



IIT ROORKEE



NPTEL ONLINE
CERTIFICATION COURSE

Molecular Genetics of Plant Development-cont..

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Approaches to the study of plant development

- **Reverse Genetics:** An approach to study function(s) of gene(s)
(Genotype to Phenotype)
 - *Identifying & isolation of gene for functional study (gene of interest)*
 - *Analyzing expression pattern of the gene (differential, spatial & temporal)*
 - *Functional characterization of the gene (gain or loss-of-function)*
 - *Gene interaction study (genetic, physical or regulatory interaction)*



Identifying gene of interest for functional study

➤ *Monitoring gene expression:*

- Organ-specific expression pattern (Differential expression pattern)
- Tissue-specific gene expression (Spatial expression pattern)
- Developmental stage-specific gene expression (Temporal expression pattern)
- Promoter trapping (expression pattern)



Analyzing expression pattern of the gene (spatial & temporal)

- **RNA *in situ* hybridization**
 - Whole-mount
 - Tissue sections
- **Protein *in situ* hybridization** (immuno-histochemistry)
- **Promoter::reporter assay**
- **Promoter trapping**



RNA *in situ* hybridization: Step 1: Tissue preparation

Sample collection



Plant samples: Root, stem, inflorescence, flowers etc.

Tissue fixation



Fixation: To terminate any ongoing biochemical reactions
Fixatives: Formaldehyde, para- Formaldehyde, glutaraldehyde

Embedding



Embedding: Dehydration (ethanol series)
Infiltration (paraffin in xylene)

Sectioning

Sections collected on coated slides and dried

Whole-mount
& sections

Sections

RNA *in situ* hybridization: Step 2: Probe preparation

Probes



Probes: Single stranded RNA probes, sense and anti-sense probes

Templates



PCR: Designed primers with flanking T7/T3 promoter sequences

Clone: Gene-specific fragments cloned in vector, linearization

Probe synthesis

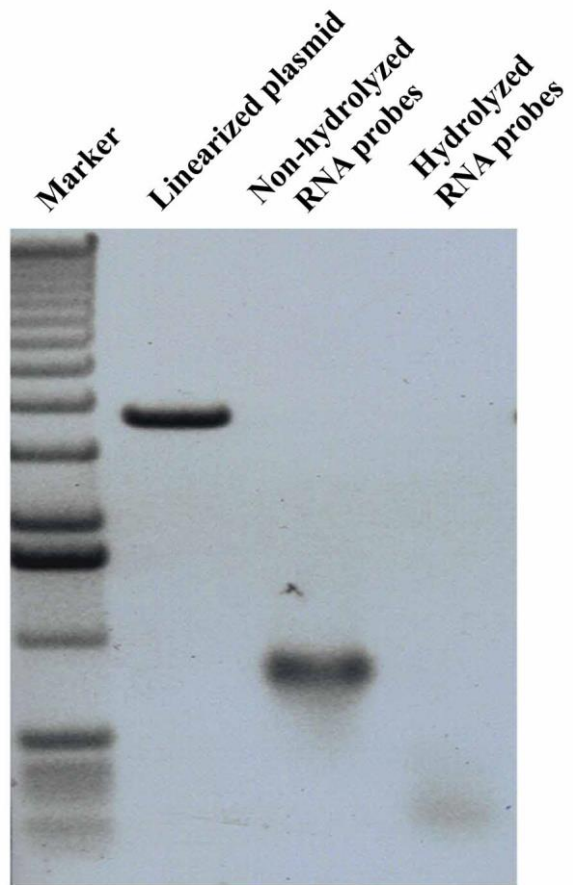


In vitro transcription: IVT with T7/T3 RNA polymerase and labeled NTPs

Radioactive (isotope-NTP) vs non-radioactive (bio-11-dUTP, Digoxigenin-11-UTP)

Probe hydrolysis

Large probes hydrolyzed to a mean length of 75-100 nt



RNA *in situ* hybridization: Step 3: Probe Hybridization

Pre-hybridization



Proteinase K: To partially digest the tissue to allow better probe penetration

Hybridization



RNA probes: Use 75-150 ng probes per slides in Hyb buffer
Incubate at 50-55 °C for about 16-20 hrs

Washing



Rnase treatment: To remove unbound ssRNAs
Washing: stringent washing to remove un-hybridized probes

Detection

RNA *in situ* hybridization: Step 4: Signal Detection

Blocking



Blocking Reagents: To decrease non-specific binding of the antibodies

Antibodies treatment



Antibodies: Anti-DIG antibodies with AP conjugates (colorimetric assays) or Anti-DIG Abs with rhodamine conjugates (fluorescent based detection)

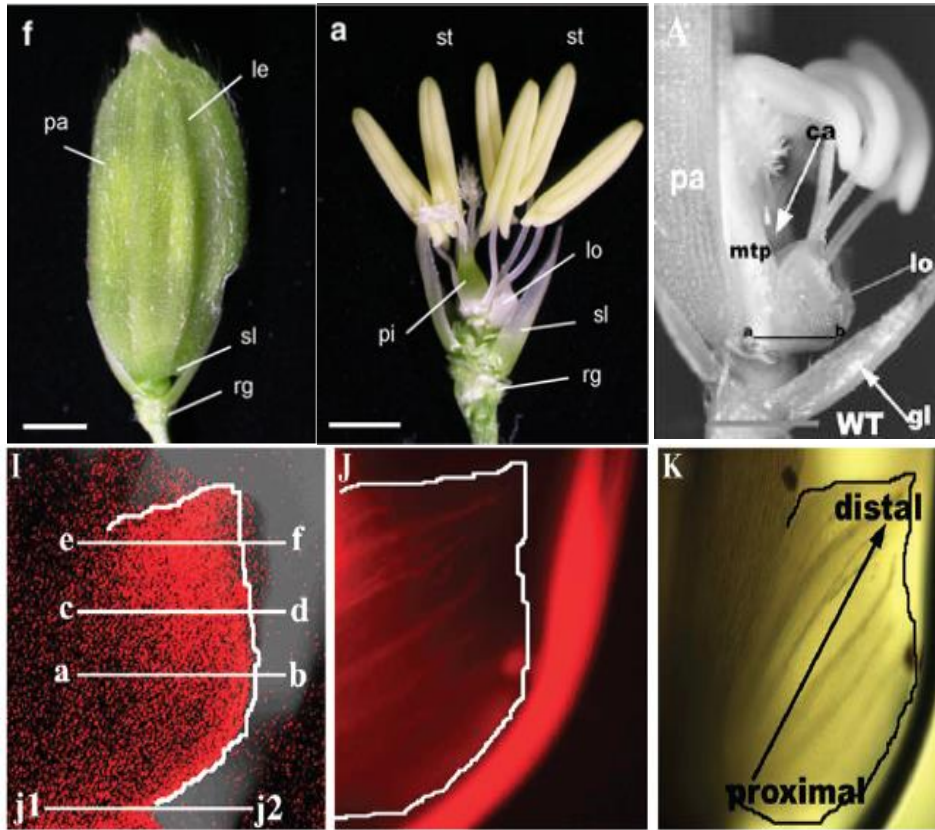
Washing



Washing: To remove unbound antibodies

Detection

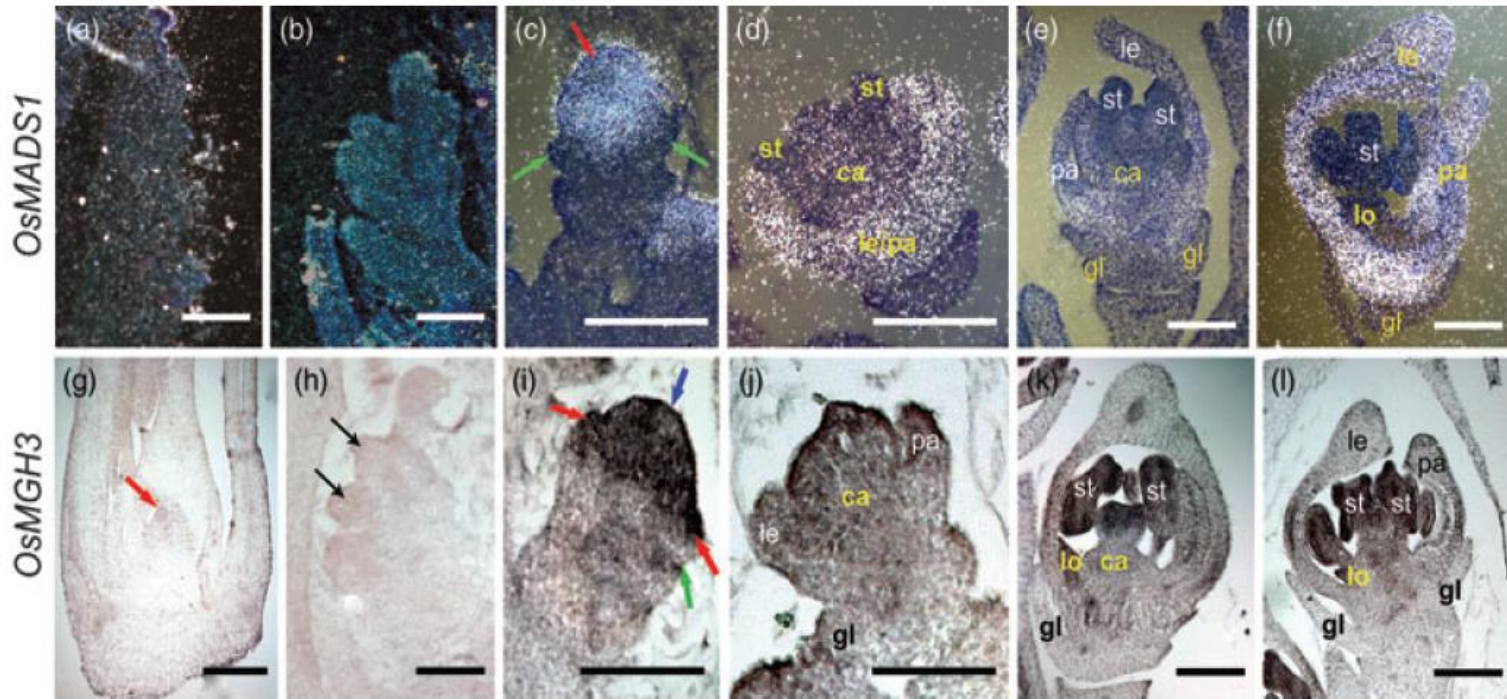
Detection: Autoradiography/color reaction/fluorescent microscopy



Whole-mount RNA *in situ* of *OsMADS2* by non-radioactive DIG-UTP labeled RNA probes and detected by fluorescent-tagged antibodies

Image source:
Hirano et al. (2014)
Methods Mol Biol. ;1110:57-84.

Yadav, Prasad et al. (2007)
Genetics, 176, 283-94.



Tissue sections RNA *in situ* of *OsMADS1* by radioactive labeled RNA probes and *OsMGH3* by DIG-UTP labeled RNA probes and detected by colorimetric assays

Image source: Prasad et al. (2005) Plant J., 43, 915-28.

Sample collection



Fixation



Membrane permeabilization



1^o antibody incubation



2^o antibody incubation



Co-staining (PI/DAPI)

Protein *in situ* hybridization (immuno-histochemistry): whole-mount

PIN1 (green), H⁺-ATPase (red) and DAPI (blue)

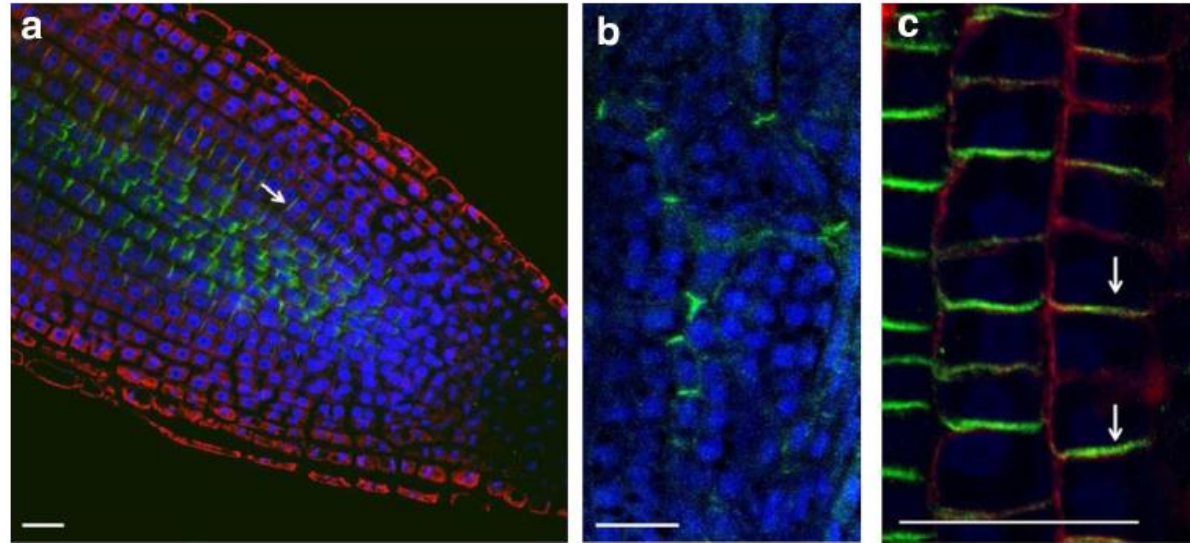


Image source: Pasternak et al. (2015) Plant Methods, 11,50.

Monitoring gene expression

➤ Promoter::reporter assay

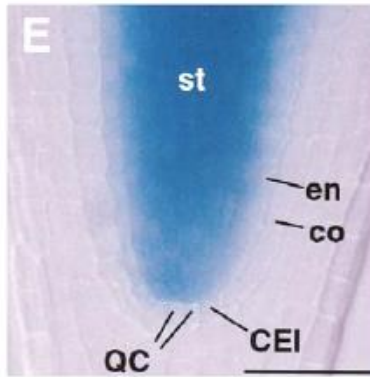
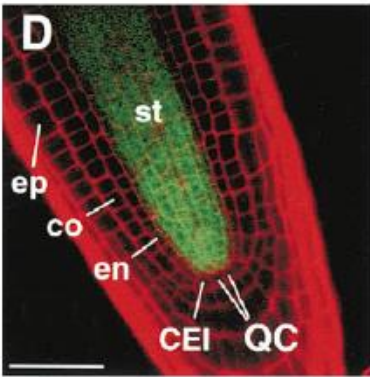
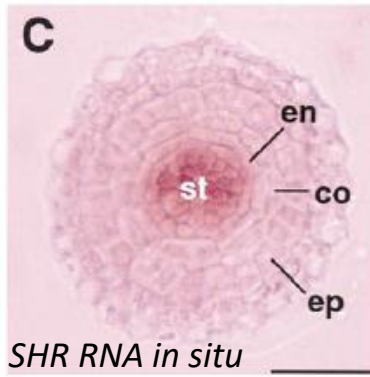
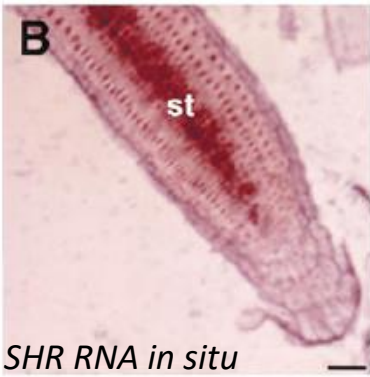
- **Transcriptional fusion construct:** Promoter activity domains

Validation by *in situ* hybridization

- **Translational fusion construct:** Domains & sub-cellular Protein localization

Validation by complementation





pSHR::GFP

Image source: Helariutta et al. (2000)
Cell, 101, 555-67.

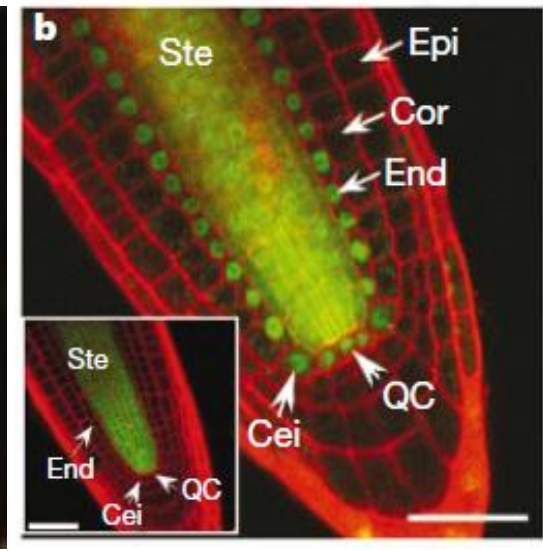
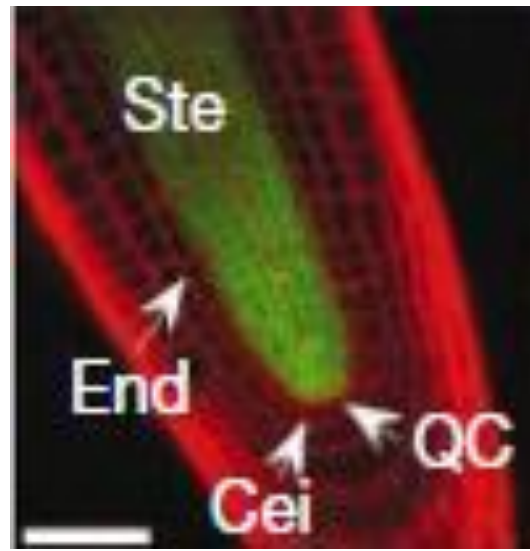


Image source: Nakajima et al. (2001)
Nature, 413, 307-11.

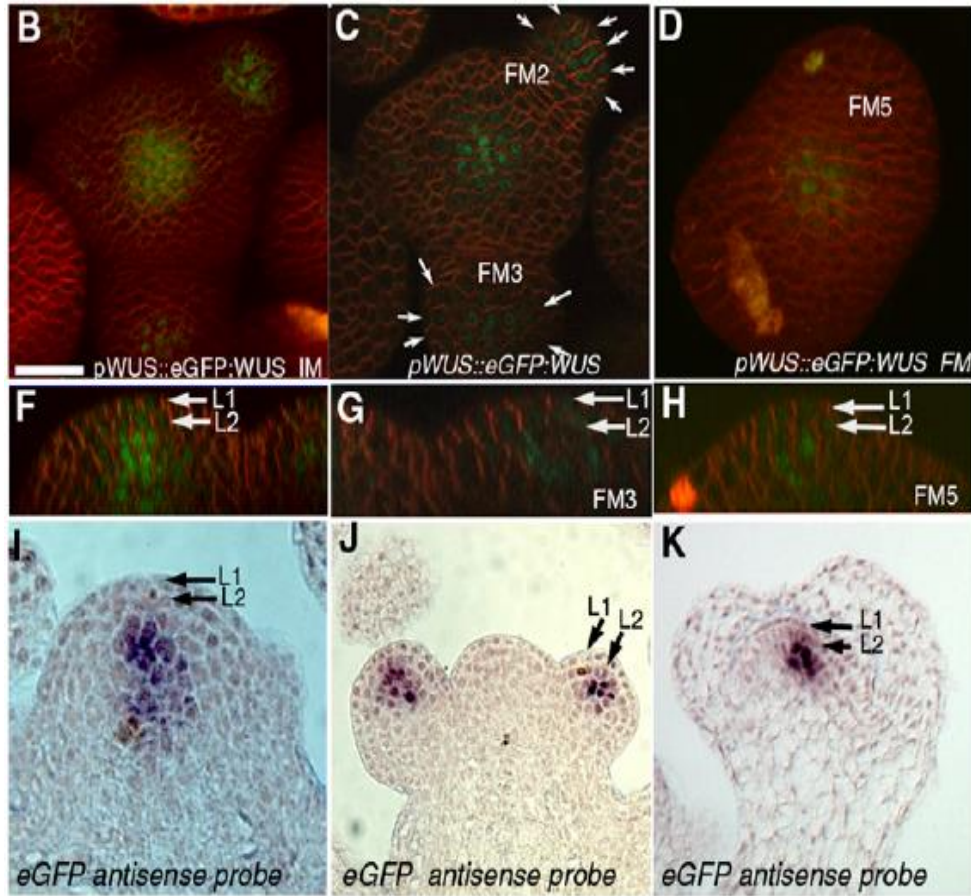


Image source: Yadav et al. (2011)
Genes Dev., 25, 2025-30.

Analyzing expression pattern of the gene (spatial & temporal)

Promoter/Enhancer/Gene trapping

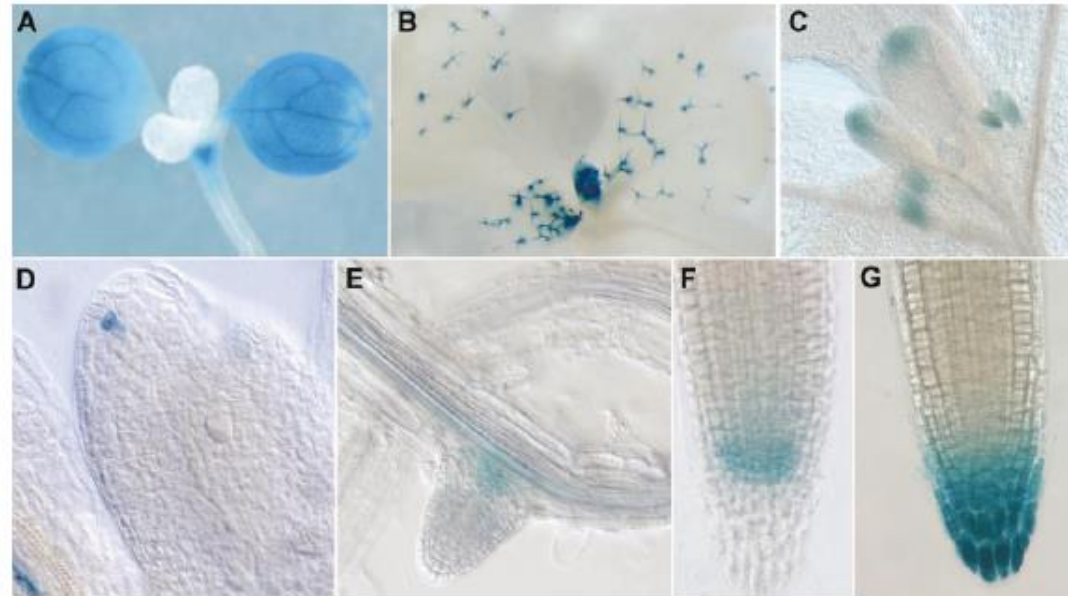
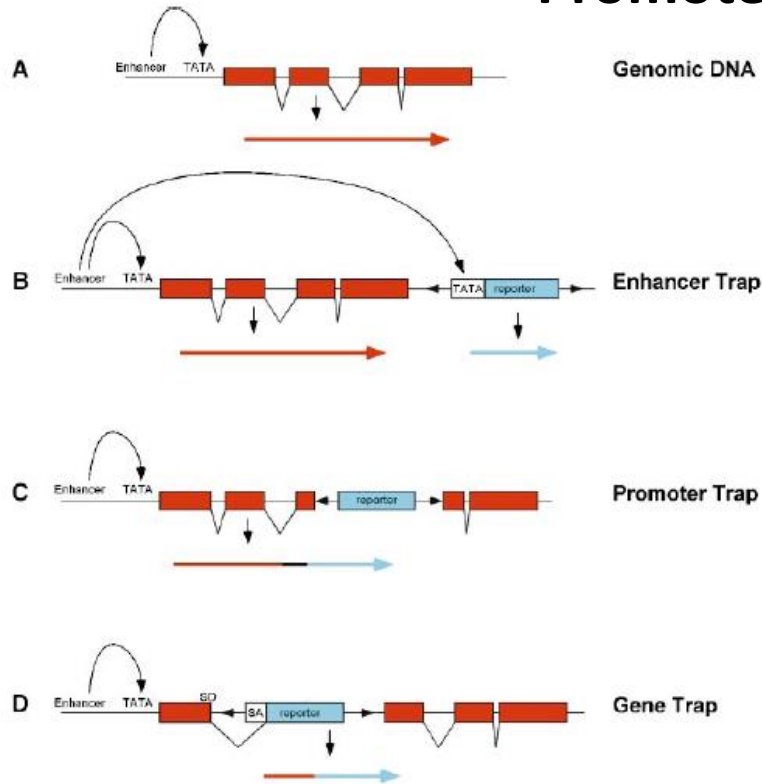
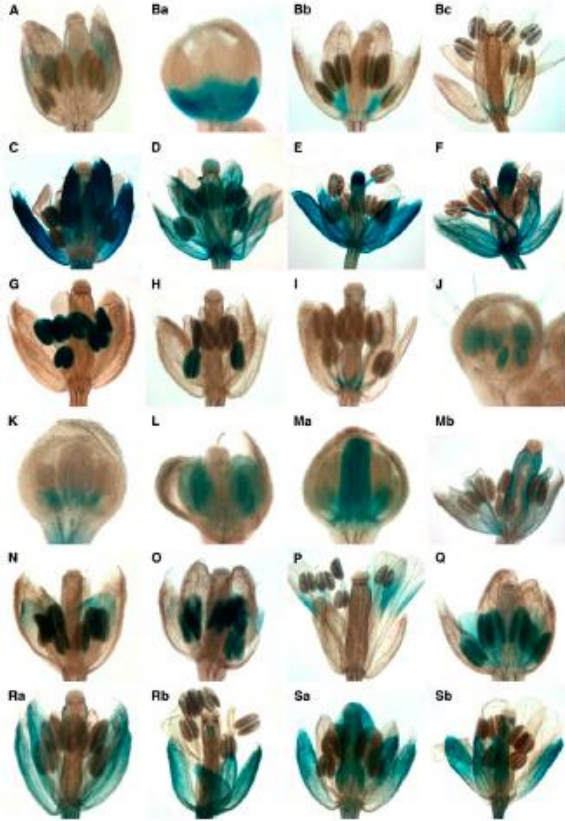


Image source: Springer, P. S. (2012)
Plant Cell, 12, 1007-20.

Analyzing expression pattern of the gene (spatial & temporal)

Promoter/Enhancer/Gene trapping



Gene Trap Lines Showing Petal- and/or Stamen-Specific Expression Patterns

Image source: Nakayama et al. (2005)
Plant Cell, 17, 2486-2506.

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