

# GENETICALLY MODIFIED MAIZ, ANTIBIOTIC RESISTANCE AND SOMETHING TO THINK ABOUT

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In recent years the incidence of multiple drug resistant pathogens has increased significantly. These unexpected increases have been reported for Haemorrhagic E. coli: 0157 food poisoning and *Salmonella* infections have increased exponentially in some countries. Similarly, the drug methloquine an antimalarial drug introduced in the mid eighties has been rendered ineffective in 60% of malaria cases after merely 5 years of use. The rates of increase and acquisition of resistance to widely used antibiotics such as penicillin, ampicillin, and antipseudomonas penicillin in the pathogen *Staphylococcus aureus* is to date almost at 100% – taking into account that antibiotics were introduced only in the 1940s, during the Second World War<sup>1</sup>.

It now obtains that most common bacterial pathogens have developed some level of antibiotic resistance. Apart from the cases already cited, the causal agent for diseases such as Pneumonia, Cholera, Meningitis, Leprosy, Rheumatic fever and Dysentery all show signs of multiple drug resistance which are very troubling<sup>2</sup>. Although the precise cause is not well known, several factors have been identified as contributing to the emergence of Multiple Drug Resistant Pathogens. These include: the misuse of antibiotics in agriculture and medicine, wars, civil unrest, natural disasters, expansion of human habitation with resulting environmental implications, population growth and urbanization and finally Genetic Engineering Biotechnology. While nearly all of these factors have been dealt with comprehensively in the literature very little has been published on the influence of Genetic Engineering Biotechnology on the resurgence of infectious diseases and multiple drug resistant pathogens via horizontal gene transfer.

This paper looks at the mechanisms for horizontal gene transfer, and the implications of using antibiotic resistance marker genes in Genetically Modified Plants and their derived products. This examination is done in the context of the controversial genetically modified maize variety Bt10 which was produced and inadvertently distributed in the U.S

and Europe between 2001 and 2004 without regulatory approval by the biotechnology company Syngenta<sup>3</sup>.

Genetic Engineering involves the identification, isolation and transfer of genes from any source to practically any target organism using recombinant DNA technology to produce genetically modified organisms or GMOs.

The production of genetically modified organisms involves the synthesis of artificial vector systems designed to invade genomes and cross species barriers. Vector systems are designed to successfully transfer genes to the desired organism. The gene of interest is not transferred alone but in stacked unit constructs known as 'expression cassettes' which are spliced into the vector systems.

In addition to the gene of interest the expression cassette also carries a number of other regulatory sequences such as control elements (promoter/enhancer) necessary for expression of the genes i.e. to produce the protein it codes for. It also contains a selectable marker gene such as an antibiotic resistance gene that allows the modified form to be selectively amplified while the unmodified organisms are eliminated. In the production of genetically modified crop plants it is used to help in the identification of cells/tissues that carry the transgene. In most cases the selectable marker gene remains in each cell of the modified plant. The promoter sequence acts as a switch for turning genes on. Every gene requires its own promoter. By nature promoters are very complex and the detailed mechanisms for regulating gene expression have not yet been fully understood. These promoter sequences are commonly derived from viruses associated with diseases. They are used because they give continuous over-expression of genes placed under their control. A commonly used promoter is the Cauliflower Mosaic Virus promoter which has a very broad host range and gene expression can be achieved in monocotyledons, dicotyledons, algae and bacteria using this promoter. At the end of the gene there is a terminator sequence that signals the end of translation.

The vectors are made by joining together parts of viruses (promoter) and other infectious genetic parasites (plasmids and transposons) that spread antibiotic and multiple drug resistance genes via a process termed Horizontal Gene Transfer.

The genetic constructs are transferred across cell membranes into the cells by several methods including 'Biolistics' (gene gun), electroporation (exposing to an electric field) chemical treatment and using the soil bacterium *Agrobacterium tumefaciens* which contains a tumour-inducing plasmid (Ti) capable of infecting plant cells and joining to its genome. In the other cases the vector systems after entering the cells find their way to the nucleus to become inserted into the chromosomes of the recipient cell and in a very imprecise and unpredictable manner they are integrated into the plant's genome.

Bt10 Maize contains a gene from the soil bacterium *Bacillus thuringiensis* (*Cry IAb* genes) which codes for a protein which when consumed by the European corn borer, Corn Ear worm and other lepidopterous pests causes damage to the cell membrane of the intestines and kills their larvae. In addition it contains an antibiotic resistance gene which codes for the production of an enzyme ( $amp^r$  –ampicillin resistance) beta-lactamase, which allows bacteria to break down the antibiotic ampicillin and so survive<sup>4</sup>. It also contains the Cauliflower Mosaic Virus 35s promoter, the PAT marker gene which gives the plant tolerance to the herbicide glufosinate (Liberty) and the *NOS* terminator.

Many questions and concerns have been raised about the health and safety of GM crops in general, including those containing antibiotic resistance marker genes such as Bt10 maize. In the Bt10 case the concerns have mainly to do with the use of ampicillin resistance genes and the potential for transference to other micro organisms in the environment and those colonizing the gastro-intestinal tract of humans and animals who consume the GM maize and its derivatives. Subsequently the genes for antibiotic resistance would eventually end up in disease causing bacteria which would render ampicillin and other related antibiotics useless. This comes against the backdrop of an increase in antibiotic resistance in bacterial populations as was mentioned previously.

There are several mechanisms which can facilitate such transfer but all can be grouped under the broad theme 'Horizontal Gene Transfer'

Horizontal gene transfer can be described as the transfer of genetic material between cells, and genomes belonging to unrelated species by processes other than reproduction<sup>5</sup>. In nature only closely related or the same species reproduce and in the process genes are transferred 'vertically' from the parent to the offspring. Horizontal gene transfer has been known to take place in bacteria with relative ease which accounts for the spread of antibiotic resistance between bacterial species. DNA sequence analysis of genes for neomycin-kanamycin resistance from *Staphylococcus aureus*, *Streptococcus* and *Campylobacter sp* provided the definitive evidence of horizontal gene transfer. The evidence revealed an identical set of genes in all the pathogens<sup>6</sup>. However, the extent to which horizontal gene transfer potentially takes place between plants and bacterial cells has not clearly been elucidated but current knowledge does call for a more precautionary approach towards Genetically Modified Organisms.

In bacteria there are three well known mechanisms for horizontal gene transfer and these are:

- ✚ Conjugation – Movement of DNA between bacteria following cell to cell contact and effected by plasmids or transposons.
- ✚ Transformation – uptake of free ('naked') DNA from the environment and its incorporation into the bacterial genome.
- ✚ Transduction – The transfer of genetic material from one bacterium to another by a bacteriophage (virus that infects bacteria)

Conjugation is considered to be the most effective mechanism of gene transmission under natural conditions<sup>7</sup>. It depends on extra-chromosomal pieces of DNA called plasmids that are capable of independently self replicating. These plasmids or circular pieces of DNA contain genes that are necessary for conjugation. Most plasmids carry one or more antibiotic resistance genes and have been classified into compatibility groups. Those who are in the same incompatibility group cannot be present in the same bacterial cell and they contain genes that code for the pilus a tube like structure

connecting the donor and recipient in conjugation through which the DNA passes from the donor to the recipient.

In other cases it has been put forward that genes playing a role in plasmid transfer their products are specific to the plasmid and its host and not interchangeable. The incompatibility group therefore comprises plasmids with broad host range as can be extrapolated from the above.

Because of the broad host range of bacteria within the incompatibility group they are able to overcome species barriers and thus transfer genes between phylogenetically distant species. Some conjugative plasmids have been used in constructing artificial vectors between *E.coli* and other distant species and has also been used in *Agrobacterium* mediated plant genetic transformation. The efficacy of conjugative plasmids resides in the fact that they have multiple origins of replication for multiple species and origins of conjugative transfer as well for lots of species. In conjugative transfer a single stranded DNA is transferred directly and escapes destruction by restriction enzymes. Shuttle vector systems are engineered to require helper transfer functions provided by other plasmids which can be present in the donor or recipient or in a third species.

During conjugation conjugative transposons mediate their own transfer to recipient cells and can insert into recipient chromosomes<sup>8</sup>. Transposons are mobile genetic elements sometimes called 'jumping genes' that can jump from one site to another on the same chromosome or from chromosomes into plasmids and *vice versa*.

Conjugative transposons also form a part of artificial vector systems used in genetic engineering. Some have suggested that some conjugative transposons called 'integrons' are involved in the evolution of multi-resistance plasmids . The integrons carries a gene which encodes an enzyme that is responsible for the integration of antibiotic resistance gene cassettes within the integron in a manner that every cassette has its own promoter.

Transformation is wide spread and in the case of genetically modified foods and crops may be the most likely mechanism for horizontal gene transfer to microorganisms. Following digestion after eating by humans and non target organisms such as earth

worms, caterpillars (non lepidopterous) herbivores etc and after decomposition of the plant material or food 'naked' DNA can be released into the environment which includes the human body.

The DNA released into the environment would then bind to the surface of bacteria, enter into the cells and recombines with the bacterial chromosome or in the case of plasmids reconstitutes the plasmids<sup>9</sup>. The uptake of 'naked' DNA by the cells will depend upon their physiological stage of competence. The stage of competence is contingent upon several factors including ontogeny, impact of stress factors and/or presence of diffusible competence factors secreted by bacteria and the species in question<sup>10</sup>. Homologous DNA sequences have a greater propensity for establishment and recombination within the recipient genome, however, DNA uptake may not depend on the presence of homologous sequences.

For plasmids their establishment will depend upon the presence of suitable origins of replication. But the question now arises what are the 'real possibilities of transformation in natural environments? The fact of the matter is there are many findings that suggest that transformation is common in natural environments and furthermore it has been shown that Transformation of endogenous bacteria in the gastrointestinal tract of *Folsomia candida* after feeding with GMOs<sup>11</sup>. It must be noted however, that environmental factors are thought to influence transformation by providing the ideal conditions which may be inimitable in laboratory conditions. Such environmental factors include presence of nutrients and minerals, ionic strength of water and temperature<sup>12</sup>.

It can be extrapolated from the above that the transformation of bacterial DNA from plant derived DNA may be very rare but what has to be factored into the equation is the volumes of GMO food consumed by individuals or animals as well as the large numbers of bacteria within the human body (animals as well) and the environment which increases exponentially the chances of successful transformation events. In that vein the methodologies used to detect rare transformation events may not be sufficiently

sensitive<sup>13</sup> and if such is really the case can we say that transformation events do not take place categorically?

When transformations are not detected it may signify that the initial transformation levels are beyond detection but this has to be taken with great caution as rare transformation events can take place and be amplified via multiplication eventually.

There is also a body of evidence that does suggest that horizontal gene transfer may take place between plants and bacteria. These include studies showing that some genes are likely to survive passage through the human small intestine and would be able to transform the bacteria in the bowel<sup>14</sup>. It has been shown that DNA can survive in the saliva of sheep for periods of time sufficient to transform bacteria<sup>15</sup> also, oral bacteria in human saliva has been transformed by naked DNA<sup>16</sup>. Additionally, it has been demonstrated that DNA from GM crops can persist in soils for two years<sup>17</sup>. What is even more compelling is gene transfer from GM soya to unidentified microorganisms in the intestines in samples taken of three of seven patients with ileostomies<sup>18</sup>.

Given the abundance of information that suggest that horizontal gene transfer may actually take place from plants to bacteria it is imperative that we analyse the implications of using antibiotic resistance genes such as ampicillin resistance ( $amp^r$ ) in genetic modifications and the repercussions thereof. Ampicillin forms part of the beta-lactams group (some of broad-spectrum penicillins) of antibiotics, these are probably the most commonly used group of antibiotics. Ampicillin is used to treat a wide range of maladies such as bronchitis, pneumonia, urinary tract infections, gonorrhoea, enteric fever, peritonitis, meningitis, ear, nose and throat infections, gynaecological infections and gastro-intestinal infections in humans. In animals it is also used for the treatment of wide range of diseases and infections which extols the importance of the beta-lactam antibiotics for both human and veterinary medicine. The ampicillin resistance gene does not only code for ampicillin resistance but also for all the other antibiotics of the beta-lactam group.

What exacerbates the problem is the fact that unlike natural ampicillin resistance genes found in nature Bt10 ampicillin resistance gene is found on an artificial vector pUC 18 which has a mutated *ori* sequence which produces up to ten times more copies of the beta-lactamase enzyme per cell<sup>19</sup>. Therefore, if this trait is transferred horizontally to gut microorganisms the high levels of beta-lactamase produced by the cells would render ampicillin useless in the treatment of infections and disease.

Transduction is mediated by bacteria infecting viruses or bacteriophages. It requires living cells unlike transformation. Bacteriophages are quite host specific and may scarcely transfer genes between unrelated species. It is more likely to occur under conditions of high metabolic activity<sup>20</sup>. Both plasmids and chromosomal DNA can be transferred via transduction.

The scope of transduction and flow of genetic material will depend on the host range of the bacteriophage. The bacteriophage consists of viral DNA wrapped in a coat protein, which binds to specific receptor sites on the bacteria. The bacteriophage then injects its DNA into the bacterium, the virus then controls the cell to either produce more bacteriophages leading to cell lysis or to incorporate its DNA into the plant chromosome to become replicated. Transduction has been observed in the natural environment<sup>21</sup> and experiments show that gene transfer occurs via transduction in an aquatic environment<sup>22</sup>. Transduction is dependent upon the concentration of phage particles and bacterial host cells. Bacteriophages have been implicated in evolution by horizontal gene transfer and recombination which led to increased host ranges<sup>22</sup>. In the context of genetically modified crops and foods this method of horizontal gene transfer does not present a great potential to take place as is the case with Transformation events.

Another factor which is of significance is the effects of environmental pollution on horizontal gene transfer. It has already been seen that many physical and chemical factors influence horizontal gene transfer. Heavy metals are used in laboratory procedures to increase cell competence for DNA uptake in genetic modification. Factors such as the presence of antibiotics are believed to increase horizontal gene transfer 10 to 10,000

fold<sup>23</sup>. The effects of xenobiotics is an area which needs to be carefully studied to determine their precise effect on horizontal gene transfer. It has been postulated that xenobiotics can affect horizontal gene transfer in two ways<sup>24</sup>. Firstly, they can affect cells by mutating DNA thus influencing uptake and affecting the cell membrane and intercellular functions thereby influencing the ability of the cell to take up foreign DNA. The importance of considering xenobiotics when designing experiments and assessing the risks associated with GMOs cannot be omitted because of the enhancing effect on the process of horizontal gene transfer these substances can have and the fact that they exist throughout our natural environment.

Given the evidence of research which demonstrates clearly, that intact DNA can survive passage through the gastro-intestinal tract, mouth and in the soils for significant periods to allow for bacterial transformation. Coupled with that the wide use of the beta-lactam antibiotics such as ampicillin throughout human and veterinary medicine dictates against introducing genetically modified organisms such as Bt10 maize into the environment. Although the probability for spread of antibiotic resistance genes via horizontal gene transfer may appear low but the risks are too enormous and there are knowledge gaps which need to be filled before we can conclusively adopt a position which favours the use of those genes. More rigorous testing methods must be employed, better animal studies and the use of truly generalizable results which may be derived from a more holistic approach to risk assessment which can take into account the other factors mentioned in this document such as xenobiotics, scale and frequency of exposure between a GMO and bacteria in humans, animals and the environment.

Against the background of increasing multiple drug resistant pathogens it is wise that countries adopt a precautionary posture and decrease from using antibiotic resistance marker genes in GMOs. They are superfluous and have no significance in the end product.

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