National Science Foundation Plant Genome

Cereal Plant Transformation Workshop

Albert Kausch

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Plant Transformation: History, Molecular and Cellular Mechanisms

NSF Plant Transformation Workshop

Albert Kausch, *University of Rhode Island*

Plant Transformation

Transformation refers to the genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings and taken up through the cell membrane(s).

In plant biology, this most commonly refers to the stable integration and expression of foreign DNA into the plant genome inherited to a subsequent generation. ***"Integrative transformation"***

Plant Transformation- Plant Biology's Unsung Hero

A fundamental but often underappreciated technology for understanding the plant genome and utilizing plants to their greatest potential involves the *capability to create, test and cultivate transgenics.*

Transgenic technology can introduce valuable agronomic genetic variation into crops, functionally link genes to biological functions and modify metabolic pathways.

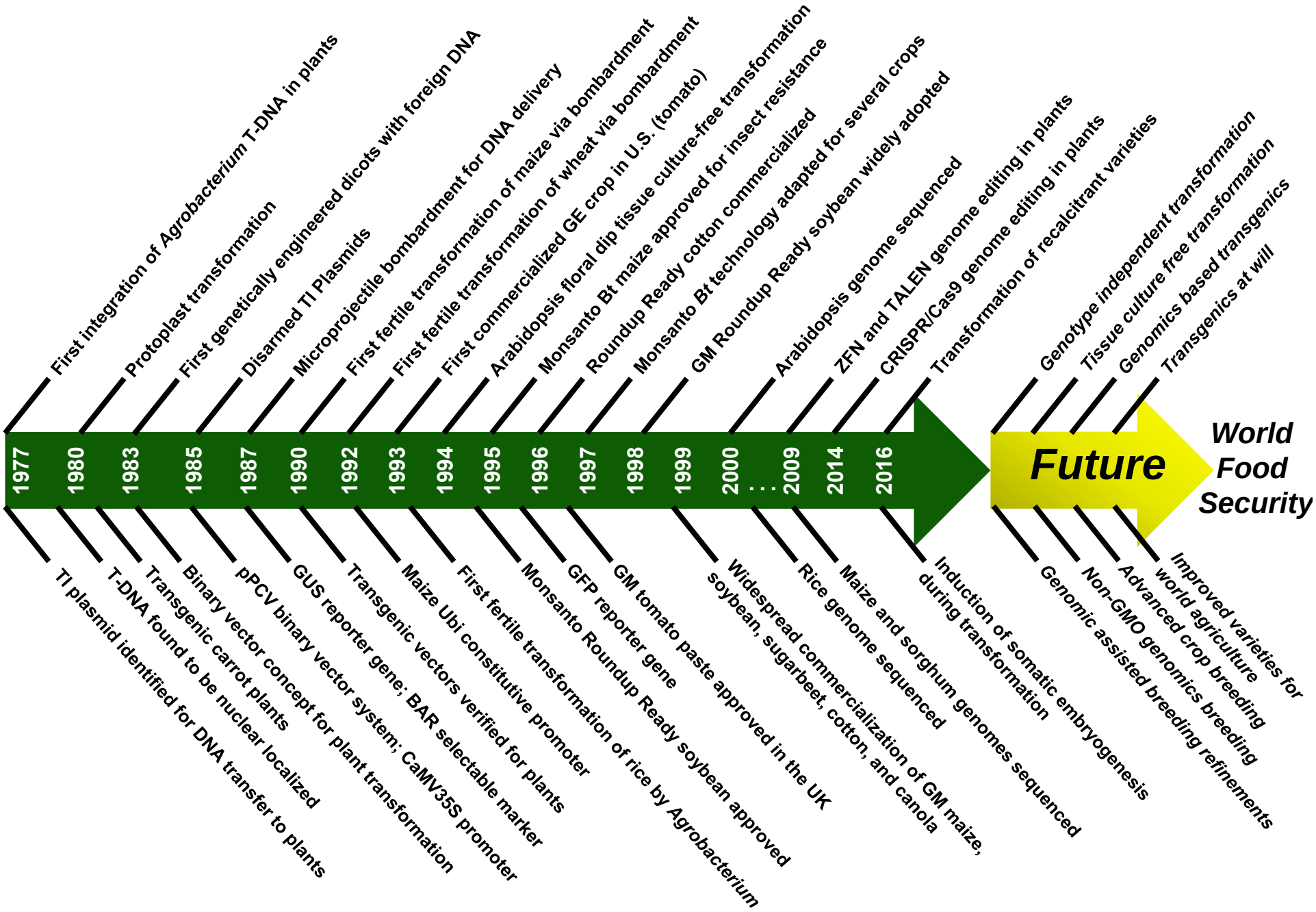


Figure 3. Important historical milestones in plant transformation.

The Importance of Plant Transformation

At the basic scientific research level, transgenic technology is an essential resource. The application of Koch's Principles renders robust transgenic capabilities an imperative genomics technology.

The ability to knock-out (down) gene expression, genome editing conduct expression analyses with reporter genes, make specific adjustments in protein structure and function, and observe over-expression and ecotopic characteristics represent a few examples of transgenics' role in basic plant biology.

Plant transgenic technology will continue to play an essential role scientific research and in the introduction of novel genes that convey traits pertinent to pest, stress and drought tolerance, as well as a host of other important agronomic characteristics.

The Importance of Plant Transformation

Transformation of dicot plants was achieved in the early 1980s However, while transformation of many dicots is now routinely used in many laboratories worldwide, cereal transformation is still considered quite difficult, cumbersome and not widely practiced or accessible. What is the fundemental difference in these systems?

Genetic transformation of the major cereal crops, such as maize, rice, wheat, barley, sorghum, oats and rye has been achieved but major problems remain that limit widespread utility.

The Importance of Plant Transformation In The Era of Genome Editing

Genome Editing

- ZFN
- TALENs
- CRISPR

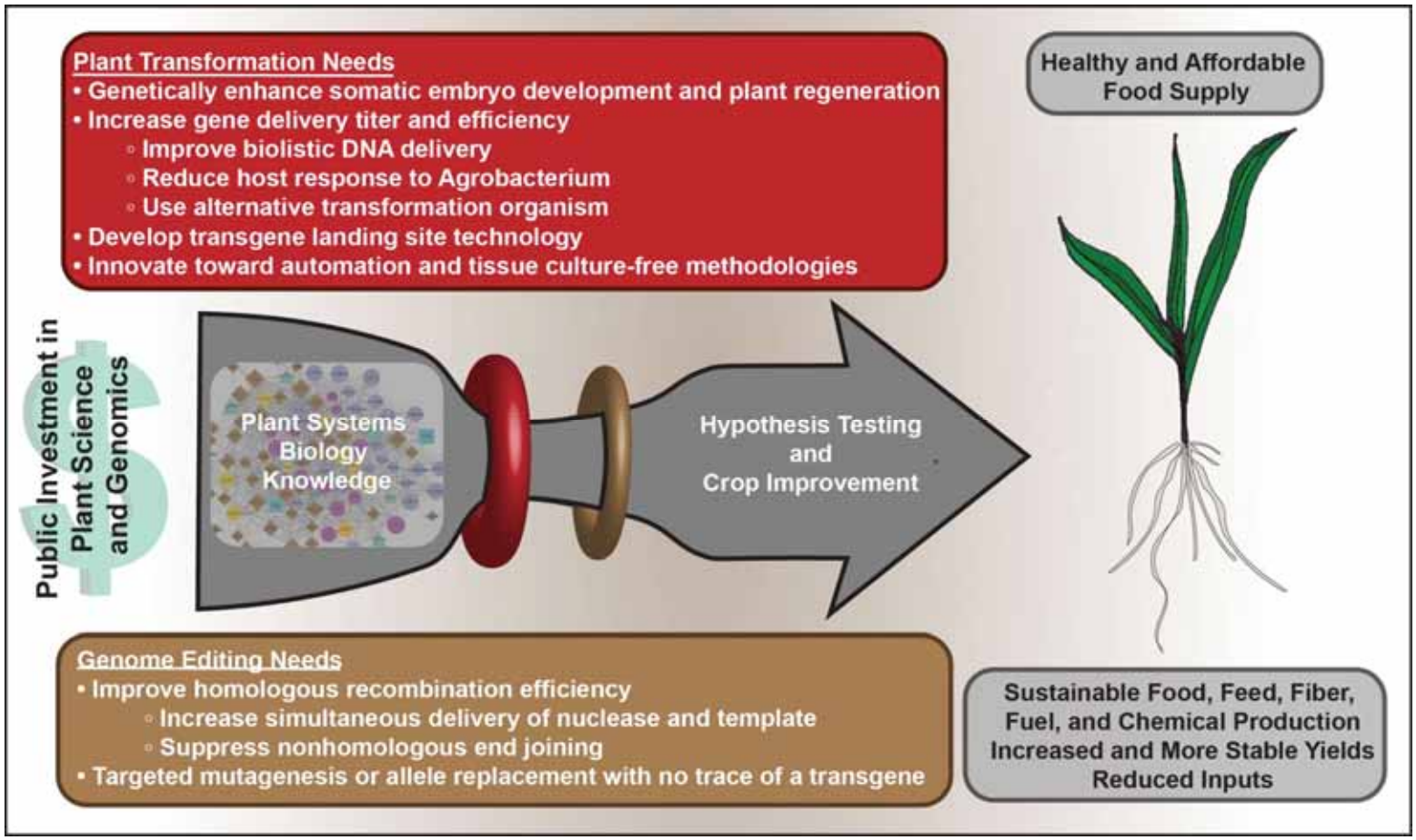


Figure 1. Current bottlenecks in applying genome editing to crop functional genomics and crop improvement. The main bottleneck is in plant transformation and regeneration. A secondary bottleneck is in the delivery of genome editing reagents to plant cells to produce the intended effects.

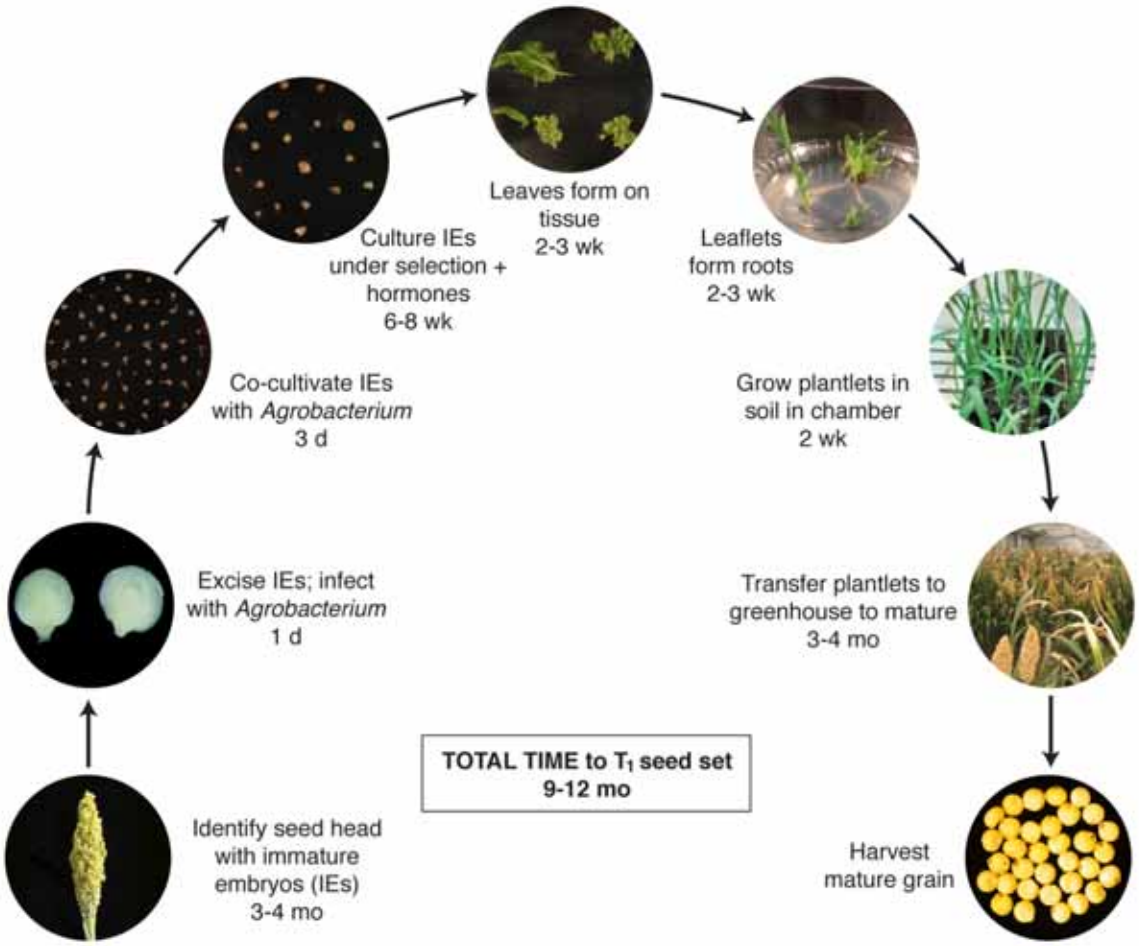


Figure 2. Sorghum (*Sorghum bicolor*) is a crop recalcitrant to transformation and regeneration. Starting left and going clockwise are representations of steps and time required to for each step in the method: from growth of donor plants to provide target immature embryos to the harvesting of mature seed. Times required at each step is indicated as d, days; wk, weeks; mo, months. Similar protocols and timelines prevent the high throughput transformation and genome editing for most important U. S. crops.

Best Practices in Plant Tissue (Cell) Culture for Genetic Transformation

Albert Kausch, *University of Rhode Island*

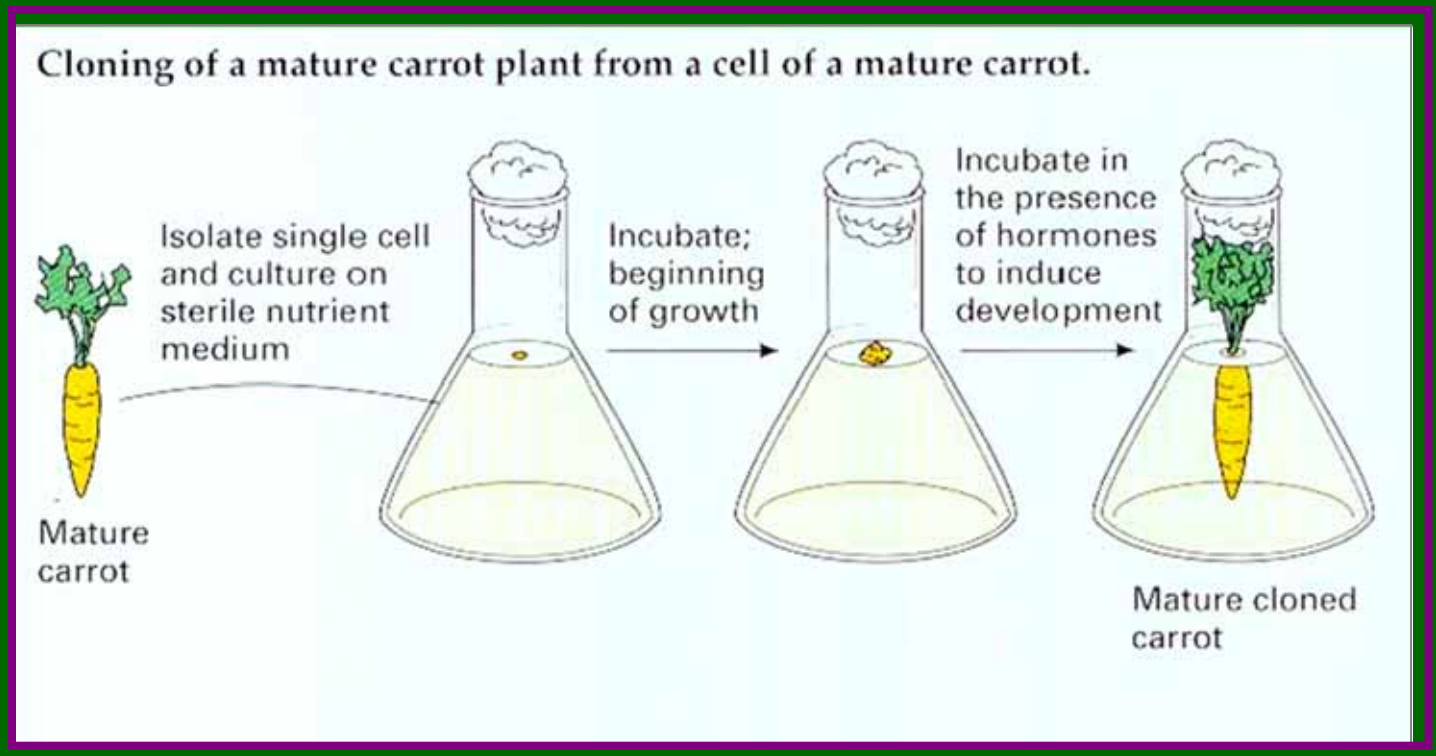
Fundamentally, plant transformation systems are complex but depend on three key processes:

1) **DNA transfer and integration into a host recipient cell;**
2) **ability to select transformed cells from non-transformed cells;**
3) **ability to regenerate adult fertile plants from single totipotent transgenic cells.**

Essential Components for Successful Plant Transformation

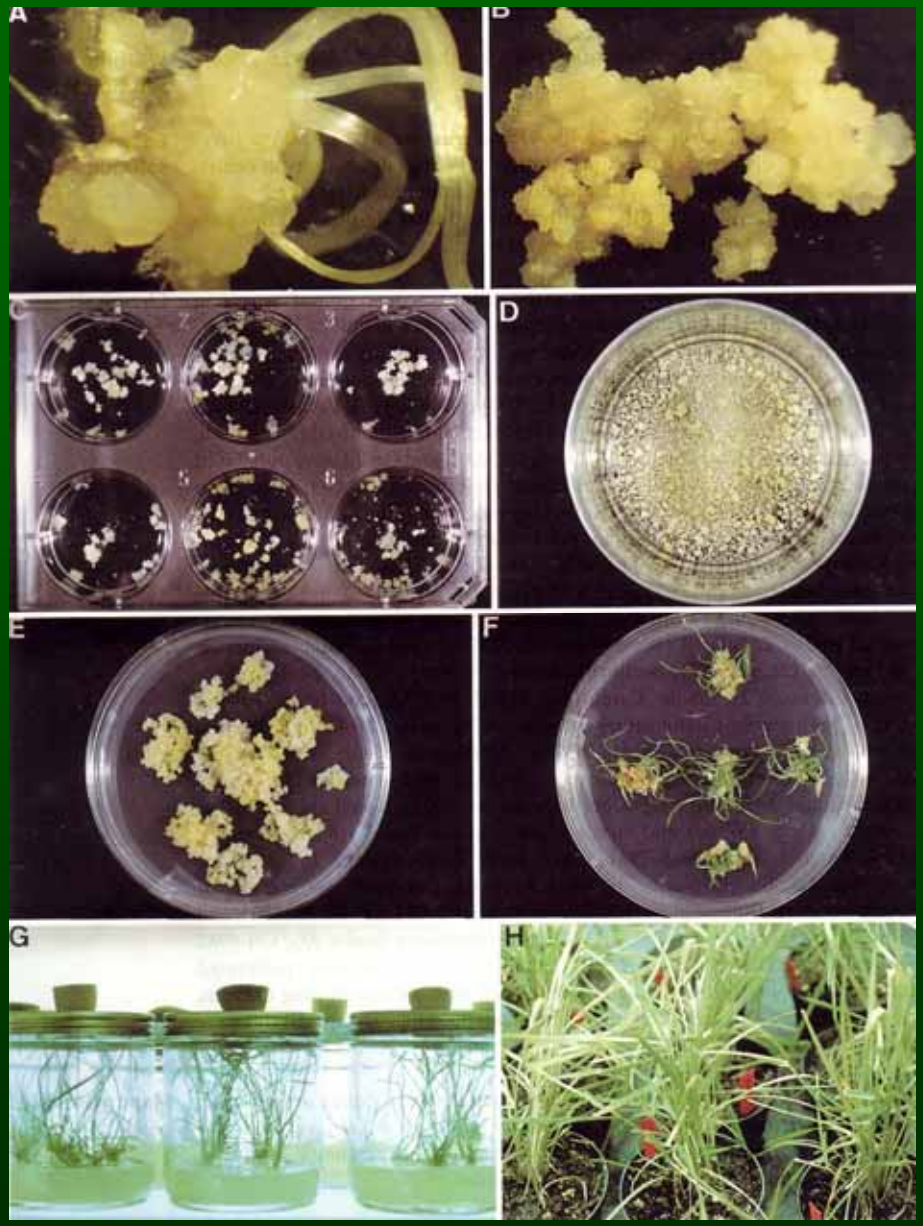
- Tissue Culture System *
  - regeneration of whole plants from single cells
- Molecular Gene Construction Technology
  - Molecular vector construct using foreign DNA
- Method for DNA Delivery
  - *Agrobacterium tumefaciens*
  - Microprojectile Bombardment
  - Direct DNA Uptake
  - Whiskers
  - Sonication
- Efficient Selection Strategy
  - differentiation between cells which receive the transgene from those that don't
    - Antibiotic Resistance
    - Herbicide Resistance

Plant Regeneration: Plants can be grown back from a single cell this ability is called "totipotency".
Steward 1958 Cornell Univ.



Many, but not all plant cells are totipotent. Tissue culture is a key element for creating transgenic plants.

Regenerable Cell Culture Systems



Organogenic

Somatic Embryogenic

Whole plants can be regenerated back from single cells or groups of cells

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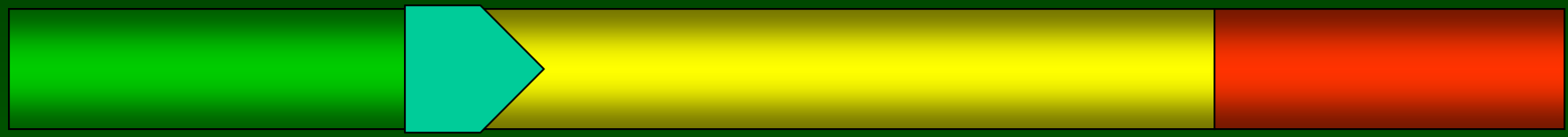
Anatomy of a Transgene

Promoter Coding Sequence Terminator



Cell specificity
Developmental specificity
Start transcription

Protein coding sequence

Stop transcription
Message stability

Gene constructs can be moved into plants and the gene is expressed driven by the promoter sequence

Anatomy of a Transgene

A method for selecting cells that have received the DNA from those that have not.

Promoter | Coding Sequence | Terminator

Constitutive expression
'on in all cells'

Herbicide resistance
Antibiotic resistance
Metabolic (i.e pmi)

Stop transcription
Message stability

Transgenes confer new traits

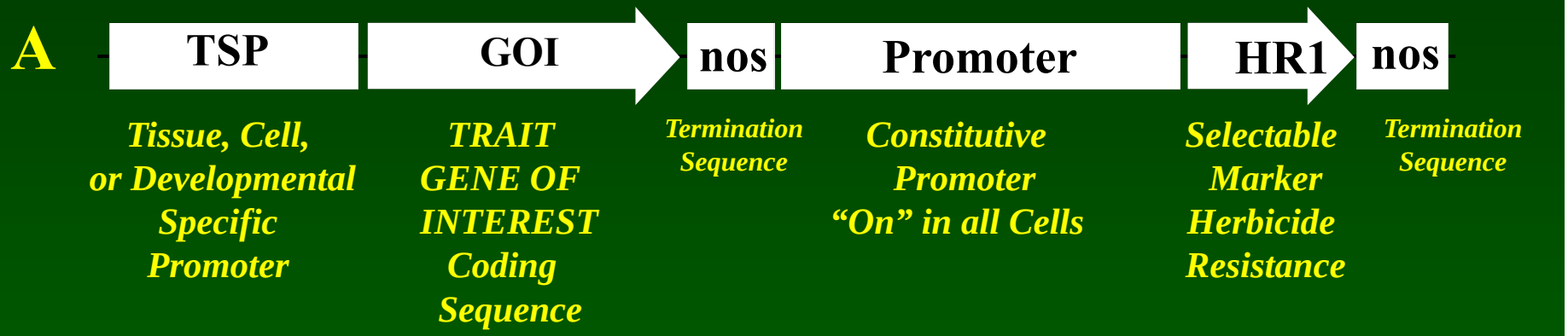
Promoter | Coding Sequence | Terminator

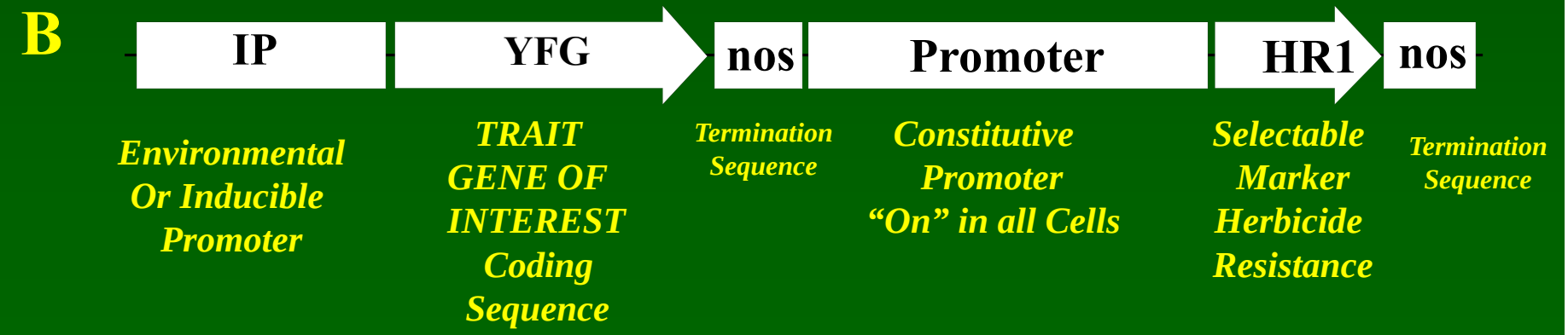
Controlled expression
'on in only specific cells'

Herbicide resistance
Pest resistance
insect, fungus, virus
Drought, salt,
and freeze tolerance
Nutritional enhancement
proteins, vitamins, oils
Specialty chemicals
pharmaceuticals, bioplastics

Stop transcription
Message stability

Genetic Modification of Plants for Traits





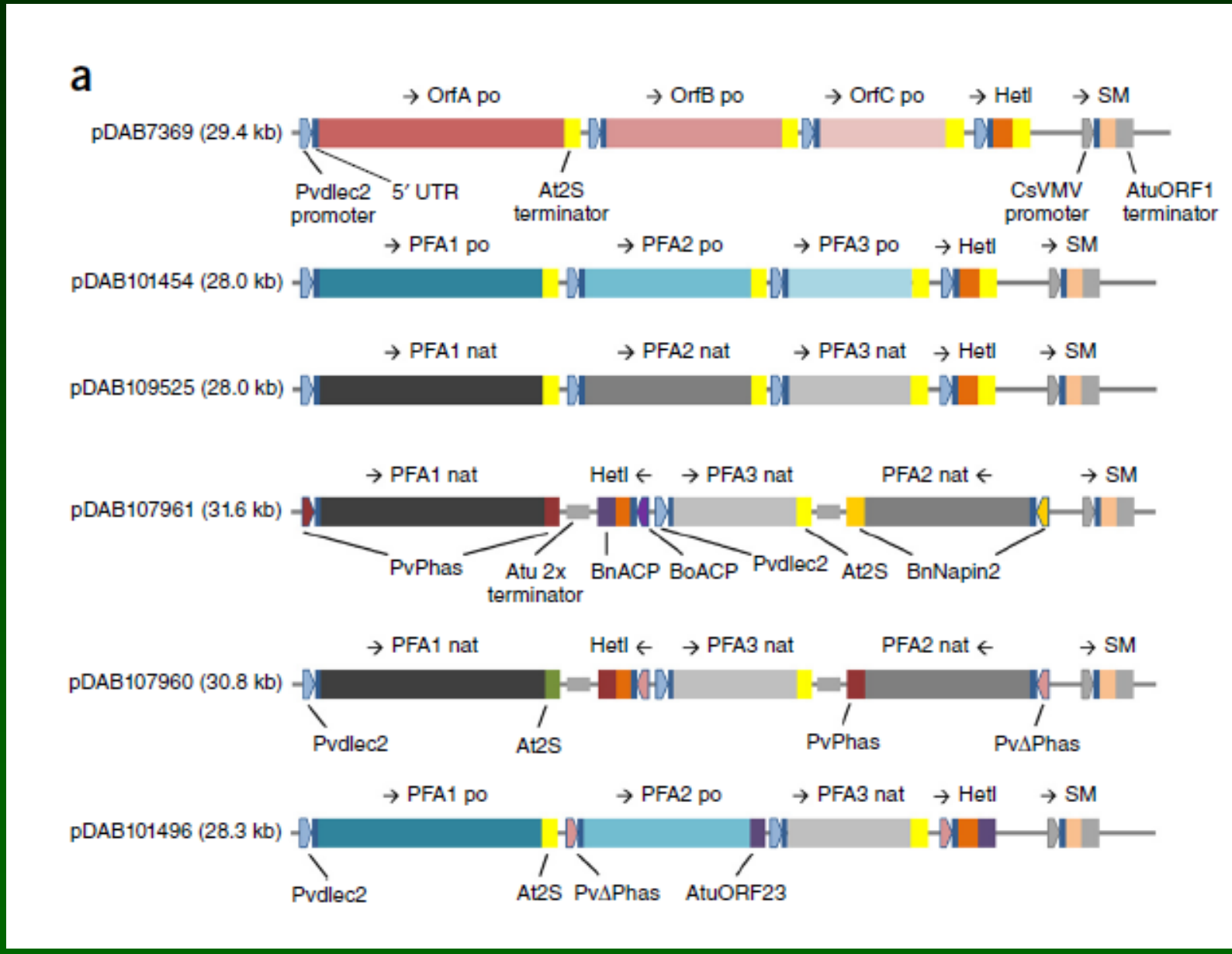
Traits and Genes

Second and additional trait genes are 'piggy-backed' in and are co-expressed with the selectable marker gene. Thus, two genes (and more) can be delivered at the same time.

In addition, genes can be stacked by crossing transgenic plants

Trait gene vectors are now constructed to be more consistent with eukayotic genes (UTRs, introns, codon preference, termination signals, cis genetics, and limiting the possibilities for recombination)

Metabolic Engineering and Multigenic Traits



Walsh et al. 2016 Nature Biotechnology

LETTERS

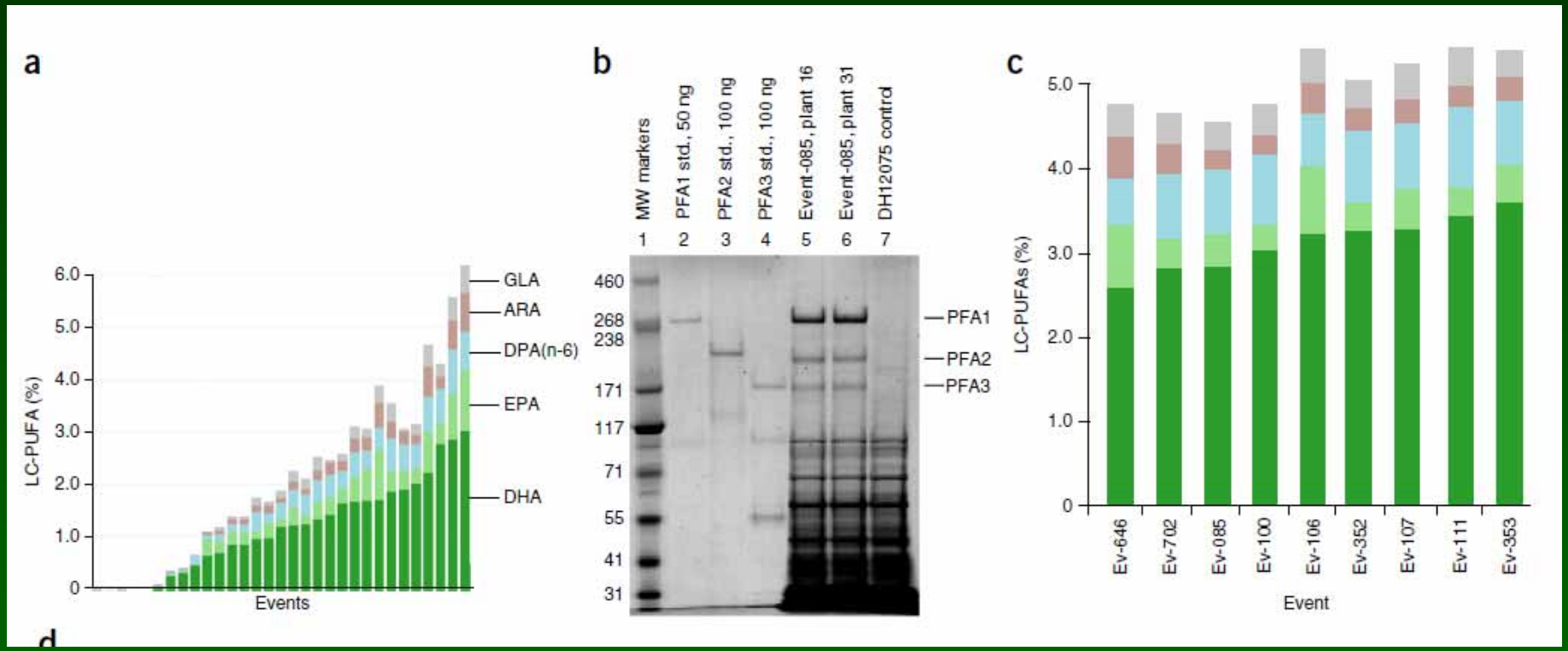
nature biotechnology

Canola engineered with a microalgal polyketide synthase-like system produces oil enriched in docosahexaenoic acid

Terence A Walsh[1], Scott A Bevan[1], Daniel J Gachotte[1], Cory M Larsen[1], William A Moskal[1], P A Owens Merlo[1], Lyudmila V Sidorenko[1], Ronnie E Hampton[1], Virginia Stoltz[1], Dayakar Pareddy[1], Geny I Anthony[1], Pudota B Bhaskar[1], Pradeep R Marri[1], Lauren M Clark[1], Wei Chen[1], Patrick S Adu-Peasah[1], Steven T Wensing[1], Ross Zirkle[2] & James G Metz[3,4]

Walsh et al. 2016 Nature Biotechnology

Random Integration and Position Effect



Walsh et al. 2016 Nature Biotechnology

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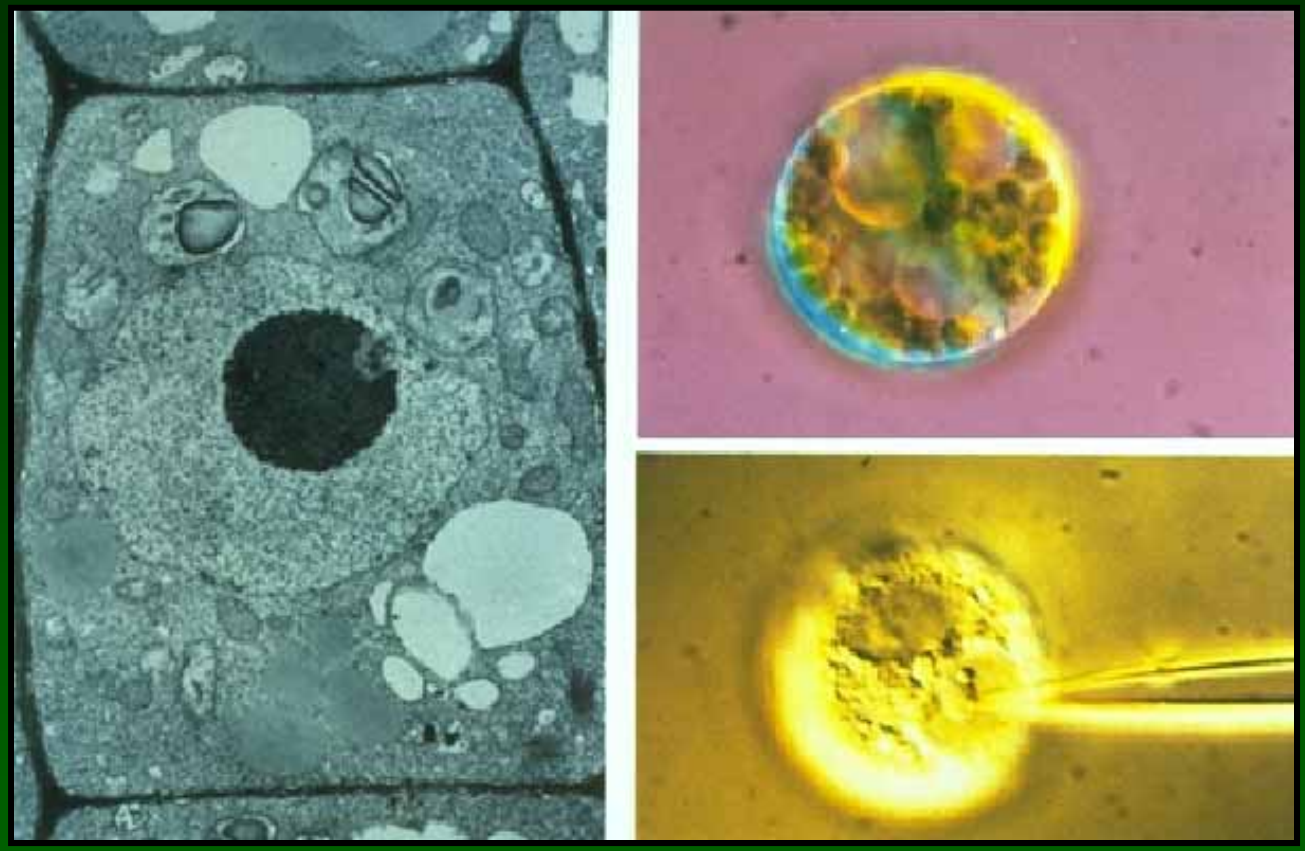
  - differentiation between cells which receive the transgene from those that don't
    - Antibiotic Resistance
    - Herbicide Resistance

How are cloned genes moved into cells?



Microprojectile Bombardment
Agrobacterium vectors
Electroporation

Microinjection
Si Wiskers

Protoplasts:

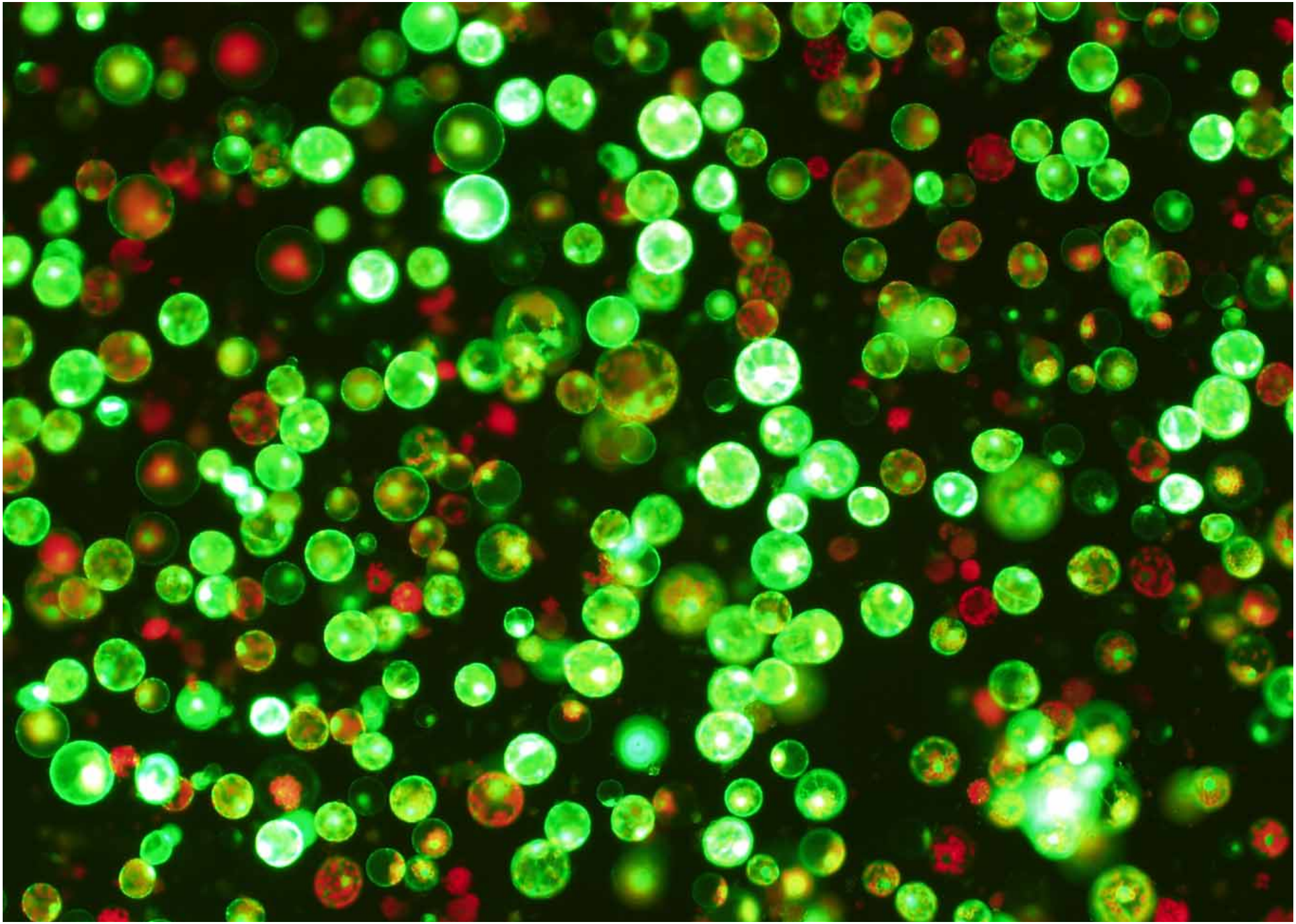
‘Naked Plant Cells’

Plant cells with their
Cell wall removed.

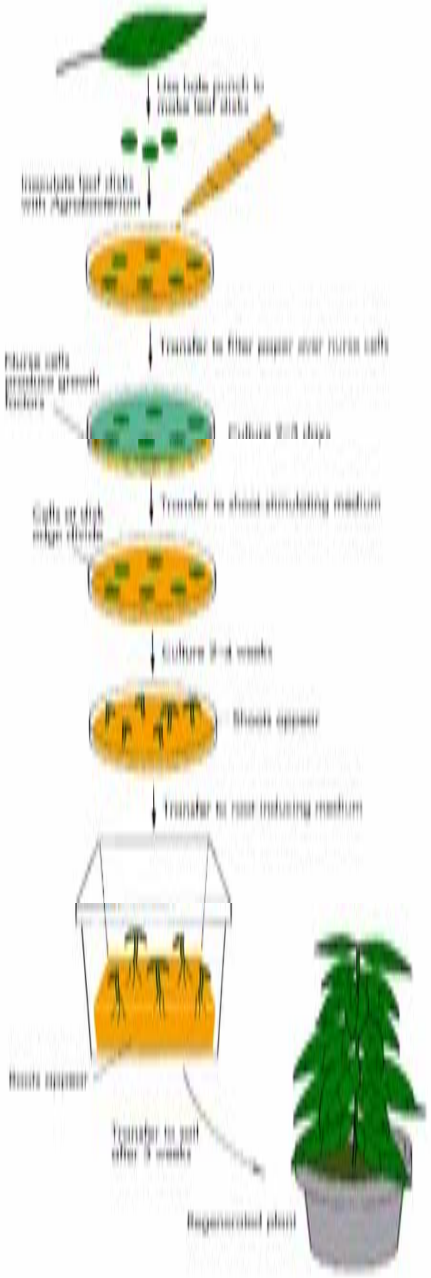
Can be used to insert DNA
- *Direct DNA Uptake*
- *Electroporation*
- *Microinjection*

Serious Drawbacks

- Very Tedious
- Cultivar limitations
- Not effective
- Non-fertile plants



Potato protoplasts expressing green florescence to demonstrate high transformation efficiency. Calyxt,



Agrobacterium tumefaciens:
a gene transfer vector for plants

- A naturally occurring soil bacterium which transfers DNA to plants
- Allowed the first genetically engineered plant (tobacco) 1983
- Used to transfer genes to dicot plants, however not readily amenable to cereal crops

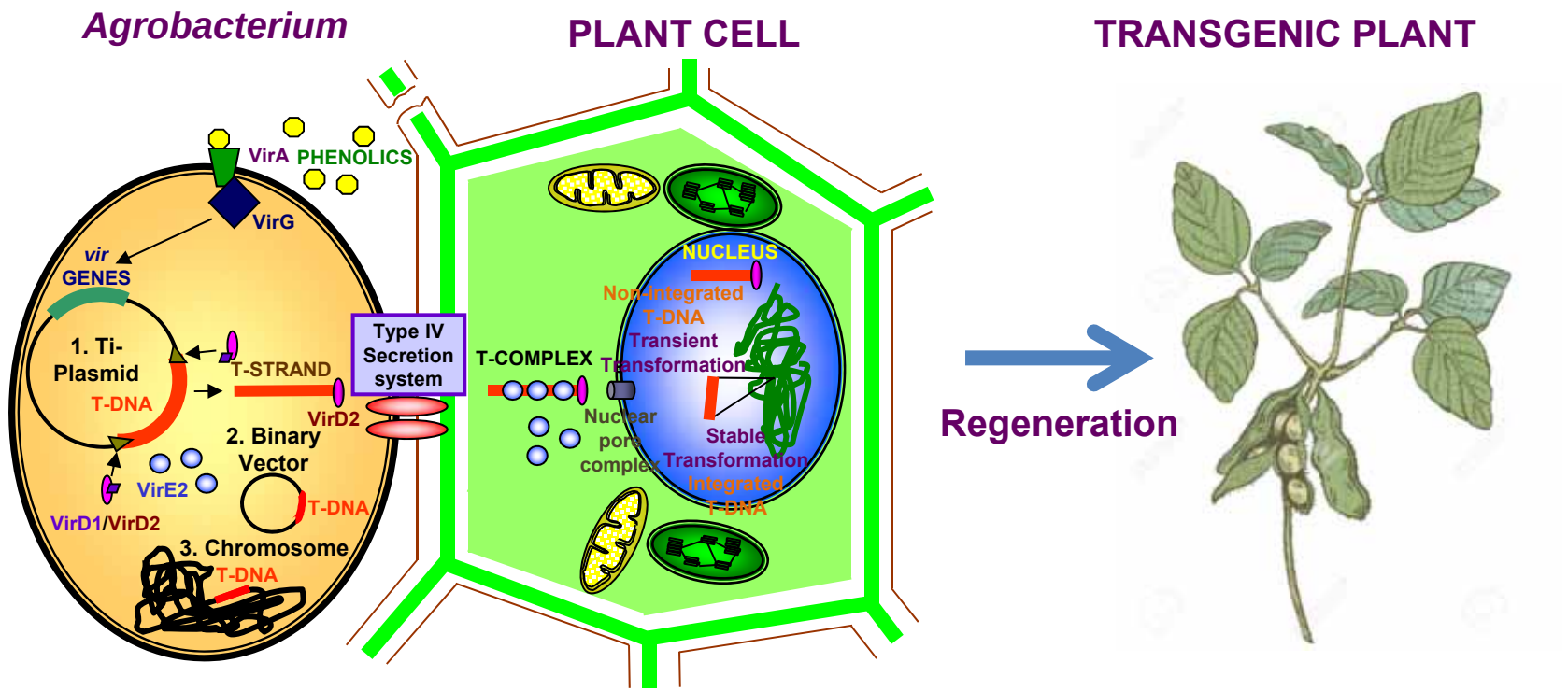
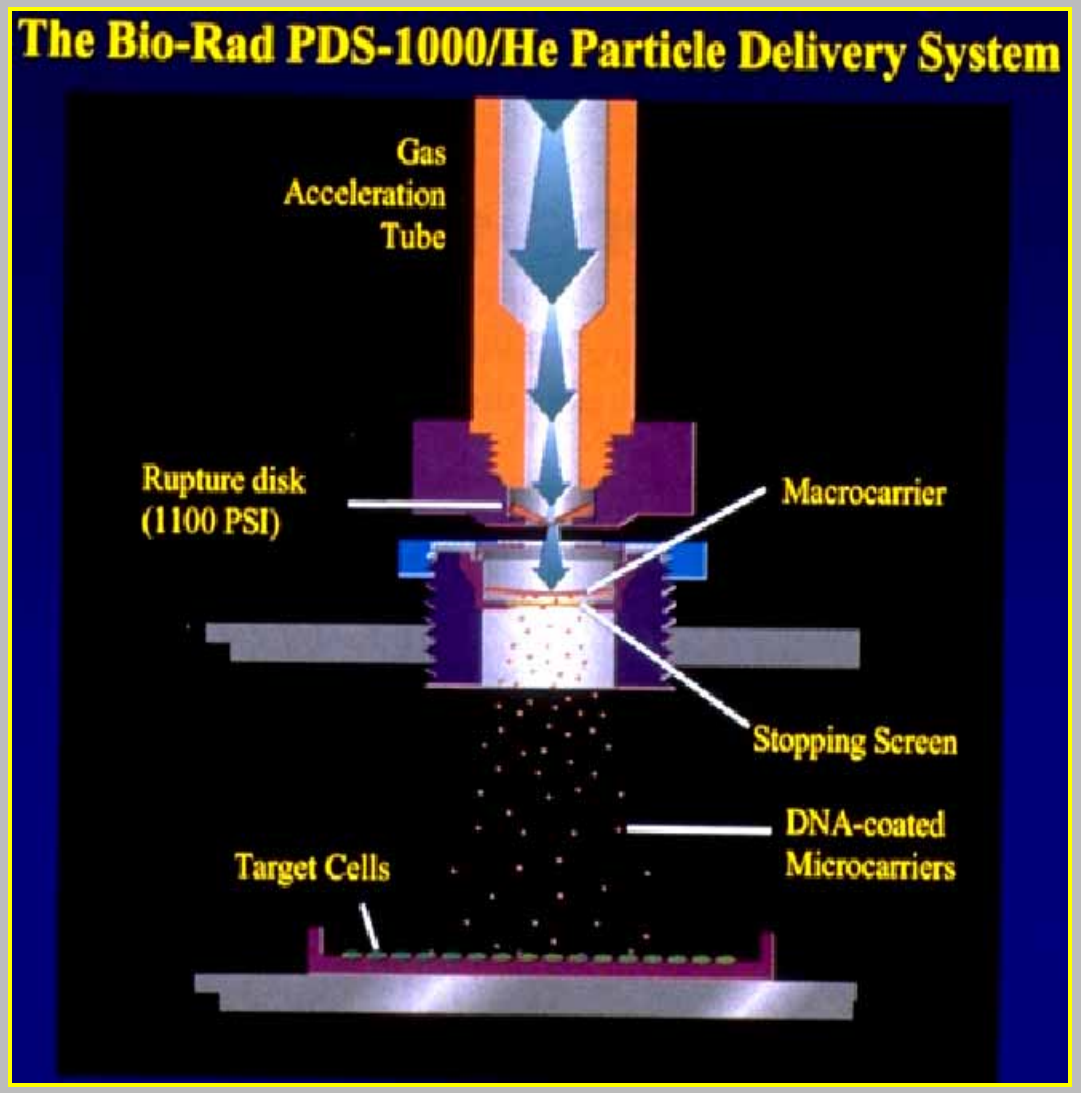


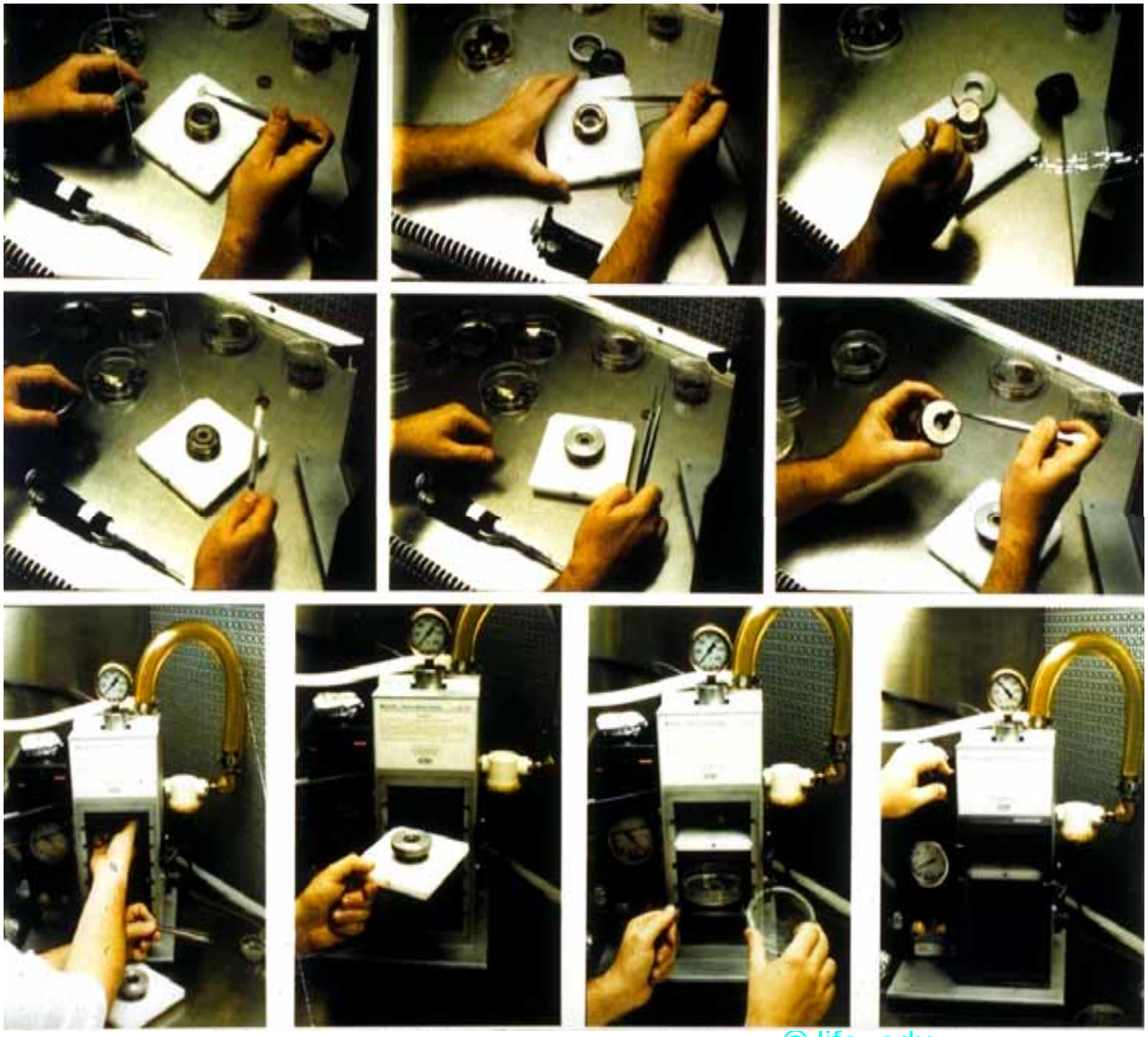
Figure 4. Overview of *Agrobacterium*-mediated transformation to generate a transgenic plant. Phenolic compounds secreted by wounded plants are perceived by the *Agrobacterium* VirA/VirG two-component sensing system, resulting in induction of virulence (*vir*) genes. Among these genes, *virD1* and *virD2* form a site-specific nuclease that nicks the T-DNA region at border sequences. In nature, T-DNA resides on the Ti-(tumor inducing) or Ri-(root inducing) plasmid (1), but in the laboratory T-DNA can be "launched" from binary vectors (2) or from the bacterial chromosome (3). VirD2 covalently links to single-strand T-DNA and leads T-strands through a Type IV secretion system (composed of VirB and VirD4 proteins) into the plant. Other transferred virulence effector proteins are VirE2 (a single-strand DNA binding protein proposed to coat T-strands in the plant cell) and VirD5, VirE3, and VirF (not pictured). Within the plant, VirD2/T-strands likely form complexes with VirE2, other Vir effector proteins, and plant proteins. These complexes target the nucleus. Once inside the nucleus, proteins must be stripped from T-strands, which can replicate to a double-strand non-integrated form (transient transformation). T-DNA can integrate into the plant chromosomes, resulting in stably transformed cells. These cells can be regenerated to plants harboring and expressing transgenes.

The Gene Gun:
Invented by John Sanford (1986)

DuPont bought the rights to Sanford's device



BIO-RAD Biolistic PDS-1000/He Particle Delivery System



Allows testable and reproducible parameters:

Gap distance
Rupture discs
Macrocarrier flt distance
Particle flt distance
Particle size
Particle density
Target cell positioning
Cell/Tissue pre-treatments:
- osmotic
- silver nitrate
- numerous others

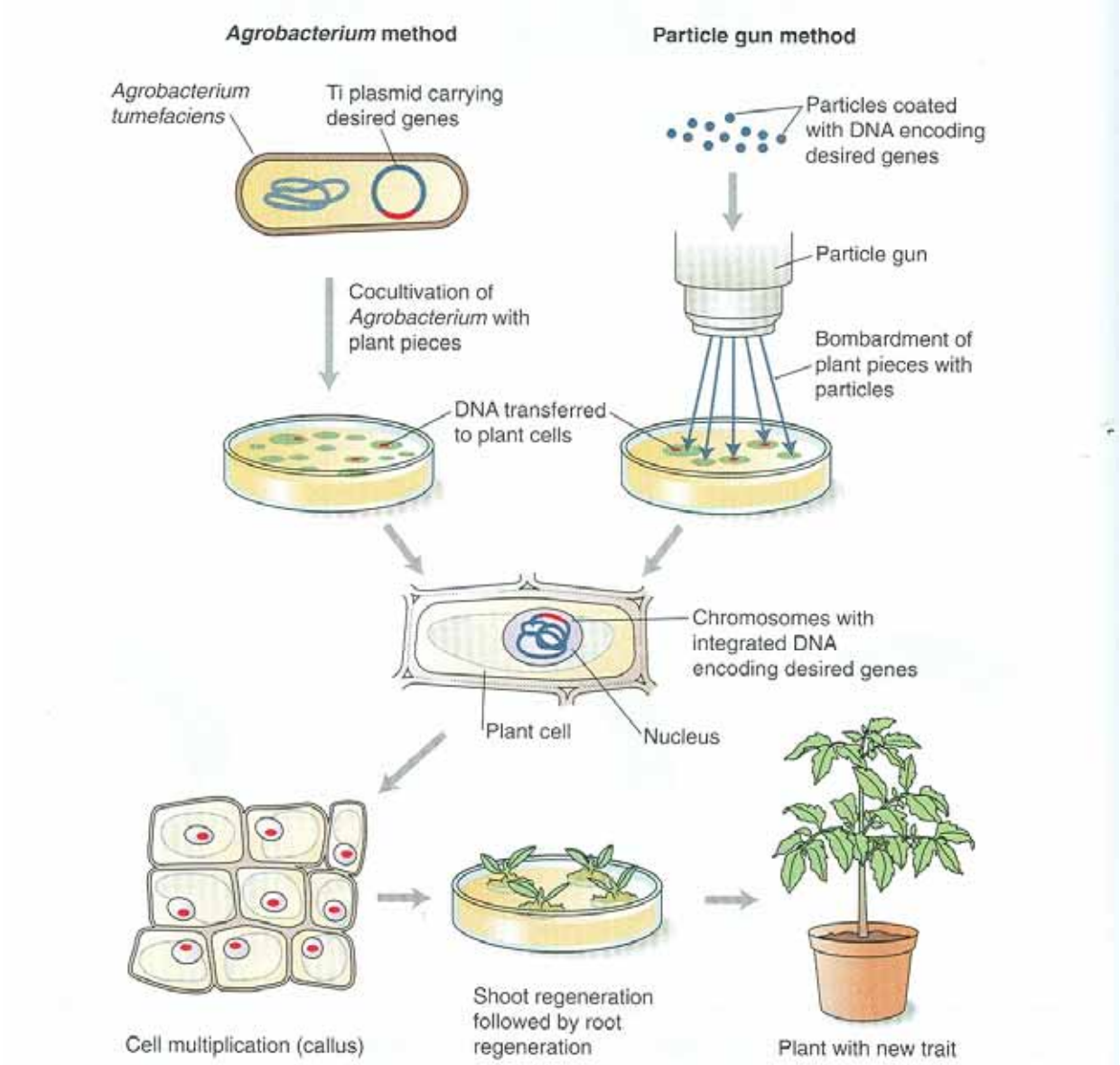


Figure 6.17 Schematic representation of two different ways to create transgenic plants. In the *Agrobacterium* method (left), the biologist inserts DNA carrying the desired genes into the tumor-inducing (Ti) plasmid of the bacterium, and when the bacterium infects a wounded tissue, it transfers this DNA to a cell nucleus and integrates it into the chromosome. In the particle gun method, metal particles coated with DNA become integrated into the plant chromosome. When a new plant regenerates from a single transformed cell, all the cells in the plant carry the new genes. *Source:* Adapted from "Transgenic Crops" by C. S. Gasser and R. T. Fraley, *Scientific American*, June 1992. Copyright 1992 by *Scientific American, Inc.* All rights reserved.

Agrobacterium is now a vector for monocots and dicots

Agrobacterium and the gene gun are viable approaches to plant gene transfer

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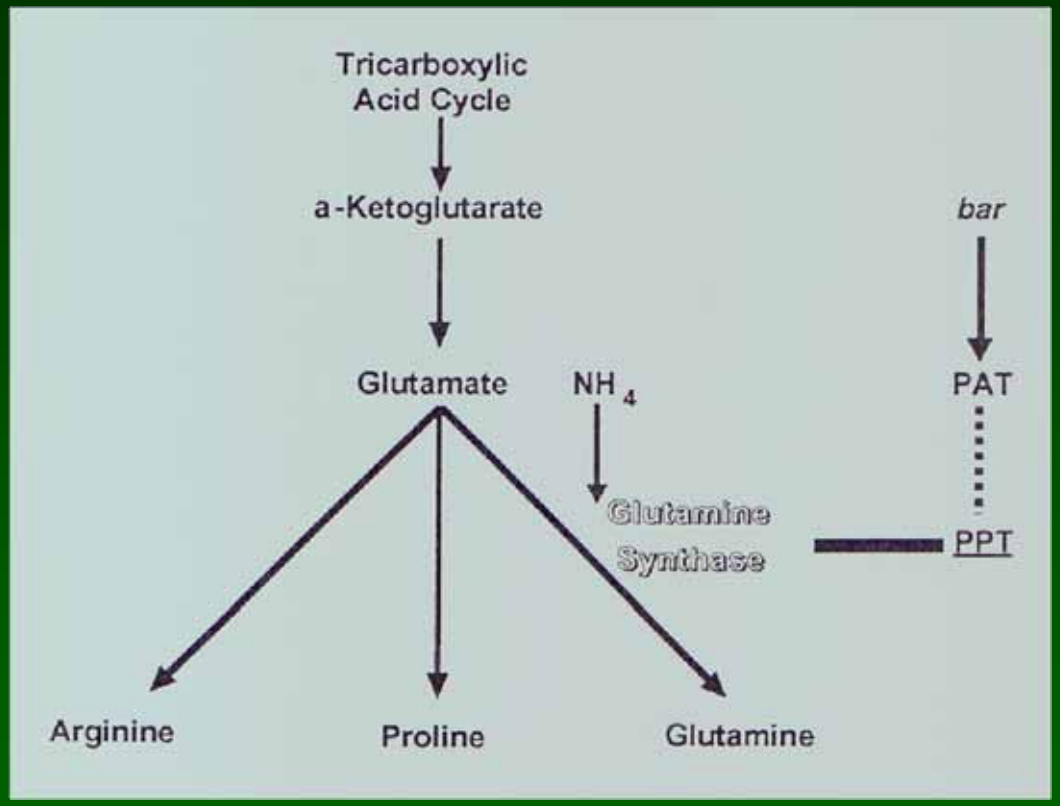
Herbicide Resistance in Crops

Engineering Detoxicification of Herbicides Which Inhibit Glutamine Synthase

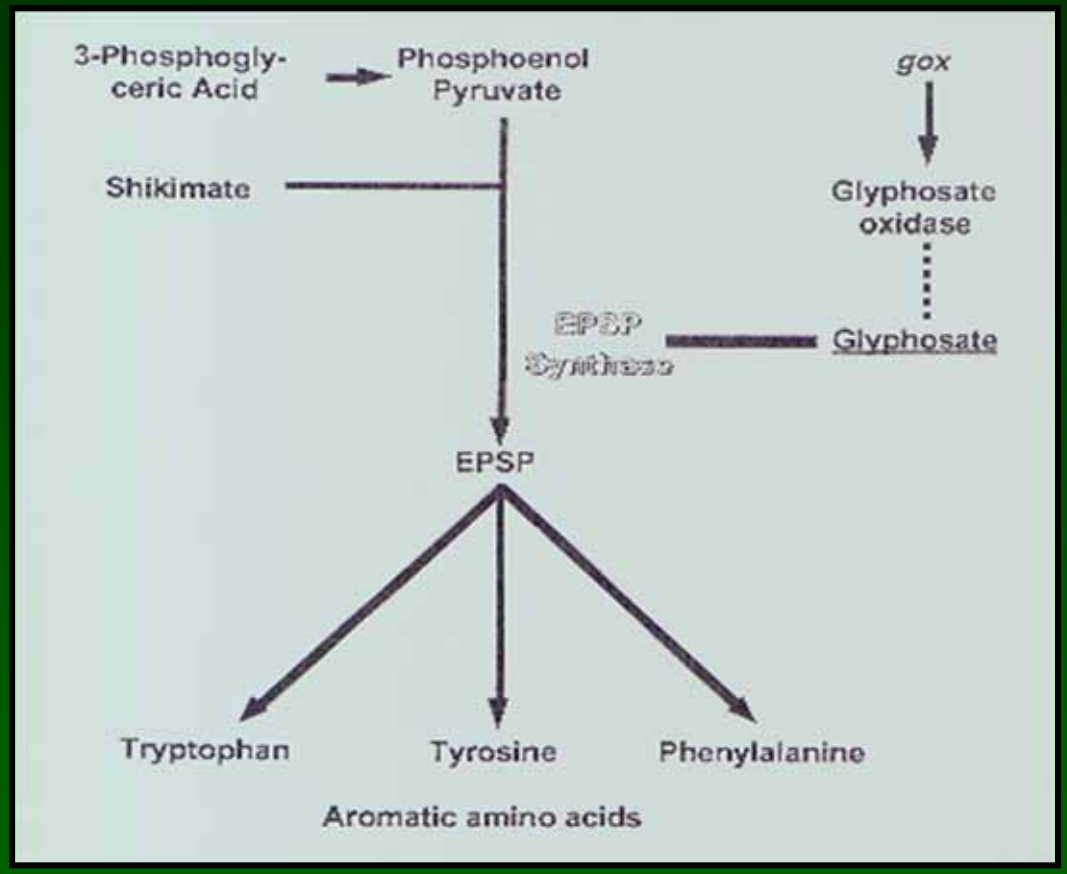


The enzyme glutamine synthase (GS) catalyses the synthesis of glutamine from glutamate and free ammonium.

Phosphinothricin (PPT) is a glutamate analogue which acts by inhibiting glutamine synthase activity resulting in a cytotoxic accumulation of ammonium.

Herbicide Tolerance as a selectable marker

Round-up Ready Plants



Engineering Tolerance to, and Detoxification of, Herbicides Which Inhibit

- ***5-Enol-Pyruvyl Shikimate-3-Phosphate Synthase***

The chloroplast-localized enzyme 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) catalyses a common step in aromatic amino acid biosynthesis. Glyphosate-containing herbicides act by specifically inhibiting EPSPS activity.

REPORTER GENES:

Promoter analysis for gene expression in transgenics

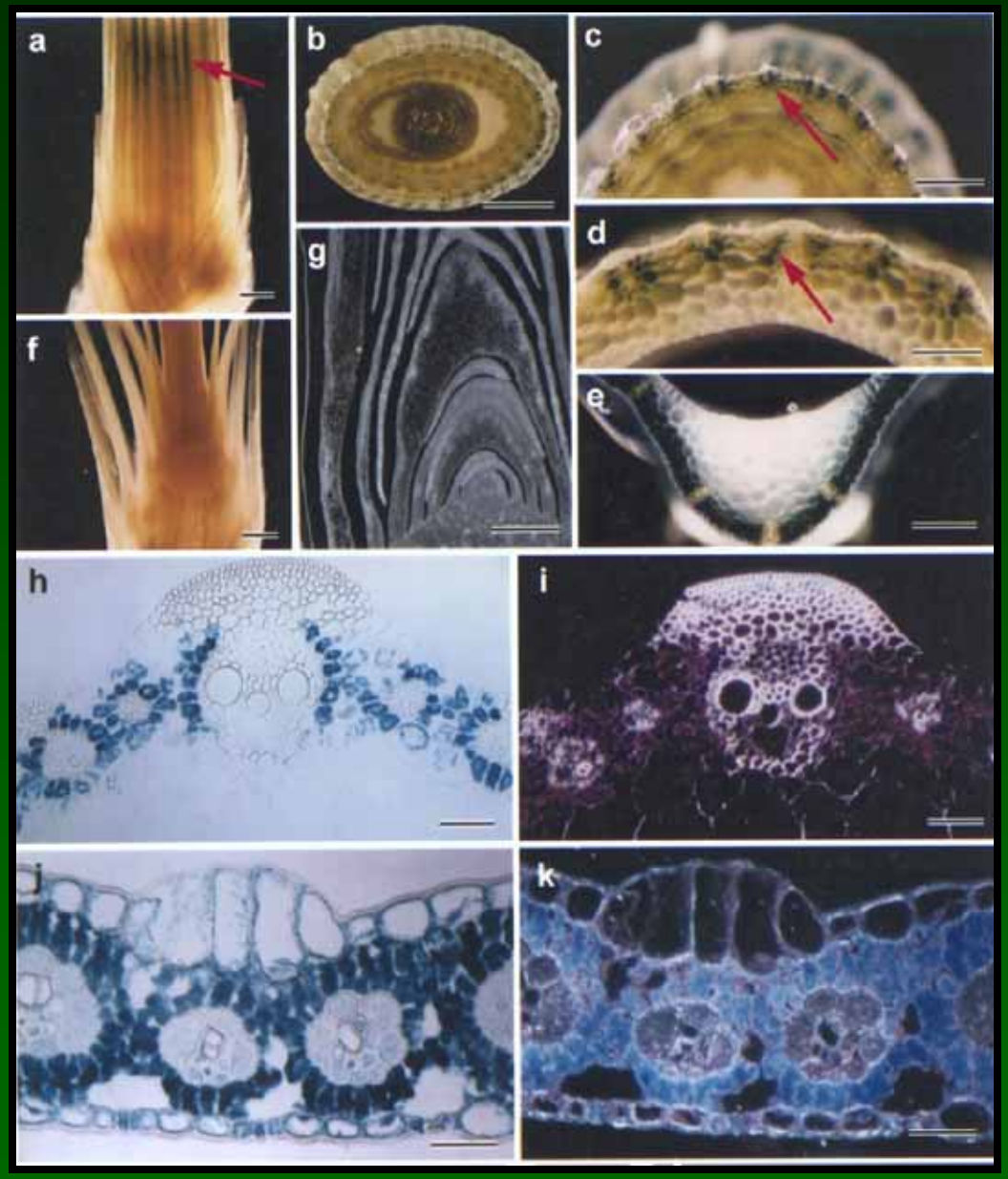
Promoter	Reporter gene	Assay
•pep carboxlyase	GUS	Stable transformants
•adh1	GFP	Histological
•CaMV 35S	C1B	mRNA *in situ*
•aldolase 1	R	Enzymatic assays
•actin 1	LUX	PCR
•endosperm specific		Southern blots

Endosperm-specific GUS expression shown as days post-pollination

Adh1 GUS in hypoxia induced maize seedlings

CaMV 35S-GUS

Promoter analysis for gene expression in transgenics



Transformability

Transformability" is now understood as a complex of interdependent and inclusive systems characteristics.

The systems comprise a number of biological processes such as:

i) accessibility to DNA introduction;

ii) and stable chromosomal integration of the transgene;

iii) genotype-specific cell culturability;

iv) selectability of totipotent transfected cells; and,

v) regeneration of fertile plant from stably transformed cells.

As a result successful transformation methods are often, *complex, multi-step protocols,* whereby small improvements are accrued over time, minute method compliance is mission-critical, and hands-on experience matters significantly. This is especially true of monocot systems.

Dicot vs Monocot

Best Practices in Plant Tissue (Cell) Culture for Genetic Transformation

Albert Kausch, *University of Rhode Island*

Dicot

- **Wound response is callus formation**
- **Recipient cells are mature cell types which undergo dedifferentiation**
- **Susceptible to *Agrobacterium* infection in early experiments**
- **Selectable using antibiotics (esp. aminoglycides)**

<u>Examples</u>

- **Tobacco**
- **Tomato**
- **potato**

Best Practices in Plant Tissue (Cell) Culture for Genetic Transformation

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Monocot

- **Wound response is cell death and necrosis**
- **Recipient cells are somatic embryogenic cultures**
- **Not Susceptible to *Agrobacterium* infection in early experiments**
- **Not Selectable using aminoglycides; Herbicide selection**

<u>Examples</u>

- **Maize**
- **Rice**
- **Sorghum**

Best Practices in Plant Tissue (Cell) Culture for Genetic Transformation

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Monocot

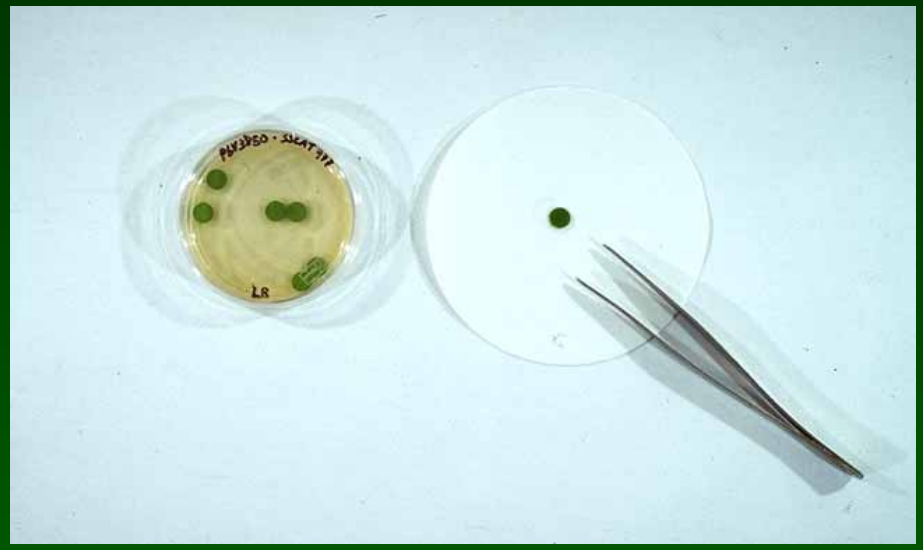
What is not as well appreciated, is that a number of synergistic factors are essential for reliable and efficient cereal transformation including:

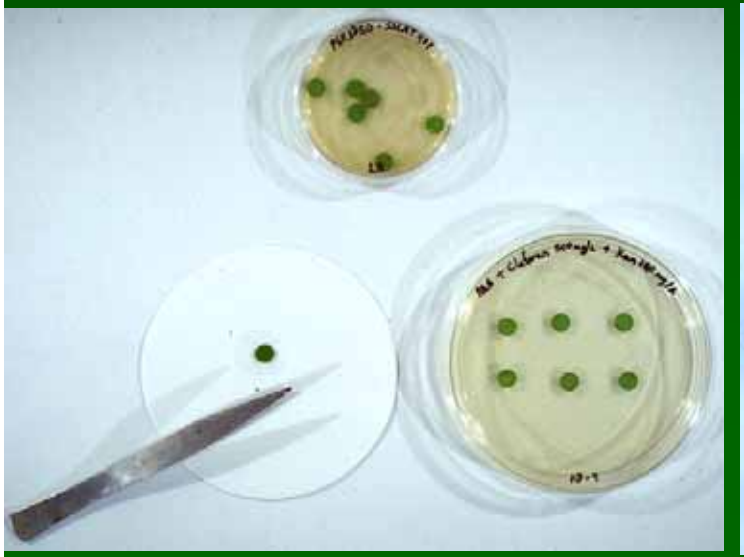
i) **advances in monocot tissue culture of "early'" embryogenic cells as transformation competent recipients**
ii) **identification of suitable explant sources that produce these types of cell cultures (e.g., immature embryos and embryogenic callus)**
iii) **careful selection of genotypes amenable to production of such embryogenic cultures**
iv) **use of appropriate selectable makers for ;**
v) **complex media alterations; and,**
vi) **a number of species-specific refinements including the identification of genotype dependent cell culture responses**

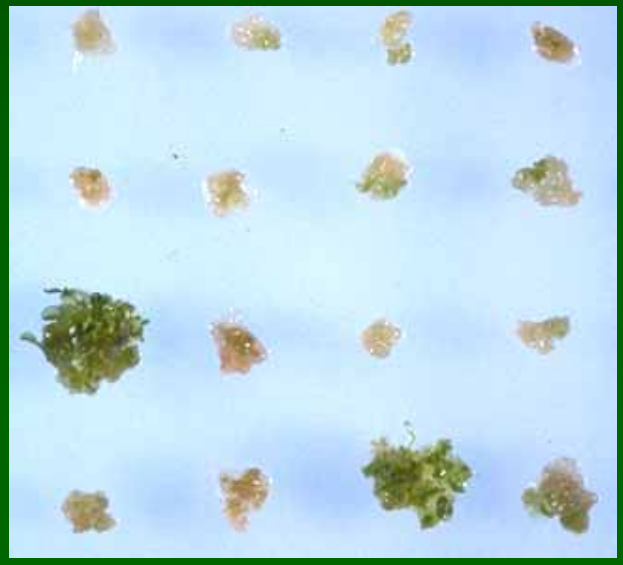
Early work on Plant Transformation:

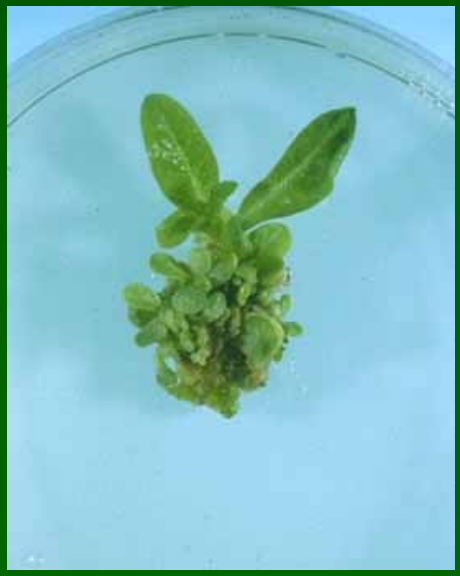
Marc Van Montague Lab, Gent, Belgium; Rob Fraley Lab, Monsanto,

***Agrobacterium tumefaciens*: Gene transfer to plants, 1982-83**

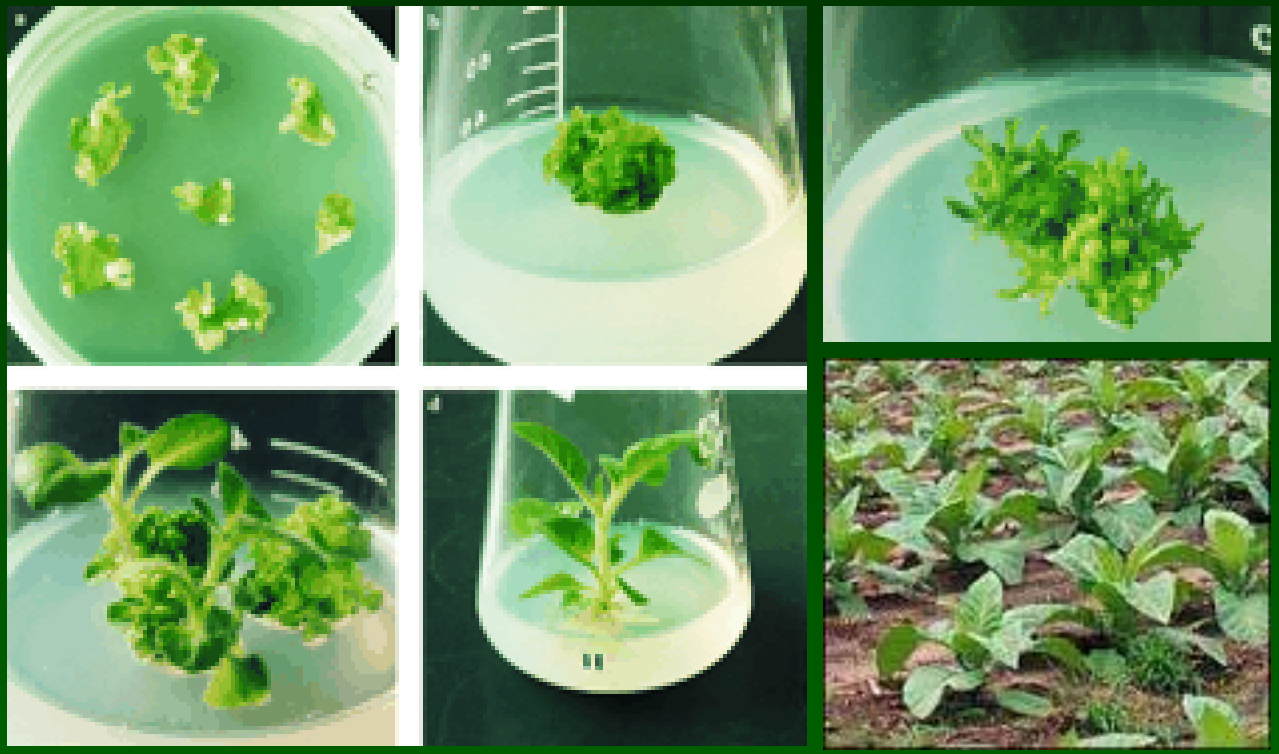








Tobacco Plant Regeneration



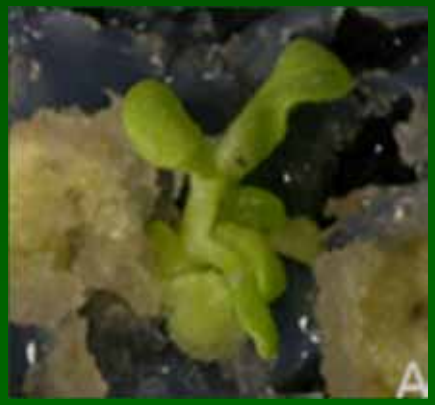
Tobacco Transformation

In vitro grown tobacco plants as leaf explant source

Tobacco disk explants infected with agrobacterium

Tobacco disks on MSD4*2 medium, containing kanamycin 100 mg/l (selected medium).

Transformed Tobacco disks



Transformed Tobacco shoots arise from meristematic domes

Transformed Tobacco plantlet

Transformed Tobacco In field experiments

Tomato Transformation

10 weeks

4 weeks

Acclimation to the Greenhouse

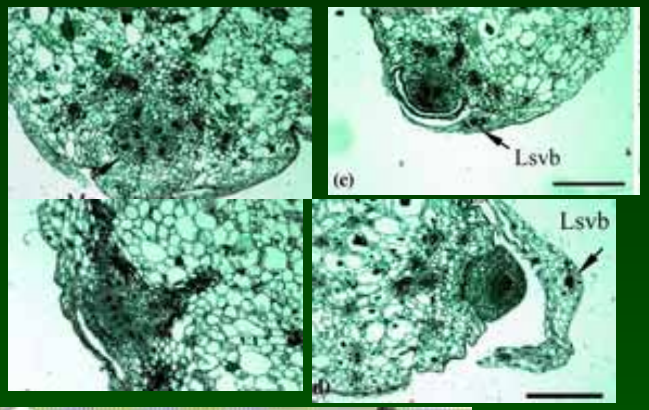
4 weeks

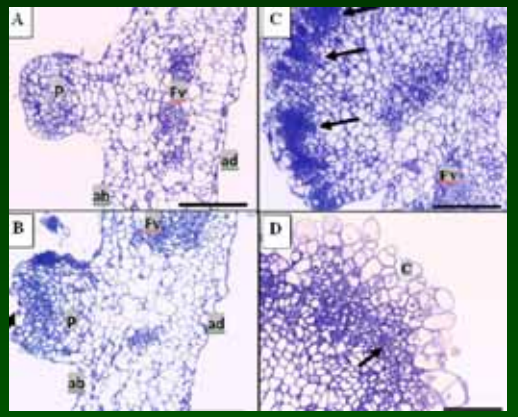
10 - 12 weeks

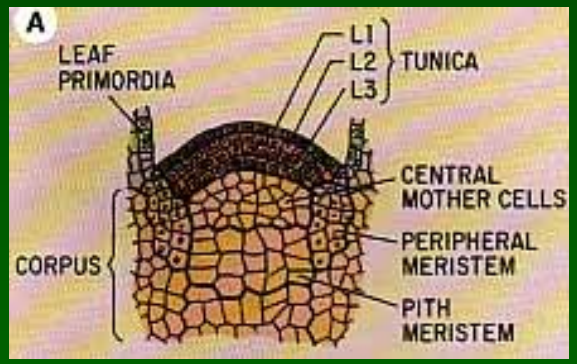
Whole Process

32 - 37 weeks

The Centrality of Organogenesis and Meristem Formation for Dicot Transformation







Dicot Wound Response generates callus that can develop shoot meristems
Transgenic Plants are Recovered from Single Cell Clonal Events

Organogenesis shoots

Organogenesis

Multiple shoots

A multi-celled events

High cytokinin

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Dicot vs Monocot

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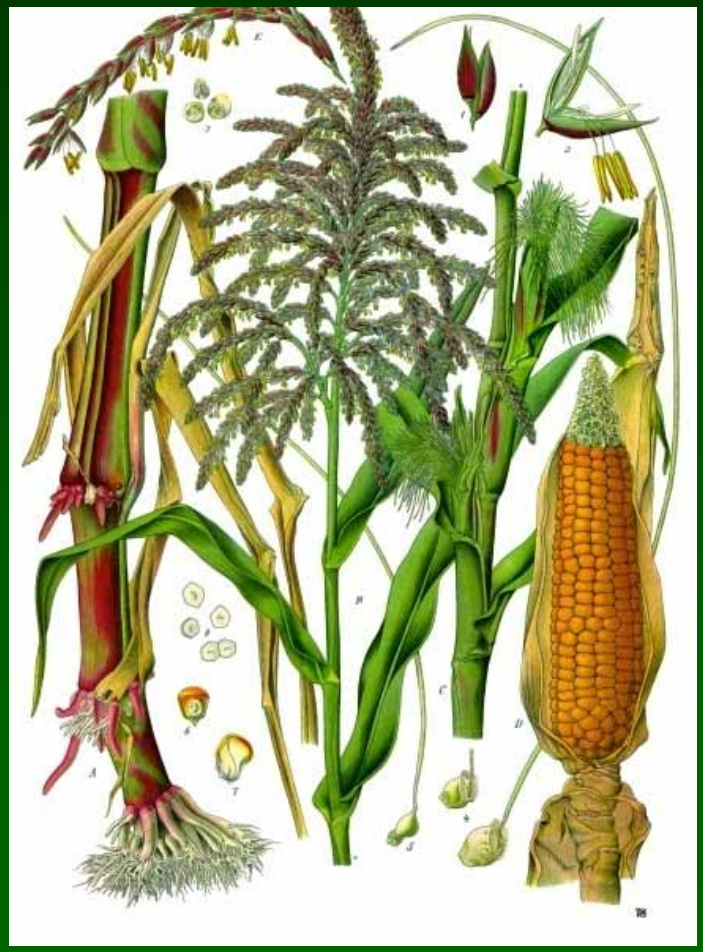
Dicot vs Monocot

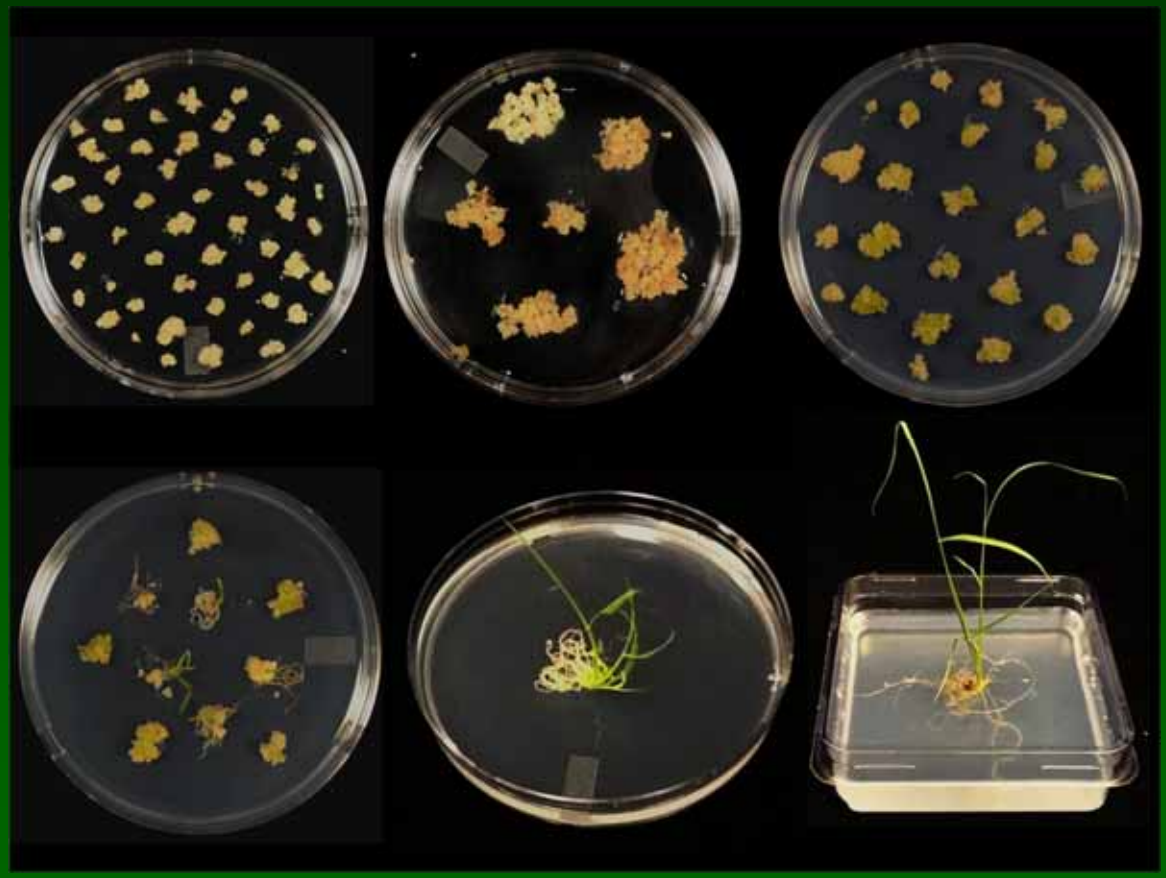
Morphogenesis

- **Dedifferentiation to regenerablity**
- **Developmental plasticity**
- **Terminal differentiation**
- **Embryogenic Stem Cells**

Monocot



Monocot



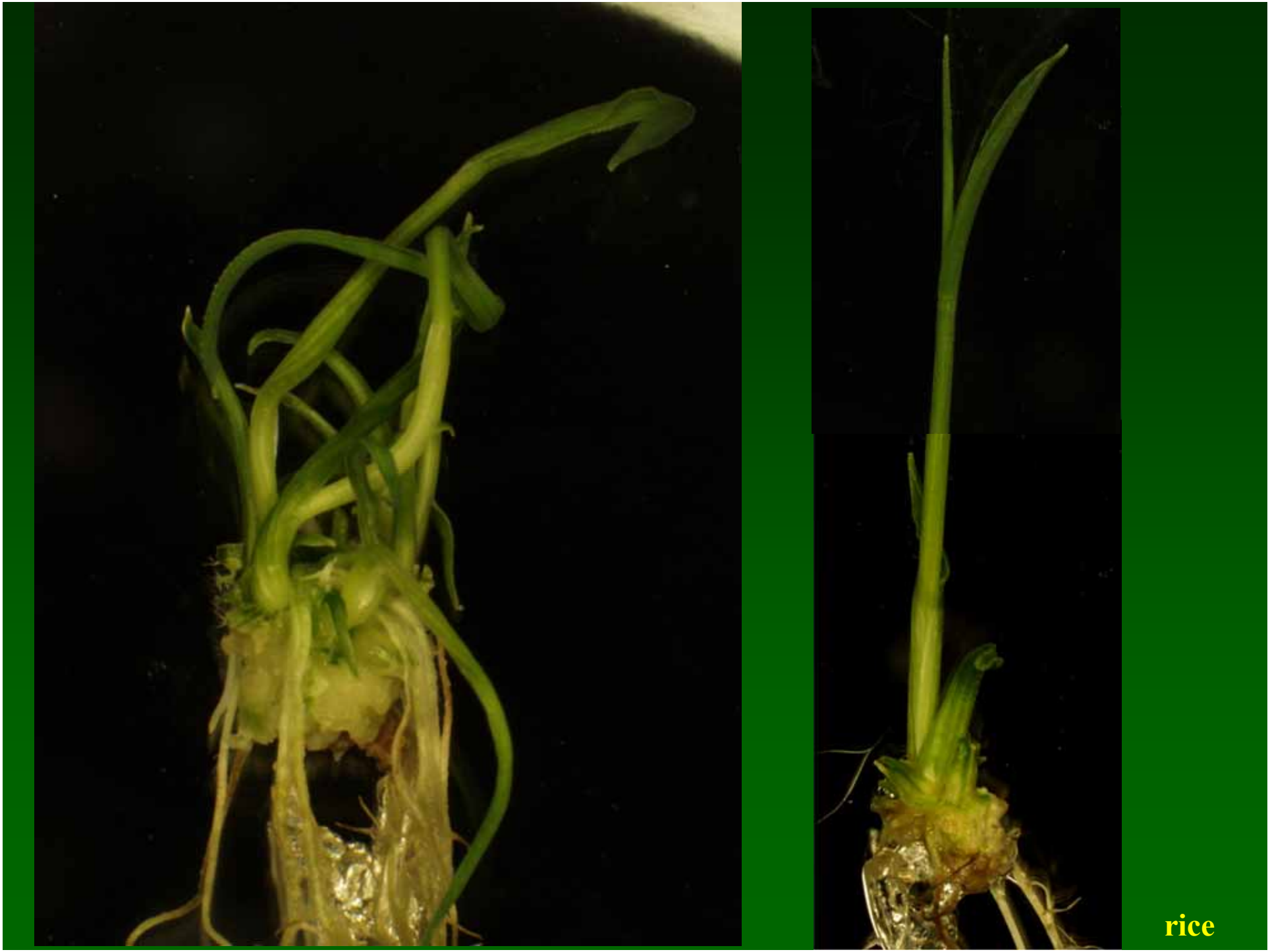
switchgrass

Monocot

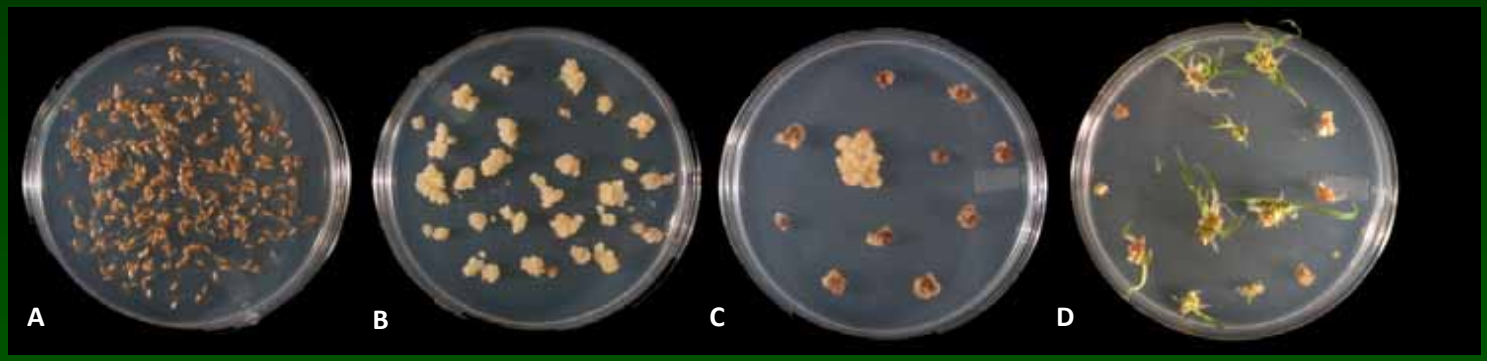
rice

Monocot

rice


rice

Monocot



switchgrass

Monocot

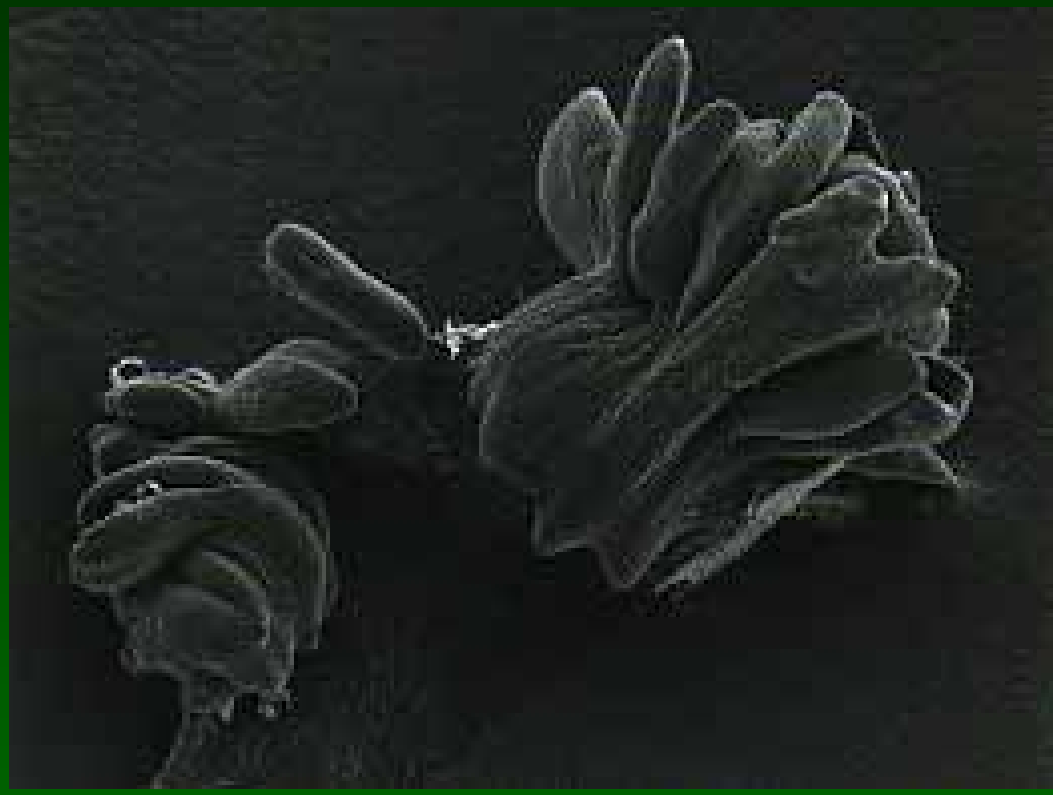
Type I vs Type II Response

Somatic Embryogenesis

In vitro embryos
go through the same stages
of development as embryos
in planta

Can be fused

Can form secondary embryos



Monocot

switchgrass

Somatic Embryogenesis

maize and other grasses

Type I And Type II Cultures

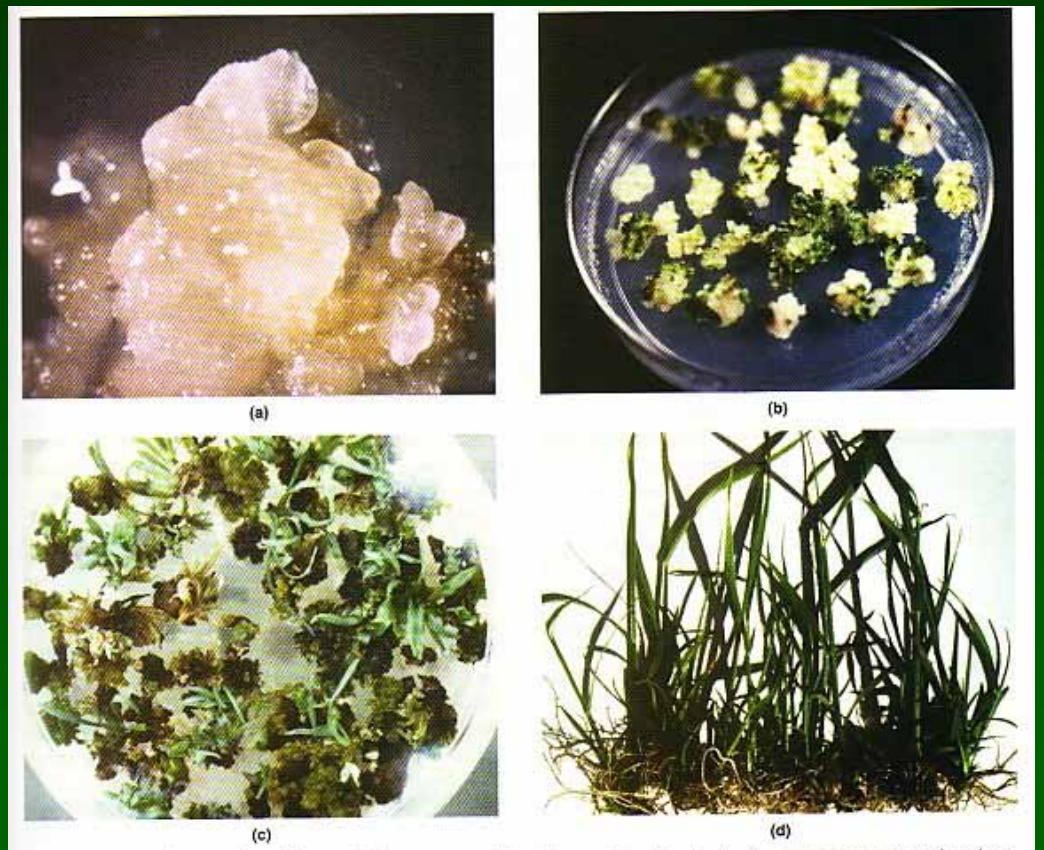
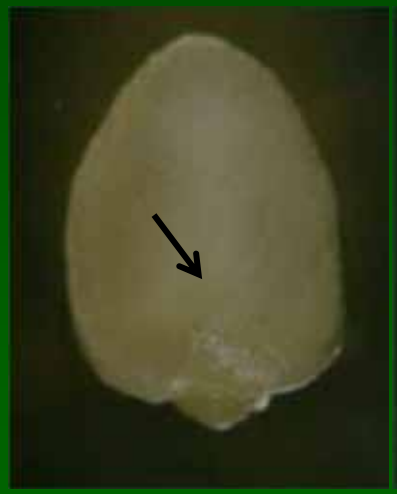


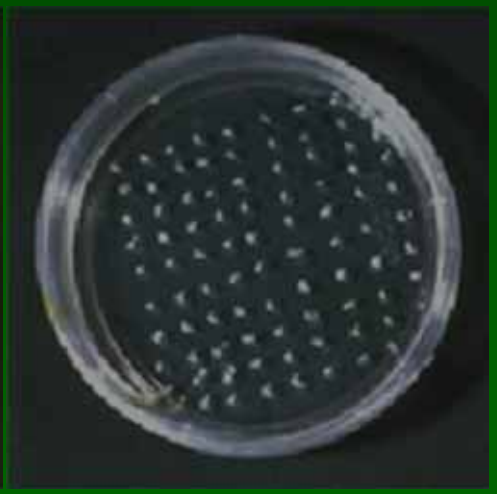
Figure 6.14 Embryogenic callus and plant regeneration. For most plants, the levels of two hormones, auxin and cytokinin, in the medium and the ratio of one to the other determine whether a small piece of tissue only forms callus (when both hormones are at high levels), sends out shoots (high cytokinin, low auxin), or sends out roots (high auxin, low cytokinin). **(a)** Proliferation of embryogenic callus derived from young leaf tissue. **(b)** Shoots began to regenerate from pieces of callus after the biologist reduced the concentration of cytokinin in the media. **(c)** When shoots have elongated, the biologist cut them off the callus and placed them on a media with a high auxin concentration. **(d)** Roots develop on media with a high auxin concentration. In this example, the plant is sugarcane. *Source:* Courtesy of James E. Irvine and T. Erik Mirkov.

Monocot

maize

Monocot





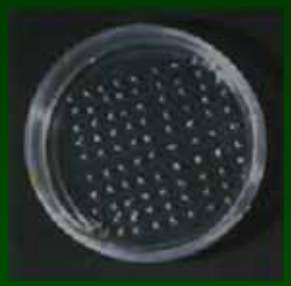
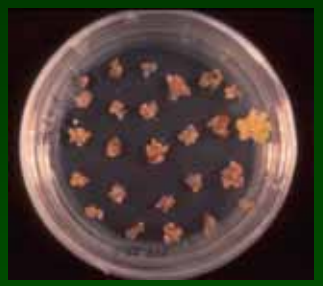
Recipient Cell Biology

Epidermal cells of abaxial side of immature embryo contribute to embryogenesis callus formation

maize


9
10
11
12 DAP

Monocot


maize

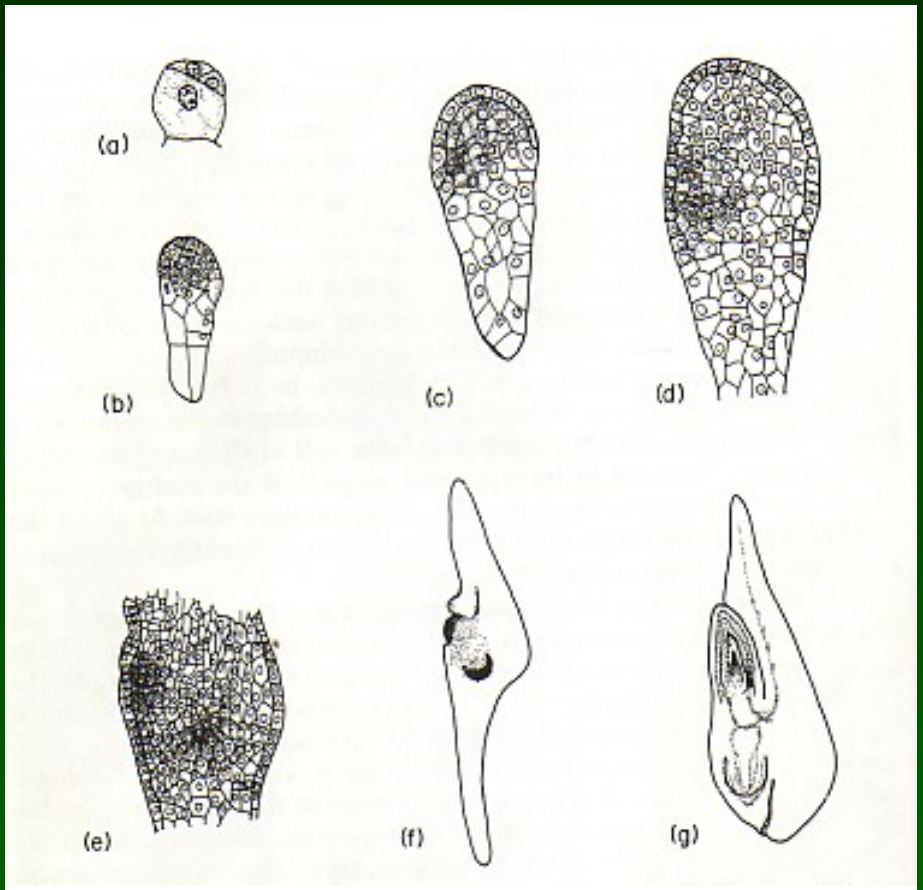
Monocot

Somatic embryogenic cultures

The Centrality of Embryogenesis for Dicot Transformation

When do monocot cells become terminally Differentiated?

S. Poethig (1980s)

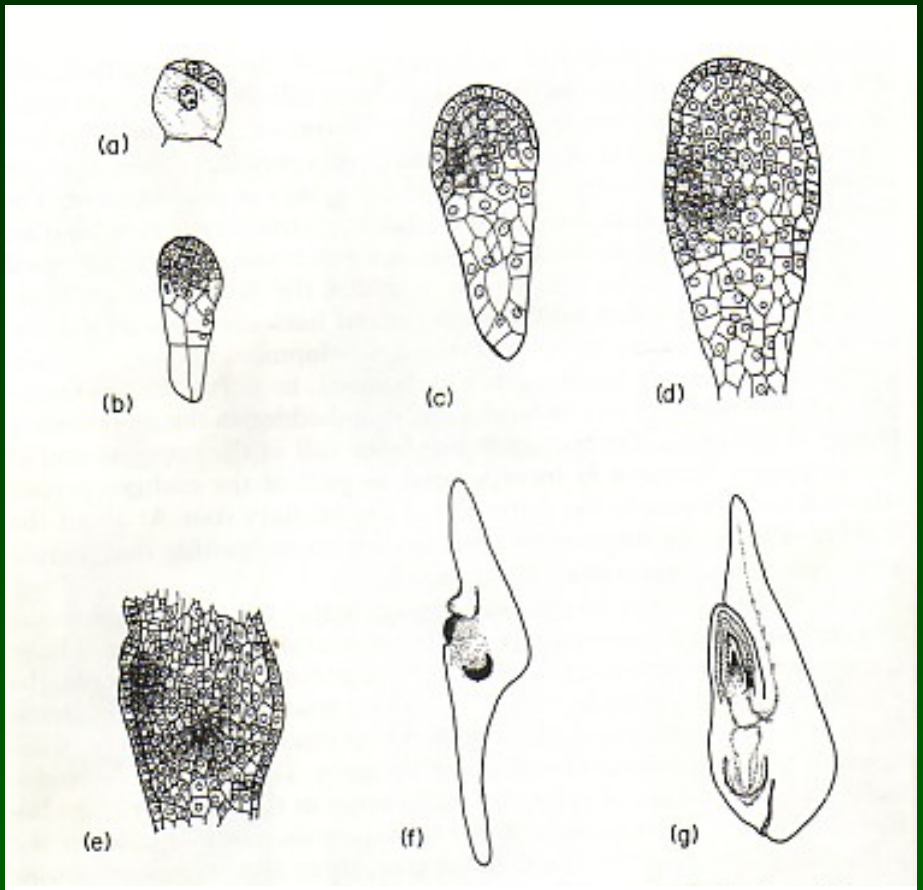


Zygotic embryo in maize Zea mays. (a) Three-celled embryo showing first division of terminal cell (b) Six day embryo showing embryo proper and suspensor. (c) Seven day embryo showing delimitation of the protoderm. (d) nine day embryo show merstem differentiation (from Randolf 1936)

maize

The Centrality of Embryogenesis for Dicot Transformation

Somatic embryogenic cultures in monocots must contain cells at stages in development similar to a six day old zygotic embryo



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maize

The Centrality of Embryogenesis for Dicot Transformation

For many monocots, reproducible and reliable transformation efficiency is still to be quite low .

The limitation of genotype dependence and/or low transformation efficiencies in some species is due to several mutually inclusive criteria:

- **capability to produce early embryogenic cultures from a single transformed cell**
- **the proliferation amidst the senescing cells of the untransformed culture during selection**
- **retaining the potential for subsequent whole plant regeneration to fertile plants (totipotency).**
- ***Agrobacterium*-mediated transformation may involve only recipient target cells at the surface of tissues.**

Thank You