AN ABSTRACT OF THE DISSERTATION OF

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Title: Characterization of the Response Mediated by the Plant Disease Susceptibility gene *LOV1*.

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Victoria blight, caused by fungus *Cochliobolus victoriae*, is a disease originally described on oats and recapitulated on Arabidopsis. Victoria blight is used as a model plant disease that conforms to an inverse gene-for-gene interaction. *C. victoriae* virulence is dependent upon its production of victorin, a host-specific toxin that induces programmed cell death in sensitive plants. In oats, victorin sensitivity and disease susceptibility is conferred by the *Vb* gene, which is genetically inseparable from the *Pc-2* crown rust resistance gene. In Arabidopsis,

victorin sensitivity and disease susceptibility is conferred by the *LOCUS ORCHESTRATING VICTORIN EFFECTS 1 (LOV1)* gene which encodes a NB-LRR protein, a type of protein commonly associated with disease resistance. LOV1-mediated cell death occurs when victorin binds Thioredoxin-h5 (TRX-h5) and LOV1 appears to "guards" TRX-h5. Together, these results suggest *C. victoriae* causes disease by inducing a resistance response.

The work presented here aimed to determine if the response mediated by LOV1 is functionally related to a resistance response. We genetically characterized the response mediated by LOV1 with virus-induced gene silencing. We determined *SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1),* a gene required for the function of many resistance genes, is required for victorin sensitivity and involved in LOV1 protein accumulation. We screened a normalized library and identified six genes that suppressed victorin-mediated cell death and cell death induced by expression of the *RESISTANCE TO PERONOSPORA PARASITICA PROTEIN 8 (RPP8)* resistance gene: a mitochondrial phosphate transporter, glycolate oxidase, glutamine synthetase, glyceraldehyde 3-phosphate dehydrogenase and the P- and T-protein of the glycine decarboxylase complex. Silencing the latter four also inhibited cell death induced by the expression of an autoactive form of the resistance gene *PTO*, and reduced *PTO*-mediated resistance to *Pseudomonas syringae* pv. *tabaci*. These results provide evidence that victorin-mediated cell death is functionally similar to a

resistance response, further supporting the hypothesis that a resistance response is exploited by *C. victoriae* for pathogenesis in Victoria blight.

Resistance function of LOV1 was evaluated by observing *Pseudomonas syringae* pv. *tomato* virulence upon LOV1 activation. The LOV1 response pathway in Arabidopsis was adapted to activate upon infection with *Pseudomonas syringae* pv. *tomato* expressing the type III-dependent effector protein AvrRpt2, a well-characterized protease. We developed a construct to express a beta-glucuronidase (GUS) and TRX-h5 fusion protein separated by an AvrRpt2 proteolytic cleavage site, in which GUS sterically inhibits TRX-h5 function in LOV1-mediated cell death. The fusion is cleaved upon infection by *P. syringae* pv. *tomato* expressing *avrRpt2*, thereby leading to TRX-h5-mediated activation of LOV1 in the presence of victorin. However, when this strain was inoculated with victorin into transgenic *LOV1 trx-h5* plants expressing the GUS/TRX-h5 fusion protein, no decrease in pathogen virulence was observed. Technical shortcomings likely prevented observable LOV1 resistance function.

# Characterization of the Response Mediated by the Plant Disease Susceptibility gene *LOV1*

by
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APPROVED:

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Brian M. Gilbert, Author

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**Characterization of the Response Mediated by the Plant Disease Susceptibility gene *LOV1***

# 1 Introduction

## 1.1 VICTORIA BLIGHT OF OATS

Victoria blight, a disease caused by the necrotrophic fungus *Cochliobolus victoriae,* was originally discovered on oats (Meehan and Murphy, 1946). Victoria blight is a useful model for disease susceptibility due to the genetic simplicity of its host-pathogen interaction,. This interaction follows an inverse gene-for-gene model. For diseases that follow the classic gene-for-gene model (Flor, 1971), resistance occurs when a dominant resistance gene in the host pairs with a dominant avirulence gene in the pathogen (Fig 1-1 A). Avirulence genes encode virulence effectors, but confer avirulence because their actions are monitored by the products of host resistance genes (Jones and Dangle, 2006). In these interactions, susceptibility occurs in the absence of pathogen recognition. For diseases that follow an inverse gene-for-gene model (Fenton et al., 2009), susceptibility occurs when a dominant susceptibility gene in the host pairs with a dominant virulence gene in the pathogen (Figure 1-1 B). *C. victoriae* virulence is completely dependent upon the production of a single effector, victorin (Meehan

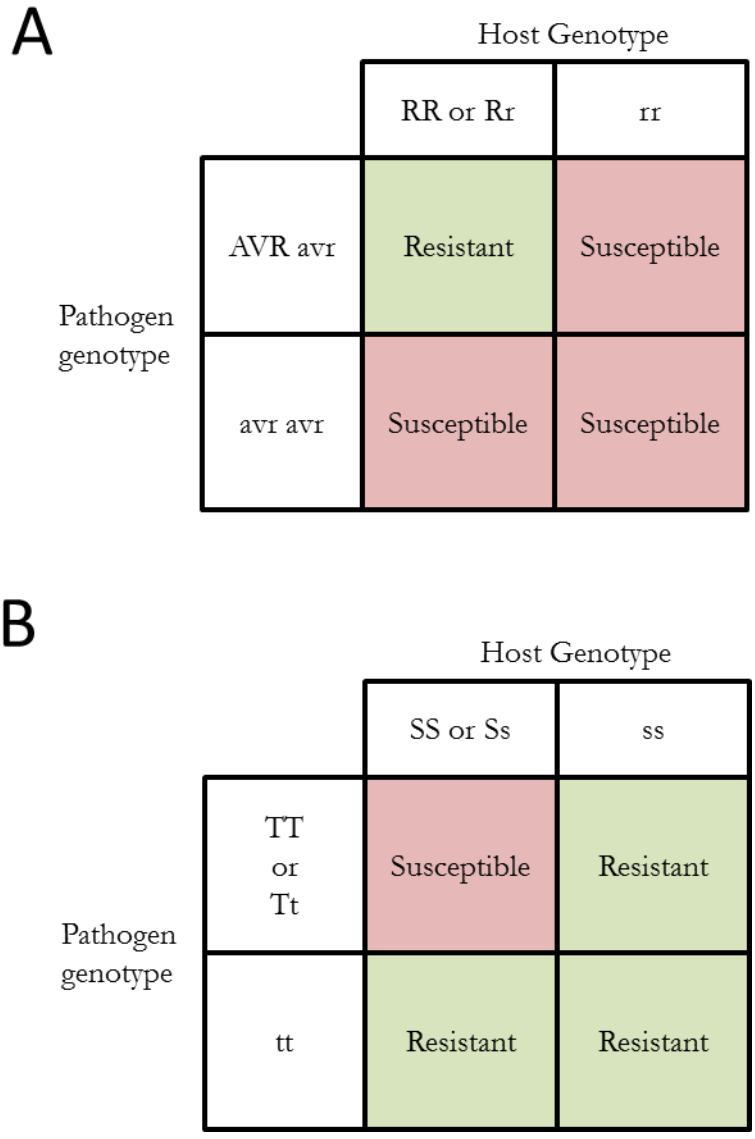


Figure 1-1: Gene-for-gene models depicting resistant and susceptible phenotypes according to host and pathogen genotype. (A) Classic gene-for-gene model. Resistance occurs only in the presence of the dominant resistance (R) gene in the host and dominant avirulence (AVR) gene in the pathogen (B) Inverse gene-for-gene model. Susceptibility only occurs in the presence of the dominant susceptibility (S) gene in the host and toxin producing (T) gene in the pathogen.

and Murphy, 1947). Disease susceptibility in oats is dependent upon the dominant host gene *Vb* (Litzenberger, 1949).

Victoria Blight is of particular interest because it emerged as a consequence of the widespread use of an oat variety resistant to the crown rust pathogen, *Puccinia coronata*. Resistance in this variety of oats is conferred by a gene called *Pc-2*, which provides gene-for-gene resistance to *P. coronata*. In 1945, approximately 75% of the oat varieties planted contained *Pc-2*, setting the scene for a disease of epidemic proportions. *C. victoriae* emerged, exclusively infecting oats that contained the *Pc-2* resistance gene (Mehaan and Muphy, 1946; Litzenberger, 1949). Numerous efforts to separate *Pc-2* from the susceptibility gene *Vb* have failed, suggesting that they could be the same gene (Welsh et al., 1954; Luke et al., 1966; Rines and Luke, 1985; Mayama et al., 1995). Therefore, susceptibility of oats to *C. victoriae* infection is associated with the presence of a resistance gene that protects against a different pathogen.

## 1.2 VICTORIN

*C. victoriae* is pathogenic due to the production of victorin, a host-selective peptide toxin (Meehan and Murphy, 1947; Macko et al., 1985; Wolpert et al., 1985). Toxin production segregates as a single gene, which is thought to encode a non-ribosomal peptide synthetase (Walton, 1996; Walton et al., 2004). Host-selective toxins are primary determinants of virulence and are termed "host-selective" because they typically only function in the host of the pathogen that is producing it (Wolpert et al., 2002). Host-selective toxins induce cell death in

susceptible hosts (Walton et al., 1996). Because both toxin production in the pathogen and toxin sensitivity in the host are required for pathogenesis, toxin-induced cell death appears to be causal to disease development. Victorin physiologically recapitulates Victoria blight symptoms in hosts susceptible to *C. victoriae* (Meehan and Murphy, 1947). Consequently, this allows investigation of the susceptible host response in the absence of the pathogen.

Although victorin causes disease, it induces host responses typical of disease resistance. This is in agreement with genetic analysis in oats that suggests a resistance gene confers susceptibility to *C. victoriae.* Effects of victorin include callose deposition, a respiratory burst, lipid peroxidation, ethylene evolution, extracellular alkalization, phytoalexin synthesis, and K+ efflux – all documented resistance responses (Walton and Earle, 1985; Romanko, 1959; Navarre and Wolpert 1999; Shain and Wheeler, 1975; Ullrich and Novacky, 1991; Mayama et al., 1986; Wheeler and Black, 1962). Importantly, victorin induces a programmed cell death (PCD) response in sensitive oats (Navarre and Wolpert, 1999). The PCD induced by victorin resembles apoptosis and involves a mitochondrial permeability transition (Navarre and Wolpert, 1999; Yao et al., 2001,2002; Curtis and Wolpert, 2002; Coffeen and Wolpert, 2004). The mitochondrial permeability transition results from a change in inner mitochondrial membrane permeability, which leads to mitochondrial dysfunction and cell death (Javadov et al., 2009). The hypersensitive response (HR) is a form of PCD that resembles apoptosis and is involved in disease resistance (Mur et al., 2007; Jones and Dangle, 2006;

Greenburg and Yao, 2004). HR is localized cell death of host tissue in the region of infection, and it serves as an effective component of resistance to biotrophic pathogens, which derive nutrients from live host tissue (Jones, 2006). The HR is thought to prevent the spread of infection by limiting water and nutrient availability to the pathogen. However, the HR may actually be beneficial to necrotrophic pathogens, which actively kill host tissue as they colonize and feed on the contents of dead or dying cells (Govrin and Levine, 2000, Wolpert et al, 2001)). The HR has been shown to aid in pathogenicity of the necrotroph *Botyrtis cineara* by providing a growth substrate (Govrin and Levine, 2000). The similarities between victorin-mediated cell death and HR suggest *C. victoriae* is utilizing a host defense response for pathogenesis.

## 1.3 VICTORIA BLIGHT OF ARABIDOPSIS

Characterizing *Vb* in oats is problematic due to its relatively uncharacterized, hexaploid genome. As a consequence, *Arabidopsis thaliana* is used as a model for victorin sensitivity. Victorin sensitive Arabidopsis ecotypes were identified and enabled the cloning of a single dominant gene that confers both victorin sensitivity and Victoria blight susceptibility (Lorang et al., 2004; Lorang et el, 2007). This gene was called *LOCUS ORCHESTRATING VICTORIN EFFECTS1* (*LOV1*). *LOV1* encodes a nucleotide binding site leucine-rich repeat (NB-LRR) protein (Lorang, 2007). The majority of NB-LRR proteins characterized are plant disease resistance (R) proteins. Therefore, as in oats, susceptibility appears to be conditioned by a resistance gene. In a gene-for-gene

manner, R proteins guard pathogen effector targets and activate effector-triggered immunity upon effector recognition (Van der Biezen and Jones, 1998; Jones and Dangl, 2006). Pathogens deploy effectors to increase virulence through inhibition of basal defense in a process known as PAMP-triggered immunity. PAMP-triggered immunity is activated by recognition of pathogen-associated molecular patterns (PAMPS), which are conserved elicitor molecules shared by classes of microbes (Bittel and Robatzek, 2007; Jones and Dangl, 2006). Effector-triggered immunity is considered a stronger and faster defense response than PAMP-triggered immunity and typically includes the HR. (Jones and Dangl, 2006). LOV1 is a member of the RPP8 (recognition of peronospora parasitica 8) family of proteins (Lorang et al., 2007), of which all other members with known functions (RPP8, HRT, and RCY1) are involved in effector-triggered immunity. RPP8, the closest homolog to LOV1 (86% similar in amino acid sequence) (Lorang et al. 2007), confers resistance to the oomycete pathogen *Hyaloperonospora arabidopsidis* (McDowell et al., 1998). HRT confers resistance to turnip crinkle virus (Cooley et al., 2000), while RCY1 confers resistance to cucumber mosaic virus (Takahashi et al. 2002).

A forward genetic screen of ethyl methanesulfonate (EMS) mutagenized Arabidopsis identified thioredoxin h5 (TRX-h5) as a second protein required for victorin-mediated cell death (Sweat and Wolpert, 2007). TRX-h5 covalently binds victorin, and this duplex is likely recognized by LOV1 (Lorang et al., In Press). TRX-h5 is a modulator of non-expressor of pathogenesis related genes 1 (NPR1),

a transcriptional co-regulator in plant defense responses (Tada et al., 2008). Upon pathogen challenge, *TRX-h5* is up-regulated, likely by salicylic acid signaling (Sweat and Wolpert, 2007; Laloi et al., 2004). TRX-h5 monomerizes NPR1 oligomers permitting NPR1 to translocate to the nucleus where it induces expression of pathogenesis-related genes (Tada et al., 2008; Kinkema et al., 2000). Pathogenesis-related genes encode proteins with antimicrobial activity (Edreva 2005) that effect local and systemic resistance (Kinkema et al., 2000). This control over defense places TRX-h5 as a potential target for pathogen effectors. Covalent binding of victorin to TRX-h5 inhibits TRX-h5 catalytic function, and as a result victorin increases susceptibility to the pathogen *Pseudomnas syringae* pv *maculicola* in Arabidopsis lacking *LOV1* (Lorang et al., In Press). These results demonstrate victorin can function as a virulence effector in the absence of *LOV1*, by inhibiting TRX-h5 and consequently, local or systemic resistance. Because guarding pathogen effector targets is a typical resistance protein function, the mechanism of LOV1 activation suggests LOV1 functions as a resistance protein. It apparently guards TRX-h5 and initiates an effector-triggered response upon victorin binding to its guardee. In addition, *LOV1* is widespread and highly conserved in Arabidopsis suggesting *LOV1* is maintained as an active resistance gene against a pathogen (Sweat et al., 2008).

## 1.4 IS LOV1 FUNCTIONALLY RELATED TO RESISTANCE PROTEINS?

As discussed above, multiple lines of evidence suggest victorin functions as an avirulence determinate, inducing effector-triggered immunity upon recognition by LOV1. LOV1 is encoded by a member of a disease resistance gene family and appears to be guarding an effector target, TRX-h5 (Lorang et al, In Press). Activation of LOV1 results in a physiological response that is similar to a defense response (Lorang et al, 2007; Wolpert et al, 2011). These data suggest effector-triggered immunity is involved in susceptibility to *C. victoriae*. However, a resistance gene function for LOV1 has not been evaluated. For other plant pathogen interactions that follow an inverse gene-for-gene model, susceptibility may also involve effector-triggered immunity. In *Sorghum bicolor*, susceptibility to *Periconia circinata* producing the host-selective, PC-toxin is dependent on a NB-LRR (Nagy and Bennetzen, 2008 ). *Tsn1* encodes an atypical NB-LRR protein that confers wheat susceptibility to two different pathogens, *Pyrenophora tritici-repentis* (*Ptr*) that produces the host-selective toxin Ptr ToxA (Faris et al., 2010) and *Stagonospora nodorum* (*Sn*) that produces SnToxA (Liu et al, 2006). Ptr ToxA and SnToxA are peptides with a high degree of similarity. Ptr ToxA has been shown to induce gene expression changes associated with disease resistance in susceptible plants (Pandelova et al., 2009; Pandelova et al., 2012).

The potential involvement of effector-triggered immunity in disease susceptibility has broad implications. The targeted deployment of R genes in

agriculture by the introduction of R genes into plants to confer disease resistance may unwittingly increase vulnerability to certain pathogens. The history of Victoria blight suggests newly introgressed R genes may result in future disease epidemics that could affect agriculture and food supply.

However, although NB-LRR encoding genes can condition disease susceptibility, the downstream function of NB-LRRs in disease susceptibility and their similarity to resistance signaling has not been fully evaluated. Do "susceptibility" NB-LRR proteins have a distinct feature that allows them to be exploited by pathogens? The work presented here will further assess whether LOV1 functions in a resistance response pathway. The approach was twofold. Virus-induced gene silencing (VIGS) was employed to identify genes involved in victorin-mediated cell death. This approach allowed a comparison of the genes required for victorin-mediated cell death to those involved in disease resistance. As an additional approach, the ability of LOV1 to confer disease resistance following pathogen invasion was evaluated in Arabidopsis.

## 1.5 GENETIC CHARACTERIZATION OF VICTORIN-MEDIATED CELL DEATH

Identifying genes other than *LOV1* and *TRX-h5* that are required for victorin-mediated cell death should reveal whether victorin-mediated cell death is functionally related to a disease resistance response. The initial screen for mutations that suppress *LOV1*-mediated cell death identified *TRX-h5,* but no additional components required for victorin-mediated cell death (Sweat and

Wolpert, 2007). The genetic requirements for effective resistance vary among resistance genes (Glazebrook, 2005). Salicylic acid (SA), jasmonic acid (JA), and ethylene are three major signal molecules utilized in defense pathways (Thomma et al., 2001). Depending on the host and pathogen, SA, JA, and ethylene may act in pathways that promote or antagonize defense. Within the *RPP8* gene family, signaling requirements for disease resistance are variable depending on the specific resistance gene (Takahashi et al., 2002). Mutant analysis has indicated SA and JA signaling pathways are not required for victorin sensitivity, and ethylene signaling pathways play a subtle role in victorin sensitivity (Lorang et al., 2007).

In this work, virus-induced gene silencing (VIGS) was used to find genes other than LOV1 and TRX-h5 involved in the victorin response. VIGS can be used as both a reverse and forward genetic tool to analyze gene function (Lu et al., 2003 b). VIGS is used to silence genes post-transcriptionally in a sequence specific manner. This allows identification of phenotypes associated with the silencing of specific genes. VIGS utilizes RNA interference (RNAi) (Figure. 1-2), a mechanism of antiviral defense (Baulcombe 1999; Lu et al., 2003 b). RNAi involves the recognition of double-stranded RNA, which is believed to be formed during replication of the virus (Dalmay et al., 2000). The dsRNA is cleaved by the ribonuclease Dicer into 21 to 25 nucleotide, small interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999; Tang et al 2003). The siRNAs associate with the RNA-induced silencing complex (RISC) (Hannon, 2002). The RISC-siRNA complex targets specific cleavage or translational suppression of mRNA

transcripts with sequence complementarity to the specific siRNA (Hannon, 2002). This anti-viral defense response can be exploited for silencing specific host genes by constructing viruses carrying fragments of a target gene. When plants are infected with unmodified virus, the response leads to partial inhibition of viral replication and spread. When infected with modified virus, the response also leads to inhibition of the corresponding plant mRNAs (Baulcombe, 1999).

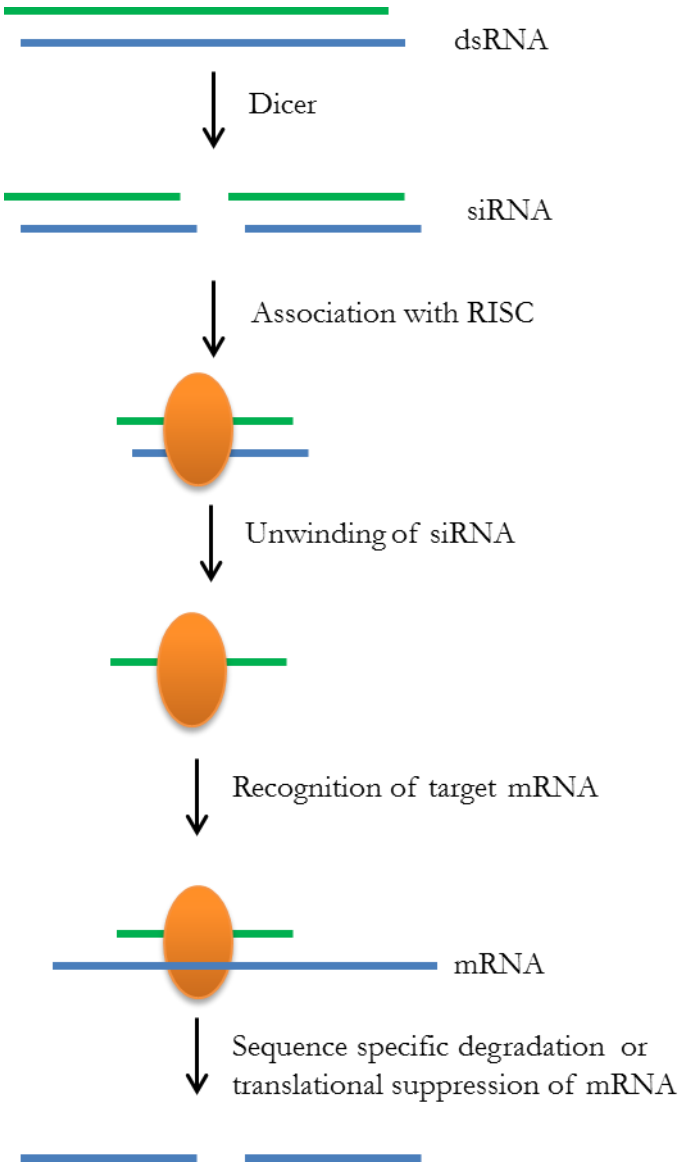


Figure 1-2: Virus induced gene silencing utilizes RNA interference for sequence specific degradation of mRNA. Double stranded RNA (dsRNA), from viral replication, containing the target host gene sequence is cleaved by the enzyme Dicer into small interfering RNAs (siRNAs). The siRNA associates with RNA-induced silencing complex (RISC) and the siRNA is unwound. RISC then uses the antisense strand of siRNA as a guide to cleave or suppress translation of complementary mRNA.

VIGS is highly efficient in *Nicotiana benthamiana.* Silencing is generally more pronounced and more persistent than in other plants (Lu et al, 2003 b). This has been attributed to a greater ability of the virus to infect and spread cell-to-cell in *N. benthamaiana (*Lu et al, 2003 b; Waigmann et al, 2000). In this work, a VIGS vector based on tobacco rattle virus (TRV) was used. TRV is highly efficient for silencing in *N. benthamiana,* attributed to the ability of TRV to invade the meristem (Burch-Smith et al, 2004; Ratcliff et al., 2001, Martín-Hernández and Baulcombe, 2007). The viral symptoms from TRV are mild which allows easy identification of phenotypes resulting from silencing (Burch-Smith et al, 2004; Ratcliff et al, 2001).

VIGS is amenable to high-throughput functional genomic studies (Lu et al, 2003 b). The use of a virus circumvents the need for stable plant transformation with RNAi inducing transgenes. The virus is introduced by expression of viral RNAs via *Agrobacterium*-mediated, transient expression. High-throughput applications are possible with VIGS in *Nicotiana benthamiana*, where sufficient infection can occur by using a toothpick to inoculate *Agrobacterium* containing the viral vector (Anand et al., 2007). An *Agrobacterium* colony is picked with a toothpick and then directly inoculated into a leaf by wounding. In other hosts such as Arabidopsis, the *Agrobacterium* must be delivered in suspension and infiltrated for efficient silencing by VIGS (Wang et al., 2006). The infiltration method is more time consuming and less applicable for high-throughput studies.

Components involved in disease resistance pathways have been identified with VIGS in high-throughput screens in *N. benthamiana*. For forward genetics, a cDNA library is first mobilized into a VIGS vector resulting in clones that silence targeted host genes. Individual plants are infected with a clone, resulting in a different gene silenced in individual plants. When an interesting phenotype is found, the cDNA responsible can be determined by sequencing the particular VIGS clone. The functional requirement for Heat Shock Protein 90 (HSP90) in disease resistance was found by screening for loss of HR elicited by bacteria carrying an avirulence determinant (Lu et al., 2003). A MAP kinase cascade involved in the signal transduction of cell death and resistance was identified by screening for the loss of HR induced upon heterologous expression of an R gene and cognate avirulence determinate (Pozo et al., 2004). We utilized VIGS to identify genes involved in the response mediated by LOV1 by screening for the loss of victorin-mediated cell death after gene silencing.

VIGS offers several advantages as a genetic tool. VIGS can occur following development, which allows analysis of genes that cause embryonic lethality when disabled. VIGS can silence multiple members of a gene family if the sequence targeted is conserved among alleles (He et al.., 2004). This can overcome the problem of finding important mutations in genes where gene products are functionally redundant. In the initial EMS mutagenesis screen for mutations that abrogate LOV1 function, important mutations in these types of genes would have

been missed. These advantages of VIGS should allow further characterization of the response mediated by LOV1.

Determining how LOV1 functions will further clarify the difference between resistance and susceptibility to biotrophic compared to necrotrophic pathogens. Understanding the molecular basis of Victoria blight could help prevent future epidemics that would impact agriculture and food systems. A screen was conducted to identify proteins required for victorin-mediated cell death to further characterize the response mediated by LOV1. We also tested whether LOV1 can function as a resistance gene. The results of these studies are presented in the following two chapters.

# 2 Characterization of Genes Required for Victorin-mediated cell death using Virus-Induced Gene Silencing

Brian M. Gilbert and Tomas J. Wolpert

Prepared for Submission

## ABSTRACT

Victoria blight, caused by *Cochliobolus victoriae*, is a disease originally described on oats and recapitulated on *Arabidopsis*. *C. victoriae* pathogenesis depends upon production of the toxin, victorin. In oats, victorin sensitivity is conferred by the *Vb* gene, which is genetically inseparable from the *Pc2* resistance gene. Concurrently, in Arabidopsis, sensitivity is conferred by the *LOV1* gene. LOV1 is a NB-LRR protein, a type of protein commonly associated with disease resistance. LOV1 appears to "guard" the defense thioredoxin, TRX-h5. Expression of *LOV1* and *TRX-h5* in *Nicotiana benthamiana* is sufficient to confer victorin sensitivity. Virus-induced-gene silencing was used to characterize victorin-mediated cell death in *N. benthamiana*. We determined *SGT1* is required for sensitivity and involved in LOV1 protein accumulation. We screened a normalized cDNA library and identified six genes that, when silenced, suppressed victorin-mediated cell death and cell death induced by expression of the *RPP8* resistance gene: a mitochondrial phosphate transporter, glycolate oxidase, glutamine synthetase, glyceraldehyde 3-phosphate dehydrogenase and the P- and T-protein of the glycine decarboxylase complex. Silencing the latter four also inhibited cell death and disease resistance mediated by the *PTO* resistance gene. Together these results provide evidence that victorin-mediated cell death is functionally similar to a resistance response.

## 2.1 INTRODUCTION

Victoria blight, a disease originally described on oats (*Avena sativa)*, is caused by the necrotrophic fungus *Cochliobolus victoriae* (Meehan and Murphy, 1946). Victoria Blight universally appeared on oats carrying the crown rust resistance gene *Pc-2*, which confers resistance to the biotrophic fungus *Puccinia coronata* (Litzenberger, 1949). *C. victoriae* pathogenesis is dependent on production of victorin, a peptide host-selective toxin (Meehan and Murphy, 1947; Macko et al., 1985; Wolpert et al., 1985). Sensitivity to victorin is conferred by the dominant *Vb* gene in oats. Only oats sensitive to victorin are susceptible to *C. victoriae,* and oats treated with victorin alone display symptoms of Victoria blight (Meehan and Murphy, 1947). Various genetic and mutagenic efforts to separate Victoria blight susceptibility from crown rust resistance have failed, suggesting that *Pc-2* and *Vb* are the same gene (Welsh et al., 1954; Luke et al., 1966; Rines and Luke, 1985; Mayama et al., 1995). Thus, genetic evaluations indicate that Victoria blight susceptibility in oats is associated with a resistance gene. Consistent with the genetic data, victorin elicits a response that resembles a defense response in sensitive oats (i.e. those containing *Vb*,). This response includes callose deposition, a respiratory burst, lipid peroxidation, ethylene evolution, extracellular alkalization, phytoalexin synthesis, and K+ efflux (Walton and Earle, 1985; Romanko, 1959; Navarre and Wolpert 1999; Shain and Wheeler, 1975; Ullrich and Novacky, 1991; Mayama et al., 1986 ; Wheeler and Black, 1962). Notably, victorin also induces a programmed cell death (PCD) response, which involves a mitochondrial permeability transition (Navarre and Wolpert, 1999; Tada et al.,

2001;Yao et al., 2001, 2002; Coffeen and Wolpert, 2004; Curtis and Wolpert, 2002, 2004). This PCD response shares characteristics with the hypersensitive response (HR), a form of PCD associated with disease resistance (Mur et al., 2007).

Victorin sensitivity and susceptibility to *C. victoriae* was identified in Arabidopsis (Lorang et al., 2004), and victorin-induced cell death was shown to share characteristics of PCD in oats (Wolpert et al, 2011) and of the HR (Lorang et al., 2007). Victorin sensitivity and disease susceptibility in Arabidopsis is conferred by a single dominant gene designated *Locus Orchestrating Victorin Effects1* (*LOV1*) (Lorang et al., 2007). *LOV1* encodes a nucleotide-binding site-leucine-rich repeat (NB-LRR) protein which is a class of proteins typically associated with disease resistance (Nimchuk et al., 2003). LOV1 is a member of the RPP8 family of proteins, of which all other members with known functions are involved in disease resistance (Cooley et al. 2000; McDowell et al. 1998; Takahashi et al. 2002; Lorang et al., 2007). NBS-LRR proteins act as immune receptors, directly or indirectly recognizing pathogen effectors (Dangl and Jones, 2001). Pathogens produce effectors to promote virulence (Dangl and Mcdowell, 2006). Recognition of pathogen effectors results in effector triggered immunity (ETI)(Jones and Dangl, 2006). Effective ETI leads to a decrease in pathogen growth and typically includes the HR (Greenberg and Yao, 2004).

The HR is thought to be an effective defense against biotrophic pathogens, which derive nutrients from living host tissue (Glazebrook, 2005;

Agrios, 1997). PCD associated with the HR surrounding the infection site halts the spread of infection by limiting access to nutrients and water (Heath, 2000; Glazebrook, 2005). *C. victoriae* is characterized as a necrotrophic pathogen, which actively kills host tissue and extracts nutrients from dead cells (Stone, 2001). For necrotrophic pathogens, the HR may actually be beneficial and could promote susceptibility (Govrin and Levine, 2000, Wolpert et al, 2001). For the necrotroph *Botrytis cinerea,* the HR has been shown to aid in pathogenicity by providing a growth substrate (Govrin and Levine, 2000). Induction of the HR by the hemi-biotrophic fungus *Mycosphaerella graminicola* is hypothesized to play an important role in its pathogenicity on wheat (Keon, et al., 2006; Hammond-Kosack and Rudd, 2008). The similarities of victorin-induced cell death with the HR, the association of *Vb* with the *Pc2* rust resistance gene and the finding that *LOV1* belongs to a family of disease resistance genes all suggest that *C. victoriae* may have co-opted the defensive HR to derive nutrients from the host.

A screen for EMS-induced loss of sensitivity in Arabidopsis identified TRX-h5 as a protein required for LOV1 function (Sweat and Wolpert, 2007). LOV1 is activated (induces cell death) when TRX-h5 binds victorin (Lorang et al., In Press). TRX-h5 is also required for the redox-mediated control of NPR1 (non-expresser of PR1 protein), a transcriptional coregulator (Tada et al., 2008). NPR1 regulates changes in defense gene expression in response to pathogens (Kinkema et al., 2000). Due to its role in defense, TRX-h5 is a likely target for pathogen effectors. In the absence of LOV1, victorin acts as a typical virulence

effector by inhibiting TRX-h5 catalytic function and thereby interfering with defense mediated by NPR1 (Lorang et al., In Press). Conversely, in the presence of LOV1, victorin binding to TRX-h5 leads to LOV1 activation and cell death (Lorang et al., In Press). These findings support the interpretation that *LOV1* functions in ETI as a resistance gene in Arabidopsis, guarding TRX-h5 from effectors that targets thioredoxins. Thus, mechanistic studies also support the contention that *C. victoriae* may be co-opting the HR.

The purpose of this work was to find genes other than *LOV1* and *TRX-h5* required for the victorin response to further characterize the mechanism of victorin-mediated cell death and clarify its relationship with ETI. Here we exploit the finding that co-expression of Arabidopsis *LOV1* and *TRX5* in *N. benthamiana* confers victorin sensitivity (Lorang et al., In Press). We use the tobacco rattle virus (TRV)-based virus induced gene silencing (VIGS) system to screen for additional genes involved in victorin-mediated cell death in *N. benthamiana*. VIGS employs RNA interference as a tool for reverse genetics to analyze gene function (Baulcombe, 1999, Lu et al., 2003b). A gene of interest is inserted into the viral vector, and upon infection, double-stranded RNA of the inserted gene is produced, leading to silencing of host mRNAs that contain sequence homology. This approach offered several advantages over the previous EMS mutagenesis screen that identified TRX-h5. Unlike EMS mutagenesis, VIGS can silence multiple members of a gene family if the insert contains sequence conserved across all family members, and VIGS can be used to study genes with roles in

both cell death and development while avoiding embryonic lethality (Lu et al., 2003b). Therefore, the use of VIGS had the potential to identify additional components required for victorin-mediated cell death.

We show that genes involved in metabolic activity, a mitochondrial phosphate transporter, and *SGT1* (for *SUPPRESSOR OF G2 ALLELE OF SKP1*) are required for victorin sensitivity. The metabolic enzymes found to be important were four photorespiratory enzymes glycolate oxidase (GOX), glutamine synthetase (GLU), P-protein of the glycine decarboxylase complex (GDC-P), T-protein of the glycine decarboxylase complex(GDC-T)) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). These metabolic enzymes were also required for cell death induced by transient expression of the *RPP8* resistance gene and an autoactive form of *PTO* (PTOY207D). Silencing of these metabolic genes also reduced PTO-mediated resistance to *Pseudomonas syringae* p.v. tabaci in *N. benthamiana,* demonstrating a role for these genes in resistance conferred by ETI. A role for SGT1 in LOV1 protein accumulation was also established. Together these results provide evidence that the victorin-mediated cell death response is functionally similar to a resistance response.

## 2.2 RESULTS

### 2.2.1 VIGS establishes that SGT1 is required for Victorin-mediated cell death

Victorin sensitivity in *N. benthamiana*, conferred by transient co-expression of *LOV1* and *TRX-h5* (Lorang et al., In Press), demonstrates that the pathway

necessary for victorin-mediated cell death is present in *N. benthamiana*. Victorin sensitivity was utilized in *N. benthamiana* to identify genes required for victorin-mediated cell death. *N. benthamiana* plants were infected with a tobacco rattle virus (TRV)-vector (Lu et al., 2003b) containing a fragment of a host gene to be silenced by VIGS. After gene silencing, *LOV1* and *TRX-h5* were transiently expressed in the leaves and the extent of the cell death following victorin treatment was assessed. An electrolyte leakage assay was developed to quantify victorin-mediated cell death in *N. benthamiana*. Leaf disks were excised from silenced plants expressing *LOV1/TRX-h5*. Negative controls employed plants infected with a construct to silence expression of *GREEN FLOURESCENT PROTEIN (*TRV:*GFP)* to control for viral effects in our VIGS experiments. Conductance was measured to quantify electrolyte leakage, an indicator of cell death, over the course of 12 hours after exposing the leaf disks to victorin (Figure 2-1). Electrolyte leakage increased over time, fitting a typical sigmoidal curve and approached saturation 12 hours after exposure to victorin (Figure 2-1A). Electrolyte leakage increased appreciably as victorin concentration was increased from 10 to 1000 ng/mL in half-log increments (Figure 2-1A). As victorin concentration was increased logarithmically, leakage increased linearly at all times tested including 2, 4, 6, 8, and 12 hours ($R^2$=0.99, 0.99, 0.99, 0.94, and 0.70 respectively), indicating a dose-response relationship which began to decay (reach saturation) around 12 hours after victorin treatment (Figure 2-1B). Therefore, electrolyte leakage at these doses and up to 12 hours after treatment appears to provide a quantitative measure of victorin-mediated cell death.

**Figure 2-1: Victorin elicits electrolyte leakage as a dose-responsive indicator of cell death, in *N. benthamiana* leaves expressing *LOV1* and *TRX-h5*.**

Effect of victorin concentration on electrolyte leakage kinetics from leaf disks from leaves of plants expressing TRV:*GFP,* a negative control used for virus induced gene silencing, and *LOV1* and *TRX-h5*. (A) Leakage over a 12 hour time-course following treatment with 0, 10, 31.6, 100, 136, or 1000 ng victorin/mL. (B) Electrolyte leakage increases as a log dose response to victorin concentration indicating a dose-response relationship for 12 hours after treatment. Means ± SE of 3 samples are plotted and are representative of three independent experiments.

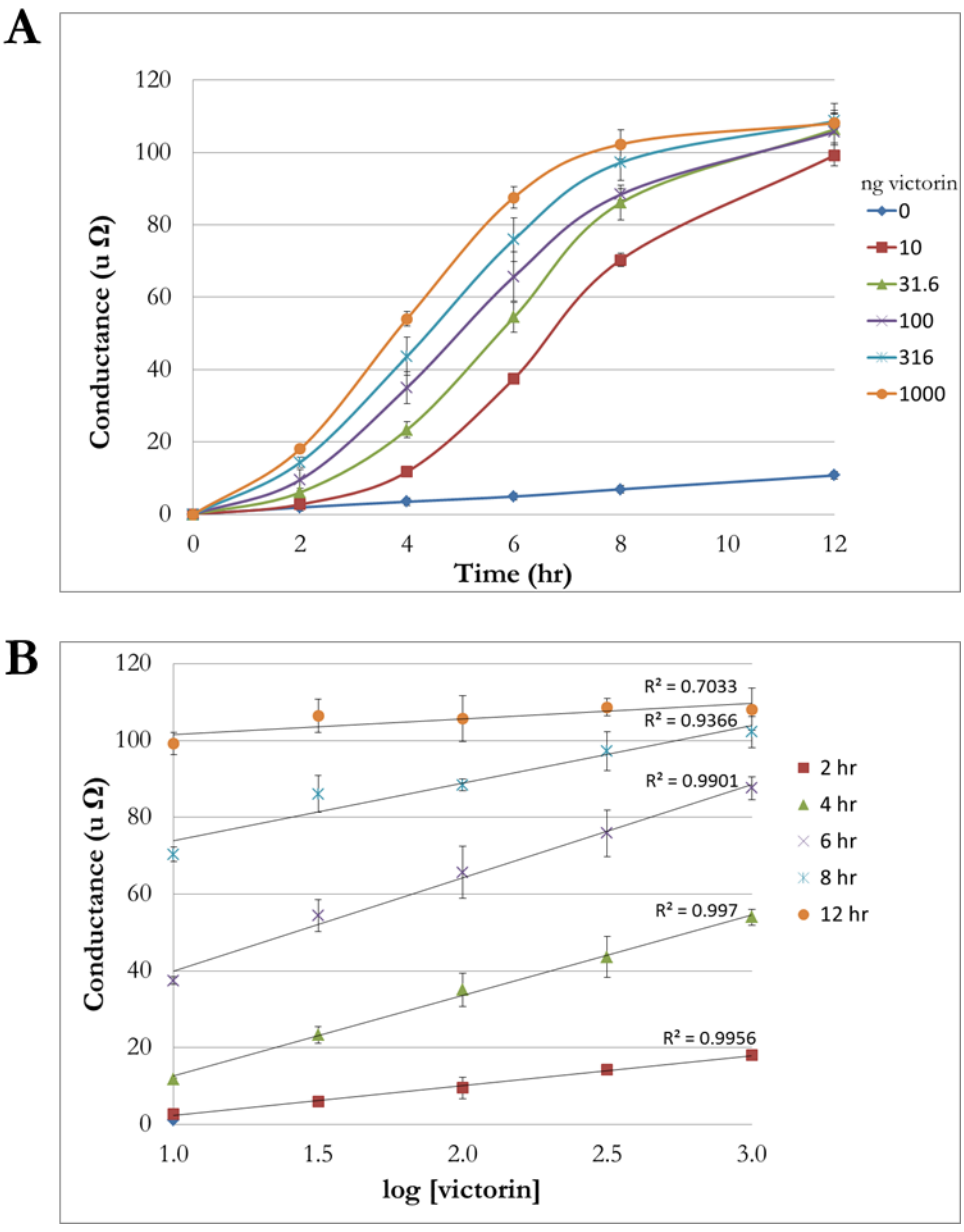


**Figure 2-1: Victorin elicits electrolyte leakage as a dose-responsive indicator of cell death, in *N. benthamiana* leaves expressing *LOV1* and *TRX-h5*.**

VIGS was used for silencing a set of plant genes that have known roles in defense signaling to determine the function of these genes in victorin-mediated cell death. We silenced *N. benthamiana* orthologs of mitogen-activated-protein (MAP)-kinases (*WIPK, SIPK, MEK1, MEK2*), the MAP-kinase kinase *MAPKKKα*, the protein kinase *ADI2*, *EDS1* and *NRC1* which have been implicated in signaling during the HR, and *SGT1*, *RAR1*, and *HSP90* (members of a chaperon complex). We routinely assayed for both victorin sensitivity and cell death induced by the expression of the *RPP8* resistance gene in leaves of plants silenced for the individual genes. By monitoring cell death induced by *RPP8* expression we could compare the signaling requirements for LOV1 to that of a highly homologous protein with known resistance function. Of the genes tested, *NbSGT1* was identified to be required for victorin-mediated cell death in *N. benthamiana* (Figure 2-2). Leaves transiently expressing *LOV1* and *TRX-h5* in plants inoculated with the control TRV:*GFP* construct showed the expected cell death phenotype following victorin treatment (Figure 2-2A). In contrast, leaves transiently expressing *LOV1* and *TRX-h5* from *NbSGT1* silenced plants showed no cell death after victorin treatment (Figure 2-2A). Cell death induced by *RPP8* expression was not affected by silencing *NbSGT1* (Figure 2-2A). The reduction in visible cell death mediated by victorin correlated with reduced electrolyte leakage. Leaf disks excised from *LOV1/TRX-h5* expressing leaves from plants silenced for *NbSGT1* exhibited considerably less electrolyte leakage after victorin treatment over an 8 hour time period compared to the control (Figure 2-2B).

**Figure 2-2: Victorin-mediated cell death is inhibited by silencing *NbSGT1*.**

(A) Leaves from plants expressing TRV:*GFP* (control) or TRV:*NbSGT1* were inoculated (in region identified by red circles) with *Agrobacterium* containing constructs to transiently express *LOV1* and *TRX-h5* or *RPP8* (control). 72 hr after inoculation, 100 ng victorin was infiltrated into the region expressing *LOV1 and TRX-h5*. Cell death was assessed 24 hr after victorin treatment. (B) Electrolyte leakage is reduced in leaves when *NbSGT1* is silenced. Electrolyte leakage kinetics were analyzed following 31.6 ng/mL victorin treatment of leaf disks from leaves of plants inoculated with TRV:*GFP* or TRV:*NbSGT1* and expressing *LOV1* and *TRX-h5*. Conductivity is reported relative to control at 8 hrs. Means ± SE of three independent experiments are plotted. (C) *NbSGT1* positively regulates LOV1 accumulation. Protein was extracted from leaves of plants inoculated with TRV:*GFP* (control) or TRV:*SGT1* and expressing *LOV1* and *myc:TRX*. Relative amounts of LOV1, myc:TRX-h5, and NbSGT1 were determined by western blotting with anti-LOV1, anti-myc, and anti-SGS antibodies respectively.

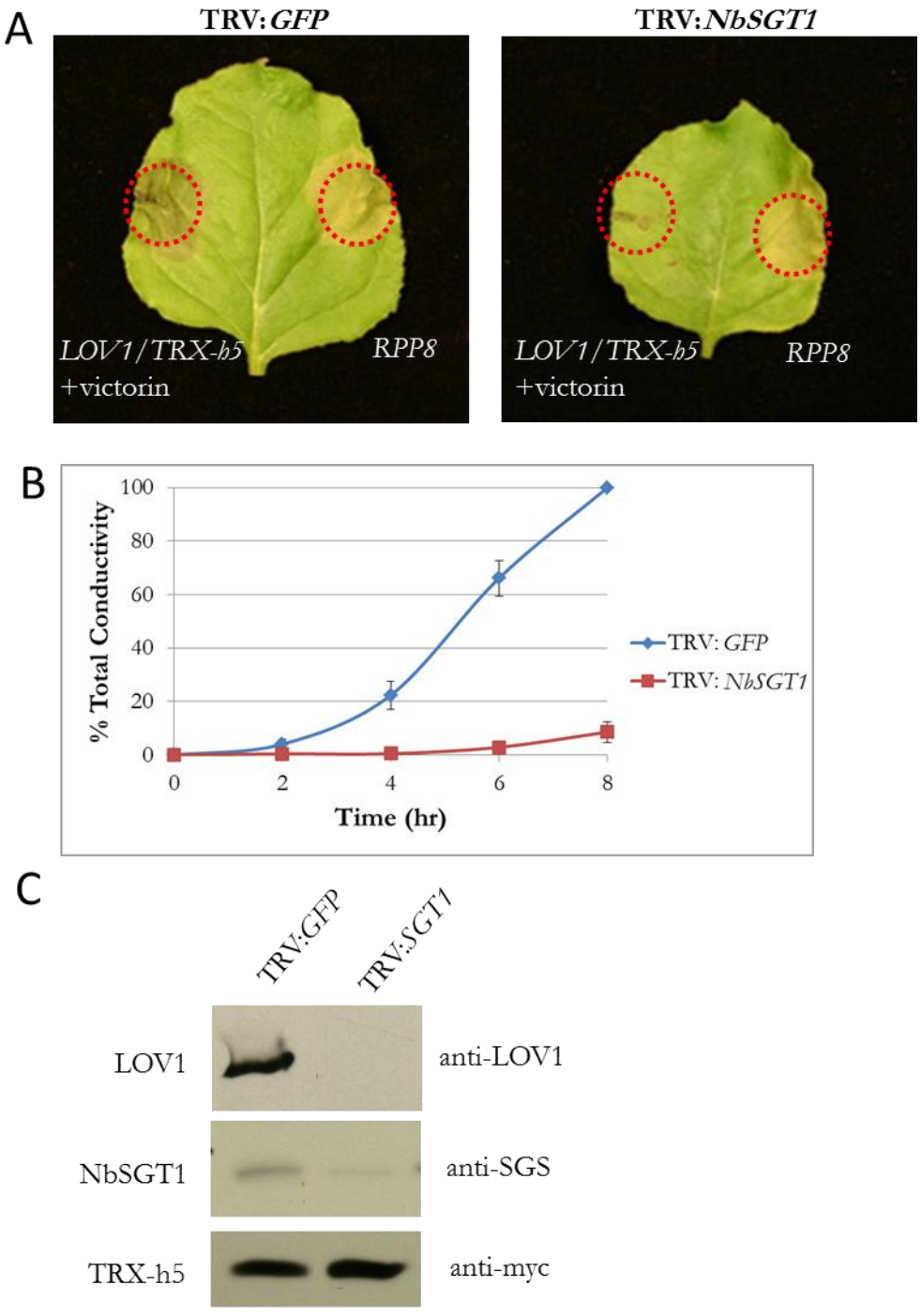


**Figure 2-2: Victorin-mediated cell death is inhibited by silencing *NbSGT1*.**

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When *NbSGT1* was silenced, electrolyte leakage was about 10% of that observed in the positive control after 8 hours of victorin treatment (Figure 2-2B).

Because SGT1 can impact the accumulation of resistance proteins (Azevedo et al., 2006), we wanted to determine if the reduction in victorin-mediated cell death in *NbSGT1* silenced plants is due to a reduced accumulation of LOV1 protein. To monitor the effect of silencing NbSGT1 on TRX-h5 protein levels, myc-tagged TRX-h5 was expressed. Myc-tagged TRX-h5 is fully functional in conferring victorin-mediated cell death when co-expressed with *LOV1* (data not shown). Total protein extracts from *LOV1*/*myc:TRX-h5* expressing leaves silenced for *NbSGT1* or expressing TRV:*GFP* (control) were analyzed by Western blotting . Probing with an antibody raised against the conserved SGS domain of SGT1 (Azevedo et al., 2002) confirmed the depletion of NbSGT1 protein when silencing *NbSGT1*. This depletion of NbSGT1 correlated with a reduction in LOV1 protein, but levels of mcy:TRX-h5 protein were not affected (Figure 2-2C).

In Arabidopsis there are two SGT1 isoforms, AtSGT1a and AtSGT1b. An Arabidopsis *LOV1* genotype containing a mutation in *AtSGT1b* was previously found to be fully-sensitive to victorin (Lorang et al, 2007), but plants having an*AtSGT1a* mutation had not been evaluated for victorin sensitivity. We obtained an *AtSGT1a* T-DNA insertion mutant (GABI_266H09) and crossed it with a wild-type *LOV1* plant. Western blot and insertion analyses indicated that GABI_266H09 is likely a null mutation (Figure SI 2-1). We found plants

homozygous for the T-DNA insertion and *LOV1* were indistinguishable from wild-type plants with regard to victorin sensitivity (Figure SI 2-2). We therefore concluded that if SGT1 is required for victorin-mediated cell death in Arabidopsis, AtSGT1a and AtSGT1b likely have redundant functions.

Because *Atsgt1a/At sgt1b* double mutants are embryo lethal (Azevedo et al., 2006), we utilized a heterologous transient complementation assay in *N. benthamiana* (Azevedo et al., 2006) to determine if either AtSGT1a or AtSGT1b is able to function in victorin-mediated cell death. The ability of *AtSGT1a* or *AtSGT1b* expression to complement silencing of *NbSGT1* and restore victorin sensitivity was tested (Figure 2-3). As expected, co-expression of the control *GFP* with *LOV1* and *TRX-h5* did not complement victorin sensitivity in plants silenced for *NbSGT1*. Concurrently, co-expression of *AtSGT1a* or *AtSGT1b* with *LOV1* and *TRX-h5* had no visible effect on victorin-mediated cell death in plants expressing the empty vector control, TRV:00 (Figure 2-3A). In contrast, co-expression of myc:*AtSGT1a* or myc:*AtSGT1b* with *LOV1* and *TRX-h5* restored victorin sensitivity in *NbSGT1* silenced plants (Figure 2-3A). Western blot analysis verified the accumulation of myc:AtSGT1a and myc:AtSGT1b protein when expressed in *NbSGT1* silenced plants (Figure 2-3B). As expected, when *LOV1* is co-expressed with the *GFP* control, levels of LOV1 protein are reduced in NbSGT1 silenced plants compared to control (Figure 2-3B). In contrast, when LOV1 is co-expressed with AtSGT1a or AtSGT1b, LOV1 levels in *NbSGT1* silenced plants are restored to levels similar to that of the unsilenced control

**Figure 2 3: Transient expression of *AtSGT1a* or *AtSGT1b* complements silenced *NbSGT1* and restores victorin-mediated cell death.**

(A) Leaves from plants silenced with TRV:*SGT1* or TRV:00 (empty-vector control) were infiltrated in the areas indicated by red circles with *Agrobacterium* to express *LOV1 (LOV)* and *TRX-h5 (TRX5)* in combination with *GFP* (control), *AtSGT1a*, or *AtSGT1b*. Cell death was assessed 24 hr after 10 ug/mL victorin treatment. (B) Relative abundance of LOV1 increases when *LOV 1* is co-expressed with *myc:AtSGT1a* or *myc:AtSGT1b* in leaves from plants silenced with TRV:*NbSGT1*. Total protein was extracted from leaves of plants silenced with TRV:00 (control) or TRV:*SGT1* expressing the indicated constructs. Relative amounts of LOV1, AtSGT1a, and ATSGT1b were determined by Western Blotting. Equal loading of the different lanes was confirmed by Ponceau S staining of Rubisco on blot membrane.

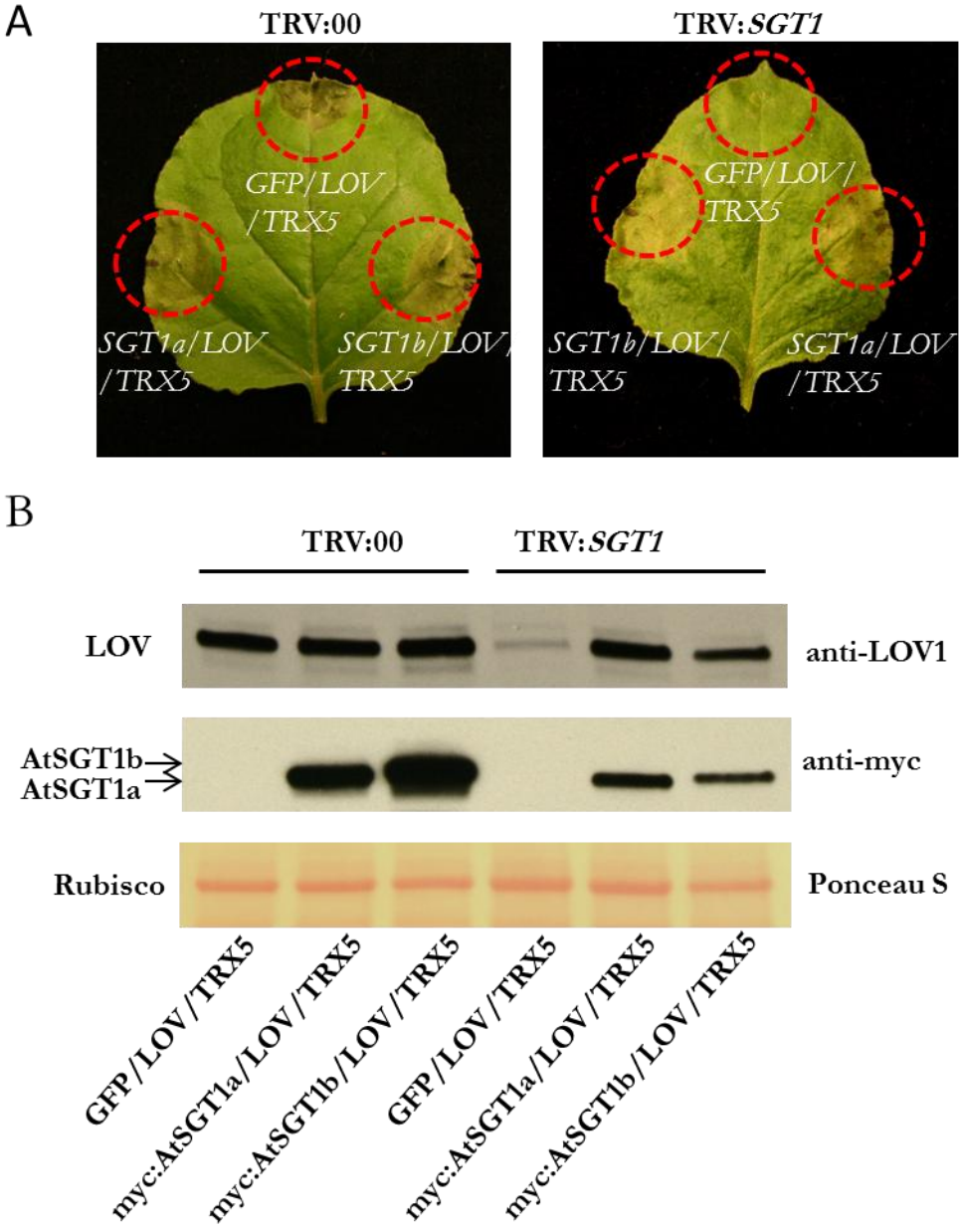


**Figure 2-3: Transient expression of *AtSGT1a* or *AtSGT1b* complements silenced *NbSGT1* and restores victorin-mediated cell death.**

(Figure 2-3B). These data demonstrate that both AtSGT1a and AtSGT1b have the capacity to function in victorin-mediated cell death and LOV1 accumulation.

### 2.2.2 A high-throughput VIGS screen identifies several genes involved in victorin-mediated cell death

To identify additional genes involved in victorin-mediated cell death, we used VIGS to silence approximately 4,000 genes from a *N. benthamiana* mixed-elicitor cDNA (cNbME) library. The cNbME library was prepared from tissue exposed to the plant pathogens *Agrobacterium tumefaciens*, *Pseudomonas syringae* pv. *tabaci*, and *Pseudomonas syringae* pv. *tomato*, and to the abiotic elicitors salicylic acid, jasmonic acid, and ethylene. (Anand et al, 2007). TRV clones (one gene per clone) from the library were individually inoculated into *N. benthamiana* plants in duplicate. After silencing, we tested for both victorin sensitivity and cell death induced by the expression of *RPP8*. For every experiment, VIGS of the non-plant gene *GFP* was used as a negative control for cell death and VIGS of *LOV1* was used as a positive control for the loss of victorin-mediated cell death (Figure 2-4A). Any clones that interfered with victorin-mediated cell death or cell death induced by *RPP8* expression in at least one replicate in the primary screen were subjected to subsequent screenings in triplicate. In these subsequent screens, *Agrobacterium* carrying a construct to express the green fluorescent protein (GFP) reporter was infiltrated to eliminate clones that silenced genes which interfere with transient expression. Four days following *Agrobacterium*

**Figure 2 4: VIGS based identification of cDNA clones that inhibit victorin-mediated, LOV1-dependent cell death and cell death induced by *RPP8*.**

(A) Controls used in the VIGS screen. Assessment of cell death after VIGS of *GFP* (negative control), VIGS of *LOV1* (positive control), and an example image demonstrating no effect on GFP fluorescence as used to confirm clones tested had no effect on *Agrobacterium*-mediated transient expression. (B) Assessment of cell death in leaves from plants silenced with the clones identified from the screen. TRV infected plants were infiltrated (in the regions identified by red circles) with *Agrobacterium* containing constructs to express *LOV1 (LOV)* and *TRX-h5 (TRX5)*, or *RPP8*. Forty-eight hrs after *Agrobacterium* inoculation, victorin was infiltrated in the region of *LOV1* and *TRX-h5* expression. Pictures were taken 24 hrs after victorin treatment

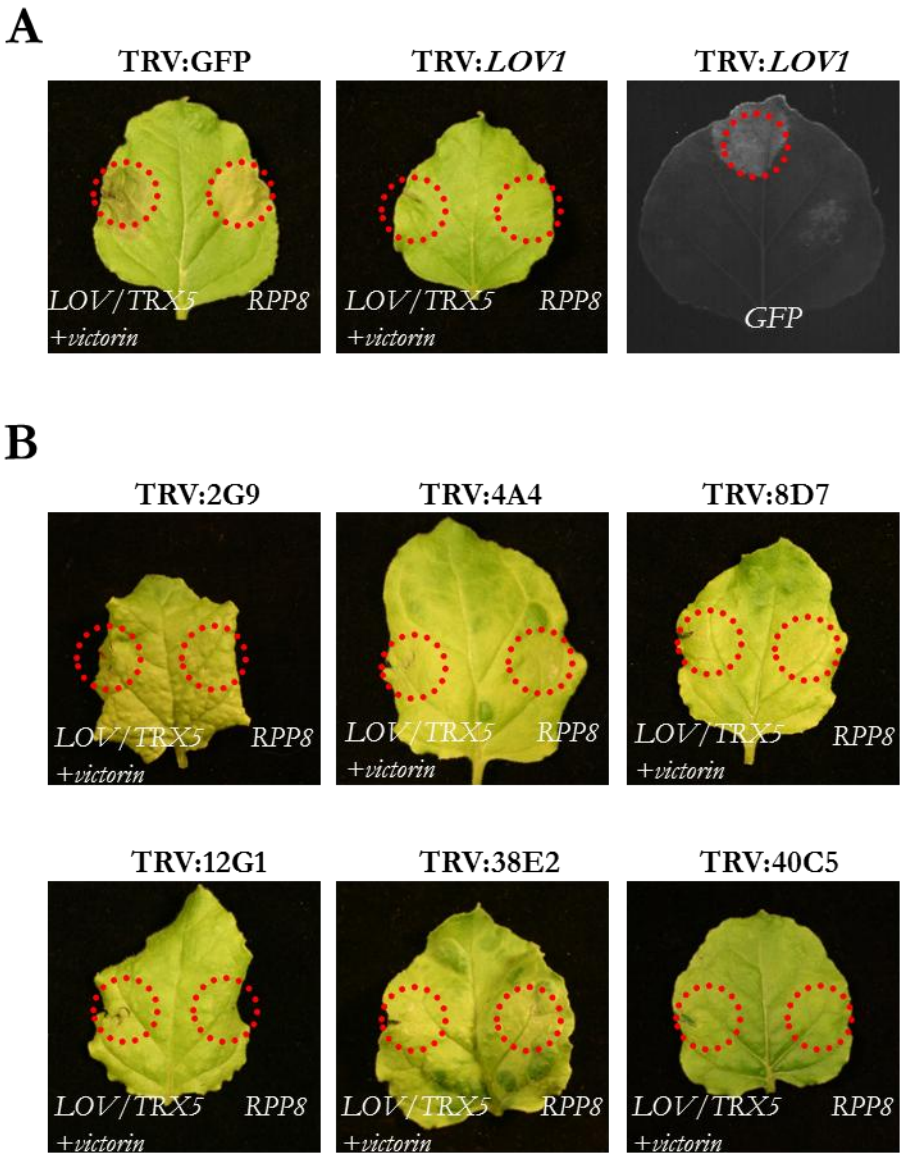


**Figure 2-4: VIGS based identification of cDNA clones that inhibit victorin-mediated, LOV1-dependent cell death and cell death induced by *RPP8*.**

inoculation, GFP fluorescence in the area inoculated was monitored under a UV trans-illuminator to verify efficient transient expression (Figure 2-4a). After four rounds of screening, we identified 6 clones which reproducibly inhibited victorin-mediated cell death (Figure 2-4B). These clones were chosen for further analyses. Five of these six clones produce a mildly-chlorotic, silencing phenotype. This phenotype was common in our VIGS screening, and does not affect victorin sensitivity per se (Figure SI 2-2).

The inserts in clones TRV:2G9, TRV:4A4, TRV:8D7, TRV:12G1, TRV:38E2, and TRV:40C5 were sequenced to infer gene function. The sequences were used in BLASTN searches of The Gene Index Project (Dana-Farber Cancer Institute, Boston, MA) (DFCI) to identify homologous putative expressed sequence tag (EST) contigs in *N. benthamiana* (Table 2-1). To confirm the DFCI annotation, the matching ESTs were used in a BLASTX search against The Arabidopsis Information Resource (Columbus, OH) (TAIR) database (Table 2-1). The cDNAs in TRV:2G9, TRV:4A4, TRV:8D7, TRV: 12G1, TRV:38E2, and TRV:40C5 correspond to the *N. benthamiana* genes: glycolate oxidase (*GOX*); T-protein of the glycine decarboxylase complex (*GDC-T*); glutamine synthetase (*GLU*); P-protein of the glycine decarboxylase complex (*GDC-P*); glyceraldehyde 3-phosphate dehydrogenase (*GAPB*); and a mitochondrial phosphate transporter (*PHT*) respectively. Gene ontology terms (Ashburner et al., 2000) annotated for the Arabidopsis homologs of the genes identified are listed in Table SI 2-1.

**Table 2-1: Clones and their corresponding gene annotations identified as required for *RPP8* and victorin-mediated cell death in a VIGS screen of the cNbME library. Listed are the cDNAs corresponding to the TRV inserts and their predicted gene function.**

| TRV clone | *N. benthamiana. Gene Index* ID * | E-value | Symbol | Arabidopsis Homolog † | Identity | Description |
|---|---|---|---|---|---|---|
| 2G9 | TC22852 | 6.E-07 | GOX | At3g14420 | 90% | Glycolate oxidase |
| 4A4 | CN742595 | 2.E-08 | GDC-T | At1g11860 | 80% | Glycine cleavage T-protein |
| 8D7 | TC19485 | 2.E-08 | GLU | AT5G35630 | 92% | Glutamine synthetase 2 |
| 12G1 | TC20514 | 1.E-07 | GDC-P | AT4G33010 | 84% | Glycine Decarboxylase P-protein 1 |
| 38E 2 | CN743656 | 1.E-04 | GAPB | AT1G42970 | 85% | Glyceraldehyde-3-phosphate dehydrogenase beta subunit, chloroplast localized |
| 40C5 | TC19080 | 3.E-08 | PHT | AT5G14040 | 86% | Phosphate transporter 3, mitochondrial |

* indicates top BLASTN results † indicates top BLASTX results

Semi-quantitative RT-PCR was conducted to confirm that VIGS of *GOX, GDC-T, GLU, GDC-P, GAPB*, and *PHT* reduced the abundance of the predicted transcripts (Figure 2-5). Transcript abundance corresponding to *GOX, GDC-T,GLU,GDC-P, GAPB,* and *PHT* was reduced in cDNA prepared from mRNA isolated from plants silenced with the respective constructs compared to control (Figure 2-5A and Figure 2-5B). In all RNA samples, abundance of 18S RNA transcripts was similar and served as an internal control (Figure 2-5C).

The electrolyte leakage assay was used to quantify suppression of victorin-mediated cell death when silencing *GOX, GDC-T, GLU, GDC-P, GAPB*, and *PHT*. Leaf disks from plants with these genes silenced, and expressing *LOV1* and *TRX-h5,* exhibited significantly reduced electrolyte leakage after victorin treatment when compared to the control (Figure 2-6). Silencing *GOX*, *GDC-P*, *GAPB*, and *PHT* inhibited cell death by approximately 80% after eight hours of victorin treatment, and silencing *GLU* inhibited cell death by approximately 90% after eight hours of victorin treatment. Silencing *GDC-T* inhibited cell death by 60% after eight hours of victorin treatment.

### 2.2.3 VIGS of GOX, GDC-T, GLU, GDC-P, and GAPB affects the PTO-mediated resistance pathway

To determine whether VIGS of *GOX, GDC-T, GLU,GDC-P, GAPB*, and *PHT* inhibits other cell death pathways associated with resistance gene activation, these genes were silenced in *N. benthamiana* expressing *PTO* and *BS2* resistance genes (Figure 2-7). As expected, transient expression of the constitutively active

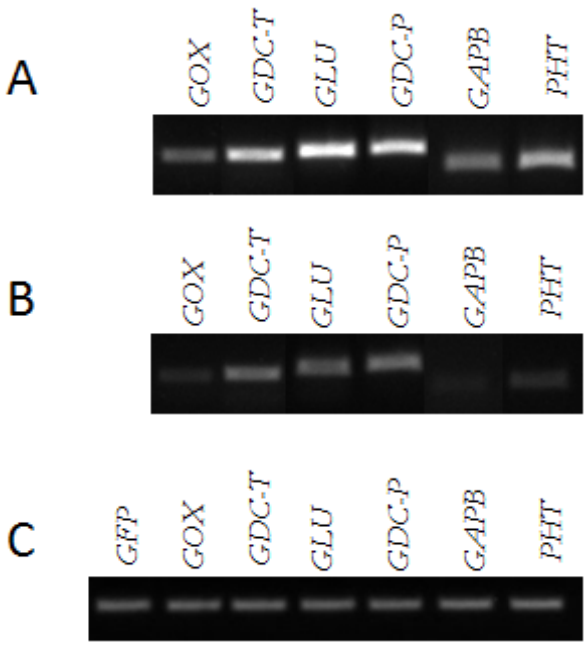


**Figure 2-5: Semi-quantitative PCR confirms reduction of transcripts in TRV silenced plants. (A) PCR products amplified with gene-specific primers from cDNA isolated from plants silenced with TRV:GFP (control). (B) PCR products amplified with the same gene-specific primers from cDNA isolated from plants silenced with the specified TRV clones. (C) PCR products amplified with 18S primers (as an internal control) from cDNA isolated from plants silenced with TRV:*GFP* and specified TRV clones.**

**Figure 2-6: Virus induced gene silencing of *GOX, GDC-T, GLU, GDC-P, GAPB*, and *PHT* inhibits victorin-mediated electrolyte leakage.**

Kinetics of electrolyte leakage following 31.6 ng/mL victorin treatment of leaf disks from *LOV1/TRX-h5*-expressing leaves silenced with controls (TRV:*GFP* or TRV:*LOV1*) or clones identified in the genetic screen. Conductivity is reported relative to control at 8 hrs. Means ± SE of 3 independent experiments are plotted

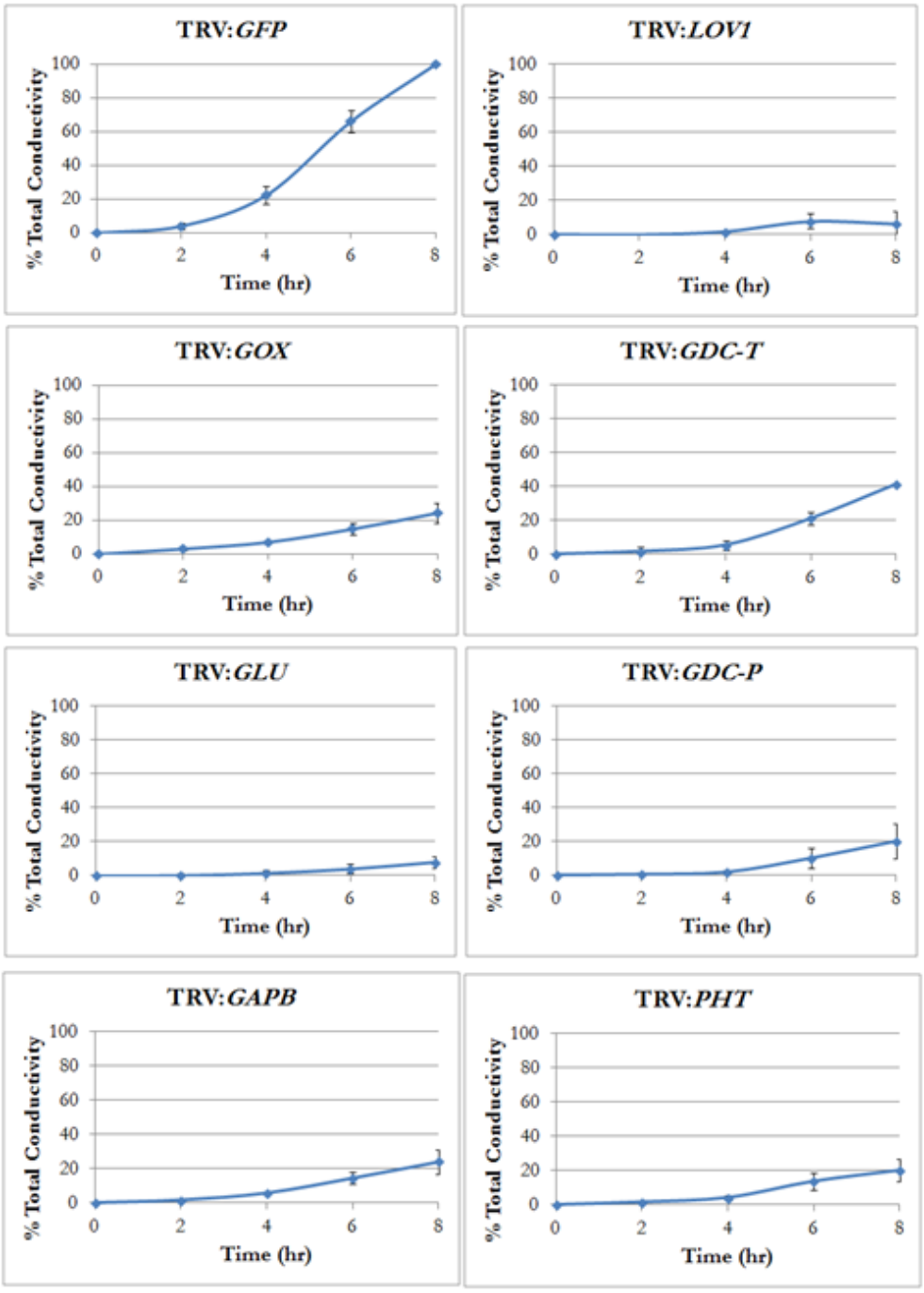


**Figure 2-6: Virus induced gene silencing of *GOX, GDC-T, GLU, GDC-P, GAPB,* and *PHT* inhibits victorin-mediated electrolyte leakage.**

form of *PTO* (*PTOY207D*) resulted in cell death in leaves from wild-type *N. benthamiana* plants expressing the TRV:*GFP* (control) (Figure 2-7A). In *GOX, GDC-T, GLU, GDC-P,* and *GAPB* silenced plants, cell death was absent in regions of leaves expressing *PTOY207D* (Figure 2-7A). *PTO*-mediated cell death was not inhibited in plants silenced for *PHT* (Figure 2-7A). In contrast, there was no reduction in cell death mediated by the expression of *AvrBs2* in *BS2*-transgenic *N. benthamiana* plants silenced with any of the constructs (Figure 2-7B).

We determined whether the loss of *PTO*-mediated cell death was accompanied by a reduction of *PTO*-mediated disease resistance. *GOX, GDC-T, GLU, GAPB,* and *PHT* were silenced in transgenic *N. benthamiana* that express the *PTO* resistance gene (G. Martin, Boyce Thompson Institute, unpublished). As a control for the loss of resistance, *PRF* was silenced, which is required for *PTO*-mediated resistance (Salmeron et al., 1996). As additional controls, TRV:*GFP* was expressed in wild-type and *PTO*-transgenic *N. benthamiana*. Silenced plants were inoculated at a low concentration (1 X $10^4$ cfu/mL) by infiltration of either compatible *Pseudomonas syrinagae* pv. *tabaci* (*Pstab*) or incompatible *Pstab* carrying *AvrPto*. Bacterial growth was monitored two days after inoculation. *PTO*-mediated resistance was observed by the differential growth of *Pstab* (*avrPto*) in TRV:*GFP* expressing, *PTO*-transgenics compared with either *PTO*-transgenics silenced for *PRF* or wildtype expressing TRV:*GFP* (Figure 2-8A). Growth of *Pstab* (*avrPto*) in *PTO* plants silenced for *PRF* was similar to wild-type plants expressing the TRV:*GFP* control, demonstrating effective

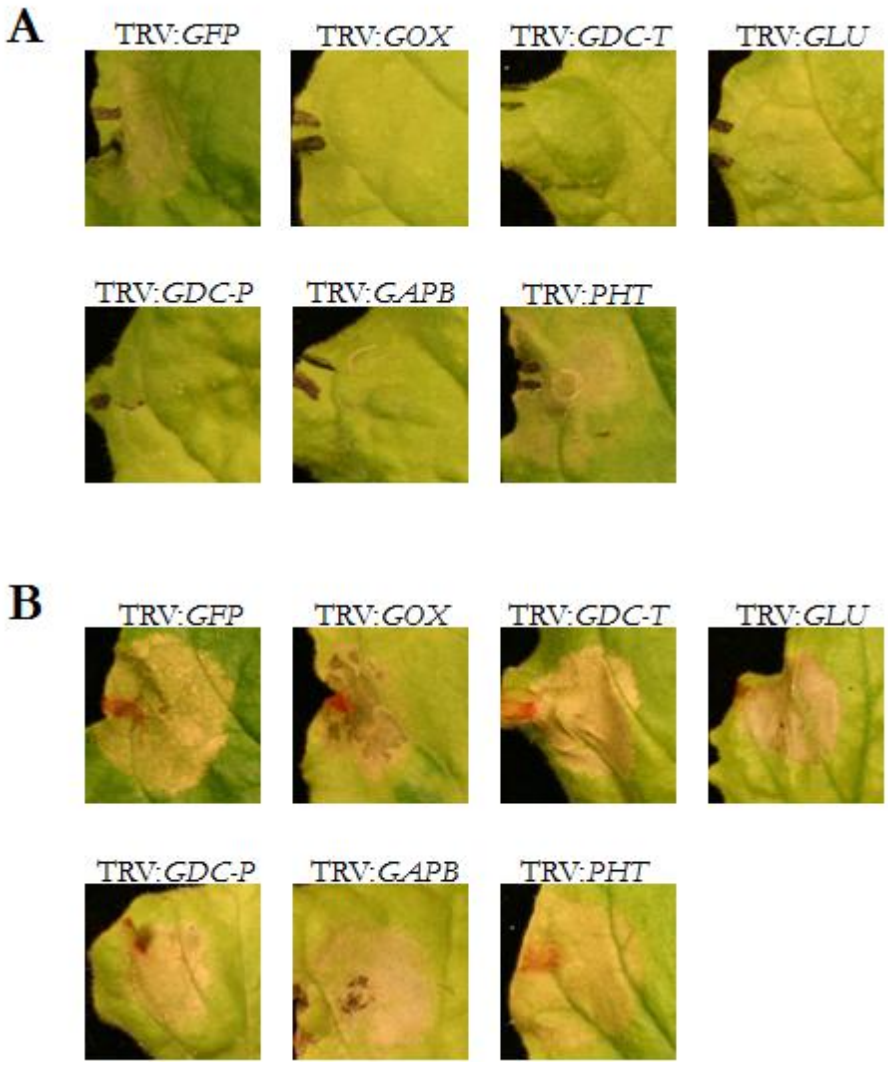


**Figure 2-7 VIGS of *GOX*, *GDC-T*, *GLU*, *GDC-P*, and *GAPB* (5 of the 6 TRV clones identified in our VIGS screen that inhibit RPP8 and victorin-mediated cell death) inhibit cell death mediated by the *PTO* resistance gene but not the *BS2* resistance gene. (A) *Agrobacterium* carrying a construct to express a constitutively-active form of *PTO* (*PTOY207D*) was infiltrated into leaves of *N. benthamiana* silenced with TRV:*GFP* (control) and the TRV clones identified from our screen (TRV:*GOX*, TRV:*GDC-T*, TRV:*GLU*, TRV:*GDC-P*, TRV:*GAPB*, and TRV:*PHT*). (B) *Agrobacterium* carrying a construct to express *AvrBs2* was infiltrated into leaves of *BS2*-transgenic *N. benthamiana* silenced with the indicated TRV constructs. Cell death was assessed 4 days after infiltration.**

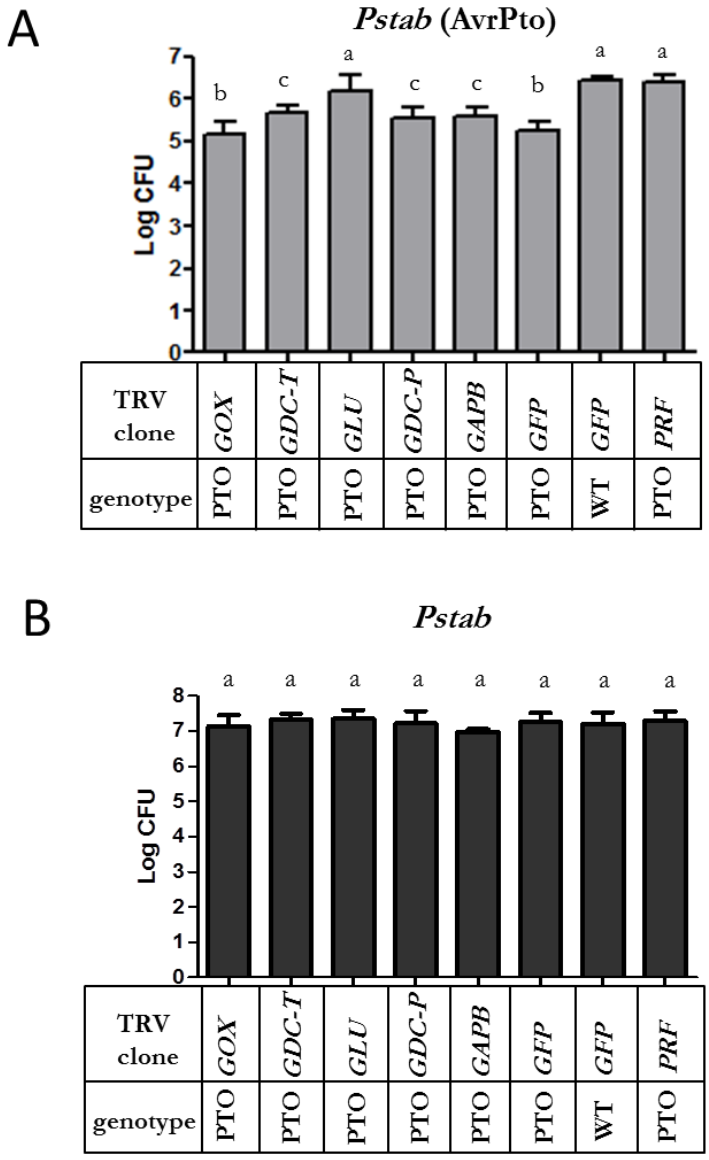


**Figure 2-8: Virus induced gene silencing of *GDC-T, GLU, GDC-P,* and *GAPB* compromises resistance mediated by the *PTO* resistance gene in *N. Benthamiana*. *Pseudomonas syringae* pv. *tabaci* (*Pstab*) growth 2 days after inoculation of either the *PTO*-transgenic, or the *PTO*-transgenic silenced for the identified TRV clones or *PRF* (control) or wild-type *N. benthamiana* expressing TRV:*GFP* (control). (A) *In planta* pathogen growth assay to asses virulence of *Pstab* carrying *AvrPto* and (B) *Pstab* lacking *AvrPto*. Error bars represent 95% confidence limits (n = 3 samples), and results are representative of at least 3 independent experiments. Significantly different means ($p < .05$, one-way ANOVA multiple comparison analysis) are indicated by different letters.**

reduction of *PTO*-mediated resistance by VIGS (Figure 2-8A). An approximate 10-fold increase in *Pstab* (*avrPto*) growth was observed in *PTO* plants silenced for *GLU* compared to *PTO* plants expressing the control TRV:*GFP* (T-test $P < 0.05$)(Figure 2-8A). There was a moderate but significant four-fold increase in *Pstab* (*avrPto*) growth in *PTO* plants silenced for *GDC-T*, *GDC-P*, and *GAPB* compared to the controls (ANOVA, $P < 0.05$) (Figure 2-8A). Growth of *Pstab* (*avrPto*) in *PTO* plants silenced for *GOX* was similar to resistant controls (Figure 2-8A), even though silencing of this gene has a consistent effect on visible cell death mediated by *PTO* (Figure 2-7). Inconsistent results were obtained when silencing *PHT* over the course of four independent experiments (data not shown). Growth of compatible *Pstab* lacking *avrPto* was similar in all silenced plants, indicating that the differences seen in the growth of *Pstab* (*avrPto*) are specific to the expression of disease resistance (Figure 2-8B).

## 2.3 DISCUSSION

To further characterize the victorin response and clarify its relationship with effector triggered immunity, we used virus induced gene silencing (VIGS) to identify genes required for victorin-mediated cell death. By silencing a set of plant genes that have known roles in defense, we identified a requirement for *SGT1* (suppressor of G-two allele of Skp1) in victorin-mediated cell death (Figure 2-2). SGT1 is a highly-conserved, eukaryotic protein associated with protein complexes involved in multiple processes (Shirasu and Schulze-Lefert, 2003). In plants, SGT1 has been shown to be involved in auxin and jasmonate responses (Gray et

al, 2003), heat shock tolerance (Noel et al, 2007), and immunity against pathogens (Shirasu 2009; Seo et al., 2008; Peart et al, 2002). Mutation analysis and gene silencing experiments have demonstrated that SGT1 is required for plant defenses conferred by diverse resistance (R) genes (Austin et al, 2002; Peart et al, 2002;). SGT1 is thought to act as a co-chaperone, interacting with the co-chaperonins HSP90 (heat shock protein 90) and RAR1 (required for MLa12 resistance) (Takahashi et al, 2003) in a molecular chaperon complex. Thus, SGT1 likely functions in protein folding and stability (Shirasu 2009; Seo et al, 2008). However, HSP90, RAR1, and SGT1 are differentially required for resistance depending on the specific R protein (Seo et al, 2008).

SGT1 is required for steady-state accumulation of NB-LRR disease resistance proteins such as Rx (Azevedo et al. 2007; Boter et al 2007) and N (Mestre and Baulcombe 2006), indicating that one way SGT1 regulates plant resistance function is by maintaining the stability of R proteins (Azevedo, et al., 2007). We show here that silencing *SGT1* results in reduced levels of LOV1 when transiently expressed in *N. benthamiana* (Figure 2-2), and conclude that SGT1 has a role in maintaining the stability of LOV1. This suggests that SGT1 would positively regulate susceptibility to *Cochliobolus victoriae*. However we were unable to test this in our heterologous, transient expression system due to the need to infiltrate *Agrobacterium*. Notably, SGT1 has been shown to positively regulate disease susceptibility to *Botrytis cineara* in *N. benthamiana* (Oirdi and

Bouarab, 2007). Like *C. victoriae*, *B. cinerea* is a necrotrophic pathogen, which is thought to manipulate defense signaling to promote cell death for pathogenesis.

In Arabidopsis, SGT1 has two isoforms, AtSGT1a and AtSGT1b, each of which are able to function in certain R-gene mediated resistance pathways (Azevedo et al, 2006). Mutations in either *AtSGT1a* only (Figure SI 2-1) or *AtSGT1b* only (Lorang et al 2007) do not affect victorin sensitivity. Because a *sgt1a/sgt1b* double mutant is lethal in Arabidopsis (Azevedo et al, 2006), we heterologously expressed *AtSGT1a* and *AtSGT1b* in *N. benthamiana* silenced for *NbSGT1* and showed that both of these isoforms are able to function in victorin mediated cell death (Figure 2-3). Therefore, the absence of an effect of *sgt1a* or *sgt1b* mutants on victorin sensitivity in Arabidopsis is likely due to redundant contributions of these genes towards LOV1 stability. The requirement of SGT1 for LOV1 function suggests that LOV1 expression is regulated similarly to other R proteins.

Because HSP90 and RAR1 are associated with SGT1, we were interested in their effects on victorin sensitivity and LOV1 accumulation. A prior study showed that treatment of victorin-sensitive Arabidopsis with the HSP90 inhibitor geldanamycin did not reduce sensitivity to victorin (Lorang et al, 2007), however, negative results with geldanamycin cannot exclude a role for HSP90. Geldanamycin treatment did not inhibit the hypersensitive response mediated by RPM1, nevertheless, HSP90 is important for RPM1 resistance function (Takahasi et al 2003; Hubert et al, 2003). Interaction between HSP90 and SGT1 is also

required for Rx accumulation and function (Boter et al., 2007). A silencing construct, which contained sequence homology to the three *N. benthamiana HSP90* isoforms identified in *N. benthamiana* was designed. However, plants silenced with this construct exhibited significantly reduced *Agrobacterium*-mediated GFP fluorescence compared to the control, indicating an inhibitory effect on transient expression (data not shown). A role for HSP0 in *Agrobacterium*-mediated transformation has not been reported in the literature. However HSP90, via its interaction with SGT1 (Takahashi et al, 2003), is associated with the SCF (Skp1–Cullin 1–F-box) E3 ubiquitin ligase complex (Zhang et al., 2008; Azevedo et al, 2002), which has been implicated in the transfer and integration of bacterial T-DNA during *Agrobacterium*-mediated transformation (Anand et al., 2012). Thus, we were unable to determine whether HSP90 is required for victorin sensitivity. Future investigations of a possible role for HSP90 in victorin sensitivity should be addressed in a system where transient expression is not required to confer victorin sensitivity. Any approach will likely require gene silencing or mutation of multiple *HSP90* alleles, because a *HSP90* requirement was not identified in a near-saturating EMS mutagenic screen for the loss of victorin sensitivity (Sweat and Wolpert, 2007).

The co-chaperone RAR1 can function to enhance the HSP90–SGT1 interaction (Boter et al, 2007), and has been shown to positively regulate levels of some R proteins, including Rx (Bieri et al., 2004; Tornero et al 2002). Victorin sensitivity was slightly attenuated in Arabidopsis *rar1* mutants (Lorang et al, 2007),

suggesting RAR1 may play a role in LOV1 accumulation. However VIGS of the *N. benthamiana* RAR1 ortholog did not affect victorin sensitivity or LOV1 protein levels in our assays (data not shown). This apparent LOV1 RAR1 independence observed in *N. benthamiana* may be the result of LOV1 overexpression in our heterologous assays, in which minor effects on stability would not be observable.

Another method we used to discover genes required for victorin-mediated cell death was VIGS to randomly silence genes from a mixed-elicitor cDNA (cNbME) library. With this screen six genes were identified that when silenced consistently inhibited victorin-mediated cell death. These genes included a mitochondrial phosphate transporter (*PHT*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and the photorespiratory enzymes glycolate oxidase (*GOX*), glutamine synthetase (*GLU*), and the P- and T-protein of the glycine decarboxylase complex (*GDC-P* and *GDC-T*) (Figure 2-5,2-6). Cell death induced by *RPP8* expression was also inhibited in plants silenced for these genes. Significantly, we did not identify any silencing constructs from the cNbME library that inhibited only one of these cell death pathways. This indicates that the cell death response mediated by the homologous LOV1 and RPP8 proteins are similar. Given that RPP8 confers resistance to *Hyaloperonospora arabidopsidis*, these results indicate that the LOV1-mediated response shares characteristics of a defense response.

Given their implication in NB-LRR mediated cell death, a role in defense was directly evaluated for the genes that were identified in our screen. Silencing these

genes did not affect cell death mediated by the resistance gene *BS2*, demonstrating different requirements for different NB-LRR proteins in cell death pathways. However, *PTO*-mediated cell death was compromised in *N. benthamiana* silenced for *GOX, GLU, GDC-P, GDC-T*, and *GAPDH* (5 of the 6 genes identified) (Figure 2-7), thus reinforcing the view that LOV1-mediated cell death is related to that evoked by other resistance proteins. PTO and BS2 have been demonstrated to utilize different signaling components (Jin et al., 2002), therefore differences in requirements for cell death mediated by these resistance genes is not unexpected. *PTO*-mediated resistance was compromised in plants silenced for *GLU, GDC-P, GDC-T*, and *GAPDH*, demonstrating a role for these genes in defense. Together, these results support the hypothesis that the victorin response is functionally related to a defense response.

Four out of the six genes that were identified (*GOX, GLU, GDC-P* and *GDC-T*) encode photorespiratory enzymes. Photorespiration is a metabolic process in plants that salvages phosphoglycolate, a product of the oxygenase activity of rubisco. Phosphoglycolate is recycled to the Calvin cycle intermediate phosphoglycerate in a process which consumes energy and reducing equivalents, and results in is a loss of carbon released as $CO_2$. One of the photorespiratory genes we identified, *GOX*, encodes glycolate oxidase, and has been previously shown to be involved in cell death and R-gene mediated resistance (Rojas et al 2012). Glycolate oxidase catalyzes the oxidation of glycolate, derived from phosphoglyate, to glyoxylate producing hydrogen peroxide in the process. This

reaction can be a major source of hydrogen peroxide in photosynthetic cells (Noctor et al, 2002, Foyer 2009). Hydrogen peroxide is an important reactive oxygen species (ROS), which can function as a signaling molecule in defense (Torres et al., 2006). Consistent with our data, Rojas et al (2012) found that VIGS of *GOX* inhibited *PTO*-mediated cell death in *N. benthamiana*, while other R-gene-mediated, cell death pathways tested were unaffected. Rojas et al (2012) also showed that resistance of Arabidopsis to *Pseudomonas syringae* pv. *tomato* mediated by *RPM1* and *RPS4* is compromised by mutants in members of the *GOX* family . The Arabidopsis *GOX* mutants were found to be defective in hydrogen peroxide production after pathogen challenge, independent of NADPH oxidase activity. The timing of this hydrogen peroxide generation followed the second phase of the oxidative bust (Rojas et al., 2012). In *N. benthamiana* silenced for *GOX, PTO*-mediated resistance against *Pseudomonas syringae* pv. *tabaci* was not affected (Figure 2-8). This is in contrast to a 10-fold reduction in RPS4 mediated resistance against *Pseudomonas syrinage* pv. *tomato* observed by Rojas et al. (2012). However these differences could be due to differences between the Arabidopsis and *N. benthamiana* pathosystems. In preliminary analyses, we were unable to detect changes in hydrogen peroxide following victorin treatment of *LOV1*- and *TRX-h5*-expressing *N. benthamiana* by either staining with 3,3'-Diaminobenzidine (DAB) or chemiluminescence with luminol (data not shown). Due to the transient nature of this signaling molecule, additional analyses will be necessary to address whether the reduction in victorin-mediated cell death in GOX silenced plants is due differences in hydrogen peroxide production.

Photorespiratory enzymes other than *GOX* have been implicated in disease resistance and associated cell death. The photorespiratory enzyme, NADH-dependent hydroxypyruvate reductase (HPR), is the proposed target of P34 (Okinaka et al., 2002), the syringolide resceptor that triggers resistance mediated by the *Rpg4* resistance gene in soybean (Ji et al., 1998). It was hypothesized that P34/syrinoglide binding to HPR inhibits HPR function, leading to *Rpg4*-mediated cell death (Okinaka et al., 2002) Interestingly, this implies that Rpg4 guards NADH-dependent hydroxypyruvate reductase thereby suggesting that it has a role in defense, a possibility consistent with our findings. Two genes encoding photorespiratory glyoxylate aminotransferases, when expressed at high levels, confer resistance to the omycete pathogen *Pseudoperonospora cubensis* in the plant family *Cucurbitaceae* (Taler et al., 2003). The enhanced expression of these enzymes correlated with higher GOX activity, which was hypothesized to play a role in the resistance to *P. cubensis* by greater production of hydrogen peroxide (Taler et al., 2003). The two peroxisomal aminotransferases are responsible for the transamination of glyoxylate (formed from the GOX reaction) to glycine (Bourguignon et al., 1999). Glycine is then converted to carbon dioxide, ammonia, and serine by the coupled reaction of the glycine decarboxylase complex (GDC) and hydroxymethyltransferase in the mitochondria.

Two components of the GDC (GDC-P and GDC-T) were identified in our screen. Notably, GDC-P was the first hypothesized target of victorin because victorin bound GDC-P only in oat genotypes that are victorin sensitive (Wolpert

and Macko, 1989). This interaction was hypothesized to play a role in victorin's mode of action, because binding apparently inhibits glycine decarboxylase activity (Navarre and Wolpert, 1995). It is now clear that the GDC is not the primary site of action for victorin. Victorin binding to GDC-P in susceptible oats occurs after the initiation of cell death when victorin gains access to the mitochondria likely through induction of a mitochondrial permeability transition, an early event in some forms of programmed cell death (Curtis and Wolpert, 2002; 2004). In Arabidopsis, the NB-LRR protein, LOV1, conforms to the "guard model" of resistance protein function and triggers cell death when victorin binds to the guardee, TRX-h5 (Lorang et al, 2007; Lorang et al, In Press). In oats, victorin sensitivity and Victoria Blight susceptibility are also associated with an R gene (Rines and Luke, 1985; Mayama et al., 1995), and physiological responses mediated by victorin in oats are similar to those of Arabidopsis (Wolpert et al., 2011). Therefore the mechanism of victorin sensitivity is likely conserved between oats and Arabidopsis. Regardless, a role for the GDC has been implicated in disease resistance. The GDC has been implicated in the hypersensitive response evoked by harpin, an elicitor produced by the plant pathogen *Erwinia amylovora*. Harpin leads to inhibition of the GDC by S-nitrosoglutathione (Palmieri et al, 2010). It was hypothesized that the GDC regulates ROS production in the mitochondria during the defense response (Palmieri et al, 2010). We demonstrate the GDC has a role in defense (Figure 2-8). Therefore binding of the GDC remains a possible effector function for victorin.

It is unclear how VIGS of *GOX, GLU, GDC-P* and *GDC-T* suppresses victorin- and *RPP8*-mediated cell death, or cell death and resistance mediated by *PTO*. Notably, victorin-mediated symptom development has been shown to be significantly delayed under environmental conditions that limit photorespiration (Navarre and Wolpert, 1999; Navarre and Wolpert, 1999 b). Interestingly, GLU catalyzes the rate-limiting step in photorespiration (Kozaki and Takeba, 1996). The greatest suppression of victorin-mediated cell death was observed in plants silenced for *GLU* compared to the other genes identified in our screen (Figure 2-6). In addition the greatest reduction in *PTO*-mediated resistance was observed in plants silenced for *GLU* compared to the other genes identified in our screen (figure 2-8). This trend could indicate that the effects that were observed are due to changes in the rates of photorespiration after gene silencing. Pleiotrophic effects on photosynthesis should also be considered. Photorespiration operates alongside photosynthesis, linking the rates of photorespiration and photosynthetic metabolism (Kangasjarvi et al., 2012). RNAi inhibition of *GOX* in rice revealed a direct relationship between GOX activity and photosynthetic rate (Xu et al, 2009). *GOX* inhibition resulted in a decrease in photosynthetic rate in a linear relationship, demonstrating how changes in photorespiratory enzyme activity could affect primary metabolism (Xu et al, 2009). Photosynthesis could impact cell death and defense by photoproduced ROS, or by affecting energy or metabolites required for induction of defense (Bolton et al, 2009; Swarbrick et al. 2006). However, victorin-mediated symptom development was reduced in sensitive oats leaves incubated under elevated $CO_2$ concentrations, a condition

that decreases photorespiration without decreasing photosynthesis (Navarre and Wolpert, 1999 b). This suggests that changes in photorespiratory activity, not photosynthesis, impact victorin-mediated cell death.

Silencing a chloroplastic *GAPDH* was found to suppress victorin- and RPP8-mediated cell death and cell death and resistance conferred by PTO, providing evidence that the metabolic state can affect symptom development and disease resistance. Chloroplastic GAPDH catalyzes a reducing step of the calvin cycle for $CO_2$ fixation, and therefore is directly involved in metabolism by its role in photosynthesis. Chloroplastic GADPH may also play a signaling role as a mediator of ROS signaling. Chloroplastic GAPDH is subject to redox regulation by glutathionylation and hydrogen peroxide (Zaffagnini et al, 2006; Zaffagnini et al 2012). In addition to regulating enzyme activity, oxidative modification of GAPDH is proposed to cause structural changes that change the proteins GAPDH interacts with, as has been shown in animals. (Kim et al., 2003; Hwang et al., 2009; Besson-Bard et al.,2008).

In our screen, a mitochondrial phosphate transporter (*PHT*) was implicated as involved in victorin-mediated cell death and cell death induced by *RPP8* expression. The mitochondrial phosphate transporter catalyzes proton co-transport of phosphate into the mitochondrial matrix. The phosphate transported into the matrix is used for ATP synthesis via oxidative phosphorylation. Recent evidence suggests that, in animal cells, a mitochondrial phosphate transporter is a component of the mitochondria permeability transition pore (MPTP) and is

involved in the regulation of pore formation (Varanyuwatana and Halestrap, 2012; Leung et al., 2008). The mitochondrial permeability transition (MPT) is considered an early and essential step of apoptosis. Victorin has been shown to induce a mitochondrial permeability transition in victorin-sensitive oats which likely influences the cell death response (Curtis and Wolpert 2002; Curtis and Wolpert 2004). The mitochondria permeability transition has been shown to play a role in cell death in plants, including Arabidopsis (Arpagaus et al, 2002; Tiwari et al., 2002; Scott et al, 2008), demonstrating this mechanism is conserved. We have also observed an inhibition of victorin-mediated cell death in *N. benthamiana* when treated with cyclosporine, an inhibitor of MPT pore opening (data not shown). The phosphate transporter that was identified in *N. benthamiana* is homologous to a family of three phosphate transporters in Arabidopsis, one of which has been confirmed to be localized in the mitochondria *in vivo* (Auken et al, 2009). Further characterization is necessary to determine if the PHT identified has a role in the MPT, or if the suppression that was observed in victorin-mediated cell death is due its other roles.

From our screen of approximately 4000 genes, six genes were identified that are involved in victorin-mediated cell death and cell death induced by *RPP8* expression. Certain aspects of our screen may have affected the number and types of genes identified. The cNbME library used for screening was derived from leaf tissues exposed to a variety of elicitors, and not specifically LOV1- or RPP8-mediated cell death. The abiotic elicitors were salicylic acid (SA), jasmonic

acid (JA), and ethylene, and the biotic elicitors *PTO*/*avrpto*-mediated HR, *P. syringe* pv. *tomato* T1-mediated non-host HR, and disease caused by *P.s.* pv. *tabaci* (Anand et al., 2004). Therefore it is not surprising that some of the genes identified inhibit *PTO*-mediated cell death, as this was one of the elicitors used for library preparation. The absence of an effect on *LOV1*- and *RPP8*-mediated cell death from silencing *NRC1, MAPKKK, MEK1, MEK2, WIPK, SIPK,* and *ADI2* (data not shown), which are signaling components required for *PTO*-mediated resistance and cell death, indicates differences between these pathways (Pozo et al., 2004, Gabriels et al.,2007). Also, victorin-mediated cell death and RPP8 function do not require SA, JA signaling components, two out of the three abiotic elicitors used for library preparation (Eulgem et al., 2004; Lorang et al., 2007). Therefore it is possible that cDNAs in the cNbME library were not enriched for genes involved in signaling pathways for LOV1- or RPP8-mediated cell death. In addition, our screen was not saturating, as only 4,000 out of an estimated 18,000 genes expressed in *N. benthamiana* (Goodin et al., 2008) were screened. Therefore, it is likely further components of victorin-mediated cell death could be identified with VIGS.

In summary, our results provide evidence that the victorin response mediated by LOV1 is functionally similar to a resistance response. We found that SGT1, a common modulator of resistance protein function, regulates victorin-mediated cell death by maintaining the stability of LOV1. Six genes were identified in a screen of a cNbME library that, when silenced, inhibit both LOV1-

and RPP8-mediated cell death, thus suggesting that LOV1 shares functions in common with the closely-related resistance protein, RPP8. In addition, silencing most of these genes inhibited both cell death and resistance mediated by the PTO resistance protein. These findings coupled with the genetic association of victorin sensitivity with the *Pc2* rust resistance gene in oats (Welsh et al., 1954; Luke et al., 1966; Rines and Luke, 1985; Mayama et al., 1995) and our recent finding that the interaction of LOV1, TRX-h5 and victorin recapitulates the guard mechanism for resistance protein activation (Lorang et al., In Press) strongly suggest that *C. victoriae* exploits R-gene mediated defense for virulence and disease susceptibility.

## 2.4 MATERIALS AND METHODS

### 2.4.1 Plant material and bacterial strains

*Nicotiana benthamiana* or transgenic *Nicotiana benthamiana* carrying the *PTO* (Greg Martin, unpublished) or *BS2* (Leister et al., 2005) resistance genes were grown in 3 1/4 inch pots at 25°C in a growth chamber under 16h light/8h dark cycle. The *Arabidopsis thaliana* T-DNA insertion mutant (GK_266H09) was obtained from The Ohio State Arabidopsis Biological Resource Center (ABRC). Plants were screened for presence of the T-DNA insertion with the forward primer 5'-CTATCACGCCGAGTGAATCC-3' and the reverse primer 5'-TATCTGGGAATGGCGAAATC-3', and screened for the absence of the T-DNA insertion with the forward primer 5'-CTATCACGCCGAGTGAATCC-3' and reverse primer 5'-GTGCCATTGAGTTGGGTTCT-3'. A homozygote containing the T-DNA insertion was crossed into COL-LOV, a victorin-sensitive

line that is near-isogenic to Col-4 (Lorang et al., 2007). F2 plants homozygous for the insertion mutation were screened for the presence of the *LOV1* gene using the 3571 SSLP marker (forward 5'-GTGGTGACCTCTCCCTCAAA-3' and reverse 5'-CCCACTTCACCGTTTCTCTC-3') as previously described (Lorang et al. 2007).

*Agrobaterium tumefaciens* strain GV2260 was used for VIGS and transient expression. *Agrobacterium* was cultured in Luria-Bertani (LB) media, re-suspended in 10 mM MES pH5.2 (10 mM MgCl, 150 uM Acetosyringone) to the indicated optical densities (OD),and incubated for three hours before syringe infiltration. *Pseudomonas syrinage* pv *tabaci* isolate 11528 was obtained from Dr. Jeff Chang, Oregon State University (Corvallis, OR, U.S.A.). *Pseudomonas syrinagae* pv *tabaci* isolate 11528 containing AvrPto (plasmid pPtDC38; Ronald et al., 1992) was obtained from Dr. Brian Staskawicz at University California, Berkley (Berkley, CA, U.S.A.) *Pseudomonas syrinage* pv *tabaci* strains were cultured in King's medium B (King et al, 1954).

### 2.4.2 TRV vectors and derivatives

The vectors pTRV1 and Gateway-ready pTRV2 (Liu et al., 2002), and the pTRV2:cNbME library in *A. tumefaciens* (Anand et al., 2007) were kindly provided by Dr. Kiran Mysore (The Samuel Roberts Noble Foundation, Ardmore, OK, U.S.A.). *A. tumefaciens* containing the pTRV2 derivatives *MEK1, MEK2, MAPKKKα, EDS1, NRC1*, and *SGT1* were obtained from Dr. Greg Martin (The Boyce Thompson Institute, Ithaca, NY, U.S.A.). For TRV:*GFP*, TRV:*RAR1*, and

TRV:*HSP90*, fragments corresponding to *GFP*, *RAR1*, and *HSP90* were amplified by PCR, cloned into pENTR/D-TOPO (Life Technologies), and subsequently recombined into Gateway-ready pTRV2 with Gateway LR clonase II enzyme mix (Life Technologies). A 535 bp fragment of GFP was amplified from Pearlygate 103 (Earley et al, 2006) with the forward primer 5'-CACCAGTGGAGAGGGTGAAGGTGA-3' and the reverser primer 5'-AAAGGGCAGATTGTGTGGAC-3'. A 508 bp fragment of HSP90 was amplified from cDNA isolated from *N. benthamiana* with the forward primer 5'-CACCGATCCTGAAGGTTATTCGCA-3' and the reverse primer 5'-CAAGCTTGAGACCTTCCTTG-3'. A 560 bp fragment of RAR1 was amplified from cDNA isolated from *N. benthamiana* with the forward primer 5'CACCCCAGAGGATCGGGTTGCAACGCCAC-3' and the reverse primer 5'-TTTCCATCCTCTCATCCGGT-3'. For TRV:*LOV1*, *LOV1* was recombined from pENTR:*LOV1* (Lorang et al., In Press) into pTRV2. The resulting pTRV2 derivatives were transformed into *A. tumefaciens*. For pTRV:00, empty Gateway-ready pTRV2 was transformed into *A. tumefaciens*.

### 2.4.3 VIGS

*Agrobacterium* infiltration for VIGS in *N. benthamiana* was done by the toothpick method (Anand et al., 2007). Briefly, individual *Agrobacterium* pTRV2 clones, which contain the fragment of the gene to be silenced, were grown on LB agar. *Agrobacterium* containing pTRV1 (Lu et al., 2003b) at OD600=0.9 was infiltrated in the second or third true leaf of 3 week old *N. benthamiana*. Then a

toothpick was used to inoculate the individual TRV2 clones into the same region TRV1 was infiltrated. The plants were grown for at least two weeks before they were used for assays.

### 2.4.4 Cell death assays

To assay for victorin-mediated cell death, *Agrobacterium* carrying 35S:*LOV1* (Lorang et al, In Press) and 35S:*TRX-h5* (Sweat and Wolpert, 2007) diluted to OD600=0.5 were mixed in equal volumes and syringe-infiltrated into fully-expanded leaves of silenced *N. benthamiana*. 100 ng/mL of Victorin, purified as described previously (Macko et al. 1985; Wolpert et al. 1985), diluted in water was infiltrated 48 hours after *Agrobacterium* inoculation. Cell death was visually assessed 24 hours after victorin infiltration. For complementation of *NbSGT1* silencing, *Agrobacterium* carrying pBIN61:*myc:AtSGT1a*, pBIN61:*mycAtSGT1b* (Kadota et al., 2008), or PSLJ:GFP diluted to OD600=0.5 were mixed with *Agrobacterium* carrying 35S:*LOV* and 35S:*TRX-h5* at OD600=0.5 in equal volumes. 10 ug/mL of victorin was infiltrated 48 hours after *Agrobacterium* infiltration. Cell death was visually assessed 24 hours after victorin treatment.

To assay for cell death induced by *RPP8* expression, silenced leaves were infiltrated with *Agrobacterium* carrying 35S:*RPP8* diluted to OD600=0.5, and cell death was monitored 4 days after *Agrobacterium* infiltration. 35S:RPP8 was constructed by PCR amplification of *RPP8* from pBAR1:*RPP8* (McDowell et al 1998 ) with the primers 5'-TATTAGCGGCCGCATGGCTGAAGCATTTGTGTCG-3' and 5'-

TTATTAGCGGCCGCTCCTCAGCCAAGAGATTGGT-3' and sub-cloning into pENTR/D-TOPO (Life Technologies). The resulting clone was recombined into pEarlyGate100 (Earley et al., 2006) with LR clonase (Life Technologies).

*PTO*-mediated cell death was assayed by infiltrating *Agrobacterium* containing 35S:*PTO*Y207D (Rathjen et al, 1999) diluted to a final OD600=0.4 into fully-expanded leaves from silenced *N. benthamiana.* Cell death was visually monitored 4 days after *Agrobacterium* infiltration. For *BS2*-mediated cell death, fully-expanded leaves from silenced *BS2*-transgenic *N. benthamiana* (Leister et al, 2005) were infiltrated with *Agrobacterium* containing 35S:*AvrBs2* (Leister et al, 2005) diluted to OD600=0.4. Cell death was visually monitored 4 days after *Agrobacterium* infiltration.

### 2.4.5 Electrolyte leakage assay

Fully-expanded leaves from silenced plants were infiltrated with *Agrobacterium* carrying 35S:*LOV1* and 35S:*TRX5* diluted to OD600=0.5 and mixed in equal volumes. 48 hours after *Agrobacterium* infiltration, 36 4mm leaf disks were excised from 3 plants silenced for each clone. The 36 disks were pooled, rinsed in 100 mL deionized water for 30 minutes, then divided into 6 sets of 6 and placed into a 24- well multiwell plate. Three sets were treated with 31.6 ng/mL victorin and three sets were treated with water, each in a final volume of 2.5mL. For each time-point, "total conductivity" is the conductivity with water treatment subtracted from the conductivity from victorin treatment. Electrolyte

leakage is expressed as the percentage of total conductivity elicited for control (TRV:*GFP*) at eight hours.

### 2.4.6 Semiquantitative RT-PCR to analyze gene expression

Total RNA was extracted from three silenced plants using Qiagen RNeasy Plant Mini Kit (Maryland, USA), including an RNase-free DNase treatment step (Qiagen). First strand cDNA was synthesized with 500 ng total RNA, oligo d(T) primer, and Superscript III reverse transcriptase (Life Technologies). cDNA was amplified by PCR with GoTaq DNA Polymerase (Promega) with primers specific for the candidate cDNAs and the 18S ribosomal gene as an internal control (Table 2-2). At least one primer of each pair was designed to anneal outside the region targeted for silencing to ensure that the gene of interest was silenced. PCR products were resolved in 1% agarose gels and the number of cycles was optimized for each transcript. 20, 20, 30, 35, 35, 35, and 30 cycles were used for *18S, GOX, GDC-T, GLU, GDC-P, GAPB,* and *PHT* respectively.

### 2.4.7 Protein analysis

Protein extracts were prepared by grinding leaf tissue in SDS buffer (.625 tris ph 6.8, SDS, 5% β-mercaptoethanol). For *N. benthamiana* protein samples, 0.8 $cm^2$ tissue was ground in 125 uL For Arabidopsis 0.5 $cm^2$ tissue was ground in 125 uL. 30 uL of each sample were loaded onto a 4-15% SDS-polyacrylamide gel (Bio-Rad) and transferred onto Protran nitrocellulose membrane with a .2 um pore size (Whatman Schleicher and Schuell, Dassel, Germany). Membranes were blocked in 5% skim milk powder in TBST (25 mM Tris–HCl (pH 7.5), 150 mM

**Table 2-2 Oligonucleotide primers used in semi-quantitative RT-PCR to assess gene silencing**

| Transcript | Primers (5' – 3') |
|---|---|
| GOX | TGTTTTGCCACCATTTTTGA |
| | AGCACCATGGTTTGACACAA |
| GDC-T | TTCCCCCAATATTTTGTCCA |
| | TGCTGTTGACCTTGCAAAAG |
| GLU | AGGTTATGCCAGGACAGTGG |
| | AACCTTTGCCTTGCTTCTCA |
| GDC-P | AAGGCTGCTGAGGCAAATAA |
| | CCAGGGAGCAGCAGAAATAG |
| GAPB | TCCAATGGATCTCCACTTCC |
| | TTAGCTGTGTGCGATGAACC |
| PHT | TCTTTGGGGACGTCAGATTC |
| | GCCCAACAAAAACCTTGAAA |
| 18S | GTGACGGGTGACGGAGAATT |
| | AGACTCATAAAGCCCGGTAT |

NaCl, 0.1% Tween-20). Mouse anti-*myc-HRP* antibody (Life Technologies) was used at a 1:5000 dilution. Rabbit anti-LOV1 antibody (Lorang et al, In Press) was used at a 1:4000 dilution. Rabbit anti-SGS antibody (Azevedo et al., 2002) was used at a 1:7500 dilution. For membranes probed with anti-LOV1 or anti-SGS, goat anti rabbit HRP conjugate at a 1:10,000 dilution was used as a secondary antibody. Proteins were visualized with ECL chemiluminescent substrate (Life Technologies).

### 2.4.8 Monitoring of bacterial disease on plants

To measure *in planta* bacterial growth, fully-expanded leaves from silenced plants were syringe-inoculated with *Pstab* strains diluted to $10^4$ colony forming units/mL (CFU/mL) in 10 mM $MgCl_2$. Two days following inoculation, nine 1.5 $cm^2$ leaf disks were excised from three inoculated leaves of each treatment. The nine discs were divided into three sets of three and were ground in 10mM MgCl2. The bacterial populations in the leaves were determined by plating serial dilutions on KMB supplemented with 100 mg/L rifampicin. Colonies were counted after 48 hours of growth at 28°C. All bacterial growth assays were repeated at least three times. Bacterial populations were log-transformed and a one-way ANOVA (Tukey's test) or an unpaired two-tailed t-test was used for statistical analysis.

## 2.5 ACKNOWLEDGEMENTS

We thank Dr. Kiran Mysore for providing the cNbME library in pTRV2, TRV1, and TRV2-gateway vector, Dr. Greg martin for providing *PTO*-transgenic *N. benthamiana*, 35S:PTOY207D, and TRV2 derivatives, Dr. Brian Stakawicz for

*Pstab* 11528 pPtDC38, Dr. Ken Shirasu for pBin61_*AtSGT1a*, pBin61_*AtSGT1b*, and anti-SGS antibody, Jim Carrington for providing pSLJ:GFP, Jennifer Lorang of the Wolpert lab for help constructing pEarleyGate100:*RPP8* and reviewing the manuscript, Ashley Chu of the Wolpert lab for purification of Victorin C and technical support, and the ABRC for providing GK_266H09 seed. This work was supported by a grant from the USDA National Research Initiative Competitive Grants Program (2007-01598).

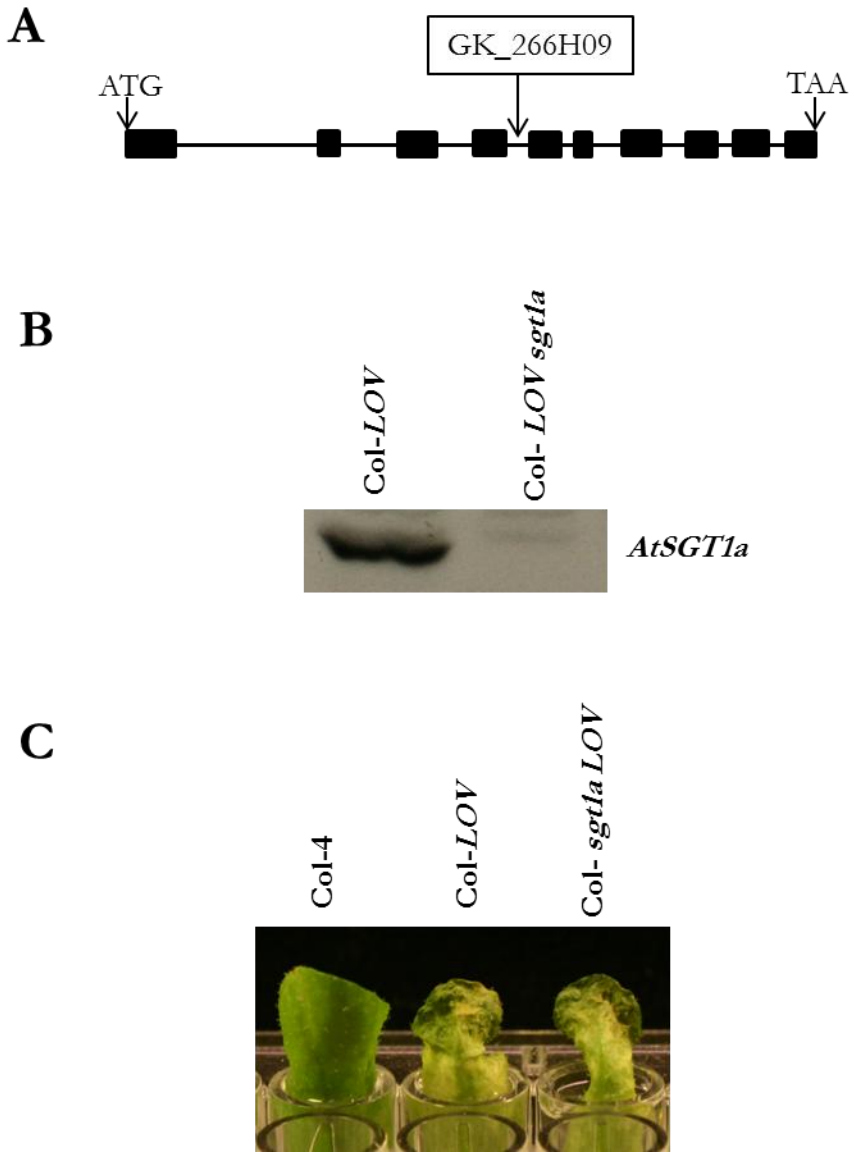


**Figure SI 2-1: *SGT1a* mutant does not affect victorin sensitivity. (A) Location of the T-DNA insertion in GK_266H09. (B) T-DNA insertion is a loss of function mutation. Total leaf protein extracts were prepared from 3-week-old plants and analyzed by immunoblotting using anti-SGS antibody to detect SGT1a. (C) Arabidopsis leaves 48 hrs after treatment with 1 ug victorin. Genotypes are Col-4 (victorin insensitive), Col-*LOV* (Col-4 near isogenic for *LOV1*), Col-*LOV sgt1a* mutant.**

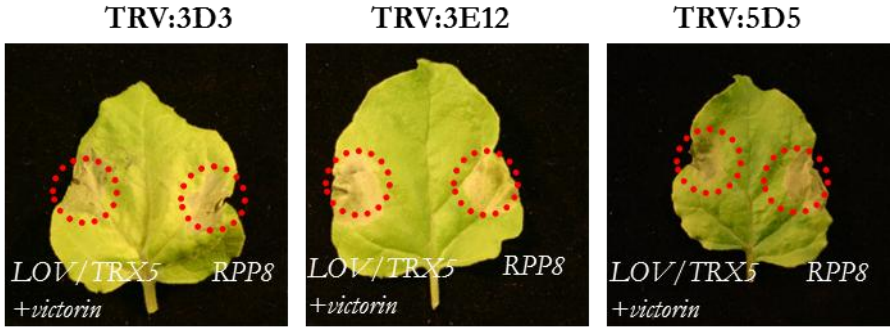


**Figure SI 2-2: A mild chlorotic phenotype resulting from VIGS does not inhibit victorin mediated cell death or cell death induced by *RPP8* expression. Assessment of cell death in leaves from plants silenced with clones from the cNbME library. TRV infected plants were infiltrated (in the regions identified by red circles) with *Agrobacterium* containing constructs to express *LOV1 (LOV)* and *TRX-h5 (TRX5)*, or *RPP8.* Forty-eight hrs after inoculation, victorin was infiltrated in the region of *LOV1* and *TRX-h5* expression. Pictures were taken 24 hrs after victorin treatme**

## Table SI 2-1: Gene ontology terms for the genes identified

| Gene | Gene Ontology Terms (Biological Process) |
|---|---|
| GOX | oxidation-reduction process |
| GDC-T | auxin polar transport, carotenoid biosynthetic process, chloroplast organization,cysteine biosynthetic process, glycine catabolic process, growth, hydrogen peroxide catabolic process, ovule development, pattern specification process,regulation of cell size, regulation of meristem growth, response to cadmium ion,root morphogenesis |
| GLU | Golgi organization, aging, ammonia assimilation cycle, cysteine biosynthetic process, glutamine biosynthetic process, glycolysis, hyperosmotic response,nitrogen compound metabolic process, pentose-phosphate shunt, regulation of proton transport, response to blue light, response to cadmium ion, response to cold, response to far red light, response to fructose stimulus, response to glucose stimulus, response to high light intensity, response to red light,response to salt stress, response to sucrose stimulus, response to temperature stimulus, water transport |
| GDC-P | PSII associated light-harvesting complex II catabolic process, aromatic amino acid family biosynthetic process, aromatic amino acid family metabolic process,cell differentiation, cell wall modification, cellular amino acid biosynthetic process, chlorophyll biosynthetic process, coenzyme biosynthetic process,cysteine biosynthetic process, glycine catabolic process, glycine decarboxylation via glycine cleavage system, glycine metabolic process, isopentenyl diphosphate biosynthetic process, mevalonate-independent pathway, jasmonic acid biosynthetic process, leaf morphogenesis, lipoate metabolic process,maltose metabolic process, nucleotide metabolic process, oxidation-reduction process, oxidoreduction coenzyme metabolic process, pentose-phosphate shunt, positive regulation of catalytic activity, positive regulation of transcription, DNA-dependent, response to cadmium ion, secondary metabolic process,serine family amino acid metabolic process, starch biosynthetic process, sulfur amino acid metabolic process, sulfur compound biosynthetic process,unsaturated fatty acid biosynthetic process, vitamin metabolic process |
| GAPB | PSII associated light-harvesting complex II catabolic process, chlorophyll biosynthetic process, chlorophyll metabolic process, chloroplast relocation,cysteine biosynthetic process, defense response to bacterium, glucose metabolic process, glycolysis, hydrogen peroxide catabolic process, isopentenyl diphosphate biosynthetic process, mevalonate-independent pathway, oxidation-reduction process, oxylipin biosynthetic process, pentose-phosphate shunt,photosynthetic electron transport in photosystem I, photosystem II assembly,plastid organization, rRNA processing, reductive pentose-phosphate cycle,regulation of lipid metabolic process, regulation of proton transport, response to blue light, response to cadmium ion, response to cold, response to far red light,response to glucose stimulus, response to high light intensity, response to light stimulus, response to red light, response to sucrose stimulus, unsaturated fatty acid biosynthetic process |
| PHT | glucose catabolic process, pentose-phosphate shunt, response to salt stress, transport |

# *3* Investigation of LOV1 activation in response to *Pseudomonas syringae* pv. *tomato*

## 3.1 ABSTRACT

Victoria blight of oats and Arabidopsis is caused by the fungus *Cochliobolus victoriae*, which is pathogenic due to its production of the host-specific toxin victorin. In oats, victorin sensitivity is conferred by the *Vb* gene, which is genetically inseparable from the *Pc-2* crown rust resistance gene. Concurrently, in Arabidopsis, victorin sensitivity is conferred by the *LOV1* gene which encodes a NB-LRR protein, a type of protein commonly associated with disease resistance. LOV1, conforms to the "guard model" of resistance protein function and triggers cell death when victorin binds to the guardee, TRX-h5. To evaluate a possible LOV1 resistance function, *Pseudomonas syringae* pv. *tomato* virulence was evaluated upon LOV1 activation. The LOV1 response pathway in Arabidopsis was adapted to activate upon infection with *Pseudomonas syringae* pv. *tomato* expressing the type III-dependent effector protein AvrRpt2, a well-characterized protease. We developed a construct to express a beta-glucuronidase (GUS) and TRX-h5 fusion protein separated by an AvrRpt2 proteolytic cleavage site, in which GUS sterically inhibits TRX-h5 function in LOV1-mediated cell death. The fusion is cleaved upon infection by *P. syringae* pv. *tomato* expressing *avrRpt2*. It was assumed that TRX-h5 processing by AvrRpt2 would lead to LOV1 activation in the presence of victorin. However, when the strain expressing AvrRpt2 was inoculated with

victorin into transgenic *LOV1 trx-h5* plants expressing the GUS/TRX-h5 fusion protein, no decrease in pathogen virulence was observed. Technical shortcomings likely prevented observable LOV1 resistance function.

## 3.2 INTRODUCTION

Victoria blight, caused by the fungus *Cochliobolus victoriae,* is a disease originally described on oats (*Avena sativa)* (Meehan and Murphy, 1946). *C. victoriae* exclusively infects oats that carry *Pc-2*, a gene that confers resistance to crown rust caused by the fungus *Puccinia coronata* (Litzenberger, 1949). *C. victoriae* pathogenesis is dependent on production of victorin, a peptide host-selective toxin (Meehan and Murphy, 1947; Macko et al., 1985; Wolpert et al., 1985). Treating oats with purified victorin elicits Victoria blight disease symptoms, and only oats sensitive to victorin are susceptible to *C. victoriae* (Meehan and Murphy, 1947). Sensitivity to victorin, and thus susceptibility to Victoria blight, is conferred by the dominant *Vb* gene in oats (Meehan and Murphy, 1947). Genetic and mutagenic efforts to separate Victoriae blight susceptibility from crown rust resistance have failed, indicating that *Pc-2* and *Vb* are the same gene or tightly linked (Welsh et al., 1954; Luke et al., 1966; Rines and Luke, 1985; Mayama et al., 1995).

Identification of the *Vb*/*Pc-2* gene in oats is difficult due to a relatively uncharacterized hexaploid genome. Victorin sensitivity and susceptibility to *C. victoriae* is recapitulated in *Arabidopsis thaliana*. Therefore, this plant species is used as a model to characterize victorin sensitivity (Lorang et al., 2004). Sensitivity to

victorin and susceptibility to Victoria blight in Arabidopsis is conferred by *Locus Orchestrating Victorin Effects1* (*LOV1*) (Lorang et al., 2007). LOV1 encodes a nucleotide-binding-site-leucine-rich-repeat (NB-LRR) protein, a class of proteins typically associated with disease resistance (Nimchuk et al., 2003). LOV1 is a member of the RPP8 family of proteins, of which all other members with known functions are involved in disease resistance (Cooley et al. 2000; McDowell et al. 1998; Takahashi et al. 2002; Lorang et al., 2007). NB-LRR proteins activate an immune response to decrease pathogen growth upon direct or indirect recognition of pathogen effectors (Dangle and Jones, 2001; Dangl and Mcdowell, 2006). Therefore, genetics in both oats and Arabidopsis suggests Victoria blight susceptibility is conferred by a resistance gene.

Consistent with the genetic data, victorin elicits a physiological response that resembles a defense response in sensitive oats and Arabidopsis. Notably, victorin induces a programmed cell death (PCD) response in both Arabidopsis and oats. This PCD response shares characteristics with the hypersensitive response, a form of PCD associated with effector-triggered immunity (Mur et al., 2007). *C. victoriae* is characterized as a necrotrophic pathogen. Necrotrophs actively kill host tissue and extract nutrients from death cells; therefore it is conceivable *C. victoriae* has co-opted the defensive hypersensitive response to derive nutrients from the host. In addition, victorin induces other responses typically associated with defense in sensitive hosts. In oats, victorin induces callose deposition, a respiratory burst, lipid peroxidation, ethylene evolution,

extracellular alkalization, phytoalexin synthesis, and K+ efflux (Walton and Earle, 1985; Romanko, 1959; Navarre and Wolpert 1999; Shain and Wheeler, 1975; Ullrich and Novacky, 1991; Mayama et al., 1986 ; Wheeler and Black, 1962; Tada et al., 2001;Yao et al., 2001, 2002; Coffeen and Wolpert, 2004; Curtis and Wolpert, 2002, 2004). In Arabidopsis, victorin induces up-regulation of PR1 gene expression, salicylic acid accumulation, phytoalexin synthesis, and camalexin production (Lorang et al., 2007).

To further characterize the response mediated by LOV1, Arabidopsis was screened for mutations that lead to a loss of victorin sensitivity. This screen identified *TRX-h5* as required for victorin sensitivity (Sweat and Wolpert, 2007). LOV1 is activated (induces cell death) when TRX-h5 binds victorin (Lorang et al., In Press). TRX-h5 also plays a role in defense, as it is required for the redox-mediated control of the defense transcriptional regulator, NPR1 (non-expresser of PR1 protein) (Tada et al., 2008; Kinkema et al., 2000). Due to this role in defense, TRX-h5 is a likely target for pathogen effectors. In the absence of LOV1, victorin acts as a typical virulence effector by inhibiting TRX-h5 catalytic function thereby interfering with defense mediated by NPR1 (Lorang et al., In Press). Conversely, in the presence of LOV1, victorin binding to TRX-h5 leads to LOV1 activation and cell death (Lorang et al,). These findings support the interpretation that *LOV1* functions in defense response, guarding TRX-h5 from effectors that targets thioredoxins.

Therefore, multiple lines of evidence suggest *C. victoriae* activates a resistance response for disease susceptibility. Conversely, *C. victoriae* may activate a response that is unique for disease susceptibility. While activating LOV1 by the effector, victorin, elicits markers of defense, resistance function can only be evaluated by the effect activation has on pathogen virulence. A decrease in pathogen virulence upon activation of LOV1 would provide unambiguous evidence that resistance expression can lead to disease susceptibility.

To assess whether LOV1 can provide resistance, it is necessary for LOV1 to be activated in direct response to a pathogen during pathogenesis. Because the gene(s) encoding victorin synthesis has not been identified, victorin must be added exogenously complicating the ability to activate LOV1 in direct response to a pathogen. Therefore, the LOV1 response pathway in Arabidopsis was modified in an effort to facilitate activation dependent on infection with *Pseudomonas syringae* pv. *tomato* DC3000 expressing the type III-dependent effector *avrRpt2*.

The Arabidopsis pathogen *Psuedomonas syringae* pv *tomato* DC3000 utilizes type III secretion to inject effector proteins into plant cells during pathogenesis (Galán and Collmer, 1999). Effectors are used by pathogens to increase virulence; however when detected by host NB-LRR resistance proteins, effectors trigger a defense response (Jones and Dangle, 2006; Dangl and Mcdowell, 2006;). *AvrRpt2* encodes a well-characterized cysteine protease that cleaves the defense regulator RPM1 interacting protein 4 (RIN4) (Mackey et al, 2003; Chisholm et al, 2004). AvrRpt2-mediated cleavage of RIN4 results in activation of defense in plants

expressing the *RPS2* resistance gene (Mackey et al, 2003; Axtel and Staskawicz 2003). We employed Arabidopsis *rps2* mutants so that *avrRpt2* does not trigger *RPS2*-mediated resistance but could be exploited to engineer LOV1 activation when this effector is introduced into cells.

Importantly, TRX-h5 positively regulates LOV1. Expression of LOV1 does not lead to a cell death phenotype in the absence of TRX-h5 in *Nicotiana benthamiana* (Lorang et al., In Press). Because TRX-h5 functions as a positive regulator of LOV1 function, we modified TRX-h5 so that it would become activated upon delivery of avrRpt2 during infection by *Pseudomonas syringae* pv. *tomato* thereby activating LOV1 if victorin is present. We developed a construct to express a beta-glucuronidase (GUS) and TRX-h5 fusion protein separated by an AvrRpt2 proteolytic cleavage site, in which GUS sterically inhibits TRX-h5 function. The fusion is cleaved upon infection by *P. syringae* pv. *tomato* expressing *avrRpt2* (*Pst (avrRpt2)*), thereby leading to active TRX-h5. The assumption was that the newly-activated TRX-h5 could facilitate LOV1 activation in the presence of victorin. *Pst (avrRpt2)* was inoculated with victorin into transgenic Arabidopsis containing *LOV1*, mutant in wildtype *TRX-h5*, and expressing the GUS/TRX-h5 fusion protein; however, no decrease in pathogen virulence was observed. Technical shortcomings likely prevented observable LOV1 resistance gene function.

## 3.3 RESULTS

### 3.3.1 GUS fusion with Trx-h5 inhibits Trx-5 function and AvrRpt2 expression results in LOV1 activation

TRX-h5 was modified such that LOV1 could be activated upon delivery of the *Pseudomonas* type III effector AvrRpt2 (Figure 3-1). AvrRpt2 cleaves the peptide sequence RSNVPKFGNWEA, which is an AvrRpt2-specific proteolytic site present in the Arabidopsis protein RIN4 (Chisholm et al., 2005). Synthetic substrates containing this amino acid sequence have been demonstrated to be proteolytically processed in plant cells following the introduction of AvrRpt2 (Chisholm et al., 2005). A construct to express a beta-glucuronidase (GUS) and TRX-h5 fusion protein (GUS:TRX-h5) was created to sterically inhibit TRX-h5 function. GUS and TRX-h5 were connected by a linker containing the AvrRpt2 proteolytic cleavage site, with the assumption that delivery of AvrRpt2 upon *Pseudomonas syringae* pv *tomato* infection in plants expressing *GUS:TRX-h5* would activate TRX-h5 and compliment LOV1 function. The ability of the GUS fusion to inhibit TRX-h5 mediated LOV1 activation was tested by heterologous expression in *Nicotiana benthamiana*. Consistent with a previous study (Lorang et al., In Press), leaves transiently expressing *LOV1*, *TRX-h5* , and the control green fluorescent protein (*GFP)* conferred a cell death phenotype after victorin treatment (Figure 3-2A). In contrast, leaves transiently expressing *LOV1*, GUS:*TRX-h5,* and *GFP* exhibited no cell death after victorin treatment, indicating that the GUS fusion inhibited TRX-h5 function in victorin-mediated cell death

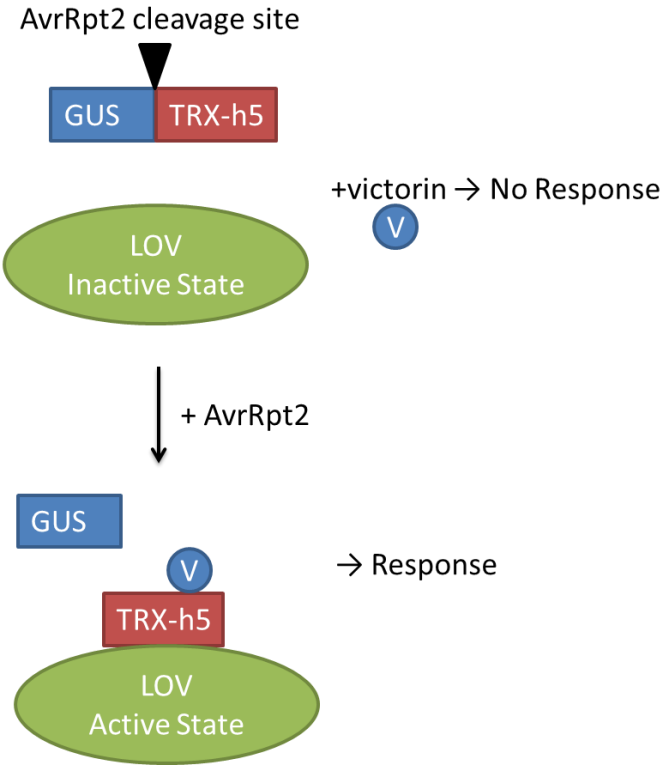


**Figure 3-1: Model depicting fusion of TRX-h5 with GUS to sterically inhibit TRX-h5 mediated LOV1 activation, and subsequent activation of LOV1 by proteolytic cleavage of GUS from TRX-h5 by AvrRpt2.**

(Figure 3-2A). The ability of AvrRpt2 to proteolytically process GUS:TRX-h5 and activate TRX-h5 was tested by co-expressing *AvrRpt2* with *GUS:TRX-h5* and *LOV1*. Leaves transiently expressing *LOV1*, GUS:*TRX-h5,* and *AvrRpt2* exhibited a cell death phenotype after victorin treatment, indicating *AvrRpt2* expression results in TRX-h5 mediated LOV1 activation (Figure 3-2A).

The proteolytic processing of GUS:TRX-h5 was monitored by western blotting. Protein extracts were sampled from leaves co-expressing *LOV1, GUS:TRX-h5* and *AvrRpt2* or the *GFP* control, and blots were probed with an antibody to detect GUS. Full-length GUS:TRX-h5 accumulated when *GUS:TRX* was co-expressed with *LOV1* and the *GFP* control (Figure 3-2B). When *GUS:TRX-h5* was co-expressed with *LOV1* and *AvrRpt2*, only the GUS cleavage product was detected, indicating that *AvrRpt2* expression results in processing of the GUS and TRX-h5 fusion (Figure 3-2B).

### 3.3.2 Infection by *P. syringae* pv. *tomato* expressing *avrRpt2* results in proteolytic processing of the GUS and TRX-h5 fusion protein

Processing of GUS:TRX-h5 was evaluated in response to *P. syringae* pv. *tomato* expressing *avrRpt2* [*Pst (avrRpt2)*]. Transgenic *LOV1/trx5/rps2* Arabidopsis expressing *GUS:TRX-h5* were inoculated at a high concentration (1 X $10^6$ cfu/mL) by infiltration with *Pst (avrRpt2)* or the control *P. syringae* pv. *tomato* expressing a non-functional allele of avrRpt2 [*Pst (avrRpt2*:: Ω)]. Protein extracts were sampled four and six hours after inoculation. Only full length GUS:TRX-h5 was detected in extracts sampled from leaves inoculated with the control *Pst*

**Figure 3 2: GUS fusion with Trx-h5 inhibits Trx-5 function and AvrRpt2 expression results in LOV1 activation in the presence of victorin.**

(A) *N. benthamaiana* leaves were inoculated with *Agrobacterium* containing constructs to transiently express *LOV1* in combination with *GUS:TRX-h5* or the control*TRX-h5*, and *avrRpt2* or the control *GFP*. 48 hr after inoculation, 1 ug victorin diluted in water or water was infiltrated into the leaves. Cell death was assessed 10 hr after victorin treatment. (B) *AvrRpt2* expression results in cleavage of the GUS:TRX-h5 fusion protein. Protein was extracted from *N. benthamiana* leaves expressing *LOV, GUS:TRX-h5*, and *avrRpt2* or the control *GFP*. GUS:TRX-h5 (83.4 kDa) and cleaved GUS (60 kDa) was detected by western blotting with anti-GUS antibody.

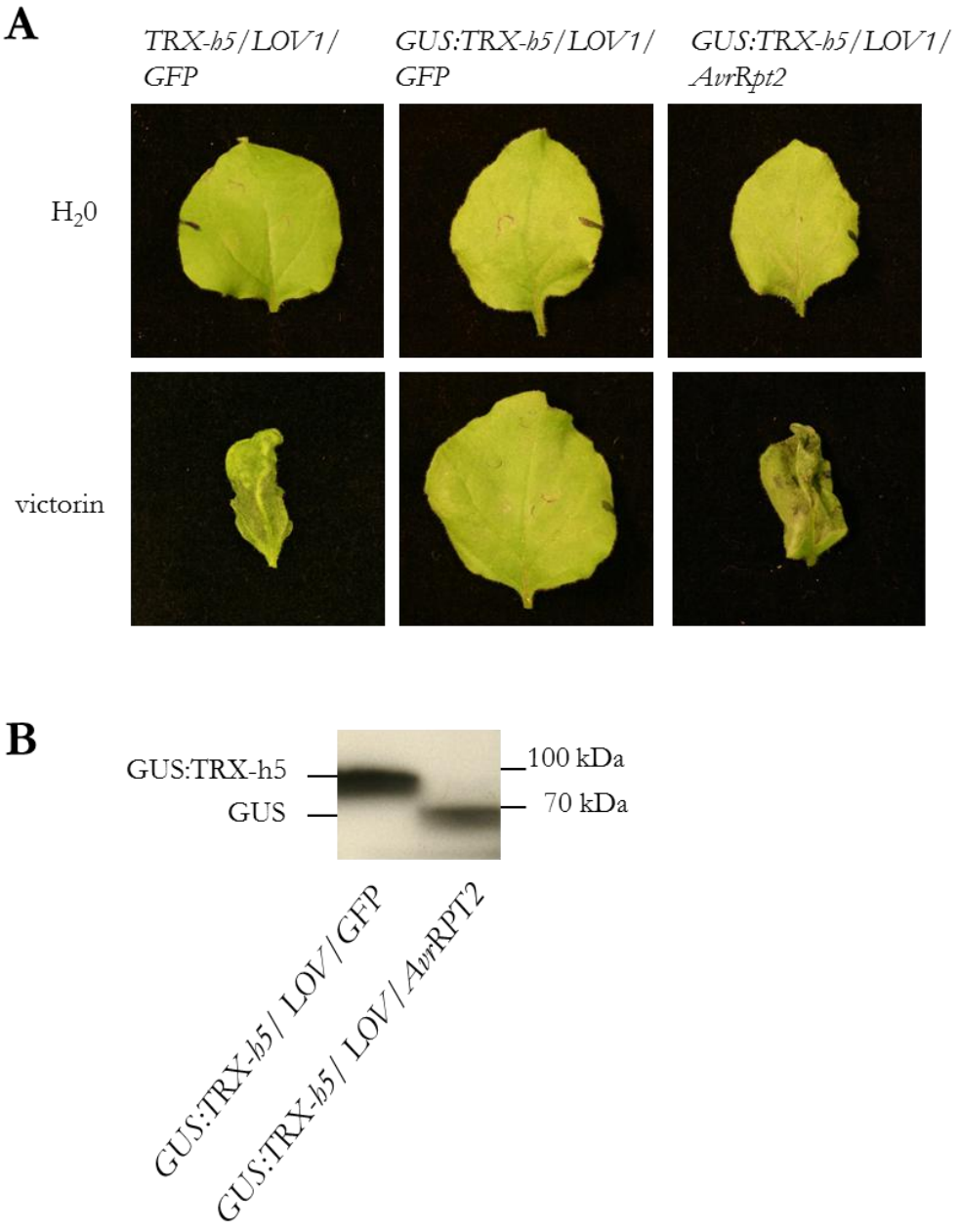


**Figure 3-2: GUS fusion with Trx-h5 inhibits Trx-5 function and AvrRpt2 expression results in LOV1 activation in the presence of victorin.**

*(avrRpt2*:: Ω) (Figure 3-3). In contrast, in extracts sampled six hours after inoculation with *Pst (avrRpt2),* the GUS cleavage product was detected indicating *Pst (avrRpt2)* infection results in GUS:TRX-h5 processing in transgenic Arabidopsis (Figure 3-3). The GUS cleavage product was not present in extracts sampled four hours after inoculation with *Pst (avrRpt2),* indicating that GUS:TRX-h5 is processed approximately six hours after infection.

### 3.3.3 Virulence of *P. syringae* pv. *tomato* expressing *avrRpt2* is not affected in Arabidopsis containing *LOV1* and expressing the *GUS:TRX-h5* in the presence of victorin.

We tested whether Arabidopsis containing *LOV1* and expressing *GUS:TRX-h5* were resistant to *P. syringae* pv. *tomato* expressing *avrRpt2* in the presence of victorin. Transgenic *LOV1/ trx-5/ rps2* plants expressing *GUS:TRX-h5*, or *LOV1/ trx-h5/ rps2* plants (control) were inoculated at a low concentration (1 X $10^5$ cfu/mL) by infiltration with *Pst (avrRpt2)* or the control *Pst (avrRpt2*:: Ω). As a disease resistant control, *LOV1/trx-h5/ RPS2* plants were inoculated, thereby activating *RPS2*- mediated resistance upon *Pst (avrRpt2)* infection. 50 μg victorin or water was infiltrated six hours after bacterial inoculation, to ensure that victorin was present at high concentrations after *GUS:TRX-h5* is processed. Bacterial growth was monitored two days after inoculation. *RPS2*-mediated resistance was observed by the differential growth of *Pstab* (*avrPto*) in *LOV1/trx-h5/ RPS2* plants compared with *LOV1/trx /rps2*plants, and the differential growth of *Pst (avrPto)* compared to *Pst (avrRpt2*:: Ω) in *LOV1/ trx-h5/ RPS2*

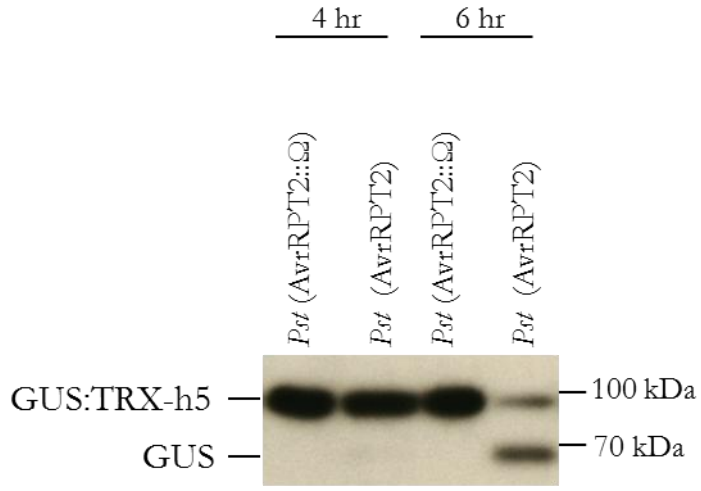


**Figure 3-3: Inoculation of transgenic *LOV1 trx-h5 rps2* Arabidopsis expressing the *GUS:TRX* fusion with *Pseudomonas syringae* pv. *tomato* carrying the type-III effector *avrRpt2* results in cleavage of the GUS:TRX fusion protein. Protein was extracted from leaves of transgenic *LOV1 trx-h5 rps2* Arabidopsis expressing *GUS:TRX-h5* at four or six hours after infiltration with 1 X $10^6$ cfu/mL *Pseudomomans syringae* pv *tomato* DC3000 carrying *avrRpt2* (*Pst (avrRpt2)*) or the control *Pseudomomans syringae* pv *tomato* DC3000 carrying *avrRpt2*::Ω, a non-functional allele of *avrRpt2* (*Pst (avrRpt2*:: Ω)). GUS:TRX-h5 (83.4 kDa) and cleaved GUS (60 kDa) was detected by western blotting with anti-GUS antibody.**

plants (Figure 3-4). However, growth of *Pstab* (*avrPto*) was similar in transgenic *LOV1/ trx-h5/ rps2* plants expressing *GUS:TRX-h5* and the control *LOV1/ trx-h5/ rps2* plants in the presence of victorin (ANOVA, $P < 0.05$) (Figure 3-4). Growth of *Pstab* (*avrPto*) in transgenic *LOV1/ trx-h5/ rps2* plants expressing *GUS:TRX-h5* was similar after victorin treatment compared to the control water treatment (ANOVA, $P < 0.05$)(Figure 3-4). These results indicate that virulence of *P. syringae* pv. *tomato* expressing *avrRpt2* is not affected in Arabidopsis containing *LOV1* and expressing *GUS:TRX-h5* in the presence of victorin.

## 3.4 DISCUSSION

In this work, the resistance function of LOV1 was evaluated in response to *P. syringae* pv. *tomato* infection. The LOV1 response was adapted to become activated upon infection with *P. syringae* pv. *tomato* that expresses the effector *avrRpt2,* a well-characterized protease (Figure 3-1). TRX-h5 was modified by the addition of an N-terminal GUS fusion (GUS:TRX-h5), which inhibited TRX-h5 mediated LOV1 activation (Figure 3-2A). The GUS fusion was designed to be cleaved by interaction with AvrRpt2, with assumption was that the newly-activated TRX-h5 could facilitate LOV1 activation in the presence of victorin. However, there was no change in virulence of *P. syringae* expressing *avrRpt2 (Pst (avrRpt2))* on transgenic *LOV1/ trx-h5* Arabidopsis expressing *GUS:TRX-h5* in the presence of victorin (Figure 3-3). While this could indicate a lack of resistance function for LOV1, technical shortcomings prevent this conclusion.

**Figure 3-4: Transgenic *LOV1 trx-h5 rps2* Arabidopsis expressing *GUS:TRX-h5* does not alter the virulence of *Psuedomonas syringae* pv. *tomato* DC3000 carrying the type-III effector *avrRpt2 (Pst (avrRpt2))* in the presence of 50 ug/mL victorin.**

The indicated genotypes were inoculated with *Pseudomomans syringae* pv *tomato* DC3000 carrying *avrRpt2* (*Pst (avrRpt2)*) or the control *Pseudomomans syringae* pv *tomato* DC3000 carrying *avrRpt2*::Ω, a non-functional allele of *avrRpt2* (*Pst (avrRpt2*:: Ω)). Plants were infiltrated with either victorin or the carrier water six hours after *Pst* inoculation. Bacterial growth (log colony forming units (cfu)) was measured two days after inoculation. The Arabidopsis genotypes were *LOV1 trx-h5 rps2* plants, transgenic *LOV1 trx-h5 rps2* plants expressing *GUS:TRX-h5*, and *LOV1 trx-h5 RPS2* plants. Error bars represent standard error (n = 3 samples), and results are representative of 3 independent experiments. Significantly different means ($p < .05$, one-way ANOVA multiple comparison analysis) are indicated by different letters.

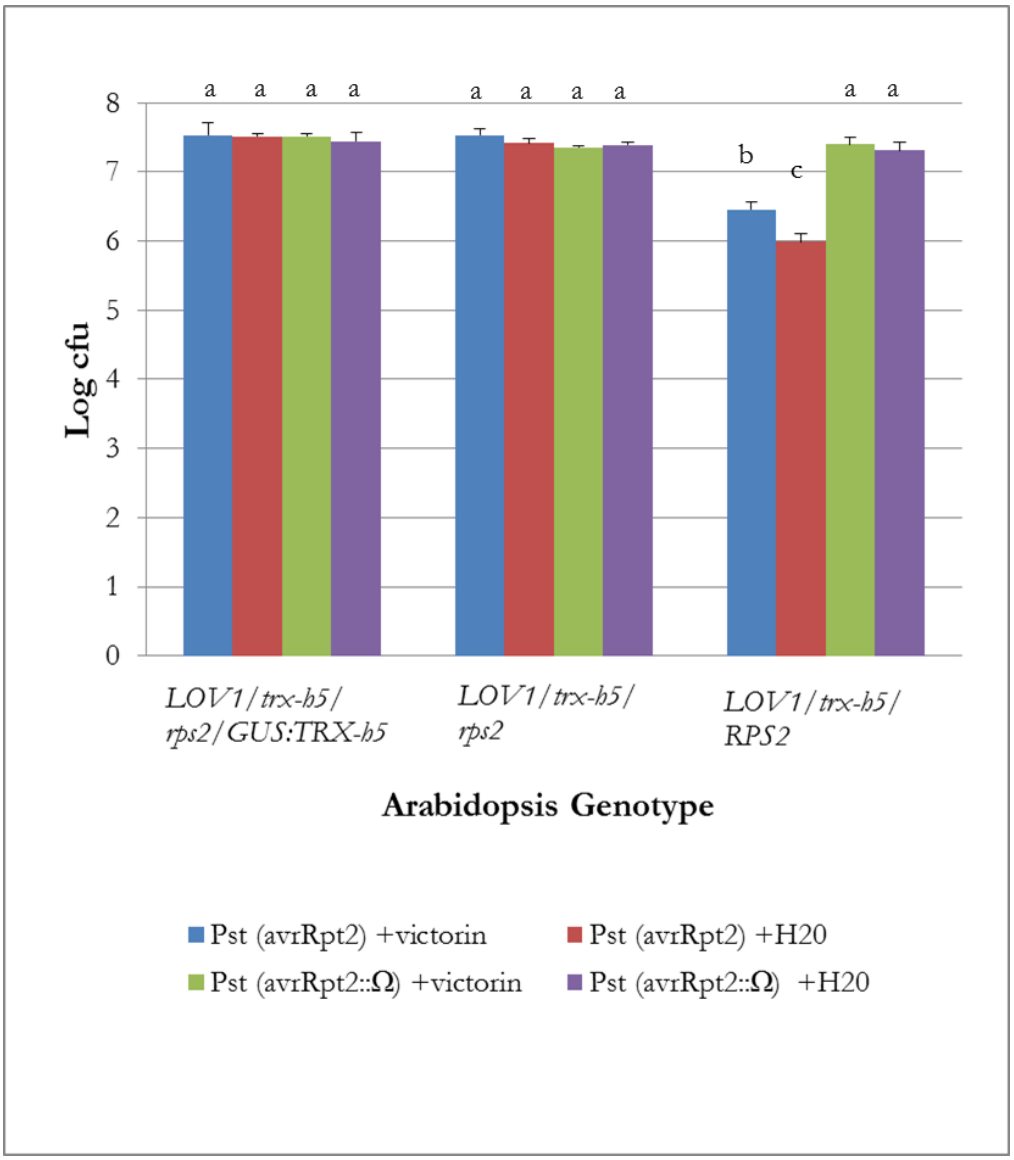


**Figure 3-4: Transgenic *LOV1 trx-h5 rps2* Arabidopsis expressing *GUS:TRX-h5* does not alter the virulence of *Psuedomonas syringae* pv. *tomato* DC3000 carrying the type-III effector *avrRpt2 (Pst (avrRpt2))* in the presence of 50 ug/mL victorin.**

Victorin-mediated cell death was observed when *GUS:TRX-h5* was co-expressed with *LOV1* and *AvrRpt2* in *N. benthamiana,* indicating that the proteolytically processed GUS:TRX-h5 can be effective in facilitating LOV1 activation (Figure 3-2). In Arabidopsis, it was demonstrated that proteolytic processing of the GUS:TRX-h5 fusion occurred upon *Pst (avrRpt2)* infection (Figure 3-3). However, we were not able to observe victorin sensitivity under these conditions (Data Not Shown). The absence of victorin sensitivity in transgenic Arabidopsis may be because proteolytically processed TRX-h5 is unable to assemble into a functional complex with LOV1 after LOV1 is expressed. In *N. benthamiana*, *AvrRpt2* was simultaneously expressed with *GUS-TRX-h5* and *LOV1*. Therefore proteolytic processing of GUS:TRX-h5 likely occurred simultaneously with *LOV1* expression, allowing processed TRX-h5 and LOV1 to assemble into a functional complex. In Arabidopsis, *GUS-TRX-h5* was processed after LOV1 expression. Therefore it is possible that the proteolytic processing of TRX-h5 upon *Pst (avrRpt2)* infection is unable to function in LOV1 activation because the majority of LOV1 occurs in a pre-existing state.

Alternatively, if TRX-h5 processing confers mild sensitivity to victorin, it is conceivable that mild decreases in pathogen virulence upon LOV1 activation would not be observable due the virulence effector function of victorin. Victorin binds TRX-h5 at a conserved thioredoxin active site and victorin binds other thioredoxins including TRX-h3 (Lorang et al., In Press). TRX-h3 is also involved in the redox control of NPR1, a transcriptional regulator of defense (Tada et al.,

2008). In *LOV1 trx-h5 RPS2* plants infected with *Pst (avrRpt2)*, we observed a half-log increase in bacterial growth in leaves treated with victorin compared to the water control (ANOVA, $P > 0.05$) (Figure 3-4), demonstrating that victorin decreases *RPS2*-mediated disease resistance in *trx-h*5 mutant plants as expected (Lorang et al., In Press). Therefore, a half-log decrease in bacterial growth due to LOV1 activation in transgenic *LOV1 trx-h5 rps2* plants infected with *Pst (avrRpt2)* may not have been observed due to the presence of the canonical effector function of victorin. In this case, victorin would suppress LOV1-mediated defense a half-log.

Using a resistance gene to confer resistance to a type of pathogen not normally affected by that resistance specificity has not been reported in literature. This is likely due to the complexity of achieving resistance protein activation in response to a "heterologous" pathogen. Cross resistance has only been demonstrated when the resistance protein is activated for pathogens of the same type. *Rx*, a gene which confers resistance to potato virus X, has been shown to confer resistance to tobacco mosaic virus expressing potato virus X coat protein (Bendahmane et al., 1995). Members of the RPP8 family of proteins with known resistance function confer resistance to viral and omycete pathogens, not bacterial pathogens (Takahashi et al., 2002; Cooley et al., 2000). Therefore, it is conceivable that LOV1 activation simply does not provide resistance to the bacterial pathogen *P syringae*.

## 3.5 MATERIALS AND METHODS

### 3.5.1 Plant material

*Nicotiana benthamiana* and Arabidopis were grown at 22°C in a growth chamber under 16h light/8h dark cycle. Plants homozygous for the *rps2* mutant allele *rps2-101c* (Mindrinos et al., 1994), were obtained from Dr. Jeff Chang, Oregon State University (Corvallis, OR, U.S.A.). A plant carrying *rps2-101c* was crossed into a line that is near-isogenic to Col-4 and is homozygous for *LOV1* and homozygous for a SALK *trx-h5* mutantation (Sweat and Wolpert, 2007). The F2 segregating population was screened for the presence of the *LOV1* gene using the 3571 SSLP marker (forward 5'-GTGGTGACCTCTCCCTCAAA-3' and reverse 5'-CCCACTTCACCGTTTCTCTC-3') as previously described (Lorang et al., 2007). Plants homozygous for *LOV1* were screened for the presence of the SALK T-DNA insertion in *trx-h5* with the primers 5'-GTGGACCGCTTGCTGCAACT-3' and 5'-TCTTGTTATGTCCAGGGCTTTT-3' and checked for presence of a wild-type allele using the primers 5'-TCTTGTTATGTCCAGGGCTTTT-3′ and 5′-TTTTCGTGTTCGTGGTTGAA-3′ as previously described (Sweat and Wolpert, 2007). Plants homozygous for *LOV1* and *trx-h5* were checked for the presence of *RPS2* by monitoring for bacterial disease upon syringe inoculation of *Pst (avrRpt2)* diluted to $10^5$ colony forming units/mL (CFU/mL). Lines homozygous for *rps2* or *RPS2* were identified by analyzing the progeny of self-fertilized F2 plants for segregation.

*Agrobacterium tumefaciens* strain GV3101 carrying 35S::*GUS:TRX-h5* was used to transform *LOV1/ trx-h5/ rps2* plants with the *Agrobacterium* floral dipping procedure (Clough and Bent, 1998). Primary transformants (T1) were selected on soil containing 25 μM Basta. T1 plants were allowed to self-fertilize to generate T2 progeny. Independent lines homozygous for the *GUS:TRX-h5* transgene were selected by identifying T2 plants that gave rise progeny that did not segregate for *GUS:TRX-h5*. GUS histological staining was used to evaluate *GUS:TRX-h5* segregation. GUS was assayed by vacuum-infiltrating leaves with GUS substrate solution (5 M NaPO4 pH 7.2, 10 mM EDTA, 10% Triton X-100, and 2mM X-GLUC (Sigma-Aldrich)) and incubating overnight at 37°C.

### 3.5.2 Constructs

For 35S::*GUS:TRX-h5*, *GUS* was amplified from pBI-505 (Dalta et al., 1991) with the primers 5'-GATCGCGGCCGCATGTTACGTCCTGTAGAAACCC-3' and 5'-GATCGCGGCCGCTTCCCAGTTTCCAAATTTTGGTACATTCGAACGTTGTTTGCCTCCCTGCTGCG-3', which added a *Not*I restriction site on the 5' end of *GUS* and a synthetic peptide containing the sequence RSNVPKFGNWEA followed by a *Not*I restriction site on the 3' end of *GUS*. The resulting PCR product was transferred as a *Not*I-*Not*I fragment into pENTR:*TRX-h5* (Sweat and Wolpert, 2007). The direction of the insert was checked with the primers 5'-TAATGTTCTGCGACGCTCAC-3' and 5'-TCAAGCAGAAGCTACAAGACCA-3'. The resulting clone was recombined

into pEarleyGate100 (Earley et al., 2006) with Gateway LR clonase II enzyme mix (Life Technologies).

For 35S:*AvrRpt2*, *AvrRpt2* was amplified from *Pseudomonas syrinage* pv *tomato* DC3000 carrying *avrRpt2* (Whalen et al., 1991) with the primers 5'-CACCATGAAAATTGCTCCAGTTGCC- 3' and 5'-GCGGTAGAGCATTGCGTGTG-3' and cloned into pENTR/D-TOPO (Life Technologies). The resulting clone was recombined into the destination vector pGWB11 (Nakagawa et al., 2007) with Gateway LR clonase II enzyme mix (Life Technologies).

### *3.5.3* Agrobacterim-mediated transient transformation of *N. benthamiana* and cell death assay

*Agrobacterium tumefaciens* strain GV2260 cultures containing 35S:*LOV1* (Lorang et al, In Press), 35S:*TRX-h5* (Sweat and Wolpert, 2007) *,* 35S:*GUS:TRX-h5,* 35S:*AvrRpt2,* and PSLJ:*GFP* were re-suspended in 10 mM MES pH5.2 (10 mM MgCl, 150 uM Acetosyringone), diluted to $OD_{600}$=0.5, and incubated for three hours. Equal volumes of the appropriate cultures were mixed before syringe-infiltration into fully-expanded *Nicotiana benthamiana* leaves. 1 ug/mL of victorin, purified as described previously (Macko et al. 1985; Wolpert et al. 1985), diluted in water was infiltrated 48 hours after *Agrobacterium* inoculation. Cell death was visually assessed 10 hours after victorin infiltration.

### 3.5.4 Protein analysis

Protein extracts were prepared by grinding leaf tissue in SDS buffer (.625 tris ph 6.8, SDS, 5% β-mercaptoethanol). *For N. benthamiana* protein samples, 2.5 $cm^2$ tissue was ground in 100 uL. For Arabidopsis, 0.6 cm2 tissue from 3 plants was pooled, ground in 150 uL, and 20 uL was loaded. Thirty uL of each *N. benthamiana* sample and 20 uL of each Arabidopsis sample were loaded onto 8% SDS-polyacrylamide gels and transferred onto Protran nitrocellulose membrane with a .2 um pore size (Whatman Schleicher and Schuell, Dassel, Germany). Membranes were blocked in 5% skim milk powder in TBST (25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween-20). Rabbit anti-GUS antibody (Sigma) was used at a 0.5 ug/mL. Anti-rabbit HRP conjugate at a 1:10,000 dilution was used as a secondary antibody. Proteins were visualized with ECL chemiluminescent substrate (Life Technologies).

### 3.5.5 Monitoring of bacterial disease on plants

*Pseudomonas syrinage* pv. *tomato* DC3000 (*Pst)* carrying avrRpt2 (*Pst avrRpt2*) and *Pseudomonas syrinage* pv. *tomato* DC3000 carrying avrRpt2::Ω (*Pst avrRpt2*::Ω) (Whalen et al., 1991) were obtained from Dr. Brian Staskawicz (University California Berkley, Berkley, CA, U.S.A.) *Pst* strains were cultured in King's medium B (King et al, 1954), diluted to $10^5$ colony forming units/mL (CFU/mL) in 10 mM $MgCl_2$, and syringe-inoculated into fully-expanded 3rd or 4th true leaves of transgenic *LOV1 trx-h5 rps2* plants expressing *GUS:TRX*, and *LOV1 trx-h5 RPS2* plants. Six hours after inoculation, the inoculated leaves were infiltrated

with either 50 μg victorin or 10mM $MgCl_2$. Three days following inoculation, nine 0.4 $cm^2$ leaf disks were excised from plants of each treatment. The nine discs were divided into three sets of three and were ground in 10mM $MgCl_2$. The bacterial populations in the leaves were determined by plating serial dilutions on King's medium B supplemented with 100 mg/L rifampicin and 30 mg/L kanamycin. Colonies were counted after 48 hours of growth at 28°C. The bacterial growth assays were repeated three times. Bacterial populations were log-transformed and a one-way ANOVA (Tukey's test) was used for statistical analysis.

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# 4 General Conclusions

The work described in this thesis has provided advances in our understanding of the victorin response mediated by the plant disease susceptibility gene *LOV1*. The results presented here provide evidence that the response mediated by *LOV1* is functionally similar to a resistance response, thus further supporting the hypothesis that effector-triggered immunity is involved in susceptibility to *C. victoriae*. The response mediated by LOV1 was genetically characterized with virus-induced gene silencing (VIGS). We found that SGT1, a common modulator of resistance protein function, regulates victorin-mediated cell death by maintaining the stability of LOV1. Six genes were identified in a screen of a cNbME library that, when silenced, inhibit both LOV1- and RPP8-mediated cell death, thus suggesting that LOV1 shares functions in common with the closely-related resistance protein, RPP8. In addition, silencing 4/6 of these genes inhibited both cell death and resistance mediated by the PTO resistance protein. These findings coupled with the genetic association of victorin sensitivity with the *Pc2* rust resistance gene in oats (Welsh et al., 1954; Luke et al., 1966; Rines and Luke, 1985; Mayama et al., 1995) and our recent finding that the interaction of LOV1, TRX-h5 and victorin recapitulates the guard mechanism of resistance protein activation (Lorang et al., In Press) strongly suggest that *C. victoriae* exploits R-gene mediated defense for virulence and disease susceptibility.

Components of the LOV1 response pathway remain to be identified. Further characterization of the LOV1 response pathway would be valuable in helping understand which resistance responses are vulnerable to necrotrophic exploitation. Here, we show that VIGS is a valuable tool to characterize the victorin response pathway. VIGS could be exploited to further characterize this response. Components can be identified by further screening of known genes involved in disease resistance, or screening libraries of genes induced during LOV1 activation. If we couple the quantitative electrolyte leakage assay with VIGS screening, components that have mild effects on victorin-mediated cell death could be identified. Finding more genes that are involved in victorin sensitivity will further our understanding of disease susceptibility.

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