AN ABSTRACT OF THE DISSERTATION OF

<u>Teresa A. Sweat</u> for the degree of <u>Doctor of Philosophy</u> in <u>Molecular and Cellular Biology</u> presented on <u>March 27, 2007</u>.
Title: <u>Genetic Dissection of Victoria Blight Disease Susceptibility in *Arabidopsis thaliana*.</u>

Abstract approved:

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Thomas J. Wolpert

The fungus *Cochliobolus victoriae* causes Victoria blight of oats and *Arabidopsis* and is pathogenic due to its production of a compound called victorin, which induces programmed cell death in sensitive plants. Victorin sensitivity in *Arabidopsis* is conferred by the dominant gene *LOCUS ORCHESTRATING VICTORIN EFFECTS1* (*LOV1*), which encodes a coiled-coil-nucleotide binding site-leucine-rich repeat (CC-NB-LRR) protein, a type of protein typically associated with disease resistance. In order to better characterize the pathway leading to victorin sensitivity in *Arabidopsis*, we undertook an EMS-mutagenesis screen to identify mutants that had lost sensitivity to victorin. We isolated 63 victorin insensitive mutants, including 59 *lov1* mutants and four *locus of insensitivity to victorin1* (*liv1*) mutants. The *LIV1* gene encodes thioredoxin *h*5 (*ATTRX5*), a member of a large family of disulfide oxidoreductases. We found that the victorin response was highly specific to ATTRX5, as the closely-related ATTRX3 could only partially compensate for loss of ATTRX5, even when overexpressed. We also created chimeric ATTRX5/ATTRX3 proteins, which identified the central portion of the protein as important for conferring specificity to ATTRX5 in the victorin response. Furthermore, we found that *ATTRX5*, but not *ATTRX3*, is highly induced in sensitive *Arabidopsis* following victorin treatment. Finally, we determined that only the first of the two active site cysteine residues in ATTRX5 is required for the response to

victorin, suggesting that ATTRX5 function in the victorin pathway does not involve its redox activity.

We sequenced the *LOV1* gene from the 59 *lov1* mutants and found that the spectrum of mutations causing loss of function of LOV1 was similar to that found to cause loss of function of RPM1, a CC-NB-LRR protein with a known function in resistance. This indicates that LOV1 functions in a manner similar to resistance proteins. A survey of victorin sensitivity in 30 *Arabidopsis* ecotypes revealed that victorin sensitivity is common in worldwide populations and that there is very little genetic variation among *LOV1* alleles. The prevalence of functional *LOV1* alleles suggests that *LOV1* functions as a resistance gene to a naturally-occurring pathogen of *Arabidopsis*.

Genetic Dissection of Victoria Blight Disease Susceptibility in *Arabidopsis thaliana*

by
Teresa A. Sweat

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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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Teresa A. Sweat, Author

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CONTRIBUTION OF AUTHORS

Dr. Thomas Wolpert was involved in the design and editing of all chapters. Dr. Jennifer Lorang constructed and analyzed the plants carrying the Cl-0/Col-4 chimeric *LOV1* gene described in Chapter 3. Dr. Erica Bakker assisted in the population genetic analyses presented in Chapter 3 and in editing of Chapter 3.

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Chapter 1

Introduction

VICTORIA BLIGHT OF OATS

The fungus *Cochliobolus victoriae* is the causal agent of Victoria blight of oats (Meehan and Murphy, 1946) and is pathogenic due to production of a "toxin" called victorin, which is composed of a closely-related group of cyclized pentapeptides, of which the most prevalent is victorin C (Macko et al., 1985, Wolpert et al., 1985). Treatment with victorin alone recapitulates the symptoms of Victoria blight (Meehan and Murphy, 1947), and only host genotypes that are sensitive to victorin are susceptible to infection by *C. victoriae* (Walton, 1996; Wolpert et al., 2002). Victoria blight was first described in the 1940's and was found only on the Victoria oat variety (Meehan and Murphy, 1946), which was introduced into the United States as a source of resistance to crown rust caused by the fungus *Puccinia coronata* (Litzenberger, 1949). Sensitivity to victorin and susceptibility to Victoria blight in oats are conferred by a single dominant gene, called the *Vb* gene, and Victoria-type crown rust resistance is conferred by the dominant *Pc-2* gene. Extensive efforts, including various genetic and mutagenic approaches, have failed to separate Victoria blight susceptibility from crown rust resistance (Welsh et al., 1954; Luke et al., 1966; Rines and Luke, 1985; Mayama et al., 1995), indicating that the *Vb* and *Pc-2* genes are likely the same. In support of this conclusion, victorin has been found to induce physiological responses in sensitive plants that are typically associated with resistance, including callose production (Walton and Earle, 1985), a respiratory burst (Romanko, 1959), ethylene evolution (Shain and Wheeler, 1975), extracellular alkalization (Ullrich and Novacky, 1991), phytoalexin synthesis (Mayama et al., 1986), and K^+ efflux (Wheeler and Black, 1962). Victorin also induces programmed cell death (PCD), a form of cellular

"suicide," in sensitive plants (Navarre and Wolpert, 1999; Yao et al., 2001, 2002; Curtis and Wolpert, 2002, 2004; Coffeen and Wolpert, 2004). Interestingly, a form of PCD known as the hypersensitive response (HR) often occurs at the site of pathogen contact during the resistance reaction (Heath, 2000; Greenberg and Yao, 2004). The genetic connection between *Vb* and *Pc-2* and the similarities between the response to victorin and a typical resistance response strongly suggest that susceptibility to Victoria blight in oats is conferred by a "resistance" gene.

VICTORIA BLIGHT OF *ARABIDOPSIS*

The possibility that susceptibility to Victoria blight may be conferred by a gene that also functions in resistance opens up an intriguing avenue for study of the relationship between plant disease resistance and susceptibility. Unfortunately, identification of the *Vb/Pc-2* gene has been hampered due to the large allohexaploid genome of oats. However, victorin sensitivity has been found to occur in some accessions of the model plant *Arabidopsis thaliana* (Lorang et al., 2004). As in oats, sensitivity in *Arabidopsis* is conferred by a single dominant gene, called the *LOCUS ORCHESTRATING VICTORIN EFFECTS1* (*LOV1*) gene. Furthermore, *Arabidopsis* plants that are sensitive to victorin are also susceptible to infection by *C. victoriae* (Lorang et al., 2004). Also similar to oats, victorin induces cell death and resistance-like responses, including phytoalexin production and induction of the gene encoding the pathogenesis-related protein PR-1, in sensitive *Arabidopsis* (T.A. Sweat, unpublished results). Therefore, *Arabidopsis* presents a suitable genetic model for studying the pathway leading to Victoria blight susceptibility and its possible association with disease resistance pathways.

NB-LRR GENES AND *LOV1*

LOV1 was recently cloned and found to encode a coiled-coil-nucleotide binding site-leucine-rich repeat (CC-NB-LRR) protein (J.M. Lorang, unpublished results). The *Arabidopsis* genome encodes 149 NB-LRR proteins, including 51 CC-

NB-LRR proteins and 83 that contain a Toll/Interleukin-1 receptor (TIR) domain at the N-terminus of the protein (Meyers et al., 2003). In contrast, the rice genome encodes 480 proteins belonging the CC-NB-LRR class and no TIR-NB-LRR proteins (Zhou et al., 2004). Absence of this latter class is typical of monocot grass species (Cannon et al., 2002). Proteins of the NB-LRR class characterized to date have been almost exclusively implicated as disease resistance proteins. Therefore, in both oats and *Arabidopsis*, victorin sensitivity and Victoria blight susceptibility are likely conferred by a type of gene typically associated with resistance. It is noteworthy that the *LOV1* gene belongs to the CC-NB-LRR class, because, as mentioned above, TIR-NB-LRR proteins have not been found in grass species (Cannon et al., 2002; Zhou et al., 2004). This further supports a relationship between the mechanisms conferring victorin sensitivity in oats and *Arabidopsis*.

NB-LRR proteins are involved in conferring gene-for-gene type resistance, which occurs when a resistance gene product in the host recognizes a pathogen avirulence (Avr) gene product, thereby triggering a strong and rapid defense response, typically including an HR (Belkhadir et al., 2004; Jones and Dangl, 2006). Avr proteins are pathogen effectors that aid in virulence in the absence of the corresponding resistance protein, and it has been proposed that resistance gene products act as receptors that directly bind Avr proteins. While this has been found to be true for a few NB-LRR resistance proteins (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006), it is beginning to appear that indirect recognition of Avr proteins may be more common. This type of recognition involves a resistance protein "guarding" another host protein, which may play a role in the basal defense of the plant. When one of these host proteins is altered by contact with an Avr protein, the resistance protein recognizes the modified host protein and triggers a resistance response (Jones and Dangl, 2006). The best known example of this is the RIN4 protein, which plays a role in basal defense in *Arabidopsis* (Kim et al., 2005) and is targeted by three Avr proteins produced by *Pseudomonas syringae*. In the presence of AvrRpm1 or AvrB, RIN4 is phosphorylated, leading to the activation of the RPM1

resistance protein (Mackey et al., 2002). AvrRpt2 is a protease that cleaves RIN4, which relieves the negative regulation of RPS2 by RIN4, leading to activation of RPS2-mediated resistance (Axtell et al., 2003; Mackey et al., 2003). Another example of a "guarded" host protein is the protein kinase PBS1, which is cleaved by the *P. syringae* effector protease AvrPphB, leading to activation of RPS5 (Shao et al., 2003; Ade et al., 2007). Like LOV1, RPM1, RPS2, and RPS5 are all CC-NB-LRR proteins, and it is possible that LOV1 functions in a similar manner by recognizing alteration of a host protein as a result of victorin treatment, although it is also possible that LOV1 is activated by direct recognition of victorin. In either case, LOV1 triggers a resistance-like response, including a cell death response reminiscent of an HR, indicating a similarity between *LOV1*-mediated and resistance gene-mediated signaling.

FUNCTIONS OF NB-LRR PROTEIN DOMAINS

NB-LRR proteins contain multiple domains that work together to recognize the presence of a pathogen effector and initiate a signaling cascade leading to a resistance reaction. The LRR domain has been implicated as the portion of the protein conferring recognition specificity (Ellis et al., 1999; Dodds et al., 2001; Zhou et al., 2006) and therefore is thought to bind pathogen effectors or other upstream activators, while the N-terminal portion of the protein (CC or TIR domain) is believed to interact with downstream signaling partners (Belkhadir et al., 2004). However, this is too simplistic of a view as the LRR domain has also been implicated in downstream signaling (Warren et al., 1998; Moffett et al., 2002; Hwang and Williamson, 2003), and the N-terminal domain has in some cases been found to be responsible for conferring recognition specificity (Ellis et al., 1999). Furthermore, it is the N-terminal domains of RPM1 and RPS5 that bind to the upstream interactors RIN4 and PBS1 (Mackey et al., 2002; Ade et al., 2007). However, it is possible that the LRR is responsible for recognition of the altered forms of RIN4 and PBS1 after exposure to pathogen effectors. Therefore, protein-protein interactions at the LRR, the N-terminal

domain, or both may be involved in direct or indirect recognition of pathogen effectors.

The NB region of NB-LRR proteins is part of a larger domain called the NB-ARC domain, which is comprised of the NB, ARC1, and ARC2 domains. The NB-ARC domain is shared within a class of P-loop NTPases that includes plant resistance proteins, as well as the protein APAF-1 and its homolog in *Caenorhabditis elegans*, CED-4, which function in the initiation of programmed cell death in animals (Leipe et al., 2004). Plant NB-LRR genes share many conserved motifs within the NB-ARC domain that, through mutagenic analyses, have been found to be important for resistance gene function (Dinesh-Kumar et al., 2000; Tao et al., 2000; Tornero et al., 2002; Takken et al., 2006). It has also been shown that the NB-ARC regions of the tomato resistance proteins I-2 and Mi-1 are capable of binding and hydrolyzing ATP (Tameling et al., 2002). Through comparison with better-characterized animal P-loop NTPases, putative roles in nucleotide binding and hydrolysis can be assigned to many of the motifs within the NB-ARC domain (Leipe et al., 2004; Takken et al., 2006). In the P-loop, a conserved lysine binds the β- and γ- phosphates of the nucleotide while a conserved serine or threonine binds the associated Mg^{2+} ion. The kinase 2 motif contains two conserved acidic residues, the first of which is involved in coordination of the Mg^{2+} ion, while the second is thought to have a catalytic function required for ATP hydrolysis. The arginine of the RNBS-B motif is thought to act as a sensor of the γ-phosphate that relays information regarding the bound nucleotide to other portions of the protein. The RNBS-C and GLPL motifs contain amino acids involved in nucleotide binding, while the RNBS-A motif has an amino acid that is part of the NTPase active site and may indirectly affect nucleotide binding. Finally, the histidine of the MHD motif directly interacts with the β-phosphate of the bound nucleotide (Leipe et al., 2004; Takken et al., 2006). While these functions have not all been confirmed in plant NB-LRR proteins, there is evidence in support of the idea that the NB-ARC regions of plant and animal proteins function similarly. Binding of ATP by the tomato I-2 protein was found to require the presence of a divalent cation, such as

Mg^{2+}, and mutation of the conserved lysine of the P-loop virtually abolished the ability of I-2 to bind ATP (Tameling et al., 2002). Furthermore, mutation of the second conserved acidic residue in the kinase 2 motif of I-2 did not affect ATP binding but did cause reduced ATP hydrolysis (Tameling et al., 2006).

GENETIC ANALYSIS OF *LOV1* AND THE VICTORIN RESPONSE PATHWAY

The discovery of victorin sensitivity in *Arabidopsis* has for the first time allowed an in-depth genetic analysis of Victoria blight. The work presented in this study includes an extensive genetic screen for mutants that have lost sensitivity to victorin, and therefore susceptibility to *C. victoriae*. We undertook this screen to identify proteins other than LOV1 that are required in the victorin response pathway and also to gain better insight into the function of the LOV1 protein in this pathway. Given the discovery that *LOV1* encodes a CC-NB-LRR protein, this second objective took on added significance because our mutant analysis allowed us to determine whether the same motifs and domains that are required for resistance protein function are also required for function of LOV1, which would imply an analogous mechanism of action. In addition, we undertook a population genetics approach to survey the diversity of *LOV1* alleles in multiple *Arabidopsis* ecotypes. This allowed us to determine the prevalence of victorin sensitivity and to compare the level of diversity found among *LOV1* alleles with that observed for NB-LRR genes with known roles in resistance. The results of these studies are presented in the following two chapters.

Chapter 2

Thioredoxin *h*5 is required for victorin sensitivity mediated by a CC-NBS-LRR gene
in *Arabidopsis*

Teresa A. Sweat and Thomas J. Wolpert

ABSTRACT

The fungus *Cochliobolus victoriae* causes Victoria blight of oats and is pathogenic due to its production of victorin, which induces programmed cell death in sensitive plants. Victorin sensitivity has been identified in *Arabidopsis* and is conferred by the dominant gene *LOCUS ORCHESTRATING VICTORIN EFFECTS1* (*LOV1*), which encodes a coiled-coil-nucleotide binding site-leucine-rich repeat protein. We isolated 63 victorin insensitive mutants, including 59 *lov1* mutants and four *locus of insensitivity to victorin1* (*liv1*) mutants. The *LIV1* gene encodes thioredoxin *h*5 (*ATTRX5*), a member of a large family of disulfide oxidoreductases. To date, very few plant thioredoxins have been assigned specific, non-redundant functions. We found that the victorin response was highly specific to ATTRX5, as the closely-related ATTRX3 could only partially compensate for loss of ATTRX5, even when overexpressed. We also created chimeric ATTRX5/ATTRX3 proteins, which identified the central portion of the protein as important for conferring specificity to ATTRX5. Furthermore, we found that *ATTRX5*, but not *ATTRX3*, is highly induced in sensitive *Arabidopsis* following victorin treatment. Finally, we determined that only the first of the two active site cysteine residues in ATTRX5 is required for the response to victorin, suggesting that ATTRX5 function in the victorin pathway involves an atypical mechanism of action.

INTRODUCTION

The fungus *Cochliobolus victoriae* causes Victoria blight of oats (Meehan and Murphy, 1946). *C. victoriae* is pathogenic due to its production of victorin, a cyclized pentapeptide that acts as a host-selective toxin. Only oat genotypes that are sensitive to victorin are susceptible to *C. victoriae* infection, and treatment with victorin alone reproduces the symptoms of Victoria blight in sensitive oats (Meehan and Murphy, 1947). These symptoms include a programmed cell death response (Navarre and Wolpert, 1999; Yao et al., 2001, 2002; Curtis and Wolpert, 2002, 2004; Coffeen and Wolpert, 2004) and induction of host plant defense responses (Wheeler and Black,

1962; Shain and Wheeler, 1975; Mayama et al., 1986; Ullrich and Novacky, 1991).

Victorin sensitivity in oats is conferred by a single dominant gene named *Vb*.

Interestingly, oat genotypes that are sensitive to victorin all carry the *Pc-2* resistance gene, which confers resistance to crown rust of oats caused by the fungus *Puccinia coronata* (Litzenberger, 1949). Extensive efforts to separate resistance to crown rust from susceptibility to Victoria blight have failed (Welsh et al., 1954; Luke et al., 1966; Rines and Luke, 1985; Mayama et al., 1995), indicating that the *Vb* and *Pc-2* genes are the same.

Efforts to identify the *Vb/Pc-2* gene have been hampered due to the large allohexaploid genome of oats. However, victorin sensitivity has been identified in some accessions of the model plant, *Arabidopsis thaliana* (Lorang et al., 2004). Genetic characterization showed that, as in oats, victorin sensitivity in *Arabidopsis* is conferred by a single dominant gene designated *LOCUS ORCHESTRATING VICTORIN EFFECTS1* (*LOV1*) (Lorang et al., 2004). Treatment of sensitive *Arabidopsis* with victorin induces disease symptoms similar to those observed in sensitive oats, including cell death and induction of defense responses. Furthermore, *Arabidopsis* lines that are sensitive to victorin are susceptible to infection by *C. victoriae* (Lorang et al., 2004). Interestingly, the *LOV1* gene was recently cloned and found to encode a coiled-coil-nucleotide binding site-leucine-rich repeat (CC-NBS-LRR) protein (J.M. Lorang, unpublished results), a type of protein typically associated with disease resistance (Belkhadir et al., 2004). Therefore, in both oats and *Arabidopsis*, victorin sensitivity is apparently dependent on a resistance-like gene. Further characterization of the pathway leading to victorin-induced cell death should give insights into the intriguing relationship between plant disease resistance and susceptibility in the response to victorin.

Thioredoxins (TRXs) are typically small (~12 to 14 kDa) proteins that act as protein disulfide oxidoreductases and are found in all free-living organisms (Buchanan and Balmer, 2005). Thioredoxins contain two cysteine residues in their active site that either form a disulfide bond or exist as thiols, depending on the oxidation state of the

protein. Reduced thioredoxin can help protect cells from oxidative stress by providing reducing power to thiol-containing antioxidant proteins. Alternatively, reduction of a disulfide bond by a thioredoxin can directly activate or inactivate the target protein (Gelhaye et al., 2005; Masutani et al., 2005). In this function, thioredoxins act as redox-dependent regulators of enzyme activity. Mammals have one cytosolic thioredoxin and one mitochondrial thioredoxin, which are both essential for survival (Matsui et al., 1996; Nonn et al., 2003). In contrast, the *Arabidopsis thaliana* genome encodes 19 thioredoxins belonging to six major groups, *f*, *m*, *h*, *o*, *x*, and *y*, that are localized to various subcellular compartments, including the chloroplast, mitochondria, and cytosol (Gelhaye et al., 2005).

In *Arabidopsis*, the largest TRX group consists of the eight *h*-type thioredoxins, which are generally thought to be cytosolic proteins. The *h*-type thioredoxins have distinct but overlapping expression patterns in *Arabidopsis* (Reichheld et al., 2002), and it remains unclear what role each TRX*h* plays in maintaining cell function. Thioredoxin *h*5 (*ATTRX5*) is somewhat unique in this group in that it has been shown to be induced by biotic and abiotic stress conditions, including wounding, senescence, exposure to pathogen-derived elicitors, and oxidative stress (Reichheld et al., 2002; Laloi et al., 2004). In contrast, thioredoxin *h*3 (*ATTRX3*), the thioredoxin most closely related to *ATTRX5*, is not induced by any of these treatments. This indicates a possible unique role for ATTRX5 in responding to stress conditions. However, it has not yet been demonstrated that ATTRX5 actually functions in this regard or that this function cannot be compensated for by other TRXs. Therefore, to date, there are no specific, non-redundant functions assigned to any of the *h*-type thioredoxins in *Arabidopsis*.

In this work, we report isolation and characterization of 63 *Arabidopsis* mutants that have lost sensitivity to victorin. Genetic analyses have shown that these mutants fall into two complementation groups. One group consists of 59 independently-isolated mutants that contain mutations in the *LOV1* gene. The other group consists of four mutants that contain mutations at a separate locus, designated

LIV1 (*locus of insensitivity to victorin1*). The *liv1* mutation results in complete loss of sensitivity to victorin and susceptibility to *C. victoriae* but has no effect on the response to other phytotoxins tested or hypersensitive cell death in response to avirulent *Pseudomonas syringae* pv *tomato*. The *LIV1* locus was mapped and found to encode the *ATTRX5* gene. Further characterization showed that ATTRX5 is required for victorin sensitivity in wild-type plants, although overexpression of ATTRX3 can partially compensate for the loss of ATTRX5 in transgenic plants. Detailed promoter fusion and chimeric gene studies confirmed the specificity of ATTRX5 versus ATTRX3 in signaling for victorin sensitivity. Additionally, *ATTRX5*, but not *ATTRX3*, is induced in sensitive *Arabidopsis* following treatment with victorin.

MATERIALS AND METHODS

Plant material and growth conditions

Plant lines used were the victorin-sensitive line LOV1, derived from the ecotype Cl-0 (Lorang et al., 2004), and Col-LOV, a victorin-sensitive line that is near-isogenic to Col-4. This line was created by crossing LOV1 and Col-4, followed by eight backcrosses to Col-4, selecting for sensitivity to victorin at each generation. A sensitive F1 from the final backcross was selfed and an F2 plant homozygous for LOV1 was used to generate the Col-LOV line. The *lov1-6*, *liv1-1*, and *liv1-4* mutants were each backcrossed to their wild-type parent three times to eliminate background mutations before being used in these studies. SALK lines 144259, 039152, and 045978 were obtained from The Ohio State University Arabidopsis Biological Resource Center (ABRC). Seed for *npr1-1* (Cao et al., 1994)*, ein2* (Guzmán and Ecker, 1990)*,* and *NahG* mutants (Delaney et al., 1994) were obtained from the ABRC. Seed for the *ndr1-1* mutant (Century et al., 1995) were obtained from Dr. Brian Staskawicz (University of California, Berkeley). Plants homozygous both for *LOV1* and for either the *NahG* transgene or the *npr1-1*, *ein2*, or *ndr1-1* mutant alleles were generated by crossing each mutant to the LOV1 line and screening F2 progeny for PCR markers linked to the loci of interest. PCR markers were 3571 for *LOV1* (F

5'-GTGGTGACCTCTCCCTCAAA-3' and R 5'-CCCACTTCACCGTTTCTCTC-3'),
gene-specific primers for *ndr1-1* (F 5'-AATCTACTACGACGATGTCCAC-3' and R
5'-GTAACCGATGGCAACTTTCAC-3') and *NahG* (F 5'-CAGAAGGTATCGCCC-
AATTC-3' and R 5'-ACCTTCCAGCACATGGCTAC-3'), or markers linked to *ein2*
or *npr1-1* selected from sequence information available at The Arabidopsis
Information Resource (TAIR) website (http://www.arabidopsis.org). Individual plants
homozygous for *LOV1* and for each mutant allele were allowed to self-fertilize and
tested in the F3 generation for presence of the appropriate PCR markers. Except for
the seedling assays described below, seeds were incubated at 4ºC for 5 days in 0.1%
agarose and then applied to soil. Plants were grown under long-day conditions (16 h
light, 8 h dark) at 22ºC.

Mutagenesis and screening

For both the LOV1 and Col-LOV lines, approximately 20,000 seed were
mutagenized in 0.2% ethyl methanesulfonate (EMS) for 11 hours. For each line, 4096
M1 plants were grown from the mutagenized seed, and M2 seed was collected in 256
families of 16 M1 plants each. The M2 seed were surface sterilized in 0.5% sodium
hypochlorite for 3 minutes and rinsed 3 times with distilled water. For each family,
approximately 800 sterile seed were placed in a petri dish lined with filter paper
moistened with nutrient solution [5 mM KNO_3, 2.5 mM KH_2PO_4 (pH 6.5), 2 mM
$MgSO_4$, 2 mM $Ca(NO_3)_2$, 50 µM Fe-EDTA, 70 µM H_3BO_3, 14 µM $MnCl_2$, 0.5 µM
$CuSO_4$, 1 µM $ZnSO_4$, 0.2 µM Na_2MoO_4, 10 µM NaCl, 0.01 µM $CoCl_2$]. After 5 days
at 4ºC, the petri dishes were placed at room temperature under fluorescent lights.
Approximately 2-3 days after germination, the lids were removed and the filter paper
was soaked with 10 µg/mL victorin C, which was purified as described previously
(Macko et al., 1985; Wolpert et al., 1985). Lids were left off during the day and the
seedlings watered with distilled water as needed. Seedlings were covered overnight.
Application of victorin was repeated at about 4-day intervals until >95% of the
seedlings died or became chlorotic. At this time, generally about 2 weeks after

germination, healthy seedlings were transplanted into soil. When plants reached sufficient size, typically 2-3 weeks after transplanting, 2 leaves of each plant were infiltrated with 10 μg/mL victorin using a blunt-ended 1 mL syringe. Plants showing no symptoms 3 days after infiltration were re-infiltrated. For plants that remained healthy after another 3 days, a leaf was removed and placed in a well of a 96-well plate with 250 µl of 10 μg/mL victorin. Distilled water was added as needed and the leaves were scored for sensitivity after 3 days. Plants which showed no symptoms on the detached leaf assay were designated as victorin-insensitive mutants and further characterized as described below. Plants with significantly reduced symptoms were allowed to self-fertilize and were retested in the next generation to confirm the phenotype. However, these individuals were not further characterized in this study.

Genetic analysis of mutants

Each victorin-insensitive mutant was allowed to self-fertilize and eight plants from the next generation were infiltrated with victorin to confirm the phenotype and check for homozygosity of the mutation. Each homozygous mutant was crossed to two victorin sensitive lines, LOV1 and Col-LOV, and to the insensitive line Col-4. For each cross, at least eight F1 plants were grown and scored for sensitivity to victorin. Crosses to the sensitive lines were used to determine whether the mutations were recessive or dominant. The crosses to Col-4, which lacks a functional *LOV1* gene, allowed determination of whether the mutated loci were allelic to the *LOV1* gene. The four mutants that produced sensitive F1 progeny when crossed to Col-4, and therefore that did not fall into the *LOV1* complementation group, were crossed to each other to determine the number of additional complementation groups represented.

Treatment with *Cochliobolus victoriae*, *Pseudomonas syringae*, fumonisin, and coronatine

Spores of *C. victoriae* were prepared as previously described (Lorang et al., 2004). Washed spores were resuspended to 10^5 spores per mL in 0.01% Tween 20 and 10 µL of the suspension was applied to the center of each expanded leaf on 3-

week old plants. Plants were placed in a moist chamber with a clear lid and incubated at 25ºC under fluorescent lights for one week. *Pseudomonas syringae* pv *tomato* DC3000 containing *avrRpt2*, *avrRpm1*, or an empty vector control (pBBR1-MCS2) were obtained from Dr. Jeff Chang (Oregon State University). Cultures were grown overnight in King's B medium with 30 µg/mL kanamycin at 28ºC. Cells were harvested and resuspended in 10 mM $MgCl_2$ to a concentration of 5 x 10^7 cfu/mL. Leaves were photographed 20 hours after infiltration. Fumonisin B1 (Sigma-Aldrich; F1147) and coronatine (Sigma-Aldrich; C8115) were resuspended in methanol to 1 mg/mL. For infiltration assays, fumonisin was diluted to 20 or 100 µM and coronatine to 5 nM, 100 nM, or 1 µM in water and infiltrated into leaves of 3 ½ -week old plants. Control leaves were infiltrated with 5% methanol, which exceeds the highest concentration of methanol used in the toxin preparations. Leaves were photographed six days after infiltration.

Mapping and cloning of *LIV1*

The *LIV1* gene was mapped using 209 victorin-insensitive F2 plants from a cross between the *liv1-1* mutant, which is in the LOV1 background, and a Col-LOV plant. DNA was prepared from the F2 plants as described by Edwards et al. (1991). Initial mapping was performed using primers flanking simple sequence length polymorphisms (SSLPs) listed on the TAIR website (http://www.arabidopsis.org). The region between nga392 and nga280 was then manually scanned for short, primarily di- and tri- nucleotide, repeats. Primers were designed flanking each repeat, and the new markers were tested for polymorphisms between the two parent lines (Table 2.1). These new SSLPs were used to map the *liv1-1* mutation to a 50 kb region between markers 17.0ssr3 and 17.1ssr1 on BAC F27F5.

BAC clone F27F5 was obtained from the ABRC. The BAC DNA was digested with *Sac*I or *Sal*I and overlapping 25 kb *Sac*I and 21 kb *Sal*I fragments spanning the majority of the region between 17.0ssr3 and 17.1ssr1 were ligated into the *Sac*I and *Xho*I (compatible with *Sal*I overhang) sites of the binary vector

Table 2.1. Polymorphic SSLP markers used to map the *LIV1* gene.

Marker	Position (kb)[a]	Forward primer (5'→3')	Reverse primer (5'→3')
12.5ssr2	12511.2	TCAACCTGAAAACCCCGTTA	AAACTCTGTGCGGGACCTTT
13.0ssr2	13048.6	AAGAATCTGCACTGCCAAAGA	GGTGGAAAGATTGGAAATGC
13.3ssr1	13303.2	GGATCATGAATCTCCATTCTCTG	GATAAATATCGGCGCGACAC
13.8ssr3	13853.5	TTCCATTGAAGTAGACGCAAC	ATTTCCCTGGGTAGGCATTT
15.8ssr1	15797.5	CTCGATCAGTAGTAATTTTCTCG	ATGATTTATATACACTTCCAATTTTCT
16.5ssr1	16510.6	AACCTTTCTCGTTGATTTCCAA	CCAAGCAAGAAGGCAAAATC
16.6ssr1	16604.2	GAAGTAATCAAACATGCAACTCAA	TGATCTCCAAGGGATAAACGA
16.7ssr2	16741.9	ATGGCGATGATGACAACAAA	GGTGGAATCGAATGGAAGAA
16.8ssr2	16847.7	CGTGTCCTAATATTTAAGGAATCCA	CCATGCAAGTTTTCCAATGA
16.9ssr3	16922.4	TCAATTCTACAAGAAAAATGCTGA	GCCCATATAATGTGCATCACG
17.0ssr2	17025.5	TGTGTTGAATAGCACACTGATGA	ACTCGCTTTGGAAGGCACTA
17.0ssr3	17052.8	CACTACAATAAAGAGCAAGTACGTTT	TCAAAGAAATCCATGAAACCAA
17.0ssr4	17067.1	AATTAATGGAAATCGAAGAAAAA	CCGAGTTGGGACCAGTGTAA
17.1ssr1	17104.3	GGGGGATAGAGAGAACAGGAG	CGATTAGTTTAGCGGTGTATGAGA
17.2ssr1	17214.7	GTGTTTGCCGGAATCATCTT	CCTTGAGACAAAAAGACCTCCA
17.3ssr1	17282.0	TACTGACGGGGAAATCTTCG	TCACAAAATCCAAACAAATCTCC
17.4ssr1	17379.9	GAACAAATACAAATTTAAGCAAACAAA	CGATCTCGAGTTGAGACCAGT
17.4ssr2	17400.6	ATTGTCCCTCCTCGTTTCTT	ACATTTTTAGTATCGAAAATTACTCAA
18.7ssr2	18751.9	TTCAGGCCCAACTTTATTTGA	GGGTAAAAATTTGGGGGAAT
19.8ssr2	19860.9	CACGGCCCATTAGTGTTCTT	ACGGAGACGAACAGGAGACA

[a]Position denotes distance in kilobases from the North end of Chromosome I.

pCLD04541 (Bent et al., 1994). The ligated DNA was electroporated into One Shot GeneHogs Electrocomp Cells (Invitrogen). Clones carrying the correct insert as determined by restriction digest were transformed into *Agrobacterium tumefaciens* strain GV3101. Plants containing the *liv1-1* mutation were transformed using the floral dip method (Clough and Bent, 1998). Seed from the dipped plants was collected and surface sterilized as described above. The seed was plated on nutrient agar made from the nutrient solution described above supplemented with 100 µg/mL kanamycin and 100 µg/ml cefatoxamine. After 5 days at 4ºC, the plates were placed at room temperature under constant light. After 1 week, surviving seedlings with well-developed roots were transplanted to soil. Presence of the transgene was confirmed by PCR and the transgenic plants were scored for sensitivity to victorin by the detached leaf assay. For all transgenic constructs used in this study, at least twenty T0 transgenic plants and at least eight T1 plants from each of eight transgenic lines (64 total T1 plants) were evaluated for their response to victorin, with the exception of the *ATTRX5/ATTRX3* chimeric construct plants (see below), which were only evaluated in the T0 generation.

The *ATTRX5* gene, including approximately 1.3 kb upstream of the start codon, was amplified by PCR from the cloned *Sac*I fragment with the primers 5'-CAGGTCAGCTTCATCTTCTCTTG-3' and 5'-ACACTCTCGGTTAGCCCTAAG-TT-3' using Platinum *Pfx* polymerase (Invitrogen). The resulting product was tailed with an A overhang by addition of Taq and incubation for 10 minutes at 72ºC. The product was cloned into pCR 2.1-TOPO using the TOPO TA cloning kit (Invitrogen). The thioredoxin gene was excised from the TOPO vector with *Sac*I and *Xho*I and cloned into pCLD04541. Transgenic plants were generated and screened as described above.

Identification of the mutated nucleotides in the *liv1* mutants

Genomic DNA was prepared from each *liv1* mutant and the two exons of *ATTRX5* were PCR-amplified using the following primers: Exon1 F, 5'-AAAAGCT-

GATCCCAACAAGAA-3'; Exon1 R, 5'-CCCTAGAGAGGAGAAGAAGAGAAAA-3'; Exon2 F, 5'-TCTTGTTATGTCCAGGGCTTTT-3'; Exon2 R, 5'-TTTTCGTGTT-CGTGGTTGAA-3'. At least two independently generated PCR products from each mutant were sequenced.

Characterization of SALK *attrx5* mutant

SALK line 144259 was obtained from the ABRC. Plants were screened for presence of the T-DNA insertion using shortened forms of the left border primers LBa1 (5'-TGGTTCACGTAGTGGGCCAT-3') or LBb1 (5'-GTGGACCGCTTGCT-GCAACT-3') in combination with the *ATTRX5* Exon2 F primer (see above). A plant carrying the mutant allele was crossed to the LOV1 line. F2 plants from this cross were screened for presence of the T-DNA insertion. Plants carrying the insertion were then checked for presence of a wild-type allele using the *ATTRX5* Exon2 F and Exon2 R primers. Plants lacking a wild-type allele, and therefore presumed to be homozygous for the insertion mutation, were screened for the presence of a functional *LOV1* gene using an SSLP tightly linked to *LOV1*, amplified with the primers 3571 F 5'-GTGGTGACCTCTCCCTCAAA-3' and R 5'-CCCACTTCACCGTTTCTCTC-3'. Individuals homozygous for both the *attrx5* insertion allele and the *LOV1* gene were tested for victorin sensitivity by the detached leaf assay.

NADPH-dependent thioredoxin reductase mutants

SALK insertion lines for *NTRA* (SALK 039152) and *NTRB* (SALK 045978) were obtained from the ABRC and screened for the presence of the T-DNA insertion with LBa1 or LBb1 in combination with NTRA R (5'-CGCCCTAAACGTATCCCT-CCT-3') or NTRB F (5'-TCGGAGCGATTCGGTACTACG-3') primers. A plant carrying the insertion allele from each line was crossed to the LOV1 line. The F2 plants were screened for presence of the insertion as described above and for lack of a wild-type allele using the flanking primers NTRA F (5'-CAAATCCGCCGTCTCTA-GCC-3') and NTRA R or NTRB F and NTRB R (5'-GACAAGCCATAGGGTCACA-

GAGC-3'). Plants homozygous for the insertion allele were screened for the presence of the *LOV1* gene as described above, and plants homozygous both for *LOV1* and for each insertion mutant were screened for victorin sensitivity by the detached leaf assay. Homozygous plants were allowed to self and the genotypes and phenotypes were confirmed in the next generation. To obtain the *ntra ntrb* double mutant plants, a *LOV1 ntra* plant was crossed with a *LOV1 ntrb* plant. The F2 plants were screened for presence of each insertion allele and absence of each wild-type allele as described above.

Creation of pEarleyGate overexpression constructs

Clones of *ATTRX5* (stock #U09186) and *ATTRX3* (stock #U16645) cDNAs were obtained from the ABRC. Each cDNA was amplified by PCR and cloned into pENTR/D-TOPO (Invitrogen) using the following primers: *ATTRX5* ENTR F, 5'-CACCATGGCCGGTGAAGGAGA-3'; *ATTRX5* ENTR R, 5'-TCAAGCAGAAGCT-ACAAGACCA-3'; *ATTRX3* ENTR F, 5'-CACCATGGCCGCAGAAGGAG-3'; *ATTRX3* ENTR R, 5'-TCAAGCAGCAGCAACAACTGT-3'. Each pENTR clone was digested with *Nsi*I and the fragment containing the cDNA was gel-purified and recombined into the binary vector pEarleyGate 100 (Earley et al., 2006) using Gateway LR Clonase II Enzyme Mix (Invitrogen) according to the manufacturer's instructions. The recombination reactions were transformed into *E. coli* strain DH5α and the resulting pEarleyGate clones were introduced into *Agrobacterium* strain GV3101. Plants containing the *attrx5-1* mutation were transformed as described above. Putative transgenic seed were planted in soil wet with 0.02% glufosinate-ammonium.

Mutagenesis of active site cysteine residues

The active site cysteine residues were mutated both individually and in combination to serine residues using the Quik-Change II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. The following primers

and their reverse complements were used for the mutagenesis reactions: C39S, 5'-CTTCACAGCATCATGGAGTCCACCTTGCCG-3'; C42S, 5'-CATGGTGTCCAC-CTAGCCGTTTCATTGC-3'; C39S/C42S, 5'- CACAGCATCATGGAGTCCACCT-AGCCGTTTCATTGC-3'. The *ATTRX5* cDNA pENTR construct was used as template for the mutagenesis reactions. Successfully mutagenized clones were digested with *Nsi*I and recombined into pEarleyGate 100. Clones were confirmed by sequencing and used to transform *attrx5-1* plants as described above.

Creation of *ATTRX5/ATTRX3* gene fusions

The Quik-Change II Site-Directed Mutagenesis Kit (Stratagene) was used to mutate nucleotides in the *ATTRX5* and *ATTRX3* cDNAs in pENTR to create restriction sites for splicing the cDNAs. The nucleotide changes did not affect the amino acid sequence at those sites. The following primers and their reverse complements were used to introduce the indicated restriction sites: *ATTRX5 Ban*I, 5'-CACAGCATCAT-GGTGCCCACCTTGCCG-3'; *ATTRX3 Eco*RI, 5'-GAACACTGTTGCTGAGGAAT-TCAAAGTTCAGGCAATGCC-3'; *ATTRX5 Kas*I, 5'-GATCGTGTTGTCGGCGCC-GCGAAAGATGAGATC-3'; *ATTRX3 Kas*I, 5'-CAAGGAGACTGTGGTTGGCGC-CGCTAAAGAAGAAATC-3'. Each cDNA clone in pENTR was digested with *Not*I and either *Ban*I, *Eco*RI, or *Kas*I to remove a 5' fragment that was replaced by the corresponding fragment from the other thioredoxin. The chimeric cDNAs were digested with *Nsi*I and recombined into pEarleyGate 100. Clones were confirmed by sequencing and used to transform *attrx5-1* plants as described above.

Creation of *ATTRX5* and *ATTRX3* promoter fusions

The *ATTRX3* promoter was amplified from Col-4 genomic DNA using the forward primer 5'-CACCAGATGCGGTTGTTGATG-3' and the reverse primer 5'-CCAGTTTCTTGGATTCGTTGG-3' and the product was cloned into pENTR/D-TOPO (Invitrogen). The resulting clone was digested with *Not*I and *Hae*III and the fragment containing the promoter was gel purified. The *ATTRX5* cDNA and the six

ATTRX5/*ATTRX3* chimeric cDNAs in pENTR were digested with *Hae*III and *Asc*I and the fragment containing the cDNA was gel purified. The pENTR vector was digested with *Not*I and *Asc*I and gel purified. These 3 fragments were mixed in a 3-way ligation resulting in each cDNA cloned downstream of the *ATTRX3* promoter in pENTR. These clones were digested with *Nsi*I and recombined into pEarleyGate 303 (Earley et al., 2006). Because the cDNAs included stop codons, the C-terminal myc tag present in pEarleyGate 303 was not translatable. The resulting *ATTRX3* promoter clones were verified by sequencing and used to transform *attrx5-1* plants.

The *ATTRX5* gene including the promoter region was amplified from the genomic *ATTRX5* construct in pCLD04541 described above using the forward primer 5'-CACCTCTCGGTTAGCCCTAAGTT-3' and the reverse primer 5'-AGCAGAAG-CTACAAGACCACC-3' and cloned into pENTR/D-TOPO (Invitrogen). The pENTR clone was digested with *Hind*III and *Asc*I to remove the coding region while leaving the promoter attached to the pENTR vector backbone. The *ATTRX3* and *ATTRX5*/*ATTRX3* chimeric cDNAs in pEarleyGate 100 were each amplified using a forward primer that added nucleotides corresponding to the 3' end of the *ATTRX5* promoter to the PCR product (including the *Hind*III site just upstream of the start codon). The reverse primer was made to the sequence of pEarleyGate 100 downstream of the *Asc*I site. The forward primer for fusions containing *ATTRX5* coding sequence at the 5'end was 5'-TCTTAAAAGCTTAAGAACAAATAATAAA-AATGGCCGGTGAAGGAGA-3'. The forward primer for *ATTRX3* and fusions containing *ATTRX3* coding sequence at the 5'end was 5'- TCTTAAAAGCTTAAGA-ACAAATAATAAAAATGGCCGCAGAAGGAGA-3'. The reverse primer for all clones was 5'-CTAGACTCACCTAGGCACCACTTTG-3'. The PCR products were digested with *Hind*III and *Asc*I and ligated to the *Hind*III/*Asc*I cut *ATTRX5* promoter in pENTR described above. The resulting cDNA clones under the *ATTRX5* promoter were digested with *Nsi*I and recombined into pEarleyGate 303. The resulting clones were confirmed by sequencing and used to transform *attrx5-1* mutant plants as described above.

RNA gel blot analysis

For time-course experiments, detached leaves from 3-week old plants were infiltrated with 30 μg/mL victorin and incubated in the dark in a petri dish lined with moistened filter paper floating in a 25°C water bath. At the indicated times, leaves were frozen in liquid nitrogen and stored at -80°C until processing. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). RNA (5 μg per lane) was separated on a 1.2% agarose MOPS/formaldehyde gel and blotted onto Hybond-N+ membrane (Amersham Biosciences). Gene-specific probes for *ATTRX5* and *ATTRX3* were made using the 3'UTRs of each gene. These regions were PCR-amplified from genomic DNA using the following primers: *ATTRX5* 3'UTR F 5'-GATGAAGCATG-GTGGTCTTG-3' and R 5'-TTTTCGTGTTCGTGGTTGAA-3'; *ATTRX3* 3'UTR F 5'-CGAGAAGCACAAGACAGTTG-3' and R 5'-GCATAGCTGCGAGTAATCA-AG-3'. For analysis of transgene expression, RNA was isolated from untreated plants. Transgene probes were made from the 3'UTR region of the pEarleyGate 100 vector (Earley et al., 2006), using the *ATTRX5* or *ATTRX3* 3'UTR F primer in combination with the pEarley R primer, 5'-GATCTGAGCTACACATGCTC-3'. Probes were synthesized using the Strip-EZ DNA labeling kit (Ambion). This kit was also used to strip blots for reuse. Blots were hybridized in Church's buffer at 65°C.

Accession numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *ATTRX5*, At1g45145; *ATTRX3*, At5g42980; *NTRA*, At2g17420; and *NTRB*, At4g11610.

RESULTS

Isolation and genetic characterization of victorin-insensitive mutants

Seed from the victorin-sensitive lines LOV1, which was derived from the sensitive ecotype Cl-0, and Col-LOV, a line near-isogenic to Col-4 into which the *LOV1* gene has been introgressed, were mutagenized in 0.2% EMS. The mutagenized

seed were used to grow 4,096 M1 plants for each line. The M1 plants were allowed to self and M2 seed was collected from families of 16 M1 plants each for a total of 256 seed pools per line. At least 800 seedlings per pool were screened for loss of victorin sensitivity by watering seedlings germinated on filter paper with a solution of victorin. All 256 LOV1 families and 10 families of Col-LOV were screened, for a total of approximately 212,000 seedlings screened. The remaining Col-LOV families were not screened due to apparent saturation of the mutant screen with regard to the number of mutated loci.

Seedlings that survived the initial screen were transplanted to soil and further tested by infiltrating leaves of the mature plants with victorin. Only about 1-2% of transplanted seedlings were found to be insensitive to victorin upon secondary screening. This result was not surprising given that seedlings are much less sensitive to victorin than adult plants, allowing many individuals to escape the initial screen. As a result of the secondary screen, 63 independent victorin-insensitive mutants were isolated (mutants were considered independent if they were isolated from different families), of which 61 were in the LOV1 background while 2 were in the Col-LOV background. Each mutant was backcrossed to its sensitive parent and the F1 progeny were scored for victorin sensitivity. All F1 progeny showed restoration of victorin sensitivity, demonstrating that all 63 mutations are recessive. Complementation tests showed that 59 mutants carry mutations in the *LOV1* gene (Lorang et al., 2004), while four mutants form a second complementation group. These four were designated as *locus of insensitivity to victorin* (*liv1*) mutants (*liv1-1* to *liv1-4*). The *lov1-1* and *liv1-4* mutants were in the Col-LOV background, while all other mutants were isolated in the LOV1 background.

All the *lov1* and *liv1* mutants characterized in this study showed complete loss of sensitivity to victorin (Figure 2.1A, top). To determine whether this corresponds to loss of susceptibility to infection by *C. victoriae*, the fungus that produces victorin, we inoculated wild-type LOV1 and the mutants *lov1-6* and *liv1-1* with *C. victoriae* by placing a droplet of spores in the center of each leaf and placing the plants in a moist

Figure 2.1. Response of victorin-insensitive mutants to toxin exposure or pathogen infection.

A) Leaves from wild-type LOV1, *lov1-6,* and *liv1-1* plants photographed 3 days after treatment with victorin (top) or 7 days after inoculation with *C. victoriae* (bottom). $n \geq 50$ leaves per line for infection assays. B) Leaves from wild-type Col-LOV or *liv1-4* plants photographed 20 hours after infiltration with virulent *P. syringae* pv *tomato* or *Pst* carrying *avrRpm1* or *avrRpt2*. $n \geq 48$ leaves per treatment per plant line. C) Leaves from wild-type LOV1 or *liv1-1* plants photographed 6 days after infiltration with 5% methanol (left) or with 100 nM coronatine (top right) or 20 µM fumonisin (bottom right). The underside of the leaves in the top row were photographed to show accumulation of anthocyanin. $n \geq 30$ leaves per treatment per line.

A
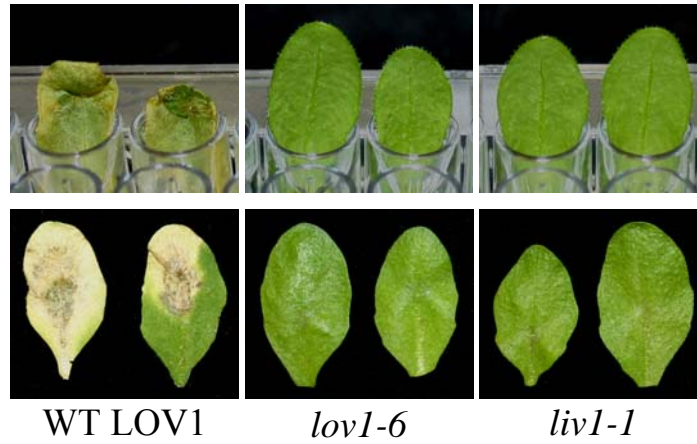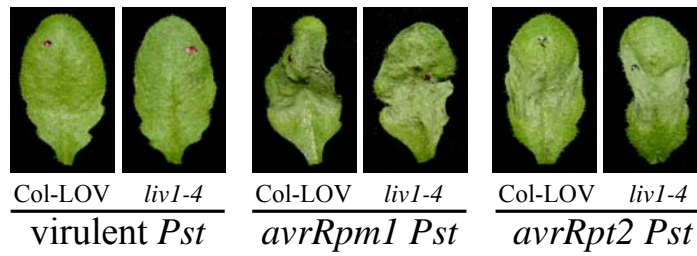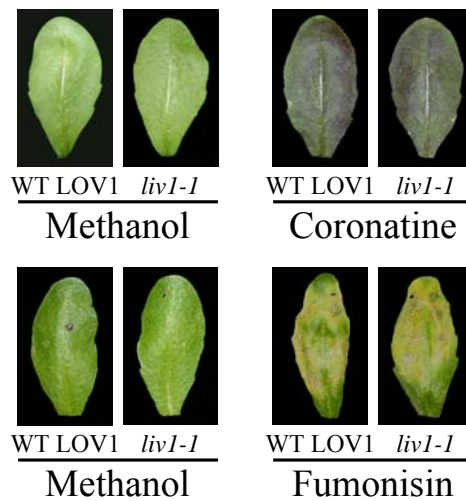
WT LOV1   *lov1-6*   *liv1-1*

B

Col-LOV   *liv1-4*   Col-LOV   *liv1-4*   Col-LOV   *liv1-4*

virulent *Pst*   *avrRpm1 Pst*   *avrRpt2 Pst*

C

WT LOV1   *liv1-1*   WT LOV1   *liv1-1*

Methanol   Coronatine

WT LOV1   *liv1-1*   WT LOV1   *liv1-1*

Methanol   Fumonisin

Figure 2.1. Response of victorin-insensitive mutants to toxin exposure or pathogen infection.

chamber for one week. Infection levels on the LOV1 plants were variable with all
older leaves (third and fourth true leaves) showing severe symptoms (Figure 2.1A,
bottom left) and younger leaves showing variable degrees of infection. No infection
was observed on any of the leaves of either the *lov1-6* or *liv1-1* plants (Figure 2.1A,
bottom right), indicating that, as in oats (Walton, 1996; Wolpert et al., 2002), there is a
strict correlation between sensitivity to victorin and susceptibility to *C. victoriae*
infection in *Arabidopsis*.

We also wanted to determine whether the *liv1* mutation affects the response to
other toxins or cell death in general. We tested the affect of mutation of *liv1* on cell
death associated with the hypersensitive response (HR) by infiltrating wild-type and
mutant plants with *Pseudomonas syringae* pv *tomato* (*Pst*) carrying the avirulence
genes *avrRpt2* or *avrRpm1* or an empty vector (virulent control). For these studies, we
used the *liv1-4* mutant because it was isolated in the Col-LOV background, which is
known to carry the corresponding resistance genes *RPM1* and *RPS2*. The *liv1-4*
mutant was indistinguishable from wild-type with regard to its response to infiltration
with *Pst* (Figure 2.1B). Nearly all inoculated leaves of both the wild-type and mutant
lines developed an HR to *Pst avrRpm1* by six hours, and an HR to *Pst avrRpt2* by
twenty hours. There was no apparent difference in either the timing or extent of the
HR between the wild-type and mutant plants. The virulent controls showed no
symptoms within this timeframe in either Col-LOV or *liv1-4* plants. The HR assays
were performed twice with equivalent results. We also looked at symptom
development in response to infiltration with the toxins coronatine, produced by *P.
syringae*, and fumonisin B1, produced by the fungus *Fusarium moniliforme*. For these
studies, we compared both LOV1 versus *liv1-1* and Col-LOV versus *liv1-4*. In all
cases, the mutant and corresponding wild-type line showed identical symptoms.
Coronatine (100 nM) caused accumulation of anthocyanin pigments on the underside
of all treated leaves (Figure 2.1C, top). We also tested coronatine at 5 nM and 1 μM.
At 5 nM, none of the lines tested showed any symptoms after one week, while at 1
μM, treated leaves showed anthocyanin accumulation on both the top and bottom of

infiltrated leaves by day 6. Treatment with 20 μM fumonisin caused development of necrotic lesions and chlorosis on all treated leaves (Figure 2.1C, bottom), while treatment with 100 μM fumonisin caused complete necrosis of all treated leaves on all lines tested. Both wild-type and mutant plants also showed necrotic lesions on systemic leaves after treatment with fumonisin. These results indicate that mutation of *LIV1* does not have a general effect on cell death or toxin response pathways. The affect of mutation of the *LOV1* gene was not evaluated because the Col-0 ecotype carries a pseudogene at this locus (J.M. Lorang, unpublished results), and therefore *LOV1* is clearly not required for *Pst*-induced HR, or the response to fumonisin B1 or coronatine, as these pathways have all been characterized in Col-0 plants.

Mapping and cloning of the *LIV1* gene

The *liv1-1* mutant, which was identified in the LOV1 background (ecotype Cl-0), was crossed to a wild-type Col-LOV plant to create a segregating population. Approximately 800 F2 plants were scored for sensitivity to victorin. These plants showed the expected ratio of 3 sensitive:1 insensitive predicted by Mendelian inheritance of a single recessive mutation. The 209 insensitive plants were used for mapping *LIV1* by PCR amplification of simple sequence length polymorphisms (SSLPs). Initial rough mapping placed the *LIV1* gene in the central portion of Chromosome I in a 31.3 cM region between nga392 and nga280 (Figure 2.2). To facilitate fine-mapping, new SSLPs were developed in this region (Table 2.1). The new polymorphic markers were then used to narrow the region containing *LIV1* to an approximately 0.4 cM (~50 kb) region between 17,053 and 17,104 kb from the North end of Chromosome I. This region was completely contained on BAC F27F5. Overlapping *Sac*I and *Sal*I fragments spanning the majority of the 50 kb region were subcloned from BAC F27F5 and introduced into the *liv1-1* mutant by *Agrobacterium*-mediated transformation. Transgenic plants were tested for victorin sensitivity by the detached leaf assay, in which a leaf is placed in a well of a 96-well plate with 250 μL of 10 μg/mL victorin. The 25 kb *Sac*I fragment was found to restore victorin

Figure 2.2. Map-based cloning of *LIV1*.

LIV1 was mapped to a 50 kb region flanked by the SSLP markers 17.0ssr3 and 17.1ssr1. Marker positions are indicated in kilobases from the north end of Chromosome I. The relative positions of the subclones spanning this region and the structure of the *ATTRX5* gene are shown. Note the orientation of *ATTRX5* is reversed with respect to the diagram of the *LIV1* region. Arrows indicate positions of EMS-generated point mutations. The *attrx5-4* allele is a SALK T-DNA insertion allele.

sensitivity to the *liv1-1* mutant. This fragment contained four annotated genes, two of which were classified as retrotransposons. The other two genes were a ß-galactosidase and a cytosolic thioredoxin. Because the thioredoxin was the most likely candidate, this gene was PCR-amplified from the *Sac*I subclone, including approximately 1.3 kb upstream of the start codon and 400 bp downstream of the stop codon. The cloned thioredoxin gene was found to restore victorin sensitivity when introduced into the *liv1-1* mutant (Figure 2.3A). This thioredoxin belongs to the *h*-type family of thioredoxins, which are thought to be localized to the cytosol. This gene has been designated as thioredoxin *h*5 (Rivera-Madrid et al., 1995), and will henceforth be referred to as *ATTRX5*. An *ATTRX5* cDNA expressed under control of the *35S* promoter was also able to restore victorin sensitivity to *liv1-1* plants (Figure 2.3C, bottom left). Overexpression of *ATTRX5* did not cause an apparent difference in the timing or extent of symptom development in comparison to wild-type plants, suggesting that ATTRX5 is not the rate-limiting factor in the induction of victorin-induced cell death. Both *liv1* mutant plants and plants overexpressing *ATTRX5* showed wild-type morphology and development under our growth conditions.

Identification of the mutations in the *liv1* mutants

Of the four *liv1* mutants, three were in the LOV1 background and one (*liv1-4*) was in the Col-LOV background. The two exons of *ATTRX5* were PCR-amplified from genomic DNA from each *liv1* mutant and from the LOV1 and Col-LOV parents. Sequencing of the LOV1 and Col-LOV *ATTRX5* exons showed no differences from the published *Arabidopsis* Col-0 genomic sequence. In contrast, each of the *liv1* mutants showed a single nucleotide change in the *ATTRX5* gene (Figure 2.2). The mutations were all G to A transition mutations, as is expected for EMS mutagenesis, and each mutation was confirmed by sequencing a second, independently generated PCR product. The *liv1-1* and *liv1-3* mutants were both found to have a mutation of the invariant G at the first nucleotide of the sole *ATTRX5* intron, presumably causing a disruption of the splicing of exons 1 and 2. This allele was named *attrx5-1*. The

Figure 2.3. Victorin sensitivity phenotypes of plants expressing wild-type or mutant *ATTRX5*, overexpressing *ATTRX3*, or mutant for the NADPH-dependent thioredoxin reductase genes *NTRA* and *NTRB*.

Detached leaves from indicated plant genotypes were treated with 10 µg/mL victorin or water. For A), C), and D), at least eight plants from each of eight T1 lines (64 plants total) were scored for sensitivity to victorin. A) Leaves from wild-type LOV1 plants, *liv1-1* mutant plants, or *liv1-1* T1 transgenics transformed with a genomic clone of *ATTRX5* photographed 2 days after treatment with victorin. B) Leaves from plants carrying the *LOV1* gene and mutant for either *ntra* or *ntrb* or both photographed 2 days after treatment with victorin. $n \geq 20$ leaves per genotype. C) Leaves from T1 transgenics of *liv1-1* plants transformed with *35S:ATTRX5*, *35S:ATTRX5*(C42S), *35S:ATTRX5*(C39S), or *35S:ATTRX5*(C39S/C42S) constructs photographed 3 days after treatment with water or victorin. D) Leaves from wild-type LOV1 plants or *liv1-1* T1 transgenics transformed with a *35S:ATTRX3* construct photographed 2 days after treatment with victorin.
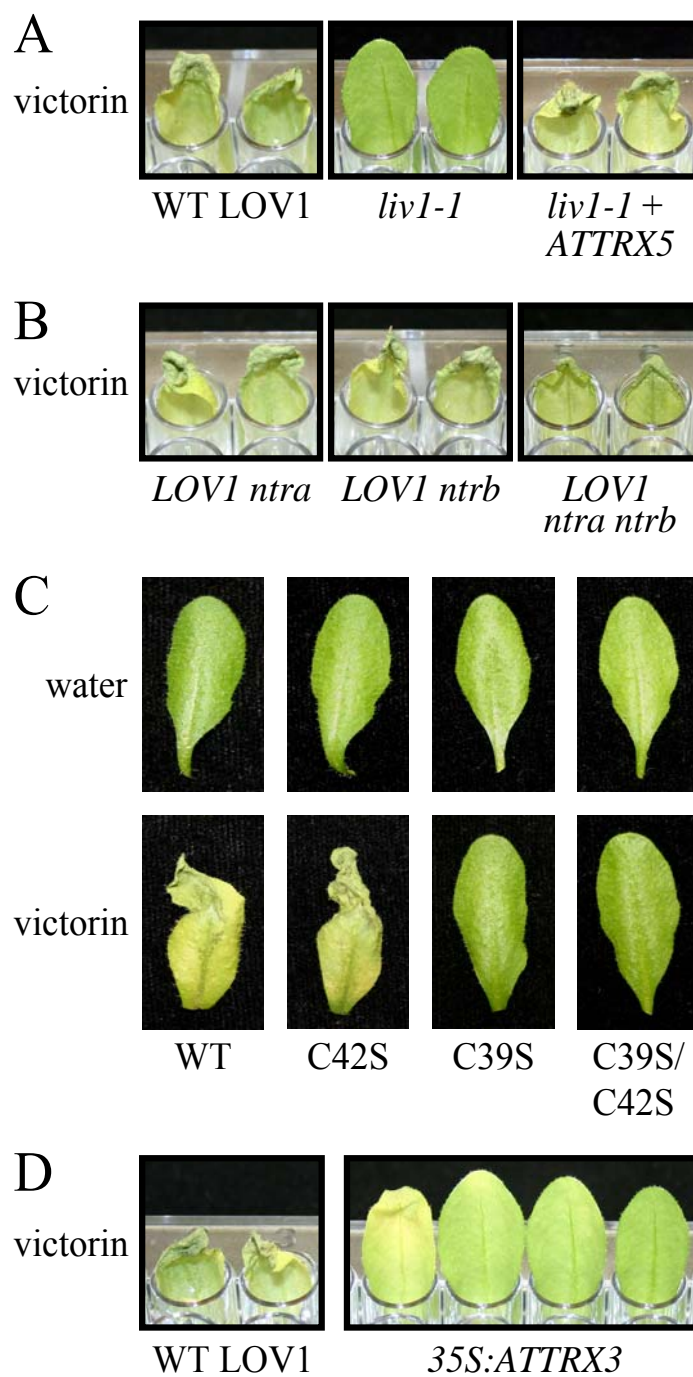
A

victorin

WT LOV1   *liv1-1*   *liv1-1 + ATTRX5*

B

victorin

LOV1 ntra   *LOV1 ntrb*   *LOV1 ntra ntrb*

C

water

victorin

WT   C42S   C39S   C39S/ C42S

D

victorin

WT LOV1   *35S:ATTRX3*

Figure 2.3. Victorin sensitivity phenotypes of plants expressing wild-type or mutant *ATTRX5*, overexpressing *ATTRX3*, or mutant for the NADPH-dependent thioredoxin reductase genes *NTRA* and *NTRB*.

mutation in *liv1-2* is a missense mutation that converts Arg-43 to a cysteine. This additional cysteine is adjacent to the conserved active site WCPPC at amino acids 38 to 42 and may interfere with active site function. This allele was designated *attrx5-2*. The *liv1-4* mutant was found to have a nonsense mutation in exon 1 of *ATTRX5*. This converts the TGG codon for Trp-16 to a TGA stop codon. This allele was designated *attrx5-3*. In addition, a SALK mutant (SALK 144259) was obtained that contained a T-DNA insertion near the end of the second exon of the *ATTRX5* coding sequence (Figure 2.2). This mutant was crossed to a wild-type LOV1 plant. The F1 plants, which were heterozygous both for *LOV1* and the insertion mutation, retained sensitivity to victorin, as is expected for a recessive mutation. However, in the segregating F2 population, all plants that were homozygous for the T-DNA insertion were completely insensitive to victorin, regardless of their genotype at the *LOV1* locus (data not shown). This indicates that this mutant allele, designated as *attrx5-4*, also results in complete loss of victorin sensitivity.

Requirement for NADPH-dependent thioredoxin reductases

Because thioredoxins generally function by reducing other proteins, a system is required to maintain the pool of reduced thioredoxin in the cell. In the cytosol, this function is performed by NADPH-dependent thioredoxin reductases (NTRs) (Florencio et al., 1988). The *Arabidopsis* genome encodes two NTR genes, *NTRA* and *NTRB*, that each encode two different mRNAs, a long transcript that encodes a mitochondrial NTR and a short transcript that encodes a cytosolic isoform (Laloi et al., 2001; Reichheld et al., 2005). However, NTRA was found to be the predominant isoform in the cytosol, whereas NTRB acts as the major mitochondrial NTR (Reichheld et al., 2005). SALK insertion lines for *NTRA* (SALK 039152) and *NTRB* (SALK 045978) show no transcript accumulation for the corresponding gene and show a large decrease in NTR protein levels in the cytosol (*ntra*) or mitochondria (*ntrb*) (Reichheld et al., 2005). This indicates an inability to compensate by increased expression of the other *NTR* gene. These SALK mutant lines were obtained and each

line was crossed to a LOV1 plant. For each mutant, a segregating F2 population was generated and scored for victorin sensitivity, as well as genotyped for both the presence of *LOV1* and the T-DNA insertion. All F2 plants from both the *ntra* and *ntrb* populations carrying at least one copy of the *LOV1* gene were sensitive to victorin, including plants that were homozygous for either of the *ntr* insertion mutations. Plants that were homozygous for both *LOV1* and the *ntra* or *ntrb* insertion were selfed and the phenotype confirmed in the next generation. These plants were indistinguishable from wild-type plants with regard to victorin sensitivity (cf. Figures 2.3B and 2.3A, left). We then crossed homozygous *LOV1 ntra* and *LOV1 ntrb* plants and screened the F2 population to isolate double mutant plants (*LOV1 ntra ntrb*). The double mutant plants were slightly smaller and darker green than wild-type plants. However, these plants showed wild-type levels of victorin sensitivity (Figure 2.3B). This indicates that loss of both NTR isoforms is insufficient to block the response to victorin.

Requirement for active site cysteine residues

The two cysteine residues in the active site of thioredoxins enable them to reduce other cysteine-containing proteins. When these cysteine residues are in the reduced form, the first cysteine in the active site (Cys-39 in ATTRX5) can form a mixed disulfide with the target protein. This intermolecular disulfide bond is quickly reduced by the second cysteine (Cys-42 in ATTRX5), resulting in release of the reduced target protein from the oxidized TRX, which then contains a disulfide bond between the two active site cysteine residues (Kallis and Holmgren, 1980). In order to test the requirement for the redox activity of ATTRX5 in victorin sensitivity, Cys-39 and Cys-42 were mutated both individually and together to serine residues by site-directed mutagenesis. The resulting cDNAs (C39S, C42S, and C39S/C42S) were cloned downstream of the *35S* promoter and introduced into a mutant carrying the *attrx5-1* allele (*liv1-1*). Multiple transgenic lines were selected and tested for restoration of victorin sensitivity by the introduced transgenes. Expression of each transgene was confirmed by RNA gel blot analysis. As mentioned above, introduction

of the wild-type *ATTRX5* cDNA completely restores victorin sensitivity to the *attrx5-1* mutant. In contrast, the C39S and C39S/C42S transgenes were unable to complement the *attrx5-1* mutation, suggesting the importance of a functional ATTRX5 active site (Figure 2.3C). Surprisingly, the C42S transgene was able to restore victorin sensitivity as effectively as the wild-type cDNA (Figure 2.3C). The C42S construct was also able to restore sensitivity when expressed from the *ATTRX5* native promoter, indicating that the complementation was not an artifact of overexpression (data not shown). It is possible that only the initial binding of ATTRX5 to a target protein (through Cys-39) is required for the victorin response, or that the reduction can be completed by another mechanism, possibly involving another thioredoxin. Alternatively, these data may indicate that the redox function of ATTRX5 is not required for victorin sensitivity.

Complementation by ATTRX3

Thioredoxin *h*3 (ATTRX3) is the most closely-related thioredoxin to ATTRX5 (Meyer et al., 2002), showing 73.7% identity and 83.9% similarity at the amino acid level. Therefore, we tested the ability of *ATTRX3* to complement the *attrx5-1* mutation when overexpressed. The *ATTRX3* cDNA was cloned behind the *35S* promoter and introduced by *Agrobacterium*-mediated transformation into plants carrying the *attrx5-1* allele. The majority of the hemizygous T0 plants (14 out of 20) showed no sensitivity to victorin by the detached leaf assay, while some plants (5 out of 20) showed slight yellowing and one showed moderate tissue collapse. These phenotypes correlated well with the expression level of the introduced transgene as determined by RNA gel blot analysis (Figure 2.4). In the T1 generation, a range of phenotypes was again observed, ranging from no sensitivity to a moderate response to victorin (Figure 2.3D), indicating that when expressed at a high level, ATTRX3 can partially compensate for loss of ATTRX5. There were no apparent morphological or developmental phenotypes associated with overexpression of *ATTRX3*.
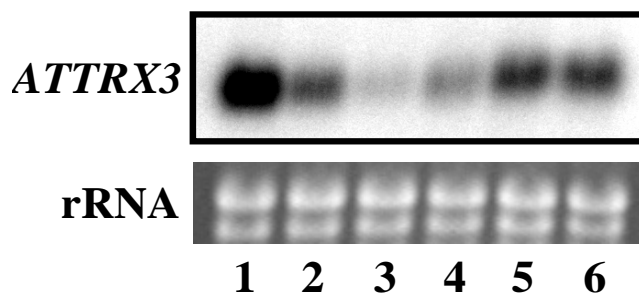
Victorin Sensitivity Phenotype:
1 = **Moderate tissue collapse**
2 = **Slight yellowing**
3 = **Insensitive**
4 = **Insensitive**
5 = **Slight yellowing**
6 = **Slight yellowing**
Remaining plants:
   2 had slight yellowing
   12 were insensitive

Figure 2.4. Correlation of *ATTRX3* transgene expression with victorin sensitivity.

Twenty *35S:ATTRX3* T0 transgenics were scored for victorin sensitivity by the detached leaf assay. Six of these plants were evaluated for level of transgene expression by RNA gel blot analysis. Note that this blot was exposed for a very short time to allow visualization of differences in band intensities between lanes. Ethidium bromide staining of the RNA gel is shown to confirm equal sample loading.

RNA gel blot analysis of *ATTRX5* and *ATTRX3* expression

In untreated leaves of wild-type LOV1 plants, RNA gel blot analysis shows undetectable levels of *ATTRX5* mRNA (Figure 2.5A). In contrast, *ATTRX3* mRNA is detectable at moderate levels in healthy leaf tissue. After infiltration of detached leaves with 30 μg/mL victorin, the levels of *ATTRX5* mRNA show strong induction, with an increase to detectable levels by 3 hours after infiltration. The levels continue to increase rapidly, showing maximum expression at 12 hours and diminishing thereafter. In contrast, *ATTRX3* mRNA levels show no induction after victorin infiltration. Instead, the level of *ATTRX3* mRNA is maintained at a relatively constant, moderate level of expression throughout the experiment with a possible slight decrease at the final 24-hour time point. In leaves from plants lacking a functional *LOV1* gene, including the victorin-insensitive Col-4 line or a *lov1* mutant line, *ATTRX5* expression was generally not induced upon treatment with victorin, although a very slight induction was sometimes observed at the later time points, possibly due to nonspecific stress caused by incubation of the detached leaves (Figure 2.5B). *ATTRX3* again showed constitutive levels of expression in these plants. The same result was obtained with LOV1 leaves infiltrated with water. We also tested *ATTRX5* and *ATTRX3* induction in the *liv1-1* mutant. *ATTRX5* showed a severe reduction in expression compared to wild-type plants (cf. Figures 2.5A and 2.5C), consistent with the expected disruption of splicing in this mutant. However, we did not observe accumulation of larger transcripts that would correspond to unspliced mRNA, suggesting that the unspliced transcript is unstable. *ATTRX3* expression in the *liv1-1* mutant again shows a moderate level of constitutive expression (Figure 2.5C). This indicates that *ATTRX3* expression is not elevated to compensate for loss of *ATTRX5*.

RNA gel blot analysis of *ATTRX5* induction was also performed with plants carrying both the *LOV1* gene and various defense response mutations. These plants were obtained by crossing LOV1 plants with plants carrying the mutations and are therefore in a mixed ecotype background. Because all the defense response mutants

Figure 2.5. RNA gel blot analysis of *ATTRX5* and *ATTRX3* gene expression.

[32]P-labeled probes were used to monitor expression of *ATTRX5* and *ATTRX3* in the indicated plant genotypes. Ethidium bromide staining of the RNA gels is also shown to confirm equal sample loading. Time points are given in hours after infiltration with 30 µg/mL victorin. The first lane of each gel contains RNA from untreated leaves (U). A) *ATTRX5* and *ATTRX3* expression in victorin-sensitive plants from the LOV1 line. B) *ATTRX5* and *ATTRX3* expression in victorin-insensitive *lov1-6* mutant plants. C) *ATTRX5* and *ATTRX3* expression in victorin-insensitive *liv1-1* mutant plants. D) *ATTRX5* and *ATTRX3* expression in victorin-sensitive plants from the Col-LOV line. E) *ATTRX5* expression in plants homozygous for *LOV1* and for the indicated defense response mutant allele.
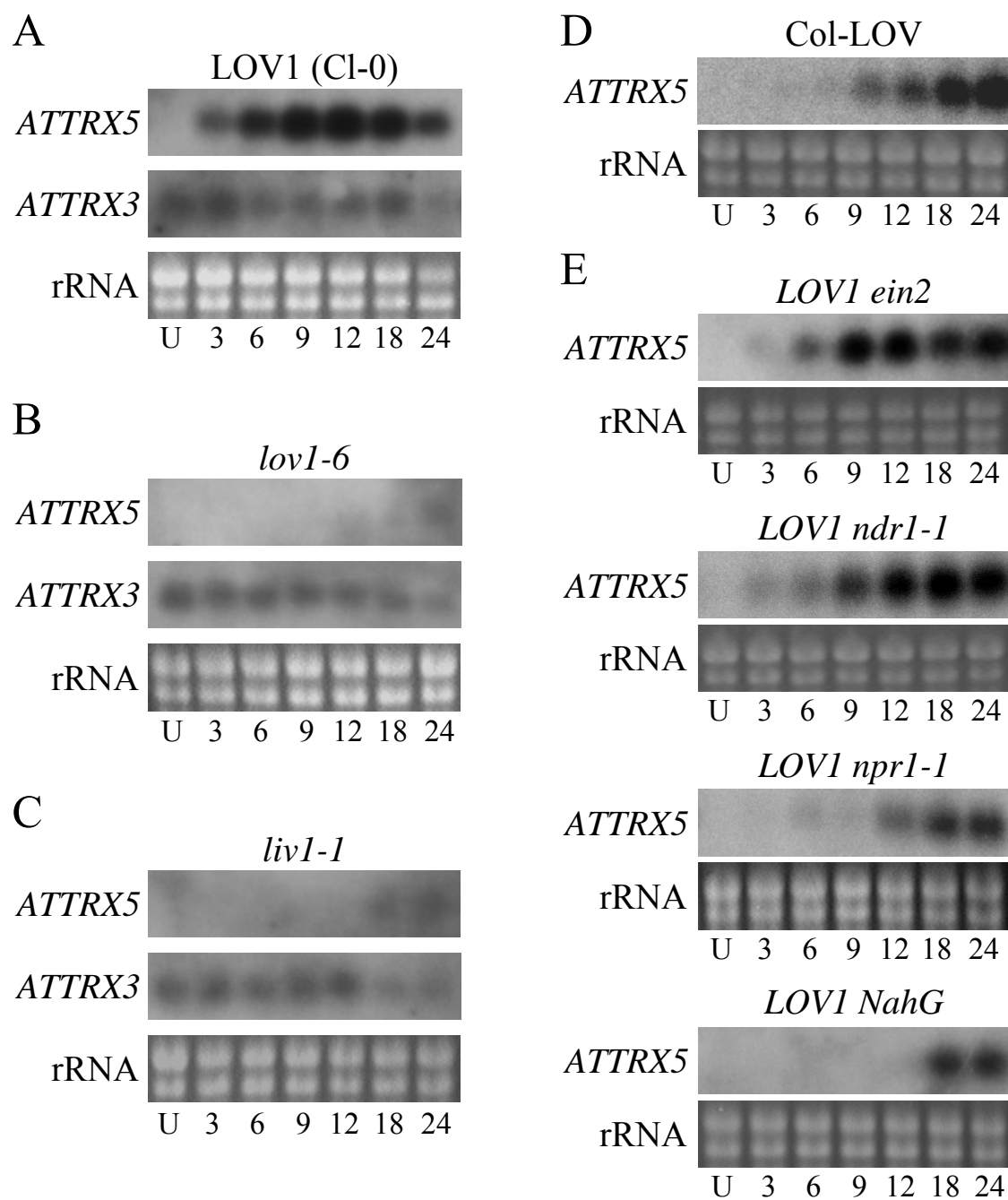
Figure 2.5. RNA gel blot analysis of *ATTRX5* and *ATTRX3* gene expression.

were in a Col-0 background, we first tested *ATTRX5* induction in Col-LOV plants (Figure 2.5D). *ATTRX5* induction was somewhat slower in this background than in the LOV1 (Cl-0) plants, consistent with the observation that Col-LOV plants are slightly less sensitive to victorin than LOV1 plants (T.A. Sweat, unpublished results). The ethylene-insensitive *ein2* mutation (Guzmán and Ecker, 1990), and the *ndr1-1* mutation, which impairs signaling from a subset of disease resistance genes (Century et al., 1995; Aarts et al., 1998), both showed a slight reduction or delay in *ATTRX5* induction compared to the LOV1 (Cl-0) parent. However, induction was not delayed in comparison to the Col-LOV plants. Therefore, we conclude that *ndr1-1* and *ein2* have little or no effect on *ATTRX5* induction by victorin treatment (Figure 2.5E). However, both the *npr1-1* mutation, which results in a defect in salicylic acid (SA) signaling (Cao et al., 1994), and expression of the *NahG* transgene, which encodes the SA-degrading enzyme salicylate hydroxylase (Delaney et al., 1994), resulted in a delay in induction of *ATTRX5* after victorin treatment even in comparison to Col-LOV plants. It has been found that *ATTRX5* expression is induced by salicylic acid treatment (Laloi et al., 2004), and these results suggest that SA plays a role in inducing *ATTRX5* after victorin treatment. However, SA is not required for victorin sensitivity in *Arabidopsis* (J.M. Lorang, unpublished results).

Characterization of *ATTRX5/ATTRX3* gene fusions

As seen in the RNA gel blot analysis described above, expression from the *ATTRX5* promoter is strongly induced in sensitive plants following treatment with victorin, while the *ATTRX3* promoter shows moderate-level constitutive expression that is not affected by victorin treatment. In addition, it was found that high levels of ATTRX3, expressed from the *35S* promoter, could partially compensate for the loss of ATTRX5. Therefore, wild-type *ATTRX5* was cloned downstream of the *ATTRX3* promoter and *ATTRX3* was placed under control of the *ATTRX5* promoter in order to better separate the effects of TRX specificity from differences due to expression levels. Additionally, in order to determine which areas of the protein confer

specificity differences between the TRX proteins in the response to victorin, a series of gene constructs was made in which portions of the *ATTRX5* gene were replaced with the corresponding sequence from *ATTRX3* (Figures 2.6A and 2.6B). The gene fusions were placed under control of either the *ATTRX5* or *ATTRX3* native promoters. All constructs were introduced into the *attrx5-1* mutant by *Agrobacterium*-mediated transformation. Transgenics were tested in the first generation, hemizygous state so that a number of different transformants for each construct ($n \geq 60$ for *ATTRX5* promoter constructs; $n \geq 34$ for *ATTRX3* promoter constructs) could be examined before segregation of the transgene in the T1 generation. Two to four leaves from each plant were tested for victorin sensitivity by the detached leaf assay. Each leaf was placed in a well of a 96-well plate with 250 µL of 10 µg/mL victorin. Leaves were scored for sensitivity at 1, 2, and 3 days following victorin treatment. A symptom rating scale was created to attempt to quantify the differences in victorin sensitivity conferred by the different constructs. Each leaf was assigned a score ranging from 0 (no symptoms) to 5 (complete dessication and collapse of all portions of the leaf projecting from the well) (Figure 2.6C). An average symptom value was determined for days 1, 2, and 3 for each plant by averaging the values of the individual leaves. The 1, 2, and 3 day averages for each plant were then used to determine an average value for all plants transformed with the same construct for each day.

Plants carrying *ATTRX5* expressed under control of the *ATTRX3* promoter showed high levels of sensitivity (average rating of 4.2 on day 3), even though the plants were not homozygous for the transgene (Table 2.2). This suggests that the high level of *ATTRX5* induction seen in wild-type plants is not required for victorin sensitivity. In contrast, plants carrying the *ATTRX3* gene under control of the *ATTRX5* promoter showed few symptoms following victorin treatment (average rating of 0.9 on day 3) (Table 2.2). Most leaves tested from *ATTRX5:ATTRX3* plants showed no symptoms, while some showed slight to moderate sensitivity. The individuals showing significant symptoms likely result from higher expression of the transgene in those individuals, possibly due to multiple insertion sites. Additionally, these plants
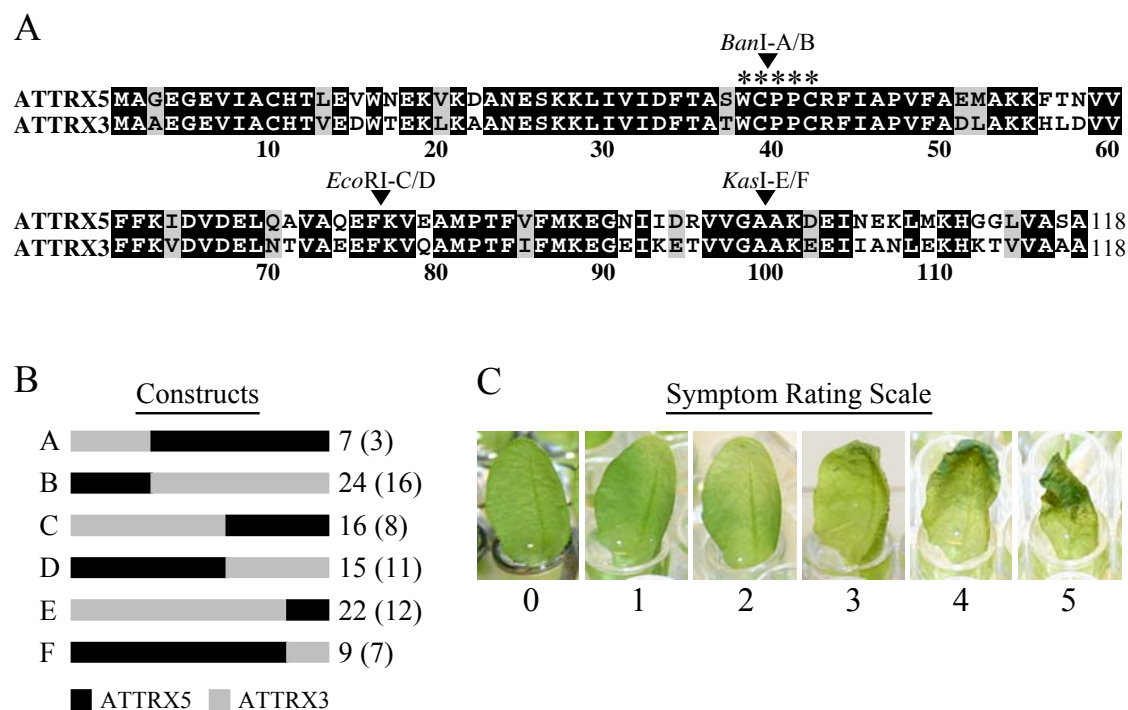
Figure 2.6. **Construction and evaluation of *ATTRX5/ATTRX3* fusion constructs.**

A) Alignment of ATTRX5 and ATTRX3 amino acid sequence by ClustalW. Conservative amino acid substitutions are highlighted in gray. Active site residues are denoted by asterisks. Arrowheads show location of restriction sites used to splice the *ATTRX5* and *ATTRX3* coding sequences for the indicated constructs. B) Diagram showing the portions of *ATTRX5* and *ATTRX3* coding sequences present in each construct. Numbers to the immediate right indicate the number of amino acid substitutions relative to wild-type ATTRX5 and the numbers in parentheses indicate the number of nonconservative substitutions relative to wild-type ATTRX5. C) Representative leaves illustrating the symptom rating scale used to evaluate the degree of victorin sensitivity conferred by each construct.

Table 2.2. Symptom ratings for *ATTRX5*/*ATTRX3* chimeric constructs.

ATTRX5 Promoter Construct	Average Symptom Rating			*ATTRX3* Promoter Construct	Average Symptom Rating			% Identity to ATTRX5	% Similarity to ATTRX5
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3		
ATTRX3	0.2	0.6	0.9	ATTRX5	2.4	3.7	4.2	(73.7%)[a]	(83.9%)[a]
A	3.3	4.5	4.9	A	2.4	3.9	4.3	94.1%	97.5%
B	0.5	1.0	1.7	B	0.2	0.7	0.9	79.7%	86.4%
C	0.6	1.2	2.0	C	0.3	0.6	1.1	86.4%	93.2%
D	2.3	3.6	4.3	D	1.1	2.2	2.9	87.3%	90.7%
E	0.3	1.1	1.9	E	0.4	0.8	1.4	81.4%	89.8%
F	3.1	4.2	4.8	F	0.6	1.6	2.4	92.4%	94.1%

[a]These values represent the % amino acid similarity and identity of ATTRX3 to ATTRX5 and apply only to the full-length ATTRX3 construct on the left side of the table. Values in all other rows denote % similarity and % identity of chimeric constructs A-F to wild-type ATTRX5.

were already carrying the native *ATTRX3* gene, so overall ATTRX3 levels may have reached relatively high levels in plants carrying multiple copies of the transgene. The chimeric *ATTRX5/ATTRX3* cDNAs expressed from the *ATTRX5* promoter showed that the more amino acids identical to ATTRX5 are present, the greater the response to victorin (Table 2.2). This suggests that amino acids in all portions of the protein play some role in determining specificity for this response. However, there is a fairly large difference between the sensitivity conferred by constructs A, D, and F in comparison to the much weaker response seen with constructs B, C, and E. This is apparent even though constructs C and D have nearly the same amount of amino acid changes from wild-type ATTRX5 (16 versus 15), and construct D has 11 nonconservative changes, while construct C has only eight nonconservative substitutions (Figure 2.6B). These data suggest that the region of ATTRX5 sequence included in constructs A, D, and F, but not in constructs B, C, and E, plays a particularly important role in determining the effectiveness of ATTRX5 versus ATTRX3 in the response to victorin. This region corresponds to the central portion of the protein C-terminal to the active site and includes nine amino acid differences (five nonconservative changes) between Ala 50 and Glu 75 (Figure 2.6A). In contrast, there is little difference in the strength of the response conferred by construct C versus E or construct D versus F. The region that differs between both of these construct pairs contains six amino acid substitutions (four nonconservative) between Val 78 and Val 96. These amino acid residues apparently have little effect on specificity.

When these gene fusions were expressed under control of the *ATTRX3* promoter, the results were similar although somewhat less consistent. There was again a fairly large decrease in sensitivity between constructs A, D, and F, in comparison to B, C, and E (Table 2.2). There was also only a small difference between constructs C and E and between D and F, again suggesting that the amino acids differing between these pairs have little effect on the protein's ability to signal for victorin sensitivity. In this experiment, the relative sensitivity of plants carrying constructs D versus F and C versus E was reversed from the *ATTRX5* promoter results,

but the differences between each pair were small compared to the overall differences seen in the experiment. There was also very little difference in sensitivity between construct A and full-length *ATTRX5* expressed from the *ATTRX3* promoter. This suggests that the seven amino acid changes in construct A, which occur in the N-terminal portion of the protein, have little effect on TRX specificity in this response. The chimeric constructs were also expressed under the *35S* promoter, and the same trend was observed although levels of sensitivity were greater for each construct, due to higher levels of expression (data not shown).

DISCUSSION

Our extensive mutant screen resulted in isolation of 63 independent mutants that are completely insensitive to victorin. Complementation tests showed that 59 carry mutations in the *LOV1* gene, which confers victorin sensitivity in *Arabidopsis* (Lorang et al., 2004), while four have mutations in the gene encoding ATTRX5. The difference in the number of mutations found in each gene likely is mainly due to the difference in size of the coding regions of these genes. The LOV1 protein consists of 910 amino acid residues, while ATTRX5 has only 118 residues. However, we also noted that all the mutations in *ATTRX5* likely cause a severe disruption of protein function as they cause a nonsense mutation early in the protein, splice site disruption, or addition of an extra cysteine residue immediately adjacent to the active site. Therefore, it is possible that ATTRX5 can sustain a number of less severe point mutations without loss of function. Our numbers are consistent with a similar screen involving loss of function of the CC-NBS-LRR gene *RPM1*. Tornero et al., (2002) isolated 95 *rpm1* mutant alleles and only 15 mutations in other genes. This may suggest that NBS-LRR genes are particularly sensitive to perturbations in function, which might be expected for a protein that has to recognize a highly-specific signal to perform its function. However, as noted above, it may be simply a function of their large size.

An additional item of note is that our mutant screen failed to turn up mutations in any genes known to be involved in disease resistance or cell death. Conversely, extensive mutant screens have been performed with other pathogen systems, resulting in elucidation of many of the genes that mediate disease resistance pathways (Glazebrook, 2005), and *ATTRX5* has not been identified in any of these studies. *ATTRX5* was also not identified in an extensive screen for loss of hypersensitive cell death mediated by RPM1, which, like LOV1, is a CC-NBS-LRR protein (Tornero et al., 2002). Screens for mutants that have lost sensitivity to other toxins, such as coronatine or fumonisin B1, have also failed to turn up mutations in the *ATTRX5* gene (Feys et al., 1994; Stone et al., 2000, 2005). These data are consistent with our finding that mutation of ATTRX5 has no effect on the RPM1- or RPS2-mediated HR to *Pst* or on the response to fumonisin B1 or coronatine. Together, these results suggest that ATTRX5 has a function specific to the victorin response pathway.

The *Arabidopsis* genome encodes 19 classic thioredoxins in six major groups (*f*, *m*, *h*, *o*, *x*, and *y*), as well as multiple thioredoxin-like and thioredoxin domain-containing proteins (Meyer et al., 2002). These include eight *h*-type thioredoxins. A few *h*-type TRXs have been found to be targeted to specific subcellular locations, including a mitochondrial TRX*h* in poplar (Gelhaye et al., 2004) and a plasma membrane-anchored TRX*h* in soybean (Shi and Bhattacharyya, 1996). However, the *Arabidopsis h*-type TRXs, with the possible exception of ATTRX8, are all predicted to be cytosolic proteins (Gelhaye et al., 2005). To date, there has been little success in assigning specific functions to individual thioredoxins, largely due to the apparent redundancy of the system. Various proteomics approaches have been developed in an attempt to identify proteins targeted by thioredoxins. One method involves immobilizing thioredoxin proteins on a resin and incubating cell lysates with the TRX resin to isolate proteins that bind to the immobilized TRX (Motohashi et al., 2001). This type of study was performed with five of the *Arabidopsis h*-type thioredoxins (ATTRX1-ATTRX5) and resulted in identification of several new potential thioredoxin targets (Yamazaki et al., 2004). However, the authors of this study were

unable to assign targets to specific TRX*h* isoforms, as these *in vitro* interactions largely showed a lack of specificity for individual TRX*h*s. This is in agreement with other studies of this type, which have shown a lack of *in vitro* specificity even between the different major groups of TRXs (Motohashi et al., 2001; Balmer et al., 2003, 2004). Other studies have attempted to define specificity for the *Arabidopsis h*-type TRXs based on expression differences. However, while differences exist in the level of expression of these eight thioredoxins among various tissues and developmental stages, there is also a large degree of overlap in their expression patterns with at least five members of this group being expressed in leaf tissue (Rivera-Madrid et al., 1995; Reichheld et al., 2002).

Of the eight *h*-type *Arabidopsis* TRXs, ATTRX3 and ATTRX5 are the most closely related based on sequence homology. In particular, both ATTRX3 and ATTRX5 contain the sequence WCPPC in their active sites rather than the much more common WCGPC, and some evidence suggests this difference is important for determining substrate specificity (Bréhélin et al., 2000; Mazzurco et al., 2001). *ATTRX3* and *ATTRX5* are also the two most highly expressed *Arabidopsis* TRX*h*s based on EST abundance, and both are expressed in the vascular tissue of leaves (Reichheld et al., 2002). However, there is a major difference between the expression patterns of these two thioredoxins. While *ATTRX5* is expressed at lower levels than *ATTRX3* in healthy leaf tissue, the expression of *ATTRX5* is highly induced in response to various biotic and abiotic stresses (Reichheld et al., 2002; Laloi et al., 2004). In contrast, *ATTRX3* is not induced under these treatments but rather shows a moderate constitutive level of expression. This is in agreement with our results showing that treatment with victorin causes a rapid increase in the levels of *ATTRX5* mRNA, while the level of *ATTRX3* expression remains constant throughout the course of the experiment (Figure 2.5A). This difference in the regulation of gene expression could be responsible for our finding that *ATTRX5* is specifically required for victorin sensitivity. However, our studies with promoter fusions clearly show that *ATTRX5* is functional for the response to victorin even when expressed under the non-inducible

ATTRX3 promoter, while *ATTRX3* is unable to fulfill this role even when expressed under the inducible *ATTRX5* promoter (Table 2.2). Furthermore, plants carrying mutations in salicylic acid signaling pathways show no decrease in victorin sensitivity (J.M. Lorang, unpublished results), even though *ATTRX5* induction is significantly delayed (Figure 2.5E). Therefore, the low basal level of expression from the *ATTRX5* promoter is sufficient for triggering a response to victorin in plants carrying a wild-type *ATTRX5* gene. However, this low level expression is not enough to initiate a response in plants carrying the *ATTRX3* gene under the *ATTRX5* promoter, even though the plants are also carrying a native *ATTRX3* gene. We found that *ATTRX3* can partially compensate for loss of *ATTRX5* when expressed at very high levels from the *35S* promoter (Figures 2.3D and 2.4). However, the data clearly indicate specificity at the protein level for ATTRX5 versus ATTRX3 in the response to victorin.

Our work with chimeric genes, in which portions of the *ATTRX5* coding sequence were replaced with the corresponding sequence from *ATTRX3*, further support the specificity of ATTRX5 over ATTRX3 in mediating victorin sensitivity. These constructs showed that the strength of the response to victorin correlates well with the percent amino acid identity to wild-type ATTRX5 (Table 2.2), suggesting that amino acid residues conferring specificity occur throughout the protein sequence. However, when the percent similarity of the amino acid sequence is considered, an interesting observation can be made. Our data show that construct D confers a much stronger response to victorin than construct C, despite having a lower similarity to wild-type ATTRX5 (Table 2.2). Construct D encodes the N-terminal portion of ATTRX5 and the C-terminal portion of ATTRX3, while construct C encodes the N-terminal portion of ATTRX3 and the C-terminal portion of ATTRX5 (Figure 2.6B). This indicates that residues particularly important for determining specificity are contained somewhere within the N-terminal two-thirds of the protein. We also observed that construct A confers approximately the same level of sensitivity as the wild-type *ATTRX5* coding sequence when expressed from the *ATTRX3* promoter,

despite containing ATTRX3 sequence in the N-terminal one-third of the protein (Table 2.2 and Figure 2.6B). Therefore, the central portion of the ATTRX5 protein that is present in constructs A and D, but not in construct C, is implicated as an important determinant for the specificity of ATTRX5 over ATTRX3 in the response to victorin (Figures 2.6A and 2.6B). The nine amino acids in this region that differ between ATTRX5 and ATTRX3 occur between Ala 50 and Glu 75 in an area C-terminal to the active site and include five nonconservative substitutions that may be largely responsible for the specificity differences conferred by this region.

An additional observation made from the *ATTRX5/ATTRX3* gene fusions is that all chimeric constructs conferred higher levels of victorin sensitivity when expressed under the *ATTRX5* promoter versus the *ATTRX3* promoter (Table 2.2). This indicates that induced expression from the *ATTRX5* promoter does act to enhance the response to victorin. Wild-type ATTRX5 and construct A, which is highly similar to ATTRX5, confer fairly high levels of sensitivity even under control of the *ATTRX3* promoter, suggesting that high protein levels are not essential if specificity is maintained. However, for the other constructs, there is a fairly large reduction in symptoms when the constructs are expressed from the *ATTRX3* promoter compared to the *ATTRX5* promoter. This suggests that high expression levels may help overcome lack of specificity, as was observed with wild-type ATTRX3 expressed from the *35S* promoter.

The primary mode of action described for thioredoxins is reducing disulfide bonds of target proteins. This requires involvement of both TRX active site cysteine residues. Initially, the first cysteine forms a mixed disulfide with the target protein. The reduction is then completed by the second cysteine residue (Kallis and Holmgren, 1980). Our data show that the first active site cysteine (Cys-39) of ATTRX5 is required for the response to victorin (Figure 2.3C). In contrast, mutation of the second cysteine (Cys-42) had no effect on the ability of ATTRX5 to mediate victorin sensitivity. We also found that plants lacking both a functional NTRA and NTRB, which are involved in regenerating reduced TRX in the cytosol, were as sensitive to

victorin as wild-type plants (Figure 2.3B). These data are consistent with a model in which the mechanism of ATTRX5 in signaling for victorin sensitivity does not involve reduction of a target protein. However, because Cys-39 is required for victorin sensitivity, ATTRX5 may still be involved in formation of a mixed disulfide with a target protein, and we cannot rule out the possibility that reduction of this disulfide is completed by another TRX or other reducing agent in the cell. Additionally, the *ntr* mutant data may simply indicate that ATTRX5 can be reduced by other reducing agents in the cytosol. It is also possible that other mechanisms, such as increased transcription of TRX genes, can replenish the supply of reduced TRX in the *ntr* mutants (Reichheld et al., 2005).

To date, there are two major examples of cytosolic thioredoxins involved in signaling in response to external stimuli in plants. The *Brassica S*-locus receptor kinase (SRK), which is involved in the self-incompatibility response, was found to interact with two *h*-type thioredoxins, THL1 and THL2 (Bower et al., 1996). THL1 binds the cytoplasmic kinase domain of SRK and inhibits autophosphorylation of SRK *in vitro*. However, in the presence of pollen coat proteins from self-pollen, the inhibition by THL1 is relieved and SRK becomes phosphorylated, which is the active state for initiation of an incompatibility reaction (Cabrillac et al., 2001). THL1 and THL2 are most similar to the *Arabidopsis h*-type thioredoxins ATTRX3 and ATTRX4, respectively (Mazzurco et al., 2001). These four thioredoxins, as well as *Arabidopsis* ATTRX5, all contain the active site WCPPC, rather than the WCGPC found in most thioredoxins. Interestingly, it was found that ATTRX3 and ATTRX4 could also interact with SRK. In contrast, *Arabidopsis* ATTRX1 and ATTRX2, which contain the WCGPC active site, failed to interact with SRK (Mazzurco et al., 2001). This suggests that the active site sequence plays a role in determining the ability of individual thioredoxins to interact with specific target proteins in the cell, which may partially explain the inability of other *Arabidopsis h*-type TRXs, such as ATTRX1 or ATTRX2, to compensate for loss of ATTRX5 in the victorin response.

The second example involves the tomato *Cf-9* resistance gene, which confers resistance to races of *Cladosporium fulvum* carrying the *Avr9* avirulence gene. *Cf-9* encodes a receptor-like protein that was found to interact with a thioredoxin (Rivas et al., 2004). This example is particularly intriguing, given the recent discovery that the *LOV1* gene encodes a resistance-like protein (J.M. Lorang, unpublished results). The thioredoxin found to interact with Cf-9, CITRX, is only distantly related to previously described thioredoxins in *Arabidopsis*. It is most closely related to the plastid-localized *x*-type thioredoxin. However, CITRX does not appear to contain a signal peptide and is believed to be located in the cytosol. Similar to the situation found for SRK, CITRX binds the C-terminal cytoplasmic portion of Cf-9, and results suggested that it acts as a negative regulator of Cf-9 activity. In this scenario, the presence of Avr9 would relieve the inhibition, resulting in activation of the hypersensitive response (Rivas et al., 2004). However, more recent results have demonstrated that CITRX likely acts as an adaptor protein between Cf-9 and the Avr9/Cf-9 induced kinase 1 (ACIK1). Because ACIK1 is a positive regulator of the Cf-9 conditioned defense response, it has been suggested that CITRX could play a positive role in regulation of Cf-9 (Nekrasov et al., 2006).

The above examples may provide some insight into the mechanism by which ATTRX5 regulates the response to victorin in *Arabidopsis*. However, there are some significant differences. Both SRK and Cf-9 are transmembrane receptor proteins, whereas *LOV1* encodes a cytosolic resistance-like protein belonging to the CC-NBS-LRR class of resistance genes (J.M. Lorang, unpublished results). Because LOV1 lacks an extracellular region, it cannot be directly activated by external stimuli in a manner analogous to SRK or Cf-9. Secondly, SRK is negatively regulated by thioredoxins, whereas ATTRX5 acts as a positive regulator in the response to victorin. Currently, it is unclear whether CITRX acts as a positive or negative regulator of Cf-9. Finally, mutation of either active site cysteine residue abolishes binding of THL1 to SRK (Mazzurco et al., 2001), whereas mutation of both active site cysteine residues has no effect on the binding of CITRX to either Cf-9 or ACIK1 (Nekrasov et al.,

2006). It is unknown whether ATTRX5 directly interacts with LOV1. However, unlike both THL1 and CITRX, ATTRX5 requires the first but not the second active site cysteine for its function in the victorin response.

In animal cells, cytosolic TRX has been found to act as a key inhibitor of cell death, both by acting as an antioxidant to prevent cell death triggered by reactive oxygen species (ROS), and by directly regulating proteins involved in programmed cell death pathways (Masutani et al., 2005). For example, mammalian TRX has been found to directly bind to and inhibit apoptosis signal-regulating kinase 1 (ASK1) (Saitoh et al., 1998). Upon oxidation of TRX, possibly by reactive oxygen species, TRX dissociates from ASK1. This relieves the inhibition of ASK1, allowing ASK1 to initiate signaling for apoptosis. Human TRX has also been found to catalyze the S-nitrosation of caspase-3 *in vitro* (Mitchell and Marletta, 2005). This involves the specific transfer of a nitrosothiol from a non-catalytic cysteine residue of TRX to the catalytic cysteine of caspase-3, resulting in inhibition of caspase-3 activity. As caspase-3 is a key protease in the cell death process (Jiang and Wang, 2004), its nitrosation by TRX results in inhibition of apoptosis.

In both plants and animals, thioredoxins tend to act as inhibitors of cell death either by providing reducing power to proteins that scavenge reactive oxygen species or by directly inhibiting proteins that trigger the cell death process (Masutani et al., 2005; Vieira Dos Santos and Rey, 2006). This is in direct contrast to the action of ATTRX5, which acts as a positive regulator of victorin-induced cell death. Victorin triggers a programmed cell death (PCD) response in sensitive oats that resembles apoptosis (Navarre and Wolpert, 1999; Yao et al., 2001, 2002; Curtis and Wolpert, 2002; Coffeen and Wolpert, 2004), and this also appears to be true in sensitive *Arabidopsis* (T.A. Sweat, unpublished results). The hypersensitive response is also a form of PCD and shares many biochemical features typically associated with apoptosis (Heath, 2000; Greenberg and Yao, 2004). Given the discovery that *LOV1* encodes a resistance-like protein, it appears likely that the hypersensitive response and victorin-induced cell death are related processes, which in the cases of Cf-9 and LOV1

are both regulated by cytosolic thioredoxins. Future work will be directed at determining the mechanism by which ATTRX5 regulates victorin-induced cell death, and whether this involves a direct interaction of ATTRX5 and LOV1, as is the case with Cf-9 and CITRX. This work should provide insight into what increasingly appears to be a close relationship between the regulation of plant disease resistance and susceptibility.

Chapter 3

Characterization of natural and induced variation in the *LOV1* gene, a CC-NB-LRR
gene conferring victorin sensitivity and disease susceptibility in *Arabidopsis*

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ABSTRACT

The fungus *Cochliobolus victoriae*, the causal agent of Victoria blight, produces a compound called victorin, which is required for pathogenicity of the fungus. Victorin alone reproduces disease symptoms, including programmed cell death, on sensitive plants. Victorin sensitivity and susceptibility to *C. victoriae* were originally described on oats but have since been identified on *Arabidopsis thaliana*. Victorin sensitivity in *Arabidopsis* is conferred by the *LOCUS ORCHESTRATING VICTORIN EFFECTS1* (*LOV1*) gene, which encodes a coiled-coil-nucleotide binding site-leucine rich repeat (CC-NB-LRR) protein. We sequenced the *LOV1* gene from 59 victorin-insensitive mutants and found that the spectrum of mutations causing loss of function of LOV1 was similar to that found to cause loss of function of RPM1, a CC-NB-LRR protein with a known function in resistance. In addition, many of the mutated residues in LOV1 are in conserved motifs found in other studies to be required for resistance protein function. These data suggest that LOV1 has a mechanism of action similar to resistance proteins. A survey of victorin sensitivity in 30 *Arabidopsis* ecotypes indicated that victorin sensitivity is the prevalent phenotype with only three accessions showing insensitive individuals. In addition, we found that there is very little genetic variation among *LOV1* alleles, which could indicate a recent selective sweep of *LOV1*. As selection would not be expected to preserve a functional *LOV1* gene to confer victorin sensitivity and disease susceptibility, we propose that *LOV1* may function as a resistance gene to a naturally-occurring pathogen of *Arabidopsis*.

INTRODUCTION

The *Arabidopsis thaliana* genome encodes 149 nucleotide binding site-leucine-rich repeat (NB-LRR) proteins, including 83 with an N-terminal Toll/Interleukin-1 receptor (TIR) domain and 51 with an N-terminal coiled-coil (CC) domain (Meyers et al., 2003). Nearly all plant NB-LRR proteins that have been characterized to date function as disease resistance proteins. These proteins directly or indirectly recognize

pathogen effectors known as avirulence (Avr) proteins (Jones and Dangl, 2006). Recognition triggers a resistance response in the host, typically including a programmed cell death (PCD) response known as the hypersensitive response (HR) at the site of pathogen contact (Heath, 2000; Greenberg and Yao, 2004). The *LOCUS ORCHESTRATING VICTORIN EFFECTS1* (*LOV1*) gene (GenBank accession EF472599) is unique in that it encodes a CC-NB-LRR protein that confers susceptibility to infection by the fungus *Cochliobolus victoriae* in *Arabidopsis thaliana* (J.M. Lorang, unpublished results). *C. victoriae* was originally described as the causal agent of Victoria blight of oats (Meehan and Murphy, 1946). Pathogenic strains of *C. victoriae* produce a cyclized pentapeptide called victorin (Macko et al., 1985; Wolpert et al., 1985), and, for both oats and *Arabidopsis*, only genotypes that are sensitive to victorin are susceptible to infection by *C. victoriae* (Walton, 1996; Wolpert et al., 2002; Lorang et al., 2004; Sweat and Wolpert, 2007). Victorin triggers a PCD response in sensitive plants (Navarre and Wolpert, 1999; Yao et al., 2001, 2002; Curtis and Wolpert, 2002, 2004; Coffeen and Wolpert, 2004) and also induces host defense responses (Wheeler and Black, 1962; Shain and Wheeler, 1975; Mayama et al., 1986; Ullrich and Novacky, 1991, T.A. Sweat, unpublished results). In oats, the gene that confers sensitivity to victorin and susceptibility to *C. victoriae* is the *Vb* gene. The *Vb* gene is believed to share identity with the *Pc-2* gene, which confers resistance to crown rust of oats caused by the fungus *Puccinia coronata* (Litzenberger, 1949), as extensive genetic and mutagenic studies have failed to separate crown rust resistance from Victoria blight susceptibility (Welsh et al., 1954; Luke et al., 1966; Rines and Luke, 1985; Mayama et al., 1995). This genetic connection to a resistance gene and the fact that victorin induces a host response similar to that mediated by resistance genes, including both induction of resistance responses and PCD, strongly suggest that susceptibility to *C. victoriae* in oats is conferred by a resistance gene. The recent cloning of *LOV1* has shown that a resistance-like protein of the NB-LRR class is indeed responsible for conferring both sensitivity to victorin and susceptibility to *C. victoriae* in *Arabidopsis*.

We previously reported isolation and genetic characterization of 63 independent ethyl methanesulfonate (EMS)-generated mutants that had completely lost sensitivity to victorin from a screen of approximately 212,000 seedlings (Sweat and Wolpert, 2007). These mutants fell into two complementation groups, consisting of 59 *lov1* mutants and four carrying mutations in the *LIV1* gene, which was found to encode thioredoxin *h*5 (ATTRX5). We showed that loss of victorin sensitivity resulted in complete loss of susceptibility to *C. victoriae* in both a *lov1* and a *liv1* mutant (Sweat and Wolpert, 2007), as was expected, given that there is a strict correlation between sensitivity to victorin and susceptibility to *C. victoriae* in both oats and *Arabidopsis* (Walton, 1996; Wolpert et al., 2002; Lorang et al., 2004). In the present study, we used a two-fold approach to better understand the function of LOV1 in conferring victorin sensitivity. First, we sequenced the *LOV1* gene from each of the 59 *lov1* mutants. This revealed that many of the mutations leading to loss of function of LOV1 are in regions that are conserved among NB-LRR proteins and are known to be required for resistance protein function. Our screen was similar in scope to another large-scale mutagenesis screen conducted on *RPM1*, a CC-NB-LRR gene that confers resistance to *Pseudomonas syringae* carrying *avrRpm1* or *avrB* (Bisgrove et al., 1994; Grant et al., 1995). By comparing the mutations leading to LOV1 loss of function with those identified in the RPM1 mutant screen, we found that a similar spectrum of mutations causes loss of function of these proteins, suggesting that LOV1 may function in a manner analogous to a known resistance protein. Second, in order to survey the prevalence of victorin sensitivity in *Arabidopsis* and the naturally-occurring variation in *LOV1* alleles, we sequenced the *LOV1* gene from 30 *Arabidopsis* ecotypes and correlated these data with the response of each ecotype to victorin treatment. This analysis showed that sensitivity to victorin is much more common than insensitivity and that there is very little genetic variation among *LOV1* alleles. Because a functional *LOV1* gene is not likely to be either selected for or maintained as a means to confer victorin sensitivity and susceptibility to *C. victoriae*, we propose that *LOV1* may be acting as a resistance gene to a naturally-occurring pathogen of *Arabidopsis*.

MATERIALS AND METHODS

Plant material, growth conditions, and treatment with victorin

All seed stocks used in this study were obtained from The Ohio State University Arabidopsis Biological Resource Center (ABRC). Stock numbers are as follows: Cl-0 (CS1082), Col-4 (CS933), Ler-0 (CS20), Sp-0 (CS1530), Sha (CS929), Tol-0 (CS8020), C24 (CS906), Rubezhnoe-2 (CS928), Bay-0 (CS22676), Cvi-0 (CS22682), En-1 (CS1137), Kas-1 (CS903), Nd-0 (CS6803), No-0 (CS1394), Ws-0 (CS1603), Ws-2 (CS22659), Mt-0 (CS1380), Bil-5 (CS22578), HR-10 (CS22597), Fei-0 (CS22645), An-1 (CS6603), Ts-1 (CS1553), Yo-0 (CS22624), Se-0 (CS6852), Bur-0 (CS1028), Ag-0 (CS901), Vind-1 (CS22560), RRS-10 (CS22565), Lov-1 (CS22574), KZ-1 (CS22606), *A. suecica* (CS22505, CS22507, and CS22511), *A. korshinskyi* (CS4653), *O. pumila* (CS22562), *A. arenosa* (CS3901), *C. lasiocarpa* (CS6191), and *C. rubella* (CS22561). Seeds were incubated at 4ºC for 5 days in 0.1% agarose and then applied to soil. Plants were grown under long-day conditions (16 h light, 8 h dark) at 22ºC. Victorin C was purified as described previously (Macko et al., 1985; Wolpert et al., 1985). Plants were treated with victorin either by infiltration of 10 µg/mL victorin using a blunt-ended 1 mL syringe or by the detached leaf assay, in which a leaf is removed and placed in a well of a 96-well plate with 250 µl of 10 µg/mL victorin and distilled water added as necessary.

DNA preparation and sequencing

Plant genomic DNA was prepared as described by Edwards et al. (1991). PCR amplification of mutant *lov1* alleles was performed in segments using the following primers: LOV1a F 5'-TCTTCTTGTCGTGACCACAC-3' and LOV1a R 5'-TTCTT-GCAGCGACATCGAAC-3'; LOV1b F 5'-CAAGAAGCATGCGAGAAGAC-3' and LOV1b R 5'-TGTGTGCGTCTTGCTTGCTT-3'; LOV2 F 5'-ACACTGGTCGCAA-ATGCCTT-3' and LOV2 R 5'-GGAAGTTCTCCTCTTTGGCT-3'; LOV3 F 5' GA-ACAACCATCCAAGATAAGG-3' and LOV3 R 5'-CACGAGGTTCTTGCCTAT-CA-3'; LOV4 F 5'-ACGCTAGTGTGATGGACCTC-3' and LOV4 R 5'-TGAGGT-

GTGCACAAGTGAGC-3'. For amplification of *LOV1* from other ecotypes and *A. suecica*, the same primers were used except that LOV1b F and LOV2 R were used to amplify a longer segment in order to obtain the complete sequence of intron 1. To amplify *LOV1* from *A. korshinskyi*, primers used were LOV1b F and LOV2 R; LOV4 F and LOV4 R; Op1a F 5'-GCTAGACAAGGTTGACTTTGG-3' and Op1 R 5'-CAT-TCTCCACCAAATGACCA-3'; and Ak3 F 5'-GCATTGCTTCCTTTACCTAGC-3' and Ak3 R 5'-CCATAGATATATGTGTGAAAGG-3'. To amplify *LOV1* from *O. pumila*, primers used were LOV1b F and LOV1b R; LOV4 F and LOV4 R; Op1a F and Op1 R; Ak3 F and Ak3 R; and Op-gap F 5'-CCACTCTCGCAAGACAAGTC-3' and Op-gap R 5'-CTTCTCCACTATCTCGGATG-3'. The *ATTRX5* coding sequence was amplified as previously described (Sweat and Wolpert, 2007). All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) before sequencing. For the *lov1* mutant alleles, any alleles showing more than one mutation were re-amplified and sequenced to confirm both mutations. For the ecotype alleles, any unique polymorphism (present in only one ecotype) was confirmed by sequencing an independently generated PCR product.

Creation of plants carrying chimeric Cl-0/Col-4 *LOV1* genes

The chimeric Cl-0/Col-4 *LOV1* genes were constructed from a 6.5 kb *Xba*I fragment containing the entire *LOV1* gene in the binary vector pCB302 (Xiang et al., 1999). The *LOV1* gene contains a single *Nco*I site that occurs between the 621 A to T (nonsense mutation) and the 1767 G to A (missense mutation) polymorphisms in the Col-4 sequence and a single *Bam*HI site that occurs between the 2152 A to C (missense mutation) and the 3042 T deletion (frameshift mutation) polymorphisms. These restriction sites were used to remove portions of the Cl-0 *LOV1* gene from the 6.5 kb *Xba*I clone. Portions of the Col-4 *LOV1* gene were PCR-amplified from Col-4 genomic DNA, digested with the appropriate enzymes, and ligated into the 6.5 kb *Xba*I clone in place of the removed portions of the Cl-0 *LOV1* sequence. This resulted in creation of three clones: one containing the nonsense mutation, one containing both

missense mutations, and one containing the frameshift mutation. Clones were electroporated into *Agrobacterium tumefaciens* strain GV3101 and used to transform Col-4 *Arabidopsis* by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected in soil wet with 0.02% glufosinate-ammonium. Plants were tested for victorin sensitivity by the detached leaf assay.

Simple sequence length polymorphism analysis

The SSLP markers used to check for polymorphisms between the sensitive and insensitive No-0 individuals and the Col-4 ecotype were nga111, nga172, nga112, nga1126, CIW6, and CIW9. None of these markers are linked to *LOV1*. Primer sequences were obtained from The Arabidopsis Information Resource (TAIR) website (http://www.arabidopsis.org). PCR products were analyzed on a 3.5% agarose gel.

Population genetic analyses

Alignments of the *LOV1* alleles were created using ClustalX (Thompson et al., 1997). Population genetic analyses, including all calculations of polymorphism and divergence, were conducted using DnaSP 4.10 (Rozas et al., 2003). Statistical significance of K_a /K_s ratios was determined by comparison to confidence intervals obtained through Monte-Carlo simulation using the program K-estimator (Comeron, 1999). The neighbor-joining tree of *LOV1* alleles is a bootstrap consensus tree (500 replicates) created using *MEGA* 3.1 (Kumar et al., 2004) using the Kimura 2-parameter model and other default settings. It was created from an alignment of the entire *LOV1* sequenced region, including both coding and noncoding regions.

RESULTS

Loss-of-function *lov1* alleles

Characterization of the 59 previously isolated *lov1* mutants showed that most of the mutants were wild-type in appearance although a few had unusual morphologies or poor seed set, likely resulting from unrelated second-site mutations. The *LOV1*

coding region of each of these mutants was amplified by PCR and sequenced. Each allele was found to carry a single point mutation in the *LOV1* gene, with the exception of alleles *lov1-1* and *lov1-44*, which each contain two point mutations (Table 3.1). All mutations were G to A (or C to T) transition mutations, as is expected with EMS mutagenesis. Five alleles had mutations that result in creation of a premature stop codon. Two alleles, *lov1-1* and *lov1-51*, contain mutations at the junction between the first intron and second exon. Allele *lov1-51* has a G to A mutation in the last nucleotide of intron 1, while *lov1-1* has a mutation of the first G of exon 2. As these positions are highly conserved at splice junctions, both mutations likely interfere with correct splicing of exons 1 and 2. Therefore, the second mutation in *lov1-1*, which is in exon 3, was not included in further analysis because its effect on LOV1 function could not be independently evaluated. The remaining 52 alleles contain missense mutations that result in amino acid substitutions. As mentioned, *lov1-44* contains two point mutations, which both seem to have an effect on the loss-of-function phenotype (see below). This leaves 51 alleles in which a single amino acid change results in complete loss of LOV1 function. Some of these alleles were found to be identical, leaving 44 unique alleles affecting 42 amino acid residues.

Characterization of mutants with reduced sensitivity to victorin

In our mutant screen, we also isolated ten mutants with reduced sensitivity to victorin. These mutants were selfed and tested through at least three generations to ensure that the phenotype was consistent and not due to heterozygosity of a mutation that would otherwise cause complete loss of function. The reduced sensitivity mutants show no or only slight symptoms after infiltration of leaves on plants with victorin. All show some symptoms by 2 to 3 days in the more sensitive detached leaf assay, in which a leaf is removed and placed in 250 μL of 10 μg/mL victorin for 3 days, but symptom development is delayed in comparison to wild-type plants. Some mutants show only yellowing after treatment with victorin for three days (e.g. *lov1-61*), while others show tissue collapse that develops more slowly than in wild-type plants. These

Table 3.1. Mutations found in loss-of-function *lov1* alleles.

Allele	Mutant	Mutation	Amino Acid Change
Loss of Sensitivity			
Point mutations			
lov1-2	262A	2809 C to T	768 P to L
lov1-3	264A	298 G to A	9 G to E
lov1-5	273A	1816 C to T	437 P to L
lov1-6	276A	2326 G to A	607 G to E
lov1-7	277A	2284 C to T	593 S to F
lov1-8	278A	2971 G to A	822 G to D
lov1-9	280A	1863 G to A	453 A to T
lov1-10	283A	1615 C to T	370 A to V
lov1-11	288A	1945 G to A	480 R to K
lov1-12	289A	2452 C to T	649 P to L
lov1-13	290A	1914 G to A	470 G to R
lov1-14	298A	303 C to T	11 H to Y
lov1-15	300B	298 G to A	9 G to E
lov1-16	305A	334 C to T	21 S to F
lov1-17	306A	2503 C to T	666 S to L
lov1-18	319A	501 G to A	77 E to K
lov1-19	335A	2137 G to A	544 G to D
lov1-20	337A	732 C to T	154 R to W
lov1-21	341A	2277 G to A	591 D to N
lov1-22	352A	2146 C to T	547 A to V
lov1-23	361A	2122 G to A	539 R to K
lov1-24	367A	2788 C to T	761 A to V
lov1-25	368A	1690 G to A	395 G to E
lov1-26	369A	868 C to T	199 T to I
lov1-27	375A	298 G to A	9 G to E
lov1-28	384A	1629 G to A	375 G to R
lov1-30	401A	2400 C to T	632 L to F
lov1-32	407A	1339 G to A	306 G to E
lov1-33	408A	334 C to T	21 S to F
lov1-34	409A	2496 C to T	664 P to S
lov1-35	411A	2437 G to A	644 G to E
lov1-37	425A	1086 C to T	272 L to F
lov1-38	427A	2043 G to A	513 E to K
lov1-39	429A	1110 G to A	280 D to N
lov1-40	435A	1806 G to A	434 A to T
lov1-41	436A	1815 C to T	437 P to S
lov1-42	443A	922 G to A	217 G to E
lov1-43	462A	2026 G to A	507 C to Y

Table 3.1 (Continued).

Allele	Mutant	Mutation	Amino Acid Change
lov1-44	470A	411 G to A	47 D to N
		2287 G to A	594 R to Q
lov1-45	471A	2835 C to T	777 L to F
lov1-46	473A	2214 G to A	570 E to K
lov1-47	476A	2788 C to T	761 A to V
lov1-48	480A	745 C to T	158 A to V
lov1-49	481A	298 G to A	9 G to E
lov1-52	490A	2037 G to A	511 A to T
lov1-53	491A	847 G to A	192 G to E
lov1-54	495A	2400 C to T	632 L to F
lov1-55	498A	2935 C to T	810 A to V
lov1-56	509A	1915 G to A	470 G to E
lov1-57	510A	2889 C to T	795 L to F
lov1-58	511A	2503 C to T	666 S to L
lov1-59	512A	1575 G to A	357 G to R
Nonsense Mutations			
lov1-4	268A	1304 G to A	294 W to stop
lov1-29	396A	932 G to A	220 W to stop
lov1-31	406A	971 G to A	233 W to stop
lov1-36	421A	327 C to T	19 R to stop
lov1-50	482A	2100 C to T	532 R to stop
Splice Site Disruption			
lov1-1	1B	1300 G to A	293 G to D or splice
		2160 G to A	552 G to R
lov1-51	485A	1299 G to A	splice site
Reduced sensitivity			
lov1-60	296	2287 G to A	594 R to Q
lov1-61	344	2884 C to T	793 P to L
lov1-62	370	2116 C to T	537 S to F
lov1-63	398	1711 C to T	402 S to F
lov1-64	467	2145 G to A	547 A to T

mutants were not genetically characterized because it is difficult to distinguish reduced victorin sensitivity due to mutation versus reduced sensitivity due to heterozygosity of wild-type *LOV1* in segregation analyses. However, we sequenced the *LOV1* gene from each of these mutants and found a single point mutation in five of the ten alleles (Table 3.1). We cannot decisively say that these mutations are the cause of the reduced sensitivity. However, because the vast majority of loss-of-function mutations uncovered in our mutant screen were in the *LOV1* gene, this is a reasonable assumption. The only other gene that was identified in our mutant screen as being required for the response to victorin was *ATTRX5* (Sweat and Wolpert, 2007). This gene was also sequenced from each of these mutants, but no mutations were found in any of the reduced sensitivity mutants. Therefore, the mutations in these five *lov1* alleles were considered to cause partial loss of LOV1 function in further analysis. Interestingly, one of the alleles, *lov1-60*, has an R594Q mutation, which is the same as one of the mutations in allele *lov1-44*. Because *lov1-44* is a completely nonfunctional allele, the other mutation in this allele, D47N, must have at least a partial effect on LOV1 function. Therefore, D47N was considered as another partial loss-of-function mutation in further analysis, although it is possible that this mutation alone causes complete loss of function. Another reduced sensitivity allele, *lov1-64*, carries the mutation A547T. In *lov1-22*, the mutation A547V results in complete loss of function, indicating that mutation of this amino acid can result in different phenotypes depending on the nature of the substituted amino acid. The five mutants with reduced sensitivity that did not contain a mutation in *LOV1* (or *ATTRX5*) may contain mutations in other genes affecting victorin sensitivity, or they may have mutations in regulatory regions of *LOV1* or *ATTRX5*, such as the promoters, which were not sequenced in this study. Two of these five mutants also had unusual leaf morphologies, which may cause an apparent decrease in victorin sensitivity due to reduced uptake of victorin or other nonspecific effects.

Comparison of *lov1* versus *rpm1* mutant alleles

Although the *LOV1* gene confers victorin sensitivity and susceptibility to *C. victoriae* (Lorang et al., 2004), it belongs to the CC-NB-LRR class of genes, which are typically associated with resistance (J.M. Lorang, unpublished results). An extensive mutagenesis study has been performed with the *RPM1* resistance gene (Tornero et al., 2002), which is also a CC-NB-LRR gene. Therefore, we compared the spectrum of mutations uncovered in their work and in another study of *RPM1* (Grant et al., 1995) with the results of our study. We reasoned that if the *LOV1* gene is functioning in a manner analogous to a resistance gene, the loss-of-function alleles uncovered for *LOV1* should be similar in spectrum to the mutant alleles found to cause loss of RPM1 function. The *RPM1* studies uncovered 61 alleles with a single G to A or C to T mutation that resulted in complete loss of RPM1-dependent HR. The other mutations turned up in these studies were not of the type expected to be found in an EMS mutagenesis screen and were therefore not included in comparisons of *rpm1* and *lov1* mutations leading to loss of function. Of the 61 alleles, ten (eight unique alleles) had mutations resulting in premature stop codons. The remaining 51 alleles all contained single missense mutations. These represent 43 unique nucleotide changes affecting 39 amino acid residues. Tornero et al., (2002) also found a single point mutation in each of four partial loss-of-function *rpm1* alleles. Therefore, the number and type of mutant alleles found for *RPM1* versus *LOV1* are very similar, allowing a direct comparison of the distribution of mutations in these two genes.

In order to compare the spectrum of missense mutations resulting in loss of function in *lov1* versus *rpm1*, we plotted the number of loss-of-function alleles containing single amino acid substitutions in each 50 amino acid window across LOV1 (Figure 3.1A). For comparison, we performed an identical analysis for RPM1 (Figure 3.1B), similar to that performed by Tornero et al. (2002). To preserve as much information as possible and prevent skewing the data due to the isolation of multiple identical alleles, we counted the mutant alleles in three different ways: the total number of alleles, the number of unique alleles, and the number of amino acids
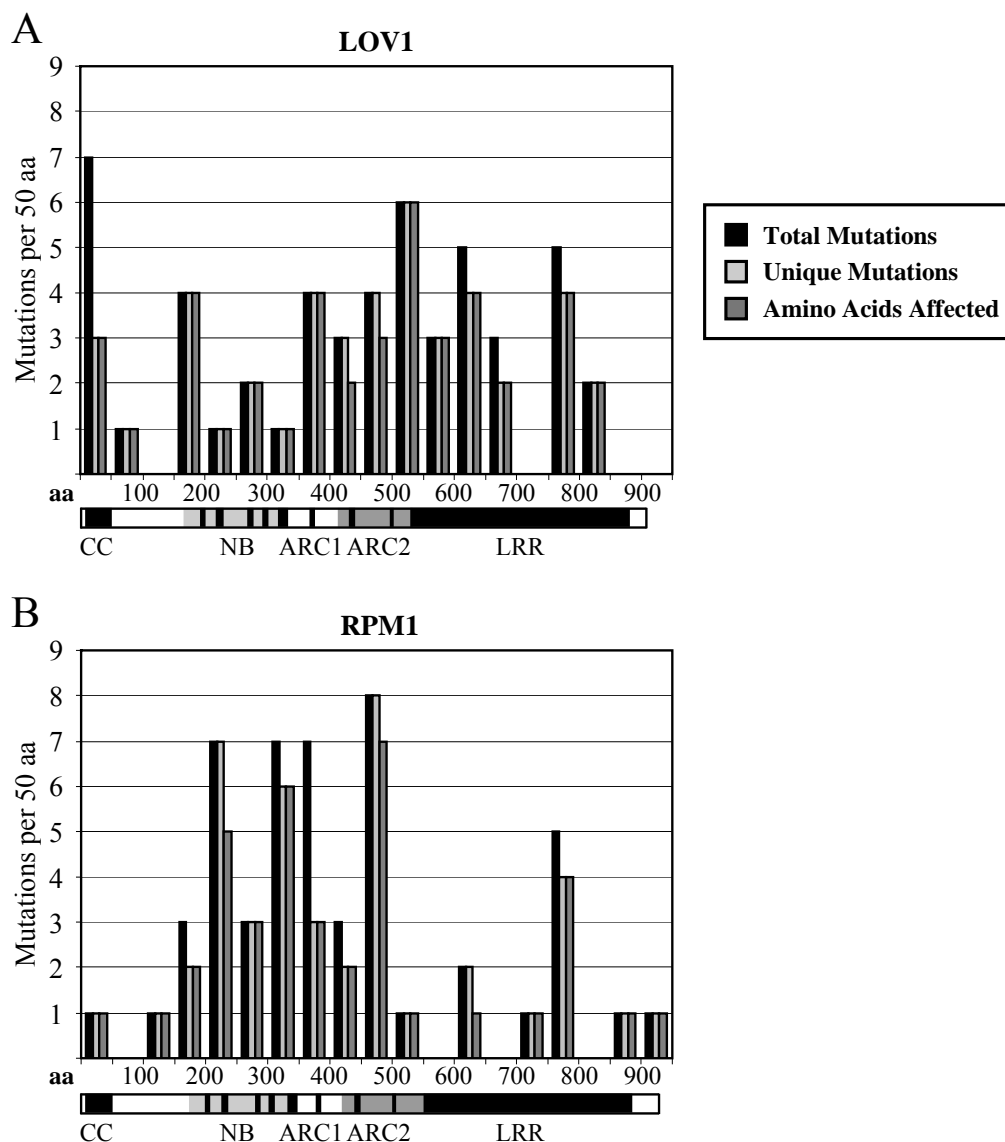
Figure 3.1. Number of mutations in each 50 amino acid window of LOV1 and RPM1.

The number of total and unique mutations and the number of amino acids affected in mutant alleles of A) LOV1 and B) RPM1 were plotted in 50 amino acid windows. Diagrams under each graph resprepsent the structure of each gene with the coiled-coil (CC), nucleotide-binding (NB), ARC1, ARC2, and leucine-rich repeat (LRR) regions indicated. The smaller black rectangles represent conserved motifs within the NB-ARC domain. From left to right they are the P-loop, RNBS-A, kinase 2, RNBS-B, RNBS-C, GLPL, RNBS-D, and MHD motifs.

affected by mutation in each window. These last two counts were only different in the few cases where two different nucleotide changes affected the same amino acid. In most cases, all three counts gave very similar numbers. However, there were notable exceptions, particularly the first window (1-50) for LOV1 and window 351-400 for RPM1. These each contained multiple mutations of a glycine codon (Gly 9 for LOV1 and Gly 384 for RPM1). EMS mutagenesis is known to exhibit site preferences, based primarily on the nucleotides at the -2 to +2 positions flanking the mutated G, possibly due to differing efficiencies of the mismatch repair system at these sites (Greene et al., 2003). For example, the nucleotide change in *LOV1* that results in the mutation of Gly 9 is in a particularly favorable context for EMS mutagenesis, as all nucleotides in the -2 to +2 positions are overrepresented in EMS-mutagenized populations. Therefore the affected guanine is expected to be mutated 2.7 times more often than an "average" G. In order to minimize the effects of EMS biases, we focused further analysis on the number of unique alleles.

The distribution of mutations in the *lov1* and *rpm1* alleles have some striking similarities. Both show a preponderance of mutations in the central portion of the protein with mostly low numbers of mutations at either end of the gene (Figure 3.1). Additionally, both have a peak of mutations in window 751-800 surrounded by windows with low numbers of mutant alleles. However, there are notable differences, such as the peak of mutations in the first window of *LOV1*, which includes the coiled-coil domain of this protein. There is also a skew in the area containing the most mutations. Both show a large number of mutations in the NB-ARC domain, but the *rpm1* alleles show a high number of mutations throughout the NB-ARC region with very few mutations in the first portion of the LRR. In contrast, the *lov1* alleles show a large number of mutations in the ARC1 and ARC2 domains, which are defined by their homology with the animal apoptosis regulators APAF-1 and CED-4 (Takken et al., 2006), and in the first portion of the LRR. LOV1 shows fewer mutations in the NB region compared to RPM1, although both genes show a peak of mutations in and around the P-loop at the beginning of the NB domain. A closer inspection of the

mutations in the NB-ARC domain shows that both genes have mutations in or immediately adjacent to the P-loop, kinase 2, GLPL, and RNBS-D motifs, which are conserved regions in the NB-ARC domain (Figure 3.2). The *lov1* alleles also included one mutation in the RNBS-A motif, while *RPM1* had mutations in the RNBS-B and RNBS-C motifs.

In several cases, amino acids that were in corresponding positions in an alignment of LOV1 and RPM1 were found to be mutated in loss-of-function alleles of both genes (Figure 3.2). These include LOV1 A370V (RPM1 A379V) in the GLPL motif and the nearby LOV1 G375R (RPM1 G384R); LOV1 A434T (RPM1 S439F) and LOV1 P437S/L (RPM1 P442L), both in the RNBS-D motif; LOV1 G470R/E (RPM1 A474T); LOV1 A511T (RPM1 S515F); and LOV1 L777F (RPM1 L781F). In two cases, an amino acid change in LOV1 that resulted in complete loss of function only resulted in partial loss of function when the corresponding amino acid was mutated in RPM1. These are LOV1 G192E (RPM1 G200E) in the P-loop and LOV1 G822D (RPM1 G824E). These nine amino acid positions that affect function of both LOV1 and RPM1 may be particularly important for the activity of CC-NB-LRR proteins, especially considering that four of them are in conserved motifs of the NB-ARC domain, and that two different amino acid substitutions at residues 437 and 470 both eliminated function of LOV1. Alternatively, some of these amino acids may be required for the stability of CC-NB-LRR proteins.

Comparison to *RPP8* family members

The genes most closely related to *LOV1* that are known to function in disease resistance are the *RPP8* family members. These include the genes *RPP8*, which confers resistance to *Hyaloperonospora parasitica* isolate Emco5 in the *Arabidopsis* ecotype Landsberg *erecta* (McDowell et al., 1998), *RCY1*, which confers resistance to the yellow strain of cucumber mosaic virus in ecotype C24 (Takahashi et al., 2002) and *HRT*, which is involved in resistance to turnip crinkle virus in the Dijon ecotype (Cooley et al., 2000). *RPP8*, *RCY1*, and *HRT* are allelic to each other and share 91-

Figure 3.2. Alignment of LOV1 and RPM1 protein sequences indicating position of substitutions causing loss of function of LOV1 and RPM1 and natural polymorphisms found in LOV1.

An amino acid alignment of LOV1 and RPM1 was created using ClustalW. The position of missense mutations leading to loss of function of each protein are indicated by triangles, black for complete loss of function and gray for partial loss of function. Arrows denote the positions of nonsynonymous polymorphisms found in *LOV1* alleles from different *A. thaliana* ecotypes in comparison to the Cl-0 allele used in the alignment. Thin arrows denote polymorphisms found in ecotypes that retain sensitivity to victorin. Bold arrows denote polymorphisms found only in insensitive ecotypes. Conserved motifs within the NB-ARC domain are marked by lines over the motifs. For this study, the major domains of LOV1 were considered to encompass the following residues: CC = 1-51; NB = 167-324; ARC1 = 325-412; ARC2 = 413-530; LRR = 531-878.

```
LOV1    1 MAEGVVLFGVHKLWELLNRESARLNGIGEQVDGLKRQLGRLQSLLKDADAKKHE------
RPM1    1 MASATVDFGIGRILSVLENETLLLSVHGEIDKMKKELLIMKSFLEDTHKHGGNGSTTTT

LOV1   55 SERVRNFLEDVRDIVYDAEDIIESFLLNEFRTKEKGIKKHARRLACFLVDRRKFASDIKG
RPM1   61 TQLFQTEVANTRDLAYQIEDILDEFGYHIHGYRSCAKIWRAFHFPRYMWARHSIAQKLGM

LOV1  115 ITKKISEVIGGMKSLGIQEIIDGASSMSLQE-RQREQKEIRQTFANSSESDLVGVEQSVE
RPM1  121 VNVMIQSISDSMKRYYHSENYQAALLPPIDDGDAKWVNNISESSLFFSENSLVGIDAPKG

                                        P-loop                    RNBS-A
LOV1  174 ALAGHLVEND-NIQVVSISGMGGIGKTTLARQVFHHDMVQRHFDGFAWVFVSQQFTQKHV
RPM1  181 KLIGRLLSPEPQRIVVAVVGMGGSGKTTLSANIFKSQSVRRHFEESYAWVTISKSYVIEDV

                                              Kinase 2
LOV1  233 WQRIWQELQPQNG-----DISHMDEHILQGKLFKLLETGRYLVVLDDVWKEEDWDRIKAV
RPM1  241 FRTMIKEFYKEADTQIPAELYSLGYRELVEKLVEYLQSKRYIVVLDDVWTTGLWREISIA

              RNBS-B                              RNBS-C
LOV1  288 FPR-KRGWKMLLTSRNEGVG-IHADPKSFGFKTRILTPEESWKLCEKIVFHRRDETGTLS
RPM1  301 LPDGIYGSRVMMTTRDMNVASFPYGIGSTKHEIELLKEDEAWVLFSNKAFP-----ASLE

                              GLPL
LOV1  346 EVRVDEDMEAMGKEMVTCCGGGLPLAVKVLGGLLATKHTVPEWKRVYDNIGPHLAGRSSLD
RPM1  356 QCRTQN-LEPIARKLVERCQGLPLAIASLGSMMSTKKFESEWKKVYSTLNWELNN----N

                                  RNBS-D
LOV1  406 DNLNSIYRVLSLSYEDLPMCLKHCFLYLAHFPEYYEIHVKRLFNYLAAEGIITSSDDGTT
RPM1  411 HELKIVRSIMFLSFNDLPYPLKRCFLYCSLFPVNYRMKRKRLIRMWMAQRFVEPIR-GVK

                                          MHD
LOV1  466 IQDKGEDYLEELARRNMITIDKNYMFLRKKHCQMHDMMREVCLSKAKEENFLEIFK-VST
RPM1  470 AEEVADSYLNELVYRNMLQVILWNPFGRPKAFKMHDVIWEIALSVSKLERFCDVYNDDSD

LOV1  525 ATSAINARSLSKSRRLSVHGGNALQSLGQTINKKVRSLLYFAFEDEFCILESTTPCFRSL
RPM1  530 GDDAAETMENYGSRHLCIQKEMTPDSIRATN---LHSLLVCSSAKHKMELLP------SL

LOV1  585 PLLRVLDLSRVKFEGGKLPSSIGDLIHLRFLSLHRAWISHLPSSLRNLKLLLYLNLGFNG
RPM1  581 NLLRALDLEDSSIS--KLPDCLVTMFNLKYLNLSKTQVKELPKNFHKLVNLETLNTKHSK

LOV1  645 MVHVPNVLKEMQELRYLQLP-----------MSMHDKTKLELSDLVNLESLMNFSTKYAS
RPM1  639 IEELPLGMWKLKKLRYLITFRRNEGHDSNWNYVLGTRVVPKIWQLKDLQVMDCFNAEDEL

LOV1  694 VMDLLHMTKLRELSLFITDGSS-DTLSSSLGQLRSLEVLHLYDRQEPRVAYHGGEIVLNC
RPM1  699 IKNLGCMTQLTRISLVMVRREHGRDLCDSLNKIKRIRFLSLTSIDEE--EPLEIDDLIAT

LOV1  753 IHLKELELAIHMPRFPDQYLFHPHLSHIYLWCCSMEEDPTPILERLLHLKSVILTFGAFV
RPM1  757 ASIEKLFLAGKLERVPSWFNTLQNLTYLGLRGSQLQENAILSIQTLPRLVWLSFYN-AYM

LOV1  813 GRRMVCSKGGFPQLCFLKLEELEELEEWIVEEGSMPLLRALTICNCRKLK-LPGGINYIT
RPM1  816 GPRLRFAQ-GFQNLKILEIVQMKHLTEVVIEDGAMFELQKLYVRACRGLEYVPRGIENLI

LOV1  872 SLKELTIVGMKWKEKLVPGGE---DYYKVQNIPNVQFINCDE----------
RPM1  875 NLQELHLIHVSNQLVERIRGEGSVDRSRVKHIPAIKHYFRTDNGSFYVSLSS
```
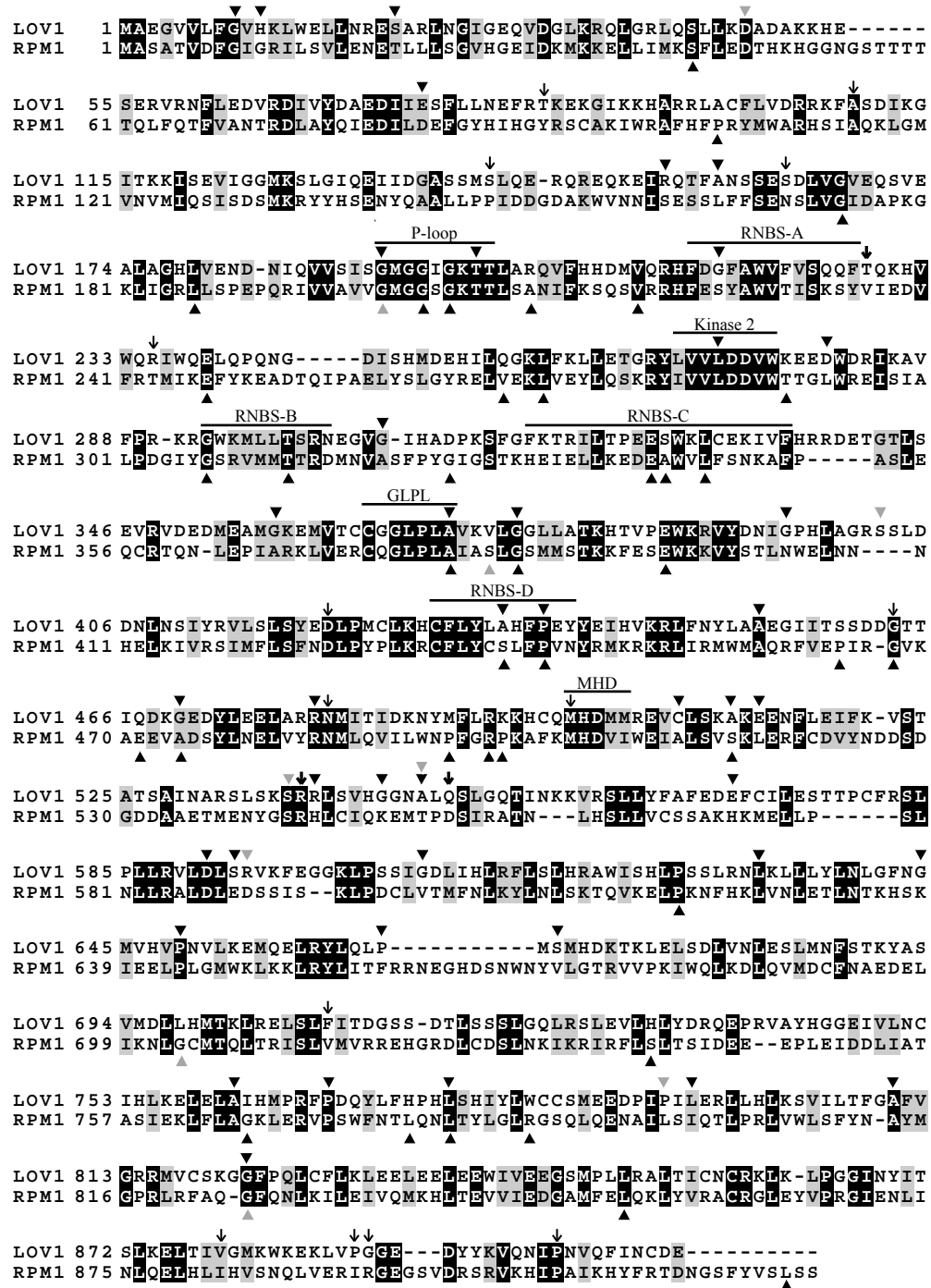
Figure 3.2. Alignment of LOV1 and RPM1 protein sequences indicating position of substitutions causing loss of function of LOV1 and RPM1 and natural polymorphisms found in LOV1.

92% amino acid identity (94-95% similarity) in pairwise alignments. *LOV1* is not allelic to these three genes, but shows approximately 70% identity (80% similarity) to each of these genes at the amino acid level. An alignment of the RPP8 family members with LOV1 shows that of the 910 amino acid residues in the LOV1 coding region, 615 (67.6%) are identical between LOV1 and all three of the RPP8 family members. These conserved residues accounted for 35 out of 42 (83.3%) of the residues that were mutated in the *lov1* loss-of-function alleles. Conversely, 195 LOV1 residues (21.4%) are not identical to the corresponding residue in any of the other three proteins. However, these residues accounted for only five (11.9%) of the mutated residues in the loss-of-function alleles. The remaining 2 mutations were in amino acid residues identical between LOV1 and at least one of the RPP8 family members. This skew towards mutation of conserved residues suggests that most of the mutations uncovered in this study might result in loss of function of any of the RPP8 family members and may affect conserved resistance gene functions or protein stability. In contrast, the five mutations in residues unique to LOV1 may define amino acids specifically important for direct or indirect perception of victorin or for interaction with unique downstream signaling partners. These five mutations (H11Y, A158V, G544D, G644E, and S666L) were not clustered together in the protein sequence. However, three of them are located in the LRR region of the protein, which is generally the most variable portion of resistance genes and in several cases has been found to determine resistance gene specificity (Ellis et al., 1999; Dodds et al., 2001; Zhou et al., 2006), and one is located in the CC domain, which is thought to be involved in interaction with signaling partners (Belkhadir et al., 2004).

Sampling of natural *LOV1* allelic diversity

The original study that identified victorin sensitivity in *Arabidopsis* suggested that sensitivity is relatively rare with only six out of 433 seed populations characterized as containing individuals that were clearly sensitive to victorin (Lorang et al., 2004). However, we have since determined that seedlings are typically much

less sensitive to victorin than adult plants (Sweat and Wolpert, 2007). As seedlings were employed in the initial screen, this raised the possibility that many sensitive ecotypes were overlooked. Therefore, in addition to the original six sensitive ecotypes and the insensitive ecotype Col-4 reported in the first study, we obtained seed from 23 additional ecotypes and tested all 30 ecotypes by the detached leaf assay, in which a leaf from a mature plant is removed and placed in 250 µL of 10 µg/mL victorin for 3 days. At least eight individuals of each ecotype were tested except in the few cases where poor seed germination precluded screening eight individuals. Leaves were scored for symptom development at 1, 2, and 3 days after victorin treatment. Somewhat surprisingly, we found that nearly all ecotypes showed some level of sensitivity to victorin (Figure 3.3). The only plants that were completely insensitive were all individuals of the Col-4 and Bay-0 ecotypes and some individuals of the No-0 ecotype, which was the only ecotype found to have both victorin sensitive and insensitive individuals. The sensitive ecotypes were compared to the ecotype Cl-0, from which the *LOV1* gene was originally cloned (J.M. Lorang, unpublished results) in order to evaluate levels of sensitivity to victorin. Because the timing of the response to victorin tends to be somewhat variable between experiments, we only attempted to define two categories of sensitivity. Nine ecotypes reproducibly showed low to moderate levels of sensitivity (mS), showing no or only very slight symptoms until at least 2 days after treatment with victorin and usually showing only yellowing or partial collapse on day 3. The remaining ecotypes consistently showed visible symptoms by day 1, similar to Cl-0, and generally showed complete collapse of the leaf by day 3. Not surprisingly, five of the six accessions originally described as being sensitive to victorin (Lorang et al., 2004) fell into the highly sensitive (S) category. We also tested for victorin sensitivity in the related species *Arabidopsis suecica*, *Arabidopsis korshinskyi*, *Olimarabidopsis pumila*, *Arabidopsis arenosa*, *Crucihimalaya lasiocarpa*, and *Capsella rubella*. All other species tested were completely insensitive to victorin with the exception of *A. suecica*, which showed very low levels of sensitivity.

Figure 3.3. Neighbor-joining tree of *LOV1* alleles.

Neighbor-joining tree (500 bootstraps) based on the entire sequenced region (coding and noncoding) of *LOV1*. The victorin sensitivity phenotype of each ecotype is indicated in bold as follows: I (insensitive), mS (moderately sensitive), S (highly sensitive). The origin of each ecotype is also indicated. Numbers above each branch indicate the number of mutations with the number of nonsynonymous substitutions in parentheses; bootstrap values >80% are indicated below the branches in italics.
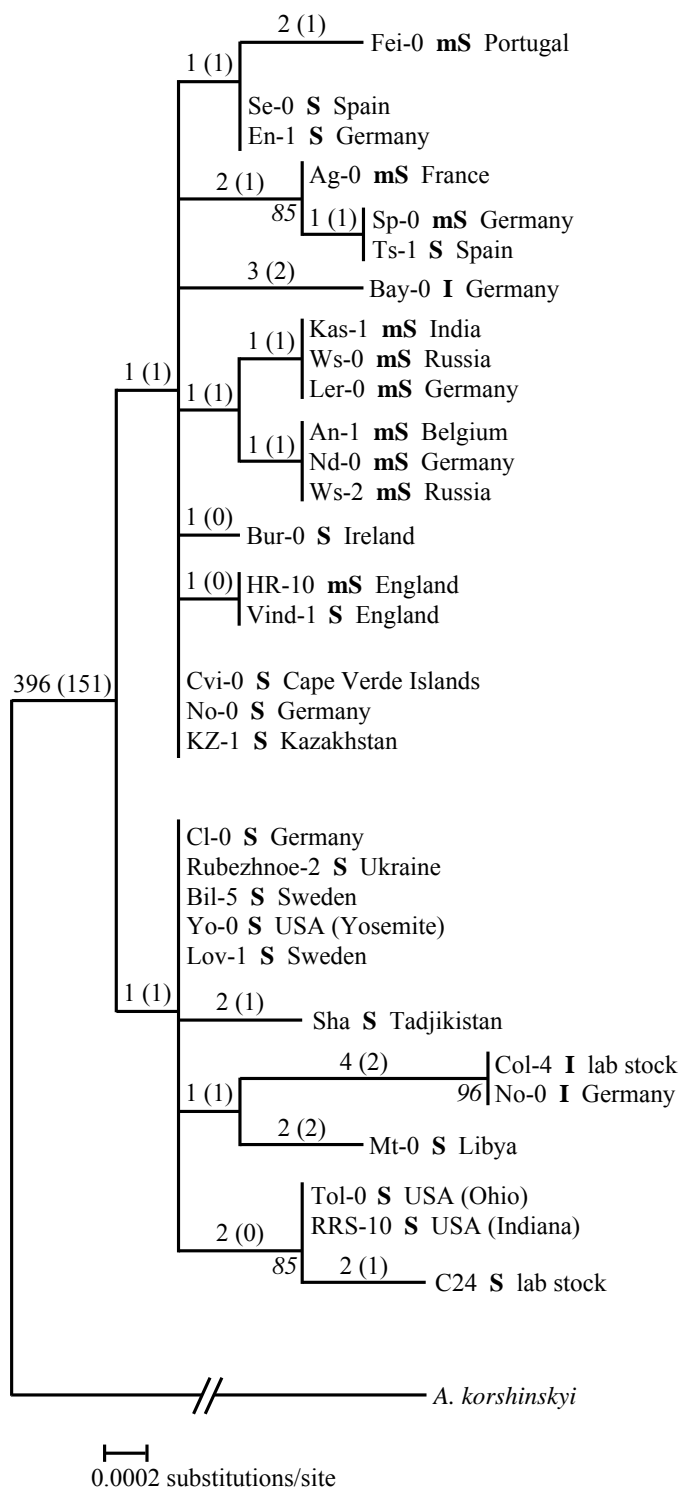
Figure 3.3. Neighbor-joining tree of *LOV1* alleles.

The *LOV1* gene was sequenced from each of the evaluated ecotypes and the polymorphisms present were compared to the Cl-0 allele (Table 3.2). The sequenced region spanned from 272 base pairs upstream of the start codon to 149 nucleotides downstream of the stop codon (3,388 base pairs total). Four ecotypes (Rubezhnoe-2, Bil-5, Yo-0, and Lov-1) were found to carry an allele identical to the Cl-0 allele and the allele found in two other ecotypes (Tol-0 and RRS-10) differed only by silent changes. The other sensitive ecotypes had two to five polymorphisms compared to Cl-0, causing one to four amino acid substitutions. The Col-4 allele, which is identical to the published Col-0 sequence, showed 6 nucleotide differences when compared to the Cl-0 *LOV1* gene (J.M. Lorang, unpublished results). The insensitive individuals from the No-0 ecotype had an allele identical to that of Col-4. We therefore considered the possibility that these insensitive No-0 individuals could be the result of seed contamination from Col-0 or Col-4. We analyzed six simple sequence length polymorphisms (SSLPs) scattered throughout the genome (see Materials and Methods) and compared the SSLP pattern of the sensitive and insensitive No-0 individuals with each other and with Col-4. One of the SSLP markers was the same size in all three individuals. The remaining SSLPs were all polymorphic between Col-4 and both No-0 individuals. In contrast, four of the five gave PCR products of the same size between the No-0 individuals while the fifth (CIW6) showed an apparent slight size difference between the sensitive and insensitive individuals (data not shown). These data suggest that the insensitive and sensitive No-0 individuals are related and not due to seed contamination. The only other insensitive ecotype, Bay-0, has an allele that encodes a full-length protein with five nucleotide polymorphisms compared to Cl-0.

Evaluation of the insensitive alleles

Of the six nucleotide changes present between the Col-4 and Cl-0 *LOV1* alleles, two do not affect the amino acid sequence (Table 3.2). Of the remaining four differences, two result in amino acid substitutions (D421N and Q549P). The other two differences cause truncation of the protein. The 621 A to T mutation results in a

Table 3.2. Polymorphisms found in *LOV1* alleles from various ecotypes compared to the Cl-0 *LOV1* allele.

Nucleotide	Amino Acid Change	Ecotype(s)
73 T to G	none (5' noncoding)	Bay-0
83 A to G	none (5' noncoding)	Tol-0[a], RRS-10[a], C24
99 T to A	none (5' noncoding)	Sp-0[b], Ts-1[b], Ag-0
121 A deleted	none (5' noncoding)	Cvi-0[c], No-0(S)[c], KZ-1[c], Bur-0
191 C to T	none (5' noncoding)	C24
528 A to G	86 T to A	Bay-0, Ler-0[d], Kas-1[d], Ws-0[d], Nd-0[e], Ws-2[e], An-1[e], Sp-0[b], Ts-1[b], Ag-0, Cvi-0[c], No-0(S)[c], KZ-1[c], Bur-0, En-1[f], Se-0[f], Fei-0, HR-10[g], Vind-1[g]
598 C to A	109 A to D	Bay-0, Ler-0[d], Kas-1[d], Ws-0[d], Nd-0[e], Ws-2[e], An-1[e], Sp-0[b], Ts-1[b], Ag-0, Cvi-0[c], No-0(S)[c], KZ-1[c], Bur-0, En-1[f], Se-0[f], Fei-0, HR-10[g], Vind-1[g]
621 A to T	117 K to stop	Col-0[h], No-0(R)[h]
696 T to C	142 S to P	Mt-0
760 G to T	163 S to I	Sp-0[b], Ts-1[b]
806 T to C	none (synonymous)	Col-0[h], No-0(R)[h]
954 A to G	228 T to A	Bay-0
977 G to T	235 R to S	Ler-0[d], Kas-1[d], Ws-0[d]
1276 C to G	none (intron)	Col-0[h], No-0(R)[h]
1715 C to T	none (synonymous)	Bur-0
1767 G to A	421 D to N	Col-0[h], No-0(R)[h], Mt-0
1894 G to A	463 G to E	Sha
1947 A to C	481 N to H	Ler-0[d], Kas-1[d], Ws-0[d], Nd-0[e], Ws-2[e], An-1[e]
2003 G to A	499 M to I	Fei-0
2118 C to A	538 R to S	Bay-0
2152 A to C	549 Q to P	Col-0[h], No-0(R)[h]
2387 T to A	none (synonymous)	HR-10[g], Vind-1[g]
2632 T to A	709 F to Y	C24
2681 G to A	none (synonymous)	Tol-0[a], RRS-10[a], C24
3042 T deleted	846 frameshift	Col-0[h], No-0(R)[h]
3141 G to A	879 V to I	Nd-0[e], Ws-2[e], An-1[e]
3158 G to A	none (synonymous)	Fei-0
3172 C to T	889 P to L	En-1[f], Se-0[f], Fei-0
3175 G to A	890 G to D	Mt-0
3208 C to G	901 P to R	Sp-0[b], Ts-1[b], Ag-0
3276 T to C	none (3' noncoding)	Sha
none	none	Cl-0[i], Rubezhnoe-2[i], Bil-5[i], Yo-0[i], Lov-1[i]

[a-i]Letters denote ecotypes with identical alleles.

stop codon at lysine 117 and the deletion of a nucleotide at 3042 results in a frameshift at codon 846, which would cause the addition of seven incorrect amino acids and a premature stop. Portions of the Col-4 *lov1* allele were swapped with portions of the Cl-0 *LOV1* gene in order to assess the individual effects of each of the four differences that result in an altered protein product. It was found that either of the nucleotide substitutions causing truncation of the N- or C-terminus of the protein were sufficient to eliminate LOV1 function when present individually. In order to evaluate the effects of the two amino acid substitutions, the central portion of the Cl-0 *LOV1* gene was replaced with the portion of the Col-4 allele carrying the two differences. This was also found to eliminate LOV1 function. These base pair changes were not evaluated individually in this experiment, and these two changes together may result in the observed loss of sensitivity. However, the D421N amino acid substitution was also found to be present in ecotype Mt-0, which is sensitive to victorin. Therefore, the Q549P change appears to be responsible for the loss of sensitivity, which is not unexpected given that it is a very nonconservative change and is in a region near other mutations that were found to result in loss of LOV1 function (Figure 3.2). Four of the five polymorphisms in the Bay-0 ecotype result in amino acid substitutions. Of these, two (T86A and A109D) are present in many other ecotypes belonging to both the S and mS phenotypic groups and apparently do not reduce sensitivity to victorin (Table 3.2). Of the other two amino acid changes, one (R538S) is between two amino acids found to affect victorin sensitivity when mutated, partial loss in the case of S537F and complete loss for R539K (Figure 3.2). This suggests that the R538S substitution may be responsible for the insensitive phenotype of Bay-0. However, the other substitution (T228A) in the Bay-0 allele is immediately adjacent to the RNBS-A domain and could lead to loss of function either on its own or in combination with R538S.

Polymorphisms in sensitive alleles

The phylogeny of *LOV1* alleles shows two major clades (Figure 3.3). However, these are separated by only two polymorphisms, both of which cause amino

acid substitutions (T86A and A109D) (Table 3.2). As mentioned above, these two polymorphisms are present in alleles belonging to each phenotypic class (S, mS, and I) and therefore do not appear to affect the victorin response. All ecotypes belonging to the mS group are found in the upper clade, but these *LOV1* alleles all contain additional polymorphisms that likely play a role in reducing sensitivity (Figure 3.3 and Table 3.2). In two cases (Sp-0/Ts-1 and HR-10/Vind-1), ecotypes with identical alleles show different levels of sensitivity. This could be due to differences in *LOV1* expression between the ecotypes or differences in other genes that may modify the phenotype. It is also possible that differences in plant morphology may cause a decrease in the appearance of symptoms due to effects not specific to the victorin response pathway. A similar situation was previously observed when the Cl-0 *LOV1* allele was introgressed into the Col-4 background. This line, Col-LOV, showed slightly reduced sensitivity to victorin compared to the Cl-0 parent, indicating that ecotype background differences can influence victorin sensitivity (Sweat and Wolpert, 2007). The HR-10/Vind-1 allele is also identical to the Bur-0, Cvi-0, No-0 (S), and KZ-1 alleles at the amino acid level. As all the other ecotypes fall into the S category, it appears that HR-10 may have reduced expression or other background differences that result in reduced sensitivity to victorin. However, in the case of the clade containing six moderately sensitive (mS) ecotypes, Kas-1, Ws-0, Ler-0, An-1, Nd-0, and Ws-2, the difference in sensitivity is likely caused by the N481H polymorphism present in all of these ecotypes, which is adjacent to the R480K loss-of-function mutation found in allele *lov1-11*, although each ecotype also contains another nonsynonymous polymorphism that could contribute to the phenotype. The moderately sensitive phenotype of the Fei-0 ecotype is likely due to the M499I polymorphism, which falls within the conserved MHD motif. None of the ecotypes tested showed polymorphisms at any of the amino acid residues mutated in the LOV1 loss-of-function screen (Figure 3.2), but the Sha ecotype showed a G463E polymorphism at the residue corresponding to the G467R substitution that resulted in loss of RPM1 function (Tornero et al., 2002). However, the adjacent residues are not

conserved between LOV1 and RPM1 so the substituted residue may fulfill different roles in the two proteins.

Geographic differentiation

We noted three pairs of ecotypes from the same region that share identical *LOV1* alleles, Lov-1 and Bil-5 from northern Sweden, Tol-0 and RRS-10 from the Midwestern United States, and HR-10 and Vind-1 from England (Figure 3.3). This is in line with work showing that individuals collected from different populations within a region tend to be more related than individuals from different geographic regions (Nordborg et al., 2005; Bakker et al., 2006a). This was particularly noted with populations from the Midwestern USA, which tend to be highly related. However, in general our results do not show a strong geographic correlation as many of the alleles are present in ecotypes from very diverse geographic regions. This is also consistent with previous studies, which found that most genetic variation occurs within geographic regions (Nordborg et al., 2005; Bakker et al., 2006a), such that geographic structure would not be expected to be evident in studies focusing on relatively small samples and a single gene. A similar study of the *RPS2* resistance gene also did not find any allelic differentiation among regions (Mauricio et al., 2003).

Sequencing of *LOV1* from other species

Using the original sets of PCR primers (see Materials and Methods), the only species besides *A. thaliana* to give a complete *LOV1* sequence was *A. suecica*, which was also the only other species to show a response to victorin. We sequenced the *LOV1* allele from three accessions of *A. suecica* (CS22505, CS22507, and CS22511) and found all three to be identical to the allele from the *A. thaliana* Sha ecotype (Table 3.2). However, while the Sha ecotype shows high levels of sensitivity, *A. suecica* is only slightly sensitive to victorin. *A. suecica* accession CS22511 showed a somewhat stronger response to victorin than the other two accessions but was still reduced in comparison to the Sha ecotype. Whether these variations in sensitivity are due to

different levels of gene expression or to the presence of other genes that modify the phenotype is unknown. We also sequenced the *ATTRX5* gene, which is required for the victorin response (Sweat and Wolpert, 2007), from *A. suecica* and found that it is identical to that found in both the Col-4 and Cl-0 ecotypes. The high similarity of the *LOV1* gene between *A. suecica* and *A. thaliana* alleles is likely due to the origin of *A. suecica* as an allopolyploid between *A. thaliana* and *A. arenosa* (Mummenhoff and Hurka, 1995). As we were unable to amplify the complete *A. arenosa LOV1* allele with our primers, the product obtained from *A. suecica* likely came from the *A. thaliana* ancestral parent.

Because the *A. suecica* allele does not represent a true outgroup, we used partial *LOV1* sequences obtained from *A. korshinskyi* to design new PCR primers to amplify the remainder of the *LOV1* sequence. We chose this species because the initial PCR of this allele resulted in the most successful amplification products of all the tested species. Using newly designed primers, we were also able to amplify the entire *LOV1* allele from *Olimarabidopsis pumila* and found it to be nearly identical to the *A. korshinskyi* allele, differing at only seven nucleotide positions. The *A. korshinskyi* allele is 85.5% identical to the Cl-0 allele across the entire sequenced region at the nucleotide level and 82.8% identical (89.9% similar) at the amino acid level. Excluding sites with gaps, there are 257 fixed differences in the coding sequence between *A. korshinskyi* and all *A. thaliana LOV1* alleles. These include 106 synonymous and 151 nonsynonymous (amino acid changing) fixed differences. The *A. korshinskyi* allele encodes a full-length protein and the domains known to be critical for NB-LRR gene function (e.g. the P-loop and kinase 2 motifs) appear to be intact. Therefore, the *A. korshinskyi* allele appears to encode a functional NB-LRR protein, which may be diverged sufficiently to prevent functioning in the victorin response pathway. In support of this is the observation that three residues found to cause partial or complete loss of victorin sensitivity when mutated in the *A. thaliana* Cl-0 allele are substituted in the *A. korshinskyi* sequence. One of these residues is located in the coiled-coil domain and the other two are in the LRR region and could

therefore disrupt victorin recognition or signaling pathways, as these regions are thought to be important for determining recognition specificity and interacting with signaling proteins (Belkhadir et al., 2004).

Population genetic analysis of *LOV1* alleles

Within the *A. thaliana* ecotypes there are 29 polymorphic sites, excluding the 2 sites with gaps (Tables 3.2 and 3.3). These consist of 18 nonsynonymous (amino acid changing) polymorphisms, 5 synonymous changes, and 6 polymorphisms that fall outside the coding region. When standardized for the sequence length (3,386 base pairs excluding gaps), this gives a value of 0.0086 segregating sites per base pair (Table 3.3). This is a relatively low number of segregating sites compared to that seen in other NB-LRR genes previously studied in *Arabidopsis* (Mauricio et al., 2003; Rose et al., 2004; Bakker et al., 2006b). However, these segregating sites result in 16 different haplotypes at the nucleotide level encoding 13 protein variants. This is also reflected in the nucleotide diversity (π), which is on the low end of the spectrum compared to other NB-LRR genes (Mauricio et al., 2003; Rose et al., 2004; Bakker et al., 2006b). Over the entire sequenced region $\pi = 0.00126$ and in the coding region $\pi = 0.00131$ (Table 3.3). At nonsynonymous sites $\pi_a = 0.00143$, while $\pi_s = 0.00093$ at synonymous sites, indicating an excess of nonsynonymous compared to synonymous diversity ($\pi_a/\pi_s = 1.54$), in line with the relatively large number of encoded protein variants. Dividing the *LOV1* gene into functional regions showed that the highest nucleotide diversity at nonsynonymous sites (π_a) fell in regions outside of those with well-characterized roles in NB-LRR gene function. In particular, $\pi_a = 0.00477$ between the CC and NB domains and $\pi_a = 0.00798$ between the last structural residue of the LRR region and the end of the protein. The ARC2 region also showed a higher than average nucleotide diversity at nonsynonymous sites ($\pi_a = 0.00228$). In contrast, the CC, NB, ARC1, and LRR domains showed relatively low values of nonsynonymous nucleotide diversity ($\pi_a = 0$-0.00066). Pairwise K_a/K_s comparisons, excluding the truncated Col-4 and No-0 (I) alleles and any pairwise comparisons with

Table 3.3. Nucleotide diversity and segregating sites in functional regions of *LOV1*.

Region	Position	Length[a]	π[b]	S_{total}[c]	π_{silent}[b]	S_{silent}[c]	π_{nonsyn}[b]	S_{nonsyn}[c]
Entire region	1-3388	3386	0.00126	29 (0.0086)	0.00098	11	0.00143	18
Coding	273-3236	2729	0.00131	23 (0.0084)	0.00093	5	0.00143	18
Coiled-coil	273-425	153	0.00000	0 (0.0000)	0.00000	0	0.00000	0
CC to NB	426-770	345	0.00375	5 (0.0145)	0.00000	0	0.00477	5
NB	771-1149 + 1300-1394	474	0.00078	3 (0.0063)	0.00126	1	0.00066	2
ARC1	1395-1458 + 1543-1742	264	0.00024	1 (0.0038)	0.00111	1	0.00000	0
ARC2	1743-2096	354	0.00179	4 (0.0113)	0.00000	0	0.00228	4
LRR	2097-3140	1043	0.00054	5 (0.0048)	0.00127	2	0.00032	3
LRR to stop	3141-3236	96	0.00699	5 (0.0521)	0.00346	1	0.00798	4
5' noncoding	1-272	271	0.00181	4 (0.0148)	0.00181	4	NA	NA
Intron 1	1150-1299	150	0.00083	1 (0.0067)	0.00083	1	NA	NA
Intron 2	1459-1542	84	0.00000	0 (0.0000)	0.00000	0	NA	NA
3' noncoding	3237-3388	152	0.00042	1 (0.0066)	0.00042	1	NA	NA

[a]Length in base pairs, excluding sites with gaps.
[b]Nucleotide diversity, as determined by DnaSP 4.10 (Rozas et al., 2003).
[c]Number of segregating sites. Numbers in parentheses are standardized by sequence length.

$K_s = 0$, showed the highest K_a/K_s ratio ($K_a/K_s = 2.06$) between the Mt-0 and Fei-0 alleles. Although this ratio significantly exceeds the threshold of $K_a/K_s > 1$, as identified by K-estimator (Comeron, 1999), which is considered to be a sign of balancing selection within a species, the result was not significant after Bonferroni correction, which accounts for the number of pairs tested. This lack of statistical significance is likely due to the low overall levels of polymorphism in the gene.

Analysis of K_a/K_s ratios between *A. korshinskyi* and *A. thaliana* alleles gave a Jukes Cantor corrected $K_a = 0.0769$ and $K_s = 0.2000$ ($K_a/K_s = 0.3842$) in the coding region (Table 3.4). A McDonald-Kreitman test of intraspecies polymorphism versus divergence between species at synonymous and nonsynonymous sites (McDonald and Kreitman, 1991) fails to reject the hypothesis of neutral evolution (p = 0.077), again likely due to the low numbers of polymorphisms present in the *LOV1 A. thaliana* alleles. In the between species comparison, no functional region was observed to have a K_a/K_s ratio >1 and the LRR region showed a slightly lower than average K_a/K_s ratio of 0.3473 (Table 3.4). The solvent-exposed, hypervariable residues (x) of the xxLxLxx motif are thought to be important for mediating the specificity of protein interactions with the LRR (Jones and Jones, 1997) and show evidence of being under positive selection in many resistance genes (Bergelson et al., 2001; Mondragón-Palomino et al., 2002). For *LOV1*, the interspecies K_a/K_s ratio equals 0.933 for these residues, which is much higher than the average across the entire gene and the LRR as a whole, but still below the threshold of $K_a/K_s > 1$ indicative of positive selection. Relatively high K_a/K_s ratios were also observed for the region between the CC and NB domains and for the ARC1 and ARC2 regions. This is somewhat similar to the within species comparisons in that lower levels of nonsynonymous substitutions were observed in the CC, NB, and LRR regions. For the intraspecies data, there were too few polymorphisms to determine whether the level of nonsynonymous polymorphisms is elevated in the solvent-exposed residues of the xxLxLxx motif.

Table 3.4. Analysis of divergence between *A. thaliana* and *A. korshinskyi LOV1* alleles.

Region	Position	N_s[a]	D_s[b]	K_s(JC)[c]	N_a[a]	D_a[b]	K_a(JC)[c]	D_{xy}[d]	K_a/K_s
Coding	1-2772	599.7	106	0.2000	2109.3	151	0.0769	0.0961	0.3842
Coiled-coil	1-153	37.3	8	0.2526	115.7	4	0.0354	0.0784	0.1402
CC to NB	154-498	71.9	10	0.1538	273.1	21	0.0859	0.0932	0.5583
NB	499-972	99.9	15	0.1684	374.1	24	0.0674	0.0827	0.4004
ARC1	973-1239	55.1	7	0.1291	196.9	13	0.0719	0.0795	0.5567
ARC2	1240-1596	75.2	16	0.2411	275.8	28	0.1127	0.1281	0.4674
LRR	1597-2670	241.2	46	0.2210	796.8	58	0.0768	0.1010	0.3473
LRR to stop	2671-2772	19.2	4	0.2472	76.9	3	0.0445	0.0766	0.1801

[a]Estimated number of synonymous (N_s) and nonsynonymous (N_a) sites.

[b]Number of fixed differences between species at synonymous (D_s) and nonsynonymous (D_a) sites.

[c]Jukes-Cantor corrected synonymous differences per synonymous site (K_s) and nonsynonymous differences per nonsynonymous site (K_a).

[d]Average number of nucleotide substitutions per site between species.

DISCUSSION

Several lines of evidence suggest that LOV1, although it confers susceptibility to infection by the fungus *C. victoriae*, functions in a manner analogous to a resistance protein. As described above, the spectrum of mutations found to result in loss of function of LOV1 (Table 3.1 and Figures 3.1 and 3.2) is similar to those found to disrupt the function of RPM1, another CC-NB-LRR protein (Tornero et al., 2002). In particular, both studies uncovered mutations in many of the domains of the NB-ARC region that are conserved among NB-LRR proteins (Figure 3.2). Also, several residues at corresponding positions of LOV1 and RPM1 were observed to be mutated in both studies, suggesting that these two proteins may function in a similar manner. Additionally, LOV1 is highly related to the RPP8 family members (RPP8, RCY1, and HRT), which all have known functions in resistance (McDowell et al., 1998; Cooley et al., 2000; Takahashi et al., 2002), and the residues that were found to be substituted in the nonfunctional *lov1* mutants were skewed towards residues that are conserved between LOV1 and all three of the RPP8 family members.

The results obtained with LOV1 are consistent with the results of mutagenesis screens performed on other NB-LRR genes that function in resistance, further suggesting that LOV1 functions in a manner analogous to resistance proteins. Therefore, our mutagenesis study of LOV1 adds to the data from other studies that have helped elucidate the structure-function relationship of NB-LRR proteins. For example, the 2 mutations in the P-loop of LOV1 that resulted in loss of function (G192E and T199I) also resulted in loss of function when the corresponding amino acids were mutated in the TIR-NB-LRR tobacco *N* gene product (G216A/E/V/R and T223A/N) (Dinesh-Kumar et al., 2000), further confirming the importance of these residues in NB-LRR protein function. Another large-scale mutant screen was performed by Bendahmane et al. (2002) on the CC-NB-LRR gene, *Rx*, which confers resistance to Potato virus X (Bendahmane et al., 1999). This screen was designed to look for gain-of-function mutations that result in an HR in the absence of elicitor. Seven such mutations were uncovered (Bendahmane et al., 2002). Of these, three

were in the RNBS-D domain, leading the authors to speculate that this serves as a negative regulatory region. However, mutation of the amino acids at two other positions in this domain led to loss of function of both LOV1 and RPM1 (see above), suggesting that the RNBS-D domain is not solely important as a negative regulatory region. Mutations within the conserved MHD motif, specifically the histidine residue in the L6 protein and the aspartic acid residue in the Rx, I-2, and L6 proteins, have been reported to cause gain-of-function phenotypes (Bendahmane et al., 2002; De la Fuente van Bentem, 2005; Howles et al., 2005). Consistent with these findings, mutation of these residues did not show up in our loss-of-function screen for LOV1 or in the RPM1 study (Tornero et al., 2002), although mutation of nearby residues in both proteins did result in loss of function. Mutation of the methionine of the MHD motif to a lysine resulted in loss of function of the L6 protein (Howles et al., 2005). This is consistent with the idea that the M499I polymorphism in the MHD motif of LOV1 is responsible for the reduced sensitivity of the Fei-0 ecotype. The other three mutations found to cause elicitor-independent activation of Rx occurred in the LRR domain (Bendahmane et al., 2002). One of these was a D543E mutation in the VLDL motif conserved in LRR3 of many resistance genes. Interestingly, the VLDL motif is conserved in LOV1 and mutation of the corresponding aspartate (D591N) caused loss of function of LOV1. Whether this difference is due to differences in the function of these genes or to the difference in the substituted amino acid is unclear. A nearby mutation in LOV1, S593F, also caused loss of LOV1 function. In the CC-NB-LRR protein RPS2, mutation of the corresponding residue (S566L) also leads to loss of function (Axtell et al., 2001). However, this *rps2* mutation was found to be semidominant. This was not observed for the *lov1-7* mutant.

In order to better evaluate the results of a large-scale mutant screen such as the one performed on *LOV1*, it is helpful to estimate the coverage of the screen. In other words, what proportion of possible EMS-generated mutations were actually created and evaluated in this study? If it is assumed that most nonsense mutations result in nonfunctional proteins, the number of nonsense mutations turned up in this study

compared to the number of nonsense mutations that could have been generated by EMS would give an estimate of the coverage of our mutagenesis screen. There are 51 amino acids that can be mutated to stop codons by G to A or C to T mutations in *LOV1*. Only five of these were mutated in our screen (Table 3.1). This would suggest that only about 10% of the amino acids that could be mutated by EMS were identified in our screen. A similar proportion (eight different amino acids out of 69 possible) were mutated to stop codons by EMS in the RPM1 screen (Tornero et al., 2002). This would suggest that the RPM1 screen also only covered about 10-15% of the mutable amino acids. However, if both screens covered only about 10% of mutable amino acids, we would expect about a 1% overlap in corresponding residues mutated in both studies, and this would actually be a high estimate because it would assume that nearly all the same residue positions are important for function of both proteins. In fact, nine residue positions were found to cause partial or complete loss of function in both LOV1 and RPM1 and this represents around 20% of the mutated amino acids in each mutant screen. An overlap of approximately 20% would suggest that each screen had around 40-50% coverage, which seems reasonable, given that many amino acids were hit more than once in each screen. If this coverage estimate is correct, it would suggest that both LOV1 and RPM1 can sustain missense mutations in many amino acids without loss of function. For LOV1, mutations at 47 amino acid residue positions were found to cause partial or complete loss of function. This represents only 8.3% of the amino acids mutable by EMS, and therefore, a 40-50% coverage rate would suggest that mutation of only about 16-20% of the mutable amino acids would lead to loss of function.

The conflicting coverage estimates discussed above could be explained by two different theories. First, EMS does not mutate every G (or C on the opposite strand) with the same probability. It exhibits certain site preferences based on local nucleotide composition (Greene et al., 2003). The positions that were affected in both LOV1 and RPM1 may be in a favorable context for EMS mutagenesis and therefore overrepresented in these studies. However, this seems unlikely as they are not in

identical nucleotide sequence contexts in the two proteins. A more likely explanation is that mutations creating nonsense codons were either created or recovered at a lower than expected rate in these studies. This is supported by the finding that nonsense mutations were recovered at about the same frequency as missense mutations in LOV1 when normalized for the possible number of each type of mutation that could be generated by EMS. This would suggest that missense mutations are just as likely as nonsense mutations to cause loss of function of LOV1, which is not what we would have expected. Only three amino acids (Arg, Trp, and Gln) can be mutated to stop codons by EMS, and these codons may have sequences less likely to be affected by EMS, which could result in creation of nonsense codons less frequently than expected. For example, mutation of TGG (Trp) to TAG (stop) is only expected to occur 49% as often as mutation of an "average" guanine based on the -1 and +1 flanking nucleotides. Two of the other four possible mutations leading to nonsense codons are also only expected to occur 40-50% as often as average based on two of the flanking nucleotides. However, as the other flanking nucleotides will vary for each occurrence of these codons, the exact effect of site preferences cannot be easily determined, although it appears that this bias may play a partial role in explaining the underrepresentation of nonsense mutations. Another possibility is that nonsense mutations were underrepresented in the LOV1 and RPM1 studies due to retention of the ability to cause cell death, as has been observed with some truncated NB-LRR genes (Tao et al., 2000; Bendahmane et al., 2002; Frost et al., 2004; Zhang et al., 2004; Ade et al., 2007). In our study, four of the five nonsense mutations occurred in the first third of the protein, and for RPM1, six of eight were in the first half. This is consistent with the finding that the N-terminal portion of the protein and the NB are generally required for HR-inducing activity. LOV1 and RPM1 truncations containing more than half of the protein may in some cases retain activity, but lack negative regulatory domains, leading to constitutive cell death or other detrimental phenotypes, such that they were not recovered in these screens. The one LOV1 and two RPM1 proteins in which smaller portions of the protein were deleted and found to cause loss

of function could either be unstable or could retain negative regulatory domains that block constitutive activity as was observed in studies of RPS2, in which an overexpressed protein lacking the entire LRR showed constitutive activity, while deletion of smaller portions of the LRR resulted in loss of function (Tao et al., 2000). However, studies showing HR-inducing activity of truncated NB-LRR proteins have generally been done with overexpressed proteins, and they are often performed in tobacco, a heterologous host. It is unclear whether truncated LOV1 or RPM1 under control of its native promoter and in its natural host would have any inducible or constitutive activity. Also, deletion of the LRR portion of some resistance genes has been shown to cause loss of function (Moffett et al., 2002; Howles et al., 2005). Therefore, we cannot definitively conclude what may have caused the apparent underrepresentation of truncated alleles in this study.

In order to better characterize the function of LOV1 in conferring victorin sensitivity, we also undertook a survey of the occurrence of victorin sensitivity in multiple *A. thaliana* accessions and related species in an attempt to define the amount of variation present in sensitive *LOV1* alleles and the nature of alleles that do not confer victorin sensitivity. This study showed that sensitivity to victorin is the prevalent phenotype within *A. thaliana* ecotypes and that the *LOV1* alleles from all ecotypes tested are highly related (Figure 3.3 and Table 3.2). The population genetic analysis of *A. thaliana LOV1* alleles showed that the level of nucleotide diversity is on the low end of the spectrum compared to other NB-LRR genes studied to date (Mauricio et al., 2003; Rose et al., 2004; Bakker et al., 2006b). For *RPS2*, which like *LOV1* encodes a CC-NB-LRR protein, $\pi_a = 0.0023$ and $\pi_s = 0.0091$ in the coding region of the gene (Mauricio et al., 2003) compared to $\pi_a = 0.0014$ and $\pi_s = 0.0009$ for *LOV1* (Table 3.3). For the 27 NB-LRR genes evaluated by Bakker et al. (2006b), the three genes with the lowest nucleotide diversity ($\pi = 0.0010$-0.0014) show similar values to that observed for the *LOV1* coding region ($\pi = 0.0013$). However, the Bakker et al. study included only the LRR portion of each gene, which for LOV1 has $\pi = 0.00054$, a value only about half that of the lowest value observed in the study.

Similarly, the number of segregating sites per base pair in the region encoding the LRR of LOV1 ($S = 0.0048$) is lower than that observed for any of the NB-LRR genes in the Bakker et al., study. These data could suggest a recent selective sweep on the *LOV1* locus, such that the overall level of polymorphisms is relatively low, even between functional alleles and the Col-4/No-0 (I) allele, which encodes a pseudogene. A selective sweep was suggested for At5g04720 in the Bakker et al. (2006b) study, and this gene shows slightly higher values for nucleotide diversity and number of segregating sites in the LRR region ($\pi = 0.0018$; $S = 0.0069$) in comparison to *LOV1*. The fact that *LOV1* encodes a relatively large number of protein variants and shows a higher level of nonsynonymous versus synonymous polymorphisms suggests that diversifying selection may have acted on this locus subsequent to the possible selective sweep. However, the level of within species polymorphisms is too low to obtain a statistically significant K_a / K_s ratio, and the excess of nonsynonymous polymorphisms may be due to random chance, particularly considering that in the LRR region, which is often found to be under positive selection in resistance genes (Bergelson et al., 2001; Mondragón-Palomino et al., 2002), the level of synonymous nucleotide diversity was found to exceed the level of nonsynonymous nucleotide diversity for *LOV1*, which is suggestive of purifying selection acting on this region (Table 3.3).

Whatever the evolutionary forces acting at the *LOV1* locus, they have resulted in most *A. thaliana* ecotypes carrying a functional *LOV1* allele. This presents an interesting conundrum. Because a functional *LOV1* gene would not be maintained in natural populations as a means to confer victorin sensitivity and disease susceptibility, we propose that the *LOV1* gene may serve as a resistance gene to a naturally-occurring pathogen of *A. thaliana*. This idea is compatible with the possibility that *LOV1* may have experienced a recent selective sweep followed by diversifying selection, as a resistance gene would be expected to be under this type of selective pressure (Bakker et al., 2006b). If *LOV1* is in fact experiencing diversifying selection, it appears that the primary functional domains (CC, NB, and LRR) are not the targets of this

selection, given the fact that most of the nonsynonymous polymorphisms occur outside of these regions. This may indicate conservation of domains required for LOV1 to function in resistance to a pathogen of *A. thaliana*. A number of resistance genes, such as RPM1, RPS2, and RPS5, show evidence for balancing selection resulting in an ancient division of "resistance" and "susceptibility" clades with relatively low genetic diversity within each clade (Stahl et al., 1999; Tian et al., 2002; Mauricio et al., 2003; Shen et al., 2006). Although *LOV1* does not show ancient differentiation of functional and nonfunctional alleles indicative of balancing selection, the low level of polymorphisms seen within functional *LOV1* alleles is similar to the variation observed within the "resistance" clade for *RPS2*, *RPM1*, and *RPS5*. These genes have been shown to function through indirect recognition of their cognate avirulence determinants (Mackey et al., 2002, 2003; Axtell et al., 2003; Shao et al., 2003). It has been proposed that functional alleles of these genes show low levels of diversity because they are recognizing ("guarding") a host target altered by an avirulence effector rather than directly recognizing an avirulence protein that may be rapidly evolving (Van der Hoorn et al., 2002; Dodds et al., 2006). This may also be true for LOV1. We have determined that LOV1 function requires ATTRX5, a cytosolic thioredoxin, for its function. *ATTRX5* expression has been found to be induced by biotic and abiotic stresses and may play a role in protecting plant cells from oxidative stress (Reichheld et al., 2002; Laloi et al., 2004), such as would occur during the plant defense response due to production of reactive oxygen species. It is therefore not unreasonable to consider the possibility that ATTRX5 could be a target of pathogen virulence effectors and guarded by an NB-LRR protein. If this is the case, it is possible that ATTRX5 is targeted both by victorin and by an avirulence effector of the putative pathogen for which *LOV1* may act as a resistance gene. The CC and LRR domains of NB-LRR proteins mediate protein-protein interactions that are involved in conferring recognition specificity and interacting with signaling partners (Ellis et al., 1999; Dodds et al., 2001; Belkhadir et al., 2004; Zhou et al., 2006), making these domains likely regions for interactions with a targeted host protein.

Overall, *LOV1* does not show an excess of synonymous versus nonsynonymous polymorphisms, as would be expected for long-term conservation of functional alleles. However, the CC domain shows no within species polymorphisms, while the LRR domain shows a higher rate of synonymous versus nonsynonymous nucleotide diversity (Table 3.3), an indicator of possible purifying selection. While these numbers are too low to establish statistical significance, they are not inconsistent with the idea that one or both of these domains could be conserved for interaction with ATTRX5, possibly with LOV1 acting as a "guard" that would recognize alteration of ATTRX5 by a pathogen effector. The interspecies data also show that the CC and LRR domains have a $K_a / K_s < 1$, and lower than the K_a / K_s for the coding sequence as a whole (Table 3.4), again possibly suggesting purifying selection acting on these regions. The finding that many mutations in the CC and LRR domains were found to cause loss of function of LOV1 is also consistent with this idea. Future work will be directed at determining how victorin is recognized by LOV1 (directly or indirectly) and whether there is a direct interaction between ATTRX5 and LOV1, which could be indicative of LOV1 acting as a guard for ATTRX5.

As a whole, this work indicates that LOV1 is likely functioning in a manner similar to a resistance protein, despite the fact that it confers sensitivity to victorin and susceptibility to *C. victoriae*. This is supported by our mutagenesis study, which showed that mutations in domains known to be conserved in resistance proteins eliminate the function of LOV1, suggesting that LOV1 functions in a manner analogous with resistance proteins. In other words, LOV1 acts in the response to victorin by directly or indirectly recognizing a pathogen effector and triggering a defense response, which includes a PCD response reminiscent of an HR. Analysis of the *LOV1* allele from other ecotypes indicates that most accessions carry a functional *LOV1* gene and that there is little genetic variation between *LOV1* alleles, particularly within domains known to be required for resistance gene function. While we cannot definitively conclude what evolutionary forces led to this result, this may indicate that functional *LOV1* alleles have been either maintained or selected for because *LOV1* is

functioning as a resistance gene to a yet undiscovered pathogen. Therefore, the *LOV1* gene could represent a situation where a gene conferring resistance to one pathogen also confers susceptibility to another pathogen. This opens up an intriguing avenue for further study of the relationship between plant disease resistance and susceptibility.

Chapter 4

General Conclusions

Victoria blight of oats, caused by *Cochliobolus victoriae*, was first described in the 1940's following introduction of oats carrying Victoria-type crown rust resistance (Meehan and Murphy, 1946). Since that time, numerous attempts to separate Victoria blight susceptibility from crown rust resistance have failed (Welsh et al., 1954; Luke et al., 1966; Rines and Luke, 1985; Mayama et al., 1995). This strongly suggests that the *Vb* gene, which confers susceptibility to Victoria blight, and the *Pc-2* gene, which confers resistance to crown rust, are the same gene. *C. victoriae* produces a compound called victorin, which alone reproduces the symptoms of Victoria blight on sensitive plants (Meehan and Murphy, 1947). Interestingly, victorin induces host defense responses (Wheeler and Black, 1962; Shain and Wheeler, 1975; Mayama et al., 1986; Ullrich and Novacky, 1991) and programmed cell death (PCD) in sensitive plants (Navarre and Wolpert, 1999; Yao et al., 2001, 2002; Curtis and Wolpert, 2002, 2004; Coffeen and Wolpert, 2004). As gene-for-gene type resistance also involves PCD in the form of a hypersensitive response (Heath, 2000; Greenberg and Yao, 2004), these physiological data further support the idea that there is a connection between induction of resistance to crown rust and susceptibility to Victoria blight in oats. The PCD response is particularly important given that *C. victoriae* is a necrotrophic fungus, whereas *Puccinia coronata*, the crown rust fungus, is a biotroph. This likely at least partially explains why similar physiological responses in the host result in very different disease outcomes.

Unfortunately, further genetic analysis of the relationship between disease susceptibility and resistance conferred by the *Vb*/*Pc-2* gene has been hampered due to the molecular genetic intractability of oats. However, the discovery that some ecotypes of the model plant *Arabidopsis thaliana* are both sensitive to victorin and susceptible to infection by *C. victoriae* opened up a new avenue for study of Victoria

blight (Lorang et al., 2004). The validity of *Arabidopsis* as a model for studying this disease is supported by data indicating that, as in oats, victorin sensitivity in *Arabidopsis* is conferred by a single dominant gene (Lorang et al., 2004) and involves induction of host defense responses and a PCD response (T.A. Sweat, unpublished results). The gene that confers victorin sensitivity in *Arabidopsis*, *LOV1*, was recently cloned and found to encode a coiled-coil-nucleotide binding site-leucine-rich repeat (CC-NB-LRR) protein (J.M. Lorang, unpublished results). This type of protein has previously only been associated with conferring disease resistance. Therefore, cloning of this gene finally provided definitive proof that a resistance-like protein is responsible for conferring victorin sensitivity and Victoria blight susceptibility, at least in *Arabidopsis*, and strengthens the idea that the *Vb* and *Pc-2* genes share identity.

The discovery of victorin sensitivity in *Arabidopsis* allowed us to take a genetic approach towards better understanding the pathway leading from recognition of victorin to induction of host cell death. The finding that victorin sensitivity in *Arabidopsis* is conferred by a CC-NB-LRR protein added an additional aspect to this work, namely, to determine whether the LOV1 protein functions in a manner similar to that of previously characterized NB-LRR resistance proteins. The method chosen to meet these objectives was a large-scale EMS mutagenesis study involving screening approximately 212,000 seedlings for loss of sensitivity to victorin. This led to isolation of 63 independent mutants in two complementation groups that had completely lost sensitivity to victorin. Of these, 59 were found to carry mutations in the *LOV1* gene and four contained mutations at a second locus, which we named the *LOCUS OF INSENSITIVITY TO VICTORIN1* (*LIV1*).

Sequencing of the 59 *lov1* mutants showed that many of the mutations leading to loss of function of *LOV1* were in conserved motifs previously found to be important for resistance gene function (Takken et al., 2006), such as the P-loop, RNBS-A, kinase 2, GLPL, and RNBS-D motifs. The *lov1* mutant alleles also contained many mutations in the CC and LRR domains, which are important for conferring recognition specificity and interacting with signaling partners of CC-NB-LRR proteins (Belkhadir

et al., 2004). Furthermore, many of the *lov1* mutations fell at residue positions that were also found to be mutated in *rpm1* loss-of-function alleles (Tornero et al., 2002). As RPM1 is known to function in disease resistance (Bisgrove et al., 1994), these results suggest that LOV1 functions in a manner analogous to a resistance protein. There was a difference in the distribution of mutations between *LOV1* and *RPM1*, with *LOV1* containing more mutations in the ARC1 and ARC2 domains and the first portion of the LRR, whereas *RPM1* shows a higher proportion of mutations throughout the NB-ARC domain and fewer mutations in the LRR. This difference may be due to random distribution of the mutations as these screens were each estimated to cover only about 40-50% of possible EMS-generated mutations. Also, some sites that can be mutated by EMS in one protein may not be mutable in the other protein due to differing nucleotide sequences, as EMS only generates G to A (C to T on the opposing strand) transition mutations. However, many of the differences are likely due to the requirements of LOV1 and RPM1 to interact with different protein partners, both upstream for recognition and downstream for transmission of the signaling response. Also, differences in the regulation of these proteins may account for some of the dissimilarities in the distribution of mutations. One major difference between these two proteins is that RPM1 is under negative regulation by RIN4 (Mackey et al., 2002), whereas ATTRX5 is required as a positive regulator of LOV1 (see below).

Our survey of victorin sensitivity in *Arabidopsis* ecotypes collected from around the world showed that victorin sensitivity is the prevalent phenotype in natural populations, with only three out of 30 ecotypes tested showing insensitive individuals. Furthermore, sequencing of *LOV1* from these ecotypes showed that there is very little sequence variation among *LOV1* alleles, even between those that are functional and those that are clearly pseudogenes. This may indicate a recent selective sweep of *LOV1* such that all alleles are highly related. This may have been followed by selection for divergent alleles, given that the number of nonsynonymous mutations exceeds the number of synonymous changes. However, these numbers are too low to

show statistical significance. Furthermore, the level of nonsynonymous diversity is higher in regions between known functional domains, such as the CC and LRR domains, than it is within these domains, suggesting that if diversifying selection is acting at this locus, it is acting in such a way as to conserve protein function. Whatever the evolutionary forces acting on the *LOV1* locus, they have resulted in most ecotypes carrying a *LOV1* allele that is capable of conferring victorin sensitivity. As this is not a phenotype expected to be selected for in a natural environment, we propose that *LOV1* may act as a resistance gene for a yet uncharacterized pathogen.

We also cloned the *LIV1* gene, which encodes thioredoxin *h*5 (ATTRX5), identifying this protein as another essential component of the victorin response pathway. This was an interesting finding for several reasons. First, the *Arabidopsis* genome encodes many thioredoxins and thioredoxin domain-containing proteins, including eight belonging to the *h*-type group of thioredoxins (Gelhaye et al., 2005). Previously, no specific functions had been defined for any of these thioredoxins and they were thought to be largely redundant. However, our data showed that *ATTRX5* was specifically required for the response to victorin as the highly-related thioredoxin *h*3 (*ATTRX3*) was unable to complement the *liv1* mutation unless it was highly overexpressed, and even then, it only partially restored the victorin sensitivity phenotype. Promoter swaps between *ATTRX5* and *ATTRX3* also indicated that it was the protein sequence and not the expression level that determined the ability to restore victorin sensitivity. Additionally, we created ATTRX5/ATTRX3 chimeric proteins, which revealed that the proteins with the highest amino acid identity to ATTRX5 were also the most active in restoring victorin sensitivity. These data clearly indicate that ATTRX5 shows specificity for the victorin response at the protein level.

Another interesting aspect of the ATTRX5 studies was that only the first of the two active site cysteines was found to be required for functioning in the victorin response pathway. The typical mechanism of action for thioredoxins is to initially bind to a target protein by the first active site cysteine and then complete the reduction with the second cysteine (Kallis and Holmgren, 1980). Our data could indicate that

only the initial binding of ATTRX5 to a target protein is required for the victorin response or that the reduction can be completed by another thioredoxin or other reducing agent in the cell. We also found that mutation of both cytosolic NADPH-dependent thioredoxin reductases (NTRs) failed to abrogate or reduce sensitivity to victorin. As NTRs are required to maintain the pool of reduced thioredoxins (Florencio et al., 1988), this again suggests that ATTRX5 is not acting through a redox mechanism in the victorin response pathway.

Recently obtained data provide further insight into the results presented in this work and begin to put together a picture of the pathway leading from victorin recognition to activation of LOV1. We have found that the ATTRX5 protein shows an increase in size upon treatment with victorin (T.A. Sweat, unpublished results). This shift in size occurs in both a *LOV1* and a *lov1* background, suggesting that it is occurring upstream of LOV1 function. We also found that the size shift occurs with the ATTRX5 C42S mutant protein, which retains the ability to confer victorin sensitivity, but not in the C39S mutant, which is unable to mediate victorin sensitivity. This suggests that the modification to ATTRX5 is required for the victorin response and that it is likely occurring at Cys-39. As the size increase is of approximately the correct size (~1 kDa), and victorin is known to bind free sulfhydryls, we considered the possibility that victorin may be binding to ATTRX5. In fact, after treatment with biotinylated victorin, the higher molecular weight ATTRX5 band was found to react with anti-biotin antibody. While this finding awaits final confirmation, it is highly likely that victorin is binding to ATTRX5. Interestingly, ATTRX3 appears to bind victorin with equal affinity to that showed by ATTRX5.

These finding brought up additional questions, namely, how does binding of victorin to ATTRX5 lead to activation of LOV1, and, if both ATTRX5 and ATTRX3 bind victorin, why is only ATTRX5 able to function in the victorin response? In order to answer these questions, ongoing work is being directed at determining whether ATTRX5 directly interacts with LOV1. Initial yeast two-hybrid results indicate that there is a direct interaction between LOV1 and ATTRX5, whereas ATTRX3 does not

appear to interact with LOV1 (T.A. Sweat, unpublished results), which would explain the specific requirement for ATTRX5 in the victorin response. Future studies in the lab will be directed at confirming this interaction through independent means, such as by co-immunoprecipitation experiments using tagged LOV1 and ATTRX5 proteins.

The idea that ATTRX5 is modified by binding of victorin to its active site, which then leads to activation of LOV1, an NB-LRR protein, is reminiscent of the "guard hypothesis." This hypothesis states that at least some NB-LRR resistance proteins function by monitoring the state of another host protein, which often functions in basal defense of the host. When this host protein is targeted and somehow altered by a pathogen effector, the resistance protein recognizes the altered host protein and triggers a resistance response (Jones and Dangl, 2006). The best known example of this is the RIN4 protein. This protein is targeted by three pathogen effectors produced by *Pseudomonas syringae*. In the presence of AvrRpm1 or AvrB, RIN4 is phosphorylated, leading to the activation of RPM1 (Mackey et al., 2002). RIN4 is cleaved by the protease AvrRpt2, which relieves the negative regulation of RPS2 by RIN4, leading to activation of RPS2-mediated resistance (Axtell et al., 2003; Mackey et al., 2003). Another example of a targeted host protein is the protein kinase PBS1, which is cleaved by the *P. syringae* effector protease AvrPphB, leading to activation of RPS5 (Shao et al., 2003; Ade et al., 2007). The N-terminal domain of the resistance protein, including the CC domain, was implicated as the site of interaction between RPM1 and RIN4 (Mackey et al., 2002) and between RPS5 and PBS1 (Ade et al., 2007). Interestingly, it is also the N-terminal domain of LOV1 that appears to interact most strongly with ATTRX5. In addition, ATTRX5 has been shown to be induced by biotic and abiotic stresses and may play a role in protecting the plant against oxidative stress (Reichheld et al., 2002; Laloi et al., 2004). Therefore, ATTRX5 may play a role in basal defense, which would make it a likely target of pathogen effectors such as victorin.

Our preliminary studies indicating that victorin binds to ATTRX5 and that ATTRX5 and LOV1 directly interact suggest that this pathway is analogous to the

indirect activation of resistance genes described by the "guard hypothesis." We therefore propose a similar model for the activation of LOV1 (Figure 4.1). In this model, ATTRX5 is bound to inactive LOV1 at the N-terminal portion of the LOV1 protein. We propose that ATTRX5 interacts with LOV1 in the absence of victorin because the yeast two-hybrid results were obtained without addition of victorin. In our model, LOV1 is held in an inactive conformation by intramolecular bonds between the domains, as was proposed for the Rx resistance protein (Moffett et al., 2002), which, like LOV1, is a CC-NB-LRR protein. ATTRX5 is not proposed to be required for holding LOV1 in an inactive conformation because loss of ATTRX5 is not lethal, as would be expected if loss of ATTRX5 led to constitutive activation of LOV1. Instead, ATTRX5 is apparently required as a positive regulator of LOV1 activity. Binding of victorin to Cys-39 of ATTRX5 triggers a conformational shift in LOV1 that disrupts the intramolecular bonds and leads to activation of LOV1. ATTRX5 may be released from the activated protein, or it may remain bound to the N-terminal portion of LOV1. It is also possible that binding of victorin to ATTRX5 changes the binding affinity of ATTRX5 such that it now binds to another portion of LOV1, such as the LRR domain, which shows a very weak interaction with ATTRX5 in the yeast two-hybrid assay in the absence of victorin. This model suggests an evolved relationship between LOV1 and ATTRX5 leading to LOV1 acting as a "guard" for ATTRX5. As previously mentioned, there is no reason for LOV1 to be functionally conserved in order to trigger cell death in response to victorin, because for *C. victoriae*, cell death favors infection. Instead, the ATTRX5/LOV1 interaction may have developed to guard *Arabidopsis* from attack by another pathogen, which may also produce an effector that targets ATTRX5, leading to activation of LOV1 through a similar mechanism to that proposed for activation by victorin.

Although much work needs to be done to confirm the pathway leading from victorin recognition to activation of LOV1, the data presented in this study provide evidence that ATTRX5 and LOV1 likely act in a pathway similar to those described for known resistance genes. This again brings up the possibility, as has long been
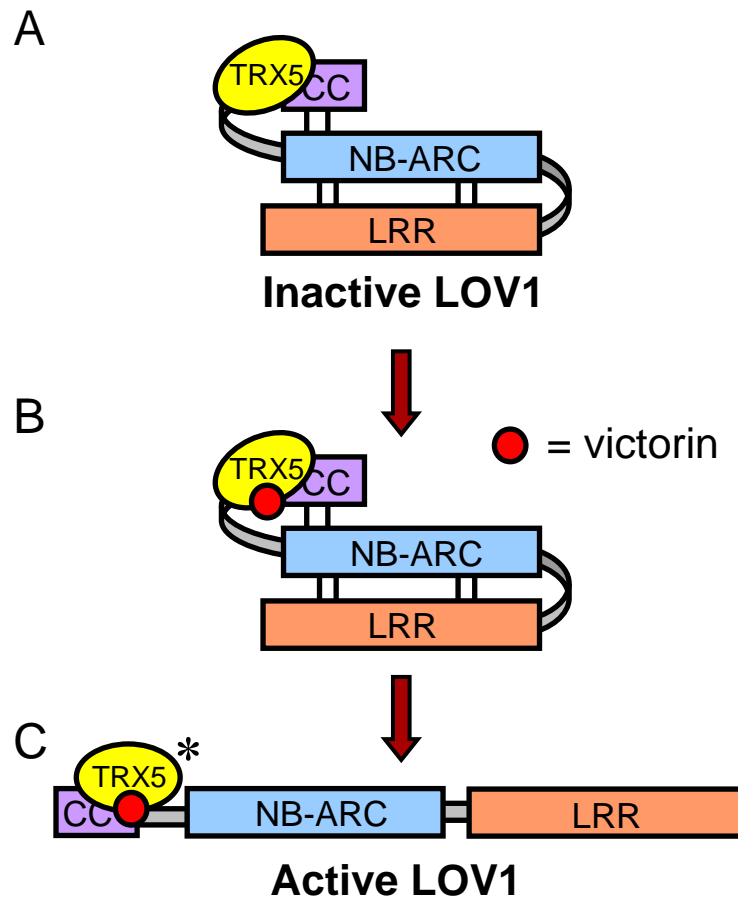
Figure 4.1. Model of LOV1 activation by victorin binding to ATTRX5.

A) LOV1 is held in an inactive conformation by intramolecular bonds between the domains. ATTRX5 is bound to LOV1 at its N-terminal region, which includes the CC domain. B) Victorin binds to Cys-39 of ATTRX5, leading to a conformational shift in LOV1. C) The conformational shift in LOV1 causes disruption of the intramolecular bonds in LOV1 and activation of the LOV1 protein. *ATTRX5 may remain bound to active LOV1 at the CC domain or its affinity may shift to the LRR domain or it may be released from active LOV1.

suspected for *Vb* and *Pc-2*, that the same gene may confer resistance to one pathogen while conferring sensitivity to another pathogen. This has implications for plant breeding and the creation of transgenic crops carrying gene-for-gene type resistance genes, as well as in natural populations. Many NB-LRR genes, including some with known roles in resistance, such as *RPM1* and *RPS5*, segregate as presence/absence polymorphisms (Stahl et al., 1999; Tian et al., 2002; Shen et al., 2006). It has been proposed that this segregation may occur because there is a fitness cost to expressing at least some of these resistance genes, as was found for *RPM1* (Tian et al., 2003). It is conceivable that triggering of these genes by necrotrophic pathogens could in some cases contribute to this fitness cost. While this remains quite speculative, additional study of the victorin response in both *Arabidopsis* and oats should provide further insight into the close relationship between disease resistance and susceptibility and possibly into how plants balance the cost and benefits of expressing NB-LRR genes such as *LOV1*.

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