Molecular Biology: Its practice and role in Crop Protection

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Preface

Molecular biology has developed as a major discipline over the past decade. It has its origins in biochemistry and molecular genetics. As such it has a vocabulary and a set of terminologies which are distinct from the experience of most of those involved in crop protection. The rate of development of molecular biology as a distinct discipline has been so rapid that most of those who have not actively followed the subject now feel so far adrift from the subject as to be unable to catch up. This is now especially the case for those in crop protection research who come from a background in chemistry, conventional biology or environmental science. Crop protection has always depended upon input from a range of disciplines because ultimately the management of pests, weeds and diseases under field conditions involves the matching of the genotype of crop, the ecology of the weed, pest or disease, the environment of the site and the attributes of the chemical or biological agent being used to effect control.

The potential contribution that molecular biology can make to crop protection, to the molecular characterisation of pests, weeds and diseases, to plant breeding or to the design of crop protection agents, means that it is essential that molecular biology is brought into the main stream of crop protection. For this to occur it is necessary for the main stream crop protection to understand the terminology of molecular biology, the practice of the major molecular methods and the potential uses of these methods in crop protection. To allow this to happen a special introductory symposium was developed to precede the 1991 Brighton Crop Protection Conference. In contrast to most BCPC Conference sessions and symposia which are dominated by presentations on recent research, this symposium deliberately set out to educate and update those who attended by breaking down the technical jargon barriers and giving the mainstream of the industry the ability to relate to those in molecular biology.

This introduction to molecular biology was designed to combine an appreciation of basic techniques e.g. the isolation, analysis, cloning and transformation of genes, together with the application of such technologies. The methodologies thus describe the integration of traditional and novel skills in chemistry, biochemistry, cell biology, tissue culture and genetics to provide new approaches at the molecular level. With this sophisticated array of techniques we require to identify target areas in crop protection where problem solving can perhaps be facilitated in an effective and more efficient manner compared to traditional approaches.

In this volume, a cross section of topical issues in weed science, plant pathology and pesticide science was presented. These include the selection of cultivars for improved disease, weed and pest control via genetic manipulation. With a focus on environmental protection, consideration is given to new techniques for measurement and detection of pesticides. In addition, the development of pesticide resistance may be better understood and improved strategies for avoidance implemented by the application of molecular-based techniques.

It is our hope that this volume will engender a sense of curiosity in crop protectionists to seek an appraisal of the ways by which molecular biology may serve as a valuable tool in their area of investigation. We gratefully acknowledge the efforts of each of the contributors have made towards sharing their knowledge of molecular biology in crop protection. It is through such interactive activities that we can seek to fully meet the future challenges in crop protection for the 1990s.

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BRIGHTON CROP PROTECTION CONFERENCE
Weeds – 1991

Introductory Session Molecular Biology – Its Practice and Role in Crop Protection

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INTRODUCTION TO DNA ISOLATION AND ANALYSIS

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ABSTRACT

Conventional plant breeding techniques have been responsible for the significant gains in yield and quality attained in crop plants this century. However modern technologies targeted at improving and protecting crop plants are now undertaken not only at the whole plant level, but also at the cellular and molecular level as well.

Nucleic acids are of vital importance because of their central position in plant cell manipulation. The importance of a precise knowledge of the structure and function of genes and the end product of gene expression, proteins, is crucial to the application in crop protection of the wide portfolio of techniques categorised under the umbrella term of genetic engineering. This has greatly enhanced the precision of developing such important traits as herbicide and insecticide resistance in modern crop species.

In this paper we set the history and current applications of nucleic acid isolation into a practical context. The techniques of gel electrophoresis, restriction enzymes, Southern blotting, Pulsed-field gel electrophoresis, DNA sequencing and gene amplification by polymerase chain reaction are described. The importance and relevance of such techniques to current and future applications in crop protection are highlighted.

INTRODUCTION

Genetic engineering has been widely defined by many authors but for the purpose of this paper we define it as the addition of a new piece of DNA to the genetic material of a cell in such a way as to allow replication of this new message, and consequent transmission to its progeny.

It is necessary to arrange for the translation of this additional message and is important to note that the source of such novel DNA may be plant, animal, bacterial or from an entirely novel source. The novel DNA has to be isolated and cloned, usually utilising plasmid or viral vectors to carry the

foreign genetic material. It also has to be presented in such a way that the host cell can recognize it, and replicate it. Thus, the novel DNA must be attached to a segment of DNA capable of being recognized and replicated in the host cell and is therefore "spliced" into a cellular element capable of such replication.

Our present knowledge of the structure of the plant genome (the complete set of chromosomes carried by a sex cell) and its constituent parts, nuclear, chloroplastic and mitochondrial has come about largely through research based on recombinant DNA or gene cloning techniques. These techniques allow the isolation and characterisation of specific pieces of DNA which can be cloned into bacterial cells where they can be replicated, and the processes and products studied offering exciting opportunities for the manipulation of genetic material.

These methods have been established only in the last twenty years, however, the story begins at a much earlier time.

Historical background

In 1839 two German microscopists, Mathias Schleiden and Theodor Schwann proposed that all plants and animals are constructed of small fundamental units called "cells" and that these cells arise from others. It was later discovered that these cells are surrounded by a membrane, and usually contain an inner body known as the nucleus which is also surrounded by a membrane. New cells arise from others by the process of division where cells split into two daughter units and at the same time the nucleus also splits into two. Each nucleus contains a fixed number of linear bodies called chromosomes. A chromosome is the structure containing DNA that carries hereditary information. Before cell division each duplicates to form two identical chromosomes. This process was first accurately observed by Walte Flemming in 1879. The result of this action is a doubling of the chromosome number, and during nuclear division one of each pair moves into each daughter nucleus. As a result of these events (known as mitosis) the chromosomal complement of each daughter cell is the same as that of the parent.

The theory thus explains that all cells come from pre-exisiting cells, and has been found to be true of bacteria, plants and animals. The cell therefore has the ability to transmit hereditary properties from one generation to another. The geneticist Gregor Mendel outlined the basic rules of heredity in the 1850s and though these were effectively ignored until 1900, in 1903 Walter S. Sutton wrote his classic paper "The Chromosomes in Heredity" and brought together the disciplines of genetics and the study of cell structure, cytology.

Further elucidation in the process and its understanding came in 1909 when Garrod postulated that genes affect the synthesis of enzymes. The crucial hypothesis of a gene:enzyme relationship resulted in more detailed analysis of gene chemical structure and mode of action. It was found from such studies that nucleic acids and proteins were both implicated in genetics

but since their own structure was unknown this did not help to clarify the understanding of the process as a whole.

Concentrated research resulted in the conclusion that proteins themselves were not the "genetic molecules", so attention was diverted towards more detailed study of the structure, function and effect of nucleic acids. These were found to be prominent features of cells but had yet to be assigned a particular function. DNA had been identified in the late 19th century as a major constituent of chromosomes and early research found RNA, a similar biological polymer to DNA but with the sugar ribose instead of deoxyribose in its structure, in yeasts. It was believed for a while that RNA was the "plant" nucleic acid and DNA the "animal" form. However it was soon discovered that both were found together. That DNA could be the key genetic molecule emerged from experiments by an English microbiologist Frederick Griffith who worked on pneumonia-causing bacteria and observed that non-virulent strains of bacteria became virulent when mixed with their heat-killed pathogenic counterparts. These results raised the hypothesis that genetic components remain undamaged, and could also be transformed (i.e. permanently or transiently taken up by a cell) into other cells and genetically recombined.

Investigations into the properties of nucleic acids by chemical means was difficult as they are, like proteins, macromolecules, in this case constructed from their own unique building blocks known as nucleotides. Each nucleotide contains a phosphate group linked to a five carbon atom sugar group which in turn is linked to a flat aromatic molecule that can be a double ringed purine or single ringed pyrimidine. DNA contains the sugar deoxyribose, and RNA ribose. DNA and RNA are both built up from two purine containing nucleotides and two pyrimidine containing nucleotides. The purine component of DNA and RNA is the same, comprising adenine and guanine, and the pyrimidine component comprises cytosine in both DNA and RNA with thymine in DNA and uracil in RNA. Early attempts at isolating nucleic acids seriously degraded them due to the lack of sophistication in the techniques used. Advances were made in elucidating the structures of nucleic acids with the advent of electron microscopy in the early 1950s where studies revealed DNA to be a very thin molecule built up from tens of thousands of nucleotide building blocks.

The development of paper chromatography techniques allowed the analysis of the nucleotide composition of DNA, and it was found that the four nucleotides are not present in equal amounts. It was also found that Adenosine with Thymine (A:T), and Guanine with Cytosine (G:C) were found in the same ratios. It was also discovered that the ratios of overall nucleotides varied from species to species and from these findings it was postulated that the precise arrangements of nucleotides within a DNA molecule could confer its genetic specificity.

The fundamental importance of this was not realised until research on the three dimensional structure of DNA was accomplished and in 1953 the work of Watson and Crick (and others) led to the celebrated double helix theory. This breakthrough offered opportunities for geneticists to analyze their

data and begin to answer some of the problems associated with the mechanisms of gene replication.

The genetic information within DNA is conveyed by the sequence of its four building blocks, and it was proved that genes, the hereditary units consisting of a sequence of DNA occupying a specific position within the genome (the complete haploid set of chromosomes) control amino acid sequences in proteins. However DNA was found not to be the template that directly orders amino acids during protein synthesis as it was discovered from cellular fractionation studies that protein synthesis took place at sites in the cell where DNA did not exist. Protein synthesis was found to take place in the cytoplasm which is separated from the nucleus by a membrane. A second "information molecule" was then found to be necessary which takes its genetic specificity from DNA and moves to the cytoplasm as a function for a template for protein synthesis, and this attribute was described as a role for RNA.

The synthesis of a utilitarian product within a cell, using the information encoded by the nucleotide sequence, is dependent on a related series of events. The gene is transcribed into a messenger RNA, processed, transported into the cytoplasm and by the process of translation the information in the mRNA is decoded into a polypeptide chain. The mechanism of protein synthesis is essentially similar for all organisms, though there are some differences in the constituents and reactions between prokaryotes and eukaryotes.

The final product of the above processes is a functional protein which is fundamental to plant growth and development. However, the necessary proteins must be produced in the appropriate tissue at the correct stage of development for normal growth to occur and so an overall regulation of the processes involved is crucial.

The area of regulation of gene expression during plant development is the subject of a great deal of current research and has critical applications in the crop protection context, for example in the tissue specific expression of herbicide resistance genes.

Current applications

There is enormous interest in the applications of molecular biology, both to understand fundamental scientific problems and to use such powerful and precise techniques to specific problems in plant biotechnology. In such areas as gene mapping, plant genetic transformation, and identification and analysis of specific agronomic traits such as disease resistance genes the importance of isolation and analysis of plant nucleic acids taken from a variety of plant types at defined stages of development is central to the success of such projects. Methods for achieving and applying this as described below.

DNA - Deoxyribonucleic acid.

There is a tremendous range of DNA content encountered even among organisms of similar biological complexity. In eukaryotic animals there is an

average of 1-5 pg DNA per haploid genome but in the salamander there is 100pg. Some primitive vascular plants have 300 pg DNA per haploid genome and even within plant groups there are large differences e.g. *Vicia* with up to seven fold differences in content between closely related species and it has been concluded that nuclear DNA content in higher plants is not positively correlated with either the amount or the sophistication of genotypic control exercised during development.

Plant cells contain large amounts of DNA, mainly in the nucleus, and even the smallest plant genome (that of the small cruciferous weed *Arabidopsis*) is five times larger than that of the widely studied fruit fly *Drosophila* (see Table 1).

TABLE 1. Comparative contents of DNA
(based on Bennet and Smith 1976)

Arabidopsis	0.5 pg DNA/haploid genome
Tobacco	2.0
Wheat	5.1
Onion	32.5
Mistletoe	107.0

Only 1-10% of the actual genome is required to account for all the known functions of DNA in development. This figure has been estimated by calculating the amount of DNA in comparison with evolutionary changes and by direct measurements of the fraction of the genome transcribed into nuclear RNA and mRNA (Leutwiler *et al.*, 1984). This provides a question, what use is this excess DNA and has it a function as yet to be perceived?

The majority of structural genes are composed of single-copy number sequences (syn. unique or non-repetitive) and repetitive sequences, which occur in substantial amounts and appear to be composed of families of related sequences. Again there is considerable variation even within closely linked groups, the example *Vicia* has four times the structural gene DNA content than mung bean. There is direct experimental evidence that suggests that much of the single copy DNA is not required to code for mRNAs. Repetitive DNA is of varied form, for example in wheat there is a range from 200 - 1000+ nucleotide pairs in repeats. The significance of these types may be in multigene families, or as regulatory elements, as there is widespread interspersion of short repetitive sequences and it is plausible that they may control elements important in transcriptional regulation. The nuclear genome is the largest in the plant cell in terms of the pg/DNA present and the resultant number of genes encoded. The nuclear DNA is found in the chromosomes, histones and non-histone proteins. Most actively-dividing plant

cells complete a cycle of growth and cell division in 15-40h depending on the species and environment.

There are other genetic domains in plant cells notably chloroplasts and mitochondria. Each has its characteristic DNA and RNA component and profile and this represents a fascinating problem in investigating the interaction between the types.

Chloroplast DNA

Chloroplasts are the site of photosynthesis in the plant where carbon dioxide is converted to carbohydrate, a simple statement covering a complex interrelated reaction whereby external energy is fixed from the biosphere. Chloroplasts are specialised organelles and synthesize their own proteins and retain the synthetic abilities via unique RNA and DNA. The presence of DNA in chloroplasts was first demonstrated by Ris and Plaut (1962). Chloroplastic DNA is double stranded, closed circular molecule with a range of sizes from 120-160bp (base pairs).

TABLE 2. Comparative sizes of chloroplast DNA

E.coli	3.8×10^6 bp
tobacco ct DNA	1.6×10^5
maize ct	1.36×10^5
broad bean	1.21×10^5
(*Arabidopsis* total genome = 2×10^6)	

Chloroplastic DNA is 85% single copy sequences and is thought to be prokaryotic in nature, the evolution of chloroplast DNA being of a relatively conserved nature.
The structural origins of cpDNA are well-characterised and the complete genomes of tobacco (Shinozaki *et al.*, 1986), rice and liverwort have been sequenced.

Mitochondrial DNA

Mitochondria are responsible for the oxidation of carbohydrates to energy via respiration. The mitochondrial genome is highly variable in size and form, though generally very large it only makes up less than 1% of the total cellular DNA. It is of vital importance however, and is thought to be neither prokaryotic or eukaryotic in evolution. Mitochondrial DNA, mainly in the form of small cyclic molecules, carries the genetic information for the

synthesis of some of the mitochondrial-specific proteins and for the ribosomal RNA present in the mitochondria.

Outside of higher plants there has been enormous scientific research into the structure, form and manipulation of the bacterial nucleic acid component. Bacteria play a vital and underpinning role in plant research. Their properties make them one of the principal vectors used in genetic manipulation. The isolation of bacterial plasmid DNA is therefore of great significance.

Plasmid DNA

A major part of the genetic material of a bacterial cell is in the form of a single DNA molecule with the double helical structure joined into a close circle. This large circular chromosome carries all the essential genes for reproduction of the bacteria. Many bacteria carry smaller additional circles of DNA, known as plasmids, which may carry genetic information for specialised functions. Plasmids also have the capacity to replicate independently of the chromosome. Thus plasmids are excellent vectors for cloning and as such are in widespread current use in all areas of molecular biology. Gene cloning can be defined as the isolation and amplification of an individual gene sequence by insertion of a sequence into a bacterium where it can be replicated (Mantell, Mathews and McKee, 1985). Artificially constructed plasmids have been made containing expedient features from a number of ingenuous plasmids such as the provision of single sites for a number of common restriction enzymes and consequent possibilities of DNA insertion. An early experiment introduced the nitrogen-fixation genes from *Klebsiella* to *E. coli* using recombinant plasmids (Cannon *et al.*, 1976). Other applications include the isolation of specific DNA fragments, routine subcloning and the generation of radiolabelled probes. Plasmid DNA is of a relatively small size and covalently closed circular nature.

RNA - Ribonucleic acid

Like DNA , RNA is a polymer, the monomer units of which are ribonucleoside monophosphates. Most RNA molecules are single-stranded, though they can form double-stranded hydrogen-bonded molecules. All living cells contain three main kinds of RNA:-

Ribosomal (rRNA) comprises about 80% of the total plant RNA and is contained in the ribosomes which are responsible for the translation of mRNA molecules transcribed from nuclear DNA. rRNA is synthesized at very high rates ingrowing tissues. Features of its production have been reviewed by Grierson, 1982.

Transfer RNA (tRNA) functions as adaptors for amino acids in the course of protein synthesis. It is of comparatively low molecular weight, and comprises about 15% of the total plant RNA.

Messenger RNA (mRNA) is a section of RNA transcribed from a DNA molecule that carries the code for the amino acid sequence of a protein, this type amounts to only around 2 - 5% of the total but is of key significance. In some specialised plant cells the major part of protein synthesis is concerned with the production of one or very few proteins and the identification and purification of mRNA is facilitated using these systems. Early knowledge of plant gene structure was derived from the study of such systems as seed storage proteins from maize (Larkins, 1983).

1. THE ISOLATION OF DNA

The initial step in the isolation of nucleic acids is the separation of nucleic acids from all other cellular constituents and associated protein. The first step often comprises a mechanical method of disruption e.g. mortar and pestle, homogeniser. If organelle DNA is required the first stage is actually isolating clean samples of the relevant organelles. These procedures all involve cell fractionation and purification of the cell contents usually by a range of centrifugation and solvent steps (phenol or chloroform:isoamylalcohol are commonly used to separate nucleic acids from proteins) to provide samples free from contaminants. In certain cases a mixture of genomic DNA is all that is required and consequently the requirement for organelle fractionation is alleviated. Certain factors have relevance to all types of DNA extraction notably:-

(i) The selection of the appropriate plant (or bacterial) tissue, as the final yield will be dependent on the number of cells. Also, the efficiency of nuclear isolation from the tissues, and the recovery of DNA from the crude nuclear or organellar preparations. Plant leaves are generally a good source (an obvious advantage is that the plant is not destroyed by the removal of its leaves) and are also virtually free of starch.

(ii) Contamination by chloroplasts and mitochondria in nuclear DNA extraction, and non-required organelles in specific DNA extracts, and also by starch and other carbohydrates that are co-extracted with DNA and cause contamination. General guidelines for good laboratory procedures include freezing the plant material in liquid nitrogen as soon as possible after harvesting. For maximum repeatability in results and for the avoidance of contamination all glassware should be washed thoroughly, and all solutions and buffers made up with great care and renewed frequently, also some of the key solutions (but not solvents!!) may benefit from sterilisation.

Specific methodology for each type of DNA required is described below.

Chloroplast DNA

Reliable preparations of chloroplast DNA have been obtained by several methods. Treatment of intact purified chloroplasts by DNase prior to DNA isolation was used by Herrmann *et al.*, 1975. The development of a non-aqueous method (Bowman and Dyer, 1982) introduced several advantages over the previously-used aqueous methods. The final yield was improved, particularly from plants from which cpDNA was difficult to isolate, and also the method facilitated the storage of plant material for long periods even at ambient temperatures. The chloroplasts are isolated from a homogenate of

freeze-dried leaf powder by isopycnic banding in a step gradient of organic solvents. A drawback of this method is the problems with contamination with nuclear DNA, and the requirement of the use of hazardous solvents such as carbon tetrachloride. A more recent protocol (Dally and Second, 1989) describes a refined procedure and avoids the use of some of the hazardous chemicals used in the original while giving a notable increase in the recovery of cpDNA
procedure.

Mitochondrial DNA

Mitochondrial DNA has been successfully isolated despite quantitatively being the smallest genetic component of a plant cell (Palmer and Thompson, 1980). Mitochondrial DNA has been studied to a lesser extent than other forms, but has several valuable applications. An example is mitochondrial DNA isolations from leaves of Brassicas by CsCl-ethidium bromide gradient methods in studies of taxonomic classification (Lebacq and Vedel, 1981) and the isolation and role of mitochondrial DNA in taxonomic studies in nematodes (Harrower., pers comm.)

Plasmid DNA

Plasmid DNA was first isolated by Marmur *et al.*(1961). Though there are several methods currently used for the extraction and purification of plasmid DNA most follow a general protocol . Bacterial cells are grown in liquid medium, harvested and gently lysed (using lysozyme or a detergent) to release their DNA. After high speed centrifugation, 20-40000 rpm for 1 hour (Davis, *et al.*, 1980), most of the high molecular weight DNA and cellular debris is sedimented leaving the plasmid DNA in the cleared lysate, along with contaminating RNA and soluble cell fragments. Denaturation of the proteinous components is achieved by shaking this supernatant with an equal volume of 1:1 phenol/chloroform. The nucleic acids are then ethanol-precipitated. The plasmid DNA is subjected to Caesium chloride density centrifugation. Added ethidium bromide binds to the DNA by intercalating between the base pairs, causing the helix to unwind. As closed circular plasmid DNA has no free ends it can only unwind to a small extent and thus the amount of dye that can bind is limited. The chromosomal fragments do not have this restraint and as ethidium bromide binds, the buoyant density of the chromosomal DNA becomes lower than the plasmid DNA and hence bands occur in the gradient. Also RNA and other contaminants will be separated allowing a good degree of purification of the plasmid DNA. Variations on this generalised procedure include different lysis procedures, the absence of ethanol or phenol/chloroform steps, and the use of column chromatography rather than centrifugation. (A good review of basic isolation and sizing of plasmids is given by Broda, 1979).

These methods are however expensive and time consuming, and consequently many alternative methods have been developed, many of which involve ion-exchange or gel-filtration chromatography or, alternatively a differential precipitation step to separate plasmid and host DNAs.

DNA miniprep

In many cases important and repeatable results can be rapidly obtained using "minipreps" of both plant and bacterial material. These DNA preparations are produced by a rapid microscale method without requiring ultra centrifugation steps. For such applications as gene amplification using Polymerase Chain reaction (PCR) techniques this method of DNA isolation is highly applicable and efficient. Only small (for example, 1 or 2 small leaves, or 1.5mls of bacterial culture) samples are required and the whole process only takes three hours and involves relatively simple laboratory reagents and equipment. In our laboratories a typical miniprep protocol has been used for the isolation of DNA from leaves and callus tissue of *Brassica*, *Solanum* and *Linum* material based on the CTAB methods of Rogers and Bendich (1988). Only 15-30 mg of plant tissue is used for these isolations. The methods are currently being adapted for use with more recalcitrant plant species such as *Arachis* and *Coriander*.

2. THE ISOLATION OF RNA

The methods for the isolation of RNA are based on the same principles as those of the isolation of DNA i.e. to prepare undegraded nucleic acids free from protein contamination by three basic stages, cell lysis, deproteinisation and the precipitation of nucleic acids.
DNA can be removed from the samples by treatment with deoxyribonuclease in the same way that RNA can be removed from DNA samples by the application of RNAse. Standard phenol methods for the isolation of total RNA may result in a poor recovery of messenger RNA, the RNA that is transcribed from a DNA molecule that carries the code for the amino acid sequence of a protein.

The mRNA component of the total RNA is the most important fraction and can be purified from the total RNA by removing protein, polysaccharides and other contaminants and then using the technique of oligo-dT cellulose chromatography (Aviv and Leder, 1972) to produce a fraction highly enriched for mRNA. Messenger RNA isolated by these methods can be used as a template for in vitro protein synthesis or the synthesis of complementary DNA (cDNA), a DNA strand synthesized on an RNA template by the action of the enzyme reverse transcriptase i.e. the reversal of the normal situation where the DNA sequence determines that of the RNA molecule. RNA is not very stable and the enzyme ribonuclease is a particular problem and thus great care must be taken for its exclusion by sterilising solutions and the use of careful handling procedures. Further steps include harvesting fresh tissue or storing tissue at -80^{o}C before use. Enrichment processes for a particular mRNA sequence can be done and an example of a combination method, using targeted plant tissue and size fractionation of the mRNA, produces a highly purified storage protein mRNA preparation from *Phaseolus* (Hall *et al.*, 1978).

3. RESTRICTION ENZYMES

Restriction enzymes are the basic tool of the molecular biologist. These enzymes bind specifically to, and cleave, double- stranded DNA at specific sites within or adjacent to a particular site of the DNA known as the recognition sequence. Each of the many hundreds of restriction enzymes available currently has its own characteristic recognition sequence which may involve between 4 and 7 bases. Some restriction enzymes cut and leave "sticky" ends which are of great importance in cloning work as the sticky ends provide the preliminary structure for inserting plasmids or gene sequences. The use of DNA ligase stabilises the hybrid structure. After cutting or "restricting" the DNA into a series of distinct fragments by the use of a specific restriction enzyme it is possible to separate the fragments and importantly, derive an estimate of their size by gel electrophoresis.

4. GEL ELECTROPHORESIS OF DNA

Electrophoresis through agarose or polyacrylamide gels is the standard method used to separate, identify and purify DNA fragments. The technique is simple, quick to perform and capable of resolving fragments of DNA that cannot be separated by other procedures such as density gradient centrifugation. In addition, the location of DNA within each gel can be directly determined by staining with ethidium bromide. Bands containing as little as 1-10ng of DNA can be visualised under ultraviolet light. Also, these bands of DNA can be recovered from the gels and used for a number of cloning or further analysis applications.

N.B. Ethidium bromide is a carcinogen and requires extremely cautious handling and disposal.

Polyacrylamide

These types of gel systems are rapid and able to utilise large samples of DNA but are, however, more difficult to prepare and handle than agarose gels. Also these types of gels are run in a vertical configuration in a constant electric field. Polyacrylamide gels have high resolving power and fragments of DNA that differ in size by as little a 1bp can be separated, subsequently this type of gel is most effective for the separation of small (5-500 bp) fragments of DNA .

Agarose

Agarose is extracted from seaweed and is a linear polymer. It has been found that the degree of purity varies from batch to batch and this can effect rates of DNA migration and the ability of recovered DNA to act as a substrate in reactions. Modern agaroses have been developed that gel and melt at low temperatures and also have a greater resolving power, but still not the resolution available from polyacrylamide based systems. The gels are made by melting the agarose in a buffer (often using a microwave) until a clear transparent solution is obtained, poured and allowed to set. As an electric field

is applied across the gel, DNA which is negatively charged at neutral pH, migrates towards the anode. The rate of migration is dependent on the following factors:-
(i) the molecular size of the DNA (larger molecules migrate more slowly)
(ii) the agarose concentration used as a linear DNA fragment of a known size migrates through gels containing different concentrations of agarose at different rates. There is a linear relationship between agarose concentration and efficient range of separation.

TABLE 3. The relationship between agarose content and efficient range of separation of linear DNA

w/v agarose	efficient range (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

In terms of size, shape, porosity and configurations the range of gel types vary and the selection of a particular system is dependent on the size of the fragments being separated. Though having a lower resolving power than polyacrylamide, agarose has a greater range of separation. DNA samples from 200bp to 50 kb can be separated using agarose systems that are usually run in a horizontal configuration in an electric field of steady energy and charge. A further consideration is the applied voltage as at low voltages the rate of migration of linear DNA fragments is proportional to the voltage applied, however as electric field strength is increased the mobility of high molecular weight fragments of DNA increases differentially, and so, the competent range of separation in agarose gels decreases as the voltage is increased. To obtain the maximum resolution of DNA fragments greater than 2kb in size, agarose gels should be run at no more than 5v/cm. The electrophoretic behaviour of DNA in agarose gels (unlike in PAGE) is not significantly altered by the temperature at which the gel is run. Agarose gels are mainly run at room temperature though 4^{o}C may be desirable for gels of less than 0.5%.

The mobility of DNA is directly influenced by the composition of the electrophoretic buffer, for example in buffers of high ionic strength electric conductance is very efficient. Buffer composition examples include TAE containing EDTA and tris-acetate, and TBE containing Tris-borate and EDTA.

It is advisable to replace the buffers at frequent intervals. Buffers are often made up as concentrated stocks and stored at room temperature.

Apparatus for gel electrophoresis

There are many proprietary and home-made systems in use. The horizontal slab gel is the most common due to the simplicity of loading, pouring and handling. Gels are poured and a comb used to create wells. The gels are submerged just below the surface of the buffer. Other considerations in the selection of apparatus includse the ease of filling and emptying buffer, the type of power pack available and safety considerations.

Minigels

Recent advances in analytical biochemistry technology have resulted in the popularisation of methods for analysing very small amounts of DNA rapidly using miniature gels and equipment. Rapid analysis of small quantities of DNA by the use of minigels is of particular application when a rapid assay prior to the "next step" in a procedure is required. This type of gel can be prepared in advance, and the smaller sizes involved (e.g. 10-12 mls of melted agarose) facilitate the use of smaller amounts of reagents and are therefore cost effective. The disadvantages however include that larger fragments (>3kb) are poorly resolved because of the high voltages and small size of the gels used.

Staining of DNA in agarose gels

Samples of DNA are loaded into the pre-formed wells in the agarose gel mixed with a small amount of gel-loading buffer. These buffers increase the density of the sample ensuring that the DNA drops evenly into the well (the sample is applied using a positive-displacement automatic pipette) and also contain dyes that move through the gel at anticipated rates. Such dyes include bromophenol blue which migrates through agarose run in 1/2 TBE buffer at about the same rate as 300bp linear strand DNA.

The most popular technique for visualising DNA in agarose gels is the use of ethidium bromide (see above for properties). For these purposes a stock of 10mg/ml is made up in water, stored at room temperature in the dark. and used at a concentration of 0.5mg/ml. Gels can be run in the presence or absence of ethidium bromide, in the absence the gel is stained after it is run. The gel is removed (carefully) from the equipment placed in running buffer or water and stained for 10 minutes. The DNA is then visualised at 302nm by using commercially available transilluminators and photographs can be taken using Polaroid type 667 film.

Pulsed field gel electrophoresis (PGFE).

This technique can be applied to the separation of larger DNAs up to 10000kb in length. Conventional methods of agarose gel electrophoresis have been the principal method for separation of DNA fragments for some years,

and has been found to be extremely successful in resolving fragments up to 20kb. Using DNA fragments of greater molecular weight results in progressively worse resolution by such techniques. Methods by which larger fragments of DNA could be resolved were developed in the mid-eighties. These techniques were based on a system of pulsing alternating orthogonal electric fields applied to an agarose gel. When molecules in a gel matrix are subjected to an electric field at an obtuse angle to their direction of migration they will reorientate themselves along the new field axis and move forward. Larger molecules are unable to reorientate themselves as quickly as smaller molecules and as a consequence migrate slower through the gel resulting in the clearer separation of the DNA than possible using conventional methods of gel electrophoresis. The early methods have been successfully modified and the size range which can now be resolved is in the range of 50kb-12mb. This has facilitated a greater flexibility in molecular genetical studies and the widening of applicability. Some examples include the determination of the genome size of some prokaryotes (Dixon and Kinghorn, 1990) and in the analysis of mammalian genetic disease (Kenwrick *et al.*, 1987). The analysis of plant DNA is also being undertaken (Ganal and Tanksley , 1989) resulting in an evaluation of the extraction methods of DNA suitable for PGFE, the use of rare-cutting enzymes, Southern transfers and hybridisation of radiolabelled probes to megabase plant DNA.

The preparation of high molecular weight DNA samples by conventional methods may present a problem because of their sensitivity. A method can be used if the cells have their cell walls removed. The resultant protoplasts (for review of plant protoplasts, see Millam and Kumar, 1991) are embedded in agarose blocks and freed of their contaminating protein and cell wall debris, as protein that has bound to DNA alters the electrophoretic capability of the DNA. A solution of EDTA, laurolyl sarcosine and proteinase K is used to lyse the cells, yielding intact chromosomal DNA samples suitable for PGFE (Smith *et al.*, 1987). The agarose matrix keeps large DNA molecules undiminished whilst permitting the free radiation of detergent and proteinase K. The large fragments of DNA which arise following the use of rare-cutter enzymes can be disassociated using PGFE and ultimately it has been speculated that PGFE will enable the generation of complete physical maps of many higher eukaryote genomes.

5. SOUTHERN BLOTTING

Southern analysis (Southern, 1975) is used to determine the presence, location and organisation of particular sequences of DNA in gel fractionated nucleic acids and is perceptive enough to map restriction sites around single copy genes in total genomic DNA. Genomic DNA is digested with restriction enzymes and the resultant fragments separated according to size by agarose gel electrophoresis (Southern, 1979). If DNA is heated beyond a critical temperature or treated with an alkali the double helix will separate into single strands (denaturation). When the rigourous conditions are removed the double helix will reanneal. An important element is that the double helix will only reform if complementary strands link up with each other. The restricted single-strand DNA is transferred to a support membrane by capillary action

(or electrotransfer) so that the relative position of the DNA remains the same. The immobilized DNA is then radioactively labelled with hybridization probes (complementary DNA) for the detection of specific DNA sequences. The blot is then washed and laid down against film in an intensifying cassette. The blots are then developed for a 24-72 hour period (often at -70^{0}C) before being developed.

The resultant bands are indicative of a hybridisation having taken place between the restricted DNA and the selected probe. Nucleic acid hybridization is a key tool in molecular biology. Almost every aspect of gene cloning, characterisation and analysis of genes involves, at some stage, the hybridization of one strand of nucleic acid to another.

Methods of hybridisation of radiolabelled probes in solution to immobilised nucleic acids vary according to solvent used, concentration of the labelled probe and its activity and the use of compounds such as dextran sulphate to increase the reassociation rate of nucleic acids. Possibly the most favoured technique in use today is the synthesis of uniformly labelled DNA probes using random oligonucleotide primers. Random primers are obtained by several methods including the synthesis of a population of a population of octamers using a DNA synthesiser. Several types of substance can be used to block the non-specific attachment of the probe to the filters including denatured salmon sperm, dried milk and heparin. It should be noted that DNA will also hybridise with complementary RNA, and hence it is possible for the probe to be a purified RNA species rather than denatured DNA.

6. DNA SEQUENCING

Until comparatively recently DNA sequencing was extremely difficult. The methods of Maxam and Gilbert (1977), based on the ability to manipulate DNA with a variety of enzymes revolutionized this vital area. Their method involved the labelling of restriction fragments at each end with radiolabelled enzymes. From a restriction map an enzyme is chosen which will remove a small section from one end of the molecule leaving one end still labelled. The DNA is then chemically cleaved at specific residues, but in these reactions the cleavages are only partly completed. These partial digestion products are then separated by PAGE and autoradiographed. Only fragments containing the radioactively labelled termini will be visible, and the sequence can then be evolved from the order of fragments from the different digestions.

Other methods exist and the subject was reviewed by Old and Primrose in 1981. The analysis of cloned DNA has resulted in the clarification and advancement of the sequencing of genes, their expression and function. Though sequencing is a key technique in molecular biology it is heavily dependent on many of the aforementioned tools such as DNA isolation, gel electrophoresis and restriction mapping.

7. GENE AMPLIFICATION USING POLYMERASE CHAIN REACTION (PCR) TECHNIQUES

Undoubtedly the most significant breakthrough in molecular biology in recent years has been the development and utilisation of gene amplification techniques by a technique known as polymerase chain reaction (PCR). This reaction utilises the ability of a small fragment of DNA to "find and bind" to another fragment of DNA. An enzyme known as polymerase constructs a new piece of DNA analogous to the region adjacent to the target. On repeating this through several cycles the target sequence is exponentially amplified.

In the PCR reaction primers (oligonucleotides), buffer and an enzyme isolated from the bacteria found in hot springs *Thermophilus aquaticus* (Taq) are mixed with the DNA isolated from plant or bacteria. The reaction mix is heated up to 90^{o}C+ and the double stand of DNA is separated. As the mix is cooled to 35-50^{o}C (according to the specific requirements of the assay) the short oligonucleotides find their complementary sequences along the strands of DNA and bind to them. The polymerase joins nucleotides into a new strand of DNA attached to the oligonucleotide primer, this new strand is complementary to the sequence found along the long strand. The next step is to reheat the mix, again the result is the separation of the DNA strands, but where previously there was one sequence there are now two. Again as the reaction is cooled the primer binds to the target sequence, the polymerase builds a new strand of DNA and there are now four sequences of the target DNA, after 30 cycles there will be approximately a billion copies of the target sequence.

The mix can then be run on an agarose gel and stained with ethidium bromide to visualise the products against controls and marker samples. These techniques can be applied for a range of applications such as cloning specific sequences, searching for a known gene sequence (possibly that of a gene that has been added to the plant by genetic transformation) and genetic fingerprinting. The principle advantages are the speed of the method (3-4 hours from initial sample to result) and the avoidance of the use of radioactive compounds. The basic equipment is relatively cheap (a programmable hotblock costs around £2000-4000) but the enzyme is relatively expensive, though new enzymes are being developed.

FUTURE PROSPECTS

The historical and current status of nucleic acid isolation and characterisation are outlined in this paper. We can speculate as to how these techniques can contribute to areas of plant research and crop protection in both the near future and the longer term.

The importance of understanding the fundamental processes involved in basic biology cannot be underestimated. The knowledge of how cells replicate, how genetic information can be transferred from cell to cell and from species to species allow a much more precise targetting of research effort. Previous methods involving the screening of large populations can be

replaced partly or in some instances wholly by the application of molecular and biochemical methods.

The use of genetic marker technology (which will be discussed in more detail by some of the following speakers) has enormous implications in the development of crop protection products. The use of rapid detection techniques using PCR has resulted in a considerable speeding-up of basic plant analysis techniques and such methods are in use for varietal identification (Wilde *et al.*, 1991), confirmation of the transfer of a new gene to a plant (Millam *et al.*, 1991) and numerous other applications.

The advent and application of gene transfer technology has considerably broadened the scope of creating plants which contain specific genes taken from another source which would have been impossible by conventional plant breeding technology, examples include the transfer of a gene that confers insect resistance to a range of crops taken from the African legume plant cowpea (Gatehouse *et al.*, 1991).

Molecular biology has been the focus of a great deal of research effort in the last ten years. Though much work has been targeted at understanding basic mechanisms the knowledge, and importantly, the techniques that have been derived from this considerable input of investigative endeavour have immense and far-reaching implications and practical value.

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MOLECULAR TECHNIQUES FOR PLANT SCIENTISTS

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ABSTRACT

Recent developments in molecular biology are having a profound impact on the plant sciences. Techniques are now available that permit the manipulation of nucleic acids in studies leading to a detailed understanding of plant processes. This report describes the techniques of subtraction hybridization, restriction fragment length polymorphism analysis, and the polymerase chain reaction. Current and potential applications of these techniques in plant biology are presented. In addition, the extraordinary power of plant mutant analysis in elucidating biochemical and developmental pathways is discussed.

INTRODUCTION

The techniques of molecular biology are being widely used to provide substantial insight into many areas of plant biology. In combination with traditional investigations of physiology, biochemistry, and genetics, molecular biologists are beginning to elucidate the fundamental aspects and regulation of basic plant physiological processes. Some of the first and most widely publicized applications of molecular biology in the plant sciences have occurred in the area of weed science, i.e., the introduction of herbicide resistance into crop plants. Accomplishments in this field are well documented (Hatzios, 1987; LeBaron et al., 1987; Mazur and Falco, 1989) and the commercial release of several resistant crop cultivars is at hand. Such developments, while highly significant because of the technological advances they represent, do not address basic aspects of weed science concerning the biology, competitiveness, and ecology of weedy plants and their interactions with crops. Therefore, this paper will not review the current status of herbicide-resistant crops, but will instead present and discuss several molecular techniques with enormous potential to provide insight into some fundamental questions of weed science.

Traditional techniques of molecular biology are based largely on the goal of isolating, characterizing, or manipulating a particular gene of known function. Techniques designed with these goals in mind are described by other contributors to this symposium and have recently been reviewed (Dyer, 1991b). In addition, comprehensive and lucid laboratory manuals for most methods of molecular biology are available (Ausubel et al., 1988; Sambrook et al., 1989). An alternative to studying a single gene is to investigate the genetic aspects and regulation of a developmental process or molecular responses to environmental stimuli. In this regard, the technique of subtraction hybridization may be used to isolate genes involved in such processes with no previous knowledge of their specific functions. Two recently developed techniques, restriction fragment length polymorphism (RFLP) analysis and the polymerase chain reaction (PCR), will be described as well as their current and potential applications in plant science. In addition, the advantages of plant mutants as experimental vehicles will be discussed.

SUBTRACTION HYBRIDIZATION

Recent advances in molecular techniques now permit the isolation of genes based solely on differential expression levels, with no underlying knowledge of gene function. In other words, the only prerequisite for this technique is that genes are expressed under one set of conditions and not under other conditions. The term subtraction hybridization refers to the process of subtracting or removing all mRNAs expressed under both conditions so that only messages unique to the induced condition are recovered. Figure 1 illustrates one of several methods for subtraction hybridization.

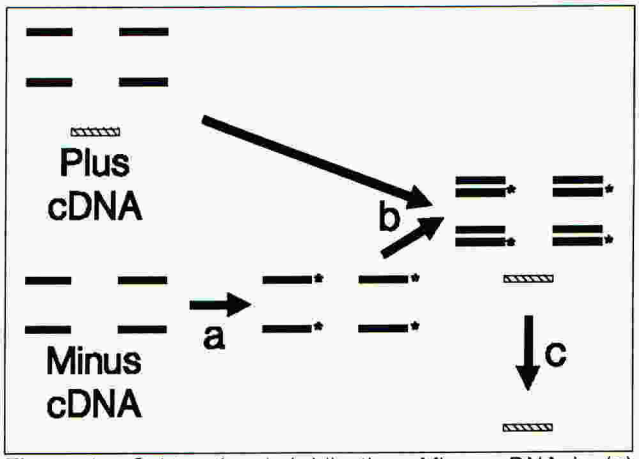

Figure 1. Subtraction hybridization. Minus cDNA is (a) photobiotinylated, (b) hybridized with Plus cDNA, and the mixture is (c) treated with streptavidin, leaving unique cDNA (hatched box) for recovery and cloning.

Complementary DNA (cDNA) is synthesized (Ausubel et al., 1988) from mRNA isolated from induced (plus) and noninduced (minus) tissue and randomly cloned into a vector that allows the isolation of single-stranded cDNA molecules. A number of specialized vectors, or DNA vehicles capable of independent replication within bacterial hosts, have recently been developed and are widely available (i.e., Short et al., 1988). Minus cDNA is photobiotinylated to chemically "tag" the nucleic acids and then hybridized with plus cDNA so that all molecules common to both conditions hybridize to their counterparts, creating duplex DNA. The biotinylated duplex cDNA is treated with streptavidin, causing it to partition into the organic phase for removal during a subsequent phenol extraction. cDNAs unique to the plus condition remain as single-stranded molecules and are retained in the aqueous phase for recovery and cloning to create a small library. An alternative method for removing double stranded molecules is via hydroxylapatite chromatography (Davis, 1986), although this step is technically very difficult and tedious. cDNAs thus isolated may be sequenced and their role(s) in the process under investigation determined.

Subtraction hybridization has been used successfully to isolate genes expressed specifically in plant organs (Medford et al., 1991) and genes involved in a number of plant developmental processes such as flowering (Sommer et al., 1990). In an interesting and commercially very useful application resulting from this technology, Mariani et al. (1990) fused a promoter region from a gene specifically expressed in the tobacco tapetum (tissue surrounding the pollen sac early in pollen development) to a ribonuclease-encoding gene and transformed tobacco and oilseed rape plants with this construction (transgenic plants). Organ-specific expression of ribonuclease during microsporogenesis destroyed the tapetum and resulted in male sterility. Similar subtraction methodology has been used to isolate genes highly expressed during early seedling growth, whose promoters may be fused to genes conferring herbicide resistance in crop plants, thus providing the maximum degree of

expression during exposure to the herbicide. Commercially acceptable levels of glyphosate resistance in transgenic crop plants have been achieved using resistance genes controlled by meristem-specific promoters.

Subtraction hybridization may have potential in weed science for investigations of herbicide safener mechanism of action. Herbicide safeners, also known as antidotes or protectants, are widely used as seed treatments and tankmixes to protect crops from normally injurious herbicides (Lamoureux and Rusness, 1986). Most safeners so far investigated induce the activity of glutathione *S*-transferase and/or increase the amount of free glutathione in cells, thus allowing elevated metabolic detoxification of the herbicide via glutathione conjugation. However, induction of other enzymes or herbicide catabolic pathways may also contribute to the safening process. Subtraction hybridization of safened *vs.* nonsafened cDNA populations may allow the isolation of genes involved in these as yet uncharacterized processes.

The physiological aspects of seed dormancy have been extensively studied, but a detailed molecular understanding of dormancy maintenance and release in mature seeds remains elusive. As a first step in using subtraction hybridization to identify regulatory genes controlling this process, mRNA and protein populations have been compared in embryos isolated from dormant and nondormant *Avena fatua* caryopses during imbibition (Dyer, 1991a). The underlying hypothesis is that changes in relative amounts of certain mRNAs or proteins may be visible during the transition from dormancy to germinability if seed dormancy maintenance and release are regulated at the level of gene expression. Briefly, poly(A^+) mRNA was isolated from imbibing embryos, translated *in vitro* using a rabbit reticulocyte system in the presence of ^{35}S-methionine, and the translation products separated using two-dimensional polyacrylamide gel electrophoresis (2-D PAGE).

2-D PAGE combines the separation of proteins based on net charge with separation by molecular weight to provide a powerful tool for establishing reproducible patterns of complex protein populations (Dunbar, 1987). *In vitro* translation products or protein extracts are subjected to isoelectric focusing in small diameter acrylamide tube gels, in which individual proteins are separated based on their isoelectric points (pI) in a steady state pH gradient. Proteins are subsequently electrophoresed through polyacrylamide slab gels in the presence of SDS, so that separation is based primarily on molecular weight. Visualization of individual polypeptides in the slab gel is accomplished through staining, or autoradiography for *in vitro* translation products. Results from the seed dormancy studies show that specific mRNA and polypeptides are associated only with dormant embryos during imbibition, indicating that dormancy may be enforced and maintained by dormancy-specific embryonic gene expression. While such results do not establish cause and effect, they provide strong initial support for subsequent attempts to isolate and identify these putative dormancy-specific genes using subtraction hybridization.

RFLP ANALYSIS

Studies of genetic variation in plants provide valuable information on genotypic diversity, mating systems, and genetic structures of populations. In particular, genetic analyses of weedy species have been useful in documenting levels of inter-and intraspecific diversity (Warwick, 1990) as well as for suggesting reasons why certain weedy taxa, such as the weed-crop complex in *Setaria* have been especially successful as colonizers (Darmency et al., 1987). Similar analyses have been combined successfully with traditional taxonomic

studies to aid in grouping many genera and species (Crawford, 1990). However, systematic classification of weedy species within some genera, such as *Euphorbia* and *Setaria*, has been especially difficult.

Traditional plant taxonomy has relied primarily on morphological and cytological markers for classification. Measurements of variation in isozyme patterns using starch gel electrophoresis (Soltis and Soltis, 1989) have also been used for plant systematic studies. Studies of allelic variation at 25 loci for North American populations of the annual weedy grass *Bromus tectorum* have been used to document levels of within-population and among-population genetic variation (Novak et al., 1991). However, in many weedy genera and species, very low levels of isozyme variation preclude their use as systematic characters. More recently, restriction fragment length polymorphism (RFLP) analysis of chloroplast DNA (cpDNA) has been shown to be of substantial utility in determining phylogenetic and systematic relationships (see Palmer, 1987 and references therein for detailed protocols). In general, cpDNA is preferred to mitochondrial or nuclear DNA since it is highly conserved among all land plants and thus polymorphisms are limited to a manageable number. The technique is based on detecting alterations in restriction endonuclease recognition sites, which are specific four- to eight-base pair (bp) DNA sequences (usually) recognized and cleaved by enzymes from various bacterial sources. Alterations due to point mutations and rearrangements are thought to be passed on to all progeny of the original mutant and are therefore group specific and stably inherited.

The simplest form of RFLP analysis involves purifying cpDNA from species or populations under study and digesting the DNA with a battery of restriction endonucleases that recognize AT-rich, six-bp sequences. Resulting DNA fragments are size-separated via agarose gel electrophoresis, stained with ethidium bromide, and photogragphed under ultraviolet illumination. Loss or gain of restriction sites results in changes in restriction fragment patterns, and when shared among individuals, populations, or species, are taken as indicators of phylogenetic relationship. This type of RFLP analysis may be adequate for studies at the interspecific or generic level.

A further refinement of RFLP analysis involves blotting DNA fragments from agarose gels onto reusable nylon membranes (Southern blotting) which are hybridized with a radioactively or chemically labeled DNA fragment from the chloroplast genome. Probe sequences are chosen from highly conserved regions of the genome and therefore will only detect a manageable number of polymorphisms. This technique is usually carried out using total genomic DNA, which is technically much simpler to isolate than purified cpDNA. Radioactive detection permits a much higher degree of sensitivity, and since blots can be rehybridized with several different probes, only one gel need be run for most determinations. In addition to RFLP analysis, a modification of the polymerase chain reaction technique (see discussion below) has potential to be used for phylogenetic and systematic studies.

Precise classification of weedy species is important not only for reasons as mentioned above, but is also being carried out to aid in the successful selection and use of biological control agents. Nissen and Masters (1991) are comparing RFLP maps of domestic and foreign accessions of *Euphorbia esula* in order to more closely determine the center of origin of types recently introduced into the western United States, which are spreading rapidly. Potential biological control agents collected from the species' center of origin should have ahigh likelihood of colonization on domestic infestations, since they are already adapted to similar ecotypes.

In other studies, genotypic variation within asexual lineages of *Taraxacum officinale* has been documented by comparison of restriction site variation in genes encoding ribosomal RNAs (King and Schaal, 1990). The results indicate that, together with processes of clonal selection, nonmeiotic recombination may play a significant role in the genetic variation and adaptive evolution of an asexual species.

Genes or linkage groups responsible for disease resistance in *Hordeum spontaneum*, a weedy relative of cultivated barley, have been located with the aid of RFLP analysis. Introgression of these genes into barley cultivars is currently being carried out. Similar applications may be possible where close relationships between wild and domesticated species have been identified.

POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR), although a very recently developed technique, is finding wide application in a number of diverse fields of science (Erlich, 1989). PCR is used to rapidly amplify specific DNA sequences from complex mixtures of nucleic acids. The basic procedure relies on the ability of thermostable DNA polymerase to replicate a specific DNA sequence through many cycles of denaturation, annealing, and strand elongation using short oligonucleotides as primers. Figure 2 schematically illustrates the PCR process.

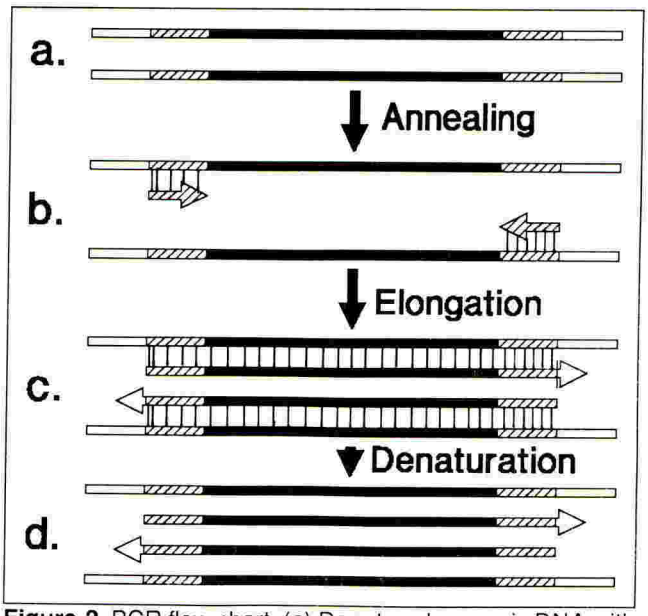

Figure 2. PCR flow chart. (a) Denatured genomic DNA with known primer sequences (striped) and region to be amplified (shaded) is (b) annealed with primers. Two new DNA strands are (c) synthesized and (d) denatured for further amplification.

One of the primary applications of PCR is for cloning in heterologous systems, in which a known gene sequence is used to clone the same gene in the desired experimental organism. During the PCR process, annealing temperature is probably the most critical parameter, since it determines the stringency of hybridization, or how easily primers may bind to nonhomologous sequences. This factor is especially important in heterologous cloning because the potential lack of sequence conservation among species may necessitate some empirical testing to determine the optimum annealing temperature. After PCR, the amplified fragment is first checked using agarose gel electrophoresis to determine if it is of expected size. If so, the fragment is usually sequenced to confirm its identity, after which it may be radioactively labeled for use in gene expression studies or to obtain the full length clone. The advantage

of this approach is that it allows a clone to be isolated from one overnight PCR run, as opposed to several months of library preparation and screening.

PCR has had a major impact in the area of diagnostic testing for the presence of pathogens or other organisms. For example, viral infection in registered crop seeds or other plant parts may be detected using PCR with primers complementary to specific virus DNA sequences. The unique specificity afforded by primer design in combination with the ability to detect extremely small amounts of DNA allows detection of any foreign organisms long before symptomology may appear. Also, PCR detection is often much faster and more reliable than traditional culturing methods.

Numerous modifications of the basic PCR protocol have been developed for specific applications (Innis et al., 1990). For example, amplification of cDNA populations may be carried out if obtaining sufficient amounts of experimental tissue is especially difficult or if mRNA or cDNA are limiting. Random amplification of polymorphic DNA (RAPD) PCR is used in combination with RFLP mapping to construct molecular genetic maps (Williams et al., 1990), and may be useful in characterizing populations or subgroups of weedy invaders. Genomic DNA is amplified using individual primers of arbitrary nucleotide sequence, which hybridize randomly and create a series of amplified fragments resolved as group-specific patterns in agarose gel electrophoresis. Polymorphisms thus detected between groups may be used as genetic markers in the absence of any specific sequence information. PCR has also been successfully applied to the technique of *in vitro* mutagenesis (Dulau et al., 1989), in which directed mutations are introduced into genes or regulatory regions to determine the effect of specific changes on gene expression or regulation. For example, mutations introduced into the regulatory regions of several genes controlled by light have identified specific DNA sequences that are involved in the phytochrome response. Additional modifications of the basic PCR technique with novel applications continue to appear regularly, and without doubt herald the firm establishment of PCR as a major methodology in molecular biology.

THE UTILITY OF PLANT MUTANTS

Genetic analysis of mutants has proven to be an exceedingly valuable tool to identify components of morphogenetic and developmental processes, as well as responses to environmental stimuli (Key, 1981). In weed science, a particularly useful application has been the determination of herbicide mechanisms of action. For example, molecular details of glyphosate and sulfonylurea herbicide action have been provided in large part through characterization of resistant mutants (Hatzios, 1987; LeBaron et al., 1987). Substantial progress has been made in defining genetic pathways for many processes in organisms amenable to genetic analysis including *E. coli*, the yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans* and *Drosophila melanogaster*. Although genetic analysis is currently used in only a few plant species, the number of available morphological and developmental mutants is rapidly increasing (Thomas and Grierson, 1987). Most mutants have been created in *Arabidopsis thaliana*, a small weedy crucifer with several desirable attributes for molecular and biochemical studies, including small genome size, short generation time, and ease of cultivation and transformation. *Zea mays* mutants with a number of altered phenotypes have also been well characterized and are now the subject of intense molecular scrutiny in several laboratories.

Genetic analysis is extremely useful because it identifies genes essential to a process which can then be cloned and sequenced, providing information necessary to understand a particular system in molecular detail. Also, genetic analysis of mutant phenotypes may reveal a genetic linkage between two physiological aspects previously thought to be unrelated. In this way, mutant analysis, in combination with traditional physiological and biochemical studies, has contributed substantially to our fundamental understanding of plant developmental, morphogenetic, and metabolic processes.

Plant mutants are most often obtained through chemical mutagenesis of seed, usually using ethyl methanesulfonate (EMS). EMS creates random point mutations in DNA, altering or inactivating gene expression. Plants resulting from EMS-treated seed (M1 generation) are selfed and the progeny (M2 generation) are screened for mutants with the desired phenotype, e.g. herbicide resistance, thus providing a means of recovering homozygous recessive mutations. Once a desired mutant has been identified and characterized, the chromosomal location of the lesion may be identified by traditional backcrossing experiments using mapping strains containing known genetic markers. More importantly, the gene of interest may be isolated by "shotgun" complementation (Klee et al., 1987), in which the mutant is transformed with random segments of wild type genomic DNA and transgenic plants in which the mutation has been complemented or reversed are recovered. Using accompanying marker genes as guides, it then becomes a fairly straightforward process to isolate and identify the gene of interest.

A second method of generating mutants involves the use of transposons or transposable elements, discrete DNA segments with the ability to integrate randomly in a host plant's genome (Wienand and Saedler, 1987). Integration usually destroys the target gene function. Genes thus inactivated are "tagged" with known transposon DNA sequences, creating a molecular label by which the affected gene may be isolated. Also, spontaneous but imprecise transposon excision may generate alleles with altered gene expression which are useful for studying gene regulation and function. Several homeotic mutations (altering the time and location at which particular organs develop) affecting floral development have recently been obtained through transposon mutagenesis of *Antirrhinum majus* (Carpenter and Coen, 1990). This and other reports from laboratories studying flowering provide strong optimism that regulatory genes controlling this fundamental plant process will soon be isolated and identified. Although transposon families have been well characterized only in *Zea* spp. and *A. majus*, the demonstration that a maize transposable element could transpose in transgenic tobacco and other dicots (Baker et al., 1986) is part of a substantial effort underway in several laboratories to develop tagging strategies for other plants.

SUMMARY AND CONCLUSIONS

Novel molecular techniques now allow the direct isolation of genes controlling fundamental plant processes with no previous knowledge of their identity or function. These advances provide exciting opportunities for researchers in all areas of plant science. Traditional biochemists and physiologists should be able to enter into fruitful collaborations with molecular biologists using this methodology. Similarly, RFLP analysis may be used by plant systematists in conjunction with weed scientists to characterize and map the spread of invading species. PCR represents a widely accepted technique for amplifying DNA that may be used to augment and streamline a number of existing molecular methods. Applications in heterologous cloning, plant disease detection, and systematic studies are but a few of the many uses for PCR. Characterization of plant mutants using recently developed molecular

techniques continues to be of immense value in elucidating developmental processes and metabolic pathways. As more mutants become available, the contribution of this field to our overall understanding of plant biology and plant interactions will increase dramatically.

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GENETIC MANIPULATION FOR CROP PROTECTION: APPLICATION OF SOMATIC CELL APPROACHES AND RECOMBINANT DNA TECHNOLOGY

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ABSTRACT

The reproducible regeneration of fertile plants from tissues, cells and isolated protoplasts is fundamental to the genetic manipulation of crop plants through genetic transformation, somatic hybridisation and the exploitation of somaclonal variation. *Agrobacterium*-mediated transformation has been used to introduce virus, insect and herbicide resistances into several dicotyledonous plants, including crop species. Direct DNA uptake into isolated protoplasts and bombardment of tissues with DNA-coated particles are methods for transforming monocotyledons and other plants which do not respond to *Agrobacterium*-mediated gene transfer. Somatic hybridisation enables genes to be mobilised between genera, species and varieties. This approach can be employed to transfer agronomically important genes from wild to cultivated plants.

INTRODUCTION

During the last 10 years, a range of somatic cell and molecular procedures have been developed for plant genetic manipulation, which can be used as an adjunct to conventional plant breeding. These approaches include plant tissue culture and recombinant DNA technology, the latter permitting the precise manipulation of individual genes. Concurrent with major advances in recombinant DNA technology has been the elucidation of many of the molecular events associated with the transformation of plant cells by Gram-negative soil bacteria of the genus *Agrobacterium*. Indeed, detailed studies of the plasmids of these micro-organisms resulted in the discovery that agrobacteria are natural plant genetic engineers. This fact has been turned to advantage by utilising the bacterial gene transfer mechanism to insert genes, which have been isolated or synthesised in the laboratory, into plant cells. It has also been established that isolated plant protoplasts can be stimulated, like animal cells, to take up foreign DNA, and this has also been used in plant transformation. Additionally, techniques such as micro-injection of cells and particle bombardment of tissues are being exploited to introduce DNA into plants. Whilst these research disciplines are distinct, their unification has been essential to ensure the production of transgenic plants expressing novel traits.

Thus, it is possible to identify and to isolate DNA sequences which control important plant physiological processes related to development and productivity, to modify existing genes or to transfer new ones into plants from other

organisms. Characteristics such as herbicide and insect resistance can be introduced into plants. As the ability to isolate and to clone large pieces of DNA carrying several genes becomes routine, so the insertion of polygenic traits, such as disease resistance, into crop plants should become a realistic proposition.

TISSUE CULTURE AS A BASIS FOR PLANT GENETIC MANIPULATION

The ability to regenerate large populations of plants reproducibly, with the minimum of effort and in the shortest period of time from excised organs, tissues, cells and isolated protoplasts, is essential for plant genetic manipulation. About 6000 papers and 100 books were published on plant tissue culture during the period 1985-1989 (Bhojwani *et al.*, 1990), and the fact that about 850 publications relate to plant genetic engineering, emphasises the interest in this subject. The concept of culturing plant cells in the laboratory, conceived at the turn of the present century, and the ideas and procedures which have developed since that time, have been reviewed extensively (Thorpe, 1990).

After surface sterilisation (e.g. by immersion in a dilute solution of sodium hypochlorite, followed by washing with sterile water), plant organs can be grown axenically in the absence of micro-organisms. The media used for culture consist of major salts and trace elements, vitamins, amino-acids, a carbohydrate source and a suitable combination of auxins and cytokinins, and may be made semi-solid with a gelling agent such as agar or agarose. The precise medium composition and growth conditions must be determined empirically, but a temperature of 25°C with low intensity daylight fluorescent illumination (0.5 - 2.0 W m^{-2}) is adequate for many plants. A range of glass and plastic vessels, in which plant material can be cultured, is available from several commercial suppliers.

Organs can serve as a source of explants for callus induction. In the case of leaves, explants about 1 cm in size are placed on the surface of the culture medium. The process of excision damages cells at the cut edge of the explants and induces a wound response, which, in the presence of growth regulators in the medium, culminates in the production of parenchymatous cells around the periphery of the explant. The resulting callus can be removed from the explant and transferred to fresh culture medium, where it can be maintained indefinitely by regular subculture every 7 - 28 days. High cytokinin levels relative to auxin in the medium normally promote organogenesis, enabling shoots to be induced from callus. Such shoots, in due course, can be excised from the parent tissue, transferred to medium lacking growth regulators, or containing very low auxin levels, for rooting and later transferred to compost prior to hardening off and maintenance in the glasshouse. Shoot production in many plants occurs by organogenesis, but with some plants, such as forage legumes and cereals, somatic embryogenesis is the main pathway of plant regeneration. Frequently, shoots can be induced directly on explants with the minimum of callus formation. Callus can be used to initiate cell suspensions by immersing the tissue in liquid medium in Erlenmeyer flasks on a horizontal rotary shaker. These suspensions can be maintained by regularly transferring an aliquot of medium containing suspended cells to fresh medium (approximately 1:4, v:v dilution) every 7 - 21 days. Callus can be re-initiated from suspensions by transferring the latter to agar-solidified medium. Cells in suspension can be induced to undergo morphogenesis to form shoots or somatic embryos, the production of somatic embryos having application in 'artificial seed' technology (Merkle *et al.*, 1990; Redenbaugh and Walker, 1990).

Leaves from glasshouse-grown plants and axenic shoots, seedling organs

(cotyledons, hypocotyls and roots), callus and cell suspensions are employed as sources of protoplasts (cells from which the walls have been removed). The latter are isolated enzymatically using mixtures of commercially available enzymes which digest cell walls. Most of the cellulase and pectinase enzymes commonly used and the general procedures for protoplast isolation have been reviewed extensively in articles which can be found in Giles (1983), Potrykus *et al.* (1983), Vasil (1986) and Puite *et al.* (1988). Isolated protoplasts, being naked cells, are bounded only by their plasma membranes, as a consequence of which they are osmotically fragile and must be protected from bursting by inclusion of an osmotic stabiliser, such as mannitol (7 - 13% w/v), in the medium. The fact that the plasma membranes of freshly isolated protoplasts are exposed, enables them to be used for direct DNA uptake and somatic hybridisation. In culture, isolated protoplasts synthesise new cell walls and divide to produce daughter cells and tissues. The latter can be handled in the same way as explant-derived callus for plant regeneration. Such protoplast-derived plants, and those regenerated from other sources such as explants, can be micropropagated prior to their establishment under glasshouse or field conditions.

Shoots which arise directly from explants, and those which are micropropagated from parental shoots by axillary bud development, generally resemble the parental material. However, plants regenerated from explant-derived callus (somaclones) or protoplast-derived tissues (protoclones) frequently exhibit variation in phenotypic and other characteristics. Such somaclonal or protoclonal variation is not usually associated with gross cytological changes but may be related to more subtle alterations in nucleic acid composition, although evidence for this is still lacking. Frequently, this variation results in detrimental characteristics such as fasciated stems and dwarfism. However, in the case of *Medicago sativa*, a plant of the cultivar Europe, which originated from experiments using leaf protoplasts described by dos Santos *et al.* (1980), exhibited increased resistance to *Verticillium* wilt (Latunde-Dada and Lucas, 1983). In sugarcane, plants derived from callus have been screened for resistance to mosaic virus, downy mildew, Fiji disease, eyespot, leaf blight and smut. Krishnamurthi (1974) identified a somaclone, Pindar 70-31, which had resistance to Fiji disease and to downy mildew. The sugar yields of Pindar 70-31 were similar to those of the parent variety Pindar, enabling the somaclone to be introduced as a commercial variety in Fiji. Thus, tissue culture can result in plants expressing novel characteristics and, as a consequence, the culture procedure can be considered as a simple form of plant genetic manipulation. Such plants can be utilised to develop new varieties, provided these traits are stably inherited.

AGROBACTERIA AS NATURAL GENE VECTOR SYSTEMS

Agrobacterium tumefaciens and *A. rhizogenes* induce crown gall and hairy root diseases respectively on a range of dicotyledonous plants. The former disease is characterised by the presence of irregularly shaped tumours at the site of infection by the bacterium (often in, or near to, the crown of the plant), while in hairy root disease, short, cone-like roots develop on the host plant at the site of infection. Both transformed 'hairy' roots and crown gall tumour cells can be excised from the parent plant and cultured on simple culture media lacking growth regulators. Tumour cells produce their own auxin and cytokinin, and hairy roots elongate rapidly and exhibit prolific root hair development under the high humidity conditions of culture. In contrast, non-transformed plant cells require exogenously supplied auxins and cytokinins for growth *in vitro*. The

inciting bacteria can be eliminated from the tumours or hairy roots by including antibiotics in the media during the first 4 - 8 weeks of culture.

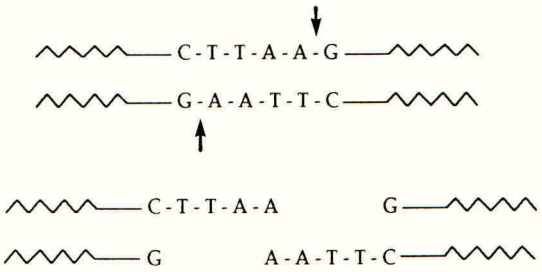
Agrobacteria are aerobic, rod-shaped (approx 1.7 x 0.5 µm) flagellate bacteria, which carry single-copy, large (180 - 220 kb) Ti (tumour-inducing) or Ri (root-inducing) plasmids. During the mid 1970s it became possible to separate the Ti plasmid from bacterial chromosomal DNA by ultra-centrifugation on caesium chloride gradients and to use the isolated plasmid for contour length measurements by transmission electron microscopy. The ability to cut DNA at defined base sequences using specific restriction enzymes (e.g. *Eco* R1 and *Hind* III, Fig. 1) and to separate the fragments of different sizes on agarose gels by electrophoresis, enabled restriction maps of Ti and Ri plasmids to be constructed. Additionally, DNA fragments could be removed from gels and ligated into small plasmids (Fig. 1), such as pBR322 from the gut bacterium *Escherichia coli*. Such small plasmids, when re-introduced into *E. coli*, enabled the DNA sequence of interest to be amplified. The use of Southern blotting procedures to transfer DNA fragments from agarose gels to nitrocellulose or nylon membranes, followed by the use of cloned Ti and Ri plasmid fragments as probes for DNA - DNA hybridisation, permitted Ti and Ri plasmid sequences to be detected in tumour cells and transformed roots. A common feature of Ti and Ri plasmids is the presence of a T-DNA region which is transferred to, and stably integrated into, the plant genome, and which carries the genes for tumourigenesis. T-DNA integration into plant chromosomes appears to be random. Crown galls and transformed roots sythesise unusual amino-acids (opines) which act as carbon, nitrogen and phosphate sources for the transforming bacteria. As a result of this phenomenon, *Agrobacterium* may be considered as a sophisticated plant parasite which utilises natural genetic engineering to create a favourable niche for its own benefit (Hookaas and Schilperoot, 1985). Ti and Ri plasmids, and the bacterial strains which carry them, are classified according to the opines synthesised by transformed cells. Wild-type Ti plasmids and strains of *A. tumefaciens* (Fig. 2), are of the octopine nopaline, agropine, succinamopine or grapevine types, the first 2 being the most studied. Ri plasmids and strains are of the agropine, mannopine or cucumopine types. Reviews of the earlier literature on Ti and Ri plasmids can be founed in Davey *et al.* (1986) and Hooykaas (1989).

The T-DNA of nopaline-type Ti plasmids is a contiguous segment of about 23kb. The corresponding T-DNA of the octopine-type plasmids is slightly shorter and sometimes integrates in two pieces; the left (T_L; 13kb) and right (T_R; 6kb) regions [not to be confused with the left (L) and right (R) border regions of the T-DNA segment of Ti plasmids; see below and Fig. 2]. The T_L-DNA is essential for transformation. The T_R-DNA can be linked to the T_L-DNA or independently integrated into the plant genome with a similar or different copy number. Sometimes, the T_R-DNA is absent in transformed cells. The octopine T_L-DNA and the nopaline T-DNA carry the oncogenes *iaaM (tms 1)* and *iaaH (tms 2)*, which encode enzymes converting tryptophan to IAA, and *ipt (tmr)*, which encodes the enzyme converting adenosime monophosphate to isopentenyl adenosine monophosphate. The nopaline synthase *(nos)* and octopine synthase *(ocs)* genes are located near the right border; the gene for agrocinopine synthase is near the middle of the nopaline T-DNA. Gene 6a encodes octopine and nopaline secretion by plant cells. Two 25 bp imperfect direct repeat sequences border the T-DNA [left (L) and right (R) border sequences] and contain recognition sites for T-DNA cleavage and excision during transfer to plant cells. The virulence *(vir)* region, of about 40 kb, outside the T-DNA of the Ti plasmid is also essential for T-DNA excision and transfer to plants. It has at least 22 genes in 7 operons *(vir* A - *vir* G). *Vir* A and *vir* G, when activated by phenolic plant molecules such as

Figure 1.

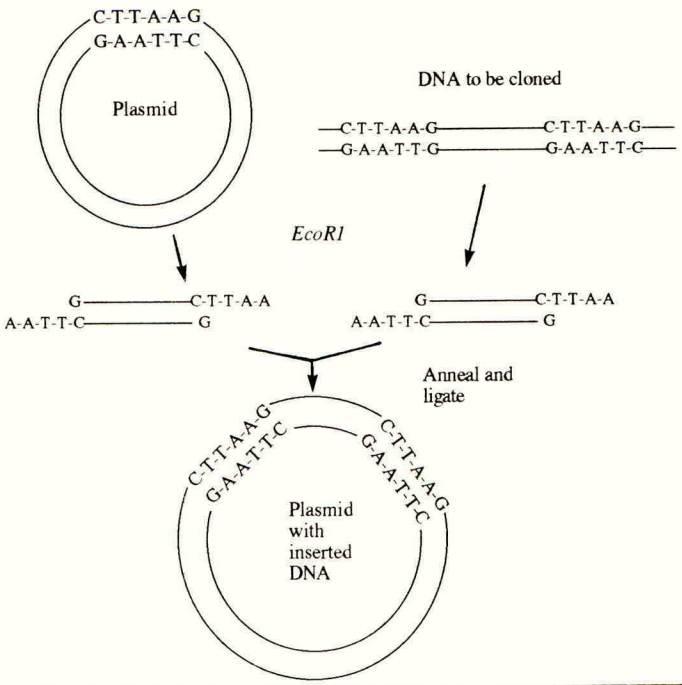
CUTTING (RESTRICTING) DNA

DNA can be cut using purified bacterial restriction endonucleases or 'restriction enzymes' which recognise particular sequences of bases and cleave the DNA within this recognition sequence. Each enzyme has its own characteristic recognition sequence, for example *EcoR1,* derived from *Escherichia coli,* cleaves DNA as shown:-



JOINING (LIGATING) DNA

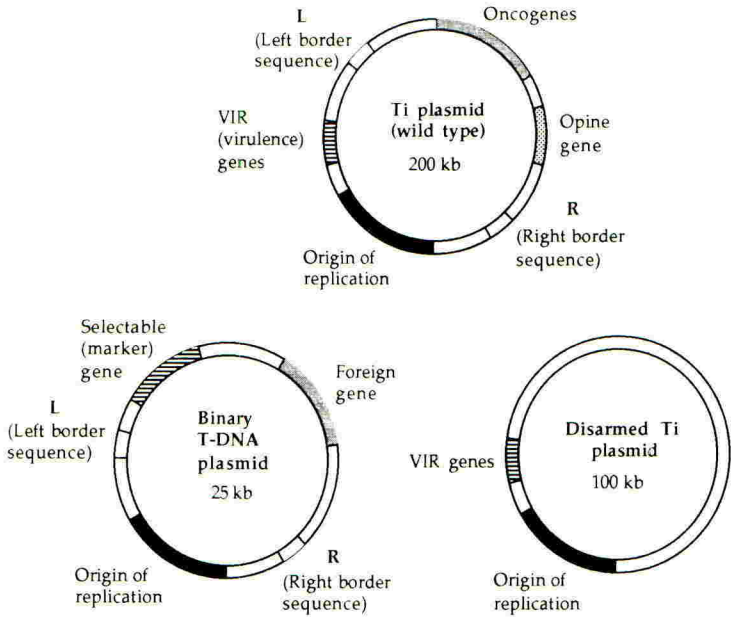
Enzymes such as *EcoR1* make staggered cuts in the two DNA strands generating single stranded tails or 'cohesive ends' whereas others generate blunt ends. Enzymes which generate cohesive ends are particularly useful for cloning work (such as inserting useful genes into plasmid vectors to be used for genetic transformation). Cohesive ends provide the initial structure for joining the plasmid and the insert DNA; the hybrid structure is stabilised by the action of a DNA ligase enzyme. The use of of *EcoR1* and DNA ligase for the cloning of a DNA insert into a plasmid vector is shown:-



acetosyringone (produced during wounding of dicotyledonous plant cells) activate other *vir* genes. Two genes on the *vir* D operon encode an endonuclease which nicks the T-DNA borders and initiates production of single-stranded T-DNA molecules. *Vir* B encodes proteins essential for T-DNA mobilisation. A *vir* C protein binds to an 'overdrive' region, next to the right T-DNA border repeat, which is essential for efficient T-DNA transfer to plant cells. The *vir* E2 gene encodes a T-strand binding protein. The *vir* region of Ri plasmids is thought to have a functional similarity with the *vir* region of Ti plasmid.

Figure 2.

WILD TYPE AND ENGINEERED BINARY TI PLASMIDS FOR GENE TRANSFER INTO PLANT CELLS



The wild type Ti plasmid from *A. tumefaciens* induces the infected plant to produce opine, and hormones which give rise to tumour formation. The engineered binary T-DNA plasmid transfers recombinant DNA to the plant utilising the virulence genes of a 'disarmed' Ti plasmid acting in trans.

Agropine Ri plasmids have left (T_L) and right (T_R) regions, each of 15 - 20 kb, separated by 15 kb of DNA which is not transferred to the plant genome. Although the T_L-DNA transferred to plants is relatively constant, the T_R-DNA is more variable. The T_R-DNA carries genes homologous to *tms* 1 and *tms* 2 of the Ti plasmids, and *ags* genes involved in agropine synthesis. The *rol* A, B, C and D loci on the T_L-DNA influence the phenotype of transgenic plants regenerated

from Ri-transformed roots, possibly by affecting cytokinin metabolism. Such phenotypic characteristics include wrinkled leaves, dwarfism and reduced fertility. Ri plasmids carrying only the T_L-DNA or only the T_R-DNA (McInnes *et al.*, 1991) can induce transformed roots, but the response is not a strong as with wild type plasmids. A gene (*rol* B^{TR}), homologous to the T_L-DNA *rol* B gene, has been identified in the T_R-DNA of the agropine pRiA4 (Bouche and Camilleri, 1990). The molecular ogranisation of the T-DNA regions of mannopine and cucumopine Ri plasmids is less well defined, although the mechanism of root induction by mannopine Ri T-DNA and agropine Ri T_L-DNA may be similar.

The mechanism of T-DNA integration into the plant chromosomes is not clear, although T-strands could be transported and integrated into the plant genome. Frequently, foreign genes transferred to plant cells are truncated, rearranged or present in tandem repeats. Often single or an average of 3 tandem copies of the T-DNA integrate into the genomes of dicotyledons; both inverted and direct repeats are found. T-DNA integration, like the generation of the transferable T-strand copy, is directed by the right T-DNA border. Integration of foreign DNA into the plant genome may occur at the first two cell division cycles during the DNA replication.

The expression and inheritance of genes in plants transformed by *Agrobacterium* have been studied in a number of species. Variability of expression may reflect differences in insertion sites, copy number and degree of methylation. Foreign genes can be transmitted to progeny in a Mendelian fashion with high meiotic stability, but there are examples of loss of the transformed phenotype during meiosis through gene deletion or inactiviation. Non-Mendelian inheritance may occur when DNA integrates at high copy number. Inactiviation of T-DNA genes in tobacco crown galls after long periods of stability has been attributed to rearrangement of DNA related to somaclonal variation during tissue culture. In potato, spontaneous deletions of T_L- and T_R-DNA from Ri transformed potato root clones were reported after prolonged culture and also in a number of plants regenerated from these roots (Hanisch Ten Cate *et al.*, 1990). However, the causes of these deletions are not known.

Ti AND Ri PLASMIDS AS VECTORS FOR DELIVERING FOREIGN GENES TO PLANTS

Vectors for *Agrobacterium*-mediated DNA transfer to plants rely upon the fact that DNA inserted between the 25 bp border repeats of the T-DNA is transferred to plant genomes. Pioneering experiments in the use of the Ti plasmid as a vector involved insertion of the bacterial transposon Tn7 into the nopaline synthase gene of pTiT37 (Hernalsteens *et al.*, 1980). Crown galls induced on tobacco plants carried Tn7, coding for resistance to streptomycin, spectinomycin and trimethoprin, were not expressed in the transformed cells. Shortly after this report, recombinant DNA technology progressed to the stage at which it was possible to construct chimaeric genes, in which a bacterial antibiotic resistance gene was linked to a suitable promoter, such as the nopaline synthase *(nos)* promoter from the Ti plasmid itself, or a promoter from cauliflower mosaic virus (CaMV). Thus, by inserting a chimaeric gene with the *nos* promoter linked to the neomycin phosphotransferase II *(npt II)* gene into the T-DNA, crown gall cells were produced which expressed resistance to the antibiotic kanamycin sulphate (Herrera-Estrella *et al.*, 1983).

A major limitation of the Ti plasmid in its wild-type form was that

transformed cells either failed to produce shoots, or regenerated shoots were phenotypically abnormal and failed to develop roots. A significant advance in vector development was the deletion of the oncogenicity genes from the T-DNA, and their replacement with DNA sequences from *E. coli* cloning vectors, such as pBR322. Such a mutated Ti plasmid (pGV3850) still contained T-DNA borders (Zambryski *et al.*, 1983). Homologous recombination between a pBR322 vector carrying a foreign gene of interest with pBR322 sequences between the T-DNA borders, permitted insertion of the foreign gene into this disarmed Ti plasmid. Such a Ti plasmid could effect transfer and integration of the foreign gene into plant cells, because it still carried the T-DNA borders and the *vir* genes.

Using *A. tumefaciens* carrying pGV3850 with chimaeric genes between the T-DNA borders, DeBlock *et al.* (1984) transformed protoplast-derived cells of tobacco to kanamycin, methotrexate and choramphenicol resistance. Phenotypically normal, fertile plants were regenerated from these antibiotic resistant tissues and the chimeric genes were transmitted to seed progeny in a Mendelian manner. Another important advance in gene transfer to plants was the use of strains of *A. tumefaciens*, carrying disarmed vectors, to infect leaf discs. Following excision, the leaf explants were immersed in a bacterial suspension for a few minutes before blotting to remove excess bacteria. The explants were transferred to culture medium containing antibiotic to kill the bacteria and the appropriate auxins and cytokinins to induce shoot regeneration with the minimum of callus proliferation. Provided a selectable marker was present in the vector, transformed shoots could be separated from non-transformed shoots by their ability to grow in the presence of antibiotic (e.g. kanamycin sulphate) included in the plant regeneration medium (see Fig. 3). This approach resulted in the production of kanamycin resistant plants of petunia, tobacco and tomato (Horsch *et al.*, 1985). The simplicity of this procedure has enabled it to be applied to a range of species, including those outside the Solanaceae.

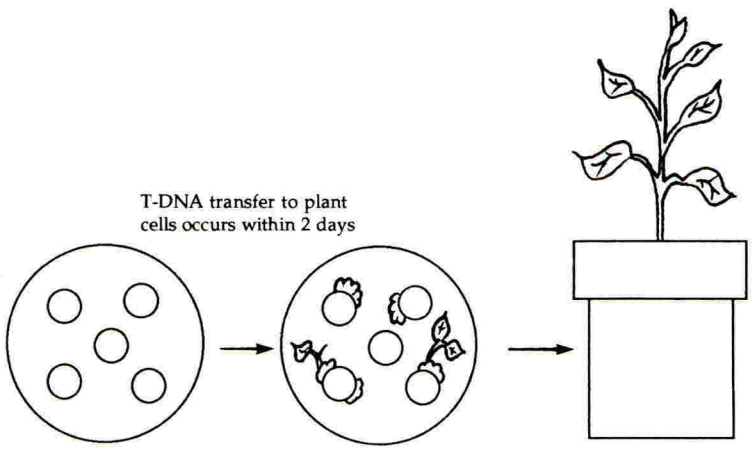
Ri plasmids have also been employed to introduce foreign genes into plants. The attraction of Ri plasmids as vectors is that roots transformed by wild type plasmids readily regenerate shoots spontaneously, as in *Lotus corniculatus*, or after transfer of root segments to shoot inducing medium (eg. *Glycine canescens;* Rech *et al.*, 1989). Although the roots of such regenerated shoots may exhibit the Ri transformed phenotype of negatively geotopic and plagiotropic growth, such root systems are usually extensive and enable regenerated shoots to be transferred easily to compost. The Ri T_L-DNA has been the site for co-integration of foreign genes, since the T_L-DNA incorporated into plant cells is less variable than the T_R-DNA. Thus, Morgan *et al.* (1987) introduced a chimaeric kanamycin resistance gene into *Hind* III fragment 11 of the T_L-DNA of pRiA4, while Pythond *et al.* (1987) introduced a similar *npt II* gene into *Hind* III fragment 21. The bacterial strain R1601 produced by Pythond *et al.* (1987) also carried pTVK291 in trans, conferring a 'supervirulent' phenotype on the bacterium. The wide host range of R1601 has enabled this strain to be used for the transformation of plants from several families. As in the case of Ti plasmids, disarmed Ri plasmids have also been produced. McInnes *et al.* (1989) removed the *rol* genes from the T_L-DNA of pRiA4, leaving only the T_L-DNA borders between which foreign genes could be co-integrated.

Binary vectors are also used extensively for plant genetic engineering and are based on the fact that gene transfer still occurs even when the *vir* and T-DNA regions of Ti or Ri plasmids are on separate plasmids (Fig. 2). Hoekema *et al.* (1983) first demonstrated that a strain of *A. tumefaciens*, harbouring two compatible plasmids, induced tumours on tomato, *Kalanchoë*, tobacco and pea.

The *vir* region was on a Ti plasmid deleted of its T-DNA; the T-DNA was carried on a small cloning vector. The advantage of this arrangement is that vectors carrying the T-DNA are much smaller than the Ti or Ri plasmids themselves. Consequently, these vectors are more amenable to genetic engineering. Foreign genes can be inserted readily into the T-DNA and the vectors can be easily introduced and amplified in *E. coli* prior to insertion into *Agrobacterium.* A logical progression in binary vector technology was the deletion of the oncogenecity genes between the T-DNA borders of the small vector and their replacement with *E. coli* sequences (Bevan, 1984). The latter usually contain unique restriction sites to permit easy insertion of foreign genes. Disarmed binary vectors with Ti T-DNA borders have been used in conjunction with resident wild-type Ri plasmids, while binary vectors with pRi T-DNA borders (disarmed micro-Ri plasmids) or pRi T-DNA borders plus parts of the pRi T-DNA (root-inducing, mini-Ri plasmids) have been inserted into agrobacteria carrying a reistant Ri or Ti plasmid lacking its entire T-DNA. Binary vectors have also been constructed with the origin of replication from agropine Ri plasmids and Ti T-DNA borders. Such vectors have been used in *A. tumefaciens.*

Figure 3.

PLANT GENETIC TRANSFORMATION USING *AGROBACTERIUM*



Surface sterilised leaf pieces inoculated with an engineered strain of *Agrobacterium tumefaciens* * and placed onto agar culture medium

Leaf discs transferred to callus/shoot regeneration medium containing antibiotic; only transformed cells/shoots can grow

Transformed (transgenic) plant containing the foreign gene in the transferred T-DNA

* contains the foreign gene of interest and an antibiotic resistance marker gene

PLANT TRANSFORMATION BY DIRECT UPTAKE OF DNA INTO PROTOPLASTS AND TISSUES

Whilst *Agrobacterium* transforms a wide range of dicotyledons, there are several genera of these plants which fail to respond to inoculation. Moreover, there is little evidence for the transformation of the major cereals and other monocotyledons, by *Agrobacterium*. Alternative approaches have been developed for transforming these plants, the most successful involving the direct uptake of DNA into isolated protoplasts. This area of research was stimulated by the discovery that T-DNA borders were unnecessary for gene integration into plant cells. Treatment with polythene glycol (PEG) of a mixture of tobacco leaf protoplasts and a plasmid carrying the *npt II* gene linked to a CaMV promoter, resulted in kanamycin resistant tissues from which phenotypically normal transgenic plants were regenerated. The *npt II* gene was transmitted to seed progeny in a Mendelian fashion (Paszkowski *et al.*, 1984). Subsequent work with tobacco showed that plasmid uptake into leaf protoplasts could also be induced by short duration (μsec or msec) high voltage electrical discharges. During electrical treatment (electroporation; see Fig. 4), pores are believed to form in the plasma membranes of the protoplasts, which permit entry of exogenously supplied DNA into the cytoplasm. Such pores are thought to re-seal soon after electrical treatment.

A range of home-constructed and commercially produced electroporators are available. The transformation frequency, following chemically or electrically induced plasmid uptake, is low, with 1 stably transformed protoplast-derived colony being produced for every 10^3 - 10^6 protoplasts treated with plasmid. Transformation is influenced by a number of parameters, including the molecular weight and concentration of PEG, plasmid size and configuration (whether supercoiled or linearised), the treatment voltage, duration and number of electrical pulses, the type of pulse (whether delivered by a capacitor discharge or by a square-wave generator) and the physiological condition and stage of the cell cycle of the recipient protoplasts. As in the case of *Agrobacterium*-induced transforation, the mechanism of foreign gene integration into the recipient plant genome remains unclear, and gene integration appears to be random. Irradiation of protoplasts prior to plasmid uptake has been shown to stimulate transformation, possibly through a DNA repair mechanism facilitating integration of foreign DNA into the plant genome. A combination of PEG and electroporation may be beneficial in transforming some protoplast systems.

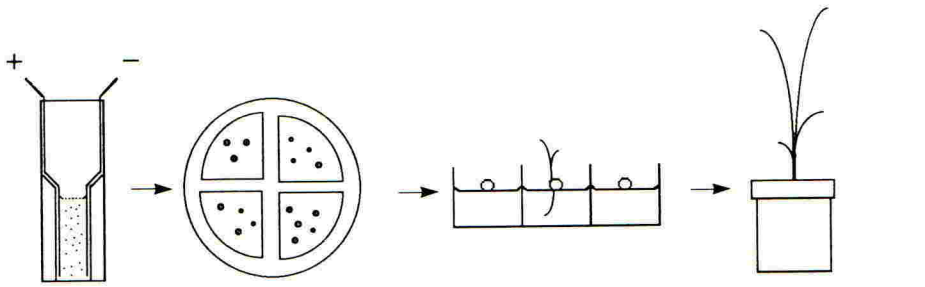
An important aspect of direct DNA uptake into protoplasts is that it is possible to transform protoplasts with plasmids carrying more than one gene, or with more than one plasmid each carrying one or more genes. In addition to the production of stably transformed tissues and plants, direct DNA uptake can be used in transient gene expression systems to study gene promoters and vector constructs. Genes such as those for β-glucuronidase (GUS), chloramphenicol acetyltransferase (CAT) and luciferase can be assayed within a few hours of uptake into protoplasts. Methods based on the detection of fluorescence by flow cytometry are also being developed for monitoring DNA uptake into protoplasts (Blackhall, Finch, Davey and Cocking, submitted).

Whilst protoplasts of tobacco and other members of the Solanaceae have been used extensively as model systems to develop the technology of direct DNA uptake, this method for gene introduction has been extended to economically important plants, including *Brassica napus* and *Vigna aconitifolia*. The most important application of this procedure is in the transformation of the cereals,

some of which, notably rice, can now be regenerated into plants from protoplasts (Finch *et al.*, 1991). Indeed, several groups have reported the production of transgenic rice plants following direct DNA uptake into protoplasts isolated from embryogenic cell suspension cultures. Some of the transgenic plants have produced seed progeny which have been evaluated for their phenotypic characteristics and for maintenance and expression of foreign genes (Davey *et al.*, 1991). More detailed discussions of transformation by direct DNA uptake can be found in Davey *et al.* (1989), Potrykus (1990 a,b) and Kumar and Davey (1991).

Figure 4.

PLANT GENETIC TRANSFORMATION BY DIRECT DNA UPTAKE INTO PROTOPLASTS INDUCED BY 'ELECTROPORATION' TREATMENT



DC pulse/s applied to protoplast suspension, in the presence of recombinant plasmid DNA,* in foil-lined cuvette 'electrode'

Protoplasts cultured in segments of agarose culture medium incubated in antibiotic-containing liquid medium (selection pressure). Only transformed cells grow into colonies.

Putative transformed cell colonies (callus) transferred to plant regeneration medium.

Regenerants transferred to the glasshouse. Plant tissues later analysed to confirm the presence of the foreign gene.

(not to scale)

N.B. DNA uptake into protoplasts can also be induced chemically through the use of polyethylene glycol (PEG).

* recombinant plasmid contains the foreign gene of interest and an antibiotic resistance marker gene

The production of transgenic plants by direct DNA uptake into protoplasts necessitates reproducible protoplast-to-plant systems. As such systems are labour intensive to maintain, workers in several laboratories have focussed attention on the development of alternative approaches. The injection of developing panicles

of cereals with large quantities of DNA, the micro-injection of protoplast-derived cells and dividing microspores with pico-gramme quantities of plasmid, and the imbibition of excised dry embryos in DNA solutions have been assessed. Recently, leaf bases of intact rice seedlings have been electroporated in the presence of DNA (Dekeyser *et al.*, 1990), while vortexing suspension cell/plasmid mixtures with silicon fibres has been used for transformation (Kaeppier *et al.*, 1990). Transformation of tissues by bombardment of tissues with tungsten or gold particles coated with DNA and accelerated to high velocity by explosive or electrical discharge (the 'biolistic' approach) is being evaluated in a number of laboratories. The advantage of the latter method is that plasmids can be introduced through intact cell walls and can be directed to target tissues, which is essential in studies of tissue-specific gene expression. Transgenic plants of soybean (McCabe *et al.*, 1988) and maize (Gordon-Kamm *et al.*, 1990) have been produced by bombardment of seedling meristems. Although such plants produced by this procedure are chimaeric for the introduced gene, stable transformants can be found in their seed progeny. Currently, particle bombardment is considered by many workers to be the best alternative to DNA uptake into protoplasts for the production of transgenic plants of species which cannot be transformed by *Agrobacterium*.

GENETIC TRANSFORMATION FOR CROP PROTECTION

During the last decade, considerable progress has been made in cloning genes with the potential to confer resistance to viruses, insect pests and non–specific herbicides, and transferring these genes into susceptible plants. In most cases transformation has been carried out utilising *A. tumefaciens* strains, containing cointegrate disarmed Ti plasmids, in conjunction with intermediate vectors carrying chimaeric foreign gene constructs.

Virus resistance in transgenic plants

The introduction of virus resistance into plants has been achieved by expression of viral coat protein genes and by the use of antisense constructs. Coat protein mediated resistance (CPMR) is due to an accumulation of the viral coat protein (CP) in transgenic plant tissues. Resistance to infection by the virus from which the CP gene was obtained and sometimes resistance to other viruses, subsequently ensues. For example, the TMV CP gene confers resistance not only to TMV but also to ORSV, PMMV, TMGMV and ToMV. The SMV and TRV CP genes also confer resistance to viruses other than that from which the gene was obtained. This cross protection occurs to varying degrees in some but not all cases (see van Dun *et al.*, 1988). The mechanism of CPMR is not fully understood, although there is some evidence that CP-gene expression interferes with an early event of the infection process. Nucleic acid release from the swelling viroid is a primary stage of infection and a number of studies have shown that virus resistance can be overcome if resistant plants are directly inoculated with viral RNA rather than with intact viroids (encapsidated RNA). There is also evidence that entry of viruses into vascular tissue is impeded in plants transformed with viral coat protein genes, thus inhibiting the spread of infection (see Beachy *et al.*, 1990).

Due to the ease with which it can be transformed, tobacco has been used extensively as a model species to test the efficacy of foreign gene constructs. Indeed, coat protein genes cloned from a wide range of plant viruses have been expressed in transgenic tobacco plants (Table 1). Additionally, resistance to a

number of agronomically important viruses has been achieved with several other important crops (Table 2).

TABLE 1. Reports of coat protein mediated resistance in tobacco

Virus to which transgenic plants were resistant	Virus from which CP gene was cloned	Reference
Alfalfa mosaic virus (AMV)	AMV	Tumer *et al.*, 1987 van Dun *et al.*, 1987 Halk *et al.*, 1989 Hill *et al.*, 1991
Cucumber mosaic virus (CMV)	CMV	Cuozzo *et al.*, 1988
Ondontoglassum ring spot-virus (ORSV)	TMV	Nejidat & Beachy, 1990
Pea early browning virus (PEBV)	TRV	van Dun & Bol, 1988
Pepper mild mottle virus (PMMV)	TMV PRV	Nejidat & Beachy, 1990 Ling *et al.*, 1991
Potato virus X (PVX)	PVX	Hemenway *et al.*, 1988
Potato virus Y (PVY)	SMV PRV	Stark & Beachy, 1989 Ling *et al.*, 1991
Tobacco etch virus (TEV)	SMV PRV	Stark & Beachy, 1989 Ling *et al.*, 1991
Tobacco mild green-mosaic virus (TMGMV)	TMV	Nejidat & Beachy, 1990
Tobacco mosaic virus (TMV)	TMV	Powell-Abel *et al.*, 1986
Tomato mosaic virus (ToMV)	TMV	Nelson *et al.*, 1987
Tobacco streak virus (TSV)	TSV	van Dun *et al.*, 1988
Tobacco rattle virus (TRV)	TRV	van Dun & Bol, 1988

PRV = Papaya ring spot virus
SMV = soybean mosaic virus

Gene constructs used to encode coat proteins in transgenic plants have, in most cases, contained a promoter sequence derived from cauliflower mosaic virus (e.g. the CaMV 35S promoter) and a *nos* terminator sequence derived from *A. tumefaciens* T-DNA. However, in several cases, a ribulose bisphosphate carboxylase terminator sequence *(rbcS)* has been utilised (see Beachy *et al.*, 1990),

or the terminator signal ORF25 from *A. tumefaciens* T-DNA (Hill *et al.*, 1991). The tobacco plants expressing the viruses listed in Table 1 were transformed using the natural gene transfer system of *A. tumefaciens*. Since the recombinant vectors used also contained the *nptII* gene, selection of transformed shoots was based on their resistance to the antibiotic kanamycin sulphate at an early stage of culture development. The level of CP gene expression has been found to vary in transgenic plants. Coat protein accounts for between 0.001% w/w (Cuozzo *et al.*, 1988) and 0.8% w/w (Tumer *et al.*, 1987) of total extractable cellular protein in transformed plants. The level of expression is influenced by a range of factors including plant genotype and the efficacy of the gene construct.

TABLE 2. Reports of coat protein mediated resistance in crops

Coat protein (CP) gene	Virus resistance	Transgenic (CP+) crop	Comparative agronomic traits of transgenic plants	Field trial of CP(+) plants	Reference
TMV	TMV ToMV	tomato	vegetative growth, development and fruit yield equal in CP(+) & CP(-) plants.	viral inoculation did not affect yield of CP(+) clone '306' compared to uninoculated 306.	Nelson *et al.*, 1988
PVX	PVX	potato	between 60 & 87% of transgenic plants grew 'true-to-type' in the field	delay in disease-symptom development & large reduction in virus accumulation in challenged CP(+) plants.	Hoekema *et al.*, 1989
Co-PVX & PVY	PVX & PVY	potato	4 different CP(+) clones showed yields similar to CP(-) controls	inoculated CP(+) line '303' showed normal yields	Lawson *et al.*, 1990 Kaniewski *et al.*, 1990
AMV	AMV	alfalfa	not described	not described	Hill *et al.*, 1991

Protection against low level inoculation with PVX (Hemenway *et al.*, 1988) and CMV (Cuozzo *et al.*, 1988) has also been achieved in tobacco by transforming plants with antisense RNA genes. Such RNA molecules, containing complementary sequences to viral CP RNA, inhibit gene expression by binding to the polynucleotide sequence and thus directly blocking translation.

Insect resistance in transgenic plants

Bacillus thuringiensis is a Gram-positive bacterium that produces

insecticidal crystals upon sporulation. These proteinaceous crystals are composed of endotoxins which are processed in the alkaline midgut of the insect to yield specific, highly active, toxic fragments. Spore preparations of *B. thuringiensis* have been used for many years as biological insecticides (Rowe and Margaritis, 1987); for example spore preparations form the active ingredient in Dipel®.

A number of groups have reported the cloning of 'insect control protein' (ICP) genes from plasmid or chromosomal DNA of various strains of *B. thuringiensis* (the specificity of toxicity differs between strains), including berliner 1715 (*bt2* gene; Vaeck *et al.*, 1987), kurstaki (*B.t.k* gene; Schnepf and Whiteley, 1981; Fischoff *et al.*, 1987) and sotto (*icp* gene; Shibano *et al.*, 1985). Early experiments suggested that the expression of full length ICP genes was in some way toxic to plant tissues, since transformants which contained unmodified toxin did not survive. Thus, chimaeric genes containing truncated variants of the ICP coding regions, in most cases under the control of the CaMV 35S promoter and *nos* terminator signals, were constructed for effective expression in a range of plant species. Control of damage caused by Lepidopteran larvae has been demonstrated in the transgenic plants produced (Table 3).

TABLE 3. Examples of insect resistance in plants expressing *Bacillus thuringiensis* endotoxin genes

Plant transformed	Pest to which resistance was conferred	Agronomic traits of transgenic plants	Glasshouse / field trial of transgenic plants	Reference
tobacco	tobacco hornworm	indistinguishable from control plants for morphology & vigour	lethal to pest with only a few mm^2 leaf damage	Vaeck *et al.*, 1987
tobacco	tobacco hornworm cotton bullworm corn earworm beet armyworm	not described	not described	Barton *et al.*, 1987
tomato	tobacco hornworm	not described	complete control	Fischoff *et al.*, 1987 Delannay *et al.*, 1989
tomato	tomato pinworm tomato fruitworm	not described	significant control → reduced fruit damage; little evidence of leaf damage	Fischoff *et al.*, 1987 Delannay *et al.*, 1989
cotton	cabbage looper beet armyworm	exhibited 'normal phenotype'	little leaf damage; up to 75% boll survival	Perlak *et al.*, 1990

Analyses of leaf protein content in insect resistant plants transformed with ICP genes have indicated that the insecticidal protein accounts for between 0.00125% w/w (Barton *et al.*, 1987) and 0.1% w/w (Perlak *et al.*, 1987) of the total extractable leaf protein, thus demonstrating its high potency. In tomato and cotton, very low levels of the protein were detected in the fruits/bolls. Although newly hatched larvae are initially foliar feeders, increased expression of the ICP gene in reproductive tissues would be desirable for complete insect control. Further improvement of chimaeric toxin genes, particularly the adoption of stronger promoter sequences, may lead to the higher levels of ICP gene expression required.

Herbicide resistance in transgenic plants

Selective herbicides play an important role in weed control, but their availability is limited by high development costs. An alternative to the costly production of novel selective herbicides is the development of genetically modified, herbicide resistant crops. There are at least 3 strategies for the production of such crops. These are (i) to induce the crop plant to overproduce the herbicide's biochemical target [e.g. transform with multiple (amplified) copies of the gene which codes for the target enzyme], (ii) to alter the target gene/enzyme (e.g. transform with a resistant-mutant target gene to reduce herbicide affinity) and (iii) to transform with a herbicide detoxification gene.

In most cases, herbicide resistance genes have been inserted into an *A. tumefaciens* T-DNA vector under the control of the CaMV 35S promoter and either the *nos* or *ocs* terminator signals. The leaf disc transformation system has been employed most extensively, with selection of kanamycin resistant transgenic shoots. Stalker *et al.* (1988) and Cheung *et al.* (1988) used a promoter derived from a light inducible tissue-specific ribulose bisphosphate carboxylase/oxygenase (rubisco) small subunit gene to control the expression of the bromoxynil-specific nitrilase gene and the resistant quinone-binding protein gene respectively, in tobacco leaves (see Table 4).

Crops transformed by herbicide resistance genes have been assessed under field conditions. Thus, De Greef *et al.* (1989) evaluated phosphinothricin (PPT) tolerance of potato and tobacco transformants (produced by De Block *et al.*, 1987) under field conditions. They observed complete resistance to field dose applications of Basta®, a commercial ammonium salt preparation of PPT. Moreover, the transformants exhibited the same agronomic characters as non-transformed control plants which had not been sprayed with the herbicide.

McHughen *et al.* (1990) field-tested sulfonylurea-resistant linseed plants over a period of two years. Transgenic plants expressing the mutant ALS gene (Table 4) varied in their degree of resistance, but most plants were resistant to field-dose applications of sulfonylurea-herbicide. Furthermore, these lines exhibited normal seed yield.

In addition to the plants listed in Table 4, several other glyphosate-tolerant crops, including soybean, alfalfa and rape, have been produced and field tested during the last three years (see Tomes, 1990).

TABLE 4. Examples of herbicide tolerance in transgenic plants.

Strategy	Gene utilised	Crop transformed	Herbicide resistance	Reference
Heterologous target gene transferred → overproduction of herbicide's biochemical target	bacterial EPSP-synthase *(aro A)* gene	tobacco	glyphosate (Roundup®)	Comai *et al.*, 1985;
Amplified target gene isolated from mutant petunia cell line and transferred → overproduction of herbicide's biochemical target	mutant EPSP-synthase gene	petunia	glyphosate	Shah *et al.*, 1986
Heterologous, herbicide-detoxification gene transferred	bacterial *bar* gene (encodes PAT)	tobacco potato tomato	PPT (Bialaphos®)	De Block *et al.*, 1987
Heterologous, herbicide-detoxification gene transferred	bacterial *bxn* gene (encodes BSN)	tobacco	bromoxynil	Stalker *et al.*, 1988
Target gene isolated and transferred from a resistant biotype of *Amaranthus hybridus* → herbicide's affinity for target reduced	heterologous *psbA* chloroplast gene (encodes Q_B)	tobacco	atrazine	Cheung *et al.*, 1988
Resistant (mutant) target gene isolated from *Arabidopsis* mutant and transferred → herbicide's affinity for target reduced	heterologous, mutant ALS gene	tobacco linseed	chlorsulfuron	Haughn *et al.*, 1988
Resistant (mutant) target gene isolated from mutant tobacco tissue cultures and transferred → herbicide's affinity for target reduced	mutant ALS gene	tobacco	chlorsulfuron (Glean®) sulfometuron-methyl (Oust®)	Lee *et al.*, 1988
Heterologous target gene transferred → overproduction of of herbicide's biochemical target	bacterial EPSP-synthase gene	hybrid-Poplar	glyphosate	Fillatti *et al.*, 1988

Continued...

TABLE 4. Continued...

Strategy	Gene utilised	Crop transformed	Herbicide resistance	Reference
Heterologous, herbicide-degradation gene transferred	bacterial *tfdA* gene (encodes DPAM)	tobacco	2,4-D	Streber & Willmitzer, 1989
Resistant target gene, from *Arabidopsis* mutant (Haughn *et al.*, 1988), transferred → herbicide's affinity for target reduced	heterologous, mutant ALS	linseed	chlorsulfuron sulfometuron-methyl	McHughen, 1989 McHughen *et al.*, 1990
Heterologous target gene transferred → overproduction of of herbicide's biochemical target	bacterial EPSP-synthase gene	linseed	glyphosate	McHughen *et al.*, 1990

ALS = acetolactate synthase (catalyzes the biosynthesis of the branched-chain amino acids, valine, leucine and isoleucine; target enzyme for sulfonylurea and imidazolinone herbicides)
BSN = bromoxynil-specific nitrilase (detoxifies bromoxynil - an inhibitor of photosynthetic electron transport - by converting it to its primary metabolite)
2,4-D = 2,4-dichlorophenoxyacetic acid (synthetic auxin may which may mimic the function of the intrinsic auxin, indole-acetic-acid, on an internal auxin receptor of the plant - selectively toxic to dicotyledonous plants at high concentrations)
DPAM = 2,4-dichlorophenoxyacetate mono-oxygenase (catalyzes the first step in the bacterial 2,4-D degradative pathway)
EPSP = 5-enolpyruvylshikimate-3-phosphate (EPSP-synthase is a key enzyme of the shikimate pathway - aromatic amino acid biosynthesis - ; target enzyme for glyphosate)
PAT = phosphinothricin acetyl transferase (detoxifies PPT by acetylating the free NH_2 group)
PPT = phosphinothricin (a potent inhibitor of glutamine sunthetase)
Q_B = photosynthetic quinone-binding protein (involved in photosynthetic electron transport; target enzyme for triazine herbicides)

SOMATIC HYBRIDISATION FOR CROP PROTECTION

The absence of a cell wall enables isolated protoplasts to be fused chemically, electrically or by a combination of these procedures (Fig. 5). The advantage of this procedure is that it overcomes sexual incompatibility barriers, enabling inter-generic, inter-specific and intra-specific hybrids to be produced through the mobilisation of genetic material between plants. As in the case of plant transformation, there is now a vast literature relating to this area of research and the general principles of this procedure have been discussed extensively (Giles 1983; Gleba and Sytnik, 1984; Puite *et al.*, 1988; Bhojwani, 1990). Essentially, the important products of chemical or electrical fusion are heterokaryons which result from the fusion of protoplasts from the two parents (Fig. 6). Such heterokaryons must be encouraged to develop in preference to homokaryons and unfused protoplasts. Various procedures, including micro-manipulation, biochemical complementation and flow cytometry have been developed to select

hybrid cells and tissues from which plants can be regenerated.

Many wild species represent important germplasm reserves for genes encoding resistances to viruses, insects and adverse environmental factors including drought, cold and salinity. It is through transfer of such genes to cultivated species that somatic hybridisation has potential in terms of crop protection. Rice, for example, is difficult to hybridise with many of its wild relatives, some of which carry disease and stress tolerance genes. A number of workers have studied somatic hybridisation as a means to transfer salt tolerance (Finch *et al.*, 1990) and insect resistance (Hayashi *et al.*, 1988) into rice accross sexual incompatibility barriers.

Figure 5. PROTOCOLS FOR PROTOPLAST FUSION

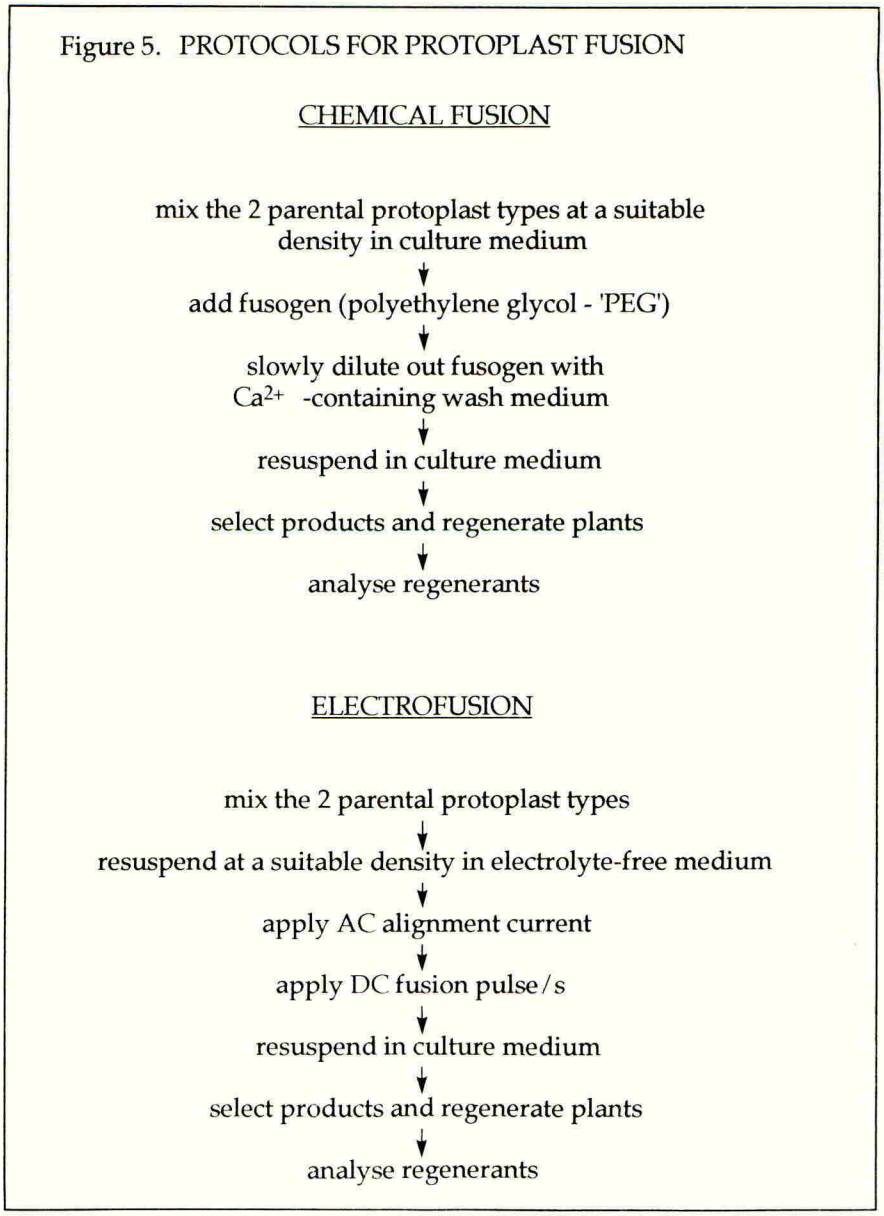
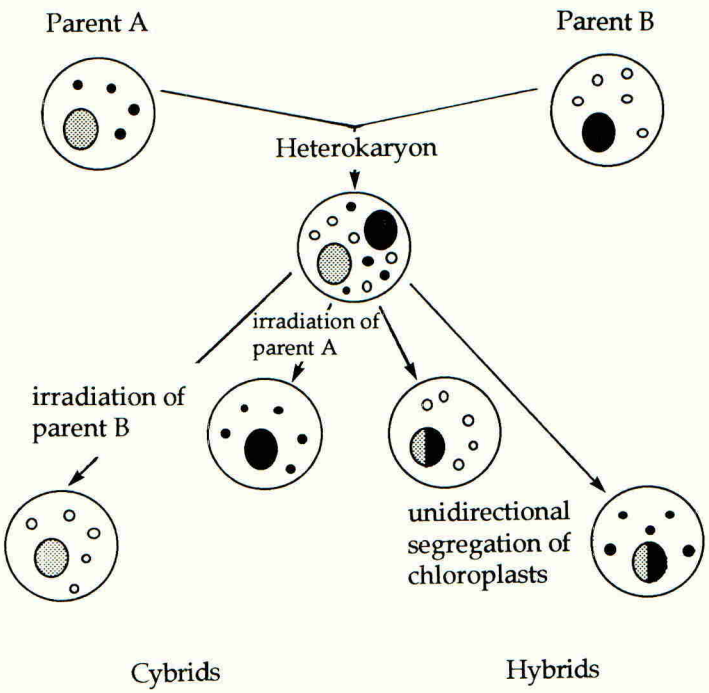


Figure 6. PRODUCTS OF PROTOPLAST FUSION



Some of the possible products which can be obtained through protoplast fusion, showing segregation of nuclear and chloroplast genomes. When one considers that similar segregation of the mitochondrial genomes also takes place and that partial nuclear hybrids may arise, it is easy to appreciate that a vast range of modified cell products can be produced.

In tomato, the wild species *Lycopersicon hirsutum* f. *glabratum* carries insect resistance because it synthesises the natural insecticide 2-tridecanone in 'type 6' trichomes on its leaves (Williams *et al.*, 1980). Other sources of agronomically useful characters are still being discovered. Sotirova and Rodeva (1990) detected resistance to *Septoria lycopersici* in *L. peruvianum, L. pimpinellifolium* and *L. racemigerum,* while Bournival and Vallejos (1991) have shown *L. hirsutum* and *L. parviflorum* to be resistant to race 3 of *Fusarium*

oxysporum f. sp. *lycopersici*. Somatic hybridisation of those wild *Lycopersicon* species which are sexually incompatible with the cultivated tomato, *L. esculentum*, could result in somatic hybrid plants carrying agronomically important traits. Sexual backcrossing of somatic hybrids to *L. esculentum* could transfer these traits into the cultivated crop. Additionally, in some combinations, irradiation of protoplasts of the wild species prior to fusion to fragment their genomic DNA, could assist the introgression of agronomically important genes to *L. esculentum* through the production of partial somatic hybrids. However, to date, somatic hybridisation has not been exploited in this way for the transfer of agronomically important genes, such as those for insect resistance, into tomato.

CONCLUSIONS AND FUTURE PROSPECTS

Whilst considerable progress has been achieved in the use of *Agrobacterium*-based vectors for introducing genes for virus, insect and herbicide resistances into several dicotyledonous plants, there are no reports, to date, of such genes being inserted into monocotyledons. However, the success in transforming monocotyledons, such as rice and maize, by direct DNA uptake into protoplasts isolated from embryogenic cell suspension cultures, should enable this approach to be employed, once the genes have been cloned into suitable vectors. An example of cereal crop protection, which is already in progress in several laboratories, is the introduction of the coat protein gene from rice Tungro spherical virus into protoplasts of Japonica rice varieties. The coat protein gene has been cloned into small *E. coli* plasmid vectors carrying chimaeric antibiotic resistance genes for selection of transformed protoplast-derived tissues. It remains to be seen whether plants regenerated from transformed tissues will exhibit resistance to Tungro disease when exposed to leaf hoppers carrying the virus. It seems likely that, in the future, particle bombardment will also be employed to introduce genes for insect, viral and herbicide resistances into monocotyledons and other crops which do not respond readily to *Agrobacterium*.

Overall, somatic cell and molecular techniques offer considerable potential for crop improvement, now that reliable plant regeneration has been established from cultured material of many of the most important species. Exploitation of these procedures will necessitate close collaboration between tissue culturalists, plant breeders and molecular biologists. At the molecular level, current advances in the mapping of restriction fragment length polymorphism (RFLP) markers to agronomically important genes, such as those for root knot nematode resistance (Klein-lankhorst *et al.*, 1991) and resistance to *Fusarium oxysporum* f. sp. *lycopersici* (Sarfatti *et al.*, 1991) in wild tomatoes, will, in the future, assist in the introduction of these genes into crop plants by somatic hybridisation or by transformation involving *Agrobacterium*-mediated gene delivery or direct uptake of DNA.

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IMMUNOASSAYS FOR THE MEASUREMENT AND DETECTION OF PESTICIDES IN THE ENVIRONMENT

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ABSTRACT

For over thirty years, immunoassays have been widely used in many scientific disciplines when high analytical sensitivity and selectivity is required but it is only recently that their potential for the detection and measurement of pesticides has been recognised. Immunoassays, which exploit the specific binding of antibodies to antigens, are known for their versatility and, depending on the assay format, can be used to provide quantitative, semi-quantitative and qualitative results either in the laboratory or in extra-laboratory sites. This paper describes with particular reference to pesticides the principles and various forms of immunoassay, the production of antibodies (both polyclonal and monoclonal), assay development and analytical characteristics. Present and future applications of immunoassays to environmental analysis will be discussed.

INTRODUCTION

The protection of crops by the application of appropriate chemicals has provided enormous benefits in increased yields of foodstuffs over the last fifty years. There are now several hundred compounds approved for use in the U.K. and additional chemicals are regularly being introduced for agricultural use. Pesticide residue analysis has been a fundamental part of safe and effective product development and registration in the past but there is now additional pressure to provide increased information on the fate of pesticides in the environment. In order to meet the needs of present legislation relating to the use and levels of pesticides in the environment and to allay public concern increased monitoring for the presence of pesticides in food, drinking water etc. needs to be undertaken. Pesticide residue analysis using traditional and approved techniques such as gas chromatography (GC) are not entirely suited to the task of rapid and frequent monitoring of large numbers of samples. Less time consuming, cheaper methods of analyses are required to complement existing techniques. Immunoassays have the potential to improve monitoring programmes by providing tests which are rapid, inexpensive and simple to perform.

The development of immunoassay began over thirty years ago when a rather cumbersome (by today's standard) method for plasma insulin was described (Yalow & Berson, 1960). Since then the principle of that assay has been used to develop a huge range of assays for the measurement of hundreds, if not thousands, of compounds with widely different molecular characteristics. Many of the developments in assay design and technology are still largely derived from the needs of the clinical biochemist, but scientists

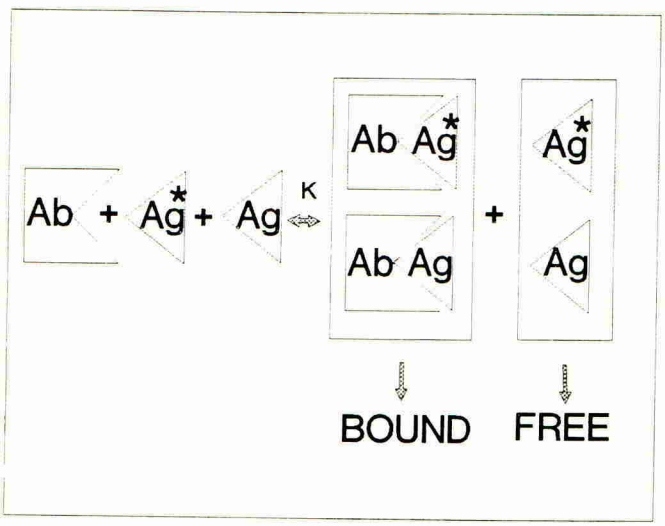
in other fields of analysis have adopted and modified the techniques to suit their own requirements. In contrast to other types of chemical methods of analysis, the major investment in immunoassays is in reagent and method development not in the day to day running of the assays. The facilities and expertise for assay development exists in various university and commercial research laboratories but detailed knowledge of the technique is not required by the end-user.

There have been many excellent reviews of immunoassay techniques and developments to which the reader is referred (Voller et al., 1981; Hunter & Corrie, 1983; Butt, 1984; and Gosling, 1990). The application of immunoassay theory and practise to the detection and measurement of pesticides will be presented here. Additional information can be obtained from earlier publications on the subject (Newsome, 1986; Harrison et al., 1988; Van Emon et al., 1989; Aherne, 1990).

PRINCIPLES OF IMMUNOASSAY

Immunoassays are based on the principle of competitive binding or saturation analysis (Figure 1). The analyte (Ag) in samples or in standards competes with a constant amount of a labelled form of the analyte (Ag*), commonly called the label or tracer, for a fixed and limited number of antibody binding sites (Ab) specific for the analyte. The reaction obeys the Laws of Mass Action. At the end of a period of incubation the antibody bound

Figure 1. The principles of competitive immunoassays



fraction is separated from the "free" fraction in a procedure known as "phase separation". The amount of the label associated with either the bound or free fraction is then measured.

As the concentration of the analyte increases the amount of labelled antigen bound to antibody decreases. A standard curve is constructed plotting the bound label against concentration and sample values can be interpolated from this curve. Conventionally the amount of label bound is expressed in terms of the binding of the zero standard (Bo).

This type of immunoassay is known as a competitive or limited reagent assay in that there is a limited amount of antibody present. Non-competitive or reagent excess assays (immunometric assays) are also commonly used (Jackson & Ekins, 1986) although these are generally less suitable for small molecular weight compounds such as pesticides.

Requirements for assay development

Antibodies

The main requirement for the development of an immunoassay is that a specific antiserum to the analyte is available. Different antibody preparations should be assessed for their potency or titre, their specificity and avidity. The titre is defined as the dilution of antiserum required to bind 50% of the added label (in the absence of analyte) and is a measure of specific antibody concentration.

It is important to determine the specificity of the antiserum i.e. the extent to which the binding is not influenced by compounds of similar structure. Cross reacting substances in the samples may compete with the analyte for the binding sites thus leading to erroneous results. The energy with which the antibody binds to its specific antigen is known as its avidity and it is equivalent to the association constant (K) in Figure 1. Highly sensitive assays are only possible with high avidity antibodies (Jackson & Ekins, 1986) with binding constants in the order of 10^{-9} - 10^{-12} L/mole. In addition assay performance in terms of speed, precision and reproducibility is increased with high avidity antibodies.

Labelled reagents

As originally described the labelled components in immunoassay were radioisotopes (^{3}H and ^{125}I) and these assays are known as radioimmunoassays (RIA) or immunoradiometric assays (IRMA). The use of radioisotopes provided high specific activity labels conferring exquisite sensitivity to the assay procedures and are still the method of choice in many laboratories. To overcome the disadvantages of using radioisotopes e.g. short shelf life of reagents, expensive counting equipment, waste disposal problems and potential health risks, non isotopic labels have been introduced over the years. The use of alternate labels including the use of enzymes (EIA), luminescence (LIA) and fluorescence (FIA) allows the production of stable labelled reagents and the development of a wide range of assay formats (Collins, 1985; 1988) some of which can be used away from the laboratory or by unskilled personnel e.g. over the counter pregnancy diagnostic kits. Probably the most widely used assay format is now the Enzyme Linked ImmunoSorbent Assay (ELISA) which will be discussed later.

Phase separation

The antibody bound fraction can be separated from the "free" fraction by any appropriate chemical, physical or immunological means. A practical yet efficient method is required and the most frequently used methods are coated charcoal adsorption of the small molecular sized "free" fraction, precipitation of the"bound" fraction using ammonium

sulphate or PEG, and immuno-precipitation using a second antibody specific for the immunoglobulin of the primary antibody. All three methods require a centrifugation step. Non-covalent or covalent binding of either the antibody or the antigen to a solid phase (plastic tubes, magnetic beads, microtitre plates) has greatly simplified the phase separation process as no centrifugation is required.

ANTIBODY PRODUCTION

An antibody is a serum protein, an immunoglobulin, produced by the immune system in response to a foreign protein or compound or organism. An immunoglobulin will bind to the compound that elicited its production (the antigen) in a classical "lock and key" fashion. This complementary fit confers specificity to the antibody/antigen reaction.

Polyclonal antibody production

Antibodies to particular antigens can be produced by immunising animals with the relevant compound and at an appropriate time the antiserum can be harvested to be used either diluted in an immunoassay or the immunoglobulin fraction can be prepared and purified further. The antibodies produced are polyclonal in nature as they will be derived from several clones of lymphocytes sensitised by the immunogen and the overall specificity of the antiserum will be determined by the characteristics of all the antibodies present.

Various immunisation schedules have evolved, each laboratory tending to use procedures which have proved successful in the past. The production of antibodies remains an imprecise science as individual animals respond very differently. High avidity antibodies are normally produced later in the immunisation schedule after one or more booster injections. In our laboratory booster injections are given only when the titre of antibodies has fallen to a low or plateau level following the previous injection. It may thus take 6-12 months or even longer to produce antisera which are suitable for the development of highly sensitive and robust immunoassays.

Production of antibodies to small molecules

Many antigens are immunogenic in their own right e.g. proteins, but compounds such as pesticides with a low molecular weight (less than approximately 5000 daltons) are not normally immunogenic. These haptens need to be covalently conjugated to a suitable carrier protein which is foreign to the animal being immunised to form an immunogen

In many cases the antigen in question is chemically unreactive and a more reactive analogue or derivative is required. Carboxyl, amino and hydroxyl containing derivatives are frequently used and the many chemical reactions available for conjugation have been reviewed (Smith, 1988). The linkage used to produce the immunogen has a major influence on the ultimate specificity of the antiserum produced because the main antigenic determinant (epitope) is normally furthest away from the point of attachment. The choice of conjugate should be carefully considered prior to immunisation if, for instance, a compound specific antiserum is required rather than a group specific one which will have a broad cross-reactivity within a chemical group of compounds or vice versa.

Monoclonal antibody production

Monoclonal antibodies are produced by fusing antibody-producing cells (lymphocytes) with malignant myeloma cells. After fusion, the hybridomas exhibit characteristics from each parent cell type i.e. specific antibody production derived from a single lymphocyte and "immortality" derived from the myeloma cells. By convention both cell types are normally obtained from murine sources. Cloning procedures allow the isolation of cell lines producing quantities of a single immunoglobulin with a single specificity. Another advantage of monoclonal antibodies is that the supply is virtually unlimited and the nature of the antibody does not change with time. For small molecules such as drugs and pesticides the advantages of monoclonal antibodies however may not always outweigh the expense and effort required to produce them.

The affinity of monoclonal antibodies derived from immunised mice is generally relatively low and assay performance may therefore be compromised. Heterohybridomas can be produced (but with more difficulty) when the lymphocytes from a rabbit or sheep known to be producing antisera of high quality are fused with a myeloma partner of murine origin (Groves et al., 1987). However, as far as small molecules are concerned there is little evidence that monoclonal antibodies provide more specificity than polyclonal antibodies. Unlike large polypeptide antigens where there are usually several different epitopes for antibody recognition there may be only one epitope that an antibody can combine with. The specificity of both types of antibodies is therefore determined by the extent to which related chemicals possess the same or similar chemical groups.

The insurance of a continual supply of monoclonal antibodies of defined characteristics may be a factor in the wider dissemination of immunoassay technology but polyclonal antibodies if they are of high titre and sufficient volumes are available (as is the case if large animals such as sheep and goats are used for immunisations) are remarkably stable. Polyclonal antisera have been stored undiluted in our laboratory at 4^{o}C in the presence of 0.1% sodium azide for 10-20 years with little or no deterioration in binding or assay performance.

IMMUNOASSAY FORMATS

To the non immunoassayist there are a seemingly bewildering number of different assay formats described in the literature. Some of these are just interesting novelties demonstrating the versatility of the antibody - antigen reaction and have not been widely applied. The popular use of acronyms to describe immunoassays is also confusing. A useful review of assay formats and nomenclature can be found in the paper by Gosling, (1990).

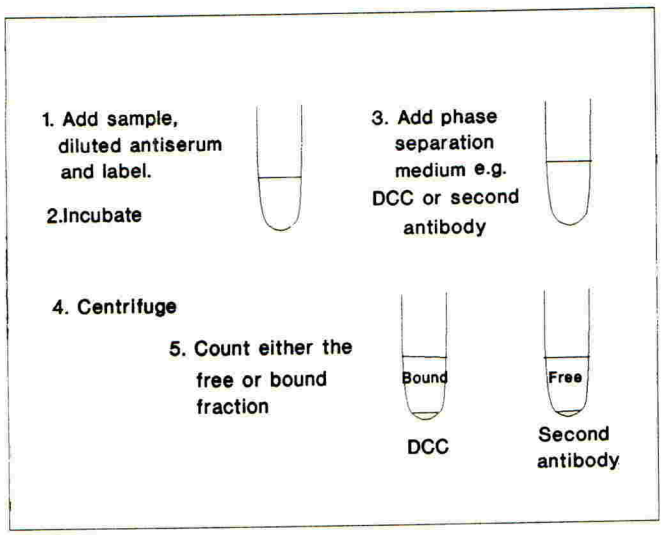
Basically, assays are either reagent (antibody) limited and competitive or antibody excess (non-competitive) in nature. The relative merits of both types of assay have been discussed (Ekins, 1985). The type of label (radioisotopes, enzymes, fluorescence etc.) used really only determines how the assay end-point is measured although labels which can be detected at very low concentrations can influence assay sensitivity (Ekins, 1985). Variations to these basic formats are included in assay design to increase sensitivity and selectivity. Assay sensitivity can be improved by amplification of the enzyme activity e.g. the use of

avidin/biotin systems or of enzyme/anti-enzyme systems (Bates, 1987) and selectivity can be improved by two-site assays (Woodhead et al., 1974) although this approach is not applicable to small molecular weight analytes. To conserve valuable primary antibodies a second antibody or capture antibody can be used to coat solid phases such as microtitre plates.

Radioimmunoassay and related procedures

Although RIAs have not been widely used for pesticides the procedures involved are described for information and as a comparison with the more generally used ELISA procedures. Figure 2 illustrates the stages in an RIA. Briefly, the appropriate standards and dilutions of samples are pipetted into tubes and antiserum (diluted to bind 20-60% of the added label as previously determined by an antiserum dilution curve) and label are added. In the majority of cases there is no need for sample preparation. If extraction of the analyte is required e.g. from soil or food, extensive time consuming procedures are unlikely to be necessary. Following a period of incubation the phase separation procedure is carried out which, unless solid phases are used, involves centrifugation.

Figure 2. Schematic diagram of RIA procedures.



The amount of label associated with either the antibody bound fraction or the free fraction is measured using appropriate equipment e.g. liquid scintillation or gamma counting. Many types of instrument are available which have been designed for immunoassay applications but these are not essential. Software packages for on-line standard curve fitting and data reduction are often incorporated into equipment. Because RIA and related assays are simple to carry out large sample numbers can be handled at one time. For small molecules equilibrium is reached in relatively short times and assays can be completed in a matter of hours.

ENZYME LINKED IMMUNOSORBENT ASSAYS (ELISA)

The advent of the ELISA (Engvall & Perlmann, 1971; Van Weeman & Schuurs, 1971) was one of the major developments in immunoassay and has been readily accepted in laboratories where there has been no tradition of immunological analysis. The most familiar type of ELISA utilises the 96-well microtitre plate format but the same principles apply to assays carried out in tubes, on dipsticks, or beads. The different forms of ELISA are now too many to describe here and the reader is referred to the literature (Kemeney & Challcombe, 1988). Two examples of ELISA formats which are suitable for the analysis of pesticides will be described. In both assays one of the components is adsorbed hydrophobically to polystyrene plastic surfaces. This procedure is known as "coating" and is carried out by simply filling the wells with a solution of the coating material for a period of time. Uncoated material is removed by washing the plate. Coating of proteins is commonly carried out at in alkaline buffers at approximately pH 9.6. Covalent binding of reagents to the solid phase has also been advocated and although this should confer greater stability for some analytes it is not a preferred option. For some analytes it may be necessary to reduce non-specific binding to the plastic by blocking uncoated sites with an inert protein such as gelatin, albumin or casein. Once the coating has taken place the next stages in the assay can be carried out with further washing steps included to remove any unbound reagents. The times and incubation periods required at each stage have to be optimised for each assay. Typically incubation times are 1-2hr at 37°C although overnight incubations are also favoured especially for the coating step. The washing procedures can be achieved using running tap water or by using one of the many plate washers which are commercially available.

At the end of the assay the enzyme is quantitated by the addition of an appropriate substrate which produces a coloured product. The amount of product formed is measured using a spectrophotometric plate reader the complexity of which can vary from a simple manual reader to complex automatic instruments which can record kinetic data on the enzymatic reaction. For some applications of ELISA a visual estimate of the colour produced is all that is necessary.

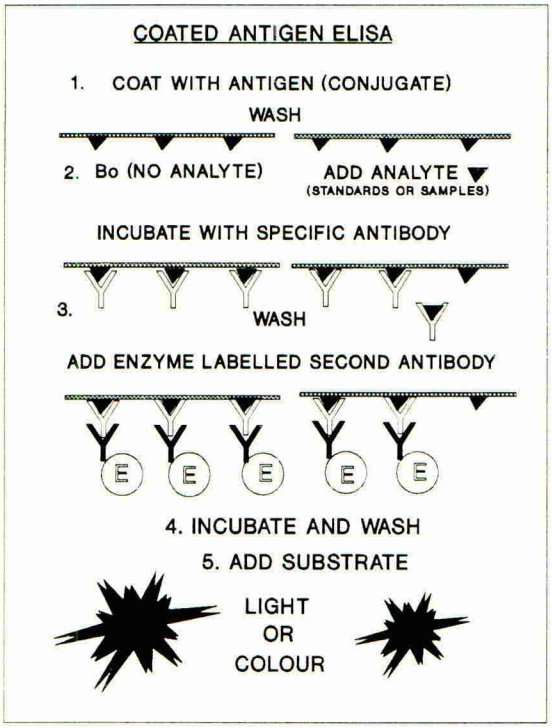
Coated antigen ELISA

In this type of assay, the antigen or a protein conjugate for small molecular sized antigens is coated on to the wells. The coating conjugate can be prepared using similar chemical reactions as used to prepare immunogens. In order to reduce non-specific immunoglobulin reactions it is important that the protein used to make this conjugate is different to that employed for the immunogen. Even when different proteins are used for the immunogen and the coating conjugate high non-specific reactions can take place resulting in reduced sensitivity.

Figure 3 shows the stages in a coated antigen ELISA. Standards and samples (or dilutions of samples) are added to the plate usually in duplicate. When specific antibody is added to the plate (at a predetermined dilution) there is competition for binding between the solid phase antigen and that in samples and standards. Antibodies not bound to the solid phase are removed by washing. A second antibody specific for the

immunoglobulin of the primary antibody which has been conjugated to an enzyme is added and following a further period of incubation and washing enzyme substrate is added and the colour measured. In those wells where only solid phase analyte was present (Bo) a strong colour signal develops and as the concentration of analyte present increases the colour signal is decreased.

Figure 3. Schematic diagram showing the stages in a coated antigen ELISA



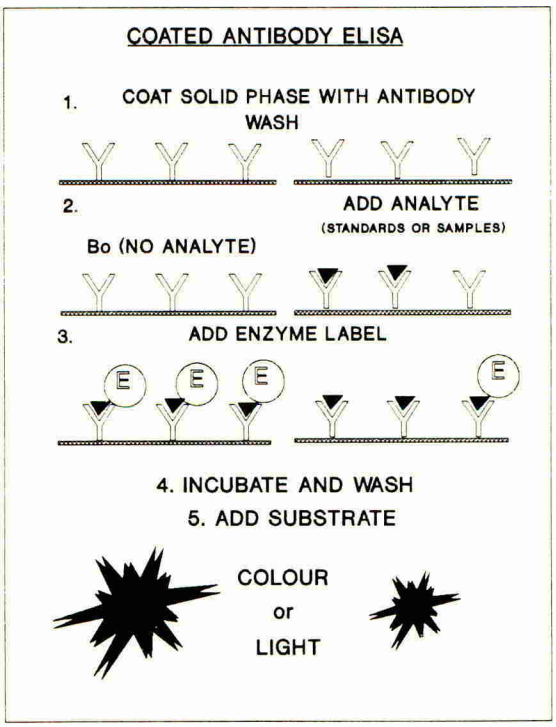
Coated antibody ELISA

Coated antibody assays are one of the simplest types of ELISA to perform. The solid phase is produced using the primary antibody the coating concentration of which is determined by initial titration experiments. Standard solutions of analyte and samples are added to the washed coated wells with the antigen-enzyme conjugate and the plates incubated. Competition takes place between the analyte and the enzyme label for the antibody binding sites. Thus following another wash step, in the absence of added analyte (Bo) no competition takes place and a high colour signal will be obtained on addition of substrate. A weaker colour signal will be obtained in the presence of competing analyte (Figure 4). This type of ELISA is comparable to the RIA procedure described earlier except that phase separation has been achieved by virtue of the solid phase.

Two-site ELISA

Another type of ELISA is the "sandwich" or two-site assay in which the analyte is "captured" by solid phase specific antibodies. The amount of antigen bound to solid phase is then reported by the addition of a specific antibody-enzyme conjugate. This assay is a non-competitive type assay and is potentially very sensitive. Unfortunately it is unsuitable for the measurement of pesticides and other haptens which may have only one epitope and because steric hindrance would prevent binding by the antibody-enzyme conjugate. This is unfortunate because unlike the assay formats previously described two-site assays generate positive signals for positive samples.

Figure 4. Schematic diagram showing the stages in a coated antibody ELISA



Preparation of enzyme conjugates

Several enzymes have been employed in ELISA but the most widely used are horseradish peroxidase (HRPO), alkaline phosphatase and ß-galactosidase. Enzyme conjugates for use as labels in ELISA can be prepared by a number of chemical coupling reactions (Blake & Gould, 1984). The reactions should be carefully chosen to retain both immunoreactivity and enzymatic activity.

In our laboratory stable high specific activity antibody and hapten HRPO conjugates have been prepared using the N-hydroxysuccinimide active ester reaction (Gadow et al.,

1984). Equal molar amounts of N-hydroxysuccinimide, dicyclo-hexylcarbodiimide (DCC) and the hapten are mixed overnight in dry, redistilled dimethylformamide. Production of an "active ester" is indicated by the formation of substituted urea crystals. An aliquot of the active ester is added to an aqueous solution of HRPO at an enzyme to hapten molar ratio of 1:10 or 1:20. Reaction is allowed to occur overnight and the conjugate is purified by gel filtration. HRPO conjugates prepared in this way are stable at 4oC for periods of time up to 12 months.

Enzyme substrates and reactions

The substrate used at the end of an ELISA will depend on the enzyme used to prepare the labelled component of the assay. Several chromagens are available for HRPO producing different coloured endpoints many suitable for visual examination. Many of them have unfortunately been shown to be carcinogenic or mutagenic in test systems. o-Phenylenediamine (OPD) previously one of the commonest substrates, producing a yellow colour changing to orange when the reaction is stopped by the addition of acid, has been largely replaced by tetramethylbenzidine (TMB) as it is thought to be less hazardous. TMB produces a pale blue colour changing to yellow on addition of acid.

Alkaline phosphatase obtained from calf intestine is more expensive than HRPO but its conjugates are very stable and non-hazardous colorimetric and fluorimetric substrates have been used. Para-nitrophenyl phosphate is one of the most widely used substrates for spectrophotometric assays and methyl umbelliferryl phosphate can be used to give a fluorescent end-point.

Enhanced luminescence in ELISA

The high sensitivity at which chemiluminescent reactions can be measured has provided the opportunity of developing other types of ELISAs. However, chemiluminescent signals are usually very short-lived which presents some practical difficulties in measurement. Enhanced luminescent reactions in which an intense, prolonged light signal is produced have now been effectively utilised in ELISA methodology with both HRPO and alkaline phosphatase labels. In the HRPO enhanced luminescent reaction luminol is oxidised by hydrogen peroxide in the presence of the enhancer, p-iodophenol to produce a signal which reaches a peak at 2 minutes and declines slowly over 20-30 minutes (Thorpe et al., 1985a). HRPO enhanced luminescence has been applied to a wide range of assays both as research tools and for routine use and several luminescent microplate readers are now available commercially. The reaction can also be used to measure immunoassay end-points in a semi-quantitative manner by recording the signal onto high-speed Polaroid film using a portable camera luminometer (Thorpe et al., 1985b).

Enhanced luminescent end-points have also been developed for alkaline phosphatase reactions (Thorpe et al., 1989). The luminescence is based on 1,2-dioxetane derivative (AMPPD) which has been stabilised with an adamantyl group and also contains a phosphate group. When the phosphate group is enzymatically cleaved, AMPPD becomes destabilised and decomposes with light emission, the rate of which is dependent on the enzyme concentration.

Extra-laboratory applications of ELISA

The simple procedures used in ELISA and the visual reading possibilities have allowed the development of numerous types of assay for use away from the laboratory. For many purposes qualitative or semi-quantitative results are acceptable and it is in this type of assay that the adaptability of ELISA excels. Reagent kits have been produced whereby the user only has to add reagents in the order specified to previously coated plates or tubes for a coloured end-point to be produced. Applications include veterinary fertility tests and diagnostic tests for animal and plant infectious diseases. Over the counter pregnancy and ovulation tests are also based on ELISA principles.

APPLICATIONS OF IMMUNOASSAY TO PESTICIDE ANALYSIS

In spite of their obvious advantages for the analysis of pesticides in the environment it is only relatively recently that the potential of immunoassay techniques has been widely considered. This may be because there is a lack of awareness and familiarity with the advantages of the assays. The expertise and facilities may also not be available to the relevant laboratories and the demands of Regulatory Authorities may at present preclude the use of data obtained by immunoassay. But it is probably the limited availability of specific antibodies to pesticides and other related compounds that is preventing a more widescale use of immunoassays for environmental analysis.

The first report of the production of antibodies to pesticides was in 1970 when antisera to DDT and malathion were described (Centeno et al., 1970). In 1971 a review on the immunochemical analysis of pesticides was published (Ercegovich, 1971) in which the "development of immunological methods of pesticide analysis as supplemental methods for rapid screening and confirmatory tests " was encouraged. The increased rate at which publications describing the production of antibodies to pesticides are appearing shows that the potential of immunoassays may now be being realised. Several commercial organisations are supplying kits and reagents for various applications and the demand for environmental immunodiagnostics is predicted to be worth millions of dollars.

Antibodies to pesticides

Antibodies have been produced to a wide range of pesticides including herbicides, insecticides and fungicides reference to which can be found in the various reviews quoted earlier. The procedures used to prepare antibodies to pesticides are the same as for any other small molecule and consideration of the likely specificity of the antisera is just as important. There have been several reports of the production of antisera to the triazine herbicides and immunogens have been prepared from haptens of different structure. None of the antisera are specific for one triazine but different spectra of cross-reactivity are obtained depending on the point of attachment of the hapten to the carrier protein (Aherne, 1991). Relatively specific antisera have been produced in our laboratory for the phenylurea herbicides isoproturon and chlortoluron. Figure 5 shows the structures of the two herbicides, the haptens used to prepare the immunogens and the percentage cross-reactivity with selected phenylureas. These results show how even small changes in chemical structure can reduce or eliminate antibody binding. Figure 5 also shows that the cross reaction between classes of herbicides is negligible.

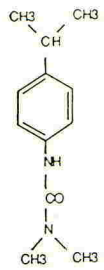
The majority of reported antisera to pesticides have been polyclonal in nature although some monoclonal reagents have also been reported e.g. for paroxon (Brimfield et al., 1985) and atrazine (Giersch & Hock, 1990). The relative merits of monoclonal and polyclonal antibodies with respect to pesticides have been discussed (Van Emon et al., 1989). It is arguable whether monoclonal antibodies presently offer significant advantages over polyclonal antibodies for the measurement of pesticides.

Pesticide immunoassay formats

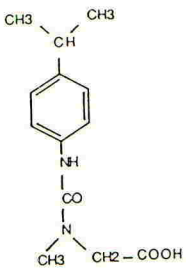
Some of the first immunoassays described for pesticides were RIAs (Vallejo et al., 1982; Knopp et al., 1985) but the preferred format is ELISA. However, if a radiolabelled form of the compound is available even at low specific activity, it can be a useful reagent for screening antisera during the initial stages of assay development.

Figure 5. Specificity of antibodies to isoproturon (Sheep 4676) and chlortoluron (Sheep 1825).

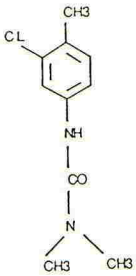
Isoproturon



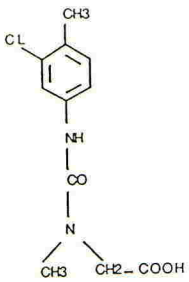
Isoproturon hapten



Chlortoluron



Chlortoluron hapten



Compound	% Cross reactivity (Sheep 4676)	%Cross reactivity (Sheep 1825)
Chlortoluron	0.08	100
Chlorsulfuron	<0.01	<0.01
Isoproturon	100	0.09
Chlorbromuron	0.01	71.0
Metsulfuron	<0.01	0.45
Metoxuron	<0.01	1.8
Atrazine	<0.01	<0.001
Simazine	<0.01	<0.001
MCPA	ND	<0.001
2,4-D	ND	<0.001
Paraquat	ND	<0.001

Several different forms of ELISA have been described for pesticides although the most universally employed enzyme system is HRPO with a colorimetric end-point. In general, the coated antibody ELISA format has been used although some assays which utilise an

antigen-coated solid phase have been described (Niewola et al., 1986). An ELISA for the fungicide fenpropimorph uses a "capture" second antibody to coat the wells of a microtitre plate conserving the use of the specific antibody which was used at a dilution of 1:200,000 (Jung et al., 1989). Various forms of solid phase have been used in addition to the microtitre plate. For example, an atrazine immunoassay has been developed using polystyrene test tubes (Bushway et al., 1988) which has been adapted for field use, results being measured on a simple portable photometer. Enhanced luminescence has also been used to provide assays which can potentially be used at extra-laboratory sites. The applications of pesticide immunoassays will be described below.

Applications of pesticide immunoassays

Water analysis

The urgent need to develop more cost effective monitoring of drinking water supplies has been a great impetus to the development of pesticide immunoassays. The legal limit for individual pesticides in drinking water in Europe is the EC Maximum Admissible Concentration (MAC) of 0.1μg/L (ppb) and total pesticide concentrations should not exceed 0.5μg/L. For some pesticides these concentrations are many times less than was allowed under previous legislation. For example, the previous UK Government guideline concentration for atrazine was 30μg/L. The MAC is not necessarily a toxicity indicator but a limit set to protect water supplies from pollution and therefore frequent monitoring is required. There are analytical problems involved with the measurement of pesticides at levels below the MAC as for some pesticides traditional methods such as GC and high performance liquid chromatography cannot achieve the desired sensitivity. In addition, extensive sample preparation, using solvent or solid phase extraction procedures, add to assay costs and reduces the number of samples that can be handled at any one time. Immunoassays offer an alternative but complementary type of assay that will facilitate the increased monitoring that is demanded by modern legislation and by the public. Rapid cost-effective screening of water supplies can be achieved for pesticides at levels which will detect breaches in the MAC. However, unlike chromatography where it is possible to carry out multi-analyte residue studies in one run, only a single compound or group of compounds can be measured with one antibody.

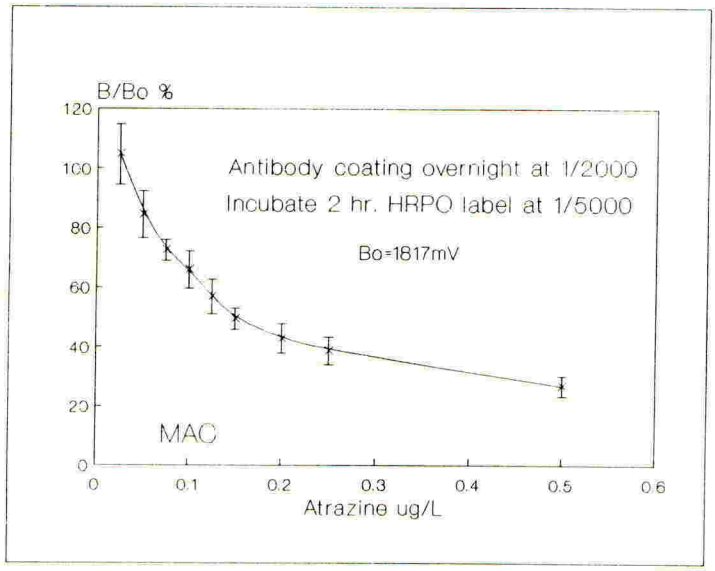
For every immunoassay developed it is essential as with any analytical technique that a complete validation is carried out. This includes determining the limit of detection or sensitivity of the method as well as obtaining precision and accuracy data. Finally it is important that the immunoassay is compared with an established technique. The sensitivity of ELISA methods for the measurement of pesticides in water are in the order of 0.01-0.1μg/L without sample preparation. In our laboratory and using an enhanced luminescent ELISA carried out in polystyrene tubes, atrazine could be measured at levels as low as 0.025μg/L and paraquat at 0.01μg/L.

The standard curve obtained using the enhanced luminescence ELISA for atrazine is shown in Figure 6. The coated solid phase is stable for some weeks if stored at 4°C and the assay can be completed in approximately 2 hours (not including the coating stage). Recovery of atrazine "spiked" into water samples ranged from 83-112% at concentrations of 0.06-0.24μg/L with a CV% of 10- 20%. A correlation coefficient of 0.915 was obtained between the immunoassay and a GC method for atrazine. Close correlations with

chromatographic methods have been obtained for other atrazine ELISAs (Wittman & Hock, 1990; Thurman et al., 1990) and for molinate in water (Harrison et al., 1989).

The samples of water in these studies were analysed without any pretreatment. However, it is important to determine that there is no effect on antibody binding by water from different sources. These matrix effects should be tested for each combination of antibody and label because water with different physico-chemical properties may influence the binding in an assay (Harrison et al., 1988; Wittmann & Hock, 1990) leading to erroneous results. Simple steps can be taken to minimise these effects e.g. the addition of small volumes of buffers of high molarity.

Figure 6. Standard curve for atrazine (n=5) obtained with an enhanced luminesence ELISA.



It is clear that immunoassays have an important role to play in the monitoring of water for the presence of pesticide micro-contamination. The assays can be formatted to give quantitative results reducing the number of samples that need to be assayed by more expensive techniques. Alternatively, assays can be designed to be qualitative to identify samples which exceed certain concentration thresholds. In both cases confirmation of immunopositive results can be obtained by chromatography if necessary. In addition, the possibility exists for "field" assays to be developed which could be used by non-scientific personnel. Commercial kits and antibodies are already available for several compounds e.g. EnvironGard ™, test kits (Millipore) which use a colour end-point, results being available within 10 minutes. The enhanced luminescence assays developed in our laboratories have also been adapted for field use. Results are recorded onto photographic film and assessed visually and a permanent record of the results is obtained (Hardcastle et al., 1989) for record keeping.

Soil and plant analysis

Many of the assays developed for water analysis have also been applied to the measurement of pesticides in soil and plants. Such assays are required to assess the persistence and effectiveness of pesticide residues. It is often essential to determine that a previously applied chemical is not present before the next crop is planted. Soil and plant samples unlike water will require some pre-assay treatment but to exploit the virtues of immunoassay these should be as simple as possible. In one reported assay for atrazine, this pre-treatment consisted of shaking or sonicating the soil sample for a short time in either water or acetonitrile (Bushway et al., 1988). Good agreement was obtained between GC and immunoassay for atrazine extracted from both plant and soil samples. The low detection limit of the ELISA was advantageous in that no atrazine enrichment of samples was necessary (Wittmann & Hock, 1990).

A more extensive soil extraction procedure was used for the analysis of paraquat in soil (Niewola et al., 1986) involving reflux of the sample in 6M acid. The neutralised extract after the addition of chelating reagent was added to the antigen coated wells of an ELISA plate and a monoclonal antibody to paraquat was used with an enzyme-labelled second antibody. The limit of detection for the assay was 0.2mg/kg soil a limit which is more than adequate for most applications. The problems of representative sampling of soil and plant material is common to both immunoassay and conventional procedures.

Food analysis

The food industry is also faced with an increased need for testing products for contamination by pesticides. Government restrictions are becoming more stringent and rapid simple tests are required to facilitate this. Official methods of analysis are often time consuming and are unlikely to cope with the increased demands. Immunoassays are again an attractive option though the problems of representative sampling and extraction may negate some of the advantages. Immunoassays have been more widely used in the food industry than in the water industry. For example, ELISA methods have been used for microbiological identification, the measurement of bacterial toxins, aflatoxin, anabolic steroids and for meat speciation (Morris & Clifford, 1985). The detection and measurement of pesticides in food by immunoassay should therefore be easily accepted and it is likely that an increased number of assays will be described in the near future.

The analysis of pesticides in food has been reviewed (Newsome, 1986) and the detection limits for pesticides in various foodstuffs varied from 0.1-2μg/kg. For example, using a relatively simple extraction procedure, paraquat could be measured by ELISA with improved detection limits in milk, beef and potatoes (Van Emon, et al., 1987). An ELISA for methyl 2-benzimidazolecarbamate, the degradation product of benomyl, has been reported (Bushway et al., 1990) in which a portable photometer is used, facilitating analysis away from the normal analytical laboratory. The limit of detection was 10ng/g for fruit juices and there was good agreement with an HPLC assay.

Human exposure studies

There is increasing evidence that occupational and domestic exposure to pesticides may have deleterious effects on health and health and safety regulations define maximum permitted exposure levels for specific compounds. Also, in cases of accidental or deliberate ingestion of pesticides it may be necessary to monitor plasma levels to ensure that the

correct treatment is given. Immunoassays have an important role to play in occupational exposure monitoring (Van Emon et al., 1986) and in the measurement of pesticides in plasma and urine. Enhanced luminescence assays for atrazine and paraquat in plasma and urine which are sensitive to less than 1ng/ml have been evaluated in our laboratory. The assays are rapid and require no sample preparation and as only 200μl of sample is required are suitable for exposure time course studies.

SUMMARY AND FUTURE PROSPECTS

The purpose of this paper was to briefly describe the principles and practise of immunoassays and to show how they can be usefully applied to the environmental analysis of pesticides. ELISA methods in particular are suitable for this purpose and offer the advantages of speed, simplicity, sensitivity, selectivity and are cost-effective requiring relatively inexpensive equipment. Once developed ELISAs are simple to perform and very little training is necessary to carry out the procedures involved. Because minimal or no sample treatment is necessary, the sample workload can be increased over presently used methods. The versatility of immunoassays provides the means of tailoring tests to the requirements of a particular application and analyte. For many applications a "yes-no" result is all that is required and this can be readily achieved with either the coloured or luminescent visual end-point although simple portable equipment is also available for semi-quantitation. The use of immunoassays is not advocated as an alternative to traditional methodologies for residue analysis. Rather, it should be seen as an adjunct to it, offering complementary characteristics which can be suited to the changing needs of the analyst.

One of the drawbacks to widespread use of immunoassay for residue analysis is the shortage of suitable antibodies but these are now being produced to a range of different compounds.The reluctance of analysts to accept immunological assays is gradually being overcome and there needs to be greater inter-disciplinary efforts during future development and evaluation of assays. For some compounds immunoassays may not be suitable either because they are not antigenic in nature or because the sensitivity required may not be possible to achieve. Immunoassays may not be justified for some sample types and compounds which require extensive extraction and the high specificity of antibodies precludes true multi-residue analysis. The speed at which immunoassays are adopted as routine methods in environmental monitoring depends on a number of factors but it is crucial that the assays are vigorously and completely validated before use.

What future developments in immunochemical detection lie ahead? There are currently tremendous efforts to develop immunosensors for clinical applications. Much work remains to be done but the implications for environmental monitoring are huge. Other possibilities are the use of continuous or semi-continuous monitors consisting of some form of antibody-coated device (dipstick, filter, column) and promising pilot studies have been undertaken in our laboratory.

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UNDERSTANDING PESTICIDE RESISTANCE THROUGH MOLECULAR BIOLOGY

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ABSTRACT

Molecular biology has provided researchers with many new techniques and has switched the focus of studies on the mechanism of pesticide resistance to identifying changes at the DNA level. Cloning, restriction mapping and sequencing techniques have provided new insights into how resistance operates and these are explored in terms of gene amplification and target site changes. The practical benefits of this work are still to emerge, but should include better diagnosis and management of resistance, development of new crop protection agents, as well as the introduction of transgenic crop plants with resistance against broad spectrum herbicides.

INTRODUCTION

Resistance is a major factor limiting the effectiveness of all classes of pesticides. Where resistance develops soon after the introduction of a new pesticide, development costs may not be recovered, and the target site may cease to be a resource available to both growers and the agrochemical industry. Consequently, surveys must be organised to monitor changes in sensitivity, and strategies implemented to combat spread should resistance emerge. A wide range of epidemiological, biochemical and genetic information about resistance must be welded together if strategies are to be effective, and knowledge of mode of action and mechanism of resistance will often be crucial.

Pesticides generally inhibit structural or enzymatic proteins, and to understand the mechanisms of resistance, traditional biochemical methods of protein separation and enzyme assays are increasingly augmented with a battery of powerful recombinant DNA techniques. These new techniques often allow answers to be found to questions which were not readily tackled before, and as a result a great deal more information is now available about the molecular events responsible for resistance. This has already led at the research level to improved methods of detection, and to the development of transgenic crop plants with resistance to cheap, broad-spectrum herbicides.

This paper illustrates through a series of case studies some of the molecular techniques that have contributed to increasing our knowledge of pesticide resistance. It emphasises how rapidly these new techniques are taken up by researchers in their attempts to find durable approaches to combating resistance.

INSECTICIDE RESISTANCE AND GENE AMPLIFICATION

Mechanism of amplification

Gene amplification is well established as a mechanism whereby organisms develop resistance to drugs, pesticides and other toxophores. Increased levels of either target proteins, or detoxifying enzymes, reaching 10% of total cell protein in some instances, result from changes in molecular organisation at the DNA level. In natural populations, amplification is reflected by stepwise geometric increases in resistance levels in response to selection, coupled with instability in otherwise genetically homogeneous individuals and reversion to susceptibility once selection is stopped. This pattern of development of resistance to a wide range of insecticides was observed in peach-potato aphids (*Myzus persicae*), being linked to changes in esterases (E4 or FE4) which sequester and hydrolyse insecticidal esters, and forming the basis of the resistance mechanism (Devonshire & Moores, 1982). It had been recognised earlier that resistance might be caused by gene amplification (Devonshire & Sawicki, 1979), a mechanism which now appears to account for insecticide resistance in mosquitoes (*Culex* sp.; Mouchès *et al.*, 1990) as well.

To confirm this amplification hypothesis, it was necessary to identify the gene involved, and show that gene copy number increased in line with resistance. The search for the gene coding for E4 esterase was begun using a highly resistant (x 64) field clone of *M. persicae*, and isolating first an RNA fraction enriched for the corresponding E4 mRNA (Field *et al.*, 1988). mRNA (poly A) fractions were screened for their ability to direct the cell-free synthesis of a protein that could be immunoprecipitated with polyclonal anti-sera to E4 esterase. Positive fractions were used to prime the synthesis of a double-stranded DNA copy (cDNA), which was then cloned in the plasmid pUC8. cDNA Clones which hybridised strongly to E4 mRNA from resistant, but not sensitive, aphids were confirmed using hybrid arrested translation (HART). Treatment with RNA-ase H destroys DNA-RNA hybrids, so that cDNA clones complementary to E4 mRNA arrested synthesis of E4 esterase protein in a cell-free rabbit-reticulocyte system.

Two E4 specific cDNAs were selected for further study and hybridised by dot-blot assays to total DNA from sensitive, R_1 (x 4), R_2 (x 16) and R_3 (x 64) aphid clones (Figure 1). Intensity of hybridisation increased in a stepwise progression, indicating that resistance was associated with increased copy number of the E4 gene and its mRNA transcription product. Southern analysis of genomic DNA from some of these aphid clones suggested amplification events were not identical for all resistant aphids. EcoRI digests probed with E4 cDNA showed two qualitative banding patterns correlating with overproduction of either E4 or FE4 esterases, and whether or not resistant clones carried a chromosomal translocation. Further work exploited differences between the isoschizomers *Msp*I and *Hpa*II, which both cleave CCGG sequences, but only *Msp*I cuts this sequence if an internal cytosine is methylated. Southern blots of genomic DNA from a sensitive, resistant, and revertant susceptible clone probed with E4 cDNA, revealed a correlation between methylation of amplified E4 sequences and insecticide resistance (Figure 2, Field *et al.*, 1989). Increased methylation it seems suppressed gene transcription.

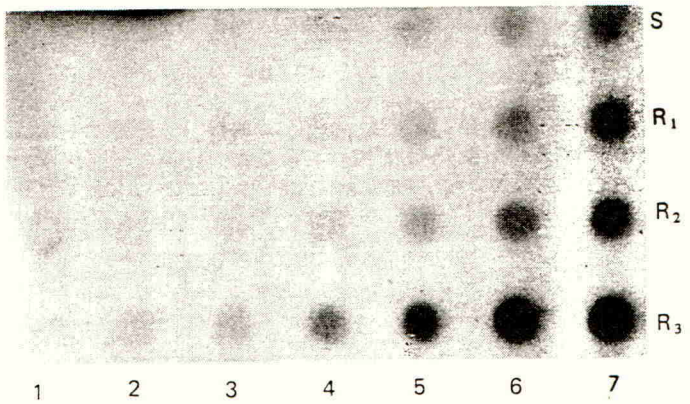

Figure 1. Dot-blot assay for E4 related sequences in genomic DNA for sensitive and resistant aphid clones. DNA prepared from S, R_1, R_2 and R_3 aphids was loaded on to a nylon filter at 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 μg (dots 1-7 respectively). The filter was hybridised with ^{32}P-labelled pMp31 DNA, washed and autoradiographed. (From Field *et al.*, 1988).

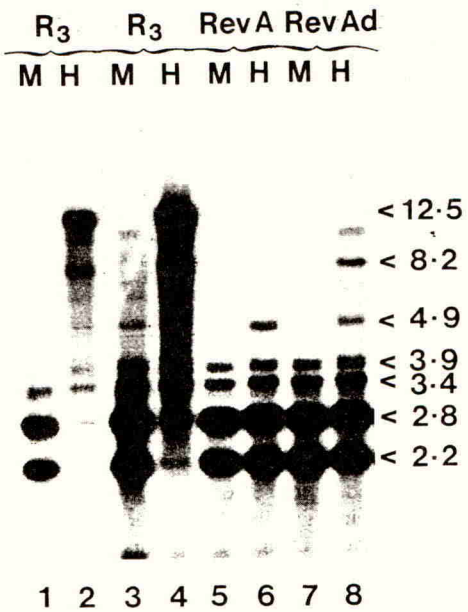

Figure 2. Southern analysis and DNA methylation. Hybridisation of the E4 cDNA (pMp24) to Southern blots of DNA from extremely resistant (R_3), revertant (RevA) and reselected revertant (RevAd) aphids, digested with either M*sp*I (M) or H*pa*II (H). Lanes: (1,2) after 3 days autoradiography; (3-8) after 10 days autoradiography. (From Field *et al.*, 1989).

Amplification in field populations of *M. persicae*

A fully susceptible revertant glasshouse clone (Rev A) no longer expressed the amplified E4 esterase gene, yet still carried the amplified, but unmethylated, sequence (Field *et al.*, 1989). The potential for mis-identifying sensitive aphids has important consequences for resistance management since, unlike susceptible wild-type aphids, revertants can readily be selected for increased esterase levels and resistance (ffrench-Constant *et al.*, 1988). Analysis of 84 aphid clones, established from a UK field population, encountered no aphids with non-methylated amplified E4 sequences, although in 9 aphid clones E4 specific DNA was only partially methylated and showed lower esterase levels as measured by immunoassay (Field, personal communication, 1991). If reversion only occurs from extremely resistant variant aphids, revertants should become more frequent as these variant aphids become more frequent in field popultions.

FUNCTIONAL ANALYSIS OF FUNGICIDE-RESISTANCE GENES BY TARGETED MUTATION

Where transformation systems are available, genes responsible for resistance may be cloned by screening a genomic library, rescuing the wild-type recipient strain from the effects of pesticide selection. Genes cloned in this way may be sequenced, and their function possibly identified by sequence homology, although data bases are still rather limited, and complementary sequences may not be available. Nevertheless, this approach has already been used to help identify the mechanism of resistance in mutants of the fungus *Aspergillus nidulans*, selected in the laboratory with a new class of fungicides (Gustaffson *et al.*, 1990). This led directly to identification of the mode of action of these fungicides. Transformation systems are not generally available for important pathogens, pests and weeds, and resistance genes are frequently cloned instead by heterologous probing using a corresponding gene cloned from another species. Often this is on the assumption that resistance results from changes in the gene coding for the target site (e.g., ß-tubulin), but this approach clearly requires rigorous confirmation of the identity of the cloned gene by some functional analysis.

Contrary to the situation described earlier for gene amplification, DNA methylation normally leads to gene inactivation. This phenomenon has been exploited in *Neurospora crassa*, which methylates only sequences homologous to any introduced DNA, to generate targeted null alleles (Repeat Induced Point Mutation, RIP; Selker, 1990). Duplicated sequences are subject to pre-meiotic disruption and are inactivated because of base-pair changes and cytosine methylation. Crosses involving *N. crassa* strains with specific copies of target sequences inserted by transformation, yield progeny with mutations in the corresponding resident *Neurospora* gene. Early uses of RIP were confined to the insertion and analysis of cloned duplicate *Neurospora* sequences (Marathe *et al.*, 1990), but recent work has extended that to include genes from other ascomycetes (Connerton, 1990; Connerton *et al.*, 1991). New mutants have been generated corresponding to the cloned sequences from these alternative species. The potential of RIP lies not only in its use in identifying the function of unknown DNA fragments, but it also provides a powerful tool to make targeted gene disruptions in filamentous fungi and so analyse gene function more thoroughly. Unlike in yeast, where in-coming DNA generally recombines with homologous resident

sequences, in filamentous fungi transformation leads to insertion of in-coming DNA at random loci involving unrelated sequences.

To understand the mechanism of resistance to fungicides (DMIs) known to inhibit sterol 14 α-demethylase (14DM), we have cloned a putative 14DM gene from barley powdery mildew (*Erysiphe graminis* f.sp. *hordei*) by heterologous DNA hybridization with the equivalent *Saccharomyces cerevisiae* structural gene (ERG 11; Kalb *et al.*, 1987). To confirm the function of this mildew clone, the resident *N. crassa* gene was disrupted pre-meiotically. The entire 3.3 kb cloned mildew fragment was inserted into a plasmid vector carrying the complete *am*$^+$ gene (NADP glutamate dehydrogenase) from *N. crassa* as a selectable marker. Conidial protoplasts of a *N. crassa* strain lacking the entire *am* gene (*am* 132) were transformed with this vector construct. Only a few transformants were mitotically stable, but all showed wild-type sensitivity to DMI fungicides despite the fact that they carried many copies of the mildew sequence. Very few of these stable transformants generated mature ascospores when back-crossed to *am* 132 of the opposite mating type, and less than 5% of progeny were derived from the transformed *am*$^+$ parent. Attempts to select 14DM-less mutants in filamentous fungi have been unsuccessful (Ziogas *et al.*, 1983), so we expected that extensive mutation and methylation generated by RIP might be lethal, and only progeny less seriously disrupted would survive. However, some of the viable progeny which no longer contained inserted mildew sequences and required glutamate for growth, did show altered sensitivity to DMI fungicides (Table 1). Surprisingly they were also resistant to the morpholine fungicides fenpropimorph and fenpropidin, even though these affect not 14DM but the next enzyme in the sterol pathway, $\Delta^{14\text{-}15}$ reductase. Subsequent genetic analysis separated DMI and morpholine sensitivity, indicating that at least two *Neurospora* genes had been mutated during RIP. We have, therefore, not only generated novel mutants in *N. crassa* with perhaps altered 14DM, but also confirmed that our mildew fragment codes for a function affecting DMI sensitivity, and may contain a second gene involved in morpholine resistance.

Fungicide resistance at the target site

MBC and related fungicides have been in use for over twenty years. In some pathogens resistance developed very rapidly (Schroeder & Provvidenti, 1969), but slowly or not at all in others (Jones, 1990). The reasons for these differences are not at all clear, but understanding of the resistance mechanisms at a molecular level should help provide an answer, and perhaps improve the durability of strategies aimed at combating the spread of MBC resistance. Pathogens resistant to one MBC fungicide invariably show cross-resistance to another, suggesting changes in the target ß-tubulin gene (Orbach *et al.*, 1986). At least three different alleles, each one conferring a different level of MBC resistance, have been identified in pathogenic fungi (Ishii *et al.*, 1990). Electrophoretic analysis of the altered ß-tubulin protein showed that mutations occurred in the structural gene (Oakley & Morris, 1981), and numerous binding experiments confirmed that the altered protein bound less MBC (Davidse & Flack, 1978). In addition, MBC resistance is often associated with increased sensitivity to phenylcarbamates (Kato *et al.*, 1984), which is also governed by mutations in the ß-tubulin gene (Ishii *et al.*, 1990). This negative-cross resistance has provided the basis for a mixture strategy to combat MBC resistance.

TABLE 1. Sensitivity of mutations induced (RIP) in *Neurospora crassa* by a barley mildew genomic DNA fragment

*Strain	**Growth in absence of glutamate	***Fungicide sensitivity ED_{50} µg/ml Tridimenol	Fenpropidin
aml32a (parental)	-	0.43 ± 0.05	0.38 ± 0.1
aml32a EMBL	+	0.27 ± 0.10	0.38 ± 0.1
aml32a TA	+	0.35 ± 0.08	0.33 ± 0.08
aml32a SP5	+	3.4 ± 0.2	1.95 ± 0.15
aml32a FR	-	1.1 ± 0.25	0.34 ± 0.07

* aml32a EMBL and aml32a TA were transformants derived from aml32a by insertion of the vector alone, or vector plus 3.3 kb mildew fragment respectively. aml32a SP5 was an ascospore progeny (RIP) from a cross between TA and aml32A. aml32 FR was a progeny from a back-cross between aml32a SP5 and aml32A.

** Growth on Vogel's medium in presence of 20 mM glycine.
*** Sensitivity tests were carried out on Vogel's medium supplemented with glutamate (3 mm) and amended with different concentrations of each fungicide. Radial growth was measured after 24 h at 30°.

These biochemical and genetic findings prompted attempts to clone both wild-type and MBC resistance ß-tubulin alleles from several fungi, including at least one plant pathogen (Cooley & Caten, 1989). Sequence analysis soon established that resistance was the result of single base changes, which mainly occurred in the region of the gene coding for amino acids 167-241 (Table 2). No crystal structure is available for ß-tubulin and the protein structure in this region is not clear. Chimeric genes constructed between an allele of *N. crassa* conferring MBC resistance, and one conferring resistance to both MBC and the phenylcarbmate diethofencarb, were inserted into a wild-type *N. crassa* strain by transformation, and screened for growth on MBC and diethofencarb amended media (Figure 3; Fujimura *et al.*, 1990). This located the region involved in binding both fungicides within an EcoRl-BamHl fragment of the ß-tubulin gene, and identified that glycine replaced glutamate at amino acid 198 in the MBC resistant allele. The imidazole residue of MBC appears to bind to the dicarboxylic amino acid but not to glycine, allowing diethofencarb to fit into the space created (Fujimura *et al.*, 1990). However, changes in ß-tubulin allowing both diethofencarb and carbendazim to bind do occur, and doubly-resistant mutants have been isolated from field populations of pathogens, threatening the success of anti-resistance strategies (Leroux, 1991). Unfortunately, these molecular studies with *N. crassa* were not extended to sequence analysis of the doubly resistant mutant allele used to construct the chimeric genes.

TABLE 2. Amino acid substitutions in ß-tubulin genes and carbendazim resistance

	MBC sensitivity	Position of the amino acid substitution			
		167	198	200	241
N. crassa	S	phe	glu	phe	arg
N. crassa	R	tyr	glu	phe	arg
N. crassa	R	phe	tyr	phe	arg
S. cerevisiae	S	phe	glu	phe	arg
S. cerevisiae	R	phe	glu	phe	his
V. inaequalis	S	?	glu	phe	?
V. inaequalis	R	?	ala	phe	?

Data from: Thomas *et al.*, 1985; Orbach *et al.*, 1986; Fujimura *et al.*, 1990; Koenraadt *et al.*, 1991.

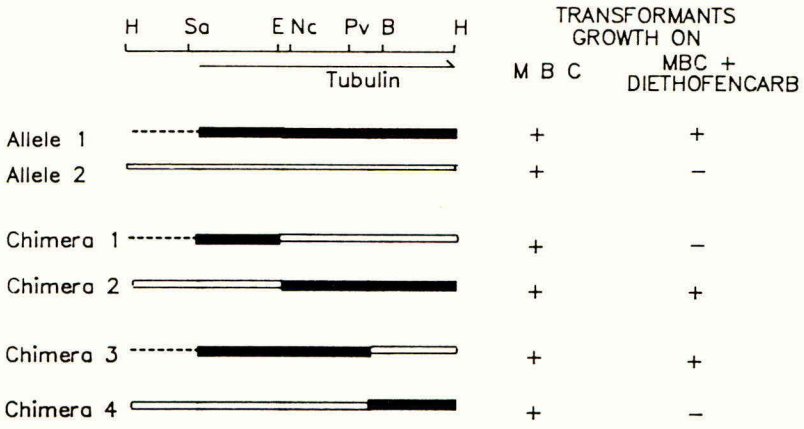

Figure 3. Characterisation of plasmids carrying chimeric ß-tubulin genes. Top line is a restriction map of a DNA fragment containing the ß-tubulin gene. Restriction sites are as follows: H, Hind III; Sa, Sal I; E, EcoRI; Nc, Nco I; Pv, PvuII; B, BamHI. Redrawn with modification from Fujimura *et al.*, (1990).

Detection of single base changes

Single base changes in genomic DNA linked to MBC resistance provide the basis for allele specific probes (Koenraadt *et al.*, 1991), which have already been used successfully to identify different levels of MBC resistance in field isolates of *Venturia inaqeualis*, *V. pirina*, *Monilinia fructicola* and *Penicillium* species. The critical 408 bp region containing point mutations governing MBC-resistance was amplified from genomic DNA template using PCR and flanking primers. Amplified DNA was dot-blotted onto membranes and probed with 18 mer oligonucleotides designed to hybridize only with complementary point mutations. This procedure allows detection of resistance within three days using already isolated mycelial cultures. Because of the degeneracy of the genetic mode, and the several different point mutations which may lead to resistance, many different probes might be needed to detect resistance on a routine basis. However, in practice very few probes seem to be required to analyse field populations, since many mutations may adversely affect fitness, and will be rare. If these diagnostic tests can be applied to diseased tissue, they should help understand the population dynamics underlying the spread of MBC resistance, and provide the monitoring information needed to effectively operate any fungicide mixture strategy to combat its spread.

GENETICALLY ENGINEERED HERBICIDE RESISTANT CROPS

Herbicide resistance is a recent practical problem but already attempts have been made to exploit it by constructing transgenic crop plants engineered for resistance to broad spectrum herbicides. Although biochemistry and genetics were used to identify the site of action of most herbicides, molecular biology has broadened understanding of these mechanisms at a molecular level, and paved the way for engineering crop plants for increased herbicide resistance (Shah *et al.*, 1986).

The selective sulphonylurea herbicides inhibit acetolactate synthase (ALS = acetohydroxyacid synthase, EC 4.1.3.18), which is involved in the biosynthesis of branched chain amino acids, valine, leucine and isoleucine. Their mode of action was largely established through studies with bacteria (Hartnett *et al.*, 1990), but the ALS gene has now been cloned from a number of other organisms including yeast, algae and higher plants. Resistant mutants were easily generated in all these organisms, and soon appeared in weed species in the field, but only to sulphonylureas with long soil persistence. Cross-resistance patterns varied considerably between different sulphonylureas, and the chemically unrelated imidazolines and triazolopyrimidines which also inhibit ALS. Sequencing resistance genes revealed that a single amino-acid substitution, often in conserved regions of the protein but away from the catalytic site, invariably caused resistance. Enzyme activity in the absence of herbicide was seldom affected by these amino-acid changes, suggesting that resistant mutants were unlikely to have deleterious effects on fitness. At least two resistant mutants from tobacco (SuRA, SuRB; Hartnett *et al.*, 1990) and one from *Arabidopsis thaliana* (csrl-1; Gabard *et al.*, 1989) have been introduced into wild-type tobacco using a binary expression vector in *Agrobacterium tumefasciens*. The genes were inserted into an EcoRI-BglII cloning site in the vector, flanked with a cauliflower mosaic virus promoter sequence (CAMV-35S) and a NOS poly A^+ site (Figure 4), and containing a neomycin phosphotransferase gene for selection with kannamycin. Tobacco leaf discs were infected with the

A. tumefasciens, selected with kannamycin and sulphonylureas, and regenerated resistant plants assayed for ALS activity in the presence of herbicides. These mutant genes were semi-dominant, and expressed in conjunction with the resident wild-type allele. Gene dosage appeared unimportant and inhibition of ALS activity was seldom more than 50% (Table 3). This was sufficient to resist field application rates, but quite unlike genetically engineered glyphosate resistance where gene amplification increased the target 5-enolpyruvylshikimate phosphate protein by at least 20-fold. By choosing the right ALS resistance allele, and from knowledge of cross-resistance patterns, scope exists for engineering resistance to less persistent sulphonylureas, so lessening the risk that resistant weeds might emerge.

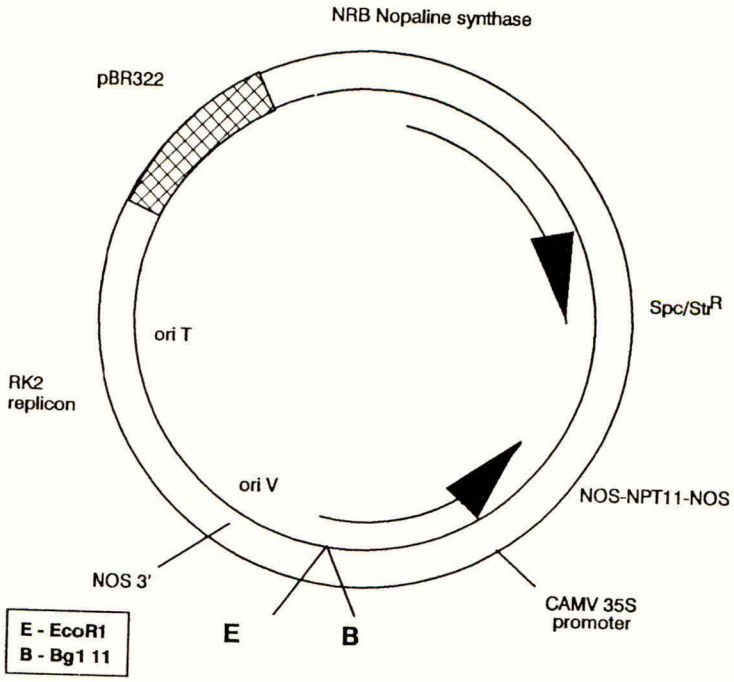

Figure 4. Binary expression vector for insertion of foreign genes into plants using *Agrobacterium tumefasciens*. (From Shah *et al.* 1986).

TABLE 3. ALS activity and inheritance of herbicide resistance in transformed tobacco lines

	% Inhition of ALs activity	Segregation ratio: resistant/sensitive
NK 326 (WT)	93	-
K 14 (WT)	93	-
NK 326/9c	53	3:1
K 14/711	48	3:1
K 14/40	59	15:1
K 14/53	63	3:1

From Hartnett *et al.* (1990).

CONCLUDING REMARKS

The four case studies discussed in this paper illustrate some of the contributions of molecular biology towards understanding pesticide resistance. The cases involve important pests and diseases, and pesticides, but their interpretation rests on knowledge accumulated from molecular studies with a wide range of organisms, many of which are not encountered in crop protection, such as *Escherichia coli*, yeast, *Arabidopsis thaliana* and Hela cells. Coupled with improvements in biochemistry, genetics and immunology, this has shifted the impetus and focus of research on resistance mechanisms to the DNA level. As yet few practical benefits have reached the grower, although thinking about resistance is beginning to be influenced by these new research findings. Better diagnosis of resistance seems possible, and this should enable better management of the problem. Understanding the mechanisms of resistance at the molecular level should stimulate chemists into generating new compounds able to combat resistance in novel ways. Transgenic crop plants with engineered herbicide resistance should broaden the range of chemicals available for weed control. The future impact of molecular biology on field practices to combat resistance is uncertain, but there is no slackening of the pace of research, and some of these techniques will undoubtedly find application in crop protection at the farm level.

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GLOSSARY

allele Two or more alternative forms of a gene which occupy the same relative position on homologous chromosomes.

amplification This refers to an increase in the copy number of a plasmid in a bacterial cell or culture.

analyte A name given to describe a substance which is being produced in a analytical procedure.

annealing The association of two single strand molecules of a nucleic acid by complementary bases on the individual strands.

antibody A protein produced in a vertebrate animal when a foreign sub stance (the antigen) gains access to the body fluids. Antibodies and antigens combine chemically in a very specific manner.

antigen A substance that is capable of stimulating the production of antibody proteins when injected into a vertebrate animal.

cDNA Complementary DNA produced via a mRNA template using the enzyme reverse transcriptase.

chimaera (chimera) An organism, tissue or recombinant DNA molecule which is composed of genes from more than one organism.

chromosomes Thread-like structures consisting of DNA and protein which occur in the nucleus of animal and plant cells. Chromosomes carry genes, the units of inheritance.

cloning In this process, one gene is isolated and inserted into a vector which has the capacity to transport genes into a host cell. Ideally, the vector replicates itself and the gene which it is to carry inside the host.

complementarity The base sequence of a nucleic acid is complementary to another if it can form a hydrogen- bonded duplex with it. Thus the mRNA strand GCAU is complementary to the DNA sequence CGTA.

copy number The number of molecules expressed per genome of a plasmid or gene which are contained in a cell.

electrophoresis A technique used to separate charged molecules which migrate in response to the application of an electric field.

ELISA Enzyme-linked immunosorbent assay. A very sensitive assay technique for the detection and measurement of antigens or antibodies in solution.

embryogenesis The simultaneous initiation of root and shoot meristems in competent cells so that bipolar structures develop into embryos and later into whole plants.

epitope During the binding of an antibody to an antigen, the epitope is the location or locations where the antibody binds.

eukarote Refers to cells of all organisms except bacteria and blue-green algae. The nucleus of such cells is separated from the cytoplasm by a nuclear membrane.

gene the unit of inheritance composed of either DNA or RNA (some viruses).

genome One complete single copy of the genetic instructions for an organism.

genomic library A collection of recombinant DNA molecules or clones which collectively comprise the total genome of an organism.

haptenes A small, separable non-protein part of certain antigen molecules which carries the chemical group that combines with the antibody. The injection of a hapten into an animal does not cause the initiation of an antibody response.

heterokaryon A cell which contains at least two nuclei from different cell types.

hybridoma An antibody-secreting cell line which is immortal.

immunoassay A technique which detects proteins by using an antibody specific to that protein.

isozymes or isoenzymes Variation of a specific enzyme in an organism, typically this subtle difference in molecular structure allows each isozyme to be separated e.g. by electrophoresis.

mRNA Messenger RNA, a molecule which is synthesised from a DNA template by the enzyme RNA polymerase.

mutation A suddenly occurring heritable change in DNA. Such mutations may be brought about by chemical or physical agents (mutagens) e.g. UV radiation.

nucleic acid A DNA or RNA molecule which may be composed of one or two strands.

operon A group of genes which function as a collective unit.

organelle A structure found within an individual cell which has a specific function e.g. mitochondion for cellular respiration.

oligonucleotide A very short length of a nucleic acid molecule.

plasmid A self-replicating piece of DNA which is found outside the chromosomes of an organism. Typically, plasmids are found in bacteria and are used in biotechnology as cloning vectors to introduce foreign DNA into a host cell.

primer A short oligonucleotide that base-pairs to a region of single-stranded template oligonucleotide. Primers are used to copy adjacent sequences of mRNA and cDNA's.

probe A piece of labeled DNA or RNA which is used to locate another piece of nucleic acid by complementary base pairing. The reaction is monitored by autoradiographic or enzymatic detection of the hybridisation.

prokaryote An organism (bacterium or blue green algae) which has its genetic material in diffuse filaments scattered within the cell and not within the confines of a nucleus.

promoter The region of DNA which binds RNA polymerase and directs the enzyme to the correct site where transcription of the gene will begin.

protoplast A plant cell from which the hard cell wall has been removed, normally by enzyme digestion.

sequencing A means of idenitfying the order of nucleotides in DNA, RNA or amino acids in a polypeptide.

Southern blotting This is a method for transferring separated DNA fragments from an agarose gel to a solid support such as a nitrocellulose membrane.

transcription The process of RNA synthesis by RNA polymerase to produce a single-stranded RNA complementary to a DNA template.

transformation A means of transferring genes from one organism to another.

transgenic An organism whose genome has been altered to include new genes from the same or different species.

translation Protein synthesis determined by the information coded by mRNA

transposon A piece of DNA which can insert itself into many different sites in other DNA sequences in the same cell

vector This is a vehicle for cloning, typically a DNA molecule (plasmid or bacteriophage DNA) capable of self-replication in a host organism.

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