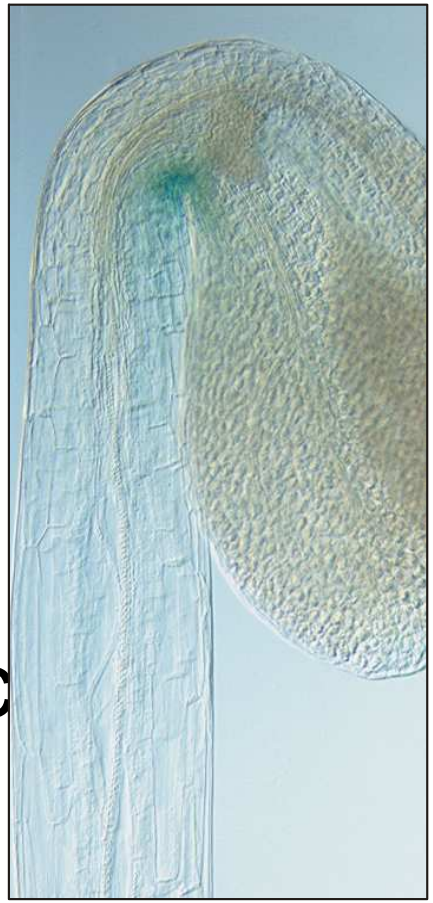
Experimental biology

Description > Manipulation > Understanding

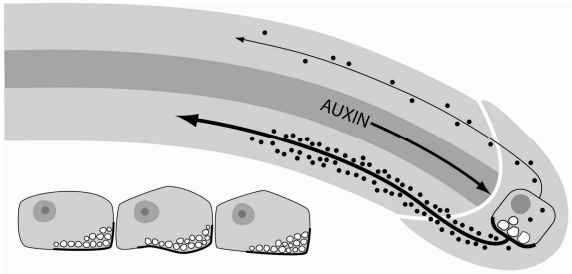
Money > Applications > Publishing

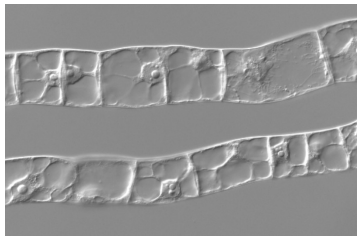


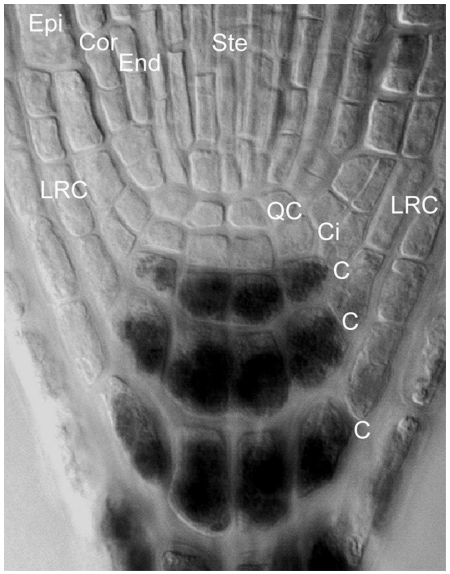
- Anatomy
- Physiology (spray and pray)
- Chemistry (identification of signals)
- Biochemistry (protein isolation/struc
- Genetics (genes/mutants)
- Cell biology (subcellular structures)
- Molecular biology (gene manipulation)

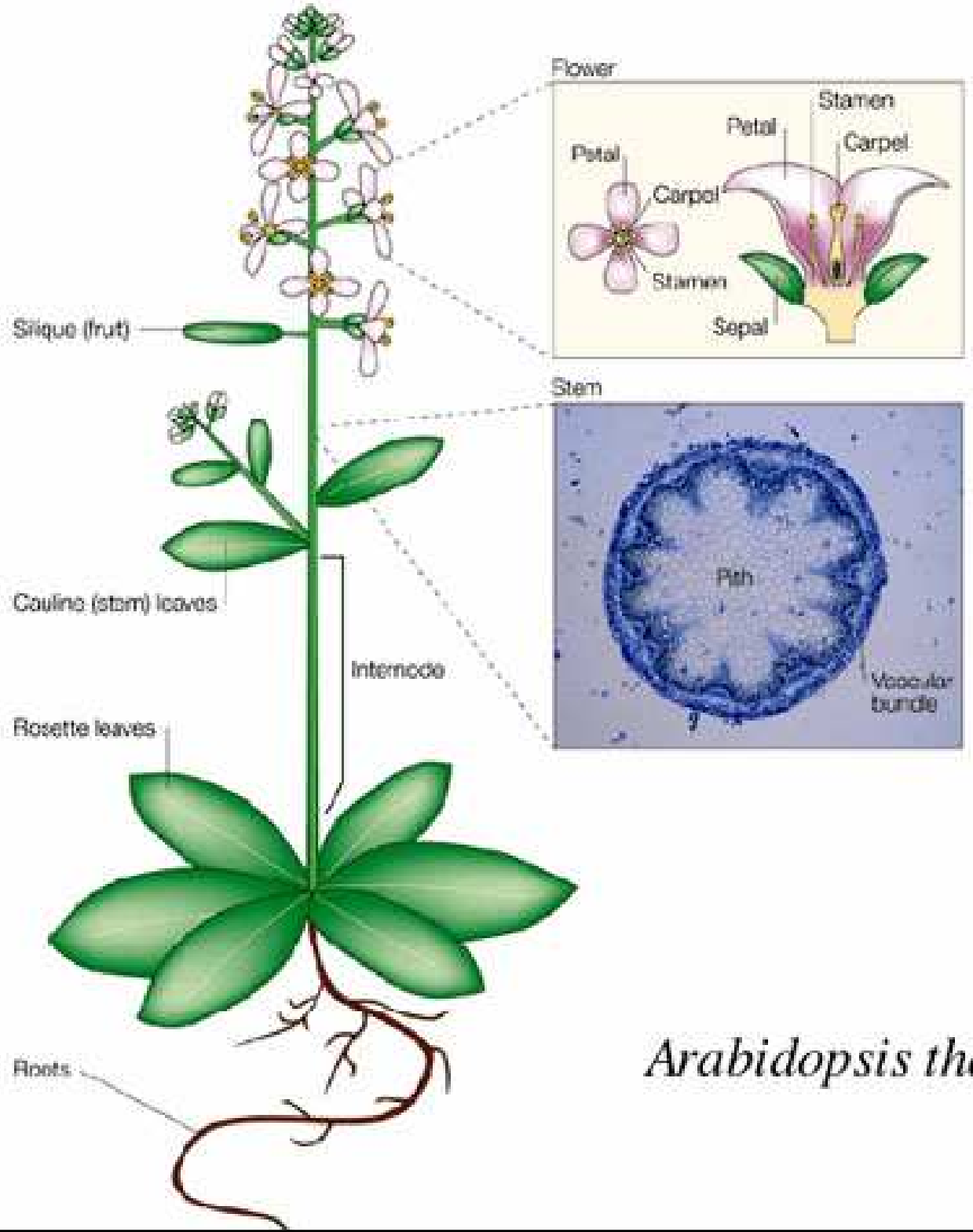
Choice of research topic?

- 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)

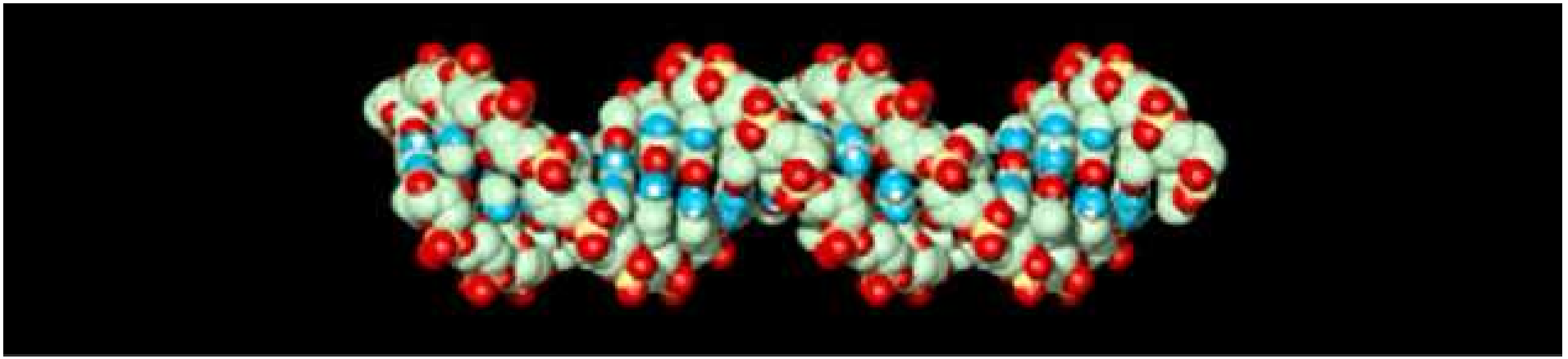
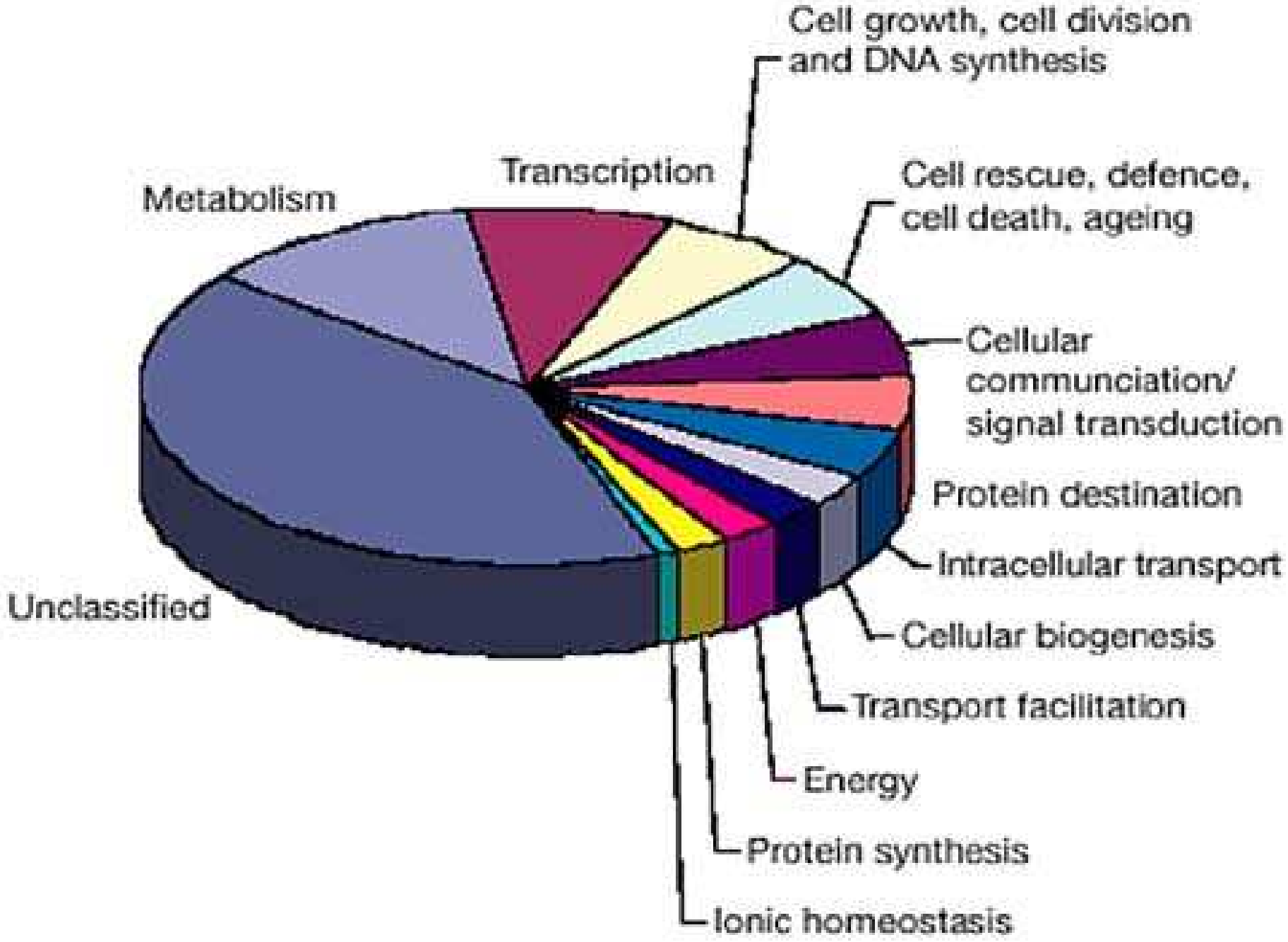
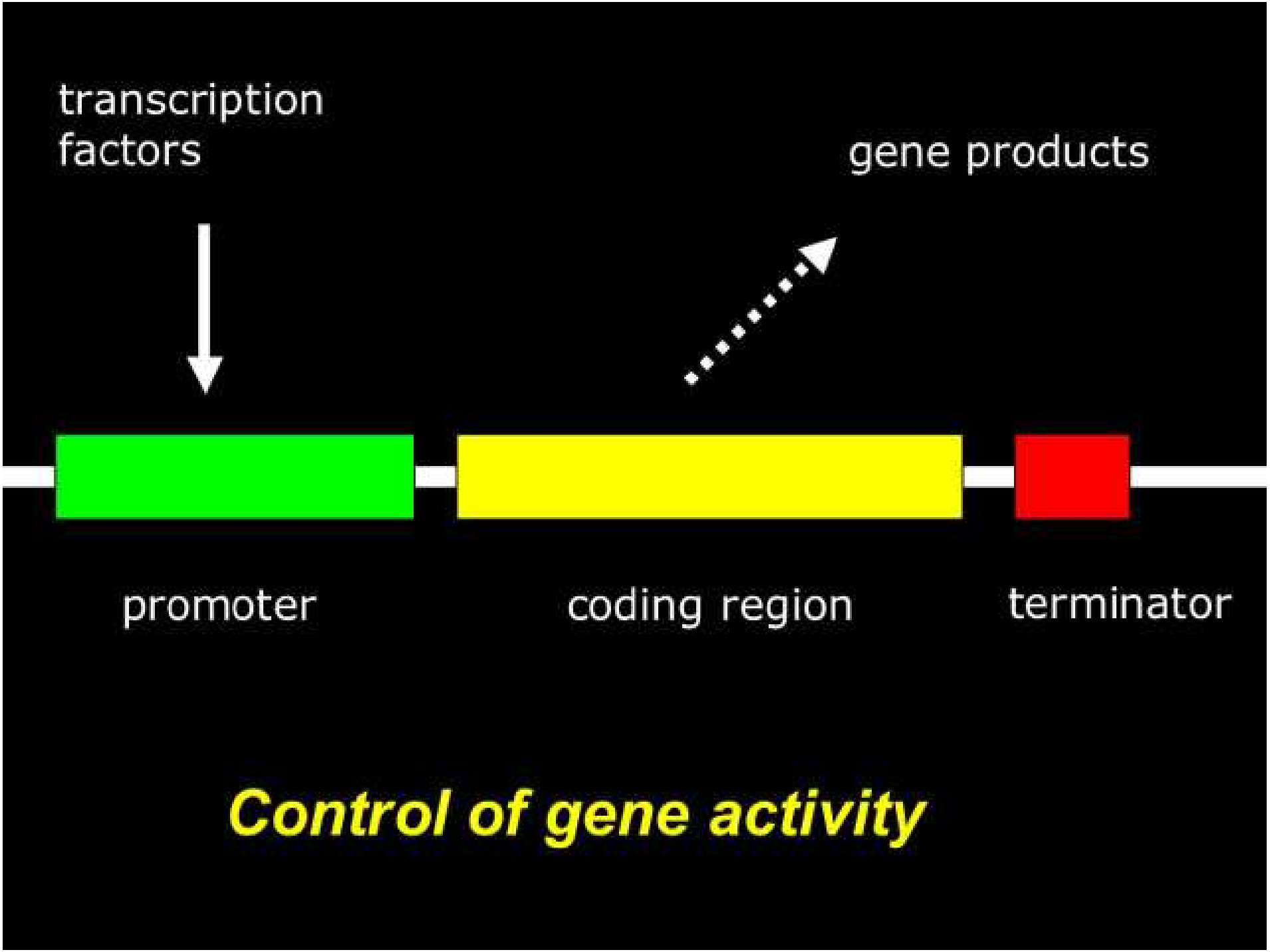


Table 4 General features of genes encoded by the three genomes in *Arabidopsis*

	Nucleus/cytoplasm	Plastid	Mitochondria
Genome size	125 Mb	154 kb	367 kb
Genome equivalent/cell	2	560	26
Duplication	60%	17%	10%
Number of protein genes	25,498	79	58
Gene order	Variable, but syntenic	Conserved	Variable
Density (kb per protein gene)	4.5	1.2	6.25
Average coding length	1,900 nt	900 nt	860 nt
Genes with introns	79%	18.4%	12%
Genes/pseudogenes	1/0.03	1/0	1/0.2–0.5
Transposons (% of total genome size)	14%	0%	4%


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

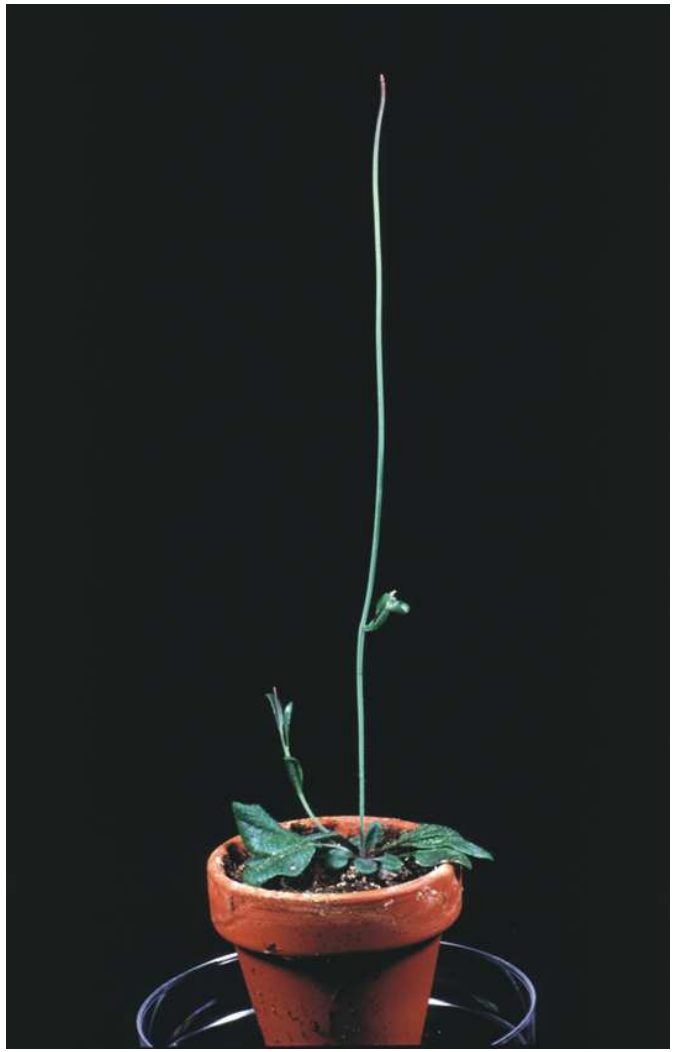

transcription
factors
gene products
promoter
coding region
terminator
Control of gene activity

How to get your favorite gene?

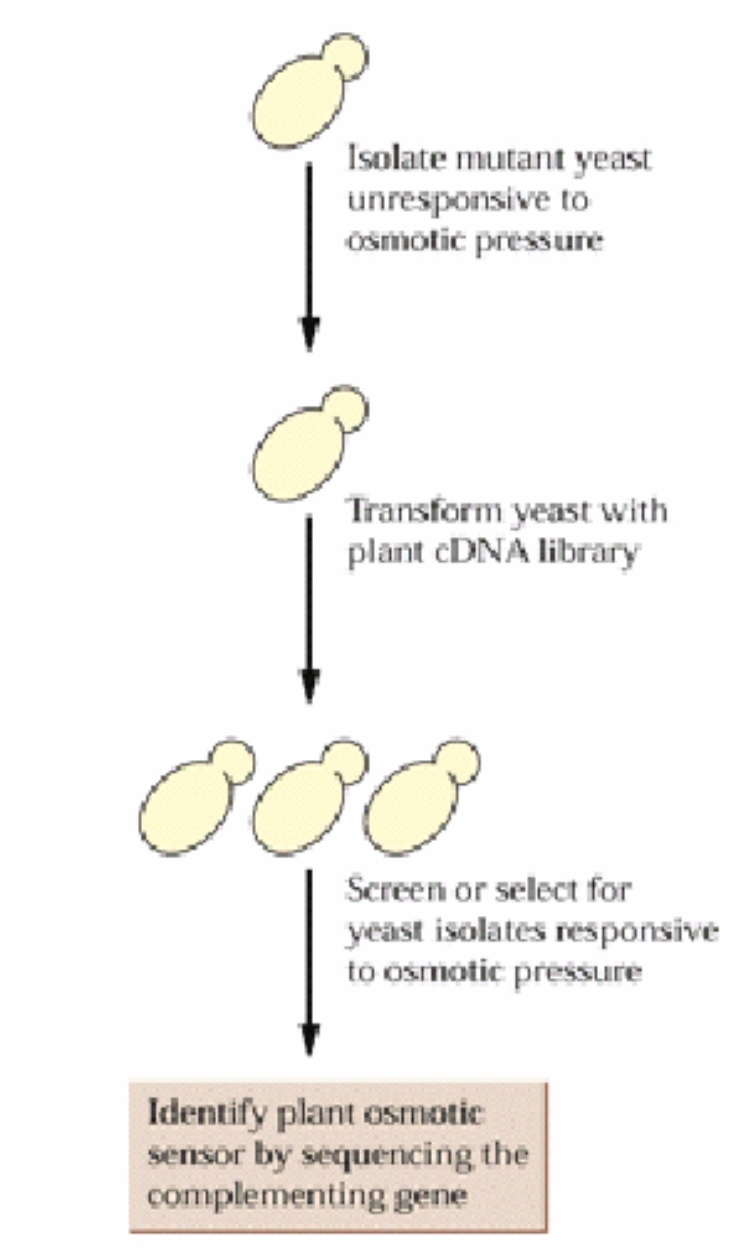
- “Monte Carlo” candidate gene approach
- Functional complementation
- From the protein back to the gene
- Expression
- Forward genetics

“Monte Carlo”

- Homology to known factors
  (trimeric G-proteins)
- Interesting domains
  (kinases, phosphatases)
- „Other“ reasons
  (serendipity)

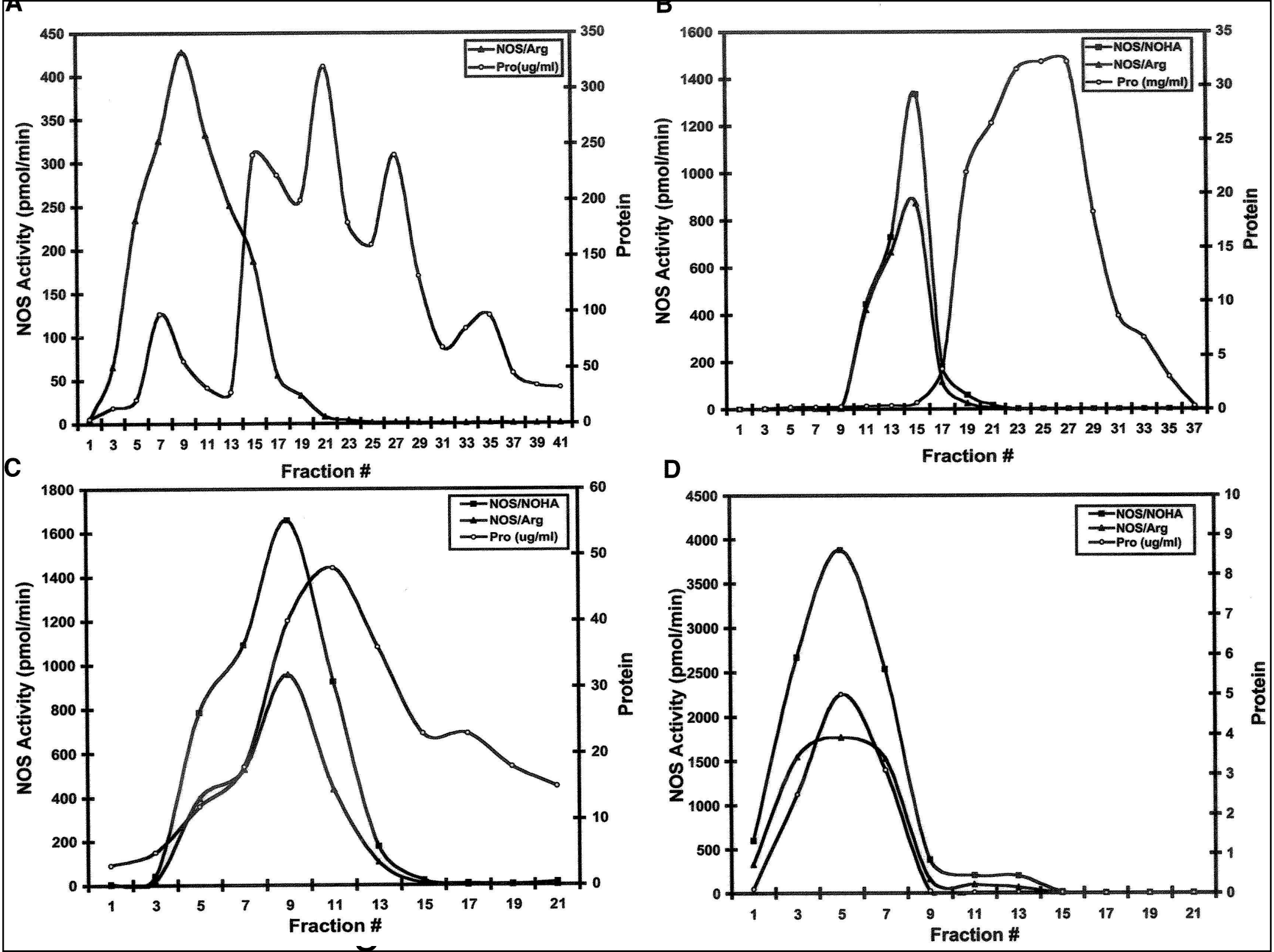


Functional complementation



Protein > gene

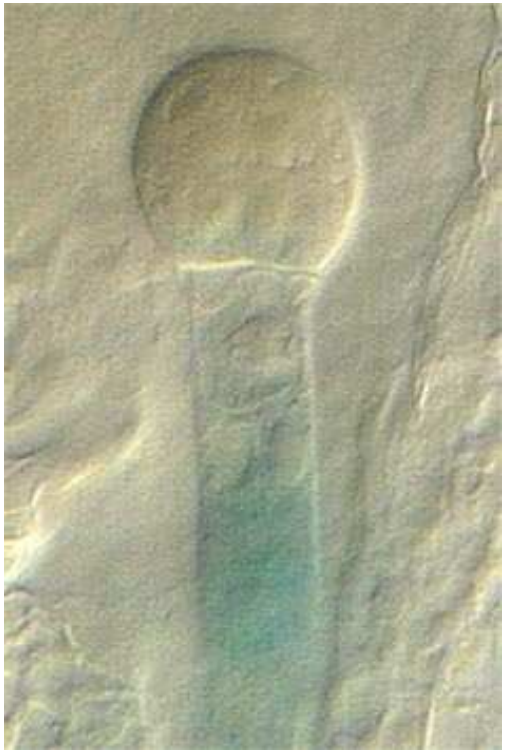
- Ligand binding (affinity chromatography, azidolabeling; ABP1, NPB, Zm-p60)
- Enzyme activity (CKX, NOS)
- Complex members
- Proteomics approaches (phosphoproteomics, differential display)
  - Microsequencing
  - Blast search:
    - amino acid > nucleotide
  - Search for a gene

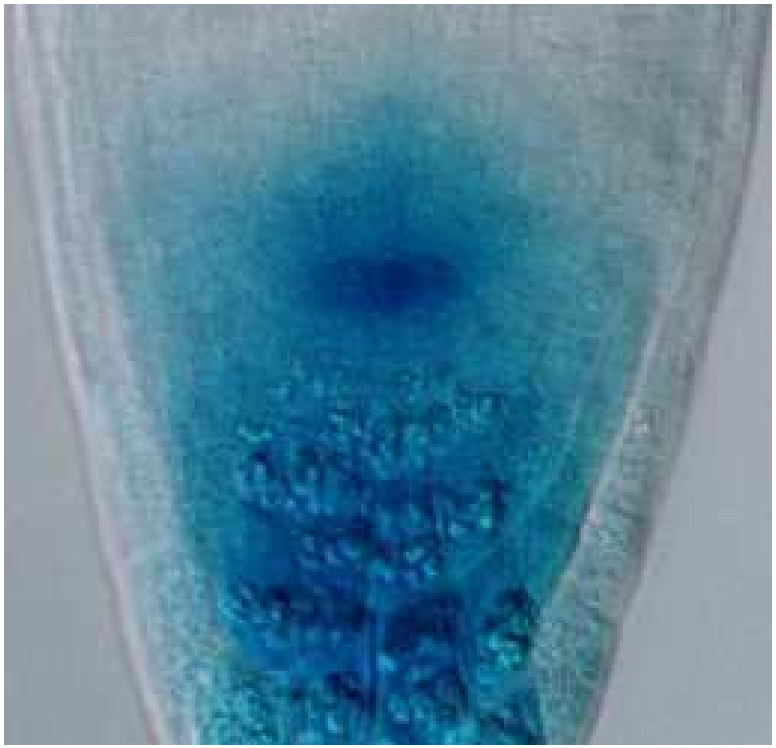

A
NOS/Arg
Pro(ug/ml)
NOS Activity (pmol/min)
Protein
Fraction #
B
NOS/NOHA
NOS/Arg
Pro (mg/ml)
NOS Activity (pmol/min)
Protein
Fraction #
C
NOS/NOHA
NOS/Arg
Pro (ug/ml)
NOS Activity (pmol/min)
Protein
Fraction #
D
NOS/NOHA
NOS/Arg
Pro (ug/ml)
NOS Activity (pmol/min)
Protein
Fraction #

Expression pattern

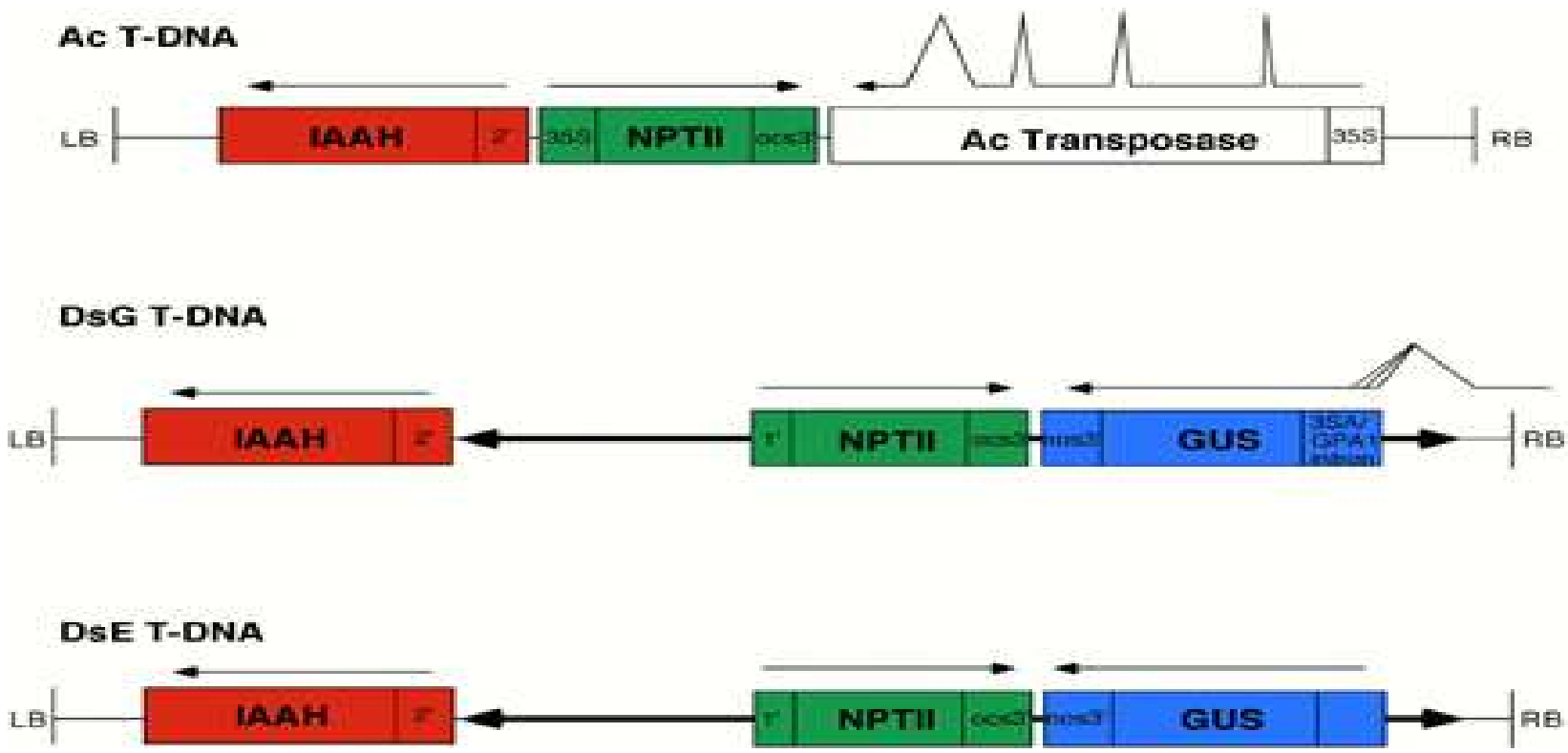
- Enhancer/Gene-trap libraries

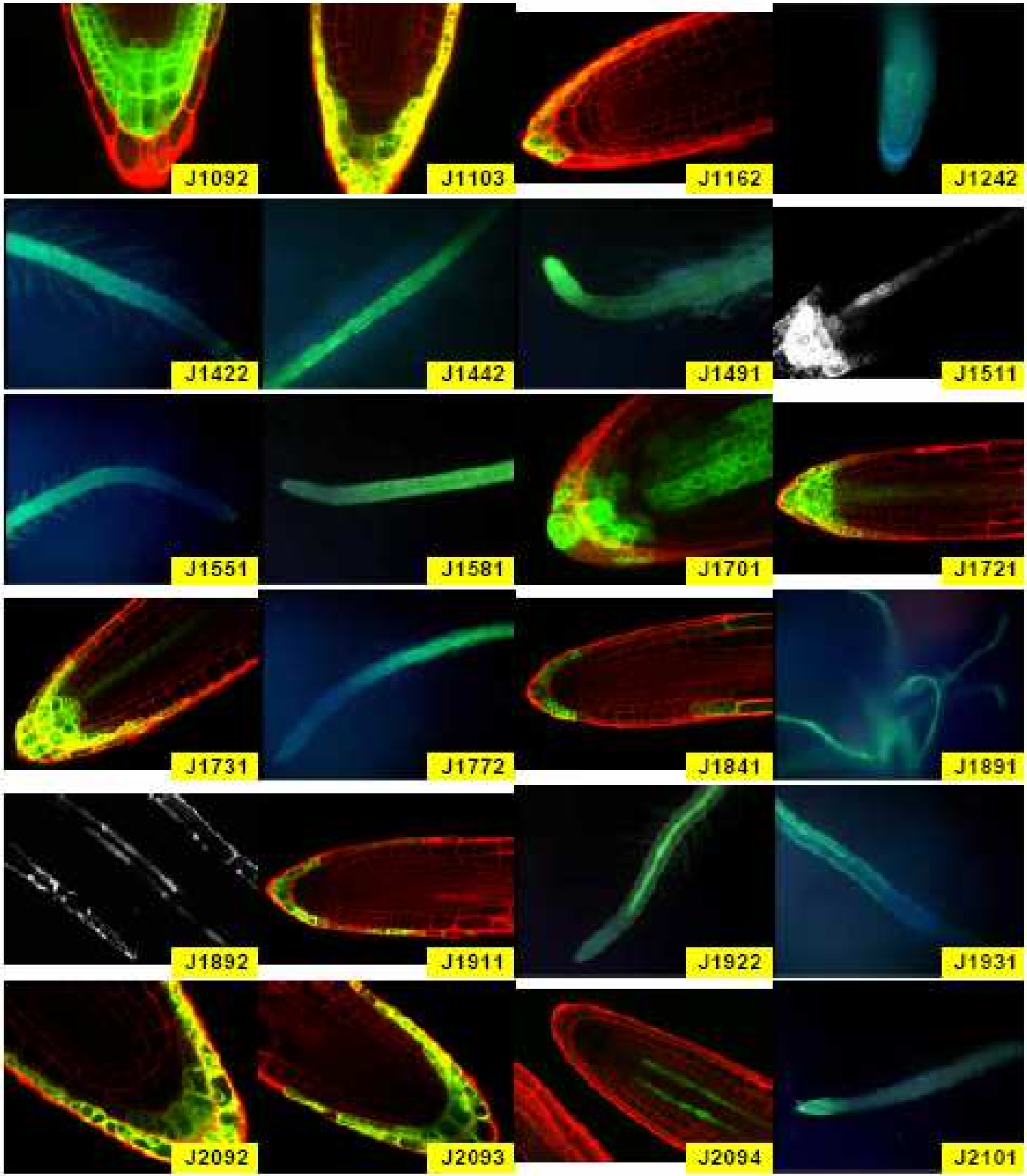
- Differential display
  - substractive hybridisation
  - microarray



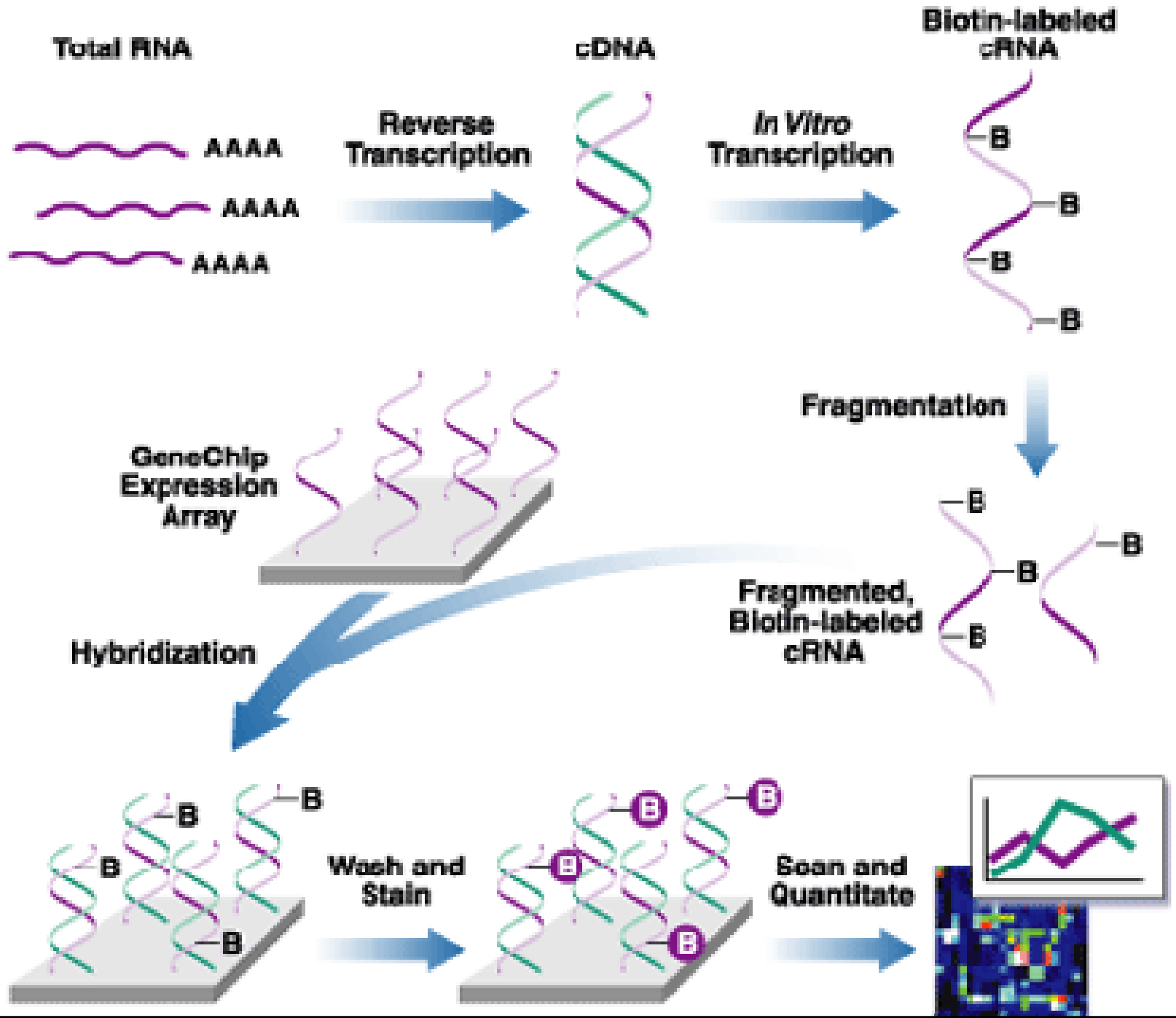


Gene and enhancer trap libraries

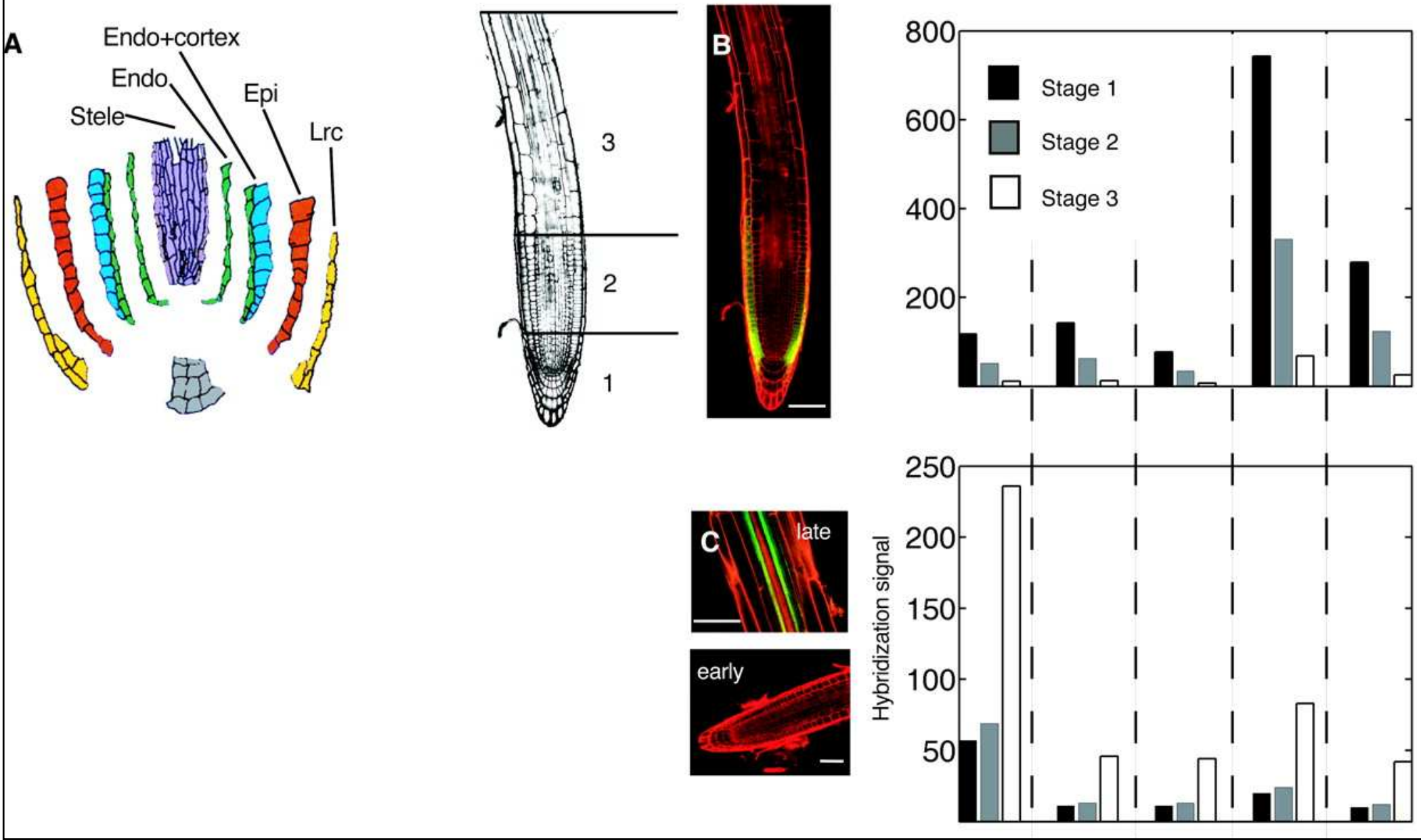



J1092
J1103
J1162
J1242
J1422
J1442
J1491
J1511
J1551
J1581
J1701
J1721
J1731
J1772
J1841
J1891
J1892
J1911
J1922
J1931
J2092
J2093
J2094
J2101

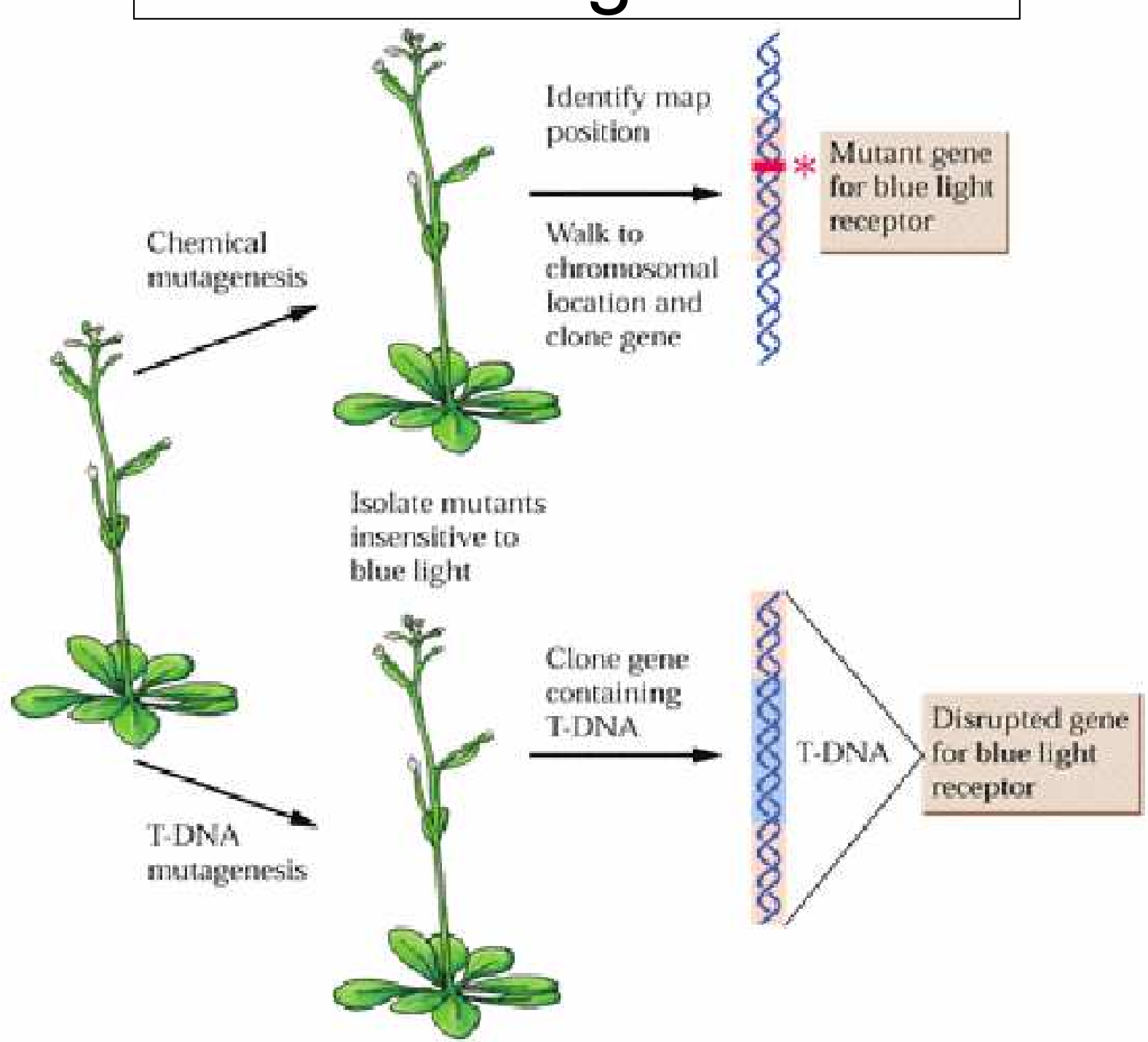
Microarray


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

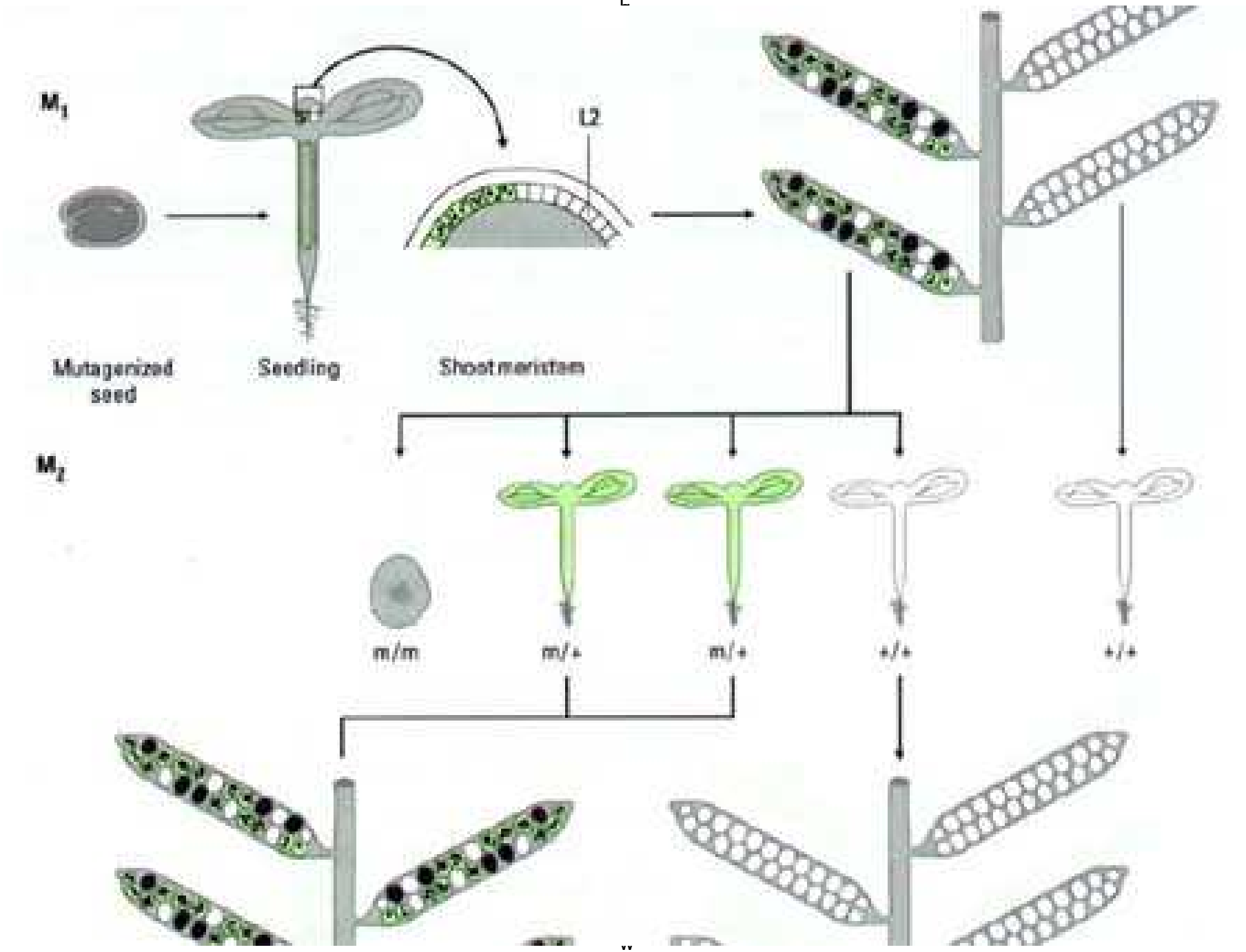
Expression map of Arabidopsis root



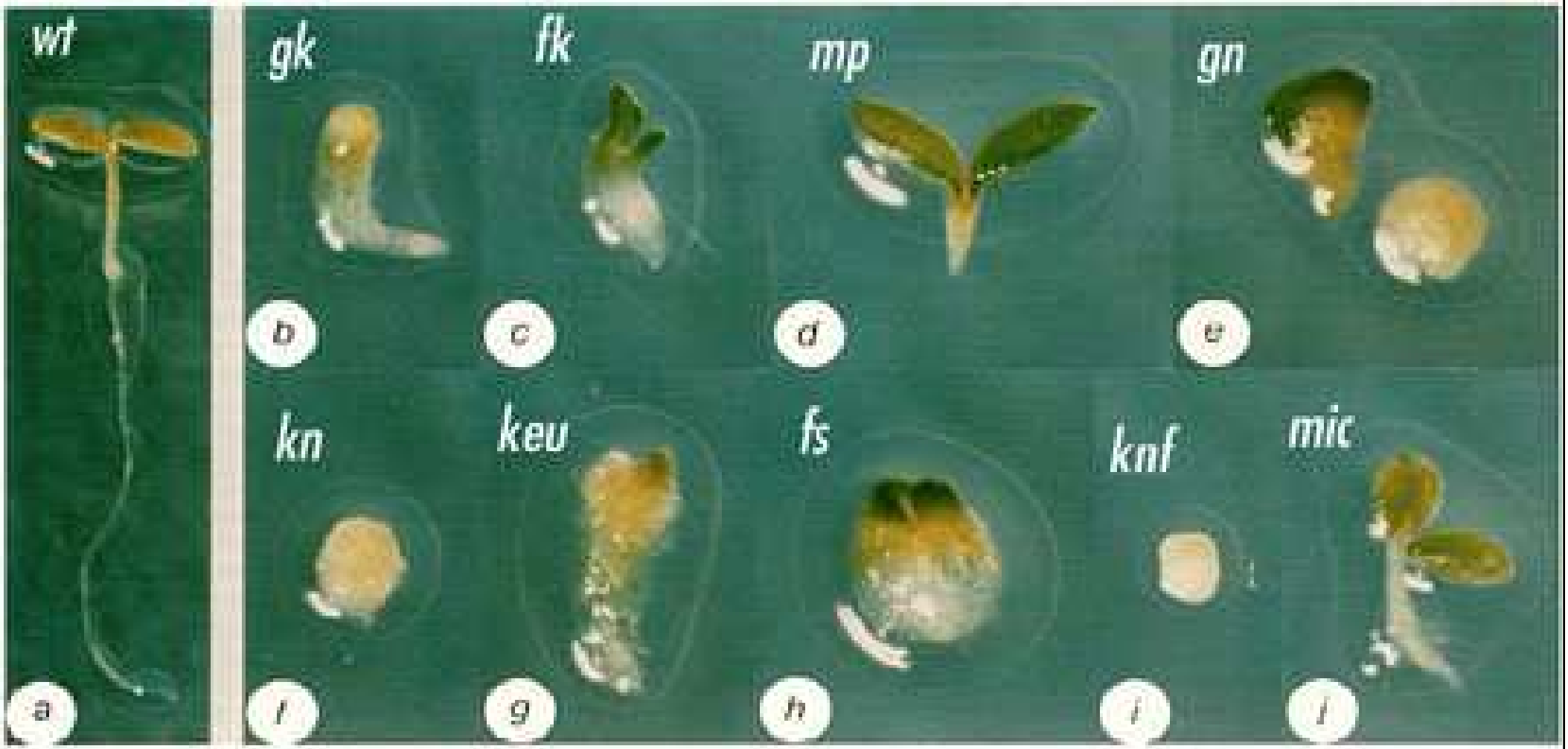
Forward genetics



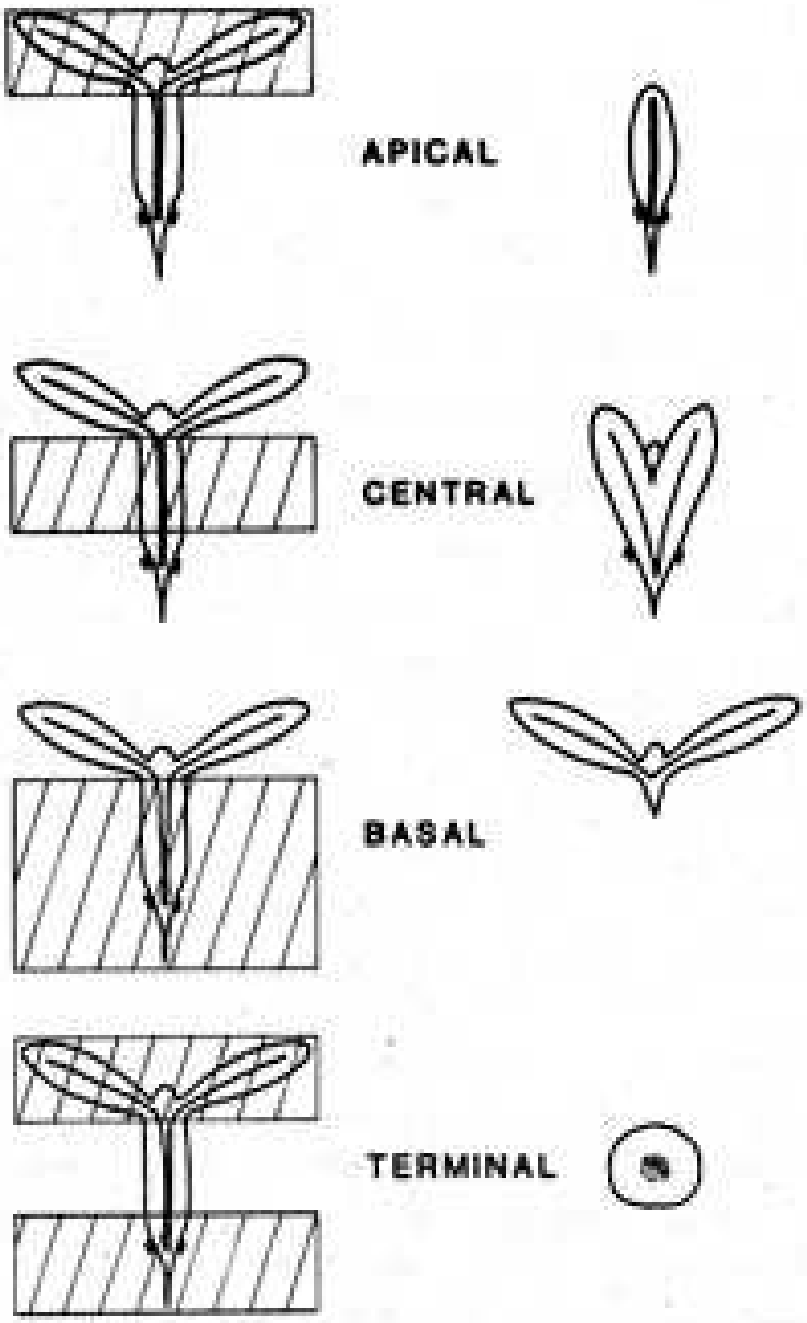
EMS mutagenesis



Mutant screen at seedling level

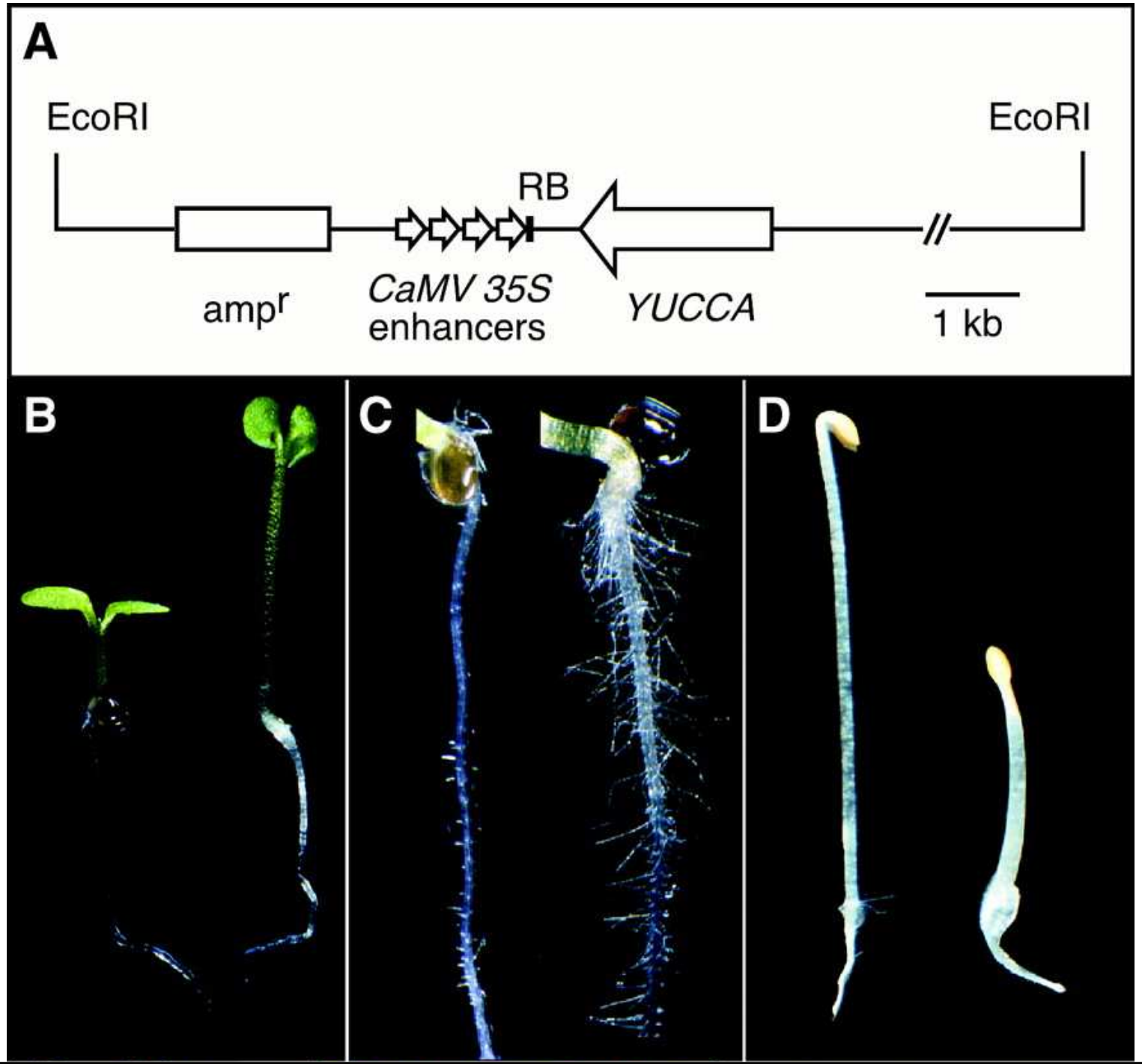


Patterning mutant types



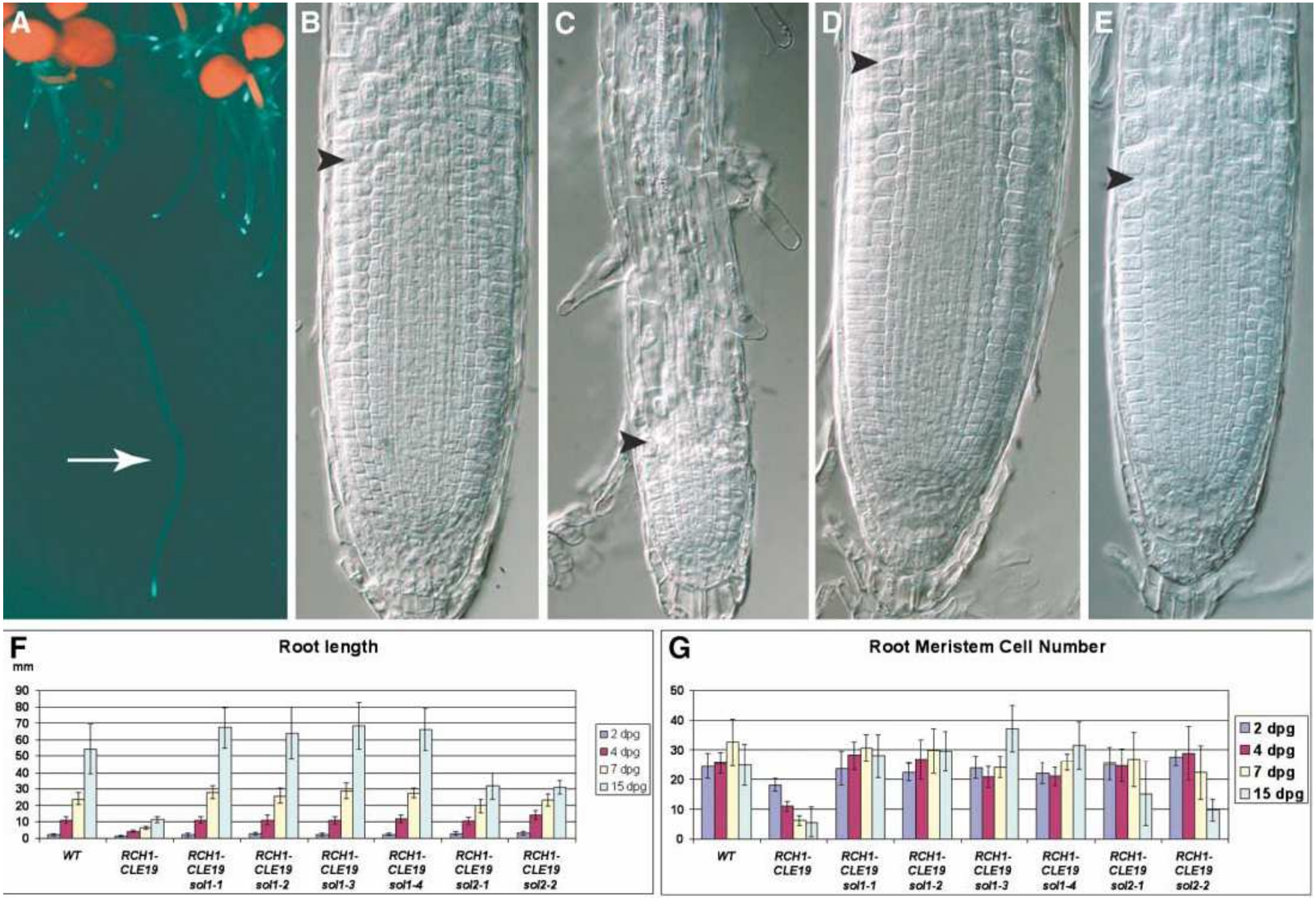
(gurke)	Fatty acids
(fackel)	Sterols
(monopterous)	Signalling
(gnom)	Vesicle traffic

Activation tagging - YUCCA

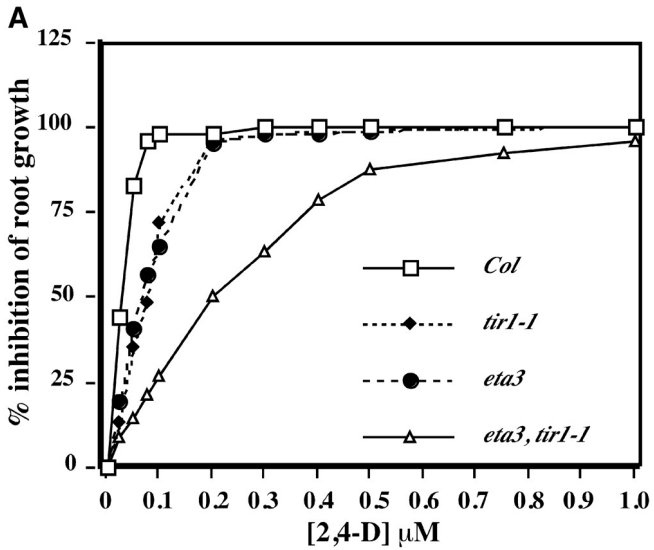


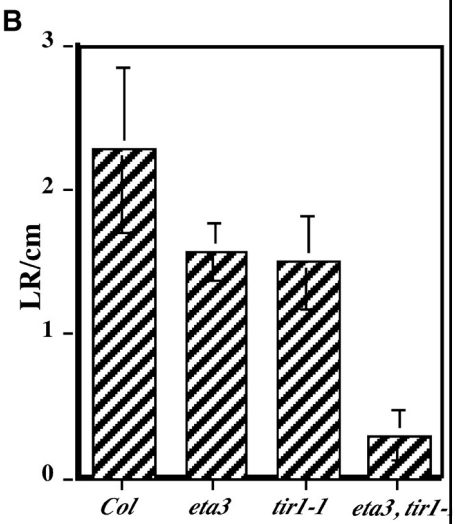
Second site mutagenesis - suppressors

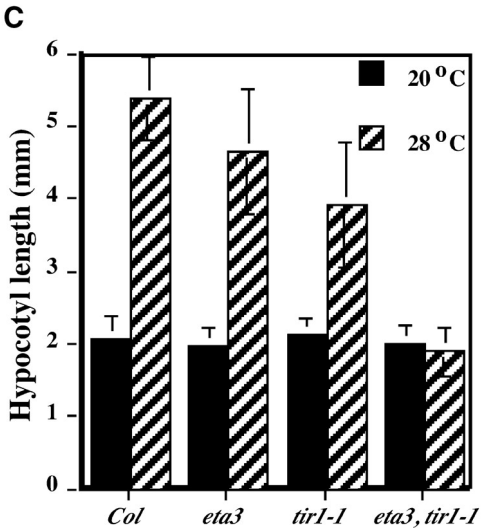
Suppressors of CLV3 overexpression



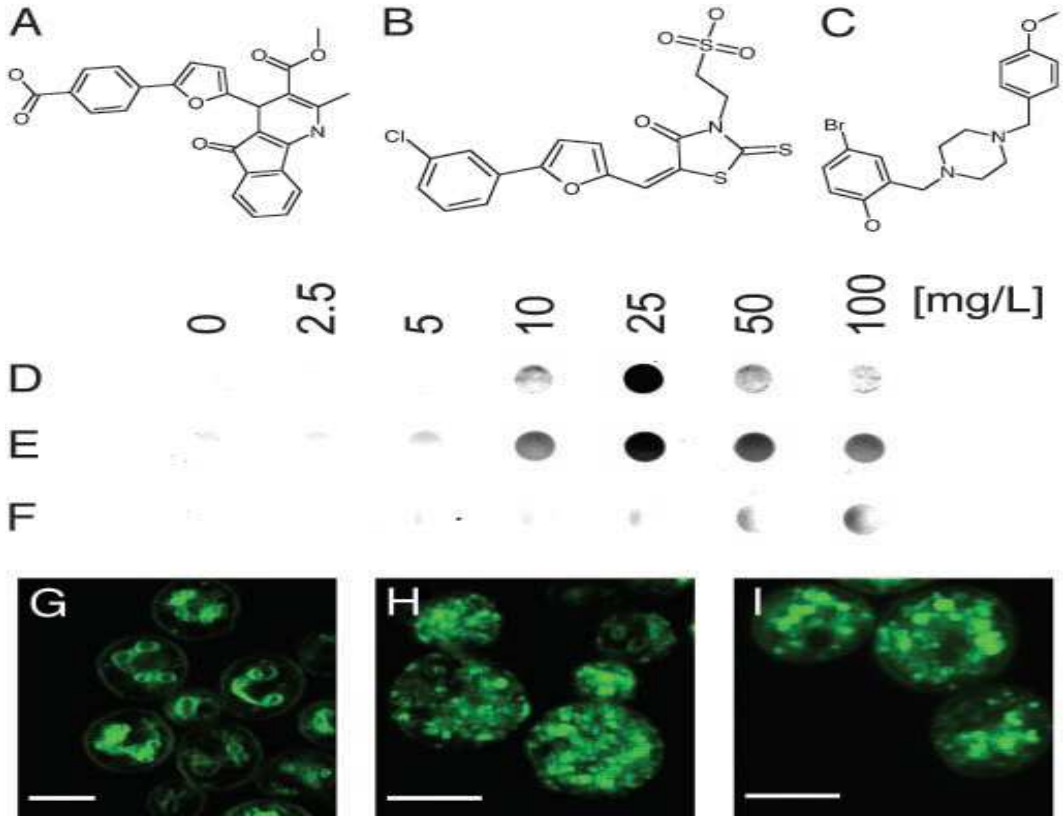
Second site mutagenesis - enhancers







Chemical genetics

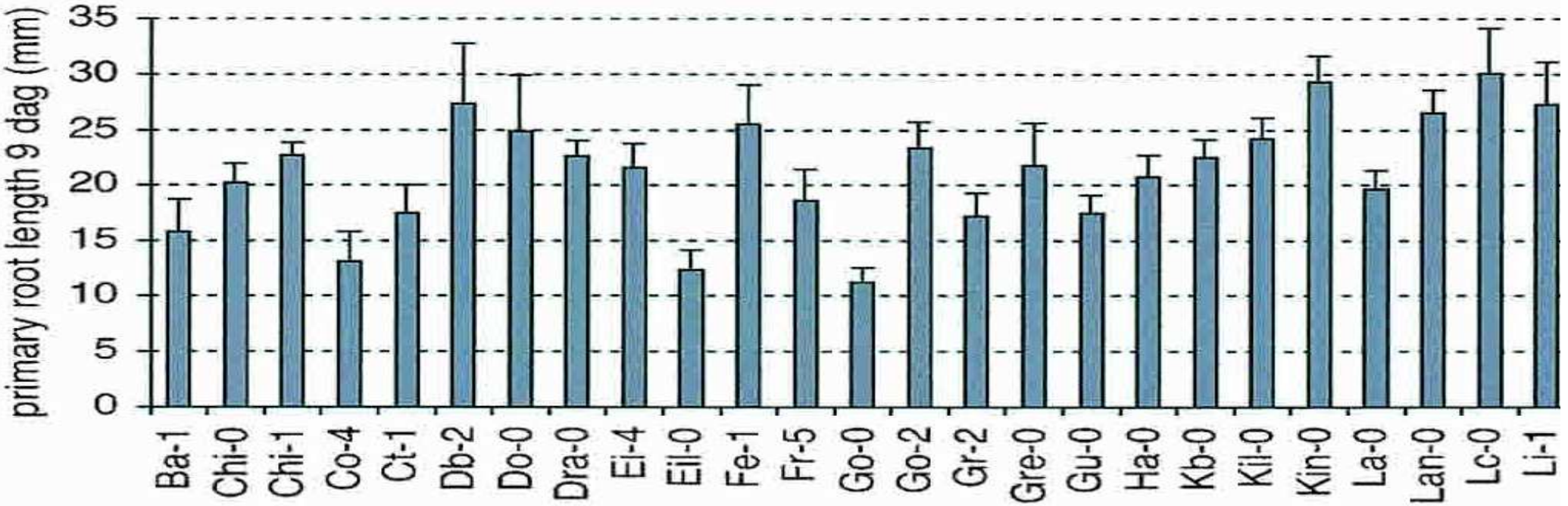


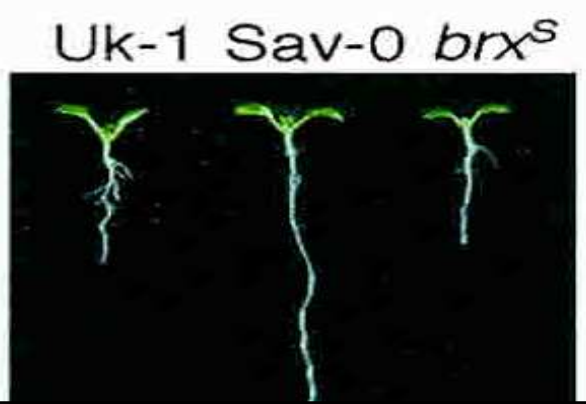
Zouhar et al., 2004

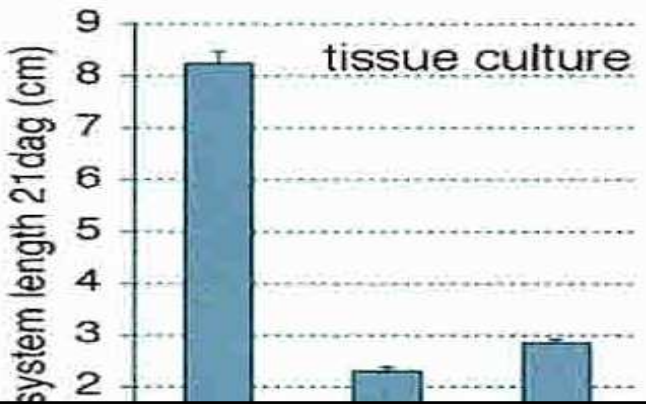
Gene verification

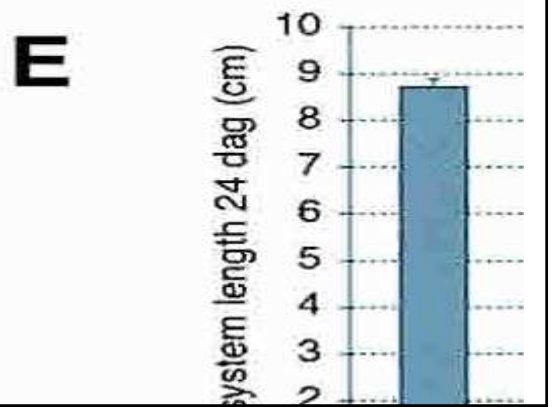
- Multiple alleles
- Transposone reversion
- Complementation

QTL


primary root length 9 dag (mm)
0
5
10
15
20
25
30
35
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


Uk-1 Sav-0 brx^s


C
tissue culture
system length 21dag (cm)
2
3
4
5
6
7
8
9


E
system length 24 dag (cm)
2
3
4
5
6
7
8
9
10

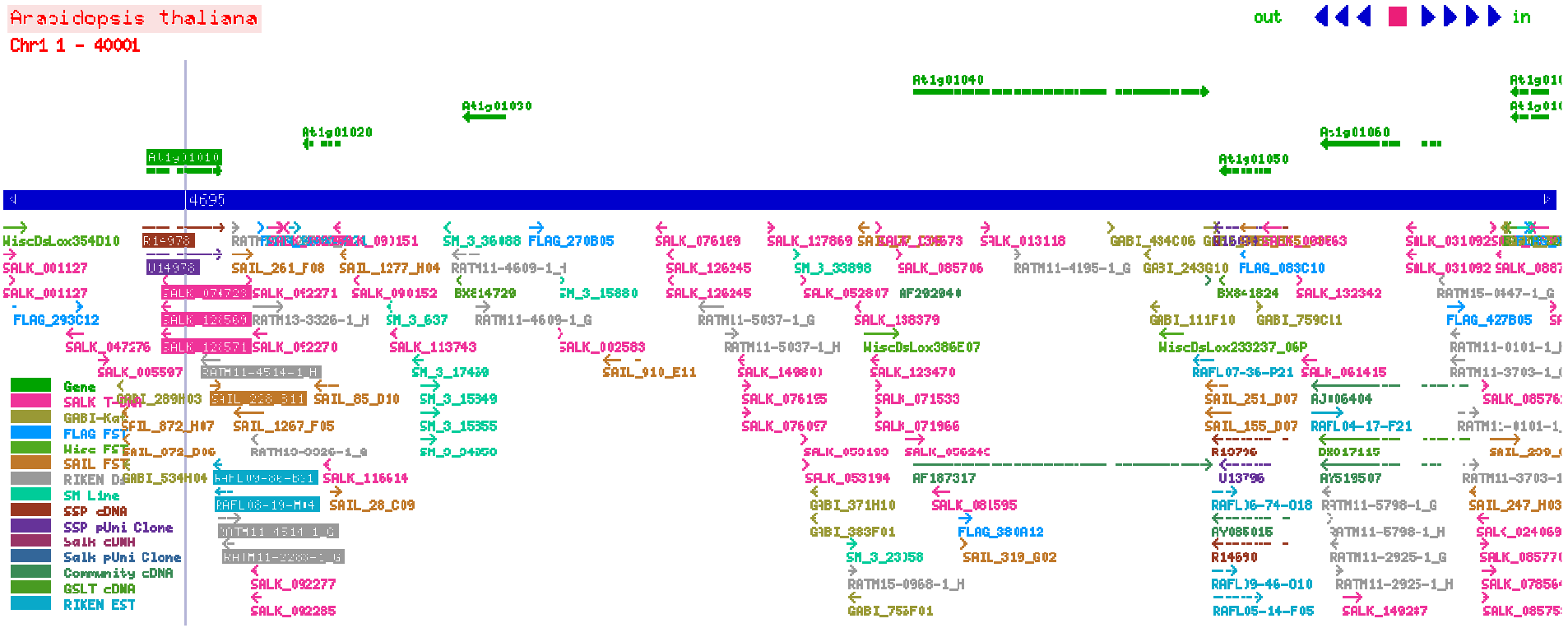
Towards a gene role

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

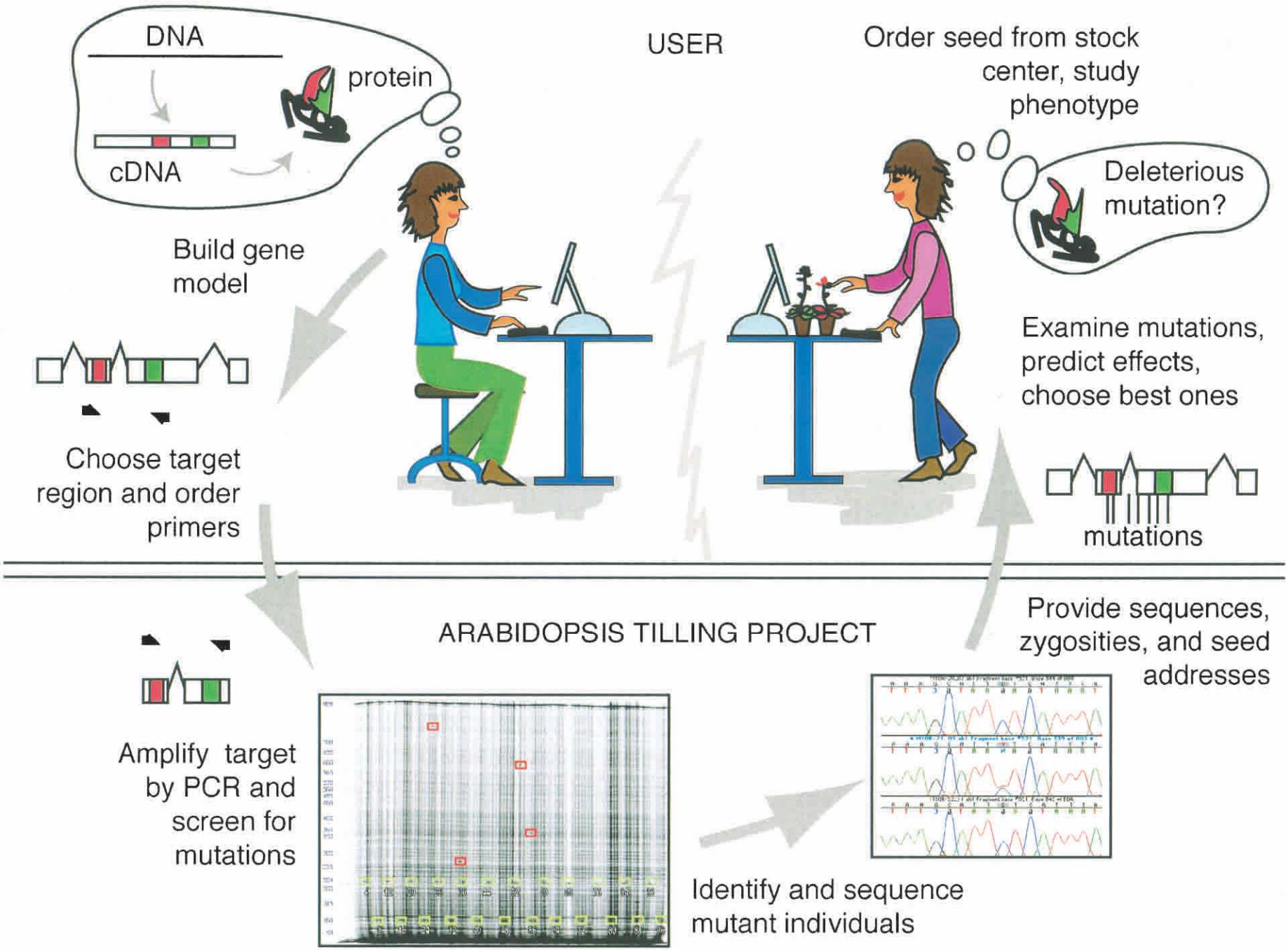
Loss of function

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

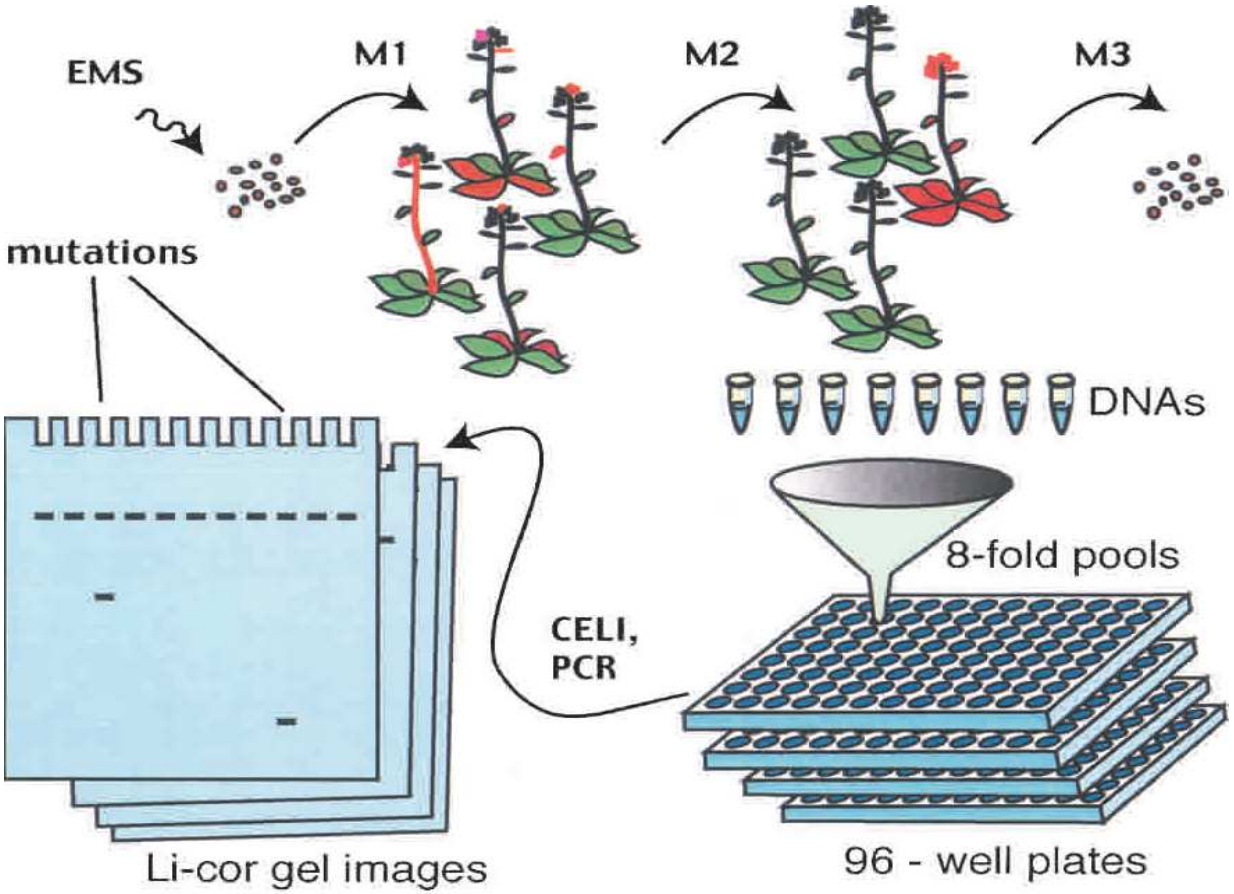
Reverse genetics – indexed mutant libraries



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

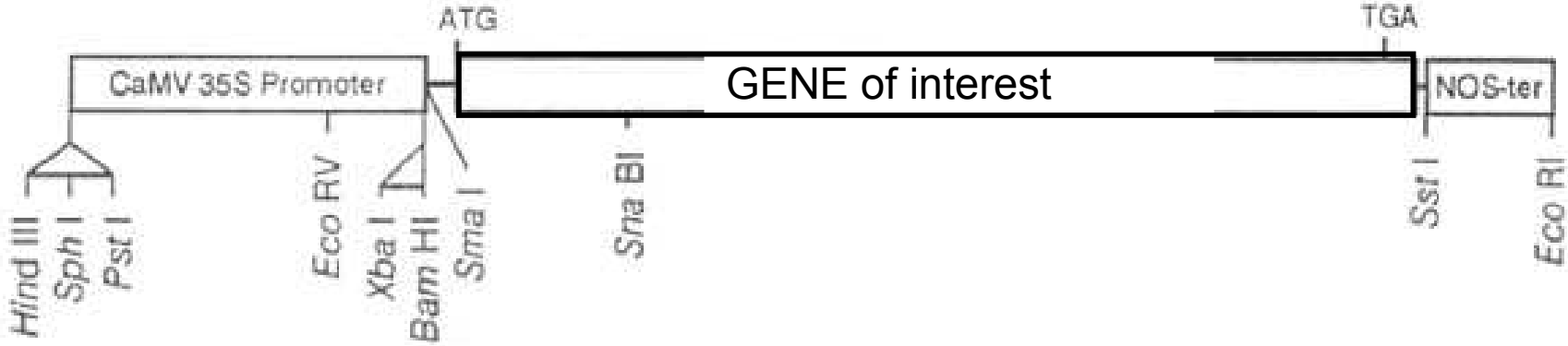
TILLING


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

Gain of function

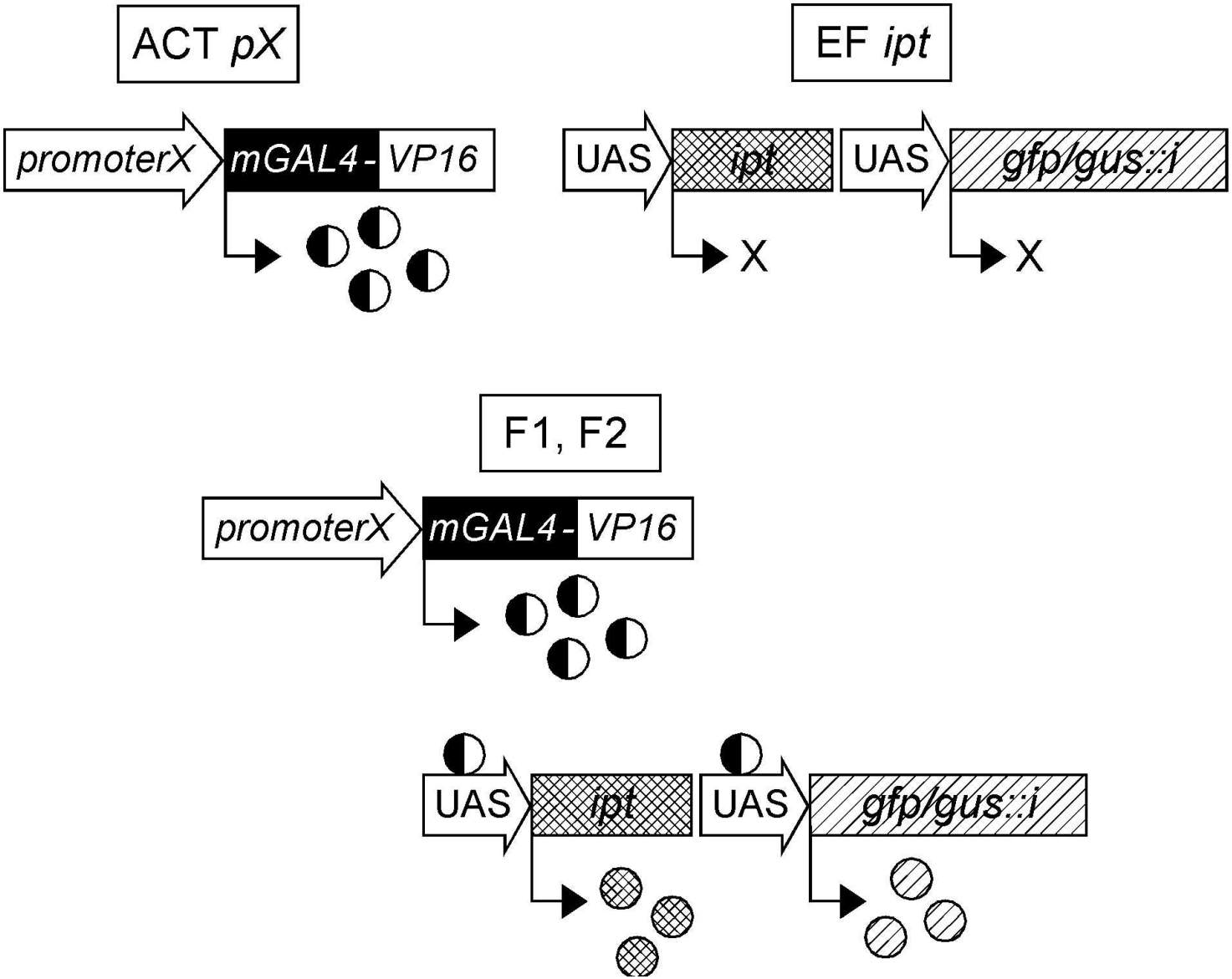
- Overexpression
- Tissue specific expression
- Conditional expression
- Protein stabilisation

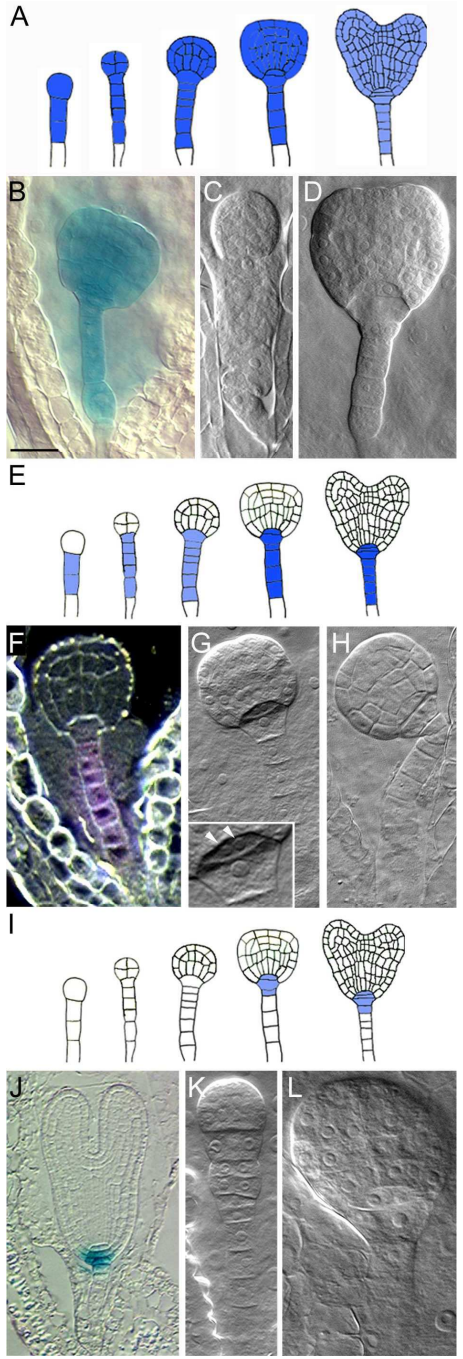
CaMV 35S Promotor

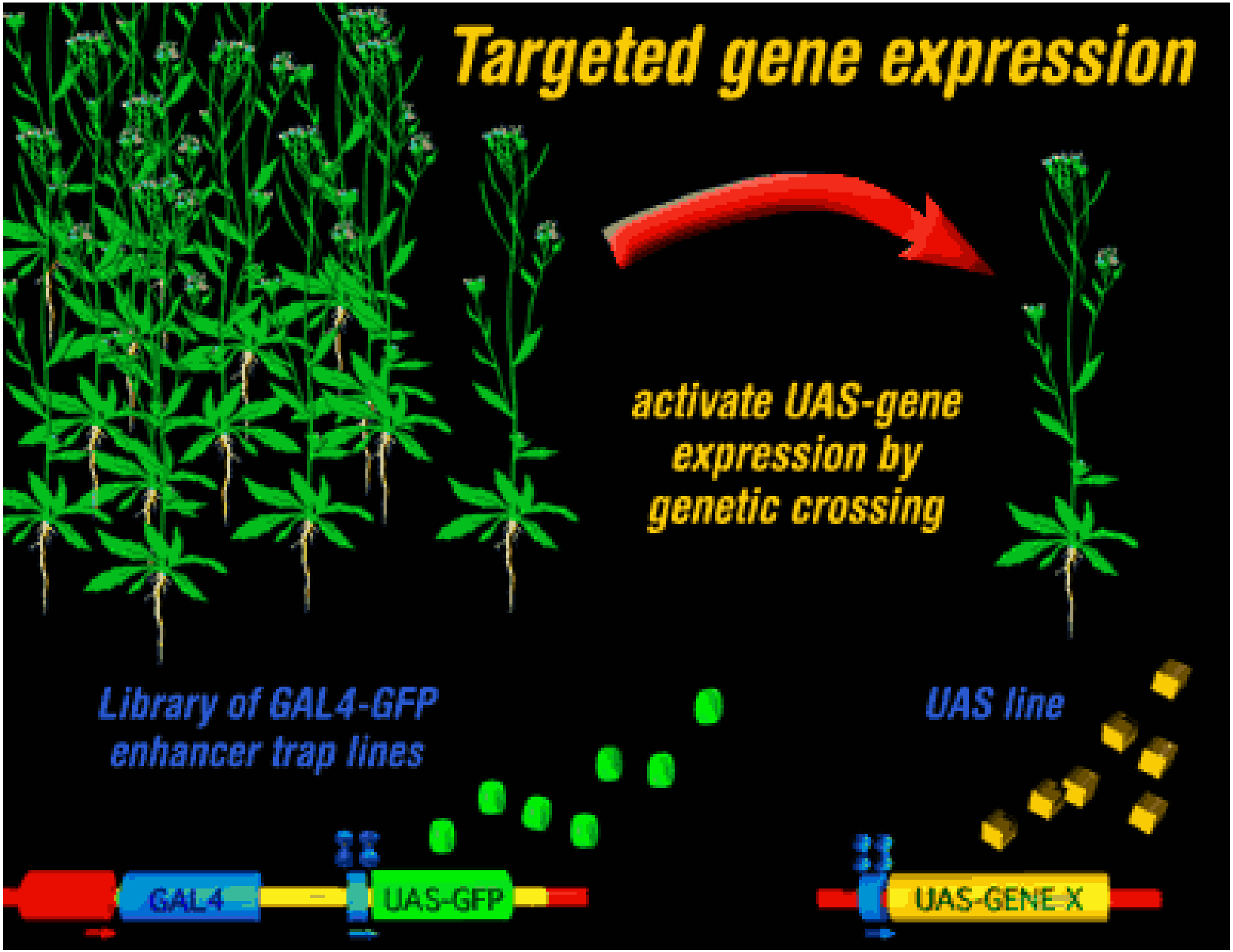


pBI221 The CaMV 35S promoter-GUS-NOS-ter portion of pBI121 was cloned into pUC19 to produce pBI221.

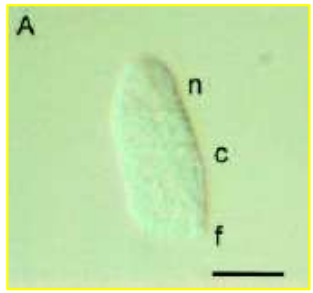
Two component system for gene expression

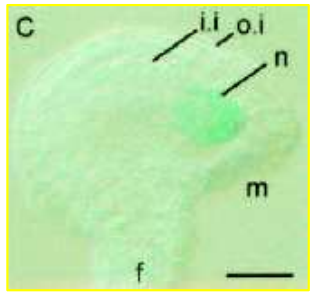


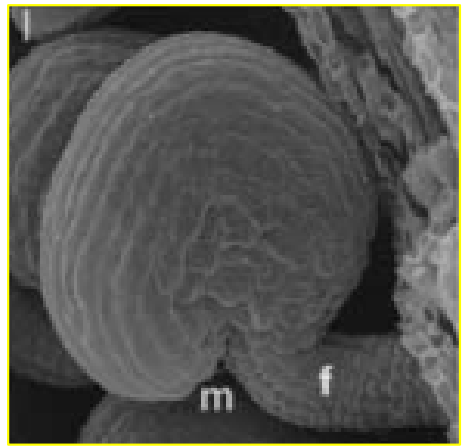


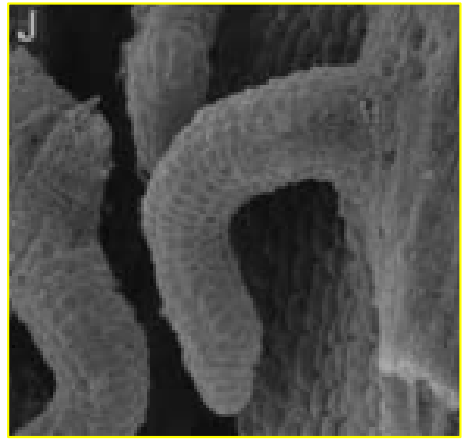

Targeted gene expression
activate UAS-gene expression by genetic crossing
Library of GAL4-GFP enhancer trap lines
UAS line
GAL4
UAS-GFP
UAS-GENE-X

The hidden function of WUSCHEL

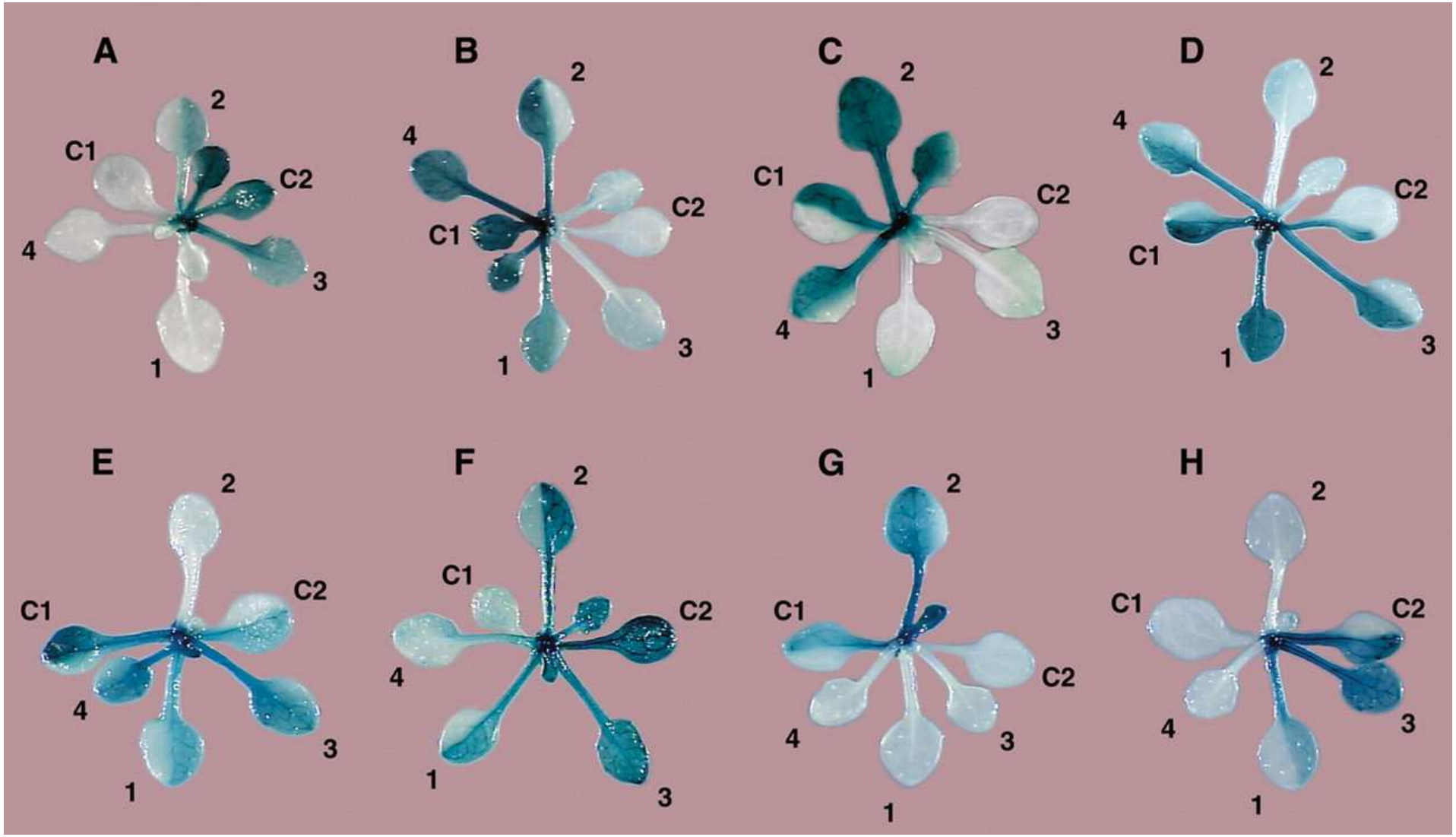








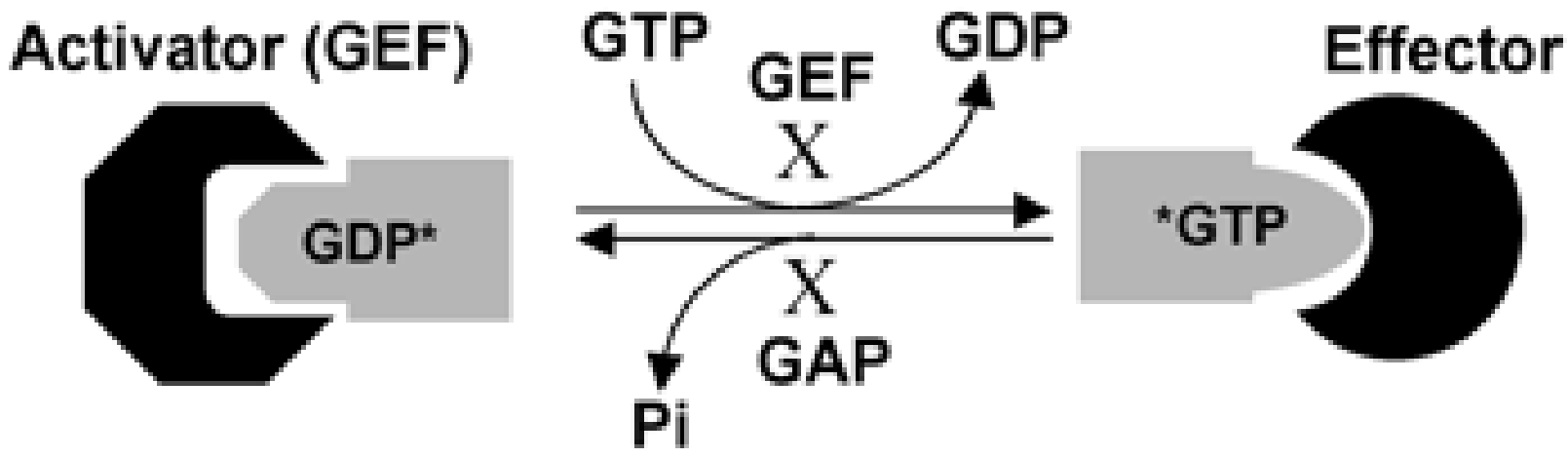
Mosaics – Cre/Lox



Sequence manipulation

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

rop GTPases mutants



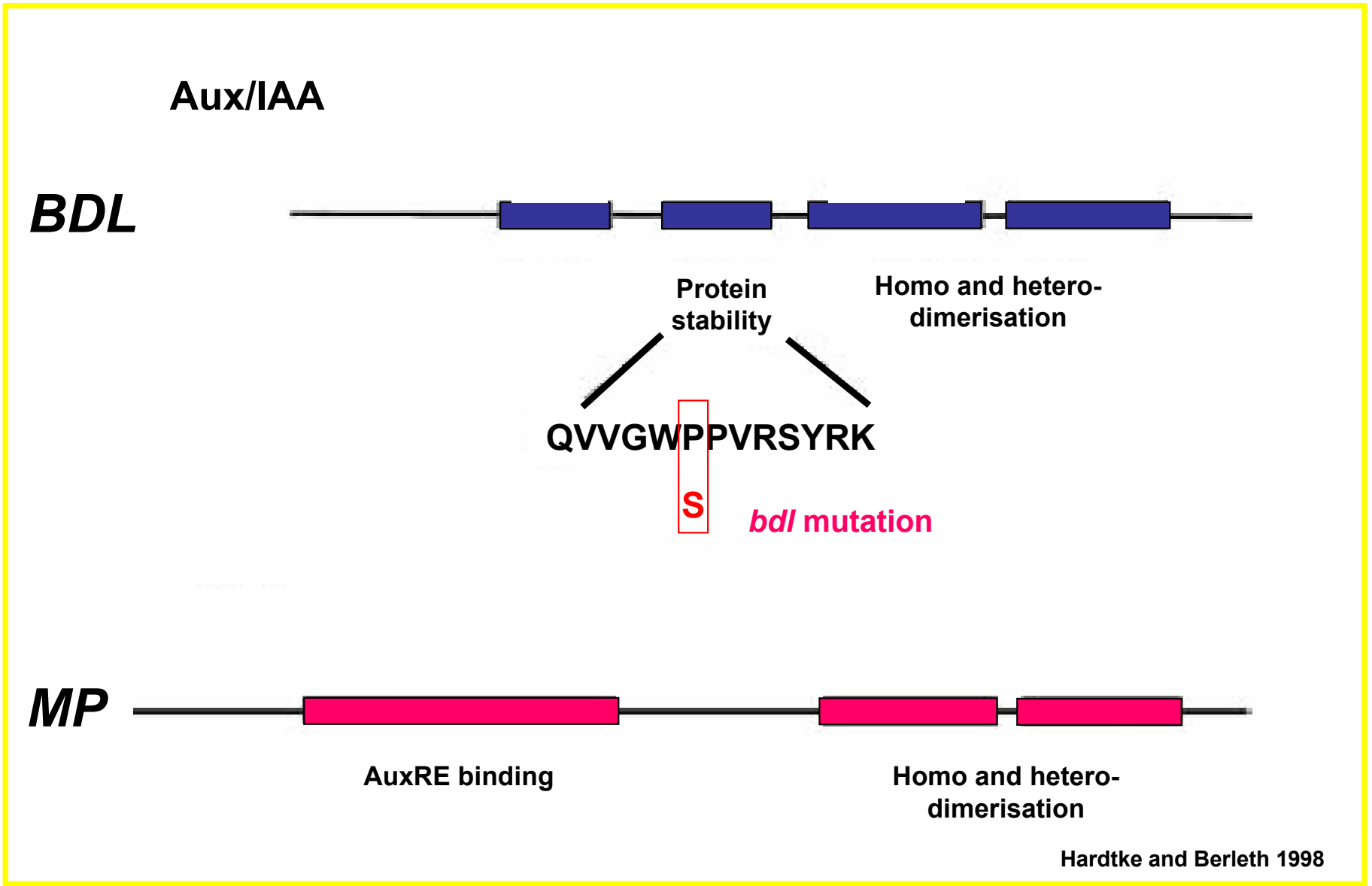
DN-rop mutants

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

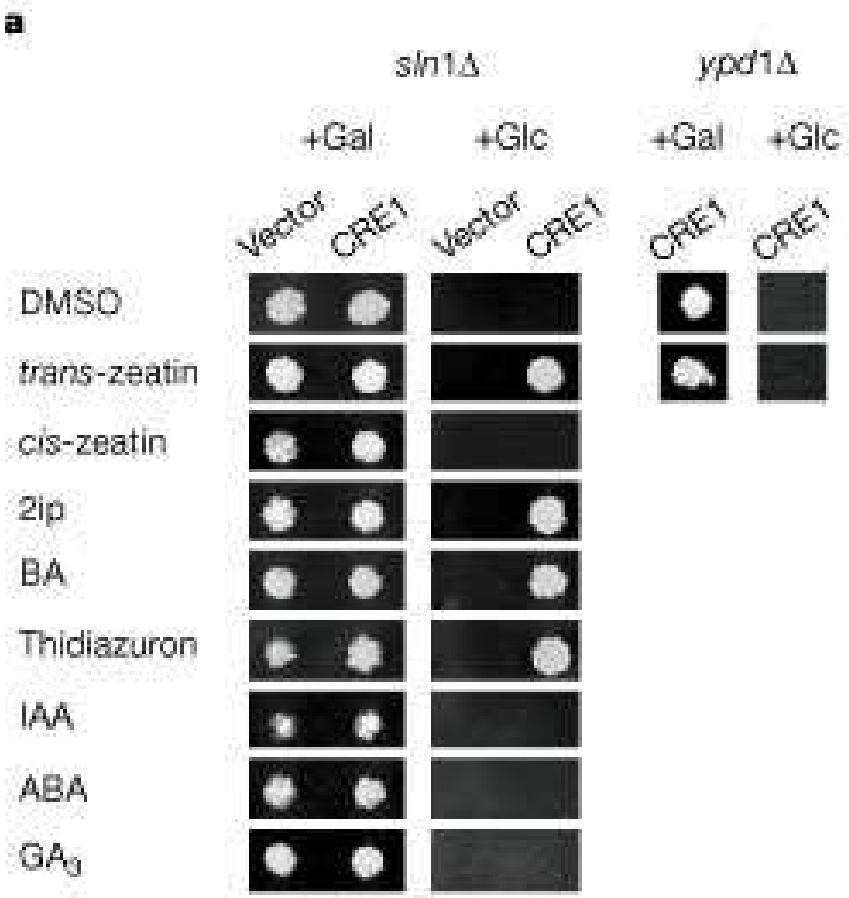
CA-rop mutants

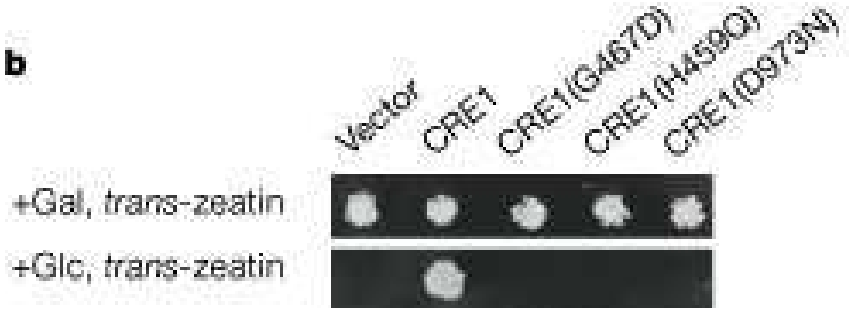
- Permanently bind GTP
- Insensitive to GAP
- Constitutively activate effectors when expressed in cells
- Examples:
  - ROP1/ROP2/ROP4/ROP6: G15V or Q64L
  - ROP5: G15V or Q64E

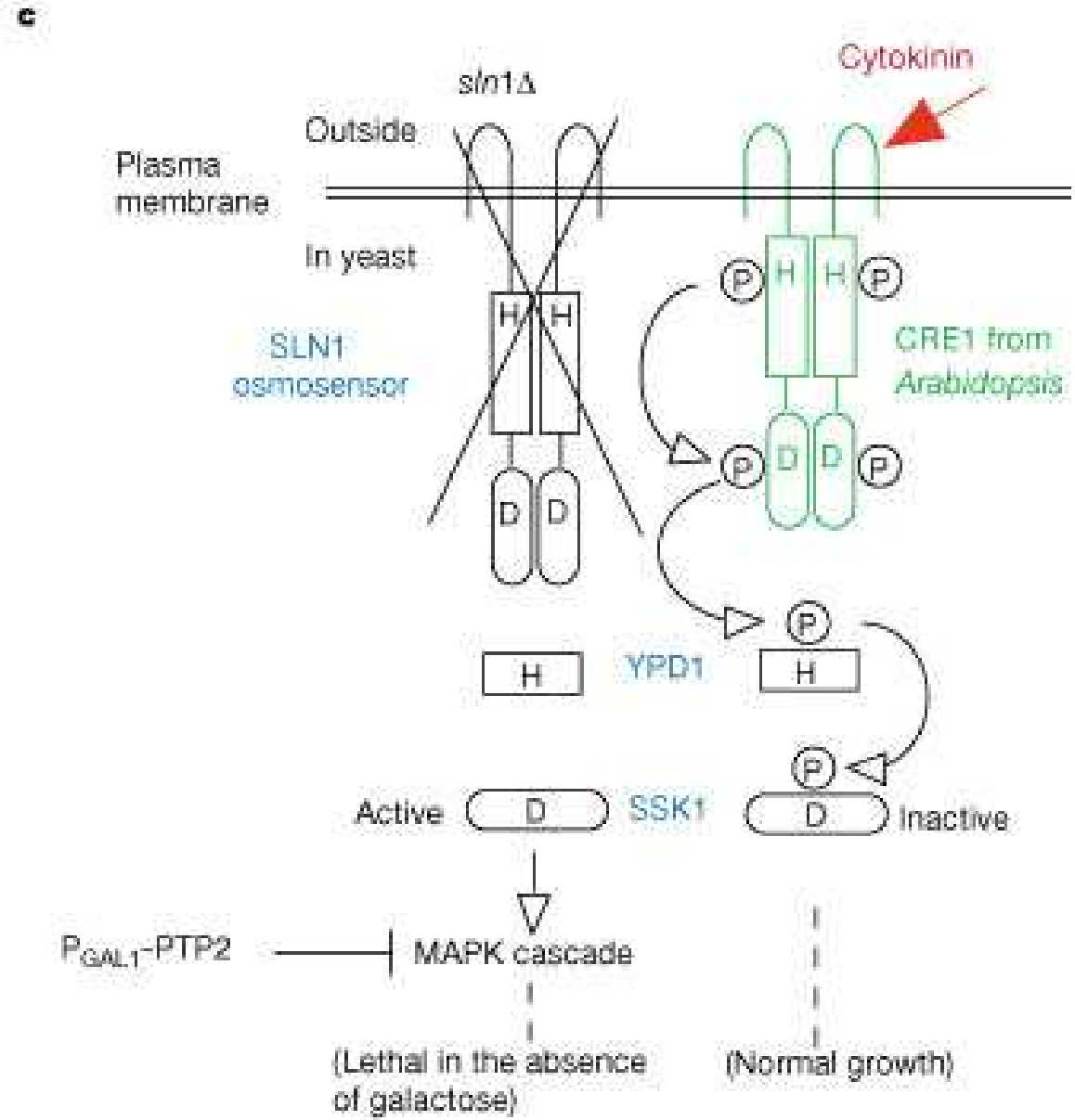
AUX/IAA and ARF proteins



CRE1 – cytokinin receptor





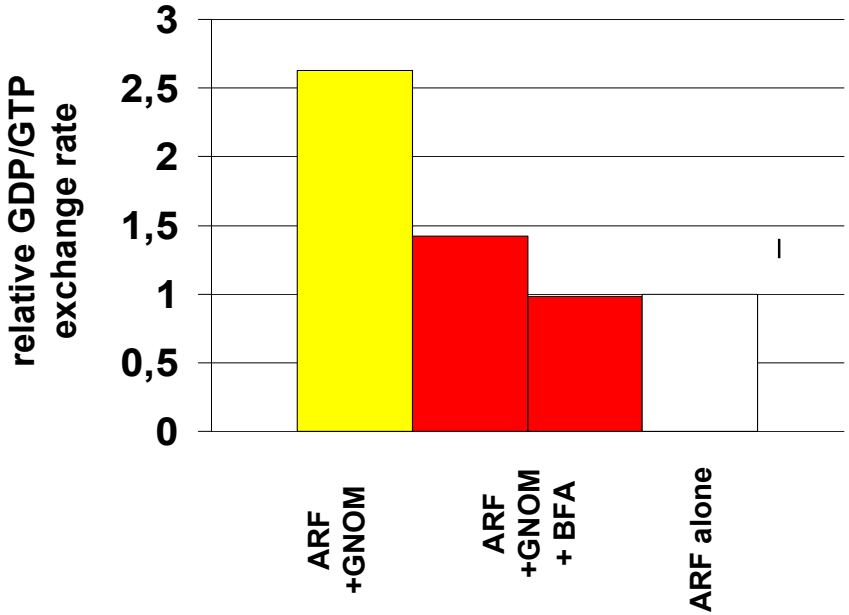


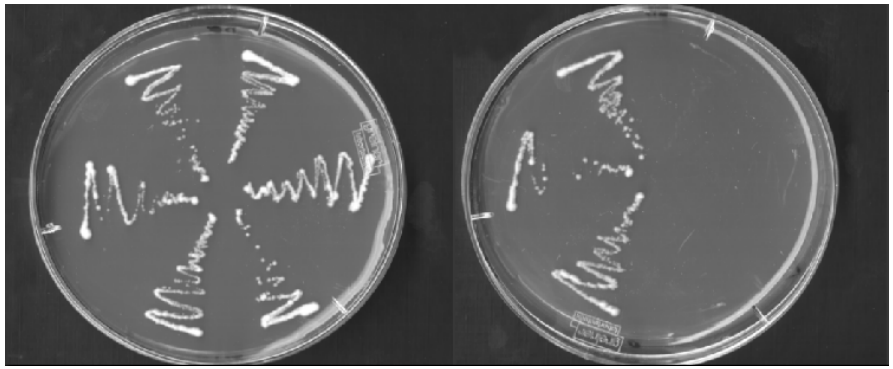
Phenotype analysis

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

Biochemical function

- Protein activity
- Yeast complementation
- Xenopus oocytes





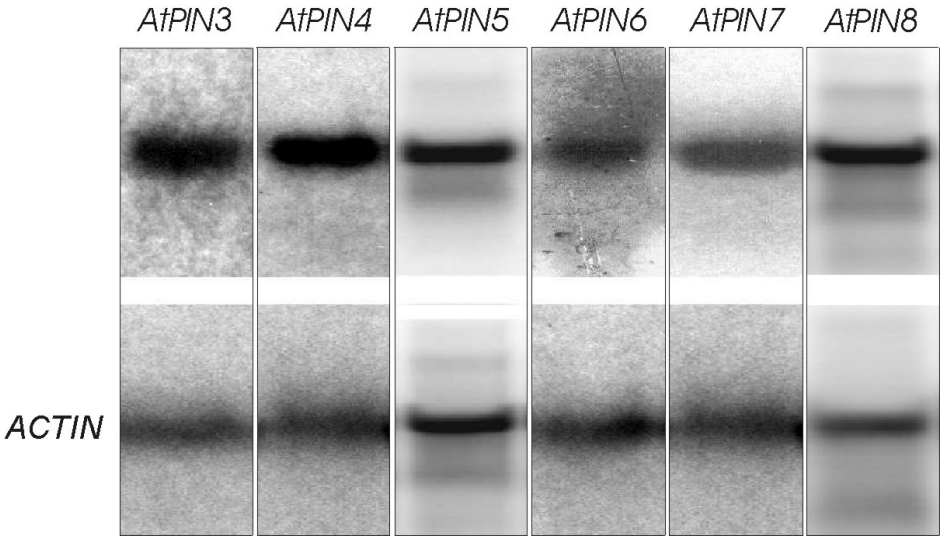
Gene Expression and Protein Localization

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

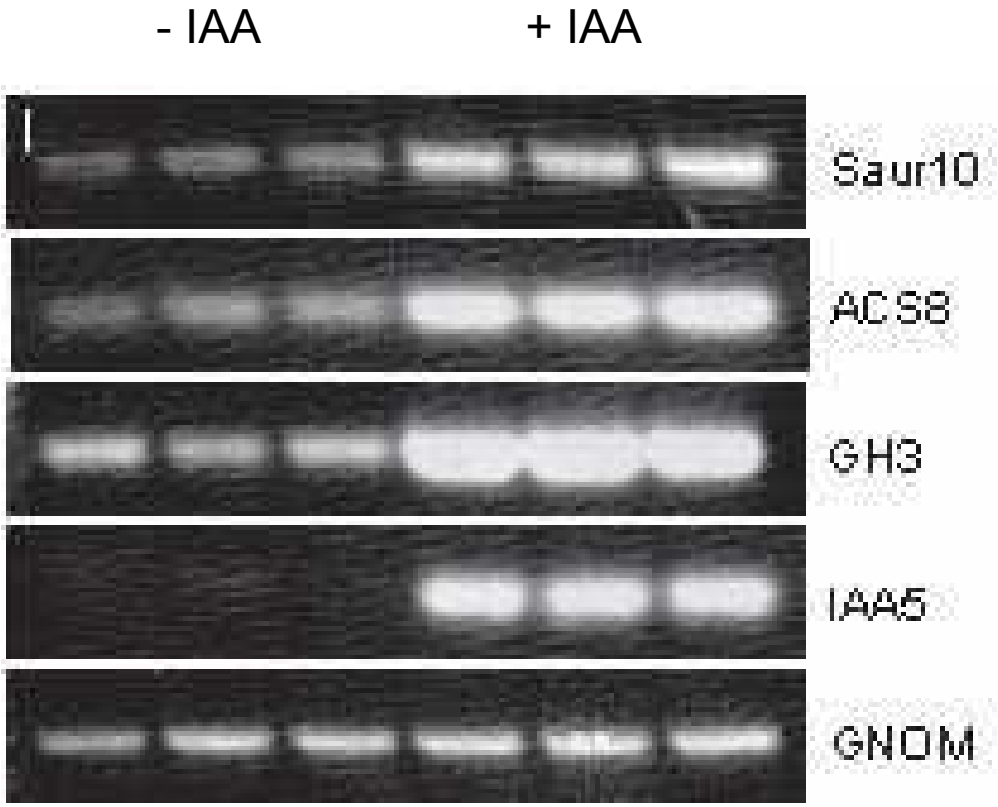
Blots and RT-PCR

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

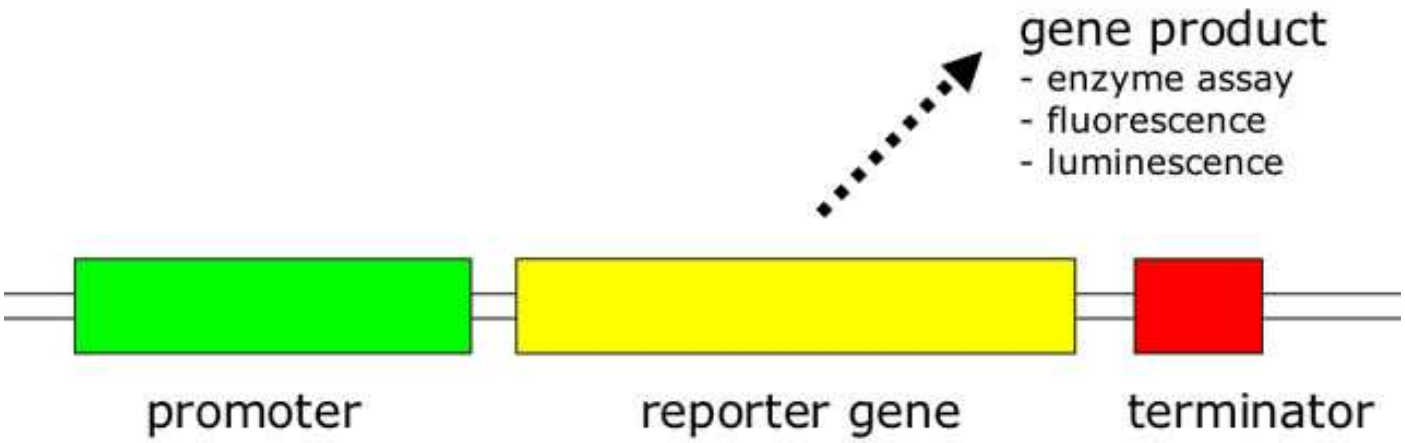


Southern and Western blots

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

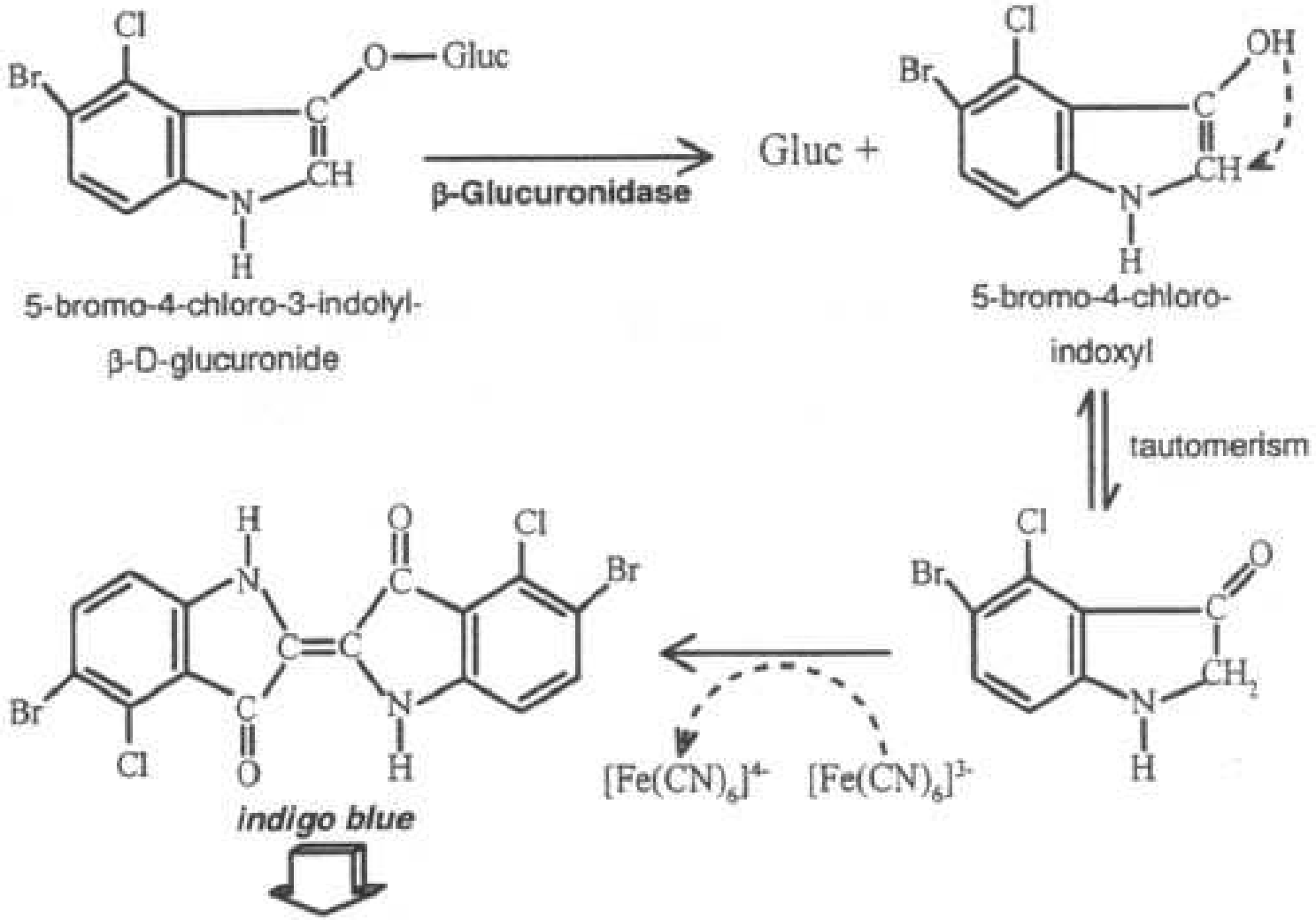
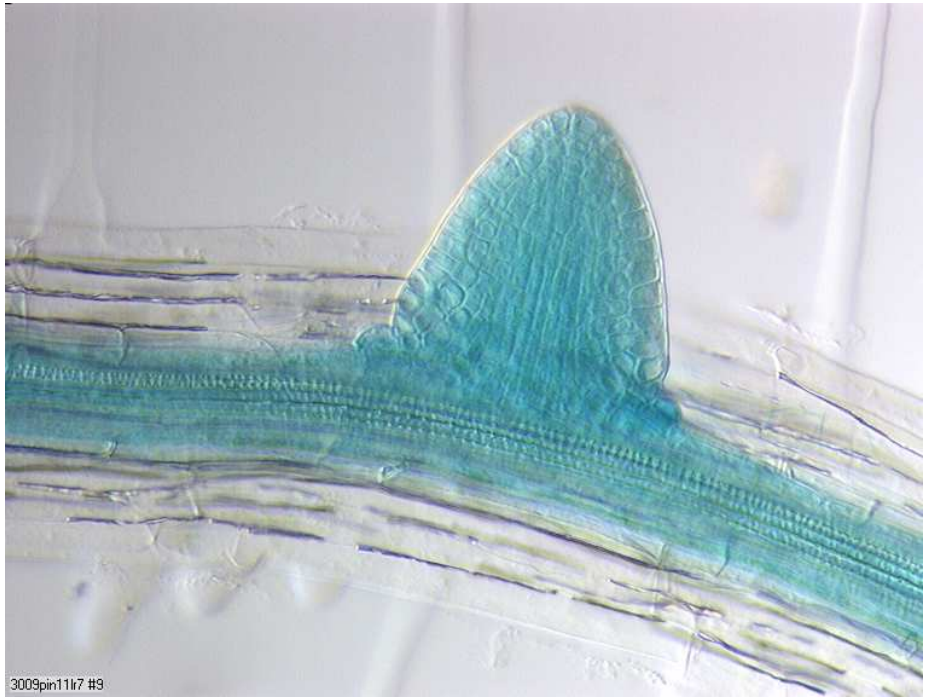
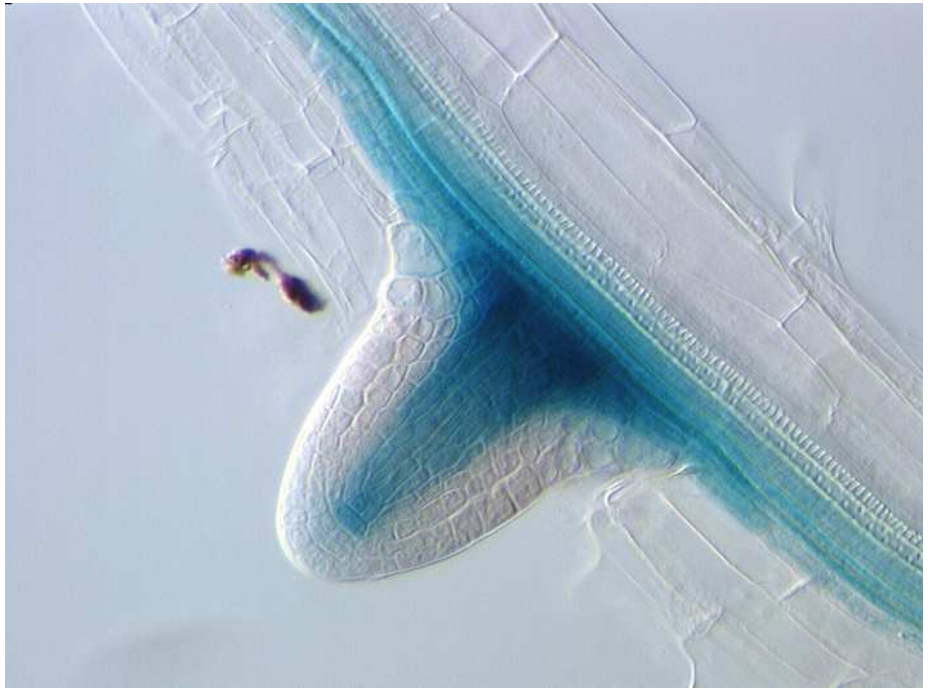
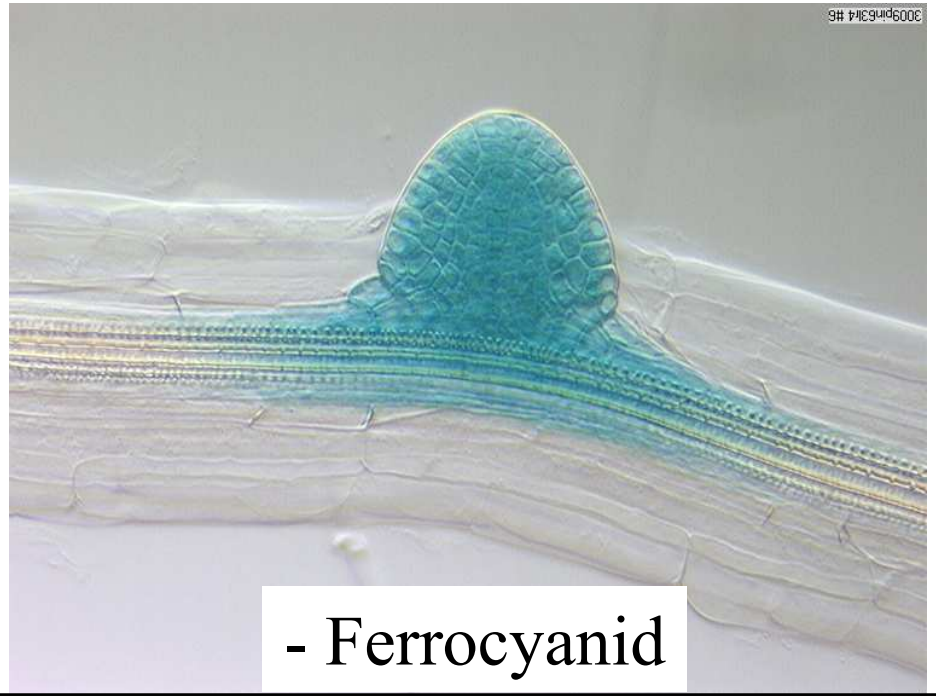


Fig. 1. Chemistry of X-Gluc reaction. Hydrolyzation of X-Gluc by the β-glucuronidase enzyme results in a reactive indoxyl molecule. Two indoxyl molecules are oxidized to indigo blue; ferri(III)cyanide enhances the dimerization.

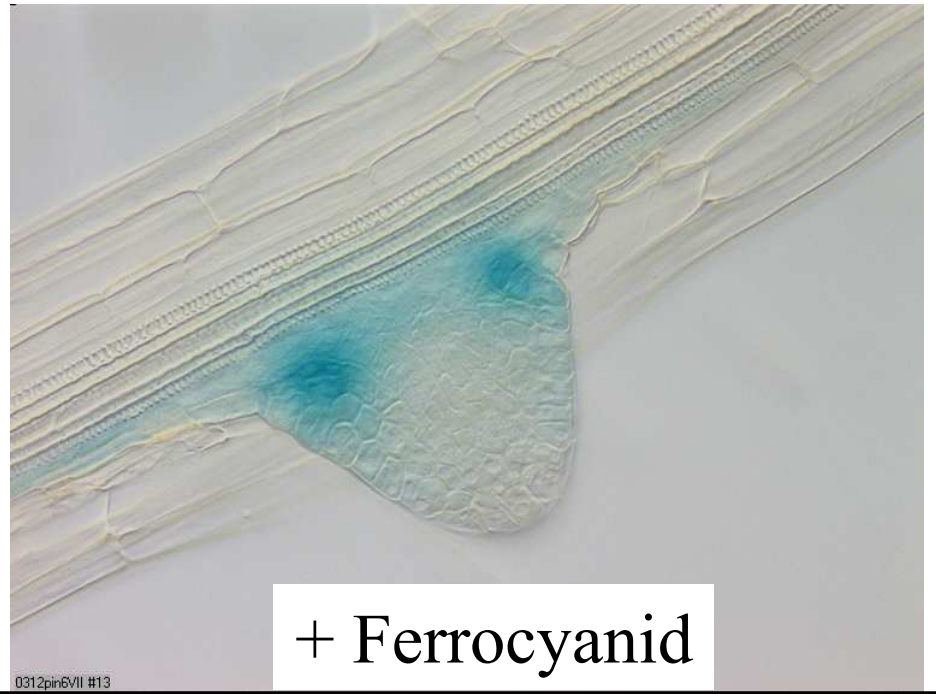
GUS – ß-Glucuronidase



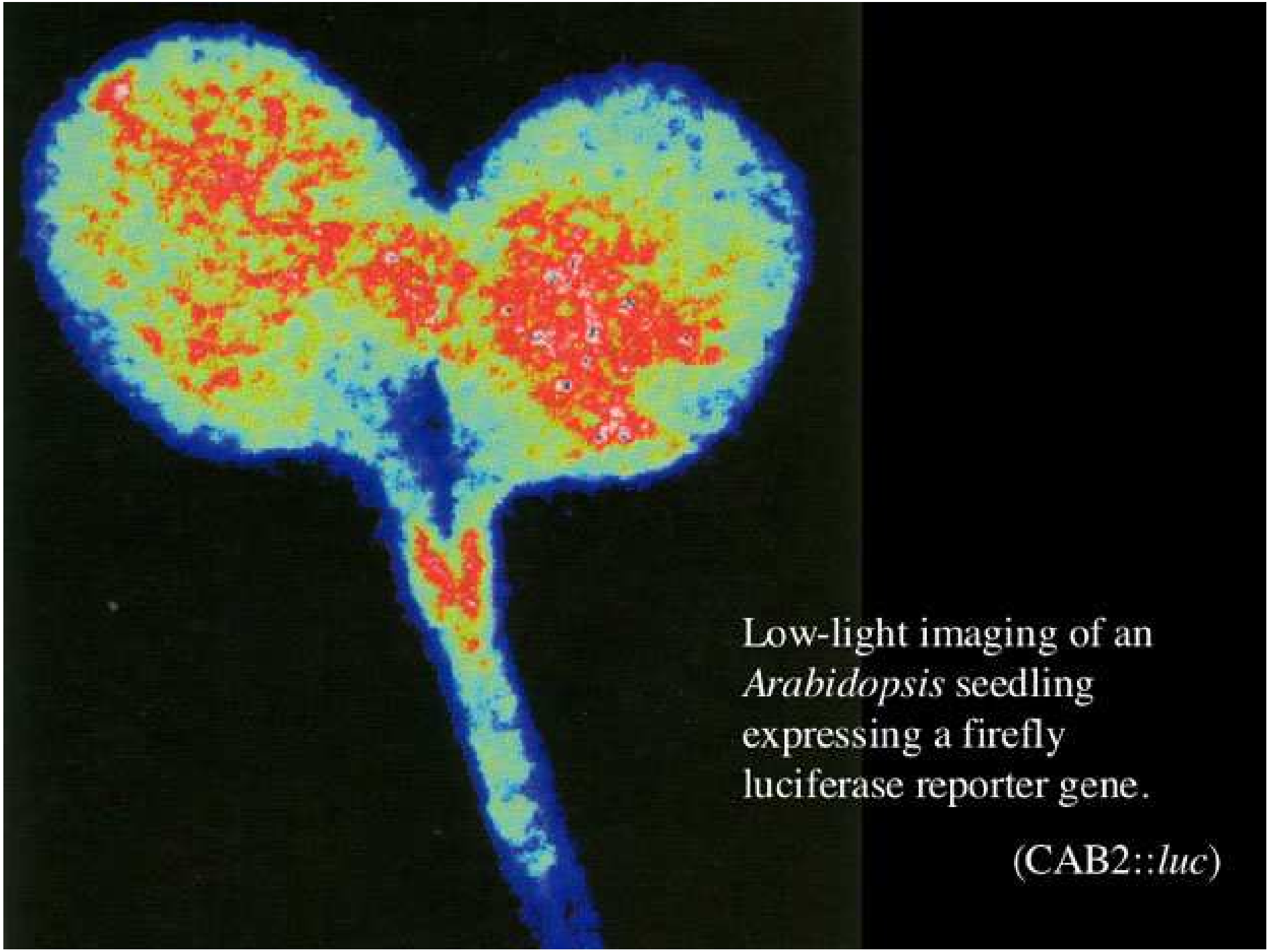




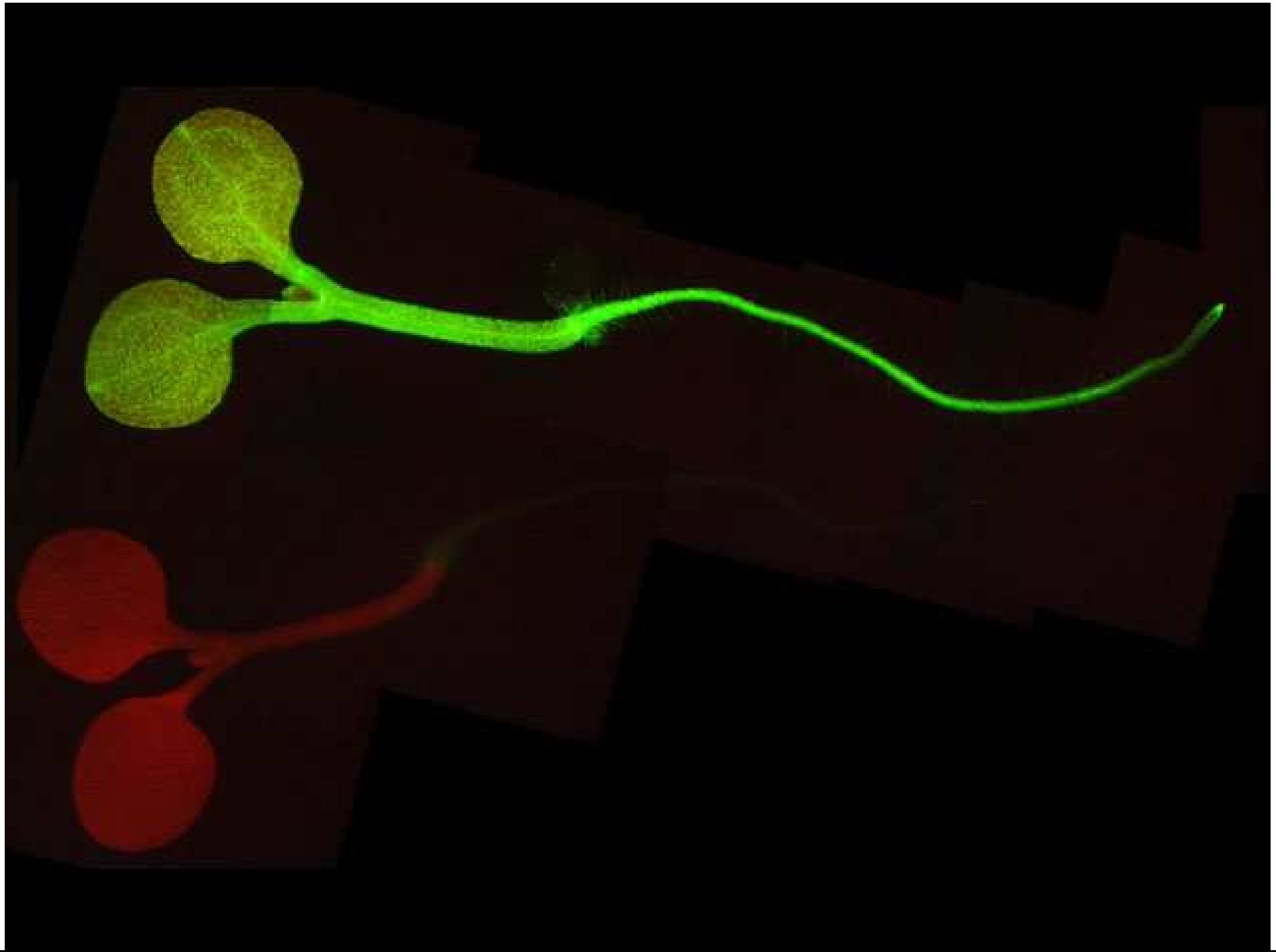
- Ferrocyanid



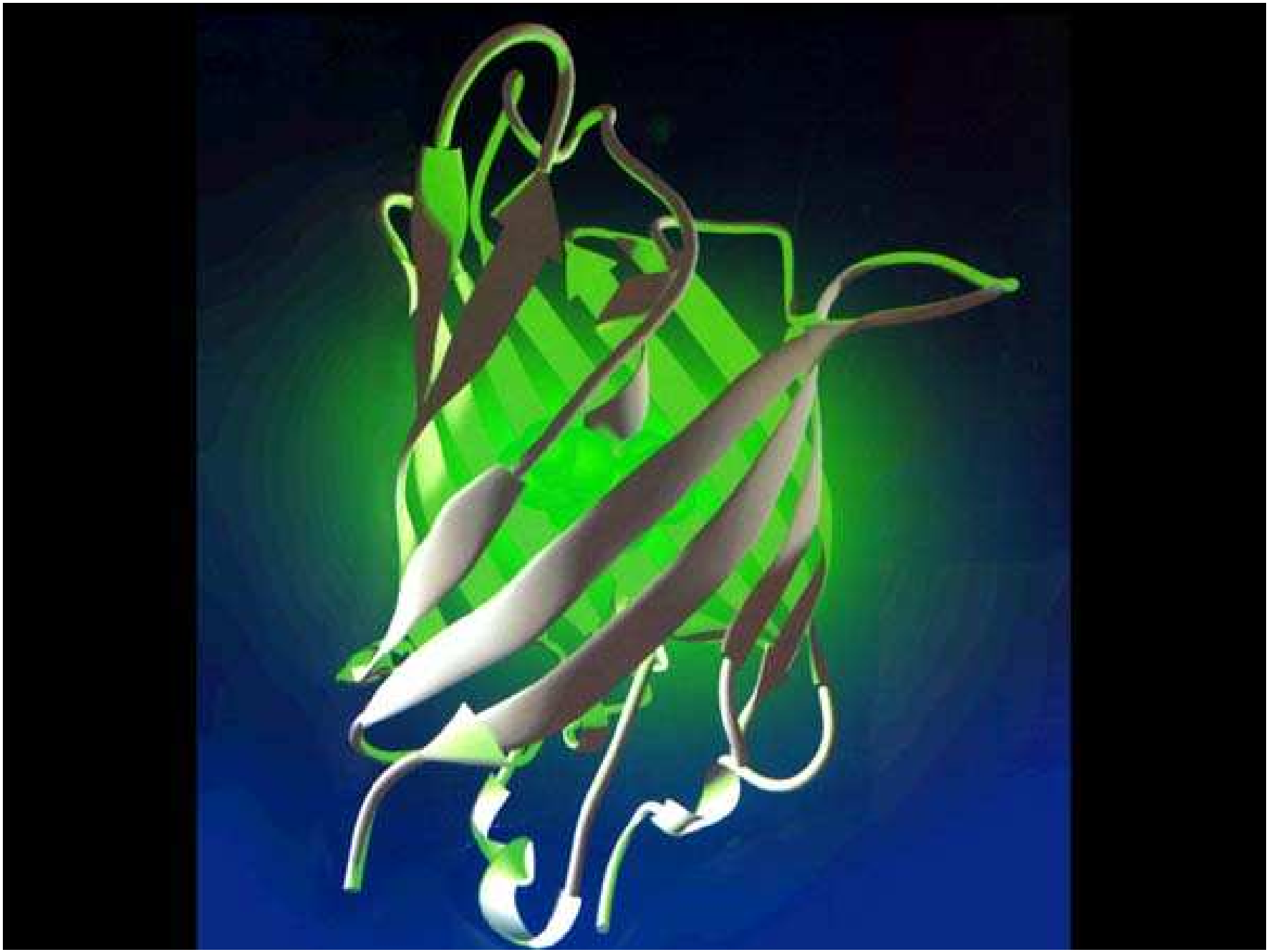
+ Ferrocyanid

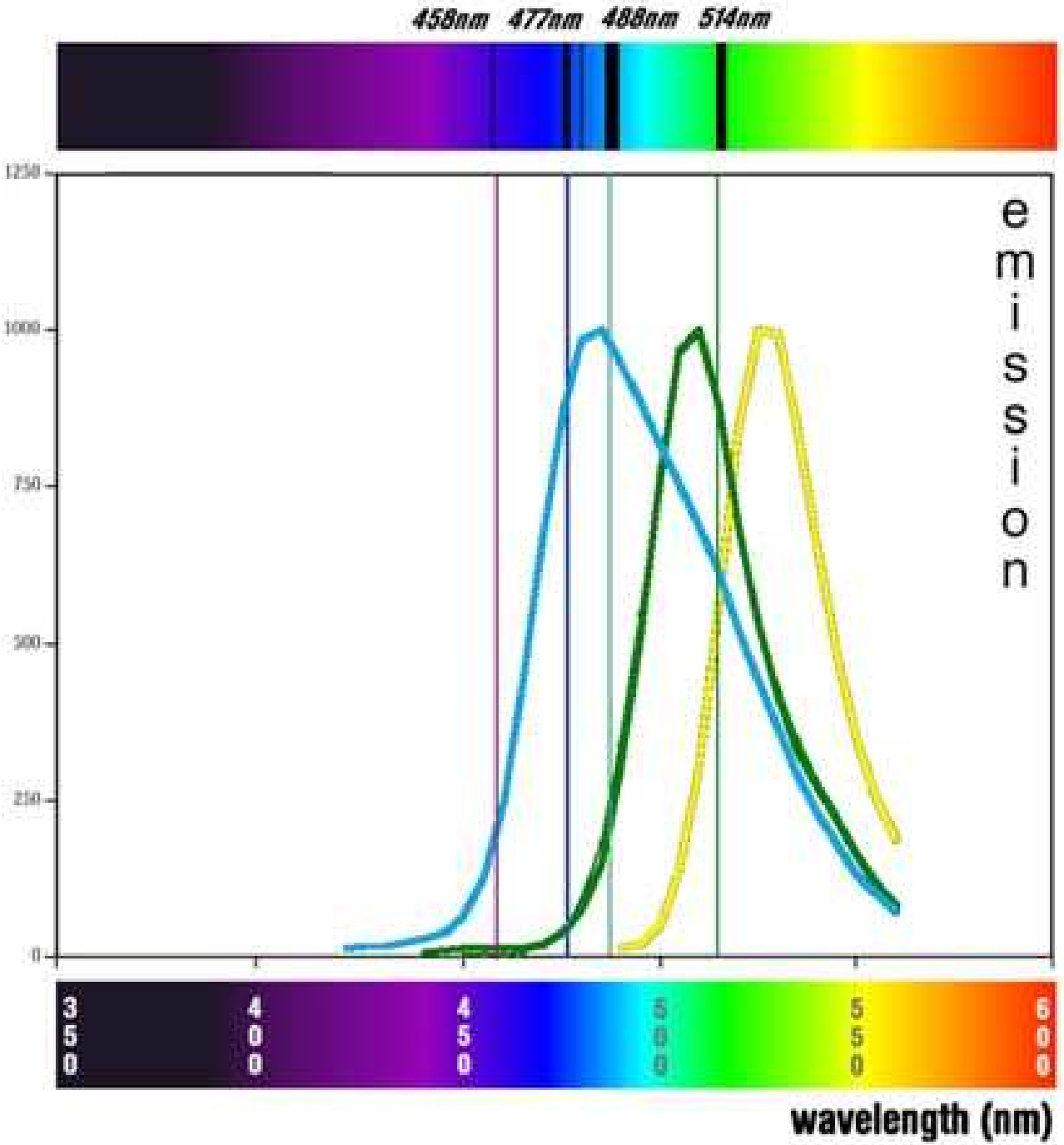

Low-light imaging of an
Arabidopsis seedling
expressing a firefly
luciferase reporter gene.
(CAB2::*luc*)

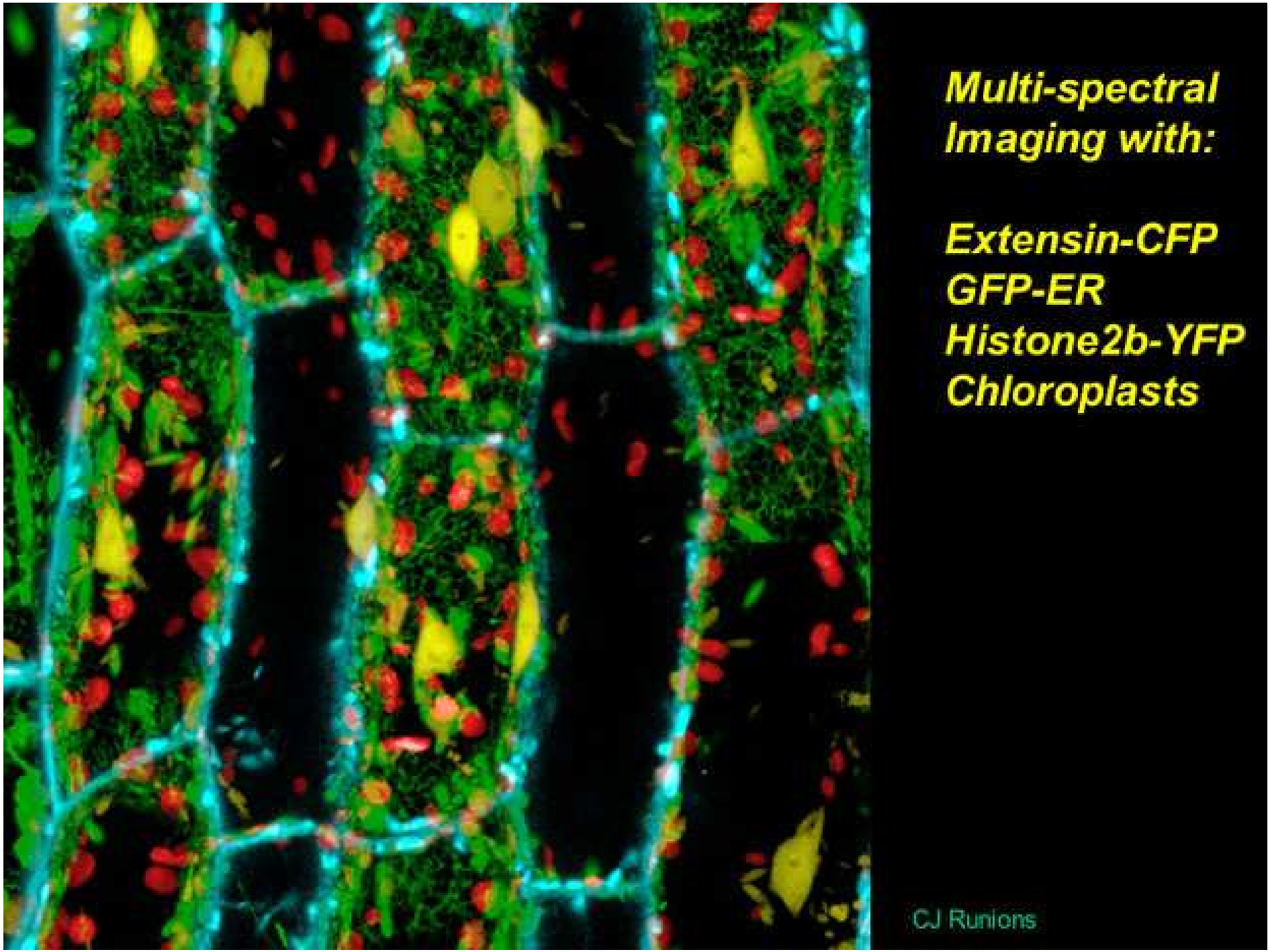
Green Fluorescence Protein



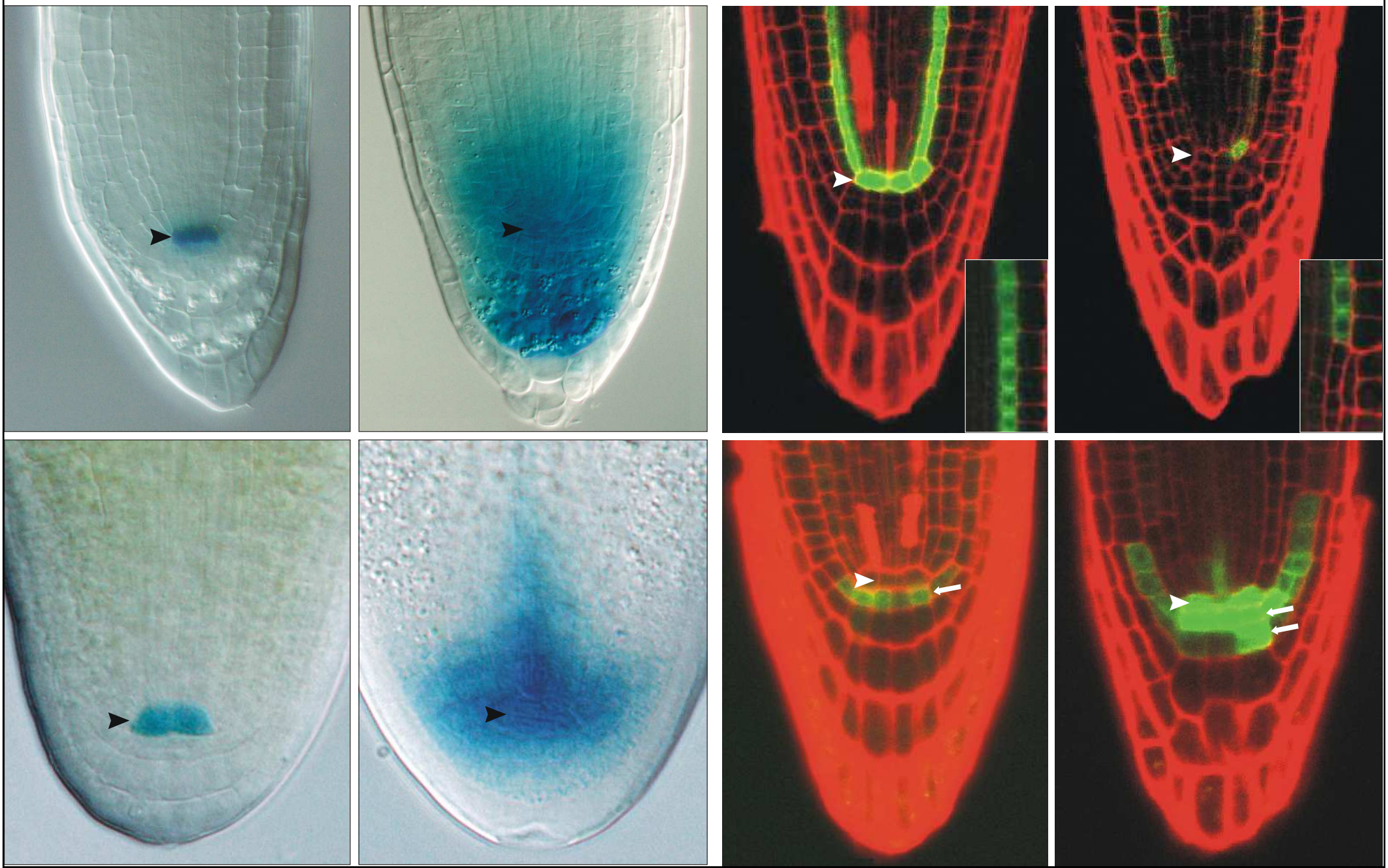

Bioluminescence in Aequoria victoria
aequorin
green fluorescent protein




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

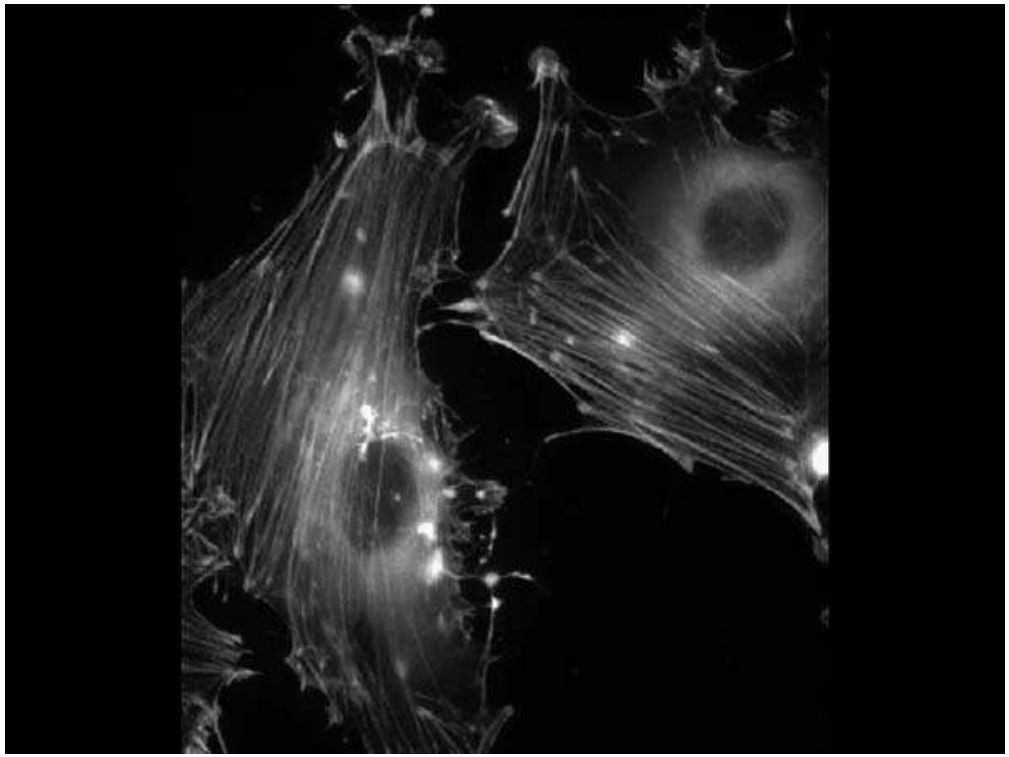

Multi-spectral
Imaging with:
Extensin-CFP
GFP-ER
Histone2b-YFP
Chloroplasts
CJ Runions

Cell identity markers

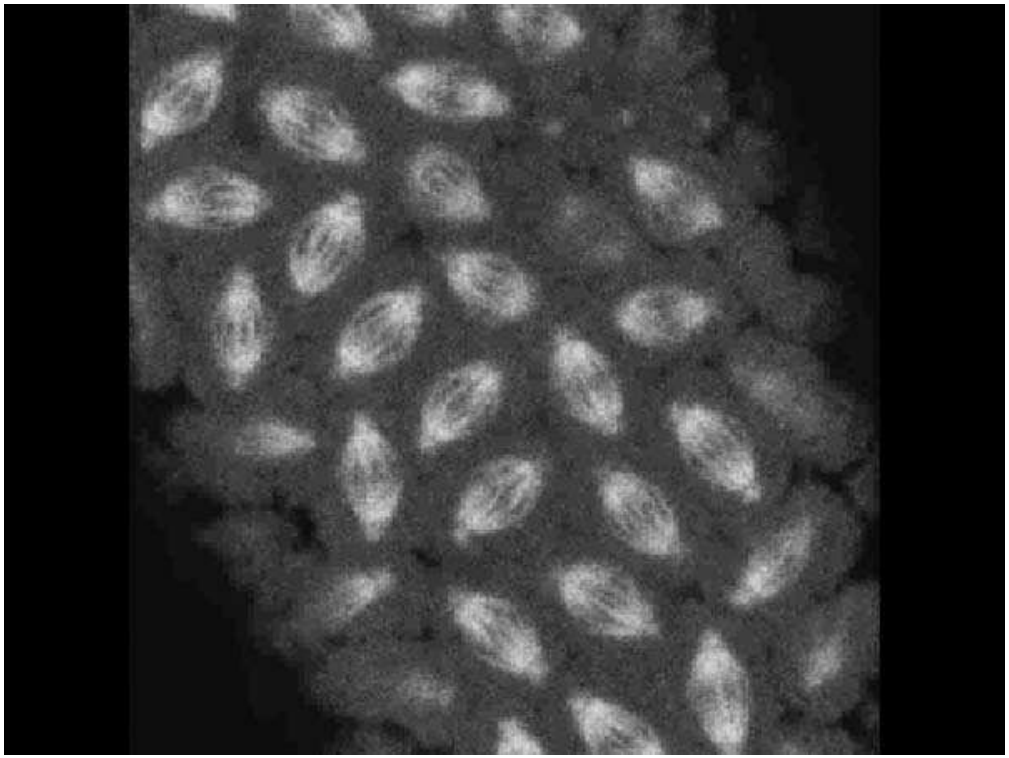


Subcellular structure markers

Actin



Tubulin

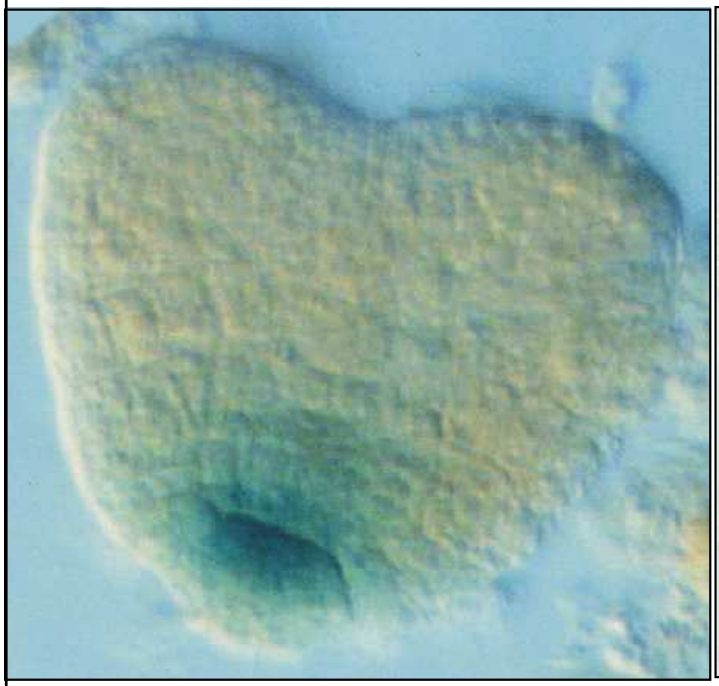


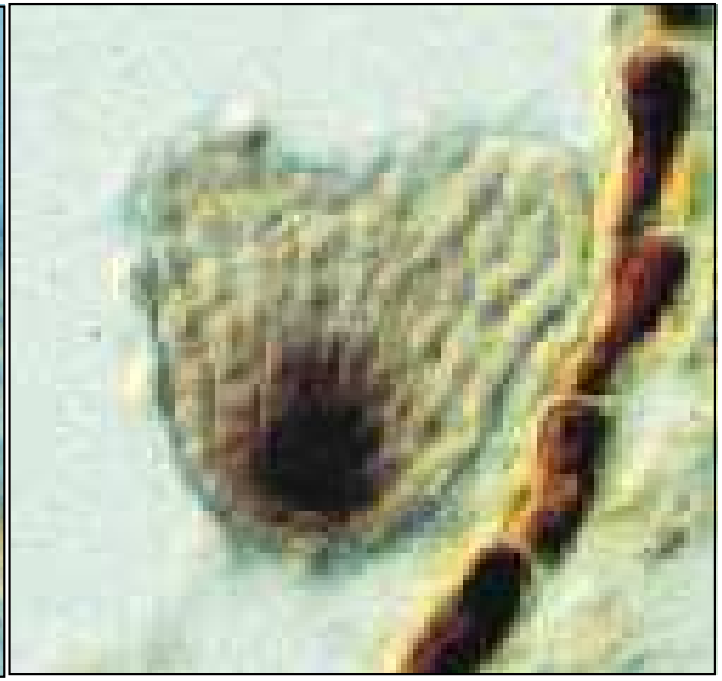
In situ mRNA/protein localisation

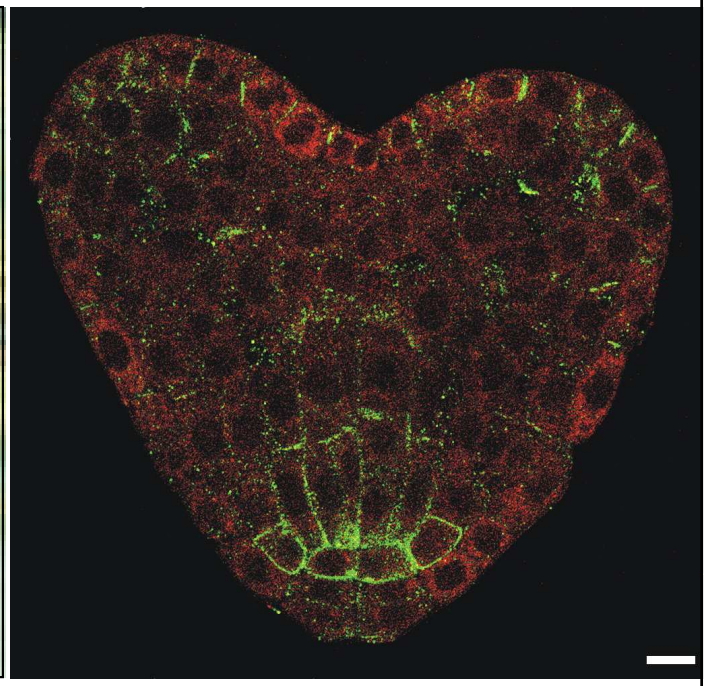
- Probe preparation
- Fixation
- Embedding
- Sectioning
- Deparafinization
- Treatment with probe
- Removal of unbound probe
- Signal visualization

Analysis of gene expression

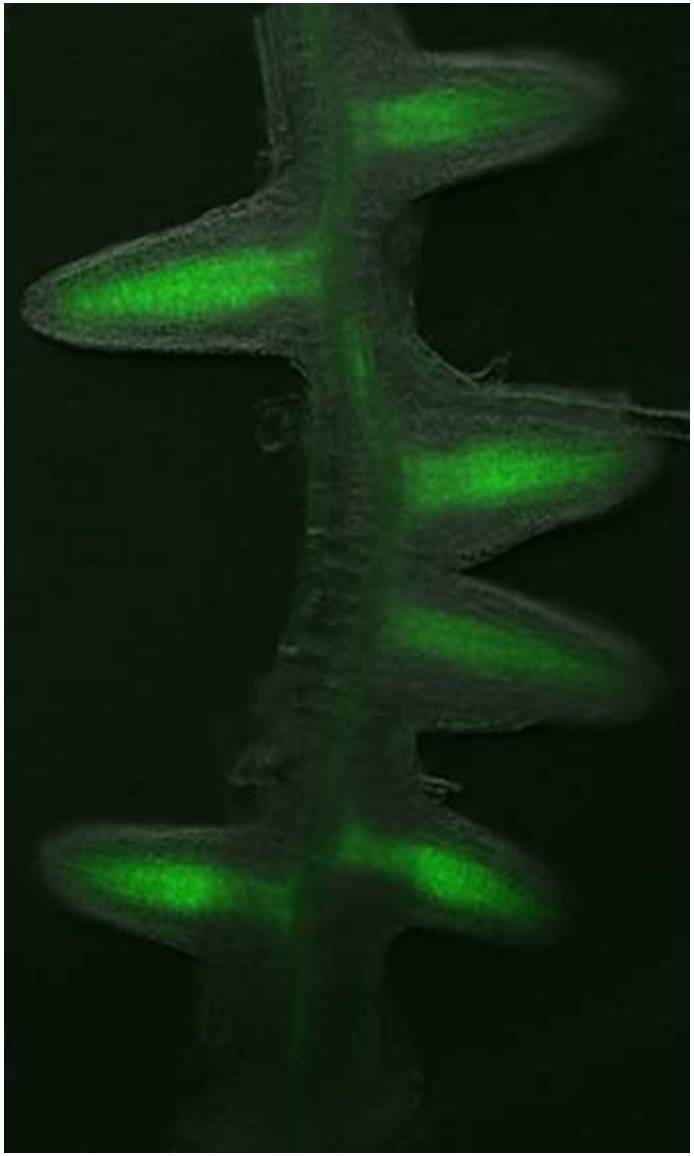
GUS **mRNA** **Protein**

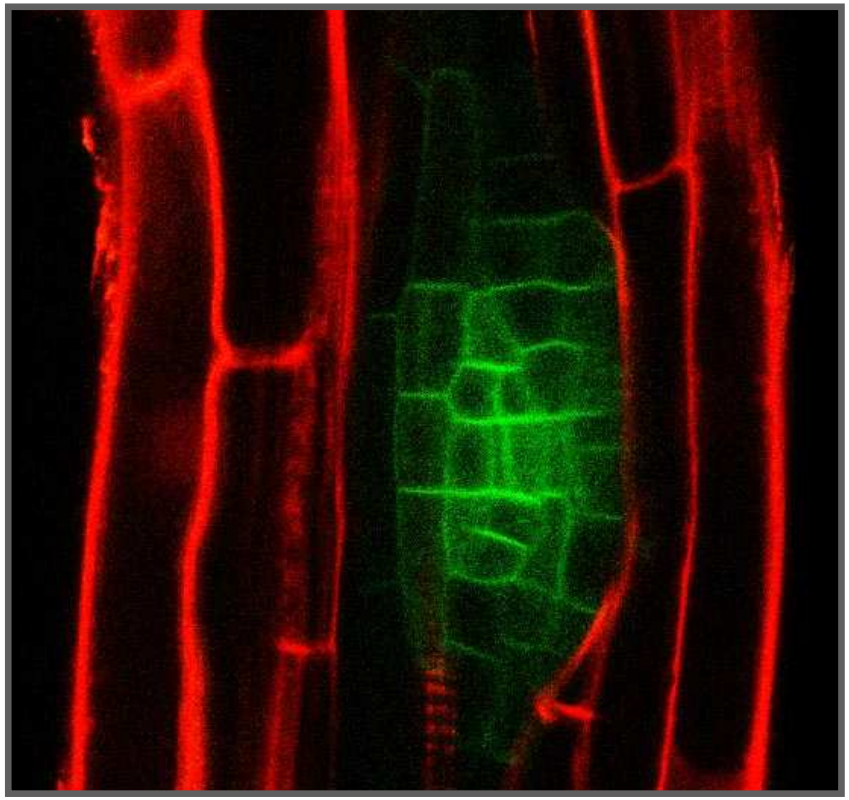


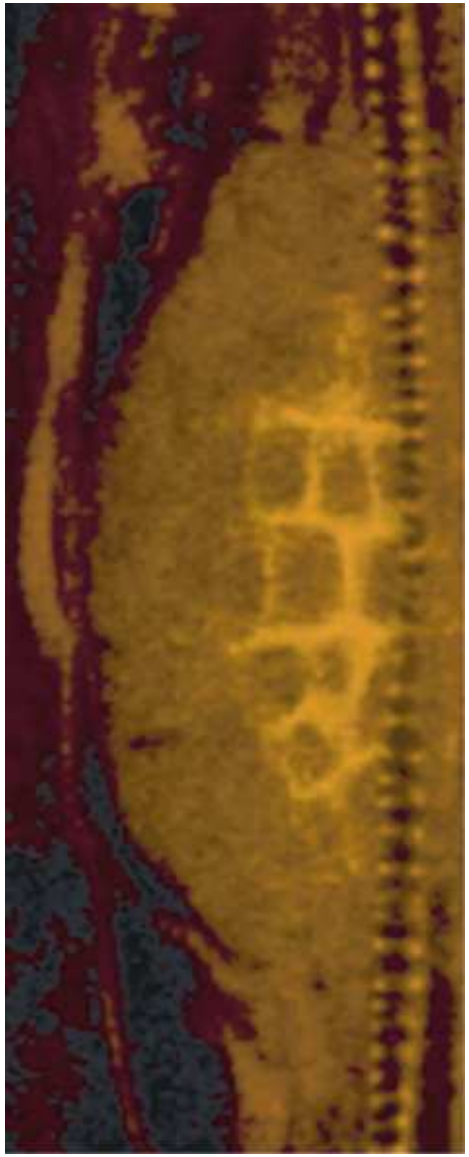




Analysis of protein localisation







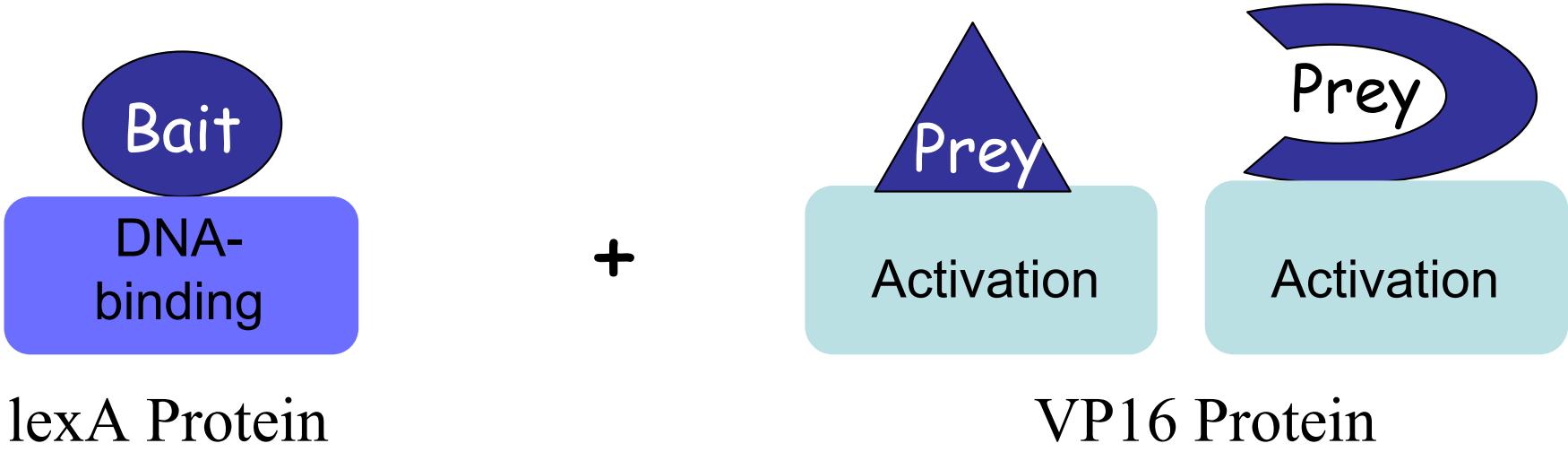
Friends and associates

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

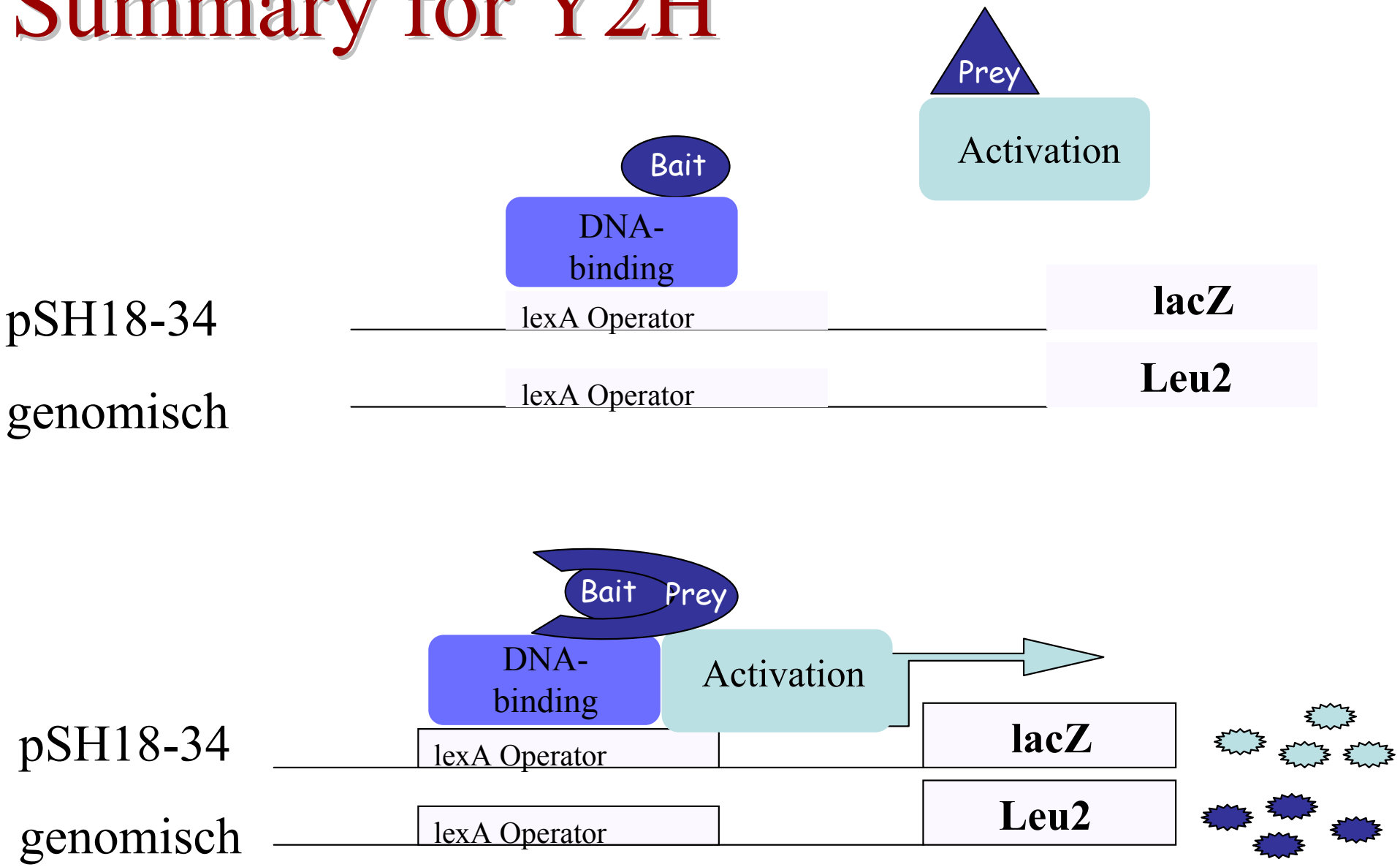
Yeast two hybrid

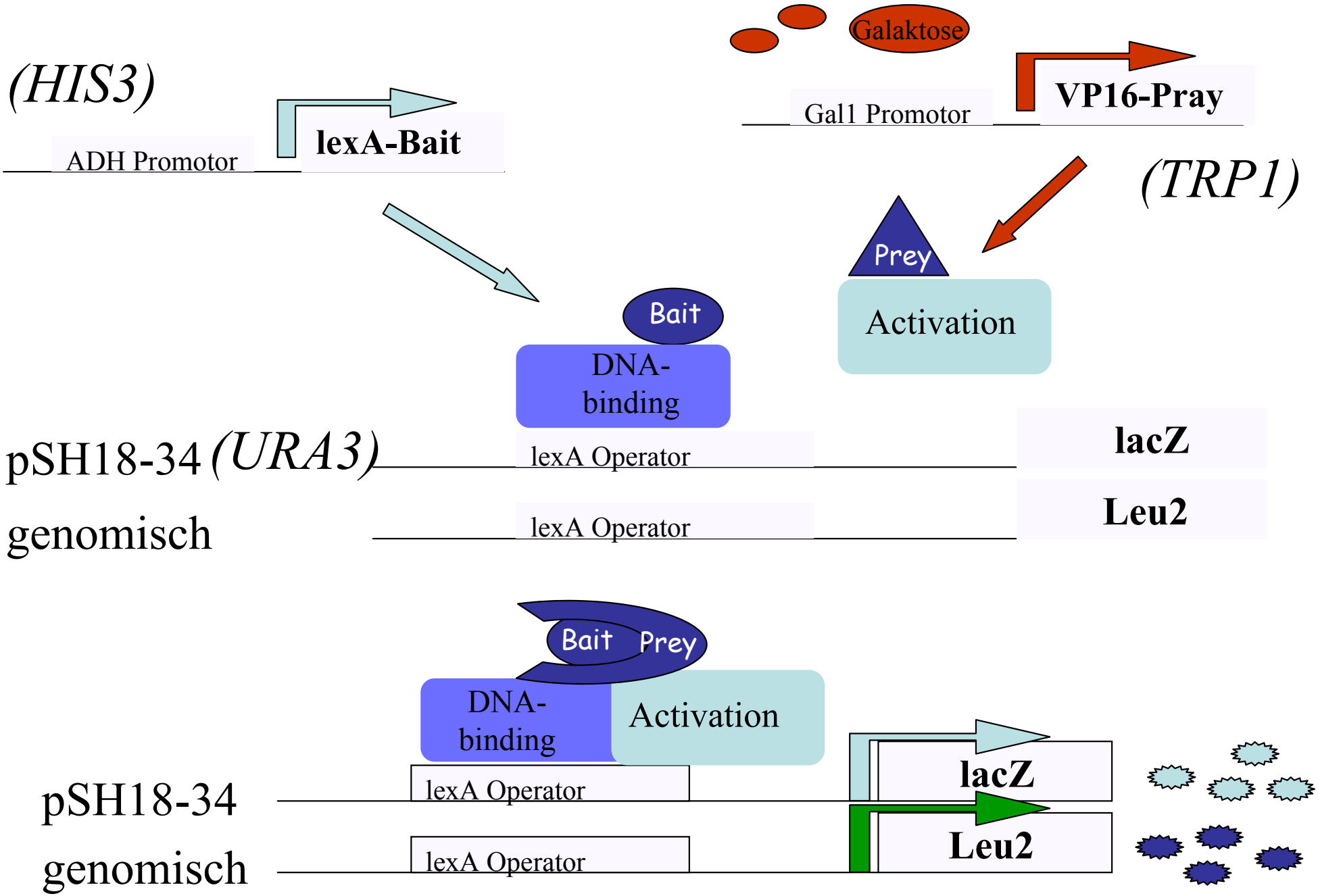
Classical transcription factor

1. DNA Binding domain
2. Activation domain



Summary for Y2H





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

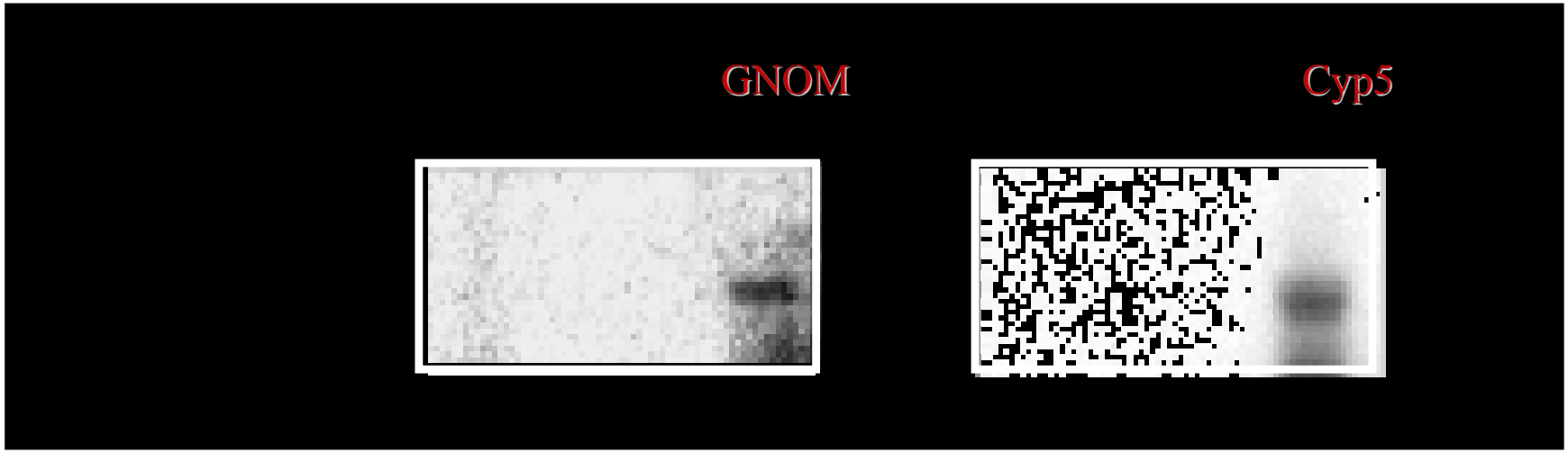
Conditions for Y2H-System

1. Proteins must be able to localize to the nucleus

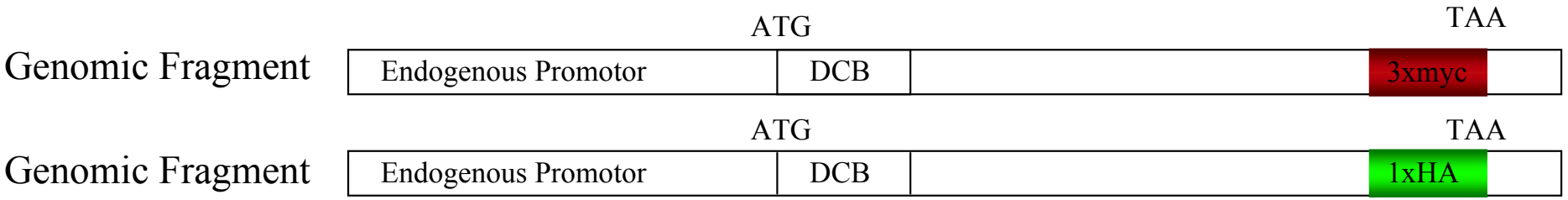
2. Bait construct must not have its own activation domain (Autoactivation)

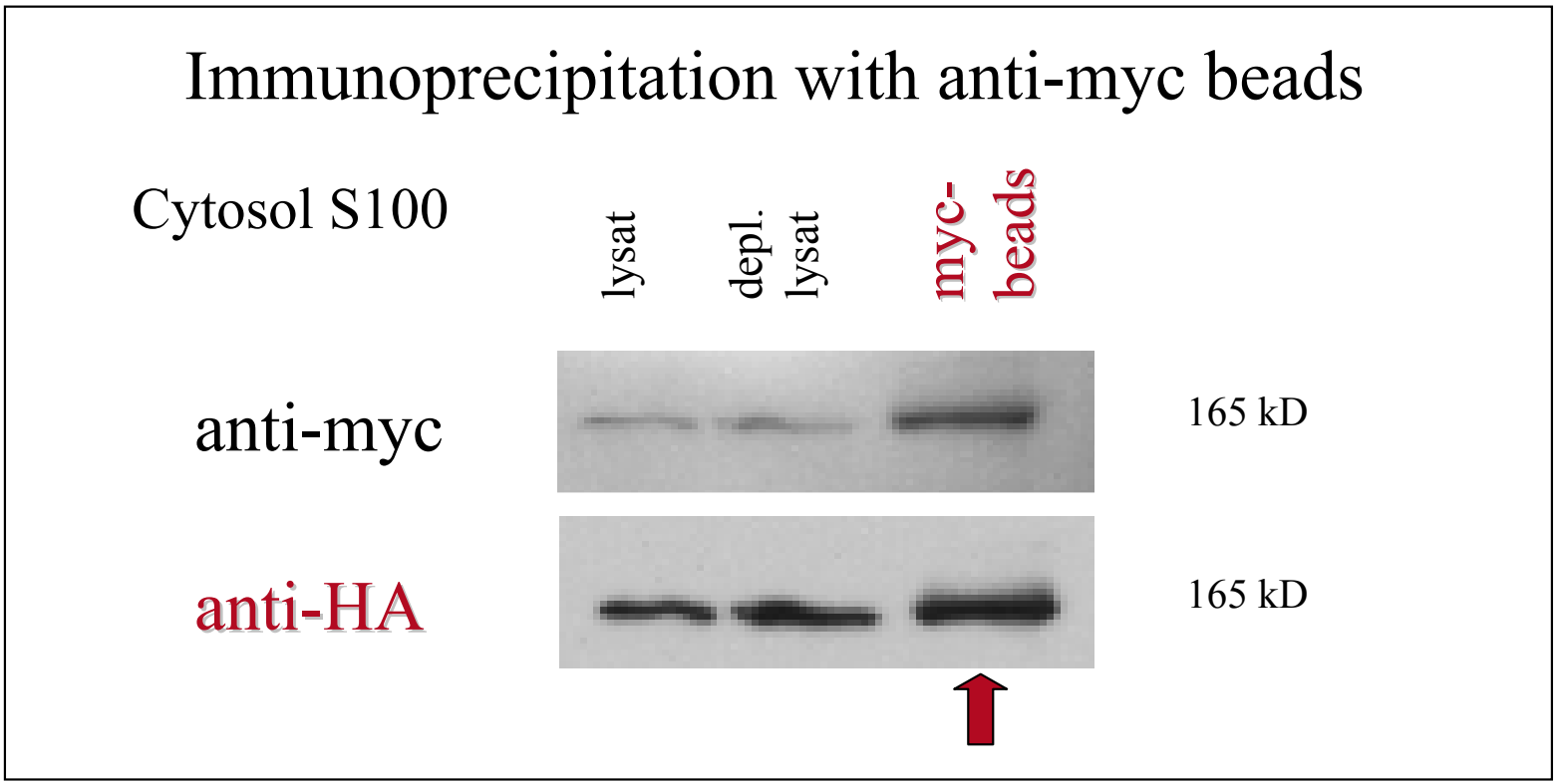
In vitro Pulldown-Assay

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

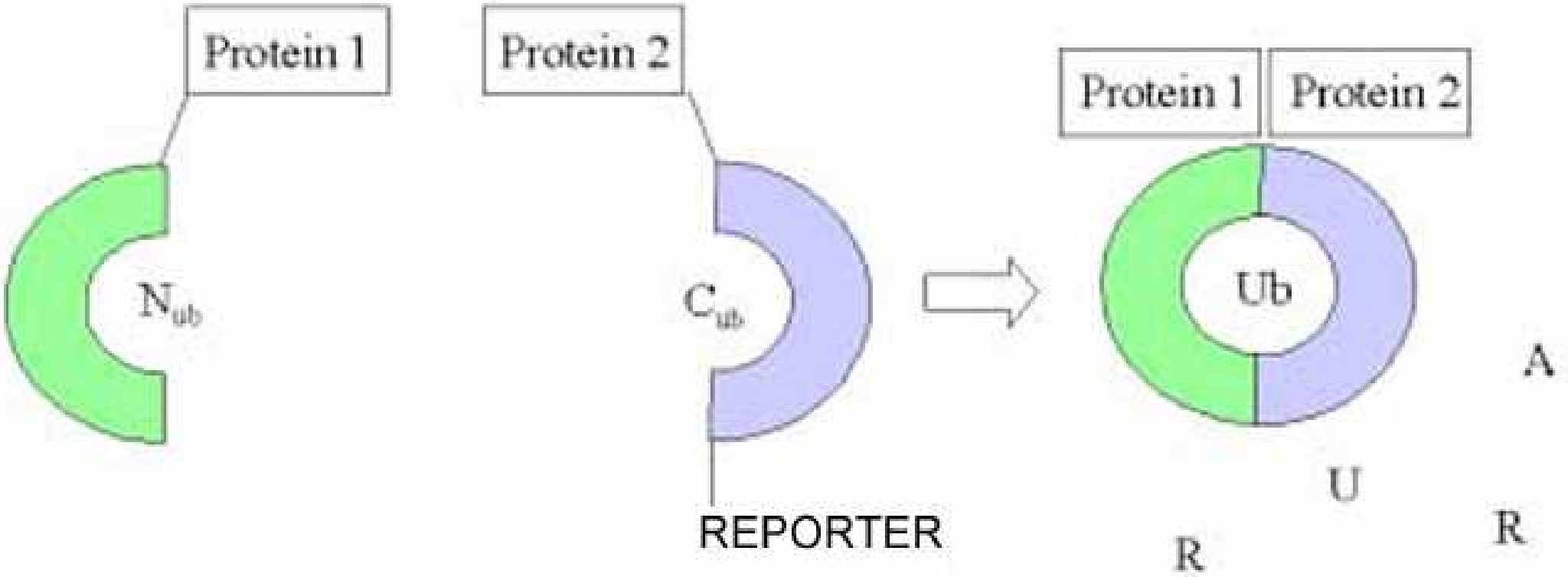


Interaction of GNOM *in vivo*





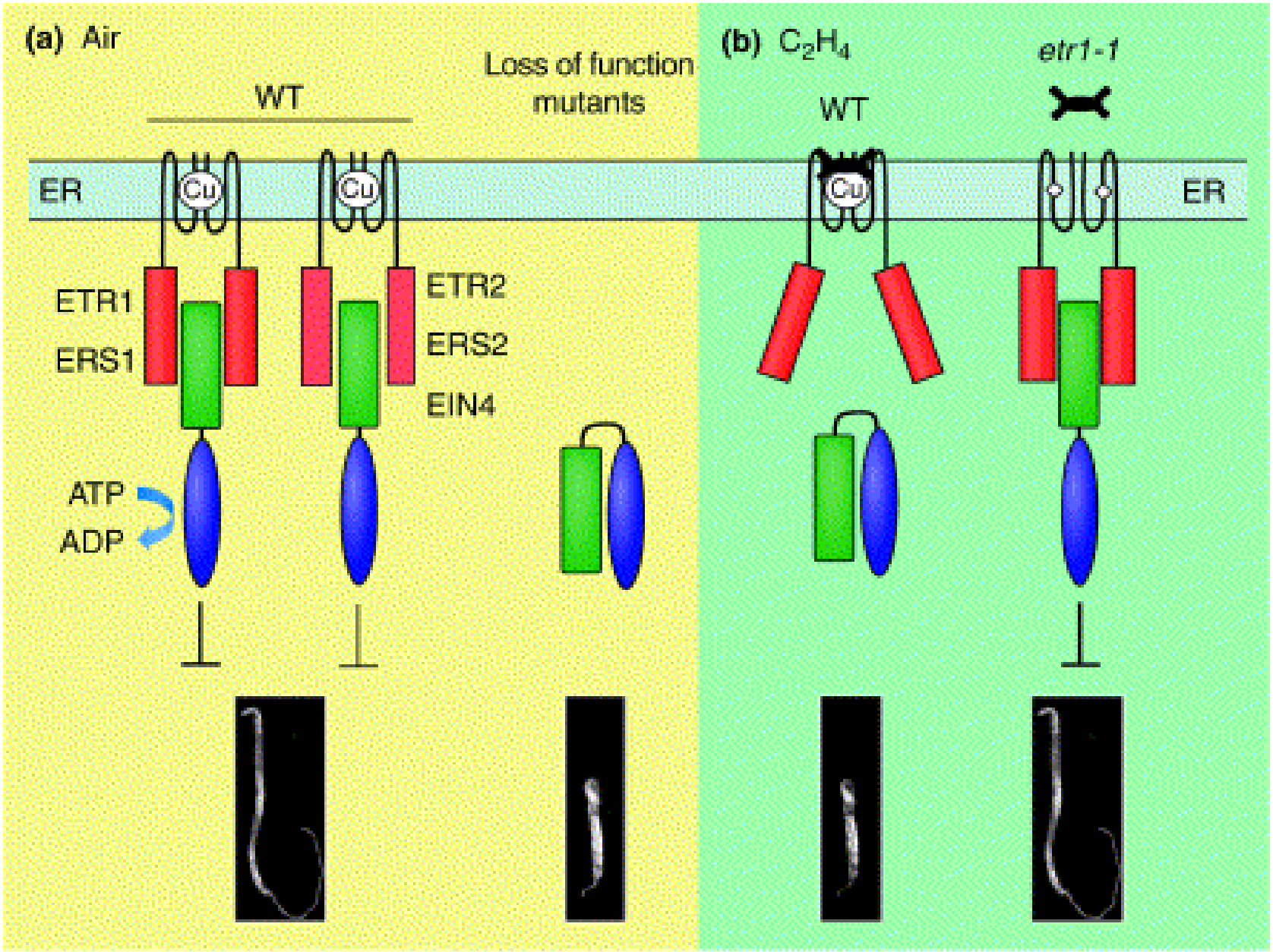
Split-Ubiquitin



Split-YFP

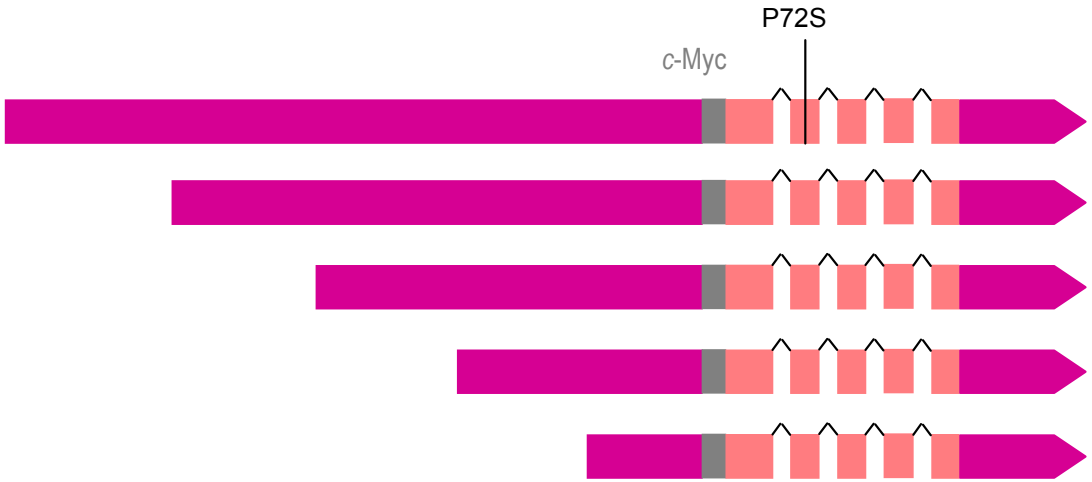
- Protoplast transfection

Genetic interactions



Current Opinion in Plant Biology

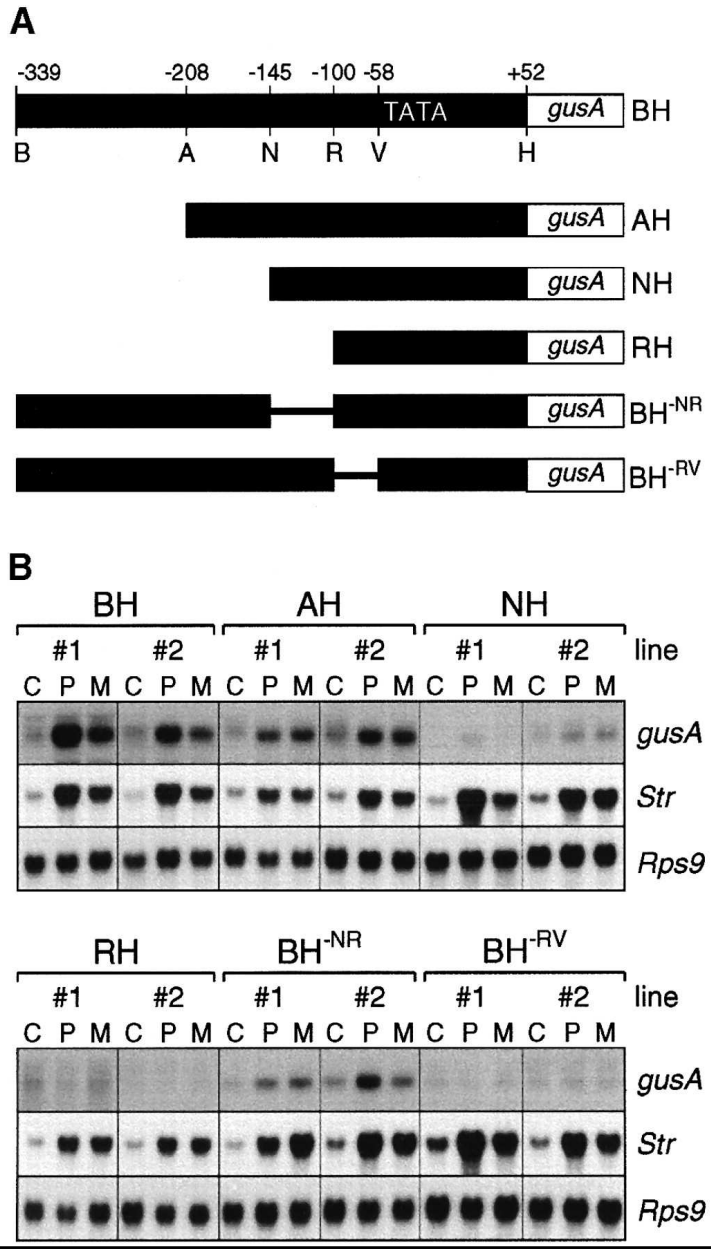
Upstream - Promotor analysis (yeast one hybrid)

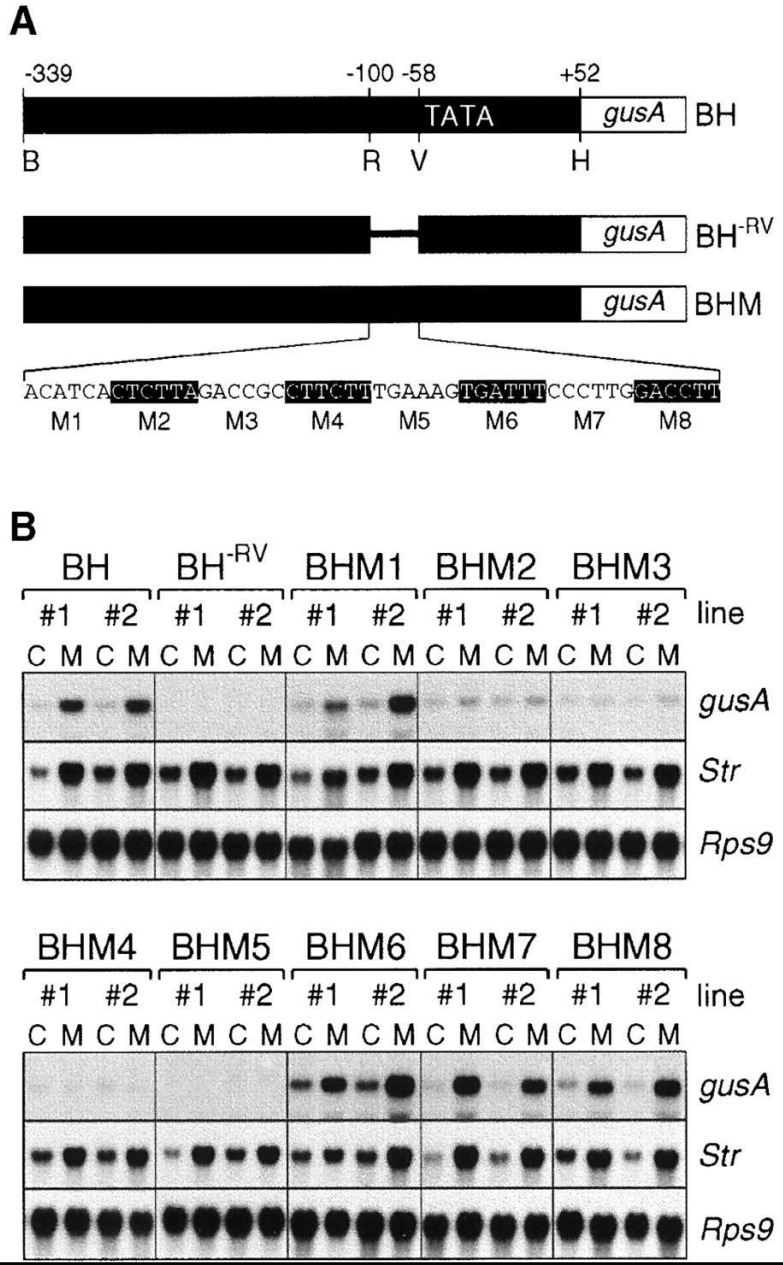


Promoter (bp)	Activity
2,400	Yes
1,500	Yes
1,000	Yes
500	No
150	No

promoter::GUS

Promotor analysis – yeast one hybrid





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

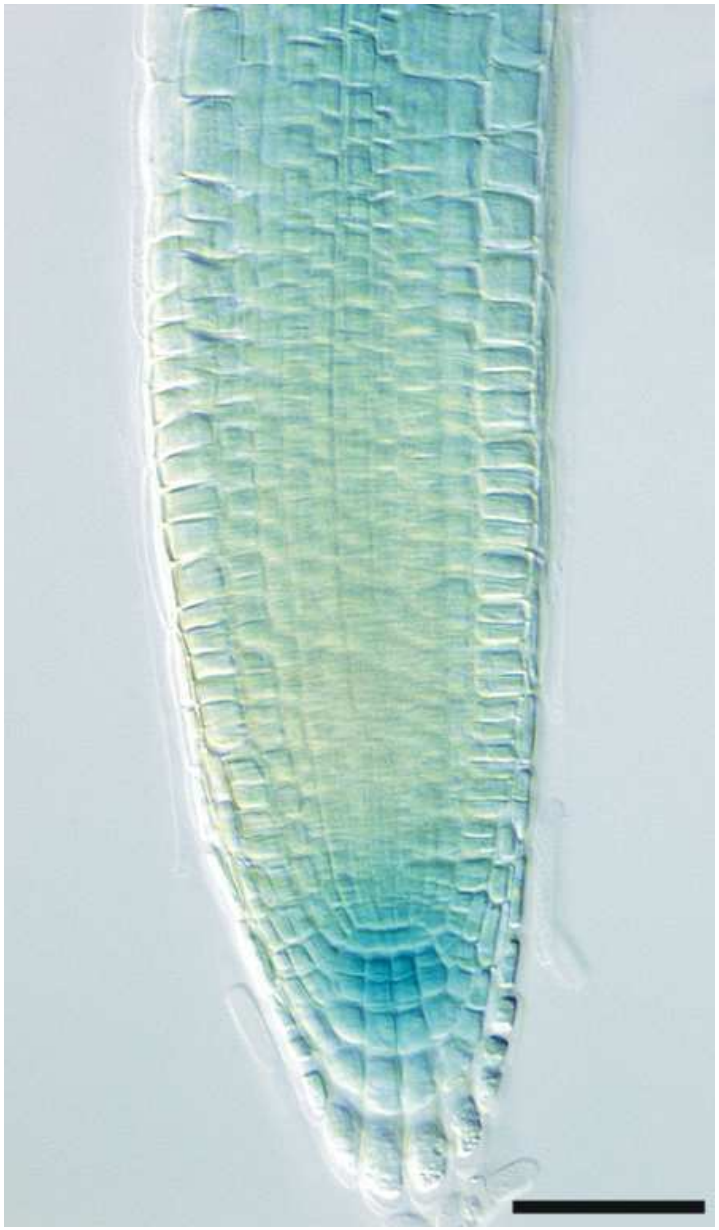
5´ CCTTT TGTCTC *3´*

9x inv.

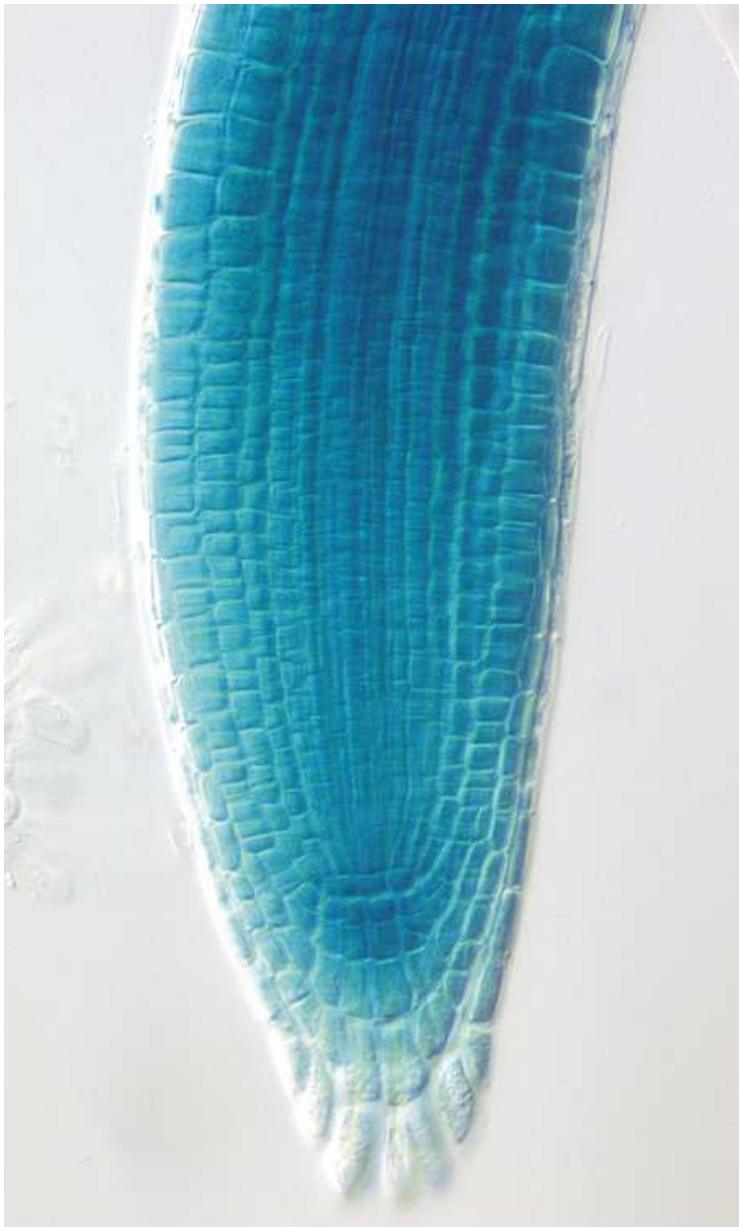
DR5: Ulmasov *et al.*, 1997

DR5::GUS

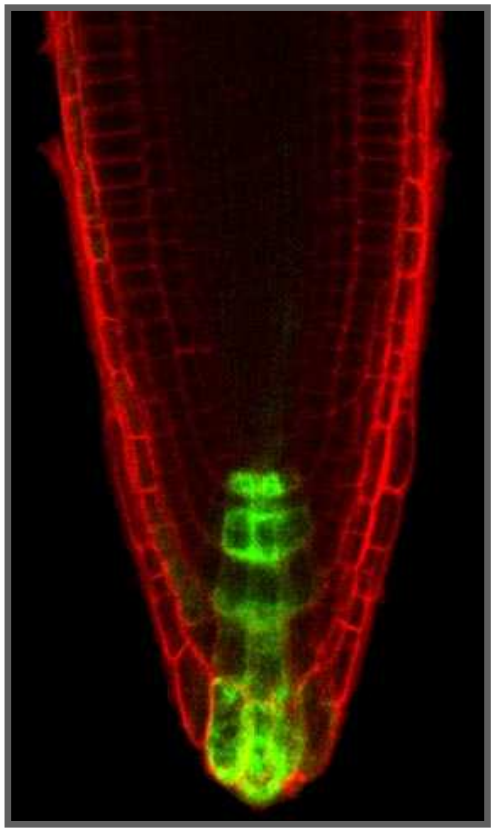
- Auxin



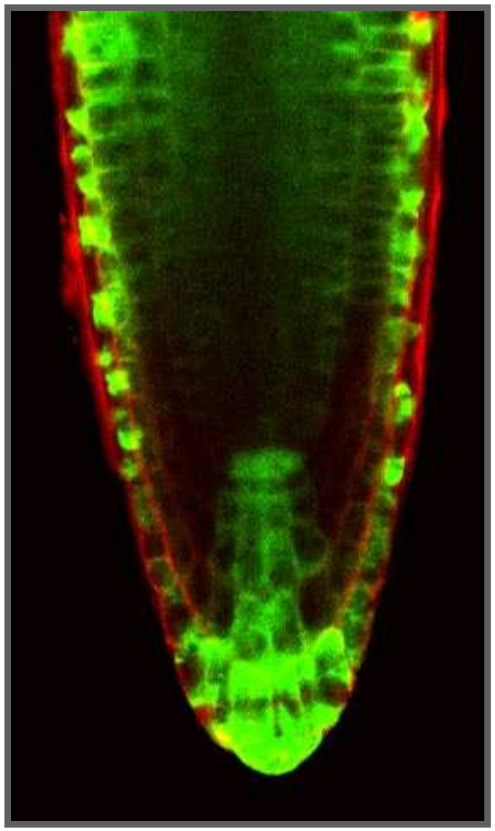
+ Auxin



DR5::GFP Auxin Reporter

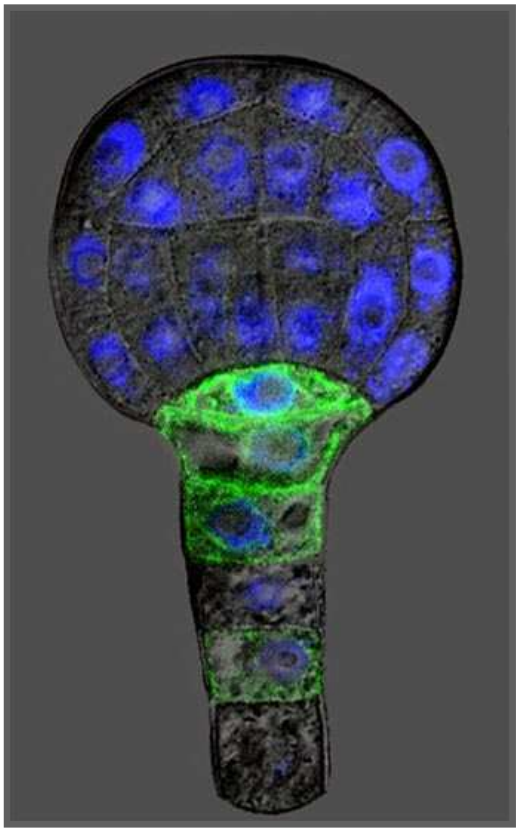


Root



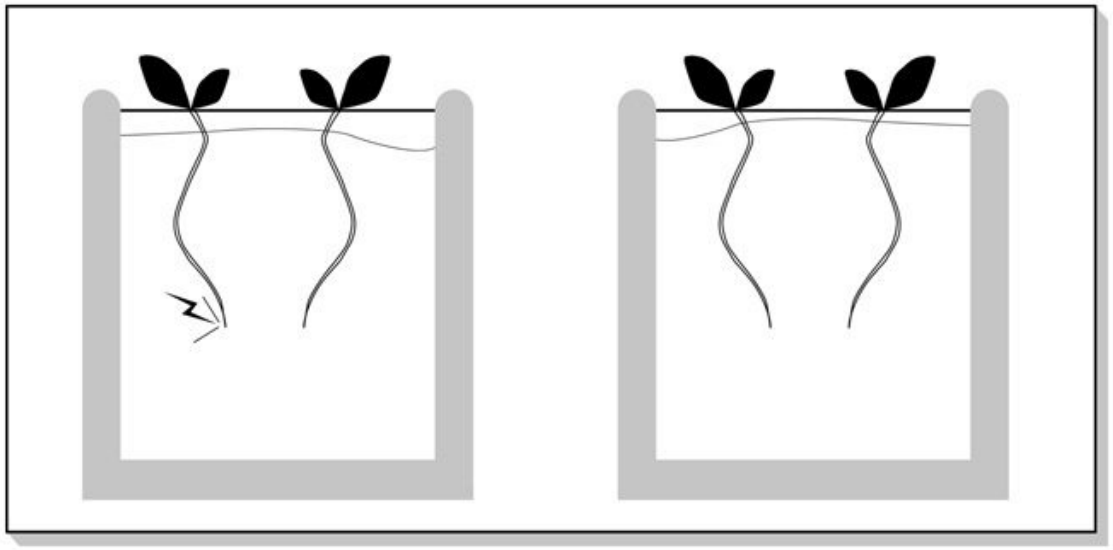
Root + Auxin

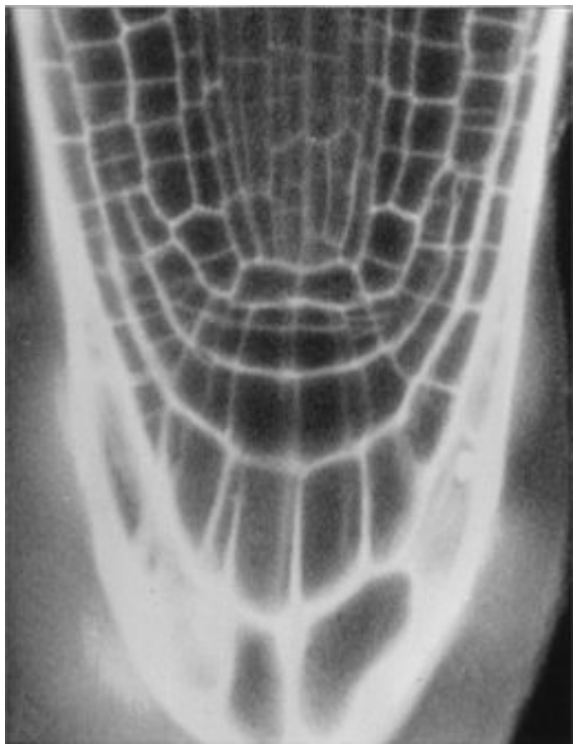
anti-IAA AB

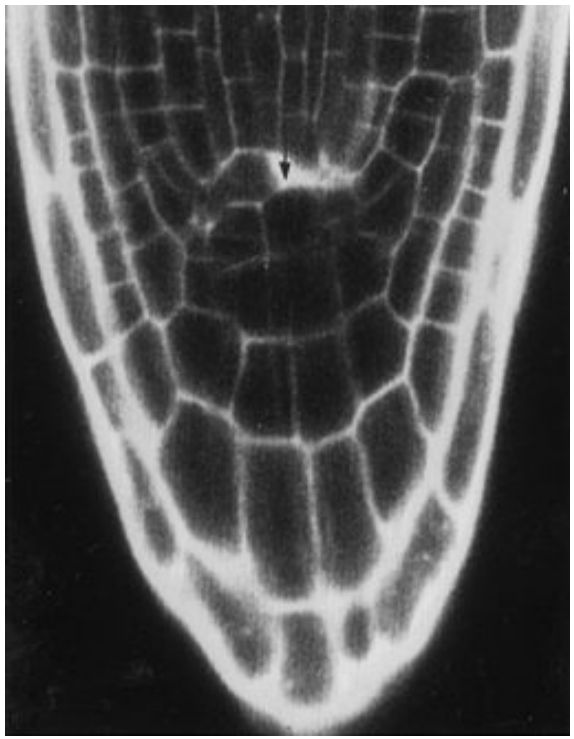


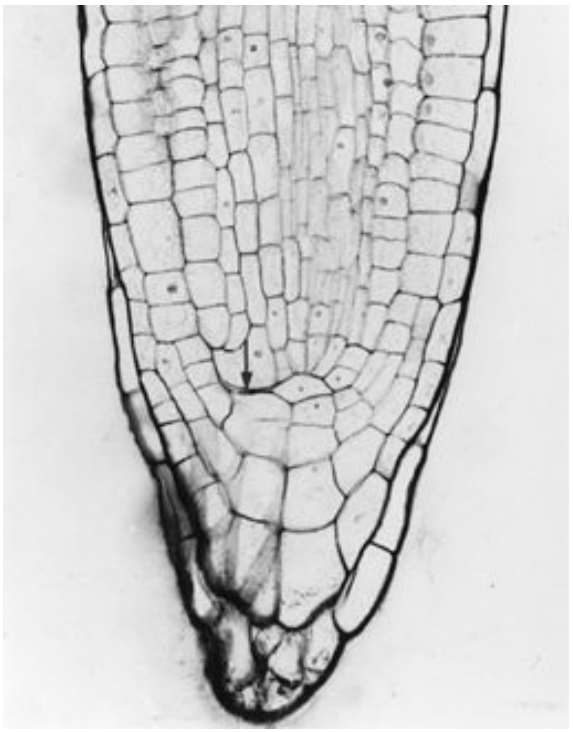
Embryos

Laser ablations

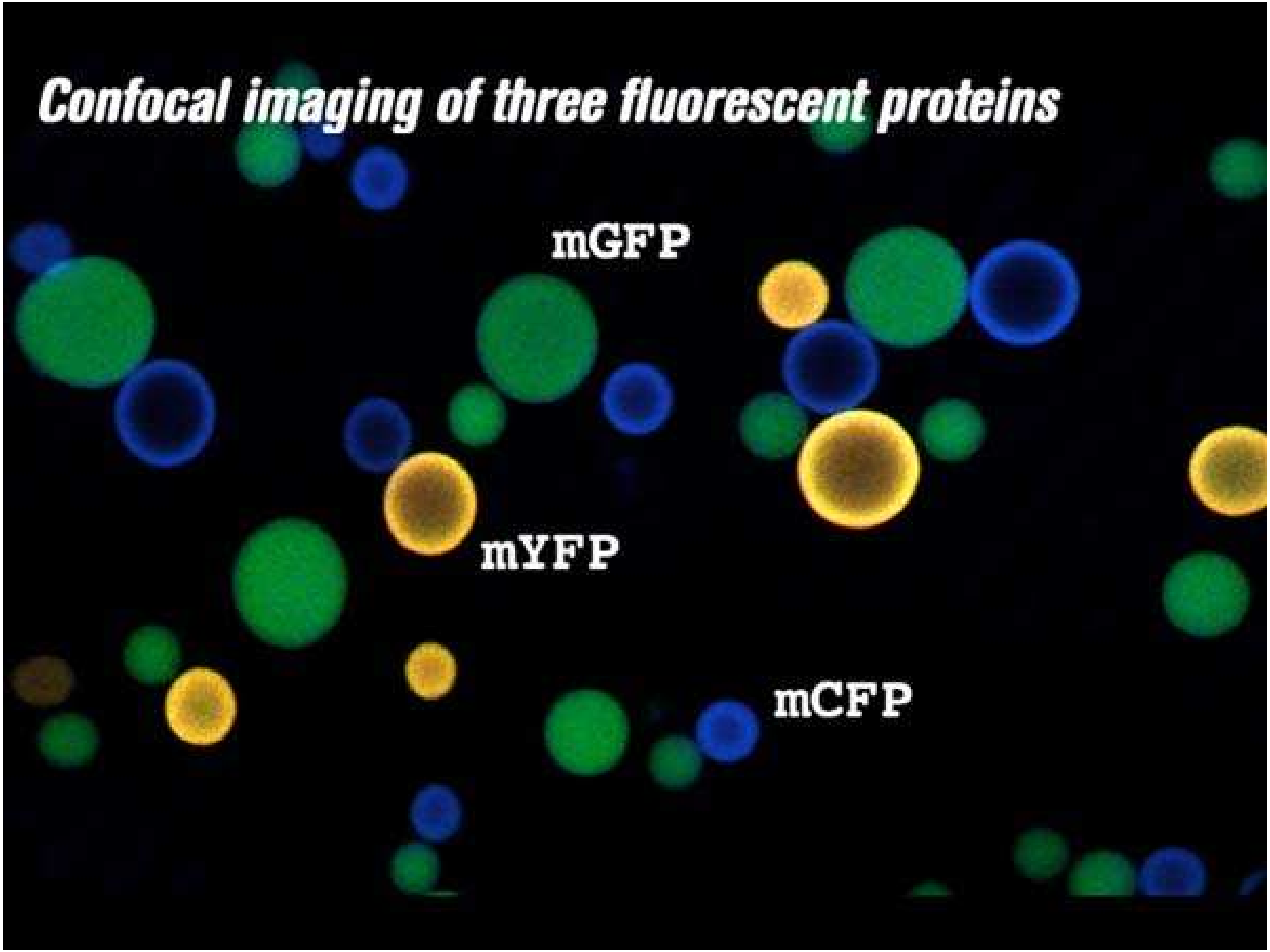






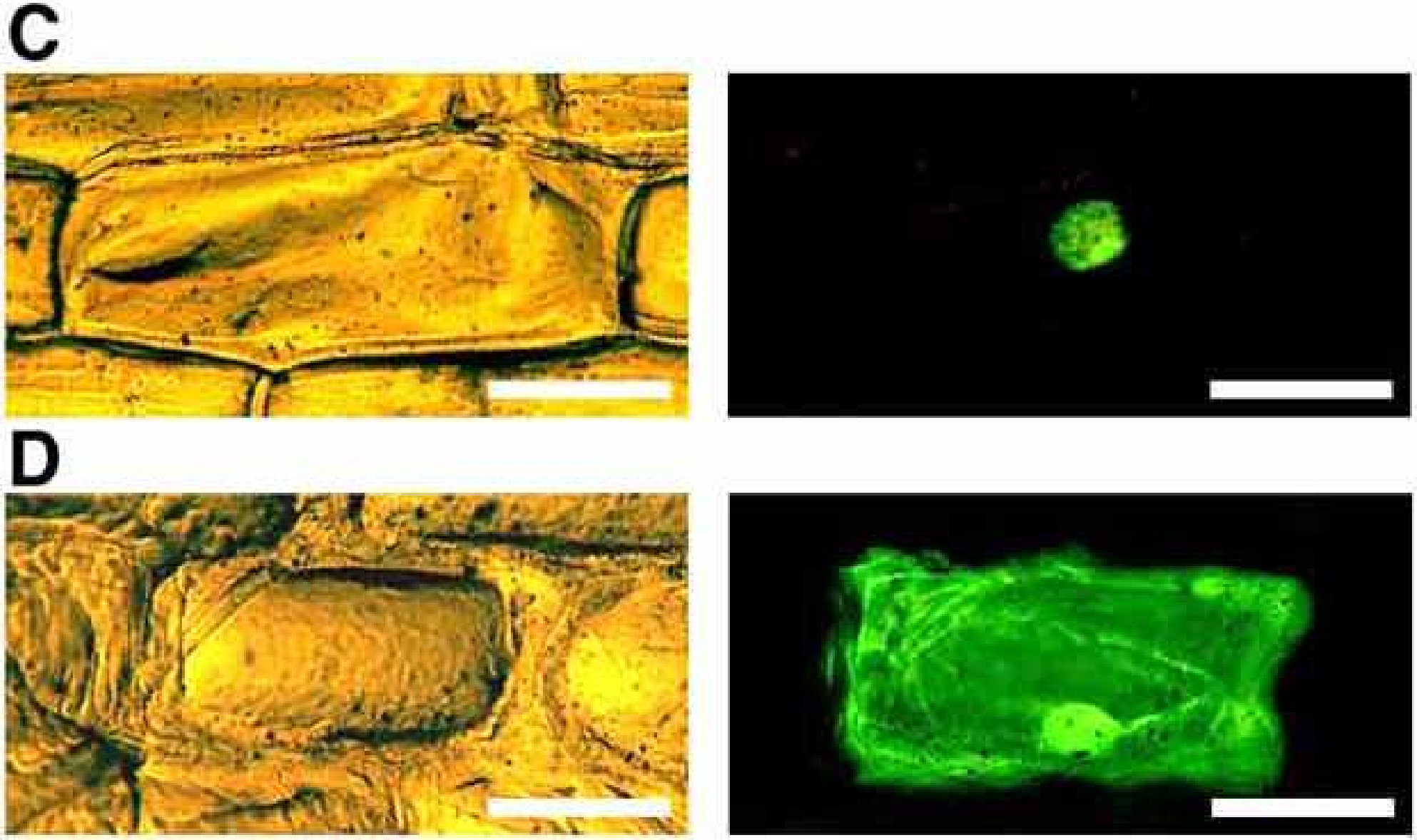


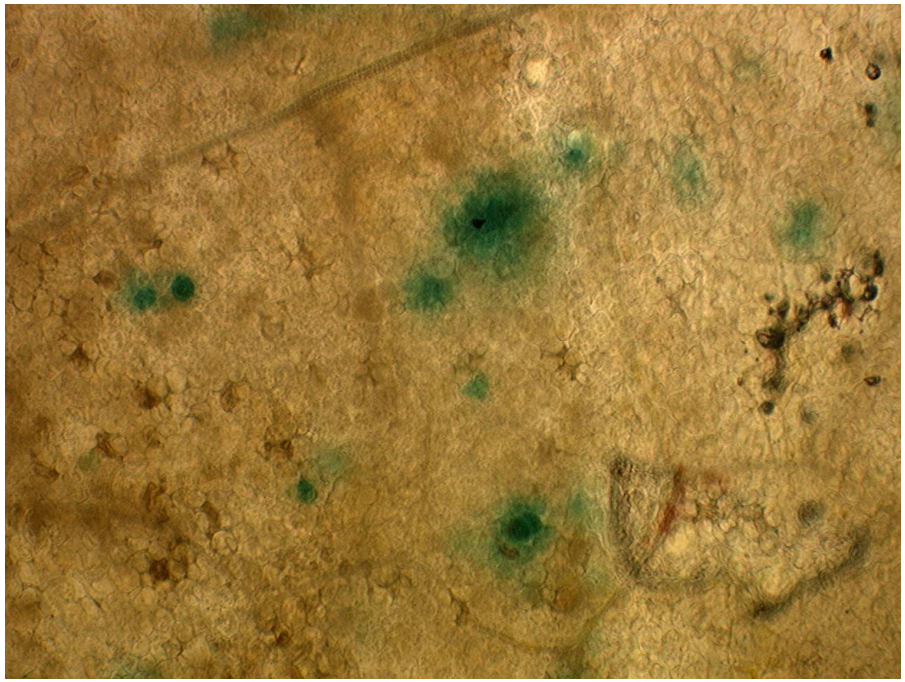
Transient transfection



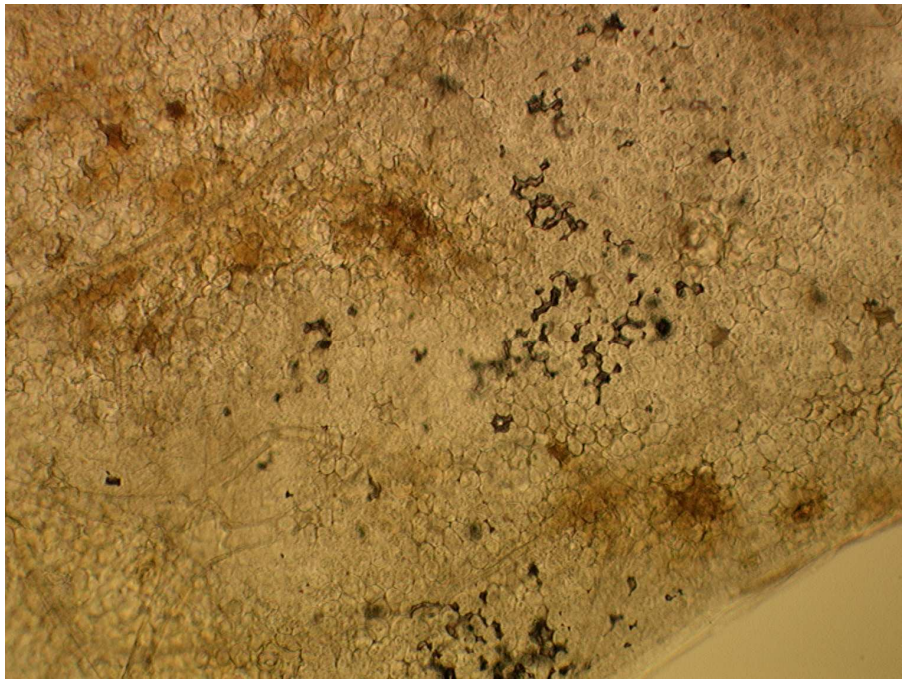
Protoplasts

Onion epidermis cells

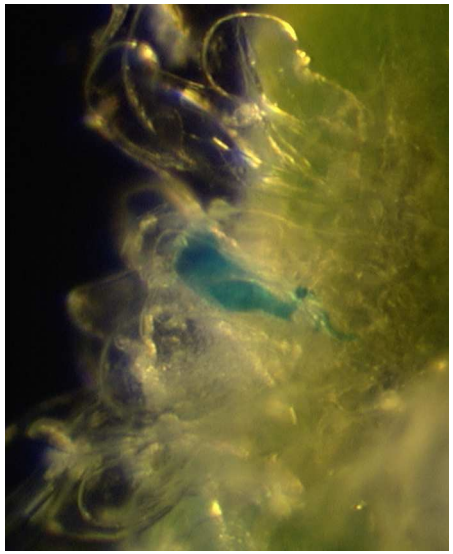




GUS

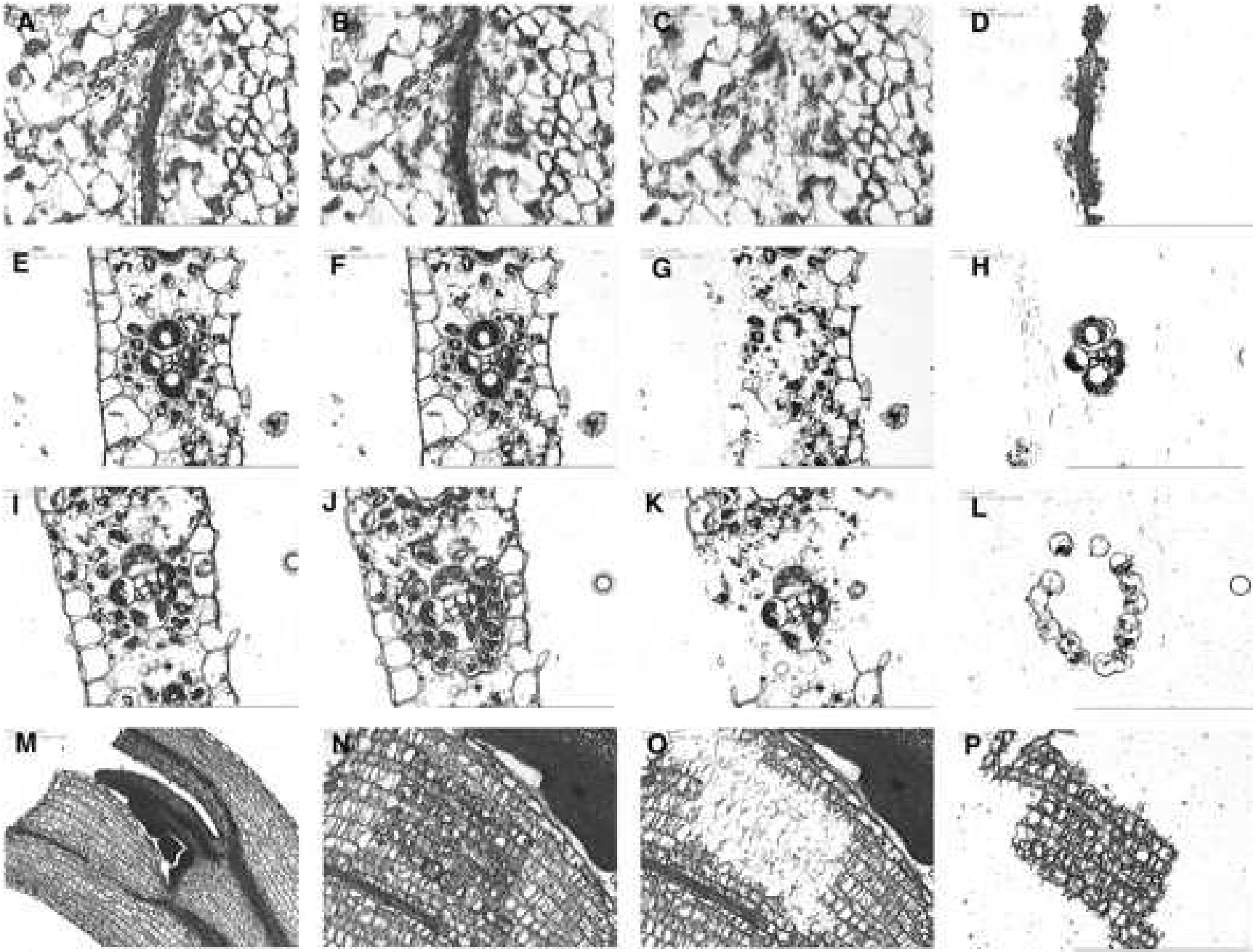


GUS + Diphteria Toxin



GUS + IPT (cytokinin biosynthesis)

Laser capture



Reconstitution of signaling pathway