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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The fungus *Cochliobolus victoriae*, the causal agent of Victoria blight, produces a compound called victorin that is required for pathogenicity of the fungus. Victorin alone reproduces disease symptoms 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 and disease susceptibility in *Arabidopsis* are conferred by *LOV1*, a coiled-coil-nucleotide-binding-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 *LOV1* loss of function was similar to that found to cause loss of function of *RPM1*, a CC-NB-LRR resistance protein. Also, many of the mutated residues in *LOV1* are in conserved motifs required for resistance protein function. These data indicate that *LOV1* may have a mechanism of action similar to resistance proteins. Victorin sensitivity was found to be the prevalent phenotype in a survey of 30 *Arabidopsis* ecotypes, and we found very little genetic variation among *LOV1* alleles. 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*.

The *Arabidopsis thaliana* genome encodes 149 nucleotide-binding-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 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 (Greenberg and Yao 2004; Heath 2000). The *LOCUS ORCHESTRATING VICTORIN EFFECTS1* (*LOV1*) gene is unusual in that it is a CC-NB-LRR protein found to confer susceptibility to infection by the fungus *Cochliobolus victoriae* in *Arabidopsis* (Lorang et al. 2007). *C. victoriae* was originally described as the causal agent of Victoria blight of oats (*Avena sativa*) (Meehan and Murphy 1946). Pathogenic strains of *C. victoriae* produce a group of cyclized pentapeptides called victorin (Macko et al. 1985; Wolpert et al. 1985), and treatment with victorin alone reproduces disease symptoms on sensitive plants (Lorang et al. 2004; Meehan and Murphy 1947). For both oats and *Arabidopsis*, only genotypes that are sensitive to victorin are susceptible to infection by *C. victoriae* (Lorang et al. 2004; Sweat and Wolpert 2007; Walton 1996; Wolpert et al. 2002). Victorin triggers a PCD response in sensitive plants (Coffeen and Wolpert 2004; Curtis and Wolpert 2002, 2004; Navarre and Wolpert 1999; Yao et al. 2001, 2002) and also induces host defense responses (Lorang et al. 2007; Mayama et al. 1986; Shain and Wheeler 1975; Ullrich and Novacky 1991; Wheeler and Black 1962). 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 (Luke et al. 1966; Mayama et al. 1995; Rines and Luke 1985; Welsh et al. 1954). The genetic connection between *Vb* and *Pc-2* and the fact that victorin induces a host response similar to that mediated by resistance genes, including both induction of defense 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 protein of the NB-LRR class is indeed responsible for conferring both sensitivity to victorin and susceptibility to *C. victoriae* in *Arabidopsis*

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Nucleotide sequence data for *LOV1* alleles from the following *Arabidopsis thaliana* ecotypes and related species are available in the GenBank database under accession numbers Cl-0, EF472599; Ler-0, EU053655; Sp-0, EU053656; Sha, EU053657; Tol-0, EU053658; C24, EU053659; Rubeshnoe-2, EU053660; Bay-0, EU053661; Cvi-0, EU053662; En-1, EU053663; Kas-1, EU053664; Nd-0, EU053665; No-0 (victorin-sensitive individual), EU053666; Ws-0, EU053667; Ws-2, EU053668; Mt-0, EU053669; Bil-5, EU053670; HR-10, EU053671; Fei-0, EU053672; An-1, EU053673; Ts-1, EU053674; Yo-0, EU053675; Se-0, EU053676; Bur-0, EU053677; Ag-0, EU053678; Vind-1, EU053679; RRS-10, EU053680; Lov-1, EU053681; KZ-1, EU053682; Col-4, EU053686; No-0 (victorin-insensitive individual), EU053687; *Arabidopsis korshinskyi*, EU053683; *Olimarabidopsis pumila*, EU053684; and *Arabidopsis suecica*, EU053685.

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(Lorang et al. 2007). Interestingly, recent work strongly suggests that the *Pc* gene, which confers susceptibility to mild disease in sorghum (*Sorghum bicolor*) caused by the fungus *Periconia circinata*, is also an NB-LRR gene (Nagy et al. 2007). Similar to the situation for *C. victor*iae, *P. circinata* produces phytotoxic peptides, called PC-toxin (Macko et al. 1992), that alone reproduce disease symptoms (Wolpert and Dunkle 1983), and both sensitivity to PC-toxin and susceptibility to the fungus are conferred by the *Pc* gene.

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 *LIVI* gene, which was found to encode thioredoxin *h5* (ATTRX5). We showed that loss of victorin sensitivity resulted in complete loss of susceptibility to *C. victor*iae 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. victor*iae in both oats and *Arabidopsis* (Lorang et al. 2004; Walton 1996; Wolpert et al. 2002). In the present study, we used a twofold 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* (Tornerio et al. 2002), 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. victor*iae, we propose that *LOV1* may be acting as a resistance gene to a naturally occurring pathogen of *Arabidopsis*.

RESULTS

Loss-of-function *lov1* alleles.

The *LOV1* coding region from each of the 59 previously isolated *lov1* mutants that had completely lost sensitivity to victorin was amplified by polymerase chain reaction (PCR) and was 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 (Supplementary Table S1). 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 51 alleles each contain a single amino-acid change (missense mutation) resulting in complete loss of LOV1 function. Some of these alleles were found to be identical, leaving 44 unique alleles affecting 42 amino-acid residues. Most of these mutants were wild-type in appearance, although a few had unusual morphologies or poor seed set, likely resulting from unrelated second-site mutations.

Characterization of mutants with reduced sensitivity to victorin.

In our mutant screen, we also isolated 10 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 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 μ l of a 10 μ g/ml solution of victorin for 3 days, but symptom development is delayed in comparison with that in wild-type plants. Some mutants show only yellowing after treatment with victorin for 3 days (e.g., *lov1-61*), while others show tissue collapse that develops more slowly than in wild-type plants. These 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. 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 the reduced-sensitivity mutants, but no mutations were found. 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. Interestingly, in *lov1-22*, the mutation A547V results in complete loss of function. 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. victor*iae (Lorang et al. 2004), it belongs to the CC-NB-LRR class of genes (Lorang et al. 2007), which

are typically associated with resistance. An extensive mutagenesis study has been performed on 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 *LOV1* is functioning in a manner analogous to a resistance protein, the loss-of-function alleles uncovered for *LOV1* should show similarities 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 *lov1* versus *rpm1* loss-of-function alleles. Of the 61 *rpm1* alleles, 10 (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 and associates (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 mutations leading to loss of function in these two genes.

In order to compare the overall distribution of missense mutations resulting in loss of function of *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* (Fig. 1A). For comparison, we performed an identical analysis for *RPM1* (Fig. 1B), similar to that performed by Tornero and associates (2002). To preserve as much information as possible and to 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 affected by mutation in each window. These last two counts were only different in the few cases in which 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 to 50) for *LOV1* and window 351 to 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 overall distribution of mutations in the *lov1* and *rpm1* alleles are only somewhat similar. 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 (Fig. 1). Additionally, both have a peak of mutations in window 751 to 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 CC 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 and in the first portion of the LRR. *LOV1* shows fewer mutations in the NB region as compared with *RPM1*, although both genes show a peak of mutations in and around the P-loop at the beginning of the NB domain.

Although the distribution of mutations in the *lov1* versus *rpm1* alleles appears only somewhat similar, 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 (Fig. 2), which are conserved regions in the NB-ARC domain that have been found to be important for resistance-protein function (Dinesh-Kumar et al. 2000; Takken et al. 2006; Tao et al. 2000; Tornero et al. 2002). The *lov1* alleles also included one mutation in the RNBS-A motif, while *RPM1* had mutations in the RNBS-B and RNBS-C motifs. Additionally, it is striking to note that in several cases, amino acids in corresponding positions of an

Fig. 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* (from Tornero et al. 2002) were plotted in 50-amino acid windows. Diagrams under each graph represent the structure of each protein 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.

alignment of LOV1 and RPM1 were found to be mutated in loss-of-function alleles of both genes (Fig. 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

Fig. 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. Data for the *rpm1* mutant alleles was obtained from Tornero and associates (2002). Arrows denote the positions of nonsynonymous polymorphisms found in *LOV1* alleles from different *Arabidopsis thaliana* ecotypes in comparison to the CI-0 allele used in the alignment. Thin arrows denote polymorphisms resulting in single amino-acid changes found in ecotypes that retain sensitivity to victorin. Bold arrows denote polymorphisms found only in insensitive ecotypes. Conserved motifs within the nucleotide-binding (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: coiled coil = 1-51; NB = 167-324; ARC1 = 325-412; ARC2 = 413-530; leucine-rich repeat = 531-878.

loss of function resulted in only 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 five of them fall in or near 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 to 92% amino-acid identity (95 to 96% similarity) in pairwise alignments. *LOV1* is not allelic to these three genes but shows approximately 70% identity (86% 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 two 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 NB-LRR 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 (Dodds et al. 2001; Ellis et al. 1999; Zhou et al. 2006), and one is located in the CC domain, which is thought to be involved in interaction with signaling partners (Belkadir 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 a solution of victorin for 3 days. At least eight individuals of each ecotype were tested, except in the few cases in which

Fig. 3. Analysis of ecotype variation in victorin sensitivity and *LOV1* allele sequence. **A**, Representative leaves showing symptoms on victorin insensitive (I), moderately sensitive (mS), and highly sensitive (S) ecotypes two days after treatment with 10 μ g of victorin per milliliter. **B**, 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. 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.

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 (Fig. 3). The only plants that were completely insensitive, besides Col-4, were Bay-0 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 with the ecotype CI-0, from which the *LOV1* gene was originally cloned (Lorang et al. 2007), 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 (Fig. 3A). Ten ecotypes reproducibly displayed low to moderate levels of sensitivity (mS), showing no or only very slight symptoms until at least two days after treatment with victorin and usually showing only yellowing or partial collapse on day 3. Sixteen ecotypes, including CI-0 and the sensitive No-0 individuals, consistently showed visible symptoms by day 1 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 this highly sensitive (S) category. Two ecotypes, Ts-1 and Vind-1, could not be placed conclusively in either of these categories because, in repeated experiments, about half the leaves from each ecotype showed symptoms on day 1 while the other leaves took longer to develop symptoms. This was true even when multiple leaves from the same individual were sampled, indicating that it is not a genotypic difference. Rather, it is likely due to these ecotypes expressing levels of sensitivity very near the threshold required for symptom development within one day. These were, therefore, categorized as mS/S. We also tested for victorin sensitivity in the related species *Arabidopsis suecica*, *Arabidopsis korshinskyi* (also called *Olimarabidopsis cabulica*), *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.

Sequencing of *LOV1* alleles from *A. thaliana* ecotypes.

The *LOV1* gene was sequenced from each of the evaluated ecotypes and the polymorphisms present were compared with the CI-0 allele (Supplementary Table S2). The sequenced region spanned from 272 bp upstream of the start codon to 149 bp downstream of the stop codon (3,388 bp total). Four ecotypes (Rubezhnoe-2, Bil-5, Yo-0, and Lov-1) were found to carry an allele identical to the CI-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 with CI-0, causing one to four amino-acid substitutions. The Col-4 allele, which is identical to the published Col-0 sequence, showed six nucleotide differences when compared with the CI-0 *LOV1* gene (Lorang et al. 2007). 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 13 simple sequence-length polymorphism (SSLP) markers scattered throughout the genome, including one SSLP tightly linked to *LOV1*, marker 3571, and 13 unlinked markers, and we compared the SSLP pattern of the sensitive and insensitive No-0 individuals with each other and with Col-4. One marker was not polymorphic between any of the three individuals. The remaining markers were all polymorphic between Col-4 and both No-0 individuals. Of these 12 markers, eight, including the SSLP linked to *LOV1*, gave PCR products of the same size

in both the No-0 individuals, while four were polymorphic between the sensitive and insensitive individuals (data not shown). These data indicate that the insensitive and sensitive No-0 individuals are related and not due to seed contamination, although there is apparently a relatively large amount of genetic variation within this accession. The only other insensitive ecotype, Bay-0, has an allele that encodes a full-length protein with five nucleotide polymorphisms compared with CI-0.

Evaluation of the insensitive alleles.

Of the six nucleotide changes present between the Col-4 and CI-0 *LOV1* alleles, two do not affect the amino-acid sequence. 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 stop codon at lysine 117, and the deletion of a nucleotide at 3,042 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 CI-0 *LOV1* gene in order to assess the individual effects of the four differences that result in an altered protein product. It was found that either of the nucleotide substitutions causing truncation 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 CI-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. 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 (Fig. 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. 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 (Fig. 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 divides them into two major clades (Fig. 3B). However, these clades are not strongly supported by the bootstrap analysis and are separated by only two polymorphisms, both of which cause amino-acid substitutions (T86A and A109D) in a comparison of the upper group with the lower group. As mentioned above, these two polymorphisms are present in alleles belonging to each phenotypic class (S, mS, and I [victorin insensitive]) and, therefore, do not appear to affect the victorin response. All ecotypes belonging to the mS group are found in the upper group, but these *LOV1* alleles all contain additional polymorphisms that likely play a role in reducing sensitivity (Fig. 3B). Although, in actuality, the sensitive ecotypes represent a continuum of phenotypes from slightly sensitive to highly sensitive to victorin, the division of these ecotypes into two primary groups (mS and S) allows information to be gleaned regarding the relationship between the sequence of *LOV1* and the level of sensitivity dis-

played. In general, there is a very strong correlation between genotype and phenotype, although there are exceptions. In two cases (Sp-0/Ts-1 and HR-10/Vind-1), ecotypes with identical alleles show slightly different levels of sensitivity. This could be due to variation in *LOVI* expression between the ecotypes or possibly due to differences in modifying genes or differences in plant morphology that cause a decrease in the appearance of symptoms due to effects not specific to the victorin response pathway. A similar situation was observed when the CI-0 *LOVI* allele was introgressed into the Col-4 background. This line, Col-LOV, showed slightly reduced sensitivity to victorin compared with the CI-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 these other ecotypes fall into the S category, it appears that HR-10 (mS) and Vind-1 (mS/S) 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 *LOVI* loss-of-function screen (Fig. 2).

Geographic differentiation.

We noted three pairs of ecotypes from the same region that share identical *LOVI* 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 (Fig. 3B). 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 (Bakker et al. 2006a; Nordborg et al. 2005). This was particularly noted with populations from the midwestern United States, 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 (Bakker et al. 2006a; Nordborg et al. 2005), 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 *LOVI* from other species.

Using the original sets of PCR primers (discussed below), the only species besides *A. thaliana* to give a complete *LOVI* sequence was *A. suecica*, which was also the only other species to show a response to victorin. We sequenced the *LOVI* 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. 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 CI-0 ecotypes. The high similarity of the *LOVI* 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* *LOVI* 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 out-group, we used partial *LOVI* sequences obtained from *A. korshinskyi* to design new PCR primers to amplify the remainder of the *LOVI* 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 *LOVI* 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 CI-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* *LOVI* 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 is likely too divergent to function 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* CI-0 allele are substituted in the *A. korshinskyi* sequence. One of these residues is located in the CC 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 *LOVI* alleles.

Within the *A. thaliana* ecotypes there are 29 polymorphic sites, excluding the two sites with gaps (Table 1). These consist of 18 nonsynonymous (amino acid changing) polymorphisms, five synonymous changes, and six polymorphisms that fall outside the coding region. When standardized for the sequence length (3,386 bp excluding gaps), this gives a value of 0.0086 segregating sites per base pair. This is a relatively low number of segregating sites compared with that seen in other NB-LRR genes previously studied in *Arabidopsis* (Bakker et al. 2006b; Mauricio et al. 2003; Rose et al. 2004). 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 with other NB-LRR genes (Bakker et al. 2006b; Mauricio et al. 2003; Rose et al. 2004). Over the entire sequenced region, $\pi = 0.00126$ and, in the coding region, $\pi = 0.00131$. At nonsynonymous sites, $\pi_a = 0.00143$, while $\pi_s = 0.00093$ at synonymous sites, indicating an excess of nonsynonymous compared with synonymous diversity ($\pi_a/\pi_s = 1.54$), in line with the relatively large number of encoded protein variants. Dividing the *LOVI* gene into functional regions showed that the highest nucleotide diversity at nonsynonymous sites (π_a) fell in regions outside of those with well-char-

acterized 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 ($\pi_a = 0.00066$). Pairwise K_a/K_s comparisons, excluding the truncated Col-4 and No-0 (I) alleles and any pairwise comparisons with $K_s = 0$, 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 2). 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 2). 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.

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* shows many similarities to those mutations found to disrupt the function of *RPM1*, another CC-NB-LRR protein (Tornero et al. 2002). In particular, several residues at corresponding positions of *LOV1* and *RPM1* were observed to be mutated in both studies (Fig. 2), suggesting that these two proteins may function in a similar manner. In addition, both studies uncovered mutations in many of the motifs of the NB-ARC domain that are conserved among NB-LRR proteins (Fig. 2) and are important for resistance protein function, likely due to a role in the binding and hydrolysis of ATP (Takken et al. 2006; Tameling et al. 2002, 2006). For *LOV1*, 8.4% of these residues (7 of 83) were affected by loss-of-function mutations, in comparison to 4.2% of all other residues (35 of 827) and 3.9% of other residues in the NB-ARC domain (11 of 281), indicating that mutation of these conserved residues is about twice as likely to cause loss of *LOV1* function, assuming equal chances of these residues undergoing mutagenesis. Additionally, *LOV1* is highly related to the *RPP8* family members

Table 1. Nucleotide diversity and segregating sites in functional regions of *LOV1*

Region ^a	Position	Length ^b	π^c	S_{total}^d	π_{silent}^c	S_{silent}^d	π_{nonsyn}^c	S_{nonsyn}^d
Entire region	1 to 3,388	3,386	0.00126	29 (0.0086)	0.00098	11	0.00143	18
Coding	273 to 3,236	2,729	0.00131	23 (0.0084)	0.00093	5	0.00143	18
Coiled-coil	273 to 425	153	0.00000	0 (0.0000)	0.00000	0	0.00000	0
CC to NB	426 to 770	345	0.00375	5 (0.0145)	0.00000	0	0.00477	5
NB	771 to 1,149 + 1,300 to 1,394	474	0.00078	3 (0.0063)	0.00126	1	0.00066	2
ARC1	1,395 to 1,458 + 1,543 to 1,742	264	0.00024	1 (0.0038)	0.00111	1	0.00000	0
ARC2	1,743 to 2,096	354	0.00179	4 (0.0113)	0.00000	0	0.00228	4
LRR	2,097 to 3,140	1,043	0.00054	5 (0.0048)	0.00127	2	0.00032	3
LRR to stop	3,141 to 3,236	96	0.00699	5 (0.0521)	0.00346	1	0.00798	4
5' noncoding	1 to 272	271	0.00181	4 (0.0148)	0.00181	4	NA	NA
Intron 1	1,150 to 1,299	150	0.00083	1 (0.0067)	0.00083	1	NA	NA
Intron 2	1,459 to 1,542	84	0.00000	0 (0.0000)	0.00000	0	NA	NA
3' noncoding	3,237 to 3,388	152	0.00042	1 (0.0066)	0.00042	1	NA	NA

^a CC = coiled-coil; NB = nucleotide binding; and LRR = leucine-rich repeat.

^b Length in base pairs, excluding sites with gaps.

^c Nucleotide diversity, as determined by DnaSP 4.10 (Rozas et al. 2003).

^d Number of segregating sites. Numbers in parentheses are standardized by sequence length.

Table 2. Analysis of divergence between *Arabidopsis thaliana* and *A. korshinskyi* *LOV1* alleles

Region	Position	N_s^a	D_s^b	$K_s(\text{JC})^c$	N_a^a	D_a^b	$K_a(\text{JC})^c$	D_{xy}^d	K_a/K_s
Coding	1 to 2,772	599.7	106	0.2000	2109.3	151	0.0769	0.0961	0.3842
Coiled-coil	1 to 1,53	37.3	8	0.2526	115.7	4	0.0354	0.0784	0.1402
CC to NB	154 to 498	71.9	10	0.1538	273.1	21	0.0859	0.0932	0.5583
NB	499 to 972	99.9	15	0.1684	374.1	24	0.0674	0.0827	0.4004
ARC1	973 to 1,239	55.1	7	0.1291	196.9	13	0.0719	0.0795	0.5567
ARC2	1,240 to 1,596	75.2	16	0.2411	275.8	28	0.1127	0.1281	0.4674
LRR	1,597 to 2,670	241.2	46	0.2210	796.8	58	0.0768	0.1010	0.3473
LRR to stop	2,671 to 2,772	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.

(RPP8, RCY1, and HRT), which all have known functions in resistance (Cooley et al. 2000; McDowell et al. 1998; Takahashi et al. 2002), and the residues that were found to be substituted in the nonfunctional *lov1* alleles 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 two 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 and associates (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 motif, leading the authors to speculate that this serves as a negative regulatory region. However, mutation of amino acids at two other positions in this motif led to loss of function of both LOV1 and RPM1 (discussed above), suggesting that the RNBS-D motif 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 conjecture 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 with 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. 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 to 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 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 to 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, mutation of 47 amino acids 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 to 50% coverage rate would suggest that mutation of only about 16 to 20% of the mutable amino acids would lead to loss of function.

The conflicting coverage estimates discussed above could be explained in two different ways. 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 to 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 (Ade et al. 2007; Bendahmane et al. 2002; Frost et al. 2004; Tao et al. 2000; Zhang et al. 2004). 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 (Howles et al. 2005; Moffett et al. 2002). 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 (Fig. 3B). 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 with other NB-LRR genes studied to date (Bakker et al. 2006b; Mauricio et al. 2003; Rose et al. 2004). 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 with $\pi_a = 0.0014$ and $\pi_s = 0.0009$ for LOV1 (Table 1). For the 27 NB-LRR genes evaluated by Bakker and associates (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 study by Bakker and associates 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 study by Bakker and associates (2006b). 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 by Bakker and associates (2006b), 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, in the LRR region, which is often found to be under positive selection in resistance genes (Bergelson et al. 2001; Mondragon-

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 1). Overall, the level of within-species polymorphisms is too low to obtain statistically significant K_a/K_s ratios, and therefore, we cannot decisively say what type of selection has occurred at the LOV1 locus.

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 (Mauricio et al. 2003; Shen et al. 2006; Stahl et al. 1999; Tian et al. 2002). 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 (Axtell et al. 2003; Mackey et al. 2002, 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 (Dodds et al. 2006; Van der Hoorn et al. 2002). 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 (Laloi et al. 2004; Reichheld et al. 2002), 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 (Belkadir et al. 2004; Dodds et al. 2001; Ellis et al. 1999; 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 1), 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 2), 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. victorinae*. 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 an as-yet-undiscovered pathogen. Therefore, the *LOV1* gene could represent a situation in which 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.

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 4°C for 5 days in 0.1% agarose, and then, were applied to soil. Plants were grown under long-day conditions (16 h light, 8 h dark) at 22°C. 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 of victorin per milliliter 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 victorin at a concentration of 10 µg/ml, and distilled water was added as necessary.

DNA preparation and sequencing.

Plant genomic DNA was prepared as described by Edwards and associates (1991). PCR amplification of mutant *lov1* alleles was performed in segments using the following primers: LOV1a F 5'-TCTTCTTGTCGTGACCACAC-3' and LOV1a R 5'-TTCTTGCAGCGACATCGAAC-3'; LOV1b F 5'-CAAGAAGCATGCGAGAAGAC-3' and LOV1b R 5'-TGTGTGCGTCTTGCTTGCTT-3'; LOV2 F 5'-ACACTGGTCGCAATGCCTT-3' and LOV2 R 5'-GGAAGTTCTCTCTTTGGCT-3'; LOV3 F 5'-GAACAACCATCCAAGATAAGG-3' and LOV3 R 5'-CACGAGGTTCTTGCCTATCA-3'; LOV4 F 5'-ACGCTAGTGTGATGGACCTC-3' and LOV4 R 5'-TGAGGTGTGCACAAGTGAGC-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'-CATTCTCCACCAAATGACCA-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'-CTTCTC CACTATCTCGGATG-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, Valencia, CA, U.S.A.) before sequencing. For the *lov1* mutant alleles, any alleles showing more than one mutation were reamplified 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 *XbaI* fragment containing the entire *LOV1* gene in the binary vector pCB302 (Xiang et al. 1999). The *LOV1* gene contains a single *NcoI* site that occurs between the 621 A to T (nonsense mutation) and the 1,767 G to A (missense mutation) polymorphisms in the Col-4 sequence and a single *BamHI* site that occurs between the 2,152 A to C (missense mutation) and the 3,042 T deletion (frameshift mutation) polymorphisms. These restriction sites were used to remove portions of the Cl-0 *LOV1* gene from the 6.5-kb *XbaI* clone. Portions of the Col-4 *lov1* allele were PCR-amplified from Col-4 genomic DNA, were digested with the appropriate enzymes, and were ligated into the 6.5-kb *XbaI* clone. This resulted in creation of three clones, one containing the nonsense mutation, one containing both missense mutations, and one containing the frameshift mutation, that otherwise encoded the Cl-0 LOV1 protein. Clones were electroporated into *Agrobacterium tumefaciens* GV3101 and were 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.

SSLP 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, CIW1, CIW4, CIW5, CIW6, CIW7, CIW9, CIW10, CIW11, and 3571. Only marker 3571 is linked to *LOV1*. Primer sequences for all markers except 3571 were obtained from The *Arabidopsis* Information Resource (TAIR) website. Primers for 3571 were previously described (Sweat and Wolpert 2007). 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 two-parameter model and other default settings. It was created from an alignment of the entire *LOV1* sequenced region, including both coding and noncoding regions.

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