

# Overexpression of a kinase-deficient form of the *EDR1* gene enhances powdery mildew resistance and ethylene-induced senescence in *Arabidopsis*

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## Summary

The *EDR1* gene of *Arabidopsis* has previously been reported to encode a Raf-like mitogen-activated protein kinase kinase (MAPKK) kinase, and to function as a negative regulator of disease resistance. A phylogenetic analysis of plant and animal protein kinases revealed, however, that plant Raf-like kinases are more closely related to animal mixed lineage kinases (MLKs) than Raf-like kinases, and are deeply divergent from both classes of animal kinases, making inferences of substrate specificity questionable. We, therefore, assayed the kinase activity of recombinant EDR1 protein *in vitro*. The EDR1 kinase domain displayed autophosphorylation activity and phosphorylated the common MAP kinase substrate myelin basic protein. The EDR1 kinase domain also phosphorylated a kinase-deficient EDR1 protein, indicating that EDR1 autophosphorylation can occur via an intermolecular mechanism. Overexpression of a kinase-deficient full-length *EDR1* gene (*35S::dnEDR1*) in wild-type *Arabidopsis* plants caused a dominant negative phenotype, conferring resistance to powdery mildew (*Erysiphe cichoracearum*) and enhancing ethylene-induced senescence. RNA-gel blot analyses showed that the *35S::dnEDR1* transgene was highly transcribed in transgenic plants. Western blot analysis, however, revealed that neither the wild-type nor mutant EDR1 protein could be detected in these lines, indicating that the dominant negative phenotype may be caused by a translational inhibition mechanism rather than by a protein level effect. Overexpression of orthologous *dnEDR1* constructs may provide a novel strategy for controlling powdery mildew disease in crops.

**Keywords:** EDR1, MAP kinase kinase kinase, senescence, disease resistance, ethylene.

## Introduction

Plants have evolved sophisticated mechanisms to defend themselves against pathogen attack. To identify genes that regulate plant defense responses, we previously screened for *Arabidopsis* mutants that displayed enhanced resistance to virulent pathogens. From this screen, we identified a single mutant that displayed enhanced disease resistance (*edr1*) to *Pseudomonas syringae* and *Erysiphe cichoracearum* (Frye and Innes, 1998). Significantly, the *edr1* mutant does not display constitutive expression of defense genes and appears phenotypically normal in the absence of pathogen, indicating that the disease resistance is not caused by constitutive activation of systemic acquired resistance (Frye and Innes, 1998).

Disease resistance in plants is regulated in part by a complex set of interactions between signaling pathways induced by the plant hormones salicylic acid (SA), jasmonic

acid (JA), and ethylene (Ryals *et al.*, 1996; Thomma *et al.*, 1998, 1999). These three compounds induce distinct but overlapping sets of defense genes (Dong, 1998; Schenk *et al.*, 2000). We have previously assessed the contribution of SA, JA, and ethylene to *edr1*-mediated resistance by double mutant analyses (Frye *et al.*, 2001). The *edr1*-mediated resistance phenotype is suppressed by mutations that block SA perception (*npr1/nim1*) or reduce SA production (*eds1* and *pad4*) (Falk *et al.*, 1999; Zhou *et al.*, 1998). Similarly, the transgene *NahG*, which lowers endogenous SA levels, also suppresses the *edr1* disease resistance phenotype. In contrast, the *ein2* mutation, which suppresses all known ethylene-induced responses (Alonso *et al.*, 1999) and a subset of JA-induced responses (Penninckx *et al.*, 1998; Thomma *et al.*, 1998, 1999), does not suppress the *edr1*-enhanced powdery mildew resistance phenotype.

These data demonstrate that the *edr1*-mediated resistance phenotype is SA dependent, but JA and ethylene independent.

SA-induced defense responses may be mediated in part by a mitogen-activated protein (MAP) kinase cascade (Innes, 2001; Zhang and Klessig, 1997). MAP kinases have been implicated in many signal transduction pathways in plants, including disease resistance, hormone responses, wounding, cold, and touch (Tena *et al.*, 2001). MAP kinase cascades typically include three functionally linked kinases. The upstream kinase, a MAPKKK, activates a dual-specificity MAPKK by phosphorylation. The activated MAPKK then activates a MAPK by simultaneous phosphorylation of threonine (T) and tyrosine (Y) in the conserved TXY motif found in the activation loop between kinase subdomains VII and VIII. Activated MAPKs then phosphorylate multiple targets, often including transcription factors. In plants, two distinct families of presumptive MAPKKs have been defined based on similarities to animal proteins, the MEKK family and the Raf family (Jouannic *et al.*, 1999). Phylogenetic analyses of plant kinases indicate that these two families of MAPKKs are less similar to each other than to many non-MAPKKs (<http://plantsp.sdsc.edu>).

Direct phosphorylation of a plant MAPKK by a plant MAPKKK has not yet been shown. However, the putative *Arabidopsis* MAPKKK protein MEKK1 interacts with the *Arabidopsis* MAPKK protein MKK2 in a yeast two-hybrid assay. Furthermore, MKK2 can complement a MAPKK mutation in yeast (*pbs2D*) only when co-expressed with MEKK1, implying that MEKK1 activates MKK2 (Ichimura *et al.*, 1998). In addition, transient expression of the *Arabidopsis* MEKK1 kinase domain in mesophyll protoplasts activates the *Arabidopsis* MAPKK protein MKK5 (Asai *et al.*, 2002), suggesting that MEKK1 may have multiple MAPKK substrates. In contrast, no interactions between Raf-like plant MAPKKs and MAPKKs have been reported to date; thus, it is presently unclear whether these kinases function as classical MAPKKs.

We have recently shown that *EDR1* encodes a putative MAPKKK belonging to the Raf family (Frye *et al.*, 2001). The only other member of this family with a known mutant phenotype is *CTR1* (Kieber *et al.*, 1993). Loss-of-function mutations in *CTR1* confer a constitutive ethylene response phenotype and *ctr1* mutant plants are severely dwarfed, indicating that *CTR1* functions as a negative regulator of ethylene responses. The *edr1* mutant, however, displays no constitutive ethylene-associated phenotypes and is of normal size, suggesting that *EDR1* does not function directly in ethylene signal transduction (Frye and Innes, 1998; Frye *et al.*, 2001).

Although *EDR1* does not appear to directly regulate ethylene responses, *edr1* mutants do display an enhanced senescence phenotype in response to exogenous ethylene (Frye *et al.*, 2001). This observation suggests that *EDR1* may

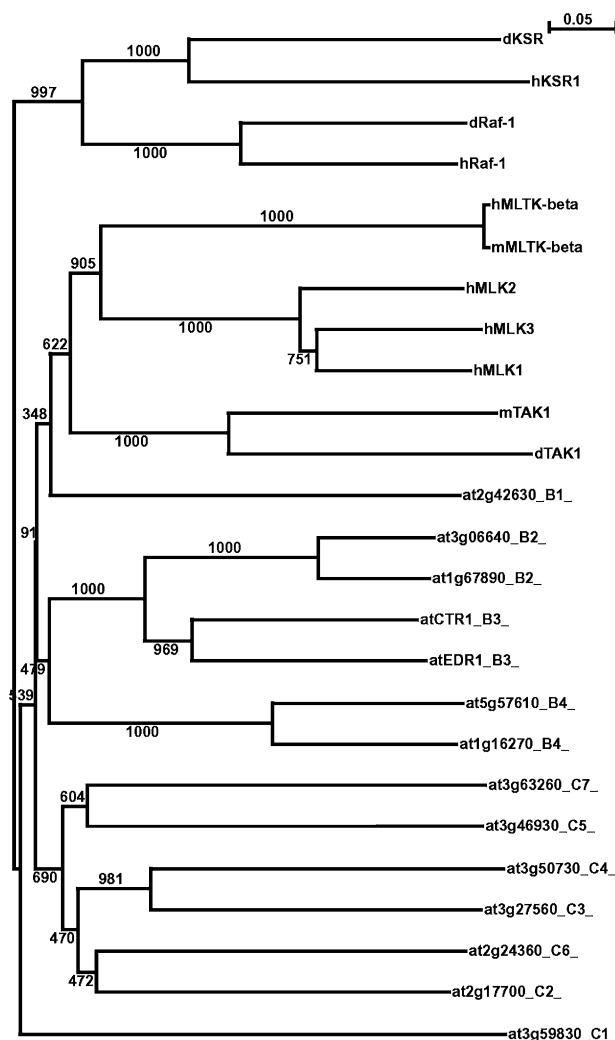
negatively regulate some aspect of the senescence program in plants. As many pathogenesis-related (*PR*) genes are upregulated during senescence (Hanfrey *et al.*, 1996; Morris *et al.*, 2000; Quirino *et al.*, 1999), *EDR1* may function in a signal transduction pathway shared between senescence- and defense-related processes.

The *EDR1* pathway is likely conserved between monocots and eudicots as clear orthologs of *EDR1* are present in maize, rice, and tomato (Frye *et al.*, 2001). Because the *edr1* mutant is phenotypically normal in the absence of pathogen but displays enhanced resistance to powdery mildew, further characterization of the *EDR1* pathway will facilitate genetic engineering of powdery mildew resistance in crops. Powdery mildews infect over 650 monocotyledonous and over 9000 dicotyledonous plant species, and cause significant economic losses in many crops (Schulze-Lefert and Vogel, 2000). Here, we report that the overexpression of a putative dominant negative form of *EDR1* in wild-type *Arabidopsis* results in enhanced powdery mildew resistance and ethylene-induced senescence. Surprisingly, the dominant negative phenotype appears to be caused by inhibition of translation. This study may have broad applications towards the engineering of disease resistance, senescence, and ethylene responses in crop plants.

## Results and discussion

### Phylogenetic analysis of the *EDR1/CTR1* kinase family

A recent analysis of all predicted MAP kinases, MAPKKs, and MAPKKKs in *Arabidopsis* places *EDR1* in the B3 subgroup of plant Raf-like kinases, consisting of six members in *Arabidopsis*, including *CTR1* (Ichimura *et al.*, 2002). Like other members of the B3 subgroup, the *EDR1* protein consists of a kinase domain in the carboxyl-terminal third and a non-kinase putative regulatory region in the N-terminal two-thirds. To gain possible insights into the function and regulation of the *EDR1* kinase domain, we aligned the kinase domains of several plant Raf-like kinases, representing all subgroups and including *EDR1* and *CTR1*, with several animal MAPKK kinases, then constructed a phylogenetic tree using the CLUSTALX program (Thompson *et al.*, 1994). The animal MAPKK kinases were selected as representative of those with the highest similarity to *EDR1* based on a BLAST search of the NCBI non-redundant protein database (Altschul *et al.*, 1997). As shown in Figure 1, all plant Raf-like kinases (representing 48 predicted kinases in *Arabidopsis*) are quite divergent from all of the animal MAPKK kinases, but are slightly more similar to the mixed lineage kinase (MLK) family (approximately 38% identity between *EDR1* and MLK3 based on a BLAST alignment (Altschul *et al.*, 1997)) than to the Raf family (approximately 35% identity between *EDR1* and hRaf). The bootstrap



**Figure 1.** Phylogenetic relationship between plant Raf-like kinases and selected animal MAPKK kinases.

This tree was derived from a comparison of the kinase domains only (see Experimental procedures). Numbers along branches indicate bootstrap values for 1000 replicates. Scale indicates amino acid substitutions per position. Prefixes on protein names indicate species of origin (at, *Arabidopsis thaliana*; d, *Drosophila melanogaster*; h, human; m, mouse). Suffixes on *Arabidopsis* proteins indicate subfamily affiliation as defined in (29).

values for this tree indicate that the relative relationships among the plant Raf-like kinases, the animal Raf kinases, and the animal MLK/TAK family cannot be resolved with confidence. Previous analyses with smaller datasets support this conclusion (Ichimura *et al.*, 1997; Jouannic *et al.*, 1999).

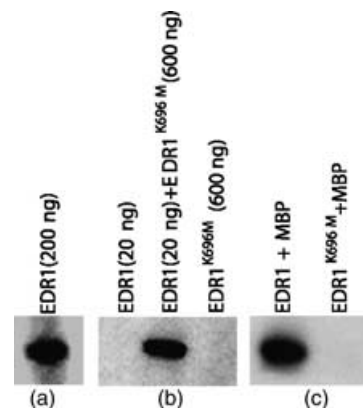
The animal Raf, MLK, and TAK protein families all possess MAPKK kinase activity (Morrison and Cutler, 1997; Shirakabe *et al.*, 1997; Tibbles *et al.*, 1996), possibly suggesting that the plant Raf-like kinases will also function as MAPKK kinases. However, members of the MLK family

also phosphorylate non-MAPKK substrates. For example, human MLK3 regulates activation of the NF $\kappa$ B transcription factor via phosphorylation of the I $\kappa$ B kinase complex (Hehner *et al.*, 2000). This observation suggests that one cannot predict substrate specificity based solely on similarity within the kinase domain.

There are no detectable regions of similarity between EDR1 and animal kinases outside the kinase domain. Searches of the GENBANK non-redundant protein database with both BLAST and PSI-BLAST algorithms (Altschul *et al.*, 1997) using the complete non-kinase portion of EDR1 failed to identify any proteins of significant similarity in organisms other than angiosperms, suggesting that the EDR1/CTR1 kinase family is unique to flowering plants. These analyses suggest that it is misleading to classify the EDR1/CTR1 kinase family as either Raf-like or MLK-like, and therefore it should not be inferred that these proteins will function as MAPKK kinases.

#### *The EDR1 kinase domain can autophosphorylate and can phosphorylate myelin basic protein*

To assay EDR1 kinase activity *in vitro*, we expressed the EDR1 kinase domain using an *Escherichia coli* expression system (NEB pMAL vector system). Using a standard *in vitro* kinase assay, we found that the EDR1 kinase domain can phosphorylate itself (Figure 2a). To determine whether the autophosphorylation site(s) was on EDR1, or on the maltose-binding protein fused to EDR1, we also tested for



**Figure 2.** EDR1 displays kinase activity *in vitro*.

(a) EDR1 autophosphorylates. Purified recombinant EDR1 kinase domain-maltose-binding protein (EDR1-kd) was incubated in a kinase assay buffer containing  $\gamma$ -<sup>32</sup>P-ATP, then separated on an SDS polyacrylamide gel and autoradiographed.

(b) EDR1 can autophosphorylate via an intermolecular mechanism. Lane 1 contains 20 ng EDR1-kd, which produces an autophosphorylation signal that is undetectable at this exposure. Lane 2 contains the same amount of EDR1-kd plus 600 ng of EDR1<sup>K696M</sup>-kd, which contains a substitution in the ATP binding domain. Lane 3 contains 600 ng of EDR1<sup>K696M</sup>-kd only.

(c) EDR1 phosphorylates myelin basic protein. Lane 1 contains 12.5  $\mu$ g myelin basic protein plus 200 ng EDR1-kd. Lane 2 contains 12.5  $\mu$ g myelin basic protein plus 200 ng EDR1<sup>K696M</sup>-kd.

phosphorylation of maltose-binding protein directly. Both the commercial maltose-binding protein and an *E. coli*-expressed maltose-binding protein (*E. coli* was transformed with the pMAL c2G vector and the protein was induced and affinity purified) were tested as potential substrates, and neither were phosphorylated by EDR1 (data not shown). These data demonstrate that EDR1 can phosphorylate itself and that the autophosphorylation site lies within the EDR1 kinase domain (amino acids 658–933).

To further characterize the kinase activity of EDR1, we investigated whether EDR1 autophosphorylation is via an inter- or intramolecular mechanism. We expressed a kinase-deficient C-terminal EDR1 maltose-binding fusion protein. In this kinase-deficient fusion protein (K696M), a conserved lysine residue in kinase subdomain II was substituted by a methionine, which disrupts the ATP binding domain. The kinase-deficient C-terminal EDR1 fusion protein cannot phosphorylate itself (Figure 2b). As shown in Figure 2(b), the C-terminal EDR1 wild-type protein was able to phosphorylate the kinase-deficient EDR1 protein, demonstrating that EDR1 can autophosphorylate via an intermolecular mechanism.

Myelin basic protein is a substrate often used to assay MAP kinase activity, and is known to be a substrate of many other kinases as well (Eichberg and Iyer, 1996). We therefore conducted a kinase assay using myelin basic protein as substrate. Myelin basic protein was strongly phosphorylated by the wild-type EDR1 fusion protein, but not by the kinase-deficient form (Figure 2c).

#### *Complementation of the edr1 mutant with only the EDR1 kinase domain*

Because the *in vitro* kinase assays were done with only the kinase domain of EDR1, we can conclude that the N-terminal two-thirds of EDR1 is not needed for kinase activity. To determine whether the non-kinase domain of EDR1 is needed for EDR1 function in whole plants, we attempted to complement the *edr1* mutation with a kinase domain-only construct under control of a strong constitutive promoter (cauliflower mosaic virus (CaMV) 35S). Tests of first and second generation (T<sub>1</sub> and T<sub>2</sub>) transformants for powdery mildew resistance indicated that this construct failed to complement the *edr1* mutation. To determine whether this was due to a failure of the protein to function properly, or a failure of the transgene to express, we performed RNA-gel blot analysis and Western blot analysis on T<sub>3</sub> homozygous lines. We were unable to detect either mRNA or protein corresponding to the EDR1 kinase domain construct. These data indicate that overexpression of the EDR1 kinase domain by itself may be lethal to *Arabidopsis* as the only transgenic lines we could recover were those in which the EDR1 kinase domain transgene failed to express.

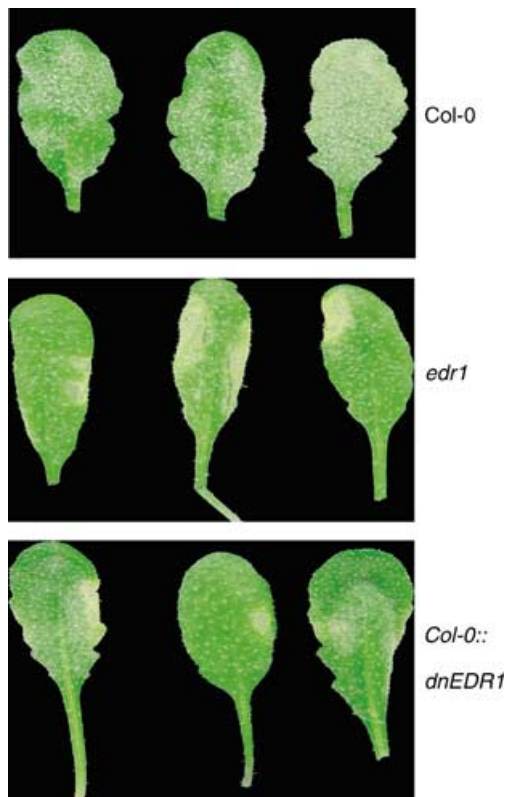
#### *Overexpression of a kinase inactive version of EDR1 enhances powdery mildew resistance and ethylene-induced senescence*

Overexpression of kinase-deficient forms of MAPKKs such as *Drosophila* and human Raf protein and tobacco NPK1 causes a dominant negative effect (Kolch *et al.*, 1991; MacNicol *et al.*, 1993; Nishihama *et al.*, 2001). To test a new strategy for genetic engineering of disease resistance in crop plants, we overexpressed a full-length, kinase-deficient *EDR1* gene (K696M) in wild-type *Arabidopsis* plants under control of a CaMV 35S promoter. To assess the dominant negative effect of EDR1<sup>K696M</sup>, three independent transgenic T<sub>3</sub> homozygous lines (35S::*dnEDR1*) in the wild-type Col-0 background were inoculated with *E. cichoracearum*. All three lines phenocopied the *edr1* mutant, showing the same necrosis and enhanced disease resistance in response to powdery mildew infection (Figure 3; data not shown). A vector-only control had no effect on the Col-0 plants (data not shown).

Previously, we observed that *edr1* mutants display an enhanced ethylene-induced senescence response (Frye *et al.*, 2001). To investigate whether the *dnEDR1* plants also phenocopy this enhanced senescence phenotype, we exposed 6-week-old Col-0 wild-type *edr1* mutant and the *dnEDR1* transgenic lines to 100 ppm ethylene for 3 days. The oldest two leaves in wild-type Col-0 begin to yellow after 3 days. As described previously, in the *edr1* mutant, this yellowing is visible in much younger leaves. All three of the independent Col-0 35S::*dnEDR1* transgenic lines showed an ethylene response similar to *edr1* plants (Figure 4a). Quantification of chlorophyll in leaves five through eight (leaf 1 being the oldest true leaf) revealed an approximately fivefold lower level of chlorophyll in Col-0 35S::*dnEDR1* than in wild-type Col-0 lines (Figure 4b). There was no significant difference between Col-0 35S::*dnEDR1* and *edr1* plants. Vector only control transgenic lines displayed no difference from wild-type Col-0 plants in this assay (data not shown).

#### *The endogenous EDR1 protein is absent from dnEDR1 transformants*

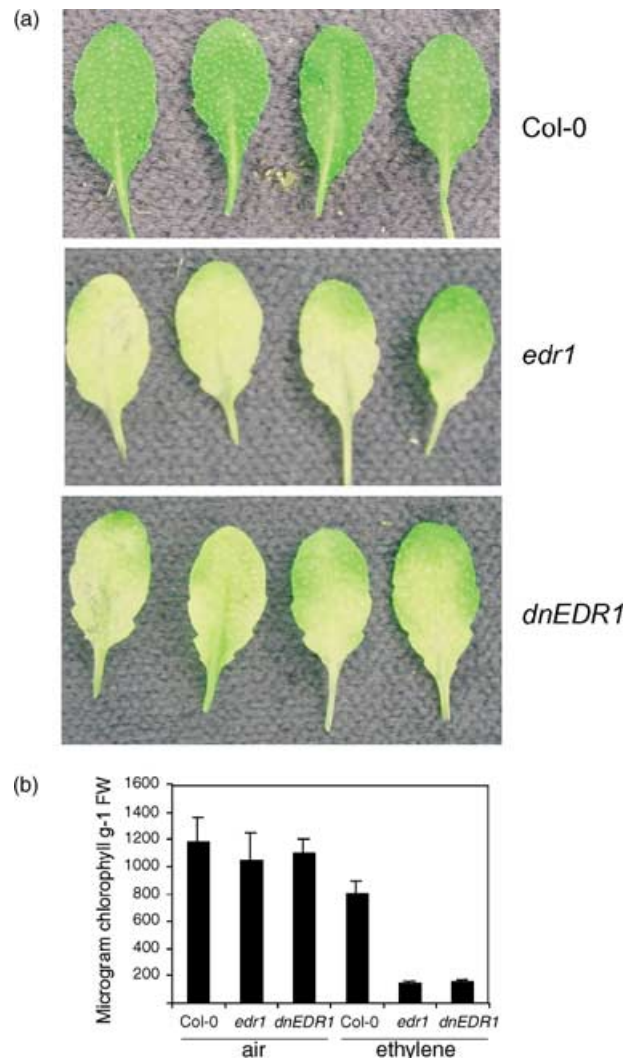
To investigate the mechanism of the dominant negative effect, we analyzed the expression of *EDR1* in the transgenic *dnEDR1* lines. RNA-gel blot analysis showed that *EDR1* was highly transcribed in all three Col-0 35S::*dnEDR1* transgenic lines (Figure 5a). In order to assess the protein expression of EDR1, anti-EDR1 antibody was raised against a synthetic oligopeptide corresponding to the last 16 amino acid residues of EDR1. This antiserum detected an approximately 100 kDa protein corresponding to the estimated molecular mass of EDR1 in Col-0 wild-type plants. As expected, the antibody detected no EDR1 protein in the *edr1* mutant,



**Figure 3.** Overexpression of a kinase inactive version of EDR1 enhances powdery mildew resistance. Leaves from 6-week-old plants were infected with powdery mildew (*Erysiphe cichoracearum*). Each set of three leaves is from a single representative plant. Leaves were removed for photography 8 days after inoculation. In wild-type plants, abundant asexual sporulation is observed as a white powder. In contrast, in the *edr1* mutant and Col-0 *35S::dnEDR1* plants, little to no sporulation is visible and necrotic patches are beginning to form.

which contains an early stop codon causing a C-terminal truncation (Frye *et al.*, 2001). This result demonstrates that the antibody is specific to EDR1. Surprisingly, we could detect no EDR1 protein in the *35S::dnEDR1* transgenic lines (Figure 5b), although the *EDR1* message was highly transcribed.

To further address the mechanism of the dominant negative effect of EDR1, we overexpressed EDR1 full-length wild-type cDNA in wild-type Col-0 plants using a construct identical to the *35S::dnEDR1* construct, except for the single amino acid substitution in the ATP-binding domain. Twelve independent *35S::EDR1* Col-0 transformants were analyzed for powdery mildew resistance. None displayed a visible difference from wild-type Col-0 plants (data not shown). To analyze EDR1 expression in these transformants, we conducted RNA-gel blot and Western blot analyses. These analyses revealed no obvious increase in EDR1 transcript and protein levels (Figure 5a; data not shown), indicating that the *EDR1* transgene was not being expressed. We also failed to obtain high-level expression of the *35S::EDR1*



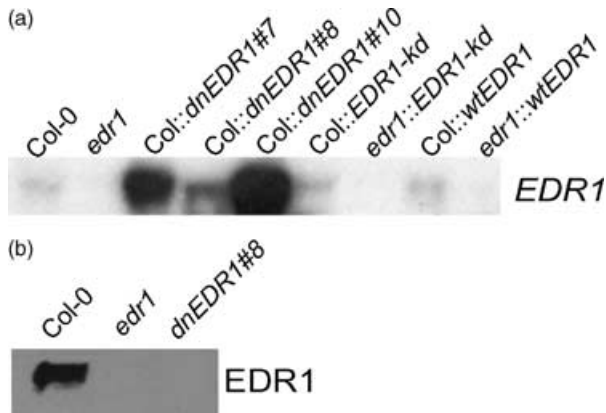
**Figure 4.** Overexpression of a kinase inactive version of EDR1 enhances ethylene-induced senescence. Six-week-old plants were placed in a sealed chamber containing 100 ppm ethylene for 3 days.

(a) Leaves five through eight (leaf 1 being the first true leaf) were removed from plants and photographed at the end of 3 days in ethylene.

(b) Chlorophyll levels in leaves five through eight. Bars represent the mean and standard deviation of values obtained from six plants.

construct when we transformed it into the *edr1* mutant genotype (Figure 5a). These data suggest that high-level expression of wild-type EDR1 protein is either lethal to *Arabidopsis*, or blocks some step in the transformation process.

The failure to obtain high-level expression of transgenes is often attributed to post-transcriptional gene silencing (PTGS) (Chicas and Macino, 2001). However, PTGS is typified by loss of mRNA corresponding to the transgene and any homologous endogenous genes (Chicas and Macino, 2001). In our *35S::EDR1* and *35S::EDR1* kinase domain transformants, the endogenous *EDR1* transcript levels appeared to be unaffected (Figure 5a), suggesting that



**Figure 5.** *EDR1* mRNA and protein levels in *dnEDR1* transgenic plants. (a) RNA-gel blot analysis of *EDR1* mRNA. Each lane was loaded with 5 µg total RNA from rosette leaves of uninoculated 6-week-old plants. Only the portion of the blot showing full-length *EDR1* message is shown; however, no signal was detected at the position expected for the kinase domain only (*EDR1*-kd) product. (b) Western blot analysis of *EDR1* protein content. Total proteins were extracted from rosette leaves of uninoculated 6-week-old plants. Transgenic lines Col::*dnEDR1*#7 and Col::*dnEDR1*#10 also displayed no detectable *EDR1* protein (data not shown).

the lack of transgene expression is caused by a pre-transcriptional block rather than PTGS. The induction of PTGS is believed to be triggered by formation of double-stranded RNA molecules homologous to the silenced gene, and likely requires an initiation phase in which the triggering RNA is expressed at high levels (Baulcombe, 2002). We speculate that during the transformation process, high-level expression of *EDR1* (either full length or kinase domain only) is not tolerated, precluding recovery of plants in which PTGS could be triggered. The apparent toxicity of the *EDR1* transgene appears to be dependent on the kinase activity of *EDR1*, as we had no difficulty recovering *35S::dnEDR1* lines that displayed high levels of *EDR1* transcript (Figure 5a).

#### Potential mechanisms leading to loss of endogenous *EDR1* protein in the *35S::dnEDR1* lines

Despite the high level of *EDR1* transcript present in the *35S::dnEDR1* lines, we failed to detect any *EDR1* protein. Although it is a formal possibility that both the *dnEDR1* and wild-type proteins are being rapidly degraded to below the detectable level in these lines, we view this as unlikely, given the abundance of the *dnEDR1* transcript. Rather, it appears that translation of both the wild-type and *dnEDR1* message is being inhibited. This would represent a here-to-fore undescribed mechanism for induction of dominant negative phenotypes.

How might translation of the *EDR1* mRNAs be inhibited in these lines? The mechanism may be similar to a recently discovered translational regulatory mechanism in animals

mediated by small temporal RNAs (stRNAs, more generally referred to as microRNAs or miRNAs) (Banerjee and Slack, 2002). stRNAs are 21–25 nucleotides long and are thought to inhibit translation by binding to complementary sequences in the 3' untranslated regions of specific target mRNAs (Reinhart *et al.*, 2000). In *Caenorhabditis elegans*, *Drosophila*, and humans, there are several different stRNA genes that are conserved (Lagos-Quintana *et al.*, 2001), each of which presumably regulates the translation of a specific target mRNA. Analogous RNAs have recently been described in plants (Llave *et al.*, 2002; Reinhart *et al.*, 2002). Both plant and animal miRNAs are transcribed as longer precursor RNAs that are processed by the RNase Dicer/DCR-1 and members of the RDE-1/AGO1 family of proteins (Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Reinhart *et al.*, 2002). These enzymes are also involved in production of small RNA molecules associated with RNA interference (RNAi) in animals and PTGS in plants (Moss, 2001). PTGS/RNAi is an evolutionarily conserved process that causes sequence-specific degradation of mRNAs, and is thought to function as a defense mechanism against RNA viruses and possibly as part of normal gene regulation during plant and animal development (Chicas and Macino, 2001). The shared requirement for Dicer and AGO proteins suggests a mechanistic relationship between PTGS/RNAi and translational inhibition by stRNAs.

There is at least one previous report of PTGS in plants where the reduction in protein levels was much more dramatic than the reduction in mRNA levels (Van Houdt *et al.*, 1997). In this study, one transgene (neomycin phosphotransferase) was silenced by the presence of an unlinked identical transgene in tobacco. Careful quantification of transgene protein and RNA levels revealed that the reduction in protein levels was significantly greater than the reduction in mRNA levels, although both were dramatically reduced. The authors inferred that much of the remaining transgene RNA in the silenced lines was non-functional, and speculated that this RNA may not be completely processed (e.g. lacking in 5' or 3' end processing). It is possible, however, that translation was being inhibited by a mechanism similar to that postulated for stRNAs in animals.

As few studies on PTGS in plants have carefully examined mRNA and protein levels in the same lines, this phenomenon may be more common than realized. Indeed, many recent studies have used green fluorescent protein (GFP) as a reporter, and have only examined the levels of product by epifluorescence, without examining RNA levels. Any discrepancies between RNA levels and protein levels would have been missed.

#### Engineering crop plants for powdery mildew resistance

Regardless of the mechanism, our data demonstrate that *EDR1* function can be effectively eliminated from *Arabidopsis*

by overexpression of a full-length kinase inactive form of EDR1. It should now be possible to eliminate EDR1 function in any crop plant by the same means. EDR1 orthologs have already been identified in tomato, rice, and barley (Frye *et al.*, 2001). A search of the EST database at NCBI revealed likely orthologs also in wheat, sorghum, soybean, and potato (data not shown). We expect that inactivation of these genes using the above strategy will produce crop plants with enhanced resistance to powdery mildew. As powdery mildew causes major losses in grape, wheat, and barley, as well as greenhouse-grown tomatoes, such resistance could be quite valuable. The potential trade-off, however, could be increased sensitivity to stresses that promote senescence. The overall benefit of suppressing EDR1 function in crop plants will need to be tested on a crop-by-crop basis.

## Experimental procedures

### Multiple sequence alignments and relationship analysis

The complete kinase domains of selected plant and animal MAPKKs were aligned using a Macintosh version of CLUSTALW (CLUSTALX 1.64b) (Thompson *et al.*, 1994) following default parameters. Relationship trees were generated using the neighbor-joining distance method and bootstrap analysis (1000 replicates) as executed by the CLUSTALX program.

### *Arabidopsis* cultivation and inoculation of *Erysiphe cichoracearum*

Plants (*A. thaliana*) were grown in growth rooms in 9 h of light per day as described previously (Frye and Innes, 1998). *E. cichoracearum* growing on wild-type Col-0 plants (8–10 days after inoculation) was used as inoculum. To inoculate plants, diseased plants were used to brush healthy 4–6-week-old plants to pass asexual spores (conidia). Presence of an *edr1*-like resistance response was scored 8 days after inoculation by assessing the presence of visible powder (conidia) and necrotic lesions.

### Ethylene-induced senescence tests

Six-week-old plants were placed in a sealed chamber containing 100 ppm ethylene for 3 days. Leaves five through eight (leaf 1 being the oldest true leaf) were then removed for photography and the chlorophyll extracted and measured as previously described (Frye *et al.*, 2001).

### Construction of EDR1 expression clones

Full-length *EDR1* cDNA (GENBANK Accession AF305913) was cloned into the pGEMT Easy vector (Promega, Madison, WI). To produce a kinase-deficient EDR1 construct, a conserved lysine residue at position 696 was substituted with a methionine (K696M) using a two-step recombinational PCR method (Higuchi, 1990). The wild-type and mutant EDR1 cDNAs were then excised from pGEMT Easy using *Apal* and *SacI* and inserted into the pBI1.4t vector, which contains a modified 35S CaMV promoter (Leister

*et al.*, 1996). For making the 35S EDR1 kinase transformation construct, primers containing *XbaI* and *SacI* restriction sites (5'-CCTCTAGAATGCCGAAACTCCACCGTGATCCTCGT-3' and 5'-CCGAGCTCCTATTGTGGTGTAGGAAGTACAAGCCG-3') were used to amplify the EDR1 residues 524–933, which includes the entire kinase domain plus 134 amino acids N-terminal of the kinase domain, from an EDR1 wild-type cDNA clone. The PCR product was digested with *XbaI* and *SacI* and inserted into the pBI1.4t vector. All clones were verified by sequencing and transformed to *Agrobacterium* strain GV3101 by electroporation.

### Plant transformation

*Arabidopsis* plants were transformed by using the floral dip method (Clough and Bent, 1998). Transgenic plants (T<sub>1</sub> generation) were selected by growing on 0.5× Murashige and Skoog salts (Gibco BRL, Rockville, MD) plus 0.7% agar and 50 µg ml<sup>-1</sup> kanamycin. T<sub>1</sub> lines were allowed to self-fertilize, and lines with single insertion loci were selected based on a 3:1 segregation of kanamycin resistance in the T<sub>2</sub> generation. Analyses were conducted on homozygous T<sub>3</sub> lines.

### Expression of recombinant EDR1 proteins

Primers containing *XbaI* and *PstI* restriction sites (5'-GCAATCTAGAGATGTTGGTGAATGTGAAATTCCTT-3' and 5'-ACTGATCTGCAGCTATTGTGGTGTAGGAAGTACAAGCCGG-3') were used to amplify the kinase domain from an *EDR1* wild-type cDNA and a kinase-deficient K696M clone. The PCR products were fused in frame to the C-terminus of the maltose-binding protein using the pMAL system (New England Biolabs, Beverly, MA). The constructs contained only the EDR1 kinase domain (from amino acids 658–933) and were confirmed by sequencing. The fusion protein constructs were transformed into *E. coli* strain DH10B. Expression of the fusion protein was induced by 0.3 mM IPTG for 2 h. *E. coli* cells were collected and suspended in column buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA) and lysed by French Press. The protein was affinity purified using amylose resin (New England Biolabs, Beverly, MA). The protein concentration was measured with a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard.

### RNA-gel blot analysis

Total RNA was isolated from rosette leaves of uninoculated 6-week-old *Arabidopsis* plants using an RNA Easy kit (Qiagen, Valencia, CA). A total of 5 µg of total RNA was separated on a denaturing formaldehyde-agarose gel. After transferring to Hybond N nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, England), RNA-gel blots were hybridized with a radiolabeled EDR1 C-terminal fragment corresponding to amino acid residues 658–933 and washed at 65°C using Church buffer (Ashfield *et al.*, 1998).

### Antibody production

A peptide corresponding to the C-terminal 16 amino acids of *EDR1* (TEVLKPLNRLVLPQP), which shows little to no similarity to the other Raf-like MAPKKs in *Arabidopsis*, was synthesized (Research Genetics, Huntsville, AL) and conjugated to keyhole limpet hemocyanin. A polyclonal antiserum in rabbit was raised against this synthetic peptide by a commercial vendor (Cocalco Biologicals, Reamstown, PA).

### Preparation of protein extracts

Leaf material was ground in liquid nitrogen and extracted in two volumes of extraction buffer (50 mM HEPES, pH 7.4, 5 mM EDTA, 5 mM EGTA, 5 mM DTT, 10 mM NaF, 10 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM β-glycerophosphate, 1 mM PMSF, 2 μg ml<sup>-1</sup> antipain, 2 μg ml<sup>-1</sup> aprotinin, and 2 μg ml<sup>-1</sup> leupeptin). The homogenate was centrifuged at 14000 rpm for 30 min at 4°C in an Eppendorf microfuge, and the supernatant used as a crude protein extract. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with BSA as the standard.

### Immunoblotting

Protein samples (20 μg total protein per lane) were separated by electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Osmonics, Westborough, MA) by wet electroblotting using a Mini-Protein II system (Bio-Rad Laboratories, Hercules, CA) in transfer buffer (10 mM Tris, 192 mM glycine, 20% methanol). The membrane was blocked by incubation in 5% non-fat dry milk in TBS buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl) for 1 h, and then incubated with a rabbit polyclonal EDR1 antibody at a dilution of 1:5000 for 2 h at room temperature and washed three times with TBS buffer. The first two washes contained 0.05% Tween 20 (TTBS). The membrane was then incubated with a horseradish peroxidase conjugated goat antirabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:10 000 for 1 h at room temperature. After two washes with TTBS and one wash with TBS, the antibody-antigen complex was visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) following the manufacturer's protocol.

### Kinase assays

All kinase assays were performed as described by Sessa *et al.* (1998) with minor modifications. Briefly, 200–500 ng of MBP fusion protein was assayed in 20 μl of kinase buffer (50 mM Tris-HCl, pH 7.0, 1 mM DTT, 10 mM MnCl<sub>2</sub>, 20 μM ATP), containing 4 μCi of γ-[<sup>32</sup>P]-ATP (6000 Ci mmol<sup>-1</sup>) (ICN Biomedicals, Costa Mesa, CA). Reactions were incubated at room temperature for 30 min. Reactions were terminated by adding 10 μl of loading buffer to the sample. The samples were then boiled for 3 min and separated on 10% SDS-polyacrylamide gels. Gels were vacuum dried at 80°C for 1 h. Kinase activity was detected by exposure to Kodak X-ray film. EDR1 intermolecular autophosphorylation was assayed by mixing MBP-EDR1 fusion protein (20 ng) and MBP-EDR1 (K696M) fusion protein (600 ng) in 20 μl of kinase buffer, and incubated at RT for 30 min as described above. Phosphorylation of myelin basic protein was assayed by mixing 12.5 μg myelin basic protein with 200 ng EDR1 fusion protein and conducting the kinase assay as described above.

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