A. K. Chakravarthy *Editor*

Genetic Methods and Tools for Managing Crop Pests

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Foreword

Intensive agricultural production systems targeting high yield are characterised by heavy reliance on chemical inputs, especially pesticides. However, rapid evolution of pesticide resistance in pests and diseases in crop plants and harmful effects of chemical pesticides on human and environmental health warrant the search for novel pest management methods. Genetic tools for pest suppression have unique and obvious merits, which is primarily due to the fact that these effects are inborn in the plant and heritable. Evidences are becoming available on the interactions between inherent resistances in plants in combination with the action of biocontrol agents that can manage pests without insecticides. Recently, population modelling has proved that it is possible to quantify the combined impacts of plant resistance and biological control on the dynamics of pests. Scientists are amazed at the profound phenotypic and genotypic variations a crop germplasm portrays and the degree of damage that the improved varieties reduce. The science of genetics has progressed from field selection of resistant varieties to the genes. Several unexplored avenues still exist to achieve sustainable pest management through genetic interventions. Genomics, gene pyramiding, gene editing, gene silencing and gene splicing are the buzzwords today. Genetics, biotechnology, biochemistry, molecular biology and other allied sciences will be the scientific fields to be increasingly researched in the future for achieving sustainable pest management. Recent trends have clearly established an imminent need to apply modern genetic methods against pests and plant pathogens. Hence, global co-operation and cutting-edge scientific developments in crop protection will benefit humankind in the future. In this pretext, the book 'Genetic Methods and Tools for Managing Crop Pests' is timely and hopefully helps those who wish to conduct research on insects as well as plant genetics. The book has five parts: the first part focuses on newer and emerging innovations in genetics that address issues in crop protection hitherto remain unresolved. This part will also help in understanding the pest and disease complicated situations. The second part deals with enhancing the efficiency and genetic fitness of beneficial organisms including useful insects as honeybees and silkworms. Can the science of genetics be useful in protecting crop plants equally in diverse habitats? The stability and suitability of tools can manifest depending on the climato-geographic and other factors, as detailed in the third part. The fourth part deals with non-insect pests such as mites and nematodes. Genetic tools designed against

insect pests may or may not be equally applicable against mite and nematode pests. The fifth part concerns about the mechanisms of genetics operating in insect pests via a myriad of pathways. This book addresses the need for a conceptual framework to realise the maximum potential of integrated pest management via genetic methods per se or in combination with other methods and reduce economic, ecological and environmental risks to farmers and human population at large.

World Vegetable Center
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Srinivasan Ramasamy

Preface

Resistant/tolerant plant varieties as a stand-alone tool provide inbuilt mechanism against pest populations sustaining modest crop yields. Resistant cultivars are compatible with most of the other pest population reduction tactics and may or may not require fewer inputs to realise economic crop yields. As a pest management tool, resistant plants also sustain populations of beneficials. In fact, it is an ideal means of pest suppression. A major breakthrough in the genetic method occurred when transgenics were developed, expressing the endotoxin of *Bacillus thuringiensis* for effective pest suppression. Bt cotton technology, as an example, has sustained cotton farming in India for the past 18 years. Sustainability of Bt cotton endured even under abiotic/biotic stress conditions. Planting upgraded Bt cotton (HTBt in Maharashtra, India) has been so successful that the use of HTBt can make Indian cotton more competitive globally, ensuring more returns to the farmers. Understanding insect genetics has paved the way for the eradication of the New World screw-worm fly *Cochliomyia hominivorax* Coquerel by sterile insect technique (SIT), from Mexico and the USA. SIT was successfully used against melon fly and Oriental fruit fly in Japan. DNA probes are being used to identify cryptic species and individual insects with genes for resistance to insecticides. Genetic engineering is utilised to break down the very narrow host races of certain nuclear polyhedrosis viruses (NPVs) and biocontrol agents. In broader context, in Israel, strains of *Escherichia coli* surviving on sugar cane have been genetically altered to consume carbon dioxide and could be harnessed to help produce regular supplies of food and fuel. Modern plant breeding techniques to enhance durability of resistance, integration of biorational plant volatiles, plant vaccination, botanicals and biocontrol agents together can result in sustainable holistic solutions.

The incidence of human vector and viral-borne diseases is far greater today than before. Worldwide effort to find an effective medicine is on. It is the science of genetics that comes to the fore. Genetics is a composite science. The emerging plant pest and disease situations are so complex that a multi-institutional and multifaced team but with tailored approach is needed. Secondly, benefits from genetic methods can be derived only when technologies embracing molecular biology, biotechnology, biochemistry and other disciplines progress.

Genomics-assisted breeding (GAB) of climate-smart inbred and hybrid rice varieties resilient to biotic and abiotic stress is the need of the hour. GAB integrating

advanced genomics tools and an improved green super rice (GSR) breeding strategy (GSR-Bt) is one of the reliable breeding strategies to develop climate-resilient varieties. This provides high-quality genome sequences and SNP's allelic variants from advanced genetic tools in Asia and Africa.

Global campaigns were thought to fizzle out the pandemic human diseases. But it did not. Certain human diseases indeed bounced back widespread. Economic analysis and environmental assessments, combined with impacts on non-target organisms with area-wide trials, will be a part of any new genetic tool. Ground was lost in Africa against tsetse flies and trypanosomiasis in livestock and man. Still, in Asia, Africa and the Middle East, the locusts, whiteflies and fruit flies remain a major menace. As advances in genetics continue bringing high-throughput techniques to the field and developing global genetic diversity maps, there is a concern about their potential to increase the rate of resistance development. Genetic tool development is time-consuming, requiring sophisticated instruments and analyses. However, the great increase in the ability to manipulate genes and transfer desired traits from one organism to another, in future, will see more number of genetically modified organisms. The prospects for managing pest situations through genetic means have tremendous potential. So, compilation and assessment on the utility of genetic tools become almost mandatory. That is why a book on genetic methods and tools was planned.

The book has five parts. The first part, Genetic Tools and Techniques, has four chapters concerning newer genetic methods and tools, vectors and markers for genetic engineering, newer molecular approaches, computer science, robotics and artificial intelligence. The second part deals with beneficial genetic tools containing three chapters involving honeybees, silkworms and natural enemies. Genetic interventions against pests in diverse geographical regions are discussed in Part III, with three chapters each for South Africa, Vietnam and Sri Lanka. Part IV is titled as Genetic Tools for the Management of Plant-Feeding Mites, Nematodes and Insect Vectors of Viral Diseases, each covering three chapters. Part V, i.e. Genetic Approaches and Mechanisms Against Insect Pests, comprises 15 chapters. This part addresses diverse aspects as genetic behaviour, gene expression, plasticity, pathways and interactions and options for mitigation of pests. For humankind to benefit, the science of genetics has to advance. It is in this context that the book on genetic methods against pests is worth.

Bengaluru, Karnataka, India A. K. Chakravarthy

Acknowledgement

The science of genetics has advanced so rapidly and divergently that each technique, concept or mechanism has grown off as a special branch. So, experts in each aspect had to be consulted while organising this work, and specialists were approached to contribute for the book. Dr. Srinivasan Ramasamy, World Vegetable Center, Taiwan, and Dr. B. Fakruddin, Department of Biotechnology and Crop Improvement, University of Horticultural Sciences Campus, G.K.V.K Post, Bangalore, India, were consulted while organising the book contents. I owe a debt of gratitude to them. Dr. Srinivasan Ramasamy is obliged to give an apt foreword for the book. I thank him immensely. Over 60 contributors from 14 countries contributed for the book on wide-ranging topics of contemporary significance. The contributions contain aspects of immense potential for their applications and use in the future. This should attract a large number of scientists to leap forward to the science of genetics to address global problems. I profoundly thank all the contributors to have given their valuable time for the book, even under the total lockdown conditions due to COVID-19 virus pandemic. I wish to especially thank Mr. Gopalkrishna H. R (IIHR, Bangalore, India), Yogendra M. Kalenahalli (University of Adelaide, Australia), Nitin K. S. (University of Cape Town, South Africa), B. Doddabasappa (UHS, Bangalore, India), Mahran Zeity (Damascus, Syria) and Dr. Prabhulinga T (CICR, Nagpur, India) for their inputs and help throughout the book preparation period. I sincerely thank the Springer Publishers, New Delhi, for their interest and enthusiasm evinced in publishing this book.

—A. K. Chakravarthy

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A. K. Chakravarthy Head and Principal Scientist (Rtd.), Division of Entomology and Nematology, is an author of many books and over 350 scientific papers and 70 chapters in entomology and natural history. His interest includes insects, birds, bats, rodents and mammals. With nearly four decades of experience in teaching, research and extension, Dr. Chakravarthy has been the investigator for over 35 research projects and has guided more than 28 students for post-graduation. With a Ph.D. from Punjab Agricultural University and being a fellow from IARI, New Delhi, he is a member of several national and international scientific academia, an adviser, a panellist, a referee, a reviewer and an editor, and he is associated with the publication of several national and international journals worldwide. A field-orientated, widely travelled biologist, he is actively working on novel approaches in integrated pest management, host plant interaction, vertebrate pest management, biodiversity and environmental conservation issues. Recently, he has published books titled *Innovative Pest Management Approaches for the 21st Century* (Foreword by Prof. Dr. Remzi ATLIHAN, Turkey), *New Horizons in Insect Science: Towards Sustainable Pest Management* (Foreword by Prof. Hiroshi Honda, Japan), *Economic and Ecological Significance of Arthropods in Diversified Ecosystems: Sustaining Regulatory Mechanisms* (Foreword by Fani Hatijina, Greece), *Arthropod Diversity and Conservation in the Tropics and Sub-tropics* (Foreword by Prof. Madhav Gadgil, India), *The Black Spotted, Yellow Borer,*

Conogethes punctiferalis Guenee and Allied Species (Foreword by Prof. Alma Solis, USA) and *Experimental Techniques in Host-Plant Resistance* (Foreword by Prof. Michael C. Smith, USA) by Springer.

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Part I

Genetic Tools and Techniques

1 Newer Genetic Tools, Techniques, Vectors, Promoters, and Molecular Markers for Genetic Engineering of Herbivorous Insects

D. Devika Rani, S. Subhash, H. R. Gopalkrishna, and A. K. Chakravarthy

Abstract

Insects can transmit major infectious diseases to crop plants. Recent advances in insect genomics and transformation technology provide new strategies for the control of insect-borne pathogen transmission and insect pest management. One such strategy is the genetic modification of insects with genes that block pathogen development. Another is to suppress insect populations by releasing either sterile males or males carrying female-specific dominant lethal genes into the environment. Newer genetic tools and methods are described.

Keywords

Vectors · Promoters · Markers · Genetic engineering

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1.1 Introduction

For a long time, genetic tools have been used to characterize populations. Many of these tools and methods were first used in the human context. Later, these tools proved useful for the conservation and management of wildlife. Currently, some of these tools and methods have proven useful for pest population suppression. This has been attempted against vector-borne mosquitoes initially, later on herbivorous insects. Besides advances in genetic methods, there has been an increase in computational power permitting the use of advanced statistical methods. Population genetic analyses suitable for pest management should be first chosen before the question of genetic markers arises. However, there are a number of issues to be considered before choosing appropriate genetic markers. Sunnucks (2000) has revived the literature and described efficient genetic markers for population biology studies. Currently novel mosquito control strategies based on genetic manipulation of mosquitoes are being developed as tools to suppress disease transmission.

Genetic pest management encompasses manipulation of an organism at the genetic level.

A transposable element (TE) is a DNA sequence that can change its position within a genome, sometime creating as reversing mutations and altering cell's genetic identity. Transposition often results in duplication of the same genetic material. They are also known as "jumping genes," DNA sequences that move from one location on the genome to another.

Since Barbara McClintock's discovery in 1945, two types of transposons have been identified: class II transposons or class 1 transposons as transposable elements and retrotransposons (clan I transposons).

1.1.1 The Meaning of Transposon

Transposon is a segment of DNA that is capable of independently replicating itself and inserting the copy into a new position within the same or another chromosome or plasmid. They are considered an important contributor pr gene and genome evolution. They represent the most abundant repeats in most pl genomes.

Oxytricha (a ciliate species) is known for highly fragmented genome.

Transposition can be classified as "autonomous" or non-autonomous in both clan I and clan II TEs. Autonomous TEs can move by themselves, whereas non-autonomous Tem requires the presence of another TEm to move.

Drosophila melanogaster is an ideal model organism for the study of eukaryotic Electron Transport System (ETS) as its genome contains a diverse array of active TEs. The regulation on DNA transposons in somatic cells is poorly understood through to some extent mechanism identified for P elements in *D. melanogaster*.

P elements are the best-studied DNA transposons in *D. melanogaster* genome. P elements play a significant role in hybrid dysgenesis syndrome, a phenomenon observed in the progeny of hybrid crosses of certain *Drosophila* strains. The

phenomenon observed in this syndrome includes high rates of mutation, recombination, and sterility in the F1 hybrids of only P-type male crosses with M-type females.

Drosophila strains are defined as P type or M type depending on weather hybrid dysgenesis results from crosses with the parental or maternal parent. Gene isolation is population of organisms that has little genetic mixing with other organisms within the same species. This may result in speciation, but this is not necessarily the case. Genes are isolated by the simplest way inserting a particular fragment into the purified DNA genome of a self-replicating genetic element, generally a virus or a plasmid. To isolate a specific gene, one often begins by constructing a DNA library, a comprehensive collection of cloned DNA fragments from a cell, tissue, or organism. There are generally three types of cloning adopted, gene cloning, reproductive cloning, and therapeutic cloning.

1.1.2 Gene Targeting Vector

This involves the construction of a piece of DNA, known as a gene targeting vector which is then introduced into the cell where it replaces or modifies the normal chromosomal gene through the process of homologous recombination. The meaning of isolation is the act of separating from other things. By isolating, cloning, and sequencing the DNA, emergence of new species occurs and is an important event in evolution.

1.1.3 Gene Cloning

To obtain information about the nucleotide sequence of the genomic DNA sequencing or restriction enzyme, cutting analysis can be used to study a gene or compare variations of a gene from different sources. There are four types of isolation: content isolation, droplet isolation, air-borne isolation, and solitary confinement.

Cloning is the process of producing individuals with identical or virtually identical DNA, either naturally or artificially. In nature many organisms produce clones. It is a common practice in molecular biology labs that is used by researchers to create copies of a particular gene for downstream applications such as sequencing, mutagenesis, gene typing, or expression of proteins. There are three steps in gene cloning:

(a) Isolation of a donor fragment or gene
(b) Selection of a suitable vector
(c) Incorporation of donor DNA fragment into the vector
    Transformation of recombinant vector into a suitable host cell. Gene cloning helps in the synthesis of proteins, hormones, and antibiotics. Different genes for treatment of diseases, and agricultural application of cloning in bacteria facilitates nitrogen fixation in plants. Clones allow farmers to upgrade the overall quality of the host animals in the herd.

A **transposable element** (**TE or transposon**) is a DNA sequence that can change its position within a genome, sometimes creating or reversing mutations and altering the cell's genetic identity and genome size. Transposition often results in duplication of the TE. Barbara McClintock's discovery of these **jumping genes** earned her a Nobel Prize in 1983.

Transposable elements make up a large fraction of the genome and are responsible for much of the mass of DNA in a eukaryotic cell. It has been shown that TEs are important in genome function and evolution. In *Oxytricha*, which has a unique genetic system, these elements play a critical role in development. Transposons are also useful to researchers as a means to alter DNA inside a living organism.

There are at least two classes of TEs: class I TEs or retrotransposons generally function via reverse transcription, while class II TEs or DNA transposons encode the protein transposase, which are required for insertion and excision, and some of these TEs also encode other proteins. Genes could not only move, but they could also be turned on or off due to certain environmental conditions or during different stages of cell development.

1.2 Class I (Retrotransposons)

Class I TEs are copied in two stages: first, they are transcribed from DNA to RNA, and the RNA produced is then reverse-transcribed to DNA. This copied DNA is then inserted back into the genome at a new position. The reverse transcription step is catalyzed by a reverse transcriptase, often encoded by the TE itself. The characteristics of retrotransposons are similar to retroviruses, such as HIV.

Retrotransposons are commonly grouped into three main orders:

- TEs with long terminal repeats (LTRs), encoding reverse transcriptase, similar to retroviruses.
- Long interspersed nuclear elements (LINEs, LINE-1s, or L1s), encoding reverse transcriptase but lack LTRs, and are transcribed by RNA polymerase II.
- Short interspersed nuclear elements do not encode reverse transcriptase and are transcribed by RNA polymerase III.

Retroviruses can also be considered TEs. For example, after conversion of retroviral RNA into DNA inside a host cell, the newly produced retroviral DNA is integrated into the genome of the host cell. These integrated DNAs are termed *proviruses*. The provirus is a specialized form of eukaryotic retrotransposon, producing RNA intermediates that may leave the host cell and infect other cells. The transposition cycle of retroviruses has similarities to that of prokaryotic TEs, suggesting a distant relationship between the two.

1.3 Class II (DNA Transposons)

The cut-and-paste transposition mechanism of class II TEs does not involve an RNA intermediate. The transpositions are catalyzed by several transposase enzymes. Some transposases non-specifically bind to any target site in DNA, whereas others bind to specific target sequences. The transposase makes a staggered cut at the target site producing sticky ends, cuts out the DNA transposon, and ligates it into the target site. A DNA polymerase fills in the resulting gaps from the sticky ends, and DNA ligase closes the sugar-phosphate backbone. This results in target site duplication, and the insertion sites of DNA transposons may be identified by short direct repeats (a staggered cut in the target DNA filled by DNA polymerase) followed by inverted repeats.

Cut-and-paste TEs may be duplicated if their transposition takes place during S phase of the cell cycle, when a donor site has already been replicated, but a target site has not yet been replicated. Such duplications at the target site can result in gene duplication, which plays an important role in genomic evolution.

Not all DNA transposons transpose through the cut-and-paste mechanism. In some cases, a replicative transposition is observed in which a transposon replicates itself to a new target site (e.g., helitron).

Class II TEs comprise less than 2% of the human genome, making the rest class I.

1.4 Autonomous and Non-Autonomous Transposition

Transposition can be classified as either "autonomous" or "non-autonomous" in both class I and class II TEs. Autonomous TEs can move by themselves, whereas non-autonomous TEs require the presence of another TE to move. This is often because dependent TEs lack transposase (for class II) or reverse transcriptase (for class I).

Activator element (*Ac*) is an example of an autonomous TE, and dissociation elements (*Ds*) is an example of a non-autonomous TE. Without *Ac*, *Ds* is not able to transpose.

1.5 Transposable Elements for Nondrosophilid Insects

The following are four different class II transposable elements used to transform species other than *Drosophila*:

- *Tc1* elements
- *mariner* elements
- *hAT* elements
- *piggyBac* elements

1.6 P Elements

These are transposable elements that were discovered in *Drosophila* as the causative agents of genetic traits called hybrid dysgenesis. The transposon is responsible for P trait of P element, and it is found only in wild flies.

All P elements have a canonical structure containing 31 bp terminal inverted repeats and 11 bp internal inverted repeats located at THAP domain of the transposase. The shorter and longest P elements are non-autonomous elements. The longest *P* elements encode transposase needed for transposition.

In hybrid dysgenesis, one strain of *Drosophila* mates with another to produce hybrid offspring causing chromosomal damage known as dysgenics. Hybrid dysgenesis requires a contribution from both parents, for example, in the *P-M system*, P strain contributing paternal *and* M strain contributing maternal. The reverse cross, with M father and P mother, produces normal offspring, as it crosses P $\times$ P or M $\times$ M manner. P male chromosome can cause dysgenesis when crossed with an M female.

P element also encodes a suppressor of transposition, which accumulates in the cytoplasm during the development of cells. Thus, in a cross of a P or M male with a P female, the female cytoplasm contains the suppressor, which binds to any P elements and prevents their transposition.

P elements are commonly used as mutagenic agents in genetic experiments with *Drosophila*. One advantage of this approach is that the mutations are easy to locate. The P element encodes for the protein P transposase and is flanked by terminal inverted repeats important for mobility. Unlike laboratory strain females, wild-type females are thought to express an inhibitor to P transposase function. This inhibitor reduces the disruption to the genome caused by the P elements, allowing fertile progeny. Evidence for this comes from crosses of laboratory females (which lack a P transposase inhibitor) with wild-type males (which have P elements). In the absence of the inhibitor, the P elements can proliferate throughout the genome, disrupting many genes and killing the progeny:

- The first step involves choosing and isolating the gene that will be inserted into/removed from the genetically modified organism.
- The gene must generally be combined with a promoter and terminator region as well as a selectable marker gene.
- Then the genes must be spliced into the target's DNA. For animals, the gene must be inserted into embryonic stem cells.
- The resulting organism must have the presence of the target gene confirmed.

First-generation offspring are heterozygous, requiring them to be inbred to create the homozygous pattern necessary for stable inheritance. Homozygosity must be confirmed in the second generation, which then becomes the final product.

1.7 History

Human-directed genetic manipulation began with the domestication of plants and animals through artificial selection in about 12,000 BC. Several techniques were developed to aid in breeding and selection. Hybridization was able to make rapid changes in an organism genome. Some plants were able to propagate by vegetative cloning. X-rays were first used to deliberately mutate plants in 1927. Between 1927 and 2017, more than 3248 genetically mutated plant varieties have been produced using X-rays.

It wasn't until the mid-1800s that DNA and genes were discovered, which would form the basis of modern genetic manipulation. Genetic inheritance was first discovered by Gregor Mendel in 1865 following experiments crossing peas. In 1928 Frederick Griffith proved the existence of a "transforming principle" involved in inheritance, identified as DNA in 1944 by Oswald Avery, Colin MacLeod, and Maclyn McCarty. Select methods of transposition are shown in Fig. 1.1. Frederick Sanger developed a method for sequencing DNA in 1977, greatly increasing the genetic information available to researchers.

Also discovering how DNA works, tools had been developed that allowed it to be manipulated. In 1970, Hamilton Smith's lab discovered restriction enzymes, enabling scientists to isolate genes from an organism's genome. DNA ligases, which join broken DNA together, had been discovered earlier in 1967, and by

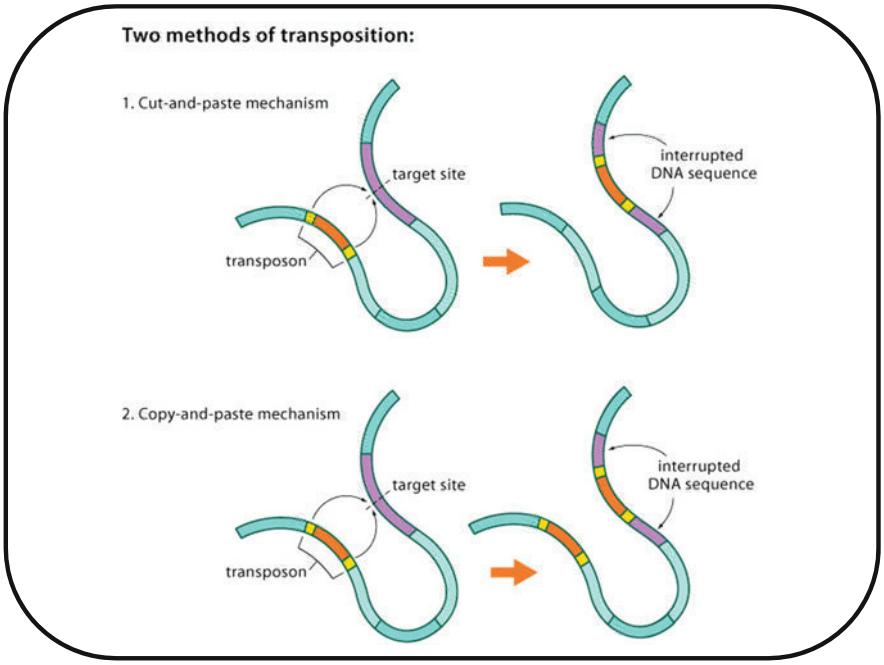

Fig. 1.1 Two commonly used methods of transposition

combining the two enzymes, it was possible to "cut and paste" DNA sequences to create recombinant DNA. Plasmids, discovered in 1952, became important tools for transferring information between cells and replicating DNA sequences. Polymerase chain reaction (PCR), developed by Kary Mullis in 1983, allowed small sections of DNA to be amplified and aided identification and isolation of genetic material.

Techniques had to be developed for gene insertion (known as transformation) into an organism's genome. Griffith's experiment had already shown that some bacteria had the ability to naturally uptake and express foreign DNA. Artificial competence was induced in *Escherichia coli* in 1970 by treating with calcium chloride solution ($CaCl_2$). Transformation using electro-oration was developed in the late 1980s, increasing the efficiency and bacterial range. In 1907, a bacterium causing plant tumors, *Agrobacterium tumefaciens*, had been discovered, and in the early 1970s, it was found that the bacteria inserted DNA into the plants using a Ti plasmid. By removing the genes in the plasmid and adding in novel genes, researchers were able to infect plants with *A. tumefaciens* and let the bacteria insert chosen DNA into the plant genomes.

An important part of genetic engineering is to identify useful genes to transform into the genetically modified organism. The bacteria *Bacillus thuringiensis* was first discovered in 1901 as the causative agent in the death of silkworms. Due to these insecticidal properties, the bacterium was used as a biological insecticide, commercially developed in 1938. The cry proteins were discovered to provide the insecticidal activity in 1956, and by the 1980s, scientists had successfully cloned the gene coding for this protein and expressed it in plants. The gene that provides resistance to the glyphosate herbicide was found. Target genes can be cloned from a DNA segment after the creation of a DNA library. The libraries generally cover the organism's genome multiple times, and its size will depend on how large the genome is.

1.8 Gene Isolation

The DNA is first digested with a random digestion method, commonly by cutting the DNA with restriction enzymes (enzymes that cut DNA). A partial restriction digest cuts only some of the restriction sites, resulting in overlapping DNA fragment lengths. The DNA fragments are put into individual plasmid vectors and grown inside bacteria. Once in the bacteria, the plasmid is copied as the bacteria divides. To determine if a useful gene is present on a particular fragment, the bacterial library is screened for the desired phenotype. If the phenotype is detected, it is possible that the bacteria contain the target gene. If the gene does not have a detectable phenotype or a DNA library, it does not contain the correct gene; other methods can be used to isolate it. If the position of the gene can be determined using molecular markers, then chromosome walking is one way to isolate the correct DNA fragment. If the gene expresses close homology to a known gene in another species, then it could be isolated by searching for genes in the library that closely match the known gene.

If the DNA sequence of the gene and the organism is known, restriction enzymes can cut the DNA on either side of the gene, and gel electrophoresis can sort the fragments according to length. The DNA band at the correct size should contain the gene, where it can be excised from the gel. Polymerase chain reaction (PCR) can be used to amplify the gene, which can then be isolated through gel electrophoresis. It is also possible to synthesize the gene.

The gene inserted into the genetically modified organism must be combined with other genetic elements to work properly. The gene can also be modified at this stage for better expression or effectiveness. The gene to be inserted the most constructs contains a promoter and terminator region as well as a selectable marker gene. The promoter region initiates transcription of the gene and can be used to control the location and level of gene expression, while the terminator region ends transcription. The selectable marker, which in most cases confers antibiotic resistance to the organism it is expressed in, is needed to determine which cells are transformed with the new gene. The constructs are made using recombinant DNA techniques, such as restriction digests, ligations, and molecular cloning.

1.9 Gene Targeting

Gene targeting uses homologous recombination to target the desired changes to a specific endogenous gene. This tends to occur at a relatively low frequency in plants and animals and generally requires the use of selectable markers. The success of gene targeting can be enhanced with the use of engineered nucleases such as zinc finger nucleases, engineered homing endonucleases, transcription activator-like effector nuclease, or CRISPR. Engineered nucleases can also introduce mutations at endogenous genes that generate a gene knockout.

1.10 Transformation

1.10.1 *Agrobacterium Tumefaciens* Is Attached to a Carrot Cell

About 1% of bacteria are naturally able to take up foreign DNA, but this ability can be induced in other bacteria. Stressing the bacteria with a heat shock or an electric shock can make the cell membrane permeable to DNA that may then incorporate into the genome or exist as extra chromosomal DNA. DNA is generally inserted into animal cells using microinjection, where it can be injected through the cell's nuclear envelope directly into the nucleus or through the use of viral vectors. In plants, the DNA is generally inserted using *Agrobacterium*-mediated recombination or biolistics.

In *Agrobacterium*-mediated recombination, the plasmid construct must also contain Transfer DNA *Agrobacterium* naturally inserts DNA from a tumor-inducing plasmid into any susceptible plant that it infects, causing crown gall disease. The T-DNA region of this plasmid is responsible for insertion of the DNA. The DNA

inserted is cloned into a binary vector containing T-DNA and can be grown in both *E. coli* and *Agrobacterium*. Once the binary vector is constructed, the plasmid is transformed into *Agrobacterium* containing no plasmids, and plant cells are infected. *Agrobacterium* naturally inserts the genetic material into the plant cells.

In biolistic transformation, particles of gold or tungsten are coated with DNA and then shot into young plant cells or plant embryos. Some genetic material enters the cells and transforms them. This method can be used on plants that are not susceptible to *Agrobacterium* infection and also allows transformation of plant plastids.

Another transformation method for plant and animal cells is electroporation, which involves subjecting cells to an electric shock, which can make the cell membrane permeable to plasmid DNA. In some cases the electroporated cells will incorporate the DNA. Due to the associated cell and DNA damage, the transformation efficiency of biolistics and electroporation is lower than with agrobacteria and microinjection.

1.11 Selection

Not all the organism cells are transformed with the new genetic material; typically a selectable marker is used to differentiate transformed from untransformed cells. Cells that have been successfully transformed with the DNA will also contain the marker gene. By growing the cells in the presence of an antibiotic or chemical that selects or marks the cells expressing that gene, it is possible to separate modified from unmodified cells. Another screening method involves a DNA probe that sticks only to the inserted gene. Multiple strategies can remove the marker from the matured plant.

1.12 Regeneration

As often only a cell is transformed with a genetic material, the modified organism must be grown from that single cell. Bacteria consist of a cell and reproduce clonally, so regeneration is not necessary for them. In plants, this is accomplished through the use of tissue culture. Each plant species has different requirements for successful regeneration. If successful, the technique produces an adult plant that contains the transgene in every cell.

In animals it is necessary to ensure that the inserted DNA is present in embryonic stem cells. Offspring can be screened for the gene. All offspring from the first generation will be heterozygous for the inserted gene and must be inbred to produce a homozygous specimen.