Molecular Techniques and Links
(Manish Raizada, University of Guelph)

A. General Links

Plant tissue types
http://www.botany.uwc.ac.za/ecotree/celltissues/tissues.htm

ISI Web of Science (tool to find papers online, on campus)
http://portal.isiknowledge.com/portal.cgi?DestApp=WOS&Func=Frame

Wikipedia, molecular biology
http://en.wikipedia.org/wiki/Molecular_biology

Bioteach UBC
http://bioteach.ubc.ca/

General Molecular Biology Protocols
Protocol Online
http://www.protocol-online.org/

Pedro's BioMolecular Research Tools
http://www.public.iastate.edu/~pedro/rt_all.html

Online Molecular Biology Techniques
http://www.mcb.uct.ac.za/manual/MolBiolManual.htm

Bionet Newsgroup
http://www.bio.net/bionet/

B. What to do after you have isolated a gene?

1.Initial analysis

a. Sequence
b. Check Genbank (see below)
c. If necessary, check copy number in nonsequenced genome using Southern blot (1 week)

Southern Blot Flash Video
http://www.dnalc.org/ddnalc/resources/shockwave/southan.html

2. in silico analysis: Genbank homology analysis (more in depth in future class)

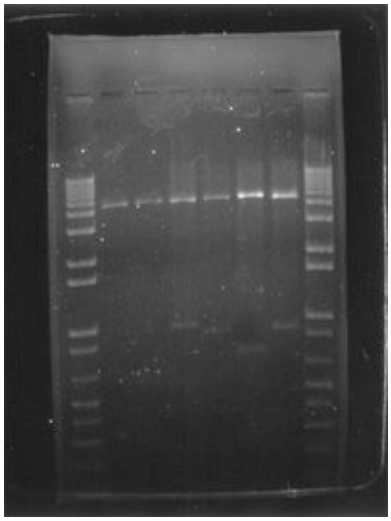
a. ORF analysis
b. Promoter or other motif analysis (to predict how regulated)
c. Analyze how gene is expressed in online microarray profiles
d. Evolutionary relationships within gene families

ENTREZ, NCBI list of databases
http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi

3. Check RNA expression
   a. Northern (most quantitative) but requires fair bit of RNA and slow (few weeks)
   b. RTPCR analysis, real-time or quantitative qRTPCR (advantages: rapid, requires very little RNA) – visualize on an agarose gel (rapid) (few days)
   c. semi-quantitative RTPCR using low PCR cycle numbers and Southern blotting (few weeks)

Wikipedia - PCR
http://en.wikipedia.org/wiki/Polymerase_chain_reaction

PCR Flash Animation
http://www.dnalc.org/ddnalc/resources/shockwave/pcranwhole.html



d. RNA Microarrays (mutant vs wild-type; overxpression vs wild-type) (whole procedure and analysis, 6mos)

a. Different tissues, stress conditions, list good sources
b. Enriching tissue for RNA or protein; Fluorescence activated cell sorter (flow cytometry) with reporter GFP clones
c. Cost $500-$1000 per chip, 3 replicates needed + negative and positive controls, correlation between RNA vs protein is moderate, then need statistics and software to analyze output data (not as sensitive as Northern blot or RTPCR, but can detect thousands of transcript types simultaneously)

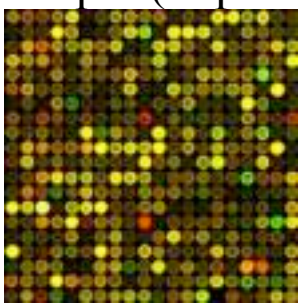
Links to Microarray Info and Protocols
http://ihome.cuhk.edu.hk/~b400559/array.html

Flash Movie on Microarrays
http://www.bio.davidson.edu/courses/genomics/chip/chip.html
Wikipedia, microarrays
http://en.wikipedia.org/wiki/DNA_microarray
Excellent Microarray Tutorial
http://www.bioteach.ubc.ca/MolecularBiology/microarray/index.htm
Flow Cytometry tutorial (to enrich for a specific cell type RNA)
http://www.bioteach.ubc.ca/MolecularBiology/FlowCytometry/

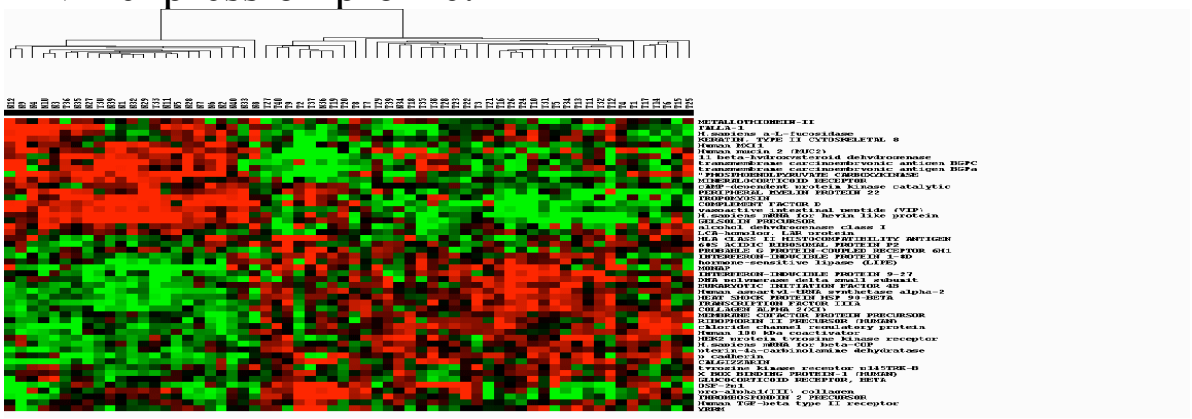
Type: microarray (in Google images)

Picture of Affymetrix gene chip (~to scale)

Output (requires $50,000-$100,000 chip reader)



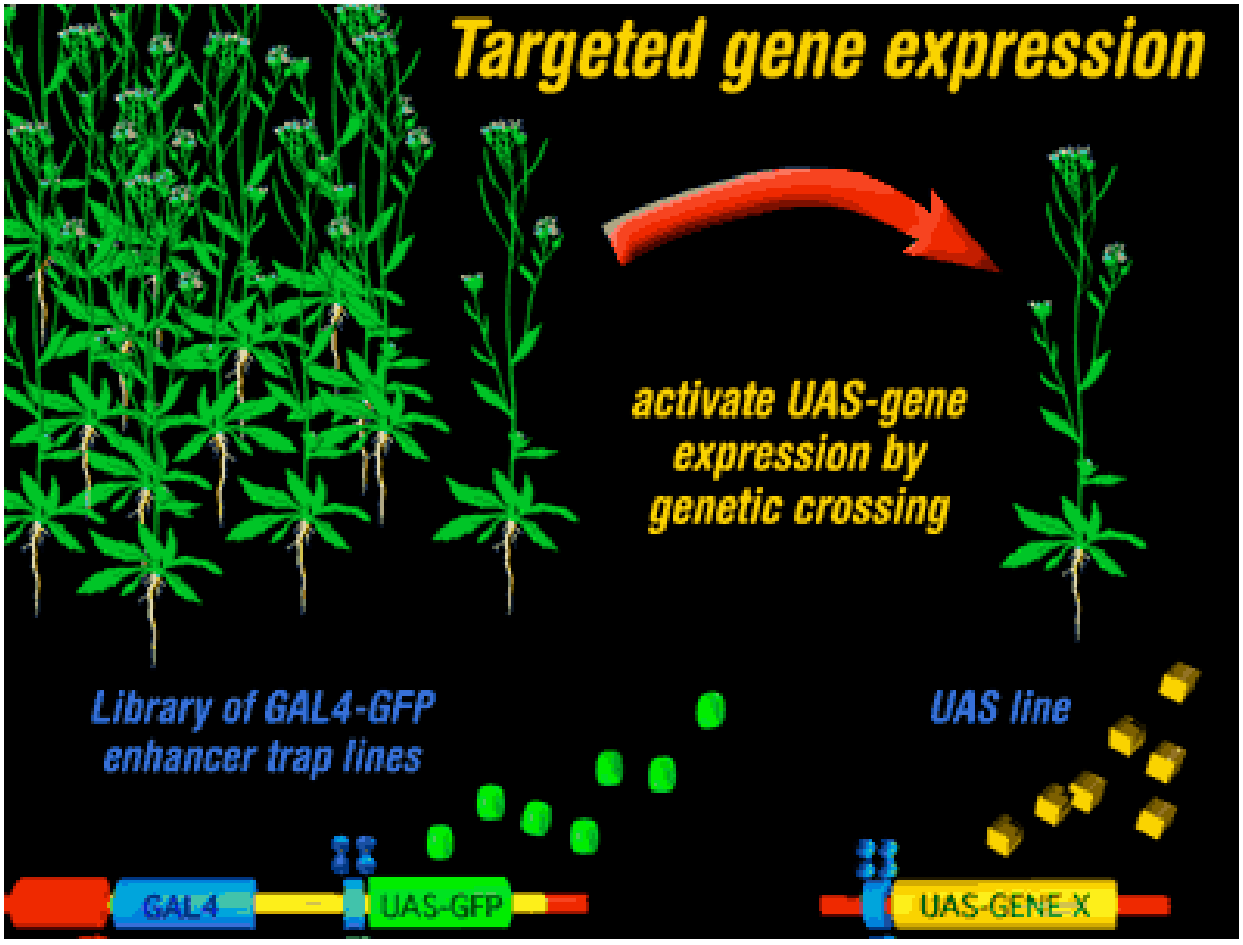
Cluster analysis: Use software to group genes by common RNA expression profile:



4. Transgene expression

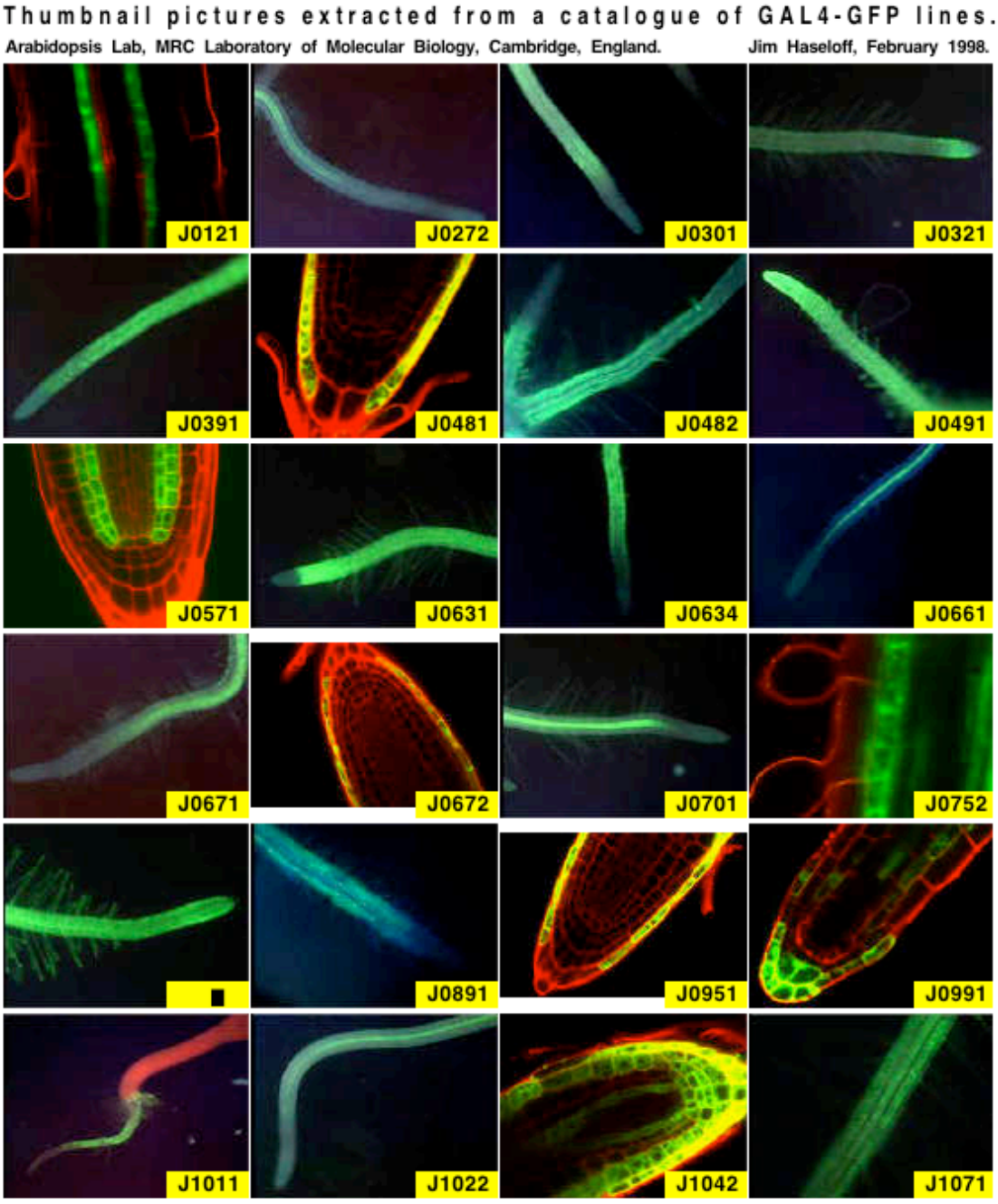
a. Complementation (4-6mos for Arabidopsis)
b. overexpression – CaMV 35S promoter, Ubiquitin promoter, actin promoter (6mos for Arabidopsis)
c. misexpression in different cell types, tissues, organs
   Use of two component gene systems to express many transgenes under the same promoter rapidly (e.g. Haselhoff GAL4) (6mos for Arabidopsis)

GAL4 two-component system in Arabidopsis
http://www.plantsci.cam.ac.uk/Haseloff/geneControl/GAL4/GALtrapscheme.html



Catalog of Available GAL4-GFP enhancer trap lines (many more)

http://www.plantsci.cam.ac.uk/Haseloff/geneControl/catalogues/GAL4_GFP_html/GAL4_GFPimages.htm



5. Western Blot analysis: to measure protein levels using a PAGE gel – requires antibody (therefore, must overexpress cDNA in yeast/bacteria, then inject animal) (need to express protein first, then animal bleeds, then purification, expect 1-2 years)
http://en.wikipedia.org/wiki/SDS-polyacrylamide_gel_electrophoresis

6. *In situ* hybridization in tissue (RNA) (6mos –1 yr)

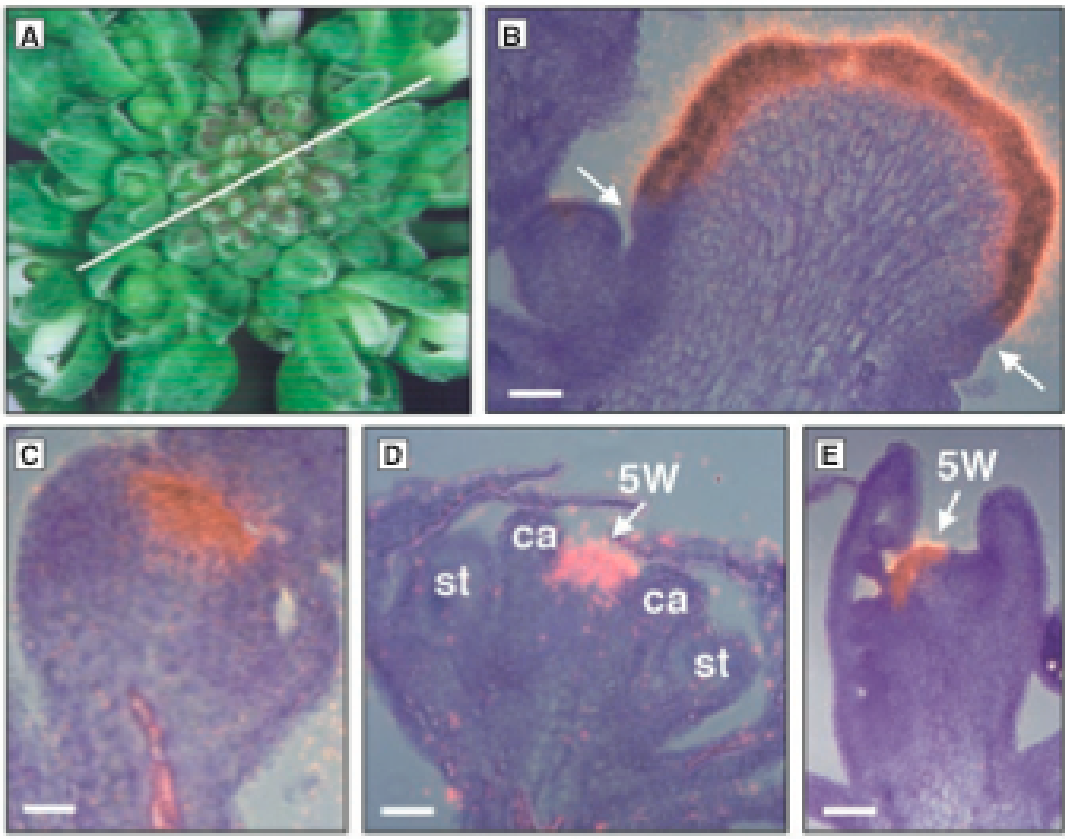


Fig. 4. *CLV3* mRNA expression patterns in *clv1* mutant tissues. (A) *clv1*-4 inflorescence meristem and flowers. The line denotes the approximate orientation of the section taken in (B). (B to E) In situ hybridization with *CLV3* radiolabeled antisense probe. Each section was photographed with brightfield-darkfield double exposure, using a red filter for the darkfield exposure. (B) Abnormally enlarged *clv1* inflorescence meristem. *CLV3* is expressed throughout the enlarged meristem except at the margins (arrows). (C) Stage 5 *clv1* flower. (D) Stage 8 *clv1* flower. (E) Gynoecium of mature

7. Immunohistochemistry using antibody (protein)
   - similar to in situ hybridization, but using antibody + detection system (need to purify protein, develop antibody first – expect 1.5-2 years)

8. Make GFP/GUS/Luc reporter fusions – analyze under different conditions (6mos-9mos for Arabidopsis)

a. GFP vs GUS vs Luciferase – which one to choose?

GFP/RFP/YFP/CFP (up to 16 colours) – nondestructive, gives cellular, subcellular resolution, great for developmental biology, can do time-lapse, but protein very stable, accumulates; great for microscopy; can quantify in fluorescence reader, but semi-quantitative
GUS (blue precipitate) – destructive, but good for developmental biology when expression level is weak, gives cellular/tissue resolution, protein is very stable and accumulates; can quantify enzyme using fluorometer machine, but semi-quantitative
Luciferase – gives tissue resolution, can visualize using expensive photon-capture cameras; most quantitative reporter, can measure photon emission in luminometer; rapid protein turnover so great for dynamic studies (transcriptional induction, circadian rhythm studies, etc.)

b. to monitor transcription: promoter-reporter fusion

c. to monitor protein: promoter-open reading frame-translational fusion (reporter at C terminus)

9. Examine subcellular location of protein

-e.g. light wavelength can cause transcription factor protein to move from cytoplasm to nucleus

Techniques: immunohistochemistry, EM, or GFP translational fusions

10. Direct microscopy

a. Light (rapid)

b. Fluorescent: to mark cell walls, nucleus, live vs dead - give URL for Handbook of Fluorescent dyes from Invitrogen Molecular Probes (rapid)

Fluorescent stain images
http://dept.kent.edu/projects/cell/images3.htm
Arabidopsis imaging
http://www.plantsci.cam.ac.uk/Haseloff/imaging/Index_imaging.htm

c. Confocal/3D imaging (few days)

Confocal 3D GFP images
http://www.plantsci.cam.ac.uk/Haseloff/imaging/gallery/Gallery.html

3D visualization of Arabidopsis
http://www.plantsci.cam.ac.uk/Haseloff/celldynamics/Index_dynamics.htm

d. SEM (few days)

SEM images
http://remf.dartmouth.edu/images/ArabidopsisSEM/index.html

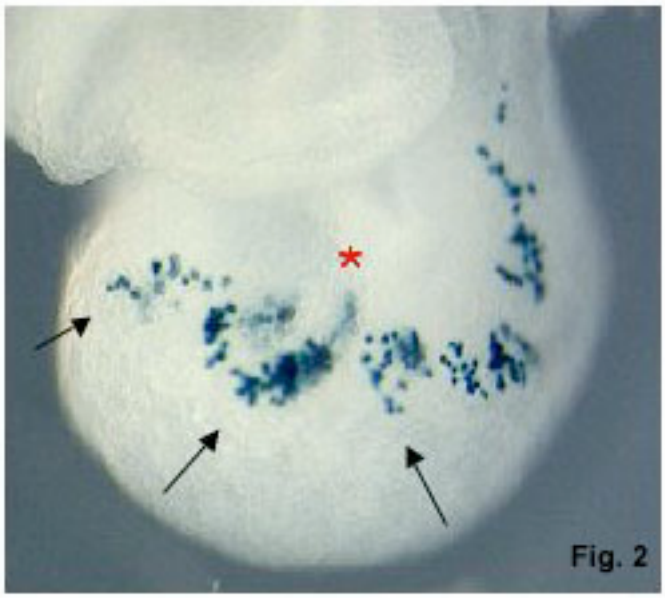
e. E.M. – electron microscopy (few days)

f. Time-lapse photography and videos (duration of growth, potentially cheap and easy)

Time Lapse Video Examples (Plants in motion)
http://plantsinmotion.bio.indiana.edu/plantmotion/starthere.html

11. Clonal analysis: to trace differentiated cell back to its progenitor cells For developmental genetics (few months)

-can use transposon exision (e.g. GUS inserted with Ac/Ds transposon)



12. Look for protein-protein interaction partners
    a. Yeast two-hybrid (Matchmaker Kit) (6months, but then need to confirm candidates using CoIP, pulldown, FRET, or YFP interaction)

http://www.bioteach.ubc.ca/MolecularBiology/AYeastTwoHybridAssay/index.htm

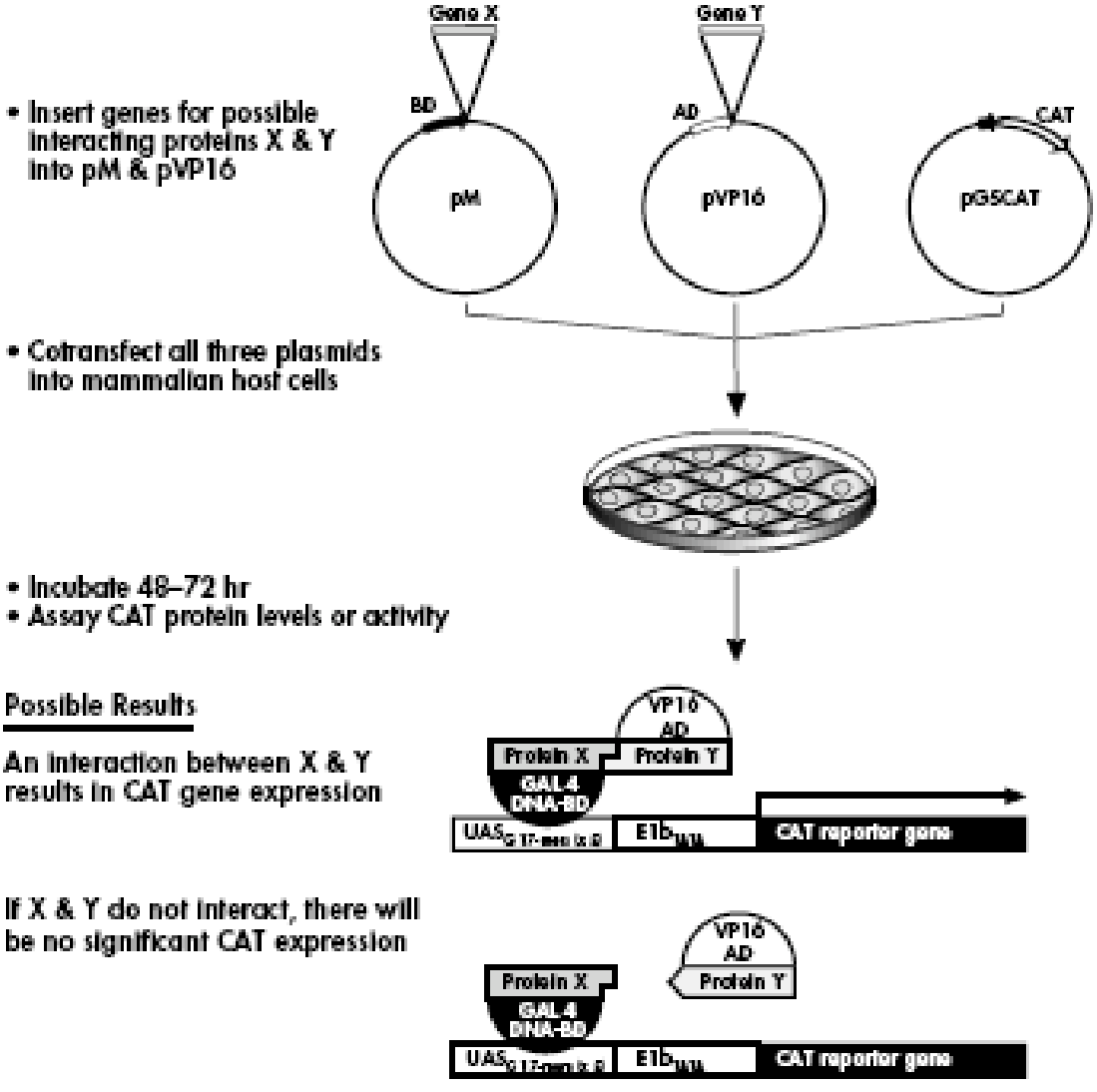


Figure 1. Using the Mammalian MATCHMAKER Two-Hybrid Assay Kit.

b. Co-Immunoprecipitation (Co-IP) (need to develop antibody first, by purifying protein, etc. so 1-2 years)

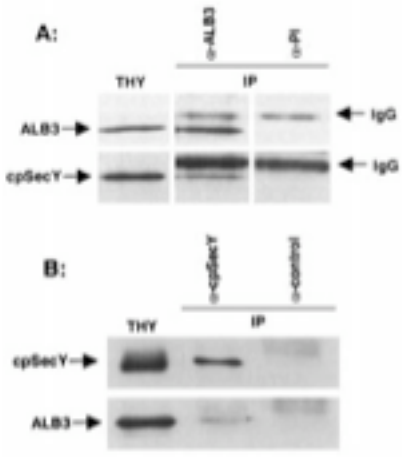
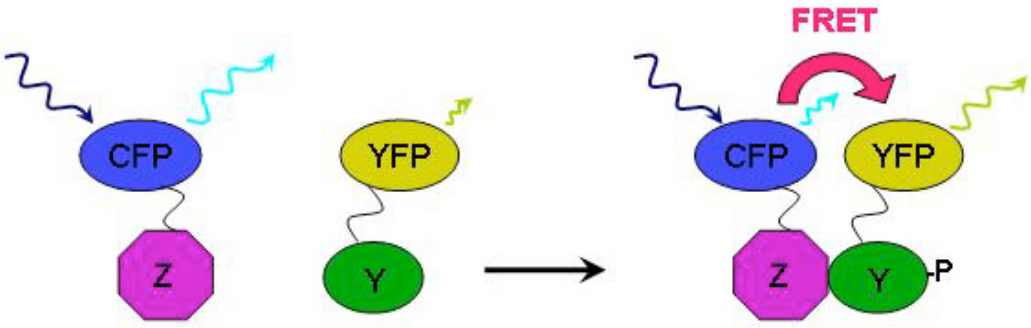


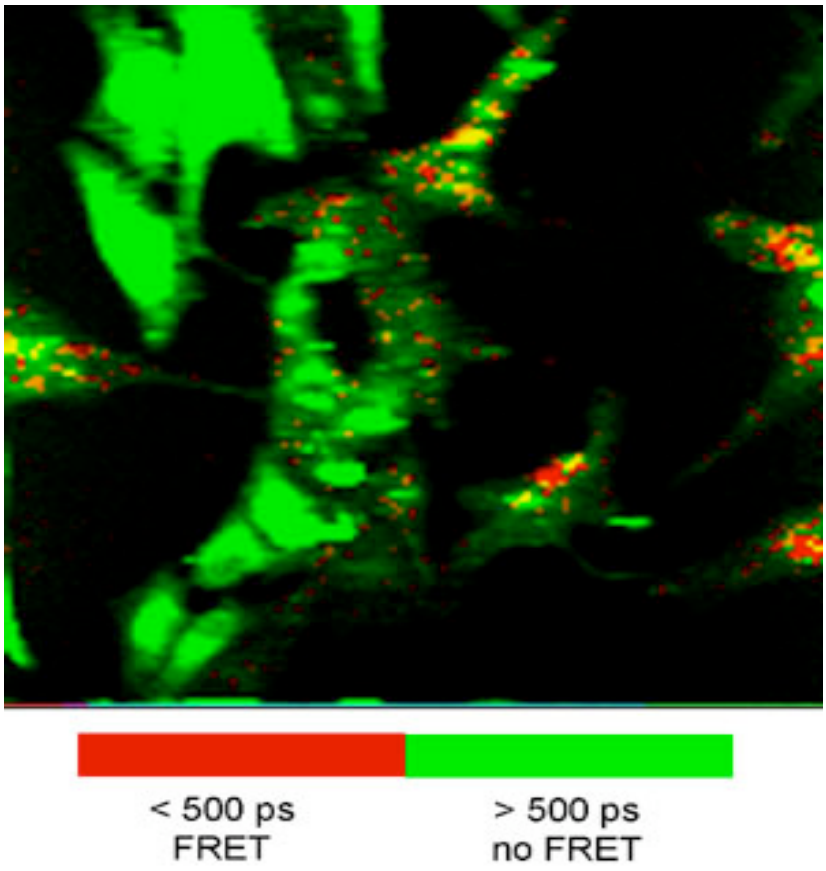
Figure 3 Co-immunoprecipitation of ALB3 and cpSecY

Thylakoid membrane proteins (THY) were solubilized with 1.5% digitonin, and immunoprecipitation (IP) was performed using anti-ALB3 antibodies (**A**) or anti-cpSecY antibodies (**B**). Preimmune serum (PI; **A**) or antibodies against an irrelevant protein (**B**) were used as controls (see the Experimental section for details). The immunoprecipitates were subjected to immunoblot analysis with anti-ALB3 and anti-cpSecY antibodies.

c. GST fusion pulldown - Overexpressing protein using transgene before doing pull-down protein expts (1-2 years)

d.FRET/BRET (need to make plasmid constructs first, then possibly generate transgenic plants, so expect 1 year)
FRET (Zeiss site) http://www.zeiss.com/c12567be0045acf1/Contents-Frame/af1e055b42a249aac1256af1003a1595





e. newer YFP (N-terminus) and YFP (C-terminus) fusion interaction technologies (only get YFP expression if two proteins interact) (need to make plasmid vectors, then possibly make transgenic plants, so expect 1 year)

13. Use RNAi to knock-out/knock down family (need to make plasmid constructs, then transform, so expect 1 year)

--design to 3'UTR" or 5'UTR to make specific to one gene, or to conserved ORF motifs for whole family

14. DNA-protein interaction studies for DNA-binding proteins

a. do gel-shift EMSA (Electromobility Shift Assay) (need to express cDNA in bacteria/yeast, purify protein, then do analysis, so expect >1 year)

b. ChiP assay – to find downstream DNA targets of known TFs (expect >1 year)

http://www.komabiotech.co.kr/product/immunology/use/chip.htm

15. To hunt for transcription factors/regulators that bind to a known promoter

Yeast one-hybrid (look for MatchMaker one-hybrid kit PDF) (6 months)

http://www.clontech.com/clontech/matchmaker/index.shtml

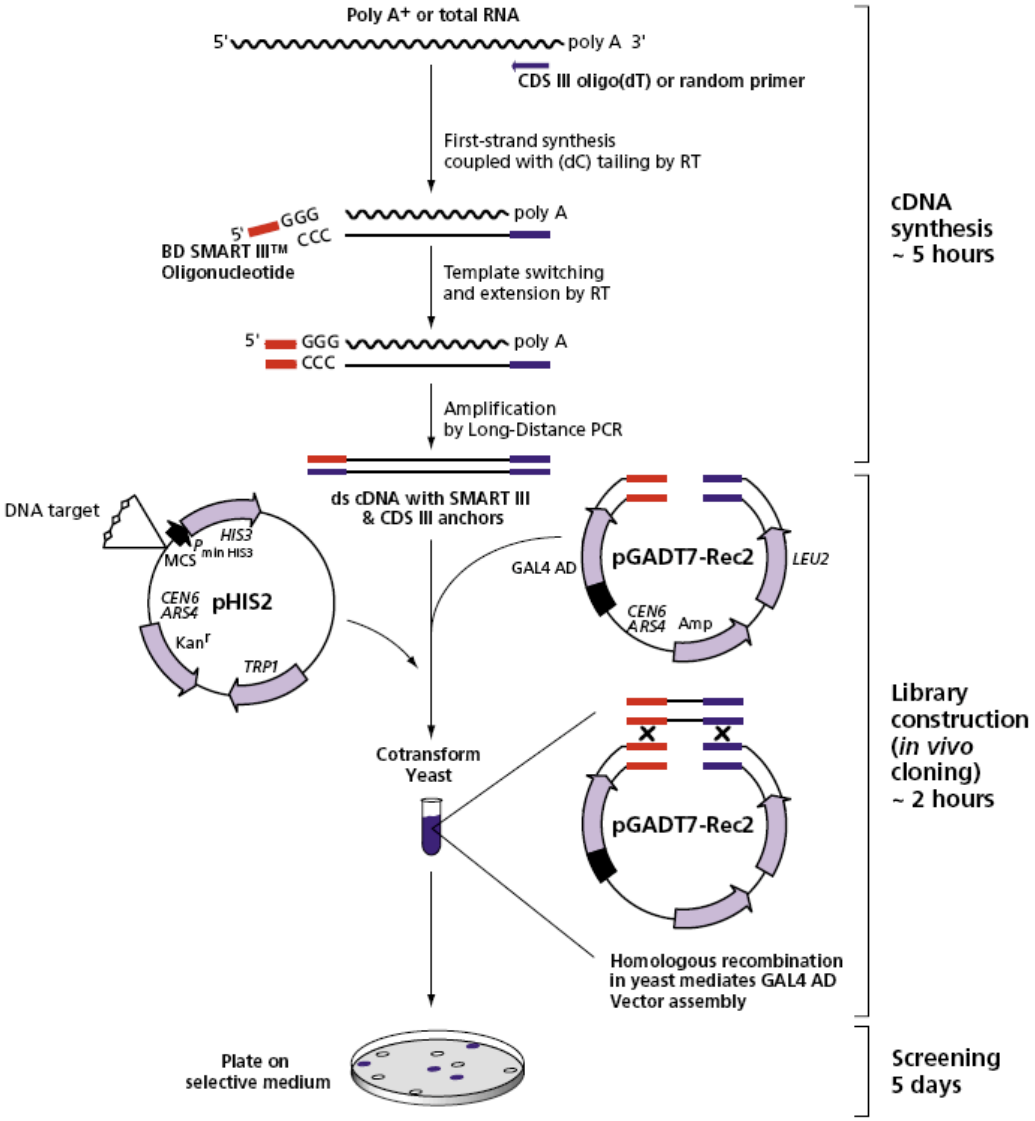


Figure 1. Construct and screen a one-hybrid library in just 7 days. First, a DNA target sequence (the bait) is inserted into pHIS2, the reporter plasmid, which encodes the nutritional marker *HIS3*. Second, a cDNA library is synthesized using the BD SMART reagents provided. Third, yeast strain Y187 is cotransformed with the library, the pHIS2 reporter, and *Sma* I-linearized pGADT7-Rec2. To screen for positive one-hybrid interactions, spread the transformation mixture on selective medium and incubate (1). *In vitro* ligation and bacterial amplification are unnecessary because the cloning takes place in yeast via homologous recombination—another time-saving feature you won't find in any other one-hybrid system.

16. Determine 3D structure of protein using
protein folding software, threading, but ultimately using
X-ray crystallography (1-3 years or more)
Protein Database (PDB)

Protein 3D structure links
http://www.cbi.pku.edu.cn/mirror/GenomeWeb/prot-3-struct.html

Wikipedia, X-ray crystallography
http://en.wikipedia.org/wiki/X-ray_crystallography

17. Determine effect of chemical inhibitors (especially
biochemical enzymes) (weeks or months)
-obtain chemicals from Sigma (St.Louis, Missouri)

C. Other gene/allele hunting strategies:

1. Gene trap/enhancer trap (few months to screen existing resource; 2-3 years to develop resource)

http://www.jic.bbsrc.ac.uk/science/cdb/exotic/

Enhancer Trap (Haseloff Lab)

http://www.plantsci.cam.ac.uk/Haseloff/geneControl/catalogFrame.html

Explanation of enhancer and transposon mutagenesis in Arabidopsis

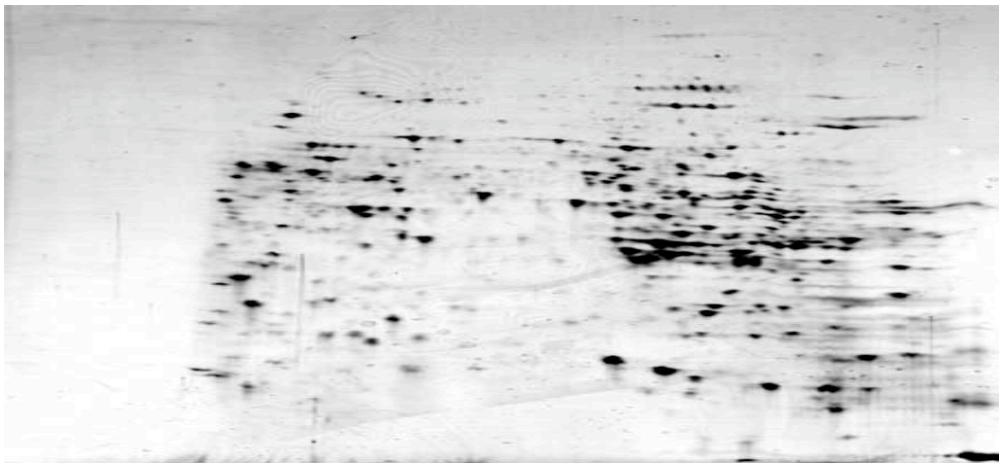
http://www.arabidopsis.org/info/springer.jsp

2. Activation tagging (strong promoter at ends of TDNAs or transposons to randomly overexpress genes, by random insertion) (few months to screen existing resource; 2-3 years to develop resource)

http://pfgweb.gsc.riken.go.jp/pjActl.html

http://www.salk.edu/LABS/pbio-w/acttag.html

3. 2D SDS PAGE gels – then cut out spots, peptide sequencing using mass-spec and identify gene (2-3 years)
2D- gel
http://en.wikipedia.org/wiki/Two-dimensional_gel_electrophoresis
ExPASY Proteomics Tools
http://www.expasy.org/
Mass Spec
http://en.wikipedia.org/wiki/Mass_spectrometry
Mass Spec Simple Tutorial
http://www.bioteach.ubc.ca/MolecularBiology/MassSpectrometry/index.htm



4. TILLING: if wish to find many mutant alleles (point mutations) in a population generated by mutagenesis ---- High throughput mutation detection for SNP analysis; Heteroduplex analysis, indels, conserved genes, wobble base (6mos-1 year to screen an existing population; 3+ years to start from scratch)
TILLING reference
http://www.plantphysiol.org/cgi/content/full/135/2/630