PROSPECTS OF BIOTECHNOLOGY IN HORTICULTURAL BREEDING

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Abstract
Plant biotechnology offers an array of techniques for the enhancement of efficiency in crop improvement by: (1) overcoming natural crossing barriers, and (2) speeding up different steps of the breeding procedure. The most promising field of application of plant biotechnology is the creation of novel idiotypes in an asexual way, often indicated as Genetic Manipulation or Genetic Engineering. Various techniques at protoplast fusion, chromosome transplantation, genetic transformation, anti-sense RNA are applied to actualize this goal.

Some early striking successes of genetic manipulation in vegetable crops are the introduction of CMS from radish into rape, the insertion of the Bacillus thuringiensis crystal gene in tomato, resulting in resistance to some lepidopterous insects, and resistance to herbicides in various crops. Several other applications will probably become available in the near future.

Another rapidly developing field of research is the use of 'molecular markers' (RFLPs). Molecular markers enable us to tag target genes and to indirectly select for agronomically important traits. Two example of research fields, viz, cytoplasmic male sterility (CMS) and resistance to pathogens, are chosen to illustrate the potential contribution of genetic manipulation to plant breeding.

1. Introduction

Plant Breeding can be defined as the sum total of activities directed at the improvement of cultivated crops to meet human needs. Defined this way plant breeding started when men gave up their nomadic existence of collector and hunter and settled in agricultural communities. For a very long time plant breeding has been performed on a sheer intuitive basis. Framers selected plants (or seeds from plants) which were bigger, stronger, better adapted and less vulnerable to pathogens and other stress factors. By repeatedly doing so, cultivated forms evolved from the wild species which only could survive by protection of man, because in natural conditions they would not be competitive to their wild relatives. This process of 'taming plants' is called domestication.

After the detection of sexuality in plants around 1600 botanists started crossing experiments and new variants, mainly interspecific crosses, came available. It took another 300 years, until the rediscovery of Mendelian laws in 1990, before plant breeding obtained a scientific base. By application of carefully cytological and

physiological tools, production potencies and many other characters of crops were greatly improved.

Notwithstanding their undeniable successes the established procedures of plant breeding have some obvious drawbacks. First of all they an initial cross between two or more parents is very laborious and time-consuming. A period of 10-15 years between initial cross and release of a new shortcoming of classical plant breeding is the impossibility of exchanging genes between distantly related taxa, caused by **crossing barriers**, usually indicated as **incongruity** or **interspecific incompatibility**.

When in 1978 Melchers et al. published their sensational article on the '**pomato**', an interspecific hybrid between the potato and the tomato, obtained after fusion of naked cells (**protoplasts**), there were many speculations about a 'breakthrough' in plant breeding. In fact it was no more than a further step in a process that had started some 20 years earlier: the application of in vitro techniques in plant breeding research. Since then several novel cellular and molecular techniques have become available, which, after some hesitation, are now readily adopted by the plant breeder as additional tools for the attainment of his aforementioned goal.

Though many of the older in vitro techniques, such as **meristem culture, ovule culture, pollen** and **microspore culture, embryo culture** and **embryo rescue** techniques, as well as large scale in vitro propagation, have been very beneficial for plant breeding and have found wide application both commercially and in breeding research. I will confine myself to discussing the recently developed techniques, usually indicated as **Genetic Manipulation** or **Genetic Engineering**.

2. Genetic Manipulation Devices

In the course of the last 20 years a host of GM devices has been developed (Table 1). The principles, applications, prospects and limitations are briefly discussed in the subsequent paragraphs.

2.1. Protoplast Fusion

Protoplast fusion implies the merging of wall-less, somatic (non-sexual) cells of different species. Depending on our objectives different 'end-products' can be pursued (Figure 1):

- Somatic hybrid: a complete fusion between two cells both genomes (chromosone sets) and cytoplasma organelles duplicated.

- Cybrid: one chromosome set and mixed or hybridized organelles.

- Nucleus substitution: nucleus of species A in cytoplasma, including organelles, of species B.

Important applications of protoplast fusion are (1) Overcoming **crossing barriers** and (2) mixing and recombination of extranuclear DNA. Point 2 deserved extra

attention as it forms a real new situation, which can not be realized by sexual fusion. In sexual fusions the male gamete in most cases contributes a naked nucleus without organelles (sometimes a few plastids are transferred), whereas the mother supplies a complete cell apparatus (the eggs cell). Consequently extranuclear genetic information is **maternally** inherited. With somatic hybrids the cytoplasm including the mitochondria and chloroplasts of the two fusion partners is blended (and the DNA possibly hybridized).

Though fusion between very unrelated cell types is usually easy to realize, e.g. man-mouse, plant-fungus/bacterium, most 'unrelated' combination function defectively as a result of disharmony together (**somatic incongruity**). Many combinations do not develop or are unstable. In the last case one of the chromosome sets and/or organelle types is gradually eliminated. Even when a fusion product is viable it will rarely be competitive for agronomic traits with superior varieties. Therefore, the initial enthusiasm about protoplast fusion has largely faded away. Nevertheless some successes worth mentioning have been obtained. Research groups in the USA and Japan have produced a number of intergeneric fusion products (**somatic hybrids**) in citrus with tolerance to the tristeza virus, cold tolerance and nematode resistance, which offer promising opportunities for use as rootstocks.

The most striking result is the creation of hybrids of Brassica realized by Pelletier and coworkers in France. They restored chlorophyll deficiency in a cytoplasmic male sterile (CMS) sexual hybrid of radish and rape by replacing the radish chloroplasts with rape chloroplasts (in combination with radish mitochondria). In a second fusion product resistance to the herbicide atrazine was transferred from turnip to CMS rape.

2.2. Chromosome Transplantation

At first sight **chromosome transplantation** seems a simple way of introducing new genetic information into an organism. Especially when about 10 years ago methods became available for: (1) mass-isolation of chromosomes and (2) separation of the target chromosome from the total genome. Isolation was made possible by enveloping contracted metaphase chromosomes in fatty substances (**liposomes**) after liberating them from the cell nucleus. Separation of chromosomes was highly facilitated by the use of a flow selectively sort out a particular chromosome from the mass (Figure 2).

However, chromosome transplantation has appeared to be an inadequate method of GM. The direct uptake of chromosomes by protoplasts is very inefficient. Even at extremely high chromosome concentrations (10^6 - 10^7/ml) only less than one percent of protoplasts of lily and tobacco took up an alien chromosome. This problem has been overcome by microinjection of chromosomes, either as liposomes or as **micronuclei**. Micronuclei develop by **treatment** of nucleus with specific chemicals. The chromosomes spread through the nucleus and are enveloped individually by cytoplasma.

But the major constraints of the method are the **instability** of the transplantation products and **linkage drag**. Usually the foreign chromosomes are rapidly eliminated when the cells start dividing. In case the extra chromosome would be able to survive, it is doubtful whether its genetic information can come into expression - especially with very distantly related organisms. And even when expression is realized the effect of a few positive genes may be counteracted by the effect of undesirable genes on the chromosome (linkage drag).

3. Genetic Transformation

Probably the most promising technique of GM is **genetic transformation**. Genetic transformation stands for the incorporation of defined pieces of DNA into a receptor genomes. The method has striking advantages compared to the foregoing techniques because:

- It allows the exchange of genetic information between very distant organisms, such as plants and fungi, bacteria or viruses.

- It makes possible the insertion of single, desired genes in a genome. Along with the **target gene** also **marker genes** can be integrated in order to enable early selection of the genetically transformed or **transgenic plants**.

- It (supposedly) leaves intact the structure of the genome adding only one or a few traits to existing commercial varieties. Whether this assumption is completely true has still to be proven. It is quite possible that the site if **insertion** of foreign DNA and the number of inserted copies will influence the performance of the transgenic plant not only for the trait controlled by the new gene but also for other traits.

Nevertheless, transformation seems a promising way to mend breeding procedures. In conventional breeding required genes are often closely linked to undesirable traits and it takes much effort (several backcross generations) to get rid of this 'linkage drag'. Transformation with pure gene is much more efficient.

To realize successful transformation the following conditions have to be met:

- The desired gene has to be identified and isolated. This condition is the main bottleneck in transformation. Though remarkable progress has been made during the last few years the number of available genes is still limited.

- A mechanism is needed to transfer the foreign DNA into the recipient cell or tissue.

- Transgenic products must be selectable in an early stage.

- The novel information has to come into expression in the regenerated plants and in further generations.

4. Gene transfer mechanisms

Two main categories of gene transfer mechanisms can be distinguished, viz, **direct gene transfer** and **vector-mediated** systems.

4.1. Direct Gene Transfer

4.1.1 Direct uptake of DNA from a liquid solution.

Direct uptake of DNA from a liquid solution by roots, embryos, and seeds or by protoplast and cell suspensions only in rare cases has resulted in transgenic plants. The chance of hitting the target is very low as the DNA may degrade in the medium or even more probably somewhere during its passage through the cell to the nucleus. But the method may be a substitute in species which do not positively respond to vector-mediated systems, such as cereal crops. Transgenic plants by direct DNA uptake have been obtained i.a. in tobacco, rice and sugar cane. Degradation of DNA can be prevented by coating with fatty substances (in the same way as we have discussed for chromosome transfer) - **liposomes**.

4.1.2. Ballistic or particle gun methods

Several **biolistic**, also called **ballistic or particle gun** methods have been developed in the last few years. Principle of biolistic methods is acceleration of heavy metal (gold, platinum, tungsten) micro-projectiles coated with DNA. Because of the apparent advantages of biolistic enormous investments have been made in this technique. Potrykus (1991) summed up the advantages as follows: (1) Easy to handle, (2) One-shot - multiple hits, (3) Cells easily survive particle intrusion, (4) Not only protoplasts but also walled cells, tissues and pro-embryos can serve as target, (5) Particles can penetrate in deeper cell layers, and (6) No host limitation.

The combination of points 4 and 6 makes biolistic techniques particularly beneficial for the transformation of 'recalcitrant' crops such as the agronomically important grain cereals, which poorly regenerate from protoplast culture and do not respond to the commonly used Ti-plasmid mediated transformation. Particle bombardment has been successfully applied on embryogenic tissue of maize and soya bean and on cell cultures of sorghum.

4.1.3. Microinjection

Microinjection in many respects is comparable to the gun shot approach, with the exception that per injection only one cell receives DNA. Microinjection also allows delivery of large copy numbers of the target DNA into the cell or even in the nucleus. As elucidated elsewhere microinjection is also applied for the transfer of whole chromosomes, chromosome fragments and organelles. As only extremely limited volumes of liquid (somewhere between 10^{-12} - 10^{-15} l) can be injection the density of the transmitted substance must be very high.

Several other techniques of direct DNA delivery have been explored, e.g. transfer of DNA via germinating pollen grain (**egg cell transformation**), direct delivery through pollen tubes, transfer of DNA through wounded cells to adjacent tissue (**microinjection**), intrusion of DNA through opening in the plasmalemma (**electropor-**

ation) or via holes burned into cell walls or membranes (**microlaser** treatment). Though there are claims of successful transformation with some of these techniques, stable and heritable changes (**integrative transformation**) as yet has not been proven to have occurred with any of them.

4.2. Vector-mediated DNA Transfer

With direct gene transfer the foreign DNA has to penetrate the host nucleus and to integrate in the genome autonomously. The major part of the DNA gets lost and the number of transformed cells is rather low. Gene transfer can become more efficient by connecting the foreign DNA to a vehicle which transmits DNA to the host cell. Two transfer systems have received major attention, 1.e. (1) plant viruses, and (2) Agrobacterium plasmids.

4.2.1. Plant viruses

Plant viruses look well-suited for transfer of genetic information, as they rapidly replicate. Besides, many viruses are very infection and easily spread through the whole plant. Consequently viral vectors would allow transformation on the plant level, thus circumventing the often critical step of cell or tissue culture.

For a long time the main limitation for viral vectors has been, that most viruses contain RNA instead of DNA and therefore were not suitable as DNA vectors. But this problem has been overcome as researchers are able to convert RNA into its matching DNA. Another drawback is that virus-transmitted DNA usually is not integrated in the plant genome but replicates autonomously with the virus. This implies that the novel genetic information is not expressed in the next generation as germ cells and consequently seeds generally are virus-free. This restriction is not applicable to vegetatively propagated crops such as sugar cane and tuberous and bulbous crops. A positive point of autonomous virus replication is that the novel DNA is available in many copies and may come into expression massively.

Two plant DNA-viruses have been more extensively studied: **cauliflower mosaic virus** (CaMV) and **gemini viruses**. CaVM and other caulimoviruses have only a limited host range; gemini viruses exhibit a wider host range and may be more suitable for general application. It is claimed that some bacterial genes, mainly resistances to antibiotics and toxins, have been transferred by viruses to i.a. wheat, maize and rape (Brassica napus).

Much more successes have been obtained with another vector: the **Ti-plasmid** of the bacterium Agrobacterium tumefaciens. It has been known for a long time that many higher plants can be infected by A. tum. The bacterium invades wounded stem parts and causes cancerous outgrowth of the infected cells named '**crown gall**' disease. It has been found that not the bacterium itself enters the wounded cells but only a little part of DNA, the so-called **T-DNA** (or **T-region**). This T-DNA is located on a **plasmid**, a circular kind of minichromosome which occurs in the bacterium along with a much larger DNA strand. The T-DNA enters the nucleus and

in a natural (but still not well-understood) way inserts into the genome of the host cell.

The T-region contains so-called **onc-genes** which are responsible for the strong cell proliferation and an **opine synthesizing gene**. The latter gene produces nutrients for the bacterium. In summary we can say that the Ti-plasmid meets all requirements for genetic transformation: it can penetrate the cell and nucleus, it integrates in the plant genome, and the genes carried by the Ti-plasmid come into expression. It can be regarded as a natural genetic engineer!

Researchers all over the world have created gene-vector system based on A. tumefaciens and its relative A. rhizogenes (which causes **hairy root** formation instead of crown gall disease). Essential in all system is the replacement of the canker or hairy root genes by the new genes which we want to introduce in the receptor cells. To detect the transgenic cells we can use the opine gene as a **marker**, but often the opine gene also is replaced by a more easily selectable marker gene, usually antibiotics resistance genes.

Agrobacterium vectors have been applied on protoplasts and leaf discs. The last method is simpler because it makes superfluous the complicated protoplast culture. It has been widely applied in Solanaceous crops and in legumes, e.g. to incorporate resistance to glyphosate-containing herbicides. **Glyphosate tolerance** of tomato, potato and petunia and other resistances or tolerances to herbicides have found wide publicity because of the potential ecological risks (e.g. incorporation of the resistance gene into wild relatives), diverting the attention from commercially even more promising results, which will be discussed in chapter 3.

Major shortcoming of the Ti/Ri-plasmid is the limited host range of Agrobacterium. With the possible exception of one (asparagus) or a few species, the Ti-plasmid is not able to infect monocots, including the major cereal crops (rice, maize, barley, sorghum).

5. Antisense RNA

Many, if not all, crop varieties contain genetic information for undesirable traits. It is not always possible to get rid of these unwanted genes by crossing procedures, e.g. because alternative alleles are not available or because of strong linkage of the minus-gene to a highly desired gene. The most effective way would be to 'cut out' DNA segment in question by gene surgery, but this approach is not yet applicable. There is, however, an elegant alternative: inactivation of the involved gene by **'antisense RNA'**. The principle of antisense RNA is demonstrated in Figure 3. The DNA segment controlling the detrimental trait (**sense DNA**) is copied in reverse (**antisense DNA**) and inserted in the genome. The messenger RNA molecules transcribed from the antisense DNA segment (**antisense RNA**) will bind with the mRNA of the corresponding sense DNA and by doing so 'turn off' the gene. The gene product (protein) is not made because the sense mRNA is inactivated by binding with the antisense mRNA.

Antisense RNA has already been successfully applied in manipulation of flower colors of petunia by a research team of the Free University Amsterdam. The USA-based biotech company Calgene has used antisense RNA to inhibit the expression of the gene for polygalacturonase (PG) which plays an important role in softening of tomato fruits by partially solubilizing the pectin fraction of the cell walls during fruit ripening.

An advantage of antisense constructs in comparison to mutants is the dominant expression of gene suppression; mutants are usually recessive and become manifest only in the absence of the dominant allele. Antisense is also to be preferred to transformation as insertion of foreign genes so far is completely random and may negatively affect other useful genes. Anti-sense does not influence other genes in any way. However, it still has to be proven that antisense suppression is stable and complete. Experiments have shown that to get effective reduction of the expression of the target gene, a high ratio of antisense to sense mRNA is required. It also has been found that the actual mechanism of inactivation of gene expression by the anti-sense mRNA is more complex than stated before.

6. Restriction Fragment Length Polymorphism

Efficient selection may be greatly hampered by the lack of knowledge of the number and location of **agronomically important genes**. This is especially the case with polygenic traits such as growth rate, yielding capacity, protein composition etc. If the breeder could recognize the individual genes, directly or indirectly, selection would become much more efficient.

Many genes are expressed only in a late stage of development, e.g. resistance to fruit-rot causing pathogens manifests itself not until fruits have developed. This constituteaa further handicap for selection efficiency. Finally, several useful traits, e.g. many resistance genes, are recessive and can not be detected when they occur in heterozygous state.

To speed up the selection process breeder try to find ways of **indirect selection**, i.e. they do not select for the target trait itself but for other characteristics, the genes of which are closely linkage to the target gene, and which are early and easily recognizable.

. In the past **morphological** and **pigmentation markers** have been used. The limited number of this kind of genes, and their late expression was a serious drawback. A step forward was the use of **isozymes markers**, based on the occurrence of different form of enzymes. These differences can often be observed already on the cellular level. A revolutionary step, however, was the so called Restriction Fragment Length Polymorphisms or **RFLPs**.

RFLPs are commonly used in human genetics, especially in prenatal diagnostics, already since 1974, but their potential application in plant breeding was only put forward in 1980. RFLPs have a great number of advantages compared to classical

genetic markers. Their number is principally unlimited though large differences in number between plant species occur, they have a simple Mendelian inheritance, show no pleiotropic effects and are completely codominant and insensitive to environmental factors. Finally they allow early detection of linkage agronomic characteristics irrespestive of the stage of development at which these characteristics become manifest.

The principle of RFLP-detection is shown in Figure 4. Any restriction enzyme cleaves DNA on specific sites by recognition of specific base sequences. Mutations in the DNA (base substitutions, inversions, deletions) resulting changes in cleavage sites compared to the original situation (**'wild type'**). These differences can be made visible by **electroforesis** and **hybridization** of the target DNA with a (radioactively or otherwise) labeled DNA probe. In Figure 4 the cleavage sites of two restriction enzymes are presented for the 'wild type' (A) and for two mutants (B and C). Mutant B lacks a cleavage site for enzyme resulting in a bigger DNA fragment and a higher position in the electropherogram (Figure 4b). Mutant C contains an insertion influencing the fragment size for both endonucleases.

The crucial step is to connect RFLPs to useful traits. If plants with a specific RFLP nearly all process a particular trait then there probably is a linkage between the RFLP and the gene responsible for that trait. As we can determine the presence of RFLPs already in every early stage they are an efficient tool of predicting the presence of a desired gene.

RFLPs can be used in plant breeding in various ways which will not be discussed in detail. The most attractive aspect is the potential use in selection for quantitative traits. In various laboratories all over the world RFLP research is executed in i.a. potato, tomato, pea, cabbage, lettuce and onion. Some preliminary results of the efforts are linkage of RFLPs with resistance genes for the TMV-virus and the fungus Verticillium in tomato. The main limitation of the RFLP procedure is constituted by the high costs connected with finding appropriate probe-restriction fragment combinations.

7. Application of GM

In the foregoing sections some successful cases of application of genetic manipulation techniques have been briefly mentioned. Instead of adding a complete list of useful results let us consider two main topics to clarity the potential contribution of GM to horticultural breeding, viz. cytoplasmic male sterility and resistance to pathogens.

7.1. Cytoplasmic Male Sterility

In the last three decades there is an increasing tendency in the horticultural seed industry to create hybrid varieties. Hybrid seed results from large scale crosses between two to four inbred lines, which have been proven to be 'well-matching', i.e. their progeny are superior to the parental types for important agronomic traits. The advantages of hybrid varieties (**HVs**) over open pollinated varieties (**OPs**) are

obvious; HVs are uniform, highly productive, often contain several resistance genes, and have a natural protection against seed production by the grower. The last character is especially beneficial in countries without legal protection of breeders' rights.

Though in maize hybrid varieties have been grown since the early 1920's, their triumphal march started around 1960, when in several crops methods became available to emasculate the female parent of a hybrid, so that contamination of the hybrid with self-or sib-seed of the maternal parent was impeded. The most attractive and most generally applied type of emasculation is cytoplasmically inherited male sterility (CMS). **CMS** has largely contributed to the common use of hybrids in various horticultural crops, e.g. carrot, onion, paprika and hot pepper. But in many major horticultural crops reliable systems of CMS are as yet not available. Therefore it is very challenging trying to transfer CMS to these crops by genetic manipulation. As in several crops, i.e., maize, sugar beet and petunia, it has been proven that mitochondria play a major part in the control of CMS, endeavors initially were focused at manipulation of these organelles. Presently three approaches are explored, viz.:
- Cybridization
- Mitochondria injection
- Insertion of a ribonuclease gene

7.1.1. Cybridization.

Cybridization after protoplast fusion looks an attractive way to transmit CMS to a crop. The principle is demonstrated in Figure 5. The simplest way of introducing CMS, indeed, would be just replacing the mitochondria of the recipient by those of the male sterile donor (**organelles substitution**) but, especially with very distantly related fusion partners, this combination might be inviable or at least non-competitive because of **nucleus-organelles incongruity**. As it has been shown that after mixing organelles hybrid mt-DNA may come into being, cybridization seems more appropriate. The main problem with this approach is how to detect hybrid DNA which is near-identical to the receptor DNA except for the CMS gene. And how to protect the hybrid mitochondria against elimination to introduce CMS from petunia and wild Solanum species into tomato have failed so far.

7.1.2. Mitochondria injection.

Mitochondria injection is a new technique, which is applied i.a. by researchers of CPRO-DLO, The Netherlands, to introduce CMS radish mitochondria into various brassica crops. Though the first results are promising it still has to be proven that stable, competitive regenerants can be obtained from the treated cells. If the method is successful it might be a welcome alternative for the protoplast fusion approach applied by Pelletier et al. in France.

Spectacular results have been obtained by researchers of the Gent, Belgium based company Plant Genetic Systems. They have succeeded in constructing a so called **chimaeric ribonuclease gene**, which expresses only in the anthers during microspore

formation, by selectively destroying the tapetal layer that surrounds the pollen sac, and thus preventing the production of functional pollen.

The gene construct has been succesfully inserted into tobacco and rape seed. By connecting the (dominant) ribonuclease gene to a dominant herbicide resistance gene a simple system has been obtained to select for uniform populations of CMS plants, which serve as the mother parent in hybrid seed production. The main advantage of the ribonulease gene construct is, that it is applicable in all crops where fruits or seed are not the harvested product, e.g. in leafy vegetables, cabbages and Allium crops.

7.2. Resistance to Pathogens

Much effort is given by plant protectionists and plant breeders to protect crops against damage or destruction by animal and plant parasites. As there is a growing awareness that chemical compounds are unreliable, inappropriate and harmful for both environment and consumer, the call for alternative biological plant protection strategies is increasingly loud. Biological control, integrated pest management (**IPM**) or integrated crop management (**ICM**) are advanced as more appropriate strategies to protect crops in an environment-friendly way.

Genetic resistance of crops to pathogens can be an important component of the new strategies. In the past breeders have succeeded in introducing hundreds of resistance genes in crop varieties by larger scale screening within crop species and, progressively, by crossing crops with their wild relatives. But

many resistances, once obtained, were rapidly undone by the occurrence of new, virulent strains of the parasite.

Apart from the fact that the established way of resistance breeding is laborious and cumbersome, the 'attainable' sources of resistance are getting existed and alternative sources can only be found in distant organisms or by applying novel approaches. The various courses taken to introduce resistance or tolerance are summarized in Table 2. Some will be briefly discussed in the following section.

7.2.1. Resistance against virus

Also succeeded in producing transgenic of two potato cv. Russet Burbank containing coat protein genes of two potato viruses PVX and PVY. Coat protein protected potato varieties (i.a. of cv. Bintje) have also been created by Mogen, Leiden, The Netherlands.

In several countries projects are underway to protect crops against virus diseases by introducing genes that code for the **viral coat protein**. The presence of the virus coat gene in the plant genome appears to give (a variable level of) resistance to infection by virulent strains of the virus (**cross protection**). TMV and ToMV resistance in tomato has been obtained by the biotech division of the chemical multinational Monsanto. In preliminary experiments nearly no symptoms nor yield reduction was found after both late and early infection (Delanay, 1991; pers. comm.).

'Defective interference' is caused by excessive production of viral satellites in a plant. **Viral satellites** are small nucleic acids that are replicated in conjunction with the regular nucleic acids of (some) viruses. They are under the control of a specific gene. When the satellite-gene has been inserted in a plant, on infection by the 'mother virus' the satellite is replicated in large amounts at the expense of the reproduction of the virus itself, thus largely reducing the disease symptoms of infected plants. Promising results have been obtained with the CMV virus satellite (**CARNA 5**) in tobacco and tomato.

7.2.2. Resistance against fungi

Thionins are small proteins occurring in seeds of cereals (wheat, barley, oats). Extractions of thionins were highly toxic to *Xanthomonas campestris*, the causal agent of an important bacterial disease of cabbage, and to *Claviceps michiganense*, which causes bacterial canker in tomato. The thionin gene of barley (**hordothionin**) has been isolated and endeavors are made at introducing it in solanaceous crops and cabbages.

After infection of plants by pathogen specific proteins, indicated as (pathogen related) proteins are produced by the plant, which most probably play a part in the defense against the pathogens in question. Two well-studied groups of PR proteins are **chitinases** and **glucanases**, hydrolytic enzymes which can respectively degrade chitin and glucan, the most elementary components of the cell wall of fungi. Cell degradation results in delay or termination of growth of the fungus.

Of some chitinases the controlling gene has been isolated and transgenic plants have been obtained, with increased chitinase production, i.a. for the host-pathogen combination cucumber-*Didymella bryonidae*. But preliminary tests have made clear that most probably plants can only be effectively protected against pathogens with joint chitinase and glucanase production.

7.2.3. Resistance against insect.

A completely different approach has been chosen to transmit **insect resistance**. Larvae of several insect groups (butterflies, flies, mosquitoes) are killed after digestion of spores of the bacterium *Bacillus thuringiensis*. The crystalline spores contain proteins which are broken down to toxic particles in the intestinal tract.

The company Plant Genetics Systems in Gent, Belgium was the first to clone a. *B. thuringiensis* crystal gene and to transfer it into tobacco cells. Part of the regenerated plants appeared to be resistant to several caterpillars. Elsewhere insect resistant tomato and potato plants have been produced in a similar way. A serious drawback of the **Bt-toxins** is their specificity. There exist at least 30 different types. Some of them are toxic to larvae of *Lepidoptera* (butterflies), whereas others only intoxicate *Diptera* (flies, mosquitoes) or *Coleoptera* (bugs).

It is generally accepted that **proteinase inhibitors (PIs)** serve as defense mechanism to bacteria and insects. PIs inhibit or delay the activity of protein degrading enzymes which are produced in the digestive organs of insects. PIs mainly occur in seeds and tubers but are also produced by the plant on mechanical injury by insects or bacteria. An interesting case of resistance on the basis of this mechanism is the **cowpea trypsin inhibitor** or CpTi. During storage cowpea is heavily damaged by the insect Callosobruchus maculatus. Large scale screening of the cowpea germ plasm at IITA, Nigeria, resulted in the obtainment of one single resistant accession. The trypsin inhibitor was found to be the causal agent. Transgenic plants of i.e. tobacco and tomato are under investigation by several research groups in Europe and the USA. CpTi appears to confer resistance to various insects, including species of Heliothis, Spodoptera, Diabrotica and Manduca. PIs, unlike Bt-toxins, may cause harmful effects in man and animals. This reduces the applicability of PI-genes.

8. Concluding remarks

In the past, expectation with respect to the contribution of biotechnology to plant breeding have been greatly exaggerated. This was, to a large extent, caused by inadequate knowledge of molecular biologists concerning the complexity of gene expression, interallic interactions, and gene-environment interactions in living organisms.

Presently expectations have become more realistic. Though field screening of transgenic plants in many countries is bound to many restrictions because of strong public opposition against transgenic plants, it is very probable that the first transgenic commercial varieties of horticultural crops will become available within 4-6 years. With respect to herbicide resistance, the first cultivar-a hybrid of maize is expected to be released as early as 1992.

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Table 1 - Outline of GM techniques with relevance to plant breeding

	Technique	Variants
1.	Protoplast fusion	- somatic hybridization - cybridization - nucleus bustitution
2.	Chromosome transplantation	- whole chromosomes - chromosome fragments
3.	Organelle transplantation	
4.	Genetic transformation	- direct DNA uptake - cel or protoplast suspension - root, seed or embryo incubation - biolistic techniques - microinjection - other mechanisms
5.	Antisense RNA Restriction Fragment Length Polymorphism (RFLP)	

Table 2 - Sources of resistance used or in study for genetic transformation of cultivated crops

Pathogen	Resistance Mechanism	Methods	Crops
Viruses	Cross protection	Virus coat protein gene	tomato, potato
	Antisense RNA	Virus gene construct	tobacco
	Defective interference	Satellite RNA-gene	tomato, tobacco
Bacteria	Thionins	Foreign thionin genes	potato, tomato
	Toxin immunity	In vitro mutant selection	
	Resistance from foreign source	Foreign gene	tomato
Fungi	Cell wall degradation	Chitinase gene	cucumber
	Toxin immunity	In vitro mutant selection	tomato, potato
	Resistance from foreign source	Foreign gene, asymmetric fusion	
Insects	Bacterial toxin	Crystal gene of Bacillus thuringiensis	tomato, cotton, chrysanthenum
	Proteinase inhibitors	CpTi gene	potato, cowpea
	Foreign defense mechanism	Foreign gene; asymmetric fusion	potato
Nematodes	Intraspecific hypersensitity	Hypersensitivity gene	tomato, potato, beet
	Toxicity from foreign source	Toxin gene	rape, seed
	Reistance from other species	Foreign gene	potato, beet

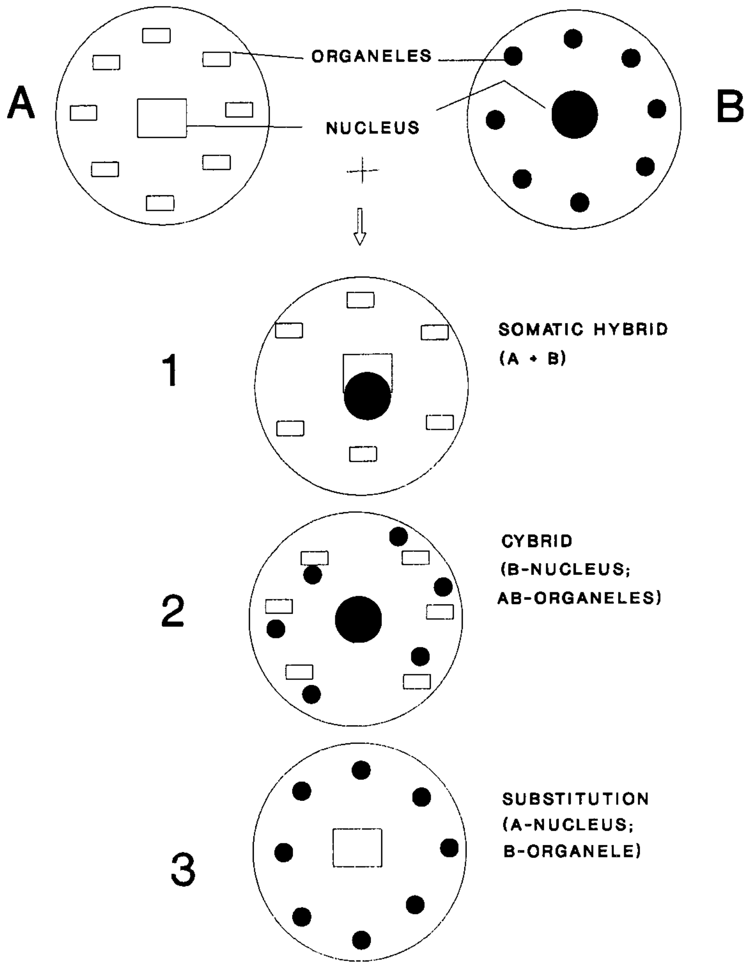


Figure 1 - Possible products of protoplast fusion

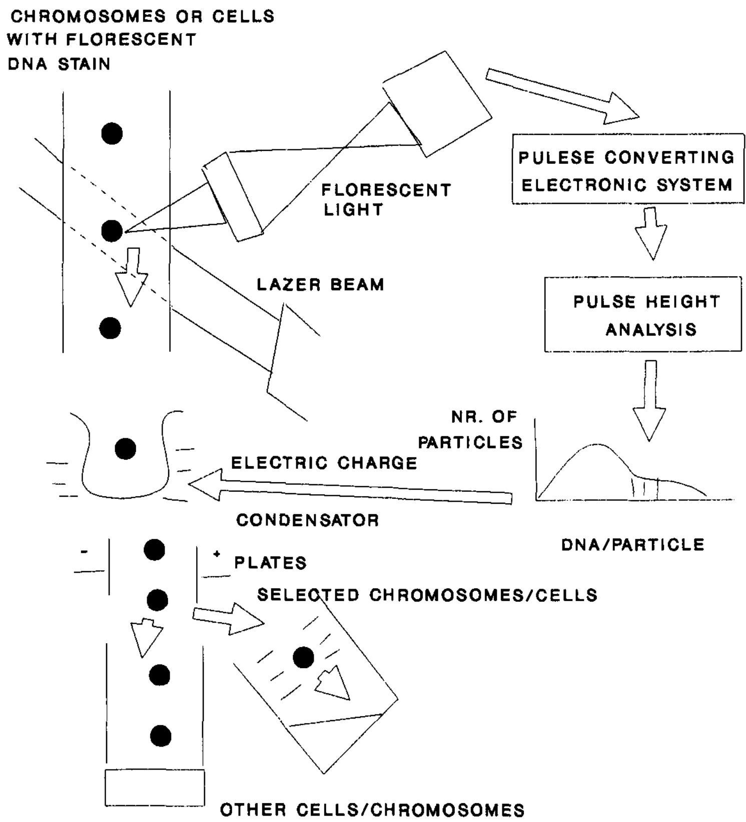


Figure 2-Principles of flow cytometer with cell or chromosome sorter. The DNA of the particles (cells, chromosomes) is stained with a non-toxic florescent stain. Individual particles pass a laser beam and produce flourescent signals of different strength depending on DNA content. The signals are electronically analysed. When a signal has a value belonging to required particle the flow is electronically charged for a short while, resulting in deflection of particle.

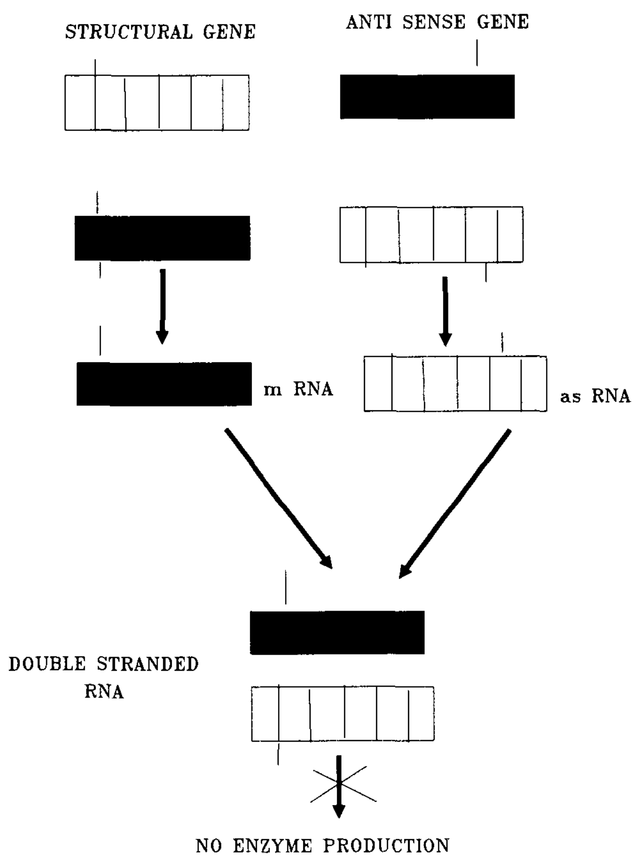
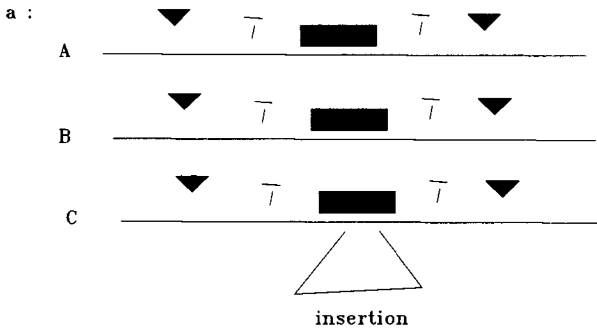


Figure 3–Selective suppression of gene expression by antisense RNA

a :



b :

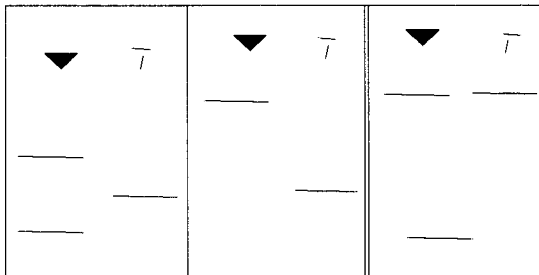


figure 4–Principle of RFLP detection. Explanation in text.
(Source: Wagenvoort & Den Nijs, 1988; Prophyta 42:275)

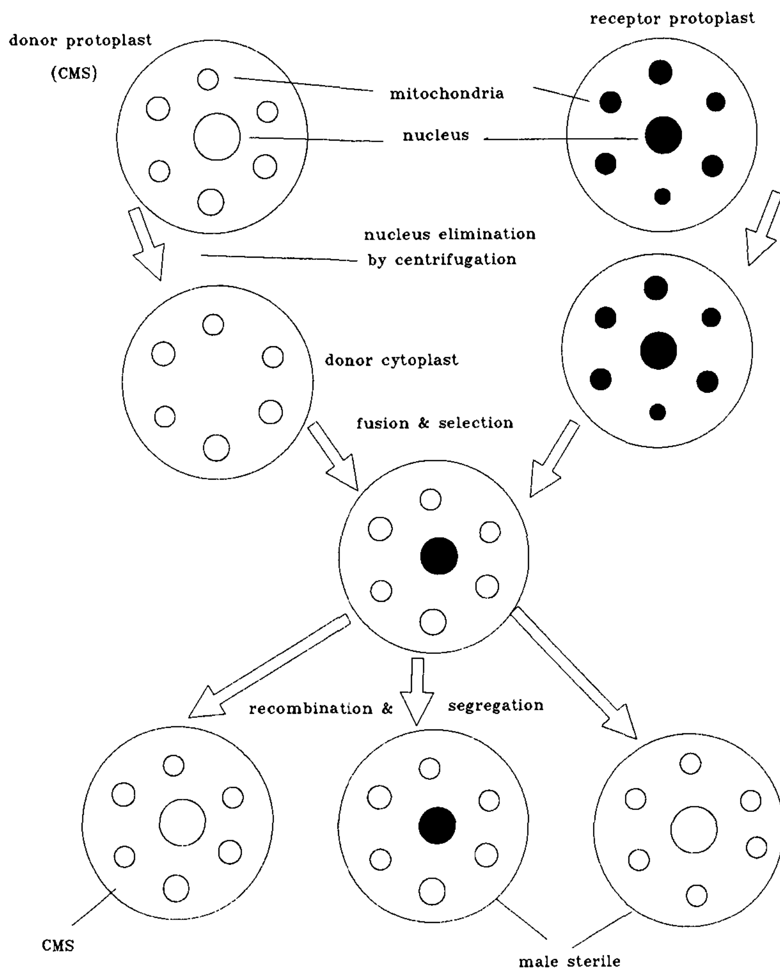


Figure 5–Theoritical pathway for the transmission of cyto-plasmic male sterility (CMS) between different species