Bharathidasan University

Tiruchirappalli- 620024,

Tamil Nadu, India

Programme: M.Sc., Botany

Course Title: Plant Biotechnology

Course Code: 22PGBOTCC204

Unit III - PLANT MOLECULAR BIOLOGY

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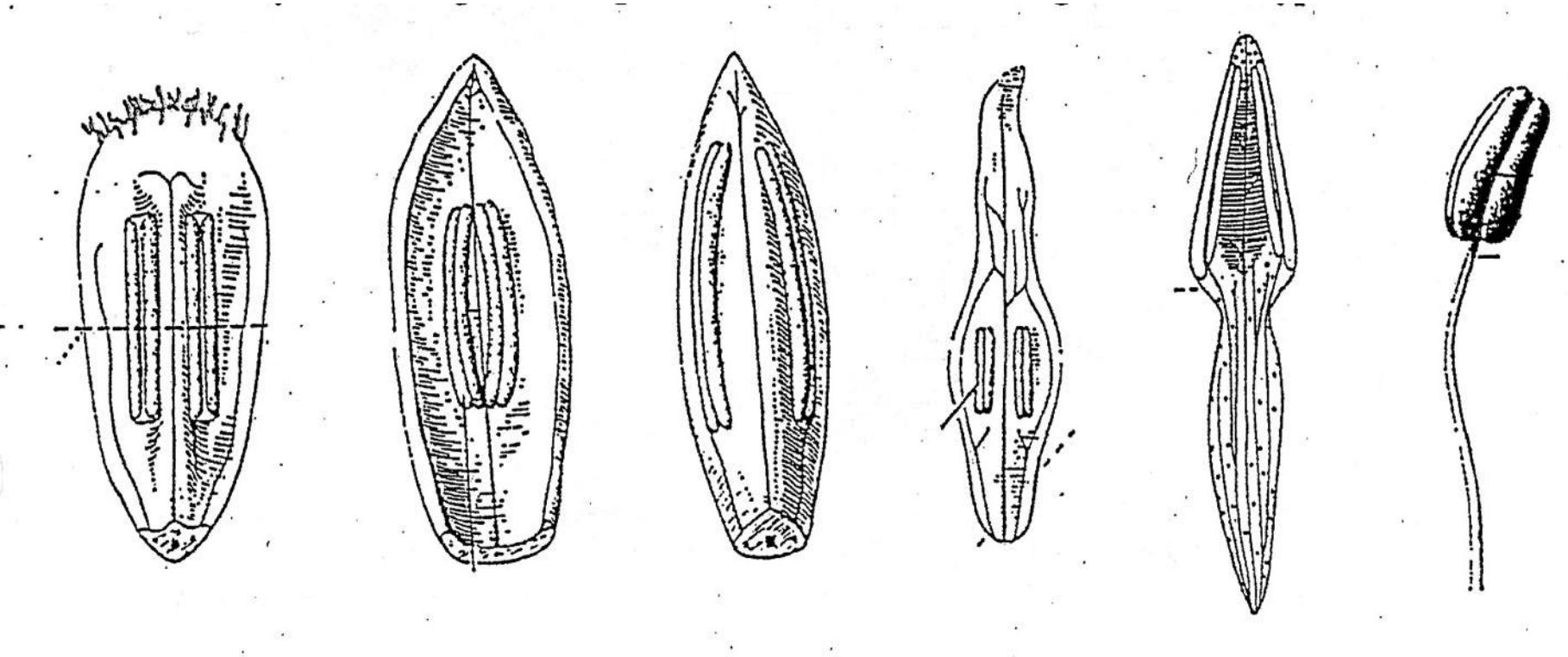
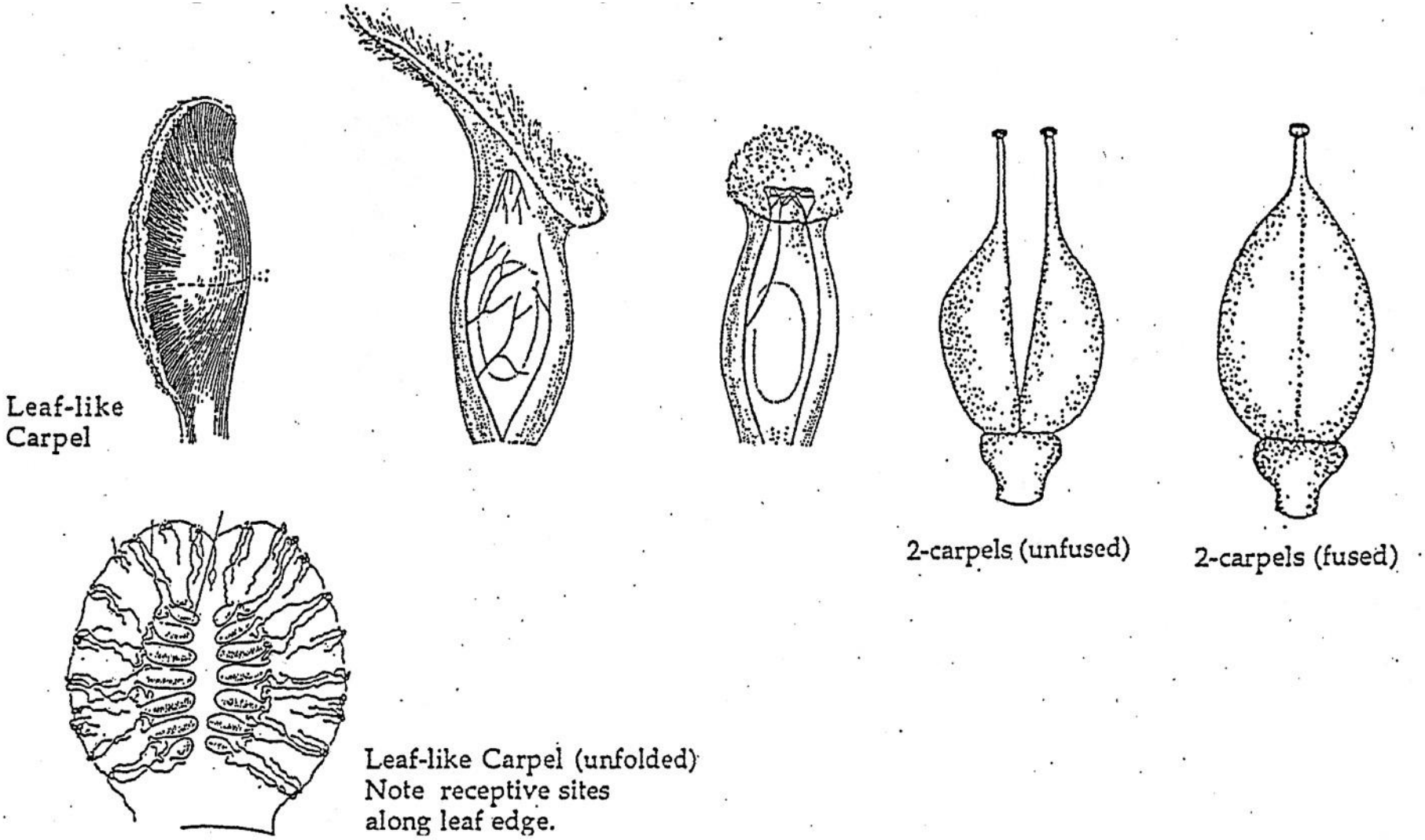
Department of Botany

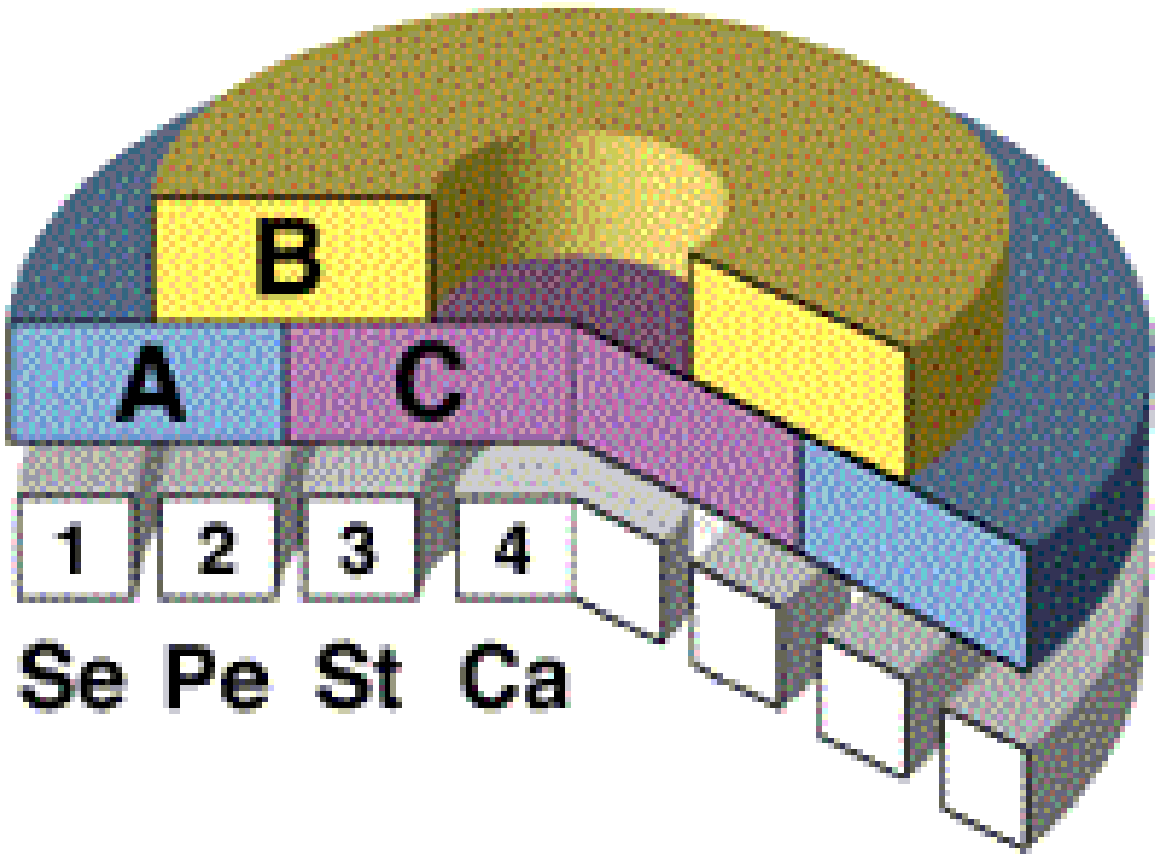
Morphological series



Stamen series from leaf-like to a slender filament.

Carpels from leaf-like (conduplicate carpel) to carpels with a highly defined stigma, style and ovary.





Basic “ABC Model of Floral Organ Development

Normal wild-type *Arabidopsis thaliana* (Brassicaceae)

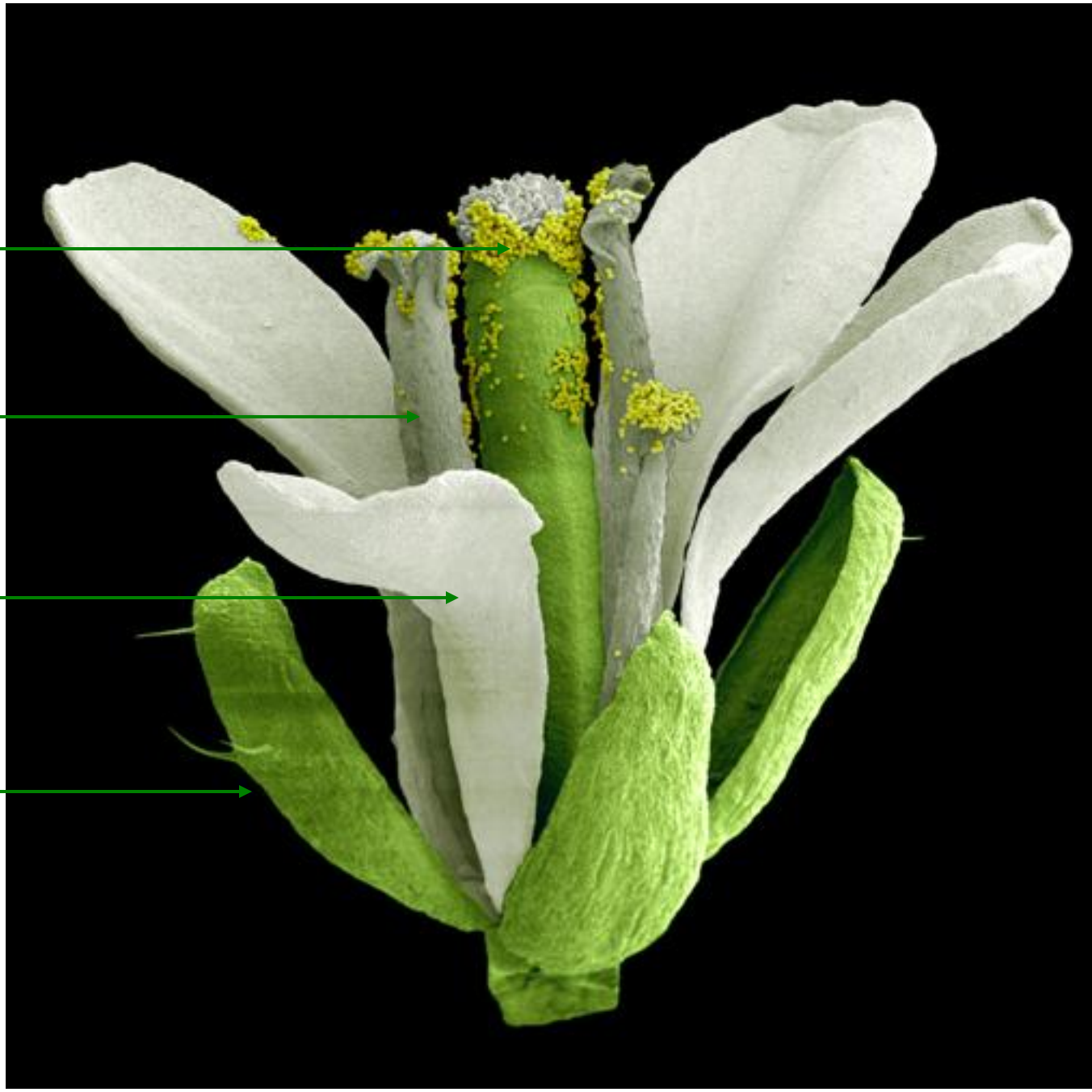
Arabidopsis
showing 4
floral organs

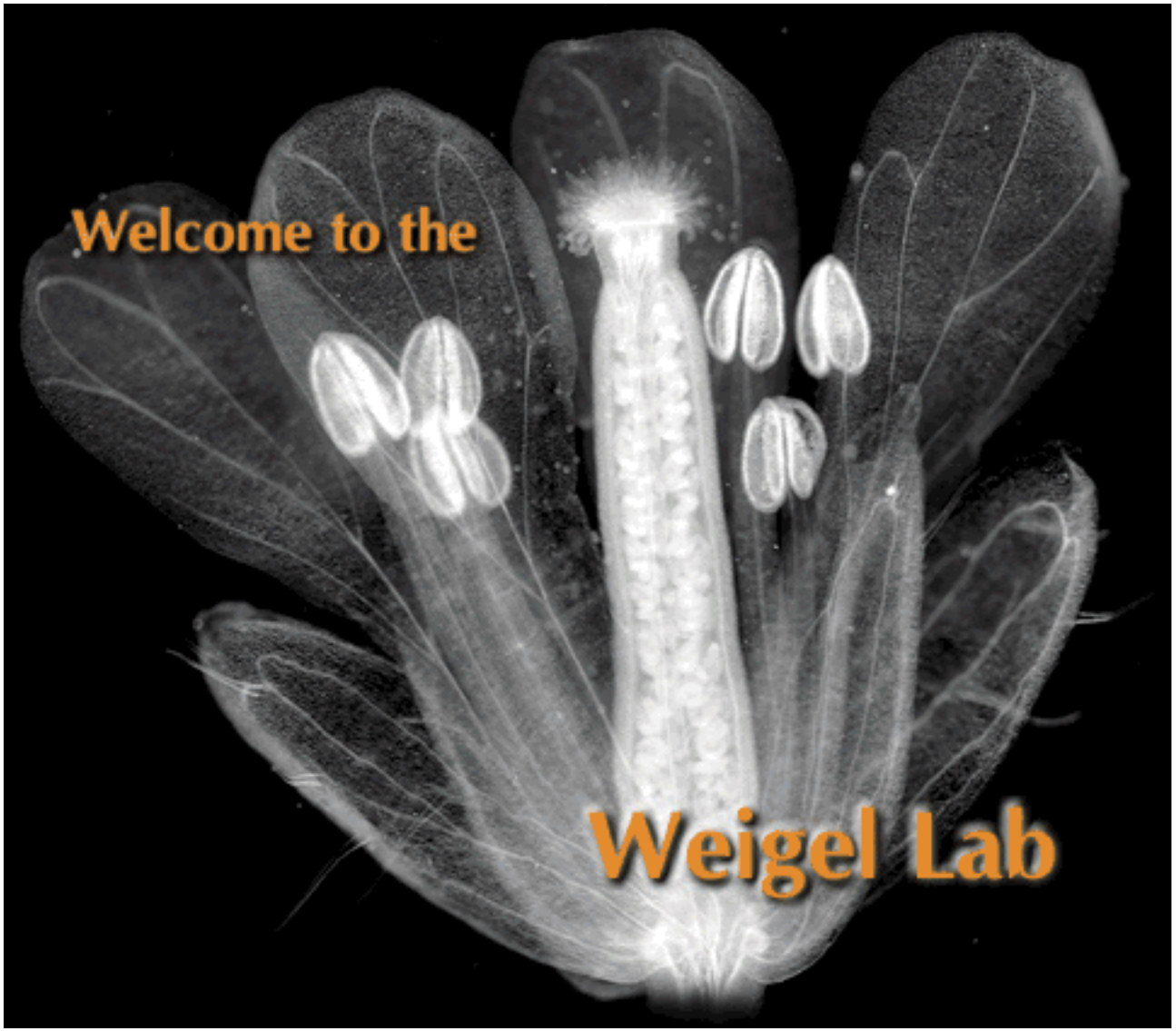
Carpels-C

StamensB&C

Petals-A&B

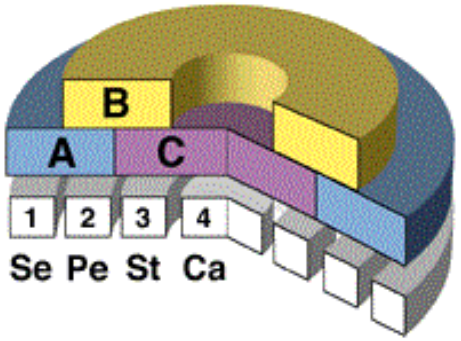
Sepals-A

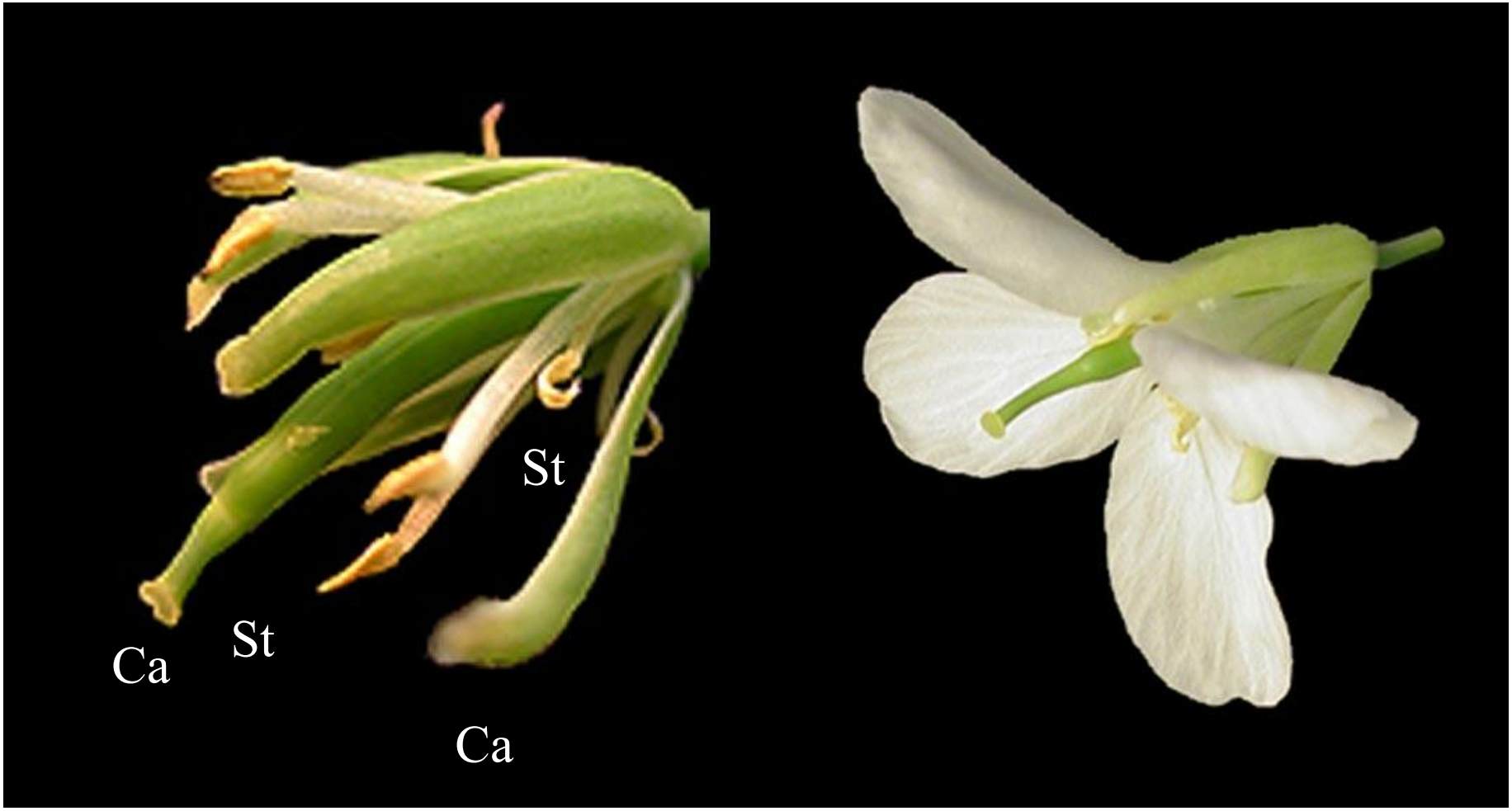



Welcome to the
Weigel Lab

apetala 2 mutant

No A(Ca -St-St - Ca)

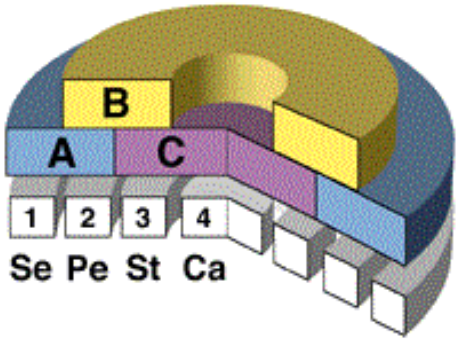




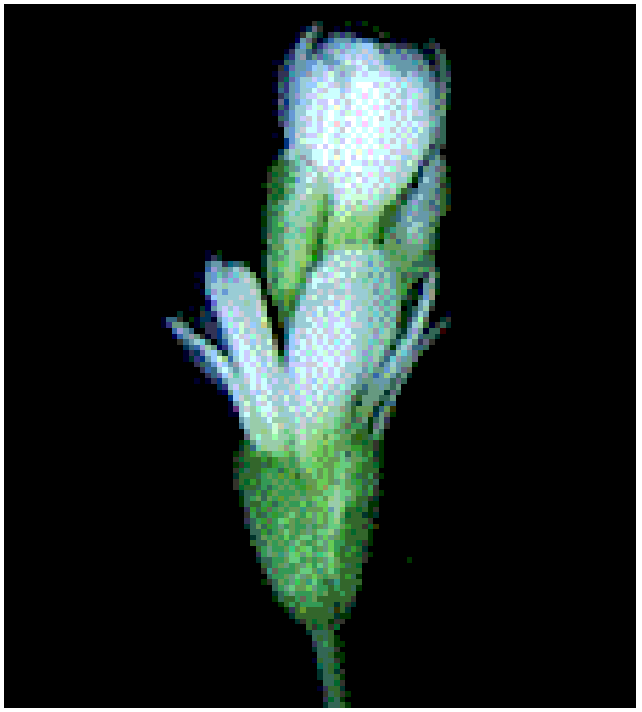
Apetala mutant
No A (Ca -St-St - Ca)

Wild Type Flower

apetala or pistillata mutant,
No B (Se-Se-Ca-Ca)



Top View



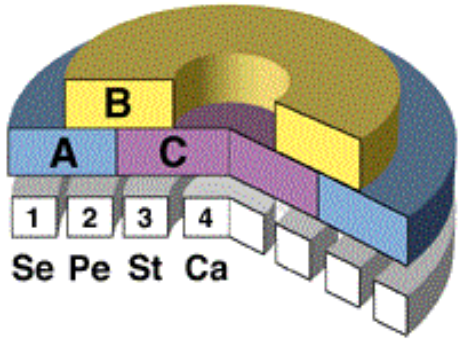
Side View

C- Class Mutants

AGAMOUS mutant

No Gametes

(Se-Pe-Pe-Se-Pe-Pe)



The name of a gene describes the flower when that gene is not functional.

A-Class Mutant

No *APETALA 1* or *APETALA2*

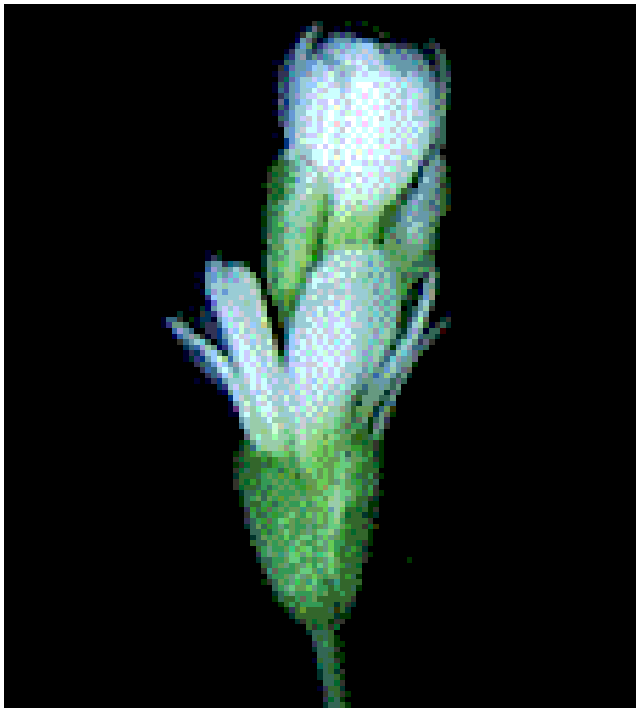
No Petals (only carpels and stamens)

B-Class Mutant

APETALA 3 or *PISTILLATA*

No petals or **a lot of Pistils**

Top View



Side View

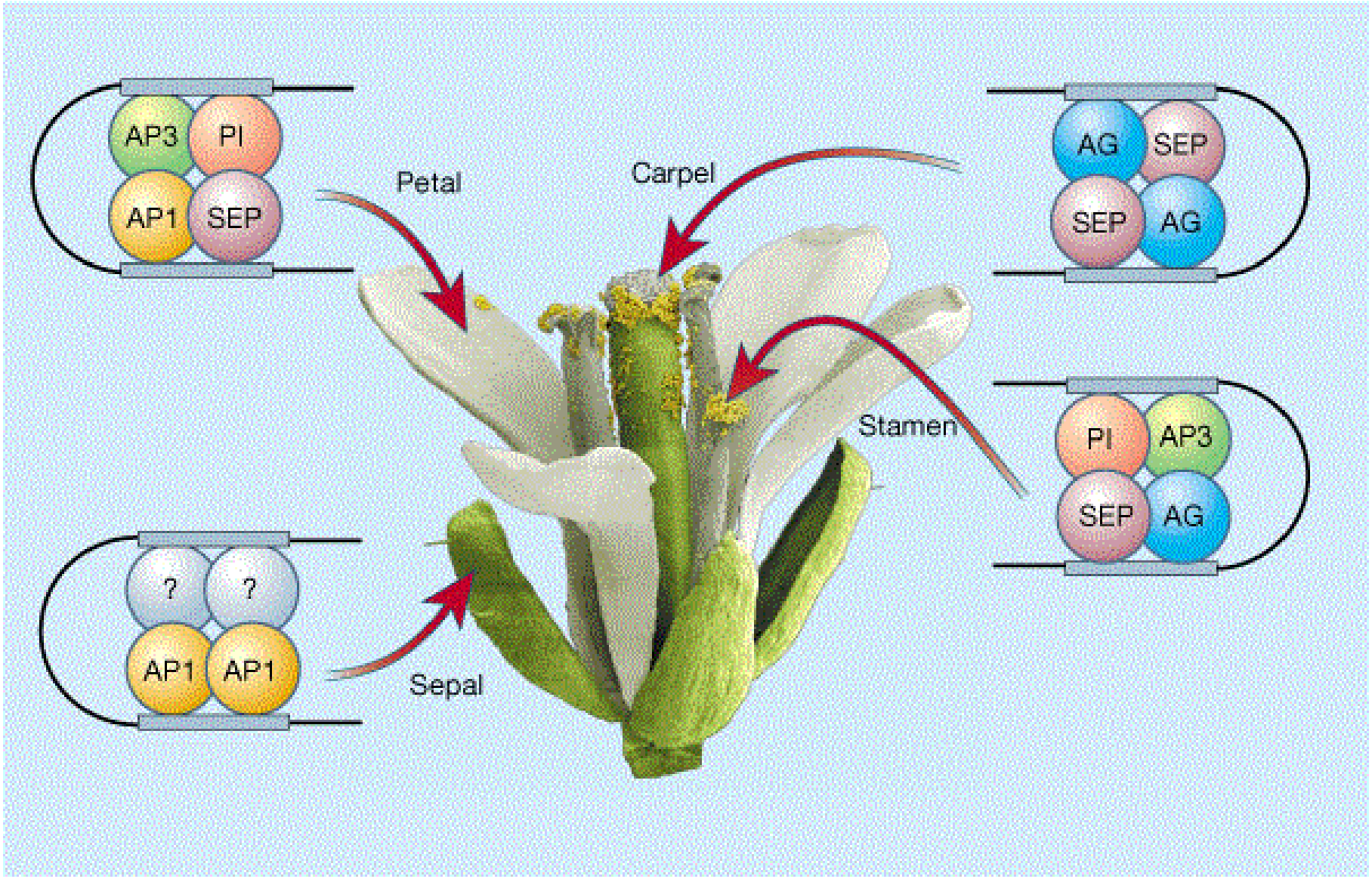
C- Class Mutants

AGAMOUS mutant

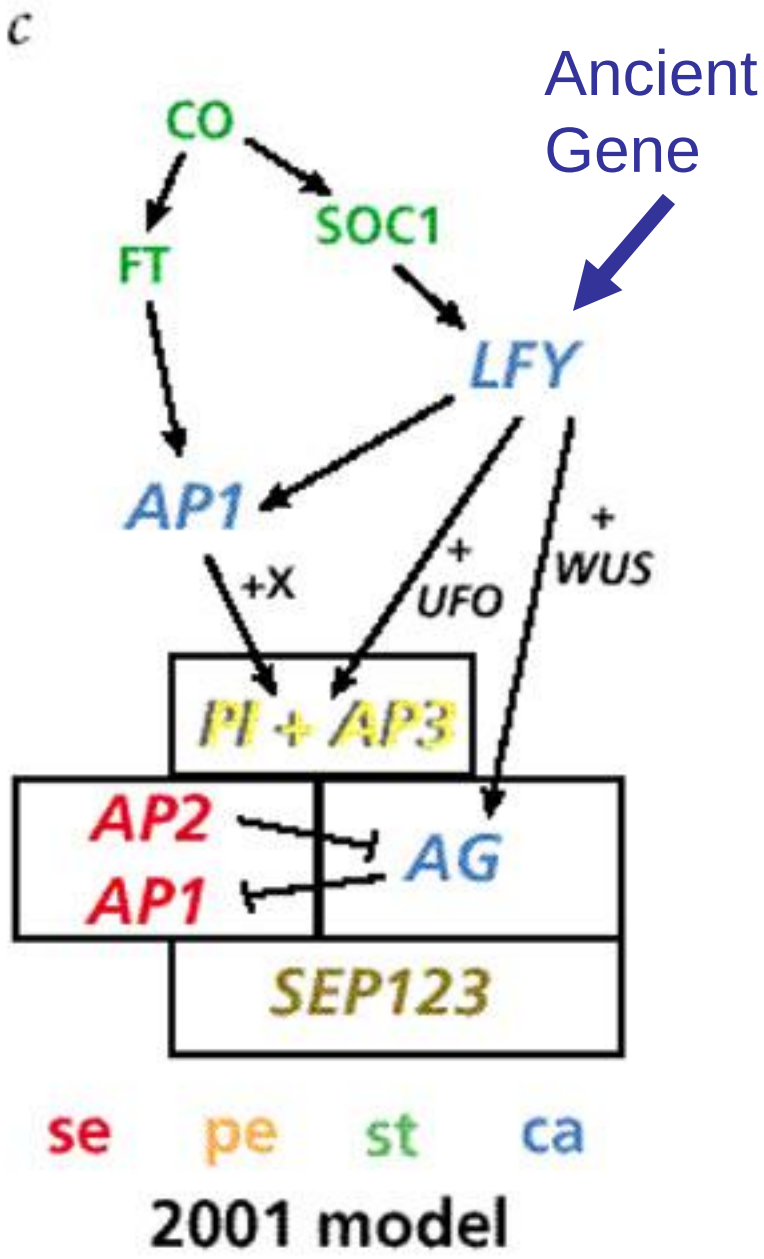
No Gametes

(Se-Pe-Pe-Se-Pe-Pe)

The *SEPALLATA* genes alone cannot direct flower development. If you turn on the *SEPALLATA* genes in vegetative plants you get petal-like structures where leaves should be, but none of the other flower parts were present. If you turn on the ABC genes without SEPALLATA genes, you also do not get a flower. So BOTH sets of genes are necessary for normal flower development.



Flower structure and the "quartet model" of floral organ specification in *Arabidopsis* (From Nature 409, 469 - 471 (2001)



ABC plus model

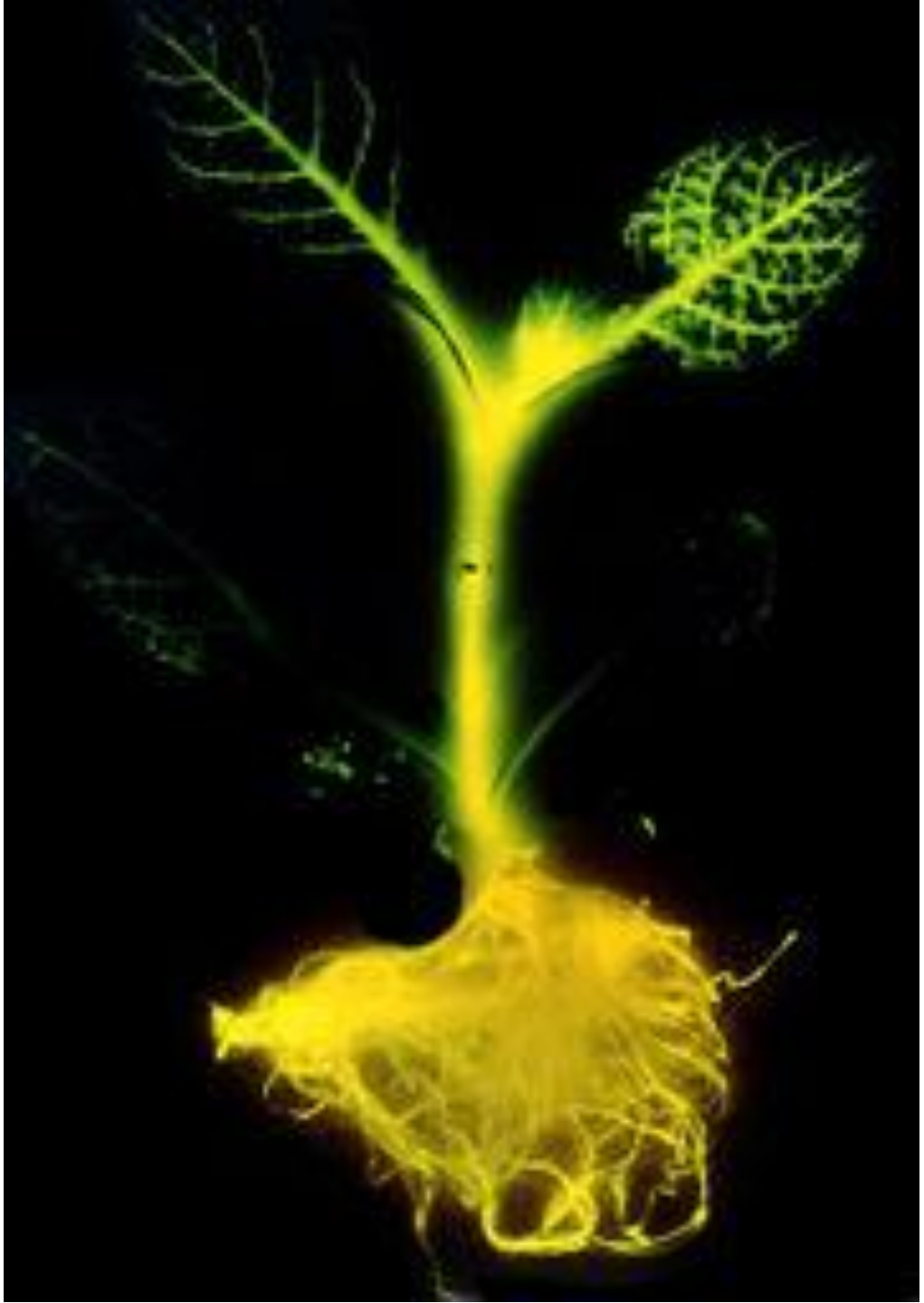
Note addition of *SEPALLATA* genes and others (green, blue and black).

Even though it is more complex-- a gene change here or there can have a profound effect on flower phenotype.

Transgenic Plants: Creation

Dr. Kamal Kumar Gupta

Associate Professor



Methods of production of Transgenic plants

Agroagbacterium tumifaciencs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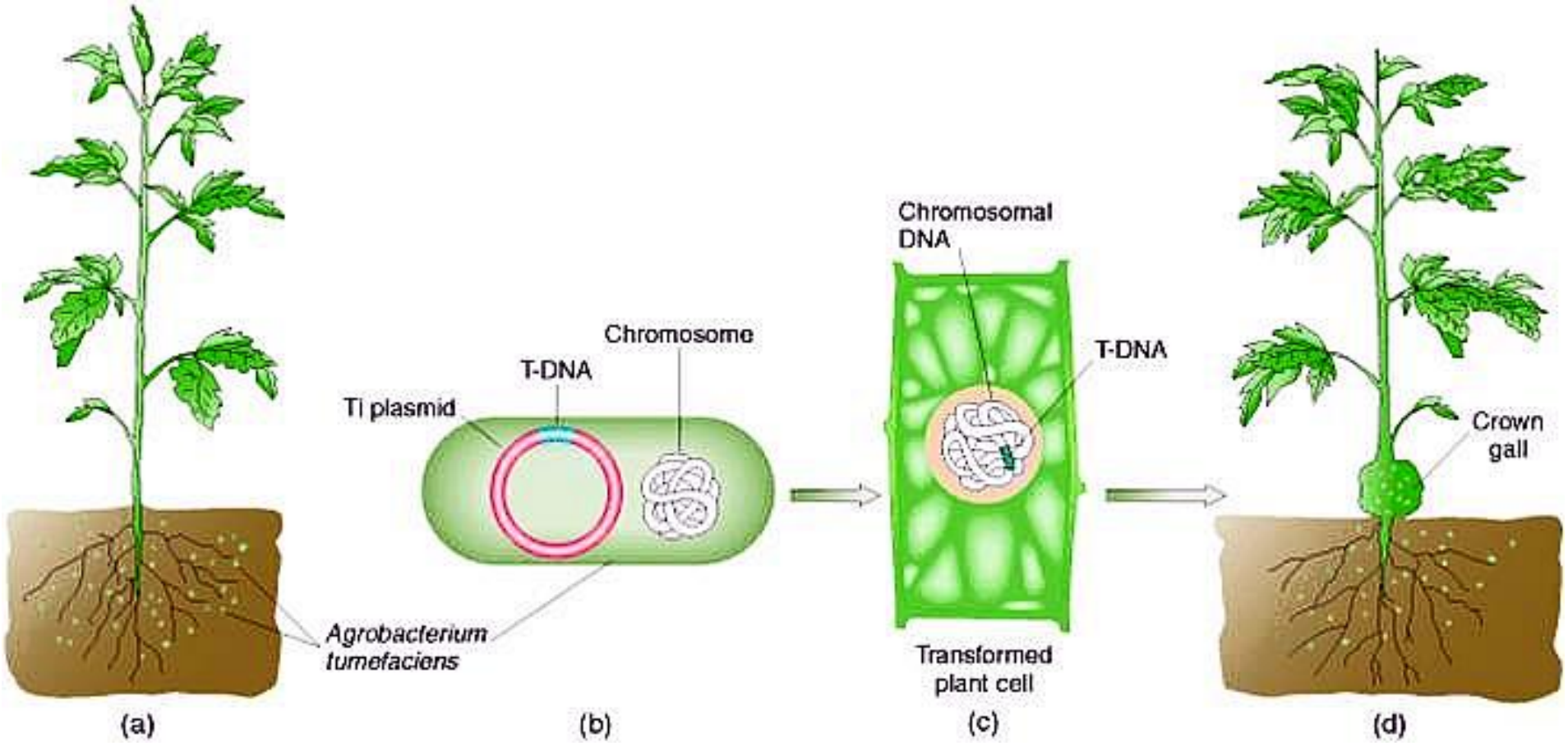
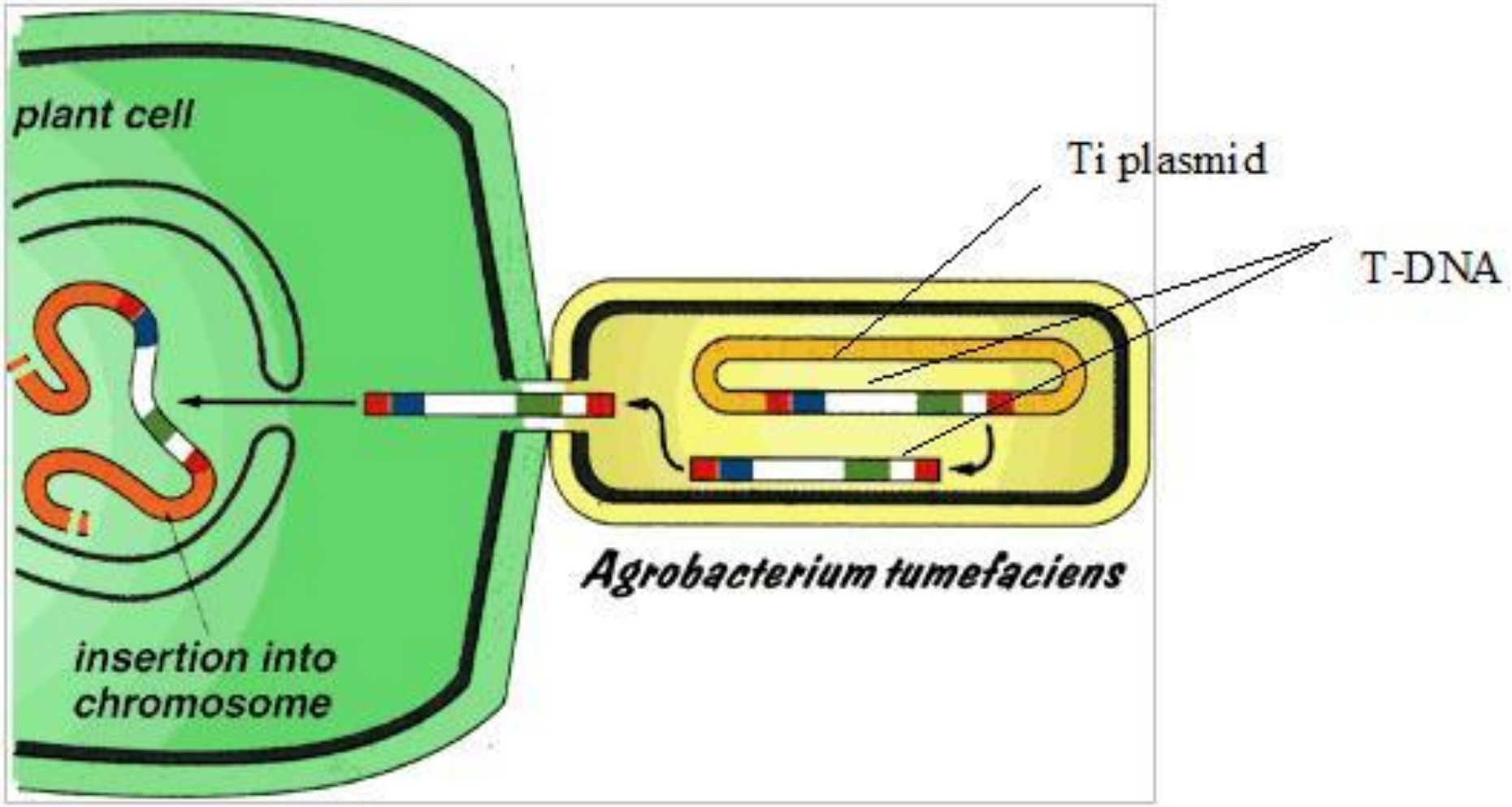
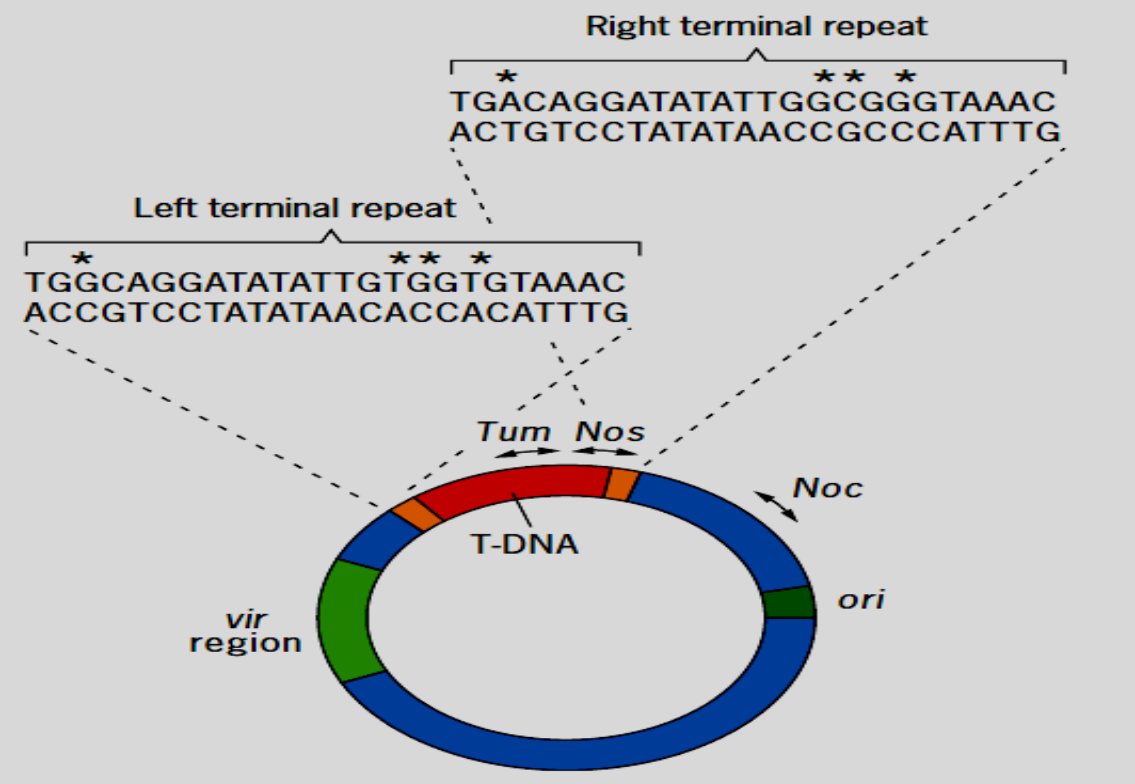
- The ability of *A. tumefaciens* to induce crown galls in plants is controlled by genetic information carried on **Ti plasmid (***t***umor-***i***nducing plasmid).**
- Ti plasmid has two components the **T-DNA** (*T*ransferred *DNA*) and
- the ***vir* region,** which are essential for the transformation of plant cells.
- During the transformation process, the T-DNA is excised from the Ti plasmid, transferred to a plant cell, and integrated into the DNA of the plant cell.
- The integration of the T-DNA occurs at random chromosomal sites. In some cases, multiple T-DNA integration events occur in the same cell.

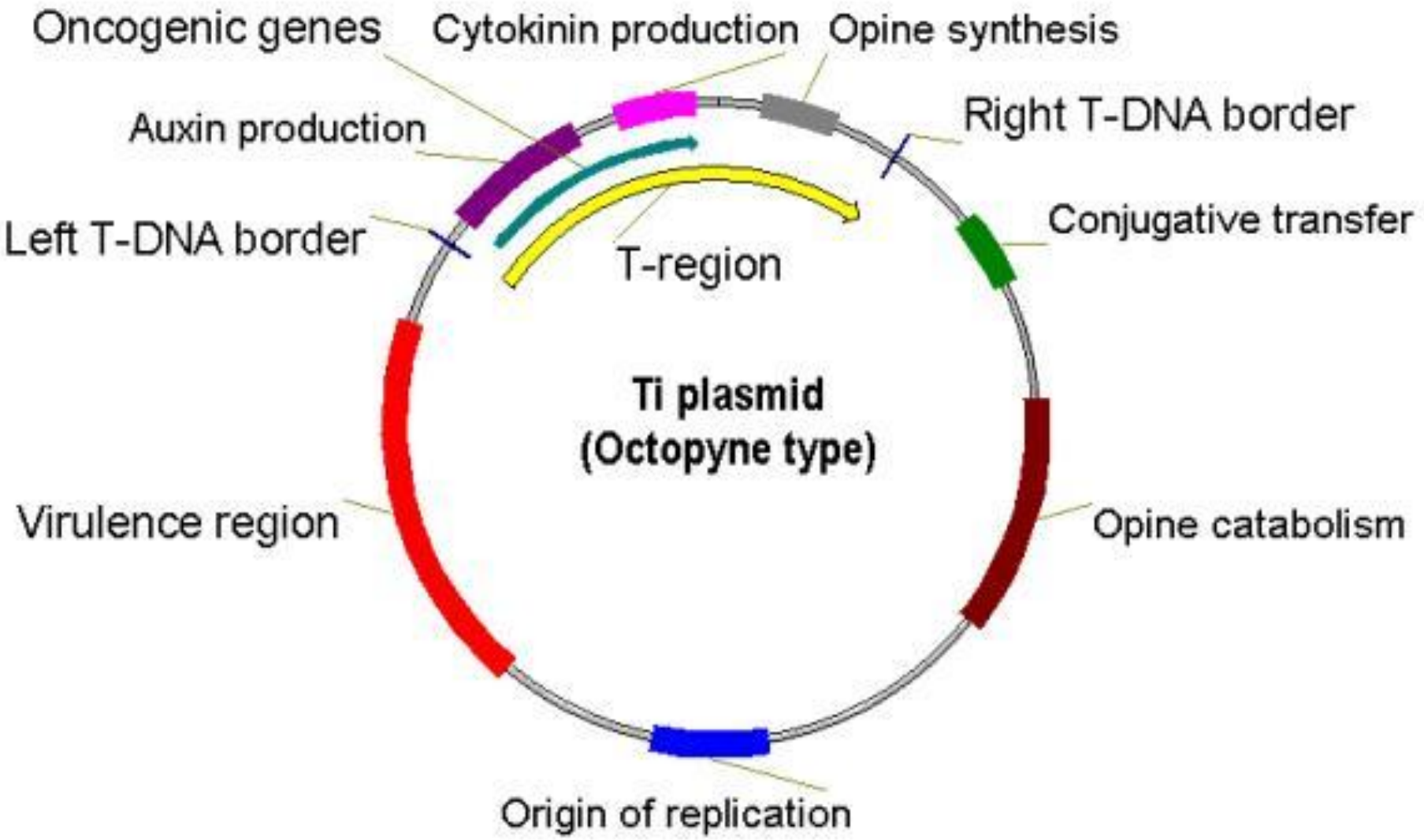

Chromosome
T-DNA
Ti plasmid
Chromosomal DNA
T-DNA
Crown gall
Agrobacterium tumefaciens
Transformed plant cell
(a)
(b)
(c)
(d)

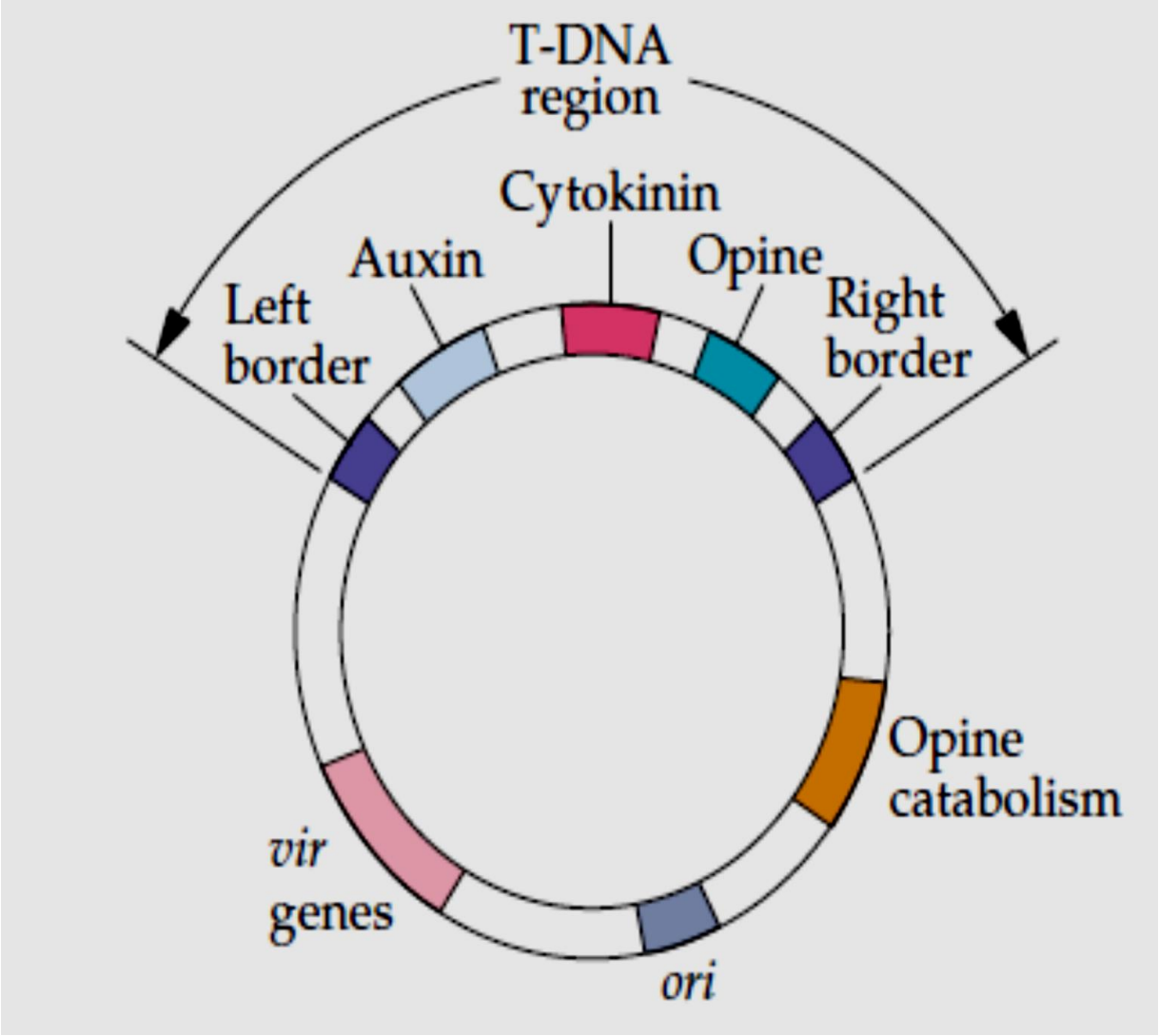
Structure of Ti Plasmid

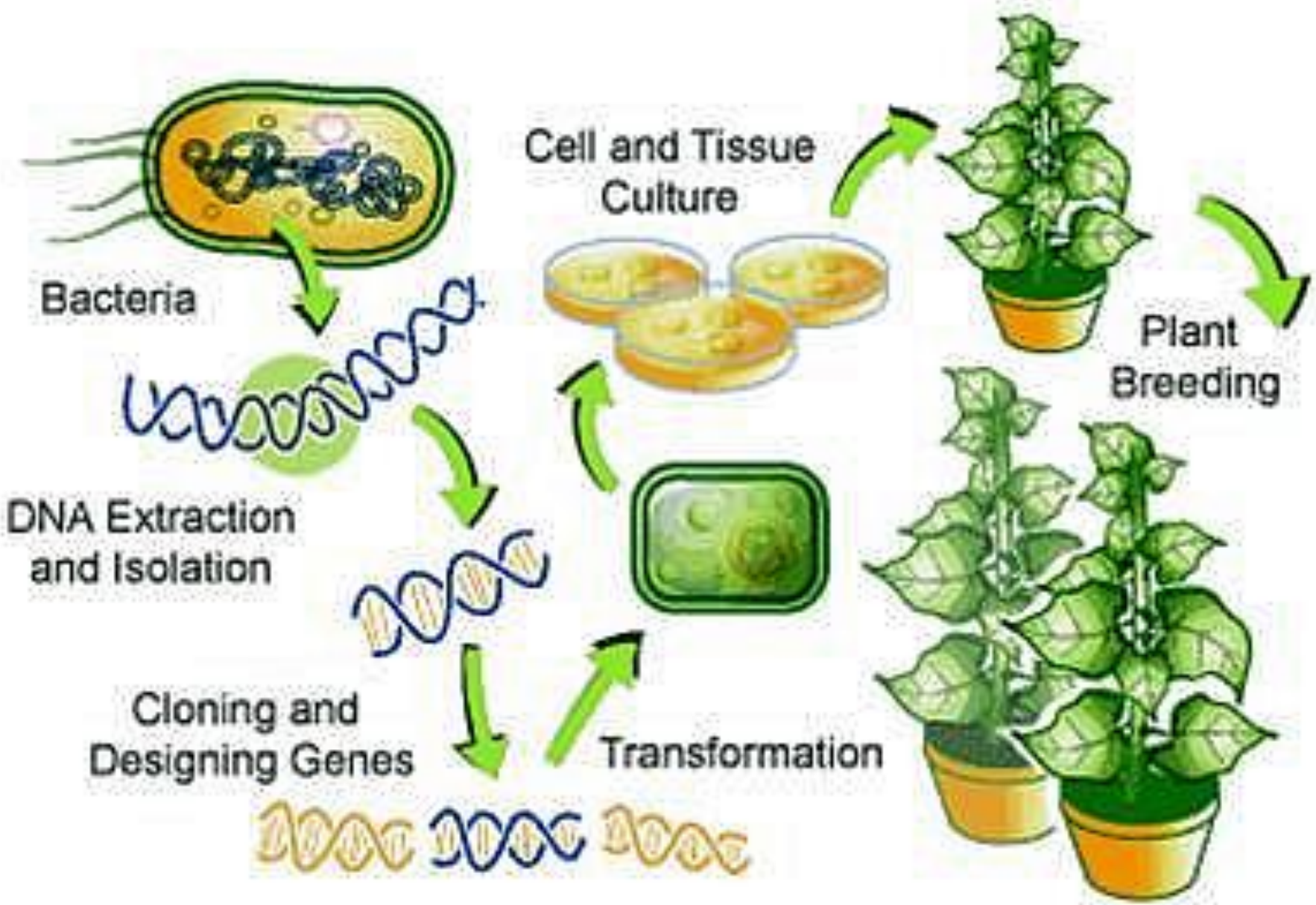


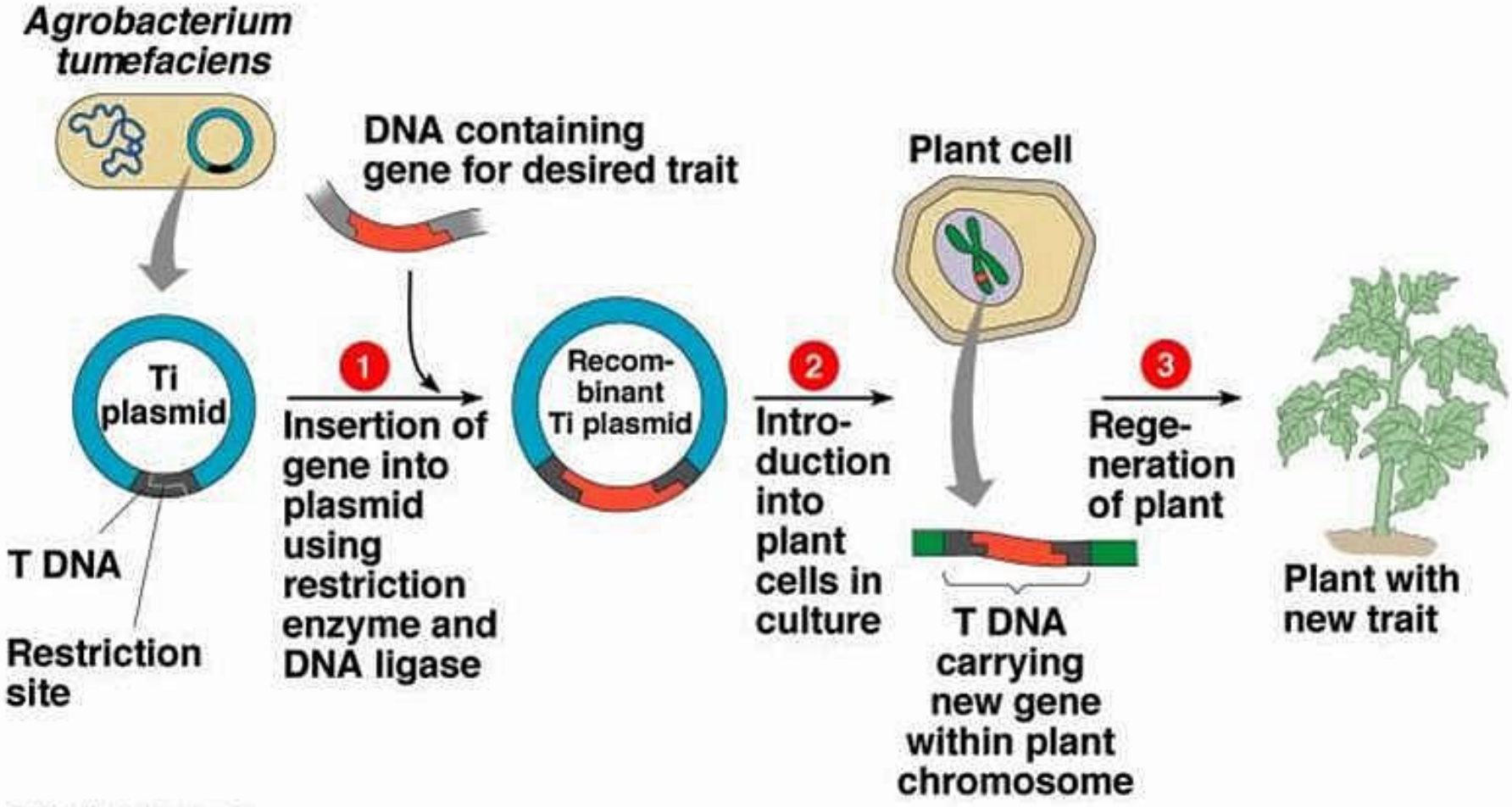
Structure of Ti Plasmid
The *vir* (virulence) region




Oncogenic genes
Cytokinin production
Opine synthesis
Auxin production
Right T-DNA border
Left T-DNA border
Conjugative transfer
T-region
Ti plasmid
(Octopyne type)
Virulence region
Opine catabolism
Origin of replication


T-DNA
region
Cytokinin
Auxin
Opine
Left
border
Right
border
Opine
catabolism
vir
genes
ori


Bacteria
DNA Extraction
and Isolation
Cloning and
Designing Genes
Transformation
Cell and Tissue
Culture
Plant
Breeding



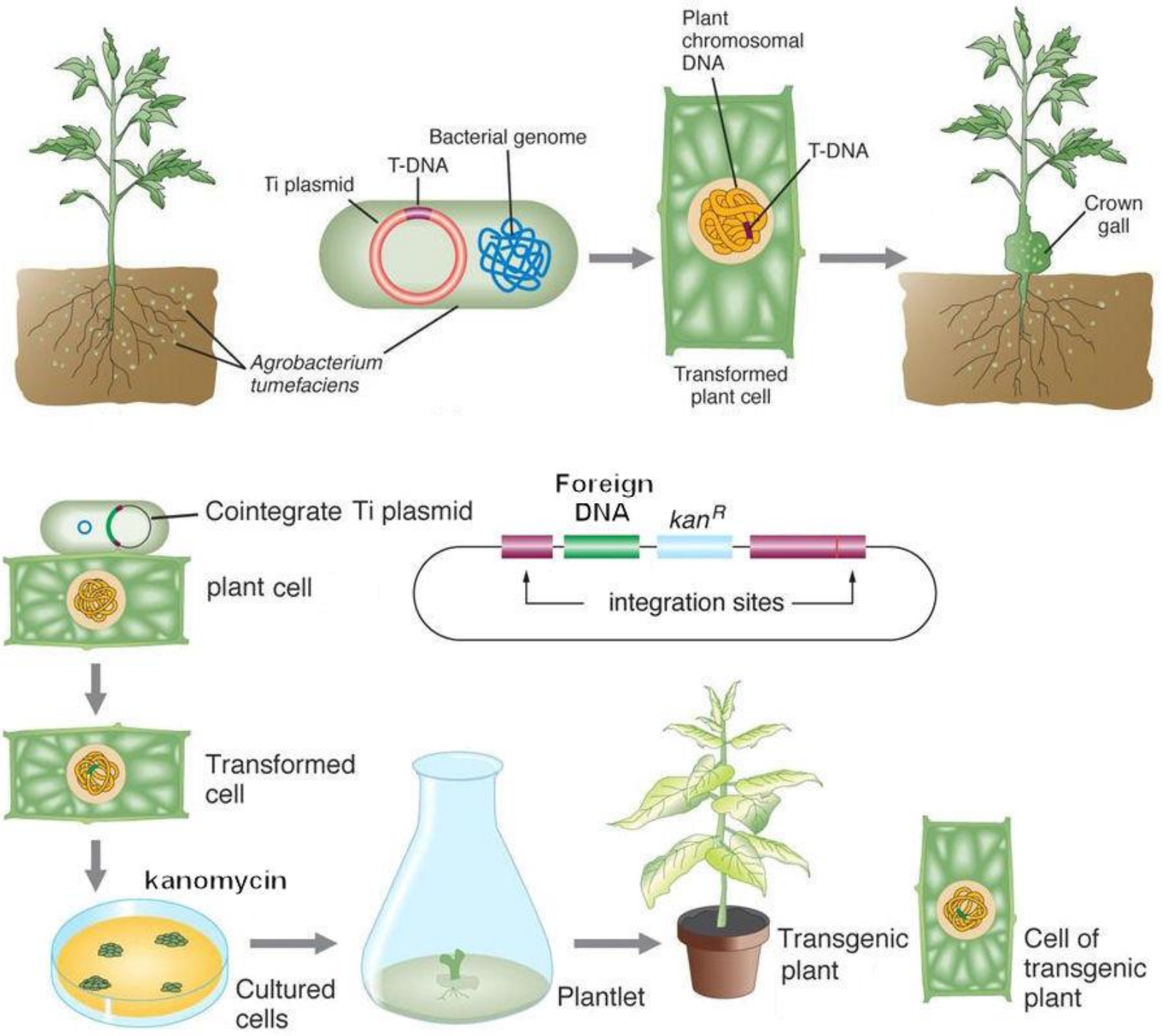
Ti plasmid vector for creating transgenic plants

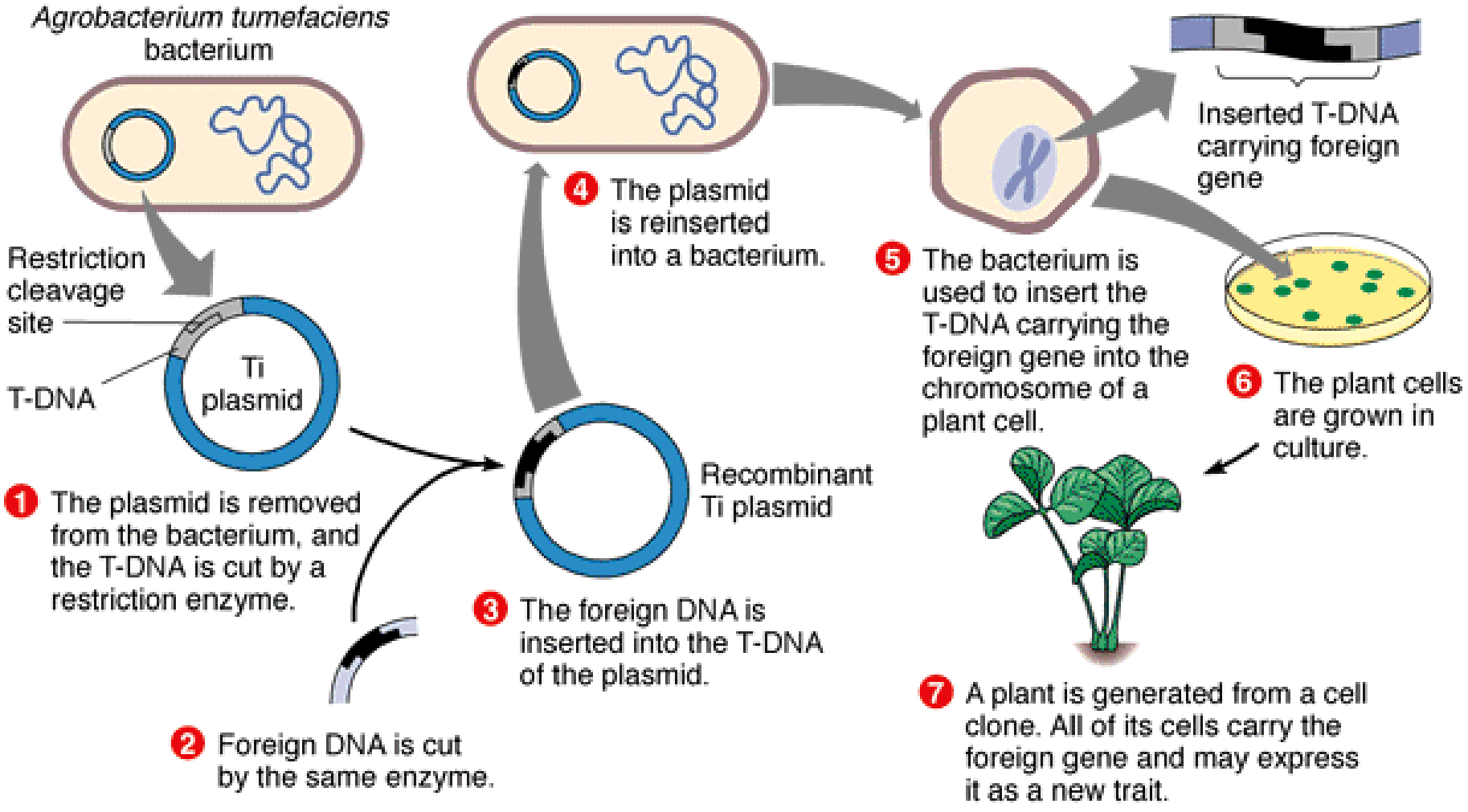
- Foreign genes could be inserted into the T-DNA and then transferred to the plant.
- In the modified Ti plasmid the genes responsible for tumor formation are deleted
- Selectable markeris added along with appropriate regulatory elements.

Ti plasmid vector for creating transgenic plants

- The *kan*^{r} gene from the *E. coli* transposon Tn*5* has been used extensively as a selectable marker.
- it encodes an enzyme called neomycin phosphotransferase type II (NPTII), it detoxify the kanamycin.
- The NPTII coding sequence are provided with a plant promoter and plant termination and polyadenylation signals.
- Such constructions with prokaryotic coding sequences flanked by eukaryotic regulatory sequences are called **chimeric selectable marker genes.**

- One widely used chimeric selectable marker gene contains the cauliflower mosaic virus (CaMV) 35S promoter, the NPTII coding sequence, and the Ti nopaline synthase (*nos*) termination sequence; this chimeric gene is usually symbolized 35S/NPTII/*nos*.
- The Ti vectors used to transfer genes into plants have the tumor-inducing genes of the plasmid replaced with a chimeric selectable marker gene such as 35S/NPTII/*nos*.


Plant chromosomal DNA
Bacterial genome
T-DNA
Ti plasmid
T-DNA
Crown gall
Agrobacterium tumefaciens
Transformed plant cell
Cointegrate Ti plasmid
plant cell
Foreign DNA
kanR
integration sites
Transformed cell
kanomycin
Cultured cells
Plantlet
Transgenic plant
Cell of transgenic plant



Limitations as routine Ti plasmid vectors

- The production of phytohormones by transformed cells prevents them from being regenerated into mature plants.
- A gene encoding opine synthesis is not useful to a transgenic plant and may lower the final plant yield
- Ti plasmids are large (approximately 200 to 800 kb).
- Ti plasmid does not replicate in *Escherichia coli,* therefore it cannot be cloned in *E. coli*.
- Transfer of the T-DNA, which begins from the right border, does not always end at the left border. Rather, vector DNA sequences past the left border are often transferred.

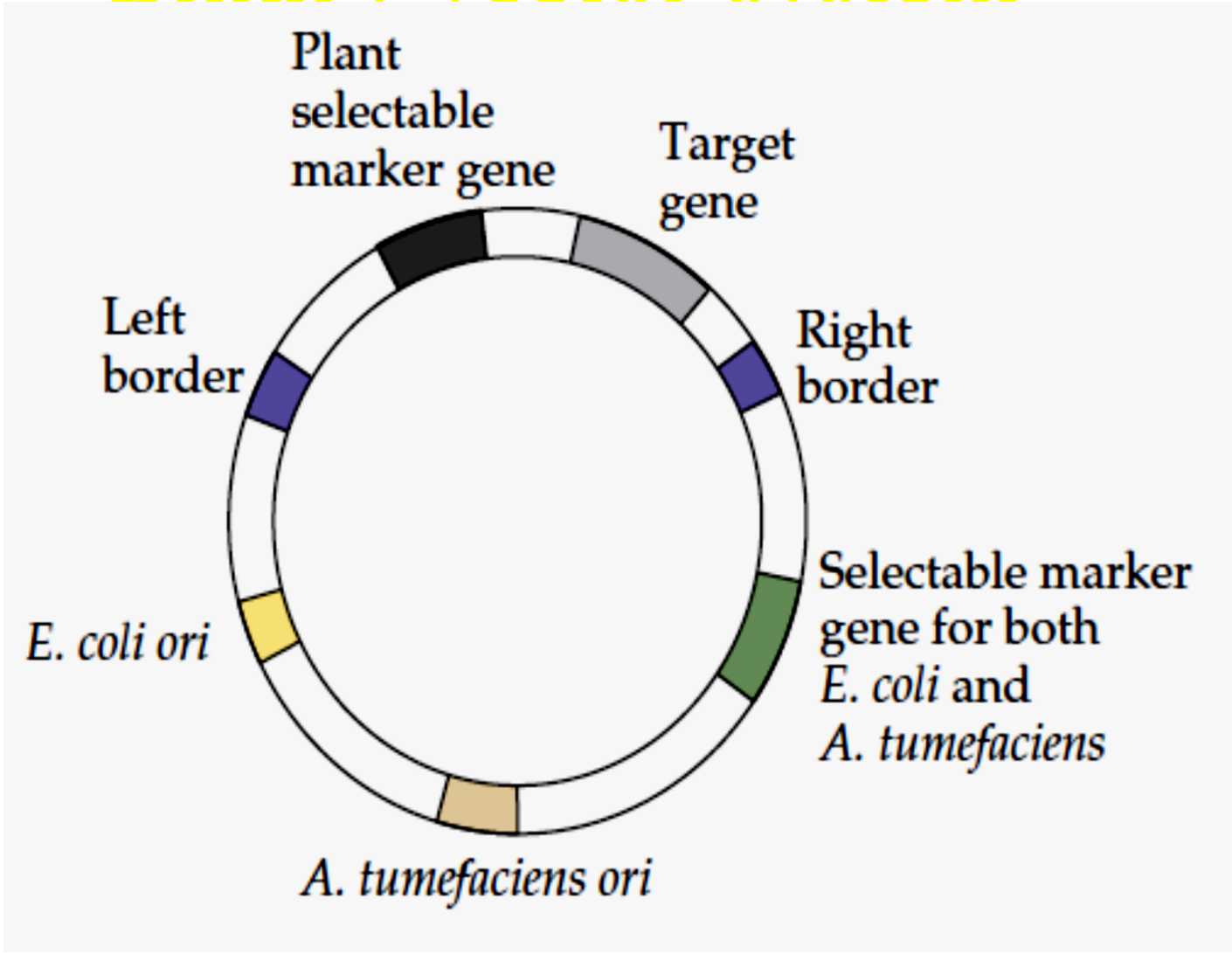
Ti plasmid-based vectors: Components

- A selectable marker gene, such as neomycin phosphotransferase, put under the control of plant (eukaryotic) transcriptional regulation signals, including both a promoter and a termination–polyadenylation sequence.
- An origin of DNA replication that allows the plasmid to replicate in *E. coli* .
- The right border sequence of the T-DNA region. Most cloning vectors include both a right and a left border sequence.
- A polylinker to facilitate insertion of the cloned gene into the region between T-DNA border sequences.
- A “killer” gene encoding a toxin downstream from the left border to prevent unwanted vector DNA past the left border from being incorporated into transgenic plants. If this incorporation occurs, and the killer gene is present, the transformed cells will not survive.

Binary vector system

- The binary cloning vector contains either the *E. coli* and *A. tumefaciens* origins of DNA replication or a single broadhost range origin of DNA replication.
- All the cloning steps are carried out in *E. coli* before the vector is introduced into *A. tumefaciens*.
- The recipient *A. tumefaciens* strain carries a modified (defective or disarmed) Ti plasmid that contains a complete set of *vir* genes but lacks the T-DNA region, so that this T-DNA cannot be transferred.
- With this system, the defective Ti plasmid synthesizes the *vir* gene products and acts as a helper plasmid.
- This enables the T-DNA from the binary cloning vector to be inserted into the plant chromosomal DNA.
- Since transfer of the T-DNA is initiated from the right border, the selectable marker, is usually placed next to the left border.
- A few binary vectors have been designed to include two plant selectable markers, one adjacent to the right border and the other adjacent to the left border.

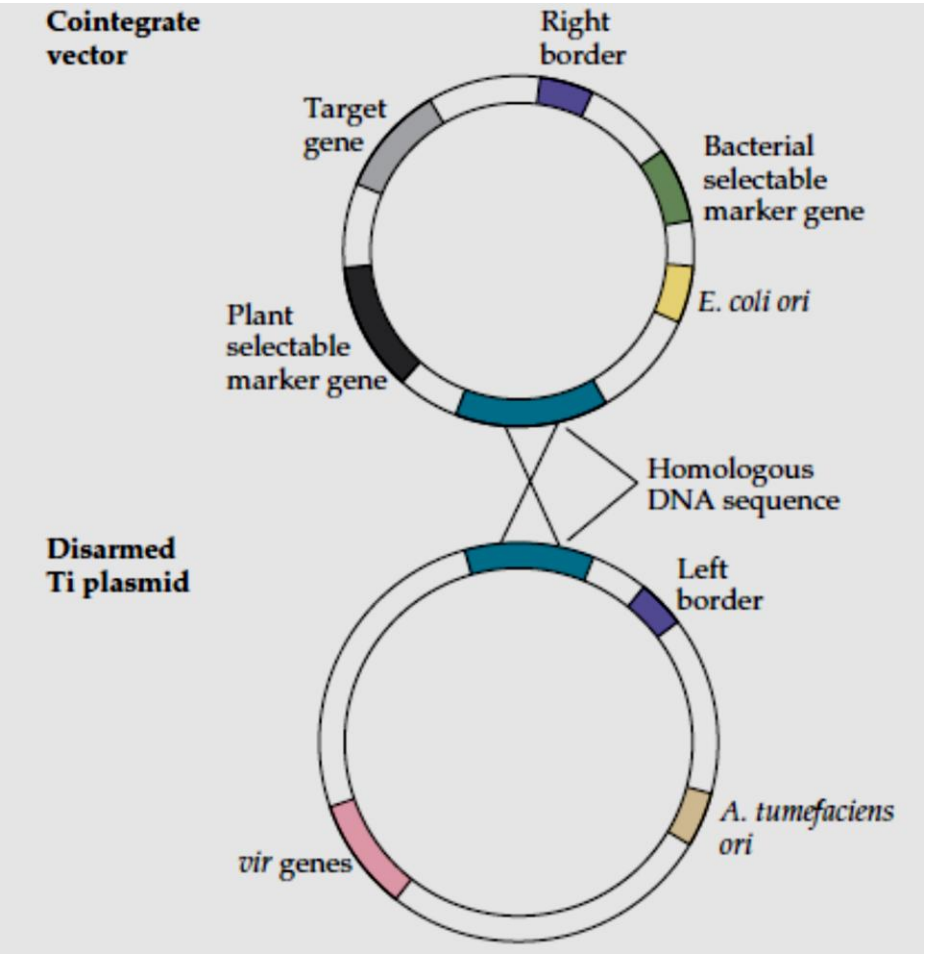
Binary vector system

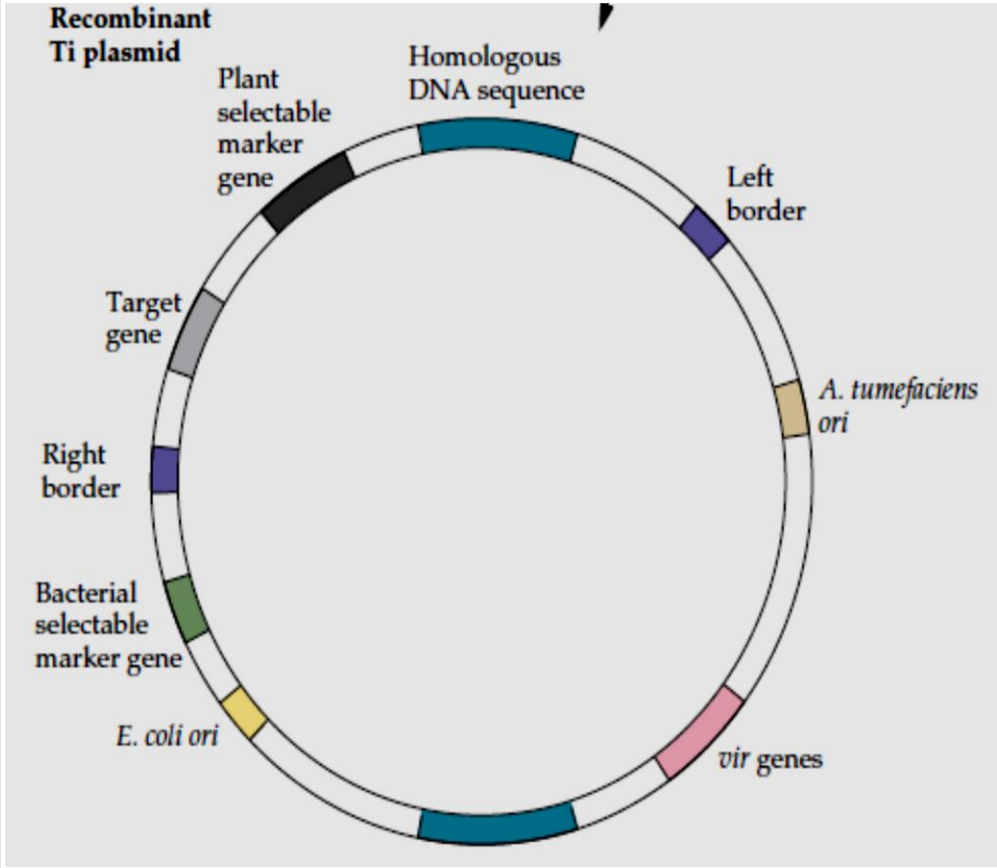


Cointegrate vector system

- The cointegrate vector has a plant selectable marker gene, the target gene, the right border, an *E. coli* origin of DNA replication, and a bacterial selectable marker gene.
- The cointegrate vector recombines with a modified (disarmed) Ti plasmid that lacks both the tumor-producing genes and the right border of the T-DNA within *A. tumefaciens*.
- The entire cloning vector becomes integrated into the disarmed Ti plasmid to form a recombinant Ti plasmid

Cointegrate vector system

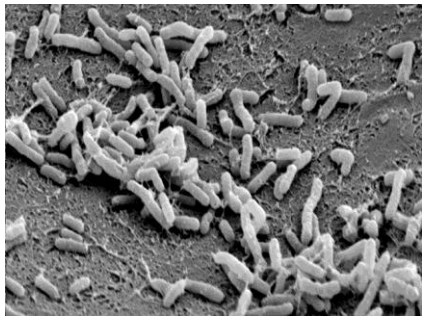




Crown gall tumors

Tumor

Agrobacterium tumefaciens



1907: Smith and Townsend demonstrated that *Agrobacterium tumefaciens* causes crown gall tumors.

Later, causative agent of hairy root disease was determined to be *Agrobacterium rhizogenes*.

Agrobacterium genetically engineers plant cells?

1958: Arnim Braun (Rockfeller Univ) showed that tumors could be excised and propagated on in vitro culture media without addition of plant hormones. It was also demonstrated that tumor could propagate even when the bacteria was removed from the tumor. He proposed that the bacteria is able to transfer "Tumor Inducing Principle" (TIP) into plant cells.

1968: Georges Morel (France) found that the tumors released compounds (opines) that *Agrobacteria* use as nutrients. Therefore, he proposed that (a) bacteria transfer opine synthesis genes into plant cell, and (2) the synthesized opine is transported back into the bacterial cell.

This suggests that *Agrobacteria* are genetic engineer!!! Unacceptable to the scientific community at that time.

TIP is a plasmid

1971

Hamilton and Fall (University of Pennsylvania) reported that a virulent strain of *Agrobacterium*, when grown at 37^{o}C lost virulence irreversibly.

Allen Kerr (Adelaide, Australia) co-inoculated “avirulent” and “virulent” strains on sunflower, and re-isolated the “avirulent” strain. He found that the “avirulent” strain had become virulent.

These results indicate that the TIP in *Agrobactrium* probably resides on a plasmid, which can be transferred between bacterial strains by conjugation.

1974: Ivo Zaenan (University of Ghent) isolated the megaplasmids of *Agrobacterium*. He called them Ti plasmids, which was later proven to be the TIP.

Agrobacterium tumefaciens were classified on the basis of the opine they catabolized such as octopine, nopaline, agropine, mannopine etc. Later it was found that such classification is faulty because most strains catabolize more than one type of opine.

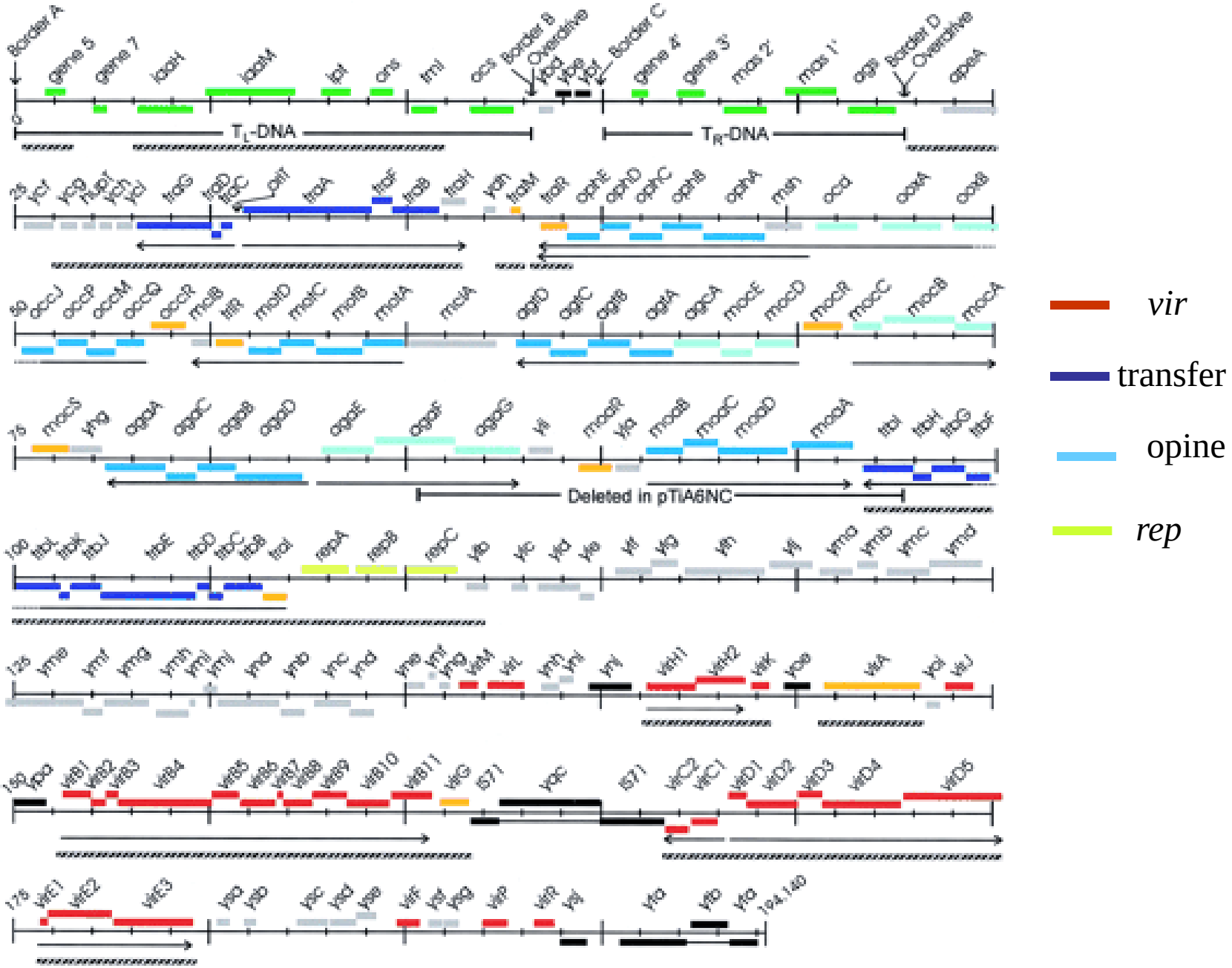
Ti plasmids of A6 strain, pTiA6, pTiAch, pTiB6S3 are referred to as octopine Ti plasmids. Ti plasmid of C58 strain (pTiC58) is a nopaline Ti plasmid.

Features of Ti plasmid

About 200 kb. 155 Open Reading Frames (ORFs).

Contains following 5 components:

1. T region (T-DNA), which codes for sequences that are transferred to plant cell.
2. The *vir* region that directs the processing and transfer of T-DNA.
3. The *rep* region that is required for the replication of Ti plasmid in bacterial cell.
4. The *tra* and *trb* loci, which direct the conjugal transfer of Ti plasmid between two bacterial cells.
5. Genes that direct the uptake and catabolism of opines.



Operons in an Octopine Ti plasmid

T-DNA Borders

Right: 5’- GXXTGXCAGGATATATXXXXXXGTXAXX-3’

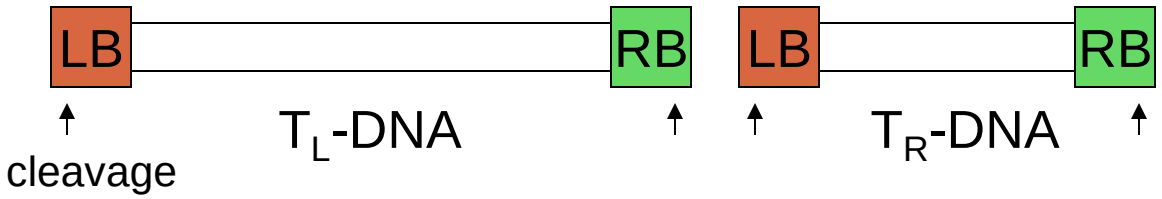
Left: 5’- XGGTGGCAGGATATATXXXXXTGTAAAX-3’

Overdrive is present in Octopine Ti Plasmids

During infection, A. t. carrying an octopine-type Ti plasmid transfers two fragments of DNA to plant cell. These fragments are designated as T_L-DNA and T_R-DNA and are 13 and 7.8 kb long, respectively. A nopaline type Ti plasmid transfers a single DNA fragment (T-DNA) that is about 20 kb long.

T_L-DNA and T_R-DNA or T-DNA is each flanked by cis-acting 25 bp direct repeats called **border sequences** (LB and RB or A, B, C and D). The left border is dispensable for T-DNA transfer but right border is essential and acts in polar fashion.

Octopine Ti plasmids often contain near RB of T_L-DNA another *cis*-acting element called **overdrive**, which is required for wild-type transfer efficiency and provides a binding site for a Vir protein called VirC1. Another possible overdrive element is present near RB of T_R-DNA but its role is unknown.



In the presence of Vir proteins, T-region undergoes following processing steps:

1. Each border is cleaved exactly 4 nt from its left end catalyzed by VirD2 protein, which remains covalently bound to the 5’ end of each cleaved strand.

2. Bottom strands are recovered as single-stranded (ss) form, referred to as T strand.

Ti plasmid (octopine type) encoded proteins required for T-DNA processing and transfer (*vir* genes)

vir operons: *virA, -B, -C, -D, -E, -G.*
vir F and *vir H*

Vir D1 and D2:

D2 is a site-specific endonuclease.

Some reports indicate that D1 probably contains topoisomerase I activity, other contradict it.

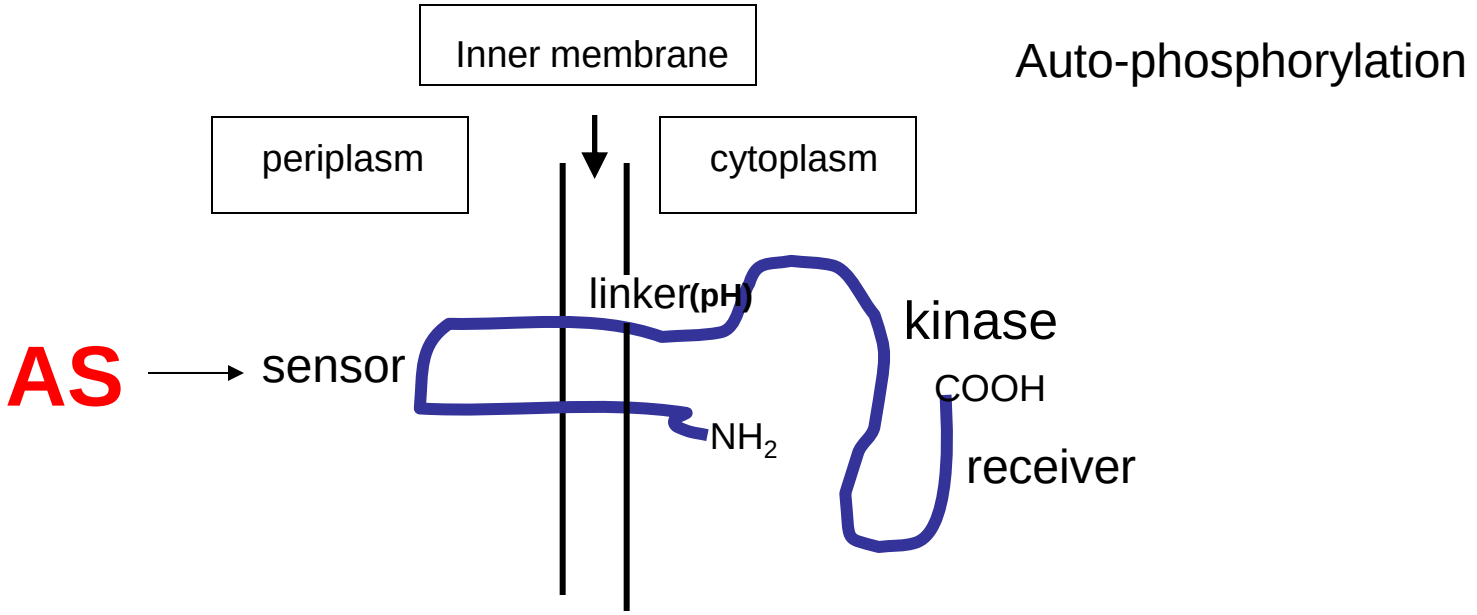
However, both D1 and D2 are required for nicking borders on a supercoiled or relaxed double stranded DNA. Whereas D2 can cleave border sequence on a single stranded DNA without the help of D1. This suggests that D1 could be involved in ripping ds DNA into ss form for D2 to act upon the ss border sequences.

Induction of Virulence Function (initiation of T-DNA transfer)

Virulence functions are transcriptionally regulated by 2 component gene regulatory system belonging to a large family of bacterial chemosensors that respond to the chemical environment. Optimal *vir* gene induction occurs at acidic pH and in the presence of phenolic inducers such as acetosyringone (AS) that are released by wounded plant cells. The *vir* gene regulatory system operates through two monocistronic virulence genes: *vir A* and *vir G.*

Vir A

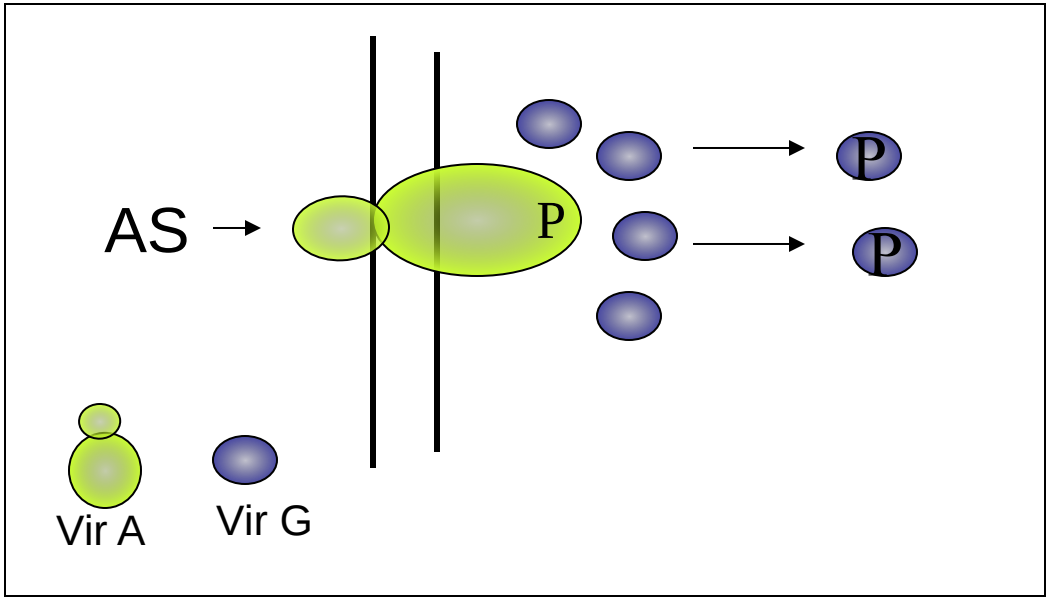
vir A gene is constitutively expressed. Vir A protein is located in the inner membrane and responds to the chemical environment [acidic pH and acetosyringone (AS)]. In the presence of the stimulants, it is auto-phosphorylated.



Linker responds to pH and interacts with ChvE (a sugar-binding protein encoded by *Agrobacterium* genome). At sub-optimal AS levels, VirA can be further stimulated by sugars, opines or amino acids.

Vir G

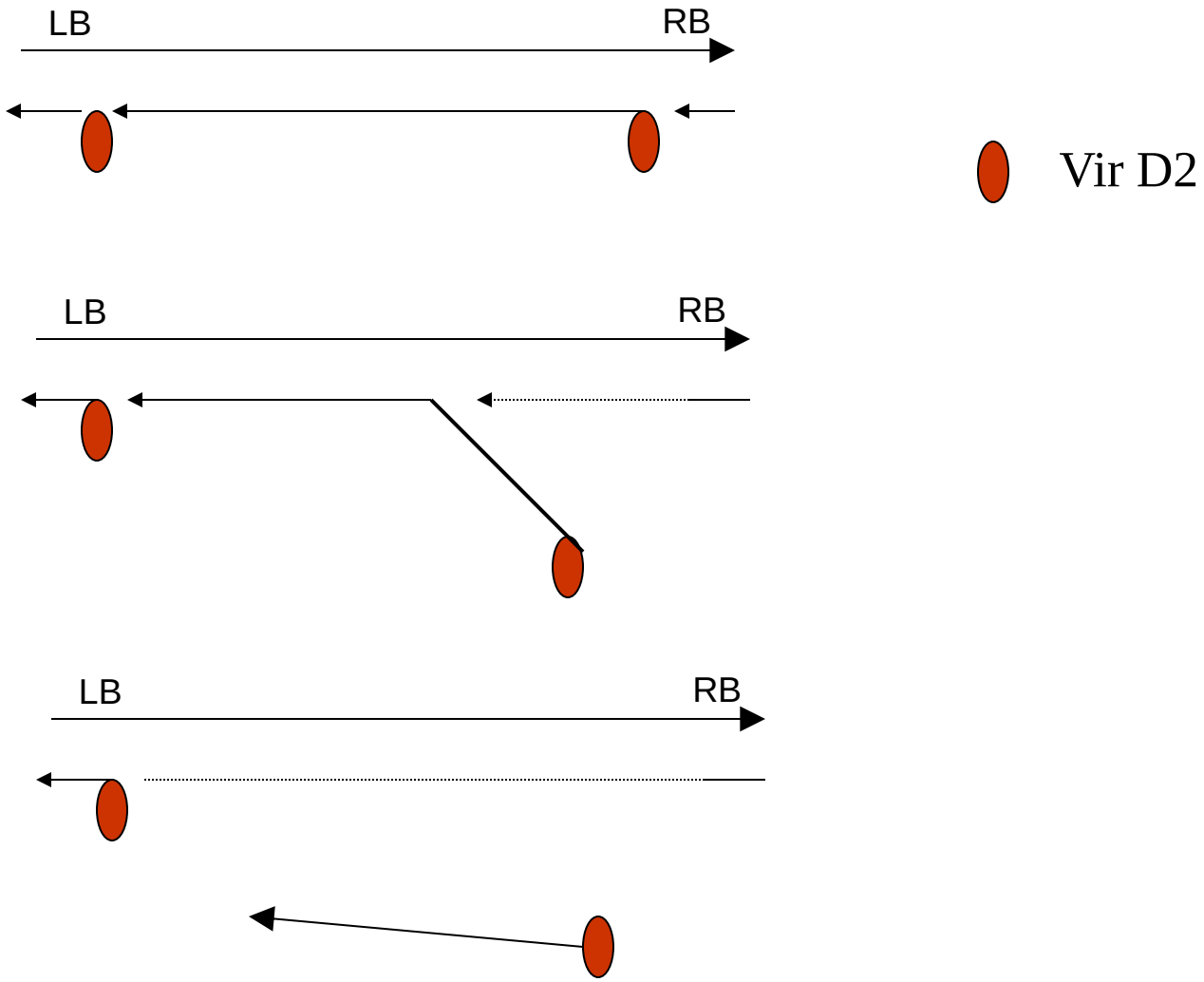
vir G gene is also constitutively expressed. Vir G protein is freely available in the cytoplasm. The activated (phosphorylated) Vir A in turn phosphorylates Vir G protein at aspartic acid residue 52. Phosphorylated Vir G becomes the transcriptional activator of the remaining *vir* genes. Promoters of *vir* genes possess one of more "*vir* box" of 12 bp sequence.



A mutant that expresses its *vir* genes constitutively, contains a *vir G* mutation called *virG*-N54D. This mutation leads to a conformation of protein that is similar to phosphorylated Vir G.

T-DNA Processing

Vir D1, D2



Vir C1 and C2

C1 mutants display lower virulence

1. C1 binds to the overdrive site.
2. C2 function is unknown.
3. It is not clear exactly how binding of C1 on overdrive helps increase the efficiency of T-strand transfer to plants.
4. Overdrive is absent in nopaline type Ti plasmid.

Vir H or *pinF*

Non-essential. May be involved in detoxification of plant phenolics. VirH exhibits sequence homology with cytochrome P450 like gene. Cytochrome P450 enzymes catalyze NADH-dependent oxidation of aromatic substrates.

Vir F

Host range factor. Possible interaction with Skp1 proteins to regulate plant cell division cycle.

Vir J

Putative T-strand binding protein. May have a role in T-strand export from *Agrobacterium.*

Attachment of *Agrobacterium* to plant cell

This is a polar two step process:

1. Mediated by cell associated acetylated, acidic polysaccharides encoded by attR locus. attR mutants are avirulent. This step is reversible because sheer forces are sufficient to dislodge bacteria.
2. Involves formation of cellulose fibrils by bacterium, which enmeshes large number of bacteria at the wound site.
3. chvA, chvB and pscA genes are involved in synthesis, processing and export of cyclic beta 1,2 glucans and other sugars, that may be involved indirectly in bacterial attachment.

Plant proteins???

RAT1 encodes an arabinogalactan protein (AGP). When AGP is blocked by Yariv reagent, transformation is blocked.

RAT4 encodes cellulose synthase like gene

Bacterial attachment

Attachment of bacteria to plant cell is a prerequisite for DNA transfer. While many genes involved in attachment process (*att* genes) have been elucidated, the mechanism of this intriguing process is not fully understood. A 20-kb block of *att* genes located on bacterial chromosome is involved in attachment.

attA1 and *attH* gene products are probably secreted because attA1 and attH mutation can be complemented by conditioned media (in which plant cell and *Agrobacteria* were growing).

attR gene product is involved in the production of acidic polysaccharides.

After initial attachment, *Agrobacterium* produces a network of cellulose fibrils that bind the bacterium with the plant cell tightly and entraps other *Agrobacterium* that are not yet attached. Cellulose production is important for efficiency of transformation but not absolutely necessary.

Chromosomal virulence genes [*chv*A (beta-1,2-glucan), *chv*B (transport protein), *psc*A] are also involved in the attachment process.

Bacterial attachment

Chromosomal virulence genes [*chv*A, *chv*B, *psc*A] are also involved in the attachment process.

ChvB is a 235 kDa protein involved in the formation of cyclic β-1,2-glucan.

ChvA is a transport protein located in the inner membrane, necessary for the transport of β-1,2-glucan into periplasm.

PscA is also involved in the production of β-1,2-glucan.

T-DNA Transfer To The Plant Cell

Until recently the following two topics were among the most debated topics on T-DNA transfer.

1. Whether T-DNA enters plant cell as ss or ds molecule? Two recent studies categorically demonstrated that T-DNA is ss molecule.
2. Whether T-DNA travels to the plant cell alone or as a complex with VirE2 protein? Some recent studies have elegantly demonstrated that VirE2 is transported into plant nucleus separately.

T-DNA Transfer Apparatus

Encoded by *virB* operons (11 genes). Each virB except virB1 is essential for tumorogenesis. All 10 VirB proteins have been localized to the inner or outer membrane and most appear either to be integral membrane protein or to be exported from cytoplasm.

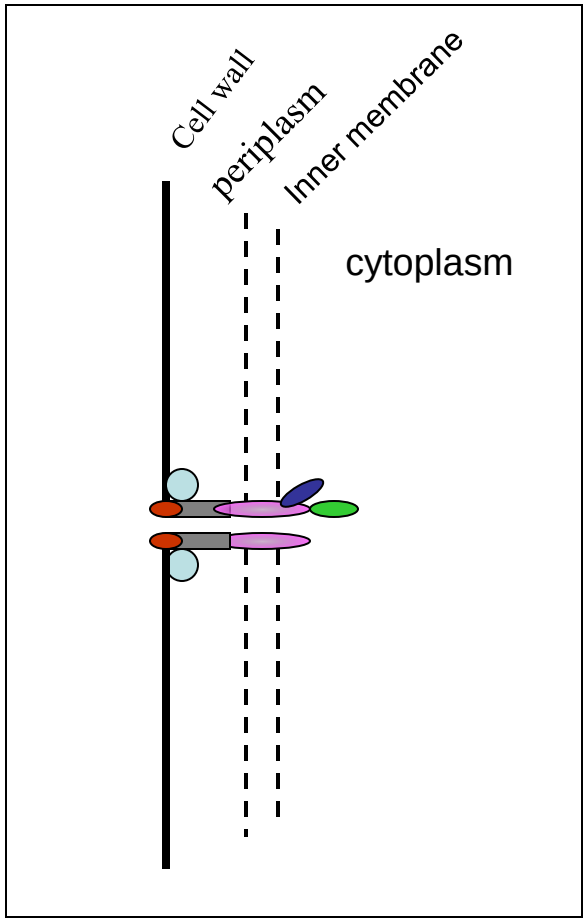
VirB1 possesses sequence motifs found in transglycosylase and eukaryotic lysozyme, suggesting a role in localized digestion of the peptidoglygan.

VirB4 and **VirB11** are peripherally bound to others and located primarily in cytoplasm and they contain ATPase activity. Therefore, they may be involved in providing energy for the export of other protein subunits, for T-strand transport or both.

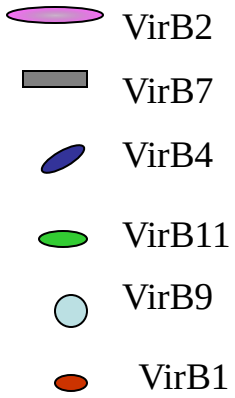
VirB proteins constitute a pilus that resembles conjugative pilus and **VirB2** is the major subunit of this pilus.

VirB7 may help anchor this pilus to the bacterial cell as it is an outer membrane lipoprotein that forms disulfide bonds with the periplasmically localized **VirB9**.

T-DNA Transfer Apparatus: pilus or pore



VirB pore



Other VirB proteins [B3, B5, B6, B8, B10] are minor constituents of the pore.

Which Vir Proteins accompany the T-strand into the plant cell?

Candidates

1. VirD2

2. VirE2 can bind to ss DNA *in vitro*

3. The AcvB protein shares homology with VirJ, which is a ss DNA binding protein. Nopaline strains carrying acvB mutation are avirulent. However, octopine strains are not, probably because this mutation is compensated by virJ gene (which is absent in nopaline strains)

3. Vir F???

virE genes

Both virE1 and E2 are essential for tumorogenesis i.e. E1 or E2 mutants are avirulent.

1. VirE2 is ss binding protein and contains nuclear localization signal.
2. *virE2* mutation can be complemented extra-cellularly.
3. Extracellular complementation is dependent on *virB* function.
4. VirE1 is required for transfer of VirE2 but not of T-strand i.e. VirE1 is an export chaperone for E2.
5. *virE2* mutant can be complemented by expressing VirE2 protein in plant cell, suggesting that VirE2 protein is required in plant cell.

VirE3:

Transferred from *Agrobacteria* to plant cell, where it interacts with E2 and facilitates its nuclear import. E2 can interact with VIP1 (a plant protein) for nuclear import. Since VIP1 is a rare protein, *Agrobacteria* has probably evolved its own counterpart of VIP1, E3, to carry out nuclear transport of E2.

Whether VirE2 travels to the plant cell alone or as a complex with T-DNA?

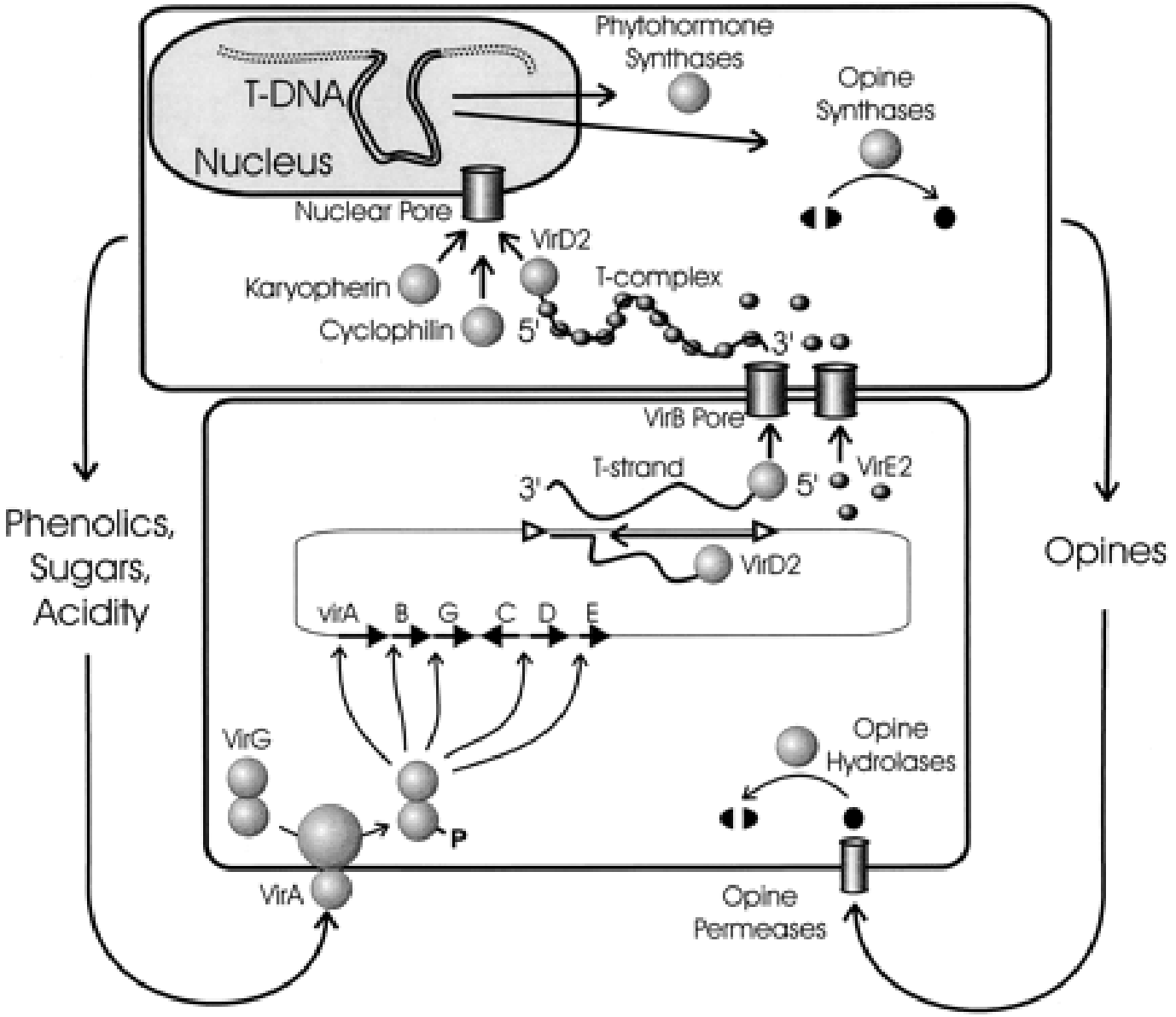
Argument 1: VirE2 should bind T-DNA in bacterial cell because

1. It is a strong ss binding molecule and present when T-DNA is generated.
2. Experimental evidence: induced agrobacteria produce some VirE2 coated T-strands (immunoprecipitation studies).

Argument 2: VirE2 and T-DNA are exported independently into plant cell where E2 binds with T-DNA to prevent nucleolytic attacks.

1. VirE1, the chaperone of E2 prevents the binding of E2 with T-DNA.
2. virE2 mutation can be complemented extra-cellularly.
   a. by VirE2 protein provided by another strain.
   b. by plant cell expressing *virE2* gene.

Current model of T-DNA transfer



Zupan et al. Plant J. 2000 23(1):11-28

Nuclear targeting of T-DNA in plant cells

VirD2 and VirE2 are bound to the T-DNA in plant cell. One or both of these proteins must be responsible for nuclear targeting of T-DNA.

VirD2 and VirE2 contain plant-active nuclear localization signal (NLS) sequence.

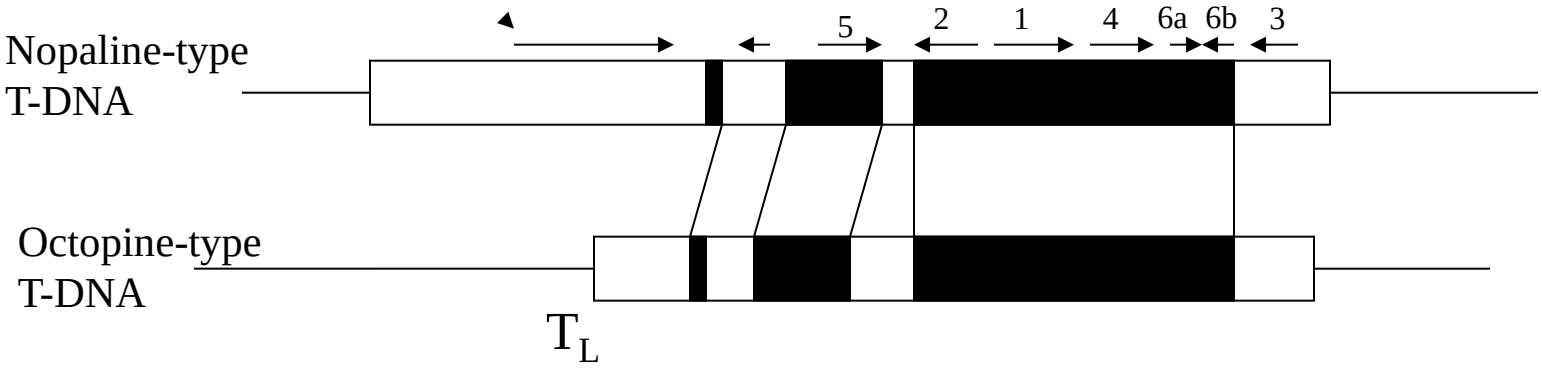
D2 contains two NLSs: one at N-terminal and the other at C-terminal. The latter is probably responsible for targeting.

E2 contains two separate NLS regions. ssDNA coated with E2 (when microinjected) can localize into nucleus!! Whereas minus E2 DNA remains in cytoplasm.

Does VirE2 play any role in NLS dependent nuclear targeting?
Or does it simply keep T-DNA protected and distended for nuclear import?

VirD2 $\triangle$*NLS*, *virE2* mutant can transform plant cell producing VirE2 protein, suggesting that VirE2 NLS is functional.

T-DNA genes carry eukaryotic regulatory sequence



1= tms1
2= tms2
3 = nos or ocs
4= tmr

5= autoregulates synthesis of auxin antagonist.
6b=auxin like?, reduces cytokinin effect?

Before the elucidation of T-DNA genes, scientists thought that plant and animal tumors must be biologically similar i.e. the basis of tumorization must be same. For this reason, NIH funded Crown gall studies. But now we know that the two types of tumors are fundamentally different.

Biochemical basis of tumorogenesis:

Auxin synthesis

tms1= Trp mono-oxygenase
tms2= IAM hydrolase

Cytokinin synthesis

tmr= IPP transferase

Transformation Vectors

1. ‘Disarmed vectors’: non-tumor inducing Ti Plasmids.
2. Co-integrative
3. Binary vectors.

Old *Agrobacterium* binary vectors: low copy

New Binary vectors: high copy

Agrobacterium host-range

WHR, LHR, Super-vir strains

Necrotic response by WHR strains on grape-vines is attributed to VirC activity

Agro-infection of maize with MSV

Strain	infection
A6 strain:	No
C58	Yes
C58 + $VirA_{A6}$	Yes
A6+$VirA_{correc}$	Yes

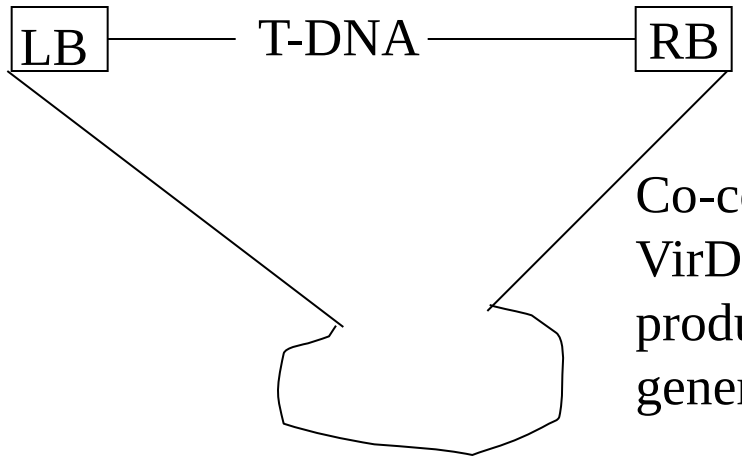
$VirA_{A6}$ defect is aggravated in A6 strain suggesting that A6 chromosome produces a repressor that interacts with $VirA_{A6}$

Monocots (considered recalcitrant to *Agrobacterium* infection)

1. “Agrolistic” system.
2. Super-binary system: extra set of virG, virC and virB.

“Agrolistic”

All major monocots were transformed by biolistic (bombardment of DNA coated gold particles) method by 1992. *Agrobacterium* produces simple integration patterns, where as biolistic produces complex patterns



Co-combardment of binary vector with VirD1 and VirD2 expression cassettes: produced integration patterns just like the ones generated by Agrobacterium.

Transformation Vectors

Basic component:

Selectable markers: *nptII*, *hpt*, *gentR*, *bar*, *EPSPS* gene.

Screenable markers:

codon usage, gene shuffling.

Introns

Some fancy stuff:
Scaffold Attachment Region (SAR) or Matrix Attachment Region (MAR):

1. normalize gene expression, especially of high copy number locus.
2. Yeast and tobacco SAR were effective, whereas human and soybean SAR were non-functional.

Site-specific integration system.

Role of the Plant Cell

Transformation efficiency:

1. Cultivar/ ecotype variation.
2. Tissue or cell type variation.

Plant factors? Plant genes?

Survey of Arabidopsis T-DNA tagged line.

1. Root explant transformation.
2. Germline transformation method.

Ecotype/ mutant	Block	Efficiency	
		Root explant	Germ-line transformation
Ws	None	87%	0.26
Aa-0	None	89%	0.63
rat-1	bacterial att.	5	0.22
rat-3	bact. Att.	9	0.22
rat5	integration	15	0.22
rat9	integration	6	0.21

rat5 mutant

1. *rat5* mutant contains a T-DNA insertion in histone H2A gene: deficient.
2. Complemented by overexpression of H2A gene.
3. Overexpression of H2A improved transformation efficiency even in WT plants.
4. *rat5* is competent for transient expression but incompetent for stable expression.

Thank you