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Regulation of plant defense responses in Arabidopsis by EDR2, a PH and START domain-containing protein

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Summary

We have identified an Arabidopsis mutant that displays enhanced disease resistance (*edr2*) to the biotrophic powdery mildew pathogen *Erysiphe cichoracearum*. Inhibition of fungal growth on *edr2* mutant leaves occurred at a late stage of the infection process and coincided with formation of necrotic lesions approximately 5 days after inoculation. Double-mutant analysis revealed that *edr2*-mediated resistance is suppressed by mutations that inhibit salicylic acid (SA)-induced defense signaling, including *npr1*, *pad4* and *sid2*, demonstrating that *edr2*-mediated disease resistance is dependent on SA. However, *edr2* showed normal responses to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000. *EDR2* appears to be constitutively transcribed in all tissues and organs and encodes a novel protein, consisting of a putative pleckstrin homology (PH) domain and a steroidogenic acute regulatory protein-related lipid-transfer (START) domain, and contains an N-terminal mitochondrial targeting sequence. The PH and START domains are implicated in lipid binding, suggesting that EDR2 may provide a link between lipid signaling and activation of programmed cell death mediated by mitochondria.

Keywords

defense responses; disease resistance; powdery mildew; programmed cell death; salicylic acid; senescence

Introduction

Powdery mildews are biotrophic pathogens that infect a large number of plant species, causing significant economic losses in crops such as grape, wheat, and barley (Schulze-Lefert and Vogel, 2000). In Arabidopsis, the process of infection with the powdery mildew pathogen *Erysiphe cichoracearum* has been described in detail (Adam and Somerville, 1996). When *E. cichoracearum* infects Arabidopsis plants, spores first produce appressorial germ tubes, which penetrate the host epidermal cells, and then the fungus forms a bag-like haustorium inside the epidermal cells of the host. Haustoria are surrounded by host cell plasma membrane and function as feeding structures for the fungus. The fungus then develops secondary hyphae that grow along the leaf surface, forming secondary haustoria in adjacent epidermal cells. Conidiophores (chains of asexual spores) are formed 4–7 days after infection. By day 7, abundant conidiation is apparent and the conidiophores produce a powdery appearance for which the disease is named. Analysis of Arabidopsis–*E. cichoracearum* interactions in various Arabidopsis genotypes is providing new insights into how plants combat infection by biotrophic pathogens (Schulze-Lefert and Vogel, 2000).

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Resistance to powdery mildew among different Arabidopsis accessions is variable (Adam and Somerville, 1996; Adam *et al.*, 1999). Among them, Arabidopsis accession Moscow-0 (Ms-0) is highly resistant. *E. cichoracearum* cannot grow on Ms-0 leaves, but instead induces small lesions on the leaves consistent with induction of a hypersensitive resistance (HR) response. The resistance to *E. cichoracearum* on Ms-0 is mediated by RPW8, a small basic protein with a putative N-terminal transmembrane domain and a coiled-coil domain (Xiao *et al.*, 2001). Unlike most characterized disease resistance genes, *RPW8* mediates a broad-spectrum resistance conferring resistance to all tested isolates of four species of powdery mildew pathogens. The *RPW8*-mediated powdery mildew resistance requires pathways induced by salicylic acid (SA), but does not require pathways induced by jasmonic acid (JA) and ethylene (Xiao *et al.*, 2001, 2005). In contrast to Ms-0, Arabidopsis accession Columbia (Col-0) does not contain a functional *RPW8* gene and is susceptible to *E. cichoracearum*, displaying abundant conidiophores on mature leaves 7 days after infection.

To investigate the interaction between *E. cichoracearum* and Arabidopsis plants, a number of Arabidopsis mutants displaying enhanced disease resistance to powdery mildew have been identified (Frye and Innes, 1998; Vogel and Somerville, 2000; Vogel *et al.*, 2002, 2004). However, the mechanisms underlying these mutations mediating powdery mildew resistance appear to be very different. Among these mutants, the *edr1* mutant develops necrotic lesions at the site of infection and displays almost no visible powder on the leaves 8 days after inoculation. The fungal growth is inhibited at a very late stage and resistance appears to be caused by an accelerated activation of host defenses, including programmed cell death (PCD), suggesting that EDR1 is a negative regulator of plant defense (Frye and Innes, 1998). Further characterization demonstrated that *edr1*-mediated disease resistance is dependent on SA but independent of JA and ethylene (Frye *et al.*, 2001). Interestingly, the *edr1* mutation enhances transcription of an *RPW8.1* transgene in the Col-0 genetic background, and this enhanced expression correlates with spontaneous HR-like lesions (Xiao *et al.*, 2005), further establishing a link between *EDR1* and regulation of PCD.

EDR1 encodes a CTR1-like protein kinase, consisting of a putative regulatory N-terminal domain and a C-terminal kinase domain (Frye *et al.*, 2001). The EDR1 kinase domain alone displays kinase activity *in vitro* and overexpression of an EDR1 kinase-deficient protein causes dominant negative phenotypes that mimic the *edr1* mutant (Tang and Innes, 2002).

Unlike the *edr1* mutant, several other resistant mutants including *pmr1* to *pmr6* do not display a powdery mildew-induced lesion phenotype (Frye *et al.*, 2001; Vogel and Somerville, 2000; Vogel *et al.*, 2002, 2004). *PMR4* encodes a callose synthase responsible for producing callose in response to biotic and abiotic stresses (Nishimura *et al.*, 2003). Although the *pmr4* mutant is more resistant to powdery mildew, it produces less callose than wild-type (WT) Col-0 plants upon infection with *E. cichoracearum* (Nishimura *et al.*, 2003). The *pmr5*- and *pmr6*-mediated powdery mildew resistance does not require the SA pathway and defense responses in *pmr6* are not constitutively expressed (Vogel *et al.*, 2002, 2004). *PMR5* encodes a member of a large plant-specific gene family of unknown function, potentially involved in the regulation of cell wall composition (Vogel *et al.*, 2004). *PMR6* encodes a pectate lyase-like protein, which is thought to function as a susceptibility factor in Arabidopsis required for the growth of powdery mildew (Vogel *et al.*, 2002).

Many plant defense responses are regulated by pathways induced by the plant hormones SA, JA and ethylene (Dong, 1998). Several genes associated with SA-induced defense responses have been identified by genetic approaches. Mutations in EDS1 (Falk *et al.*, 1999; Parker *et al.*, 1996) and PAD4 (Glazebrook *et al.*, 1997; Jirage *et al.*, 1999) affect SA accumulation. Mutations in NPR1/NIM1 (Cao *et al.*, 1997; Delaney *et al.*, 1995; Ryals *et al.*, 1997) block SA-induced responses. Mutations in EDS5 (Nawrath *et al.*, 2002; Rogers and Ausubel,

1997) and SID2 (Wildermuth *et al.*, 2001) reduce SA production. All of these mutations compromise SA-induced defense responses against pathogen attack and enhance susceptibility to biotrophic pathogens. In contrast, mutants that constitutively accumulate high levels of SA are more resistant to a variety of biotrophic pathogens (Bowling *et al.*, 1994, 1997; Clarke *et al.*, 1998, 2000; Maleck *et al.*, 2002).

Besides the SA pathway, some defense responses are controlled by ethylene and JA pathways (Alonso *et al.*, 1999; Clarke *et al.*, 2000; Penninckx *et al.*, 1998; Staswick *et al.*, 1998; Thomma *et al.*, 1999). Among the mutants resistant to powdery mildew, *edr1*- and *pmr4*-mediated disease resistance requires SA but not ethylene, while *pmr5*- and *pmr6*-mediated resistance does not require SA, ethylene or JA (Nishimura *et al.*, 2003; Vogel *et al.*, 2002, 2004). Although both *edr1*- and *pmr4*-mediated resistance to powdery mildew are dependent on SA but independent of ethylene, their strategies for defense are largely different, as the *edr1* mutant displays significant callose accumulation 3 days after infection (Frye and Innes, 1998) while the *pmr4* mutant produces dramatically less callose in response to inoculation (Nishimura *et al.*, 2003).

In an effort to characterize the signaling pathway regulated by EDR1, and to possibly identify substrates of the EDR1 kinase domain, we screened for additional *edr1*-like mutants that displayed *E. cichoracearum*-induced lesions and a reduction in formation of conidia.

Results

Isolation of Arabidopsis mutants resistant to *E. cichoracearum*

To identify Arabidopsis mutants with enhanced disease resistance, we inoculated ethyl methane-sulfonate mutagenized Col-0 plants with the UCSC strain of *E. cichoracearum* and scored for disease responses 8 days after inoculation. Plants displaying no visible powder were selected. Approximately 12 000 M_2 Col-0 plants, derived from approximately 3000 M_1 parents, were screened, and mutants that displayed enhanced disease resistance were selected. Three mutants were identified that displayed strongly enhanced disease resistance in the M_3 generation. Here, we describe the characterization of one of these, *edr2*. Characterization of the other mutants will be described elsewhere.

Compared with Col-0 WT plants, *edr2* mutant plants were much more resistant to *E. cichoracearum*. Figure 1a shows that visible necrotic lesions formed on *edr2* leaves by 8 days after inoculation and little to no powder were produced. To further characterize the *edr2*-mediated resistance to powdery mildew, we monitored the development of *E. cichoracearum* and host cell death using trypan blue staining. Spores of *E. cichoracearum* germinated and produced appressorial germ tubes on *edr2* leaves and WT leaves 1 day after inoculation (data not shown). By 3 days after inoculation, *E. cichoracearum* developed extensive branched hyphae on both Col-0 and *edr2* leaves (data not shown). By 5 days, extensive hyphae nearly covered both Col-0 and *edr2* leaves (Figure 1b,c). However, many conidiophores formed on Col-0 leaves while significantly fewer formed on *edr2* leaves. By day 7, abundant conidiophores developed on Col-0 leaves while the number of conidiophores was largely reduced on the *edr2* leaves (Figure 1d,e). These observations demonstrated that the growth of *E. cichoracearum* was affected at a late stage of the infection process on *edr2* plants. Instead of producing abundant conidia, *edr2* mutants displayed large patches of dead mesophyll cells 5 days after infection (Figure 1c) with cell death becoming dramatic by 7 days after infection (Figure 1e). This massive mesophyll cell death was not observed on Col-0 leaves (Figure 1d).

The above phenotypes are highly similar to those of the *edr1* mutant (Frye and Innes, 1998). F_1 plants derived from a cross between the *edr1* and *edr2* mutant displayed WT susceptibility

to *E. cichoracearum*, however, indicating that these mutations are in different genes. We therefore proceeded with detailed characterization of the *edr2* mutant phenotype and isolation of the *EDR2* gene.

The *edr2* mutant displays normal responses to *Pseudomonas syringae* pv. *tomato* strain DC3000

To determine whether the *edr2* mutation mediates a broad-spectrum disease resistance, we challenged *edr2* mutant plants with the bacterial pathogen *P. syringae* pv. *tomato* strain DC3000 with or without the avirulence gene *avrRpt2*. Col-0 WT plants are susceptible to strain DC3000 and resistant to DC3000 (*avrRpt2*). We observed no significant differences in bacterial growth between WT and *edr2* mutant plants, nor any differences in symptoms (data not shown). These data suggest that EDR2 does not play a role in regulating disease resistance against virulent or avirulent *P. syringae* strains.

PR-1 gene expression is enhanced in *edr2* plants

To determine whether the *edr2* mutation affects SA-induced gene expression, we monitored mRNA levels of the defense gene *PR-1* at various time points after inoculation with *E. cichoracearum*. As shown in Figure 2, no or very little *PR-1* transcript was detected prior to inoculation. *PR-1* expression gradually increased after infection in both WT and *edr2* mutant plants. By 3 days after inoculation, however, higher levels of *PR-1* expression were observed in *edr2* plants than in WT plants. The *PR-1* expression was further increased at day 5 after inoculation in both WT and *edr2* mutant plants; however, there was no observable difference between wild type and *edr2*. These data suggest that SA-induced defenses are more rapidly induced in the *edr2* mutant than in WT Col-0 plants upon infection with *E. cichoracearum*.

Enhanced disease resistance mediated by *edr2* is dependent on SA signaling, but not JA or ethylene signaling

To gain more insight into how EDR2 regulates defense responses in Arabidopsis, we assessed the roles of SA, ethylene and JA in *edr2*-mediated disease resistance using double-mutant analysis. A mutation in *NPR1* (*nim1-1*), which reduces responsiveness to SA (Delaney *et al.*, 1995), and mutations in *PAD4* (*pad4-1*) and *SID2* (*sid2-2*), which reduce levels of pathogen-induced SA (Nawrath and Metraux, 1999; Zhou *et al.*, 1998), suppressed *edr2*-mediated enhanced resistance to powdery mildew (Figure 3), indicating that this phenotype is dependent on SA. In contrast, mutations in *EIN2* (*ein2-1*) and *COI1* (*coi1-1*), which block all known ethylene and JA responses, respectively (Alonso *et al.*, 1999; Feys *et al.*, 1994), did not suppress the *edr2*-mediated resistance (Figure 3), indicating that *edr2*-mediated resistance to powdery mildew does not require ethylene- and JA-induced defense responses.

The *edr2* mutant displays an enhanced ethylene-induced senescence phenotype

In addition to resistance to powdery mildew, the previously identified *edr1* mutant also displays an enhanced ethylene-induced senescence phenotype (Frye *et al.*, 2001). To test whether the *edr2* mutant also has this trait, we exposed *edr2* plants to ethylene (100 μl l^{-1}) for 3 days. Interestingly, *edr2* plants displayed an enhanced senescence phenotype indistinguishable from *edr1* plants (Figure 4a). Ethylene induced visible chlorosis (yellowing) on the oldest two leaves of WT Col-0 plants after 3 days' exposure to ethylene. However, in *edr2* mutant plants, chlorosis occurred on much younger leaves (Figure 4a). Quantification of chlorophyll levels revealed significant differences between ethylene-treated WT Col-0 and *edr2* mutant plants (Figure 4b).

The *edr1* and *edr2* mutations are not additive

The *edr2* mutant phenotypes are very similar to those of the previously identified *edr1* mutant in response to both *E. cichoracearum* and ethylene (Frye and Innes, 1998; Frye *et al.*, 2001). To gain more insight into the relationship between the *edr1* and *edr2* mutations, we crossed *edr1* with *edr2* and characterized the phenotypes of the *edr1*/*edr2* double mutant. The double mutant displayed a resistant phenotype similar to that of *edr1* and *edr2* single mutants when inoculated with *E. cichoracearum*, including a lack of conidia formation and development of similar necrotic lesions (data not shown). We also assayed the *edr1*/*edr2* double mutant for its response to ethylene. Figure 4 shows that the double mutant displayed an enhanced ethylene-induced senescence phenotype similar to that of *edr1* and *edr2* single mutants. The similar phenotypes of *edr1* and *edr2* in response to both powdery mildew and ethylene and the observation that *edr1* and *edr2* do not display additive or synergistic effects suggest that *EDR1* and *EDR2* may function in the same signal transduction pathway.

Genetic mapping and identification of *EDR2*

Genetic mapping was accomplished using an F_2 population derived from a cross between the *edr2* mutant (Col-0 genotype) and Landsberg *erecta* (L*er*). Genomic DNA was isolated from 46 resistant F_2 plants and scored with published simple sequence length polymorphism (SSLP) markers. This initial mapping localized *edr2* between molecular markers T6K21 and M4I22 on chromosome 4. We then developed our own molecular markers at intervals between these two markers using Monsanto Col-0 and L*er* polymorphism data (http://www.arabidopsis.org/Cereon/index.jsp). Five hundred and four resistant F_2 plants representing 1008 meioses were scored. Ultimately, *edr2* was localized between bacterial artificial chromosome (BAC) end sequence F13C5 (GenBank accession AL021711) and an internal sequence of BAC clone T18B16 (GenBank accession AL021687) (Figure 5). This analysis defined a 120 kb region containing 28 predicted genes that co-segregated with *edr2*.

To identify the *EDR2* gene, we constructed a cosmid library using F13C5 BAC DNA. Twelve cosmid clones that overlapped and covered the whole BAC were identified and used to rescue the *edr2* phenotype. Among the 12 clones, only cosmid clone 3 complemented the *edr2* mutation (Figure 5b). This clone contained two full-length genes, *At4g19030* (encodes a nodulin-26-like protein) and *At4g19040* (encodes an unknown protein). No mutation was found in *At4g19030*, but a C → G transversion was found in *At4g19040*, which caused an early stop (P246stop) in the predicted open-reading frame (ORF). Taken together, these data indicated that *EDR2* corresponds to *At4g19040*.

To confirm that *At4g19040* is *EDR2*, and to identify more *edr2* alleles, we obtained four T-DNA insertion lines (Alonso *et al.*, 2003) from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH, USA). We identified homozygous plants for each T-DNA insertion line, confirmed the location of insertion sites by direct sequencing of flanking PCR products (Figure 6a), and then tested their resistance to *E. cichoracearum*. All four lines displayed the *edr2*-like phenotype, including a lack of visible powder and dramatic lesion production 8 days after inoculation (data not shown).

Overexpression of *EDR2* complements the edr2 mutant phenotype

To gain more insight into how EDR2 regulates the defense responses of plants, we overexpressed a full-length *EDR2* gene in both the WT and *edr2* background under control of a cauliflower mosaic virus (CaMV) 35S promoter. Thirty independent T_1 transgenic plants from WT or *edr2* were inoculated with *E. cichoracearum*. There were no significant differences between WT plants and the WT plants carrying the 35S::*EDR2* transgene. However, 27 out of 30 35S::*EDR2* transgenic plants in the *edr2* background displayed a WT-like phenotype. These transgenic plants were susceptible to *E. cichoracearum* and showed extensive conidiation and

no necrotic lesions 8 days after infection, demonstrating that the 35S::*EDR2* construct complemented the *edr2* mutation (Figure 5b). The transgene did not cause any growth phenotype, as all transgenic lines were indistinguishable from WT plants prior to inoculation (data not shown).

Analysis of *EDR2* expression

To gain insight into *EDR2* expression, we searched Arabidopsis microarray data available through the AtGeneExpress Web interface (http://www.arabidopsis.org/info/expression/atgenexpress.jsp) for *EDR2* expression. Microarray analyses showed that *EDR2* is expressed in various tissues and organs at all developmental stages. To investigate *EDR2* expression more directly, we expressed a GUS reporter gene in WT Arabidopsis plants under control of the *EDR2* promoter, which was assumed to be contained within a 1040 base-pair fragment 5′ to the *EDR2* start codon. We obtained a number of *EDR2* ::GUS transformants and analyzed a total of 20 transgenic lines. Figure 7 shows typical GUS staining found in all plants analyzed. Consistent with the microarray data, GUS staining was observed in all tissues and organs tested, including leaves, roots, flowers, stems and siliques, demonstrating that *EDR2* is ubiquitously expressed.

To investigate whether *EDR2* is induced by *E. cichoracearum*, we conducted real-time RT-PCR analysis to examine *EDR2* expression. The time course of *EDR2* transcription was determined in Col-0 plants inoculated with *E. cichoracearum*. The levels of *EDR2* transcription were not significantly affected by infection with powdery mildew (data not shown).

EDR2 encodes a novel protein containing a PH and a steroidogenic acute regulatory protein-related lipid-transfer (START) domain

EDR2 is predicted to encode an unknown protein of 718 amino acid residues with a mass of 77.6 kDa and an isoelectric point (pI) of 7.18. The annotation data predicted that *EDR2* consists of 22 exons and 21 introns (Figure 6a). To confirm this prediction, we isolated RNA from WT Col-0 plants and performed RT-PCR followed by direct sequencing of the PCR product. The sequence data confirmed the predicted splice sites.

Sequence analysis indicates that EDR2 contains a pleckstrin homology domain (PH), a START domain, and a DUF1336 domain (PFAM accession nos PF00169, PF01852 and PF07059, respectively) (Figure 6b). The PH domain is approximately 120 amino acid residues and was first identified in pleckstrin (Haslam *et al.*, 1993; Mayer *et al*., 1993). Several PH domains are known to function as lipid-binding domains, and facilitate membrane localization (Maffucci and Falasca, 2001).

The START domain is about 200 amino acids long and has also been implicated in lipid binding (Soccio and Breslow, 2003). It is found in many signaling proteins. The START domain is believed to have important roles in lipid transport, lipid metabolism and cellular signaling (Soccio and Breslow, 2003).

The DUF1336 domain is a plant-specific domain of unknown function and is approximately 250 amino acids in length. Four other Arabidopsis proteins, At5g45560, At3g54800, At2g18320 and At5g35180, contain PH, START and DUF1336 domains, but none of these proteins have a known function.

We also analyzed the EDR2 protein sequence for potential subcellular targeting signals. Both the iPSORT algorithm (Bannai *et al.*, 2002; http://hc.ims.u-tokyo.ac.jp/ipsort/) and the TARGETP algorithm (Emanuelsson *et al.*, 2000; http://www.cbs.dtu.dk/services/targetp/) identified a probable mitochondrial targeting peptide at the N-terminus of EDR2, suggesting that EDR2 may function inside mitochondria.

The C-terminal DUF1336 domain of EDR2 is highly conserved among homologs in other plant species

To determine whether EDR2 is conserved among plant species, we performed a BLAST search of the Institute for Genome Research (TIGR) plant gene indices database (http://www.tigr.org/tdb/tgi/ego/orth_search.shtml) using the full-length EDR2 protein sequence as a query. We identified highly similar proteins in many plant species, including several distantly related species such as rice and barley. Alignment of EDR2 with its homologs revealed that the EDR2 C-terminal DUF1336 domain is particularly well conserved (about 80% identical to the barley and rice homologs; Figure 6c), suggesting that this domain is critical to the function of EDR2-like proteins, and that EDR2 may play a fundamental and conserved role in the regulation of plant defense responses and cell death.

Discussion

Because loss-of-function mutations in the *EDR2* gene confer enhanced disease resistance to powdery mildew, EDR2 probably functions as a negative regulator of powdery mildew resistance. Loss of EDR2 may lower the threshold of activation for host defenses such as PCD and PR gene expression. In this scenario, *E. cichoracearum* normally activates host defenses only weakly, but in the *edr2* mutant defense responses are induced more rapidly and to a greater level.

The *edr2* mutant phenotypes are very similar to *edr1* mutants. Both display enhanced resistance to powdery mildew and enhanced ethylene-induced senescence. Furthermore, both *edr1*- and *edr2*-mediated resistances require pathways induced by SA, but not by JA or ethylene. In addition, the *edr1*/*edr2* double mutant displays phenotypes indistinguishable from *edr1* and *edr2* single mutants. Combined, these observations strongly suggest that EDR1 and EDR2 may function in the same pathway(s) to regulate senescence and cell death.

EDR2 encodes a novel protein containing a PH and a START domain. The PH domain was first identified in pleckstrin, the major substrate of protein kinase C in platelets (Haslam *et al.*, 1993; Mayer *et al.*, 1993). The PH domain occurs in a wide range of proteins involved in intracellular signaling, cytoskeletal organization, membrane transport and modification of phospholipids (Lemmon *et al.*, 2002; Rebecchi and Scarlata, 1998). In the human genome, 252 different PH domain-containing proteins have been found (Consortium, 2001). In *Saccharomyces cerevisiae*, 33 different proteins with PH domains have been identified (Yu *et al.*, 2004). To date, a number of PH domain structures have been solved by nuclear magnetic resonance and X-ray crystallography. Despite the low sequence similarity among different PH domains, the three-dimensional structure of the PH domain is remarkably conserved (Maffucci and Falasca, 2001).

Although a large number of PH domains have been identified in different genomes, the function of PH domains is not yet clear and may vary from one protein to another (Yu *et al.*, 2004). When it was first identified, the PH domain was thought to be a protein-binding domain (Maffucci and Falasca, 2001), and several protein ligands have been identified, such as the beta/gamma subunit of heterotrimeric G proteins, WD40 repeat-containing proteins and tyrosine kinase (Maffucci and Falasca, 2001). The best-known feature of PH domains, however, is their ability to bind to phospholipids, such as phosphoinositides or inositol phosphates (Lemmon, 2003). Phospholipid binding is believed to play an important role in targeting PH domain-containing proteins to cellular membranes. To date, the best-characterized PH domains are from the phospholipase C (PLC) family of proteins. PLC enzymes hydrolyze phosphatidylinositol 4,5- bisphosphate (PIP2), a key regulator of several cellular processes. The products of the hydrolysis are two second messengers 1,4,5-trisphosphate and diacylglycerol, which regulate release of Ca^{2+} from intracellular stores and

activate protein kinase C (Philip *et al.*, 2002). By analogy to these proteins, the PH domain of EDR2 may function in subcellular localization of EDR2 via lipid-binding or protein– protein interactions.

In addition to the PH domain, EDR2 contains a START domain. The START domain is a lipid/sterol-binding domain first found in StAR (steroidogenic acute regulatory protein), which transfers cholesterol to the inner mitochondrial membrane in steroid-hormone-producing cells (Stocco, 2001). Proteins with START domains can bind various ligands such as sterols (StAR protein) and phosphatidylcholine (PC-TP) (Ponting and Aravind, 1999; Soccio and Breslow, 2003). In multiple-domain proteins, ligand binding by the START domain can regulate the activities of other domains that co-occur with the START domain, such as Rho-gap, the homeodomain and the thioesterase domain. In the human and mouse genomes, 15 genes have been identified that encode START domains (Soccio and Breslow, 2003). In Arabidopsis, there are 35 START domain-containing genes, 21 of which are fused to homeodomains, suggesting important roles for these START-domain containing proteins in plant development (Ponting and Aravind, 1999; Schrick *et al.*, 2004).

Proteins containing both a PH domain and START domain are rare; however, the human ceramide transport protein CERT contains a PH and a START domain (Hanada *et al.*, 2003). CERT mediates the intermembrane transfer of ceramide from the endoplasmic reticulum to the Golgi apparatus. The START domain of CERT specifically binds ceramide, while the PH domain targets the Golgi apparatus by binding to phosphatidylinositol-4-monophosphate (PtdIns4p). In addition, the remaining middle region of CERT contains a motif for targeting to the endoplasmic reticulum (Loewen *et al.*, 2003). Ceramide has been shown to regulate various cellular processes (Hannun and Luberto, 2000; Mathias *et al.*, 1998). Interestingly, a mutation in a ceramide kinase, ACD5, in Arabidopsis leads to spontaneous cell death, indicating that ceramide plays an important role in modulating PCD in Arabidopsis (Liang *et al.*, 2003). By analogy, EDR2 may function in a way similar to CERT, mediating the intermembrane transfer of a lipid signal molecule such as ceramide to regulate defense responses and cell death.

Many studies have implicated lipid signaling in disease resistance. For instance, the EDS1 and PAD4 proteins, two positive regulators of SA signaling, contain lipase-like domains (Falk *et al.*, 1999; Jirage *et al.*, 1999). Significantly, mutations in EDS1 and PAD4 compromise *edr1*-mediated resistance to powdery mildew (Frye *et al.*, 2001). In addition, a mutation in *DIR1*, which encodes a putative apoplastic lipid transfer protein, abolishes induction of systemic acquired resistance, although the *dir1* mutant exhibits WT local resistance, indicating that a lipid signal may be involved in systemic acquired resistance (Maldonado *et al.*, 2002). Furthermore, a mutation in *SSI2*, which encodes a stearoyl-ACP desaturase, suppresses the *npr1* mutation and displays constitutive *PR* gene expression, spontaneous lesions and enhanced resistance to *Peronospora parasitica* (Kachroo *et al.*, 2001; Shah *et al.*, 2001). Several of these *ssi2*-mediated phenotypes, including constitutive *PR* gene expression, are suppressed by mutations in the *FAD6* or *SFD1* genes. *FAD6* encodes a plastidic ω6-desaturase that is involved in the synthesis of lipids containing polyunsaturated fatty acids (Nandi *et al.*, 2003), while *SFD1* encodes a putative dihydroxyacetone phosphate reductase and may play an important role in glycerol lipid metabolism (Nandi *et al.*, 2004). In another report, both SA- and JA-mediated phenotypes of *ssi2* plants are restored by a mutation in glycerol-3-phosphate dehydrogenase (Kachroo *et al.*, 2004). These findings demonstrate that lipid signaling may interact with the SA pathway and play an important role in defense responses and PCD. EDR2 may be an important component that connects SA and lipid signaling.

Both iPSORT and TARGETP predict that mitochondria are the target for EDR2. If true, this would provide an intriguing functional link between lipid signaling, mitochondria and PCD in

plants. In animals, mitochondria play a central role in integrating cellular stress signals in the activation of PCD (Ferri and Kroemer, 2001). Release of cytochrome *c* from mitochondria leads to the activation of caspases, a family of cysteine proteases that serve as the essential switch for most forms of PCD in animal cells (Green, 2000). Release of cytochrome *c* from mitochondria in animal cells is regulated by several different proteins including Bax, which associates with the outer mitochondrial membrane and modifies its permeability (Ferri and Kroemer, 2001). Although plants do not contain a recognizable homolog of Bax, expression of murine Bax in plant cells induces PCD and this PCD is correlated with targeting of Bax to mitochondria (Lacomme and Santa Cruz, 1999), suggesting that release of mitochondrial proteins and/or loss of mitochondrial membrane potential may be key activators of PCD in plant cells. Indeed, loss of mitochondrial membrane potential has recently been shown to be an early indicator of PCD in Arabidopsis protoplasts induced by diverse stimuli, including ceramide (Yao *et al.*, 2004). It will be interesting to determine whether the *edr2* mutation affects mitochondrial function, particularly the permeability of the mitochondrial outer membrane.

Regardless of the mechanism of *edr2*-mediated disease resistance, the *EDR2* gene identified in this study may serve as an important entry point for understanding the function of plant PH and START domains and possible links between lipid signaling, mitochondria and the activation of PCD in plants.

Experimental procedures

Plant growth

Plants (*Arabidopsis thaliana*) were grown in growth rooms under 9 h light/15 h dark cycles at 22–24°C as described previously (Frye and Innes, 1998).

Powdery mildew infections

Erysiphe cichoracearum strain UCSC1 was maintained by growing on hyper-susceptible *pad4-1* mutant plants. To inoculate plants, diseased *pad4-1* plants (8–10 days after inoculation) were used to brush healthy 4–6-week-old plants to pass conidia (asexual spores) onto new plants. The disease phenotype was scored 8 days after inoculation. Fungal structures and dead plant cells were stained using alcoholic trypan blue (Koch and Slusarenko, 1990). Samples were observed and photographed using a Nikon e800 microscope.

Mutant screening

Mutagenized Col-0 plants (M_2 generation) were inoculated with *E. cichoracearum* and scored for disease responses 8 days after inoculation. Plants displaying no visible powder were selected and allowed to set seeds. Approximately 12 000 M_2 plants from ethyl methylsulfonate-mutagenized Col-0 plants, derived from 3000 M_1 parents, were screened.

P. syringae infections

Plants were grown in a growth room under 9 h light/15 h dark cycles (150 μmol m^{-2} sec^{-1} of light) at 22–24°C. Plants that were 4–6 weeks old were inoculated by dipping leaves in a suspension of 2×10^8 colony-forming units ml^{-1} of strain DC3000 or DC3000 carrying *avrRpt2* suspended in 10 mM $MgCl_2$ supplemented with 250 μl l^{-1} L77 Silwet (OSI Specialties; Danbury, CT, USA). Inoculated plants were covered with a dome for 2 days. Disease symptoms were scored 3 days after inoculation. To monitor bacterial growth inside plant leaves, leaf samples were removed from plants using a number 2 cork borer (three discs per sample) and macerated in 200 μl of 10 mM $MgCl_2$. Dilutions were made in 10 mM $MgCl_2$ and plated on trypticase soy agar containing 50 mg l^{-1} kanamycin sulfate. Colonies were counted 48 h after incubation at 30°C.

Ethylene-induced senescence assay

Five-week-old plants were placed in a sealed chamber containing 100 μl l^{-1} ethylene for 3 days. Leaves five to eight (leaf one being the oldest true leaf) were removed and chlorophyll was extracted and measured as previously described (Frye *et al.*, 2001).

Cosmid library construction

Bacterial artificial chromosome clone F13C5 (GenBank accession AL021711) was obtained from the ABRC. The binary vector pCLD04541 (Bancroft *et al.*, 1997) and F13C5 BAC DNA were isolated using the Hi-Speed kit from Qiagen (Valencia, CA, USA) following the manufacturer's protocol. F13C5 BAC DNA was partially digested with the restriction enzyme *Sau*3A and ligated to *Bam*HI-digested pCLD04541. The ligation mix was packaged using a GigapackIII XL packaging extract (Stratagene, La Jolla, CA, USA) and transfected into *Escherichia coli* strain DH5α. Positive clones were selected on Luria–Bertani (LB) agar medium with 10 mg l^{-1} tetracycline.

Assembly of cosmid contigs

Overlapping cosmid clones were identified by PCR-based library screening with specific primer pairs derived from internal sequences of BAC F13C5. A total of 12 cosmid clones that covered all of BAC F13C5 were selected. These cosmids were purified using a plasmid miniprep kit (Qiagen) and transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Positive *Agrobacterium* clones were selected on 50 mg l^{-1} kanamycin and further confirmed by PCR using specific primer pairs.

Complementation of the *edr2* mutation

Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected by growing on half-strength Murashige and Skoog salts plus 0.8% agar and 50 mg l^{-1} kanamycin. Transformants were transplanted to soil 7 days after germination and were inoculated with *E. cichoracearum* when 5 weeks old. Disease resistance was scored 8 days after inoculation. The cosmid that complemented the *edr2* mutant phenotype was analyzed by sequencing of the junctions between the insert and vector using the flanking primers T3 and T7. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 3730 automated sequencer.

DNA sequence analysis of Arabidopsis *EDR2*

We amplified the intact *EDR2* ORF by PCR using an Arabidopsis Col-0 cDNA library (Frye *et al.*, 2001) as template. The PCR product was sequenced directly using the ABI BigDye Terminator Cycle Sequencing Kit. DNA sequences were assembled using the SEQUENCHER program (Gene Codes; Ann Arbor, MI, USA). Our experimentally determined cDNA sequence was identical to the predicted full-length coding sequence for EDR2 (GenBank accession NM118022).

Construction of double mutants

Double mutants were created by standard genetic crosses. The *edr1*, *edr2*, *pad4-1*, *sid2-2* and *ein2-1* mutations were all in the Col-0 genotype of Arabidopsis, while *npr1* (*nim1-1*) was in the Ws genotype and *coi1-1* was in the Col-6 genotype. The double mutant of *npr1*/*edr2* was identified by PCR-based molecular marker screening of F_2 progeny. To identify *edr1*/*edr2*, *pad4-1*/*edr2*, *sid2-2*/*edr2*, *coi1- 1*/*edr2*, *ein2-1*/*edr2* double mutants, we used PCR to amplify the respective genes followed by direct sequencing to identify plants that were homozygous for the mutations. All double mutants were verified to contain the *edr2* mutation using a cleaved

amplified polymorphic sequence (CAPS) marker designed to detect the *edr2* mutation. CAPS primers (5′-AGACAAGAACCATCATTATAGTGCTA- 3′ and 5′-AACAACACAACTTCACAGAAAGAGCA-3′) were used for PCR amplification and the PCR product was digested by *Bsm*AI to detect the *edr2* mutation.

Identification of *EDR2* T-DNA insertion mutants

Four T-DNA insertion lines were ordered from the ABRC (Salk-010966, Salk-048099, Salk-052496 and Salk-080753). Seeds from each line were sowed and 5-week-old plants were inoculated with *E. cichoracearum*. Defense responses were scored 8 days after infection. All four lines segregated for resistant and susceptible phenotypes. To confirm the phenotype, three plants from each line displaying a resistant phenotype were selected and self-fertilized, and the self-progeny tested for resistance to powdery mildew. T-DNA insertion sites, shown in Figure 7a, were confirmed by PCR amplification using a gene-specific primer and a T-DNA left border primer followed by direct DNA sequencing of the PCR products.

RNA gel blot hybridization

Total RNA was isolated from Arabidopsis leaf tissue using an RNeasy Mini Kit (Qiagen). A total of 5 μg of RNA was separated on a denaturing formaldehyde–agarose gel and transferred to Hybond N nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). RNA gel blots were hybridized with a [^{32}P]-labeled *PR-1* DNA probe and washed at 65°C using Church buffer (Ashfield *et al.*, 1998).

RT-PCR analysis

Plants were grown and inoculated with *E. cichoracearum* as described above. Leaves were removed from plants at different time points. Total RNA was isolated using the RNeasy kit. First strand cDNA from 2 μg of total RNA was synthesized using a SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed using *EDR2*-specific primers (5′-ATGTCTAAGGTAGTGTACGAAGG- 3′ and 5′-GTCCTCTTCATCCTCTGCCGCA- 3′). A tubulin gene (*At5g19770*) was used as a control for normalizing the amount of cDNA using the following primers: 5′-GCGAGAAATCATAAGCAT-3′ and 5′-ACCATCAAACCTCAAAGA-3′.

Overexpression of *EDR2*

EDR2 full-length cDNA was amplified by PCR using primers that incorporated restriction sites for *Nhe*I and *Xho*I. The PCR products were cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). The EDR2 cDNA was then excised from pGEM-T Easy and inserted into *Xba*I- and *Xho*I-digested pBI1.4t vector, which contains a modified 35S CaMV promoter (Leister *et al.*, 1996). The construct was verified by sequencing and transformed into *Agrobacterium* strain GV3101 by electroporation. Plant transformation, transgenic plant selection and phenotyping were performed as described above.

Construction of the EDR2 promoter::GUS reporter and GUS activity assay

A 1040 bp promoter fragment of *EDR2* was amplified by PCR from genomic DNA of WT Col-0 using primers containing *Xba*I and *Bam*HI restriction sites: (5′-AAGGTCTAGACAAAACCCAAATCCTCTGTCCAAT- 3′ and 5′-ACTTGGATCCCTGTCCCCAGAAATTACAAAAAATCT- 3′). The PCR product was digested with *Xba*I and *Bam*HI and inserted into the pCB308 vector (Xiang *et al.*, 1999). The clone was verified by sequencing and transformed into *Agrobacterium* strain GV3101 by electroporation. Plant transformation was conducted as described above. GUS activity analysis was performed as described (Jefferson *et al.*, 1987). Samples were observed and photographed using a Nikon SMZ1500-dissecting microscope.

Protein sequence alignments

EDR2 protein homologs were identified by searching the GenBank and TIGR databases using the BLASTP program (Altschul *et al.*, 1997). Putative EDR2 orthologs were identified in the TIGR website (http://www.tigr.org/tdb/tgi/ego/orth_search.shtml). Protein alignments were performed using CLUSTAL X with manual corrections (Thompson *et al.*, 1997).

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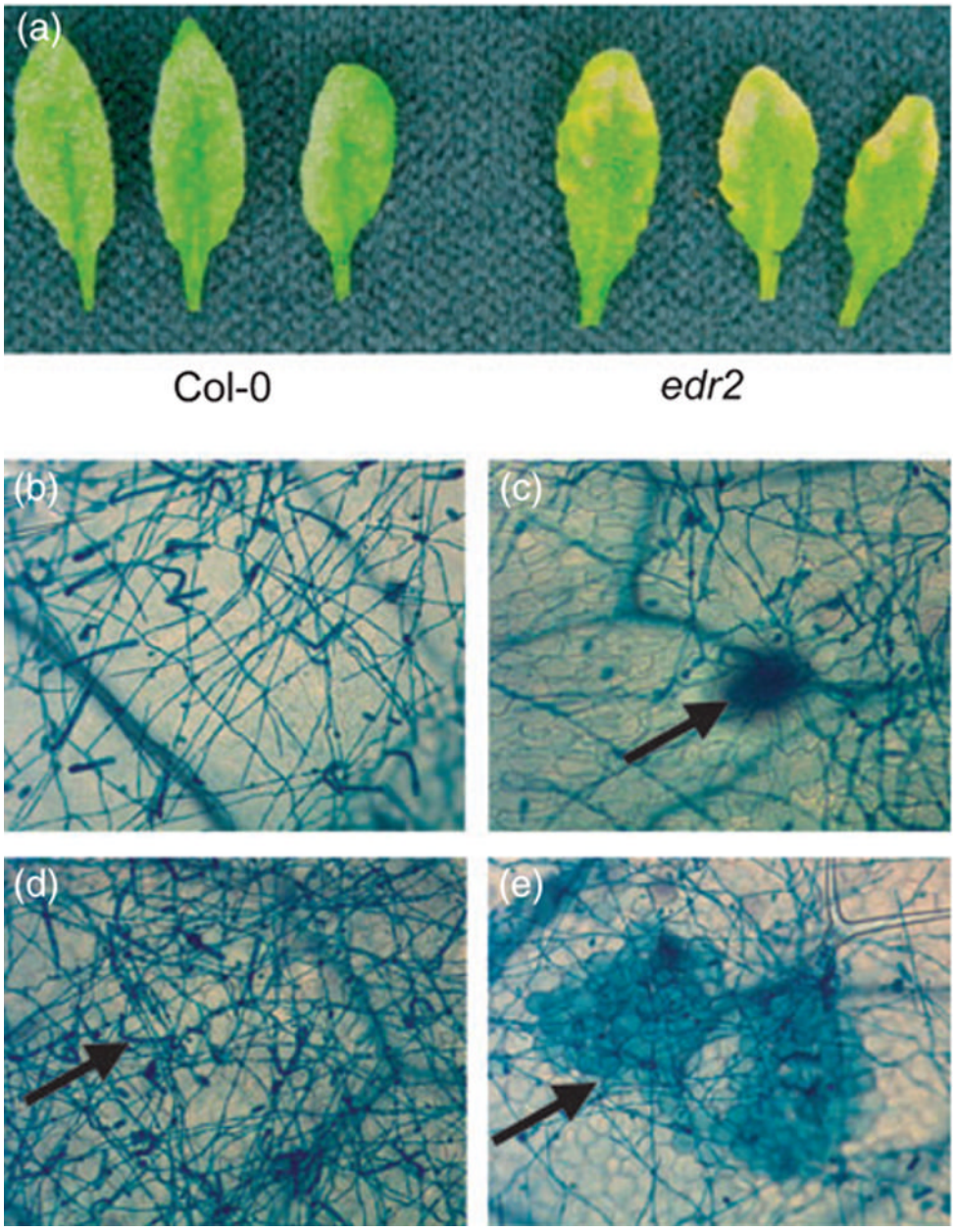

Figure 1.
Response of Arabidopsis WT and *edr2* mutant plants to *E. cichoracearum*.
(a) Col-0 and *edr2* plants were inoculated with *E. cichoracearum* and leaves detached for photography 8 days after inoculation.
(b) Fungal hyphae growing on the surface of Col-0 leaves 5 days after infection, stained with trypan blue.
(c) Fungal hyphae growing on the surface of the *edr2* mutant leaves 5 days after infection. Mesophyll cell death (arrow) is apparent at this stage.
(d) Extensive conidia (arrow) produced on Col-0 leaves 7 days after infection.
(e) Extensive mesophyll cell death (arrow) in *edr2* mutant leaves 7 days after infection, but very few conidia.

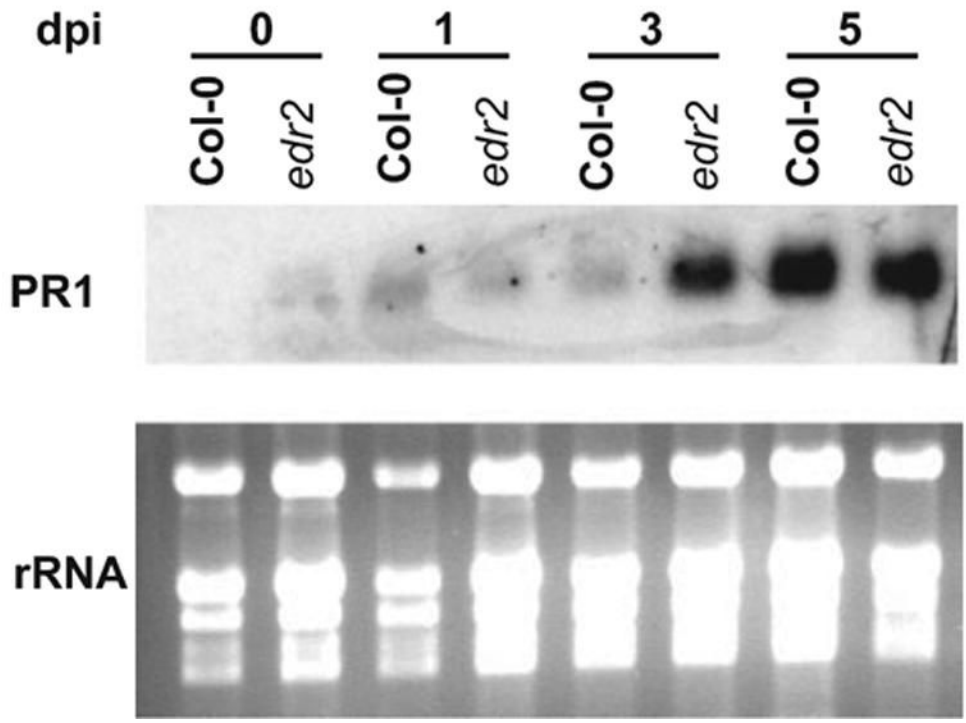

Figure 2.
PR-1 transcripts accumulate more rapidly in the *edr2* mutant than in WT plants after infection with *E. cichoracearum*. Top: an RNA gel-blot hybridized with a radiolabeled *PR-1* cDNA probe. Bottom: the corresponding ethidium bromide-stained gel to show the relative amounts of RNA loaded in each lane.

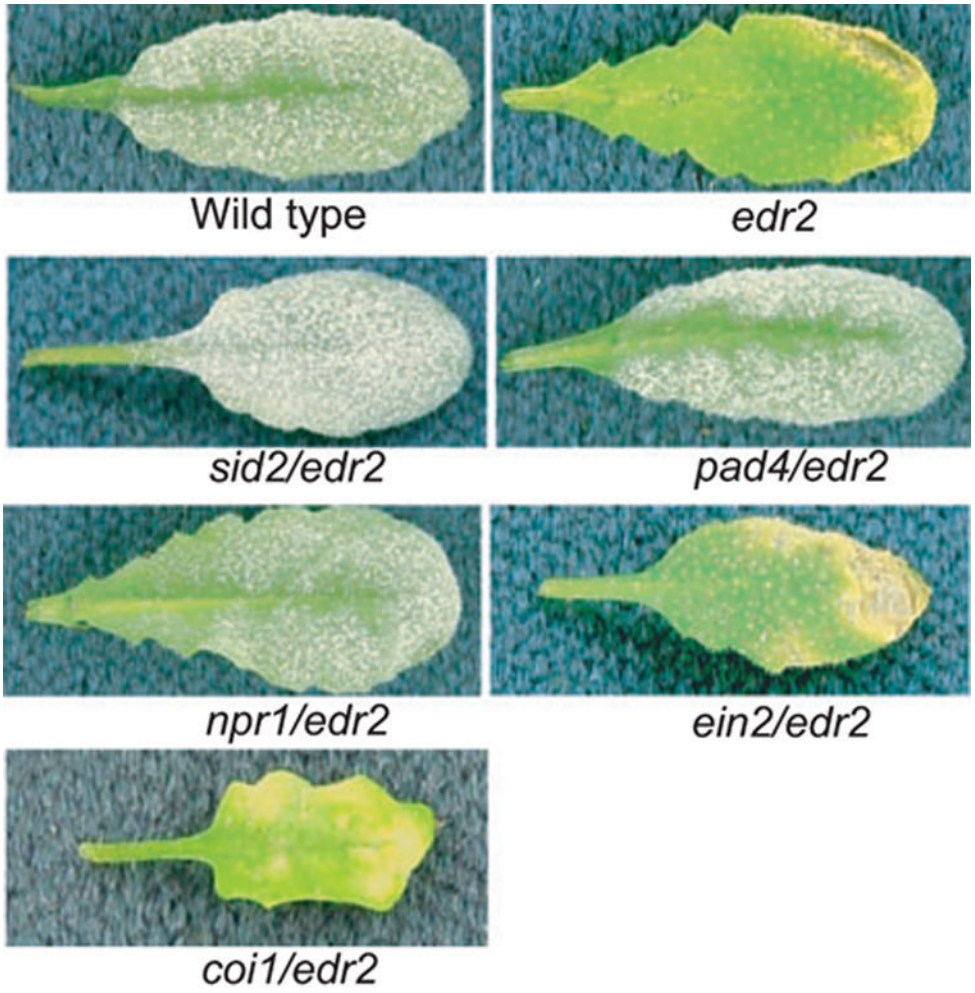

Figure 3.
Resistance to powdery mildew mediated by the *edr2* mutation is dependent on SA signaling, but not JA or ethylene signaling. The indicated mutants were inoculated with *E. cichoracearum* and disease phenotypes scored 8 days after infection. Single representative leaves were removed from intact plants for photography.

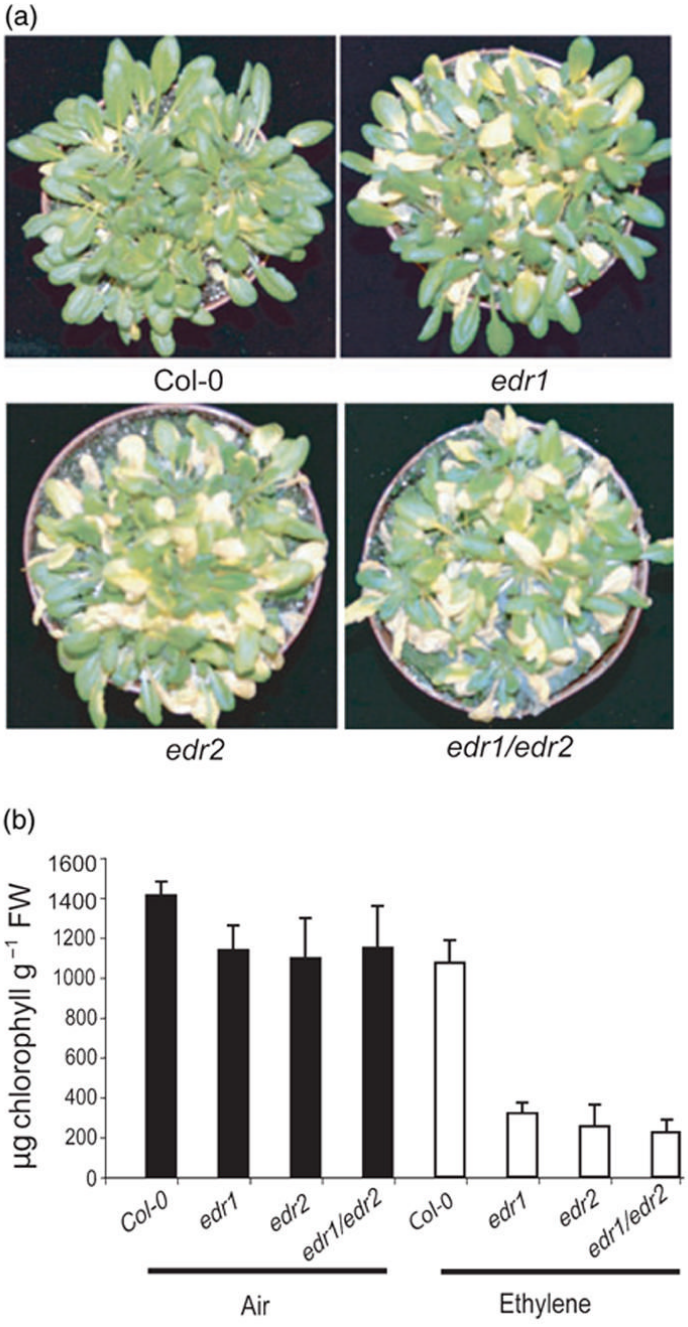

Figure 4.

Enhanced ethylene-induced senescence in *edr1* and *edr2* mutant plants.

(a) Increased chlorosis after ethylene treatment. Plants were photographed after 3 days of exposure to 100 µl l^{-1} ethylene.

(b) The chlorophyll content in leaves five to eight (leaf one being the first true leaf) of the plants shown in (a). Bars represent the mean and standard deviation of values obtained from four plants.

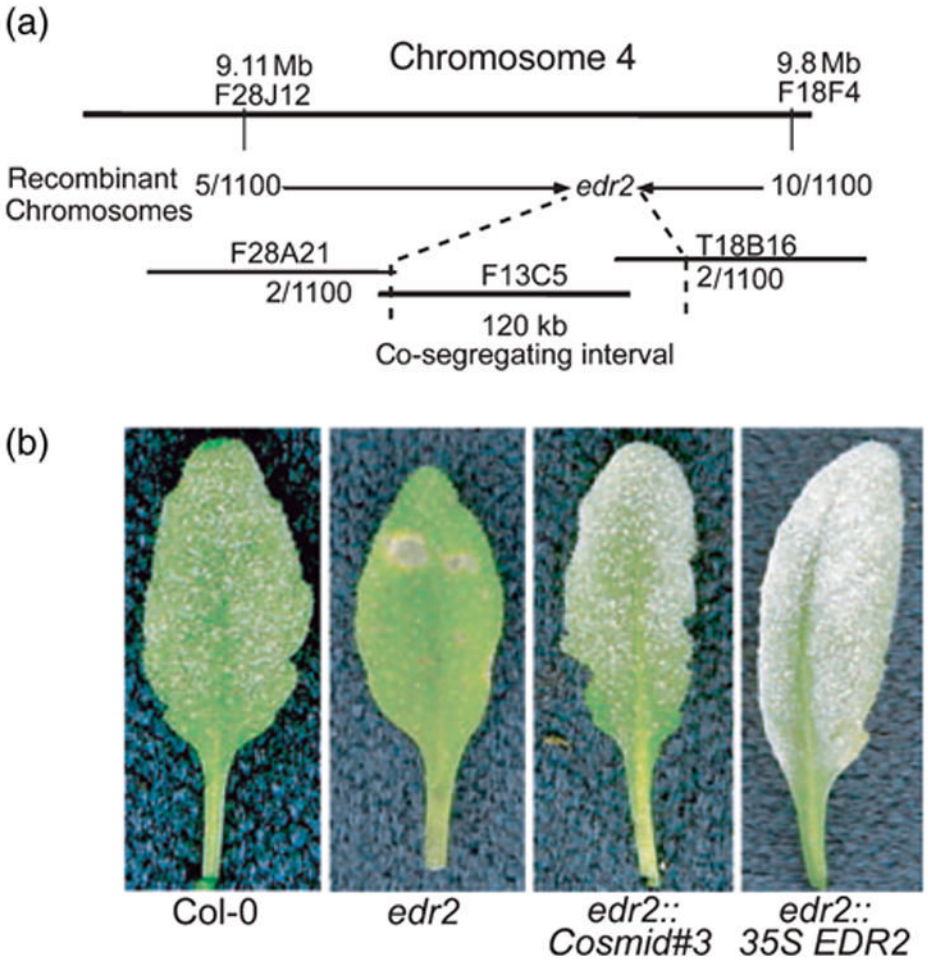

Figure 5.

Positional cloning of the *EDR2* gene.

(a) Genetic and physical map of the region flanking *EDR2*. Shorter horizontal lines indicate BAC clones spanning the region to which *edr2* was mapped.

(b) Complementation of the *edr2* mutation by *Agrobacterium*-mediated transformation. The indicated plants were inoculated with *E. cichoracearum* and representative leaves removed for photography 8 days after inoculation. Both a genomic cosmid clone (#3) and a cDNA clone (35S *EDR2*) containing the *At4g19040* gene were able to complement the mutation.

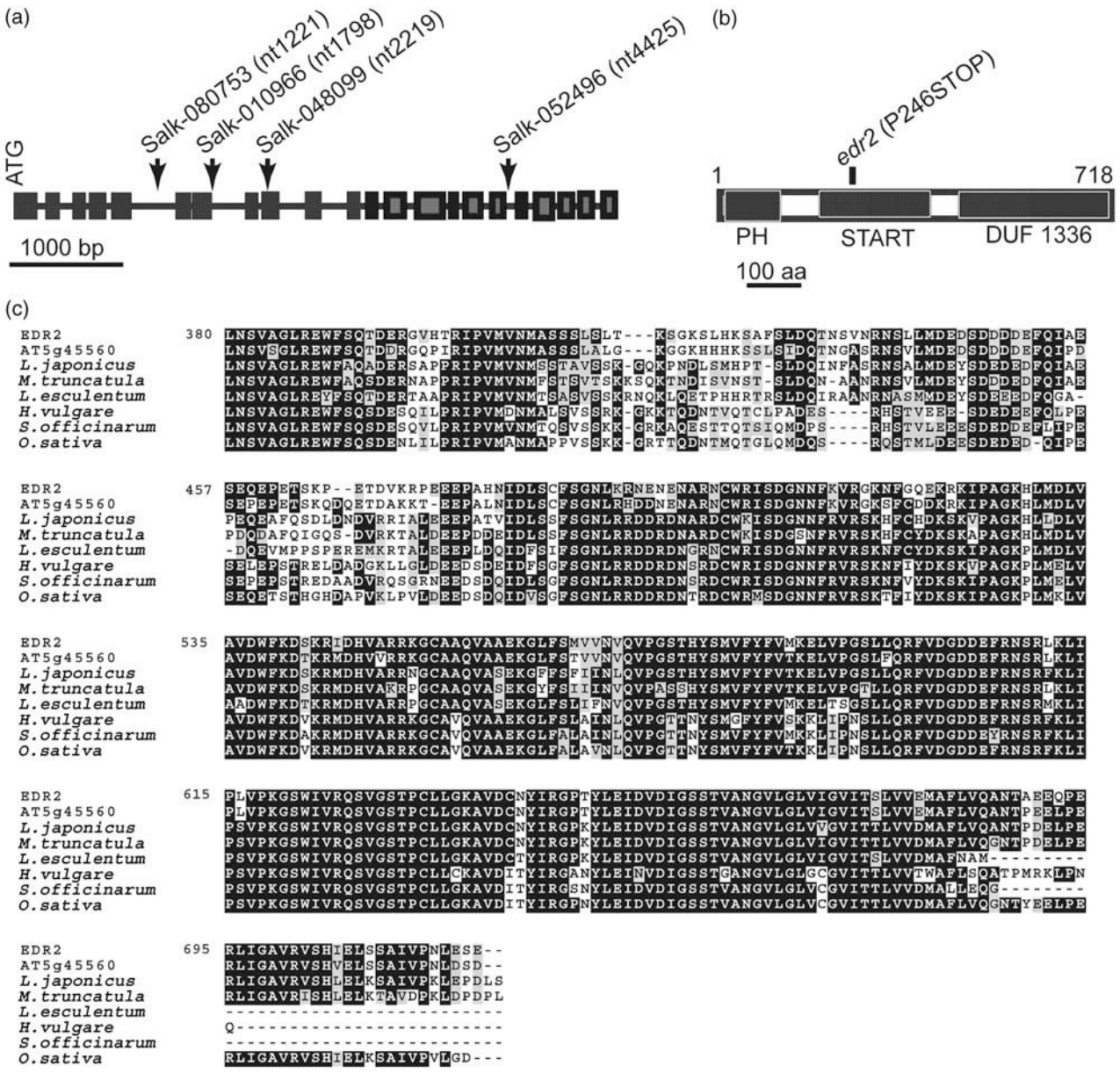

Figure 6.

Characterization of *EDR2*.

(a) Structure of the *EDR2* gene. Insertion sites in the Salk T-DNA lines are indicated by arrows. Numbers in parentheses indicate the nucleotide position in the genomic sequence relative to the start of the coding region.

(b) The predicted EDR2 protein contains 718 amino acids and includes a PH domain, a START domain and a C-terminal DUF1336 domain, which is highly conserved among homologs in other plant species.

(c) Alignment of the EDR2 C-terminal DUF1336 domain with the most similar Arabidopsis protein (At5g44560) and homologs from other plant species identified in the TIGR Plant Gene Indices database. Tentative consensus numbers for the homologs are *Oryza sativa* (rice), TC251404; *Saccharum officinarum* (sugarcane), TC58737; *Hordeum vulgare* (barley),

TC150750; *Lycopersicon esculentum* (tomato), TC144758; *Medicago truncatula* (medicago), TC88033; *Lotus japonicus* (lotus), TC15086.

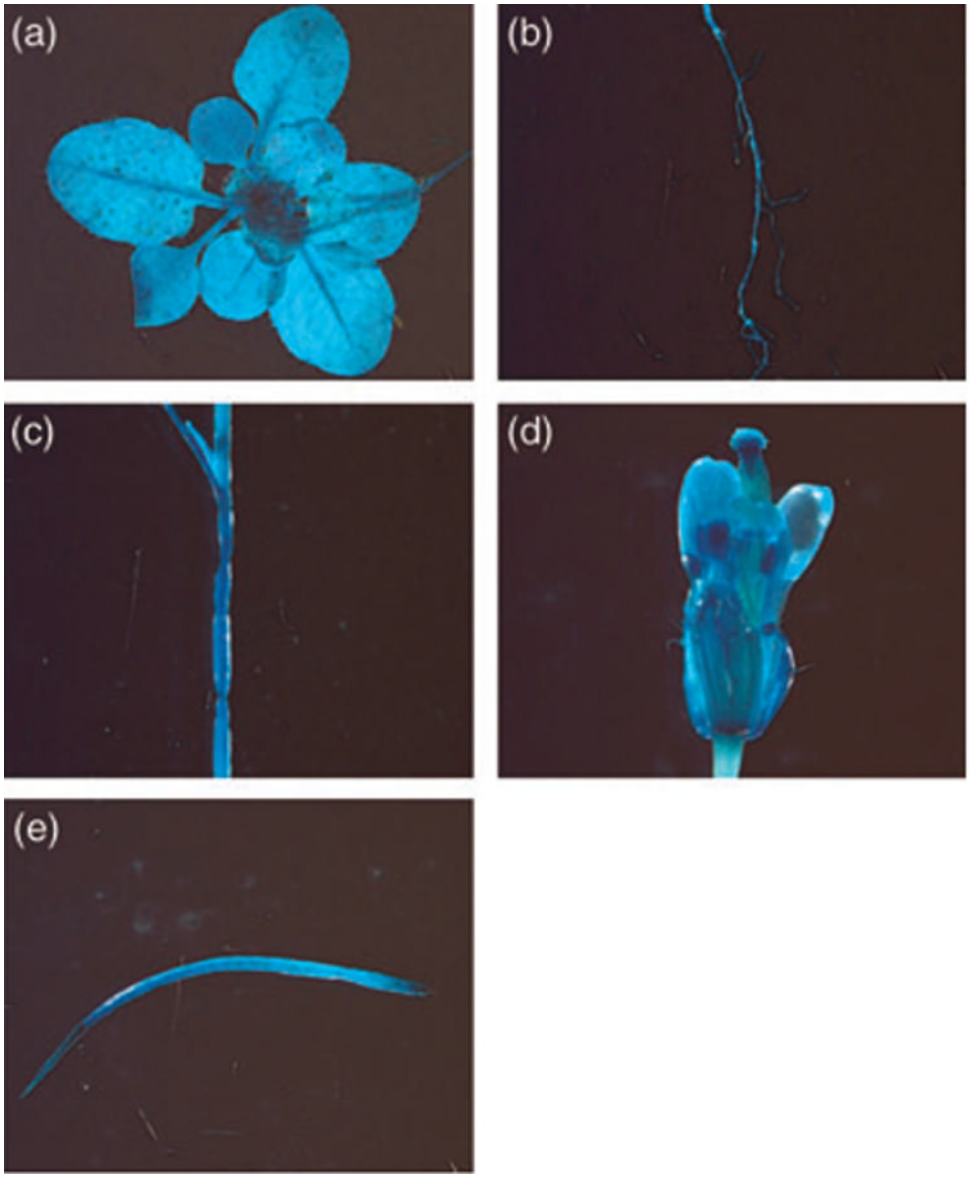

Figure 7.
EDR2 is expressed in all tissues and organs examined. *EDR2* promoter–GUS expression in (a) seedlings, (b) roots, (c) stem, (d) flower and (e) silique.