EFFECTORS AND PLANT IMMUNITY

TSL SUMMER SCHOOL 2017, NORWICH

www.kamounlab.net

August 2017

Program Overview

Thursday 3rd August

Effectors and Plant Immunity. Led by Sophien Kamoun

Time	Activity	Venue
0930	PEDAGOGICAL LECTURE – Sophien Kamoun	
1100	Tea Break and Discussion	
1130	Practical Session	
1230	Lunch NRP Venues	
1330	KEYNOTE LECTURE - JENS BOCH – A battle for life and death - Evolution of TALEs in plant-pathogenic Xanthomonas bacteria	JIC G34/35
1430	Tea Break and Discussion	
1500	Practical Session	

NB. All activities will take place in the Chris Lamb Training Suite, unless otherwise stated

Friday 4th August

Effectors and Plant Immunity. Led by Sophien Kamoun

Time	Activity	Venue
0930	Practical Pickup Session and Discussions	

Specific Program

Session I	**Identification of effector-plant protein complexes by coIP/MS**	
1130-1230	coIP	Training Lab
1500-1700	coIP continued	Training Lab
1700-1730	MS dataset analysis	Training Theatre
1730-1830	Presentation by Juan Carlos De La Concepcion on *in vitro* methods to characterize protein-protein interaction	Training Theater
Session II	**Identification of protein-protein interaction partners by Yeast-two-hybrid (Y2H)**	
1130-1230	Yeast-two-hybrid Theory and experimental design	Blue Sky Room
1500-1730	Yeast Co-transformation and Y2H assay	Training Lab
1730-1830	Presentation by Juan Carlos De La Concepcion on *in vitro* methods to characterize protein-protein interaction	Training Theater
Session III	**Agroinfiltration and virus-induced gene silencing**	
1130-1230	Designing VIGS and Complementation experiments	Training Theatre
1500-1800	VIGS and Agroinfiltration	Kamoun Lab

Session I

Identification of effector-plant protein complexes by coIP/MS

Erin Zess

Background

Host-translocated effectors manipulate host cells by interacting with host protein complexes. To better understand effector functions it is necessary to identify the proteins they associate with. In the first session, we will use co- immunoprecipitation (coIP) of tagged effectors transiently overexpressed in *Nicotiana benthamiana* leaf cells to purify interactors. In the second session, we will analyze data from a large-scale coIP/MS experiment, try to identify specific effector interactors, and discuss potential follow-up experiment

Buffers:

GTEN: 10% glycerol, 25 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl
Extraction Buffer: GTEN, 2% w/v PVPP, 10 mM DTT, 1x protease inhibitor cocktail (Sigma), 0.1% Igepal
Immunoprecipitation (IP) buffer: GTEN, 0.1% Igepal

Morning session

Anti-FLAG co-immunoprecipitation

1. Weigh out the leaf powder and re-suspend it with ice-cold extraction buffer (2 mL of buffer for 1 g of powder). Vortex thoroughly until the solution is homogenous and keep on ice. Be cautious with dry ice.
2. Centrifuge at full speed in a microcentrifuge for 10 min at 4°C. Transfer the supernatant into syringe and filter through 0.45 µm membranes. Keep the filtrate (~1.0 mL) in a new tube and place on ice.
3. Re-suspend the anti-FLAG resin by tapping the side of the vial several times and mix with a cut pipette tip.

4. Pipette enough resin (20 µL per sample) into a 1.5 mL Eppendorf tube. Centrifuge at 800 × *g* for 1 min and carefully remove the supernatant (take care not to aspirate the resin).
5. Re-suspend the resin in 1 mL of IP buffer.
6. Centrifuge at 800 × *g* for 1 min and remove the supernatant.
7. Re-suspend the resin to 2x original volume with IP buffer and add 40 µL of diluted resin to the leaf extract prepared above (step 2).
8. Incubate the resin and the leaf extract over lunchtime at 4°C.

Afternoon session

Anti-FLAG co-immunoprecipitation continued…

9. Centrifuge at 800 × *g* for 30 s. Discard supernatant and add 1 mL of fresh IP buffer. Repeat four more times but always leave about 50 µL at the bottom of the tube to avoid aspirating the beads. After the last wash, remove as much supernatant as possible without touching the beads. Beads are ready for trypsin digestion (not to be performed during this practical; can be performed on beads, or after running gel).

Analysis of a coIP/MS dataset

1. Open the file 'Petre_coIPMS_RustEffectors_TSLSummerSchool' (.xlsx). The
2. table has 674 lines (N. benthamiana proteins identified by LC-MS/MS) and 36 columns (GFP-tagged effectors used as baits for the co-immunoprecipitation assays). Numbers indicate peptides matching a given plant protein (total spectrum count method). Leaf cell compartments in which fusion proteins accumulate are indicated.
3. Answer the questions below, and try to be critical about their significance from a methodological and a biological point of view. You will need to use scoring methods and graphics.
4. On average, how many plant proteins co-immunoprecipitated with a single effector?
5. On average, how many effectors co-immunoprecipitated a single plant protein?
6. Which plant proteins represent are reliable and specific interactors?
7. Which plant proteins are non-specific interactors (potential artifacts)?
8. What conclusions can you draw from this analysis?

Discussion of follow-up experiments

Presentation by Juan Carlos De La Concepcion on *in vitro* methods to characterize protein-protein interactions

References

Win J, Kamoun S, Jones AME (2011) Purification of Effector–Target Protein Complexes via Transient Expression in Nicotiana benthamiana. In Plant Immunity, *Methods Mol Biol* McDowell JM (ed) pp 181-194-194. Humana Press

Petre B, Saunders DG, Sklenar J, Lorrain C, Win J, Duplessis S, Kamoun S (2015) Candidate Effector Proteins of the Rust Pathogen Melampsora larici-populina Target Diverse Plant Cell Compartments. *Mol Plant Microbe Interact* 28: 689-700

Contact

erin.zess@tsl.ac.uk

joe.win@tsl.ac.uk

emilie.chanclud@tsl.ac.uk

juan.de-la-concepcion@jic.ac.uk

Session II

Identification of protein-protein interaction partners by Yeast-two-hybrid (Y2H)

Thorsten Langner

Background

Pathogens secrete effector proteins to manipulate their host. Once translocated into the host cell effectors can interact with host proteins (form complexes) to manipulate important host pathways. Yeast-two-hybrid (Fig. 1) is an easy technique to identify protein-protein interaction partners. Here, we will learn how to transform yeast cells with our desired construct, select for positive transformants, and select for positive interaction partners.

Morning Session – Yeast-two-hybrid Theory and experimental design

1) Theoretical introduction into Y2H
2) Preparation of material for afternoon session
   - Preparation of carrier DNA
   - Preparation of plates for Y2H assay

Afternoon Session – Yeast Co-transformation and Y2H assay

Yeast Transformation

1) Grow Y2H Gold cells in YPD (+Adenine) Medium
2) Spin cells at 1100 x *g* for 5 min; wash with H2O twice
3) Resuspend cells in sterile 100 mM LiAc / 100mM TE-buffer
4) Mix 100 ng of plasmid(s) DNA with 10 µl of carrier DNA
5) Add 100 µl Cells to DNA mix
6) Add 500 µl sterile LiAc/PEG solution (100 mM LiAc; 40% PEG, 100 mM TE-buffer) → mix well and shake at 28 ºC
7) Add 70 µl DMSO (sterile), mix gently
8) incubate at 42 ºC for 15 min, cool on ice for 2 min
9) spin cells at full speed (16000 x *g*) for 30 sec
10) resuspend cells in 250 µl 100 mM TE-buffer, plate on SD (-leu/-trp) plates for selection of transformants

Y2H assay

1) Inoculate overnight culture with a single transformant (single colony) from the SD –leu/-trp selection plate

2) Measure the OD_{600} of the overnight culture (dilute culture 1:10 in SD medium for measurement)

3) Set the OD_{600} of all test strains to 1

4) Prepare serial 1:10 dilutions (1:10 / 1:100 / 1:1000 i.e. OD_{600} 1 / 0.1 / 0.01 / 0.001). Mix well

5) Prepare replica drop plates of all dilutions on SD –leu/-trp and SD –leu/-trp/-ade/-his

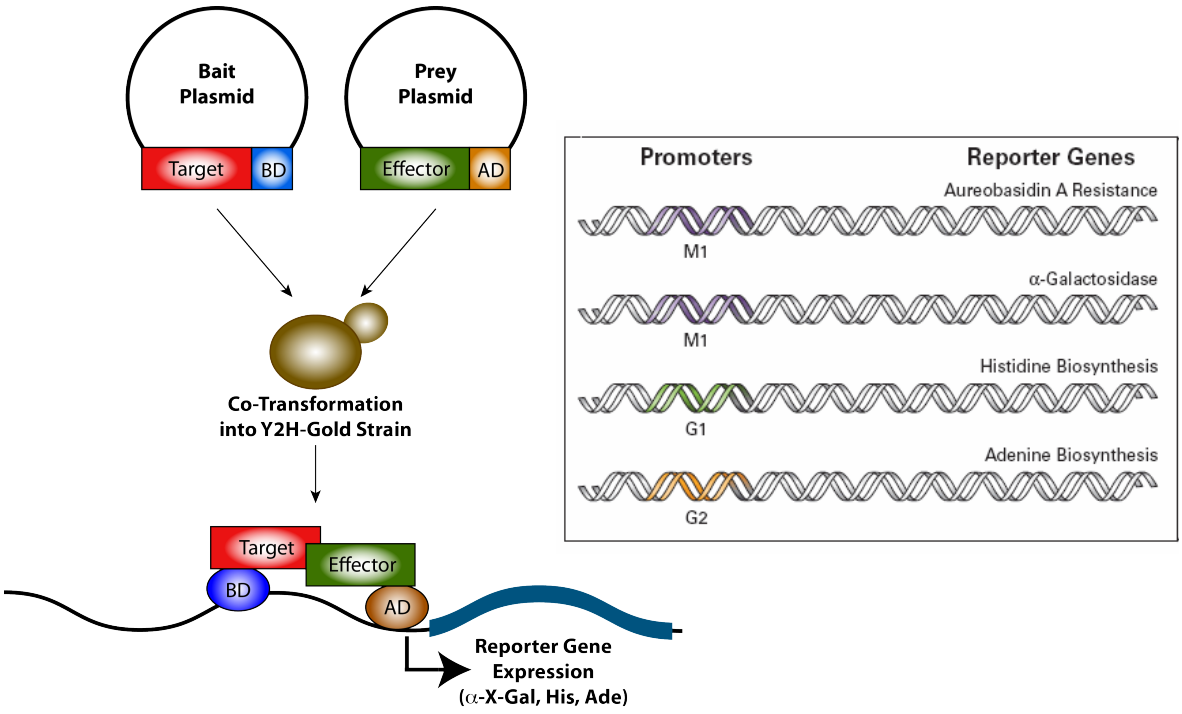


Fig. 1: Yeast-two-hybrid at a glance. A bait plasmid carrying the putative target fused to the gal4-binding domain and a prey plasmid carrying the effector fused to the gal4-activation domain are co-transformed into the Y2H-Gold strain. If the effector protein interacts with the host target protein an effector-target complex is formed bringing the DNA-binding domain and the activation domain in close proximity. This complex is able to bind to upstream activating elements (UAE) of promoter regions driving the expression of different reporter genes. Activity of the reporter genes can be visualized on selective medium lacking essential amino acids and by α-galactosidase activity.

Session III

Agroinfiltration and virus-induced gene silencing

Chih-Hang Wu

Background

Agroinfiltration and virus-induced gene silencing (VIGS) are widely used for understanding the function of gene-of-interest, including components involved in effector-immune receptor recognition. In this session, we will use Rpi-blb2/AVRblb2 and NRC4 as an example to illustrate how to design VIGS experiments. We will use the SGN-VIGS tool and *N. benthamiana* genome databases to select the regions for silencing, and introduce how to use synthetic gene strategy to validate targeted genes through complementation assays. This experimental design is powerful for functional analysis of genes involved in effector-immune receptor recognition as well as other cellular processes.

Designing VIGS and complementation experiments

Selecting region for VIGS experiment:

1. Go to the SGN (http://solgenomics.net) website and open the page of VIGS tool.
   Tools → VIGS tool
2. Copy and paste your sequence.
3. Set parameters: n-mer, fragment length, mismatches
4. Select database: v0.4.4 or v1.0.1
5. Run VIGS analysis

Designing synthetic fragment for complementation:

Manual editing

1. Copy and paste sequence in ExPASy translate tool (http://web.expasy.org/translate/)
2. Select output format “include nucleotide sequence”
3. Translate sequences
4. Copy and paste result to word file

5. Edit sequence manually
6. Check similarity by sequence alignment or blastn

Useful tips

No alternative codon: M/ATG; W/TGG

Tolerate more than one change: L/TTA → CTG; R/CGT → AGA; S/ TCT → AGC

Codon table (https://en.wikipedia.org/wiki/DNA_codon_table)

VIGS and transient expression of effectors and immune receptors in *N. benthamiana*

TRV inoculation by agroinfiltration:

1. Collect *Agrobacterium tumefaciens* cells (harboring TRV1 or TRV2) from fresh plates (1-day-old) or spin down bacteria from liquid culture.
2. Re-suspend cells in infiltration buffer.
3. Spin down cells at 1100xg for 5 minutes.
4. Remove supernatant and re-suspend the cells in infiltration buffer.
5. Measure the concentration (OD_{600}).
6. Calculate the final concentration (OD_{600}) for TRV1 = 0.2 and TRV2 = 0.1
7. Mix TRV1 with TRV2 bacterial solutions to the final concentrations calculated above
8. (optional) Incubate the cells at room temperature around 30 to 60 minutes.
9. Infiltrate 2-3 leaves of 14-day-old *N. benthamiana* seedlings with needle-less syringe.
10. Keep *N. benthamiana* in growth chamber for 2-3 weeks until the size is ideal for the follow-up experiment.

Transient expression of Rpi-blb2, AVRblb2 , $NRC4^{syn}$, $NRC4^{syn\_K190R}$ in NRC4-silenced N. benthamiana

1. Collect *Agrobacterium tumefaciens* cells from fresh plates (1-day-old) or spin down bacteria from liquid culture.
2. Re-suspend cells in infiltration buffer.
3. Spin down bacteria at 1100 x *g* for 5 minutes.

4. Remove supernatant and re-suspend cells in infiltration buffer.
5. Measure the concentration (OD600).
6. Mix cells harboring different expression constructs to the indicated concentration: Rpi-blb2, 0.2; AVRblb2, 0.1; EV, 0.2; $NRC4^{syn}$ 0.4.
7. Use needle-less syringe to infiltration the solution into the leaves of 2-3 week old silenced *N. benthamiana* leaves.
8. Keep infiltrated *N. benthamiana* plants in growth chamber for few days until the cell death is clear.

Buffer

Infiltration buffer: 10 mM MES, 10 mM $MgCl_2$, and 150 µM acetosyringone, pH5.6

Note: Add 15 µl 0.5 M acetosyringone into 50 mL MES-$MgCl_2$ buffer before use.

References

Fernandez-Pozo N, Rosli HG, Martin GB, & Mueller LA (2015) The SGN VIGS tool: user-friendly software to design virus-induced gene silencing (VIGS) constructs for functional genomics. *Mol Plant* 8(3):486-488.

Wu CH, et al. (2017) NLR network mediates immunity to diverse plant pathogens. *Proc Natl Acad Sci* U S A 114(30):8113-8118.

Contact

chih-hang.wu@tsl.ac.uk

Calculations:

Total volume = 2 mL
Final OD600 = 0.1
Starting OD600 = x
Amount to add = y mL

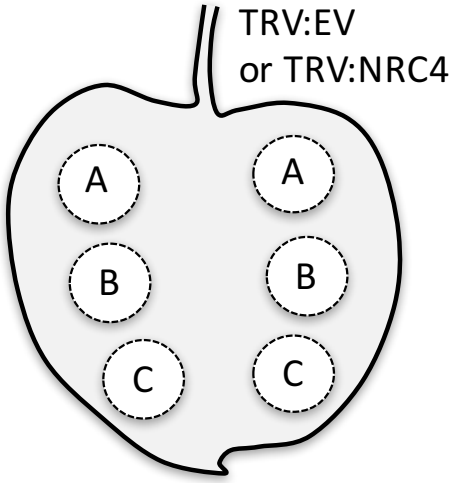
$$\frac{Final\ OD\ \mathrm{x}\ Total\ volume}{Starting\ OD\ (x)} = y\ \mathrm{mL}$$

TRV inoculation by agroinfiltration

	TRV1	TRV2-EV	TRV2-NRC2/3	TRV2-NRC4	TRV2-NRC-Triple
Starting OD					
Final OD	0.2	0.1	0.1	0.1	0.1
TRV1+ TRV2-EV					
TRV1+ TRV2-NRC2/3					
TRV1+ TRV2-NRC4					
TRV1+ TRV2-NRC-Triple					

Transient expression and complementation

	Rpi-blb2	AVRblb2	EV	NRC4	NRC4^{K190R}
Starting OD					
Final OD	0.2	0.1	0.4	0.4	0.4
A. Rpiblb2+AVRblb2+EV					
B. Rpiblb2+AVRblb2+NRC4					
C. Rpiblb2+AVRblb2+NRC4^{K190R}					



All data and documents are available to download from
https://github.com/joewinnz/TSL_Summer_School_2017