Characterization of the *LOV1*-Mediated, Victorin-Induced, Cell-Death Response with Virus-Induced Gene Silencing

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Submitted 14 January 2013. Accepted 11 April 2013.

Victoria blight, caused by *Cochliobolus victoriae*, is a disease originally described on oat and recapitulated on *Arabidopsis*. *C. victoriae* pathogenesis depends upon production of the toxin victorin. In oat, 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 *LOCUS ORCHESTRATING VICTORIN EFFECTS1 (LOV1)* gene. *LOV1* encodes a nucleotide-binding site leucine-rich repeat protein, a type of protein commonly associated with disease resistance, and *LOV1* “guards” 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-induced cell death in *N. benthamiana*. We determined that *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 *LOV1*-mediated, victorin-induced cell death and cell death induced by expression of the closely related *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 the victorin response mediated by *LOV1* is a defense response.

Victoria blight, a disease originally described on oat (*Avena sativa*), is caused by the necrotrophic fungus *Cochliobolus victoriae* (Meehan and Murphy 1946). Victoria blight exclusively appeared on oat plants carrying the crown rust resistance (*R*) gene *Pc2*, 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 (Macko et al. 1985; Meehan and Murphy 1947; Wolpert et al. 1985). Sensitivity to victorin is conferred by the dominant *Vb* gene in oat. Only oat plants sensitive to victorin are susceptible to *C. victoriae*, and oat plants 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 *Pc2* and *Vb* are the same gene (Luke et al. 1966; Mayama et al. 1995; Rines and Luke 1985; Welsh et al. 1954). Thus, genetic evaluations indicate that Victoria blight susceptibility in oat is associated with an *R* gene. Consistent with the genetic data, victorin elicits a response in sensitive oat plants (i.e., those containing *Vb*) that resembles a defense response. This response includes callose deposition, a respiratory burst, lipid peroxidation, ethylene evolution, extracellular alkalization, phytoalexin synthesis, and K⁺ efflux (Mayama et al. 1986; Navarre and Wolpert 1999a; Romanko 1959; Shain and Wheeler 1975; Ullrich and Novacky 1991; Walton and Earle 1985; Wheeler and Black 1963). Notably, victorin also induces a programmed cell death (PCD) response, which involves a mitochondrial permeability transition (MPT) (Coffeen and Wolpert 2004; Curtis and Wolpert 2002, 2004; Navarre and Wolpert 1999a; Tada et al. 2001; Yao et al. 2001, 2002). This PCD response shares characteristics with the hypersensitive response (HR), a form of PCD associated with disease resistance (Mur et al. 2008).

Victorin sensitivity and susceptibility to *C. victoriae* was identified in *Arabidopsis* (Lorang et al. 2004), and victorin-induced cell death in *Arabidopsis* was shown to share characteristics of PCD in oat (Wolpert et al. 2011) and 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; Lorang et al. 2007; McDowell et al. 1998; Takahashi et al. 2002). NB-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). PCD associated with the HR surrounding the infection site halts the spread of infection by limiting access to nutrients and water (Glazebrook 2005; Heath 2000). *C. victoriae* is characterized as a necrotrophic pathogen, which actively kills host tissue and extracts nutrients from dead cells. For necrotrophic pathogens, the HR may actually be beneficial and could promote susceptibility (Govrin and Levine 2000; Wolpert et al. 2002). For the necrotroph *Botrytis cinerea*, the HR has been shown to aid in pathogenicity by providing a

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary figures and one supplementary table are published online.

growth substrate (Govrin and Levine 2000). Induction of the HR by the hemibiotrophic fungus *Mycosphaerella graminicola* is hypothesized to play an important role in its pathogenicity on wheat (Keon et al. 2007; Rudd et al. 2008). The similarities of victorin-induced cell death with the HR, the association of *Vb* with the *Pc2* rust *R* gene, and the finding that *LOV1* belongs to a family of disease *R* genes all suggest that *C. victoriae* co-opts the defensive HR to derive nutrients from the host.

A screen for ethylmethane sulphonate (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. 2012). TRX-h5 is also required for the redox-mediated control of NPR1 (nonexpresser of pathogenesis-related [PR] protein 1), a transcriptional co-regulator (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 displays properties of a “typical” virulence effector by inhibiting TRX-h5 catalytic function and, thereby, interfering with defense mediated by NPR1 (Lorang et al. 2012). This property of victorin increases the virulence of *Pseudomonas syringae* pv. *maculicola* but appears to provide no advantage to *C. victoriae* (Lorang et al. 2012). Conversely, in the presence of *LOV1*, victorin binding to TRX-h5 leads to *LOV1* activation and cell death and pathogenesis by *C. victoriae* (Lorang et al. 2012). These findings support the interpretation that *LOV1* functions in ETI as an R protein guarding TRX-h5 from effectors that targets thioredoxins. Thus, mechanistic studies also support the contention that *C. victoriae* is 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 nature of victorin-mediated cell death and clarify its relationship with ETI. Here, we exploit the finding that co-expression of *Arabidopsis LOV1* and *TRX-h5* in *Nicotiana benthamiana* confers victorin sensitivity (Lorang et al. 2012). We used 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. 2003). 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 (Sweat and Wolpert 2007). Unlike EMS mutagenesis, VIGS can silence multiple members of a gene family if the insert contains sequences 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. 2003). 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 (PHT), and the *SUPPRESSOR OF G2 ALLELE OF SKP1* (*SGT1*) are required for victorin sensitivity. The metabolic enzymes found to be important were glyceraldehyde 3-phosphate dehydrogenase (GAPB) and four photorespiratory enzymes: glycolate oxidase (GOX), glutamine synthetase (GLU), the P protein of the glycine decarboxylase complex (GDC-P), and the T protein of the GDC (GDC-T). These metabolic enzymes were also required for cell death induced by transient expression of the *RPP8* *R* gene and an auto-active form of *PTO* (PTOY207D). Silencing of these metabolic genes also reduced *PTO*-mediated resistance to *P. syringae* pv.

tabaci in *N. benthamiana*, demonstrating a role for these genes in resistance conferred by ETI. Together, these results provide evidence that the victorin-mediated response is functionally equivalent to a resistance response.

RESULTS

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. 2012), demonstrates that any additional genes necessary for victorin-mediated cell death are present in *N. benthamiana*. Victorin sensitivity in *N. benthamiana* was utilized to identify other genes required for victorin-mediated cell death. *N. benthamiana* plants were infected with a TRV vector (Liu et al. 2002) containing a fragment of a host gene to be silenced by VIGS. After gene silencing, *LOV1* and *TRX-h5* were transiently over-expressed in the leaves and the extent of the cell death following victorin treatment was visually assessed. Additionally, 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 FLUORESCENT PROTEIN* (TRV:*GFP*) to control for viral effects in VIGS experiments. Conductance was measured to quantify electrolyte leakage, an indicator of cell death, over the course of 12 h after exposing the leaf disks to victorin (Fig. 1). Electrolyte leakage increased over time, fitting a typical sigmoidal curve, and approached saturation 12 h after exposure to victorin (Fig. 1A). Electrolyte leakage increased appreciably as victorin concentration was increased in half-log increments from 10 to 1,000 ng/ml (Fig. 1A). As victorin concentration was increased logarithmically, leakage increased linearly at all times tested, including 2, 4, 6, 8, and 12 h ($R^2 = 0.99, 0.99, 0.99, 0.94,$ and 0.70 , respectively), indicating a dose-response relationship which began to decay (reach saturation) approximately 12 h after victorin treatment (Fig. 1B). Therefore, electrolyte leakage at these doses and up to 12 h after treatment appears to provide a quantitative measure of victorin-mediated cell death.

VIGS was used for silencing a set of plant genes that have known roles in defense signaling to determine whether they serve a function in victorin-mediated cell death. We silenced *N. benthamiana* orthologs of the mitogen-activated-protein (MAP)-kinases *WIPK* and *SIPK*; the MAP-kinase kinases (MAPKK) *MEK1*, *MEK2*, and *MAPKKKα*; the protein kinase *ADI2*; the lipase-like protein *EDS1*; the NB-LRR *NRC1*; and *SGT1*, *RAR1*, and *HSP90* (members of a chaperon complex), each of which has been implicated in signaling during the HR (Bogdanove and Martin 2000; Calderini et al. 2001; Gabriëls et al. 2007; Liu et al. 2002; Pozo et al. 2004; Seo et al. 2008; Shirasu 2009; Yang et al. 2001; Zhang et al. 2000). We routinely assayed for both victorin sensitivity and cell death induced by the overexpression of the *RPP8* *R* gene in leaves of plants silenced for individual genes. *RPP8* is constitutively active when overexpressed (Luo et al. 2009). 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* (Fig. 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 (Fig. 2A). In contrast, leaves transiently expressing *LOV1* and *TRX-h5* from *NbSGT1*-silenced plants showed no cell death after victorin treatment (Fig. 2A).

Cell death induced by *RPP8* expression was not affected by silencing *NbSGT1* (Fig. 2A). The reduction in visible cell death mediated by victorin correlated with reduced electrolyte leakage. Leaf disks excised from *LOV1*- or *TRX-h5*-expressing leaves from plants silenced for *NbSGT1* exhibited considerably less electrolyte leakage over an 8-h time period after victorin treatment compared with the control (Fig. 2B). When *NbSGT1* was silenced, electrolyte leakage was approximately 10% of that observed in the positive control 8 h after victorin treatment (Fig. 2B).

Because *SGT1* is a chaperone that can impact the accumulation of R proteins (Azevedo et al. 2006), we wanted to determine whether the reduction in victorin-mediated cell death in *NbSGT1*-silenced plants was 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* (Lorang et al. 2012). Total protein extracts from *LOV1*- or *myc:TRX-h5*-

Fig. 1. Victorin elicits electrolyte leakage as a dose-responsive indicator of cell death in *Nicotiana benthamiana* leaves expressing *LOV1* and *TRX-h5*. Effect of victorin concentration on electrolyte leakage kinetics from leaf disks from leaves of plants expressing *Tobacco rattle virus* (TRV):*GFP*, a negative control used for virus-induced gene silencing, and *LOV1* and *TRX-h5*. **A**, Leakage over a 12-h time-course following treatment with victorin at 0, 10, 31.6, 100, 316, or 1,000 ng/ml. **B**, Electrolyte leakage increases as a log dose response to victorin concentration indicating a dose-response relationship for 12 h after treatment. Means $\pm$ standard error of three samples are plotted and are representative of three independent experiments.

Fig. 2. Victorin-mediated cell death is inhibited by silencing *NbSGT1*. **A**, Leaves from plants expressing *Tobacco rattle virus* (TRV):*GFP* (control) or TRV:*NbSGT1* were inoculated (in region identified by red circles) with *Agrobacterium* containing constructs to transiently overexpress *LOV1* and *TRX-h5* or *RPP8* (control). At 72 h after inoculation, 100 ng of victorin was infiltrated into the region expressing *LOV1* and *TRX-h5*. Cell death was assessed 24 h after victorin treatment. **B**, Electrolyte leakage is reduced in leaves when *NbSGT1* is silenced. Electrolyte leakage kinetics were analyzed following victorin treatment at 31.6 ng/ml 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 h. Means $\pm$ standard error 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.

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. Levels of myc: TRX-h5 protein, which serves as an internal control, were not affected (Fig. 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 (Supplementary Fig. S1). We found that plants homozygous for the T-DNA insertion and *LOV1* were indistinguishable from wild-type plants with regard to victorin sensitivity. Therefore, we concluded that, if SGT1 is required for victorin-mediated cell death in *Arabidopsis*, AtSGT1a and AtSGT1b likely have redundant functions.

Fig. 3. Transient expression of *AtSGT1a* or *AtSGT1b* complements silenced *NbSGT1* and restores victorin-mediated cell death. **A**, Leaves from plants silenced with *Tobacco rattle virus* (TRV):*SGT1* or TRV:00 (empty-vector control) were infiltrated in the areas indicated by red circles with *Agrobacterium* to overexpress *LOV1* (LOV) and *TRX-h5* (TRX5) in combination with *GFP* (control), *AtSGT1a*, or *AtSGT1b*. Cell death was assessed 24 h after victorin treatment at 10 µg/ml. **B**, Relative abundance of LOV1 increases when *LOV1* 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.

Because *Atsgt1a/Atsgt1b* 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 whether 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 (Fig. 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 (Fig. 3A). In contrast, co-expression of myc:*AtSGT1a* or myc:*AtSGT1b* with *LOV1* and *TRX-h5* restored victorin sensitivity in *NbSGT1*-silenced plants (Fig. 3A). Western blot analysis verified the accumulation of myc:*AtSGT1a* and myc:*AtSGT1b* protein when expressed in *NbSGT1*-silenced plants (Fig. 3B). As expected, when *LOV1* is co-expressed with the *GFP* control, levels of LOV1 protein are reduced in *NbSGT1*-silenced plants compared with the control (Fig. 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 (Fig. 3B). These data demonstrate that both *AtSGT1a* and *AtSGT1b* have the capacity to function in victorin-mediated cell death and LOV1 accumulation.

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 an *N. benthamiana* mixed-elicitor cDNA (cNbME) library. The cNbME library was prepared from tissue exposed to the plant pathogens *Agrobacterium tumefaciens*, *P. syringae* pv. *tabaci*, and *P. syringae* pv. *tomato*, and to the abiotic elicitors salicylic acid (SA), jasmonic acid (JA), and ethylene. (Anand et al. 2007). TRV clones (one gene per clone) from the library were individually inoculated into duplicate *N. benthamiana* plants. 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 (Fig. 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 GFP reporter was infiltrated to identify and eliminate clones that silenced genes that interfere with transient expression. Four days following *Agrobacterium* inoculation, GFP fluorescence in the area inoculated was monitored under a UV transilluminator to verify efficient transient expression (Fig. 4A). After four rounds of screening, we identified six clones which reproducibly inhibited both victorin-mediated cell death and cell death induced by *RPP8* expression (Fig. 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 (Supplementary Fig. S2).

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 [DFCI], Boston) to identify homologous putative expressed-sequence tag (EST) contigs in *N. benthamiana* (Table 1). To confirm the DFCI annotation, the matching EST were used in a BLASTX search against The *Arabidopsis* Information Re-

source (Columbus, OH, U.S.A.) database (Table 1). The cDNAs in TRV:2G9, TRV:4A4, TRV:8D7, TRV: 12G1, TRV:38E2, and TRV:40C5 correspond to the *N. benthamiana* genes *GOX*, *GDC-T*, *GLU*, *GDC-P*, *GAPB*, and *PHT*, respectively.

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 with the control (Fig. 5). Silencing *GOX*, *GDC-P*, *GAPB*, and *PHT* inhibited cell death by approximately 80% after 8 h of victorin treatment, and silencing *GLU*

inhibited cell death by approximately 90% after 8 h of victorin treatment. Silencing *GDC-T* inhibited cell death by 60% after 8 h of victorin treatment.

Semiquantitative reverse-transcriptase polymerase chain reaction (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 (Fig. 6). 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 with the control (Fig. 6A). In all RNA samples, abundance of 18S RNA transcripts was similar and served as an internal control (Fig. 6B).

Fig. 4. Virus-induced gene silencing (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. *Tobacco rattle virus* (TRV)-infected plants were infiltrated (in the regions identified by red circles) with *Agrobacterium* containing constructs to overexpress *LOV1* and *TRX-h5*, or *RPP8*. At 48 h after *Agrobacterium* inoculation, victorin was infiltrated in the region of *LOV1* and *TRX-h5* expression. Pictures were taken 24 h after victorin treatment.

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 *R* gene activation, these genes were silenced in *N. benthamiana* expressing PTO and BS2 *R* genes (Fig. 7). As expected, transient expression of the constitutively active form of PTO (PTOY207D) resulted in cell death in leaves from wild-type *N. benthamiana* plants expressing the TRV:GFP (control) (Fig. 7A). In GOX-, GDC-T-, GLU-, GDC-P-, and GAPB-silenced plants, cell death was absent in regions of leaves expressing PTOY207D (Fig. 7A). PTO-mediated cell death was not inhibited in plants silenced for PHT (Fig. 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 (Fig. 7B).

We determined whether the loss of PTO-mediated cell death was accompanied by a reduction of PTO-mediated disease resistance (Fig. 8). GOX, GDC-T, GLU, GDC-P, and GAPB were silenced in transgenic *N. benthamiana* that expressed the PTO *R* gene (G. Martin, Boyce Thompson Institute, unpublished). As a control for the loss of resistance, PRF (NB-LRR required by Pto) 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 (1×10^4 CFU/ml) by infiltration of either compatible *P. syringae* pv. *tabaci* or incompatible *P. syringae* pv. *tabaci* carrying AvrPto. Bacterial growth was monitored 2 days after inoculation. PTO-mediated resistance was observed by the differential growth of *P. syringae* pv. *tabaci* (avrPto) in TRV:GFP expressing PTO transgenics compared with either PTO transgenics silenced for PRF or the wild type (no PTO) expressing TRV:GFP (Fig. 8A). Growth of *P. syringae* pv. *tabaci* (avrPto) in PTO plants silenced for PRF was similar to wild-type plants expressing the TRV:GFP control, demonstrating effective reduction of PTO-mediated resistance by VIGS (Fig. 8A). An approximate 10-fold increase in *P. syringae* pv. *tabaci* (avrPto) growth was observed in PTO plants silenced for GLU compared with PTO plants expressing the control TRV:GFP (*t* test, $P < 0.05$) (Fig. 8A). There was a moderate but significant fourfold increase in *P. syringae* pv. *tabaci* (avrPto) growth in PTO plants silenced for GDC-T, GDC-P, and GAPB compared with the controls (analysis of variance [ANOVA], $P < 0.05$) (Fig. 8A). Growth of *P. syringae* pv. *tabaci* (avrPto) in PTO plants silenced for GOX was similar to resistant controls (Fig. 8A), even though silencing of this gene has a consistent effect on visible cell death mediated by PTO (Fig. 7). Inconsistent results were obtained when silencing PHT over the course of four independent experiments (data not shown). Growth of compatible *P. syringae* pv. *tabaci* lacking avrPto was similar in all silenced plants, indicating that the differ-

ences seen in the growth of *P. syringae* pv. *tabaci* (avrPto) are specific to the expression of disease resistance (Fig. 8B).

DISCUSSION

To further characterize the victorin response and clarify its relationship with ETI, we used 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* in victorin-mediated cell death (Fig. 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 (Peart et al. 2002; Seo et al. 2008; Shirasu 2009). Mutation analysis and gene silencing experiments have demonstrated that *SGT1* is required for plant defenses conferred by diverse *R* genes (Austin et al. 2002; Peart et al. 2002). *SGT1* is thought to act as a co-chaperone, interacting with the co-chaperonins heat shock protein 90 (HSP90) and required for MLA12 resistance (RAR1) (Takahashi et al. 2003) in a molecular chaperon complex. Thus, *SGT1* likely functions in protein folding and stability (Seo et al. 2008; Shirasu 2009). 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 *R* proteins such as Rx (Azevedo et al. 2006; Botër 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. 2006). We show here that silencing *SGT1* results in reduced levels of LOV1 when transiently expressed in *N. benthamiana* (Fig. 2), and conclude that *SGT1* has a role in maintaining the stability of LOV1. This suggests that *SGT1* would positively regulate susceptibility to *C. 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 cinerea* 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 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 each of these isoforms is able to function in victorin-mediated cell death (Fig. 3). While it is possible that complementation re-

Table 1. Clones and their corresponding gene annotations identified as required for *RPP8* and victorin-mediated cell death in a virus-induced gene silencing screen of the cNbME library^a

TRV clone	Gene Index ID ^b	E value	Symbol	<i>Arabidopsis</i> homolog ^c	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
38E2	CN743656	1.E-04	GAPB	AT1G42970	85	Glyceraldehyde-3-phosphate dehydrogenase β subunit, chloroplast localized
40C5	TC19080	3.E-08	PHT	AT5G14040	86	Phosphate transporter 3, mitochondrial

^a Listed are the cDNAs corresponding to the Tobacco rattle virus (TRV) inserts and their predicted gene function.

^b *Nicotiana benthamiana* Gene Index, top BLASTN results.

^c Top BLASTX results.

quires overexpression of AtSGT1a or AtSGT1b, these data suggest that 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 re-

quirement for SGT1 by LOV1 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

Fig. 5. Virus-induced gene silencing of glycolate oxidase (*GOX*), the T protein of the glycine decarboxylase complex (*GDC-T*), glutamine synthetase (*GLU*), the P protein of GDC (*GDC-P*), glyceraldehyde 3-phosphate dehydrogenase (*GAPB*), and mitochondrial phosphate transporter (*PHT*) inhibits victorin-mediated electrolyte leakage. Kinetics of electrolyte leakage following victorin treatment at 31.6 ng/ml of leaf disks from *LOV1*- or *TRX-h5*-expressing leaves silenced with controls (*Tobacco rattle virus* [TRV]:*GFP* or TRV:*LOV1*) or clones identified in the genetic screen. Conductivity is reported relative to control at 8 h. Means $\pm$ standard error of three independent experiments are plotted.

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 HR mediated by RPM1; nevertheless, HSP90 is important for RPM1 resistance function (Hubert et al. 2003; Takahashi et al. 2003). Interaction between HSP90 and SGT1 is also required for Rx accumulation and function (Botër 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 with the control, indicating an inhibitory effect on transient expression (data not shown). A role for HSP90 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 Skp1–Cullin 1–F-box E3 ubiquitin ligase complex (Azevedo et al. 2002; Zhang et al. 2010), 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 an *HSP90* require-

ment 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 (Botër 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 that 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 to randomly silence genes from a mixed-elicitor cDNA (cNbME) library with VIGS. With this screen, six genes were identified that, when silenced, consistently inhibited victorin-mediated cell death. These genes included *PHT*, the β subunit of photosynthetic *GAPB*, and the

Fig. 6. Semiquantitative polymerase chain reaction (PCR) confirms reduction of transcripts in *Tobacco rattle virus* (TRV)-silenced plants. **A**, PCR products amplified with gene-specific primers from cDNA isolated from plants silenced with TRV:GFP (control) or from plants silenced with the specified TRV clones (silenced): glycolate oxidase (*GOX*), the T protein of the glycine decarboxylase complex (*GDC-T*), glutamine synthetase (*GLU*), the P protein of GDC (*GDC-P*), glyceraldehyde 3-phosphate dehydrogenase (*GAPB*), and mitochondrial phosphate transporter (*PHT*). **B**, PCR products amplified with 18S primers (as an internal control) from cDNA isolated from plants silenced with TRV:GFP and specified TRV clones.

Fig. 7. Virus-induced gene silencing (VIGS) of glycolate oxidase (*GOX*), the T protein of the glycine decarboxylase complex (*GDC-T*), glutamine synthetase (*GLU*), the P protein of GDC (*GDC-P*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPB*) (five of the six *Tobacco rattle virus* [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 *Nicotiana 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:mitochondrial phosphate transporter [*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.

photorespiratory enzymes *GOX*, *GLU*, *GDC-P*, and *GDC-T* (Figs. 4 and 5). 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 further support the inference 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 *R* 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 *GAPB* (five of the six genes identified) (Fig. 7), thus reinforcing the view that *LOV1*-mediated cell death is related to that evoked by other *R* 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 *R* genes are not unexpected. *PTO*-mediated resistance was compromised in plants silenced for *GLU*, *GDC-P*, *GDC-T*, and *GAPB*, 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 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 a Calvin cycle intermediate, phosphoglycerate, in a process which consumes energy and reducing equivalents, and results in a loss of carbon released as CO_2 . One of the photorespiratory genes we identified, *GOX*, encodes *GOX* and has been previously shown to be involved in cell death and *R*-gene-mediated resistance (Rojas et al. 2012). *GOX* catalyzes the oxidation of glycolate, derived from phosphoglycolate, to glyoxylate, producing hydrogen peroxide in the process. This reaction can be a major source of hydrogen peroxide in photosynthetic cells (Foyer 2009; Noctor et al. 2002). 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 and associates (2012) found that *VIGS* of *GOX* inhibited *PTO*-mediated cell death in *N. benthamiana*, whereas other *R*-gene-mediated cell death pathways tested were unaffected. Rojas and associates (2012) also showed that resistance of *Arabidopsis* to *P. 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 burst (Rojas et al. 2012). In *N. benthamiana* silenced for *GOX*, *PTO*-mediated resistance against *P. syringae* pv. *tabaci* was not affected (Fig. 8). This is in contrast to a 10-fold reduction in *RPS4*-mediated resistance against *P. syringae* pv. *tomato* observed by Rojas and associates (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 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 to 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 receptor that triggers resistance medi-

Fig. 8. Virus-induced gene silencing of the T protein of the glycine decarboxylase complex (*GDC-T*), glutamine synthetase (*GLU*), the P protein of GDC (*GDC-P*), and glyceraldehyde 3-phosphate dehydrogenase (*GAPB*) compromises resistance mediated by the *PTO* resistance gene in *Nicotiana benthamiana*. *Pseudomonas syringae* pv. *tabaci* (*Pstab*) growth 2 days after inoculation of either the *PTO*-transgenic or the *PTO*-transgenic silenced for the identified Tobacco rattle virus (TRV) clones or *PRF* (control) or wild-type *N. benthamiana* expressing TRV:*GFP* (control). **A**, In planta pathogen growth assay to assess 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 three independent experiments. Significantly different means ($P < 0.05$, one-way analysis of variance multiple comparison analysis) are indicated by different letters.

ated by the *Rpg4* *R* gene in soybean (Ji et al. 1998). It was hypothesized that P34 or syringolide binding to HPR inhibits HPR function, leading to *Rpg4*-mediated cell death (Okinaka et al. 2002). Interestingly, this implies that *Rpg4* guards NADH-dependent HPR, 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 oomycete pathogen *Pseudoperonospora cubensis* in the plant family *Cucurbitaceae* (Taler et al. 2004). 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. 2004). 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 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 oat occurs after the initiation of cell death when victorin gains access to the mitochondria, likely through induction of an MPT, an early event in some forms of PCD (Curtis and Wolpert 2002, 2004). In *Arabidopsis*, the NB-LRR protein, LOV1, conforms to the "guard model" of R protein function and triggers cell death when victorin binds to the guard, TRX-h5 (Lorang et al. 2007; Lorang et al. 2012). In oat, victorin sensitivity and Victoria blight susceptibility are also associated with an *R* gene (Mayama et al. 1995; Rines and Luke 1985) and physiological responses mediated by victorin in oat are similar to those of *Arabidopsis* (Wolpert et al. 2011). Therefore, the mechanism of victorin sensitivity is likely conserved between oat and *Arabidopsis* and, thus, involves *R* gene activation. Regardless, a role for the GDC has been implicated in disease resistance. The GDC has been implicated in the HR 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). Our results implicate a positive role for the GDC in the HR and defense. Therefore, binding to 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 1999b). 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 with the other genes identified in our screen (Fig. 5). In addition, the greatest reduction in *PTO*-mediated resistance was observed in plants silenced for *GLU* compared with the other genes identified in our screen (Fig. 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 oat leaves incubated under elevated CO₂ concentrations, a condition that decreases photorespiration without decreasing photosynthesis (Navarre and Wolpert 1999b). This suggests that changes in photorespiratory activity, not photosynthesis, impact victorin-mediated cell death.

Silencing the β subunit of photosynthetic *GAPB* 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. *GAPB* catalyzes a reducing step of the Calvin cycle for CO₂ fixation and, therefore, is directly involved in metabolism by its role in photosynthesis. Photosynthetic *GAPB* may also play a signaling role as a mediator of ROS signaling. Photosynthetic *GAPB* is subject to redox regulation by glutathionylation and hydrogen peroxide (Zaffagnini et al. 2007, 2012). In addition to regulating enzyme activity, oxidative modification of *GAPB* is proposed to cause structural changes that change the proteins the enzyme interacts with, as has been shown in animals (Besson-Bard et al. 2008; Hwang et al. 2009; Kim et al. 2003).

In our screen, a mitochondrial phosphate transporter (*PHT*) was implicated as involved in victorin-mediated cell death and cell death induced by *RPP8* expression. *PHT* 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, mitochondrial phosphate transporter is a component of the mitochondria permeability transition pore and is involved in the regulation of pore formation (Leung and Halestrap 2008; Varanyuwatana and Halestrap 2012). The MPT is considered an early and essential step of apoptosis. Victorin has been shown to induce an MPT in victorin-sensitive oat, which likely influences the cell death response (Curtis and Wolpert 2002, 2004). The MPT has been shown to play a role in cell death in plants, including *Arabidopsis* (Arpagaus et al. 2002; Scott and Logan 2008; Tiwari et al. 2002), demonstrating that this mechanism is likely 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 (Aken et al. 2009). Further characterization is necessary to determine whether the *PHT* identified has a role in the MPT or if the suppression that was observed in victorin-mediated cell death is due to its other roles.

From our screen of approximately 4,000 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 SA, JA, and ethylene, and the biotic elicitors *PTO*/*AvrPto*-mediated HR, *Pseudomonas syringe* pv. *tomato*

T1-mediated nonhost HR, and disease caused by *P. syringae* pv. *tabaci* (Anand et al. 2007). Therefore, it is not surprising that some of the genes identified inhibit PTO-mediated cell death, because 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 (Gabriels et al. 2007; Pozo et al. 2004). Also, victorin-mediated cell death and RPP8 function do not require SA or JA signaling components (Eulgem et al. 2004; Lorang et al. 2007), two out of the three abiotic elicitors used for library preparation. 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, because only 4,000 of an estimated 18,000 genes expressed in *N. benthamiana* (Goodin et al. 2008) were screened. Therefore, it is likely that 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 R 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 R protein RPP8. In addition, silencing most of these genes inhibited both cell death and resistance mediated by the PTO R protein. These findings, coupled with the genetic association of victorin sensitivity with the *Pc2* rust R gene in oat (Luke et al. 1966; Mayama et al. 1995; Rines and Luke 1985; Welsh et al. 1954) and our recent finding that the interaction of LOV1, TRX-h5, and victorin recapitulates the guard mechanism for R protein activation (Lorang et al. 2012), strongly suggest that *C. victoriorum* exploits R-gene mediated defense for virulence and disease susceptibility.

MATERIALS AND METHODS

Plant material and bacterial strains.

N. benthamiana or transgenic *N. benthamiana* carrying the PTO (G. Martin, unpublished) or BS2 (Leister et al. 2005) R genes were grown in 3 1/4-in. pots at 25°C in a growth chamber under a cycle of 16 h of light and 8 h of darkness. 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'-CTATCACGCCGA GTGAATCC-3' and the reverse primer 5'-TATCTGGGAATG GCGAAATC-3', and screened for the absence of the T-DNA insertion with the forward primer 5'-CTATCACGCCGAGTG AATCC-3' and reverse primer 5'-GTGCCATTGAGTTGGG TTCT-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 simple sequence length polymorphism marker (forward 5'-GTGGTGACCTCTCCCT CAAA-3' and reverse 5'-CCCACTTACCGTTTCTCTC-3') as previously described (Lorang et al. 2007).

Agrobacterium tumefaciens strain GV2260 was used for VIGS and transient expression. *Agrobacterium* was cultured in Luria-Bertani (LB) media; resuspended in 10 mM, MES, pH 5.2 (10 mM MgCl and 150 µM acetosyringone) to the indicated optical densities (ODs); and incubated for 3 h before

syringe infiltration. *P. syringae* pv. *tabaci* isolate 11528 was obtained from J. Chang, Oregon State University (Corvallis, U.S.A.). *P. syringae* pv. *tabaci* isolate 11528 containing *AvrPto* (plasmid pPtDC38) (Ronald et al. 1992) was obtained from B. Staskawicz at University California, Berkley (Berkley, U.S.A.) *P. syringae* pv. *tabaci* strains were cultured in King's medium B (King et al. 1954).

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 K. 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 G. 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, Eugene, OR, U.S.A.), 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 reverse primer 5'-AAAGGGCAGATTGTGTGGAC-3'. A 508-bp fragment of *HSP90* was amplified from cDNA isolated from *N. benthamiana* with the forward primer 5'-CACCGATCCTGAA GGTTATTCGCA-3' and the reverse primer 5'-CAAGCTTGA GACCTTCCTTG-3'. A 560-bp fragment of *RAR1* was amplified from cDNA isolated from *N. benthamiana* with the forward primer 5'-CACCCCAGAGGATCGGGTTGCAACGCC AC-3' and the reverse primer 5'-TTTCCATCCTCTCATCCG GT-3'. For TRV:*LOV1*, *LOV1* was recombined from pENTR:*LOV1* (Lorang et al. 2012) into pTRV2. The resulting pTRV2 derivatives were transformed into *A. tumefaciens*. For pTRV:00, empty Gateway-ready pTRV2 was transformed into *A. tumefaciens*.

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 (Liu et al. 2002) at an OD at 600 nm (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 in which TRV1 was infiltrated. The plants were grown for at least 2 weeks before they were used for assays.

Cell death assays.

To assay for victorin-mediated cell death, *Agrobacterium* samples carrying 35S:*LOV1* (Lorang et al. 2012) 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*. Victorin (100 ng/ml), purified as described previously (Macko et al. 1985; Wolpert et al. 1985) and diluted in water, was infiltrated 48 h after *Agrobacterium* inoculation. Cell death was visually assessed 24 h after victorin infiltration. For complementation of *NbSGT1* silencing, *Agrobacterium* samples carrying pBIN61: *myc: AtSGT1a*, pBIN61:*mycAtSGT1b* (Kadota et al. 2008) or PSJL:*GFP* diluted to OD₆₀₀ = 0.5 were mixed with *Agrobacterium* carrying 35S:*LOV* and 35S:*TRX-h5* at OD₆₀₀ = 0.5 in equal volumes. Victorin (10 µg/ml) was infiltrated 48 h after *Agrobacterium* infiltration. Cell death was visually assessed 24 h 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 subcloning 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:*PTOY207D* (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.

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. At 48 h after *Agrobacterium* infiltration, 36 4-mm leaf disks were excised from three plants silenced for each clone. The 36 disks were pooled, rinsed in 100 ml of deionized water for 30 min, then divided into six sets of 6 and placed into a 24-well multiwell plate. Three sets were treated with victorin at 31.6 ng/ml and three sets were treated with water, each in a final volume of 2.5 ml. 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 8 h.

Semiquantitative RT-PCR to analyze gene expression.

Total RNA was extracted from three silenced plants using a Qiagen RNeasy plant mini kit (Germantown, MD, U.S.A.), including an RNase-free DNase treatment step (Qiagen). First-strand cDNA was synthesized with 500 ng of total RNA, oligo d(T) primer, and Superscript III reverse transcriptase (Life Technologies). cDNA was amplified by PCR with GoTaq DNA Polymerase (Promega, Madison, WI, U.S.A.) with primers specific for the candidate cDNAs and the 18S ribosomal gene as an internal control (Supplementary Table S1). 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. For *18S*, *GOX*, *GDC-T*, *GLU*, *GDC-P*, *GAPB*, and *PHT*, 20, 20, 30, 35, 35, 35, and 30 cycles were used, respectively.

Protein analysis.

Protein extracts were prepared by grinding leaf tissue in sodium dodecyl sulfate (SDS) buffer (0.625 Tris, pH 6.8; 2% SDS; and 5% β-mercaptoethanol). For *N. benthamiana* protein samples, 0.8 cm² of tissue was ground in 125 μl. For *Arabidopsis*, 0.5 cm² tissue was ground in 125 μl. Each sample (30 μl) was loaded onto a 4 to 15% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, U.S.A.) and transferred onto Protran nitrocellulose membrane with a 0.2-μm pore size (Whatman Schleicher and Schuell, Dassel, Germany). Membranes were blocked in 5% skim milk powder in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween-20. Mouse anti-myc-HRP

antibody (Life Technologies) was used at a 1:5,000 dilution. Rabbit anti-*LOV1* antibody (Lorang et al. 2012) was used at a 1:4,000 dilution. Rabbit anti-SGS antibody (Azevedo et al. 2002) was used at a 1:7,500 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).

Monitoring of bacterial disease on plants.

To measure in planta bacterial growth, fully expanded leaves from silenced plants were syringe inoculated with *P. syringae* pv. *tabaci* strains diluted to 10⁴ CFU/ml in 10 mM MgCl₂. Two days following inoculation, nine 1.5-cm² 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 10 mM MgCl₂. The bacterial populations in the leaves were determined by plating serial dilutions on KMB supplemented with rifampicin at 100 mg/liter. Colonies were counted after 48 h 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.

ACKNOWLEDGMENTS

We thank K. Mysore for providing the cNbME library in pTRV2, TRV1, and TRV2-gateway vector; G. Martin for providing *PTO*-transgenic *N. benthamiana*, 35S:*PTOY207D*, and TRV2 derivatives; B. Staskawicz for *P. syringae* pv. *tabaci* 11528 pPtDC38; K. Shirasu for pBin61_AtSGT1a, pBin61_AtSGT1b, and anti-SGS antibody; J. Carrington for providing pSL1:GFP; J. Lorang of the Wolpert lab for help constructing pEarlyGate100:*RPP8* and reviewing the manuscript; A. 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, in part, by the Agriculture and Food Research Initiative Competitive Grants Program from the United States Department of Agriculture National Institute of Food and Agriculture grant 2008-35319-18651.

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