The protein encoded by the *Arabidopsis* homeotic gene *agamous* resembles transcription factors

Martin F. Yanofsky, Hong Ma, John L. Bowman, Gary N. Drews,
Kenneth A. Feldmann & Elliot M. Meyerowitz

Published : Nature, July 1990

Presented by Team Awesome
a.k.a. Simon Bedard & Jason Chen

Plant Development

- The meristem is the region of cell differentiation and extension of the growing shoot.
- As it grows, it sends off leaves, producing junctions referred to as axils. In each axil is a ball of cells that will become a new meristem, starting a new shoot.
- Flower development is the final stage in a shoot's growth, and results in the cessation of its meristematic activity.

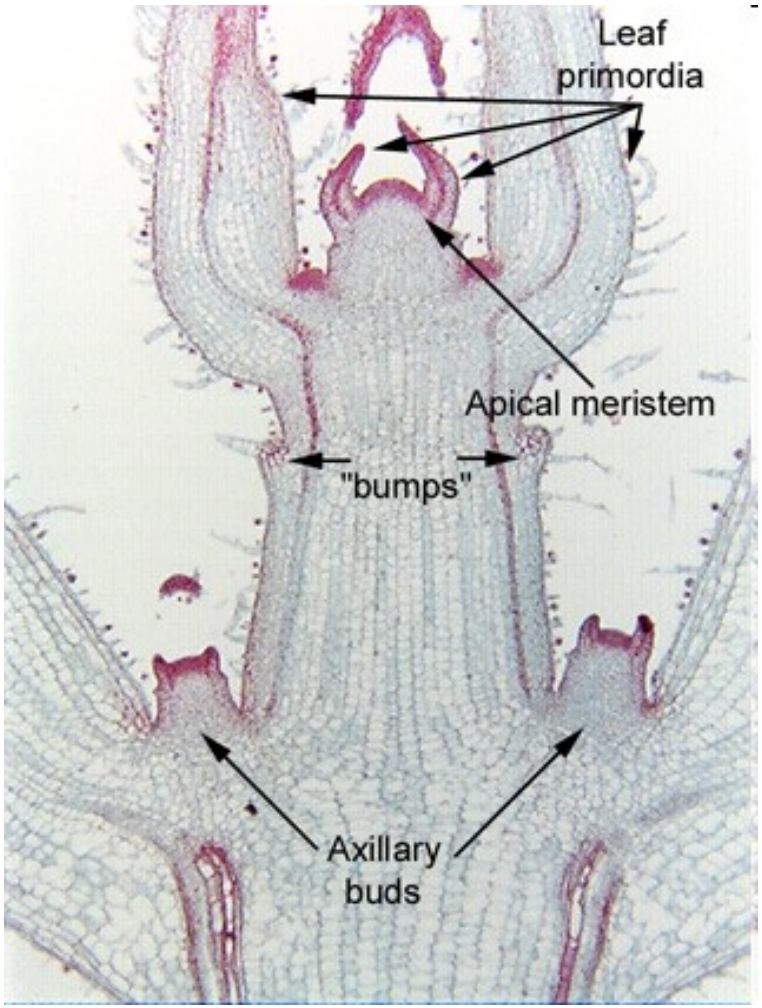


image from: http://www.sbs.utexas.edu/mauseth/weblab/webchap6apmer/6.1-1.htm

Plant Development

- The flower itself is the reproductive organ of the plant
- The basic structures of the flower and the order in which they develop :
    1) Sepals
    2) Petals
    3) Stamens (Androecium)
    4) Carpels (Gynoecium)

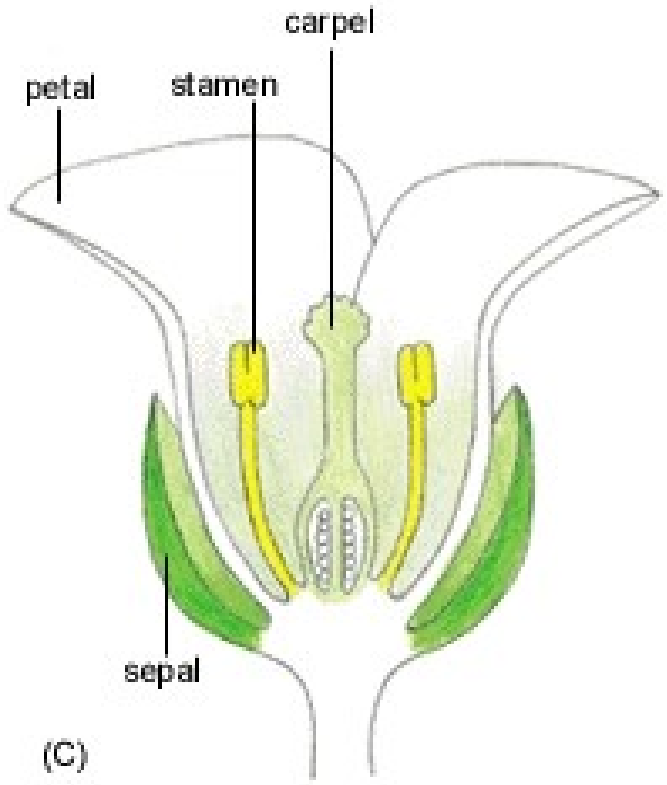
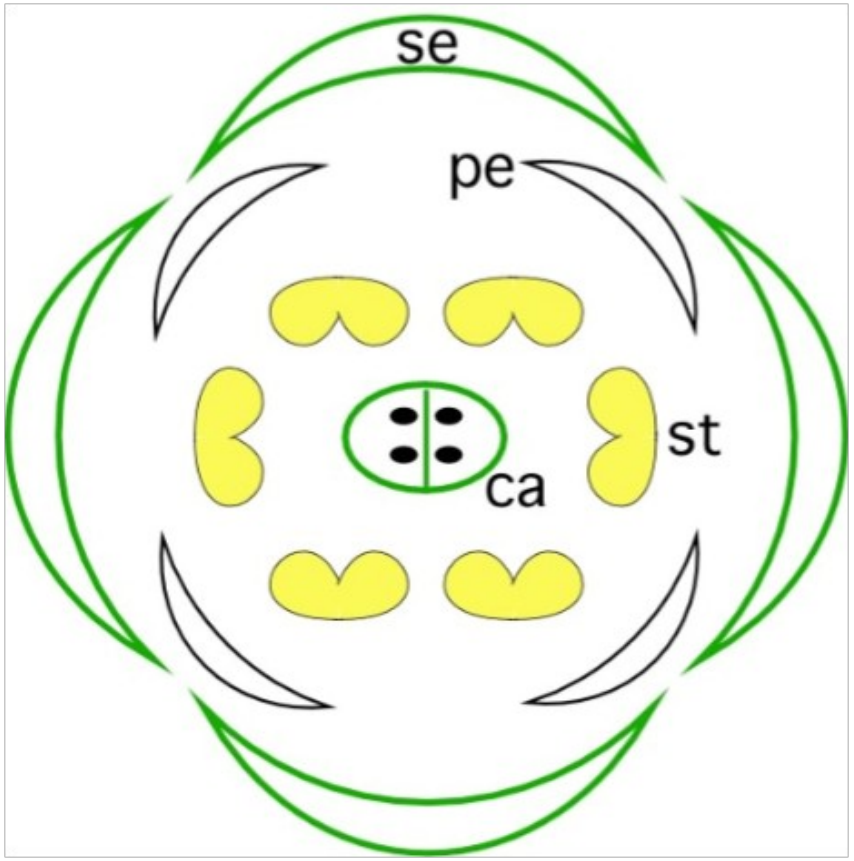


image from http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=cell&part=A5697

Plant Development

- The floral parts may be arranged in whorls, as is the case in *A.thaliana*.
- Wild-type *A.thaliana* has four green sepals (whorl 1), four white petals (whorl 2), six stamens (whorl 3), and two fused carpels (whorl 4)
- The differentiation of the meristematic tissue into these floral structures is regulated by homeotic genes and their products.



Arabidopsis thaliana flower, from above

image from :http://www.biol.sc.edu/~krizek/krizek.html

Arabidopsis thaliana Development

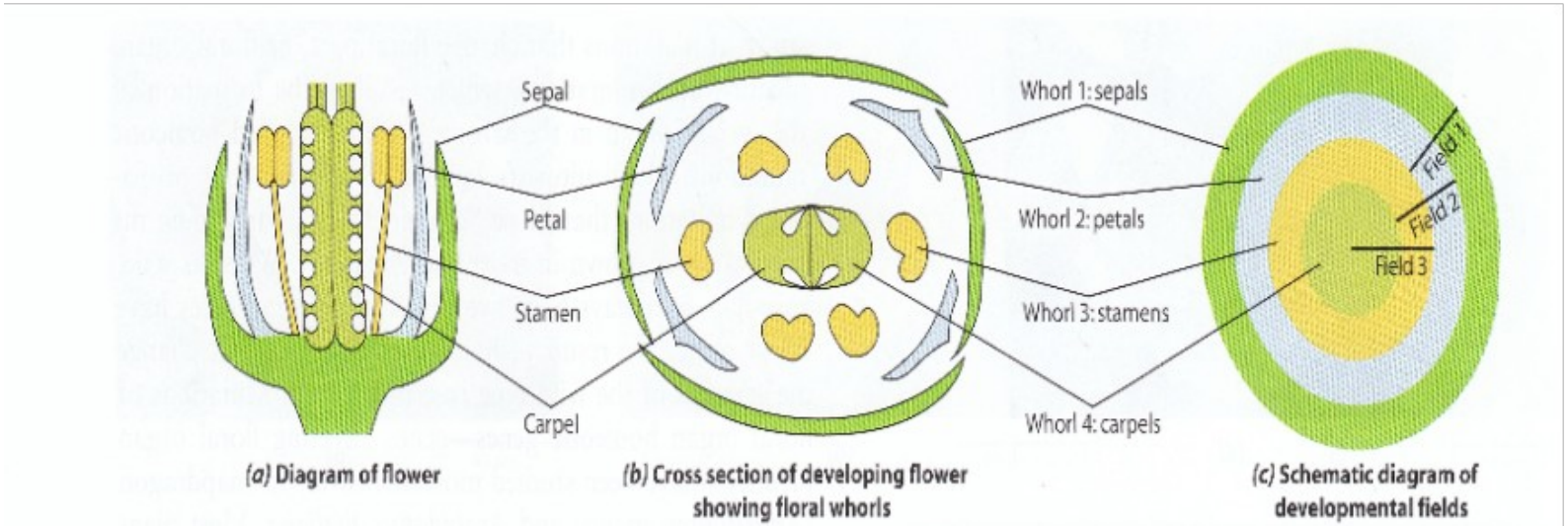
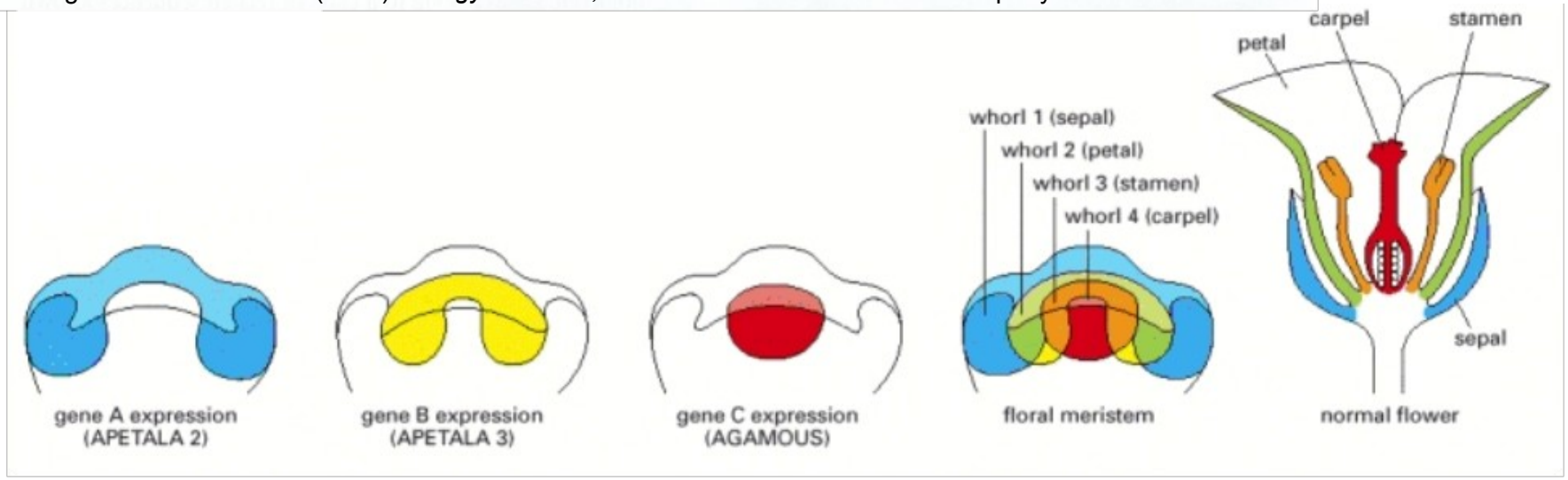
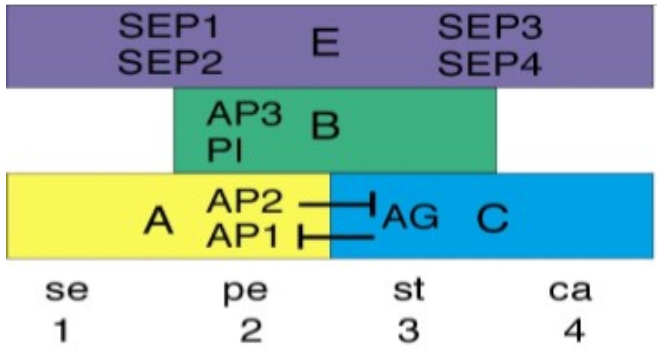


image from: Raven et al. (2005) *Biology of Plants, 7th Ed.*. W.H. Freeman and Company.



Arabidopsis thaliana Development



***apetala2* mutant**
sepals → carpels
petals → stamens

***apetala3* mutant**
petals → sepals
stamens → carpels

***agamous* mutant**
stamens → petals
carpels → floral meristem

Images from:
http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=cell&part=A5697
http://www.biol.sc.edu/~krizek/krizek.html

Define : Homeotic

- Homeosis : the replacement of a part of one segment of an animal by a structure characteristic of another segment.
- Homeotic gene : a gene that affects the proper structural development of an organism, whose mutation may result in homeosis.
- Homeobox : A sequence motif shared by homeotic genes.

Image from: http://science-egroup.xanga.com/

Changes to homeotic genes can cause beautiful flowers to become...

Horribly disfigured *MUTANTS*!!!

Images from:
http://www.picable.com/Nature/Bushes/Alberta-Wild-Rose.872599
http://www.digitalphotoartistry.com/roses/cwdata/Imgp0152.html

Historical context

- Up until this point, although almost a century of work had characterized homeotic genes and developmental pathways in animal models, such work in plants had not been pursued to the same degree.
- With the work of researchers such as Somerville, the relative ease of application and power of mutant analysis as a tool for characterizing metabolic pathways in plants had been clearly demonstrated.
- This work (along with that of others) launched a revolution in plant genetics, with *A.thaliana* emerging as the model organism.

Historical context

- Up until the late ’80’s, gene characterization was done with classic genetic analysis techniques : laborious years of crossing plants, raising progeny, and calculating recombination frequencies to produce genetic maps.

- However, we all know genetic maps are not physical maps. A genetic map cannot narrow down the location of a gene on the physical chromosome, and so the leap from identification to sequence was extremely difficult.

Why *AGAMOUS*, why now?

- This research was carried out in 1990, when little was known about the specific molecular mechanisms involved in regulating the complex process of flower development.

- Several mutations affecting floral morphogenesis, such as *apetala2* had been identified examined using genetic analysis, but had not yet been fully characterized at the molecular level.

- *Agamous* mutants had been reported in the scientific literature for over a century, and had also been genetically characterized and mapped, but had not been characterized at the molecular level. i.e. sequence was unknown.

Why *AGAMOUS*, why now?

- Around this time, a fellow named Feldman figured out that the *WS* ecotype of *A.thaliana* was better able to endure the process of transfection with *Agrobacterium tumefaciens* than some other ecotypes.

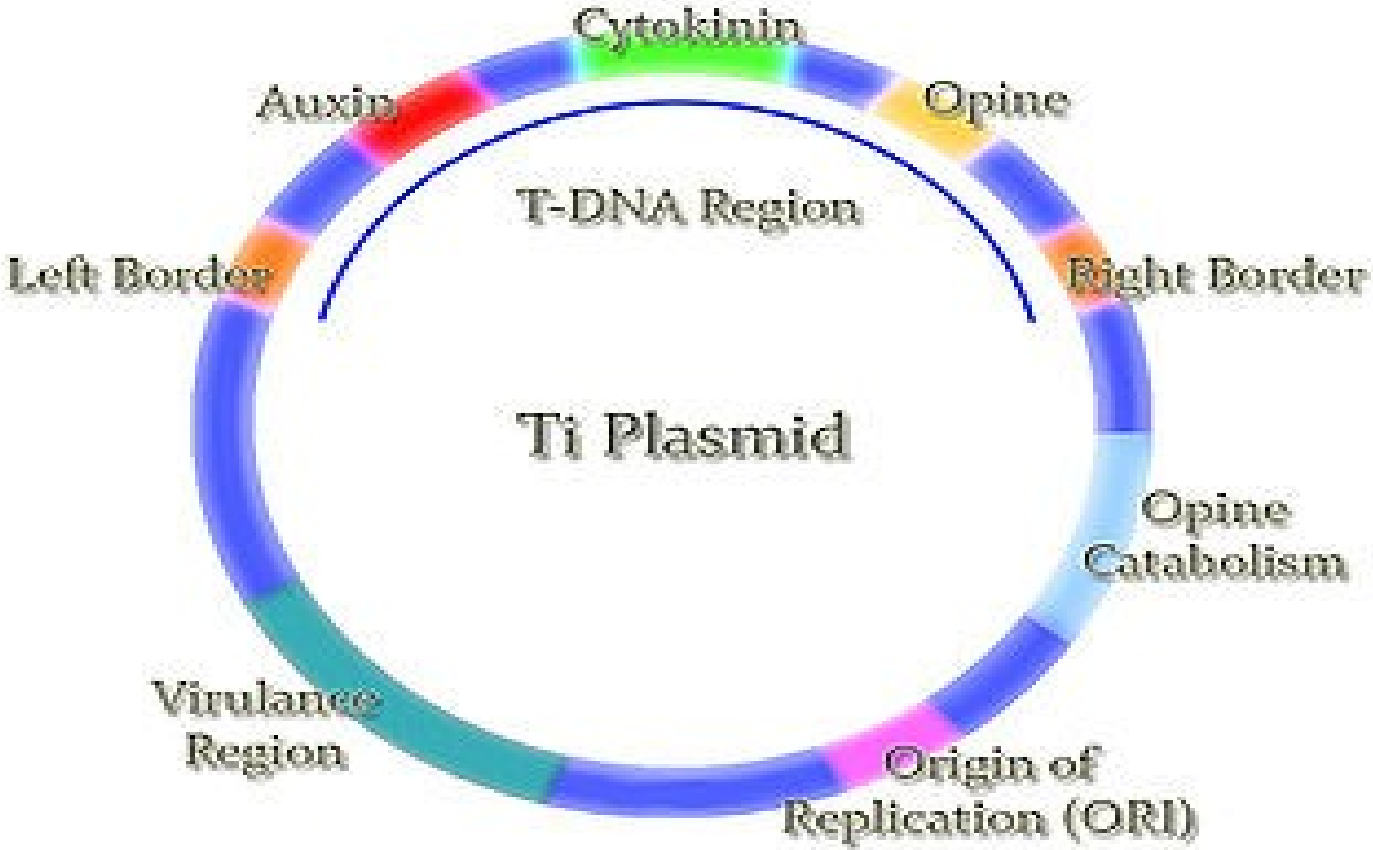
  - He was the only one who could do it (this was before the whole “dipping” method) and had recently created a population of T-DNA insertion lines.

  - By chance, one of these T-DNA insertion lines had a phenotype resembling *ag-1*, the EMS-mutagenized line that had originally been used to characterize the *AG* gene. This T-DNA insertion line was named *ag-2*...

Insertion mutant of agamous

- Studied using T-DNA insertion
- T-DNA mutagenesis carried out by Kenneth A. Feldmann created a stock of mutant plants

T-DNA Insertion



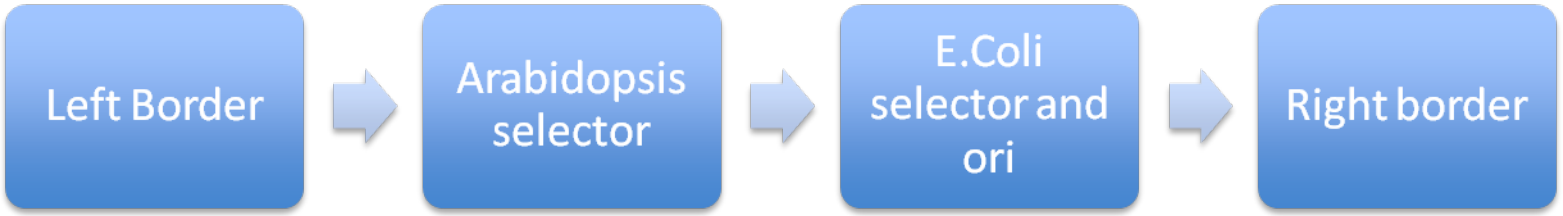
http://upload.wikimedia.org/wikipedia/commons/thumb/8/89/Ti_Plasmid.jpg/350px-Ti_Plasmid.jpg

Question

- What are the necessary elements that the binary vector MUST have?
- Left and right border
  - So that the bacterial proteins can move the T-element
- Origin of replication
  - So that the plasmid can replicate in the bacteria

T-DNA

- Contains TWO selector genes. One for the plant and one for Ecoli.



T-DNA

- In the T-DNA infected stock a mutant phenotype found:
  - Lack of stamens and carpels
  - Flower within a flower

- Showed a difference with original ag-1 in length of greater separation of internal flowers due to the pedicels of inner flowers elongated.
- Hypothesis: The T-DNA is inserted in the partially characterized *agamous* gene

Proving it is the *agamous* gene

- What are the 3 things we need to do to show about the TDNA insert before moving forward:

- Heritable
- Single gene
- Dominant/Co Dominant/Recessive

Proving it is the *agamous* gene

Methods for Genetic analysis

- Ways we learned in class:

- Cross with wild type to determine if it's a dominant or recessive
  - Self progeny and look at ratios for gene number
- Cross with known mutant to show complementation

Proving it is the *agamous* gene

Complementation like test

- Crossed heterozygote mutant to heterozygote ag-1
- Produced ¼ ag mutant flowers

Questions

- **Why did they cross heterozygotes and not homozygotes?**
- Because homozygotes are sterile
- **Why are they sterile?**
- Because they lack reproductive organs!
- **What does the ¼ ag mutant result show about the relation of the 2 genes?**
- They are allelic

Proving it is the *agamous* gene

Have we proved:

- Heritable
  - Yes 1/4th from the heterozygous were homozygous showing that the phenotype is inherited
- Single gene
  - Not exactly but we know it's shares a single gene mutation with ag-1 (allelic)
- Dominant/Co Dominant/Recessive
  - Showed to be recessive from the heterozygous cross (1/4th mutants)

Proving it is the *agamous* gene

- Also did co segregation test with the kanamycin resistance that was located on the T-DNA insert (data not shown)
- Kanamycin resistance co segregates with the mutant phenotype
- Shows that the insert is in or very close to the agamous gene

Proving it is the *agamous* gene

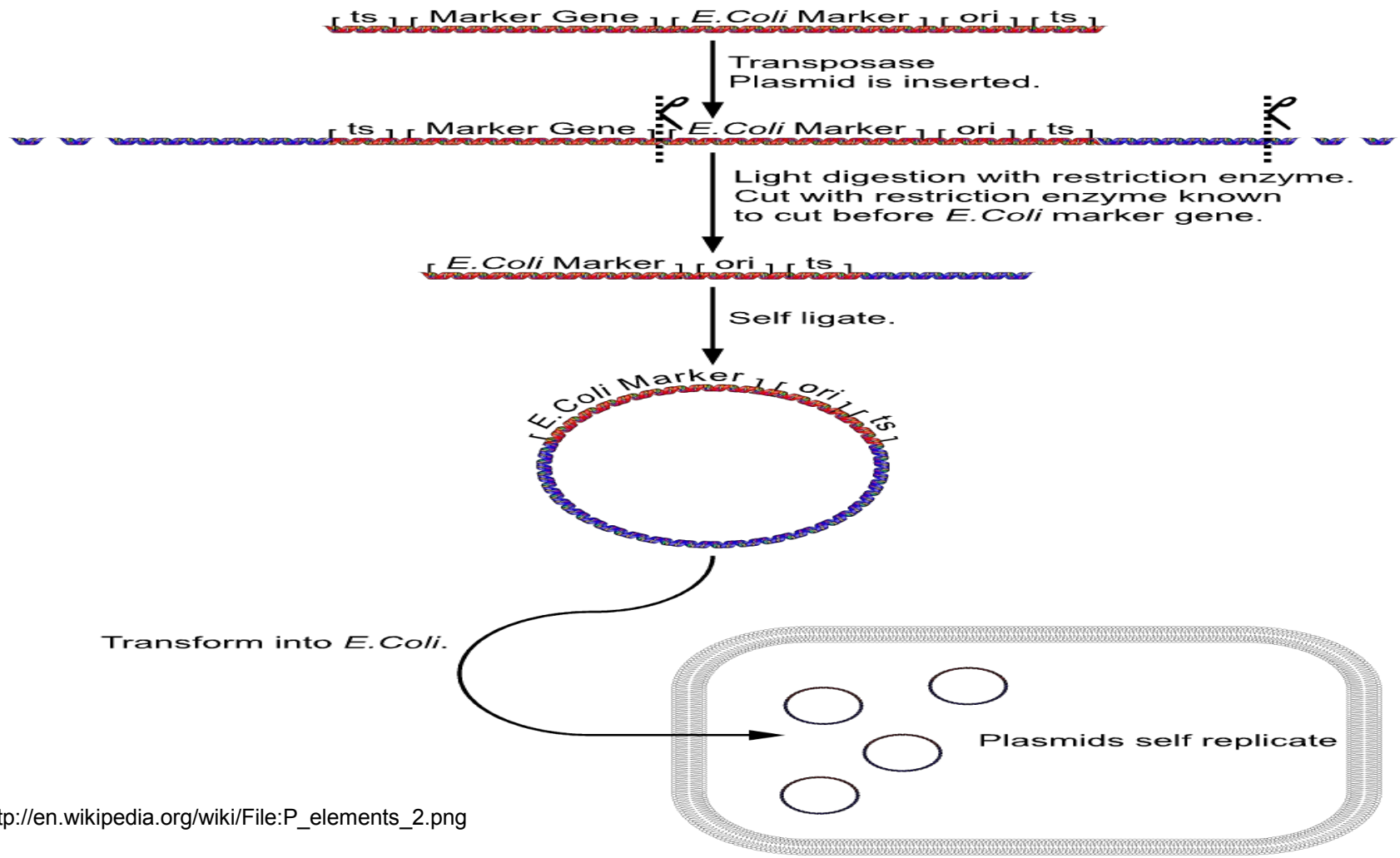
Evidence of T-DNA insertion

- What evidence is there that the T-DNA was inserted into the *agamous* gene?
- Heritable
- Allelic
- Kanamycin resistance co segregates with *agamous* phenotype

Plasmid rescue

- After invading enemy territory how do we get our T-DNA insert back?
- PLASMID RESCUE!
- LEAVE NO MAN BEHIND

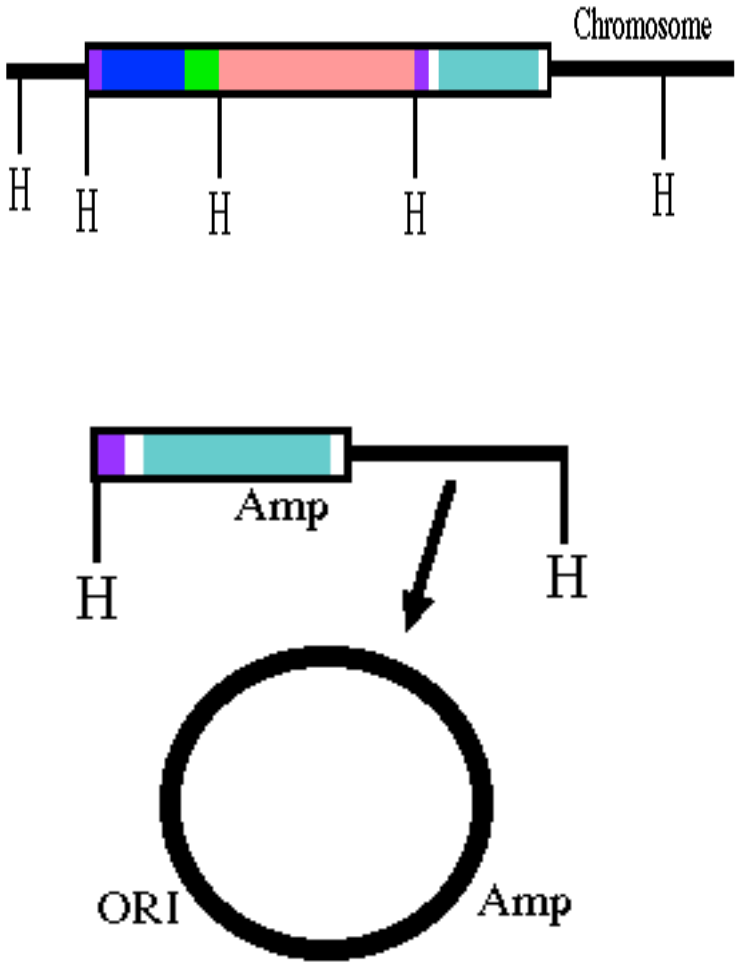
Plasmid rescue



http://en.wikipedia.org/wiki/File:P_elements_2.png

Plasmid Rescue Method

The purpose of plasmid rescue is to isolate the chromosomal DNA adjacent to an inserted piece of DNA. There are 4 steps to this process.



1) At the beginning, a piece of DNA has inserted into the chromosome
2) Isolate the genomic DNA (gDNA) and digest it with a restriction enzyme that will cut at the edge of the plasmis protion of the insert. In this example, you would use Hind III.
3) Clean and concentrate the DNA, and do a ligation on the digested gDNA

4) Transfrom the ligation into E. coli and look for colonies. Any cells that survive antibiotic selection must have some flanking chromosomal DNA in the plasmid.

http://www.bio.davidson.edu/Courses/Molbio/plasres/plasres.html

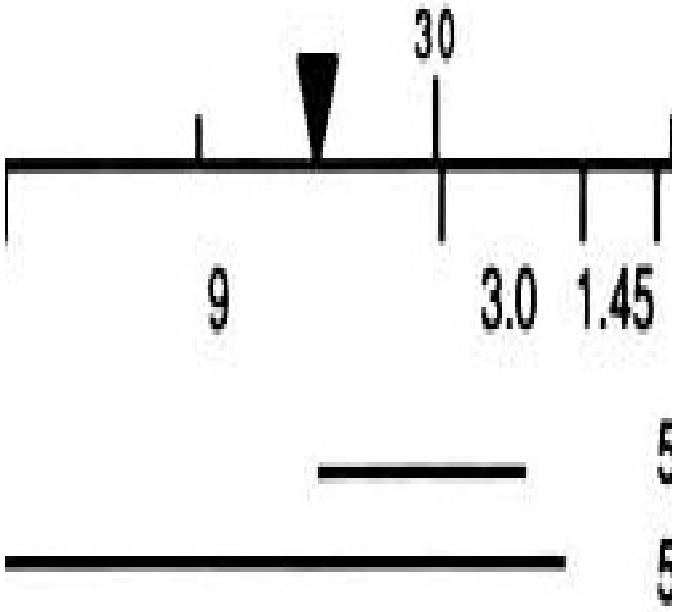
Proving it is the *agamous* gene

- Now that we have saved the plasmid how do we know it is the *agamous* gene?
- We need to compare the sequence of our recovered section and map it along the chromosome

Proving it is the *agamous* gene

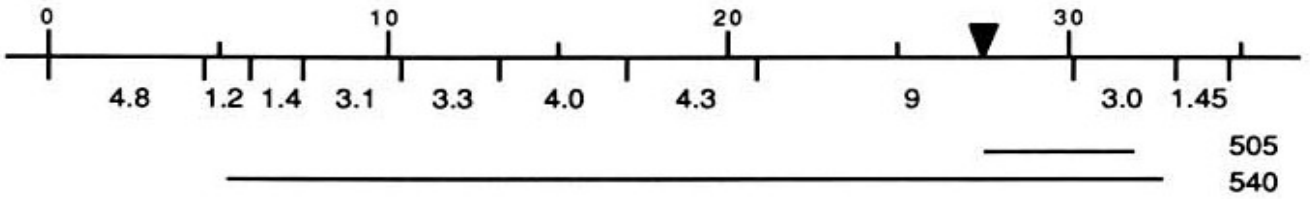
RFLP analysis

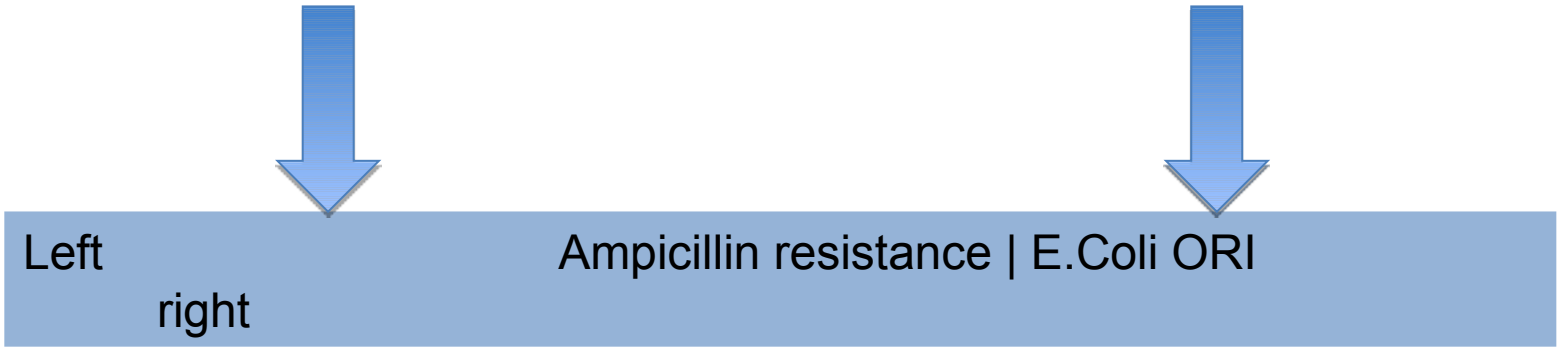
- Using restriction fragment length polymorphism one can map genes
- When cut with restriction enzymes the cut patterns that result on a gel can be used to map pCIT505



Question

Based on the results and the mapping of the plamid which hypothetical cut site did they use?

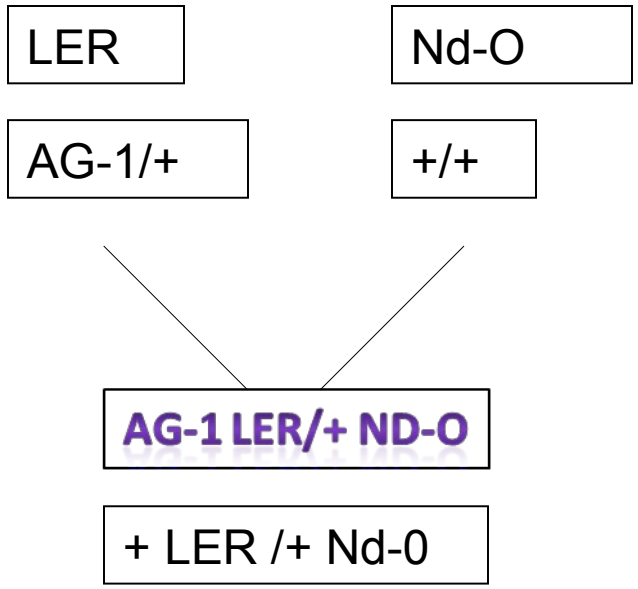




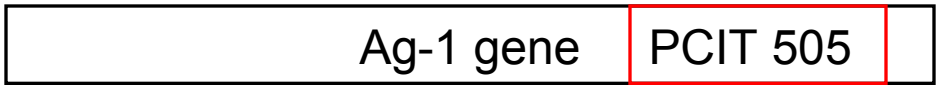
Proving it is the *agamous* gene

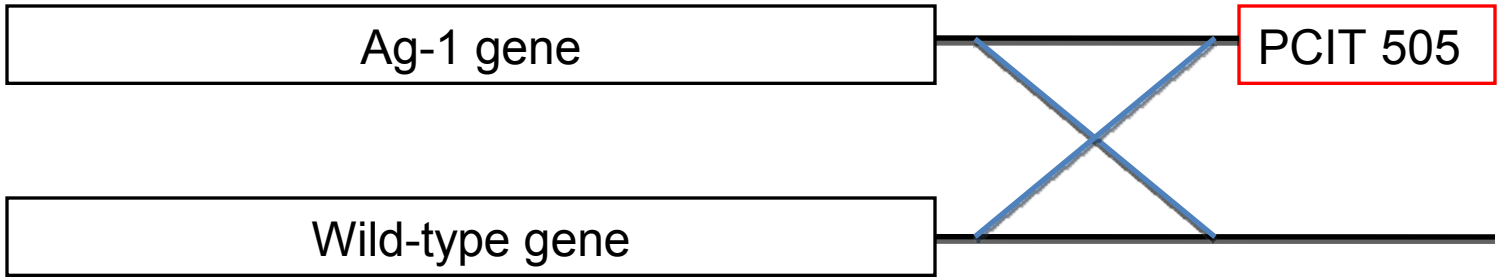
RFLP analysis

- They now know the region that the rescued plasmid spanned
- Next they need to make sure that the T-DNA inserted in the ag gene
- Could be inserted in another gene
  - Multiple copy from tandem duplication
  - homologue



Co segregation analysis of pCIT505 linked polymorphism and the ag-1 gene





Proving it is the *agamous* gene

- NO crossovers observed in 118 meitoic products scored leading to the conclusion that the cloned section and the *agamous* gene are less than 2 centimorgans apart
- So the polymorphism associated with the pCIT505 plasmid co segregate with the mutant phenotype

Getting the full gene

- Now that part of the gene is isolated it can be used to probe for the whole gene
- Using a southern blot and a cosmid library

Getting the full gene

Cosmid library

- Entire genome is cut and placed into vectors
- Each cosmid may contain part, whole or multiple genes
- Cosmids are able to contain 37 – 52 kb of DNA while normal plasmids can only carry about 1-20kb

- Several cosmids were obtained
  - One of which was called pCIT540

Getting the full gene

Restoration of wildtype phenotype

- The cosmids that were isolated were then introduced into a homozygous ag-2 plant
- What are the expected results?
- Some with the mutant phenotype still
- A few with wild-type phenotype

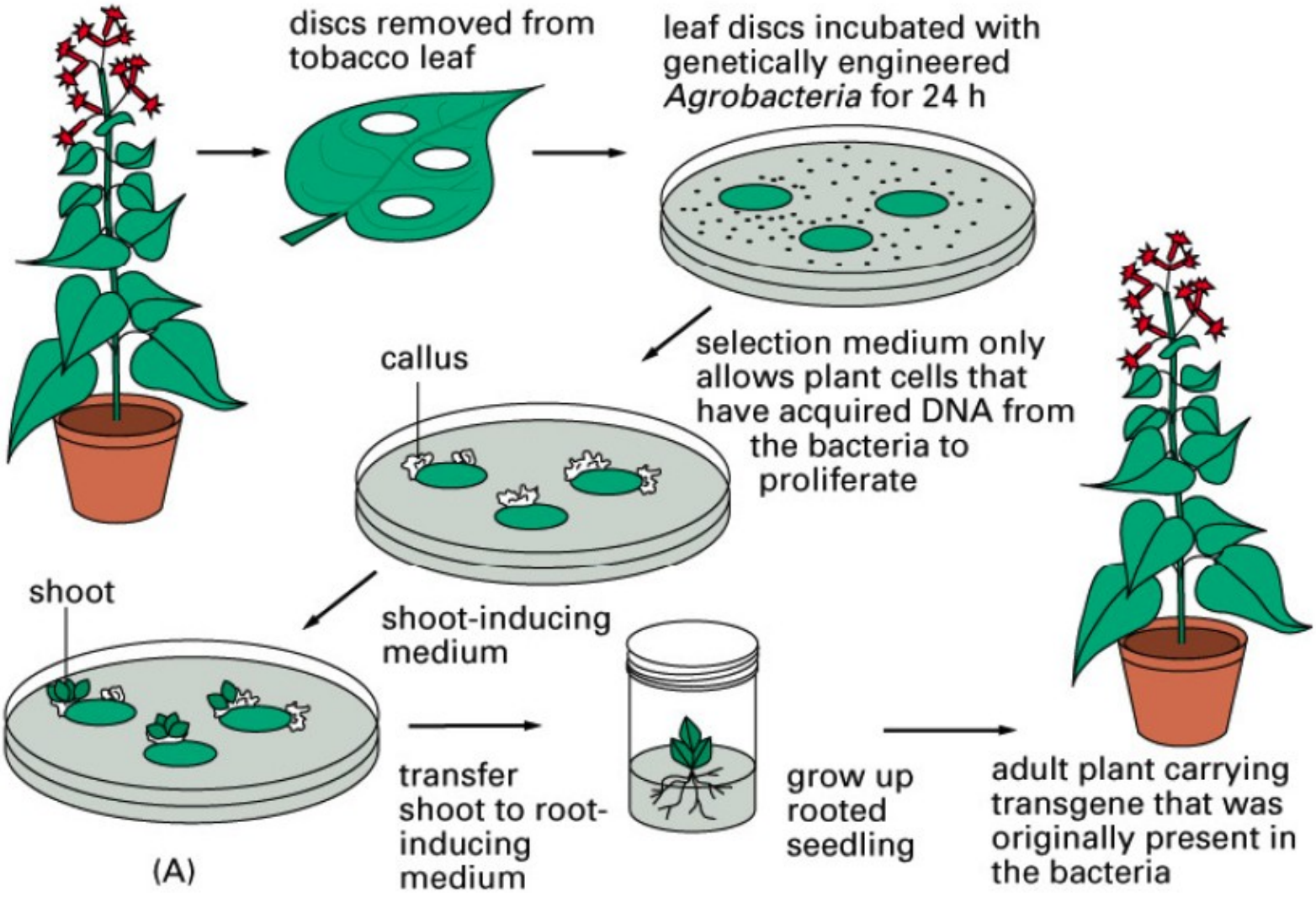


Figure 8–72 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

Getting the full gene

- pCIT540 is placed into a T-DNA vector
- Samples of mutant ag-2/ag-2 are infected with the T-DNA insert
- Shoots are selected for based on the E.Coli marker and grown

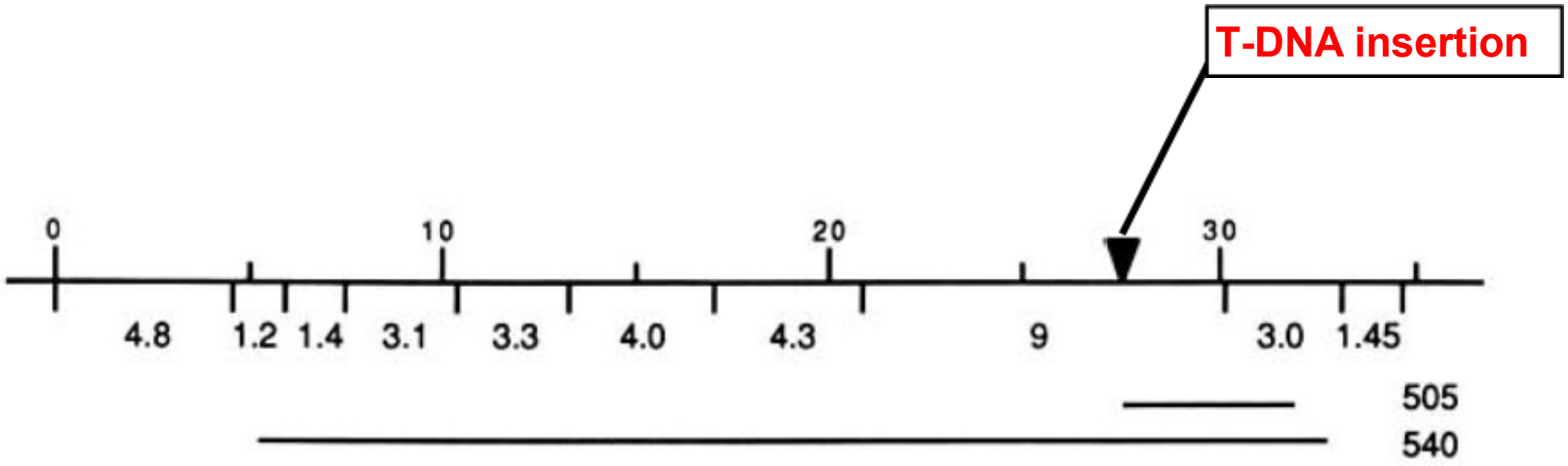
Getting the full gene

pCIT540

- When introduced into homozygote mutants wildtype phenotype was restored
- Means that the pCIT540 contained a working copy of the *agamous* gene

Nucleotide analysis

- All right, we have the gene! Now what?
- How do we find the sequence?
- What information do we have?



From T-DNA to cDNA

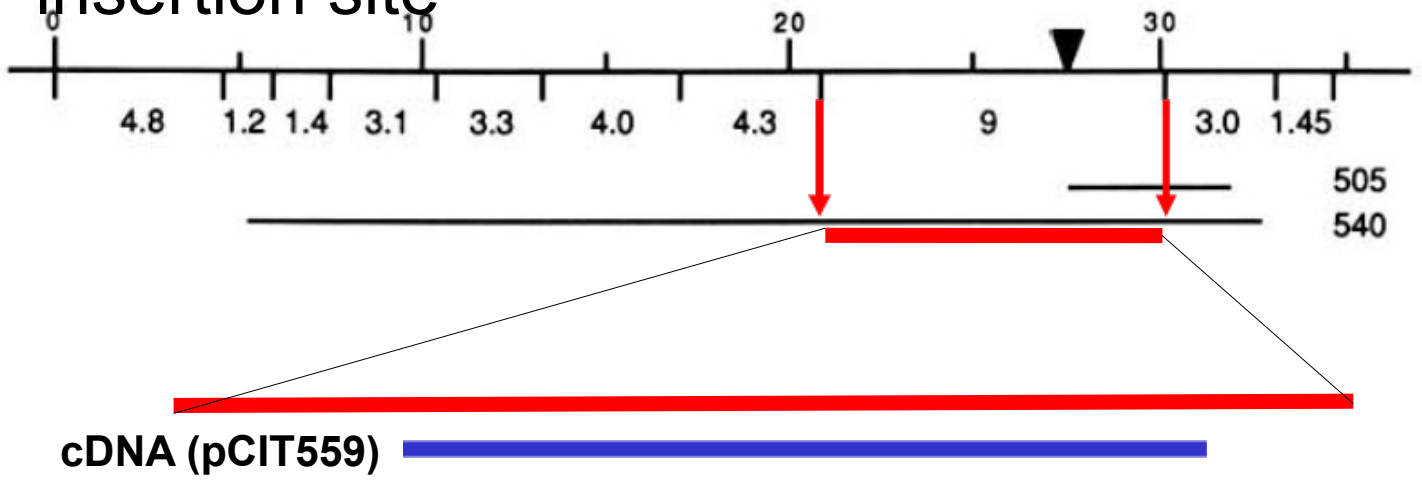
- Yanofsky et al. had a cDNA library from their previous research

- What ecotype would this library have been?
  - *Landsberg erecta*

- What ecotype was the T-DNA insertion line *ag-2* from?
  - WS

From T-DNA to cDNA

- They probed the cDNA library using a restriction fragment that spanned the T-DNA insertion site

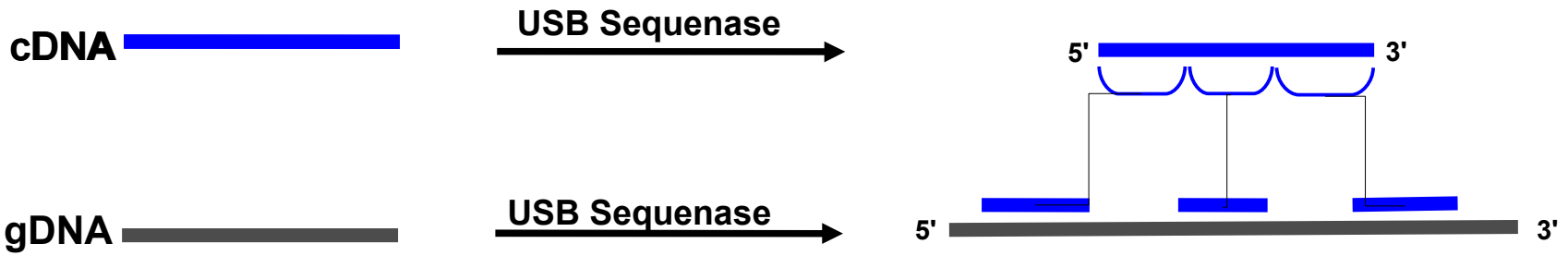


- Only one class of cDNA clones had sequences complementary to the section of the genome that flanked the insertion site.

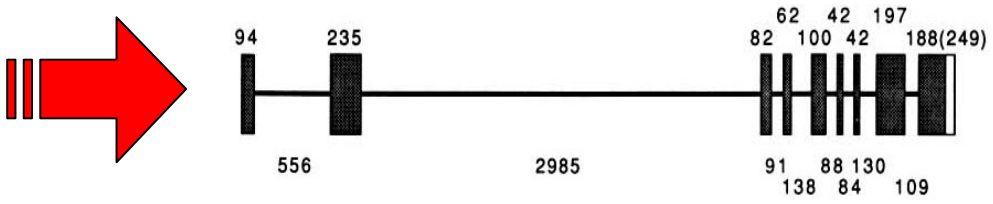
From cDNA to gDNA to the gene

- They now know what the mature mRNA transcript of the gene looks like, and have isolated its position on the chromosome to within a few kb!

- Time to get sequencing...

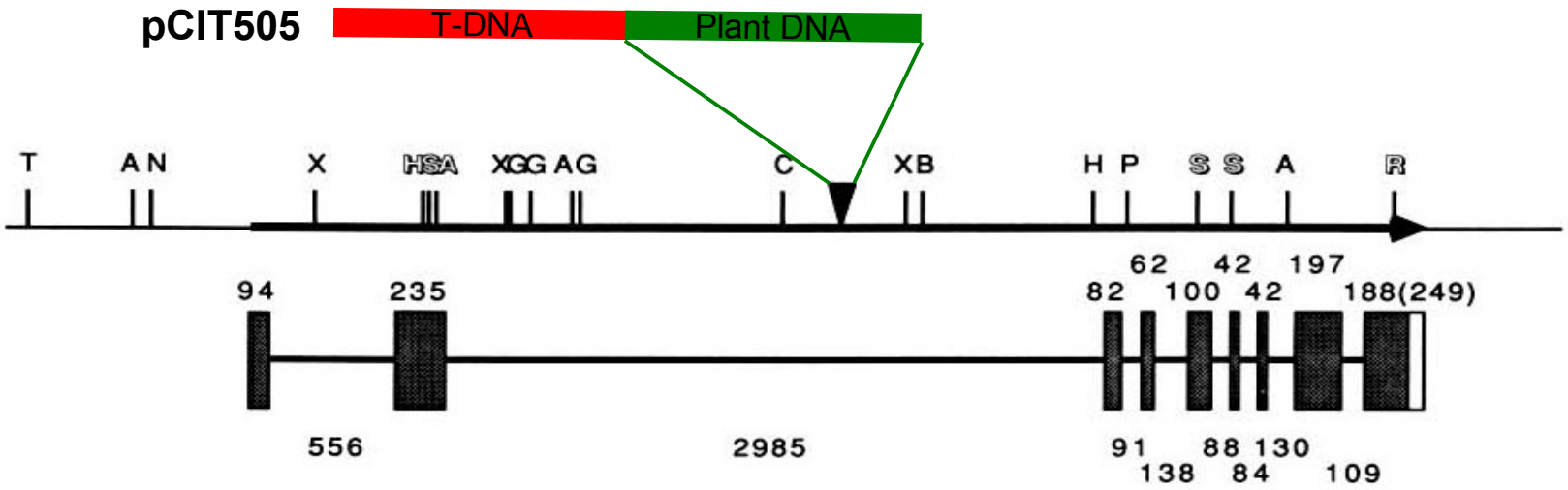


- ...and then they can draw a map of the gene!!!!



Locating the T-DNA insert

- Since they now had the complete sequence of the gene, it was time to get a sequence for the plant DNA in pCIT505 and match it up to the gene, showing that this was the gene that the *ag-2* mutant's T-DNA insert had landed in.



Confirming the identity of the gene

- They had to confirm the identity of the gene that had been cloned and sequenced as being *AG*. Why?
- Up until now, they had only shown that the T-DNA insert had landed within 2 cM of *AG*. They needed to show explicitly that the cDNA clone they isolated was complementary to the mRNA produced by *AG*, by showing that the T-DNA insert had landed within *AG*.
- If the T-DNA insert had indeed landed in *AG*, then it would be expected that the sequence of the T-DNA tagged gene would be the same as previously isolated Ag mutants.
- Sequence analysis of the appropriate fragments of the *ag-1* mutant showed two things:

  1) The cDNA library had indeed identified *AG*. (Yay!)
  2) The specific mutation that had occured in *ag-1* to produce the mutant phenotype. (Bonus!)

Hang on a second...

- From sequence analysis, they noticed that the deduced open reading frame of the gene extended beyond the scope of the largest cDNA clone isolated!

- The difference between the longest cDNA and the polyadenylated mRNA was only ~50bp... pretty close, but they were not happy...

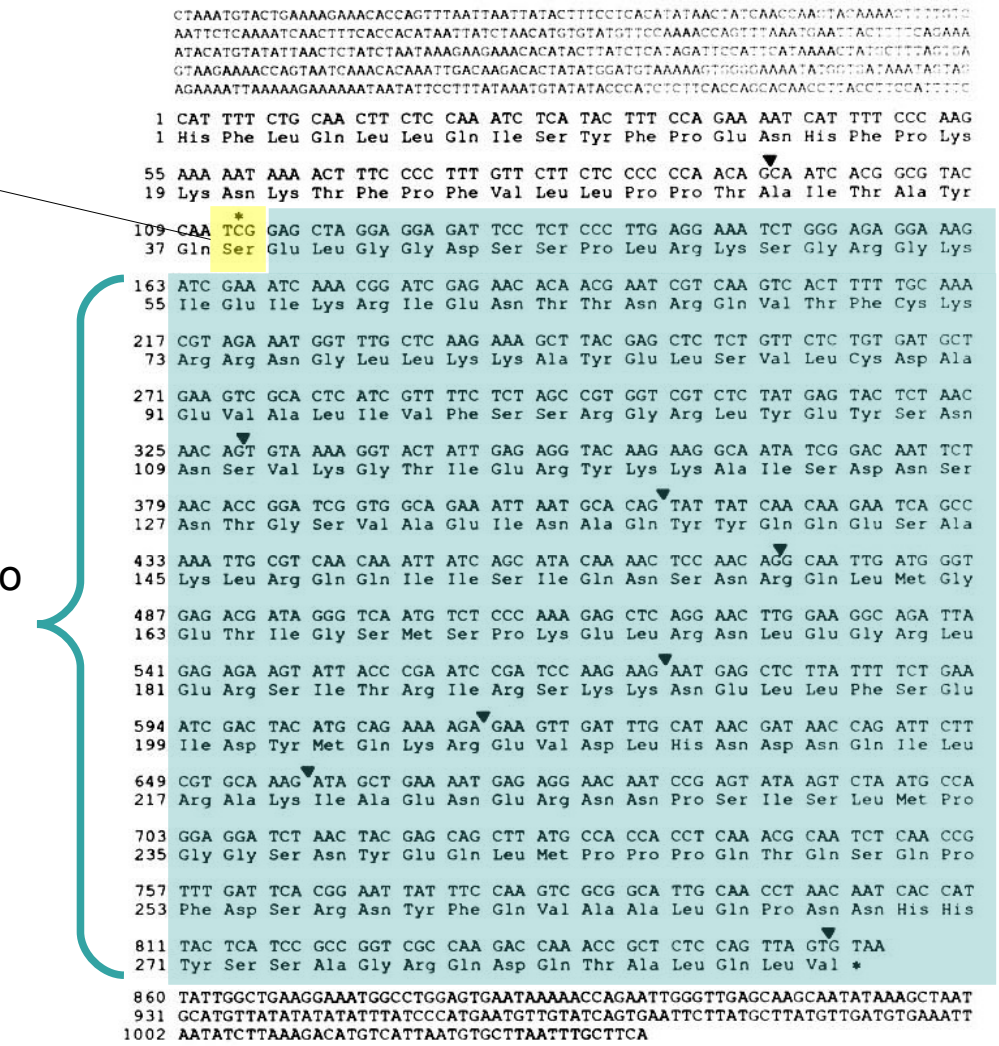
- They tried several approaches to getting a longer cDNA, including creating another library! Nothing worked.

- They eventually had to get moving forward, as they were racing to publish before they got scooped. They rationalized this with the fact that *AG* homologues in *Brassica napus* seemed to indicate that all the important parts of the AG protein were present.

Hang on a second...

Position of start codon in two *AG* homologues in *B.napus*

Region of evolutionarily conserved amino acid sequence between *A.thaliana* and *B.napus*.



How could they possibly have known...?

- As it turned out, their cDNA clones WERE full length!!
- Any ideas???

- Subsequent work by another group of researchers showed that *AGAMOUS* was one of a very few genes that may initiate translation at a **non-AUG** codon!!! Whaaaat????

Don't believe me? Check it out for yourself:

MOLECULAR AND CELLULAR BIOLOGY, Dec. 1999, p. 8505–8512 Vol. 19, No. 99
0270-7306/99/$04.00+0
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Non-AUG Initiation of *AGAMOUS* mRNA Translation in *Arabidopsis thaliana*

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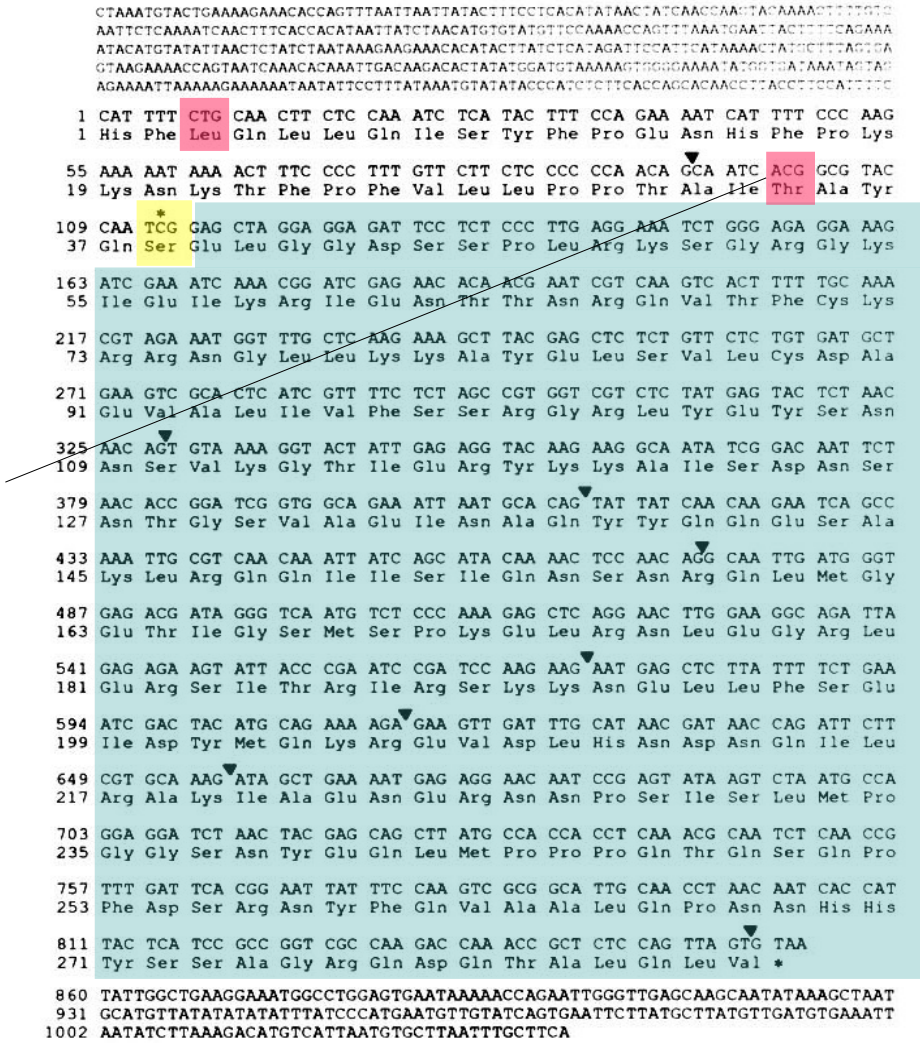
Received 11 June 1999/Returned for modification 3 August 1999/Accepted 18 August 1999

How could they possibly have known...?

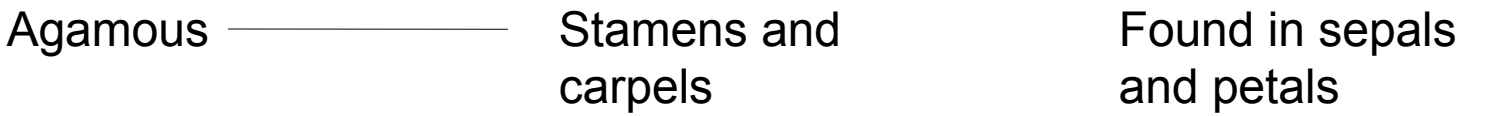
CUG and ACG are both non-AUG start codons that are present in *AG.*

As it turns out, the latter is the start codon.

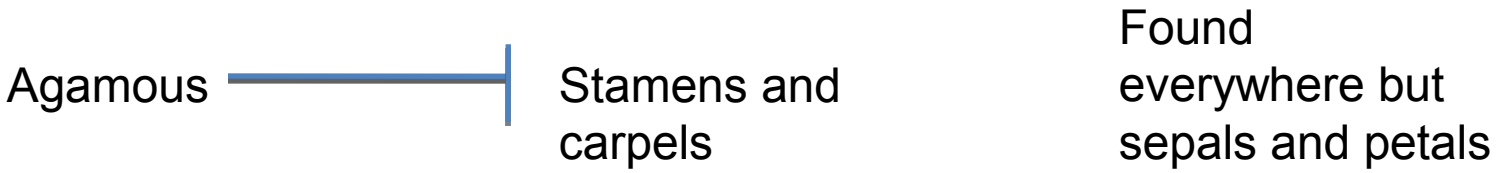


RNA Analysis

- Study gene expression in the plant
- Positive regulator

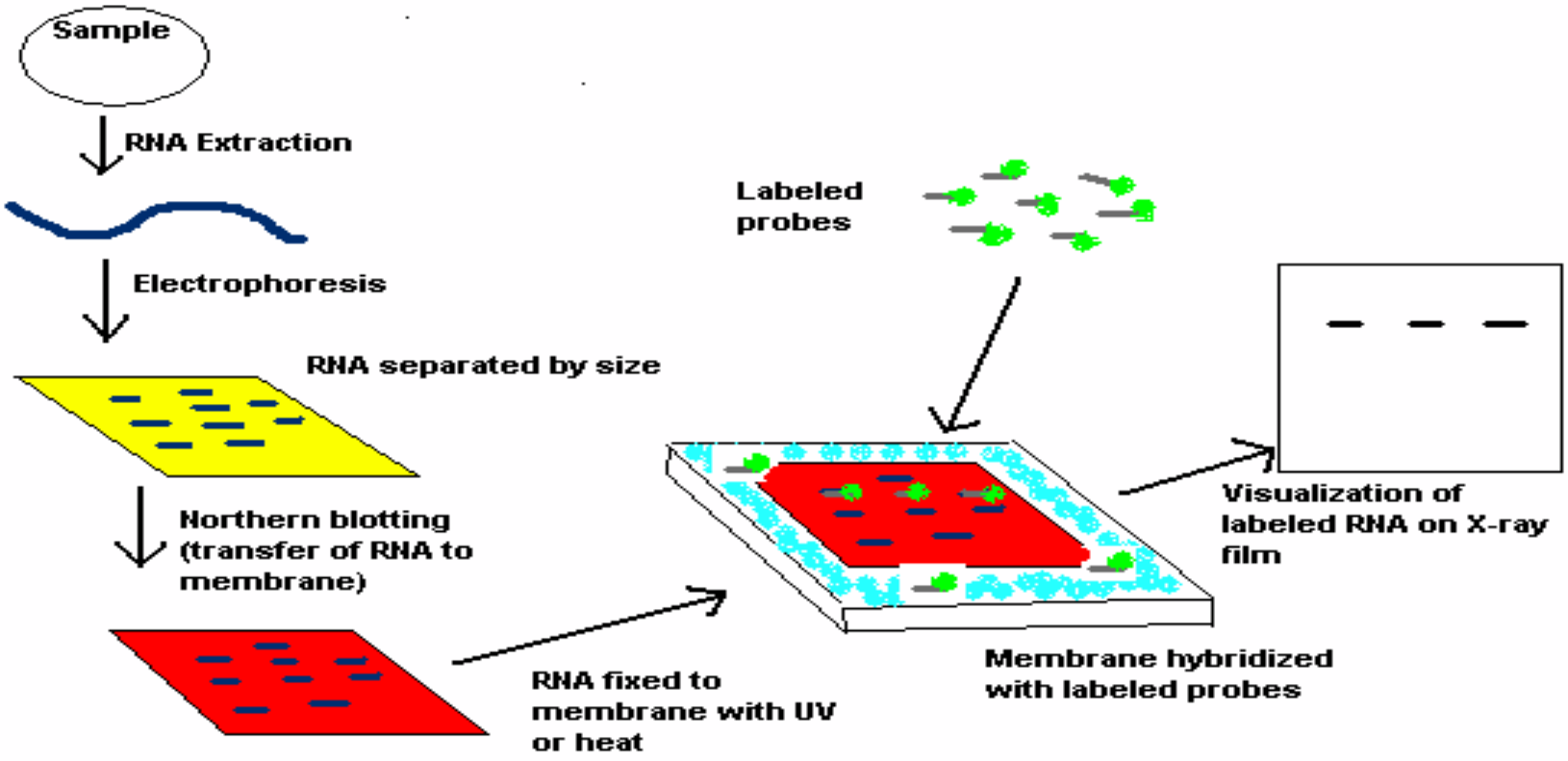


- Negative regulator



RNA Analysis

Northern Blot

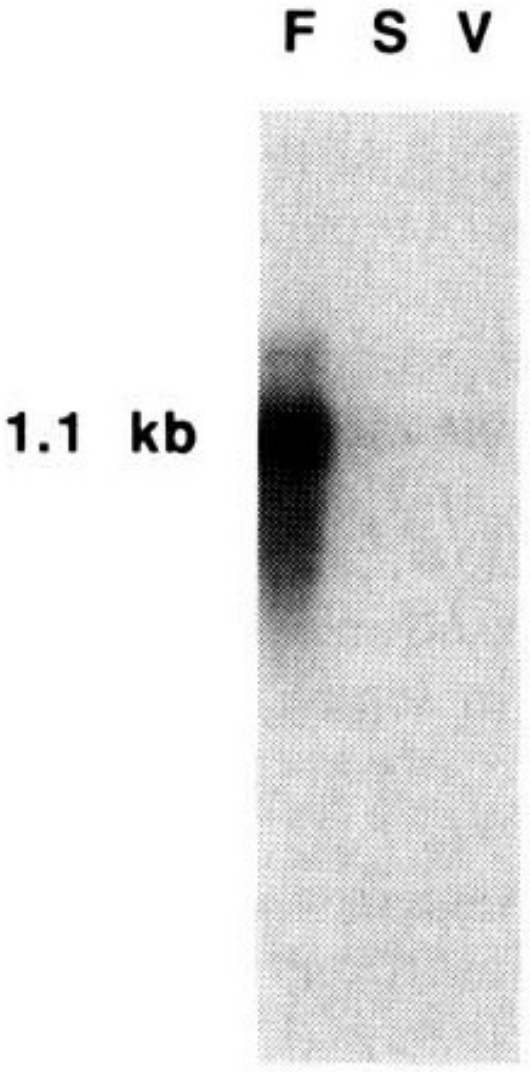


RNA Analysis

- Northern Blot of Poly A RNA isolated from different tissues of the cell
- From the results we can see where the *agamous* gene is transcribed and where it is not

RNA Analysis

- An RNA from flowers (length ~ 1.1 kb) hybridized with the AG cDNA probe (lane F)
- After much longer exposure similar band found in inflorescence stems and whole plants



RNA Analysis

Northern Blot

- Isolated RNA from
  - Floral parts,
  - Inflorescence stem
  - Whole plants (with small flowers)

RNA Analysis

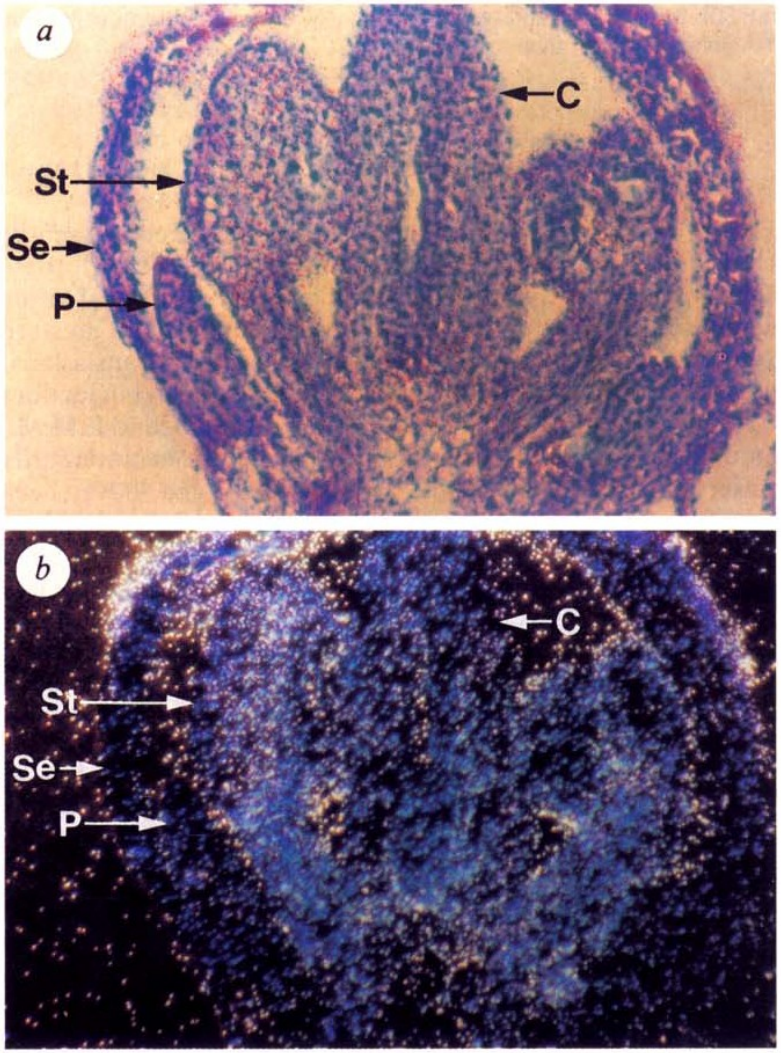
- Northern Blot shows that agamous expression is specific to the flowers
- Which parts of the flowers?

RNA Analysis

In Situ Hybridization

- Hybridization of labelled radioactive antisense RNA so to be seen in tissue
- Used to show tissue specific RNA expression

RNA Analysis



- a. diagram of the floral organs
- b. Radioactive label
  - Stamens and carpels show RNA hybridization
- NOTE: signal above sepal is seen in sense and antisense RNA

RNA Analysis

Organ specific expression

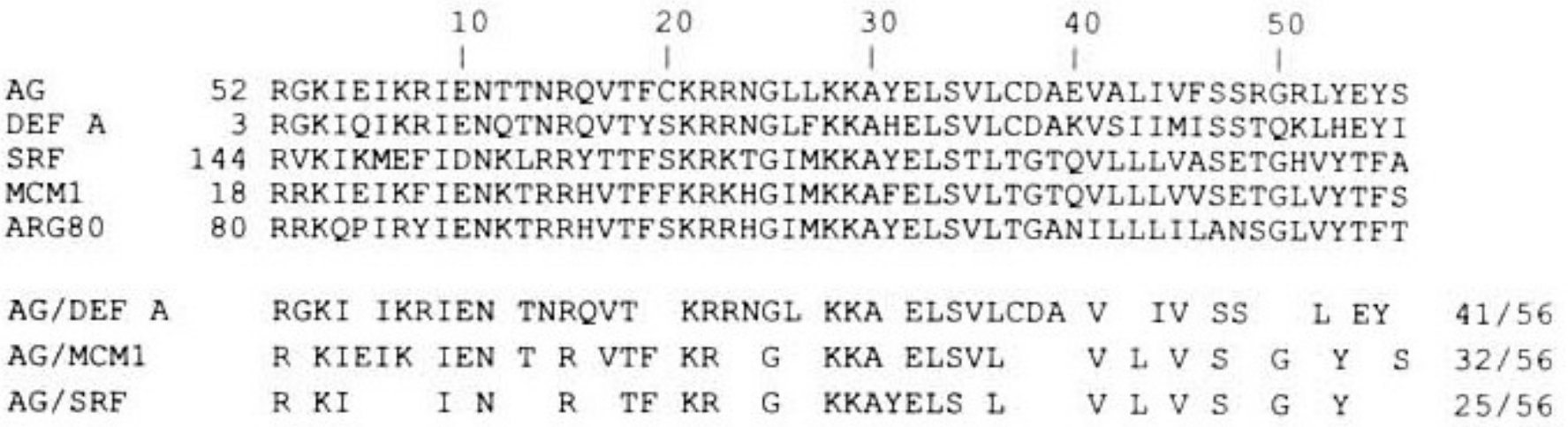
- Organ specific analysis showed it was expressed in stamens and carpels exclusively
- Shows positive regulation of stamen and carpel developement

Discussion – Gene similarity

Gene Similarity

- Striking sequence similarity to transcription factors
  - Human SRF
  - Yeast MCM1 and ARG80
- Protein similarity tt def A homeotic gene in plant *Antirrhinum majus*

Discussion – Gene similarity



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                        10        20        30        40        50
                        |         |         |         |         |
AG          52 RGKIEIKRIENTTNRQVTFCKRRNGLLKKAYELSVLCDAEVALIVFSSRGRLYEYS
DEF A        3 RGKIQIKRIENQTNRQVTYSKRRNGLFKKAHELSVLCDAKVSIIMISSTQKLHEYI
SRF        144 RVKIKMEFIDNKLRRYTTFSKRKTGIMKKAYELSTLTGTQVLLLVASETGHVYTFA
MCM1        18 RRKIEIKFIENKTRRHVTFFKRKHGIMKKAFELSVLTGTQVLLLVVSETGLVYTFS
ARG80       80 RRKQPIRYIENKTRRHVTFSKRRHGIMKKAYELSVLTGANILLLILANSGLVYTFT

AG/DEF A      RGKI IKRIEN TNRQVT  KRRNGL KKA ELSVLCDA V  IV SS   L EY   41/56
AG/MCM1       R KIEIK IEN T R VTF KR  G  KKA ELSVL    V L V S  G  Y  S  32/56
AG/SRF        R KI    I N   R  TF KR  G  KKAYELS L    V L V S  G  Y     25/56
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FIG. 3 Amino-acid sequence comparison (single-letter code). An alignment is shown for the deduced amino-acid sequences for the gene products from *Arabidopsis thaliana* (AG), *Antirrhinum majus* (DEF A), humans (SRF) and yeast (MCM1, ARG80). Identical residues between AG and each of DEF A, MCM1 and SRF are shown below, as are the fractions of identical amino acids shared by these proteins. Numbers to the left of each sequence indicate the position of the first amino acid shown for each protein.

Discussion – Gene similarity

Similar genes

- Human serum response factor (SRF)
  - serum-inducible transcriptional activation of genes such as c-fos a nuclear proto-oncogene in mammals
- MCM1 transcriptional regulator of mating type specific genes.
  - Shares 80% homology include DNA binding domain
- Taken as evidence to suggest *agamous* gene encodes a transcriptional factor that determine stamen and carpel development in wild-type flowers

Discussion – Gene similarity

Similar Mutation

- Antirrhinum
  - 4 concentric whorls (sepals, petals, stamens and carpels)
- Mutation in *Def A* homeotic gene causes conversion of petals and stamens into sepals and carpels

Discussion – Gene similarity

Antirrhinum def A

- Phenotype similar to AP3 or PI gene in Arabidopsis
- Homologues
- High degree of similarity to Agamous in DNA binding domain. Def A and Agamous are more similar than the other found genes.
- Family of genes that control flower developement

Synthesis and Discussion

- The researchers had managed to quickly and efficiently characterize the *AGAMOUS* gene using a T-DNA insertion line to expedite the process.
- Although their findings would eventually form an integral part of our current understanding of the genetic processes involved in regulationg floral development and morphogenesis, it was the use of T-DNA to quickly and easily locate and sequence a gene that made this work so seminal.
- Moreover, they had quickly been able to resolve and sequence the gene, allowing them to analyze its putative protein product (AG) and begin to deduce a potential function.

Synthesis and Discussion

- Sequence homology with known transcription factors seemed to indicate a similar role for *AG*'s gene product.
- Yanofsky et al. had by this time isolated a family of genes in *A.thaliana* that shared *AG*'s sequence homology with transcription factors. (i.e. a specific DNA-binding motif)
- Their hypothesis, one which would be borne out by subsequent research, was that *AG* and the other members of this gene family would function very much as the *HOX* genes were known to in *Drosophila melanogaster*, *Saccharomyces sp.*, and *Homo sapiens* (amongst other metazoans…).

Synthesis and Discussion

- On what did they base this hypothesis? Primarily on the sharing of highly specific DNA-binding motifs and structural motifs shared by the *A.thaliana* gene family and others.
- This trend towards a specialization in DNA-binding regions was known to characterize regulatory genes in *Drosophila*.
- The region of sequence homology that Yanofsky et al. had identified would eventually come to be known as the MADS-box, a sequence that encodes the MADS domain, a critical DNA-binding domain for certain transcription factors.
- MADS? ***M****CM1* (Yeast), ***A****GAMOUS (A.thaliana)* , ***D****EF* (*Antirrhinum majus), **S**RF* (Humans).

Synthesis and Discussion

- The preferential expression of *AG* in the stamens and carpels of wild-type *A.thaliana*, as demonstrated by *in situ* hybridization, demonstrates that this gene has a critical role to play in the determination of proper organ identity in floral morphogenesis. But why is this important?
- This demonstrates how we can use mutant analysis to deduce developmental pathways in the same way that we can characterize biosynthetic pathways!
- More importantly, this showed how quickly it was possible to identify and characterize the gene responsible for an observed phenotype using T-DNA insertions!

Conclusions

- This paper is relevant for its historical significance. (T-DNA insertional tagging!)
- This paper is very dense (Nature papers...), but it describes a set of experimental procedures that have become standard operating procedure in this type of genetic examination.
- This paper shows how we can attack the most complex, difficult, and fundamental biological questions using the geneticist's tools.
- It also has significance for the role that these findings played in enhancing our understanding of the genetic and molecular mechanisms involved in floral development