Activation of a Floral Homeotic Gene in *Arabidopsis*

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In *Arabidopsis* flowers, appearance is based, in part, upon the expression of floral meristem-identity genes and their interaction with homeotic genes.
What’s a *meristem-identity* gene?

- A gene which codes for a transcription factor that regulates other genes that contribute to phenotype.

What’s a *homeotic* gene?

- A gene that controls the pattern of body formation during development, contributing to the overall phenotype.
Floral meristem-identity genes which contribute to the appearance of Arabidopsis include *APETALA1 (AP1)*, *CAULIFLOWER* and *LEAFY (LFY)*. These genes are expressed early to control flower initiation throughout the floral primordium.
Growth Phases of *Arabidopsis*

- Vegetative phase = plants grow leaves and shoots.
- Reproductive phase = flowers produced.
Inactivation and overexpression experiments had been done which demonstrated the control these genes had on flower initiation.

For example, loss-of-function experiments which used *lfy* mutants saw leaves with associated shoots instead of early flowers.
What more they knew at the time...

Homeotic genes are expressed in distinct domains of the flower and specify the identity of the floral organs which include sepals, petals, stamens and carpels. Section C is specified by the *AGAMOUS (AG)* gene.
Loss-of-function experiments demonstrated how the genes of the ABC model specify floral organ identity.

For example, when A function is disrupted (which is specified by the gene *APETALA2*), mutant phenotype plants lack sepals and petals.
How are LFY and AG related?

Besides playing a part in initiating floral development, a similar study conducted by Parcy et al. (1999) found that LFY plays a role in the activation of AG.

In order to demonstrate this, they developed a version of LFY with increased transcriptional-activation ability by fusing it with a strong activation domain taken from the viral transcription factor VP16 to make LFY:VP16.

Question: Why use a viral transcription factor?
Answer: because viral-proten 16 (VP16) is a strong activator and can be used to amplify the effects of LFY on AGAMOUS (AG).
What they found in LFY:VP16 plants with respect to AG RNA:

- detected earlier
- found ectopically, meaning gene was expressed in cell types in which it is normally inactive.
- produced more RNA

...all in comparison with wild type.

- This suggests not only that AG is activated in some way by LFY, but also that AG expression is normally region-specific.
- Parcy et al. (1999) state that coregulators interact with LFY protein to restrict AG expression.
So if they knew all this, what was left to prove?
What they didn’t know at the time…

...was the “directness” of interaction which in the case of this study, was between LFY and the AGAMOUS (AG) gene of the ABC model.
In order to better understand the interaction between *LFY* and *AG*, they first identified enhancers responsible for *AG* activation.

why?
They isolated enhancers to see *if* and how they interact with the LFY protein produced by the *LFY* gene.

Does LFY protein bind to the AG gene or does it bind elsewhere?
To illustrate the LFY – AG interaction...

They created a translation fusion of *AGAMOUS (AG)* to β-glucuronidase (GUS). This is where a gene is constructed to produce a protein that can be tagged in order to locate where in the cell it is produced and at what levels.

It was found (regarding *AG*) that the second intron (of *AG*) was required for expression of this chimeric (artificial) gene because it contained enhancers necessary and sufficient for wild type *AG* expression.

It was also found that this intron was spaced mostly within a 3kb Hind-III restriction fragment.
This was demonstrated using the KB9 fragment, which includes the whole Hind-III fragment:

When arranged in the forward position (3’ closest to promoter), transformants showed intermediate-to-zero staining.

Oppositely, when arranged in the reverse position (5’ closest to promoter) transformants showed weak-to-strong staining.
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Oppositely, when the Hind-III fragment was removed, there was no response of the artificial gene. This was *with* the use of LFY:VP16 to amplify the interaction.
At the same time, the KB9 reporter also illustrated how AG responds to LFY, suggesting AG contains LFY-responsive enhancer sequences.

In *lfy* mutants, GUS expression was delayed (and reduced) as indicated by the stain:
In LFY:VP16 subjects, GUS was expressed earlier (than normal) and ectopically, illustrated by the stain:
Ag-Enhancers

- Deletion analysis of 3-kb fragment revealed two enhancers that drive the GUS expression in Arabidopsis Flowers

- Combined these two enhancers could drive GUS expression in young wild type flowers in a similar way to those under the control of the 3kb fragment
Ag-Enhancers

- The enhancers KB14 and KB31 both are expressed in the centre of young flowers.
- These enhancers are in the 5' end and 3' of the fragment respectively
- This is very similar to expected endogenous AG expression in the early stages of flower development
Ag-Enhancers

- Two main findings from studying LFY mutants
  - One, the appearance of what appears to be enhancers that help to mediate AG activation independent of LFY
  - As evident from how expression of smaller reporters is reduced more than full length reporters.
- What does this say about LFY mediated activation of AG?
Ag-Enhancers

- Two main findings from studying LFY mutants

- One, the appearance of what appears to be enhancers that help to mediate AG activation independent of LFY

- As evident from how expression of smaller reporters is reduced more than full length reporters.

- What does this say about LFY mediated activation of AG?
  - That other factors play a role in AG activation as well.
Ag-Enhancers

• Second, that the reporters appear to have a regulatory role in AG expression.

• Several reporters are only expressed in certain regions even when exposed to the activated protein LFY:VP16

• Particularly evident in KB18's and KB14's Outer whorls, Floral stem, and Pedical tissues
Ag-Enhancers Analysis

- The 3' Enhancer was chosen for as a main focus, Why?
Ag-Enhancers Analysis

- The 3' Enhancer was chosen for as a main focus, Why?
  - Size, the 3' enhancers were considerably smaller than those of the 5' end ones
  - Their GUS activity was reduced in older flowers which would make them easier to interpret their expression
Ag-Enhancers Analysis

- Using LFY:VP16 activated protein, certain reporters which normally either have very low expression or no expression in wild type can be detected in the LFY:VP16.

- As these reporters were expressed in the presence of LFY:VP16 it must mean that there is an interaction between the reporter and the protein.

- Using KB30 as a guide the responsive element for LFY:VP16 was mapped.
Finding the Binding Sites

- The binding sites for LFY were found by performing an immunoprecipitation of the protein-DNA complex.

- This done by using an antibody which binds to the transcription factor protein LFY and allows for the isolation of the DNA that it's bound to which can then be sequenced.
Finding the Binding Sites

- From an electromobility assay it was found that using a 160 bp fragment from the 3' enhancer that the protein bound to the DNA at two binding sites.

- As the DNA isolated from the immunoprecipitation only 31 bp long looking for common motifs which resemble LFY binding sites in AP1 it was possible to isolated the two different binding sites in AG's 3' enhancer.
Using this method, it was found that LFY did bind to AG at two sites named AG I AND AGII.

LFY binds to both sites with equal levels.

To test whether these binding sites are needed to express the 3' enhancer these binding sites were deleted, to see its effect on GUS expression.
**LFY binding sites**

- The first experiment was to delete the AG1 site.

- This resulted in the KB45 mutant, this mutant has no detectable GUS expression in the Wild type and has a reduced level of expression in the LFY:VP16.
LFY binding sites

- This indicates that both AG sites are needed for normal levels of in vivo 3' AG enhancer expression.

- A further double deletion was also made which both of the AG sites deleted, called KB46.

- In KB46 expression in both the wild type and those exposed to LFY:VP16 showed no expression of the AG enhancer.

- This appears to show that without the binding sites LFY can't activate the 3' enhancer.
LFY binding sites

- To prove that the lost of enhancer activity was indeed due to loss of function of the binding site, a small 2 bp mutation was induced at the beginning of the conserved region of the binding site.

- A similar mutation renders the binding site of AP1 non-functional.

- Another mutation was also induced this time a single bp in the center of the binding site.

- These mutations were named m1 and m2 respectively and in an electromobility assay, the m1 mutation did indeed appear to prevent binding of LFY, while m2 mutation had no effect.
LFY binding sites

- To show this the case in actually plants these mutation were introduced into two plants
- One MX68 had the m1 mutation in both binding sites

KB30-FLY:VP16

MX68-Wild type

MX68-FLY:VP16
LFY binding sites

• While the other mutant was given the m2 mutation in both binding sites

• This mutation however as expected has no effect on expression as MX 100 as a very similar level of expression as the KB 31 reporter which contains the 3' enhancer.
• Unlike ABC and other transcription factors LFY is not part of a gene family

• Only one homolog of LFY is present in the haploid genome

• What this means is that the binding sites on the AG enhancer is unlikely to bind any other protein than LFY.
Conclusions

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• How did they prove this?
  - First they isolated the 5' and 3' enhancers in the 3 kb fragment
  - Then show that the LFY made a difference to the level of expression of the the reporter, and therefore AG
  - Then they isolated the binding sites of the 3' enhancer
  - Using these they deleted them to prove that with the ability to bind to AG the gene could not be expressed.
  - They also proved how specific and conserved the LFY binding motif is.
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- As you may remember even without LFY some expression of AG was occurring.

- As well IFY in certain enhancer elements was only expressed in certain regions.

- So now that the main regulator for AG is known, it is important to find the CO-regulators that help govern the expression of AG in flowers.

- We now know of two possible co-regulators of LFY the region specific genes UFO (Unusual Floral Organ) and WUS (Wuschel).
Conclusions

Why is this paper so important?
Conclusions

Why is this paper so important?

-As it proved that the AG an ABC was directly mediated by LFY

-Prior this it was unclear if it was a direct link or that it went through intermediates

\[ \text{IFY} \quad \longrightarrow \quad \text{ABC} \]

With the work also outline in this paper it proved that IFY bound to and regulated the expression of AG and AP1, and would likely regulated expression of all ABC genes.

A genetic framework for floral patterning.

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A molecular link between stem cell regulation and floral patterning in Arabidopsis.

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