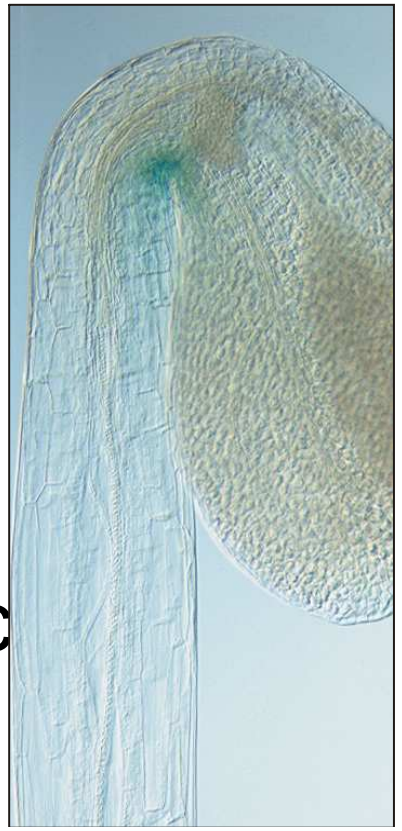
# Experimental biology

Description > Manipulation > Understanding

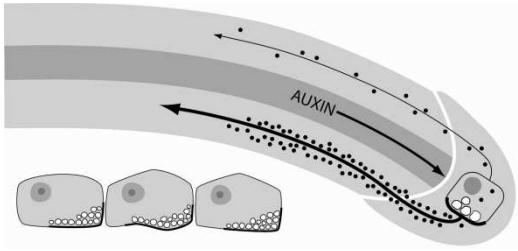
Money > Applications > Publishing

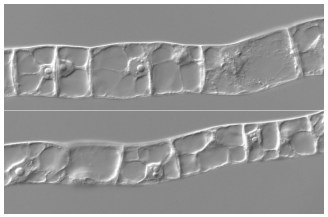


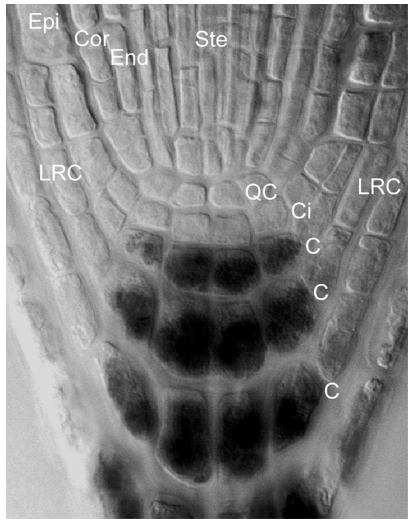
- Anatomy
- Physiology (spray and pray)
- Chemistry (identification of signals)
- Biochemistry (protein isolation/struc
- Genetics (genes/mutants)
- Cell biology (subcellular structures)
- Molecular biology (gene manipulation)

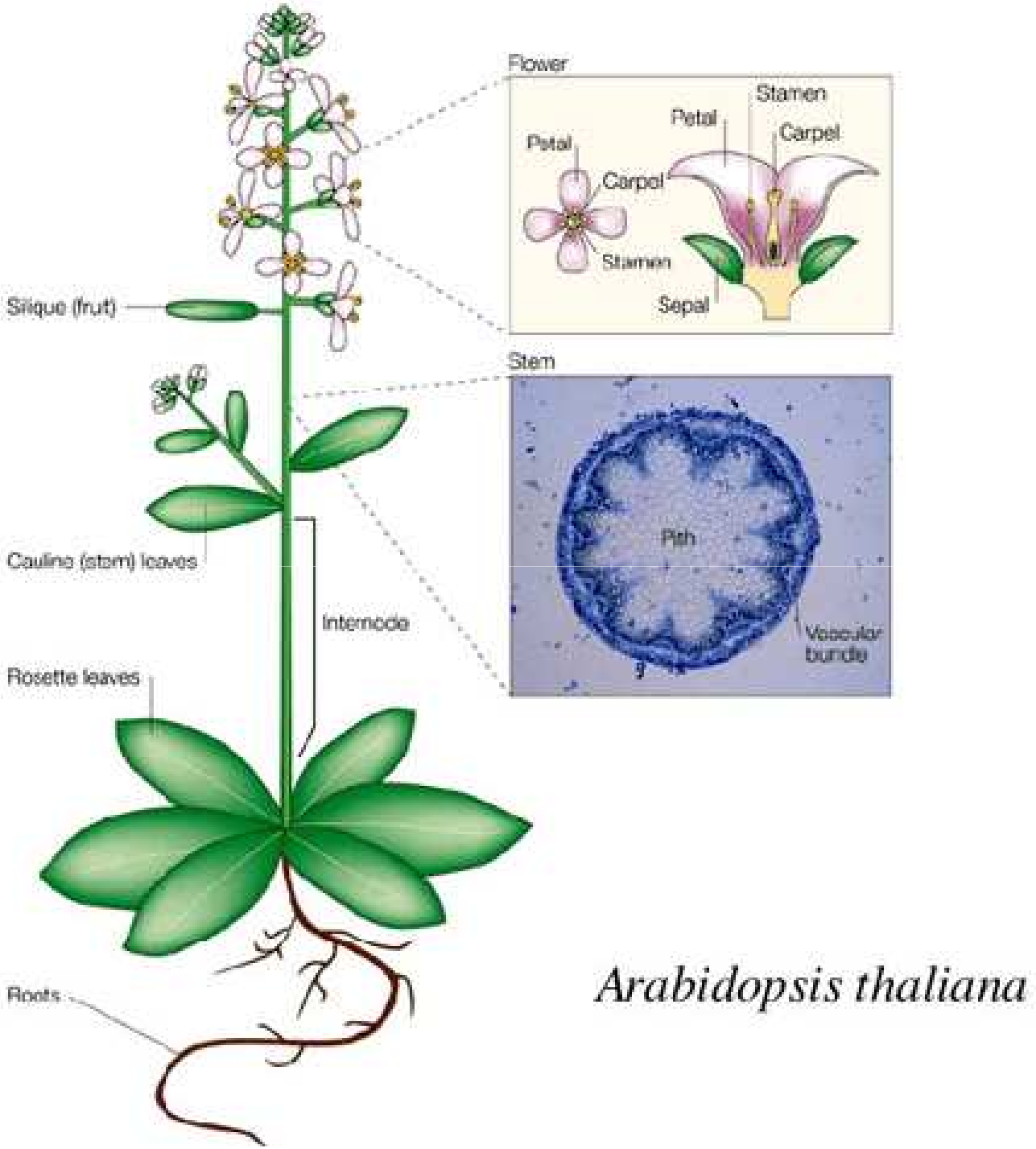
# Choice of research topic?

- Gene/Gene family
- Biological process
- Signaling pathway
- Model system
- Available methods
- „Trendy topic“
- Serendipity






*Arabidopsis thaliana*

# *Arabidopsis thaliana*

- Small, fully sequenced genome

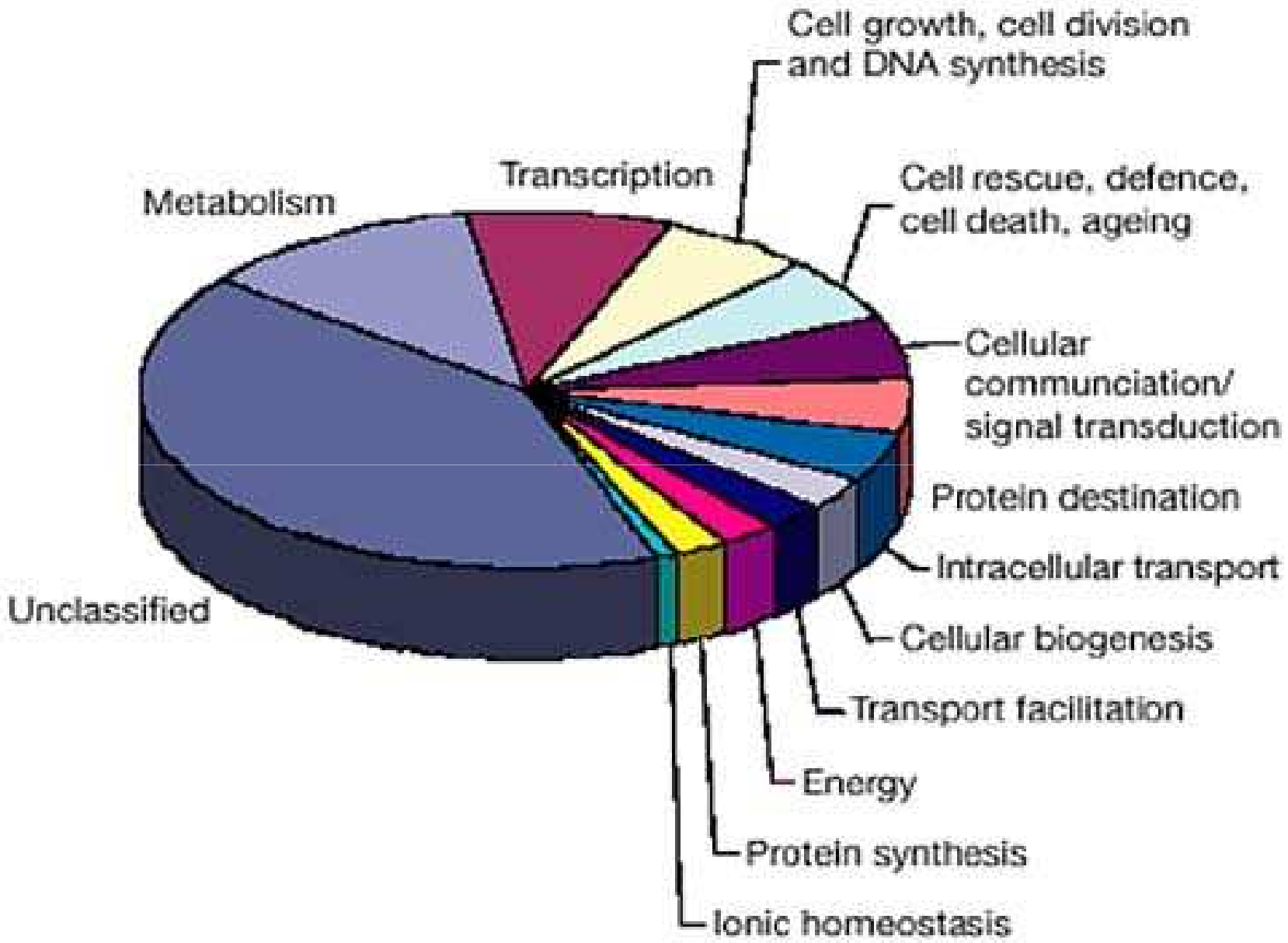
- Easy genetics (diploid/self-polinater)

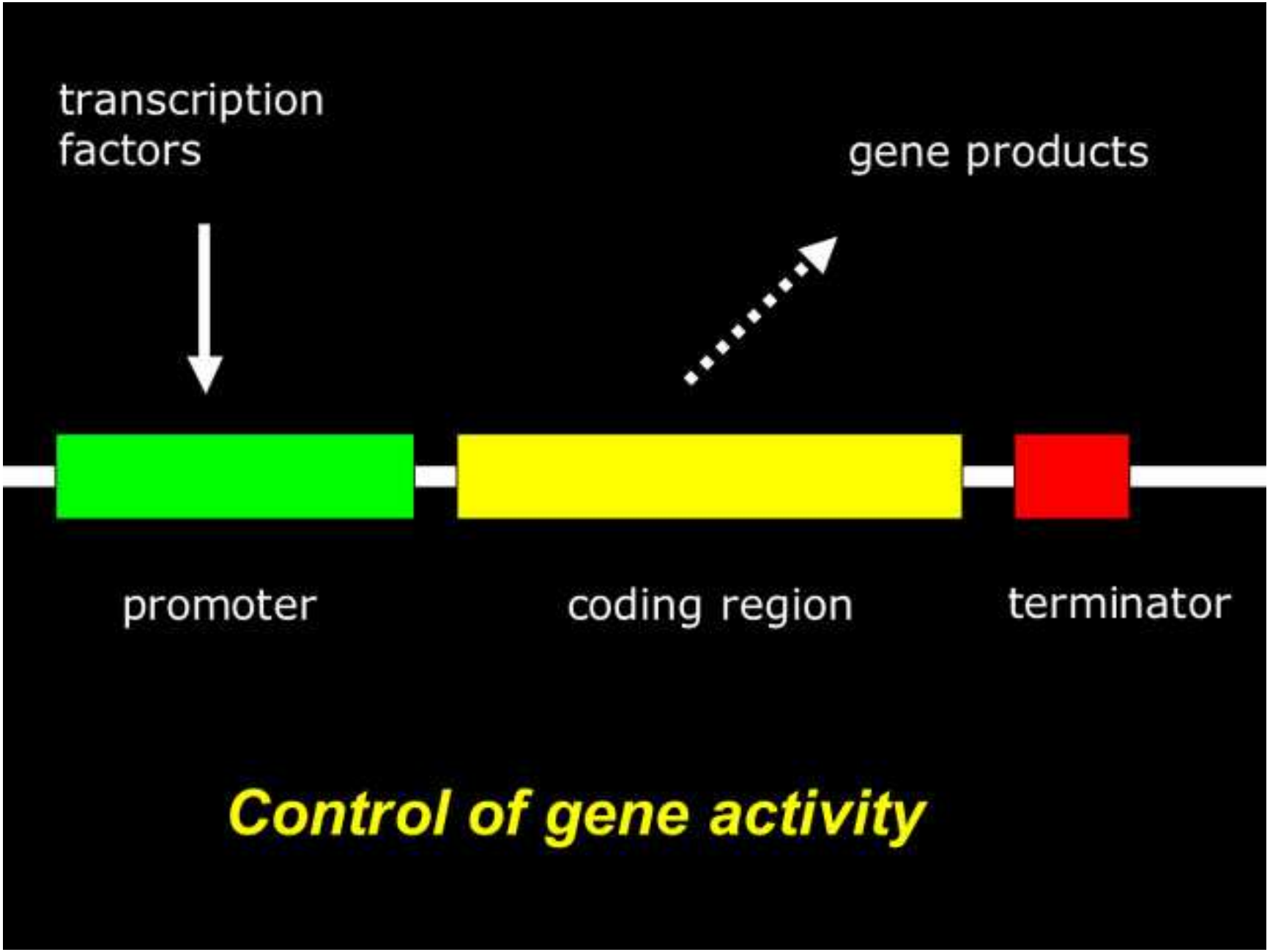
- Short vegetation time

- No large space requirement

- Simple organ and tissue structure

- Many established tools and facilities
  (transformation, libraries, databases)


Cell growth, cell division and DNA synthesis
Transcription
Metabolism
Cell rescue, defence, cell death, ageing
Cellular communciation/ signal transduction
Protein destination
Intracellular transport
Unclassified
Cellular biogenesis
Transport facilitation
Energy
Protein synthesis
Ionic homeostasis


transcription
factors
gene products
promoter
coding region
terminator
Control of gene activity

# How to get your favorite gene?

- “Lottery” candidate gene approach

- Functional complementation

- From the protein back to the gene

- Expression

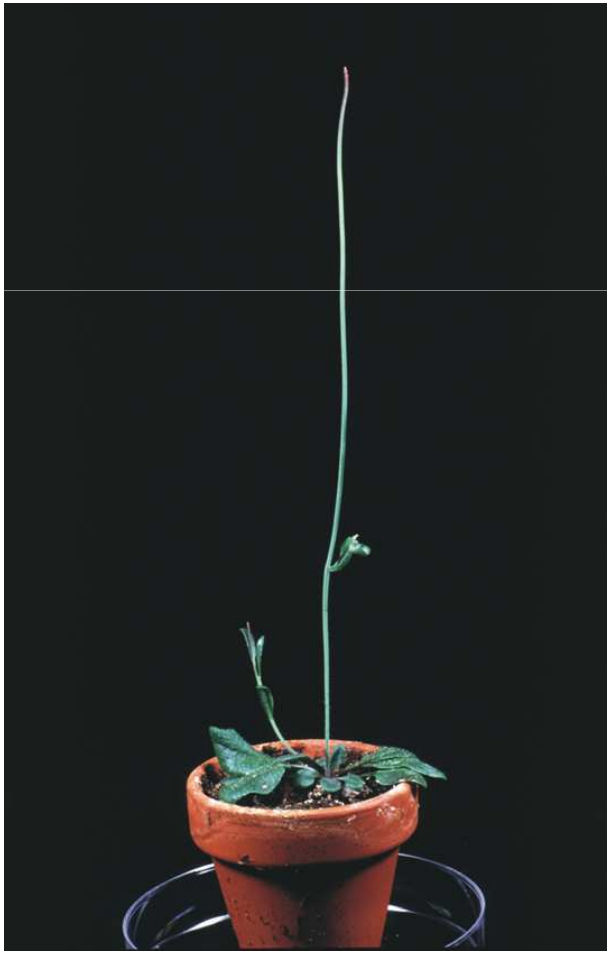
- Forward genetics

# “Lottery”

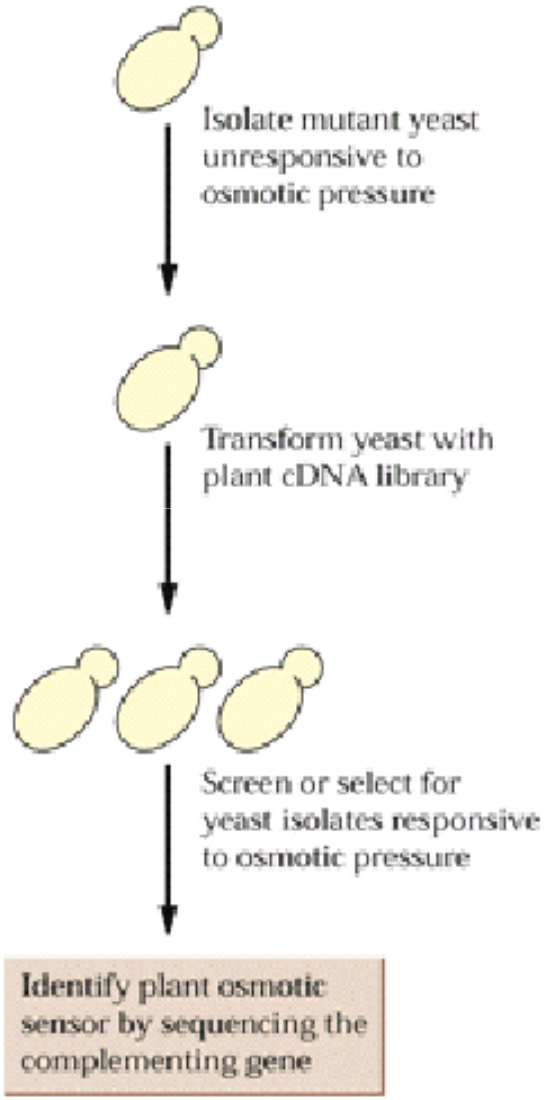
- Homology to known factors
  (trimeric G-proteins)

- Interesting domains
  (kinases, phosphatases)

- „Other“ reasons
  (serendipity)

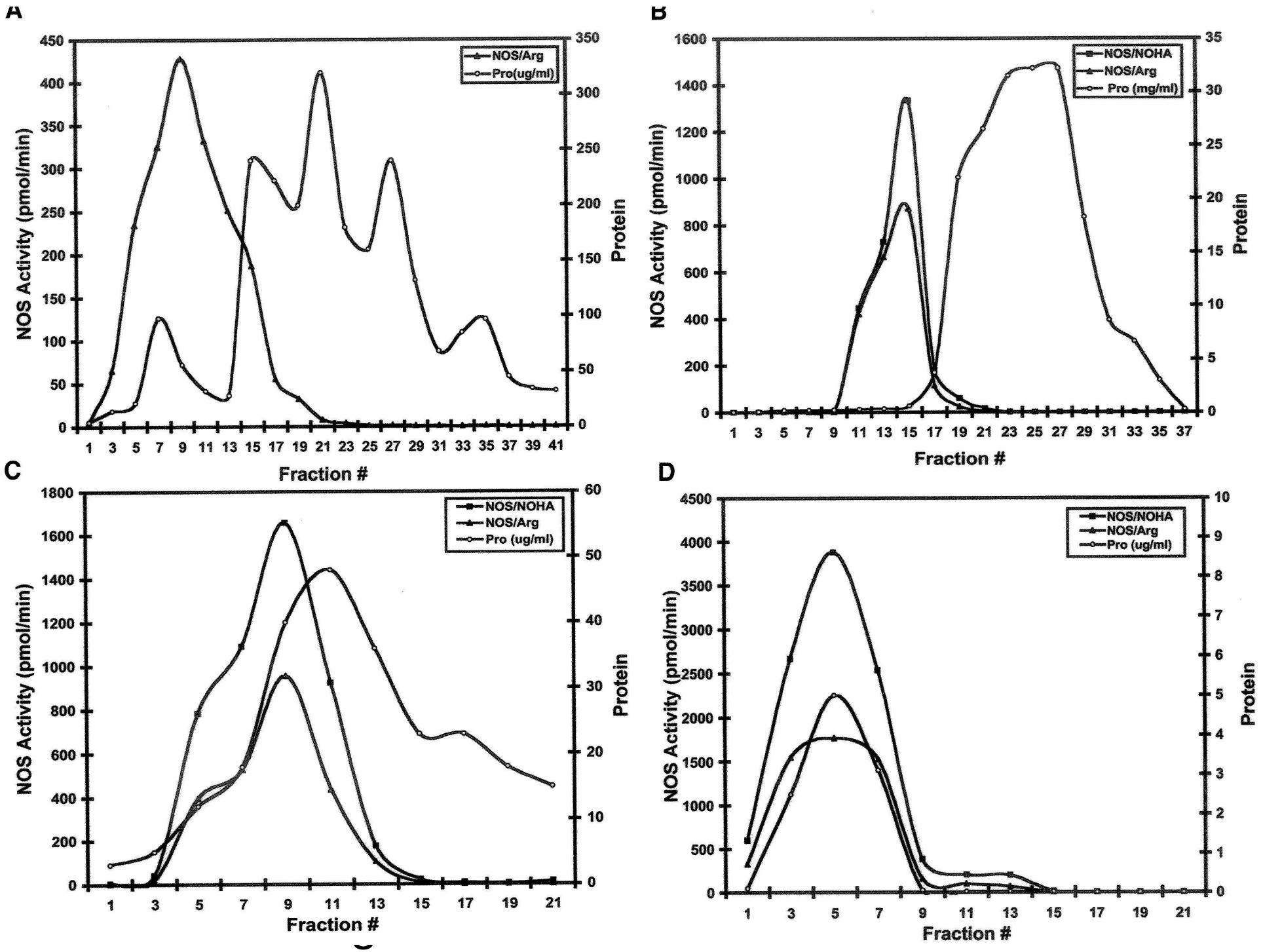


# Functional complementation



# Protein > gene

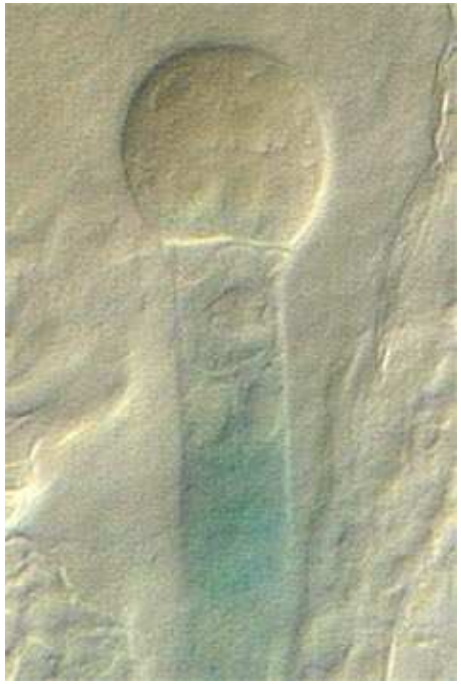
- Ligand binding (affinity chromatography, azidolabeling; ABP1, NPB, Zm-p60)
- Enzyme activity (CKX, NOS)
- Complex members
- Proteomics approaches (phosphoproteomics, differential display)
  - Microsequencing
  - Blast search:
    amino acid > nucleotide
  - Search for a gene

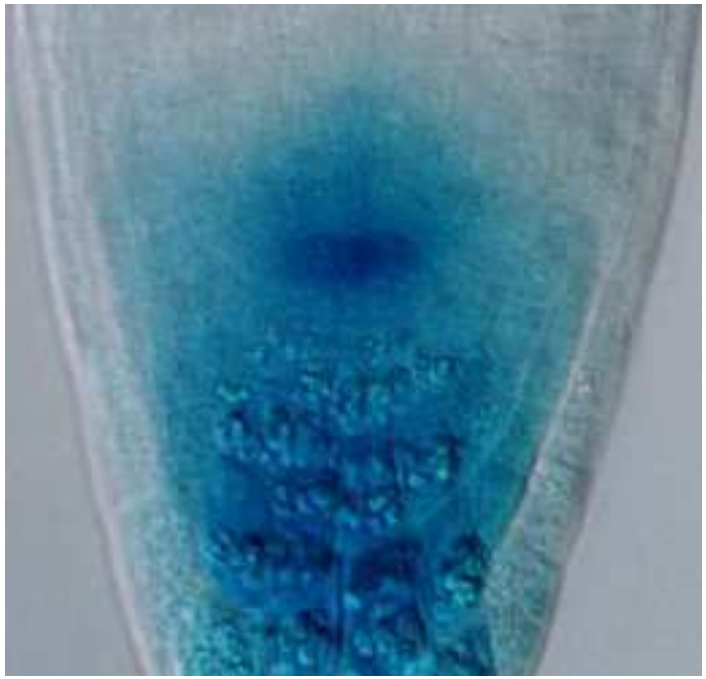

A
NOS/Arg
Pro(ug/ml)
NOS Activity (pmol/min)
Protein
Fraction #
B
NOS/NOHA
NOS/Arg
Pro (mg/ml)
NOS Activity (pmol/min)
Protein
Fraction #
C
NOS/NOHA
NOS/Arg
Pro (ug/ml)
NOS Activity (pmol/min)
Protein
Fraction #
D
NOS/NOHA
NOS/Arg
Pro (ug/ml)
NOS Activity (pmol/min)
Protein
Fraction #

# Expression pattern

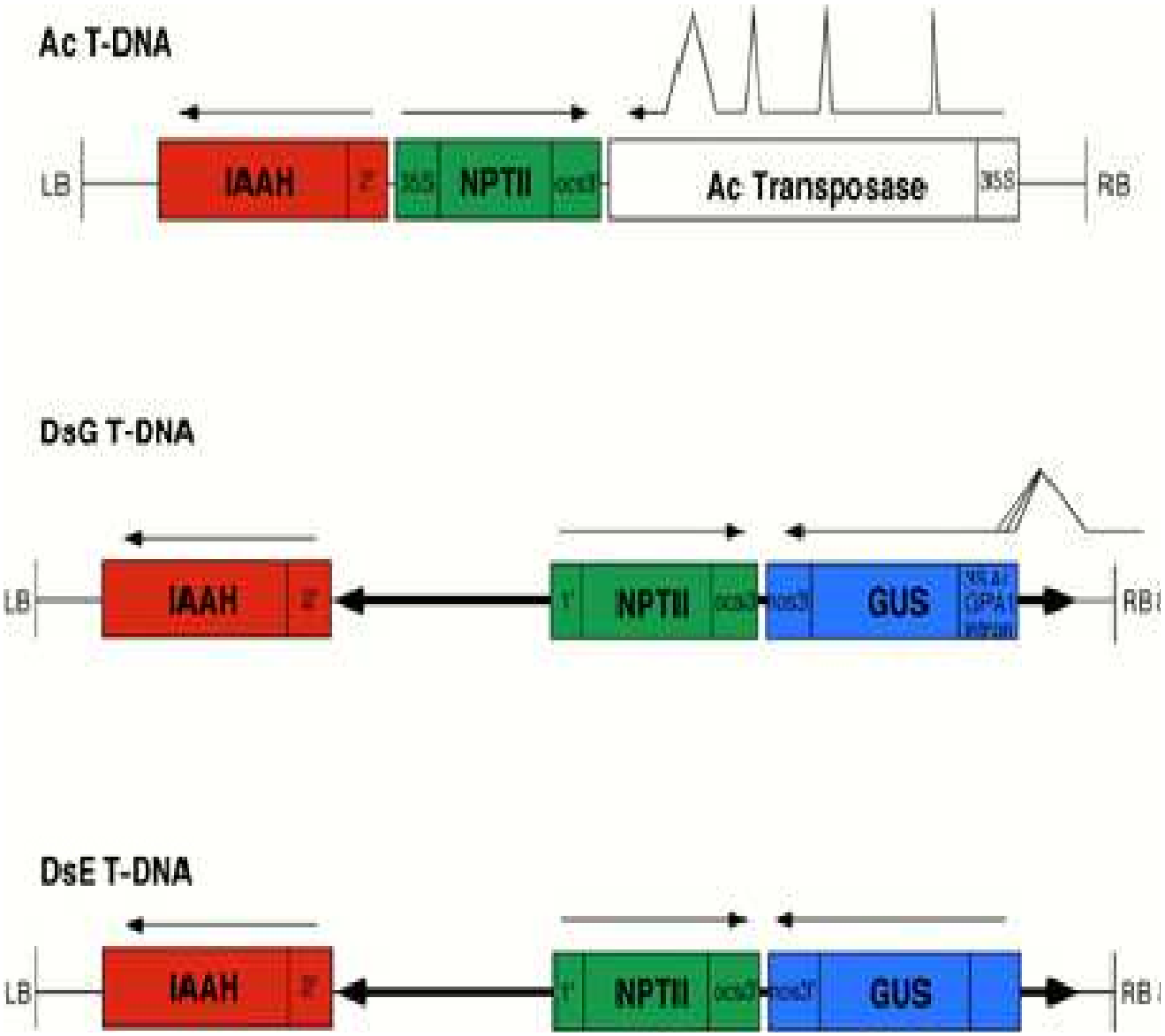
- Enhancer/Gene-trap libraries

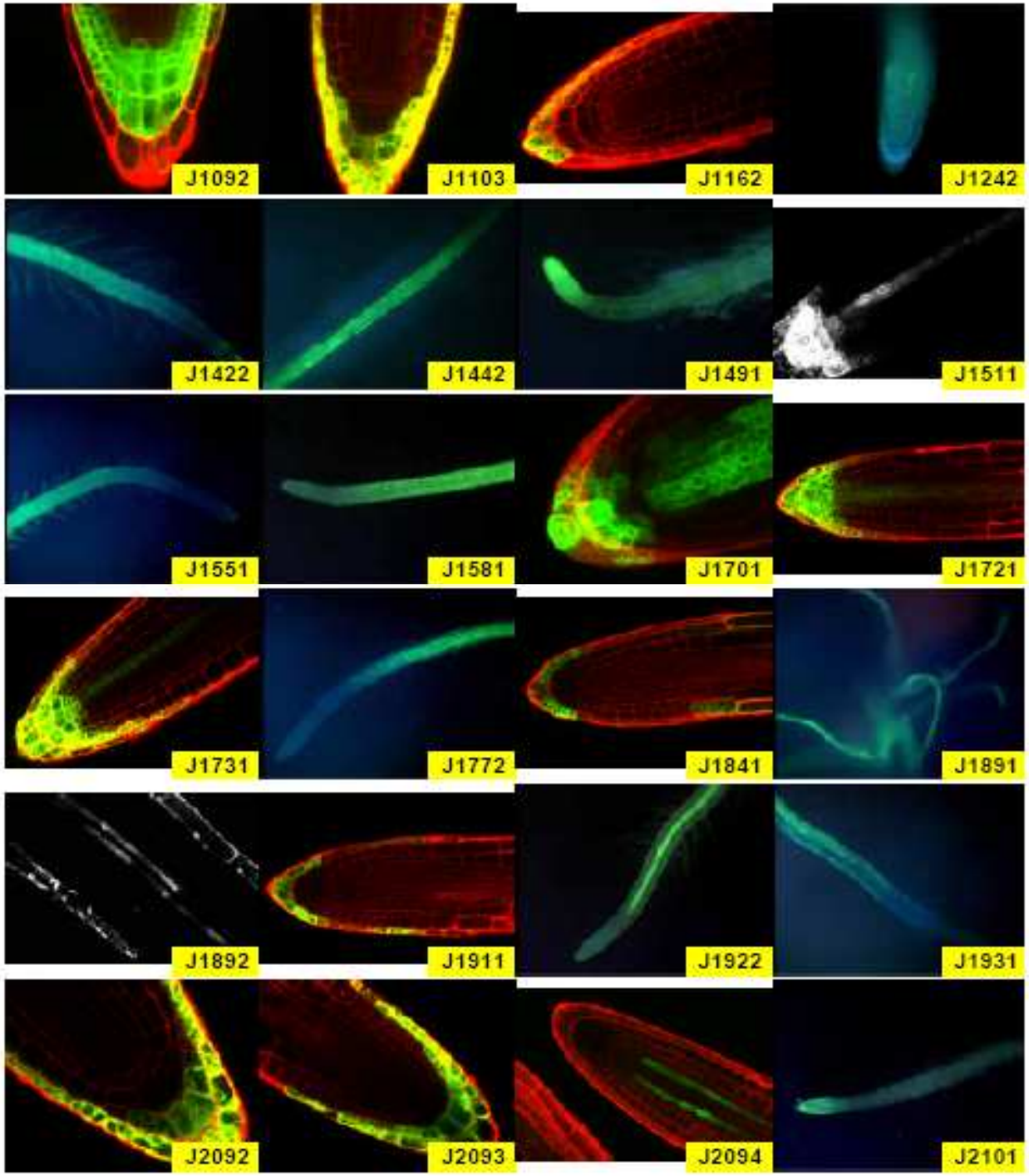
- Differential display
  - substractive hybridisation
  - microarray



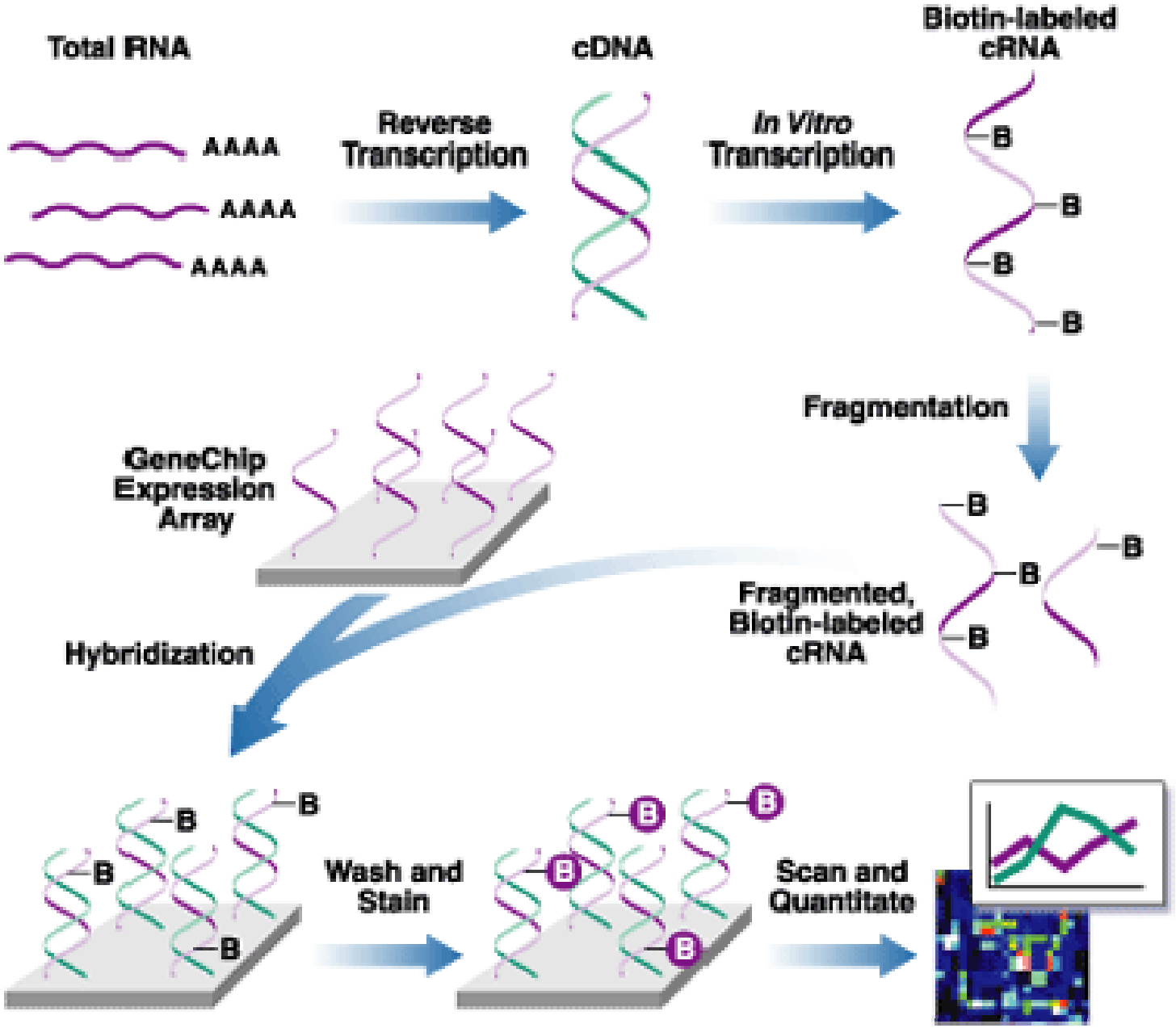


# Gene and enhancer trap libraries

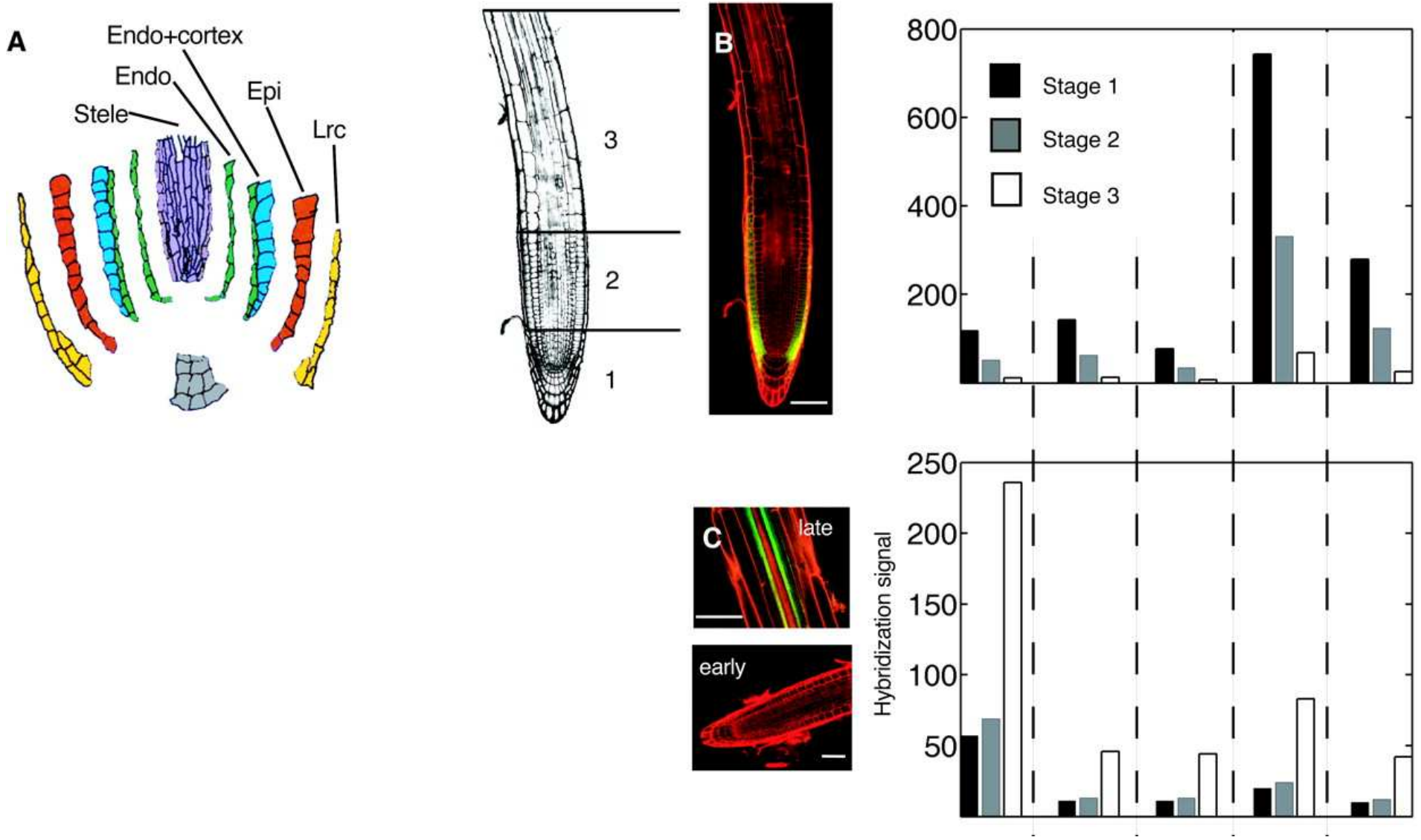



J1092
J1103
J1162
J1242
J1422
J1442
J1491
J1511
J1551
J1581
J1701
J1721
J1731
J1772
J1841
J1891
J1892
J1911
J1922
J1931
J2092
J2093
J2094
J2101

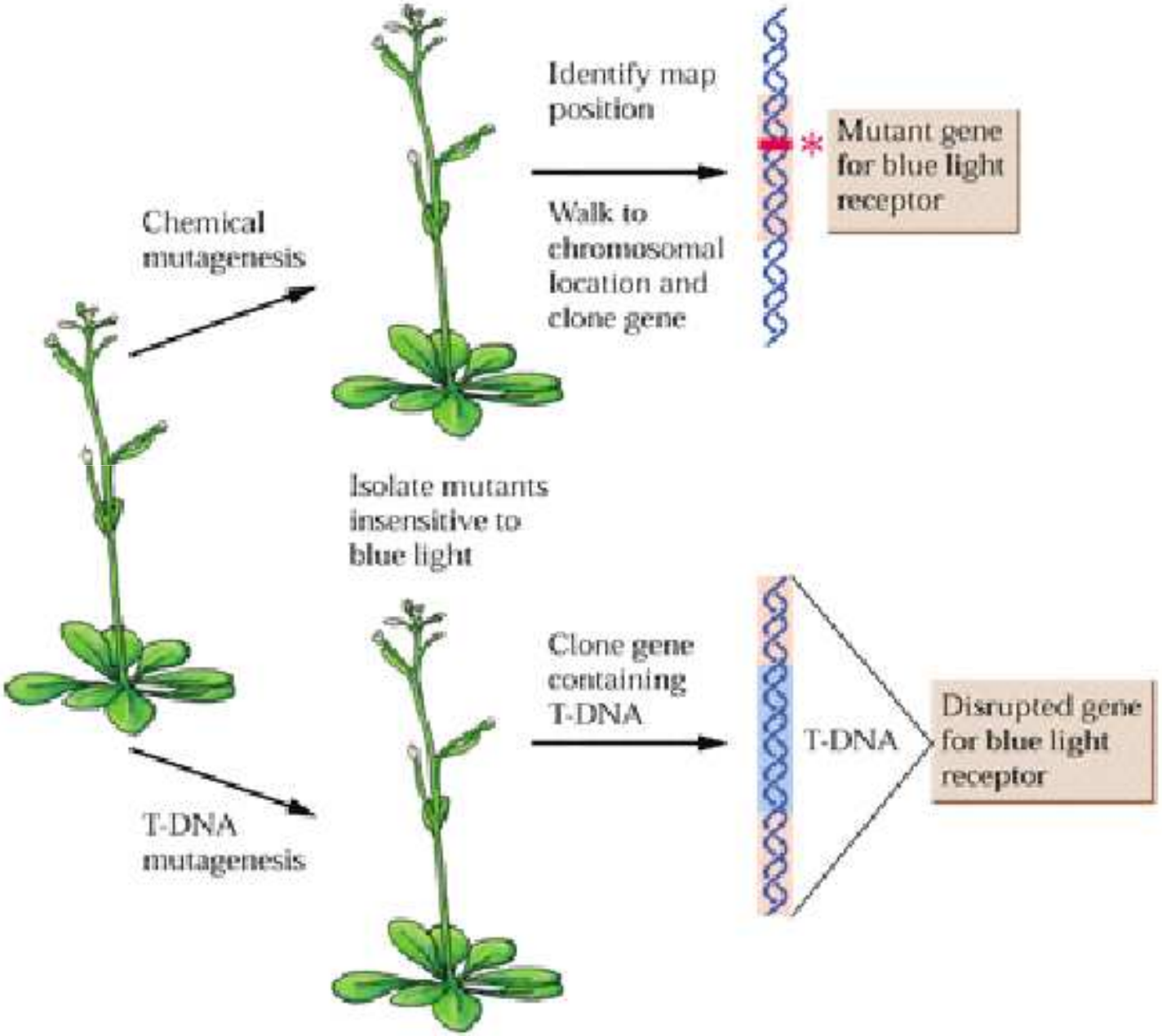
# Microarray


Total RNA
AAAA
AAAA
AAAA
Reverse Transcription
cDNA
In Vitro Transcription
Biotin-labeled cRNA
B
B
B
B
Fragmentation
GeneChip Expression Array
Hybridization
Fragmented, Biotin-labeled cRNA
B
B
B
B
B
B
B
B
B
B
Wash and Stain
Scan and Quantitate

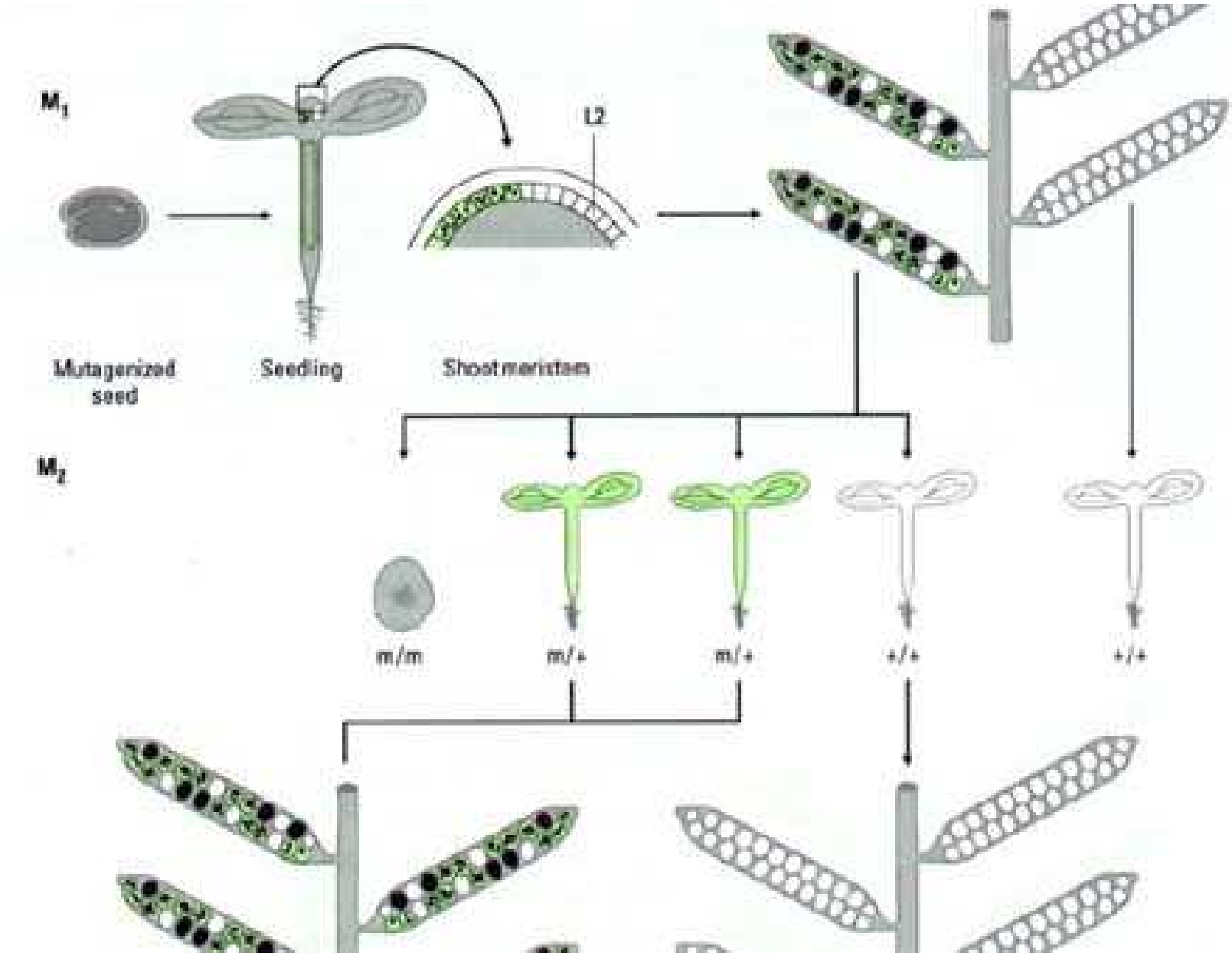
# Expression map of Arabidopsis root



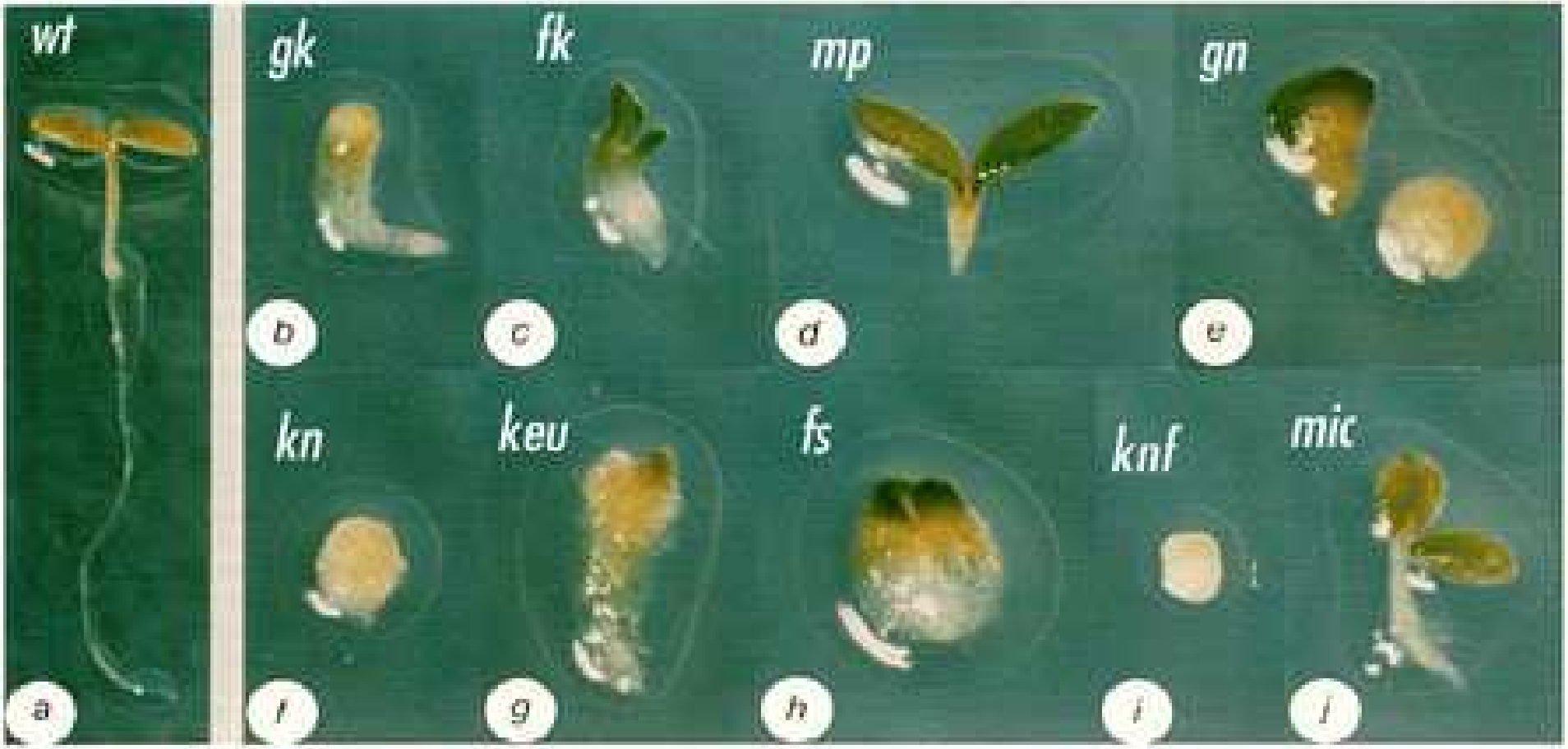
# Forward genetics



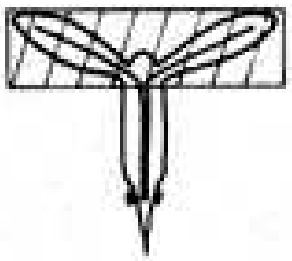
# EMS mutagenesis



# Mutant screen at seedling level



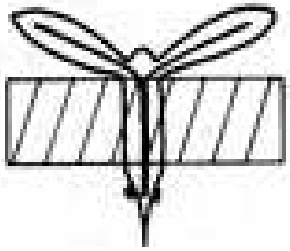
# Patterning mutant types



APICAL

*(gurke)*

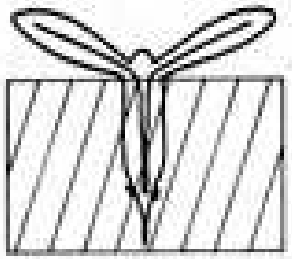
Fatty acids



CENTRAL

*(fackel)*

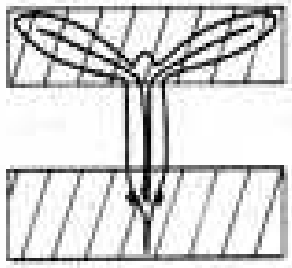
Sterols



BASAL

*(monopterous)*

Signalling

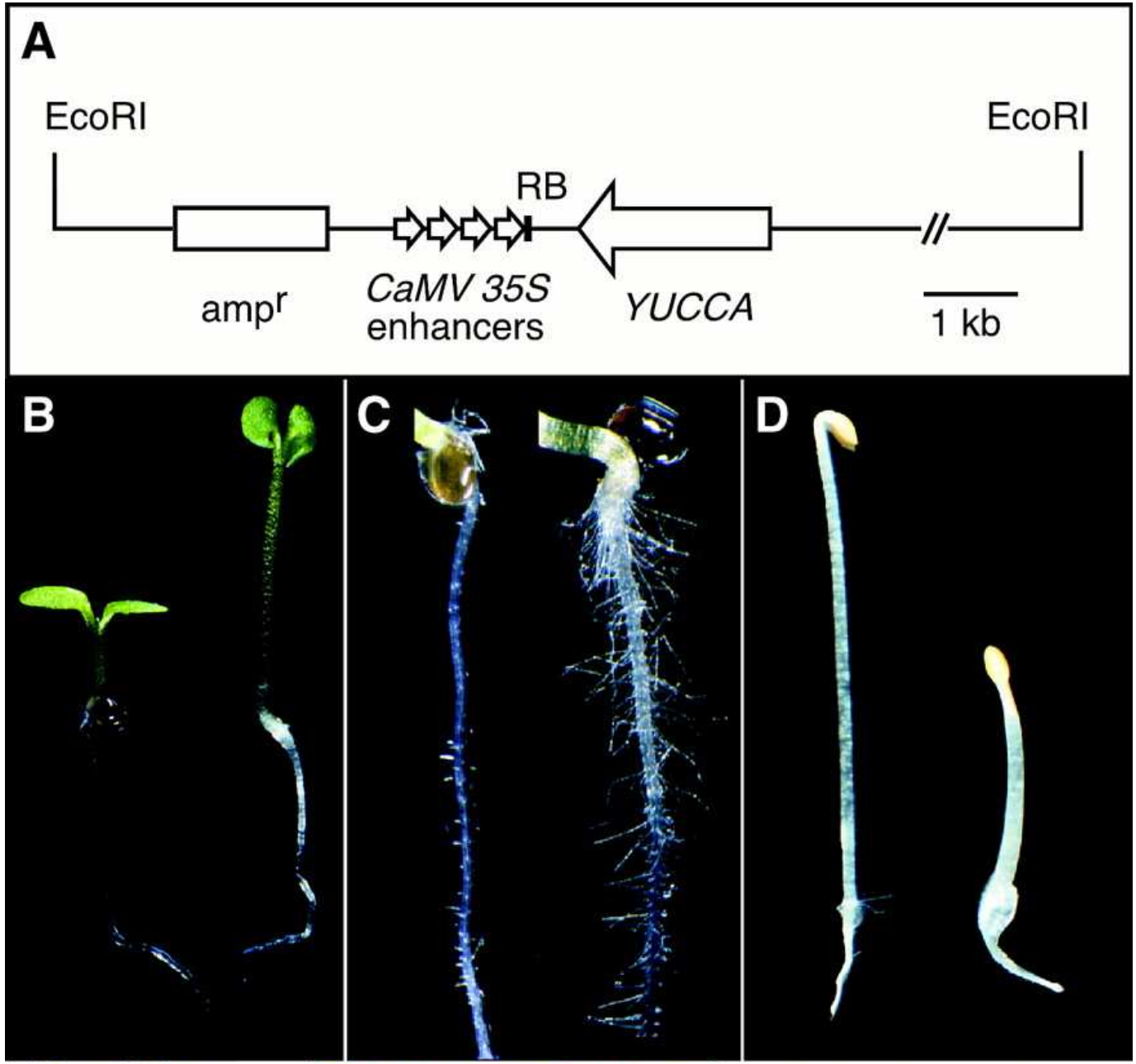


TERMINAL

*(gnom)*

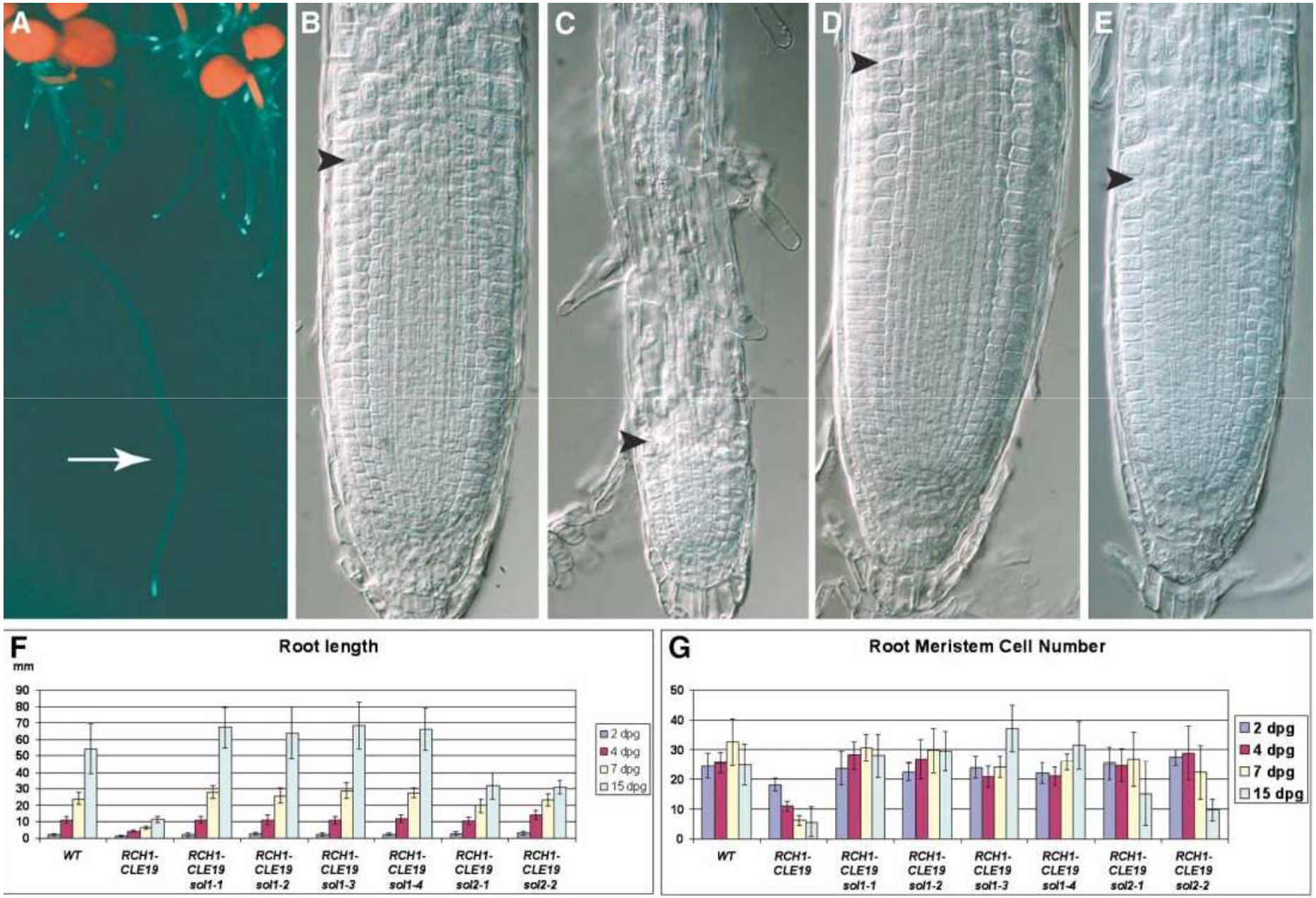
Vesicle traffic

# Activation tagging - YUCCA

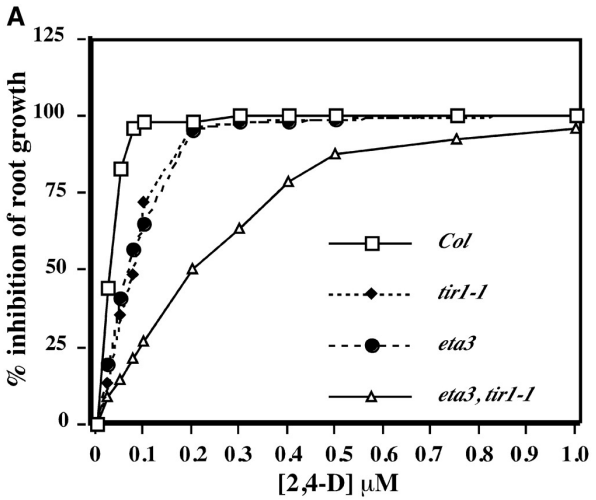


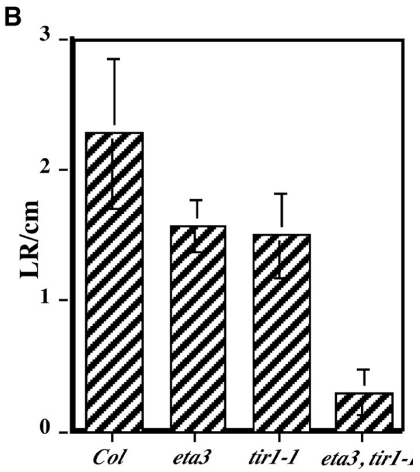
# Second site mutagenesis - suppressors

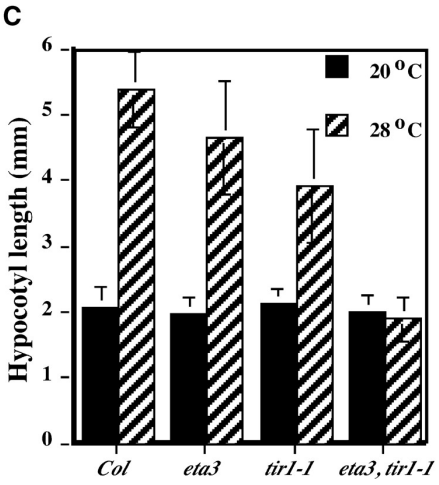
# Suppressors of CLV3 overexpression



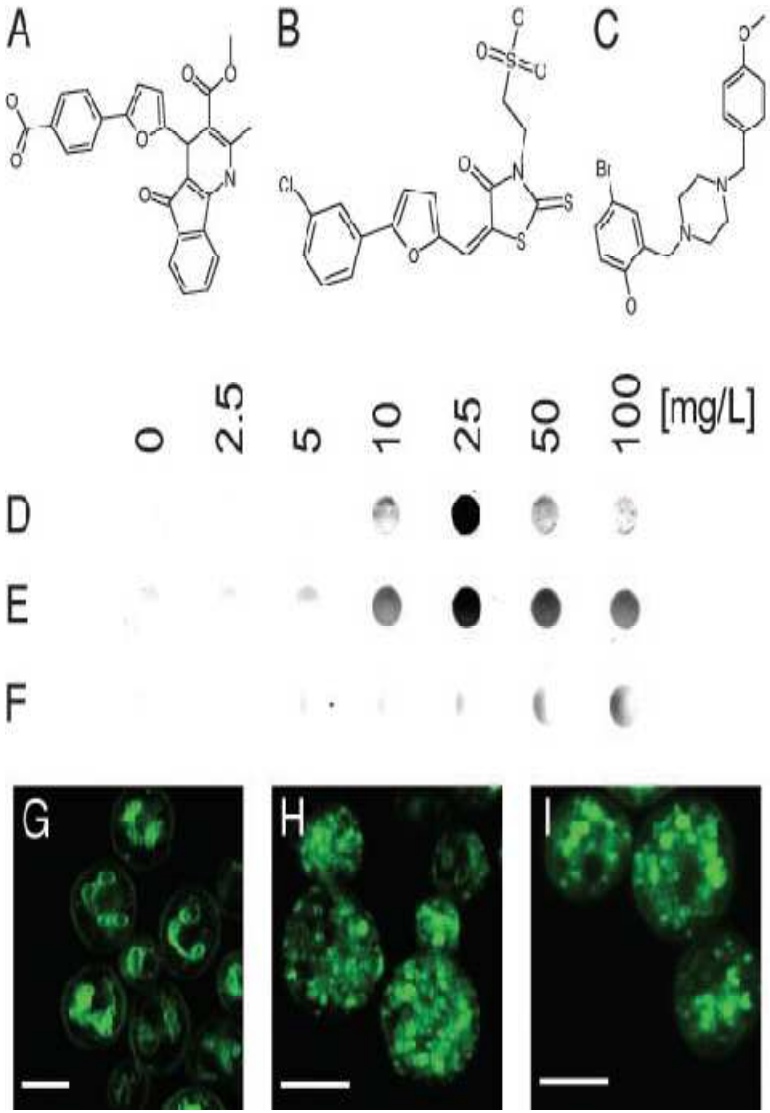
# Second site mutagenesis - enhancers

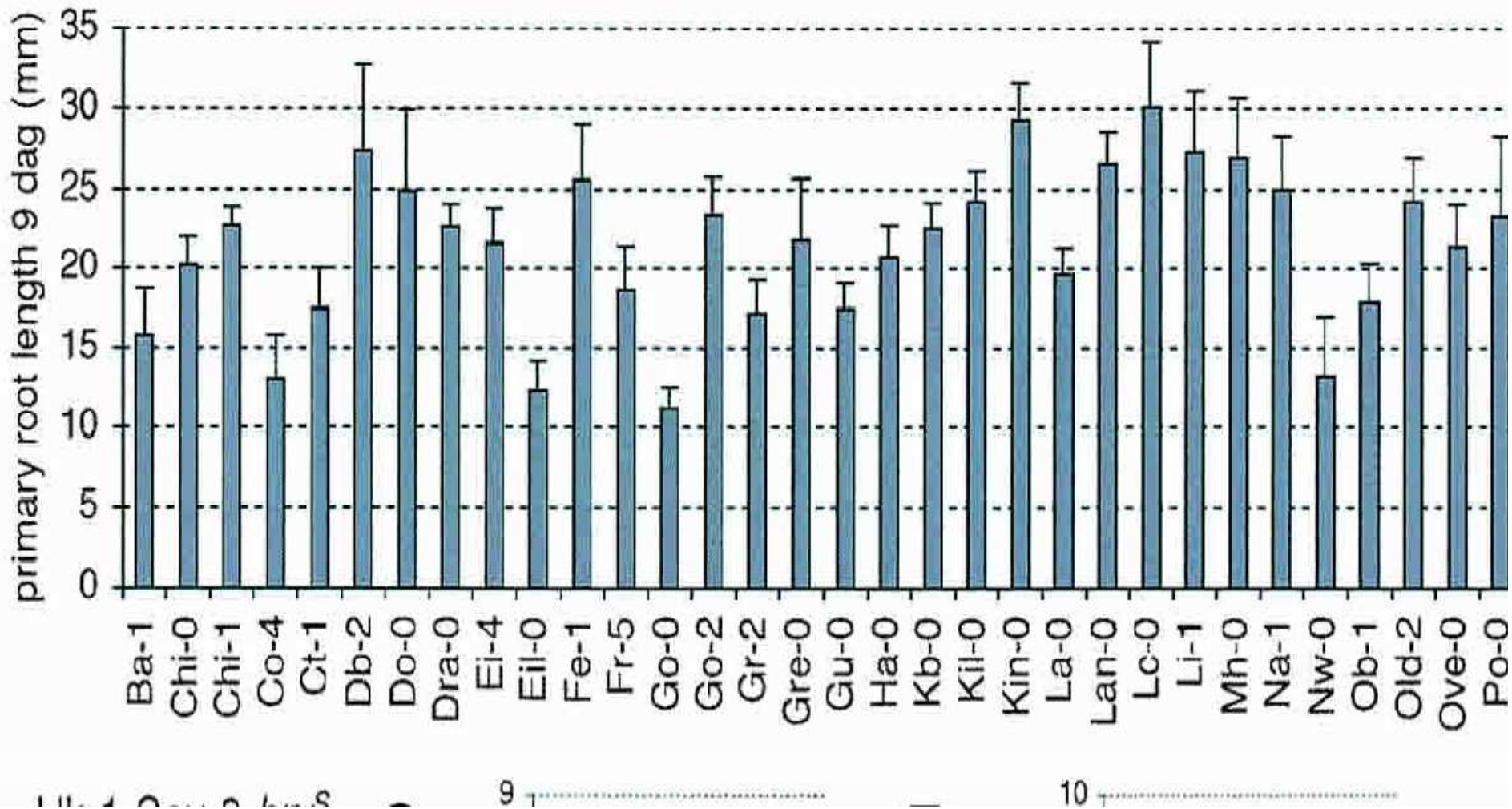



# Chemical genetics




Zouhar et al., 2004

# QTL


primary root length 9 dag (mm)
35
30
25
20
15
10
5
0
Ba-1
Chi-0
Chi-1
Co-4
Ct-1
Db-2
Do-0
Dra-0
Ei-4
Eil-0
Fe-1
Fr-5
Go-0
Go-2
Gr-2
Gre-0
Gu-0
Ha-0
Kb-0
Kil-0
Kin-0
La-0
Lan-0
Lc-0
Li-1
Mh-0
Na-1
Nw-0
Ob-1
Old-2
Ove-0
Po-0
9
10

# Gene verification

- Multiple alleles
- Transposone reversion
- Complementation

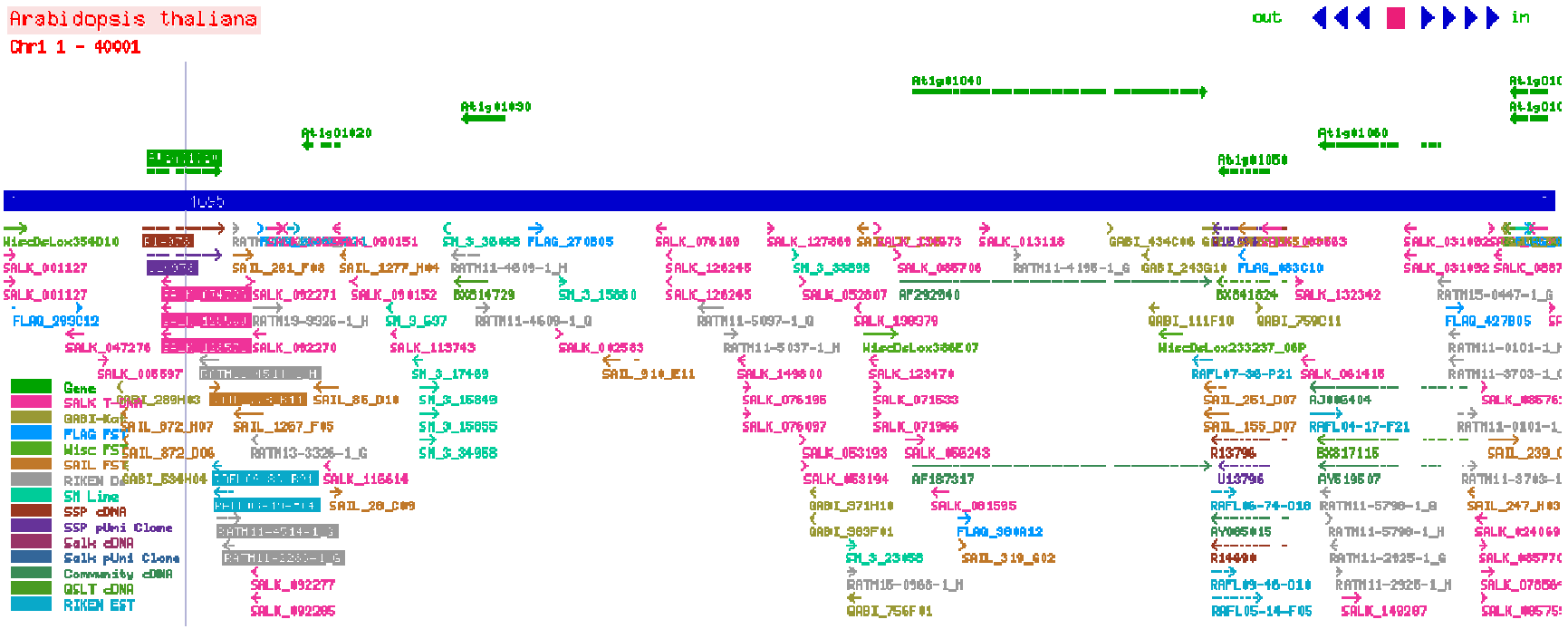
# Towards a gene role

- Loss of function: Reverse genetics
- Gain of function: Ectopic expression
- Mosaics
- Sequence manipulations
- Phenotype analysis
- Biochemical function

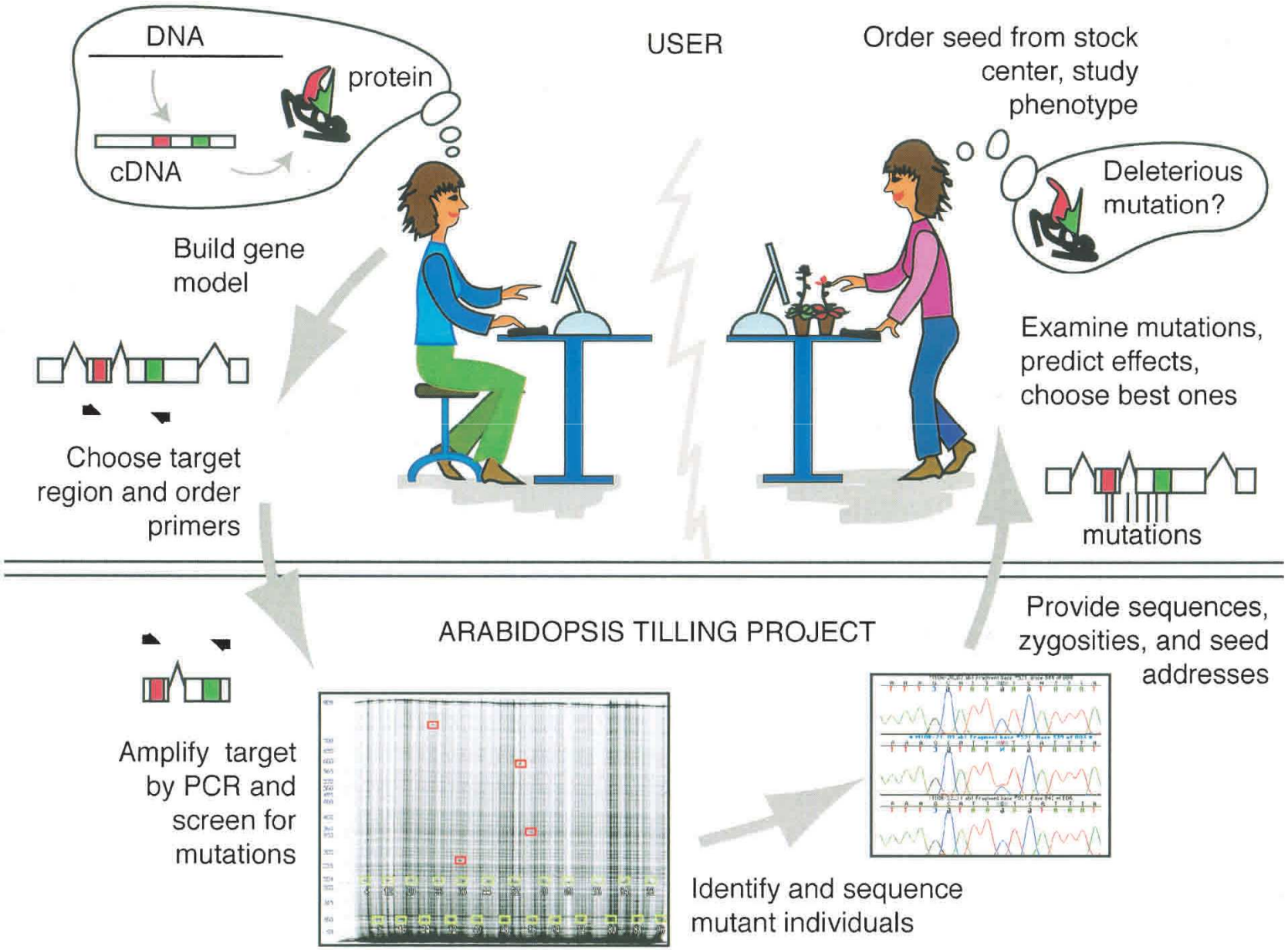
# Loss of function

- Reverse genetics/TILLING
- Antisense and RNAi approaches
- Immunomodulation
- Repression domain
- Titration

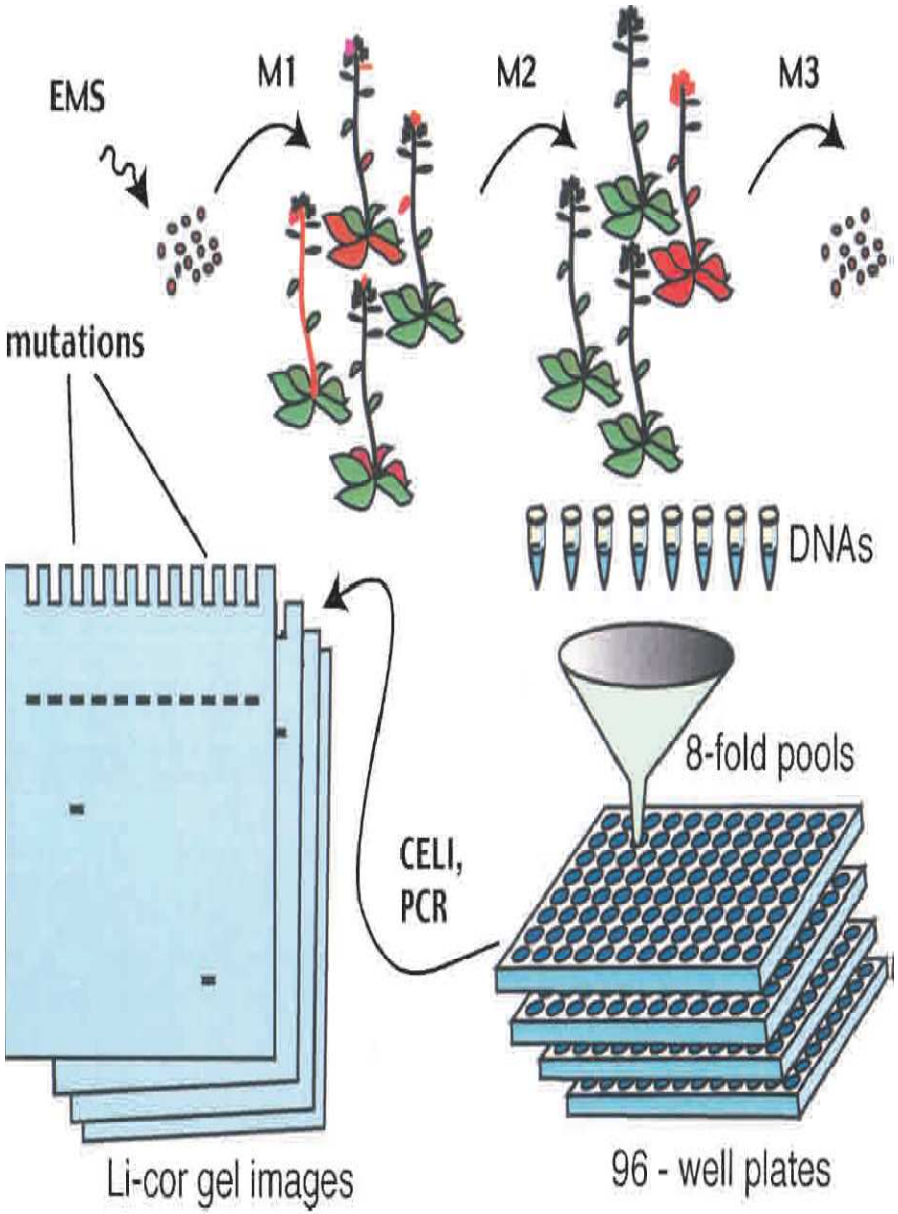
# Reverse genetics – indexed mutant libraries



# TILLING


DNA
protein
cDNA
USER
Order seed from stock center, study phenotype
Deleterious mutation?
Build gene model
Examine mutations, predict effects, choose best ones
Choose target region and order primers
mutations
ARABIDOPSIS TILLING PROJECT
Provide sequences, zygosities, and seed addresses
Amplify target by PCR and screen for mutations
Identify and sequence mutant individuals

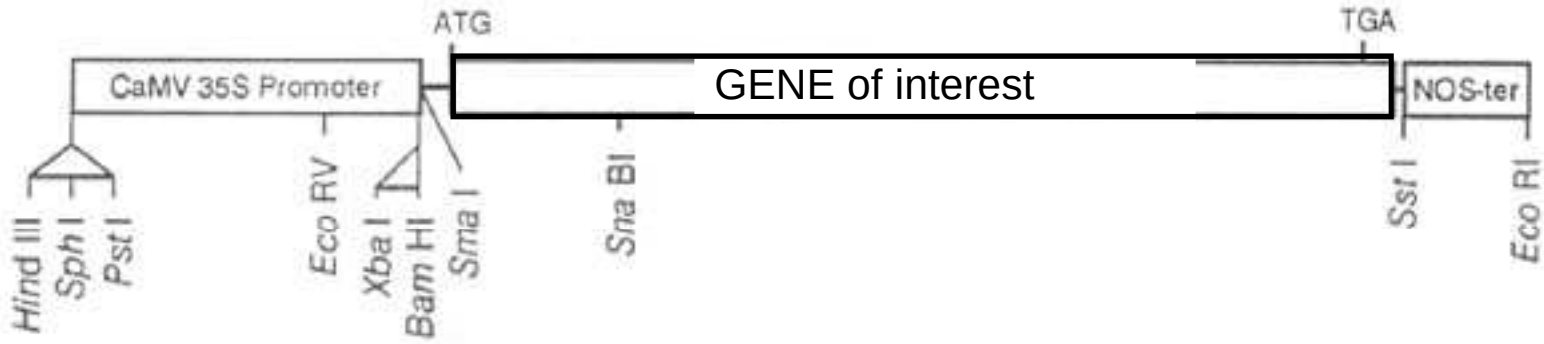
# TILLING


EMS
M1
M2
M3
mutations
DNAs
8-fold pools
CELI,
PCR
Li-cor gel images
96 - well plates

# Gain of function

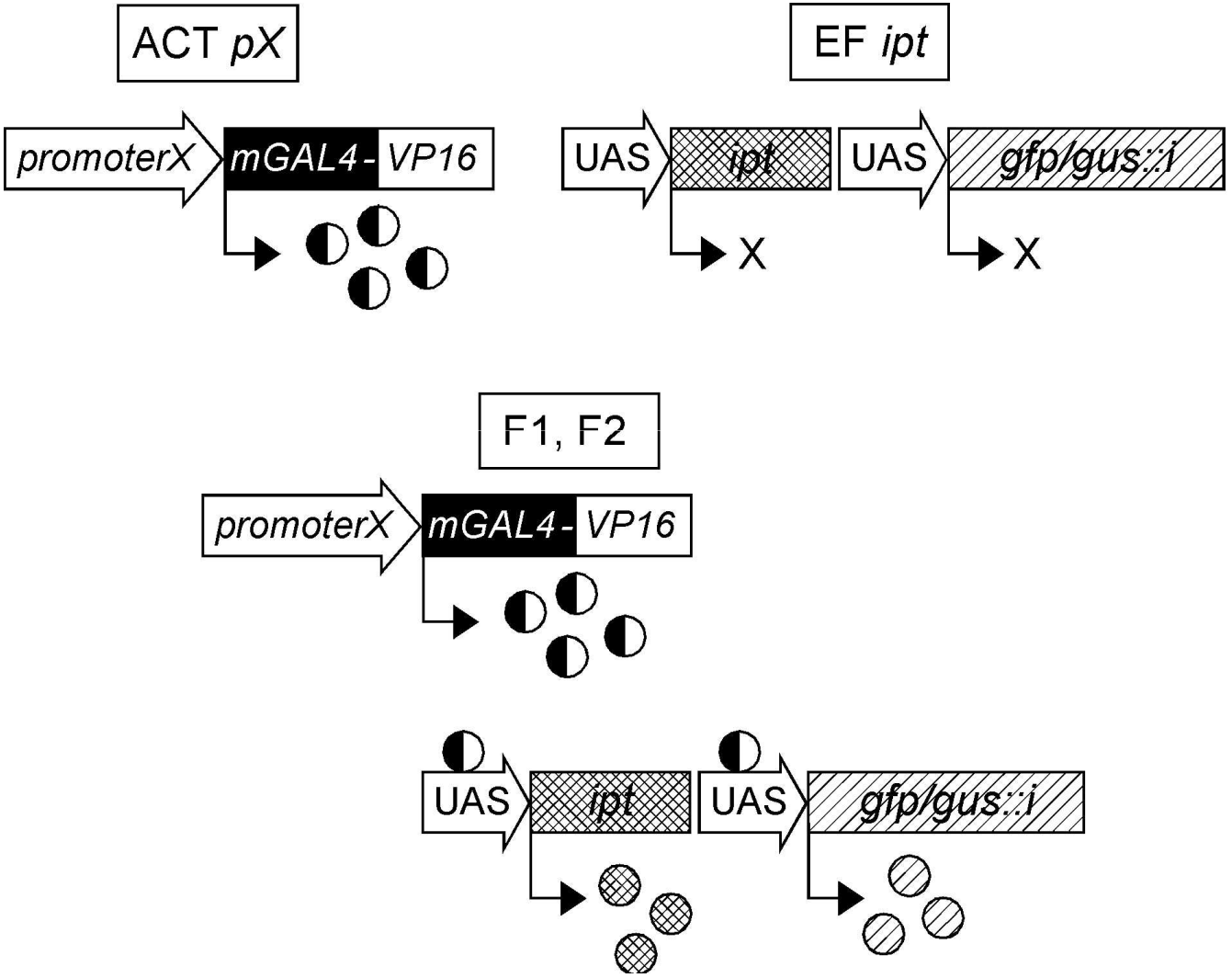
- Overexpression
- Tissue specific expression
- Conditional expression
- Protein stabilisation

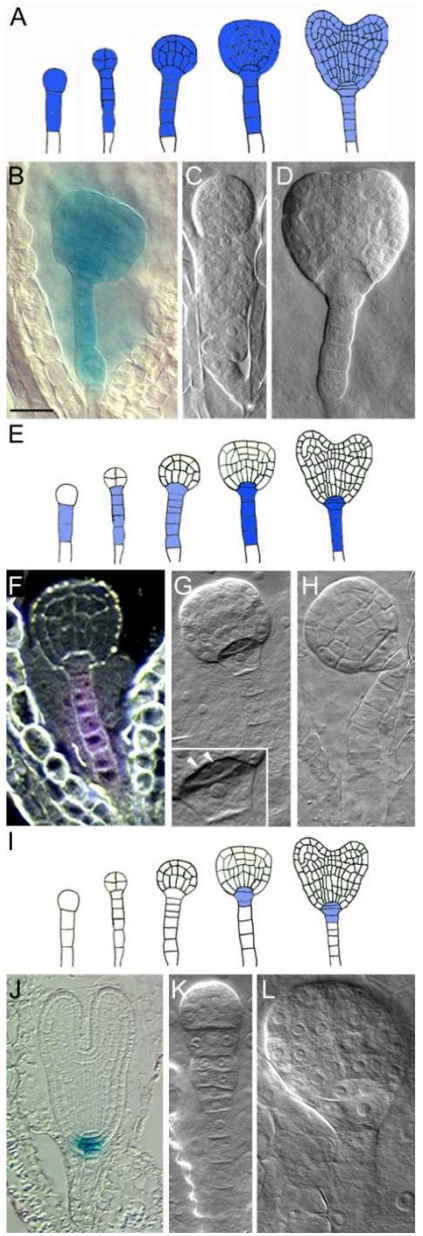
# CaMV 35S Promotor

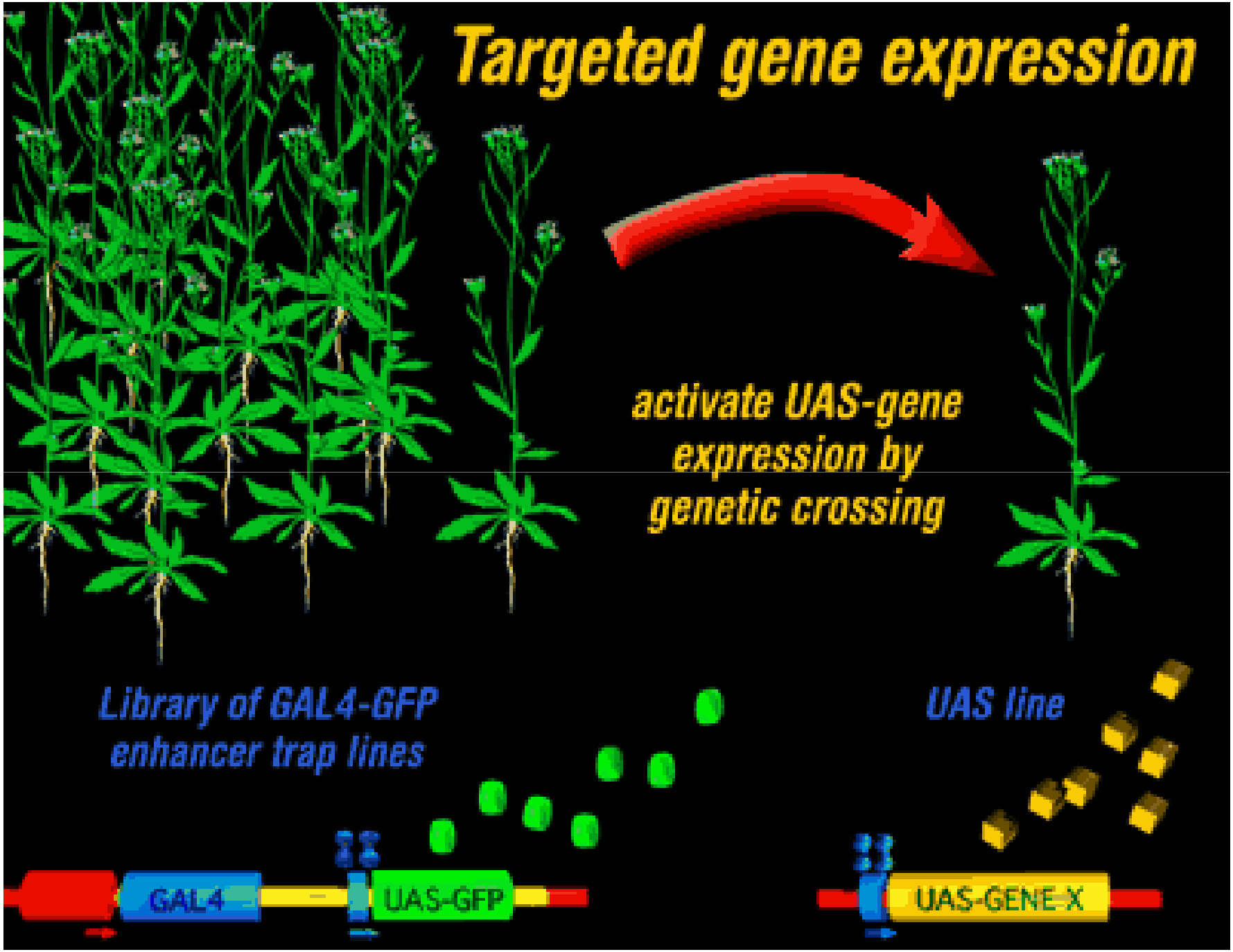



**pBI221** The CaMV 35S promoter-GUS-NOS-ter portion of pBI121 was cloned into pUC19 to produce pBI221.

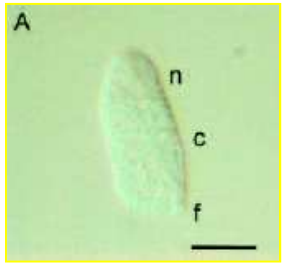
# Two component system for gene expression

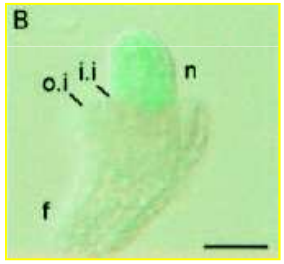


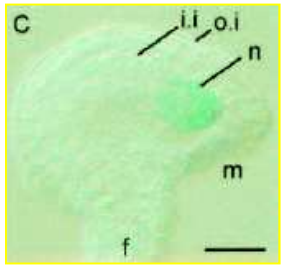



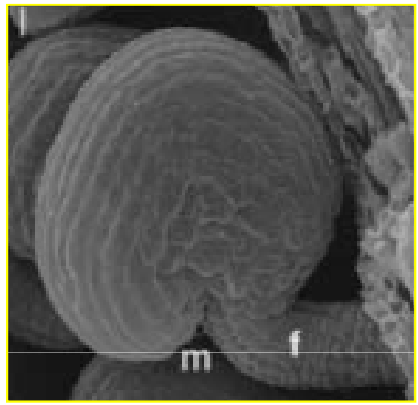

Targeted gene expression
activate UAS-gene expression by genetic crossing
Library of GAL4-GFP enhancer trap lines
UAS line
GAL4
UAS-GFP
UAS-GENE-X

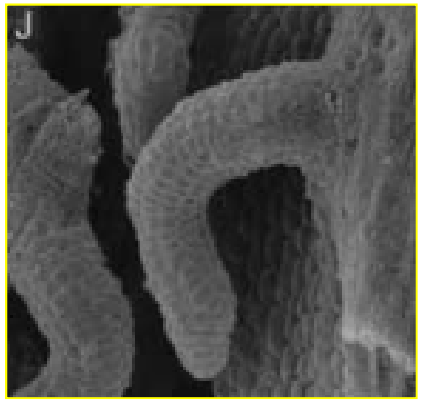
# The hidden function of WUSCHEL



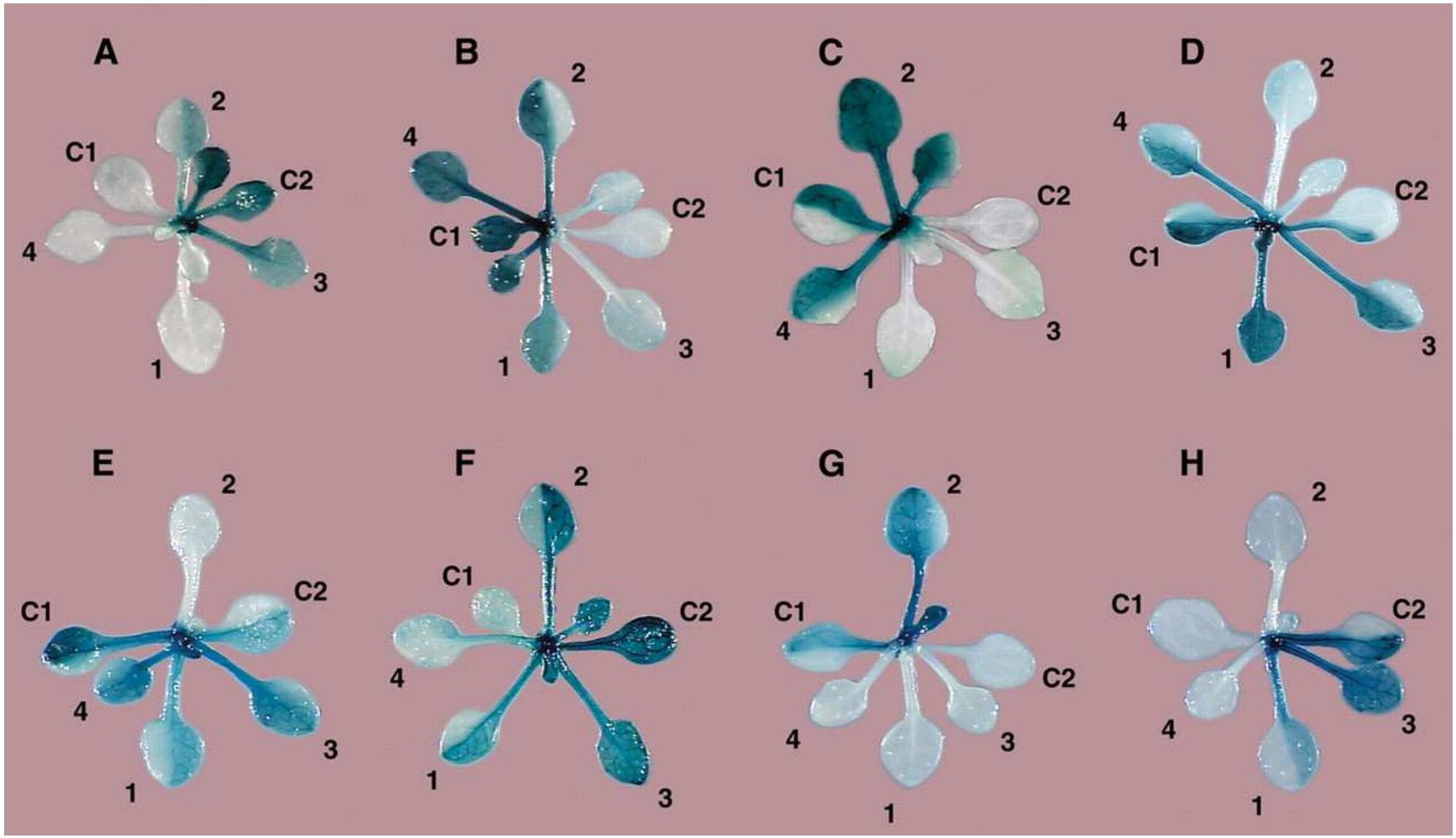



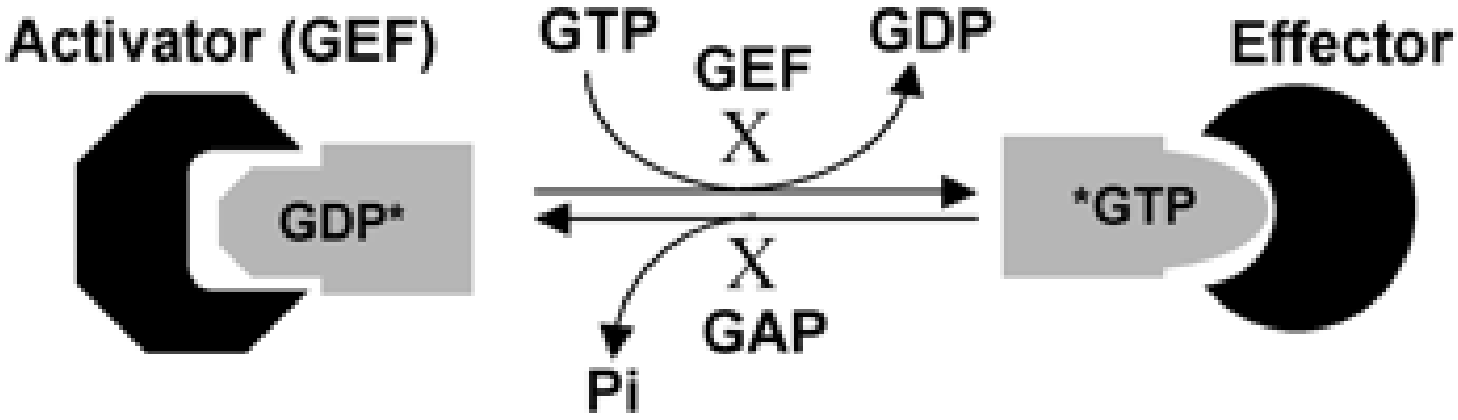

# Mosaics – Cre/Lox



# Sequence manipulation

- Site-directed mutagenesis
- Domain deletions and swaps
- Chimeric proteins

# rop GTPases mutants



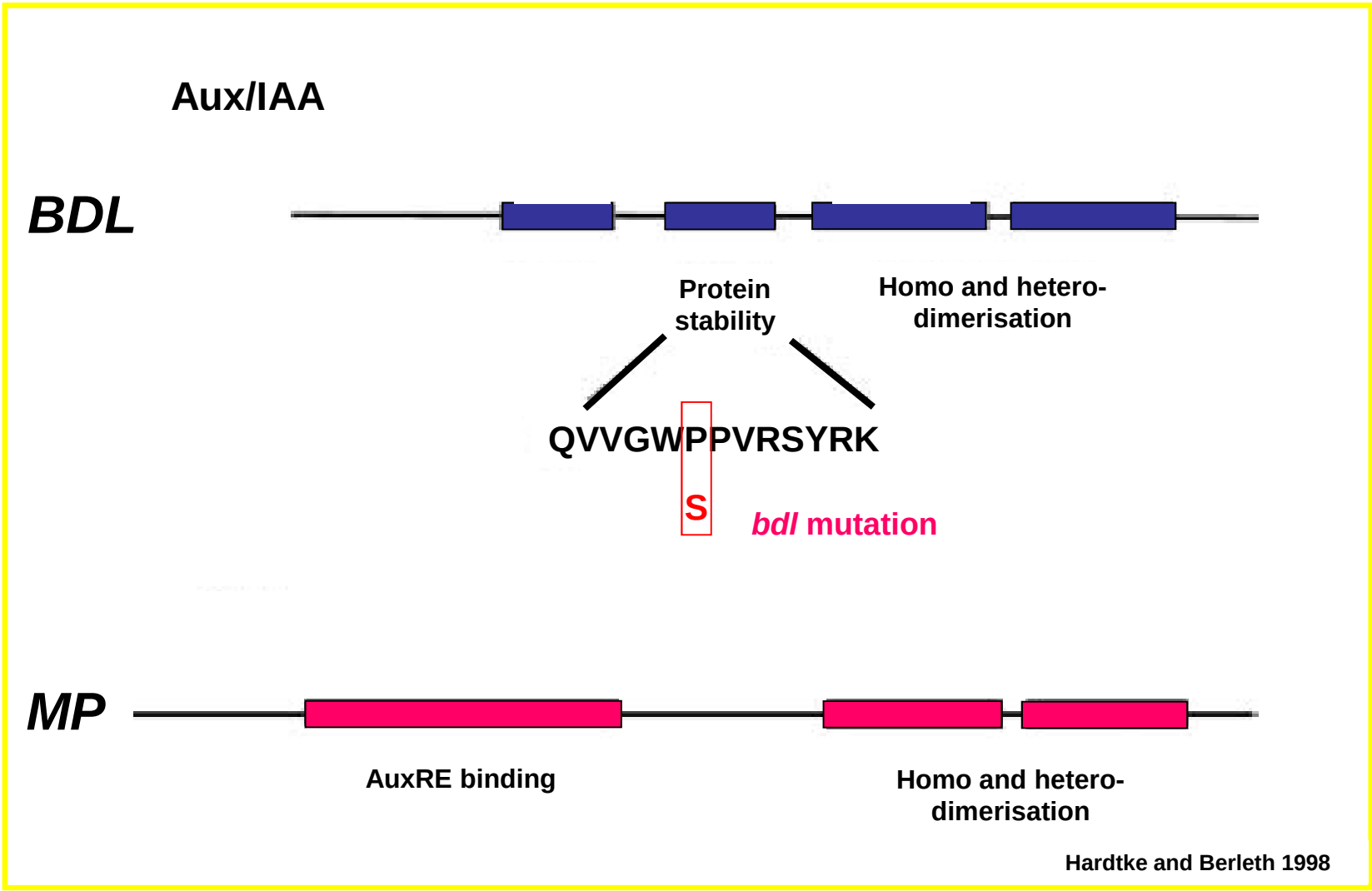

**DN-rop mutants**

- Permanently bind GDP or nucleotide-free
- Sequester activator (GEF) when overexpressed
- Examples:
  - ROP1/ROP2/ROP4/ROP6: T20N, A121D
  - ROP5: T20N

**CA-rop mutants**

- Permanently bind GTP
- Insensitive to GAP
- Constitutively activate effectors when expressed in cells
- Examples:
  - ROP1/ROP2/ROP4/ROP6: G15V or Q64L
  - ROP5: G15V or Q64E

# AUX/IAA and ARF proteins

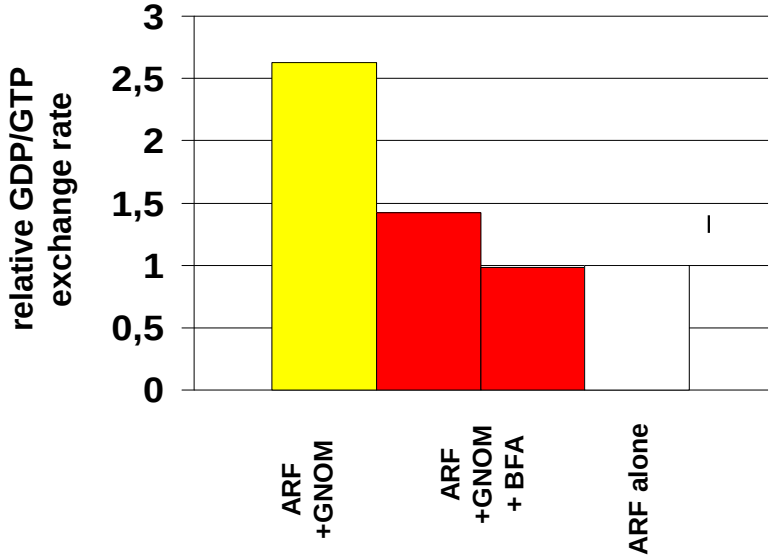


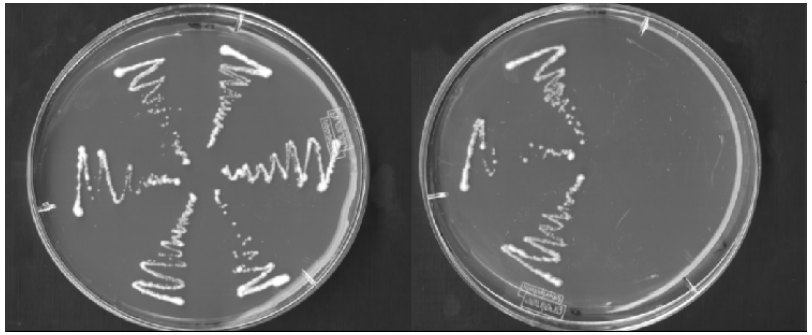
# Phenotype analysis

- Visual evaluation
- Ultrastructure (EMS)
- Use of markers
- Treatments

# Biochemical function

- Protein activity
- Yeast complementation
- Xenopus oocytes



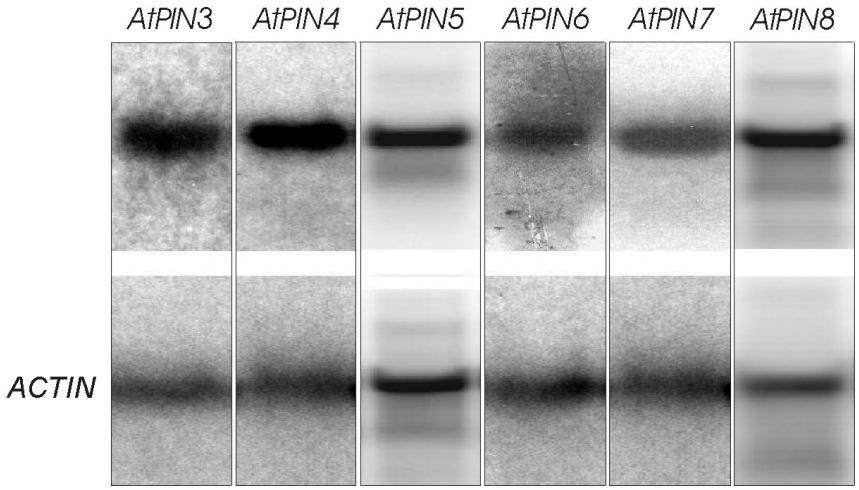
# Gene Expression and Protein Localization

- Blots, RT-PCR
- Reporter genes
- In situ mRNA hybridization
- In situ protein localization
- In situ protein activity detection

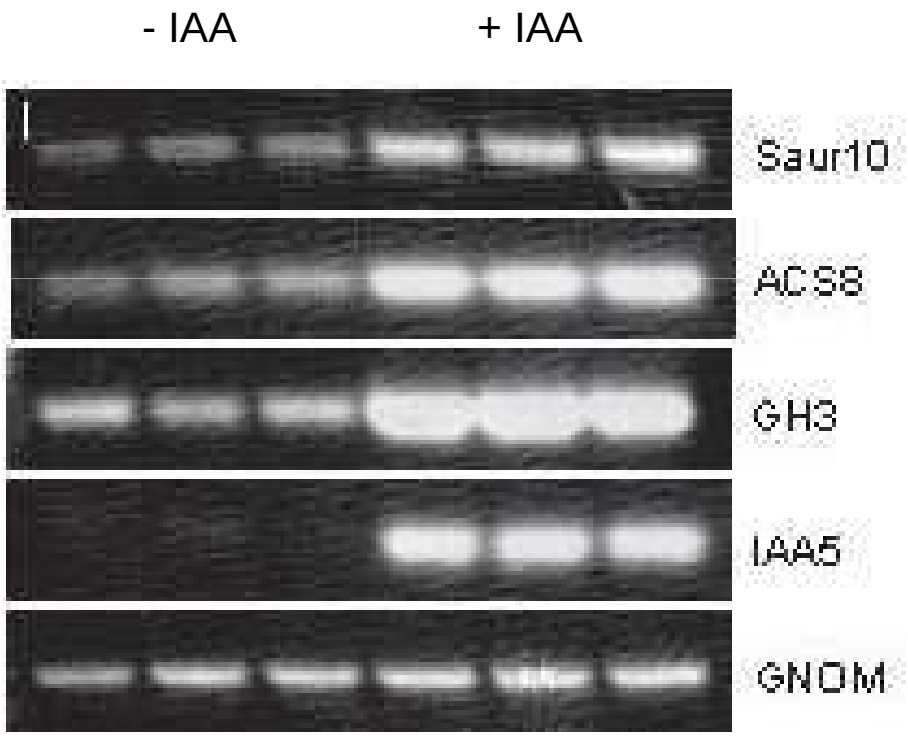
# Blots and RT-PCR

## Northern blots

**Figure 8. Expression of *AtPIN* genes.**
Northern blot and RT PCRanalysis of *AtPIN3* - *AtPIN8* genes. *AtPIN3* transcript was found in stem, *AtPIN4*, *AtPIN6* and *AtPIN7* in root and *AtPIN5* and *AtPIN8* in seedling.
In the second line *ACTIN* signal is depicted.




## RT-PCR

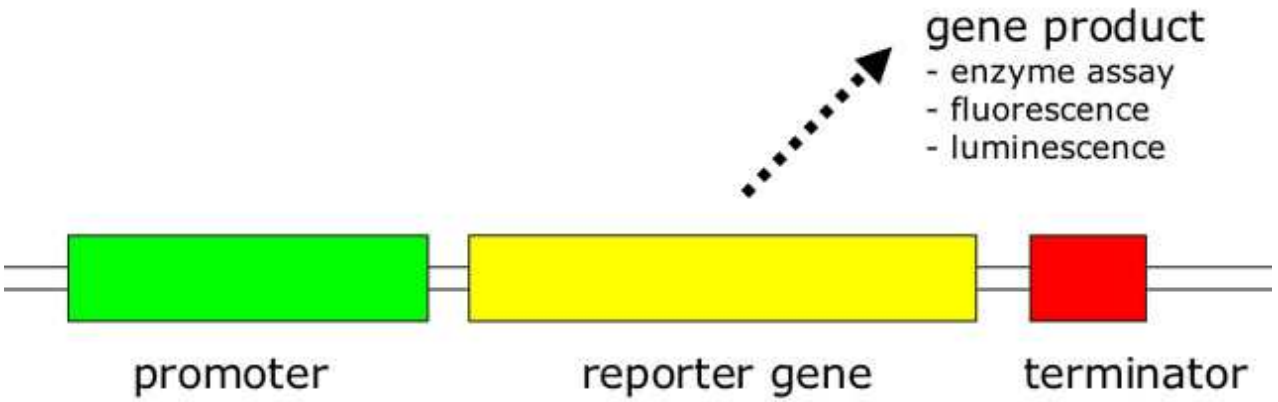



## Southern and Western blots

# Reporter genes

- Transcriptional fusions
- Translational fusions
- GUS, Luciferase, GFP
- Applications

# Transcriptional fusion




***Reporter genes: markers for gene expression***

***ß-glucuronidase***
***green fluorescent protein***
***luciferase***

# GUS – ß-Glucuronidase

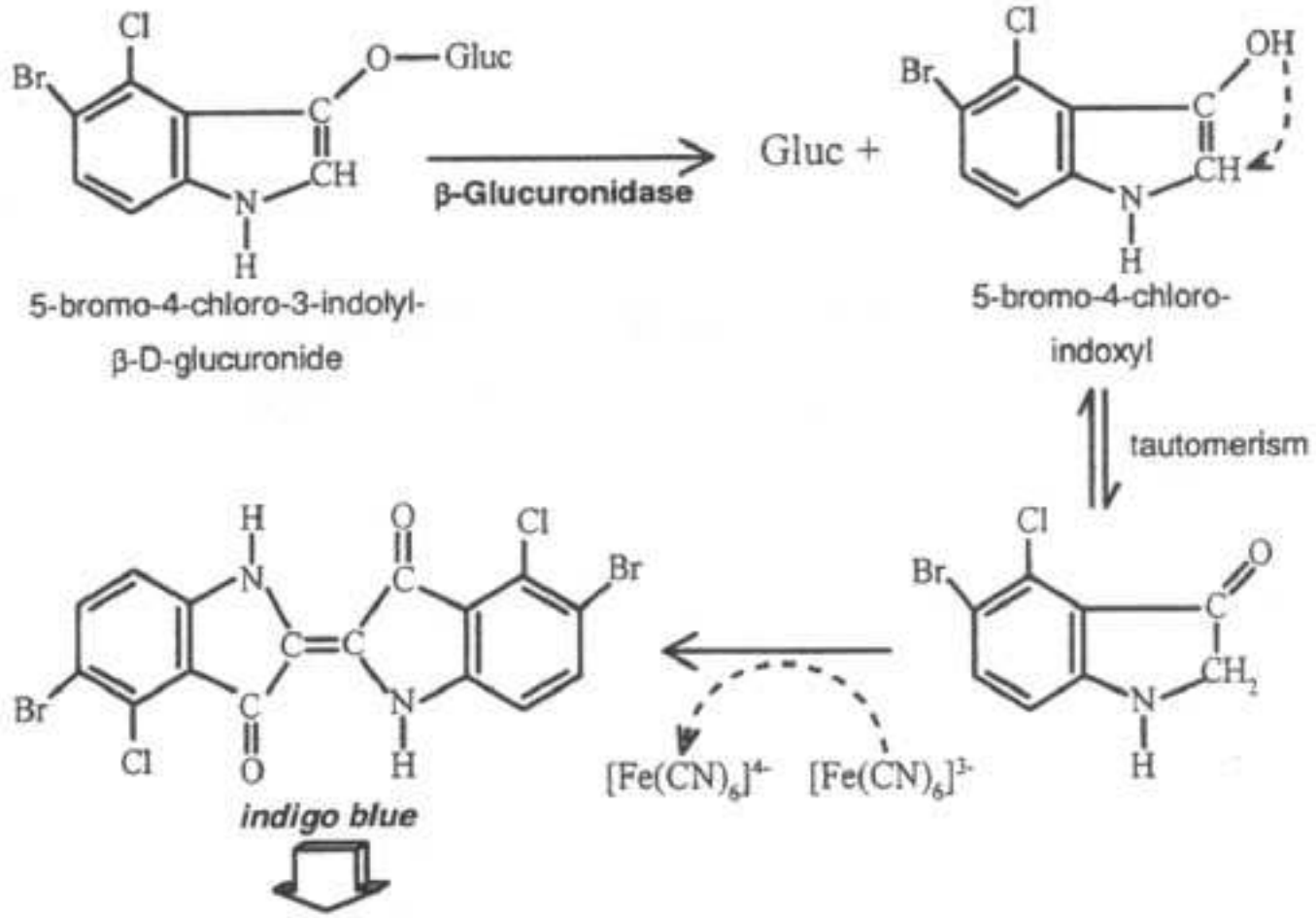
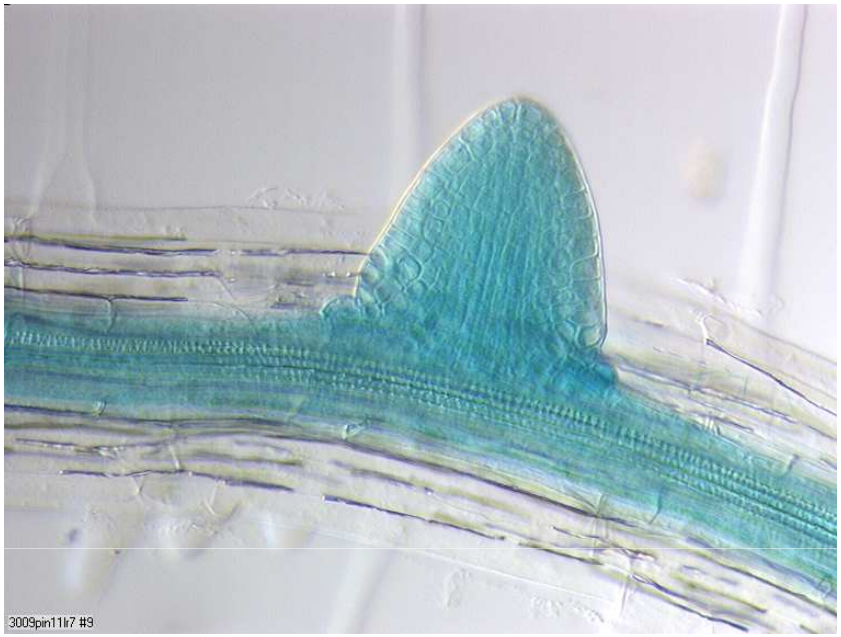
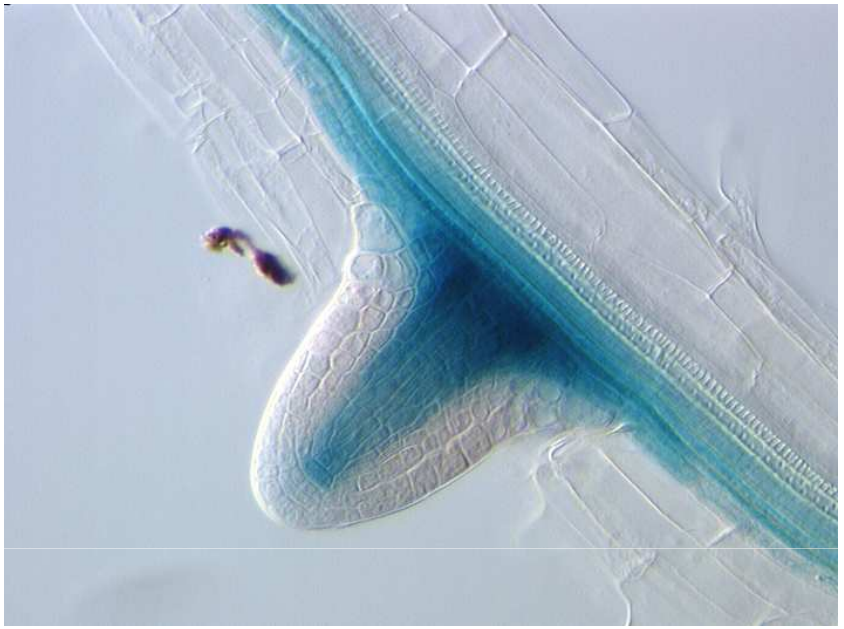
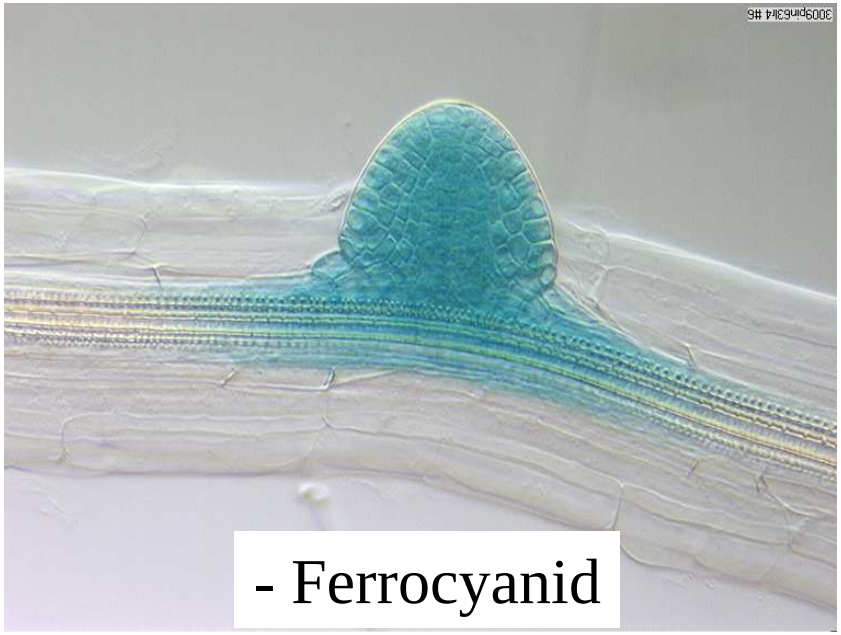


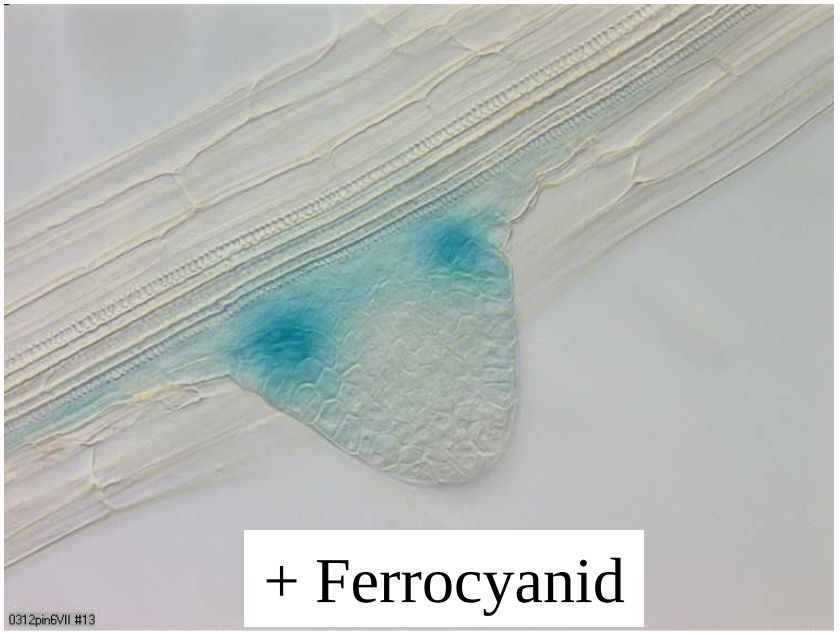

Fig. 1. Chemistry of X-Gluc reaction. Hydrolyzation of X-Gluc by the β-glucuronidase enzyme results in a reactive indoxyl molecule. Two indoxyl molecules are oxidized to indigo blue; ferri(III)cyanide enhances the dimerization.

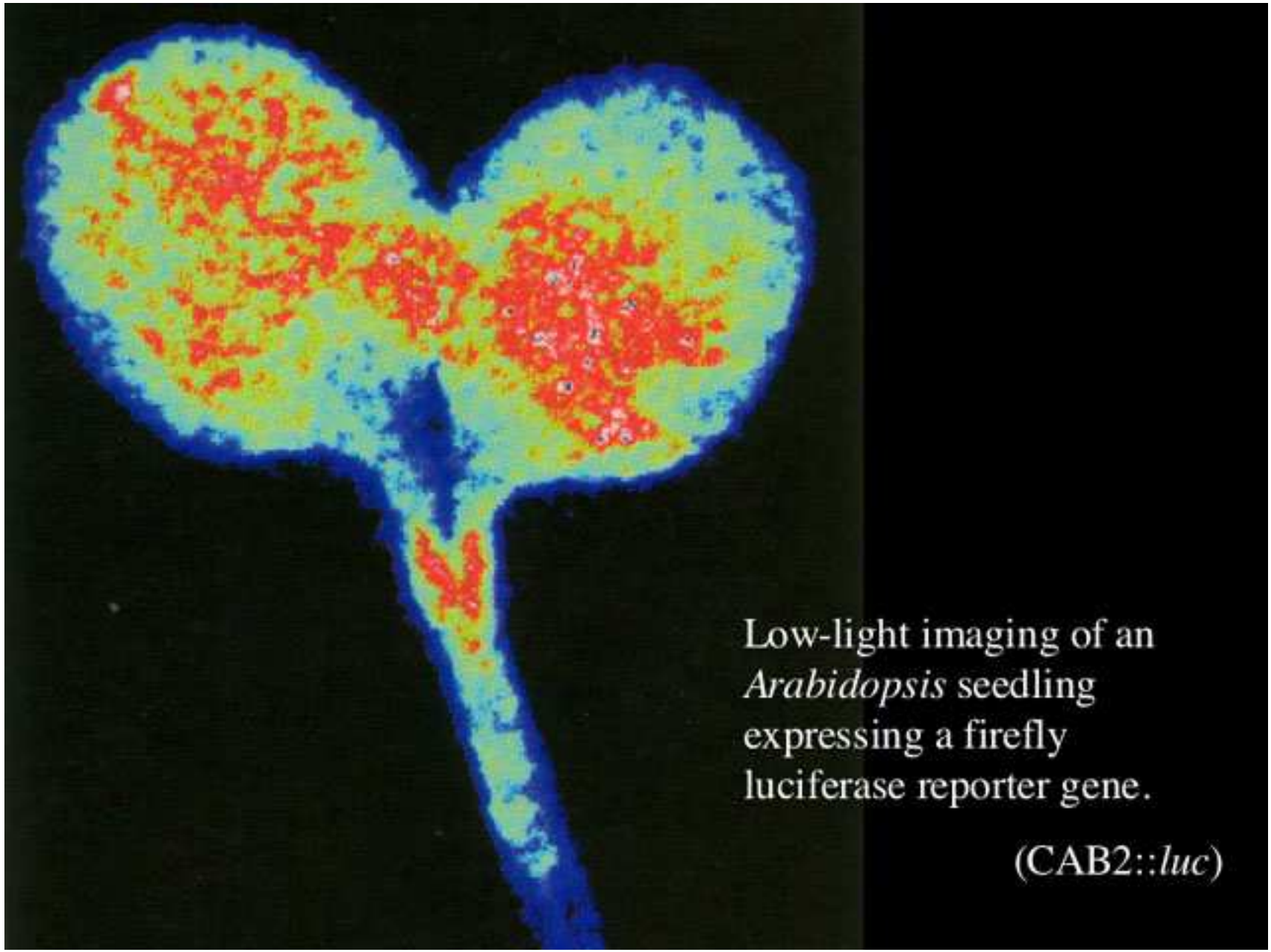
# GUS – ß-Glucuronidase



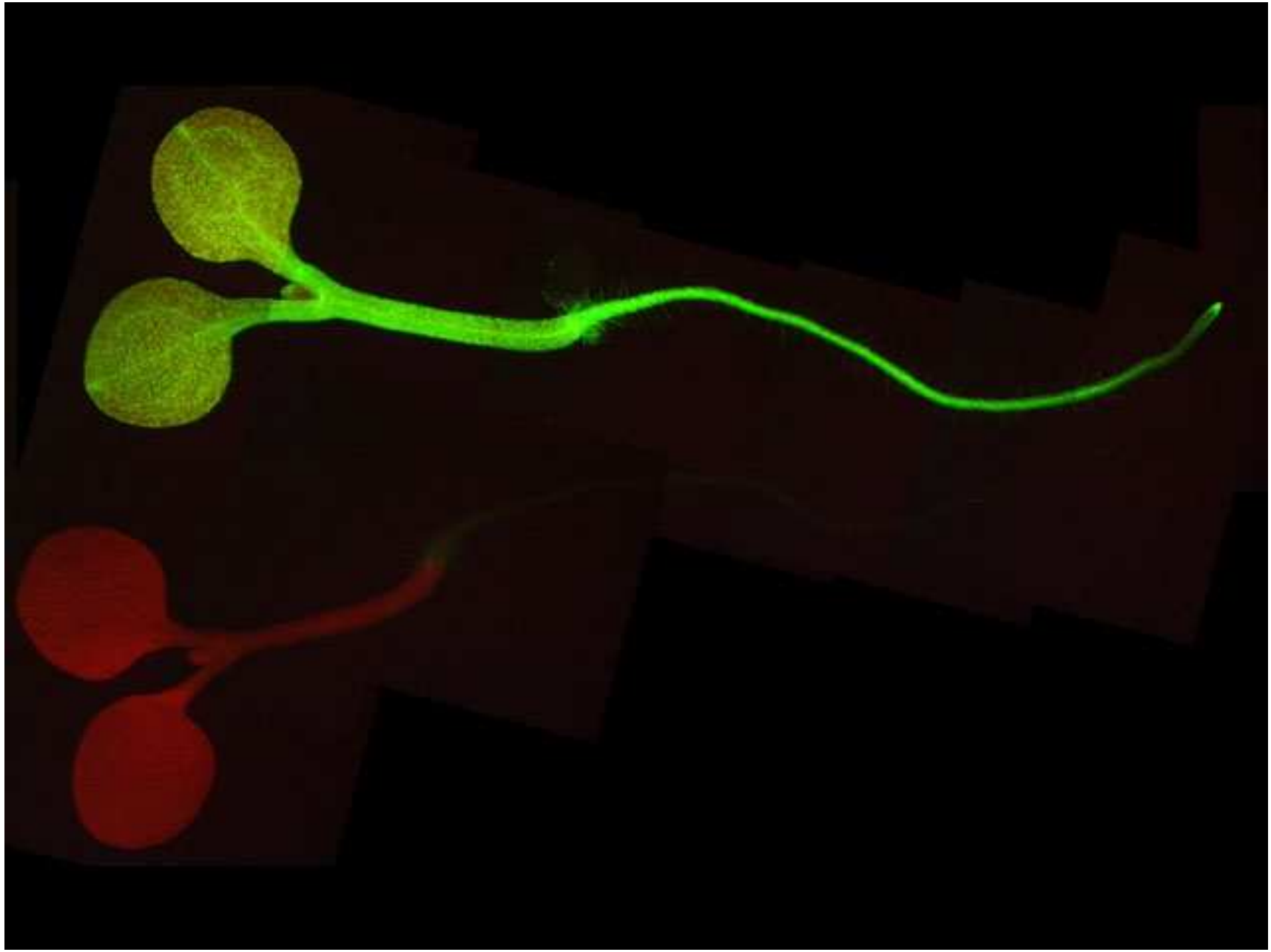





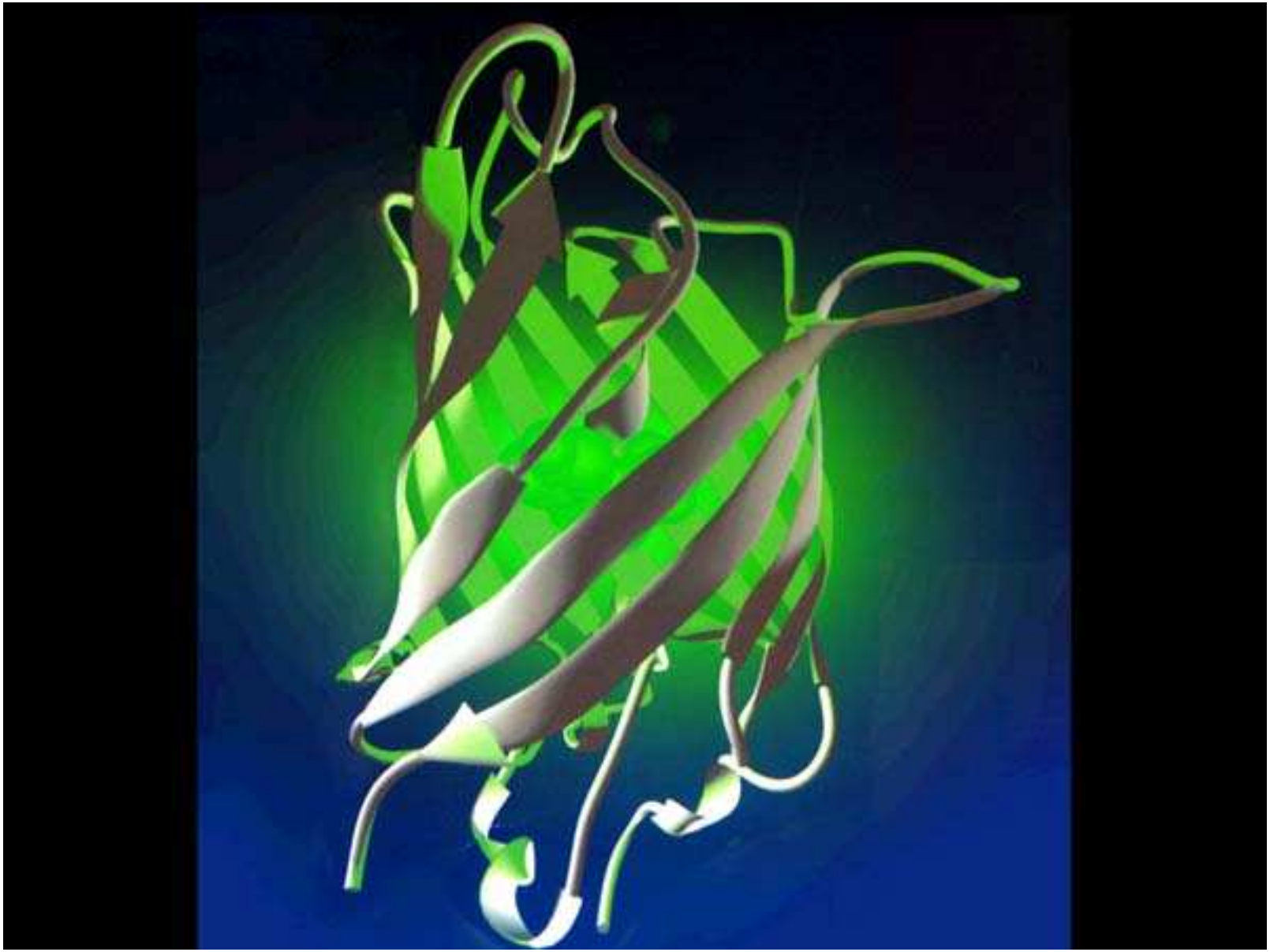



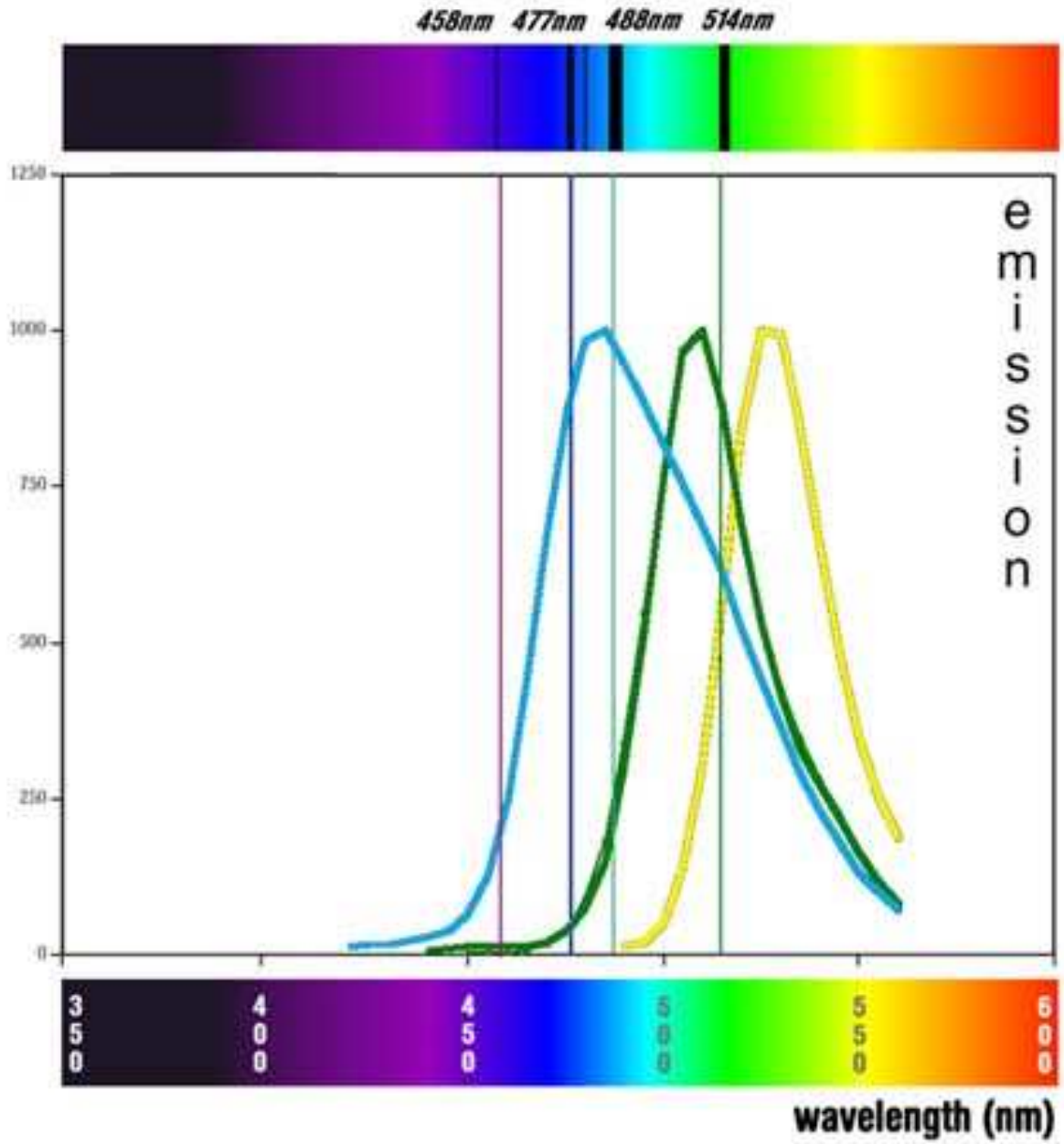

Low-light imaging of an
*Arabidopsis* seedling
expressing a firefly
luciferase reporter gene.
(CAB2::*luc*)

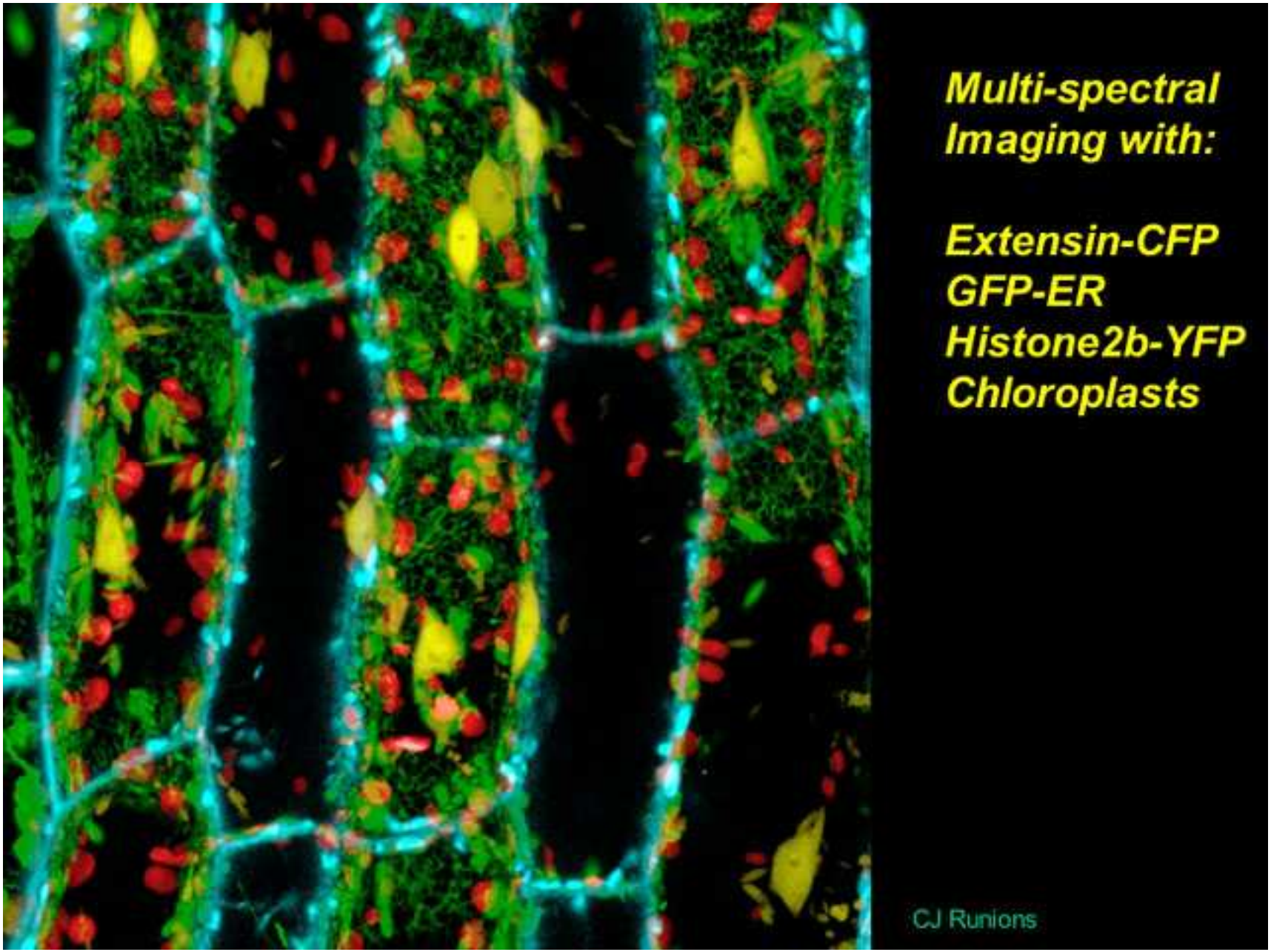
# Green Fluorescence Protein



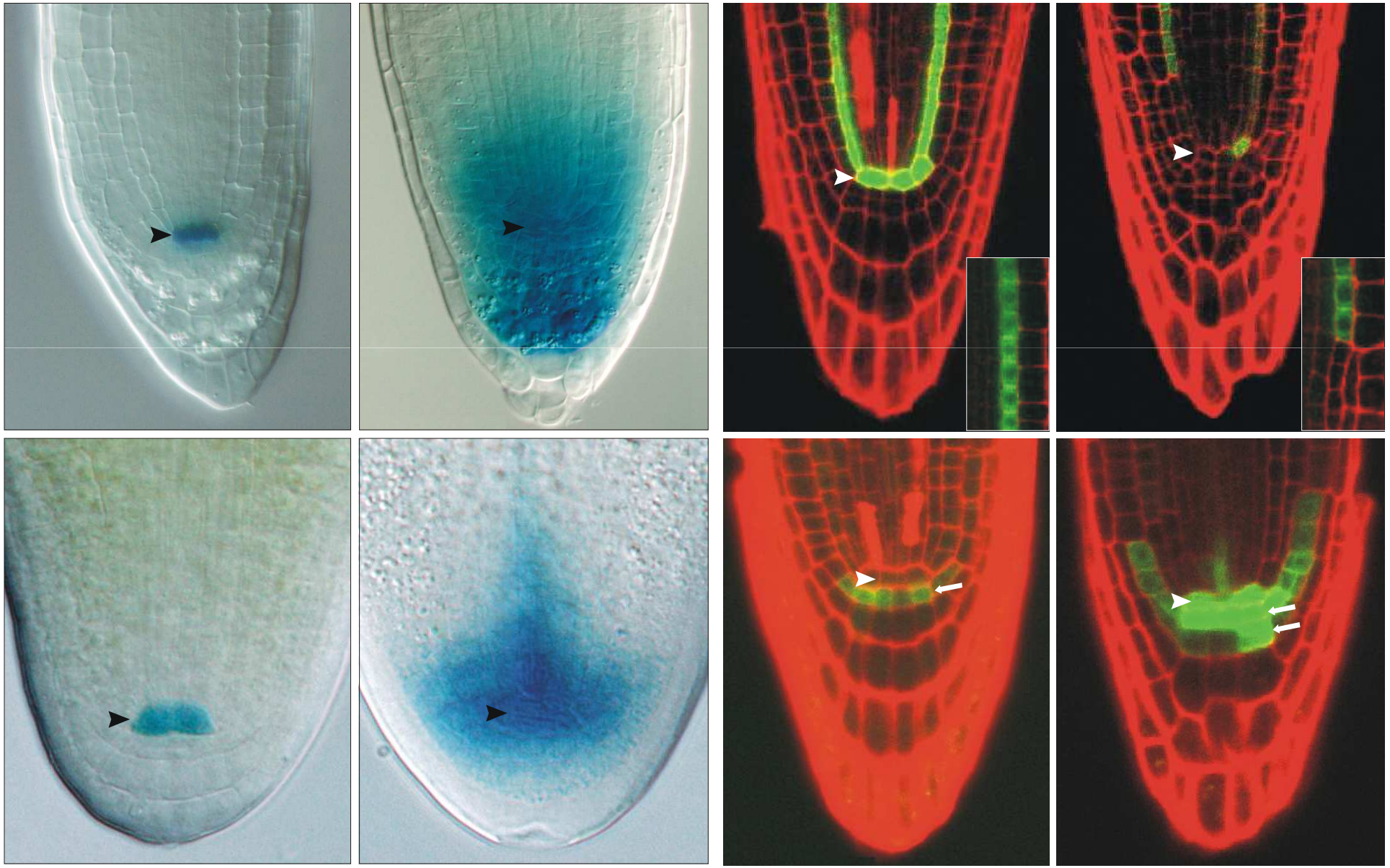

Bioluminescence in Aequoria victoria
aequorin
green fluorescent protein




458nm
477nm
488nm
514nm
1250
1000
750
500
250
0
emission
350
400
450
500
550
600
wavelength (nm)

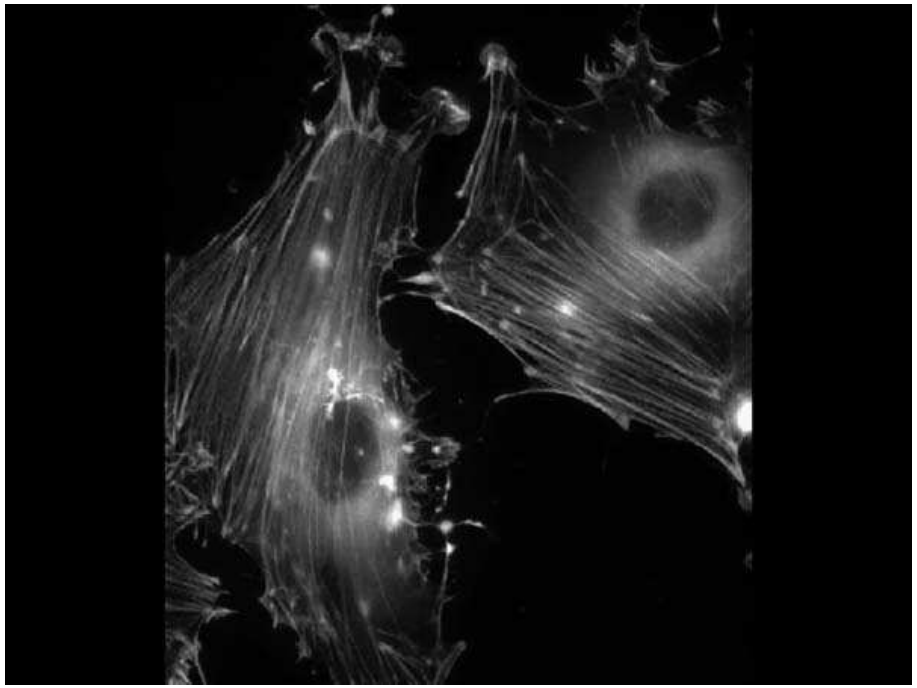

Multi-spectral
Imaging with:
Extensin-CFP
GFP-ER
Histone2b-YFP
Chloroplasts
CJ Runions

# Cell identity markers

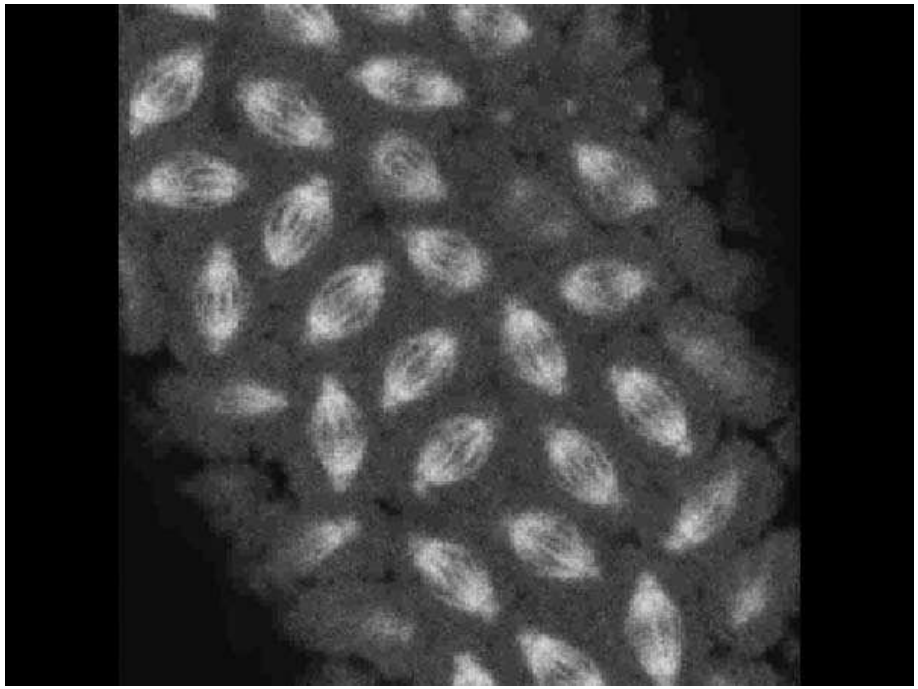


# Subcellular structure markers

Actin



Tubulin

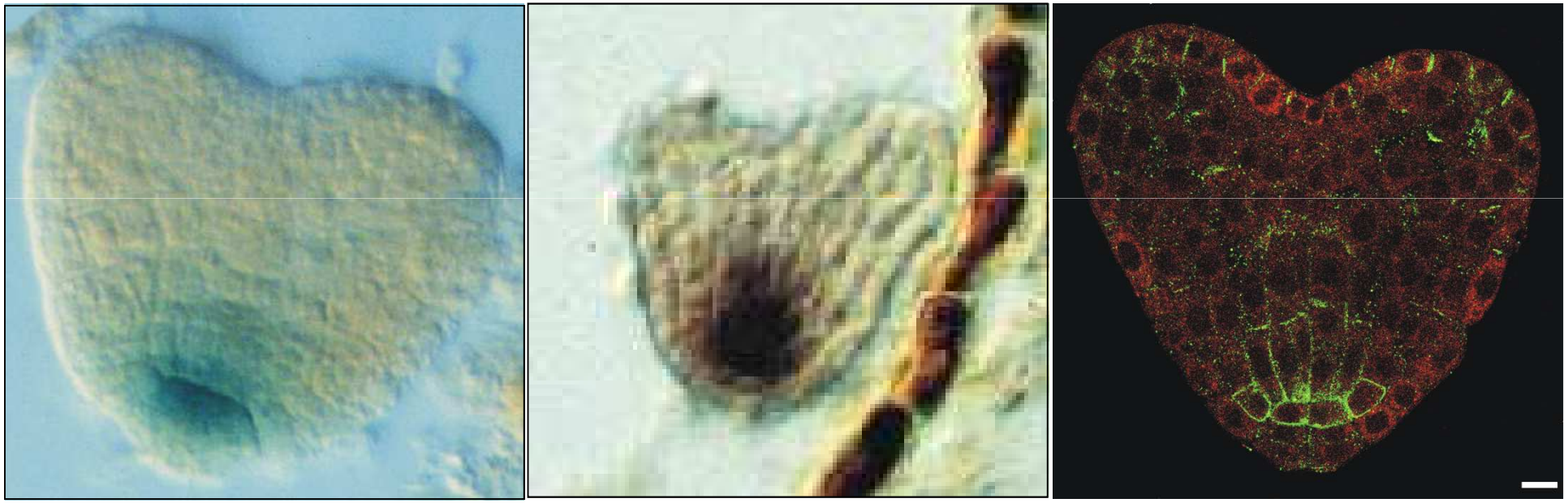


# In situ mRNA/protein localisation

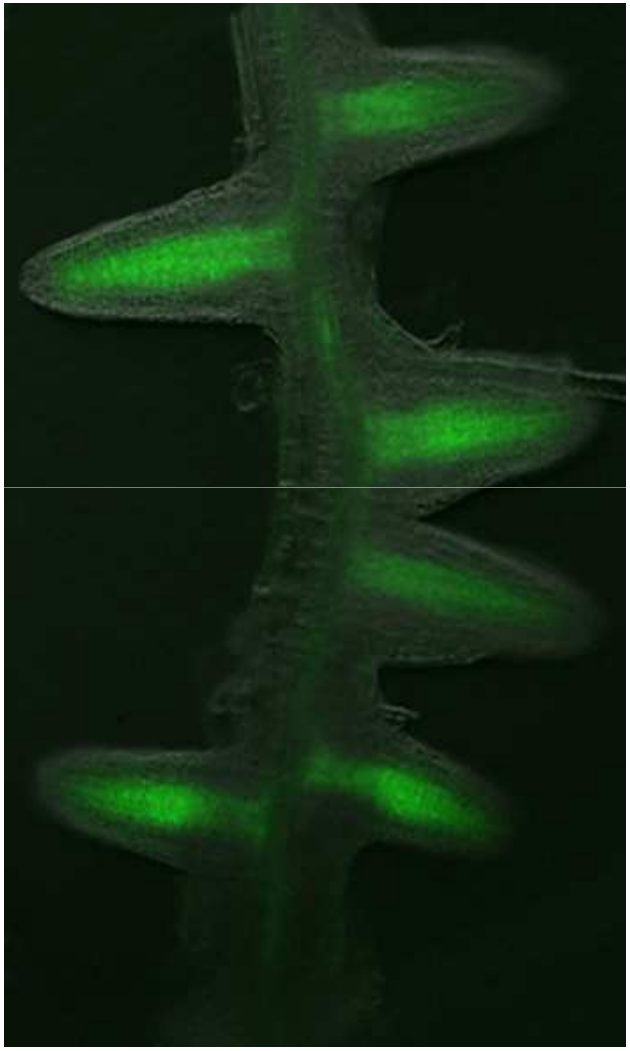
- Probe preparation
- Fixation
- Embedding
- Sectioning
- Deparafinization
- Treatment with probe
- Removal of unbound probe
- Signal visualization

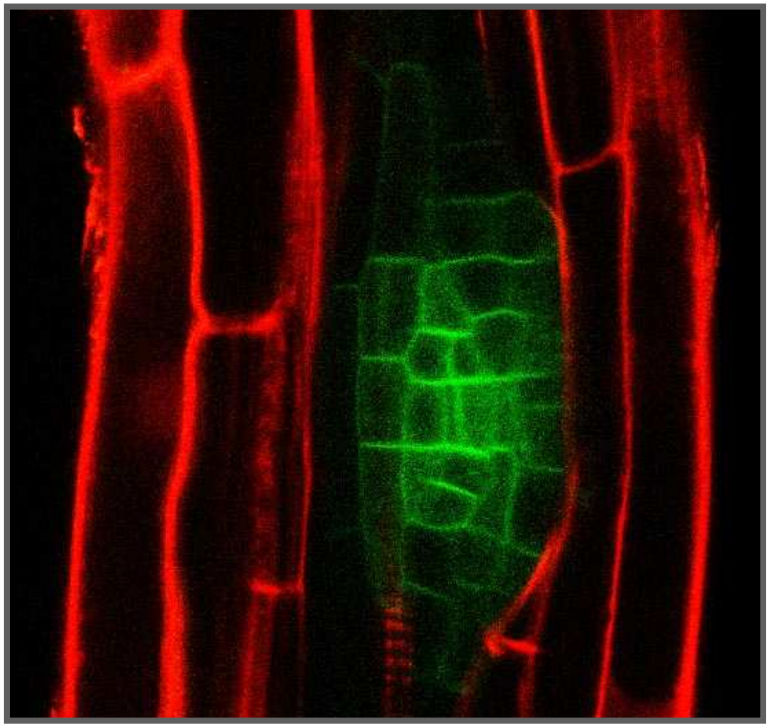
# Analysis of gene expression

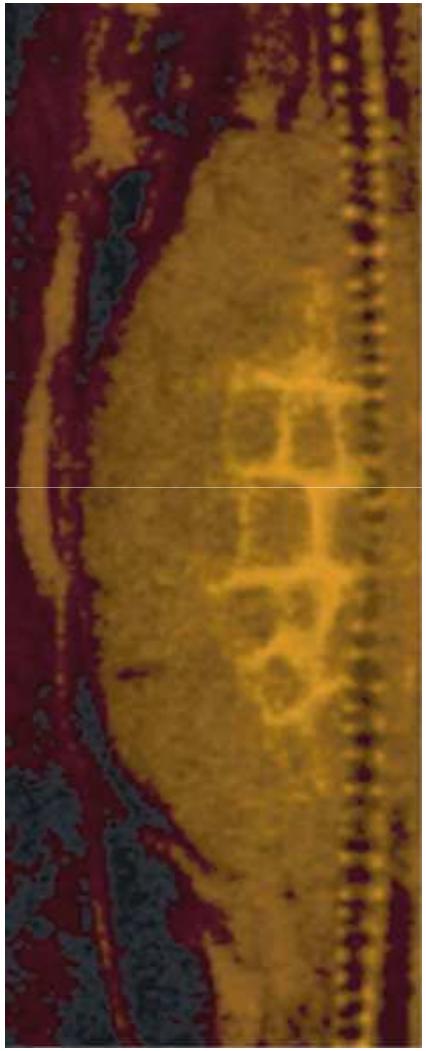
**GUS** **mRNA** **Protein**



# Analysis of protein localisation







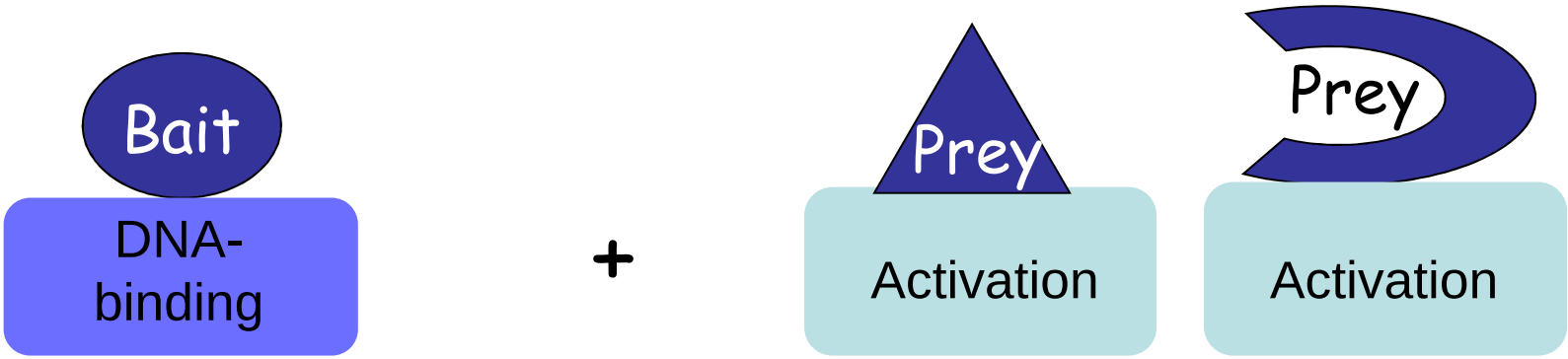
# Friends and associates

- Yeast-two-hybrid
- Split ubiquitin, split YFP
- Genetic interactions
- Upstream and downstream

# Yeast two hybrid

## Classical transcription factor

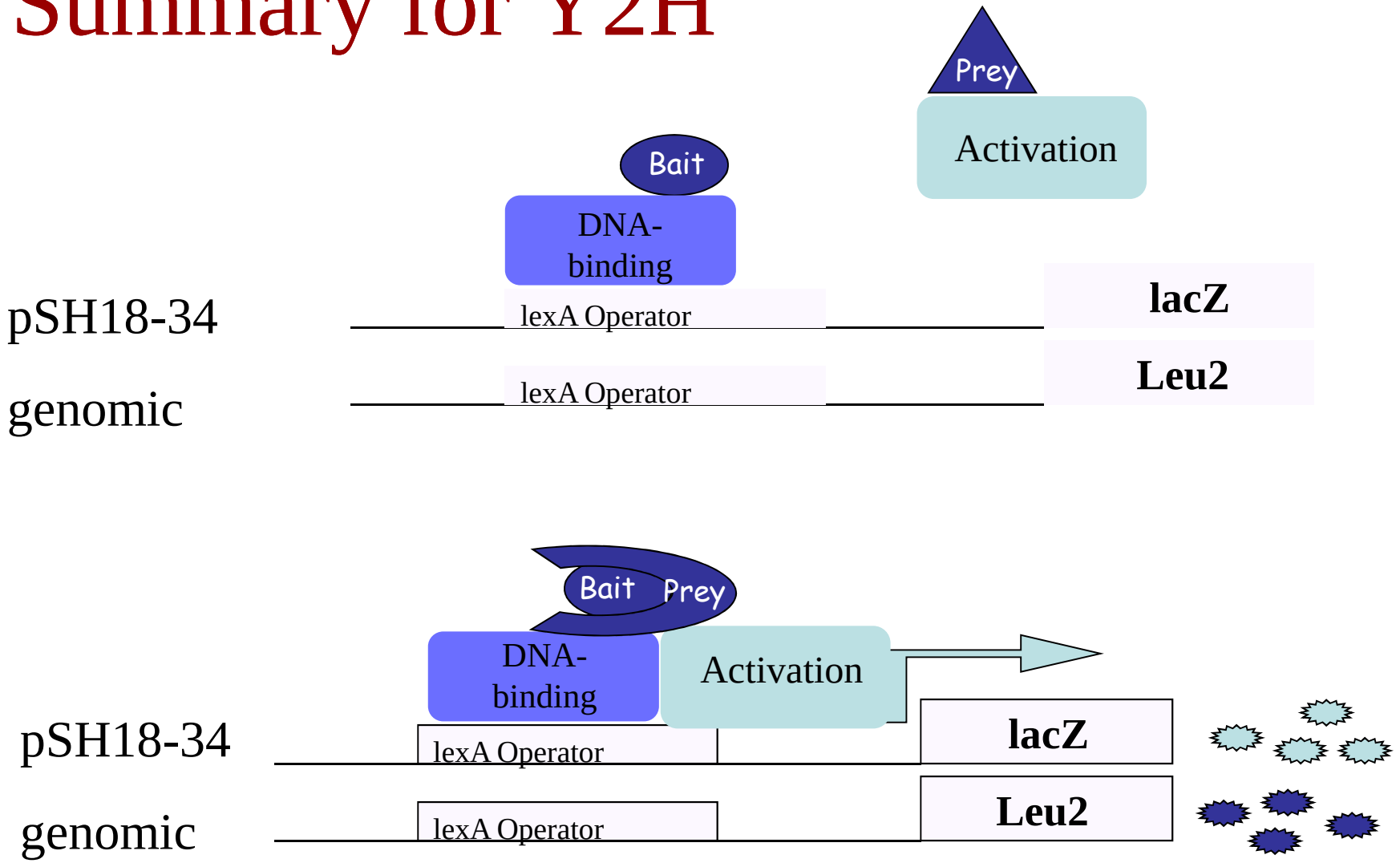
1. DNA Binding domain
2. Activation domain

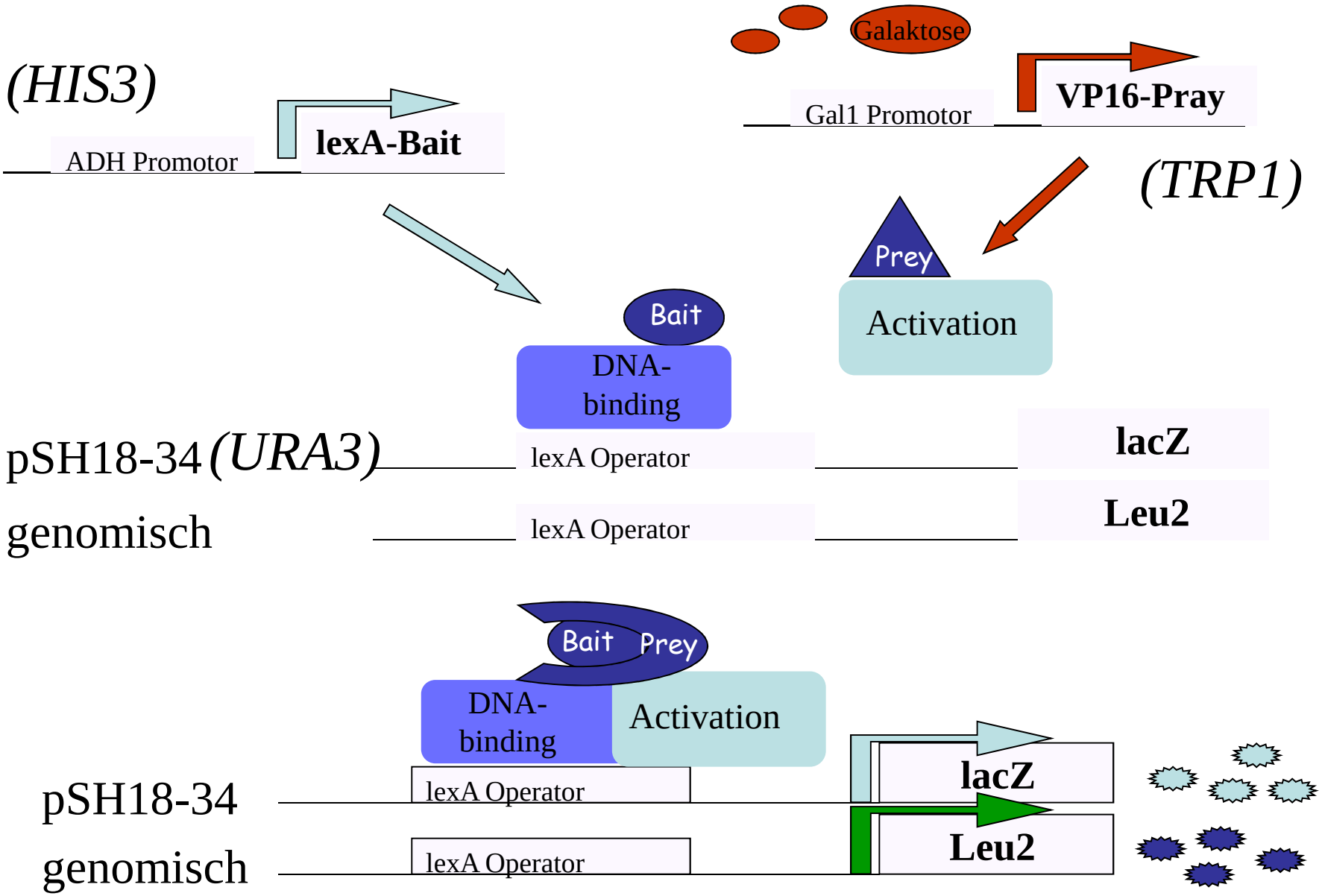




lexA Protein

VP16 Protein

# Summary for Y2H






EGY48: Mutant for *HIS3*, *TRP1*, *URA3* und *LEU2*

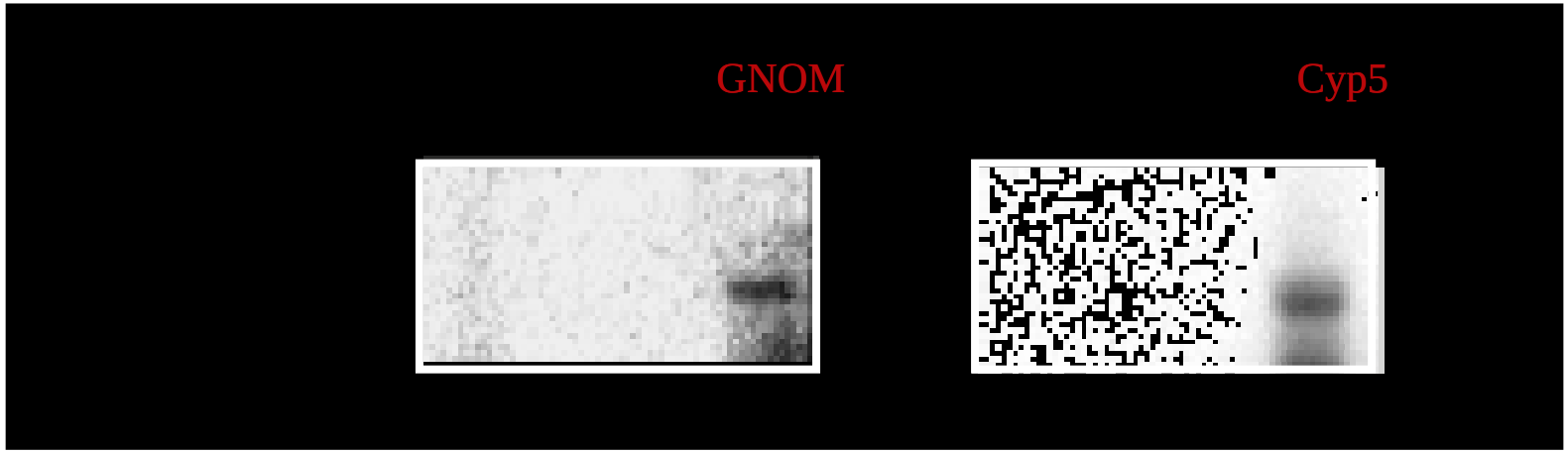
# Conditions for Y2H-System

1. Proteins must be able to localize to the nucleus

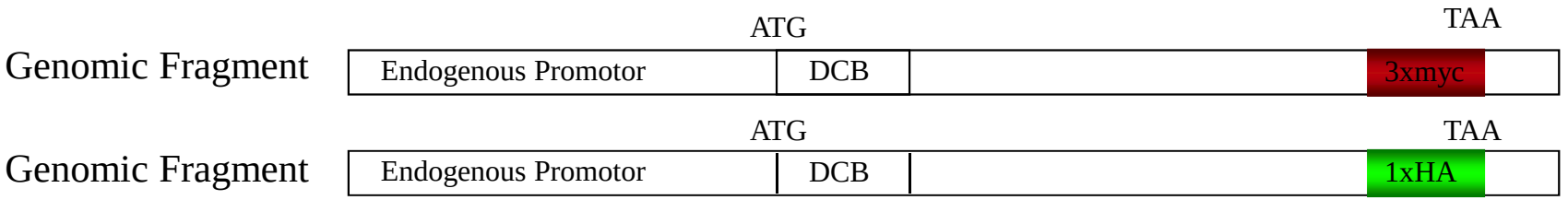
2. Bait construct must not have its own activation domain (Autoactivation)

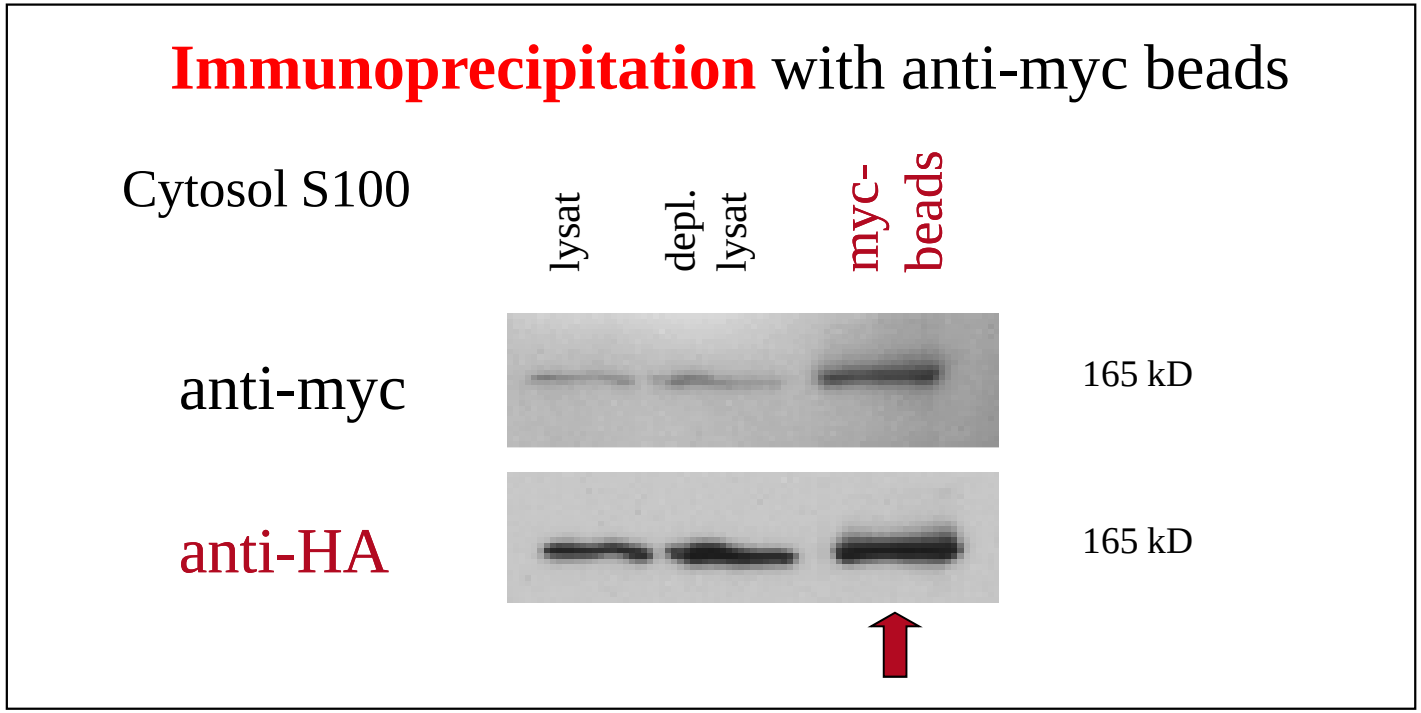
# *In vitro* Pulldown-Assay

GST-Cyp5 and GST-GNOM$_{1-246}$ bind GNOM from *Arabidopsis* protein extract

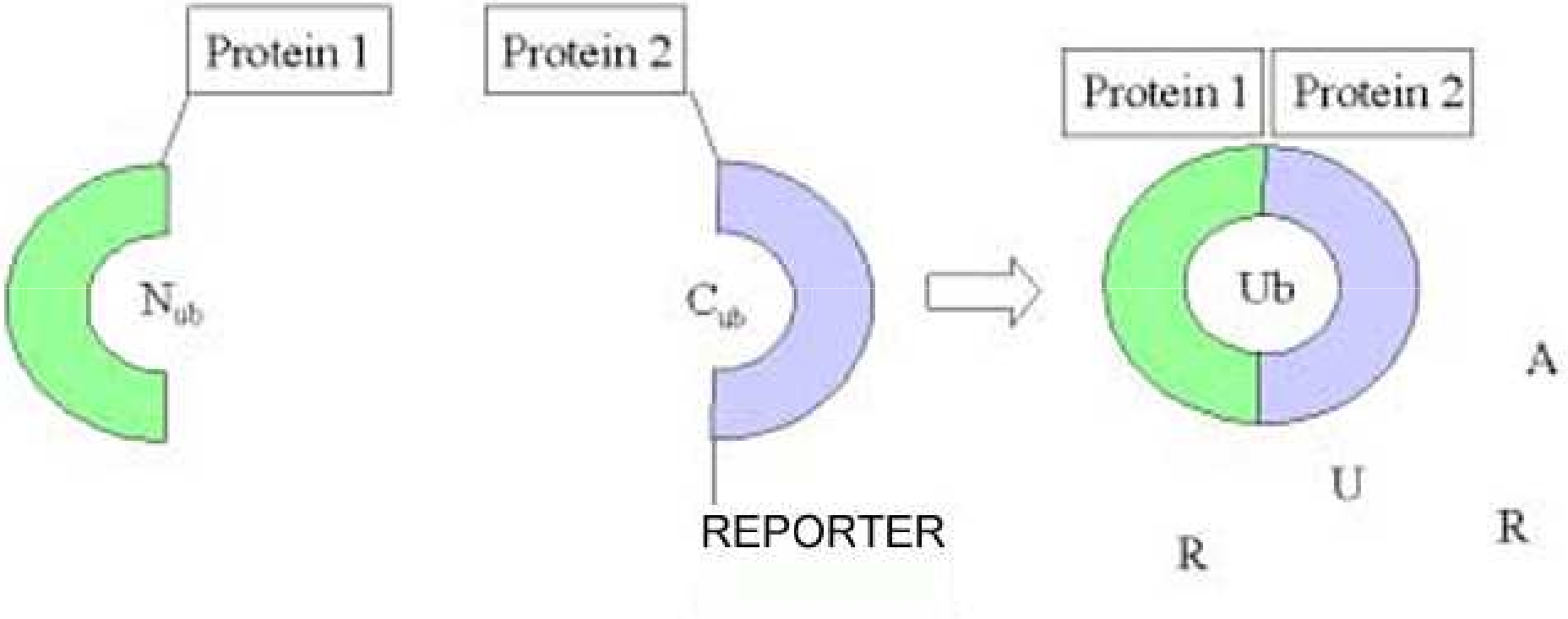


# Interaction of GNOM *in vivo*



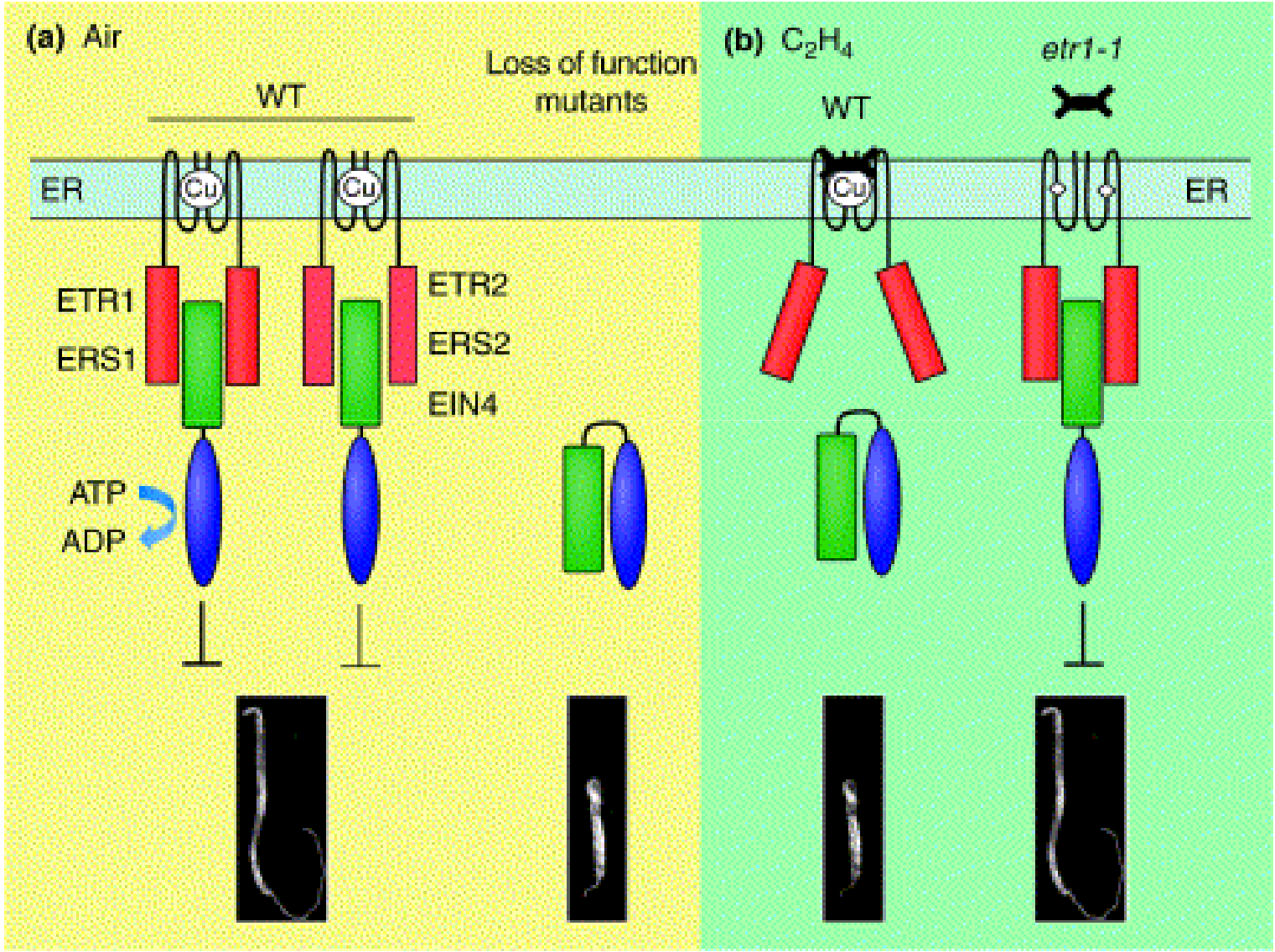
# Split-Ubiquitin



# Split-YFP

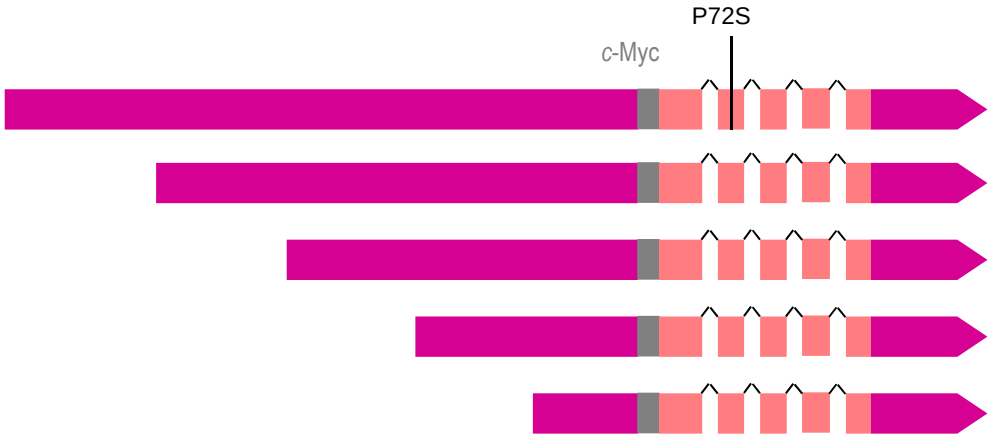
- Protoplast transfection

# Genetic interactions

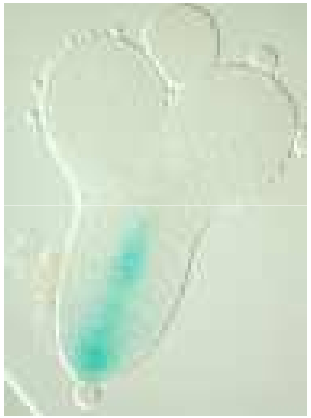



Current Opinion in Plant Biology

# Upstream - Promotor analysis (yeast one hybrid)




| Promoter (bp) | Activity |
|---|---|
| 2,400 | Yes |
| 1,500 | Yes |
| 1,000 | Yes |
| 500 | No |
| 150 | No |



promoter::GUS

# Downstream targets

- expression profiling
- proteomics
- second site mutagenesis
- educated guess

# Special methods and tools

- DR5 auxin response reporter
- Transient transfection
- Laser ablations and laser capture

# DR5 (Auxin) Response Reporter

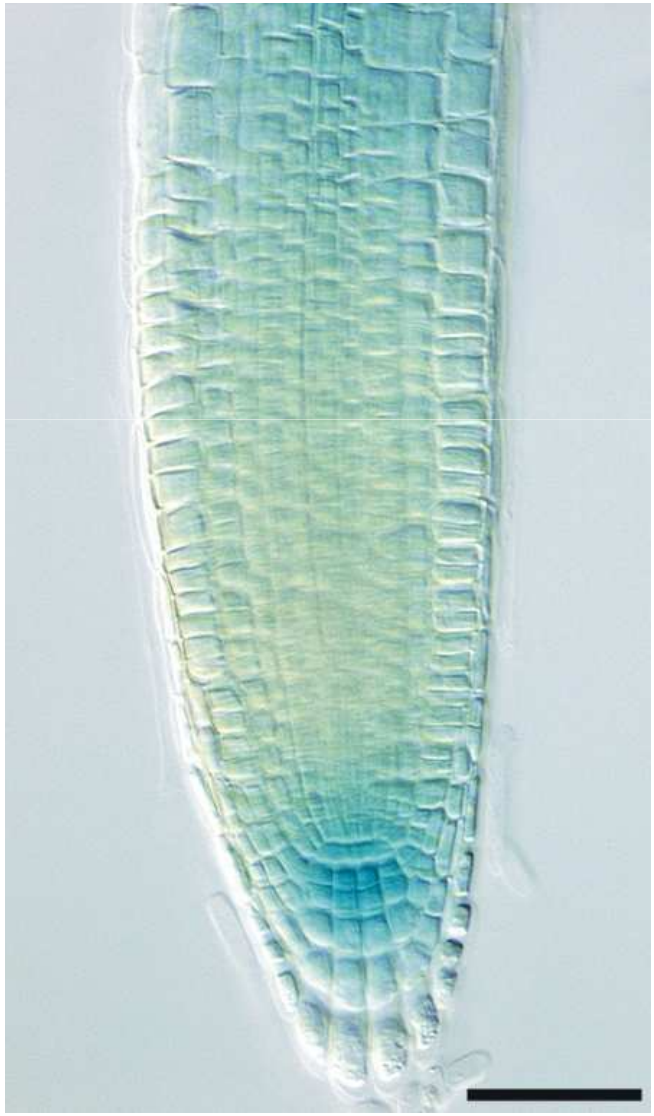
*5´ CCTTT* TGTCTC *3´*

9x inv.

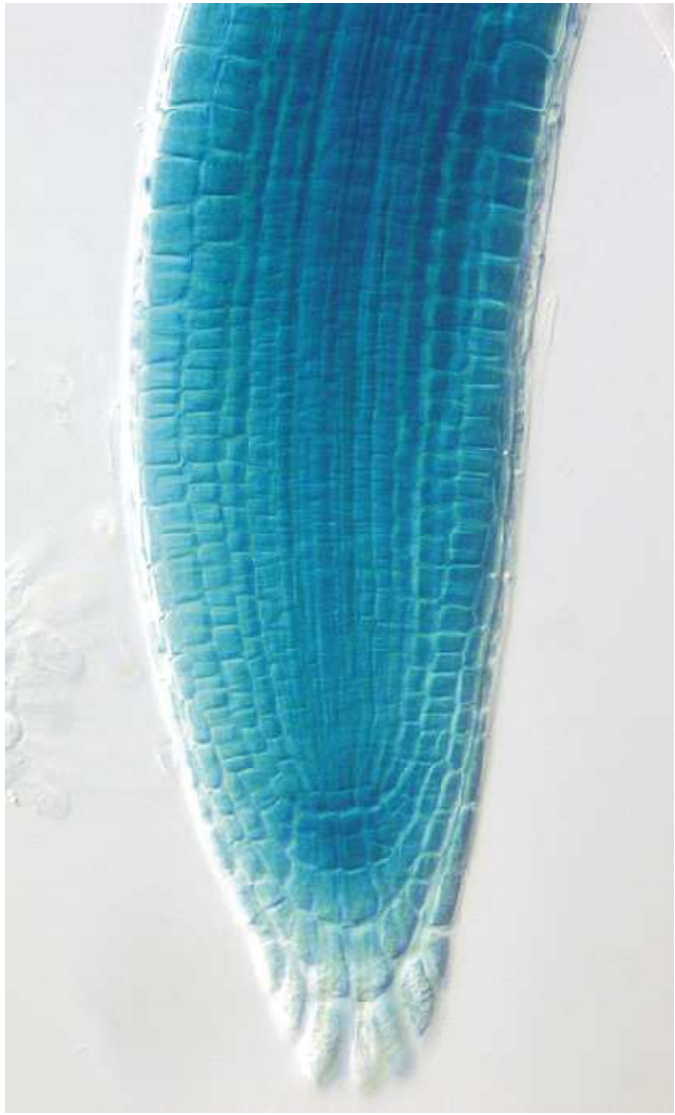

**DR5: Ulmasov *et al.*, 1997**

# *DR5::GUS*

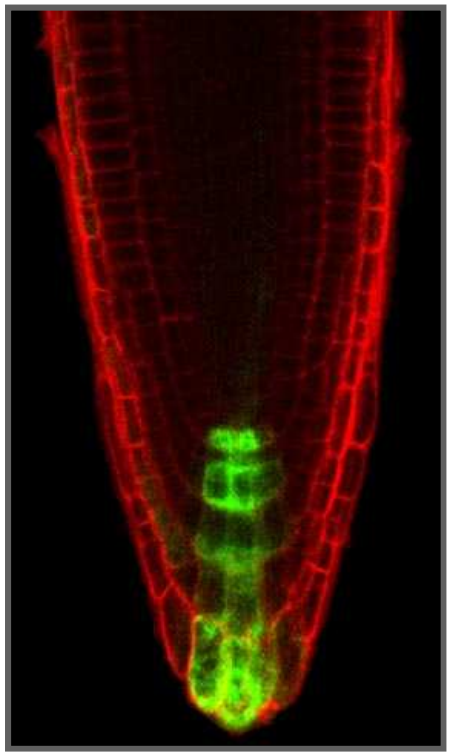
- Auxin



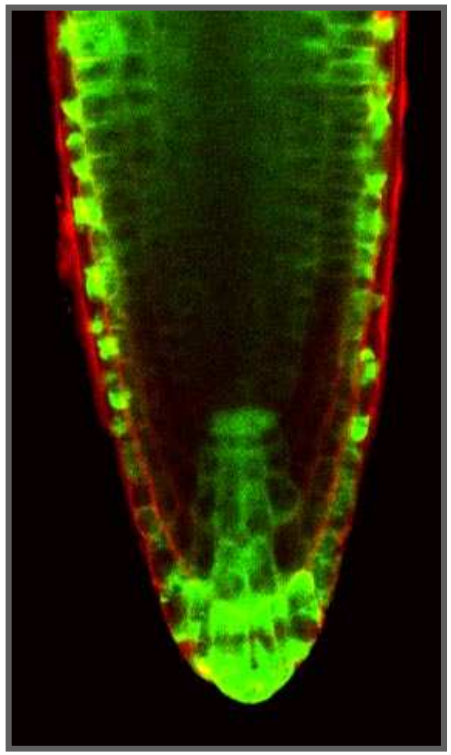
+ Auxin



# *DR5::GFP* Auxin Reporter

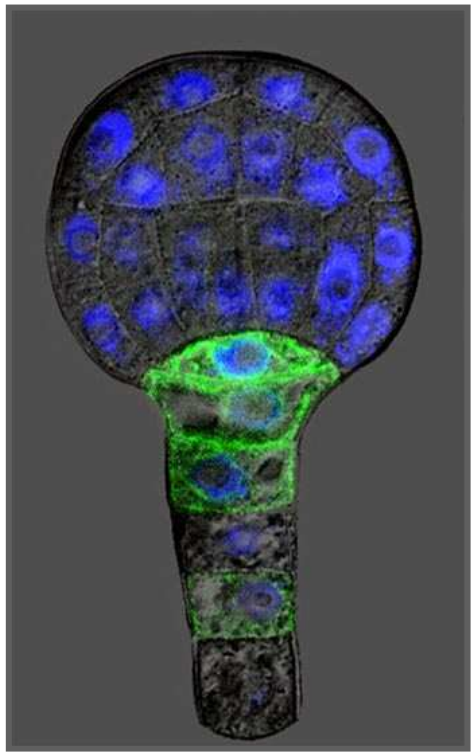



Root



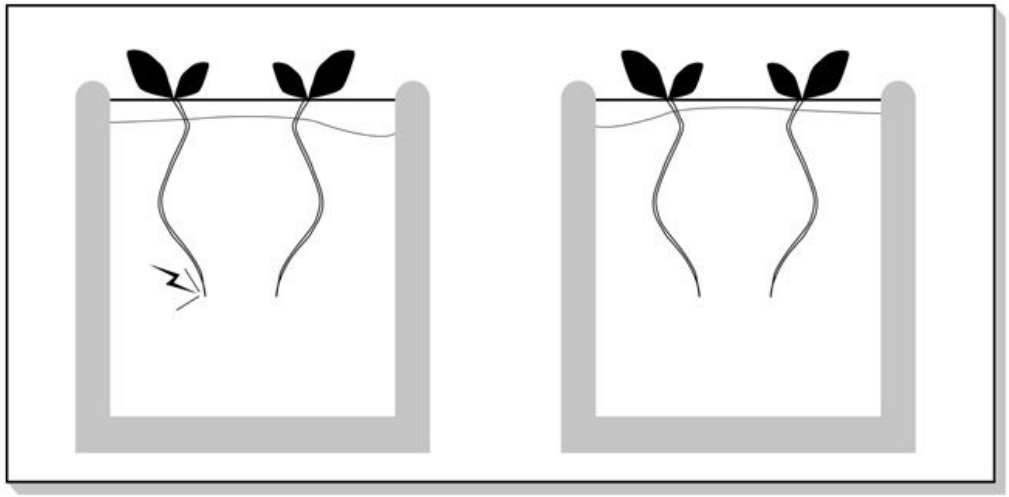
Root + Auxin

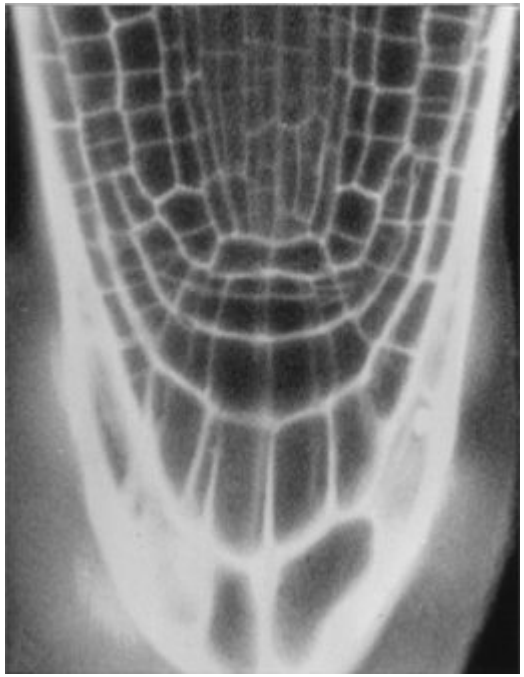
anti-IAA AB

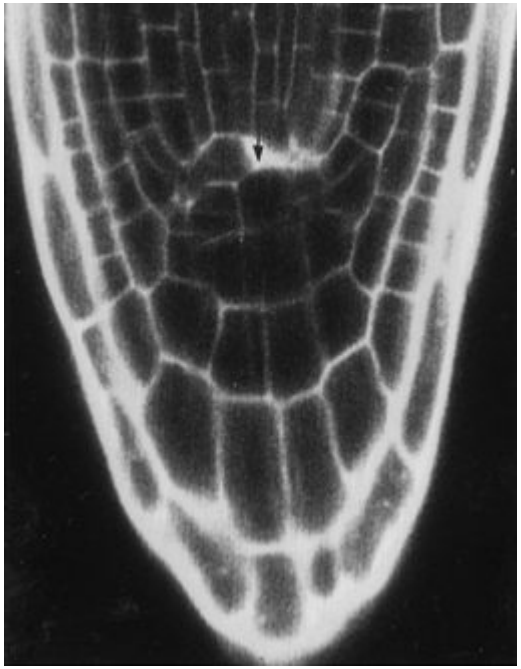


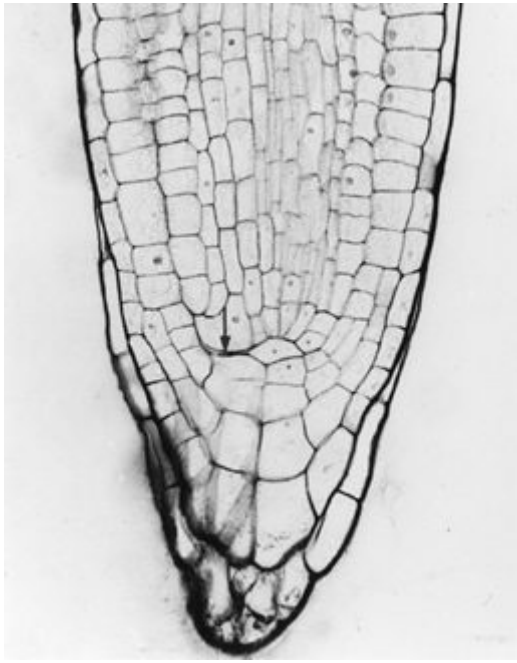
Embryos

# Laser ablations

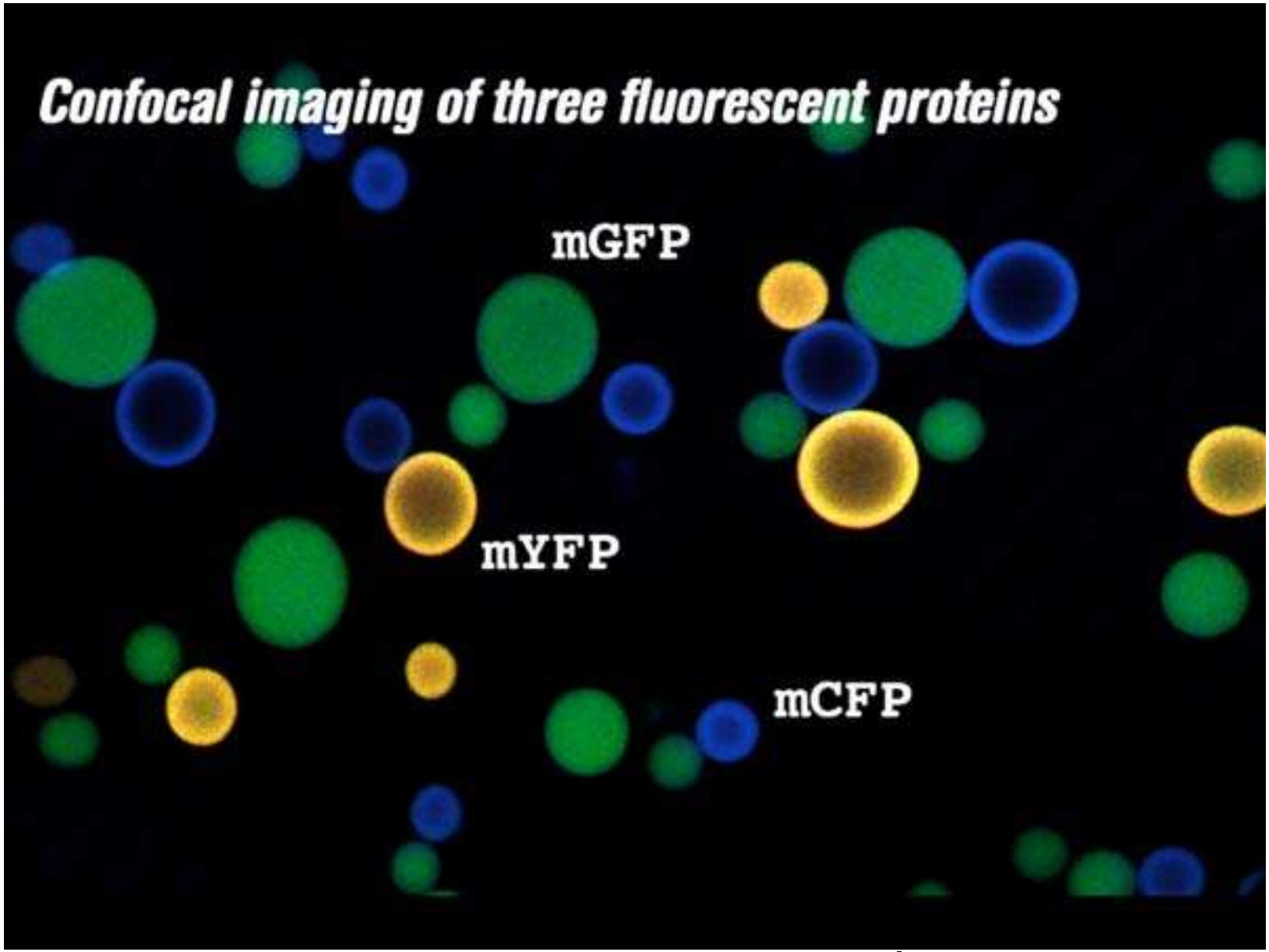






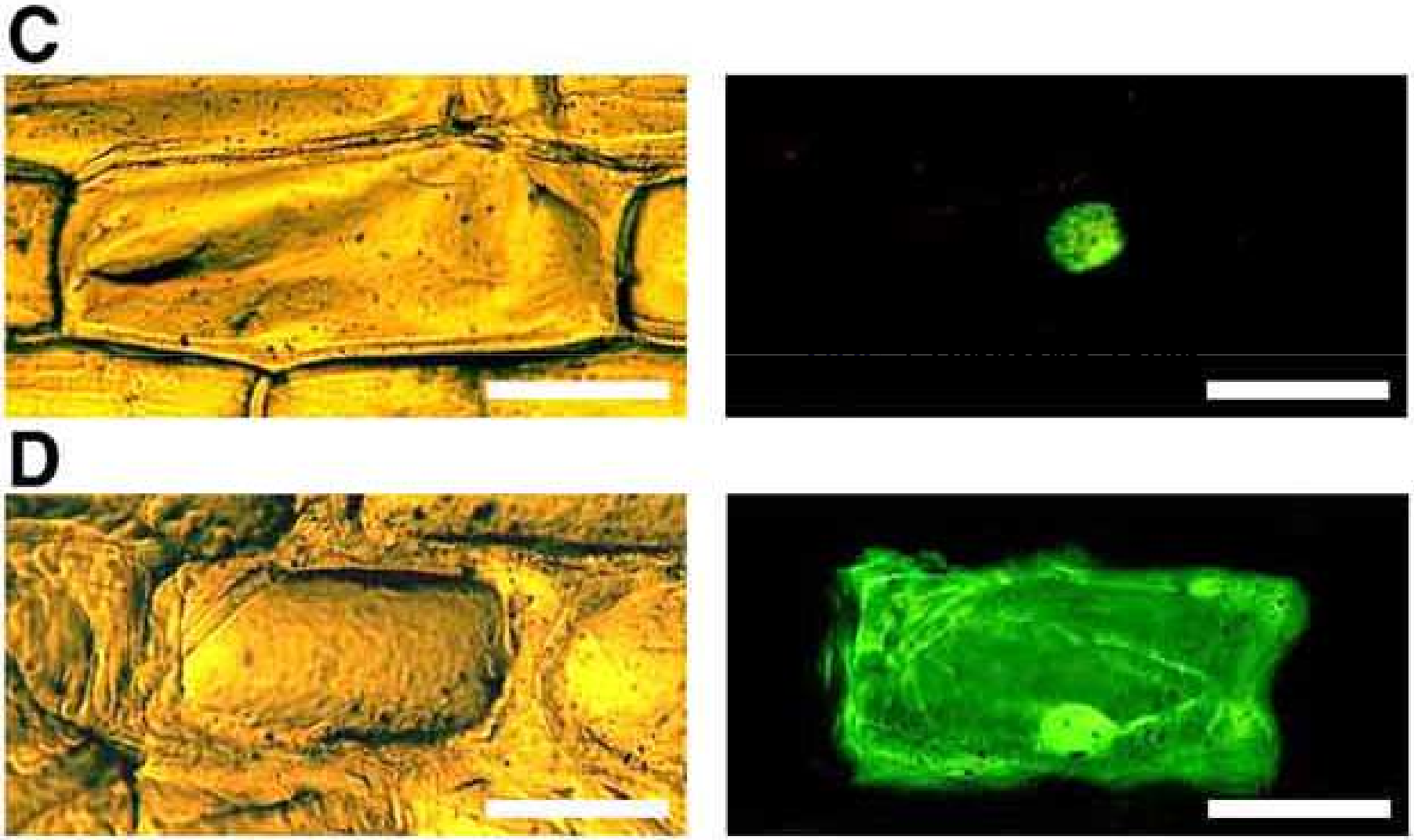


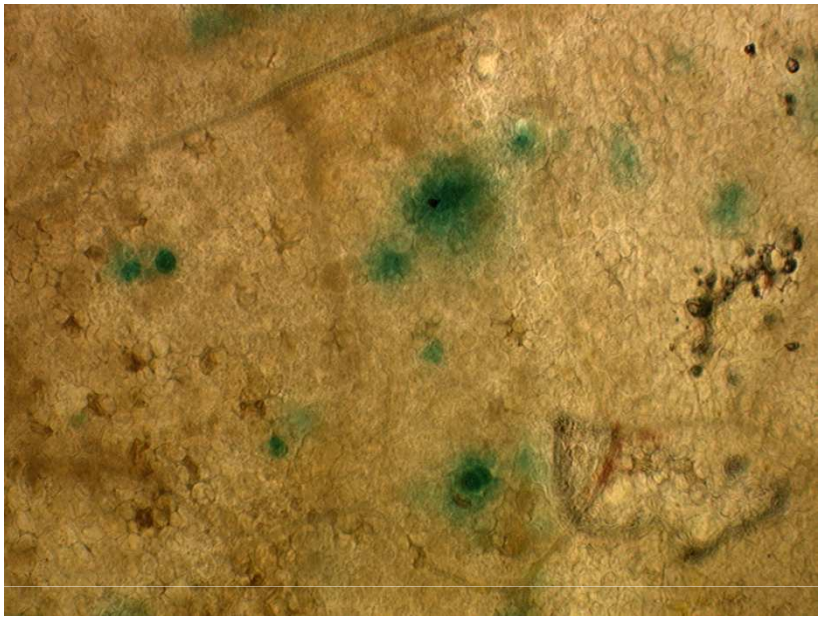
# Transient transfection



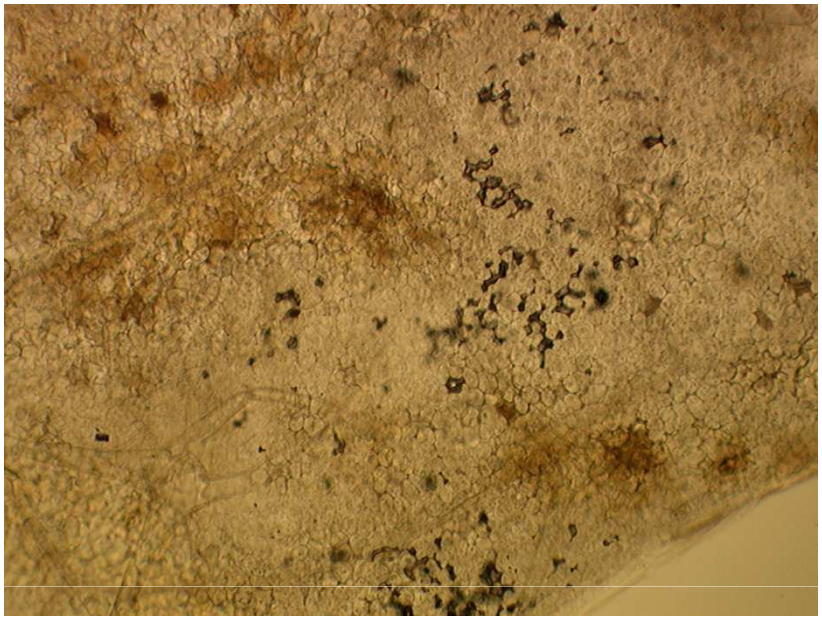

Protoplasts

# Onion epidermis cells

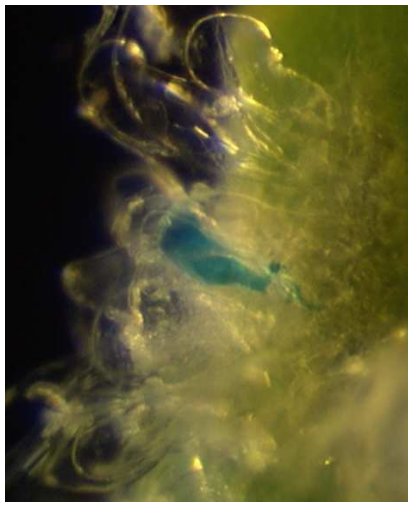




GUS



GUS + Diphteria Toxin



GUS + IPT (cytokinin biosynthesis)

# Laser capture