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Tomato: a crop species amenable to improvement by cellular and molecular methods

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Summary

Tomato is a crop plant with a relatively small DNA content per haploid genome and a well developed genetics. Plant regeneration from explants and protoplasts is feasable which led to the development of efficient transformation procedures.

In view of the current data, the isolation of useful mutants at the cellular level probably will be of limited value in the genetic improvement of tomato. Protoplast fusion may lead to novel combinations of organelle and nuclear DNA (cybrids), whereas this technique also provides a means of introducing genetic information from alien species into tomato. Important developments have come from molecular approaches. Following the construction of an RFLP map, these RFLP markers can be used in tomato to tag quantitative traits bred in from related species. Both RFLP's and transposons are in the process of being used to clone desired genes for which no gene products are known. Cloned genes can be introduced and potentially improve specific properties of tomato especially those controlled by single genes. Recent results suggest that, in principle, phenotypic mutants can be created for cloned and characterized genes and will prove their value in further improving the cultivated tomato.

Introduction

Although tomato was cultivated by Indians in Mexico and introduced in Europe as early as the 16th century, it lasted until the second half of the nineteenth century before *Lycopersicon esculentum*, became generally recognized as a highly valuable and nutritious food crop. Nowadays, tomato is one of the major vegetable crops throughout the world. It is grown in both tropical, sub-tropical and temperate areas with an annual production of approximately 50 million metric tons (see Atherton & Rudich, 1986).

The commercial tomato (*L. esculentum* Mill., $2n = 2\times = 24$) is a member of a relatively small genus, *Lycopersicon*, within the large family Solanaceae. This genus consists of nine species with Peru as its main centre of diversity. Phylogenetic relationships on the basis of morphological and crossability studies reveal two species complexes,

viz. the 'esculentum complex' consisting of *L. esculentum, L. pimpinellifolium, L. cheesmanii, L. hirsutum, L. pennellii, L. chmielewskii* and *L. parviflorum* and the 'peruvianum complex' with its members *L. peruvianum* and *L. chilense*. The species of both complexes, which show a high degree of homosequentiality in their chromosomes, have served as an invaluable source of genetic variation and disease resistance genes in improving the cultivated tomato (Rick, 1982; Stevens & Rick, 1986; Atherton & Rudich, 1986).

As tomato turned out to be highly amenable to basic genetic and cytogenetic analysis, tomato breeding and genetics have gone hand in hand during the first half of this century. Thus, a wealth of genetic information and knowledge has been gathered on tomato that is now starting to be assimilated by cell- and molecular biologists/geneticists. With the advent of DNA recombinant technology, restriction fragment length polymorphism (RFLP)-mapping, effective DNA transformation procedures and cell- and tissue culture techniques, tomato has become also amenable to cellular and molecular analysis and manipulation. As a result, new trends in basic tomato research emerged in the early eighties, which promise to be of enormous potential also to practical breeding. Unfortunately, many of the molecular and cellular data and concepts gathered from research laboratories fail to reach those involved in commercial tomato production and *vice versa*. Accordingly, the integration of newly developed techniques and concepts into classical breeding proceeds slowly.

In this review, an attempt is made to condense the various lines of molecular and cellular tomato research that may be pertinent to breeding, but remained so far fragmented and outside the scope of the excellent monograph 'The Tomato Crop' (Atherton & Rudich, 1986) which is covering in detail the biology and cultivation of tomato.

Plant genetics

When compared to other crop plants, a relatively large amount of single gene determined traits have been described in tomato. Stevens & Rick (1986) estimated the total number of available monogenic mutants at 1200. These mutants include both spontaneous genetic variants (from inter- and intraspecific origin), as well as those induced by irradiation and chemical treatment. Genes in tomato have been characterized by their stable variant phenotype as compared with the cultivar Marglobe, representing the standard wildtype (+) allele and by monogenic inheritance of this variant phenotype. Lists with the name, symbol and if known the map position of these genes are published every few years in 'Genetic maps' (O'Brien, ed. Cold Spring Harbor Lab, 1987) and the annually appearing Report of the Tomato Genetics Cooperative. The majority of the genetic stocks are being maintained by the Tomato Genetic Stock Center, Department of Vegetable Crops, University of California at Davis, USA.

By repeated backcrossing of monogenic mutants with the English cultivar Ailsa Craig near isogenic lines for many of the well known marker genes have been developed at the Glasshouse Crops Research Station at Littlehampton, UK (Maxon-Smith & Ritchie, 1983).

Several examples in which monogenic traits have contributed to tomato breeding can be found in recent reviews (Stevens & Rick, 1986; Stevens, 1986; Tigchelaar, 1986). The respective genes affect the growth habit of the tomato plant (e.g. *sp, br, d)*, flower characteristics such as jointless pedicel *(j-l, j-2)*, they may cause male sterility (*ms* mutants), a delay in fruit ripening (*alc, nor, Nr, rin*) or they affect fruit pigmentation (*u, hp, gs, B, og, r, t, y*). In addition to these 'useful' genes, many well defined biochemical variants are known in tomato e.g. for genes involved in anthocyanin synthesis, allozymes, plant hormones and photoreceptors, which are especially useful for basic plant research.

Monogenic traits are of interest because they can be used relatively easily in breeding programs and also because the respective genes, in principle, can be isolated molecularly. Recently, cloning strategies are being developed, also for tomato, that allow the cloning of a gene for which only a variant phenotype and a map position is known (see sections Molecular markers and Transposontagging). A special challenge for tomato molecular biologists

will be the cloning of monogenic disease resistances (Table 1), many of which are dominant and derive from other *Lycopersicon* species (Rick, 1982).

Cytogenetics

The cultivated tomato is one of the few crop plants in which a critical and intensive cytogenetic analysis has been conducted. Among several factors that have favoured such an analysis, the following are prominent: (I) tomato is a naturally self-pollinated diploid species; (II) despite their rather small size, the chromosomes can be accurately identified; (III) numerous easily recognizable genetic markers are available; and (IV) the plants are highly prolific so that genetic analysis is easy. Utilizing these advantages, cytogeneticists have built up a vast amount of information regarding the genome of tomato. Some of the salient features of this information will be outlined below:

Despite the large basic chromosome number (n = 12), tomato must be considered a true diploid species from the following lines of evidence. Firstly, all the 12 pairs of chromosomes are morphologically distinct from each other (Barton, 1950; Ramanna & Prakken, 1967), thus ruling out the possibility of polyploidization in its recent evolutionary history. Secondly, analysis of chromosome pairing in tomato monohaploids has indicated the absence of intragenomic homology (Ecochard et al., 1969). Thirdly, extensive genetic and molecular analysis has revealed that, with only a few exceptions, duplicate loci are absent in the genome (Rick, 1971; Tanksley et al., 1987). In addition, there is evidence that the karyotypes of *Lycopersicon* species are strikingly similar to those of diploid tuberous *Solanum*, a genus closely related to the former (Gottschalk, 1954; Ramanna, 1979), indicating that the karyotypes of the species of these two genera have been highly conserved during evolution. In fact, Hawkes & Smith, (1965) have argued that the genus *Solanum* and its relatives have evolved at least for 100 million years. The similarities between the karyotypes of *Solanum* and *Lycopersicon* species are also evident from the cytology of intergeneric hybrids (Menzel, 1962; Klush & Rick, 1963).

The chromosomes of tomato have the unique characteristic of being linearly differentiated into distinct segments of euchromatin and heterochromatin. As in many other organisms, heterochromatin in tomato is relatively darkly stained as compared to euchromatin. This differentiation can be cytologically demonstrated in both mitotic and meiotic cells by treating them with common nuclear stains such as acetocarmine or Feulgens reagent (Ramanna & Prakken, 1967).

Table 1. Diseases for which monogenic resistances are present

Disease	Pathogen	Type	Resistance genes*
Alternaria stem canker	*Alternaria alternata* f.sp. *lycopersici*	F	Asc
Early blight	*Alternaria solani*	F	ad
Leaf mold	*Cladosporium fulvum*	F	*Cf* series
Fusarium wilt	*Fusarium oxysporum*	F	*I, I-2*
Late blight	*Phytophtora infestans*	F	*Ph*
Corky root	*Pyrenochaeta lycopersici*	F	*pyl*
Verticillium wilt	*Verticillium* sp.	F	*Ve*
Gray leaf spot	*Stemphylium* sp.	F	*Sm*
Septoria leaf spot	*Septoria lycopersici*	F	*Se*
Bacterial Speck	*Pseudomonas syringae*	B	*Pto*
Tobacco mosaic virus	*TMV*	V	*Tm-1, Tm-2*
Root knot nematodes	*Meloidogyne* spp.	N	*Mi*

F: Fungus; B: Bacterium; V: Virus; N: Nematode.

* Capital indicates dominance of the resistant allele.

The karyotypes can be analysed both at metaphase stage of mitosis and pachytene stage of meiosis. However, identification of metaphase chromosomes of tomato is more difficult and less accurate than those of pachytene chromosomes. For this reason, all the cytogenetic analysis has been possible in tomato only because of the ease and accuracy with which pachytene chromosomes can be identified. A common feature to both mitotic and meiotic chromosomes is that the heterochromatic segments are invariably present in the proximal positions of the centromeres. This also means, that the position of euchromatic segments is invariably distal. The only exceptional chromosome arm is the short arm of chromosome 2 which is entirely heterochromatic. This arm possesses the nucleolar organizing region (NOR) which can be recognized as a constriction in one pair of chromosomes at mitotic metaphase and as a nuclear bivalent at pachytene stage both of which are marked as chromosome 2.

In order to localize genes on chromosomes, the identification of different types of aneuploids is one of the important pre-requisites. A significant step in this direction was achieved through the identification of a complete primary trisomic series in tomato (Rick & Barton, 1954; Rick et al., 1964). Subsequently, the detection and use of various secondary and tertiary trisomics facilitated the localization of genes to specific arms of chromosomes (for detailed review see Gill, 1983). Especially with the help of tertiary trisomics and telotrisomics, the centromere positions have been rather accurately mapped on the chromosomes. However, a singular aspect of gene localization in tomato is the application of the so called 'pseudodominant' method which has facilitated the most accurate delimitations of some of the loci in the genome (see Khush & Rick, 1968). This technique involves the irradiation of the pollen of a parent that carries certain normal genes and the subsequent pollination to a parent with known recessive homozygotes. In the F_1, those recessive genes that show 'pseudo-dominance' are selected and analysed cytologically at pachytene stage in order to detect the deletions corresponding to the pseudo-dominant gene. By this method Khush & Rick (1968) approximated 35 loci on 18 of the 24 chromosome arms. Among these, some of the loci could be delimited to very narrow chromosome segments of 5 μ or less (Rick, 1971).

In addition to the classical linkage map, the development of a molecular map of the genome (see section Molecular markers) had added a new dimension to the cytogenetics of the tomato. The molecular maps are developed on the basis of allelic differences that exist for the lengths of restriction fragments (restriction fragment length polymorphisms or RFLPs) for certain loci which can be detected through DNA hybridization techniques. These analyses have been carried out by using segregation data of interspecific hybrids between *L. esculentum* and *L. pennellii*. Although the chromosomes of these two species are supposed to be homosequential (Khush & Rick, 1963), it is by no means certain that the crossing over in the interspecific hybrids is strictly comparable to that in pure *esculentum*. In fact, Rick (1969) has pointed out reduced recombination and marked deviations in segregation for specific loci in the back-cross segregations of *L. esculentum* × *L. pennellii* hybrids when compared to normal tomato. In view of this, the map distances estimated in the case of molecular maps ought to be treated with caution and quantitative comparisons between molecular and classical maps should await more critical data.

Molecular biology

It is not within the scope of this section to review all the gene systems currently being studied at the molecular level. They range from housekeeping genes to genes involved in reproduction and in adaptation to abiotic and biotic stress. Some of them are studied to understand the fundamental biology of tomato, while others are examined because of their supposed economic importance. The reader is referred to a book entitled 'Tomato Biotechnology', the Proceedings of a Symposium held at the University of California, Davis, August 20–22 1986 (Eds. D.J. Nevins & R.A. Jones; Alan R. Liss Inc., New York) for an extensive discussion of the various gene systems. In this section we will

restrict ourselves in discussing the basic molecular features of the chloroplast-, mitochondrial and nuclear genome. In the following sections, specific genes and molecular approaches as they apply to practical breeding will be discussed.

Chloroplast DNA

Chloroplast DNA molecules from a wide variety of plants have many features in common and are remarkably conserved in size and structure. They are relatively small (120–200 kbp), circular double-stranded molecules containing a pair of inverted repeat sequences which are flanking large single copy and small single copy regions (Palmer & Thompson, 1982; Palmer, 1985; Umesono & Oreki, 1987).

Recently, the complete sequence of two chloroplast genomes has been established, viz. *Nicotiana tabacum* (Shinozaki et al., 1986) and *Marchantia polymorpha* (a liverwort; Ohyama et al., 1986). Sequence comparison of the two chloroplast genomes of these evolutionarily very distant plant species reveals a remarkably similar organization, strongly suggesting that chloroplast genomes in all green plants may have originated from a unique ancestor (Umesono & Oreki, 1987).

Though no complete nucleotide sequence is yet available for tomato chloroplast DNA, all the evidence derived from complete restriction maps and clone banks point to a molecular organization which is in agreement with the picture described above (Phillips, 1985a, 1985b; Piechulla, et al., 1985; Hanson & McClean, 1987; Gruissem et al., 1987). The tomato chloroplast DNA is a circular molecule of 156.6–159.4 kbp (Phillips, 1985a; Piechulla et al., 1985) with two identical sequences of 23.3–27.7 kbp which are arranged as an inverted repeat and are located at similar positions as in other plant species. Comparison of the tomato restriction map with those of tobacco and petunia shows a high conservation of restriction sites (Phillips, 1985a). By using heterologous probes from pea, wheat and spinach and by using coupled *in vitro* transcription/translation on tomato plastid DNA clones, 14 protein-coding genes and the rDNA genes could be mapped on the tomato restriction map (Phillips, 1985b). The results show that all studied tomato chloroplast genes are in similar positions as those in spinach, tobacco and petunia.

One of the first studies to be carried out on the phylogenetic relationships of chloroplast DNA sequences among *Lycopersicon* and *Solanum* species were those by Palmer & Zamir (1982). Though a limited amount of sequence divergence was found, by using a large number of restriction enzymes, sufficient sequence variation was detected among the 484 restriction sites surveyed to allow the construction of a chloroplast DNA phylogeny. The phylogenetic tree thus established, reflected the evolutionary relationships based on morphology and crossability, except for *L. chmielewski* which is closer to *L. peruvianum* and *L. chilense* according to chloroplast DNA polymorphisms than other species of the *esculentum* complex.

The restriction site polymorphisms between the chloroplast DNA of *Lycopersicon* species has been applied successfully to identify the parental origin of chloroplast DNAs found in somatic hybrid plants (O'Connell & Hanson, 1985, 1986; Tan, 1987).

Mitochondrial DNA

Unlike chloroplast genomes, mitochondrial genomes from higher plants exhibit wide variation in size, ranging from approximately 218 kb in *Brassica campestris* (Palmer & Shields, 1984) via 570 kb in maize (Lonsdale et al., 1984) to 2500 kb in muskmelon (Ward et al., 1981). In addition, they contain many direct repeats. Restriction fragment length analysis of tomato mitochondrial DNA gave an estimate of 300 kb as the minimal size (McClean & Hanson, 1986; Hause et al., 1986; Hanson & McClean, 1987). For *Brassica campestris* it was shown, that the mitochondrial genome is organized into three physically distinct circular molecules (218 kb, 135 kb and 83 kb), one of which bears the entire sequence complexity of the genome. The two smaller circles contain distinct subsets of the 'master' chromosome (Palmer & Shields, 1984). A

similar but more complex pattern exists in maize, with a master chromosome of 570 kb and multiple circular species of DNA (47 to 503 kb) which are derived from each other by recombination (Lonsdale et al., 1984). So far, it is not known whether multiple DNA species are present within the tomato mitochondrion. Intergenomic recombination has been suggested to occur in generating novel genomes in tomato somatic hybrids following fusion (O'Connell & Hanson, 1985, 1986).

Mitochondrial DNA sequence divergence among *Lycopersicon* and closely related *Solanum* species has been studied by McClean & Hanson (1986). As compared to chloroplast genomes, mitochondrial genomes of tomato species are less conserved. They show, however, much less divergence than mammalian mitochondrial genomes. The mitochondrial DNA-derived phylogenetic relationships resemble the relationships based on 'classical' taxonomic data, except for the grouping of *L. pennellii* with *L. peruvianum* and *L. chmielewskii* and the close relationship between *L. hirsutum* and *L. esculentum*.

Nuclear DNA

Among the higher plants, tomato has a relatively small genome. Using chemical methods and flow cytometric analysis of nuclei, the nuclear DNA content of a wide variety of plants has been determined and shown to range from 0.5 pg to over 200 pg per haploid genome (Bennett et al., 1982; Galbraith et al., 1983). Within this range, tomato ranks low with 0.74 pg per haploid nucleus. As one pg of double stranded DNA corresponds to 0.965×10^9 nucleotide pairs, this amount represents approximately 0.71×10^9 nucleotide pairs, which is roughly 180 times as much as *E. coli*, 10 times as much as *Arabidopsis thaliana* but 5 and 13 times as less as maize and rye, respectively. The genetic content of the tomato genome has been taken as approximately 1200 cM (Tanksley, 1983). Thus, on average, one cM in tomato corresponds to about 600 kb.

A large proportion of the nuclear DNA of higher eukaryotes is composed of repetitive, non-protein coding, sequences and tomato is, in this regard, no exception. On basis of their chromosomal distribution, repeated DNA sequences can be divided into four major classes: repeated sequences (I) located at or around centromeres (II) associated with telomeres (III) clustered at specific domains on chromosome arms and (IV) interspersed, intra- and interchromosomal, among other genomic (single copy) sequences (see Flavell, 1982; Ganal et al., 1988; Manuelidis, 1982; Zabel et al., 1985). Recently, a systematic survey has been conducted in tomato on the proportion and distribution of single-copy sequences and the various repetitive sequence classes (Ganal et al., 1988; Tanksley et al., 1987; Schweizer et al., 1988; Zabel et al., 1985; Zamir & Tanksley, 1988). Unlike the genome of various monocots (wheat, rye and maize) and other dicots like pea, in which repeated sequences account for 60–80% of the genome, the tomato genome is comprised largely of single copy and low copy number (2–20 copies) sequences. Among the four major classes of repetitive DNA sequence families identified, the majority is specific for *Lycopersicon* species and *Solanum lycopersicoides*.

Application of the RFLP-technology (see section Molecular markers) has been a major tool in studying the chromosomal position of protein-coding genes. By linkage analysis the position of more than 100 random cDNA clones as well as various cloned genes of known function has been established (Mutschler et al., 1987; Tanksley et al., 1987). From these studies, the following conclusions can be drawn.

1. The majority of genes in tomato are represented by a single copy at a single locus (Bernatzky & Tanksley, 1986) and are dispersed throughout the genome.
2. Approximately 30–35% of the cDNA clones correspond to duplicated genes, the majority of which are at genetically independent loci. A minority (10–15%) of the clones are represented by 3–5 loci which reside on different chromosomes. So far, at least 20 sets of unlinked, duplicate genes have been detected.
3. Two of the proteins involved in photosynthesis – small subunit of ribulose-bis-phosphate carboxylase (RBCS) and a chlorophyl a/b binding poly-

peptide (CAB) – are encoded by multigene families. The RBCS – multigene family consists of five genes which are distributed among three loci at chromosome 2 and chromosome 3 (Vallejos et al., 1986; Pichersky et al., 1987). The CAB-gene family consists of 11–12 genes distributed among five loci at chromosomes 2, 8, 7 and 12 respectively (Vallejos et al., 1986; Pichersky et al., 1987).

4. No internal duplication of chromosome segments has been detected except for chromosome 2, which contains a duplication of a chromosomal fragment with an RBCS-gene and two anonymous cDNA sequences.

In vitro culture methods

Like other Solanaceae, such as *Nicotiana* and petunia, tomato is relatively favourable for *in vitro* culture of various types of tissues and organs. Procedures have been developed for the *in vitro* culture of embryos, shoots, roots, leaf explants, and protoplasts. These methods have opened the possibilities for various applications, e.g. embryo rescue, *in vitro* mutant selection, basic biochemical and virus research, the isolation of mitotic chromosomes, protoplast fusion and genetic transformation. In this section some of the *in vitro* methods will be briefly outlined.

Callus and suspension cultures

Tomato tissue can develop callus or shoots depending upon the composition of the medium (especially hormonal balance), culture conditions and factors such as age, vigour and the genotype of the donor plant. In order to initiate the culture, any one of the following tissues or organs have been used: roots, cotyledons, hypocotyls, stems, leaves, anthers and embryos.

Callus is generally induced on media with relatively high auxin contents combined with moderate levels of cytokinins. When callus of most *L. esculentum* cultivars is repeatedly transferred on to the same medium, the ability to regenerate plants from such calli upon transfer to cytokinin-containing media is very limited (Morgan & Cocking, 1982). In contrast, callus of *L. peruvianum* maintains its regeneration capacity for a much longer time (Mühlbach, 1980; Thomas & Pratt, 1982). This 'regeneration capacity' has been bred into *L. esculentum* genotypes by backcrossing hybrids of *L. peruvianum* × *L. esculentum* with *L. esculentum* (Koornneef et al., 1986; Koornneef et al., 1987). These genotypes facilitate the application of cellular techniques that aim at obtaining genetically modified plants.

Shoot regeneration on explants

Direct regeneration of adventitious shoots on explants placed on media with a high cytokinin/auxin ratio has been studied by many authors (Padmanabhan et al., 1974; Kartha et al., 1976; Behki & Lesley, 1976; Frankenberger et al., 1981a; Locky 1983; Zelcer et al., 1984). In general, the observation of Kartha et al., 1976 that media with benzyladenine (BA) or zeatin (Z) at concentrations of 1–5 mgl-l combined with indoleacetic acid (0.1–0.5 mgl-l) are most effective for shoot regeneration has been confirmed. Shoot formation by *L. esculentum* genotypes is often accompanied by callus formation on the explant. However on *L. peruvianum* explants, shoots often appear without the simultaneous appearance of callus tissue. Also within *L. esculentum* genotypes, genetic differences for shoot forming capacity have been described (Ohki et al., 1978; Frankenberger et al., 1981b; Tan et al., 1987b).

Plant regeneration from protoplasts

Unlike *L. peruvianum* protoplasts, those of *L. esculentum* were found recalcitrant to regenerate (Mühlbach, 1980; Morgan & Cocking, 1982). Recently, however, (Shahin, 1985; Niedz et al., 1985; Tan et al., 1987a) procedures have been developed which allow a more efficient regeneration of tomato protoplasts from a wide range of genotypes. The importance of a proper pretreatment of the plant

material to be used as a source for protoplasts (Tabaeizadeh et al., 1984; Shahin, 1985; Tan et al., 1987a) and a relatively low ammonium ion content in the media (Zapata et al., 1981) are generally recognized as being critical.

Transformation

In this section methods are reviewed to transfer well characterized DNA sequences into the plant genome and to select for transformed plant cells. Two fundamentally different delivery systems are described. Firstly, direct (physical) DNA delivery systems to plant cells will be discussed. Secondly, transformation methods based on the natural interaction between the soil bacterium *Agrobacterium tumefaciens* and plants will be in focus.

Selection markers

Upon introduction of new genetic information in plant cells, it is crucial to be able to distinguish between transformed and non-transformed plant cells. To this end, vectors have been developed in which the gene of interest is carried along with a gene which confers resistance to an antibiotic that is lethal to plant cells. Following introduction of the vector into a population of plant cells, transformed plant cells can be selected for by growth in the presence of the antibiotic. Genes coding for antibiotic resistance have been isolated from bacterial strains and brought under control of plant gene expression signals to allow proper expression in plant cells. Thus, dominant selectable markers have been constructed which confer resistance to the antibiotics kanamycin (Bevan et al., 1983; Herrera-Estrella et al., 1983a), chloramphenicol (Herrera-Estrella et al., 1983b), hygromycin (van den Elzen et al., 1985b; Waldron et al., 1985) and bleomycin (Hille et al., 1986), to the herbicide phosphinotricine (PTT; De Block et al., 1987) and to the drug methotrexate (Herrera-Estrella et al., 1983b), an inhibitor of dihydrofolate reductase. Among these markers, kanamycin resistance is the most popular in tomato, although hygromycin resistance is equally effective (Weide, pers. comm.).

Direct gene transfer

In the past few years methods have been developed to directly deliver characterized DNA sequences into plant cells. These procedures have commonly be called direct gene transfer. In general, plant protoplasts are incubated with calciumphosphate-DNA precipitates in conjunction with polymers like polyethylene glycol (Paszkowski et al., 1984; Negrutiu et al., 1987) or polyvinylalcohol (Hain et al., 1985). More recently, conditions have been established for electrically introducing DNA into plant protoplasts (so-called electroporation, Shillito et al., 1985), and also microinjection of DNA into plant protoplasts, either cytoplasmic or intranuclear, has been successfully applied (Crossway et al., 1986; Miki et al., 1987). A new development is the application of high velocity micro projectiles, coated with DNA, in transformation of plant cells (Klein et al., 1988). This approach has the advantage of not being limited to protoplasts.

Transformation of tomato protoplasts has been performed using the calciumphosphate DNA transformation procedure (Koornneef et al., 1986; Pieterse & Koornneef, 1988). Selection for kanamycin resistance (50 mg/l) encoded by the plasmid DNA used in transformation, was started two weeks after protoplasts transformation. Using this procedure, kanamycin resistant calli were obtained at frequencies up to 10^{-3}.

Six tomato transformants were chosen for further detailed molecular analysis (Jongsma et al., 1987). By Southern blot analyses it was shown that the transformants contain up to three copies of the kanamycin resistance gene integrated into the tomato nuclear DNA. Less than full length plasmid copies were found to be integrated into the nuclear genome suggesting that nuclease activity had linearized and degraded the plasmid DNA to different extents prior to integration. Evidence was found for physical linkage of integrated plasmid DNA fragments in transformants containing more than one integrated fragment. A similar linkage was observed in tobacco transformants obtained fol-

lowing electroporation (Riggs & Bates, 1986).

Agrobacterium tumefaciens – mediated transformation methods

A remarkable example of naturally occurring plant genetic engineering is provided by the soil bacterium *Agrobacterium tumefaciens*. This phytopathogen transforms susceptible plant cells to cause crown gall, a neoplastic disease of dicotyledonous plants. The transfer, integration and expression of T (transferred)-DNA from its Ti (tumor-inducing)-plasmid into the nuclear genome of plant cells is now known to be the molecular basis for pathogenicity of *A. tumefaciens* (for reviews see: Hille et al., 1984; Schell et al., 1984; Koukolilova-Nicola et al., 1987).

Two kinds of plant gene vectors have been developed to introduce new genetic information in plant cells (for a recent review: see Rogers & Klee, 1987). In the so-called cointegrate type T-DNA vector, all oncogenic functions located on the T-DNA of the Ti-plasmid have been deleted and replaced by a DNA sequence, e.g. a copy of plasmid pBR322 which functions as a target for homologous recombination. All sequences, cloned in *E. coli* on a pBR322 like plasmid, can then be transferred to *A. tumefaciens* and placed in the T-DNA of the Ti-plasmid following homologous recombination between the pBR322 sequences (Zambryski et al., 1983; Van Haute et al., 1983).

In the binary type T-DNA vector, the Ti-plasmid has been split into two independently replicating plasmids: one carrying virulence functions and the other the T-DNA. The plasmid containing the T-DNA (from which the oncogenes have been deleted) is now sufficiently small and can be used in *E. coli* for cloning the desired genes. This plasmid is then transferred back to *A. tumefaciens* harboring the other plasmid containing the virulence functions and the resulting strain can be used for plant cell transformation (Hoekema et al., 1983; De Framond et al., 1983).

Currently, the most widely used techniques for the efficient introduction of new genetic information into plant cells mediated by *A. tumefaciens* are the cocultivation method (Marton et al., 1979) and the leaf disc method (Horsch et al., 1985). In the first method, purified plant protoplasts are incubated for 2–4 days to allow cell wall regeneration to occur. Then, as the first cell divisions are visible, *A. tumefaciens* is added in a ratio of 100 bacteria per protoplast and the mixture is incubated (cocultivated) for 2–4 days. Subsequently, the bacteria are removed by washing and the plant cells are further cultured in the presence of antibiotics to kill the remaining bacteria. (Fraley et al., 1984; Van den Elzen et al., 1985a; Hille et al., 1986). Using this procedure, transformation frequencies up to 50% of the regenerating micro-calli have been reported. However, both the cocultivation method and the direct gene transfer methodology have their limitations in genetic manipulation of plants, since they are both based on protoplast regeneration. Far from all plant species can be regenerated from protoplasts and plants derived from protoplasts often show variation due to mutations and/or chromosomal abnormalities (so-called somaclonal variation).

A method that bypasses most of these limitations is the leaf disc method as developed by Horsch et al., (1985), which combines the efficient plant cell transformation capacity of *A. tumefaciens* with regeneration capacity of leaf explants. To this end, leaf discs are cut from leaves, dipped in a culture of *A. tumefaciens* and incubated for two days. Subsequently, the leaf explants are transferred to a shoot-inducing medium containing antibiotics to kill the bacteria and to select for transformed plant cells. Using the leaf disc transformation procedure all plant species, which can be regenerated from leaf explants and which can be infected by *A. tumefaciens*, can be transformed and regenerated into transgenic plants. In case of the cultivated tomato all genotypes tested, including some cultivars, could be transformed and regenerated into fertile plants. The newly introduced kanamycin resistance gene was shown to transmit through meiosis in a Mendelian manner (Koornneef et al., 1986; McCormick et al., 1986).

Molecular markers

Over the past decades, a linkage map has been constructed containing loci for morphological markers, resistance genes and mutants affecting physiological functions. Though this map has served well in genetic studies, its applications in plant breeding programs is rather limited. Therefore, more recently, particular attention is paid to the construction of a detailed molecular map based on protein and restriction fragment length polymorphism (RFLP) markers which do not affect the phenotype. Protein markers are loci encoding proteins (isozymes) that can be separated by electrophoresis and subsequently visualized by specific staining to establish the presence or absence of specific alleles. The term restriction fragment length polymorphism has been introduced to describe the variation in length of DNA restriction fragments from two genetically distinct individuals that are homologous to a labeled cloned DNA sequence. Actually, both types of markers, protein and RFLP, are based on polymorphisms which are due to differences in the primary sequence of genomic DNA. With protein markers, the polymorphisms are usually detected as electrophoretic or antigenic polypeptide variants, whereas DNA markers directly reflect the variation at the DNA level, being either in a coding or noncoding region. In the human genome the variation in restriction fragment length is mainly due to single basepair changes (Barker et al., 1984; see also Gusella, 1986) while in plants, like maize, changes by insertions and deletions seem to be common (Helentjaris et al., 1985).

As discussed by Tanksley (1983) and many others (Beckmann & Soller, 1983, 1986; Soller & Beckmann, 1983; Burr et al., 1983; Helentjaris et al., 1986; Landry & Michelmore, 1987b) molecular markers possess several distinct advantages over morphological and other conventional 'classical' markers. The following discussion was given by Tanksley (1983).

1. While phenotypes of most morphological markers can only be recognized at the plant level, genotypes of molecular loci can be established at cellular, tissue and plant levels.
2. The number of distinguishable alleles at morphological or biochemical loci is rather limited and often dependent on the application of exogenous mutagens. RFLPs, however, provide a virtually unlimited number of markers, as the polymorphism does not need to alter the phenotype or the charge of a protein to become detectable. With markers for all chromosomes at intervals of 10–15 map units, the flow of any gene of interest through segregating generations in a breeding program can be monitored, using a linked (anonymous) RFLP-marker as 'tag',
3. Morphological markers are usually associated with unwanted phenotypic effects, which is not the case with molecular markers.
4. As alleles of most molecular markers are codominant, all possible genotypes can be distinguished in any segregating generation.
5. Only a few epistatic or pleiotropic effects occur with molecular markers, allowing a virtually unlimited number of segregating markers to be monitored in a single population.

Among the molecular markers, isozyme markers have proven their usefulness already in genetic studies and breeding programs of various crops (Tanksley & Rick, 1980; Tanksley & Orton, 1983). For tomato, an isozyme linkage map has been constructed containing 41 isozymic genes corresponding to 15 unique enzyme reactions. Thirty six of these genes have been mapped to their respective chromosomes (Tanksley & Bernatzky, 1987). Thus, tomato isozymes have served many purposes (see Tanksley & Bernatzky, 1987), like e.g., verifying the purity of hybrid seed (Tanksley & Jones, 1979), tagging genes of economic importance (Rick & Fobes, 1977; Tanksley et al., 1984), detecting genes and chromosomes from wild species following introgression (Rick et al., 1986), identifying somatic hybrids and mapping genes underlying quantitative variation (Vallejos & Tanksley, 1983). The great potential power of using isozyme markers might best be inferred from the publication of a two volume manual entitled 'Isozymes in Plant Genetics and Breeding' (Tanksley & Orton, Eds. 1983) which comprises an extensive discussion of both theoretical and practical aspects for many crops.

Nevertheless, isozymic loci suffer several limitations which are not inherent to the use of DNA markers (RFLPs). The great potential usefulness of RFLPs in basic plant genetic studies and plant breeding programs has been advocated in the early eighties by Beckman & Soller (1983), Burr et al., (1983), Helentjaris et al., (1985) and Tanksley (1983), following a thorough description of the theoretical basis of using RFLPs as a new source of markers for the diagnosis of human diseases (Botstein et al., 1980; Little et al., 1980; Phillips et al., 1980; Gusella et al., 1983; Gusella, 1986). Since then, RFLP markers have found their way as powerful tools in human genetics and cloning genes which are linked to them and would otherwise not have been clonable (see Gusella, 1986). In plants, the integration of RFLP-technology in basic and applied research proceeds more slowly but should have a similar impact. Thus far, genetic linkage maps based on RFLPs have been constructed for tomato (Bernatzky & Tanksley, 1986a, b; Helentjaris et al., 1985. Mutschler et al., 1987; Tanksley et al., 1987), lettuce (Landry et al., 1987a, b) maize (Helentjaris et al., 1986; Helentjaris, 1987) and *Arabidopsis thaliana* (Chang et al., 1988).

For an elaborate discussion of the methodology and the prospects of the applications of RFLP analysis in plant systems, the reader is referred to reviews by Landry & Michelmore (1987a, b) and Tanksley et al. (1987).

In a first series of experiments on RFLPs in tomato, random cDNA clones, derived from leaf mRNAs, were used successfully in genetic mapping (Bernatzky & Tanksley, 1986a, 1986b; Helentjaris et al., 1985). These studies have been extended more recently with genomic clones representing single/low copy number sequences (Helentjaris et al., 1986; Tanksley et al., 1987; Young et al., 1987).

By linkage analysis, the chromosomal position of more than 200 DNA markers, including random (anonymous) cDNA clones, genomic clones and various cloned genes of known function, has now been established (Mutschler et al., 1987; Tanksley et al., 1987, Young et al., 1987). Thus, a molecular linkage map of tomato has been constructed in which each of the 12 chromosomes is represented with a characteristic set of DNA markers.

With a molecular linkage map containing more than 200 DNA markers now available, how many more markers are needed or desirable to saturate the tomato genome? The answer to this question depends on the type of application being pursued, namely whether the RFLP markers are considered to be used as genetic markers to tag chromosome segments in breeding programs or as molecular tools for isolating nearby genes of interest. For most purposes in plant breeding, the current map is already sufficient in tagging genes or chromosomes of interest or detecting quantitative trait loci (QTLs), as was shown for the loci controlling fruit mass (Paterson et al., 1988), fruit pH (Paterson et al., 1988) and the soluble solid content (Osborn et al., 1987; Paterson et al., 1988 Tanksley & Hewitt, 1988). Another example of the successful application of current RFLP markers in tomato concerns the determination of the chromosomal location of foreign DNA sequences introduced in the genome by *A. tumefaciens* (Chyi et al., 1986). For the other type of application – RFLP markers as molecular tools for isolating genes linked to them – more markers should be mapped to fill in the gaps. An alternative to mapping RFLP's in the proximity of the desired gene is to identify DNA markers revealing polymorphisms between near isogenic lines as was recently described for the Tm-2a (TMV-resistance) gene (Young et al., 1988).

Though gaps are certainly not the plant molecular biologist's wish-dream, the development of new types of clone libraries (chromosome jumping and linking libraries, Poustka & Lehrach, 1986) and new techniques for generating and fractionating restriction fragments up to 4000 kb. (Barlow & Lehrach, 1987) makes even these distances no longer elusive. Currently already, the gap in resolution between classical genetics and molecular DNA techniques is being bridged in mammalian systems (Barlow & Lehrach, 1987; Poustka & Lehrach, 1986). Genetic loci which were only known from their mutant phenotype and genetic map position have become amenable to molecular analysis and cloning, following the construction of physical maps at the cM-level. Application of RFLP-markers in conjunction with these long-range cloning

and mapping techniques could have a similar impact on tomato genetics. Disease resistance loci are obvious candidates for such a 'reverse genetic' approach, since their protein products are as yet unknown and identification of coding sequences corresponding to the locus remains otherwise elusive. As a prelude to constructing a physical genetic map of the nematode resistance Mi region on chromosome 6 of tomato, we have recently developed a procedure for the isolation of megabase-sized chromosomal DNA and the subsequent generation and fractionation of restriction fragments in the range 100–1500 kb (van Daelen et al., 1989). These conditions should allow the construction of long-range restriction maps and the isolation of genes in close proximity to RFLP markers. Given the pace of these new mapping and cloning techniques, it is anticipated, that physical genetic maps of various chromosomal regions of tomato will be established with the next couple of years.

Transposon tagging

Isolation of plant genes and their introduction in crops gives a new dimension in crop improvement. However, it is often difficult to isolate these useful genes molecularly, since, in most cases, gene products are not known. Two general approaches are currently being developed for their molecular isolation: RFLP tagging and transposon tagging.

As described earlier, a close linkage between known genes and RFLPs can be used as starting points for the molecular isolation of certain genes. An inherent problem associated with this RFLP-tagging approach is that it is difficult to associate the appropriate DNA sequences with the corresponding phenotype of the desired gene. A solution to this problem can be found in some cases. For example, if the cloned DNA sequences represent the wild type, such sequences might be introduced into a recessive mutant through genetic transformation. In this case, the sequence or sequences that restore wild type in the transformants can be easily associated with the corresponding mutant gene.

Transposon tagging might be a more straight forward approach for the molecular isolation of useful genes. Application of endogenous plant transposable elements has shown to be a useful method to obtain plant mutants. The insertion of a transposable element into a genetic locus can give rise to mutations. Using a transposable element from maize, the Ac-element, Fedoroff et al. (1984), were able to clone a locus which until then was only characterised genetically. Their results indicate that in principle any locus in maize, identifiable with a transposable element, can be cloned and analysed at the molecular level. This approach, called transposon tagging, has successfully been applied for the isolation of various maize genes (Wienand & Saedler, 1987).

The subsequent demonstration that the maize transposable element Ac can transpose in dicots has opened the possibility to use this well-characterised element for gene-isolation in plants other than maize (Baker et al., 1986, 1987; Van Sluys et al., 1987). Using the *A. tumefaciens* mediated leaf explant transformation procedure, the transposable element Ac has been introduced into various tomato lines and shown to transpose within these lines (Yoder et al., 1988; Haring et al., 1989).

In maize and also in tobacco it has been shown that Ac has a tendency to transpose in the vicinity rather than to far away positions (Greenblat, 1984; Dellaporta & Chomet, 1986). Therefore, to obtain a transposon insertion in a particular gene, it is advantageous to start with a plant containing the transposable element close to the position of the desired gene. Currently, the utility of this transposon tagging system for tomato gene isolation is under study in various research groups.

Finally, it must be pointed out that in spite of extensive genetic and cytogenetic investigations, there has not been any indication so far for the presence of transposable elements in tomato. It would be highly attractive to detect and characterize transposable elements, if any, in tomato for two reasons: Firstly, they could be useful for enhancing the knowledge regarding the genome organization at the molecular level, and secondly, they might be useful as tools in isolating desirable genes through 'transposon tagging' (Federoff et al., 1984). In this connection, attempts are underway (Ramanna et

al., 1985) to induce genetic instability in tomato through 'breakage-fusion-bridge-cycle' (BFBC), a method that was originally used by McClintock in maize (for a review, see McClintock, 1984). For this purpose, the iso-chromosome line of tomato that was described by Moens (1965) has been especially useful. The iso-chromosomes in these lines are entirely heterochromatic and many copies (1–9) can survive in plants without affecting vigour and fertility. A remarkable feature of these extra chromosomes is that they are structurally unstable and generate chromosomes with 'sticky ends' which cause BFBC. Plants with such unstable chromosomes can give rise to progeny that contain a low frequency of genetically unstable plants as wel as with chromosomal abnormalities (Ramanna et al., 1985 and unpublished).

Applications

Anther and pollen culture

Although the production of a tomato haploid by anther culture has been reported as early as 1972 (Greshoff & Doy, 1972) and the application of pollen culture was reported in the same year (Sharp et al., 1972), haploids are still difficult to obtain. The response to anther culture was found to be genotype dependent (Zagorska et al., 1982) and especially the positive effect of genotypes homozygous recessive for the *ms-10*^{35} allele has been reported by several laboratories (Zagorska et al., 1982; Zamir et al., 1980; Ziv et al., 1984). However, from such cultures haploid plantlets were rarely recovered. Probably the rapid polyploidization of haploid callus cells of tomato and the outgrowth of anther wall tissue (Levenko et al., 1977, Zamir et al., 1981) are the main reasons for this failure.

Haploid plants originating by parthenogenesis can be found spontaneously at frequencies of approximately 2×10^{-4} (Ecochard et al., 1969; Koornneef et al., 1989) and can be propagated vegetatively. When protoplasts from such haploids can be induced to divide and regenerated into plants, they might be used for the selection of recessive mutants at the cell level.

Embryo culture

In some of the interspecific and intergeneric crosses, the embryos abort and prevent the formation of hybrid seeds. Such premature embryos can be rescued by cultivating them *in vitro* (embryo rescue). One early example is the interspecific cross *L. esculentum* × *L. peruvianum* which was instrumental for the introduction of resistance against root knot nematode into the cultivars (Smith, 1944). The other examples are the hybrids of *L. esculentum* × *L. chilense* (Rick & Smith, 1953; Rick, 1963), and the intergeneric hybrid *L. esculentum* × *Solanum lycopersicoides* (Rick, 1951; De Verna et al., 1987).

An improvement of embryo culture technique was published by Thomas & Pratt (1981) who induced callus formation on very young embryos subsequently followed by shoot induction.

Somaclonal variation

Variation among plants derived from tissue culture (called somaclonal variation) is considered a drawback by those interested in the uniform multiplication of a particular genotype. In contrast, plant breeders looking for more variation consider somaclonal variation as an advantage of tissue culture. However, one should realize that most variants found will not be useful. Plants with a higher ploidy level than in the original plant material occur depending on the type of tissue culture system employed. Plants derived from diploid explants by adventitous shoot formation show predominantly the original ploidy level. However, plants regenerated from established callus cultures or protoplasts are in majority tetraploid (O'Connell et al., 1986; Koornneef et al., 1989).

Sibi (1982) reported for the first time in tomato the occurrence of 'epigenetic variation' among regenerated plants derived from tissue culture. Evans & Sharp (1983) studied the progeny of 230 plants derived from leaf explants and found 13 tentative nuclear mutations. Four of these mutants

showed allelism with mutants that were described earlier (Evans & Sharp, 1986). Buiatti et al., (1985) found that 17% of 88 progenies segregated monogenic mutants. Another striking example of somaclonal variation in tomato has been published by Shahin & Spivey (1986) who found 5 out of 100 plants that were resistant to *Fusarium oxysporum* race 2 (a dominant resistance) following regeneration of protoplasts. Darden et al. (1986) reported the isolation of somaclonal variants with maternally inherited TMV resistance. Sibi et al., (1984) regenerated plants from cotyledons of F_1 hybrids heterozygous for several linked genes and found an increase in recombination frequencies as compared with control F_1 hybrids. The increase was 5–7% for genes located at approximately 20 cM from each other. A comparison of chemical mutagenesis with tissue culture induced mutants indicated differences in both mutant spectrum and mutant frequency (Gavazzi et al., 1987).

Mutant selection at the cellular level

Selection at the cellular level for resistance against toxic compounds seems a feasible and attractive approach to obtain mutants. Indeed, various variants have been described (Table 2). There are, however, several major problems associated with this type of mutant selection. First, variant plant cells are often found difficult to regenerate. Secondly, chromosomal instability can occur in the regenerated plants. Thirdly, there is a lack of haploid plants or cell cultures which can be efficiently regenerated in order to facilitate the isolation of recessive mutants.

Protoplast fusion

In comparison to sexual crossing, protoplast fusion offers several unique possibilities including: (1) sexual barriers can be overcome, (2) different cytoplasms can be mixed which cannot normally be accomplished, and (3) parts of a genome can be transferred through asymmetric fusion.

The demonstration of protoplast fusion as a technique to hybridize species that cannot be crossed sexually was reported for the first time with tomato/potato hybrids (Melchers et al., 1978). Recently, somatic hybrids have been produced between other *Lycopersicon* species and related *Solanum* species (Table 3).

The efficiency for the selection of hybrids is so far limited, however, mainly due to the lack of suitable cell selection markers. Selection criteria thus far applied in fusion experiments are the capacity of hybrids to regenerate (Adams & Quiros, 1985); Wijbrandi et al., 1988), kanamycin resistance (Adams & Quiros, 1985), the ability to survive

Table 2. Variants isolated at the cell level in *Lycopersicon*

Variants phenotype resistant to	Parental genotype	Regeneration of variant plants	References
Glyphosate (Herbicide)	*L. peruvianum* × *L. esculentum* hybrid	–	Smith et al. (1986)
Paraquat (Herbicide)	*L. peruvianum* × *L. esculentum* hybrid L2	+*	Thomas and Pratt (1982)
Cadmium	*L. peruvianum* suspension	–	Bennetzen and Adams (1984)
Aluminum	*L. esculentum* 'Marglobe'	–	Meredith (1978)
Polyethylene glycol	*L. esculentum*	–	Bressan et al. (1981)
Fusaric acid	*L. esculentum*	+	Shahin and Spivey (1986)
Fusarium elicitor	*L. esculentum*	–	Buiatti et al. (1987)

* Resistance was expressed at the callus level (also on callus derived from regenerated plants) but not at the plant level.

particular media and treatments (Handley et al., 1986) and morphological selection (O'Connell & Hanson, 1985; Kinshara et al., 1986; Tabaeizadeh et al., 1985). Recently, nuclear markers selectable at the cell level, such as antibiotic resistances introduced by transformation, became available and allow a more efficient selection of hybrids. Also, chloroplast-encoded albino mutants (Hosticka & Hanson, 1984) and chloroplast-encoded antibiotic resistances (Jansen et al., in preparation) are now available in tomato. Thus far, use of the somatic hybrids in breeding programs has not been reported.

In order to transfer a limited amount of desirable genetic variation from a wild species into cultivated tomato, asymmetric protoplast fusion is a useful tool.

Asymmetric hybrids were obtained by fusing irradiated protoplasts of *L. peruvianum* with untreated *L. esculentum* protoplasts and have been isolated by Wijbrandi et al. (1988) by selecting the hybrids on the basis of their better regeneration capacity as compared with *L. esculentum*.

Table 3. Somatic hybrids obtained with *Lycopersicon* species

Parental species	References
L. esculentum (+) *L. peruvianum*	Kinshara et al., 1986; Wijbrandi et al. 1988
L. esculentum (+) *S. tuberosum*	Melchers et al., 1978, Shepard et al., 1983
L. esculentum (+) *S. lycopersicoïdes*	Handley et al., 1986; Tan, 1987
L. esculentum (+) *S. rickii*	O'Connell et al., 1986
L. esculentum (+) *L. pennellii*	O'Connell et al., 1985; 1987
L. peruvianum (+) *L. pennellii*	Adams & Quiros, 1985; Tan, 1987
L. peruvianum (+) *Petunia hybrida*	Tabaeizadeh et al., 1985
L. esculentum (+) *S. nigrum*	Guri et al., 1988

Some representative examples of tomato genetic manipulation

Chromosomal location of introduced genes

The genetically well characterized tomato has been used as a plant species to test whether T-DNA inserts can be used as chromosome specific markers and whether there is site-specificity in T-DNA

Table 4. Genes introduced into the tomato genome by genetic transformation

Trait	Specification	References
direct gene transfer		
kanamycin resistance		Koornneef et al. 1986 Jongsma et al. 1987
A. tumefaciens mediated		
kanamycin resistance		Chyi et al. 1986 Koornneef et al. 1986 McCormick et al. 1986
herbicide tolerance	glyphosate	Filatti et al. 1987
	L-phosphinothricin	De Block et al. 1987
virus resistance	TMV-coatprotein	Nelson et al. 1988
	AMV-coatprotein	Tumer et al. 1987
insect resistance	lepdidopteran insects	Vaeck et al. 1987
fruit ripening	down regulation of polygalacturonase activity	Smith et al. 1988
transposon activity	Activator (Ac) element from maize	Yoder et al. 1988 Haring et al. 1989
protein composition in chloroplasts	plastocyanin imported in chloroplasts	De Boer et al. 1988

insertion. To this end, the site of T-DNA insertions has been determined for 7 tomato transformants using a molecular genetic approach (Chyi et al., 1986) and for 8 tomato transformants using a genetic approach (Weide et al., in preparation). The 15 mapped T-DNA inserts were located on 8 different chromosomes. It was concluded that the T-DNA does integrate into various tomato chromosomes and can indeed be used as a chromosome specific marker. Similar results have been presented for T-DNA inserts in *Petunia* chromosomes (Wallroth et al., 1986) and in *Crepis capillaris* chromosomes (Ambros et al., 1986). With respect to a particular chromosome, the T-DNA can be integrated at various positions, as was shown for tomato, petunia and *Crepis capillaris*.

Herbicide tolerance

In modern agriculture the use of herbicides has become essential for weed control. However, most of the new herbicides, that combine effectiveness with safety for animals and environment do not distinguish between weeds and crops. Therefore, three independent strategies have been employed to obtain plants tolerant to these herbicides.

(1) Overproduction of the sensitive target enzyme. It has been demonstrated in petunia that overexpression of the protein, on which the herbicide acts, confers herbicide tolerance (Shah et al., 1986).
(2) Modification of the sensitive target enzyme. The protein on which the herbicide acts is modified in the engineered tomato plants so as to render its activity insensitive to the herbicide. (Fillatti et al., 1987).
(3) Detoxification of the herbicide. A gene is introduced into tomato encoding a protein which detoxifies the herbicide (De Block et al., 1987).

All three strategies have both their advantages and disadvantages. However, it might be expected that especially the third strategy will prove most useful since quite often, the detoxifying enzymes can be isolated from bacteria and transformed crops display a relatively high level of herbicide resistance (for a recent review see: Botterman & Leemans, 1988).

Virus resistance

Cross-protection, in which plants are infected with a mild virus strain to protect against infection with virulent strains, has since long been used to control yield of plants. Following the development of genetic manipulation procedures, it has been tested whether virus resistance can be induced in alternative ways. Several strategies have been followed, including (1) expressing viral coat protein in transgenic plants (Tumer et al., 1987; Nelson et al., 1988), (2) expressing antisense viral RNA in transgenic plants (Cuozzo et al., 1988) and (3) expressing satellite RNA's in transgenic plants (Harrison et al., 1987; Gerlach et al., 1987). The coat protein strategy results in symptom retardation and is dependent on the amount of coat protein expressed in the transgenic plants and on the strengh of the viral inoculum. Apparently, the effect of the expressed coat protein can be overcome when the viral RNA accumulates above a certain concentration. The antisense RNA approach, using antisense coat protein message, results in a low protection level against virus infection, that is less effective compared to the coat protein mediated protection. The satellite RNA strategy depends on amplification of the satellite RNA molecules by the incoming virus, resulting in suppression of symptom development. Therefore, protection is independent of the strengh of the viral inoculum and insensitive to the amount of satellite transcripts in the transgenic plants, but limited, however, to those viruses which contain a satellite RNA.

Insect resistance

In agriculture a wide variety of insecticides is used to control insect damage. Since chemical control is expensive and sometimes undesirable, effort is focused on improving plant defences against insect attack.

A single gene has been identified in the bacterium *Bacillus thuringiensis* which codes for a polypeptide which is specifically toxic to a variety of insect species. This gene has been cloned, made suitable for expression in plant cells and introduced into plants. Transgenic plants, producing this new pro-

tein, were shown to be protected against feeding damage by larvae of the insect species (Vaeck et al., 1987).

In nature several mechanisms exist by which plants protect themselves against insect damage. One such mechanism involves trypsin inhibitors which act at the catalytic site of enzymes, thereby interfering with digestion in the insect gut. A trypsin inhibitor gene has been cloned from cowpea, manipulated in such a way that the expression of the gene was constitutive and relatively high, and introduced into tobacco. Transgenic tobacco plants were shown to have an enhanced resistance to one of its major insect pests (Hilder et al., 1987).

Down-regulation of specific plantgene expression

Genes are universally expressed via RNA transcripts giving rise to either messenger RNA's which are subsequently translated into proteins, or structural RNA's. Recently, strategies have been developed in plants to down-regulate endogeneous gene expression by interfering at the RNA level. These approaches involve the introduction into plants of either an antisense gene or a very specific endoribonuclease activity.

Antisense RNA inhibition has been shown in petunia at the flower colour level (van der Krol et al., 1988) and in tomato at the level of polygalacturonase activity in ripe fruits (Smith et al., 1988). General rules have been established for the design of RNA enzymes (so-called ribozymes) capable of cleaving RNA highly specific. This has been applied against a model target sequence and shown to be successful *in vitro* (Haseloff & Gerlach, 1988). Ribozymes have not yet been tested in plants.

Both approaches will enable to create mutant phenotypes for any molecularly characterized gene and might prove valuable in e.g. manipulating biochemical pathways in plants in both a quantitative and a qualitative manner.

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