

**PLANT-PATHOGEN INTERACTIONS:  
*TURNIP CRINKLE VIRUS* SUPPRESSION OF THE HYPERSENSITIVE  
REPOSE IN *ARABIDOPSIS THALIANA***

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**ABSTRACT:**

The presence of *turnip crinkle virus* (TCV) in *Arabidopsis thaliana* plants has previously been shown to suppress the ability of these plants to produce a hypersensitive response (HR) upon inoculation with pathogens that would normally elicit this defense response. The ecotype Columbia-0 was examined using wildtype TCV and non-pathogenic strains of *Pseudomonas syringae* pv. *glycinea* Race 4 containing virulence genes *avrRpt2*, *avrRpm1* and *avrRps4*. Transgenic lines of *A. thaliana* that express the TCV proteins p8, p9 or CP were also examined in an attempt to determine if these proteins play a role in suppression of the HR. Crosses of these transgenic lines were made in order to determine if binary combinations of these proteins were sufficient for HR suppression. In addition, assays were completed to determine if the inhibition of the HR correlated with suppression of resistance to the virulent *Pseudomonas syringae* pv. *maculicola* ES4236 *avrRpt2* growth in the plant. Finally, PR-1 protein expression was inspected by visual and quantitative GUS reporter gene assays to determine if TCV also played a role in inhibition of the plants ability to develop systemic acquired resistance (SAR).

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# 1. Introduction

## 1.1. Plant-Pathogen Interactions and the Gene-for-Gene Hypothesis

The importance of autotrophic life forms, like plants, for the survival of the rest of life on the planet is well understood. Perhaps equally significant is our reliance on plants, not only for food, but also for building supplies, clothing, paper, furniture, and a multitude of other effects. The industries are affected by loss of productivity caused by plant-pathogens. Therefore, plant-pathogens are a major problem for society.

Throughout history plant-pathogens have plagued humans by damaging crops grown for food and in many cases, in combination with other factors, have led to the starvation and death of millions of people. The Irish Potato Blight in the 1840's is a commonly known example but there are numerous others. In addition to the loss of human life, plant diseases cost millions of dollars in crop losses each year. The exact amount of loss is extremely difficult to estimate because our ability to measure loss is limited by sampling methods and the incidence of disease in crop fields. Costs may include such things as the added cost of quality control or harvesting and processing time.

The problem is compounded by insufficient and costly methods of controlling plant-pathogens through conventional methods such as pesticides. Indirect costs of using pesticides may actually outweigh the perceived benefits of using these toxic compounds. A 1992 study of pesticide costs in the US (Pimental *et al.*, 1992) reveals that pesticides cost at least \$8 billion per year.

Costs include many factors: the loss of fish, bird, wildlife, and natural predators of pests; increased resistance of pests to pesticides, and public health effects. It was estimated that \$787 million is spent on the 67,000 poisonings and 27 deaths in the United States annually. These figures plus the extent of potential plant disease associated with agricultural crops are compelling arguments for controlling plant disease.

Most plants are naturally resistant to a plethora of pathogens found in the wild. By understanding this natural resistance and the sub-cellular interactions that occur when a plant system is attacked by a pathogen, one may be able to prevent disease by increasing the resistance of plants to pathogens. It may be possible to alter susceptible plants by genetically engineering them to express the genes that confer resistance. Before this can be accomplished, we must understand how gene-products interact *in vivo* and what exactly leads to resistance.

After entry of the pathogen into the plant system, the plant may respond to infection with one of three general responses. If the plant is not able to support the growth of a pathogen, it will not be able to replicate, thrive, and cause disease in the plant. This general response is known as passive non-host resistance (Heath, 2001). On the opposite end of the spectrum, pathogens may be able to cause a systemic infection where the pathogen replicates and spreads from the initially infected cells to other cells of the plant. The plant may show physical characteristics of disease. This disease may interfere with normal growth and reduce the plant's lifespan and/or productivity. Another interaction

that could occur between plant and pathogen is the ability of the plant to actively combat the pathogen through activation of specific disease resistance compounds. In this process of active resistance, the plant recognizes that it is being invaded by foreign material and immediately initiates a response by which the growth of the pathogen is suppressed.

In the gene-for-gene resistance hypothesis, bacteria, viruses and fungi contain certain gene-products that host systems are able to recognize and initiate defense responses. One response is called the hypersensitive response (HR) (Keen *et al.*, 1990): at the point of entry, the cells immediately surrounding the area initiate a programmed cell death response causing necrotic lesions. The purpose of this cell death response is, in the case of viruses, to localized the virus in the dead tissue and prevent movement cell to cell to cause systemic infection. In the case of larger life-forms like bacteria that cannot cross the cell wall/membrane and live in the extracellular space, HR prevents the bacteria from obtaining the nutrients needed to grow and multiply.

HR seems to be induced by particular gene-products made by pathogens called *Avr* genes, the first of which was isolated from *Pseudomonas syringae* pv. *glycinea* (Staskawicz *et al.*, 1984). Gene-products can be recognized *in planta* by plant resistance genes (*R* genes) that produce gene-products (*R* proteins). It has been proposed there is direct or indirect interaction between the *R* gene-products and *Avr* gene-products, which elicits the HR (Flor, 1971). The model is comparable to the receptor-ligand model: the *R* gene-product acts as receptor and the *Avr* gene-product acts as the ligand. However, if the interaction is not

direct there must be a more complex model to explain the interaction between AVR and R factors. The Guard Hypothesis proposed by Van der Biezen and Jones (1998) suggests that an elicitor protein binds and inhibits the activity of a basal defense activator. The *R* gene-product then recognizes this complex and the defense mechanisms are activated.

## **1.2. Plant Resistance Gene-products (R proteins)**

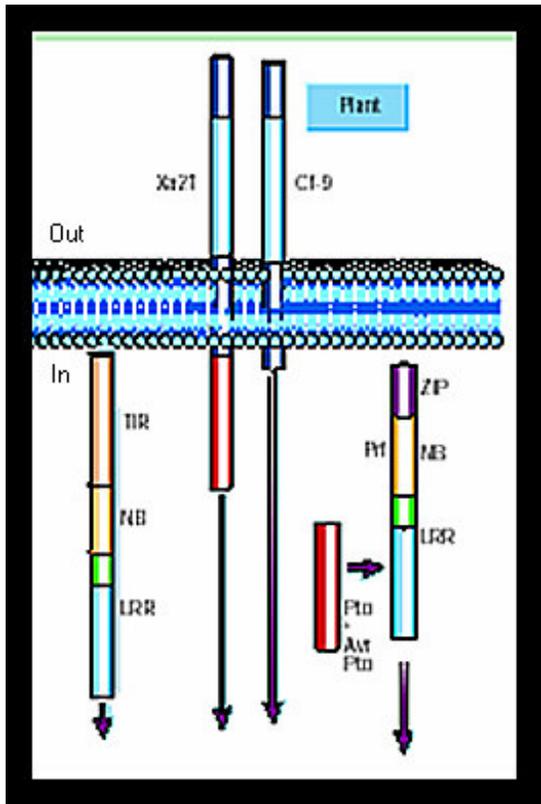
Currently there are five known classes of R proteins (Dangle and Jones, 2001). There are several physical characteristics that are common among almost all plant resistance genes. These are coiled coil (CC) or Toll-interleukin-1-resistance (TIR) domains, nucleotide binding sites (NBS), leucine rich repeats (LRRs), and kinase domains. The largest class of these proteins has an NBS followed by LRRs. RPS2 discussed in section 1.6, is an example of such an R protein. The RPS2-201 allele has a mutation at amino acid 668 from threonine to proline within the LRR region that is successful in preventing the AvrRpt2/RPS2 interaction that elicits an HR (Wolfe *et al.*, 2000). LRRs seem to be responsible for protein-protein interactions while the CC and TIR domains located on the N terminal end of the protein may play some role in transcription factor activation (Bent, 1996). Recently a new TIR domain protein, RRS1-R, has been identified and may extend the known classes of these proteins. This protein has a C-terminal extension with a putative nuclear localization signal (NLS) and a WRKY domain (Deslandes *et al.*, 2002). The other four existing R protein classes have varying structures. Two of these also contain LRRs that are contained outside

the plasma membrane, extracellularly, and are characterized by the presence or absence of kinase domains in the cytoplasm. Of the last two R protein subclasses, one has a cytoplasmic CC and is bound to the plasma membrane and the other has kinase domains and is free to move about the cytoplasm (Dangle and Jones, 2001). See Table 1.1 and Figure 1.1 for visual clarification.

**Table 1.1 R Protein Classifications**

Protein Class/Domains	Location
CC--NBS-LRR TIR-NBS-LRR	Membrane Bound or Intercellular
CC-LRR-Kinase	Extra-Intercellular
CC-LRR	Membrane Bound Extracellular
NBS-LRR-Kinase	Free Cytoplasmic

**Figure 1.1 Visual representation of R Protein Classifications**



*Figure 1.1* Depicts four of the five known classes of R-proteins. Extracellular signals can be transmitted into the cell through the interaction with LRRs of membrane bound proteins. Signals may also travel freely within the cell by using the free cytoplasmic R-proteins.

From Dangle and Jones, 2001

### **1.3. Biochemical Associations of the Hypersensitive Response**

One event associated with the hypersensitive response is the oxidative burst: the production of active oxygen species (AOS). These compounds include  $O_2^-$ ,  $H_2O_2$ , and perhaps  $\cdot OH$  (Apostol *et al.*, 1989). Genetic evidence has been given that supports the idea that the oxidative burst is a downstream component of the *RPS2* (*R* gene) / *avrRpt2* (*Avr* gene) gene-for-gene signal cascade that leads to the HR (Wolf *et al.*, 2000). The oxidative burst occurs before the HR and is not a byproduct of HR but seems to be a signaling factor for HR. It may be possible that this oxidative burst is responsible for general disease resistance (Yahraus *et al.*, 1995). There is also an association of salicylic acid (SA) accumulation with the defense responses to pathogens including the HR and systemic acquired resistance (SAR) (Delaney *et al.*, 1994; Keen, 1990). In addition, nitric oxide (NO) also seems to play an important role in HR cell death (Delledone *et al.*, 1994; Durner *et al.*, 1998). Other events that are associated with HR are increases in defense gene expression and strengthening of the cell walls with auto-fluorescent compounds (Glazebrook *et al.*, 1997).

### **1.4. Systemic Acquired Resistance**

Plants that have been induced to form HR by avirulent pathogens can also show resistance to subsequent inoculations of virulent pathogens. Such resistance to later attack by pathogens is called SAR. (Kuc, 1995; Sticher, *et al.*, 1997). SAR has three stages: the induction/immunization stage that begins with

AOS accumulation during HR, the establishment stage, which is accompanied by systemic micro-HR, and the manifestation stage, which occurs when the plant is challenged by a normally virulent pathogen (Alvarez *et al.*, 1998). *Arabidopsis* requires the accumulation of salicylic acid to induce SAR (Vernooj *et al.*, 1994; Dong, 1998). Repression of salicylic acid accumulation by expression of a bacterial salicylic acid hydroxylase gene (*NahG*) abolishes SAR (Delaney *et al.*, 1994). SAR is also associated with the induction of several pathogenesis-related (PR) proteins (Lawton *et al.*, 1995; Uknes *et al.*, 1993) and is regulated by NIM1/NPR-1 (Cao *et al.*, 1994; Delaney *et al.*, 1995). *PR-1* gene expression is considered a marker for general disease resistance and SAR.

### **1.5. Pathogen Avirulence Gene (*Avr* genes)**

*Avr* genes produce elicitors that act either directly or indirectly as signal molecules interacting with the R proteins in the host to elicit the defense response. In viral systems the elicitor is usually the direct product of the *Avr* gene whereas in bacterial systems the elicitor is usually a secondary messenger that is activated with the expression of the *Avr* gene. This stems from the idea that bacteria use different pathological mechanisms than their viral counterparts. Viruses attack cells from within the host cell whereas bacteria cannot typically penetrate the plant cell wall and must remain in the extracellular space (apoplast). Therefore bacterial elicitor proteins must enter the cell through a completely different mechanism (Buchanan *et al.*, 2000). This system is called the Type III effector secretion system where avirulence and virulence proteins

are delivered to the host cell through *hrp* pili from the apoplastic space (He, 1998; Hueck, 1998; Galan and Collmer, 1999). *Pseudomonas syringae* pv. *glycinea* Race 4 (*Psg* R4) is considered non-virulent in *A. thaliana* because growth of these bacteria are naturally inhibited after infiltration with these bacteria. Additionally, *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*) are virulent in *A. thaliana* (Dong *et al.*, 1991).

### **1.6. *RPS2/AvrRpt2* Gene-for-Gene HR Model**

The gene in Col-0 required for recognition of the avirulence factor AvrRpt2 and elicitation of HR is *RPS2*, which encodes for the RPS2 protein. There appears to be a direct interaction of RPS2 and AvrRpt2 inside the plant cell based on *in vitro* assay (Leister *et al.* 1996). It was thought based on these same *in vitro* studies that RPS2 was located in the cytosol. However, recent direct *in vivo* evidence suggests that RPS2 is actually membrane bound (Boyes *et al.*, 1998; Axtell and Staskawicz, 2003).

Bacteria which contain the plasmid vector expressing the avirulence gene *avrRpt2* elicit the normal gene-for-gene HR in *A. thaliana* plants carrying the corresponding R gene *RPS2* (Yu *et al.* 2000). The biochemical basis for HR and the corresponding resistance conferred by R proteins is just beginning to be understood. RIN4 is a factor that is physically associated with RPS2 *in vivo* and is required for proper RPS2 function (Mackey *et al.*, 2003). RPS2 may act as a guard of RIN4 which is consistent with information showing that over-expression of RIN4 inhibits RPS2 function and that elimination of RIN4 activates RPS2. In

addition, AvrRpt2 induces, independently of RPS2, the disappearance of RIN4. Another factor, NDR1, is required for RPS2-mediated HR and resistance (Century, 1995).

### **1.7. *RPM1/AvrRpm1* Gene-for-Gene HR Model**

RPM1 is similar to RPS2 in that they are both CC-NBS-LRR proteins (Grant *et al.*, 1995). Like RPS2, RPM1 may be a guard of RIN4 (Mackey *et al.*, 2002). RPM1 is a peripheral membrane protein that is degraded upon HR formation (Boyes *et al.*, 1998). Resistance conferred by the *RPM1* gene is mediated through interaction with bacterial avirulence factor AvrRpm1 or a sequence unrelated protein AvrB (Grant *et al.*, 1995). These effector molecules cause hyperphosphorylation of RIN4 independent of RPM1 which may reflect on these proteins' virulence activity (Mackey *et al.*, 2002).

### **1.8. *Arabidopsis thaliana* as a Plant Model for Pathogenesis Research**

*Arabidopsis thaliana* is an ideal candidate for research on plant-pathogen interactions. The plant is compact in size, spanning only a few inches in diameter when mature, and has a relatively short life cycle of 5-6 weeks from germination to seed. It contains a genome of about 125 Mb in five chromosomes making it the smallest genome among known flowering plants. The total number of genes the plant contains is 25,498 (Sparrow *et al.*, 1972; Leutwiler *et al.*, 1984; Meinke *et al.*, 1998; The Arabidopsis Genome Initiative, 2000). There are extensive amounts of natural variation in wild populations known as ecotypes making the

plant suitable for molecular genetic studies. By comparing normal development of this plant to morphology of mutant strains, the normal functioning of its genome can be elucidated (Bowman, 1994).

Three of the ecotypes used in this laboratory, Colombia-0 (Col-0; Li *et al.* 1990), Wassilewskija-1 (Ws-1) and Landsberg erecta (Ler) are susceptible to TCV. The Dijon-0 (Di-0) ecotype is partially resistant to TCV (Simon *et al.*, 1992). Di-0 was further segregated into Dijon-17 (Di-17) which was found to be completely resistant to TCV (Dempsey *et al.*, 1993) and the ecotype Dijon-3 (Di-3) which is completely susceptible (Dempsey *et al.*, 1993).

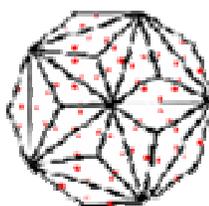
Many *R* genes have been identified and their loci determined in *A. thaliana* (Kunkel, 1996). These *R* genes respond to many pathogens including bacteria, fungi and viruses. Understanding gene interactions in *A. thaliana* will facilitate understanding of plant resistance in other species. *A. thaliana* responds to several studied viral pathogens including *tobacco mosaic virus* (Ishikawa *et al.*, 1993) and TCV (Simon *et al.*, 1992; Dempsey *et al.*, 1993). The *R* gene specific for TCV, HRT (Hypersensitive Response to ICV), was cloned by Cooley *et al.* (2000).

### **1.9. Turnip Crinkle Virus as Pathogen Model in Plant Disease Resistance**

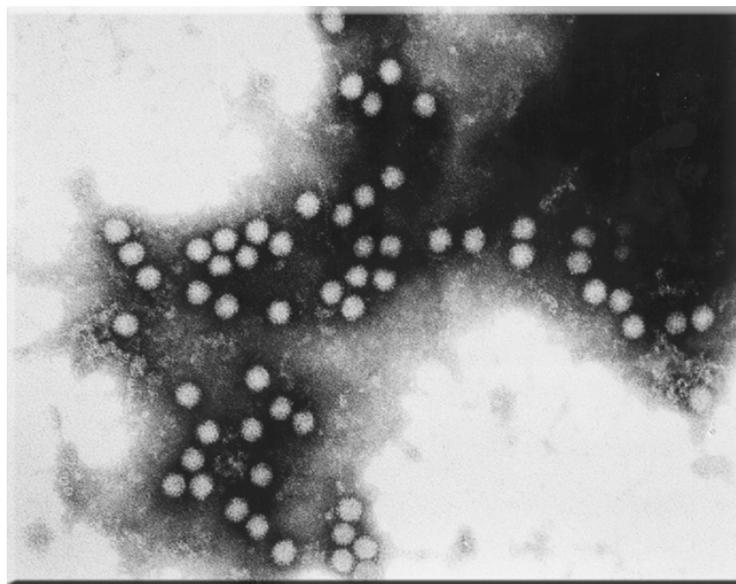
TCV belongs to a viral genus *Carmoviridae*. It has a positive sense single stranded (ss) RNA genome that consists of 4054 bases of known sequence and encodes five proteins p28, p88, p8, p9, the coat protein (CP; p38) and several sub-viral RNAs. Open reading frames (ORFs) 1 and 2 encode the replication

proteins p28 and p88 (White *et al.*, 1995). ORFs 3 and 4 encode the movement proteins p8 and p9 and ORF 5 encodes the coat protein (Carrington *et al.*, 1989; Hacker *et al.*, 1992). Virion particles are composed of 180 subunit copies of the coat protein as dimers arranged into an icosahedral three dimensional structures. An electron micrograph of the 34 nm virion particles can be seen in Figure 1.2 below.

**Figure 1.2 Structure of TCV particle**



(<http://www.tulane.edu/~dmsander/WWW/335/335Structure.html>)



Taken from Brunt *et al.* (1996)

(<http://biology.anu.edu.au/Groups/MES/vide/>)

### **1.10. Turnip Crinkle Virus and *Arabidopsis thaliana* Interactions**

TCV has been previously shown to reduce or eliminate the HR in *A. thaliana* due to subsequent inoculation of TCV (Hammond, 2001). A small percentage of TCV-inoculated Di-17 plants display HR upon initial inoculation of

TCV and subsequently develop systemic infection. A challenge inoculation with the same virus on systemically infected tissue fails to produce the HR seen upon initial inoculation. It appears as though the presence of TCV is suppressing the HR to itself. The ability of TCV to accomplish this is perhaps an example of the constant struggle for plants and their pathogens to develop new methods for effective survival. The mechanism(s) by which TCV initiates the suppression of the HR is not known; however, the answer must lie in the interaction of one or more TCV proteins with the resistance pathways of *A. thaliana*. In order to study this HR suppression Hammond devised a system that would be more efficient, since only a small number of these Di-17 plants became systemically infected. Work done by Hammond (2001) suggested Col-0 plants, which are 100% susceptible to TCV, systemically infected with TCV have a suppressed ability to form the HR. Additional experiments are needed to confirm this result. If confirmed, further research will be needed to identify the component of TCV responsible for the suppression and it will be necessary to elucidate the active defense response that is affected using transgenic plants.

### **1.11. The use of Transgenic Organisms in Research**

Transgenic *A. thaliana* plants created from Ws-1 ecotype to contain TCV proteins p8 and p9 and from Col-0 to contain CP are used in this thesis. These transgenic plants are used to determine if any of these proteins may be responsible for the suppression of HR. Use of PR-1::GUS transgenics will allow determination of the *PR-1* induction status of HR suppressed plants. The reporter

gene, *β-glucuronidase* (GUS), allows the histological detection of *PR-1* expression. The enzyme catalyzes cleavage of substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide (X-gluc) into dichloro-dibromoindigo (CIBr-indigo) (Pearson, *et al.*, 1961). The pigment immediately precipitates out of solution, which may be useful in structural studies. Another substrate, *p*-nitrophenyl  $\beta$ -D-glucuronide (PNG), may also be used to quantitatively measure GUS activity spectrophotometrically.

## **2. Materials and Experimental Techniques**

### **2.1. Plant Growth Conditions**

*A. thaliana* was grown on a 14 day/10 night hours cycle at 23.0°C and 21.0°C respectively. Flats with eight pots each containing non-sterilized Pro Mix Bx (Premiere Horticulture Inc., Red Hill, PA) were used. The chambers used for growth were Percival Scientific AR-60L growth chambers. Each pot contained nine plants spaced to minimize crowding and competition for light and nutritional resources giving a total of about seventy-two plants per flat. When the seedlings were fourteen days old the flats were fertilized with “Miracle Grow”™ once a week at a concentration of 0.35 g/L. Plants were watered every three to four days or as needed.

### **2.2. Seed Stocks**

Seeds were collected via two methods depending on the number of seeds required for propagation. Individual siliques were cut onto white paper and opened with wooden skewers. After harvest the paper was rolled up several times to prevent seeds from falling out and the seeds were allowed to dry in the paper for several days. Mass collection of seeds was done by allowing a flat of plants to self fertilize and develop mature seed stalks. When the majority of the stalks began to dry the stalks of all plants were cut off onto several sheets of white paper overlapped. The semi-dry stalks were rubbed together and the seeds and siliques halves were allowed to fall onto the papers. When the desired

amount of seed was obtained the seed stalks were discarded and the siliques with seeds transferred to one sheet of paper to be dried as described above. After drying, the siliques would open naturally and the seeds would fall onto the paper. Being denser than the siliques, the seed would drop to the bottom of the pile of siliques. The siliques were discarded and the seeds filtered over a #30 mesh before being transferred to labeled eppendorf tubes for storage.

### **2.3. Crosses**

Crosses of transgenic plants were made by allowing plants to mature until seed production. When the stalks were about six inches high and producing a constant supply of flowers three to four unopened flower buds were carefully isolated. All open flowers and buds that showed any white were considered too mature and were removed. The remaining buds that were too small to be utilized were also removed. Each bud was then carefully opened, using precision tweezers (Ted Pella Inc. Model # 505-NMX3, Redding, CA) removing the outer protective layers first. Then all the petals and undeveloped stamen were removed leaving only the pistil. Mature stamens from plants that were desired to be crossed were removed and gently brushed against the tip of the pistil. This was repeated with several stamens of different flowers of the same plant to ensure an adequate dusting. The pistil was then wrapped loosely in plastic wrap until the seed pod had matured to about a centimeter in length. The wrap was removed while the siliques were still green and the cross was labeled for identification.

## 2.4. Bacterial Transformation by Electroporation

*E. coli* DH5 $\alpha$  electro-competent cells were obtained from -80°C freezer and thawed on ice. DH5 $\alpha$  cells were mixed with 1  $\mu$ g or less DNA plasmid. GIBCO BRL Electroporation Apparatus (Life Technologies., Rockville, MD) was used for electroporation and the procedures were carried out following the manual supplied by the manufacturer. The cells were electroporated at 4 kv and allowed to sit for 10 min. The cells were then added to 1.0 ml of S.O.C. media (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl<sub>2</sub>, 10mM MgSO<sub>4</sub>, 20mM glucose) and incubated at 37°C for 1 hr on a rotor set to maximum speed. The incubated mixture was then plated in two aliquots on LB plates containing 100  $\mu$ g/ml ampicillin and incubated at 37°C for 16 hrs.

## 2.5. DNA Maxi Prep

DNA maxi preparation was carried out essentially as described in Sambrook *et al.* (1989). One transformed colony isolated from the LB plate obtained above was transferred into 500 ml of L-broth medium containing 100  $\mu$ g/ml of ampicillin and incubated for 16 hrs at 37°C with shaking (220 rpm) after the initial 3 hours of growth 2.5 ml of 34.0 mg/ml chloramphenicol was added to the culture. The next day the culture was poured into a 500 ml centrifuge bottles and centrifuged at 4000 rpm for 15 min at 4°C. The pellet was washed by resuspending in 50 ml of ice-cold STE solution (0.1M NaCl, 10 mM Tris.HCl (pH 8.0), 1mM EDTA (pH 8.0) and centrifuged again. The pellet was resuspended in 10.0 ml GTE in a 50

ml centrifuge tube (Solution 1: 50 mM Glucose, 25 mM Tris.HCl (pH 8.0), 10 mM EDTA (pH 8.0)) 1.0 ml lysozyme solution (10 mg/ml) was added to the culture and mixed. Then 20.0 ml of Solution II (0.2 N NaOH, 1.0% SDS w/v) was added and the solution mixed by inversion. The tube was placed on ice for 7.5 min. 15 ml of ice-cold Solution III (60.0 ml 5M KOAc, 11.5 ml galacial acetic acid, 28.5 ml DI water) lysis solution was added, thoroughly mixed and incubated on ice for 10 min. The solution was then centrifuged at 4000 rpm at 4°C for 15 min and the rotor was allowed to stop without breaking. The supernatant was transferred to a fresh tube and centrifuged again for 18 min. The mixture was then poured through four layers of cheesecloth into another new centrifuge tube and the temperature was allowed to warm to room temperature (RT). 27 ml of isopropanol was added to the supernatant and kept at RT for another 10 min. The tube was then centrifuged at 5,000 rpm for 10 min. The supernatant was drained and the pellet rinsed with 70% EtOH. The pellet was then resuspended in 3.0 ml of TE buffer and transferred into a 30 ml oaks ridge tube and 3.0 ml ice cold 5 M LiCl was added. The solution was mixed and centrifuged for 15 min at 10,000 rpm and 4°C. The supernatant was transferred to a new oak ridge tube and an equal volume of isopropanol was added. The tube was centrifuged at RT for 15 min at 10,000 rpm. The supernatant was decanted and the pellet rinsed with 70% EtOH. The pellet was air dried and resuspended in 500 µl TE buffer with RNase A (20 µg/ml) and transferred to a microfuge tube. The tube was allowed to sit at RT for 30 min before adding 500 µl PEG (13%) and 1.6 M NaCl. The tube was centrifuged at 14,000 rpm for 8 min and the pellet resuspended in

300 µl TE buffer. This was Phenol:Chloroform extracted and the aqueous layer transferred to a new tube. 1.0 ml of 100% EtOH was added and the tube stored for 10 min at RT. The tube was centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatant removed. To this was added 200 µl 70% EtOH and vortexed briefly. The tube was centrifuged again for 2 min and the supernatant removed. The remaining EtOH was evaporated and 500 µl TE buffer (pH 8.0) was added. The concentration was measured by spectrophotometer. The DNA was stored at -20 °C.

## **2.6. *In Vitro* Transcription**

Plasmid DNA (20 µg) was cut with *Xba*I in 100 µl NE BioLabs buffer 2 and ethanol precipitated. The following reagents were allowed to thaw on ice: 10x transcription buffer, 10 mM each NTP: ATP; GTP; CTP; UTP, RNasin (Promega, Madison, WI), *Xba*I cut DNA, T7 RNA polymerase (New England BioLabs Inc., Beverly, MA) and DEPC treated water. To 28 µl DEPC treated water was added in the following order: 10 µl 10x transcription buffer, 10 µl each NTP, 2 µl RNasin, 12 µg DNA template, and 8 µl (400 units) T7 RNA polymerase. The reaction was incubated at 37 °C for 2 hours before adding RQ1 DNase (1 µl) (Promega, Madison, WI). The reaction was further incubated for an additional 15 min. To the reaction was added 25 µl 5M ammonium acetate before one phenol/chloroform/isoamyl alcohol extraction. The supernatant was removed to a fresh tube and 250 µl ice cold 100% EtOH was added. The RNA was precipitated for a minimum of two hours before centrifugation at 14,000 rpm for 10 min. The

RNA was resuspended in 100  $\mu$ l DEPC treated water and the concentration determined by spectrophotometry.

## **2.7. RNA Protocols**

### **2.7.1. RNA Extraction from Plant Tissue**

For 0.1 gram of plant tissue (approximately two *Arabidopsis* leaves): Tissue was ground in liquid nitrogen in a mortar and pestle that had been treated to remove RNase. The tissue should be an extremely fine powder. To this was added 500  $\mu$ l GTC buffer (4.0 M guanidium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% sodium lauryl sarcosine) containing 5.0  $\mu$ l  $\beta$ -mercaptoethanol added to the buffer freshly. After the buffer began to thaw the mixture was homogenized further until the buffer was completely melted. The mixture was then removed to an eppendorf tube and 50.0  $\mu$ l of 2.0M sodium acetate was added and the tube vortexed. All samples were processed to this point and place on ice before adding 500  $\mu$ l phenol/chloroform/isoamyl alcohol and vortexing for 30 seconds. The tubes are centrifuged at 7000 rpm for 10 min and the aqueous phase was removed to a fresh tube. To this was added 500  $\mu$ l of ice cold isopropanol and the tubes placed at -20°C for at least 10 min. The tubes were then centrifuged at 14,000 rpm for 10 min and the supernatant was discarded. The pellet was then resuspended in 75  $\mu$ l DEPC treated water as efficiently as possible (the pellet will not dissolve completely). 75  $\mu$ l 4M LiCl was added and the tubes incubated on ice for two to four hours. The tubes were then centrifuged at 14,000 rpm for 10 min and the pellet resuspended in 75  $\mu$ l resuspension buffer (0.5% SDS, 10 mM

Tris.HCl pH 7.5, and 1.0 mM EDTA). The solution was extracted twice with 50  $\mu$ l phenol/chloroform/isoamyl alcohol and the aqueous phase was removed to a fresh tube. To this was added 10  $\mu$ l 1M sodium acetate and 60  $\mu$ l isopropanol. The RNA was allowed to precipitate overnight at -20°C. The next day the RNA was isolated by centrifuging at 14,000 rpm for 10 min. The resulting pellet was resuspended in 20  $\mu$ l DEPC treated water.

### **2.7.2. RNA Gel Electrophoresis**

10x MOPS buffer was prepared as stock solution. (For 500 ml use 20.9 g MOPS, 13.3 ml 3M sodium acetate, 10.0 ml 0.5 M EDTA, and 440 ml DI water, adjust pH to 7.0 and bring volume to 500 ml. Store covered with foil at RT.) 1.0% RNA gel was prepared by melting 0.5 grams agarose in 5.0 ml 10x MOPS and 36.075 ml dH<sub>2</sub>O. The temperature was brought to 60°C and 8.925 ml 37% formaldehyde was added in a hood and the flask was swirled to mix. Gels were poured in the hood and allowed to solidify for at least one-half hour. Just before loading sample the gel was run empty for 5 min at 50 volts in 1X MOPS buffer.

While the gel was solidifying the samples were treated in the following manner. For a final volume of 10.0  $\mu$ l combine 5.0  $\mu$ l deionized formamide, 1.75  $\mu$ l 37% formaldehyde, 1.0  $\mu$ l 10X MOPS buffer, and 2.25  $\mu$ l RNA (at least 1.0  $\mu$ g of RNA should be loaded per lane to ensure visualization). The mixture was incubated for 15 min at 55°C and then 2.0  $\mu$ l bromophenol blue (500  $\mu$ l 100% glycerol, 2  $\mu$ l 0.5M EDTA, 25  $\mu$ l bromophenol blue, and dH<sub>2</sub>O to 1.0 ml) and 1.0

$\mu$ l ethidium bromide was added to each sample. The gel was loaded and run at 47 volts for 2 hours and 45 min.

### **2.7.3. Northern Blot**

Northern hybridization was performed essentially as described by Sambrook *et. al.*, (1989). After RNA gel electrophoresis was performed samples were transferred to a Magnacharge 0.45 Micron nylon membrane (Osmonics, Westborough, MA) overnight (12-18 hours). Then the RNA was cross-linked to the membrane with a Fisher (Pittsburgh, PA) UV cross linker model # FB-UVXL-1000 using the optimum crosslink setting. The membranes, wrapped in mesh, were pre-hybridized at 68°C for two hours with 6X SSC, 2X Denhart's Reagent and 0.1% SDS. The pre-hybridization solution was removed and hybridization solution (6X SSC, 0.5% SDS, 100  $\mu$ g/ml denatured fragmented salmon sperm DNA) was added with  $\alpha$ -<sup>32</sup>P labeled probe at 68°C for 24 hours. The membranes were then washed with 1X SSC, 0.1%SDS for 15 min at RT and twice with 0.2 X SSC, 0.1%SDS for 20 min each at 68°C. The blots were exposed to Kodak X-OMAT AR film at -80°C for two days with intensifying screens. The film was developed per standard protocol.

## **2.8. DNA Extraction from Plant Tissue**

DNA was extracted using Plant DNAzol ® Reagent (Life Technologies, Carlsbad, CA) and their proprietary protocol which can be found at [www.stratagene.com](http://www.stratagene.com). The protocol was modified slightly. The reagent was added directly to the mortar as described above for RNA extraction. All

centrifugation was done at 12,500 rpm at 4 °C. It is important to note that after the initial precipitation of DNA the pellet was usually spread out along the side of the centrifuge tube. The pellet was recovered by scraping the inside of the tube with the tip of the pipette. When finished, the DNA was allowed to dissolve for several hours at RT before removing the insoluble material by centrifugation. The concentration of DNA was then quantified by spectrophotometer. The DNA was stored at -20 °C until needed.

### **2.8.1. Southern Blot**

DNA samples (10 µg) were digested with 20-40 units (1-2 µl) EcoRI at 37 °C overnight. An additional 1 µl of enzyme was added after the initial six hours. DNA was then precipitated with 100% EtOH by adding 1 µl glycogen, 1/10<sup>th</sup> volume 3M sodium acetate pH 5.2 and 2.5 volumes EtOH. DNA was allowed to precipitate for 3 hours to overnight. The DNA was centrifuged at 14,000 rpm for 10 min and the pellet washed once with 80% EtOH and repelleted. The DNA was resuspended in 10 µl sterile water. The DNA samples were loaded with orange G on 0.8% agarose gel containing ethidium bromide. The gel was run overnight (about 10 hours) at 23 volts and photographed. The gel was allowed to shake on a platform with ten volumes of denaturing solution (1.5M NaCl, 0.5M NaOH) for 45 min. The gel was rinsed with distilled water and shaken with neutralization solution (1.5M NaCl, 1M Tris.HCl (pH 7.4) for another 45 min. While the gel was neutralizing a transfer pyramid was set up by placing a support in a larger container and filling with 20x SSC to just below the top of

the support. A piece of Whatman paper was placed on top of the support and allowed to soak up the 20x SSC. A fresh single edged razor blade was used to cut a section of Magnacharge 0.45 Micron nylon membrane (Osmonics, Westborough, MA) 1mm wider than the gel and the lower left hand corner was removed for later orientation with the gel. The membrane was floated on the surface of a volume of water and allowed to submerge for 5 min after which time the membrane was transferred to a volume of transfer buffer for at least 5 min. When the gel was neutralized it was placed bottom side up on the wetted Whatman paper so that no air bubbles were between the gel and the papers. Plastic wrap was placed over the edges of the gel before placing the wet membrane on the gel as to avoid letting any air bubbles become trapped beneath the membrane. Two pieces of Whatman paper the same size as the gel soaked in 2x SSC were placed on top of the membrane and a stack of paper towels just smaller than the paper were placed on top of that. A weight was placed on top of the paper towels and the DNA was allowed to transfer overnight. The next day the pyramid was disassembled and the well positions marked with a ball point pen. The membrane was rinsed with 2x SSC and allowed to dry on a stack of paper towels. The DNA was immobilized by irradiating DNA side up with a UV crosslinker set to optimal. Dry membranes were stored at RT between two sheets of Whatman paper until needed for hybridization.

## 2.9. Virus Inoculation

Inoculations of *A. thaliana* were carried out using a concentration of 0.1 µg/µL total RNA in 1x inoculation buffer (0.05M Glycine, 0.03M K<sub>2</sub>HPO<sub>4</sub>, and 1% celite, modified from Dempsey et al., 1993). Plants were inoculated 21-25 days post planting (dpp) depending on experiment via a procedure modified from Dempsey et al. (1993). Total volume of inoculant was determined by the number of leaves per flat to be inoculated. On average two leaves per plant were inoculated (n = 80-149 +/- 8 leaves). 1.0 µL was pipetted onto the surface of leaves and gently rubbed with a glass rod. The glass rod was disinfected with 50% bleach solution and rinsed with distilled water prior to each set of inoculations. Mock inoculations for controls were done with 1x inoculation buffer alone prior to all inoculations with TCV to prevent accidental inoculation of mock plants with infectious RNA. Because the leaves that become systemically infected are the newest leaves to arise after TCV inoculation, the smallest leaves that are present at the time of TCV inoculation are marked with a Sharpie™. All leaves that arise after the marked leaf will be systemically infected with TCV.

## 2.10. Bacterial Growth Conditions

The bacterial cultures used were *Pseudomonas syringae* pv. *glycinea* Race 4 (*Psg* R4) and *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*). Bacterial cultures were grown using NYG media for *Psg* and King's B media for *Psm* (Table 2.1(Turner *et al.*, 1984)). Media was prepared by dissolving all

components except antibiotics, adjusting the pH to 7.0 and autoclaving for 30 to 60 min. It is important to note that for King's B media one should not add the sterile  $\text{MgSO}_4$  until after autoclaving the rest of the components and the temperature cools to  $50^\circ\text{C}$ . Sterile kanamycin stock solution (10 mg/ml) was added to the autoclaved NYG media for a final concentration of  $25\ \mu\text{g/ml}$  using aseptic technique. Kanamycin stock solution was made using sterile  $0.22\ \mu\text{m}$  filters (Gelman No 4192, Ann Arbor, MI) and 20 ml syringes (BD No. 309661, Franklin Lakes, NJ). Streptomycin and tetracycline were added to King's B media after cooling to  $50^\circ\text{C}$  for a final concentration of  $100\ \mu\text{g/ml}$  and  $10\ \mu\text{g/ml}$  respectively. Streptomycin stock was made by dissolving 10 mg/ml water and sterile filtering as above. Tetracycline stock was made in 50% EtOH at a concentration of 5 mg/ml. All antibiotic stocks were stored in the dark under aluminum foil. Liquid media was dispensed in 5 ml aliquots to culture tubes and stored at  $4^\circ\text{C}$ .

**Table 2.1 Media Preparation**

Preparation of media (1 liter)	Liquid King's B (solid)	Liquid NYG
Reagents		
Proteose Peptone 3	20.0 g (10.g)	5.0 g
Yeast Extract	n/a	3.0 g
Glycerol	40.0 ml 25% glycerol	80.0 ml 25% glycerol
$\text{K}_2\text{HPO}_4$ (anhydrous)	6.0 ml 1.43M $\text{K}_2\text{HPO}_4$	n/a
$\text{MgSO}_4$	6.0 ml 1.0M $\text{MgSO}_4$	n/a
Agar	12.0 g (solid only)	15.0 g (solid only)
Kanamycin	n/a	2.5 ml 10 mg/ml stock
Streptomycin	10 ml 10 mg/ml stock	n/a
Tetracycline	2 ml 5 mg/ml stock	n/a

Bacterial preparation for infiltration into *Arabidopsis* was modified from Stone et al. (2000). Plates containing the appropriate media and antibiotic were streaked from bacterial stocks one at a time. Careful attention was given to plate the bacteria with speed as to avoid thawing of stock bacteria. The plates were then incubated at RT for three to four days after which time the plates were kept in the refrigerator at 4°C for one to two weeks of use. These plates were periodically re-plated from frozen stocks to ensure viability of bacterial cultures. Several individual colonies were taken from the plates and placed in culture tubes containing 5 ml of liquid media and antibiotic. The bacteria in these tubes were incubated overnight on a roller drum at ambient temperature. 200-500 µL of saturated culture solution was placed into 5 ml of fresh media plus antibiotic the following day and the new tubes placed onto the roller drum. The bacteria were allowed grow for approximately 8 hours and the absorbance read at 595 nm after blanking with fresh media. The absorbance was typically between 0.5 and 0.9. The cultures were then poured into 15 ml Falcon tubes and centrifuged at 3000 rpm using a Sorvall™ GSA rotor at 4°C (Dupont, Wilmington, DE). The supernatant was poured off and the pellet resuspended in 5 ml of 10 mM MgSO<sub>4</sub>. The bacteria were spun down once more to wash the bacteria of any remaining media and the supernatant was poured off. The resulting pellet was resuspended in a few ml of 10 mM MgSO<sub>4</sub> and the absorbance measured at 595 nm after blanking with fresh 10 mM MgSO<sub>4</sub>. This solution was then used to make a suspension with an absorbance reading of 0.2 A<sub>595</sub> for visual HR experiments and 0.002 A<sub>595</sub> for *in planta* bacterial growth assays and GUS assays. Only one

lateral half of each leaf was infiltrated in visual HR experiments otherwise infiltration was done throughout the leaf.

### **2.11. Infiltration of Bacteria**

After TCV inoculation the plants were allowed to become systemically infected. On the appearance of fully matured mildly symptomatic leaves (approximately 7-10 dpi) about 0.1 ml of bacteria solution was pressure infiltrated using a 1 ml syringe without the needle into the backside of these symptomatic leaves. Visual data was collected at the 24-48 hrs post infiltration.

### **2.12. *In planta* Bacterial Growth Assays**

Leaves are TCV or mock inoculated with 10 mM MgSO<sub>4</sub>. TCV inoculated plants are allowed to become systemically infected and at 7-10 dpi are infiltrated with bacteria. Leaves that have been infiltrated with 0.002 A<sub>595</sub> inoculum were harvested at 0,2,3 and 4 dpinf. Two 0.4 cm diameter leaf disks were excised using a cork borer and placed in eppendorf tubes containing 100 µl 10 mM MgSO<sub>4</sub>. The tissue was homogenized and the green solution 10x serially diluted to 10<sup>-4</sup> on a 96 well microtiter plate. Pipette tips must also be ejected after each dilution to prevent contamination. 10 µl of dilutions 10<sup>0</sup> to 10<sup>-3</sup> were plated in duplicate on media plates for day 0-4 for *Psg* R4 experiments and day 0 for *Psm* experiments. For *Psm* experiments day 2-3 10<sup>-1</sup> to 10<sup>-4</sup> dilutions were plated. (These guidelines should be sufficient but dilutions to be plated should be determined empirically). Non-virulent bacteria (*Psg* R4) will not grow more than about one log whereas virulent bacteria (*Psm*) should grow 3-4 logs. Avirulent

bacteria (*Psm*) may grow 1-2 logs or more if resistance is suppressed.) The plates were sectioned into quarters to facilitate ease in counting of bacteria. At least five leaves per group per time point were sampled. Duplicate plates received one leaf's worth of data. Careful attention to serial dilution and plating must be made to ensure reproducible results. Pipette tips were wiped clean with a Kim™ Wipe (Kimberly-Clark, Dallas, TX) after drawing the first 10  $\mu$ l of  $10^0$  dilution. Contamination of dilutions was kept to a minimum by using this technique.

### **2.13. Histological Detection GUS Assay**

GUS transgenic plants were TCV or mock inoculated at 21 dpp. Plants were allowed to develop systemic infections and systemically infected leaves were infiltrated with *Psg* R4 at a concentration of 0.002  $A_{595}$ . Whole leaves that have been infiltrated with bacteria were excised at several time points, 0, 18, 24, and 30 hours post infiltration. These leaves were then submerged in 0.25 ml visual GUS assay buffer (Table 2.2) for 12-24 hours in a 24 well micro titer plate at 37°C. Leaves were removed to a fresh plate containing several ml of 100% EtOH to remove the chlorophyll pigment. After about 24 hours the leaves were photographed and qualitatively rated for GUS activity by the extent of blue color.

**Table 2.2 X-gluc Reagent Mix**

Stock Solution	Final Concentration	Reagent ( $\mu$ l)
1.0 M NaPO <sub>4</sub> Buffer, pH 7.0	0.1 M	100
0.25 M EDTA, pH 7.0	10 mM	40
0.005 M Kferrocyanide, pH 7.0	0.5 mM	200
0.01 M X-Gluc	1.0 mM	50
10% Triton X-100	0.10%	10
Deionized Water	NA	600

(Stomp 1992)

## 2.14. Quantitative GUS Assay

GUS transgenic plants were TCV or mock inoculated at 21 dpp. Plants were allowed to develop systemic infections and systemically infected leaves were infiltrated with *Psg* R4 at a concentration of 0.002 A<sub>595</sub>. Whole leaves that were infiltrated with bacteria were then excised at time points 24 and 48 hours post infiltration. Leaf tissue was ground in liquid nitrogen in an eppendorf to form a fine powder and 200  $\mu$ l of ice cold Homogenization Buffer I (See Appendix A: Soluble GUS Assay Reagent Preparation) was added. The powder was further homogenized and an additional 200  $\mu$ l of Homogenization Buffer I was added to rinse the pestle. The samples were then centrifuged at 7000 rpm for 10 min and the supernatant collected as total protein extract. Samples were kept on ice until all samples were processed.

The spectrophotometer was allowed to warm up for at least 15 min. While the spectrophotometer was warming 5  $\mu$ l of each sample was placed (in triplicate) in a disposal cuvette pre-labeled with sample type and number. A standard curve was made by adding the appropriate amount of 0.2 mg/ml Human

IgG (Fisher #BP268550, Pittsburg, PA) (in triplicate) for 0, 1, 4, 8, 12, 16 and 20  $\mu\text{g}$  and the volume brought up to 200  $\mu\text{l}$  with Soluble GUS Assay Buffer (without the DTT and PNG) (see Appendix A: Soluble GUS Assay Reagent Preparation) in a disposable cuvette. 800  $\mu\text{l}$  of 1x Bradford Reagent (BioRad, Hercules, CA) prepared according to the manufacturer's directions was added to each cuvette and vortexed starting with the standard curve samples through total protein samples. Samples were then read at  $A_{595}$  against a blank Bradford reaction containing 200  $\mu\text{l}$  GUS Assay Buffer. Fresh GUS Assay Reagent was prepared by adding DTT and PNG to GUS Assay Buffer. PNG must be kept on ice prior to adding to buffer. GUS Assay Reagent (800  $\mu\text{l}$ ) was added to pre-labeled eppendorf tubes and placed at 37°C. 200  $\mu\text{l}$  of each protein extract sample was then added to the eppendorf tubes and the time noted after each addition. 100  $\mu\text{l}$  of each reaction was removed and placed into a cuvette containing 800  $\mu\text{l}$  of Stop Solution and the time noted. The cuvette was vortexed briefly and returned to the 37°C water bath. This was repeated for a total of three time points approximately 15 min apart. When all samples were collected the absorbance was read at 405 nm against a stopped blank reaction. The slope of the time points (absorbance/time) was determined and used to calculate the rate of reaction in nanomoles product formed per minute per mg of protein. The equation to determine this is  $\text{Rate} = S / (0.02 \times V \times \text{protein concentration})$ , where S equals the slope of the time points and V is the volume of reaction assayed (in this case  $V=0.02$  ml). The 0.02 in the equation comes from the molar extinction coefficient of *p*-nitrophenyl which is 18,000 (Lessard, 2001).

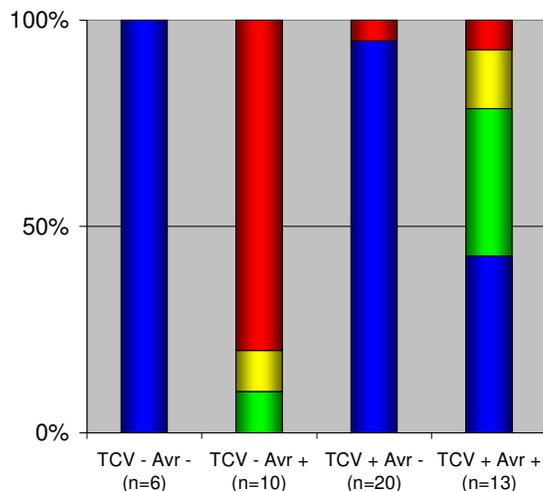
### 3. Results and Discussion

The basis for this thesis comes from the following observation: a small number of Di-17 plants inoculated with TCV become systemically infected even though they initially form HR lesions indicative of an active resistance response. Additionally, this systemically infected tissue when challenge inoculated with a second round of TCV displayed no HR (Hammond, 2001). This suggested TCV was suppressing the plant's defense mechanisms. Since only a few Di-17 become systemically infected, in order to study this HR suppression phenomenon in greater detail, a system was devised that was much more amenable to laboratory research. By using a plant that was susceptible to the virus such as Colombia-0 and bacteria that normally elicit HR, the phenomenon could be studied more effectively (Hammond, 2001).

#### 3.1. TCV Suppresses RPS2 Controlled HR in Col-0

Col-0 plants produce a very strong HR within 24 hours after infiltration with *Pseudomonas syringae* pv. *glycinea* Race 4 (*Psg* R4) expressing *avrRpt2*. However, systemic TCV infection greatly reduces or eliminates this HR formation. In this experiment, Col-0 plants are inoculated with TCV. After systemic symptoms are observed, the leaves showing mild symptoms are infiltrated with bacteria expressing the *Avr* or carrying an empty plasmid. The response is compared to similarly infiltrated, mock virus infected plants. The HR is assessed on a 0-3 scale for each condition and the results are plotted in Figure 3.1.

**Figure 3.1 TCV Suppression of HR Elicited by AvrRpt2 in Col-0**



*Colombia-0 plants are inoculated with TCV (TCV +) or mock inoculated (TCV -) at 21 days post planting and allowed to become systemically infected. Systemically infected or equivalent leaves are then infiltrated with isogenic *Pseudomonas syringae* pv. *glycinea* Race 4 that are expressing *avrRpt2* (Avr +) or not (Avr -). HR is rated at 24 hours post infiltration.*

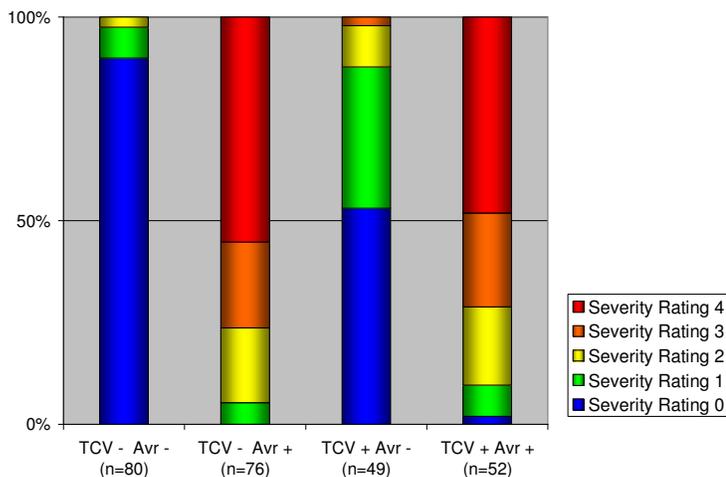
Normally when leaves are infiltrated with bacteria that are isogenic but do not express *avrRpt2*, the leaves remain unaffected and healthy. However, the data clearly shows that 80% of leaves systemically infected with TCV display little to no HR when infiltrated with bacteria expressing *avrRpt2*, whereas in that absence of TCV the bacteria normally elicit a very strong HR with over 95% affected.

### 3.2. Test for Suppression of HR to Other Elicitors

Systemic TCV infection has been shown to suppress the HR to two different effector molecules, the TCV-CP (Hammond, 2001) and AvrRpt2 (Hammond, 2001; see above). To determine if TCV causes global suppression of the HR, the same experiment above was repeated with the same bacteria *Pseudomonas syringae* pv. *glycinea* Race 4 expressing either *avrRpm1* or *avrRps4*, instead of *avrRpt2*. The scale was extended to four qualitative points

because the range of HR intensity was broader than in the *avrRpt2* experiment above. Data for the experiment using *avrRpm1* can be seen in Figure 3.2.

**Figure 3.2 Hypersensitive Response of Col-0 to Bacteria Expressing *avrRpm1***



*Colombia-0* plants are inoculated with TCV (TCV +) or mock inoculated (TCV -) at 21 days post planting and allowed to become systemically infected. Systemically infected or equivalent leaves are then infiltrated with isogenic *Pseudomonas syringae* pv. *glycinea* Race 4 that are expressing *avrRpm1* (Avr +) or not (Avr -). HR is rated at 24 hours post infiltration. This experiment was done twice and the results combined in this graph. (See appendix B for individual experiment data).

In the case of bacteria infiltrated that express the avirulence factor *avrRpm1* the suppression of HR by TCV is less significant. What is most interesting is the greater incidence of HR formation to bacteria that do not contain *avrRpm1* in TCV infected plants. In this set of experiments it seems as though the presence of TCV may actually be activating the plants' defense responses since the HR is produced in response to bacteria that are normally considered non-virulent. This may be due in part to a heightened sensitivity to proteins expressed by the bacteria detected as foreign by the plant or it may be due to an increased sensitivity of the plant tissue to the physical manipulation of bacterial infiltration. However, this was not seen to the same extent in the 1<sup>st</sup> experiment with bacteria expressing *avrRpt2*.

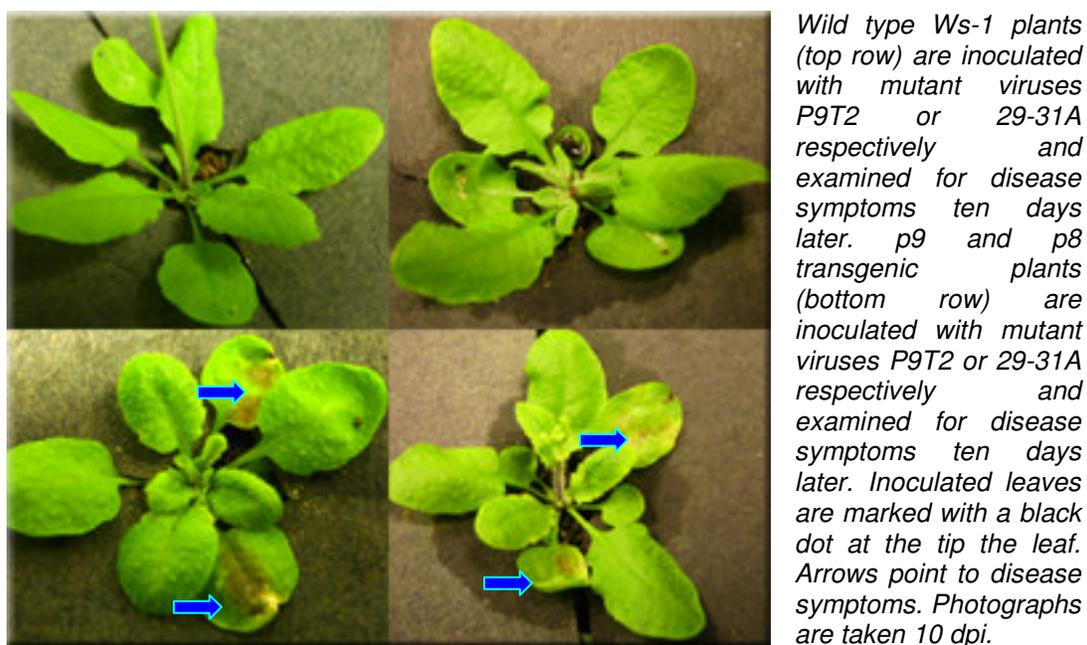
The plant R proteins RPS2 and RPM1 which confer resistance to pathogens containing avirulence factors AvrRpt2 and AvrRpm1, respectively, are both CC-NBS-LRR domain proteins and are 21% identical and 51% similar (Bent *et al.*, 1994; Grant *et al.*, 1995; Mindrios *et al.*, 1994). For this reason it is interesting that in one case HR is suppressed and in the other case it is not. It is then possible to hypothesize that whatever factor is suppressed is not common to the pathways of both R proteins. Because RIN4 has been shown to be associated with both RPM1 (Mackey, 2002) and RPS2 (Mackey, 2003) I would suggest that this convergent point in these pathways would not be where TCV suppression of HR is occurring. Interestingly, a null mutation of *NDR-1* abolishes RPS2-mediated HR but not RPM1-mediated HR yet resistance as measured by *in planta* bacterial growth was eliminated in both (Century *et al.*, 1995; 1997).

To test for TCV suppression of HR to *avrRps4* it was necessary to use a different host plant. Col-0 does not display a visible HR but Ler does (Gassmann *et al.*, 1999). Ler was tested; however, no HR was seen even in the absence of prior TCV infection and even at extremely high inoculum levels ( $A_{595}$  1.0, data not shown). It is possible that the variety available to us, Ler-glabrous a mutant variety, was not ideal. Therefore, original Ler seed was ordered but received too late to perform this experiment again. AvrRps4 is recognized by RPS4, a TIR-NBS-LRR R protein (Gassmann *et al.*, 1999). It will be interesting to see if the different N-terminal domain and the resulting signaling differences will have the same HR suppression effect by TCV as AvrRpt2.

### **3.3. *Arabidopsis* Transgenics Containing TCV Proteins**

In order to determine what part of the TCV genome was responsible for the suppression of HR several transgenic lines of *Arabidopsis* were obtained. Each transgenic line contained one of the following TCV proteins: Coat Protein, p8 and p9. Before any conclusions could be made about HR suppression in these plants we needed to determine if the TCV genes in these plants were being expressed and more importantly if they were functional. Transgenic plants were inoculated with TCV mutants 29-31A and P9T2. The mutant 29-31A lacks a functional p8 protein (Akgoz *et al.*, 2001) whereas P9T2 lacks a functional p9 protein (Li *et al.*, 1998). These mutants are unable to move normally through the plant and therefore plants do not become systemically infected (see Figure 3.3 top). However, transgenic plants that contain the respective genes do become systemically infected (see Figure 3.3 bottom). It can therefore be concluded that the p8 and p9 transgenic lines do express these proteins and that they are functional because these proteins are able to complement the mutant virus *in trans*.

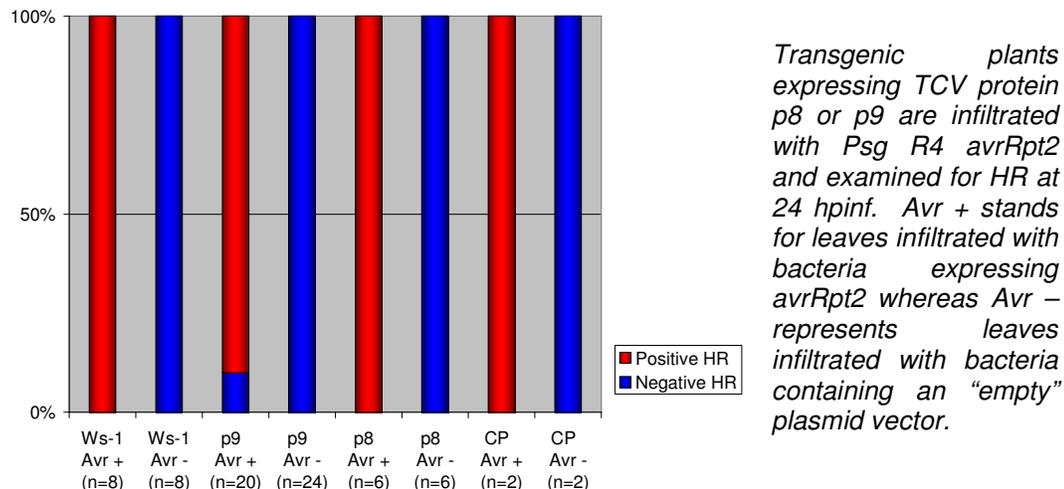
**Figure 3.3 Ws-1 Background and Transgenic Leaves Inoculated with Mutant Virus**



The TCV-CP transgenic line was not verified as expressing a functional protein using a mutant virus.

Transgenic lines expressing TCV proteins were grown to maturity (about 4 weeks) at which time they were infiltrated with *Pseudomonas syringae* pv. *glycinea* with or without the avirulence factor AvrRpt2 at a concentration of 0.2 A<sub>595</sub>. The leaves were then examined 24 hours post infiltration (hpinf) for HR.

**Figure 3.4 Transgenic Lines Display HR upon Inoculation with *avrRpt2* Bacteria**



Of the p8 and CP transgenics 100% displayed a strong HR to *Avr*-expressing bacteria whereas, of the p9 transgenics 90% showed a strong HR and 10% (note that this comes from two leaves of the same plant, see Figure 3.4) of these leaves did not respond with HR to the *Avr*-expressing bacteria. We do not believe this is significant because the discrepancy is most likely due to the human error of overlooking one plant in the infiltration process. It is apparent from these results that these single protein inclusions in *Arabidopsis* are not sufficient to suppress the HR elicited by bacteria expressing *avrRpt2*. It is still possible that the one or both of the two remaining proteins of TCV, p28 and p88 are responsible for the observed HR suppression.

A further possibility is that the TCV proteins acting in combination are required for HR suppression. A first step to test this possibility was to make crosses of the transgenic lines of *Arabidopsis* containing TCV proteins. A number of combinations of the three TCV protein transgenics were produced p8 X p9;

p9 X p8; p8 X CP; p9 X CP. The seeds from these crosses were collected, dried and planted. The assay to demonstrate conclusively that the crosses were successful was not unequivocal. Therefore, the F2 generation will need to be tested. However, in a preliminary assay, total of about 30 F1 plants were screened for HR suppression by infiltrating with *Psg* R4 expressing *avrRpt2*. The number of leaves tested was small (two leaves per plant) for the plants were few and needed to produce F2 seed. No crosses were completely HR suppressed in response to infiltration of *Psg* R4. Only leaves of four plants may have been slightly reduced (severity rating 3 out of 4) in their HR intensity however the reduction was not consistent in both leaves tested per plant suggesting that the reduction was due to natural variation and not HR suppression by TCV proteins. These data suggest that the binary protein combinations of TCV proteins p8, p9 and CP are also not sufficient for HR suppression. The crosses which may have been reduced in their ability to form HR are p9 X CP crosses: 9C2 and 9C4; and p8 X CP crosses; 8C7 and 8C8. One plant (9C4) was consistently HR reduced in both leaves. Therefore, it may be of some value to examine the F2 generation of this cross further.

### **3.4. HR Suppression: Suppression of Resistance or Suppression of Symptoms**

The HR is one of many defense responses induced by avirulent pathogens, the sum of which, result in resistance or reduction in pathogen replication and systemic spread. We were interested to see if the growth of normally avirulent pathogens is increased in HR suppressed plants. However,

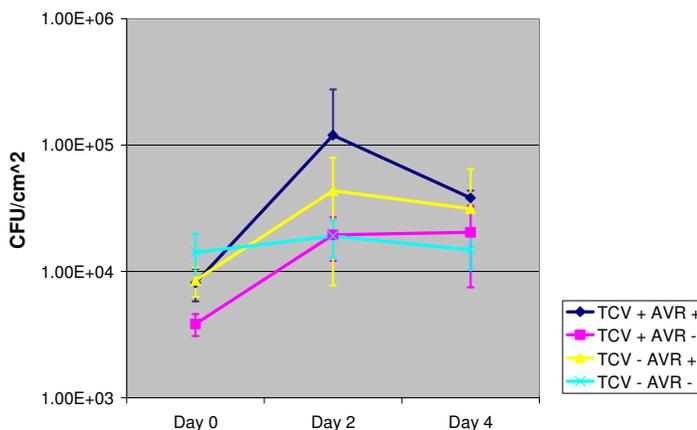
since the HR and resistance can be unlinked (Tao *et. al.*, 2003) it is also possible that TCV only interferes with HR production and that the plant will maintain its resistance to pathogens.

To distinguish between these possibilities we use bacterial growth assays. Virulent bacteria will grow to significant levels in infiltrated leaves, whereas avirulent bacteria will not multiply at all or grow only a small amount over the course of several days. The bacteria *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 is quite virulent in *Arabidopsis* tissue growing as much as 5 logs over the course of several days. Yet the same bacteria expressing avirulence genes *avrRpm1*, *avrRpt2* or *avrRps4* have an attenuated growth pattern of perhaps 2 logs of growth at best (Century *et. al.*, 1995; Whalen *et al.*, 1991; Hinsch and Staskawicz, 1996; Musket *et. al.*, 2002)

*Arabidopsis* Col-0 plants were TCV or mock inoculated at 21 dpp. The systemically infected tissue was then infiltrated with *Pseudomonas syringae* pv. *glycinea* Race 4 at a concentration of 0.002 A<sub>595</sub> after 7-10 dpi. Immediately after infiltration and two and four days post infiltration a standard amount of leaf tissue was excised and the bacteria extracted from the tissue. The extracted bacteria were serial diluted and plated on selective medium. The numbers of colonies that develop were quantified after 2-3 days for each time point. Figure 3.5 shows the population growth of these bacteria over the course of time.

**Figure 3.5 Growth of Non-virulent and Avirulent *Pseudomonas syringae* pv. *glycinea* in Col-0 Plants in the Presence or Absence of TCV**

*Colombia-0* plants are TCV- or mock-inoculated at 21 dpi. *Pseudomonas syringae* pv. *glycinea* Race 4 with or without avirulence gene *avrRpt2* is infiltrated into systemically infected tissue at a concentration of 0.002  $A_{595}$  at 7-10 dpi. A standard amount of leaf tissue is excised and bacteria extracted by grinding in 10 mM  $MgSO_4$ . The extracted bacteria are diluted and plated on selective medium. Colonies are counted after 2-3 days and the colony forming units (CFU) are determined per  $cm^2$  of leaf tissue. Error bars are one standard deviation from the mean of five leaf samples.

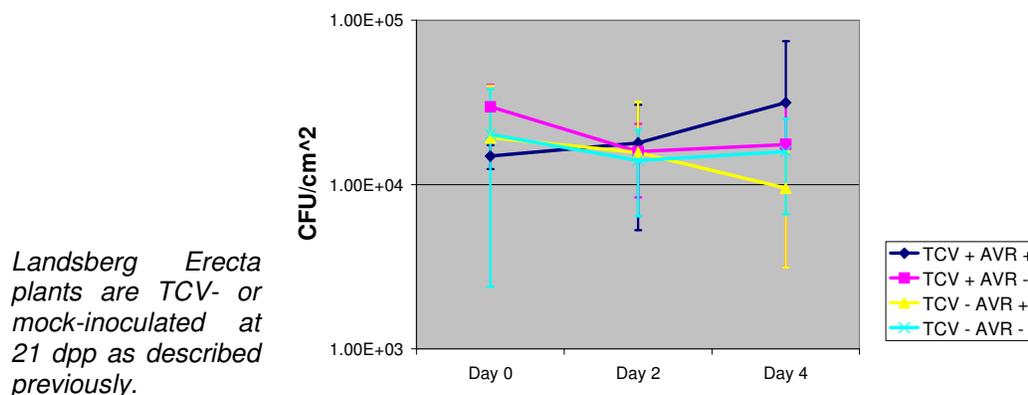


Since *Arabidopsis* is a non-host for *Pseudomonas syringae* pv. *glycinea* Race 4 the bacteria are unable to grow to significant levels. These data show that the bacteria are able to grow slightly, reaching a maximum at about 48 hpinf. It may be worth noting that *avrRpt2*-expressing bacteria grow slightly better than bacteria that do not express the avirulence factor, especially in the presence of TCV. This experiment was completed three times with similar results.

In earlier experiments with *Landsberg erecta* to determine if the HR induced by *avrRps4* was suppressed, the data was unclear and the symptoms produced in these plants seemed similar to those produced by virulent bacteria. If these bacteria were actually virulent in these plants it could explain why we did not see a typical HR. Therefore, the same experiment was conducted as above

using Ler plants and *Psg* expressing *avrRps4*. These results can be seen in Figure 3.6 below.

**Figure 3.6 Growth of Non-virulent and Avirulent *Pseudomonas syringae* pv. *glycinea* Race 4 in *Landsberg erecta* in the Presence or Absence of TCV**



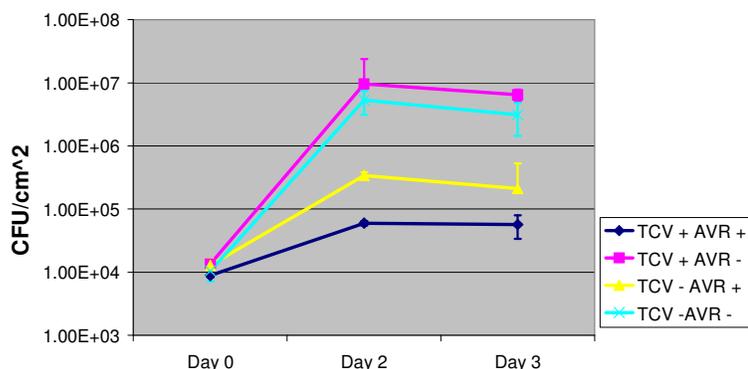
It is apparent that *Psg* R4 is not virulent in *Landsberg erecta* plants. In fact it would appear from Figure 3.6 above that the growth of these bacteria is even more limited than the growth of these bacteria in *Col-0*, since there is almost no increase in population over the course of 4 days.

In order to properly determine if resistance is compromised by systemic TCV infection we switched to a virulent strain, *Pseudomonas syringae* pv. *maculicola* ES4326. These bacteria normally grow to high populations in nonresistant plant hosts after infiltration (Hendrickson *et al.*, 2000). Our results are consistent with this (see Figure 3.7 below). The virulent bacteria increase by almost three logs in the presence or absence of prior TCV infection. Interestingly though, the growth pattern in the presence or absence of TCV (TCV +/- Avr -) is similar yet the small difference is highly significant ( $p=.01$ ) by day three. The

presence of TCV seems to facilitate the growth of virulent bacteria. Bacteria containing the avirulence gene *avrRpt2* showed significantly less growth ( $p = 0.02$ ) over time. This occurrence can be observed in the TCV – Avr + group below. In the presence of TCV (TCV + Avr +) there appeared to be an even lower accumulation of bacteria than in the absence of TCV, suggesting an increase in resistance. However, the difference approaches significance but is not statistically significant. This may be due to the variance of the data. A much larger data set is required to determine if the difference between these two groups is significant. A repeat of this experiment gave similar results.

**Figure 3.7 Growth of Avirulent and Virulent *Pseudomonas syringae* pv. *maculicola* in Col-0 Plants In the Presence or Absence of TCV**

Experiment was performed essentially the same as the experiment in Figure 3.5 however, using *Pseudomonas syringae* pv. *maculicola* ES4326 as the bacterial vector.



These results provide further evidence that the HR is not required for resistance. Despite the suppression of the HR in the presence of TCV the growth of avirulent bacteria is inhibited. Other factors certainly are responsible for this resistance. For instance NDR-1 is essential for most CC-NBS-LRR proteins' function and *EDS1* and *PAD4* are necessary for resistance conferred by TIR-NBS-LRR genes (Aarts *et al.*, 1998). Another resistance signaling gene, *RAR1*,

seems to act early in the plant signaling cascade. The barley *rar1* mutant is impaired in its ability to accumulate AOS and formation of HR to *Mla12*-specified resistance (Freialdenhoven *et al.*, 1994; Shirasu *et al.*, 1999). It is possible to have HR without resistance as well as to have resistance without HR.

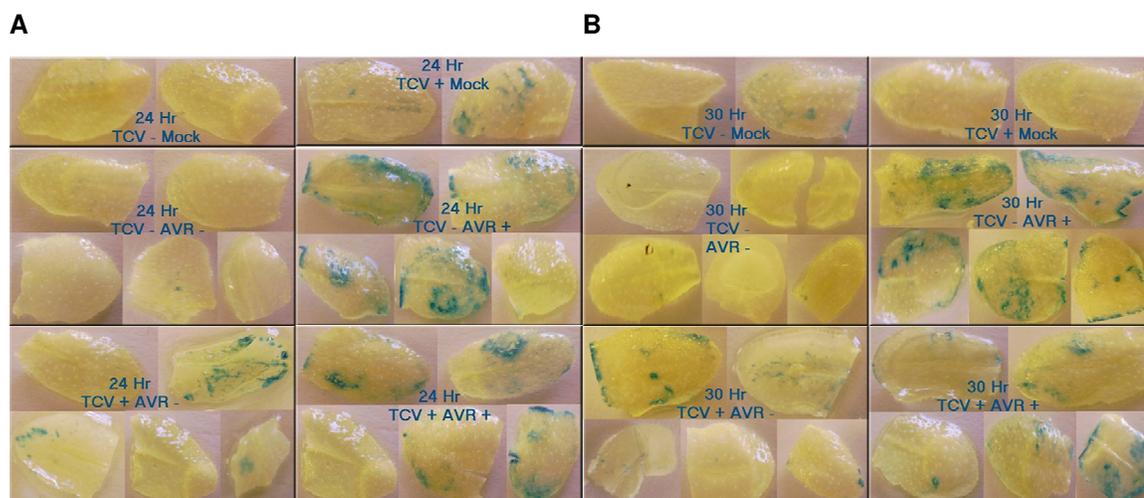
### **3.5. TCV influence on Other Components of Active Defense Responses**

An additional active defense response is the induction of the *PR-1* gene. This gene is induced as part of the systemic acquired resistance (SAR) response typically induced following infection with an avirulent pathogen (Ryals *et al.*, 1998). SAR is a long lasting, broad spectrum resistance response triggered through the activation of a set of pathogenesis related (PR) proteins (Dong, 1998). To determine if this pathway was affected by systemic TCV infection we obtained another transgenic line of *Arabidopsis* in a Col-0 background. This transgenic line carries the  $\beta$ -glucuronidase (GUS) gene under the control of the *PR-1* promoter. Thus, induction of PR-1 protein expression results in production of the GUS protein (Shapiro and Zhang, 2001). It is assumed that there is a direct correlation between the expression of GUS and the expression of PR-1 protein. There are several commercially available substrates for this enzyme that can be used for histochemical localization of GUS activity or for spectrophotometrically quantifiable measurement of soluble GUS activity.

The activation of SAR in response to avirulent pathogens has been well documented (Ryals, 1998). We wanted to determine if SAR induction was repressed in the presence of TCV. GUS transgenic plants were inoculated with

TCV or mock buffer at 21 days post planting; at 7-10 days post inoculation the plants were infiltrated with *Pseudomonas syringae* pv. *glycinea* Race 4. Whole leaves were then excised at several time points to determine the level of GUS activity. The experiment was repeated twice with similar results. These data can be seen in Figure 3.8 as well as tabulated data in Appendix B: Data from Individual Experiments.

**Figure 3.8 Histological Detection GUS Assay**



*PR-1::GUS* transgenic leaves are TCV or mock inoculated at 21 days post planting and allowed to develop systemic infection. Systemically infected leaves are infiltrated with *Pseudomonas syringae* pv. *glycinea* Race 4 *avrRpt2* or empty vector bacteria at a concentration of 0.002  $A_{595}$  7-10 dpi. Whole leaves are excised and soaked in GUS substrate buffer containing *x-gluc* overnight at 37 °C. Chlorophyll is decolorized in 100% ethanol and leaves photographed.

GUS activity was induced by several treatments: TCV + Avr +; TCV – Avr +; TCV + Avr – and sometimes TCV + Mock. What was interesting about these data and quite unexpected was the induction of GUS activity in the presence of TCV in response to non-virulent bacteria as well as mock buffer infiltration. It seems as though the presence of TCV is somehow potentiating SAR, such that upon further manipulation of the tissue *PR-1* is induced. It is

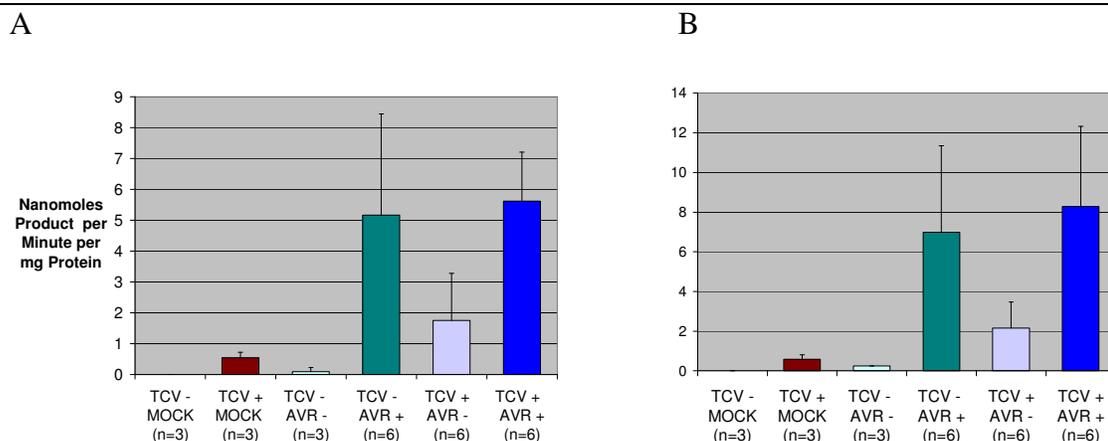
possible that the plant is able to sense what may be virulent-like effector proteins that the bacteria are inserting into the plant cell through the Type III effector secretion system. The reduced amount of GUS activity visualized in mock infiltrated leaves may be due to the physical trauma of forcing fluid into the intercellular spaces of the leaf.

Comparison of the 24 hour time point to the 36 hour time point suggested a reduction in GUS activity in the presence of TCV after infiltration with *avrRpt2* bacteria (TCV + Avr +). This result correlates with the suppression of HR phenotype. Furthermore, because the bacteria grow to their peak level inside the plant by 48 hpinf one would think that the most intense signal would develop closer to 48 hours rather than 24 hours.

It is clear that the level of GUS expression varied quite a bit even within a single experimental group and that some leaves seemed to have more expression than others. In order to obtain more quantifiable data the same experiment was performed using spectrophotometric quantification of GUS activity. In this assay, the soluble protein is extracted from tissue and the assay conducted using a substrate conjugated to *p*-nitrophenyl. Upon cleavage of the substrate the *p*-nitrophenyl creates a distinctive yellow color that has peak absorbance at 405 nm (Naleway, 1992). The rate at which the absorbance at this wavelength increases over time is proportional to the amount of GUS enzyme present in the reaction. By standardizing the rate to the protein concentration one can determine the specific activity of the GUS enzyme and therefore the level of PR-1 production in the leaf. The experiment was completed

three times with varying results yet some fairly obvious trends were common to all replicates. These data can be viewed in Figure 3.9 and Appendix B: Data from Individual Experiments.

**Figure 3.9 Soluble GUS Assay: 26 and 56 hours post infiltration**



*PR-1::GUS transgenic leaves are TCV or mock inoculated at 21 days post planting and allowed to develop systemic infection. Systemically infected leaves are infiltrated with *Pseudomonas syringae* pv. *glycinea* Race 4 *avrRpt2* or empty vector bacteria at a concentration of 0.002  $A_{595}$  7-10 dpi. Whole leaves are excised 24-48 hpi and total soluble protein extracted. Cell lysate was spectrophotometrically measured for GUS activity using PNG. Error bars represent one standard deviation from the mean of  $n$  samples indicated.*

The first experiment suggested induction of GUS activity induced by AvrRpt2 was reduced in the presence of TCV. This is consistent with the observation that TCV suppresses HR formation. However, there was a large amount of variance in the data. Later independent replicates conflicted with the observation that GUS activity was reduced in the presence of TCV. These experiments suggest the opposite is true, that GUS activity is stimulated more in the presence of TCV than in the absence of TCV in response to AvrRpt2. Given

these circumstances the data shown above was considered most representative of this assay.

For all replicates there is a 10% possibility that the difference seen between TCV+ Mock and TCV- Mock groups is due to chance. It may be possible that this difference will be significant if more than three leaves are assayed. The presence of TCV seems to be potentiating SAR in response to the infiltration procedure for both 10 mM MgSO<sub>4</sub> and the Avr – bacteria, which is consistent with histological data above. The GUS activity induced by AvrRpt2 alone in the absence of TCV is significant ( $p=0.04$ ) at 26 hours and approaches significance at 56 hours. It is expected that GUS should be activated by AvrRpt2 and generally it is. However, the activation of GUS activity in the presence of TCV to AvrRpt2 is statistically very highly significant to very significant ( $p=0.0002$  to  $p=0.002$ ) over the course of experiment above. These data suggest that in the presence of TCV SAR induction is enhanced.

## Major Conclusions:

As a result of the experiments conducted during this work we can conclude that TCV is more efficient at suppressing the HR to TCV-CP and AvrRpt2 than AvrRpm1. Since *avrRpt2* and *avrRpm1* use much of the same signaling system the reason for this discrepancy is unclear.

The TCV proteins p8, p9 and CP are individually insufficient to suppress the HR. Some combinations of these proteins may be slightly more competent for this suppression.

The effect of systemic TCV infection on the ability to induce *PR-1* and therefore, presumably SAR, is unclear. While TCV seems to potentiate a response to infiltration, the induction by *Avr*-expressing bacteria seems to be relatively less in the absence of TCV. This enhancement of SAR in the presence of TCV seems to suggest an increased defense response.

## Future Research:

The original intent of testing for HR suppression in NahG transgenic plants was to see if SA was important in the suppression. The NahG transgene compromises RPS2-mediated resistance (Delaney *et al.*, 1994) so we thought that SA was necessary for HR in this system. We chose to use *avrRpm1* carrying bacteria because RPM1 is not SA dependent. However, Tao *et al.* (2003) had consistent reproducible HR in NahG plants in response to bacteria expressing *avrRpt2*. Therefore, I would be very interested to know if HR elicited by *P. syringae* pv. *Glycinea* Race 4 *avrRpt2* is still suppressed in our recently obtained NahG transgenics in Col-0 background. Tao *et al.* (2003) also reports that not only do they still see HR but that the minimum bacterial dose of *P. syringae* pv. *tomato* carrying *avrRpt2* is lower for NahG than for wild type plants.

The TCV-CP transgenic needs to be tested for a functional protein using a mutant virus lacking CP in order to validate data in this thesis. It will also be interesting to examine p88 and p28 proteins' possible involvement in HR suppression.

Further verification of the crosses experiment should be completed to determine if crosses were successful. It may also be of interest to further examine the p9 X CP cross 9C4 F2 generation for HR suppression due to a consistent slight reduction of HR in both leaves tested in this plant.

Because the requirements for NDR-1 in RPS2 and RPM1 pathways differ in their ability to form an HR (Century 1995, 1997), it may prove beneficial to examine this protein's involvement in HR suppression further.

In addition, it will be necessary to assay more *Avr* genes of various other pathogens in order to form a clearer view of which families of R proteins fail to produce HR in the presence of TCV. I believe that this will help to further elucidate where in the resistance pathways TCV is interfering with HR formation. By examining areas of convergence and comparing these to areas of divergence in the resistance pathways of the various R genes one may be able to select the common factor(s) that correspond to HR suppression. These factors may be where TCV is interfering HR signaling.

## Appendix A: Soluble GUS Assay Reagent Preparation

### Homogenization Buffer I

[250 mM Sucrose, 25 mM HEPES (pH 7.5), 1 mM DTT (added freshly), 1 mM MgCl<sub>2</sub>]  
Proteinase inhibitors are added freshly to final concentration of: 2 µg/ml aprotinin (Stock in diH<sub>2</sub>O), 1 µg/ml leupeptin (in diH<sub>2</sub>O), 1 µg/ml pepstatin A (in MetOH), 1 mM PMSF (in MetOH). Store proteinase inhibitors at -20°C and PMSF at 4°C.

### GUS Assay Reagent Buffer

[50 mM NaPO<sub>4</sub>, pH7, 1mM EDTA, 5mM DTT, 1.25mM PNGG]  
for 50 ml:  
49.13mL GUS Buffer Stock Solution  
250 µl 1M DTT  
625 µl 100mM PNG  
Prepare **fresh** and pre-warm to 37°C

### GUS Buffer Stock Solution

[50 mM NaPO<sub>4</sub>, pH7, 1mM EDTA]  
for 200 ml:  
7.8 ml 0.5M NaH<sub>2</sub>PO<sub>4</sub>  
12.2 ml 0.5M Na<sub>2</sub>HPO<sub>4</sub>  
0.4 ml 0.5M EDTA, pH8  
180mL distilled H<sub>2</sub>O  
Prepare ahead and store at room temperature.  
DTT and PNG need to be added before use.

### 0.5M NaH<sub>2</sub>PO<sub>4</sub>

for 500 ml:  
34.5g NaH<sub>2</sub>PO<sub>4</sub> anhydrous  
dH<sub>2</sub>O to 500mL  
Prepare ahead and store at room temperature.

### 0.5M Na<sub>2</sub>HPO<sub>4</sub>

for 500 ml:  
35.5g Na<sub>2</sub>HPO<sub>4</sub> anhydrous  
dH<sub>2</sub>O to 500mL  
Prepare ahead and store at room temperature.

### 0.5M ethylenediamine tetraacetic acid (EDTA)

for 200 ml:  
dissolve 37.22g Na<sub>2</sub>EDTA·2H<sub>2</sub>O in 140 ml dH<sub>2</sub>O  
Adjust pH to 8.0 with 10N NaOH (~10 ml) dH<sub>2</sub>O to 200 ml  
Prepare ahead and store at room temperature.

### 1M dithiothreitol (DTT)

for 10 ml:  
1.54g DTT  
dH<sub>2</sub>O to 10ml  
Prepare ahead and store 1 ml aliquots at -20°C.

### 100mM *p*-nitrophenyl β-D-glucuronide (PNG)

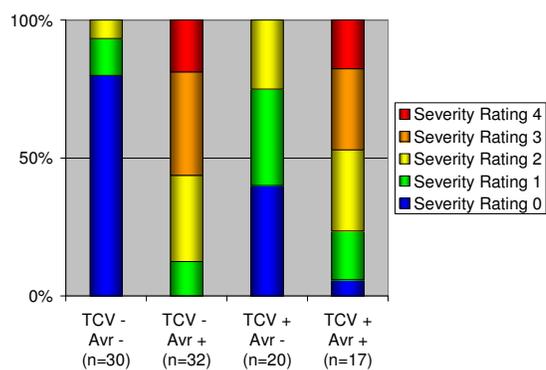
for 1 ml:  
0.032g PNG  
GUS Buffer Stock Solution to 1 ml  
Prepare **fresh** and keep on ice.

### 0.4 M Na<sub>2</sub>CO<sub>3</sub> (Stop Solution)

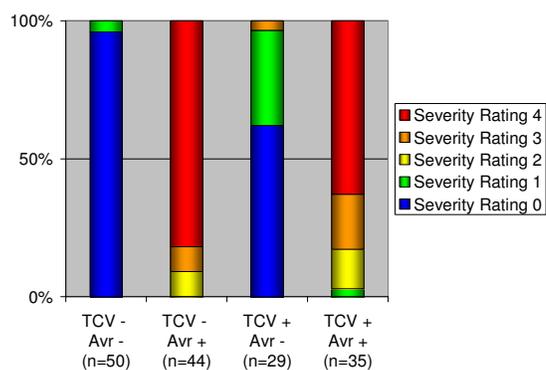
for 500ml:  
21.2g Na<sub>2</sub>CO<sub>3</sub>  
dH<sub>2</sub>O to 500ml  
Prepare ahead and store at room temperature.

## Appendix B: Data from Individual Experiments

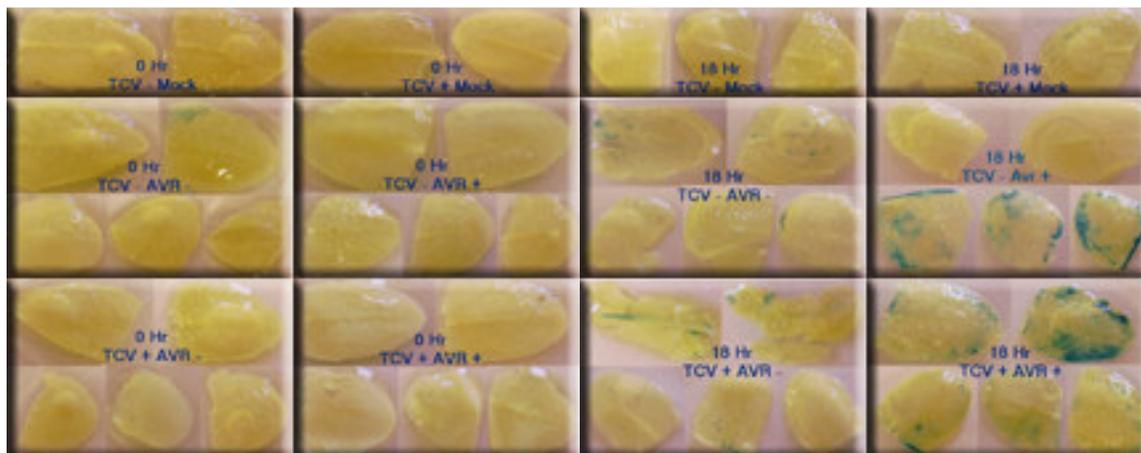
**Figure 0.1 HR in Col-0 to Bacteria Expressing avrRpm1, Experiment #1**



**Figure 0.2 HR in Col-0 to Bacteria Expressing avrRpm1, Experiment #2**



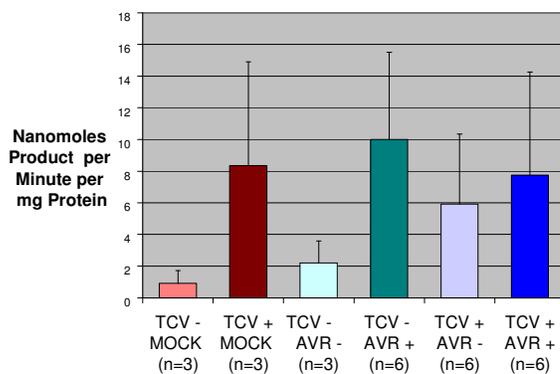
**Figure 0.3** Histological GUS Detection Assay #1



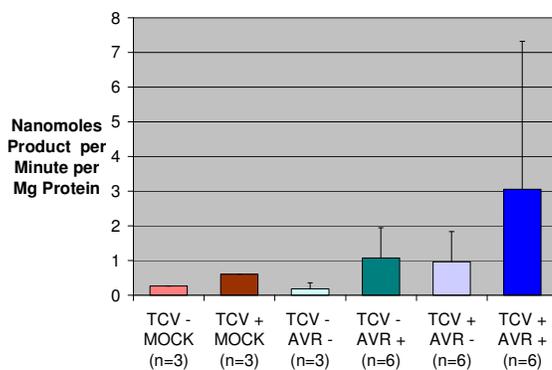
**Figure 0.4** Histological GUS Detection Assay #2



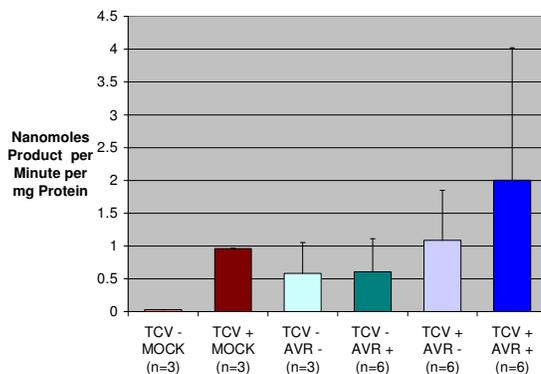
**Figure 0.5 Soluble GUS Assay: 48 Hour Time Point, Experiment #1**



**Figure 0.6 Soluble GUS Assay: 24 Hour Time Point, Experiment #3**



**Figure 0.7 Soluble GUS Assay: 48 Hour Time Point, Experiment #3**



Visual GUS Assay # 1							
Time Point	TCV	Bacteria	Extent of Blue	Time Point	TCV	Bacteria	Extent of Blue
0 hr	-	M	0	24 hr	-	M	0
	-	M	1		-	M	1
	+	M	0		+	M	1
	+	M	0		+	M	2
	-	-	0		-	-	0
	-	-	0		-	-	0
	-	-	0		-	-	0
	-	-	0		-	-	0
	-	-	1		-	-	0
	-	+	0		-	+	2
	-	+	0		-	+	2
	-	+	0		-	+	2
	-	+	0		-	+	3
	-	+	0		-	+	3
	+	-	0		+	-	0
	+	-	0		+	-	0
	+	-	0		+	-	1
	+	-	0		+	-	1
	+	-	0		+	-	2
	+	+	0		+	+	0
+	+	0	+	+	1		
+	+	0	+	+	2		
+	+	0	+	+	2		
+	+	0	+	+	3		
18 hr	-	M	0	30 hr	-	M	0
	-	M	0		-	M	0
	-	M	0		+	M	0
	+	M	0		+	M	1
	+	M	1		-	-	0
	-	-	0		-	-	0
	-	-	0		-	-	0
	-	-	1		-	-	0
	-	-	1		-	-	0
	-	-	1		-	-	0
	-	+	0		-	+	2
	-	+	0		-	+	2
	-	+	3		-	+	3
	-	+	3		-	+	3
	-	+	3		-	+	3
	-	+	4		+	-	0
	+	-	0		+	-	1
	+	-	0		+	-	1
	+	-	1		+	-	1
	+	-	2		+	-	1
+	-	2	+	+	1		
+	+	1	+	+	1		
+	+	1	+	+	1		
+	+	2	+	+	1		
+	+	2	+	+	1		
+	+	4	+	+	4		

Visual GUS Assay # 2

Time Point	TCV	Bacteria	Extent of Blue	Time Point	TCV	Bacteria	Extent of Blue
0 hr	-	M	0	24 hr	-	M	0
	-	M	0		-	M	1
	+	M	0		+	M	1
	+	M	0		+	M	2
	-	-	0		-	-	0
	-	-	0		-	-	0
	-	-	0		-	-	0
	-	-	0		-	-	0
	-	-	0		-	-	0
	-	-	0		-	-	0
	-	-	3		-	-	0
	-	+	0		-	+	0
	-	+	0		-	+	0
	-	+	2		-	+	2
	-	+	2		-	+	2
	-	+	2		-	+	3
	-	+	2		+	-	3
	-	+	3		+	-	3
	+	-	2		+	-	3
	+	-	2		+	-	3
+	-	3	+	-	3		
+	-	3	+	-	3		
+	+	0	+	+	2		
+	+	0	+	+	2		
+	+	2	+	+	2		
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+	+	2	+	+	3		
+	+	2	30 hr	-	M	1	
+	+	2		-	M	1	
+	+	2		+	M	0	
+	+	3		+	M	0	
-	M	0		-	-	0	
-	M	0		-	-	0	
+	M	0		-	-	0	
+	M	1		-	-	0	
-	-	0		-	-	0	
-	-	0		-	-	0	
-	-	0	-	-	0		
-	-	0	-	+	0		
-	-	0	-	+	0		
-	-	0	-	+	1		
-	-	1	-	+	1		
-	-	1	-	+	1		
-	+	0	-	+	2		
-	+	0	+	-	2		
-	+	2	+	-	2		
-	+	2	+	+	1		
-	+	2	+	+	2		
-	+	2	+	+	2		
-	+	3	+	+	2		
+	-	2	+	+	2		
+	-	2	+	+	2		
+	-	3	+	+	2		
+	-	3	+	+	2		
+	-	3	+	+	2		
+	+	0					
+	+	0					
+	+	2					
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+	+	2					
+	+	2					
+	+	3					

18 hr

## Appendix C: Glossary of Terms

- Active oxygen species (AOS)—Oxidizing compounds like  $O_2^-$  or  $H_2O_2$
- Active resistance—Disease resistance due to biochemical interactions
- Arabidopsis thaliana*—A small flowering plant
- Avirulent—Non-disease causing due to active defense responses
- Avr genes—Genes that encode for avirulence factors found in pathogens
- AvrRpm1*-- An Avr gene found in bacteria
- AvrRps4*-- An Avr gene found in bacteria
- AvrRpt2*—An Avr gene found in bacteria
- Coiled Coils (CC)—Domain of R proteins
- Colombia-0 (Col-0)—Susceptible ecotype of *A. thaliana* from USA
- Dpi—days post inoculation
- Dpinf—days post infiltration
- Dpp—days post planting
- Dijon-0 (Di-0)—Partially resistant ecotype of *A. thaliana* originated from France.
- Dijon-3 (Di-3)—Susceptible ecotype of *A. thaliana*
- Dijon-17 (Di-17)—Resistant ecotype of *A. thaliana*
- Ecotype—Varieties of plants originating from a particular geographic location
- Elicitor—A signal molecule that interacts with R proteins to elicit a defense response
- Gene-for-gene resistance hypothesis—The idea that hosts can recognize gene-products through activation of its own gene-products.
- $\beta$ -glucuronidase (GUS)—An enzyme that cleaves glycosidic bonds of sugar substrates.
- Hpinf—hours post infiltration
- Hypersensitive Response (HR)—Localized programmed cell death in plants in response to pathogen attack.
- In Planta*—In the plant.
- Leucine rich repeats (LRRs)—Domain of R proteins.

Landsberg erecta (Ler)—Susceptible ecotype of *A. thaliana* originating from Germany

NahG—Salicylic acid hydroxylase

Nitric Oxide (NO)—A chemical associated with HR.

Non-host resistance—Passive disease resistance

Non-virulent—Pathogens that do not cause disease due to passive resistance

Nuclear Localization Signal (NLS)—Sequence of nucleotides that codes for protein localization in the nucleus.

Nucleotide binding sites (NBS)—Domain of R proteins that binds nucleotides

Open reading frame (ORF)—A sequence of DNA/RNA that codes for a protein.

Oxidative burst—A biochemical event involving a sudden increase in AOS.

p8—TCV movement protein

p9—TCV movement protein

p38—TCV coat protein.

p28—TCV replication protein

p88—TCV replication protein

Pathogenesis-related (PR) proteins—Proteins that are expressed in response to pathogen attack.

*p*-nitrophenyl  $\beta$ -D-glucuronide (PNG)—Spectrophotometric GUS substrate

*PR-1*—A *PR* gene that is considered a marker for SAR

*Pseudomonas syringae* pv. *glycinea* Race 4 (*Psg* R4)—non-virulent bacteria in *A. thaliana*

*Pseudomonas syringae* pv. *Maculicola* ES4326 (*Psm*)—Virulent strain of bacteria in *A. thaliana*

R genes—Genes that encode for resistance factors in a host

*RPS2*—An *R* gene found in plants that confers resistance to pathogens carrying *avrRpt2*

Salicylic acid (SA)—A compound associated with defense responses in plants

Systemic acquired resistance (SAR)—General resistance to a range of pathogens after exposure to an avirulent pathogen

*Turnip Crinkle Virus* (TCV)—A small icosohedral carmovirus which infects plants

Virulent—Disease causing

Wassilewskija (Ws-1)—Susceptible ecotype of *A. thaliana* originating from Russia

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