Thioredoxin *h*5 Is Required for Victorin Sensitivity Mediated by a CC-NBS-LRR Gene in *Arabidopsis* [W]

Teresa A. Sweat and Thomas J. Wolpert[1]

Department of Botany and Plant Pathology, Center for Genome Research and Biocomputing, Oregon State University, Corvallis, Oregon, 97331-2902

The fungus *Cochliobolus victoriae* causes Victoria blight of oats (*Avena sativa*) and is pathogenic due to its production of victorin, which induces programmed cell death in sensitive plants. Victorin sensitivity has been identified in *Arabidopsis thaliana* 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, nonredundant 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 Cys 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 (*Avena sativa*; 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 kD) proteins that act as protein disulfide oxidoreductases and are found in all free-living organisms (Buchanan and Balmer, 2005). TRXs contain two Cys residues in their active site that either form a disulfide bond or exist as thiols, depending on the oxidation state of the protein. Reduced TRX can help protect cells from oxidative stress by providing reducing power to thiol-containing antioxidant proteins. Alternatively, reduction of a disulfide bond by a TRX can directly activate or inactivate the target protein (Gelhaye et al., 2005; Masutani et al., 2005). In this function, TRXs act as redox-dependent regulators of enzyme activity. Mammals have one cytosolic TRX and one mitochondrial TRX, which are both

essential for survival (Matsui et al., 1996; Nonn et al., 2003). By contrast, the *Arabidopsis* genome encodes 19 TRXs 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 TRXs, which are generally thought to be cytosolic proteins. The *h*-type TRXs 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). By contrast, thioredoxin *h*3 (*ATTRX3*), the TRX 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, nonredundant functions assigned to any of the *h*-type TRXs 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* (for *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* (*Pst*). 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.

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% ethyl methanesulfonate (EMS). The mutagenized seed were used to grow 4096 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 ~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 ~1 to 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 two 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 *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 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 chamber for 1 week. Infection levels on the LOV1 plants were variable with all older leaves (third and fourth true leaves) showing severe symptoms (Figure 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 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 effect of mutation of *liv1* on cell death associated with the hypersensitive response (HR) by infiltrating wild-type and mutant plants with *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 the wild type with regard to its response to infiltration with *Pst* (Figure 1B). Nearly all inoculated leaves of both the wild-type and mutant lines developed an HR to *Pst avrRpm1* by 6 h and an HR to *Pst avrRpt2* by 20 h. 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
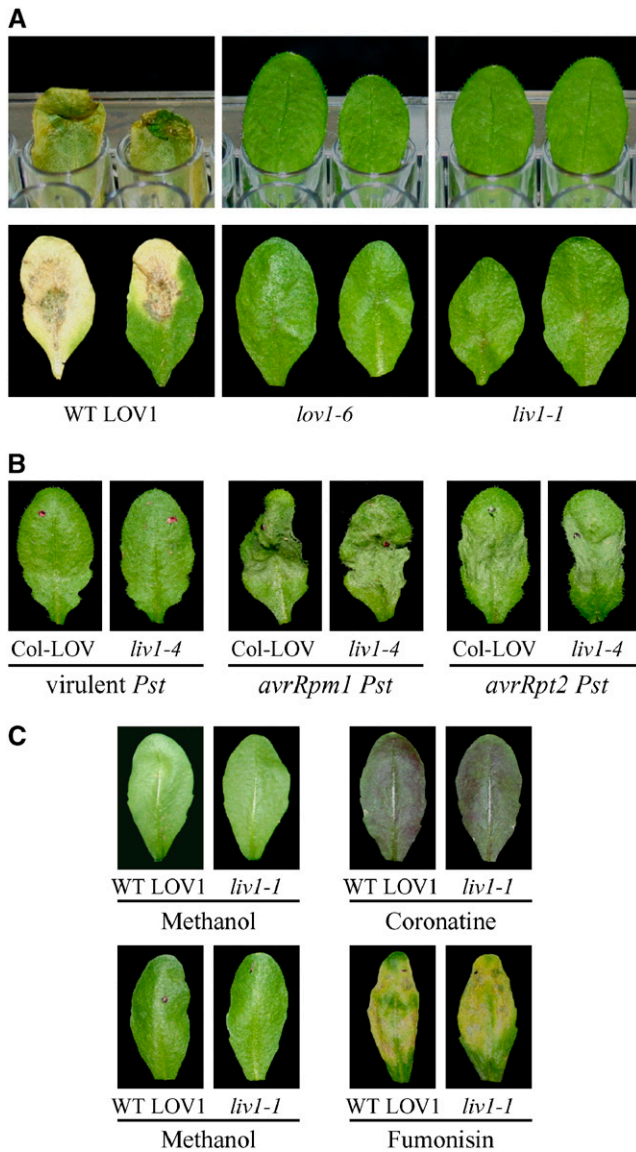
Figure 1. Response of Victorin-Insensitive Mutants to Toxin Exposure or Pathogen Infection.

(A) Leaves from wild-type LOV1, *lov1-6*, and *liv1-1* plants photographed 3 d after treatment with victorin (top) or 7 d after inoculation with *C. victoriae* (bottom). *n* ≥ 50 leaves per line for infection assays.

(B) Leaves from wild-type Col-LOV or *liv1-4* plants photographed 20 h after infiltration with virulent *Pst* or *Pst* carrying *avrRpm1* or *avrRpt2*. *n* ≥ 48 leaves per treatment per plant line.

(C) Leaves from wild-type LOV1 or *liv1-1* plants photographed 6 d after infiltration with 5% methanol (left), 100 nM coronatine (top right), or 20 μM fumonisin (bottom right). The underside of the leaves in the top row was photographed to show accumulation of anthocyanin. *n* ≥ 30 leaves per treatment per line.

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 1C, top). We also tested coronatine at 5 nM and 1 μM. At 5 nM, none of the lines tested showed any symptoms after 1 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 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); 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 three sensitive to one 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-centimorgan region between nga392 and nga280 (Figure 2). To facilitate fine-mapping, new SSLPs were developed in this region (see Supplemental Table 1 online). The new polymorphic markers were then used to narrow the region containing *LIV1* to an ∼0.4-centimorgan (∼50 kb) region
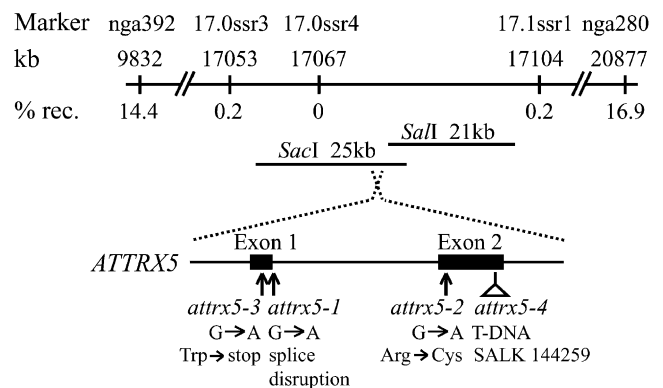

Figure 2. Map-Based Cloning of *LIV1*.

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.

between 17,053 and 17,104 kb from the north end of Chromosome I. This region was completely contained on BAC F27F5. Overlapping SacI and SalI fragments spanning the majority of the 50-kb region were subcloned from BAC F27F5 and introduced into the *liv1-1* mutant by *Agrobacterium tumefaciens*–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 SacI fragment was found to restore victorin 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 β-galactosidase and a cytosolic TRX. Because the TRX was the most likely candidate, this gene was PCR-amplified from the SacI subclone, including ~1.3 kb upstream of the start codon and 400 bp downstream of the stop codon. The cloned TRX gene was found to restore victorin sensitivity when introduced into the *liv1-1* mutant (Figure 3A). This TRX belongs to the *h*-type family of TRXs, which are thought to be localized to the cytosol. This gene has been designated as TRX *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 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. By contrast, each of the *liv1* mutants showed a single nucleotide change in the *ATTRX5* gene (Figure 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 mutation in *liv1-2* is a missense mutation that converts Arg-43 to a Cys. This additional Cys 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). 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
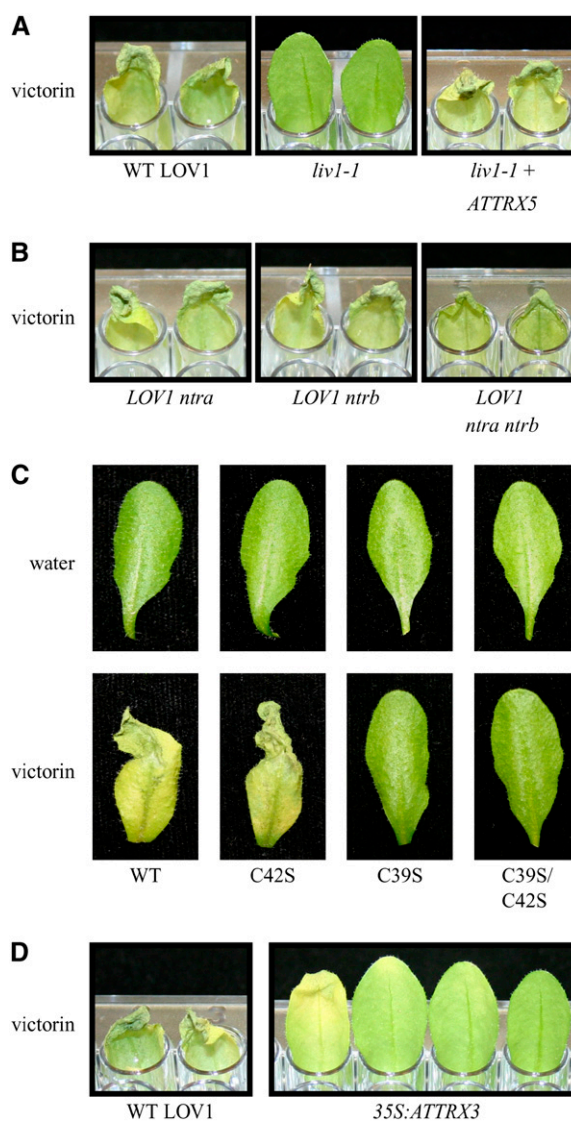

Figure 3. Victorin Sensitivity Phenotypes of Plants Expressing Wild-Type or Mutant *ATTRX5*, Overexpressing *ATTRX3*, or Mutant for the NADPH-Dependent TRX Reductase Genes *NTRA* and *NTRB*.

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 d after treatment with victorin.

(B) Leaves from plants carrying the *LOV1* gene and mutant for either *ntra*, *ntrb*, or both photographed 2 d after treatment with victorin. n ≥ 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 d 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 d after treatment with victorin.

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 TRX Reductases

Because TRXs generally function by reducing other proteins, a system is required to maintain the pool of reduced TRX 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 3B and 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 3B). This indicates that loss of both NTR isoforms is insufficient to block the response to victorin.

Requirement for Active-Site Cys Residues

The two Cys residues in the active site of TRXs enable them to reduce other Cys-containing proteins. When these Cys residues are in the reduced form, the first Cys 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 Cys (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 Cys residues (Kallis and Holmgren, 1980). 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 Ser 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. By 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 3C). Surprisingly, the C42S transgene was able to restore victorin sensitivity as effectively as the wild-type cDNA (Figure 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 TRX. Alternatively, these data may indicate that the redox function of ATTRX5 is not required for victorin sensitivity.

Complementation by ATTRX3

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 (Figure 6A). 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 4). In the T1 generation, a range of phenotypes was again observed, ranging from no sensitivity to a moderate response to victorin (Figure 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*.

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 5A). By 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 h after infiltration. The levels continue to increase rapidly, showing maximum expression at 12 h and diminishing thereafter. By 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-h 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
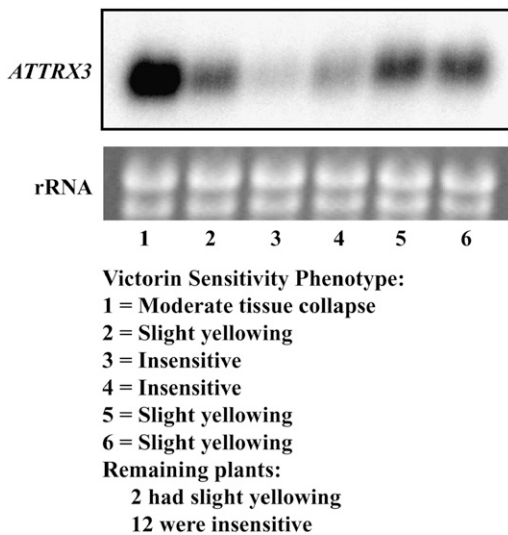
Figure 4. Correlation of *ATTRX3* Transgene Expression with Victorin Sensitivity.

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.

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 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 with wild-type plants (cf. Figures 5A and 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 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 were in a Col-0 background, we first tested *ATTRX5* induction in Col-LOV plants (Figure 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 with 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 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 SA 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 to better separate the effects of TRX specificity from differences due to expression levels. Additionally, 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 6A and 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 d 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 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 d 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 1). This suggests that the high level of *ATTRX5* induction seen in wild-type plants is not required for victorin sensitivity. By contrast, plants carrying the *ATTRX3* gene under
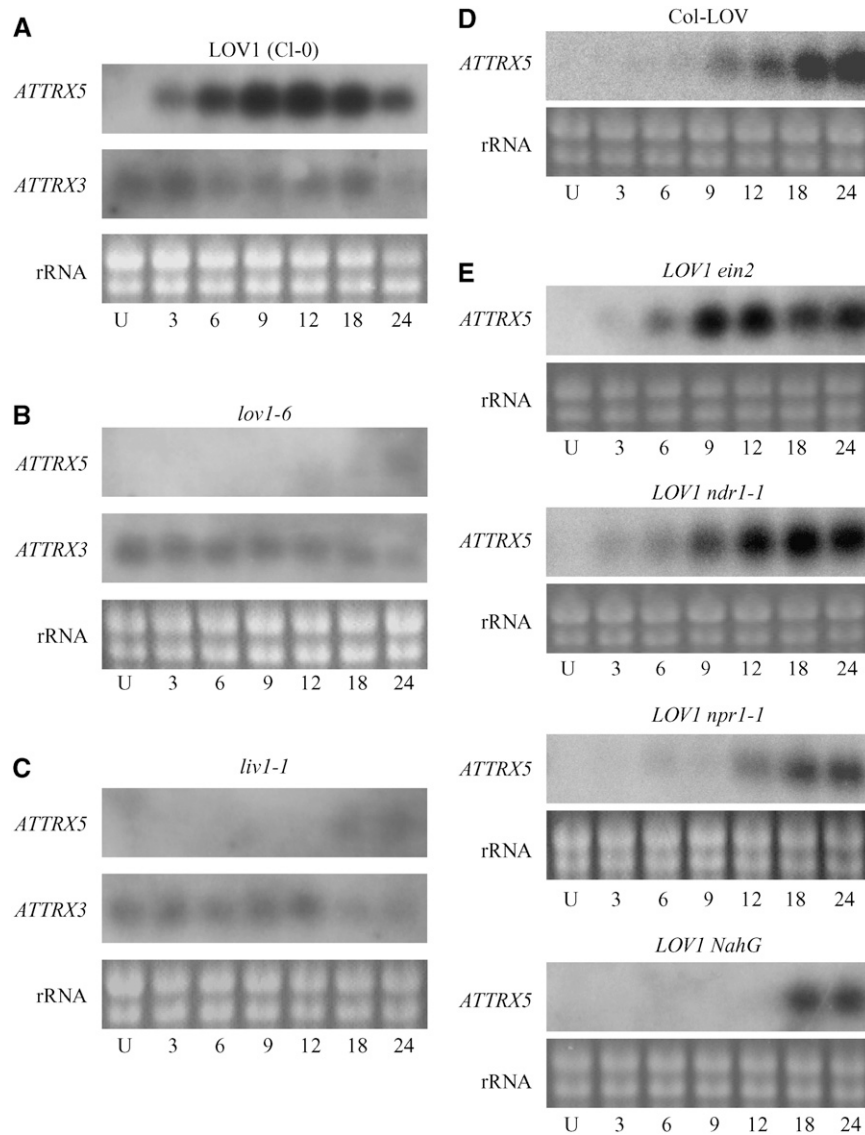
Figure 5. RNA Gel Blot Analysis of *ATTRX5* and *ATTRX3* Gene Expression.

[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.

control of the *ATTRX5* promoter showed few symptoms following victorin treatment (average rating of 0.9 on day 3) (Table 1). 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 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 1). 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
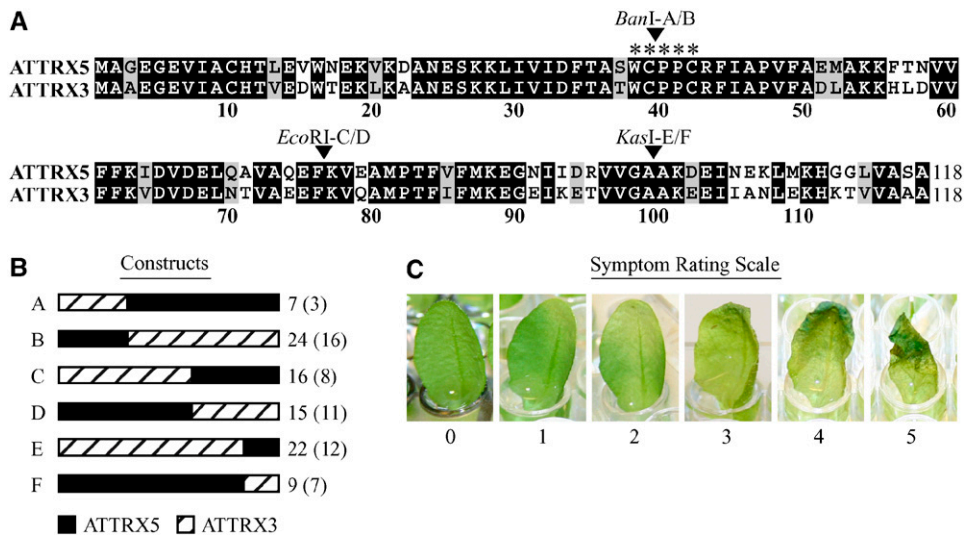
Figure 6. Construction and Evaluation of *ATTRX5/ATTRX3* Fusion Constructs.

(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.

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 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 6A). By 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 1). 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 com-pared with 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 con-structs 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 indepen-dent mutants that are completely insensitive to victorin. Com-plementation 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 Cys 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

Table 1. 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 percentage of 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 percentage of similarity and percentage of identity of chimeric constructs A to F to wild-type ATTRX5.

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 TRXs in six major groups (*f*, *m*, *h*, *o*, *x*, and *y*) as well as multiple TRX-like and TRX domain–containing proteins (Meyer et al., 2002). These include eight *h*-type TRXs. A few *h*-type TRXs have been found to be targeted to specific subcellular locations, including a mitochondrial TRX*h* in poplar (*Populus* spp; Gelhaye et al., 2004) and a plasma membrane–anchored TRX*h* in soybean (*Glycine max*; 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 TRXs, largely due to the apparent redundancy of the system. Various proteomics approaches have been developed in an attempt to identify proteins targeted by TRXs. One method involves immobilizing TRX 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 TRXs (ATTRX1-ATTRX5) and resulted in identification of several new potential TRX 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 TRXs 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 TRXs. 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). By 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 remained constant throughout the course of the experiment (Figure 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 noninducible *ATTRX3* promoter, while *ATTRX3* is unable to fulfill this role even when expressed under the inducible *ATTRX5* promoter (Table 1). Furthermore, plants carrying mutations in SA signaling pathways show no decrease in victorin sensitivity (J.M. Lorang, unpublished results), even

though *ATTRX5* induction is significantly delayed (Figure 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 3D and 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 percentage of amino acid identity to wild-type ATTRX5 (Table 1), 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 1). 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 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 1, Figure 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 6A and 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 1). 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 with 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 TRXs is reducing disulfide bonds of target proteins. This requires involvement of both TRX active-site Cys residues. Initially, the first Cys forms a mixed disulfide with the target protein. The reduction is then completed by the second Cys residue (Kallis and Holmgren, 1980). Our data show that the first active-site Cys (Cys-39) of ATTRX5 is required for the response to victorin (Figure 3C). By contrast, mutation of the second Cys (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 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 TRXs 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 TRXs, 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 phoshphorylated, 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 TRXs ATTRX3 and ATTRX4, respectively (Mazzurco et al., 2001). These four TRXs, as well as *Arabidopsis* ATTRX5, all contain the active-site WCPPC, rather than the WCGPC found in most TRXs. Interestingly, it was found that ATTRX3 and ATTRX4 could also interact with SRK. By 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 TRXs 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 (*Solanum lycopersicum*) *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 TRX (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 TRX found to interact with Cf-9, CITRX, is only distantly related to previously described TRXs in *Arabidopsis*. It is most closely related to the plastid-localized *x*-type TRX. 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 HR (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 TRXs, 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 Cys residue abolishes binding of THL1 to SRK (Mazzurco et al., 2001), whereas mutation of both active-site Cys 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 Cys 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 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 noncatalytic Cys residue of TRX to the catalytic Cys 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, TRXs 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 with the action of ATTRX5, which acts as a positive regulator of victorin-induced cell death. Victorin triggers a programmed cell death 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 HR is also a form of programmed cell death 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 HR and victorin-induced cell death are related processes, which in the cases of Cf-9 and LOV1 are both regulated by cytosolic TRXs. 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.

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 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 Brian Staskawicz (University of California, Berkeley, CA). 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* (forward 5′-GTGGTGACCTCTCCCTCAAA-3′ and reverse 5′-CCCACTT-CACCGTTTCTCTC-3′), gene-specific primers for *ndr1-1* (forward 5′-AAT-CTACTACGACGATGTCCAC-3′ and reverse 5′-GTAACCGATGGCAAC-TTTCAC-3′) and *NahG* (forward 5′-CAGAAGGTATCGCCCAATTC-3′ and reverse 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 4°C for 5 d in 0.1% agarose and then applied to soil. Plants were grown under long-day conditions (16 h light/8 h dark) at 22°C.

Mutagenesis and Screening

For both the LOV1 and Col-LOV lines, ~20,000 seed were mutagenized in 0.2% EMS for 11 h. 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 min and rinsed three times with distilled water. For each family, ~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, and 0.01 μM $CoCl_2$]. After 5 d at 4°C, the Petri dishes were placed at room temperature under fluorescent lights. Approximately 2 to 3 d 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 ~4-d intervals until >95% of the seedlings died or became chlorotic. At this time, generally ~2 weeks after germination, healthy seedlings were transplanted into soil. When plants reached sufficient size, typically 2 to 3 weeks after transplanting, two leaves of each plant were infiltrated with 10 μg/mL victorin using a blunt-ended 1 mL syringe. Plants showing no symptoms 3 d after infiltration were reinfiltrated. For plants that remained healthy after another 3 d, 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 d. Plants that 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 25°C under fluorescent lights for 1 week. *Pst* DC3000 containing *avrRpt2*, *avrRpm1*, or an empty vector control (pBBR1-MCS2) were obtained from Jeff Chang (Oregon State University). Cultures were grown overnight in King's B medium with 30 μg/mL kanamycin at 28°C. Cells were harvested and resuspended in 10 mM $MgCl_2$ to a concentration of 5×10^7 colony-forming units/mL. Leaves were photographed 20 h 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 3.5-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 6 d 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 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 trinucleotide repeats. Primers were designed flanking each repeat, and the new markers were tested for polymor-

phisms between the two parent lines (see Supplemental Table 1 online). 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 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 were 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 d at 4°C, 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 20 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 ~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′-ACACTCTCGGTTAGC-CCTAAGTT-3′ using Platinum *Pfx* polymerase (Invitrogen). The resulting product was tailed with an A overhang by addition of Taq and incubation for 10 min at 72°C. The product was cloned into pCR 2.1-TOPO using the TOPO TA cloning kit (Invitrogen). The TRX 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 forward, 5′-AAAAGCTGATCCCAACAAGAA-3′; Exon1 reverse, 5′-CCCTAGAGA-GGAGAAGAAGAGAAAA-3′; Exon2 forward, 5′-TCTTGTTATGTCCAGG-GCTTTT-3′; Exon2 reverse, 5′-TTTTCGTGTTCGTGGTTGAA-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′-GTG-GACCGCTTGCTGCAACT-3′) in combination with the *ATTRX5* Exon2 forward 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 forward and Exon2 reverse 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 forward 5′-GTG-GTGACCTCTCCCTCAAA-3′ and reverse 5′-CCCACTTCACCGTTTCT-CTC-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 TRX 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 reverse (5′-CGCCCTAAACGTATCCCTCCT-3′) or NTRB forward (5′-TCGGAGC-GATTCGGTACTACG-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 forward (5′-CAAATCCGCCG-TCTCTAGCC-3′) and NTRA reverse or NTRB forward and NTRB reverse (5′-GACAAGCCATAGGGTCACAGAGC-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 number U09186) and *ATTRX3* (stock number 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 forward, 5′-CACCATGGCCGGTGAAGGAGA-3′; *ATTRX5* ENTR reverse, 5′-TCAAGCAGAAGCTACAAGACCA-3′; *ATTRX3* ENTR forward, 5′-CACCATGGCCGCAGAAGGAG-3′; *ATTRX3* ENTR reverse, 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 *Escherichia 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 Cys Residues

The active-site Cys residues were mutated both individually and in combination to Ser 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′-CTTCACAGCATCATGGAGTCCA-CCTTGCCG-3′; C42S, 5′-CATGGTGTCCACCTAGCCGTTTCATTGC-3′; C39S/C42S, 5′-CACAGCATCATGGAGTCCACCTAGCCGTTTCATTGC-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 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<u>GGTGCCC</u>ACCTTGCCG-3′; *ATTRX3 Eco*RI, 5′-GAACACTGTTGCTGAG<u>GAATTC</u>AAAGTTCAGGCA-ATGCC-3′; *ATTRX5 Kas*I, 5′-GATCGTGTTGTC<u>GGCGCC</u>GCGAAAGAT-GAGATC-3′; *ATTRX3 Kas*I, 5′-CAAGGAGACTGTGGTT<u>GGCGCC</u>GC-

TAAAGAAGAAATC-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 TRX. 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 three fragments were mixed in a three-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′-AGCAGAAGCTACAAGACCACC-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 pEarley-Gate 100 downstream of the *Asc*I site. The forward primer for fusions containing *ATTRX5* coding sequence at the 5′end was 5′-TCTT-AAAA<u>AGCTT</u>AAGAACAAATAATAAAAATGGCCGGTGAAGGAGA-3′. The forward primer for *ATTRX3* and fusions containing *ATTRX3* coding sequence at the 5′end was 5′-TCTTAAAA<u>AGCTT</u>AAGAACAAATAATA-AAAATGGCCGCAGAAGGAGA-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′ untranslated regions (UTRs) of each gene. These regions were PCR-amplified from genomic DNA using the following primers: *ATTRX5* 3′UTR forward 5′-GATGAAGCATGGTGGTCTTG-3′ and reverse 5′-TTTTCGTGTTCGTGGTTGAA-3′; *ATTRX3* 3′UTR forward 5′-CGAGA-AGCACAAGACAGTTG-3′ and reverse 5′-GCATAGCTGCGAGTAATC-AAG-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 forward primer in combination with the pEarley reverse 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.

Supplemental Data

The following material is available in the online version of this article.

   Supplemental Table 1. Polymorphic SSLP Markers Used to Map the *LIV1* Gene.

REFERENCES

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E. (1998). Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95:** 10306–10311.

Balmer, Y., Koller, A., del Val, G., Manieri, W., Schürmann, P., and Buchanan, B.B. (2003). Proteomics gives insight into the regulatory function of chloroplast thioredoxins. Proc. Natl. Acad. Sci. USA **100:** 370–375.

Balmer, Y., Vensel, W.H., Tanaka, C.K., Hurkman, W.J., Gelhaye, E., Rouhier, N., Jacquot, J.-P., Manieri, W., Schürmann, P., Droux, M., and Buchanan, B.B. (2004). Thioredoxin links redox to the regulation of fundamental processes of plant mitochondria. Proc. Natl. Acad. Sci. USA **101:** 2642–2647.

Belkhadir, Y., Subramaniam, R., and Dangl, J.L. (2004). Plant disease resistance protein signaling: NBS-LRR proteins and their partners. Curr. Opin. Plant Biol. **7:** 391–399.

Bent, A.F., Kunkel, B.N., Dahlbeck, D., Brown, K.L., Schmidt, R., Giraudat, J., Leung, J., and Staskawicz, B.J. (1994). *RPS2* of *Arabidopsis thaliana*: A leucine-rich repeat class of plant disease resistance genes. Science **265:** 1856–1860.

Bower, M.S., Matias, D.D., Fernandes-Carvalho, E., Mazzurco, M., Gu, T., Rothstein, S.J., and Goring, D.R. (1996). Two members of the thioredoxin-h family interact with the kinase domain of a Brassica *S* locus receptor kinase. Plant Cell **8:** 1641–1650.

Bréhélin, C., Mouaheb, N., Verdoucq, L., Lancelin, J.-M., and Meyer, Y. (2000). Characterization of determinants for the specificity of *Arabidopsis* thioredoxins h in yeast complementation. J. Biol. Chem. **275:** 31641–31647.

Buchanan, B.B., and Balmer, Y. (2005). Redox regulation: A broadening horizon. Annu. Rev. Plant Biol. **56:** 187–220.

Cabrillac, D., Cock, J.M., Dumas, C., and Gaude, T. (2001). The *S*-locus receptor kinase is inhibited by thioredoxins and activated by pollen coat proteins. Nature **410:** 220–223.

Cao, H., Bowling, S.A., Gordon, S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6:** 1583–1592.

Century, K.S., Holub, E.B., and Staskawicz, B.J. (1995). *NDR1*, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. Proc. Natl. Acad. Sci. USA **92:** 6597–6601.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Coffeen, W.C., and Wolpert, T.J. (2004). Purification and characterization of serine proteases that exhibit caspase-like activity and are associated with programmed cell death in *Avena sativa*. Plant Cell **16:** 857–873.

Curtis, M.J., and Wolpert, T.J. (2002). The oat mitochondrial permeability transition and its implication in victorin binding and induced cell death. Plant J. **29:** 295–312.

Curtis, M.J., and Wolpert, T.J. (2004). The victorin-induced mitochondrial permeability transition precedes cell shrinkage and biochemical markers of cell death, and shrinkage occurs without loss of membrane integrity. Plant J. **38:** 244–259.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science **266:** 1247–1250.

Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K., and Pikaard, C.S. (2006). Gateway-compatible vectors for plant functional genomics and proteomics. Plant J. **45:** 616–629.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. **19:** 1349.

Feys, B.J.F., Benedetti, C.E., Penfold, C.N., and Turner, J.G. (1994). Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6:** 751–759.

Florencio, F.J., Yee, B.C., Johnson, T.C., and Buchanan, B.B. (1988). An NADP/thioredoxin system in leaves: Purification and characterization of NADP-thioredoxin reductase and thioredoxin h from spinach. Arch. Biochem. Biophys. **266:** 496–507.

Gelhaye, E., et al. (2004). A specific form of thioredoxin *h* occurs in plant mitochondria and regulates the alternative oxidase. Proc. Natl. Acad. Sci. USA **101:** 14545–14550.

Gelhaye, E., Rouhier, N., Navrot, N., and Jacquot, J.P. (2005). The plant thioredoxin system. Cell. Mol. Life Sci. **62:** 24–35.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. **43:** 205–227.

Greenberg, J.T., and Yao, N. (2004). The role and regulation of programmed cell death in plant-pathogen interactions. Cell. Microbiol. **6:** 201–211.

Guzmán, P., and Ecker, J.R. (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell **2:** 513–523.

Heath, M.C. (2000). Hypersensitive response-related death. Plant Mol. Biol. **44:** 321–334.

Jiang, X., and Wang, X. (2004). Cytochrome *c*-mediated apoptosis. Annu. Rev. Biochem. **73:** 87–106.

Kallis, G.-B., and Holmgren, A. (1980). Differential reactivity of the functional sulfhydryl groups of cysteine-32 and cysteine-35 present in the reduced form of thioredoxin from *Escherichia coli*. J. Biol. Chem. **255:** 10261–10265.

Laloi, C., Mestres-Ortega, D., Marco, Y., Meyer, Y., and Reichheld, J.-P. (2004). The Arabidopsis cytosolic thioredoxin *h*5 gene induction by oxidative stress and its W-box-mediated response to pathogen elicitor. Plant Physiol. **134:** 1006–1016.

Laloi, C., Rayapuram, N., Chartier, Y., Grienenberger, J.M., Bonnard, G., and Meyer, Y. (2001). Identification and characterization of a mitochondrial thioredoxin system in plants. Proc. Natl. Acad. Sci. USA **98:** 14144–14149.

Litzenberger, S.C. (1949). Nature of susceptibility to *Helminthosporium victoriae* and resistance to *Puccinia coronata* in Victoria oats. Phytopathology **39:** 300–318.

Lorang, J.M., Carkaci-Salli, N., and Wolpert, T.J. (2004). Identification and characterization of victorin sensitivity in *Arabidopsis thaliana*. Mol. Plant Microbe Interact. **17:** 577–582.

Luke, H.H., Murphy, H.C., and Petr, F.C. (1966). Inheritance of spontaneous mutations of the Victoria locus in oats. Phytopathology **56:** 210–212.

Macko, V., Wolpert, T.J., Acklin, W., Jaun, B., Seibl, J., Meili, J., and Arigoni, D. (1985). Characterization of victorin C, the major host-selective toxin from *Cochliobolus victoriae*: Structure of degradation products. Experientia **41:** 1366–1370.

Masutani, H., Ueda, S., and Yodoi, J. (2005). The thioredoxin system in retroviral infection and apoptosis. Cell Death Differ. **12** (suppl. 1): 991–998.

Matsui, M., Oshima, M., Oshima, H., Takaku, K., Maruyama, T., Yodoi, J., and Taketo, M.M. (1996). Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. Dev. Biol. **178:** 179–185.

Mayama, S., Bordin, A.P.A., Morikawa, T., Tanpo, H., and Kato, H. (1995). Association of avenalumin accumulation with co-segregation of victorin sensitivity and crown rust resistance in oat lines carrying the Pc-2 gene. Physiol. Mol. Plant Pathol. **46:** 263–274.

Mayama, S., Tani, T., Ueno, T., Midland, S.L., Simms, J.J., and Keen, N.T. (1986). The purification of victorin and its phytoalexin elicitor activity in oat leaves. Physiol. Mol. Plant Pathol. **29:** 1–18.

Mazzurco, M., Sulaman, W., Elina, H., Cock, J.M., and Goring, D.R. (2001). Further analysis of the interactions between the *Brassica S* receptor kinase and three interacting proteins (ARC1, THL1, and THL2) in the yeast two-hybrid system. Plant Mol. Biol. **45:** 365–376.

Meehan, F., and Murphy, H.C. (1946). A new *Helminthosporium* blight of oats. Science **104:** 413–414.

Meehan, F., and Murphy, H.C. (1947). Differential phytotoxicity of metabolic by-products of *Helminthosporium victoriae*. Science **106:** 270–271.

Meyer, Y., Vignols, F., and Reichheld, J.P. (2002). Classification of plant thioredoxins by sequence similarity and intron position. Methods Enzymol. **347:** 394–402.

Mitchell, D.A., and Marletta, M.A. (2005). Thioredoxin catalyzes the S-nitrosation of the caspase-3 active site cysteine. Nat. Chem. Biol. **1:** 154–158.

Motohashi, K., Kondoh, A., Stumpp, M.T., and Hisabori, T. (2001). Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc. Natl. Acad. Sci. USA **98:** 11224–11229.

Navarre, D.A., and Wolpert, T.J. (1999). Victorin induction of an apoptotic/senescence-like response in oats. Plant Cell **11:** 237–249.

Nekrasov, V., Ludwig, A.A., and Jones, J.D.G. (2006). CITRX thioredoxin is a putative adaptor protein connecting Cf-9 and the ACIK1 protein kinase during the Cf-9/Avr9- induced defence response. FEBS Lett. **580:** 4236–4241.

Nonn, L., Williams, R.R., Erickson, R.P., and Powis, G. (2003). The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice. Mol. Cell. Biol. **23:** 916–922.

Reichheld, J.-P., Mestres-Ortega, D., Laloi, C., and Meyer, Y. (2002). The multigenic family of thioredoxin *h* in *Arabidopsis thaliana*: Specific expression and stress response. Plant Physiol. Biochem. **40:** 685–690.

Reichheld, J.-P., Meyer, E., Khafif, M., Bonnard, G., and Meyer, Y. (2005). AtNTRB is the major mitochondrial thioredoxin reductase in *Arabidopsis thaliana*. FEBS Lett. **579:** 337–342.

Rines, H.W., and Luke, H.H. (1985). Selection and regeneration of toxin-insensitive plants from tissue cultures of oats (*Avena sativa*) susceptible to *Helminthosporium victoriae*. Theor. Appl. Genet. **71:** 16–21.

Rivas, S., Rougon-Cardoso, A., Smoker, M., Schauser, L., Yoshioka, H., and Jones, J.D.G. (2004). CITRX thioredoxin interacts with the tomato Cf-9 resistance protein and negatively regulates defence. EMBO J. **23:** 2156–2165.

Rivera-Madrid, R., Mestres, D., Marinho, P., Jacquot, J.-P., Decottignies, P., Miginiac-Maslow, M., and Meyer, Y. (1995). Evidence for five divergent thioredoxin *h* sequences in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **92:** 5620–5624.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J. **17:** 2596–2606.

Shain, L., and Wheeler, H. (1975). Production of ethylene by oats resistant and susceptible to victorin. Phytopathology **65:** 88–89.

Shi, J., and Bhattacharyya, M.K. (1996). A novel plasma membrane-bound thioredoxin from soybean. Plant Mol. Biol. **32:** 653–662.

Stone, J.M., Heard, J.E., Asai, T., and Ausubel, F.M. (2000). Simulation of fungal-mediated cell death by fumonisin B1 and selection of fumonisin B1-resistant (*fbr*) Arabidopsis mutants. Plant Cell **12:** 1811–1822.

Stone, J.M., Liang, X., Nekl, E.R., and Stiers, J.J. (2005). Arabidopsis *AtSPL14*, a plant-specific SBP-domain transcription factor, participates in plant development and sensitivity to fumonisin B1. Plant J. **41:** 744–754.

Tornero, P., Chao, R.A., Luthin, W.N., Goff, S.A., and Dangl, J.L. (2002). Large-scale structure-function analysis of the Arabidopsis RPM1 disease resistance protein. Plant Cell **14:** 435–450.

Ullrich, C.I., and Novacky, A.J. (1991). Electrical membrane properties of leaves, roots, and single root cap cells of susceptible *Avena sativa*. Plant Physiol. **95:** 675–681.

Vieira Dos Santos, C., and Rey, P. (2006). Plant thioredoxins are key actors in the oxidative stress response. Trends Plant Sci. **11:** 329–334.

Walton, J.D. (1996). Host-selective toxins: Agents of compatibility. Plant Cell **8:** 1723–1733.

Welsh, J.N., Peturson, B., and Machacek, J.E. (1954). Associated inheritance of reaction to races of crown rust, *Puccinia coronata avenae* Erikss., and to Victoria blight, *Helminthosporium victoriae* M. and M., in oats. Can. J. Bot. **32:** 55–68.

Wheeler, H., and Black, H.S. (1962). Changes in permeability induced by victorin. Science **137:** 983–984.

Wolpert, T.J., Dunkle, L.D., and Ciuffetti, L.M. (2002). Host-selective toxins and avirulence determinants: what's in a name? Annu. Rev. Phytopathol. **40:** 251–285.

Wolpert, T.J., Macko, V., Acklin, W., Jaun, B., Seibl, J., Meili, J., and Arigoni, D. (1985). Structure of victorin C, the major host-selective toxin from *Cochliobolus victoriae*. Experientia **41:** 1524–1529.

Yamazaki, D., Motohashi, K., Kasama, T., Hara, Y., and Hisabori, T. (2004). Target proteins of the cytosolic thioredoxins in *Arabidopsis thaliana*. Plant Cell Physiol. **45:** 18–27.

Yao, N., Tada, Y., Park, P., Nakayashiki, H., Tosa, Y., and Mayama, S. (2001). Novel evidence for apoptotic cell response and differential signals in chromatin condensation and DNA cleavage in victorin-treated oats. Plant J. **28:** 13–26.

Yao, N., Tada, Y., Sakamoto, M., Nakayashiki, H., Park, P., Tosa, Y., and Mayama, S. (2002). Mitochondrial oxidative burst involved in apoptotic response in oats. Plant J. **30:** 567–579.