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EDS5, an Essential Component of Salicylic Acid–Dependent Signaling for Disease Resistance in Arabidopsis, Is a Member of the MATE Transporter Family

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The *eds5* mutant of Arabidopsis (earlier named *sid1*) was shown previously to accumulate very little salicylic acid and PR-1 transcript after pathogen inoculation and to be hypersusceptible to pathogens. We have isolated *EDS5* by positional cloning and show that it encodes a protein with a predicted series of nine to 11 membrane-spanning domains and a coil domain at the N terminus. EDS5 is homologous with members of the MATE (multidrug and toxin extrusion) transporter family. *EDS5* expression is very low in unstressed plants and strongly induced by pathogens and UV-C light. The transcript starts to accumulate 2 hr after inoculation of Arabidopsis with an avirulent strain of *Pseudomonas syringae* or UV-C light exposure, and it stays induced for ~2 days. *EDS5* also is expressed after treatments with salicylic acid, indicating a possible positive feedback regulation. *EDS5* expression after infection by certain pathogens as well as after UV-C light exposure depends on the pathogen response proteins EDS1, PAD4, and NDR1, indicating that the signal transduction pathways after UV-C light exposure and pathogen inoculation share common elements.

INTRODUCTION

Plants react to an attack by phytopathogenic microorganisms with an array of inducible defense responses. Whether a plant is resistant or susceptible to a potential pathogen depends largely on how fast a pathogen is recognized and defense responses are activated. For instance, in gene-for-gene resistance, the product of an avirulence gene of the pathogen is recognized by a corresponding resistance gene product of the plant, leading to the rapid activation of various defense responses. Such a pathogen is avirulent to the plant, its invasion can be stopped, and the plant is resistant. Disease ensues when the pathogen is not recognized rapidly and defense mechanisms are activated too slowly to stop the infection process. In this case, the pathogen is virulent and the plant is susceptible. In addition, defense responses can be induced systemically in all parts of the plant by pathogens, soil-borne microorganisms, chemicals, or certain forms of stress. This form of induced resistance is referred to as systemic acquired resistance (Ryals et al., 1994; Sticher et al., 1997).

Salicylic acid (SA) is synthesized after inoculation of plants with pathogens or exposure to certain abiotic stresses, such as ozone and UV-C light. SA was found to be essential for gene-for-gene resistance, systemic acquired resistance, and reduction of disease development after inoculation with virulent pathogens (Delaney et al., 1994; Nawrath and Métraux, 1999).

In Arabidopsis, the elucidation of the signal transduction pathway downstream of SA leading to the expression of a number of pathogenesis-related (PR) proteins, such as *PR-1*, *PR-2*, and *PR-5*, has been centered on the characterization of the *npr1/nim1* mutant (Cao et al., 1994; Delaney et al., 1995). The *npr1/nim1* mutant does not express *PR-1*, *PR-2*, and *PR-5* after treatment with SA analogs, such as isonicotinic acid (Cao et al., 1994; Delaney et al., 1995). However, when infected with pathogens such *as Pseudomonas syringae* pv *maculicula*, only *PR-1* expression is reduced strongly, indicating that pathogens may induce *PR-2* and *PR-5* in a NPR-independent manner (Glazebrook et al., 1996). The *NPR1/NIM1* gene encodes a novel protein with ankyrin repeats (Cao et al., 1997; Ryals et al., 1997) that is translocated to the nucleus upon SA treatment (Kinkema et al., 2000). NPR1/NIM1 likely acts as a transcriptional coactivator that enhances the binding of basic leucine zipper protein transcription factors of the TGA family to the as1 element of the PR-1 promoter (Zhang et al., 1999; Zhou et al., 2000).

Several mutants of Arabidopsis have been isolated that are unable to establish defense responses (Glazebrook, 1999). NDR1, a small membrane-associated protein, is essential for the establishment of resistance after inoculation with certain avirulent pathogens, such as *P. syringae* carrying the *avrRpt2* or *avrRpm1* gene. In this case, effective activation of defense depends on resistance genes containing a

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nucleotide binding site and a leucine-rich repeat domain (Century et al., 1995, 1997). EDS1, a protein with homology with lipases, is necessary for activation of the defense pathway after inoculation with virulent and certain avirulent pathogens, such as *P. syringae* carrying the *avrRps4* gene (Parker et al., 1996; Aarts et al., 1998; Falk et al., 1999; Feys and Parker, 2000). PAD4 also contains a lipase domain (Glazebrook et al., 1997; Jirage et al., 1999) and is required for the activation of the SA-dependent defense pathway, the production of the phytoalexin camalexin, and the reduction of disease development after inoculation with virulent *P. syringae* and virulent as well as avirulent *Peronospora parasitica* isolates (Glazebrook et al., 1997; Zhou et al., 1998).

The *eds5*/*sid1* (*eds5* is allelic to *sid1*) (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999) and *sid2* mutants of Arabidopsis do not accumulate SA after inoculation with either virulent or avirulent pathogens or after abiotic stresses and demonstrate strongly reduced expression of *PR-1*. Both mutants display pathogen-induced increases in *PR-2* and *PR-5* expression, similar to that observed in the *npr1*/*nim1* mutant (Glazebrook et al., 1996), and accumulate high levels of camalexin (Nawrath and Métraux, 1999; Dewdney et al., 2000). In contrast, SA-degrading NahG plants, despite endogenous SA levels as low as those in *eds5* and *sid2*, show strong reduction in *PR-1*, *PR-2*, and *PR-5* expression as well as reduced camalexin accumulation after pathogen inoculation (Delaney et al., 1995; Zhao and Last, 1996). The susceptibility to pathogens of the *eds5* and *sid2* mutants is intermediate between that of wild-type and NahG plants (Nawrath and Métraux, 1999). Here, we describe the isolation of the *EDS5* gene by positional cloning. The predicted protein is a membrane protein that shows homology with MATE (multidrug and toxin extrusion) transporters.

RESULTS

Identification and Characterization of the *EDS5* Gene

EDS5 was mapped ~2 centimorgan (cM) from the simple sequence length polymorphism (SSLP) marker nga1107 (Nawrath and Métraux, 1999). Analysis of 48 *eds5* plants selected from a Landsberg *erecta* (L*er*) × *eds5* F2 population identified the SSLP markers F20D10-45.5 and F23K16-28.4 as the closest markers flanking the *eds5* mutation on each side. Screening of 1060 randomly chosen plants from a L*er* × *eds5-3* F2 mapping population with the SSLP markers F20D10-45.8 and F23K16-28.4 identified 39 plants having a recombination in the interval. By using these 39 recombinant plants, *EDS5* was found to be located 0.38 cM from nga1107 and 0.2 cM from CCR1. By using additional markers, *EDS5* was positioned at an equal distance of 0.09 cM between two SSLP markers located at positions 49.5 and 79.3 kb of the bacterial artificial chromosome (BAC) F19H22, defining a 30-kb interval. Four of six genes present on the annotated sequence of the 30-kb region of BAC F19H22 were examined by RNA gel blot analysis. Gene 130 (At4 g39030) was identified as the putative *EDS5*, because this gene was pathogen inducible and showed a lower transcription level in all *eds5* mutant alleles compared with wild-type plants, as shown below. An overview of the mapping strategy is given in Figures 1A and 1B.

To identify the structure of the *EDS5* gene, a 1.4-kb fragment was amplified by reverse transcriptase–mediated polymerase chain reaction (RT-PCR) according to the annotated sequence of gene F19H22.130 (At4 g39030). Direct sequencing of the RT-PCR product identified an additional intron at the 3′ end of the gene, indicating that the end of the coding region had not yet been identified. Four additional introns were identified using an internal forward primer close to the predicted end and several reverse primers designed from the genomic sequence. The stop codon and 250 bp of the 3′ untranslated region of *EDS5* were characterized with a reverse primer positioned 780 bp downstream of the annotated stop codon of the F19H22.130 gene. The 5′ untranslated region was characterized by RT-PCR using primers at positions −100, −150, −220, and −320 bp from the putative ATG start site. Products of the expected lengths were obtained in all reactions, except

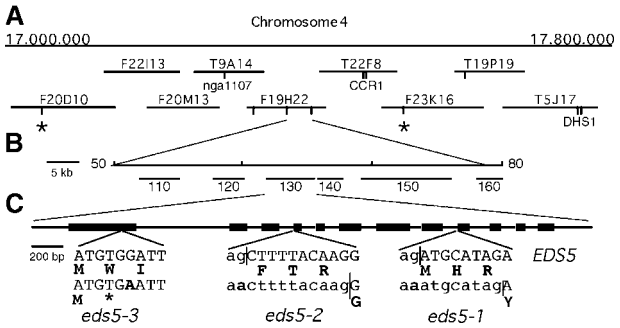

Figure 1. Positional Cloning and Structure of *EDS5*.

(A) Region of 800 kb on the bottom of chromosome 4 with overlapping BACs. Positions of known cleaved amplified polymorphic sequence and SSLP markers used for mapping are indicated. Positions of SSLP markers developed for this work from sequence data are labeled by vertical lines. Flanking markers used for the screening of recombinations in 2120 chromosomes are marked by asterisks.

(B) Location of *EDS5* on the sequenced BAC clone F19H22. *EDS5* was positioned at an equal distance of 0.09 cM between two SSLP markers located at positions 49.5 and 79.3 kb of BAC F19H22. Annotated genes are indicated by their numbers.

(C) Exon/intron structure of *EDS5*. The coding regions are indicated with thick lines. The inserts present the nucleotide exchanges and their influence on the protein sequences of all three mutant alleles indicated below the wild-type sequence. Lowercase letters mark intron sequences, uppercase letters indicate exon sequences, boldface letters mark amino acids, and the asterisk indicates a stop codon. Splice junctions are indicated by vertical lines.

when using the primer at −320 bp, which did not result in any RT-PCR product. The ATG of F19H22.130 is the correct start of EDS5 translation because it is the first start codon resulting in an open reading frame and stop codons are present in all three reading frames of the transcript before this ATG. In agreement with the sequence data, a transcript of ~2.0 kb was identified by RNA gel blot analysis. Thus, the *EDS5* gene consists of a gene spanning a 3.4-kb genomic sequence with 12 exons and 11 introns (Figure 1C) and an open reading frame of 1632 bp encoding a protein of 543 amino acids (Figure 2).

The mRNAs of the three mutant alleles of *EDS5* were sequenced. As indicated in Figures 1C and 2, *eds5-1* and *eds5-2* carry short deletions of 8 and 10 bp in the cDNA as a result of abnormal splicing at the border of intron 8/exon 9 and intron 3/exon 4, respectively, resulting in a frameshift and a premature stop codon. These changes are caused by a G-to-A transition in the AG from the splice acceptor site in intron 8 and intron 3, respectively. The *eds5-3* allele carries a transition mutation converting a TGG to a premature stop codon (TGA) at nucleotide 339 of the coding region. The changes in all three alleles lead to nonsense mutations. The reduction in transcript accumulation that was observed by RNA gel blot analysis (see below) therefore might have been caused by a nonsense-mediated RNA decay system (Hentze and Kulozik, 1999).

Finally, the identity of EDS5 was tested by complementation analysis. The *eds5-3* mutant was transformed with a 4.4-kb (pEDS5-1) or 4.6-kb (pEDS5-2) genomic sequence of the wild type, which included 970 or 830 bp, respectively, upstream of the ATG and 200 bp downstream of the stop codon. Of six independent lines transformed with pEDS5-1 or pEDS5-2, all but one showed complementation of the *eds5* mutant phenotype to wild-type or nearly wild-type amounts of SA after pathogen inoculation. A detailed analysis of two representative lines of transgenic plants carrying pEDS5-1 or pEDS5-2 is shown in Figure 3. In addition to increased amounts of SA, these plants also showed a reduced level of camalexin that was even slightly lower than in wild-type plants and increased expression of PR-1 (Figure 3). The *eds5-3* plants complemented with the wild-type *EDS5* gene were not significantly different from wild type in any of the phenotypes identified previously in the *eds5* mutant (Nawrath and Métraux, 1999).

EDS5 Belongs to the MATE Family of Transporters

BLAST analyses with the full-length *EDS5* sequence indicate a significant homology of EDS5 with DINF (DNA damage–inducible gene F) of *Escherichia coli*. *DinF* is induced by treatments with DNA damage–inducing agents, such as UV-C light (Kenyon and Walker, 1980). However, the DINF function has not yet been identified (Walker, 1995; G.C. Walker, personal communication). Recently, DINF was shown to be homologous with NorM of *Vibrio parahaemolyticus* (Brown et

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ATGCTAATCAAATCCCAAAGATTGACTCTTTTCTCTCCTCTCTTGTCCAAA   51
M  L  I  K  S  Q  R  L  T  L  F  S  P  L  L  S  K    17

ACAAGACGGATCCCGGTTAACTCCCACCAAACACTGGTCGCAGAATCGGTG  102
T  R  R  I  P  V  N  S  H  Q  T  L  V  A  E  S  V    34

ATAACTCGGAGAACACTCGGTGCGATCACCGCAACTCCGAGTTTTCATAAA  153
I  T  R  R  T  L  G  A  I  T  A  T  P  S  F  H  K    51

AATCCGGTGGTGATTCGACGGAGGATCAAATTAGAGAGAGTTACGAGGAAC  204
N  P  V  V  I  R  R  R  I  K  L  E  R  V  T  R  N    68

TGCGTCAGAATTGATCGGGAAATCGACGAAGAAGAAGAAGAGGAGAAG    255
C  V  R  I  D  R  E  I  D  E  E  E  E  E  E  E  K    85

GAGAGAGGGGATCTGGTGAAACAGAGCATATGGGAACAGATGAAAGAGATA  306
E  R  G  D  L  V  K  Q  S  I  W  E  Q  M  K  E  I    102

GTGAAGTTCACAGGTCCGGCGATGGGGATGTGGATTTGTGGACCGTTGATG  357
V  K  F  T  G  P  A  M  G  M  W  *I  C  G  P  L  M    119

AGTCTCATCGACACGGTCGTCATCGGCCAAGGAAGCTCCATCGAACTCGCT  408
S  L  I  D  T  V  V  I  G  Q  G  S  S  I  E  L  A    136

GCTCTTGGACCGGGAACAGTACTATGCGACCACATGAGTTATGTCTTCATG  459
A  L  G  P  G  T  V  L  C  D  H  M  S  Y  V  F  M    153

TTCCTCTCCGTGGCTACATCCAATATGGTTGCTACTTCTCTTGCCAAACAG  510
F  L  S  V  A  T  S  N  M  V  A  T  S  L  A  K  Q    170

GACAAGAAAGAAGCGCAACATCAAATCTCTGTTTTACTCTTTATTGGATTG  561
D  K  K  E  A  Q  H  Q  I  S  V  L  L  F  I  G  L    187

GTTTGTGGACTAATGATGCTTCTGCTCACAAGATTATTCGGTCCTTGGGCT  612
V  C  G  L  M  M  L  L  L  T  R  L  F  G  P  W  A    204

GTTACTGCTTTTACAAGGGGGAAGAACATTGAGATTGTCCCTGCAGCTAAT  663
V  T  A  F  T  R  G  K  N  I  E  I  V  P  A  A  N    221

AAGTATATTCAGATTCGAGGCTTAGCGTGGCCGTTTATCCTTGTTGGATTG  714
K  Y  I  Q  I  R  G  L  A  W  P  F  I  L  V  G  L    238

GTTGCTCAAAGTGCAAGTCTTGGAATGAAGAACTCATGGGGACCTCTTAAA  765
V  A  Q  S  A  S  L  G  M  K  N  S  W  G  P  L  K    255

GCATTAGCTGCAGCAACGATCATTAACGGTCTTGGCGATACAATCCTATGC  816
A  L  A  A  A  T  I  I  N  G  L  G  D  T  I  L  C    272

TTGTTTCTTGGACAAGGTATCGCTGGAGCTGCTTGGGCAACAACTGCTTCA  867
L  F  L  G  Q  G  I  A  G  A  A  W  A  T  T  A  S    289

CAGATTGTTTCGGCTTATATGATGATGGATTCTCTGAACAAAGAAGGCTAC  918
Q  I  V  S  A  Y  M  M  M  D  S  L  N  K  E  G  Y    306

AATGCTTACTCTTTTGCGATCCCTTCACCGCAAGAACTATGGAAGATCTCT  969
N  A  Y  S  F  A  I  P  S  P  Q  E  L  W  K  I  S    323

GCACTCGCTGCACCTGTTTTTATCTCCATTTTCTCCAAGATTGCTTTCTAC 1020
A  L  A  A  P  V  F  I  S  I  F  S  K  I  A  F  Y    340

TCTTTCATAATTTACTGTGCTACTTCCATGGGAACTCACGTTTTAGCCGCT 1071
S  F  I  I  Y  C  A  T  S  M  G  T  H  V  L  A  A    357

CATCAGGTGATGGCTCAGACGTATAGGATGTGCAATGTATGGGGTGAGCCA 1122
H  Q  V  M  A  Q  T  Y  R  M  C  N  V  W  G  E  P    374

CTCTCTCAAACGGCTCAGTCATTTATGCCAGAGATGTTATATGGTGCGAAC 1173
L  S  Q  T  A  Q  S  F  M  P  E  M  L  Y  G  A  N    391

CGTAATCTTCCCAAGGCTAGAACGTTGCTTAAGTCCCTGATGATTATTGGA 1224
R  N  L  P  K  A  R  T  L  L  K  S  L  M  I  I  G    408

GCCACGCTTGGATTAGTCTTGGGAGTTATCGGAACAGCGGTTCCAGGCCTG 1275
A  T  L  G  L  V  L  G  V  I  G  T  A  V  P  G  L    425

TTTCCTGGTGTCTACACACATGATAAAGTCATCATATCCGAGATGCATAGA 1326
F  P  G  V  Y  T  H  D  K  V  I  I  S  E  M  H  R    442

CTGCTTATACCTTTCTTCATGGCGTTGTCTGCATTGCCCATGACAGTATCC 1377
L  L  I  P  F  F  M  A  L  S  A  L  P  M  T  V  S    459

CTCGAGGGTACATTGCTGGCGGGACGTGATCTCAAATTCGTCAGCTCAGTG 1428
L  E  G  T  L  L  A  G  R  D  L  K  F  V  S  S  V    476

ATGAGTTCAAGCTTCATAATTGGTTGTCTCACACTAATGTTCGTGACAAGA 1479
M  S  S  F  I  I  G  C  L  T  L  M  F  V  T  R    493

AGTGGCTATGGTTTACTCGGCTGCTGGTTCGTTCTCGTCGGATTTCAATGG 1530
S  G  Y  G  L  L  G  C  W  F  V  L  V  G  F  Q  W    510

GGCCGGTTTGGTCTATATCTACGACGGCTTCTTTCGCCTGGAGGCATACTT 1581
G  R  F  G  L  Y  L  R  R  L  L  S  P  G  G  I  L    527

AATTCAGATGGGCCGAGTCCATACACGGTGGAGAAGATTAAATCCATTTAA 1632
N  S  D  G  P  S  P  Y  T  V  E  K  I  K  S  I  *    543
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Figure 2. cDNA Sequence of *EDS5* and the Corresponding Protein Sequence.

The amino acid sequence is indicated below the nucleotide sequence in boldface letters. Deletions caused by mutations in the slice acceptor sites in introns 8 and 3 of alleles *eds5-1* and *eds5-2*, respectively, are underlined. The G-to-A transition in nucleotide 339 changing the codon TGG to TGA in *eds5-3* is indicated by an asterisk.

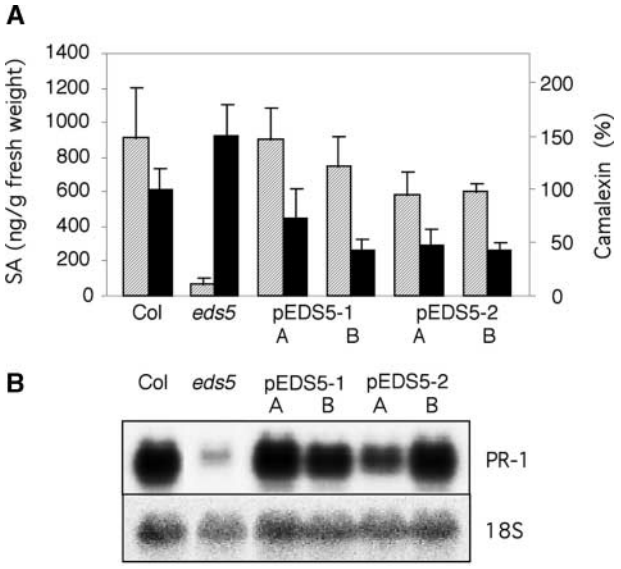

Figure 3. Complementation of *eds5*.

SA and camalexin accumulation **(A)** and PR-1 transcript accumulation **(B)** from two independent transgenic *eds5-3* plants harboring plasmid pEDS5-1 or pEDS5-2, respectively (pEDS5-1 A and B, pEDS5-2 A and B) were analyzed compared with wild type and *eds5-3*. Plants were inoculated with *P. syringae* carrying the *avrRpt2* gene and analyzed 2 days after inoculation.

(A) SA accumulation is shown in a striped pattern (left scale), and camalexin accumulation is shown in black (right scale); each bar represents the mean and standard error of four to six replicate samples. Camalexin amounts were measured relative to wild type (Col is equal to 100%).

(B) An 18S rDNA probe was used to evaluate uniform loading of RNA. The experiment was repeated once with similar results.

al., 1999). NorM is the only biochemically characterized member of the MATE protein family, and it has been found to pump antimicrobial agents out of bacterial cells in exchange for sodium (Morita et al., 1998, 2000). A second MATE protein to which a possible transporter function could be assigned is the ethionine resistance protein ERC1 from *Saccharomyces cerevisiae* (Shiomi et al., 1991; Brown et al., 1999). So far, 56 MATE proteins have been identified in the Arabidopsis genome and are presented in the Arabidopsis membrane protein library (www.cbs.umn.edu./Arabidopsis). Dual sequence alignments with the T-coffee program among EDS5, ERC1, NORM, and DINF showed that all of the proteins share a limited sequence homology, having between 15 and 25% identical amino acids and between 50 and 60% homologous amino acids in the protein part covering the membrane-spanning domains of the protein. Both NORM and DINF are predicted to have 12 transmembrane domains (TMDs), whereas EDS5 and ERC1 have an additional hydrophilic domain at the N terminus. Interestingly, although these N-terminal domains share no explicit sequence homology, both contain stretches rich in glutamic acid (ERC1, 10 Glu in 18 amino acids; EDS5, 9 Glu in 12 amino acids). Further sequence comparisons have been restricted to the region homologous with the TMDs of NORM/DINF. A comparison of EDS5 with DINF and NORM is shown in Figure 4. The homology of EDS5 with DINF was the highest (19% identical, 55% homologous), followed by NORM (18% identical, 53% homologous). The ERC1 of yeast shows a lower homology with EDS5 than with the bacterial proteins (16% identical, 51% homologous), although ERC1 also shows close homology with NORM (22% identity, 69% homologous) and DINF (22% identical, 55% homologous), indicating that ERC1 and EDS5 might have diverged independently from DINF and NORM.

The protein structure of EDS5 is predicted to include a coil domain at the hydrophilic N terminus, whereas the rest of the protein forms nine to 11 membrane-spanning domains (see Methods for the programs used). Program PRED-TMR identified 11 membrane-spanning domains correlating to TMDs 1 to 7 and 9 to 12 in the NORM protein (Pasquier et al., 1999). TMD 8 of NORM was not identified in EDS5 by any program, despite good sequence homology in this region (Figure 4).

EDS5 Expression and Accumulation of SA after Exposure to UV-C Light

SA has been reported to accumulate after exposure of tobacco to UV-C light (Yalpani et al., 1994). UV-C light also is a good inducer of SA biosynthesis in Arabidopsis. Figure 5A shows the accumulation of free SA in Arabidopsis leaves after 20 min of UV-C light exposure. At the start of the experiment, in plants having a low amount of free SA, the expression level of *EDS5* also was very low, but detectable, after prolonged exposure to x-ray film (Figures 5A and 5B). The accumulation of free SA started ~4 hr after the beginning of the UV-C light treatment, increased during the next 8 hr, and stabilized for at least 2 days. Conjugated SA increased in parallel but continued to accumulate even when the amount of free SA did not increase further (data not shown). The *EDS5* transcript started to accumulate 1.5 to 2 hr after UV-C light treatment, was maximal after ~6 hr, and decreased during the next 42 hr (Figure 5B). Thus, the *EDS5* transcript increased 2 hr before the first increase in SA was observed, indicating that increased *EDS5* expression might be part of the functional mechanism for the induction of SA biosynthesis. In contrast, PR-1 expression started 12 to 24 hr after UV-C light exposure once SA had accumulated to higher levels (Figure 5B).

Expression of *EDS5* after Treatment with SA

The expression of several defense genes, such as *PAD4* or *EDS1* (Falk et al., 1999; Jirage et al., 1999), has been shown to be regulated by SA. Therefore, we tested the effect of SA on *EDS5* expression. Applied either as a soil drench or a leaf

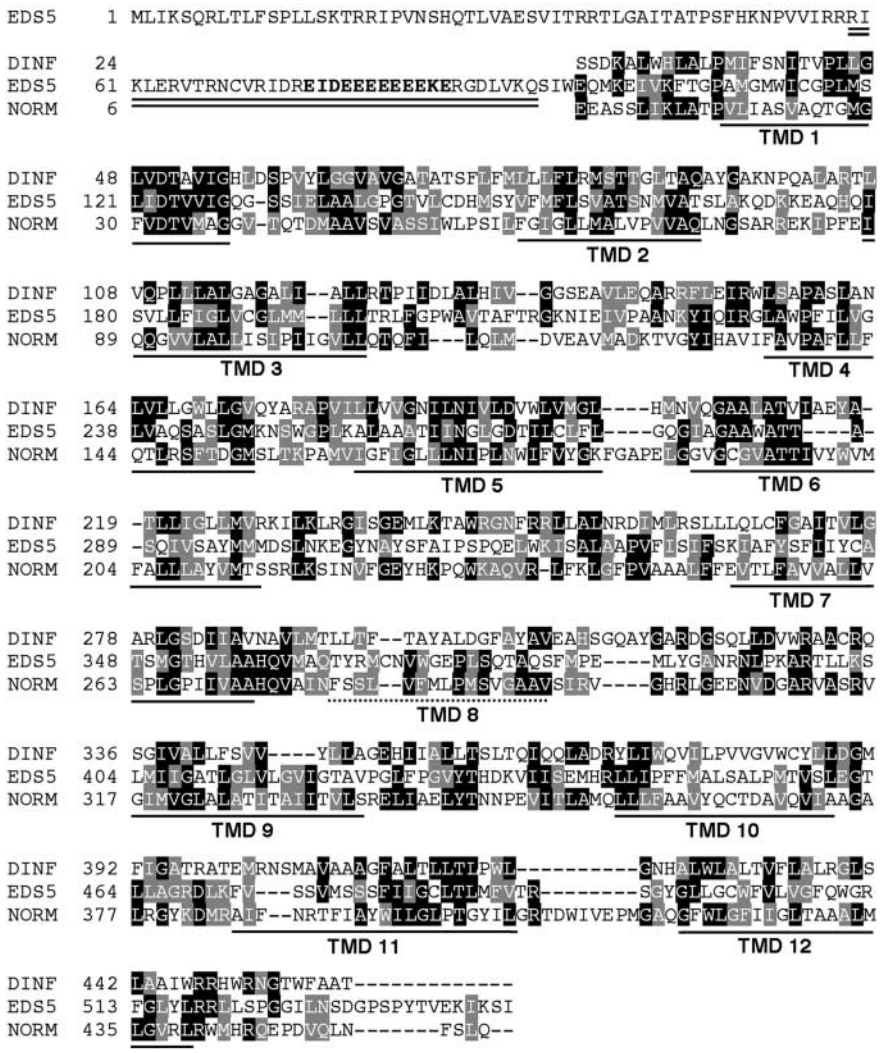

Figure 4. Analyses of the Predicted Protein Sequence of *EDS5*.

The double line indicates a predicted coiled-coil structure at the N terminus of the protein. Amino acids of a characteristic Glu stretch are indicated in boldface letters. Sequence comparisons of the EDS5 protein with the DINF protein of *E. coli* (amino acids 24 to end) and the NORM protein of *V. parahaemolyticus* (amino acids 6 to end) are shown over the entire conserved region of EDS5 (amino acids 105 to 543). Invariant amino acids are highlighted in black, and conserved amino acids are highlighted in gray. The locations of the TMDs in EDS5 predicted by the PRED-TMR program are underlined with solid lines. TMD 8 of NORM, which has not been identified in EDS5 by any program, is indicated by a dotted line.

spray, SA enhanced *EDS5* expression between 6 and 12 hr after treatment followed by a decrease in transcript accumulation (Figure 5C). Thus, as in *PAD4* and *EDS1*, the transcriptional regulation of *EDS5* might involve a positive feedback regulation loop by SA.

Expression of *EDS5* after Inoculation with *P. syringae*

Inoculation with the avirulent strain of *P. syringae* carrying the *avrRpt2* gene led to an increase in the expression of *EDS5* starting 2 hr after pathogen inoculation, reaching a maximum at 8 hr and decreasing slowly during the next 40 hr (Figure 6A). *EDS5* was induced earlier than *PR-1*, whose transcription level began to increase only 6 hr after pathogen inoculation and was maximal between 24 and 32 hr (Figure 6A).

After inoculation with the isogenic virulent strain of *P. syringae*, transient expression of *EDS5* could be observed starting ~8 hr after inoculation, with a maximum between 12 and 24 hr and decreasing during the next 24 hr (Figure 6B). *EDS5* was less induced than after inoculation with the

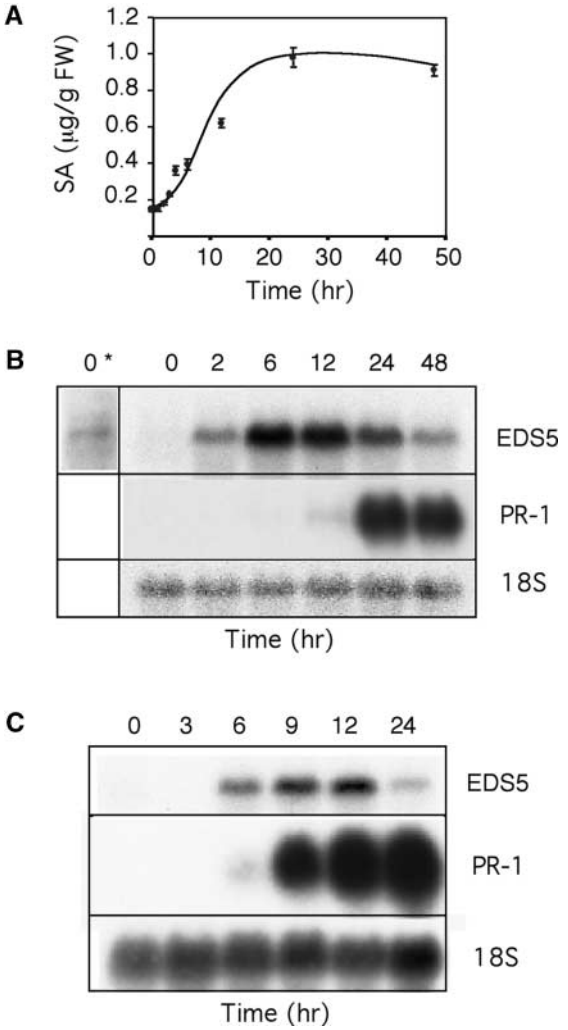

Figure 5. SA Accumulation and *EDS5* Expression after Treatment with UV-C Light and *EDS5* Expression after Exogenous Application of SA.

(A) and **(B)** Accumulation of free SA **(A)** and expression of *EDS5* and *PR-1* **(B)** in Col plants were measured in parallel after UV-C light treatment at the indicated times. In **(B)**, a long-term exposure to x-ray film is indicated by an asterisk. FW, fresh weight.

(C) Col plants were treated by drenching the soil with 0.2 mM Na-SA, and the plants were harvested after the times indicated. Values represent means of three to five measurements ±SE from one representative experiment.

In **(B)** and **(C)**, *PR-1* expression was studied in parallel to *EDS5* expression. The 18S rDNA probe was used to evaluate uniform loading. The experiments were repeated twice with similar results. For **(A)**, the absolute amounts of the SA accumulated varied somewhat from experiment to experiment, although the general shape of the curve was identical.

avirulent strain. In comparison, *PR-1* expression began to increase only 12 hr after pathogen inoculation and was maximal at ~32 hr (Figure 6B).

In all three *eds5* mutant alleles, the expression level of *EDS5* after inoculation with both strains of *P. syringae* was much lower than in wild-type plants but followed similar kinetics (Figure 6C and data not shown).

Expression of *EDS5* in Mutants Affected in the Disease Resistance Responses after Pathogen Inoculation or UV-C Light Exposure

The expression of *EDS5* after inoculation with avirulent and virulent *P. syringae* strains or UV-C light treatment was further characterized in several mutants affected in their response to pathogens, as presented in Figure 7.

sid2 is a SA induction–deficient mutant with a very similar phenotype to *eds5* (Nawrath and Métraux, 1999; Dewdney et al., 2000). The level of expression of *EDS5* in *sid2* is not significantly different from that of the wild-type plants after inoculation with pathogens (Figures 7A and 7B) or after UV-C light exposure (Figure 7C), showing that SA is not essential for the accumulation of the *EDS5* transcript.

Interestingly, the expression of *EDS5* is nearly absent in SA-degrading NahG plants (Delaney et al., 1994) 6 and 12 hr after inoculation with avirulent or virulent *P. syringae*, respectively (Figures 7A and 7B), but it is normal after UV light exposure (Figure 7C). This finding is in sharp contrast to the expression of *EDS5* in inoculated *sid2* plants. However, 24 and 48 hr after inoculation with *P. syringae* carrying the *avrRpt2* gene, NahG and Columbia expressed *EDS5* to approximately the same extent (data not shown). This indicates that NahG plants might display some uncharacterized differences in addition to having very low SA levels, for example, by the unspecific action of the SA hydroxylase on potential signaling compounds other than SA (Cameron, 2000). These hypothetical differences might delay *EDS5* accumulation in NahG plants.

The *npr1* mutant has a block in the resistance pathway downstream of SA and accumulates higher amounts of SA than wild-type plants after inoculation with *P. syringae* DC3000 pv *tomato* carrying the *avrRpt2* gene and possibly upregulating SA biosynthesis in a feedback loop (Shah et al., 1997). The *npr1* mutation did not influence the expression level of *EDS5* significantly after inoculation with this pathogen (Figures 7A and 7B) or UV-C light exposure (Figure 7C). Thus, *NPR1* does not affect the control of *EDS5* expression.

The *pad4* mutant displays low amounts of SA and camalexin as well as enhanced disease susceptibility to the virulent strain of *P. syringae* DC3000 pv *tomato* (Glazebrook et al., 1997). *EDS5* expression was found to be very low after inoculation with virulent *P. syringae* (Figure 7A) and reduced after inoculation with avirulent *P. syringae* (Figure 7B) or exposure to UV-C light (Figure 7C), indicating that PAD4 is in-

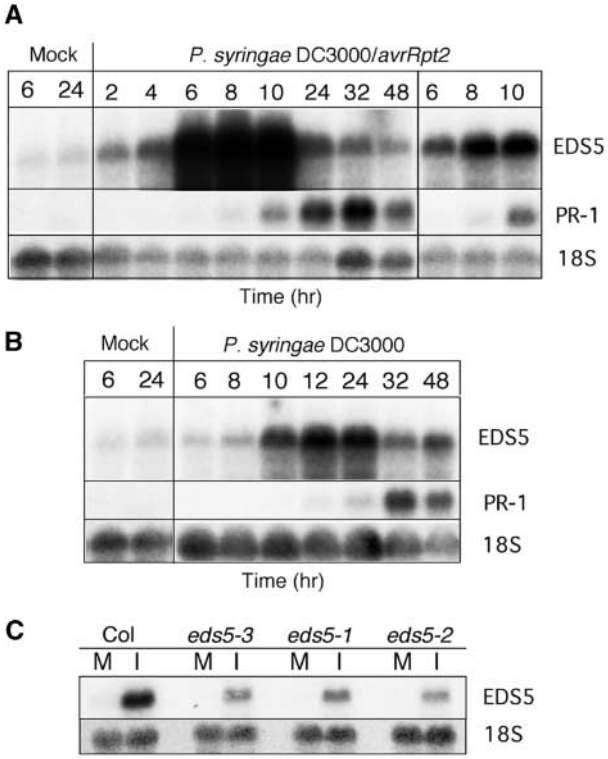

Figure 6. *EDS5* Expression after Pathogen Inoculation.

EDS5 and *PR-1* transcript accumulation in wild-type plants at different times after inoculation with an avirulent strain of *P. syringae* carrying the *avrRpt2* gene **(A)** and after inoculation with the isogenic virulent *P. syringae* **(B)**. In **(A)**, a short exposure time is shown at right to clarify overexposed parts of the film to the left. **(C)** shows *EDS5* transcript accumulation in wild-type and *eds5* mutant alleles 8 hr after inoculation with *P. syringae* carrying the *avrRpt2* gene. The 18S rDNA probe was used to evaluate uniform loading. The experiments were repeated once with similar results. M, mock inoculation; I, pathogen inoculation.

volved in the transcriptional regulation of EDS5 after either pathogen inoculation or UV-C light exposure.

The *ndr1* mutant is impaired in resistance to *P. syringae* carrying the *avrRpt2* gene and accumulates low levels of SA (Century et al., 1995; A. Shapiro, personal communication). In *ndr1*, *EDS5* gene expression was very low after inoculation with *P. syringae* carrying *avrRpt2*, and the induction was reduced after inoculation with the isogenic virulent strain or after UV-C light treatment, indicating that NDR1 also is involved in the transcriptional regulation of EDS5 after either pathogen inoculation or exposure to UV-C light.

The *eds1* mutant shows increased susceptibility to *P. syringae* carrying the *avrRps4* gene (Aarts et al., 1998). In *eds1*, *EDS5* gene expression was nearly absent after inoculation with *P. syringae* carrying the *avrRps4* gene as well as after exposure to UV-C light, as shown in Figure 7D. The expression of *EDS5* was not altered significantly in the *jar1* and *etr1* mutants, indicating that *EDS5* is not regulated transcriptionally by the ethylene/jasmonate pathway after inoculation with pathogens or UV-C light exposure (data not shown).

Thus, PAD4, EDS1, and NDR1, which are believed to act early in the response to pathogens (Glazebrook, 1999), are involved in the regulation of *EDS5* transcription after pathogen inoculation as well as after exposure to UV-C light, indicating that the signaling pathways share common elements. A particularly strong reduction in *EDS5* expression was observed in these mutants after inoculation with a pathogen strain to which the respective mutant has enhanced susceptibility. Thus, this reduction can be seen in *pad4* inoculated with the virulent *P. syringae* strain or in *ndr1* inoculated with *P. syringae* carrying *avrRpt2*. *EDS5* expression is independent of SID2 and NPR1, which are in the SA-dependent pathogen response pathway as well as in the ethylene and jasmonate response pathways.

DISCUSSION

To characterize the biosynthesis of SA, we have used a mutational approach whereby Arabidopsis plants infected with an avirulent *P. syringae* were screened for their inability to accumulate SA. This led to the discovery of two mutants, *sid1* (allelic to *eds5*; Rogers and Ausubel, 1997) and *sid2*, both of which are characterized by low levels of SA, low PR-1 expression, and enhanced disease susceptibility after infection (Nawrath and Métraux, 1999). Therefore, *EDS5* encodes a protein with an essential function in the SA-dependent pathway of plant defense against pathogens.

Using a positional cloning strategy, we have identified the *EDS5* gene. *EDS5* complemented the *eds5-3* mutation, as shown by the high SA accumulation and PR-1 expression in pathogen-inoculated transgenic *eds5* plants expressing *EDS5* compared with inoculated *eds5* mutants (Figure 3). Thus, the epistasy of EDS5 to SA accumulation and PR-1 expression was reconfirmed. The expression of *EDS5* in *eds5* also reduced the high levels of camalexin observed in the *eds5* mutant (Nawrath and Métraux, 1999) (Figure 3). This reduction might be attributable to reduced growth of the bacteria in the transgenic *eds5* plants complemented with the wild-type *EDS5* gene.

The transcription of *EDS5* is induced rapidly by pathogens and abiotic stresses, such as UV-C light, which induce accumulation of SA. The time course of *EDS5* expression is similar to that of *PAD4* (Jirage et al., 1999), *EDS1* (Falk et al., 1999), and *NDR1* (Century et al., 1997). However, we showed that the transcript accumulation of EDS5 after exposure to UV-C light and certain pathogens depends on PAD4, EDS1, and NDR1. The onset of expression of *EDS5* after exposure to UV-C light was 2 hr earlier than the onset of the increase in SA. Similarly, *EDS5* transcript appeared as

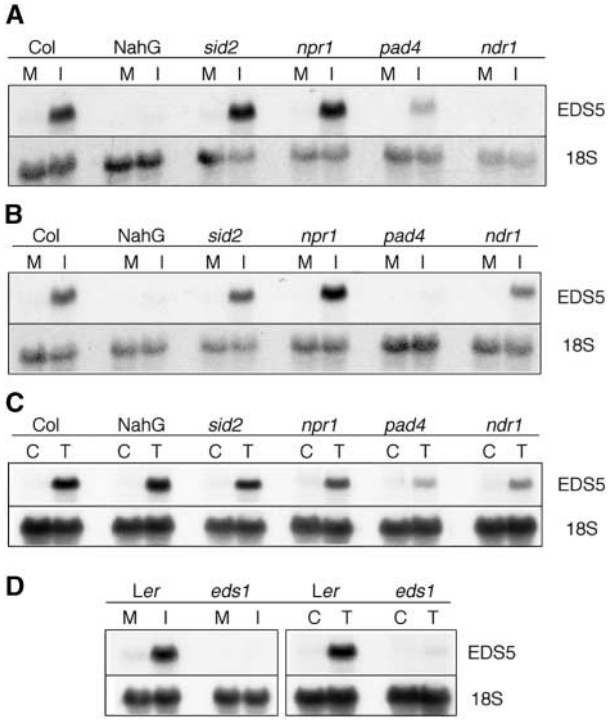

Figure 7. *EDS5* Transcript Accumulation in Different Arabidopsis Mutants.

Mutants in the Col background were inoculated with *P. syringae* DC3000 pv *tomato* carrying the *avrRpt2* gene **(A)**, with the isogenic virulent strain **(B)**, or exposed to UV-C light **(C)** and compared with treated Col plants. In **(D)**, the *eds1* mutant in the L*er* background was inoculated with the isogenic *P. syringae* strain carrying the *avrRps4* gene (left) and was treated with UV-C light (right) and compared with treated L*er* plants. The plants were harvested at the time of nearly maximal expression: 8 hr after inoculation with *P. syringae* carrying the *avrRpt2* gene **(A)** or *avrRps4* **(D)**, 24 hr after inoculation with the isogenic virulent *P. syringae* strain **(B)**, and 7 hr after exposure to UV-C light (**[C]** and **[D]**). The 18S rDNA probe was used to evaluate uniform loading. M, mock inoculation; I, pathogen inoculation; C, control; T, UV-C light treatment.

early as 2 hr after inoculation with *P. syringae* carrying the *avrRpt2* gene, whereas SA started to accumulate 3 to 4 hr after inoculation (D. Lieberherr and J.-P. Métraux, unpublished data). These kinetics suggest that the accumulation of the *EDS5* transcript might be involved functionally in the accumulation of SA and plant defense after both UV-C light exposure and pathogen induction. The expression data are summarized in the following model. After either UV-C light exposure or pathogen inoculation, the increase in the transcription of *EDS5* depends on functional *EDS1*, *PAD4*, and *NDR1* genes. This increase in *EDS5* transcription is strongly correlated to a subsequent increase in SA and *PR-1* transcript accumulation in a timely, coordinated manner (Figure 8). The overall speed of these events, however, depends strongly on the type of signal. Whether the same hierarchy of regulation is found at the protein level will have to be investigated in the future.

EDS5 expression also was induced by relatively high concentrations of exogenous SA. The effect of SA was slower than that of UV-C light (onset of expression 6 and 2 hr after treatment, respectively). Interestingly, the transcript accumulation of *EDS5* occurred only transiently during the first hours after induction by UV-C light and pathogens and returned close to basal levels after 48 hr, despite high endogenous concentrations of SA (Figures 5 and 6). In addition, *EDS5* transcript accumulation was normal in the *sid2* mutant, which accumulated low amounts of SA after induction. Therefore, the biological relevance of the SA-mediated expression of *EDS5* is not obvious. At optimal concentrations, it might regulate the extent of transient *EDS5* expression by a positive feedback loop.

EDS5 encodes a protein of 543 amino acids with a predicted structure that includes nine to 11 membrane-spanning domains and a coiled coil at the N terminus. Its protein structure and its sequence homology with MATE proteins provide evidence that EDS5 might be a transporter. Of the 56 proteins that have been classified as MATE proteins in the Arabidopsis Membrane Protein Library database, none had a biological function assigned until very recently. The mutant *transparent testa 12* blocked in flavonoid biosynthesis has been shown to be defective in a MATE protein potentially acting as a vacuolar flavonoid transporter (Debeaujon et al., 2001). Furthermore, AFL5 is a recently identified MATE transporter that renders Arabidopsis resistant to toxins (Diener et al., 2001). Thus, MATE proteins are involved in the transport of different kinds of organic molecules in plants.

The nature of the substances transported by EDS5 remains unknown. NORM, the only biochemically characterized member of the MATE proteins, is a Na^+-driven antimicrobial efflux pump (Morita et al., 2000). It is possible that EDS5 transports organic molecules against Na^+. These organic molecules could be part of the signal transduction cascade, possibly products of EDS1 and PAD4. It also may be possible that EDS5 has evolved to transport phenolic compounds that are precursors for the biosynthesis of SA.

Interestingly, only six of the 56 MATE proteins of Arabidopsis have an extended hydrophilic region of ~100 amino acids at the N-terminal end. The MATE proteins of yeast also have this feature. It is possible that the transport activity is regulated by factors binding to this hydrophilic domain, possibly forming a coiled coil in EDS5.

Among the different classes of MATE proteins, EDS5 is most closely related to DINF proteins. In *E. coli*, *DINF* is induced after exposure to DNA-damaging agents, including UV-C light, an interesting parallel to *EDS5*. *DinF* is part of an operon comprising genes involved in the save our souls (SOS) response (Kenyon and Walker, 1980), a mechanism leading to DNA damage tolerance in prokaryotes (Little and

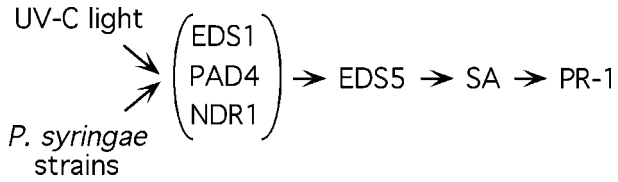

Figure 8. Model for the Transcriptional Regulation of *EDS5*.

Treatment with UV-C light or inoculation with different strains of *P. syringae* induce the transcript accumulation of *EDS5* when EDS1, PAD4, and NDR1 are functional. An increase in *EDS5* expression is followed by the accumulation of SA and PR-1 expression. The gene names shown in parenthesis have different strengths of impact on *EDS5* transcription depending on the inducer; their order of action has not been determined.

Mount, 1982; Walker, 1995). The genes belonging to this operon are involved in recombination and DNA repair (Walker, 1995). DINF has been found in Gram-negative bacteria, Gram-positive bacteria, and archaebacteria (Bouyoub et al., 1995). Such conservation during evolution suggests that DINF proteins fulfill an important physiological role. In *Streptococcus pneumoniae*, *DinF* belongs to an operon induced by competence, also a process requiring DNA recombination events. It is hypothesized that DINF might function in increasing genetic exchanges that allow a better adaptation to environmental conditions (Mortier-Barrière et al., 1998), but the potential transport function of DINF has not been characterized further and it is not known if DINF is involved directly in processes requiring DNA recombination. Because *eds5* mutants have not been found to be more sensitive to UV-C light irradiation (data not shown), it remains to be seen if EDS5 is involved in decreasing DNA damage in plants.

Plants have evolved to use functions for building the defense network against pathogens that have parallels in all kingdoms of organisms: R genes and the transcriptional coactivator NPR1 share similarities in mammalian innate immunity, and defensins are found in the defense system of lower vertebrates. This study shows that plants also have recruited proteins for pathogen defense that have structural homologs in prokaryotes, *Archea*, and lower eukaryotes. It will be interesting to discover the specific functions of EDS5 in the network of defense against pathogens in plants and to determine if functional parallels exist in other organisms.

METHODS

Plants and Growth Conditions of Plants and Bacteria

Arabidopsis thaliana plants, accessions Columbia (Col) and Landsberg *erecta* (L*er*), were used in these experiments. The isolation of the mutant alleles *eds5-1* and *eds5-2* was as described by Rogers and Ausubel (1997) and Volko et al. (1998), respectively. The isolation of *eds5-3* (*sid1*) and the allelism tests to *eds5-1* and *eds5-2* were reported by Nawrath and Métraux (1999). *sid2* was described by Nawrath and Métraux (1999). Other mutants/plants were obtained from the following persons/institutions: *pad4-1*, *ndr1-1*, and *npr1-1* from J. Glazebrook (Torrey Mesa Research Institute, San Diego, CA), A. Shapiro (University of Delaware, Newark), and X. Dong (Duke University, Durham, NC), respectively; *etr-1* and *jar-1* from the Arabidopsis Biological Resources Center (Columbus, OH); NahG plants from J. Ryals (Paradigm Genetics, Research Triangle Park, NC); and *eds1-2* and L*er* from Jane Parker (Sainsbury Laboratory, John Innes Centre, Norwich, UK). All mutants mentioned above are in the Col background, except *eds1*, which is in the L*er* background. *Pseudomonas syringae* DC3000 pv *tomato* and the isogenic strains carrying the *avrRpt2* or *avrRps4* gene were used for pathogen inoculations. Plants and *P. syringae* were grown as described by Nawrath and Métraux (1999). *Agrobacterium tumefaciens* strain GV3101 MP90 was used for the transformation of Arabidopsis.

Isolation of RNA and DNA

For RNA gel blot analysis, RNA was isolated as described previously (Nawrath and Métraux, 1999). For reverse transcriptase–mediated polymerase chain reaction (RT-PCR), RNA was isolated from plants 24 hr after inoculation with *P. syringae* as described by Nawrath and Métraux (1999), except that the RNA was treated with DNase in the presence of the RNase inhibitor rRNAsin (Promega) for 1 hr at 37°C and purified by phenol/chloroform followed by ethanol precipitation. Genomic DNA used for mapping was prepared by fast isolation methods for PCR (Edwards et al., 1991; Klimyuk et al., 1993).

Inoculation with Bacteria and Treatments with UV Light and Salicylic Acid

For RNA gel blot analysis, 4- to 5-week-old plants were syringe inoculated with a suspension of 2×10^6/mL *P. syringae* DC3000 pv *tomato* or of the isogenic strains carrying the *avrRpt2* or *avrRps4* gene. Two- to 3-week-old plants were exposed to UV-C light (254 nm) at 30 cm for 20 min in the dark and then placed in continuous light until harvest. Two- to 3-week-old plants were treated with salicylic acid (SA) either by adding Na-SA to the soil at a final concentration of 0.2 mM or by spraying a 0.01% Silwet L-77 solution (OSi Specialties, Inc., Meyrin, Switzerland) containing Na-SA at 1.0 or 3.3 mM on the shoots. As controls for the second type of treatment, plants were sprayed with 0.01% Silwet L-77 alone.

Mapping, Isolation, and Identification of the *EDS5* Gene

EDS5 was mapped to the lower arm of chromosome 4 near the simple sequence length polymorphism (SSLP) marker nga1107 (Nawrath and Métraux, 1999). Genetic fine mapping of the *EDS5* gene was performed using additional SSLP and cleaved amplified polymorphic sequence markers (published at http://www.arabidopsis.org). Additional markers were generated by analysis of sequenced bacterial artificial chromosomes (BACs) for simple sequence islands and tested for SSLPs between the Arabidopsis accessions Col and L*er*. A total of 1060 randomly selected F2 plants from the cross L*er* ×

eds5-3 were screened with the flanking markers F20D10-45.8 and F23K16-28.4. The marker F20D10-45.8 was amplified using the oligonucleotides 5′-GTTTGTTCCCAATGCGAAAG-3′ and 5′-TTCGTATGTTACAAGCAAAATC-3′, resulting in 186- and 175-bp fragments in Col and L*er*, respectively, whereas the marker F23K16-28.4 was amplified using oligonucleotides 5′-CGCATTTTGTAATCGTTTCAT-3′ and 5′-AGGTTATCATGCGTGTATTTA-3′, resulting in 205- and 220-bp fragments in Col and L*er*, respectively. The genotype of the F2 plants having a recombination in this interval was determined in the F3 population after selfing by measurements of total SA 3 days after inoculation with *P. syringae* carrying the *avrRpt2* gene. The *EDS5* gene was determined to be flanked by the SSLP markers at position 49.4 and 79.3 kb of BAC F19H22. The marker F19H22-49.4 was amplified using the oligonucleotides 5′-TCCTATTATGACAAAATTGGT-3′ and 5′-CACTGATTATCTCCTTAAGA-3′, giving fragments of 226 bp (Col) and 216 bp (L*er*), whereas the marker F19H22-79.3 was amplified using the oligonucleotides 5′-AATACATGTCAAGATCTAAT-3′ and 5′-AAAATACACGACTAGGGTTC-3′, giving fragments of 236 bp (Col) and 240 bp (L*er*).

A genomic sequence was amplified from BAC F19H22 with the ExpandR High Fidelity PCR Amplification System (Boehringer, Mannheim, Germany) using the oligonucleotides 5′-GGAATTCAGAAGGATTTCTCAAT-3′ and 5′-GGAATTCAACGGTCTGAAAGAGGA-3′, located 970 and 830 bp upstream from the ATG, respectively, and the oligonucleotide 5′-CCGAATTCTCCTTTGCTGGGAAG-3′, located 200 bp downstream of the stop codon. These primers introduced EcoRI sites that were used to clone the 4.6- and 4.4-kb PCR fragments into the binary vector pPZP112 (Hajdukiewicz et al., 1994). The *eds5-3* mutant was transformed by the floral dip transformation method (Desfeux et al., 2000).

Characterization of the *EDS5* cDNA

RT-PCR was performed with the Access RT-PCR system from Promega. The 5′ end of the cDNA was determined by RT-PCR using primers located 100, 150, 220, and 320 bp upstream from the predicted ATG on the annotated genomic sequence. The 3′ end of the cDNA was determined by RT-PCR using primers located 140, 380, 660, and 780 bp downstream of the stop codon of the annotated genomic sequence. The cDNAs of wild-type and mutant alleles were sequenced directly as RT-PCR products. The sequence data were analyzed using the BLAST program against the genomic DNA. The following programs and databases contributed information to the presented work: for functional analyses, SMART (Simple Modular Architecture Research Tool; www.smart.embl-heidelberg); for sequence alignments, Clustal W (decypher2.stanford.edu/algo-cw/clustalW_ax) and T-coffee (www.ch.embnet.org/software/Tcoffee); for protein structure, PRED-TMR (www.O2.db.oua.gr/PRED-TMR), TMHMM (www.cbs.dtn), HMMTOP (www.enzim.hn/hmmtop), and AMPL (Arabidopsis Membrane Protein Library; www.cbs.umn.edu/Arabidopsis).

RNA Gel Blot Analyses

RNA gel blot analyses were performed as described previously (Nawrath and Métraux, 1999). A 500-bp fragment comprising the first exon of the *EDS5* gene was taken as a specific probe for *EDS5*.

Accession Number

The GenBank accession number for the full-length EDS5 cDNA described in this article is AF416569.

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