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Negative regulation of defence signalling pathways by the EDR1 protein kinase

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SUMMARY

The *enhanced disease resistance 1* (*edr1*) mutant of Arabidopsis confers enhanced resistance to bacterial and fungal pathogens. To better understand how *edr1*-mediated resistance occurs, we performed transcriptome analyses on wild-type and *edr1* plants inoculated with the fungal pathogen *Golovinomyces cichoracearum* (powdery mildew). The expression of many known and putative defence-associated genes was more rapidly induced, and to higher levels, in *edr1* plants relative to the wild-type. Many of the genes with elevated expression encoded WRKY transcription factors and there was enrichment for their binding sites in promoters of the genes upregulated in *edr1*. Confocal microscopy of transiently expressed EDR1 protein showed that a significant fraction of EDR1 was localized to the nucleus, suggesting that EDR1 could potentially interact with transcription factors in the nucleus. Analysis of gene ontology annotations revealed that genes associated with the endomembrane system, defence, reactive oxygen species (ROS) production and protein kinases were induced early in the *edr1* mutant, and that elevated expression of the endomembrane system, defence and ROS-related genes was maintained for at least 4 days after infection.

INTRODUCTION

Plants have evolved complex mechanisms to defend themselves against pathogens (Bent and Mackey, 2007). To identify the genes regulating plant defence responses, we have previously screened for Arabidopsis (*Arabidopsis thaliana*) mutants with enhanced resistance to virulent pathogens (Frye and Innes, 1998). The *enhanced disease resistance 1* (*edr1*) mutant displays enhanced resistance to the fungus *Golovinomyces cichoracearum*, an obligate biotroph and causal agent of powdery mildew on Arabidopsis and many cucurbit species (Adam *et al*., 1999; Adam and Somerville, 1996). Although *G. cichoracearum* forms conidiophores (stalks of asexual spores) on the surface of susceptible leaves, resistance in *edr1* is manifested as necrotic lesions at the site of infection and a reduction in conidiophores (Frye and Innes, 1998). In addition, *edr1* mutants show greater callose deposition and form more papillae, and at an earlier time, than do wild-type Col-0 plants. *EDR1* encodes a protein with a C-terminal kinase domain and a putative N-terminal regulatory domain (Frye *et al*., 2001). A recombinant protein containing the EDR1 kinase domain only is able to autophosphorylate and can phosphorylate the common kinase substrate myelin basic protein *in vivo*, demonstrating that EDR1 does, indeed, have kinase activity (Tang and Innes, 2002).

The enhanced resistance of the *edr1* mutant is suppressed by mutations that reduce salicylic acid (SA) production (*sid2*, *eds1* and *pad4*) or block SA perception (*npr1/nim1*) (Frye *et al*., 2001; Tang *et al*., 2005). Transgenic expression of *NahG*, which lowers endogenous SA levels, also eliminates the *edr1*-mediated enhanced disease resistance phenotype (Frye *et al*., 2001). In contrast with the requirement for SA signalling in *edr1*-mediated resistance, neither ethylene (ET) nor jasmonic acid (JA) appears to be necessary, as mutations in the *ETHYLENE INSENSITIVE 2* (*EIN2*) or *CORONATINE INSENSITIVE 1* (*COI1*) gene do not alter *edr1*-mediated disease resistance (Frye *et al*., 2001).

In addition to regulating responses to pathogens, EDR1 also regulates responses to abiotic stresses, such as drought. When grown under drought conditions, the *edr1* mutant is dwarfed and forms lesions, whereas growth is normal under optimal conditions (Tang *et al*., 2005). These phenotypes are suppressed by mutations in the SA signalling pathway (*eds1*, *pad4* or *npr1*), indicating that the drought response is also dependent on SA and may share similarity with the pathogen response in *edr1*. In addition, the F-box protein mutant, *ore9*, which shows delayed senescence in response to ET, restores wild-type growth under drought conditions to the *edr1* mutant, but does not abate the drought-induced lesion phenotype (Tang *et al*., 2005).

EDR1 is most similar to the ET response regulator, CTR1, and four other proteins of unknown function in Arabidopsis. Despite

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Accession numbers: Table S1 (see Supporting Information).

this similarity, *edr1* mutants have a normal triple response, unlike *ctr1* mutants. However, when *edr1* mutants are treated with ET, they senesce more rapidly than wild-type Col-0 (Frye *et al*., 2001). This response can be abolished by the presence of *ein2*, an ET signalling mutation, but it does not require SA responses (Tang *et al*., 2005). Taken together, the responses to pathogen, drought and ET in *edr1* imply that EDR1 negatively regulates cell death in response to various stimuli.

A mechanism for CTR1-mediated ET regulation proposes that two F-box proteins, EBF1 and EBF2, target ET-inducible transcription factors for proteasome-mediated degradation (Guo and Ecker, 2003; Potuschak *et al*., 2003). This degradation is dependent on an active CTR1 protein and, in the absence of CTR1, the transcription factor EIN3 can accumulate. ET represses CTR1 activity, preventing the activity of EBF1/2, and this allows EIN3 to accumulate and activate ET responses. It is possible that EDR1 may negatively regulate cell death responses in a similar manner. Mutations in the F-box protein ORE9 can block ET-induced cell death in the *edr1* mutant, as well as drought-induced growth inhibition, suggesting that a repressor of these phenotypes accumulates in the *ore9* mutant. However, not all *edr1*-mediated responses can be blocked by *ore9*, indicating that ORE9 regulates only a subset of *EDR1*-mediated responses.

All known *edr1* mutant phenotypes can be suppressed by a specific missense mutation in the *KEEP ON GOING* (*KEG*) gene, which encodes an E3 ubiquitin ligase that is responsible for the degradation of the abscisic acid (ABA)-inducible transcription factor ABI5. This result suggests that EDR1 may mediate cell death via a mechanism similar to the regulation of ET responses by CTR1, namely the targeting of transcription factors to the proteasome. Consistent with this model, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses have revealed that some ABA-inducible genes are expressed more highly in *edr1* mutant plants, and this enhanced expression is abolished by the *keg-4* mutation (Wawrzynska *et al*., 2008).

Despite the extensive work performed on the *edr1* mutant, there is still little information on how EDR1 negatively regulates cell death, particularly in response to *G. cichoracearum*. To investigate the control of cell death in the *edr1* mutant, we performed microarray experiments to identify the genes whose regulation was affected by the *edr1* mutation in the presence of powdery mildew. As expected, many of the genes upregulated in the *edr1* mutant were defence response genes, indicating that EDR1 negatively regulates defence signalling pathways and that the removal of such repression in the *edr1* mutant results in enhanced resistance. Significantly, the EDR1 protein was found to localize, at least part of the time, to the nucleus, suggesting that EDR1 may regulate directly the stability and/or activity of defence-related transcription factors.

RESULTS

Identification of genes regulated by EDR1

Wild-type Col-0 and *edr1* mutant plants were inoculated with *G. cichoracearum* and tissue was collected at 18, 36 and 96 h post-inoculation (hpi). By 18 h, the fungus has germinated, penetrated the epidermal cells and begun to form haustoria (Fabro *et al*., 2008). By 36 h, infected cells have begun to form papillae and deposit callose. By 96 h, stalks of asexual spores (conidia) begin to form on wild-type leaves, but very few formed on *edr1* leaves; however, no cell death is observable in wild-type or *edr1* plants, even at 96 hpi, and visible powder has not begun to form (Frye and Innes, 1998). Tissue was also collected from plants immediately prior to inoculation as an uninfected control (0 h). High-quality RNA was prepared from the collected tissue, including four biological replicates per genotype per time point, and analysed using Affymetrix ATH1 gene chips.

To identify genes that were negatively regulated by EDR1, we first selected genes that were upregulated by more than two-fold in *edr1* relative to wild-type Col-0 at any time point and that were determined to be significantly different ($P \leq 0.05$) using the Benjamini–Hochberg correction (Benjamini and Hochberg, 1995). This correction should reduce the false discovery rate to less than 5%. In addition, genes that were upregulated by more than two-fold in *edr1* or wild-type Col-0 after inoculation relative to uninoculated plants were selected. These datasets were then compared to identify genes that were upregulated in an *edr1*- and pathogen-dependent manner. Genes whose expression was higher in *edr1* than in Col-0 at any time and was also higher in either Col-0 or *edr1*, or both, after pathogen inoculation were selected (areas bounded by the yellow oval in Fig. 1; Table S1, see Supporting Information). This subset of genes contained 553 probe sets corresponding to 545 annotated genes. We refer to this subset as the *edr1&pm*-upregulated gene set. It should be noted that, because of cost issues, we did not include an uninoculated control at each time point; thus, it is a formal possibility that some of the genes included in the powdery mildew-induced gene set are upregulated as a result of circadian changes in gene expression instead of, or in addition to, powdery mildew infection. Nevertheless, all genes included in the *edr1&pm*-upregulated gene set were more highly expressed in the *edr1* mutant than in wild-type Col-0 during at least one time point.

Upregulation of defence genes in the *edr1* mutant

Many of the genes identified in the *edr1&pm*-upregulated gene set (Table S1) are known to be involved in plant defence responses. For example, *PBS3* and *PAD4* are both required for SA accumulation (Glazebrook *et al*., 1996; Nobuta *et al*., 2007), and *PR-3*, *PR-4*, *THI2.1*, *PDF1.4* and *ATTI1* are associated with

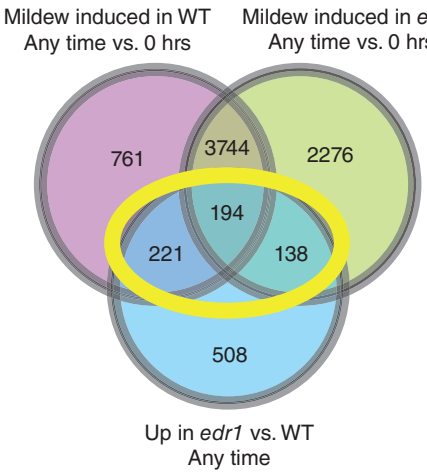

Fig. 1 Venn diagram showing overlap between powdery mildew-induced and *enhanced disease resistance 1* (*edr1*)-induced gene sets. The yellow oval indicates the set of *edr1&pm*-upregulated genes used for the majority of the analyses. WT, wild-type.

JA-inducible defences. Commonly, SA and JA defences have antagonistic modes of action (Li *et al*., 2004), but may also have additive effects depending on hormone concentration and the type of pathogen encountered (Mur *et al*., 2006). *PR-3* and *PR-4* encode a chitinase and chitin-binding protein with antifungal activity, respectively (Potter *et al*., 1993; Verburg and Huynh, 1991). *THI2.1*, *PDF1.4* and *ATTI1* are all induced by JA and may also have antifungal or antimicrobial activity (Epple *et al*., 1995; Silverstein *et al*., 2005). Other defence-associated genes in the *edr1&pm*-upregulated gene set include 12 genes with leucine-rich repeat (LRR) domains, including three encoding a Toll/interleukin-1 receptor (TIR) class nucleotide-binding leucine-rich repeat (NB-LRR) disease resistance protein.

The *edr1&pm*-upregulated gene set also contains five receptor-like kinases (RLKs), including *RLK5* and *RLK6*. *RLK5* and *RLK6* were identified in a search for genes that are regulated by pathogen-inducible transcription factors and are known to be induced by SA treatment and by pathogens (Du and Chen, 2000). In addition to the RLKs identified, there are also eight putative LRR kinases. LRR kinases can act as receptors to transmit signalling information, often during defence responses (Shiu and Bleecker, 2001). An additional 11 kinases are present in the *edr1&pm*-upregulated gene set, indicating that phosphorylation cascades are important for the defence mechanism induced by *G. cichoracearum* in *edr1*.

Significantly, the *edr1&pm*-upregulated gene set contains at least 28 genes encoding transcription factors. The largest family of transcription factors in this gene set is the WRKY family, of which there are eight members, or 1.5% of the annotated genes in this gene set, compared with 61 of the 22 810 genes on the ATH1 chip (0.27%). There are at least 75 WRKYs in the Arabidopsis genome and many WRKYs have been implicated in controlling aspects of plant defence responses (Bhattarai *et al*., 2010; Eulgem *et al*., 2000). WRKY transcription factors have a conserved DNA-binding domain, which contains a WRKY motif that is required for DNA binding (Ciolkowski *et al*., 2008). WRKYs also contain a zinc-binding region in the DNA-binding domain. WRKYs bind to the sequence (T)TGAC(C/T), known as the W-box, in the promoter sequence of target genes (Ciolkowski *et al*., 2008).

A second class of transcription factors over-represented in the *edr1&pm*-upregulated gene set is the AP2/ERF family. There are seven AP2/ERFs present, comprising 1.28% of the genes, compared with 89 on the ATH1 chip, or 0.39% of the genes. AP2/ERF transcription factors were originally identified as genes that were induced in response to the hormone ET, but have since been shown to include genes that are induced in response to pathogen and during JA-inducible defences (Gutterson and Reuber, 2004). Indeed, one AP2/ERF, ORA59, has been found to integrate JA- and ET-mediated signalling pathways (Pre *et al*., 2008). AP2/ERF family transcription factors bind to the GCC-box (GCCGCC) to activate transcription.

The *edr1&pm*-upregulated gene set is also enriched for genes involved in reactive oxygen species (ROS) accumulation and turnover. Eleven genes annotated with putative peroxidase function were identified. Peroxidases act to oxidize other molecules through the use of H_2O_2 or O_2, either as a way of preventing toxicity or to signal (Yoshida *et al*., 2003). One of the peroxidase genes identified in the *edr1&pm*-upregulated gene set, *ATP2a*, has also been shown to be upregulated in response to wounding and may play a role in pathogen responses (Cheong *et al*., 2002).

Other genes that are present in the *edr1&pm*-upregulated gene set include six genes that encode small heat shock proteins (sHSPs). sHSPs can act as molecular chaperones and have been identified in the regulation of responses to various stresses and developmental processes, including apoptosis (Basha *et al*., 2006). Genes encoding glutathione *S*-transferase (GST) genes are also present. GSTs are involved in the regulation of the cellular redox state and are often induced during defence responses (Wagner *et al*., 2002).

Nine genes encoding flavin adenine dinucleotide (FAD)-binding domain-containing proteins were also identified as part of the *edr1&pm*-upregulated gene set. These genes are closely related to a sunflower gene that encodes an antimicrobial protein with carbohydrate oxidase activity, Ha-CHOX (Custers *et al*., 2004). Ha-CHOX catalyses the production of H_2O_2 using glucose as a substrate. When Ha-CHOX is overexpressed in tobacco, it confers greater resistance to *Pectobacterium carotovorum*. The nine FAD-binding domain-containing genes in the *edr1&pm*-upregulated gene set are all members of the same family of proteins, sharing similarity across their entire length (Fig. S1, see Supporting Information).

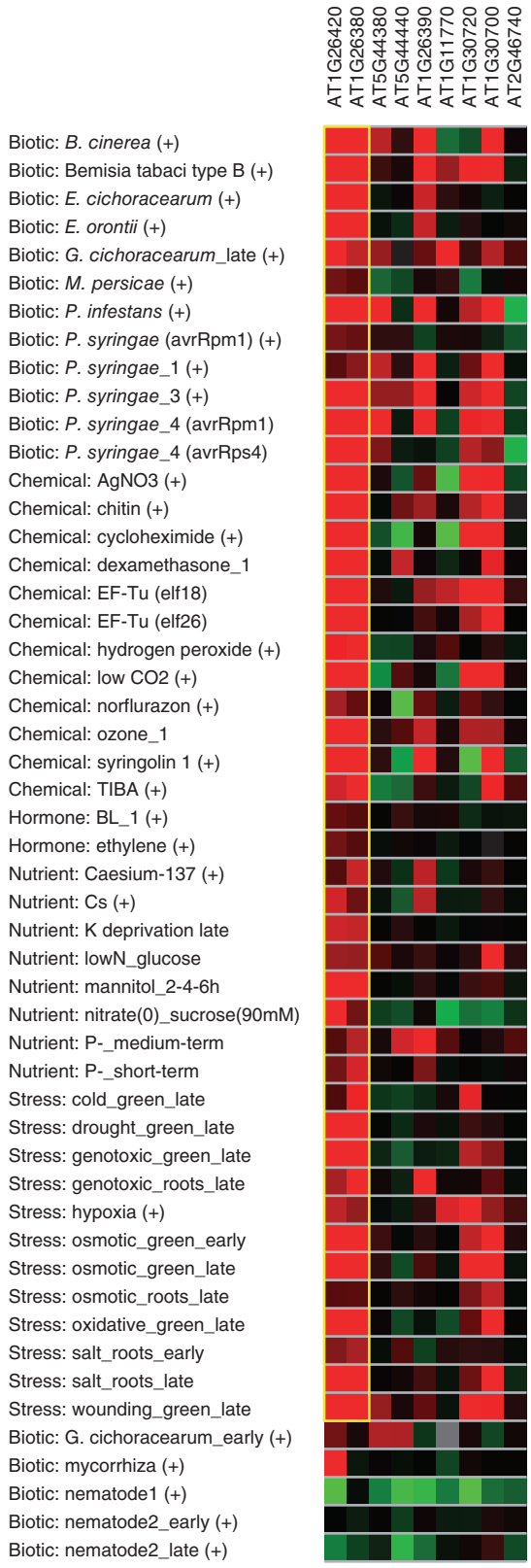

Fig. 2 BiMax clustering of flavin adenine dinucleotide (FAD)-binding domain-containing genes. Red indicates induction and green indicates suppression, with brighter colours indicating a greater effect of the indicated treatment. The yellow box indicates the two genes with highly similar expression patterns over the indicated treatments.

To determine whether the FAD-binding domain genes were induced by other pathogens, data from publicly available microarrays were analysed using the Genevestigator web-based interface (https://www.genevestigator.ethz.ch/gv/index.jsp) (Hruz *et al*., 2008; Zimmermann *et al*., 2004). Using BiMax clustering, the available high-quality arrays were analysed for conditions in which the FAD genes from our dataset were expressed in similar patterns (Fig. 2). The majority of these genes were induced by multiple pathogens, including fungi (e.g. *Botrytis cinerea*), oomycetes (*Phytophthora infestans*) and bacteria (*Pseudomonas syringae*). In addition, several of these genes were also induced by microbial-associated molecular patterns (MAMPs) elf18, elf26 and chitin, and by some abiotic stresses, including osmotic and oxidative stresses. These results point to a role for this family of genes in controlling defence and stress responses, perhaps through the production of H_2O_2.

Analysis of transcription factor motifs

To determine whether the *edr1&pm*-upregulated gene set was enriched for genes that are induced by WRKY family transcription factors, a kilobase region of sequence upstream of the start codon was collected for all genes with AGI numbers in our dataset. These regions were then searched for W-boxes to determine the frequency of this element. As a control, we analysed the equivalent upstream regions from all genes that remained unchanged (<1.155 fold up or down) in *edr1* versus wild-type, and also remained unchanged at all time points after infection in both *edr1* and wild-type plants (a total of 472 genes). Significantly, the upstream regions of genes from our dataset were enriched for W-boxes, with 910 elements, or a frequency of 1.59 elements per gene, compared with the unchanged dataset, where there was a frequency of 0.79 elements per gene (Table 1). These data strongly suggest that WRKY-regulated genes are upregulated in the *edr1* mutant after pathogen treatment.

Using the same promoter scanning analysis as for the WRKY transcription factors, the number and frequency of GCC-boxes in the promoter regions of genes from the *edr1&pm*-upregulated gene set were calculated. There were 52 GCC-boxes, a frequency of 0.091 per gene, compared with a frequency of 0.127 per gene in the unchanged dataset and 0.11 for all Arabidopsis genes (Table 1), indicating that the majority of the genes in the *edr1&pm*-upregulated gene set were not regulated by AP2/ERF family transcription factors. Indeed, the lower than average

Table 1 Frequencies of transcription factor binding sites in promoter regions of *edr1&pm*-upregulated genes. A 1-kb region 5′ of the start codon for each gene was analysed. This information was also calculated for genes that were unchanged in *enhanced disease resistance 1* (*edr1*) mutants relative to Col-0, and unchanged after pathogen infection in either genotype, and for all available upstream sequences in The Arabidopsis Information Resource (TAIR) (33 518 sequences).

	No. of elements (*edr1&pm*-upregulated)	No. of elements (unchanged)	No. of elements (genome)	Frequency (*edr1*)	Frequency (unchanged)	Frequency (genome)
W-box (TTGACC/T)	910	514	40 948	1.59	0.79	1.22
GCC-box (GCCGCC)	52	60	3 528	0.091	0.127	0.11
MYC2 (CACATG)	267	204	16 556	0.47	0.43	0.49
ARF (TGTCTC)	270	284	17 690	0.47	0.60	0.53
MYB2 (C/TAACG/TG)	591	535	36 727	1.03	1.11	1.1
GATA (T/AGATAG/A)	1968	1404	107 927	3.43	2.98	3.2
DREB (CCGAC)	544	570	37 212	0.998	1.21	1.11

frequency of GCC-boxes suggests that the *edr1&pm*-upregulated gene set is enriched in genes that lack AP2/ERF binding sites.

EDR1 localizes to the endoplasmic reticulum (ER) and the nucleus

The enrichment for WRKY transcription factors and genes containing their binding sites in the *edr1&pm*-upregulated gene set suggests that EDR1 negatively regulates the activity of these transcription factors. To determine whether this could be occurring directly, we analysed the subcellular localization of full-length EDR1 protein fused to super yellow fluorescent protein (sYFP2; Kremers *et al*., 2006) using confocal microscopy. This fusion protein was shown to be functional as EDR1-sYFP expressed under the EDR1 native promoter was able to complement an *edr1* Arabidopsis mutant in stable transgenic plants (Fig. S2, see Supporting Information). Unfortunately, we were unable to detect EDR1-sYFP using confocal microscopy in these plants, probably because of the low level of expression from the native EDR1 promoter. To visualize EDR1-sYFP, we thus transiently expressed it in *Nicotiana benthamiana* leaves using a dexamethasone-inducible promoter. All transformed cells gave a similar pattern, displaying localization to internal membranes and to the nucleus (Fig. 3A). To confirm the nuclear localization, we co-expressed EDR1-sYFP with the nuclear protein GCN5-mCHERRY (Bhat *et al*., 2004). GCN5-mCHERRY fluorescence was confined to the nucleus and appeared to be excluded from the nucleolus (Fig. 3B). The nuclear portion of the EDR-sYFP fluorescence co-localized with GCN5. To determine whether the membrane localization was associated with ER, we co-expressed EDR1-sYFP with an ER marker consisting of the signal peptide of AtWAK2 (*Arabidopsis thaliana* wall-associated kinase 2) at the N-terminus of mCherry and the ER retention signal His–Asp–Glu–Leu at its C-terminus (Nelson *et al*., 2007). Figure 3D–F shows that EDR1-sYFP co-localized with this ER marker outside of the nucleus. To assess whether the nuclear signal from EDR1-sYFP could be a result of the degradation of EDR1-sYFP, we performed immunoblot analyses using an anti-green fluorescent protein (GFP) antibody (Fig. 3G). We observed a band of approximately 130 kDa, the expected size for intact EDR1-sYFP, and a second band at approximately 70 kDa. Untagged EDR1 is readily cleaved, releasing a C-terminal 50-kDa fragment (without GFP) during extraction from either plant cells or from *Escherichia coli* (data not shown), despite the use of protease inhibitors in the extraction buffer. It is thus unclear whether this 70-kDa protein is present in live cells, but, regardless, this EDR1-YFP fragment should be too large to diffuse into the nucleus on its own. These observations indicate that a portion of the EDR1 pool accumulates in the nucleus.

Gene ontology analysis of genes in the *edr1*-upregulated dataset

Gene ontology (GO) annotations have been assigned to nearly every gene in the Arabidopsis genome (Berardini *et al*., 2004). These annotations provide information about putative structure, function and cellular localization for the predicted protein products. By analysing the GO annotations of the *edr1*-upregulated genes at each time point, it is possible to determine which categories of genes are enriched, and how these change temporally after infection with powdery mildew. For this analysis, genes whose expression was at least two-fold higher in *edr1* compared with wild-type Col-0 at any time point were selected (blue circle in Fig. 1). At 0 h, prior to inoculation with pathogen, the GO categories that were most significantly enriched ($P \leq 0.0001$) were 'endomembrane system', 'cellulase activity', 'cell wall', 'external encapsulating structure', 'extracellular region', 'membrane', 'nutrient reservoir', 'response to heat' and 'apoplast' (Table S2, see Supporting Information), suggesting that, in the absence of pathogen, the *edr1* mutation primarily affects the expression of genes associated with secretion and the cell wall. Notably, the endomembrane system category remained highly enriched at 18, 36 and 96 hpi, suggesting that the secretory system may play an important role in *edr1*-mediated defences. This is consistent with the ER localization of EDR1.

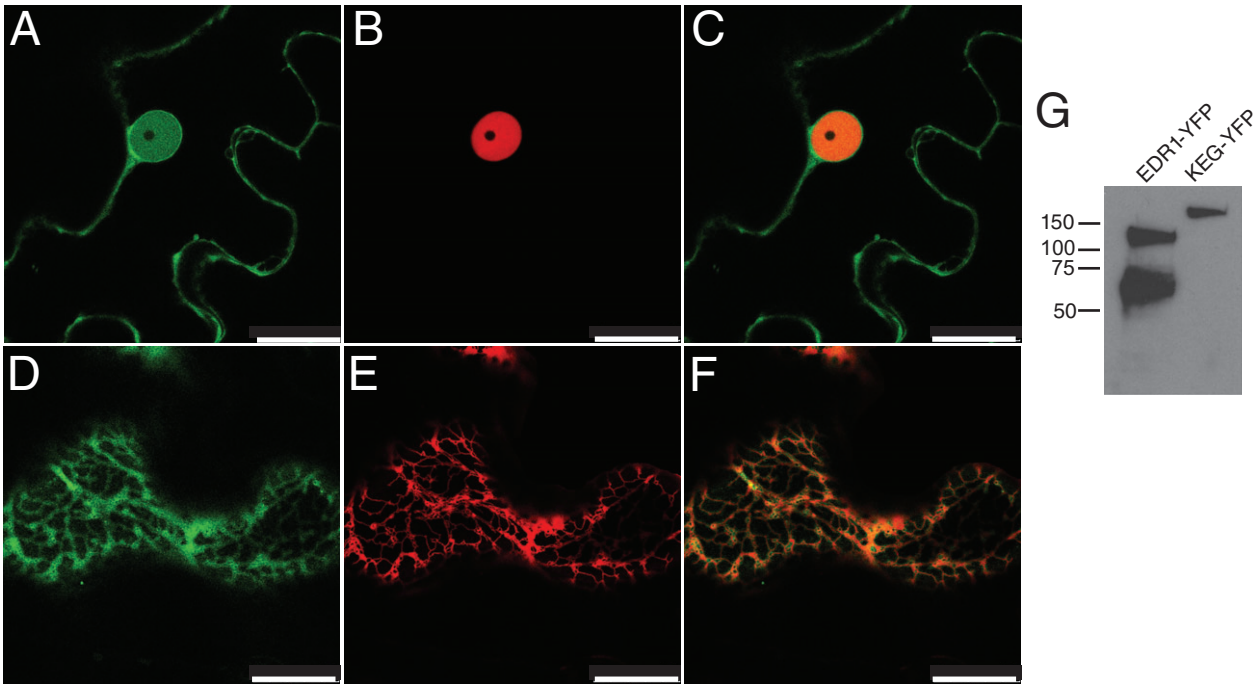

Fig. 3 Subcellular localization of enhanced disease resistance 1 protein (EDR1). (A–C) EDR1-sYFP and GCN5-mCherry were transiently co-expressed in *Nicotiana benthamiana* leaves and imaged using confocal laser scanning microscopy. (A) EDR1-sYFP (a single optical section taken through the nucleus of an epidermal cell). (B) GCN5-mCherry expressed in the same cell. (C) Overlay of (A) and (B). (D–F) EDR1-sYFP and mCherry ER marker (see Experimental procedures) were transiently co-expressed in *N. benthamiana*. (D) EDR1-sYFP (a single optical section taken through the cell cortex of an epidermal cell). (E) mCherry-HDEL. (F) Overlay of (D) and (E). (G) Immunoblot of EDR1-sYFP extracted from *N. benthamiana* leaves. KEG-sYFP is an unrelated yellow fluorescent protein (YFP) fusion protein included to show the specificity of the antibody. Scale bar, 25 μm.

At 18 hpi, the number of categories that were enriched increased dramatically. Of the 25 GO categories enriched with $P \leq 0.0001$, the majority were associated with defence responses (e.g. 'defence response', 'response to other organism', 'response to biotic stimulus', 'response to fungus', 'immune system process', 'response to chitin', 'innate immune response', 'systemic acquired resistance', etc.). In addition, the 'kinase activity' category was highly enriched ($P = 1.85 \times 10^{-4}$). These observations suggest that the early response to *G. cichoracearum* in *edr1* plants is a specific defence response regulated in part by kinase cascades.

At 36 hpi, many of the same categories were still enriched, with the notable exception of the kinase activity-related categories (Table S2). In addition, at 36 hpi, the three new categories with the highest significance were 'peroxidase activity', 'oxidoreductase activity' and 'antioxidant activity'. This implies that, by 36 hpi, *edr1* plants produce significantly more ROS than do wild-type plants. After 96 h, there was a reduction in the number of GO categories significantly enriched in *edr1* plants because gene expression in wild-type Col-0 had caught up with the levels of expression in *edr1* (Table S2). The GO analysis suggests that the response to *G. cichoraceaum* in *edr1* mutant plants is primarily a more rapid and robust activation of defence genes, probably mediated by kinase signalling cascades that include increased ROS production.

BiMax clustering of *edr1*-upregulated genes

Genes that are involved in defence pathways are induced in response to many different stimuli. To determine whether the *edr1&pm*-upregulated gene set included genes that were also induced by other pathogens or stimuli, we used the BiMax clustering algorithm within the Genevestigator V3 web toolbox to analyse the expression of these genes across all Arabidopsis datasets involving biotic or abiotic stimulation, or comparing mutant plants with the wild-type (Prelic *et al*., 2006). These analyses revealed a subset of genes from the *edr1&pm*-upregulated gene set that were regulated in a similar manner in 10 different treatments (Fig. 4A). These 10 different experiments included infection with various virulent and avirulent pathogens, and responses to abiotic stresses such as ozone and wounding. Many of the genes that share regulation in these different categories are defence associated, including a flavin monooxygenase, a TIR-NB-LRR gene, two GSTs, a WRKY transcription factor and a cytochrome P450 gene that has been associated with defence responses (Fig. 4A). In addition, the FAD-binding

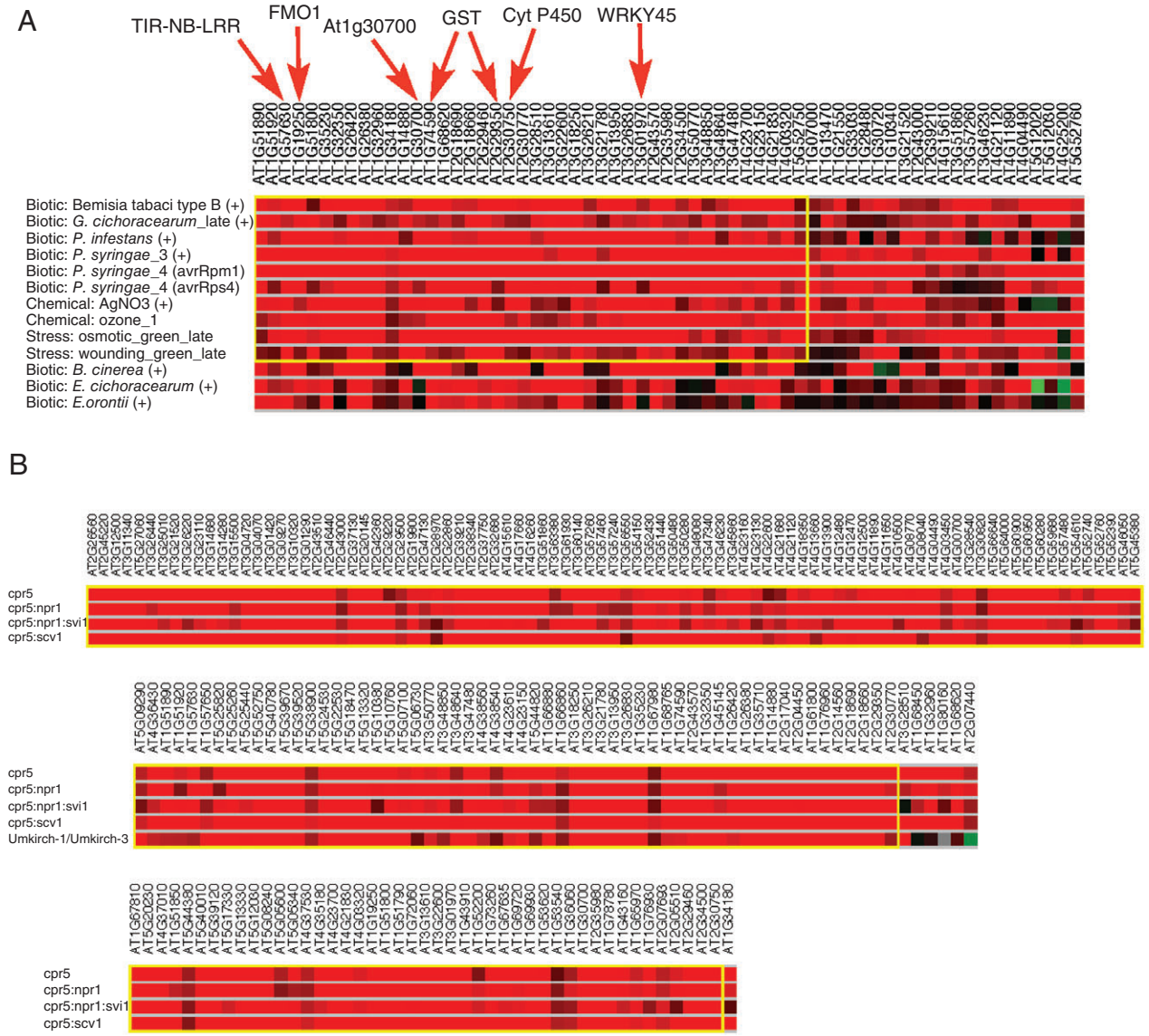

Fig. 4 BiMax cluster analysis of *edr1&pm*-upregulated genes. (A) Cluster analysis across the set of experiments housed within Genevestigator under the 'stimulus' category. (B) Cluster analysis across the set of experiments housed under the 'mutant' category. Only the largest clusters are shown, bounded by the yellow box in each.

domain-containing gene At1g30700, the gene most similar to Ha-CHOX, was present in this BiMax cluster (Fig. 4A).

BiMax clustering was also performed to compare gene regulation in different mutant backgrounds (Fig. 4B). Interestingly, the largest BiMax mutant cluster included the constitutive defence mutant *cpr5* (Bowling *et al*., 1997). Over one-third of the genes from the *edr1&pm*-upregulated gene set were also upregulated in *cpr5*. This implies that enhanced resistance controlled by *edr1* and *cpr5* may be mediated by many of the same genes. *CPR5* was identified in a screen for plants with enhanced resistance to pathogens. Unlike the *edr1* mutant, however, the expression of defence genes, such as *PR-1*, in the *cpr5* mutant was high in the absence of pathogen.

DISCUSSION

The EDR1 kinase appears to negatively regulate cell death in response to pathogen infection, as well as in response to abiotic stress. Although it has been established that EDR1 is a functional kinase (Frye *et al*., 2001), we still have little information on how EDR1 regulates responses to pathogen infection. The transcriptome analyses described above revealed a set of genes that were

upregulated in the *edr1* mutant relative to wild-type Arabidopsis after inoculation with *G. cichoracearum*. Of the 545 genes identified in this dataset, many are known to be involved in disease resistance from previous work. The presence of these genes indicates that the enhanced resistance to pathogen infection in *edr1* is at least partly the result of the derepression of defence-associated genes. This result supports the previous finding that *edr1*-dependent enhanced resistance requires an intact SA signalling pathway (Frye *et al*., 2001), as many of these known genes are involved in SA pathways.

In addition, the genes that are induced in *edr1* after pathogen inoculation encode for many signalling proteins, including putative NB-LRR proteins and RLKs. The higher expression of these genes indicates that signalling and, possibly, the perception of pathogens are elevated in the *edr1* mutant, and that one function of EDR1 may be to prevent unnecessary signalling. This may also serve to limit the perception of pathogen in the absence of immediate threat.

It should also be noted that, although the *edr1* mutation enhances the expression of numerous defence and signalling genes following powdery mildew inoculation, this set of genes represents only a small percentage of the total number of genes induced by at least two-fold following inoculation (Fig. 1). We identified nearly 4000 genes that were induced in both wild-type and *edr1* mutant plants during at least one time point following inoculation. The great majority of these (>95%) were not significantly affected by the *edr1* mutation (i.e. they were induced similarly in wild-type and *edr1* plants), indicating that the *edr1* mutation does not simply enhance the expression of all powdery mildew-induced genes.

The number of genes identified as being induced by powdery mildew infection (4920 in wild-type and 6352 in *edr1*) is larger than reported previously. For example, Zimmerli *et al*. (2004) examined gene expression in wild-type Arabidopsis infected with the same strain of powdery mildew as used in this study and identified only 13 genes that were upregulated significantly at 24 hpi. More recently, Fabro *et al*. (2008) examined gene expression in wild-type, *npr1-1* mutant and *jar1-1* mutant Arabidosis at 18 h following infection with the same powdery mildew strain and identified 117 induced genes. It is difficult to make direct comparisons between our study and those of Zimmerli *et al*. (2004) and Fabro *et al*. (2008), however, as their studies employed cDNA microarrays containing only about one-half of the genes present on the ATH1 Affymetrix gene chip used in our study, two-colour dye hybridization and different statistical tests. The reduced variability associated with Affymetrix chips relative to cDNA microarrays probably increased the sensitivity of our analyses. In addition, we sampled at later time points (36 and 96 h), allowing us to identify genes induced later in the infection process. Finally, many of the genes identified as upregulated in both *edr1* and wild-type plants at 18 and 36 h in our study may be under circadian regulation, which is one reason why genes that were not also upregulated in *edr1* relative to wild-type plants were excluded from our analyses (Fig. 1).

The analysis of GO annotations for the *edr1*-upregulated gene set (blue circle in Fig. 1) revealed a distinct temporal pattern of gene induction during powdery mildew infection. At 18 hpi, numerous defence-associated gene categories were highly enriched, including the 'kinase' category, whereas, at 36 hpi, the kinase category was no longer enriched, but several ROS-related categories appeared. These results suggest that, immediately following pathogen inoculation, genes involved in signalling and defence responses are expressed more highly, and that, as the response continues, there is a shift from initial induction of signalling to a more sustained response, perhaps through the use of ROS as signalling molecules. By 96 h, most of the categories were no longer enriched, because gene expression in wild-type plants had caught up.

To determine whether the genes in the *edr1*-upregulated dataset were also regulated in response to other pathogens, we used the publicly available microarray data and the web-based analysis tool Genevestigator V3 (Hruz *et al*., 2008; Zimmermann *et al*., 2004). Using the BiMax algorithm within Genevestigator (Prelic *et al*., 2006), we identified subsets of genes that were regulated in a similar manner in other microarray experiments (Fig. 4). Interestingly, many of the genes upregulated in *edr1* were also induced in response to different pathogens as well as abiotic stress. That these genes are regulated by EDR1 suggests that, in the absence of pathogen, EDR1 serves to keep the transcription of these genes low or off, and, once a pathogen has been detected, EDR1 function is repressed, allowing for higher levels of transcription of pathogen-inducible genes. Interestingly, a subset of *edr1*-upregulated genes was also induced at greater levels during attack by the whitefly *Bemisia tabaci*. The induction of defence-associated genes in response to *B. tabaci* may be caused by a wounding response, which is consistent with the observation that these same genes are also regulated in a similar manner in response to mechanical wounding.

BiMax clustering also revealed that many of the *edr1*-upregulated genes were also more highly expressed in *cpr5* mutants. Mutations in *CPR5* cause constitutive expression of defence genes, such as *PR* genes and *PDF1.2*, elevated ROS in leaves and the formation of lesions that display the deposition of autofluorescent compounds (Bowling *et al*., 1997). Lesions induced on *edr1* mutant leaves also show the deposition of autofluorescent compounds and elevated ROS (unpublished observations). In addition, like the *edr1* mutant, *cpr5* mutant plants display enhanced senescence (Jing *et al*., 2007; Yoshida *et al*., 2002). Interestingly, the majority of genes expressed in common between *cpr5* and *edr1* were not suppressed by the *npr1* mutation in the *cpr5* mutant (Fig. 4B). Previous work has demonstrated that *edr1*-mediated disease resistance is depen-

dent on *NPR1* (Frye *et al*., 2001). It is possible that CPR5 functions downstream of NPR1, or that this subset of genes is not central to *edr1*-mediated resistance. In addition, although these genes may be independent of *NPR1* in a *cpr5* background, they may still require *NPR1* in the *edr1* background. Further experiments are required to understand the signalling pathways controlling the expression of this subset in *edr1*.

The elevated levels of ROS and ROS-associated gene expression in *edr1* plants suggest that ROS may play a role in *edr1* phenotypes, including enhanced sensitivity to drought (Tang *et al*., 2005). Recently, mutations in an *EDR1*-like gene in rice, designated *DSM1* (*drought-hypersensitive mutant1*), have been shown to confer a similar enhanced sensitivity to drought (Ning *et al*., 2010). This drought sensitivity correlated with an increased sensitivity to oxidative stress and a reduction in the expression of two peroxidase genes and in peroxidase activity during drought stress. Similar to the *edr1* mutant, transcriptome analysis of the *dsm1* mutant revealed a large number of genes (678) whose expression was significantly upregulated during stress, suggesting that the *dsm1* mutation may cause large disruptions to cellular homeostasis under stress conditions.

The set of *edr1*-upregulated genes should include the genes directly responsible for the enhanced disease resistance phenotype of *edr1* mutant plants. A particularly intriguing gene family identified in our dataset is the FAD-binding domain family, which is related to the sunflower *Ha-CHOX* gene. Ha-CHOX was identified for its antimicrobial properties and was found to have carbohydrate oxidase activity, which is the ability to convert glucose into H_2O_2 (Custers *et al*., 2004). This class of protein may act to produce ROS that can act as either signalling molecules or as agents of cell death. ROS are produced in the cell during many different processes, including photosynthesis and defence responses (Apel and Hirt, 2004). During defence responses, ROS can be produced by a variety of proteins, including NADPH oxidases [also known as respiratory burst oxidase homologue (Rboh)] in the plasma membrane and peroxidases present in the apoplast (Allan and Fluhr, 1997; Torres *et al*., 2005; Vera-Estrella *et al*., 1992). H_2O_2 production has been linked to programmed cell death (PCD) in response to pathogen (Dangl and Jones, 2001). However, overexpression of the Rboh protein AtRbohD limits PCD in response to *P. syringae* DC3000 and *Botrytis cinerea* (Torres *et al*., 2005). It appears that, in this case, ROS may act instead as a signalling molecule, delineating the area of infection. At the time of its discovery, Ha-CHOX represented a new class of oxidase for the production of ROS with glucose as a substrate (Custers *et al*., 2004). This family of proteins represents another pathway for the production of ROS, and may contribute to the observed resistance of *edr1* plants to powdery mildew infection.

Two classes of transcription factor were over-represented significantly in the *edr1*-upregulated dataset: WRKYs and AP2/ERFs. Both WRKY and AP2/ERF family transcription factors have been shown previously to be induced during defence responses and are known to induce defence-related genes (Buttner and Singh, 1997; Eulgem and Somssich, 2007; Song *et al*., 2005). Consistent with the over-representation of WRKY transcription factors, promoter scanning of the *edr1*-upregulated genes showed them to be enriched for W-boxes. Surprisingly, however, the same gene set had a lower than average frequency of AP2/ERF binding sites (GCC-boxes), possibly indicating that the upregulated AP2/ERFs function as transcriptional suppressors.

WRKY transcription factors have long been associated with the control of defence gene induction. The WRKY-box was originally identified in the promoters of PR genes from parsley (Rushton *et al*., 1996). Most WRKYs are transcriptional activators, but there is evidence that some can also act as repressors of transcription (Eulgem and Somssich, 2007). Two of the WRKY genes identified in the *edr1*-upregulated dataset, *WRKY38* and *WRKY59*, have also been shown to be induced by the overexpression of NPR1 and by treatment with the SA analogue benzothiadiazole *S*-methylester, supporting a role for these two transcription factors in defence responses (Wang *et al*., 2006). Another WRKY present in the dataset, *WRKY75*, has been shown to be a regulator of phosphate uptake in roots and is induced under phosphate-deficient conditions, indicating a role for this gene in nutritional stress responses (Devaiah *et al*., 2007).

AP2/ERF transcription factors were originally identified as proteins that modulated transcription in response to ET. In addition to proteins that are responsive to ET, ERF domain-containing transcription factors can also be activated in response to pathogen, such as Pti4 from tomato (Chakravarthy, 2003), and in response to drought, such as DREB2A (Sakuma *et al*., 2006). Although none of the AP2/ERF genes identified in our dataset have a function yet ascribed, their similarity to other AP2/ERF transcription factors suggests that they may also be involved in defence- or drought-related responses.

The promoters of several of the *edr1*-upregulated transcription factors contain W-boxes, indicating that they may be regulated by a positive feedback loop (data not shown), enabling a rapid response to even slightly elevated levels of these proteins. We hypothesize that EDR1 may function to regulate the level of these proteins by phosphorylation, which would then target them for proteasome-mediated degradation. This model is supported by our localization studies, which showed that at least a fraction of the EDR1 protein was localized to the nucleus (Fig. 3), where it could interact with these transcription factors directly. A similar model has been proposed for the CTR1 kinase (Gagne *et al*., 2004; Guo and Ecker, 2003; Potuschak *et al*., 2003), which belongs to the same kinase subfamily as EDR1 (Frye *et al*., 2001). CTR1 regulates the level of the EIN3 transcription factor via direct or indirect phosphorylation of EIN3 on a specific threonine residue (T592; Yoo *et al*., 2008), which promotes its degradation

by the proteasome (Gao *et al*., 2003). In this context, it is worth noting that, like EDR1, CTR1 has been localized to the ER, where it is associated with ET receptors (Gao *et al*., 2003); thus, if CTR1 phosphorylates transcription factors directly, it may also need to move between a membrane complex and the nucleus. Alternatively, it has been proposed that CTR1 may activate a mitogen-activated protein kinase pathway which then leads to the phosphorylation of EIN3 on T592, but this remains to be shown (Yoo *et al*., 2008). A function for EDR1 and CTR1 in the nucleus is further supported by the finding that DSM1 from rice, which belongs to the same subfamily of kinases as EDR1 and CTR1, is primarily located in the nucleus (Ning *et al*., 2010).

Like CTR1, the majority of EDR1 protein appears to be associated with ER (Fig. 3). The significance of this localization is not yet clear, but is consistent with the GO analyses, which showed that genes associated with secretion and the endomembrane system are highly enriched in the *edr1*-upregulated dataset (Table S2).

That EDR1 may regulate the levels of transcription factors by targeting them to the proteasome is supported by our previous finding that all *edr1*-mediated phenotypes can be suppressed by a missense mutation in the *KEG* gene, which encodes a RING-finger E3 ubiquitin ligase (Wawrzynska *et al*., 2008). Null mutations in KEG have been shown to cause elevated levels of the ABI5 transcription factor, a central regulator of ABA signalling during post-germinative growth, and KEG and ABI5 can physically interact (Stone *et al*., 2006). These data suggest that KEG may ubiquitinate ABI5, targeting it for proteasome-mediated degradation. ABI5 cannot be the only target of KEG, however, as an *abi5* null mutation only partially suppresses a *keg* null mutation (Stone *et al*., 2006). We have proposed a model whereby EDR1 is responsible for the phosphorylation of at least a subset of transcription factors that are KEG substrates, and it is this phosphorylation that promotes the association with KEG (Wawrzynska *et al*., 2008). The transcription factors identified in the present study as being upregulated by the *edr1* mutation represent candidates for testing this model.

EXPERIMENTAL PROCEDURES

Plant growth and inoculation conditions

Arabidopsis thaliana Col-0 and *edr1* seeds were sown on Metromix soil and placed at 4 °C for 3 days. Plants were then transferred to a growth room and grown under 9 h of daylight at a temperature of 23 °C. After 4 weeks, the plants were inoculated with *G. cichoracearum* using a settling tower approximately 1 m tall. Plants to be inoculated were placed at the bottom of the tower, which contained a Nytex mesh screen at the top. Four *pad4* mutants with heavy powder growth were passed over the mesh 20 times each to transfer the spores to the plants below. The spores were allowed to settle for 30 min and the plants were transferred to growth chambers.

Tissue collection and RNA preparations

Tissue was collected for each time point (0, 18, 36 and 96 h) by harvesting four full rosettes per genotype per biological replicate. Four biological replicates were collected and placed in liquid nitrogen. Tissue was ground with a mortar and pestle and used for RNA preparations. High-quality RNA was prepared using the Spectrum Plant Total RNA Kit (Sigma, St. Louis, MO, USA) and concentrated to >0.75 μg/μL with the RNEasy MinElute Cleanup Kit (Qiagen). RNA was then frozen in liquid nitrogen and shipped to the Center for Medical Genomics at the Indiana University School of Medicine, Indianapolis, IN, USA.

Transcriptome analyses

First-strand cDNA synthesis, biotinylated cRNA synthesis, hybridization to Affymetrix ATH1 GeneChips® and chip scanning were carried out using the facilities of the Center for Medical Genomics at the Indiana University School of Medicine, Indianapolis, IN, USA. Data were processed using the Affymetrix MAS5 algorithm. Data analysis was carried out using ArrayAssist software (now sold under the name GeneSpring GX from Agilent Technologies, Santa Clara, CA, USA) and Genevestigator (https://www.genevestigator.ethz.ch/) (Hruz *et al*., 2008). Data were normalized using the GC-RMA algorithm and $\log_2$-transformed using ArrayAssist. Genes whose expression was at least two-fold greater in *edr1* than wild-type Col-0 for any time point with $P \leq 0.05$ using the asymptotic computation were selected. We also generated separate lists of genes that were induced at least two-fold after inoculation with *G. cichoracearum* in wild-type and the *edr1* mutant. Each list of genes was then subjected to correction for multiple testing errors using the Benjamini–Hochberg method, and genes with a corrected $P \leq 0.05$ were selected, representing a false discovery rate of less than or equal to 5% (Benjamini and Hochberg, 1995). As a control for our promoter analyses, we also selected a set of genes unresponsive to either *G. cichoracearum* or the *edr1* mutation (the unchanged dataset), defined as all genes whose fold change was less than 1.155 (up or down) in all comparisons. The 1.155-fold change value was chosen in order to create a gene set of approximately the same size as the *edr1*-upregulated gene set.

For GO analyses, we used the ArrayAssist program to identify GO terms that were significantly enriched ($P \leq 0.05$) in the set of genes upregulated in *edr1* at each time point by at least two-fold relative to the wild-type (blue circle in Fig. 1). For bicluster analysis of the *edr1&pm*-upregulated genes, we used the BiMax algorithm within the web-based program Genevestigator V3 (Hruz *et al*., 2008; Prelic *et al*., 2006). Because the BiMax

algorithm is limited to the analysis of 100 genes at a time, we divided our set of *edr1&pm*-upregulated genes (yellow circle in Fig. 1) into five groups and analysed each group independently. Each group was subjected to BiMax cluster analysis with discretization set to 1.0 (Prelic *et al*., 2006). The 'stimulus' and 'mutant' microarray datasets within Genevestigator were analysed separately.

Promoter analyses

One kilobase regions upstream of the ATG start codon were collected for all the genes in the *edr1*-upregulated dataset and the unchanged dataset using The Arabidopsis Information Resource (TAIR) bulk sequence retrieval tool (http://arabidopsis.org/tools/bulk/sequences/index.jsp). The promoter regions were scanned for six letter words using the TAIR motif analysis tool (http://arabidopsis.org/tools/bulk/motiffinder/index.jsp).

Construction of EDR1-sYFP fusion proteins and subcellular marker proteins

To make translational fusions of EDR1 to sYFP2 (Kremers *et al*., 2006), a full-length *EDR1* cDNA without the stop codon and an *sYFP2* cDNA with a stop codon were cloned into pDONR P1-P4 and pDONR P4r-P2 Gateway-compatible vectors (Invitrogen, Carlsbad, CA, USA), respectively. The sequences of EDR1 and sYFP in the respective vectors were verified and the two pDONR vectors with EDR1 and sYFP were recombined into the pTA7002-GW destination vector (Aoyama and Chua, 1997; McNellis *et al*., 1998), using multisite Gateway cloning technology from Invitrogen, to generate a dexamethasone-inducible EDR1-sYFP fusion protein construct. A similar cloning strategy was used to generate the dexamethasone-inducible nuclear marker protein GCN5-mCherry (Bhat *et al*., 2004). An ER marker was created by combining the signal peptide of AtWAK2 at the N-terminus of mCherry and the ER retention signal His–Asp–Glu–Leu at its C-terminus (Nelson *et al*., 2007). To generate an EDR1-sYFP construct expressed under the native EDR1 promoter, approximately 1.5 kb of *EDR1* 5′ sequence was inserted into the binary vector pMDC32-HPB in place of the 35S promoter in this Gateway-compatible vector (Qi and Katagiri, 2009). EDR1-sYFP was then recombined into the resulting vector as described above. A stop codon was included after the sYFP sequence; thus the HPB tag was not added.

Subcellular localization of EDR1

Fusion proteins were transiently expressed in leaves of *N. benthamiana* using agroinfiltration as described previously (Ade *et al*., 2007). For dexamethasone-inducible constructs, leaves were imaged 24 h after the application of 50 μM dexamethasone. Intracellular fluorescence was observed by confocal laser scanning microscopy using a Leica SP5 AOBS inverted confocal microscope (Leica Microsystems, Bannockburn, IL, USA) equipped with argon ion (458-, 476-, 488-, 496- and 514-nm laser lines) and He-Ne (561-nm laser line) lasers and a Leica 63X NA1.2, HCX PL APO, water objective (Part# 506279). sYFP (excited by the 514-nm argon laser) fluorescence was detected using the Leica AOBS system and a custom 522–545-nm bandpass emission filter, whereas mCherry (excited using the 561-nm He-Ne laser) fluorescence was detected using the Leica AOBS system and a custom 595–620-nm bandpass emission filter.

The integrity of the EDR1-sYFP protein within *N. benthamiana* leaves was assessed by immunoblot analysis using rabbit polyclonal anti-GFP antisera (Thermo Scientific, Waltham, MA, USA).

Complementation of the *edr1* mutation with *EDR1-sYFP*

The Arabidopsis *edr1* mutant was transformed with the *EDR1* native promoter *EDR1-sYFP* construct described above using the floral dip transformation procedure (Clough and Bent, 1998). Plants containing the transgene were selected on agar plates using 30 μg/mL hygromycin. T2 generation plants were tested for complementation of drought-induced senescence and lesion phenotypes of the *edr1* mutant. Plants were grown in Metromix 360 in 4-in plastic pots under 9 h of daylight for 3 weeks with watering as needed to keep the soil moist. At 3 weeks, watering was stopped. Ten days after cessation of watering, *edr1* plants began to show yellow and brown lesions on the leaves and severe chlorosis on older leaves, whereas all leaves on wild-type Col-0 plants and *edr1* plants transformed with EDR1-sYFP remained green.

Data deposition

The raw and normalized gene expression data generated in this study have been deposited in the National Center for Biotechnology Information (NCBI) GEO expression database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE26679.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Boxshade alignment of flavin adenine dinucleotide (FAD)-binding domain proteins.

Fig. S2 Complementation of the Arabidopsis *enhanced disease resistance 1* (*edr1*) mutation with *EDR1-sYFP*.

Table S1 *edr1&pm*-upregulated genes. All genes listed showed at least two-fold higher expression in the *enhanced disease resistance 1* (*edr1*) mutant compared with the wild-type for at least one time point, and were induced at least two-fold following inoculation by *Golovinomyces cichoracearum* in wild-type and/or *edr1* mutant plants.

Table S2 Gene ontology (GO) analysis. The *enhanced disease resistance 1* (*edr1*)-upregulated gene set was analysed for enrichment of GO terms at each time point. Only GO terms that were significantly enriched for at least one time point are listed. Expanded definitions of GO terms can be found at http://www.arabidopsis.org/servlets/Search?action=new_search&type=keyword

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