



RECOMBINANT DNA TECHNOLOGY



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By

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PREFACE

The progressing scientific revolution is resulting in changes in the established rules of human-biotic world interactions. Researchers are now able to identify and characterize genes for almost all biological functions, modify and incorporate them into living cells, exchange genetic materials between species and produce clones (genetic blueprints) of all living beings through the art of Recombinant DNA Technology. Genetic engineering has emerged as the ultimate solution to most everyday life problems, from food safety/security to improving health. A plethora of life's daily issues are now dependent on genetic engineering, either through the development of genetically modified organisms or of new drugs/vaccines to combat diseases. All this is possible due to emerging knowledge in genetic engineering.

This book details different Recombinant DNA Technology techniques and their applications. It is intended to address the approaches of current genetic engineering and their wizardly applications in laboratories, as well as in the field. The book is aimed at professional biologists, university students and scientists in general. This book will have an evocative impact on the minds of its readers and will provide comprehensive knowledge on the further potential of Recombinant DNA Technology. In this book, we have tried to leave no stone unturned to present the complex field of genetic engineering in a fluid way, by using simple and reader-friendly language without compromising its scientific value.

The book is comprised of seven chapters. The first chapter is on Gene Cloning, while the second is about the Construction of Gene Libraries. The third chapter concerns principles and methods for the genetic Transformation of Plants, and light has been shed on Marker Genes and their excision in the fourth chapter. Techniques in Molecular Biology and Approaches in Gene Editing are discussed in the fifth and sixth chapters respectively. The last chapter is about the Applications of Recombinant DNA Technology. We are hopeful that by the end, this effort will lead to the creation of some opportunities to add a little to the already existing ocean of genetic engineering.

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ABBREVIATIONS

A	Adenine
AdoMet	S-adenosyl methionine
Amp	Ampicillin
ATP	Adenosine Tri Phosphate
attB	Bacterial attachment Site
attP	Phage attachment Site
BAC	Bacterial Artificial Chromosome
bar	A gene, confers resistance to bialaphos herbicide
BCP-T	5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt
BT	Bacillus thuringiensis
C	Cytosine
C2H2	Cys2His2
CaMV35S	Cauliflower mosaic virus 35S
Cas 9	CRISR-associated protein 9
cDNA	Complementary DNA
Cre	Cyclic recombinase
CRISR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPRRNA
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
DSBs	Double stranded Breaks
EDTA	Ethylenediamine tetra acetic acid
FLP	Flippase
G	Guanine
GDS	Gel Documentation System
GFP	Green Fluorescent Protein
GM●	Genetically Modified ●organisms
HART	Hybrid Arrest Translation
HCl	Hydrochloric acid
HDR	Homology Directed Repair
He	Helium

HR	Homologous Recombination
HRP	Horseradish peroxidase
HRT	Hybrid Release Translation
Int	Integrase
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IR	Illegitimate Recombination
ISSR	Inter Simple Sequence Repeat
KJ	kilojoule
LoxP	Locus of X-over in P1 bacteriophage
LSC	Large single copy
M	Molar
MCSs	Multiple Cloning Sites
MgCl ₂	Magnesium Chloride
mRNA	Messenger RNA
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NBT	Nitro Blue Tetrazolium
NHEJ	Non Homologous End Joining
NLS	Nuclear localization sequence
NPC	Nuclear pore complex
ORF	Open Reading Frame
ori	Origin of Replication
PAGE	Polyacrylamide Gel Electrophoresis
PAGE	Polyacrylamide Gel Electrophoresis
PAM	Protospacer adjacent motif
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PI domain	PAM interacting domain
qPCR	Quantitative PCR
R gene	Resistance gene
RAMP	Repair associated mysterious protein
RAPD	Random Amplified Polymorphic DNA
RBS	Recombinase Binding site
Ri plasmid	Root inducing plasmid
RNA	Ribonucleic acid
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl Sulphate
SSC	Small single copy
SSC	Sodium Chloride-Sodium Citrate
SSCP	Single Strand Conformational Polymorphism

SSR	Simple Sequence Repeat
ssTDNA	single stranded transfer DNA
T	Thymine
TAE	Tris acetate EDTA
TAL	Transcription activator-like
TALEN	Transcription activator-like effector nucleases
Taq polymerase	DNA polymerase from <i>Thermus aquaticus</i>
T-DNA	Transfer DNA
Ti plasmid	Tumor inducing plasmid
tracrRNA	transactivating CRISPR RNA
UV	Ultraviolet
Vir gene	Virulence gene
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YAC	Yeast Artificial Chromosome
ZFNs	Zinc Finger Nucleases

CHAPTER ONE

GENE CLONING: AN OVERVIEW

In gene cloning, a set of experiments to assemble DNA fragments is used to generate a recombinant DNA molecule. Gene cloning is the core aspect of Recombinant DNA Technology. In principle, it involves the joining of DNA fragments from different sources. In this technique, the DNA fragment is ligated with a vector to develop a recombinant DNA molecule. Then this recombinant DNA molecule is delivered into the host cell for multiplication, so as to produce multiple identical copies of this molecule.

Methods for the cloning of a particular segment of DNA into a vector are categorized as either (1) ligase dependent or (2) ligase independent methods. However, ligase dependent methods are generally practiced for cloning. Ligation dependent cloning methods are further subdivided into two groups, (1) cohesive ended ligation and (2) blunt ended ligation. These approaches require restriction enzymes to generate cohesive or sticky ends, and multiple enzymatic modifications for blunt ended ligation. PCR development has made this task quite simple and fast.

PCR amplified fragments are cloned through different methods, such as through engineering restriction enzymes sites at the 5' end of primers, ligase independent cloning and TA cloning. Among all these, TA cloning is the more efficient cloning strategy. The TA vector has a 3'-T overhang, while *Taq* polymerase has the ability to generate a 3'-A overhang in amplified PCR products, by introducing a single 3'-A into them. These overhangs are complementary to each other, thus facilitating the direct cloning of a PCR product into the vector. Therefore, in this chapter, an overview of the general cloning strategies is briefly presented.

BASIC FEATURES OF VECTORS

A cloning vector must have the ability to replicate independently in the host cell. Vectors to be used in cloning experiments must have the following features:

- They must be a circular DNA molecule.
- They must have their origin of replication (*ori*) for their autonomous replication within a host.
- They must contain selectable marker gene sequences.
- They must have multiple cloning sites (MCSs) region.

VECTORS FOR CLONING

Different vectors are used in molecular biology for cloning purposes, e.g. plasmid, λ phage, cosmids, BAC (bacterial artificial chromosome) and YAC (yeast artificial chromosome). They accept different sizes of DNA fragments/inserts to carry them as recombinant. A list of cloning vectors and insert sizes which they may carry is given in the table below (Table 1.1).

Table 1.1: Cloning vectors

Vector	Insert size
Plasmid	~ 10 Kb
λ phage	~ 23 Kb
Cosmid	~ 45 Kb
BAC	~ 350 Kb
YAC	~ 1000 Kb

BASIC STEPS OF GENE CLONING

DNA manipulative and DNA modifying enzymes with diverse properties, as well as a series of experimentations involved in gene cloning, are described in detail below. The general steps involved in gene cloning are also illustrated pictorially in Fig. 1-1.

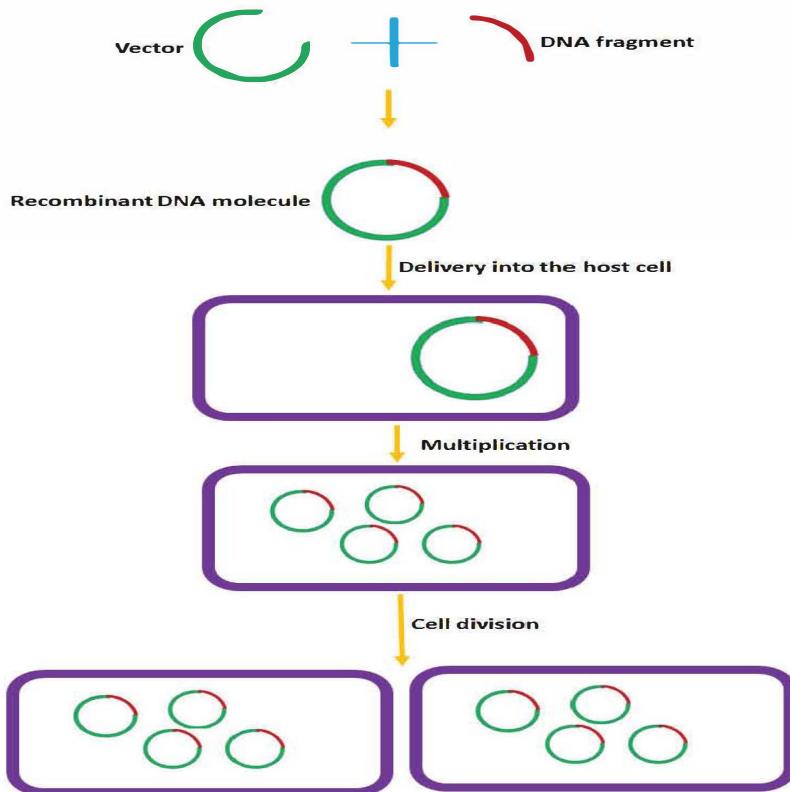


Figure 1.1: A schematic diagram of gene cloning, representing generation of recombinant DNA molecule and its multiplication (idea taken from Brown 2006).

Gene isolation:

In the first step, a gene, or any DNA fragment to be used in cloning is isolated using different methods, such as a PCR based on sequence-specific primers, to amplify DNA fragments from extracted genomic DNA, RNA, organellar DNA, gene libraries etc. This step in gene cloning is mediated by polymerases.

Restriction digestion

Restriction enzymes (molecular scissors) are involved in the restriction digestion step of gene cloning. These restriction enzymes are categorized into two broad classes based on their mechanism of action; (1) Exonucleases (2) Endonucleases. A diverse range of restriction enzymes is available. The vector and DNA fragment must both be cut with the same kind of restriction enzymes for generating compatible ends in order to facilitate their proper joining.

Multiple cloning sites (MCSs) or polylinkers in a vector are the restriction sites that are specific to restriction enzymes (restriction endonucleases). The sites at which these molecular scissors make the cut are known as recognition sites or restriction sites. These sites may be made up of 4, 5, 6 or 8 base pair-long symmetrical inverted repeats (Palindromic) as well as of asymmetrical sequences; hence, based on the sequence length of recognition sites, endonucleases are referred to as either four cutters, five cutters, six cutters or eight cutters. Among them, four cutters and six cutters are the most commonly used molecular scissors in recombinant DNA technology. The restriction enzymes are cut in different ways and thereby may generate sticky ends or staggered ends (5' protruding ends or 5' phosphate overhangs, and 3' protruding ends or 3' hydroxyl overhangs) as well as blunt ends or flush ends (Fig. 1-2). The ligation efficiency of compatible sticky ends is more than that of blunt ended DNA molecules. Thus, to turn blunt ended DNA into a sticky ended molecule, linkers and adapters are used. Adapters are the short stretches of synthetic oligonucleotides with sticky ends, whereas linkers are blunt ended synthetic oligonucleotides but with restriction enzymes sites, which generate sticky ends.

Classification of restriction endonucleases

Restriction endonucleases are generally classified into four broad categories based on sequence specificity, cleavage position, the composition of their subunits and the requirements of their co-factors etc. These are classified as:-

- Type I restriction endonucleases
- Type II restriction endonucleases
- Type III restriction endonucleases
- Type IV restriction endonucleases

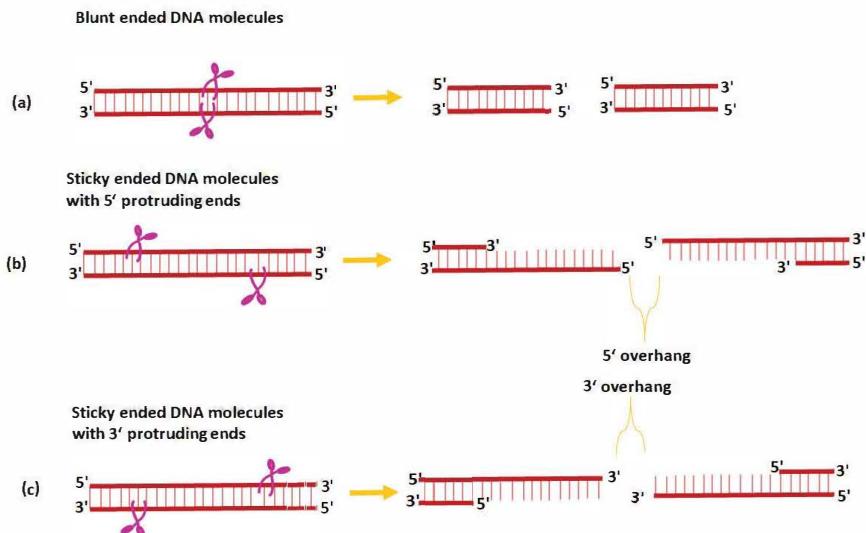


Figure 1.2: Cleavage action of restriction endonucleases (a) blunt ended DNA molecule (b) 5' protruding sticky ended DNA molecule (c) 3' protruding sticky ended DNA molecule (idea taken from Russell 2009).

Type I Restriction Endonucleases

Type I restriction enzymes are made up of multi-subunits and are complex in nature. They have independent subunits for recognition, cleavage of DNA molecules at specific sites and their modifications. The location of these endonucleases' recognition sites is far from their cleavage sites, which make these restriction enzymes no longer suitable for biotechnological applications. These restriction enzymes require AdoMet (S-adenosyl methionine), an Mg^+ ion and ATP (Adenosine triphosphate) for their activity.

Type II Restriction Endonucleases

Type II restriction enzymes' recognition sites are located within or close to their restriction sites. This feature gives them specificity and defined positions for cutting. They recognize symmetrical palindromic sequences. This class of restriction endonucleases is of practical value for use in gene cloning and other molecular analyses in laboratories. However, they don't have any AdoMet and ATP requirements for their activity. A list of restriction enzymes which are being used in recombinant DNA technology is given in the Table 1.2.

Type III Restriction Endonucleases

These restriction enzymes make a cleavage at DNA molecules outside their recognition sites. They recognize asymmetrical sequences of inverse orientation like the Type I class of restriction enzymes. This group of enzymes also requires AdoMet and ATP for their functioning.

Type IV Restriction Endonucleases

These restriction endonucleases act on modified methylated DNA molecules as recognition sites.

DNA modification

DNA molecules are modified by the addition or removal of their chemical groups according to the cloning strategy's needs. Various types of DNA modifying enzymes, such as alkaline phosphatase (the mediating removal of the phosphate group at the 5' terminal of a DNA molecule), polynucleotide kinase (the mediating addition of the phosphate group at the 5' terminal of a DNA molecule) and terminal deoxynucleotidyl transferase (the mediating addition of more than one nucleotide at the 3' terminal of a DNA molecule) are used in gene cloning experimentation.

Ligation reaction

Restriction digestion as well as modification (in some cases) of a DNA molecule and vector is followed by a ligation reaction. In ligation reactions, the ligase enzyme (molecular scissors) is used to mediate the joining of the DNA fragment and vector molecule together into a recombinant DNA molecule. A ligation mixture contains,

- 1) Unligated molecules
 - a) Unligated vectors
 - b) Unligated DNA fragments
- 2) Self-ligated vector molecules
- 3) A recombinant DNA molecule

Delivery to host and clone selection

The ligation mixture is delivered into the host cell for the selection of a desired clone (a recombinant DNA molecule), and then to produce multiple copies of this clone. However, the selection of the clone is made using a selective agent (in the culture medium required for the growth of a host cell) against a selectable marker gene present in the vector backbone.

Restriction Enzymes	Ends generated								
AloI	Sticky ends	AccB7I	Sticky ends	BfrI	Sticky ends	BseJI	Blunt ends	BspLU11I	Sticky ends
AluI	Blunt ends	AccBSI	Blunt ends	BfrBI	Blunt ends	BseLI	Sticky ends	BspMI	Sticky ends
AlwI	Sticky ends	AciI	Sticky ends	BfuI	Sticky ends	BseMI	Sticky ends	BspPI	Sticky ends
Alw21I	Sticky ends	AclI	Sticky ends	BfuAI	Sticky ends	BseMII	Sticky ends	BspTI	Sticky ends
Alw26I	Sticky ends	AclWI	Sticky ends	BaeI	Sticky ends	BseNI	Sticky ends	BspT104I	Sticky ends
Alw44I	Sticky ends	AcsI	Sticky ends	BfuCI	Sticky ends	BsePI	Sticky ends	BspT107I	Sticky ends
AlwNI	Sticky ends	AcuI	Sticky ends	BglI	Sticky ends	BseRI	Sticky ends	BspXI	Sticky ends
Ama87I	Sticky ends	AcyI	Sticky ends	BglII	Sticky ends	BseSI	Sticky ends	Eco57I	Sticky ends
Aor13HI	Sticky ends	AdeI	Sticky ends	BisI	Sticky ends	BseXI	Sticky ends	BspLU11I	Sticky ends
Aor51HI	Blunt ends	AfaI	Blunt ends	BlnI	Sticky ends	BseX3I	Sticky ends	BsrI	Sticky ends
ApaI	Sticky ends	AfeI	Blunt ends	BlpI	Sticky ends	BseYI	Sticky ends	BsrBI	Blunt ends
ApaLI	Sticky ends	Afl II	Sticky ends	Bme18I	Sticky ends	BsgI	Sticky ends	BsrDI	Sticky ends
ApeKI	Sticky ends	Afl III	Sticky ends	Bme1390I	Sticky ends	Bsh1236I	Blunt ends	BsrFI	Sticky ends
ApoI	Sticky ends	AgeI	Sticky ends	Bme1580I	Sticky ends	Bsh1285I	Sticky ends	BsrGI	Sticky ends
AscI	Sticky ends	AhdI	Sticky ends	BmgBI	BLUNT	BshNI	Sticky ends	BsrSI	Sticky ends
AseI	Sticky ends	AhlI	Sticky ends	BmrI	Sticky ends	BshTI	Sticky ends	BssECI	Sticky ends
AsiGI	Sticky ends	AjiI	Blunt ends	BmtI	Sticky ends	BsiEI	Sticky ends	BssHII	Sticky ends
AsiSI	Sticky ends	AjnI	Sticky ends	BmyI	Sticky ends	BsiHKAI	Sticky ends	BssKI	Sticky ends
AspI	Sticky ends	AjuI	Sticky ends	BoxI	Blunt ends	BsiWI	Sticky ends	BssNAI	Blunt ends

Asp700I	Blunt ends	AleI	Blunt ends	BpiI	Sticky ends	BsiYI	Sticky ends	BssSI	Sticky ends
Asp718I	Sticky ends	AlfI	Sticky ends	BplI	Sticky ends	BslI	Sticky ends	BssTII	Sticky ends
AspA2I	Sticky ends	BanII	Sticky ends	BpmI	Sticky ends	BslFI	Sticky ends	Bst6I	Sticky ends
AspEI	Sticky ends	BanIII	Sticky ends	Bpu10I	Sticky ends	BsmI	Sticky ends	Bst98I	Sticky ends
AspHI	Sticky ends	BauI	Sticky ends	Bpu14I	Sticky ends	BsmAI	Sticky ends	Bst1107I	Blunt ends
AspLEI	Sticky ends	BbeI	Sticky ends	Bpu1102I	Sticky ends	BsmBI	Sticky ends	BstACI	Sticky ends
AspS9I	Sticky ends	BbrPI	Blunt ends	BpuAI	Sticky ends	BsmFI	Sticky ends	BstAPI	Sticky ends
AsuC2I	Sticky ends	BbsI	Sticky ends	BpuEI	Sticky ends	Bso31I	Sticky ends	BstAUI	Sticky ends
AsuHPI	Sticky ends	BbuI	Sticky ends	BsaI	Sticky ends	BsoBI	Sticky ends	BstBI	Sticky ends
AsuNHI	Sticky ends	BbvI	Sticky ends	Bsa29I	Sticky ends	Bsp13I	Sticky ends	Bst2BI	Sticky ends
AvaI	Sticky ends	Bbv12I	Sticky ends	BsaAI	Blunt ends	Bsp19I	Sticky ends	BstBAI	Blunt ends
AvaII	Sticky ends	BbvCI	Sticky ends	BsaBI	Blunt ends	Bsp68I	Blunt ends	Bst4CI	Sticky ends
AviII	Blunt ends	BccI	Sticky ends	BsaHI	Sticky ends	Bsp106I	Sticky ends	BstC8I	Blunt ends
AvrII	Sticky ends	BceAI	Sticky ends	BsaJI	Sticky ends	Bsp119I	Sticky ends	BstDEI	Sticky ends
AxyI	Sticky ends	BcgI	Sticky ends	BsaMI	Sticky ends	Bsp120I	Sticky ends	BstDSI	Sticky ends
AarI	Sticky ends	BciVI	Sticky ends	BsaWI	Sticky ends	Bsp143I	Sticky ends	BstEII	Sticky ends
AasI	Sticky ends	BclI	Sticky ends	BsaXI	Sticky ends	Bsp143II	Sticky ends	BstENI	Sticky ends
AatI	Blunt ends	BcnI	Sticky ends	Bsc4I	Sticky ends	Bsp1286I	Sticky ends	BstF5I	Sticky ends
AatII	Sticky ends	BcuI	Sticky ends	Bse1I	Sticky ends	Bsp1407I	Sticky ends	BstFNI	Blunt ends
AccI	Sticky ends	BdaI	Sticky ends	Bse8I	Blunt ends	Bsp1720I	Sticky ends	BstH2I	Sticky ends

AccII	Blunt ends	BfaI	Sticky ends	Bse21I	Sticky ends	BspCI	Sticky ends	BstHHI	Sticky ends
AccIII	Sticky ends	BfiI	Sticky ends	Bse118I	Sticky ends	BspCNI	Sticky ends	BstKTI	Sticky ends
Acc16I	Blunt ends	BfmI	Sticky ends	BseAI	Sticky ends	BspDI	Sticky ends	BstMAI	Sticky ends
Acc36I	Sticky ends	BalI	Blunt ends	BseDI	Sticky ends	BspEI	Sticky ends	BstMBI	Sticky ends
BaeGI	Sticky ends	BtsCI	Sticky ends	BcoDI	Sticky ends	BspQI	Sticky ends	CviQI	Sticky ends
Acc65I	Sticky ends	BamHI	Sticky ends	Bse3DI	Sticky ends	BspHI	Sticky ends	BstMCI	Sticky ends
AccB1I	Sticky ends	BanI	Sticky ends	BseGI	Sticky ends	BspLI	Blunt ends	BstMWI	Sticky ends
BstNI	Sticky ends	DseDI	Sticky ends	FspBI	Sticky ends	MfeI	Sticky ends	PauI	Sticky ends
BstNSI	Sticky ends	EaeI	Sticky ends	Fsp4HI	Sticky ends	Mfl I	Sticky ends	PceI	Blunt ends
BstOI	Sticky ends	EagI	Sticky ends	GsuI	Sticky ends	MhII	Sticky ends	PciI	Sticky ends
BstPI	Sticky ends	Eam1104I	Sticky ends	HaeII	Sticky ends	MlsI	Blunt ends	PctI	Sticky ends
BstPAI	Blunt ends	Eam1105I	Sticky ends	HaeIII	Blunt ends	MluI	Sticky ends	PdiI	Blunt ends
BstSCI	Sticky ends	EarI	Sticky ends	HapII	Sticky ends	MluNI	Blunt ends	PdmI	Blunt ends
BstSFI	Sticky ends	EciI	Sticky ends	HgaI	Sticky ends	MlyI	Blunt ends	PfeI	Sticky ends
BstSNI	Blunt ends	Ecl136II	Blunt ends	HhaI	Sticky ends	Mly113I	Sticky ends	Pfl23II	Sticky ends
BstUI	Blunt ends	EclHKI	Sticky ends	Hin1I	Sticky ends	MmeI	Sticky ends	Pfl FI	Sticky ends
Bst2UI	Sticky ends	EclXI	Sticky ends	Hin1II	Sticky ends	MnlI	Sticky ends	Pfl MI	Sticky ends
BstV1I	Sticky ends	Eco24I	Sticky ends	Hin4I	Sticky ends	Mph1103I	Sticky ends	PfoI	Sticky ends
BstV2I	Sticky ends	Eco31I	Sticky ends	Hin6I	Sticky ends	MroI	Sticky ends	PhoI	Blunt ends
BstXI	Sticky ends	Eco32I	Blunt ends	HinP1I	Sticky ends	MroNI	Sticky ends	PinAI	Sticky ends
BstX2I	Sticky ends	Eco47I	Sticky ends	HincII	Blunt ends	MroXI	Blunt ends	PleI	Sticky ends
BstYI	Sticky ends	Eco47III	Blunt ends	HindII	Blunt ends	MscI	Blunt ends	Ple19I	Sticky ends
BstZI	Sticky ends	Eco52I	Sticky ends	HindIII	Sticky ends	MseI	Sticky ends	PmaCI	Blunt ends

BstZ17I	Blunt ends	Eco72I	Blunt ends	HinfI	Sticky ends	MslI	Blunt ends	PmeI	Blunt ends
Bsu15I	Sticky ends	Eco81I	Sticky ends	HpaI	Blunt ends	MspI	Sticky ends	PmlI	Blunt ends
Bsu36I	Sticky ends	Eco88I	Sticky ends	HpaII	Sticky ends	Msp20I	Blunt ends	PpiI	Sticky ends
BsuRI	Blunt ends	Eco91I	Sticky ends	HphI	Sticky ends	MspA1I	Blunt ends	PpsI	Sticky ends
BtgI	Sticky ends	Eco105I	Blunt ends	Hpy8I	Blunt ends	MspR9I	Sticky ends	Ppu21I	Blunt ends
BtgZI	Sticky ends	Eco130I	Sticky ends	Hpy99I	Sticky ends	MssI	Blunt ends	PpuMI	Sticky ends
BtrI	Blunt ends	Eco147I	Blunt ends	Hpy188I	Sticky ends	MunI	Sticky ends	PscI	Sticky ends
BtsI	Sticky ends	EcoICRI	Blunt ends	Hpy188III	Sticky ends	MvaI	Sticky ends	PshAI	Blunt ends
BveI	Sticky ends	Eco57MI	Sticky ends	HpyCH4III	Sticky ends	Mva1269I	Sticky ends	PshBI	Sticky ends
Cac8I	Blunt ends	EcoNI	Sticky ends	HpyCH4IV	Sticky ends	MvnI	Blunt ends	PsiI	Blunt ends
CaiI	Sticky ends	EcoO65I	Sticky ends	HpyCH4V	Blunt ends	MwoI	Sticky ends	Psp5II	Sticky ends
CciNI	Sticky ends	EcoO109I	Sticky ends	HpyF3I	Sticky ends	NaeI	Blunt ends	Psp6I	Sticky ends
CelII	Sticky ends	EcoP15I	Sticky ends	HpyF10VI	Sticky ends	NarI	Sticky ends	Psp1406I	Sticky ends
CfoI	Sticky ends	EcoRI	Sticky ends	Hsp92I	Sticky ends	NciI	Sticky ends	PspAI	Sticky ends
CfrI	Sticky ends	EcoRII	Sticky ends	Hsp92II	Sticky ends	NcoI	Sticky ends	Psp124BI	Sticky ends
Cfr9I	Sticky ends	EcoRV	Blunt ends	HspAI	Sticky ends	NdeI	Sticky ends	PspCI	Blunt ends
Cfr10I	Sticky ends	EcoT14I	Sticky ends	ItaI	Sticky ends	NdeII	Sticky ends	PspEI	Sticky ends
Cfr13I	Sticky ends	EcoT22I	Sticky ends	KasI	Sticky ends	NgoMIV	Sticky ends	PspGI	Sticky ends
Cfr42I	Sticky ends	EcoT38I	Sticky ends	KpnI	Sticky ends	NheI	Sticky ends	PspLI	Sticky ends
ClaI	Sticky ends	EgeI	Blunt ends	Kpn2I	Sticky ends	NlaIII	Sticky ends	PspN4I	Blunt ends
CpoI	Sticky ends	EheI	Blunt ends	KspI	Sticky ends	NlaIV	Blunt ends	PspOMI	Sticky ends
CseI	Sticky ends	ErhI	Sticky ends	Ksp22I	Sticky ends	NmuCI	Sticky ends	PspPPI	Sticky ends
CspI	Sticky ends	Esp3I	Sticky ends	Ksp632I	Sticky ends	NotI	Sticky ends	PspXI	Sticky ends
Csp6I	Sticky ends	FalI	Sticky ends	KspAI	Blunt ends	NruI	Blunt ends	PsrI	Sticky ends

Csp45I	Sticky ends	FaqI	Sticky ends	Kzo9I	Sticky ends	NsbI	Blunt ends	PstI	Sticky ends
CspCI	Sticky ends	FatI	Sticky ends	LguI	Sticky ends	NsiI	Sticky ends	PsuI	Sticky ends
CviAII	Sticky ends	FauI	Sticky ends	LweI	Sticky ends	NspI	Sticky ends	PsyI	Sticky ends
CviJI	Blunt ends	FauNDI	Sticky ends	MabI	Sticky ends	NspV	Sticky ends	PvuI	Sticky ends
DdeI	Sticky ends	FbaI	Sticky ends	MaeI	Sticky ends	OliI	Blunt ends	PvuII	Blunt ends
DpnI	Blunt ends	FblI	Sticky ends	MaeII	Sticky ends	PacI	Sticky ends	RcaI	Sticky ends
DpnII	Sticky ends	Fnu4HI	Sticky ends	MaeIII	Sticky ends	PaeI	Sticky ends	RgaI	Sticky ends
DraI	Sticky ends	FokI	Sticky ends	MalI	Blunt ends	PaeR7I	Sticky ends	RsaI	Blunt ends
DraII	Sticky ends	FriOI	Sticky ends	MamI	Blunt ends	PagI	Sticky ends	RsrII	Sticky ends
DraIII	Sticky ends	FseI	Sticky ends	MbiI	Blunt ends	PalI	Blunt ends	Rsr2I	Sticky ends
DrdI	Sticky ends	FspI	Blunt ends	MboI	Sticky ends	PalAI	Sticky ends	SacI	Sticky ends
DriI	Sticky ends	FspAI	Blunt ends	MboII	Sticky ends	PasI	Sticky ends	SacII	Sticky ends
SalI	Sticky ends	Sfr3•3I	Sticky ends	SrfI	Blunt ends	TliI	Sticky ends	XagI	Sticky ends
SanDI	Sticky ends	SalI	Sticky ends	Sse9I	Sticky ends	Tru1I	Sticky ends	XapI	Sticky ends
SapI	Sticky ends	SanDI	Sticky ends	Sse8387I	Sticky ends	Tru9I	Sticky ends	XbaI	Sticky ends
SatI	Sticky ends	SapI	Sticky ends	SsiI	Sticky ends	TscI	Sticky ends	XceI	Sticky ends
Sau96I	Sticky ends	SfuI	Sticky ends	SspI	Blunt ends	TseI	Sticky ends	XcmI	Sticky ends
Sau3AI	Sticky ends	SgfI	Sticky ends	SspBI	Sticky ends	TsoI	Sticky ends	XhoI	Sticky ends
SbfI	Sticky ends	SgrAI	Sticky ends	StuI	Blunt ends	Tsp45I	Sticky ends	XhoII	Sticky ends
ScaI	Blunt ends	SgsI	Sticky ends	StyI	Sticky ends	Tsp509I	Sticky ends	XmaI	Sticky ends
SchI	Blunt ends	SinI	Sticky ends	StyD4I	Sticky ends	TspEI	Sticky ends	XmaCI	Sticky ends
ScrFI	Sticky ends	SmaI	Blunt ends	SwaI	Blunt ends	TspGWI	Sticky ends	XmaJI	Sticky ends
SdaI	Sticky ends	SmiI	Blunt ends	TaaI	Sticky ends	TspRI	Sticky ends	XmiI	Sticky ends
SduI	Sticky ends	SmiMI	Blunt ends	TaiI	Sticky ends	TstI	Sticky ends	XmnI	Blunt ends

SexAI	Sticky ends	SmlI	Sticky ends	TaqI	Sticky ends	Tth111I	Sticky ends	XspI	Sticky ends
SfaNI	Sticky ends	SmoI	Sticky ends	TaqII	Sticky ends	Van91I	Sticky ends	ZraI	Blunt ends
SfcI	Sticky ends	SmuI	Sticky ends	TasI	Sticky ends	Vha464I	Sticky ends	ZrmI	Blunt ends
SfI	Sticky ends	SnaBI	Blunt ends	TatI	Sticky ends	VneI	Sticky ends	Zsp2I	Sticky ends
SfoI	Blunt ends	SpeI	Sticky ends	TauI	Sticky ends	VpaK11BI	Sticky ends	HpyAV	Sticky ends
Sfr274I	Sticky ends	SphI	Sticky ends	Tfi I	Sticky ends	VspI	Sticky ends	TspMI	Sticky ends

Table 1.2: A List of restriction enzymes along with ends generated by these endonucleases, used in recombinant DNA technology. Made after consulting information provided by <http://rebase.neb.com/rebase/rebase.html>

Protocols in Gene Cloning

DNA molecules generated from different sources may be used in gene cloning for diverse downstream applications. This section will explain the general cloning workflow and protocols involved in gene cloning experimentation, and describe how the DNA fragment of interest (the insert) is amplified through a Polymerase Chain Reaction (PCR). Restriction sites of specific restriction enzymes are engineered at the 5' ends of specific primers to the DNA fragment of interest. PCR analysis is performed to amplify the DNA fragment of interest flanked by specific sites against particular restriction enzymes. The PCR product (amplicon) is resolved on agarose using gel electrophoresis. Agarose gel is prepared in a 0.5X TAE buffer (Appendix A). The gel is visualized and photographed using the gel documentation system (GDS). Subsequent to this procedure, the following methods are employed.

DNA Elution

For DNA elution, agarose gel containing a DNA fragment of the required size is excised with a clean scalpel. Gel extraction is performed by using available gel extraction kits (according to the manufacturer's protocol) for eluting the required fragment.

PCR purification

Instead of DNA elution, PCR purification may also be performed if the PCR product is a lone specified amplicon. For purification, PCR purification kits are available. Therefore, PCR purification is performed according to the manufacturer's protocol which gives instructions on which PCR purification kit to use.

Competent Cell Formation

Competent *Escherichia coli* (E. coli) cells are prepared so as to build up their ability to take DNA up. For cloning purposes, plasmidless strains of E. coli are used. The general protocol for the preparation of bacterial competent cells is given below.

- Take a loop of E. coli fresh culture and transfer into L.B liquid for broth cultures (Appendix B).
- Incubate this culture at 37°C @180rpm overnight and then transfer 2ml from overnight culture into 100ml of LB liquid in a flask.

- Incubate it at 37°C @ 180 rpm to get a fresh culture.
- Centrifugate (for 5 minutes, at 3000-4000 rpm, 4°C) to harvest E. coli cells.
- Discard the supernatant.
- Add 0.1M MgCl₂ to pellet
- Resuspend it by gentle mixing and then centrifugation (5 minutes, 3000-4000 rpm, 4°C).
- Discard the supernatant.
- Add 0.1M CaCl₂ to pellet.
- Resuspend it by gently swirling and incubating on ice for 15-30 minutes followed by centrifugation (for 5 minutes, at 3000-4000 rpm, 4°C).
- Discard the supernatant.
- Add 0.1M CaCl₂ to pellet and resuspend it.
- Discard supernatant, add 2ml 0.1M of CaCl₂ and resuspend pellet into it.
- Prepare the aliquots by adding glycerol and keep in storage at -80°C.

Restriction digestion and Ligation reaction

The restriction digestion of an eluted DNA or PCR purified product (insert) and vector is performed with the same restriction enzymes. Subsequently the ligation reaction is performed using T4 DNA ligase.

Heat Shock Method and Blue White Selection

The delivery of a ligation product into a bacterial cell (a competent E. coli cell) is generally and commonly executed by the heat shock method. Heat shock treatment is given to the competent cell culture after the addition of a ligated product to it at 42°C, for 2 minutes. After heat shock treatment, transformed bacterial cells are incubated in a small quantity of liquid medium without antibiotics, for a short time period. Because of this incubation, the expression of the resistance gene (an enzyme) reaches the level at which this enzyme may detoxify the antibiotic in the culture regime and thereby allows the growth of the transformed bacterial cell on culture plates. The bacterial culture is spread onto an L.B. solid medium of ampicillin along with X-Gal and IPTG. This is done for blue white selection to select a recombinant clone. Cultured plates are incubated at 37°C overnight, and bacterial colonies appear on the culture plate if bacterial transformation is achieved. Keeping the culture plates for a long period of time results in the formation of satellite colonies.

In cloning vectors, an antibiotic resistance gene (commonly the *amp* or *bla* gene encoding β -lactamase enzyme against ampicillin, an antibiotic) and partial region *LacZ* gene (*LacZ_a*, encoding a subunit of the polypeptide chain of the *LacZ* gene) which encodes the functional region of β -galactosidase is present for the selection of a recombinant clone. Restriction sites are present within the *LacZ* gene, and even adjacent to it. In most of the cloning vectors, multiple cloning sites (MCS) or polylinkers are present within the *LacZ* gene. Thus, disruption of the open reading frame (ORF) of *LacZ*, due to the insertion of a DNA fragment results in its inactivation and hence is referred to as the insertional inactivation of the *LacZ* gene.

X-Gal is the substrate of the β -galactosidase enzyme because it is a lactose analog, whereas IPTG is the inducer. When the *LacZ* gene is intact, then β -galactosidase is produced, which, upon the availability of X-gal, hydrolyzes it. Hence, this breakdown of X-gal generates blue color pigments. Otherwise, the interruption of the *LacZ* gene by the insertion of a DNA fragment renders it nonfunctional and thus no blue color pigments are produced. Therefore, those bacterial cells which take up a self-ligated vector (ligation product) give blue color colonies. However, bacterial cells which take up a recombinant vector (ligation product) give white color colonies, because in recombinant vector a DNA fragment (insert) is inserted into the *LacZ* gene region, thereby disrupting its ORF. Hence, no pigments are produced, resulting in bacterial colonies on the plate which are white in appearance (Fig. 1-3).

Moreover, positive selection cloning vectors are also available in those expressing a lethal gene, for instance a restriction enzyme, which on expression starts digesting the genomic DNA of the host cell. The expression of this gene is disrupted by the ligation of an insert into the cloning site. Consequently, only those bacterial cells which have recombinant clones grow to form colonies; otherwise, no colony appears on the culture plate.

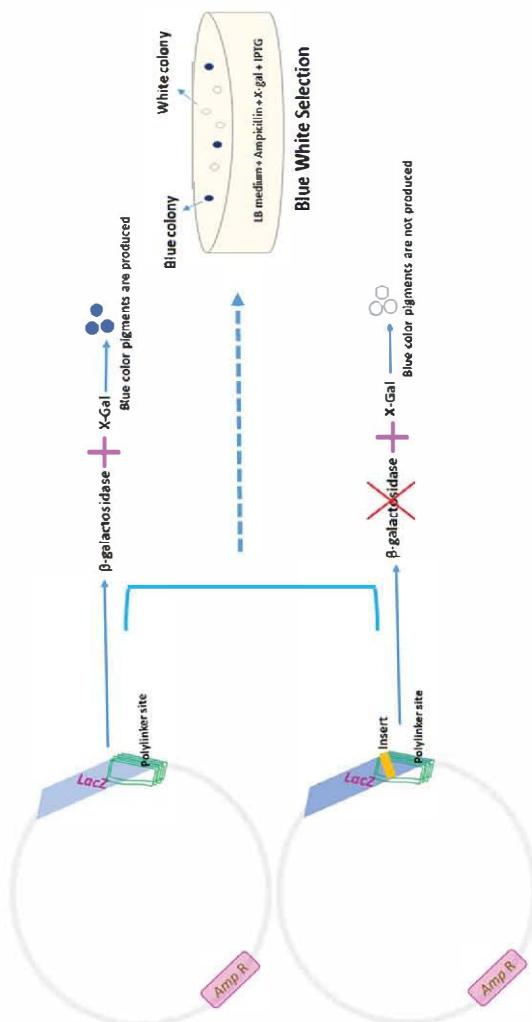


Figure 1.3: Blue White Selection for selecting the recombinant molecule (clone). Appearance of blue colonies on plate showing intact ORF of LacZ gene in a vector resulting in the production of β -galactosidase in a cell, which breaks down x-gal on culture regime and results in blue color pigmentation. Meanwhile the appearance of white colonies on the plate show interrupted ORF of LacZ gene in a vector molecule due to the insertion of a DNA fragment (insert). Therefore, there is no production of β -galactosidase in a cell and no breakdown of x-gal; hence no blue color precipitation. This is why white colonies appear on culture plates. (Idea taken from Gupta et al. 2017).

Plasmid DNA isolation

For plasmid isolation, white colonies are picked and are then cultured in an L.B. liquid medium containing ampicillin. For bacterial growth, it is incubated at 37°C, @180rpm overnight. For plasmid DNA isolation, kits are used according to the manufacturer's protocol. Then the recombinant clone is verified by restriction digestion analysis, colony PCR and/or sequencing.

Key points:

- ❖ Gene cloning comprises a set of experiments involved in the assembling of DNA fragments to generate a recombinant DNA molecule.
- ❖ A cloning vector must have the ability to replicate independently in the host cell.
- ❖ Both the vector and DNA fragment must be cut with the same kind of restriction enzyme for generating compatible ends to facilitate their proper joining.
- ❖ In a ligation reaction, the ligase enzyme is used to mediate the joining of a DNA fragment and vector molecule together into a recombinant DNA molecule.
- ❖ The recombinant DNA molecule is delivered into a host cell for making multiple identical copies.
- ❖ Blue white selection is done to select a recombinant clone.

CHAPTER TWO

GENE LIBRARIES CONSTRUCTION AND SCREENING

A gene library is the set of DNA sequences in an organism made by cloning these collections of DNA sequences into vectors. Gene libraries are divided into two categories based on the DNA source, (1) Genomic libraries and (2) cDNA libraries. In the case of genomic libraries, the source is an entire genomic DNA, while in the case of cDNA libraries the mRNA population of a cell is a source for synthesizing complementary DNA (cDNA). The size of the library (number of recombinants) can be predicted using the following formula;

$$N = \frac{\ln(1-P)}{In(1-a/b)}$$

P = probability a = insert size b = genome size

The number of recombinants (N) varies according to the type of vector used. If a vector carries a large insert size then a lower number of recombinants will be produced, and vice versa.

GENOMIC LIBRARIES

A genomic library is the collection of DNA sequences from an organism's entire genome. For constructing genomic libraries, isolated DNA can be fragmented by using one of two approaches, namely, the physical shearing of genomic DNA or enzymatic fragmentation. The fragmentation of DNA using the physical shearing approach is done by sonication, nebulization, and hydrodynamic shearing (using a Hydroshear instrument), while in the case of the enzymatic approach, non-limit restriction digestion of genomic DNA using restriction enzymes is performed.

Heterogeneous DNA fragments of varying lengths with 5' or 3' protruding ends could be gained in fragmentation. Hence, to make them blunt ended,

nuclease activity is performed using S1-mungbean nuclease (single stranded specific exonuclease). Hence, prior to the ligation of linkers (having restriction enzymes' recognition sites), with the aid of T4-DNA ligase, the restriction sites of blunt ended DNA fragments are methylated (so as to block them).

Example

If linkers have *Eco*RI restriction sites, then *Eco*RI restriction sites in blunt ended DNA fragments are methylated using the *Eco*RI methylase enzyme. Then linkers are ligated to *Eco*RI methylated blunt ended DNA fragments. Therefore, for efficient blunt ended ligation, large numbers of linkers are used in this reaction and it is followed by restriction digestion with *Eco*RI restriction enzyme to make their ends sticky. For generating compatible cohesive ends, vectors in which these DNA fragments are to ligate are also cut with the same restriction enzyme that is used to cut DNA fragments. Then a ligation reaction is performed to generate recombinant molecules for creating libraries (Fig. 2.1).

Alternatively, *Taq* DNA polymerase can also be used to generate cohesive ends by adding adenine (A) at the 3' end of DNA fragments (A-tailed DNA) that can be ligated into vectors which have a 5' T-overhang. If fragmentation is achieved using restriction enzymes which generate blunt ends, then there is no need for end repair using single stranded exonuclease or T4 DNA polymerase.

cDNA LIBRARIES

A cDNA library is the representation of the mRNA population of a specific cell at a specific stage and time. For constructing a cDNA library, the total RNA population of a cell is extracted followed by first strand cDNA synthesis, which is primed by either an oligo (dT) primer, gene specific primers or random primers. The oligo (dT) primer is used when the poly (A)-tailed mRNA population is subjected to first strand cDNA synthesis. For this, the total extracted RNA is passed down to a column with magnetic beads are linked to oligo (dT). So when the total RNA is passed down to the column, then poly (A)-tailed mRNA is bound to oligo-dT linked beads and the mRNA is recovered. This mRNA is primed with an oligo (dT) primer in the presence of a reverse transcriptase enzyme and dNTPs, and thereby the first strand of cDNA is synthesized.

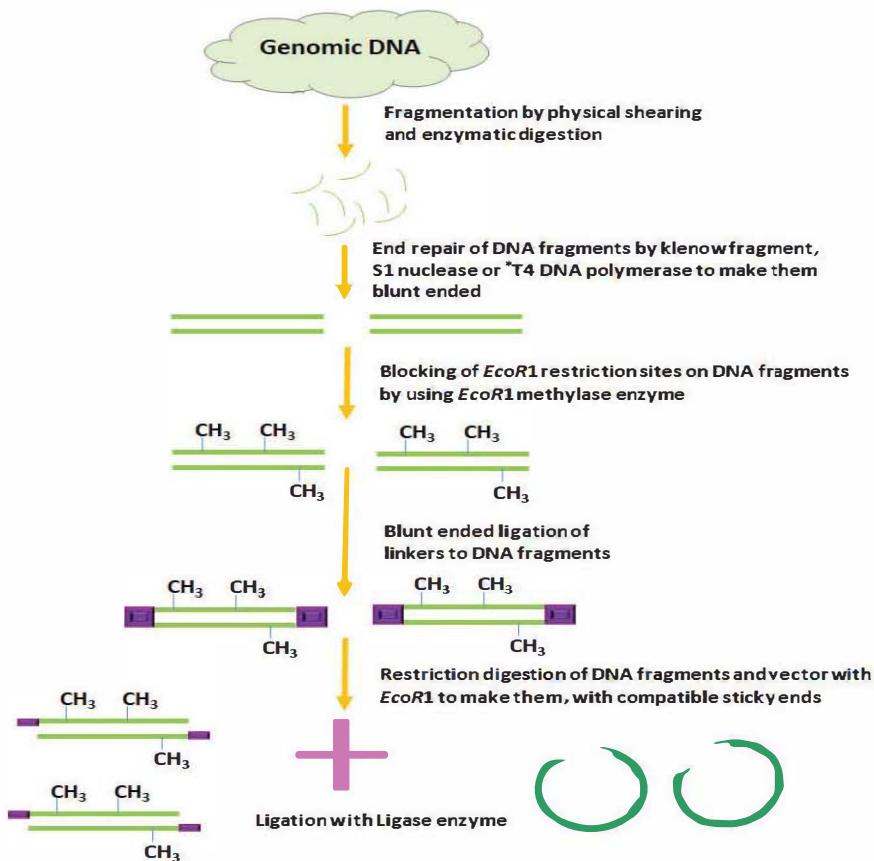


Figure 2.1: A schematic representation of the general procedure for genomic library construction (idea taken from Maniatis *et al.* (1978). *T4 DNA polymerase may perform end repair because it possesses single stranded exonuclease activity (whereby the 3' overhang is removed), as well as polymerase activity to fill in the 5' overhang. However, Klenow polymerase fills in recessed 3' ends.

There are various approaches for second strand cDNA synthesis. One of them is the priming of the first strand of cDNA with an oligo (dG) primer in which the terminal deoxynucleotidyl transferase enzyme is used, which polymerizes the poly (dC)-tail at the 3' end of cDNA: mRNA duplex when dNTPs are provided in a reaction. The mRNA strand is then degraded by alkali treatment and oligo (dG) is provided in a reaction along with Klenow polymerase or reverse transcriptase, and dNTPs, to synthesize the second

strand of cDNA (Fig. 2.2). Subsequently ends of the cDNA duplex are protruded because of the different length of the poly (dC)-tail and oligo (dG) primer. So for making a cDNA duplex suitable for cloning, it must be blunt ended, which is achieved by treatment with S1 nuclease.

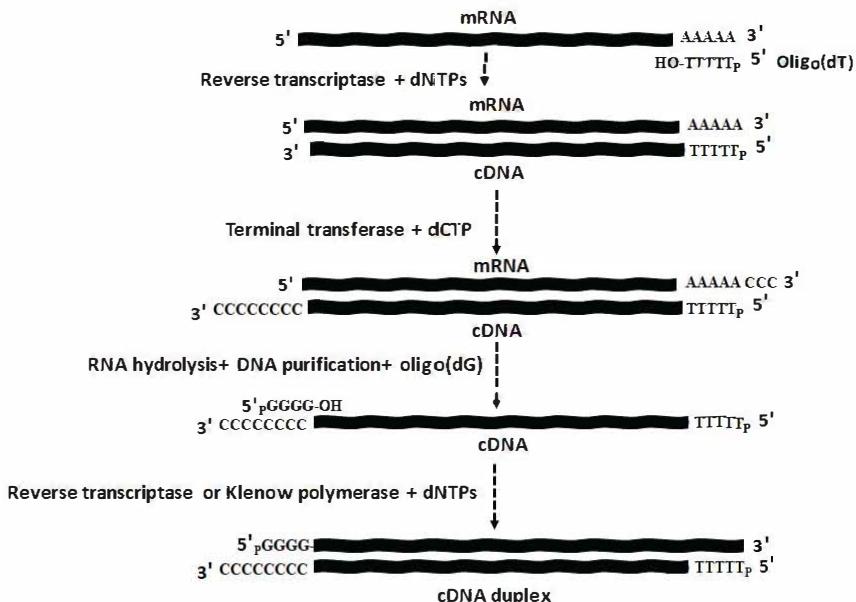


Figure 2.2: An illustrative diagram of the general procedure for cDNA library construction, showing the first and second strands of cDNA synthesis for cDNA library construction (idea taken from Turner *et al.* 2005).

CLONE SCREENING

Screening is the process of identifying a particular clone from gene libraries. There are various ways to screen the desired clone e.g., the PCR technique, Hybrid arrest translation (HART), Hybrid release translation (HRT), colony hybridization or plaque hybridization, and chromosome walking.

- In screening through the PCR technique, a gene specific primer pair is used. If the sequence is present, the PCR product is detected. This way the desired colony can be screened and identified.
- In Hybrid release translation (HRT), cDNA clones are denatured and are transferred onto a nylon or nitrocellulose membrane. Then the

membrane is incubated with the mRNA population to achieve its hybridization with cDNA, and unbound mRNAs are removed by washing the membrane. Subsequently hybrids are purified and mRNAs (hybridized mRNA) are released and translated into a cell free expression system. Thereby proteins encoded by a particular cDNA can be identified.

- In Hybrid arrest translation (HART), the process is the same as HRT up until the hybridization of cDNA and mRNA. But in this case, hybrids (mRNA bound to cDNA) are neither purified nor denatured. Here, unbounded mRNA and hybrids are both subjected to translation in a cell free expression system. Thus, protein not translated due to the inhibition is identified, as is the cDNA which has arrested the translation (protein synthesis).
- In colony hybridization, bacterial colonies of a plate (from which a particular clone is to be screened) are replicas plated onto a nylon membrane (a nitrocellulose membrane can also be used) and cell lysis to release DNA onto a membrane is achieved by dipping it in SDS (Sodium dodecyl sulfate) and protease solutions. Subsequently [single stranded] DNA is allowed to denature by soaking the membrane in an alkali solution followed by its exposure to UV-irradiation or baking it at 80°C at which single stranded DNA makes a covalent linkage with the nylon membrane. Following that, hybridization is achieved by exposing the membrane to a solution containing a probe (it could be a radiolabeled probe or a chromogenic probe) and it is incubated so as to hybridize the probe to its complementary DNA sequence. After hybridization, unhybridized or unbound probes are removed by washing the membrane (the complete procedure will be described in detail in chapter five). The membrane is visualized by autoradiography (if the probe is radiolabeled) or by immersing it in the solution containing a substrate respective to the enzyme/antibody linked to a probe (if the probe is chromogenic) and the colony is identified by comparing the membrane with the original plate.

Key points

- ❖ A genomic library is the collection of DNA sequences of an organism's entire genome.
- ❖ Cloning vectors used in molecular biology are, plasmids, λ phage, cosmids, BAC (Bacterial artificial chromosome) and YAC (yeast artificial chromosome).

- ❖ A cDNA library is the representation of the mRNA population of a specific cell at a specific stage and time.
- ❖ Synthesis of the first strand of cDNA is primed by either an oligo (dT) primer, gene specific primers or random primers.
- ❖ For the screening of a particular clone from gene libraries, PCR technique, Hybrid arrest translation (HART), Hybrid release translation (~~HRT~~), colony hybridization or plaque hybridization and chromosome walking are employed.

CHAPTER THREE

CONCEPTS AND APPROACHES IN PLANT GENETIC TRANSFORMATION

Genetic transformation is an adaptation or change in genetic background through the addition or deletion of a DNA segment. In plants, genome tailoring and engineering endow genetic transformation with new prospects for improving its quantitative and qualitative traits. Plant genetic transformation using genetic engineering tools is achieved through the introduction of genes from different sources (transgenes) into the plant genome. The introduction of transgenes into a plant genome modifies its genetic background and genetically transforms the plant. This approach has broken species-, genera- and even kingdom- barriers by the introduction of a gene into a plant from unrelated organisms. However, transgene stable integration into a plant genome is used to bring nutritional improvement to plants, as well as to make them resistant against, or tolerant to, biotic and abiotic stresses. The genetic engineering approach is also used for the spatial and temporal regulation of a complex genetic pathway by adding or deleting a genetic component(s). Both gene introduction and deletion are the domains of genetic engineering. For the delivery and transfer of a gene into a plant genome, there are different approaches that may be used. Among these, the agrobacterium mediated plant transformation and microprojectile bombardment/gene gun/biolistic approaches are of the more common used in routine practices in plant genetic transformation. Both these techniques have their merits as well as their shortfalls. This chapter is aimed at providing precise, unequivocal and comprehensive knowledge on these gene delivery methods in plants.

PLANT TRANSFORMATION APPROACHES

The plant genetic transformation approaches are divided into two methods; direct or physical methods, and indirect methods or biological methods. In direct methods, different physical approaches are practiced, such as microprojectile bombardment or the gene gun, silicon carbide fibers or whiskers, embryo electrophoresis, electroporation, Polyethylene glycol (PEG) mediated plant transformation and microinjection. A proper

understanding of the physics involved in all physical methods is necessary for enhancing transgene penetration into the cell wall and integration into the genome. In indirect methods, bacterial cells are used as mediators e.g., Agrobacterium mediated transformation. Hence, an understanding of the natural infection route chosen to infect plants with agrobacterium in order to make a plant diseased is required for revealing the philosophy behind this natural route of gene transfer across the plant kingdom; this became an inspiration to plant molecular biologist for delivering the gene into a plant genome.

Agrobacterium natural infection pathway

Agrobacterium is a genus of plant associative bacteria of the Rhizobiaceae family. This genus has different species based on diverse host plants and diseased symptomology, namely, *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Agrobacterium radiobacter*, *Agrobacterium rubi*, and *Agrobacterium vitis*. All these species are virulent except *Agrobacterium radiobacter* (avirulent) and cause different diseases in plants such as crown gall disease, hairy root disease, galls on grapes and cane gall disease, amongst others. Among these species, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* are the two most common species that have been used and studied extensively in the realm of plant molecular biology. However, in this chapter, the infection pathway of *Agrobacterium tumefaciens* will be explained comprehensively.

Agrobacterium tumefaciens is a phytopathogenic soil bacterium, and the rhizosphere of dicotyledonous plants is its natural habitat. A mega-plasmid (approximately > 250 kb size) is present in it, which induces crown gall disease in plants, and so is named ‘Ti plasmid’ (Tumor inducing plasmid). This plasmid has different regions containing different genetic elements, namely, a T-DNA (transfer DNA) region, a *vir* region (virulence region containing *vir* genes), an opine catabolism gene determined region and a conjugative transfer involved region (Fig. 3.1). The T-DNA region is delimited or flanked by left border and right border sequences (a region of 25bp imperfect/direct repeats is transferred to the plant cell). This region is approximately 20-30kb in size and one Ti plasmid contains either only one T-DNA region or multiple T-DNA regions. Both the left and right border regions are the cis-elements of T-DNA and act as a signal for its transfer to, and integration into, the plant nuclear genome. The transfer of the T-DNA region is the cumulative action of both Ti plasmid virulence genes/*vir* genes (Ti plasmid determined genes) and chromosomal virulence genes/*chv* genes (chromosomal determined genes).

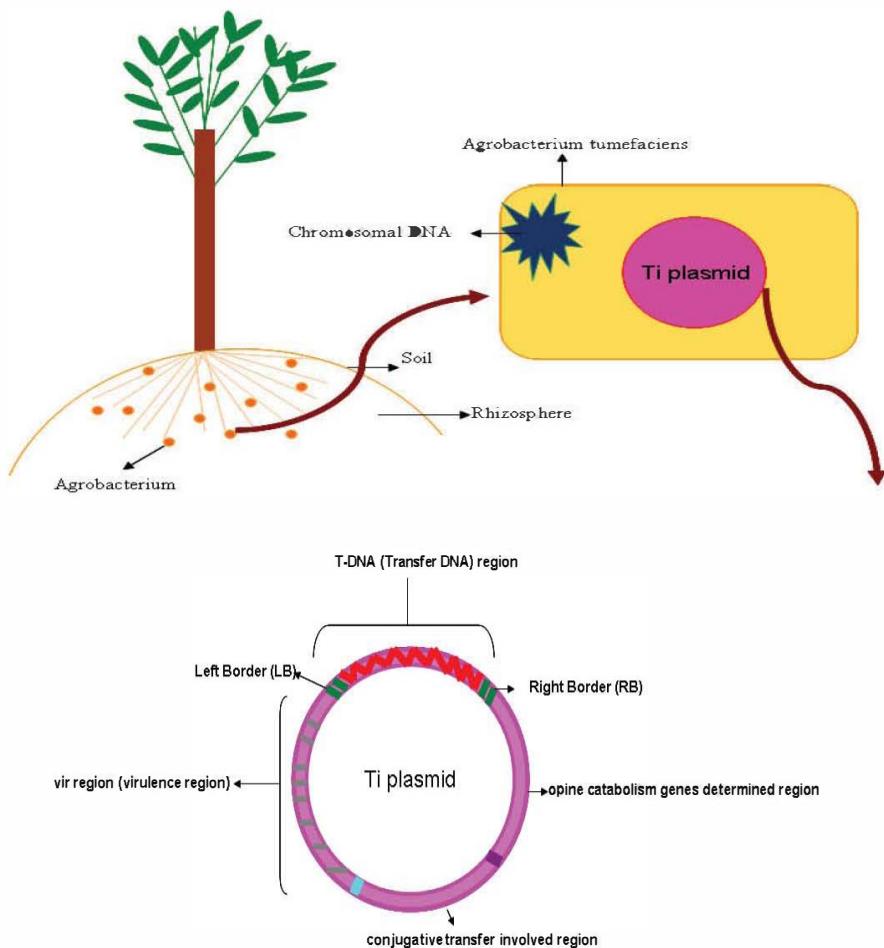


Figure 3.1: An illustration of *Agrobacterium tumefaciens* and Ti plasmid.

The preliminary step involved in the agrobacterium infection pathway is bacterial colonization, in which agrobacterium is attached to a plant cell. In the plant cell-bacterial cell attachment process, chromosomal virulence genes (*chv* genes) are involved, which include *chvA*, *chvB*, *chvD*, *chvE*, *pscA*, *cel*, *att* and *acvB*. Agrobacterium, while attaching itself to the host plant, starts to secrete capsular polysaccharides of a strong anionic nature which are termed 'K antigens'. They are deprived of a lipid anchor. This is the common characteristic of all rhizobiaceae bacteria. The mesh

established in the plant-bacterial attachment is made up of cellulose and polysaccharide fibers, synthesized from different chromosomal virulence genes. The genes *chvA* and *chvB* are involved in the synthesis and secretion of the β -1, 2 glucan, whereas *chvE* encodes the sugar binding protein (a glucose/xylose/galactose transporter). These sugars (glucose/xylose/galactose) enhance the induction of vir genes. The regulation of vir gene induction is the function of a protein encoded by the *chvD* gene. However, the *cel* gene is involved in the production of cellulose fibrils, while *pscA* is responsible for the synthesis of succinoglycan. Likewise, another *chv* gene, *att*, is involved in the synthesis of cell surface proteins. Mutagenesis analysis studies have suggested that the *att* gene region (20kb in size) is made up of two functional units, 10kb on the left side and 10 kb on the right. In this, the former is responsible for molecular signaling events whereas the latter is involved in the synthesis of essential components. However, the chromosomal virulence gene *acvB* is associated with the transport of T-DNA.

The second step of this infection pathway is linked with the induction of vir genes present in the vir region of the Ti plasmid which is 30-40kb in size. This region is a regulon comprised of eight operons, namely, *vir A*, *vir B*, *vir C*, *vir D*, *vir E*, *vir G*, *vir F* and *vir H*. In these operons, the number of open reading frames varies; in *virA*, *virF* and *virG* each has only one open reading frame, while *vir E* has three open reading frames in which two are present in the *vir C* and *vir H* operons respectively, and the *vir B* and *vir D* operons have eleven and four open reading frames respectively.

When the plant is injured, the phenolic compound acetosyringone is secreted from the wounded region and acts as a signal whose receiver is the product of the *virA* gene present on the membrane of the agrobacterium. As a result, a cascade of events indispensable for the transfer of T-DNA from *Agrobacterium tumefaciens* to the plant genome is begun (Fig. 3.2).

The protein *vir A* is a sensor protein that has three domains; one is a periplasmic domain and two are transmembrane domains (TM1 and TM2). The TM1 domain acts as transmitter (signaling), the TM2 domain acts as a receiver (sensor) while the periplasmic domain acts as a periplasmic antenna, which smells and senses the presence of the phenolic compound (acetosyringone) which is secreted from the wounded part of the plant. It also detects the monosaccharide sugars that are involved in the enhancement of the *vir* gene induction. The detection of monosaccharides is a vital amplification system and its induction is associated with the ChvE protein that is a periplasmic sugar (glucose/xylose/galactose) binding transporter

protein. The TM2 domain is an amphipatic helix (having both hydrophilic and hydrophobic regions) adjacent to the periplasmic domain. It is basically a histidine kinase involved in the autophosphorylation of the Vir A protein that takes place in response to acetosyringone sensing. Thus, the sensing of plant-derived xenobiotics (acetosyringone and monosaccharides) by the Vir A protein is of fundamental importance for the activation of vir genes.

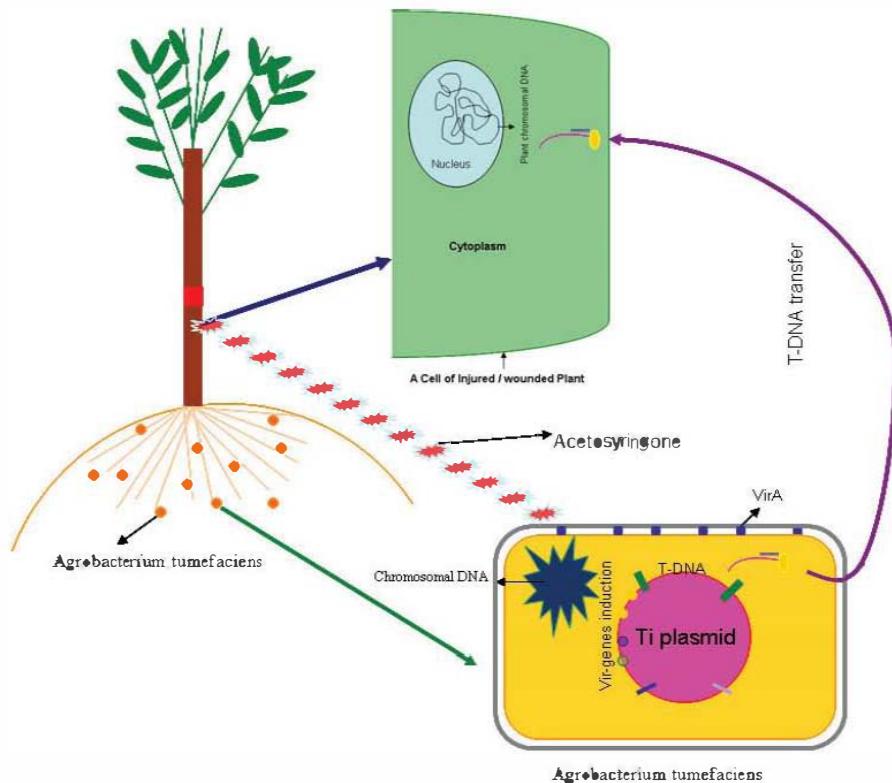


Figure 3.2: Signal cascading for the transfer of T-DNA from *Agrobacterium tumefaciens* to the plant genome.

The activated Vir A protein transphosphorylates the Vir G protein by transferring the phosphate group to it and making the Vir G protein active. The nonphosphorylated form of the Vir G protein is its inactive state, while on phosphorylation it becomes active. The Vir G protein is basically a transcription factor that recognizes the Vir box regions present in the promoters of Vir genes and activates as well as regulates their expression,

thereby inducing their transcription. The C-terminal region of the vir G protein is involved in the DNA binding activity, whereas the N terminal of this protein is the phosphorylation domain and has homology with the TM2 domain (receiver domain) of the vir A protein. Thereby the *virA* and *virG* operons (constitutive genes) encoded proteins function as the members of a two-component genetic system.

Subsequent to the induction of vir genes, an ssT-DNA transfer complex is generated. The *virD1* and *virD2* proteins of the *vir D* operon are the key components of this step, and play an important role by recognizing and nicking the T-DNA borders that are the cis elements of the T-DNA transfer system. Then a 5' to 3' single stranded (ss) copy of T-DNA is synthesized which is covalently linked with a *virD2* protein that prevents the exonucleolytic cleavage of the 5' end of ssT-DNA, and also labels it as the leading end of this transfer complex. Subsequently, this ssT-DNA-*virD2* complex is coated by the *virE2* protein, and this association not only prevents this ssT-DNA from nucleases activity but also reduces the diameter of the ssT-DNA complex that makes the translocation through the membrane easier, and results in its safe export to the plant cell.

A type IV secretion system is established by 11 *vir B* proteins along with a *virD4* protein, and this system is necessary for the export and transfer of the ssT-DNA complex and its other associated *vir* proteins to a plant cell. The *virD4* protein upholds the interaction between the T-DNA/*vir D2* complex with the *vir B* proteins. The majority of *Vir B* proteins are involved in the formation of the membrane channel and may also function as ATPases for providing energy to the channel assembly as well as to export processes. Numerous *vir B* proteins, namely *vir B2*, *vir B5*, and *vir B7* establish the T-pilus. Among these, *vir B2* that is processed and cyclized, is the chief pilin protein. The T-pilus may act as a “hook” for grasping the plant cell and bacterium cell in close proximity for molecular transport.

After the transfer of the ssT-DNA complex to a plant cell, *Vir D2* directs this complex to the nucleus for the integration of T-DNA into a nuclear genome. The *Vir D2* protein has one nuclear localization signal (NLS) while *Vir E2* has two NLSs that are necessary for the export and translocation of the ssT-DNA complex and its integration into the nuclear genome by illegitimate recombination (R). *Vir E1* is recognized and identified as an essential protein for *Vir E2* export to the plant cell. As mentioned above, *Vir E2* also contains nuclear localization sequences (NLSs) but they are inactive and occluded because *Vir E2* is a single strand DNA binding protein, and on binding to ssT-DNA its DNA binding domain and NLS

domain overlap.

In the cytoplasm of a plant cell, there are different NLS-binding specific proteins such as importins; VIP1, VIP2 etc. These groups of proteins facilitate the transfer of proteins containing nuclear localization sequences (NLS) through a nuclear pore complex (NPC). Importins recognize the NLS of the Vir D2 protein while VIP1 and VIP2 proteins interact with the NLS of Vir E2 and facilitate its interaction with importins. These proteins guide the ssT-DNA to the nuclear genome for integration.

The last and critical step of the Agrobacterium infection pathway is the integration of T-DNA into the plant genome. The integration of foreign DNA into a plant genome is mediated by a genetic mechanism known as Illegitimate Recombination (IR). For this process, micro-homologies of the 1-8bps sequence are required between the recipient genome and the transgene (the process of T-DNA transfer and integration from *Agrobacterium tumefaciens* to a plant genome is shown in Fig. 3.3).

In an illegitimate recombination mechanism, the 3'-end of ssT-DNA (ssT-DNA: Vir D2 complex) encounters microhomologies with the 5'---->3' strand of the chromosomal DNA of a plant cell, and a synapsis is established. This interaction results in the displacement of the plant's DNA's 3'---->5' strands and a gap is formed between the 5'---->3' and 3'---->5' strands of the genomic DNA of plant cell. The 3'overhang region of T-DNA displaces the 3'---->5' strand (bottom strand) of plant chromosomal DNA at its 3'end, and the 5'---->3' strand (upper strand) is cleaved by endonucleases. Then, DNA strand synthesis is primed and started from micro-homologies and ends at a nucleotide covalently linked to Vir D2 using an inserted T-DNA strand as a template. The 3'end of T-DNA then joins the displaced 3'--->5' strand at nick and is thereby integrated into this strand and Vir D2 is removed. Then the 3' end of upper strand (5'--->3') anneals to micro-homology in the bottom strand (3'--->5') and extraDNA is cleaved. This introduction brings about torsion and generates nicks, which stimulate a repair mechanism that fills single stranded gaps, and a complete locus is formed (Fig. 3.4).

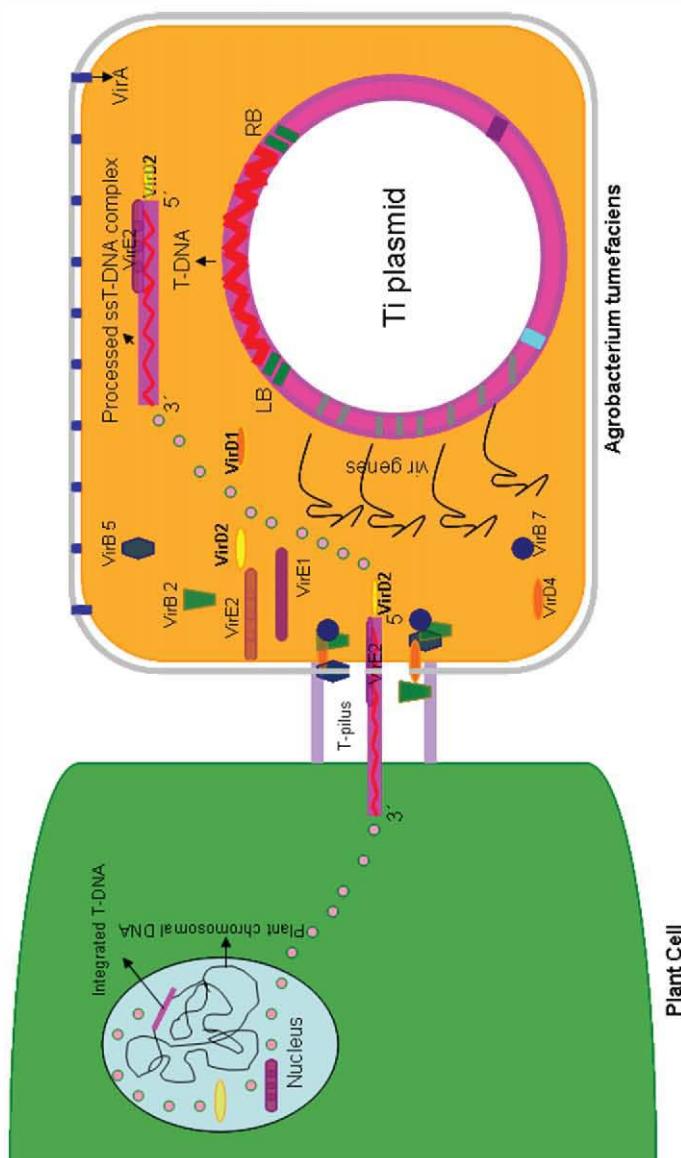


Figure 3.3: A pictorial representation of T-DNA transfer and integration from *Agrobacterium tumefaciens* to plant genome.

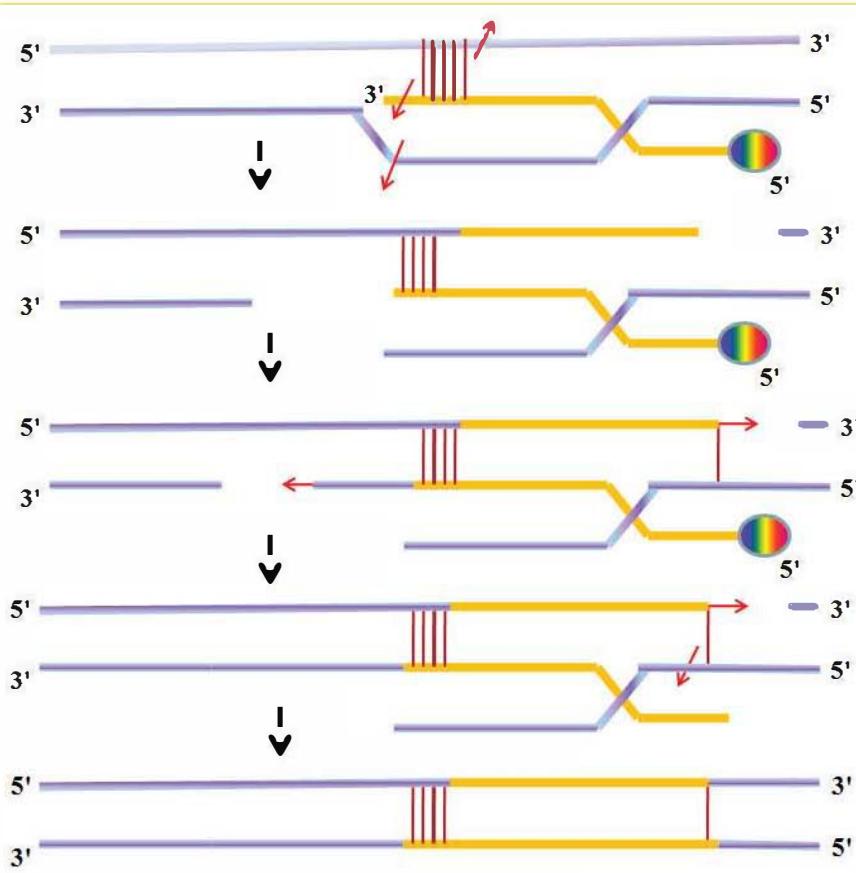


Figure 3.4: A schematic diagram of illegitimate recombination.

Agrobacterium mediated plant transformation:

The natural infection cycle of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* is the motivation or inspiration for their use as natural plant genetic engineers. Agrobacterium-based plasmids are used as vectors for introducing the gene of interest which confers particular traits. In agrobacterium-mediated plant transformation, its plasmid is engineered in such a way that its tumor inducing regions are removed and replaced by the gene of interest, conferring particular traits.

Two types of vector systems, namely, the co-integrated vector system and binary vector system, are used for Agrobacterium mediated plant transformation. In the co-integrated vector, the Ti plasmid is disarmed by removing the T-DNA region along with the right border, so only the left border is retained in a plasmid. An intermediated vector is constructed containing the gene for a desired trait as well as selectable marker genes, and this construct is flanked by cis acting in the left and right border sequences. Subsequently this intermediated vector is spliced in the disarmed vector by homologous recombination between the left borders of both the disarmed and intermediated vector. In the binary vector system, however, Vir genes and T-DNA reside on separate vectors. In the binary vector system, there are two disarmed vectors; one is devoid of Vir genes but has T-DNA (the DNA or gene of interest to be transferred to a plant cell) while the other has Vir gene regions but is devoid of a T-DNA region. For delivering these vectors to the target genome, agrobacterium is used as a mediator, which is why this approach is called the indirect method. As it is clear that agrobacteria are the bacteria of dicotyledonous plants and have biological incompatibility with monocotyledons plants, the monocots are recalcitrant for genetic transformation using the Agrobacterium mediated approach. Even in this case, the approach has been attempted in the genetic transformation of monocots.

Gene gun mediated plant transformation

Gene gun mediated plant transformation is also known as microprojectile bombardment, the biolistic or bioballistic approach. This is the most common, versatile, and effective physical method for plant transformation. It has an unlimited host range, which is the beauty of this technique. This physical approach is employed for the delivery of DNA into a target genome. As far as the agrobacterium mediated transformation is concerned, it is used only for nuclear genome transformation while the gene gun is not just used for nuclear genome transformation, but is also used for organellar genome transformation (plastome and mitochondrial genomes). This is also used for a variety of purposes such as DNA vaccination, gene therapy, genetic immunization, and so on. The gene gun PDS-1000/He is most commonly used for the routine practices of plant genetic transformation. In the biolistic technique, DNA to be delivered into the target genome is coated onto gold particles (tungsten particles can also be used). These particles are used because they are inert in nature, therefore no cellular toxicity is caused.

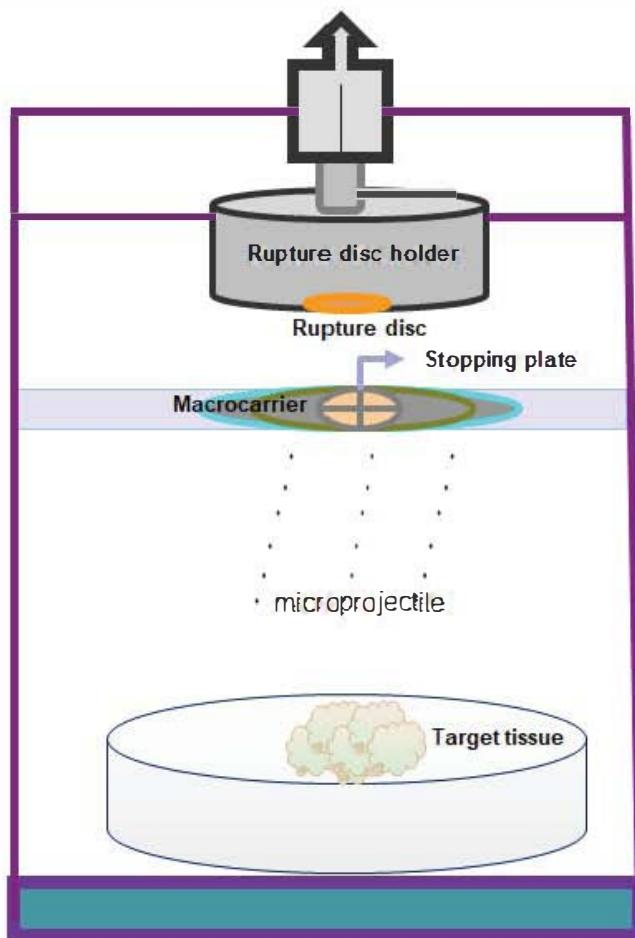


Figure 3.5: Gene gun: a physical approach for gene delivery into the plant genome.

The gold particles (micro-carrier or microprojectile) are about 0.6 microns in size. These microprojectiles are accelerated by establishing high pressure and low pressure (partial vacuum) zones separated by a diaphragm (rupture disc). A vacuum is created in a barrel or lower chamber that is an acceleration path. In a vacuum chamber or acceleration path, a macrocarrier covered with DNA coated microcarriers (gold particles) is assembled in apparatus along with a stopping screen (like a sieve) which is underneath the macrocarrier. Target tissue is placed below this assembly (Fig. 3.5) and a vacuum is created in a vacuum chamber which is followed by the release

of Helium (He) gas from a cylinder. Helium (He) gas is lighter and expands faster with high diffusivity and hence is used for accelerating microprojectiles in the gene gun mediated DNA delivery method. Through this process, a pressure gradient is established above (high pressure) and below (low pressure) the rupture disc. Rupture discs burst at the respective pressures (1100 psi, 1300 psi and 1500 psi) for which they are designed.

When the pressure reaches a particular level, a fire bursts the rupture disc and pressure is moved downward, or to a low pressure zone. This pressure gradient accelerates the projectile (macrocarrier) and they are rushed towards the stopping screen. This screen stops the macrocarrier, while microcarriers (DNA coated gold particles) pass through it, hit the target tissue and penetrate into the cell. Thereby, DNA is released from the microcarriers and is integrated into the genome by illegitimate recombination (IR).

PLANT GENOME ENGINEERING

In plants, genetic information is dispersed among three cellular compartments; the nucleus, plastids and mitochondria. Plant nuclear transformation is a conventional transgenic technology that can be achieved either by direct or indirect methods. In plant nuclear transformation, the integration of a transgene or foreign gene into a host genome occurs at random sites. Site specific integration of a transgene however, is only possible in chloroplast transformation, where the transgene is integrated into a genome via two homologous recombination events. Therefore, the position effect, epigenetic effect and gene silencing are absent; they are encountered in cases of plant nuclear transformation where site-specific integration of the transgene is not possible and the transgene is integrated randomly in the genome through illegitimate recombination.

The method of organellar genetic transformation was restricted due to the double membrane; a physical barrier for transgene delivery as well as the fact no virus or bacteria as a vehicle were known to infect these organelles. This bottleneck for organellar genome transformation was broken after the introduction of the gene gun approach. In higher plants, plastome (the plastid genome) is extraordinarily conserved in terms of organization as well as coding capacity, and possesses diminutive interspecific variations as compared to mitochondria. In plastid transgenic technology, chloroplast transformation is becoming an attractive target. The plastome is a circular double-stranded DNA molecule containing two inverted repeats, which separate a large single copy (LSC) region from a small single copy (SSC) region.

As mentioned earlier, in a plastome, transgene integration for developing transplastomic (stably transformed plastids) plants is achieved through homologous recombination. So when a transgene is introduced into the target tissue, it is integrated into an inverted repeat by homologous recombination which is followed by a homologous copy correction mechanism. This mechanism is a remarkable feature of chloroplast where the identical DNA sequence is maintained in both inverted repeat regions. However, to successfully develop transplastomic plants, the transgene must be present in each copy of the plastome in each cell, because in a cell, 1000 plastome copies are present. The state is called homoplasmy, when each copy of plastome in a cell acquires a transgene; if not, then it is called heteroplasmy which reverts back to wild type easily. Homoplasmy is achieved by keeping the target tissue for a repeated round of selection (25-30 cell divisions) on the tissue culture regime.

Chloroplast transformation technology offers several other advantages, such as a higher level of transgene expression (due to a high copy number of plastome) and transgene containment (because they are maternally inherited in most crop plants, hence the risk of genes escaping through pollens can be avoided, which is the main concern in nuclear transformation). However, horizontal gene transfer from engineered chloroplast to the nucleus has been reported. Thus, various strategies have been demonstrated for overcoming this concern, and intein-mediated protein trans-splicing is one of them.

***IN PLANTA* TRANSFORMATION**

For the development of transgenic plants, an efficient tissue culture system is considered indispensable and a pre-requisite for the genetic engineering of plants. However, *in planta* transformation methods have been practiced for the generation of transgenic plants by avoiding a tissue culture system. There are different techniques that use agrobacterium-based plant transformation without the aid of a tissue culture system, such as vacuum infiltration, floral dip culturing, the clip 'n' squirt method, agroinfiltration (injecting an agrobacterium suspension into intact plant leaves or other plant parts through a syringe) etc.

Vacuum infiltration method

In the vacuum infiltration method, germinating seeds and/or saplings are treated with a suspension culture of agrobacterium and then vacuum infiltrated and left to grow until maturity for further analyses and assays.

Floral dip culture

In floral dipping, plants are grown in pots, and during emerging inflorescence the first flower is clipped off to encourage secondary bulbs. An agrobacterium solution is only applied to newly emerging bulbs and, after treatment, plants are wrapped in plastic bags for a day and then left to grow until maturity for further analyses and assays.

Clip ‘n’ squirt method

In the clip ‘n’ squirt method, first the flower of a plant is clipped off, followed by uprooting the plant and then submerging this wounded plant into an agrobacterium suspension culture with mild agitation for a few minutes. Subsequently the treated plant is replanted and left to grow until maturity for further analyses and assays.

Key points

- ❖ Genetic transformation is the adaptation or change in genetic background by adding or deleting a DNA segment.
- ❖ The introduction of a transgene into a plant genome modifies its genetic background and renders the plant genetically transformed.
- ❖ The genetic engineering approach is also used for the spatial and temporal regulation of a complex genetic pathway by adding or deleting a genetic component(s).
- ❖ Plant genetic transformation approaches are divided into two methods: direct or physical methods, and indirect methods or biological methods.
- ❖ Direct methods involve different physical approaches such as microprojectile bombardment or the gene gun, silicon carbide fibers or whiskers, embryo electrophoresis, electroporation, Polyethylene glycol (PEG) mediated plant transformation, microinjection etc.
- ❖ Indirect methods use bacterial cells as mediators, such as Agrobacterium mediated transformation.
- ❖ In the illegitimate recombination mechanism, the 3'-end of ssT-DNA (ssT-DNA: Vir D2 complex) encounters microhomologies with a 5'--->3' strand of the chromosomal DNA of plant cell, and a synapsis is established.
- ❖ Two types of vector systems, specifically, the co-integrated vector system and binary vectors system are used for Agrobacterium-mediated plant transformation.
- ❖ Agrobacterium-mediated plant transformation is used only for nuclear genome transformation.

- ❖ The gene gun is used for nuclear as well as organellar genome transformation.
- ❖ The tissue culture system is considered indispensable, and a requisite strategy for the genetic engineering of plants.
- ❖ *In planta* transformation methods have been practiced for the generation of transgenic plants by avoiding tissue culture systems.
- ❖ Vacuum infiltration, floral dip culturing, the clip ‘n’ squirt method, agroinfiltration etc. are examples of *in planta* transformation.

CHAPTER FOUR

MARKER GENES: TOOLS FOR TRACKING PLANT TRANSFORMATION EVENTS

In the development of transgenic plants as well as transplastomic plants, *in vitro* selection, through giving selection pressure in a tissue culture regime or by visualizing a transformation event, is the first step of putative plant(s) selection. This is achieved by using selectable marker genes and/or screenable marker genes which have co-transformed along with the gene of interest conferring desired traits. Selectable marker genes confer resistance against different compounds like antibiotics or herbicides etc., while screenable marker genes are used for visual monitoring of a transformation event. These marker genes facilitate the identification and isolation of transformed cells from untransformed cells. Hence, a comprehensive description of selectable marker genes and screenable marker genes has been attempted in this chapter, in order to delineate and explain their potential role in tracking plant transformation events.

SELECTABLE MARKER GENES

Several selectable marker genes have been reported which are being used for the selection of putative transgenic plants. They are categorized under positive as well as negative selection systems and as either conditional or non-conditional (based on whether they require an external substrate or not). Positive selectable marker genes promote and encourage transformed cells while inhibiting the growth of untransformed ones. However, the situation is otherwise in the case of negative selectable marker genes.

POSITIVE SELECTION SYSTEM

Selectable marker genes in this category are subdivided into two groups based on the substrate requirement.

- Positive-conditional selectable marker genes

- Positive non-conditional selectable marker genes

Positive-conditional selectable marker genes

For the *in vitro* selection of transgenic plants having marker genes belonging to this category, an external substrate (either toxic or non-toxic in nature) is provided in a tissue culture regime for giving selection pressure. The positive selection system encourages the growth of transformed cells while discouraging the growth of untransformed cells. Antibiotic resistant selectable marker genes (*nptII*, *aad4* etc.) and herbicide resistant selectable marker genes (*bar* gene/*pat* gene) are examples of positive-conditional selectable marker genes in which the substrate is toxic to untransformed cells. However, the *manA* gene is also an example of this selection system, though in this case the external substrate—in which the growth of transformed cells is induced or enhanced as compared to untransformed cells—is non-toxic.

The *bar* gene/*pat* gene confers resistance against herbicides, Basta (Phosphinothricine is its active ingredient) while the *nptII* gene and *aad4* gene give resistance against kanamycin and spectinomycin antibiotics respectively. The *nptII* gene encoding the enzyme aminoglycoside phosphotransferase inactivates the kanamycin (an antibiotic) through its phosphorylation. By contrast, the *aad4* gene encodes for aminoglycoside adenyltransferase, inactivating the spectinomycin through its adenylation. Hence, upon their co-transformation along with the transgene of interest into targeted tissues, cells that get a selectable marker gene start to produce proteins that provide resistance to transformed cells by inactivating the selective agent in the tissue culture regime. This selective agent is an external substrate which is provided for giving selection pressure to select transformed cells. In this case, untransformed cells die, while transformed cells survive and develop into plantlets.

The shortfall of this system is the death of transformed cells due to the secretion of compounds (toxic for surviving transformed cells) from untransformed dead tissue that may inhibit the transformed cells' proliferation and growth.

Even then, the use of these marker genes is common in transgenic technologies in plants because their efficacy and success is greater than that of other selectable marker genes. However, the gene *manA* is a positive-conditional selectable marker gene whose substrate is non-toxic for untransformed cells or tissues. When the *manA* gene is used as a selectable

marker, marmose is provided in the selection medium. Therefore untransformed cells are unable to get carbon from marmose for their growth, as is otherwise the case for transformed cells, so their proliferation and growth is enhanced.

Positive non-conditional selectable marker genes

For these marker genes, no external substrate is required in a tissue culture regime; the *ipt* gene is an example of this. The transformed cells containing this gene show morphological, physiological and developmental alterations. In transformed cells, shoot development is enhanced and there is vigorous growth, while untransformed material is emaciated, having withered and weakened shoots.

NEGATIVE SELECTION SYSTEM

In the negative selection system, transformed cells are killed. This selection system is the alternative version of the positive selection system. This selection system includes negative-conditional and negative non-conditional selectable marker genes based on their requirement of substrates. The products of negative-conditional marker genes transform the non-toxic compounds to toxic compounds which lead to the death of transformed cells. Examples of negative-conditional selectable marker genes include: the *cod4* gene (Enzyme, Cytosine deaminase; Substrate, 5-Fluorocytosine), the *dhl4* gene (Enzyme, Dehalogenase; Substrate, Dihaloalkane), the *cue* gene (Enzyme, Alcohol dehydrogenase; Substrate, Allyl alcohol) and the *CYP105A* gene (Enzyme, Cytochrome P450 monooxygenase; Substrate, Sulfonylurea). The non-conditional negative system however includes genes whose expression products are toxic for transformed cells, e.g. ribonucleases.

SCREENABLE MARKER GENES

Screenable marker genes, or reporter genes, are the companion class of selectable marker genes. The reporter gene system is a valuable tool for screening the transformed tissues visually. With the help of reporter genes, transformed tissue can be manually separated and isolated. Their products can be visualized and become easily assayable; therefore they are used for the visual monitoring of the transformation event. A reporter gene can also be used to characterize the regulatory sequences (such as promoters) for monitoring their activities. Reporter genes are also conditional or non-conditional based on their external substrate requirement. There are a

number of reporter genes that are being used in plant molecular biology research, such as GUS, GFP, LUC and Lac Z.

GUS (β -glucuronidase) reporter gene

The *uidA* (*gusA*) gene of *E. coli* is extensively used as a reporter gene system in plants. This is a conditional screenable marker gene. This gene encodes the β -glucuronidase enzyme. When GUS is used as a reporter gene, different assays such as histochemical, fluorimetical and spectrophotometrical assays can be performed using different glucuronides. For a histochemical assay, the target tissue (containing the *gusA* gene as the reporter gene) is stained with an X-gluc (5 bromo-4-chloro-3-indolyl β - glucuronide) substrate through incubation at 37 °C. It is a chromogenic substrate, which on cleaving by the activity of β -glucuronidase, gives an insoluble blue color precipitation. For spectrophotometrical and fluorimetical assays, p-nitrophenyl β - D-glucuronide and 4-methylumbellifery β - D-glucuronide are used as substrates for β -glucuronidase enzyme activity respectively.

GUS intrinsic expression is encountered in almost all microbes as well as vertebrates and invertebrates, while a minute background signal is found in higher plants. Therefore, in plant transgenic technologies, it is extensively employed as a reporter gene system. The β -glucuronidase enzyme is not harmful to plants, but enzyme activity assays (with histochemical staining) are destructive to plant cells or tissues. This is the major flaw in this reporter gene system. There are no biosafety issues regarding β -glucuronidase enzyme toxicity in humans or animals because they are continuously exposed to the GUS enzyme due to their intestinal resident bacterium, *E. coli*, so producing no hazardous effects. So, when humans or animals consume genetically modified crops containing the *gusA* gene in the genome, their stomach conditions do not support this enzyme and it is degraded.

GFP (Green fluorescent protein) reporter gene

The GFP is a non-conditional reporter gene, thus no external substrate is required for applying target tissues (containing the *gfp* gene) to visualize its activity. As a result, its activity assay is non-destructive to plant cells/tissues. The GFP gene from jellyfish has become an attractive and popular reporter system in plants. By using this reporter gene, the transformation event is directly visualized under UV light. It has no cytotoxic effects in plants, and thus is extensively used as a reporter gene. The philosophy of GFP activity is: in the presence of oxygen, the

chromophores of GFP are formed autocatalytically, which then fluoresce green in color on absorbing UV light or blue light. For enhancing GFP reporter gene activity and efficiency, its fusion with the *aadA* gene (conferring resistance against spectinomycin) has been shown to result in a fluorescent antibiotic resistant marker, FLARE-S. So far, different versions of GFP with enhanced stable expression have been generated using codon optimization and/or by deleting a few base pairs from its sequence.

Lac Z reporter gene

Lac Z is also a conditional reporter gene which encodes the β -galactosidase enzyme. This is not a useful reporter gene for plants because they have the β -galactosidase enzyme endogenously. Therefore, the *Lac Z* gene is not usually used as a screenable marker gene because the intrinsic background signals of the β -galactosidase enzyme are encountered in plants. The Lac Z gene, is used as a reporter gene in lacZ⁻ bacteria as well as in animals, because they have no endogenous expression of β -galactosidase. This is extensively used as a reporter gene in molecular cloning practices for identifying recombinant plasmid DNA. When a plasmid containing the Lac Z gene is introduced into lacZ⁻ bacteria the β -galactosidase enzyme starts to be produced in these bacterial cells. So when these bacteria are cultured or grown in a medium having X-gal (the chromogenic substrate of β -galactosidase) along with IPTG (an inducer of X-gal), then this enzyme breaks down the X-gal and blue colored precipitation is formed. Thereby, bacterial colonies that have a LacZ containing plasmid appear blue in color, while those have no such plasmid are white in color.

LUC reporter gene

The *luc* gene from fireflies is also used as a reporter gene. A yellow colored light (560nm) is emitted when the enzyme luciferase is encoded by the *luc* gene which is exposed to luciferin. This enzyme mediates the reaction in the presence of ATP, in which the oxidative decarboxylation of luciferase takes place. LUC is a conditional reporter gene but its assay is non-destructive to plant cells. Background free assays can be performed in plants using this gene as the reporter because of its absence in plants. After the delivery of the LUC reporter gene into the target tissue, the luciferin (a substrate of the luciferase enzyme) is sprayed and applied onto this target tissue, and a bioluminescence is analyzed using spectroscopy.

Selectable Marker Gene Excision Approaches

The presence of marker genes in transgenic plants has aroused public concern with regard to biosafety. Consumers are not tolerant of transgenic plants harboring selectable marker genes in their genome. In the scenario where consumers remain reluctant to accept genetically modified plants as a source of nourishment, the presence of these genes in genetically modified organisms (GMO) presents a dilemma. There could be biosafety issues and ecosystem concerns regarding their presence in transgenic plants, but as yet no scientific bases exist to support this.

Considering consumers' or end-users' preferences and demand, selectable marker genes-free transgenic plants may encourage consumers' acceptance. No doubt, selectable marker genes may result in a metabolic burden to a cell. Apart from their use in tracking transformation events for selecting putative transgenic plants, they are not of any value to transgenics. Hence, different strategies have been practiced for developing marker free transgenic plants: the segregation of marker genes, site-specific recombinase based selectable marker gene excision, intrachromosomal recombination based selectable marker gene excision, transposable element mediated selectable marker gene excision and so on. Among them, site-specific recombinase-based selectable marker gene excision has become the most popular approach. This system has also been used in reverse genetics studies to find out the function of any given DNA sequence in a genome. In the following section, the principles and mechanism of the action of the site-specific recombinase based gene excision system are explained.

Site-specific recombinase based gene excision system

Site-specific recombinase based systems for selectable marker gene excision involve a dual component-mediated gene excision. Different site-specific recombination systems have been documented nevertheless; among them the Cre-loxP recombination system, the FLP/FRT recombination system and the phage phiC31 based system are the more commonly used in gene excision studies. These site-specific recombinases are grouped into two classes based on their catalytic amino acid residues; either tyrosine or serine. The Cre and Flp recombinases belong to the tyrosine group, however, phiC31 integrase is from the serine group. Recombinases of the tyrosine group recognize identical target sites and thereby mediate bidirectional recombination. As far as recombinases of serine groups are concerned, such as phiC31 integrase, they recognize non-identical target sites, thereby mediating a unidirectional recombination.

These recombinases share a similar principle for their action mechanism as they interact with their specific target sites and mediate the recombination event, which could be any one of excision, inversion or translocation, depending on the orientations of the target sites. The cre-loxP recombination system is from bacteriophage P1, while the FLP/FRT based recombination system is from *Saccharomyces cerevisiae*.

The cre recombinase enzyme recognizes specific sites—loxP sites—in pairs. There is no record of these sites in plants and animals yet, therefore, they are safe to use in gene excision from their genomes. Cre recombinase consists of two domains, with a total of ~343 amino acids. However, each loxP site is 34bp in length, consisting of two palindromic repeats of 13bp each, with an 8bp spacer region sandwiched between them. When Cre recombinase finds loxP sites in a pair, then it establishes an interaction with these target sites and induces a break at a spacer region. This enzyme catalyzes recombination at a holiday junction present within the spacer region.

The outcome of the recombination reaction depends on their orientation. If loxP sites are present in a direct orientation then floxed DNA (an intervening DNA segment flanked by loxP sites) is excised and the halves of both sites are joined (stitched), resulting in one loxP site which is 34bp in length (Fig. 4.1a). The direction of loxP sites, opposite to each other, results in the inversion of intervening DNA sequences (Fig. 4.1b). However, the presence of loxP sites on different chromosomes results in translocation (Fig. 4.1c).

The FLP/FRT recombination system also works on the principle of the Cre-loxP recombination system. FLP recombinase, on recognizing FRT sites in pairs (each FRT site is of 34bp), mediates the recombination process based on the orientation of FRT sites. In the case of the phage PhiC31-based gene excision system, the integrase (Int) enzyme (of ~613 amino acids) mediates the recombination between attP (PP) which is 39bp long and attB (BB) which is a 34bp long non-homologous site and results in an attR (PB) site after the excision of a stuffer fragment (a DNA segment flanked by attP and attB target sites). Therefore, in marker free transgenic plant development or gene excision for functional genomics studies, loxP sites, FRT sites or attP/attB sites are cloned as the flanking region of the gene(s). Hence, any gene encoding Cre recombinase, a gene encoding FLP recombinase or a gene (*int*) encoding Integrase are tethered with an inducible promoter to control the expression of recombinases.

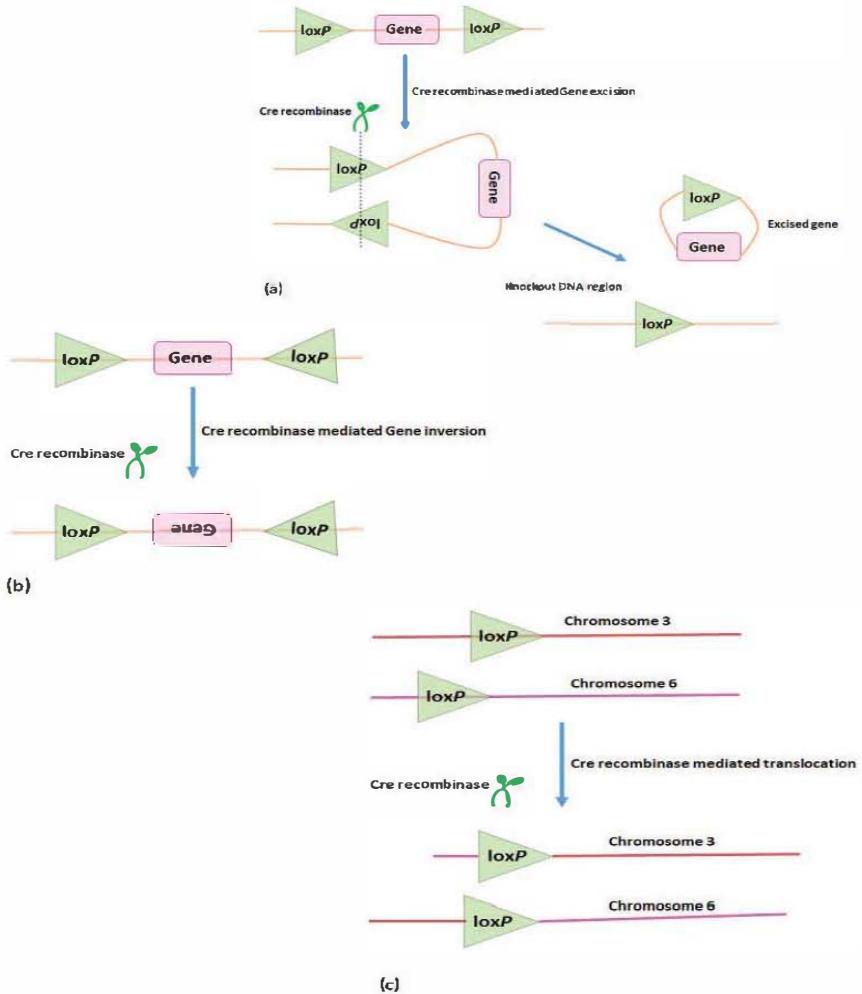


Figure 4.1: Cre-loxP recombination system (a) Gene excision because loxP sites are present in direct orientation (b) Gene inversion because loxP sites are present in opposite direction (c) Translocation because loxP sites are present on different chromosomes (idea taken from Home, 2017).

Key points

- ❖ Selectable marker genes are categorized based on a positive and negative selection system and whether the selection system is conditional or non-conditional (whether they require an external substrate or not).
- ❖ The antibiotic resistant selectable marker gene (*nptII*, *aadA* etc.) and herbicide resistant selectable marker genes (*bar* gene/*pat* gene) are examples of positive-conditional selectable marker genes in which an external substrate is toxic for untransformed cells.
- ❖ In the positive non-conditional selection system, no substrate is required in the tissue culture regime.
- ❖ Products of negative-conditional marker genes transform the non-toxic compounds to toxic compounds that lead to the death of transformed cells.
- ❖ Screenable marker genes are used to visually monitor the transformation event.
- ❖ GUS intrinsic expression is encountered in almost all microbes, as well as vertebrates and invertebrates, while minute background signals are found in higher plants.
- ❖ The β -glucuronidase enzyme is not harmful to plants but enzyme activity assay (histochemical staining) is destructive to plant tissues/cells.
- ❖ GFP is a non-conditional reporter gene, thus no external substrate is required for applying to target tissues (containing the *gfp* gene) to visualize its activity.
- ❖ The lac Z reporter gene (which encodes β -galactosidase) is not a useful reporter gene for plants because they have the β -galactosidase enzyme endogenously.
- ❖ Consumers highlight biosafety issues and ecosystem concerns regarding marker genes in transgenic plants.
- ❖ Selectable markers may bring a metabolic burden to a cell.
- ❖ Site-specific recombinase based systems for selectable marker gene excision involve two component-mediated gene excision.
- ❖ The cre-loxP recombination system, FLP/FRT recombination system and phage phiC31 based system are more commonly used in gene excision studies
- ❖ The Cre and Flp recombinases belong to the tyrosine group; however, phiC31 integrase is from the serine group.
- ❖ The Cre and Flp recombinases recognize identical target sites and thereby mediate bidirectional recombination.

- ❖ The phiC31 integrase performs the recognition of non-identical target sites, thereby mediating unidirectional recombination.
- ❖ Recombinase enzymes recognize their specific sites in pairs.
- ❖ Recombinase enzymes interact with their specific target sites and mediate recombination events, which could be excision, inversion or translocation, depending on the orientations of the target sites.

CHAPTER FIVE

TECHNIQUES IN MOLECULAR BIOLOGY

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a primer based approach for the enzyme mediated amplification of a DNA segment (either genomic DNA or cDNA). It is the core molecular biology technique for amplifying a small segment of DNA into its myriad copies. A specific set of primers could be either arbitrary, gene specific or a Simple Sequence Repeat (SSR) etc. Random or arbitrary primers used in PCR analysis include RAPD and ISS. Primer design is the fundamental criterion for getting optimal results from PCR analysis. The subtle output of PCR analysis is based on the PCR reaction set up, which includes an accurate template (input DNA) concentration, an optimized reagent profile and the thermal profile. The thermal profile of a PCR reaction consists of three steps; 1) Denaturation, 2) Annealing and 3) Extension.

Primer design:

For PCR, a set of specific primers is designed from the flanking region of the DNA sequence to be amplified. While designing primers, it should be taken into account that neither primer (the forward or reverse primer) of a pair should have: yielding primer dimer, self-complementation or the propensity to anneal, and nor should they have great differences in melting temperatures (T_m). The optimal level of GC contents in primers is within the range of 40% to 60%. For better priming ability, the 3' ends of the primer are of key importance and must have C or G as a first nucleotide in order to avert DNA breathing. This end of a primer should not have complementation within a primer or with other primers. Different software packages are available for primer design and primer analyses.

Types of polymerase Chain Reaction (PCR)

- Standard or conventional PCR
- Gradient PCR
- Arbitrary primed PCR
- Alu-PCR
- In situ PCR
- Touch down PCR
- Hot Start PCR
- Nested PCR
- Nested RT-PCR
- Multiplex PCR
- Multiplex RT-PCR
- Inverse PCR
- Long PCR (LA-PCR)
- RACEPCR
- PCR-RFLP
- Digital PCR
- Differential Display PCR
- Degenerate PCR
- Asymmetric PCR
- SSCP-PCR
- RT-PCR
- Real
- time Quantitative PCR

However, in this chapter, a detailed working principle of Real time Quantitative PCR is explained based on different kinds of primers.

Real-Time Quantitative PCR

For transcript profiling or expression profiling, the use of Real-Time quantitative PCR technology is common. This technique provides information/data about the detection and quantification of the target PCR product simultaneously. The PCR product is quantitatively measured as it is amplified and accumulated at each PCR cycle. The data regarding the PCR product is quantitatively measured and analyzed at an exponential phase of a PCR reaction, which is an optimal point at which data is analyzed before distressing amplification efficiency by inhibitor accumulation, exhausting reagents and polymerase inactivation. These factors are the

pitfalls of traditional or ‘end point’ PCR in which the detection of a PCR product is at the end of a reaction through gel electrophoresis after the plateau of a PCR reaction has been achieved.

The Real-Time PCR technique is widely practiced for numerous research applications such as testing transgenic plants or genetically modified organisms, expression profiling, gene expression quantification, microarray data validation, allele discrimination, pathogen detection, viral load monitoring and SNP detection. Fluorescent reporter molecules such as fluorescent dye-labeled sequence-specific probes (TaqMan probe, Molecular Beacons, Scorpions) and non-specific double stranded DNA binding dyes (SYBER Green and Eva Green) are used for monitoring the amplification progress during PCR reactions.

In each PCR cycle, an increase in fluorescence intensity is relative to the increase in concentration of the PCR product. The number of PCR cycles at which fluorescent signals start to accumulate but are not yet at that level i.e. the level required for signal detection by an instrument, is called the baseline. However in baseline correction processes, the measure of fluorescence versus the number of cycles is plotted for all samples in a reaction, and set at a common starting point with their background fluorescence. Subsequently, an arbitrary level of fluorescence is set based on baseline variability and is known as the threshold level. At this level, fluorescence is above the background but remains within the linear phase of the amplification reaction. The number of PCR cycles at which the fluorescent signal has crossed the threshold level is referred to as the threshold cycle (C_t). The fluorescent signal at that point is the real signal for defining the threshold cycle. There is an inverse relation between the amount of target PCR product and the C_t value.

Real-Time quantitative PCR approaches

Real-Time quantitative PCR approaches are based on the fluorescent reporter dye/molecule used. There are different approaches that are commonly employed for Real-Time qPCR, namely, SYBER Green dye based Real-Time qPCR, TaqMan probes based Real-Time qPCR, Molecular Beacons based Real-Time qPCR and Scorpion probes based Real-Time qPCR.

SYBER Green dye based Real-Time qPCR

SYBER Green is a non-specific double stranded DNA (dsDNA) binding dye. This is commonly used for monitoring the amplification progress based on fluorescence. As DNA concentration is increased after each PCR cycle, the intensity of fluorescence is also increased, because SYBER Green dye fluoresces upon binding to dsDNA. The SYBER Green dye intercalates between dsDNA and the beginning of fluorescence. Free SYBER Green dye has a low fluorescence signal, while on intercalating/binding to dsDNA, high fluorescent signals are emitted from the dye. Hence, when the DNA template is amplified, the dye is bound to the double stranded PCR product and fluorescence is released from it. But when this amplified PCR product is melted for the next cycle, the dye is released and fluorescence is decreased. The only pitfall in using this dye is that, as it is not sequence specific, it binds to non-specific DNA such as the primer dimer and other non-specific DNA sequences. Thus, in this case, PCR reaction specificity only depends on the primers to be used. Due to its non-specific feature, signal contamination is a problem, but the presence of a non-specific signal can be detected through a melt curve analysis. Hence, primer designing and optimization for this approach is highly critical for avoiding primer dimer formation or non-specific DNA binding. Primers should yield a single homogenous PCR product.

The melting point of DNA is based on its length and sequence. If during a reaction, a single homogenous PCR product in terms of length and sequence is present, then a single thermal transition is achieved, otherwise getting multiple thermal transitions would reflect the presence of a heterogeneous population of PCR products. In this way, a dissociation curve is obtained based on the melting temperature of PCR end products, and discriminates specific and non-specific PCR products. Discriminating the binding of SYBER Green dye to dsDNA may result in erroneous data which are additionally attributable to the artifacts of primer dimmers. Therefore this approach is not suitable for multiplex qPCR analysis. The SYBER Green dye based Real-Time qPCR approach is not used where a high level of specificity is required.

Hence, in this case, sequence specific dye-labeled probes are used that hybridize to their specific target sequence for detecting and monitoring the required PCR product, which yields fluorescence when hybridized to its specific sequence; otherwise no fluorescence is achieved. The level of fluorescence is proportionate to the quantity of amplicon at each cycle of PCR. The multiple sequence specific probes are to be used in multiplex

qPCR analysis, where more than one PCR product is detected and quantified in a single reaction. In multiplex PCR, there may be spectral overlaps that result in cross-talk issues because in a multiplex qPCR reaction, different probes labeled with different reporters and quenchers are used; consequently fluorophores and quenchers should be sufficient to avoid this problem.

TaqMan probes based Real-Time qPCR

The TaqMan probe is a linear probe where the 5' end is labeled with reporter dye while at its 3' end, a quencher is attached. At close proximity, a quencher inhibits the fluorescence of the reporter dye. At a short distance between the reporter and quencher, the reporter dye's fluorophore is quenched. When a PCR reaction begins, the probe is bound to its specific sequence (target sequence). So when *Taq* polymerase performs polymerization and reaches the TaqMan probe, then it tries to break this hurdle in its way with its 5'--->3' exonuclease activity and cleaves the 5' end of probe. Thereby the reporter dye is released, fluorescence is emitted from it and is detected by instruments. The quenching mechanism involves the FRET (Fluorescence Resonance Energy Transfer) principles. In this mechanism, when the distance between the high-energy dye (reporter) and low energy dye (quencher) is short, then energy is transferred from the reporter to the quencher resulting in the suppression of fluorescence from the reporter when the two do not maintain close proximity due to probe cleavage by *Taq* polymerase. As a result, distance between the reporter and quencher is increased, and fluorescence emission from the reporter dye begins (Fig. 5-1). In the TaqMan approach, FAM fluorophore and the TAMRA quencher are commonly used. However, this TAMRA fluorescent quencher is now replaced with a dark quencher, which provides results even with a low background.

The energy emitted by the dark quencher (a non-fluorescent quencher) is energy that it absorbs from the reporter as heat energy rather than light energy as in the case of a traditional fluorescent quencher (the TAMRA quencher). A dark quencher's acceptance of emitted fluorescence from the reporter dye is not followed by a re-emission of it. Therefore it avoids noise stemming as generated by a fluorescent quencher, which impedes the accurate fluorescence quantitation of the reporter. The dark quencher is valuable in multiplex PCR analysis, where light emitted from the quencher results in a cross-talk signal with one of the reporter dyes. In a TaqMan probe based Real-Time qPCR reaction, three different oligonucleotides are present, specifically, a primer pair and a probe. Thus, this reaction's thermal

profile should be adjusted to facilitate the annealing of the primer pair as well as the probe to template DNA.

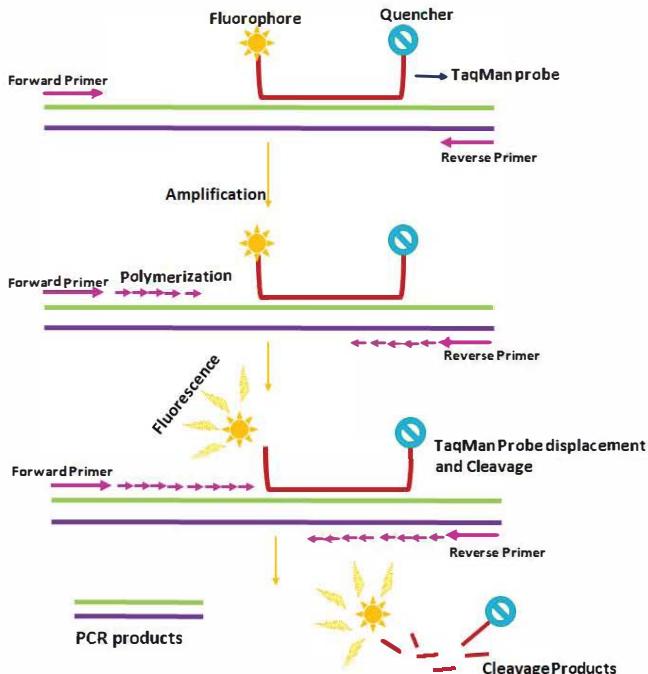


Figure 5.1: TaqMan probe based qPCR assay (idea taken from Botes *et al.* 2013).

Molecular Beacon based Real-Time qPCR

Molecular beacons are stem looped or hairpin-like structured probes, whose loop region is complementary to their target region while sequences of its stem arms are complementary to each other. The 5' stem arm carries reporter/fluorophore dye while its 3' stem arm is modified with quenchers such as Black hole quenchers (BHQs), methyl Red, DABCYL and so on. A molecular beacon is used; either static quenching or ground state quenching. When the target sequence is present, a loop sequence of the molecular beacon probe makes the complementary base pairing with the target DNA sequence. The hybridization between the loop sequence and target sequence put the fluorophore and quencher far apart, which allows the emission of fluorophore from the reporter molecule (Fig. 5-2). Otherwise, this probe thermodynamically favors the quenching of the reporter and quencher at

close proximity. Molecular beacon probes are not cleaved and remain intact throughout the PCR reaction. When the perfect match of molecular beacon probe is absent then it thermodynamically favors the hairpin structure formation rather than mismatched hybridization. This feature of molecular beacon probes results in improved and unequivocal discrimination of mismatches and makes these molecular beacons the most suitable toolbox for allele discrimination as well as SNP detection applications.

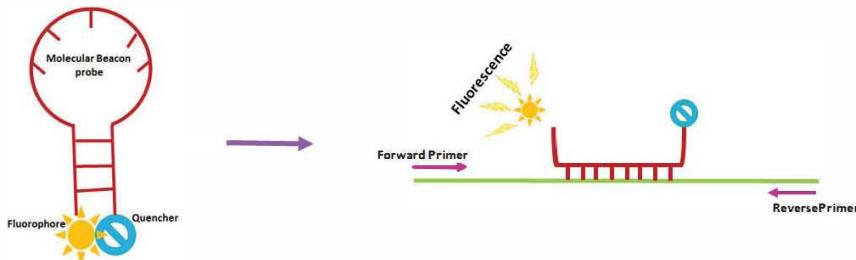


Figure 5.2: Molecular Beacon: A probe of qPCR technique (idea taken from Robert and Farrell 2009).

Scorpion probe based Real-Time qPCR

Scorpion probes in real time qPCR are based on the working chemistry of beacon probes. In scorpion probes, the probe is not physically separated from primers. The sequence of stem-looped probes is engineered in the primer sequence in such a way that the probe sequence is at the 5' end of the primer and the 3' end of the primer is complementary to the target site. Therefore the outcome is configured in such a manner that the fluorophore or reporter dye is at the 5' end, the primer is at the 3' end, and a quencher is between fluorophore and the primer. A hairpin probe structure supports the close proximity of quencher and fluorophore, but the extension followed by denaturation results in the complementation of the loop region sequence to an extended strand which parts the fluorophore from the quencher such that fluorescence is emitted. A sequence “PCR blocker” is also engineered in the hairpin structure of the scorpion probe to prevent its stem’s loop structure from being copied (Fig. 5-3).

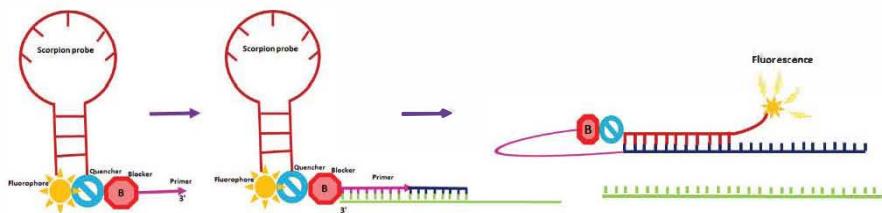


Figure 5.3: Scorpion Probe: A probe of the qPCR technique (idea taken from Arya et al. 2005).

GEL ELECTROPHORESIS

The electrophoresis technique is employed to separate biomolecules (nucleic acids and proteins) in an electric field. Electrostatic interaction triggers these biomolecules to move against their charges and facilitates their separation depending on the charge/mass ratio. Various factors such as buffer viscosity, compactness grade of biomolecules, net charge, temperature, and matrix porosity have a strong influence on the biomolecule mobility in this system. A hydrated gel network gives mechanical stability to the electrophoresis format; either horizontal gel electrophoresis or vertical gel electrophoresis. Different types of matrices, such as agarose gel, metaphor gel and polyacrylamide gel, with varying levels of resolving power, are used for a size based separation of biomolecules. The agarose matrix is polysaccharide in nature and its concentration in suspension is inversely proportional to the gel's pore size. However, in the polyacrylamide gel matrix, acrylamide is cross-linked to bisacrylamide, resulting in increased porosity and mechanical stability, and a higher resolution power of PAGE gel than agarose.

BLOTTING TECHNIQUES

Blotting techniques are used to identify or visualize a particular piece or segment of nucleic acid (DNA, RNA) and proteins from their total extracted population by transferring their electrophoretic products onto a membrane (nylon or nitrocellulose membranes). Various blotting techniques are being practiced. However, in this chapter, three main blotting techniques are briefly described: (1) southern blotting (for DNA detection), (2) northern blotting (for RNA detection) and (3) western blotting (for protein detection). A comprehensive knowledge and understanding of the principles of each technique is essential for scientists before starting work on them. Hence, in this chapter an effort has made to give a clear overview of the principles and

procedures of these blotting techniques.

SOUTHERN BLOTTING

Southern blot analysis involves the detection of specific DNA fragments from a complex DNA mixture as far as molecular analyses for the confirmation of transgenic plants are concerned. Southern blot analysis is the second step following PCR analysis for the detection of the transgene(s) at the genomic level. By employing this technique, integration patterns and the copy number of transgene(s) in transgenic plants may be determined. The steps involved in this technique are described in detail as follows, with a schematic diagram of the procedure (Fig. 5-4).

Restriction digestion

The genomic DNA of a plant is digested with restriction endonucleases. The restricted product is separated on agarose gel through electrophoresis, at 20V overnight.

Denaturation and Neutralization

After electrophoresis, the gel is immersed in a denaturation buffer (Appendix C) in a tank for 15 minutes under moderate shaking at room temperature. This step is performed for making the DNA single stranded. After denaturing the gel, a neutralization step is performed by incubating the gel in a neutralization buffer (Appendix D) for 15 minutes. This step prevents the rehybridization of the DNA and facilitates the transfer of DNA from the gel to the nylon membrane, because electrostatic interruption is at its lowest under neutral conditions. A depurination step can also be performed for making small fragments from large ones, but this is an optional step.

Blotting on nylon membrane and UV Cross linking

In this step, DNA from gel is transferred onto a nylon membrane by capillary action. For this purpose, Whatmann papers are used. 20x SSC (Sodium chloride and Sodium citrate) is used as a solvent to establish the concentration gradient for capillary action. After the transfer of DNA onto the nylon membrane, it is cross linked (a covalent bond is formed between negatively charged DNA and the positively charged nylon membrane) using a UV cross linker at an exposure of 1200KJ. Instead of exposure to a UV cross linker, gel can be baked or cooked at 80°C for this purpose.

Pre-hybridization

The nylon membrane is placed in a hybridization bottle containing a prehybridization solution (Appendix E) and denatured herring sperm DNA. This pre-hybridization step is performed at 42°C for two hours while shaking in a hybridizer. This step reduces the risk of non-specific binding of the probe to the nylon membrane.

Hybridization

Radiolabeled/radioactive (^{32}P labeled) and chromogenic probes (biotin labeled) are used to probe the nylon membrane for specific sequence detection, complementary to the probe sequence. Consequently, the probe is added to the pre-hybridization solution present in the hybridization bottle and incubated overnight at 42°C with shaking for hybridization.

Washing

After this step, the nylon membrane is taken out from the hybridization bottle containing a hybridization solution and is washed with a washing buffer (2x SSC and 0.1% SDS) twice at room temperature (for 10 minutes each time) followed by washing with a washing buffer (0.1x SSC and 0.1% SDS), twice at 65°C (for 20 minutes each time). Then the membrane is left to dry by placing it on filter paper.

Chromogenic detection procedure

A nylon membrane is washed with Blocking/Washing Buffer at room temperature (for 5 minutes). Then the nylon membrane is blocked by incubation in Blocking Solution at room temperature (for 30 minutes). Then it is incubated in Streptavidin-Alkaline phosphate conjugate solution for 30 minutes at room temperature. Streptavidin is a tetrameric protein that binds to biotin. After this incubation, the nylon membrane is washed using a Blocking/Washing Buffer, followed by its incubation for 10 minutes in a detection buffer.

Enzymatic reaction

A nylon membrane is incubated in a substrate solution (BCIP-T/NBT) for enzymatic reaction at room temperature under dark conditions to develop colored bands. In this reaction, alkaline phosphate cleaves its substrate BCIP-T, and, as a result, blue precipitations are formed. In this step, a signal

is developed and documented after an incubation period ranging from one hour to overnight.

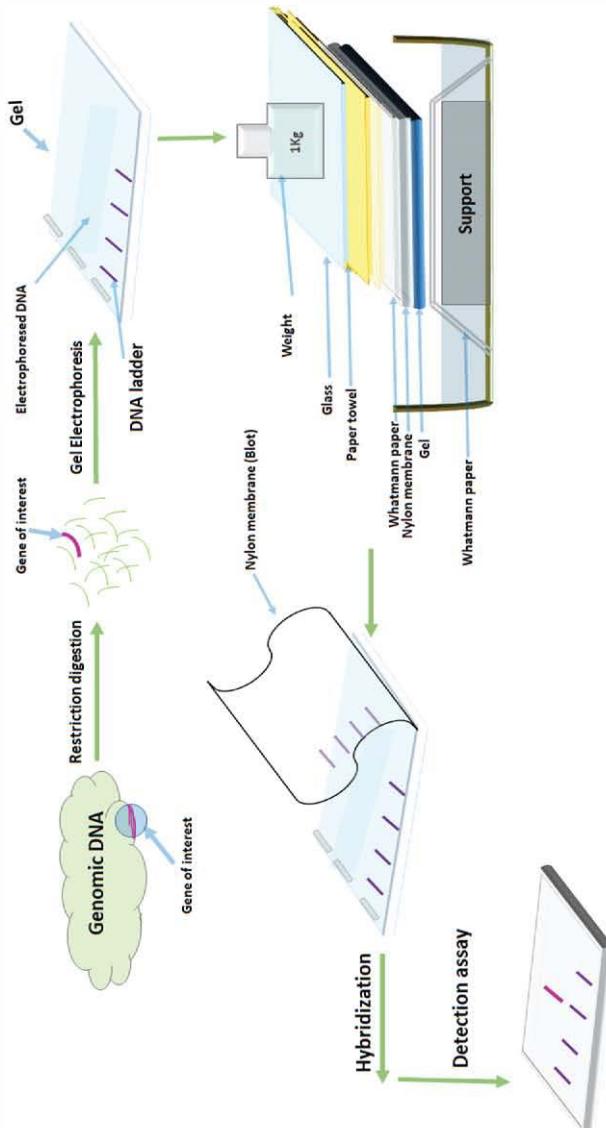


Figure 5.4: A schematic diagram of southern blotting, showing the identification of the gene of interest from a total genomic DNA.

Detection through autoradiography

When a radiolabeled probe is used to detect a specific DNA fragment, hybridization is observed or detected on X-ray film in a process called autoradiography.

NORTHERN BLOTTING

The northern blotting technique is used to determine the expression of a specific gene under particular conditions at the transcriptional level. This technique gives information about gene expression at the RNA level, for determining or evaluating a given DNA sequence's expression level, which could be either a transgene or any targeted gene, while applying particular stimuli (in case of inducible expression), and under normal conditions (in case of constitutive expression).

The general steps involved in northern blotting with a schematic diagram (Fig. 5-5) are:

- Extraction of total RNA from tissue.
- Separation of mRNA from the total RNA population, using oligo (dT) primer or oligo (dT) column.
- Gel electrophoresis of mRNA population.
- Transfer of electrophoresed mRNA from gel to a nylon membrane through capillary action or using a trans-blotter.
- Cross linking of mRNA onto nylon membrane using an UV-cross linker or baking at 80°C.
- Synthesis of radiolabeled or chromogenic probe (biotin or digoxigenin or fluorescein labelled) for the hybridization process.
- Preparation of pre-hybridization solution.
- Incubation of nylon membrane (blot) into a prehybridization solution for two hours at 42°C.
- For hybridization, a labelled probe (it could be either oligonucleotide or cDNA) is added into a prehybridization solution and incubated overnight at 42°C.
- Post-hybridization washing is carried out, followed by drying the blot with filter paper.
- In the final stage, a detection procedure is performed as described in this chapter under the section 'southern blotting'.

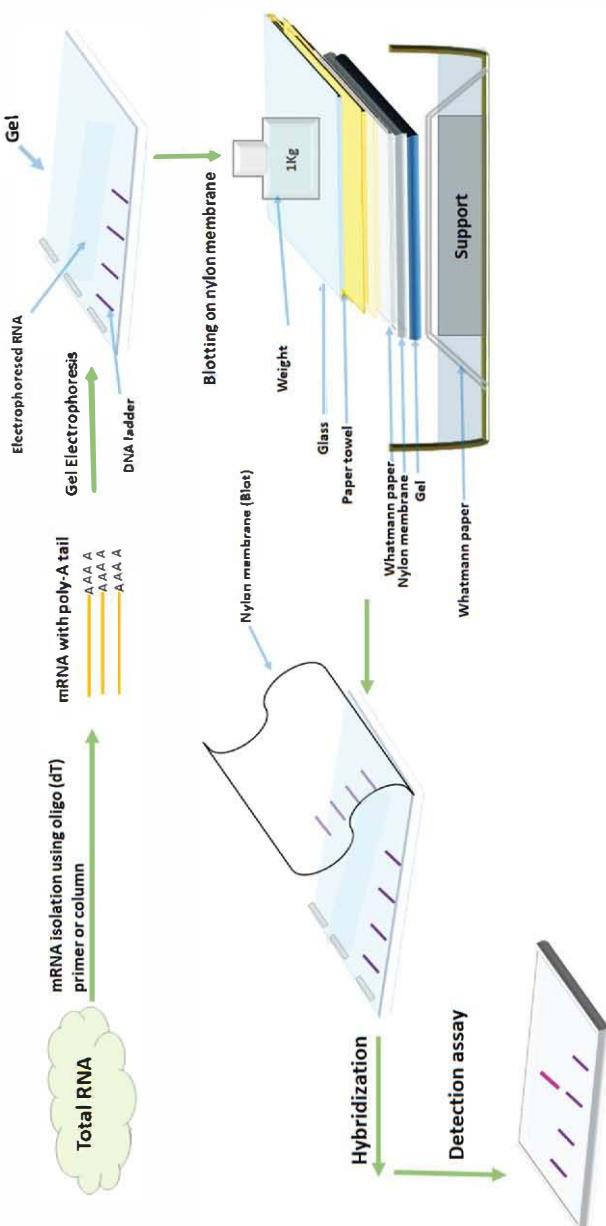


Figure 5.5: A schematic diagram of northern blotting, showing the identification of the mRNA of interest from the total RNA population of a particular condition or stage.

WESTERN BLOTTING

The western blotting technique deals with the identification of a particular protein molecule from a given mixture of proteins. It is achieved through the interaction of an antibody with the protein under investigation. The steps generally involved in western blotting are:

- Extraction of a protein from a sample, followed by heating and treatment with 2-mercaptoethanol to break disulfide bridging.
- SDS-PAGE is performed to separate or resolve protein molecules on gel.
- Transferring of electrophoretic products onto a nylon membrane (to make a blot).
- Primary antibody is synthesized against particular protein of interest.
- Secondary antibody is synthesized against primary antibody and is then conjugated with enzyme (horseradish peroxidase, HRP); in this way antibody-enzyme conjugate is prepared.
- Incubation of blot in solution containing a primary antibody followed by stringency washing.
- Subsequently, incubation of blot in secondary antibody-enzyme conjugate solution is carried out followed by stringency washing. If the protein of interest is present, then a sandwich of protein: primary antibody: secondary antibody: enzyme, is made.
- Detection of the protein of interest by incubating the blot into substrate (luminol) solution and luminescence is observed on film as a spot.

Key points

- ❖ Polymerase chain reaction (PCR) is used to amplify a small segment of DNA using either gene specific or simple sequence Repeat (SSR), Random primers etc.
- ❖ Primer design is the fundamental criterion for optimal results from PCR analysis.
- ❖ Thermal profile of a PCR reaction consists of three steps; 1) Denaturation, 2) Annealing and 3) Extension.
- ❖ For PCR, a set of specific primers is designed from the flanking regions of the DNA sequence to be amplified.
- ❖ Optimal level of GC contents in primers is within the range of 40% to 60%.

- ❖ For better priming ability, 3' ends of primer are of key importance and must have C or G as their first nucleotide to avert DNA breathing.
- ❖ For transcript profiling or expression profiling of transgenic plants, Real-Time quantitative PCR technology is used.
- ❖ The PCR product is quantitatively measured as it is amplified and accumulated at each PCR cycle.
- ❖ The pitfalls of traditional or end point PCR is that detection of PCR product is at the end of a reaction through gel electrophoresis after plateau of PCR reaction is achieved.
- ❖ Fluorescent reporter molecules such as fluorescent dye-labeled sequence-specific probes (TaqMan probe, Molecular Beacons, Scorpions) and non-specific double stranded DNA binding dyes (SYBER Green and Eva Green) are used for monitoring the amplification progress during PCR reaction.
- ❖ The number of PCR cycles at which fluorescent signal crosses the threshold level is called the threshold cycle (C_t).
- ❖ There is an inverse relation between the amount of target PCR product and C_t value.
- ❖ SYBER Green is a non-specific double stranded DNA (dsDNA) binding dye, which intercalates between dsDNA molecules and starts to show fluorescence.
- ❖ TaqMan probe is a linear probe where the 5' end is labeled with a reporter dye while at its 3' end a quencher is attached.
- ❖ When *Taq* polymerase performs polymerization and reaches a TaqMan probe, it tries to break this hurdle by 5'--->3' exonuclease activity and cleaves the 5' end of the probe, whereby reporter dye is released and fluorescence is emitted from reporter dye and detected on the instrument.
- ❖ Molecular beacons are stem looped (hairpin like), structured probes.
- ❖ Loop of molecular beacon probe has complementarity with targeted region, while sequences of its stem arms are complementary to each other.
- ❖ The 5' stem arm carries reporter/fluorophore dye, while its 3' stem arm is modified with quenchers.
- ❖ The hybridization between the loop sequence and target sequence place the fluorophore and quencher far apart, allowing for the emission of fluorophore from the reporter molecule.
- ❖ Scorpion probes are based on the working chemistry of beacon probes.
- ❖ In scorpion probes, the probe is not physically separated from primers, and sequence of stem-looped probe is engineered in the primer sequence.

- ❖ In scorpion probes, probe sequence is at the 5' end of the primer, and 3' end of the primer is complementary to target site, resulting in the configuration of either fluorophore or reporter dye at 5' end, primer at 3' end, and quencher between fluorophore and primer.
- ❖ A sequence “PCR blocker” is also engineered in the hairpin structure of scorpion probe to prevent the copying of its stem loop structure.
- ❖ Blotting techniques are applied to identify a particular segment of DNA, RNA and proteins from their total extracted populations by transferring them onto a membrane (nylon or nitrocellulose membranes) after gel electrophoresis.
- ❖ Southern blot analysis involves the detection of a specific DNA fragment from a complex DNA mixture.
- ❖ Southern blotting may be used to determine the integration pattern and copy the number of transgene(s) in transgenic plants.
- ❖ The northern blotting technique is for determining the expression of a specific gene under particular conditions at the transcriptional level.
- ❖ The Western blotting technique deals with the identification of a particular protein molecule from the mixture of proteins.

CHAPTER SIX

GENOME EDITING APPROACHES

Genome editing approaches based on sequence specific nucleases (SSNs) allow genetic manipulation in a highly precise and programmable manner. Due to the extremely specific nature of the action, these SSNs have emerged as powerful tools, which have brought about a breakthrough in the field of genome engineering. These engineered sequence-specific nucleases introduce double stranded breaks (DSBs) in targeted DNA at a specific site in a chromosome, thereby stimulating DNA repairing mechanisms in a cell. Two cellular DNA repair pathways that constitute homologous recombination (**H**R) based homology directed repair (**HDR**) and non-homologous end joining (NHEJ), are involved in repairing double stranded breaks in DNA. When overhangs are generated due to DSB in DNA, an error prone mechanism, NHEJ, mediates the targeted integration of the DNA molecule with compatible overhangs. However, **HDR** is in action when the DNA templates of a homologous region exist at the point at which a double stranded break is formed.

ZINC FINGER NUCLEASES (ZFNs)

Zinc finger nucleases (ZFNs) are synthetic proteins which are engineered in a modular fashion to target specific sequences in a genome. This feature makes ZFNs an important emerging tool in the field of genetic engineering. Zinc finger nucleases are engineered by fusing two independent domains, (1) the DNA binding domain, and (2) the DNA cleavage domain. The DNA binding domain is a zinc finger (C_2H_2 , C₄, C₆, etc.) present in most DNA binding proteins while the cleavage domain is from the *FokI* enzyme. An individual ZFN possess three to six repeats of zinc fingers in the DNA binding domain that are specific to its binding site. Each zinc finger repeat binds to 3 base pairs of DNA, hence, a pair of ZFNs with 3 zinc finger repeats each can recognize 18bps of DNA. The cleavage domain of ZFNs dimerizes to induce double stranded breaks (DSBs) on target sites, surrounded by DNA binding domains (Fig. 6-1). For the repair of double stranded DNA, these DSBs-trigger cellular repair mechanisms could either be NHEJ or **HDR**.

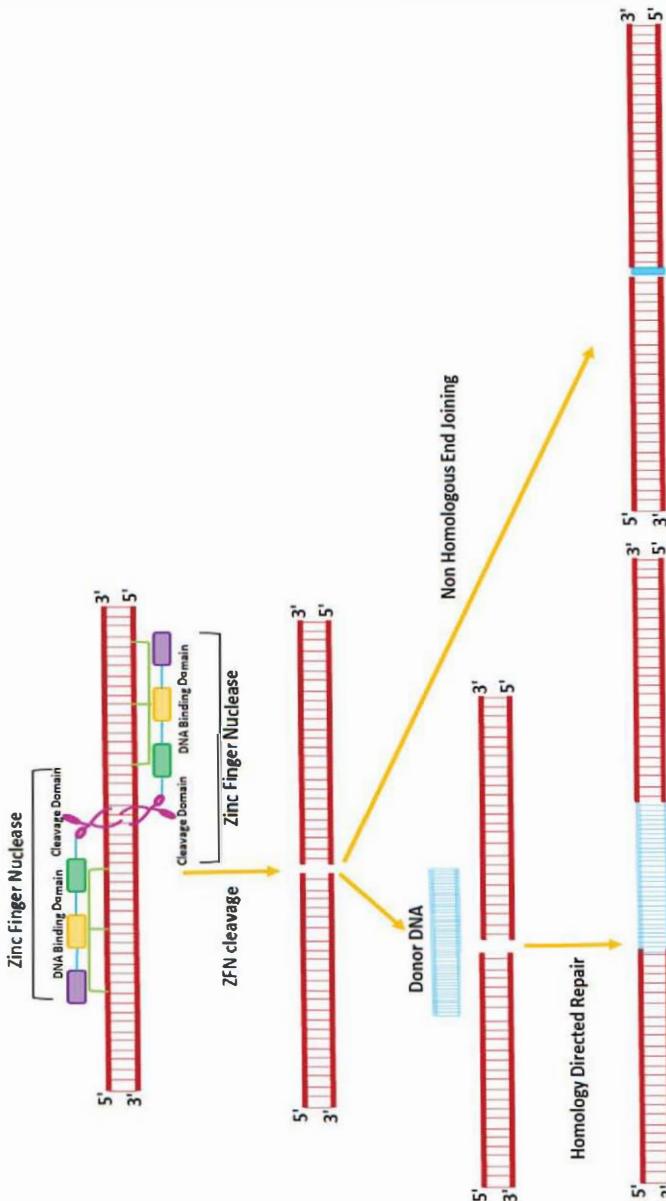


Figure 6.1: Zinc Finger nucleases (ZNFs): Mechanism of action for site directed genome editing (idea conceived by Carroll, 2011).

TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES (TALENs)

Transcription activator-like effector nucleases (TALENs) are synthetic bipartite proteins like ZFNs. These are engineered chimeric proteins which develop by the fusion and adaptation of conserved repeat regions of transcription activator-like effectors (TALE) and the endonuclease domain of the *FokI* protein. Transcription activator-like effectors are the proteins that are secreted by *Xanthomonas* (phytopathogenic bacteria). The conserved repeats of TAL effectors are the DNA binding domain of TALENs. The working principle of TALENs is identical to the ZFNs' but their mechanism of action is different.

As far as the mechanism of action is concerned, in TALENs, an array of highly conserved tandem repeats is present for DNA recognition, and each repeat is 33-35 amino acids long. Each repeat in an array recognizes a single nucleotide, and thereby the number of repeats in an array corresponds to the length of a target sequence in a genome. These repeats recognize a directed site, and a nuclease domain generates DSBs in this specific region, after which either an error prone repair pathway, NHEJ or a homologous recombination (HR) based repairing mechanism activates DNA repair in a cell.

CRISPR/CAS SYSTEMS

CRISPR is the acronym for Clustered Regularly Interspaced Short Palindromic Repeats, which belong to the family of direct repeats of DNA in a genome. CRISPR regions are found in the genome of just over half the members of both domains (bacteria and archaea) of prokaryotae. The sequences of repeats are approximately within a range of 21-37 nucleotides. These repeats of dyad symmetry are separated by various non-repetitive intervening sequences, known as spacers. These spacers are unique sequences of a regular structure and consistent size, which are positioned between each pair of direct repeats in a CRISPR region. Various well-conserved Cas loci have been found adjacent to CRISPR regions. These Cas regions encode for CRISPR-associated proteins which act as site-specific nucleases. However, these CRISPR regions do not encode for polypeptide chains, but rather they transcribe, and then are processed into short RNA transcripts and serve in the adaptive immune system of prokaryotes.

Classification

There are different classes of CRISPR systems with varied repeat patterns, different sets of loci, and diverse ranges of species. The history of CRISPR/Cas systems classification began with the trials of Daniel H. Haft and his scientific team in 2005. They described a guild of 45 Cas protein (CRISPR-associated protein) families. They categorized these families into six core proteins: Cas1, Cas2, Cas3, Cas4, Cas5 and Cas6; eight subtypes of CRISPR/Cas; and a repair associated mysterious protein (RAMP) module (a CRISPR associated module) in prokaryotes. However, Makarova *et al.* (2011) proposed another classification, which was based on the presence of Cas3, Cas9 and Cas10 protein signatures. They classified CRISPR/Cas systems into three types (the type I CRISPR/Cas system, type II CRISPR/Cas system and type III CRISPR/Cas system,) and ten subtypes based on additional signature proteins. Makarova *et al.* (2015) documented a modification leading them to the classification of two class-five type classification systems based on CRISPR loci and kinds of signature proteins.

Class-I of CRISPR exhibits multi-subunit protein effectors and thus possesses variant species of nucleases for different tasks, such as the processing of pre-crRNA, the loading of spacer sequences and processing targeted cleavage, while Class-II is comprised of single-subunit protein effectors. Therefore all these functions are performed by a single effector.

CRISPR/Cas9: a genome editing technology

Among all the CRISPR/Cas systems, CRISPR/Cas9 has gained much popularity as a genome editing tool. The CRISPR/Cas9 approach is comprised of three principle components, namely the Cas9 gene (which encodes for the Cas9 protein), target sites and sgRNA. These are the key parts of the CRISPR/Cas9 cellular machinery for cutting and stitching the genome in a directed and programmed fashion. In 2011, the CRISPR/Cas9 system's mechanism was unraveled, in which Cas9 nuclease works in juxtaposition with crRNA (CRISPR-RNA) and tracrRNA (trans-activator crRNA), to attack invasive DNA. In CRISPR/Cas9, as a three-component-based biological phenomenon, invasive DNA encounters CRISPR repeats, and starts the transcription of the CRISPR region which is followed by the processing of CRISPR transcript into small interfering crRNAs (CRISPR RNAs) which is ~40 bases long. Then, these cRNAs are combined with transactivating CRISPR RNA (tracrRNA) for the activation of Cas9 (Fig. 6-2a) which starts the cleavage of protospacer DNA in the invasive DNA.

The shift of the CRISPR/Cas9 system from the three component-based-biological mechanism to a two-component-based genome editing tool occurs upon the unraveling of the reprogramming of target DNA sequence—which is just the game of alteration in 20 bases of crRNA,—while its targeting specificity may be combined with the configurational features of tracrRNA in a chimeric single guide RNA (sgRNA) (Fig. 6.2b). For the activity of Cas9, a conserved region of PAM must be present downstream of the target DNA sequence. A seed sequence of ~12 nucleotides upstream of a PAM sequence of target DNA imparts the specificity to this system, and it must match gRNA.

As has already been emphasized, the target sites for the activity of Cas9 nuclease must be followed by a protospacer adjacent motif (PAM). The PAM region is an indispensable targeting component, because the Cas9 protein cannot bind to and cleave those targeted DNA sequences which are not followed by PAM. Engineering the sgRNA involves the introduction of a target sequence into its 5' terminal for its base pairing to a target site in the genome. A target sequence introduced into the sgRNA does not contain a PAM sequence. This target sequence is the complementary sequence of a targeted DNA sequence in the genome that is adjacent to a protospacer adjacent motif. Thus the sgRNA: Cas9 complex recognizes the target site (followed by a PAM sequence) and then Cas9 nuclease melts the DNA strand from the target region to make it single stranded, where sgRNA establishes the base pairing to it.

The Cas9 protein has a conserved core region and bi-lobed motif for nucleic acid binding that consists of a large recognition lobe (REC) and a small nuclease lobe (NUC), which are tied by a helix bridge. Under natural conditions, Cas9 is inactive while changing into an active state on interaction between its REC lobe and sgRNA, which determines the specific function of Cas9, while its NUC lobe is comprised of RuvC and HNH (nuclease domains), as well as a PAM interacting (PI) domain. Cas9 nuclease domains, HNH and RucV, are involved in the generation of a double stranded break (DSB) in the target DNA. They cut the target sequence at 3bps upstream of PAM. This double stranded break is then repaired by NHEJ and/or HRD mechanisms, resulting in either a knock-in or knock-out of the DNA sequence, depending on the repair pathway involved.

For their expression in plants, sgRNAs are tethered by U3 or U6 promoters (small nuclear RNA gene promoters). A sgRNA including 20 nucleotides of the target sequence is approximately 100-mer long. However, the Cas9

gene requires the fusion of nuclear localization signal(s) to its coding region (of ~4107bp) for its integration into eukaryotes' nuclear genome. While entering the cell, the sgRNA:Cas9 complex starts to scan the proper PAM region and on recognition, double stranded DNA melts, and base pairing is achieved between complementary bases of sgRNA and the target sequence. Then, the HNH domain of Cas9 breaks the target DNA: sgRNA hybrid, whereas the RuvC nuclease domain cleaves opposite strands and thereby a double stranded break is generated.

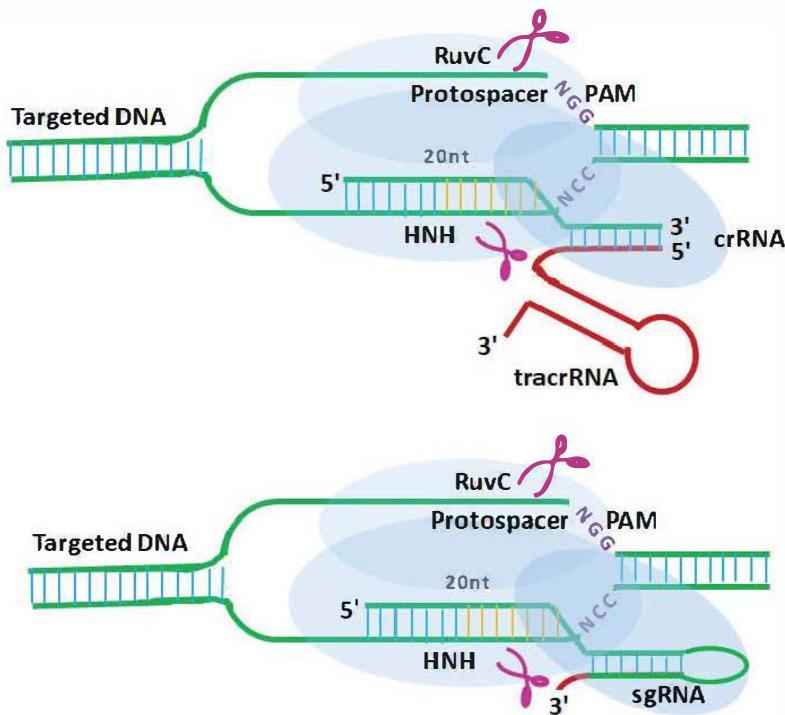


Figure 6.2: CRISPR/Cas9 system (top) Mechanism of action as a three component based biological phenomenon (bottom) Mechanism of action as two component based genome editing tool (idea taken from Bortesi and Fischer, 2015).

Another important feature of CRISPR/Cas9 based genome editing is multiplex genome editing for targeting multiple loci simultaneously, which could be achieved by cloning multiple sgRNA with different target sequences in a single construct. In dicotyledonous and monocotyledonous

plants, the CaMV35S promoter and *Ubiquitin* promoters from Arabidopsis, rice and maize ('constitutive promoters') have served as strong promoters for deriving the Cas9 gene for proficient gene editing. In different cases of genome editing in plants, a higher efficiency of *Ubiquitin* promoters as compared to the CaMV35S promoter has been documented.

Key points

- ❖ Genome editing approaches based on sequence specific nucleases (SSNs) allow genetic manipulation in a highly precise and programmable manner.
- ❖ These engineered sequence specific nucleases introduce double stranded breaks (DSBs) in targeted DNA at a specific site in the chromosome, and thereby stimulate DNA-repair mechanisms in a cell.
- ❖ Two cellular DNA repair pathways that are homologous recombination (HR) based homology directed repair (HDR) and non-homologous end joining (NHEJ) are involved in repairing the double stranded breaks in DNA.
- ❖ Zinc finger nucleases (ZFNs) are synthetic proteins which are engineered in a modular fashion to target specific sequences in a genome.
- ❖ Zinc finger nucleases are engineered by fusing two independent domains, (1) a DNA binding domain (zinc finger), and (2) a DNA cleavage domain (domain is from the FokI enzyme).
- ❖ Individual ZFNs possess three to six repeats of zinc fingers in a DNA binding domain that is specific to its binding site.
- ❖ Each zinc finger repeat binds to three base pairs of DNA, hence, a pair of ZFNs with three zinc finger repeats each can recognize 18bps of DNA.
- ❖ Transcription activator-like effector nucleases (TALENs) are synthetic bipartite proteins, as are ZFNs.
- ❖ These are engineered chimeric proteins, which develop from the fusion and adaptation of conserved repeat regions of Transcription activator-like effectors (TALE) and the endonuclease domain of the FokI protein.
- ❖ The conserved repeats of TAL effectors are the DNA binding domain of TALENs.
- ❖ Each repeat in an array recognizes a single nucleotide and thereby the number of repeats in an array corresponds to the length of a target sequence in a genome.
- ❖ CRISPR regions are found in the genome of just over half the members of both domains (bacteria and archaea) of prokaryotae.
- ❖ Various well-conserved Cas loci have been found adjacent to CRISPR

regions.

- ❖ These Cas regions encode for CRISPR-associated proteins which act as site specific nucleases.
- ❖ The CRISPR/Cas9 approach is comprised of three principal components: the Cas9 gene (encodes for Cas9 protein), target sites and sgRNA.
- ❖ Target sites for the activity of Cas9 nuclease must be followed by a protospacer adjacent motif (PAM).
- ❖ The Cas9 protein cannot bind to and cleave those targeted DNA sequences which are not followed by PAM.
- ❖ A seed sequence of ~12 nucleotides upstream of a PAM sequence of target DNA imparts the specificity to this system and must match gRNA.
- ❖ Another important feature of CRISPR/Cas9 based genome editing is multiplex genome editing for targeting multiple loci simultaneously which could be achieved by cloning multiple sgRNAs with different target sequences in a single construct.

CHAPTER SEVEN

APPLICATION OF RECOMBINANT DNA TECHNOLOGY

Recombinant DNA Technology is a remarkable development in the domain of science and technology. The emergence of this science has stimulated the invention of diverse techniques in the field of genetic engineering, each with subtle differences. This technology has illuminated the merits of genome manipulation in improving traits, producing therapeutic proteins, novel metabolites, recombinant polypeptides, recombinant insulin, and more. In short, this technology is like a vehicle that you can drive at will, over which you have control, and whose steering wheel you can turn to go where you wish.

The engineering of cellular pathways by introducing gene(s) or genetic elements to knock in and knock out a particular DNA segment to and from the genome is an important emerging field. This approach has unraveled the hidden stories of cells and their regulated functioning at the molecular level. Recombinant DNA technology is of fundamental importance for dissecting the genome for either the heterologous or homologous expression of genes, as well as editing the genome for adapting gene expression. Genome engineering in plants, to make them resistant against biotic as well as abiotic factors and to improve their yield and nutrition, is becoming a pressing need of our times for the purposes of feeding a rising world population.

The advent of microbial genome engineering has improved microbe efficiency as cellular factories for synthesizing new metabolites and products, by modulating their genetic systems. These microbes are genetically altered to synthesize recombinant polypeptides, compounds, therapeutic products and so on. The recombinant insulin (Lispro), epoetin alfa (a recombinant protein used to cure anemia), cytokine myeloid progenitor inhibitory factor-1 and recombinant hGH, are just a few of this approach's success stories. Among the microbes, fungi (yeast and true filamentous fungi) are important for use in industrial biotechnology as well as agriculture. Fungi are tamed by modulating their genetic architecture for agro-industrial use. Hence, recombinant DNA technology is playing its part

in all spheres of life, though this chapter has focused on delineating the application of recombinant DNA technology in plants and fungi.

RECOMBINANT DNA TECHNOLOGY: APPLICATION IN PLANTS

Genetic engineering deals with the incorporation of a gene into a genome to achieve upregulation in gene expression, downregulation in gene expression, overexpression, or ectopic expression of a gene. Transgenic plants and cisgenic plants are developed against biotic (as in insect pests and diseases) and abiotic (heat, cold, drought, salinity) factors. Among these factors, fungal diseases bring about myriad losses in plant productivity. Different approaches are being practiced to avoid plant damage by fungal diseases. Breeding and agronomic practices, and chemical and biological control, are all traditional strategies in the fight against phytopathogenic fungi.

Enzymes involved in the cell wall degradation of plant pathogenic fungi are an integral part of the plant defense mechanism. As a result, the genetic transformation of plants for disease resistance has been achieved by incorporating antifungal genes into them. These antifungal gene-encoding cell wall-degrading enzymes have proved to be strong weapons against fungal disease. Different transgenic plants have been developed by incorporating antifungal genes into their genome. Antifungal genes such as chitinases, chitosanases, and glucanases, have been documented to be strong inhibitors of virulence proteins in fungal pathogens. These antifungal genes have been isolated from different organisms and all of them have showed varying levels of antifungal activity in plants.

Insects are also plants' bitter enemies, and pose a severe threat to agriculture. The application of insecticides and breeding plants for insect resistance are conventional approaches to handling insect attack. Recombinant DNA technology is a subtle approach that strengthens plants in their capacity to combat insects. Employing this strategy involves genetically modifying plants by the introduction of gene(s) into their genome, which either express protein inhibitors (such as α -amylase and protease inhibitors) or express insecticidal proteins. These inhibitors interfere with insects' digestive enzymes upon eating the plant expressing this protein inhibitor. However, the expression of insecticidal proteins into transgenic plants is a more efficient approach to fighting insects, as it results in insect death, while the former just weakens the insect. Amongst the popular success stories of insect resistant transgenic plants are Bt crops (Bt

cotton, Bt maize, Bt brinjal etc.).

These Bt crops have been developed through genetic engineering in which the Bt genes (*Cry* genes; *Cryl Ab*, *Cryl Ac*, etc.) were introduced into their genome. *Cry* genes encoding crystal proteins (prototoxins) are present in *Bacillus thuringiensis*. Different strains of this bacteria exhibit variants of *Cry* genes with different insecticidal potential. Thus, when an insect bites the leaves of Bt plants, it stops eating, but while chewing the leaves, the Bt toxin protein interacts with the receptors present on the insect's gut wall cell surface. Bt toxin proteins are very specific in their action; they only interact with their specific receptors. On receptor recognition, toxin proteins enter the cell membrane of midgut cells. The (alkaline) pH of the insect gut supports, and provides the medium, to this toxin for action. This toxin perforates the insect gut by breaking the gut lining's cell walls and allowing bacterial spores to germinate there along with the gut's resident bacteria. Subsequently they enter into the insect body and their proliferation results in insect death (Fig. 7-1). As mentioned above, the alkaline pH of the insect gut supports the toxin in exerting or showing its toxic effect on insects. Therefore, there is no risk to human or animal life if they are exposed to, or eat, Bt crops and their products, because they have an acidic gut.

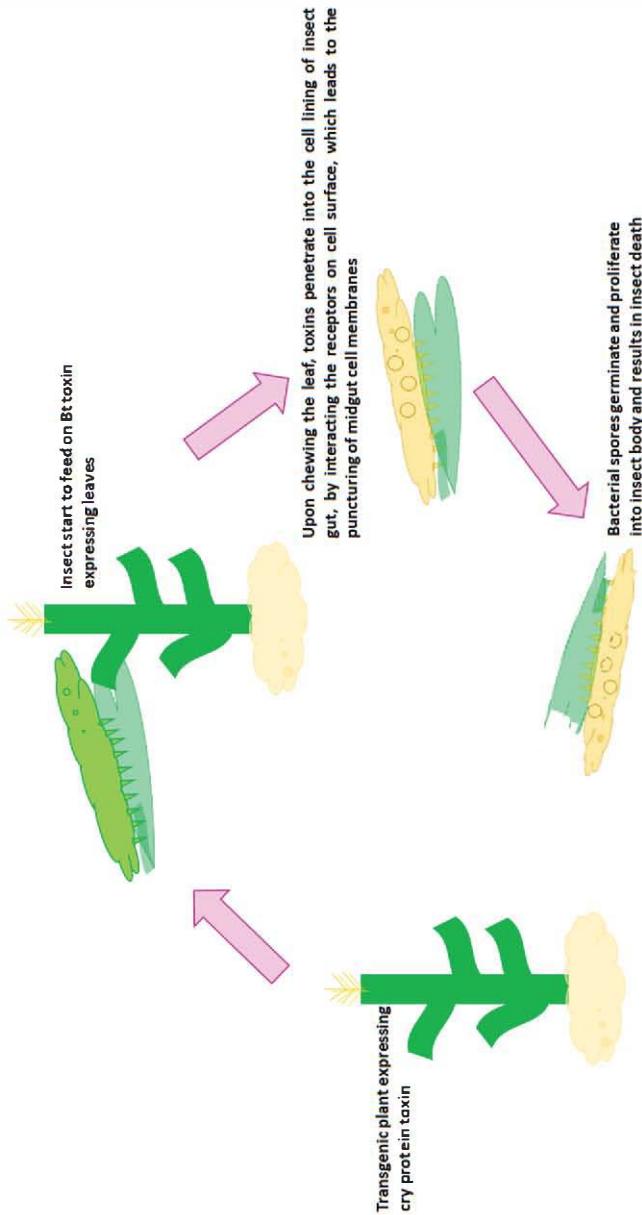


Figure 7.1: An illustrated diagram of a Bt toxin's mechanism of action on an insect after feeding on a Bt crop plant expressing Cry protein toxin.

Likewise, transgenic technology and genome editing approaches are also being applied to the genetic modification of plants. Among these genome editing techniques, CRISPR/Cas9 has taken a prime position in the site-directed targeting of genomes. An ethylene responsive factor gene, *osERF922*, in *Oryza sativa* (rice) has expression contribution towards the susceptibility of this crop to *Magnaporthe oryzae*, a phytopathogen which causes Rice blast disease. Hence, CRISPR/Cas9 based targeting of this gene sequence results in mutant rice plants, which showed resistance against *Magnaporthe oryzae* (for rice blast disease). In rice, another attempt at the CRISPR/Cas9 approach has been made by targeting the promoter of the *osSWEET13* gene, for developing resistance against bacterial blight in rice. In wheat (*Triticum aestivum*) three loci of *MLE* homoeologous genes encode proteins, which contribute to the repression of defense mechanisms against *Blumeria graminis* which causes a powdery mildew disease in wheat. Thus, CRISPR/Cas9 based genome editing has been attempted for targeting three loci of *MLE* homoeologous genes in wheat. The resultant *TaMLE* mutants showed resistance against powdery mildew in wheat.

RECOMBINANT DNA TECHNOLOGY: APPLICATION IN FUNGI

Fungi are of immense importance to industries for the production of enzymes, pigments, vitamins, lipids, saccharides and various other secondary metabolites. Secondary metabolites of fungal origin possess tremendous nutritional value for humans. Both yeasts and true filamentous fungi have been utilized for the production of wine, beer, cheese, bread etc. since the Prebiblical period. Advances in industrial microbiology have given rise to myriad fermented products, such as amino acids, antibiotics, solvents etc. However, developments in Recombinant DNA technology open up new horizons for the market of these microbial factories (filamentous fungi and yeasts). Fungi's genetic systems are engineered for the heterologous and homologous expression of genes for synthesizing recombinant proteins, recombinant insulins, pharmaceutical as well as therapeutic compounds, novel metabolites and other recombinant molecules. Genome editing in fungi has been initiated as a mutational approach to promote the discovery of new drugs and to enhance titers and the output of microbial processes.

Genome engineering in yeast

Saccharomyces cerevisiae, as a baker's yeast, is a safe production unit for the synthesis of pharmaceutical products. Advanced genetic information, rapid growth and cell density have become yeast's potential tools for the production of heterologous proteins and their extracellular secretion into a broth medium. Advances in molecular biology have made possible the heterologous expression of cloned mammalian genes into yeast, such as human epidermal growth factors (hGH), interferon, hemoglobin and more. Cloning surface antigens which encode the genes of the hepatitis B virus for the production of the hepatitis B vaccine are the first example of a recombinant vaccine produced in a yeast cell. This technology also aids in the improvement of fungal strains through engineering their genome.

Genetically engineering yeast by integrating the endoglucanase gene (of *Trichoderma reesei*) into its genome has imparted a remarkable characteristic to yeast strains, whereby it became capable of hydrolyzing the β -glucans of barley, resulting in reduced beer filterability. In the same way, the *Saccharomyces cerevisiae* strain was engineered to develop starch utilizing ability in it. The genetic transformation of wine yeast has been achieved by delivering gene- (from *Lactobacillus delbrueckii*) encoding enzymes involved in malolactic conversion into its genome, resulting in less acidic, enhanced flavored wine. Engineering the *Saccharomyces cerevisiae* genome has also been documented for lactic acid production by transferring a gene (from bovine muscle) encoding lactate dehydrogenase into its genome. Scientists of fungal biology have also attempted the synthesis of β -carotene in *Candida utilis*.

Genome engineering in filamentous fungi

Genetic engineering in filamentous fungi for synthesizing heterologous recombinant proteins is an intricate process compared to that of yeasts. In filamentous fungi, the level of heterologous protein production is much lower than homologous proteins. This may be due to the inefficiency of its cellular machinery for this purpose. Therefore, modulation of the cellular machinery of filamentous fungi has been attempted, in order to enhance the production of heterologous proteins in it. In order to improve the efficiency of engineered filamentous fungi for the enhanced heterologous expression of a cloned gene(s) into their cellular system, strategies have been established including the use of strong secretion signals, strong promoters of fungal origin, maximum copies of genes, and by constructing their protease deficient strains.

Genome engineering *Aspergillus nidulans* by replacing the promoter of a gene encoding ACVS has brought a 30-fold increase in penicillin production. The development of a recombinant strain of *Penicillium chrysogenum* by expressing the *cefEF* gene (from *Aspergillus chrysogenum*) and the *ceFE* gene (from *Streptomyces clavuligerus*) were able to produce adipyl-7-ADCA and cephalosporin intermediates adipyl-7-ACA. However, disruption of the *cefEF* gene adapted this strain to be able to produce high level penicillin titers. Another enzyme, recombinant amyloglucosidase, has been produced by dissecting the genome of *Aspergillus niger*. This recombinant enzyme breaks down unfermented dextrins and facilitates light beer production.

Key points:

- ❖ Recombinant DNA Technology is a remarkable development in the field of science and technology of genome manipulation for improving traits, and producing therapeutic proteins, novel metabolites, recombinant polypeptides, recombinant insulin and so on.
- ❖ Recombinant DNA technology has its part to play in all spheres of life.
- ❖ Genome engineering in plants, to make them resistant against biotic, as well as abiotic factors and to improve their yield and nutrition, is becoming a pressing need of this age, to feed a rising world population.
- ❖ Microbial genome engineering has improved microbe efficiency, such as cellular factories for synthesizing new metabolites and products by modulating their genetic systems.
- ❖ Microbes are genetically altered to synthesize recombinant polypeptides, compounds, therapeutic products etc. The recombinant insulin (Lispro), epoetin alfa (recombinant protein, used to cure anemia), cytokine myeloid progenitor inhibitory factor-1 and recombinant hGH, are a few of the success stories of this approach.
- ❖ Fungi (yeast and true filamentous fungi) are important in industrial biotechnology as well as agriculture. Genetic engineering in plants deals with incorporating the gene(s) into the genome to achieve upregulation in gene expression, downregulation in gene expression, and overexpression or the ectopic expression of a gene.
- ❖ Transgenic and cisgenic plants are developed against biotic (as in insect pests and diseases) and abiotic (heat, cold, drought, salinity) factors.
- ❖ Breeding and agronomic practices, chemical and biological control, are all traditional strategies for fighting phytopathogenic fungi.
- ❖ Enzymes involved in cell wall degradation in plant pathogenic fungi are an integral part of the plant defense mechanism.

- ❖ Antifungal gens, such as chitinases, chitosanases, and glucanases have been documented to be strong inhibitors of virulence proteins in fungal pathogens.
- ❖ Plants are genetically modified for insect resistance by introducing gene(s) into their genome, which either express protein inhibitors (such as α -amylase- and protease inhibitors) or express insecticidal proteins.
- ❖ Bt crops have been developed through genetic engineering, in which the Bt gene (*Cry* genes; *Cryl Ab*, *Cryl Ac*, etc.) were introduced into their genome.
- ❖ *Cry* genes encoding crystal proteins (prototoxins), are present in *Bacillus thuringiensis*.
- ❖ The (alkaline) pH of the insect gut supports and provides the medium to this toxin for action.
- ❖ This toxin perforates the insect gut by breaking the cell walls of the gut lining.
- ❖ The genome editing technique CRISPR/Cas9 has taken up a prime position in site-directed targeting of genomes. CRISPR/Cas9 based targeting of the *osERF922* (Ethylene responsive factor) gene sequence resulted in mutant rice plants, which showed resistance against *Magnaporthe oryzae* (for rice blast disease).
- ❖ CRISPR/Cas9 based targeting of the promoter of the *osSWEET13* gene for developing resistance against bacterial blight in rice.
- ❖ CRISPR/Cas9 based genome editing has been attempted for targeting three loci of *ML* homoeologous genes in wheat to develop resistance against powdery mildew disease.
- ❖ The genetic engineering of yeast by integrating the endoglucanase gene (of *Trichoderma reesei*) to hydrolyze β -glucans in barley reduces beer filterability.
- ❖ *The saccharomyces cerevisiae genome* has also been engineered by transferring a gene (from bovine muscle), and encoding lactate dehydrogenase, into its genome.
- ❖ Genetic engineering in filamentous fungi for synthesizing heterologous recombinant proteins is an intricate process compared to the analogous process in yeast.
- ❖ Strategies have been established for improving the efficiency of engineered filamentous fungi for enhanced heterologous expression of a cloned gene(s) into their cellular system, including the use of strong secretion signals, strong promoters of fungal origin, maximum copies of genes and by constructing their protease deficient strains etc.

APPENDICES

Appendix A

TAE Buffer

Na-EDTA

Tris-Base

Glacial acetic acid

d₃H₂O

Appendix B

LB Broth

Trypton

Yeast Extract

NaCl

d₃H₂O

Appendix C

Denaturation buffer

1.5 M NaCl

0.5 M NaOH

Appendix D

Neutralization buffer (pH 7.2)

NaCl

Tris HCl

EDTA

Appendix E

Prehybridization solution

6x SSC

5X Denhardt's solution

0.5 % SDS

50 % (v/v) deionized formamide

GLOSSARY

Adenylylation

Adenylylation is a process by which adenosine monophosphate is covalently linked to a protein side chain.

Alu PCR

In this type of PCR, Alu repeats based primers are used to characterize loci flanked by these repeats.

Arbitrary primed PCR

This type of PCR involves an arbitrary or random primer to amplify genome regions.

Asymmetric PCR

In this PCR, a primer pair is used in unequal concentrations, resulting in single stranded DNA as a PCR product.

Cisgenic Plants

These are the genetically modified plants developed by introducing the gene which is taken from the same species, e.g., if a gene is isolated from one variety of *Triticum aestivum* and is delivered into another variety of *Triticum aestivum*, then outcome is a cisgenic plant.

Constitutive promoter

Constitutive promoters are not influenced or regulated by any stimuli and derive genes by establishing a constant level of their expression in a cell.

Degenerate PCR

In this PCR, a degenerate set of primers of a known polynucleotide sequence are used to amplify an unknown polynucleotide sequence.

Ectopic expression

The expression of a gene at a site, or in an organism, other than its normal functioning site.

Forward genetics

Forward genetics is a functional genomics approach used to identify a DNA sequence related to a particular phenotype.

Gene Knock down

This is an approach in molecular biology by which the expression of a gene is reduced to the level at which expression as a phenotype is absent, although the gene is present into the genome.

Heterologous gene expression

Heterologous gene expression is the term given to the expression of those genes in an organism where it does not naturally occur. In other words, the introduction, and subsequent expression of a gene into a cell in which it is not naturally intended to be.

Holiday Junction

A holiday junction is a structure developed into a cross shape while homologous DNA strands separate for genetic recombination to exchange their segments.

Homologous gene expression

Homologous gene expression is the term given to the expression of those genes in an organism where they naturally occur. In other words, the introduction of a gene from one organism into another organism of same species, where it is then expressed.

Horizontal gene transfer

When a genetic exchange takes place asexually between unrelated organisms; this process is also known as *lateral* gene transfer. The relocation of genetic material from prokaryotes to eukaryotes and vice versa also comes under the term horizontal gene transfer. Agrobacterium infection is the classic model of this transfer.

Inversion

In cell biology, inversion is a phenomenon in which a segment of a chromosome breaks, rotates at 180° and then rejoins.

Satellite colonies

These are the small and weakened bacterial colonies (not having an antibiotic resistant gene, such as the bla gene containing plasmid), and surround large colonies that grow due to resistance against an antibiotic in a medium (which has an antibiotic resistant gene, such as bla gene containing plasmid). These untransformed bacterial colonies grow due to extracellular release of the β -lactamase enzyme from transformed colonies to the growth medium containing antibiotic, such as ampicillin. This enzyme degrades the antibiotic ampicillin in the medium and thereby supports the growth of satellite colonies.

Translocation

Translocation is a biological mechanism which deals with the swap of segments between two non-homologous chromosomes.

Vertical gene transfer

The transfer of hereditary material from the parent gene to the succeeding generation is referred to as vertical gene transfer.

Transgenic plants

These are genetically modified plants in which a gene(s) which is taken from a different source(s) is introduced into their genome to improve their genetic background for the purposes of increasing their economic worth.

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