« Introduction to GMO: technique and safety »

Barbara Bordogna Petriccione

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Rue Rothschild, 24 - CP 136 - 1211 Genève 2
www.ribios.ch

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1.1. WHAT IS BIOTECHNOLOGY APPLIED TO AGRICULTURE ?

The term biotechnology refers to a large set of techniques using properties of living organisms. It includes traditional processes used in every day life for millenniums such as fermentation in bread, wine, and beer making. In such processes, living organisms are used to transform one substance into another e.g. sugar into alcohol.

But today biotechnology refers mainly to modern techniques currently applied in medicine, pharmacy and agriculture. In that latter field, genetic engineering and tissue culture are the most important ones and we will only consider those in this module.

Genetic engineering techniques enable scientists to identify a particular DNA sequence corresponding to a selected gene, to excise it and to transfer it into another organism, for example a plant cell. Through genetic engineering techniques the genome of an organism can be modified.

Tissue culture is another important technique for the development of transgenic plants. It enables us to grow and multiply cells outside an organism and to regenerate a whole plant from a single cell. This regeneration process is a key step and is often a limiting factor. In rice for example, the difficulty to regenerate plants from a single cell has for a long time prevented the production of transgenic rice.

Both genetic engineering and tissue culture are necessary to create a transgenic plant; genetic engineering to introduce the transgene (gene from another species) into the plant cell and tissue culture to regenerate the transformed cell into a whole plant.

1.2. WHAT ARE CELLS, CHROMOSOMES, DNA AND PROTEINS ? (Fig. 1 Intro, p. 48)

The basic functional unit of every living organism is the cell.

There are unicellular organisms composed of a single cell (bacteria, protozoa, etc.) and multicellular organisms made up of many cells (human beings, animals, plants, etc.).

Most of the cells contain a nucleus in which are located the chromosomes. Bacteria however do not have nucleus and their chromosomes are found in the cytoplasm.

A chromosome is in fact a very long DNA (DeoxyRibonucleic Acid) molecule maintained in a contracted form by the assemblage of various proteins. The DNA is the material containing the genetic information and it is made of the same physical and chemical components in the cells of every living organism. It is this similarity that permits the transfer of a DNA sequence from one species to another, thereby producing a transgenic organism (or genetically modified organism - GMO)

The DNA is composed of four different molecules called bases (A=Adenine, C=Cytosine, G=Guanine, T=Thymine) arranged in a particular sequence. It is the order of this particular side-by-side arrangement of bases along the DNA strand (e.g., ATTCCGGA) that spells out the exact instructions required to create an organism with its own unique traits.

Human beings have approximately 35 '000 genes but this represents only about 2% of the total DNA. From the remaining 98%, 2% are regulatory sequences and introns and the rest is referred to as "junk DNA"[1] because its functions are largely unknown.

A gene is a particular DNA sequence that codes for a given protein. The gene is first transcribed into a transient molecule called mRNA (messenger RiboNucleic Acid) and then translated into a protein. Proteins are very important molecules and they represent about half the dry weight of a cell. They play different roles in an organism: they can have a structural function, like in muscle, or a metabolic one like for all enzymes. They can also have a regulatory role like hormones, and can even be used as an energy source on various occasions.

1.3. WHERE IS DNA ? (Fig. 2 Intro, p. 49)

DNA is not present only in the nucleus of the cell. We mentioned above that bacteria do not have nucleus. Bacteria and all the organisms without nucleus are called prokaryotes (no nucleus) and so bacterial DNA doesn't lie in the nucleus but in the cytoplasm. Bacteria have in general two different types of DNA: chromosomal DNA and plasmid DNA. Chromosomal DNA is a very long molecule arranged in a chromosome, like in cells with nucleus (eukaryotes) and plasmid DNA is a small and circular DNA molecule.

Plasmids are very useful for genetic engineering. They have indeed several characteristics that make them a perfect tool for molecular biologists:

- They are small and can be handled easily by molecular techniques

[1] See hereafter: 4.1 « Junk DNA », p. 43

- They are able to self-replicate in the bacteria
- Bacteria may contain several copies of the same plasmid
- Bacteria are naturally capable to transfer a plasmid to other bacteria through a process called conjugation.

For these reasons, plasmids are the ideal means to introduce the transgene (gene from another species) and to have it multiplied in the bacteria. Furthermore, the bacterium *Agrobacterium tumefaciens* is also able to transfer a part of its plasmid DNA into a plant cell (See hereafter p. 12 "1.4.1. "How to transform a plant with the bacterium *Agrobacterium tumefaciens ?*)

1.4. WHAT DO WE NEED TO MAKE A TRANSGENIC PLANT ? (Fig. 3 Intro, p. 50)

To make a transgenic plant, we first need to assemble the DNA sequence we want to introduce into the plant. This sequence should include the transgene(s) conferring the required characteristic and a marker gene for selection of the transformed cells. As we will see further on, the marker gene for selection is necessary to isolate among many untransformed cells the few ones having effectively integrated the transgene. The most commonly used marker genes are either antibiotic resistance genes or herbicide resistance genes, but alternative marker genes also exist[2].

For each gene we need a promoter and a terminator. The promoter is a DNA sequence preceding the gene. It regulates the activity of the gene. A promoter can be eukaryotic (active in plants or animals), prokaryotic (active only in bacteria), ubiquitous (active both in plants, animals and bacteria). It can also be tissue specific (active only in leaves or in roots for example) or development specific (active only during embryogenesis, during flowering, etc.). The terminator is a DNA sequence following the gene. It determines the end of the gene and stops its transcription into mRNA.

Once we have the required DNA sequence, we need to transfer it into the plant cell. There are mainly three different ways to transform plants:

- **Using a bacterium called *Agrobacterium tumefaciens***
  Agrobacterium tumefaciens is a bacterium naturally able to transfer a part of its plasmid DNA into the cells of wounded plants. This extraordinary property –the ability to transfer DNA from one kingdom (bacteria) to another one (vegetal)– is unique and molecular biologists have used it to transfer selected genes into plant cells. For a long time, this

[2] To learn more about alternative marker genes, see: Bordogna Petriccione B. (2003) «Plantes transgéniques: quelles alternatives aux gènes marqueurs de résistance aux antibiotiques ? », RIBios, url: http://www.ribios.ch/documents/marqueurs_alternatifs.pdf

technique was not applicable to monocotyledonous plants (rice, wheat, maize, bananas, and generally all cereals) due to the specific host range of *Agrobacterium*, but it now works for at least both rice and maize.

- **With gold or tungsten particles coated with DNA and shot into the plant nucleus**
  DNA shotgun is a technique, which uses very small particles of gold or tungsten coated with the chosen DNA sequence. These particles are then shot onto the plant embryo and the DNA sequence eventually integrates into the plant genome. DNA shotgun has the advantage to be applicable to monocotyledonous plants. It is through this technique that many transgenic cereals are produced. In order to get the amount of DNA sequences necessary to coat the gold or tungsten particles, the DNA sequence is first introduced into the plasmid of a bacterium. The bacterium then multiplies and the plasmid DNA is extracted from the resulting bacterial culture[3].
- **By a technique using electric shocks (electroporation).**
  As it is rarely used to develop transgenic plants, this method will not be described here.

1.4.1. How to transform a plant with the bacterium *Agrobacterium tumefaciens* (Fig. 4 Intro, p. 51) ?

As noted previously, *Agrobacterium tumefaciens* is a bacterium naturally able to transform plant cells with a part of its plasmid DNA. The particular DNA sequence transferred into the plant is called T-DNA and is bordered by two particular DNA sequences called left border (LB) and right border (RB). The T-DNA contains genes that will induce the production of particular nutrients and the development of tumorous tissues in the plant which lead to the development of the disease called crown gall tumor. The nutrients produced will then be used as a precious food source by *Agrobacterium*. So the relationship between *Agrobacterium* and the plant is of the parasite-host type: *Agrobacterium* receives nutrients from the plant whereas the plant develops Crown Gall Tumor and loses energy through feeding *Agrobacterium*.

The transforming capacity of *Agrobacterium* is used by molecular biologists to transfer target genes into plant cells. The DNA sequence between the left

[3] It is worth noting that, to select the transformed bacteria, a marker gene different from the one used for selection in the plant is necessary, as it has to be under the control of a prokaryotic promoter. Consequently, in the first transgenic plants produced with DNA shotgun, the plasmid DNA used to coat the gold or tungsten particles often contained not only the marker gene for selection of the plant but also the marker gene used for selection of the bacteria. This was the case of the Bt maize from Novartis (now Syngenta) that contained the gene for ampicillin resistance.

border (LB) and the right border (RB) of the T-DNA is excised so that it does not have the ability to cause crown gall tumor anymore and it is replaced by the target genes. In the example shown in Fig. 4 Intro (p. 51), the gene for insect resistance (*Bt* gene) and the marker gene for antibiotic resistance are inserted. The gene for antibiotic resistance is called a marker gene because it doesn't modify the plant in any beneficial way but it is there only for the selection of the transformed cells (see below: "1.6. How to select for the transformed cells ?"). *Agrobacterium* will then transfer into the plant cell the DNA segment between LB and RB that now contains the target genes[4].

1.5. HOW DOES THE TRANSGENE INTEGRATE INTO THE PLANT GENOME ? (Fig. 5 and Fig. 6 Intro, pp. 52 et 53)

Once inside the plant cell, the DNA sequence has yet to be inserted into the chromosome of the plant. The process by which such integration is achieved is called *recombination*.

Recombination is defined as an exchange of nucleotides between two molecules of RNA or DNA. It occurs commonly in all organisms. During meiosis for example recombination plays an important role in creating genetic diversity.

There are two types of recombination: homologous recombination and heterologous recombination.

Homologous recombination is the exchange of a nucleotide between two DNA or RNA molecules with a high sequence similarity. It is the most frequent mechanism in bacteria but in plants and animals it does not work well. Homologous recombination enables us to precisely replace a gene, or to insert a DNA sequence into a precise locus. As it doesn't work in the nuclear genome of plants and animals, it is not possible to predict the site and number of transgene insertions in those organisms whereas in bacteria the precise insertion of the transgene can be achieved.

Heterologous recombination is a nucleotide exchange between two unrelated RNA or DNA molecules. Transgene integration into the plant normally occurs by heterologous recombination and it is therefore not possible to predict where it will be inserted neither in how many copies.

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[4] The actual technique used in *Agrobacterium*-mediated transformation is more complex than what is described here. However, we did not consider it useful at this point to go into more details. For further insight, see: Watson J.D., Gilman M., Witkowski J. and Zoller M. (2001), « Recombinant DNA », 2nd ed., New York, pp. 277-281.

1.6. HOW TO SELECT FOR THE TRANSFORMED CELLS ? (Fig. 7 Intro, p. 54)

The ability to select transformed cells is crucial in developing transgenic plants. Without selection, the screening of all potential transformed cells would take so much time that DNA recombinant techniques would not be feasible. The transformation frequency is indeed very low[5] so that it is absolutely necessary to be able to identify the cells that have been effectively transformed.

The selection is carried out mediating a marker gene that is often an antibiotic resistance gene. This marker gene is co-introduced into the plant cell along with the functional gene we want to insert, for example the insect resistance gene (Bt gene). So if the presence of this antibiotic resistance marker gene can be detected, this means that the insect resistance gene is also present in the same cell. This selection of the antibiotic resistance marker gene is achieved by culture of all potentially transformed cells in presence of antibiotic. The cells effectively transformed will be able to grow as they contain the antibiotic resistance gene. On the contrary, non-transformed cells will not be able to grow on antibiotic because they have not integrated the marker gene conferring antibiotic resistance.

Other commonly used markers are herbicide resistance genes. Herbicide resistance genes have this characteristic of being both the marker gene and the selected gene to introduce. Indeed, herbicide resistance has been introduced into plants as a valuable trait and with a precise agronomic purpose. Apart from having been the first transgenic plant developed, herbicide resistant plants are still the most widely sawn and commercialized ones[6].

For a few years, a controversy concerning the impact of antibiotic resistance genes on the development of resistance in bacterial pathogens has been going on[7]. It has accelerated the development of alternative markers such as those enabling the cells to use alternative carbon sources for growth. These are now the most commonly used alternative markers[8].

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[5] It depends both from the species and from the variety of the plant, but in the best case, the transformation frequency is of 10^{-3}, this means that one would have to screen one thousand potentially transformed plants to find one plant effectively transformed.

[6] Further details about herbicide resistant plants are given hereafter p. 20 and p. 39.

[7] See Bordogna Petriccione B. (2003) « Développement des résistances aux antibiotiques: quel rôle pour les plantes transgéniques », RIBios, url:http://www.ribios.ch/documents/resistances_antibiotiques.pdf

[8] See : Bordogna Petriccione B. (2003) «Plantes transgéniques: quelles alternatives aux gènes marqueurs de résistance aux antibiotiques ? », RIBios, url: http://www.ribios.ch/documents/marqueurs_alternatifs.pdf

1.7. HOW TO REGENERATE A PLANT FROM A SINGLE CELL ?

The transformed cells are then grown successively on various mediums containing different concentrations of growth hormones. This process ends up in the regeneration of a whole plant from a single transformed cell.

Regeneration is a key step in producing transgenic plants. Indeed the growth conditions are very specific for each species or variety and can be established only empirically. Transgenic rice for example could be produced only recently not because of transformation problems, but because regeneration was very difficult to achieve.

1.8. WHAT IS A HYBRID ? (Fig. 8 Intro, p. 55)

After understanding what is a transgenic plant, questions often arise about hybrids. What are they exactly ? And which are the differences between a hybrid and a transgenic plant ? As plant hybrids are important elements in agriculture, we will try to answer these questions even if they do not strictly fit the topic of this module.

Hybrids can be divided in two different types:

- Genus and species hybrids
- F1 hybrids

Genus hybrids are the result of a cross between two genera (for example a horse and a donkey) and species hybrids are the result of a cross between two species (for example wheat and rye). Individuals from two different genus or species are usually not sexually compatible that is why genus and species hybrids are often sterile, like in horse and donkey cross. However, triticale - the result of wheat and rye cross - and wild hog - the result of wild boar and domestic pig cross - are examples of fertile species hybrids[9].

When using the term hybrid in plant crops, we usually refer to F1 hybrids, i.e. the result of a cross between two varieties of the same plant species[10]. A variety is defined as a plant with a particular trait, for example a special flower colour. This trait has to be homogenous (all plants of the variety should have the same special colour) and stable (when planted, all subsequent generations

[9] In reality, the first generation (F1) of triticale is sterile and it is only after the total number of its chromosomes has been doubled that triticale become fertile.

[10] This is true for self-pollinated plants, like tomato. In cross-pollinated plants however, F1 hybrids are the result of a cross between two *lines* of the same plant species. An example of cross-pollinated plant is maize. A line is created by autofecondation until the plant is homozygote.

should express the same flower colour). Unlike in genus and species crosses, plant varieties have no sexual incompatibility among them.

F1 hybrids may be less or more fit than either parent; the former condition is termed *outbreeding depression* and the latter is called *heterosis*. F1 hybrids with a good heterosis are largely used in agriculture. Heterosis is characterized, in the first generation, by an increase in yield and vigour. However, in the following generations, the benefits of heterosis decrease abruptly and very different phenotypes among the hybrids appear. As a consequence, these hybrids are usually planted only once in order to benefit from the heterosis effect and new seeds are bought from the breeder every year.

1.9. WHAT ARE THE DIFFERENCES BETWEEN A HYBRID AND A GMO ?

	GMO	**Hybrid**
What is it ?	An organism that contains one or a few genes from another species (transgenes)	An organism resulting from a cross between two different genus, species or varieties: two different genomes have been brought together.
How fertile is it ?	GMO are usually as fertile as other plants, except for V-GURTs (Variety Genetic Use Restriction Technologies) like Terminator technology, which are sterile (see: 2.1.5. GURTs, p. 24)	Genus and species hybrids are often sterile but F1 hybrids are normally fertile.
How many generations the seeds can be sawn ?	A GMO can be sawn for many generations without alteration of its characteristics (except in T-GURTs - Trait Genetic Use Restriction Technologies. See 2.1.5. GURTs, p. 24). The restriction to use saved seed for the next season (if any) comes from the intellectual property rights (patents) granted on the transgene, the promoter, etc.	In plants, F1 hybrids can express an increase in yield and vigour during the first generation (heterosis phenomenon) but this effect decreases in the subsequent generations. This decrease in yield and vigour brings farmers to buy seeds every year, as saved seeds no longer have a good heterosis.

II. GMO APPLICATIONS IN FOOD AND AGRICULTURE

2.1. WHICH ARE THE POTENTIAL APPLICATIONS OF GMO IN FOOD AND AGRICULTURE ?

As in theory any gene from any organism could be transferred into a plant, the potential applications of genetic engineering to food and agriculture are virtually unlimited. We will consider here only some of the most important ones. It is worth noting that not all the possibilities presented here have been developed: many are still out of reach for the moment, some are subject to fundamental research, some are at an experimental stage and some have already been commercialized.

2.1.1. Fight against pests, pathogens and weeds (crop protection traits)

Worldwide production losses due to pests, pathogens and weeds amount to about 40% of the expected harvest in the absence of control measures. In 1988-1990 for example, global losses for eight major food and cash crops (rice, barley, maize, potato, soybean, cotton and coffee) accounted for 42.1% of the expected harvest, where 15.6% was due to animal pests, 13.3% to pathogens and 13.2% to weeds[11].

Insects resistance

In transgenic plants, the main strategy to fight insect pests uses a gene from the bacterium *Bacillus thuringiensis* (*Bt*) coding for a crystal protein (*Bt* toxin hereafter). This protein is toxic for some insects, especially for lepidopterian larvae. There are many different *Bt* toxins and each one has a very narrow range of target insects. Furthermore, the mechanism of action of this toxin does not exist in mammals and no toxicity for human or animals has been reported.

The mode of action of the *Bt* toxin is the following: the *Bt* toxin is first ingested by the lepidopterian insect and once in the intestinal tract, digestive enzymes cleave it. Then, the toxin binds to specific receptors on the intestinal tract surface producing its disruption and the subsequent death of the insect.

[11] Oerke, E.C., H.W. Dehne, F. Schoenbeck and A. Weber (1994), "Crop production and crop protection", Elsevier, Amsterdam.

The *Bt* toxin has been used for decades in agriculture by normal spraying, but its low efficiency compared to other chemical pesticides has limited its use[12]. In organic farming however, it still plays an important role because it is one of the only pesticides allowed as it comes from a natural source, i.e. from bacteria.

One of the advantages of producing transgenic plants containing the Bt gene is that insects attacking the plant from inside the stem (European corn borer for example) can be reached because the toxin is present in every tissue of the plant, while with normal spraying of pesticide, this is much more difficult. The absence of reported toxicity, the narrow host range of target insects and the long experience acquired using this toxin are also positive elements of *Bt* transgenic plants, especially if compared to conventional crops sprayed with pesticides.

Nematodes resistance

Nematodes are very small worms that affect the roots of most cultivars. Worldwide crop damage due to plant-parasitic nematodes causes important losses each year. The traditional means to fight this pest involve the application of nematocides as well as cultural methods. However, most nematocides are neurotoxic and water-soluble pesticides that pose hazards to farmers, to other animals, to groundwater and to food safety. Regarding cultural methods such as crop rotation, they are frequently used but of limited efficiency.

Transgenic strategies to control plant-parasitic nematodes are therefore seen as a new and less chemical demanding way to reduce the losses due to this pest. Currently, researches are conducted on crops like rice, potato, banana and tomato. But no commercial applications have been developed yet.

Fungi resistance

Fungi are the most important and widespread pathogens in plants. They affect almost every plant species and the traditional means to control this pathogen usually involve enormous quantities of fungicides. Transgenic plants resistant to fungal disease would therefore potentially provide important benefits in term of reduction of pesticide use. Various research groups are working on the development of such transgenic plants, but no commercial varieties have reached the market yet.

[12] When sprayed on the plant, the Bt toxin is indeed degraded very quickily and thus require multiple applications.

Viruses resistance (Fig. 1 Application, p. 56)

Viruses are plant pathogens that cannot be controlled via the classical means used for fungi, insects, nematodes or bacteria. Indeed viruses use the plant cell machinery for their development, multiplication and propagation. So all the pesticides used to control other pests are ineffective against viruses as they are inside the plant cell and no chemical product can eliminate them without damage to the plant itself. The only means to control virus development are disinfection of the tools, burning of dead leaves and branches, seed control, etc.

The transgenic approach to the fight against viruses offers interesting perspectives. It has been shown since 1986 that a plant containing a fraction of the genetic material of a virus can develop a resistance to this virus. Such kind of resistance is called *cross protection* and is based on gene silencing mechanisms[13] that involve complex interactions between the genetic material of the virus (usually RNA) and the transgene introduced into the plant. These interactions lead then to the degradation of the genetic material of the virus. It is thought this mechanism is in fact a natural defense reaction of the plant against viral infections.

Today, most virus resistant transgenic plants contain only a portion of the gene from the envelope of the virus called capside gene (instead of the whole capside gene like in the first transgenic plants developed). Indeed, it has been shown that the gene silencing mechanism is also effective with only a fraction of the capside gene. Hence, in these plants, as the gene is not complete, no capside protein is produced, but only a fraction of its RNA.

Some commercial varieties of virus resistant crops are already available, especially for potato, squash and papaya.

[13] Gene silencing is discussed in more detail hereafter p. 44.

Herbicide resistance

Resistances to four different herbicides have been genetically engineered, but most herbicide resistant transgenic crops commercialized are resistant either to glyphosate (commercial name: Roundup) or to phosphinothricin[14] (commercial name: Liberty, Basta or Finale).

Herbicide	**Herbicide resistant transgenic crops**
Glyphosate	maize, soya, cotton, sugar-beet, oilseed rape
Phosphinothricin	maize, soya, cotton, oilseed rape, rice, sugar-beet, radicchio
Bromoxynil	cotton, oilseed rape
Sulfonylurea	cotton, flax

(FDA, 2002)

Glyphosate is a broad-spectrum herbicide: it kills all kind of plants. It is quickly degraded in the environment compared to other herbicide used in crop protection. However, as it is very difficult to detect, we might have underestimated the level of residues present in soil, groundwater and food. Indeed, until a few years ago, only a few laboratories had the technical means to detect glyphosate residues in the environment. However, recent data have not confirmed these fears.

Glyphosate has a low toxicity for humans and mammals in general. But apart from the active substance - glyphosate -, herbicides are normally commercialized as a mixture containing other compounds that might be very aggressive for skin, eyes, etc. This explains why glyphosate is the first cause of medical complaint from farmers in the United States[15].

Phosphinothricin is also a broad-spectrum herbicide that burns the plant when it enters in contact with it. It is degraded even faster than glyphosate in the environment and has also a low toxicity for humans and mammals in general.

In conventional farming, phosphinothricin and glyphosate are frequently used to replace ploughing by "chemical ploughing" thus contributing to avoid soil erosion.

[14] Phosphonothricin is also called gluphosinate ammonium.

[15] It is worth noting that glyphosate is also the pesticide more used in the United States and all over the world.

Transgenic plants resistant to glyphosate or phosphinothricin bring in different advantages[16] compared to non-GMO crops sprayed with other herbicides commonly used in agriculture like triazine.

Indeed, with non-GMO crops, broad-spectrum herbicides cannot be used directly on the plant as they kill it. They are therefore only applied before crop germination, without knowing which kind and how many weeds will be growing. Regarding the control of *post-emergence* weeds (grown after crop germination), it can be achieved only using different mixtures of selective herbicides or by mechanical means.

Conversely, with herbicide resistant transgenic plants, the farmer is not obliged to treat the field before germination as glyphosate or phosphinothricin can be sprayed directly on the transgenic crop. The weeds can therefore be controlled in a more adapted way and soil erosion can be reduced, as no hoeing is required anymore. Furthermore, glyphosate and phosphinothricin are more effective than most mixture of selective herbicides used to control post-emergence weeds on conventional crops and in the meantime, they are far less toxic and remanent. It seems that, in definitive, weed management is simplified by the adoption of herbicide resistant transgenic crops. This aspect seems to be appreciated by some farmers, in particular in the United States where herbicide resistant plants have been widely adopted.

2.1.2. Agronomic traits

Nitrogen fixation

Nitrogen is a limiting factor in plant growth. Its availability in soil is limited and plants are not able to fix N_2 from the air. Usually, cultivated plants are provided with nitrogen through fertilizers. This is true except for legumes that are plants naturally provided with nitrogen through a symbiosis they establish with nitrogen fixing bacteria. When the genetic mechanisms controlling this symbiosis will be understood, it might be possible to engineer either non-legume plants either nitrogen-fixing bacteria in order to establish such symbiosis in agronomically important crops. These plants would not need nitrate fertilizer supply any longer, which would have a positive impact on the environment.

[16] The advantages mentioned here are only true in the context of intensive or extensive farming, where the use of less polluting herbicides is a progress for the environment. These advantages can obviously not be transposed to other contexts such as organic farming, where herbicide resistant plants would just be a non-sense.

Drought resistance

Drought is an important problem for agriculture in many regions where small variations in rainfall can easily destroy an entire harvest. Furthermore, the advance of the desert is turning more and more land sterile. The perspective of developing transgenic plant resistant to drought is therefore very attractive and would possibly contribute to solve an important agronomical problem. However, even if some laboratories have undertaken researches, no drought resistant transgenic plants have reached the market yet.

Salt tolerance

High salt concentration in soil is also an important agronomic problem. Indeed, in areas of intensive irrigation, soils tend to accumulate salts. As plants are not able to grow on high salt concentration, entire landscapes are turned sterile. Plants tolerant to high salt concentration would thus be able to grow on these salty deserts and restore farming activities. However, as for drought resistance, no salt resistant transgenic plants have been commercialized yet.

2.1.3. Quality traits

Delayed softening (Flavr Savr tomato)

Improving the firmness of a fruit should normally facilitate its transport, stocking and distribution as well as allow a longer ripening directly on the plant. Firmer transgenic tomatoes (Flavr Savr) have been developed using an antisense construct strategy. An antisense construct is a DNA sequence complementary to a particular gene. When such construct is introduced in the genome, it will be transcribed in RNA and will be paired with the RNA of the gene it is complementary to. This complex of two RNA (double stranded RNA) will then be degraded so that no protein of the gene will be produced. In the case of the tomato, an antisense construct complementary to the gene responsible for cell wall degradation (polygalacturonase - PG - gene) has been introduced. The resulting transgenic tomato does not produce the PG protein any longer so that cell walls are not degraded anymore and the tomato maintains its firmness much longer.

The Flavr Savr tomato of Calgene (now Monsanto) has been commercialized for a brief period in 1994-1995 in the USA, but it has now been withdrawn from the market, due to its poor success. Another tomato from Zeneca had been commercialized in the USA and United Kingdom as tomato concentrated, but it has also been withdrawn.

Delayed ripening

Fruit ripening occurs under the action of a hormone, ethylene, which is produced by the fruit itself. The introduction of an antisense gene corresponding to the enzyme responsible for ethylene production is able to delay ripening of the fruit by reducing ethylene production. Transgenic tomatoes with this characteristic have already been developed and approved for marketing but they have not been commercialized.

Nutritional value (fatty acids, vitamins, etc.)

In oilseed rape, the composition has been modified in order to produce oil with higher nutritional value. In particular, oilseed rape varieties with higher content in some fatty acids like laurate, mystritate and oleic acid and with lower level of linoleic acid have been developed and commercialized.

Regarding rice, a variety with a higher content in provitamin A has been developed by a public research group. Vitamin A-deficiency is indeed a major nutritional problem in developing countries, with severe consequences such as blindness, vulnerability to infections and even death, especially among young children. Rice enriched in provitamin A has been called "golden rice" because it has a slightly yellow colour. It is now in the last stages before its commercialization[17].

Iron deficiency is also a major nutritional disorder; it is in fact the most common one in the world. It causes anemia, reduced mental and physical capacities and in infants, it can impair intellectual development. In women, iron deficiency can cause deaths during pregnancy as well as hemorrhage and sepsis during childbirth. Rice with higher iron content has been developed by the same group as the one that developed the golden rice. Normally, it should also be commercialized in the near future.

Specific substances production

Plants have always been used to produce particular substances such as fibers, colorants, starch, lipids, paper, etc. With the development of genetic engineering, the diversity of these productions will be considerably enlarged. Some transgenic plants are for example being developed to produce biodegradable plastics.

Regarding medicines, substances like insulin have been produced since a long time by transgenic mico-organisms. But transgenic plants offer an interesting advantage. Indeed, when a protein is produced, it undergoes secondary modifications that can be very different according to the organism. In plant,

[17] For further details, see for example: Potrykus I. (2000) « the "golden rice" tale », Ed. I.K. Vasil, url : http://www.biotech-info.net/GR_tale.html

these secondary modifications are quite similar to what occurs in humans whereas in micro-organims they are not.

Vaccines as well as recombinant plasmatic proteins are already produced in transgenic soya, tobacco, potato and banana.

2.1.4. Environmental traits

The decontamination of industrial sites where important residues of heavy metals such as zinc, cadmium, mercury, copper or lead have been released in the soil is another possible application of transgenic plant. Some plants able to decontaminate soil from heavy metals have been developed, but they are still at an experimental stage.

2.1.5. GURTs-Genetic Use Restriction Technologies (Fig. 2 and Fig. 3 Application, pp. 57 et 58)

Genetic Use Restriction Technologies (GURTs) are a set of technological means allowing the production of transgenic plants with a genetic switch mechanism. Such switch can be used in very different ways, but it has been developed mainly to confer a commercial protection (a form of technological property right) to new varieties of transgenic plants.

The genetic switch mechanism can operate at two different levels. First, it can be applied at the level of the entire variety (Variety-level Genetic Use Restriction Technologies, V-GURTs). In that case, the transgenic plant sawn by the farmer will only produce sterile seeds, so that the farmer is obliged to purchase new seeds every year.

Second, the genetic switch may be applied only to a particular trait (Trait-specific Genetic Use Restriction Technologies, T-GURTs). In such occurrence, only the new transgenic trait (for example insect resistance) would be protected. The first year, the farmer would plant the transgenic seeds that would express the insect resistance trait. Next year however, if he plant the seeds he has kept from last harvest, the plant would not be insect resistant anymore.

The first GURTs ever produced was a V-GURT that became famous after being renamed "Terminator technology" [18]. How has it been constructed and what are the mechanisms of its genetic switch ?

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[18] This technology has been developed jointly by the United States Department of Agriculture and the company Delta & Pine Land, which was later acquired by Monsanto. The NGO RAFI (Rural Advancement Foundation International, now ETC group - Action Group on Erosion, technology and concentration) has dubbed this first V-GURTs "Terminator technology" in order to denounce it as a potential threat for food safety. The Terminator technology is described in US patent No 5,723,765.

To develop a "terminator" plant, three genes are necessary. First, a gene coding for a toxin is introduced into the plant. This gene is under the control of a promoter, which is active in the late stages of embryogenesis, i.e. in the seeds. The gene is separated from the promoter by a blocking sequence preventing the expression of the toxin. However this sequence can be removed by a recombinase, a particular enzyme able to excise a DNA fragment and to reassemble the two ends together.

Second, the gene coding for the recombinase is inserted into the plant. This gene is under the control of a promoter that is maintained inactive by a repressor protein. So, as long as there are repressor proteins, the promoter will be inactive and no recombinase will be synthesized.

Third, the gene coding for the repressor protein is introduced into the plant. This gene is under the control of a constitutive promoter, so that the repressor protein is always expressed.

At this stage, the transgenic plant, with its three transgenes, is normally similar to other plants: no toxin is expressed and its seeds are fertile. This is the stage in which the seeds are produced for commercialization.

However, before commercialization, the seeds are treated with a substance that will interact with the repressor protein and prevent it from binding the repressible promoter. In turn, the repressible promoter will be activated and the recombinase will be expressed. The recombinase then plays its special role i.e. excises the blocking sequence located between the toxin gene and the late embryogenesis promoter. At this stage, the toxin is not yet expressed but it will be so as soon as the late embryogenesis promoter is activated. This will occur once the seed has been sawn, the plant has grown and has formed seeds. It is in those seeds that, eventually, the toxin will be expressed, disrupting the seed's tissues and rendering them sterile.

The Terminator technology is not the only sophisticated mechanism developed to sterilize harvested seeds. Many different V-GURTs exist, but in their principles, they are essentially identical.

As for the T-GURTs, the technical possibilities are even larger and could potentially be applied to other field than commercial protection. For example, T-GURT could be used to achieve a greater specificity in the expression of the resistance to a pathogen. But it has to be acknowledged that, until now, most of the research on T-GURTs has been concentrated on the development of commercial protection techniques.

2.2. WHICH KIND OF GMO HAVE BEEN DEVELOPED ?

The wide range of potential applications of genetic engineering contrasts sharply with the uniformity of transgenic crops sawn and commercialized. Indeed, 99% of all transgenic plants are either herbicide tolerant or insect resistant or both.

Global area of transgenic crops in 2003: 67.7 million hectares

Trait	**Percentage of the total area of transgenic crops sawn in 2003**
Herbicide tolerance	73%
Insect resistance (Bt)	18%
Insect resistance + Herbicide tolerance	8%
Others	1%

(James C., 2003)

There is no simple explanation for this surprising situation and the answer is probably a mixture of economic, social as well as technical factors. We will not discuss the first two factors here, but we will mention some technical points that might be useful to understand the direction taken by transgenic plant research and development.

Depending on the transgenic plant that is being developed, the degree of technical complexity required can vary greatly. It is possible to make a broad distinction between on the one hand single gene traits, i.e. a characteristic controlled by only one gene and on the other hand multiple gene traits, controlled by several genes sometimes in different locations in the genome and possibly having complex interactions with other functions. The technical gap between the development of a transgenic plant with a single gene trait and the development of another with a multiple gene trait is enormous. To date, almost all transgenic plants contain a trait controlled by a single gene. This is the case for herbicide tolerance, for insect resistance as well as for virus resistance and for delayed softening (tomato Flavr Savr). An example of a transgenic plant with a trait controlled by several genes is the transgenic rice enriched with pro-vitamin A (golden rice). In this rice, the genes for the whole biosynthetic pathway of provitamin A have been inserted. This plant has however not been commercialized yet and it is still undergoing further studies. Other multiple genes traits are likely to be even more complex to develop than golden rice: for example nitrogen fixation, drought resistance or salt tolerance that are technically out of reach for the moment.

These few points are worth bearing in mind while considering the potentials of genetic engineering for food and agriculture.

Global area of transgenic crops in 2003 by crop

	Million of hectares	**%**
Soybean	41.4	61
Maize	15.5	23
Cotton	7.2	11
Canola	3.6	5
Potato	<0.1	<1
Squash	<0.1	<1
Papaya	<0.1	<1

(James C., 2003)

Global area of transgenic crops in 2003 by countries and by crops

	Million of hectares	**%**	**Crops**
USA	42.8	63	Soybean, maize, cotton, oilseed rape
Argentina	13.9	21	Soybean, maize, cotton
Canada	4.4	6	Oilseed rape, maize soybean
Brazil *	3	4	Soybean
China	2.8	4	Cotton
South Africa	0.4	1	Maize, soybean, cotton
Australia	0.1	<1	Cotton
India	0.1	<1	Cotton
Romania	> 0.05	<1	Soybean
Uruguay	> 0.05	<1	Soybean, maize
Spain	<0.05	<1	Maize
Mexico	<0.05	<1	Cotton, soybean
Philippines*	<0.05	<1	Maize
Colombia	<0.05	<1	Cotton
Bulgaria	<0.05	<1	Maize
Honduras	<0.05	<1	Maize
Germany	<0.05	<1	Maize
Indonesia	<0.05	<1	Cotton

(James C., 2003)
* Countries that approved planting of GM crop for the first time in 2003

Which GMO have been commercialized in the United States ?

The United States are the most important producer of GMO worldwide accounting for 63 % of the total area of transgenic plants. Not surprisingly, in the US marketplace, many food products contain GMO.

The main transgenic crops found in US food products are soya, maize, oilseed rape and cotton.

Crop	**Transgenic production in the US**	**Is this GMO present in the food ?**
Soya	81% of total US soya production was transgenic in 2003	Transgenic soya is likely to be found in many processed foods. Indeed, many products like oil, flour, lecithin, protein extracts, etc. are soya derivatives.
Maize	40% of total US maize production was transgenic in 2002	Transgenic maize is found in many processed foods. As soya, maize-based ingredients enter the composition of a wide range of foods. Furthermore, there is no separation between conventional and transgenic maize during growth and processing stages.
Oilseed rape	Most US oilseed rape is imported from Canada, where 60% canola is transgenic.	Transgenic oilseed rape is mainly used to produce oil and it is likely to be present in a wide array of foods, from margarines to chocolates, soaps and detergents.
Cotton	71% of the total US cotton production is transgenic	Cotton, apart from being used for textile is also used to produce oil from its seeds. Transgenic cottonseed oil is therefore likely to be found in many processed foods.

(Cornell University, 2004)

The other transgenic plants approved for marketing in the US are potato, papaya, zucchini, tomato, rice, sugar-beet, radicchio, but their presence in food is unlikely.

Papaya	Transgenic papaya are only produced in Hawaii, where they account for more than 50% of total production
Potato	Distribution to farmers stopped in 2001. Not found on the market anymore.
Rice	Herbicide resistant transgenic rice (Aventis Crop science) is waiting approval from EPA (Environment Protection Agency)
Radicchio	A radicchio variety has been approved for breeding in 1997, but it never reached the market since it was withdrawn in 1999 by its producer.
Sugar-beet	Two sugar-beet varieties have been approved in the US but they have not been grown yet, mainly due to farmer's concern over international markets.
Tomato	Only the tomato Flavr Savr has been present on the market from 1995 but it has been retired since 1997.
Zucchini	Different varieties have been marketed, but they have been poorly adopted by farmers.

(Cornell University, 2004)

Which GMO have been commercialized in France ?

In France, three varieties of maize and one variety of tobacco have been authorized for importation, culture and industrial transformation. The tobacco variety however has not been commercialized.

Transgenic crops	**Inserted trait**	**Company**
Maize Bt-176	Insect resistance	Novartis
Maize MON 810	Insect resistance	Monsanto
Maize T-25	Herbicide resistance (phosphinothricin)	AgrEvo (Aventis)
Tobacco "ITB-1000-0X"	Herbicide resistant	Seita

(Ministère de l'Economie, des Finances et de l'Industrie, 2004)

Two other transgenic plants have been approved for industrial transformation only: a maize variety (only for feed use) and a soya variety.

Transgenic crops	Inserted trait	Company
Soya	Herbicide resistance (glyphosate, commercial name Roundup)	Monsanto
Maize Bt-11	Insect resistant and herbicide resistant (phosphinothricin)	Novartis

(Ministère de l'Economie, des Finances et de l'Industrie, 2004)

Which GMO have been commercialized in Switzerland ?

In Switzerland, there are no authorizations to grow transgenic plants for commercial purpose. But three varieties of maize and one variety of soya have been approved for commercialization only.

Transgenic crops	**Inserted trait**	**Company**
Maize Bt-11	Insect resistant	Novartis
Maize MON810	Insect resistant	Monsanto
Maize Bt-176	Insect resistant	Novartis
Soya	Resistance to the herbicide glyphosate (commercial name: Roundup)	Monsanto

(OFEFP, 2004)

These products are authorized either as food or feed products. It is worth noting that as they cannot be grown in Switzerland, these transgenic crops have to be imported. In Switzerland, transgenic foods must be labelled[19], but a threshold of 1% is applied for approved transgenic crops. For unauthorized crops, their presence, even in infinitesimal quantity, is not tolerated.

The two main supermarket chains in Switzerland have refused to sell GMO. Furthermore, to date, there are no transgenic food products submitted to labelling in the Swiss market. If control measures are well applied, the presence of GMO in food products would therefore only result from "tolerated" contaminations under the 1% threshold for authorized crops.

[19] "Ordonnance sur les denrées alimentaires (ODAL)", 1st March 1995, modified 14th June 1999.

Estimated value of the transgenic seed market (1995-2003)

Year	Market value (Million of $)
1995	1
1996	156
1997	858
1998	1970
1999	2947
2000	3044
2001	3839
2002	4000
2003	4500 to 4750

(James C., 2002 and 2003)

III. ENVIRONMENTAL AND SANITARY RISKS OF GMO IN FOOD AND AGRICULTURE

3.1. PRELIMINARY REMARKS

Some preliminary remarks seem to be necessary before starting the discussion about risks. We will consider here all the risks frequently mentioned in the literature associated with transgenic plant whether proved and hypothetical. We will not consider to which extent each risk has been scientifically proven and we will limit ourselves to describe the mechanisms involved. As we will see further on, a risk cannot be considered from a general point of view as it is tightly associated with the situation considered and depends on many elements often strictly contingent, for example the species of plant, the nature of the transgene, where the transgene has been inserted in the genome, where the plant will be sawn, what are the wild related species, what are the cultivated plants in the nearby area, what is the pollen distance diffusion, etc.

As a consequence, the risks associated with transgenic plants should be assessed on a case by case basis only[20]. The only pretence a general classification could have is to signal that a particular risk, for example gene flow to a wild relative with subsequent increase in its weed-like character, is proved to occur in the particular situations studied. Obviously, this is a precious piece of information to bear in mind when analyzing another context and it could be the basis for applying the precautionary principle. However, it cannot be taken as proof that this risk can realize in other situations.

Another point concerns the type of risk taken into account. As mentioned in the title above, only environmental and sanitary negative impacts are considered here, as opposed to economic or social ones. This is a deliberate choice based on two main reasons. Firstly, the topic of this brochure is focused on technical aspects of biotechnology and secondly, economic and social impacts are generally not considered as "risks" from a legal point of view.

Finally, apart from the risks identified, both hypothetical and proven, there are other areas that need to be considered to get the full picture about transgenic plant safety (see chapter IV. Scientific uncertainties, p. 43). These areas need to be highlighted, not merely because they cause some safety concerns, but because we know so little about what is going on there, that it is

[20] All regulatory framework dealing with transgenic crops require risk assessment on a case by case basis before release authorisation.

not possible to make any statement about either safety or risks. A good example of such knowledge blind spot concerns the role of non-coding DNA (junk DNA) in the genome. The present lack of knowledge and data could lead us to miss some very important point. Therefore, it is essential to address these issues and take them into account when making any statement of safety.

3.2. GENERAL RISKS

General risks refer to the potential adverse effects of transgenic plants that cannot be linked to a particular trait. Transgene dissemination for example can occur independently of the character of the transgene introduced. General risks however still need to be assessed on a case by case basis and it cannot be said in any way that the same risk will be associated with all transgenic plants.

3.2.1. Transgene dissemination

The transgene(s) present in a transgenic plant could be disseminated in different ways.

Transgene dissemination via pollen

Transgenes can be disseminated to nearby fields of the same crop or to wild relatives by sexual cross (outcrossing) through contact with the transgenic pollen.

Three main conditions are required for this to occur.

- Firstly, it should be possible for the crop to be fertilized by the pollen of other individuals of the same species or by the pollen of related plants. Indeed, some plants, like tomato, soya, wheat and oat are autofertilized i.e. they use their own pollen to fertilize their egg. Other plants, like maize, beetroot or oilseed rape can be fertilized by the pollen of other plants of the same species. These latter plants are therefore the most likely to form hybrids with non-GMO individuals of the same species as well as with wild relatives.
- Secondly, plants of the same species or related plants with which the transgenic plant can cross and form fertile hybrids must be present in the same area.
- And thirdly the pollen must be able to reach these plants.

According to these conditions, outcrossing of transgenic plants with non-GMO fields of the same crop is likely to be a problem at least for maize, beetroots and oilseed rape. The reduction of such risk requires the settelment of minimal distances between GMO and non-GMO fields as well as a careful

monitoring of all the area. However, insects, birds and climatic variations are parameters very difficult to control but very likely to play an important role in pollen dissemination. An effective prevention will terefore be difficult to achieve when GMO and non-GMO of the same crop are present in the same area.

Concerning the presence of wild relatives, it is worth considering the biodiversity centre of the crop i.e. its geographic origin (see table below and Fig. 1 Risk, p. 59). Indeed, when a plant is cultivated far away from its biodiversity centre, it is likely that no wild relatives will be growing in that region. This is the case for example of maize whose biodiversity centre is in Mexico and Central America and for which there are no wild relatives in Europe.

Main crops	**Origin of the crop**
Barley	Middle East
Canola *	Europe, USA, Canada
Cotton	America (Mexico, Equador)
Maize *	Mexico and Central America
Oat	Kasoian See (Middle East)
Oilseed Rape *	North Mediterranean
Potato *	Andine region
Rice *	South East Asia
Rye	Kaspian see
Soya *	East Asia
Sorghum	Central Africa
Sunflower	North America
Tobacco *	America
Tomato *	South America (coastal region of Peru)
Wheat *	Middle East

* Transgenic varieties already developed.

A potential reduction of the risk of transgene dissemination to other plants has been proposed by transforming chloroplasts instead of nuclear DNA (see: Fig. 2 Risks, p. 60). Transgenic plants of that type are called transplastomic. In many plants, chloroplasts are indeed inherited maternally i.e. there are no chloroplasts in pollen[21]. The transgene propagation would thus be suppressed, as no transgene would be present in the pollen. Another means

[21] This is true for the main cultivated crops, but not for gymnosperm plants for example (pine, Christmas tree, etc.).

of reducing that risk would be to have male sterile plants i.e. plants that do not produce pollen, the male gamete in plants.

Some recent experimental data

Studies have shown that in the case of oilseed rape in Europe, transgene dissemination (herbicide resistance gene) to nearby fields of the same species can occur and outcrossing with wild relatives seems also to be possible, even if only at low frequencies (Chèvre *et al.*, 1998). Transgene dissemination has also been observed between transgenic and non-transgenic beetroots and between transgenic beetroots and its wild relatives. Furthermore, outcrossing has been observed between transgenic and non-transgenic maize (Gasquez, 1998), but not with wild relatives, as there are none in Europe. In Mexico however, which is the center of biodiversity for maize, local varieties seems to have been already contaminated with GM maize (BRIDGES, 2003)

Transgene dissemination to soil or gut bacteria

Another way of transgene dissemination is the transfer to bacteria in the soil or in the intestine. In these cases, the mechanism involved is called transformation. Transformation is the process by which bacteria take up free DNA from the environment and incorporate it into their genome. The main conditions required for a transformation to occur are:

Persistence and availability of free DNA from the transgenic plant and in particular of the transgene(s) in the environment;

Presence of competent bacteria - competence is the ability of certain bacteria, under particular conditions, to take up free DNA from the environment. Competence is encountered only in a small portion of all bacteria present in the soil or in the intestine.

Recent experimental data

Experiments have shown that under optimized laboratory conditions, the transgene of a plant could be transferred to soil bacteria, but this has not been observed under natural conditions (Schluter *et al.,* 1995, Gebhard and Smalla, 1998, De Vries *et al.* 2001). As for the transfer of transgenic DNA to gut bacteria, very few data are available and theoretical probability of such transfer is very low (Thomson, 2001). However, a recent study has shown evidence that the transgenic DNA material from crops can in fact be transferred to human gut bacteria (UK Food Standards Agency, 2002). Furthermore, during in vitro experiments with human saliva, transformation of naturally competent oral bacteria with plasmid DNA has been observed (Mercer *et al.* 1999). These results suggest that such transformation might also be possible in vivo, but at frequencies that still need to be determined by further experiments.

Transgene dissemination through seeds

Transgene can also be disseminated through seeds fallen from the harvested plants and growing the subsequent years. This has been observed with different plants, in particular with oilseed rape, with beetroot and with wheat. The seeds are easily removed from those plants sand can fall and survive in the soil until the following year.

3.2.2. Fitter and more competitive hybrids can become new weeds

Another consequence of transgene dissemination could be, apart from the genetic contamination itself, the development new weeds, i.e. of fitter and more competitive hybrids (the result of the cross between the transgenic crop and a wild relative) compared to the rest of the plant population. A hybrid with such characteristics would be likely to develop and reproduce better than other plants and thus cause an imbalance in the ecosystem. It might even be able to replace other plants and become a new weed for agriculture. Such hybrid would be considered to have a « reproductive advantage ».

What is a weed?

The Oxford English Dictionary defines a *weed* as:

" A herbaceous plant not valued for use or beauty, growing wild and rank, and regarded as cumbering the ground or hindering the growth of superior vegetation.."

Technically *any* plant could be considered to be a weed, as long as it is growing where people don't want it to grow. But some plants are more likely to become weeds than others. There are certain characteristics that make some plants more troublesome than others, and much more likely to grow where they are not wanted. These traits that tend to make a plant more "weedy" include (among others):

- long-lived seeds that don't all germinate at the same time
- rapid seedling growth
- high tolerance to changes in environment, and ability to grow in different environments
- competes aggressively with other plants
- produces new seeds continuously
- produces a large number of seeds
- can disperse its seeds long distances

Although weeds don't always have all of these characteristics, most weeds do have some combination of them. Weeds also tend to be plants that are happy growing without help in areas disturbed by man (as opposed to natural habitats), such as gardens, fields, along highways, and in vacant lots.

However, what is referred to as « reproductive advantage » and that confer its weed-like trait to a plant is in fact a very complex characteristic, which depends on multiple factors, the most important two being:

- The type of transgene inserted and the character it codes for. There are indeed characters that would probably not confer any reproductive advantage, like a higher content in provitamin A for example. On the contrary, a gene for frost resistance, a gene lengthening the life period of seeds in soil, a gene modifying the flowering period, the resistance to low temperatures, the capacity to fix nitrogen, etc. would be likely to influence positively the development of this plant population.
- The fact that the plant has already an invading tendency (weed-like characteristics) will increase the likelihood of enhancing its reproductive advantage when an additional gene is introduced. On the contrary, in a plant with scarce capacity to survive in a natural environment, the addition of a single gene influencing positively the reproductive capacity will not be enough to create a plant with a reproductive advantage.

A well-known example of a transgene potentially conferring a reproductive advantage is the herbicide resistance gene. This case is analysed hereafter in the section 3.3. "Particular risks".

3.2.3. Unexpected features and genetic instability

During plant transformation, the transgene inserts randomly in the genome[22]. So neither the locus nor the number of copies of the transgene can be known in advance. However, the locus where the transgene is inserted might have an influence on the expression of other genes and on the stability of the genome.

For example, if the gene inserts inside the promoter of another gene that activates the expression of a repressor protein, the repressor protein would not be expressed anymore. In turn, the gene the repressor protein is in charge to inhibit would be over-expressed with several possible consequence such as modification of the nutritional value, toxicity if the over-expressed protein is toxic, etc.

The consequences of random insertion can thus vary greatly; from having no effect as in the case where the transgene is inserted in a non-coding region (a DNA region where there are no genes and thus presumably less interference problems with their expression)[23] to the over-expression of a toxic substance.

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[22] See above: 1.5. "How does the transgene integrate into the plant genome ?", p.13

[23] The fact that a transgene insertion in a non-coding region would have no influence on the expression and stability of the rest of the genome is in reality an assumption based on our ignorance of the function of 95% of all DNA in the genome, the so-called "junk DNA". Indeed, as we haven't found yet what is the role of this junk DNA, it is often assumed that it has no function at all. The importance of this topic is addressed hereafter, p. 43

To reduce the risks linked to randomly inserted transgene, a careful analysis of the transgenic plants should permit the elimination of the plants with abnormal characteristics. However, some locus-related effects might not be visible immediately and be revealed only once the plant is grown in open-air for example or appear only under some particular climatic conditions. At present, the extent of such consequences can only be assessed through field trials, thus emphasizing the importance of these open-air experiments to fully determine the characteristics of transgenic plants.

Using transplastomic plants would be another way to avoid this problem[24]. Indeed, in chloroplasts, it is possible to decide where to introduce the transgene and therefore to chose a site well-known for its stability and lack of disrupting effect.

3.2.4. Food allergy

Food allergy problems are not specific to transgenic plants. Allergies are caused by some substances - usually proteins or polypeptides - that can be recognized as antigens by a fraction of the population and thus induce an immunological response in these persons. The allergic reaction can vary greatly in seriousness and localisation (digestive, respiratory, skin, etc.). Allergens are present in normal foods and most allergies (90% in the United-States) are caused by the following food classes: peanut and other nuts (walnut, hazelnut, etc.), soya, milk, egg, fish, shellfish and wheat.

The risk of transgenic plants to cause allergies is due to the presence of a new protein in a food that did not contain such substance originally. Indeed, when the transgene inserted into the plant codes for a known allergenic protein, it is very likely that it will also have an allergenic effect in the transgenic plant. This is what happened with the transgenic soya of the firm Pioneer Hi-Bred that contained the albumin protein 2S from Brazil nut, a known allergen.

The risk of such allergenic effect can be reduced significantly, even if not completely, by carefully analyzing the properties of the transgene. The physico-chemical properties of the protein coded by the transgene, its 3D structure, the sequence homologies with other known allergens, etc. are all means to predict the allergenic character of a protein.

However, even if it can be reduced, the risk of a transgenic food to cause allergic reactions in a fraction of the population cannot be excluded. This is especially true in the case where the transgene comes from an organism that is not usually eaten and thus not index-linked in any database recording allergens.

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[24] See above: 3.2.1. "Transgene dissemination" p. 33 and Fig. 2 Risks, p. 60.

3.3. PARTICULAR RISKS

Particular risks refer to the adverse effects that might be associated with a trait in particular as opposed to the section "General risks" where the risks are not directly linked to the trait introduced.

3.3.1. Herbicide resistance

As mentioned above, herbicide resistance is one of the traits that might confer a reproductive advantage to a plant. Indeed, the hybrid resulting from a cross between a wild relative (or a non-GMO culture) and the herbicide tolerant transgenic plant will be able to grow in presence of the herbicide and this could lead to an increase of its weed-like character. However, not all the plants resulting from such a cross will form a new weed. Only the wild relatives that already had weed-like characteristics would be likely to become a weed. Oilseed rape is a good example of crop for which this could happen. In Europe, several wild relatives of oilseed rape grow naturally (wild radish, charlock, white mustard, black mustard, etc.) and they all have weed-like characteristics *per se*.

Another risk of transgenic plants resistant to herbicide is due to the potential toxicity of the herbicide itself, independently of the genetic modification. This includes the impact on human health if food with herbicide residues is ingested as well as the impact on the fauna feeding on the treated plants. This question is obviously not directly linked to the transgenic plant, but it has to be addressed as the transgenic plant has been developed with the sole purpose of being planted using the herbicide.

Most herbicide tolerant transgenic plants are resistant either to glyphosate or to phosphinothricin. These two herbicides have been used long before their application to transgenic plants, but never directly on food products. Their use on herbicide resistant plants is therefore a new application that requires further testing. The degradation substances produced by transgenic plants treated with the herbicide might indeed be different from those expected and from those already encountered in a traditional context.

If the degradation products induced by the transgenes might be predicted to a certain extend, the secondary metabolite that might result from modifications induced by the random introduction of the transgene cannot be predicted in any way. This means that only direct testing on the transgenic plant treated with the herbicide as well as toxicological and nutrition tests on mammals would be able to fulls assess the sanitary risks linked these plants.

Recent experimental data

Farm scale evaluations of genetically modified herbicide-tolerant crops have been recently conducted in the UK on a period of four years. The purpose of this study was to evaluate the impact of the weed management practices associated with three different herbicide resistant crops (maize, beetroot and spring-sown oilseed rape) on the farmland wildlife, compared with the impact of weed control in non-GM crops. The conclusions of this study were the following. Herbicide-resistant maize had no adverse effect on farmland biodiversity compared to conventionally managed maize. On the contrary, both beetroot and spring-sown oilseed rape had adverse effects on arable weed populations compared with conventionally managed beetroot and spring-sown oilseed rape. It is worth noting however, that the standard of comparison (i.e. the conventional practice of weed management) had a major influence on the results of this study. Indeed, if the standard is negative (i.e. the conventional practice of weed management has a negative impact on farmland wildlife), then the comparison with herbicide resistant crops is likely to be positive and *vice versa*. (Squire *et al*., 2003; Champion *et al*., 2003; Heard *et al*., 2003a; Heard *et al*. 2003b; Brooks *et al*., 2003; Haughton *et al*., 2003; Roy *et al*., 2003; Hawes *et al*., 2003)

3.3.2. Insects resistance (Bt Gene)

A major risk linked to transgenic *Bt* crops, in addition to the general risks mentioned above, is the development of resistance among the target insects. Resistance is a phenomenon that tends to appear whenever there is a selection pressure on a population. The greater the pressure, the quicker the resistance appears. In the case of the *Bt* transgenic plants already commercialised, the *Bt* gene is always under the control of a constitutive promoter. The *Bt* toxin is thus always expressed and in all plant tissues. The resulting selection pressure is obviously very high and especially more important than when the *Bt* toxin is sprayed on the crop.

If no resistance management strategies such as refuge areas are implemented, it is likely that *Bt* transgenic plants will lead to the development of resistance among the target insect populations. This means that the *Bt* toxin could be turned inefficient, not only in transgenic plants, but also for organic farming where *Bt* toxin is an important tool to fight insect pests.

Another risk of *Bt* transgenic plants is the possible adverse effects they could have on non-target or beneficial insects. Although *Bt* toxin is a narrow-spectrum pesticide, some experiments have observed unexpected impacts on non-target insects (see hereafter "Recent experimental data").

Recent experimental data

In 1999, a laboratory study showed that Bt pollen had a negative impact of on monarch butterfly larvae (Danaus plexippus) (Losey *et al.*, 1999), a non-target lepidopterian. Next year, another study reported a significantly greater mortality of monarch butterfly larvae feeded in laboratory with foliage orginating from the field and naturally contaminated with Bt pollen (Jesse *et al.* 2000). The first research under field conditions examined another non-target lepidoterian, the black swallowtail (Papilio polyxenes) and no detrimental effect of Bt pollen from maize MON810 was observed (Wraight *et al.*, 2000). However, the same study examined the effect in laboratory of Bt pollen from maize Bt-176 and found it causes a greater mortality on black swallowtails. A serie of reserch conducted by the University of Illinois[25] confirmed these laboratory results and showed a significant reduction in growth rate of black swallowtails larvae exposed to pollen from maize Bt-176 (Zangerl *et al.*, 2001). It also proved the negative impact of maize Bt-176 pollen on monach butterfly larvae whereas maize Bt-11 and MON810 pollen had no significant impact on this lepidopterian (Stanley-Horn *et al.*, 2001). It is worth noting that maize Bt-176 expresses the Bt-toxin at a higher concentration (40 times more) than maize MON810 but that fortunately, maize Bt-176 accounts only for less than 2 % of all maize acreage in 2000 in the USA (Hellmich et Siegfried, 2001).

3.3.3. Virus resistance

One of the risks linked to virus resistant transgenic plants is, as in any fight against a pest through a single mechanism, the development of resistance among the target viruses. Another risk is transgene dissemination, but this is a risk common to all transgenic plants.

The risk specific to virus resistant plants containing a part of a capside gene is recombination. Recombination is a mechanism, common in all living organisms (see: 1.5. "How does the transgene integrate into the plant genome ?", p. 13) by which sequences of DNA are exchanged between two organisms. When an invading virus - different from the target virus the transgenic plant is resistant to - enters the transgenic plant cells, an exchange of genetic material is possible between the invading virus and the portion of capside gene inserted into the plant. New viruses could therefore be produced (see Fig. 3 Risks, p. 61).

It is worth noting that recombination is also a phenomenon occurring in non-GMO plants, when they are infected by two different virus strains. The question is thus to evaluate if recombination is more frequent in transgenic

[25] Six articles have been published in 2001 in the Proceedings of the National Academy of Science, Vol. 98, pp. 11908-11942 by the Departement of Entomology of the University of Illinois.

plants than in conventional crops and if these recombinated viruses are more virulent than the initial ones.

3.3.4. Antibiotic resistance gene as selection marker

The use of antibiotic resistance genes as selection markers for transgenic plant production is a very controversial issue. As already mentionned (see: 1.6. "How to select for the transformed cells ?", p. 14), the presence of an antibiotic resistance gene in the transgenic plant is not a desired trait but a technical constraint inherent to the process of transgenic plant development.

The risks of using antibiotic resistance genes are related to the possible consequences this could have on the development of bacterial strains resistant to antibiotic used in human beings to fight infectious diseases. As antibiotic resistance in pathogen bacteria is a serious and increasing public health problem, there is great concern that antibiotic resistance marker genes used in transgenic plants might contribute to this phenomenon.

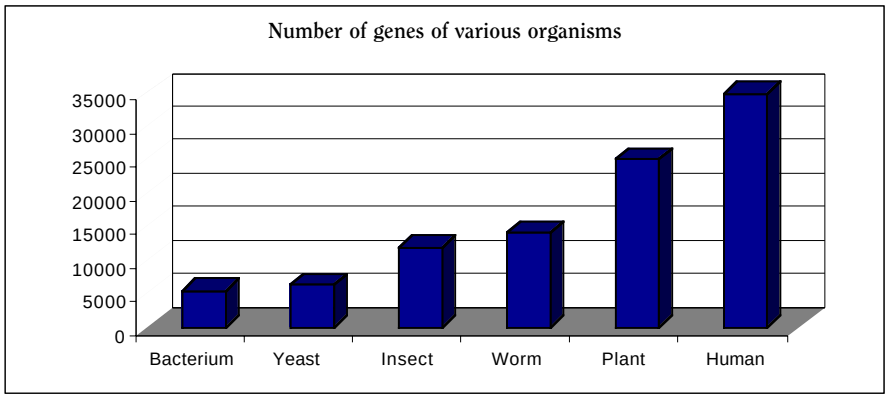
Most scientists agree that the main problem in the development of antibiotic resistance is not the marker gene used in transgenic plants, but rather the excessive use of antibiotics in medicine and agriculture. However, as marker genes other than antibiotic resistance are available, it is usually recommended to use those ones[26].

[26] For more details about the question of antibiotic markers, see: Bordogna Petriccione B. (2003) « Développement des résistances aux antibiotiques: quel rôle pour les plantes transgéniques », RIBios, url:http://www.ribios.ch/documents/resistances_antibiotiques.pdf. See also : Bordogna Petriccione B. (2003) «Plantes transgéniques: quelles alternatives aux gènes marqueurs de résistance aux antibiotiques ? », RIBios, url: http://www.ribios.ch/documents/marqueurs_alternatifs.pdf

IV. SCIENTIFIC UNCERTAINTIES

4.1. JUNK DNA

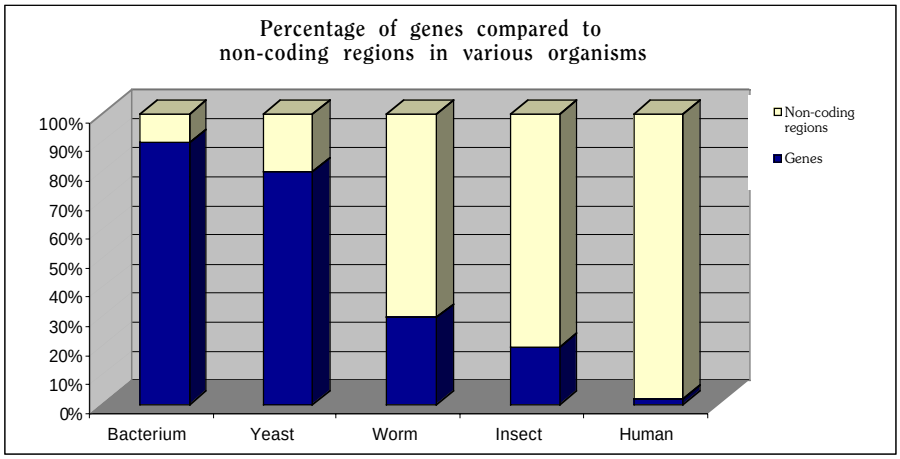
Genes represent only 2-5% of the whole DNA present in mammals. Regulatory sequences and introns account for another 2%. But very little is known about the possible function of the 93-96% DNA left. As the role of this DNA has not been discovered yet, the assumption that it might have no real function is expressed in the name it has been given i.e. "junk DNA". But in reality, there is no proof that this DNA is actually "junk" and the on-going discoveries about the complexity of the genome regulation (gene silencing, RNA interference, genome instability, etc.) could well indicate otherwise. Considering the overwhelming presence of junk DNA in all genomes, and in particular in those of higher organisms (see graphics hereafter), we are indeed forced to acknowledge the fact that we are lacking some very important knowledge in this field.



This aspect is of great significance for genetic engineering in general and for transgenic plants in particular. Indeed, should a role be effectively found for this junk DNA, it would change the assumption currently made that a transgene inserted in such a DNA region would cause no alteration in other functions of the organism. This would mean for example that when developing a transgenic plant, the insertion of the transgene, apart from the new trait it brings, could also cause other modifications in the normal functions of the recipient organism. The fact that a transgenic plant shows no obvious disruption of a coding or regulatory region would no longer be sufficient to assume that the plant's full functionality is preserved.

It is necessary to recognize the uncertainty regarding the potential role of junk DNA and to take it into account when trying to identify the risks of transgenic

plants. Further researches would also be needed in order to try reducing this gap in our current knowledge.



4.2. GENE SILENCING

Gene silencing has been briefly mentioned in the section "Virus resistance" (p.19) but as it is a very good illustration of the role "junk DNA" sequences might play in gene regulation and expression, it seems worth examine this aspect a little further.

With gene silencing, we are facing very complex and subtle interactions between DNA (transcriptional gene silencing) or RNA (post-transcriptional) sequences based on the homology between these sequences, their place into the genome, the possible modifications of their chemical structure, etc. These interactions may stop the expression of a gene or lead to the degradation of its transcription products (RNA), thus "silencing" the gene as no proteins are produced anymore.

In transgenic plants, the introduction of a transgene might be subject to gene silencing due to the presence somewhere in the "junk DNA" of sequences homologue to the transgene. Other parameters such as the place where the transgene is inserted seems also to have an influence on a possible silencing.

An example of how gene silencing might interfere with transgenic plant production has been reported with oilseed rape resistant to herbicide (Al-Kaff *et al.*, 2000). The herbicide resistance gene (the transgene) introduced in this oilseed rape was under the control of the 35S promoter, which is in fact a sequence of the cauliflower mosaic virus. The transgenic oilseed rape was behaving as expected (i.e. it was herbicide resistant) until it has been infected

with the cauliflower mosaic virus. At that point, the 35S promoter sequence started to interfere with the virus, leading to two different consequences. Firstly, the oilseed rape became resistant to the cauliflower mosaic virus (the replication of the virus has been blocked, as in virus resistant transgenic plants. See above: "Virus resistance" p.19). Secondly, the herbicide resistance gene under the control of the 35S promoter was no longer expressed: it was silenced.

Gene silencing is only one visible example of the multiple and complex interactions occurring at different levels to control gene expression and regulation. It should therefore encourage us to consider the genome in its full complexity and to go beyond the far too simplistic view that usually prevails while considering the safety of transgenic plants.

4.3. LONG TERM EFFECTS ON THE ENVIRONMENT AND COMPLEX INTERACTION CHAINS

The long-term effects of transgenic plants and their complex interactions with the environment have not been extensively documented to date (Wolfenbarger and Phifer, 2000).

The study of complex interactions between transgenic plants and the environment is a real challenge given that the knowledge available at present is very limited. Some studies of tri-trophic interactions (Bt plant - insect - predator) have been carried out (Birch *et al.*, 1997; Birch *et al.*, 1999). In addition, the potential adverse effects of Bt plants on beneficial insects (Losey *et al.*, 1999; Saxena *et al.*, 1999; Hilbeck *et al.*, 1998a; Hilbeck *et al.*, 1998b; Wraight *et al.*, 2000; Jesse et Obrycki, 2000; Zangerl *et al.*, 2001; Oberhauser *et al.*, 2001; Pleasants *et al.*, 2001; Hellmich *et al.*, 2001; Stanley-Horn *et al.*, 2001; Sears *et al.*, 2001; Losey *et al.*, 2002) and the possibility of transgene dissemination to soil bacteria have also been investigated to some extent. However, more complex and less obvious interactions have not been studied yet.

Regarding potential long-term adverse effects, it has to be emphasized that, given their very nature, there will necessarily be a delay their identification. Furthermore, such risk identification would require specific long-term monitoring strategies in order to allow an early detection of potential adverse effects.

The combination of long-term effects and complex environmental interactions constitute a real challenge for the full risk assessment of transgenic plants. Of course, further researches need to be carried out but it is still unclear whether the scientific uncertainties in this field can be significantly reduced given the technical means presently available.

4.4. HUMAN HEALTH

Regarding potential adverse effects of transgenic plants on human health, it is often argued that millions of Americans are eating GMO every day and this has not killed anybody yet. This statement aims at showing that there is no evidence of harm occurring as a result of GMO consumption. However this cannot in any way be considered a proof of safety. Indeed, "no evidence of harm" is not to be interpreted as "evidence of no harm". The first relates to a lack of research and data about the possible occurrence of an adverse effect whereas the latter is concerned with an extensive research and monitoring plan which proves that GMO can be consumed without causing prejudice to health.

It has to be stressed that, given the current lack of a monitoring strategy, any unexpected adverse effects of GMO consumption on human health would need to be extremely serious in order to be detectable (Butler et Reichhardt, 1999). There are indeed no records kept of who is eating what, no post-market monitoring and no channel segregation between GM crops and conventional crops.

Recent experimental data

Regarding experimental data, only few long-term nutritional studies have been carried out on animals in laboratory. Furthermore, some experiments have shown unexpected effects that could have implications for GM food. For instance, in the case of mice fed with foreign DNA (M13 bacteriophage), not all DNA was digested, as it was commonly thought to be, and DNA fragments passed through the intestinal wall and reached blood cells and several other organs. Some fragments were also covalently linked to the mouse DNA (Schubbert *et al.*, 1997). Further studies showed that foreign DNA fed to pregnant mice had crossed the placental barrier and was found in different organs of the fetus and new born animal (Schubbert *et al.*, 1998).

Furthermore, studies on rats fed with GM potatoes containing a lectin gene under the control of CaMV35S promoter showed variable modifications of the gastrointestinal tract (Ewen et Pusztai, 1999). According to the authors, these effects could be linked both to the lectin gene, to other parts of the construct[27] or to the transgene insertion site. It has also be shown recently that human oral bacteria can be transformed by free DNA (Mercer *et al.*, 1999) and that gene transfer from GM food to certain human gut bacteria can occur (UK Food Standards Agency, 2002).

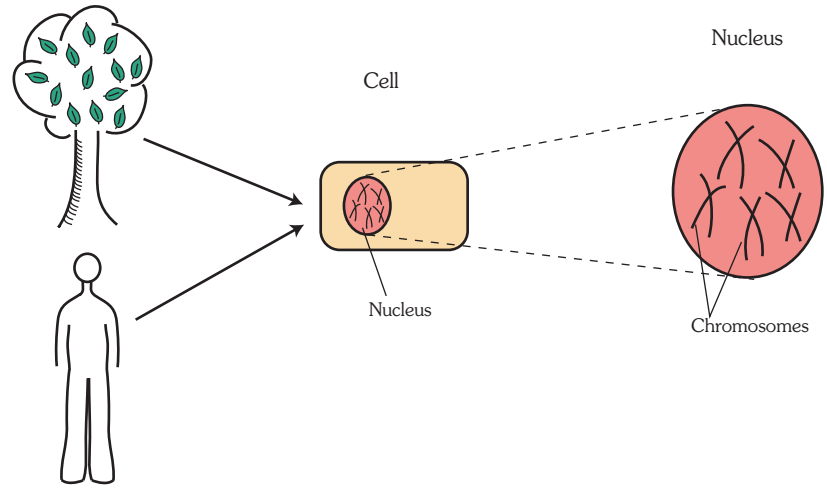
[27] It is worth noting that the destabilizing potential of the CaMV35S promoter on the genome is subject to controversy. As this promoter has been used in almost all transgenic plant produced until now, this particular uncertainty is of great concern about the safety of GM crops. For detail on this matter, see:
Cummins J. (2000) "Hazardous CaMV promoter?", Nature Biotechnology, Vol. 18, p. 363.
Warrington J. A. and Mahadevappa M. (2000) "Integrated pararetroviral sequences", Nature Biotechnology, Vol. 18, p. 579.

It is to be noted that most of these results cannot be applied directly to transgenic plants as they have mainly been performed under optimized experimental conditions that are very different from *in vivo* conditions. In addition, as for all scientific experiments, they also need to be further supported and critically reviewed. However, they all highlight a very important point: our theoretical framework about the fate and behaviour of ingested DNA is likely to be over-simplistic.

Fig. 1 Intro

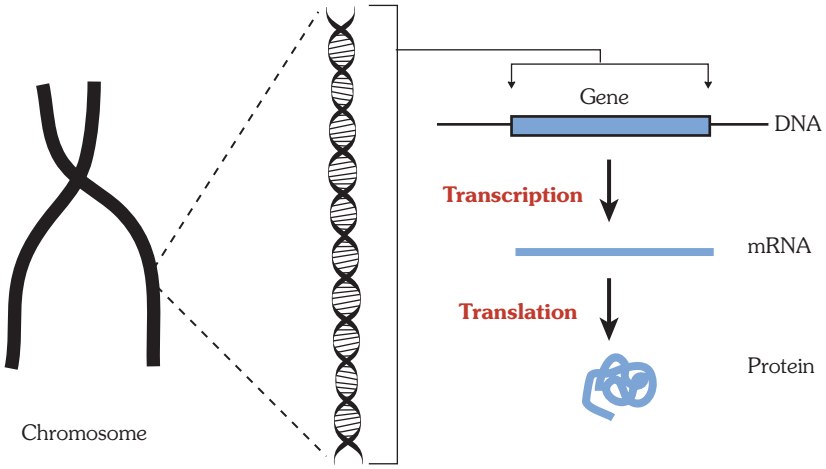
What are cells, nucleus, chromosomes and proteins ?

Multicellular organisms



What are chromosomes, DNA and proteins ?

The DNA of all living organisms is composed of the same phyical and chemical elements. It is this similarity that enabled biologists to transfer genes from one organism to another.



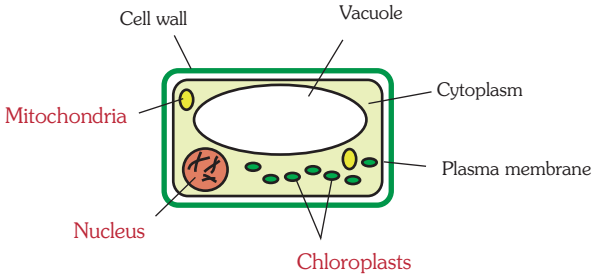
DNA (DesoxyriboNucleic Acid)

Fig. 2 Intro

Where is DNA ?

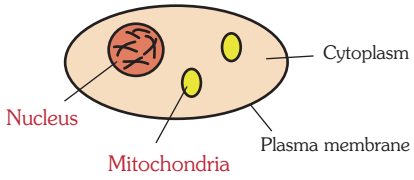
Plant cell (eukaryote, i.e. with nucleus)

In plant cells, there is DNA:
- in the nucleus
- in mitochondria
- in chloroplasts



Animal cell (eukaryote, i.e. with nucleus)

Animal cells contain DNA:
- in the nucleus and
- in mitochondria



Bacteria (prokaryotes, i.e. without nucleus)

In the cytoplasm, bacteria have:
- chromosome DNA
- often also plasmid DNA (very small circular DNA)

Bacteria do not have nucleus.

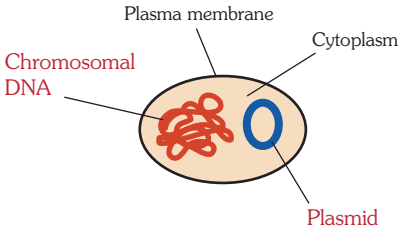


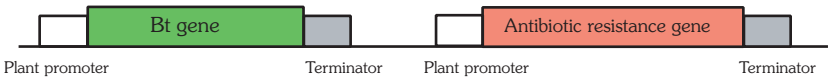
Fig. 3 Intro

What do we need to do a transgenic plant ?

1. The DNA sequence to transfer, that includes:

a) the selected gene (for example Bt gene for insect resistance)
b) a marker gene (for example antibiotic resistance or herbicide resistance gene)
c) two eukaryotic promoters (i.e. actif in the plant) one for the selected gene and one for the marker gene.
d) two terminators, one for the selected gene and one for the marker gene

DNA sequence to be inserted in the plant genome:



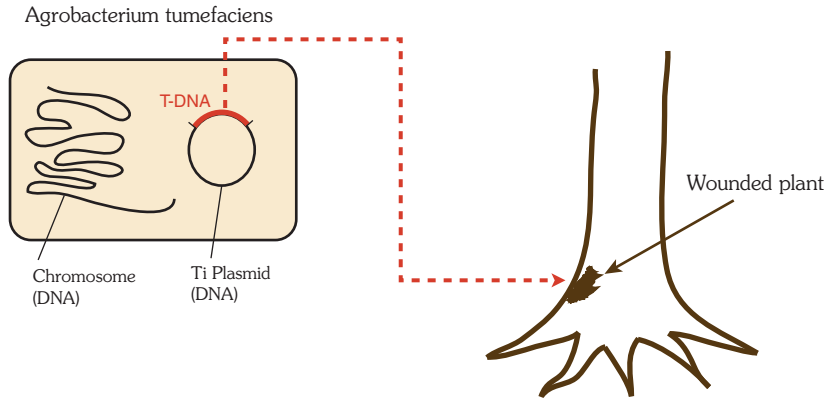
2. A technical tool for transformation that can be either:

a) a bacterium called Agrobacterium tumefaciens
b) gold or tungsten particles coated with DNA and shooted into the plant nucleus (DNA shotgun)
c) a technique using electric shocks (electroporation)

Fig. 4 Intro

How to transform a plant with the bacterium *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is naturally able to transfer its T-DNA into the cells of a wounded plant, inducing the formation of a tumor disease called Crown Gall Tumor



This natural capacity of Agrobacterium to transform plant cells is used in genetic engineering to produce transgenic plants. For this prupose, the T-DNA is excised and replaced by the genes we want to transfer.

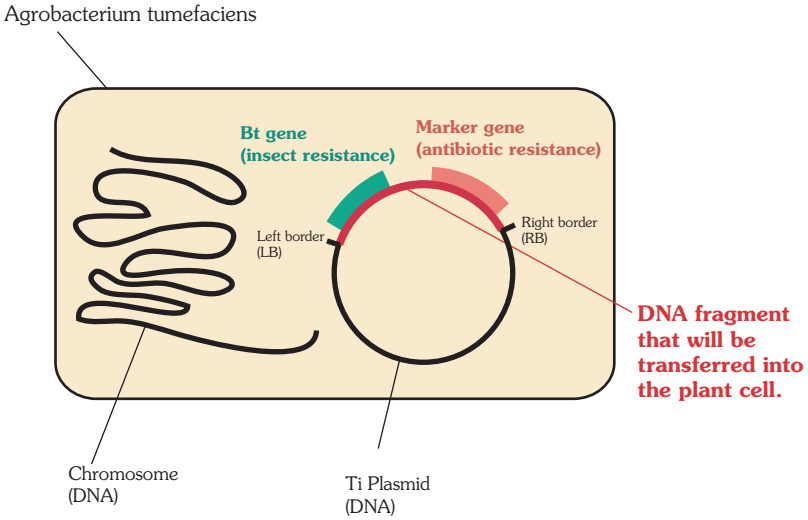
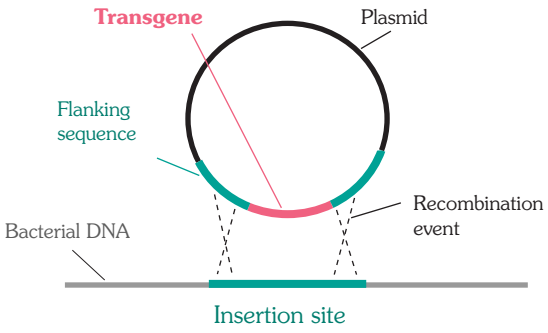


Fig. 5 Intro

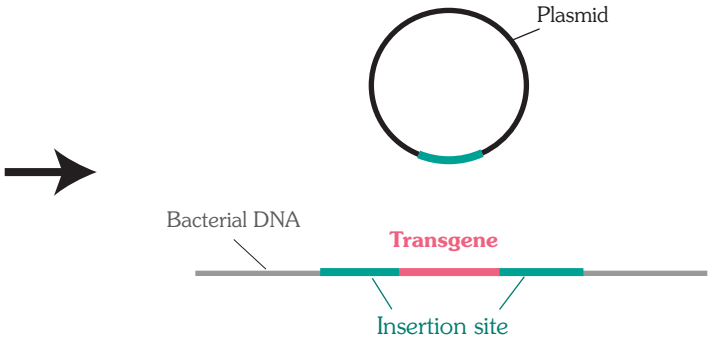
How does the transgene integrate into the plant genome ? (1)

Homologue recombination

Homologue recombination works well in bacteria but very poorly in plants and animals.



The transgene can be integrated into a precise locus: one only has to flank the transgene with sequences similar to that of the insertion site desired.



Mediating two recombination events between the flanking sequences on the plasmid and their homologue in the bacterial DNA, the transgene has been inserted into the desired locus.

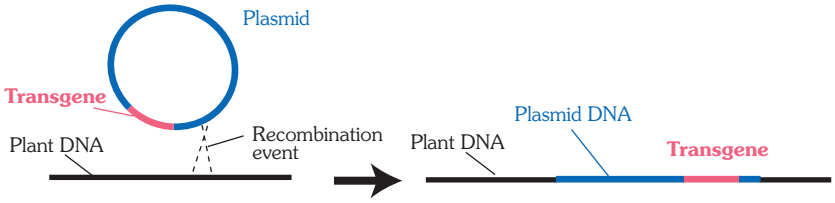
Fig. 6 Intro

How does the transgene integrate into the plant genome ? (2)

Heterologue recombination

In eukaryotic cells (plants and animals) recombination occurs randomly in the genome, between two DNA sequences without sequence homology.

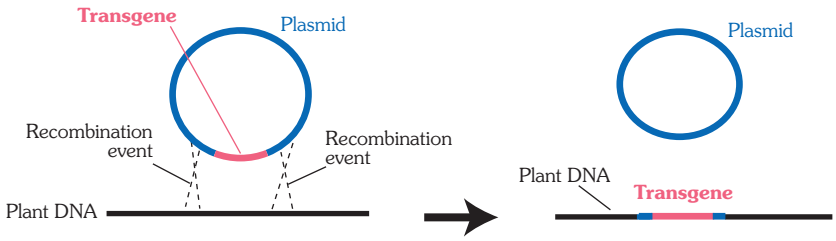
One recombination event



The recombination event occurs randomly in the genome with an unrelated DNA sequence

The entire plasmid is intergrated into the plant genome. Most heterologous recombinations occur in this way.

Two recombination events:



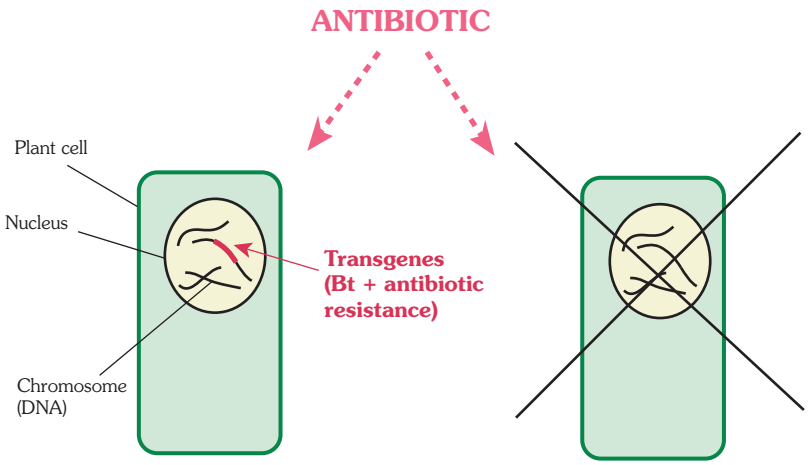
In some rare cases, two recombination events occur between the plasmid and unrelated sequences in the plant genome.

Only the DNA sequence between the two recombination events is integrated randomly into the plant genome.

The occurrance of this recombination scheme is rare.

Fig. 7 Intro
How to select for the transformed cells ?

The application of antibiotic will select the transformed cells that contain the antibiotic resistance gene; the non-transformed cells will die.



YES

Transformed plant cell : contains the transgene

NO

Non-transformed plant cell: does not contain the transgene

Regeneration

Whole transgenic plants are then regenerated from the transformed cells

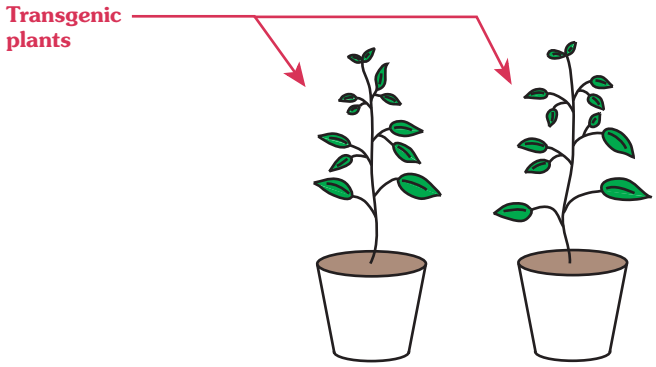
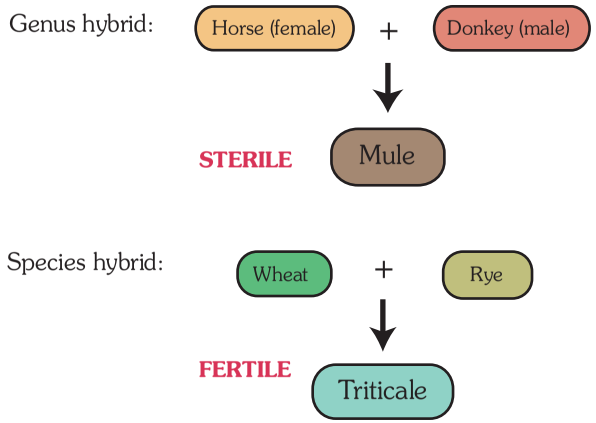


Fig. 8 Intro
What is a hybrid ?

There are mainly two types of hybrids:
1. Genus and species hybrids
2. F1 hybrids

1. Genus and species hybrids

Genus hybrids and species hybrids are the result of a cross between two different genera or two different species respectively. Species and genus hybrids are often sterile, like in horse and donkey cross. But there are exceptions like triticale (the result of wheat and rye cross) or in wild hog (the result of wild boar and domestic pig cross).



2. F1 hybrids

F1 hybrids are the result of a cross between two vartieties of the same species. F1 hybrids are always fertile.

F1 hybrid in self-pollinated plants:

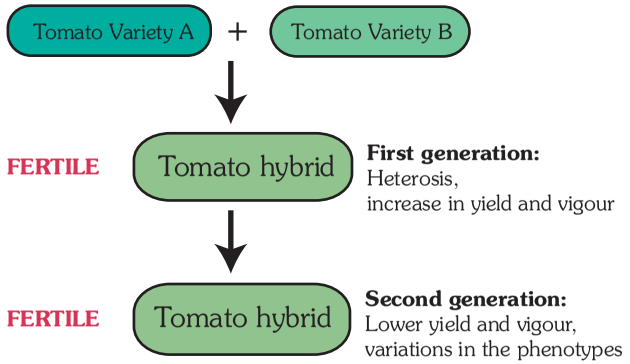


Fig. 1 Application

Virus resistant transgenic plants

To do a plant resistant to the Virus A, a portion of the gene coding for the coat-protein of this virus is inserted into the plant. This portion of coat-protein gene from Virus A is transcribed into RNA by the plant, which is consequently turned resistant to Virus A. This resistance mechanism is called cross protection and is probably due to RNA interferences into the life cycle of the virus.

Cell of a transgenic plant resistant to Virus A (before infection):

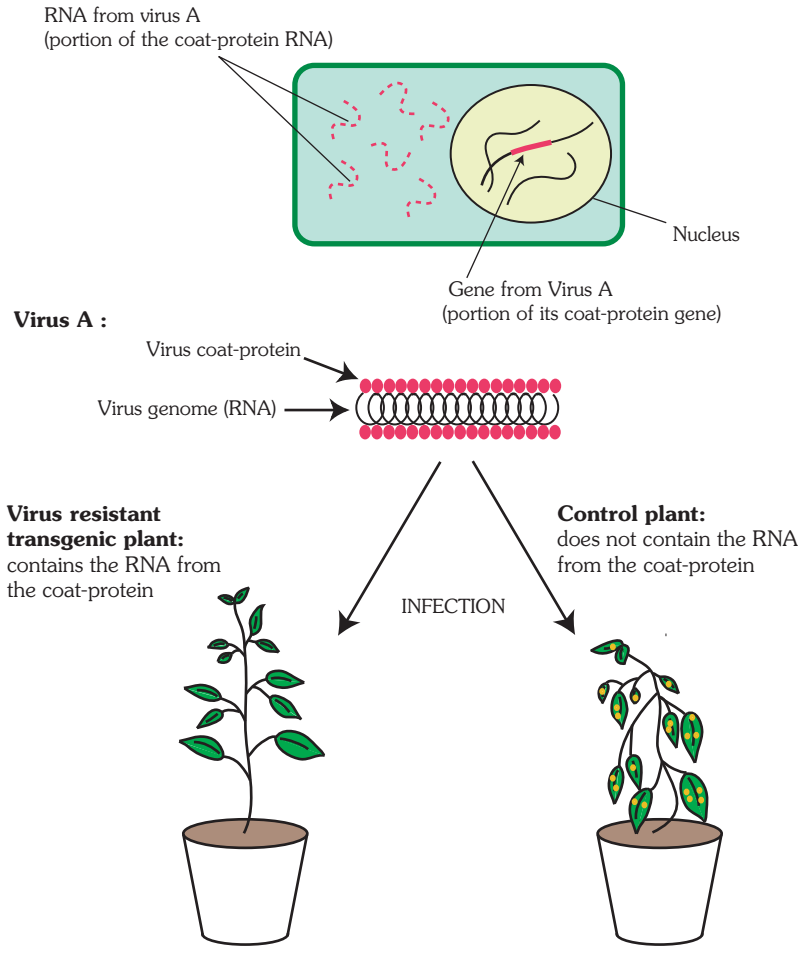


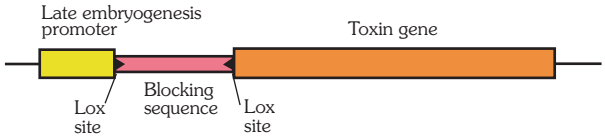
Fig. 2 Application

V-GURTs (Variety-level Genetic Use Restriction Technologies), also referred to as "Terminator technology" (1)

To produce the V-GURT, three constructs have been inserted into the plant. At the stage preceding commercialization, the situation is such as described here: no toxin is expressed and the seeds produced by the plant are fertile.
This is the phase when the stocks of seeds for marketing are produced.

Three constructs are inserted into the plant:

1.

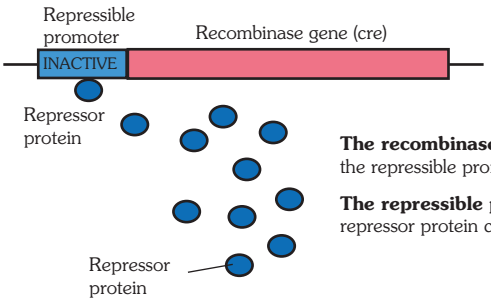


The late embryogenesis promoter is active (i.e. it activates the expression of the toxin gene) only during the late stages of embryogenesis, that is to say in seeds.

The blocking sequence prevents the expression of the toxin gene by disrupting the sequence continuity between the promoter and the gene. As long as this sequence is present, the toxin gene will not be expressed, even in seeds,when the late embryogenesis promoter is active.

The toxin gene codes for the toxin which will sterilize the seeds by disrupting their tissues.

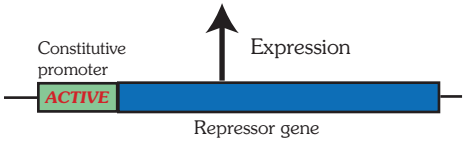
2.



The recombinase gene is not expressed as long as the repressible promoter is inactived.

The repressible promoter is inactivated by the repressor protein coded by the repressor gene.

3.



The constitutive promoter is always active and the gene under its control, the repressor gene, is always expressed.

The repressor gene always expresses the repressor protein. This protein will bind to the repressible promoter, thereby preventing the expression of the recombinase.

Fig. 3 Application V-GURTS (2)

Once the amount of seeds required is obtained, a particular substance interacting with the repressor protein is sprayed onto the seeds. The repressor protein is thereby prevented from binding to the repressible promoter. This promoter is consequently activated and the recombinase gene expressed. The recombinase excises the blocking sequence, restoring the sequence functionality of the late embryogenesis promoter coupled with the toxin gene.
This is the stage at which the plants are sold to the farmer.
Once this seeds are sawn by the farmer, they will grow normally until producing new seeds. At this point, the late embryogenesis promoter will be activated and the toxin will be produced in the seeds that will consequently be sterilized.

1. Interacting substance treatment

2. The Interacting substance binds to the repressor protein

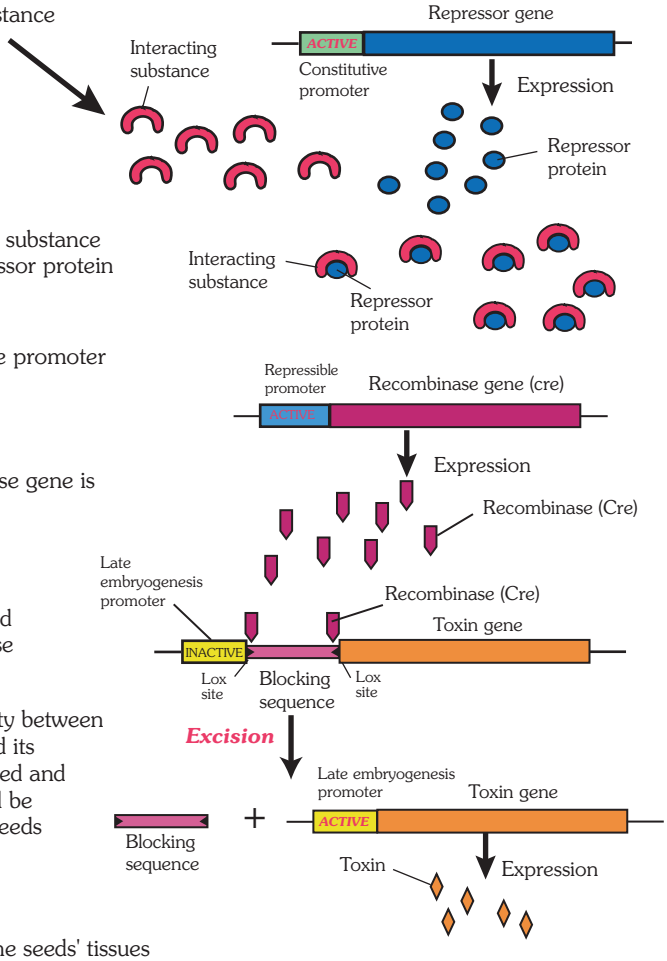
3. The repressible promoter is acitvated

4. The recombinase gene is expressed

5. The blocking sequence is excised by the recombinase

6. The functionality between the toxin gene and its promoter is restored and the toxin gene will be expressed in the seeds

7. Disruption of the seeds' tissues that consequently turn sterile



The seeds are turned sterile by the toxin

Fig. 1 Risks Main crops geographical origin

Region of origin

Region of cultivation

Barley

Maize

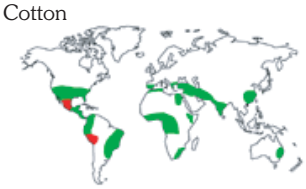
Oilseed rape

Rice

Sorghum

Triticale

Cotton



Oat

Potato

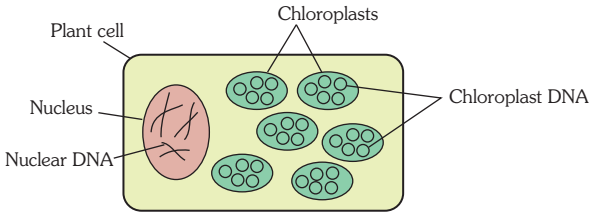
Rye

Sunflower

Wheat

Fig. 2 Risks Transplastomic plants

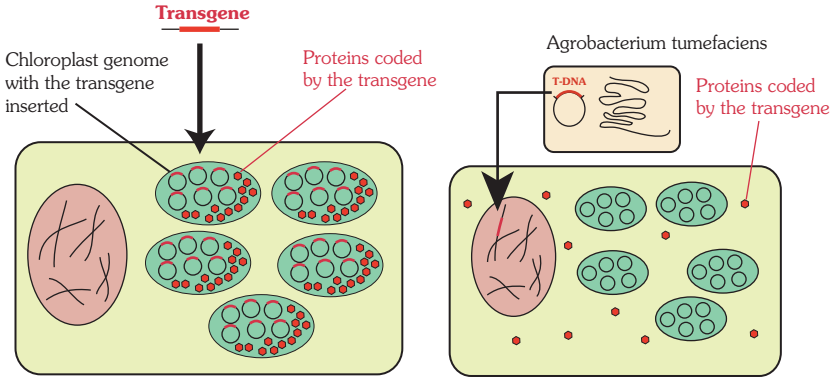
Transplastomic plants are transgenic plants whose chloroplast DNA has been transformed instead of their nuclear DNA.



N.B. In each chloroplast, there is many copies of the chloroplast genome, which is a small circular DNA sequence. There is about 1'000 to 10'000 copies of chloroplast genome per cell distributed among 10 to 100 chloroplasts.

Transformation of the chloroplasts with a selected transgene

Nuclear transformation (i.e. the usual transformation type)



Transplastomic plant cell

Transgenic plant cell

• The number of copies of the transgene as well as the quantity of proteins it codes for is much higher in transplastomic plants than in normal transgenic plants.

• In chloroplasts as in all bacteria, the homologue recombination works well so that it is possible to choose the insertion site of the transgene.

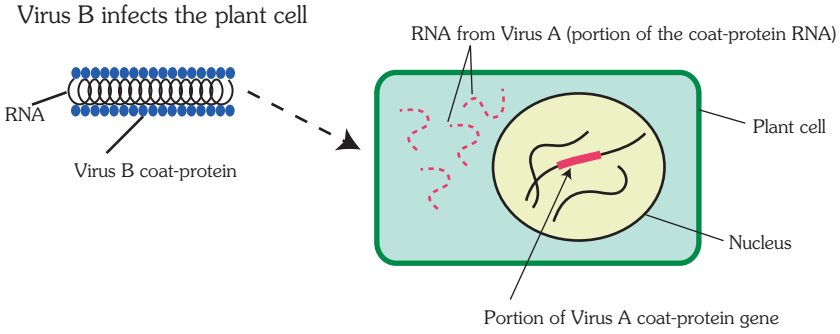
• In general, pollen does not contain chloroplasts. This means that transgene dissemination via the pollen should normally not occur in transplastomic plants.

Fig. 3 Risks Recombinant viruses

When a transgenic plant resistant to Virus A (containing a portion of the Virus A coat-protein gene and transcribing it into RNA) is infected by another virus strain (Virus B), the RNA from the two viruses may recombine and form a new recombinant virus.

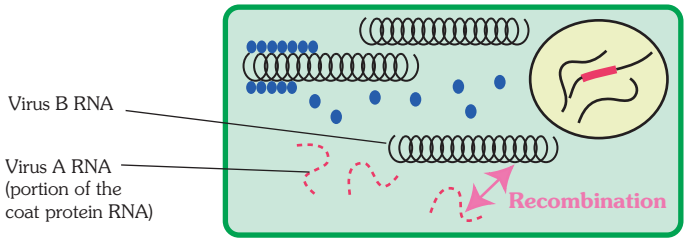
1. Infection

Virus B infects the plant cell

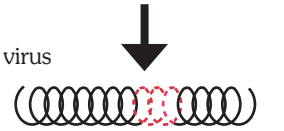


2. Multiplication and recombination

Virus B replicates and synthesizes its own coat-proteins. At that point, the RNA from Virus B may recombine with the RNA from Virus A, thus creating a new virus (recombinant) with a different genome.

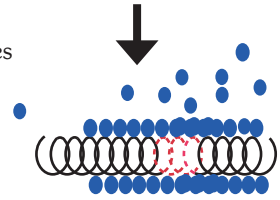


Recombinant virus



3. Reassembly

The recombinant virus reassembles with its coat-proteins.



GLOSSARY

Agrobacterium tumefaciens is a bacterium infecting wounded plants and causing a disease called Crown Gall Tumor. The bacterium transfers a fraction of its Ti plasmid, called T-DNA into the plant. The T-DNA inserts into the genome of the plant and induces a tumor-like development of the plant tissue and the production of substances the bacterium will feed on. *Agrobacterium* is used in genetic engineering to transform plant cells. The T-DNA is replaced by the DNA sequence we want to transfer into the plant. *Agrobacterium* does not naturally infect monocotyledonous plants and until recently this was a serious limitation in the use of this technique.

Allele: one of the two or more alternate forms of a gene occurring at the same position (locus) on a chromosome, which control the expression of a gene in different ways. A cell or an organism is homozygous when it contains identical alleles at the same locus, or heterozygous when there are two different alleles present. A gene for height, for example, may exist in two allelic forms, one for short and one for tall.

Allogamy refers to a plant, which is fertilized by the pollen of other plant of the same species. Maize, beetroot and oilseed rape are examples of allogamy.

Antisense construct refers to a DNA sequence complementary to a particular gene. When such construct is introduced in the genome, it will be transcribed in RNA and will be paired with the RNA of the gene it is complementary to. This complex of two RNA (double stranded RNA) will then be degraded so that no protein of the gene will be produced. This approach has been used for example in tomato to suppress the expression of the gene responsible for cell wall degradation and therefore enhance the firmness of the fruit.

Autogamy refers to a plant, which is fertilized by its own pollen. It is self-pollinated. Soya, tomato, soya, wheat, oat are all examples of autogamy.

Base: see **nucleotide**.

***Bt* gene** comes from the bacterium *Bacillus thuringiensis*. It confers resistance against Lepidopterian insects. The *Bt* protein acts as a toxin when ingested by insects. It is hydrolyzed by enzymes in the insect's digestive tract and then binds to a particular receptor on the intestinal wall, thereby causing its disruption and the subsequent death of the insect.

Cells are the basic functional working units of every living system. All the instructions needed to direct their activities are contained within the DNA (DeoxyriboNucleic Acid).

Chloroplasts are organelles in charge of the photosynthesis and they are present in many copies in plant cells. It is thought chloroplasts were originally prokaryote organisms that established a symbiotic relation with eukaryotic

cells during evolution. This would explain why chloroplasts have their own DNA, which replicates autonomously and is very similar to bacterial DNA. There are normally no chloroplasts in pollen (except in the case of gymnosperms, i.e. Christmas tree, pine, etc.), and chloroplasts are therefore inherited only from the female plant. Transgenic plants whose chloroplast DNA has been transformed are called transplastomic plants.

Chromosome: very long duplex DNA chain. The characteristic X-shape of the chromosomes is only visible during cell division. Each cell of an organism contains normally two sets of chromosomes (human beings have two sets of 23 different chromosomes = 46 chromosomes). Plants are exceptions as they often have more than two sets of chromosomes (four, six, etc.). These plants are called polyploid.

Competence is the state in which a bacterium is able to take up DNA from the environment and to integrate it into its genome. This means that only bacteria in a competent state can be transformed. Only some bacterial strains are naturally competent and this state is often temporary and depends on various parameters such as availability of food, temperature, pH, etc.

Cross-pollinated: *see* **Allogamy.**

Cytoplasm describes all the material located inside the cell, between the plasma membrane and the nucleus. In the case of a prokaryotic cell (no nucleus), the cytoplasm represents all the material inside the cell.

Dicotyledonous: plants whose seeds have two cotyledons (meaning "seed leaf"). These plants are easier to manipulate than monocotyledonous plants because they can be more easily transformed with *Agrobacterium tumefaciens.*

DNA (DeoxyriboNucleic Acid) contains the genetic information (hereditary) of an organism. DNA is invariably made of the same physical and chemical components. This similarity is what enables us to transfer a DNA sequence from one species to another, thereby producing transgenic organisms (genetically modified organisms - GMO).

DNA sequence: specific side-by-side arrangement of bases along the DNA strand (e.g., ATTCCGGA). This order is a code that spells out the exact instructions required to creating an organism with its own unique traits.

Eukaryote: organism whose cells contain a nucleus. Plants and animals are eukaryotes; many mushrooms too. On the contrary, bacteria are not eukaryotes; they are prokaryotes.

Gene: a gene is a particular DNA sequence that codes for a corresponding protein. Human beings have approximately 35 '000 genes but this represents only about 2% of their total DNA.

Gene silencing refers to complex interactions between DNA (transcriptional gene silencing) and RNA (post-transcriptional gene silencing) based on the homology between these sequences, their place into the genome, the possible

modifications of their chemical structure, etc. These interactions may stop the expression of a gene or lead to the degradation of its transcription products (RNA), thus "silencing" the gene as no proteins are produced anymore.

Genetic engineering refers to the techniques used to identify, isolate and transfer a gene into another organism.

Genome is the total genetic information contained in an organism.

Genotype refers to the genetic characteristics of an organism, independently of their expression and visibility.

Germplasm refers to the characters of an organisms that are inherited form one generation to another and that are located in the germ at the beginning of the organism development. Also used to describe the plants, seeds, or other plant parts useful in crop breeding, research, and conservation efforts, when they are maintained for the purpose of studying, managing, or using the genetic information they possess.

GMO: genetically modified organism. An organism in which a gene from another species (transgene) has been introduced.

Heterosis refers to the effect observed in the first generation of species hybrids. It is characterized by an increase in yield and robustness. The heterosis effect decreases in the following generations while the genetic variation in the population increases. For that reason, species hybrids are usually used only once and most farmers buy new seeds every year.

Homozygous refers to an individual with the same allele at corresponding loci on the homologous chromosomes.

Heterozygous refers to an individual with different alleles at some particular locus.

Intron is a DNA segment located inside the gene sequence. The intron is transcribed into RNA but it is removed before the RNA is translated into protein.

Marker gene: during transformation, only a small fraction of the cells integrate the transgene. It is therefore necessary to identify the transformed cells among all the non-transformed ones. To screen them one by one would be so time-costly that it would make transformation unpractical. The marker gene is specially designed to solve this problem. The marker gene is co-integrated into the plant cell along with another chosen gene. Let us take the case in which an antibiotic resistance marker gene is used. When all the potentially transformed cells are brought into contact with the antibiotic, only the small fraction of cells that have integrated both the chosen gene and the antibiotic resistance marker gene, i.e. the transformed cells, will be resistant to the given antibiotic. All the non-transformed cells will die or stop growing.

Meiosis refers to the two successive cellular divisions resulting in the reduction by half of the number of chromosomes in a cell. It is through

meiosis that the germ cells (sperm or eggs) are finally produced. During fecundation, the germ cells will fuse to form a new cell with the original number of chromosomes.

Mitochondria are the organelles, present in all eukaryotic cells, in charge of the production of the energy. Mitochondria contain their own DNA, replicate independenlty of nuclear DNA and are inherited maternally. As for chloroplasts, it is thought mitochondria were initially bacteria that established a symbiotic relation with eukaryotic cells during evolution.

Monocotyledons: plants whose seeds have a single cotyledon (meaning "seed leaf"). Wheat, rice, maize, palm and all other grasses and cereals are monocotyledons. Initially, transformation with *Agrobacterium tumefaciens* did not work with monocotyledons, because these are not natural hosts of *Agrobacterium*. This is why transgenic rice and wheat were so difficult to produce. However recently, new techniques have enabled us to successfully transform rice and maize by *Agrobacterium*.

Nucleotide: DNA is made of a succession of nucleotides that form the basic unit of DNA. There are four different nucleotides in DNA: adenine (A), cytosine (C), guanine (G), and thymine (T). These nucleotides are also called bases.

Nucleus: part of an eukaryotic cell, separated from the cytoplasm by a membrane and in which is localized most of the DNA (some DNA is also present in the chloroplasts and in the mitochondria). Bacteria do not have a nucleus and their DNA is found in the cytoplasm.

Phage: a phage is a virus infecting bacteria.

Phenotype is the appearance or other characteristics of an organism, resulting from the interaction of its genetic constitution with the environment. Example of a person with brown eyes: "brown eyes" is the phenotype, while the genotype is both blue and brown allele for eye colour. Indeed, as brown is dominant over blue, the brown is visible and the blue is not. So the genotype is not directly visible and is related to the genetic constitution of an individual whereas the phenotype is usually visible or at least concerns an expressed characteristic linked to the production of a particular protein.

Plasmid: very small and circular DNA present in many bacteria. Plasmids are able to self-replicate independently of the rest of the genome. Plasmids are used in genetic engineering as cloning tools.

Polyploid: an organism with more than the normal two sets of chromosomes. Plants are often polyploid.

Prokaryote: organism without nucleus. Bacteria are prokaryotes. Prokaryotes are generally unicellular.

Promoter: DNA sequence preceding the gene. The promoter regulates the activity of the gene. A promoter can be eukaryotic (active in plants or animals), prokaryotic (active only in bacteria), ubiquitous (active both in plants,

animals and bacteria): It can also be tissue specific (active only in pollen or in roots for example) or development specific (active only during embryogenesis or flowering).

Protein: very important and diversified class of molecules representing about half the dry weight of a cell. They are made up of amino acids and have many functions: structural like in muscle, metabolic like for all enzymes and regulatory for hormones, surface receptors, etc. Each protein is synthesized according to the information coded in its particular corresponding gene. Between the gene and the protein, another molecule, the mRNA (messenger RiboNucleic Acid) plays the role of intermediary.

Recombination is defined as a nucleotide exchange between two molecules of RNA or DNA. It occurs commonly in all organisms. During meiosis for example, recombination plays an important role in creating genetic diversity. There are two types of recombination: homologous recombination and recombination. Homologous recombination is the exchange of nucleotides between two DNA or RNA molecules with a high sequence similarity. It is the most frequent mechanism in bacteria but in plants and animals it doesn't work well. Homologous recombination enables us to precisely replace a gene, or to insert a DNA sequence into a precise locus. On the other hand, heterologous recombination is a nucleotide exchange between two unrelated RNA or DNA molecules. Transgene integration into the plant always occurs by illegitimate recombination and it is therefore not possible to predict the place where the transgene will be inserted neither in how many copies.

Recombinant DNA refers to the techniques used in bacteria by which the identification of a gene, its isolation and its transfer into another organism through homologous recombination are achieved.

Recombinase: An enzyme or enzyme system which promotes genetic recombination.

mRNA (messenger RiboNucleic Acid) is the intermediate molecule between the gene and the protein. The gene is first transcribed into mRNA and then translated into protein. The RNA structure is only slightly different from that of DNA so that the information coded in the gene can be preserved and this results in the synthesis of the correct protein. Contrary to DNA, mRNA is a single stranded molecule and its lifetime is very short because cytoplasmic enzymes readily degrade it.

Self-pollinated: see **Autogamy.**

Terminator: DNA sequence following the gene. The terminator sequence marks the end of the gene and stops its transcription into mRNA

Terminator technology or V-GURTS: see "2.1.5. GURTs - Genetic Use Restriction Technologies", p. 24.

Transformation is the acquisition and incorporation of new and/or foreign DNA into a cell. Transformation is usually followed by a change in the

characteristics of the organism regenerated from that cell, as it will have integrated a new gene or DNA sequence. That is why this mechanism is called transformation.

Transgenic is said of an organism in which a gene from another species (a transgene) has been introduced.

Transgene is the gene from another species that has been introduced in an organism thus rendering it transgenic.

Transplastomic refers to plants having integrated a transgene inside their chloroplasts (chloroplasts also contain DNA) instead of inside their nucleus. As there are many chloroplasts per cell, the transgene will normally be expressed at a higher frequency than if it was inserted into nuclear DNA. Furthermore, in many plants the chloroplasts of the pollen (the male gamete in plants) are not transmitted to the female plant. In these plants, chloroplasts are inherited maternally. This is seen as a technique susceptible to lower the risk of transgene dissemination to other plants via the pollen.

Unicellular: organism composed of a single cell: bacteria and protozoa are unicellular.

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