Following the question posed by Dr. J. Ferguson recently on this Website and the previous debate, quite a while ago, on this same site, you will find below some thoughts on the putative impact of the use of antibiotic resistance genes in transgenic plants on antibiotic resistance in bacteria. This text was published in the 1998 May issue of the French monthly " La Recherche " (La Recherche, 1998, **309** : 36-40). Certain references have been added or updated. It has been translated into English by Dr. Michelle Storrs, a former post-doctoral fellow in the laboratory, whom I wish to thank and congratulate for the work she has accomphished. This text is the result of the long-term interest of my laboratory in the extent and means of dissemination as well as heterologous expression of antibiotic resistance genes under natural conditions. It contains facts and hypotheses that were presented, at least in part, at the " Comité de Précaution et Prévention " of the Ministère de l'Environnement, September 5th, 1997, Paris, France and at the " Expert Consultation on Safety Assessment of Gene Modified Foods ", November 12th, Copenhagen, Denmark.

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On transgenic plants and antibiotic resistance

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Even though bacteria have developed most efficient systems for dissemination of resistance to antibiotics, is it conceivable that large scale introduction of transgenic plants harbouring resistance genes will contribute to this phenomenon ? Many Genetically-Modified Organisms (GMO) have a gene which confers resistance to antibiotics integrated in their genome which serves as a marker. The risk associated with the use of such genes has been, on my point of view, superficially dealt with by experts. However, it is even more serious than that since, at the same time, bacterial resistance to antibiotics is been facilitated by large scale use of antibiotics as supplement in animal feed. Therefore, before releasing GMO's into the environment it would be advisable to develop genetic constructions that do not utilise antibiotic resistance genes.

The construction and large scale production of genetically-modified plants has generated much hope but also given rise to much questioning. One of the most serious of which concerns what is termed " resistance " which covers two distinct questions. First, resistance to the product of the gene transferred into the plant which requires competence other than my own. Second, resistance to antibiotics. The latter could spread due to the use of bacterial genes, in order to create the transgenic plant, which remain there even though they are no longer of use. In addition it is possible that these resistance genes could migrate from the transgenic plant to bacteria. Will this " return to sender " increase the phenomenon of resistance to antibiotics of bacteria pathogenic to man ?

Transgenesis involves the introduction of a foreign gene, termed *trans*gene, into the genome of a living organism. Once inside, the gene should confer an ecological, nutritional or other advantage on its new host, termed GMO (genetically-modified organism). The isolation and purification of the *trans*gene of interest is carried out in the laboratory by cloning into a bacterium, generally *Escherichia coli*. Cloning requires the use of " vectors " which allow the introduction of the gene into the plant. Bacterial cloning vectors carry genes which confer resistance to antibiotics in order to facilitate the selection of genetic constructions. For certain GMO's, these bacterial genes are transferred with the *trans*gene, even though they are of no advantage to the plant. They are therefore simply a residue of one of the steps of the genetic construction. These genes may or may not be expressed in the plant, depending on the type of construction carried out. For example, they are expressed in the tomato developed by Calgene, but in the majority of cases, such as Novartis maize, they are not expressed.

In order to survive in the presence of antibiotics, bacteria have developed several mechanisms of resistance (1). One of the most efficient and common in nature is the synthesis of enzymes which inactivate antibiotics. The production of these enzymes is generally due to the acquisition of genes originating in other bacteria by " horizontal " transfer (*i.e.*, from one bacterium to another belonging a different species of genus ; as opposed to " vertical " transfer which involves transfer from one generation to the next). The high incidence of antibiotic resistance in pathogenic bacteria is mainly due to the fact that they have developed systems for the transfer of DNA which are extremely efficient and have a large host range (2). Several mechanisms of transfer exist : conjugation, which requires direct physical contact between a donor and a recipient bacterium cell (implying an ecosystem common to the donor and to the recipient) and by which a plasmid or a transposon transfers from one cell to the other ; transformation, by which a " competent " bacterium incorporates naked DNA present in the environment ; transduction, during which DNA is transported into the bacterial cell by a bacteriophage.

Some of the resistance genes are used during the construction of transgenic plants. The two criteria which determined the choice, amongst the very high number of antibiotic resistance genes, were either their high incidence in nature, or the fact that they confer resistance to antibiotics no longer used in clinical medicine. As we will consider, these choices show an ignorance of the ecology of resistance to antibiotics and confirm superficial knowledge of the mechanisms of antibiotic resistance and of their evolution.

The introduction of penicillin G into clinical practice during the 1940's was quickly followed by the emergence of pathogenic strains, in particular of *Staphylococcus aureus*, resistant to this antibiotic. Resistance in these strains was due to the production of an enzyme, penicillinase, which hydrolyses the antibiotic. Two strategies were followed by the pharmaceutical industry in order to circumvent this resistance mechanism. First, synthesis of molecules derived from penicillin G and refractory to the action of the enzyme (methicillin, cephalosporins). Second, synthesis of inhibitors of this enzyme (clavulanic acid, sulbactam, tazobactam, etc..) which restore sensitivity to penicillins of strains producing a penicillinase. These inhibitors are therefore used in combination with these antibiotics. The $bla_{\text{TEM-1}}$ gene is widely used in the genetic modification of plants, such as the recently authorised Novartis maize, and also in molecular biology in general. This gene is responsible for the production of a

penicillinase capable of very efficiently degrading penicillins (penicillin G, ampicillin, amoxycillin). It therefore confers resistance to one of the classes of antibiotics most commonly used in human therapy. It is known that alterations to the gene which directs synthesis of this enzyme, can considerably widen the range of resistance and increase the number of antibiotics rendered ineffective. In fact, point mutations (*i.e.*, a change in one pair of bases) in several sites of this gene can confer on the enzyme either the ability to inactivate the most recent cephalosporins (3), or becoming refractory to the action of inhibitors of penicillinase (4). Therefore, the simplest genetic event, which occurs at a relatively high frequency and whose occurence is unavoidable, can ruin dozens of years of effort on the part of the pharmaceutical industry and can confer high-level resistance to antibiotics used in clinical medicine, particularly in the case of serious infections, and by far the most prescribed in the world. The $bla_{\text{TEM-1}}$ gene, which is frequently borne by plasmids self-transferable by conjugation, is wide-spread amongst the enterobacteria that are largely responsible for nosocomial (hospital-acquired) infections. This gene is also found in approximately half of *E. coli* which is a commensal of the digestive tract, and which can, under certain circumstances, be responsible for human infections. However, it would be incorrect to suggest that the $bla_{\text{TEM-1}}$ gene is present in " 50% of bacteria that are pathogenic for the human digestive tract ". The presence of the $bla_{\text{TEM-1}}$ gene in pathogenic bacteria responsible for diarrhoea (*Salmonella*, *Shigella*, *E. coli* strains capable of producing certain virulence factors, *Vibrio cholerae*, and *Campylobacter fetus)* varies depending on species but is never higher than a few percent. Although, penicillinase-producing enterococci have already been described in both North and South America, the production of penicillinase by enterococci in Europe has not yet been reported. These bacteria are intestinal pathogens for immuno-compromised patients and are isolated with increased frequency in human pathology. Therefore, $bla_{\text{TEM-1}}$ gene is neither innocuous nor ubiquitous amongst bacteria which are pathogenic to man.

Other bacterial genes have been used for the genetic modification of plants that also originate from Gram-negative bacteria. The *aph3'-2* gene, also known as NPTII, is one of the most frequently used. This gene is found for example in the Calgene tomato and both the PGS and Calgene rape. It confers resistance to certain antibiotics belonging to the aminoglycoside class, especially kanamycin and neomycin (5). These antibiotics are rarely used in human therapy due to their toxicity ; neomycin being used only topically. However, in a similar way to the

bla$_{\text{TEM-1}}$ gene, a point mutation in this gene can confer amikacin resistance on the host bacterium (6). This antibiotic, a derivative of kanamycin, is frequently used for the treatment of nosocomial infections and has recently found use in the treatment of tuberculosis. It has been observed that Koch's bacillus is becoming more and more resistant to antibiotics normally used against it.
The *aph3'-3* gene, from Gram-positive bacteria (8) and related to the above gene, specifies resistance to amikacin (7). However, and most unfortunately, currently used techniques for *in vitro* susceptibility testing are ineffective at detecting resistance by this mechanism (9). This gene is part of bacteria-plants shuttle vectors (10) and can introduced into the plant.

A fourth resistance gene, *aad3''-9*, is used in the construction of GMO's. *aad3''-9*, present in another variety of Monsanto cotton, confers resistance to streptomycin and to spectinomycin (5). The latter antibiotic is almost exclusively, and less and less frequently, used for the treatment of gonorrhoea. However, interest in streptomycin is increasing despite the occurrence of undesirable secondary effects (toxicity, pain at the point of injection). This aminoglycoside, unlike other members of this class of drugs, does not lead and is not subject to cross-resistance with gentamicin in Gram-positive cocci (staphylococci, streptococci and enterococci). Since resistance to gentamicin is increasing in frequency in enterococci, streptomycin is used in combination with penicillins for severe infections such as endocarditis (11).

The principal risk of the presence of resistance genes in genetically-modified plants is to contribute to the dissemination of antibiotic resistance in bacteria pathogenic for humans and animals. Whereas in the case of *trans*genes of agricultural interest (*e.g.* resistance to the *Bacillus thuringiensis* toxin or to herbicides) resistance could disseminate by sexual means to closely-related species (12, 13), *trans*kingdom transfer of antibiotic resistance from plants to bacteria could only result from a horizontal transfer of DNA.

Knowledge on the transfer of genetic information between organisms distanly related on the phylogenetic level is recent and only scant ; however, it is increasing at a fast rate. The existence of gene flow from Gram-positive cocci towards Gram-negative bacteria (2) as well as the existence of a system of genetic transfer between bacteria and plants (14) under natural conditions were recognized more than 15 years ago. However, the laboratory demonstration of a transfer of DNA from Gram-negative bacteria to Gram-positive cocci (2), from bacteria to fungi (15) or to mammalian cells, including human cells (16), is much more recent.

Today, it is established that the transfer of DNA can affect even the most distant kingdoms. The opposite transfer, *i.e.* from eucaryotes to procaryotes, which represents our current concern is quite conceivable. It has been proposed in certain instances (17, 18) notably, and in very convincing manner, for the *Pgi* gene which codes for the enzyme glucose phosphate isomerase (19) and also for the genes for type III domains of fibronectin proteins (20). The direct transfer of DNA from plants to bacteria, which has not been extensively studied, has not been reproduced. This negative result, however, should not be interpreted as evidence that such a transfer does not occur. One should always bear in mind that opportunities to exchange genetic material between living organisms in nature are immense and that imitating these conditions in the laboratory, or even in the field, is extremely difficult, or even impossible today. This emphasizes the poor predictability of experiments carried out in the laboratory.

Due to the required steps, each of which has a low probability of occurrence, as well as the species barrier, the possibility of a transfer of genes from plants to bacteria, if is exists, is, at least in theory, probably rare (21) ; unless, as we will see later, both the genetic constructions and the conditions which favour such transfer are deliberately increased. In fact, contrary to claims, it is unavoidable that the intensive culture of a plant harbouring a resistance gene results in an increase in the number of copies of this gene in nature, which, in turn, favours its evolution and dissemination.

The horizontal dissemination of genetic information requires three steps : DNA transfer, stabilisation in the new host (in order to insure its vertical transmission to descendants) and its expression (2).

The retrotransfer (return) of an antibiotic resistance gene from a genetically-modified plant to bacteria could occur under two distinct circumstances. The first is transfer in the digestive tract of animals or man to bacteria commensals of the tract. The thermal stability of DNA is such that, under certain circumstances, resistance genes are not denatured during food preparation prior to ingestion. Bacteria of the intestinal ecosystem belong to a very large number of species, the vast majority of which have not yet been described. They are present in extremely high numbers and in different physiological states. Some of them may be in a competent state, *i.e.* capable of incorporating DNA freed from the plant during digestion ; in particular, the fragment carrying $bla_{\text{TEM-1}}$ which, given its small size (858 base pairs), has a high risk of remaining intact. In addition, since bacteria are in very intimate contact with one another, the digestive tract is an

ecosystem which favours genetic exchange between bacteria belonging to different genera (22, 23). Under these conditions the resistance gene could be taken up by transformation by naturally-competent bacteria, transmitted vertically to subsequent generations but also transmitted horizontally to other micro-organims. During the last twenty years, studies on the evolution of bacterial resistance to antibiotics have shown that due to the gigantic size of the bacterial populations involved, an event, even extremely rare, can not only happen but also disseminate provided that the conditions for selection are present (21). Along this line, the massive and wide-spread use of antibiotics as growth promoters in animal feed creates the most favourable conditions for the selection of transfer and dissemination of resistance. Recent studies on the use of antibiotics as supplements in animal feeds have shown the possibility of colonisation of the human digestive tract by bacteria of animal origin (24) as well as the possibility of the transfer of antibiotic resistance genes from these microorganims to human bacterial commensals (25). Since transgenic maize is mainly intended for animal feed, the use of antibiotics as supplements, as well as the use of GMO's, can only increase the risk of dissemination.

The second circumstance under which genes may " return to sender " concerns the passage of DNA from decomposing transgenic plants, in particular their roots, to soil bacteria (26, 27). This is favoured by the fact that DNA, contrary to recent opinion, is an extremely stable molecule in soil even non sterile (28) and that certain species of soil bacteria are spontaneously highly competent in their natural environment (28). In addition, these micro-organisms, for example *Acinetobacter* spp., which are opportunistic pathogens, are also associated with infections in immuno-compromised patients (AIDS patients, patients with leukaemia or cancer undergoing chemotherapy, organ recipients, those in intensive care, or the elderly) who are increasing part of the population.

In addition to the retrotransfer of the gene, it is necessary that, in a second step, the DNA is stabilised in the new host to insure its transmission to new generations and its continued expression. Stabilisation occurs by homologous recombination between sequences flanking the resistance gene and DNA of the bacterial recipient. The efficiency of this process increases with the size of the interacting homologous sequences. Interestingly, many transgenic plants, including those recently approved for cultivation in France, are constructed either by electrotransformation (use of an electric field) or by biologistics (bombardment of cells with microbeads coated with the transgene). During such processes, they have acquired not only the

antibiotic resistance gene but also large flanking regions of bacterial DNA, and possibly the entire plasmid. Therefore, these constructions are not only inelegant but they are also " genetically incorrect " as they result from very crude experiments. Upon stabilisation, the gene can integrate into the chromosome where it becomes part of the bacterial genome and is stably transmitted to the progeny. Alternatively, and more worryingly, it can integrate into a mobile genetic element such as a plasmid or a transposon. In this case, it will be transmitted both vertically and horizontally, from one bacterium to another, by conjugation, mobilisation or transformation.

The third and final step necessary for sucessful transfer to occur is the expression of the resistance gene in the new bacterial host. For genes conferring resistance to ampicillin, to streptomycin-spectinomycin, which remained in the constructs under the control of a bacterial promoter, expression will be complete and immediate in Gram-negative bacteria. However, in the case of the gene conferring resistance to kanamycin, the situation is more complex since its expression is linked to its integration downstream from a bacterial promoter. This is due to the fact that, during the genetic constructions, the gene was placed under the control of a eucaryotic promoter which is, *a priori*, non functional in bacterial hosts. Therefore, the probability of expression of the kanamycin resistance gene is much weaker than that for other resistance genes.

Even though the efficiency of horizontal transfer of genes from plants to bacteria is obviously much less than that of the genetic exchange systems developed by bacteria, it still remains a risk.

Is it therefore prudent to allow genes in transgenic plants which are useless for them and which confer resistance to major classes of antibiotics in human or animal therapy or to antibiotics which are recently increasingly prescribed because of multidrug resistance ? In addition, is it prudent to leave such genes as part of sloppy genetic constructions that accumulate structures which favour an eventual return of the gene to bacteria ? All this, when techniques exist which allow " genetically correct " constructions which are perfectly defined and which avoid the use of resistance genes.

Is it advisable not to apply the precautionary principle in allowing the dissemination of " first generation " constructions, which were perhaps useful in developing transgenic techniques, but are not adapted for release in nature ? And, in addition to all that, a " biovigilance system "

will not be able to evaluate the impact of these constructions on the dissemination of antibiotic resistance since a non-labelled (non-tagged) gene is not traceable under natural conditions.

Is it wise to create a precedent which could incite seed producers to neglect the most basic precautions ?

Is all of the above judicious considering that for the last twenty five years, no new class of antibiotics has been introduced into clinical use ?

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