Lecture

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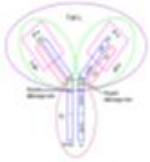
Immune system of domestic animals: An overview

Host' immune system in fight against foreign threats

When a clinician succeeds to treat a patient with an infectious disease	• Credit: 50% to the clinician & 50% to the patient's immune system
When a patient recovers from a viral disease under doctor's care	• Credit: 10% to the doctor & 90% to the patient's immune system
When we are being continuously exposed to pathogens and do not show clinical signs & symptoms	• Credit: 100% to our IS, almost all to the innate IS
When, despite threats, most of us do not suffer from cancers	• Credit: 100% to our IS, almost all to immunosurveillance
THE BOTTOMLINE:	• Immune system is indispensable to fight against 'foreign' threats

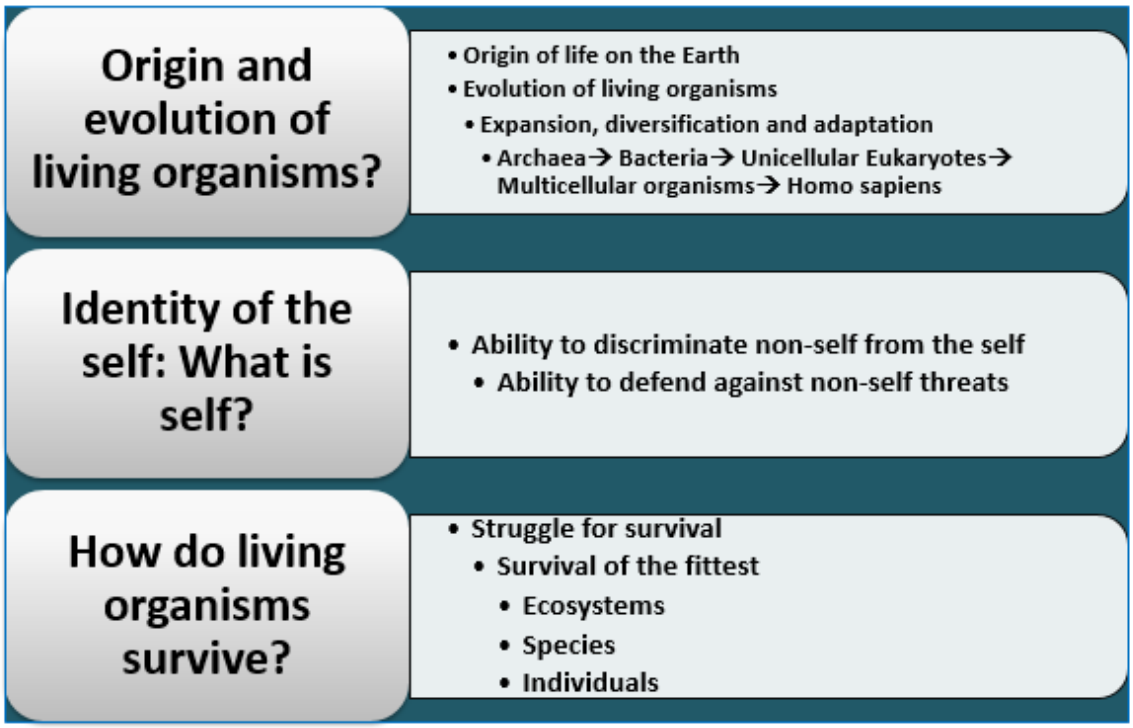
History repeats *mutantis mutandis*



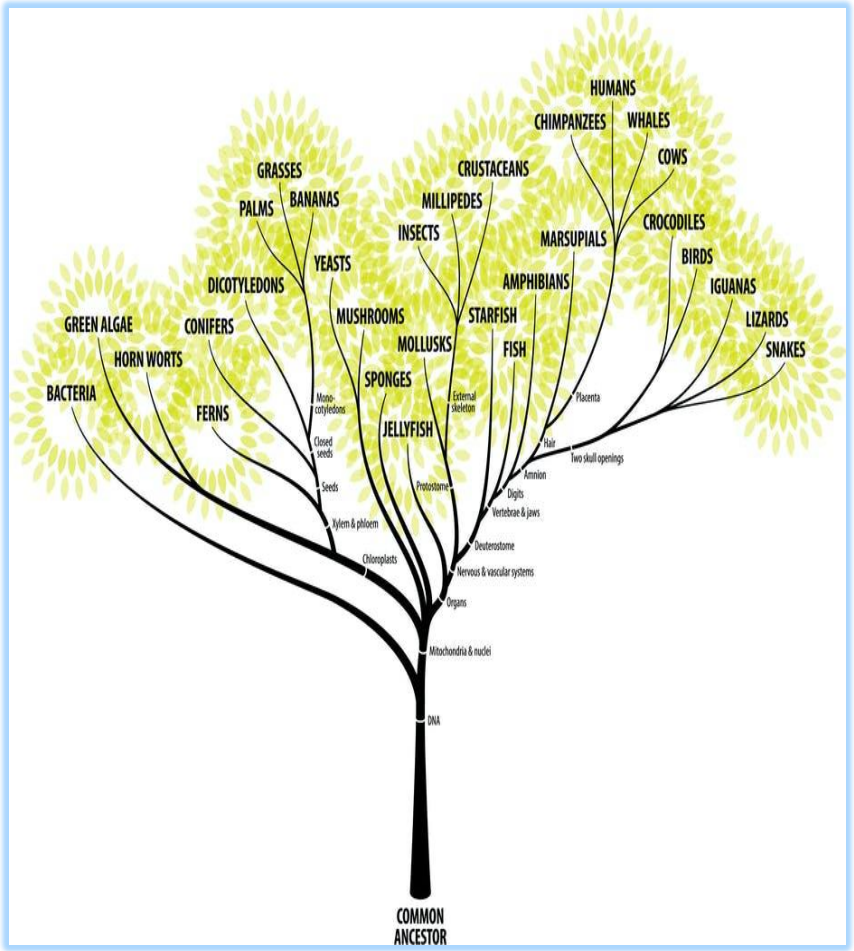
The Nobel Prize in Physiology or Medicine 1901 went to Emil Adolf von Behring

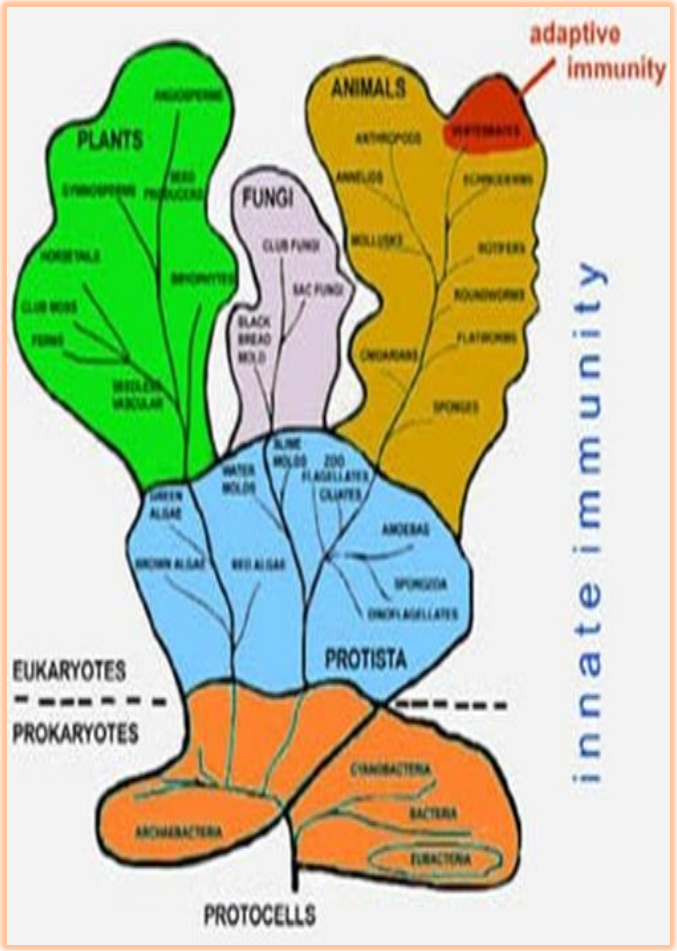
"for his work on serum therapy, especially its application against diphtheria, by which he has opened a new road in the domain of medical science and thereby placed in the hands of the physician a victorious weapon against illness and deaths"

Some basics questions about life are linked to the immunological phenomena



Higher the organism, more elaborate the immune system





Immunology: 'The study of the immune system and the threats encountered'

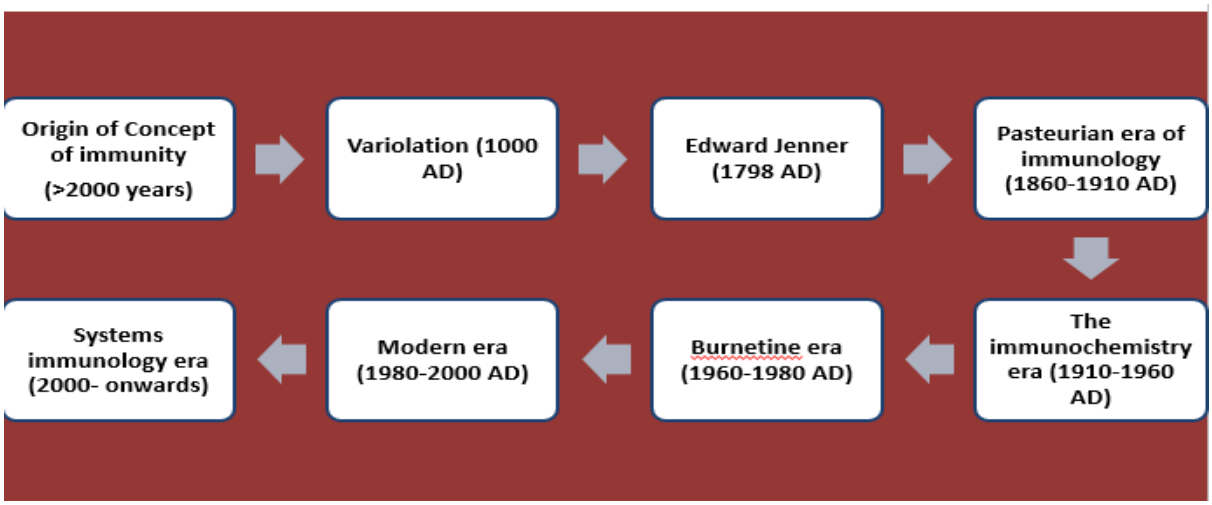
Immune system

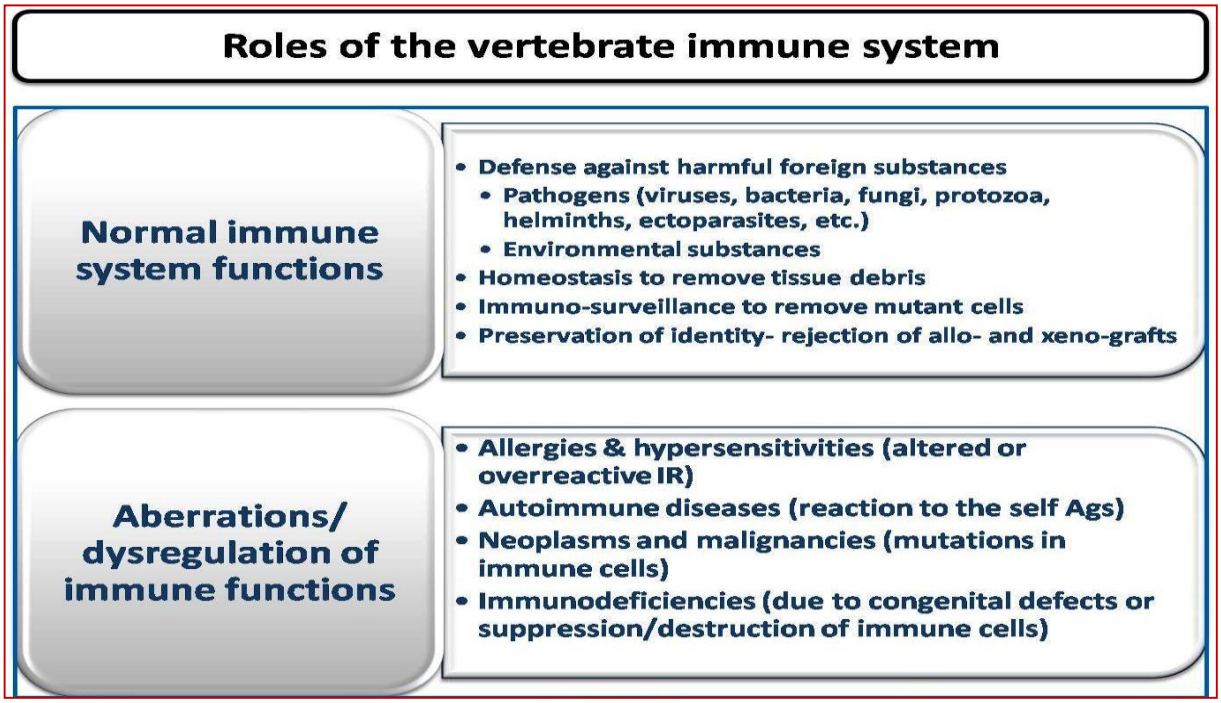
- Defense system of living organisms
- Has innate and adaptive arms
- Both arms present in only in vertebrates

Perceived threats

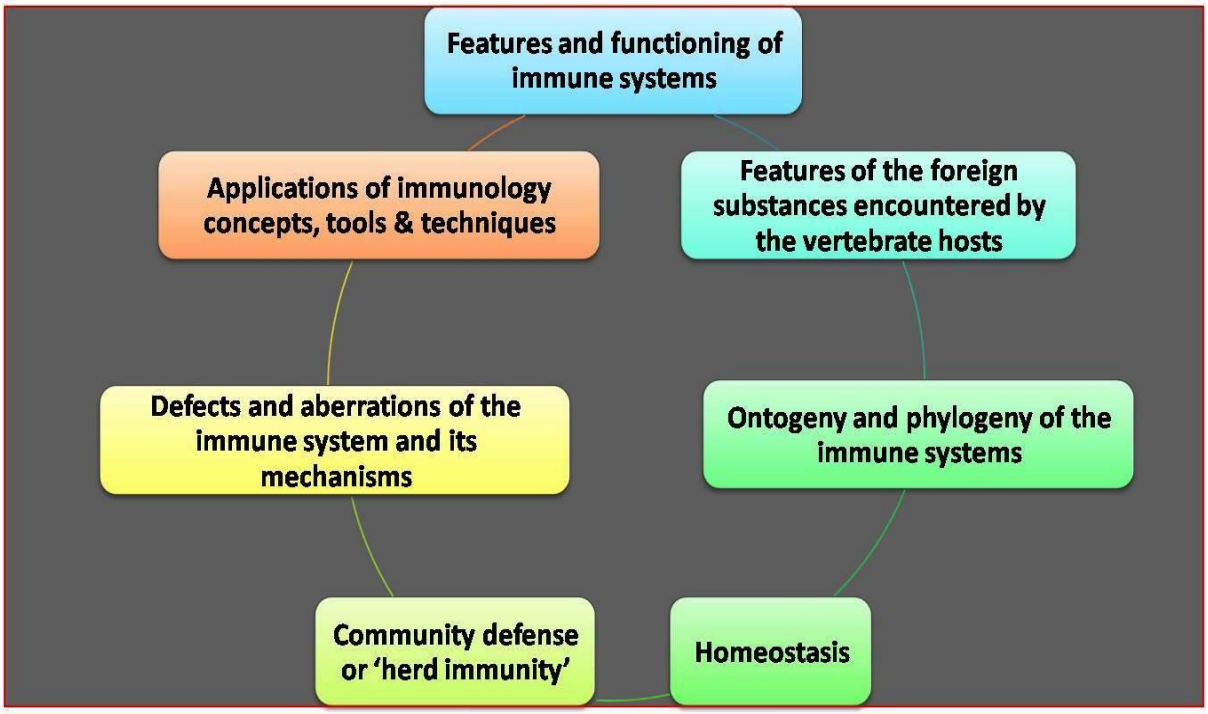
- Parasites (viruses, bacteria, fungi, protozoa, helminths, insects, etc.)
- Environmental injurious substances
- 'Not-self' threats residing or produced within immunologically marked 'self' (by education)

Conceptual growth of Immunology





The scope of Immunology



The first principles of immune phenomenon in vertebrates

- The vertebrates possess multilayered, multi-component and cooperative immune system
- Immunity is dichotomous: Innate and adaptive
- Immune system develops during fetal and neonatal life, and lymphocyte repertoires learn to be tolerant to the self
- Adaptive humoral and cellular immune responses are induced against foreign antigens
    - Involve complex molecular and cellular interactions
    - Become superior in magnitude and quality after repeated encounters with the same antigen.
    - Antigen-specific antibodies and T lymphocytes are the end products.
- Immune responses are under regulatory checks
- The immune system might go wayward and become aberrant in some circumstances

Immunology principles at work

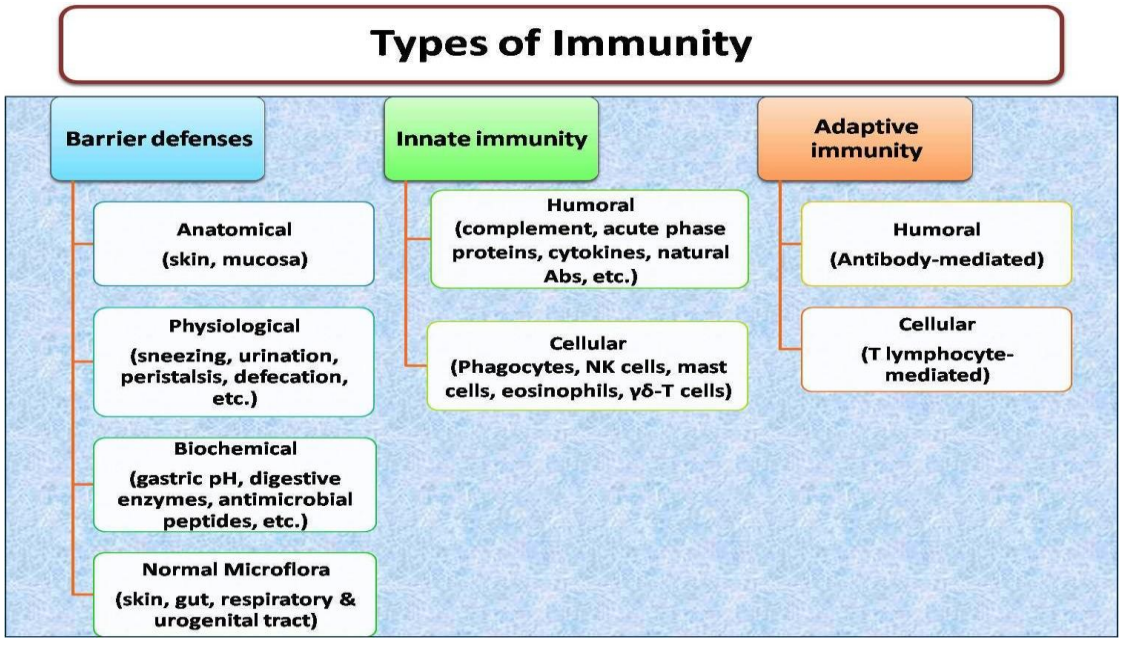
- Vaccines work through induction of protective or desired immunity (with memory response) in the host.
- Immunity is transferable from immune to non-immune animals.
- Immune responses can be altered, deviated, suppressed or deleted by drugs or other interventions.
- Innate and adaptive immune response parameters serve as phenotypic or functional markers of health, infection or disease state

- Immune responsiveness and disease resistance are ‘fitness’ traits controlled by multiple immune response alleles.
  Targets of breeding programme of farm animals.
- Pain and environmental stressors adversely affect immune functions through nervous-endocrine-immune interactions in animals
  Restored by drugs and other measures.
- Immune functions and immunocompetence i.e., ability to mount immune responses are affected by
  - Age, microflora, nutrition, production and reproductive stage of the animal
  - Require appropriate feeding and management practices**.**

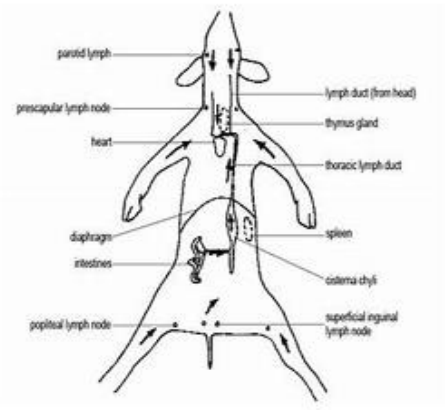
Sensing & responding to the ‘not-self’ is an essential feature of host’s immune system

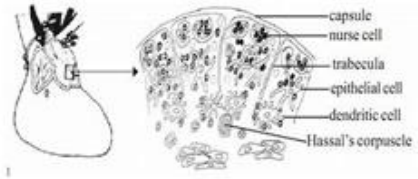
Not-self molecules/ Molecular patterns	Host’s response /sensor molecules				
	Innate (Non-clonal, germline-encoded)			Adaptive (Clonal repertoire)	
	Cell surface	Intracellular	Humoral	Cell surface	Humoral
B cell antigens	-	-	-	Ag-specific BCR clones	Antibodies
T cell antigens	-	-	-	MHC/non-MHC-Ag specific TCR clones	-
PAMPs/ MAMPs/ DAMPs	TLR1,2,4-6,10, Dectins, NK2GD	TLR3,7-9, RLRs, NLRs, DNA sensors	Comple-ment, APP, etc	-	-

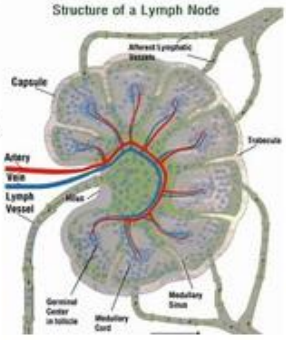
The multilayered, multi-component and cooperative immune system

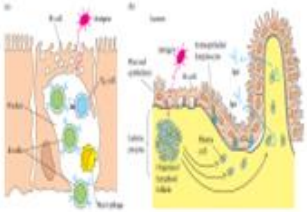


Immune system components: Organs, tissues, lymphatics, cells and molecules









Lymphoid organs & tissues:

- **Primary (Thymus & Bursa/Bone marrow**
- **Secondary: Spleen, Lymph nodes, Peyer's patches, etc.**

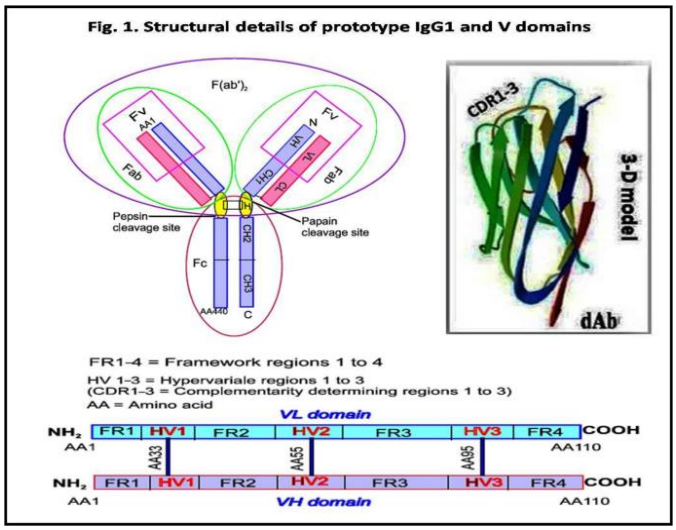
Cells and molecules

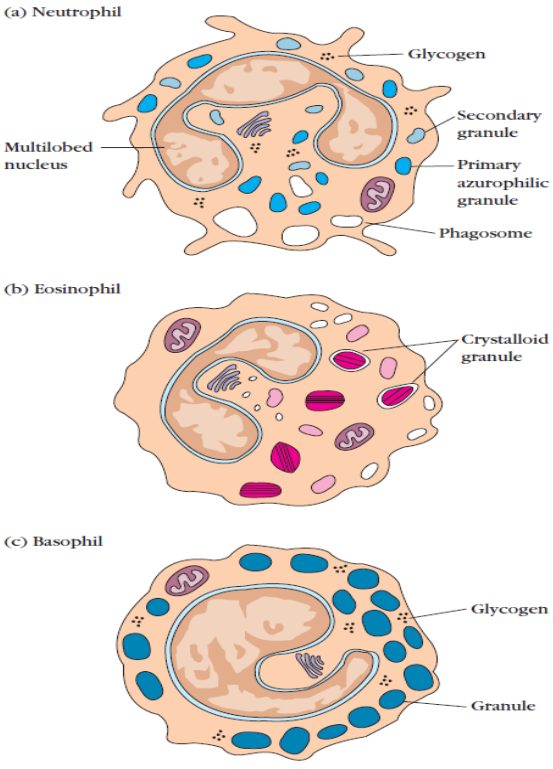
Cells of the immune system:

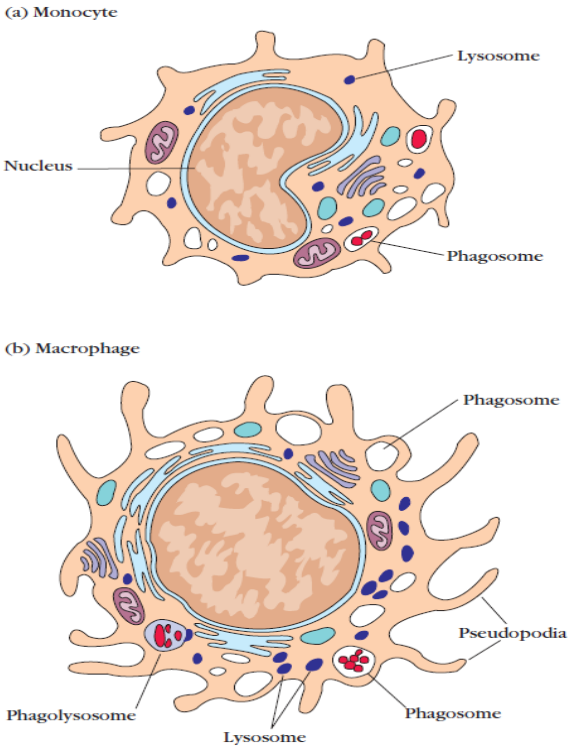
- Lymphocytes: B, T, NK
- Monocytes, Macrophages, Dendritic cells
- Granulocytes: Neutrophils, eosinophils, basophils, mast cells

Molecules of immune system:

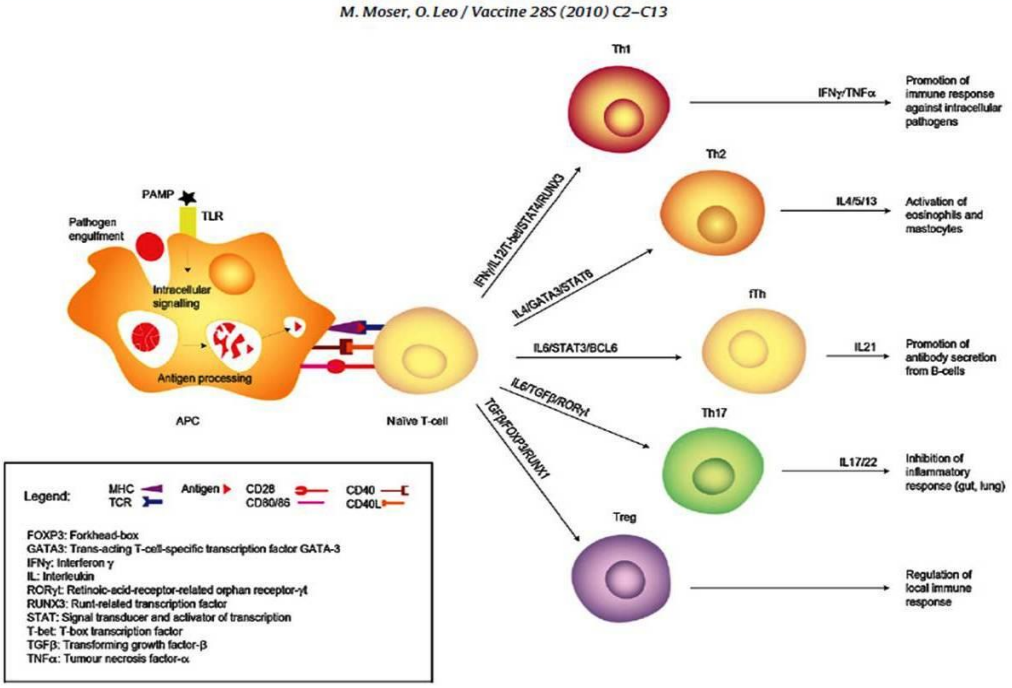
- Immunoglobulins
- TCR, MHC, Cytokines, C, so many
- PRRs
- Antigen



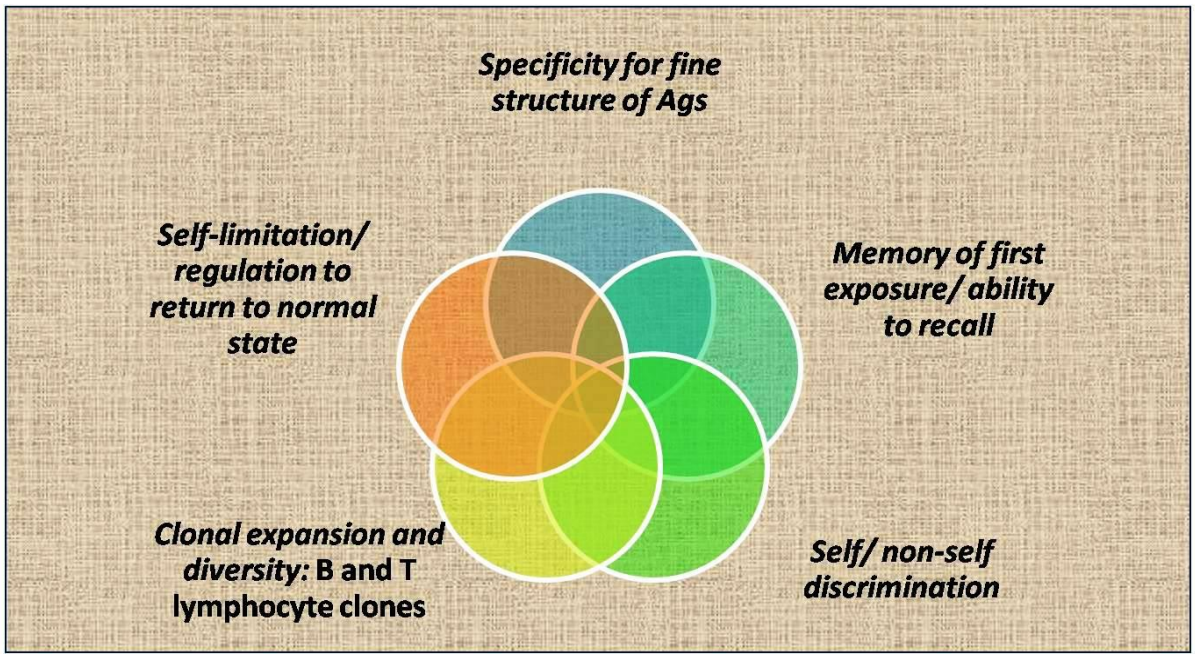




Linking IIS to AIS via PAMP-PRR interaction



Hallmarks/cardinal/essential features of adaptive immune responses



Types of Adaptive Immunity

Humoral immunity (Antibody-mediated)

Active
- Natural (e.g. recovery from infection)
- Artificial (e.g. tetanus toxoid vaccination)

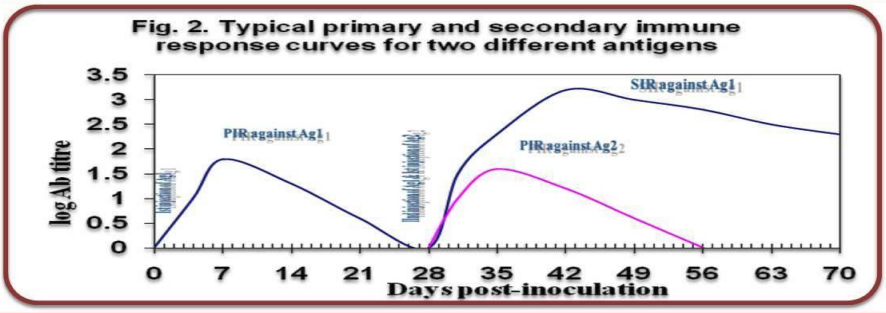
Passive
- Natural (e.g. suckling of colostrum and milk from dam)
- Artificial (e.g. Antiserum injection for treatment)

Cell-mediated immunity (T lymphocytes-mediated)

Active
- Natural (e.g. recovery from infection)
- Artificial (e.g. BCG Vaccination)

Passive
- Adoptive transfer of primed T lymphocytes

Types and nature of immune responses

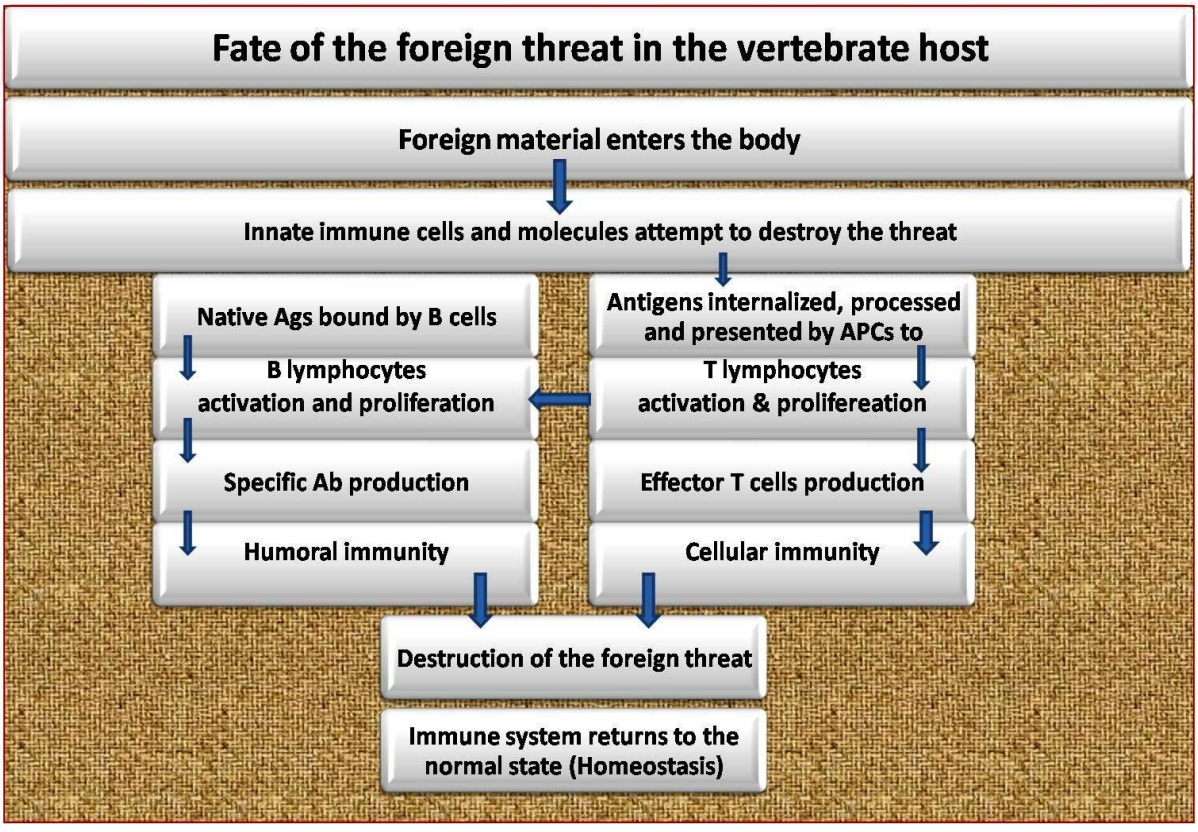


Adaptive response of a vertebrate host against an Ag leading to

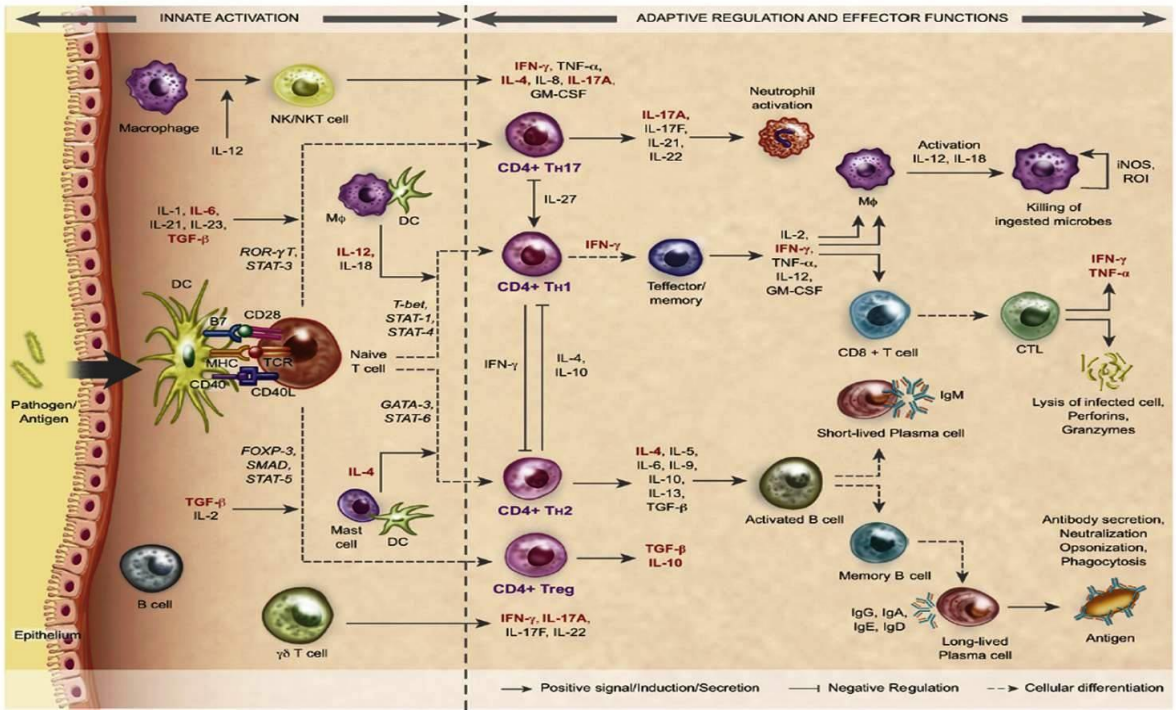
- Productive immune response: Specific Abs and/ or effector T lymphocytes are the end-products.
  - Primary IR
  - Secondary/anamnestic IR
- Null immune response: Ag-specific tolerance
- Autoimmune response: Abs and/or T cells against auto-Ags

Innate immune system vis-à-vis Adaptive immune system

Point of difference	*Innate immunity*	*Adaptive immunity*
Recognition system	Pattern recognition receptors: specific to conserved molecular patterns on pathogens	BCRs, TCRs, antibodies: specific to a chemical configuration i.e., epitopes on Ags
Manner of development	Germline-encoded, determined at species level, non-clonal	Somatically shaped by gene rearrangement of BCRs & TCRs in primary lymphoid organs
Extent of diversity	Limited no. of conserved molecules	Vast, in the order of $>10^{16}$ different clones
Immune tolerance to self	Not applicable	Clonal deletion & clonal anergy
Operation level	First line of defense	Appears later
Cellular immunity elements	Phagocytes, NK cells, etc.	Effector T lymphocyte subsets
Molecular immunity elements	Complement, cytokines, acute phase proteins, etc.	Antibodies
Memory	Absent	Present in form of memory B and T cells
Secondary response to the same foreign substance	Unchanged, because of lack of memory	Mostly of higher magnitude and different quality due to memory
Cognitive, activation and effector phases of response	Within a few minutes	Span a few days
Found in lower forms of life	Yes, in invertebrates	No, only in vertebrates



Different stages of immune response development against pathogens



Present status of understanding of the immune system

- **Historically, immunology principles mainly learnt from studies on mouse, other lab animals and humans**
- **Immune components and functions of domestic animals are phylogenetically related. For example, 'Buffalo is largely Cattle' for a traditional immunologist, vaccinologist and diagnostician**
- **Whole genome sequences & whole exome sequences of different species are becoming available**
- **Epigentic mechanisms for immune regulation are being explored**
- **Era of Computational Immunomics**
- For systems levels understanding of immune responses in individuals
- To predict immune outcomes of the antigenic/vaccine challenge
- 'Buffalo is Buffalo' for a genomicist, reverse vaccinologist and systems immunologist

Major applications of products, processes and concepts of immunology: Immunotechnology

Concept/product/process	Technology/ approaches	Applications	Impact
Antibodies, the 'magic bullets' & bio-imaging	**Animal immunization, hybridoma, recombinant DNA technologies, nanotechnologies**	**Serotherapy, serodiagnosis, passive immunization, theranostics**	**Protection and promotion of human and animal health**
Antigens/ Immunogens	**Culture, rDNA technologies, chemical synthesis**	**Modern vaccines, serodiagnosis**	**Eradication and control of infectious diseases of humans and animals**
Cells	**CAR-T cells, Dendritic cells**	**Cancer therapy**	**Fight against cancers strengthened**
Tissue antigens & MHC restriction	**Tissue matching, immunosuppression, immune tolerance**	**Organ/tissue transplantation**	**Growth of modern medicine**
Microbiomes & immunity	**Metagenomics, bioinformatics**	**Probiotics & immunobiotics**	**New approaches to protection and promotion of health**
Immune responsiveness & fitness traits	**WGS, Ir alleles, SNPs microarrays, NGS technologies**	**Genetic improvement programmes**	**Improved animal breeds**
Immune mechanisms at systems levels	**High throughput technologies, microarrays, NGS bioinformatics**	**Holistic view of immunity**	**Personalized medicine possible**

Summary

Immune systems evolved to defend against pathogens.
Immune systems have the capacity to discriminate between 'self' and 'not-self'.
Innate immunity precedes adaptive immunity
Adaptive immunity is dichotomous: a) 'humoral', b) 'cellular'
Fine specificity and memory are the distinguishing hallmarks of adaptive immunity.
Repertoires of B and T lymphocyte clones develop during fetal and neonatal life of animals
Antigens are 'not-self' organic substances recognized by cognate receptors on B and T lymphocytes.
Immune response is induced by Ag entry and develops in sequential phases: cognitive phase→ activation phase→ effector phase.
'Ag presenting cells' process and present Ags to specific T cells that help specific B cells to produce Abs and also to T cells for effector functions.
Clonal selection and expansion during activation phase is to produce sufficient Abs and effector cells for antigen disposal.
Immune mechanisms are under regulatory controls of cells and molecules operating at various steps.
Aberrant immune responses may occur in certain situations.

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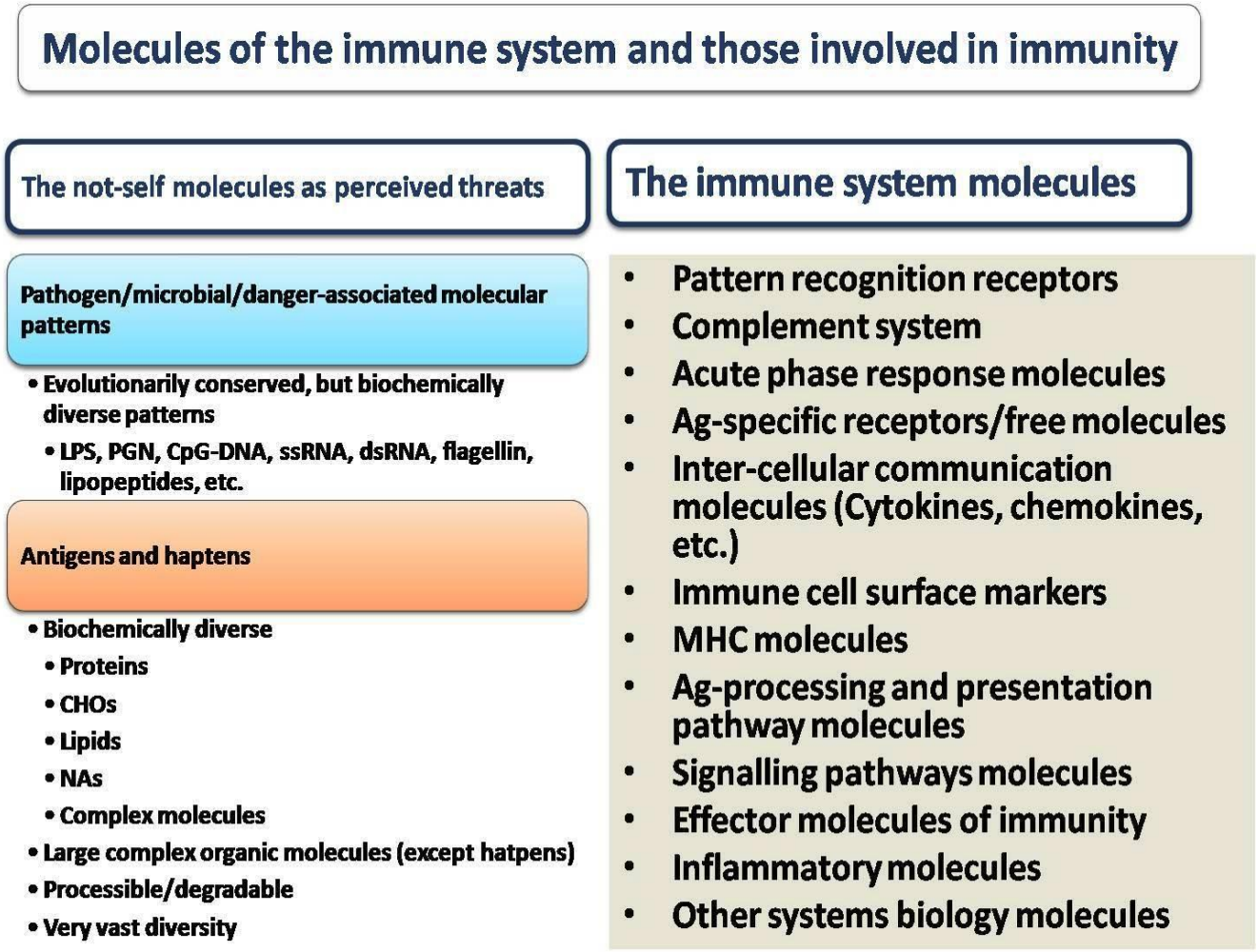
The extent and nature of the 'immunologically' foreign world



Sensing & responding to the 'not-self' is an essential feature of host's immune system

Not-self molecules/ Molecular patterns	Host's response /sensor molecules				
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	Cell surface	Intracellular	Humoral	Cell surface	Humoral
B cell antigens	-	-	-	Ag-specific BCR clones	Antibodies
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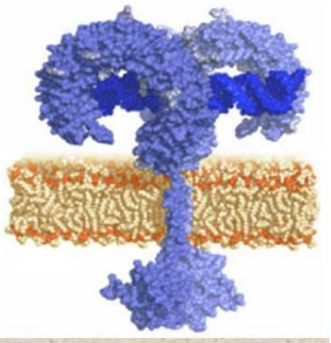
PAMPs/ MAMPs/ DAMPs	TLR1,2,4-6,10, Dectins, NK2GD	TLR3,7-9, RLRs, NLRs, DNA sensors	Comple-ment, APP, etc	-	-

Major PAMPs, their sources and TLRs for their binding

PAMP/MAMP	Source	TLR
Triacyl lipopeptides	Bacteria	TLR1-TLR2
Diacyl lipopeptides, LTA, Zymosan	Mycoplasma, bacteria, fungus	TLR2-TLR6
Peptidoglycan, lipoarabinomannan, Porins, tGPI-mucin, HA protein	Bacteria, Mycobacteria, Neisseria, Trypanosoma spp., Measles virus	TLR2
dsRNA	Viruses	TLR3
LPS, envelope proteins	Bacteria, MMTV, RSV viruses	TLR4
Flagellin	Bacteria	TLR6
ssRNA	RNA viruses	TLR7, TLR8
CpG-DNA, DNA, Malaria hemozoin	Bacteria, virus, Plasmodium	TLR9
?, Profilin-like molecules	Uropathogenic bacteria, Toxoplasma gondii	mTLR11

Toll-like receptors: 'Not-self' recognizing innate molecules

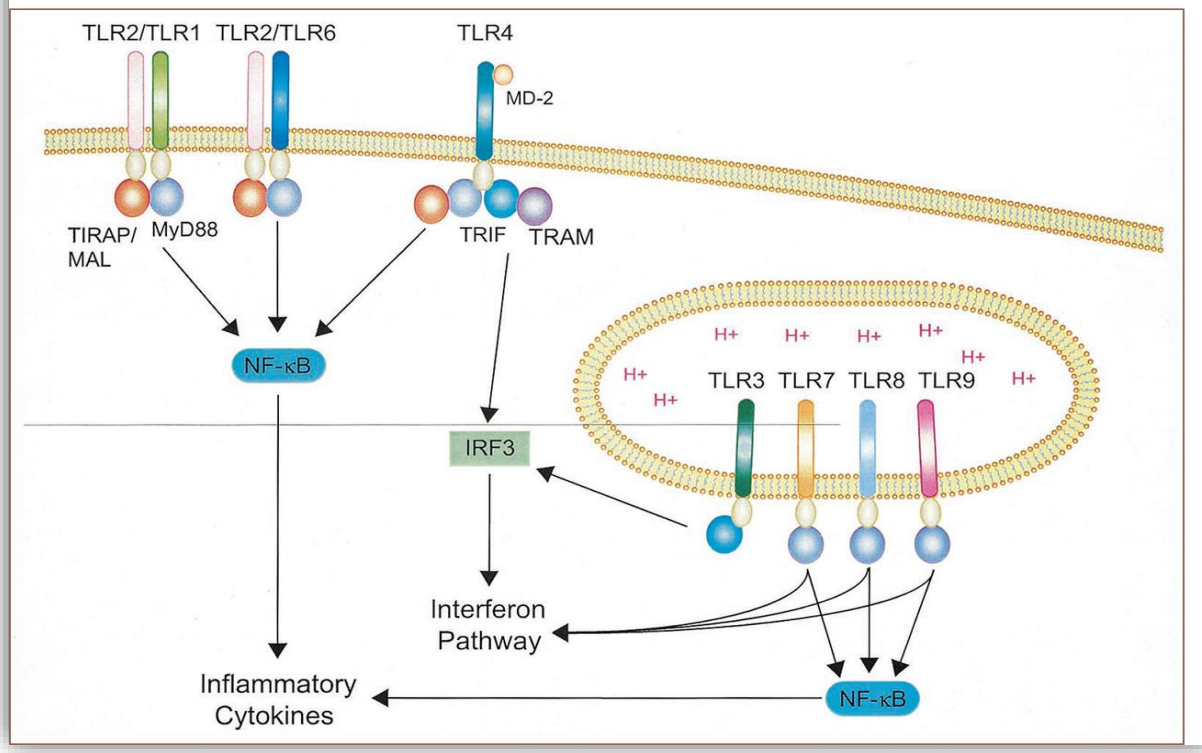
TLR discovery led to Paradigm Shift in Immunology



- **TLRs are**
  - **Evolutionarily conserved molecules**
  - **Cell surface or intracellular, preponderant on/in the innate cells**
  - **Pattern recognition receptors (PRRs) for PAMPs/MAMPs/DAMPs**
  - **Contain leucine-rich repeats in ectodomain for PAMP ligand-binding, transmembrane domain and intracytoplasmic domain for signal transduction**
  - **Involved in cellular activation, inflammatory response, enhanced phagocytosis, Ag presentation, T cell polarisation, etc.**
- **TLR agonists modulate innate & adaptive immune responses**
  - ✓ **Applications in vaccine and immunotherapeutic interventions**
  - ✓ **Species-specific TLR-mediated immunomodulation**

TLR SIGNALING PATHWAYS

[Boehme, KW & Compton, T. (2004). J.Virol. 78:7867-73]



Innate immune system in linked to the adaptive immune system

Takeda, K. & Akira, S. (2005). *Internatl. Immunol.* 17(1): 1-14.

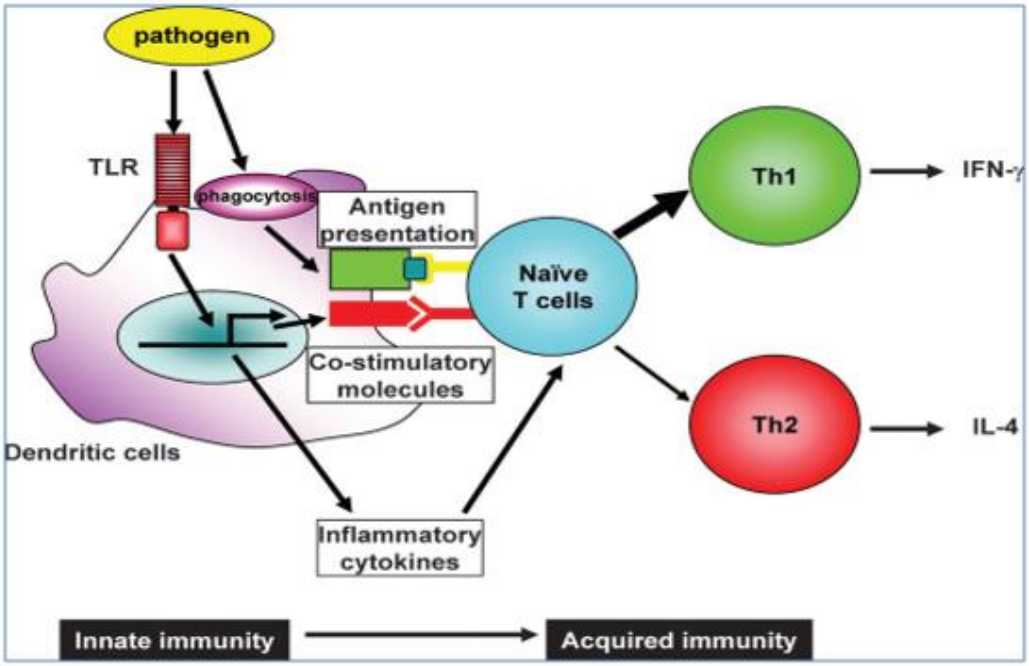


Table 1. Common PRRs in innate immunity

Items	PRR	Domains	Cellular distribution	PAMP	Sources	Signaling pathways
Toll-like receptors (TLRs)	TLR1 (TLR1–TLR2)	LRR domain–transmembrane domain–TIR domain (extracellular to intracellular)	Mo, DC, Ma, Eo, Ba	Triacyl lipopeptide	Bacteria	Most TLRs: MyD88-dependent pathways; TLR3: TRIF-dependent pathways; TLR4: MyD88-dependent pathways and TRIF-dependent pathways
	TLR2 (TLR1–TLR2, TLR2–TLR6)		Mo, DC, Ma, Eo, Ba	Lipoteichoic acid	Bacteria	
				Arabinomannan	Mycobacterium	
				Peptidoglycan	Bacteria	
				Zymosan	Fungi	
				Lipoprotein	Mycoplasma	
				Pore protein	Neisseria	
	TLR3		Mφ, DC, IEC	dsRNA	Virus	
	TLR4 (MD-2/CD14)		Mφ, DC, Ma, Eo	Lipopolysaccharides	Bacteria	
				Heat-shock proteins	Host	
	TLR5		IEC	Flagellin	Bacteria	
	TLR6 (TLR2–TLR6)		Mo, DC, Ma, Eo, Ba	Lipoteichoic acid	Bacteria	
				Peptidoglycan	Bacteria	
	TLR7		pDC, Mφ, Eo	ssRNA	Virus	
				Imidazoquinoline	Artificially synthesized	
	TLR8		Mφ, N	ssRNA	Virus	
	TLR9		pDC, Eo, Ba	Non-methylated CpG DNA	Bacteria, Virus	
	TLR10 (human)		pDC, Eo, Ba	dsRNA	Virus	
	TLR11 (mouse)		Mφ, DC	Profilin and related proteins	*Toxoplasma gondii*	
	TLR12 (mouse)		DC	Profilin and related proteins	*Toxoplasma gondii*	
	TLR13 (mouse)		Unknown	23s ribosomal RNA	Bacteria	
Nucleotide-binding oligomerization domain-like receptors (NLRs)	NOD1	LRR domain–NBD–effector domains	IEC, cytosol of Mφ	iE-DAP	Gram negative bacteria	RIP2-TAK1-NF-κB pathways
	NOD2			MDP	Gram-negative bacteria, Gram-positive bacteria	
RIG-I-like receptors (RLRs)	RIG-I	(RD)-CTD-DexD/H helicase domain–CARD	Cytosol	5′-triphosphorylated RNA, short-chain dsRNA	Virus	MAVS-TRAF6-NF-κB/TBK1 pathways
	MDA5			poly IC, long-chain dsRNA	Virus	
	LGP2			dsRNA	Virus	
C-type lectin receptors (CLRs)	Dectin-1	CTLD–ITAM	DC, Mφ	β-Glucan	Fungus	Tyrosine kinase-dependent and non-tyrosine kinase-dependent pathways
	Dectin-2			α-Mannan	Fungus	
Absent in melanoma-2-like receptors (ALRs)	ALRs	HIN-200-PYD	Cytosol	dsDNA	Bacteria	Inflammasome–pyroptosis

Antigens, immunogens, tolerogens, etc.

Antigen: A substance that can specifically bind with theproducts of the immune response, Ab and/or T cells.

- **Antigenicity**

Immunogen: A substance that provokes productive immune response.

- **Immunogenicity**

Tolerogen: An antigen against which a state of specific unresponsiveness or hypo-responsiveness develops in the host.

Allergen: Antigens that cause allergic reactions of immediate or delayed type.

Antigenic determinant/epitope: A small region or chemical configuration as an integral part of the antigenic molecule to which the Ab/immunocytes specifically bind.

- **Linear Ag determinants**
- **Conformational Ag determinants**
- **Neoantigenic determinants**

Hapten/incomplete antigen: A small synthetic or naturally occurring organic molecule that is unable to induce the production of antibody but can bind with specific antibody.

Superantigens: Antigens that stimulate multiple B or T cell clones to proliferate leading to extensive protective or harmful immune responses.

Heterophile Ags: Ag that stimulates the production of an Ab that cross-reacts with Ags present in diverge animal species, plants and microbial world.

Essential features of antigens

Diverse organic molecules of complex chemical nature:

- **Proteins, carbohydrates, lipids, nucleic acids as constituents of viruses, bacteria, fungi, parasites, plants, animals, etc. Inorganic salts are not antigens.**

Large size: Generally of >10 kDa size.

Structurally rigid or stable

Processible or degradable by host's enzymatic machinery

Non-self/ foreign

Shape: Globular proteins have conformational epitopes and fibrous protein have linear epitopes

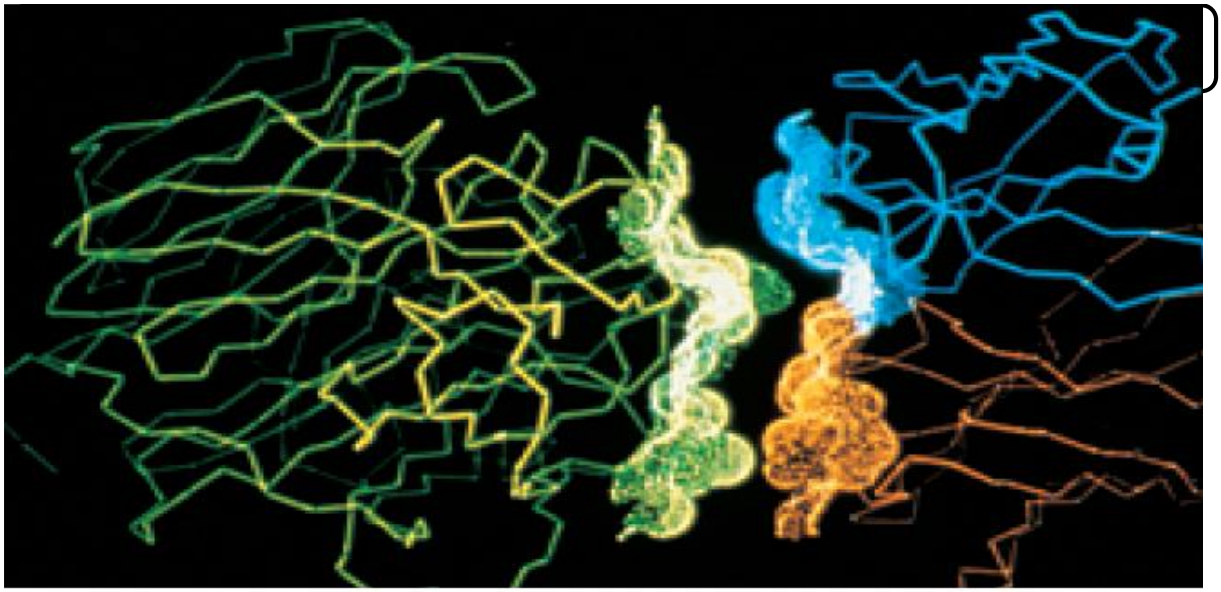
Chemical or physical treatment alters the immunogenicity with or without altering the antigenic specificity.

Trigger productive or null specific lymphocyte clonal immune response.

Recognized in their native state by B lymphocytes and in processed form bound to MHC molecules on APCs by T lymphocytes.

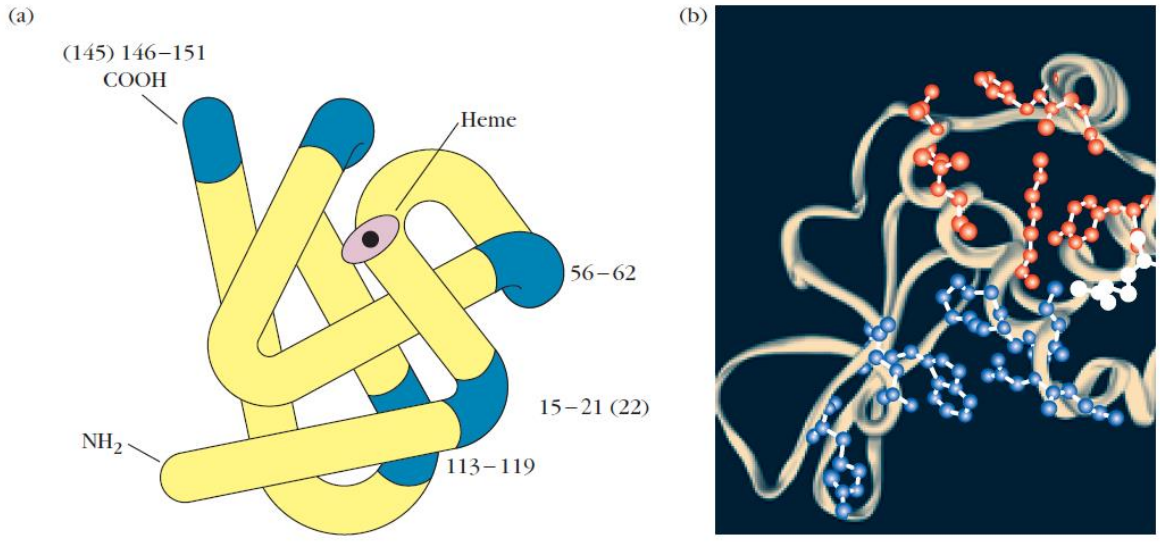
Classification of antigens

Antigen interaction with antibody: Epitope-Paratope

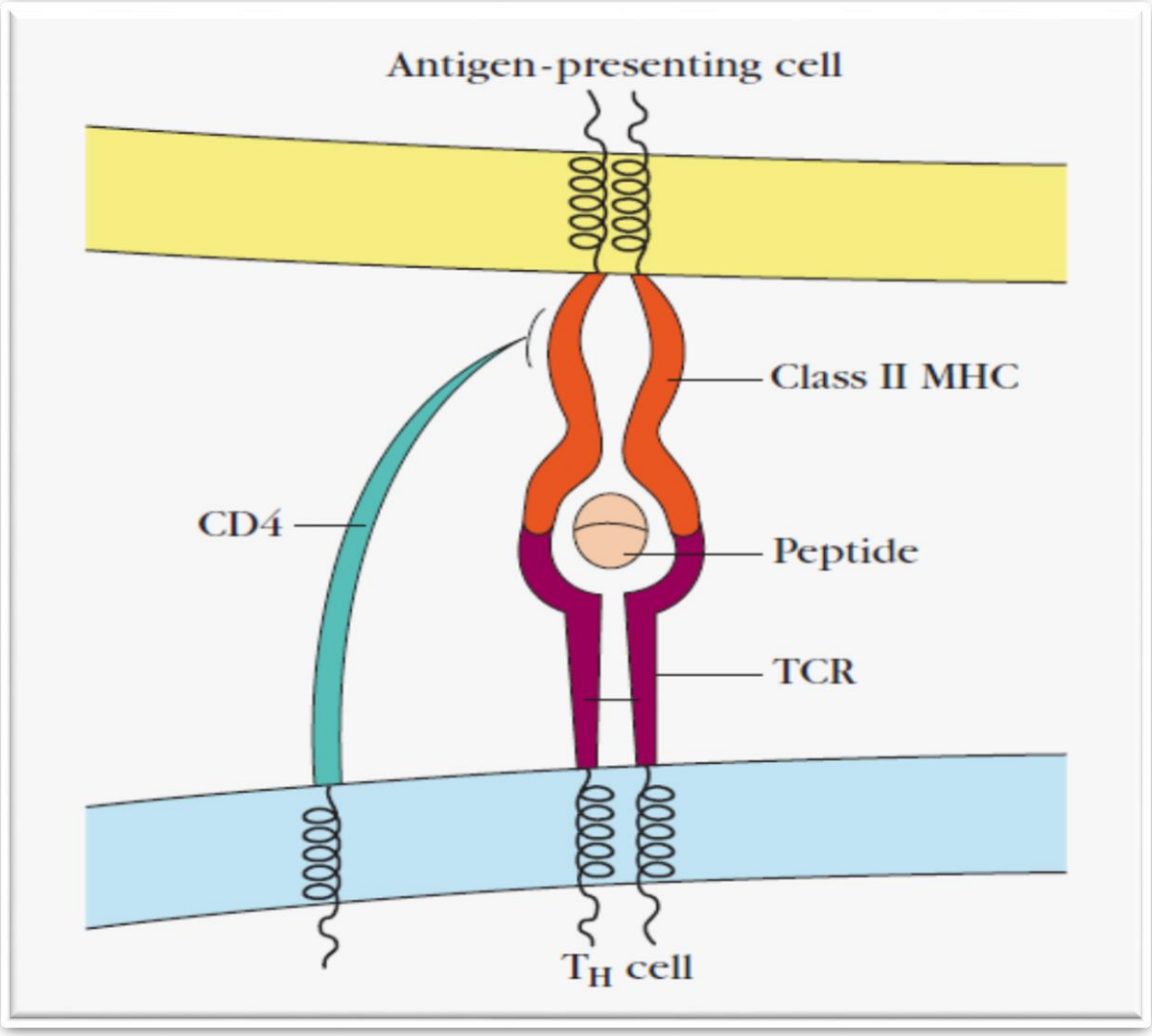


Complementarity of Interacting Surfaces of Antibody (left) and Antigen (right)

B cell epitopes: Linear epitopes (on myoglobin) and conformational epitopes (on lysozyme)



T cell epitopes: Antigen peptide presented by MHC on APC to be recognized by TCR on Th cell



Differences between T_{dep} and T_{ind} antigens

Point of difference	T_{dep}	T_{ind}
Chemical nature	Complex, degradable, mostly proteins	Simple, poorly degradable, repeating Ag determinants, mostly carbohydrates (LPS, levans, dextrans), D-amino-acid polymers, polymerized flagellin, etc.
T cell help for Ab production	Required	Not required
Memory developed	Yes, so secondary response produced	No, always a primary immune response
Ab class produced	IgG predominant	IgM predominant
Affinity maturation	Yes	No
Delayed type hypersensitivity	Produced	Not produced

Differences between B cell epitope and T cell epitope

Point of difference	B cell epitope	T cell epitope
Chemical nature	Small part of proteins, polysaccharides, glycolipids, nucleic acids, etc.	Peptides processed from proteins, rarely glycolipids or lipids
Size	4-6 amino acids; 2-4 monosaccharides, 2-3 nucleotides, haptens	8-10 amino acid peptide-MHC class I complex for CTLs; 12-18 amino acid peptide-MHC class II complex for Th cells
Specific receptors	BCRs on B cell clones as well as secreted Igs	TCR on Th, Tc, Tregs cell clones for dual recognition of epitope & MHC molecule
Structure	Linear or Conformational	Linear processed peptides
Binding site	BCR paratope made of 3CDRs each from VH & VL	TCR paratope made of 3CDRs each from Vα & Vβ or Vγ & Vδ
Recognition for immune response	Native form of antigen on B cells	Ag peptide-MHC class I or II association on Ag presenting cells required

Haptens are incomplete antigens

Small size chemicals, equivalent to epitopes on typical antigens

Bind with pre-formed Ab, but unable to induce its production unless bound to a carrier molecule

Examples

- Environmental chemicals
- Drugs, including antibiotics

Cross-reactive antigens

If two antigens of distinct origin possess common or structurally similar antigenic determinants, the antibodies produced against one tend to react with the other antigen.

The homologous antigen that induces Ab and the cross-reacting Ag (heterologous) antigen.

- Cross-reactions occur between phylogenetically related antigens
- Between substances of phylogenetically remote origin or even between substances that bear no relationship (heterophile antigens). A few examples of cross-reacting are:

Weil-Felix reaction is a cross-reaction between *Rickettsia* sp. and *Proteus sp.* OX-19.

Cross-reaction between *Treponema pallidum* and cardiolipin is used for diagnosis of syphilis by using cardiolipin as antigen.

Cross-reaction between feline infectious peritonitis virus and pig transmissible gastro-enteritis virus for diagnosis of FIP

Cross-reaction between pneumococcal polysaccharides and oxidized cotton cellulose. The presence of cello-biuronic acid is the basis of this cross- reaction.

Types of heterophile Ags

The Forssman system: (Forssman 1911)

Rabbit anti- g. pig kidney Abs cross-reacted with sheep erythrocytes. Chemically, Forssman Ag has ceramide tetra saccharide epitope.

- **F+ species: g. pig, hamster, mouse, sheep, goat, horse, dog, cat, chickens, pigeon, quail, some humans, *Bacillus anthracis*, spinach, etc.**
- **F- species: rabbit, rat, apes, monkeys, cattle, pig, frog, goose, beans, *Salmonella typhimurium*, etc.**

The Paul-Bunnel system: IgM Abs in infectious mononucleosis patients cross-react against sheep & bovine erythrocytes.

Two Ags: one on bovine erythrocytes only and the other on sheep & bovine erythrocytes have been detected.

The Hanganutziu-Deicher (H-D) system: Found in tissues of all mammals except humans. Chemically, it is N-glycosylneuraminic acid and usually on glycoprotein of sheep and cattle erythrocytes.

Blood group antigens of domestic animals

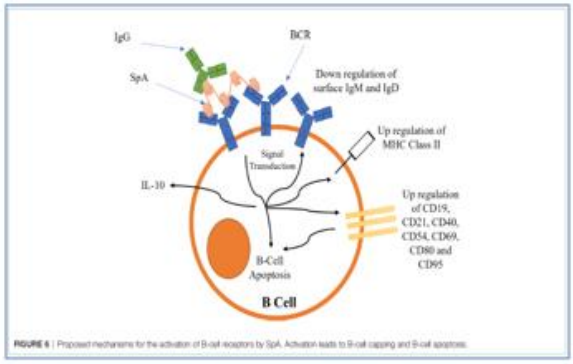
Glycoprotein or glycolipid antigens on erythrocyte surface
Exist as alleles and inherited in Mendelian manner.
Multiple systems exist in each animal species, each animal expressing a combination of blood group Ags. At population level, millions of different sets of blood group antigens.
Some blood group Ags are more potent (so more important clinically) than others.
Physiological functions of most are unknown; some are involved in transport of ions or other small molecules through erythrocyte membranes.
Natural antibodies to some blood group Ags in animals lacking those Ags, can cause transfusion problems in recipient animals.
The hemolytic disease of foals is more important than other animals.
May also be used for parentage testing in forensic medicine.
Serological tests such as hemagglutination, hemolysis and Coombs' test are often used to detect blood group Ags in various animal species.

Blood group antigens of domestic animals

Animal species	No. of blood groups	Important blood group	Natural Abs	Detection method(s)
Cattle	11	B, J	Anti-J in J- animals	C'-mediated Hemolysis
Sheep	7	B, R	Anti-R in R- animals	C'-mediated Hemolysis, Agglutination
Pig	15	A, O	Anti-A in A- animals	C'-mediated Hemolysis, Agglutination, Coombs' test
Horse	7	A, Q		C'-mediated Hemolysis, Agglutination
Dog	11	A, Tr	Anti-A in only some A- animals	C'-mediated Hemolysis, Agglutination, Coombs' test
Cat	1	AB	Anti-A in most B+ cats & Anti-B in some A+ cats	C'-mediated Hemolysis, Agglutination
Chicken	12	B		Agglutination

Super antigens

- **T cell superantigens**
  - ***S. aureus* enterotoxins**
  - ***S. aureus* toxic shock syndrome toxin (TSST)**
- **B cell superantigens**
  - ***S. aureus* protein A**



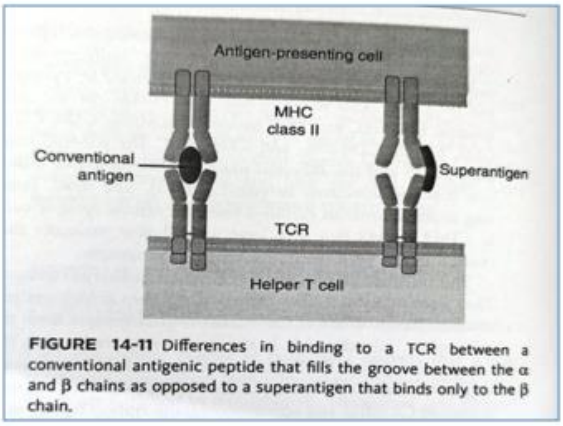


FIGURE 14-11 Differences in binding to a TCR between a conventional antigenic peptide that fills the groove between the α and β chains as opposed to a superantigen that binds only to the β chain.

Mechanism of super antigen action on T and B cells

Super antigens are unconventional antigens

- Recognize immune receptors outside their usual recognition sites e.g. CDRs, to elicit a response within the target cell.

T-cell super antigens crosslink T-cell receptors and MHC Class II molecules on APCs, leading to

- lymphocyte recruitment
- induction of cytokine storms
- T-cell energy or apoptosis among many other effects.

B-cell super antigens bind Igs on B-cells, affecting

- Opsonisation
- IgG-mediated phagocytosis
- Driving apoptosis.

Antibody-binding super antigens have been exploited extensively in industrial antibody purification

- Protein engineering to optimize the use of super antigens various areas of biotechnology.

Finding suitable Ags of the infectious agents is technologically challenging for diagnostics and vaccinology

Ags may be shared among phylogenetically related organisms

- Specific immunoassay should target unshared Ags
- Broadly specific IAs should target shared/cross-reactive Ags
- Each Ag from infectious agents are often large and multi-valent, carrying several epitopes recognized by B, Th and CTLs
- Some epitopes of each Ag may be shared and others are unique

Subunit vaccine Ags (or NA) should be conserved and non-variants or

- Target broadly cross-reactive epitopes on the variable Ags

Multi-epitope vaccines and structural biology are modern concepts

- Include B, Th, CTL epitopes predicted by AI and MLAs

Ags being largely proteins can be produced by rDNA techniques or chemically synthesized

Lecture

Dr. Ajit Singh, PhD, PDF(UK), Former Professor & Emeritus Scientist (ICAR), LUVAS, Hisar-125004 (Haryana)

Current status and challenges in diagnosis of infectious diseases of animals

Infectious diseases

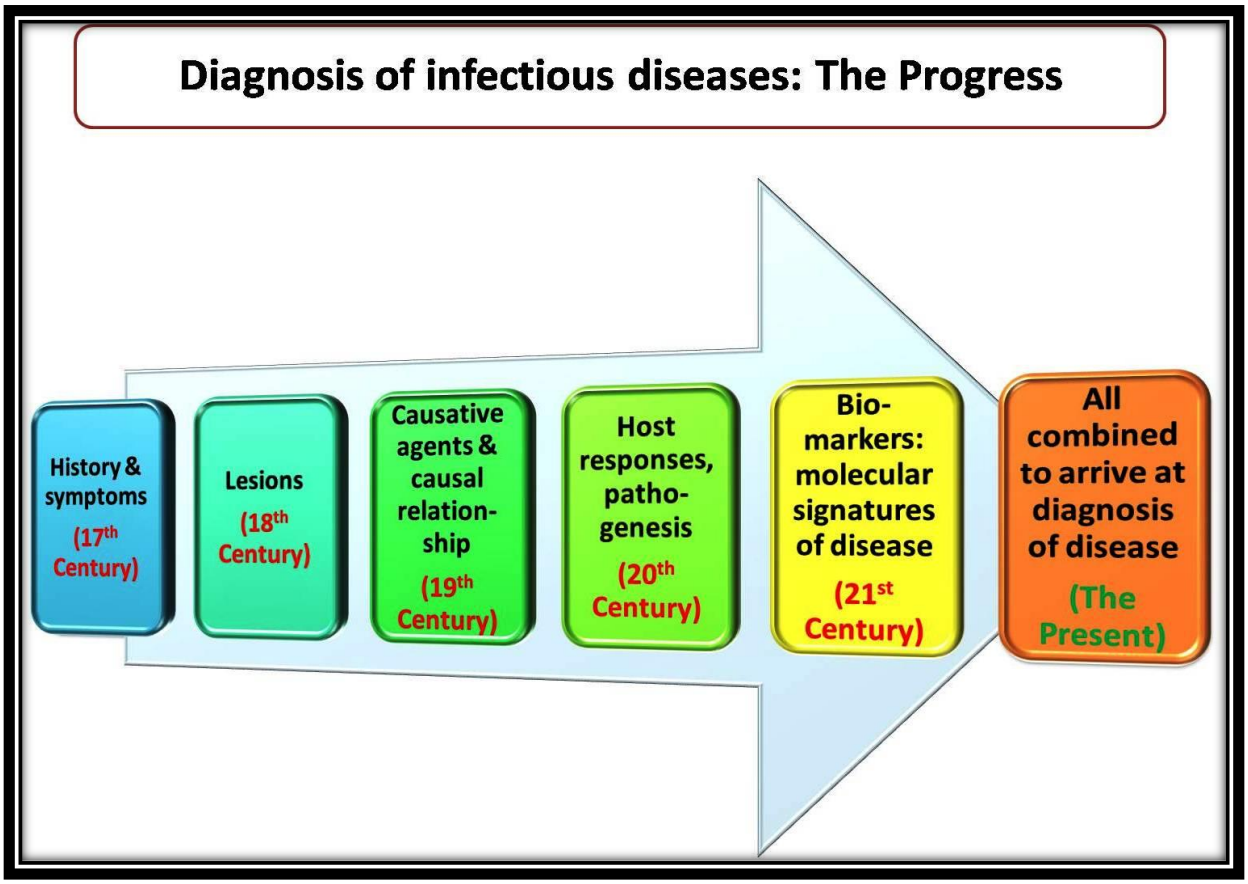
Viruses, bacteria, fungi, protozoa, etc. as causative agents

Leading cause of loss of life, suffering, and production

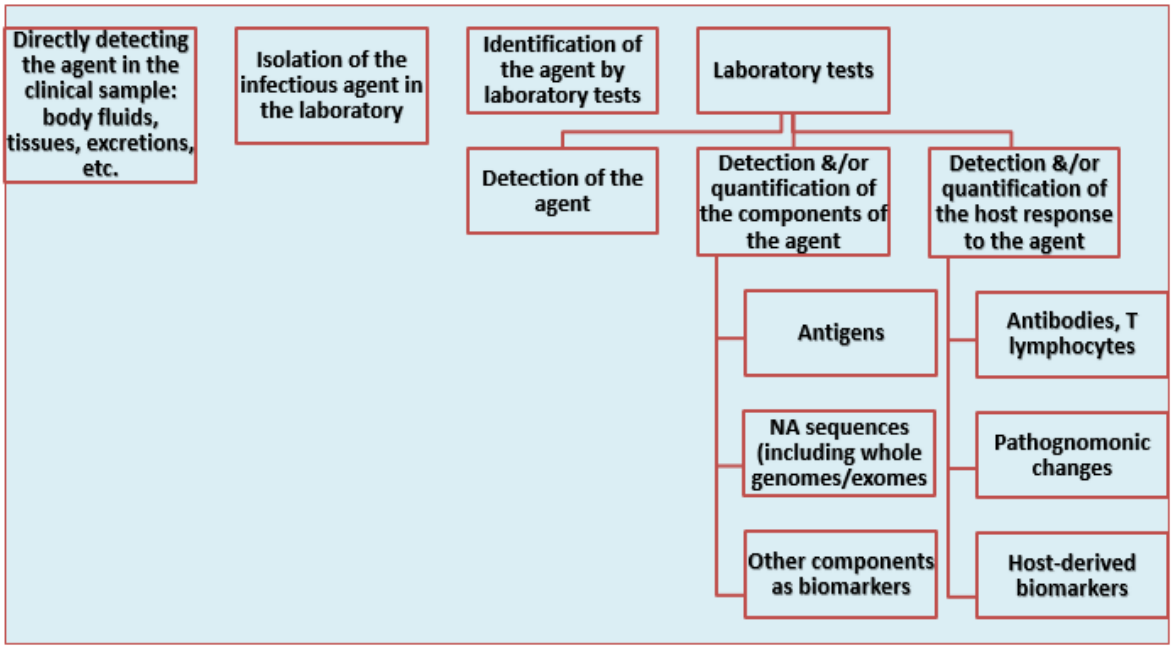
- Disease burden is increasing with newly emerging and re-emerging pathogens and antimicrobial resistance.
- Nature and human activities are fueling the increased pace of emergence and spread of these biothreats

Preparedness is required for facing these challenges

- Diagnostic tests are part of the toolkit for managing and controlling the infectious diseases
- Immunodiagnostic tests are a major contributor to the infectious disease management and control



The current scenario of diagnosis of infectious diseases



Immunological methods of infectious disease diagnosis: History is witness to their central role

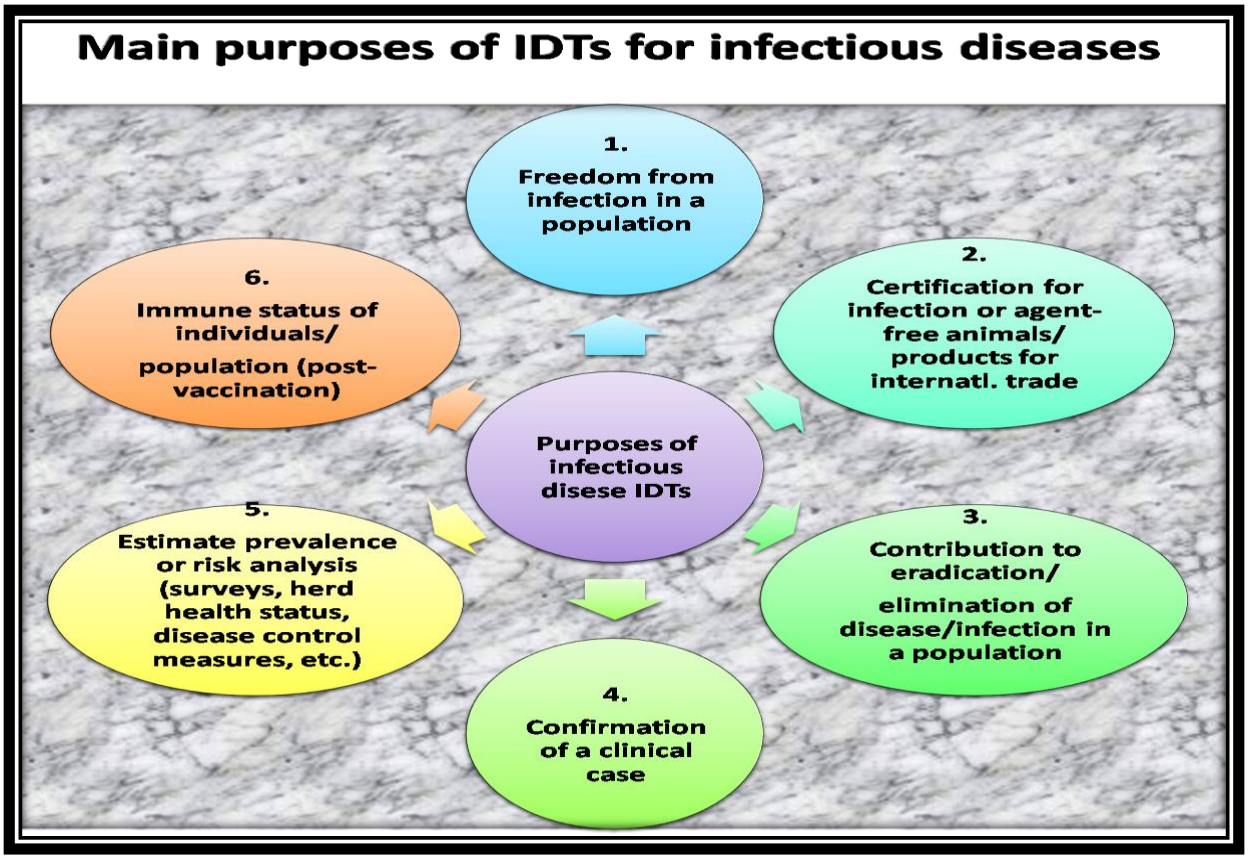
Among the oldest of all methods for definitive diagnosis

Established conceptual framework

Straightforward to do sampling of antisera

Played/continue to play role in

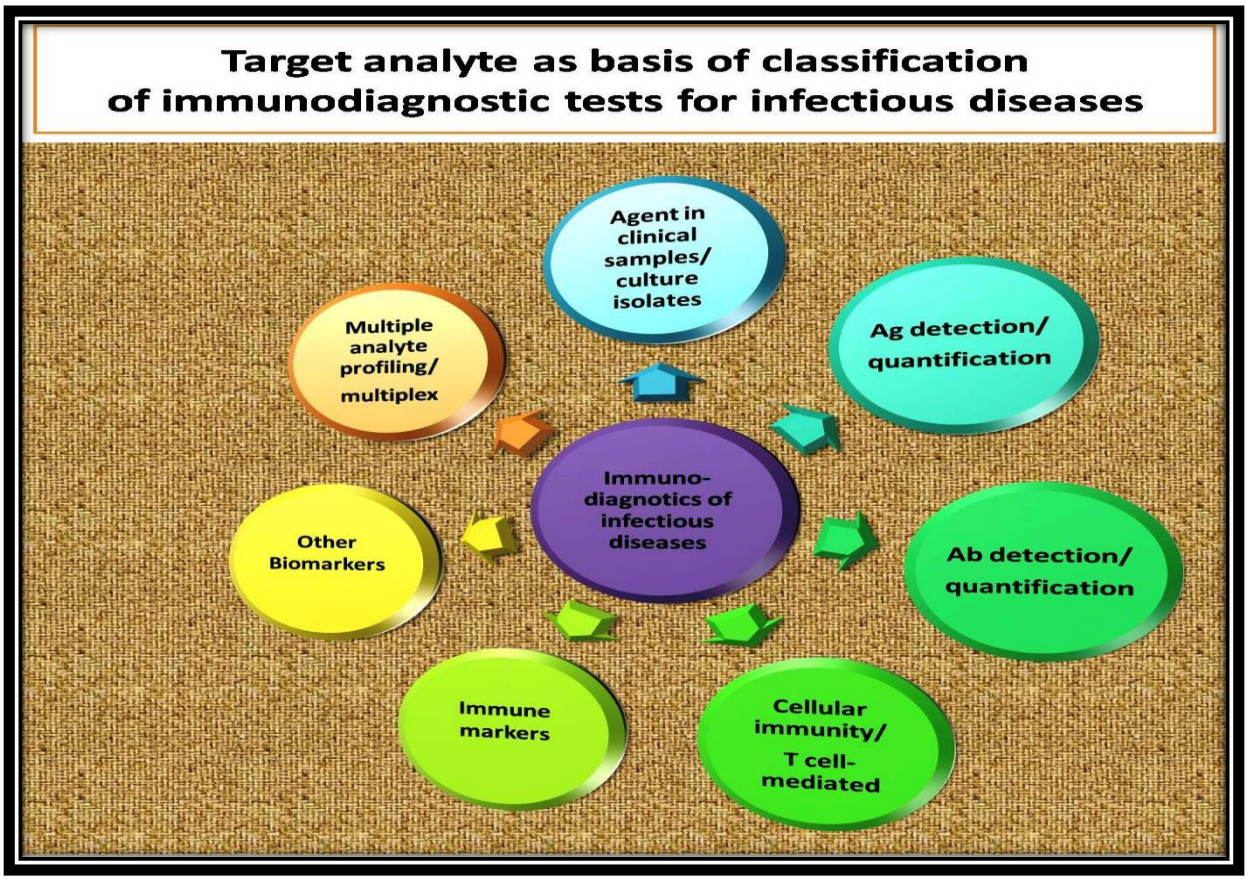
- Management of clinical cases
- Containment of outbreaks, epidemics & pandemics
- Epidemiology of the diseases
- Vaccination monitoring and herd immunity status
- Certification for freedom from disease/infection
- Implementation of control, elimination and eradication of infectious diseases
- Food safety & public health
- Environmental screening, risk analysis, etc.

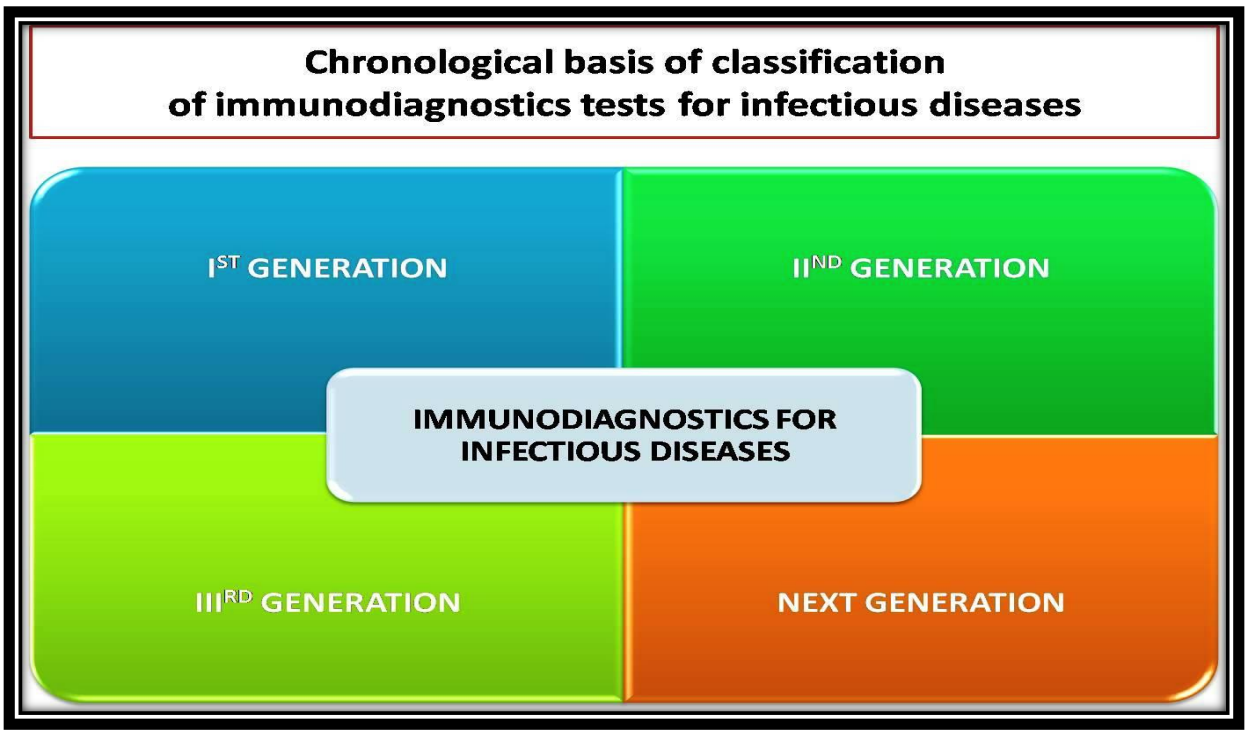


Immunodiagnostic tests (prescribed & alternative) for OIE listed infectious diseases required for export/import or movement of animals

ID test	Infectious diseases of domestic animals
AGID	BT,, Enz. Bov. leukosis, CAE, Maedi-visna, EIA, IBD, MD, ILT, AI, Myxomatosis
AGGL.	Brucellosis in bov., cap., ov. & pig, *M. gallisepticum*, Fowl typhoid, Pullorum
MAT	Leptospirosis
HI	Rift Valley fever, EEE, WEE, VEE, Einf, *M. Gallisepticum*, IB, AI, ND, Rab. Haemor. Dis.
CFT	FMD, VS, Brucellosis, CBPP, CCPP, Enz. ewe abortion, EEE, WEE, VEE, AHS, Glanders, Myxomatosis
VN	Auj. Dis., Rabies, FMD, VS, RP, BT, Rift Valley fever, IBR, PPR, Sheep pox and goat pox, Eq. rhinopneum, Eq. viral arteritis, AHS, TGE, Swine vesic. Dis., IB, ILT
PRN	EEE, WEE, VEE
FAVN	CSF
NPLA	CSF
FPA	Brucellosis,
IFAT	ASF, Myxomatosis
ELISA	Auj. Dis., Rabies, Paratuberculosis, FMD, VS, RP, BT, Rift Valley fever, Brucellosis, Enz. Bov. leukosis, IBR, CBPP, CAE, Maedi-visna, PPR, EIA, AHS, TGE, Swine vesic. Dis., ASF, CSF, IBD, IB, ILT, Rab. Haemor. Dis.
DTH	Paratuberculosis, Bov. tuberculosis, Cap. & ov. Brucellosis (-*B. ovis*), Glanders
IFN-γ	Bovine tuberculosis

Classification of various immunodiagnostic tests

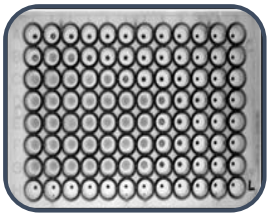


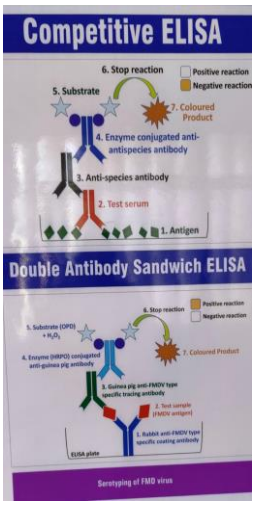


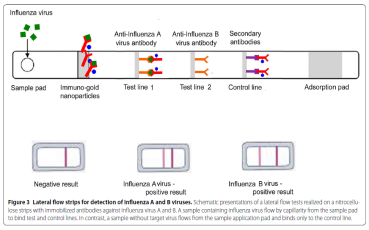
Immunodiagnostic tests

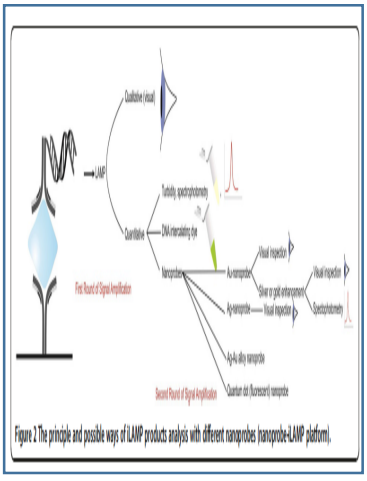
Ist Generation	IInd Generation	IIIrd Generation	Next Generation
•AGID, CIEP •Bact. Agg., HA/HI, LAT, Co-agg. •CFT •DTH	• FIAs: FAT, FLISA, TR-FIA, etc. • RIAs • EIAs: EMIT & ELISAs • IHC: IPT staining, etc. • VNTs: CPE red. PR/PI, FFI • IEM	• WB • IFN-γ assay • Simple 'POC' tests: • Dot-ELISA, LAT, LFDs • Flow cytometry • ELISPOT assay • Immuno-PCR/Novel ELISA-like IAs • FPA, FRETIAs • Particle-enhanced turbid. or Nephel. agg. reaction-based assays: PETIA, PETINIA, PACIA, QUELS, etc.	• Immuno-proteomics & MS • LFB-based POCs • PAD-based POCs • Immunosensor-based POCs • Microarrays & Nanoarrays • Micro-bead based MAPs • Smartphone-based POCs • Microfluidics LOC/POC- IAs • Single molecule detection IAs

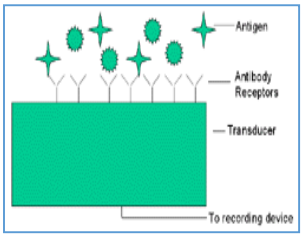
Progress in development of immunodiagnostic tests: Representative examples of generation I-IV IDTs

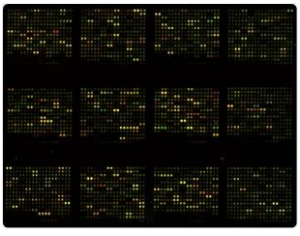












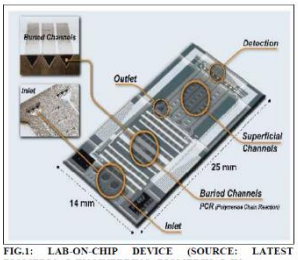


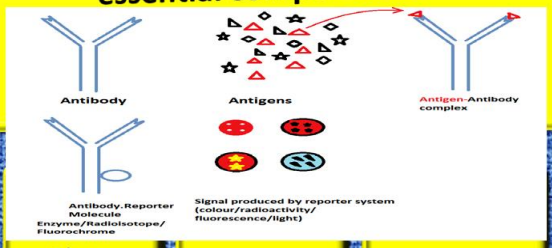
FIG.1: LAB-ON-CHIP DEVICE (SOURCE: LATEST BIOMEDICAL ENGINEERING: BIOMEDIKAL.IN)

ESSENTIAL FEATURES OF AN IMMUNODIAGNOSTIC TEST

Definition:

'Detection and quantification of an analyte (Ag, Ab, T cells, biomarker) via a signal generated by immunological interaction'

An immunodiagnostic test or immunoassay has five essential components:



- Ag (or biomarkers in some cases)
- Ab or specific T lymphocytes in some cases
- The reporter system
- Signal
- Signal recording and analysis device/ equipment

Immunological tools for diagnosis of infectious diseases: A. Basic immunological reactants

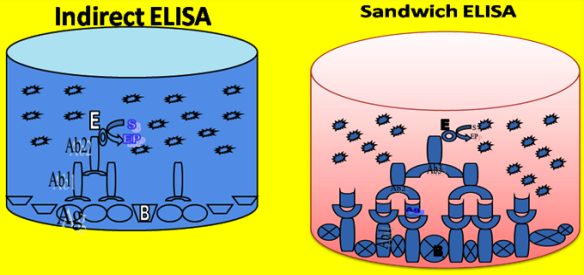
Antigen	Immunologically detectable biomarker (Host-origin)	Antibody	Ag-specific T lymphocytes
Infectious agent	Cellular: Leukocyte/ lymphocyte phenotypic/ functional subsets	HIS, antisera, convalescent sera	Helper T lymphocytes: Th1, Th2, Th17, Tregs, Memory T cells, etc.
Extracted/lysate/ released Ag	Molecular: Cytokines, other molecules	Monoclonal antibodies	CTLs
Recombinant Ag/ peptide	-	Recombinant Abs/Ab fragments (scFv, dAbs, etc.)	γδ-T lymphocytes
Aptamer/Mimotope/ synthetic peptide	-	Affibodies/ aptamers	-

Immunological tools for diagnosis of infectious diseases: B. Various labels and the signal generating/reporter systems

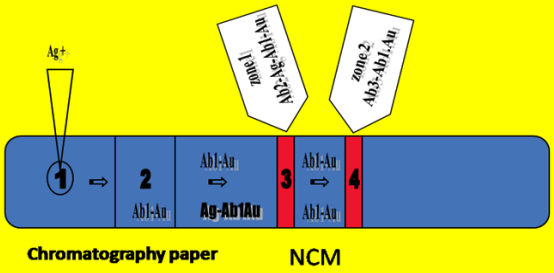
Labels	Signal generating system	Signal	Signal detector & recorder Equipment/ Device
None	Lattices of Ag-Ab complexes; Signal transducer platforms (Biosensors); Affinity MS	Agg. (HA, LAT, Bacterial), Pptn. In tubes, AGID; Biosensor-based signal	Naked eyes; Biosensor (different platforms:, optical, SPR, micro-balance, electro-chemical, etc.)
Radioisotope: ^{125}I	Radio-labelled Ag/Ab as reporter	Radioactivity (cpm)	Gamma counter
Fluorochromes: FITC, PE, Cy, Alexafluor, Eu3+ chelates, FRET pairs	Fluorochrome-labelled Ab; Eu3+ labelled Ab; FRET pair of Abs	Fluorescence intensity; quenching polarization	Fluorimeter; Flow cytometer; confocal/ fluorescence microscope
Enzymes: HRPO, AP	E-Substrate for colour; E-S for light emission; E-S for fluorescence	Colour intensity; photons; fluorescence intensity	Spectrophotometer/ ELISA reader; Luminometer; Fluorimeter;
Colloidal gold	Au- labelled detector Ab	Intense colour	Naked eyes
Nanomaterials	NP labelled detector Abs	Intense colour; fluorescence; light	Naked eyes; luminometer; fluorimeter; SECM

ELISA: The mainstay immunodiagnostic test for infectious diseases

- **Traditional ELISA formats:**
  - Direct ELISA
  - Indirect ELISA
  - Double-antibody sandwich ELISA
  - Competitive ELISA

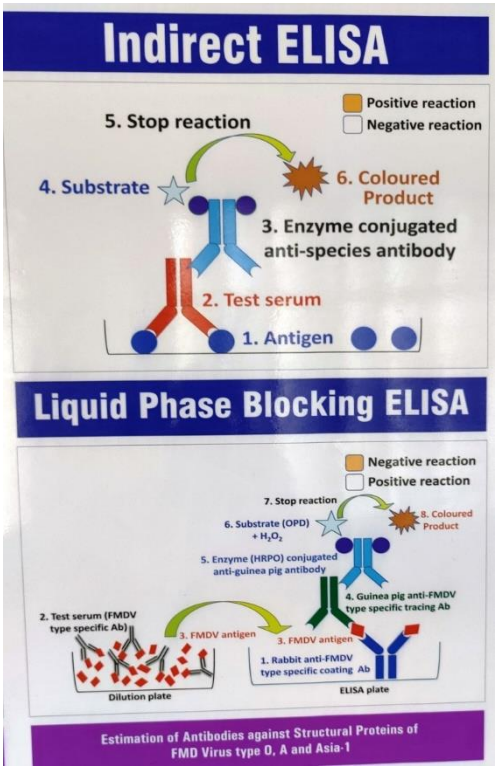


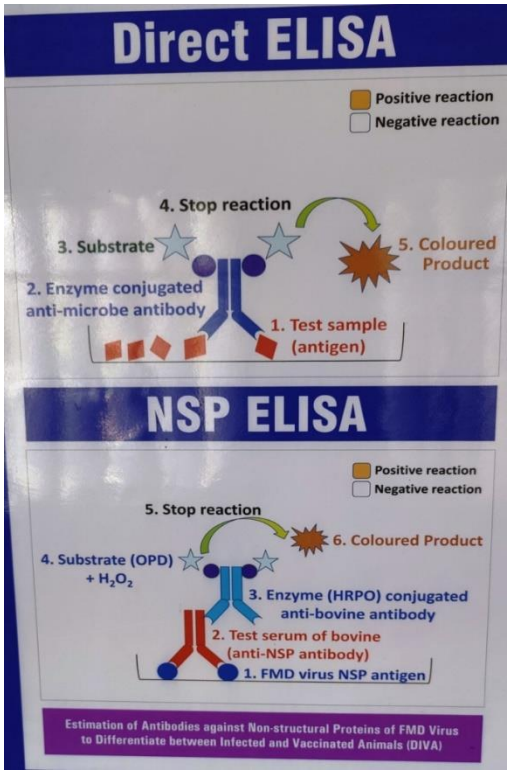
- **Tests that use ELISA principle at some stage**
  - Dot-ELISA
  - Immunoblotting/Western blotting
  - ELISPOT assay
  - 'Lateral flow' ELISA
  - Novel ELISA-like techniques
    - Immuno-PCR
    - Immuno-LAMP
    - Immuno-NASBA

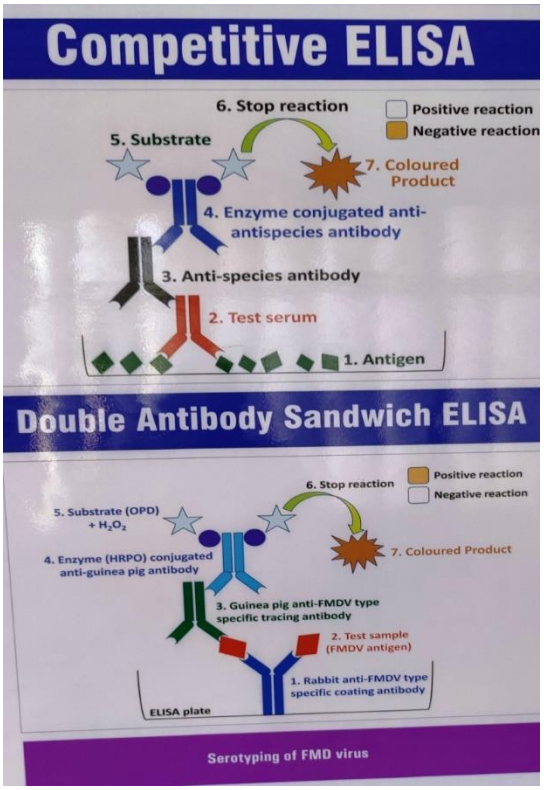


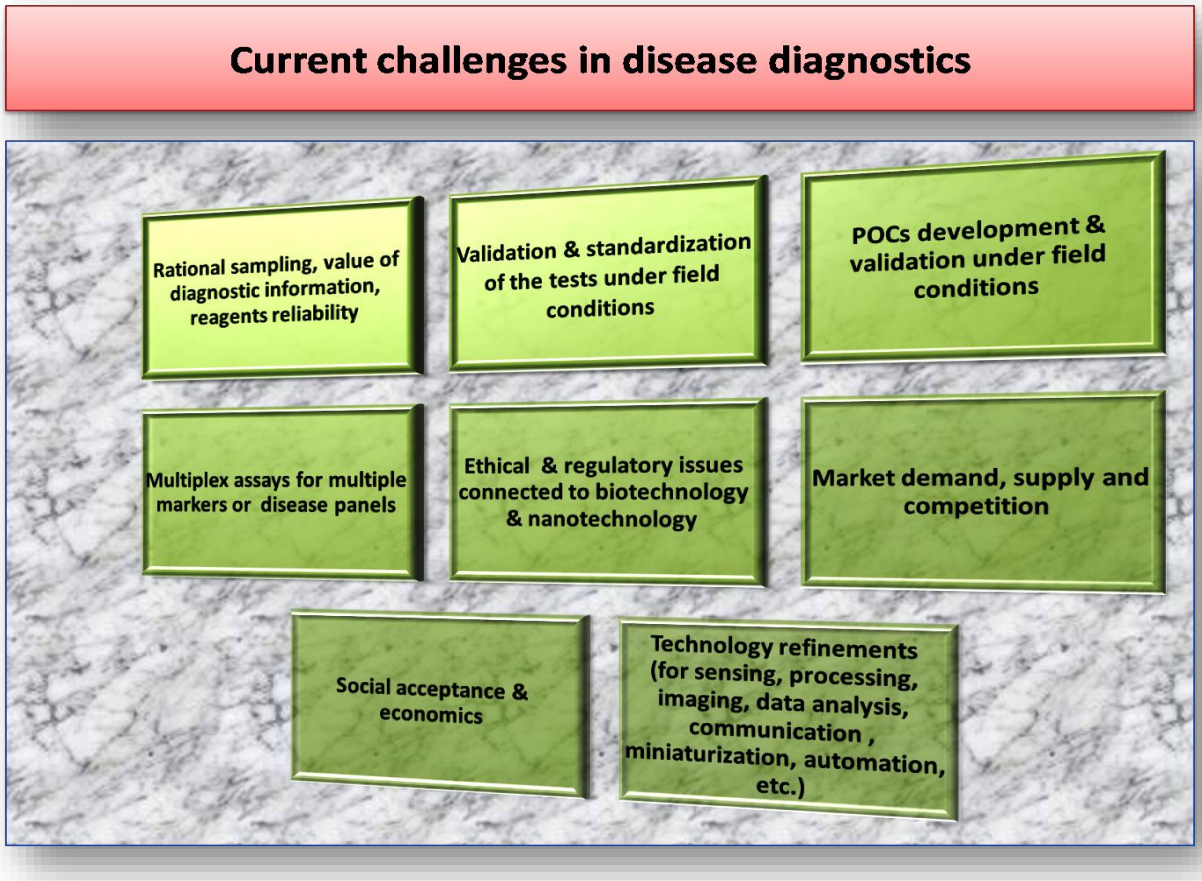
Lateral flow immunoassay device

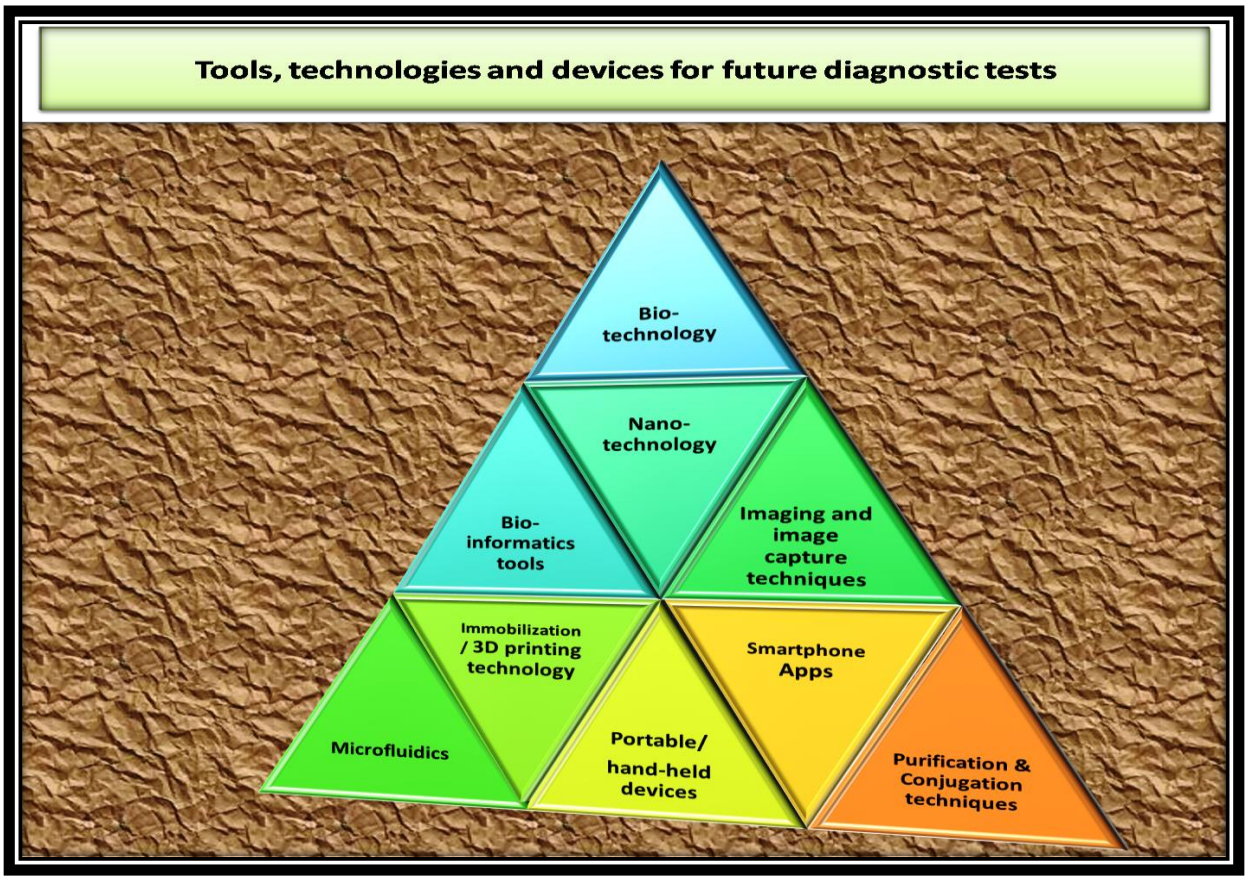
Various formats of ELISA











What major advances in immunodiagnostic tests?

Miniaturization

- Test tube > Microtitre plate > Microarray chips > Nanoarray chips

Automation and robotics

- Sample handling, processing, testing

Multiplexing

- Microfluidic Flow cytometry > Mass cytometry > Microbead-based Luminex > Microfluidics-based LOC
- Planar Microarrays > Nano-arrays

Point-of-care tests

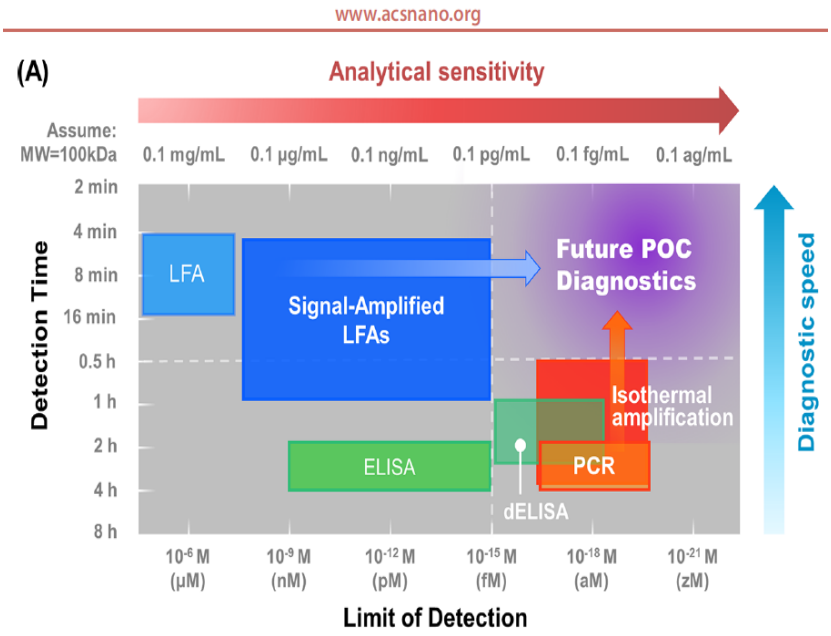
- Qualitative LFDs > Quantitative LFBs
- Smartphone-based portable devices

Increased LoD/analytical sensitivity

- Better Ab probes, nanoparticles, QDs
- Better signal capture, imaging and detector devices
- Signal amplification by ‘hybridizing’ immuno- with molecular assays

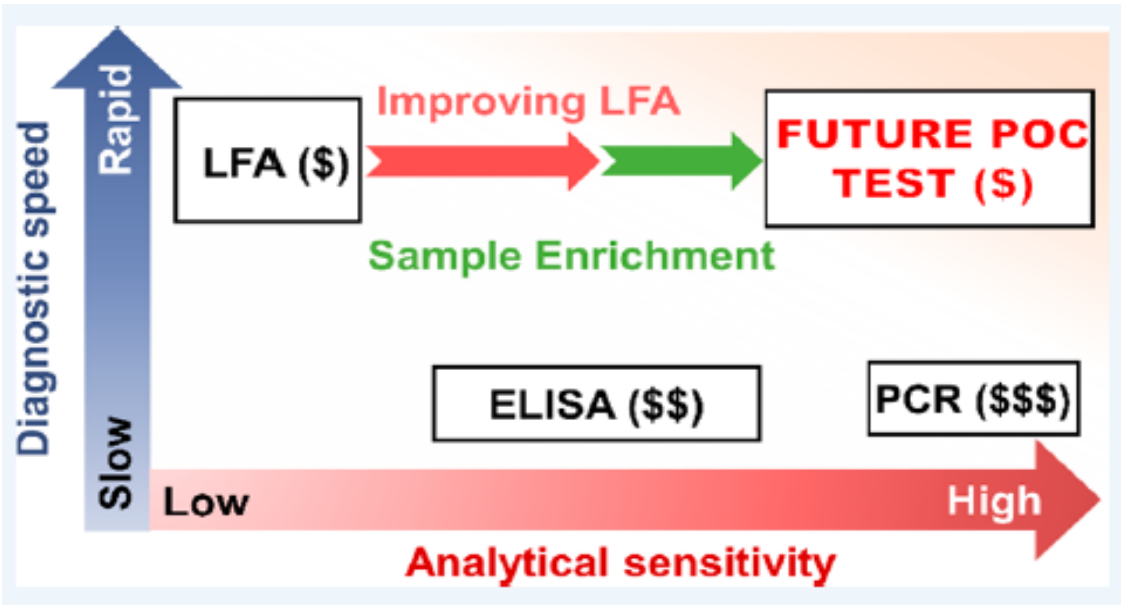
Increased specificity

- Specific Ag discovery using functional genomics & immunomics
- Biomarker discovery using omics-based technology platforms



(B)

LFAs	Limit of Detection (M)	Time (min)
Electrochemical	10^{-9} ~ 10^{-15}	10 ~ 60
SERS	10^{-9} ~ 10^{-15}	15 ~ 30
Fluorescence	10^{-8} ~ 10^{-15}	10 ~ 60
Chemically enhanced	10^{-9} ~ 10^{-14}	5 ~ 60
Photothermal	10^{-7} ~ 10^{-13}	10 ~ 25
Magnetic	10^{-7} ~ 10^{-13}	10 ~ 30



Merging of immunodiagnostics with molecular diagnostics

HYBRID DIAGNOSTIC TESTS

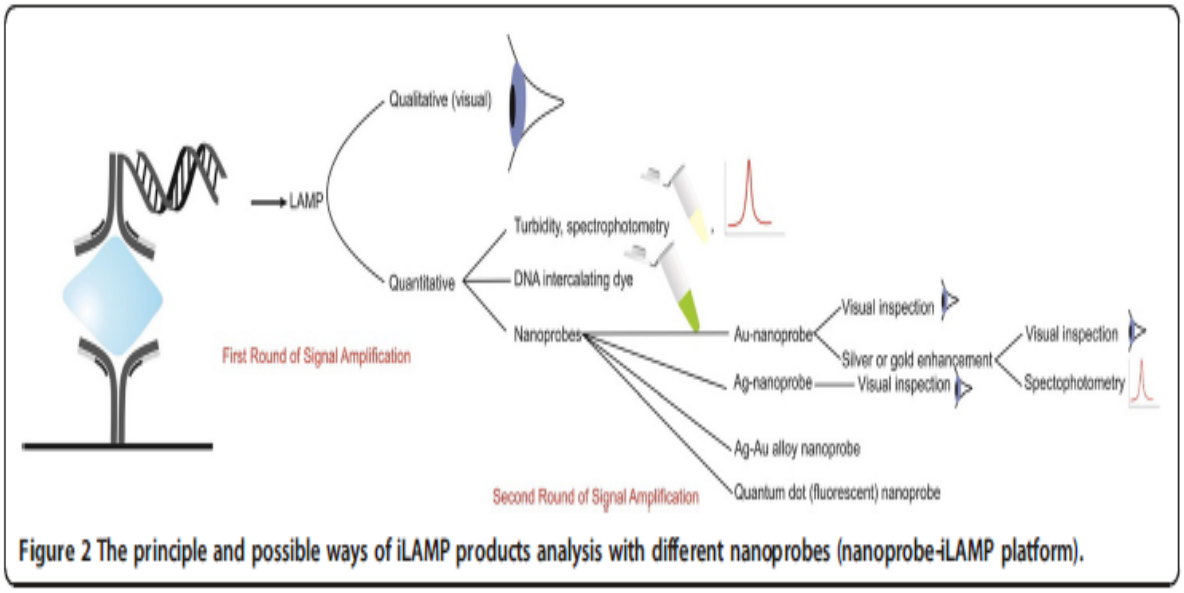
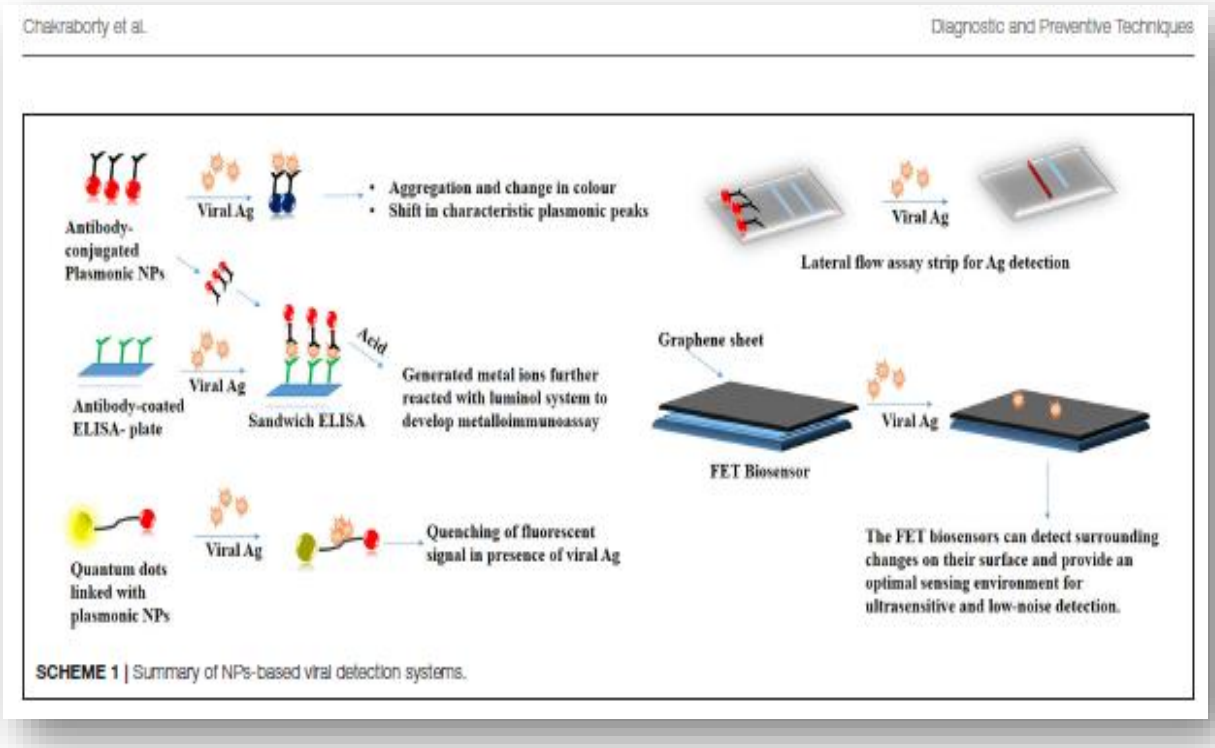


Figure 2 The principle and possible ways of iLAMP products analysis with different nanoprobes (nanoprobe-iLAMP platform).

Nanotechnology for advances in IAs



SCHEME 1 | Summary of NPs-based viral detection systems.

Integrated portable diagnostic laboratory on the 'wish list' of veterinary practitioners!

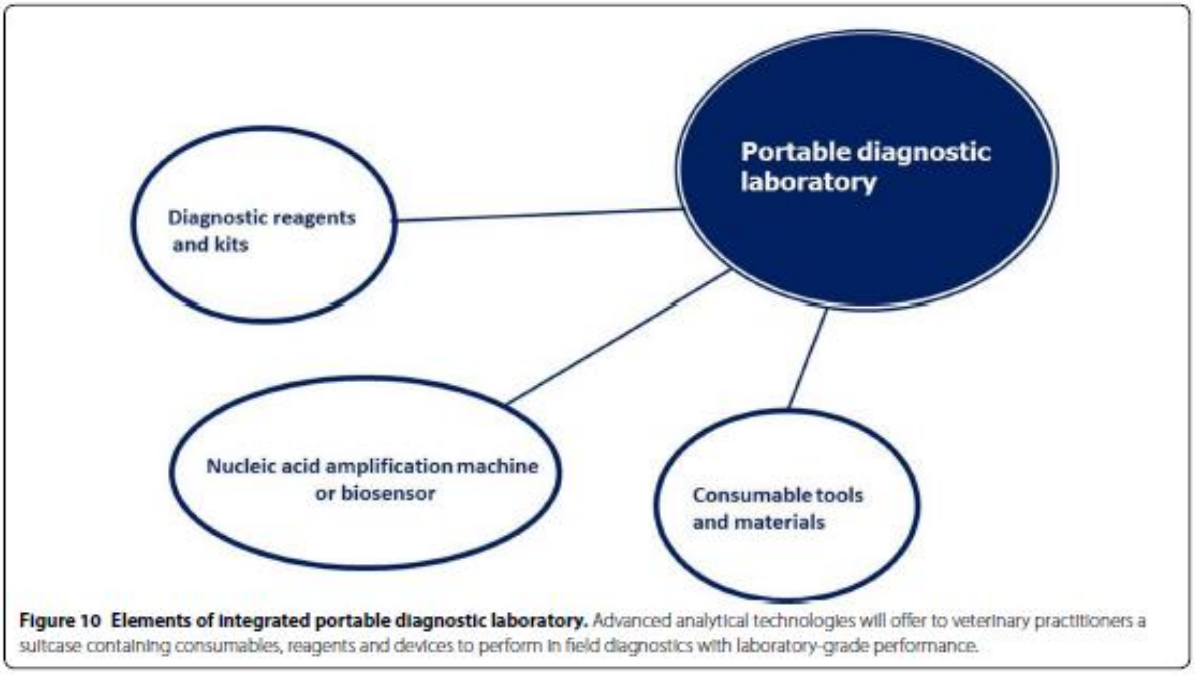
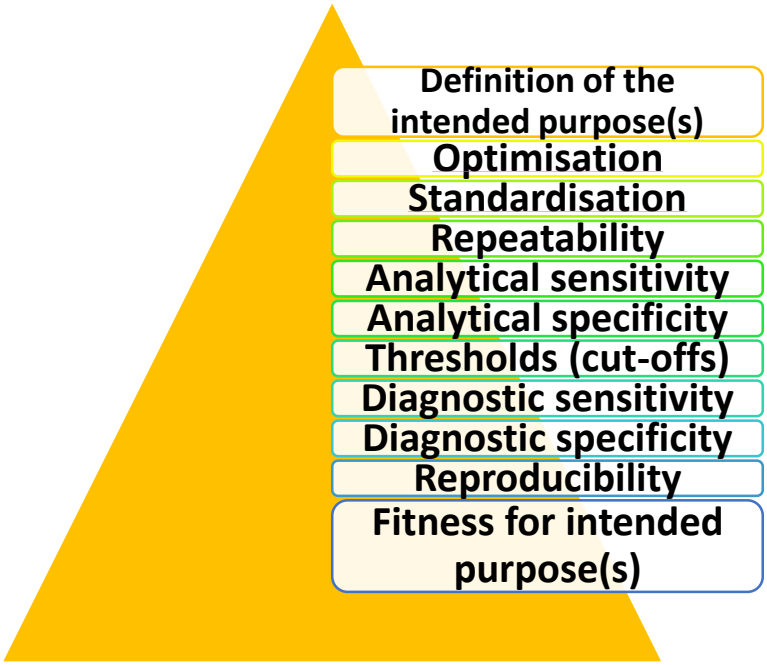
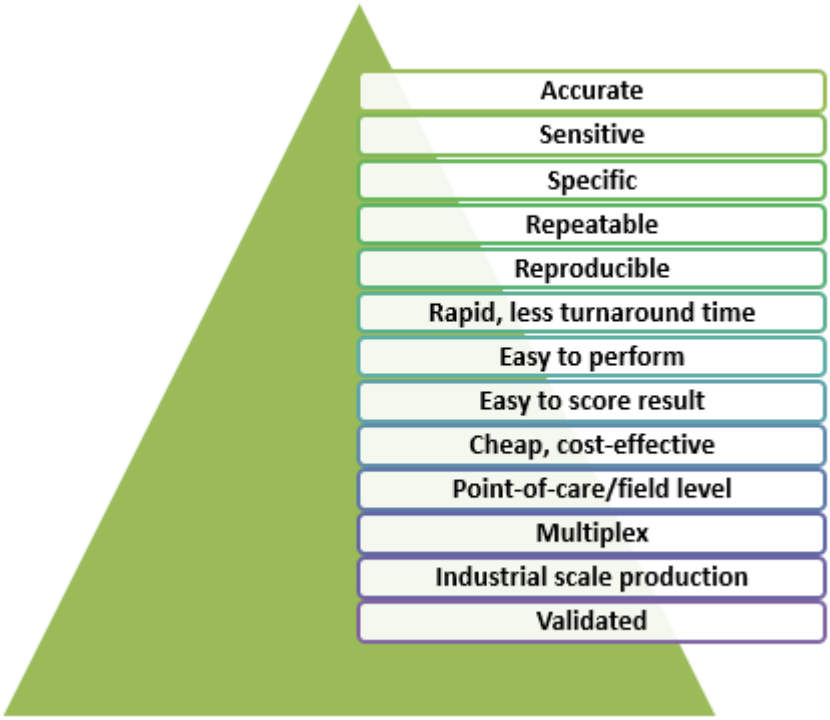


Figure 10 Elements of integrated portable diagnostic laboratory. Advanced analytical technologies will offer to veterinary practitioners a suitcase containing consumables, reagents and devices to perform in field diagnostics with laboratory-grade performance.

Want to develop an immunodiagnostic test?Satisfy OIE defined criteria for assay development and validation



No test is an ideal test! Use a 'fit-for-purpose' IDT



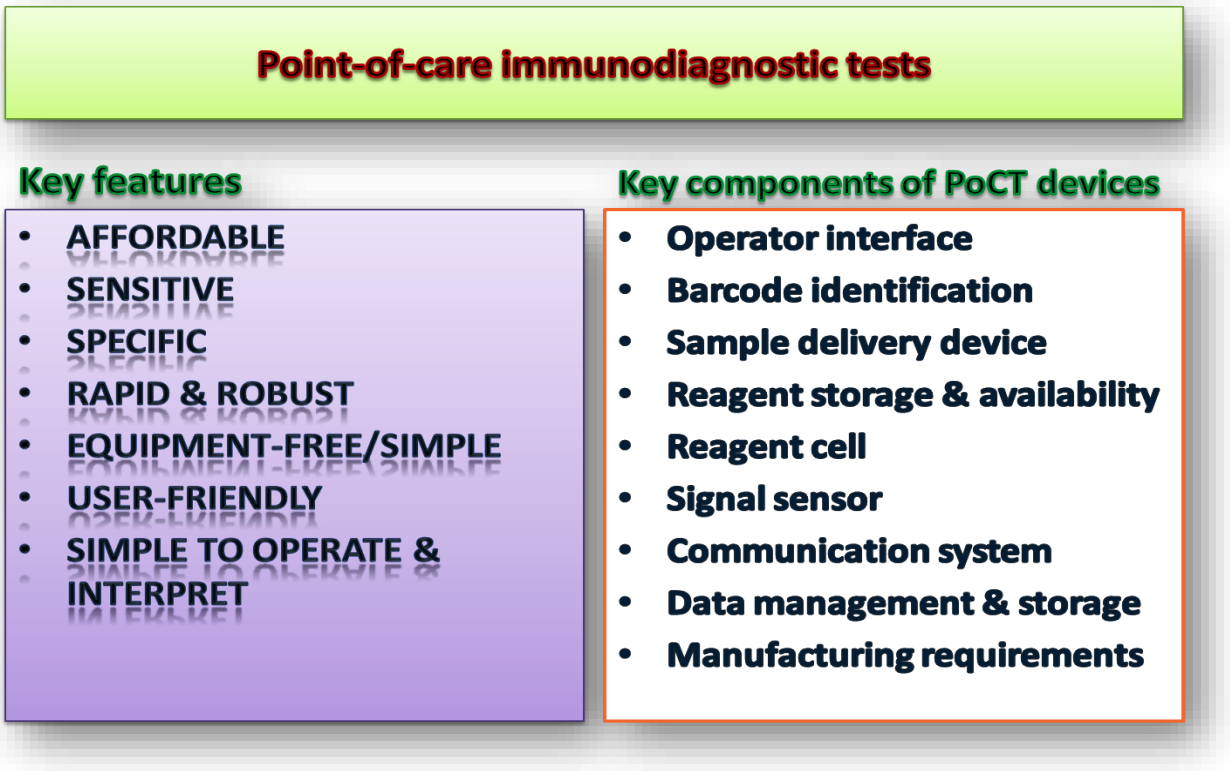
Concluding remarks

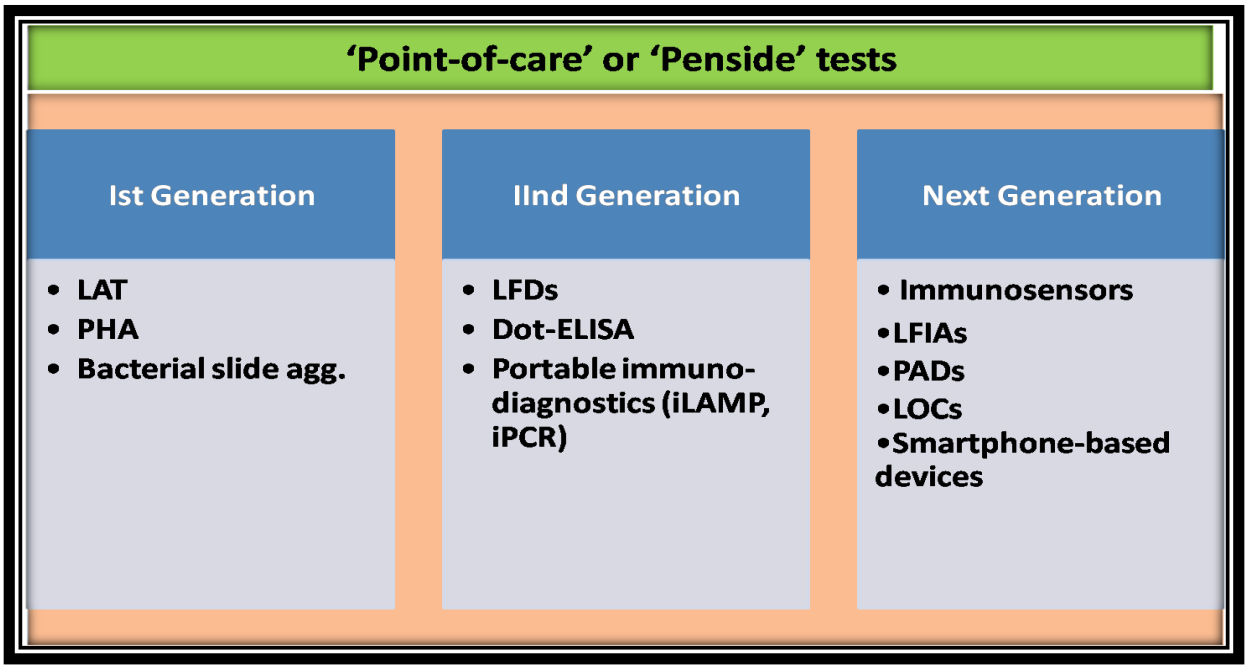
- Traditional immunodiagnostic tests served well for control and management of infectious diseases
- ELISA-based immunoassays have remained the mainstay in infectious disease diagnostics, despite impressive progress in molecular diagnostics of infectious diseases
- NGDTs are aimed at developing rapid, economic and sensitive POCs, enabled by tremendous parallel developments in microfluidics, sensing & imaging, processing, data analysis, telecommunication apps, etc.
- Veterinary diagnostics will also draw benefits from these developments in future
- Effective containment of outbreaks and epidemics in developing countries will become possible

Lecture

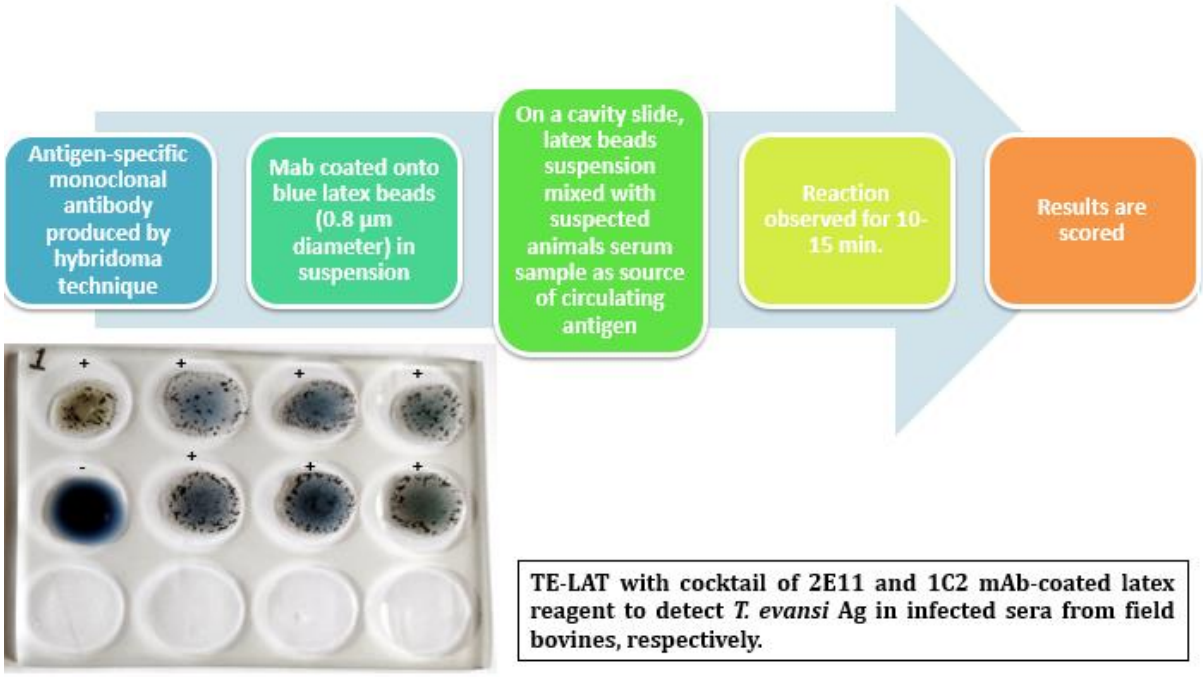
Dr. Ajit Singh, PhD, PDF(UK), Former Professor & Emeritus Scientist (ICAR), LUVAS, Hisar-125004 (Haryana)

Point-of-care immunodiagnostic tests





Latex agglutination test to detect antigen using monoclonal antibody-coated latex beads



TE-LAT with cocktail of 2E11 and 1C2 mAb-coated latex reagent to detect *T. evansi* Ag in infected sera from field bovines, respectively.

Immunodiagnosis with lateral flow device (LFD)

Essays in Biochemistry (2016) **60** 111–120
DOI: 10.1042/EBC20150012

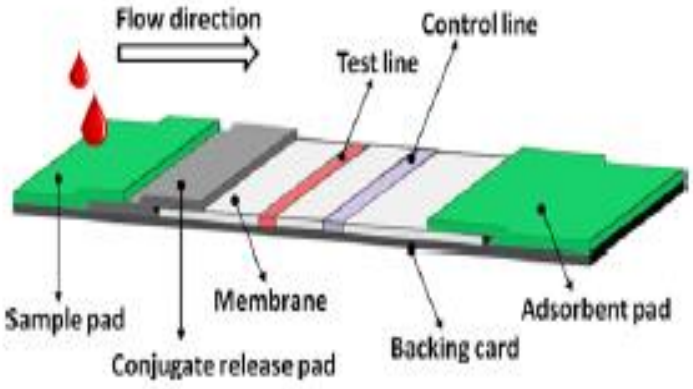


Figure 2. Typical configuration of a lateral flow immunoassay test strip

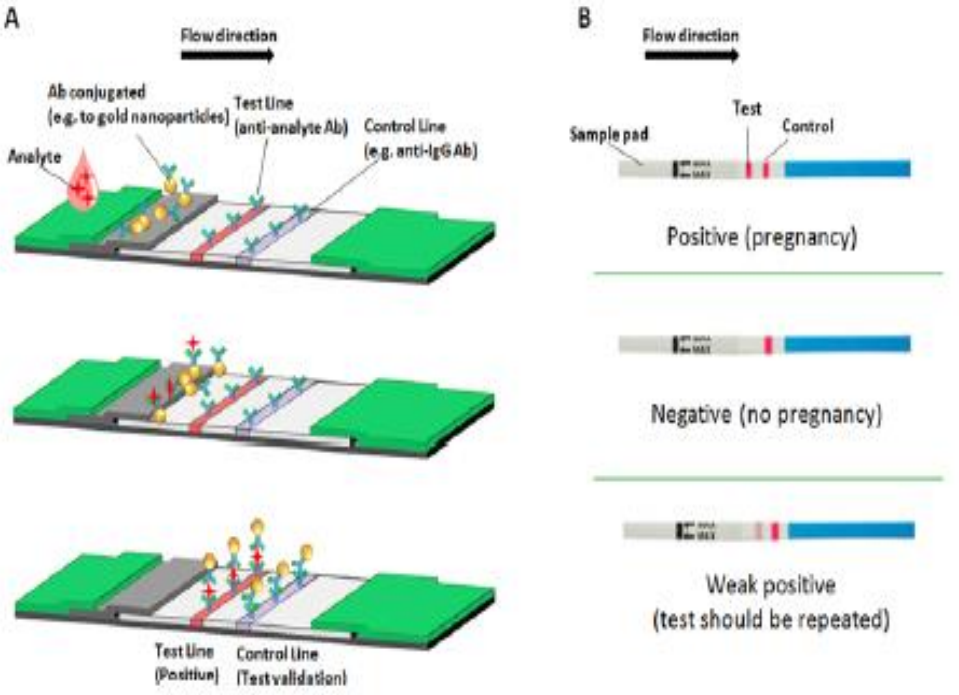
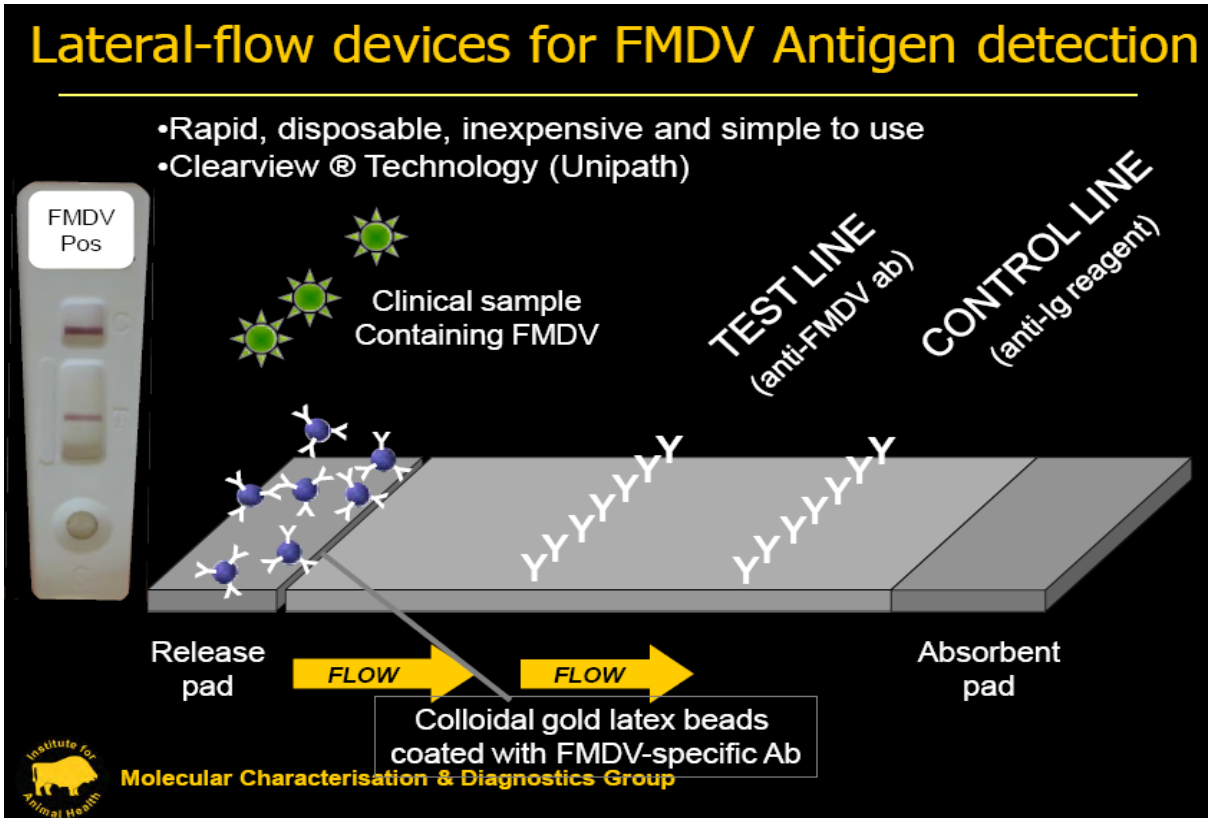


Figure 3. Operation of a lateral flow immunoassay



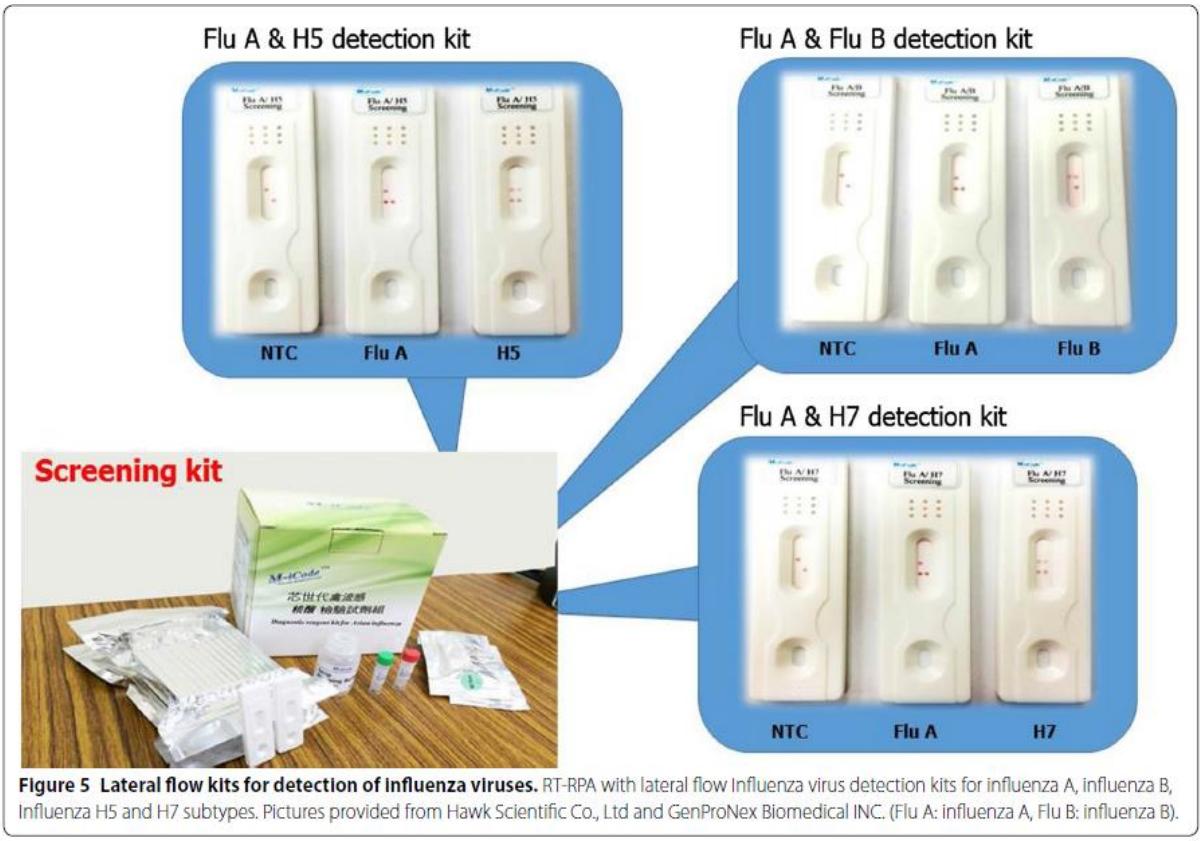


Figure 5 Lateral flow kits for detection of influenza viruses. RT-RPA with lateral flow Influenza virus detection kits for influenza A, influenza B, Influenza H5 and H7 subtypes. Pictures provided from Hawk Scientific Co., Ltd and GenProNex Biomedical INC. (Flu A: influenza A, Flu B: influenza B).

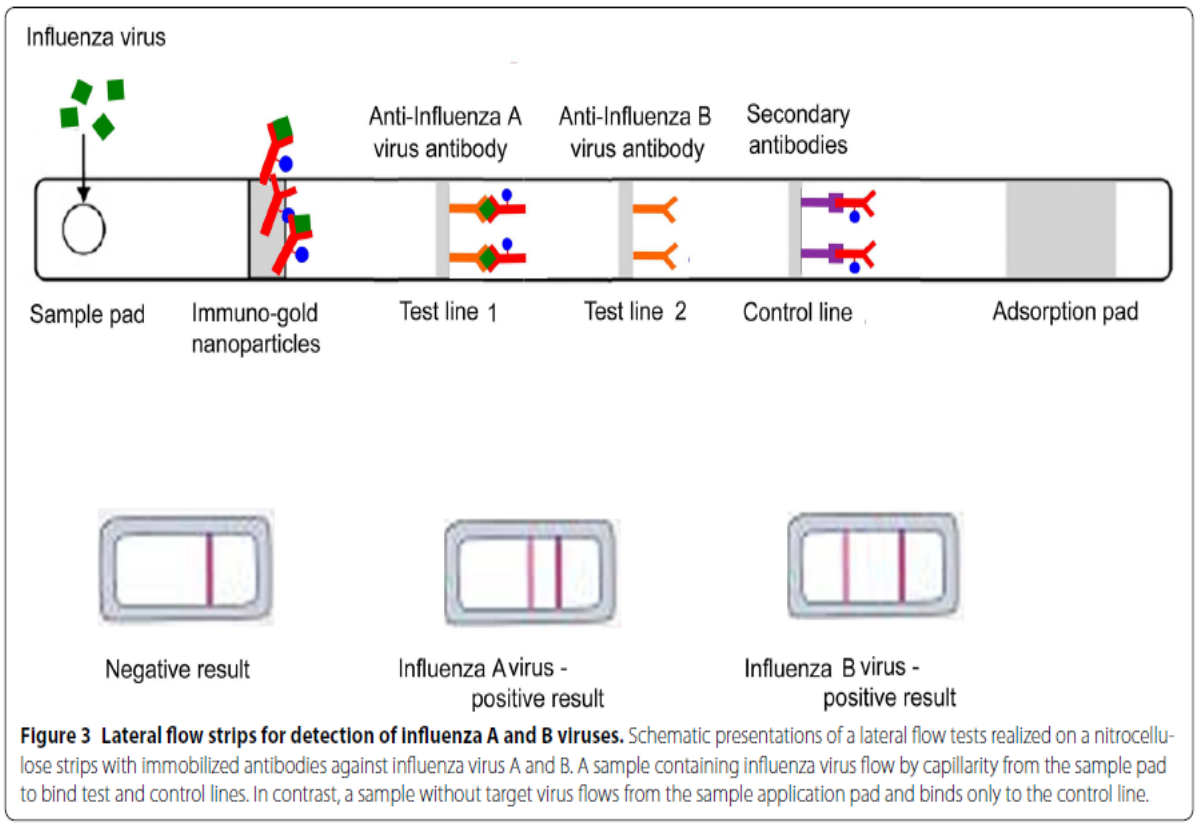


Figure 3 Lateral flow strips for detection of influenza A and B viruses. Schematic presentations of a lateral flow tests realized on a nitrocellulose strips with immobilized antibodies against influenza virus A and B. A sample containing influenza virus flow by capillarity from the sample pad to bind test and control lines. In contrast, a sample without target virus flows from the sample application pad and binds only to the control line.

Table 1. The most commonly used detection methods employed in lateral flow assays

Examples of applications of these systems can be found in optical readers [45,46], camera readers [47], ladder bars [26], fluorescent readers [48], chemiluminescent readers [49] and electrochemical readers [50]. Examples of labels include: fluorescent [51,52], paramagnetic [47,53], enzyme [54,55] and carbon nanoparticles [34].

Labels \ Results	Quantitative	Semi-quantitative	Qualitative
Colour labels e.g. gold nanoparticles, coloured latex	Optical strip readers or camera (with imaging software) for measurement of the intensity of colours produced at test and control line	Visual inspection of ladder bar, where the number of coloured lines is an indication of analyte concentration	Visual inspection of line colours
Fluorescent labels e.g. quantum dots, ruthenium complexes	Fluorescent strip reader, recording fluorescence intensity		
Other labels e.g. paramagnetic labels, enzyme labels, carbon nanoparticles	Magnetic strip readers; Electrochemical detectors; Chemiluminescence readers		

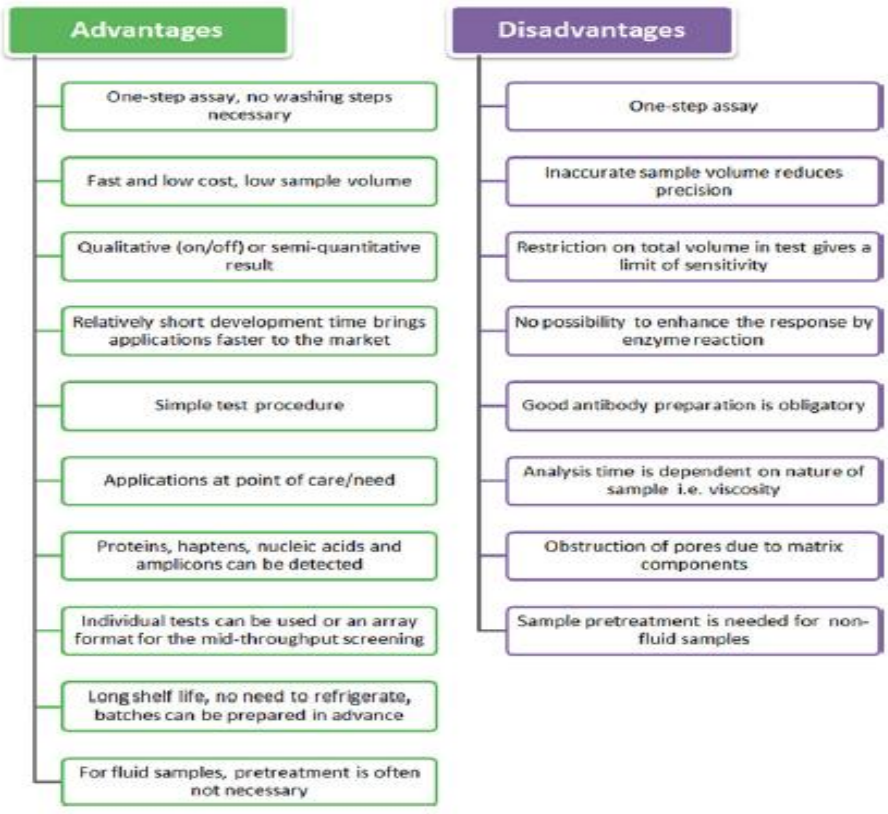


Figure 5. Advantages and disadvantages of the lateral flow assay

Point-of-care' next-generation immunoassays for emerging infectious diseases

POC technology platform/Device	Target infectious agent or disease
Biosensor/Immunosensor **(Various transducer types, such as SPR, optical waveguides, field effect transitor, quartz crystal micro-balance, electrochemical, etc.)**	**Viruses, bacteria, toxins, biomarkers in clinical, environmental and food samples. Early and sensitive detection of agents of disease outbreaks. [e.g., *E. coli, Pseudomonas aeruginosa, Acinetobacter baumannii strains, Mycoplasma*, Salmonella, epsilon toxin of *Clostridium perfringens,* pathogenic *S. aureus* and toxins, avian (H5N1) influenza viruses, etc.]**
Lateral flow device/ Paper-based device/Aptamers-based LFBs	**Examples: *Bacillus anthracis, Brucella sp, Yersinia pestis, Francisella tularensis, Burkholderia mallei, B. pseudomallei*, Salmonella spp., Orthopoxviruses, etc.**
Portable Immuno-LAMP	**Early and sensitive detection of bacterial and viral agents in outbreaks.**
QD-labelled Ab coated LOC with a portable fluorometer	**As above. e.g., Salmonella Typhimurium in chicken meat extract; food safety screening.**
Smartphone-based device	**Biomarkers, viral and bacterial antigens, toxins, etc. (e.g., LFB-based avian influenza virus detection by smartphone device)**
Abbreviations= LFB: lateral flow biosensor, LAMP: loop-mediated DNA amplification, LOC: lab-on-a-chip, QD: quantum dot, Ab: antibody	

Lateral flow biosensor

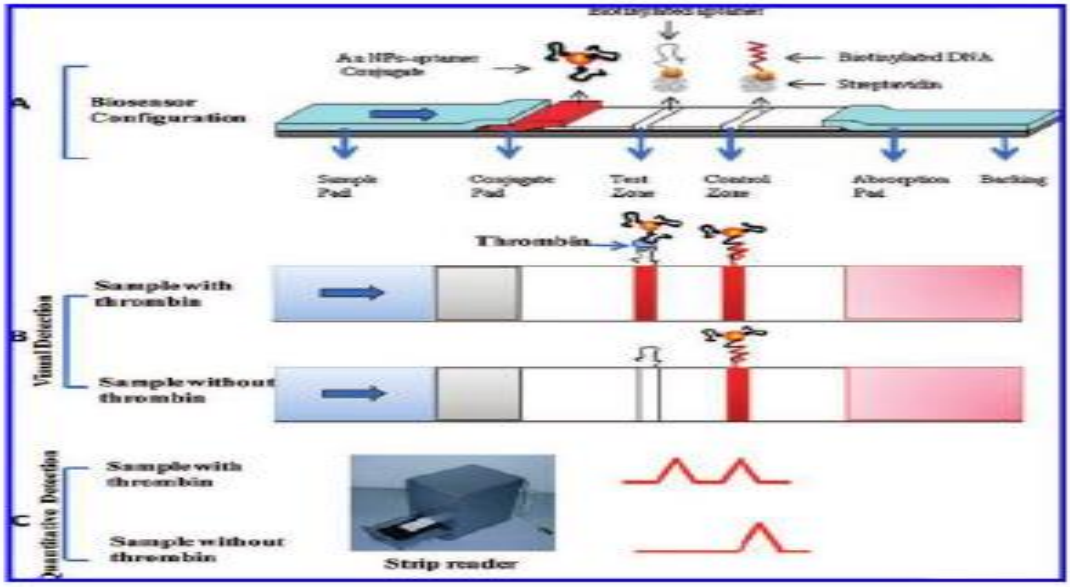


Figure 2. The sandwich format based LFB for thrombin detection [33].

Paper-based analytical device (PAD)

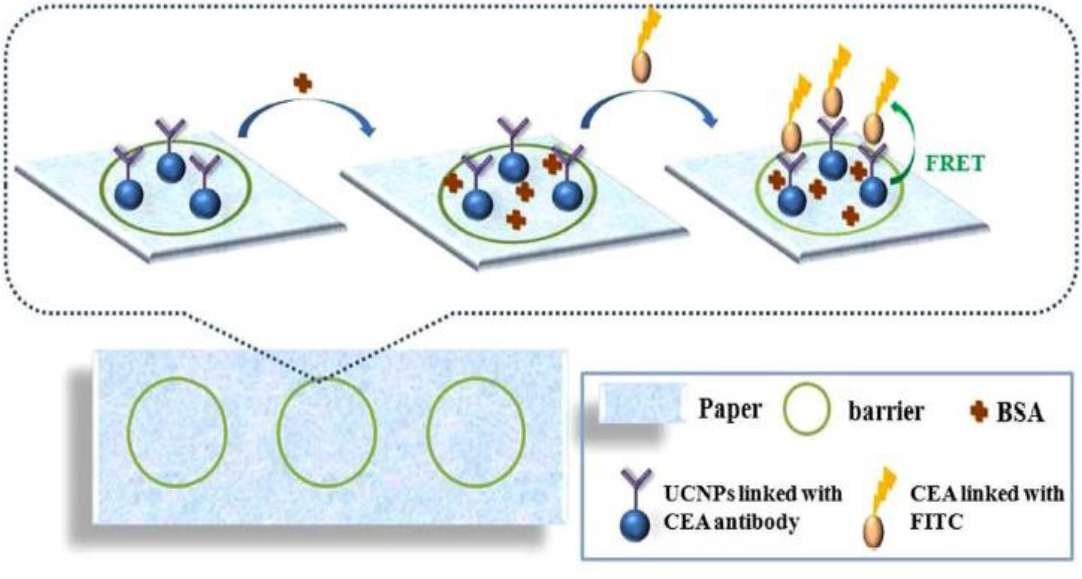


Figure 1. Procedure of the PAD based on the FRET using UCNPs as donors and FITC as acceptor.

Biosensors: a. Optical biosensors: Basic components

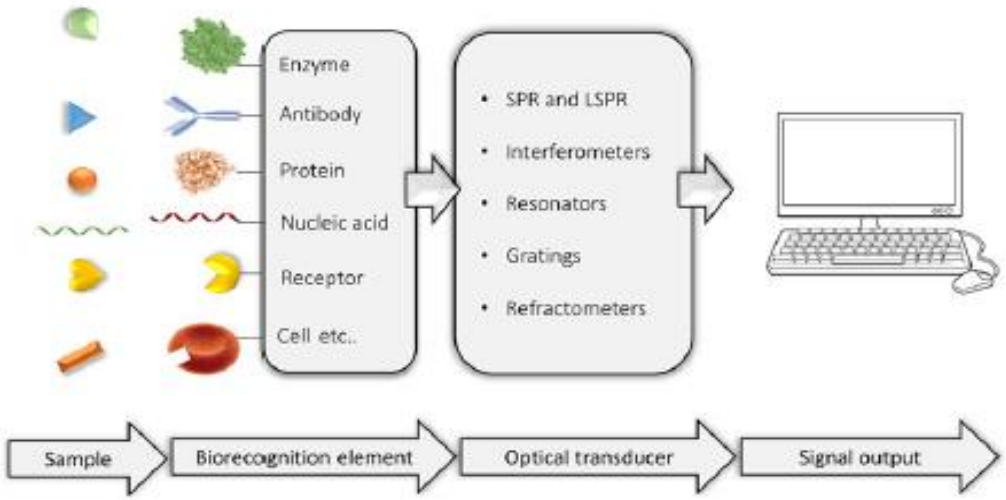
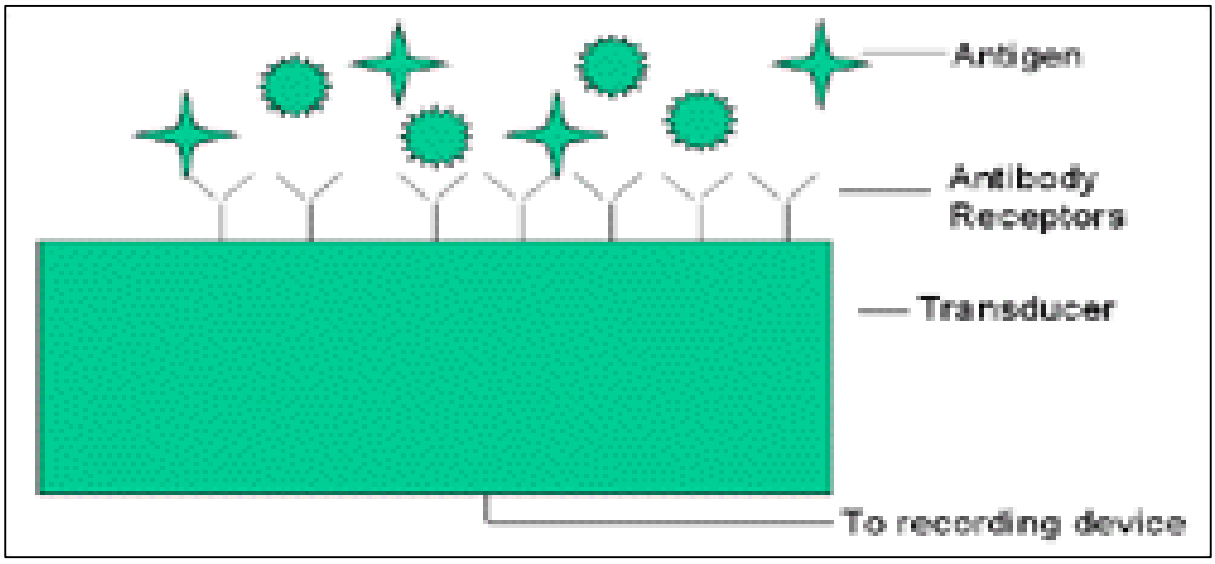


Figure 1. Optical biosensors

Immunosensor device: schematic diagram



Smartphone-based devices and applications

- SBDAs with cost effective remote sensing for delivering mobile healthcare.
- Provide real-time on-site analysis and telemedicine opportunities in remote areas.

- Very useful for developing countries, with limited access to basic healthcare service.
- Useful for the monitoring and effective management of infectious disease epidemics and outbreaks.
- Current smartphones use a variety of sensors, including light detectors, cameras, proximity sensors and fingerprinting.
- The applications of smartphone-based devices are growing in conventional immunoassays, LFA, electrochemical sensing, SPR-based biosensing, microscopy, flow cytometry and colorimetric detection.
- Mobile fluorescence imaging devices to read DNA or Ab-based IAs in POC formats.
- Also as microscopy platforms to detect nanoscale materials.
- The field-portable mobile phone based on surface-enhanced fluorescence microscopy platform- new prospects for POC sensing and molecular diagnostics in resource-limited environments.

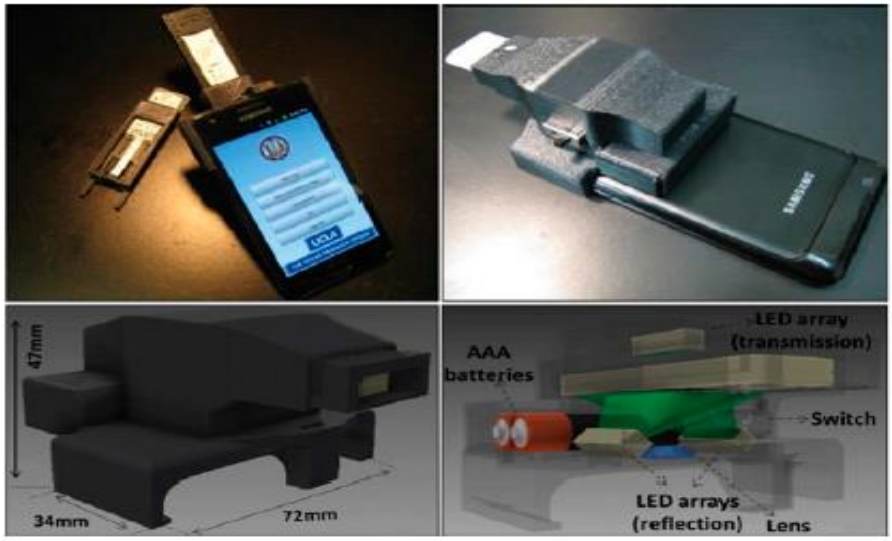
Smartphone-based devices

Figure 3. (A) NFC smart cell phone and (B) NFC SensorLinker by GENTAG, Inc. Images provided by Dr. John P. Peeters, GENTAG, Inc.

Figure 4. The mobile diagnostic reader (mReader™) by Mobile Assay, Inc. Image provided by Michael Williams, Mobile Assay, Inc.

Smartphone-based rapid diagnostic test reader

Figure 6. Smartphone-based Holomic Integrated rapid diagnostic test reader (HRDR-200) developed by Holomic, LLC [11]. Reproduced with permission from the Royal Society of Chemistry.



Smartphone-based fluorescence detector design

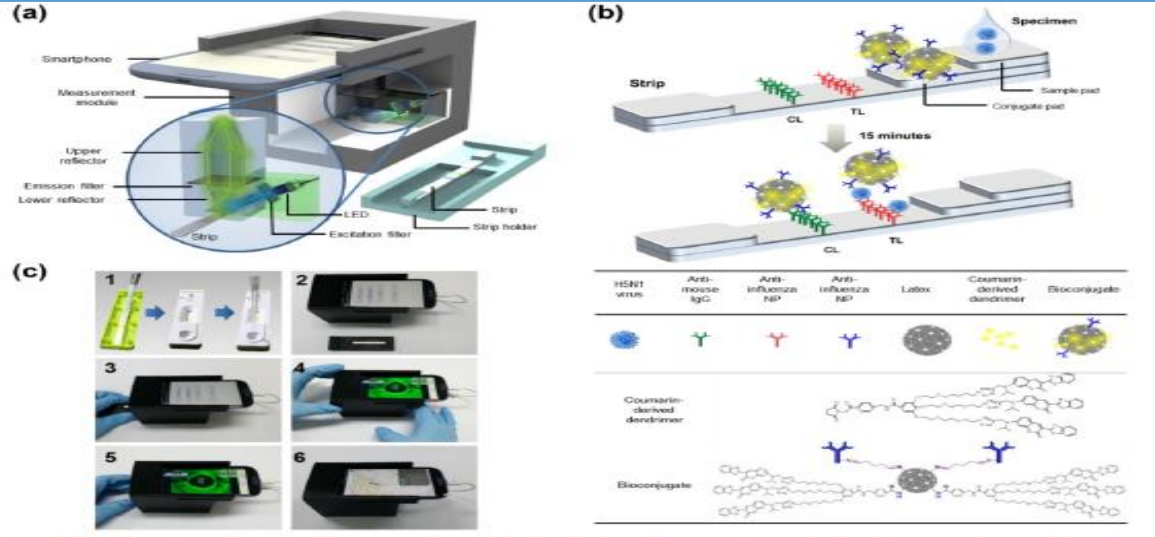


Figure 1. Overview of smartphone-based fluorescence detector design. (a) Schematic description of a smartphone-based fluorescence detector with a reflective light concentrator module. When the fluorescent lateral flow strip, shown separately, is inserted into the measurement module and excited by an LED through an excitation filter, the fluorescence emission from the strip is filtered by an emission filter, collected by the non-imaging reflector module, and detected by the smartphone's camera module. (b) Schematic of a fluorescence detector flow strip. As a base of strip, a nitrocellulose membrane is coated with anti-influenza A nucleocapsid (NP) antibody on the test line (TL) and anti-mouse IgG on the control line (CL). Bioconjugate (latex conjugated with antibody and coumarin-derived dendrimer) that is reactive to the A/ virus is captured by the anti-influenza NP on the TL. Meanwhile, the unreactive bioconjugates flow laterally further and are caught by the anti-mouse IgG on the CL. The fluorescent intensity, with an excitation/emission wavelength of 460/560 nm, is measured by the smartphone-based diagnostic device. (c1) Bioconjugate and specimens were dropped onto the conjugate pad of the diagnostic strip to induce lateral flow reaction toward the test and control lines. (c2) After 15 min, the strip was transported to the smartphone fluorescence reader. (c3, c4) A user started the measurement application on the smartphone, and followed the step-by-step instructions. (c5) The smartphone displayed the measurement results for the fluorescence intensity of the TL and CL. (c6) After finishing the measurements, the test result was displayed on the smartphone and also transmitted via wireless communication using SMS to the database. The test result appeared on the webpage with a binary diagnostic decision based on the cut-off value.

Lecture

Dr. Ajit Singh, PhD, PDF(UK), Former Professor & Emeritus Scientist (ICAR),
LUVAS, Hisar-125004 (Haryana)

Recent advances in vaccine technologies

The Roots of Vaccinology

Vaccinology is the study of art and science of vaccine construction and its use in intended human and animal populations.

Vaccine may be defined as an immunogenic formulation to induce protective immunity against the disease in the host.

Jenner's cowpox vaccine in 1796 A.D. followed by Pasteur's discovery of the principle of vaccine construction in the 19th century A.D. laid the foundations of Vaccinology.

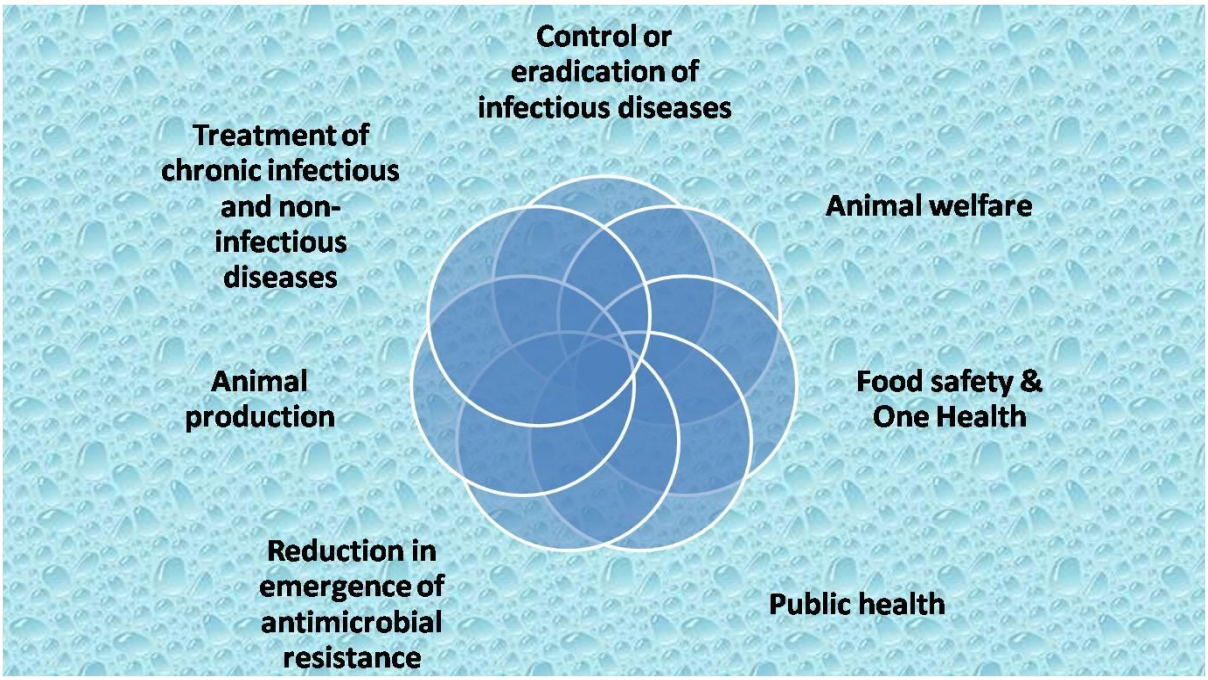
Vaccinology is intimately linked to Immunology, as vaccines work through induction of immune responses in the host.

Success of traditional vaccines

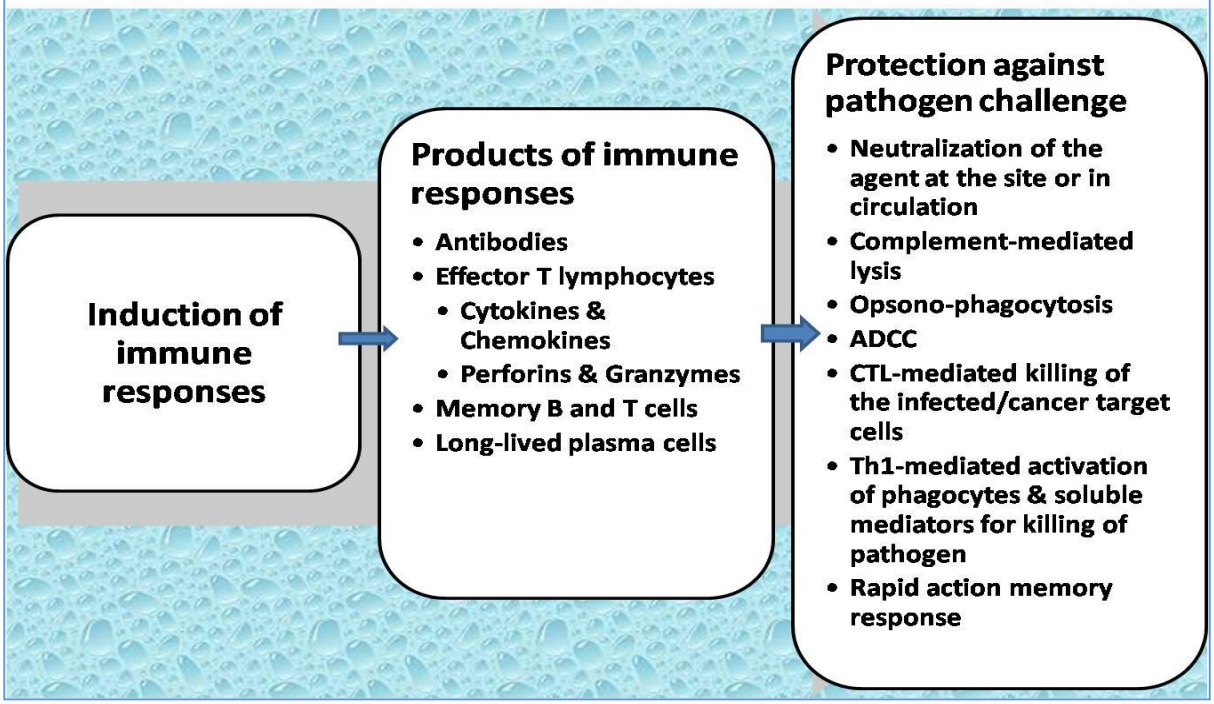
Impressive success stories

- Smallpox eradicated (1980)
- Rinderpest eradicated (2011)
- Live attenuated human and animal vaccines eliminated diseases in various countries/regions
- Killed vaccines eliminated diseases in various countries/regions
- Toxoids eliminated various toxinosis
- Millions of human and animal lives saved
- Human and animal suffering reduced
- Among the most valuable assets created by mankind

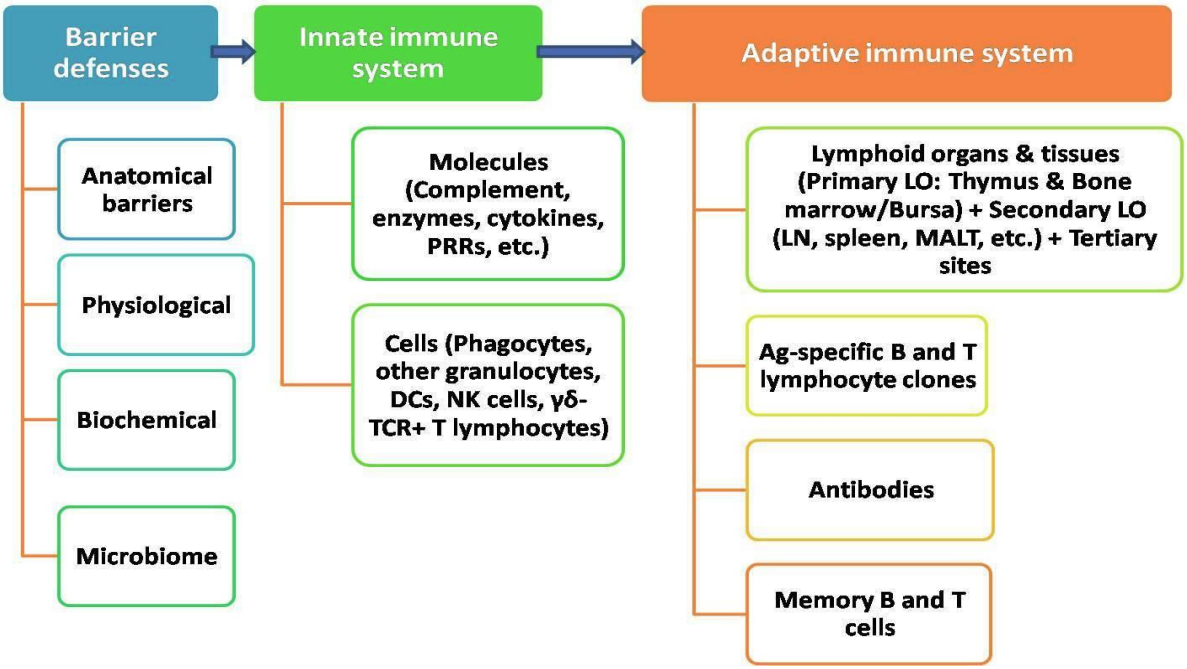
What purposes can vaccines serve?



How do vaccines work?



Immunity against infections guides vaccine design:
A. Vertebrate immune system components



Immune markers as potential correlates of protection

Immune markers of humoral response

- Neutralizing Abs
- Serum IgG
- Mucosal IgG & IgA

Immune markers of cellular response

- IFN-γ, TNF-α, IL-2 (Th1 signatures)
- IL-17 (Th17 signature)
- IL-1, -4, -10 (Th2 signatures)
- CD27+FCRL4+ (Memory B cells)
- $CCR27^{hi}CD45RA+CD27+CD28+$ T_{CM}
- $CCR27^{low}CD45RA-CD27+/-CD28+$ T_{EM}
- Some others

The Impact of the Microbiome on Immunity to Vaccination in Humans
De Jong, SE et al. Cell Host & Microbe 28, August 12, 2020

Vaccine-induced immune responses are highly variable between individuals and between populations

Several factors for variations in vaccine responses

- A key role for the gut microbiome in controlling immune responses to vaccination.
  - Studies in mice
  - Correlation evidence on human immunity
  - Broad-spectrum antibiotics for causal evidence and mechanistic insights

Advances in Vaccine Technologies

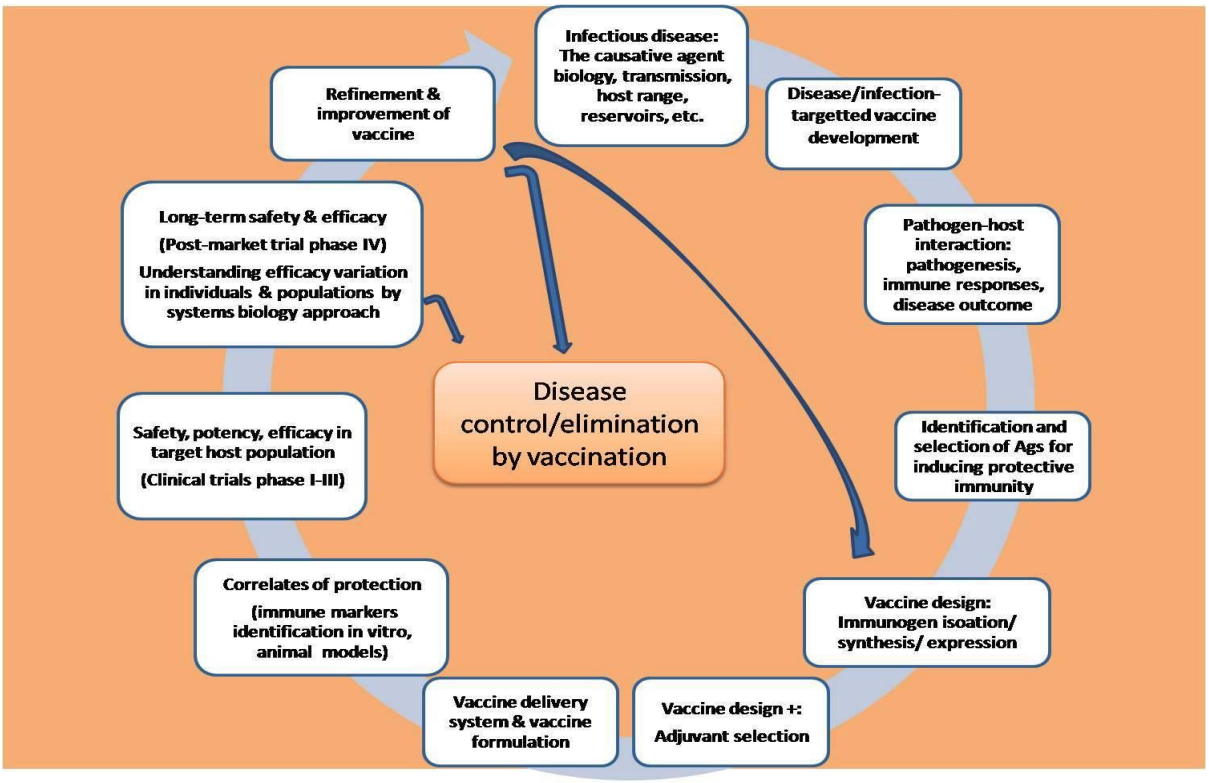


Fig. 1. Pathway for development of vaccine to control or eliminate an infectious disease.

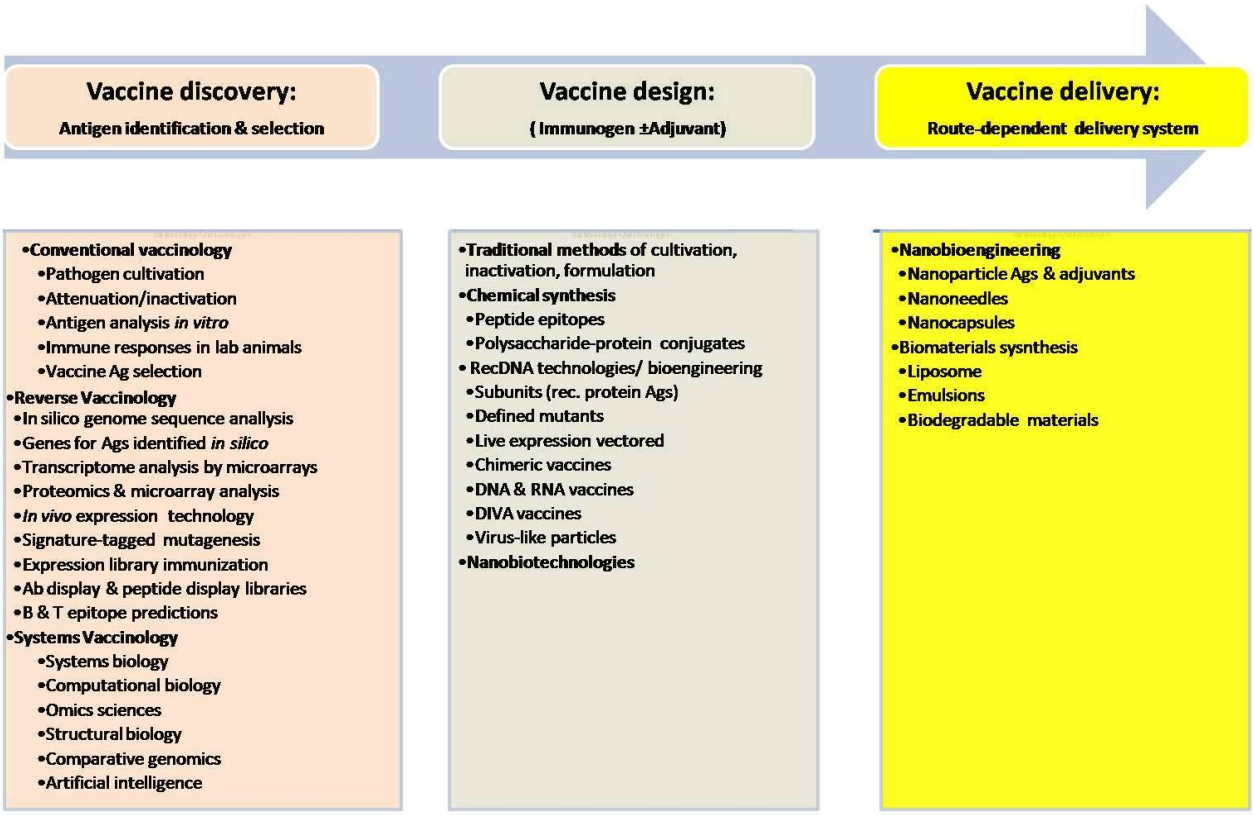


Fig. 2. Methodologies and technologies employed in Stage I (Pre-clinical) of vaccine development pathway.

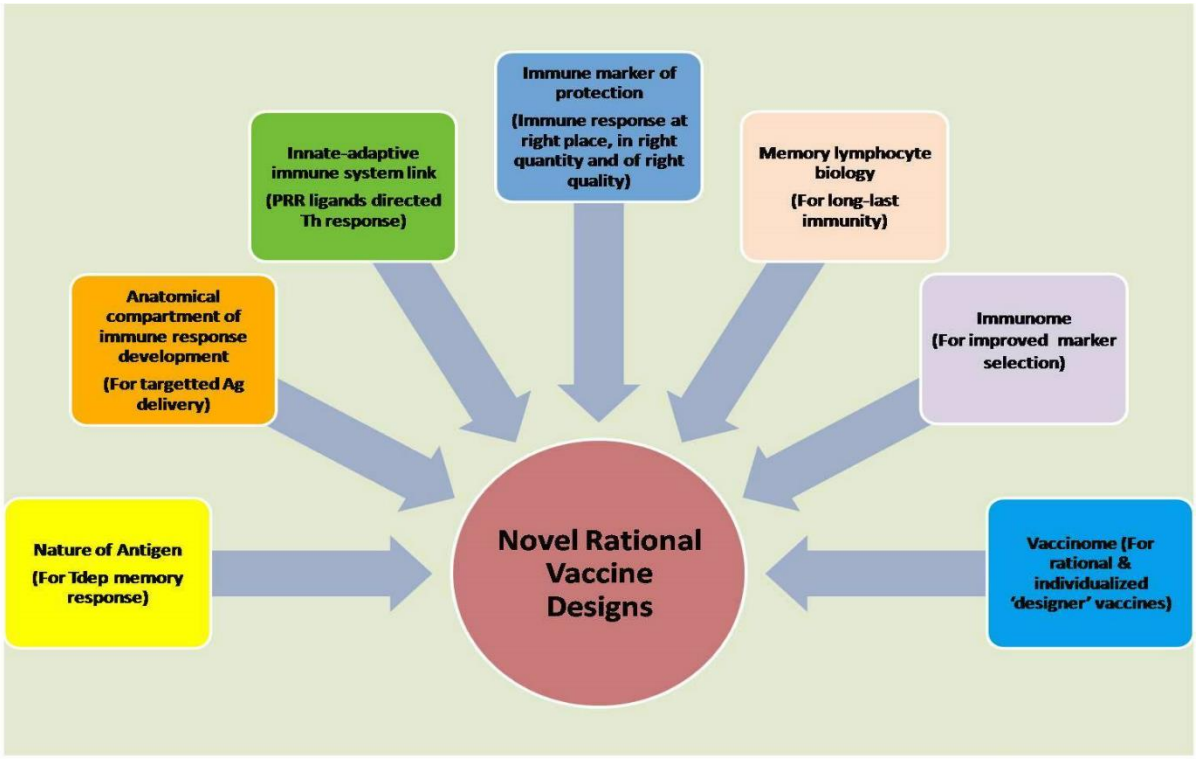


Fig. 3. Various immune determiners to guide novel rational vaccine designs

Table 1: Design of Licensed Novel Rationalized Veterinary Vaccines.

Vaccine design	Selected example(s) of licensed veterinary vaccines (target species)
Subunit	Inactivated baculovirus expressed PCV2 ORF2 protein, adjuvanted (pig); Recombinant ApxII, TbpB, CysL, OmlA(1), and OmlA(2) proteins of *Actinobacillus pleuropneumoniae* (pig); *In vitro*-cultured supernatant Ags of *Babesia canis* (dog); Native fucose-mannose-ligand Ag complex of *Leishmania donovani* (dog); Gametocyte antigen(s) of *Eimeria maxima* (poultry); recombinant Bm86 Ag of *Boophilus microplus* tick (cattle)
DNA	DNA vaccine of WNV (horse); DNA vaccine of IHNV of salmon (fish)
Live/inactivated expression vector	Live or inactivated gE-deletion marker vaccine of BHV-1 (bovine)); Baculovirus recombinant E2 protein without emulsion of CSFV (pig); gE- and TK-deleted marker vaccine of Pseudorabies virus (pig); Canarypox virus-vectored vaccine of Equine influenza virus (horse)); Canarypox virus-vectored vaccine of WNV (horse); Vaccinia virus recombinant rabies virus G (dog); Canarypox virus-vectored vaccine (HA and F Ags of CDV) (dog); Canarypox virus- vectored vaccine for FPLV (cat); Canarypox virus- vectored vaccine for rabies (cat); Fowlpox virus vectored NDV (Poultry); Canarypox vector equine flu/tetanus (horse)
Chimeric	Inactivated PCV1-2 chimera; adjuvanted (pig); Live flavivirus chimera vaccine of WNV (horse); Chimera H5N3 virus, inactivated in oil-based adjuvant (AIV) (poultry); Live recombinant chimera virus expressing VP2 gene of IBD on HVT virus for MD & IBD (poultry); Chimera virus on NDV backbone for NDV & AIV (poultry)
Defined mutants	Spontaneous rifampin-resistant rough mutant *(Brucella abortus* RB-51); Live submucosal vaccine; deletions in *aroA* gene (*Streptococcus equi* (Horse); Double gene-deleted *S. enterica* serovar Typhimurium strain (poultry)
DIVA vaccines	NSPs-free FMDV (bovines); gE-deletion mutant of BHV-1 (bovine); gE- and TK- deletion mutants of pseudorabies virus (pigs); E-deletion mutant of CSFV (pigs); H5N3 RG vaccine for H5N1 (poultry); PCV-2 capsid gene chimera into the PCV-1 backbone (pigs); WNV structural genes for PreM and E proteins in attenuated YF-17D backbone (horse)
Mucosal vaccines (oral; intra-nasal; intra-ocular; spray)	Oral: rotavirus (bovine, pig), coronavirus (bovine), turkey adenovirus, IBV, NCDV, IBDV, chicken & turkey herpes virus, reovirus, *Bordetella avium, Pasteurella multocida* (poultry); Intranasal: BHV-1, TGE, *B. bronchiseptica,* Equine influenza virus, *Streptocoocus equi,* CADV-2, PIV, *B. bronchiseptica;* Intra-ocular: Feline calicivirus, feline rhinotracheitis; above poultry virus vaccines; spray: above poultry virus vaccines, *B. avium* (poultry)

Abbreviations: PCV (*Porcine circovirus*); ORF (Open reading frame); WNV (West Nile virus); IHNV (Infectious hematopoietic necrosis virus); BHV (*Bovine herpes virus*); CSFV (*Classical swine fever virus*); TK (thymidine kinase); CDV (*Canine distemper virus*); F (fusion protein); FPLV (*Feline panleukopenia virus*); NDV (New Castle disease virus); HVT (Herpes virus of turkey); MD (Marek's disease); IBDV (*Infectious bursal disease virus*); AIV (*Avian influenza virus*); CADV (*Canine adenovirus virus*); PIV (*Parainfluenza virus*).

Reverse vaccinology: Genome-based approach to vaccine design

J. Adu-Bobie et al. / Vaccine 21 (2003) 605–610

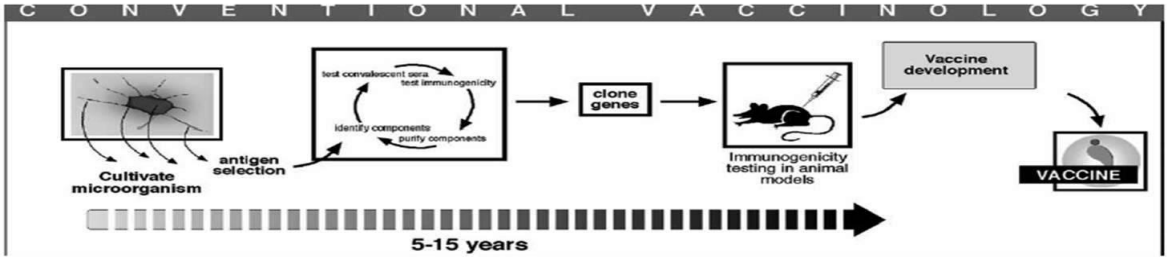


Fig. 1. Schematic representation of the conventional approach to vaccine development.

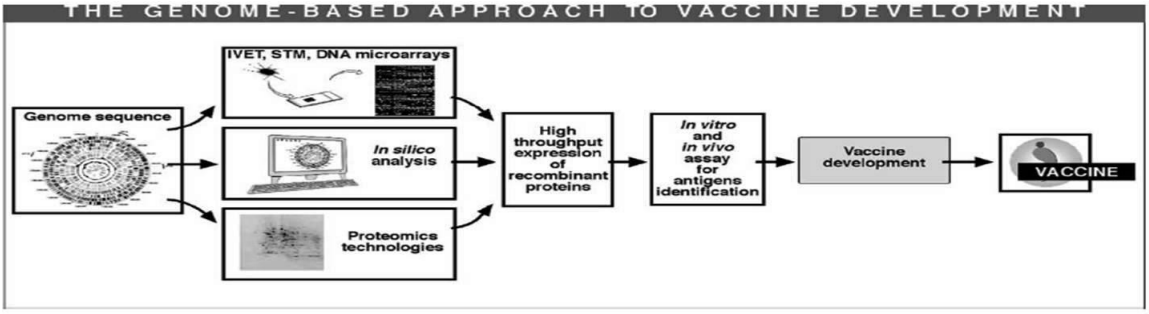
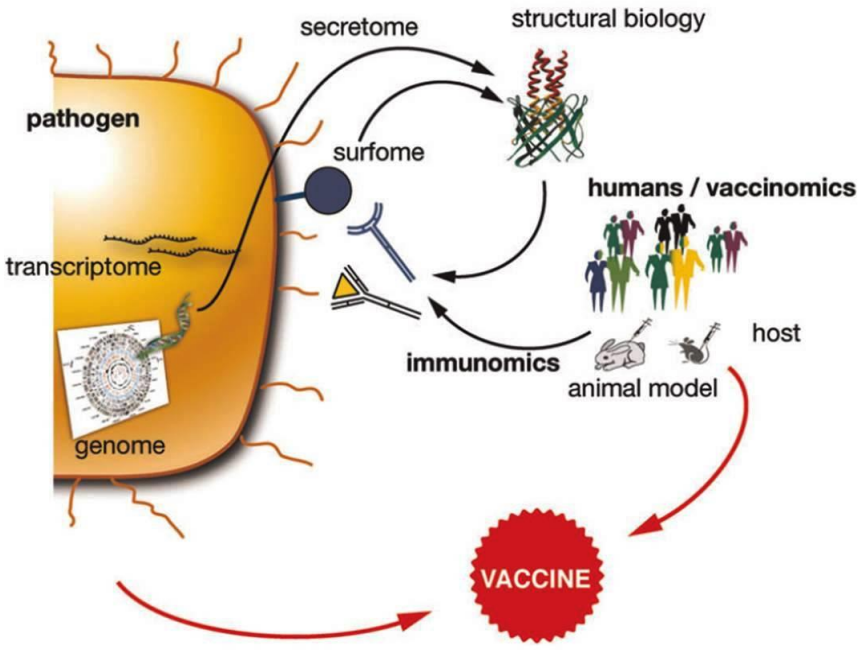
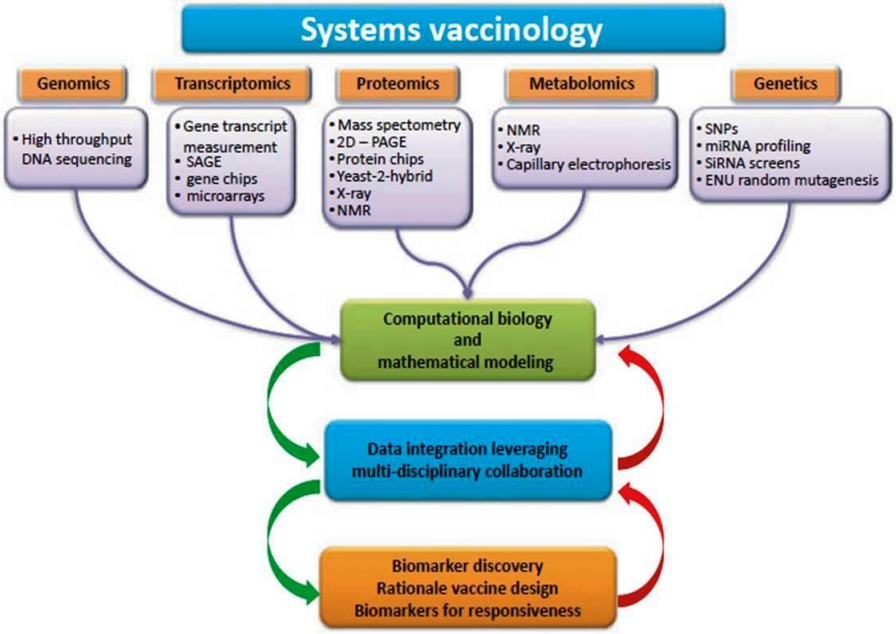


Fig. 2. The genome-based approach to vaccine development.

Application of omics sciences in vaccine design



Systems vaccinology in vaccine discovery, design and identification of immune markers



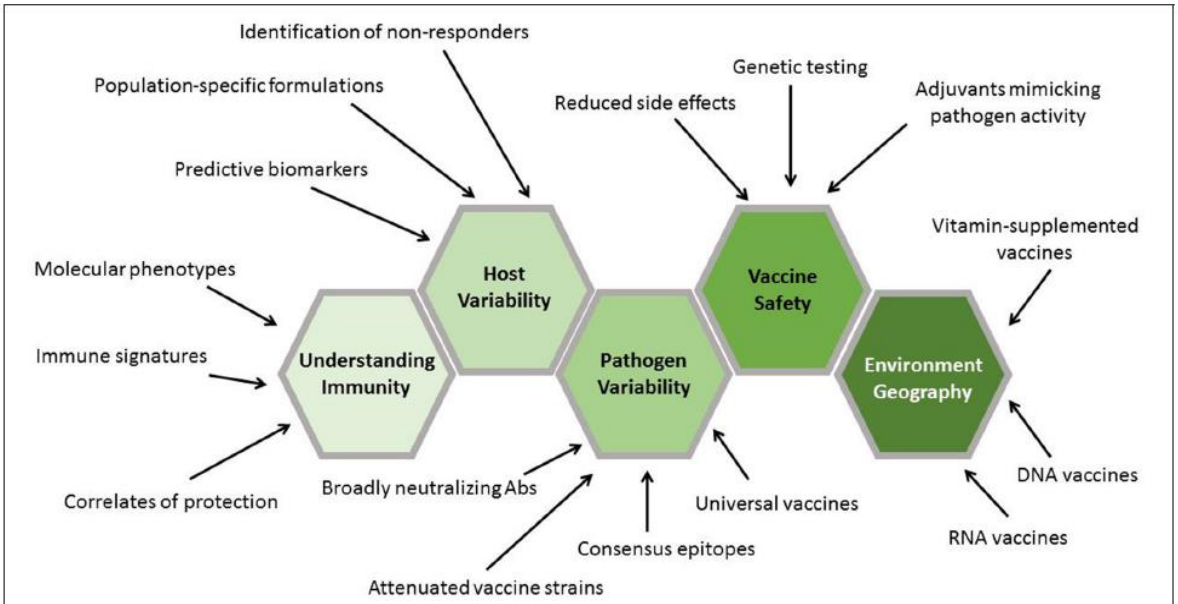


FIGURE 3 | Contribution of genomics to vaccine development stages. The results generated by genomic approaches inform each stage of vaccine development. Initial genomics work plays a critical role in discovering new vaccine targets. This is followed by the characterization of these targets in terms of their ability to generate protective immune responses. Validation of the findings in genetically diverse populations is aided by information gained through genomics approaches. Finally, application of the findings assists in the design and completion of phase I–IV clinical trials.

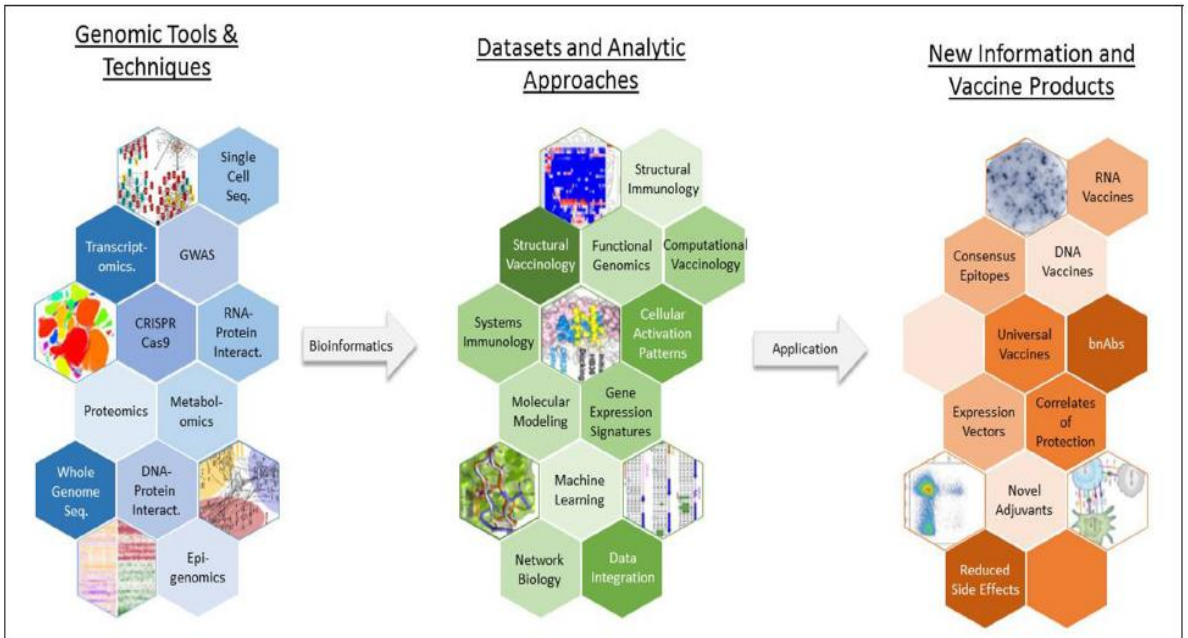


FIGURE 2 | The use of vaccinomics in new vaccine development. A wide array of genomic tools and techniques are available for researchers to study various aspects of pathogen biology and host physiology and immune response. Vaccinomics and similar approaches represent toolboxes that contain a specific assortment of laboratory assays, statistical analysis routines, bioinformatic methods, and computational models that can be used to generate an appropriate dataset and extract biologically meaningful results from the data. GWAS, genome wide association study; bnAbs, broadly neutralizing antibodies.

Table 1. List of servers used for the B-cell epitope prediction.

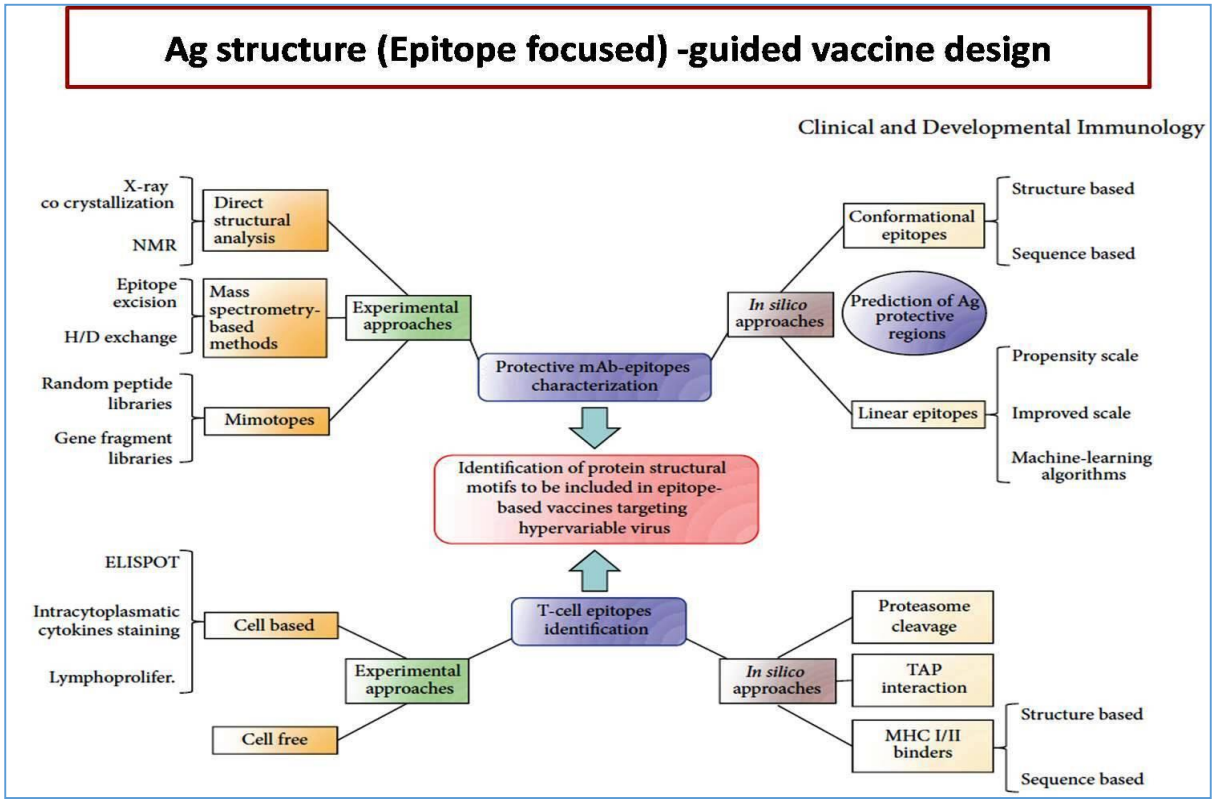
Prediction tool	Types of prediction	Web address	Reference
BCPreds	Continuous B-cell epitopes	http://ailab.ist.psu.edu/bcpreds/predict.html	[26–28]
BepiPred	Continuous B-cell epitopes	http://www.cbs.dtu.dk/services/BepiPred/	[53]
ABCPred	Continuous B-cell epitopes	http://www.imtech.res.in/raghava/abcpred/	[29]
BcePred	Continuous B-cell epitopes	http://www.imtech.res.in/raghava/bcepred/	[54]
LBtope	Continuous B-cell epitope	http://crdd.osdd.net/raghava/lbtlbt/	[55]
SVMTrip	Continuous B-cell epitope	http://sysbio.unl.edu/SVMTriP/	[30]
EPCES	Discontinuous B-cell epitope	http://sysbio.unl.edu/EPCES/	[56]
DiscoTope	Discontinuous B-cell epitope	http://www.cbs.dtu.dk/services/DiscoTope/	[32]
BEPro (PEPITO)	Discontinuous B-cell epitope	http://pepito.proteomics.ics.uci.edu/	[57]
Ellipro	Discontinuous B-cell epitope	http://tools.iedb.org/ellipro/	[31]
CBTOPE	Discontinuous B-cell epitope	http://crdd.osdd.net/raghava/cbtope/	[33]
PEASE	Discontinuous B-cell epitope	http://www.ofran-lab.org/PEASE	[58]
EpiPred	Discontinuous B-cell epitope	http://opig.stats.ox-.ac.uk/webapps/sabdab-sabpred/EpiPred.php	[59]

Table 2. List of servers used for the T-cell epitope prediction.

Prediction tool	Types of prediction	Web address
EpiJen	MHC I	http://www.ddg-pharmfac.net/epijen/EpiJen/EpiJen.htm
nHLAPred	MHC I	http://crdd.osdd.net/raghava/nhlapred/
BIMAS	MHC I	https://www-bimas.cit.nih.gov/molbio/hla_bind/
NetMHC	MHC I	http://www.cbs.dtu.dk/services/NetMHC/
Propred 1	MHC I	http://crdd.osdd.net/raghava/propred1/
MMBPred	MHC I	http://www.imtech.res.in/raghava/mmbpred/
MHC2Pred	MHC II	http://crdd.osdd.net/raghava/mhc2pred/
Propred	MHC II	http://www.imtech.res.in/raghava/propred/
netMHCIIPan	MHC II	http://www.cbs.dtu.dk/services/NetMHCIIpan/
SYFPEITHI	Both MHC I & II	http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm
MHCPred	Both MHC I & II	http://www.ddg-pharmfac.net/mhcpred/MHCPred/
RANKPEP	Both MHC I & II	http://imed.med.ucm.es/Tools/rankpep.html
SVRMHC	Both MHC I & II	http://us.accurascience.com/SVRMHCdb/
IEDB	Both MHC I & II	http://www.iedb.org

Table 3. Studies performed using *in silico* techniques to predict potential vaccine candidates.

Type	Organism	Immunoinformatics tool
Parasite	*Schistosoma haematobium*	Bepipred, RankPep
	Trypanosoma cruzi	IEDB MHC Tools
Bacteria	*Staphylococcus aureus*	Bcepred, Discotope
	Enterotoxigenic E. coli, enterohemorragic E. coli and Shigella	BCPred, Discotope, ElliPro, VaxiJen, SYFPEITHI, ProPred,
	Escherichia coli	BepiPred, ABCpred, DiscoTope, SYFPEITHI, VaxiJen, Algpred
	Treponema pallidum	Vaxign
Virus	Ebola virus (EBOV)	VaxiJen, NetCTL, IEDB, AllerHunter
	Hepatitis B	IEDB MHC Tools, MHC-NP, netCTLpan, RANKPEP, and netMH-Cpan, ToxinPred, NetMHC
Cancer	Human papillomavirus (HPV)-caused cervical cancer	NetMHC 4.0, IEDB MHC Tools, CTLPred, PAComplex, NetMHCII-pan 3.1, RANKPEP, TepiTool, MHCPred V.2.0, LBtope, EPMLR, BCPREDS, and BepiPred 1.0b, IFNepitope, Allerdictor
	Breast cancer	ABCpred



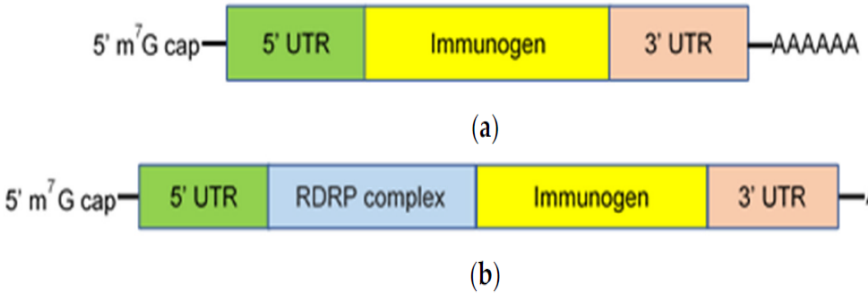


Figure 1. mRNA vaccine constructs. Constructs are classified as either nonreplicating (**a**) or self-replicating (**b**) and composed of a 5′ m^7G cap, 5′ and 3′ untranslated regions (UTR) which flank the nucleotide sequence that encodes the immunogen of interest, and a 3′-poly(A) tail. Additionally, self-replicating mRNA constructs encode an RNA-dependent RNA polymerase (RDRP) complex that transcribes and amplifies the message.

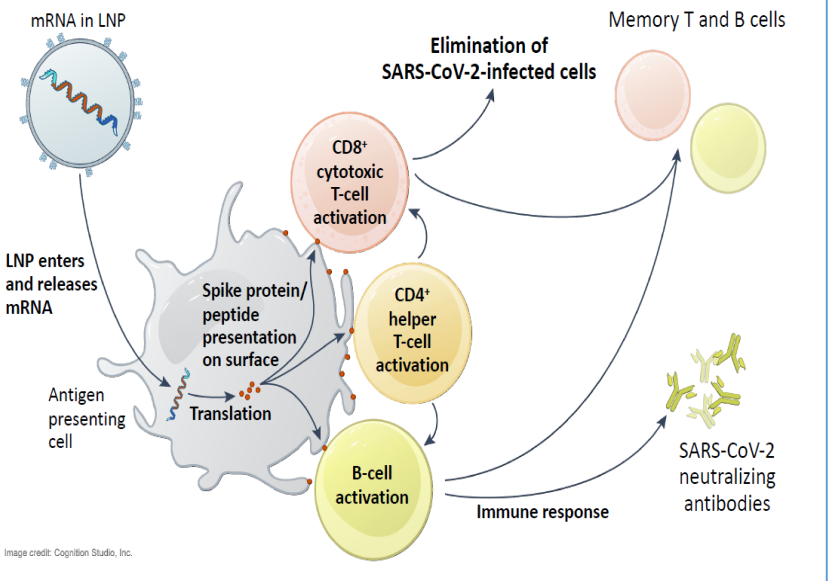
Box 1 | Strategies for optimizing mRNA pharmacology

A number of technologies are currently used to improve the pharmacological aspects of mRNA. The various mRNA modifications used and their impact are summarized below.

- Synthetic cap analogues and capping enzymes[26,27] stabilize mRNA and increase protein translation via binding to eukaryotic translation initiation factor 4E (EIF4E)
- Regulatory elements in the 5′-untranslated region (UTR) and the 3′-UTR[23] stabilize mRNA and increase protein translation
- Poly(A) tail[25] stabilizes mRNA and increases protein translation
- Modified nucleosides[9,48] decrease innate immune activation and increase translation
- Separation and/or purification techniques: RNase III treatment (N.P. and D.W., unpublished observations) and fast protein liquid chromatography (FPLC) purification[13] decrease immune activation and increase translation
- Sequence and/or codon optimization[29] increase translation
- Modulation of target cells: co-delivery of translation initiation factors and other methods alters translation and immunogenicity

Immune Response to COVID-19 mRNA Vaccines

- The immune system responds to the antigens on the surface of the cell produced by the COVID-19 mRNA vaccines
- The vaccines generate cellular immune responses (T-cell) and and humoral responses (B-cell)
- The immune response includes:
  1. Activation of cytotoxic $CD8^+$ T cells that can destroy cells infected with SARS-CoV-2
  2. Activation of $CD4^+$ T cells that augment both $CD8^+$ T-cell and B-cell responses
  3. Generation of memory T and B cells that can quickly respond to future SARS-CoV-2 infection
  4. Activation of B cells to produce antibodies against SARS-CoV-2



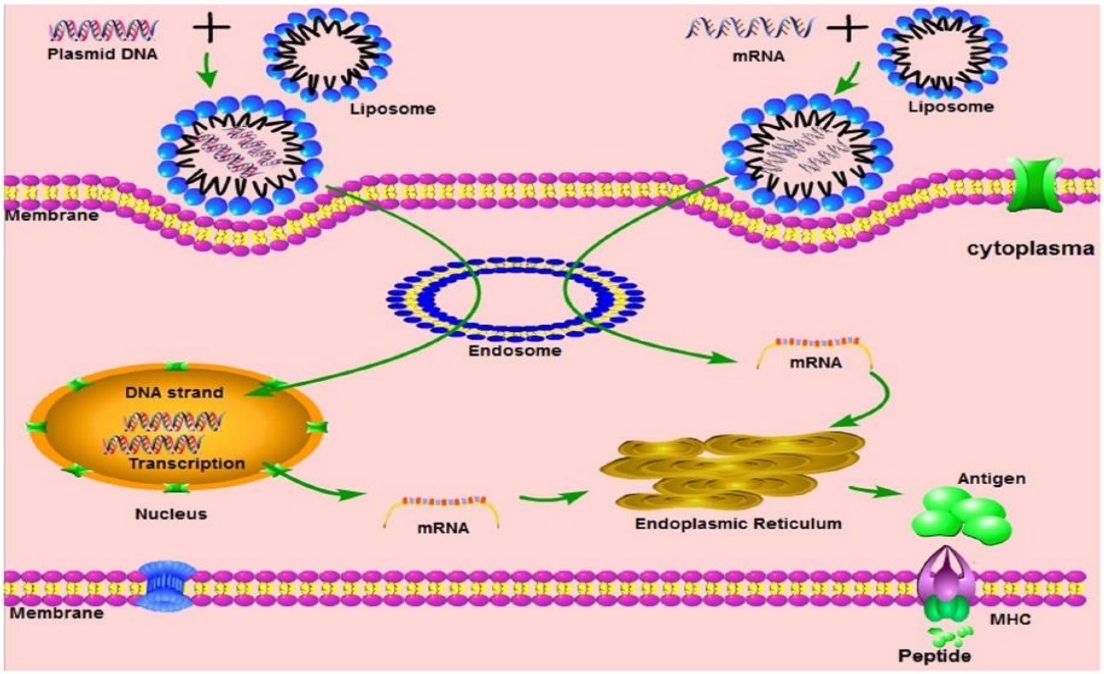


FIGURE 1 | The mechanisms of different nucleic acid vaccines, including DNA vaccines, mRNA vaccines. MHC, Major histocompatibility complex.

TABLE 1 | Advantages and disadvantages of viral vectored vaccines, DNA vaccines and RNA vaccines.

Vaccines	Advantages	Disadvantages
Viral vectored vaccines	Stimulation of innate immune response; induction of T and B cell immune response.	induction of anti-vector immunity: cell based manufacturing
DNA vaccines	Non-infectious; stimulation of innate immune response; egg and cell free; stable, rapid and scalable production; induction of T and B cell immune response.	Potential integration into human genome; poor immunogenicity in humans.
RNA vaccines	Non-infectious, non-integrating, natural degradation, egg and cell free, rapid and scalable production; stimulation of innate immune response; induction of T and B cell immune response.	Concerns with instability and low immunogenicity.

In silico analysis and computational vaccine design: iVAX toolkit for pig vaccines

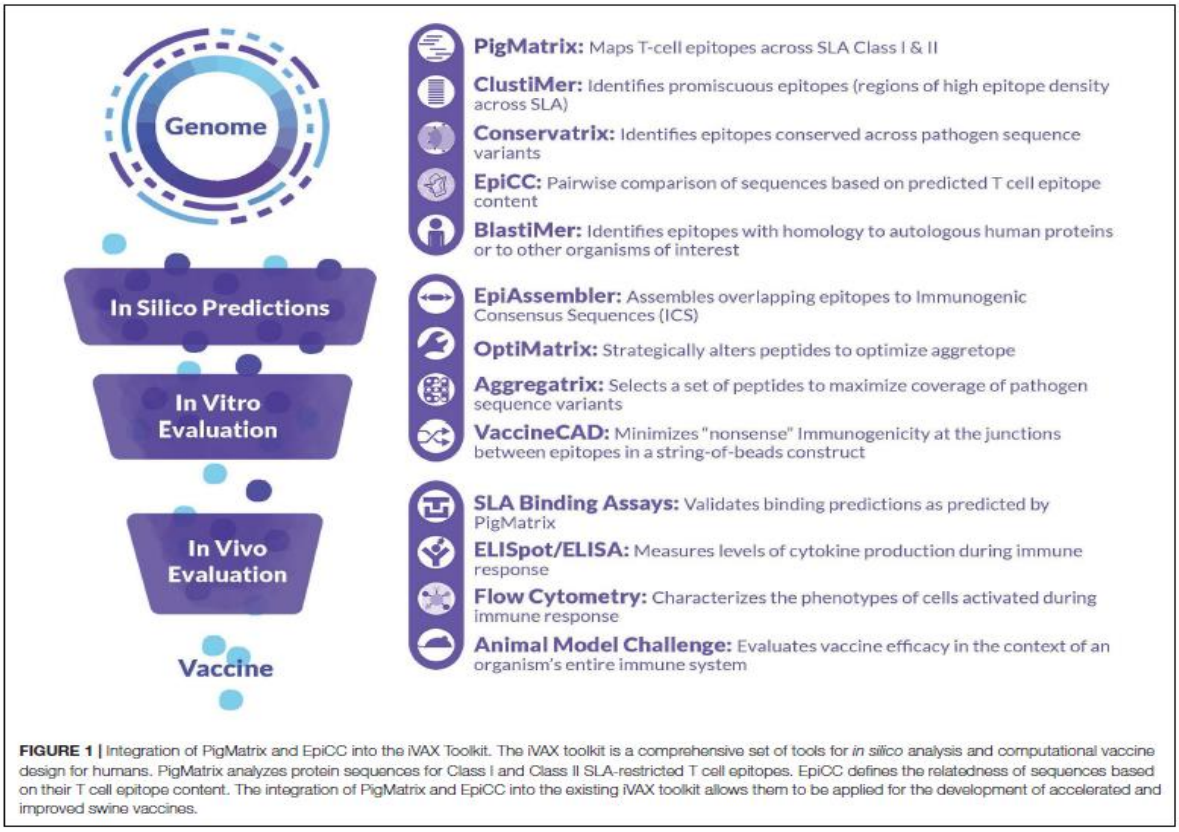
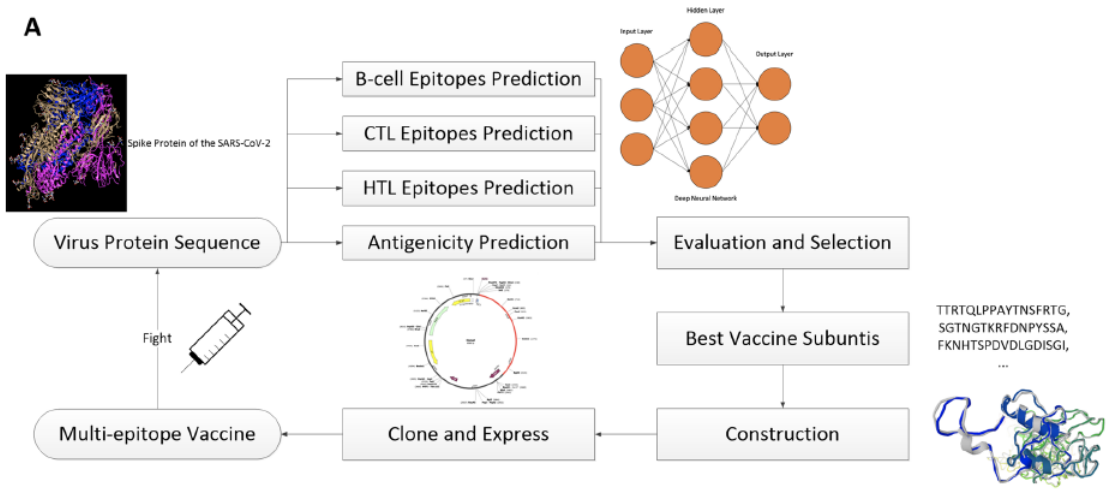


FIGURE 1 | Integration of PigMatrix and EpiCC into the iVAX Toolkit. The iVAX toolkit is a comprehensive set of tools for *in silico* analysis and computational vaccine design for humans. PigMatrix analyzes protein sequences for Class I and Class II SLA-restricted T cell epitopes. EpiCC defines the relatedness of sequences based on their T cell epitope content. The integration of PigMatrix and EpiCC into the existing iVAX toolkit allows them to be applied for the development of accelerated and improved swine vaccines.

In silico vaccine design toolkit versus DeepPredVac tool



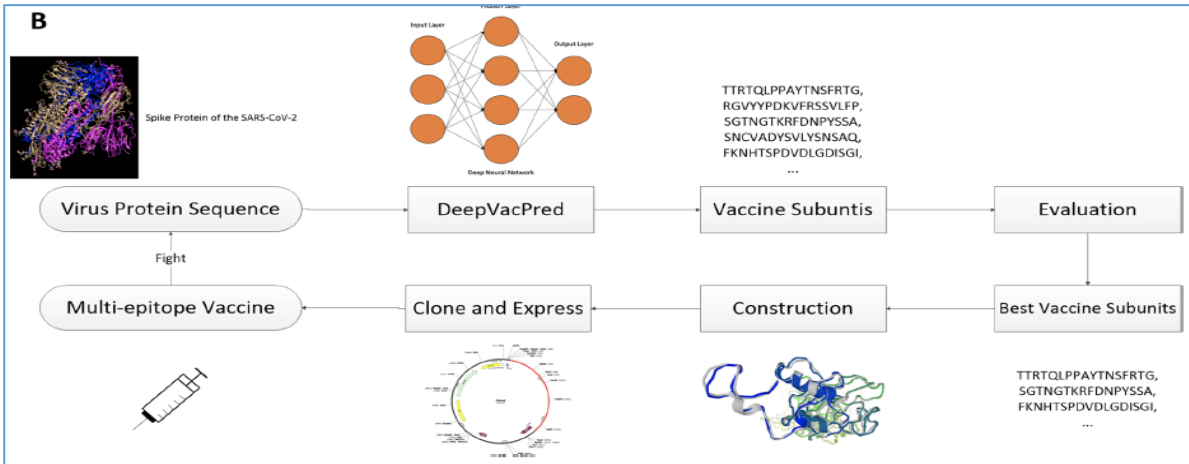


Figure 1. Schematic Diagram of In Silico Vaccine Design Process. (**A**) Traditional in silico vaccine design process. We have to use numerous vaccine design tools. The evaluation and subunits selection is very time consuming. No current tool is able to include all the predictions to comprehensively analyze and select out the best vaccine subunits directly. (**B**) In silico vaccine design by DeepVacPred framework. By replacing the many predictions, evaluations and selections with a DNN architecture inside the DeepVacPred framework, we are able to directly predict a very small number of potential vaccine subunits within a second and start the following evaluation and vaccine construction on a much smaller amount of data.

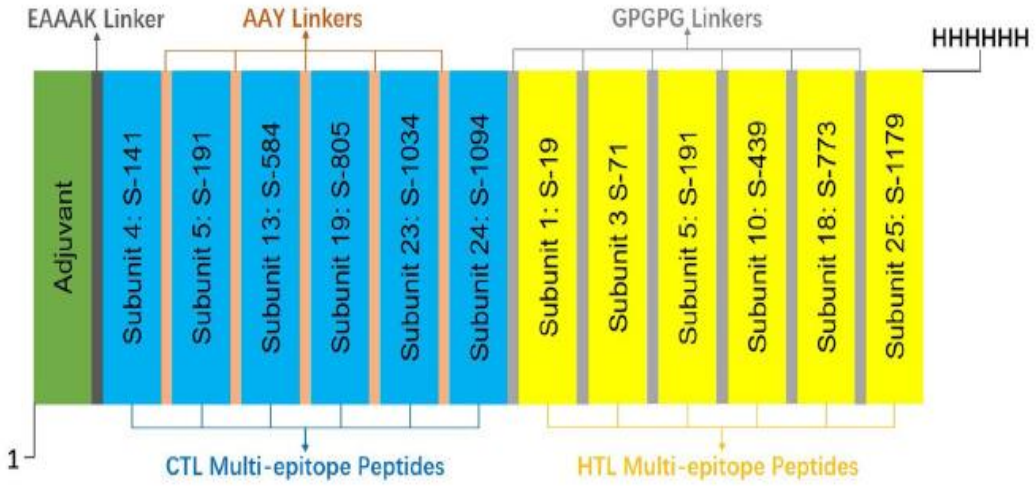


Figure 4. Schematic Presentation of the final Multi-epitope Vaccine. The vaccine is constructed by 11 subunits (Subunit 5 is used twice in both CTL and HTL region for its good performance), an adjuvant and a 6xHis tag, linked by EAAAK, AAY and GPGPG linkers. The final vaccine consists of 694 amino acid residues. It contains 16 B-cell epitopes, 82 CTL epitopes and 89 HTL epitopes.

Structural biology for vaccine design

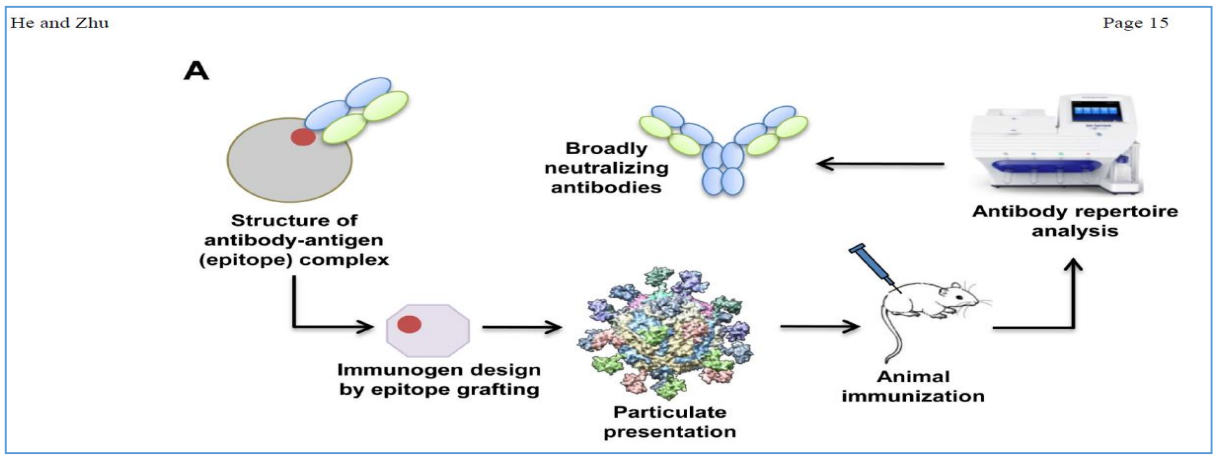


Figure 7. Vaccine 3D Structure Modeling by RaptorX based on the template with PDB ID 3j3vC. All the 694 amino acids in the final vaccine are modeled. The P-value of this model is $4.13 \times 10 - 14$ and this very low value indicates high quality of this 3D model. The unnormalized Global Distance Test (uGDT) score of this model is 506 (> 50), indicating good absolute model quality.

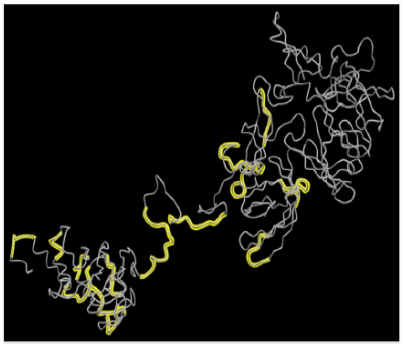


Figure 8. Refined Vaccine 3D Structure Model by GalaxyRefine. This model has a Global Distance Test—High Accuracy (GDT-HA) score of 0.900, a Root Mean Square Deviation (RMSD) score of 0.580, a MolProbity score of 2.618, a clash score of 33.5 and a Ramachandran plot score of 87.5%, showing great overall model quality. The B-cell epitopes in this final vaccine 3D model are highlighted in yellow.

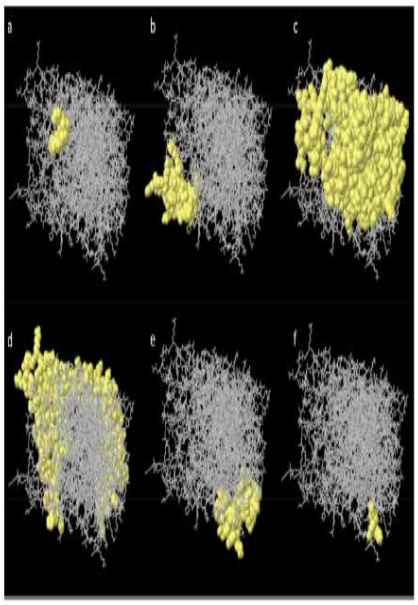
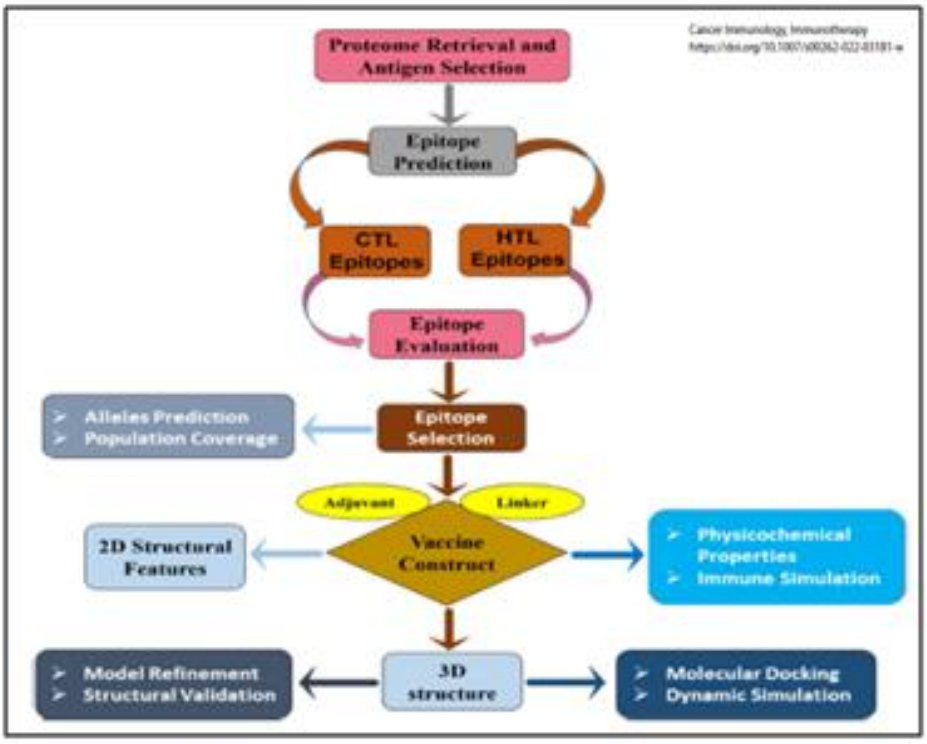
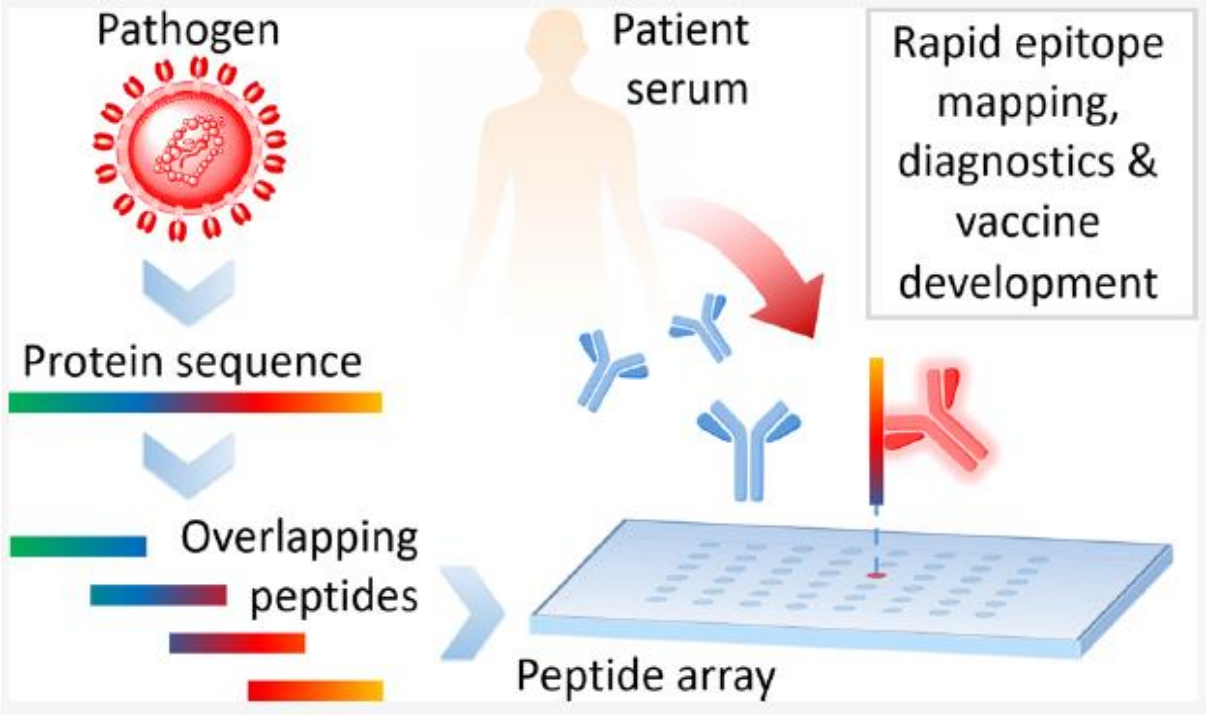


Figure 10. The 3D model of the 6 predicted conformational B-cell epitopes in the refined final vaccine structure. The yellow parts are the conformational B-cell epitopes and the grey parts are the rest of the residues. (a) 3 residues with a score of 0.963. (b) 30 residues with a score of 0.757. (c) 167 residues with a score of 0.711. (d) 161 residues with a score of 0.688. (e) 23 residues with a score of 0.59. (f) 3 residues with a score of 0.531.

Molecular dynamics and immune simulation approaches to vaccine designing (for bovine leukemia virus)



Rapid epitope detection for diagnostics and vaccine design



Conclusions

- Traditional vaccines have been successful for some, but not all, thereby requiring refinement, replacement or rationalization.
- Technological advances have brought fast, accurate and low-cost vaccine prediction, designing, delivery and evaluation tools
- Protein structure-based multi-epitope, gene-based (mRNA and DNA) platforms, and expression vectored vaccines are produced against pathogens, cancers, other diseases
- Better positioned for preparedness to fight against any new emerging infectious diseases.
- 'Personalized designer' vaccine will strengthen people's trust (& reduce vaccine hesitancy) in vaccines.
- Veterinary vaccines are also benefitting from advances in technological advances.

Lecture

Dr. Ajit Singh, PhD, PDF(UK), Former Professor & Emeritus Scientist (ICAR), LUVAS, Hisar-125004 (Haryana)

DIVA vaccines and immunodifferentiating tests for control of veterinary infectious diseases

DIVA strategy: Terminology

- DIVA is an acronym for 'Differentiation of the Infected from the Vaccinated Animals'.
- A 'marker' vaccine devoid of or possessing an Ag that differentiates it from the pathogenic agent.
- A 'negative-marker' is the native Ag/epitope of the pathogenic agent excluded from the vaccine, often by deleting its encoding gene from the wild-type pathogen so as to make a 'negative-marker' vaccine.
- A 'positive-marker' is a heterologous marker Ag/epitope included in the vaccine.
- A 'dual- marker' is the native Ag/epitope of the pathogenic agent substituted by a heterologous marker Ag/epitope in the vaccine.
- DIVA diagnostic test is a companion to the marker vaccine for immunological differentiation of vaccinated from the infected animals.
- DIVA strategy employed as an alternative to 'the slaughter and stamping-out' for control and elimination of infectious diseases of domestic animals in a country or a region, and to gain or re-gain disease-free status for international trade.

DIVA vaccine designs: Aims and objectives

- Immunological protection of the host and the immunological differentiation of the infected from the vaccinated animals (DIVA) with a companion immunodiagnostic test.
- Used in countries that follow the vaccination path for elimination of an infectious disease & to achieve the disease-free status for international trade.
- Also adopted in the disease-free countries in emergencies to contain outbreaks and to regain the disease-free status.

Essential steps in development of a DIVA toolkit

1. Selection of the appropriate marker gene/Ag for the vaccine
2. Construction of the marker vaccine
3. Development and validation of the marker Ag-based immuno-differentiation test.

Where DIVA strategy is indispensable

- Countries that follow 'the vaccination path' to gain the disease-free status for export of animals and animal products.
- An alternative to the slaughter and stamping-out policy of the disease-free countries.

- The purpose of using DIVA vaccines in emergency situations is to slow down the spread of the pathogen from the site of the outbreak.
- Regions of high-risk, high density of the infected species and existence of multiple reservoirs of disease, the use of DIVA vaccine is the best option and represents a ‘humane practice’’.
- As ‘vaccines for life’, DIVA vaccines should encourage rethinking by the policy planners, economists and those concerned with animal welfare.
- The DIVA toolkit can help strike the right balance between animal welfare on one hand, and trade and economic considerations on the other for infectious disease control programmes.
- DIVA vaccines viz., pseudorabies, CSF, FMD, and HPAI used as prophylactic or in emergency of outbreaks in various countries.
- DIVA vaccines are recommended by FAO for use against still prevalent H5N1 or H7N3 serotypes of HPAI virus, in high-risk areas in Bangladesh, India, China, Indonesia, Brazil and Vietnam.
- Successful inspired the development and refinement of DIVA vaccines and diagnostics for several infectious diseases of farm animals, including the transboundary, zoonotic and public health diseases.

Limitations of currently available DIVA vaccines

- Currently available DIVA vaccines do not provide sterile immunity, with less than desired potency and efficacy

- Development of carrier state

FMD virus is a notorious example; about 50% of all infected animals become carriers, which shed the virus for a period from >3 weeks up to 3 years or more.

FMDV infection of the DIVA vaccinated animals can lead to FMDV carrier status, particularly four months after first vaccination

The immune response against NSPs in such cases could be too weak to be detected by NSPs-based diagnostic test.

FMDV NSPs-based DIVA diagnostics are unable to differentiate the unvaccinated naturally infected animals that turned carriers from those that remained non-carriers.

- Development of life-long latent infection in the nervous system

Infection with bovine herpes virus-1.

IBR DIVA vaccine does neither cure latency nor prevent development of latency in the host exposed to the wild-type pathogen after vaccination.

- Better DIVA vaccines need to be developed

Conceptual approaches to DIVA vaccine design

- The landscape of vaccinology has changed during past three decades and vaccines based on several novel concepts have been designed.
- Recombinant DNA technology has been employed for construction of several categories of vaccines with DIVA capability.
- Mutants having deletion of non-essential genes of various infectious agents were developed as vaccines.
- The non-essential gene encoded for the marker antigen in the wild-type pathogen.
- The marker antigen elicited antibody production in the host, when infected with the pathogen.
- This approach has worked for live attenuated viral and bacterial vaccines with DIVA capability.
- Insertion of an epitope-coding foreign gene segment in the genome of the infectious agent.
    - Insertion of the gene segment in a non-essential genetic region of the virus genome and in either plasmid or chromosomal region in the bacteria.
    - DIVA is achieved by detecting anti-foreign antigen/epitope antibodies in the vaccinated animal.
- Chimeric and live expression vectored DIVA vaccines.
- Purified virus particles lacking NSPs as DIVA vaccines.
- Recombinant subunits, VLPs, synthetic (multi)-peptide systems and DNA/RNA constructs.
    - Have inherent DIVA potential and are called ‘negative-marker’ DIVA vaccines ‘by default’.
- Simple mixing of a foreign marker antigen with a whole organism vaccine makes a ‘positive marker’ DIVA vaccine.

Licensed and Commercialized DIVA Vaccines

- Several DIVA vaccines and companion diagnostic tests have been licensed and employed successfully during past 25 years.
- The first DIVA vaccine: gE-gene deletion mutant (live attenuated) of Aujesky’s disease (or pseudorabies) virus of pigs, used in combination with gE-ELISA.
    - Most of the vaccines used at present for pseudorabies are the live attenuated gene gE-deletion or gE- and thymidine kinase (TK).
- The gE-gene deletion live attenuated vaccine for bovine herpes virus-1 disease, with gE-ELISA for DIVA.
- CSF virus E-gene deletion vaccine with E2-based DIVA ELISA was constructed, but not commercialized.

    - Then, baculovirus/insect cell system recombinant E2 subunit with water-in-oil emulsion was licensed, for 'vaccination-to-live' strategy to control new outbreaks in CSF disease-free countries.
    - An effective live attenuated vaccine is used in countries where the CSF disease is endemic.
    - But chimeric DIVA vaccines are being developed with an aim to use them for CSF eradication programme. A live chimeric CSF DIVA vaccine, designated as CP7-E2gif, has been reported to be safe and effective.
- Other commercialized vaccines were introduced in the beginning of the 21st century, notable among which were:
    - NSP-free purified FMD virus particles as DIVA vaccine, when used in combination with NSP-ELISA
    - Avian influenza virus (AIV) vaccines: (a) Inactivated H5N1 marker vaccine (used in China), (b) H5 and N1 genes expressed in fowlpox virus vector (used in China and some other countries)
    - Recombinant New Castle disease virus (NDV) (La Sota strain)
    - AIV H5 live vectored bivalent vaccine (used in China, Italy and USA)
    - PCV-2 capsid gene chimera into the non-pathogenic PCV-1 backbone is a protective vaccine in pigs
    - A West Nile virus (WNV) chimera vaccine constructed by replacing structural genes (coding for PreM and E proteins) of attenuated yellow fever (YF-17D) backbone with WNV; NS1 is a reliable marker of past infection with WNV.

Proof-Of-Concept DIVA Vaccines

- Gene deletion/insertion/substitution live attenuated and killed mutants
- Chimeras and live expression vectored vaccines
- Subunit vaccines
- Virus-like particles (VLPs)
- DNA/RNA vaccines
- Multi-antigen/epitope peptide (MAP) systems
- Heterologous marker-added inactivated vaccines.
- Notable CSF DIVA vaccines:
    - recombinant chimeras [E2/Erns -substituted with bovine viral diarrhea virus (BVDV) sequences],
    - E2- live expression viral vectors, gene E2/Erns -deletion mutants, E2 DNA construct, E2 antigenic peptides, and 'dual marker' live attenuated vaccines.
    - CP7-E2alf is a chimeric marker vaccine based on BVDV strain CP7 expressing the E2 of CSFV strain Alfort/187, with Erns -based DIVA ELISA.
    - It is suitable for EU-approved emergency vaccination programme to control new outbreaks in countries having CSF disease-free status .

Futuristic vaccines with biomarker-based DIVA potential

- Molecular signatures of infection (other than the immune markers) persisting long after clearance of the pathogen from the host are being identified.
- Omics sciences and other next-generation approaches to reveal host-origin signatures (biomarker) for DIVA.
- Biomarker-based DIVA approaches for chronic intracellular bacterial infections in future.

DIVA biomarkers of *Brucella abortus* S19 vaccine have been revealed recently by employing immunoproteomics tools.

Table 1: Various approaches to construction of DIVA vaccines

S. No.	Basic Conceptual Approach for DIVA Vaccine	Basis of DIVA Diagnostics	Selected Example(s) with Refs. in Brackets
1	Deletion of a non-essential gene from the pathogen (First launched, several marker vaccines in use)	Marker antigen-specific antibodies in the infected host, but not in the vaccinated animals (Marker antigen absent in the vaccine, but present in the pathogen to produce antibodies in infected host).	gE- and TK- deletion mutants of pseudorabies virus [10, 49], gE-deletion mutant of IBR virus [25, 33]; E-deletion mutant of CSFV[8, 27]; VP1 G-H loop deletion mutant of FMDV [32]; deletion of amino acids 93–143 (an immunodominant epitope) in the NSP-3A of FMDV [31]; BCG RD1-deletion for bovine TB [5].
2	Chimeric and live vectored vaccines expressing one or more protective antigens from another pathogen (In use and Proof-of-concept vaccines)	-As above- (Marker antigen from vector or those absent in the vaccine, but present in the pathogen to produce antibodies in infected host).	Poulvac FluFend I AI H5N3 RG vaccine for H5N1 in poultry [19]; PCV-2 capsid gene chimera into the non-pathogenic PCV-1 backbone [20, 37]; West Nile structural genes for PreM and E proteins in attenuated yellow fever YF-17D backbone [58]; gE-pseudorabies virus vector expressing E2 subunit of CSFV [21]; VLPs expressing HA and M1 proteins of H9N2 AIV [44]; PPRV-Capripoxvirus [38].
3	Vaccine strain devoid of the differentiating marker antigen (In use and Proof-of-concept vaccines).	-As in S. No. 1	Purified FMD virus, free from NSPs [2]; Salmonella Typhimurium LPS mutant [60]; H9N2 AIV VLPs as vaccine [44].
4	Subunit and Nucleic acid vaccines with or without marker antigens/epitope tags (In use and Proof-of-concept vaccines).	-As in S. No. 2	Viral envelope gE2 of CSFV produced in baculovirus/insect cell culture system [11, 22].
5	Extraneous gene insertion in vaccine strain (Proof-of-concept vaccines).	Marker antigen-specific antibodies in the vaccinated animals, but not in the infected host (Marker antigen present in the vaccine produces antibodies only in the vaccinated host).	Green Fluorescent Protein inserted in Rinderpest virus [35]; NCDV with deletion of native or a foreign epitope replacing the native epitope [34].
6	Vaccine strain with added marker antigen (Proof-of-concept vaccines).	-As in S. No. 3-	Tetanus toxoid in H6N2 Avian Influenza vaccine [48]; KLH in *P. multocida* B:2 vaccine [47].
7	*Vaccines with differential biomarkers/correlates of protection (Theoretically possible; Proof-of-concept lacking).*	*Biomarkers that persist long after resolution of the infection in the host.*	*Vaccines against chronic infections and intracellular pathogens (?) [6, 73]*

MARKER ANTIGENS

- The development of a DIVA strategy requires that:

The marker antigen or epitope(s) has been identified
The kinetics, quality, magnitude and duration of immune response(s) against the marker have been clearly established
The marker-based DIVA vaccine has been constructed
The DIVA diagnostic test based on the marker has been developed and validated according to the method of OIE

- The markers are characteristic of the DIVA vaccines and on that basis, they may be divided into three major categories:

The negative markers
The positive markers
The 'dual' or 'negative-positive' markers.
Biomarkers are such immunogenic or non-immunogenic signatures of the vaccine or the vaccinated host that have the DIVA capability.

DIVA DIAGNOSTICS

- The differentiation of the infected from the vaccinated animals is achieved by a sensitive and specific immunological test based on the marker antigen(s)
- Basically, the immunological differentiation is achieved by detecting:
    - In the post-exposure host, the presence of antibodies produced against the antigens/antigenic determinants (epitopes) of the infectious agent (antibodies as serological markers of infection). These antibodies are absent in the vaccinated individuals, because the marker antigen/epitope(s) are lacking in the vaccine, or
    - In the vaccinated individuals the presence of antibodies produced against the foreign antigen/epitope(s) inserted or added in the vaccine formulation. These antibodies are absent in the post-exposure host, because the foreign antigens/epitope(s) are lacking in the infectious agent and the host's natural environment.
- The DIVA diagnostics associated with the 'negatively marked' vaccines provide more valuable information than those associated with 'positively marked' vaccines.
- The former reveal the infected animals, while the latter detect only the vaccinated animals in the population.
- Probably for this reason, number of negatively marked vaccines exceeds the positively marked ones.
- Standardization and validation of DIVA diagnostic tests requires the systematic approach similar to that used for conventional diagnostic tests.
- DIVA diagnostic tests should have high analytical sensitivity, analytical specificity, diagnostic sensitivity and diagnostic specificity.
- Differential diagnostic tests already developed for use in combination with different DIVA vaccines include

- Indirect ELISA,
- Blocking ELISA,
- Hem agglutination inhibition,
- Latex agglutination test,
- DTH,
- IFNγ assay in vitro, etc.
- Point-of-care DIVA diagnostic tests: LFDs, LAT, etc. desired

ELISA-based differentiation tests

- Several ELISA-based DIVA tests are available commercially for various DIVA vaccines.
- Rec Ags expressed in E. coli or insect cell culture system are used in indirect ELISA or competitive ELISA with mAbs or a combination of polyclonal and mAbs for anti-negative marker antibody levels in the infected hosts
- ELISA based on synthetic peptide and other peptides have been employed in FMD DIVA
- The positive marker-based ELISAs detect anti-marker antibodies in the vaccinated animals.
- The commercial tests from different manufacturers provide results in two to several hours.
- ELISA based on various NSP Ags, individually or as panels are available for FMD DIVA
- Sorensen and others have developed blocking ELISA using baculovirus expressed FMDV NSPs as antigen and g. pig antibodies or mAbs for capture and detecting.
- It has now been replaced by mAbs for high throughput studies.
- 3ABC-binding single chain Fv (scFv) antibody fragments selected from phage display library have been successfully used in competitive ELISA format for DIVA.
- However, tests for detection of NSP antibodies cannot be used reliably for detection of carrier animals as some persistently infected animals do not show sero-conversion against NSPs, and the carrier animal status may occur in previously vaccinated animals in which only limited virus replication occurs.

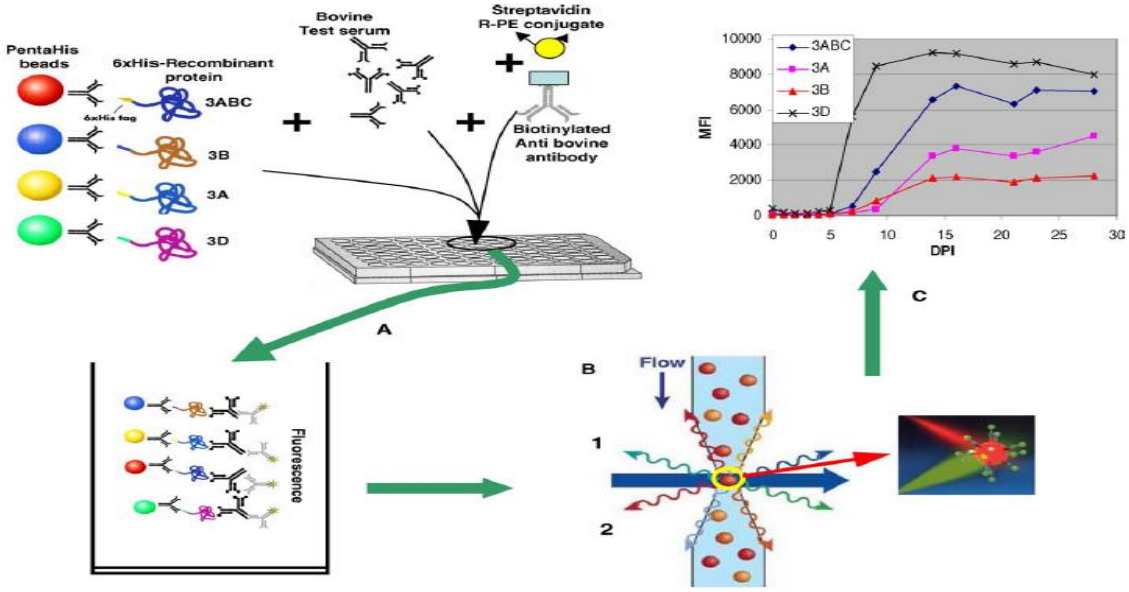
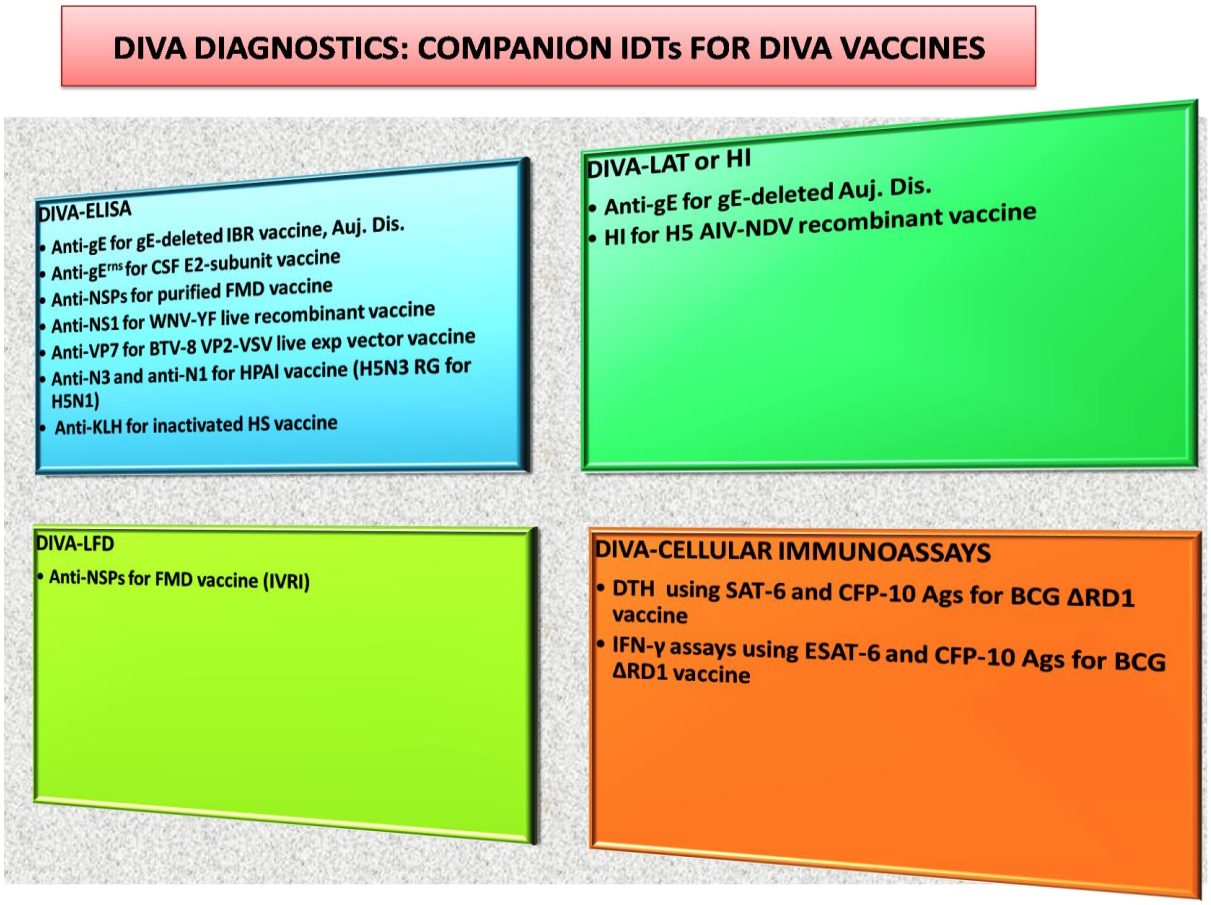


Fig. 1. The Luminex multiplex immunoassay can detect four non-structural proteins (NSPs) simultaneously. This assay consist of a bead-bound capture molecule coupled with a monoclonal antibody that react with a histidine tagged recombinant protein (6×His). Bovine test serum and a biotinylated anti-bovine antibody is added, followed by a streptavidin conjugated to the fluorophore R-phycoerythrin (R-PE) (A). By using different beads sets (each with its own unique colour code) all four reactions are measured in a single sample by irradiation of the beads with two lasers of different wavelengths (B). Fluorescence signals from the bead colour-coding dyes and the reporter-molecule dyes are recorded simultaneously and digitally processed to translate the signals into quantitative data for each reaction (C).



Marker antigens of DIVA vaccines with companion immunodiagnostic tests

Animal Species	DIVA Vaccine	Marker Antigen	Differentiating Diagnostic Test
Bovine	IBR virus vaccine [gE-deletion] live or killed	gE	Anti-gE-ELISA
	FMD virus vaccine (NSP-free purified virus)	NSPs (3ABC/3BC-most suitable), 3A, 3B, 3D, 2B, 2C, G-H loop epitope	Blocking ELISA, using rNSPs and monoclonal Abs
	Rinderpest virus-GFP vaccine	GFP	Anti-GFP ELISA
	BCG ΔRD1	ESAT-6, CFP-10, Rv3615c & MPB83	IFN-γ assay *in vitro;* DTH response
	Brucella abortus S19 vaccine	Biomarkers (InvB, Sod, Dps, Ndk, Bfr polypeptides)	Mass spectrometry (Antigens in naturally infected animals)
	Pasteurella multocida B:2 bacterin+KLH+Al_2O_3 nanoparticles	KLH	Anti-KLH-ELISA
Sheep	BTV-8 VP2-VSV live expression vector	VP7	Anti-VP7-ELISA
	PPRV-Capripoxvirus live expression vector		
Equine	West Nile virus- Yellow fever virus live expression vector	NS1	Anti-NS1-ELISA

Pig	Pseudorabies (Aujesky's disease) gE- and TK-gene deletion	gE	Anti-gE-ELISA; Anti-gE-LAT
	Classical swine fever virus vaccine (E2 subunit)	E^{ms}	Anti-E^{ms}-ELISA (suitable for herds, not individuals)
	CSFV E2-BVDV chimeric vaccine	E^{ms}	Anti-E^{ms}-ELISA
	PRRV/PCV-2	ES4 epitope of NSP2 + GFP	Anti-ES4- and Anti-GFP-ELISA
	LPS-mutant Salmonella Typhimurium ΔrfaJ' vaccine	Smooth LPS	Anti-LPS-O Ag-ELISA
Poultry	Salmonella Enteritidis SPI-*lon-fliC* mutant	Flagellin	Anti-flagellin-ELISA
	HPAIV vaccine (Poulvac FluFend I AI H5N3 RG for pathogenic H5N1)	N3 and N1	Anti-N3- and anti-N1-ELISA
	H9N2 AIV vaccine comprising of HA and M1 protein VLPs	Nucleocapsid (NC)	Anti-NC-ELISA
	Inactivated H6N2+TT vaccine	Tetanus toxoid	Anti-TT-ELISA
	NDV-nucleoprotein epitope deletion vaccine	Nucleoprotein epitope	Anti-NP epitope-ELISA

CONCLUSIONS AND FUTURE PERSPECTIVES

- DIVA vaccines with accompanying diagnostic tests have already been proven as invaluable tools for control, elimination or eradication of major infectious diseases of domestic animals, namely, CSF, pseudorabies, FMD, AIV, IBR, etc.
- The DIVA strategy has become essential in the countries that follow 'the vaccination path'
- This strategy offers a humane alternative to 'the slaughter policy' in those disease-free countries
- A DIVA vaccine is designed by deleting or inserting a marker gene/antigen in the infectious agent and the host's immune response against the DIVA vaccine differs from that against the natural infection due to the marker antigen.
- Non-whole organism rDNA-based vaccines have inherent DIVA capability, with more options for the selection of marker antigen.
- DIVA vaccines have some limitations too.
    - Less potency and efficacy of several proof-of-concept DIVA vaccines.
    - The DIVA capability of some commercial FMDV vaccine for detecting carrier animals is also questionable in some situations.
    - Development of mucosal DIVA vaccines for domestic animals and *in ovo* DIVA vaccination of poultry is highly desired.
    - Point-of-care DIVA diagnostic tests are desired for early containment of fast-spreading infectious disease.

- Modern tools of vaccinomics, immunomics, reverse vaccinology, rDNA technology, genomic sequencing, etc. are very promising
- Search for biomarkers of infection and DIVA vaccinated hosts should be continued

Lecture

(BD Singh, Ex-Emeritus professor, School of Biotechnology, Banaras Hindu University**)**

The Five Dreams of Plant Breeders That Are No Longer Mere Dreams

Dream 1: Reading the Genotype: Genotype-Based Selection

Status: Selection in based on trait phenotype

Problem: Phenotype has a non-heritable component due to the environmental effect.

Solution: *Marker-assisted selection (MAS)*

Molecular Markers

Markers which are based on differences in base sequences of the same genomic regions of different individuals or lines. These differences in base sequences can be detected by:
1). Amplification (RAPD, SSR). 2). Hybridization after restriction digestion (RFLP, DArT). 3). DNA sequencing (SNP).

Types of Markers:

Based on the marker location in relation to the gene, they are divided into:
1. Random marker: It is located outside the gene and shows partial linkage
2. Gene-based marker: It is located within the gene and shows partial linkage
3. Functional marker: The marker allele is based on the sequence difference between the two alleles of the gene and shows complete linkage.

Marker-assisted Selection and Genomic Selection

Marker-assisted selection (MAS): It is the selection for a gene based on marker genotype and not phenotype of the trait; suitable for genes having large effects on trait phenotypes
Genomic selection (GS): It is the selection for all genomic regions that affect the trait; highly effective but very demanding breeding scheme.

Markers in Plant Breeding

- Mostly used by the private sector
- In public sector, generally used for marker-assisted backcrossing
- Handling of several-fold larger amount of data and fast decision making require strong statistical and bioinformatics resources
- Use of off-season nurseries reduces time needed for development of new varieties
- Increased cost of breeding programmes

Dream 2: Gene Isolation and Transfer

Status: ***Primary gene pool***, readily used

Secondary gene pool, often used with some to considerable difficulty

Tertiary gene pool, use is possible but very difficult

Problem: Many useful genes are not available to breeders

Solution: Genetic engineering/transgenic technology (**offers limitless gene pool**)

Genetic Engineering: Transgenic Plants

- Genes and other sequences from any source, even synthetic sequences, transferred into plants
- Biosafety concerns due to:

1. ***Foreign DNA sequences***, *e.g*., the transgenes, antibiotic resistance markers, etc., and the proteins encoded by them (***relatively easier to evaluate***)
2. ***Random transgene integration*** in the genome might lead to unintended inactivation of desirable, even essential, genes and/or activation of undesirable genes (***assay very demanding*.**

Regulation of Transgenic Plants

Status: Low consumer acceptance
Problem:

1. Generation of biosafety data requires about 5.5 years
2. Biosafety dossier (file) preparation costs, on an average, 35 million US dollars
3. Approvals by competent authorities do not necessarily satisfy the consumers

Response: *cis*-genic and *intra*-genic plants

cis-genic plants

- Native (unmodified) genes with their own regulatory sequences from only the primary gene pool are used
- They are similar to backcross products
- However, gene integration is random
- Biosafety evaluation requirements less rigid

Intra-genic Plants

- Same as *cis*-genic plants, except that the coding sequence of a gene are usually combined with regulatory sequences from other genes from the *primary gene pool*
- Biosafety evaluation would be more rigid than that for cis-genic plants.

cis-genics with Targeted Transgene Insertion

- Gene integration at precisely targeted sites in the genome achieved by site-directed nuclease technology
- *Cis*-gene integration occurs by homologous recombination
- No risk of accidental inactivation of desirable genes or activation of undesirable genes
- Biosafety evaluation may be greatly relaxed
- But biosafety requirements for cis-genic and intragenic plants are the same as those for transgenic plants

Dream 3: Directed Mutagenesis
Status: Mutations are random events
Problem:

1.The recovery of desired mutants is not assured
2.Unintended and undesirable mutations are often associated with the desired mutations

Solution: Site-directed mutagenesis

Site-directed Mutagenesis

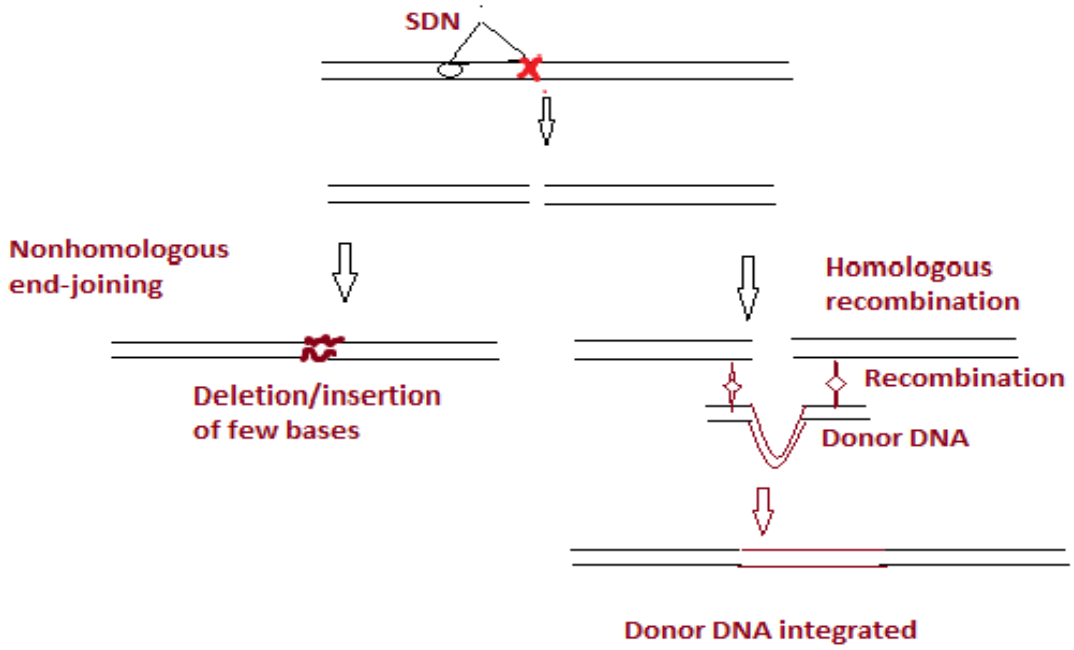
- Induction of specified mutations at the desired genomic sites (= genes)
- Site-directed mutations are induced by:

1. Site-directed nucleases (SDNs)
2. Chimeric (DNA: RNA hybrid) oligonucleotides

Site-directed Nucleases

- SDNs are designed by genetic engineering
- CRISPR is the easiest to design and the most widely used SDN
- *SDN induced double-strand breaks at the selected genomic sites, e.g., within a gene*
- Mutations are induced during repair of the breaks by the cellular machinery

Mechanism of Mutagenesis by SDNs:



Dream 4: Reliable Reconstruction of an outstanding hybrid

Objective: Isolation of a pair of inbreds from an outstanding hybrid plant that would yield the same hybrid when mated together
Problem: A very large number of different inbreds produced; their phenotypic evaluation for hybrid development is extremely demanding
Solution: Reverse breeding

Reverse Breeding

- Crossing over in the hybrid suppressed by a chemical or a transgene
- Doubled haploid (DH) plants obtained from the hybrid plant

- The number of different genotypes in the DH population will be merely 2^n (*n* is the number of chromosomes of the species)
- In case $n = 7$, the total number of different inbreds will be only $2^7 = 128$

2^{n-1} Complementing Pairs of Inbred

- The 2^n inbreds will form 2^{n-1} complementing pairs of inbreds, *i.e*., 64 different pairs when $n = 7$
- This identification becomes simple with the use of genome-wide molecular markers

(eliminates phenotypic evaluation)

Dream 5: Breeding perennials like annuals.

- *FT* (*flowering time*) genes, e.g., *FT* from Arabidopsis, *PtFT* and *PtFT2* from poplar, and *CiFT* from citrus, etc., induce early flowering
- *FT* genes transferred in perennials like fruit trees; the transgenic plants flower in one year
- Marker-assisted backcross scheme is used to transfer the desired gene in the early flowering transgenic line.

The Improved Variety is Perennial

- In the end, the transgene for early flowering is removed by segregation.
- The normal flowering end product of the backcross programme is the improved version of the recurrent parent; it is released for commercial cultivation.

Conclusions

- The transgenic, *cis*-genic and *intra*-genic plants commercialized or close to commercialization
- Products from SDN technologies are being commercialized
- Reverse breeding is in experimental stages
- Early flowering perennial scheme being used in breeding programmes

Lecture

(BD Singh, Ex-Emeritus professor, School of Biotechnology, Banaras Hindu University)

Gregor Johann Mendel and His Impact on Biology

Gregor Johann Mendel

- Born in 1822 in Morovia near Brunn in Austria, now Brno in Czechoslovakia, in a poor farmer family
- Faced financial and health problems
- Had to leave studies and join St. Augustinian monastery of Brunn in 1843 mainly due to financial problems
- Became a priest in 1847
- In 1851, went to University of Vienna and studied physics, mathematics, philosophy, etc.
- He was a sincere and hardworking student but he performed poorly in mathematics and physics
- He returned to Brunn in 1854 and was appointed as a substitute science teacher
- He turned out to be an excellent teacher.
- In 1857, Mendel began collecting seeds of pea varieties from commercial growers
- Conducted all his studies in the kitchen garden of the church
- He presented his paper in February, 8 and March 8, 1865 meetings of the Natural History Society of Brunn.

The Mendel's Paper

- The paper was published in the annual proceedings of the Society in 1866
- The proceedings was distributed to libraries in Europe and USA
- The paper: Versusche uber Pflanzenhybriden (**Experiments on plant hybrids**)
- He continued studies on honeybees, *Phaseolus* and *Hieracium*
- Mendel passed away in 1884 at the age of 62 years

The 7 Genes of Pea

- **Seed shape (*R*/*r*):** starch branching enzyme; *r* allele has transposon (*Ips*-*r*) insertion
- **Cotyledon color (*I/i*):** SGR protein (chlorophyll degradation); *i* has 2 amino acid insertion
- **Seed coat color (*A/a*):** transcription factor; *a* has G A transition that affects splicing
- **Plant height *(Le*; internode length):** GA 3-oxidase; G A transition (Thr replaces Ala)
- **Pod color (*Gp/gp*):** possibly chlorophyll accumulation; candidate genes identified
- **Pod shape (*P/p* or *V/v*):** Possibly transcription factors involved in secondary cell wall deposition
- **Flower position (*Fa/fa* or *Fas/fas*):** Perhaps a transmembrane signaling protein like CLV1.

(Ellis *et al*. 2011. Trends Plant Sci. 16: 590-596)

Reciprocal Crosses Resolved A Long Standing Controversy

- **Controversy**: The contribution of male and female parents to the features of progeny
- **Reciprocal crosses**: Round (male) x wrinkled (female) yielded Round seeded *F1*; 3:1 ratio in *F2*, Round (female) x wrinkled (male) also yielded Round seeded *F1*; 3:1 ratio in *F2*

Conclusion: Male and female parents contribute equally to the features of progeny

Mendel Was Lucky

- All the seven traits showed monogenic control
- There was no lethal gene action (risk was eliminated due to the initial growing of the parental varieties for 2 years)
- Complete dominance in all the cases
- Did not encounter linkage in dihybrid crosses
- Problem cases (honeybees, *Phaseolus* and *Hieracium*) encountered only after the principles were discovered.
- Some Genes Were Located on The Same Chromosome.
- Seed coat colour *(a)* and cotyledon colour *(i)* located on chromosome 1.
- Pod shape *(v)*, flower position *(fa)* and stem length *(le)* located on chromosome 4.
- Only *v* and *le* show linkage and **Mendel did not study this character pair.**

The Luck Runs out

- *Phaseolus* was a mixed bag: qualitative (both single and multiple gene control) as well as quantitative traits (flowering time and peduncle length)
- *Hieracium* is an apomict
- Honeybees have diploid queen and haploid males
- Contemporary biologists failed to appreciate the significance of Mendel's findings
- His work was rediscovered in 1900 independently by de Vries, Correns and Tschermak

Mendel Was Virtually Crucified

- Defamatory attacks by RA Fisher in 1936
- This was followed by spate of criticisms:

1. Findings opposed to Darwin
2. Detected linkage but did not report
3. Fictitious description of experiments, *e.g*., selective reporting of results
4. Falsification of data
5. Did not explain segregation and independent assortment although it is generally believed

R.A. Fisher's Attack on Mendelism

'These defamatory criticisms include imputations on the scope of his experimental work, his understanding of what he wrote and statistical interpretations of his results' (Quotes from Ellis *et al*. 2011. Trends Plant Sci. 16: 590-596)

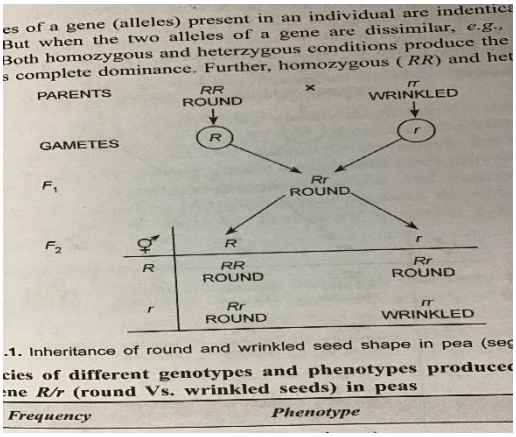
Some Misconceptions About Mendel's Work

- Mendelian inheritance' = Oligogenic inheritance

- 'Mendelian genetics'= Genetics of qualitative traits
- 'Mendelian ratios' = 3:1, 9:3:3:1 etc. Example: In several crosses, the ratio of late flowering to early flowering in *F2* plants and their *F3* progeny (3:1 or 15:1) was similar to the ratio expected from Mendel's law.
- (Hori et al. 2016 Theor Appl Genet DOI 10.1007/s00122-016-2773-4)
- **'One-gene-one-character**': Mendel suggested multigenic control of flower color in *Phaseolus*
- Even the **title of Mendel's paper is usually mistranslated** as 'Experiments in Plant Hybridisation' in the place of the correct translation: 'Experiments on Plant Hybrids' (Versuche uber Pflanzen-Hybriden')

Segregation

1. The two alleles of a gene remain together in the *F1* hybrid
2. They do not affect each other
3. They separate during gamete formation to produce two types of gametes in equal proportion
4. Phenotypic ratio in *F2* is 3:1
5. Genotypic ratio in *F2* is 1:2:1

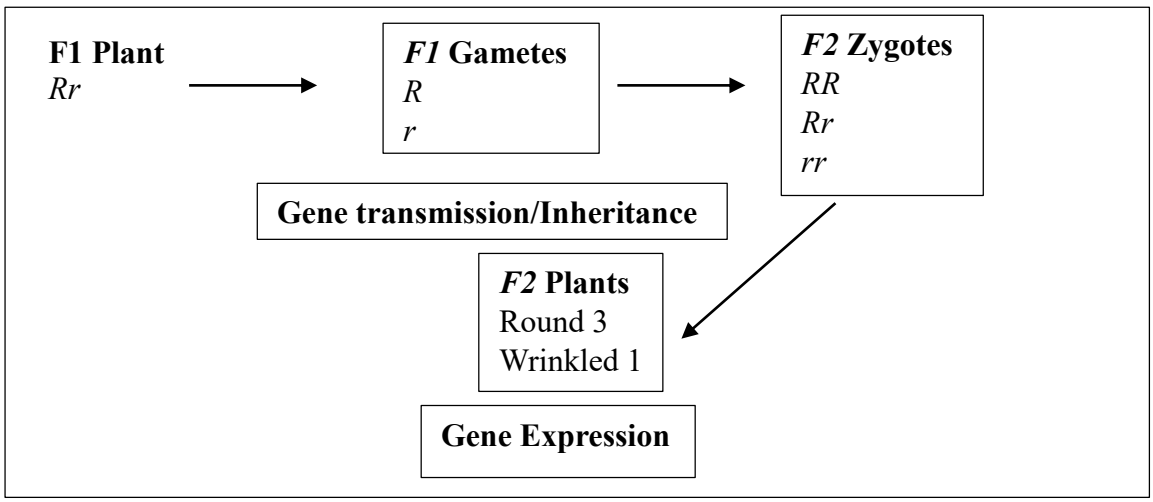


Binomial Expansion Suggests Explanation

- Mendel observed 3:1 ratio in *F2* and 1:2:1 ration in *F3*
- 1, 2, 1 are coefficients of the binomial $(a+b)^2$ $(= 1a^2 + 2ab + 1b^2)$
- Therefore, a single gene with two alleles controls seed shape in pea
- Each plant has two copies of a gene: these may be identical (*AA*, *aa*) or different (*Aa*)
- The genotypes of *F2* plants can be written as *aa*, *ab*, *bb* (today we write, *AA*, *Aa*, *aa*)

Law of Segregation

- The two alleles of a gene remain together in the *F1* hybrid without affecting each other
- They separate during gamete formation to produce two types of gametes in equal proportion.
- As a result, in *F2* Phenotypic ratio is 3:1 and Genotypic ratio in is 1:2:1



Assumptions Involved in Segregation

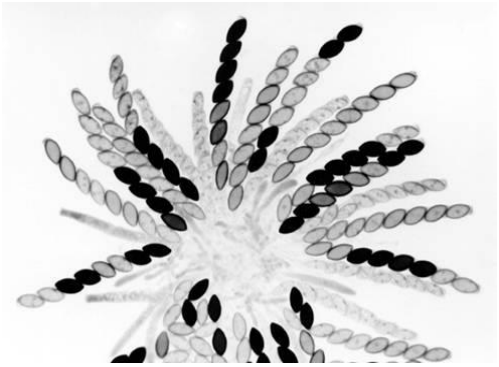
1. Two gametes in 1:1 ratio (cf. gene conversion)
2. Equal survival of gametes (cf. male sterility)
3. Equal function of gametes (cf. X and Y carrying human sperms)
4. Random union among male and female gametes (cf. gametic self-incompatibility)
5. Equal survival of zygotes (cf. lethal genes)
6. Complete dominance (cf. incomplete dominance)

Test Cross

- *F1* hybrid x recessive parent
- 1:1 phenotypic ratio
- Phenotype of test cross progeny determined by *F1* gametes only
- Therefore, F1 produces two gametes in 1:1 ratio
- But the assumptions involved in segregation leading to 3:1 *F2* ratio apply here as well

Neurospora crassa Spore Colour

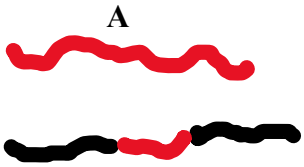
Spore colour determined by *cys3* gene
Cys3, black spore
cys3, white spore



Gene Conversion

- Neurospora produces 4:4 spores in one ascus
- Occasionally, the spores are other than 4:4, e.g., 5:3
- This results from copying of a sequence from one homologous chromosome into the other

Chromosome with *A* allele

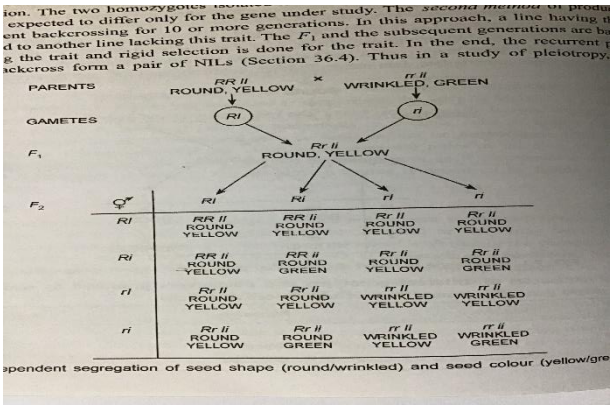


Chromosome with *a* allele

***A* allele copied into this Chromosome in the place of *a* allele**

Independent assortment

Produces four gametes in 1:1:1:1 ratio
In *F2* 9:3:3:1 phenotypic ratio is obtained



The 9:3:3:1 Ratio

- *F2* ratio: Seed shape: 3 round : 1 wrinkled; Cotyledon colour: 3 yellow : 1 green

- Probability of two independent evets occurring together is the product of their individual probabilities
- Round yellow, 3 x 3 = 9
- Round green, 3 x 1 = 3
- Wrinkled yellow, 1 x 3 = 3
- Wrinkled green, 1 x 1 = 1

The Gene Concept Emerged Out of Mendel's Discoveries

- Particulate (now known to be DNA)
- Function: Development of a trait (current view, encodes a diffusible product)
- Inheritance:
  1. Alleles remain pure and segregate (exceptions: paramutation, plasmagenes)
  2. Different genes segregate independently (exception: linkage; a logical suggestion that recombination occurs between linked genes)
- Mutation: Produces two alleles (exception: multiple alleles, which is the rule)

Lecture

(BD Singh, Ex-Emeritus professor, School of Biotechnology, Banaras Hindu University)

The Gene Concept

- Genes are particulate (Mendel; now known to be DNA)
- Unit of function: one-gene-one-character (Mendel)
- Unit of inheritance: Different genes assort independently (Mendel)
- Unit of mutation: one gene has two alleles (Mendel)
- Unit of recombination: crossing over occurs between genes (proposed later by Morgan and coworkers)

Gene as Unit of Function

- One gene specifies one character.
- For example, gene ***r*** governs seed shape in peas.
- But Mendel himself suggested that flower colour in *Phaseolus vulgaris* (rajma) may be controlled by more than one gene.

Gene Interaction

- In 1905, the first case of gene interaction was described by Bateson and Punnet.
- They reported that comb shape in poultry shows 9:3:3:1 ratio in *F2*.
- Thus, a single character, *viz*., comb shape, was governed by two genes.
- Later other types of gene interactions were described.
- It was established that more than one gene may specify a single character.
- **Conclusion**: A single gene does not produce one character.

Mendelian Inheritance Is Opposed to Evolution

- Mendelian inheritance pertained to qualitative traits that showed discontinuous variation and were not important for survival, *e.g*., flower colour, seed colour, pod shape, etc.
- In *F2* and *F3*, only combinations of the parental characters are observed and no new types are produced
- Evolution, on the other hand, acted on continuous variation that were relevant for fitness and survival
- It was argued that Mendelian principles cannot explain the inheritance of quantitative traits
- Therefore, Mendelian inheritance was thought to be inconsistent with the theory of evolution
- Mendelians argued that quantitative traits are not inherited
- Galston, a statistician, showed significant parent-offspring correlation, which revealed an inherited component in quantitative traits
- Statisticians countered that Mendelian laws cannot explain the inheritance of quantitative traits

Multiple Factor Inheritance

- In 1906, Yule suggested the idea of multiple factor inheritance
- Experimental evidence for this hypothesis was presented by Nilsson-Ehle in 1908 (seed colour in wheat)

- Wheat seed colour was governed by two genes, say, *R1* and *R2*, and their effects were small and additive
- The alleles *R1* and *R2* produced colour, while *r1* and *r2* alleles did not
- The seed colour depended on the total number of *R1* and *R2* alleles present in a seed
- Thus, the continuous variation in quantitative traits can be produced by multiple genes with additive effects governing the trait and the effects of the environment on the trait phenotype

One-Gene-One-Enzyme

- In 1941, Beadle and Tatum published their results from studies on auxotrophic mutants of *Neurospora crassa*
- Each mutant was deficient in biosynthesis of a single vitamin or amino acid
- They proposed that each gene controlled one biochemical reaction by specifying one enzyme involved in the reaction
- This was popularly described as **one-gene-one-enzyme** hypothesis

One-Gene-One Polypeptide

- Human haemoglobin has two alpha and two beta chains (=α2β2)
- Sickle cell disease is due to a single recessive gene *s*
- In 1957, Ingram and coworkers reported that haemoglobin of sickle cell disease patients differed from that of normal persons by a single amino acid
- In sickle cell patients, a valine was present at position 6 of the β-chain of haemoglobin in the place of glutamic acid found in the normal haemoglobin β-chain
- Thus, one gene specifies one polypeptide

One-Gene-One-Diffusible Product

- Many genes do not produce proteins; they encode RNAs like ribosomal RNA, transfer RNA, guide RNA, etc.
- Many other genes produce regulatory RNAs like microRNAs
- Small interfering RNAs suppress gene expression by RNA interference (RNAi)
- **Conclusion:** *The function of a gene is to produce a diffusible product*, viz., *either an RNA or a polypeptide*

Genes as Unit of Mutation

- Mendel reported two alleles for each of the seven genes he studied
- This was taken to mean that each gene can have only two alleles
- Therefore, existence of more than two alleles for a locus was considered by many to indicate the locus to be a complex locus, *i.e.*, having more than one gene
- In 1901, Landsteiner described the human A, B, O blood groups governed by three alleles (I^A, I^B, *i*) of a single gene, *i*.
- Since then, many cases of multiple alleles have been described
- **Conclusion:** each gene mutates many times (multiple alleles)

Gene as Unit of Inheritance

- Mendel showed that different genes segregate independently and produce 9:3:3:1 ratio in *F2*
- In 1902, Walter Sutton proposed that genes are located in chromosomes

- Sutton reasoned that since each organism has hundreds of different genes but only a limited number of chromosomes, each chromosome must carry several genes
- He further argued that since chromosomes are most likely inherited as a unit, the genes located in one chromosome may tend to be inherited together
- This tendency has been named as linkage

Linkage Encountered but Misunderstood

- In 1906, Bateson and Punnet encountered the first case of linkage in sweet pea between pollen shape and flower colour
- The ratio in *F2* showed large deviation from 9:3:3:1
- They misinterpreted the data: they suggested that pollen grains with some genotypes underwent additional rounds of division after meiosis leading to deviation from the expected 1:1:1:1 ratio among the four types of gametes
- They coined the terms *coupling phase* and *repulsion phase* that are used even today

Morgan Describes Linkage and Crossing over

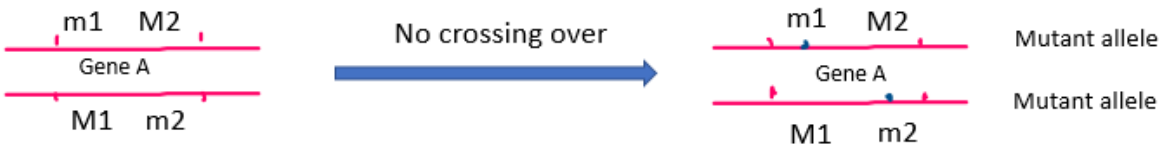
- In 1910, Morgan reported that the *white eye* gene of *Drosophila* was located on the X chromosome (*sex-linkage*)
- The single white-eyed male escaped but was recaptured since it was winter and all windows were closed
- He also described linkage among three sex-linked genes and proposed that crossing over occurs *between* linked genes
- Awarded Nobel Prize in 1933
- The phenomenon of linkage and crossing over is used to prepare linkage maps including molecular maps
- **Conclusion:** genes are not units of inheritance. The unit of inheritance is a linkage block containing more than one gene

Gene as a unit of recombination

- When two mutations, *viz*., *m1* and *m2* affect the same trait, the question arises whether they are alleles of a single gene or two different genes
- For a long time, this was answered by a simple test: if there was recombination between *m1* and *m2* and the wild type allele was recovered in the progeny of their heterozygote, they were considered to be located in two different genes

Recombination test for alleles

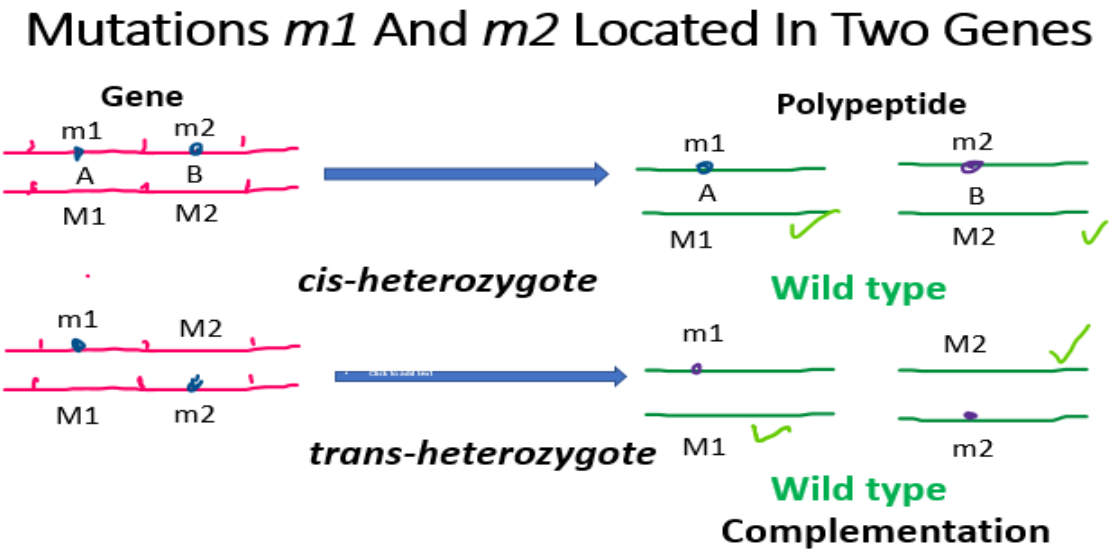
***m1* and *m2* located in different genes**



***m1* and *m2* located in the same gene**

The *rII* Locus of T4 Phage

- The *rII* locus of T4 phage causes rapid lysis and produces large plaques
- In 1955, Benzer published his findings on the *rII* locus
- He isolated **3,000 independent *rII* mutants**
- He used *cis-trans* test to show that these mutants were located in two different genes at the *rII* locus



*Cis- trans- T*est

m1 and *m2* located in	cis-heterozygote	trans-heterozygote
Two genes	Wild type	**Wild type**
One gene	Wild type	**Mutant phenotype**
	Extremely difficult in eukaryotes	**Complementation test**

The Fine Structure of a Gene

- Benzer mapped all the mutants of *rII* locus
- He used deletion mapping for rough localization of the mutants in the different regions of the *rII* locus
- Finally, he estimated recombination between pairs of mutants to fine map them in the two genes of the *rII* locus
- Fine map of a locus is linkage map of its mutant alleles
- Base sequence of a locus/gene is the ultimate fine map of that gene/locus

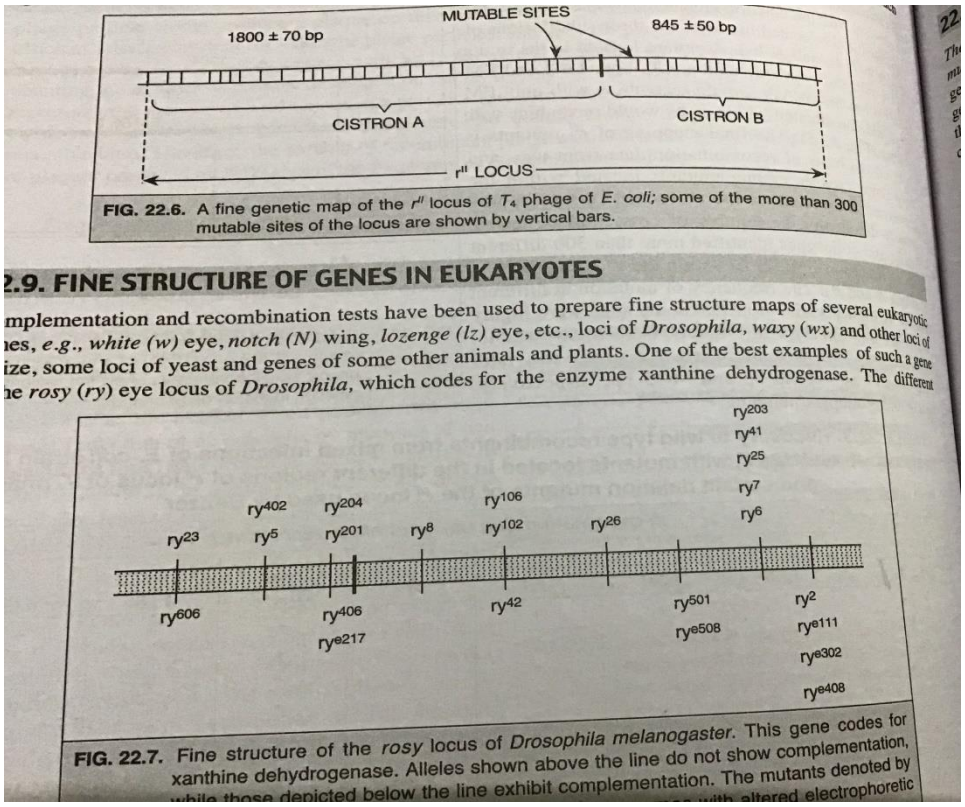


FIG. 22.6. A fine genetic map of the *rII* locus of T_4 phage of *E. coli;* some of the more than 300 mutable sites of the locus are shown by vertical bars.

2.9. FINE STRUCTURE OF GENES IN EUKARYOTES

mplementation and recombination tests have been used to prepare fine structure maps of several eukaryotic nes, *e.g., white (w)* eye, *notch (N)* wing, *lozenge (lz)* eye, etc., loci of *Drosophila*, *waxy* (*wx*) and other loci of ize, some loci of yeast and genes of some other animals and plants. One of the best examples of such a gene he *rosy* (*ry*) eye locus of *Drosophila*, which codes for the enzyme xanthine dehydrogenase. The different

FIG. 22.7. Fine structure of the *rosy* locus of *Drosophila melanogaster*. This gene codes for xanthine dehydrogenase. Alleles shown above the line do not show complementation, while those depicted below the line exhibit complementation. The mutants denoted by with altered electrophoretic

Intragenic Recombination

- Clearly, crossing over occurs within genes (**intragenic recombination**)
- Oliver (1940) reported intragenic recombination in the *lozenge* eye colour gene of *Drosophila*
- Benzer concluded:

1. **Unit of mutation**: one nucleotide
2. **Unit of recombination**: one nucleotide
3. **Unit of function**: cistron (same as gene of today); rarely used

Gene Is Particulate

- Sutton (1902): genes located in chromosomes
- Morgan (1910): evidence for location of *white* eye gene in X chromosome of *Drosophila*
- Griffith (1928): transformation of living avirulent cells of *Streptococcus pneumoniae* by dead virulent cells
- Avery and coworkers (1944): transforming principle is DNA
- Hershey and Chase (1952): only DNA is transmitted to progeny T2 phage
- DNA is the genetic material
- In some viruses, RNA is the genetic material

A Definition of a Gene

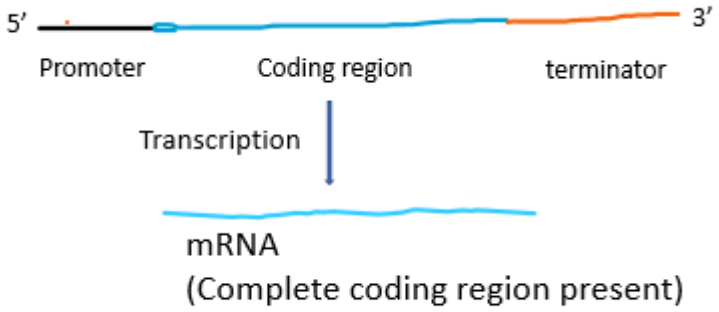
- Gene is a sequence of DNA that encodes a diffusible product (the organisation of this sequence varies)
- In addition, 5’ (promoter) and 3’ (transcription terminator) regulatory sequences are needed for gene function. In case of split genes, introns are present between exons (expressed sequences). But they do not affect properties of the gene product
- **Conclusion**: A sequence of DNA that encodes a diffusible product along with its 5’ and 3’ regulatory sequences as well as noncoding introns present within nearly all eukaryotic genes and some prokaryotic genes

Gene organization

- Uninterrupted genes (prokaryotes, some eukaryotic genes)
- Interrupted or split genes (eukaryotes, some prokaryotic genes)
- Overlapping genes (overlapping, alternative, nested)
- Antibody gene segments (vertebrates)

Uninterrupted Gene

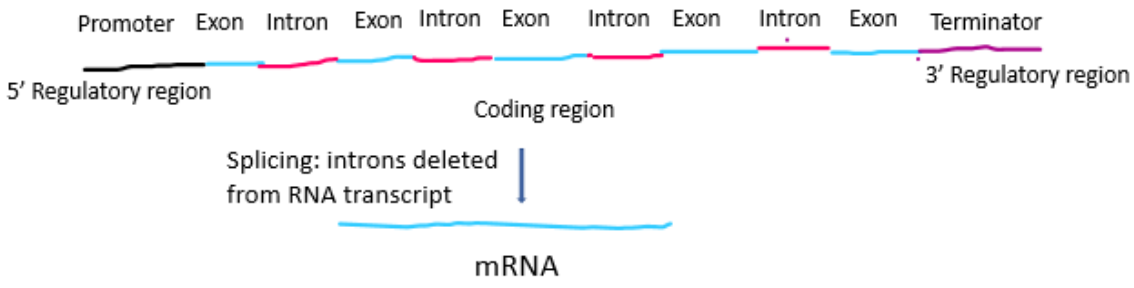
- Most prokaryotic genes; some eukaryotic genes (histone genes).
- Prokaryotes have transcription units



Eukaryotic gene

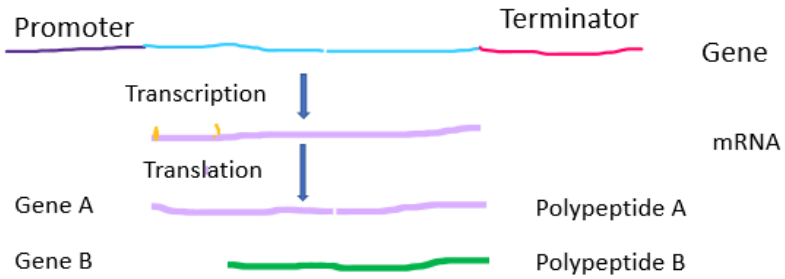
Interrupted Gene

Some genes in Archaea; most eukaryotic genes



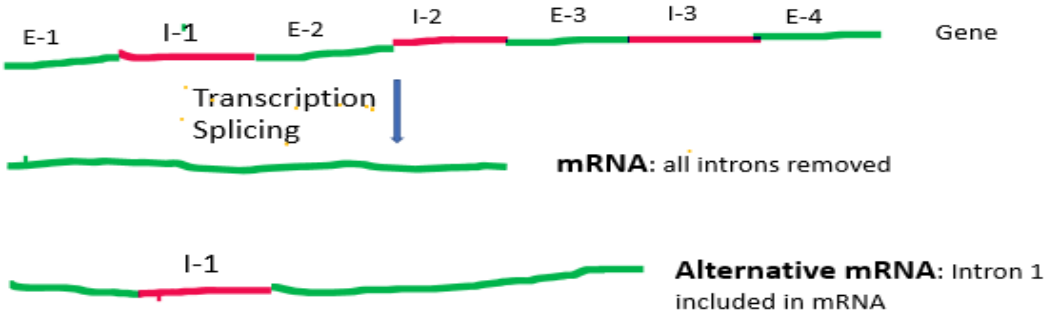
Overlapping Gene

Few genes in some viruses

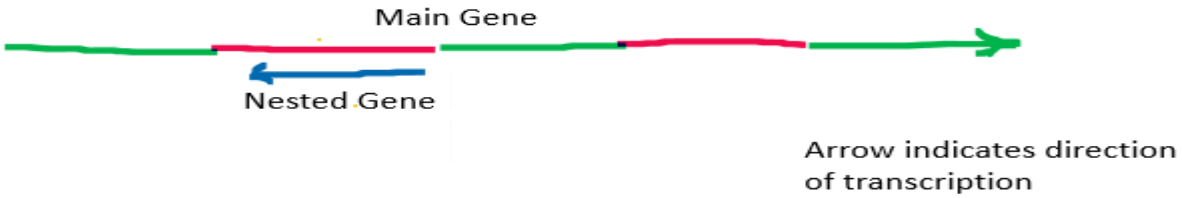


Alternative Genes (eukaryotes)

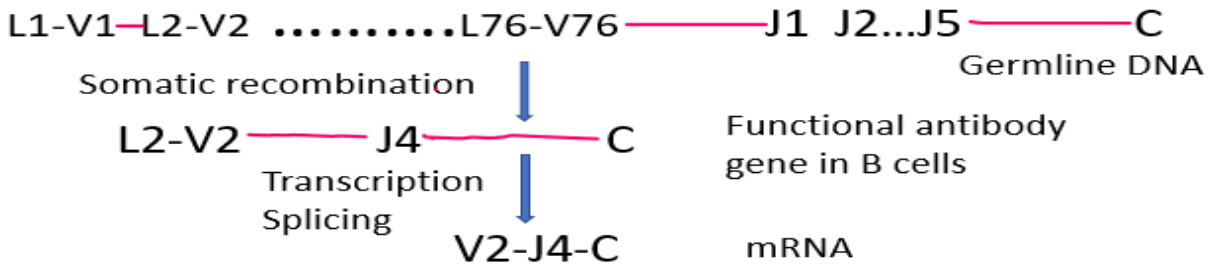
E = Exon
I = Intron



Nested Gene (Eukaryotes)



Antibody Gene Segments (Vertebrates)



Lecture

(BD Singh, Ex-Emeritus professor, School of Biotechnology, Banaras Hindu University)

Evolution of Plant Breeding Since Mendel

Plant Breeding Began Early

- **Domestication** of a relatively small number of wild species was the first plant breeding activity
- It began about 11,000 years ago
- Selection due to human activity as well as natural forces
- Specific sets of traits selected in different crops under domestication (**domestication syndrome traits**)

Recent domestications

- *Hevea* rubber, tea, coffee, etc.
- Tree species like eucalyptus
- Spices like kala jeera (*Bunium persicum*) domesticated in Himachal Pradesh
- Many medicinal plants
- Energy crops like jatropha, jojoba, perennial grasses, etc.

Domestication Syndrome Traits

- Almost same group of traits selected for in a given crop although it was domesticated in different areas of the world
- Example traits:

1. Increased seedling vigour
2. Increased self-pollination
3. Adoption of vegetative reproduction
4. Increased seed yield
5. Elimination of dormancy
6. Elimination of or reduction in shattering
7. Reduction in toxins
8. Changed plant type, *e.g*., reduced or increased plant height
9. Shorter life cycle
10. Increased bisexual forms in fruit trees and elimination of self-incompatibility
11. Reduction in variability within a crop

Domestication Vs. Evolution

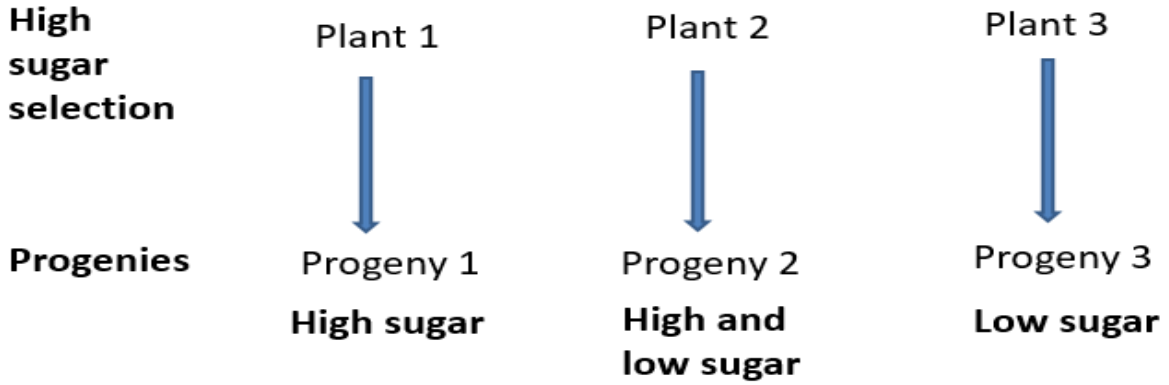
- Both domestication and evolution involve selection of desirable genotypes from among those present in the population
- Evolutionary selection depends on natural forces, while domestication uses both natural and artificial selection
- Evolution favours fitness traits while domestication favours traits relevant to human use and cultivation as crop
- In any case, changes under domestication are faster than those under evolution

Selection and Hybridization

- Farmers have practised selection since ancient times and developed important crop varieties, especially during late 18th century
- In 1843, Le Couteur reported that progenies from different superior plants differ for performance and homogeneity (progeny test)
- Systematic selection efforts began early in 19th century in Europe

Louis Vilmorin Selects for High Sugar in Sugar Beet

- Sugar beet is a cross-pollinated crop



Progeny test

- Based on his observations, he proposed the well-known 'Vilmorin isolation principle' that is the basis of progeny test
- *The value of a selected plant is revealed by the performance of its progeny: (i)* whether the selected plant is homozygous and (ii) whether its superiority is heritable
- ***Progeny test is an integral component of all breeding schemes***

Pureline theory

- Patrick Shireff practised individual plant selection (useful activity) during mid-nineteenth century
- He selected the best plant, the best spike from this plant and the best grain from this spike (a waste of time)
- In 1903, Johannsen proposed the pureline theory: ***selection within a pureline is ineffective***
- Pureline is progeny of a homozygous self-pollinated crop

Hybridization-Selection

- Hybridization combined with selection remains the most widely used method for the development of crop varieties
- Hand-pollination of date palm around 700 BC for improved fruit quality
- In 1717, Fairchild produced interspecific hybrid in *Dianthus:* hybrid called ***Fairchild's mule.***

Early Work on Hybridization

- Koelreuter reported hybrid vigour in *F1* (1760-1766)
- Many varieties of apples, peaches, grapes, etc. developed before Mendel (1750 and 1850)

- Mendel's work provided the scientific basis for selection of parents for hybridization, development of breeding schemes, and for selection of superior plants/lines

Challenges in Hybridization and Selection

- Hybridization is limited by sexual compatibility
- Selection based on phenotype
- Phenotype = Genotype + Environment
- A large effort is devoted to determining the environmental effects on quantitative traits

Distant hybridization

- Extremely important in breeding for disease and insect resistance
- Many crops would have become virtually uneconomical without the genetic support from their wild relatives
- Problems: Sexual incompatibility and linkage drag limit the usefulness of this approach

Somatic Hybridization

- Sexual incompatibility overcome by somatic hybridization
- Most successful in Solanaceae and Cruciferae
- It has been used to develop some commercial varieties of tobacco
- Transfer of cytoplasm to crop species
- Gene transfers from wild relatives
- Defect correction of *CMS-Ogu*

Hybrid varieties

- Hybrid varieties are one of the most wonderful developments; they are used wherever hybrid seed production is feasible
- The development of hybrid varieties was triggered by the failure of selection to improve maize yields
- Main challenge: Prediction of outstanding parental combinations remains an empirical exercise

Mutation breeding

- Began around 1950s
- Mutant varieties have been isolated in over 200 different plant species
- **3,281 varieties** were developed till 2018
- **1,043** varieties developed during 1980-1990
- Only 94 varieties registered during 2010-2018
- Mutation breeding activity has drastically declined
- Both physical and chemical mutagens used
- Mutants released as varieties as well used as parents in breeding programmes
- Interest declining because: **mutations are random, low frequency events**

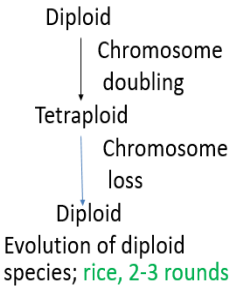
Polyploidy

- Diploid ($2x$; x = a single genome) = two copies of a single genome
- Disomic ($2n$; n = gametic chromosome number) = somatic chromosome complement of a species
- More than two copies of a genome present (autopolyploidy)

- More than one genome present (allopolyploidy)

Autopolyploidy

- Crops like potato, coffee, etc. regarded as autopolyploid species
- Few useful varieties, *e.g*., triploid sugar beet, watermelon, tea
- Autotetraploids: larger size, higher water content
- Contributed to evolution of diploid species, including humans (2 rounds; the 2R hypothesis)



Allopolyploidy

- An important contributor to evolution of plant species
- Many of our crops are allopolyploids, some are apparently autopolyploid
- It was once thought allopolyploidy will generate novel crops
- One successful human-made crop: *Triticale hexaploide*
- Limitation: Massive breeding effort required

Genotype-Based Selection

- **Conventional strategy**: Selection based on phenotype
- **Problem**: Phenotype has non-heritable environmental and nonfixable components
- **Solution**: *Marker-assisted selection (MAS)*

Marker-Assisted Selection

Molecular markers are random or gene based polymorphic sequences that can be reliably, easily and rapidly scored and analysed

Interspecific Gene Transfers

Status: *Primary gene pool*, readily used; *Secondary gene pool*, often used with some to considerable difficulty; *Tertiary gene pool*, use is possible but very difficult
Problem: Many useful genes are not available to the breeders
Solution: Transgenic technology; gene from any source can be used

Directed mutagenesis

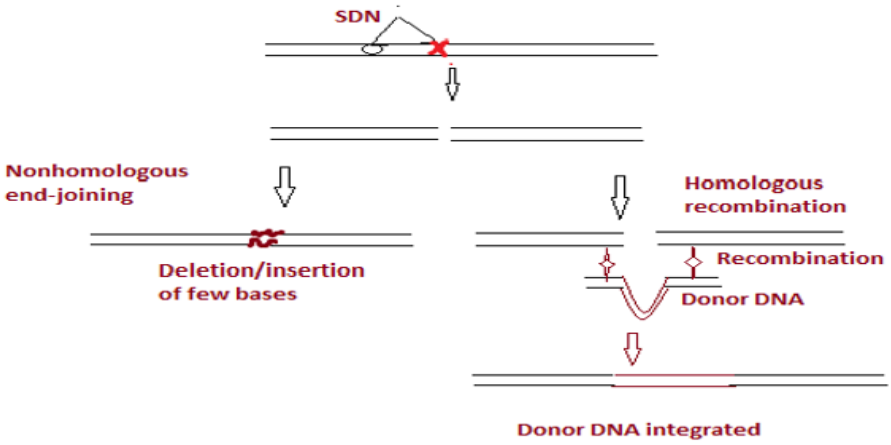
Status: Mutations are low frequency random events

Problem:

1. The recovery of desired mutants is not assured
2. Generally, unintended and undesirable mutations are associated with the desired mutations

Solution: Site-directed mutagenesis

Mutation induction by SDNs



Classical versus Modern Plant Breeding

P = G + E (applies to both)

	Action point	Expected change	Action target
Classical :	**P**	**G***	**P***
Modern:	**G**		**P***

(In both the cases, a clear understanding of correspondence between *G* and *P* is essential)

A Revisit of the Familiar Formula

Conventional:
P = G + E
P, Phenotype
G, Genotype
E, Environment

Modern:
P = G + E
P, Phenomics
G, Genomics
E, Envirotyping

Bernardo 2016. Theor Appl Genet
DOI 10.1007/s00122-016-2772-5

Envirotyping: evaluation of all possible genotypes under all possible environments

DNA Has Phenotype: The Chargaff's Rules

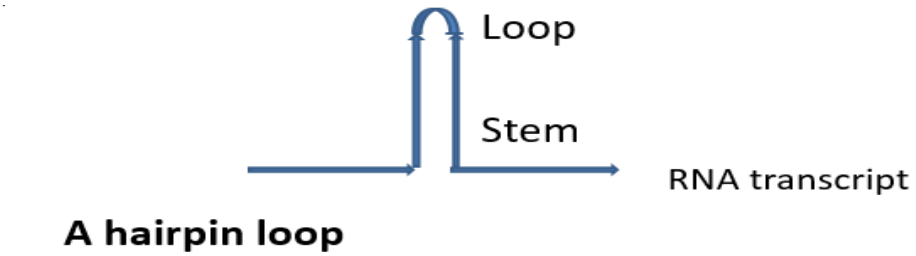
- *First parity rule*: in duplex DNA, A equals T, and G equals C; well known and easily appreciated
- *Second parity rule*: in a single DNA strand, A roughly equals T, and G almost equals C; little known and difficult to explain (possibly due to the need for stem-loop formation)
- *Cluster rule*: one strand tends to be purine-rich, and the other is pyrimidine-rich; difficult to explain
- *GC rule*: GC content of genome is species-specific; exons tend to have higher GC content than introns

Validated by extensive genome sequence analyses

The Genome Phenotype

- Fold pressure: the need for RNA transcript to form stem-loops
- Purine (AG) loading: one stand in DNA duplex is purine rich, and the other is pyrimidine rich
- GC pressure: species-specific GC content of the genome

RNA Transcripts Form Extensive Hairpin Loops



A hairpin loop

Genomic Biases

- **Mutational bias**: Mutational events biased toward higher AT content
- DNA repair tends to have GC bias (higher GC)
- **Result***: **Recovered mutations show much lower bias in favour of AT***
- **Gene conversion bias**: biased toward GC
- **Codon bias**: one codon of a synonymous set used at high frequency and it ends in G or C; possibly driven by gene conversion bias

Possible Restriction Due to Genome Phenotype

The genome phenotypes and biases might restrict the achievable genome modification

Lecture

(BD Singh, Ex-Emeritus professor, School of Biotechnology, Banaras Hindu University)

Gene/Genome Editing and Its Regulation

Plant Breeding

Creation and selection of novel gene combinations to enhance the usefulness of crop varieties to humans.

Plant Breeding Tools

Tool	**Creation of genetic variation**	**Selection**
Conventional	Hybridization	Phenotype-based selection
	Mutagenesis	
Molecular	Transgenic technology	Marker-assisted selection
	Gene/genome editing	

Conventional Mutagenesis

- Random mutations in low frequency
- Frequency of useful mutations ~0.1% of the mutants
- No control on the gene that is mutated and the type of mutation induced
- Activity has declined drastically: only 94 varieties added to IAEA database since 2010; only 9 varieties in 2019
- Very high background sequence changes (415,000 per plant after EMS treatment)

Gene Editing (Site-Directed Mutagenesis)

- Almost complete control on the gene mutated and the type of mutation induced
- Specified mutations induced when donor DNA provided
- High frequency (typically, 3-10%)

Gene/Genome Editing Tools

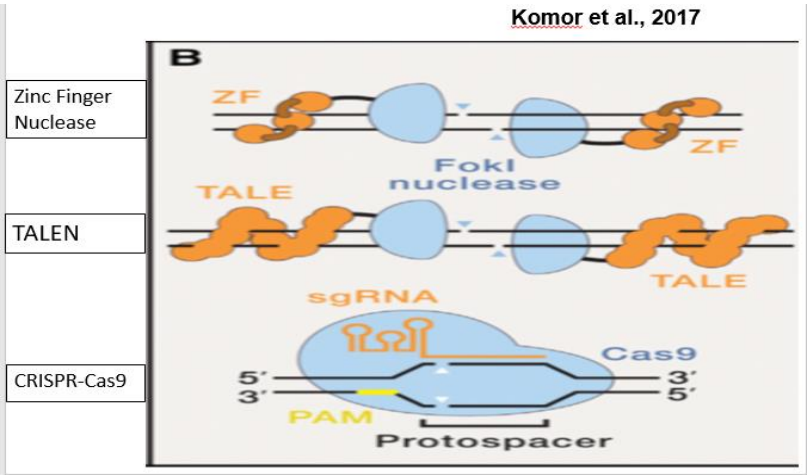
- Specified genetic changes in base sequences of genes of interest are induced by protein or nucleic acid reagents:
- Site-directed nucleases
- Base editors
- Chimeric oligonucleotides
- The technology was developed for biomedical applications

Site-Directed Nucleases

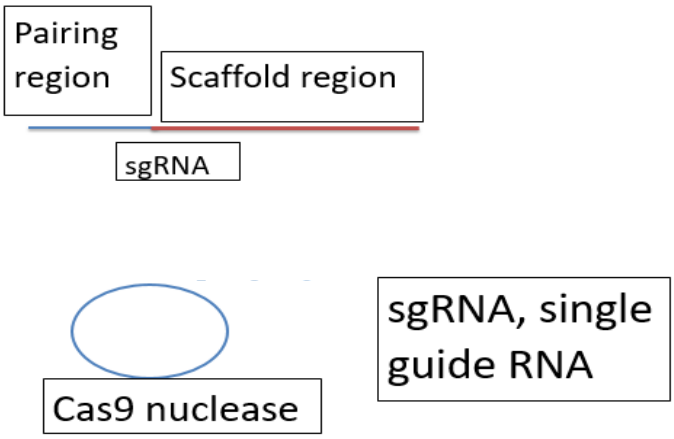
- Two functional domains:

1. **DNA recognition domain**: seeks out and binds to the targeted genome sequence
2. **Nuclease domain**: induces double-strand break (DSB) at the targeted genomic site

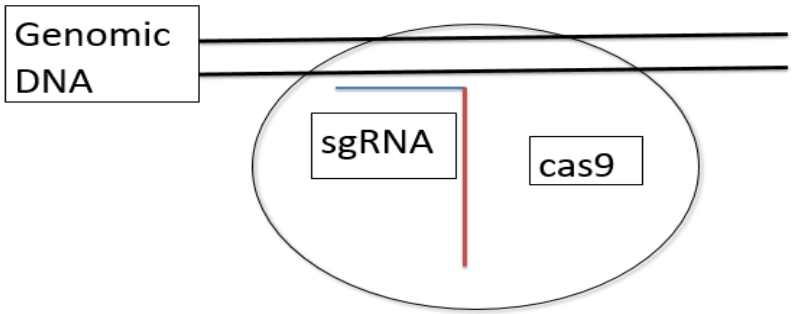
Common Site-Directed Nucleases



CRISPR-Cas9



CRISPR-Cas9 Associated with Genomic DNA



Prerequisites for Gene Editing

- Identification of the critical gene for targeting
- Selection of the target site within the gene to be mutated
- Decision on the type of change to be induced
- Designing of the appropriate SDN: easy in case of CRISPR-Cas9 system
- Implementation of the gene editing protocol

Method of Gene Editing

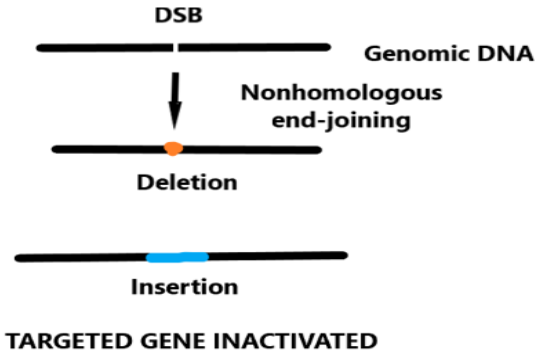
- Target genomic sequence determined
- SDN specific for the target sequence designed
- The SDN gene construct transformed into plants
- Selfed progeny produced and the desired mutant selected
- Mutant selection based on phenotype or molecular techniques

Mechanism of Gene Editing by SDNs

- Site-directed nucleases (SDNs) induce double-strand breaks
- Breaks repaired by cellular DNA repair processes; **this generates genetic changes (mutations)**

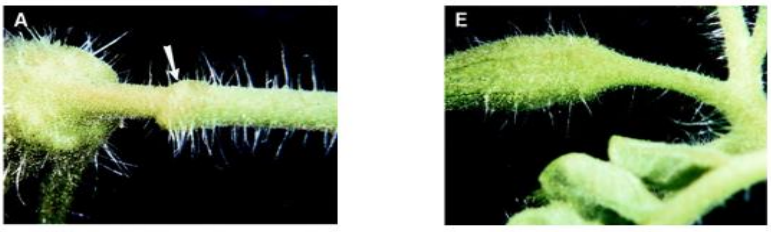
Nonhomologous End-Joining (NHEJ)

- Bulk of DSBs are repaired by NHEJ
- Only SDN is used
- DSB repair generates insertion/deletion of generally few bases (even complete genes)
- Leads to inactivation of the targeted gene (SDN-1 events)



Jointless Pedicel Tomato (**approved for cultivation in USA**)

- *J2* gene specifies jointed pedicel



Jointed pedicel
(normal tomato)

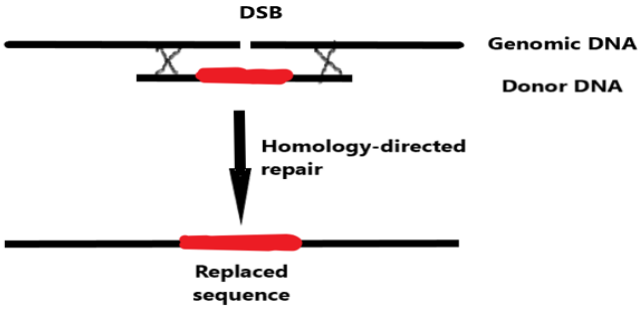
Jointless pedicel
(Gene *J2* deleted)

Wheat Powdery Mildew

- Mlo function needed for infection by the pathogen
- Wheat has three *Mlo* genes, one gene in each genome (A, B, D)
- TALEN used to inactivate all the three genes
- Gene-edited lines show broad-based RESISTANCE to the disease
- Resistance to powdery mildew is not available in germplasm; simultaneous mutation in all 3 genes very difficult

Homology-Directed Repair

- Donor DNA used as template for repair
- Donor DNA has the sequence located on either side of the break plus the sequence change desired in the mutant gene
- Donor DNA sequence copied into the genome
- Sequence replacement either
  - corrects a mutation
  - induces the specified mutation
- No other way of achieving the above **(SDN-2 events)**



Disease Resistant Maize

- Disease: Northern leaf blight of maize
- Donor DNA: sequence of a resistance allele of the same gene from another maize line
- Gene-edited line is resistant to Northern Leaf Blight
- Approved by APHIS, USDA for commercial cultivation

SDN-3 Events

- Integration of complete gene constructs at predecided or specified genomic sites
- The donor DNA has the complete transgene construct
- Normally transgenes are integrated at random genomic sites
- This may inactivate undesirable genes of inactivate desirable genes

Downside of Gene Editing

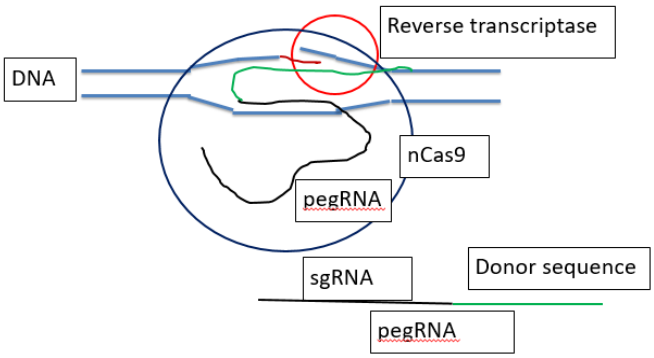
- Off-target cleavage: an evolutionary obligation
- NHEJ predominates
- Low proportion of HDR; this is a great problem in human gene editing
- Donor DNA-plasmid integrated at target site

Prime Editing

- NHEJ-mediated gene inactivation greatly reduced
- No risk of donor DNA integration into the genome

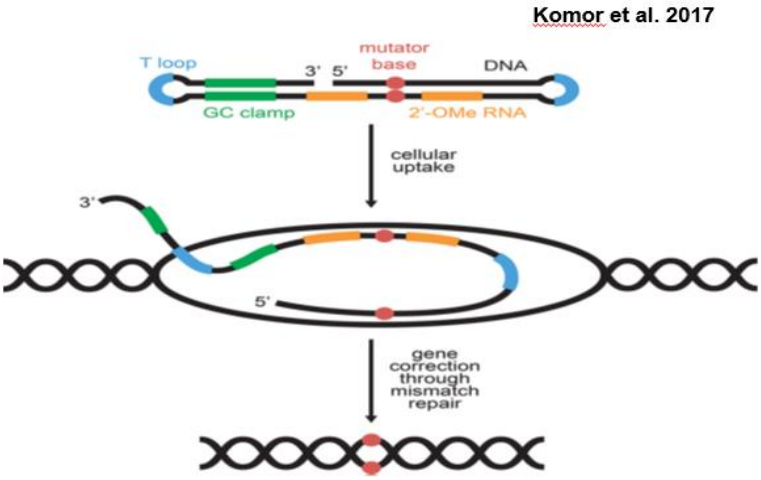
- HDR frequency comparable to that obtained with DSB induction

Anzalone et al., 2019



Oligonucleotide-Directed Mutagenesis (SU Canola)

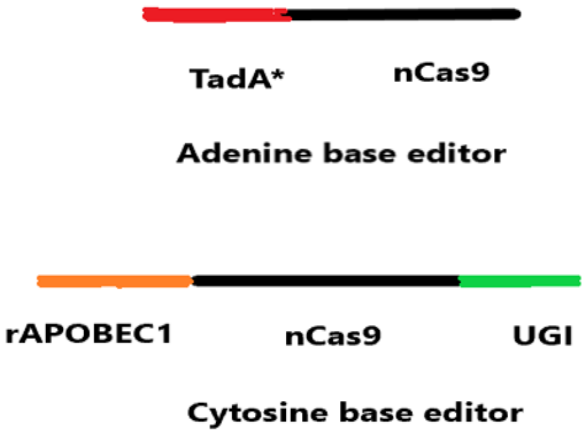
- Canola is sensitive to sulfonyl urea (herbicide)
- The herbicide acts on the enzyme acetolactate synthase (ALS)
- ALS mutated by oligonucleotide-directed mutagenesis
- The gene-edited line resistant to the herbicide
- Approved for cultivation as SU Canola in Canada and USA



Base Editing

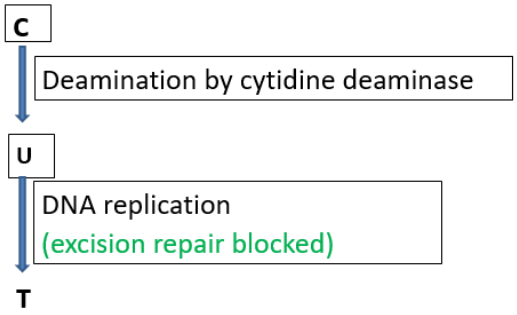
- *In vivo* permanent conversion of one base into another by chemical modification in a site-specific manner
- Catalysed by base editors based on CRISPR-Cas9 SDN system
- First report in 2016 by Komor and co-workers (Komor et al. 2016. Nature 533, 420−424)

Base Editors

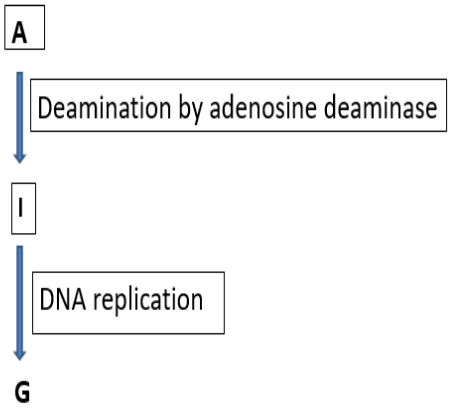


C-to-T Base Editing

- Herbicide (imazamox) resistance
- Gene encoding acetolactate synthase
- C at position 287 converted to T (Ala replaced by Val)
- Base-edited rice plants tolerant to the herbicide



A-to-G Base Editing



Uses of Base Editing

- Base transition mutations (A-to-G and C-to-T and *vice-versa*) to:

1. Correct a mutant gene (many genetic diseases produced by G:C to A:T transitions)
2. Induce a desired mutation by a single base substitution
3. Inactivate the targeted gene by producing a premature stop codon

Lecture

(BD Singh, Ex-Emeritus professor, School of Biotechnology, Banaras Hindu University)

Breeding for Resistance to Abiotic Stresses: Relevance of Molecular Markers and Novel Breeding Schemes

Stress Factors

Stress: A factor that interferes with the optimum performance

Abiotic stress: A non-living stress factor

Biotic stress: A biotic factor causing stress (pathogens, insect pests, parasitic weeds)

Abiotic Stresses

- Mainly drought, mineral toxicity/deficiency, high/low temperature, high/low pH
- They determine distribution of crop species
- They together affect >90% of the global arable land area
- Climate change and population pressure will increase the impact of abiotic stresses

Stress Resistance or Tolerance

- Stress resistance: the resistant genotype has one or more mechanisms to reduce water stress within cells, *e.g*., reduced water loss, increased water uptake
- Stress tolerance: the tolerant genotypes can tolerate water stress within cells, *i.e*., their cellular functions are little affected by water stress within the cells
- Almost all traits/mechanisms relate to stress resistance

Resistance to Abiotic Stresses

Abiotic stress resistance is a complex trait:

a. It involves several component traits
b. It shows quantitative inheritance pattern (many genes with small effects)
c. It shows large environmental influence

- Therefore, phenotypic selection for abiotic stress resistance has generally been frustrating
- Creation of reliable selection environment is a demanding task

Quantitative Traits

- Quantitative traits generally involve few large effect and several small effect quantitative trait loci (QTLs)
- Large effect QTLs are likely to be fixed during domestication and breeding
- As a result, only small effect QTLs are likely to differ among elite breeding lines
- Therefore, the differences in complex traits like yield, abiotic stress resistance, horizontal disease resistance, etc. among the breeding materials in most crops would be governed by many small effect QTLs

Quantitative Trait Locus (QTL)

- A QTL is a genomic locus involved in the control of a quantitative trait
- A QTL is detected and mapped using molecular markers
- Selection for QTLs generally with the help of molecular markers
- A QTL may have one or more genes

Relevance of Marker-Assisted Selection

- Molecular markers facilitate indirect selection for linked genes/QTLs
- MAS is quite useful when a trait:

a. Is difficult to evaluate
b. Is affected by the environment
c. Has a threshold requirement (*e.g.*, resistance to abiotic stresses)

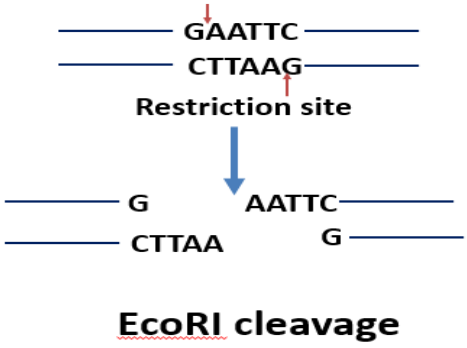
Threshold Requirement

- A **threshold character** requires a specific environmental condition for its expression
- For example, a disease can be scored only when the plants are exposed to the concerned pathogen
- Similarly, drought tolerance can be scored only when they are exposed to drought
- Result: Many susceptible plants may be scored resistant simply because they were not exposed to the threshold environment (escapes)

Molecular (DNA) Markers

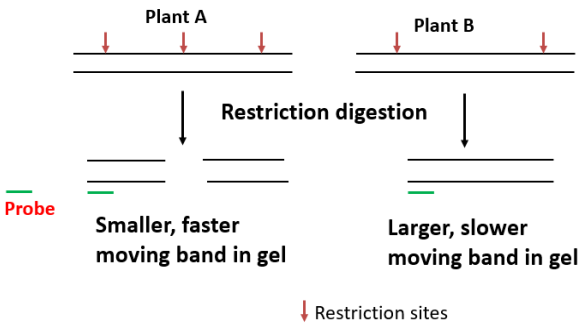
- Molecular markers are based on genomic sites with polymorphic DNA sequences
- A polymorphic genomic site shows sequence variation in different individuals/lines
- Examples: RFLP, RAPD, AFLP (amplified fragment length polymorphism), DArT (diversity array technology), SSR, SNP, etc.

Restriction Enzyme

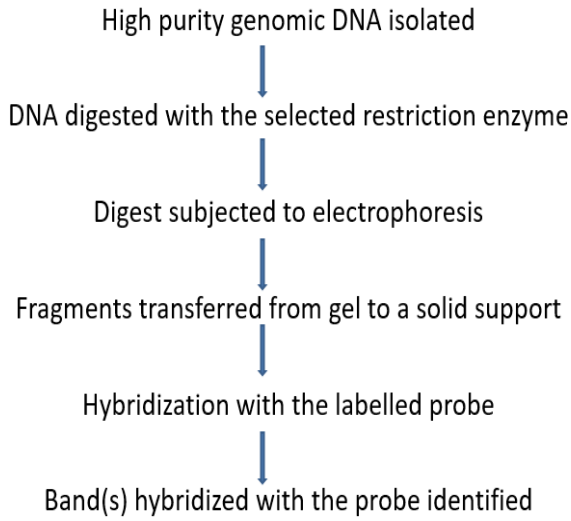


Restriction Fragment Length Polymorphism (RFLP)

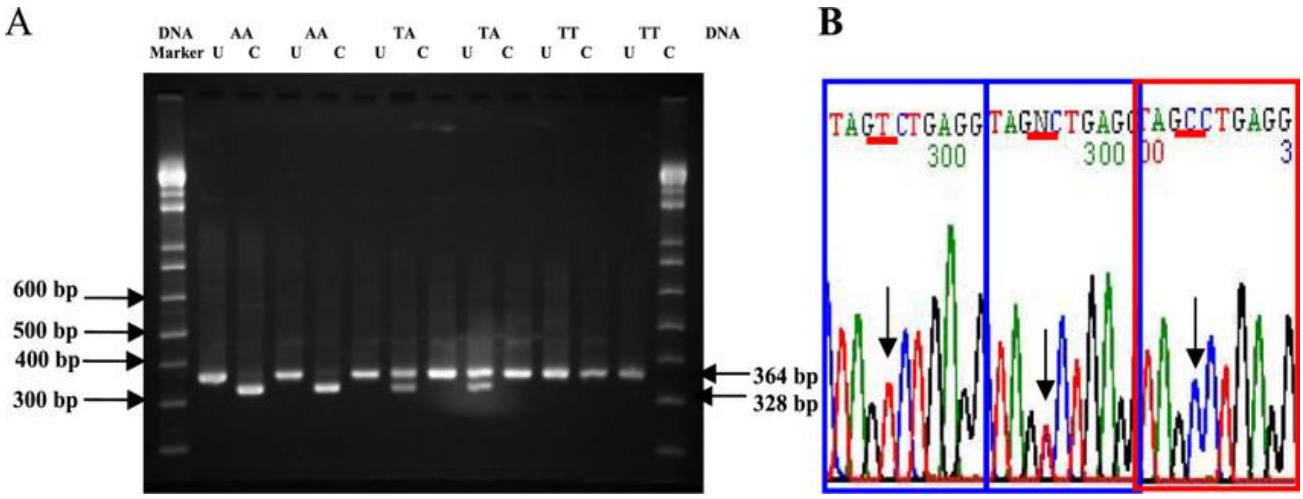
- First molecular marker
- Specific RFLP locus determined by restriction enzyme and probe combination
- Probe is labelled single-stranded DNA sequence homologous to a part of the genomic region being analysed
- Polymorphism detected as slow and fast moving bands
- Polymorphism due to abolition/creation of restriction sites
- The Gold Standard marker



RFLP Procedure

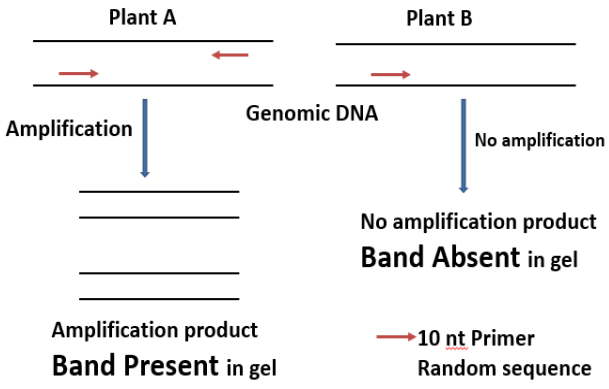


RFLP Gel Image

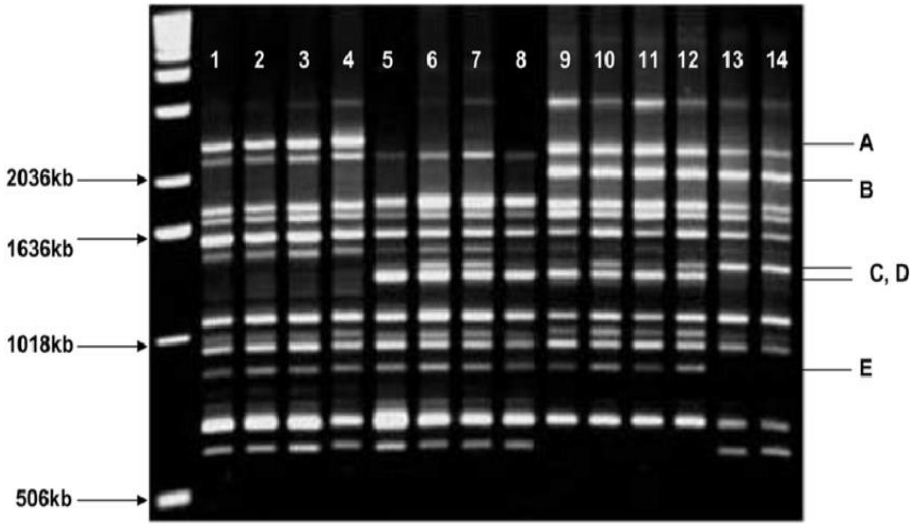


Randomly Amplified Polymorphic DNA (RAPD)

- The first PCR-based marker
- A single 10 nt primer with random sequence used for amplification
- For amplification, the primer must pair at two sites up to 2 kb apart and in opposite orientation
- Easiest to use but poor reproducibility

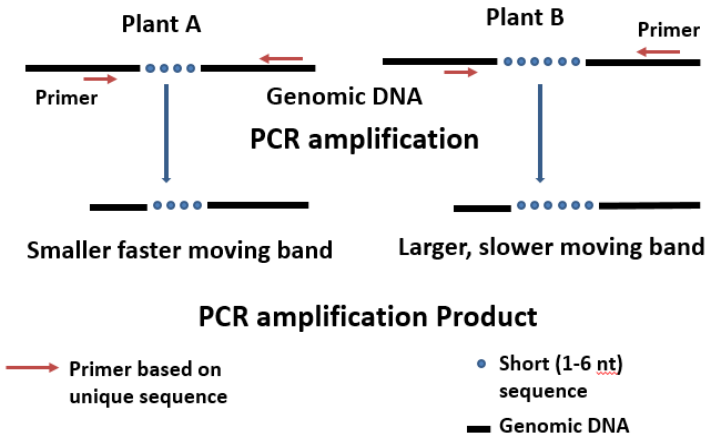


RAPD Gel Image

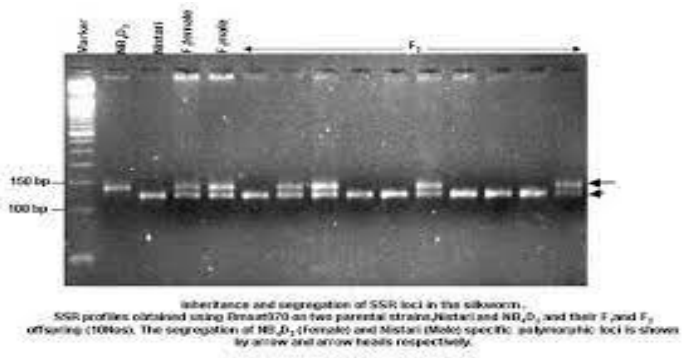


Simple Sequence Repeat (SSR)

- Polymorphism due to variation in the number of copies of repeat sequences of generally up to 6 nucleotides, *e.g.*, CA, GTC, etc.
- Marker development, is a demanding task
- Genotyping is PCR-based, relatively easy
- Some degree of automation
- Most widely used marker about a decade ago, *i.e.*, before the arrival of SNPs



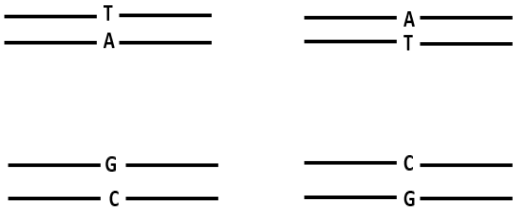
SSR Gel Image



Inheritance and segregation of SSR loci in the silkworm.
SSR profiles obtained using Bmsat070 on two parental strains,Nistari and NB_4D_2, and their F_1 and F_2 offspring (100nos). The segregation of NB_4D_2 (Female) and Nistari (Male) specific polymorphic loci is shown by arrow and arrow heads respectively.

Single Nucleotide Polymorphism (SNP)

- Often pronounced as ‘snips’
- Detected by genome sequencing and analysis of genome sequences
- Genotyping based on PCR amplification
- Automated high throughput genotyping platforms
- Genotyping services commercially available
- Most abundant and most widely used marker system



The four possible alleles at one SNP locus

Reliability of A Marker

- A marker is linked to the desired gene
- Therefore, marker allele reveals the identity of the allele of desired gene
- Crossing over between the marker and the gene will change linkage between the marker and the gene alleles
- This reduces reliability of the marker and efficacy of the marker-assisted selection
- Therefore, markers should be tightly linked to the gene of interest

The Three Classes of Molecular Markers

1. **Random markers:** they have no relation to the function of the genomic sequences constituting the marker; a vast majority of markers
2. **Gene-based markers:** Markers based on sequences of the concerned genes
3. **Functional markers:** A subclass of gene-based markers; based on sequences involved in the allelic differences of the concerned genes (also, allele-specific/perfect markers)

Marker-Assisted Selection: Indirect Selection for The trait

- Markers associated with the desired QTLs are identified in a suitable mapping population created for the purpose
- The population under selection is genotyped for these markers and desired plants are selected on the basis of marker genotype
- Phenotypic evaluation of the selected lines usually at the end of breeding scheme
- Problem: marker-trait association identified in one population may not be useful in other populations (solution: **marker-assisted recurrent selection)**

Some Innovative Breeding Schemes

- The optimum utilization of the information from molecular markers requires innovative breeding schemes, *e.g.*,
- Marker-assisted recurrent selection (MARS)
- Development of segment substitution lines
- Single large scale marker-assisted selection
- Marker-evaluated selection (MES)
- Genomic selection (GS)
- Breeding by design

Marker-Assisted Recurrent Selection

- Conventional recurrent selection schemes use phenotypic selection; take 2-3 yrs/selection cycle
- MARS, on the other hand, is based on molecular marker genotype
- *F2/BC1* population is phenotyped for the target trait and genotyped with a suitably large number of markers
- Multiple regression analysis identifies markers significantly associated with the trait phenotype
- The effects of these markers on the trait phenotype are estimated
- Selection in this generation is based on an index estimated from the trait phenotype values and the marker genotype data
- In the next generation, only the markers showing significant association with the trait are used to estimate marker effects
- Selection is based on marker scores of plants estimated from their marker genotype data
- The selected plants are inter-mated in the same generation
- Efficiency of selection depends on trait heritability, the proportion of additive genetic variance associated with markers, and the recurrent selection scheme used
- MARS is much more effective than phenotypic selection for traits with low heritability
- The chief advantage of MARS: completion of 2 – 3 selection cycles per year with the use of off-season nurseries/green-house facilities
- It is extensively used in private sector

Development of Segment Substitution Lines

- Information about the QTLs to be introgressed is not needed
- The *F1* from the cross between donor and recipient parents is backcrossed with the recipient parent to develop a set of near-isogenic lines (NILs)
- Each NIL has a distinct genomic segment from the donor parent (segment substitution lines)
- The sum total of donor genomic segments in the NILs ideally represents the entire donor genome (introgression line library)

- The NILs are evaluated for the trait of interest in replicated trials (over locations/years)
- NILs with improved target trait identified and used for evaluation/breeding

Single Large Scale Marker-assisted Selection

- Elite germplasm lines having complementing sets of QTLs for the target trait are identified
- These lines are crossed and a very large *F2/F3* generation is raised
- Markers flanking (at <5 cM) the target QTLs are used to select plants homozygous for the favorable alleles at the target QTLs
- The selected population is now handled as per the breeding objective; there is no more MAS

Marker-evaluated Selection (MES)

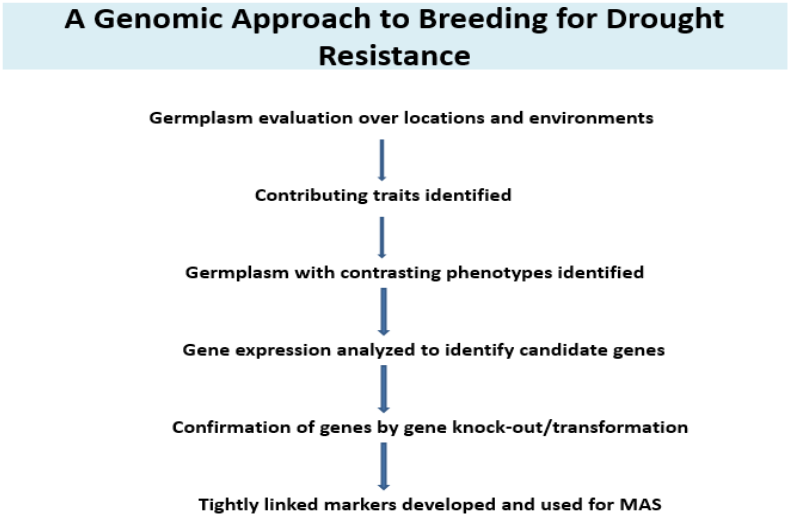
- Segregating generations from a suitable cross are grown as bulks in different agro-ecosystems
- Mass selection is carried out for agronomic performance in the different agro-ecosystems
- Selection may be done at research stations and/or farmer's fields and may involve farmers in participatory breeding
- Marker allele frequencies of the selected populations are compared
- Genomic regions involved in adaptation to and agronomic performance in specific agro-ecosystems are identified
- MAS can be used to construct genotypes having the identified genomic regions for high performance in a specific agro-ecosystem

Genomic Selection

- Problem: Marker-assisted backcrossing (MABC) and other breeding strategies exploit only large effect QTLs
- Solution: Genomic selection (GS) or genome-wide selection attempts to exploit all small effect QTLs for the trait
- A training population is used to develop the mathematical model used for genomic selection
- The training population is precisely phenotyped
- It is genotyped with a large number of markers covering the whole genome
- Marker-trait association is estimated using a suitable statistical model
- Each marker is assigned a value based on this association whether or not the association is statistically significant
- The breeding population is genotyped for the markers evaluated in the training population
- Marker data are used to estimate the genomic breeding value for each plant (using the estimates of effect of the marker alleles)
- Plants are selected on the basis of estimated genomic breeding values (GEBVs)
- GS has been very successful in improvement of dairy cattle
- CIMMYT, Mexico, in collaboration with Cornell University, USA is in the process of using GS for the improvement of horizontal resistance to stem rust in wheat

Breeding by Design: An Ambitious Scheme

- Identification of all the QTL alleles involved in control of all the relevant traits of the crop species and estimation of their effects
- Identification of donors for these QTL alleles
- Construction of the ideal genotype *in silico*
- Creation of this genotype by assembling the desirable QTL alleles and its evaluation



Three-step Screening of Germplasm for Drought Resistance Sources

First screen: Simple, less accurate; ~ 10% of the entries representing the entire diversity selected (diversity set)

Second screen: More sophisticated, more accurate, includes physiological traits; ~ 10% of diverse lines from the diversity set selected

Third screen: Highly precise, includes physiological and biochemical traits; desired genotypes selected for analysis/breeding

Some Examples of QTL Introgression for Abiotic Stress Tolerance

Common bean: Multiple QTLs for drought tolerance related traits
Cotton: Seven QTLs for drought tolerance related traits
Maize: One major QTL (*root-ABA1*) for drought tolerance
Maize: Five genomic regions for flowering traits and yield under water-stress
Pearlmillet: One major QTL for grain yield under terminal drought stress
Rice: Multiple QTLs for root traits (yield and grain quality under stress improved)
(The rice variety Birsa Vikas Dhan 11 released for cultivation in Jharkhand)
Rice: One major QTL (*Sub1*) for submergence tolerance (variety Swarna Sub1 in cultivation)

Stay-green Trait

- Sorghum plants with stay-green trait have more green leaves as well as stem under drought conditions than the normal non-stay-green plants.
- They have stronger stems and yield more grain under moisture stress than the normal sorghum plants/lines.
- Four genes, viz., *Stg1*, *Stg2*, *Stg3* and *Stg4*, are associated with the stay-green trait in grain sorghum.
- The stay-green sorghum line B35 was introduced from Ethiopia

Integration of MAS in Breeding Programmes

- **Additional work:** Marker genotyping and related activities are added to breeding work
- **Additional information:** Marker data and increased amount of breeding related information is generated
- **Additional skill:** Marker related activities, statistical analyses, quick decision making
- **Bioinformatics tools:** Data acquisition, storage and retrieval, information sharing

Limiting Factors

- High cost, particularly, related to marker genotyping
- Technical expertise for generating and using marker data
- Separate mapping and breeding activities add to cost and effort
- Reorganization of the breeding programs to accommodate marker data

Lecture

(BD Singh, Ex-Emeritus professor, School of Biotechnology, Banaras Hindu University)

Opportunities in Plant Biotechnology

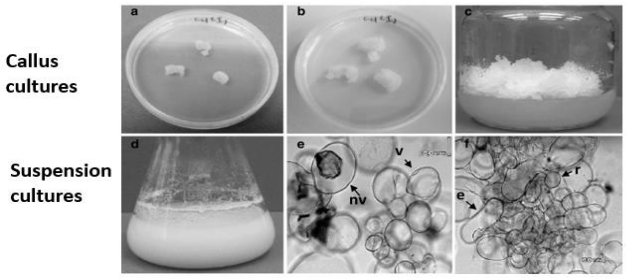
Biotechnology: Generation of useful products/services using biological agents, such as microorganisms, plant/animal cells or their subcellular constituents

Plant biotechnology: Generation of useful products/services using plant cells, tissues or organs or their subcellular constituents. Plant Biotechnology depends heavily on plant tissue culture technology

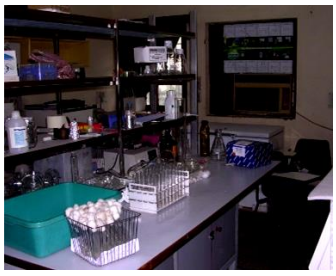
Plant Tissue Culture

- Culture of plant cells/tissues/organs *in vitro* on, usually, a synthetic medium to recover either biomass or complete plantlets
- Callus/suspension culture (biochemical production)
- Organ culture (meristem, anther, ovary, embryo)
- Complete plantlet regeneration from single cells is essential for producing transgenic plants

***Scrophularia striata* callus and cell suspension culture**



The Laboratory and some equipment



Medium Preparation Lab

Sterilizer for dry heat sterilization

Laminar air-flow cabinet

Scalpel & forceps

Shaker for suspension culture

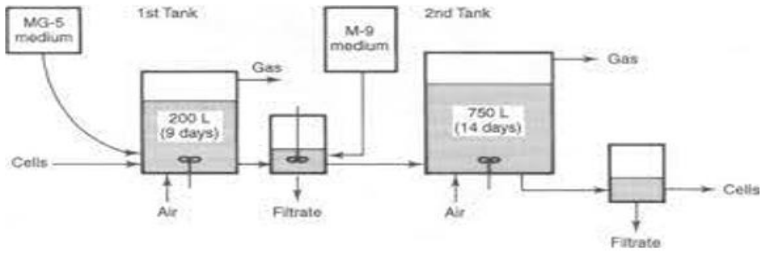
Culture room for callus cultures

Microbial contamination: the biggest irritant

Biochemicals Obtained from Plants

- **Alkaloids :** morphine, codeine, hyoscyamine, etc.
- **Steroids :** diosgenin, sterols, ferruginol, etc.
- **Terpenoids :** menthol, camphor, carotenoids, etc.
- **Phenylpropanoids :** anthocyanins, coumarins, flavonoids, etc.
- **Quinones :** anthraquinones, benzaquinones, etc.

Shikonin production from cultured *Lithospermum erythrorhyzon* Cells

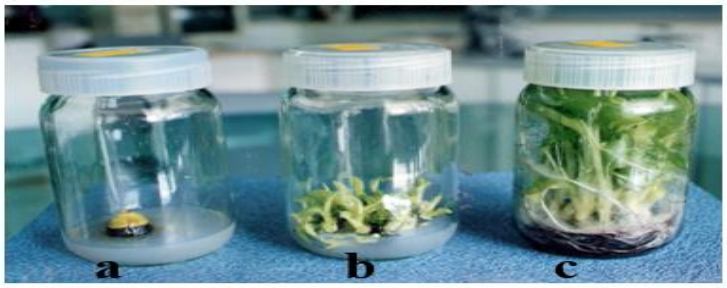


Chief Advantages and Limitation

- Protects natural plant species from over-exploitation/ extinction
- Biochemical production needs less time and space, and may be done round the year
- High breakeven (= no profit no loss) cost, ~US $ 1,500 per kg biochemical
- Relatively much lower market prices of most of the biochemicals obtained from plant cells

Micropropagation

- Rapid production of asexual progeny through:
- Enhanced axillary branching (banana)
- Adventitious shoot regeneration (geranium)
- Protocorm/bulblet regeneration (lily)
- Somatic embryogenesis (oilpalm)



A shoot tip explant (a), multiple shoots produced by axillary branching (b), and shoots rooted in bunch (c)

Micropropagation of banana using shoot tip

Plantlets transplanted in pot trays and placed in a polythene tunnel

Hardened plantlets kept in a net house

Micropropagation of Banana

Banana plantlets raised through micropropagation established in the field

Micropropagation of Banana

Aseptic transfer area for subculturing

Growth room

Commercial Micropropagation Laboratory

Embryo Rescue

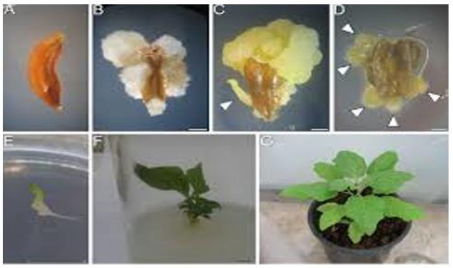
- Recovery of complete plantlets by culturing young embryos that would otherwise die

1. Recovery of interspecific hybrids (triticale, wheat x barley hybrids)
2. Recovery of haploid plantlets from interspecific crosses (*Hordeum bulbosum* x *H. vulgare*; wheat x maize)

Anther/Pollen Culture

- Anthers/pollen grains cultured on suitable media to produce haploid plantlets originating from pollen grains
- In most species, the optimum stage for culture is around the first pollen mitosis
- Pretreatment with low/high temperature promotes androgenesis
- Homozygous doubled haploid plants obtianed by chromosome doubling of haploid plants

Androgenic Haploids of Brinjal



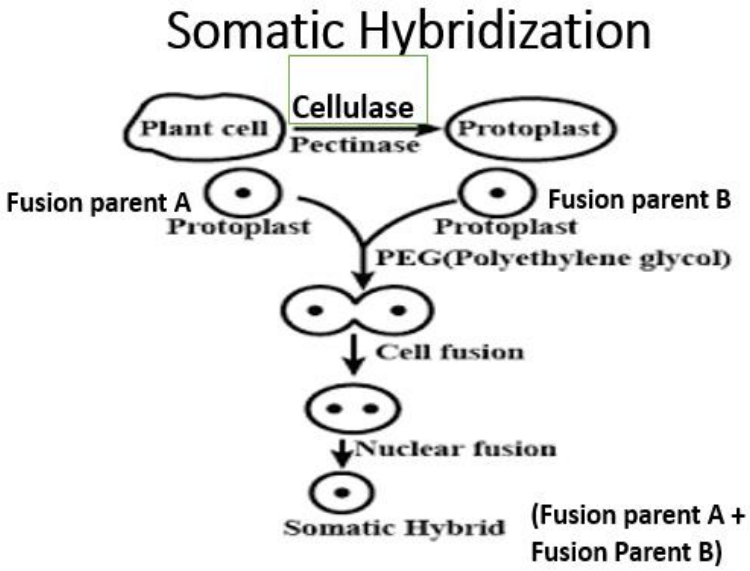
Gynogenesis

- Culture of unfertilized ovaries to recover haploid plants
- Haploids generally originate from egg cells but synergids and antipodals may also contribute
- The only route for haploid production in male sterile lines

Somatic Hybrids and Cybrids

- Protoplasts produce by digestion of cell wall using pectinase and cellulase
- Protoplasts fused by a fusogen treatment, *e.g.*, PEG (polyethylene glycol), electrofusion, etc.
- Hybrid cells give rise to:

1. Somatic hybrids (nuclear gene transfers, new species)
2. Cybrids (cytoplasm transfers, CMS)



Somaclonal Variation

Somaclonal Variation in Strawberry

- Genetic variation present among cultured cells or plants regenerated from them
- Produced due to spontaneous mutation and possibly mutagenic action of the culture environment, including errors by DNA polymerase, increased transposition, DNA methylation, etc.
- Several somaclones released as varieties, *e.g.*, Pusa Jaikisan *Brassica juncea*

Germplasm Conservation

- Germplasm of vegetatively propagated species and species producing recalcitrant seeds
- Germplasm conserved as:

1. Slow growth cultures
2. Cryopreservation
3. DNA banks

Virus-free Plants

- Virus eliminated by shoot meristem culture alone or combined with

1. Thermotherapy (heat treatment; ~40^{o}C)
2. Cryotherapy (cold treatment; 4-10^{o}C)
3. Chemotherapy (*e.g.*, ribavirin)

- Virus-free breeding stocks/nucleus seed of clonal crops
- Germplasm exchange of asexually propagated crops

Transgenic Plants

- Plants in which gene(s) has(ve) been transferred from another organism (plant, animal, bacteria, virus), through genetic engineering.
- Such a gene is called transgene. The transgene is integrated in the plant genome at random sites.
- Transgenic plants appear to have the greatest commercial potential

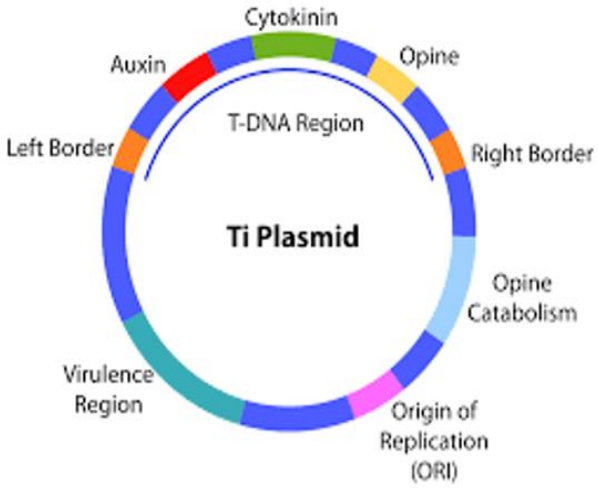
Transgenic Plants Produce Novel Proteins

1. The protein is commercial product (Hirudin, avidin, Gus, etc.)
2. It generates the phenotype (Cry protein)
3. Modifies endogenous pathway
   a. End product modified
   b. Increased efficiency of the pathway
   c. Creates a new branch in the pathway
4. Creates a new pathway (PHB production)
5. Endogenous pathway blocked (RNAi)

Transgenic Plants: Production

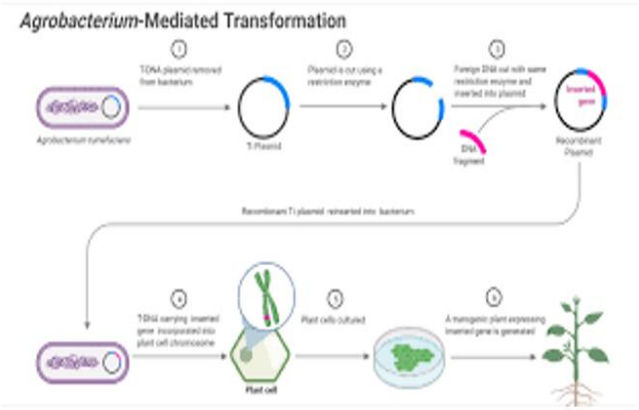
- Isolation of the desired gene

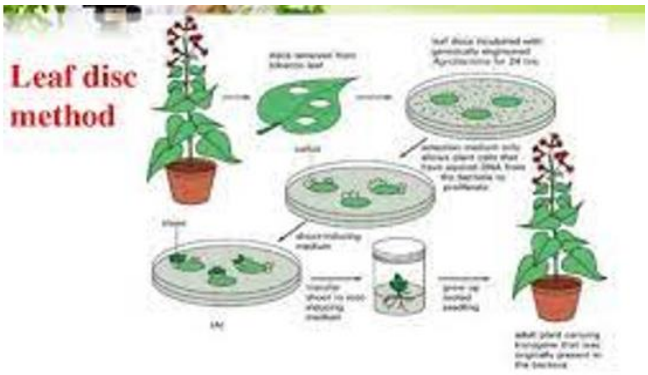
- Integration of the gene into a Ti plasmid vector (*Agrobacterium tumefaciens*); the vector has an antibiotic resistance gene
- Recombinant T-DNA is introduced into *Agrobacterium* cells, which are co-cultured with plant tissues
- Selection on a suitable medium, *e.g.*, a medium having carbenicillin and kanamycin
- The plants regenerated from the tissues are expected to have the desired gene; these are transgenic plants



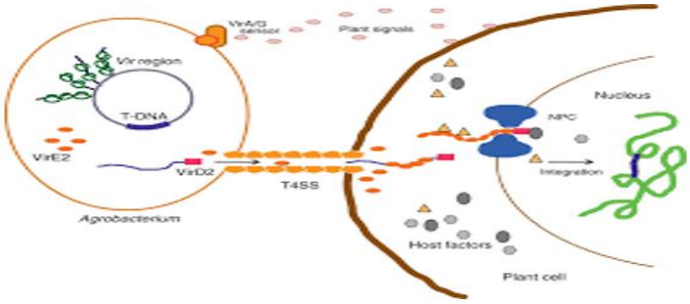
Tumor induction due to Ti plasmid of *Agrobacterium tumefaciens*

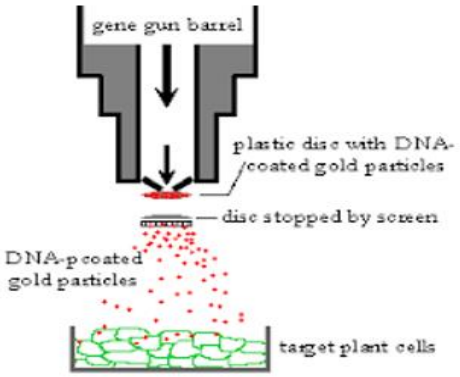
Hairy roots on stem segment due to the Ri plasmid of *Agrobacterium rhizogenes*

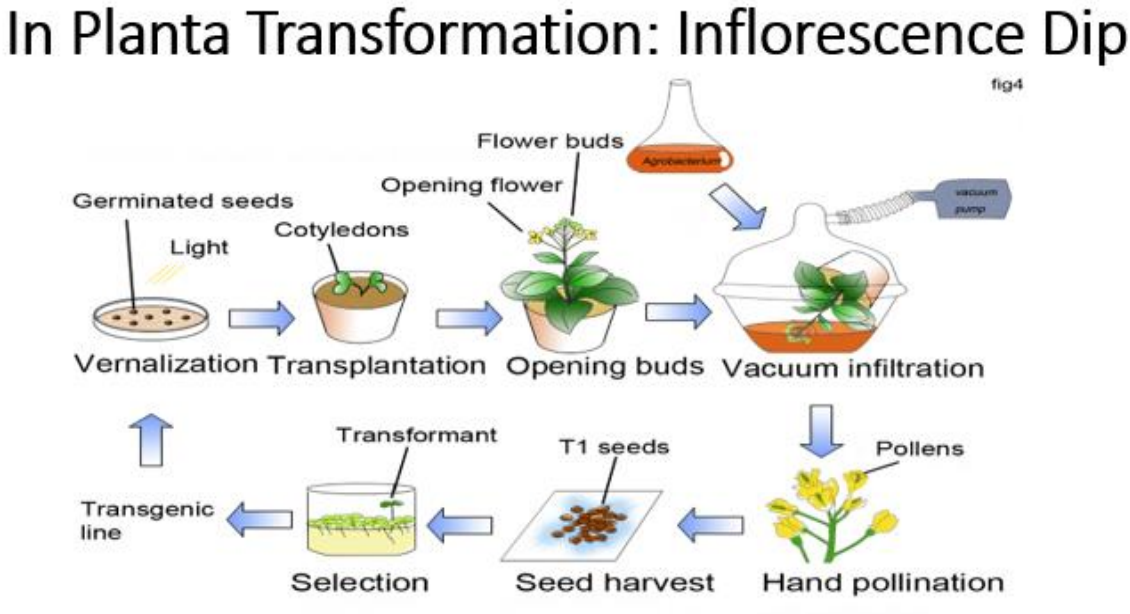




T-DNA Transfer



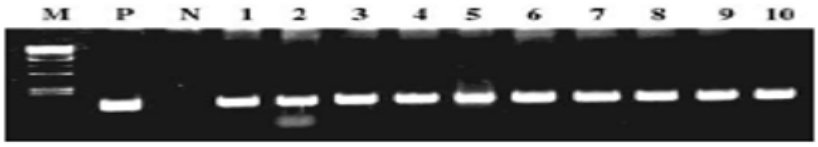




Transformation of Soil-grown Plants

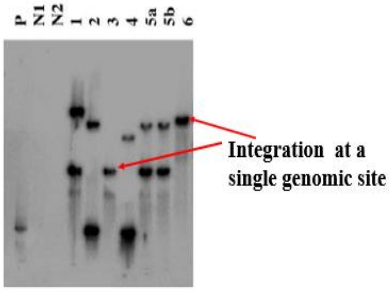
- Shoot meristems induced in soil-grown dicot plants by *A. tumefaciens*-mediated delivery of developmental regulator genes like *WUSCHEL* (*WUS*), *SHOOT MERISTEMLESS* (*STM*), etc.
- The developmental regulator genes along with the transgene(s) of interest are introduced into somatic cells of plants growing in soil.
- The expression of developmental regulator genes would lead to formation of adventitious shoots which will also contain and express the transgenes of interest.
- The seeds produced on the adventitious shoots are harvested and tested for the transgene expression
- The developmental regulator genes are removed form the transgenic plants using a suitable strategy

Identification of putative transgenic plants
PCR Amplification of Inserted Gene



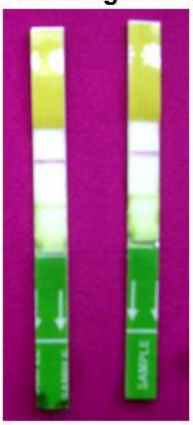
Agarose gel image of PCR amplification performed with primers for the *nptII* gene. *Lanes*: *M* Molecular size marker, *P* plasmid, *N* non-transformed plant, *1–10* transgenic plants

Confirmation of Transgene Integration into Genome
Southern Blot Analysis



Southern blot analysis of DNA isolated from leaves of two non-transformed control plants and seven plants transformed plants. *Lanes*: *P* nptII fragment of plasmid, *N1 & N2* non-transformed plant, 1-6 transgenic plant

Detection of Transgene Expression



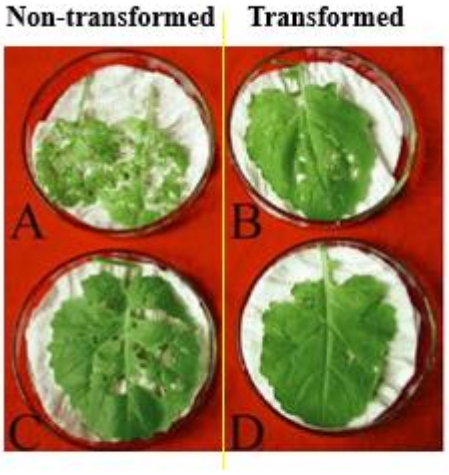
A test strip for quick detection of Cry protein

Insect Resistant Plants

Bacillus thuringiensis **is a soil bacterium, and produces insecticidal crystal (Cry) protein**

Gene encoding this protein has been transferred into plants to produce insect resistance

Bt cotton, being grown in India, has this gene



Insect bioassay with leaf pieces

Transgenic Plants: Some Applications

- Improved quality: Flavr Savr tomato, Golden Rice

- Insect resistance: Bt cotton hybrids
- Herbicide tolerance (Glyphosate)
- Virus resistance: CMV resistant squash
- A biopharmaceutical molecule: Hirudin
- Drought tolerance: maize and sugarcane
- And many other exciting examples

Lecture

(BD Singh, Ex-Emeritus professor, School of Biotechnology, Banaras Hindu University)

Transgenic Plants: Bio-safety and Social Issues

Designer Crops

- Crop varieties expressing specific novel traits, including a specific quality trait and production of a novel biochemical
- First generation GM crops: resistance to herbicides, insects, etc. (Bt-cotton resistant to bollworm)
- Second generation GM crops: quality modification (Oil from the 'Laurical' variety of *Brassica napus* has as high lauric acid as coconut oil.

New Generation GM Crops

- **Third generation GM crops**: yield improvement (a transgenic soybean expressing *bbx32*** from Arabidopsisshows altered growth/yield)
- **Fourth generation GM crops**: biochemical production (PMF, plant molecular farming)
  1. A transgenic variety of *B. napus* accumulates Hirudin, an antithrombin protein, in its seeds; Hirudin is being produced from *B. napus* in Canada
  2. In USA, research chemicals (proteins) like 'Avidin', 'ß- glucuronidase' are being produced from transgenic maize and marketed by Sigma Chemical Co., USA

Impacts of Transgenic Crops

- Animal and human health
- Environment
- Non-target organism
- Agriculture
- Socioeconomic conditions

Animal and Human Health

- Toxic action of transgene product
- Allergenic action of transgene product
- As far as possible, transgenes are taken from nonpathogenic organisms that are generally regarded as safe
- Transgenes suspected to cause toxicity or allergenic response are avoided
- Toxicity and allergenicity tests carried out on animals using purified transgene products

Toxicity and Allergenicity

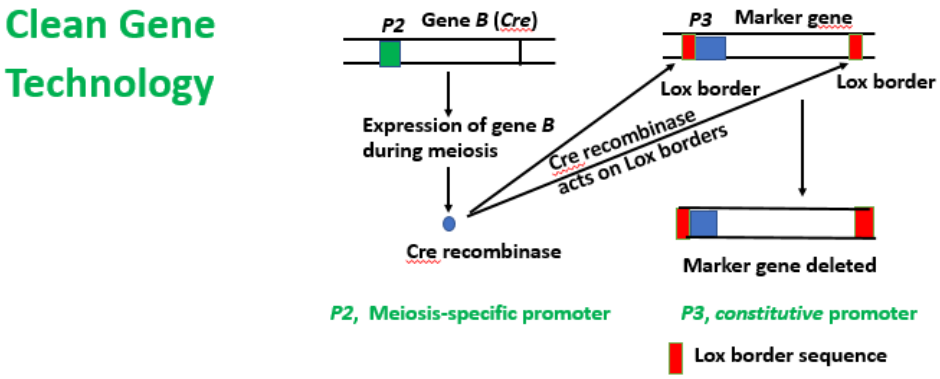
- Transgenes from even food crops may cause allergy, *e.g., alpha-amylase* from *P. vulgaris* transferred into pea for insect resistance
- NAAS, India recommends toxicity and allergenicity tests on a case-by-case basis, taking into consideration the transgene, the crop and the economic product(s) (NAAS = National Academy of Agricultural Sciences)

Antibiotic Resistance Markers

- Transfer of antibiotic resistance genes to gut microflora making them resistant to the concerned antibiotics
- This would create difficulties in management of diseases caused by these microbes

NAAS, India (2004) recommends strengthening of research to develop 'clean gene' technologies to produce antibiotic resistance marker-free transgenics (NAAS = National Academy of Agricultural Sciences)

Deletion of Antibiotic Resistance Marker



Non-antibiotic Selectable Markers (Clean Gene Technology)

uidA (beta-glucuronidase)	Acts on benzyladenine N-3 glucuronide (used in culture medium) to release BAP, which promotes shoot regeneration from transformed cells
Mannose-6-phosphate isomerase (*manA*	Converts mannose-6-phosphate present in culture medium) to fructose-6-phosphate; only transformed cells can grow
Xylose isomerase (*xylA*)	Converts xylose to xylulose; xylose used in selection medium
ipt (isopentenyltransferase)	Transformed cells produce isopentenyl adenine and proliferate on cytokinin-free medium

Nutritional Composition

- Transgene integration may activate some harmful genes or inactivate some beneficial genes located close to the transgene integration site
- Transgene expression may affect the composition of GM crop produce
- These necessitate elaborate animal feeding experiments
- Transgene silencing may reduce or abolish transgene expression. This will be cause of concern if the transgene was used to suppress a harmful endogenous gene, *e.g.*, suppression of gossypol production in cotton seed by RNAi
- This necessitates extended evaluation over locations and years

Environmental Issues

- Persistence of transgenic volunteers – increased fitness, invasiveness, competitiveness
  1. Domestication history of the crop is important

2. The trait that is modified is critical, *e.g.*, stress tolerance in forage crops may increase competitiveness

- Genetic trait control technology: to solve the problem of increased persistence, etc.
- Development of invasion may take 50-150 years
- Susceptibility of non-target organisms
- Example: Monarch butterfly larvae to Cry proteins (contradictory reports)
- Accumulation of transgene products in the environment
- Example: Cry proteins in the soil
- Increased use of chemicals in agriculture
- Example: Inducers applied to realize transgene expression

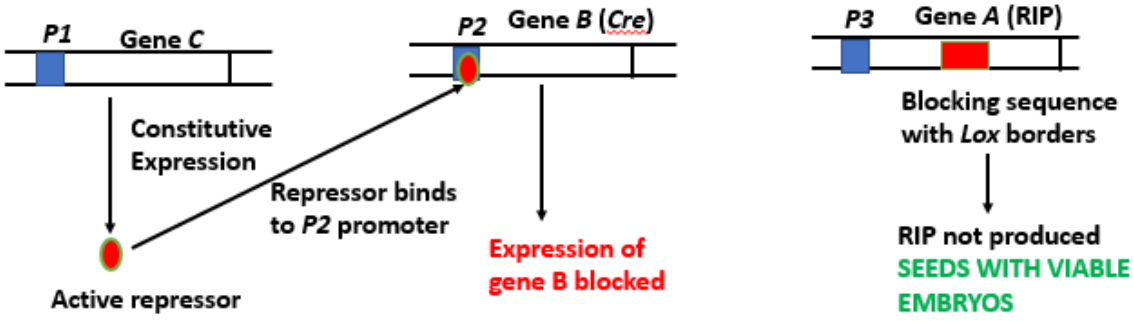
Horizontal Gene Transfers: Transgene Dispersal Through Pollen Grains

- Transfer of the transgene to nontransgenic varieties growing nearby
- Problem especially for organic farming, and in case of PMF (plant molecular farming) crops

Transfer of The Transgene to Related Wild Species

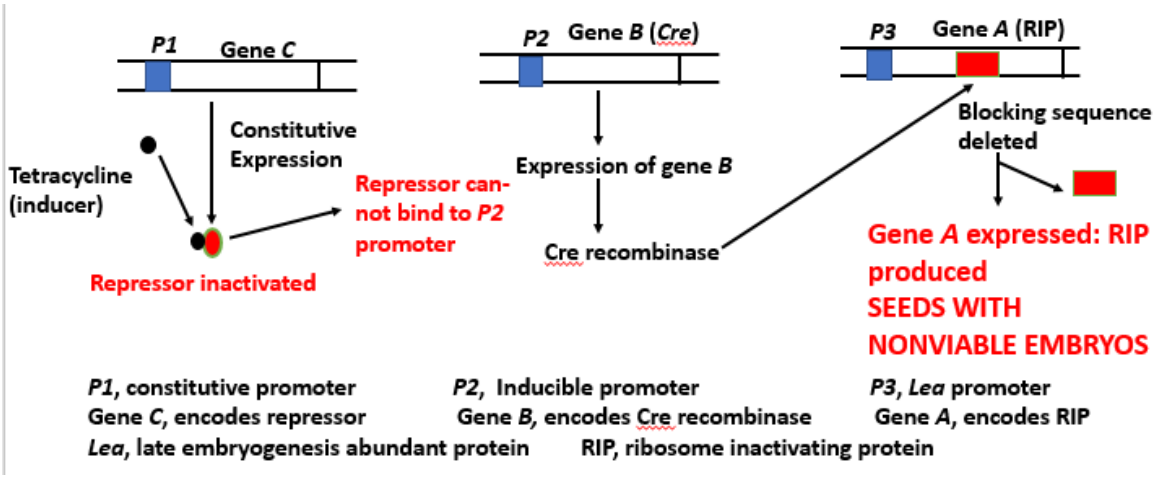
- Transgene transfer via pollen to sexually compatible wild species growing in the vicinity
- This may make the species more persistent and noxious
- Solution 1: transgene integration in cpDNA
  But pollen transmission of cpDNA (~0.5% in transgenic tobacco)
- Solution 2: genetic use restriction technology (GURT), *e.g.*, terminator seed technology

Terminator Seed Technology (No Seed Treatment)



P1, constitutive promoter — *P2*, Inducible promoter — *P3*, *Lea* promoter
Gene *C*, encodes repressor — Gene *B*, encodes Cre recombinase — Gene *A*, encodes RIP
Lea, late embryogenesis abundant protein — RIP, ribosome inactivating protein

Terminator Seed Technology (Seed Treatment with Tetracycline)



Horizontal Gene Transfers: Gene Transfer to Microorganism

- Occurs naturally by DNA uptake
- But the scale would be extremely large in case of transgenic plants
- DNA is stabilized by its association with humus/clay

Generation of New Virus Strains

- In case of coat protein gene-mediated virus resistance
- Can occur by recombination/heteroencapsidation
- Can affect virus translocation, *i.e.*, systemic spread of the virus in the host plants
- Can occur in nature in cases of mixed infections
- The scale will be the difference between nature and the transgenic situation

Designer Crops and Food Security

- The PMF crops will generate high value produce intended for nonfood uses
- These crops may replace food crops from a significant proportion of the cultivated area
- This coupled with the incessantly rising human population pressure may adversely affect 'food security', particularly in developing countries

Designer Crops and Social Concerns

- Crops producing specific biochemicals like therapeutic proteins, or having modified quality not suitable for human consumption (*e.g.*, 'Laurical' *B. napus*) may end up in the human food/animal feed chain
- This is a very relevant issue for India NAAS, India (2004) has recommended monitoring of this aspect and analysis of linkages of industry with agriculture
- It has already happened: a tuber producing medicinal plant (*Dioscorea*) was used as human food in Tamil Nadu leading to toxicity problems **(NAAS = National Academy of Agricultural Sciences)**

Agricultural issues

- Yield drag: Example, ~5% yield drag for 'Roundup Ready' soybean (Glyphosate resistant soybean)
- Improved transgenic technology has ameliorated this limitation

- Target insect pests generally develop resistance to transgene encoded Cry protein toxin
- Strategies to overcome this problem: 'refugia' concept, use of multiple *cry* genes, hybrid *cry* genes, other inset resistance genes, *e.g*., Vip encoding genes

Refugia Concept

- Insect resistant GM crops express *cry* gene from *Bacillus thurigiensis*, giving them the popular prefix 'Bt', *e.g.*, Bt-cotton
- Refugia strategy designed to manage the emergence of resistance to Cry proteins in the target insect population
- Refugia strategy: Planting of a part of the field to non-Bt varieties of the crop, *e.g*., 25% in case of maize; this dilutes the Cry protein resistance allele in the pest population
- Refugia implementation mandatory in U.S.A.
- In India, refugia implementation seems to be the responsibility of seed companies

Other Agricultural issues

- Pests may be driven to new host plants due to the deployment of insect resistant transgenic varieties, *e.g*., Armyworm
- Farmers must pay for the use of their GM crop produce as seed
- Seed replacement rate is merely nearly 10% in India; remaining are planted with farmers produce
- It will have to become 100% with 'genetic use restriction technologies' (GURT)

Illegal Seeds

- Illegal seed: seed of an unapproved or a nameless variety
- The problem of illegal seeds started in 2002 when unlicensed Bt (*cry1Ac*) cotton hybrid was found growing in ~ 10,000 acres in Gujarat
- Illegal Bt-cotton seed is in demand because of,
  1. The demonstrated ability to resist bollworm attack
  2. Relatively much lower price (Rs. 600 for 450g as compared to Rs. 1600 for 450g of the approved variety).
- 80% of all 'Bt cotton' growing in India were nameless, unapproved varieties
- The private companies may stop investing in transgenic research
- No refugia is implemented when illegal seed is grown
- Many illegal traders pose as farmers to protect themselves
- This would generate pressure for abolishing farmer to farmer seed exchange in India, which is a very important tool for spread of a new variety. Nature Biotechnology 22:1333-1334 (2005)

Intellectual Property

- **Physical property**: Land, goods, money
- **Intellectual property**: publications, software, equipment, formulae, biological agents, etc.

  Intellectual properties can be copied, improved upon

Intellectual Property Rights (IPR)

The right of an inventor/ innovator to exclude other from driving economic benefits from his invention.

Protection of IPR

- Trade secret
- Patent
- Plant breeder's rights
- Copyright
- Geographical indications

Why IPR protection?

- Promotes inventiveness by rewarding the inventors
- Encourages dissemination of technologies/ideas
- Encourages investment in R & D efforts
- Consumers get superior products
- Promotes economic development of the nation

Protection of Biotechnological Inventions	
Method/processes/concepts	**Patent**
Products	**Patent**
Application (process/product)	**Patent**
Biological agents (produced through biotechnology)	**Patent**
DNA sequences/proteins	**Patent**
Processes for genetic modification	**Patent**

The Patent Imbroglio

Development of a transgenic variety involves

- A crop variety
- Promoters and other — CaMV 35S promoter
- Regulatory sequences
- Reporter genes — *npt* II
- Genes for specific traits (ethylene biosynthesis) — ACC synthase
- Gene transfer method — *Agrobacterium*-mediated
- A specific technology (Enzo Biochem., Inc.) (sometimes) — Antisense RNA

Broad Patents in Plant Biotechnology

Development of transgenic plants requires several technologies and gene constructs, etc. These are protected by patents. Some of the patents are very wide in their coverage.	
DNA Plant Technology	**All transgenic pepper**
Plant Genetic Systems	**All transgenic plants with *cry* gene**
Mycogen Corporation	**Any method of modifying *cry* gene**
Such patents would foster monopolies, restrict plant biotechnology exploitation in developing countries and ultimately be detrimental to global food security	

Plant Breeders Rights

- The rights granted by a government to a plant breeder, originator or owner of a variety to exclude others from producing or commercializing the propagating material of the concerned variety for a period of 15-20 years
- In India, plant varieties are protected by 'The Protection of Plant Varieties and Farmers' Rights Act, 2001', a form of plant breeders rights

Plant Breeders Rights: Disadvantages

- PBR encourages monopolies for specific traits
- PBR regime hinders free exchange of germplasm
- The above are in contradiction to the FAO resolution stating that 'germplasm is common heritage of man'
- PBR may lead to a dilution of farmer's privilege
- It may lead to increased seed costs

Gene Patents and Genetic Resources

- Developing countries are technology-poor but gene-rich
- Developed countries are technology-rich but gene-poor
- Developed countries have extensive germplasm collections of all important crops, most of them from developing countries
- They are also cloning and patenting important genes, *e.g.*, *Xa*21, which specifies resistance to bacterial leaf blight of rice
- *Xa21* was discovered in India in *Oryza longistaminanta,* which is native of Male (Africa). It was cloned and patented by University of California, Davis (USA)

Farmer's Privilege

- Farmers are allowed to use the material of a protected variety produced on their farm to resow their fields without any obligation to the PBR title holder
- In India:
  1. Over 90% of fields are planted with seeds produced by the farmers themselves
  2. About 80% farmers exchange seeds
  3. The existing laws allow farmers to exchange seeds of protected varieties
- Can this provision be sustained? This concern is expressed in view of TRIPs and the problem of Illegal seeds
- India is obliged under GATT provisions to conform to TRIPs guidelines, *i.e.*, protection either by patent or a protection mechanism comparable to patent

Lecture

(BD Singh, Ex-Emeritus professor, School of Biotechnology, Banaras Hindu University)

Male Sterility and Hybrid Seed Production

Hybrid Varieties

- **Hybrid Variety:** The *F1* generation used as a variety
- These varieties exploit heterosis and are superior performing
- But their seed must be produced every year
- For this, the female parent must be emasculated before pollen shed

Hybrid Seed Production: Emasculation

- Hand emasculation has long been used, *e.g*., detasseling in maize
- Genetic emasculation:
  1. Male sterility: pollen nonfunctional (many plants)
  2. Pistillate condition: only female flowers produced (castor)
  3. Self-incompatibility: functional pollen fails to effect self-fertilization (*Brassica* spp.)
- Chemical emasculation: male sterility induced by specific chemicals

Male Sterility

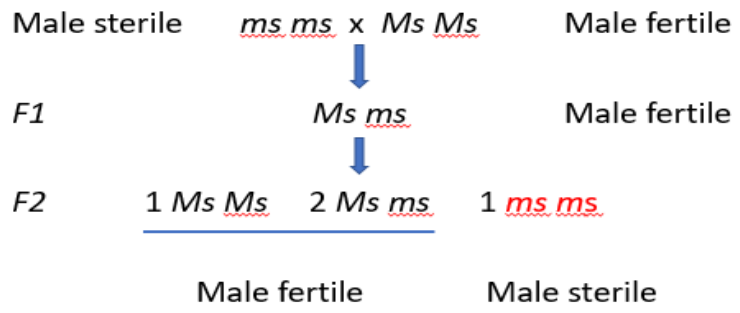
- **Genic male sterility**: male sterility generally caused by single recessive nuclear genes; typical Mendelian inheritance (3:1 ration in *F2*)
- **Cytoplasmic male sterility**: male sterility typically determined by mitochondrial genome; maternal transmission

Genic male Sterility

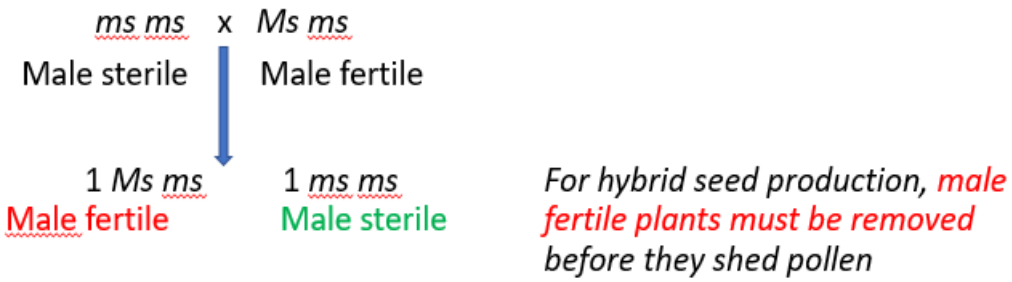
1. **Environment-insensitive male sterility**: Expression of male sterility not affected by the environment
2. **Environment-sensitive male sterility**: Expression of male sterility depends on environmental factors, especially temperature and photoperiod

3**. Transgenic male sterility:** Male sterility caused by transgenes

Genic Male Sterility: Inheritance



Genetic Male Sterile Line: Maintenance



Stage at Which Some Tomato *ms* Genes Act

Stage	*ms* gene
Premeiotic	*ms2, ms3*
Zygotene	*Ms10, msk7*
Diplotene	*ms1, ms18a*
Metaphase I	*msk3*
Tetrad stage	*ms6, ms31*
Microspore development	*ms2b, ms13*
Anytime between prophase I and tetrad stage	*ms5, ms8*

Environment-Sensitive Genic Male Sterility

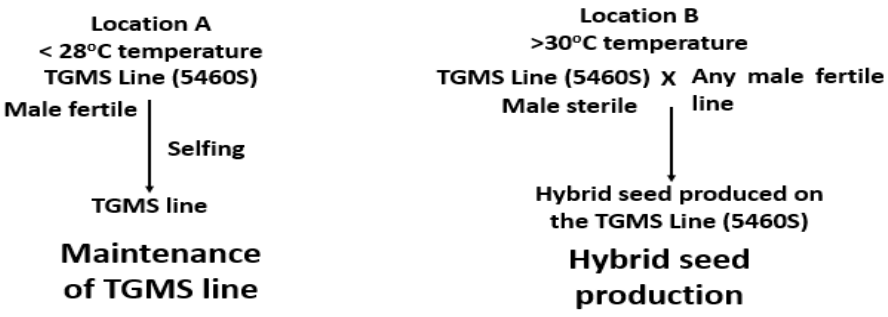
a. **Temperature-sensitive male sterility**: Male sterility produced when the temperature is higher than the threshold temperature (in case of PA64S rice, 23.5°C)
b. **Reverse temperature-sensitive male sterility**: Male sterility produced when the temperature is lower than the threshold temperature (in case of J207S rice, 31°C)
c. **Photoperiod-sensitive male sterility**: Male sterility produced when the photoperiod is longer than the threshold (in case of NK58S rice, 13 hr 45 min)
d. **Reverse photoperiod-sensitive male sterility**: Male sterility produced when the photoperiod is shorter than the threshold

Some rice PGMS and TGMS Lines

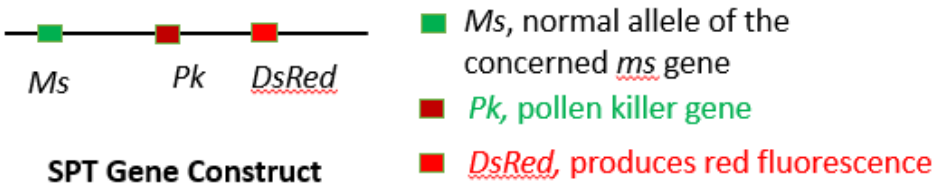
* I, Indica; J, Japonica
**developed in India

Line	Origin	Remarks
Nongken 58S (J)*	Spontaneous mutation (1973)	Male sterile under photoperiods longer than 13 hr 45 min
MG 201 (J)*	EMS mutagenesis	Male sterile under photoperiods longer than 13 hr 45 min
-	Gene editing	Male sterile under short-day conditions; rPGMS
Peiai 64S (I)*	Hybridization with NK58S	Male sterile at temperatures >23.5°C; fertile at temperatures <21-23°C
JP-38S**	-	Male fertile at >30.5°C; rTGMS
Mutants *tms5–1* and *tms5–2*	Gene editing	TGMS line

TGMS-Based Hybrid Seed Production in Rice

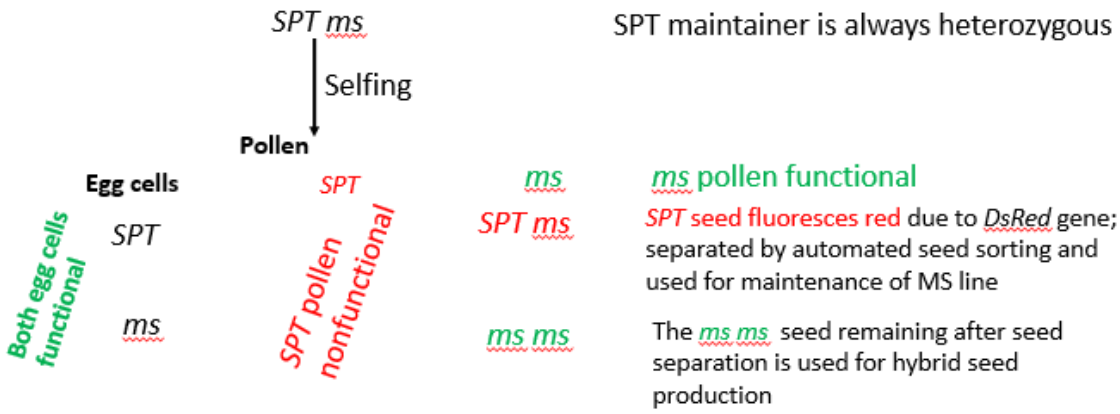


Seed production technology (SPT): Maintainer for Genic Male Sterility



1. **The SPT maintainer is transgenic**
2. **But the male sterile (*ms ms*) plants are nontransgenic**

Maintenance of Genic Male Sterility



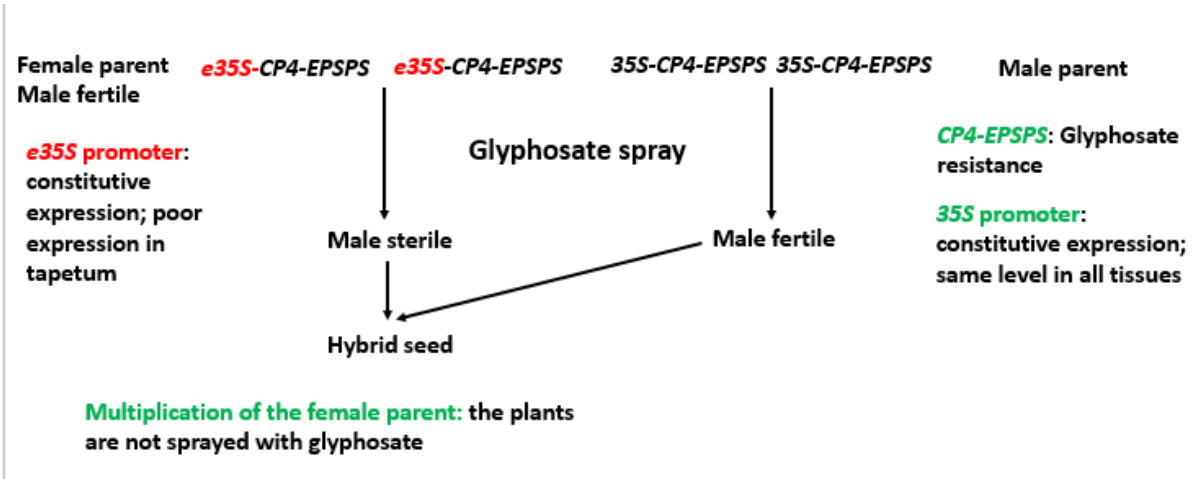
Barnase-Barstar System

1. Barnase is bacterial Rnase
2. Expression of *barnase* in tapetum cells produces male sterility
3. Barstar inhibits Barnase action; restores fertility

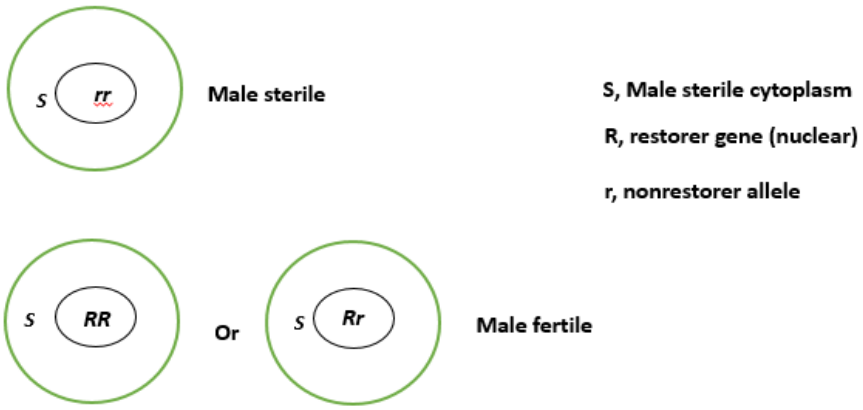
bar specifies phosphinothricin resistance; it is physically linked with *bar* gene

barnase-bar / -	**Male sterile, Phosphinothricin resistant (transgenic)**
- / -	**Male fertile, Phosphinothricin sensitive (nontransgenic)**
barstar / barstar	**Male fertile, restorer (transgenic)**

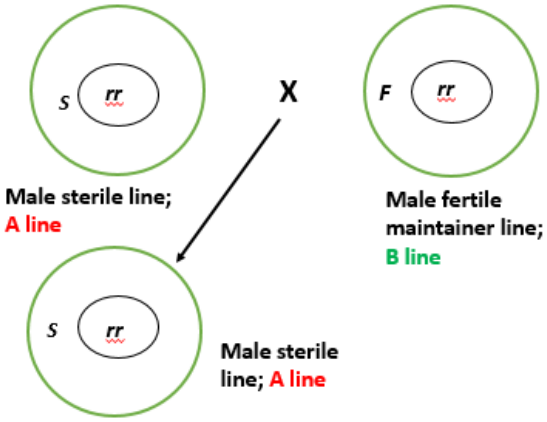
Inducible Transgenic Male Sterility (Roundup Hybridization System (RHS) in Maize)



Cytoplasmic-genetic Male Sterility



Maintenance of Cytoplasmic-genetic Male Sterility



S, Male sterile cytoplasm

F, Male fertile cytoplasm

r, nonrestorer nuclear gene

B line has the same nuclear genotype as the A line

Some male sterile cytoplasms

Crop (nucleus)	Cytoplasm	Source	Remarks
Brassica juncea	CMS-*Ogu*	Japanese wild radish	Low temperature chlorosis; lack of nectaries: corrected by somatic hybridization
	CMS-*mori*	*Moricandia arvensis*	Produced by somatic hybridization; used for hybrid seed production in India
Sunflower	PET-1	*Helianthus petiolaris*	Commercially used
Maize	CMS-C (charrua)	Spontaneous mutation	High spontaneous reversion, currently, the most widely used
	CMS-S USDA)	Spontaneous mutation	Commercially used; some CMS plants may shed pollen
	CMS-T (Texas)	Spontaneous mutation	Slight (2-4%) reduction in growth and yield; susceptibility to Southern corn leaf bight (*Bipolaris maydis*); once used almost exclusively; still in use
Rice	CMS-WA	*O. rufipogon*; wild abortive strain	Most widely used: 90% of 3-line hybrids in China and 100% in rest of the world
	CMS-BT	*O. sativa* subsp. *indica*	From Chinsurah Boro II (subsp. *indica*) x Liming (subsp. *japonica*); widely used; pollen starch deficient
	CMS-HL	Hong Lian, red-awned wild rice *O. rufipogon*	Widely used; pollen starch deficient
Triticum aestivum	*T*	*T. timopheevii*	Commercially used; requires two restorer genes for restoration

Bases of sterility and restoration

1. *orf*, open reading frame
2. CMS is due to novel *orfs* not found in normal cytoplasms
3. PPR protein, pentatricopept-ide repeat

Cytoplasm	CMS due to	Restorer (*Rf*) genes	Restoration mechanism
CMS-*Ogu*	*orf138* (cotranscribed with *atp8*; chimeric transcript)	*Rfo/Rfk1* (from Japanese radish, *Raphanus sativus*)	Regulation of *orf138* mRNA translation
PET-1)	*orf522* (programmed cell death of tapetum)	Two polymeric genes (*Rf1*, *Rf2*)	
CMS-C* (*Z. mays*; charrua)	-	*Rf4*** (full restoration), *Rf5*, *Rf6*	*Rf4*** (tassel-specific transcription factor)
CMS-WA	*WA352c* (*orf284*, *orf224*, *orf288*, and *orf126*)	*Rf3*, *Rf4* and modifying genes	*Rf4* encodes PPR protein
CMS-BT	*orf79* (transcribed as B-*atp6-orf79*)	*Rf1a* (gametophytic action) and *Rf1b* located in *Rf1* locus	cleavage of *orf79* mRNA
CMS-HL	*orfH79* (toxic peptide)	*Rf5*, *Rf6*	Encode PPR proteins
T. timopheevii	*orf279*	*Rf1* and *Rf3*)	PPR proteins cleave *orf279* transcript

Limitations of CMS systems

- Undesirable side-effects of male sterile cytoplasm
- Unsatisfactory fertility restoration
- Restoration affected by modifying genes
- Effects of environment on male sterility
- The need for developing CMS, maintainer and restorer lines which may take 5-7 years

CMS Limitations: Remedies

- Search for alternative CMS sources
- Defect correction, *e.g.*, *CMS-Ogu*
- Improving nuclear gene combinations to alleviate the defects of male sterile cytoplasm, *e.g.*, cotton
- Production of cytoplasmic blend hybrids
- Use of alternative methods of emasculation

Defect Correction of *CMS-Ogu*

- *CMS-Ogu*, *Ogura* CMS of *Brassica*; associated with low temperature chlorosis and poor nectary development
- *CMS-Ogu* has chloroplasts and mitochondria from wild radish
- The defects of *CMS-Ogu* could be corrected by replacing the chloroplasts of wild radish with those of *B. napus*, which does not show this defect
- Therefore, *CMS-Ogu* protoplasts were fused with protoplasts of normal *B. napus*

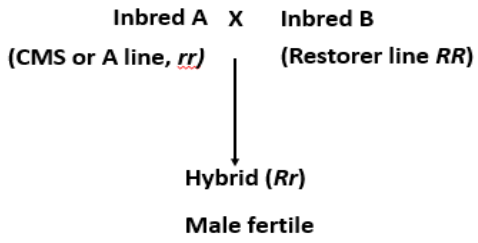
Improved *CMS-Ogu* Line

- Somatic hybrids were regenerated
- Some somatic hybrids were male sterile, free from low temperature chlorosis, and improved nectary development
- Somatic hybrids generally have chloroplasts and mitochondria of only one the two fusion parents
- In addition, often mitochondria have recombinant genomes
- Improved *CMS-Ogu* has *B. napus* chloroplasts and recombinant mitochondrial genome

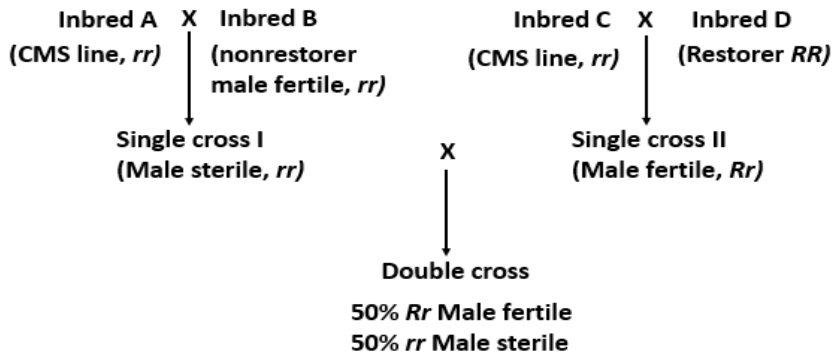
Cytoplasmic Blend Hybrids

- In 1970, CMS-T-based maize hybrids devastated by Southern leaf blight (*Bipolaris maydis*)
- For next three years, CMS not used in USA and Canada
- A **cytoplasmic blend hybrid** is a mixture of *F1* hybrid seeds obtained from more than one type of CMS; all the CMS lines have the same nuclear genotype. Similarly, and all the restorer lines have the same genotype, except for the restorer genes that would differ with the CMS source

CMS-Based Single Cross Hybrid Seed Production



CMS-Based Double Cross Hybrid Seed Production



Pistillate Condition in Castor

- Castor is monoecious: it bears male and female flowers
- Pistillate castor produces only female flowers (generally)
- **N Type Pistillate Lines**: only female flowers; a single recessive gene (*f*); similar to genic male sterility; roguing essential
- **S Type Pistillate Lines**: only up to 70% plants pistillate; later branches produce male flowers also; polygenic inheritance; roguing essential; used for hybrid seed production
- **NES Type Pistillate Lines**: only female flowers at temperatures below 35ºC; two genes, *f* and *s* (temperature sensitivity); similar to temperature-sensitive genetic male sterility; used for hybrid development

Chemical Hybridizing Agents (CHAs)

- Chemicals that specifically induce, ideally, 100% male sterility
- **First generation CHAs**: GA3, ethephon, etc.; developed for some other purpose; phytotoxicity, need for application during specific developmental stage
- **Second generation CHAs**: RH-0007 (Hybrex); developed specifically as CHAs; effective during a narrow range of developmental stages and in limited number of genotypes; Hybrex used for hybrid wheat seed production; discontinued
- **Current generation CHAs**: WL 84811, clofencet (Genesis); act as pollen suppressants; used in wheat; WL 84811 discontinued due to residues in *F1* seed; Genesis is nearly ideal CHA

- CHAs are used for commercial scale hybrid seed production in rice (China) and wheat (USA and some EU states)
- CMS is available in both rice and wheat
- CMS is the preferred system in rice
- CHAs are preferred in wheat; interest in CMS has declined drastically

CHAs: Advantages and Limitations

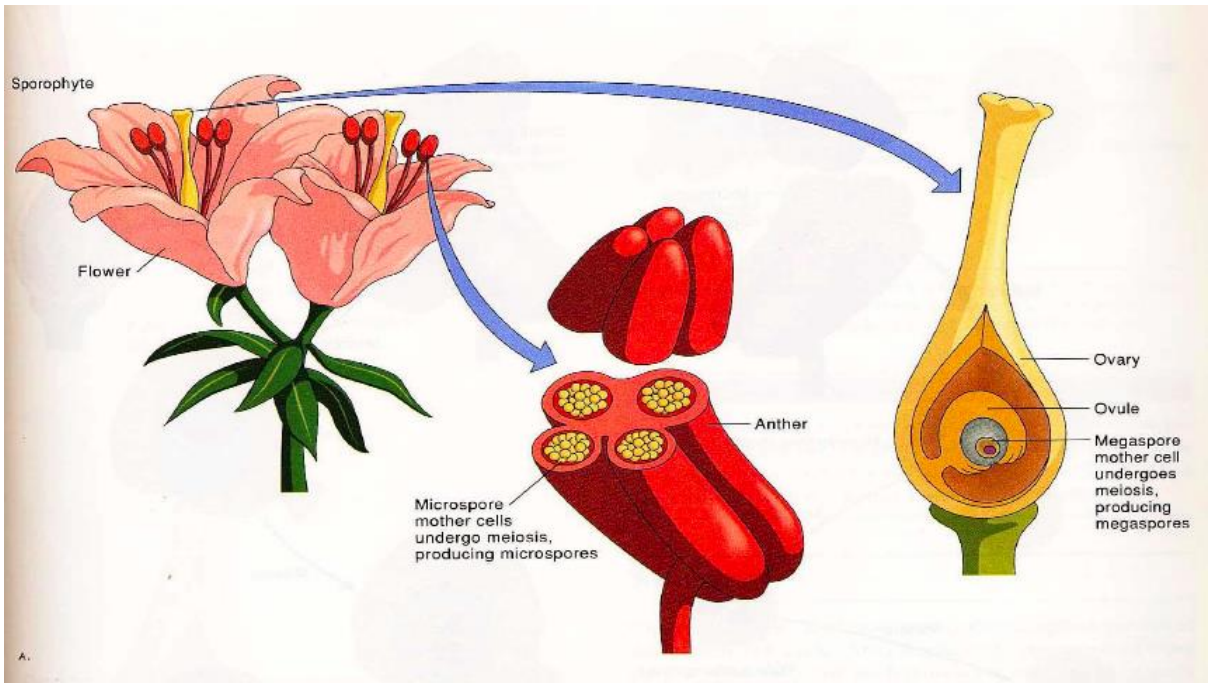
- The chief advantage is that any line can be used as female or male parent provided it yields an outstanding hybrid
- Further, there is no need to develop male sterile and/or restorer lines
- The chief limitation is the genotype-, developmental stage- and environment-specific action of CHAs
- In addition, considerable effort is needed to standardise the protocol for use of CHAs in hybrid seed production

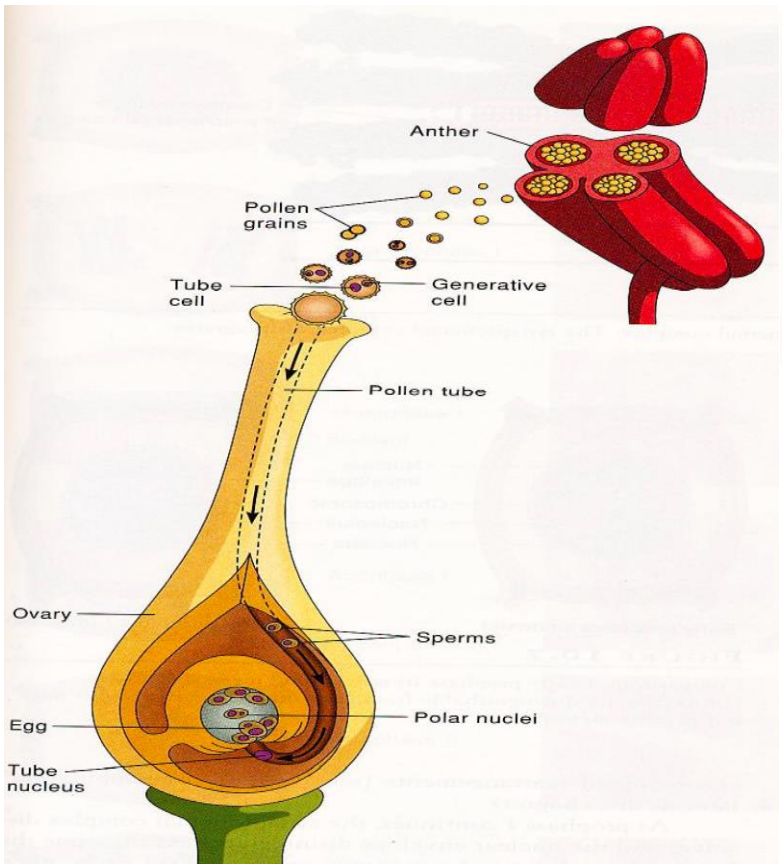
Lecture

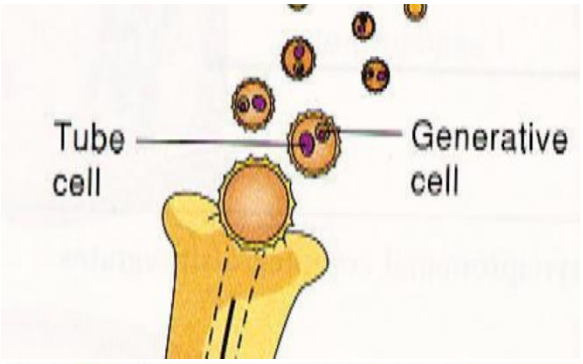
(Madan K. Bhattacharyya, Ph.D. Professor, Iowa State University)

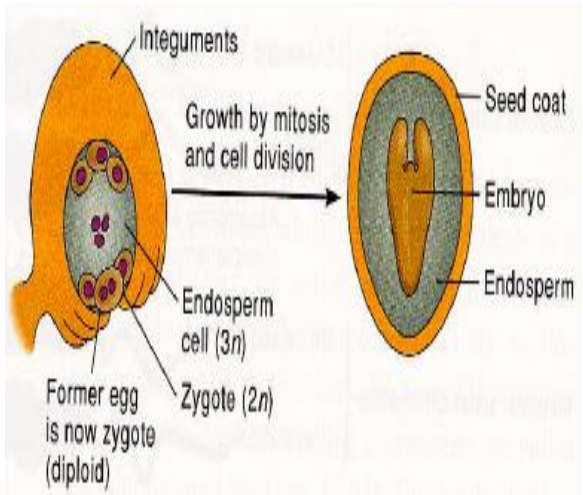
Genetics

Flowers - where the journey begins for generating novel genetic variation

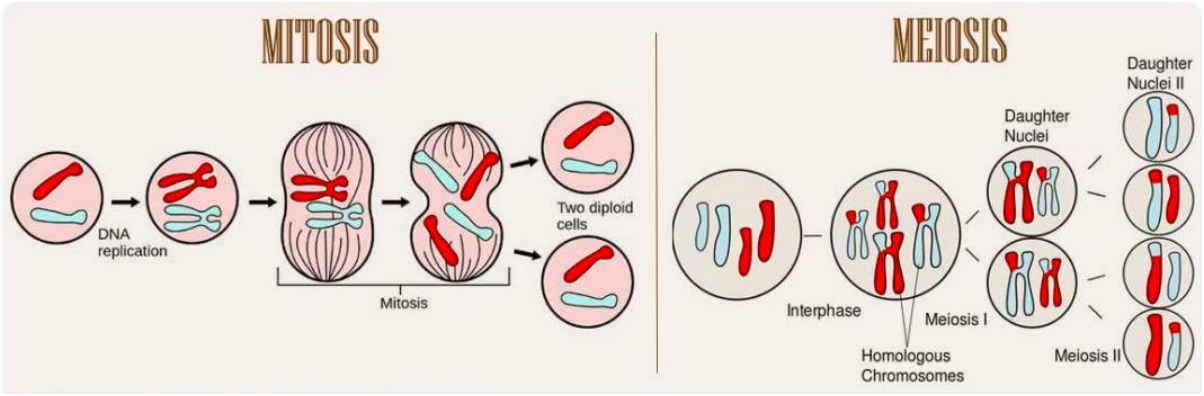




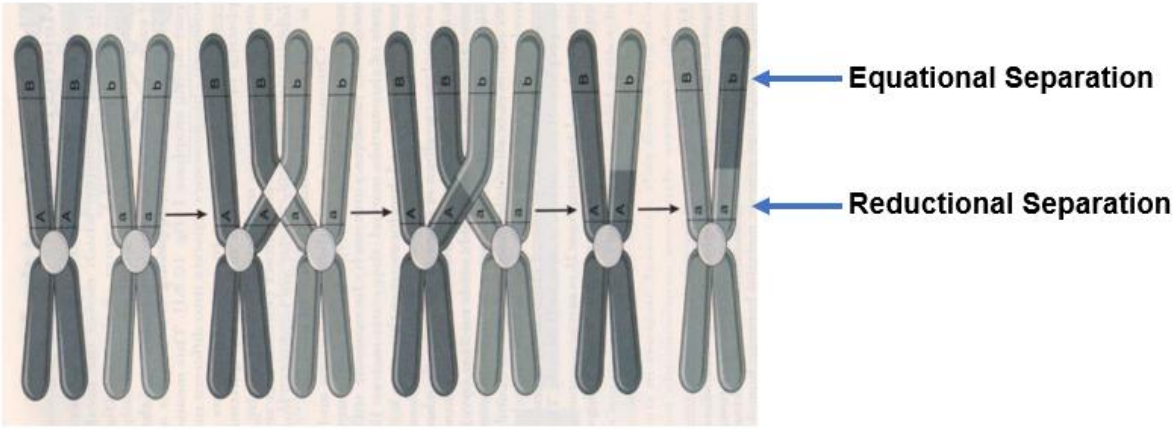




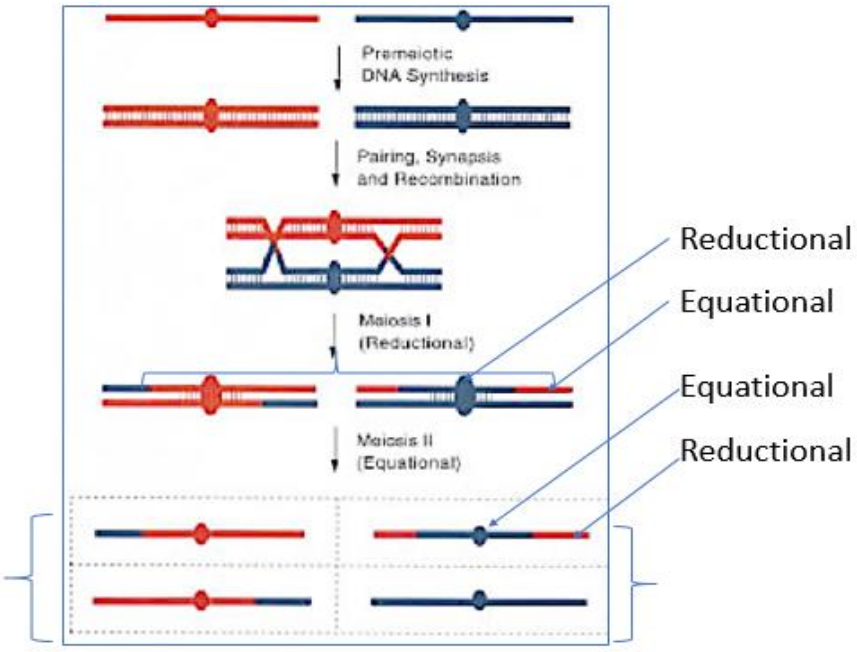
Major differences between Mitosis and Meiosis



Generation of novel chromatids through crossing over at the end Meiosis I



Type of Separations During Meiosis I and Meiosis II: Centromere



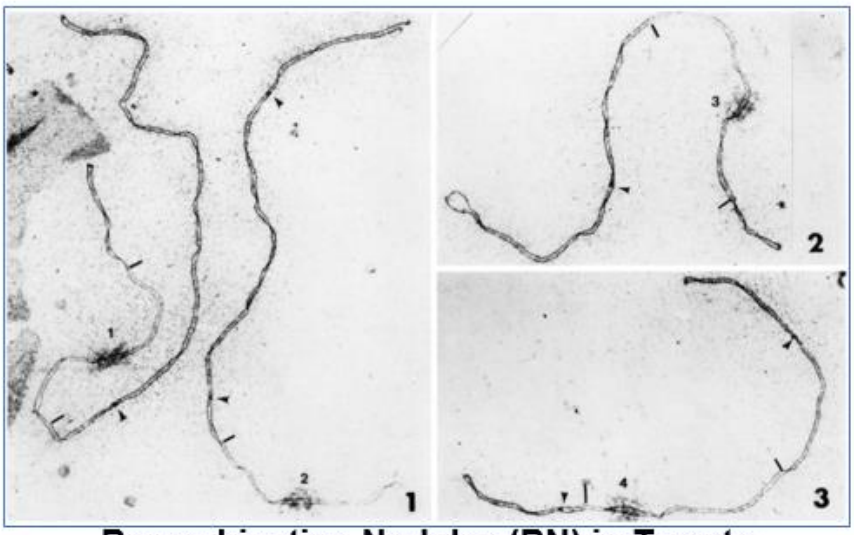
Chromosomal Separations in Meiosis

- Sister chromatids go to same pole at anaphase I.
- The kinetochore ("spindle attachment") always separates reductionally at anaphase I

- A locus some distance from the centromere will separate reductionally or equationally depending on the number of cross-over events between the locus and the centromere.
- Thus, a locus separated by one crossover from the centromere will separate equationally at anaphase I and reductionally at anaphase II.

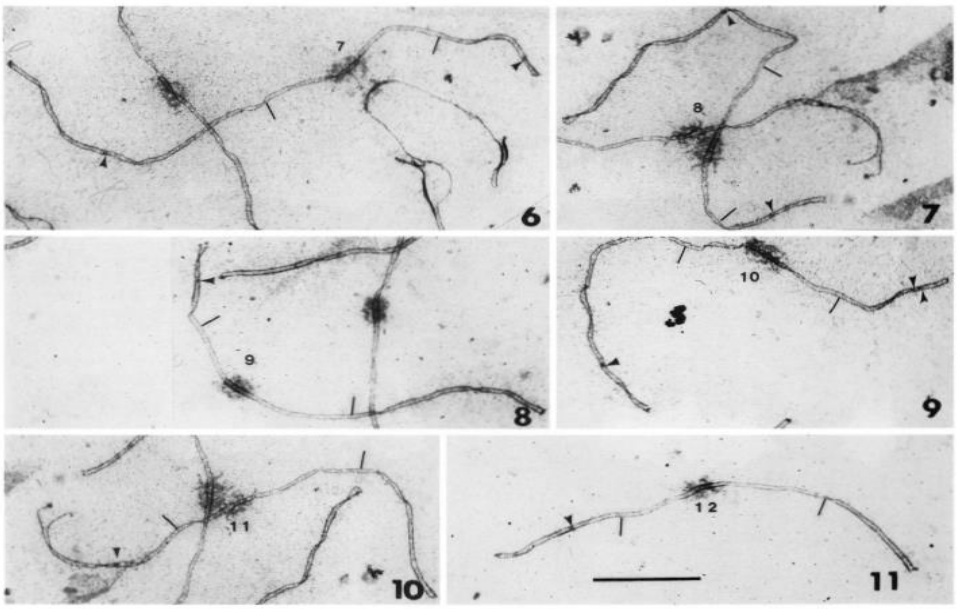
Recombination

A high-resolution physical recombination map for tomato chromosomes

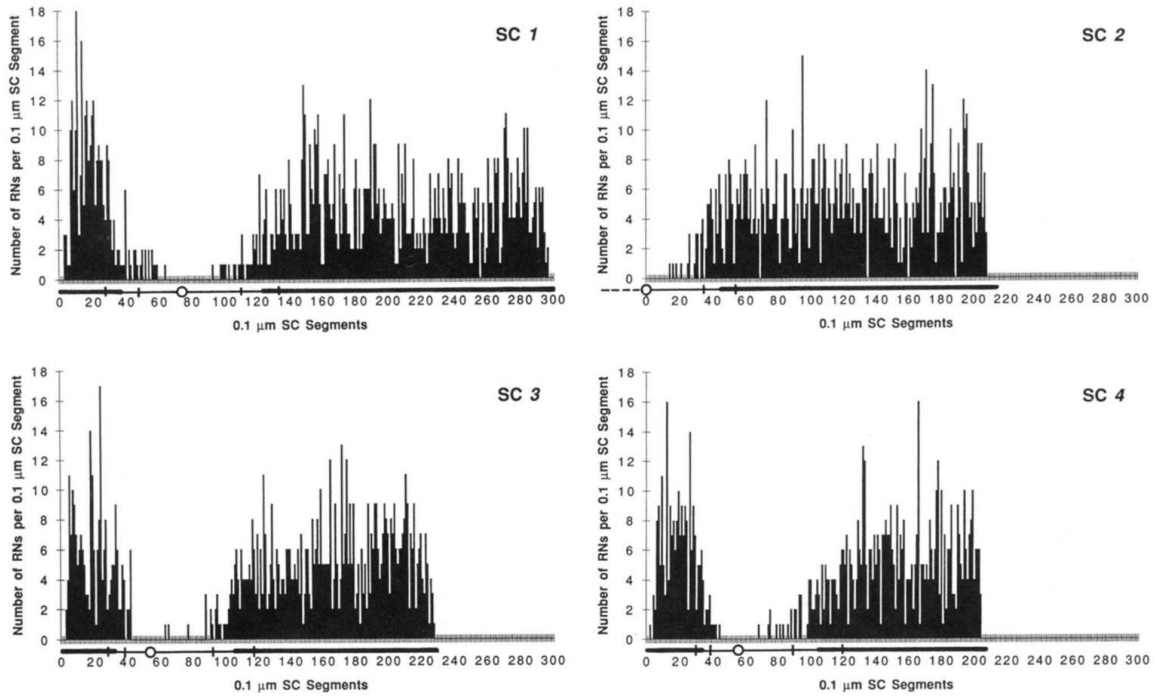


Recombination Nodules (RN) in Tomato

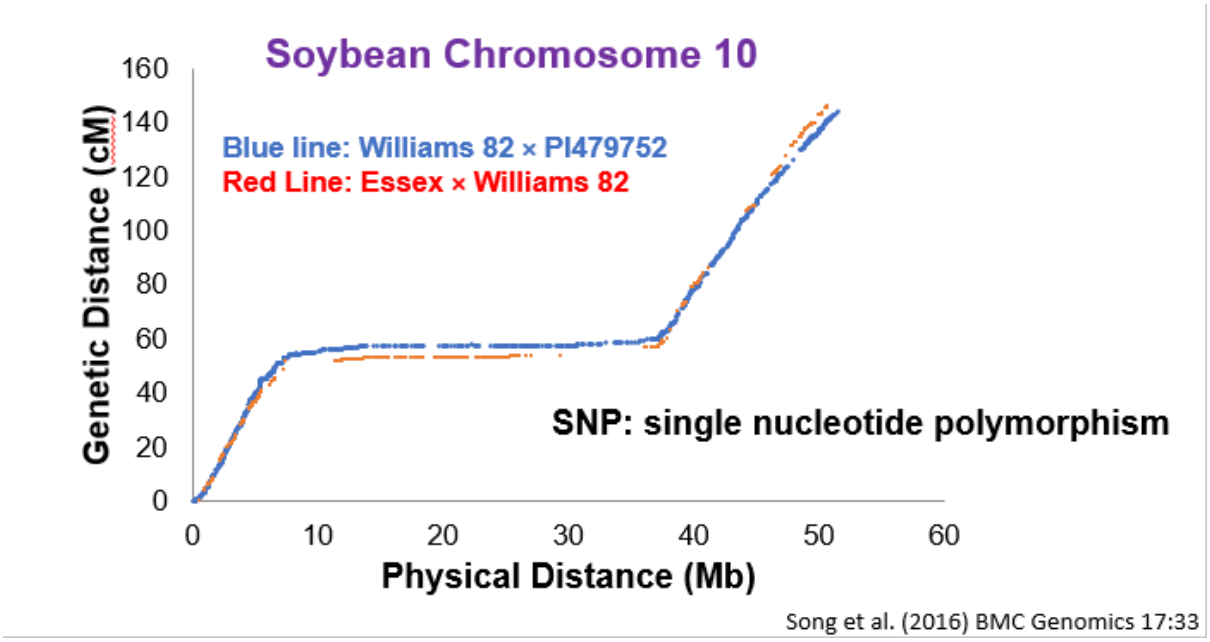
Sherman and Stack 1995



Distribution of RNs on tomato synaptonemal complexes (SCs).



Recombination is suppressed in the heterochromatic region containing the centromere.



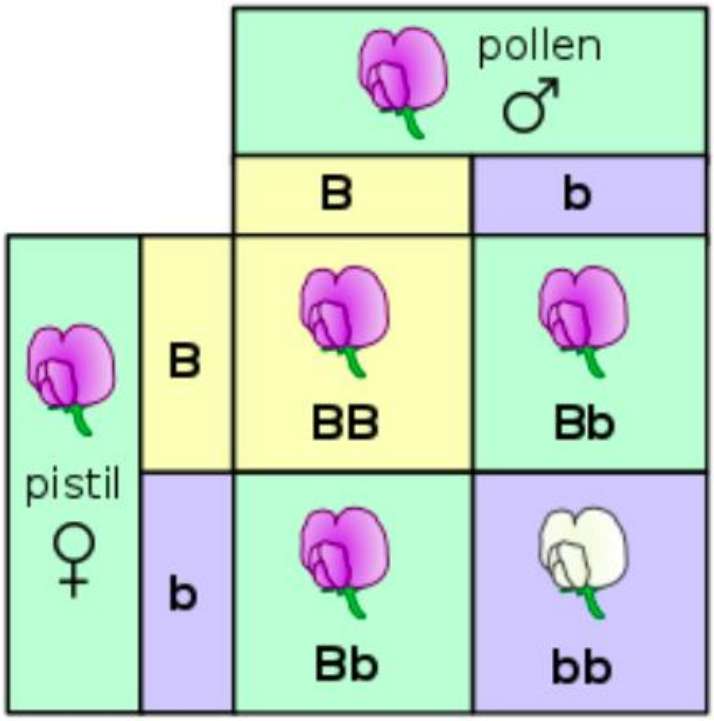
Different recombination rates across the chromosomes.

- Recombination rates are highly suppressed in the heterochromatic regions containing the centromeres.
- Genetic and physical maps do not have any linear relationships.
- Rare recombination rates in heterochromatic areas depict tight linkages between genes and/or markers. In fact, 10 -100 times more physical distances/cM in heterochromatic regions observed as compared to that in the euchromatic regions.

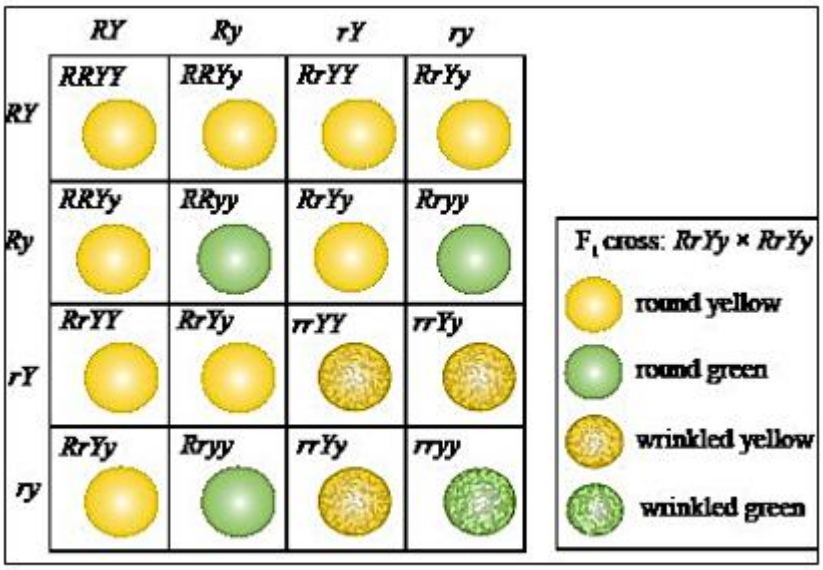
Mendel's laws of inheritance

Law	Definition
Law of dominance	Some alleles are dominant while others are recessive; an organism with at least one dominant allele will display the effect of the dominant allele.
Law of segregation	During gamete formation, the alleles for each gene segregate from each other so that each gamete carries only one allele for each gene.
Law of independent assortment	Alleles of genes governing different traits can segregate independently during the formation of gametes.

Law of segregation



Law of independent assortment



R-Y-:R-yy:rrY-:rryy:: 9:3:3:1

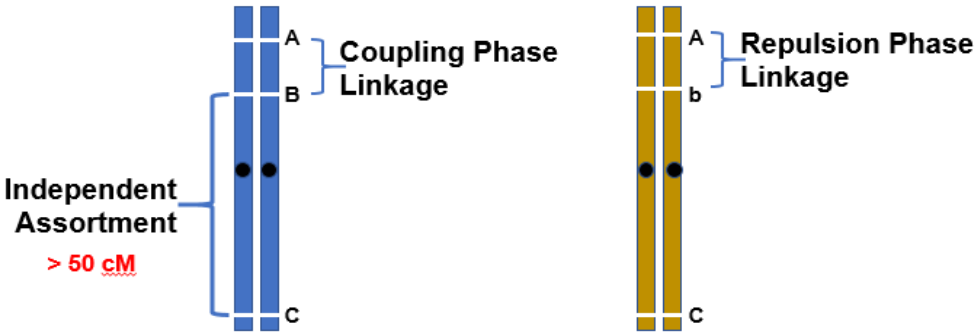
Segregation of genetic loci

Analysis of single gene segregations–determine what type of genetic control exists for a single trait–i.e., single gene dominant, codominant, two genes with partial dominance, etc.

- Need single genes in order to conduct linkage analyses.
- Testcross: Cross heterozygote Aa to homozygous recessive aa
  Intercross: Cross two heterozygotes Aa and Aa to produce an F_2 progeny
- Single genes in diploids: backcross will yield a 1:1; intercross 3:1 if dominant, 1:2:1 if codominant

- Alleles of genetic loci segregates and generates recombinants.
- Recombinants are novel genotypes with new combination of genes or alleles.
- Chi-square test allows defining segregating ratios and gene actions.

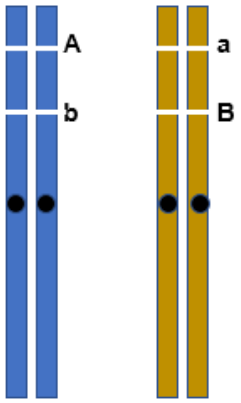
Independent Assortment & Coupling and Repulsion Phase Linkages



Detection of Linkage

Suppose we have two genes, A and B, both segregating in our population.

A & B each segregates 3:1:



	Phenotypes in F_2			
	AB	Ab	aB	ab
Recombination value	(a)	(b)	(c)	(d)
0%, Repulsion (R)	2	1	1	0
0%, Coupling (C)	3	0	0	1
50%, C or R (independance)	9	3	3	1

$\frac{ad}{bc}$ quick test: ⟶ independence = 1; repulsion linkage < 1; coupling linkage > 1.

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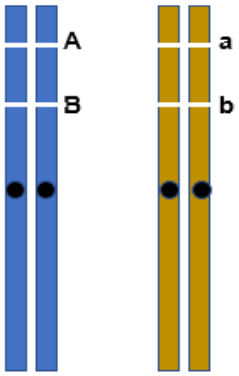

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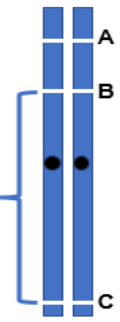


Independent Assortment
> 50 cM

	Phenotypes in F_2			
	AB	Ab	aB	ab
Recombination value	(a)	(b)	(c)	(d)
0%, Repulsion (R)	2	1	1	0
0%, Coupling (C)	3	0	0	1
50%, C or R (independance)	9	3	3	1

$\frac{ad}{bc}$ quick test: ⟶ independence = 1; repulsion linkage < 1; coupling linkage > 1.

Detection of Linkage: Example

F_2 data for *cr* (crinkly dwarf) vs. *ms* (male sterile)

***cr-ms* x Normal** (double mutant was crossed with the wild-type) (coupling phase cross)

a = 181, b = 33, c = 35, d = 30; N = 279

AB (a)

Ab (b)

aB (c)

ab (d)

	Phenotypes in F_2			
	AB	Ab	aB	ab
Recombination value	(a)	(b)	(c)	(d)
0%, Repulsion (R)	2	1	1	0
0%, Coupling (C)	3	0	0	1
50%, C or R (independance)	9	3	3	1

$\frac{ad}{bc}$ quick test: → independence = 1; repulsion linkage < 1; coupling linkage > 1.

Quick test: (181 x 30)/(33 x 35) = 4.7 (coupling linkage as > 1).

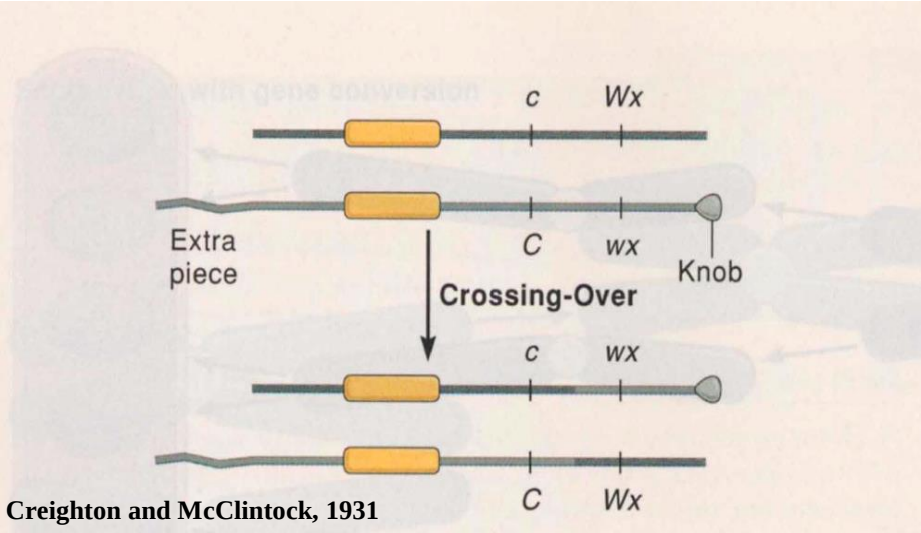
Independent Assortment and Linkage

- Relative positions of two genetic loci determines if independent assortment or linkage will prevail between them.
- Coupling and repulsion phase linkages produce different proportions of recombinants.
- Coupling phase linkage is more sensitive to determine the recombination percentage as compared to repulsion phase linkage.

	Phenotypes in F_2			
	AB	Ab	aB	ab
Recombination value	(a)	(b)	(c)	(d)
0%, Repulsion (R)	2	1	1	0
0%, Coupling (C)	3	0	0	1
50%, C or R (independance)	9	3	3	1 ← Repulsion

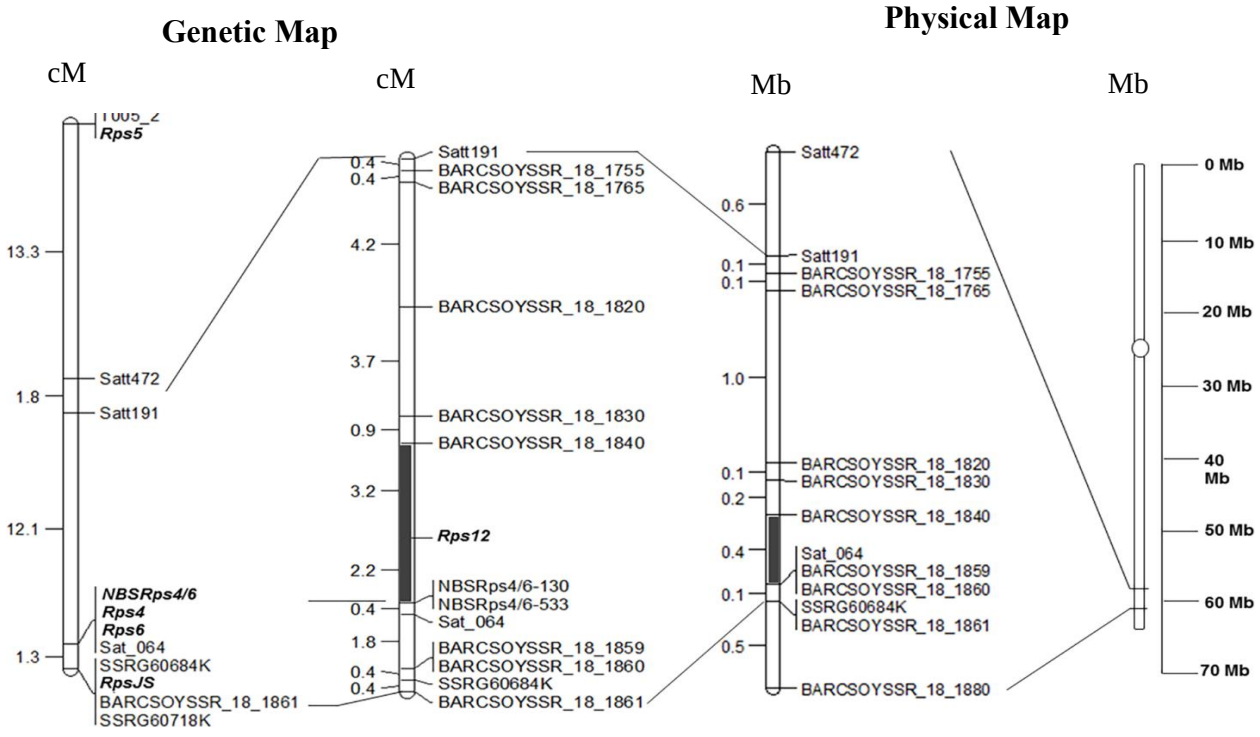
Coupling

Map of the cytological and morphological markers of maize chromosome 9 based on crossing over frequencies



Creighton and McClintock, 1931

Genetic and Physical Maps



Sahoo DK, Abeysekara NS, Cianzio SR, Robertson AE, Bhattacharyya MK (2017) A Novel Phytophthora sojae Resistance Rps12 Gene Mapped to a Genomic Region That Contains Several Rps Genes. PLOS ONE 12(1): e0169950. https://doi.org/10.1371/journal.pone.0169950
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0169950

- Genetic map is based on recombination percentage; cM units.
- Physical map denotes actual physical distances in Kb (1,000 base pairs) or Mb (1 million bp) units.
- Relationship between the two maps varies across a chromosome; with more crossing overs in the euchromatic regions and much reduced number in the heterochromatic regions.
- 1 cM could be around 100 Kb or less in euchromatic regions; whereas, ~1-4 Mb or more in the heterochromatic regions.

Metacentric Chromosomes

Metacentric chromosomes have the centromere in the center, such that both sections are of equal length.

Submetacentric Chromosomes

Submetacentric chromosomes have the centromere slightly offset from the center leading to a slight asymmetry in the length of the two sections.

Acrocentric Chromosomes

Acrocentric chromosomes have a centromere which is severely offset from the center leading to one very long and one very short section.

Telocentric Chromosomes

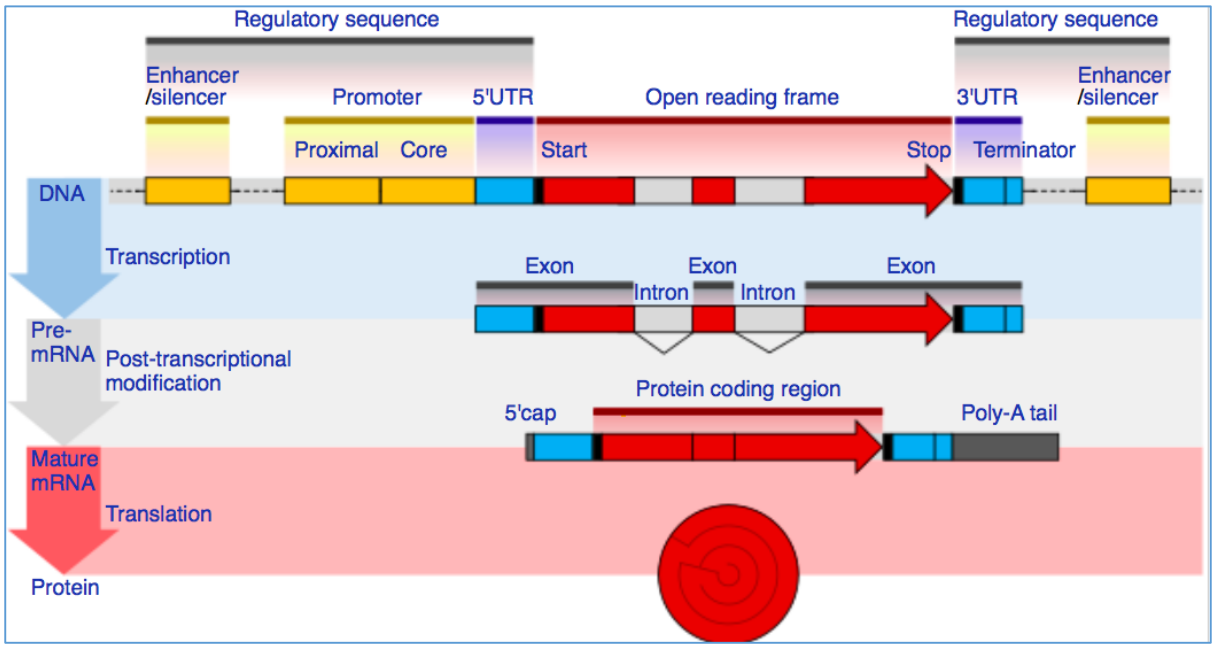
Telocentric chromosomes have the centromere at the very end of the chromosome.

Lecture

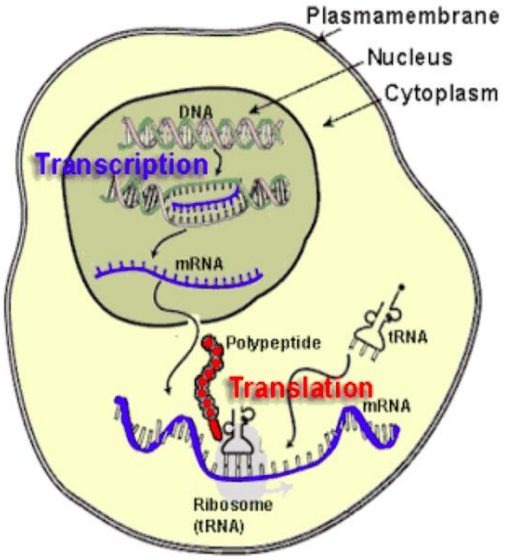
(Madan K. Bhattacharyya, Ph.D. Professor, Iowa State University)

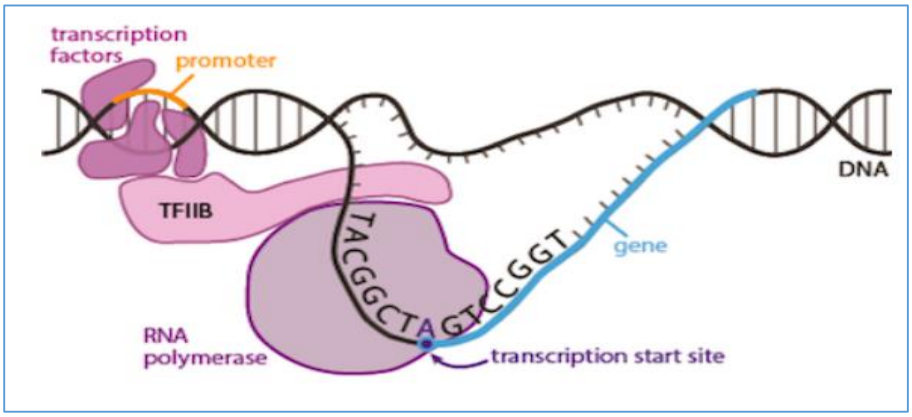
Molecular Genetics

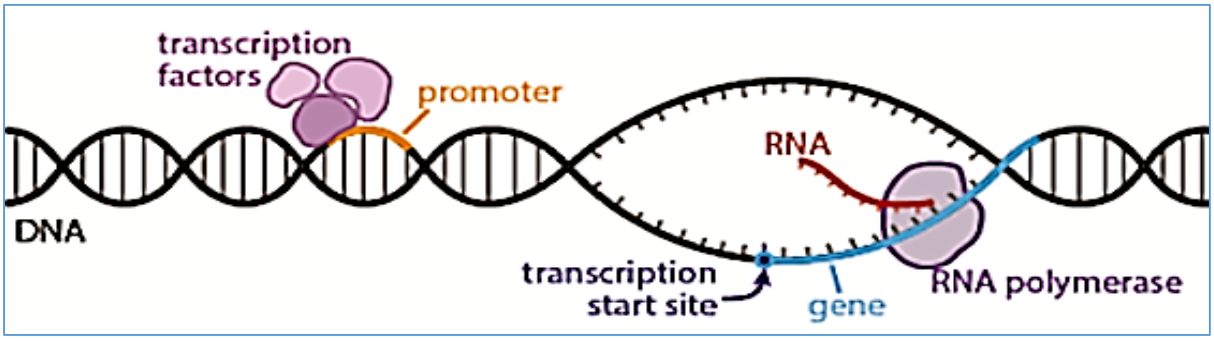
Gene Structure in Eukaryotes

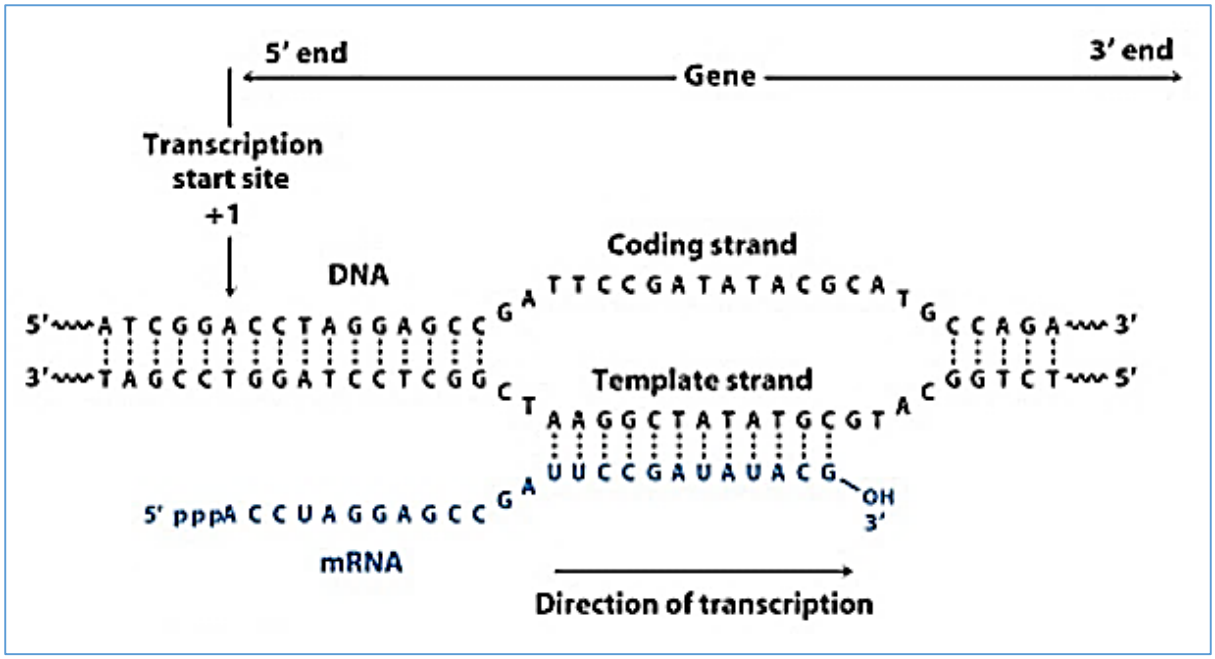


Transcription

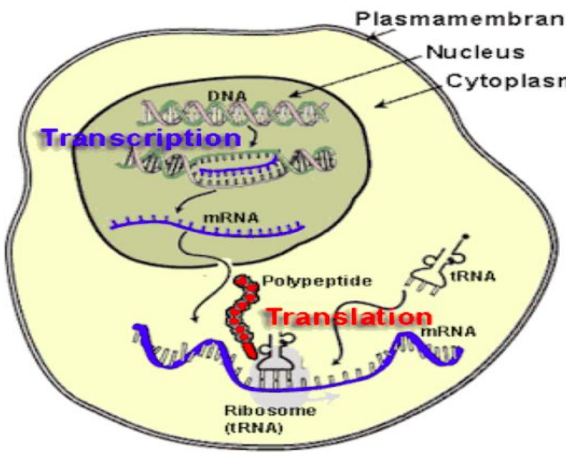


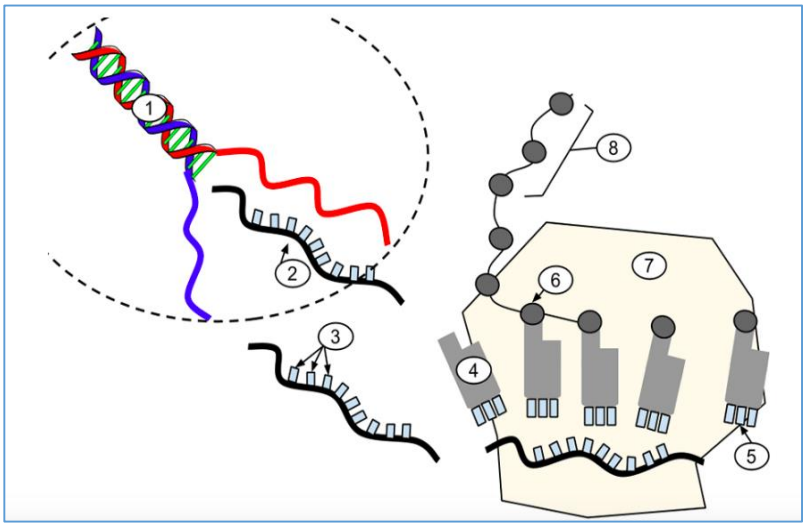


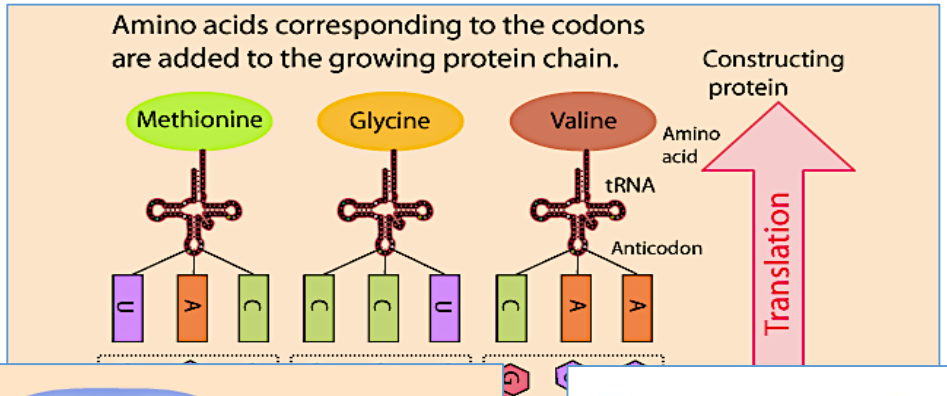


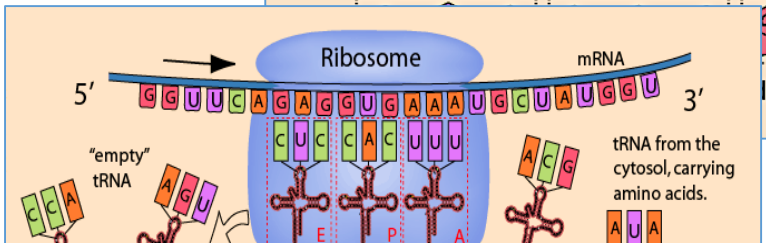


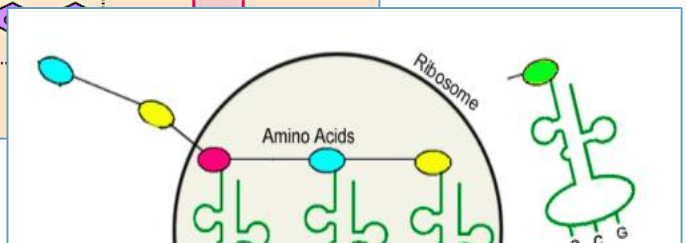
Translation





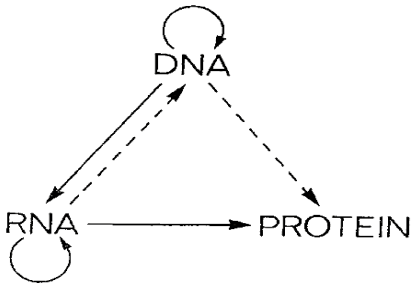






The Central Dogma in Molecular Biology

The **central dogma of molecular biology** is an explanation of the flow of genetic information within a biological system.

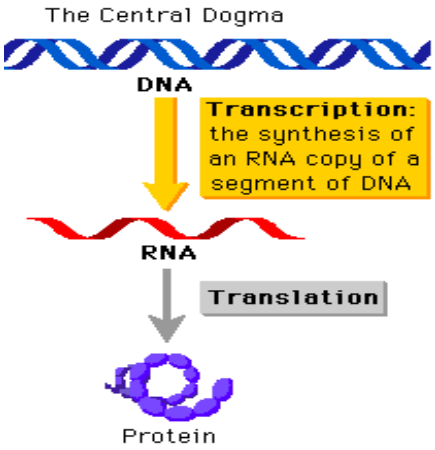


Francis Crick (1958, 1970)

"The central dogma of molecular biology deals with the detailed residue-by-residue transfer of sequential information. It states that such information cannot be transferred from protein to either protein or nucleic acid."

Francis Crick (1970)

Jim Watson (1965) in the first edition of The Molecular Biology of the Gene described this simple version.



What is a gene?

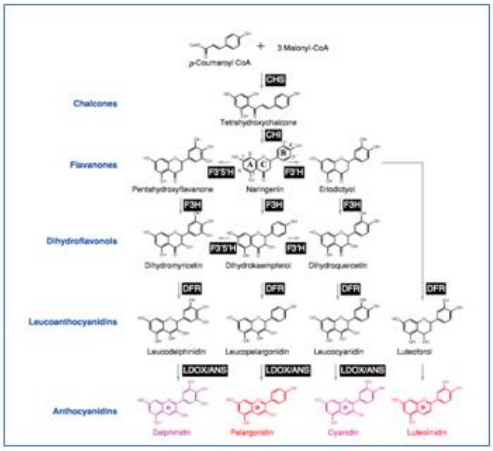
- The first mention of gene was made by Mendel.
- He explained his results in terms of **discrete inherited units (now known as genes)** that give rise to observable physical characteristics.
- In 1905, Johannsen introduced the term 'gene' and in 1908, Bateson termed genetics.
- Today you have learned that a gene, a DNA segment, contains information for a protein that mediates gene-function.
- This is the **central dogma of molecular biology** "DNA makes RNA and RNA makes protein."

Metabolites and Metabolic Pathway

Metabolites are closest to phenotypes

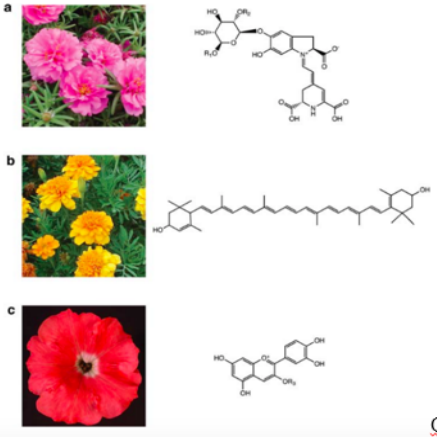
Genes

Biosynthetic pathway of four major classes of anthocyanins



Biosynthesis of the 4 major classes of anthocyanins (Grotewold, 2006). Names of compounds are indicated and enzymes, in black boxes, are as follows: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavanone 3'-hydroxylase; F3',5'H, flavanone 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX/ANS, leucoanthocyanidin dioxygenase/anthocyanidin synthase. **Grotewold E. 2006, Annu. Rev. Plant-Biol. 57:761-80.**

Metabolites provide the petal color phenotypes



Flower displaying the three major types of pigments and the corresponding structures.

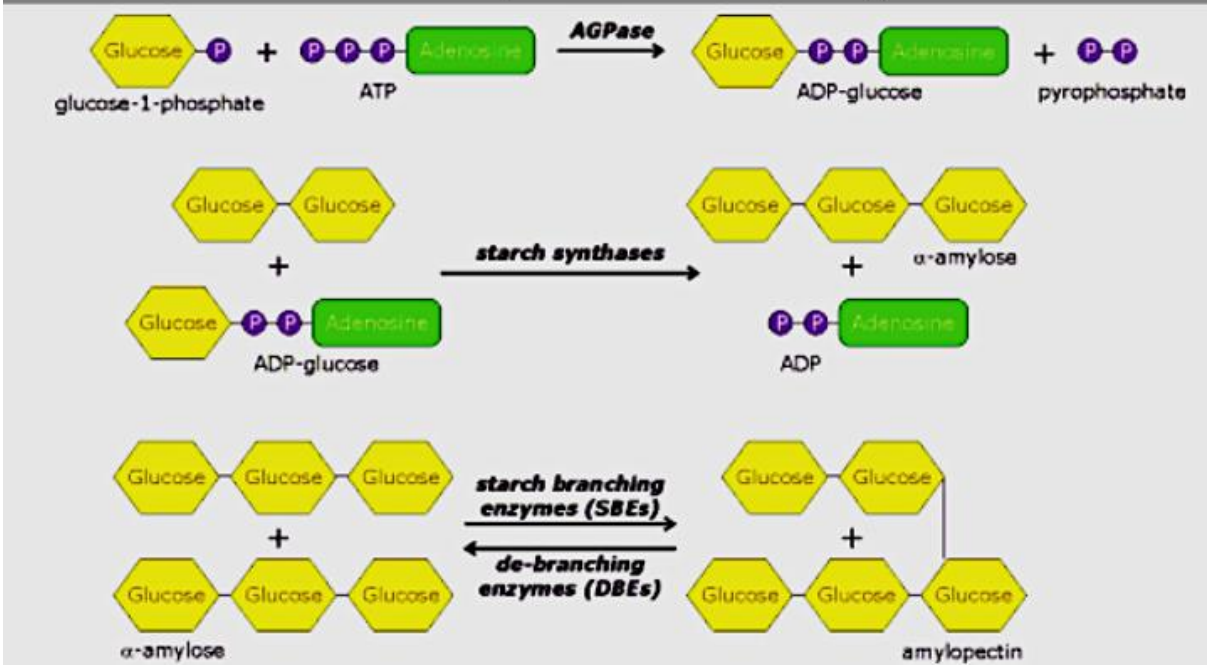
(*a*) Portulaca (*Portulaca grandiflora*) flowers accumulating primarily the **betalain** pigment, betanin (R1 = R2 = H).

(*b*) Marigold (*Tagetes patula*) flowers accumulating the **carotenoid** pigment, lutein.

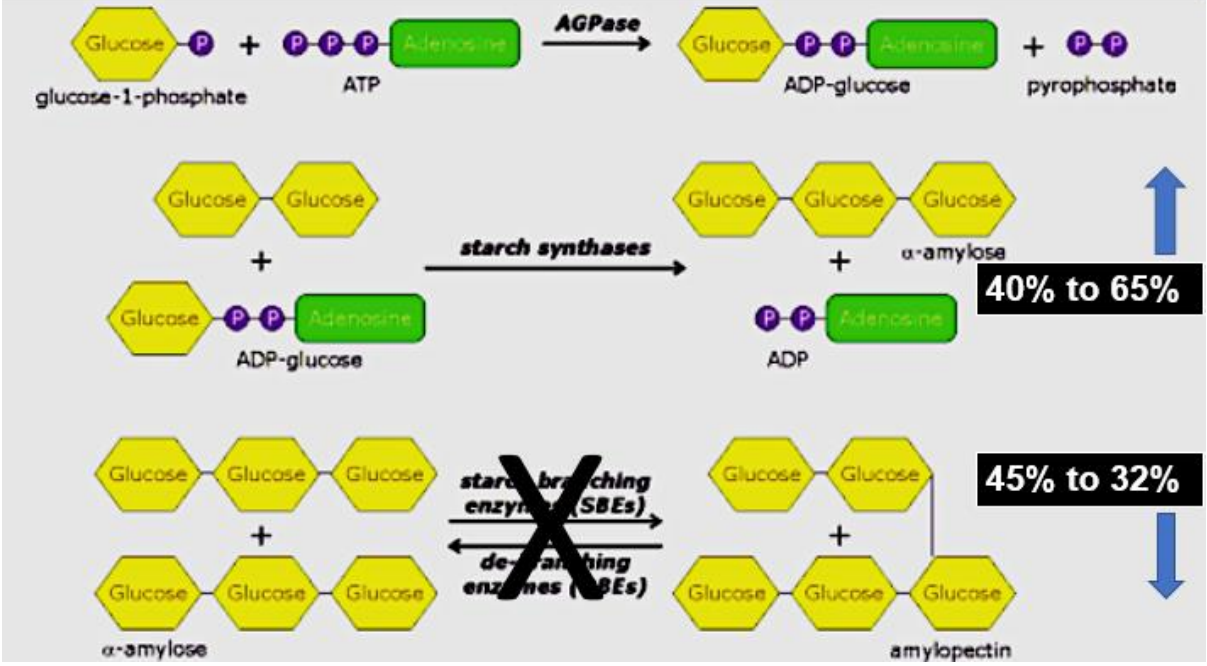
(*c*) Petunia (*Petunia hybrida*) flower accumulating an anthocyanidin, **cyanidin**.

Grotewold E. 2006, Annu. Rev. Plant-Biol. 57:761-780

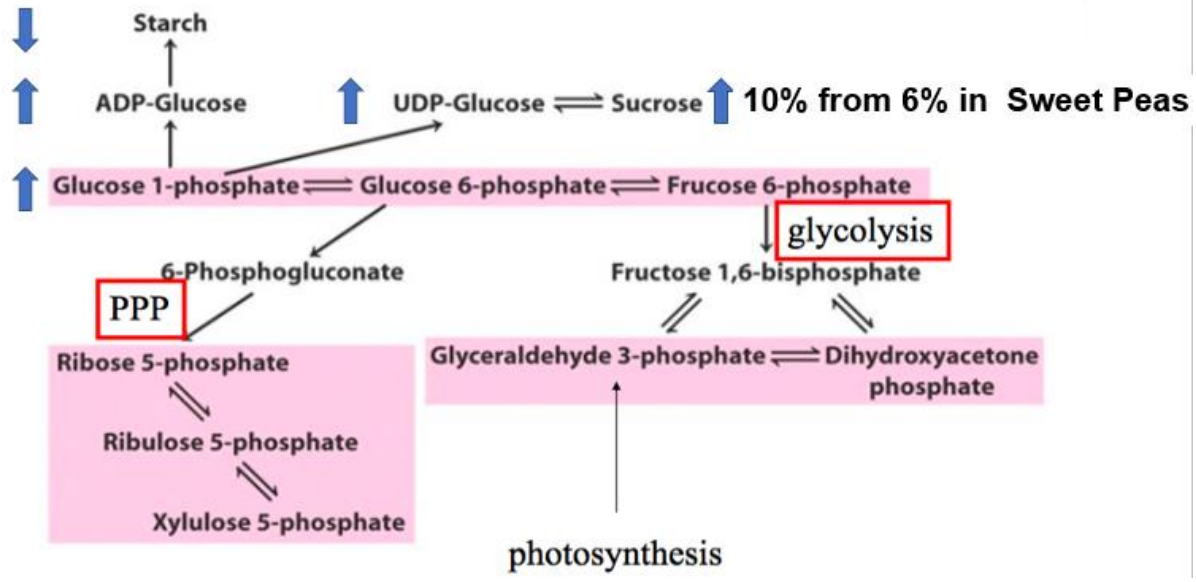
Starch Biosynthetic Pathway



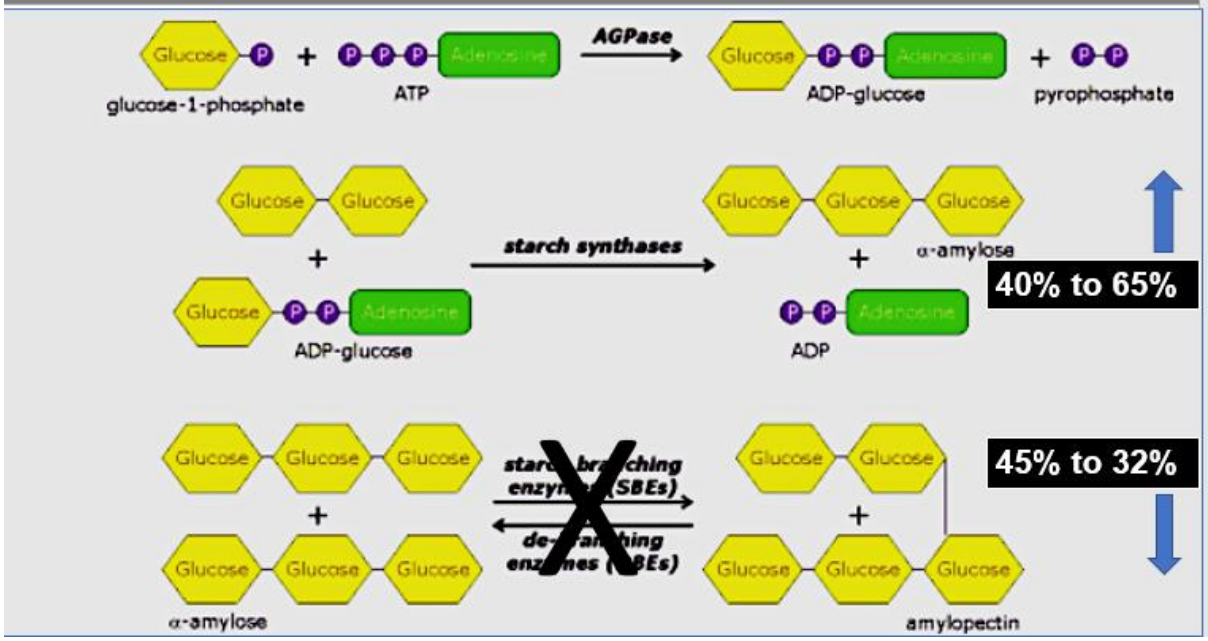
Starch Biosynthetic Pathway in *r* Pea Mutant



The *r* pea mutant

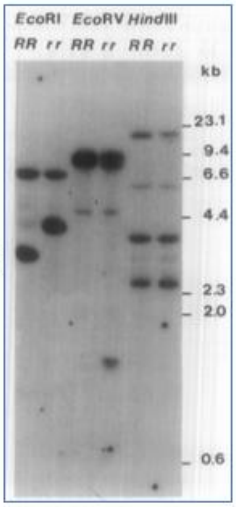


Starch Branching Enzyme I is Impaired in the *r* Pea Mutant

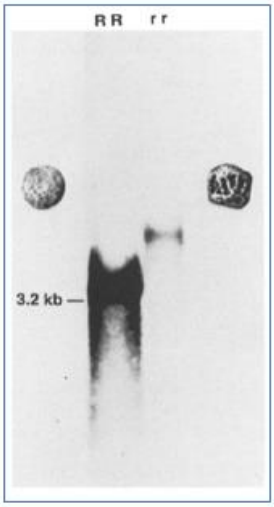


A transposon-like insertion caused mutation in a starch branching enzyme gene located in the *r* locus studied by Gregor Mendel.

DNA blot



Southern blot



RNA blot

Northern blot

Bhattacharyya et al. 1990

How does wrinkled seed phenotype develop in the *r* mutant?

Lack of SBEI

↓

Low Amylopectin and High Amylose

↓

Sugars Increased from ~ 6 to 10%

↓

Increased Osmotic Potential

↓

Higher Water Uptake During Seed Development

↓

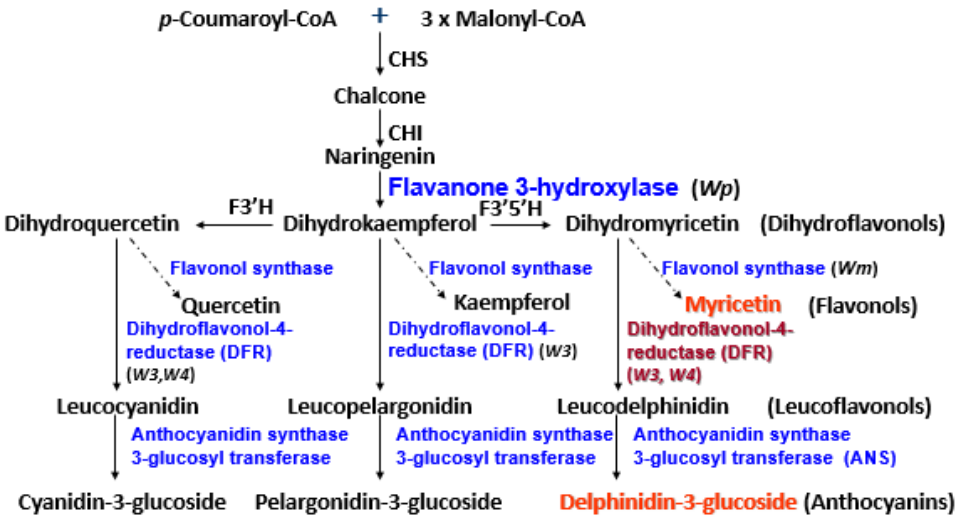
Cell Shrinking During Seed Maturation

↓

Wrinkled Seeds

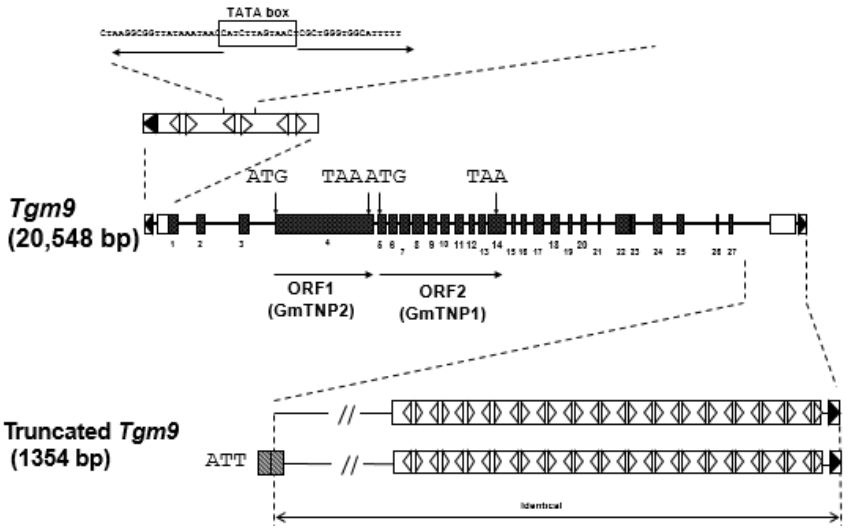
Bhattacharyya et al. 1993

The *W4* locus



Xu et al. 2010

A CACTA-type Transposable Element *Tgm9* in the *DFR2* Intron II causes loss of DFR2 function in Soybean.



Xu et al. 2010

What have you learned today?

- With new knowledge about genes available, the central dogma became irrelevant.
- Despite the information flow from DNA to RNA and then RNA to protein is universal; there are many exceptions discovered in later years.
- Gene does not work alone. A gene is a team player; work in a group. As you have seen, mutation in a single gene can have profound effect on phenotype.
- Wrinkled seed shape and sweetness in sweet peas are the result of a mutation in a starch branching enzyme gene leading to reduced amylopectin and increased amylose as well as sucrose contents.

- Variegated flowers in soybean is induced by excision of *Tgm9* from the *DFR2* gene, a member of the group of genes that control the anthocyanin biosynthetic pathway.

Plant Breeding Implications

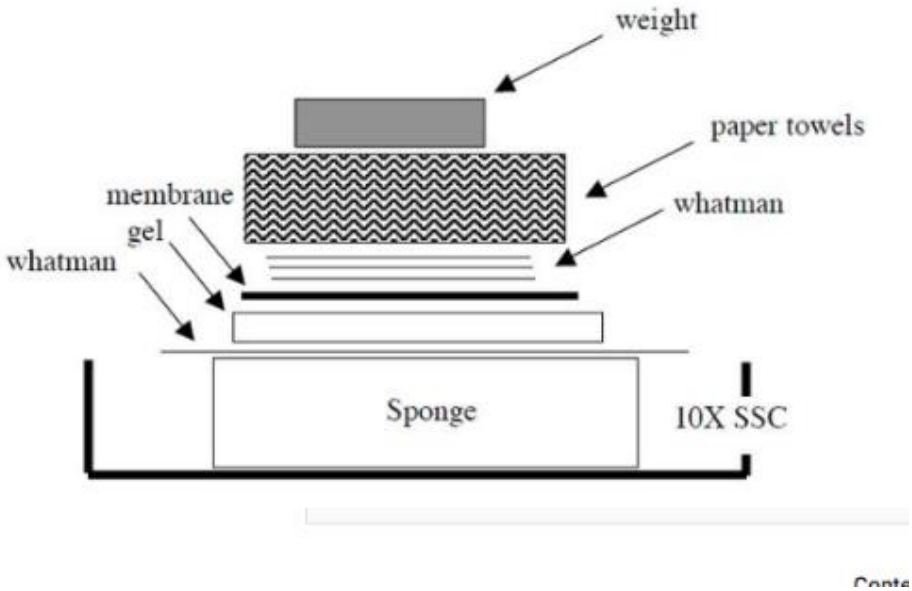
- Traits are governed by a large number of genes. Genes we target to breed in are usually regulatory genes that control the entire pathway.
- Disease resistance (*R*) genes are a good example. *R* genes are single genes; they however need a large number of genes to manifest the resistance.
- Two metabolic pathways shown here also show how structural genes are important in manifesting phenotypes.
- Mutants are the keys to studying the phenotypes.
- Metabolic markers are better predictors of phenotypes. Why not apply?
- Metabolite panels are used to check our health!

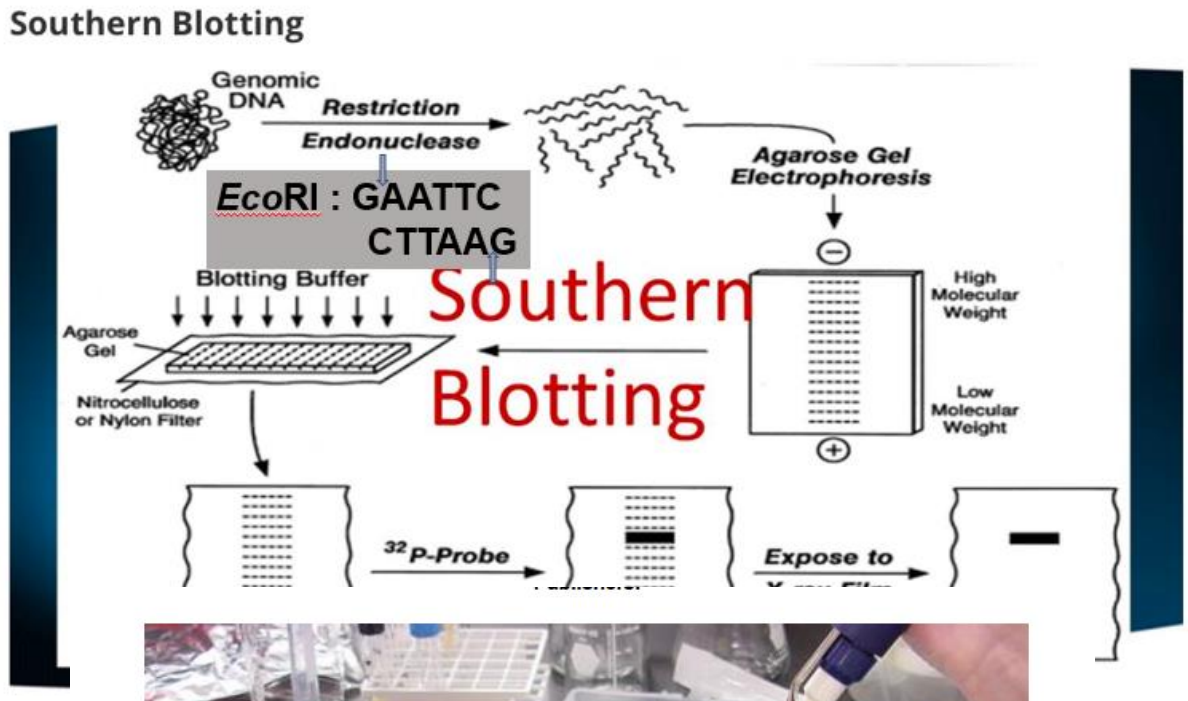
Lecture

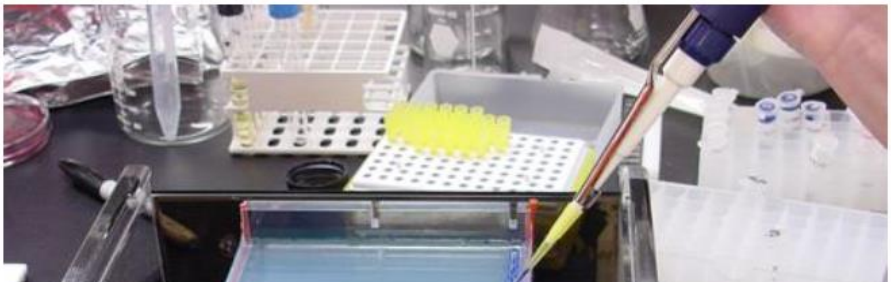
(Madan K. Bhattacharyya, Ph.D. Professor, Iowa State University)

Molecular Markers and Molecular Breeding

Southern Blotting







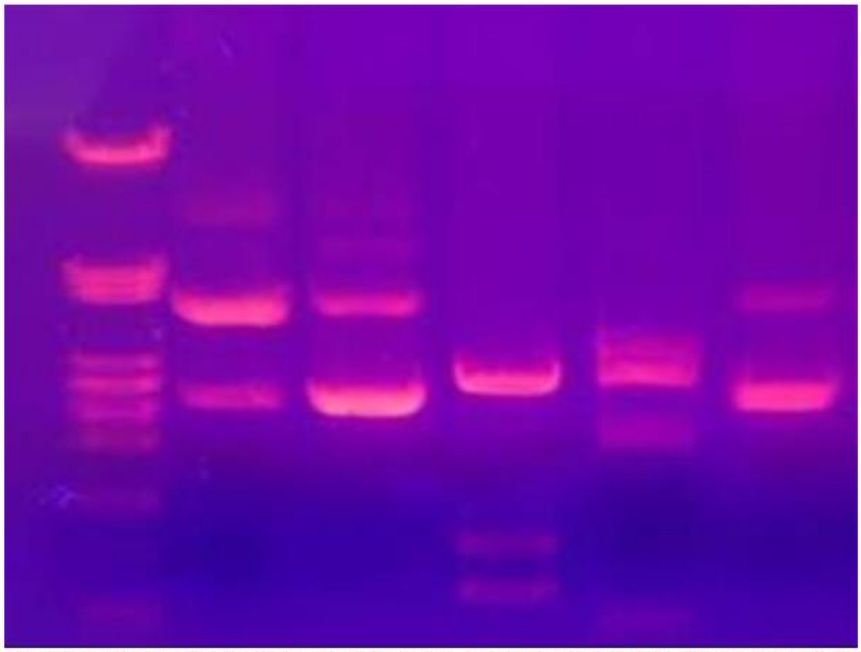
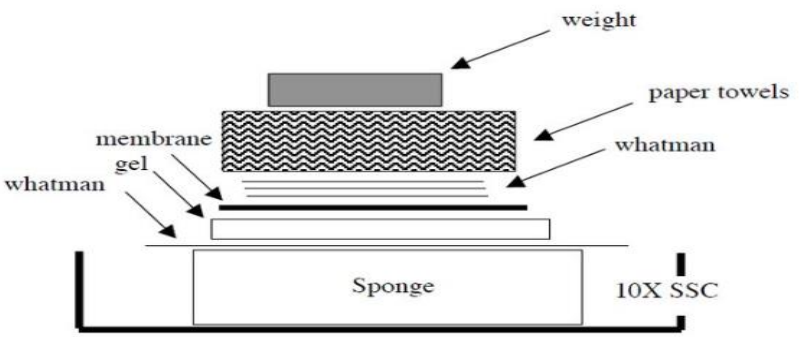


Fig 7.5 Visualization of PCR products on an agarose gel. A stain called ethidium bromide (EtBr) is added to the gel that binds to the DNA. When EtBr-treated DNA is exposed to UV light, it emits an orange-colored fluorescence.



RFLP gel showing DNA fragment length polymorphisms

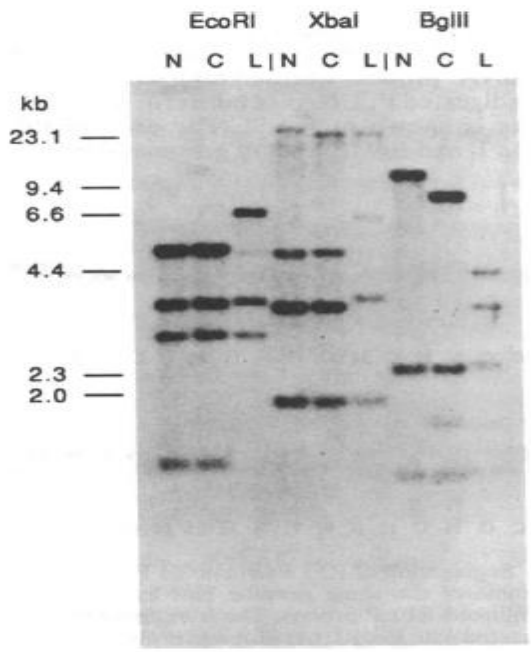
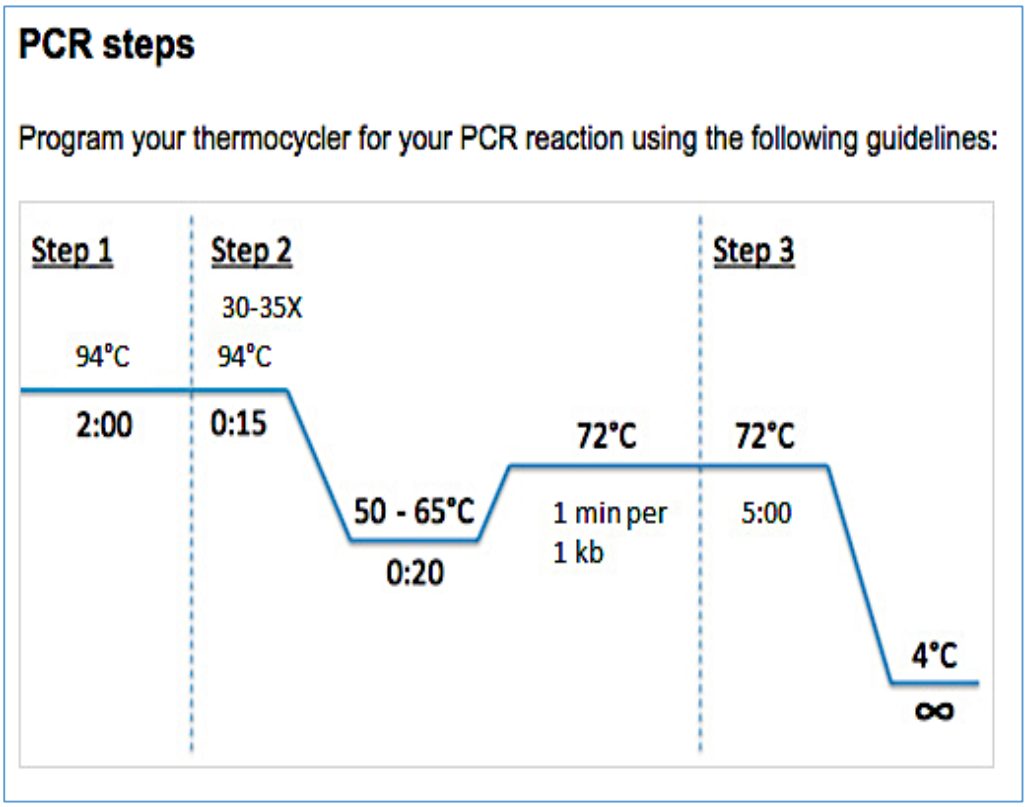
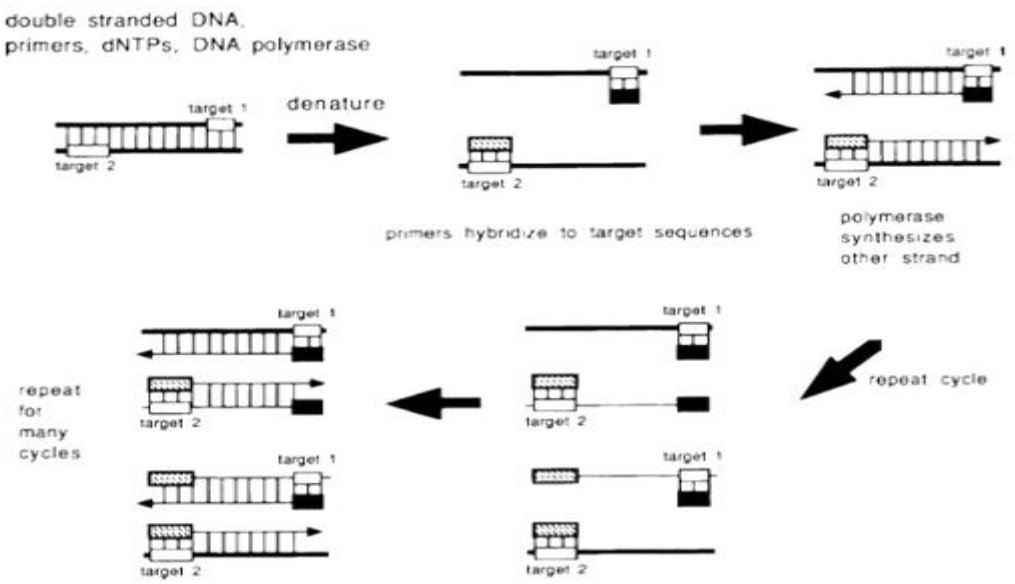
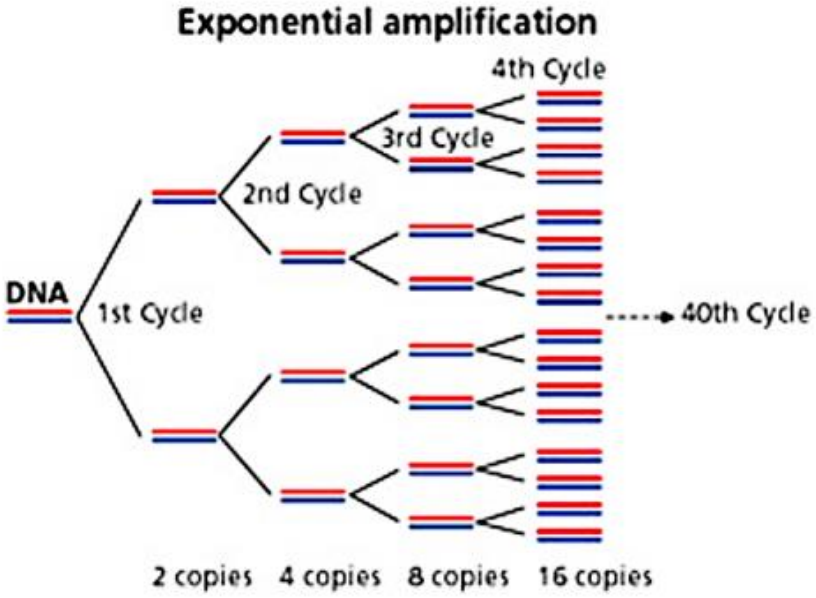


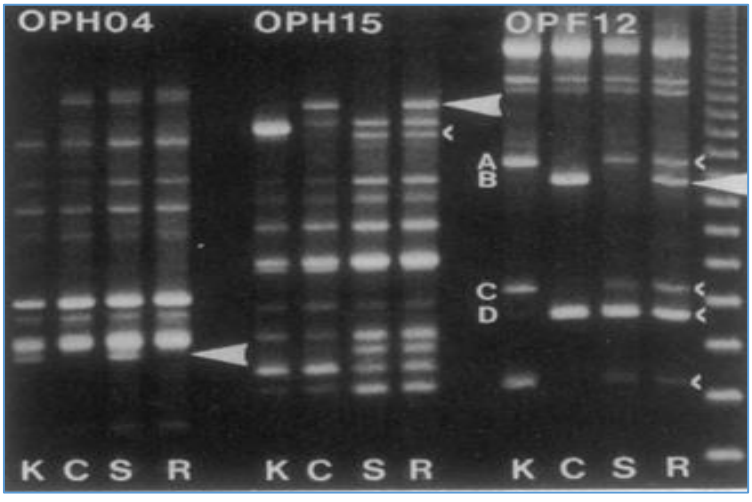
FIG. 1. Autoradiogram of a typical genomic blot to screen for RFLPs between different parental lines. The figure shows several RFLPs detected by clone 305 hybridized to Nd-0 (N), C (C), and La-0 *er* (L) DNA digested with *Eco*RI, *Xba* I, or *Bgl* II.

Polymerase Chain Termination Reaction (PCR)



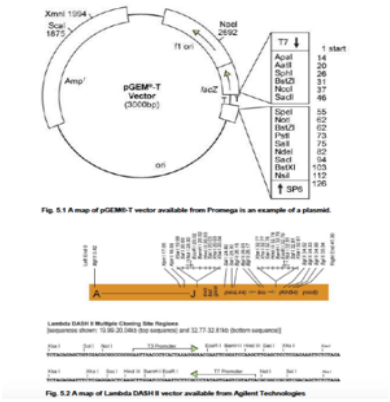

double stranded DNA,
primers, dNTPs, DNA polymerase
target 1
target 2
denature
primers hybridize to target sequences
polymerase
synthesizes
other strand
repeat cycle
repeat
for
many
cycles


Exponential amplification
4th Cycle
3rd Cycle
2nd Cycle
DNA
1st Cycle
40th Cycle
2 copies
4 copies
8 copies
16 copies


OPH04
OPH15
OPF12
A
B
C
D
K C S R
K C S R
K C S R

Recombinant DNA Technology

Plasmid (circular) and Lambda (linear) vectors for cloning DNA fragments.



Cut & Paste

- Cut with restriction endonucleases: say E*coR*I which cuts the GAATTC palindromic site.
- Same enzyme can be used to digest both the vector or DNA molecules so that DNA fragments will be compatible with the cut site released in the vector.
- Then ligate the molecules with T4 DNA ligase to paste the DNA fragments with the vector before transforming *E. coli.*

Complementary DNA (cDNA) molecules from mRNAs for cloning

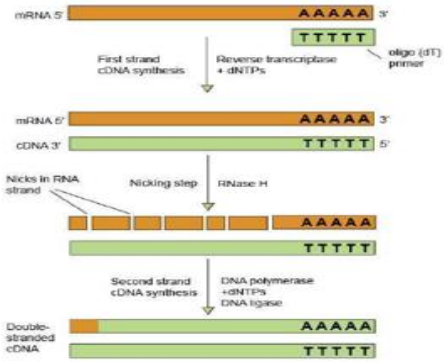
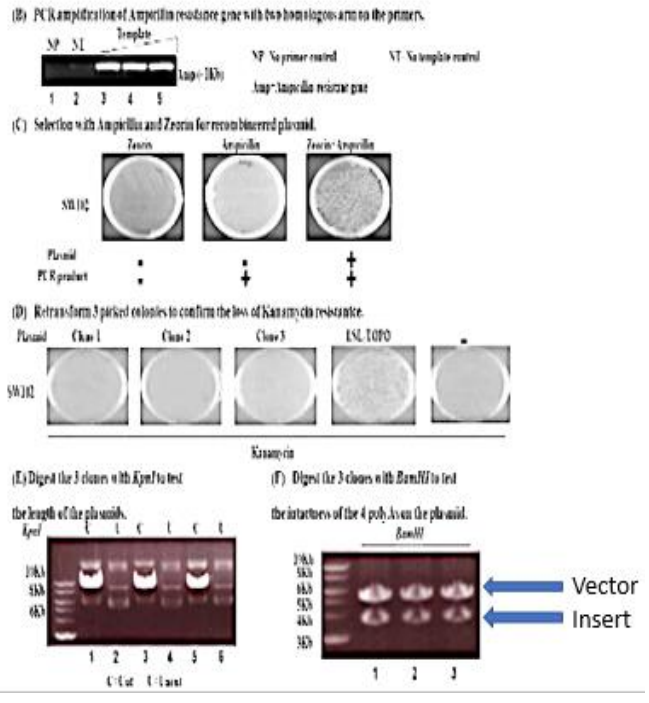
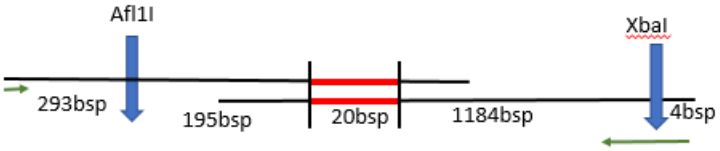


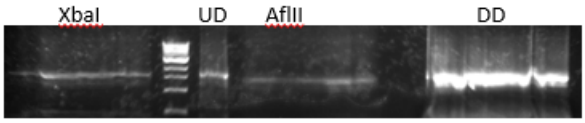
Fig. 5.5 Steps in the conversion of mRNA to double-stranded cDNA by reverse transcription in a test tube. A population of mRNA isolated from plant tissues is combined with oligo(dT) primers that anneal to the poly(A) tail of the mRNAs to initiate the reverse transcription of cDNA from mRNA template with dNTPs. The result is hybrid molecules (mRNA-DNA). Treatment with RNAse H causes degradation of the mRNA leaving an intact single stranded DNA (first strand). DNA polymerase synthesizes the second strand by adding complementary dNTPs to the growing chain. The reagents for RT usually come in pre-mixed kits making such an assay easy to carry out routinely in most laboratories. Most mRNAs from cells in tissues analyzed are converted to cDNAs. Thus, using primers specific to a sequence, that particular double stranded cDNA can be amplified by PCR for various purposes for example, gene cloning.

Cloning DNA fragments



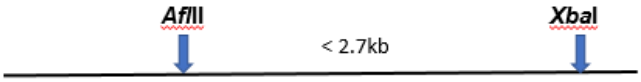
Double digestion (DD) of PCR products (Insert for Cloning)

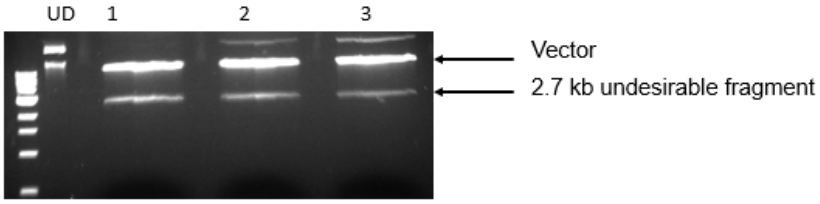




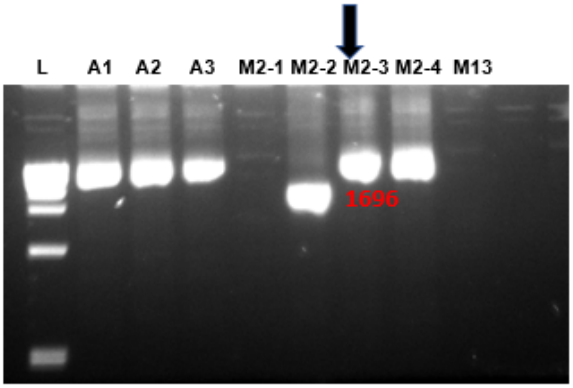
Insert before digestion: 1,696 bp
Insert after digestion: 1,399 bp

Double digestion of pHSE401 vector with *XbaI* and *AflII*



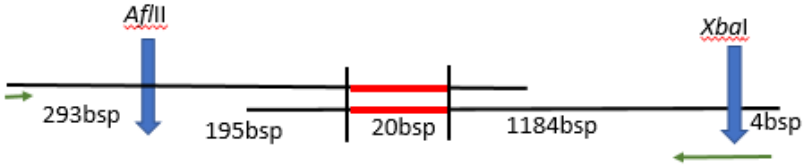


Colony PCR-MYB42g2-2

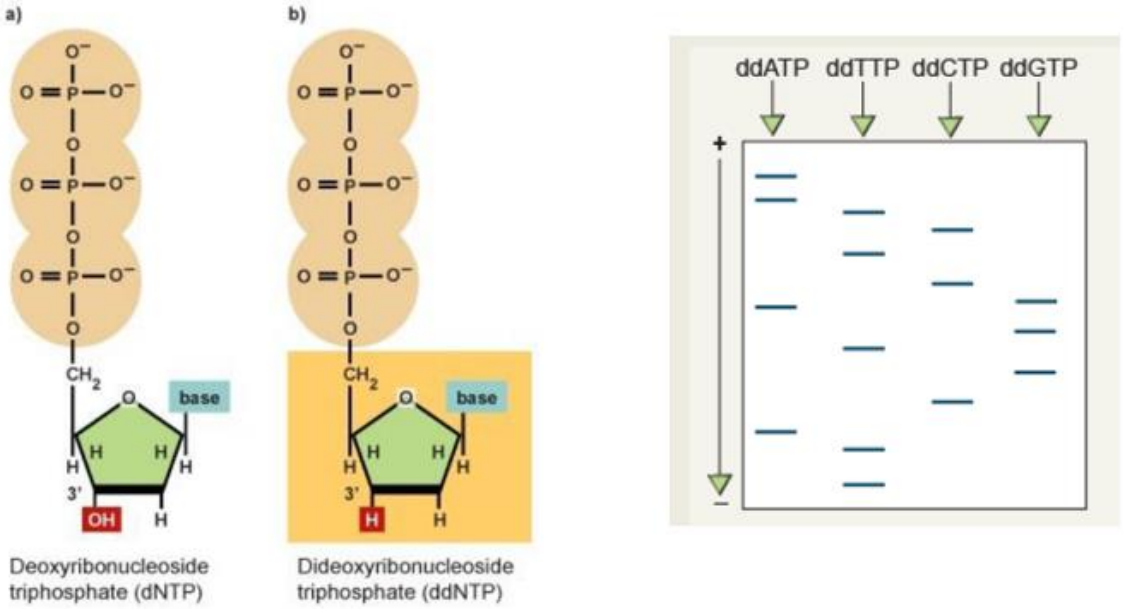


Sequence the clone just to make sure that there is no error.

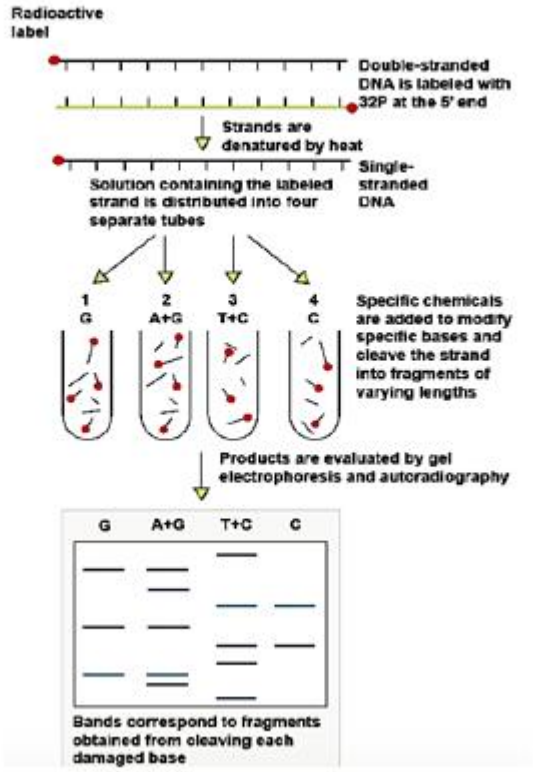
Using the green forward and reverse primers cloned inserts in E. coli were evaluated.



Sanger's Dideoxy DNA-Sequencing Procedure



Maxam & Gilbert DNA-Sequencing Procedure



Molecular Markers

RFLP: Restriction Fragment Length Polymorphism

RAPD: Random Amplified Polymorphic DNA

SSR: Simple Sequence Repeat

AFLP: Amplified Fragment Length Polymorphism

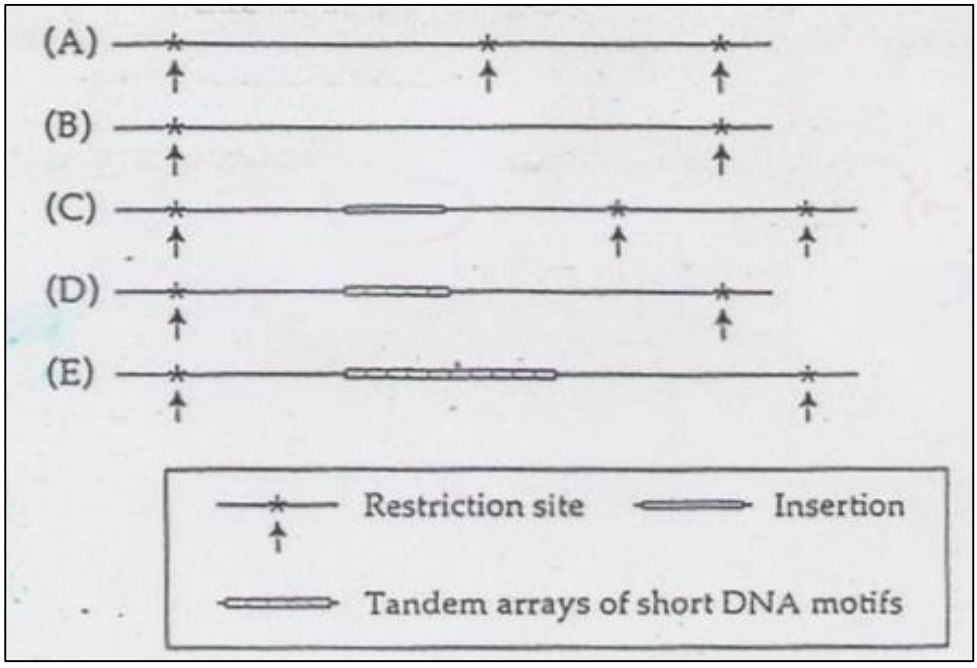
SNP: Single Nucleotide Polymorphism

GBS: Genotype by Sequencing

CAPS: Cleaved Amplified Polymorphic Sequences

SBP: Sequenced-based Polymorphic marker

Sources of RFLPs



RFLPs among three Arabidopsis ecotypes

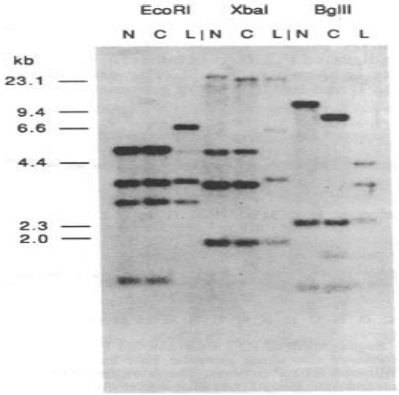


FIG. 1. Autoradiogram of a typical genomic blot to screen for RFLPs between different parental lines. The figure shows several RFLPs detected by clone 305 hybridized to Nd-0 (N), C (C), and La-0 *er* (L) DNA digested with *Eco*RI, *Xba* I, or *Bgl* II.

Segregation of two RFLP markers in F_3

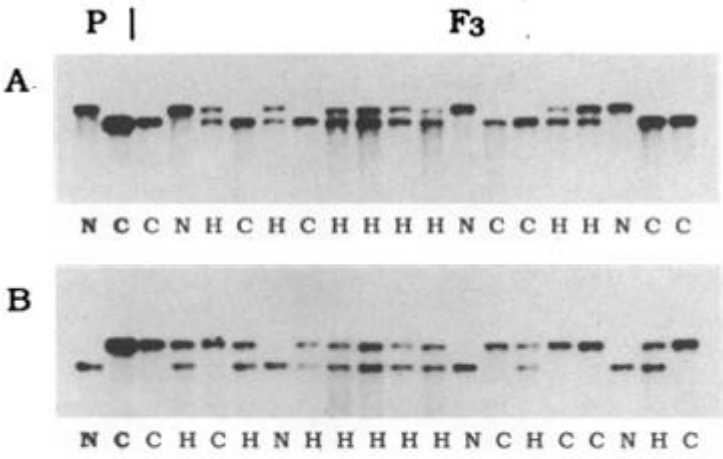


FIG. 2. Segregation of RFLPs in pooled F_3 progeny is shown in autoradiograms of the same genome blot hybridized sequentially with two unlinked RFLP probes. The blot contains genomic DNA samples digested with *Eco*RI. (*A*) Blot was probed with clone 322. (*B*) Blot was probed with clone 214. The first and second lanes contain DNA of the parental lines Nd-0 (N) and C (C), respectively. The 18 lanes to the right contain DNA of pooled F_3 progeny derived from the cross between Nd-0 and C, scored as either N, C, or H (heterozygous).

RFLP linkage map in Arabidopsis ecotypes

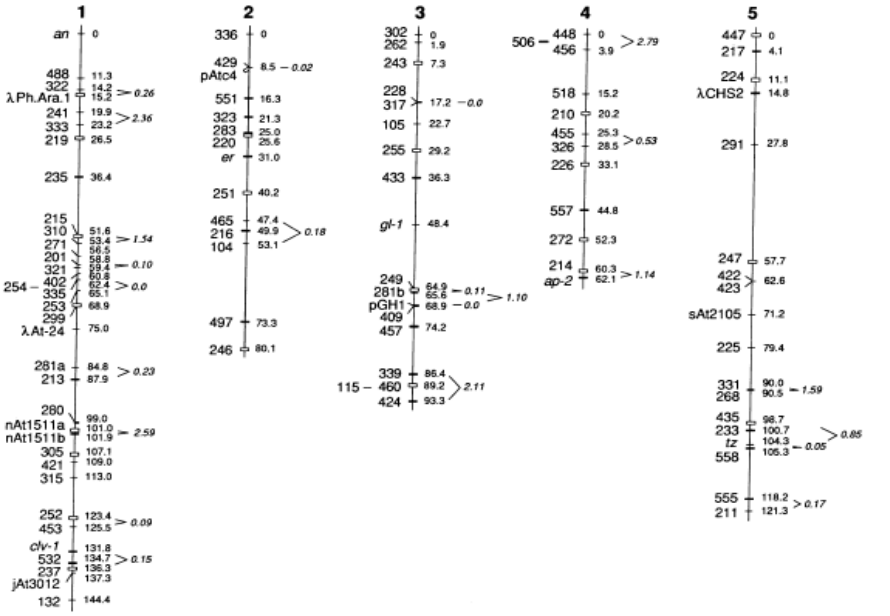
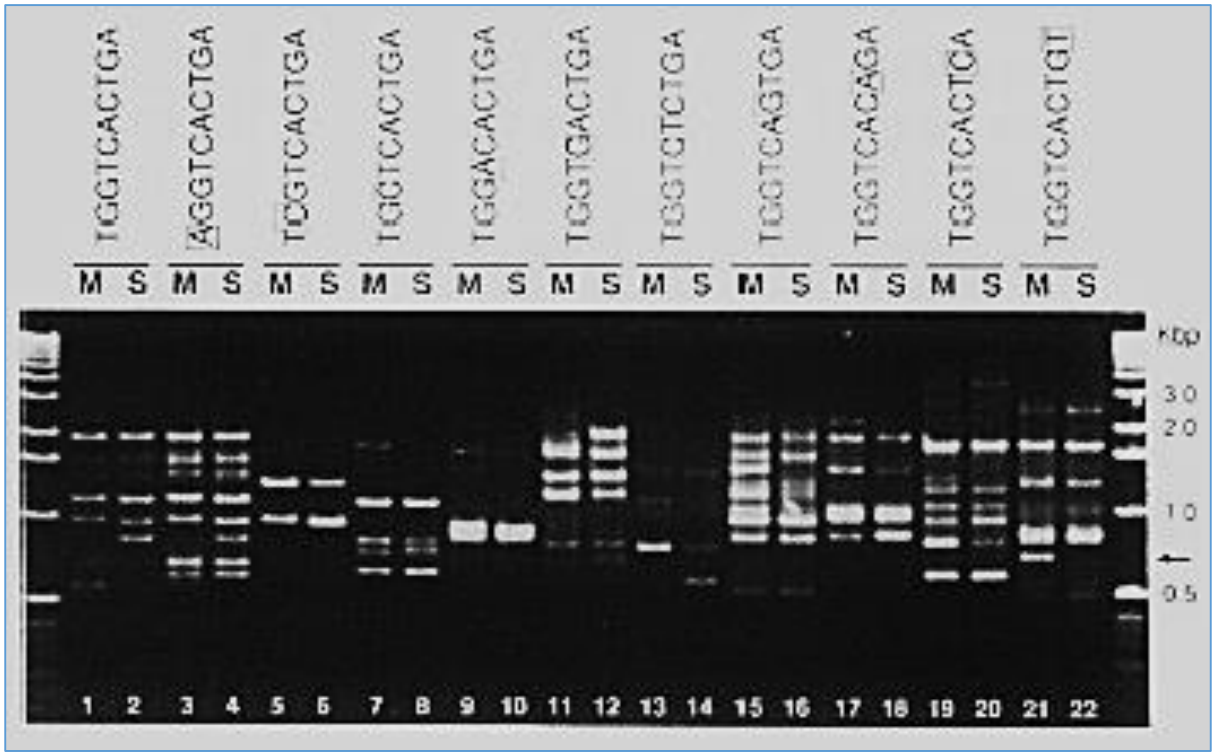


FIG. 3. An RFLP linkage map of the five chromosomes of the *Arabidopsis thaliana* genome. Map distances are shown in centimorgans. (We arbitrarily assigned a position of zero to the top-most marker on each chromosome.) Markers are designated by a clone number for the random low-copy-number clones and by a clone name for the known genes. The letter a or b after the marker name indicates that the clone detects more than one map locus. Markers scored in both crosses are indicated by an open box. Markers scored only in the cross between Nd-0 and La-0 are indicated by a solid box. The remaining markers were scored only in the cross between Nd-0 and C. Markers, placed in an order that is <1000 times more likely to have given rise to the data than any alternative order, are bracketed to the right of the map distances; the

Random Amplified Polymorphic DNA (RAPD)

A single 10 nucleotide oligo anneal in opposite orientation and amplifies DNA



Simple Sequence Repeat (SSR)

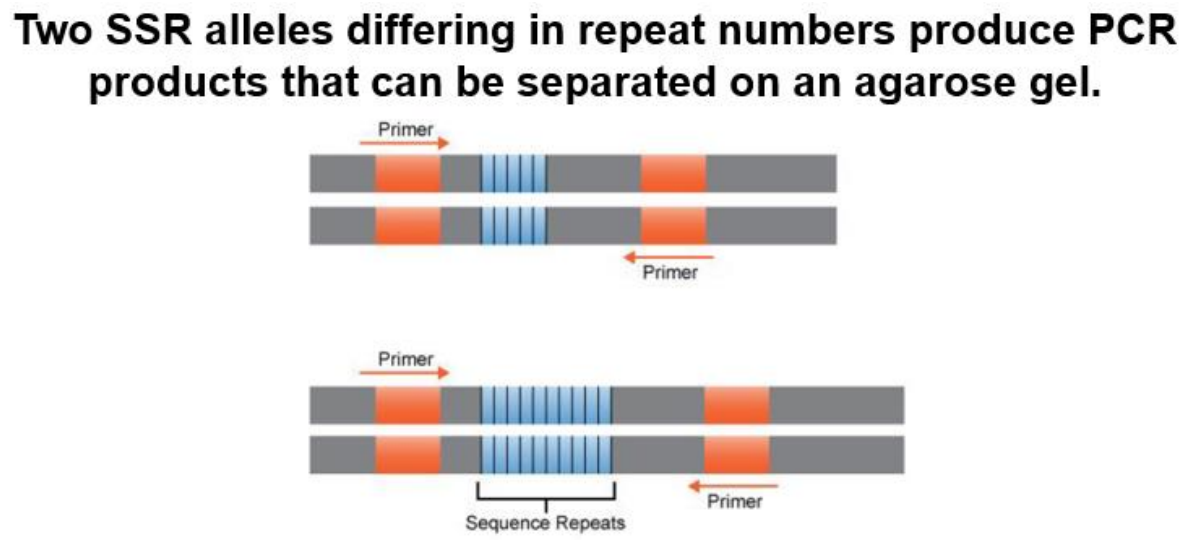


Fig 7.3 The SSR alleles 1 and 2 differ by the number of repeating DNA units. PCR primers are designed to amplify the region containing the DNA repeats. The difference in number of repeats in the two alleles causes a difference in the length of their PCR product, which can be distinguished when the PCR products are analyzed on a gel. An SSR marker should be closely linked to an allele for an important trait. Adapted from Chrispeels, M.J., & Sadava, D.E. (2003). Plants, Genes, and Crop Biotechnology. London. Jones and Bartlett Publishers.

SSR markers are usually co-dominant

Lane 10 and 25 carry the allele linked to the soybean aphid resistance *Rag1* gene in homozygous condition.

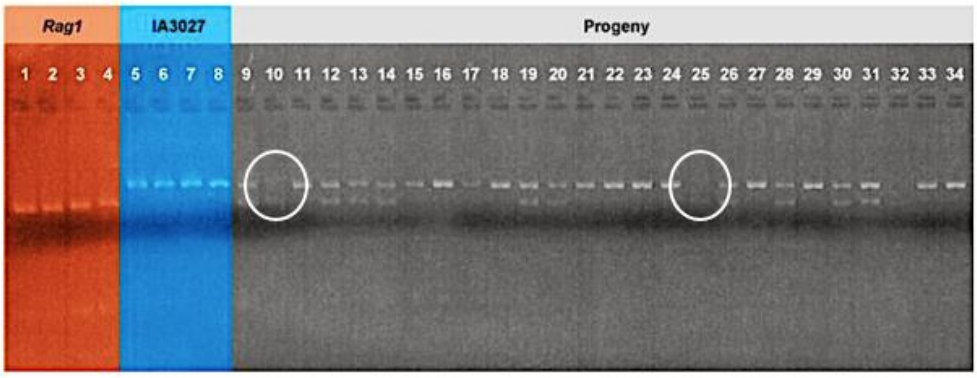
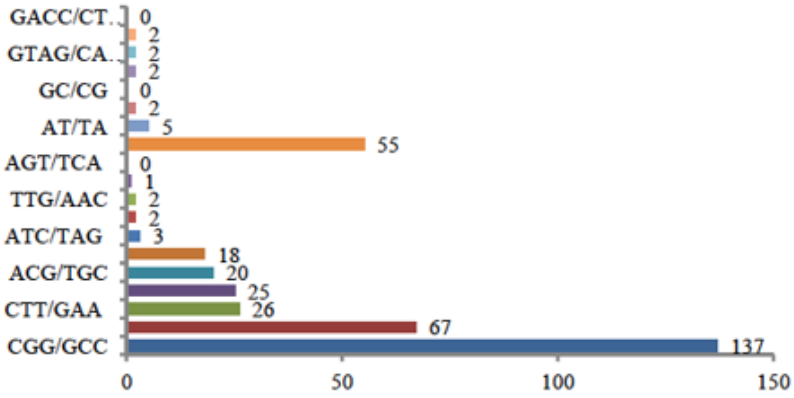


Fig 7.6B Molecular analysis of the *Rag1* allele. DNA bands for the *Rag1* parent that was homozygous for the 20 ATT repeats linked to *Rag1* (lanes1 to 4), for IA3027 that was homozygous for the 26 ATT repeats linked to *rag1* (lanes 5 to 8) and their progeny (lanes 9 to 34). Progeny homozygous for the SSR with 20 ATT repeats (lanes 10, 25, 32), progeny homozygous for the SSR with 26 ATT repeats (lanes 15-17, 21-24, 27, 33 and 34), and the heterozygous individuals with both SSRs (lanes 9, 12-14, 19, 20, 26, 28, 30 and 31).

SSR: Type and their frequencies in rice



P1: CGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCGG

P2: CGGCGGCGGCGGCGGCGGCGG (15 nt shorter)

Miah et al. 2013

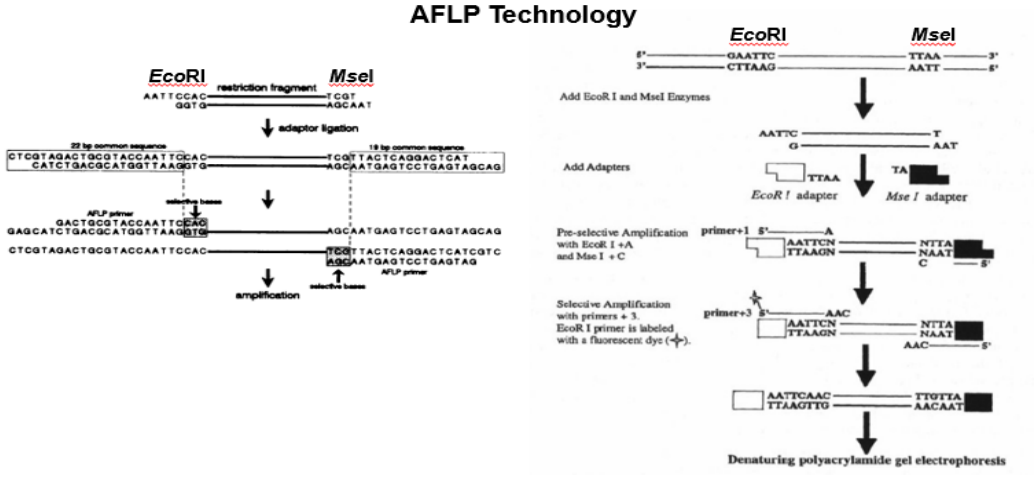
Table 5. Abundance of DNA markers discovered and developed in rice.

Crop	Genome size (MB)	RFLP	RAPD	AFLP	SSR	SNP
Rice	415–460 [b]	3,553 [b]	133 [b]	1,062 [b]	12,992 [b]	5,418,373 [a]

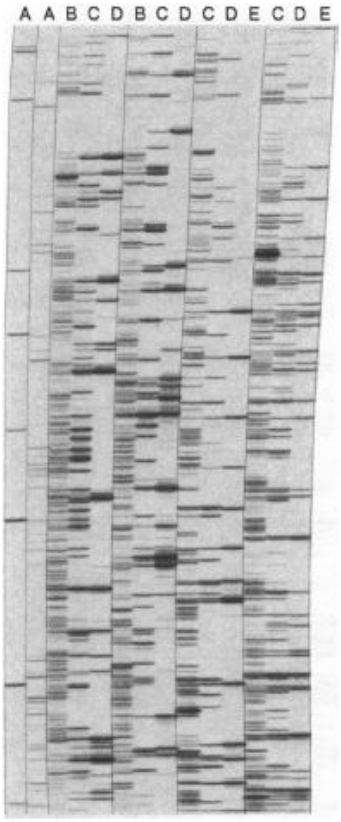
[a] www.ncbi.nlm.nih.gov; [b] Gramene web browser (http://www.gramene.org). Source: [82].

Miah et al. 2013

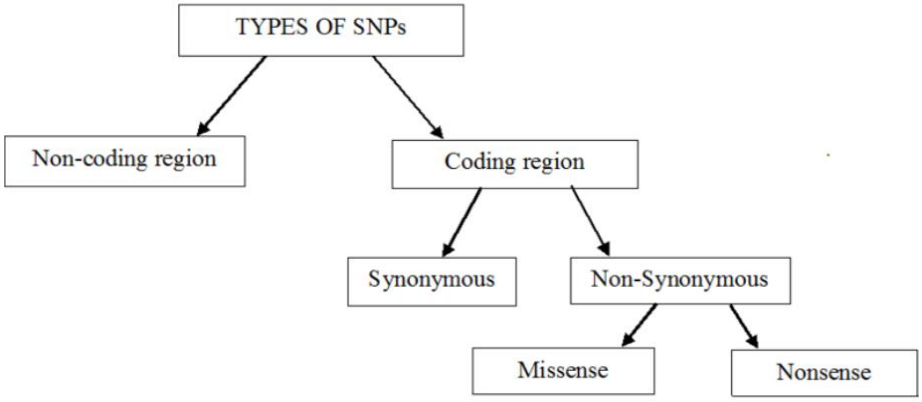
AFLP: Amplified Fragment Length Polymorphism



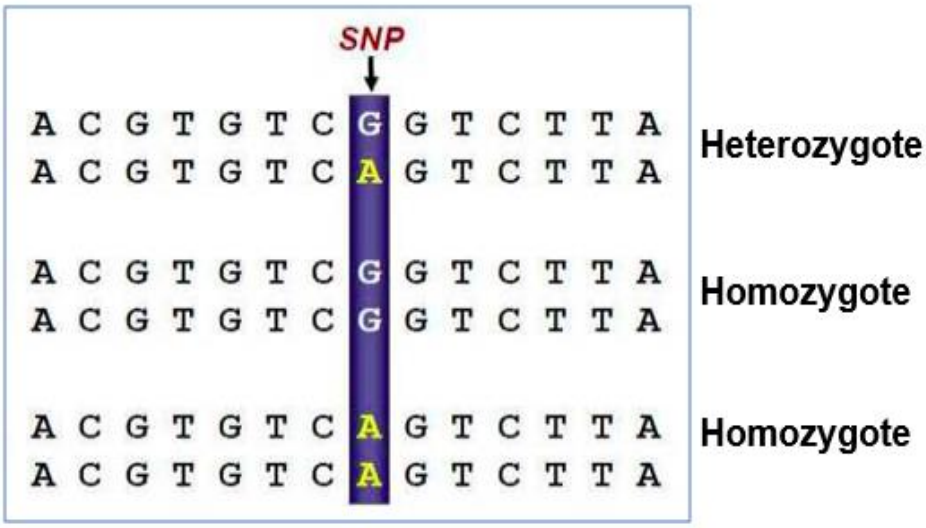
An AFLP Gel: Radioactive label is used to detect the PCR amplified fragments.



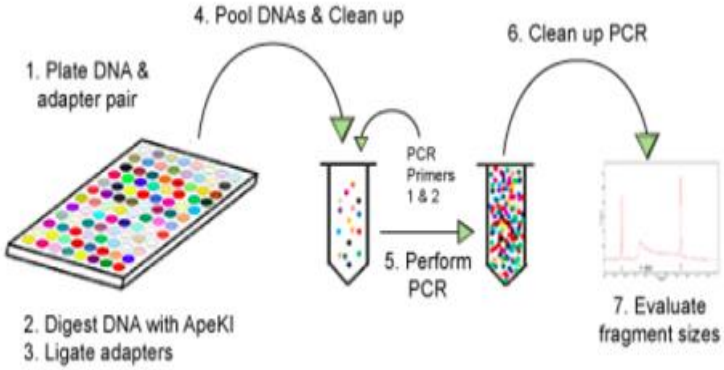
Single Nucleotide Polymorphism (SNP)



An example of SSR marker



Genotype by Sequencing



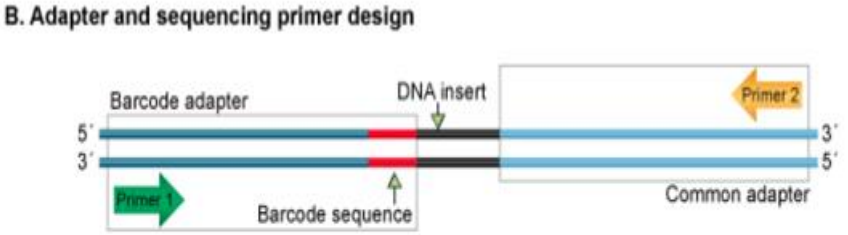


Fig. 9.1 Genotyping-by-sequencing in plants. (A) Library construction involves plating the DNA and adapter pair, digestion with a restriction enzyme (in other cases two restriction enzymes are used), and ligation of adapters to the ends of DNA fragments. Samples are pooled and cleaned up before PCR. The PCR products are also cleaned up and evaluated for quality by a DNA analyzer. (B) A barcode adapter and a common adapter flank the DNA insert to be sequenced. Primers 1 and 2 bind specific sequences on the 3' ends of the barcode and common adapters, respectively. Adapted from Elshire et al. (2011).

CAPS: Cleaved Amplified Polymorphic Sequences

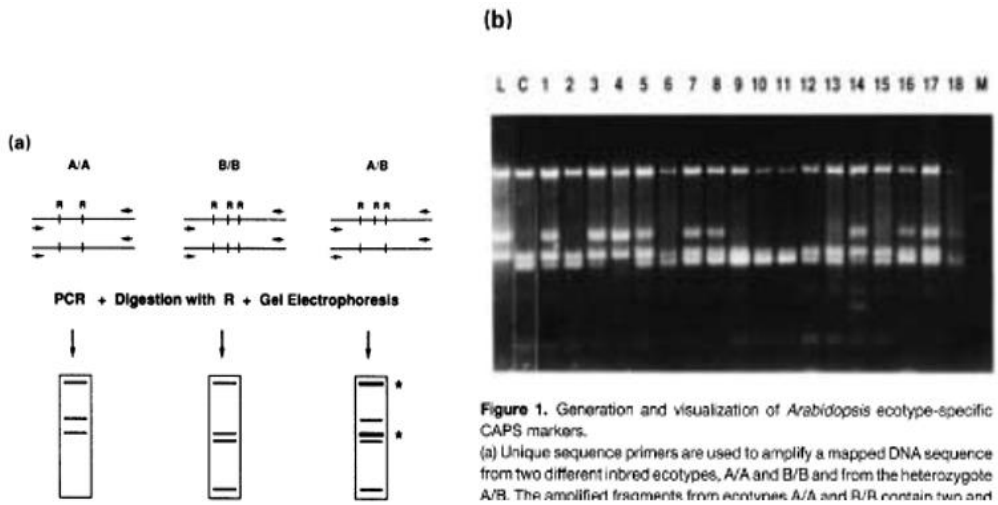


Figure 1. Generation and visualization of *Arabidopsis* ecotype-specific CAPS markers.
(a) Unique sequence primers are used to amplify a mapped DNA sequence from two different inbred ecotypes, A/A and B/B and from the heterozygote A/B. The amplified fragments from ecotypes A/A and B/B contain two and

SBP: Sequenced-based Polymorphic marker

Comparison of sequences from two Arabidopsis ecotypes.

```
Query: 4590 cggagcttccaaaaaagccataaacttcatccaatcttcttgcaaaaccaccacataccc  4649
            |||||||| || |||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1    cggagcttctaagaaagccataaacttcatccaatcttcttgcaaaaccaccacataccc  60

Query: 4650 tgccttatgtgtcca 4664
             |||||||| |||||
Sbjct: 61   agccttatgcgtcca 75

Query: 4581 ctcacagaccggagcttccaaaaaagccataaacttcatccaatcttcttgcaaaaccac  4640
            |||||||||||||||| || |||||||||||||||||||||||||||||| ||||||
Sbjct: 1    ctcacagaccggagcttctaagaaagccataaacttcatccaatcttcttgcataaccac  60

Query: 4641 cacataccctgcctt 4655
            ||||||||| |||||
Sbjct: 61   cacatacccagcctt 75

Query: 4561 ctcacggcggccaccgcagcctcacagaccggagcttccaaaaaagccataaacttcatc  4620
            |||||||||||||||||||||||||||||||||||| || ||||||||||||||||||
Sbjct: 1    ctcacggcggccaccgcagcctcacagaccggagcttctaagaaagccataaacttcatc  60

Query: 4621 caatcttcttgcaaa 4635
            |||||||||||||||
Sbjct: 61   caatcttcttgcaaa 75
```

SBP Markers that are polymorphic between Columbia -0 and Neiderzenz ecotypes

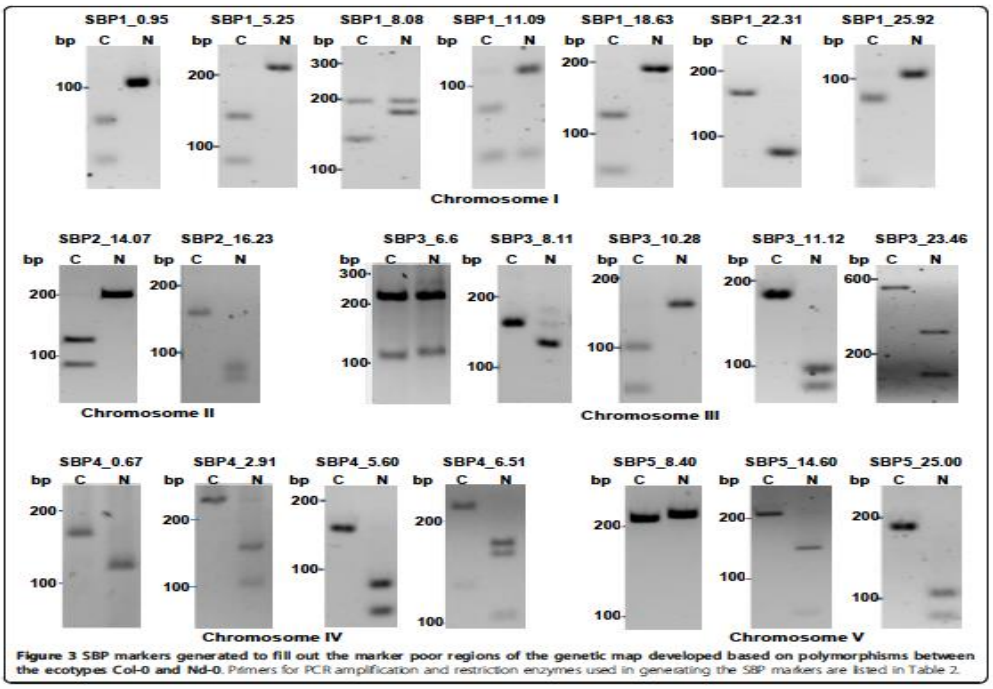


Figure 3 SBP markers generated to fill out the marker poor regions of the genetic map developed based on polymorphisms between the ecotypes Col-0 and Nd-0. Primers for PCR amplification and restriction enzymes used in generating the SBP markers are listed in Table 2.

Rapid Identification of Linked Molecular Markers

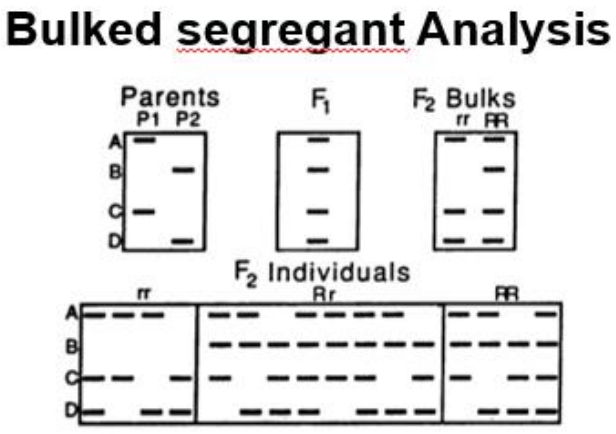


FIG. 1. Genetic basis of bulked segregant analysis. The schematic shows genotypes of four RAPD loci (A–D) detected by a single primer in two parents (P1 and P2), their F_1 and F_2 progeny, and bulks derived from F_2 individuals homozygous for resistance or susceptibility. The dominant allele at locus B is linked in cis to the *R* allele and therefore is polymorphic between the bulks. The other three loci that are polymorphic between the parents are unlinked to the resistance locus and therefore appear monomorphic between the bulks. This is an interpretation of the pattern obtained with primer OPF12 in Fig. 4.

Michilemore et al. 1991

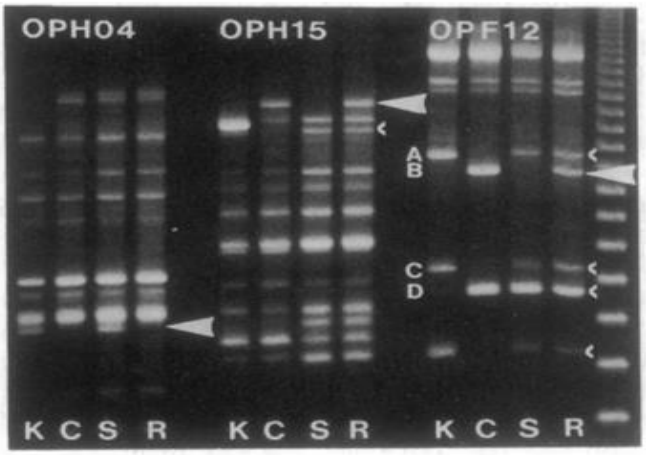


FIG. 4. RAPD markers detecting polymorphisms between bulks made for alternate alleles of *Dm5/8*. Each set of four lanes results from PCR amplification with a different 10-mer oligonucleotide primer: OPH04, OPH15, or OPF12. In each set, the first and second lanes contain parental DNA from Kordaat (K) and Calmar (C). The third lane contains bulked DNA from the homozygous susceptible individuals (S), and the fourth lane contains bulked DNA from the homozygous resistant individuals (R). The polymorphisms distin-

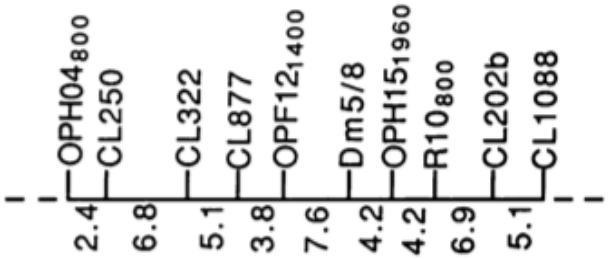
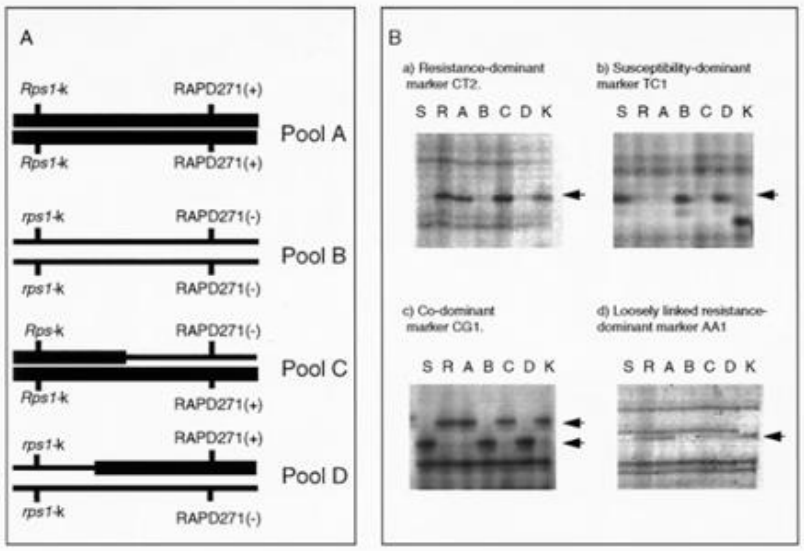


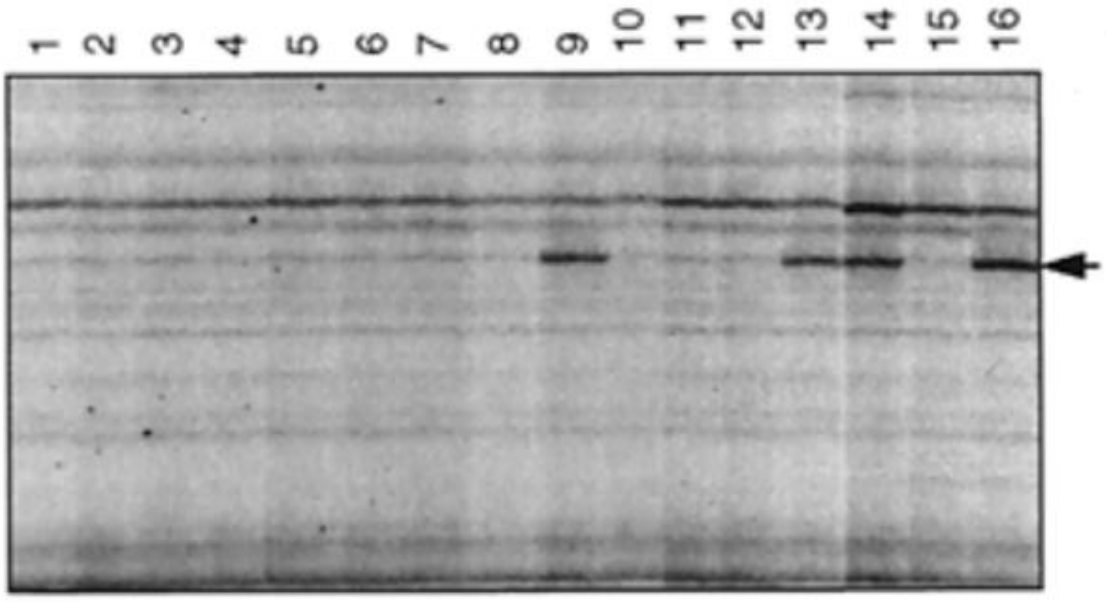
FIG. 5. Genetic map of the region of the lettuce genome containing *Dm5/8*. Three RAPD markers, $OPH04_{800}$, $OPF12_{1400}$, and $OPH15_{1960}$ were identified by bulked segregant analysis. The CL prefix designates a RFLP locus detected by a cDNA clone. Markers flanking this region have been described (5, 6). Genetic distances were derived by multipoint analysis and are shown in centimorgans. Pairwise recombination distances between the markers and *Dm5/8* are reported in the text.

BSA in Soybean for the *Rps1*-k gene

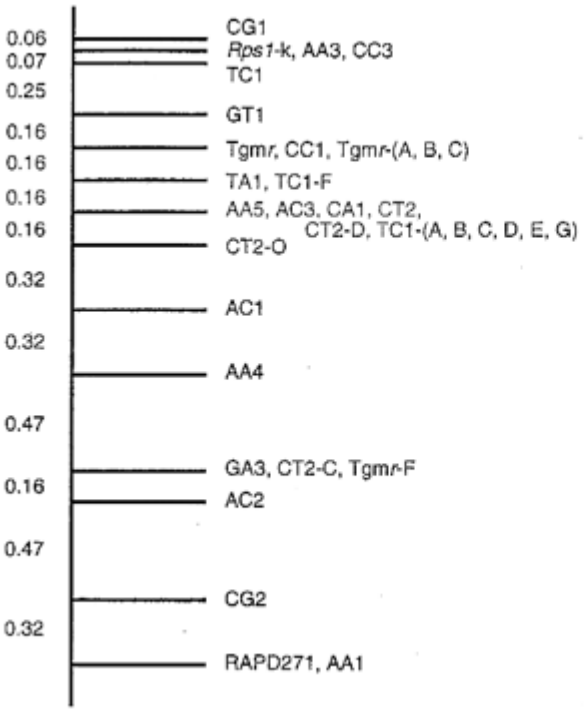


Kasuga et al. 1997

AFLP mapping of a marker CC1 linked to *Rps1*-k.



Mapping the *Rps1*-k gene that confers Phytophthora resistance in soybean



Kasuga et al. 1997

SHORE mapping to identify genetic loci (BSA)

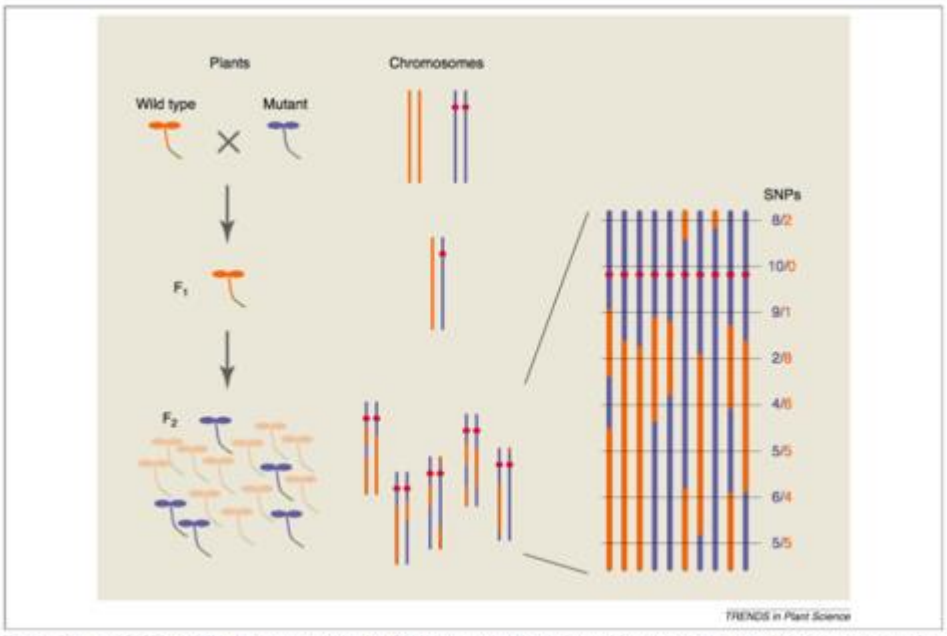
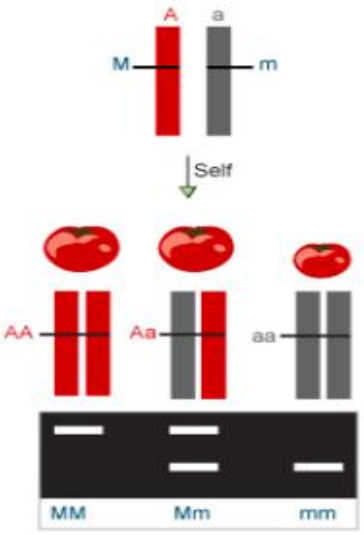


Figure 3. Diagram of SHOREmap [36] bulked segregant sequencing. Red indicates a mutation that is causal for the mutant phenotype. Note that a metric that reflects deviation from the expected 1:1 SNP ratio is needed to robustly identify the final mapping interval, rather than merely plotting raw SNP ratios.

Molecular marker assisted selection or breeding

Molecular marker linked tightly to the fruit size trait



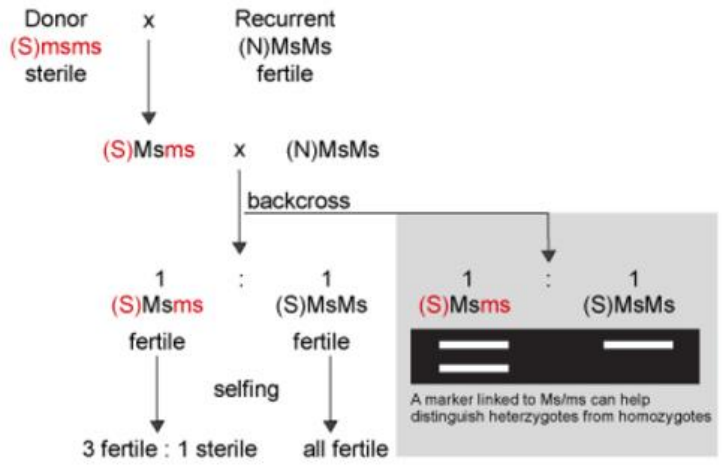


Fig. 8.16 The use of molecular markers for foreground selection. Backcross of (S)Msms to (N)MsMs produces fertile plants, but of different genotypes (Msms or MsMs). Selfing the MsMs BC1 progeny will produce all MsMs fertile plants. Selfing of BC1 Msms progeny will produce fertile and sterile plants in the ratio of 3:1. The use of a linked marker will help eliminate additional work to self and phenotypic screening of the plants.

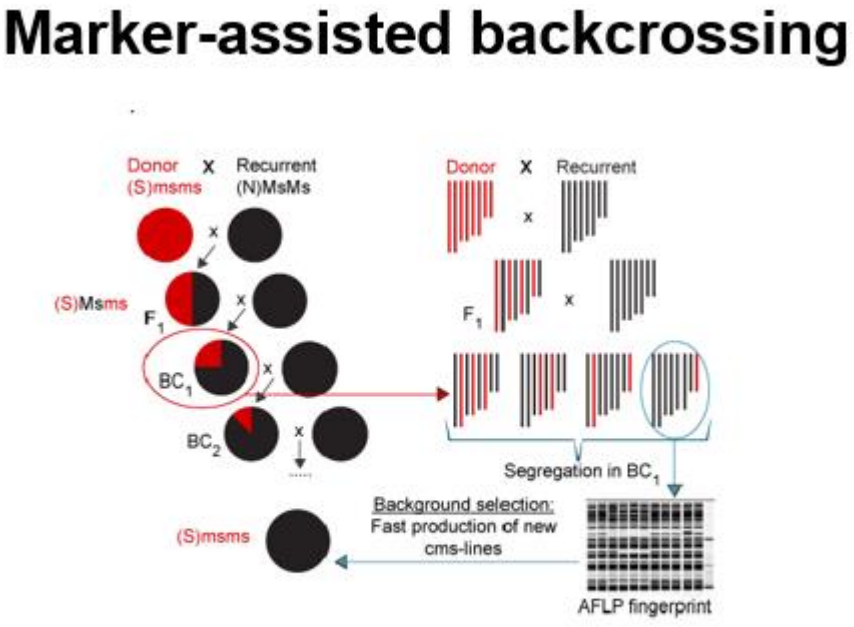


Fig. 8.15 Development of male-sterility by marker-assisted backcrossing in maize. A male sterile donor is crossed with a fertile recurrent parent. Red and black charts depict the proportions of donor and recurrent parent genomes respectively. Red and black bars depict chromosome segments of donor and recurrent parent respectively. Progeny containing largest proportion of recurrent parent genome can be detected as early as in the BC1 generation (red circle) using molecular markers and genetic fingerprinting (blue circle). Overall, the use of markers helps increase the pace of production of new male sterile lines.

Lecture

(Madan K. Bhattacharyya, Ph.D. Professor, Iowa State University)

Transgenic Technology

Recombinant DNA Technology, Cloning Vectors and Libraries

Plasmid (circular) and Lambda (linear) vectors for cloning DNA fragments

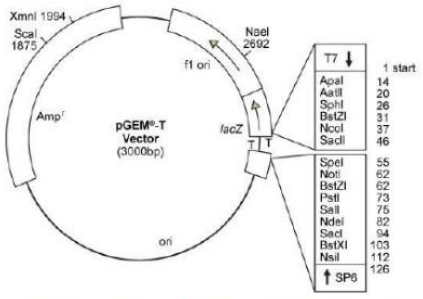


Fig. 5.1 A map of pGEM®-T vector available from Promega is an example of a plasmid.

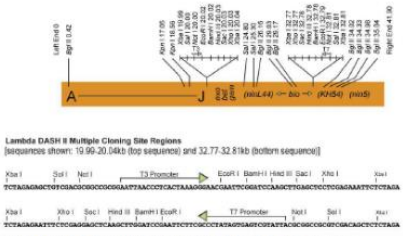
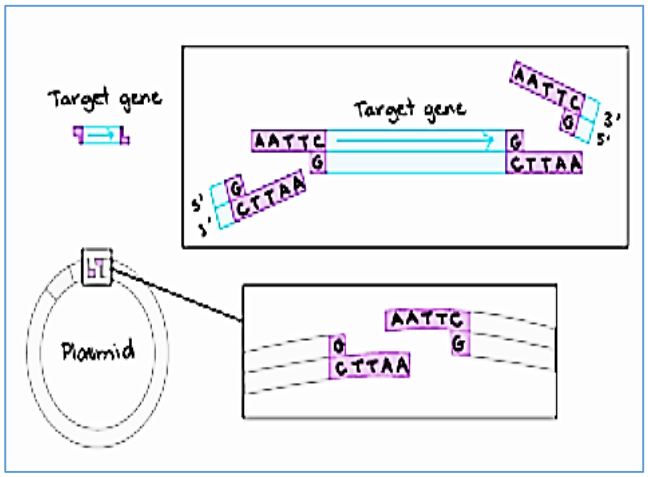


Fig. 5.2 A map of Lambda DASH II vector available from Agilent Technologies

Cut & Paste

- Cut with restriction endonucleases: say E*coR*I which cuts the GAATTC palindromic site.
- Same enzyme can be used to digest both the vector or DNA molecules so that DNA fragments will be compatible with the cut site released in the vector.
- Then ligate the molecules with T4 DNA ligase to paste the DNA fragments with the vector before transforming *E. coli.*

Cut & Paste: Cohesive-end ligation



Ligase Enzyme and Gene Cloning

- Cutting and joining of vector and DNA fragments of different origin results in recombinant DNA (rDNA) molecules. Recall that restriction endonucleases are used to cut DNA. To join DNA molecules together, an enzyme called DNA ligase is used. The enzyme DNA ligase is used to seal together restriction fragments by forming new phosphodiester bonds. The ligated vector and DNA fragment can now be transformed into a host cell for replication and expression.

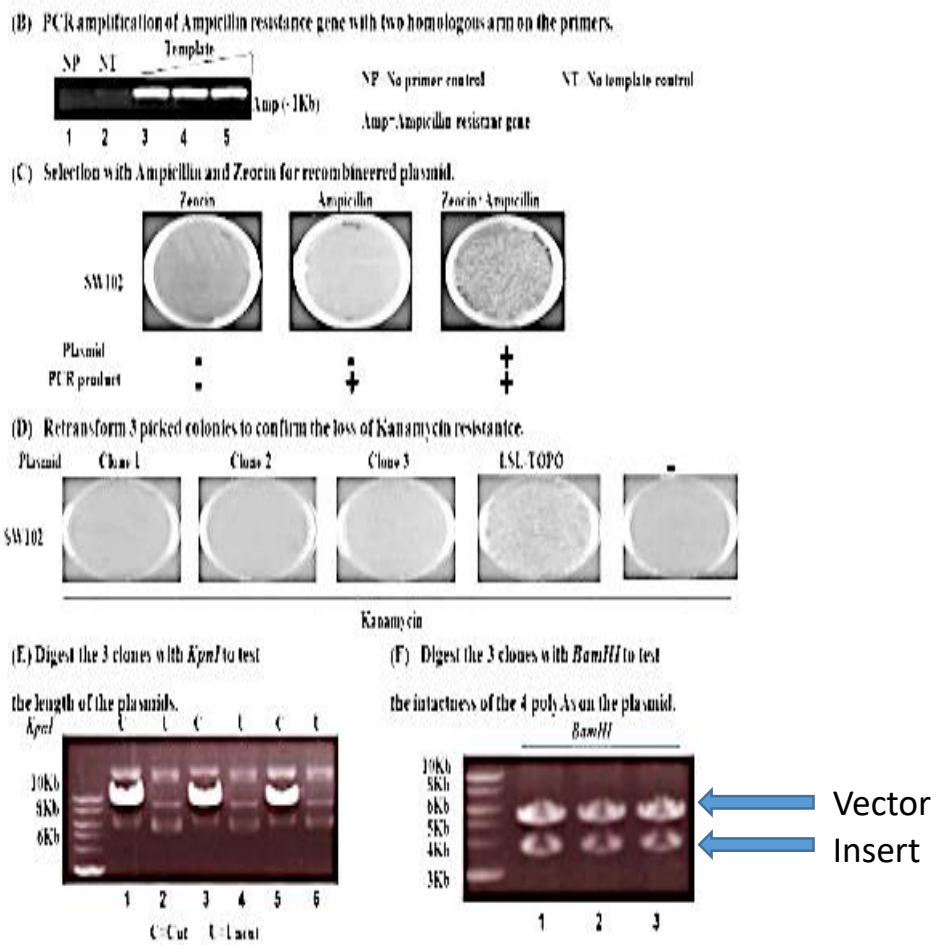
- Transformation of *E. coli* takes several steps. First, a gene of interest is inserted into a plasmid that contains a selectable marker usually encoding for resistance to an antibiotic. Second, the plasmid construct containing the gene of interest is transformed into bacterial

cells by briefly exposing the mixture of ligated plasmid-DNA fragment (rDNA molecule) and bacterial cells to cold (0^{o}C) and heat (37-42^{o}C). The next step is to grow the transformed cells on selection media containing an antibiotic. Only the cells that have been transformed with the plasmid containing the gene of interest and the marker for resistance to the antibiotic will survive.

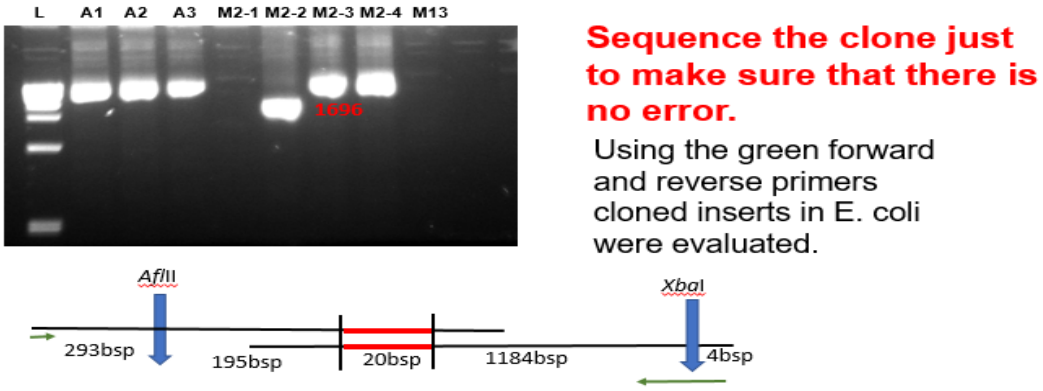
- In addition to using an antibiotic, plasmid vector systems that contain the *lacZ* gene encoding β-galactosidase allow for easier selection of positive colonies that may harbor the rDNA molecule of interest.

White colonies most likely carry the insert because *lacZ* function is disrupted to produce blue color

Cloning DNA fragments



Colony PCR-MYB42g2-2



Cloning Vectors

Cloning Vector Definition and Requirements

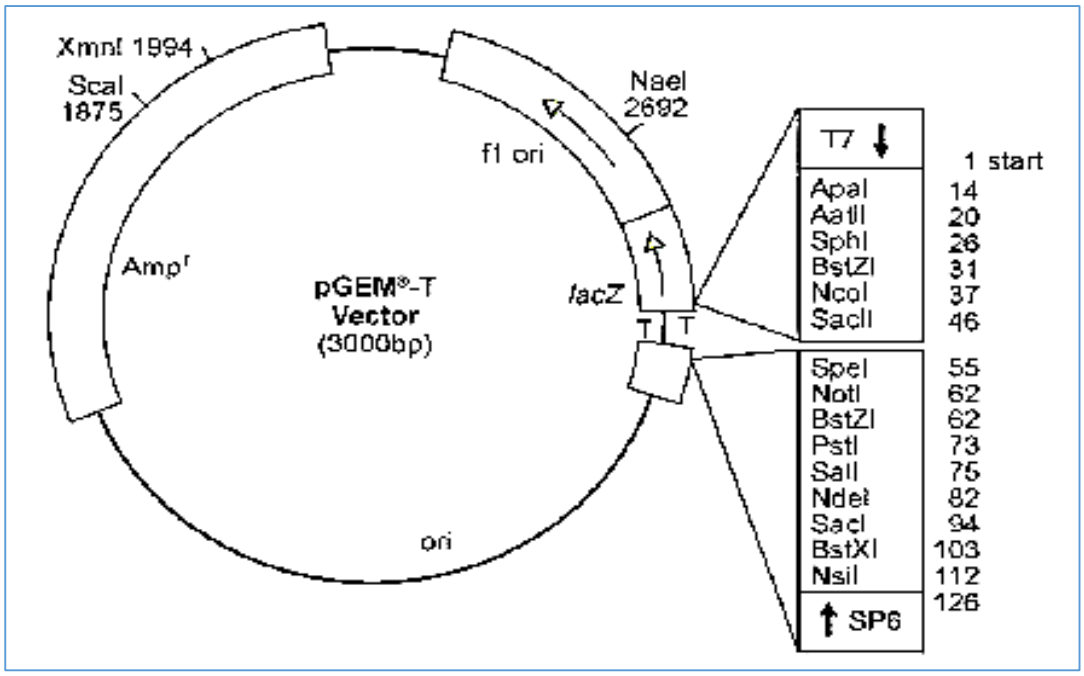
1. Should maintain itself in the new cellular environment following transformation:
2. Means of self replication – origin of replication
3. Selectable marker (e.g. resistance to antibiotics) to for selecting the cells carrying the vector.
4. Unique sizes to physically distinguish them and allow their purification.

Types of Cloning Vectors

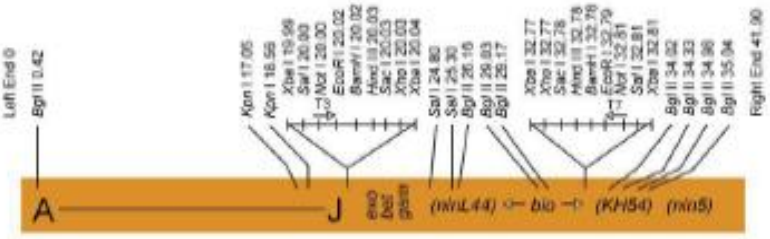
Plasmid

1. Plasmids are defined as autonomously replicating extra chromosomal circular DNA molecules which are faithfully passed on to progeny.
2. Plasmids are double stranded circular DNA and range in size from about 1 kb – 200 kb. The most useful for cloning are 2 – 10 kb because smaller plasmids are easier to manipulate and usually produce higher copy number when grown in bacterial host cells.

A Plasmid Vector



Lambda Vectors: Are developed from the Lambda phage. Cloning efficiency of DNA fragments is very high and desirable making cDNA or genomic libraries. Easy to grow on plates and transfer the DNA to nylon or nitrocellulose filters for screening the library. Can screen at a very high density (10,000 pfu/plate). Following ligation of the insert DNA molecules to the two arms, packaged to mature lambda phage particles *in vitro*



Lambda DASH II Multiple Cloning Site Regions
[sequences shown: 19.99-20.04kb (top sequence) and 32.77-32.81kb (bottom sequence)]

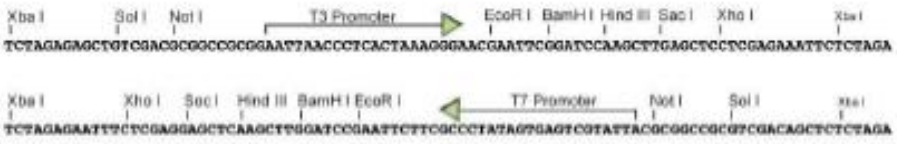


Fig. 5.2 A map of Lambda DASH II vector available from Agilent Technologies

BAC (Bacterial Artificial Chromosome) and YAC (Yeast Artificial Chromosome) vectors

These vectors can clone over 100 kb DNA fragments and suitable for making large insert genomic library for genome assembly and map-based cloning genes.

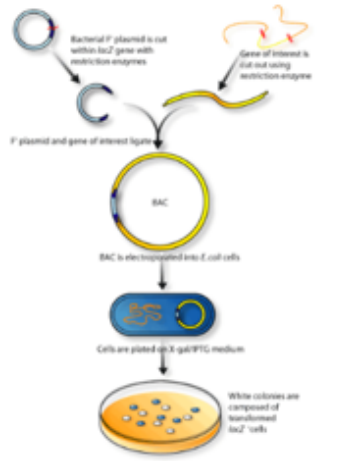
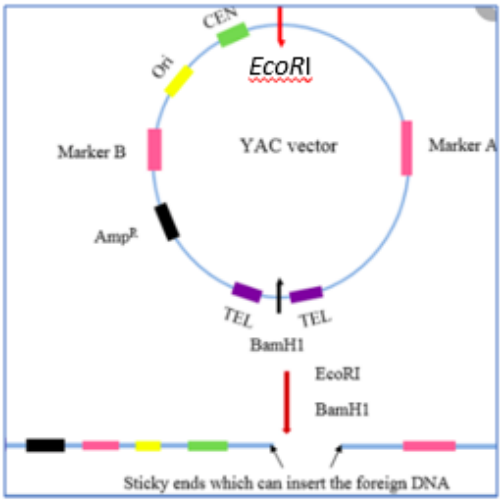
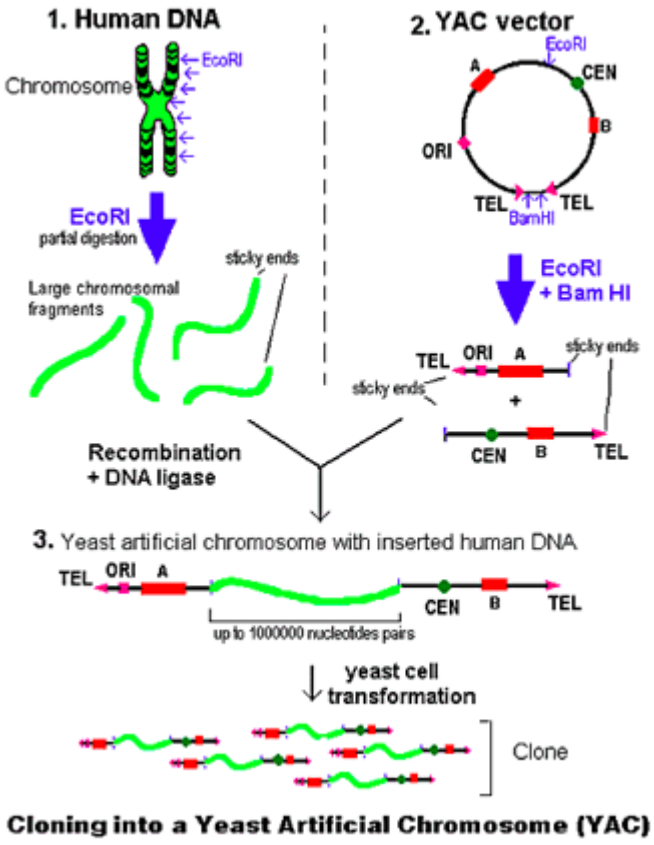


Figure I. Transforming a bacterium using a BAC vector





Cloning into a Yeast Artificial Chromosome (YAC)

cDNA and Genomic libraries

There are two types of gene libraries,

(i) cDNA

(ii) genomic

A **library** is a collection of clones that contain sequences of the entire genome of an organism. Each clone contains only a single genomic DNA fragment. Genomic libraries are made by digesting genomic DNA with a restriction enzyme followed by ligation of every fragment to a vector followed by transformation of such rDNA molecules into host cells. A cDNA library can be constructed in plasmid or lambda vectors.

Probably screen at least 100,000 insert containing cDNA clones to get the target gene sequence. If you do not, repeat more.

Cloning large DNA fragments using a BAC vector

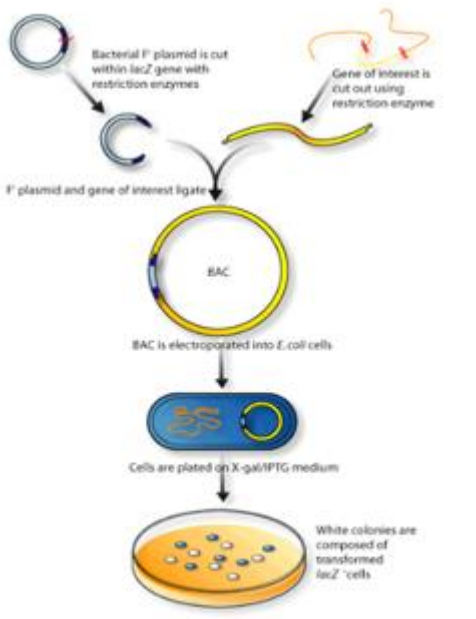


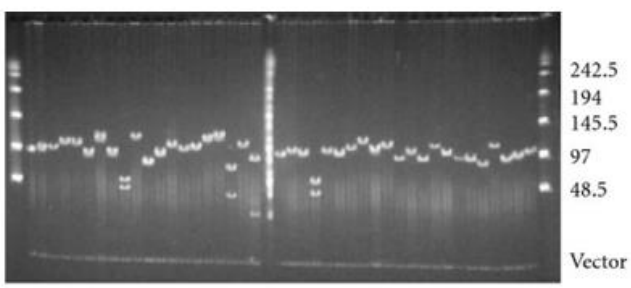
Figure 1. Transforming a bacterium using a BAC vector

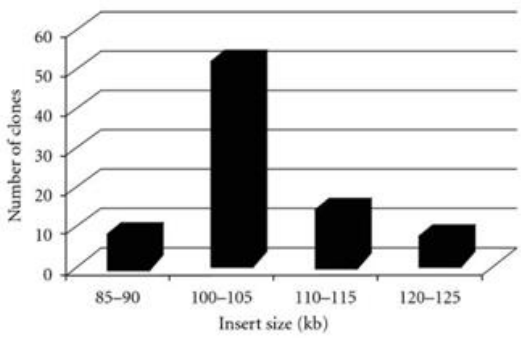
Library size to screen

It is estimated that the maize genome contains 2,500 Mb (2,500,000 kb). If you were to reliably digest a 2,500,000 kb fragment from a maize cell into a series of 100 kb restriction fragments, you would generate 2,500,000/100 = 25,000 unique genomic fragments. Clone them in a BAC vector, **pBeloBAC11**. If you clone each and every one of the 25,000 genomic fragments into this vector and transform them into *E. coli*, you would obtain a perfect genomic library. The number of clones in this "perfect" library represents what is known as a **genomic equivalent**. One genome equivalent maize library contains 25,000 BAC clones.

In reality, we cannot create a perfect library. Proportion of clones are random and some regions are under-represented and some are overrepresented. Usually, the chance or probability of finding any random DNA fragment in a three-genomic equivalent library is 0.95; while in the six-equivalent library is 0.99. In general, six-genomic equivalent library is constructed to start with

Analysis of Bacterial Artificial Chromosomes





Yeast artificial chromosome (YAC)

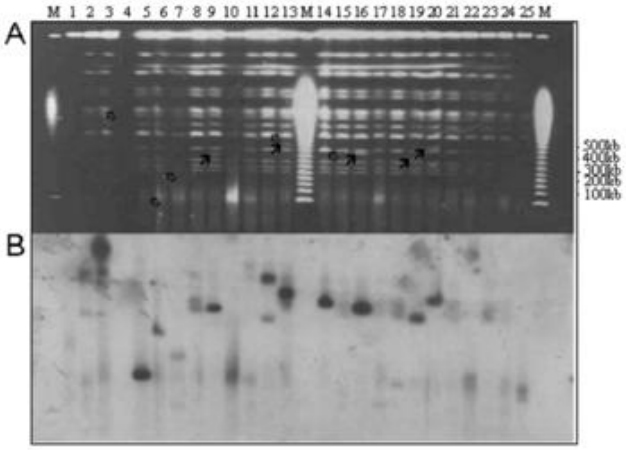
- In addition to BAC vectors, one can use yeast artificial chromosome (YAC) vector to clone large plant DNA fragments in individual yeast cells. The size of YAC clones are much bigger and can accommodate large DNA inserts up to 10 Mb.
- Difficult to prepare the library; but you have to screen a much smaller of clones than in the BAC library to isolate a target gene.

Funct Integr Genomics (2003) 3:153–159
DOI 10.1007/s10142-003-0092-8

ORIGINAL PAPER

Dipak K. Santra · Devinder Sandhu · Thomas Tai ·
Madan K. Bhattacharyya

Construction and characterization of a soybean yeast artificial chromosome library and identification of clones for the *Rps6* region



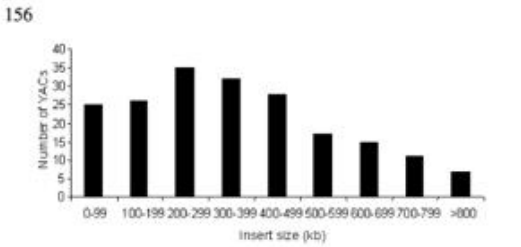
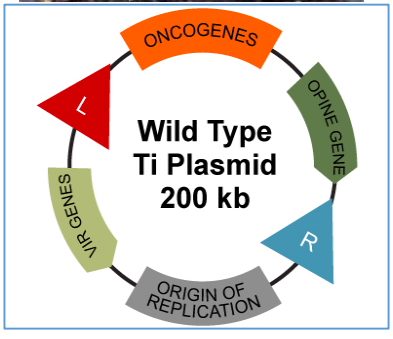


Fig. 2 Size distribution of randomly selected YAC clones. Randomly selected YAC clones from each ligation reaction were sized in CHEF gels by carrying out DNA-blot hybridization with soybean genomic DNA as the probe

Ti Plasmid Vector for Plant Transformation

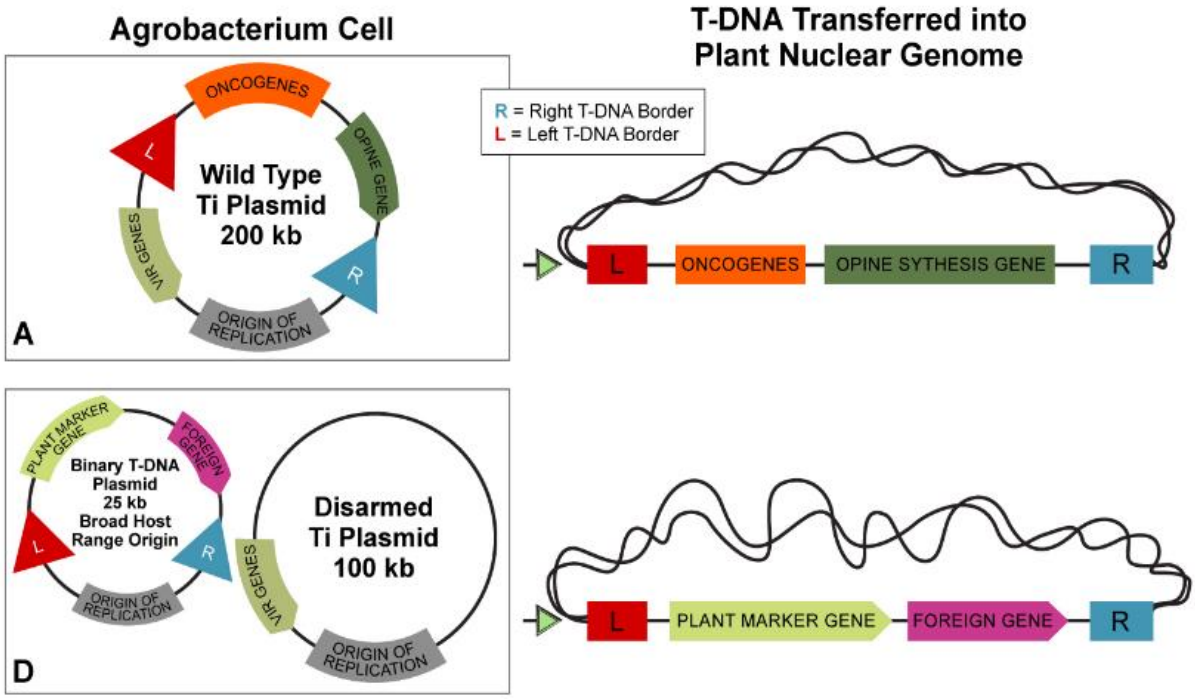
1. Crown galls are tumors of plants that arise at the site of infection by some species of the *Agrobacterium*.
2. *Agrobacteria* do not enter the plant cells, but transfer a DNA segment called T-DNA from their circular extra chromosomal *tumor-inducing* (Ti) plasmid into the genome of the host cells.
3. The T-DNA also encodes genes that affect host plant hormone physiology resulting in induced growth of the infected cells and tumor formation.
4. Oncogenes and opine synthesis genes were removed and replaced with gene of interests and selectable marker genes for plant transformation.



Engineering of the T-DNA Vector

Ti plasmid-mediated T-DNA transfer.

- Natural wild-type Agrobacterium Ti plasmid.
- Binary T-DNA plasmid in conjunction with a "disarmed" Ti plasmid. Since the "disarmed" Ti plasmid does not contain a T-DNA region, the *vir* genes can only act to transfer the T-DNA on the binary T-DNA plasmid. Note: The wavy lines represent plant DNA. Click the image to view a larger size.



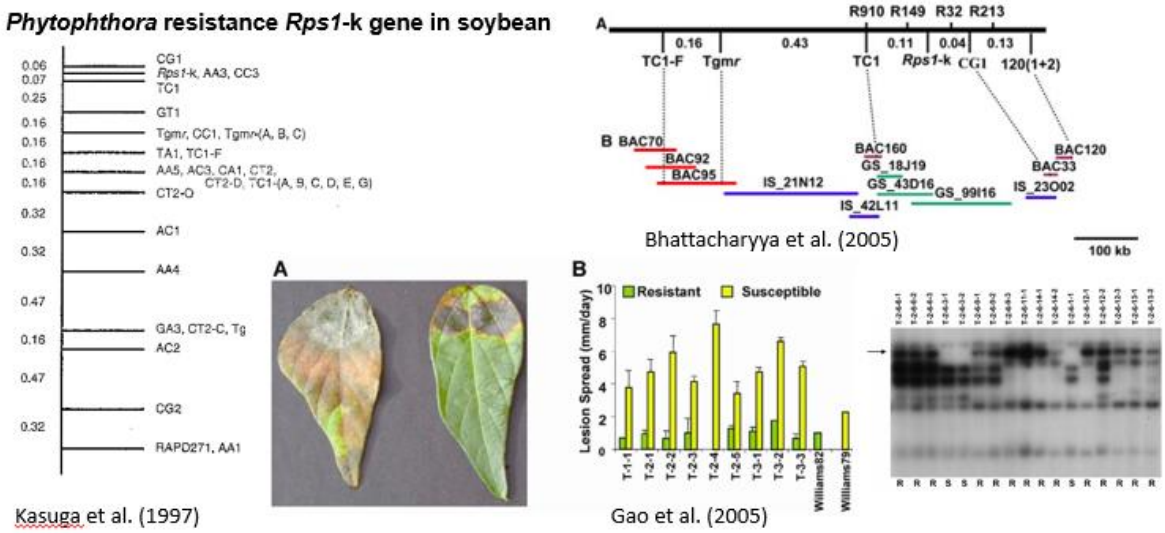
Gene Cloning

The first step is to have the gene cloned from a species of interest

The major methods of gene cloning are:

- **Homology based cloning gene:** Use a radiolabeled probe of a rice gene to screen a library of cDNA molecules created from maize mRNA molecules. Identify similar, orthologous genes – with same function.
- **PCR approach cloning homologues**: The above approach of gene cloning using homologous gene sequences has been subsequently modified to PCR approach, in which primers are being designed from the highly conserved domains of sequences gathered from several species.
- **Differential screening**: You make a library of cDNA molecules in a lambda vector (a virus based) and screen using two radiolabeled probes. Say library is from infected tissues and probes from control and infected tissues and then look for the clones hybridizing only to the infection-specific probe. A popular method during 1980s and also 1990s.

- **Immunoscreening**: In this case, mRNAs are cloned into lambda expression vector to express the proteins from individual clones with correct reading frames. An antibody raised against your protein of interest is used to screen the library – *r* locus cloning
- **Transposon tagging**: If the mutants revert back to wildtype, then it could be due to transposon induced mutation – you may have to isolate the transposon first if many versions of a transposon available in the plant. Snapdragon or *Antirrhinum* is an example. You use all types with mutants and revertant to wildtype plants for co-segregation of bands with a particular transposon; presence in the mutant; but absent in the revertant. Then use the transposon sequences to identify the adjacent gene sequences of the transposon (See Baumbach et al. 2016).
- **Map-based or positional gene cloning**: It is based map position of a gene – no information available about its DNA or protein sequences.



Methods of Plant Transformation

Most methods use these five basic steps:

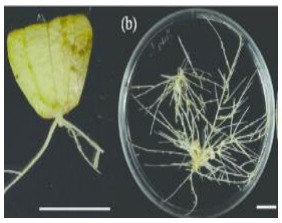
1. Preparation of the target plant tissue
2. Introduction of DNA into cells of the target tissue
3. Selection for transformed cells
4. Regeneration of new plants from tissue culture
5. Verification for transformation success and stability in subsequent generations

Examples of tissue types used for transformation of plants:

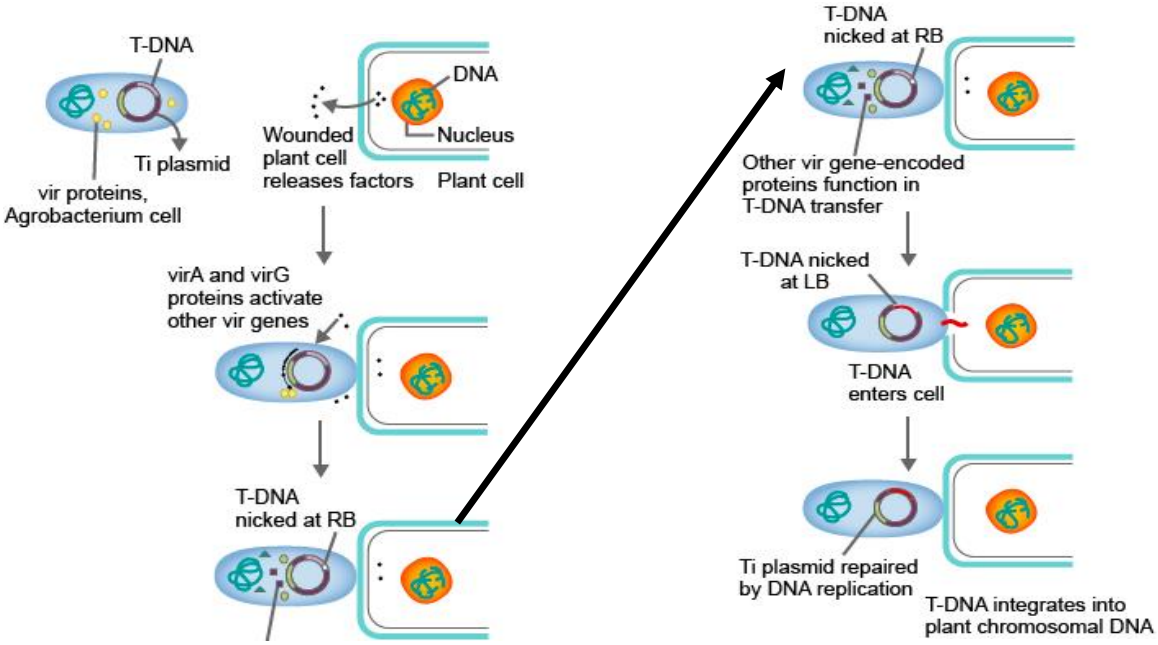
. Protoplasts from whole tissue or suspension cells
2. Intact cells from suspension cultures
3. Leaf discs
4. Cotyledon sections
5. Embryogenic tissue
6. Adventitious embryo

The methods used to conduct plant transformation are listed below. Use of a specific method depends solely on the type recipient species or type of transformations: (i) stable transformation or (ii) transient expression of genes.

1. **Direct DNA uptake** - DNA cannot be taken directly into cells because of the cell wall; therefore, protoplasts are used. DNA is imported into the protoplasts with polyethylene gycol.
2. **Microinjection** - DNA is injected directly into the host nucleus using micro capillaries.
3. ***Agrobacterium*-**mediated gene transfer – *Agrobacterium tumefaciens* or *A. rhizogenes* are used to deliver DNA into the recipient plant cells.
4. **Particle bombardment or biolistic transformation** - a physical means of transfecting cells by bombarding tissue with high velocity DNA coated particles. This method can be used on protoplasts, intact cells that are separated such as from a suspension culture, or on whole tissues or organs.
5. **Electroporation** – DNA is electroporated into protoplasts.



***Agrobacterium*-mediated gene transfer:** Two species of the *Agrobacterium* are used to generate transformed plants; *A. tumefaciens* and *A. rhizogenes. A. tumefaciens* is the most commonly used species for generating stable transgenic plants. *A. rhizogenes* produces transformed adventitious "hairy roots" and usually applied in rapid functional analyses of some traits that are expressed in roots. *A. tumefaciens* is a plant pathogen and it produces crown gall or tumors following infection.



Transfer of T-DNA from Agrobacterium into a plant cell. A wounded plant cell releases phenolic signals that induce the expression of *vir* genes on the Ti plasmid that facilitate the transfer of the T-DNA into the plant cell. The T-DNA enters the plant cells by yet unclear mechanisms, and integrates randomly into the chromosomal DNA. The Ti plasmid is repaired by DNA replication, so the Agrobacterium does not lose any information following transfer of the T-DNA to the plant cells

The Molecular Basis for the Crown Gall Disease

Agrobacterium tumefaciens is the causative agent of crown gall disease characterized by the formation of tumors on plant stems at or near the soil surface. *Agrobacterium* belongs to a

family of soil bacteria that also includes ***Rhizobium***, which is involved in symbiotic nodule formation in leguminous plants. The **crown gall** disease is caused by integration of a DNA fragment (T-DNA) of a tumor-inducing plasmid (Ti plasmid). *A. tumefaciens* tumors result from the rapid proliferation of plant cells after infection. Tumor cells isolated from an infected plant can continue to grow in culture, even when the culture is cured of he *Agrobacterium*, and in the absence of added plant hormones **cytokinin** and **auxin**. Tumor production is caused by transfer of T-DNA located in a large plasmid (about 200 kb) called **Ti (tumor inducing) plasmid**.

Ti plasmid contains genes that control four major processes associated with *Agrobacteria* infection.

1. virulence (infection), determination of host specificity and T-DNA transfer
2. phytohormone production in plant
3. opine synthesis by the plant
4. opine uptake and utilization by bacteria

Wounded plant tissue produces phenolic signal molecules that are detected by sensors produced by *Agrobacterium* genes called *VirA* and *VirG*. **Acetosyringone** is one such phenolic compound that acts as a recognition signal for *Agrobacterium*.

The genes regulating the phytohormone production are also located on the T-DNA and transferred to the plant genome. Examples of these genes are *aux, cyt1*; also called *iaaM and H, iptZ*. Enzymes encoded by these genes convert existing plant compounds to naturally occurring plant hormones auxin and cytokinin to induce the gall or hairy roots.

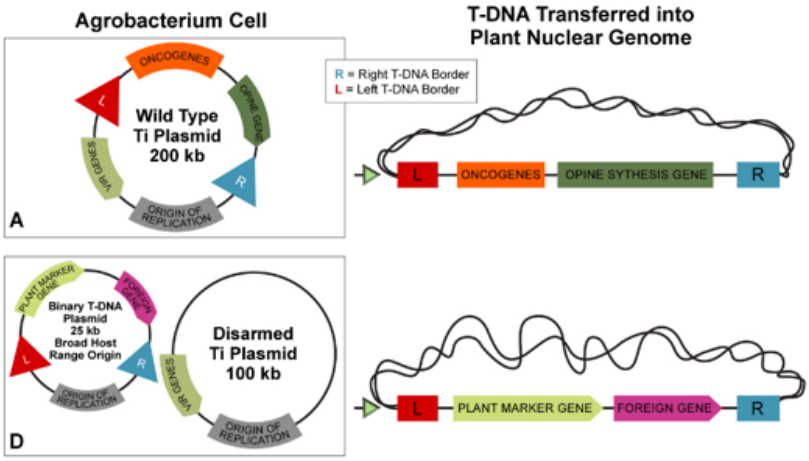
The promoters of these genes are always turned on (constitutive) and highly active in plant tissues. Thus, unlike normal plant cells, the production of these hormones in transformed cells is under no controls, leading to tumor formation

Opines are novel compounds derived from amino acids and are rich in carbon and nitrogen. **Octopine** (carboxylarginine) and **nopaline** (dicarboxypropylarginine) are the most common opines. Opines cannot be metabolized by plant cells or most other microorganisms, but can be utilized by *Agrobacteria* because the Ti plasmid contains additional genes for opine metabolism. Opine synthesis genes are located on the T-DNA that gets integrated into the plant genome, whereas opine metabolism genes remain on the Ti plasmid. This creates a favorable environment for bacterial growth because plant cells are programmed to produce N- and C-rich compounds **that can only be utilized** by *Agrobacterium*.

Use of *A. tumefaciens* in Plant Transformation

In the early days of genetic engineering, scientists modified the *Agrobacterium* Ti plasmid by removing the genes that give the ability to the pathogen cause disease but retaining its ability to transfer foreign DNA into plant cells. The transferred DNA (T-DNA) has a **left (L) and a right (R) border** sequence and all DNA in between these two borders are transferred to the plant genomes.

Modified Agrobacterium strain and Ti plasmid



Ti plasmid-mediated T-DNA transfer. (A) Natural wild-type Agrobacterium Ti plasmid. (B) Binary T-DNA plasmid in conjunction with a "disarmed" Ti plasmid. Since the "disarmed" Ti plasmid does not contain a T-DNA region, the vir genes can only act to transfer the T-DNA on the binary T-DNA plasmid. Note: The wavy lines represent plant DNA. Click the image to view a larger size.

The Ti plasmid has been engineered to enhance its usefulness for plant transformation

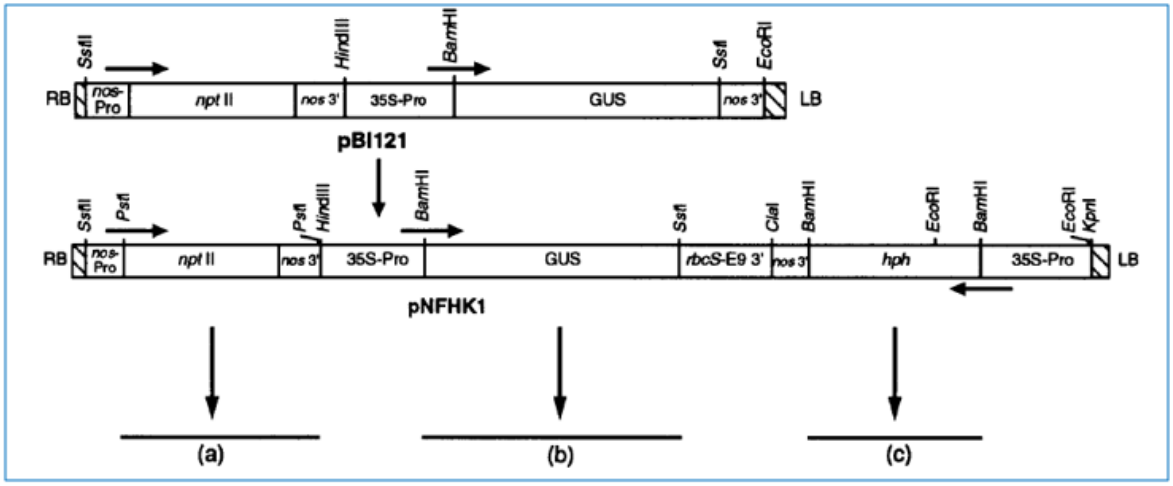
Genes responsible for altered plant hormone production (oncogenes) and other genes (opine synthesis) unnecessary for DNA transfer can be eliminated so that no tumor formed.

A binary T-DNA plasmid vector can be constructed. It contains the left and right T-DNA borders and **restriction endonuclease sites** between the borders that allow foreign DNA to be cloned into the T-DNA region.

A **plant selectable marker** (often an antibiotic resistance gene or herbicide tolerance gene) is placed in the T-DNA. Right border goes into the plant cells first and selection of T-DNA molecules using a selectable marker next to right border leads to partial integration of T-DNA molecules.

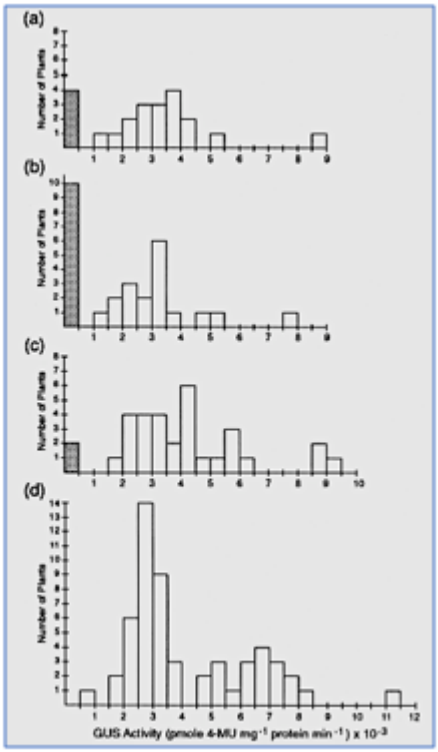
Placing the selectable marker next to the left border one can improve the complete integration of T-DNA and expression of the transgenes (Bhattacharyya et al. 1994).

Reduced variation in transgene expression from a binary vector with selectable markers at the right and left T-DNA borders



Bhattacharyya et al. 1994

Dual selection reduced the number of transformants with no GUS expression



Kanamycin

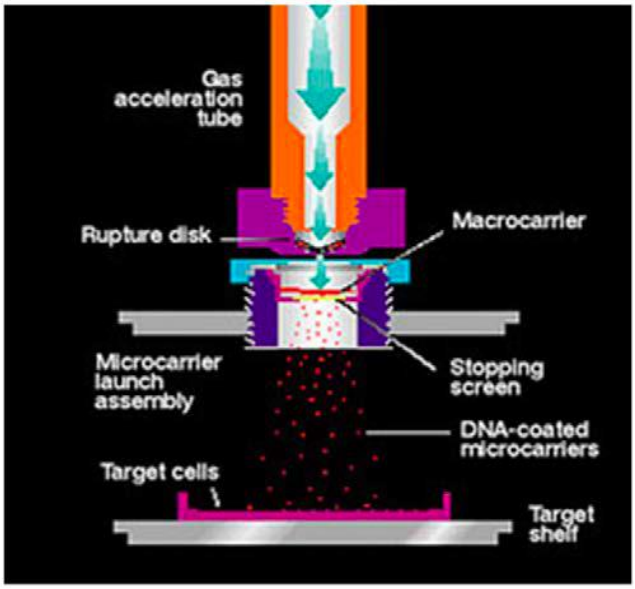
Kanamycin

Hygromycin

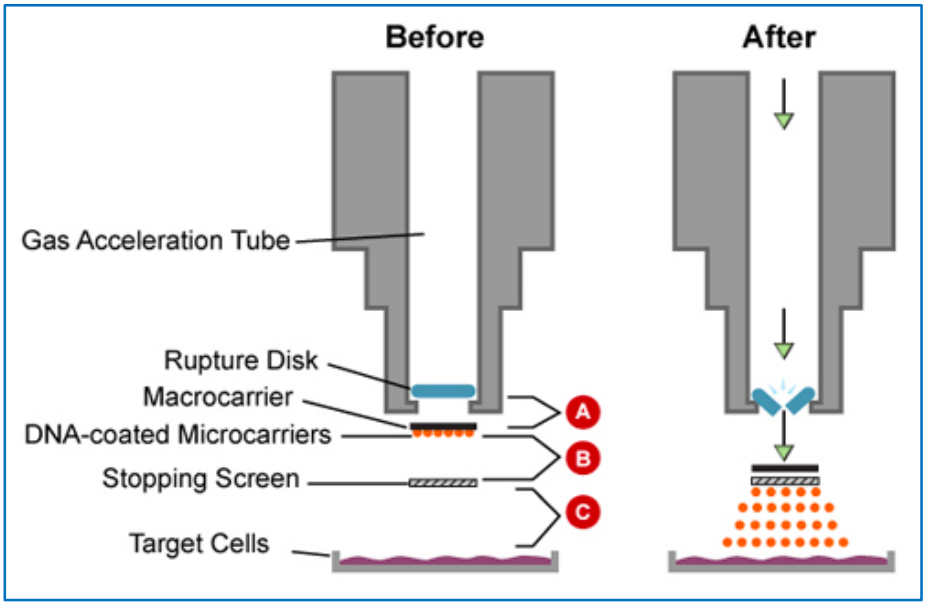
Kanamycin and Hygromycin

Bhattacharyya et al. 1994

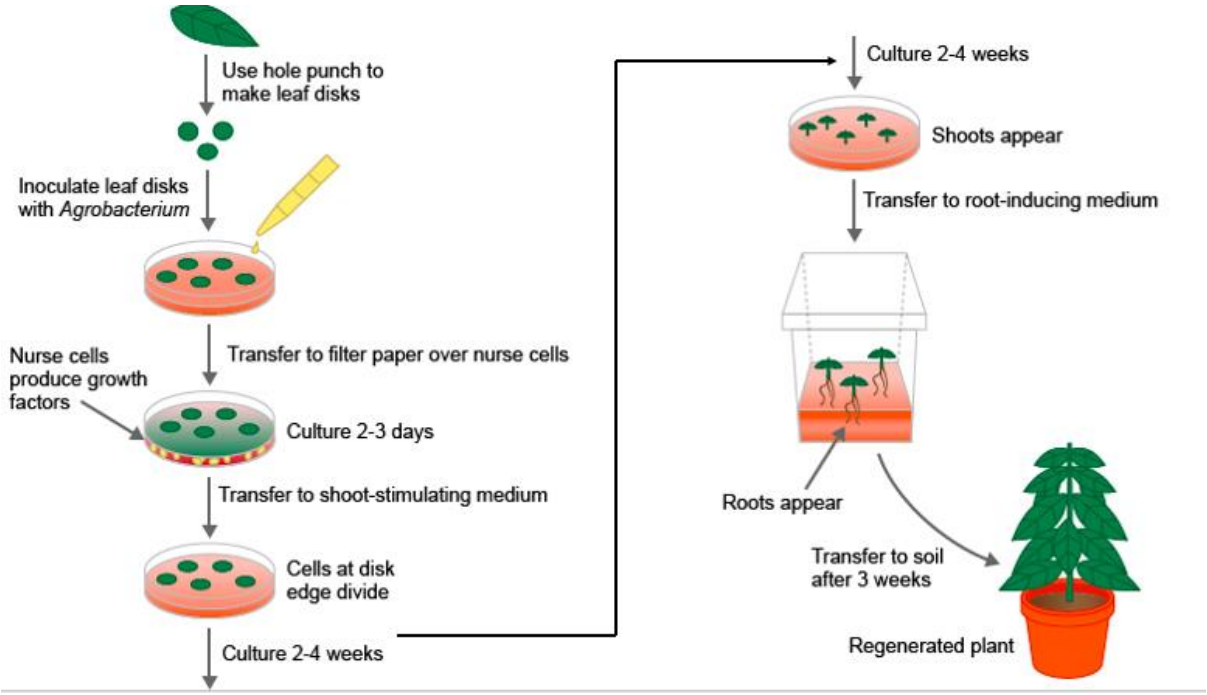
Biolistic PDS-1000/He System from Bio-Rad



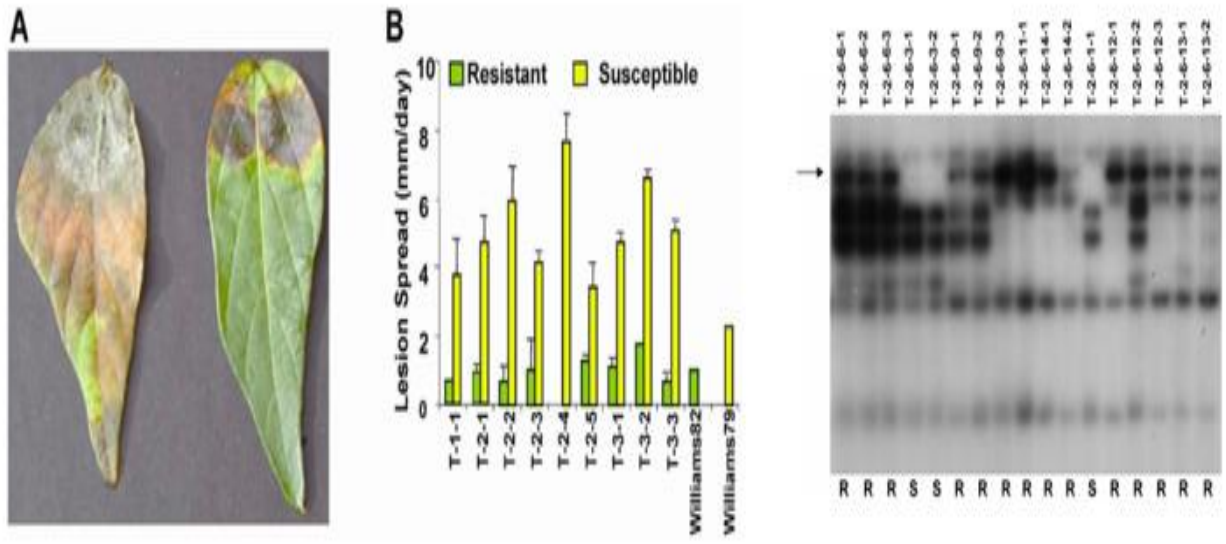
The biolistic transformation process



Transformation and regeneration of leaf disks by *Agrobacterium*.

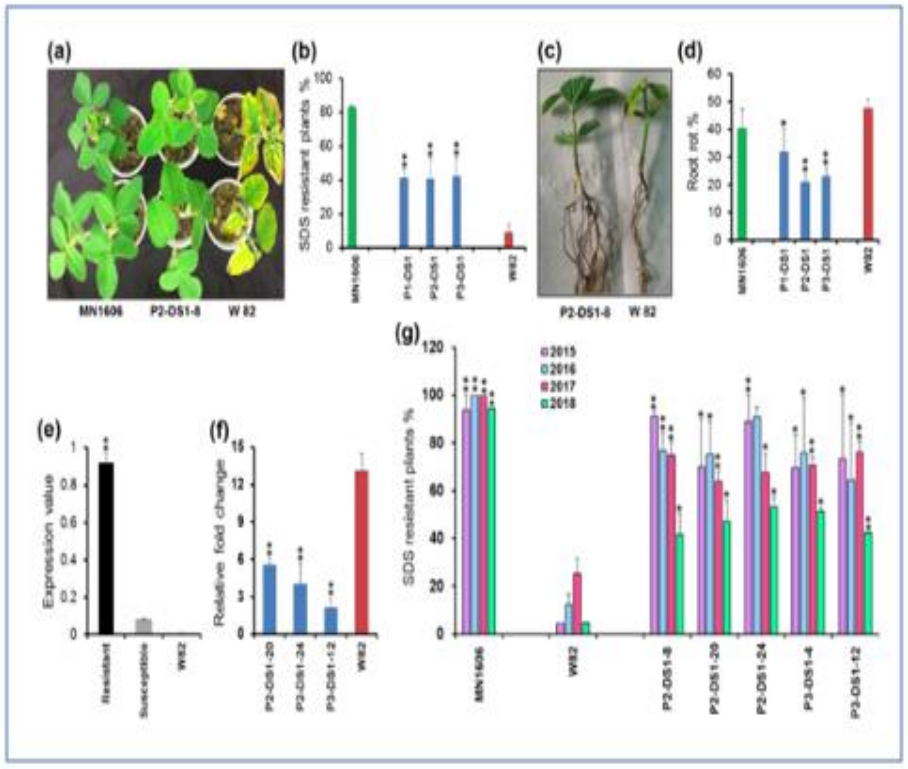


Analyses of transgenic soybean lines transformed with the Rps1-k gene



Gao et al. (2005)

Analyses of transgenic soybean lines with the overexpressed *GmDS1* gene



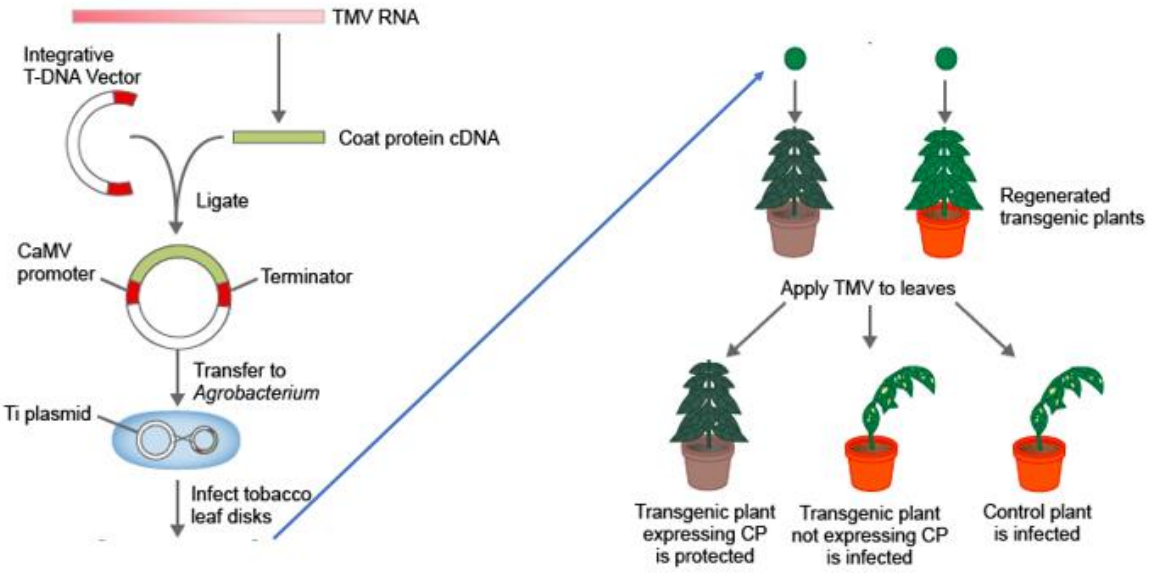
Ngaki et al. 2020; under review

Lecture

(Madan K. Bhattacharyya, Ph.D. Professor, Iowa State University)

Engineering Traits

Expression of the TMV CP Protected tobacco from TMV infection



Possible Mechanism

- Inhibition of virion disassembly by direct coat protein effect.
- For multiplication of the invading TMV molecules, the viral RNA must be unwrapped from the capsid made of coat proteins.
- As soon as the RNA molecules are released to the cell cytoplasm for replication, the coat proteins generated from the the transgene start to wrap up the RNA molecules – no replication – no increase in the number of the virus particles.

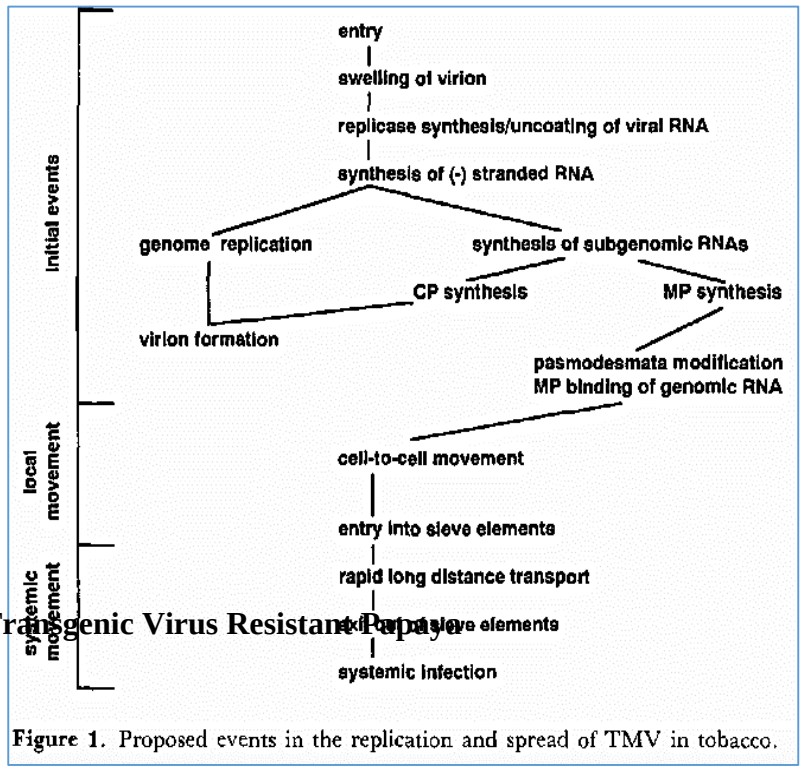


Figure 1. Proposed events in the replication and spread of TMV in tobacco.

Reimann-Philipp and Beachy 1993

Transgenic Virus Resistant Papaya

Papaya ringspot virus (PRSV) caused serious devastation to the papaya industry in Hawaii during the early 1990s. Infection by PRSV results in systemic rotting and wilting along with yellow spots and loss of chlorophyll.

To address the PRSV problem, Fitch et al. (1992) developed PRSV resistant transgenic papaya over expressing the PRSV *CP* gene. The intervention to rescue the papaya industry in Hawaii demonstrates that transgenic virus resistance is a useful approach for controlling viral diseases in horticultural crops.

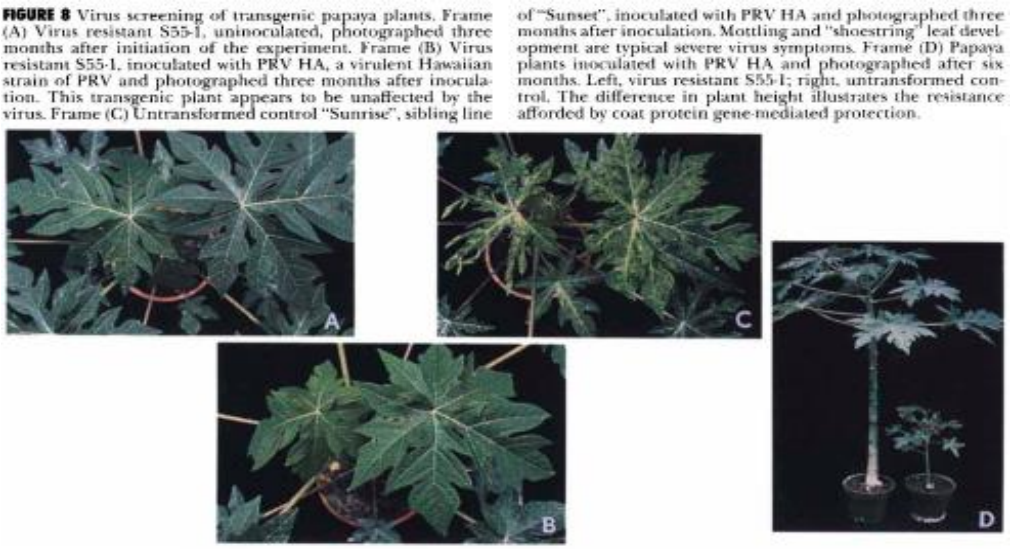
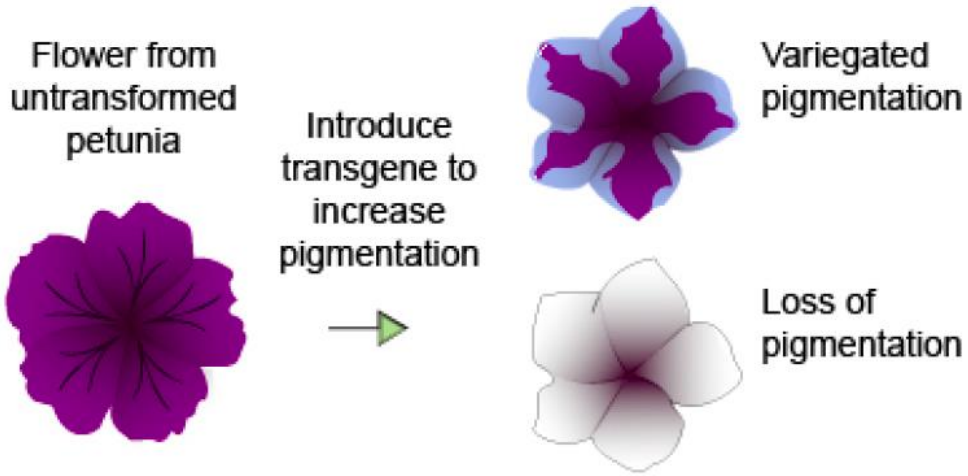


FIGURE 8 Virus screening of transgenic papaya plants. Frame (A) Virus resistant S55-1, uninoculated, photographed three months after initiation of the experiment. Frame (B) Virus resistant S55-1, inoculated with PRV HA, a virulent Hawaiian strain of PRV and photographed three months after inoculation. This transgenic plant appears to be unaffected by the virus. Frame (C) Untransformed control "Sunrise", sibling line of "Sunset", inoculated with PRV HA and photographed three months after inoculation. Mottling and "shoestring" leaf development are typical severe virus symptoms. Frame (D) Papaya plants inoculated with PRV HA and photographed after six months. Left, virus resistant S55-1; right, untransformed control. The difference in plant height illustrates the resistance afforded by coat protein gene-mediated protection.

Fitch et al. 1992

Discovery of RNAi and gene silencing

In the early 1990s, Richard Jorgensen and co-workers in an attempt to increase flower pigmentation in petunia by transgenic approaches, they observed that overexpression of transgenes did not deepen pigmentation. Instead, they observed flowers that showed variegated pigmentation, with some lacking pigments altogether as show below.

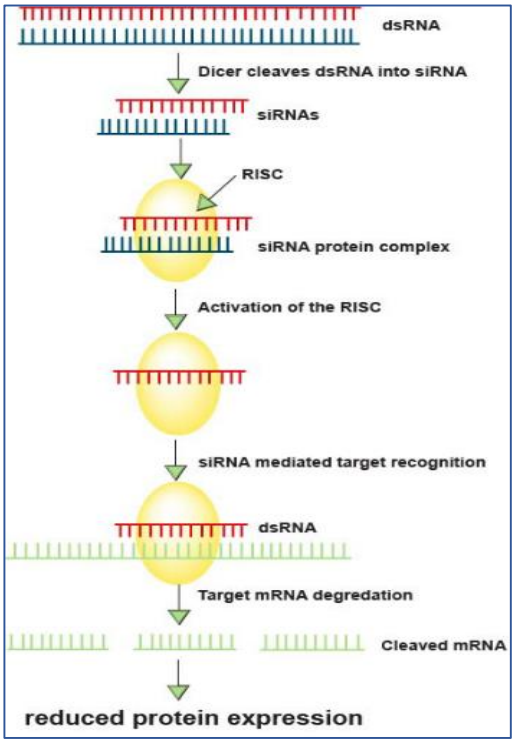


- The researchers concluded that the transgenes and endogenous sequences homologous to the transgenes must have been inactivated, a process they coined **co-suppression** (Napoli et al., 1990).

- Other laboratories later found that plants respond to RNA viruses by destroying viral RNAs to prevent viral genes from being expressed inside plant cells and cause disease.
- Co-suppression and viral RNA targeting processes in plant cells are referred to as **post-transcriptional gene silencing** (PTGS).
- RNA interference (RNAi) is a natural mechanism for moderating gene expression in the cells of all eukaryotic organisms from plants to humans.
- Although researchers working with worms received full credit for the discovery of RNAi (Fire et al., 1998), related phenomena had been previously observed in plants (Napoli et al., 1990).

Mechanism of Action of Post-Transcriptional Gene Silencing

- PTGS in plants is an RNA-degradation mechanism, similar to RNA interference (RNAi) in animals.
- RNAi refers to RNA-mediated regulation of gene expression - decreased expression, or silencing through:
    - ❖ transcriptional silencing,
    - ❖ translational silencing, or
    - ❖ mRNA degradation.
- These processes are mediated by small RNAs that target mRNA or chromosomal genes through sequence homology.
- The formation of small interfering RNA (siRNAs) or micRNA (miRNAs) is initiated by the formation of double-stranded RNA (dsRNA) molecules which are processed by dicer endonucleases to form the small RNAs of 21-24 nucleotides.
- Small RNAs then associate with a protein complex called RISC (RNAi silencing complex) and target cellular mRNAs with homologous sequences resulting in the degradation of the endogenous transcripts.



Knockdown of *MLO* genes reduces susceptibility to powdery mildew in grapevine

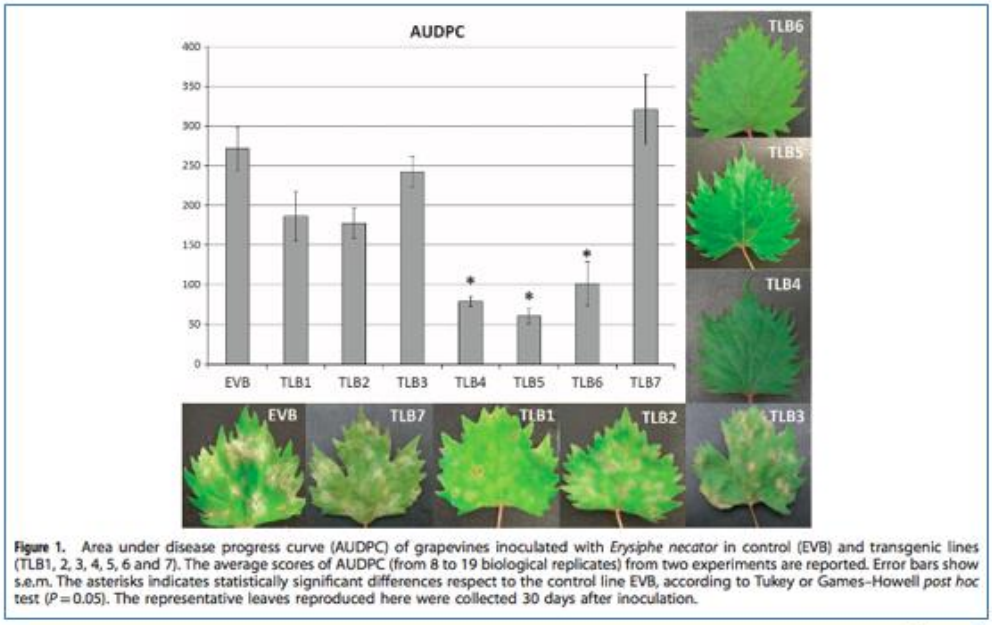
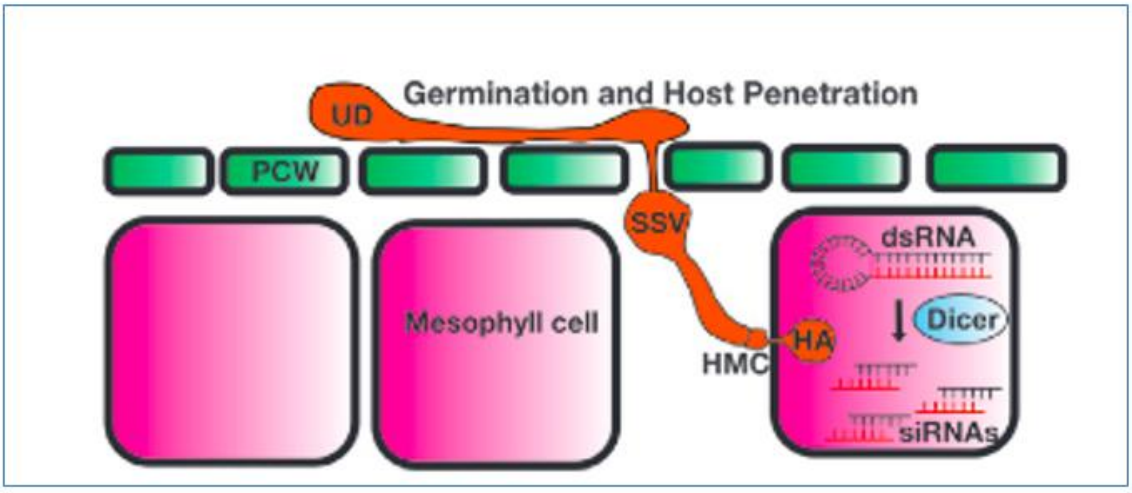


Figure 1. Area under disease progress curve (AUDPC) of grapevines inoculated with *Erysiphe necator* in control (EVB) and transgenic lines (TLB1, 2, 3, 4, 5, 6 and 7). The average scores of AUDPC (from 8 to 19 biological replicates) from two experiments are reported. Error bars show s.e.m. The asterisks indicates statistically significant differences respect to the control line EVB, according to Tukey or Games–Howell *post hoc* test ($P = 0.05$). The representative leaves reproduced here were collected 30 days after inoculation.

Pessina et al. 2016

Host Induced Gene Silencing



Schematic presentation of possible *in planta*-induced transient gene silencing (PITGS) mechanisms by which siRNA molecules generated in host cells could be delivered into fungal cells – adapted from Panwar et al. 2013.

Host-induced gene silencing of cytochrome P450 lanosterol C14α-demethylase encoding genes confers strong resistance to *Fusarium* species

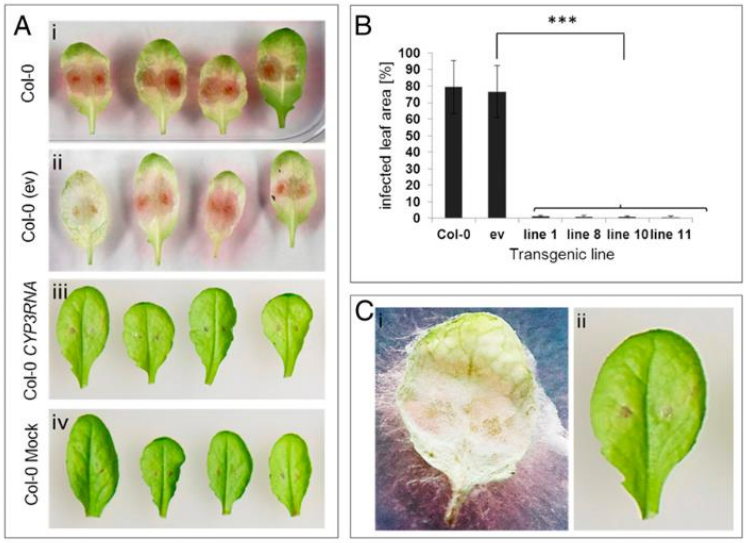


Fig. 1. Infection symptoms on *Arabidopsis* leaves following inoculation with *F. graminearum*. (*A*) Detached leaves of 5-wk-old plants were treated with 5×10^4 macroconidia mL^{-1} and evaluated for necrotic lesions at 3 dpi. (*i*) wild-type (Col-0), (*ii*) Col-0 ev control, (*iii*) Col-0 expressing *CYP3RNA* (representative line L8), and (*iv*) wild-type treated with Tween water (mock). (*B*) Quantification of infected leaf area at 3 dpi; typical infection symptoms are recorded as a percent of the total leaf area. Bars represent mean values ± SDs of three independent experiments, each using 20 leaves collected from 15 different plants of each transgenic line, as well as wild-type and Col-0 ev plants. The reduction in infection symptoms on *CYP3RNA*-expressing leaves compared with the wild-type and Col-0 ev control was statistically significant (***$P < 0.0001$; Student's *t* test). (*C*) *Fg*-inoculated *Arabidopsis* leaves at 5 dpi. (*i*) The Col-0 ev leaf is heavily infected with *Fg*; (*ii*) Col-0 expressing *CYP3RNA* does not show infection symptoms.

Endogenous silencing of *Puccinia triticina* pathogenicity genes through *in planta* expressed sequences leads to the suppression of rust diseases on wheat

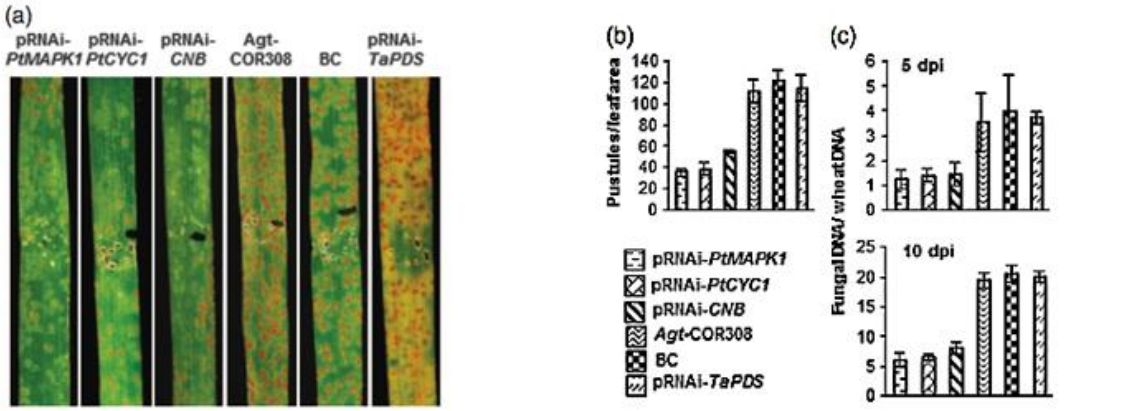


Figure 4. Effect of the presence of gene-specific siRNA molecules in wheat cv. Thatcher on *Pt* infection.

Panwar et al. 2013

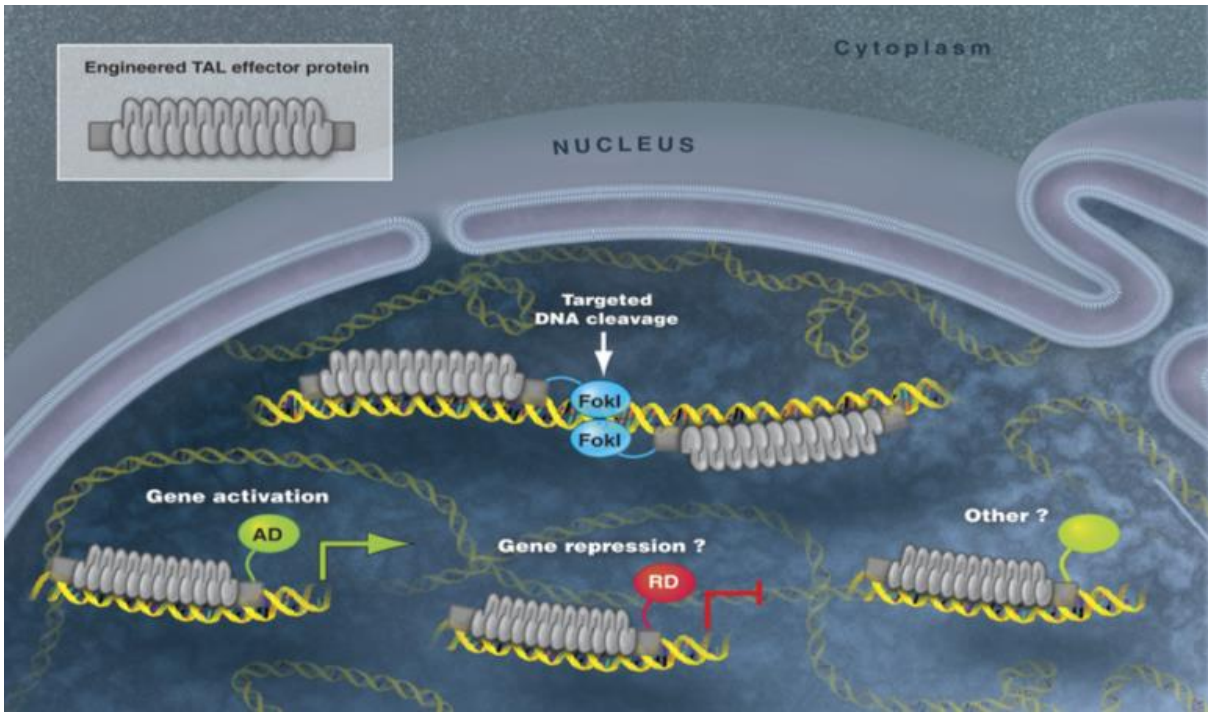
Gene Editing

- TALENs
- CRISPR-Cas9

Transcription activator–like (TAL) effector nucleases (TALENs)

It is based on discovery in plant pathology. Transcription activator–like (TAL) effectors of plant pathogenic bacteria regulate host gene expression to cause susceptibility. TAL factors carry tandem, polymorphic amino acid repeats that individually specify contiguous nucleotides in DNA as target for binding. The code of effector amino acid sequence binding to specific host DNA sequence in promoters were discovered and applied in TALEN technology.

TAL factors carry repeats of amino acids that recognize nucleic acid in a consistent pattern. Once this was known, it was feasible to design artificial TAL factors to conduct mutagenesis as shown in the next slide adapted from Bogdanove and Voytas 2011.



Fusion of TAL effector proteins to *Fok*I creates sequence-specific nucleases that enable targeted DNA cleavage for gene knockouts and genome editing

- TAL effector proteins fused to transcriptional activation domains (AD) and putatively to repression domains (RD) provide artificial switches for gene regulation in vivo. These days we can conduct mutation breeding for candidate or known genes of interest.
- *Mlo* gene in wheat (http://www.nature.com/nbt/journal/v32/n9/full/nbt.2969.html) has recently been edited using TALEN technology to enhance disease resistance.

Simultaneous editing of three homoeoalleles of *Mlo* in hexaploid bread wheat confers heritable resistance to powdery mildew

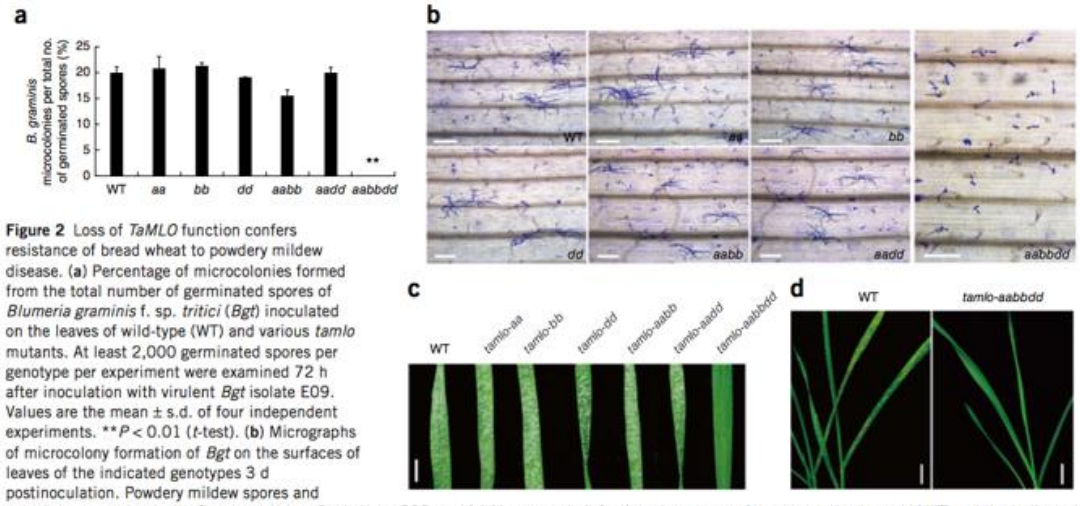
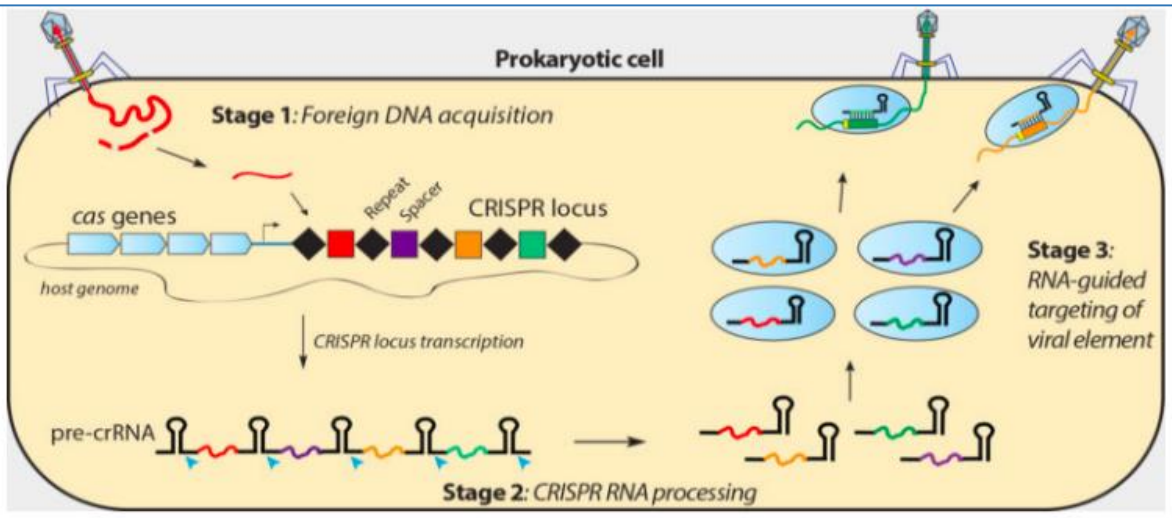


Figure 2 Loss of *TaMLO* function confers resistance of bread wheat to powdery mildew disease. (**a**) Percentage of microcolonies formed from the total number of germinated spores of *Blumeria graminis* f. sp. *tritici* (*Bgt*) inoculated on the leaves of wild-type (WT) and various *tamlo* mutants. At least 2,000 germinated spores per genotype per experiment were examined 72 h after inoculation with virulent *Bgt* isolate E09. Values are the mean ± s.d. of four independent experiments. $^{**}P < 0.01$ (*t*-test). (**b**) Micrographs of microcolony formation of *Bgt* on the surfaces of leaves of the indicated genotypes 3 d postinoculation. Powdery mildew spores and colonies were stained with Coomassie blue. Scale bars, 200 µm. (**c**) Macroscopic infection phenotypes of representative leaves of WT and the indicated *mlo* mutants 7 d after inoculation of detached leaves with *Bgt*. Scale bar, 1 cm. (**d**) Disease symptoms of wild-type (WT) and *tamlo-aabbdd* mutant plants. The photograph was taken 7 d after inoculation *in planta*. Scale bars, 2 cm.

Wang et al. 2014

CRISPR-Cas9 mediated gene editing

Innate Immunity in Prokaryote



The Clustered Regularly Interspaced Short Palindromic Repeats, or CRISPR

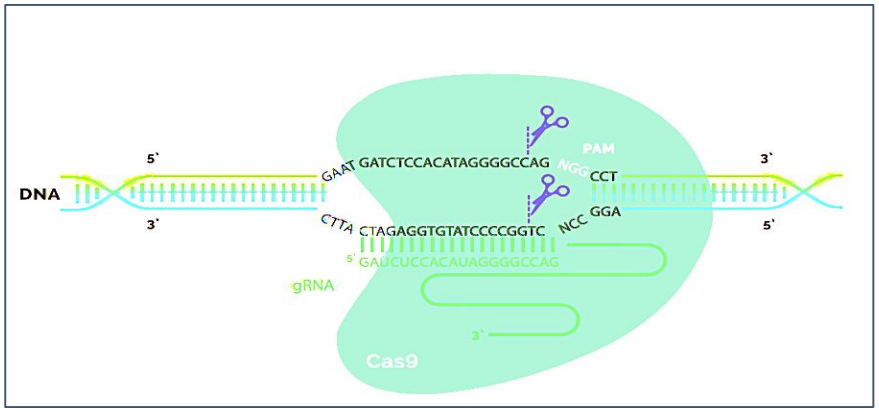
Image source: The Doudna Lab http://rna.berkeley.edu/crispr.html

- The **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats, or **CRISPR** was first discovered in 1993 through sequence analyses.
- Significance of CRISPR was unknown until 2007, when immunity function of this element was uncovered.
- The mechanism used by the element to confer immunity became known after five years
- The bacteria and archaea use CRISPR to defend against the invading viruses termed as bacteriophages. Following viral infection, they employ a special CRISPR-associated nuclease 9 (Cas9) to generate a double-strand break (DSB) in its target loci of the bacteriophages' DNA molecules. Thus, viruses become ineffective. Cas9 is directed to the target sequence by a short RNA fragment known as a guide RNA (gRNA), complementary to a segment of the viral genome for generating DSB. Parallel to viral DNA cleavage, a short viral DNA is stored between the palindromic CRISPR sequences.

This sequence is being used to generate the gRNA for rapid activation of this defense mechanism against any subsequent infection by the same bacteriophage. This system is kind of similar to ***antibody production*** in humans

Two steps to consider in designing a CRISPR-Cas9 system for editing a gene:
(i)The design of guide RNA (gRNA)
(ii)Choice of the nuclease depends on the desired application – Cas9 for plant work.
The gRNA that targets specifically to your gene of interest should be designed. Best scenario would be 0 non-specific cut and highly specific to the target site in exons. You can edit members of a gene family simultaneously. Following double stranded breaks (DSB), cells repair the DNA by non-homologous end joining (NHEJ) mechanism. During this process indels (insertions and deletions) are created leading to frameshift mutations and ***gene knockouts*** (KO).

- We can also conduct ***knock-in*** (KI) mutation by repairing the DSB through homology directed repair (HDR) mechanism.
- To facilitate or induce HDR-mediated repair, copies of homologous DNA molecules with a desirable mutation are provided to use as a template. The mutation can be a single point mutation to change an amino acid.
- It can be used to correct a disease-causing mutation in humans and generating herbicide resistance in plants.
- For example, in plants, we can replace an amino acid in the gene encoding acetolactate synthase (ALS) involved in biosynthesis of branched-chain amino acids to make the enzyme resistant to several herbicides, including imidazolinones and sulfonylureas. Outcome herbicide resistant plants.



The CRISPR-Cas9 system comprises a guide RNA (gRNA) and Cas9 nuclease, which together form a ribonucleoprotein (RNP) complex. The gRNA binds to the genomic target upstream of a protospacer adjacent motif (PAM), enabling the Cas9 nuclease to make a double-strand break in the DNA (denoted by the scissors). Adopted from: https://www.synthego.com/resources/crispr-101-ebook

- You can also generate gRNA and Cas9 protein in test tubes and apply the two as ribonucleoprotein (RNP) complexes into the cells; this approach has recently been reported to be the most effective strategy (Liang et al. 2015). The complex is unstable and does not continue to function to cause additional off-target mutations.
- Alteration of function through homologous recombination can also be accomplished through Crispr/Cas9 system.

- Point or deletion mutations through Crispr/Cas9 is predictable. The only weakness is the mutations caused by the system in non-targeted genomic regions. There are many sequences similar to **16 bp target** sequence in complex genome. Therefore, we have to investigate the genome sequence for target sequence just to make sure that the target site is either absent or is not present in coding sequences. One can always breed the desirable Crispr/Cas9-induced mutations through backcrossing.

Establishing a CRISPR–Cas-like immune system conferring DNA virus resistance in plants

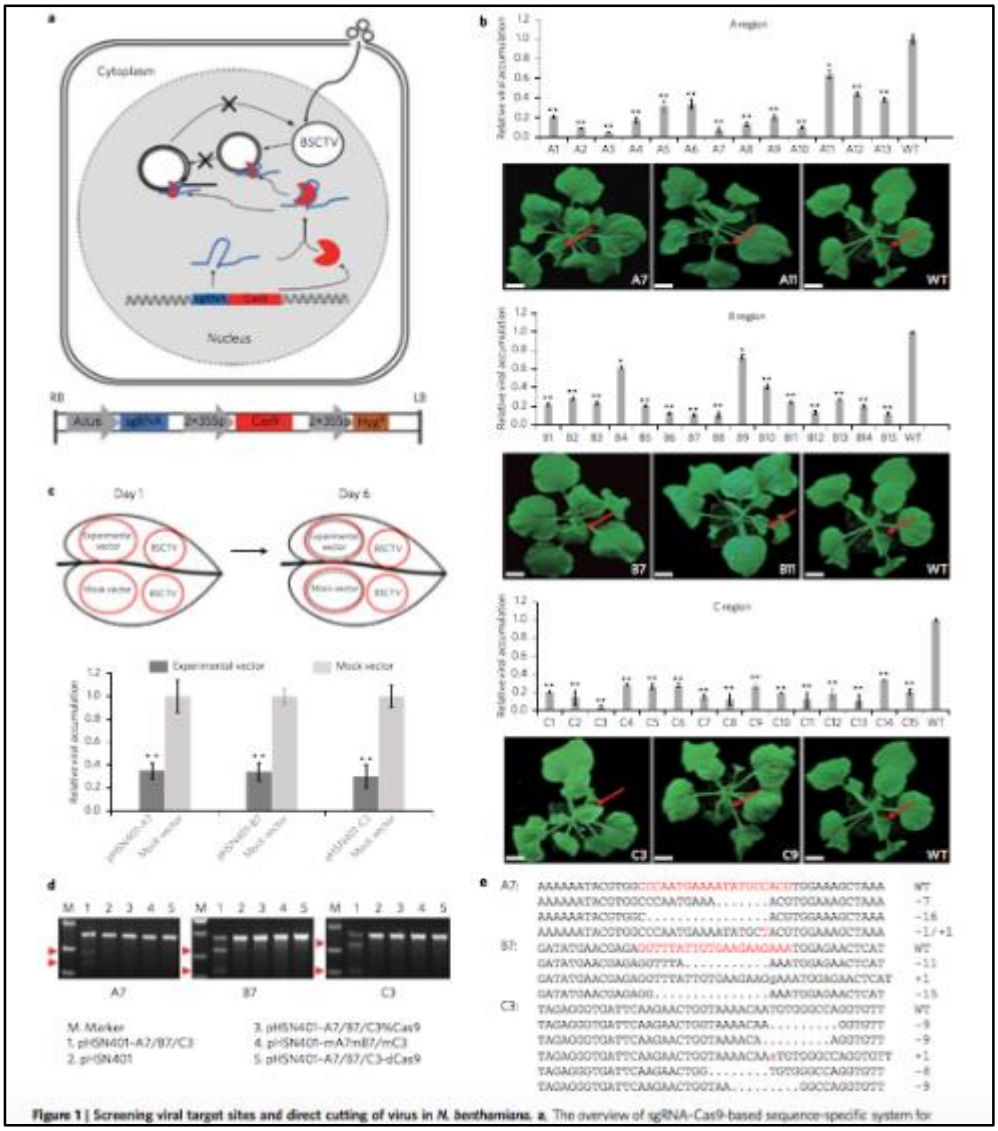


Figure 1 | Screening viral target sites and direct cutting of virus in *N. benthamiana*. a, The overview of sgRNA-Cas9-based sequence-specific system for

Ji et al. 2015

CRISPR/Cas9-mediated viral interference in plants

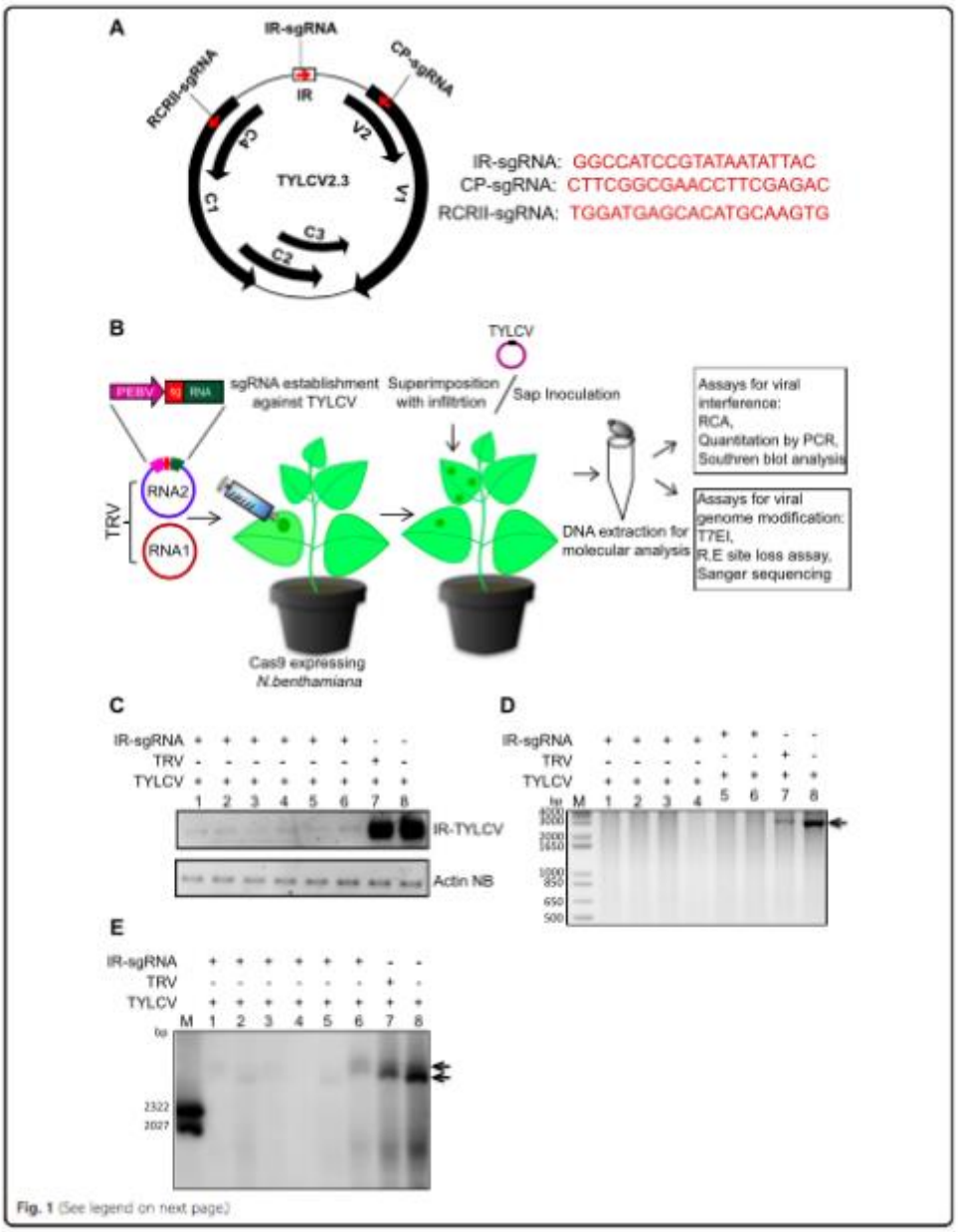
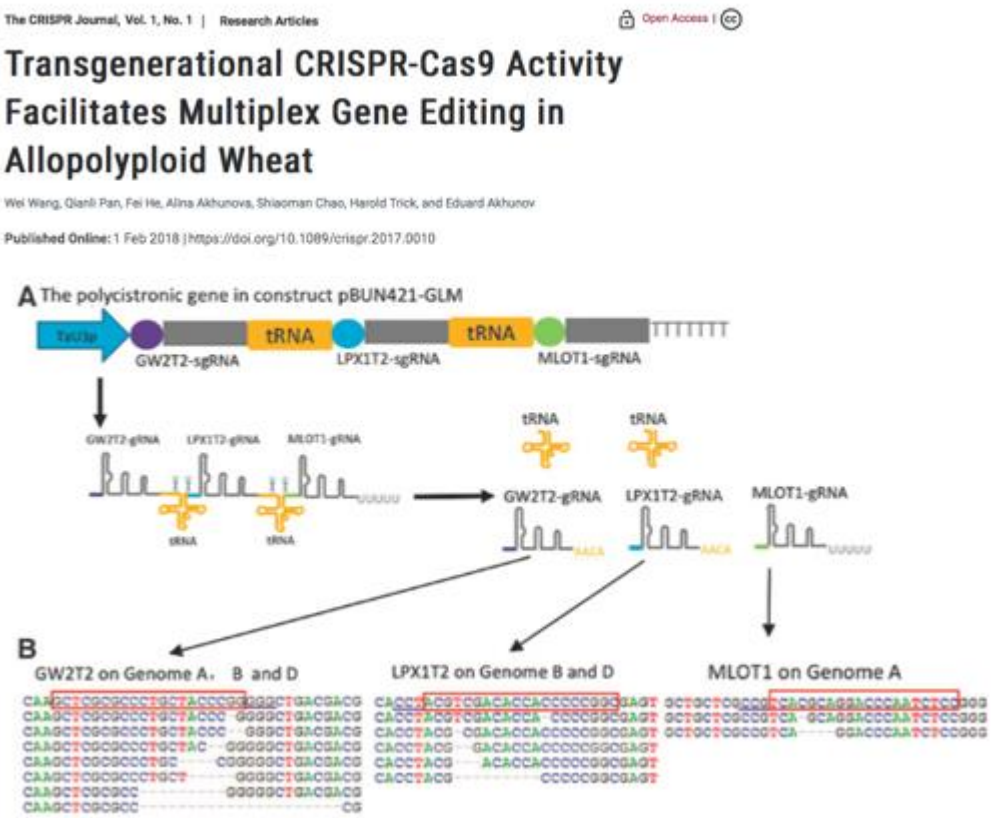
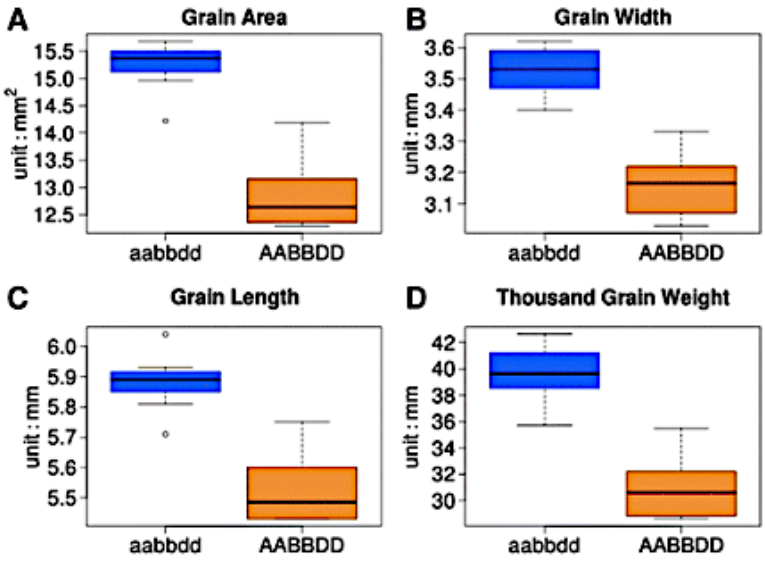


Fig. 1 (See legend on next page.)

Ali et al. 2015



The knockout mutations in all three homoeologous copies of one of the target genes, *TaGW2*, resulted in a substantial increase in seed size and thousand grain weight

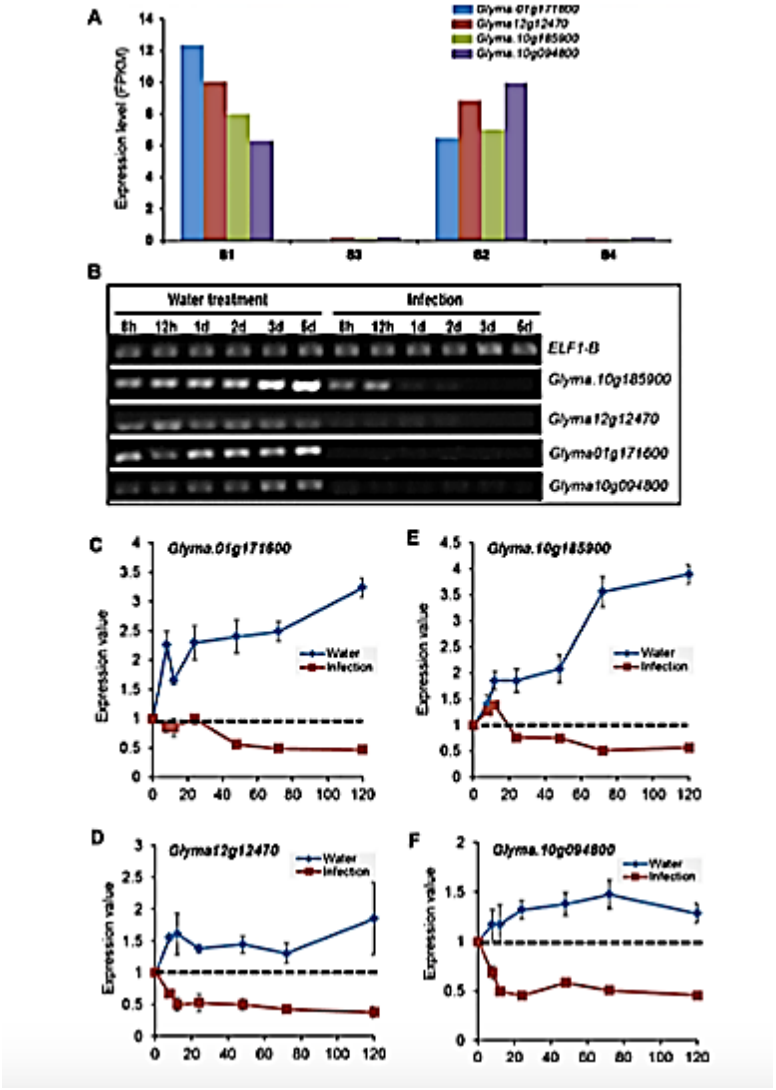


Wang et al. 2018

FIG. 3. Phenotypic effects of CRISPR-Cas9-induced mutations in the *TaGW2* gene. Box and whisker plots are used to show **(A)** grain area, **(B)** grain width, **(C)** grain length, and **(D)** thousand grain weight (TGW) of *gw2* knockout (*aabbdd*) and wild-type plants (*AABBDD*).

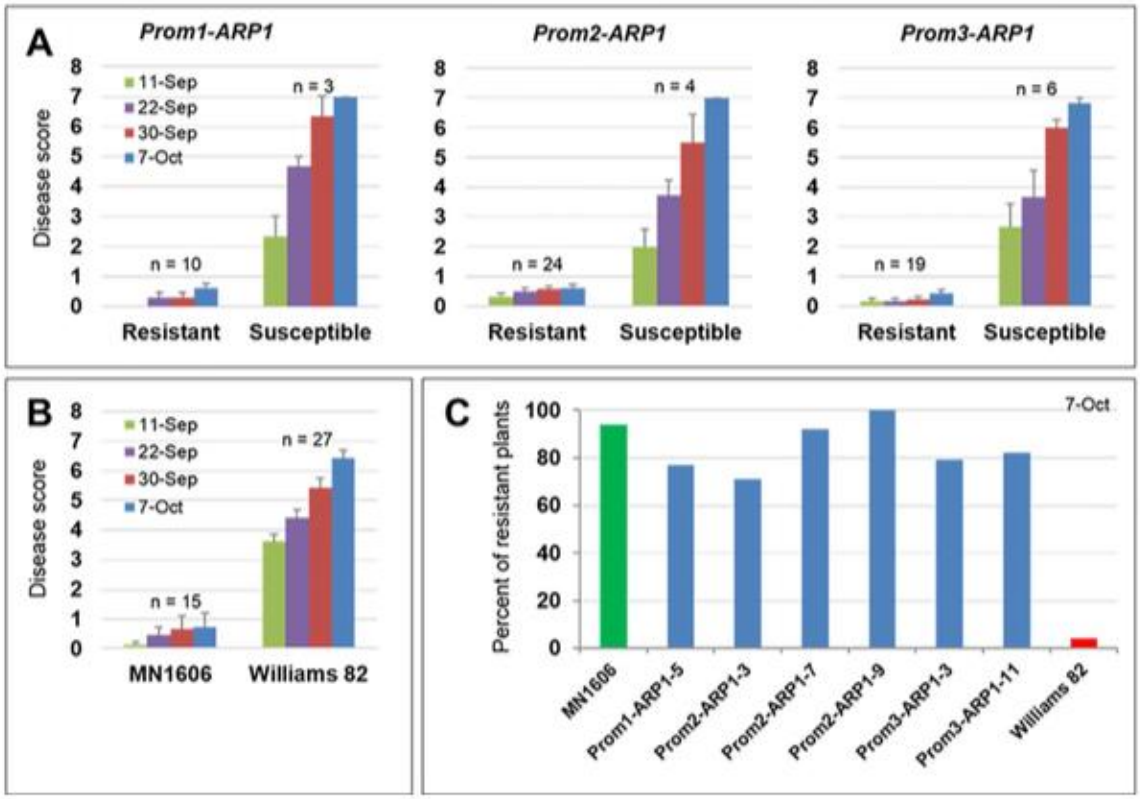
Overexpression of Genes

Only four classes of soybean genes are suppressed by *Fusarium virguliforme* that causes sudden death syndrome (SDS) in soybean.



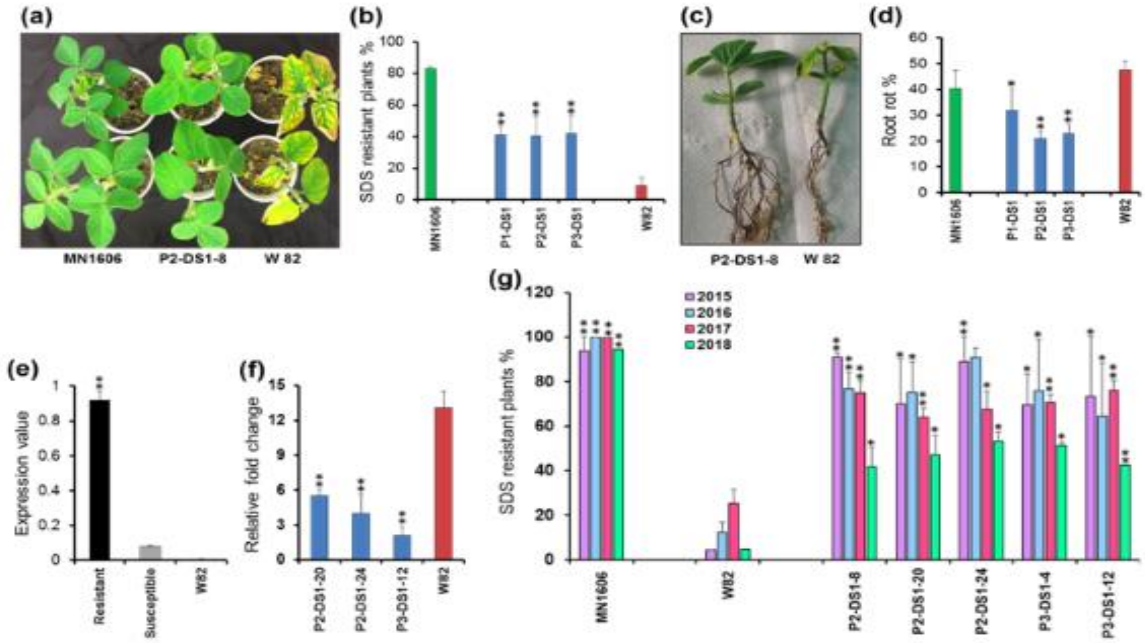
Ngaki et al. 2016

Overexpression of *GmARP1* enhances SDS resistance in transgenic soybean plants

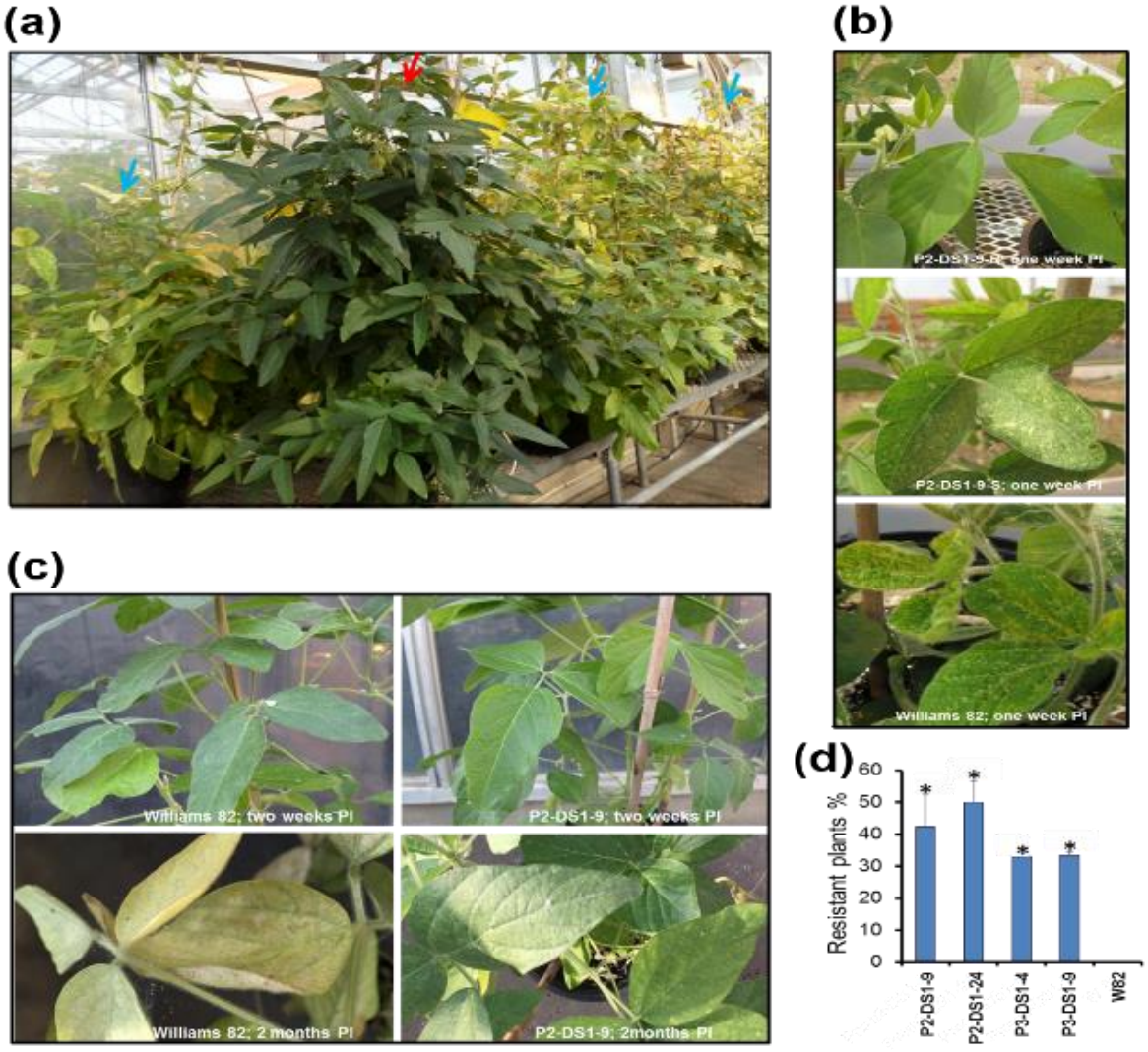


Ngaki et al. 2016

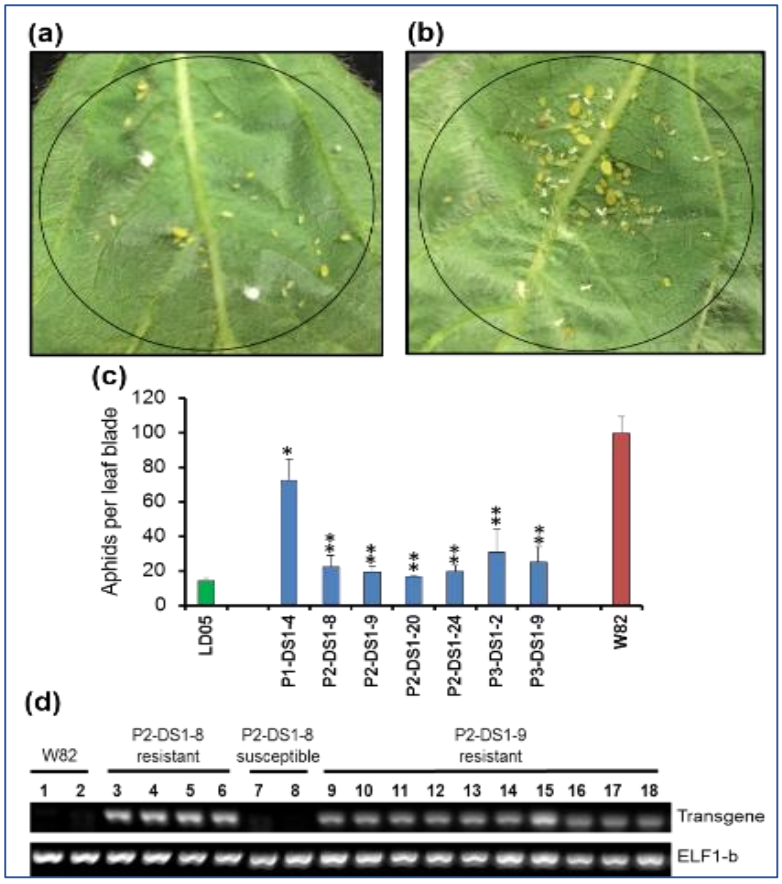
Overexpression of *GmDS1* gene led to generation of broad-spectrum disease and pest resistance



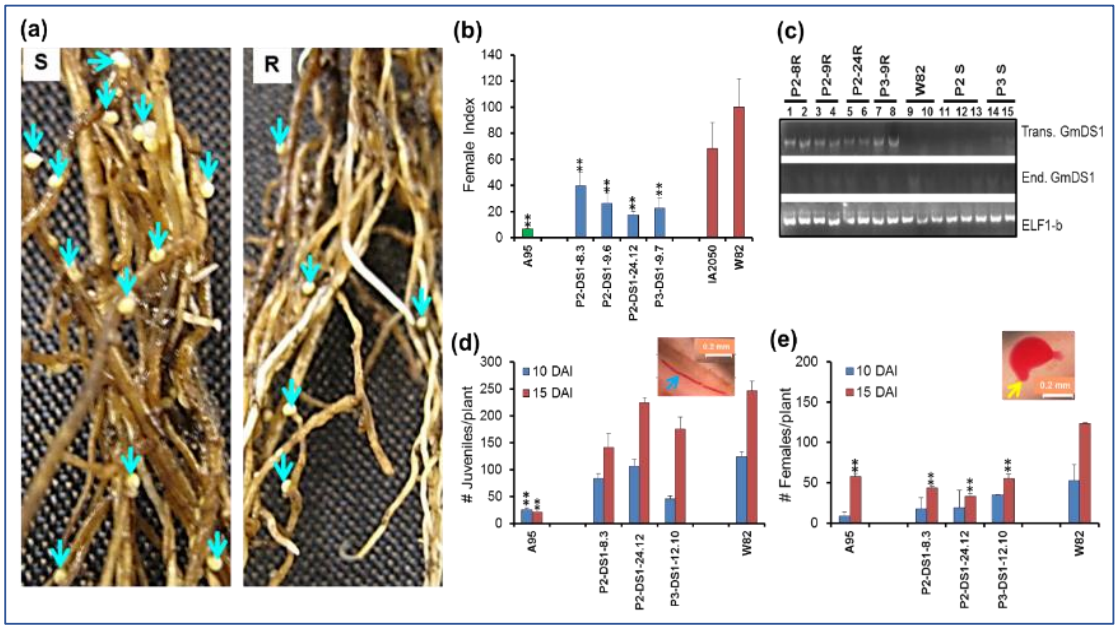
Overexpression of *GmDS1* conferred immunity of transgenic soybean plants against the spider mites



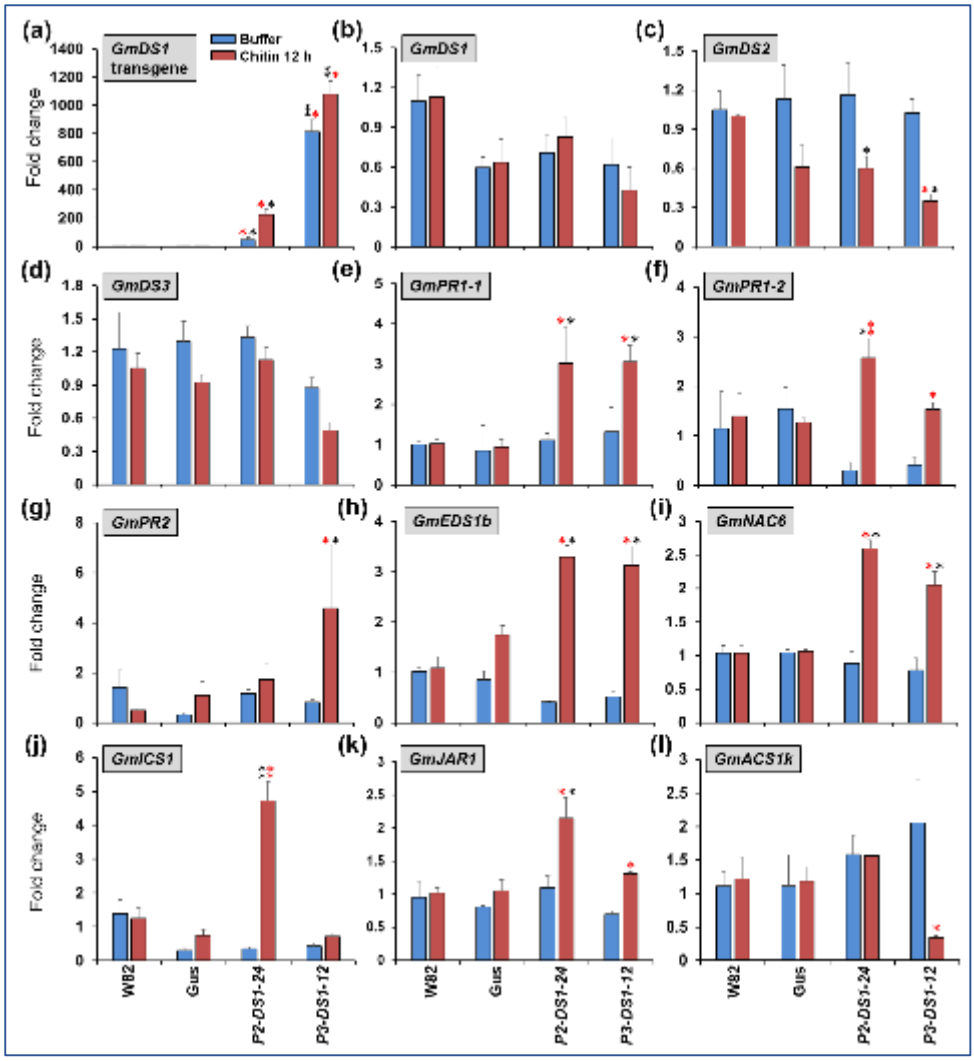
Overexpression of *GmDS1* conferred immunity of transgenic soybean plants against the soybean aphids



Overexpression of *GmDS1* conferred immunity of transgenic soybean plants against the soybean cyst nematode



Regulation of defense-related genes 12 h following treatment with chitin



Overexpression of *GmDS1*: A novel trait

Lecture

(Madan K. Bhattacharyya, Ph.D. Professor, Iowa State University)

Recent Advances in Plant Breeding

Restriction Fragment Length Polymorphism (RFLP)

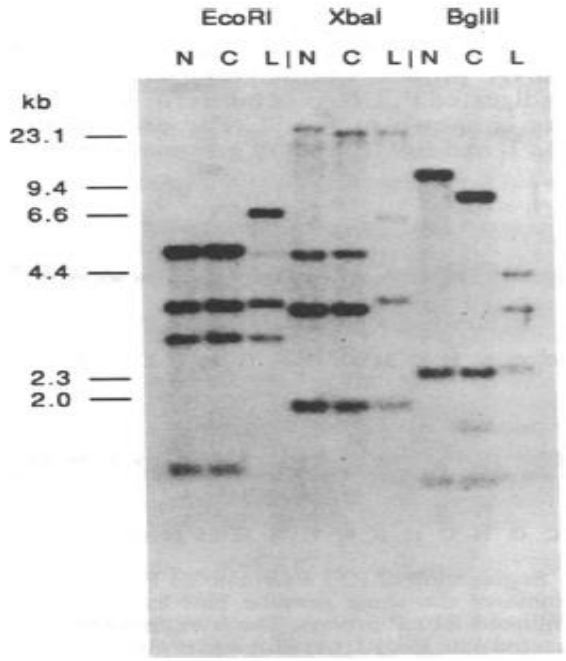
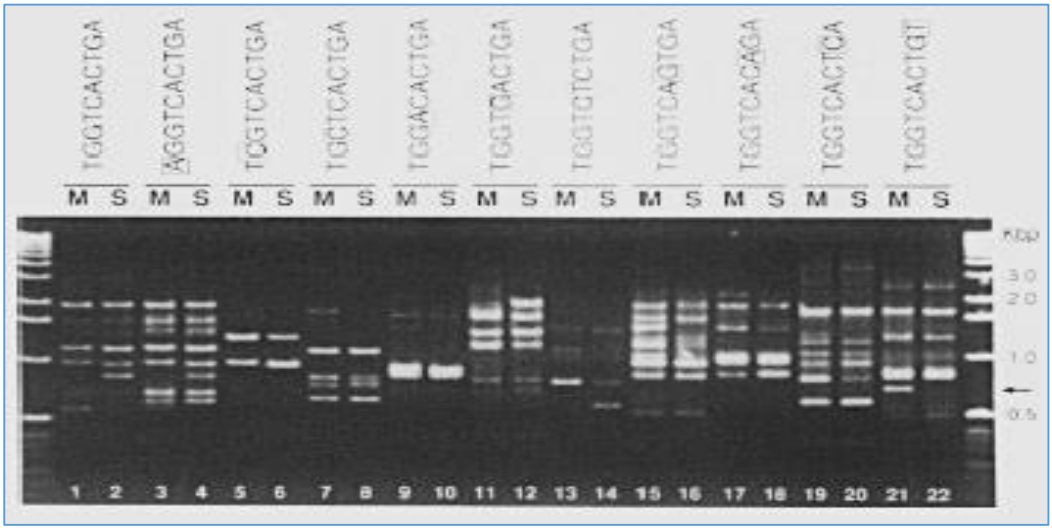
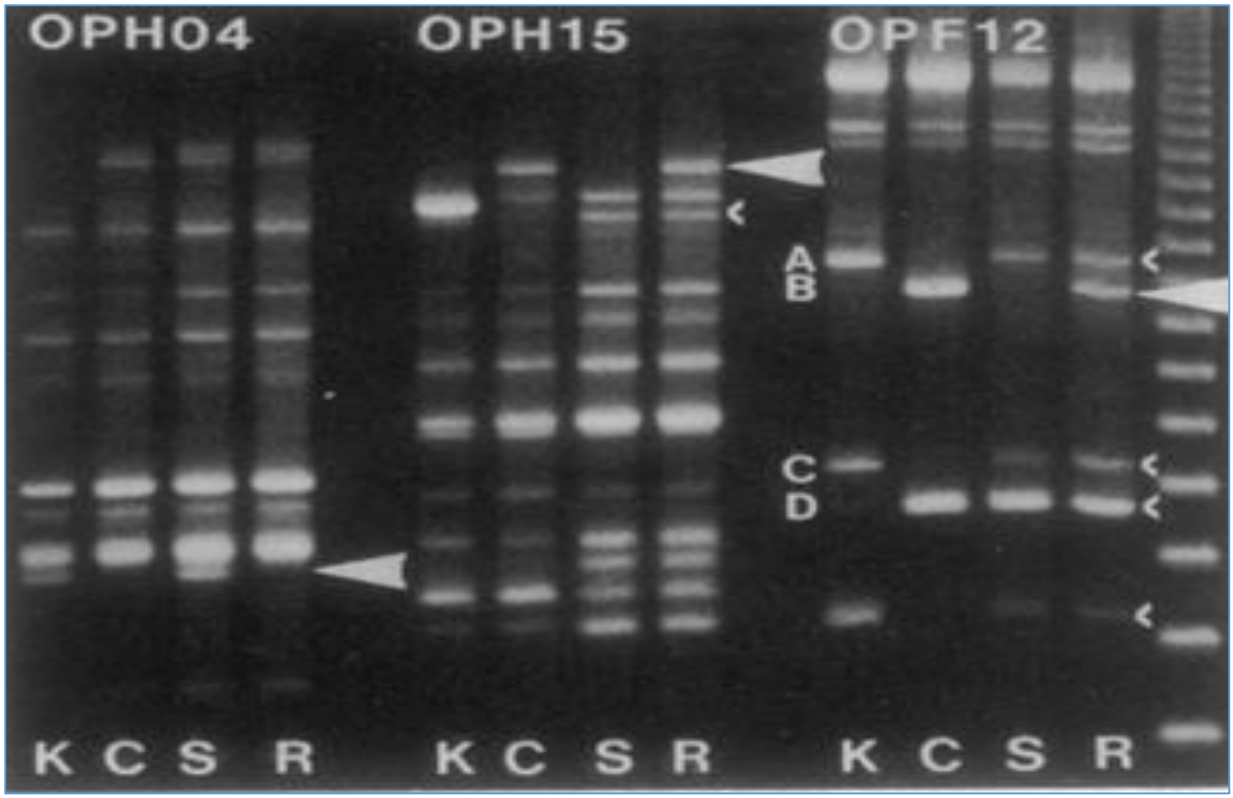


FIG. 1. Autoradiogram of a typical genomic blot to screen for RFLPs between different parental lines. The figure shows several RFLPs detected by clone 305 hybridized to Nd-0 (N), C (C), and La-0 *er* (L) DNA digested with *Eco*RI, *Xba* I, or *Bgl* II.

Random Amplified Polymorphic DNA (RAPD)

- A single 10 nucleotide oligo anneal in opposite orientation and amplifies DNA.





Simple Sequence Repeat (SSR)

SSR markers are usually co-dominant

Two SSR alleles differing in repeat numbers produce PCR products that can be separated on an agarose gel

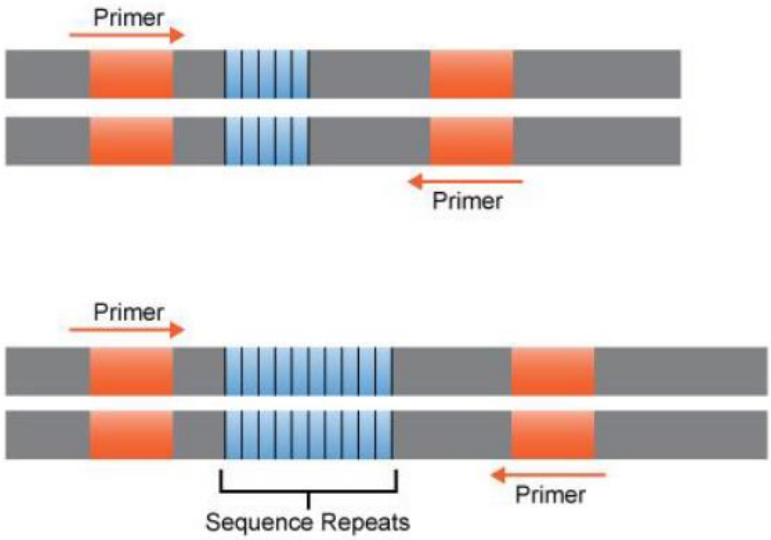
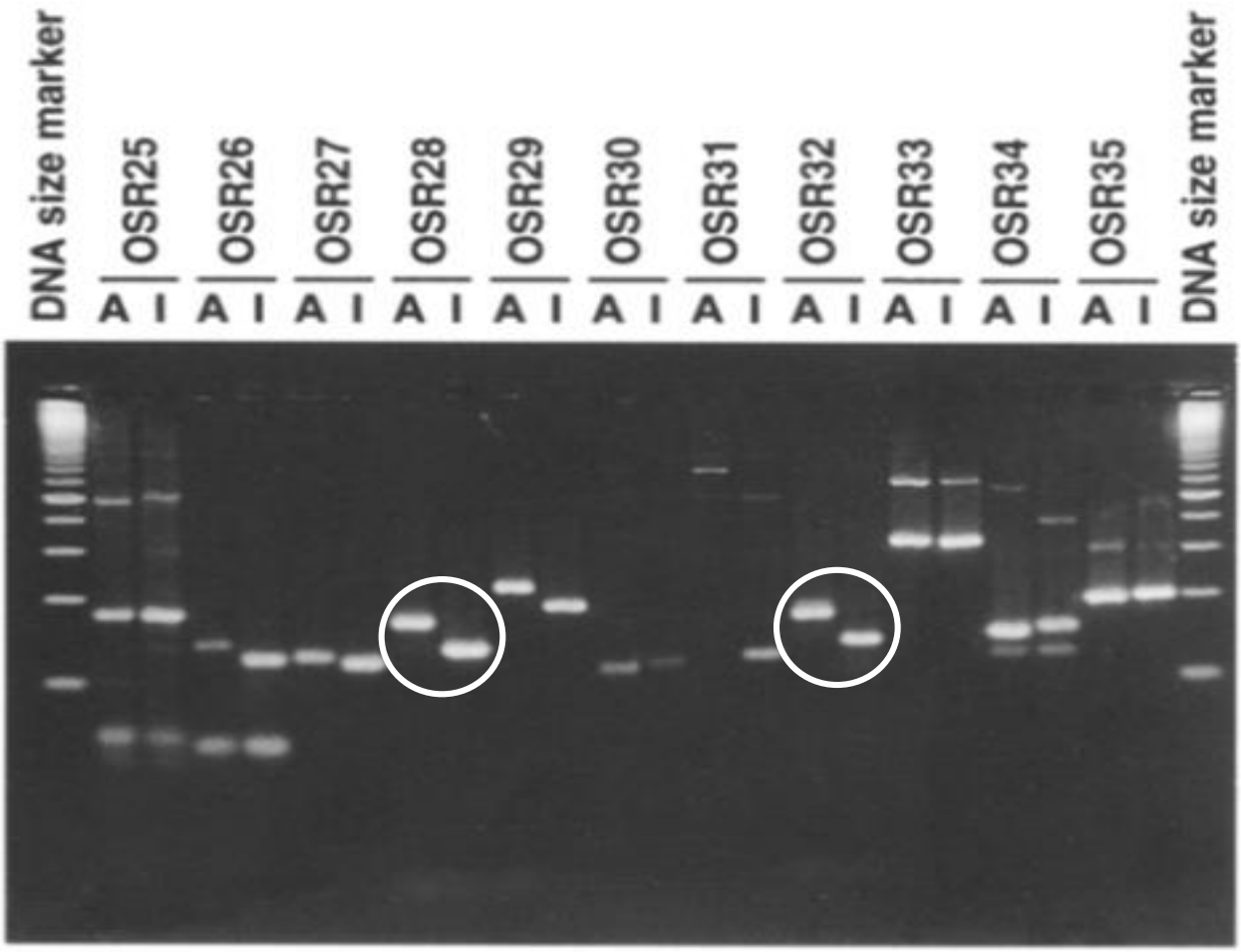
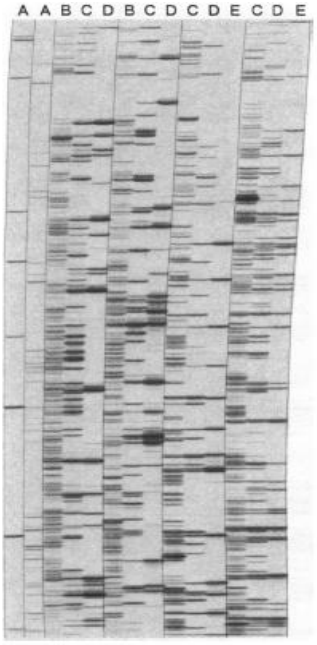


Fig 7.3 The SSR alleles 1 and 2 differ by the number of repeating DNA units. PCR primers are designed to amplify the region containing the DNA repeats. The difference in number of repeats in the two alleles causes a difference in the length of their PCR product, which can be distinguished when the PCR products are analyzed on a gel. An SSR marker should be closely linked to an allele for an important trait. Adapted from Chrispeels, M.J., & Sadava, D.E. (2003). Plants, Genes, and Crop Biotechnology. London. Jones and Bartlett Publishers.

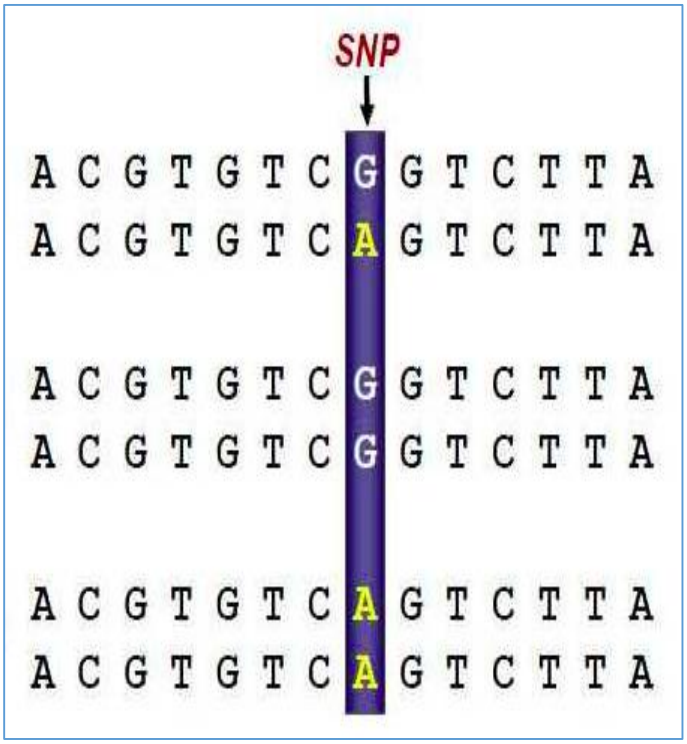


AFLP: Amplified Fragment Length Polymorphism



An AFLP Gel: Radioactive label is used to detect the PCR amplified fragments

Single Nucleotide Polymorphism (SNP)

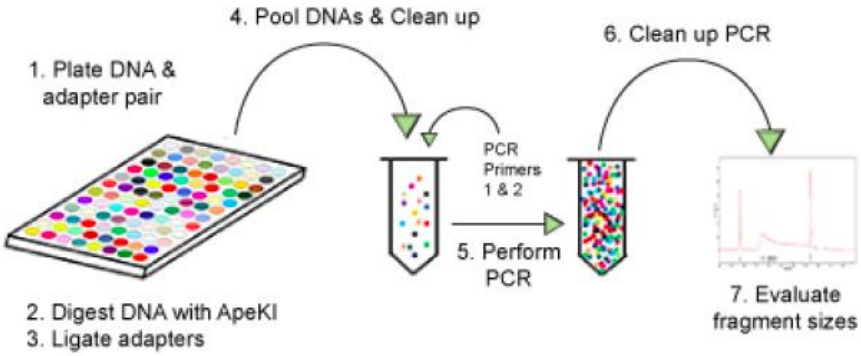


Heterozygote

Homozygote

Homozygote

Genotype by Sequencing



B. Adapter and sequencing primer design

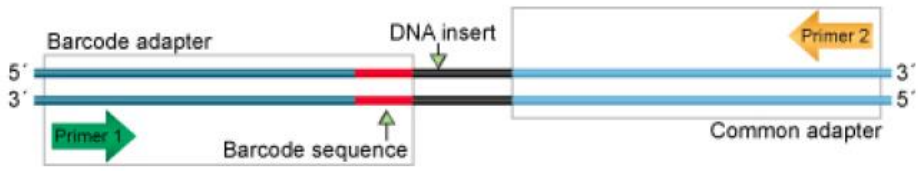


Fig. 9.1 Genotyping-by-sequencing in plants. (A) Library construction involves plating the DNA and adapter pair, digestion with a restriction enzyme (in other cases two restriction enzymes are used), and ligation of adapters to the ends of DNA fragments. Samples are pooled and cleaned up before PCR. The PCR products are also cleaned up and evaluated for quality by a DNA analyzer. (B) A barcode adapter and a common adapter flank the DNA insert to be sequenced. Primers 1 and 2 bind specific sequences on the 3' ends of the barcode and common adapters, respectively. Adapted from Elshire et al. (2011).

SBP: Sequenced-based Polymorphic marker

Comparison of sequences from two Arabidopsis ecotypes

```
Query: 4590 cggagcttccaaaaaagccataaacttcatccaatcttcttgcaaaaccaccacataccc  4649
            ||||||||| || ||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1    cggagcttctaagaaagccataaacttcatccaatcttcttgcaaaaccaccacataccc  60

Query: 4650 tgccttatgtgtcca 4664
             ||||||||| |||||
Sbjct: 61   agccttatgcgtcca 75

Query: 4581 ctcacagaccggagcttccaaaaaagccataaacttcatccaatcttcttgcaaaaccac  4640
            |||||||||||||||||| || ||||||||||||||||||||||||||||| ||||||
Sbjct: 1    ctcacagaccggagcttctaagaaagccataaacttcatccaatcttcttgcataaccac  60

Query: 4641 cacataccctgcctt 4655
            ||||||||| |||||
Sbjct: 61   cacatacccagcctt 75

Query: 4561 ctcacggcggccaccgcagcctcacagaccggagcttccaaaaaagccataaacttcatc  4620
            |||||||||||||||||||||||||||||||||||| || ||||||||||||||||||
Sbjct: 1    ctcacggcggccaccgcagcctcacagaccggagcttctaagaaagccataaacttcatc  60

Query: 4621 caatcttcttgcaaa 4635
            |||||||||||||||
Sbjct: 61   caatcttcttgcaaa 75
```

Sequence-based polymorphic (SBP) Markers that are polymorphic between Columbia - 0 and Neiderzenz ecotypes

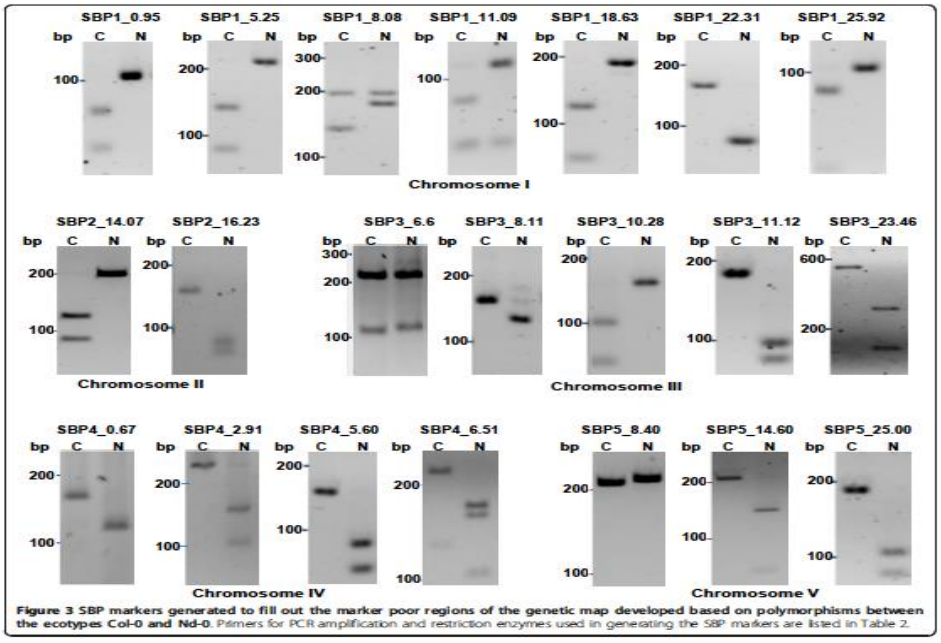
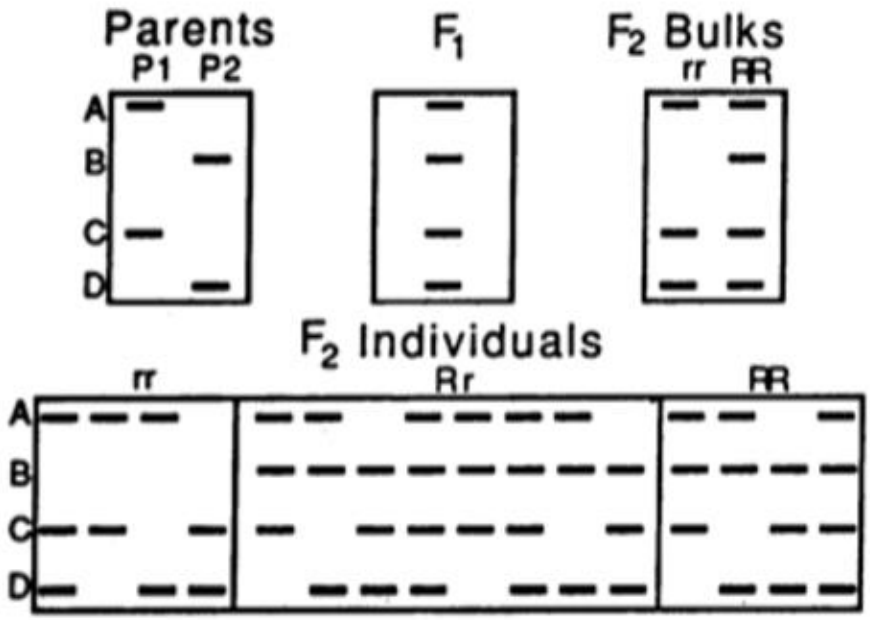


Figure 3 SBP markers generated to fill out the marker poor regions of the genetic map developed based on polymorphisms between the ecotypes Col-0 and Nd-0. Primers for PCR amplification and restriction enzymes used in generating the SBP markers are listed in Table 2.

Sahu et al. 2012

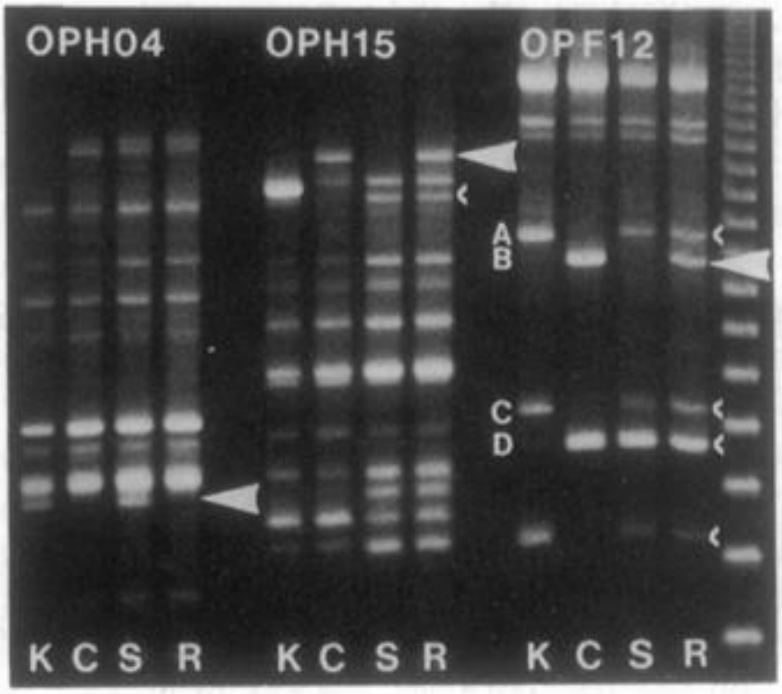
Rapid Identification of Linked Molecular Markers

Bulked segregant Analysis



Michilemore et al. 1991

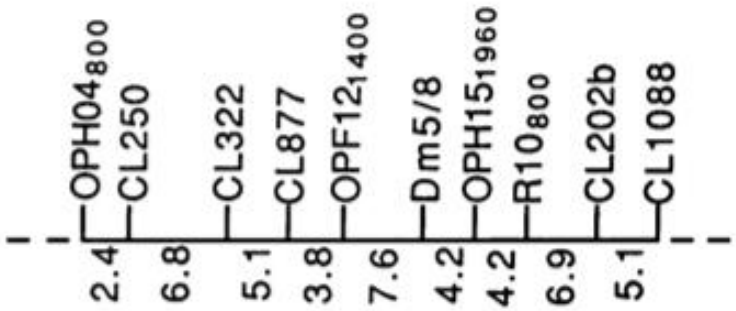
RAPD markers identified for the *Dm5/8* region using BSA method in lettuce



Segregation of five SSR markers among 17 recombinant inbred lines RIL (F_7)

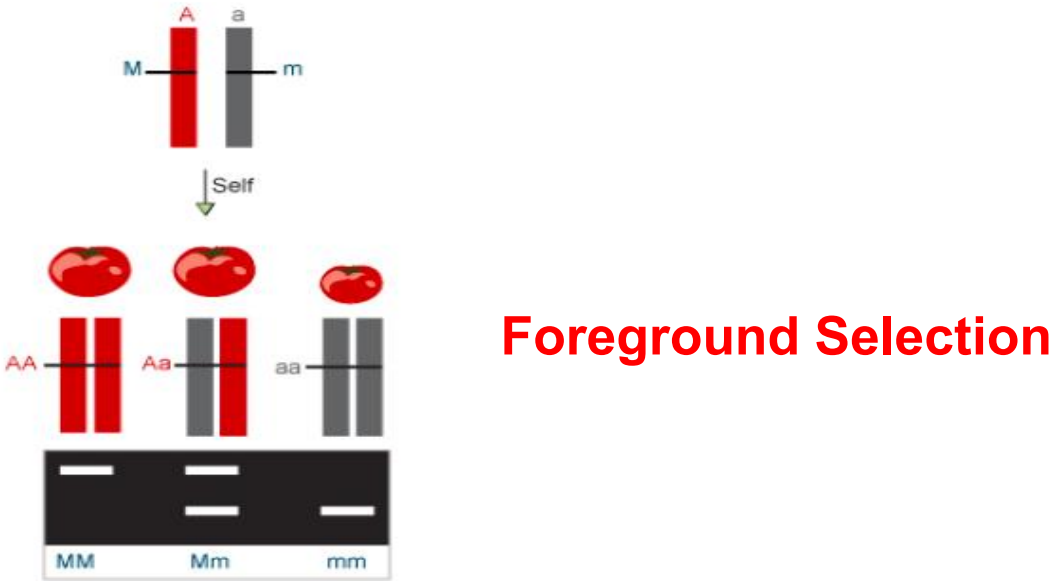
Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Marker 1	A	A	A	A	A	A	A	B	B	B	B	A	A	A	A	A	A
Marker 2	A	A	A	A	A	A	B	B	B	B	B	A	A	A	A	A	A
Marker 3	B	B	B	B	A	A	A	A	A	A	A	A	B	B	B	B	B
Marker 4	B	B	B	B	A	A	A	A	A	A	A	A	B	B	A	B	B
Marker 5	B	B	A	A	A	B	B	A	A	A	B	B	B	A	A	A	B

Genetic map developed for the *Dm5/8* region using BSA method in lettuce



Molecular marker assisted selection

Molecular marker linked tightly to the fruit size trait for identifying homozygous (*AA*) lines



Molecular marker linked tightly to the male sterility trait for identifying the heterozygous (*Msms*) lines

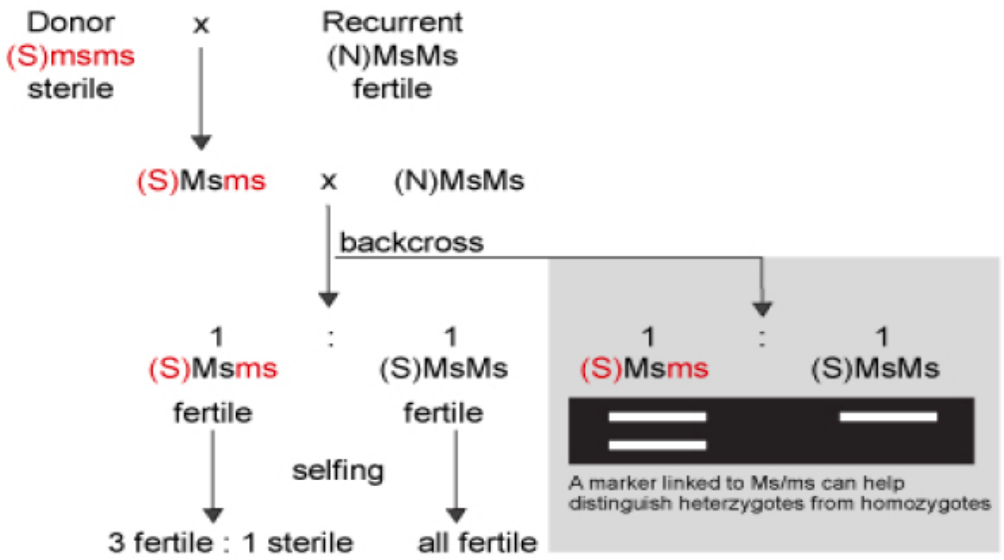
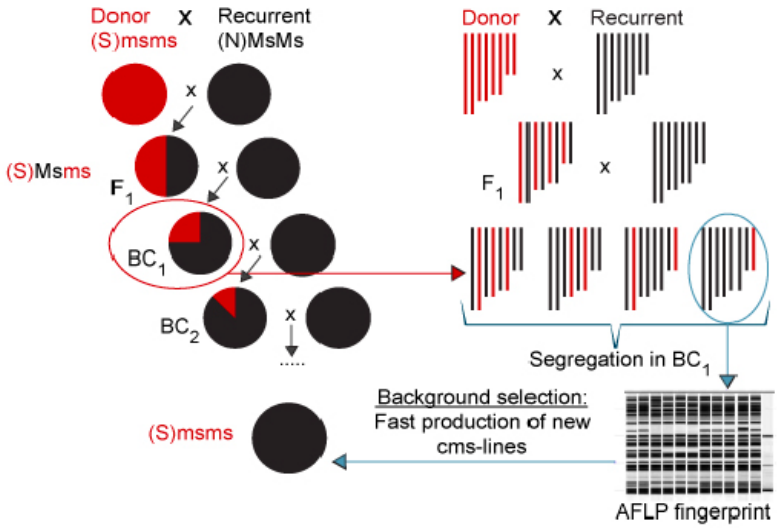


Fig. 8.16 The use of molecular markers for foreground selection. Backcross of (S)Msms to (N)MsMs produces fertile plants, but of different genotypes (Msms or MsMs). Selfing the MsMs BC1 progeny will produce all MsMs fertile plants. Selfing of BC1 Msms progeny will produce fertile and sterile plants in the ratio of 3:1. The use of a linked marker will help eliminate additional work to self and phenotypic screening of the plants.

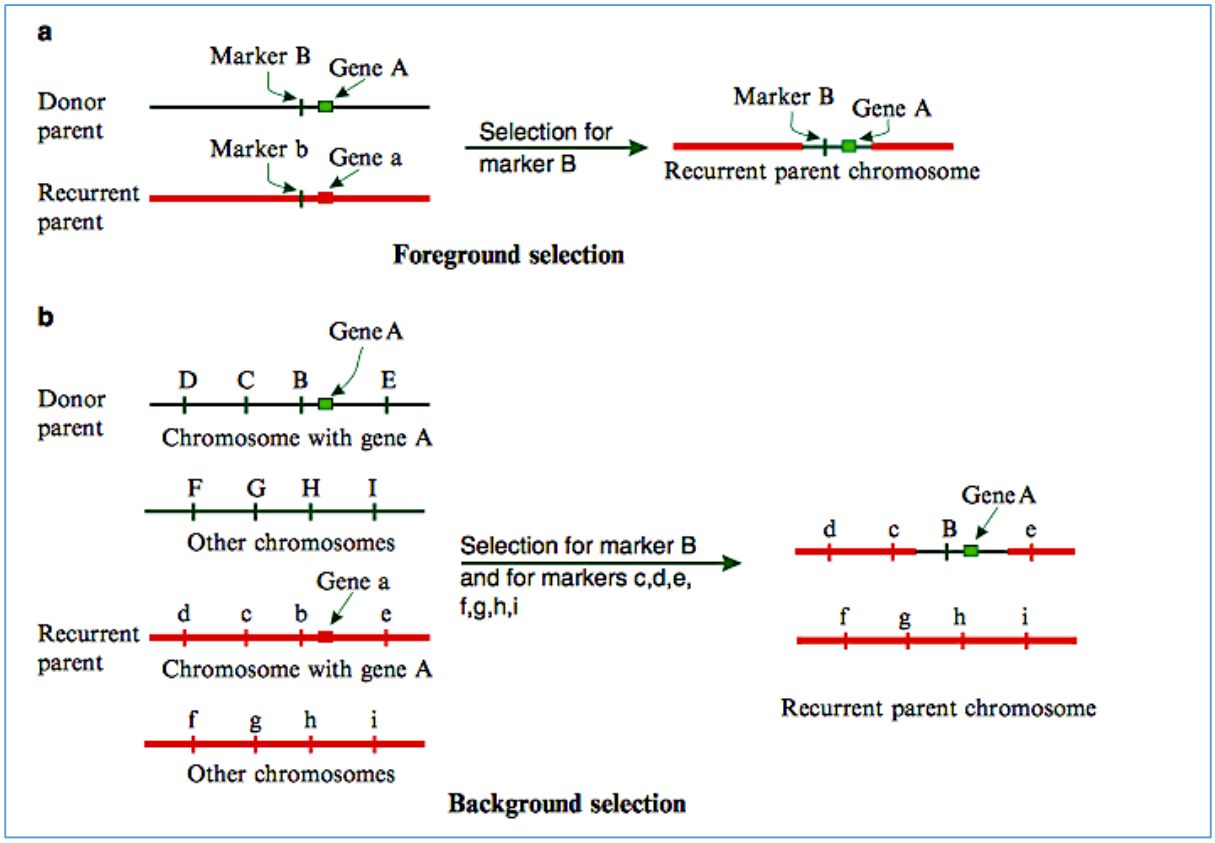
Marker-assisted backcrossing



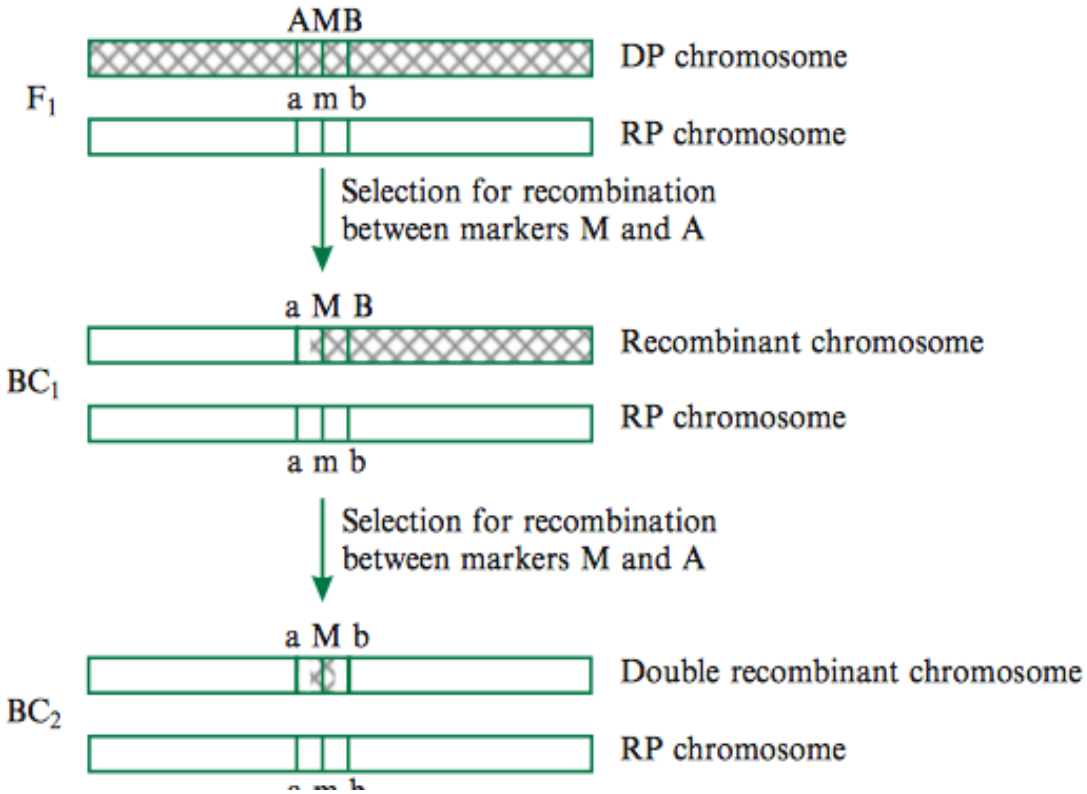
Background Selection

Fig. 8.15 Development of male-sterility by marker-assisted backcrossing in maize. A male sterile donor is crossed with a fertile recurrent parent. Red and black charts depict the proportions of donor and recurrent parent genomes respectively. Red and black bars depict chromosome segments of donor and recurrent parent respectively. Progeny containing largest proportion of recurrent parent genome can be detected as early as in the BC1 generation (red circle) using molecular markers and genetic fingerprinting (blue circle). Overall, the use of markers helps increase the pace of production of new male sterile lines.

Foreground and Background Selection



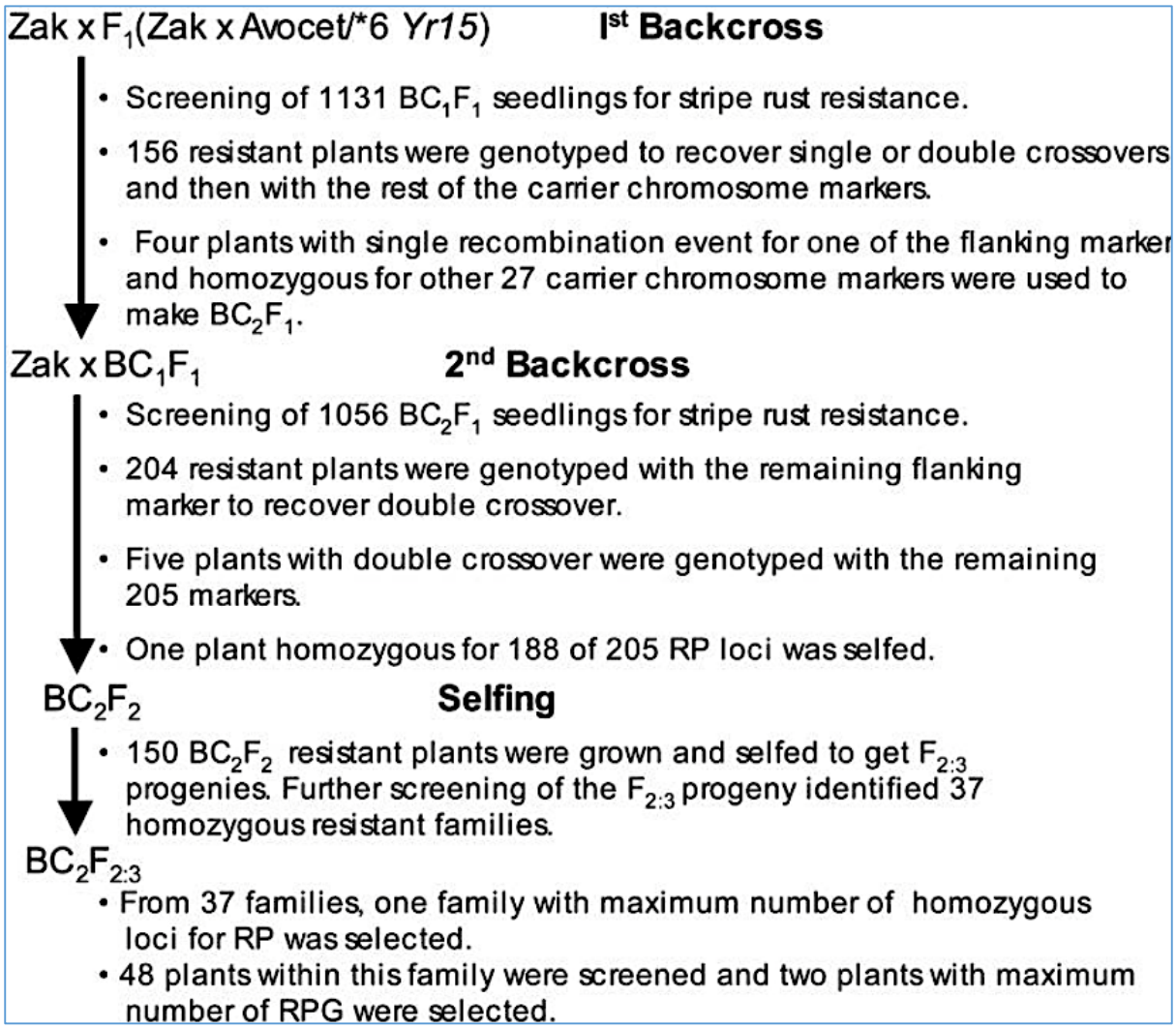
Recover the Double Recombinant to Eliminate any Linkage Drag



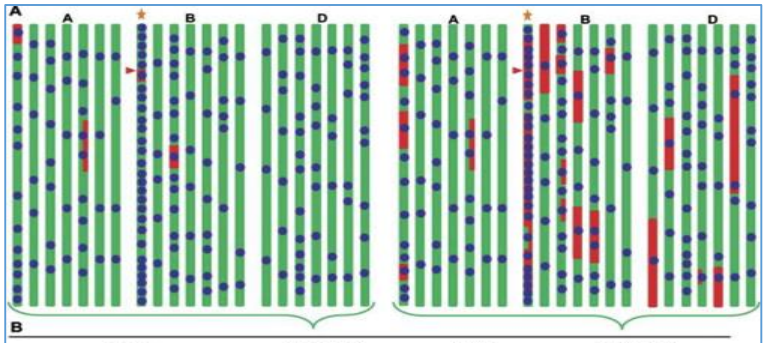
Marker-assisted Selection

- In this study Randhawa et al. 2009 identified a **$BC_2F_{2:3}$** plant with **97%** of the recurrent parent genome through marker-assietd background selection (MABS).
- In contrast, only **82%** of the recurrent parent genome was recovered in phenotypically selected **BC_4F_7** plants developed without MABS.

Marker-assisted selection can also be applied to expedite pedigree or single seed descent method of breeding, if most desirable trait loci linked markers are known

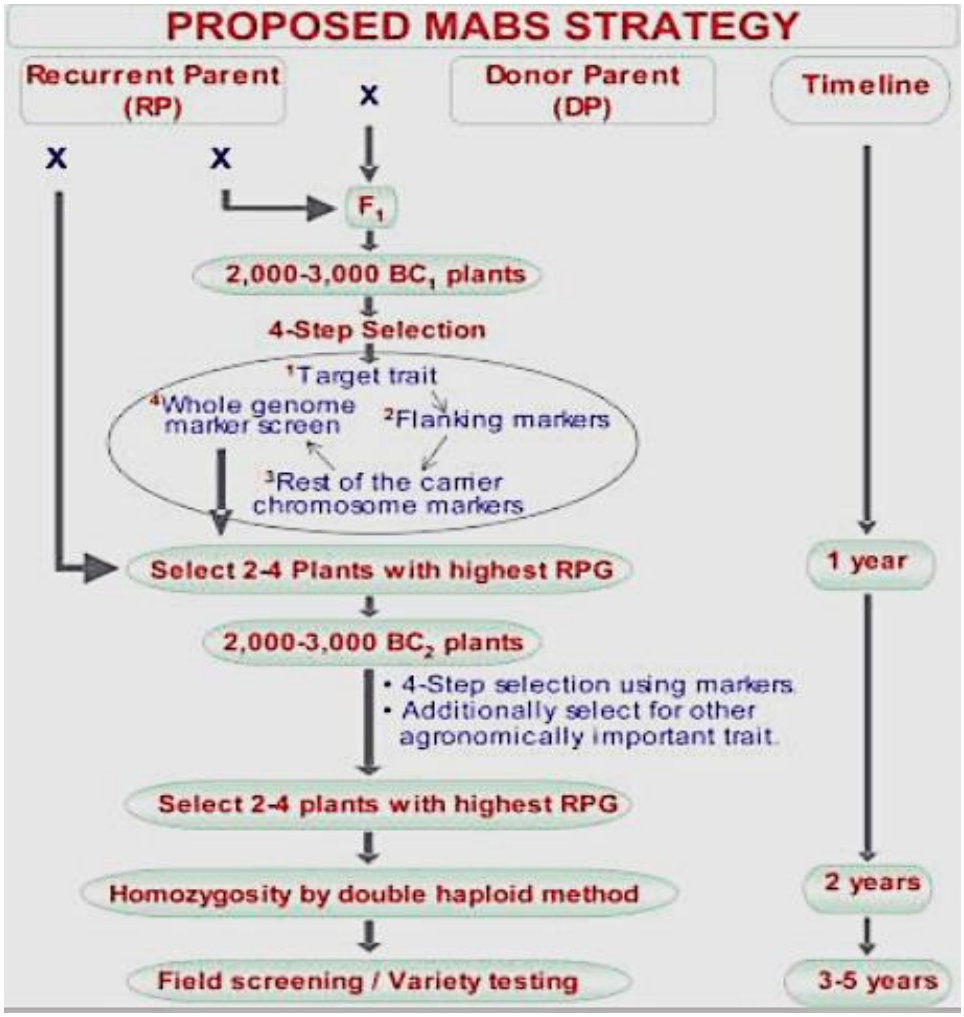


Comparison of cultivar 'Zak' derivatives carrying stripe rust resistance gene *Yr15*developed with (WA8059) and without (WA8046) MABS



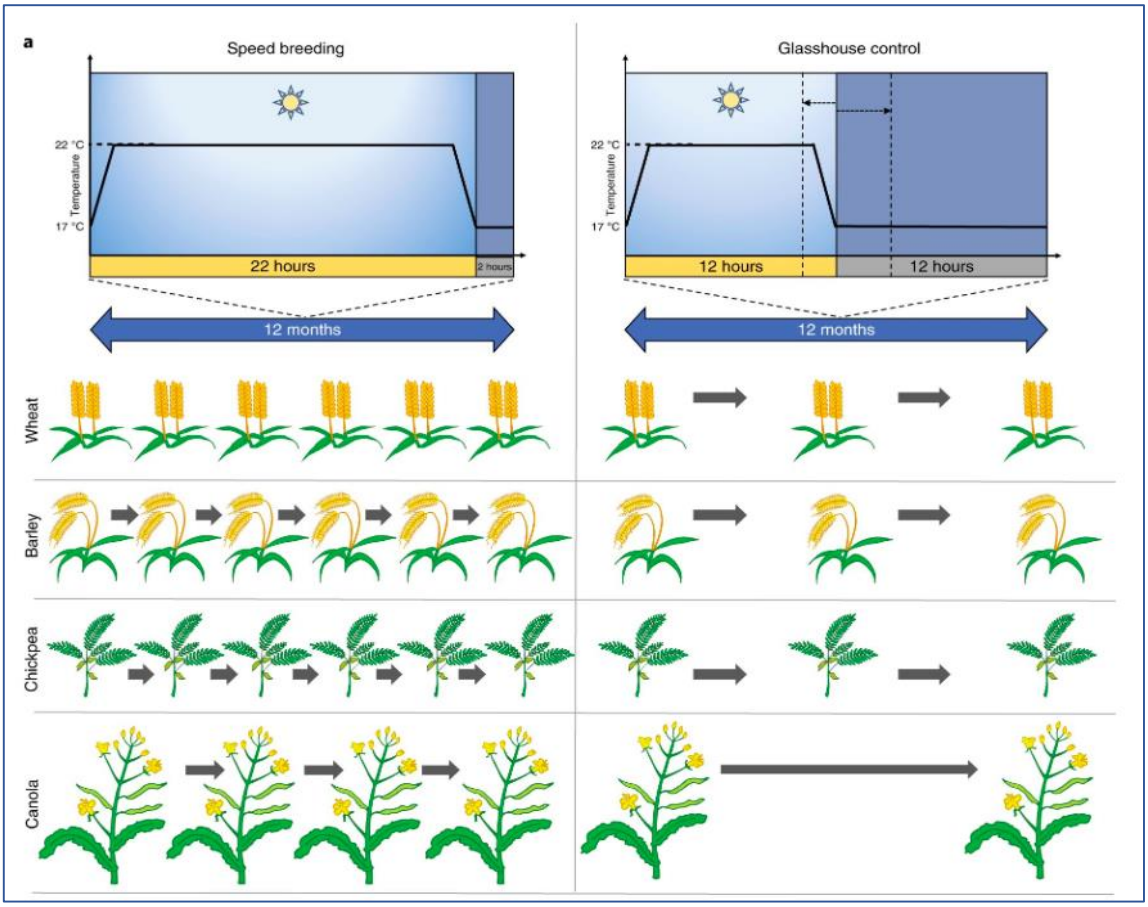
Trait	WA8059	ZAK	WA8046
Yield*	81	78.1	74.4
Test Weight*	60.6	59.1	58.7
Wheat protein*	11.7	11.1	11.4
Single kernel hardness †	25.8	20.2	20.1
Single kernel weight †	35.2	34.4	33
SKWTSD †	7.2	7.7	8.3
Flour yield †	70.1	70.2	68.4
Milling score †	87.8	87	82.4
Flour ash †	0.36	0.37	0.41
Flour protein †	9.8	8.5	9.3
Flour swelling volume †	18.4	20.4	18.3
Mixograph absorption †	53.3	52.8	55.4
Cookie diameter †	9.6	9.9	9.6

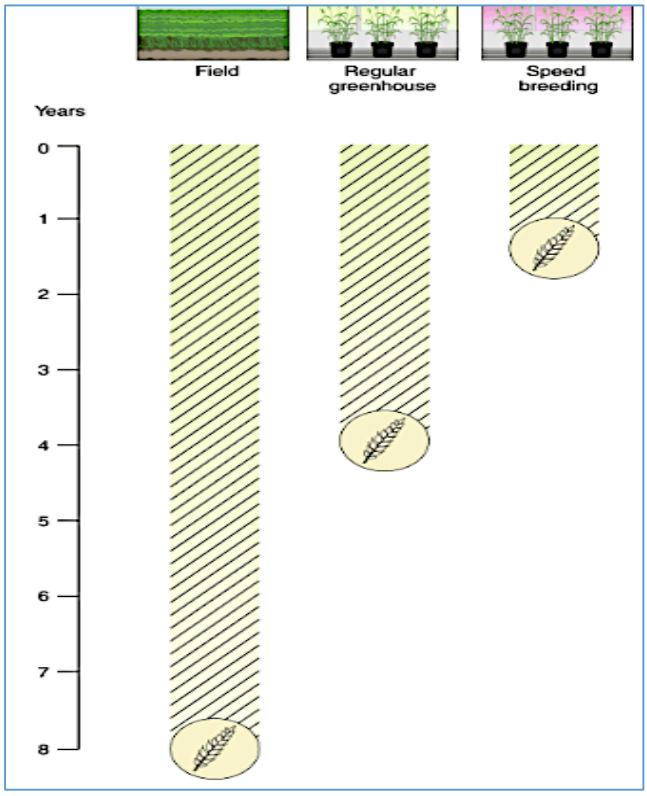
*Corresponds to the value averaged over three locations, † Corresponds to the value averaged over two locations, SKWTSD: Single kernel weight standard deviation.



Speed Breeding

Speed breeding accelerates generation time of major crop plants for research and breeding

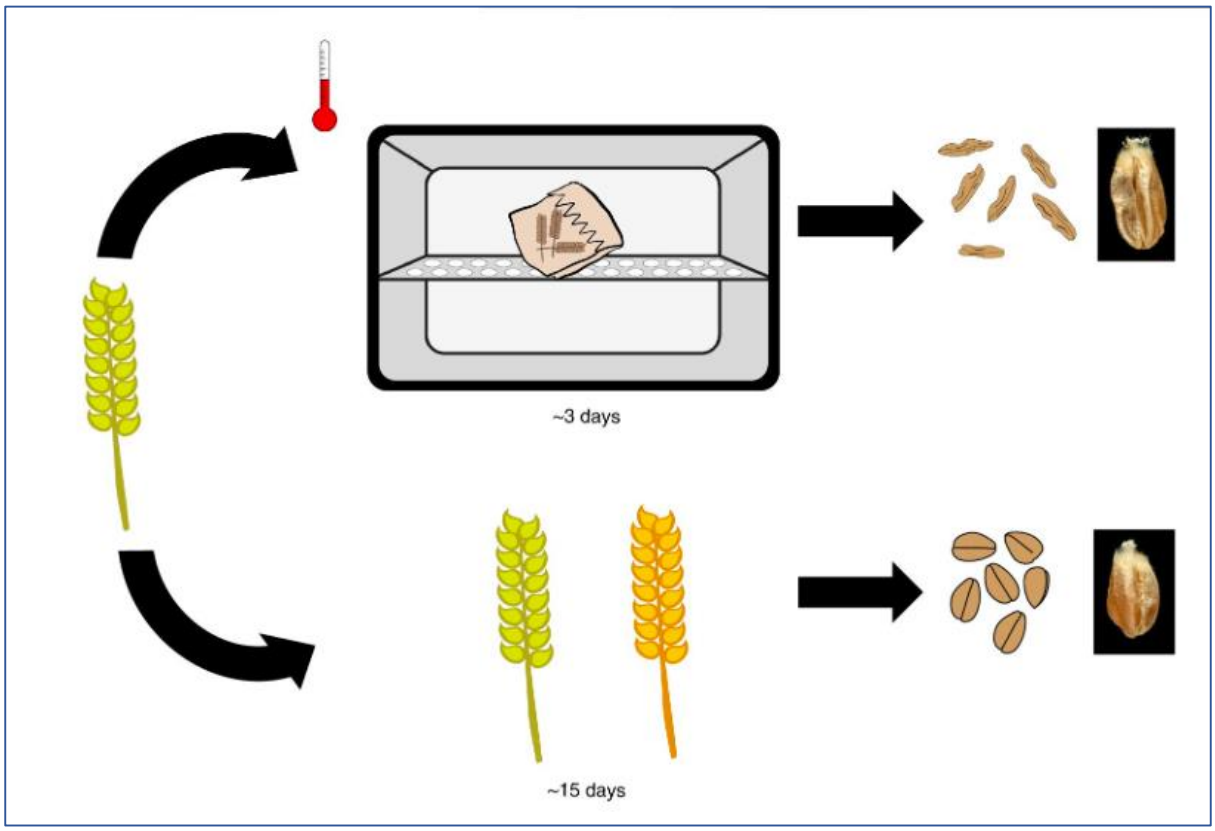




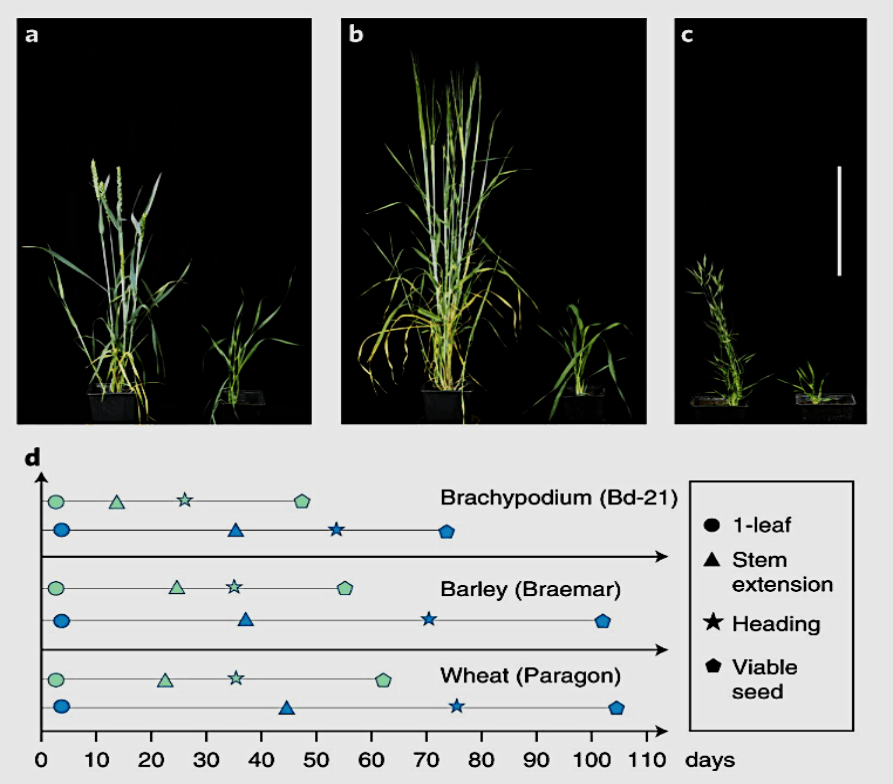
Steps to follow in Speed Breeding

1. **Lights Quality:** Light that produces a spectrum covering the PAR region (400–700 nm), with particular focus on the blue, red and far-red ranges, is suitable to use for SB.
2. **Light Quantity:** Intensity should very high: ~450–500 µmol/m2/s at plant canopy height effective for a range of crop species.
3. **Photoperiod:** We recommend a photoperiod of 22 h with 2 h of darkness in a 24-h diurnal cycle. The dark period slightly improves plant health.
4. **Temperature:** The optimal temperature regime (maximum and minimum temperatures) should be applied for each crop. A higher temperature should be maintained during the photoperiod, whereas a fall in temperature during the dark period can aid in stress recovery. A 12-h 22 °C/17 °C temperature cycling regime with 2 h of darkness occurring within 12 h of 17 °C has proven successful. A temperature cycling regime of 22 °C/17 °C for 22 h of light and 2 h of dark, respectively also work fine.
5. **Humidity:** Most controlled-environment chambers have limited control over humidity, but a reasonable range of 60–70% is ideal. For crops that are more adapted to drier conditions, a lower humidity level may be advisable

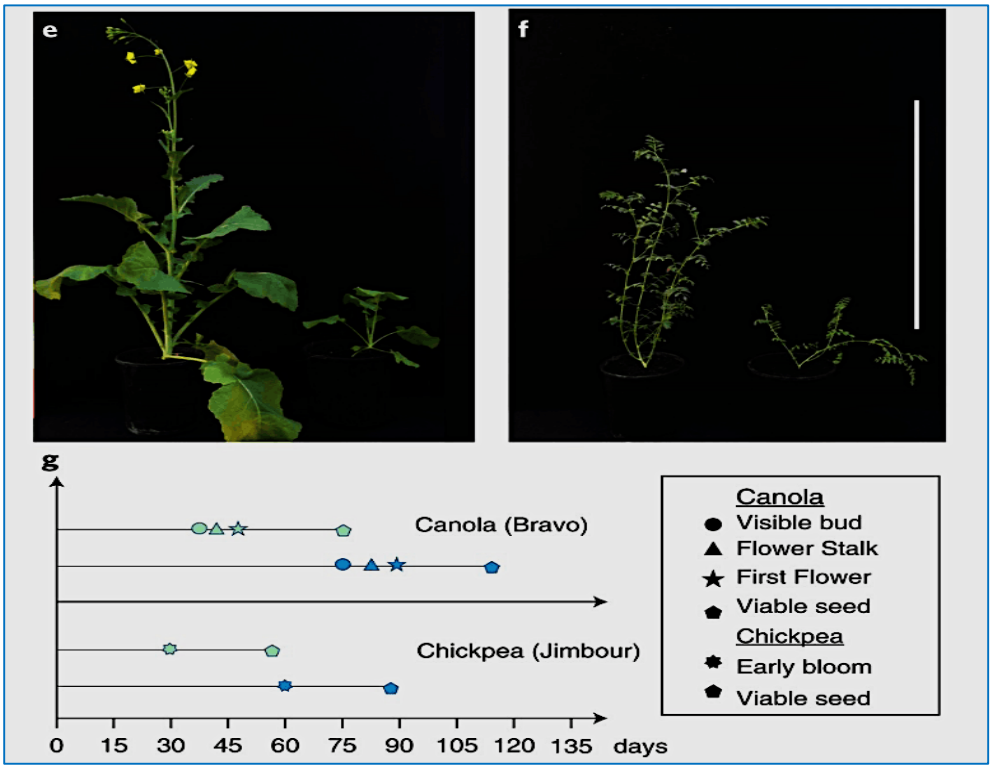
Harvesting of immature spikes and drying them in an oven/dehydrator saves 12 days (3 vs. 15 days)



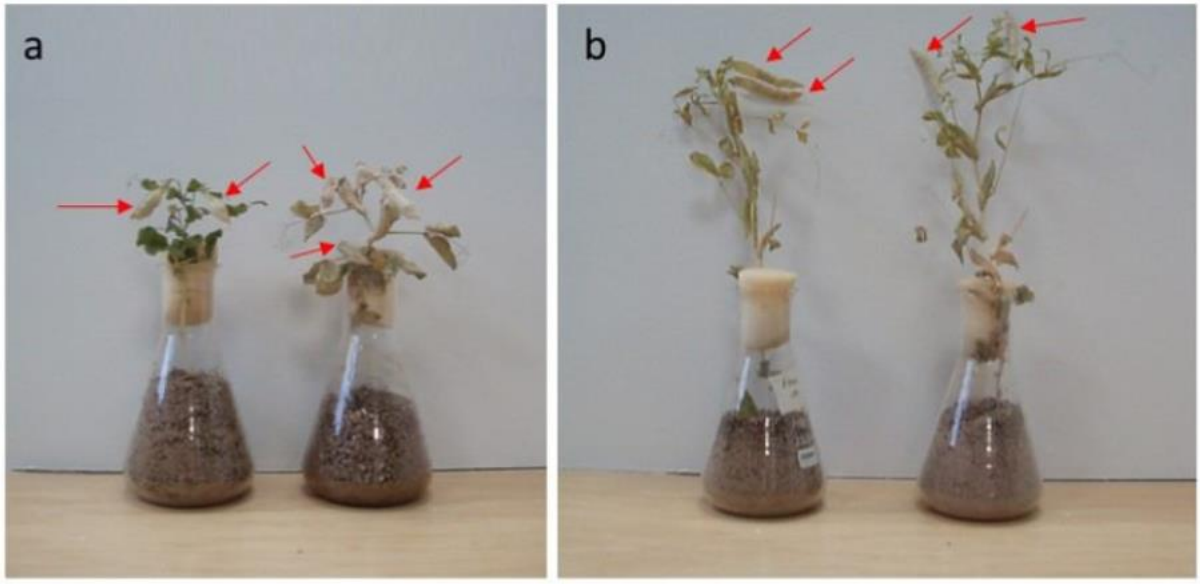
Accelerated plant growth and development under speed breeding (left) compared to control conditions (right)



Accelerated plant growth and development under speed breeding (left) compared to control conditions (right)



Peas mature in 8 instead of 12 weeks in greenhouse under normal condition



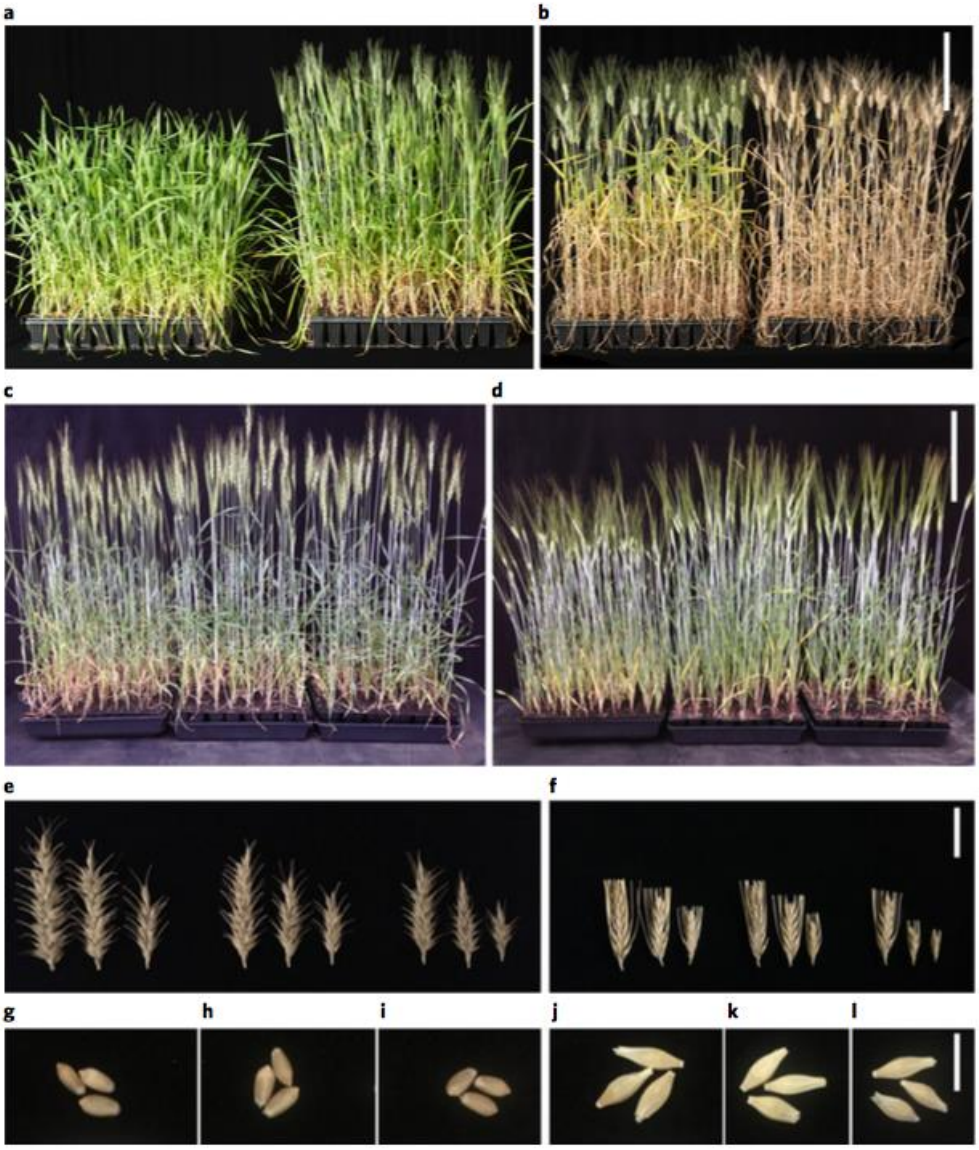
Pea plants grown in limited media and nutrition (“flask method”) in order to achieve rapid generation advancement

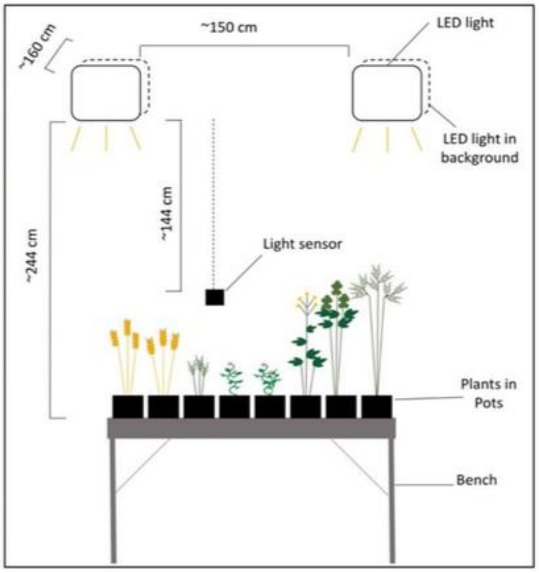
Pods harvested from *Brassica napus* RV31 grown in LED-supplemented glasshouses at the John Innes Centre, UK

22-hour photoperiod

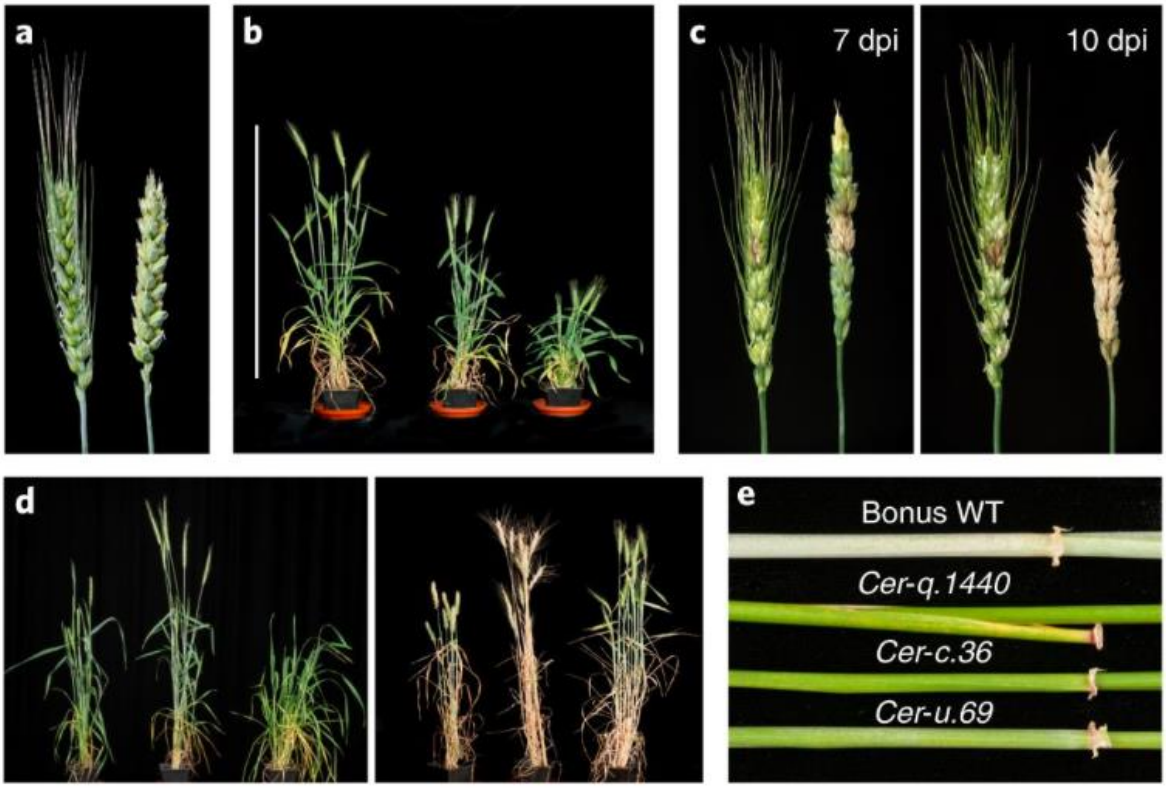
16- hour photoperiod

Single-seed descent sowing densities of spring wheat (bread and durum) and barley





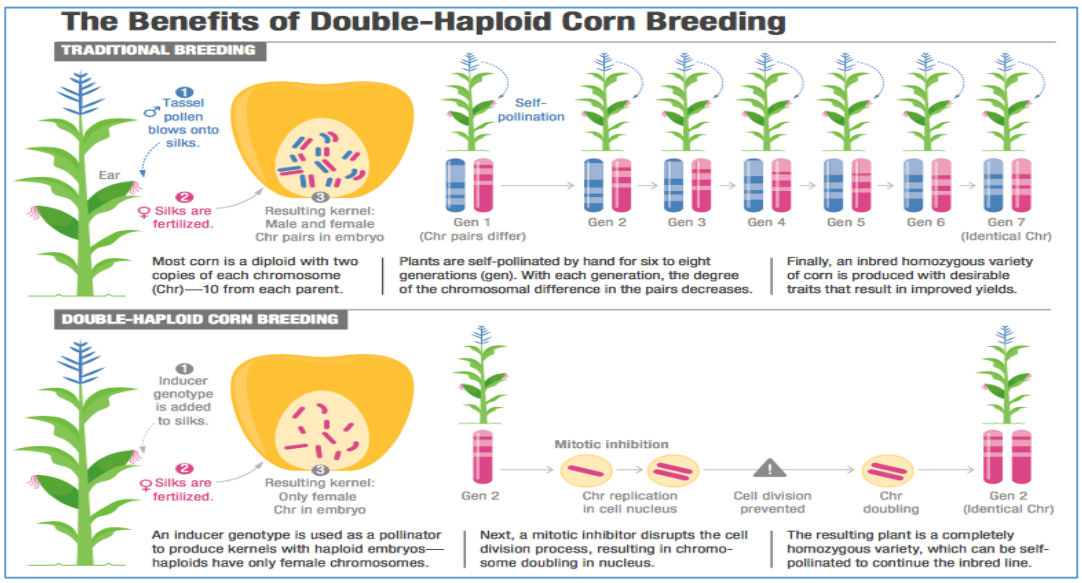
Adult plant phenotypes in wheat and barley under speed breeding conditions



Speed Breeding Expedites the Development of Novel Cultivars

- One can now generate novel cultivars very rapidly. Four to six crops a year?
- Single seed descent (SSD) method can be pursued under speed breeding without any trade off.
- In fact, becomes less expensive to grow thousands of lines densely under greenhouse condition for SSD.
- Selection can be made for disease resistance in adult plants.
- General methodologies work for most if not all crop plants; but modification can help.

Double Haploid

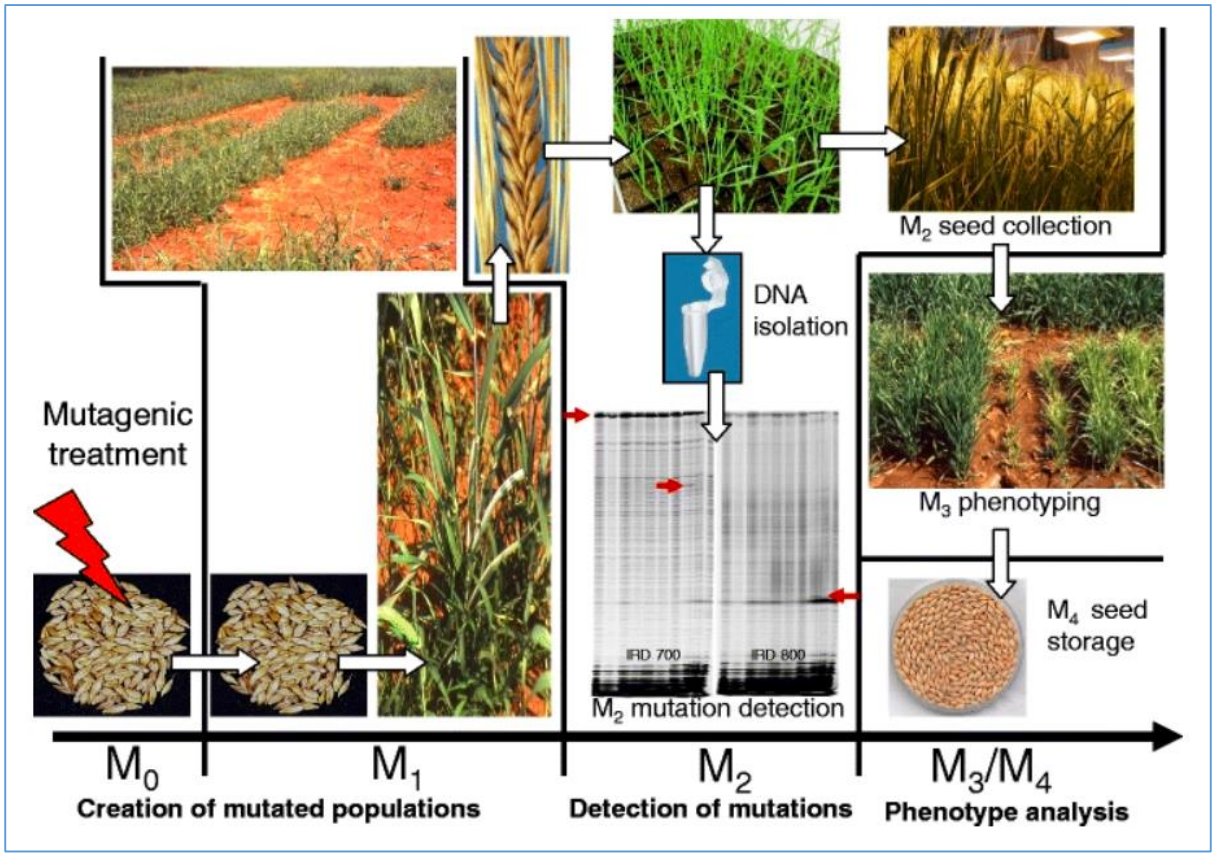


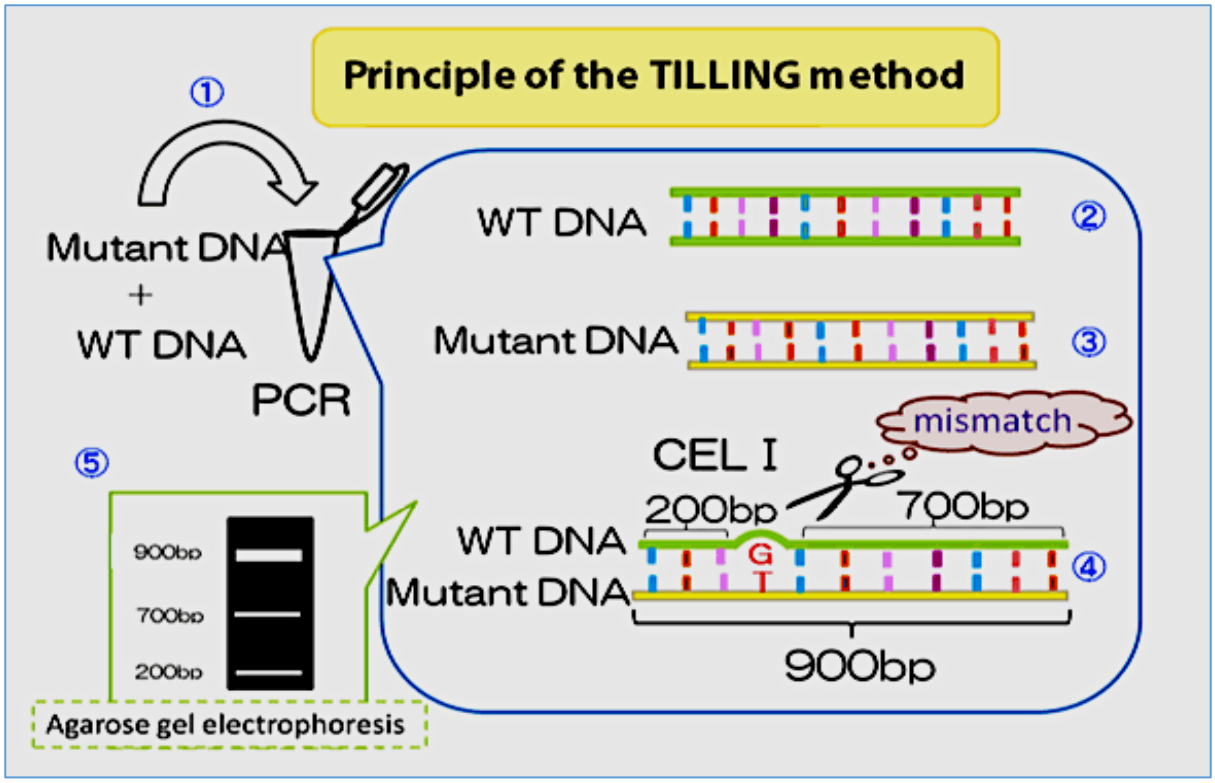
Doubled-haploid to Expedite the Breeding Program

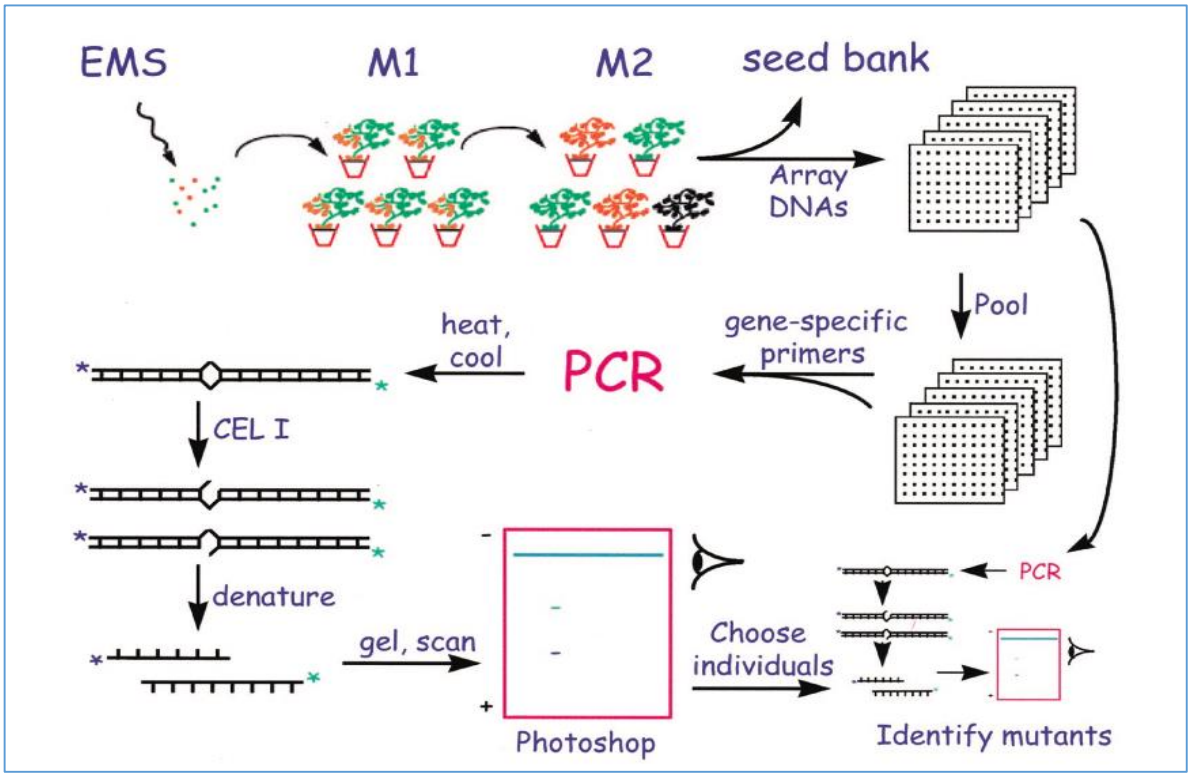
- We need to fix the inbred lines for heterosis breeding.
- Doubled-haploid approach fix the genome in one generation.
- Process is time and labor-intensive.
- Recombination in one step as opposed to additional recombination in selfing generations of the pedigree/SSD method.

Targeting Induced Local Lesions IN Genomes (TILLING)

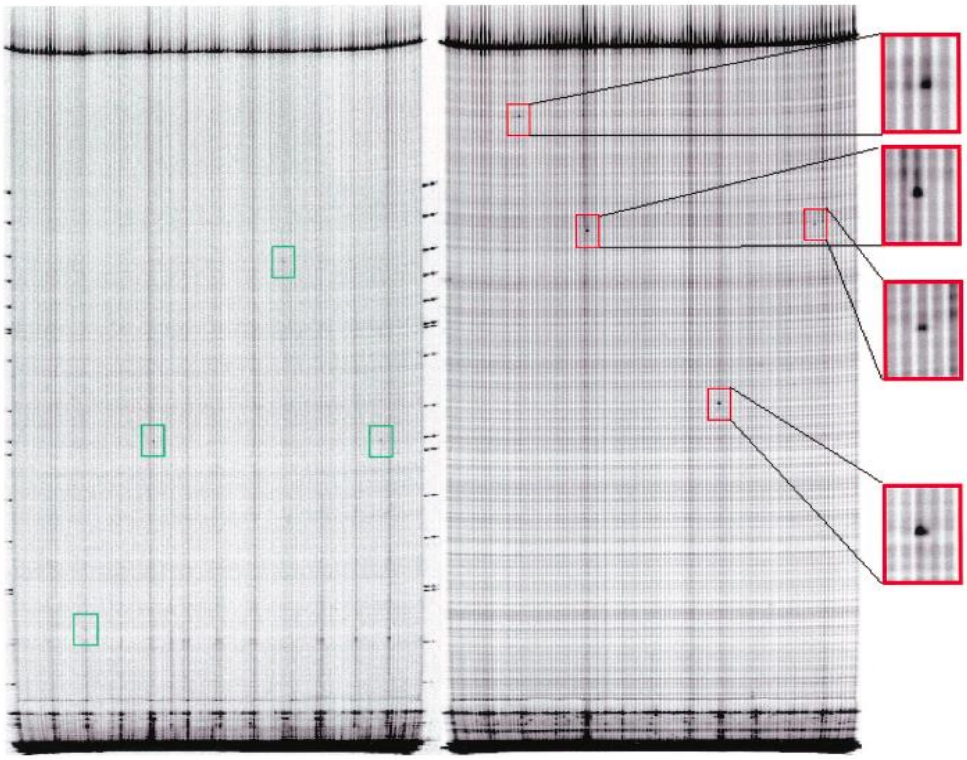
1. Intended for reverse genetics – means we look for mutations in the target gene.
2. The resource can also be screened/phenotyped for traits of interest and apply positional gene cloning – forward genetics.
3. Applied extensively across crop species.
4. One can create desirable mutants for a trait gene instead of gene silencing or knockout or editing – GMO free



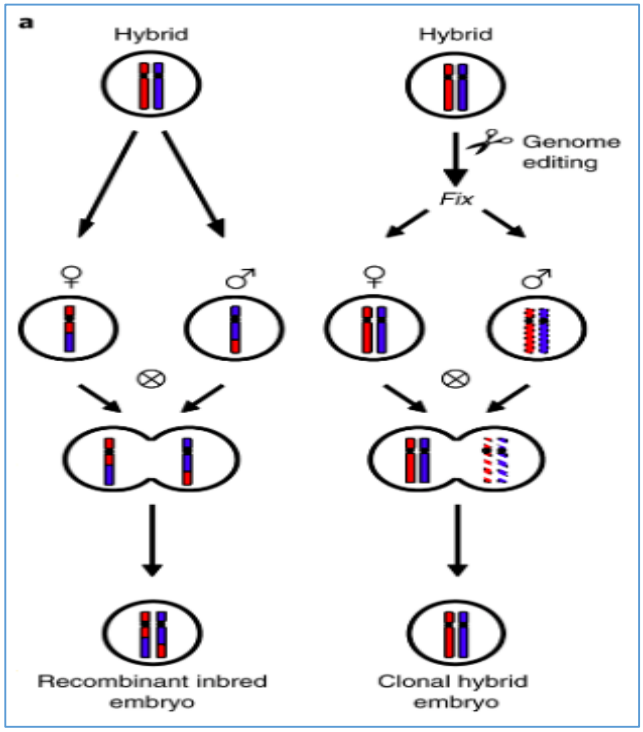

Principle of the TILLING method
①
Mutant DNA
+
WT DNA
PCR
WT DNA
②
Mutant DNA
③
mismatch
CEL I
200bp
700bp
WT DNA
G
T
④
Mutant DNA
900bp
⑤
900bp
700bp
200bp
Agarose gel electrophoresis


EMS
M1
M2
seed bank
Array
DNAs
Pool
heat,
cool
PCR
gene-specific
primers
CEL I
denature
gel, scan
Photoshop
Choose
individuals
PCR
Identify mutants

TILLING Results for A Target Gene



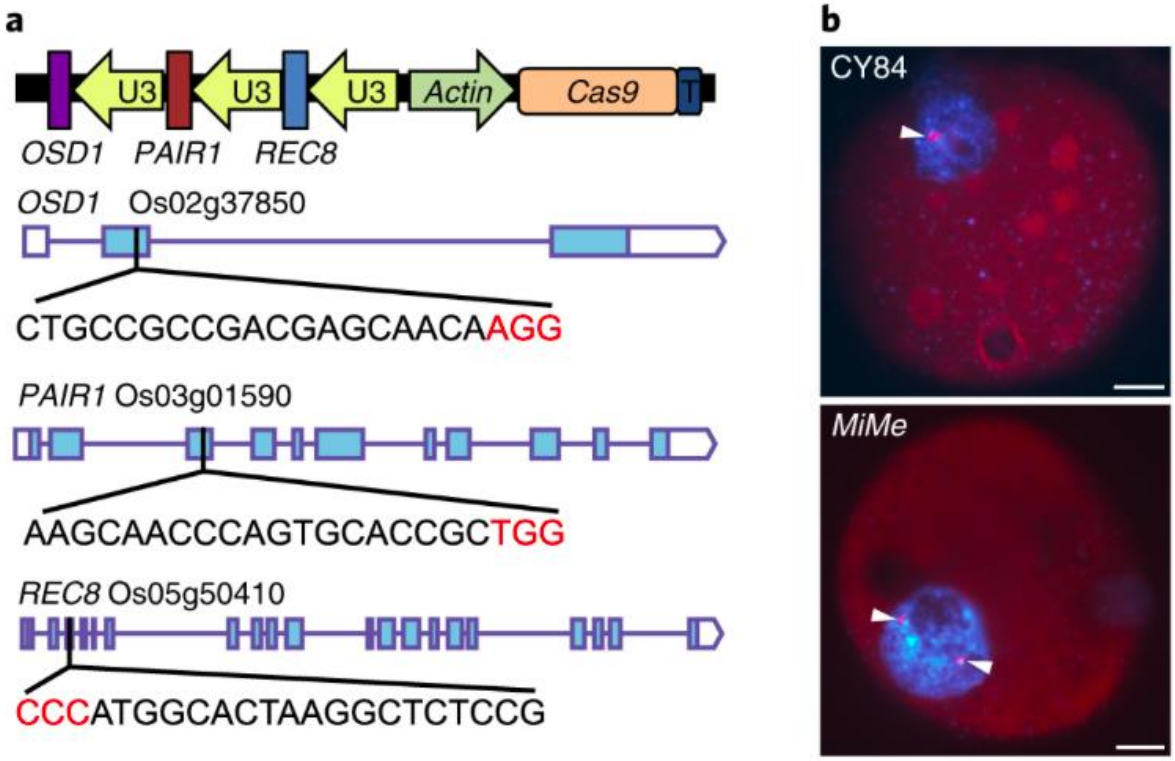
Fixation of Heterozygosity in Rice Through Editing Four Genes



- Heterosis or hybrid vigor enhances crop yield. Example; maize
- Unfortunately, most staple food and legume crops are self-pollinated.
- Generation and identification of male sterility gene have been long attempted with a view to exploit heterosis.
- Clonal propagation through seeds would enable self-propagation of F_1 hybrids in self or cross pollinated crop species.
- Wang et al. (2019) reported a strategy to enable clonal reproduction of F_1 rice hybrids through seeds.
- Conducted multiplex CRISPR–Cas9 genome editing of the ***REC8, PAIR1*** and ***OSD1*** meiotic genes to produce clonal diploid gametes and tetraploid seeds.
- Next, they editing the ***MATRILINEAL* (*MTL*)** gene (involved in fertilization) to induce formation of haploid seeds in hybrid rice.
- Finally, simultaneous editing of all four genes (*REC8*, *PAIR1*, *OSD1* and *MTL)* in hybrid rice led them to propagate F_1s clonally through seeds.

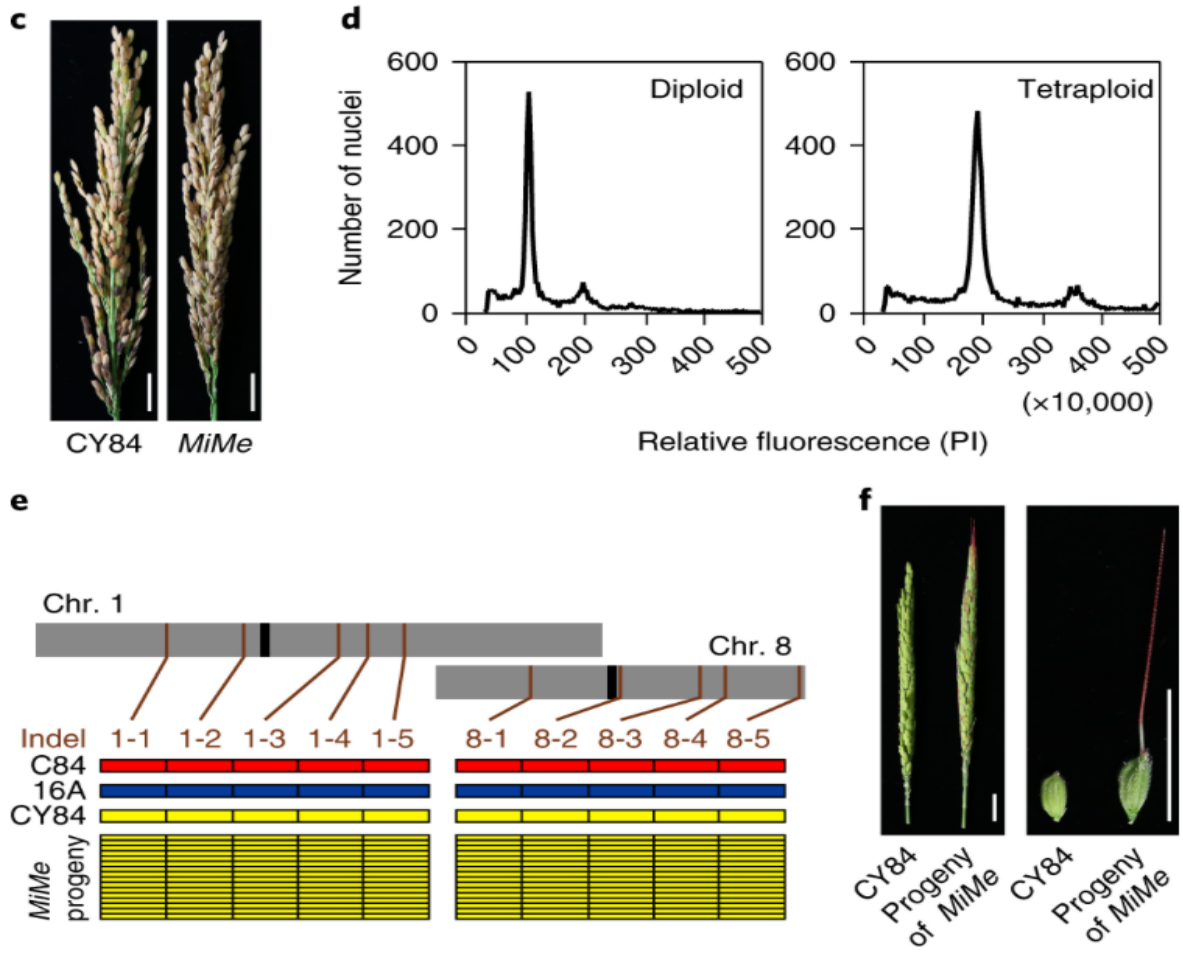
Fig. 1: Turning meiosis into mitosis in hybrid rice variety CY84.

From: Clonal seeds from hybrid rice by simultaneous genome engineering of meiosis and fertilization genes



a). The structure of CRISPR–Cas9 vector targeting *OSD1*, *PAIR1* and *REC8*.

b). The chromosomes of CY84 and *Mitosis instead of Meiosis* (*MiMe)* were probed by digoxigenin-16-dUTP-labeled 5 S rDNA (red signal, indicated with a white arrow) in spores, showing one signal in wild-type CY84 and two signals in *MiMe*. The DNA is stained with 4′,6-diamidino-2-phenylindole (DAPI, blue signal). Scale bars, 5 μm.



c, Panicles of wild-type CY84 and *MiMe*. The fertility of *MiMe* was as high as that of wild-type CY84.

d, Ploidy analysis of CY84 (left) and the progeny of *MiMe* (right) by flow cytometry, which were found to be diploid and tetraploid, respectively.

e, Genotype analysis of the paternal C84, maternal Chunjiang 16 A (16 A), hybrid variety CY84 and the progeny siblings of *MiMe*. Ten indel markers distributed on chromosomes 1 and 8 were used to identify the genotype of the offspring of *MiMe*. Positions of markers (brown) and centromeres (black) are indicated along the chromosomes. For each marker, plants carrying the C84 allele are in red, plants carrying the 16 A allele are in blue, and plants with both C84 and 16 A alleles appear in yellow. Each row represents one plant, and each column indicates a locus.

f, Panicles and grain shape of CY84 and the progeny of *MiMe*. The progeny of *MiMe* displayed reduced fertility, increased glume size and elongated awn length. Scale bars, 2 cm.

Generation of a haploid inducer line by editing the MTL gene involved in fertilization in hybrid rice variety CY84

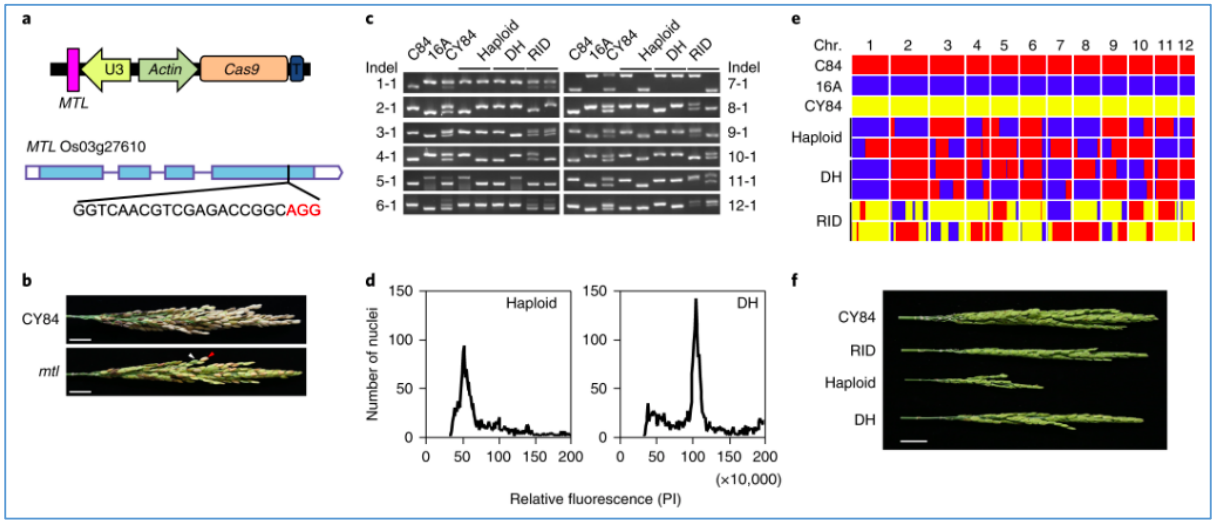
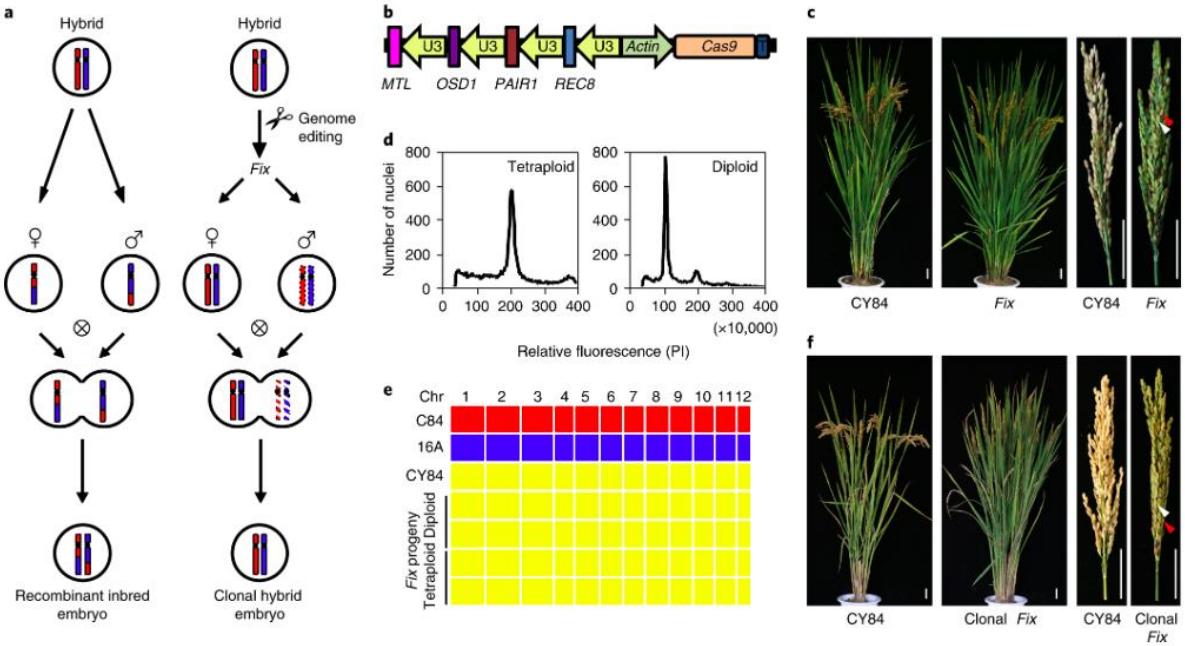
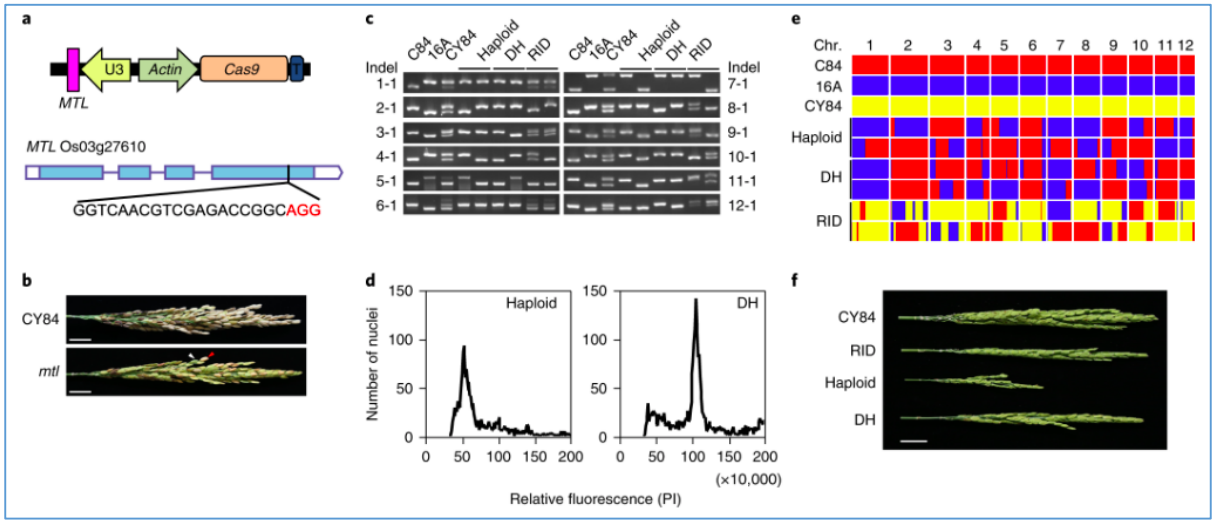


Fig. 3: Fixation of rice heterozygosity by multiplex gene editing in hybrid rice variety CY84.

From: Clonal seeds from hybrid rice by simultaneous genome engineering of meiosis and fertilization genes



Generation of a haploid inducer line by editing the MTL gene involved in fertilization in hybrid rice variety CY84



Fixation of Heterozygosity in Rice Through Editing Four Genes

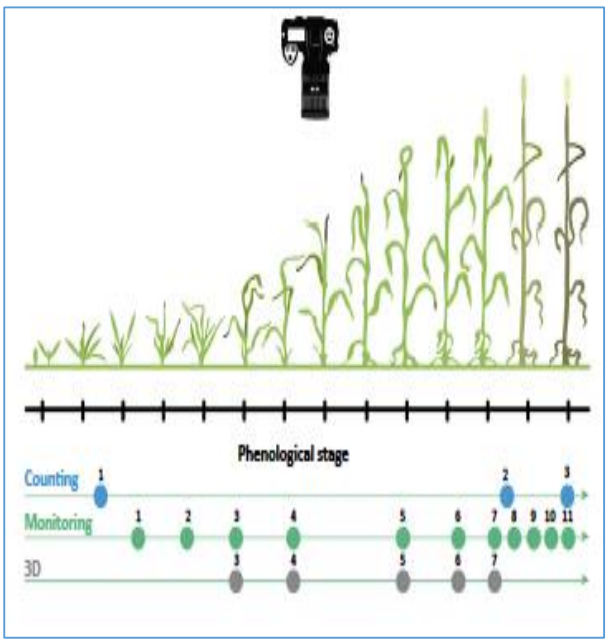
1. Two biological steps were modified to fix heterozygosity in rice.
2. In step 1, three genes edited to generate a mutant known as "*Mitosis instead of Meiosis (MiME).*"
3. In the Step 2, a single mutation to avoid fertilization is generated.
4. Gene editing for all four genes resulted in seeds that carry the genotype of the F_1.
5. You end up two copies of the haploid F_1 genome; without going through meiosis (crossing over, etc.).
6. The gametes are as a result diploids.
7. Mutation in the fertilization gene suppressed the fusion of male gamete with the novel diploid ovum.
8. The diploid ovum generates the embryo and then the seeds as in apospory.
9. Poor seed setting of these novel F_1s will require some more work to improve fertility. Okay to use in fodder crops grown for foliage only.

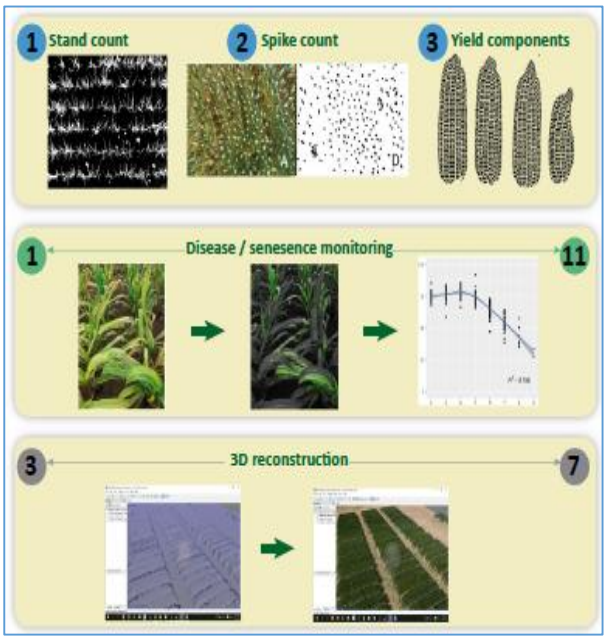
Why Phenomics?

1. Cost of high-throughput phenotyping using drones or other means under the field condition reduced the cost of phenotyping of individual genotypes significantly
2. Therefore, we can phenotype a larger segregating population.
3. Objective phenotyping using machines is consistent with much reduced errors. Therefore, phenotypes are consistent improving the heritability values.
4. Thus, we can screen a larger segregating population with improved heritability values of the traits.

Field-based Phenomics

Field Phenotyping with Red–Green–Blue (RGB) Images Produced by Conventional Digital Cameras





Genetic gain: Indicator of breeding success

$$R_t = \frac{ir\sigma_A}{y}$$

Where,
R_t is genetic gain over time,
i is selection intensity,
r is selection accuracy,
σ_A is genetic variance,
y is years per cycle

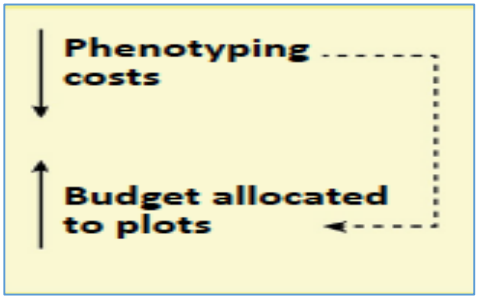
Genetic gain within a breeding program can be accelerated in a number of ways:

1. Increasing the size of the breeding program to enable higher selection intensity
2. Enhancing the accuracy of selection (higher repeatability)
3. Ensuring adequate genetic variation
4. Accelerating the breeding cycles
5. Improving decision support tools.

1. Increasing the size of the breeding program to enable higher selection intensity

High-through field phenotyping:

1. Reduced costs in phenotyping
2. Can phenotype more genotypes
3. Increased selection intensity

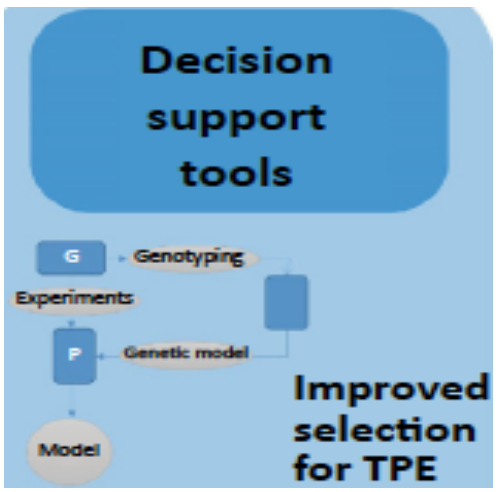


2. Enhancing the accuracy of selection (higher repeatability)

- Predictive phenotyping is objective and much more accurate than the convention phenotyping.
- As a result, heritability is improved and so is the accuracy of selection.

3. Improving decision support tools

- The approach we can apply is to generate a large segregating population – say 4,000 recombinant inbred lines through Fast Breeding.
- We select say 400 from this 4,000 lines randomly to phenotype and identify markers linked to the trait loci.
- Once the trait loci and associate markers are identified, we can use these markers to select the desirable genotypes from the entire population of 4,000 RILs.



TPE: target populations of environments

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

Herbicides: Boon or a Bane?

What is a weed?

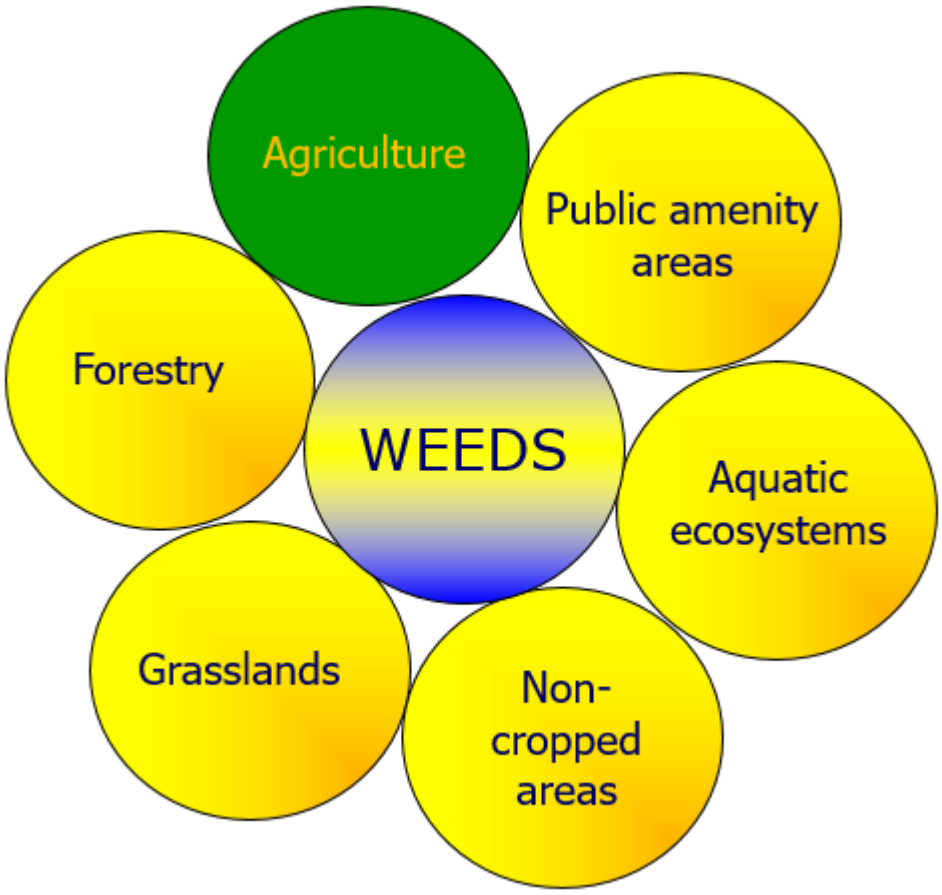
- Any plant that is out of place
- Plant which is not desired
- Plant which causes economic losses to farmers

Why weeds are a problem?

- Weeds are prolific producers of seeds
- Weeds have efficient mechanisms of seed dispersal
- Weeds have dormancy and survive is soil for many years
- Huge & inexhaustible seed bank in soil

Types of Weeds

- Come in all shapes and sizes
- They can be classified as Grasses, Broadleaved weeds & Sedges
- They can be Annuals, Biennials or Perennials



Weed problems have increased in modern agriculture

- Large scale adoption of dwarf HYV and hybrids.

- Increased use of irrigation.
- Increased use of fertilizers.
- Altered Agronomy of crops.
- Mono-cropping and multiple cropping
- Reduced tillage

Why control weeds?

- Compete with crop plants for nutrients, water & space
- Reduce crop yield
- Interfere with agricultural operations
- Increase cost of cultivation
- Affects human and animal health
- Reduce crop quality
- Act as alternate hosts for diseases, insects and nematodes

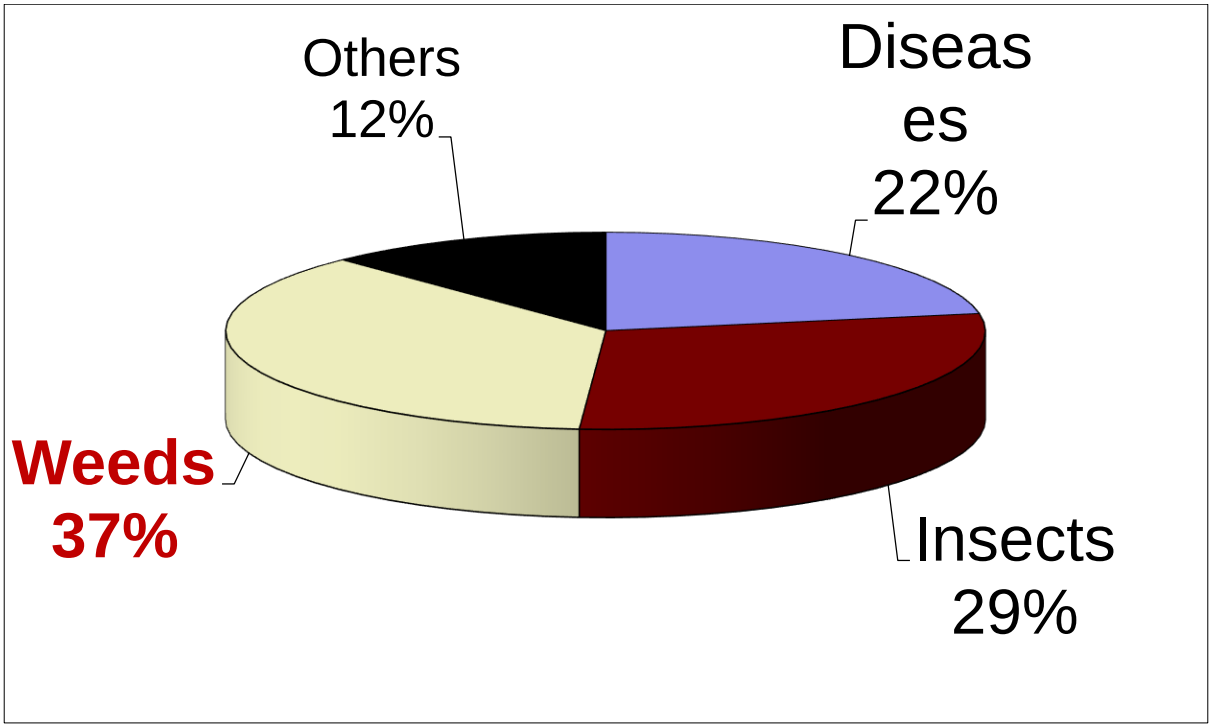
Potential and actual yield losses due to weeds (Garde et al, 2018)

Crop	Potential (%)	Actual (%)
Transplanted Rice	48.2	13.8
Direct Seeded Rice		21.4
Wheat	30.3	18.6
Maize	43.4	25.3
Soybean	62.5	31.4
Pigeon pea	33.6	23.9
Finger millet	62.0	41.5
Groundnut	57.9	35.8
Sugarcane	67.8	21.9
Sorghum	42.3	25.1
Blackgram	50.9	30.7
Pearlmillet	41.1	27.6

Equivalent to USD 11 billion (= Rs 80,000 crores) per annum

Extrapolated, the economic losses due to poor weed control in 10 major crops alone in India totals to USD 11 billion per year (= Rs 80,000 crores)

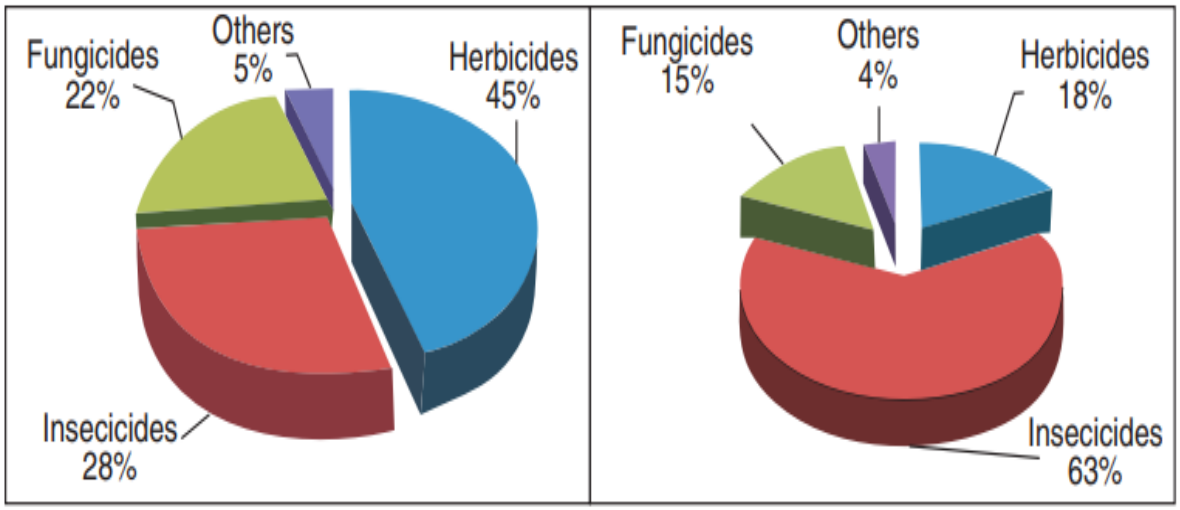
Economic losses caused by pests in India



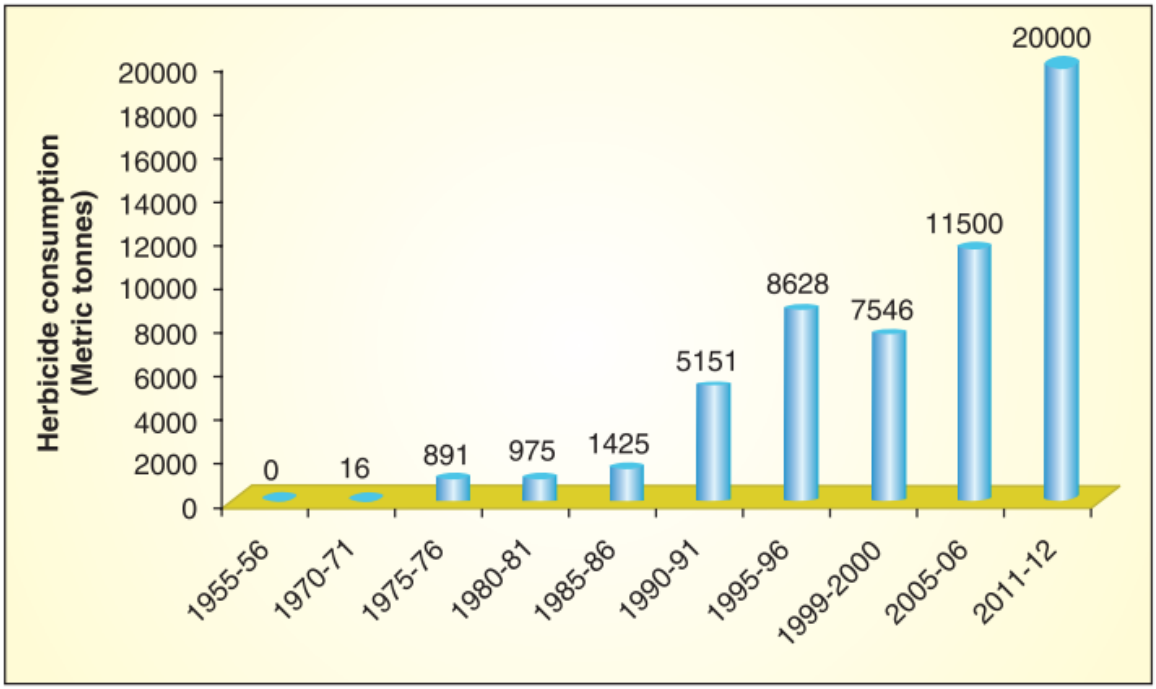
Why then, the weeds are largely ignored?

- Omnipresent- all crops, all seasons, all fields
- Damage to crop - not visible
- Fatalistic attitude
- Weed control receives last priority
- Weeds used as greens & as fodder

Pesticide use pattern



Herbicide consumption in India over the years (ICAR-DWR, 2015)



Types of herbicides

Based on selectivity
- *Selective*
- *Non-selective*

Based on mode of action
- *Contact*
- *Translocative*

Time of herbicide application

- Before planting *(Pre-planting)*
- After planting but before seed emergence *(Pre-emergence)*

- After emergence of crop *(Post-emergence)*

Chemical weed control – Benefits

- Saves labour, easy & convenient to use
- Selective control of weeds that are morphologically similar to crop
- Offers wide window of application
- Timely, efficient and economical control of weeds
- Well suited to min. and no-till conditions and under CA
- Prevents mechanical damage to crop plants
- Controls perennial weeds
- Aids in diversifying farming

The burden of manual weeding

Gross cropped area: 186 mha
Vegetables crops: 5.8 mha
Fruit crops: 3.68 mha
Plantation crops: 5.80 mha
Total : 200 mha (Approx)

Economic impact of herbicides

Total area: 200 mha
Total labour requirement: 4-6 billion man-days
Total cost (Rs 200/man-day) of manual weeding: Rs 80,000 – 120,000 crores
Chemical weeding saves 40-60% cost (Av. 50%)
Saving with chemical weeding: Rs 40,000- 60,000 crores.
Further, crop yields are higher with herbicides as they offer better control

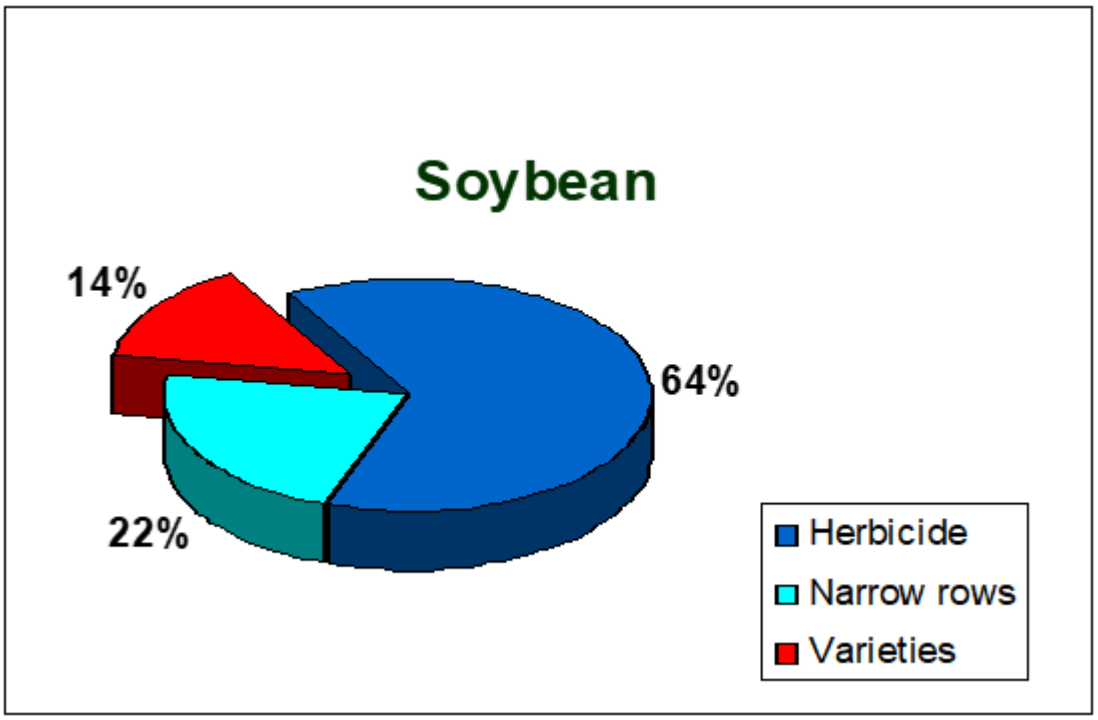
Source of Energy in weed control in USA (as % of total)

Year	Human Energy	Animal Energy	Mechanical Energy	Chemical Energy
1920	**40**	**60**	**-**	**-**
1947	**20**	**10**	**70**	**-**
1975	**5**	**TR**	**40**	**55**
1990	**<1**	**TR**	**24**	**75**

Number of persons supported by a US farmer

Year	Number
1830	**1.25**
1937	**10**
1965	**40 (much was due to mechanization)**
1990	**90(Largely due to herbicides)**

Contribution to increased production (USA) (1964-79)



Herbicide use: Concerns

- Pesticide poisoning
- Contamination of food & water
- Toxic residues in soil
- Effect on non-target organisms
- Resurgence of HR weeds

How to reduce pesticide poisoning

- Herbicides are poisonous
- Use protective clothes while spraying
- Do not touch the chemical by hand directly
- Do not spray against the wind
- Keep them away from children, livestock and food items
- Dispose the empty containers properly

Pesticides classification based on toxicity (WHO)

	LD_{50} for the rat (mg/kg body weight)

	Oral	Dermal
Ia Extremely hazardous	< 5	< 50
Ib Highly hazardous	5–50	50–200
II Moderately hazardous	50–2000	200–2000
III Slightly hazardous	2000- 5000	Over 2000
U Unlikely to present acute hazard	5000 or higher	

Number of pesticides registered in India belonging to different classes in toxicity

	Toxicity class					
	1a	**1b**	**II**	**III**	**U**	**Total**
Pesticide	**Extremely hazardous**	***Highly hazardous***	***Moderately hazardous***	***Slightly hazardous***	***Unlikely to present acute hazard***	
Insecticide	**4 (4.7)***	**12 (14.3)**	**41 (48.8)**	**10 (11.9)**	**17 (20.3)**	**84**
Fungicide	**0 (0.0)**	**1 (2.0)**	**21 (42.8)**	**6 (12.2)**	**21 (43.0)**	**49**
Herbicide	**0 (0.0)**	**0 (0.0)**	**16 (32.0)**	**12 (24.0)**	**22 (44.0)**	**50**

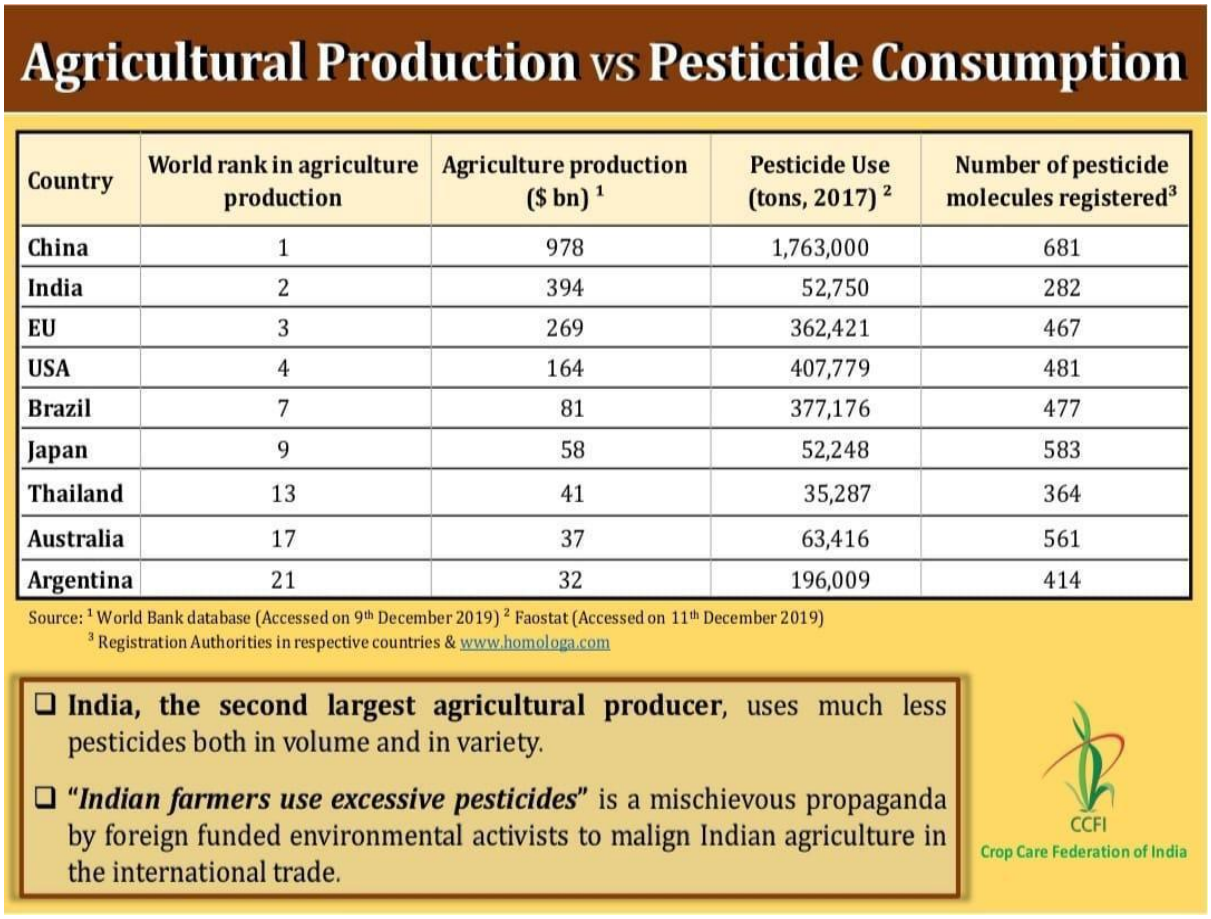
Herbicides safer than other Pesticides

- Lower mammalian toxicity (Higher LD_{50} Values)
- Lesser effect on non-target organisms
- Used early in crop season (longer waiting period)
- Never applied directly on grains/fruits
- Normally dissipated in soil before crop harvest
- Dose could be reduced easily by integrating with other methods of control
- Very little/no chance of residue reaching the food chain

Strong Regulatory System in Place

- Registered under Central Insecticides Act only after careful scrutiny of the data
- Safety to human and animal health is ensured
- Safety on non-target organisms is taken in to account
- Environmental safety is ensured
- Periodical review on pesticide risks is made

- Bad ones are – restricted/banned



Average Consumption of Pesticide in Selected Countries (g/ha)

Taiwan	**17000**
Japan	**12000**
Europe	**3000**
USA	**2500**
Argentina	**960**
Mexico	**750**
India	**570**
Africa	**127**

Fight negative propaganda

- However, it is unfortunate that the anti-pesticide lobby is busy misleading farmers and the Govt by spreading misinformation and facts not based on science on pesticides
- No technology is fool-proof. Every technology has RISKS and BENEFITS

The problem of HR weeds

Continuous use of the same herbicide for a long period leads to the development of herbicide resistance in some weeds.

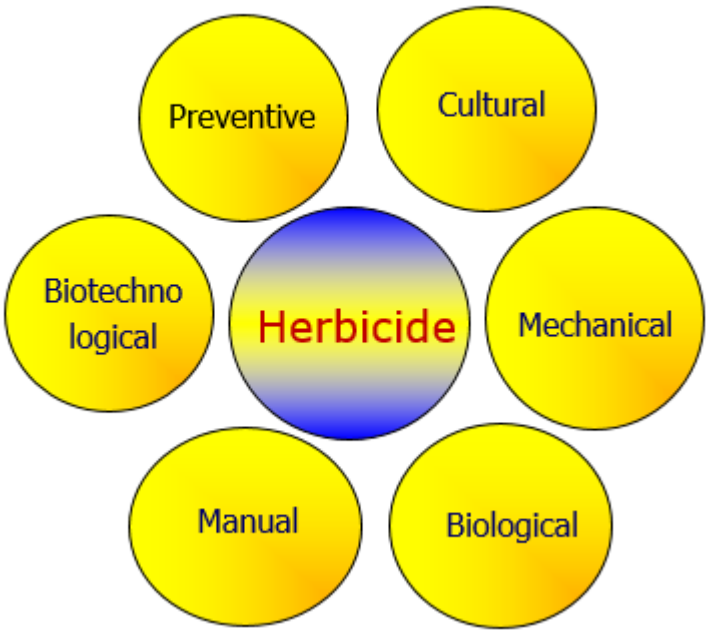
Herbicide resistance in weeds –A global challenge

Currently, 250 weed species infesting 86 cropping and non-cropping systems in 66 countries have developed resistance to 160 different herbicides belonging to 23 families with as many sites of action [Heap 2016]

Alternative herbicides

- Herbicide mixtures or sequences
- Judicious use of herbicides
- Crop diversification/rotation
- Exhausting of soil seed bank
- Integrated weed management

Herbicide-centric IWM – The realistic model



Conclusions

- Manual weeding is neither effective nor economical
- More and more farmers will opt for herbicides
- Judicious herbicide use is unlikely to lead to any undesirable consequences
- Integrate herbicides with other non-chemical methods for sustainable WM
- Create awareness and train farmers on safe use of pesticides
- Resist negative propaganda by activists

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

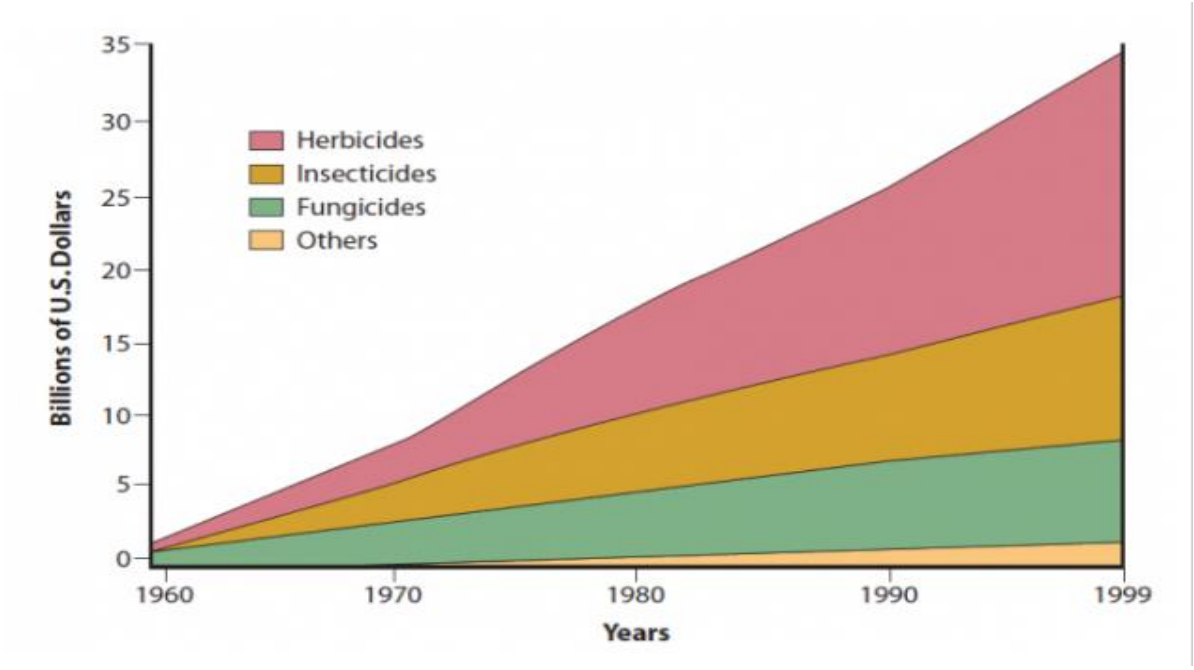
Herbicide Resistant Weeds and Their Management

Methods of Weed Control

- Chemical
- Biological
- Biotechnological
- Manual
- Cultural
- Mechanical
- Preventive

Chemical Weed Control: Chemical weed control is the most sought after method in all the developed countries. Discovery of *2,4-D* in the late 40's paved the way for chemical weed control and significantly revolutionized world agriculture

Global pesticide segment over years



Since the discovery and commercialization of 2,4-D in the 1940s, the herbicides have been able to provide efficient, economical and easy way of controlling weeds in different crops and cropping systems

They have done well in enhancing and maintaining the productivity levels of crops during the last 70 years in much of the developed countries

Herbicidal use: Concerns

- Pesticide poisoning
- Contamination of food & water
- Toxic residues in soil
- Effect on non-target organisms
- Resurgence HR weeds

Tolerance vs Resistance

Tolerance: Normal variability in response to herbicides that exists among plant species (selectivity) and that can build up quickly in a population.

Herbicides are the chemicals used to suppress/kill herbs (plants)

Selective herbicides: Kill/suppress only few plant, (Ex: All herbicides used in crops). Selective herbicides may be effective on:

- Grasses (*Graminicides*)
- Broad leaved weeds
- Sedges
- Many weeds (broad-spectrum)

Non-selective herbicides: Kill/suppress all vegetation, (Ex: Glyphosate, paraquat, glufosinate)

Selectivity is species-specific

- Different herbicides will have to be used in different crops often to control the same weed
- A herbicides effective on one grass or a dicot weed may not be effective on other weed(s) of the same category
- Unlikely that a herbicide will control all weeds in a given crop
- Continuous use of the same herbicide will result in dominance of tolerant weeds
- A minor weed may become a major weed
- Require a cocktail of two or more herbicides to control many weeds
- Use of different herbicide(s) in rotation is desirable to prevent weed flora shift

Herbicide Resistance:

- Involves the altered response to a herbicide by a formerly susceptible weed species to the extent that some individuals in that species are no longer susceptible
- Decreased response of population of a species to a herbicide as a result of herbicide application

Don’t confuse poor control with resistance:

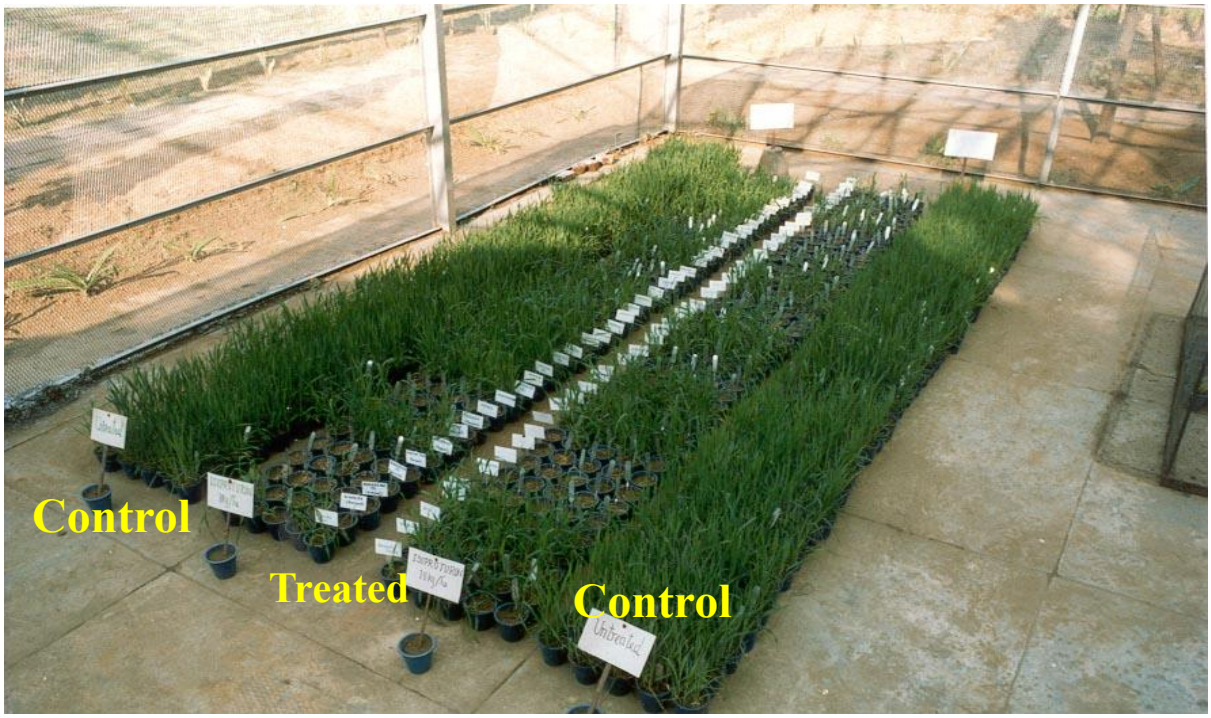
Poor weed control could be due to:

- Spurious herbicide
- Dose - lower than recommended
- Non-uniform application
- Adverse soil conditions
- Climatic conditions- rainfall, cloud cover

How to confirm resistance development?

- Herbicide use history
- Rule out all factors responsible for poor WC
- A single weed not controlled?
- Collect weed seeds from different fields
- Grow under similar soil & climatic conditions
- Expose to uniform application of the herbicide
- Measure the response.

Littleseed canary grass *Phalaris minor,* is a major weed in Rice-Wheat system in NW parts of India. Resistance development to herbicide isoproturon in 1990s threatened wheat production



Response of *Phalaris minor* biotypes collected from farmer's field of Punjab and Haryana to isoproturon (Yaduraju, 1995)

Resistance

Decreased response of population of a species to a herbicide as a result of herbicide application

Cross resistance

Resistance to other herbicides belonging to the same class (ex: Triazines & Ureas)
Resistant to two or more herbicides due to a single resistance mechanism (ex: PS II)

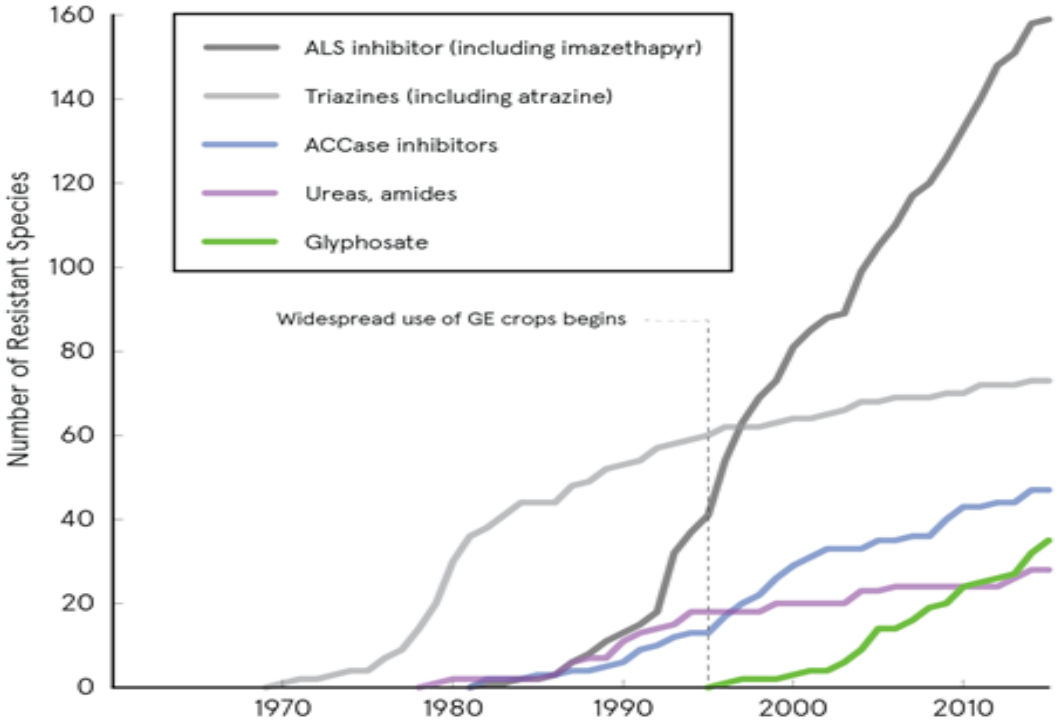
Multiple resistance

Resistance to chemically dissimilar herbicides with different modes of action
Resistance due to two or more distinct mechanisms

Herbicide resistance in weeds - A global challenge

Currently, 255 weed species (148 dicots and 107 monocots) infesting 92 crops in 70 countries have developed resistance to 163 different herbicides belonging to 23 of the 26 families with as many sites of action (Heap 2018).

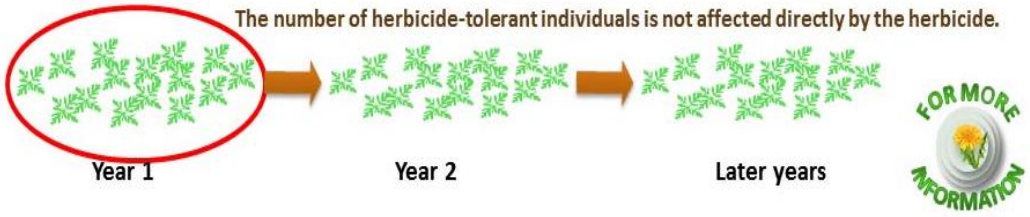
Herbicide resistance development over time



Herbicide Resistance Defined

Herbicide resistance can be defined as the acquired ability of a weed population to survive a herbicide application that previously was known to control the population.

Herbicide tolerance is the inherent ability of a species to survive and reproduce after herbicide treatment. There has been no selection acting on the tolerant weed species, and there has been no change in the weed species lack of response to the herbicide over time.



Build-up of Resistant Weed Population of with Repeated Use of a Single Herbicide

Year	Susceptible	Resistant
1.	1,000,000,	1
2.	100,000,	4
3.	10,000,	16
4.	1000	64
5.	100	256
6.	10	1024
7.	1	4026

*Assuming 90% control

Factors that regulate development of HR

1. Initial frequency of resistant population

- Triazines - 1 x 10^{-7} to 1 x 10^{-9}
- ALS-inhibitors - 1 x 10^{-3}
- ACCace inhibitors - 1 x 10^{-3}

Herbicides classified based on their risk to development of resistance in weeds

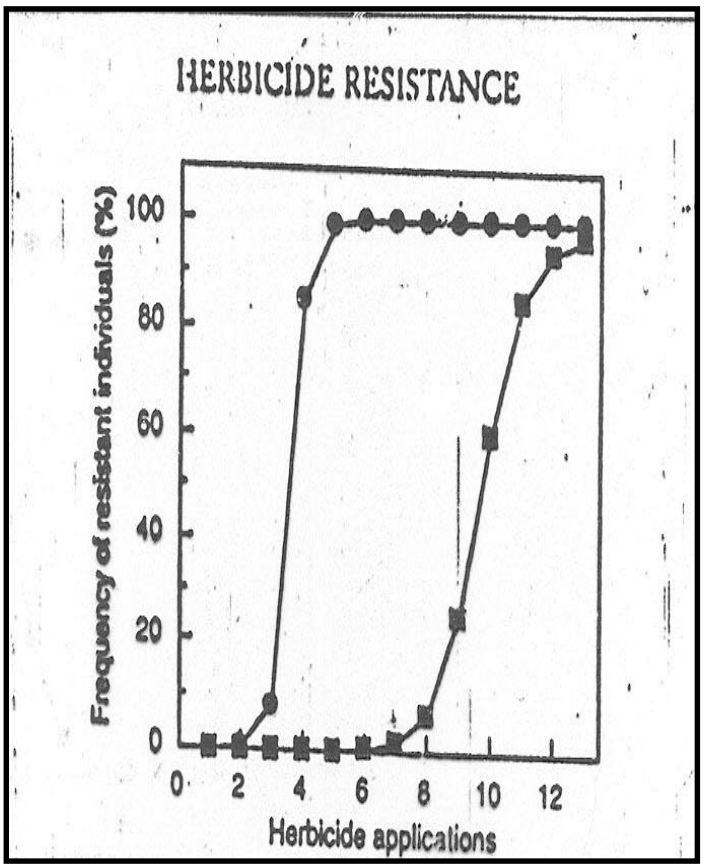
Risk	Mode of action	Herbicides
High	Amino Acid Synthesis Inhibitors (ALS Inhibitor)	Chlorimuron, Nicorsulfuron Primisulfuron, Imazethapyr
	Lipid Synthesis Inhibitors	Fluazifop-butyl, Sethoxydim
	Cell Membrane Disrupter	Lactofen, Paraquat, Nicosulfuron
Medium	Contact PS Inhibitors	Bentazone, Bromorynil
	Pigment Inhibitors	Clomozone
	Root Growth Inhibitors	Pendimethalin, Trifluralin
	Systemic Photosynthesis	Atrazine, Metribuzine, Linuron
Low	Amino Acid Derivatives	Glyphosate, Sulfosate
	Growth Regulators	Dicamba, MCPA, 2,4-D
	Shoot Growth Inhibitors	Alachlor, Metolachlor, EPTC

2. Selection pressure

- Repeated use of same/same class of herbicide
- Longer residual herbicide

- Higher Potency
- Mono-culture

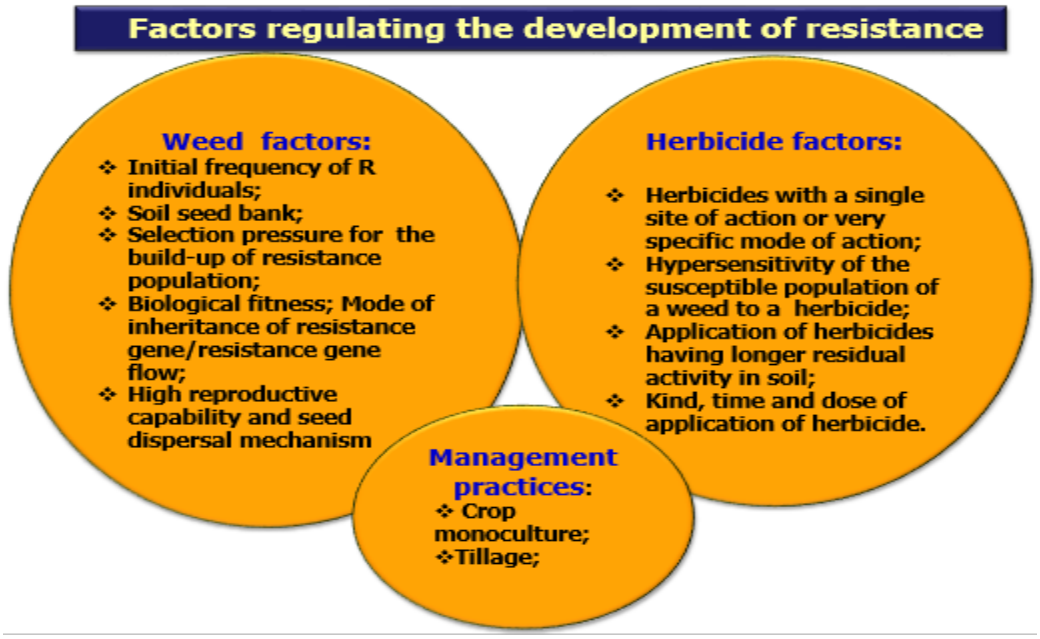
Influence of herbicidal efficacy on predicted appearance of herbicide resistance



Herbicidal control was set at 95%(●) or 80% (■) mortality of susceptible individuals. Other model parameters are an unlimited population size, an initial frequency of resistance genes of 1 X 10^{-7}, an outcrossing species with dominant mode of inheritance. 20% of seed remaining dormant. 20% mortality of seed in the soil, and 50% nonherbicidal mortality of seedlings

3. Biological fitness

4. Seed bank in the soil



Resistance mechanism

1. **Target site resistance**

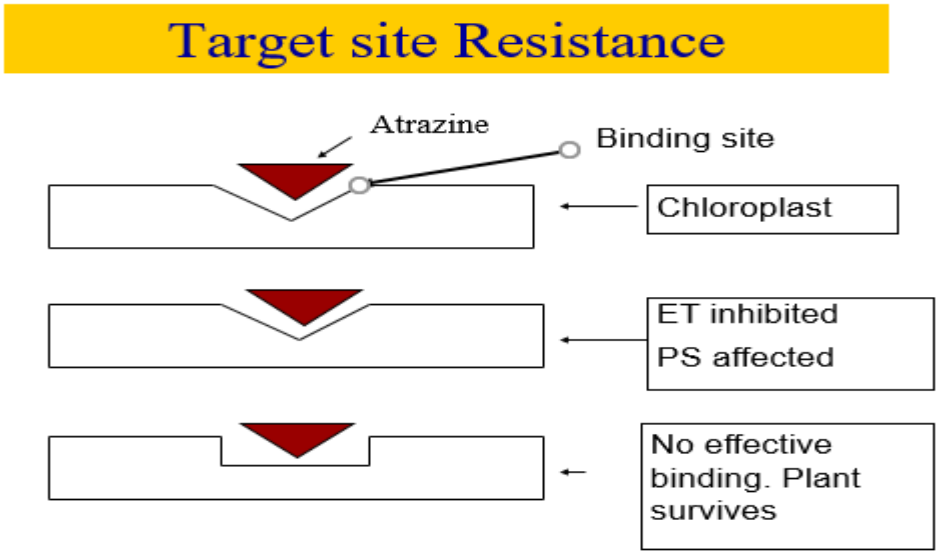
   - Triazines (D1 protein)
   - ALS-inhibitors
   - Dinitranilines (Tubulin protein)
   - ACCace inhibitors (Fops & Dims)

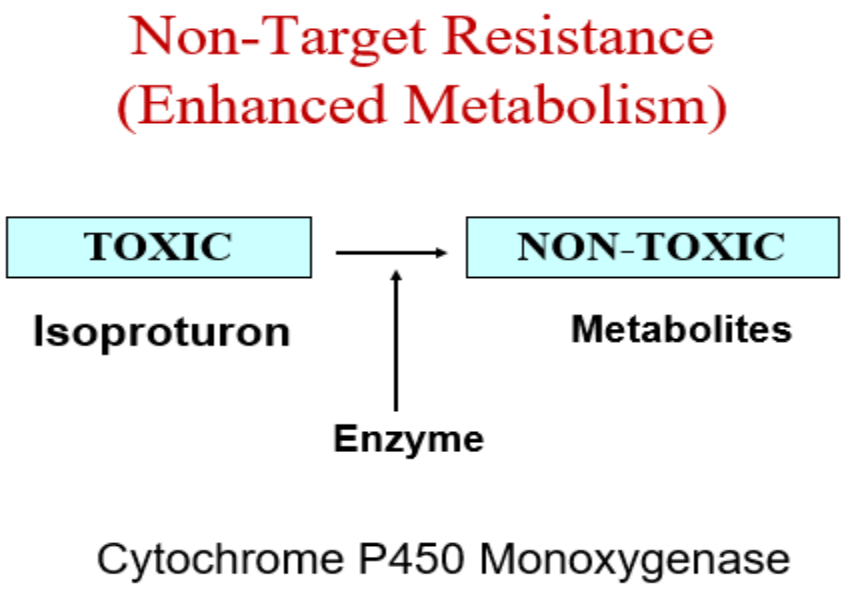
2. **Metabolic resistance (Non-target site)**

   - ACCace inhibitors
   - ALS-inhibitors
   - Phenylureas

3. **Sequestration and Compartmentation**

   - Paraquat





Response of HR *P minor* to isoproturon

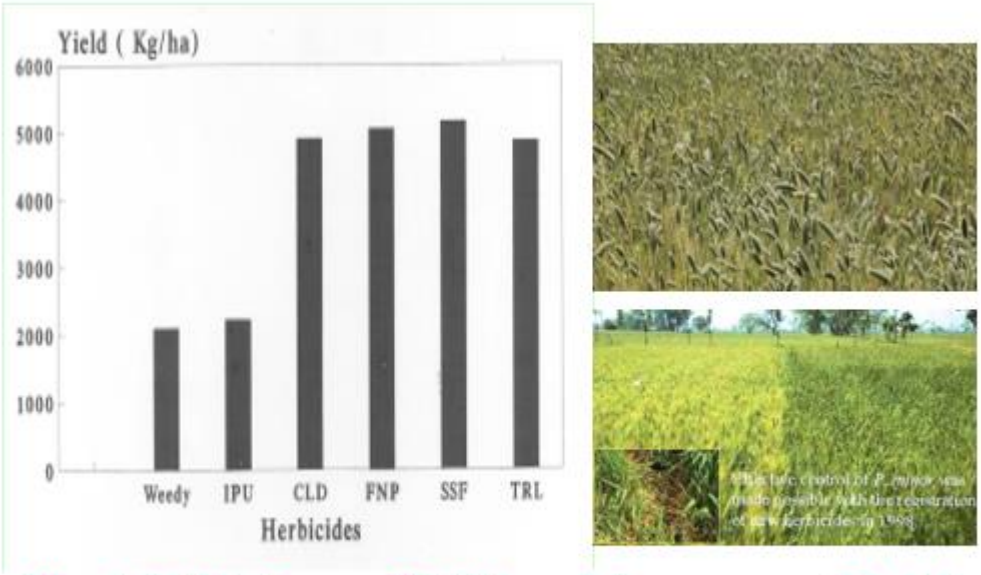
Biotype	r^2	*GR 50 (ppm)	R-factor
Susceptible	0.96	0.0556	-
Resistant	0.95	0.7262	13.1
Wheat	0.95	0.8337	15.0

* The concentration at which the growth is reduced by 50% of the control

Yaduraju & Bhowmik (1998)

New herbicides for the control of Isoproturon-resistant *Phalaris minor*

Herbicide	Dose (g/ha)	Application
Clodinofop (Topik)	**50-60**	**4-5 WAS**
Fenoxaprop (Puma super)	**100-120**	**4-5 WAS**
Sulfosulfuron (Leader)	**25-30**	**4-5 WAS**

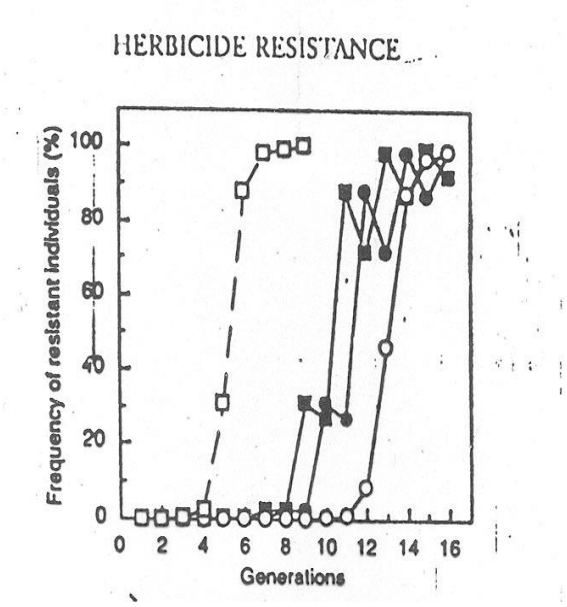


Wheat yield in farmers' field in resistance areas of Punjab & Haryana (1996-97, Mean of 17 locations)

Herbicide Resistance Management

- Alternate herbicides with different MoA
- Herbicide mixtures or sequences
- Judicious use of herbicides
- Herbicide resistant crops
- Crop diversification/rotation
- Exhausting of soil seed bank
- Integrated weed management

Predicted appearance of herbicide resistance following repeated selection with herbicide rotations



Predictions for resistance to herbicides A or B used alone (□). A (■) and B (●) used in rotation. Or for the two herbicides used as a mixture (O). Model parameters are an unlimited population size, an outcrossing species a dominant mode of inheritance of each resistance gene for each mode of action of 1×10^{-7}, 95% control of susceptible individuals by each herbicide, no cross resistance or negative cross-resistance. 20% of seed remaining dormant. 20% mortality of seed in the soil, and 50% nonherbicide mortality of seedlings

HRAC mode of action classification

Group A	Inhibition of acetyl CoA carboxylase (ACCase)
Group B	Inhibition of acetolactate synthase ALS (acetohydroxyacidsynthase AHAS)
Group C1	Inhibition of photosynthesis at photosystem II
Group C2	Inhibition of photosynthesis at photosystem II
Group C3	Inhibition of photosynthesis at photosystem II
Group D	Photosystem-I-electron diversion
Group G	Inhibition of EPSP synthase
Group H	Inhibition of glutamine synthetase
Group I	Inhibition of DHP (dihydropteroate) synthase
Group K1	Microtubule assembly inhibition
Group K3	Inhibition of cell division

But how practical is herbicide rotation?

HTCs in USA- crop rotated

- RR corn – RR soybean – RR cotton
- But the herbicide (glyphosate) remained the same

In Australia- colour coded labelling done to help farmers to make out herbicides belonging to different MoA.

India- colour coding is suggested in the new Pesticide Management bill approved by the Govt. recently

Registered Pre-mix herbicides in India

	Herbicides	Crop	Dosage (g ai/ ha)	Formulation (g / ml/ha)	Trade name
1	Anilofos 24% + 2,4-D ethyl ester 32% EC	Transplanted Rice	240+ 320 to 360 + 480	1000-1500	Arozine, Topshot
2	Bensulfuron methyl 0.6% + pretilachlor 6% GR	Transplanted Rice	60+600`	10 kg	Londex Power, Rizal
3	Carfentrazone ethyl 20% + sulfosulfuron 25% WG	Wheat	20+25 (+750 ml surfactant)	100	Affenative Force
4	Clodinafop propargyl 15% + metsulfuron methyl 1% WP	Wheat	60+4	400	Vesta
5	Clodinafop propargyl 9% + metribuzin 20% W/W	Wheat	54+120	600	Clomet
6	Clomazone 20%+2,4-D EE 30% EC	Transplanted Rice	250-375	1250	
7	Fenoxaprop-p-ethyl 7.77% w/w + metribuzin 13.6% w/w EC	Wheat	100_175	1250	Accord Plus
8	Hexazinone 13.2% + diuron 46.8 % WP	Sugarcane	264-936	2000	Velpar K-4
9	Imazamox 35% + imazethapyr 35% WG	Soybean	35+35	100	Bingo,Pyromax
10	Mesoulfuron methyl 3% + iodosulfuron methyl sodium 0.6% WG	Wheat	12+2.4	400	Atlantis
11	Metsulfuron methyl 10% + chlorimuron ethyl 10% WP	Transplanted Rice PE	2+2	20	Almix, Pimix,Cormix
12	Oxyflurofen 2.5% + glyphosate (isopropyl amine salt)41% SC (w/w)	Tea	50+820	2000	Zoomer
13	Pendimethalin 30%+ imazethapyr 2% EC	Soybean	750+50 to 900+60	500-600	Valor
14	Pretilachlor 6% + pyrazosulfuron ethyl 0.15% (G)	Rice	600-15	10 kg	Swachh

15	Sulfosulfuran 75% + metsulfuron-methyl 5%WG	Wheat	30+2	40	Total, Satsung
16	Sodium aceflourofen 16.5% + clodinafop propargyl 8% EC	Soybean	80+165	1000	Irish, Patela

Herbicide tolerant crops (HTCs) - a solution to HR weeds

- HTCs are genetically modified to tolerate applications of non-selective herbicides
- Kill all weeds including HR weeds
- HTCs form 80% of the total biotech area of 190 mha since their commercial cultivation in 1996.
- Major HTCs developed include corn, soybean, cotton, canola

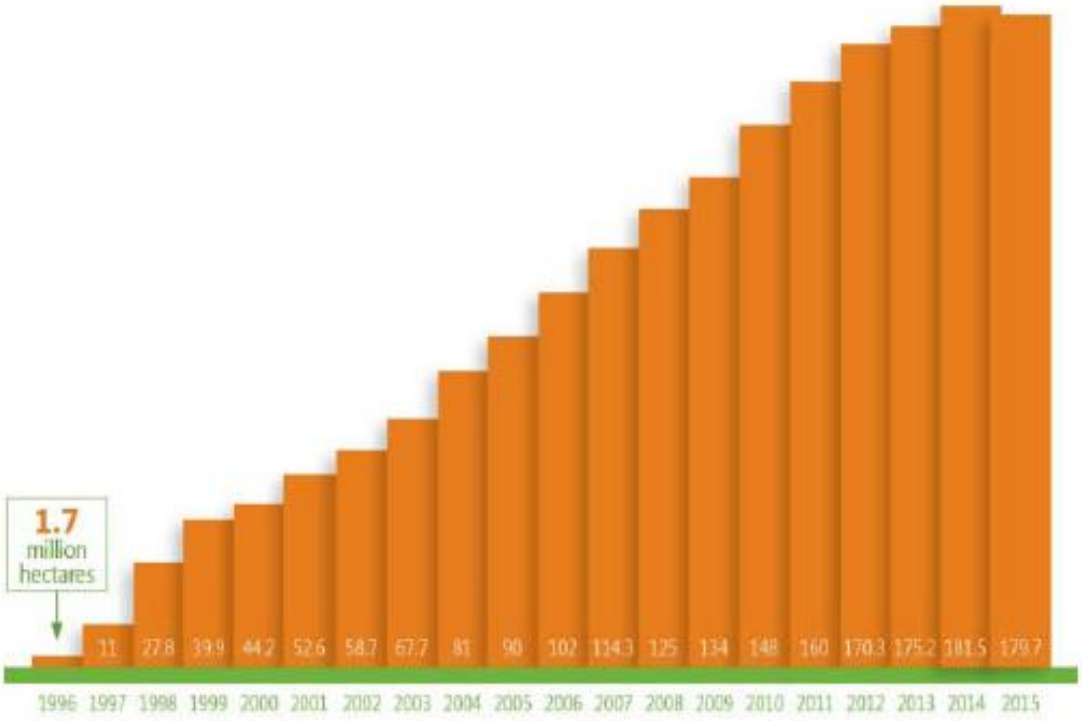


Figure 1. Global Area of Biotech Crops, 1996 to 2015 (million hectares).

Source: Clive James, 2015.

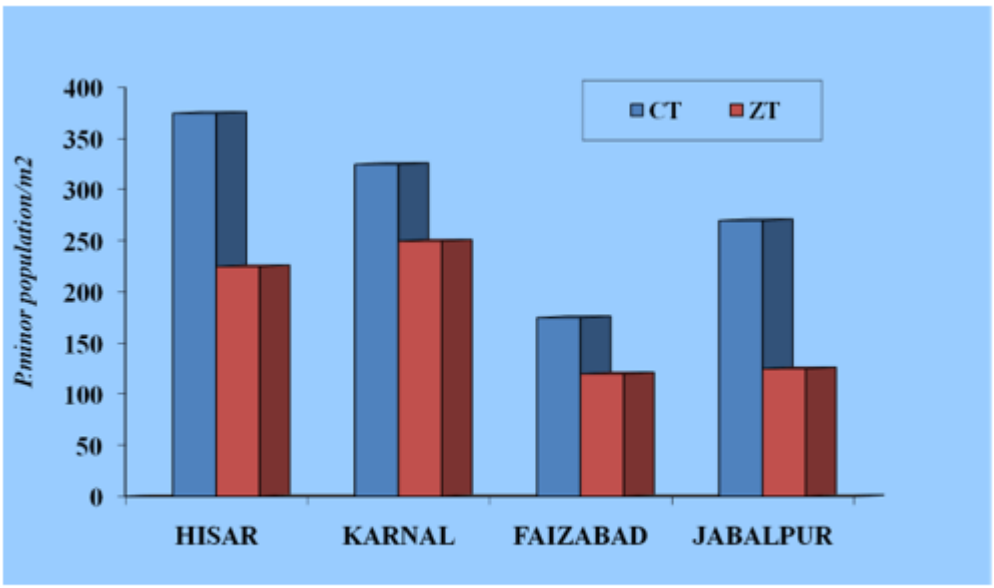
But ended up with problem of Glyphosate-Resistant weeds.

- **38 weed species in 34 crops from 37 countries have developed resistance** (Heap & Duke 2017)
- GR caused an increase of about $40-50/a in US
- WSSA estimates $ 43 billion annual loss due to uncontrolled weeds in corn and soybean in US and Canada- largely be due to GR-weeds

Weed population (No./m^2) under different cropping systems

Cropping system	Grasses	*Phalaris minor*	Broad leaf
Rice-Wheat	**58**	**22**	**20**
Maize-Wheat	**10**	**6**	**15**
Maize- Wheat- Mungbean	**8**	**4**	**11**
Maize- Potato- Mungbean	**16**	**2**	**15**
Maize- Potato- Onion	**18**	**6**	**18**
Cotton- Wheat	**14**	**6**	**12**
Cotton –African Sarson	**20**	**4**	**12**
Cotton –Gobhi Sarson	**16**	**4**	**12**
G.nut-Toria + Gobhi Sarson	**8**	**5**	**8**
G.nut-Potato-Bajra (F)	**14**	**5**	**14**

Effect of tillage on *Phalaris minor* in wheat



Mishra (2004)

Multiple resistance in weeds to herbicides - A new challenge

- HR reported in 92 crops in 70 countries for 163 different herbicides
- Currently 255 species (148 dicots & 107 monocots) of HR weeds
- Resistance to 23 of the 26 known herbicide sites of action
- *Multiple resistance*: 100 weed species resistant to two site of action (SOA)
- The number weeds showing resistance to three, four and five SOAs are 20, 12 and 6 respectively
- *Lolium rigidum* - resistance for 13 SOAs, fb *E. crus-galli* with ten sites.

Source: Heap (2018)

Risk of Resistance on a Per Species Basis			
Management Option:	**Low**	**Moderate**	**High**
Herbicide mix or rotation in cropping system	> 2 modes of action	2 modes of action	1 modes of action
Weed control in cropping system	Cultural, mechanical, and chemical	Cultural and chemical	Chemical alone
Use of same mode of action per season	Once	More than once	Many times
Cropping system	Full rotation	Limited rotation	No rotation
Resistance status to mode of action	Unknown	Limited	Common
Weed infestation	Low	Moderate	High
Control in last 3 years	Good	Declining	Poor
			Moss 1998

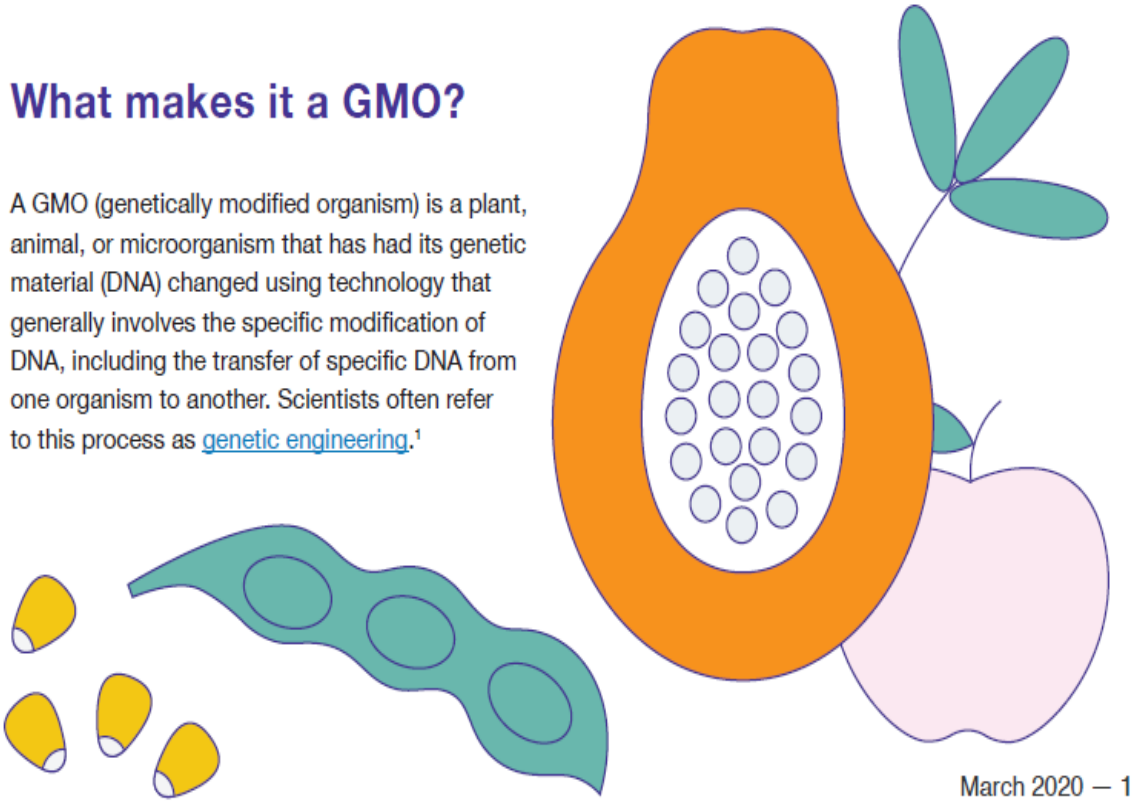
Conclusions

- Herbicide use in India is set to rise substantially
- Ensure judicious and safe use
- DO NOT rely on chemicals alone
- Adopt practices that prevent/delay development of HR in weeds
- Monitor closely for resistance development
- Follow herbicide rotation with different MoA
- Follow IWM
- Educate farmers and extension personnel on HR weeds

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

Herbicide Resistant Crops: A new tool in weed management



Why do we have GMOs?

- Traditional ways to modify crops and animals to suit their needs and tastes have been used for more than 10,000 years
- Selections, cross-breeding, selective breeding, and mutation breeding are some examples
- These breeding methods often involve mixing all of the genes from two different sources
- Modern technology now allows scientists to use genetic engineering to take just a beneficial gene (like insect resistance or drought tolerance) & transfer it into a plant.
- The reasons for genetic modification today are similar to what they were thousands of years ago: higher crop yields, less crop loss, longer storage life, better appearance, better nutrition etc.

How the GM plant is produced?

- Identify what trait they want that plant to have, such as resistance to drought, herbicides, or insects.
- Find an organism (plant, animal, or microorganism) that already has that trait within its genes
- Copy that gene. Use tools to insert the gene into the DNA of the target plant
- Grow in the laboratory, to ensure it has adopted the desired trait. If successful, grow and monitor the new plant in greenhouses and then in small field tests before moving it into larger field tests.

- GMO plants go through in-depth review and safety tests before they are ready for use by farmers.
- For example, scientists wanted to create insect-resistant corn to reduce the need to spray pesticides. They identified a gene in a soil bacterium called 2 *Bacillus thuringiensis* (Bt), which produces a natural insecticide that has been in use for many years in traditional and organic agriculture. By inserting the Bt gene into the DNA of the corn plant, scientists gave it the insect-resistance trait. **This new trait does not change the other existing traits**

Why do we need crops that are resistant to herbicides?

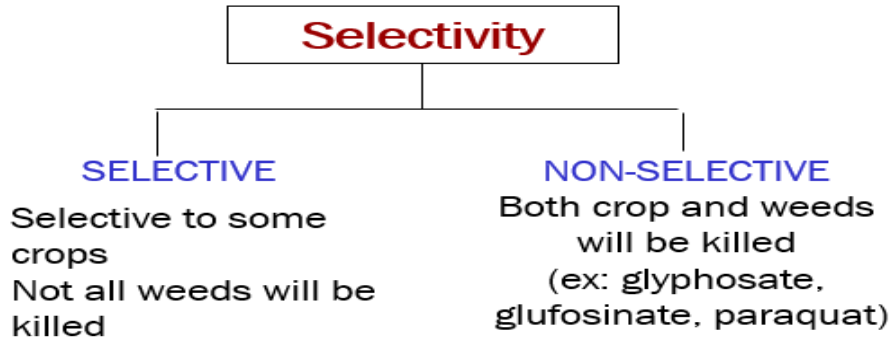
- Host resistance is an important component in IPM
- Unfortunately, no crops are resistant to weeds
- Allelopathy has not been commercially exploited yet – may not control all weeds
- Way out: Manipulate crops response to herbicides using GE techniques.

Limitations of Herbicide use

- Not all weeds are controlled by a single herbicide
- Herbicides belonging to different groups need to be used
- Weeds germinate in several flushes
- Necessitates sequential / repeated application
- Toxicity to crop / crop cultivars
- Toxicity to non-target organisms
- Residues in soil, water and food/feed
- Development of herbicide resistance in weeds

Ideal Herbicide: A pipe dream?

- Control all weeds with a single application of a herbicide
- Selective control of perennial and parasitic weeds
- Least or no phytotoxicity to crop
- Least propensity for development of resistance to herbicides
- Least or no residues left in the soil
- Safety to the user and the environment

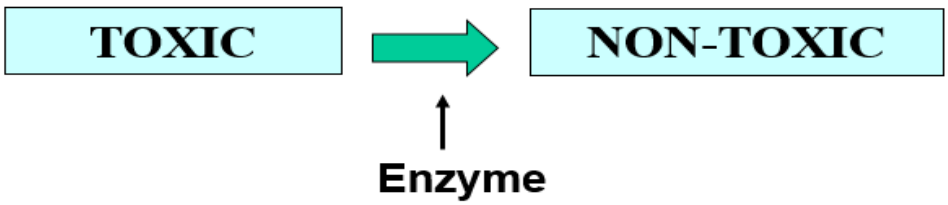


Using non-selective herbicides for selective weed control

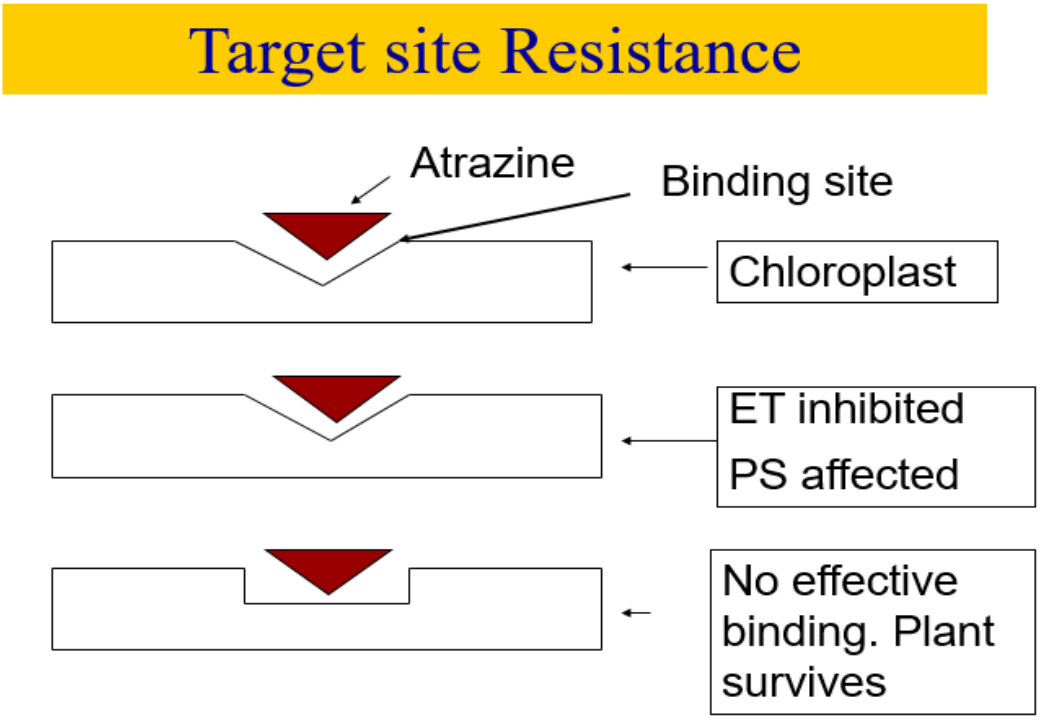
Making crop resistant to non-selective herbicide, ensure control of ALL weeds with no injury to crop. This is achieved through genetic engineering by inserting a gene resistant to the herbicide

This is what you get to see with GM HTCs

Mechanism of herbicide resistance



- Overproduction of target site of action (Non-target resistance/enhanced metabolism)
- Altered target site (Target site resistance)



Commercialized HRCs

Herbicide	Crops resistant
Atrazine	Potato, rape, tobacco
Bromoxynil	Cotton
Glufosinate	Tomato, sugarbeet, wheat, rape, rice, potato, peanut, maize
Glyphosate	Soybean, maize, cotton, rape
Imidazolines	Maize, rape, sugarbeet, tobacco

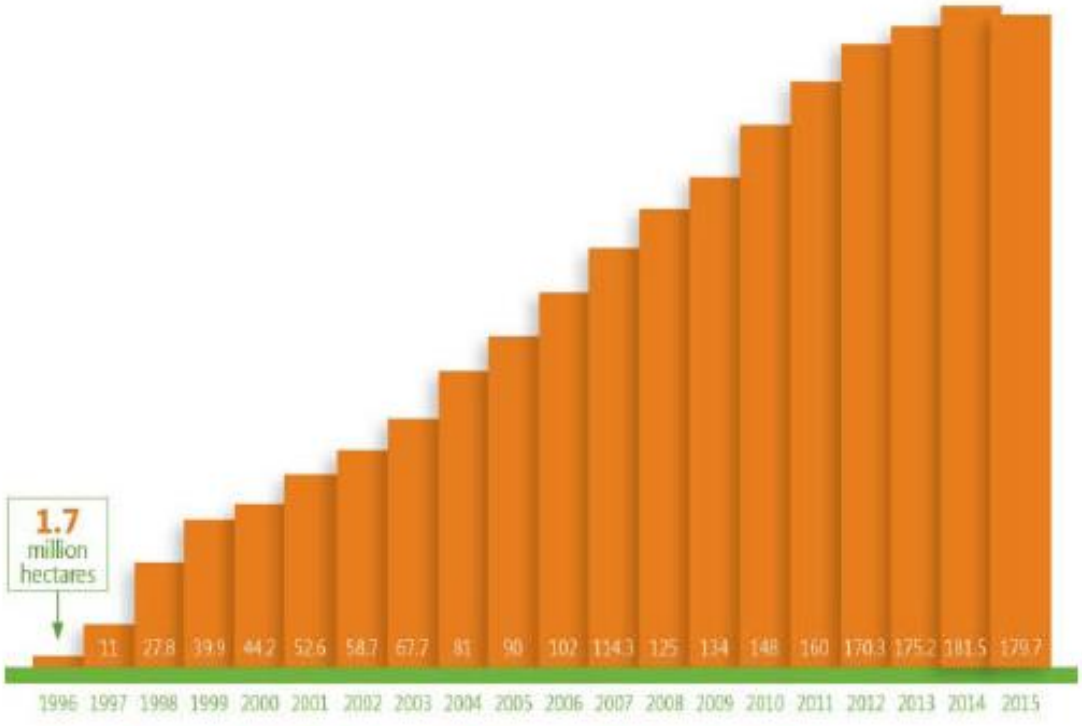


Figure 1. Global Area of Biotech Crops, 1996 to 2015 (million hectares).

Source: Clive James, 2015.

Where are Biotech Crops Grown in the World?

26 countries planted 191.7 million hectares of biotech crops in 2018, the 23rd year of global commercialization of biotech crops

21 DEVELOPING COUNTRIES

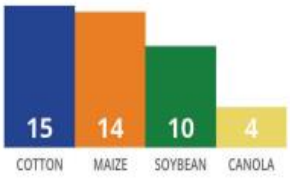
5 INDUSTRIAL COUNTRIES

TOP 5 COUNTRIES GROWING BIOTECH CROPS IN 2018 (MILLION HECTARES)

of biotech crops in the world is grown in **NORTH AMERICA** with the **USA** as the top producer

BIOTECH CANOLA's adoption rate in Canada has reached

NUMBER OF COUNTRIES GROWING MAJOR BIOTECH CROPS IN 2018



BRAZIL

is the top developing country planting biotech crops in 2018 with **51.3 MILLION HECTARES**

10 countries in Latin America planted **79.4 MILLION HECTARES BIOTECH CROPS** in 2018

2 countries in Europe planted **BIOTECH MAIZE** in 2018, led by

SPAIN

which grew ~95% of total biotech crops in Europe

ESWATINI

planted Bt Cotton for the first time in 2018. **SOUTH AFRICA and SUDAN** also planted Bt cotton in 2018.

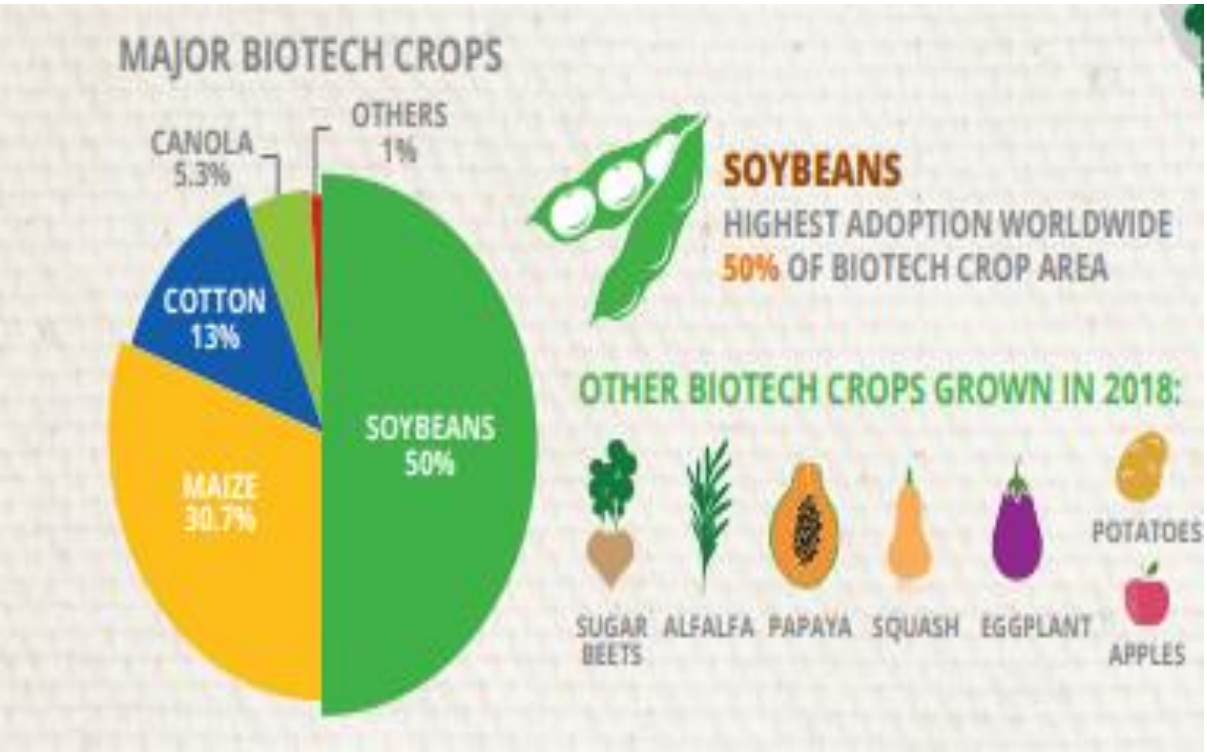
17M

small, resource-poor farmers and their families benefited from biotech crops in 2018

6 Million FARMERS

in **INDIA** planted 11.6 million hectares of **Bt COTTON** in 2018

BIOTECH SUGARCANE WAS PLANTED IN **INDONESIA** FOR THE FIRST TIME IN 2018



Source: ISAAA, 2021

- Among the HRCs, glyphosate-resistant crops dominate
- Popularly known as Roundup Ready (RR) crops

Area under glyphosate-resistant crops as % of total area (2014)

Crop	Global	USA
Soybean	**82**	**94**
Corn	**30**	**89**
Cotton	**68**	**89**

Reasons for wider adoptability of HTCs

- Easier, cheaper & better weed control
- Number & frequency of herbicide use reduced
- Control of HR weeds
- Higher crop yield & returns
- Enables ZT/MT and promotes CA- reduced soil erosion

HRCs - Concerns

- Increase (over-reliance) in herbicide use
- Abandonment of alternative methods
- Limit bio-diversity
- Problem of Volunteer (self-sown) weeds
- Development of "Super Weeds"
- Development of HR in weeds
- Food safety
- Infringement on farmers' rights
- Prohibits organic production of crops

HR crop as a volunteer weed (Ex:- HR canola in Canada)

- Shattering
- Harvest losses 5-10%
- Seeds viable for 4-5 years
- Could become a major weed (16-24th position) in Canada

Gene Flow

Through pollen

- Intra-specific (contamination of non-GM crops)
- Inter-specific (wild relatives and weeds)
- Inter-generic (wild relatives and weeds)

Through seed

- Volunteers in subsequent crops
- Dissemination through transportation

Herbicide resistance in weeds- A global challenge

Currently, 250 weed species (145 dicots and 105 monocots) infesting 86 cropping and non-cropping systems in 66 countries have developed resistance to 160 different herbicides belonging to 23 families with as many sites of action [Heap 2016]

Herbicides classified based on their risk to development of resistance in weeds

Risk	Mode of action	Herbicide
High	Amino Acid Synthesis Inhibitors (ALS Inhibitor)	Chlorimuron, Nicorsulfuron Thiefensulfuron, Primisulfuron Imazethapyr
	Lipid Synthesis Inhibitors	Fluazifop-butyl, Sethoxydim
	Cell Membrane Disrupter	Lactofen, Paraquat Nicosulfuron
Medium	Contact Photosynthesis Inhibitors	Bentazone, Bromorynil
	Pigment Inhibitors	Clomozone
	Root Growth Inhibitors	Pendimethalin, Trifluralin
	Systemic Photosynthesis	Atrazine, Metribuzine Linuron
Low	Amino Acid Derivatives	Glyphosate, Sulfosate
	Growth Regulators	Dicamba, MCPA, MCPB, 2,4-D, 2,4-DB
	Shoot Growth Inhibitors	Alachlor, Metolachlor EPTC

Exponential increase in glyphosate use

- Globally, use risen almost 15-fold since 1996
- GM-HTCs now account for about 56% of global glyphosate use
- In 2014, the use was equivalent to glyphosate at ~1.0 kg/ha on every hectare of cultivated cropland in US & nearly 0.53 kg/ha on all cropland worldwide
- Did not rotate herbicides
- Rotating Roundup-Ready soybean with RR-corn and RR-cotton was no rotation at all.

Glyphosate-Resistant weeds

- 38 weed species in 34 crops from 37 countries have developed resistance (Heap & Duke 2017)
- GR caused an increase of about $40-50/a in US
- WSSA estimates $ 43 billion annual losses due to uncontrolled weeds in corn and soybean in US and Canada- largely be due to GR-weeds

Food safety and health

- The presence of foreign gene in GM crop is "expected" to affect food quality and human & animal health adversely
- GM foods are better analyzed than any in human history
- A GM food is no more likely to cause a problem than the same non-GM food
- 75% of processed foods in the US have GE ingredients
- Millions have been consuming GM food for years with no single adverse effect
- The US National Academy of Sciences, reviewing the studies conducted globally over the past two decades concluded that that '***Genetically-engineered crops are as safe to eat as their non-GE counterparts, they have no adverse environmental impacts, and they have reduced the use of pesticides***' (NAS, 2016)

Bt-Cotton in India: A great success story

- Approved in 2002
- Cultivated on 9.4 mha (86%)
- Involving 5.6 million farmers
- Over 1000 hybrids and a variety covering 6 events available
- 23-43% increase in crop yield
- 50-130% increase in farmers' profits
- Added Rs 315 billion (since introduction) to national income
- 40-60% reduced pesticide use amounting to Rs 11 billion
- Increased employment opportunities, particularly to women

Despite the success of Bt-cotton, the future of other GM Crops in India is uncertain

The power of protests

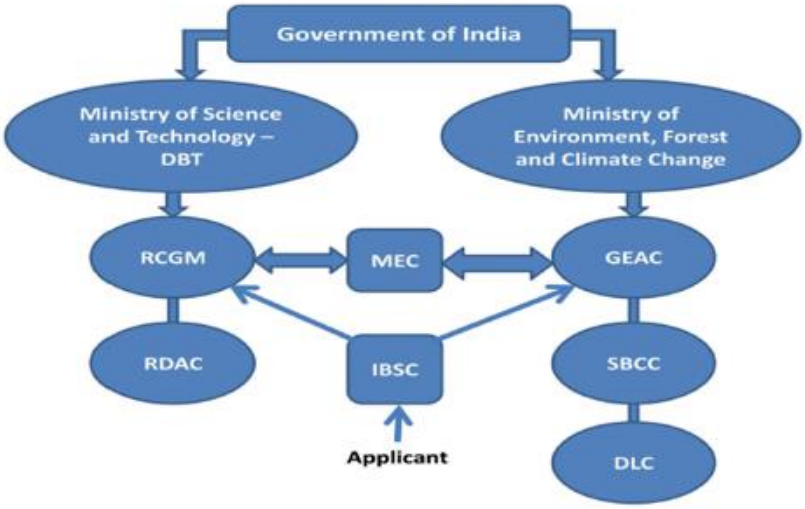
- **Bt-brinjal**: after successful trials for eight years and recommendations by the GEAC the technology was dropped (2009), because of pressure from the anti-GM lobby
- **GM mustard** developed by Delhi University (2016) also met the same fate
- Glyphosate-resistant cotton & maize have undergone controlled field trials for 4-5 yrs & waiting for approval
- Govt is coming under pressure from anti- GM activists

- Spreading misinformation and fanning tension and mistrust
- **Parliamentary Standing Committee (2012) expressed** concern over the potential and actual impacts of GM crops on farming, health & environment
- Concluded that **GM crops are just not the right solution for the country**
- Further stated that Bt-cotton has not improved the socio-economic condition of cotton farmers in the country but further deteriorated - especially in the rainfed areas
- SC appointed Technical Expert Committee sought 10 year moratorium on GM field trials

The academia ignored

- The National Academy of Agricultural Sciences (NAAS), under the Chairmanship of Prof M. S. Swaminathan, appealed the Government to lift the embargo on controlled field trials on GM crops and to approve the environmental release of the GE varieties, which have been tested to be bio-safe (NAAS, 2016)
- A group of top 17 agricultural scientists produced a paper arguing in favour of GM technology, stressing the need for ensuring food and nutrition security in the country (Datta et al., 2019)
- In 2016, 107 Nobel laureates appealed to *Greenpeace,* an environmental organisation, to rethink its long-standing opposition to GMOs

Regulatory framework for GM crops in India



Each committee is constituted by several experts from various public institutions who exercise their collective experience in evaluating the safety of a product

Conclusions

- Overwhelming support for HT technology by farmers & scientists
- The concerns on impact on environment & health & safety of GM food are scientifically unfounded
- Discourage biased, unsubstantiated & unscientific allegations
- It should not be an issue of emotion & politics
- Engage in public debate and share the right knowledge with all stakeholders, including decision makers
- No technology can solve all problems; no technology can last forever & problems do not remain the same
- Must give farmers access to new technologies that help them
- Learn from the mistakes done by other countries
- Never wage a war on weeds with chemicals only

Lecture

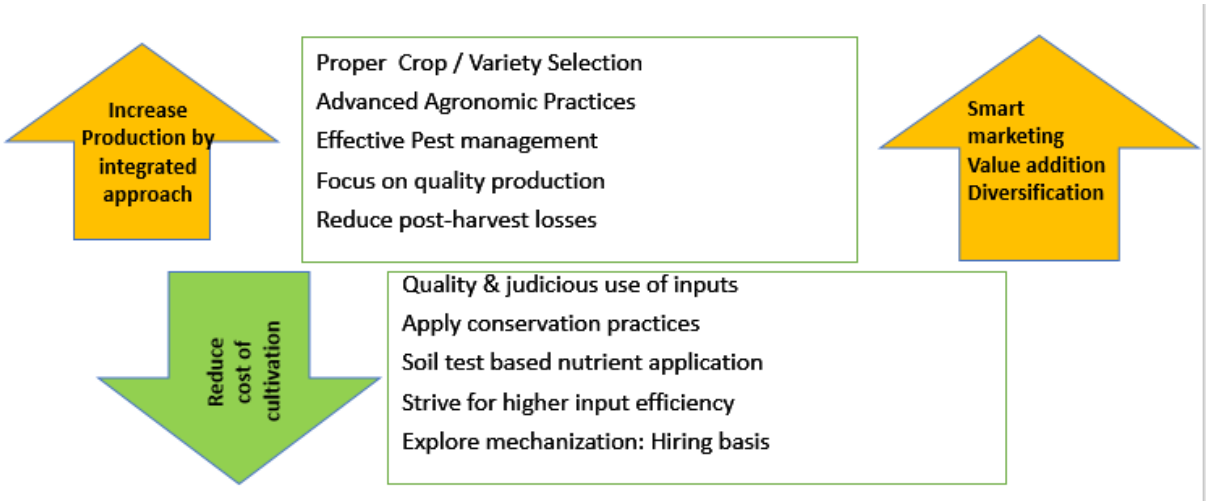
(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

Precision Agriculture

Farming no longer profitable

- Most acute is of fertilisers: between 1991-92 and 2013-14, price of urea increased by 69%, DAP by 300% and potash by 600%
- Labour is becoming unavailable and expensive
- Agricultural workforce has been consistently declining (65% in 1993-94 to 49% in 2011-12) resulting in net reduction of over 35 million
- Rural wages have been growing by 17% on average since 2006-07 - outstripping the urban wages
- Market volatility
- Climate change impacts

How to Improve Farmers' Income ?



Input use efficiency (IUE) is currently too low

- Currently output per unit of inputs- water, nutrients, pesticides, labour etc) is very low
- Need to increase IUE for higher profits
- Need-based application of nutrients, water and pesticides is vital
- Labour - scarce and expensive
- Mechanization is the need of the hour

Attempts made towards increasing FUE

- Placement vs broadcasting
- Split application
- Application based on soil testing (SSNM)
- Foliar application
- Applications based on tissue nutrient content

However:

- Does not take into account variability in the field
- Crop and cultivar

- Soil and climatic conditions
- Biotic stresses

Attempts made towards increasing WUE

- Furrow irrigation vs flooding
- Alternate furrow irrigation
- Basin application
- Drip irrigation
- Mulching
- Protected cultivation
- Hydroponics (soil-less)
- Scheduling irrigation
- Soil moisture conservation practices in RF conditions

Pest management

- Prophylactic/preventive approach
- Not dependent on pest load
- Improper identification of pests
- Blanket application

- **Resulting in:**
- Overuse
- Pesticide poisoning
- Adverse effect on environment/biodiversity
- Safety of food & water

Need to leverage technology for:

- Improving productivity
- Increasing IUE
- Reducing cost
- Increasing profit
- Reducing adverse effects on environment, biodiversity
- Safeguarding the health of humans and animals
- Strong case for adopting precision farming

Precision Agriculture

- **Precision agriculture** refers to the precise application of agricultural inputs with respect to soil, weather and crop **need** in order to improve productivity, quality & profitability in agriculture
- Also referred to as **site specific crop management** (**SSCM**): Farming management concept based on **observing, measuring** and **responding** to inter- and intra-field variability in crops

ICT tools/technologies in Agriculture

- Radio, Television: Technology info, Advisories, alerts, forecasts
- Geospatial technology: Remotely sensed information, Weather information
- Mobile Technology: Advisories, alerts, forecasts, market info

- Web application: App on Google store / IOS network
- Digital repositories:
- Internet of things (IoTs): Wireless Sensors
- Un-manned vehicles (UMVs)/Drones
- Self-driven tractors and smart machineries
- Robots
- Multispectral cameras, smart cameras- Imaging technologies

Internet of Things (IoT)

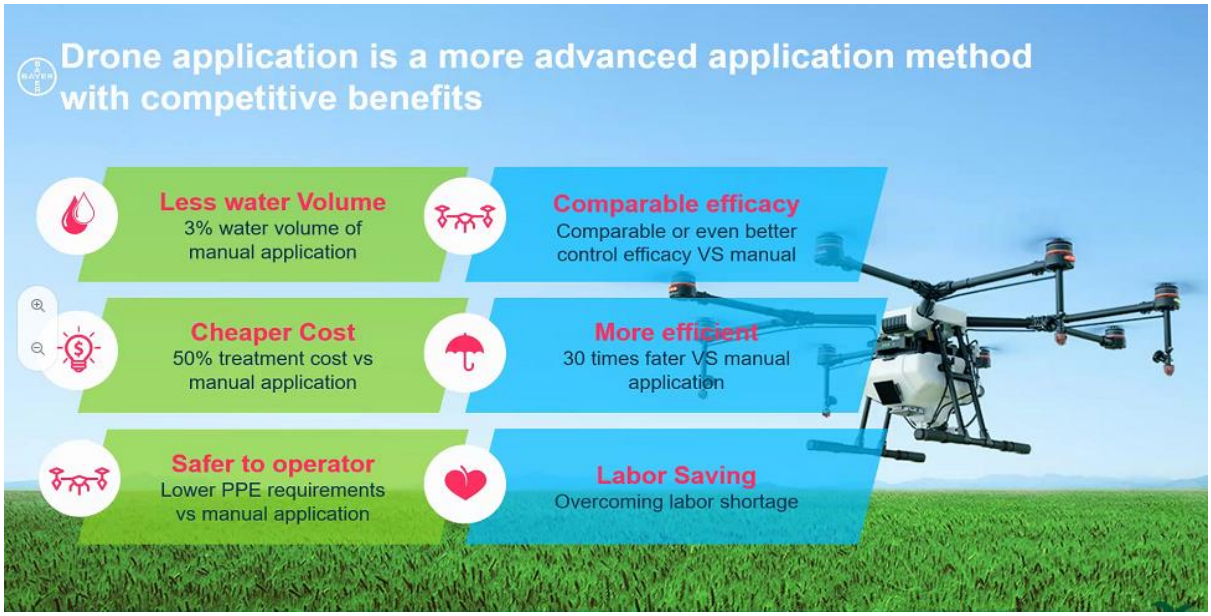
- Refers to a system of interrelated, internet-connected objects that are able to collect and transfer data over a wireless network without human intervention.
- It describes physical objects (or groups of such objects), that are embedded with sensors, processing ability, software, and other technologies, and that connect and exchange data with other devices and systems over the internet or other communications networks
- Applications in every field including agriculture
- Examples: home automation, wearable technology, connected health, and appliances with remote monitoring capabilities
- In agriculture: sensors for measuring and monitoring of soil moisture, pH, nutrients, temperature, wind speed etc
- satellite-connected guidance and tracking systems
- Share info in real time, alert for any deficiencies on user's mobile phones
- A host of sensors at ground and crop level can provide real-time information on soil health, water availability, crop fertility needs, and pest and disease pressure, while satellites and drones monitor weather changes, field conditions and crop health from above.
- Complex software will analyze this constant influx of data and package it into usable information, delivered to the farmer's cell phone in real-time, for making informed decisions
- Come harvest time, a network of sensors will track crop yield along with quality values, like water or nutrient content, in real-time. RFID will make it possible to track the movement of agricultural produce

Satellite based remote sensing

- In the past two decades, there has been significant progress made in utilizing remote sensing technologies in agricultural production
- More suitable than ground-based sensors for gathering broad-scale spatial information
- Useful in soil mapping, fertility assessments, crop monitoring, yield estimation etc
- Not beneficial for effective crop management applications at individual field-scales

Unmanned aircraft vehicles (UAV)/Drones

- Used for specific tasks where high-resolution images are required
- Captures images at low altitudes
- Fitted with sensors and a geospatial data handling framework that includes data acquisition, data storage, and possibly data transfer using communication protocols
- Drones are increasingly being used for seeding and application of fertilizers and pesticides



Mechanical weed control revisited

Problem of HR weeds and demand for organically grown food have led to the design and development of smart machines for mechanical removal of weeds.

Precision Hoeing

- Tractor guidance and auto-steer Precision hoes
- Operated by tractor that is guided by GPS
- Utilizes RTK-GPS tracking technology
- Implements are equipped with simple row-following vision technology, enabling them to actively and precisely follow rows
- Crop must be planted in straight rows with equal spacing
- Inter-row and in-row weeding smart machines are commercially available

Significant breakthrough in crop and weed differentiation

- Through artificial intelligence (AI), advanced image processing techniques and machine learning technologies, it has been made possible to identify weeds and to differentiate them from crop plants
- The system is better than Agronomists!!
- Blue River Technology company has developed **Sense and spray or see and spray systems.** The technology has been commercialized in the USA.
- This is taking weed management to a new level
- Saves herbicides by up to 80%

Robotic Weeders

- Aided by advanced vision technology, they move autonomously in the field scouting for weeds
- Capable of distinguishing between crops and weeds & take site-specific weeding action
- Versions with mechanical plucking tools, burning weeds with lasers/ steam or micro-dose of herbicide spray are being tested
- Operated by batteries powered through electricity or solar energy
- Prototypes of highly intelligent robotic implements are under trial in many countries

Conclusions

- Technology will continue to contribute to make agriculture more profitable and sustainable
- Agriculture is undergoing massive transformation - attracting serious attention and massive investments in Agtech companies
- Ensure that small farm holders are not left out. Prevent technology bias
- Many start-ups are helping farmers by providing required services for a small fee (SAAS)

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

IWM with special reference to biological control

WM in India - the reality check

- WM comprises 20-30% of total cost of cultivation
- Manual weeding- the most predominant method
- Weeding often takes last priority
- Highly laborious, back - breaking and inefficient
- Requires over 6 billion man-days
- Mostly women are engaged
- use of draught animals decreasing
- Cultural methods rarely used
- Herbicides – limited use but is on increase

WEED PREVENTION

- Use weed-free crop seed
- Use well decomposed manure
- Clean machinery/implements
- Inspect nursery stock/transplants
- Remove weeds near irrigation ditches, fence rows, rights-of-way etc.
- Prevent reproduction of weeds
- Use screens to filter irrigation water
- Restrict livestock movement
- Scouting, early detection and eradication of new weeds

Viable weed seeds in FYM (Pleasant and Schiather, 1994)

Weed species	Seeds/t.
Amaranthus spinosus L.	2700
Echinichloa crus-galli (L.) Beauv.	1900
Stellaria media (L.) Vill.	3500
Setaria viridis (L.) Beavois	1600
Setaria glauca (L.) Beauv.	4500
Polygonum aviculare L.	2700
Chenopodium album L.	42000
Brassica kaber (DC)	4700
Dactylis glomerata L.	5200
Amaranthus retroflexus L.	23900
Planto major L	5100
Capsellaburasa-pastoris (L.)	2700
Cirsium arvense (L.) Scop.	2700
Panicum capillare (L.)	2100

Manual Weeding

- Most common method

- Most labour intensive
- Mostly done by women
- Back-breaking and inefficient (perennials)
- Fails to remove weeds similar in appearance

Mechanical Control

- Timely weeding – not possible always
- If done timely effective
- Cost-effective
- Supplement with HW

CULTURAL METHODS

- Crop rotation
- Stale seed bed
- Optimum plant population
- Competitive crop cultivars
- Optimum planting date
- Optimum planting geometry
- Selective stimulation of crop
- Use of 'live mulches' or smother crops
- Intercropping

Stale seed bed preparation

- Encourage weed germination by pre-planting irrigation/rainfall
- Killing them with shallow cultivation
- Ideally through use of herbicides
- More than 50% annual weeds are controlled

Effect of crop diversification on population of *P.minor* and wheat yield (Banga *et al*, 1997)

Rice-wheat (continuous for 10 years)	2350
Rice-berseem, Rice-wheat	255
Rice-wheat, Rice-cotton, Cotton-wheat	2125
Rice-potato, rice-wheat	255
Cotton-wheat, (for four years)	38
Rice-berseem, Rice-berseem, rice-wheat	28

WM through crop competition

- Raise a good crop with full package of practices
- Understand crop-weed competition
- Give special emphasis on practises which shift balance of crop-weed competition in favour of crops
- Higher seed rate, bi-directional sowing, intercropping,
- Selective stimulation of the crop with correct method and time of fertilizer application and irrigation

Use of intercrops and cover crops

Some effective cover crops in plantation crops:

- *Calopogonium mucunoides*
- *Pueraria phaseoloides*
- *Centrosema pubescens*
- *Desmodium ovaifilium*
- *Stylosanthus gracilis*

Intercropping cowpea in maize

Treatments	Weed		Cowpea (t/ha)	Maize (t/ha)
	No/m^2	Kg/ha		
Maize-sole	96	940	-	3.59
Maize-2 HW	3	23	-	**5.76**
Maize + Cowpea	56	340	13.9	**5.51**
M + C + 1HW	46	320	17.3	5.36
LSD (P = 0.05)	24	110	NS	0.62

Selective stimulation of the crop to shift crop-weed competition in favour of crop

- Time of application of fertilizers
- Method of application- Placement against broadcasting
- Irrigating alternate ridges
- Drip irrigation

Biological Control

- Environmentally benign and ecologically acceptable
- Can be achieved through:
  - Insects and pathogens
  - Bioherbicides
  - Allelopathy

Biological Control with biocontrol agents

- A high degree of specificity for the target weed required
- No effects on non-target and beneficial plants or livestock and humans
- Not suitable for use in arable crops
- Promising in non-crop situations
- There are at least 5-6 major weeds in arable agriculture
- No bio-control agent will be effective against all of them
- Control of one weed will result in dominance of others

- Successful where a single weed dominates
- Ideal in non-crop areas, aquatic systems etc.

Bioherbicides:

- Use of microbial toxins or secondary plant metabolites
- They could be used as leads for the chemical synthesis of new herbicides
- Currently only few bio-herbicides have been registered & are in limited use

Commercialized Mycoherbicides

Name	Fungal pathogen	Target weed
De Vine	*Phytophthora palmivora*	*Morrenia odorata* in citrus
Collego	*Collatotrichum gloeosporoides f.sp. aeschynomene*	*Aeschynomene* spp. in rice and soybean
Luboa-2	*C.gloeosporoides f.sp. cuscutae*	*Cuscuta* spp. in China
Velgo	*C.coccodes*	*Abutilon theophrastii* in corn and soybean
Casst	*Alternaria cassiae*	*Cassia occidentolis* in soybean and peanut
Biomal	*C.gloeosporoides f.sp. malvae*	*Malva pusilla* in wheat and lentil

Mycoherbicides - limitations

- Finding host-specific pathogens difficult
- Require ideal environmental conditions for infection
- Require ideal conditions for storage and transport
- Has limited shelf-life
- Make way for other tolerant weeds

Allelopathy

- Host resistance is an important component in IPM
- In WM, this is almost impossible to accomplish
- Limited research has shown that few crops/crop cultivars exhibit inhibitory effect on some weeds through alleged allelopathic effect
- Despite considerable research, not much headway has been made
- However, it is an exciting area

Weed resistant crop?

By exploiting allelopathy, it is theoretically possible to develop crop cultivars resistant to weeds

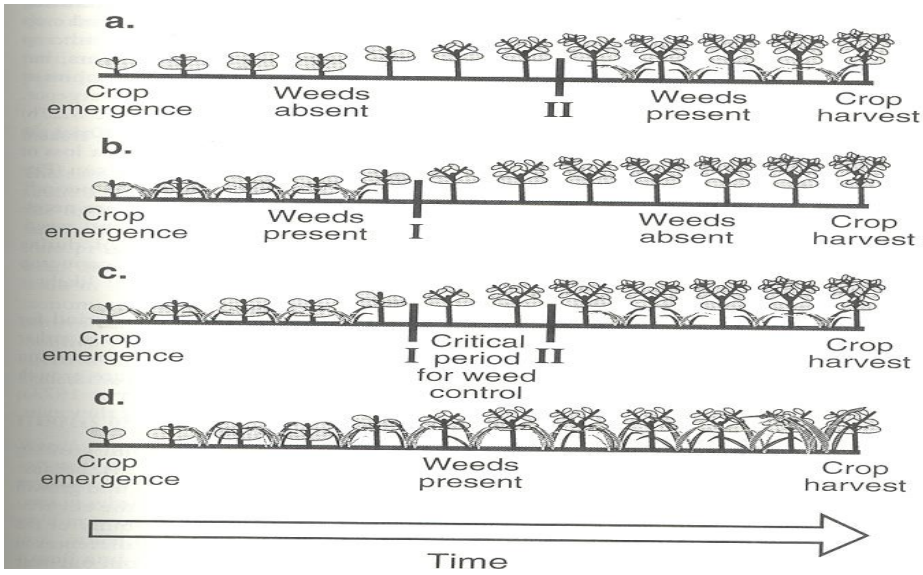
Herbicides- Benefits

- Save labour
- Easy and convenient to use
- Control perennial and parasitic weeds
- Efficient and economical control of weeds
- Well suited to min. and no-till conditions
- Mechanical damage to crop is prevented
- Offer gender equality
- Enthuse youth to take up farming
- Aid in diversifying farming

Herbicide use: Concerns

- Pesticide poisoning
- Contamination of food & water
- Toxic residues in soil
- Effect on non-target organisms
- Resurgence HR in weeds
- No single fool-proof method of weed control
- Long-term use of any single method runs the risk of failure
- Integrate two or more methods/approaches for effective, economical and sustainable control of weeds

Critical period of weed competition



IWM approach should aim at:

- Prevent introduction of new weeds
- Prevent spread of existing weeds
- Reduce weed seed bank in soil
- Minimise germination, establishment and competition by weeds
- Reduce weed seed return

Conclusions

- Weeds are known to evolve, adopt and survive any attempts to supress them
- Attempts to control weeds using a single method in a single crop cycle is not sustainable

- WM is not planned, but taken as an afterthought
- Reactive approach and not proactive
- Tendency is for a simplistic method – easy and convenient
- Long –term impact never considered
- Importance of weed seed bank is not given its due
- Knowledge of weed biology and ecology is vital in developing IWM strategies

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

Organic Agriculture: Opportunities and Challenges

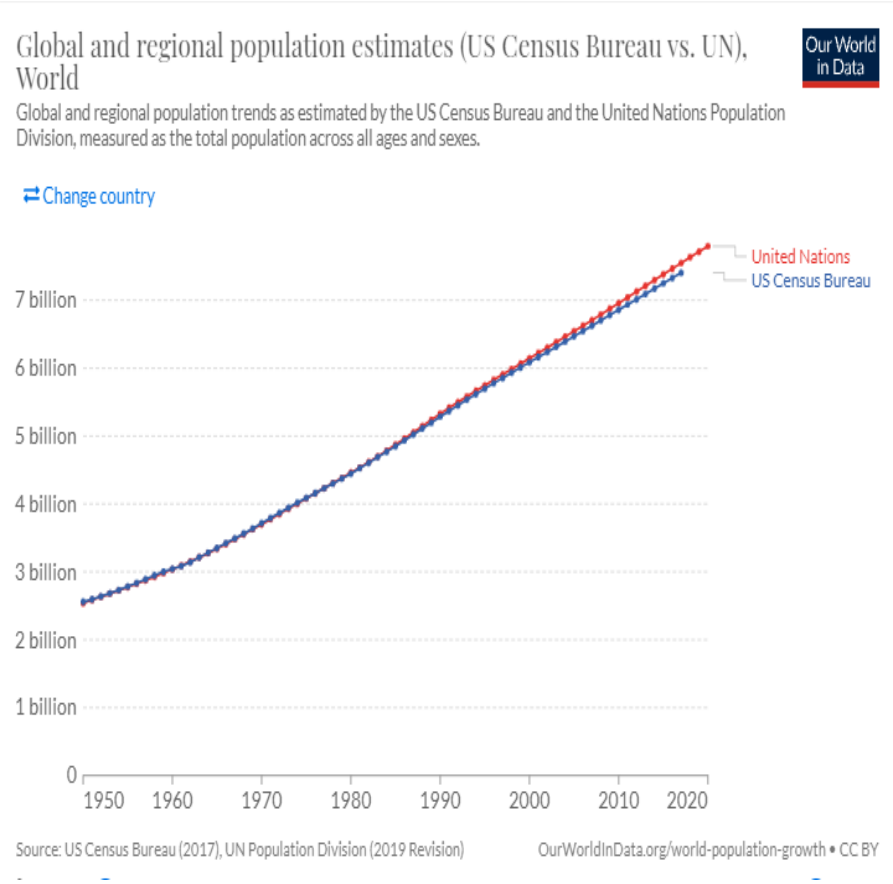
But what does "organic" mean, really?

At its core, organic refers to food that's been managed, raised, and farmed in a manner that conserves natural resources and biodiversity.

According to the United States Department of Agriculture (USDA), certified organic foods are grown and processed according to specific federal guidelines, including those that address soil quality, animal raising practices, pest control, weed control, and the use of additives. Most of the time, organic farmers use natural substances as well as physical, mechanical, or biologically based methods as often as possible.

In short, it refers to raising crops or animals without the use of synthetic fertilizers, pesticides and antibiotics. It excludes the use of GMOs also.

- Agriculture is over 10,000 years old
- Slash and burning (*Jhoom*) agriculture
- With increase in population and settlements of humans, they started practicing subsistence agriculture
- Agriculture was less intensive- no use of external inputs
- There was enough cultivable land, and sufficient food produced to feed the population
- It was organic by default
- Pests and diseases were controlled by crop rotation and use of local resources



Took:

2 million yrs to reach 1 billion mark (1803)

125 yrs to reach 2 billion (1928)

Population doubled from 2.5 to 5.0 billion in just 37 years (1950 and 1987)

- India experienced acute food shortages until 1960s
- Famines and hunger were very common

- Dependent on large-scale food imports
- Ship-to-mouth existence
- Until the Green Revolution in the mid-1960s

THE GREEN REVOLUTION & INDIA'S AGRICULTURE

Parameter	1970-1971	1980-1981	1990-1991	2000-2001	2011-2012	2016-2017
Net Sown Area (Mha)	140	140	143	141	141	141
Gross Sown Area (Mha)	165	172	186	185	196	198
Net Irrigated (Mha)	31	39	48	55	66	68
Fertilizer Use Nutrients (Mt)	2.2	5.5	12.5	16.7	27.8	25.9

6

INDIAN AGRICULTURE: A GLOBAL SUCCESS STORY
PRODUCTION INCREASE 1947 - 2020

Commodity (ME)	1947	2020	Factor
Food Grains	50	298	6
Horticulture	~32	320	10
Milk	20	184	9
Fish	1	12.6	13

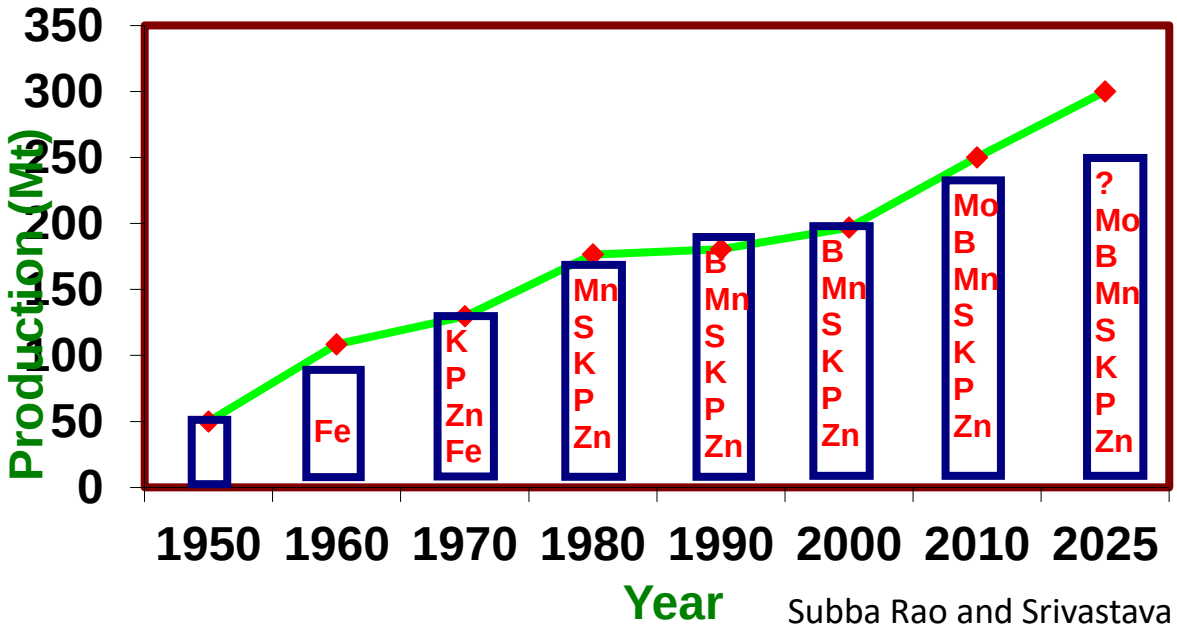
- Grain Export: 20 Mt/y (US $40B)
- Grain Buffer Stock: 50 Mt
- Poverty Reduction (%): 70 to 20

7

But Green revolution has come with a cost

- Poor soil health – low organic carbon, erosion, degradation
- Depletion of ground water
- Mono-cropping, Cereal-centric farming - Loss of biodiversity
- Increased environmental pollution (soil, air and water)
- Poor quality of food - human health

Green Revolution helped in achieving higher yields but led to multi nutrient deficiencies

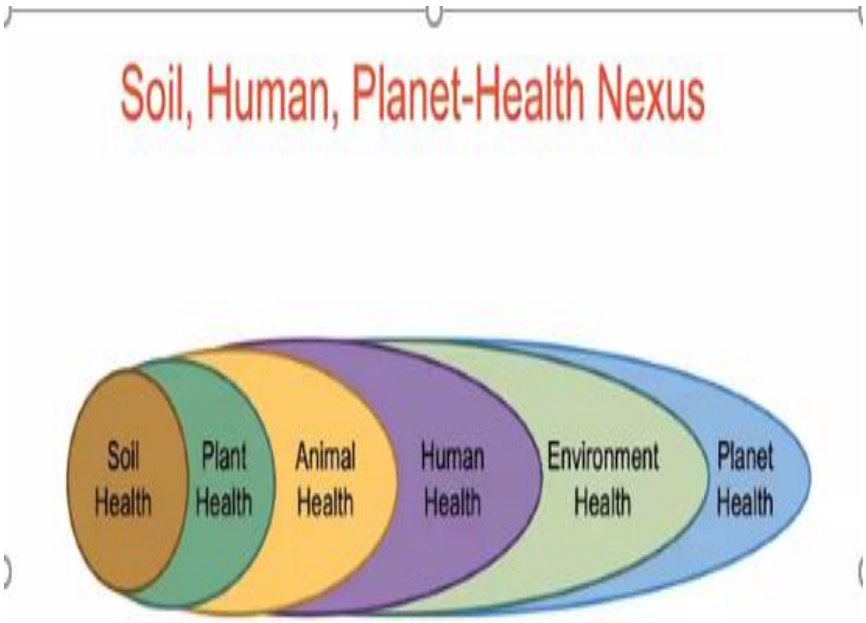


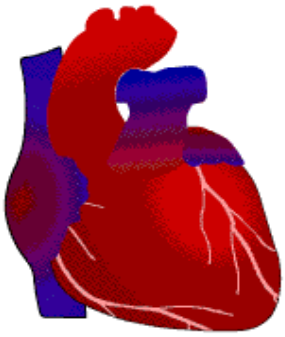
Subba Rao and Srivastava (2007)

Changes in mineral content of different types of vegetables (27 varieties), fruit (17 types) and meat (10 cuts) measured between 1940 and 1991.

Mineral	Vegetable	Fruit	Meat
Sodium	-49%	-29%	-30%
Potassium	-16%	-19%	-16%
Phosphorou	+9%	+2%	-28%
Magnesium	-24%	-16%	-10%
Calcium	-46%	-16%	-41%
Iron	-27%	-24%	-54%
Copper	-76%	-20%	-24%

Ref: 'A study on the mineral depletion of the foods available to us as a nation over the period 1940 to 1991' by David Thomas. Nutrition and Health 2003; 17: 85-115.





Soil Organic Matter is the

Life of Soil

(Lal, 2021)

Negative impact of high input agriculture on environment; growing public awareness about quality of food; increased household income are the main reasons for the increasing popularity of organic food and it has taken off strongly.

Organic Agriculture some statistics – (ICAR-IIFSR, Modipuram)

- 184 countries practicing
- 72.3 mha global area
- 3.1 million global farmers
- Over USD 100 billion market
- 44% in India
- 4.7% of the global area
- India- 2.4% of the net cultivated – Target 4.0%
- 22% growth
- 9000 cr export (69 countries) - 1000 cr domestic market
- Commodities- almost all

Plant nutrition under OF

- FYM
- Cow dung & urine used extensively
- Vermicomposting (*Eisemia foetida, Eudrilus eugeniae, Perionyx excavates*)
- Pospho-compost (rock phosphate, Pyrites with PSB)
- Biofertilizers (Rhizobium, Azospirillum, PSB, PGPR)
- Inclusion of legumes

Pest control under OF

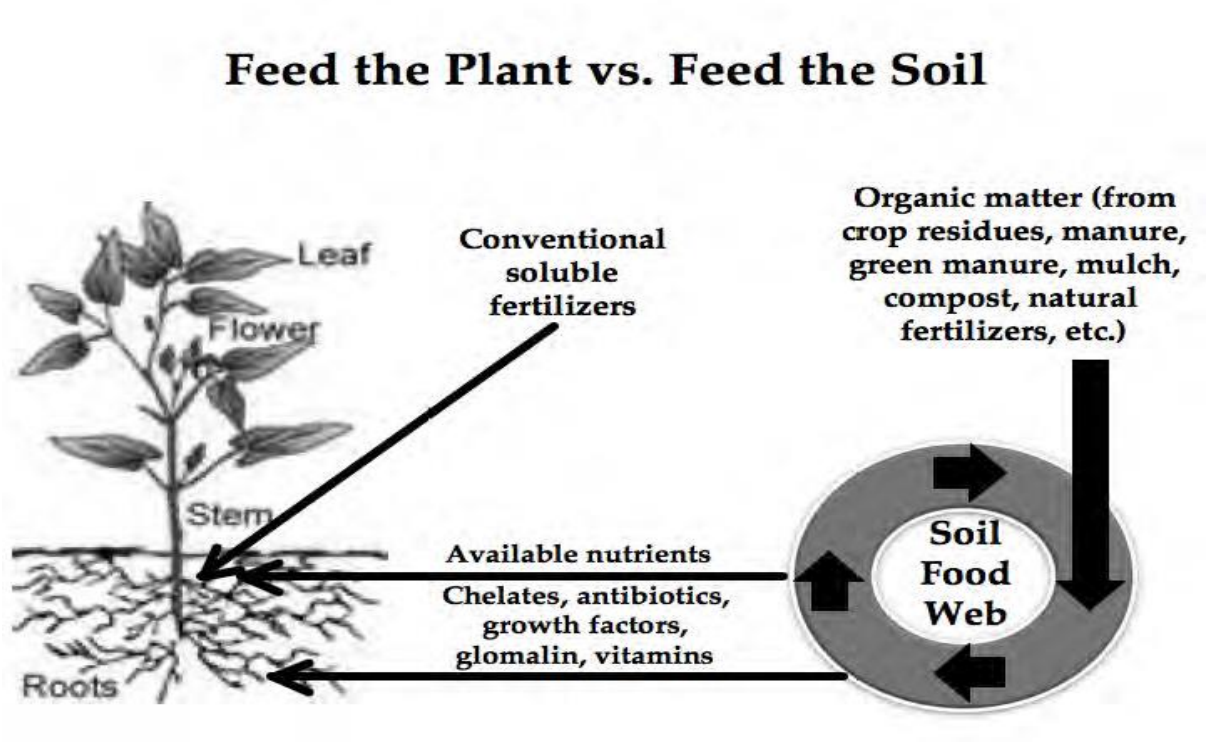
- Preventive methods
- Cultural methods –tillage, trap crops, crop rotation
- Resistant crops
- Insect pheromones
- Extensive use of Biocontrol agents
- Parasitoids (Trichogramma, Goniozus)
- Predators (Cryptolaemus, Chrysoperla)
- Pathogens (Bt, NPV, Fungi)
- Antagonists
- Cow dung, urine
- Neem extract/ cake

Soil solarization

- Covering soil with thin transparent PE films for 2-6 weeks during hot summer months
- Raises the surface soil temperature by 8-12^{o}C (55-60^{o}C actual)
- Lethal to soil pathogens, nematodes and weed seeds.

Organic Farming: Benefits

- Environmentally benign and ecologically acceptable
- Ensures sustainability and enriches soil fertility and health
- Enriches soil fertility and biodiversity
- Provides healthy food
- Growing market for organic produce



Organic Farming: Limitations

- Less productivity

- Organic food is expensive
- Involves lot of labour
- Pest management is a challenge- Spurious product in the market
- Certification is cumbersome, expensive
- Animal manure used as domestic fuel
- Crop residues used as fodder and fuel
- Reduction in livestock- Livestock resources
- Marketing
- Public perception- sceptical

Organic farming: Opportunities

- Growing market – domestic and global
- Growing consumer acceptance
- Contract farming
- Farmer producing organizations/FPCs
- Digital technologies, precision farming
- Govt. initiatives- Digital India, Organic India, *Pradhan Mantri Fasal Bima Yojana*
- Concentrate on niche regions/export orientated crops
- Ideal for spice crops, plantation crops, fruits, vegetables

Conclusions

- Environmentally benign and ecologically acceptable technology
- Growing consumer acceptance and market for organic food
- Move cautiously- keeping in mind the food security of the country (example: Sri Lanka)
- Focus on selected commodities
- Widen affordability- use technology reduce cost/increase production
- Greater sensitization among all stakeholders of the potential of OF
- Use technology (traceability) to instil confidence with exporters & consumers

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

Global climate change and its impact on agriculture

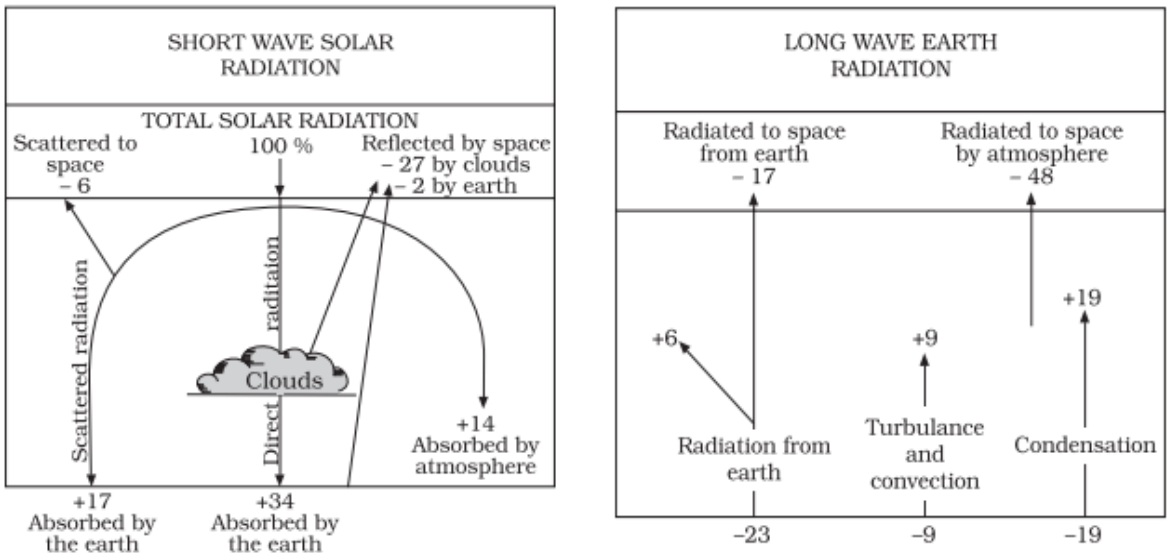
Weather vs Climate

Weather is the state of the atmosphere at a particular place and time as regards heat, cloudiness, dryness, sunshine, wind, rain, etc.

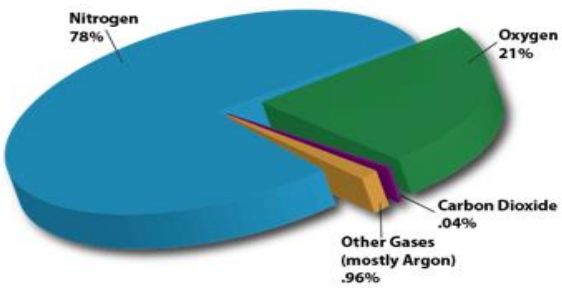
Climate is the synthesis of weather conditions in a given area, characterized by long-term statistics (mean values, variances, probabilities of extreme values, etc.) for the meteorological elements in that area (WMO, 1992)

Climate affects human life on Earth. It regulates food production and water resources and influences energy use, disease transmission, and other aspects of human health and well-being (US National Research Council, 2010).

Earth's Heat Budget



Composition of Earth's atmosphere



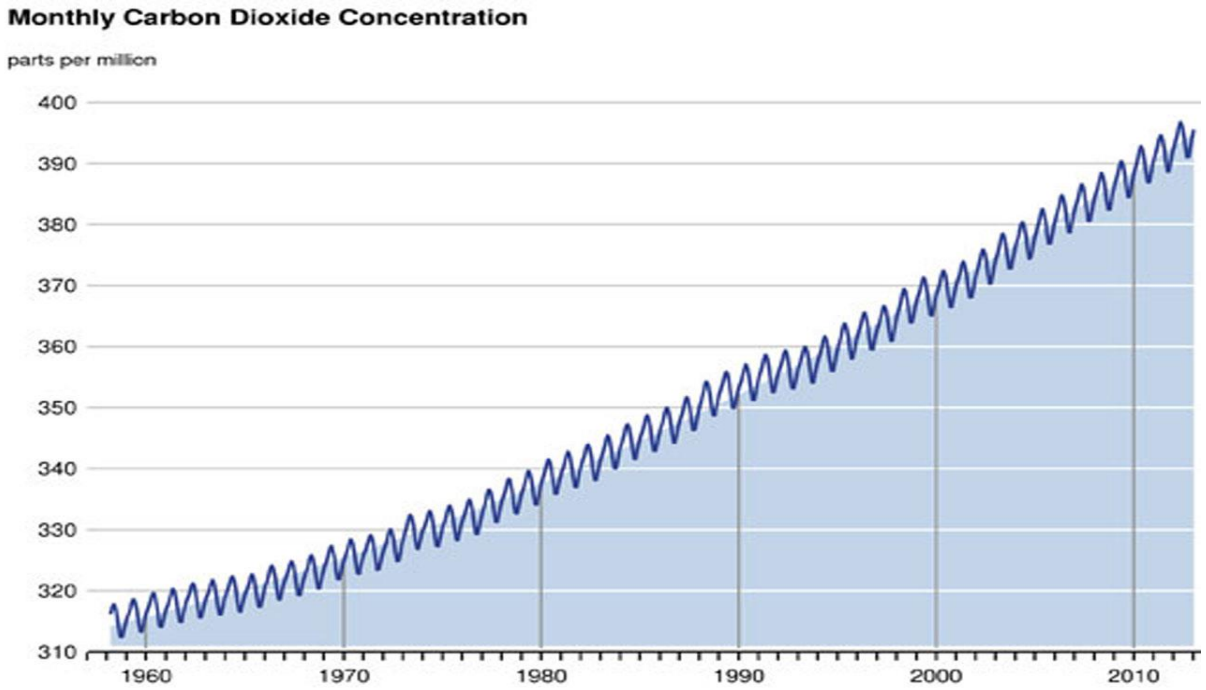
0.1 percent other gases include trace amounts of carbon dioxide, methane, water vapor, and neon are some of the other gases

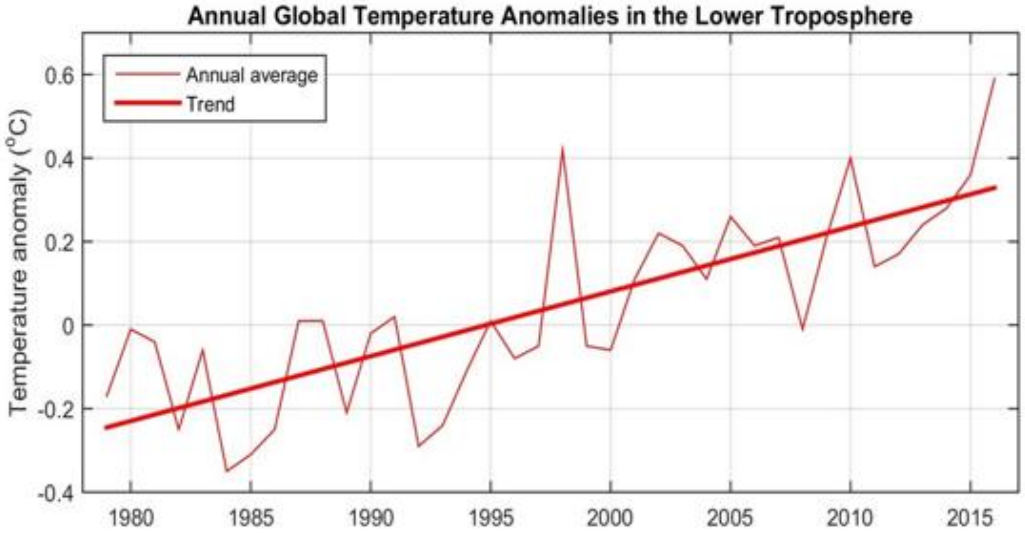
Earth's atmosphere is mainly composed of Nitrogen and oxygen have little or no influence on radiation coming from the sun or that emitted by Earth's surface. However, water vapor,

carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and chlorofluorocarbons (CFCs), absorb and re-emit infrared radiation emitted by the surface of the Earth and trap heat in the atmosphere. These are called greenhouse gases (GHGs)

What is Greenhouse effect?

Greenhouse effect is the phenomenon whereby Earth's atmosphere traps solar radiation, caused by the presence in the atmosphere of GHGs, which allow incoming sunlight to pass through but absorb heat radiated back from Earth's surface.





Earth's average surface air temperature has increased by **about 1 °C (1.8 °F)** since 1900, with over half of the increase occurring since the mid-1970s

What is Climate change?

Climate change represents a significant change, that is, a
change with important economic, environmental, and social effects, in the mean values of a

meteorological element, such as temperature and amount of precipitation during a certain period, for which the means are computed across a decade or longer (World Meteorological Organization, 1992). Greenhouse gases like carbon dioxide (CO_2) are released when we burn oil, gas and coal for our homes, factories and transport. The estimated dry matter production from agriculture in India is almost 800 million t year. This is equivalent to fixation of 320 Tg of C or 1000 Tg of CO_2 per annum. Only a part of this is retained over time, while the rest is released back to the atmosphere

Methane: The total annual output of CH_4 into the atmosphere from all sources in the world is estimated to be 535 Tg year. India's total contribution is only 18.5 Tg year. The increase in annual load of CH_4 in the atmosphere is much less than that of CO_2, but its higher absorption accounts for a major contribution to global warming. Flooded rice fields and ruminant animals are the major (68%) sources of CH_4 emissions.

Nitrous oxide: N_2O, which is present in the atmosphere at a very low concentration (310 ppbv), is increasing at 0.02% per year. But important because of its longer lifetime (150 years) and greater global warming potential than CO_2 (about 300 times more than CO_2). Both fertilized and unfertilized soils contribute to the release of this gas.

Factors contributing to Global warming

- Increased human activity
- Rapid industrialization
- Combustion of fossil fuels
- High input agriculture
- Deforestation
- Increased number of vehicles
- Rapid changes in life style
- The driving force behind these factors is the ever-increasing human population

The major contributors to global warming:

- Energy sector (which includes fossil fuel burning) - 61%
- Agriculture and its allied activities - 28%
- Industrial sector - 8%
- Others - 3%

Consequences of global warming

- Abnormal weather conditions- flooding, droughts, forest fires etc
- The quantity of rainfall and its distribution
- Melting of glaciers - Rising of sea levels
- Oceans and its habitats are under threat, example, the Great Barrier Reef in Australia has already lost half of its coral since 1995
- Frozen ground melts in places like Siberia, GHG trapped for centuries will be released into the atmosphere, worsening climate change
- Threatens biodiversity – many plants and animals may become extinct
- Decreases in agricultural productivity- hunger and malnutrition
- Huge social and economic cost

What are governments doing?

- Intergovernmental Panel on Climate Change (IPCC) created in 1988 by the WMO and the UNEP

- The objective to provide governments scientific information that they can use to develop climate policies and negotiations
- Provide a comprehensive summary of what is known about the drivers of climate change, its impacts and future risks, and how adaptation and mitigation can reduce those risks
- The UN Framework Convention on Climate Change (UNFCCC), adopted at the 1992 Earth Summit is one of the first international treaties on the topic.
- It stipulates that parties should meet regularly to address climate change, at the Conference of Parties or COP. It forms the foundation to future climate agreements.
- The Kyoto Protocol (1997) regulated greenhouse gas reductions for a limited set of countries from 2008 to 2012. The protocol was extended until 2020 with the Doha Amendment in 2012.

The Paris Agreement (2015)

- A landmark agreement- Covers climate change mitigation, adaptation, and finance.
- The Agreement was negotiated by 196 countries. USA withdrew in 2016 but rejoined in 2021
- The goal is to keep the rise in mean global temperature to well below 2 °C (3.6 °F) above pre-industrial levels
- Emissions should be reduced as soon as possible and reach net-zero by the middle of the 21st century
- It aims to increase the ability of parties to adapt to climate change impacts, and mobilize sufficient finance.
- Under the Agreement, each country must determine, plan, and regularly report on its contributions.
- No mechanism forces a country to set specific emissions targets, but each target should go beyond previous targets.

What can individuals do?

Some small changes in the way we live could have a significant impact, For example:

- Switch to renewable sources of energy
- Take fewer flights, car pool, cycling, taking the stairs,
- Use electric car/scooter
- Avoid wastage- water, food, energy
- Buy energy efficient products- AC, lights (LED), machines
- Reuse, recycle, repurpose
- Insulate your home

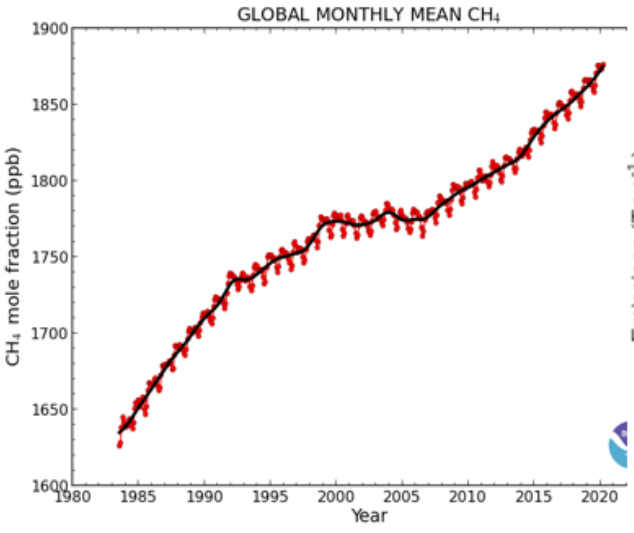
IPCC: Sixth Assessment Report (2021)- highlights

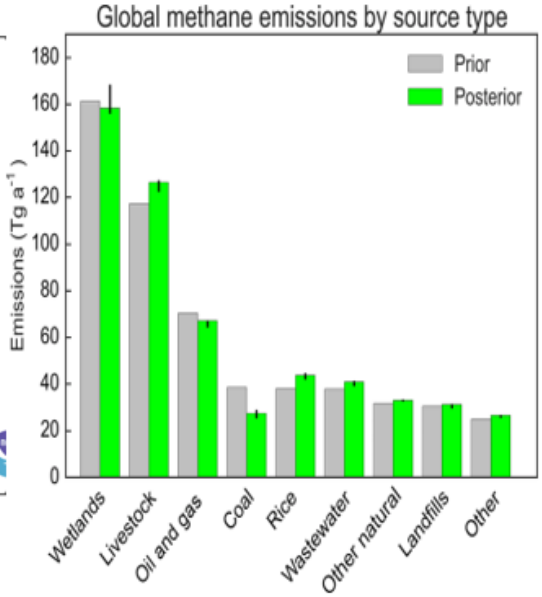
- Mitigation strategy agreed upon in 2015 Paris Summit is insufficient
- More climatic extremes – floods in China, Germany
- Scorching heat in Wester USA, Canada (BC – 49.6^0 C)
- More frequent, some would be irreversible
- India – more vulnerable, Indian ocean warming up at fastest rate
- > 90% of the heat is originating from oceans
- Unplanned development, deforestation etc.

Global share of some countries in CO2 emissions(%)

Country	1990	2003
Canada	2.19	2.39
China	10.41	14.07
France	1.80	1.63
Germany	4.24	3.35
India	2.63	4.07
Italy	1.91	1.85
Japan	5.54	4.79
Russia	9.67	6.38
United Kingdom	2.76	2.24
United States	23.04	23.06
Rest of the world	38.61	36.17

(Sathaye et al., 2006)





Impact on agriculture

Simulation models: forecasts- diverse, inconsistent, difficult to quantify
Some generalizations include:

- Elevated CO2 concentration may increase crop growth and yield due to increased photosynthesis
- The increase in temperature, however, may decrease grain yields due to the shorter duration of crop growth
- More crop losses will occur due to frequent unseasonal weather events- cyclones, frost, heat, hailstorms etc

- Loss in productivity, example according to the FAO, 1^0C rise reduces wheat yield by 6 million tons (US$ 1.5 billion at current prices)
- All other crops the loss would be US$ 20 billion each year
- Shortened crop cycle – limited choice of crops
- News pests, altered severity

Mitigation and adaptation strategies

Mitigation strategies aim at reducing GHG emissions into
the atmosphere
Adaptation strategies aim at enabling crops and livestock to perform optimally under adverse climatic conditions through management practices and genetic manipulations
Climate-smart agriculture or
Climate resilient agriculture

How can climate-smart agriculture mitigate climate change?

It includes **stopping emissions at their source**—such as reducing fossil-fuel dependence and deforestation
Carbon sequestering - drawing carbon down out of the air and storing it in trees and soils.
For agriculture, there are three key greenhouse gases: carbon dioxide, methane and nitrous oxide.

Climate-resilient agriculture (CRA) is an approach that includes:

- Using existing natural resources through crop and livestock production systems sustainably to achieve long-term higher productivity and farm incomes under climate variabilities.
- Equipping farmers to absorb and recover from shocks and stresses to their agricultural production and livelihoods.
- **National Innovations on Climate Resilient Agriculture** (NICRA) was launched in 2011 by ICAR with the objectives of strategic research, technology demonstrations and capacity building.

Conservation Agriculture

- Tillage leads to erosion, oxidation of soil organic matter resulting in release of CO_2 to atmosphere
- Removing plant residues for fodder, fuel or burning releases CO_2 air
- CA retains photosynthetically fixed CO_2 in soil, builds up OM through microbial decomposition - improves soil health
- OM supply major and micronutrients for plant and microbial growth
- We expect soil organic matter to provide fertility, but we forget that soil organic matter also needs fertility to be restored
- Soil carbon decline could be arrested and reversed when crop residue inputs were balanced with supplemental nutrients irrespective of tillage

Animal farming: some points to ponder

- Globally, 33% of the land is devoted to livestock farming
- 60% of the grain produced is fed for animals
- 75% of the soybean is used for livestock feeding
- Would have fed 3.5 billion people

To produce one kg of meat, the quantity of grain needed is:

- 5 kg for chicken
- 9 kg for pork
- 25 kg for beef

5000 litres of water needed to produce one kg meat

Some mitigation measures:

- Use more of natural resources as far as possible
- Minimize use of non-renewable sources of energy
- Use crop varieties/breeds developed for specific situations
- Adopt efficient crop/livestock management practices
- Use precision farming techniques
- Lay more emphasis on increasing Soil Health
- Adopt agri-silvi-pastoral system wherever possible
- Be prepared for adverse weather events
- Cover risks with Insurance
- Take help from experts

Conclusions

- Climate change is for real - Extreme weather events are already more intense, threatening lives and livelihoods
- People in poorer countries will suffer the most as they do not have the resources to adapt to climate change
- If nothing is done, global warming could exceed 4.0^0 C by 2100, leading to devastating consequences
- World leaders must join hands to fight this global challenge
- Experts believe that attaining global targets (under 1.5^0 C by 2100 and net zero by 2050) is achievable, but it will require governments, businesses and individuals to make big changes
- Think Global, act Local

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

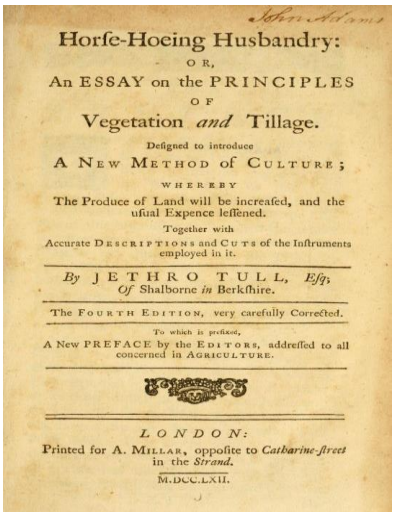
Conservation Agriculture

Tillage- The first operation in crop production

The objectives of tillage:

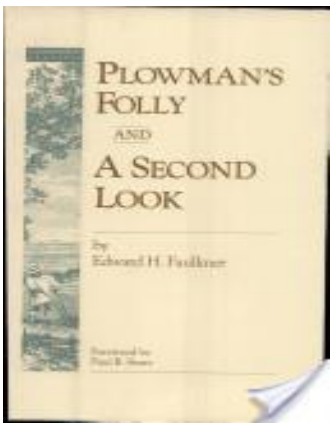
- To prepare fine seed-bed for sowing/planting
- To ensure proper germination and initial establishment of crop
- Improve moisture conservation
- Control weeds and other pests
- Mixing of fertilizers and organic manures

Jethro Tull (1671-1741) – The father of tillage


Horſe-Hoeing Husbandry:
OR,
An ESSAY on the PRINCIPLES
OF
Vegetation *and* Tillage.
Deſigned to introduce
A NEW METHOD of CULTURE;
WHEREBY
The Produce of Land will be increaſed, and the uſual Expence leſſened.
Together with
Accurate DESCRIPTIONS and CUTS of the Inſtruments employed in it.
By JETHRO TULL, *Eſq;*
Of Shalborne *in* Berkſhire.
The FOURTH EDITION, very carefully Corrected.
To which is prefixed,
A New PREFACE by the EDITORS, addreſſed to all concerned in AGRICULTURE.
LONDON:
Printed for A. MILLAR, oppoſite to *Catharine-ſtreet* in the *Strand.*
M.DCC.LXII.

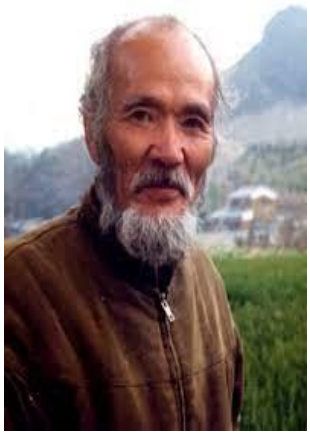
- Carried out numerous experiments dealing with cultural practices
- Believed that soil should be finely pulverized to provide proper pabulum for the growing plant
- Propounded a theory that ‘Soil particulars are ingested through openings in plant roots due to the processes caused by the swelling of growing roots’
- His experiments led to development of drill and horse drawn cultivator and a book on **“Horse Hoeing Husbandry’**
- Regarded as ‘Father of Tillage’

Edward H. Faulkner (1886-1964)


PLOWMAN'S FOLLY
AND
A SECOND LOOK
by
Edward H. Faulkner

- In the early 1940s, Faulkner dropped what *Nature* magazine termed **"an agricultural bombshell"** when he blamed the then universally used moldboard plow for disastrous pillage of the soil.
- He questioned the use of plough for cultivation of crops, and showed that all standard wisdom used as a rationale for ploughing and working the soil was invalid
- His ideas were considered 'mad' and without merit, until after his death when soil experts and scientists began to admit 'We didn't pay attention, and we should have.'
- **He is regarded as one of the first true conservationists**

Fukuoka (1975)



Fukuoka (1975) worked for more than 65 years at his farm in Japan and developed a system of **Natural Farming**. He did not plough his fields, used no agricultural chemicals nor prepared fertilizers, did not flood his rice fields as farmers have done in Asia for centuries, and yet his yields equalled or even surpassed the most productive farms in Japan. His book **'One Straw Revolution**' – one of the bestselling book in agriculture - contained the spiritual memoir of a man whose innovative system of cultivating the earth reflects a deep faith in the wholeness and balance of the natural world.

Consequences of excessive tillage

- Soil compaction- root growth, water penetration
- Soil erosion
- Wind erosion

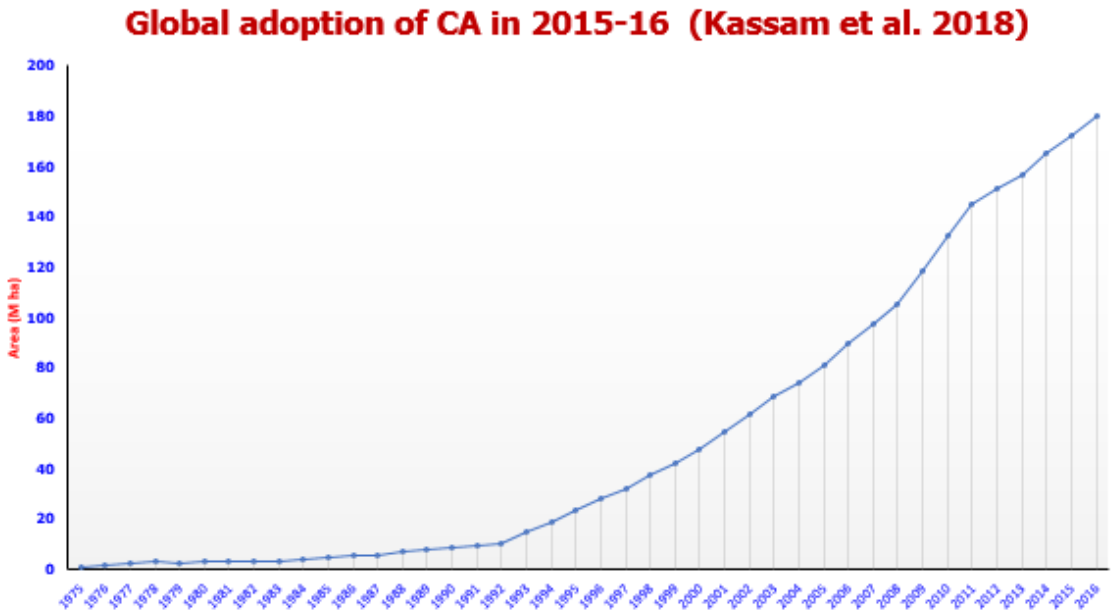
The Birth of Conservation Agriculture(CA)

- Farmers to beginning to practice Zero tillage in the USA in the 1960s to reduce soil erosion losses
- Farm machineries went in for a huge transformation
- Brazil started recognizing the merits of ZT in the 1970s
- Started experimenting with ZT with crop residues on the surface
- Stepping stone for a technology now called Conservation Agriculture
- It took another 20 years for the CA to make a mark as a novel technology

Leading countries adopting CA (area in Mha)

Country	2008-09	2015-16	% increase
USA	26.5	43.2	63.0
Brazil	25.5	32.0	25.5
Argentina	19.7	31.0	57.4
Canada	13.4	19.9	48.5
Australia	12.0	22.3	85.8
Paraguay	2.4	3.0	25.0
Kazakistan	1.3	2.5	92.3
China	1.3	9.0	592.3
India	-	1.5	-
Others	4.4	16.1	265.9
Total	106.5	180.5	69.5

Source: Kassam *et al.* (2019)



What is Conservation Agriculture?

- Planting crops continuously with no or minimal mechanical soil disturbance
- Maintenance of permanent biomass soil mulch cover on the ground surface
- Two major innovations in the latter half of 20th century that led to the popularity of CA are:
  - Availability of new farm machinery capable of planting crops and placing fertilizers without tillage and in the midst of crop residues - anchored or loose
  - Availability of range of herbicides to manage weeds effectively

Three principles of CA are:

- Continuous no or minimal mechanical soil disturbance
- Maintenance of permanent biomass soil mulch cover on the ground surface
- Diversification of crop species

All to be applied together. If applied separately, they do not constitute a CA system

Brown Manuring: Live mulch

- Crop and *Sesbania* sown together for 25-30 days
- Subsequently co-culture *Sesbania* crop is knocked down with 2,4 D or Bispyribac (rice)
- Can achieve ~50% weed control; reduce perennial *Cyperus rotundus*

What does CA to soil?

The decomposition of plant residues retained continuously on the surface will enhance microbial activity leading to significant changes in **physical, chemical** and **biochemical properties** of the soil. Overall, the **soil fertility** and **productivity** is increased

Benefits of CA

- Moderates soil temperature
- Reduce soil compaction, erosion, and OM loss
- Increases soil microbial and earthworm activity
- Increases porosity & infiltration and retention of water

- Increase soil organic matter/carbon
- Suppresses weeds
- Saves water by 25-30%
- Increased availability of nutrients & FUE
- Saves fuel cost by 60-80%
- Increases crop yield by 10-15%

Performance of crops under CA and CT at ICAR-DWR (2012-16)

Season/crop	Grain yield (t/ha)		% increase under CA over CT
	*Conventional agriculture (CT)	**CA-based practices	
Rainy season			
Rice (10)	4.02	4.06	0.0
Maize (12)	4.03	4.39	8.9
Soybean (4)	0.94	1.06	11.3
Pigeonpea (5)	2.15	2.23	3.7
Winter season			
Wheat (10)	4.19	4.56	8.8
Chickpea (8)	1.89	2.01	6.3
Mustard (4)	1.83	1.94	6.0
Gobhi sarson (4)	3.97	3.72	6.3
Field pea (4)	1.56	1.67	7.2
Summer season			
Greengram (9)	1.05	1.04	0.0

Sharma (2021)

Requirement of fuel (Diesel) for different tillage operations

Particulars	ZT	Conventional tillage					
	Sowing only	Disc plough	Disc harrow	Cultivator	Rotavator	Sowing	Total
No. of operation	1	1	2	2	1	1	7
Time required (hrs/ha/operation)	3	5	3	3	4	3	18
Total time required (hrs/ha)	3	5	6	6	4	3	24
Diesel consumption (l/hr)	3	4	3	3.5	4	3	-
Diesel consumption (l/ha)	9	20	18	21	16	9	84

At current rate: cost will be : ZT- Rs 900, CT- Rs 8400. Saving Rs 7500/ha. (=1125 crs/yr)

CA addresses emerging Challenges in Agriculture

- Declining factor productivity (water, nutrient, energy, pesticide, labour)
- Increasing production cost & declining/stagnating yields & income
- Deteriorating soil health

- Receding groundwater table
- Energy crisis
- Secondary salinization, sodicity and pollution hazards
- Declining biodiversity
- Vulnerability to climatic change
- Changing land-use pattern

CA- The constraints

- Machines Dependence and Unavailability at Farmers Level
- Fragmented and Small Land Holdings
- Availability Residues – used for fodder, fuel etc
- Problem of weeds & dependence on herbicides
- Nematodes Infestations, Fe and Zn deficiency
- Birds, Rodents and Snakes problems
- CA-based technologies are knowledge-intensive requiring skill for successful adoption
- Change of mindset - biggest stumbling block

Potential impact of CA

- Environment-Positive technology – supports resilience against climate change
- Supports soil health and productivity
- Protects soil, water and land resources
- Prevents burning of crop residues
- Lowers burning of fossil fuel thereby reducing air pollution
- Lowers cost of cultivation and increases net income of farmers

Conclusions

- Environment positive technology- low adoption, needs to be promoted
- Necessary to sensitize policymakers, R&D leaders - something similar to organic farming lobby
- Knowledge-intensive technology – need extensive awareness-raising activities and training for all stakeholders
- Front Line Demonstrations (FLDs) on permanent sites at all KVKs and SAUs
- Machinery is a prerequisite make them available to small and medium farmers on custom hiring basis
- Incentivize adoption of CA

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

Advances in Direct Seeded Rice

Rice- a staple food crop of India

- 2020-21 – a historic year with a record food production of 308.65 mt
- Record output of rice with 122 mt followed by 108 mt of wheat
- A staple food crop grown and consumed in all states
- Unique crop grown in diverse ecologies
- Rice is life – role in many rituals

Establishment methods

- Direct seeded (DSR)
    - Dry seeded – seeded in dry soil
    - Wet seeded – seeded (sprouted) on puddled soil
    - water-seeding
- Puddled transplanted (PTR)

Puddled transplanted rice

- Raise nursery, Puddling, transplanting
- Practiced under assured rainfall or under irrigated conditions
- Normally gives higher yield
- Resource- (water, labour and energy) intensive
- Deteriorates the physical properties of soil and adversely affects the succeeding upland crops performance.
- Contributes to greenhouse gas (methane) emissions

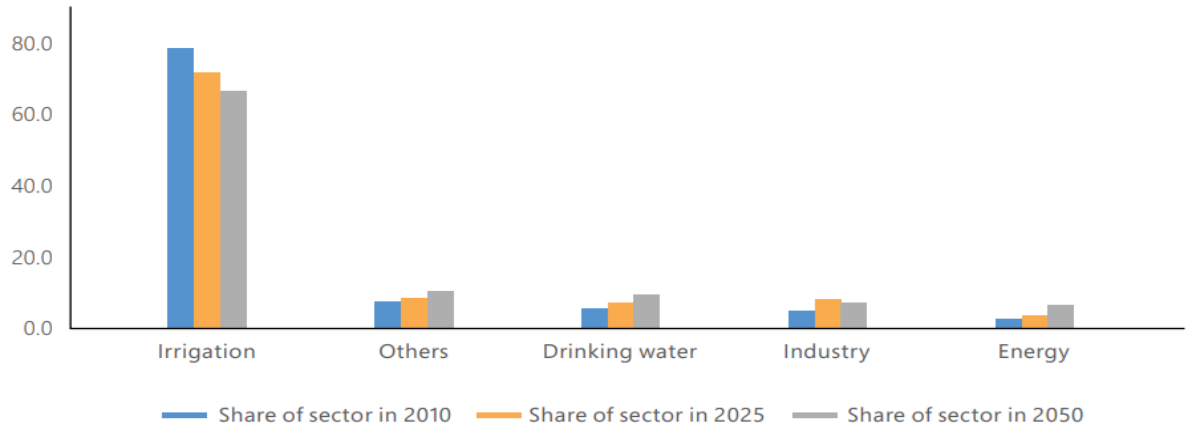
DSR to PTR

- Dry direct-seeding is probably the oldest method of rice establishment.
- Initial periods of rice domestication, rice was known to be dry sown as a mixed crop with other dryland crops under the shifting cultivation system
- Transplanting during the 1970s in most parts of the world
- With the abundant labour, water and land, farmers shifted to PTR under irrigated ecosystems.
- The rapid shift to transplanting was mainly due to the problem of weeds and the non-availability of herbicides for control of weeds
- Introduction of HYV dwarf rice cultivars, replaced the traditional practice of DSR

India’s water resources

- India has 18% of world population, having 4% of world's fresh water 80% is used in agriculture.
- 4,000 billion cubic meters as precipitation - 52% is unutilized
- Has 68.38 Mha (2015) under irrigation
- >30% of irrigated land in the world
- 48% the gross cropped area is under irrigation.
- Rice, wheat & sugarcane consume almost 80 % of irrigation water
- Leaving most of the other crops water deprived

- India is already categorised as water stressed country with 1544 cubic meter water availability (2011)
- The area under irrigated rice is about 22 mha- about 49.5% of the total rice area
- "***Har Khet ko Pani* – Water for every field"** and **"per drop, more crop"**
- Besides existing policies, programs & technologies, a change in mindset of people is very essential



Source: (CWC, 2015)
Note: Values as projected by NCIWRD: National Commision on Integrated Water Resources Development

DSR – The major drivers

- Depletion of ground water
- Labour scarcity and increasing cost
- Increasing energy cost
- North- West India which relies on migrant labour from for agriculture is hard hit
- Example, Rice transplanting in Punjab needs 500 million man-days which is 12 times more than the labour requirement for wheat harvesting

DSR – Revisited

- Savings in resources - labour (40–45%), water (30–40%), fuel/energy (60–70%)
- Mechanization- Faster and easier seeding that helps in timely sowing
- Lowers drudgery
- Availability of effective herbicides
- Better soil physical conditions for the succeeding crop
- Crop yields comparable to PTR
- Decreased cost of production and increased farmers' net profit
- Matures early: timely sowing of succeeding wheat crop, higher yields
- Lower green-house gas (GHG) emission and greater resilience to climatic risks

DSR – Limitations

- Weed management
- Moisture stress
- Availability of nutrients
- Suited to medium to heavy soils only

- Increase in some pests and diseases (neck blast and foot rot in basmati rice crop)

Weed management - a major limitation in DSR

- Infested with diverse and competitive weed flora
- Weeds and crop grow simultaneously
- Alternate wetting and drying favours weed growth
- Huge losses in yield- ranging from 40% to total crop failure
- Physical removal is ineffective, costly and not practical
- Over the years new herbicides are available, cost- effective
- Herbicide mixtures and applications in sequence required

Punjab: A case study

- 2.6 mha in rice with 8.6 mt of production
- Punjab is heavily dependent on migrant labour for agricultural operations
- The cost of manual transplanting of rice has increased from INR 1500/ha in 2005 to >INR 5000 in 2012 and to >10,000 in 2020
- DSR was introduced in 2009-2010, as an alternative to PTR to save labour, water and energy costs.
- The adoption of DSR was rapid. By 2014, the DSR was grown on 115,000 ha
- Government provided subsidies for purchase of seed drills
- Accompanied by improvements in rice seeding machinery, HYVs, agronomic practices including weed management technology
- Enhancement of farmers' skills through training programs accelerated the adoption
- But the real change came in 2020 following the outbreak of COVID19 pandemic.
- Acute labour scarcity due to reverse migration of labour forced many farmers to take up DSR
- Duly complimented by the Agricultural Dept and the SAU
- The govt provided subsidies on machinery
- The area went up to 600,000 ha in 2020
- In Haryana 25,000 ha
- Impact: Saving of over Rs 600 crs in labour
- Water saving 15-20%
- 2021, DSR adopted despite no labour shortage

DSR technology developed by PAU, Ludhiana

- Popularly called *Tar-Wattar* method, recommended in 2020
- The new DSR technique involves agronomic, genetic and mechanical interventions
- In this technique, pre-sowing irrigation is applied in a laser levelled field
- Seedbed is prepared under *tar-wattar* conditions (sufficiently high but workable soil moisture) by shallow cultivations and 2-3plankings in the evening hour
- Imbibed & treated seed sown immediately with 'Lucky Seed Drill'
- Unique machine designed and developed at PAU, which does sowing and herbicide spray simultaneously
- The major departure from earlier practices is the delay in timing of first post-sowing irrigation which is applied at 21 days after sowing which offers:

    1) higher saving in irrigation water (15-20%),

    2) lesser weed problem,

3) reduced incidence of nutrient deficiency especially iron, owing to deeper root development

Laser leveller

Laser leveling is the **process of smoothening the land surface ± 2 cm from its average elevation by using laser** equipped drag buckets to achieve precision in land leveling - altering the fields in such a way as to create a constant slope of 0 to 0.2%

How does a laser level work? A laser transmitter transmits a laser beam, **which is intercepted by the laser receiver mounted on the leveling bucket**. The control panel mounted on the tractor interprets the signal from the receiver and opens or closes the hydraulic control valve, which will raise or lower the bucket

Lucky Seed Drill- a unique tractor mounted machine, which does sowing and herbicide spray simultaneously for enhanced herbicide efficacy

Tractor-mounted herbicide sprayer for post-em application of herbicides

Herbicides in sequence or mixtures for diverse weed flora control DSR

	Pre-emergence		EPOST	POST
1	**Pendimethalin**/Oxadiargyl	fb	Bispyribac/penoxsulam + ethoxysulfuron/ pyrozasulfuron	
2	**Pendimethalin**/Oxadiargyl	fb	Penoxsulam + cyhalofop	
3	**Pendimethalin**/Oxadiargyl	fb	Fenoxaprop + ethoxysulfuron	
4			**Penoxsulam/bispyribac + ethoxysulfuron**	Fenoxaprop +S
5			**Triafamone + Ethoxysulfuron**	Bispyribac/penoxsulam/ fenoxaprop+S
6			Bispyribac+ metamifop	Ethoxysulfuron/pyrozasulfuron/Met+Chlori

Weedy (wild) rice problem may increase under DSR

- Morphologically similar to rice
- Shatter early and add to soil seed bank
- Rice herbicides do not control weedy rice
- GM HR-rice is not permitted in India
- IARI has developed rice varieties (Pusa Basmati 1979 & PB 1985) resistant to imadazolinone herbicides (through non-GM approach) which will kill weedy rice
- Released by PM Modi on 29th Sept, 2021

Conclusions

- DSR - potential to provide similar productivity/economic returns as that of PTR

- RWCS is a major production system in the IGP covering nearly 10.5 mha. Good scope for enlarging the area. Map the suitable area for adoption of DSR
- *Tar-wattar* technology should be tested in other parts of IGPs for validation
- Availability of DSR machines, laser leveler etc. to be encouraged through policy support (subsidy) and establishment of village level Custom Hiring Centres for timely and affordable access of machines by the small holder farmers
- Growers are to be provided with required level of knowledge and training
- F2F extension is ideal
- Policy support and incentives to help upscaling of DSR
- Quantification of public and private benefits of DSR based systems especially carbon foot prints in different geographies and for providing incentives to farmers for ecosystem services.

Lecture

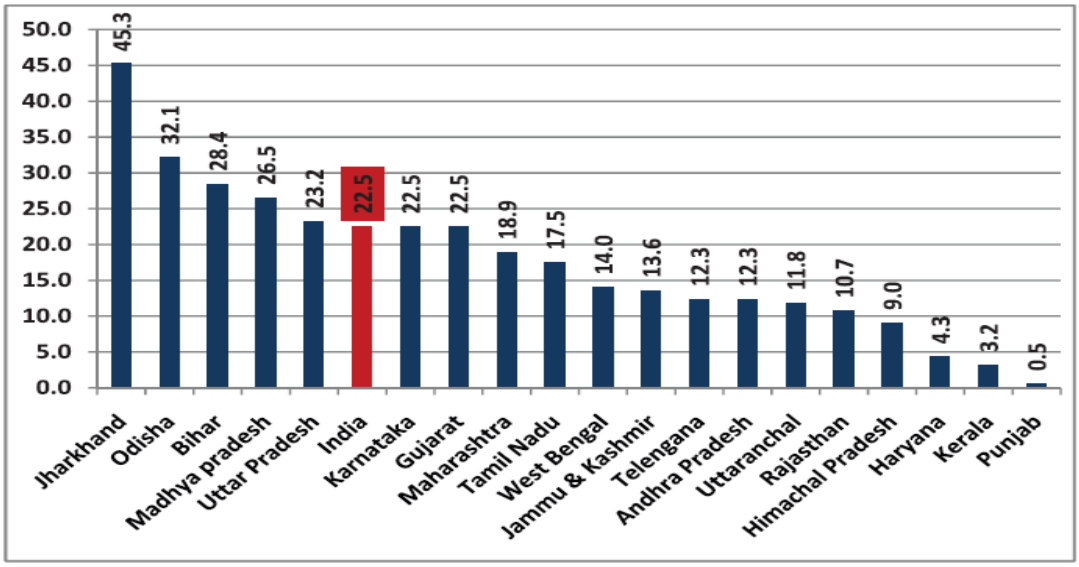
(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

Doubling Farmers' income: Challenges and Opportunities

The Background

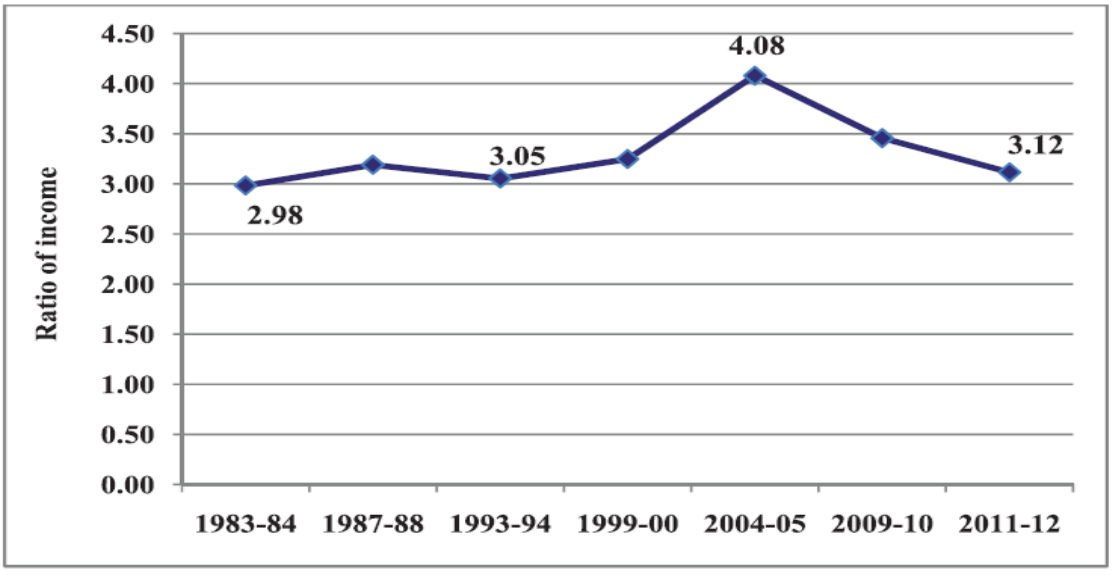
- Agrarian economy - 50% dependent on agriculture
- Agriculture not economical
- The productivity of Ag worker lower than non-ag worker
- A large proportion of farm households live BPL
- Most of the central and eastern states (23%–45%) live below the poverty line (BPL)

Fig.1.1: Farm households with income below poverty line, 2011-12



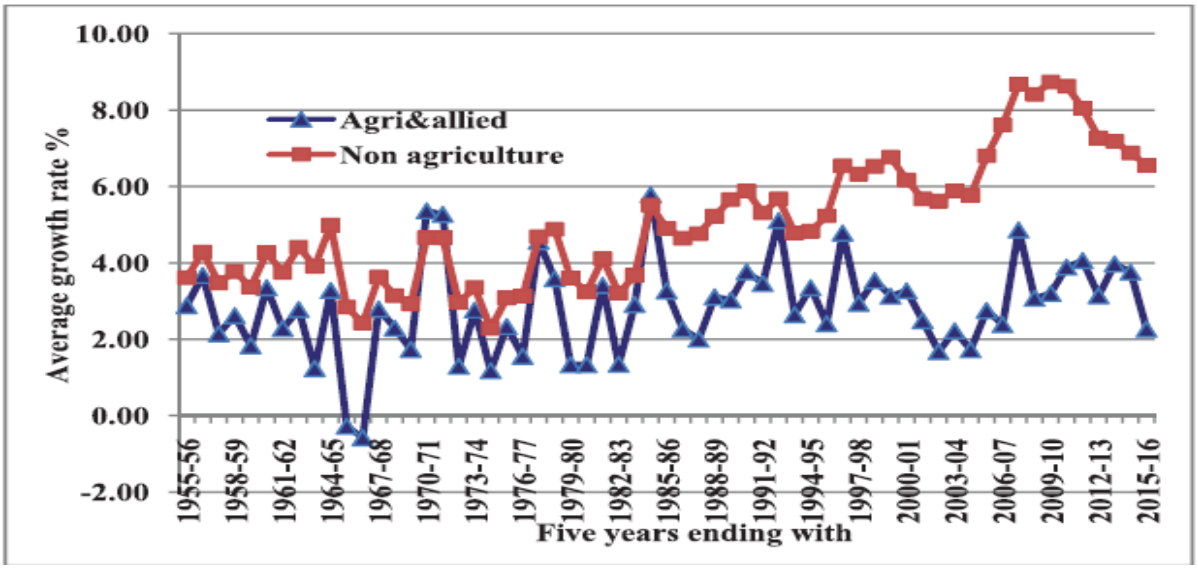
Source: Estimated from unit level Consumption Expenditure Survey data 2011-12, NSSO.

Fig. 1.2: Ratio of income per non agriculture worker to income per cultivator



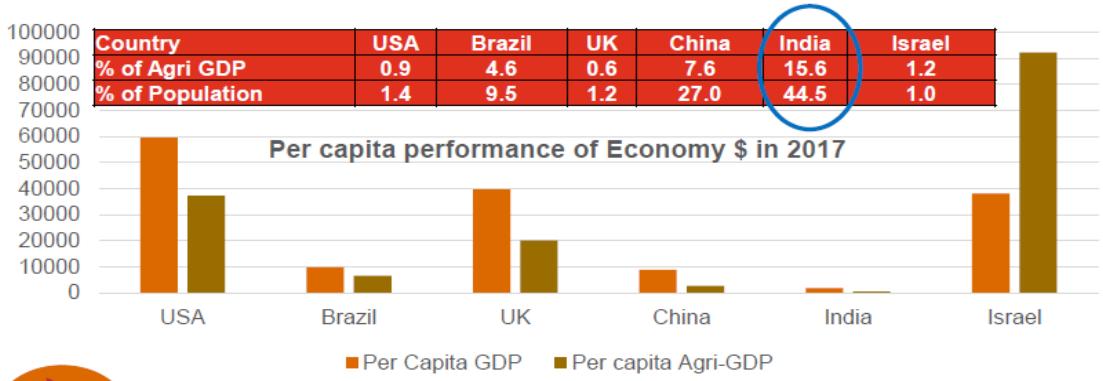
Source: Authors estimation using data from CSO and NSSO.

Fig. 4.1: Average annual rate of change in five years period in GDP agriculture and non- agriculture sectors, 1955-56 to 2015-16



Performance of Agri-Sector - a Global Perspective

Country	USA	Brazil	UK	China	India	Israel
% of Agri GDP	0.9	4.6	0.6	7.6	15.6	1.2
% of Population	1.4	9.5	1.2	27.0	44.5	1.0



Per capita GDP is more than per capita GDP of population involved in Agriculture & allied activities except Israel

15.6% of national GDP is distributed among 44.5% population of the country – not a desirable status

Source- Calculated from World Bank Meta Data

DFI: Is it doable?

- Annual income per cultivator increased from Rs 12,365 in 1993–94 to Rs 1,20,193 by 2015–16, at current prices
- However, at real prices, the increase has been from Rs 21,110 to Rs 44,027 during this period
- Real incomes doubled only over a period of 22 years
- Low & unrealistic MSPs & non-remunerative prices in the market
- The low share of farmers in the consumer rupee
- Poor penetration of crop insurance
- High and increasing input costs

- The absence of market infrastructure
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Opportunities

- Large yield gaps in all crops/commodities compared to dev countries
- Large disparities among States
- Poor resources-use efficiency
- Yield losses due to pests, weeds and diseases
- Post-harvest losses
- Focus on rainfed areas, the eastern region and small farmers
- 43% of the population & >60% of the agricultural area in India is in dry or rainfed regions.

Yield gaps

Rice:
Area- 44 mha
Production- 106 mt
Productivity- 2.4 t/ha, 27th position globally. China 4.7 t/ha

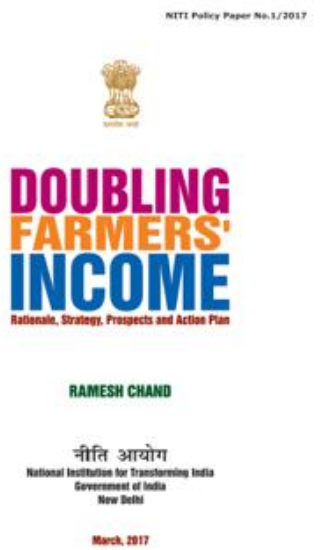
Wheat:
Area- 30 mha
Production- 93 mt
Productivity- 3.1 t/ha, 19th position globally. China 4.9 t/ha

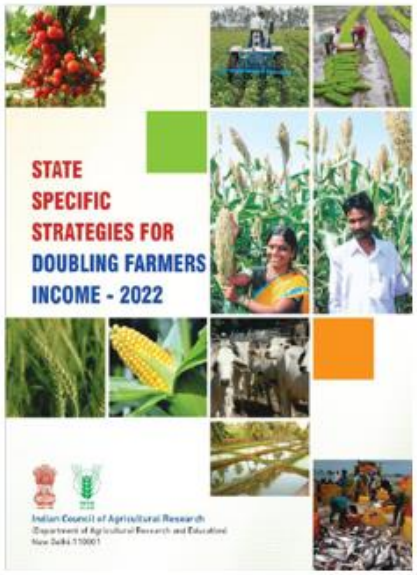
With China's productivity levels: we can save 50% area under rice and 36% area under wheat

Post-Harvest Loss Estimates in India (including on-farm & handling losses)	Post Harvest Losses Rs. 95,000 crores every year (up to 18%)

CROP	% Loss, Minimum	% Loss, Maximum
Cereals	3.9 (Sorghum)	6.0 (Wheat)
Pulses	4.3 (Chickpea)	6.1 (Blackgram)
Oilseeds	2.8 (Cotton seed)	10.1 (Groundnut)
Fruits	5.8 (Sapota)	18.0 (Guava)
Vegetables	6.8 (Cabbage)	12.5 (Tomato)
Spices, Condiments & Plantation Crops	3.9 (Black Pepper)	7.4 (Turmeric)

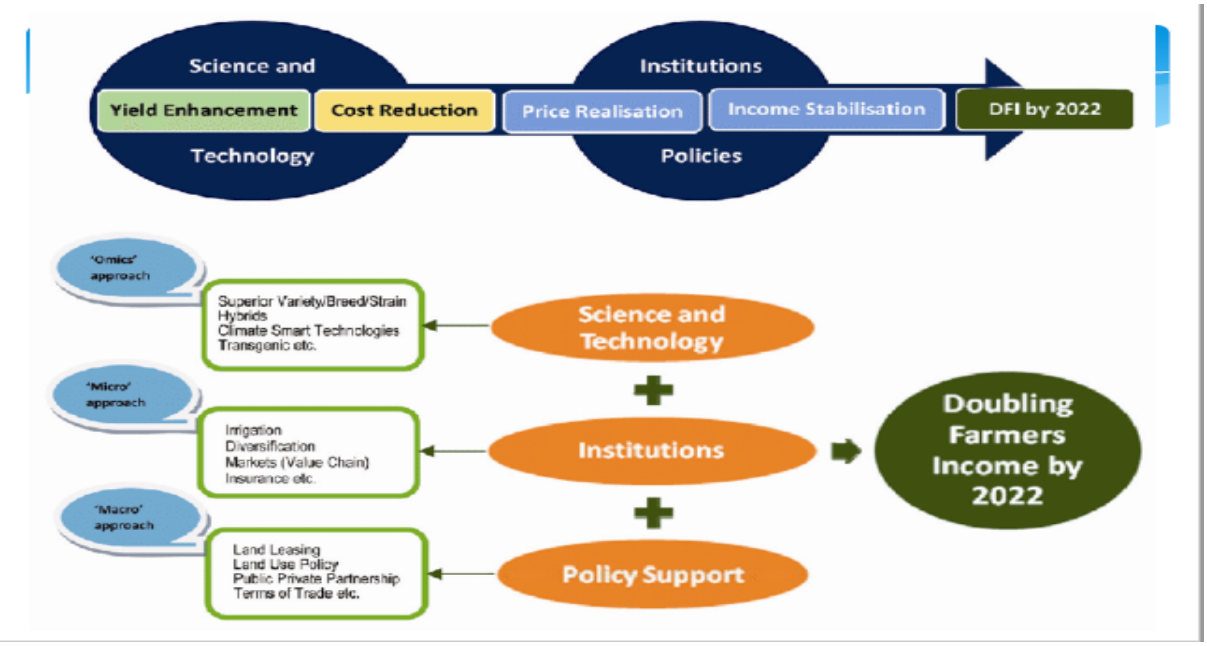
Idea set in motion...





The major sources of growth

- Improvement in productivity
- Improving Resource-use efficiency- decreasing cost of production
- Increasing crop intensity – only 40% area in under 2nd crop
- Diversification towards high value crops
- Realizing higher market price
- Shifting cultivators from farm to non-farm occupation



Diversification of agriculture

- Productivity of fruits and vegetable 4 times cereals, pulses and oilseed.
- In ten years (after 2004-05) area under fruits and vegetables increased from 12 mh to 17 mh.
- It is 8.6 per cent of Gross cropped area now
- Value chain, contract farming, food processing, market reforms – sampada scheme.

- Post harvest value addition
- Allied activities
  - Pisciculture, mushroom, bee keeping, vermiculture, forestry,
- Input and Service activities
  - Quality seed production; nursery raising: custom hiring services
- Bio waste to bio wealth. Compost, ethanol, energy

Livestock and agricultural wages are significant sources of income for farming households in India, especially for marginal and small farmers.

For example, in Bihar, on average, a farming household earns only 47% of its income from farming, 29% from livestock, and 13% from casual labour.

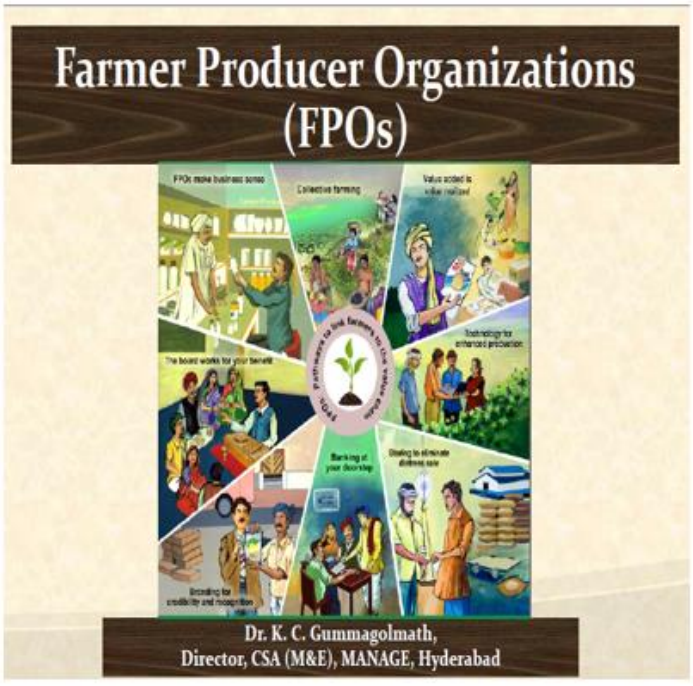
Non-farm employment

- Ag sector engages 64% of work force, but contributes only 39% of total rural net domestic production
- Over-dependence on Ag reflecting significant under-employment

- Lower worker productivity in Ag sector- Rs 62,325 compared to Rs 1,71,587 in non-farm sector (2011-12)
- Shift towards non-farm vocation is already happening
- Workforce in rural areas, fell by 34 million between 2004-05 and 2011-12
- Expected to fall by 55 million by 2022-23
- Skill India is helping find better employment

New Initiatives and their Expected Contribution

1. **Developmental actions**
   - **Routine and regular**
   - **Special: PMKSY; PKVY; PMFBY; Soil Health Card; E-NAM; and Sampada and operation Green**
2. **Policies and reforms: identified five areas**
   - **Direct Price Intervention**
   - **Model APLM (Promotion and Facilitating) Act 2017**
   - **eNAM**
   - **Model Agri Produce and Livestock Contract Farming Act 2018**
   - **Land lease reforms**
   - **Removal of restrictions on tree felling and wood based industries**
   - **New trade policy**
3. **R&D, Technology**



- Best way to empower the small and marginal farmers
- Provides scale of operation
- Better bargaining power
- Cheaper inputs
- Higher prices
- Better market linkages
- Access to modern technology
- Opportunity for value addition/exports
- Higher profits

FPO Academy – a concept nursery for promotion and implementation of FPOs

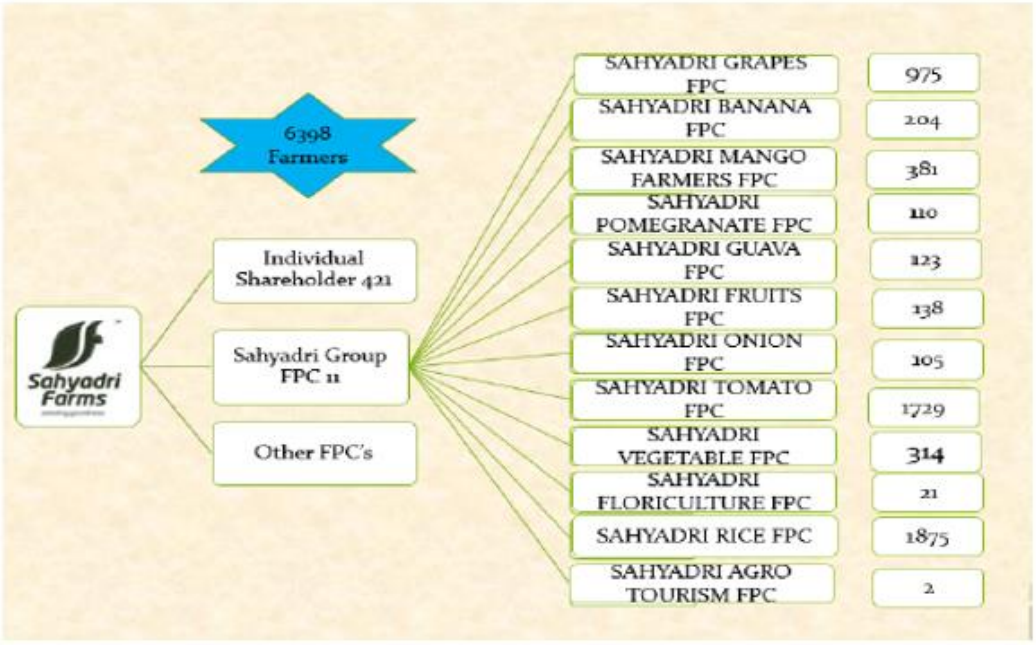
MANAGE-FPO Academy – Concept Note

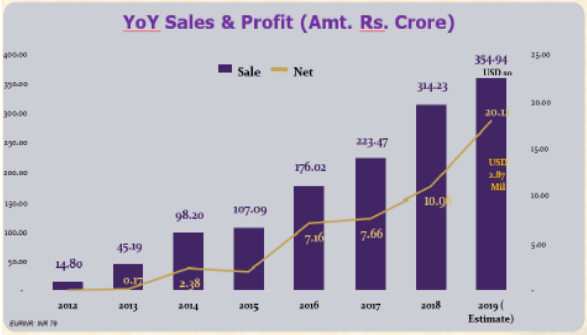
- The focus of the government: "Doubling of Farmers Income by 2022".
- One of the main strategy: **Aggregation of farmers into FPOs.**
- Solution for most of the problems faced by **small and marginal farmers**.
- Since 2002, around **8000 FPOs** have been promoted by various agencies.
- Govt. of India to **promote 10000 FPOs** over next five years.
- It is estimated that, it would require capacity building of **nearly 46 lakhs stakeholders** covering
  - Board of Directors, CEOs, Farmers
  - Bankers, CBBOs, Officers from agriculture and line departments,
  - Resources from NGO, Universities, Cooperative organizations, apex institutes dealing with agriculture and other institutes including stakeholders in the private sector.
- In order to address this huge challenge of aggregating farmers, MANAGE has setup an Academy

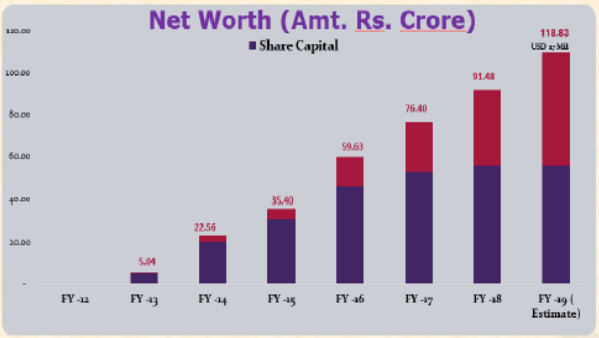
Top 20 Producer Companies With the Largest Paid-Up Capital

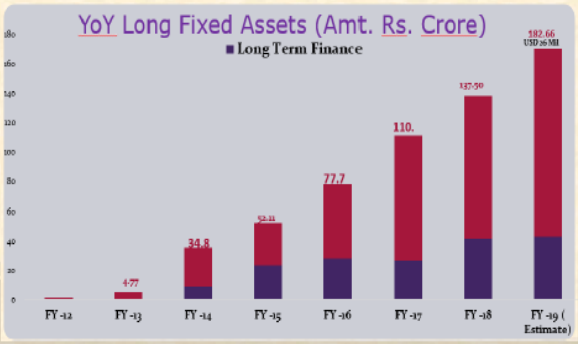
Company Name	Paid UP Capital (Rs. Crore)	Sector	Women Only	Registration	State
Sri Vijaya Vishaka Milk Producers Co.	213	Dairy		FY06	Andhra Pradesh
Sahyadri Farmers Producer Co.	55	F&V		FY11	Maharashtra
Sangam Milk Producer Co.	50	Dairy		FY14	Andhra Pradesh
Paayas Milk Produer Co.	37	Dairy		FY13	Rajasthan
Maahi Milk Producer Co.	35	Dairy		FY13	Gujarat
Saahaj Milk Producer Co.	23	Dairy		FY15	Uttar Pradesh
Karimnagar Milk Producer Co.	16	Dairy		FY13	Telangana
Shreeja Mahila Milk Producer Co.	14	Dairy	Yes	FY15	Andhra Pradesh
Baani Milk Producer Co.	10	Dairy		FY15	Punjab
Shree Chhatrapati Shahu Milk & Agro Producer Co.	10	Dairy		FY09	Maharashtra
Madhya Pradesh Women Poultry Producers Co.	6	Poultry	Yes	FY07	Madhya Pradesh
Karimnagar Milk Farmers Development Producer Co.	5	Dairy		FY17	Telangana
Vadakara Coconut Farmers Producer Co.	4	Coconut		FY16	Kerala
Begoti Tea Producer Co.	4	Tea		FY14	Assam
Palakkad Coconut Producer Co.	4	Coconut		FY14	Kerala
Perambra Coconut Producer Co.	3	Coconut		FY15	Kerala
Thirukochi Coconut Producer Co.	3	Coconut		FY14	Kerala
Tirur Coconut Producer Co.	3	Coconut		FY15	Kerala
Onattukara Coconut Producer Co.	3	Coconut		FY15	Kerala
Kaipuzha Coconut Producer Co.	3	Coconut		FY14	Kerala

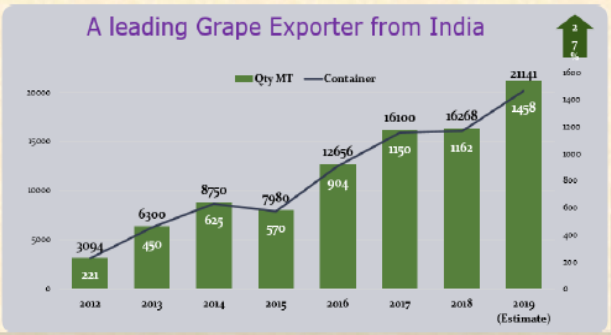
Sahyadri Farmer Producer co.



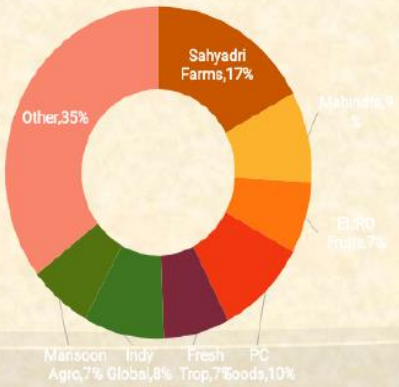








Europe Grape Market Share



Focused Crops

1.	Grapes	1.	Tomato
2.	Banana	2.	Onion
3.	Mango	3.	Potato
4.	Pomegranate	4.	Cucurbits
5.	Strawberry	5.	Leafy
6.	Guava	6.	Cole Crops
7.	Papaya	7.	Okra
8.	Watermelon	8.	Chilli
9.	Muskmelon	9.	Capsicum
10.	Custard Apple	10.	Drumstick

Infrastructures available

- ✔ Aggregate handling capacity of 850 MT per Day
- ✔ 3.50 Lac Sq. Ft Construction completed, 1.50 lac Sq. Ft. is in progress
- ✔ Precooling Rooms (6) – Each 50 MT Capacity
- ✔ Vacuum Precooling Facility for Leafy Vegt
- ✔ Advanced Ripening Chambers for Fresh Domestic Marketing – 8 Each 25 Mt Capacity
- ✔ Semi Controlled Ripening Chambers for processing – Each 250 Mt Capacity
- ✔ IQF Process Plant (Frozen) – 50 Mt / Day
- ✔ Aseptic Process Plant – 150 Mt/Day for Mango, Tomato, Banana, Papaya pulp
- ✔ Advanced FMCG Product Lines - Aseptic Juice, Hot fill Juice, Ketchup, Sauces & Jams – 250 Mt/Day
- ✔ 2000 Mt 0 Degree Storages & 2000 Mt –Ve Cold Storages
- ✔ 4000 Mt Dry Warehouses

Need to Expand Development Agenda for New India to Agriculture

- India is changing, lot of push is being given to accelerate this process. See recent initiatives:
- Investments: Make in India
- Better workforce: Skill India
- Application of technology: Digital India
- Innovation: Atal Innovation Mission
- Enterpreneurship: Start up India
- Ease of doing business: E commerce, labour reform
- Difficulty in creating jobs in non farm sectors require fresh look at theories of economic transition and role of agriculture in creating attractive jobs.

Table 6.2: Base level and target for development initiatives to double farmers' income

Source	Base level and year	Target 2022-23
Quality seed: million tonne	3.03 (2014-15)	7.97
Fertilizer: million tonne	25.58 (2014-15)	36.24
Irrigation: million hectare	92.58 (2012-13)	110.40
Electricity for agri purposes: 000GWH	147.48 (2012-13)	307.39
Are under more than one crop: per cent	40.00 (2012-13)	53.00
Area under fruits and vegetables: million hectare	16.75 (2013-14)	26.38
Area under High Yielding Varieties: %	69.3 (2014-15)	90.0

Table 6.1: Sources of growth in farm income: Achievements and required growth rate for doubling farmers' income

S.No.	Source	Recent achievements		Required growth rate for DFI
		Period	Growth rate/change	
1	Crop productivity 70% segment	2001-2013	3.1	4.1
2	Livestock value added 30% segment	2005-2014	4.5	6.0
3	Improvement in resource use efficiency	2005-2012	2.26	3.0
4	Crop intensity (70% segment)	2001-2012	1 %age	1.3
5	Crop diversification towards fruits and vegetables (70% seg)	2003-2014	3.89	5.17
6	Better price realisation: Crops	Karnataka experience. Reforms	13% total (in real terms)	17.0
7	Shift to non - farm occupation	2005-2012	1.81	2.4

Chand, 2017

Conclusions

- The PM's call to DFI by 2022 has brought a major change in the focus of agricultural sector from the traditional production orientation to income orientation &created renewed interest among all major stakeholders in the country
- The Government has taken several reformative steps, which target; re-orientation of focus from intensification to diversification, from sustenance to commercialization, and turning the agricultural units to enterprises
- A series of programs/initiatives have been floated to execute & monitor the outreach of technologies, soil health card scheme, simplification of agriculture credit, PM Krishi Sinchayee Yojana, Paramparagat Krishi Vikas Yojana etc

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

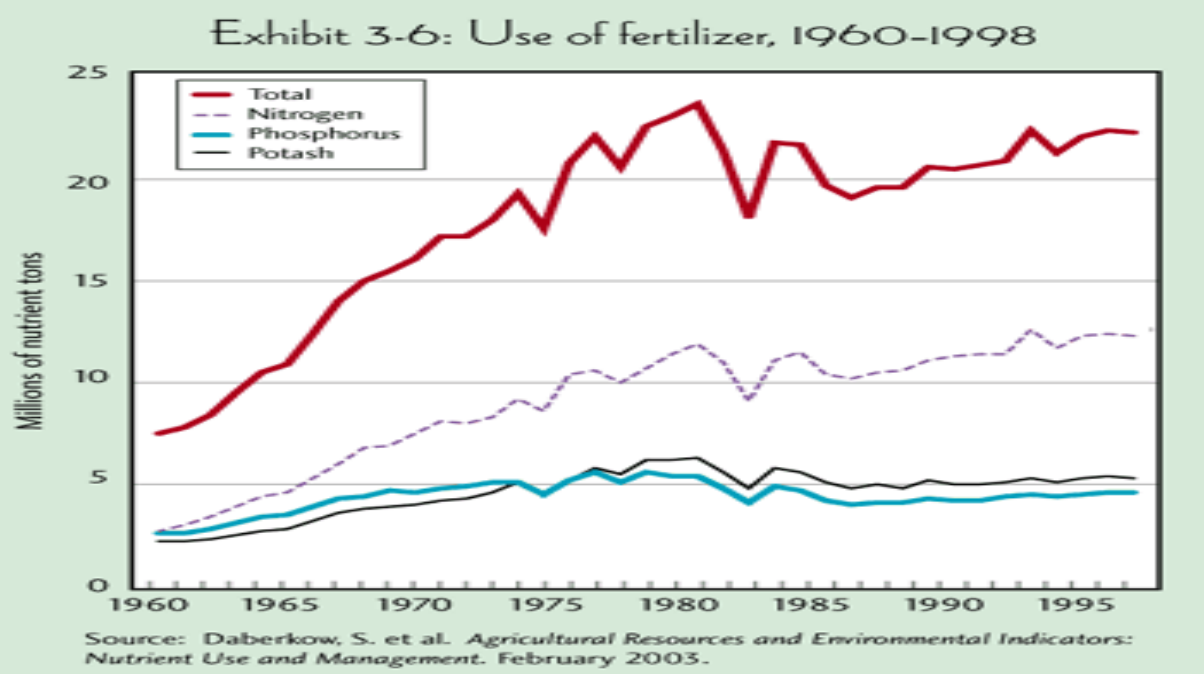
Integrated Plant Nutrition Management (IPNM)

Essentiality of nutrients

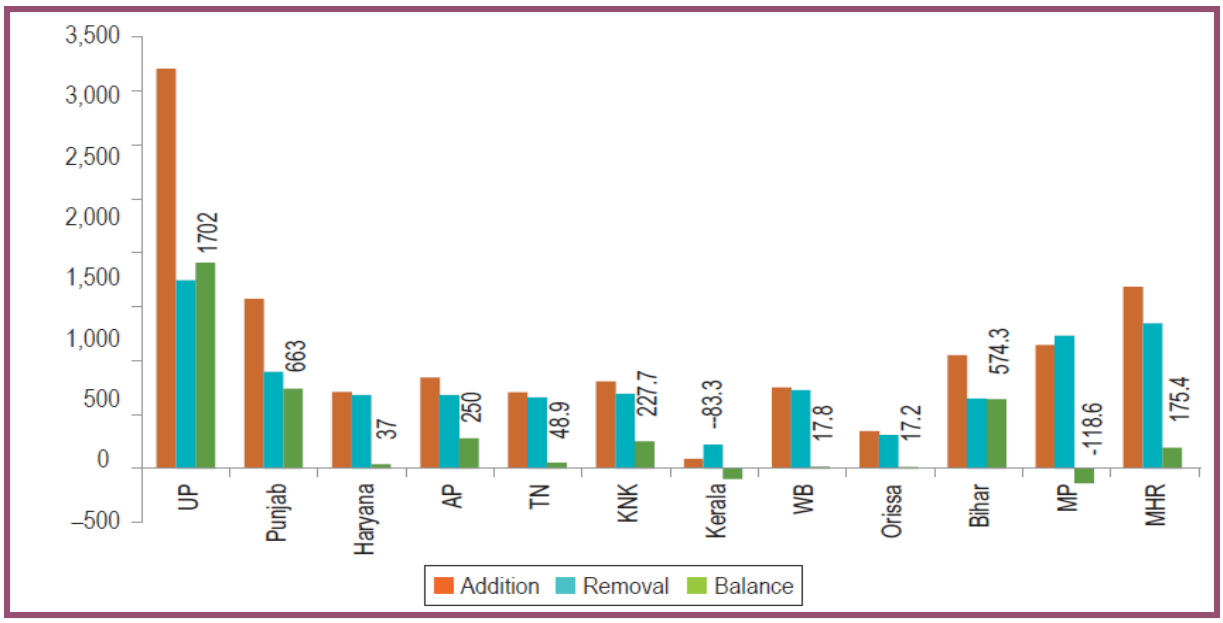
- This concept was propounded by Arnon and Stout (1939) and they considered 16 elements essential for plant nutrition
- The criteria required:
  - ✓ A plant cannot complete its life cycle without the element
  - ✓ No other element can perform the function of the element
  - ✓ The element is directly involved in plant nutrition
- Plants require 17 essential elements for growth: carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), potassium (K), sulphur (S), calcium (Ca), magnesium (Mg), boron (B), chlorine (Cl), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), and zinc (Zn)

Impact of Green Revolution

- Enhancing food production – hunger, malnutrition, death
- Over-dependence on fertilizers, irrigation and agrochemicals
- Encouraged mono-culture, overlooked crop rotation (legumes)
- Ground water pollution
- With respect to plant nutrition:
  - Over emphasis on NPK, particularly N
  - Skewed NPK ratio
  - Widespread deficiency of other nutrients
  - Low priority on organic manures

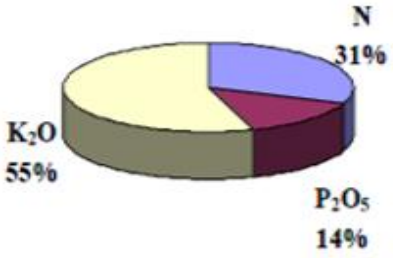


N balance (000t) in some agriculturally important states of India

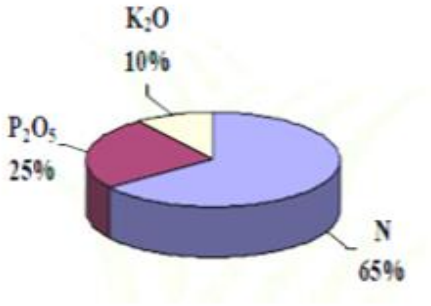


Skewed nutrient additions – Negative nutrient balance

Nutrient removal



Fertilizer consumption



Balanced Fertilizer Application Is Imperative For Sustained Productivity

Optimum N:P:K ratio**: 4:2:1**

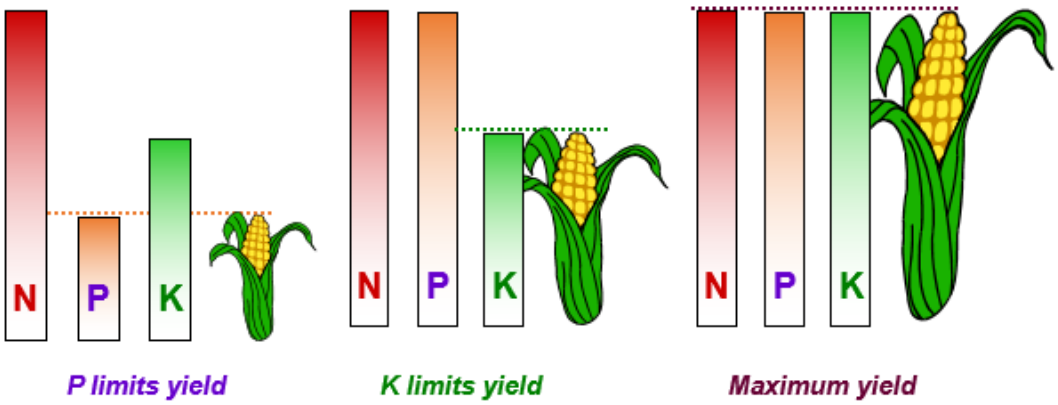
1991-92	1995-96	1996-97
5.9:2.4:1	8.5:2.5:1	10:2.9:1

Nutrient Status of Indian Soils and Their Extent of Deficiencies

Nutrient	Soil fertility Status
N	Low in 63% samples, medium in 26%, high in 11% samples
P	Low in 42% samples, medium in 38%, high in 20% samples
K	Low in 13% samples, medium in 37%, high in 50% samples
S	Deficiencies of variable degree found in over 240 districts
Mg	Kerala, other southern and north-eastern states, very acid soils are deficient
Zn	48% of 2, 50, 000 soil samples analysed found to be deficient
Fe	12 % samples tested deficient. Important on upland calcareous soils
B	33 % samples tested deficient. Important in Bihar, Karnataka, Jarkhand, Madhya Pradesh, Uttar Pradesh, Chattishgarh and West Bengal

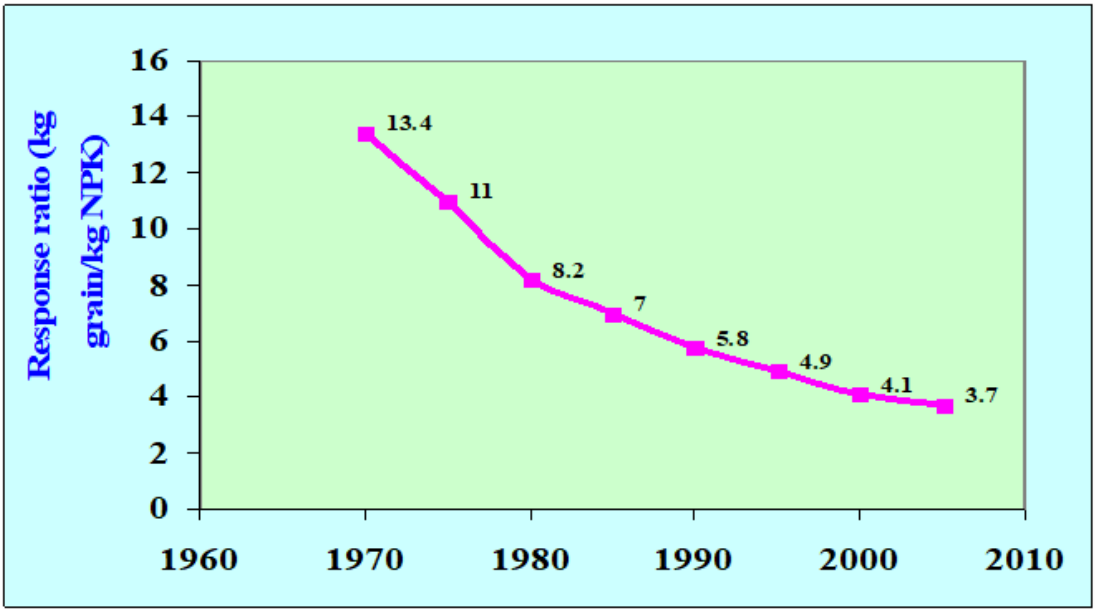
*Based on 3.65 million soil samples analysed during 1977-1999 (Motsara, 2002)

Balanced Fertilizer Application

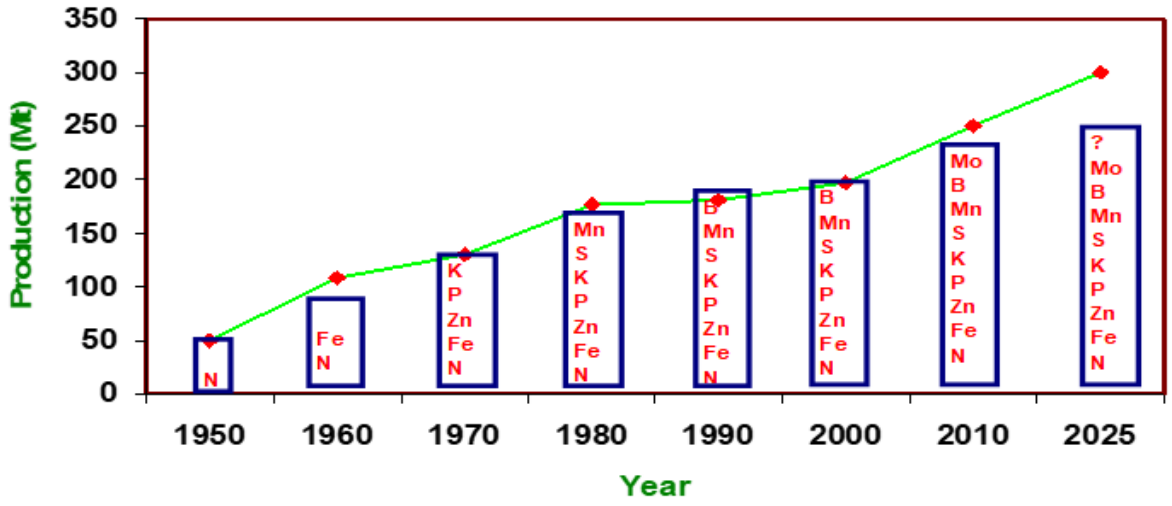


- A productive soil should contain all the essential plant nutrients in Sufficient quantity
- Inadequate availability of any nutrient will inhibit plant growth from its full potential

Declining Fertilizer Response

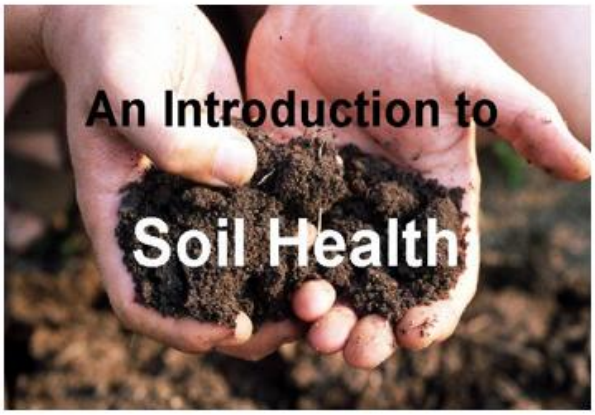


Trends in deficiency of plant nutrients in relation to food grain production

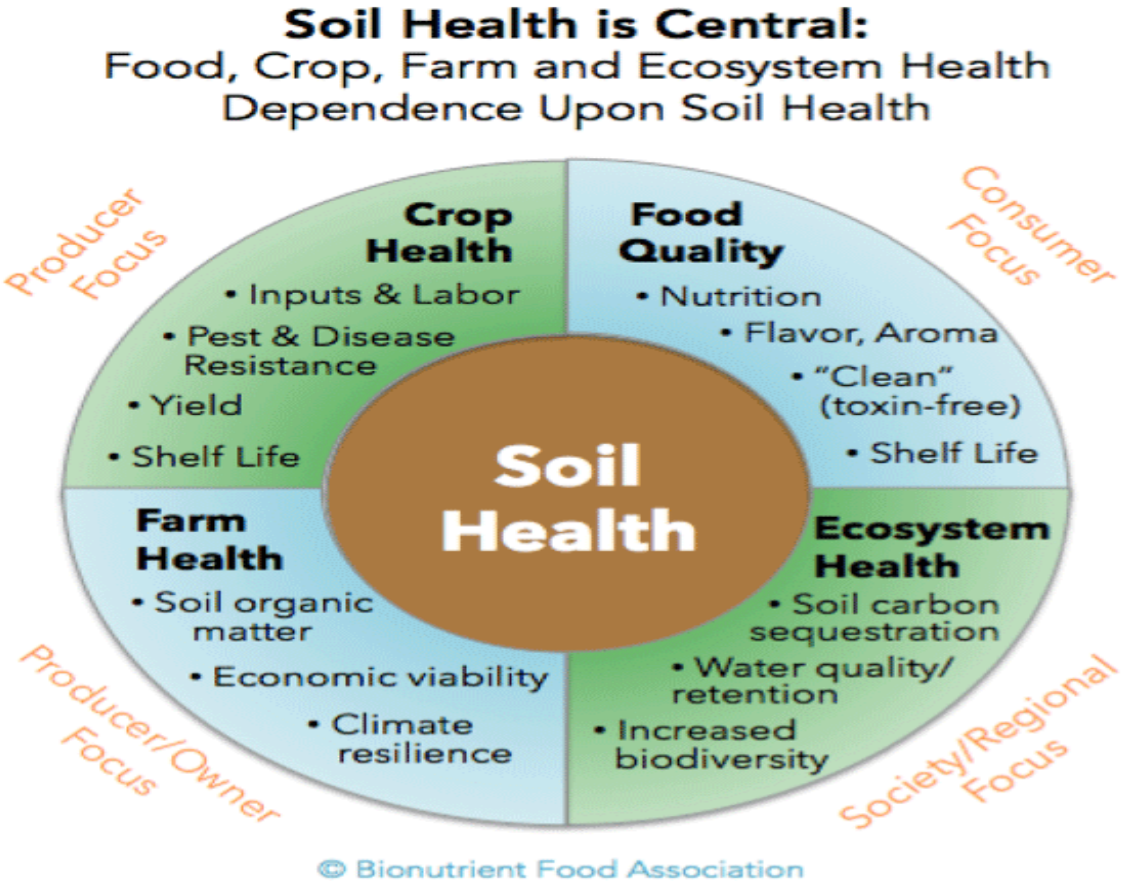


Subba Rao and Srivastava (2007)

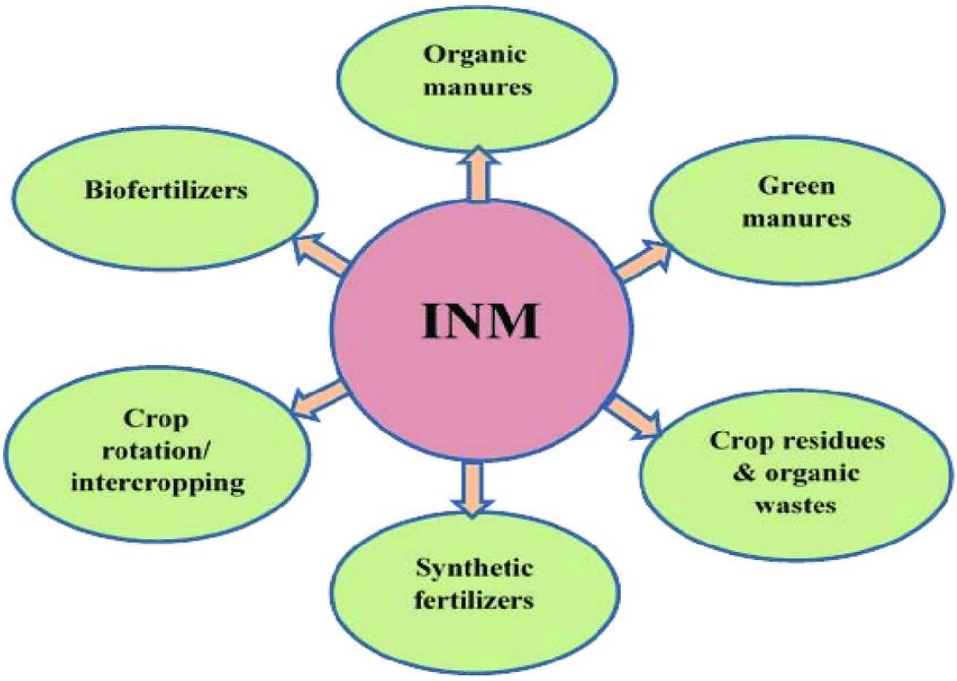
Therefore, Soil productivity needs to be enhanced which in turn is linked to soil health or quality…



Soil health is the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity and maintain water quality as well as plant, animal and human health.



- It refers to the supply of plant nutrients through different sources, through right source in the right proportion and in the right combination of sustaining soil quality and productivity
- It envisages use of chemical fertilizers along with organic manures, bio-fertilizers, legumes and locally available resources for sustainable soil health and productivity



One objective- Many names

Integrated Nutrient Management (INM)

Integrated Plant Nutrient Management (IPNM)

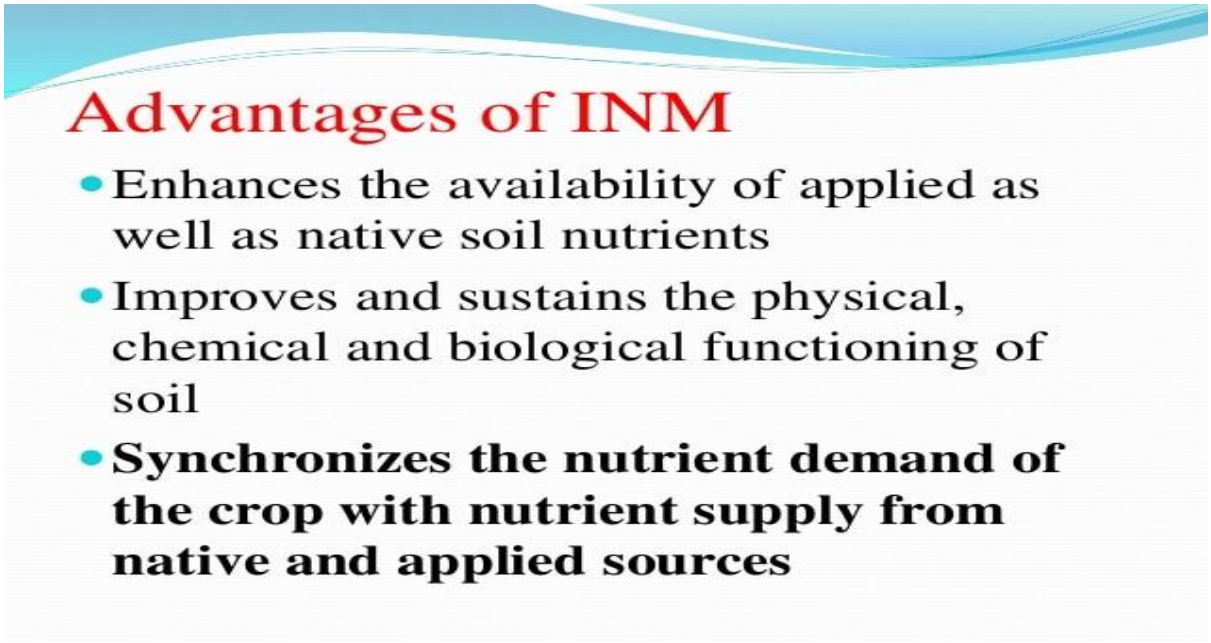
Integrated Plant Nutrition Management (IPNM)

Integrated Nutrient Supply and Management (INSAM)

Objectives

Aims at efficient and judicious use of all the major sources of plant nutrients in an integrated manner, to get maximum economic yield without any deleterious effect on physio-chemical and biological properties of soil.

* Maintains yield stability
* Correction of marginal deficiencies of secondary and micronutrients
* Enhances the efficiency of applied nutrients
* Provides a favourable soil environment



Organic Manures

- Time tested materials
- Average availability 2t/ha
- Vast untapped potential
- Nutrient supply - Direct effect, Residual effect
- Non-nutrient benefit rarely given any due economic importance
- Need to integrate different organic sources available in specific agro-eco regions with chemical fertilizers for cropping systems involving oilseeds

Potential and Possible Nutrient (NPK) Availability from Major Organic Resources

Resources	**Nutrients(million tonnes)**	
	Potential	**For Agricultural Use**
Crop residues	**5.6 – 8.7**	**1.7-2.6**
Animal dung	**3.4 – 5.7**	**1.0 – 1.7**
Night soil	**1.5 – 1.8**	**1.2 – 1.4**
Total	**10.5 – 16.2**	**3.4 – 5.7**

Crop Residues and Recyclable Wastes

- Needed for livestock, burnt to clear the field
- Some residues available in specific locations/situations can be made use of as a source of plant nutrients
- Nutrient supply-direct, residual
- Improves soil properties
- Inconsistent response due to varying pace of immobilization-mineralization cycle, particularly N
- Huge potential in making vermicompost

Nutrient Potential of Wastes

Material	Quantity(m.t.)	Nutrients/year(000't)			
		N	P_2O_5	K_2O	Total
Forest litter	**18.7**	**99.7**	**37.4**	**99.7**	**236.8**
Non edible oil cakes	**0.4**	**12.0**	**5.0**	**12.0**	**29.0**
Sewage sludge	**0.5**	**5.1**	**2.9**	**2.8**	**10.8**
Press mud	**3.2**	**33.3**	**79.4**	**55.4**	**168.1**
Water hyacinth	**73.0**	**72.0**	**14.0**	**84.0**	**170.0**
Domestic waste water*	**6351.0**	**317.6**	**139.7**	**190.5**	**647.8**
Industrial waste water*	**66.2**	**2.9**	**0.9**	**1.3**	**5.1**
*** Million cubic meters / annum; Source: Juwarkar *et al*, 1992**					

Integrating legume crops

- Enrichment of soil N due to N fixation and N conserving effect of legumes
- Improvement in soil physical, chemical and biological properties
- Reduced weeds and diseases severity and insect infestation
- Could be grown as intercrop, in rotation or as green manure

Biofertilizers on crop yield

- *Rhizobium* seed treatment in groundnut and soybean saves 20-25 kg N /ha
- Seed treatment with *Azospirillum* and *Azotobacter* saves 20 to 30 kg N/ha in sesame, mustard, sunflower and safflower
- Application of phosphorus solubilising bacteria (PSB) mobilizes fixed P in soil and reduces P requirement

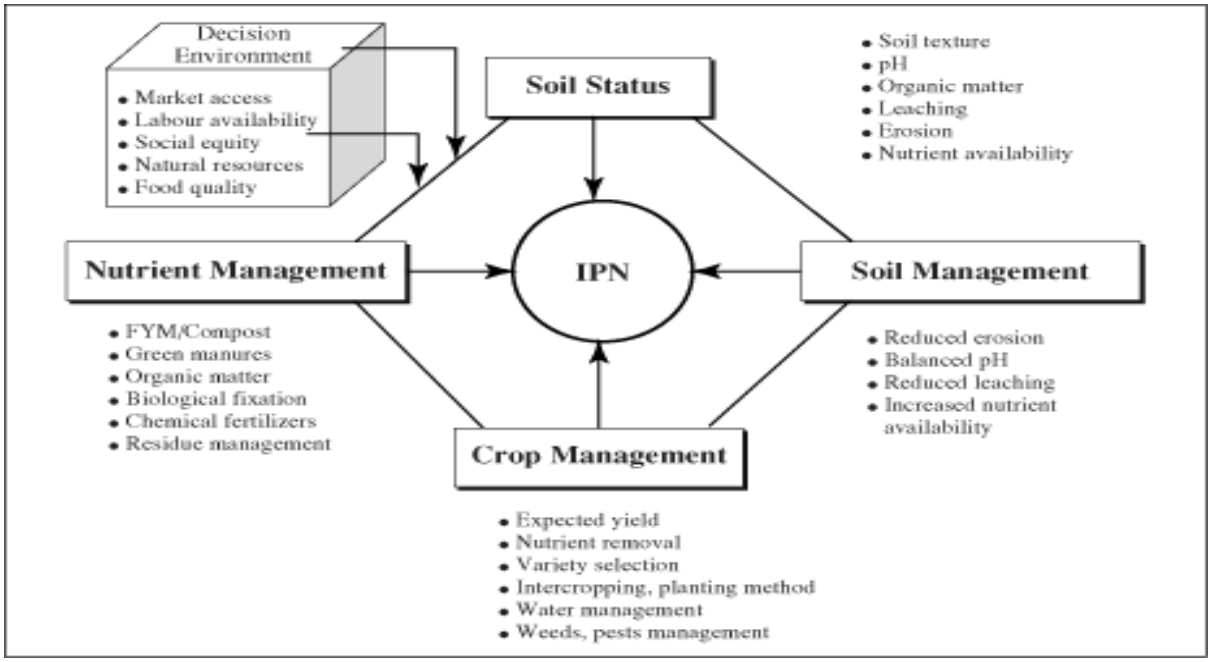
Effect of Green Manuring on Seed Yield of *Toria* (Mean Data of 3 years)

N applied (kg/ha)	Seed yield of *toria* (q/ha)		
	Without green manuring	With green manuring	Increase (q/ha)
0	2.17	10.48	8.31
20	6.10	13.64	7.54
40	8.53	15.62	7.09
60	10.49	16.10	5.61

Source: Pasricha *et al.*, 1986

Constraints

- Cattle dung as fuel
- Crop residues as animal feed
- Cost and time required for raising green manure crop
- Poor and inconsistent response to bio-fertilizers
- Knowledge-intensive
- Need training of extension personnel and farmers alike



Lecture

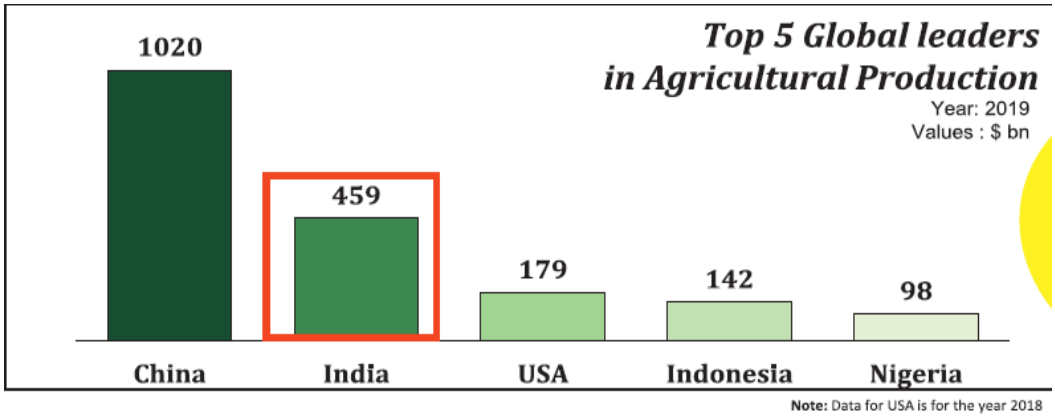
(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

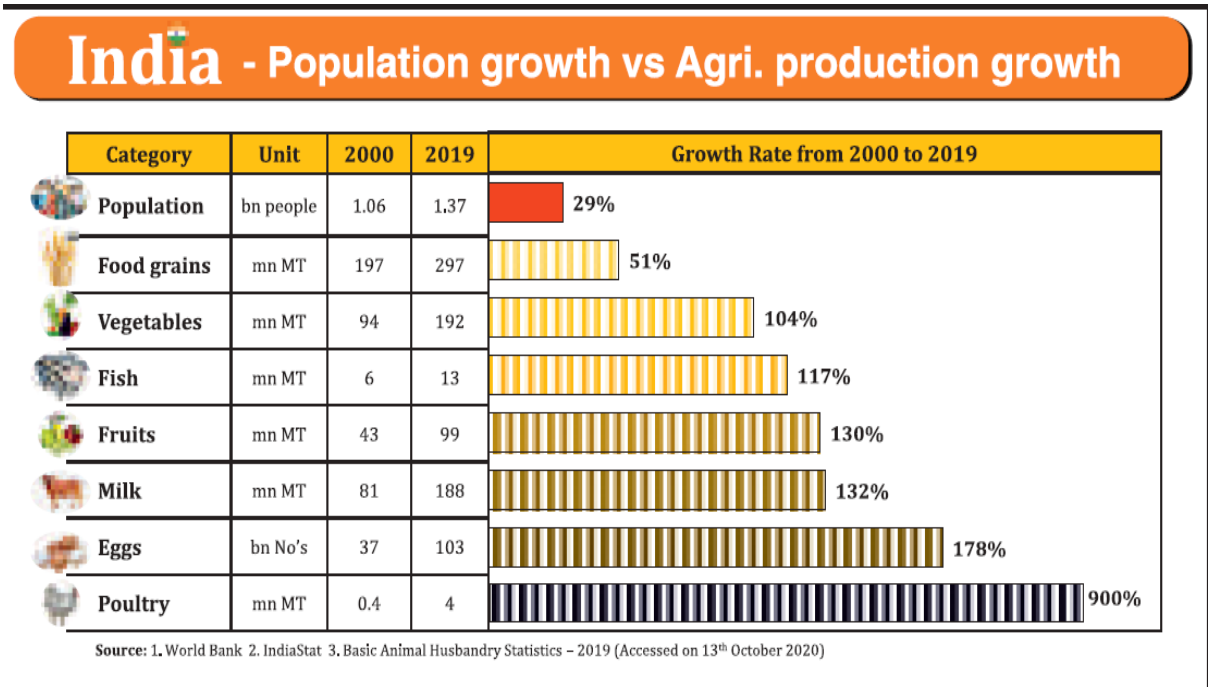
Agri-entrepreneurship and Start-ups in Agriculture

The Background

- Agrarian economy - 50% dependent on agriculture
- Agriculture not economical
- Ag sector engages 64% of work force, but contributes only 39% of total rural net domestic production
- The productivity of Ag worker lower than non-ag worker
- A large proportion of farm households live BPL
- Most of the central and eastern states (23%–45%) live below the poverty line (BPL)
- **The economy will not grow without the success of agriculture sector**

India: An agricultural powerhouse of the world





India's Global position

- First in – Milk, Jute, Pulses.
- Second - Rice, Wheat, Sugarcane, Cotton, Groundnut, Fruits, Vegetables
- The agriculture industry in India reached a value of **INR 63,506 Billion (63,50,600 crores) in 2020**. The market is expected to INR 125,350 Billion by 2026, exhibiting a CAGR of 12% during 2021-2026 (IMARC Group)
- **Agriculture has a huge potential for business opportunities and offer employment to many**

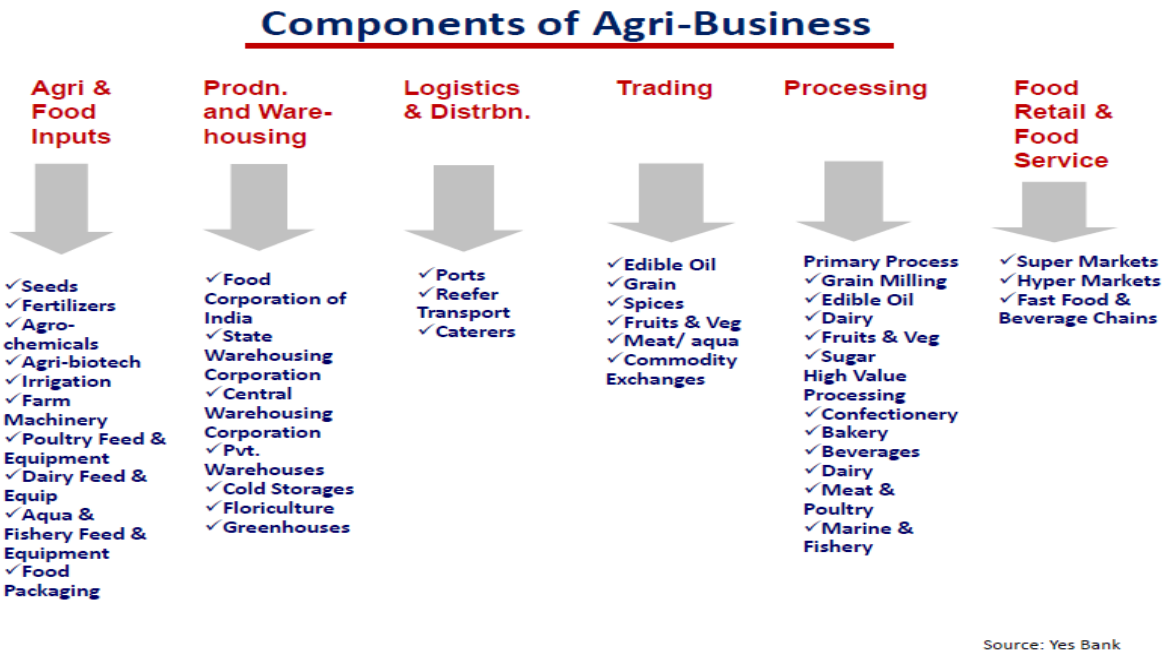
The major sources of growth for doubling farmer's income

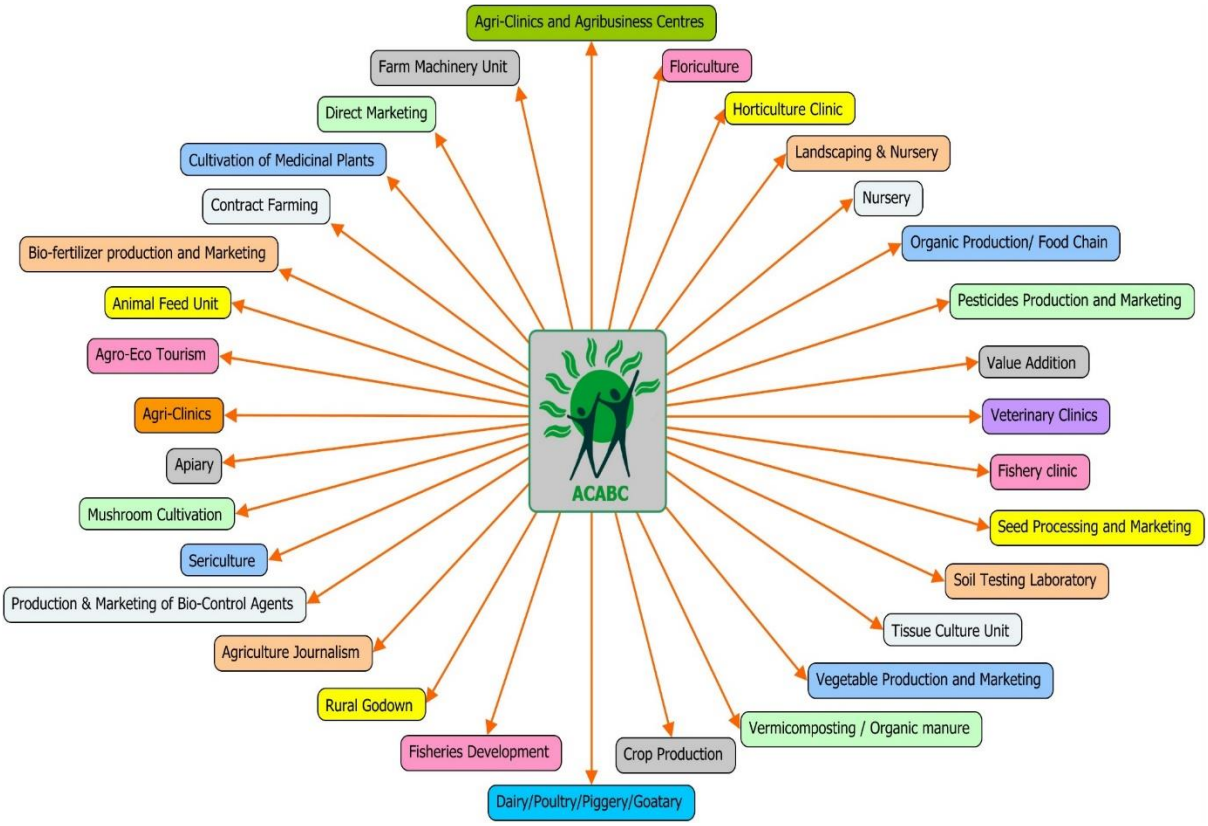
- Improvement in productivity
- Improving Resource-use efficiency- decreasing cost of production
- Increasing crop intensity – only 40% area in under 2nd crop
- Diversification towards high value crops
- Realizing higher market price
- Shifting cultivators from farm to non-farm occupation

The transformation requires:

- New skill set
- New approach
- Investments
- Enabling policy environment

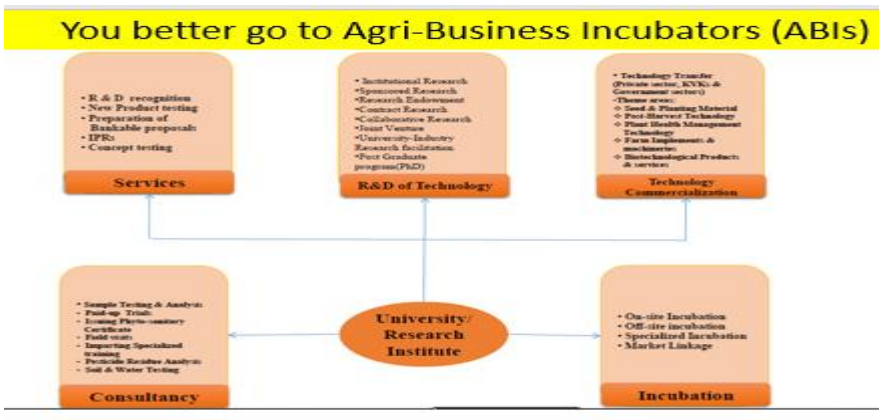
An entrepreneur is an individual who develops a business model, acquires the necessary resources to start a new venture, and operationalizes it and is responsible for its success or failure. The process of creation of enterprise is called entrepreneurship. Target youth the median age of India's population is between 28 to 29 years.





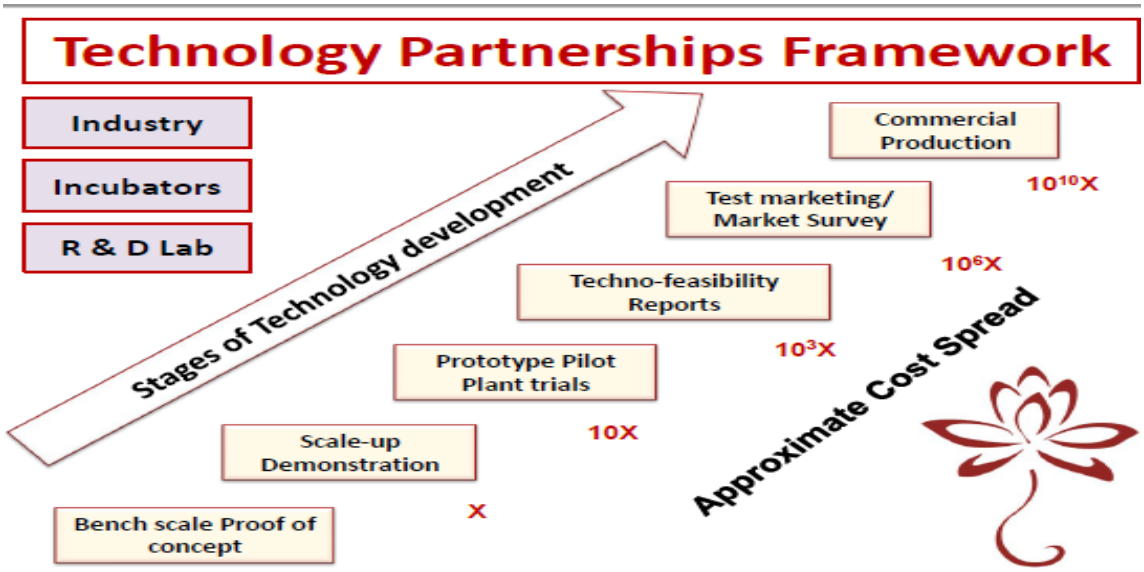
- Best way to empower the small and marginal farmers
- Provides scale of operation
- Better bargaining power
- Cheaper inputs
- Higher prices
- Better market linkages
- Access to modern technology
- Opportunity for value addition/exports
- Higher profits

How to transform an idea/concept into a product of value?

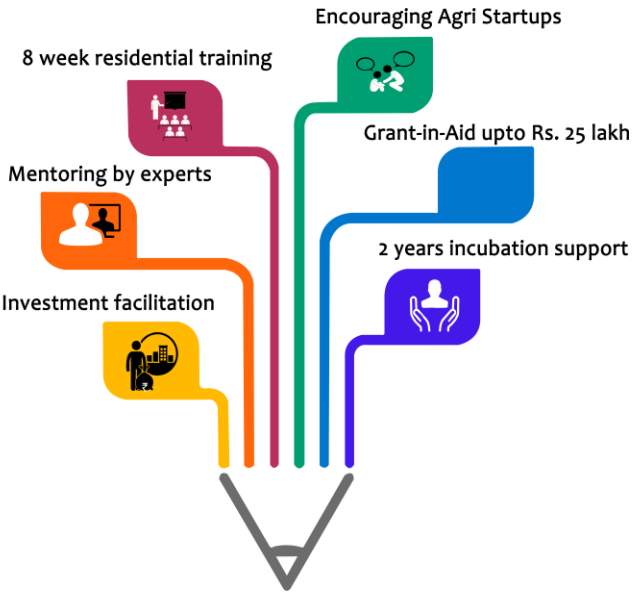


Advantages of being incubated ..

- OFFICE SPACE
- ABLE TO FOCUS ON CORE-BUSINESS - on day 1
- PRODUCTION SPACE CHEAPER
- ACCESS TO LAB & MACHINERY
- MARKETING & BRANDING HELP
- BEING WITH OTHER tech start-up BUSINESSES
- SUPPORT FROM MENTORS/ INNOVATORS
- ACCESS TO WORKSHOPS and BUSINESS TRAINING
- MORE FUNDING/ VC OPPORTUNITIES



Agripreneurship Orientation Program



Startup Agribusiness Incubation Program

Innovation In Agriculture: What Is It ?

- **Innovation** is the process and outcome of creating something new, which is also of ***value.***

- **Innovation** involves the **whole process** from opportunity identification, ideation or invention to development, prototyping, production, marketing and sales.

What is a Start-up ?

*"A Start-up is an organization formed to search for a repeatable and scalable **business** model"*

*"An organization dedicated to creating innovative solution under conditions of extreme uncertainty & do **business**" – Lean Start-up*

What you should do ?

- Connect with people: "Network Is Your Net-Worth"
- Get a Mentor.
- Trigger thoughts – Innovate !
- Be Passionate
- Understand latest trends
- Come up with ideas and solutions
- Talk to potential customers
- Develop the Ecosystem
- Focus..Focus..Focus !

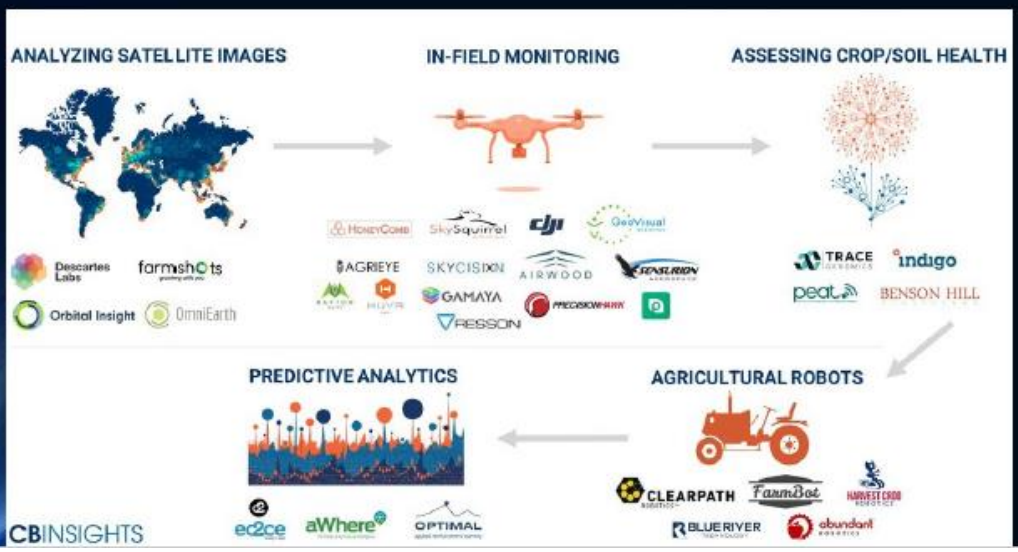
Top 10 AgriTech that are Changing Agriculture Sector

- - Agri data moves to the cloud
- - Internet Of Things (IoT)
- - Robots on the Farm (AgriBots)
- - Autonomous Farm Equipment
- - Precision Farming
- - Vertical Farming
- - Aerial Imagery using Drones / Satellites
- - Agri Retail Online (Ecommerce)

FOCUS AREAS FOR AGRI-TECH STARTUPS

- **BIG DATA:** Eg.: Agrostar, RML Agtech are investing INR 5 Cr each in building ground-breaking image recognition technology that enables farmers to receive real-time data on the pest or disease that has affected a crop.
- **FARMING-AS-A-SERVICE (FAAS):** Eg.: EM3 Agriservices offers farming services and machinery rentals to farmers on a pay-for-use basis, Goldfarm, Ravgo, Oxen Farm Solutions and Farmart.
- **MARKET LINKAGE MODELS:** Eg.: MeraKisan.com helps consumers in India to order fresh food and goods sourced from local farmers.
- **FINTECH FOR FARMERS:** Digitization of payments for farmers through payment gateways linked to their accounts. Such startups can also create the credit profile environment for funders and lenders Eg : Adityta Birla Payments Bank, Paytm
- **IOT FOR FARMERS:** Eg.: Stellapps leverages cloud computing, data analytics and wearables to improve agri-supply chain parameters, including milk production, procurement, cold chain, animal insurance and farmer payments.

Robotics & Artificial Intelligence Are Disrupting The Farms



Unlike other sectors, Agritech is make in India for the world

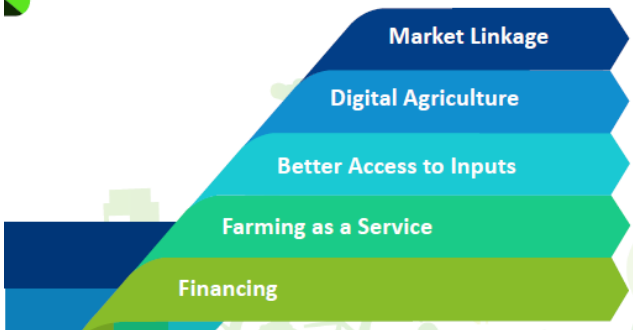
	Global	India
Start-ups	3103	450
Technology Unicorn	1	0
Funding Growth	2X*	10X*
Market exploration	<5	~25

- Every 9th Agritech startup in the world is from India.
- With recent rise in funding, 48% Agritech CEOs believe to have next Agritech unicorn in coming 3 years
- Global funds have directly invested in Agritech startups and sector focused funds. Multilaterals, such as, WEF and ADB have shown interest too.
- <5 global Agritech companies ventured in India, as compared to >25 Indian Agritech companies with global presence

* from 2013-14 to 2017-18

Source : Tracxn, News Articles, NASSCOM Analysis

New emerging business opportunities in Agritech



Improving Supply Chain and taking farmers product directly to consumers

Driving transparency, traceability and real time access to information

Taking quality inputs to the farmers for better productivity

Making services affordable to small and marginal farmers

Innovations taking roots in farmer financing

Market Linkage: Improving Supply Chain

Illustrative

Market Linkage provides a digital platform which connect farm output with the customer, it is one of the most easy way to take farmers products directly to the end customer

Landscape

ninjacart, Farm Tracs, ecoZen solutions, AGROWAVE, agricx, OUR FOOD, GOBASCO, crofarm, FarmLink, WAYCOOL

Tech Intervention	App based platform, image sensing for quality grading, IoT based storage monitoring
Value Proposition	Efficient Supply Chain

Business Attractiveness

Directly taking farm output to consumer → Huge customer traction can be seen in this business area → Received highest funding among all other business areas → Helps in reducing post harvest losses with stand at 4% to 16% of total output

ninjacart

Funding	End Customer
USD 165 mn	B2B

Business Strategy

Strong 4,500 farmer – 9,500 retailer connect

Impact

Received biggest funding in any Indian start-up till date from the US investment firm

crofarm

Funding (2018-19)	End Customer
USD 509K	B2B

Business Strategy

Offers efficient supply chain between farmers and businesses (online and offline retailers)

Impact

Crofarm supplies fruits and vegetable to over 300 plus retailers including small and medium retailers through its multiple distribution centers.

Source : Tracxn, News Articles, NASSCOM Analysis

Digital Agriculture: Driving transparency, traceability and real time access to information

Illustrative

Digital / Precision Agriculture based businesses offers innovative technology solutions for increasing crop productivity and farming process efficiency

Landscape

Tech Intervention: Drones, IoT Sensors, blockchain, image sensing, analytics, machine learning

Value Proposition: Traceability and Crop Scanning

Business Attractiveness

Improve yield and increases productivity → Leads to data generation which can be used for forecasting → Cost saving by offering better prediction techniques

Cropin

Funding (2018-19): USD 8 mn

End Customer: Corporate / Government

Business Strategy

Use Big Data analytics, artificial intelligence and remote sensing to analyze data for 265 crops for agriculture processors, distributors, inputs providers, lenders and insurers through its APIs

Impact

Engaged with nearly 2 mn farmers holding 3 mn acres of land in farm management, crop cycle monitoring, harvest and product traceability

AGNEXT

Funding (2018-19): USD 2 mn

End Customer: Large Enterprise

Business Strategy

Offers cost effective and unique technology that can help farmers save money during the crop pre and post harvest stage

Impact

AgNext has raised around USD 4 mn in funding in the last 12 months

Source : Tracxn, News Articles, NASSCOM Analysis

Better Access to Inputs

Illustrative

Providing farmers **better access to agricultural inputs** at there doorsteps, it helps farmers to understand the best input product to increase the yield and productivity

Landscape

Tech Intervention: Mobile apps, Analytics, ecommerce

Value Proposition: Quality Product Supply

Business Attractiveness

Access to Quality seeds: Quality of seed accounts for 20%-25% of productivity → Quality Fertilizers: 70% agriculture growth depends on fertilizer quality → Offer door to door delivery, makes farmers more interested in this business area

AgroStar

Funding: USD 27 mn

End Customer: Farmers

Business Strategy

Offers "one stop solution" for farmer needs like increase crop production, manage orders and buy supplies online

Impact

Engaged with more than mn farmers
400,000 active users at present

Gramophone

Latest Funding: USD 2 mn (2019)

End Customer: Farmers

Business Strategy

Allows farmers buy genuine crop inputs. They Offer toll-free number, where the farmer can give a missed call and receive a call-back from a Gramophone Agriculture expert

Impact

Received more than 60,000 calls from farmers
Improved productivity by 20 percent to 30 percent, going up to 50 percent improvement in many cases.

Source : Tracxn, News Articles, NASSCOM Analysis

Farming as a Service: Making services affordable to small and marginal farmers

Farming as a Service offers affordable technology solutions to farmers for efficient farming by converting fixed cost to variable cost

Landscape

Oxen | Gold Farm | EM3 Agri Services | KHETHINEXT | AGRI BOLO | TRRINGO

Tech Intervention | App based platform

Value Proposition | Easy Access to expensive equipment's

Business Attractiveness

Buying agricultural equipment's requires huge money making it difficult for farmer → A cyclic business model, as farmers need equipment's on regular basis → 75% of farmers own less than 1 hectare of land hence making it difficult to own equipment's

Gold Farm

Latest Funding (2017)	End Customer
USD 2 mn	Farmer

Business Strategy

Uber for farm equipment' platform to help agri equipment fleet owners realize better ROI on their farm equipment

Impact

Lead to 25% improvement in ROI, 20% reduction in field team required, 20% in turnaround time

EM3 Agri Services

Funding	End Customer
USD 10 mn	Farmers

Business Strategy

Provides farm services for the entire cultivation cycle on a pay-per-use basis

Impact

10 districts covered in Madhya Pradesh
300 acres land served daily

Source : Tracxn, News Articles, NASSCOM Analysis

Financing: Innovations taking roots in farmer financing

Illustrative

Farmers in India struggle to get finance but Agritech based **financing** start-ups helps such under served community of farmers to get loans quickly

Landscape

farMart | Samunnati | Jai Kisan | GRAMCOVER

Tech Intervention | App based platforms, analytics, data science

Value Proposition | Easy Access to Finance

Business Attractiveness

Raising funds, the biggest challenge of Indian farmers → A solution for millions, hence scalable → Farmers do not have good credit history which makes it difficult for them to raise funds through traditional channels

Jai Kisan

Latest Funding	End Customer
USD 1.5 mn (2019)	Farmer

Business Strategy

Provide low cost and timely financing for agricultural equipment

Impact

Established 25 channel partner locations across 12 districts in Maharashtra and Karnataka
Have over 60 vendor partners selling more than 1,500 SKUs on the platform

farMart

Latest Funding	End Customer
Undisclosed (2017)	Farmer

Business Strategy

Allow access to low-cost digital loans to allow them to purchase products using virtual credit card

Impact

Company's services has been availed in over 300 villages and by over 1,500 farmers
Target to reach 1 lakh farmers by 2020

Source : Tracxn, News Articles, NASSCOM Analysis

Need to Expand Development Agenda for New India to Agriculture

- India is changing, lot of push is being given to accelerate this process. See recent initiatives:
- Investments: Make in India
- Better workforce: Skill India
- Application of technology: Digital India
- Innovation: Atal Innovation Mission
- Enterpreneurship: Start up India
- Ease of doing business: E commerce, labour reform
- Difficulty in creating jobs in non farm sectors require fresh look at theories of economic transition and role of agriculture in creating attractive jobs.

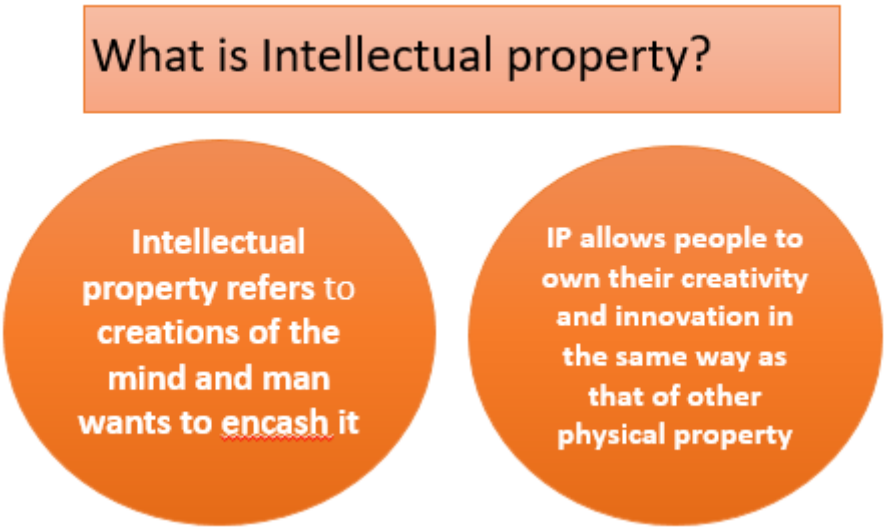
Conclusions

- Changing consumer preferences and emerging technologies are driving innovations and business opportunities
- Agriculture is attracting Business houses and technology people in equal measure
- India is emerging new area for agri- startups
- Need to integrate different domain knowledge and skills in ag innovation with Many are from non-agri background
- Framers are sceptical/ mindset- need to have more awareness-raising activities
- Agri-hackathons and Grand challenges are needed for bringing people from diverse backgrounds
- Start-ups need catalystic capital and support to make their products mainstream
- Agriculture infrastructure fund a good move by the Centre
- Regulation needs change – Agril being a state subject conflict with centre
- Govt policies – wrong impression (poverty alleviation/business)

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

Intellectual Property Right and Technology Commercialization



They could be a:

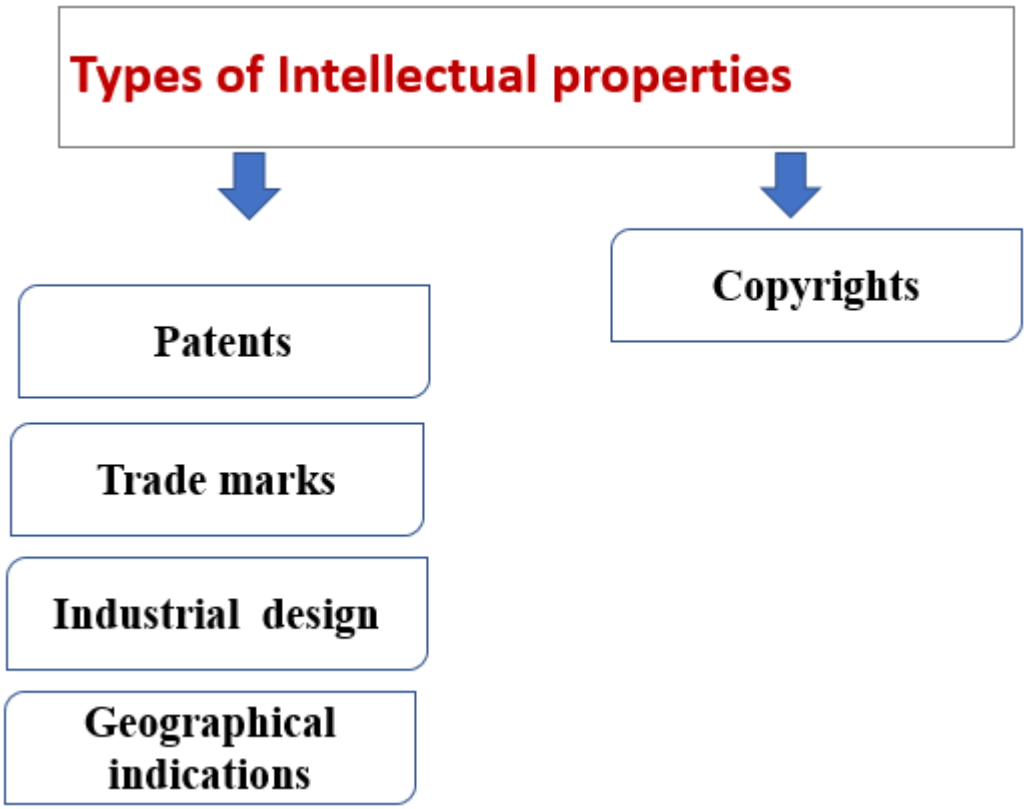
- Bio-pesticide
- Bio-stimulant
- Mobile application
- Novel method of fertilizer formulation – neem coated urea
- Novel method of cultivation
- New piece of machinery
- Novel method of food storage/avoiding wastage
- Nutraceutical value of food
- Medicinal value of plants, etc.

Intellectual Property Rights (IPR)

- Intellectual Property Rights (IPR) - refers to the right of any party to safeguard and legally prevent others from using an original brand, trade secrets or inventions
- IPRs are "private rights", ordinarily protected under different legal theories- Patent, copyright, trade mark, trade secret, plant variety rights, geographical indications, etc.
- Purpose of rights – promote innovation and progress of science by securing for limited times, to inventors and authors, the exclusive right to their respective creations

Why Intellectual Property Rights?

- To get the propriety on ones own creations
- To establish the supremacy/ownership for their creations
- To share economic benefit on the sale of the intellectual property
- To stop others using this and making illegal earnings
- To protect it from impingement by others



Patents:

Exclusive right granted for an invention by a designated authority which is a product or a process that provides, in general, a new way of doing something, or offers a new technical solution to a problem

What kind of protection does a patent offer?

- In principle, the patent owner has the exclusive right to prevent or stop others from commercially exploiting the patented invention
- It cannot be commercially made, used, distributed, imported or sold by others without the patent owner's consent
- Patents are territorial rights
- The protection is granted for a limited period, generally 20 years
- It is a long and complex process

Different patent types:

- **Utility patent:** This is what most people think of when they think about a patent. It's a long, technical document that teaches the public how to use a new machine, process, or system
- **Provisional patent: A** less formal document that proves the inventor was in possession of the invention and had adequately figured out how to make the invention work. The inventor must file a formal utility patent within a year, else the patent is lost
- **Design patent.** This patent offer protection for an ornamental design on a useful item. For example- the shape of a bottle or the design of a machinery
- **Plant patent.** Protects new kinds of plants produced by cuttings or other nonsexual means. Do not cover seeds and genetically modified organisms (GMOs)

What is not patentable?

Diamond v Chakrabarty case:

In 1980, the Supreme Court of the United States ruled that a micro-organism that had been genetically modified for use in cleaning oil spills was patentable on the grounds that it did not constitute a **"product of nature "**

The US Court set an important precedent in the area of patentability by ruling:

"The laws of nature, physical phenomena, and abstract ideas are not patentable. Thus, a new mineral discovered in the earth or a new plant found in the wild is not a patentable subject matter … Such discoveries are 'manifestations of . . . nature, free to all men and reserved exclusively to none'. "

A M Chakrabarty
Indian American microbiologist, notable for his work in developing a genetically engineered organism using plasmid transfer for cleaning oil spills, the patent for which led to landmark Supreme Court case- *Diamond v. Chakrabarty*.

Top countries in filing Patents

Country	2020 Rank[7]	2020 Patent applications[7]
China	1	1497159
U.S.	2	597172
Japan	3	288472
South Korea	4	226759
European Patent Office	5	180346
Germany	6	62105
India	7	56771
Russian Federation	8	34984
Canada	9	34565
Australia	10	29294
Brazil	11	24338
United Kingdom	13	20649

Patents signify the scientific advancement as it promotes innovations and bring prosperity through their industrial/commercial application

If not exploited commercially, however, patenting may prove counter-productive

Geographical Indications (GI)

A geographical indication (GI) is **a sign used on products that have a specific geographical origin and possess qualities or a reputation that are due to that origin**

Examples: Basmati Rice, Alphanso Mango, Nagpur Orange, Kolhapuri Chappal, Bikaneri Bhujia, Agra Petha, Paithani and Mysore silk Saree, etc

The prime purpose of registering a GI is to seek protection for specific products produced in a particular geographical region, which further encourages and motivates the marketers to expand their business at a global level

Plant Variety Protection

- Protection of crop cultivars developed by plant breeders
- HYV and hybrids contribute significantly in enhancing food production
- The improved varieties give protection against pests and diseases, improve quality etc
- Takes several years of hard work to develop a variety
- Reasonable to give IPR to plant breeders and to involve them in sharing profits from the commercialization of the IP
- Important to provide an effective system of plant variety protection with an aim to encourage the development of new varieties of plants and to prevent misuse of the IP
- **As per TRIPS (Trade-Related Aspects of IPR) agreement, the member countries are required to grant protection on plant varieties either by patents or by an effective *sui generis* system – minimum IP standards in the global trading system**

The Protection of Plant Varieties and Farmer's Rights Act, 2001 (PPV&FR)

An act to provide for the establishment of an effective system for protection of plant varieties, the rights of farmers and plant breeders, to encourage the development of new varieties of plants

This act is unique:

- It recognises the importance of farmers in conserving and improving crop varieties
- It provides farmers the right to produce, sell, share the seeds they produce

What is plant variety protection?

- Grant of right to the breeder by a designated authority
- Provide Protection against misuse/commercial exploitation
- Deriving income to the breeders/farmers and benefit sharing
- Enables multiplication of seed involving public/private parties for large scale use

Do farmers' have rights?

- Same privilege as that of a breeder
- Declaration that the initial variety is developed lawfully
- Use, exchange or sell his seed of a protected variety
- Seeds cannot be sold in special packages and under any brand name
- Should not involve any intermediaries/ traders

Conditions for grant of protection

A certificate of grant of protection to the applicant will be issued to the grantee when the registrar has approved the following criteria.
The new variety should fulfil the following:

- Novelty – with newer traits/performance
- Distinct (D)- morphological, physiological, commercial characteristics
- Uniform (U) – with describable, predictable, commercially acceptable
- Stable (S) – Remain unchanged following reproduction

Compensation Cover Available to Farmers: Only On Crop from Seed Procured through Authorised Seller/Licensee/Agent/Registered Breeder

- Right of claiming compensation is available to a farmer in the event of variety not performing as per label claim on a registered variety after adopting standard production protocols
- But, a farmer who has used his own saved seed, or who has purchased or procured unbranded seed from another farmer does not have any right to seek compensation

"The proof of the pudding is in the eating"

- The real benefit of an IP is achieved when it reaches majority of the targeted population and bring prosperity to them and to the society at large
- Cannot be done by the innovator or an organization
- The institutional capacity and competence is limited
- Best it is done by people/ agencies who have expertise and experience in the field
- For seeds- it is best done by the seed companies
- The whole process of taking the IP to the consumers is done through **Technology Commercialization**

Technology Commercialization

- Technology commercialization is more important than IP protection
- Support and handholding necessary in patenting, technology assessment, licencing, negotiations, agreement, etc
- Better done by professional and competent persons
- Ideally, each institution should have a business development cell to handle this activity
- Should have regular interactions with industry partners
- Should publicise regularly the technology available for commercialization
- Sensitize scientists about the nuances of TC
- Develop an effective system for benefit sharing

Technology Commercialization

1.Identification of Variety/Technologies

2.Testing of the technology at lab level

3.Validation of technology

4.Developing DUS guide lines in case of varieties

5.Valuation of the technology by cost:benefit ratio

6.Costing of the technology

7.Approval by the designated authority and Registration

8.Applying for patenting

9. Licensing either by direct advertisement or through an approved agency

9.Presenting the technology to the agency

10.Discussion and negotiations with the interested party

11.Release of the technology after receiving Initial royalty or fees

Conclusions

- It is mandatory under TRIPS agreement to protect IP through the provisions provided for by the Government
- IPR encourages innovation, protects from misuse and ensures economic benefits
- Necessary to create awareness about IPR with all stakeholders
- Each institution should have a business development cell to handle this activity, which requires diverse skill sets
- Institutions must put in place a robust policy for IP protection and technology commercialization
- Build capacity of all stakeholders through regular trainings

Lecture

(N T Yaduraju, Former Director, Directorate of Weed Research, Jabalpur, India)

Biodiversity: Importance and Management

Biodiversity

Biodiversity, the diversity of life on Earth, is the variability among living organisms from all sources, including diversity **within species, between species, and of ecosystems.**

Why is biodiversity important?

"It is reckless to suppose that biodiversity can be diminished indefinitely without threatening humanity itself" (Edward O. Wilson, The Diversity of Life, 1992).

- Everything that lives in an ecosystem is part of the web of life, including humans.
- Plants, animals, insects, and microorganisms interact and depend upon one another for what each offers, such as food, shelter, oxygen, and soil enrichment.
- Biodiversity mitigates and provides resilience to climate change
- All producers consume carbon dioxide for their food production, helping the intake of greenhouse gas emissions.

Ecological services attributed to biodiversity

- Balance of life in nature
- Biological productivity
- Regulation of climate
- Degradation of waste
- Cleaning of air and water
- Cycling of nutrients
- Control of potential pest and disease causing organisms
- Detoxification of soil and sediments
- Soil and water conservation
- Stabilization of river and sea coasts against erosion
- Carbon sequestration and global climate change
- Maintenance of Soil fertility
- Biodiversity also boosts the economy.

From Brundtland Commission to the Earth Summit

- The **World Commission on Environment and Development (**popularly, the **Brundtland Commission)** was a sub-organization of the United Nations founded in 1983 aimed to unite countries in pursuit of sustainable development under the chair of **Gro Harlem Brundtland**, former Prime Minister of Norway.
- The Brundtland Commission produced the report , **Our Common Future,** also known as the **Brundtland Report.** The document popularized the term **"sustainable development".**
- The commission redefined "economic development" in terms of "sustainable development', and crafted the most popular definition of sustainability:
- **"Development that meets the needs of the present without compromising the ability of future generations to meet their own needs."**

The Earth Summit

The United Nations Conference on Environment and Development (UNCED), also known as

- The Rio de Janeiro Earth Summit
- The Rio Summit
- The Rio Conference, and
- The Earth Summit
- It was a major UN conference held in Rio de Janeiro from 3 June to 14 June 14, 1992.

Legally binding agreements that were signed at the Earth Summit

- Convention on Biological Diversity (CBD)
- Framework Convention on Climate change (UNFCCC)
- United Nations Convention to Combat Desertification

The Convention on Biological Diversity

- At the 1992 Rio Earth Summit, the Convention on Biological Diversity was conceived as a practical tool for translating the principles of Agenda 21 into reality.
- The Convention recognized that the biological diversity is about more than plants, animals and microorganisms, and their ecosystems – it is about people and our need for food security, medicines, fresh air and water, shelter, and a clean and healthy environment in which we live**.**

The CBD has 3 main objectives

- The conservation of biological diversity
- The sustainable use of the components of biological diversity.
- The fair and equitable sharing of the benefits arising out of the utilization of genetic resources
- The convention was signed on 5 June 1992 and entered into force on 29 Dec.1993. So far, 196 nations signed it. It has two supplementary agreements, the Cartagena Protocol and Nagoya Protocol.

How many Species the earth inhabits?

- Estimates of the total number of species vary considerably from 3 million to 30 million, but most put the range between 5 and 10 million (Wilson, 1999).
- According to a recent estimate, 8.7 million(±1.3 million SE) eukaryotic species exists globally (6.5 million terrestrial and 2.2 million marine (Mora et al., 2011)
- Only about 20% species have been described.
- Each year, about 13,000 species are discovered and added to the 1.7 million species that have been catalogued so far.

Threats to Biodiversity

- Natural causes- Narrow geographical area, low population, low breeding rate, natural disasters, Volcano, earthquakes, floods, droughts, etc.
- Natural cycles- onset of spring, onset of winter
- Environmental Pollution
- Climate change
- Anthropogenic causes – greatest threat
    - Deforestation and habitat modification
    - Overexploitation of selected species of plants and animals

- Poaching
- Agriculture
- Invasive alien species
- Co-extinctions

Deforestation and habitat modification:

- *Destruction*, *degradation* and *fragmentation* of habitat are the three principal categories of habitat modification
- Tropical rain forests, once covering more than 14% of the earth's land surface, now cover no more than 6%
- Human- induced bush fires, forest fires, desertification, soil degradation etc
- Marine biodiversity is also under serious threat due to large scale destruction of the fragile breeding and feeding grounds of our oceanic fish and other species

Overexploitation of plants and animals

- Humans have always depended on nature for food and shelter, but when **'need' turns to 'greed**', it leads to over-exploitation of natural resources.
- Hunting activities exert a considerable burden on wildlife, provoking immense downturn of wildlife, leading to disturbed and inefficient ecosystems.
- Overhunting can lead to the elimination of large mammals in contrarily healthful unharmed habitat, compelling transition in forest structure.
- **Poaching:** Illegal trade of wildlife products by killing prohibited endangered animals i.e. poaching is another threat to wildlife.
- **Agriculture:** Since the 1900s, about 75 percent of plant genetic diversity has been lost as farmers worldwide have left their multiple local varieties and landraces for genetically uniform, high-yielding varieties.
- **Invasive alien species:** When alien species are introduced into a new habitat, unintentionally or deliberately, some of them turn invasive, and cause decline or extinction of indigenous species
- **Co-extinctions:** When a species becomes extinct, the plant and animal species associated with it in an obligatory way also become extinct.

Human- animal conflicts

- Dwindling habitats of tigers, elephants, rhinos, monkeys, wild boars, and peacocks due to shrinking forest cover compels them to move outside the forest and attack the field or sometimes even humans.
- Human encroachment into the forest areas raises a conflict between human and the wildlife, as it is an issue of survival of both.
- Earlier there used to be wild-life corridors through which the wild animals used to migrate seasonally in groups to other areas. Due to development of human settlements in these corridors, the path of wildlife has been disrupted and the animals attack the settlements.

Measures to Curb the Conflict

- Cropping pattern should be changed near the forest borders and adequate fodder, fruit, and water should be made available for the elephants within forest zones.
- Adequate crop compensation and cattle compensation scheme must be started, along with substantial cash compensation for loss of human life.

- Solar powered fencing should be provided along with electric current proof trenches to prevent the animals from straying into fields.
- Wild life corridors should be provided for mass migration of big animals during unfavorable periods. About 300 km^2 area is required for elephant corridors for their seasonal migration.
- Culling excess animals

Climate change and bio-diversity

- If temperature rises by 2ºC, **15-40%** species may face extinction
- Ocean acidification, a direct result of rise in CO_2 will have major effects on marine ecosystems
- Loss of land races, wild relatives, etc.

Biodiversity hotspots

- A biodiversity hotspot is an area with unusual concentration of species, many of which are endemic. It is marked by serious threat to its biodiversity by humans.
- These sites now support nearly 60% of the world's plant, bird, mammal, reptile, and amphibian species

Two conditions for qualifying as a Hotspot:

- The area must have at least 0.5% of the world's total vascular plant species as endemic flora (World's total vascular plant species is estimated to be 0.3 million, 0.5% of this comes to 1500)
- It has to have lost at least 70% of its original habitat (or it must have 30% or less of its original natural vegetation). In other words, it must be threatened

Biodiversity hotspots in India

1. **Eastern Himalaya:** Includes the entire Indian Himalayan region (and that falling in Pakistan, Tibet, Nepal, Bhutan, China and Myanmar)

2. Indo-Burma: Includes entire North-eastern India, except Assam and Andaman group of Islands (and Myanmar, Thailand, Vietnam, Laos, Cambodia and southern China)

3. Sundaland: Includes Nicobar group of Islands (and Indonesia, Malaysia, Singapore, Brunei, Philippines)

4. Western Ghats and Sri Lanka: Includes entire Western Ghats (and Sri Lanka)

Biodiversity conservation

- **In situ conservation-** on-site conservation or the conservation of genetic resources in natural populations of plant or animal species, such as forest genetic resources in natural populations, and in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties.
- **Ex situ conservation -** conservation outside its natural habitats. In ex situ conservation, the germplasm is removed from the place where it is naturally growing and stored off-site as seeds in genebanks, vegetative materials in in vitro storage, plant accessions in a botanical garden or field genebank, zoological gardens, captive breeding, and aquariums.

In situ conservation methods

Biosphere reserves

Biosphere reserves cover very large areas, often more than 5000 km^2. They are used to protect species in natural habitat for a long time. Currently, there are 18 Biosphere Reserves in India.

National parks

A national park is an area dedicated for the conservation of wildlife along with its environment. It is usually a small reserve covering an area of about 100 to 500 square kilometers. Within biosphere reserves, one or more national parks may also exist. Currently, there are 103 national parks in India.

Wildlife sanctuaries

A wildlife sanctuary is an area which is reserved for the conservation of animals. Currently, there are 551 wildlife sanctuaries in India

Community reserves

It is the type of protected area introduced in Wildlife Protection Amendment Act 2002 to provide legal support to community or privately owned reserves which cannot be designated as national park or wildlife sanctuary.

Special projects to conserve threatened animals

Project Tiger, Project Elephant, Project Dolphin, Project Snow leopard are special projects.

Special efforts are also launched for conserving Hangul (Kashmir stag), Indian (one horned) rhino, Indian crocodile, and sea turtle.

Sacred groves

They are tracts of forests, often near a temple, maintained as such where all the vegetation including trees and wildlife within are venerated and given total protection.

Gene sanctuary

Often, a gene sanctuary is an area where specific plants are conserved. India has set up its first gene sanctuary in the Garo Hills of Meghalaya for wild relatives of citrus.

On farm conservation

A strategy to conserve crops and various land races and cultivars in the farm itself, that is, in situ conservation of genetic resources, focusing on conserving cultivated plant species in farmers' fields.

Ramsar wetland sites

Wetlands are one among the most important identified ecosystems of the world.

As of July 2021, there are 2424 Ramsar sites around the world, protecting 254 million ha

India has 46 wetland sites designated as Ramsar Sites with a total area of about 1.09 mha

Ex situ conservation strategies

- **Zoological gardens**
- **Captive breeding**
- **Aquarium**
- **Botanical Gardens**
- **Biodiversity Parks**
- **Gene Banks - *Plant Seed Bank, Animal Seed Bank, Field gene banks, in vitro gene banks***

Gene bank/seed bank facilities in India

- National Bureau of Plant Genetic Resources (NBPGR), New Delhi
- National Bureau of Animal Genetic Resources (NBAGR), Karnal (Haryana
- National Bureau of Agriculturally Important Micro-organisms(NBAIM), Mau (UP)
- National Bureau of Agricultural Insect Resources(NBAIR), Bengaluru
- National Bureau of Fish Genetic Resources(NBFGR), Lucknow

Agrobiodiversity defined

- Agrobiodiversity is defined as the variety and variability of plants, animals, and micro-organisms that are used directly or indirectly for food and agriculture, including crops, livestock, forestry, and fisheries.
- It comprises of the diversity of genetic resources (cultivated varieties, breeds) and species used for food, fodder, fibre, fuel, and pharmaceuticals.
- It also includes the diversity of non-harvested species that support production (soil micro-organisms, predators, pollinators), and those in the wider environment that support agro-ecosystems

100 Years of Agriculture:

- About 75 % plant genetic diversity has been lost as farmers have left their multiple local varieties and landraces for genetically uniform, high-yielding varieties.
- About 30 % livestock breeds are at risk of extinction
- Presently, 75 percent of the world’s food is generated from:
- 12 plants: Rice, wheat, maize, potato, soybean, cassava, tomato, banana, onion, apple, grapes, sugarcane
- 5 animal species - cattle, sheep, goats, pigs, and chickens

- The world has over 27, 000 edible plants but 3 of them – rice, maize, and wheat – supply 60% of the world's food energy intake.
- Not good - less resilient to threats like disease, pests, and climate change.

Sustainability of food systems

- Using agricultural practices based on biodiversity – genetic, species, soil, and landscapes – can increase yields, and reduce waste and dependencies on external inputs.
- Soil erosion can be reduced by using agricultural biodiversity practices such as hedgerows, cover crops, agroforestry, or intercropping.
- Pest and diseases can be controlled by selecting plants that increase the number of pest predators, or by using pest or disease resistant varieties.
- Human activities in the name of development have inadvertently or deliberately contributed to huge losses of biodiversity.
- Various impacts such as imbalances in the cyclic pathways of ecosystems:
- Nutrient exchange
- Pollution
- Loss of food diversity leading to global food security issues
- Land degradation causing natural disasters as well reducing the quality of soil
- Encroachment of wild habitats
- Increased probability of novel diseases (like the ongoing pandemic) are evident.
- Climate change. Changes in global temperatures have proven to be detrimental to many life forms and disrupt ecological processes

Look differently

- Humans are part of biodiversity. We have no special rights to behave as the supreme being at the top of the hierarchy of all life forms.
- Conservation of biodiversity is pivotal for the management and restoration various of ecosystem services.
- The encephalization of humans with the passage of time has placed a greater responsibility of taking care of ourselves along with others on the planet.
- At collective level: Numerous governmental as well as non- governmental organizations at national and global levels have been working hard to spread awareness about biodiversity
- Many individuals, particularly the younger generation are taking great interest in conservation of biodiversity
- At individual levels too, we can be part of the solution.
- By reading more about biodiversity,
- Appreciating and spreading awareness about it,
- Planting more trees,
- Consuming a variety of foods in our diets to promote crop diversity,
- Not wasting food and water
- Generating less waste by reusing and recycling products