

## ***In planta* expression systems of pathogen effectors**

IMMUNOMODULATION ASSAYS OF EFFECTORS: AGROINFILTRATION AND AGROINFECTION ASSAYS FOR ACTIVATION AND SUPPRESSION OF IMMUNITY.

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### **1. INTRODUCTION**

Large-scale genome sequencing projects have generated a wealth of sequence information for plant pathogenic microbes. Functional genomic approaches are essential to exploit the sequence information to identify pathogen effector genes that trigger cellular and molecular responses in plant cells. In this regard, transient expression assays are valuable tools for studying the activation and suppression of plant immunity by plant pathogen effectors.

Typically, ectopic expression of single effector genes in plant cells leads to phenotypic effects. Expression of avirulence (*Avr*) genes in plant cells that contain the matching resistance (*R*) gene usually results in a local cell death called the hypersensitive response (HR). This phenomenon is extrapolated to functional genomic approaches for identifying effector candidates with AVR function, for example, by expressing *Avr* candidates *in planta* and functional profiling resistant host plants for occurrence of the HR, or by co-expressing both the *Avr* and *R* candidate genes in a heterologous model system. Also, expression of effector genes in susceptible hosts can lead to phenotypic responses that may reflect virulence function.

The use of transient expression systems to accomplish ectopic gene expression in plants has a number of advantages over stable transformation. These assays are rapid and simple to perform, and they can be applied to fully differentiated plant tissues, allowing the analysis of cell death-inducing genes without inducible promoters. In addition, these assays are not influenced by chromosomal positional effects and can be used in high-throughput functional analyses in plants.

In this practical exercise we will perform two functional assays: *Agrobacterium tumefaciens*-based transient transformation (agroinfiltration) and binary *Potato virus X* (PVX) expression (PVX agroinfection) in *Nicotiana benthamiana* plants.

## 2. AGROINFILTRATION

Agroinfiltration is an *Agrobacterium tumefaciens* – based method for transient expression of genes of interest. Because of the broad host range of the bacterium, this assay works well on numerous dicot plant species.

*A. tumefaciens* enters its host through natural wounds and causes tumors (crown galls) at infection sites. Translocation of transfer DNA (tDNA) from a Ti plasmid (i.e., tumor-inducing plasmid) occurs after the virulence machinery of the bacterium is activated by low-molecular-weight phenolic compounds and monosaccharides that are released from wounded plant cells. This natural mechanism is exploited for development of transient (and stable) plant transformation. A range of strains has been disarmed for their tumor-inducing properties, and diverse binary Ti vectors have been developed for laboratory use.

The agroinfiltration assay involves incubations of *A. tumefaciens* cell suspensions with 3'-5'-dimethoxy-4'-hydroxy acetophenone (acetosyringone). This phenolic compound mimics plant wounding, thereby inducing *vir* gene expression. This treatment is followed by the infiltration of cell suspensions into leaf panels, allowing transformation of accessible plant cells and leading to expression of the transgene(s) contained in the tDNA region.

Because *A. tumefaciens* is a plant pathogen, various defense responses from the host can be initiated, and numerous strains cannot perform sufficient gene transfer during infiltration in certain hosts. Phenotypic changes such as necrosis or chlorosis at infiltration sites do occur as well. Therefore, the choice of strains in relation to the host is essential.

Virtually, agroinfiltration can be combined with many assays, which include disease tests and agroinfection assays.

### 2.1 Agroinfiltration protocol

#### Plant Material

Typically, 4-week-old *N. benthamiana* plants are well suited for agroinfiltration. For *Solanum*, 4 to 5-week-old transplants after transfer from *in vitro* to pots are recommended. To ensure good transgene expression, leaves must be young but well developed.

## Strain

The choice of the *A. tumefaciens* strain is often key to successful transformation. We regularly use GV3101 for *N. benthamiana*. This strain electroporates at high frequency and this streamlines the cloning procedure, as ligation mixtures can be directly electroporated into *Agrobacterium*. For *Solanum*, the AGL1 strain is well suited.

## Medium preparation

- LB Medium (1L): 10 g bacteriological peptone, 10 g NaCl, 5 g yeast extract, (15 g agar)

- YEB medium (1L): 5 g beef extract, 1 g yeast extract, 5 g bacteriological peptone, 5 g sucrose, and 2 mL of 1 M MgSO<sub>4</sub>

- MMA infiltration medium (1L): 5 g MS salts, 1.95 g MES (2-[N-Morpholino] ethane sulfonic acid), 20 g sucrose, pH adjusted to 5.6 with 1 M NaOH, and 200 μM acetosyringone (3'-5' Dimethoxy-4'-hydroxy acetophenone). Add the acetosyringone just before using the medium.

- Stocks:

*MES 1M: 195 g/L*

*MgSO<sub>4</sub> 1M: 246 g/L*

*Acetosyringone 500 mM in DMSO (Dimethyl sulfoxide)*

*Antibiotics according to A. tumefaciens strain and constructs*

## *Agrobacterium* culture preparation

1. Streak recombinant *A. tumefaciens* strains onto LB solid agar media plates supplemented with antibiotics and incubate at 28°C for 2 to 3 d.
2. Inoculate 3 mL of LB medium containing antibiotics, with the recombinant *A. tumefaciens* strains and grow for 24 h at 28°C and 200 rpm.
3. Inoculate part of the LB culture into the YEB medium (10-15 mL) containing antibiotics, 2 μM acetosyringone and 10 mM MES. Grow cultures overnight at 28°C and 200 rpm to an OD<sub>600</sub> of approx 1.
4. Harvest the cells by centrifugation (4000g for 10 min), pour off the supernatant and resuspend the pellet in MMA medium supplemented with 200 μM acetosyringone.
5. Measure the OD<sub>600</sub> of the suspensions and dilute to the desired density.
6. Incubate the cells at room temperature for 1 to 3 h.

### Inoculation

Place *A. tumefaciens* suspensions into a 1 mL syringe. Carefully invert the leaf and hold the lower (abaxial) side up. Support the infiltration site with your index finger and place the syringe against the leaf and index finger. While applying gentle pressure to the leaf, inject the suspension slowly from the syringe. Successful infiltration can be seen as the *Agrobacterium* suspension spreads from the infiltration site into the leaf. During the infiltration procedure always wear safety glasses and gloves. Also, change the syringe and the gloves before infiltrating a new clone, and dry the infiltrated area with tissue to avoid cross-contamination between infiltrated spots.

### Incubation

Incubate the plants in a growth chamber or confined space at 22°C. *A. tumefaciens* transformation efficiency and transgene expression peaks at this temperature. Incubation temperatures of infiltrated plants should not exceed 28°C.

### Scoring

Responses should be visible in 2 to 3 d after infiltration for Avr-R interactions, but timing can vary depending on the tested effectors and host plants.

### Notes

- *Co-agroinfiltration assays*. Several transgenes can be delivered into the same cell with the agroinfiltration system facilitating simultaneous expression of interacting proteins (i.e., Avr and R proteins) or assembly of multimeric proteins.

- *Concentration of A. tumefaciens suspension*. Normally, we use OD<sub>600</sub> 0.3 for single infiltrations and OD<sub>600</sub> 0.3 to 0.5 for co-infiltration assays in *N. benthamiana*. For other plant species, dilution series can be made to select the best concentration. The OD<sub>600</sub> ratio between constructs in co-infiltration assays is usually 1:1, but can be changed depending on the characteristics of the constructs. Infiltration with dense *A. tumefaciens* suspensions can lead to background necrosis. This problem can be avoided by using suspensions with lower OD<sub>600</sub> values.

- **Controls.** It is critical to include proper controls for each experiment. An *A. tumefaciens* strain containing a vector without gene insert is recommended as a negative control and HR-inducing genes can be used as positive control. Binary vectors containing genes expressing marker proteins, such as green fluorescent protein, can be used to verify the level of transformation by agroinfiltration. It is also recommended to infiltrate the constructs of interest in different positions on the leaves to avoid position effects.

- **Additional compounds.** Transient *Agrobacterium*-mediated gene expression is often hampered by posttranscriptional gene silencing (PTGS), which can be overcome by co-infiltration with silencing inhibitors like P19. In the case of *R* genes, care should be taken with using silencing suppressors, because over-expression can induce elicitor independent cell death.

## **2.2 Practical exercise**

In this practical exercise we will analyze the interaction between different variants of the *Phytophthora infestans* AVR3a effector (namely, AVR3a<sup>KI</sup> and AVR3a<sup>EM</sup>) and the *Solanum demissum* R3a resistance protein by co-agroinfiltration experiments. We will also perform infiltration assays to demonstrate the ability of AVR3a<sup>KI</sup> to suppress the hypersensitive response induced by the *P. infestans* elicitor INF1. For this, we will use a simplified protocol that is regularly used in our lab to perform agroinfiltrations in *N. bentamiana* plants.

1. Streak recombinant *A. tumefaciens* strains onto LB solid agar media plates supplemented with antibiotics and incubate at 28°C for 2 to 3 d.
2. Inoculate 3-10 mL of LB medium containing the antibiotics, with the recombinant *A. tumefaciens* strains and grow for 24 h at 28°C and 200 rpm.
3. Measure the OD<sub>600</sub> of the *A. tumefaciens* cultures using LB with antibiotics as blank, and calculate the amount of culture to centrifuge (see table below).
4. Transfer the initial volume (Vi) to a new tube and harvest the cells by centrifugation at 4000g for 10 min.
5. Prepare the infiltration buffer (10 mM MgCl<sub>2</sub> – 10 mM MES) by adding acetosyringone to a final concentration of 200 μM (add 400 μL of the acetosyringone stock 0.5 M to 1 L of infiltration buffer).

Constructs	Initial OD <sub>600</sub>	Final OD <sub>600</sub>	Final volume (Vf)	Initial volume (Vi)
pGR106::ΔGFP		0.3	10 mL	
pGR106::AVR3a <sup>EM</sup>		0.3	10 mL	
pGR106::AVR3a <sup>KI</sup>		0.3	10 mL	
pGR106::INF1		0.3	10 mL	
pCB302-3::R3a		0.6	10 mL	
pK7::Avr-vnt1		0.4	10 mL	
pGRAB::Rpi-vnt1		0.4	10 mL	

6. Pour off the supernatant and resuspend the pellet in the infiltration buffer supplemented with acetosyringone (Vf = 10 mL).

7. Prepare the mixtures for the co-agroinfiltration assay:

a) 1 mL of pCB302-3::R3a + 1 mL of pGR106::ΔGFP (negative control)

b) 1 mL of pCB302-3::R3a + 1 mL of pGR106::AVR3a<sup>EM</sup>

c) 1 mL of pCB302-3::R3a + 1 mL of pGR106::AVR3a<sup>KI</sup>

d) 1 mL of pK7::Avr-vnt1 + 1 mL of pGRAB::Rpi-vnt1 (positive control)

The OD<sub>600</sub> of R3a in the mixture will be 0.3 and that of ΔGFP, AVR3a<sup>EM</sup> and AVR3a<sup>KI</sup> will be 0.15. For the positive control the OD<sub>600</sub> will be 0.2 for each construct.

7. Incubate the cells at room temperature for 1 to 3 h.

8. Infiltrate *N. benthamiana* plants according to the diagrams below (the diagrams show the abaxial side of the leaves).

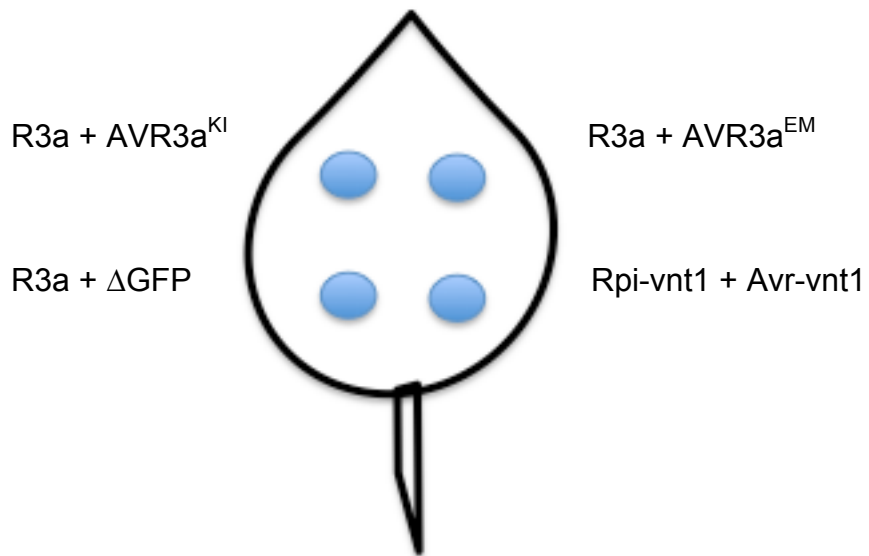
#### *Infiltration Buffer*

10 mM MgCl<sub>2</sub> (2.03 g / L)

10 mM MES (1.95 g / L)

Autoclave

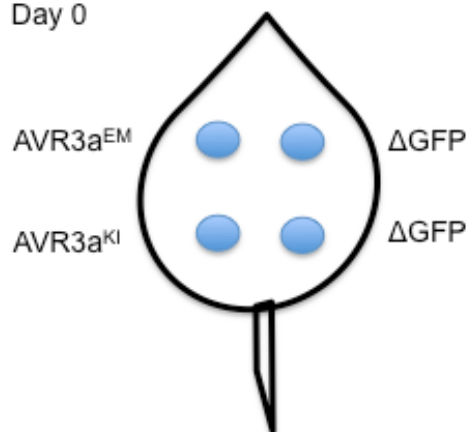
**A. Recognition of AVR3a by R3a**



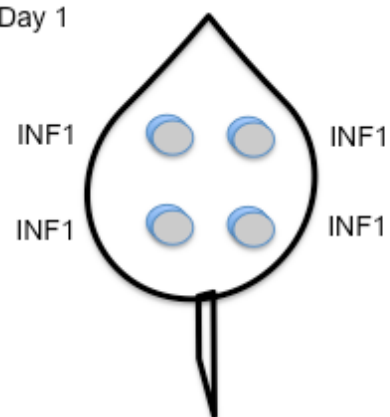
**B. Suppression of INF1-induced cell death**

**OPTION 1**

Day 0

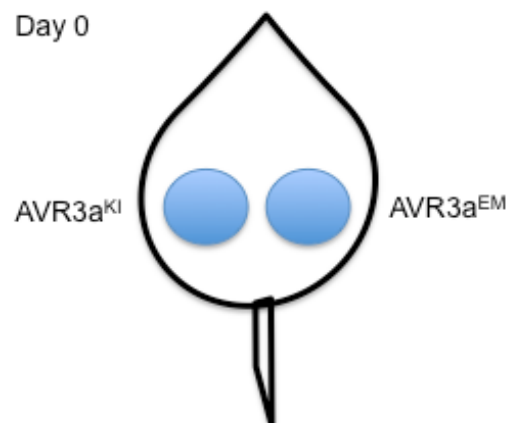


Day 1

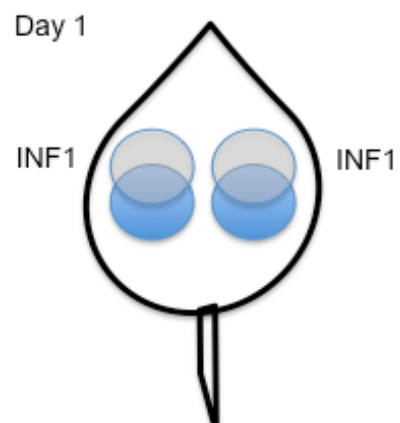


**OPTION 2**

Day 0



Day 1



### 3. AGROINFECTION

A number of plant viruses can be used as vehicles for transient gene expression in plants. RNA viruses can multiply to very high levels in infected plants, which makes them ideal vectors for gene expression. Among plant RNA viruses, PVX (Potato virus X) is widely used for expressing virulence and avirulence genes from viruses, bacteria, fungi, and oomycetes.

The PVX genome was modified by incorporating a duplicated coat protein promoter sequence followed by a multiple cloning site for insertion of the gene of interest. Original constructs required in vitro transcription of PVX RNA followed by rubbing inoculation onto plant leaves. A breakthrough in developing high throughput in plant expression systems was achieved by constructing binary PVX expression vectors in which the full-length PVX genome, flanked by the Cauliflower mosaic virus (i.e., CaMV) 35S promoter and the nopaline synthase terminator, was cloned in the tDNA of an *A. tumefaciens* binary vector. In this PVX agroinfection system, numerous candidate genes or cDNAs of a library can efficiently be inserted into the PVX backbone, and the binary vector, such as the commonly used vector pGR106, facilitates transfer of the expression construct to the plant cells through local transformation by *A. tumefaciens*. Viral infection is initiated by wound inoculation of the recombinant *A. tumefaciens* strain onto leaves of host plants resulting in transfer of the tDNA containing the PVX genome into plant cells. The PVX genome is then transcribed from the 35S promoter, resulting in virus particles that can move from one plant cell to another and spread systemically in the inoculated plants. Expression of the inserted gene is achieved during viral replication and occurs at high levels. This way, genes of interest are systemically expressed in the plant, which can then be monitored for altered phenotypes.

Current applications of PVX agroinfection involve large-scale initiatives to identify HR-inducing genes. The method can be used for over-expressing plant genes to identify positive regulators of cell death, or pathogen genes with HR-inducing activity. The unbiased approach of screening pathogen cDNA libraries has proven successful for identifying avirulence genes and other necrosis-inducing effectors. In this case, the expected plant symptoms range from a systemic mosaic (no response to the inserted gene) to systemic necrosis (hypersensitive response to an effector) or no symptoms (extreme resistance to an effector).

This assay is useful for various plant species that allow PVX replication and spread, such as *Nicotiana benthamiana*, tobacco (*Nicotiana tabacum*), tomatoes (*Solanum lycopersicum*), potatoes (*Solanum tuberosum*) and other *Solanum* species.



### **3.1 Agroinfection protocol**

#### Plant material

For *N. benthamiana* use 2-4 week-old, medium-sized plants. For *Solanum* plants generated from *in vitro* material, 2-3 weeks after transplanting to pots is appropriate. In general, if systemic symptoms are sought plants at three to four leaf stages are preferable for inoculation. Plants should be healthy, not stressed and not infected or challenged with pathogens before inoculation.

The young fully stretched leaves are recommended for inoculation. Younger leaves often show a tumor-like formation (possibly due to *A. tumefaciens*) and older leaves may senesce or drop down before scoring is finalized.

It is recommended to use a minimum of four plants for each construct. For local responses, multiple clones can be inoculated on a single leaf and three leaves per plant can be used to serve as triplicates.

#### Strain

In general, *A. tumefaciens* strain GV3101 is recommended for *N. benthamiana* and *Solanum*.

Grow the bacteria for 2-3 days at 28°C on solid agar LB medium with antibiotics. It is advisable to use fresh cultures that are not older than 4 days.

#### Inoculation

1. Toothpick-inoculate individual clones by dipping a wooden sterile toothpick in a culture of the recombinant *A. tumefaciens* strain and piercing the leaves on both sides of the mid vein.
2. For quantitative local scoring, make multiple inoculations of each construct on different leaves of different plants to exclude leaf position effects and plant effects.

#### Incubation

Incubate the plants in a growth chamber or confined space. During the infection a temperature of 22°C is advised. Higher temperatures inhibit PVX replication and lower temperatures inhibit *A. tumefaciens* infection.

### Scoring

Score 2-3 times per week during 2-3 weeks. Differences in timing usually occur between tested clones and plant genotypes. Response should be visible starting from 7 days after inoculation in *N. benthamiana*. In *Solanum*, symptoms usually become visible at approximately 10 dpi, and 1-2 weeks later most responses are very clear.

Phenotypes of the response may vary from an intense black cell death zone surrounding the wound until faint necrotic trails near veins near the inoculation spot. For a quantitative analysis of local responses, calculate the percentage of responding sites and compare with controls.

### Notes

- *Positive and negative controls.* For local scoring, good positive controls are general necrosis-inducing genes such as *Cnr2*, *Npp1* or *INF1*, and the empty vector can serve as negative control. In the case of a systemic PVX agroinfection experiment, marker genes such as *Gfp* are preferred as a negative control because PVX replication is reduced in the presence of alien inserts. Plants inoculated with the negative control show systemic mosaic symptoms, while those inoculated with a positive control (like pGR106-PVX::INF1) develop local HR lesions.

- The PVX agroinfection assay can also be performed in detached leaves.

- PVX vectors typically cannot accommodate inserts larger than 2 kb or intron-containing gene sequences.

- Virus-induced gene silencing may occur. Plants should be inoculated only once.

### Interpretation of results

For interpreting the results, a few features should be considered:

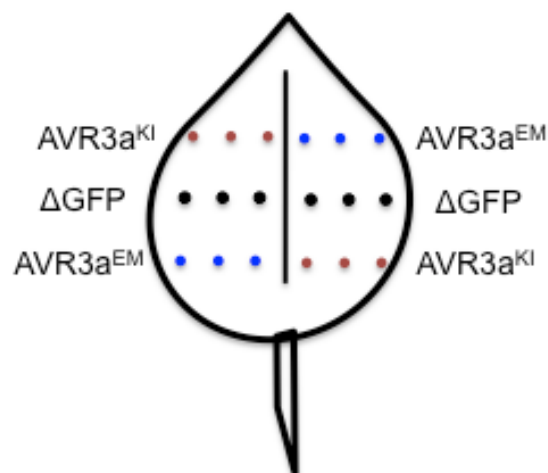
- Resistance to PVX is known to occur in some *Solanum* species, including *S. stoloniferum*, *S. brevidens*, *S. tuberosum* subsp *andigena*, *S. chacoense*, *S. acaule* and *S. sparsipilum*. This interferes with functional profiling by binary PVX.

- The size of the necrotic lesion is no measure for R-AVR activity, but depends on other aspects such as the virulence of PVX or the transformation efficiency of *A. tumefaciens* in the genetic background of the plant.
- High expression levels of single effector candidates may lead to false positives (e.g. by homologues of AVR proteins), perhaps by mimicking the homologous effector.
- In local scoring, extreme resistance can be misinterpreted as absence of response, and false negatives will occur. Switching to a systemic experiment can solve this. In the absence of cell death response, the virus will spread and cause mosaic symptoms, whereas the occurrence of cell death will either prevent viral spread (no systemic symptoms) or cause systemic cell death.
- During the genuine plant-pathogen infection, other effectors may interfere, and phenotypes of single applied effectors may differ from the natural situation.

### 3.2 Practical exercise

In this practical exercise we will express different alleles of *AVR3a* (i.e. *AVR3a<sup>KI</sup>* and *AVR3a<sup>EM</sup>*) by PVX agroinfection in WT and transgenic *N. benthamiana* plants that over-express the cognate resistance gene *R3a*. We will perform local scoring of the symptoms and will also analyze the different outputs of this assay: presence / absence of hypersensitive response and extreme resistance.

We will inoculate the leaves according to the diagram below (the diagram shows the adaxial side of the leaf) using binary pGR106-PVX-based constructs:



When performing the inoculation it is important to change gloves between different constructs since cross-contamination can easily occur and mislead the interpretation of the results.

#### 4. REFERENCES

***This booklet is based on the following book chapters (and references therein):***

- Kanneganti TD, Huitema E, Kamoun S. 2007. *In planta* expression of fungal and oomycete effectors. In 'Plant-Pathogen Interactions' P. Ronald, ed. Humana Press, 35-43.
- Vleeshouwers VGAA, Rietman H. 2009. *In planta* expression systems. In 'Oomycete Genetics and Genomics' K. Lamour and S. Kamoun, ed. John Wiley & Sons, 455-475.

***Other aspects and examples of the techniques described can be found in:***

- Bos JIB, Kanneganti TD, Young C, Cakir C, Huitema E, Win J, Armstrong M, Birch PRJ, Kamoun S. 2006. The C-terminal half of *Phytophthora infestans* RXLR effector AVR3a is sufficient to trigger R3a-mediated hypersensitivity and suppress INF1-induced cell death in *Nicotiana benthamiana*. *Plant Journal*, 48:165-176.
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- Vleeshouwers VG, Driesprong JD, Kamphuis LG, Torto-Alalibo T, Van't Slot KA, Govers F, Visser RG, Jacobsen E, Kamoun S. 2006. Agroinfection-based high-throughput screening reveals specific recognition of INF elicitors in *Solanum*. *Molecular Plant Pathology*, 7(6):499-510.
- Vleeshouwers VG, Raffaele S, Vossen JH, Champouret N, Oliva R, Segretin ME, Rietman H, Cano LM, Lokossou A, Kessel G, Pel MA, Kamoun S. 2011. Understanding and exploiting late blight resistance in the age of effectors. *Annual Review of Phytopathology*, 49:507-31.
- Vleeshouwers VG, Rietman H, Krenek P, Champouret N, Young C, Oh SK, Wang M, Bouwmeester K, Vosman B, Visser RG, Jacobsen E, Govers F, Kamoun S, Van der Vossen EA. 2008. Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. *PLoS One*, 3(8):e2875.

Additional notes:

- Other species (tomato, tomato fruit, Arabidopsis)
- Possibility of infiltrating other organisms
- VIGS
- Advantages and disadvantages of each technique