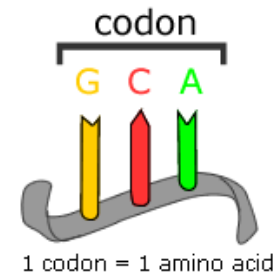
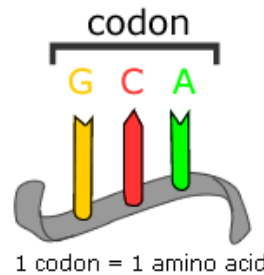
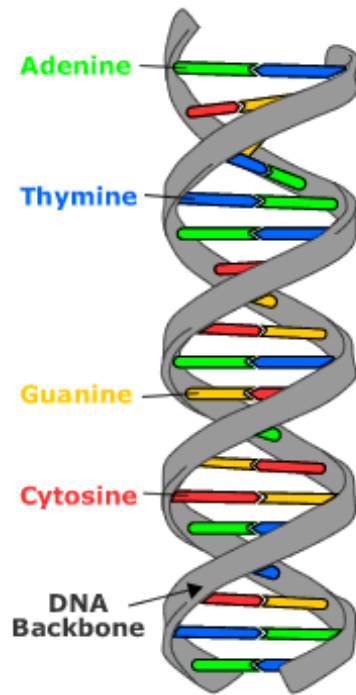


Advanced Studies on Plant Molecular Biology



Deoxyribonucleic acid, or DNA, is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms

More information on DNA

The main role of DNA molecules is the long-term storage of information

DNA contains the instructions needed to construct other components of cells, such as proteins and RNA molecules. It is often called the blue print of life

The DNA segments that carry this genetic information are called genes

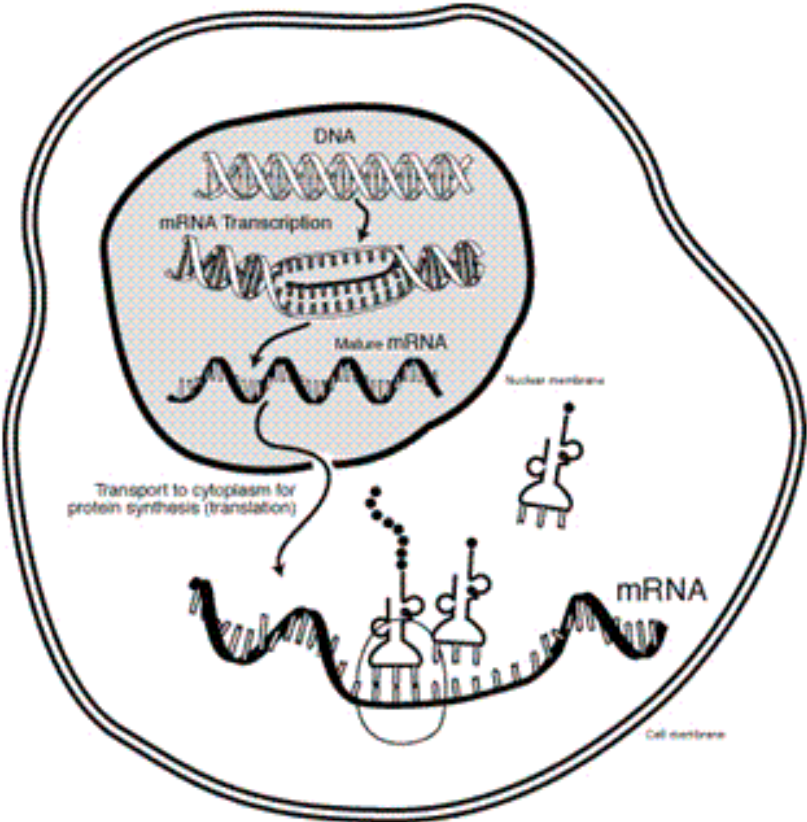
The other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information

Within cells, DNA is organized into structures called chromosomes and the set of chromosomes within a cell make up a genome






Arabidopsis chromosome

Mechanism of transfer of genetic information





Genetic approach of identifying genes involved in particular biological processes

-  Forward genetics approach
-  Reverse genetics approach
-  Forward chemical genetics approach

 Screening and characterizing the mutants

How to obtain a mutant?

❖ Mutagenizing the seeds

Different available mutagens:

EMS- Preferably induces a single base substitution (G-A); can cause variety of effects including complete loss of function, partially reduced function, constitutive function

Advantage: The high rate of mutagenesis, mutant allele of one specific locus can be found at a rate of approximately 1 in 2000-5000 M2 plants.
Because of various possible mutations can be induced by EMS, this allows identification of genes unlikely to be identified using other mutagens

Disadvantage: EMS induces many mutations, and mutants must be cleaned up by repeated backcrossing (five to ten times) to wild-type.

 **Fast Neutron**- Small deletions; Loss of function mutant

Advantage: Relatively easy to detect the deletion by using DNA blot hybridization

Disadvantage: Low frequency of mutation, need a nuclear reactor to generate fast-neutron mutagenized seeds

 **Insertional Elements**

T-DNA- Insertion in the gene causes loss of function mutants

Advantage: The Arabidopsis sequences flanking the insertion sites can be isolated easily by various PCR technique

Disadvantage: Generating the mutant plants are tedious. Large number of plants need to be screened to obtain mutant

Multiple insertions is possible which needs to be taken care of while characterizing the mutant.

The antibiotic resistance gene often does not integrate in plant genome, which sometimes makes it difficult to screen for the phenotype.

Activation tagging-Insertional alleles are generated using T-DNA or transposons that carry either a constitutive promoter, such as cauliflower mosaic virus (CaMV) 35s promoter, or multimeric enhancers from the CaMV 35s promoter

Advantage: Gain of function mutants can be generated by this approach. Relatively easy to detect the deletion by using DNA blot hybridization. The other advantage of enhancer activation tagging is that enhancers can insert both upstream and downstream from a gene to cause activation.

Disadvantage: Activation-tagged mutant phenotype is not always stable. It is assumed that there are factors that limit the interaction of 35s promoter with endogenous genes. Hence, large number of plants need to be screened

Enhancer and Gene Traps- Enhancer or gene traps identify genes that display a specific expression pattern. The general principle behind this approach is to integrate a reporter gene that either lacks a promoter (gene trap) or carries a only a minimal promoter (enhancer trap) at various sites in the wild-type genome

Advantage: Cellular localization and expression of genes

Disadvantage: It may be difficult to identify which gene's enhancers cause the specific expression pattern

Reverse genetics approach

Identifying a mutation in the gene of your interest and to test mutant plants for phenotypes that are predicted to result from loss of function of that gene

T-DNA insertion lines

RNAi approach

Creating knock out line

Advantage: One can directly approach the gene of interest and can bypass the whole mutant screening steps

Disadvantage: This approach does not always work with the multi copy genes. Finding a single-gene knock out may not always be sufficient to identify the true loss of function situation because of redundancy

■ Forward chemical genetics approach

A recently developed technique for identifying new genes that could not be identified by conventional screening

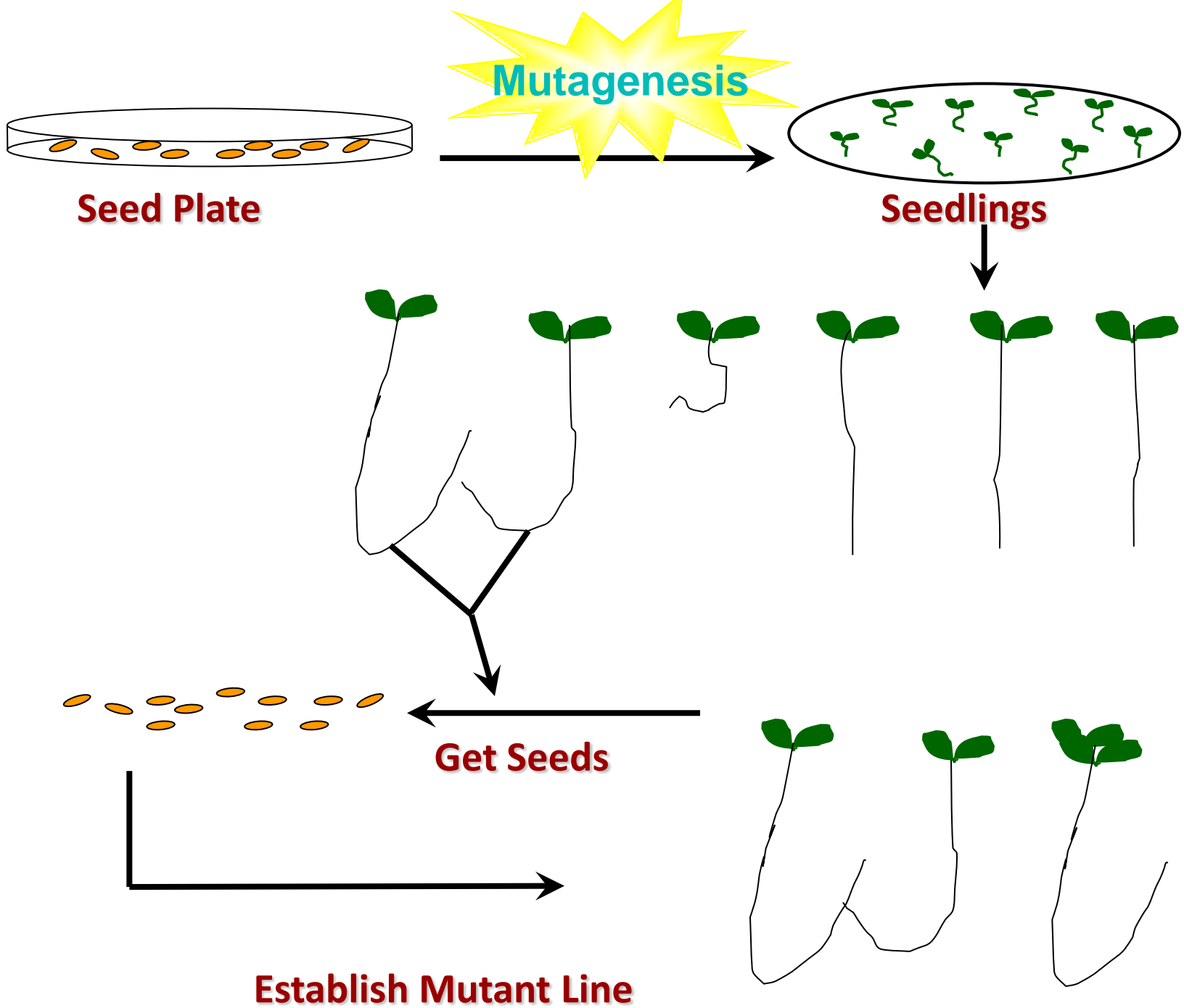
Isolation of mutants against the chemicals which are supposed to work in some regulatory pathway

Advantage: Helps to discover new factors/genes involved in a particular pathway

Disadvantage: Sometimes non-specific mutants, which are not related to the particular pathway may be obtained

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Segregation Analysis

Segregation analysis is used to determine whether a mutant phenotype is caused by Dominant or recessive mutations, how many mutations are required for the phenotype, and whether the mutations are in cytoplasmic or nuclear genes

Self progeny:

Recessive mutations are first identified in mutant screens as homozygous. If the mutant Phenotype is fully penetrant each of the self-progeny of such plants will be mutant

Dominant mutations can be identified as heterozygous or homozygotes. If heterozygous one quarter of the self progeny will exhibit a wild-type phenotype. Homozygous mutant can be identified as plants that do not yield wt offspring after crossing with wild-type

F1 progeny:

Reciprocal crosses should be done to know whether the mutation can be transmitted through both male and female gametes

wt carpels x mutant pollen	}	F1- wt- mutation is recessive and nuclear
wt pollen x mutant carpels		F1- mutant- mutation is nuclear and dominant

F2 progeny:

Grow around 100 F2 plants to determine what fractions of plants show mutant phenotype

One quarter of plants mutant- single, recessive, nuclear mutation (1:3)

Three quarters of plants mutant- single, dominant, nuclear mutation (3:1)

Segregating as wt, mutant and intermediate phenotype- semidominant (1:2:1)

The Chi-square (χ^2) statistical test can be used to determine how well a set of segregation data can fit a particular hypothesis

$$= \text{sum of } \frac{(O-E)^2}{E} \quad \text{Over all classes}$$

O- observed number of plants having a mutant phenotype

E- expected number of plants having mutant phenotype

Over all classes- classes are different phenotypic groups

The degrees of freedom (df)= (number of classes-1)

Table of Chi square

df	P = 0.05 (95%)	P = 0.01 (99%)	P = 0.001 (99.9%)
1	3.84	6.64	10.83
2	5.99	9.21	13.82
3	7.82	11.35	16.27
4	9.49	13.28	18.47
5	11.07	15.09	20.52
6	12.59	16.81	22.46
7	14.07	18.48	24.32
8	15.51	20.09	26.13
9	16.92	21.67	27.88
10	18.31	23.21	29.59

Table: *Genetic analysis of Arabidopsis aar mutants*

All χ^2 values indicate $P > 0.1$.

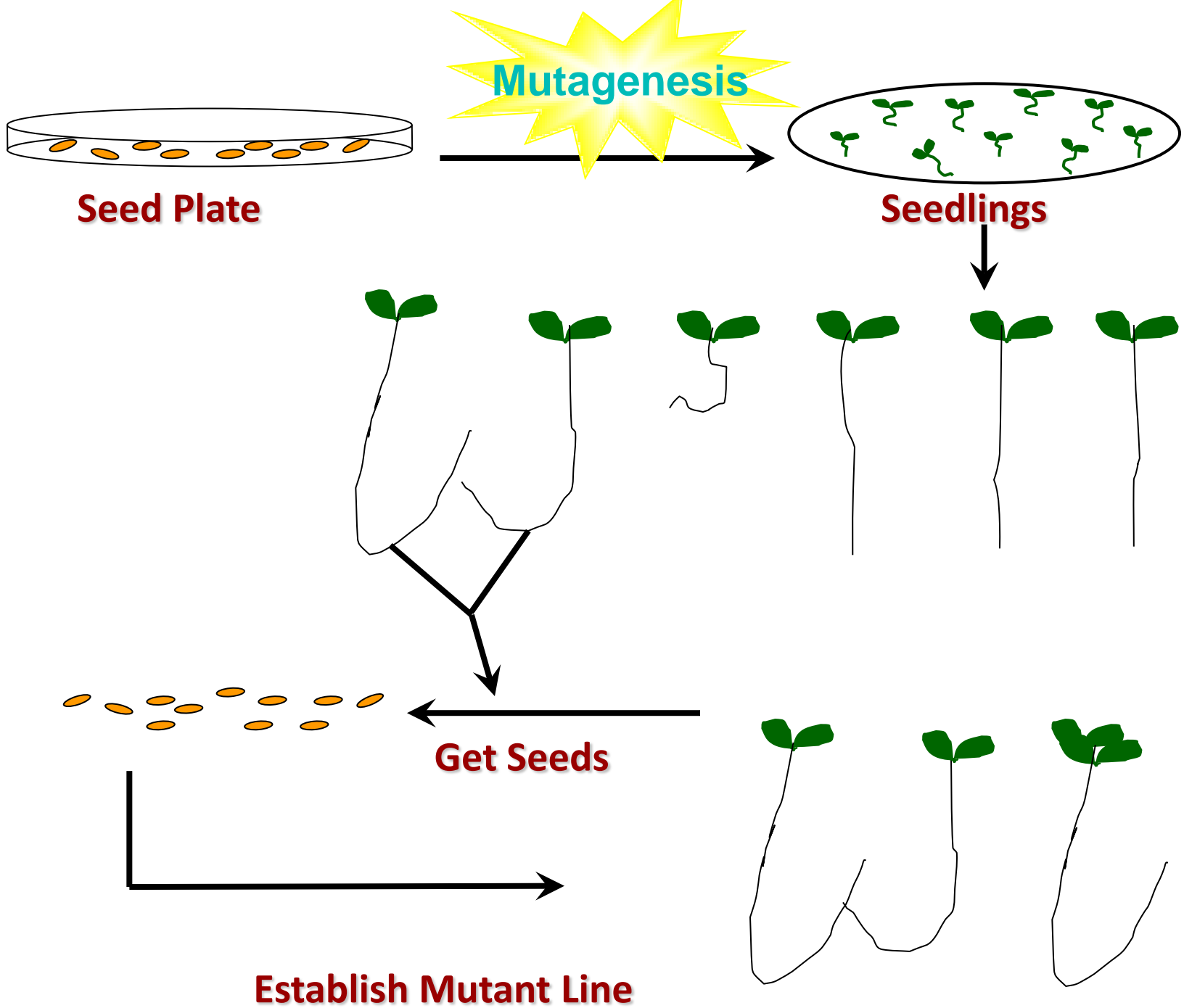
Genotype of parents (female × male)		Progeny		χ^2 ^a (Hypothesis)		
		PCIB-sensitive	PCIB-resistant			
<i>AAR2/AAR2</i> × <i>aar2/aar2</i>	F ₂	57	(weak) 111	(strong) 45	1.73 (1:2:1)	Semi dominant
<i>aar3/aar3</i> × <i>AAR3/AAR3</i>	F ₁	77		0	-	
	F ₂	70		24	0.01 (1:3)	recessive
<i>aar4/aar4</i> × <i>AAR4/AAR4</i>	F ₁	60		0	-	
	F ₂	42		19	1.23 (1:3)	recessive
<i>aar5/aar5</i> × <i>AAR5/AAR5</i>	F ₁	0		42	-	
	F ₂	28		70	0.67 (3:1)	dominant
<i>m31/m31</i> × <i>M31/M31</i>	F ₁	0		63	-	
	F ₂	26		84	0.11 (3:1)	dominant
<i>m34/m34</i> × <i>M34/M34</i>	F ₁	0		69	-	
	F ₂	32		92	0.04 (3:1)	dominant
<i>m35/M35</i> × <i>m35/M35</i>	self	32		85	1.89 (2:1)	
<i>m35/M35</i> × <i>M35/M35</i>	F ₁	27		29	0.07 (1:1)	dominant/homozygous lethal
<i>m36/M36</i> × <i>m36/M36</i>	self	44		101	0.58 (2:1)	
<i>m36/M36</i> × <i>M36/M36</i>	F ₁	35		37	0.06 (1:1)	dominant/homozygous lethal

Back crossing

Backcross of the mutant to wt is important to clean up the mutant. Theoretically, each back cross removes half of the unlinked secondary mutations.

The probability that a particular unlinked mutation remains after n rounds of back crossing is $(1/2)^n$

For pleiotropic mutants, it is necessary to determine whether the phenotypes result from same or from two different mutations. This can be determined by multiple round of backcrossing or by co-segregation analysis





Identifying and cloning the gene

Model Plant



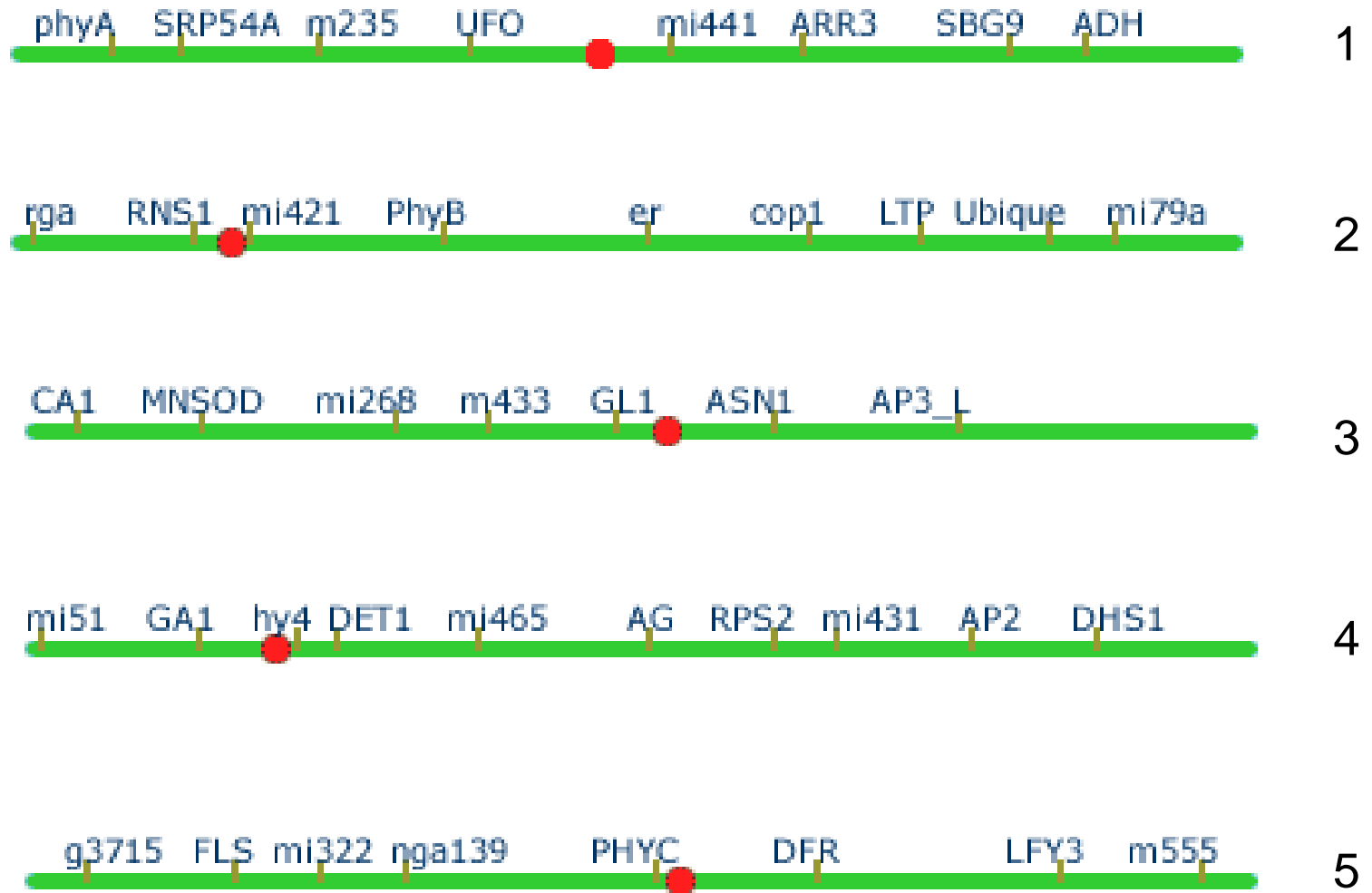
Arabidopsis thaliana

- ★ *Arabidopsis thaliana* has a small genome size
- ★ The whole genome has been sequenced
- ★ The genome of this plant is composed of 5 chromosomes which contains 125 Mb of DNA and 25,948 identified proteins
- ★ It has a shorter life cycle relative to other plants; Takes only six weeks from germination to produce seeds
- ★ It is easy to grow. Virtually it grows everywhere
- ★ Finally transferring the gene in this plant is relatively easy compared to other plants

Arabidopsis chromosomes



Chromosome



Mapping:

Mapping a novel mutation to a well defined chromosomal region is an essential step in the genetic analysis of the mutant and also a prerequisite for cloning of the corresponding gene

Once it was recognized that distantly related individuals differ in DNA sequencing, the molecular marker concept came into mapping strategy. In Arabidopsis, molecular markers exploit the natural differences between distinct ecotypes. For instance, it has been estimated that the widely used Landsberg erecta and Columbia ecotypes differ approximately 0.5 to 1% at the DNA sequence level. These local differences or polymorphisms of the DNA sequence are due to point mutations, insertions or deletions that randomly occurred in one ecotype but not in other

First step for mapping a mutation starts with crossing the mutant ecotype with a ecotype having a difference in the sequence at genome level

Mutant in columbia background X Ler wild-type



Self F1 progeny



Use resulting F2 progeny to analyze the linkage between the mutation of interest and any DNA Marker that distinguishes ecotypes columbia and landsberg



Use a PCR based approach to map the mutation

Example of difference in genomic sequences between Columbia and Landsberg ecotypes

SNP_Name	Chromosome	BAC_Name	BAC_Accession	BAC_Length	Left_Coord	Right_Coord	SNP_Type	Indel_Size	SNP_Base	Left_Flank	Right_Flank	Restriction_sites (Col)	Restriction_sites (Ler)
CER457348	5	MUK11	AB008271	79073	57195	57251	IND	55/-55		CGAGAGAGAGAGAGAGAGAGAG	GAGGAGGAGGAACCCGATCA	-	-
CER457349	5	MUK11	AB008271	79073	59113	59148	IND	34/-34		CGATTTTATTAATACTTAT	TGGAGTGAGGAGTAAAAGTC	-	-
CER457350	5	MUK11	AB008271	79073	59159	59179	IND	19/-19		CAAATTATTGGAGTGAGGAG	AAGACATTATGAACATCAAA	-	-
CER457351	5	MUK11	AB008271	79073	68828	68829	IND	-1		ATTGCCTCTGCCACGTCCTG	ATCAAGTAAACATCCAAGGA	-	-
CER457352	5	MUK11	AB008271	79073	70343	70347	IND	3/-3		TTAACTCTCTCTCTCTTT	AAATCTGCTTAAAAGCGTTT	-	-
CER457353	5	MUK11	AB008271	79073	74360	74376	IND	15/-15		ATTCTAATAGACAAATTCTC	TTCTTGAAATTAAGTCACAT	-	-
CER440680	5	MUK11	AB008271	79073	52329	52331	SNP		A/T	ACGAGGCCCTTGATTGAATT	AAAATCTCTGGTTAAACAAC	MseI Tru1I Tru9I	MseI Tru1I Tru9I AclI AhaIII ApoI DraI XapI
CER440638	5	MUG13	AB005245	86630	5374	5376	SNP		T/G	GGTTTTGTTATTATTACCTT	ACAGATGTAGAAGTGGTAAA	BfmI BstSFI SfcI SfeI	TaqI TthHB8I
CER440639	5	MUG13	AB005245	86630	5631	5633	SNP		T/C	AATCAAACCTTTTCTTTTG	TCTTCTAACGAAATTGTCG		Bsu6I Eam1104I EarI Ksp632I SapI
CER440640	5	MUG13	AB005245	86630	5718	5720	SNP		G/T	AGCAAAACAAAAACAGGCA	ACATGTAAGTAATGTATTAT	-	-
CER440641	5	MUG13	AB005245	86630	5778	5780	SNP		G/A	AGACCTTTTTATCCCTTG	CCAAGCGGGCGTTGTATCAA	BglI MwoI BshI BsuRI CviJI CviTI HaeIII Pali Balli BsaJI BseDI BssECI BssT1I CfrI EaeI Eco130I EcoT14I ErhI HaeI MliI MluNI MscI SseI Styl	
CER440642	5	MUG13	AB005245	86630	6557	6559	SNP		G/A	TTCTCCAGATTTCTCTAT	GAGTAACCAGCCTAATCCTT		BfmI BstSFI SfcI SfeI
CER440643	5	MUG13	AB005245	86630	6774	6776	SNP		T/C	GTATCTGACTCCGAAATCAA	ACTAACCACCTCTAGAAAT	-	-
CER440644	5	MUG13	AB005245	86630	7015	7017	SNP		A/G	TGATTGCATTATTGTCATAT	CGTCAAGGGTCTCGAACC	MaellI TailI TscI	FauNDI Ndel
CER440645	5	MUG13	AB005245	86630	7426	7428	SNP		C/T	TTTTATTATTATATGAAATT	AAATGCAAATAACAACCAAG	AclI ApoI XapI	MseI Tru1I Tru9I AclI AhaIII ApoI DraI XapI SmaI SnaI
CER440646	5	MUG13	AB005245	86630	8013	8015	SNP		C/T	TTTGTGTTTTTCGACCTCT	TCTCCTTCTGTGTGATTT	-	-

Predominantly two forms of molecular markers are being used for PCR based mapping

Simple Sequence length polymorphisms(SSLPs): The polymorphism that arises from microsatellite sequences. Usually the microsatellite sequences differ in various ecotypes because of the variations in the number of repeat unit. SSLPs are used as co-dominant genetic markers.

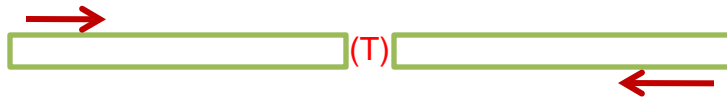


Gel electrophoresis
of PCR products

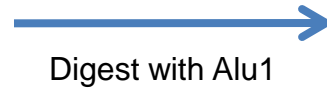


F7D8-1: 9354750 Mb

Cleaved amplified polymorphic sequences(CAPS): The polymorphism that arises from a single base change in the DNA sequences in different ecotypes. The change of that particular base must have to generate a unique restriction site



PCR product size 600

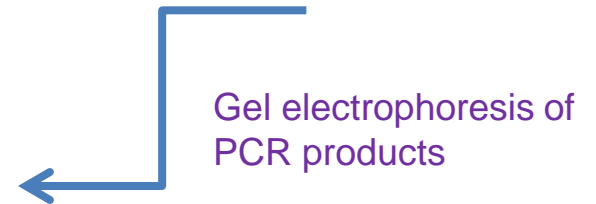
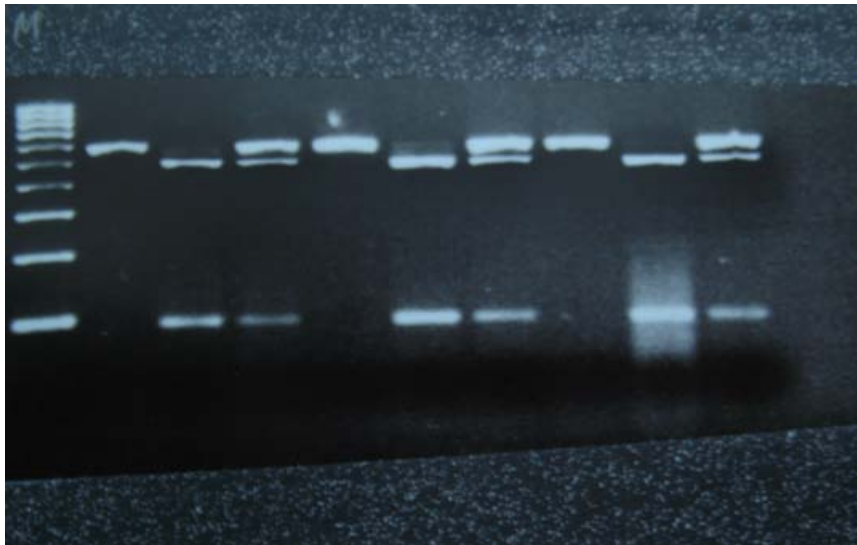


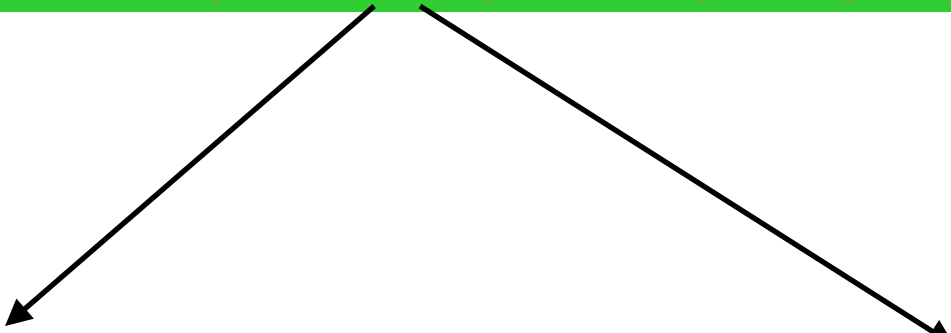
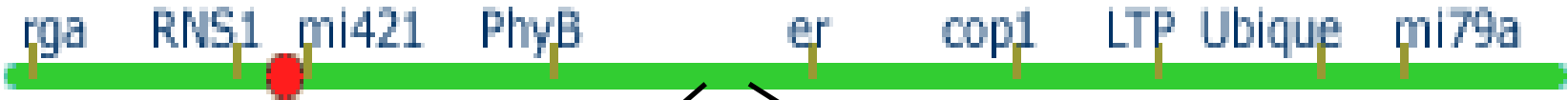
Columbia – no cut

Landsberg- cut once at 480

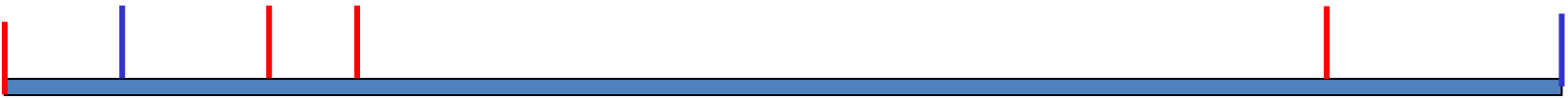


Col- 600
Ler- 480& 120





88-C 88-C 88-C 88-H
 165-H 165-H 165-H 165-C



1/350 9312804 9317160 9315340 9330750 9333691 9334430 9343320 1/350

F2G1-4 F7D8-7 F7D8-5 F7D8-1

1) Identify the mutation by sequencing

EIR1- chromosome 5 Marker- CTR1 and CIW14

Gene ID- At5g57090

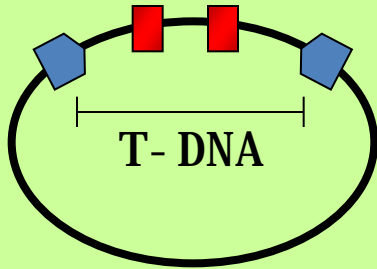
2) Complementation test

Inserting the wild-type gene in the mutant to see whether it can restore the abnormal phenotype

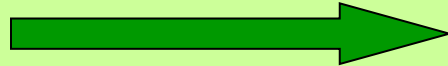
Once the gene is cloned in a vector, next step is to transfer the gene to the mutated plants

For transferring the gene, we use a natural soil bacteria, Agrobacterium and the process is called agrobacterium mediated transformation

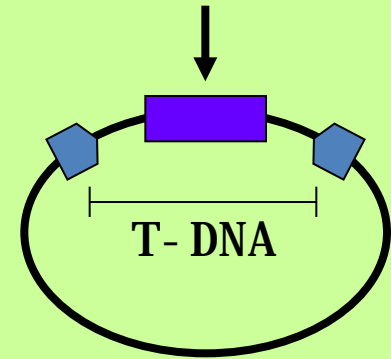
“ONC” genes



- a. Removal of “ONC” genes
- b. Replace with antibiotic resistance gene



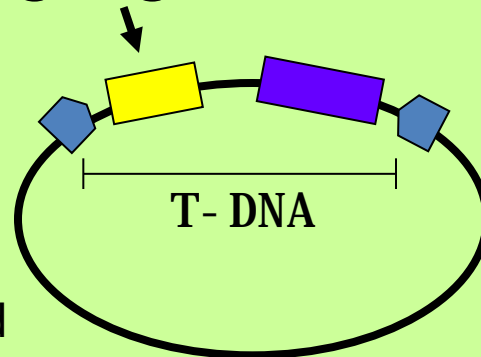
Antibiotic resistance gene



Insert chosen foreign gene into T-DNA



Foreign gene

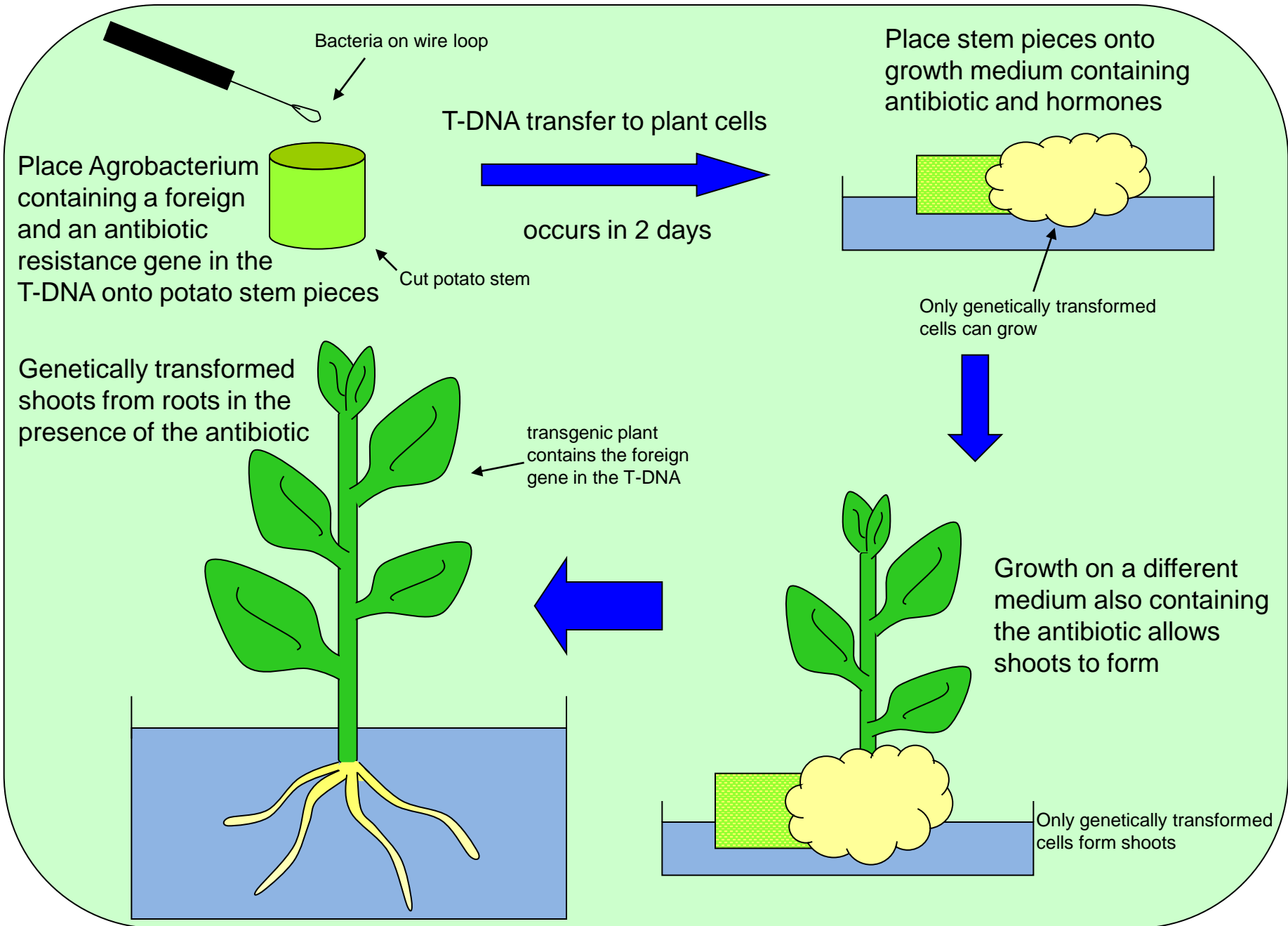


Infect wounded plant cells

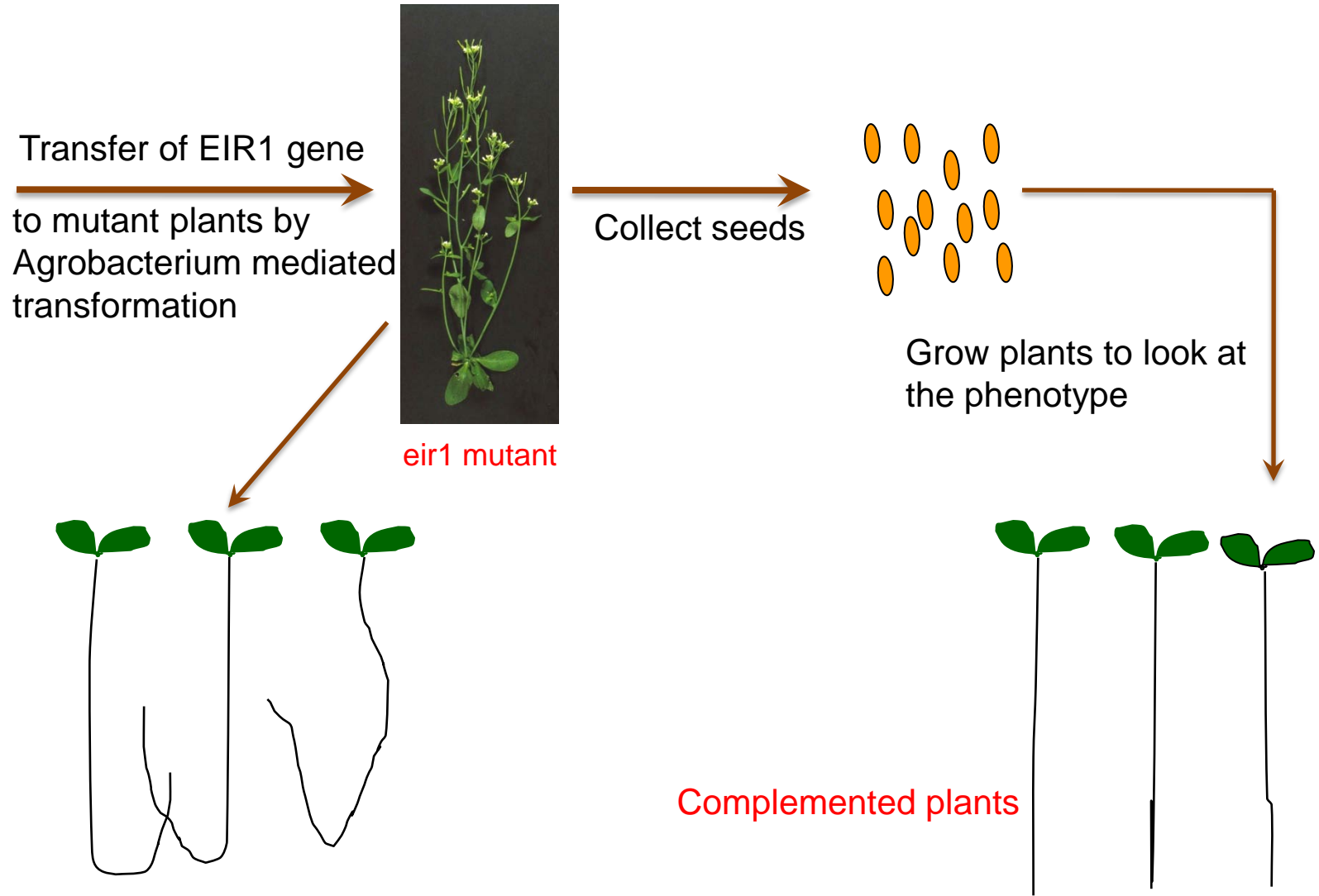


with Agrobacterium containing modified Ti-plasmid

T-DNA carries foreign gene into plant cells which are also resistant to the antibiotic



Final step: to validate the gene function we need to restore the mutant phenotype



References:

Arabidopsis A laboratory Manual: Detlef Weigel and Jane Glazebrook
CSHL press, Cold Spring Harbor, New York

EMBO COURSE Practical course on genetic and molecular analysis of Arabidopsis
Jerome Giraudat, Nathalie Beaudoin, Carine Serizet

