Negative regulation of defense responses in plants by a conserved MAPKK kinase

Catherine A. Frye*†, Dingzhong Tang*, and Roger W. Innes‡

Department of Biology, Indiana University, Bloomington, IN 47405-3700

Edited by Noel T. Keen, University of California, Riverside, CA, and approved October 30, 2000 (received for review August 22, 2000)

The *enhanced disease resistance 1* (*edr1*) mutation of *Arabidopsis* confers resistance to powdery mildew disease caused by the fungus *Erysiphe cichoracearum.* Resistance mediated by the *edr1* mutation is correlated with induction of several defense responses, including host cell death. Double mutant analysis revealed that all *edr1*-associated phenotypes are suppressed by mutations that block salicylic acid (SA) perception (*nim1*) or reduce SA production (*pad4* and *eds1*). The *NahG* transgene, which lowers endogenous SA levels, also suppressed *edr1*. In contrast, the *ein2* mutation did not suppress *edr1*-mediated resistance and associated phenotypes, indicating that ethylene and jasmonic acid-induced responses are not required for *edr1* resistance. The *EDR1* gene was isolated by positional cloning and was found to encode a putative MAP kinase kinase kinase similar to CTR1, a negative regulator of ethylene responses in *Arabidopsis*. Taken together, these data suggest that EDR1 functions at the top of a MAP kinase cascade that negatively regulates SA-inducible defense responses. Putative orthologs of EDR1 are present in monocots such as rice and barley, indicating that EDR1 may regulate defense responses in a wide range of crop species.

Plants defend themselves against infectious diseases by using both preformed and induced defenses. The latter comprise a complex suite of physiological changes, including a form of programmed cell death called the hypersensitive resistance response (HR) (1). In an effort to identify plant genes that regulate defense responses, we screened for *Arabidopsis* mutants that displayed enhanced resistance to normally virulent pathogens. The *edr1* mutant was identified in a screen for mutants that had become resistant to the bacterium *Pseudomonas syringae* and was subsequently shown to be resistant to *Erysiphe cichoracearum* (powdery mildew) (2). Significantly, *edr1* mutant plants do not display constitutive expression of the defense gene *PR-1*, indicating that resistance is not caused by constitutive activation of systemic acquired resistance-associated defenses (3).

Although known defense responses are not constitutively expressed in *edr1* plants, several defense responses are induced by *E. cichoracearum* more rapidly in *edr1* plants than in wild-type *Arabidopsis* variety Col-0 (2). These include deposition of autofluorescent compounds and callose [a β-(1→3) glucan] in mesophyll cell walls, accumulation of *PR-1* mRNA, and mesophyll cell death. In wild-type Col-0 plants infected with *E. cichoracearum*, these defenses are induced more slowly, and very little cell death is observed (2, 4). Because the *edr1* mutation is recessive, the *EDR1* gene appears to function as a negative regulator. Because these defenses are not expressed in *edr1* plants in the absence of pathogens, however, there must be pathogen-associated signals required to induce these defenses. In the absence of *EDR1* function, even presumably weak signals from virulent *Erysiphe* strains are sufficient to induce strong responses.

Because the *edr1* mutant appears phenotypically normal in the absence of pathogens but displays enhanced resistance, understanding the molecular basis of this resistance may lead to significant commercial applications. We have therefore assessed the contribution of known plant defense pathways to *edr1*-mediated resistance and have identified the *EDR1* gene. Here we report that *EDR1* encodes a putative mitogen-activated protein kinase kinase kinase (MAPKKK) and show that *edr1*-mediated resistance depends on salicylic acid (SA)-inducible defenses.

Materials and Methods

Construction of Double Mutants. Double mutants were created by standard genetic crosses. In all cases, *edr1* was derived from the Col-0 genotype of *Arabidopsis*. The *eds1–1* and *pad4–2* mutations were in a Landsberg *erecta* genotype, the *nim1–1* mutation in a Ws-0 genotype, and the *ein2–1* mutation in a Col-0 genotype. For all but *ein2–1*, double mutants could be identified by PCR-based molecular marker screening of F2 progeny. To identify *edr1/ein2–1* double mutants, F2 plants were inoculated with *E. cichoracearum*, and plants displaying resistance were selected. We then used PCR to amplify the *EIN2* gene and identified plants that were homozygous for the *ein2–1* mutation by direct sequencing of PCR products. All double mutants were verified to contain the *edr1*mutation by PCR amplification of *EDR1* followed by direct sequencing.

Disease Assays. Plants were inoculated with *E. cichoracearum* by transferring conidia (asexual spores) directly from previously infected plants via direct leaf-to-leaf contact. Suppression of the *edr1* phenotype was scored by looking for production of visible powder (i.e., abundant conidia) and absence of necrotic lesions 8 days after inoculation.

Chlorophyll Measurements. Chlorophyll was extracted from *Arabidopsis* leaves by incubating in 96% ethanol at 50°C for 2 hours. The chlorophyll content of the ethanol extract was then quantified spectrophotometrically, as previously described (5).

Genetic and Physical Mapping of *EDR1*. Genetic mapping was accomplished by using a F2 population derived from cross between the *edr1* mutant (Columbia genotype, Col-0) and Landsberg *erecta* (L*er*). Genomic DNA was isolated from 239 F2 plants representing 478 meioses and scored with published simple sequence length polymorphism and cleaved amplified polymorphic sequence markers (6, 7). This initial mapping

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: SA, salicylic acid; HR, hypersensitive resistance response; JA, jasmonic acid; MAPKKK, mitogen-activated protein kinase kinase kinase; RACE, rapid amplification of cDNA ends; BAC, bacterial artificial chromosome.

Data deposition: The nucleotide and protein sequences reported in this paper have been deposited in the GenBank database (accession nos. AF305913 for the *Arabidopsis EDR1* gene, AF305911 for the rice *EDR1* gene, and AF305912 for the barley *EDR1* gene).

*C.A.F. and D.T. contributed equally to this work.

†Present address: Novartis Agriculture Biotechnology, Inc., 3054 Cornwallis Road, Research Triangle Park, NC 27709.

‡To whom reprint requests should be addressed at: Department of Biology, Jordan Hall 142, Indiana University, 1001 East Third Street, Bloomington, IN 47405-3700. E-mail: rinnes@bio.indiana.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.011405198. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.011405198

localized *edr1* between molecular markers *Ateat1* and *nga63* (2). We then developed our own molecular markers at intervals between these two markers by amplifying and sequencing 1-kb fragments from both Col-0 and L*er*. These analyses identified several additional sequences from this region that were polymorphic between Col-0 *edr1* and L*er*, which were then mapped relative to *edr1* (marker information available on request). Ultimately, *edr1* was localized between bacterial artificial chromosome (BAC) end sequence F4014 and an internal sequence from BAC F7G19. This analysis defined a 120-kb region that cosegregated with *edr1*. This region was fully sequenced and annotated by the *Arabidopsis* genome project and contained 26 predicted genes. Three of these were selected for amplification and sequencing from the *edr1* mutant on the basis of predicted functions that were consistent with the *edr1* mutant phenotype (F22013.21, a MAPKKK, F22013.32, a myb-like transcription factor, and F22013.34, a superoxide dismutase). A C→G transversion was identified in gene F22013.21, which produced an early stop codon.

Complementation of the *edr1* Mutation. BAC clone F22013 was partially digested with *Sau*3A restriction enzyme and ligated to *Bam*HI-digested binary cosmid vector pCLD04541 (8). The ligation mix was packaged by using a Gigapack III Gold packaging extract (Stratagene) and transfected into *Escherichia coli* strain DH5α. Clones containing *EDR1* were identified by colony hybridization by using a PCR-amplified fragment internal to the predicted *EDR1* ORF (9). The inserts of these cosmids were then analyzed by restriction enzyme mapping and sequencing of junctions between inserts and vector. Two clones were selected for transformation of *Arabidopsis edr1* plants. Clone 1 contained a full-length *EDR1* gene, whereas clone 2 was truncated 76 bases 5′ of the *EDR1* stop codon but contained all other genes present on clone 1 (predicted genes F22013.19 and F22013.20). Sequence analysis of these two genes amplified from the *edr1* mutant revealed no mutations. Both clones were transferred to *Agrobacterium tumefaciens* strain GV3101 by conjugation. *Arabidopsis edr1* plants were transformed by using the floral dip method (10), selecting for kanamycin resistance (50 μg/ml).

DNA and RNA Gel Blot Hybridizations. DNA and RNA gel blots were hybridized and washed at 55°C (DNA blots) or 65°C (RNA blots) by using Church buffer as described in ref. 11. Total RNA was isolated from *Arabidopsis* leaf tissue by using TriPure Isolation Reagent (Boehringer Mannheim) following the manufacturer's protocol and poly(A) RNA subsequently purified from the total RNA by using a Qiagen Oligotex midi kit (Qiagen, Chatsworth, CA).

DNA Sequence Analysis of *Arabidopsis EDR1*. *EDR1* mRNA was amplified by reverse transcription–PCR by using overlapping sets of primers and total RNA as template. During this phase, we were unable to obtain a product by using a primer complementary to the predicted start of the *EDR1* ORF. To obtain the 5′ end of the *EDR1* cDNA sequence, we used 5′-rapid amplification of cDNA ends (RACE) PCR by using the Marathon 5′-RACE kit from CLONTECH. PCR products were sequenced directly by using an ABI377 automated DNA sequencer and Applied Biosystems BigDye dideoxy terminator reagents (Perkin–Elmer). DNA sequences were assembled by using the SEQUENCHER program (Gene Codes, Ann Arbor, MI). Assembly of the full-length *EDR1* cDNA sequence revealed that the cDNA sequence predicted from genomic sequence (accession no. AC003981) included an extra exon and intron at the 5′ end that are absent from our experimentally determined cDNA sequence. Our EDR1 protein sequence (accession no. AF305913) thus lacks the first 82 amino acids present in the predicted protein derived from genomic sequence (PIR-T00726).

Isolation of Barley *EDR1*. The kinase domain of a putative barley *EDR1* gene was amplified by using the degenerate oligonucleotides: 5′-gcIgtIaaIaaIttI(t/c)tIga(t/c)ca(g/a)ga-3′ and 5′-gcIaaIgaIggIc(g/t)IaI(g/a)ttIgg(g/a)tc-3′. Amplification was performed on cDNA from barley variety Ingrid by using the following temperature program: 94°C for 2 minutes followed by 35 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 1 min, followed by 10 min at 72°C. The resulting PCR product was cloned into the vector pGEMT-Easy (Promega) and several independent clones sequenced by using an ABI377 automated sequencer (Applied Biosystems). On the basis of this sequence, two gene-specific primers were designed (5′-TGCGTCGGCTCCGTCATCCAAATATTGT-3′ and 5′-TCTCGTCAATTTGGCAATTAGGGCGATG-3′) for performing 5′ and 3′-RACE PCR by using the Marathon kit (CLONTECH). The 5′ and 3′ RACE PCR products, which overlapped each other, were then cloned into the vector pGEMT-Easy and sequenced.

Results and Discussion

To gain insight into how *EDR1* regulates defense responses in *Arabidopsis*, we assessed the relative contribution of SA, jasmonic acid (JA), and ethylene to *edr1*-mediated resistance. These three compounds are plant hormones that induce distinct but overlapping sets of defense genes (12, 13). To assess their role in *edr1*-mediated resistance, we crossed the *edr1* mutant to mutants that are blocked at specific steps in the SA, JA, or ethylene response pathways. Double homozygous mutants were then infected with *E. cichoracearum* and the *edr1* mutant phenotype (absence of visible powder and presence of lesions) scored 8 days after infection.

Role of SA, JA, and Ethylene Pathways in *edr1*-Mediated Resistance. Mutations that block SA-associated defense responses suppressed *edr1*-mediated resistance (Fig. 1). The dependence on SA-induced responses was verified by mutations in two distinct parts of the SA pathway and by use of a transgene, *nahG*, that reduces endogenous SA levels (14, 15). The *nim1–1* mutation, which blocks a subset of SA-induced responses (16), appeared to completely suppress *edr1*-mediated resistance, including the cell death response induced by *E. cichoracearum*. Similarly, the *pad4–2* and *eds1–2* mutations, which reduce production of SA induced by pathogens (17, 18), also suppressed *edr1* (Fig. 1). Finally, the *nahG* transgene suppressed all *edr1*-associated phenotypes (Fig. 1).

In contrast to mutations that block SA-mediated defense responses, the *ein2* mutation did not suppress *edr1*-mediated resistance (Fig. 1). Mutations in *ein2* block all known ethylene-mediated responses (19) as well as most JA-mediated responses (20–22). Taken together, these data indicate that resistance mediated by the *edr1* mutation depends on SA-induced defense responses and is independent of JA- and ethylene-induced defenses.

Molecular Isolation of *EDR1*. We isolated the *EDR1* gene by using a combination of fine-structure genetic mapping and candidate gene analysis. The *edr1* mutant was crossed to wild-type *Arabidopsis* of the Landsberg *erecta* genotype and the resulting F2 population used for genetic mapping. This work localized *edr1* to an approximately 120-kb interval on chromosome 1 (Fig. 2*A*), which had been fully sequenced as part of the *Arabidopsis* genome project. Located within this region were several genes that were reasonable candidates to function as negative regulators of defense responses. Three candidate genes (a myb-like transcription factor, a superoxide dismutase, and a MAPKKK) were amplified from the *edr1* mutant by using PCR and were sequenced. Analysis of these sequences revealed a C→G transversion in the MAPKKK candidate (F22013.21) that produced

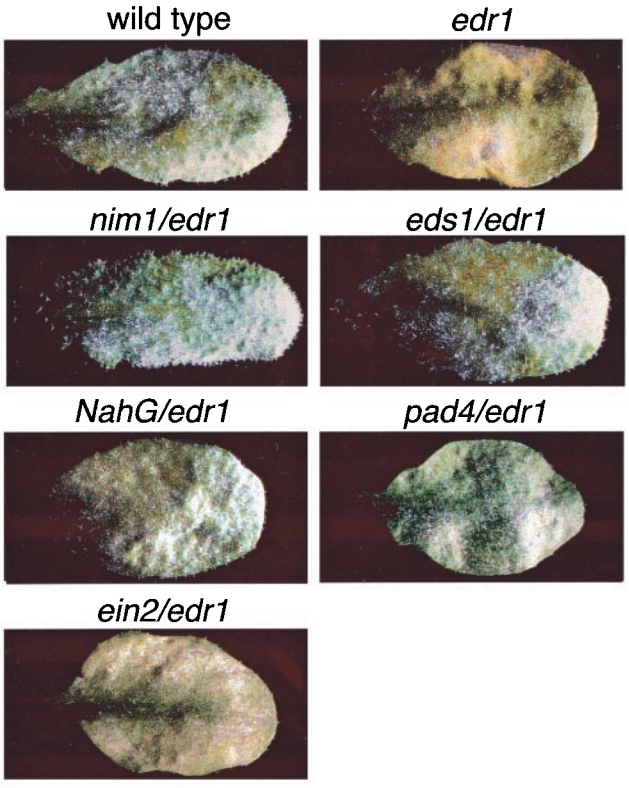

Fig. 1. Suppression of the *edr1* resistance phenotype by mutations that block SA-mediated defense responses. Double mutants containing *edr1* and the indicated mutations were constructed by standard genetic crosses. Plants were inoculated with *E. cichoracearum* and disease phenotypes scored 8 days after inoculation. Single representative leaves were removed from intact plants on the eighth day for photography. In the wild-type and double mutant plants, except for *ein2*/*edr1*, abundant asexual sporulation is observed as white patches. Also note the absence of yellow/brown necrotic patches observed in the *edr1* mutant leaf.

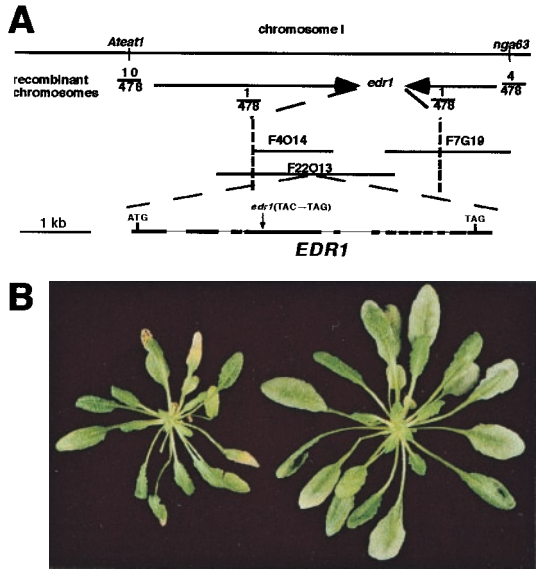

Fig. 2. Positional cloning of the *EDR1* gene. (*A*) Genetic and physical map of the region flanking *EDR1*. The vertical dotted lines indicate the position of the closest markers that defined the *edr1* genetic interval. Sequencing of three candidate genes in this interval revealed a C→G transversion in gene F22013.21, which produced an early stop codon. Comparison of the genomic sequence to the cDNA sequence obtained by reverse transcription–PCR revealed the indicated exon (boxes)-intron (lines) structure. (*B*) Complementation of the *edr1* mutation by Agrobacterium-mediated transformation. Shown are sibling plants from the T2 generation that lack the wild-type *EDR1* transgene (*Left*) or that contain the *EDR1* transgene (*Right*). The apparent difference in size is because of necrosis of the larger leaves in the *edr1* mutant (resistant) plant.

an early termination codon (Fig. 2*A*). No mutations were identified in the other two candidate genes.

To confirm that the MAPKKK gene corresponded to EDR1, we transformed two cosmid subclones of BAC F22013 (accession no. AC003981) into the *edr1* mutant (see *Materials and Methods*). Clone 1 contained a full-length *EDR1* gene, whereas clone 2 was truncated 76 bases 5′ of the *EDR1* stop codon but contained all other genes present on clone 1. Self progeny from kanamycin-resistant transformants were obtained and assayed for resistance to *E. cichoracearum.* Phenotypically wild-type (i.e., susceptible) plants were observed in 4 of 5 lines transformed with clone 1 (Fig. 2*B*), and the susceptible phenotype was shown to cosegregate with the T-DNA insert by PCR. No susceptible plants were observed with lines transformed with clone 2, indicating that the MAPKKK gene was responsible for complementation of the *edr1* mutant phenotype.

EDR1 encodes a putative MAPKKK consisting of a 276-aa C-terminal kinase domain and a 657-aa N-terminal nonkinase domain (Fig. 3*A*). The *edr1* mutation truncates the predicted EDR1 protein 265 amino acids before the kinase domain. RNA gel blot analysis showed that *EDR1* is expressed in uninoculated leaves but is moderately induced (≈5-fold) during infection by *E. cichoracearum* (Fig. 3*B*).

EDR1 Belongs to the CTR1 Family of MAPKKKs. Searches of the GenBank database by using the BLASTP and TBLASTN programs (23) uncovered five *EDR1* homologs in *Arabidopsis* with extensive sequence similarity to both the kinase and nonkinase regions (Fig. 3*C* and Fig. 6, which is published as supplemental data on the PNAS web site, www.pnas.org). The sequence similarity of EDR1 to these five putative kinases was very high in the kinase domain (>65% identity) but much lower in the nonkinase domain (≈25% identity with numerous gaps and insertions). Although the overall similarity among the nonkinase domains is low, there are several blocks of highly conserved sequence (see supplemental Fig. 6), suggesting a conserved function for this domain. No other *Arabidopsis* proteins displayed large blocks of amino acid sequence similarity to the nonkinase domain, indicating that EDR1 belongs to a distinct subfamily of MAPKKKs consisting of at least six members in *Arabidopsis*.

Of these six MAPKKKs, only CTR1 has been previously characterized. Loss-of-function mutations in *CTR1* confer a constitutive ethylene response phenotype (24); phenotypes include a *c*onstitutive *t*riple *r*esponse (a short fat hypocotyl and an exaggerated apical hook when grown in the dark), constitutive expression of ethylene inducible genes, and severe dwarfing. CTR1 thus functions as a negative regulator of the ethylene-response pathway.

EDR1 Does Not Directly Regulate Ethylene Responses. The high similarity between the CTR1 and EDR1 protein sequences led us to investigate whether *edr1* mutants display any constitutive ethylene responses. Unlike *ctr1* mutants, no visible effect on etiolation was observed, and plants were not stunted (data not shown). Furthermore, the ethylene-inducible genes *CHIB* and *HEL* were not constitutively expressed and displayed normal ethylene inducibility (data not shown). In addition, *edr1* seedlings displayed a normal triple response on exposure to 10 ppm ethylene (data not shown). These data, combined with the lack

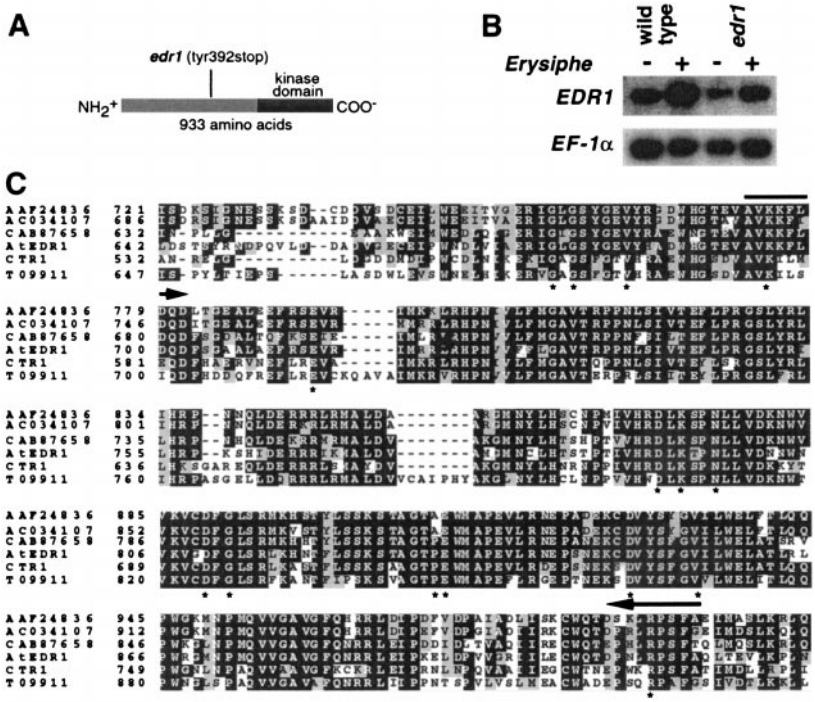

Fig. 3. EDR1 encodes a MAPKKK with high similarity to CTR1 and four additional kinases present in *Arabidopsis.* (*A*) The predicted EDR1 protein contains 933 amino acids, the carboxyl-terminal third of which contains a kinase domain. (*B*) RNA gel blot analysis of the *EDR1* mRNA. Wild-type and *edr1* mutant plants were inoculated with *E. cichoracearum* (+) or mock inoculated (−) and poly(A) RNA isolated from leaves 3 days after inoculation. The blot was hybridized with an *EDR1* cDNA probe corresponding to amino acid residues 180–461. Relative amounts of RNA loaded in each lane were estimated by using an *EF-1α* cDNA probe. PhosphorImager quantitation of this blot, normalized to the *EF-1α* signal, revealed that the *edr1* message is induced approximately 5-fold by infection with *E. cichoracearum*. This analysis was repeated three times with similar results. (*C*) Alignment of the kinase domains of the six known members of the *CTR1* kinase family in *Arabidopsis*. The entire protein for each of the indicated proteins was aligned by using the default parameters of CLUSTALX, a Macintosh version of the CLUSTALW program (47). Only the kinase domain is shown. The full-length alignment can be viewed in supplemental Fig. 6. The alignment file produced by CLUSTALX was formatted by using the BOXSHADE WWW server (http://www.ch.embnet.org/software/BOX_form.html). White type on a black background indicates a residue that is identical in at least half of the proteins shown, whereas a gray background indicates conservative substitutions as defined by the BOXSHADE 3.21 default parameters. Asterisks indicate residues that are conserved in nearly all protein kinases (48). Arrows indicate regions conserved in the *Arabidopsis*, tomato, and rice *EDR1* orthologs (see supplemental Fig. 6) but divergent in paralogs. Accession nos. for CTR1 and EDR1 are A45178 and AF305913, respectively. Accession no. AC034107 refers to the BAC DNA sequence, from which we derived the indicated protein sequence by using the EDR1 and CTR1 sequences as guides.

of suppression by the *ein2* mutation, indicate that *EDR1* functions in a pathway separate from the ethylene-response pathway.

In the course of investigating ethylene responses in *edr1* mutants, we observed that *edr1* plants displayed an enhanced ethylene-induced senescence response (Fig. 4 *A* and *B*). Exposure of 6-week-old wild-type *Arabidopsis* plants to 100 ppm ethylene induces chlorosis (yellowing) in the oldest 2 leaves starting after 3 days of ethylene exposure. However, in *edr1* mutant plants, this yellowing was visible on much younger leaves (Fig. 4*A*). Quantification of chlorophyll in leaves 3 through 8 (leaf 1 being the oldest true leaf) revealed an approximately 5-fold lower level of chlorophyll in ethylene-treated *edr1* plants compared with wild-type Col-0 (Fig. 4*B*). There was no significant difference between *edr1* and wild-type plants not exposed to ethylene.

The enhanced senescence response of *edr1* plants may reflect an overlap between the genetic control of senescence and SA-induced defense responses. The senescence process is regulated in part by SA, as plants containing *npr1*or *pad4* mutations or the *nahG* transgene display delayed yellowing and reduced necrosis during senescence (25). In addition, several defense related genes are known to be induced during leaf senescence, including the well-characterized defense gene *PR-1* (25, 26). Induction of *PR-1* by pathogens or during senescence is blocked by the expression of the *nahG* transgene, and by mutations in *NPR1* or *PAD4* (25, 27). We used RNA gel-blot analysis to examine expression of *PR-1* in ethylene-treated plants. *PR-1* was highly expressed in the *edr1* mutant plants after 3 days of ethylene exposure, whereas this message is undetectable in wild-type plants (Fig. 4*C*). This result is consistent with the enhanced senescence phenotype and suggests that both the enhanced disease-resistance phenotype and the enhanced-senescence phenotype may be related to enhanced signaling through the SA pathway.

Identification of *EDR1* Orthologs in Crop Species. Powdery mildew is an economically important disease on many monocot (e.g., wheat and barley) and dicot (e.g., tomato and grape) crops. The enhanced resistance conferred by the *edr1* mutation may thus provide a novel means of conferring resistance to this disease, provided that the *EDR1* pathway is conserved in crop plants. We therefore searched the available DNA and protein databases for homologs of EDR1. A BLASTP search of the GenBank nonredundant database identified a predicted protein sequence from tomato (TCTR2, accession no. AJ005077) that has high similarity to the *Arabidopsis* EDR1 protein over its full length (see supplemental Fig. 6), including 86% identity in the kinase domain. We also identified a partial cDNA sequence from rice in the dbEST database (accession no. D41138) that was 85% identical to the kinase domain of EDR1. We obtained a nearly full-length cDNA sequence for this rice gene by using 5′ and 3′-RACE methods (CLONTECH). Both the rice and tomato genes encode proteins that are more similar to the *Arabidopsis* EDR1 protein than are any of the 5 *Arabidopsis* EDR1 paralogs,

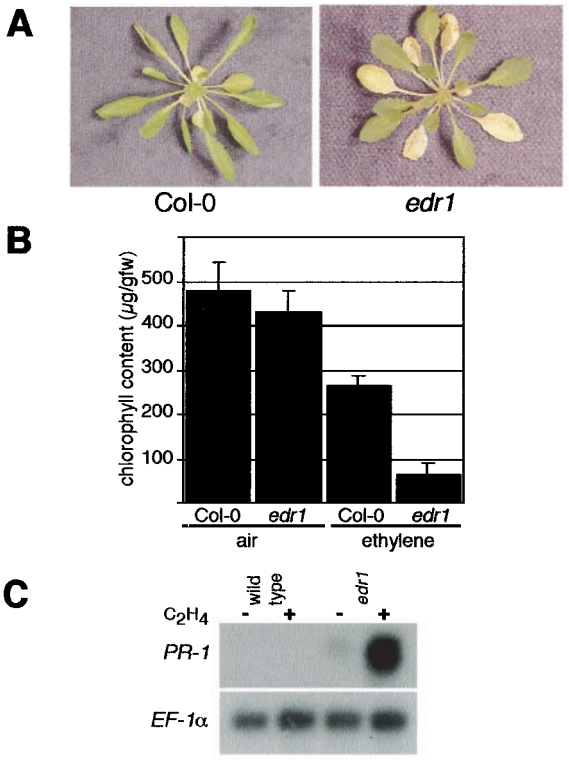

Fig. 4. Ethylene sensitivity of *edr1* mutant plants. (*A*) Ethylene-induced senescence. Wild-type and *edr1* mutant plants were grown in pots for 6 weeks in a growth cabinet set for 9-h days then placed in a sealed chamber containing 100 ppm ethylene. Plants were removed for photography after 72 h. Note the increase in the number of yellow leaves in the ethylene-treated *edr1* mutant. (*B*) Quantification of chlorophyll levels. Leaves 3 through 8 (leaf 1 being the oldest true leaf) were removed from 6-week-old plants after 72 h of exposure to 100 ppm ethylene and chlorophyll levels (microgram/gram fresh weight) measured as described (5). Bars represent the mean and standard deviation of values obtained from six plants. (*C*) Ethylene-induced expression of *PR-1*. RNA gel blots were performed with 10 μg of total RNA isolated from rosette leaves (both green and yellow) after 72 h of exposure to ethylene (+) or air (−). Blots were sequentially probed with *PR-1* and *EF-1α*. The latter demonstrates approximately equal RNA loading in all lanes.

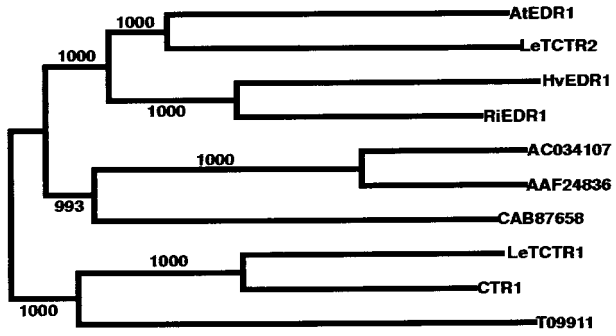

Fig. 5. Conservation of *EDR1* among diverse angiosperms. Full-length protein sequences were aligned by using the CLUSTALW program (see supplemental Fig. 6) and a phylogenetic tree derived by using the neighbor-joining method (47). Numbers indicate bootstrap values supporting branch points based on 1,000 replicates. T09911, CAB87658, ACO34107, and AAF24836 are *Arabidopsis* CTR1/EDR1 homologs of unknown function. Putative EDR1 and CTR1 orthologs are preceded by a two-letter prefix to indicate species of origin: Hv, *Hordeum vulgare* (barley), Ri, *O. sativa* (rice), Le, *Lycopersicon esculentum* (tomato), At, *A. thaliana*.

including CTR1, whose kinase domain is only 65% identical to EDR1 (Fig. 5). This relationship strongly suggests that the rice and tomato genes are orthologs of EDR1 and that this gene family came into existence before the divergence of monocots and eudicots. Consistent with this view is the presence of a second tomato sequence, TCTR1 (accession no. AF096250) that is most similar to the *Arabidopsis* CTR1 protein (Fig. 5). Furthermore, it indicates that EDR1 has been conserved during angiosperm evolution, presumably because it is performing a conserved function.

Alignment of the rice and tomato EDR1-like sequences with the six *Arabidopsis* homologs enabled us to identify regions that were conserved among the putative *EDR1* orthologs but divergent in the five *Arabidopsis* paralogs (indicated by arrows in Fig. 3*C* and supplemental Fig. 6). On the basis of these amino acid sequences, we designed degenerate oligonucleotide primers for amplification of EDR1 orthologs from other plant species (see *Materials and Methods*). Using these primers, we successfully isolated an EDR1 ortholog from barley that is highly similar to the rice EDR1 ortholog (86% identical over the kinase domain; Fig. 5 and supplemental Fig. 6). It should thus be possible to isolate EDR1 orthologs from most angiosperms.

Relationship of EDR1 to Other MAPKKKs. EDR1 and CTR1 belong to the Raf subfamily of MAPKKKs, one of three MAPKKK subfamilies found in *Arabidopsis.* The other two subfamilies have been designated AtMEKK1 and ANPs (28). The *AtMEKK1* family has been implicated in mediating touch/cold/drought signal transduction (29, 30), and the ANP family has recently been shown to respond to H_2O_2 and to negatively regulate auxin responses in protoplast assays (28). The role of these MAPKKKs in intact plants is poorly understood, however, as no mutations in endogenous genes have been described. To date, EDR1 and CTR1 are the only plant MAPKKKs with known mutant phenotypes. The observation that EDR1 and CTR1 function in distinct response pathways suggests that the remaining Raf-like MAPKKKs may function in as yet to be discovered pathways distinct from the ethylene and defense response pathways.

Yeast two-hybrid and *in vitro* analyses indicate that CTR1 physically interacts with the ethylene receptor protein ETR1 (31). On binding of ethylene to ETR1, the receptor is believed to be inactivated (32), which in turn inactivates CTR1, which then allows the ethylene response pathway to activate. How CTR1 negatively regulates ethylene inducible genes is not known; however, transient expression of the CTR1 kinase domain in maize protoplasts activates endogenous MAP kinases (33), suggesting that CTR1 functions at the top of a classical MAP kinase cascade that is conserved between monocots and eudicots.

A Model for How EDR1 Functions in Disease Resistance. Because EDR1 and CTR1 belong to the same family of MAPKKKs and because both function as negative regulators, it is tempting to speculate that EDR1 will also interact with an upstream receptor protein and will activate a MAP kinase cascade. By analogy to the CTR1 model, one would predict that a pathogen-associated signal (either induced by or produced by the pathogen) would inactivate this putative receptor, which would lead to inactivation of EDR1, which would then allow activation of SA-mediated defenses by a separate pathway. In essence, before SA-associated defenses are turned on, two signals must be perceived, one to remove the negative regulators (the EDR1 pathway) and one to activate positive regulators (e.g., EDS1/PAD4 pathways). Such a mechanism would reduce the likelihood of inappropriate deployment of defenses and would explain why *edr1* plants do not constitutively express defense responses. This mechanism would also explain why *eds1* and *pad4* mutations suppress *edr1* resistance.

The Relationship of *EDR1* to Known MAP Kinase Pathways. *Arabidopsis* contains at least nine MAP kinases (34), one or more of which could be part of the EDR1 pathway. To date, no mutant phenotypes have been ascribed to any MAP kinase gene of *Arabidopsis* or of any other plant. However, several plant MAP kinases have been identified that are activated by pathogen infection and by related signal molecules (35–41). For example, the tobacco MAP kinase SIPK is activated by tobacco mosaic virus infection, SA, by several pathogen-derived elicitors and by wounding (42–44). The tobacco WIPK MAP kinase is also activated by all of these stimuli except for SA (44, 45). For reasons described below, however, we do not believe that EDR1 directly regulates either WIPK- or SIPK-like MAP kinases in *Arabidopsis*.

Orthologs of WIPK and SIPK exist in *Arabidopsis* and have been designated AtMPK3 and AtMPK6, respectively (41, 46). Recently, Sheen and colleagues used a protoplast assay to show that both of these MAP kinases can be activated by the *Arabidopsis* MAPKKK ANP1 (28). Furthermore, this ANP1-dependent activation could be triggered by exogenous application of H_2O_2 but not by cold treatment or the plant hormones auxin and abscisic acid. Because ANP1 belongs to a different subclass of MAPKKKs than EDR1 and CTR1 (28), it is unlikely that EDR1 would also activate AtMPK3 or AtMPK6. This supposition is supported by the observation that AtMPK6 is activated within 2 min of bacterial elicitor application (41), suggesting that it positively regulates primary defense responses, whereas we have shown that EDR1 functions as a negative regulator (2).

Conclusions

We have shown that a loss-of-function mutation in the EDR1 MAPKKK causes enhanced disease resistance to powdery mildew infection of *Arabidopsis.* The *edr1* mutant phenotype indicates that this MAPKKK negatively regulates defense responses. This finding is somewhat unexpected, as there exists a large body of correlative data suggesting that MAP kinase activation is required for induction of plant defenses. We speculate that plant defense responses are regulated at least in part via antagonistic MAP kinase pathways. Because *EDR1* and many of the MAP kinases are well conserved in crop plants, it is expected that they function similarly in these species. A clear understanding of how the balance of signaling through these pathways is regulated may provide new approaches to engineering disease resistance in crops.

We thank P. Schulze-Lefert (the Sainsbury Laboratory) for providing cDNA from rice and barley, the Sainsbury Laboratory for providing research support to R.W.I. during a sabbatical visit, J. Parker (the Sainsbury Laboratory) for providing F2 seed from crosses between *edr1* and *pad4–1* and between *edr1* and *eds1–2*. J. Danzer, R. Cameron, S. Ludwig, A. Henk, and K. Christiansen for help with genetic mapping and DNA sequencing, and the *Arabidopsis* Biological Resource Center at Ohio State for providing *ein2–1* seed. This work was supported by National Institutes of Health Grant GM46451 and by Novartis Agribusiness Biotechnology Research, Inc.

1. Greenberg, J. T. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 525–545.
2. Frye, C. A. & Innes, R. W. (1998) *Plant Cell* **10,** 947–956.
3. Ryals, J. A., Neuenschwander, U. H., Willits, M. G., Molina, A., Steiner, H.-Y. & Hunt, M. D. (1996) *Plant Cell* **8,** 1809–1819.
4. Adam, L. & Somerville, S. C. (1996) *Plant J.* **9,** 341–356.
5. Lichtenthaler, H. K. & Wellburn, A. R. (1983) *Biochem. Soc. Trans.* **11,** 591–592.
6. Konieczny, A. & Ausubel, F. M. (1993) *Plant J.* **4,** 403–410.
7. Bell, C. J. & Ecker, J. R. (1994) *Genomics* **19,** 137–144.
8. Bancroft, I., Love, K., Bent, E., Sherson, S., Lister, C., Cobbett, C., Goodman, H. M. & Dean, C. (1997) *Weeds World* **4,** 1–9.
9. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
10. Clough, S. J. & Bent, A. F. (1998) *Plant J.* **16,** 735–743.
11. Ashfield, T., Danzer, J. R., Held, D., Clayton, K., Keim, P., Saghai Maroof, M. A., Webb, P. M. & Innes, R. W. (1998) *Theor. Appl. Genet.* **96,** 1013–1021.
12. Dong, X. (1998) *Curr. Opin. Plant Biol.* **1,** 316–323.
13. Schenk, P. M., Kazan, K., Wilson, I., Anderson, J. P., Richmond, T., Somerville, S. C. & Manners, J. M. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 11655–11660.
14. Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Ney, G., Uknes, S., Ward, E., Kessmann, H. & Ryals, J. (1993) *Science* **261,** 754–756.
15. Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S. & Ryals, J. (1995) *Mol. Plant–Microbe Interact.* **8,** 863–870.
16. Delaney, T. P., Friedrich, L. & Ryals, J. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6602–6606.
17. Falk, A., Feys, B. J., Frost, L. N., Jones, J. D., Daniels, M. J. & Parker, J. E. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 3292–3297.
18. Zhou, N., Tootle, T. L., Tsui, F., Klessig, D. F. & Glazebrook, J. (1998) *Plant Cell* **10,** 1021–1030.
19. Alonso, J. M., Hirayama, T., Roman, G., Nourizadeh, S. & Ecker, J. R. (1999) *Science* **284,** 2148–2152.
20. Penninckx, I. A., Thomma, B. P., Buchala, A., Metraux, J. P. & Broekaert, W. F. (1998) *Plant Cell* **10,** 2103–2113.
21. Thomma, B., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B. P. A. & Broekaert, W. F. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 15107–15111.
22. Thomma, B. P. H. J., Eggermont, K., Tierens, K. F. M.-J. & Broekaert, W. F. (1999) *Plant Physiol.* **121,** 1093–1101.
23. Altschul, S. F., Gish, W., Miller, W., E. W., M. & Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410.
24. Kieber, J. J., Rothenberg, M., Roman, G., Feldmann, K. A. & Ecker, J. R. (1993) *Cell* **72,** 427–441.
25. Morris, K., MacKerness, S. A., Page, T., John, C. F., Murphy, A. M., Carr, J. P. & Buchanan-Wollaston, V. (2000) *Plant J.* **23,** 677–685.
26. Hanfrey, C., Fife, M. & Buchanan-Wollaston, V. (1996) *Plant Mol. Biol.* **30,** 597–609.
27. Delaney, T. P., Uknes, S. J., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. & Ryals, J. (1994) *Science* **266,** 1247–1249.
28. Kovtun, Y., Chiu, W. L., Tena, G. & Sheen, J. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 2940–2945.
29. Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K. & Shinozaki, K. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 765–769.
30. Mizoguchi, T., Ichimura, K., Yoshida, R. & Shinozaki, K. (2000) *Results Probl. Cell Differ.* **27,** 29–38.
31. Clark, K. L., Larsen, P. B., Wang, X. & Chang, C. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5401–5406.
32. Hua, J. & Meyerowitz, E. M. (1998) *Cell* **94,** 261–271.
33. Kovtun, Y., Chiu, W. L., Zeng, W. & Sheen, J. (1998) *Nature (London)* **395,** 716–720.
34. Ligterink, W. (2000) in *MAP Kinases in Plant Signal Transduction*, ed. Hirt, H. (Springer, Heidelberg), pp. 11–27.
35. Liu, Y., Zhang, S. & Klessig, D. F. (2000) *Mol. Plant–Microbe Interact.* **13,** 118–124.
36. Romeis, T., Piedras, P., Zhang, S., Klessig, D. F., Hirt, H. & Jones, J. D. (1999) *Plant Cell* **11,** 273–287.
37. Schoenbeck, M. A., Samac, D. A., Fedorova, M., Gregerson, R. G., Gantt, J. S. & Vance, C. P. (1999) *Mol. Plant–Microbe Interact.* **12,** 882–893.
38. Takezawa, D. (1999) *Plant Mol. Biol.* **40,** 921–933.
39. Zhang, S., Du, H. & Klessig, D. F. (1998) *Plant Cell* **10,** 435–450.
40. Ligterink, W., Kroj, T., zur Nieden, U., Hirt, H. & Scheel, D. (1997) *Science* **276,** 2054–2057.
41. Nuhse, T. S., Peck, S. C., Hirt, H. & Boller, T. (2000) *J. Biol. Chem.* **275,** 7521–7526.
42. Zhang, S. & Klessig, D. F. (1997) *Plant Cell* **9,** 809–824.
43. Zhang, S. & Klessig, D. F. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 7225–7230.
44. Kumar, D. & Klessig, D. F. (2000) *Mol. Plant–Microbe Interact.* **13,** 347–351.
45. Seo, S., Sano, H. & Ohashi, Y. (1999) *Plant Cell* **11,** 289–298.
46. Zhang, S. & Klessig, D. F. (2000) in *Map Kinases in Plant Signal Transduction*, ed. Hirt, H. (Springer, Heidelberg), pp. 65–84.
47. Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680.
48. Hanks, S. K. & Quinn, A. M. (1991) *Methods Enzymol.* **200,** 38–62.