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In planta Expression of Oomycete and Fungal Genes

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Summary

Large-scale genome sequencing projects have generated a wealth of sequence information for plant pathogenic microbes such as oomycetes and fungi. Functional genomic approaches are essential to exploit the sequence information to identify pathogen effector genes that trigger cellular and molecular responses in plant cells. This chapter describes two functional assays, agroinfiltration and agroinfection. These assays allow rapid functional expression of pathogen genes in plants and can be used in high-throughput screens.

Key Words: Transient gene expression; *Potato virus X*; functional genomics; oomycetes; fungi; effectors; *Nicotiana benthamiana*; *Agrobacterium tumefaciens*; agroinfiltration; agroinfection.

1. Introduction

Advances in sequencing technologies resulted in extensive collections of gene sequences from a plethora of eukaryotic plant pathogens, including oomycete and fungal species. One major thrust in this post genomics era is to identify genes that are important for pathogenesis and virulence. One class of such genes encodes so-called effectors that manipulate host cell structure and function either by facilitating infection (virulence factors) or by triggering defense responses (avirulence factors or elicitors). Typically, ectopic expression of single effector genes in plant cells leads to phenotypic effects. For example, expression of avirulence (*Avr*) genes in plant cells that contain the matching resistance (*R*) gene usually results in the hypersensitive response (HR ***[1]***). Also, expression of effector genes in susceptible hosts can lead to phenotypic responses that may reflect virulence function ***(2–6)***.

The use of plants for heterologous gene expression traditionally involved integration of a transgene into the plant genome ***(7)***. The main setbackof this approach is the considerable time required for generating stable transgenic

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plants. The time interval may vary from weeks to months depending on the plant species. Alternatively, ectopic gene expression also can be accomplished using such transient expression systems as *Agrobacterium tumefaciens*-based transient transformation (agroinfiltration), viral expression systems (agroinfection), and particle bombardment. Transient expression systems have a number of advantages over stable transformation. These assays are rapid and simple to perform. They can be applied to fully differentiated plant tissues, thus allowing the analysis of cell death-inducing genes without inducible promoters. Additionally, these assays are not influenced by chromosomal positional effects ***(8)***. Therefore transient expression assays have become popular in the study of plant–microbe interactions and also have been applied to high-throughput analyses ***(2,5,6,9,10)***.

This chapter describes two methods, agroinfiltration and binary *Potato virus X* (PVX) expression (PVX agroinfection), that have proved successful in our studies on effectors of *Phytophthora* ***(5,6,10)***. Despite some limitations, these assays are crucial to modern molecular plant pathology research. In addition, they meet the demand for efficient and robust high throughput functional analysis in plants.

1.1. Agroinfiltration

Agrobacterium tumefaciens is the most commonly used agent in plant transformation experiments. This bacterium is a ubiquitous pathogen of plants. It enters 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, combined with a slightly acidic environment ***(11)***. 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. Although chromosomal integration of tDNA elements takes place during transformation, it is not known whether this is required for expression to occur. Nevertheless, the majority of plant cells in the infiltrated region express the transgene.

Ectopic expression of single pathogen genes in plant cells often leads to phenotypic effects. For instance, expression of bacterial, fungal, or oomycete *Avr* genes in plant cells that contain the matching *R* gene results in the HR ***(1)***.

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In situations in which an expressed effector gene is not recognized, other phenotypic changes, such as chlorosis, cell enlargement, cell division, or necrosis, can be observed ***(4)***. In either case, phenotypic assessments of infiltrated leaf areas can help identify effector genes and aid in subsequent functional characterizations.

1.2. PVX 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. To engineer viral vectors, viral RNA genomes are reverse transcribed in vitro and cloned as full-length complementary DNAs in transcription vectors. Insertion of foreign genes into plant viral genomes can be achieved using the following methods:

1. Gene replacement, in which nonessential viral genes such as the coat protein gene are replaced by the gene of interest.
2. Gene insertion, in which the gene of interest is placed under the control of an additional strong subgenomic promoter.
3. Gene fusion, in which the gene of interest is translationally fused with a viral gene ***(8,12)***.

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Among plant RNA viruses, PVX 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 ***(13)***. Original constructs required in vitro transcription of PVX RNA followed by rubbing inoculation onto plant leaves. However, more recently, David Baulcombe and collaborators (Sainsbury Lab, Norwich, UK) developed binary PVX 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 ***(14)***. 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.

The PVX agroinfection assay has emerged as a robust and reliable system to identify virulence and *Avr* genes from microbial and viral pathogens. Expression screens using the PVX vector facilitated the isolation and study of *Avr* and effector genes from fungal, oomycete, bacterial, and viral plant pathogens ***(2,3,5,6,9,15–18)***.

2. Materials

2.1. Agroinfiltration

1. *Nicotiana benthamiana* seeds.
2. LB solid agar media plates supplemented with 50 µg of kanamycin and 25 mg of rifampicin/mL.
3. *A. tumefaciens* strain GV3101 (*see* **Note 1**).
4. *A. tumefaciens* strain GV3101 containing binary vector constructs (*see* **Notes 2–5**).
5. YEB medium: 5 g of beef extract, 1 g of yeast extract, 5 g of bacteriological peptone, 5 g of sucrose, and 2 mL of 1 *M* $MgSO_4$/L.
6. 3'-5' Dimethoxy-4'-hydroxy acetophenone (acetosyringone): 100 m*M* stock in dimethyl formamide or 70% ethanol.
7. 2-[*N*-Morpholino] ethane sulfonic acid (MES).
8. MMA infiltration medium: 5 g of MS salts, 1.95 g of MES, 20 g of sucrose, pH adjusted to 5.6 with 1 *M* NaOH, and 200 µ*M* acetosyringone/L (*see* **Note 6**).
9. 1-mL Syringe.

2.2. PVX Agroinfection

1. *N. benthamiana* seeds (*see* **Note 14**).
2. LB solid agar media plates supplemented with 50 µg kanamycin/mL.
3. *A. tumefaciens* strain GV3101 (*see* **Note 15**).
4. GV3101 harboring pGR106 or pGR106 carrying a reporter gene as a negative control.
5. GV3101 harboring pGR106-INF1 ***(17)*** as a positive control.
6. Sterile toothpicks.

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3. Methods

3.1. Agroinfiltration

3.1.1. Growing N. benthamiana Plants for Agroinfiltration

1. Germinate *N. benthamiana* seeds in soil in a pot at 22 to 25°C with high light intensity. Cover the pots with cheesecloth to prevent drying and to provide adequate moisture.
2. After germination, remove cheesecloth and allow plants to grow for approx 1 to 2 wk.
3. Transplant 2-wk-old seedlings individually into separate Styrofoam cups containing soil and allow them to grow until they reach eight-leaf stage (*see* **Note 7**).

3.1.2. Agroinfiltration Assay Procedure

1. Streak recombinant *A. tumefaciens* strains onto LB solid agar media plates supplemented with 50 µg kanamycin and 25 µg rifampicin/mL and incubate at 28°C for 2 to 3 d.
2. Inoculate 3 mL of YEB cultures containing 50 µg of kanamycin and 25 µg of rifampicin/mL, with the recombinant *A. tumefaciens* strains and grow overnight (28°C, approx 225 rmp).

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3. Inoculate large YEB media suspensions (25–300 mL; *see* **Note 8**), containing 50 µg of kanamycin, 25 µg rifampicin/mL, and 2 µ*M* acetosyringone with the overnight culture. Grow cultures overnight at 28°C to an OD600 of approx 1.
4. Harvest the cells by centrifugation (4000*g* for 10 min), pour off the supernatant and resuspend the pellet in MMA medium to an OD of 2 (*see* **Note 9**).
5. Incubate and shake cells at room temperature for 1 to 3 h.
6. Place *A. tumefaciens* suspensions into a syringe. Carefully invert the leaf and hold the lower 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 (*see* **Note 10**). A movie on "how to agroinfiltrate" is availableat (http://www.sainsbury-laboratory.ac.uk/david-baulcombe/Services/AgroInfiltrationHP.htm).
7. Incubate the plants in a growth chamber or confined space at 22°C (*see* **Note 11**).
8. Response should be visible in 2 to 3 d after infiltration (*see* **Notes 12** and **13**).

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3.2. PVX Agroinfection

3.2.1. Growing N. benthamiana Plants for Agroinfection

1. Germinate *N. benthamiana* seeds in soil in a pot at 22 to 25°C with high light intensity. Cover the pots with cheesecloth to prevent drying and to provide adequate moisture.
2. After germination, remove cheesecloth and allow plants to grow for approx 1 to 2 wk.
3. Transplant 2-wk-old seedlings individually into separate Styrofoam cups containing soil.
4. Allow plants to grow until they reach four-leaf stage (*see* **Note 16**).

3.2.2. Agroinfection Assay Procedure

1. Streak recombinant *A. tumefaciens* strains onto LB solid agar media plates supplemented with 50 mg of kanamycin/mL and incubate at 28°C for 2 to 3 d (*see* **Notes 17** and **18**).
2. Toothpick-inoculate individual clones on the lower leaves of *N. benthamiana* plants 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 (*see* **Notes 19–21**).
3. Incubate the plants in a growth chamber or confined space at 22°C (*see* **Note 22**).
4. Response should be visible starting from 7 d after inoculation (*see* **Note 23**).
5. Strains carrying the recombinant constructs should be examined for altered viral symptoms and compared with the control strains. Strains carrying the vector pGR106 induce systemic mosaic symptoms, and strains carrying pGR106-INF1 induce local HR lesions *(5)*.

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4. Notes

4.1. Agroinfiltration

1. We prefer to use GV3101 because it electroporates at high frequency (>10^8 cfu/µg of DNA). This streamlines the cloning procedure, as ligation mixtures can be directly electroporated into *Agrobacterium*.
2. Several binary vectors can be used. Vectors based on the pCB300 series ***(19)*** or the pAvr9 vector ***(1)*** allow high expression of the candidate gene and have worked well in our hands.
3. Agroinfiltration can be used with large (>2 kb) genes. PVX does not permit expression of genes exceeding 2 kb.
4. 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. Binary vectors containing genes expressing marker proteins, such as β-glucuronidase (i.e., GUS) or green fluorescent protein, can be used to verify the level of transformation by agroinfiltration.
5. Several transgenes can be delivered into the same cell with agroinfiltration system facilitating simultaneous expression of interacting proteins (i.e., Avr and R proteins) or assembly of multimeric proteins.
6. It is recommended to make fresh MMA media by adding acetosyringone just before washing and incubation of the cell suspensions.
7. *N. benthamiana* plants that have healthy and fully developed leaves are desired in this assay. Infiltration of senescing leaves can lead to necrosis and reduced transformation rates.
8. The amount of infiltration media to be used depends on the size of the experiment and infiltration efficiency.
9. Infiltration with dense *A. tumefaciens* suspensions can lead to background necrosis. These problems can be avoided by using suspensions with lower OD600 values.
10. It is strongly recommended to practice and refine one's infiltration technique with water. Some users prefer to cause a slight wound on the leaf using a needle or a razor blade at the site of injection, which will facilitate infiltration of the bacterial solution.
11. Incubation temperatures of infiltrated plants should not exceed 28°C. *A. tumefaciens* transformation efficiency and transgene expression peaks at 22°C ***(20)***.
12. Detectable transgene expression should occur 2 to 3 d after infiltration. However, the timing of phenotypic changes varies depending on the effector tested.
13. The agroinfiltration system, unlike viral vectors, does not permit systemic expression of the foreign gene.

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4.2. PVX Agroinfection

14. PVX agroinfection is limited to host plants, such as *N. benthamiana*, *Nicotiana tabacum*, *Lycopersicon esculentum*, and *Solanum tuberosum*.

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15. We use GV3101 because it electroporates at high frequency (>10^8 cfu/μg of DNA). This streamlines the cloning procedure since ligation mixtures can be directly electroporated into *Agrobacterium*.
16. Younger plants at three to four leaf stages are preferable for inoculation if systemic symptoms are sought. For local responses, multiple clones can be inoculated on a single leaf. *See also* the method described by Takken et al. ***(9)*** for inoculation of 96 clones in tobacco leaves.
17. Always use pGR106 empty vector or a pGR106 carrying a reporter gene, such as *gfp*, as a negative control. The presence of an insert slows down virus infection so the use of a control vector carrying an insert of the same size as the candidate gene might be more appropriate than the empty vector.
18. It is advisable to use fresh cultures that are not older than 4 d.
19. Excess amount of *A. tumefaciens* can be used for toothpick inoculation.
20. Three leaves per plant can be used to serve as triplicates.
21. Inoculate a minimum of four plants for each construct.
22. Incubation temperatures of infected plants should not exceed 28°C. *A. tumefaciens* transformation efficiency peaks at 22°C. This is also the optimal temperature for virus replication.
23. Symptoms should be scored every day from 7 d postinoculation and until 15 to 21 d after inoculation.

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