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A "Whirly" Transcription Factor Is Required for Salicylic Acid-Dependent Disease Resistance in *Arabidopsis*

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Summary

Transcriptional reprogramming is critical for plant disease resistance responses; its global control is not well understood. Salicylic acid (SA) can induce plant defense gene expression and a long-lasting disease resistance state called systemic acquired resistance (SAR). Plant-specific "Whirly" DNA binding proteins were previously implicated in defense gene regulation. We demonstrate that the potato StWhy1 protein is a transcriptional activator of genes containing the PBF2 binding PB promoter element. DNA binding activity of AtWhy1, the *Arabidopsis* StWhy1 ortholog, is induced by SA and is required for both SA-dependent disease resistance and SA-induced expression of an SAR response gene. *AtWhy1* is required for both full basal and specific disease resistance responses. The transcription factor-associated protein NPR1 is also required for SAR. Surprisingly, AtWhy1 activation by SA is NPR1 independent, suggesting that AtWhy1 works in conjunction with NPR1 to transduce the SA signal. Our analysis of AtWhy1 adds a critical component to the SA-dependent plant disease resistance response.

Introduction

The perception of an invading pathogen by a plant cell leads to the activation of signal transduction pathways that globally alter the gene expression pattern of the host (Dangl and Jones, 2001). A large set of defense genes with various biochemical functions, including pathogenesis-related (*PR*) genes, are activated or repressed in response to pathogen attack. In addition, *PR* genes are also induced in uninfected parts of the plant to generate a long-lasting, broad-spectrum disease resistance throughout the entire plant termed systemic acquired resistance (SAR) (Ryals et al., 1996). The pathogen-induced accumulation of salicylic acid (SA), both at the infection site and distally, is necessary and sufficient for activation of SAR in *Arabidopsis* (Ryals et al., 1996). SAR induction requires the SA-dependent nuclear translocation and activation of the NPR1 protein, identified by its non-PR expression mutant phenotype (Cao et al., 1994; Delaney et al., 1995; Mou et al., 2003). NPR1 seems to act as a modulator of transcription, but does not appear to directly bind DNA. Numerous defense-associated transcription factors and their cognate *cis* acting elements have been identified (Rushton and Somssich, 1998). Yet, the functional requirement for these proteins in defense responses is largely unknown.

Transcriptional activation of the potato pathogenesis-related gene *PR-10a* by the oomycete pathogen *Phytophthora infestans* requires a 25 base pair (bp) promoter element termed the elicitor response element (ERE) (Després et al., 1995). Single-stranded ERE is bound by the nuclear factor PBF-2. Single-stranded DNA binding activity correlates with *PR-10a* expression, suggesting that PBF-2 functions as a transcriptional activator of *PR-10a* (Desveaux et al., 2000).

PBF-2 is a tetramer of 24 kDa protomers (StWhy1; *Solanum tuberosum* Whirly 1, formerly p24). Orthologs of StWhy1 are found throughout the plant kingdom, but not in animals or yeast. However, limited sequence similarity does exist between StWhy1 and the *Tetrahymena thermophila* TIF1 protein (Saha et al., 2001). The crystal structure of PBF-2 revealed that StWhy1 monomers assemble in a cyclic arrangement to produce a whirligig-like quaternary structure, inspiring the name Whirly (Why) for this family of proteins (Desveaux et al., 2002). This three-dimensional arrangement of StWhy1 molecules is predicted to be strongly conserved among plant orthologs.

Here, we confirm the role of StWhy1 as a transcriptional activator using transient expression assays and chromatin immunoprecipitation (ChIP) analysis. The DNA binding activity of AtWhy1 in *Arabidopsis* was revealed by treatment of plants with SA and is, surprisingly, NPR1 independent. To examine the function of the Whirly family in disease resistance, we obtained mutants of *AtWhy1*, the *Arabidopsis* Whirly ortholog most similar to StWhy1, from the *Arabidopsis* TILLING (Targeting Induced Local Lesions in Genomes) Project (McCallum et al., 2000; Colbert et al., 2001). Two *atwhy1* mutant alleles were severely compromised in SA-induced defenses, establishing AtWhy1 as an important downstream component of the SA-signaling pathway whose activation is independent of *NPR1*. Furthermore, *Arabidopsis atwhy1* mutants exhibited enhanced susceptibility to infection by both virulent and avirulent isolates of the oomycete *P. parasitica*. The degree of susceptibility to infection correlated directly with the decreased levels of AtWhy1 binding activity recovered from each mutant allele. These results establish a causal role for Whirly proteins in plant defense responses.

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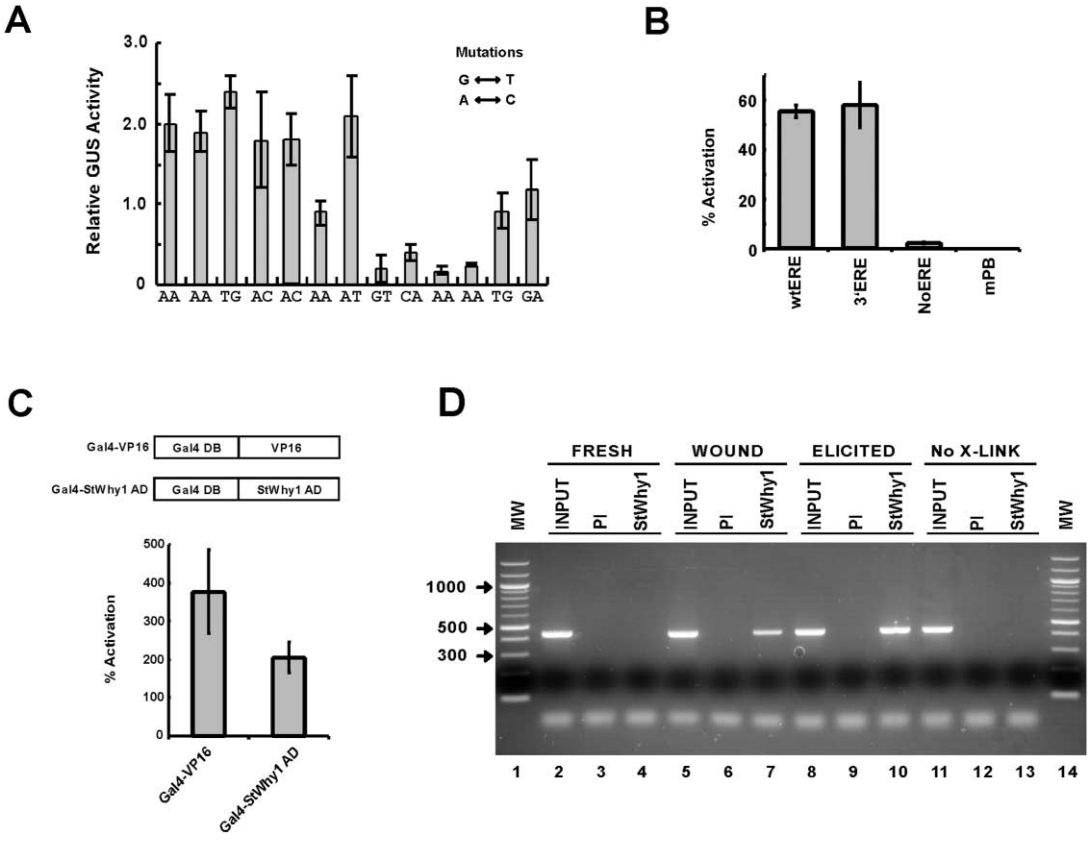

Figure 1. The PB Element (GTCAAAAA/T) Is Necessary and Sufficient for ERE Activity

(A) The ERE was mutated two nucleotides at a time and fused to the *uidA* reporter gene encoding β-glucuronidase. The wild-type ERE sequence is indicated below the *x* axis with each bar representing the GUS activity measured for each corresponding two nucleotide mutation relative to the wild-type construct. Each nucleotide was mutated according to the legend presented with G to T; T to G and C to A; A to C. The results represent the electroporation of at least three separate batches of potato protoplasts, done in triplicate within each batch. Bars indicate the standard deviation of the mean.

(B) The sequence of StWhy1 from amino acids 68–274 was fused to the 35S promoter and coelectroporated along with the β-glucuronidase reporter fusion gene constructs wtERE, 3′ERE (AgcttgattCtagAATGTCAAAAATG), noERE (cgAcgcaGtgcgAAgctTgAttctaG), and mPB (5′AAAAT GACAAAATtgCAAAAAT3′). mPB has the first two nucleotides (GT) of the PB element mutated and results in a reduction of over 80% in reporter gene activity relative to wild-type (Desveaux et al., 2000). Bars indicate the percent increase in reporter gene activity obtained in the presence of StWhy1, compared to results from coelectroporation of each reporter plasmid with the control human immunophilin protein FKBP plasmid. The results represent at least two independent experiments, with each electroporation conducted in triplicate within each experiment. Bars indicate the standard deviation of the mean.

(C) Diagrammatic representation of the Gal4 DNA binding domain (Gal4DB) fusion proteins. "Gal4-VP16" represents the Gal4DB fused to the VP16 acidic transactivation domain of herpes simplex virus; "Gal4-StWhy1 AD" represents the Gal4DB fused to StWhy1 activation domain beginning at residue 55 and ending after the polyglutamine stretch (residue 99). Expression of each fusion protein is driven by a double 35S promoter. Plasmids containing the Gal4 fusion constructs were coelectroporated into leaf protoplasts with the luciferase reporter gene fused to 5XGal4 DNA binding sites. The histogram represents the luciferase activity from the reporter gene cotransfected with the corresponding effector plasmid relative to reporter gene when cotransfected with plasmid expressing the Gal4DB alone. Transfection efficiencies were corrected by coelectroporating a β-glucuronidase reporter gene. Results are for two batches of protoplasts prepared at different times, each plasmid having been electroporated three times within each batch of protoplasts. Error bars represent the standard deviation of the mean.

(D) Chromatin immunoprecipitation (ChIP) was conducted using StWhy1 antibodies on extracts from potato tubers. Tissues were either fresh (lanes 2–4), wounded 18 hr (lanes 5–7), or elicited 12 hr with arachidonic acid with a 6 hr wound period prior to elicitor treatment (lanes 8–10). PCR was conducted using *PR-10a* specific primers on the total chromatin extracts of each tissue (+), and on extracts subjected to immunoprecipitation using either preimmune serum (PI) or StWhy1 antibodies (StWhy1). Lanes 11 to 13 represent the PCR products from elicited tissue extract not subjected to formaldehyde crosslinking. Molecular weight standards (lanes 1 and 14) are indicated at the left in bp.

Results

Functional Characterization of the Elicitor Response Element

In order to define the minimal ERE sequence required for *PR-10a* expression, a detailed two-nucleotide exchange scanning mutational analysis was conducted (Figure 1A). Only mutations affecting the sequence GTCAAAAA significantly reduced reporter gene expression in transient assays, with no significant reduction observed by mutations 5′ or 3′ of this sequence. The closely related element GTCAAAAT is also sufficient for function in vivo

Table 1. Occurrence of the PB Element in *Arabidopsis* Promoters

SOM	# of Promoters	Occurrences of PB	Average PB/Promoters	Expected PB/Promoters[c]	Fold Enrichment	# of Promoters with PB	P Value[d]
Som c1[a]	26	15	0.58	0.17	3.4	11	0.001
Som c7[a]	15	1	0.07	0.17	0.4	1	0.21
Random 20-1[b]	20	6	0.3	0.16	1.9	4	0.19
Random 20-2[b]	20	4	0.2	0.16	1.2	4	0.19
Random 20-3[b]	20	2	0.1	0.16	0.6	2	0.21

[a] According to Maleck et al., 2000.
[b] See Supplemental Figure S3 for list of promoters.
[c] Based on promoter lengths of 1100 bp for Som c1 and Som c7 and 1000 bp for the random sets.
[d] Probability of seeing the observed number of promoters with n PB elements by chance.

(data not shown). Therefore, the sequence GTCAAAAA/T is required for optimal activity of the ERE; we term this element the PB (PBF-2 binding) core element.

PB Element-Dependent *PR-10a* Gene Induction by StWhy1

To examine whether StWhy1 can induce *PR-10a* expression, we coexpressed StWhy1, or a control protein (human FK506 binding protein [FKBP]), in potato protoplasts together with β-glucuronidase reporter gene constructs containing the wild-type ERE (wtERE), the 3′ half of the ERE containing the PB element (3′ERE), no ERE (noERE), or a mutant of the PB element (mPB) (Figure 1B). β-glucuronidase reporter gene expression from each reporter plasmid was unaffected by the control FKBP-expressing plasmid (data not shown). A 55% increase in reporter gene activity was only observed when the wtERE or 3′ERE constructs were expressed in the presence of StWhy1 (Figure 1B). Neither the noERE nor the mPB mutant constructs were affected by the expression of StWhy1, demonstrating that the increase in reporter gene activity observed was dependent on an intact PB element. The low percent activation observed in these protoplasts reflects the fact that they are already activated for defense gene transcription (Desveaux et al., 2000).

The polyglutamine stretch (residues 74–83) at the N-terminal region of StWhy1 represents a potential transactivation domain (Desveaux et al., 2000). To test this, we fused the polyglutamine-containing region of StWhy1 to the Gal4 DNA binding domain (Gal4DB) and analyzed its ability to transactivate a reporter gene construct consisting of five repeats of the Gal4 DNA binding domain fused to the luciferase gene in potato protoplasts (Figure 1C). The VP16 activation domain of the herpes simplex virus was used as a positive control and increased reporter gene activity by 3.8-fold (Figure 1C). The N-terminal region of StWhy1 (amino acids 55–99) containing the polyglutamine stretch transactivated gene expression by 2.1-fold. Thus, this region functions as a transactivation domain. Further confirmation of the polyglutamine-dependent transactivation properties of StWhy1 was obtained in yeast (see Supplemental Figure S1 [http://www.developmentalcell.com/cgi/content/full/6/2/229/DC1]).

StWhy1 Is Associated with *PR-10a* in Stressed Potato Tubers

Chromatin immunoprecipitation (ChIP) has been used to demonstrate the association of transcription factors with the genes they regulate. We used ChIP to determine if the StWhy1 protein is associated with the potato *PR-10a* gene in vivo (Figure 1D). Nuclei were isolated from fresh, wounded, and arachidonic acid elicited tubers. Fragmented chromatin was immunoprecipitated using anti-StWhy1 antibodies (StWhy1; lanes 4, 7, and 10) or preimmune serum (PI; lanes 3, 6, and 9) after crosslinking by formaldehyde treatment. PCR was performed on input DNA before immunoprecipitation (Input; lanes 2, 5, 8, and 11) and on immunoprecipitated DNA using primers to amplify the potato *PR-10a* (m29041). These primers were designed to amplify nucleotide positions 496–895 of the *PR-10a* coding region (Matton and Brisson, 1989) to avoid the high A/T content of the *PR-10a* promoter and the presence of an inverted repeat at the ERE.

The ChIP experiment demonstrated that elicited tubers show increased StWhy1 association with *PR-10a* relative to wounded tubers (Figure 1D, compare lanes 8–10 to lanes 5–7), while no binding was observed in fresh tubers (lanes 2–4) or in the absence of crosslinking (lanes 11–13). Sequencing of the 400 bp PCR product confirmed it to be *PR-10a* (data not shown). The levels of StWhy1 association with *PR-10a* identified by chromatin immunoprecipitation correspond to recovery of StWhy1 DNA binding activity from these tissues (Després et al., 1995; Desveaux et al., 2000). Furthermore, the lower level of StWhy1 association with *PR-10a* in wounded tuber relative to elicited extracts correlates with *PR-10a* mRNA accumulation in these tissues under the same conditions (Matton and Brisson, 1989). StWhy1 did not associate with the potato *PoAc97* actin gene in fresh, wounded, or elicited tubers in this assay (see Supplemental Figure S2). In summary, StWhy1 is maximally associated with the *PR-10a* gene in elicited potato tubers when *PR-10a* expression is maximal (Figure 1D), and is capable of inducing *PR-10a* gene expression in an ERE-dependent manner (Figure 1B), confirming its role as a transcriptional activator.

PB Elements Are Enriched in the Promoters of *Arabidopsis* Defense Genes

To identify regulons that potentially could be controlled by Whirly proteins, we examined an *Arabidopsis* microarray database established by Maleck et al. (2000) for enrichment of the PB element in the promoters of coregulated genes relevant to defense responses. Analysis of promoters from coregulated genes contained in self-organizing maps (SOMs) from that data set revealed

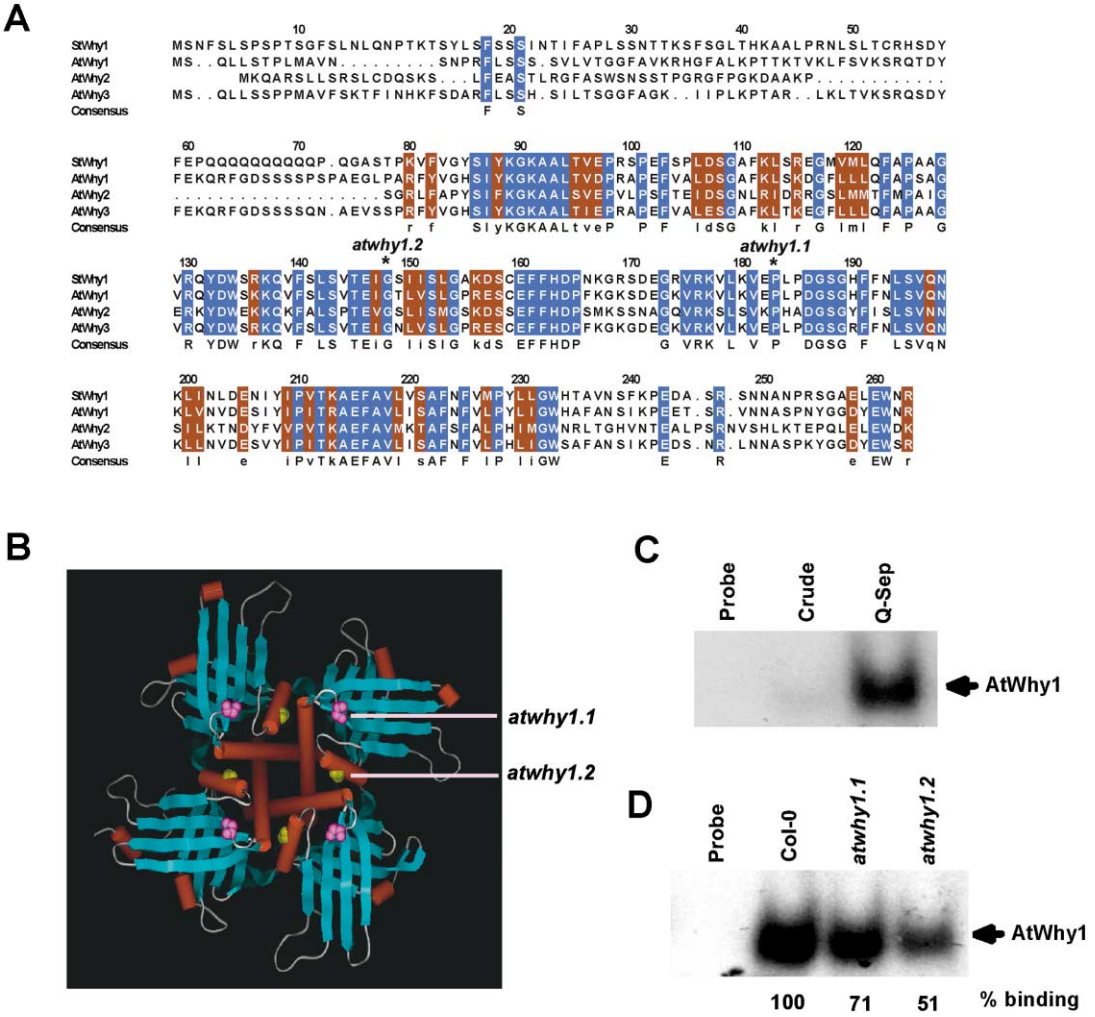

Figure 2. *Arabidopsis* Whirly Proteins Are Closely Related to StWhy1

(A) Sequence alignment of the potato StWhy1 protein sequence and the three *Arabidopsis* Whirly proteins AtWhy1 (At1g14410), AtWhy2 (At1g71260), and AtWhy3 (At2g02740). The alignment was initially performed using ClustalW (Thompson et al., 1994) and was manually modified. Numbering corresponds to the AtWhy1 protein sequence. Conserved identical residues are boxed in blue and positions with conserved similar residues are boxed in red. The consensus protein sequence obtained from the alignment is indicated at bottom. Two *Arabidopsis* mutants, *atwhy1.1* and *atwhy1.2*, possess the point mutations indicated by an asterisk giving rise to Pro183Ser and Gly148Glu, respectively.

(B) Ribbon diagram of the StWhy1 tetramer highlighting the amino acids altered in *atwhy1.1* and *atwhy1.2,* Pro183 (pink), and Gly148 (yellow), respectively. β strands are colored blue and α helices are colored red.

(C) Activation of AtWhy1 binding activity from *Arabidopsis* nuclear extracts by anion exchange chromatography. Nuclear proteins from wild-type and *atwhy1* mutant plants were subjected to Q-Sepharose anion exchange chromatography, and 1 μg of protein was incubated with radiolabeled 4xPB probe. AtWhy1 ssDNA binding activity was examined by EMSA.

(D) Nuclear proteins from wild-type Col-0 plants and the two *atwhy1* mutants were subjected to Q-sepharose anion exchange chromatography and subsequently examined for AtWhy1 ssDNA binding activity by EMSA. 1 μg of protein was incubated with the 4xPB probe and loaded in each lane. The band corresponding to AtWhy1 was cut out from the gel, and the amount of radioactive probe shifted was counted. Levels of AtWhy1 binding activity in nuclear extracts of the indicated *atwhy1* alleles are presented relative to wild-type. This experiment was repeated five times, in two different laboratories, and the results presented are typical.

a 3.4-fold enrichment of PB elements in 11/26 SAR-associated genes (SOM c1 from Maleck et al., 2000) compared to the expected chance occurrence ($p = 0.001$; Table 1; Supplemental Figure S3). Thus, at least a subset of these coregulated genes might be directly regulated by factors bound to PB elements. However, the element is not enriched in the promoter of genes in a control group (SOM c7) that do not show significant defense-related induction, nor in three groups of randomly chosen promoters from the *Arabidopsis* genome (Table 1 and Supplemental Figure S3). The enrichment of the PB element in promoters of SAR associated genes suggests that it could play an important role in the regulation of SA-induced gene expression in *Arabidopsis*.

Arabidopsis AtWhy1 DNA Binding Activity Is Similar to that of StWhy1

The *Arabidopsis* genome encodes three members of the Whirly family: AtWhy1 (At1g14410), AtWhy2 (At1g71260), and AtWhy3 (At2g02740; Figure 2A). AtWhy1 and AtWhy3

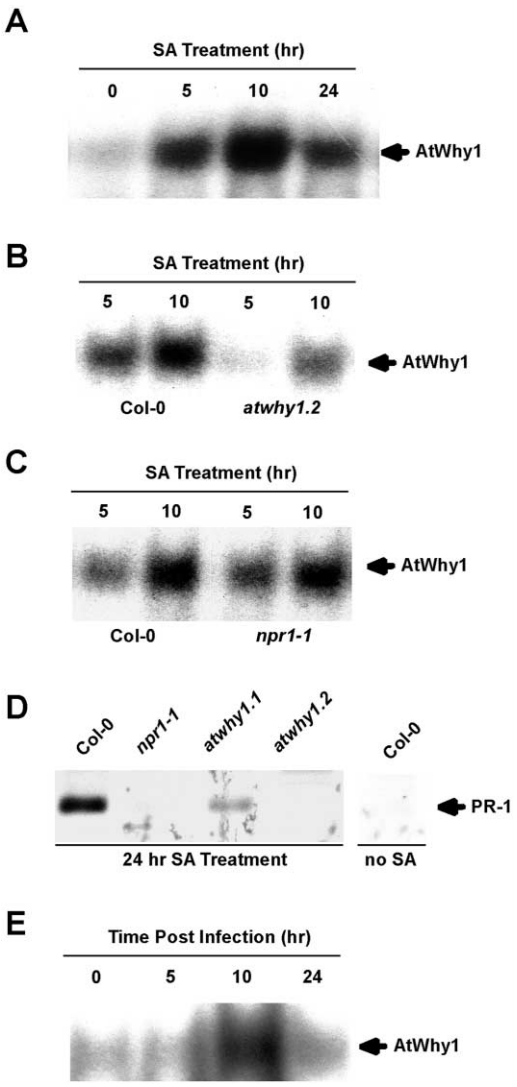

Figure 3. SA-Induced AtWhy1 DNA Binding Activity Is NPR1 Independent

(A) Induction of AtWhy1 ssDNA binding activity by SA treatment. Three-week-old wild-type Col-0 *Arabidopsis* plants were sprayed with 2 mM SA in 0.02% Silwet. Tissues were harvested before spraying and 5 hr, 10 hr, and 24 hr after SA treatment. 10 μg of nuclear protein was used for each EMSA reaction with the 4xPB oligonucleotide as probe.

(B) SA induction of AtWhy1 DNA binding activity in wild-type Col-0 and *atwhy1.2* plants. Tissues from Col-0 and *atwhy1.2* plants were harvested 5 hr and 10 hr after SA treatment. 10 μg of protein from total nuclear extracts was then used for EMSA with the 4xPB oligonucleotide as probe.

(C) SA induction of AtWhy1 DNA binding activity in wild-type Col-0 and *npr1-1* plants. Tissues from Col-0 and *npr1-1* plants were harvested 5 hr and 10 hr after SA treatment. 10 μg of protein from total nuclear extracts was then used for EMSA with the 4xPB oligonucleotide as probe.

(D) SA-induced PR-1 expression is compromised in *atwhy1* mutant plants. Col-0, *npr1-1*, *atwhy1.1*, and *atwhy1.2* plants were treated with SA for 24 hr, and total protein was examined for the presence of PR-1 by Western blot analysis. Protein from Col-0 plants not treated with SA was also examined to confirm the lack of PR-1 expression in untreated tissues.

(E) Induction of AtWhy1 DNA binding activity by Emoy2 infection. Col-0 plants were infected with the incompatible *P. parasitica* isolate Emoy2, and tissues were harvested 5 hr, 10 hr, and 24 hr postinfection. 10 μg of protein from total nuclear extracts was then used for EMSA with the 4xPB oligonucleotide as probe.

are more similar to each other (77% identity) and both share 58% identity to StWhy1. AtWhy1 is the *Arabidopsis* protein most similar to StWhy1 (75% identity excluding the variable N-terminal region up to His85). Recombinant AtWhy1 protein binds to the single-stranded ERE (Supplemental Figure S4).

To examine the role of the Whirly family in the defense response, we obtained homozygous plants carrying two different *atwhy1* missense alleles from the *Arabidopsis* TILLING Project using the structurally defined ssDNA binding domain from Leu77 to Ser252 as a target. The *atwhy1.1* mutation is Pro183Ser and the *atwhy1.2* allele is Gly148Glu (Figure 2A). Sequencing of the *AtWhy1* cDNA from these lines confirmed that these were the only mutations present in the gene (data not shown). Neither of these alleles displayed any obvious morphological phenotypes relative to wild-type plants. We could not obtain viable homozygous *atwhy1* null plants from two independent heterozygous T-DNA insertion lines (data not shown; Experimental Procedures).

Mapping the two *atwhy1* mutations onto the StWhy1 crystal structure revealed that they should have different effects on AtWhy1 function (Figure 2B). The Pro183Ser exchange in *atwhy1.1* falls on a β sheet surface harboring the ssDNA binding domain. Exchange of a structurally rigid proline for a more flexible serine could have deleterious effects on the β sheet surface and affect AtWhy1 DNA binding activity. The Gly148Glu exchange in *atwhy1.2* resides in the central α-helical region that makes intermonomeric contacts within the tetramer. Tetramerization of recombinant atwhy1.2 protein is disrupted (data not shown).

We examined the DNA binding activity of each atwhy1 mutant protein from *Arabidopsis* nuclear extracts by EMSA. Crude *Arabidopsis* nuclear extracts possessed very low levels of ssDNA binding activity (Figure 2C). However, the ssDNA binding activity of AtWhy1 was revealed by subjecting the proteins to Q-Sepharose anion exchange chromatography, as previously observed in potato nuclear extracts (Figure 2C, Desveaux et al., 2000). The activation of AtWhy1 binding activity by Q-Sepharose treatment suggests that AtWhy1 DNA binding activity could be induced in response to stimuli that presumably act to remove a negative regulator.

Nuclear extracts from the two *atwhy1* mutant alleles possessed less ssDNA binding activity than wild-type extracts (Figure 2D). *atwhy1.1* and *atwhy1.2* possessed 71% and 51% of wild-type ssDNA binding activity, respectively. The reductions in AtWhy1 DNA binding activity we observed in *atwhy1* nuclear extracts correlate with the DNA binding activity of purified recombinant atwhy1.1 and atwhy1.2 mutant proteins expressed in *E. coli* (see Supplemental Figure S4).

Regulation of AtWhy1 DNA Binding Activity by SA

Our analysis of the Maleck et al. (2000) expression data revealed an enrichment of the PB element in genes coregulated during SAR (SOM c1; Table 1). We therefore

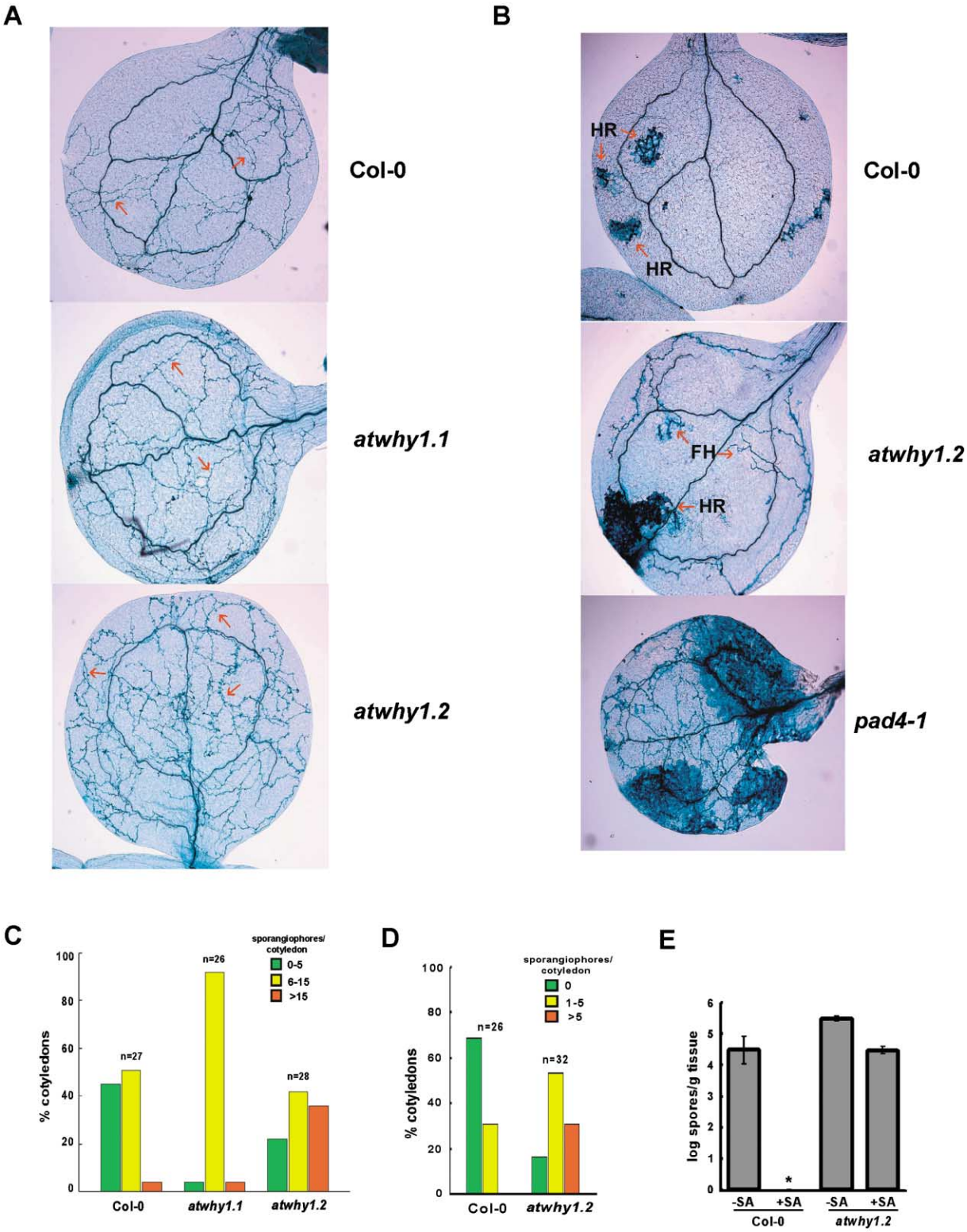

Figure 4. Enhanced Disease Susceptibility of *atwhy1 Arabidopsis* Plants to *P. parasitica* Isolates

(A) Wild-type Col-0 *Arabidopsis* plants and *atwhy1* mutants were infected with the *P. parasitica* isolate Noco2, which causes disease on Col-0. Hyphal growth was examined by trypan blue staining leaves 2 days after infection, and is indicated by arrows.

(B) Wild-type Col-0, *atwhy1.2* plants, and *pad4-1* plants were infected with the *P. parasitica* isolate Emoy2, which triggers resistance on Col-0. Hyphal growth was examined by trypan blue staining leaves 4 days after infection. Typical hypersensitive responses, HR, stained deep blue on Col-0 and *atwhy1.2*, are indicated by arrows. Arrows also indicate free hyphae (FH).

(C) *P. parasitica* isolate Noco2 infection was quantified by counting sporangiophores on cotyledons 7 days after infection. Cotyledons were scored as either having 0–5, 6–15, or greater than 15 sporangiophores per cotyledon. Bars of the histogram represent the number of cotyledons

treated wild-type Col-0 *Arabidopsis* plants with SA and examined AtWhy1 DNA binding activity from crude nuclear extracts. AtWhy1 DNA binding activity was induced by SA treatment (Figure 3A) within 5 hr, reached a peak at ~10 hr after treatment, and declined thereafter. SA treatment of *atwhy1.2* resulted in markedly less induction of DNA binding activity, confirming that the observed increase in DNA binding activity is due to AtWhy1 (Figure 3B). Importantly, SA-induction of AtWhy1 DNA binding activity was not altered in extracts from the *npr1-1* mutant (Figure 3C). Therefore, SA-induction of AtWhy1 DNA binding activity is independent of *NPR1*.

The SA-induced gene *PR-1* is commonly used as a marker for SA-induced defenses. PR-1 induction 24 hr after SA application was very low in *atwhy1.1* (Figure 3D), and undetectable at this time point in either *atwhy1*.2 or in the SAR mutant *npr1-1*, compared to wild-type. We could, however, detect very low levels of PR-1 accumulation in *atwhy1.2* by 72 hr after SA treatment (data not shown). Together, these data demonstrate that neither *AtWhy1* nor *NPR1* is sufficient for full SA-induced PR-1 expression, and that induction of AtWhy1 DNA binding activity is NPR1 independent.

We additionally examined AtWhy1 DNA binding activity recovered in crude nuclear extracts of *Arabidopsis* Col-0 plants infected with the incompatible *P. parasitica* isolate, Emoy2. This parasite triggers an SA-dependent defense response in Col-0 via the *RPP4* disease resistance (*R*) gene (Holub et al., 1994; van der Biezen et al., 2002). Emoy2 infection, similar to SA treatment, induced maximal AtWhy1 DNA binding at about 10 hr (Figure 3E). DNA binding declined by 24 hr after treatment. A similar pattern of induction was also observed using the compatible, disease causing, *P. parasitica* isolate Noco2 (data not shown). The induction of AtWhy1 DNA binding activity by *P. parasitica* infection suggests that it plays a role in defense responses against this pathogen.

AtWhy1 Is Required for Optimal Disease Resistance Responses

We tested the response of the two *atwhy1* alleles to infection with the same two *P. parasitica isolates*. Following infection with the compatible pathogen *P. parasitica* isolate Noco2, hyphal growth was examined by trypan blue staining. An obvious increase in hyphal growth was observed in both *atwhy1* mutants relative to wild-type (Figure 4A). *atwhy1.2* appears more susceptible than *atwhy1.1* as measured by the increase in hyphal growth. To quantify the levels of infection, we counted the number of sporangiophores produced per cotyledon 7 days after Noco2 infection (Figure 4C; Holt et al., 2002). We scored according to the number of cotyledons having either 0–5, 6–15, or greater than 15 sporangiophores per cotyledon. Ninety-five percent of the wild-type, susceptible Col-0 cotyledons scored in the 0–5 (45%) or 6–15 (51%) sporangiophores per cotyledon category. On the other hand, 92% of *atwhy1.1* cotyledons carried 6–15 sporangiophores and 78% of *atwhy1.2* cotyledons carried 6–15 (42%) or >15 (36%) sporangiophores. These increases in sporangiophore counts on *atwhy1* cotyledons confirm their increased susceptibility to Noco2 infection. The degree of enhanced disease susceptibility directly correlated with the decreased levels of AtWhy1 DNA binding activity recovered from each allele (Figure 2D). We found three additional control TILLING alleles, two with mutations in *atwhy1* introns and one with a conservative mutation in the coding region. None displayed differences in susceptibility compared to wild-type (data not shown). Thus, *AtWhy1* is required for full basal defense in *Arabidopsis*.

The more susceptible *atwhy1.2* allele was also tested following infection with the incompatible *P. parasitica* isolate Emoy2. Wild-type Col-0 plants are resistant to Emoy2 infection via the action of the *RPP4* disease resistance gene (Holub et al., 1994) as demonstrated by the lack of hyphal growth, and the presence of a number of sites where the hypersensitive response (HR) occurred (Figure 4B). The *atwhy1.2* mutant displayed intermediate susceptibility to Emoy2 infection as compared to the fully resistant Col-0 and the fully susceptible *pad4-1* mutant (Glazebrook et al., 1997). This intermediate phenotype was manifested by increased hyphal growth and subsequent trailing host cell necrosis, indicative of a quantitative loss of *RPP4* function (Holt et al., 2002). Quantification of sporangiophores on cotyledons corroborated this phenotype (Figure 4D). Wild-type Col-0 plants had 69% of their cotyledons with no sporangiophores on them, 31% with 1–5 sporangiophores, and no cotyledons with >5 sporangiophores. The *atwhy1.2* plants had only 16% of their cotyledons with no sporangiophores, 53% with 1–5 sporangiophores per cotyledon, and 31% with >5 sporangiophores per cotyledon. These results demonstrate the importance of *AtWhy1* in at least one specific *Arabidopsis* disease resistance response.

To confirm the role of AtWhy1 in SAR, wild-type and *atwhy1.2* plants were treated with SA for 6 hr and subsequently infected with Noco2 (Figure 4E). As previously observed with the SA analog INA (Li et al., 1999), SA treatment of Col-0 plants leads to induction of complete

falling into the three categories of sporangiophore counts for each genotype infected. Each category is represented by a different bar color: green, 0–5 sporangiophores; yellow, 6–15 sporangiophores; red, more than 15 sporangiophores per cotyledon. n represents the number of cotyledons counted for each genotype.

(D) *P. parasitica* isolate Emoy2 infection was quantified by counting sporangiophores on cotyledons 7 days after infection. Cotyledons were scored as either having 0, 1–5, or greater than 5 sporangiophores. Bars of the histogram represent the number of cotyledons falling into the three categories of sporangiophore counts for each genotype infected. Each category is represented by a different bar color: green, 0 sporangiophores; yellow, 1–5 sporangiophores; red, more than 5 sporangiophores per cotyledon. The *pad4-1* plants were too damaged to accurately count sporangiophores after 7 days of infection, but all infected cotyledons had more than 5 sporangiophores. n represents the number of cotyledons counted for each genotype.

(E) Col-0 and *atwhy1.2* plants were sprayed with 2 mM SA in 0.02% Silwet and incubated for 6 hr before spraying with *P. parasitica* isolate Noco2. Spores were counted 9 days after spraying with Noco2. The asterisk (*) indicates that no spores were observed from SA-treated Col-0 plants.

resistance against Noco2 infection. However, SA-induced resistance was fully compromised in *atwhy1.2* as demonstrated by the *P. parasitica* growth observed confirming the importance of *AtWhy1* for optimal SA-induced defenses.

Discussion

The Whirly family of plant transcription factors regulate gene expression through the PB element GTCAAAAA/T. We used reverse genetics to isolate *atwhy1* missense alleles and to demonstrate functions for *AtWhy1* in basal (Figures 4A and 4C), *RPP4*-mediated (Figures 4B and 4D), and SA-induced (Figure 4E) disease resistance. Importantly, *AtWhy1* function does not require the global SAR regulator NPR1 for its SA-dependent activation (Figure 3C). It does, however, function with NPR1 to control SA-regulated gene expression (Figure 3D). Our data add significantly to the understanding of transcriptional reprogramming in the *Arabidopsis* immune system following infection.

Arabidopsis AtWhy1 Is Required for Full Basal and Induced Defense Responses

The enhanced susceptibility of *atwhy1* plants to infection with *P. parasitica* Noco2 indicates that *AtWhy1* is required for full basal resistance. In addition, the increased susceptibility of *atwhy1.2* to Emoy2 infection demonstrates a role for *AtWhy1* in *R*-dependent defense responses. AtWhy1 is therefore required for both optimal basal and specific defense responses, suggesting at least partial convergence of these signaling pathways at the transcription level. This notion is supported by the observation that gene expression profiles are similar during compatible and incompatible interactions (Tao et al., 2003).

The induction of AtWhy1 DNA binding activity by SA treatment suggests a function for AtWhy1 in SA-regulated defenses (Figure 3A). This was confirmed by the observation that SA-induced resistance to *P. parasitica* Noco2 was fully compromised in *atwhy1.2* plants (Figure 4E). SA-induced PR-1 expression is also severely compromised in the *atwhy1* mutants. The rapid and transient increase in DNA binding activity within 10 hr of SA treatment and the presence of AtWhy1 in the nuclei of untreated tissues suggests that AtWhy1 may modulate early expression changes. PB elements are, in fact, enriched in genes of the SA-dependent *RPP4* pathway that are induced early and transiently upon SA treatment, but not in the promoters of genes that are induced later and display sustained levels of expression (T. Eulgem and J.L.D., unpublished data). PB elements are present in the promoters of additional defense genes from a number of species (see Supplemental Figure S5). PB elements are also found in the promoters of aminocyclopropane-1-carboxylate synthase (ACC synthase) genes (see Supplemental Figure S5). Since ACC synthase catalyzes the first committed and generally rate-limiting step in ethylene biosynthesis, the presence of this element may provide a regulatory mechanism for ethylene production during pathogen infection (Ohme-Takagi et al., 2000).

NPR1 is also required for SA-induced defense responses and *PR-1* expression (Cao et al., 1994; Delaney et al., 1995). However, SA-induced defenses and *PR-1* expression are reestablished in *npr1* plants also carrying a loss of function, *npr1* suppressor mutation in the *SNI1* gene (Li et al., 1999). These data, and the analysis of mutants that trigger *PR-1* expression in *npr1* plants (Clarke et al., 1998), indicate the presence of an NPR1-independent signaling pathway capable of inducing, either directly or indirectly, *PR-1* expression. AtWhy1 DNA binding activity is still induced by SA in *npr1-1* plants (Figure 3C). The NPR1-independent induction of AtWhy1 DNA binding activity and the abrogation of SA-induced disease resistance in *atwhy1* mutant plants indicate that AtWhy1 is an important component of the SA-signaling pathway. The AtWhy1 function in PR-1 regulation is probably indirect, since the PB element was not identified in linker scan experiments on the *PR-1* promoter (Lebel et al., 1998).

Figure 5 presents a model that explains the current data and suggests how NPR1 and AtWhy1 may participate in SA-regulated gene expression. SA activates NPR1 to remove inhibition imposed by SNI1. This allows activation of gene expression through the combined action of the SA-activated AtWhy1 and TGA factors (Johnson et al., 2003). This suggests interplay between the ssDNA binding Whirly family and the dsDNA binding TGA family to induce gene expression in response to SA. In this regard, it is interesting to note that StWhy1 carries a potent activation domain in addition to its DNA

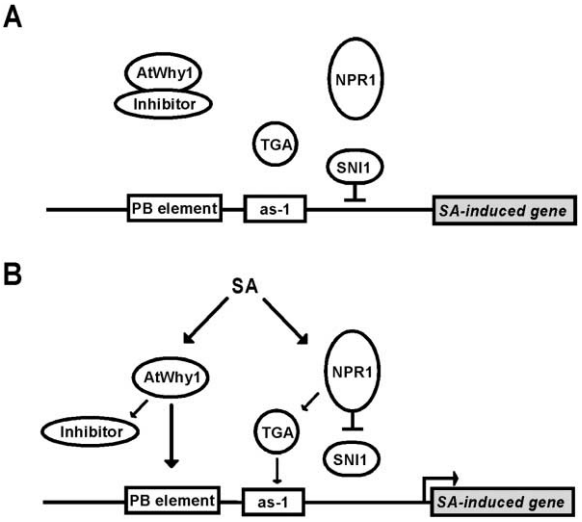

Figure 5. AtWhy1 Is Required for Maximal SA-Induced Gene Expression

(A) SA-induced gene expression involves the concerted action of two pathways: an NPR1-dependent pathway and an AtWhy1-dependent pathway. In uninduced tissues, AtWhy1 DNA binding activity is repressed by an inhibitor whose removal can be mimicked by subjecting nuclear extracts to Q-Sepharose anion exchange chromatography. SNI1 inhibits gene expression through an unknown mechanism (Li et al., 1999).

(B) Upon SA treatment, AtWhy1 DNA binding activity is induced by removal of the inhibitor. NPR1 is activated and removes the repression imposed by SNI1 (Li et al., 1999). AtWhy1 binds to PB elements to activate gene expression. NPR1 also contributes to gene activation, likely by interacting with, and enhancing the DNA binding activity of, TGA transcription factors to as-1 like elements (Després et al., 2000).

binding activity (Figure 1C and Supplemental Figure S1). It will be interesting to address whether AtWhy1 is a component of a postulated oligomeric complex containing NPR1 in the cytosol before SA treatment (Mou et al., 2003).

Transcriptional Activation by StWhy1

Most transcriptional activators can be divided into a DNA binding domain and an activation domain (Ptashne, 1988). StWhy possesses a whirligig-like ssDNA binding domain and an N-terminal transactivation domain containing the polyglutamine region. Once bound to melted promoter regions, StWhy1 could recruit general transcription machinery or coactivators to their templates. AtWhy1 lacks the polyglutamine stretch found in StWhy1. However, the N-terminal region of AtWhy1 possesses the characteristics of a serine-rich transactivation domain (Triezenberg, 1995). StWhy1 and AtWhy1 may function in a similar way as the FUSE binding protein which senses promoter activity by binding to melted regions resulting from torsionally strained DNA (He et al., 2000). The PB element would thus act as a transcription sensor "opened" by a critical level of transcriptional activity, thereby allowing StWhy1 or AtWhy1 to bind and amplify gene expression. Also, stabilization of melted DNA regions by Whirly factors could increase the potential for the formation of loops or bends facilitating the interaction of distal *cis*-regulatory elements with the downstream promoter (Kahn et al., 1994).

The PB Element Overlaps with Other Important Plant *Cis*-Regulatory Elements

The PB element (GTCAAAAA/T) potentially overlaps with the opposite strand sequence of the *cis*-element for two major classes of defense-related transcription factors: the W-box (T/G)TGAC(C/T) sequence recognized by the WRKY family of transcription factors (Rushton et al., 1996; Eulgem et al., 1999) and the TGACG element recognized by the TGA/OBF family of proteins (Ramachandran et al., 1994). An important difference between the Whirly and the WRKY or TGA/OBF families is that the former recognizes single-stranded and the other two double-stranded DNA, respectively. This may facilitate interplay between the Whirly proteins and members of these two important families of transcription factors, depending on promoter architecture. For example, the binding of WRKY or TGA/OBF transcription factors could inhibit the formation of melted duplex DNA at certain PB elements. Upon pathogen infection, the binding of AtWhy1 to PB elements would require a derepression involving the removal of dsDNA binding proteins. In support of this, transgenic plants expressing transdominant mutations that eliminate the DNA binding activities of TGA factors exhibit higher levels of *PR* gene induction by pathogen challenge and an enhanced SAR (Pontier et al., 2001). In mammals, such an interplay of single-stranded and double-stranded DNA binding factors on overlapping elements was observed for the regulation of the human *c-myc* gene (Tomonga and Levens, 1995; Michelotti et al., 1995), the mouse μ-opioid receptor gene (Ko and Loh, 2001), and the rat gelatinase A gene (Mertens et al., 1998).

In conclusion, the AtWhy1 Whirly transcription factor is an important component of the SA signaling pathway that is activated independently of the key SAR regulator NPR1. However, both NPR1 and AtWhy1 are required for optimal SA-induced defenses. AtWhy1 is also required for optimal defense responses against both compatible and incompatible interactions, adding an important component to both basal and specific plant immune responses.

Experimental Procedures

Plant Material

Potato tubers were obtained as described in Desveaux et al. (2000). *Arabidopsis* plants were grown as described in Holt et al. (2002).

Transient Expression Assays

All DNA manipulations were performed according to standard procedure (Sambrook et al., 1989). The two by two mutant ERE constructs were made from the pLP9 vector (wtERE) as described in Desveaux et al. (2000). The 3′ERE and noERE constructs were made using a modification of the ExSite PCR-based site-directed mutagenesis (Stratagene, La Jolla, CA). The StWhy1 plasmid for transient expression was constructed by excising the StWhy1 cDNA sequence described in Desveaux et al. (2000), from pBluescript vector SK- (Stratagene) followed by ligation into pBIN19 containing two copies of the cauliflower mosaic flower 35S promoter (CaMV; a gift from Dr. Daniel P. Matton). StWhy1 and the 35S promoters were digested from pBIN19 and cloned into the pBI223 expression vector replacing its 35S promoter. The FK506 binding protein (FKBP; Pelletier et al., 1998) control vector was expressed from the same double 35S promoter as StWhy1 (Subramaniam et al., 2001). Leaf mesophyll protoplasts isolation and transient expression were performed as described in Desveaux et al. (2000) and Subramaniam et al. (2001).

For cotransfection assays, 5 μg of luciferase plasmid (pWB216; Desveaux et al., 2000) containing the luciferase gene under control of the CaMV 35S promoter was coelectroporated with 30 μg of β-glucuronidase reporter plasmids and 5 μg of effector plasmids. Reporter gene activity was determined as described by Desveaux et al. (2000). Results were standardized using luciferase activity to measure transformation efficiency.

Constructs for Interaction Assays in Yeast

All plasmids for expression in yeast were obtained from Origene Technologies (Rockville, MD). StWhy1 was amplified from the full-length cDNA clone and cloned as a translational fusion to the LexA DNA binding domain (in pEG202), to the B42 transcriptional activation domain HA-tagged (in pJG4-5), or to the HA-tag (in pJG4-6). These constructs were then introduced into yeast strain EGY48 containing the pSH18-34 lacZ reporter plasmid. The polyglutamine deletion was made by PCR to remove amino acids 73 to 90.

Interaction and activation assays were performed on X-gal-containing media as described in the DupLEX-A yeast two-hybrid system (Origene Technologies, Rockville, MD).

Yeast Cultures, Protein Extraction, and Immunoblot Assays

Yeast strain EGY48 containing the pSH18-34 reporter plasmid and the appropriate StWhy1 plasmids were grown at 30°C in selective YNB media containing 1% raffinose for 48 hr and then transferred to selective YNB media containing 1% raffinose and 2% galactose. Cultures were then incubated at 30°C for 24 hr. For uninduced cultures, cells were grown in selective YNB media containing 2% glucose for 24 hr. 1 ml of yeast culture at an OD_{600} of 2.0 was used for immunoblot assays. The cells were resuspended in 100 μl of 1X protein sample buffer and 50 mg of acid washed glass beads was added. The cells were vortexed 2 min, boiled 5 min, vortexed again for 45 s, and boiled 5 min. 20 μl was then loaded on a 12% SDS-PAGE gel. A 1/20,000 dilution of the anti-LexA primary antibody (Invitrogen, Burlington, Ontario) was used to detect the LexA DB fusion proteins, and a 1/1000 dilution of the anti-HA primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to detect the HA fusion proteins.

Analysis of *Arabidopsis* SOMs

Informatic analysis of coregulated genes was conducted according to Maleck et al. (2000), using the cluster analysis data obtained from their study. The promoter sequences 1 kb upstream from each translational start were examined for the presence of the PB element (GTCAAAAA/T). The calculation used to obtain the expected number of PB elements per promoter was developed using an observed G/C content of *Arabidopsis* promoters of 32% (T. Eulgem and J.L.D., unpublished data). Therefore, the expected occurrence of the PB element in both orientations per 1100 bp of promoter sequence from SOM c1 or SOM c7 (Maleck et al., 2000) is: (G or C at two positions) $\times$ (A or T at 5 positions) $\times$ (A and T allowed at the last position) $\times$ 2 strands $\times$ 1100 bp of promoter scanned or: $(0.16)^2 \times (0.34)^5 \times (0.68)\ 2 \times 1100 = 0.17$. For simplicity, we used 1000 bp for promoters of random control promoter sets. The probability *p* of seeing *m* promoters with *n* PB elements in a set of promoters was calculated as in Maleck et al. (2000).

Chromatin Immunoprecipitation

1.5 g of potato tuber slices was fixed for 15 min in 1% formaldehyde. These were extensively rinsed with water, dried, frozen in liquid nitrogen, and stored at −80°C until processing. Tissues were ground in IP buffer (0.1% SDS, 1% Triton X-100, 50 mM HEPES [pH 7.9], 150 mM NaCl, and 1 mM EDTA) supplemented with protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). The lysate was centrifuged for 5 min at 14,000 rpm at 4°C in a microcentrifuge before filtering through siliconized glasswool. The chromatin-containing supernatant was sonicated to yield DNA fragments between 0.5 and 1.3 kb in size. After sonication, a 20 μl aliquot was set aside as the input material and stored at −20°C until processing. The chromatin solution was split into two 500 μl aliquots and combined with 20 μl of protein-A sepharose beads (BIO-RAD, Hercules, CA) that had been preadsorbed with 10 μl of preimmune sera or 10 μl of anti-StWhy1 antibodies (Desveaux et al., 2000). After overnight incubation at 4°C on a rotation wheel, the beads were washed three times with IP buffer and twice with TE (10 mM Tris-HCl [pH 8.0]; 1 mM EDTA). The immunoprecipitated material was released from the beads by heating at 65°C for 15 min in 200 μl of TE supplemented with 1% SDS. 40 μl of immunoprecipitate or 20 μl of input was combined with 80 μl (immunoprecipitate) or 100 μl (input) of TE/1% SDS and incubated at 65°C for 6 hr. After precipitation with one volume of isopropanol, 0.3 M sodium acetate, and 2 μg of glycogen, the DNA was resuspended in 20 μl of TE (immunoprecipitate) or 200 μl of TE (input). PCR amplifications were carried out in a 50 μl volume with 50 pmol of each primer, 0.2 mM dNTPs, and 5 units of Taq polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) along with 2 μl of immunoprecipitated DNA or diluted input. The primer pair 5′GTGAGGTCTGGGTAAACGGATGTA3′ and 5′CAGAAG GATTGGCGAGGAGGTA3′ was used to amplify the 400 bp fragment comprising nucleotides 496–895 of the *PR-10a* gene. The cycling conditions were 5 min initial denaturation at 95°C followed by 35 cycles with 30 s at each temperature (95, 55, and 72°C). Under these conditions, the amount of PCR product was proportional to the amount of template DNA added as was determined by serial dilutions of the input DNA. The *S. tuberosum* actin gene PoAc97 (X55751) was amplified using the primers 5′ACTATTATTCAATT TATCTGCGGCC3′ and 5′AAAAATGGCAGGCCAACTCT3′. For sequencing, the PCR fragments were generated as described above except that *Taq* polymerase was replaced by the proof-reading enzyme ExTaq (PanVera, Madison, WI). The PCR fragments were gel-excised, cloned, and sequenced.

Preparation of Nuclear Proteins from *Arabidopsis* and EMSA Analysis

500 mg of frozen tissue was ground in 1 ml of buffer C (0.33 M sucrose, 10 mM NaCl, 10 mM MES-NaOH [pH 4.6], 1 mM EDTA, and 10 mM DTT) using a mortar and pestle. The mixture was then filtered through Miracloth (Calbiochem, San Diego, CA) and centrifuged at 14,000 rpm, 15 min at 4°C. The pellet was resuspended in 1 ml buffer C containing 1% NP-40 to lyse chloroplasts and then spun at 14,000 rpm for 5 min at 4°C. The NP-40 wash was repeated followed by a wash with 1 ml of Q-Sepharose buffer (20 mM HEPES/KOH [pH 7.9], 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 200 mM NaCl) to remove any remaining detergent. The pellet was then resuspended in 150 μl of Q-Sepharose buffer, and the nuclear suspension was sonicated with a microtip sonicator to break open the nuclei. Membranes were subsequently centrifuged (14,000 rpm, 15 min, 4°C) and the supernatant was used for EMSA. Anion exchange chromatography was performed as described previously using 200 μl of Q-Sepharose Fast Flow resin (Amersham Pharmacia Biotech, Uppsala, Sweden) per 500 mg of tissue (Desveaux et al., 2000). EMSAs were performed as described in Desveaux et al. (2000) using an optimized probe designed according to the StWhy1 structure (4xPB; 5′TTTTTGTCATTTTTGTCATTTTTGTCATTTTTGTCATTTT3′). This oligonucleotide possesses four modified PB elements (GTCATTTT) to accommodate each protomer of the StWhy1 tetramer. This 4xPB probe had 10-fold more affinity than the single strands of the ERE (data not shown).

Recombinant AtWhy1 protein was amplified from an *Arabidopsis* Col-0 cDNA library cloned into the pET-21a vector creating a fusion protein with a histidine tag at the C terminus and expressed in the bacterial strain BL21 pLysS (Novagen, Germany). Mutations corresponding to *atwhy1.1* and *atwhy1.2* were introduced by PCR. Purification of recombinant proteins was performed as described by Desveaux et al. (2002).

Arabidopsis TILLING Mutants

Arabidopsis AtWhy1 (At1g14410) mutants were obtained from the *Arabidopsis* TILLING Project (ATP) for the region between Leu77 and Ser252 encompassing the predicted DNA binding domain: *atwhy1.1*, ABRC Stock CS93163 and *atwhy1.2*, ABRC Stock CS91310. *atwhy1.1* and *atwhy1.2* mutants were backcrossed once. Two control TILLING lines had mutations in introns (ABRC stocks; CS91195 and CS91419), and a third control line had a conservative mutation that did not alter the amino acid sequence (ABRC stock CS89610). We also screened exonic T-DNA insertions in *AtWhy1* (lines SALK_023713 and SALK_039000) and were unable to recover homozygous mutant progeny from segregating populations (~60 individuals from each line screened).

Peronospora parasitica Culture, Infection, Trypan Blue Staining, and Sporangiophore Counts

Propagation, infection, trypan blue staining, and spore counts on cotyledon by *P. parasitica* on Noco2 and Emoy2 were performed as described by Holt et al. (2002).

Protein

Western blot analysis was prepared as described in Mackey et al. (2003). Western blots were conducted according to standard methods with the PR-1 antibodies used at 1:5000 dilution.

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