The yeast two-hybrid assay to discover if known proteins in the ethylene signaling pathway can physically interact with each other

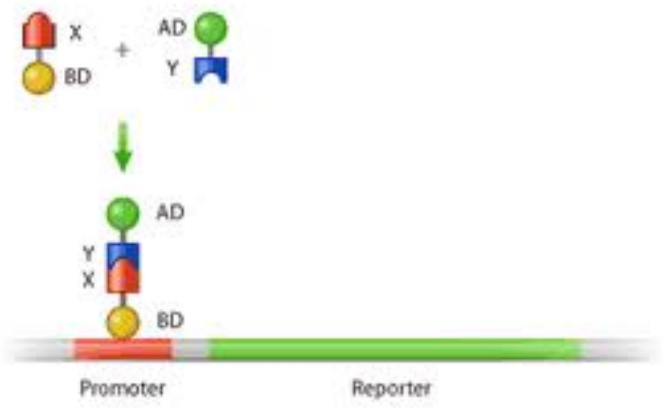
Objective

To perform the yeast two-hybrid assay, which is a powerful technique in molecular biology that is used to test whether two proteins can physically interact with each other. The assay utilizes yeast cells, but the proteins can come from any organism. In this lab, we will perform the yeast two-hybrid assay on various pairs of *Arabidopsis* proteins that are known to be in the ethylene signalling pathway, in order to see if they can interact with each other or not. Having such information on physical interactions between proteins helps scientists to put together pieces of a puzzle in order to understand the signalling pathway.

What is the yeast two-hybrid assay?

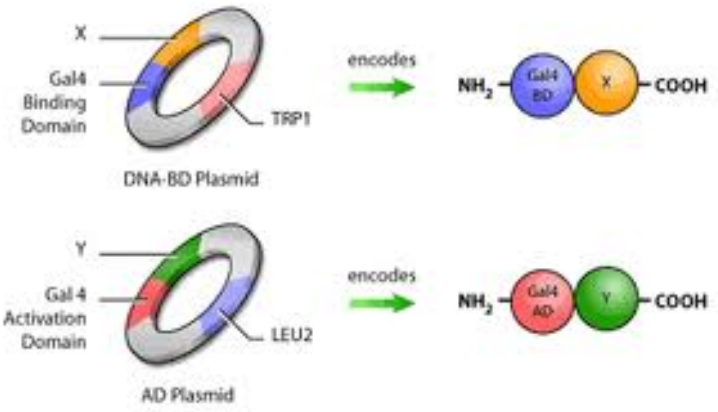
Interactions between proteins, known as "protein-protein interactions", are essential in all cellular processes. Therefore, a critical aspect of understanding almost any cellular process involves identifying these interactions. As we have discussed in this workshop, in order to understand the molecular events in a signalling pathway, a key step is to identify the genes in the pathway as well as the proteins encoded by those genes. Once this is accomplished, one of the next critical pieces of information in figuring out how the pathway works is to see which proteins can interact with each other.

One of the simplest ways to test for protein-protein interactions was first developed by geneticists about 2 decades ago and is called the yeast two-hybrid assay. The yeast two-hybrid assay is based on activation of transcription by a reconstituted transcription factor in yeast. Transcription factors bind to the promoter sequences of genes and activate their transcription. It was discovered that the two domains of eukaryotic transcription factor domains – the DNA-binding domain (BD) and the activation domain (AD) – can be physically separated, yet can by reconstituted and activate transcription if they become connected again. Thus, when a protein of interest (called "X") is fused with just the BD, while another protein of interest (called "Y") is fused with just the AD, a physical interaction of the X and Y proteins within the nucleus will bring together the BD and AD, resulting in activation of transcription, as shown in this figure.



To test for the interaction of X and Y proteins (which can come from any organism), the proteins must be expressed from plasmids that are engineered to express the DNA-binding domain (BD) fragment fused with X, while a second plasmid is engineered to express the activation domain (AD) fragment fused with Y. The protein fused to the BD may be referred to as the bait protein (encoded by

the plasmid at the top of the figure below), and is typically a known protein that the scientist is using to identify new binding partners. The protein fused to the AD may be referred to as the prey protein (encoded by the plasmid at the bottom of the figure below).



If the bait and prey proteins (X and Y) physically interact with each other, then the BD and AD of the transcription factor become indirectly connected such that transcription of **reporter gene(s)** can occur. If the two proteins do not interact, then there is no transcription of the reporter gene.

A **reporter gene** is a gene that reveals ("reports") that interaction of the bait and prey proteins has occurred. The reporter gene typically encodes a protein involved in the biosynthesis of an essential cell nutrient, such as an amino acid or nucleic acid that the cell needs to survive. The reporter gene is genetically engineered into the yeast genome and expression of the reporter gene is under the control of a promoter that the reconstituted transcription factor (BD and AD) binds to. The yeast strain used in the assay cannot grow on media that lacks the nutrient (e.g., the amino acid histidine), unless there is interaction of the bait and prey proteins, which leads to expression of the reporter protein (e.g., a protein that results in histidine biosynthesis). Yeast strains often carry more than one reporter gene for interaction. The yeast strain that we will use in this lab has two reporters: 1) a HIS3 reporter that allows cells to grow in the absence of histidine and 2) a lacZ reporter that causes cells to turn blue when a chemical called X-gal is provided.

For this lab, we will continue to focus on the ethylene signalling pathway in *Arabidopsis.* We will be testing various proteins that are known to function in the ethylene signalling pathway (shown on your separate Datasheet handout). We will re-enact the same types of experiments that researchers have carried out to elucidate the ethylene signalling pathway. The same methods can be applied to any protein pairs that act in any signalling pathway from any organism.

Materials (Part I): Plating the cells on selection plates

Per pair of students:
- Agar plates containing streaks of yeast that have been transformed with pairs of plasmids (called the "bait" and "prey" plasmids)
- sterile toothpicks for streaking out yeast cells
- 2 agar plates of selective media called "-LW"
- 2 agar plates of selective media called "-LWH"
- gloves
- a marker pen for labeling the plates
- waste container for discarding used toothpicks
- "Datasheet for Yeast Two-Hybrid Lab" containing your assigned yeast transformants

Procedure I (carry out in student pairs)

1. Each student in the pair should have 1 "-LW" plate and 1 "-LWH" plate. Label each plate with your name on the bottom of the plate (not on the lid).

2. For each student pair, decide who will be "Student A" and who will be Student B". Follow the datasheet containing your assigned yeast transformants to determine which 7 or 8 transformants you will be working with.

3. Label the plates with the code numbers: Each student will label 1 plate of "-LW" and 1 plate of "-LWH" using the number code from the datasheet. Label the bottom of the plate (not the lid). Each student should LABEL THEIR TWO PLATES IN THE SAME PATTERN.

4. "Patch" the cells onto the selective media: Working in the laminar flow hood, pick up a sterile toothpick using a gloved hand, touching only the narrow end of the toothpick. Use the untouched flat and wide end to scoop up a small amount of cells (the size of a matchstick head) from a plate of transformed yeast cells with the correct number code that you have been assigned.

5. Use the toothpick to make a "patch" of cells on your plate at the position that you labelled on your plate in step 3. The patch should be the size of the fingernail on your pinky finger, or a little smaller than a letter key on a computer keyboard. Try to hold the toothpick horizontally as you patch the cells so that you do not gouge or tear the agar. Discard the toothpick.

6. Use ***a new toothpick*** to patch the same yeast strain onto your second plate in the same position as on the first plate. Do not cross-contaminate the cells and plates by re-using the same toothpicks!

7. Carry out steps 4-6 for each of your assigned transformants. Remember to use a new toothpick **each time**! Do not cross-contaminate the cells and plates by re-using the same toothpicks!

8. When you are done, incubate your plates at 28-30C for 5-7 days to allow the yeast to grow.

9. Examine the growth and record the results on your datasheet.

Materials (Part II): Testing for lacZ reporter gene activity (several days later)

For each student:
- the -LW plate prepared in Part I above, after growth of patchat 30C for several days
- 3 ml of "Z buffer" (60 mM Na2HPO4 / 40 mM NaH2PO4 /10 mM KCl / 1 mM MgSO4, pH 7.0, 0.1% Triton X-100, b-mercaptoethanol, X-gal)
- 1 nitrocellulose circle per student
- empty petri dishes
- 2 Whatman filter paper circles
- containers of liquid nitrogen
- Forceps
- an 8"x8" square of aluminium foil

Procedure II

1. Prepare the "reaction chamber": Lay the Whatman filter paper in the bottom of an empty petri dish. Add ~3 mL of Z buffer to the filter paper. You do not want any liquid sloshing around. If necessary, pour off excess liquid.

2. Prepare the nitrocellulose membrane: Handle the nitrocellulose membrane carefully and only with the forceps. Mark the top of your nitrocellulose membrane with a pencil or ink mark, so that you can orient the membrane afterwards.

3. Lift the yeast cells onto the membrane: Lay the nitrocellulose membrane carefully on top of the yeast patches. Push down gently. Then use forceps to gently remove the filter from the plate. ONCE THE MEMBRANE HAS TOUCHED THE PLATE, YOU CANNOT LIFT IT UP TO READJUST IT.

4. Break open the yeast cells: Use forceps to dip the nitrocellulose membrane with the yeast on it into liquid nitrogen for about 10 seconds. Remove and allow to defrost.

5. Repeat step 4 once.

6. Start the reaction: Carefully put the membrane onto the damp Whatman filter paper that you prepared in Step 1. Make sure that the yeast cells are facing upward!!! You can push down the nitrocellulose membrane with tweezers to make sure it gets damp, but do not touch the yeast spots.

7. Incubate the reaction: Cover the reaction plate loosely with foil to protect the reaction from light. It may take anywhere from 2 minutes to several hours (even overnight) in order to see the results.

8. Determine which transformants are blue and which ones are white. Record the results on your datasheet.

Is your data consistent with the known ethylene signalling pathway?

Questions to think about

1. What are "-LW" and "-LWH" plates? What is the difference between them? What is each one used for?

2. Which plate should be used for the lac Z assay and why?

3. What is a "reporter gene", and what are the reporter genes in this assay?

4. In terms of your results, should there be a correlation between the growth of transformants on -LWH plates and the blue color in the lacZ assay? Why?

5. What is a negative control, and why is it important in the yeast two-hybrid assay?

6. In the experiment above, which yeast transformants are the negative controls?

7. Are any negative controls missing in our experiment?

8. If you have a known protein that serves as your bait protein, can you think of how the yeast two-hybrid assay be used to find the unknown protein that interacts with this bait?