

Regulation of Plant Disease Resistance, Stress Responses, Cell Death, and Ethylene Signaling in Arabidopsis by the EDR1 Protein Kinase¹

Dingzhong Tang, Katy M. Christiansen, and Roger W. Innes*

Department of Biology, Indiana University, Bloomington, Indiana 47405–7107

ENHANCED DISEASE RESISTANCE 1 (EDR1) encodes a *CTR1*-like kinase and was previously reported to function as a negative regulator of disease resistance and ethylene-induced senescence. Here, we report that the *edr1* mutant displays enhanced stress responses and spontaneous necrotic lesions under drought conditions in the absence of pathogen, suggesting that EDR1 is also involved in stress response signaling and cell death regulation. Double mutant analysis revealed that these drought-induced phenotypes require salicylic acid but not ethylene signaling pathways. In addition, the *edr1*-mediated ethylene-induced senescence phenotype was suppressed by mutations in *EIN2*, but not by mutations in *SID2*, *PAD4*, *EDS1*, or *NPR1*, suggesting that EDR1 functions at a point of cross talk between ethylene and salicylic acid signaling that impinges on senescence and cell death. Two *edr1*-associated phenotypes, drought-induced growth inhibition and ethylene-induced senescence, were suppressed by mutations in *ORE9*, implicating ubiquitin-mediated protein degradation in the regulation of these phenotypes. However, the *ore9* mutation did not suppress *edr1*-mediated enhanced disease resistance to powdery mildew or spontaneous lesions, indicating that these phenotypes are controlled by separate signaling pathways. To investigate the function of the EDR1 kinase domain, we expressed the C-terminal third of EDR1 in wild-type Columbia and *edr1* backgrounds under the control of a dexamethasone-inducible promoter. Overexpression of the EDR1 kinase domain in an *edr1* background had no obvious effect on *edr1*-associated phenotypes. However, overexpression of the EDR1 kinase domain in a wild-type Columbia background caused dominant negative phenotypes, including enhanced disease resistance to powdery mildew and enhanced ethylene-induced senescence; thus, the overexpressed EDR1 kinase domain alone does not exert EDR1 function, but rather negatively affects the function of native EDR1 protein.

Plants defend themselves against pathogens by many different mechanisms (Dangl and Jones, 2001). To identify genes regulating plant disease responses, we previously screened for Arabidopsis (*Arabidopsis thaliana*) mutants that displayed enhanced disease resistance to virulent pathogens. The *enhanced disease resistance 1 (edr1)* mutant displays enhanced disease resistance to the bacterium *Pseudomonas syringae* and the ascomycete fungus *Erysiphe cichoracearum* (Frye and Innes, 1998). EDR1 encodes a protein kinase with similarity to CTR1 (Frye et al., 2001), a negative regulator of ethylene responses (Kieber et al., 1993; Cao et al., 1997). The original *edr1* mutation causes a premature stop codon that eliminates the kinase domain, suggesting that EDR1 is a negative regulator of disease resistance (Frye et al., 2001). This resistance is suppressed by mutations that block salicylic acid (SA) perception (*npr1/nim1*; Cao et al., 1997; Ryals et al., 1997) or reduce SA production (*eds1* and *pad4*; Zhou et al., 1998; Falk et al., 1999), and is also suppressed by the transgene *NahG*, which lowers endogenous SA

levels (Frye et al., 2001). In contrast, the *ein2* mutation, which disrupts all known ethylene responses (Alonso et al., 1999), does not suppress *edr1*-mediated enhanced disease resistance. These data demonstrate that the *edr1*-mediated powdery mildew resistance phenotype requires an intact SA signaling pathway, but not ethylene-induced pathways (Frye et al., 2001).

In addition to the enhanced disease resistance, *edr1* also shows an enhanced ethylene-induced senescence phenotype (Frye et al., 2001). Although it has been shown that SA and ethylene signal transduction pathways may cooperate to control certain processes (Geffroy et al., 1999; Clarke et al., 2000; Greenberg et al., 2000; Wang et al., 2002), the contributions of SA and ethylene signaling pathways to the *edr1*-enhanced senescence phenotype have not been defined. Ethylene-induced senescence in Arabidopsis appears to be regulated at least in part by the F-box protein ORE9, as *ore9* mutants display a delayed ethylene-induced senescence phenotype (Woo et al., 2001). ORE9 physically interacts with one of the SCF complex components ASK1 (Arabidopsis Skp1-like 1) in vitro, suggesting ORE9 may regulate the plant senescence process by protein ubiquitylation (Woo et al., 2001). Recently, the F-box proteins EBF1 and EBF2 were shown to regulate levels of the EIN3 protein, which regulates transcription of several ethylene response element binding proteins (Guo and Ecker, 2003; Potuschak et al., 2003). Because *ctr1* mutations cause an increase in

¹ This work was supported by the National Institutes of Health (grant no. R01 GM063761).

* Corresponding author; e-mail rlnnes@indiana.edu; fax 812–855–6082.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.060400.

steady-state levels of EIN3 protein (Guo and Ecker, 2003; Potuschak et al., 2003), CTR1 may be functioning at least indirectly to regulate the rate of EIN3 ubiquitylation by EBF1 and EBF2. By analogy, EDR1 may regulate the ubiquitylation rate of a yet-to-be identified substrate of ORE9. To test this hypothesis, and to gain insight into the relationship between the ethylene-induced and pathogen-induced phenotypes in *edr1* plants, we used double mutant analyses involving *edr1*, *ore9*, and various SA pathway mutants. These analyses revealed that *edr1*-mediated disease resistance and ethylene responses are independent pathways and that ORE9 is required for a subset of the *edr1*-mediated phenotypes.

To gain additional insight into how EDR1 regulates disease resistance and ethylene-induced senescence, we transiently overexpressed the EDR1 kinase domain in planta. EDR1 consists of an N-terminal putative regulatory domain that comprises the first two-thirds of the protein and a C-terminal kinase domain that comprises the last one-third. Previously, we showed that overexpression of a kinase deficient form of the full-length *EDR1* gene enhances powdery mildew resistance and ethylene-induced senescence in Arabidopsis (Tang and Innes, 2002). However, we could not detect any EDR1 protein, although the transgene was highly transcribed, suggesting that these phenotypes resulted from inhibition of translation (Tang and Innes, 2002). The C terminus of EDR1 alone displays kinase activity (Tang and Innes, 2002). We thus attempted to complement an *edr1* mutant with the kinase domain alone (kdEDR1). We first attempted to overexpress kdEDR1 under control of the cauliflower mosaic virus (CaMV) 35S promoter. However, we were unable to recover plants that overexpressed kdEDR1 at the RNA or protein level. From this, we hypothesized that the EDR1 kinase domain may be toxic to the plant (Tang and Innes, 2002). In this paper, we overexpress kdEDR1 in both the ecotype Columbia (Col-0) and *edr1* backgrounds under the control of a dexamethasone-inducible promoter. The kdEDR1 protein did not rescue *edr1* phenotypes and surprisingly, it disrupted the native EDR1 function in wild-type Col-0 plants.

RESULTS

Drought Stress Induces Hypersensitive Response-Like Lesions on *edr1* Mutant Plants

Previously, we reported that the *edr1* mutant is phenotypically normal in the absence of pathogen when grown under optimal conditions, displaying neither microscopic nor macroscopic lesions (Frye and Innes, 1998). However, we observed that *edr1* plants appeared more sensitive than wild-type plants to underwatering and overwatering, often growing slower than wild-type plants. To characterize *edr1*-mediated stress response phenotypes, we grew plants under standard growth conditions (see "Materials and

Methods") for 3 weeks and then stopped watering the plants for 2 weeks. The *edr1* plants were significantly smaller than wild-type Col-0 plants at the end of the 2-week-drought period, despite being nearly identical in size at the start (Fig. 1A). Control *edr1* plants grown with the standard watering regime did not significantly differ in size from Col-0 at 5 weeks (Fig. 1B). In addition, *edr1* plants also developed spontaneous yellow-brown necrotic lesions under drought conditions that were similar in appearance to those induced by powdery mildew infection (Fig. 1C). To quantify the *edr1*-mediated drought-induced growth phenotype, we weighed the individual plants (fresh weight) grown under standard or drought conditions. Figure 1B shows that *edr1* mutant plants weighed almost the same as Col-0 wild-type plants when grown under standard conditions but weighed significantly less than the Col-0 wild-type plants when grown under drought conditions. These data suggest that EDR1 may also regulate plant growth and cell death in response to drought stress, in addition to its previously reported roles in disease resistance and senescence.

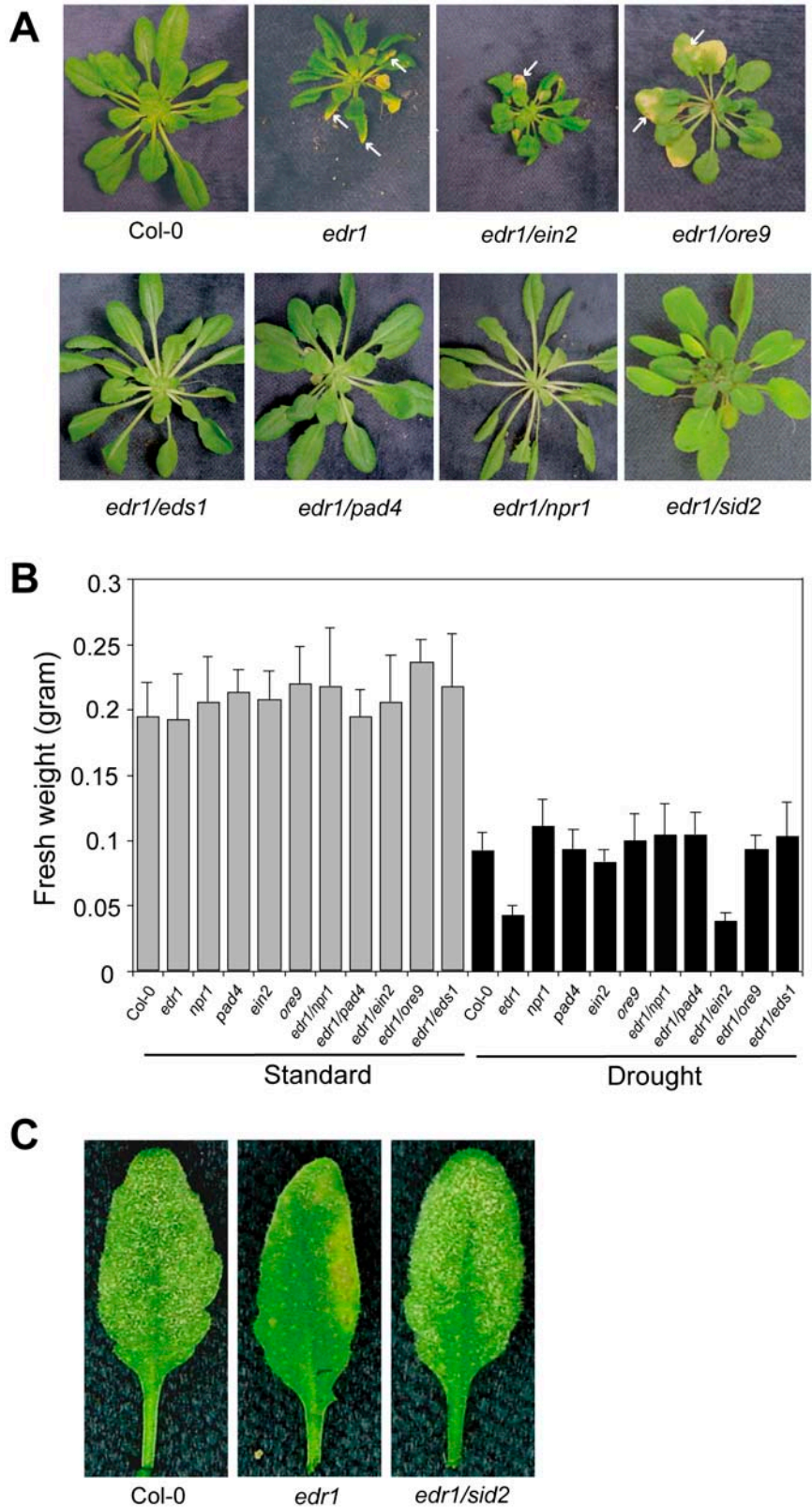
The Spontaneous Hypersensitive Response-Like Lesions in *edr1* Are SA Dependent But Ethylene Independent

To gain insight into how EDR1 regulates plant growth and cell death, we analyzed whether mutations in the SA and ethylene signal transduction pathways suppress the *edr1*-associated growth and spontaneous hypersensitive response (HR)-like phenotypes. Through double mutant analyses, we found that *eds1-1*, *nim1-1* (an allele of *npr1*), *pad4-2*, and *sid2-2* all suppressed both the *edr1*-mediated drought-induced growth and the spontaneous HR-like phenotypes, while *ein2-1* did not. These data indicate that *edr1*-mediated growth inhibition and cell death phenotypes are also SA dependent and ethylene independent (Fig. 1). This was consistent with our previous finding that *edr1*-mediated enhanced disease resistance requires SA, but not ethylene (Frye et al., 2001). We confirmed the SA requirement for *edr1*-mediated resistance by assaying resistance in the *edr1/sid2-2* double mutant, which we had not tested previously. As expected, the *sid2* mutation, which dramatically reduces SA levels in Arabidopsis (Nawrath and Metraux, 1999), suppressed the *edr1*-mediated resistance to powdery mildew (Fig. 1C). Taken together, these observations suggest that the *edr1*-mediated enhanced disease resistance, drought-induced growth, and cell death phenotypes may share the same signal transduction pathways.

edr1-Mediated Ethylene-Induced Enhanced Senescence Is EIN2 Dependent, But NPR1, PAD4, and SID2 Independent

To understand why *edr1* mutant plants display ethylene-induced enhanced senescence and to gain

Figure 1. The *edr1* mutant displays drought-induced growth inhibition and cell death phenotypes. A, Plants were grown under standard growth conditions for 3 weeks, and then watering was withheld for 2 weeks. Compared to Col-0 wild-type plants, *edr1* mutant plants were smaller and displayed spontaneous yellow-brown necrotic patches (arrows). The *edr1*-mediated drought-induced growth inhibition and cell death phenotypes were suppressed by mutations that block SA-mediated defense responses (*eds1*, *pad4*, *npr1*, and *sid2*), but not by a mutation that blocks ethylene response (*ein2*). The *ore9* mutation suppressed *edr1*-mediated drought-induced growth inhibition, but not spontaneous lesions. B, Quantification of drought-induced growth inhibition. Aerial portions were weighed immediately after removal from soil. Bars represent the mean and sd of values from 10 plants. C, *SID2* is required for *edr1*-mediated resistance to powdery mildew infection. Leaves were removed from plants for photography 8 d after infection. Abundant white powder visible on the leaves in the left and right sections indicates asexual sporulation, thus a susceptible response. The middle leaf displays yellow-brown necrotic patches and little powder, typical of an *edr1*-mediated resistance response.



insight into the cross talk between the ethylene and SA pathways, we tested the ethylene phenotypes of double mutants *edr1/ein2-1*, *edr1/npr1(nim1-1)*, *edr1/pad4-2*, and *edr1/sid2-2*. As shown in Figure 2, the *edr1*-mediated ethylene-induced senescence phenotype was suppressed by the *ein2* mutation, but not by the *npr1*, *pad4*, or *sid2* mutations. The *edr1/ein2* double mutant displayed a delayed senescence phenotype similar to the *ein2* single mutant when plants were treated with 100 ppm ethylene for 3 d. While older leaves in Col-0 plants yellowed, whole plants of *edr1/ein2* stayed green. These data suggest that the *edr1*-mediated ethylene-induced senescence phenotype functions independent of SA signaling.

A Subset of the *edr1*-Mediated Stress-Induced and Ethylene-Induced Phenotypes Require the F-Box Gene *ORE9*

To investigate whether *ORE9* plays a role in *edr1*-mediated phenotypes, we crossed *edr1* with *ore9-1* and tested the phenotypes of *edr1/ore9* double mutants. Interestingly, the *ore9* mutation suppressed the *edr1*-mediated ethylene-induced senescence (Fig. 2) and drought stress response (growth inhibition; Fig. 1), but did not affect *edr1*-mediated spontaneous lesions (Fig. 1) and the enhanced powdery mildew resistance phenotype (data not shown). These data indicate that *ORE9* is involved in at least a subset of *edr1*-mediated phenotypes and that protein degradation may play a role in EDR1 regulation of drought stress responses and ethylene-induced senescence. *ORE9* also controls shoot lateral branching, as *ore9* mutations show an enhanced shoot branching phenotype (Stirnberg et al., 2002; *ORE9* is called *MAX2* in this publication). The *edr1* mutation had no effect on the *ore9*-mediated shoot branching phenotype (data not shown).

edr1-Mediated Disease Resistance Does Not Require *NDR1* or *COI1*

To gain more insight into *edr1*-mediated disease resistance signaling, we also tested two other signaling components, *NDR1* and *COI1*, in the *edr1*-mediated disease resistance pathway. *NDR1* encodes a small, highly basic, putative membrane protein, and is required for disease resistance mediated by some CC-NBS-LRR type R genes (Century et al., 1997). *COI1* encodes a protein containing an F-box motif and is a central component of the jasmonic acid response pathway (Xie et al., 1998). Loss-of-function *coi1* mutants also display a male sterile phenotype (Xie et al., 1998). The *edr1* mutation had no effect on the *coi1*-associated male sterile phenotype (data not shown). Neither *ndr1* nor *coi1* suppressed the *edr1*-mediated enhanced powdery mildew resistance (data not shown). The failure of *coi1* to suppress *edr1*-mediated resistance is consistent with our earlier observation that *ein2* mutations also do not suppress this phenotype (Frye et al., 2001); thus, we conclude that this resistance phenotype functions independent of the jasmonic acid pathway. Table I summarizes the data obtained from the double mutant analyses.

Overexpression of the EDR1 C Terminus in Transgenic Plants

The EDR1 C terminus alone displays kinase activity *in vitro* (Tang and Innes, 2002), suggesting that the *edr1* C terminus (kdEDR1) may be responsible for EDR1 function and that the N-terminal portion of EDR1 might function as a regulator to control kinase activity. To investigate the function of the EDR1 kinase domain in planta, we overexpressed kdEDR1 in both the Col-0 and *edr1* backgrounds. Previously, we attempted to overexpress kdEDR1 using a CaMV 35S promoter, but

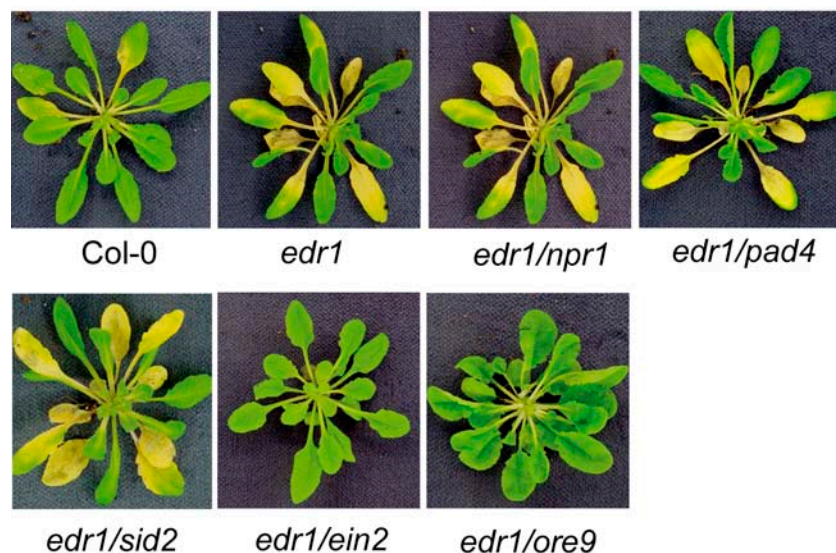


Figure 2. The enhanced ethylene-induced senescence phenotype of *edr1* mutants is *EIN2* and *ORE9* dependent, but *NPR1*, *PAD4*, and *SID2* independent. Five-week-old plants grown under standard conditions were placed in a sealed chamber containing 100 ppm ethylene for 3 d and then photographed. The *ein2* and *ore9* mutations blocked the *edr1*-mediated ethylene-induced senescence phenotype, but the *pad4*, *npr1*, and *sid2* mutations had no effect.

Table 1. Suppression of *edr1*-mediated phenotypes by other characterized *Arabidopsis* mutations

–, Did not suppress the *edr1*-mediated phenotype; +, suppressed the *edr1*-mediated phenotype; NA, not analyzed.

Mutation	Ethylene-Induced Senescence	Powdery Mildew Resistance	Drought-Induced Lesions	Drought-Induced Growth Inhibition
<i>npr1</i>	–	+	+	+
<i>pad4</i>	–	+	+	+
<i>eds1</i>	–	+	+	+
<i>ore9</i>	+	–	–	+
<i>ein2</i>	+	–	–	–
<i>ndr1</i>	–	–	–	–
<i>coi1</i>	NA	–	NA	NA
<i>sid2</i>	–	+	+	+

we were unable to detect any expression at either the RNA or protein levels in all examined Col-0 and *edr1* transgenic lines, suggesting that the EDR1 kinase domain may be toxic to plants. To overcome this problem, we used a dexamethasone-inducible system to overexpress kdEDR1. The dexamethasone-inducible promoter was fused to the C-terminal domain of EDR1, and a start codon was added in frame (see “Materials and Methods”). The construct was then transformed into Col-0 and *edr1*. Thirty and 35 T2 lines were recovered from the Col-0 and *edr1* backgrounds, respectively. The number of T-DNA insertion sites was estimated by the segregation of hygromycin resistance in T2 lines. Transgenic lines containing only one site of insertion were selected and used for further characterization. Neither Col-0 nor *edr1* transgenic plants showed any morphologic or developmental defects prior to treatment with dexamethasone.

To investigate whether overexpression of the EDR1 kinase domain is toxic, we treated transgenic plants with dexamethasone at different developmental stages. Eight Col-0::kdEDR1 lines were sown and grown on one-half Murashige and Skoog medium with or without dexamethasone. Seeds from these transgenic lines all germinated and grew normally on plates without dexamethasone. In dexamethasone containing plates (200 nM), the growth of all eight Col-0::kdEDR1 transgenic lines was inhibited to various degrees. Three of these eight lines displayed severe growth inhibition. The seedlings of these three lines became yellow and died 5 d after germination, while control nontransgenic Col-0 and vector control plants were still green and viable (Fig. 3A), demonstrating that overexpression of the EDR1 kinase domain is toxic to plants. This observation is consistent with our previous results, in which we failed to recover any stable transgenic lines overexpressing the kinase domain under the control of a CaMV 35S promoter. However, transient overexpression of the EDR1 kinase domain had no obvious effects on older plants. Five-week-old Col-0::kdEDR1 transgenic plants were sprayed with 50 μ M dexamethasone and no obvious phenotypes were observed, although the EDR1 kinase

domain was highly expressed at the protein level (data not shown).

To assess the function of the EDR1 kinase domain in powdery mildew resistance, Col-0 and *edr1* transgenic lines containing the dexamethasone-inducible kdEDR1 construct were inoculated with *E. cichoracearum*. To induce the expression of the EDR1 kinase domain, the plants were treated with 50 μ M dexamethasone after inoculation with *E. cichoracearum*. Without dexamethasone treatment, the Col-0 and *edr1* transgenic plants displayed normal powdery mildew disease responses, which were indistinguishable from untransformed Col-0 and *edr1* plants. Surprisingly, transient overexpression of the EDR1 kinase domain enhanced powdery mildew resistance on Col-0 wild-type plants, phenocopying the *edr1* mutant (Fig. 3B). Transgenic Col-0 plants carrying the vector alone were not altered in susceptibility to powdery mildew (Fig. 3B). The *edr1*::kdEDR1 lines did not show any phenotype changes. These results conflict with the hypothesis that removal of the N-terminal non-kinase domain will produce a constitutively active kinase that can exert the function of the full-length protein. Rather, these results indicate that the EDR1 N-terminal domain is required for EDR1 function and that the overexpressed EDR1 N-terminal deletion form might interfere with native EDR1.

Previously, we observed that *edr1* plants display an enhanced ethylene-induced senescence (Frye et al., 2001). When 5-week-old plants are treated with ethylene for 3 d, the oldest two leaves become yellow, however, in the *edr1* mutant, this yellowing occurs on much younger leaves. To investigate whether overexpression of the C-terminal domain of EDR1 can affect Col-0 and *edr1* ethylene responses, we exposed 5-week-old Col-0 wild-type, *edr1* and Col-0::kdEDR1 transgenic lines to 100 ppm ethylene for 3 d under 9 h light. The oldest two leaves in wild-type Col-0 yellowed. However, in *edr1*, this yellowing was visible in much younger leaves as described previously (Frye et al., 2001). Without dexamethasone treatment, the Col-0 and *edr1* kdEDR1 transgenic plants displayed ethylene responses similar to untransformed Col-0 and *edr1* plants. Dexamethasone-treated transgenic plants, however, showed an ethylene response similar to the *edr1* plants (Fig. 3C), while Col-0 lines carrying the vector only showed no significant difference from nontransgenic Col-0 plants (Fig. 3C). The dexamethasone-inducible kdEDR1 construct did not complement the *edr1*-mediated ethylene-induced senescence phenotype. These data are consistent with the results we obtained from powdery mildew tests and indicate that transient overexpression of the EDR1 kinase domain causes a dominant negative phenotype.

To investigate the mechanisms underlying the dominant negative phenotypes of the Col-0::kdEDR1 lines, we assessed EDR1 protein levels in the transgenic lines using immunoblot analyses. Figure 3D shows that the EDR1 kinase domain was highly expressed at the protein level in the kdEDR1 transgenic plants treated with

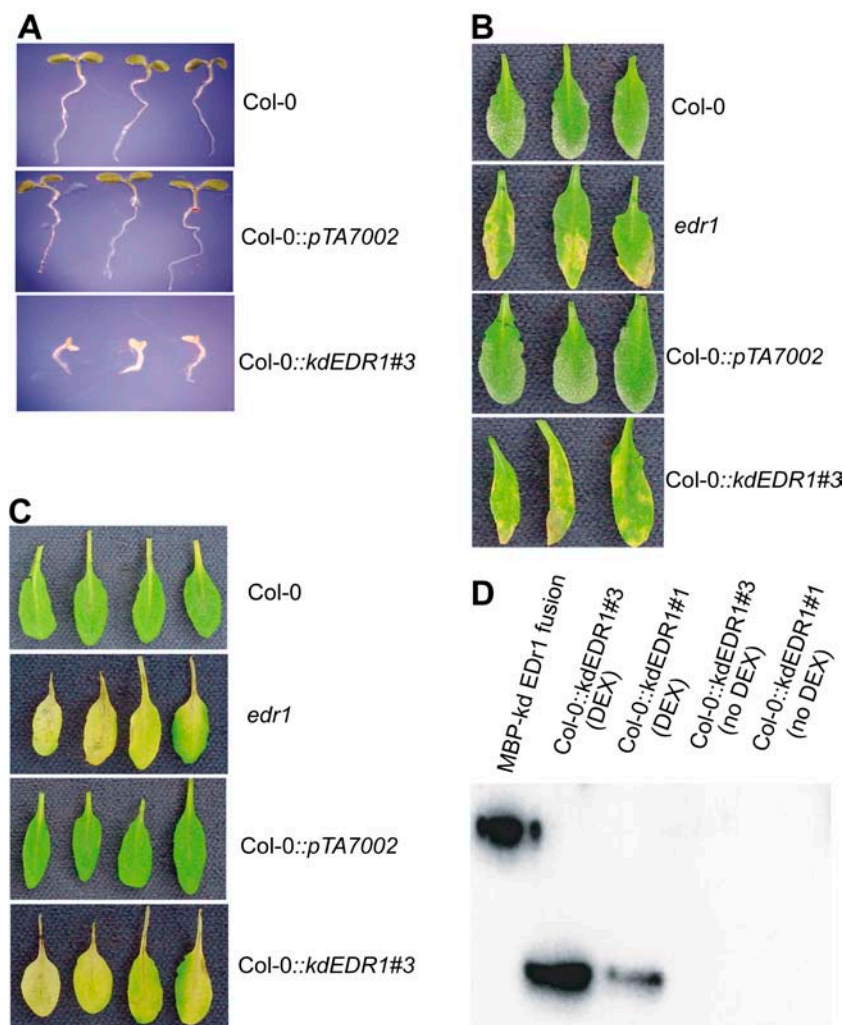


Figure 3. Overexpression of the EDR1 kinase domain. **A**, Overexpression of the EDR1 kinase domain is toxic to plants in the early development stage. Seeds from wild-type Col-0, Col-0::kdEDR1#3, and Col-0::pTA7002 vector control plants were sown and grown on one-half Murashige and Skoog medium containing 200 nM dexamethasone. Three seedlings from each line were removed from the plates and photographed 5 d after germination. **B**, Overexpression of the EDR1 kinase domain enhances powdery mildew resistance. Transgenic plants carrying the dexamethasone-inducible EDR1 kinase domain were inoculated with *E. cichoracearum* and then sprayed with 50 μ M dexamethasone. The phenotype was scored 8 d after inoculation. Wild-type and vector control plants displayed abundant spores (visible as a white powder) and lacked the yellow-brown necrotic patches observed in the *edr1* mutant and transgenic Col::kdEDR1 plants. **C**, Overexpression of the EDR1 kinase domain enhances ethylene-induced senescence. Transgenic plants were sprayed with 50 μ M dexamethasone and placed in a sealed chamber containing 100 ppm ethylene for 3 d. **D**, Immunoblot analysis of EDR1 protein content. Five-week-old plants were sprayed with 50 μ M dexamethasone and total protein was extracted from rosette leaves 3 d after spraying. As a positive control, a purified recombinant EDR1 kinase domain-maltose-binding protein fusion was included in the blot. EDR1 was detected using antiserum raised against the C-terminal 16 amino acids of EDR1 (Tang and Innes, 2002).

dexamethasone, suggesting that the dominant negative phenotypes of these lines are caused by the kinase domain interfering with full-length EDR1 function. In the absence of dexamethasone treatment, the EDR1 KD protein was not detected, demonstrating the efficacy of the EDR1 induction by dexamethasone (Fig. 3D).

DISCUSSION

While the *edr1* mutant is phenotypically wild-type in the absence of pathogen under optimal growth conditions, we observed that *edr1* mutants were stunted and displayed spontaneous lesions under drought conditions. This observation suggests that *edr1* mutants are more sensitive to environmental conditions than wild-type Col-0 plants and that EDR1 might be involved in regulating stress induced growth and cell death in addition to the previously reported disease resistance and ethylene responses.

To date, a number of mutants that display enhanced disease resistance to pathogens have been identified. Many of them constitutively display defense response

phenotypes, including spontaneous lesions and defense gene expression. Several of these are also smaller in size, indicating a correlation between defense induction and growth inhibition (Dietrich et al., 1995; Rate et al., 1999). Several mutants conditionally display microscopic lesions. These pleiotropic phenotypes also suggest a connection between defense responses and stress responses.

The link between stress and defense responses is also supported by analysis of ozone-induced responses in Arabidopsis (Rao and Davis, 1999; Overmyer et al., 2000; Rao et al., 2002). Ozone induces HR-like lesions in Arabidopsis, along with ethylene and SA biosynthesis. Significantly, ozone-induced lesions are attenuated by mutations in either the SA or ethylene signal transduction pathways, and blocks in the SA signaling pathway block ozone-induced ethylene production (Rao and Davis, 1999; Overmyer et al., 2000; Rao et al., 2002). Thus, SA appears to potentiate lesion formation directly and by enhancing ethylene biosynthesis. Drought treatment in Arabidopsis induces ethylene biosynthesis (Rao and Davis, 1999; Rao et al., 2002), but the failure of the *ein2-1* mutation to suppress

drought-induced phenotypes in *edr1* plants indicates that ethylene is not causally related to these phenotypes.

edr1-Mediated Enhanced Disease Resistance and Ethylene-Induced Senescence Are Controlled by Separate Pathways

The high similarity between the CTR1 and EDR1 protein sequences led us to investigate whether EDR1 directly regulates ethylene responses. Unlike *ctr1* mutants, *edr1* plants display a normal triple response and the ethylene-inducible genes *CHIB* and *HEL* display normal ethylene inducibility (Frye et al., 2001). However, *edr1* plants do display an enhanced ethylene-induced senescence phenotype. In addition, the defense gene *PR-1* is highly induced by ethylene in *edr1* mutant plants (Frye et al., 2001). These data suggest there is an overlap between the genetic control of ethylene-induced senescence and SA-induced defense responses. However, the *ein2* mutation has no effect on *edr1*-mediated disease resistance, although it suppresses the *edr1*-mediated ethylene-induced senescence. Furthermore, the *ore9* mutation has no effect on *edr1*-mediated disease resistance, but abolishes the enhanced ethylene-induced senescence phenotype. These data demonstrate that the *edr1*-mediated enhanced senescence and enhanced disease resistance are separate pathways requiring different components. However, it is unclear whether EIN2 and ORE9 function in the same pathway; the *ein2* mutation affects only the *edr1*-mediated ethylene phenotype, while the *ore9* mutation also affects *edr1*-mediated drought-induced growth phenotypes. This observation suggests that EIN2 may only function in the ethylene signal transduction pathway, while ORE9 may also function in other developmental processes involving EDR1.

The *edr1*-mediated disease resistance and drought-induced spontaneous lesion phenotypes appear to share the same signal transduction pathway. The *npr1*, *pad4*, *eds1*, and *sid2* mutations all suppressed these two phenotypes, while *ein2*, *ore9*, and *ndr1* mutations did not. However, the drought-induced growth phenotype was separable from the drought-induced HR-like phenotype, because the growth phenotype, but not the lesion phenotype, was suppressed by the *ore9* mutation. This observation suggests that inhibition of growth requires the function of the ORE9 protein and is a response that occurs downstream, or independent from, the cell death phenotype. Because disease resistance is maintained in the *edr1/ore9* double mutant, it appears that growth inhibition is not an obligatory cost of enhanced disease resistance.

Protein Degradation May Be Involved in *edr1*-Associated Stress Responses and Ethylene Responses

In the double mutant analysis, we demonstrated that an F-box protein, ORE9, is involved in the drought stress responses and ethylene-induced enhanced senescence. This suggests that protein degradation may

play a role in the EDR1-mediated signal transduction pathway since ORE9 has been shown to interact with ASK1, a component of the SCF ubiquitin ligase complex, in vitro (Woo et al., 2001). EDR1 may negatively regulate both stress and ethylene responses by affecting the ORE9 dependent protein degradation pathways. In this scenario, *edr1* plants can respond to stress and ethylene more readily, and these processes require ORE9 to degrade regulators involved in stress and ethylene responses. In the simplest model, wild-type EDR1 protein would function normally to phosphorylate ORE9, keeping it inactive. Loss of EDR1 function would activate ORE9, causing degradation of negative regulators of senescence, making plants more prone to environmental signals that promote senescence. Removal of ORE9 then stabilizes these negative regulators, preventing premature senescence. Alternatively, EDR1 may phosphorylate these negative regulators directly, which then prevents their binding to ORE9 and subsequent degradation. Loss of EDR1 function would thus promote binding of the regulators to ORE9 and their subsequent degradation. Loss of ORE9 would then restore stability to these negative regulators. This latter scenario is somewhat at odds with data on F-box proteins in yeast and animals, in which phosphorylation of substrates promotes binding to F-box proteins, rather than inhibiting binding (Reed, 2003).

EDR1 Function Requires More Than Just the Kinase Domain

EDR1 has significant similarity to the CTR1 protein, which encodes a negative regulator of the ethylene

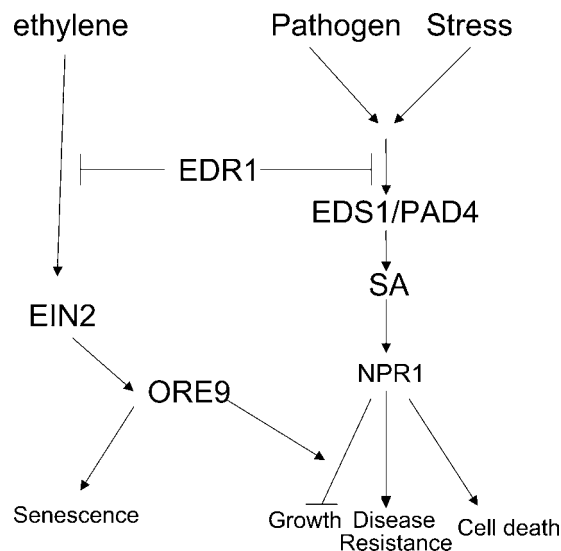


Figure 4. A model summarizing EDR1's role in programmed cell death, drought tolerance, and senescence. *E. cichoracearum* and drought induce SA accumulation, which promotes HR-like lesions. This process is negatively regulated by EDR1 to prevent inappropriate initiation of stress responses. These stress responses inhibit growth, and such growth inhibition requires both NPR1 and ORE9 function. EDR1 also negatively regulates ethylene-induced senescence, which requires the function of EIN2 and ORE9, but not NPR1, EDS1, or PAD4.

response pathway. CTR1 and EDR1 belong to the B3 subgroup of plant Raf-like kinases (Ichimura et al., 2002). Like the EDR1 protein, CTR1 displays a Ser/Thr protein kinase activity in vitro. However, unlike Raf-1, deletion of the N-terminal domain of CTR1 does not elevate the kinase activity, indicating that the N terminus does not inhibit kinase function (Huang et al., 2003). In addition, overexpression of the CTR1 N terminus causes a dominant negative phenotype. Further analysis of several mutant alleles demonstrates that the CTR1 kinase activity is required for CTR1 function (Huang et al., 2003).

Although the EDR1 C-terminal domain is highly similar to the CTR1 kinase domain and the EDR1 C-terminal domain displays protein kinase activity in vitro (Tang and Innes, 2002), it is not known whether kinase activity is required in the EDR1-mediated signal transduction pathway. The EDR1 C terminus is similar to the catalytic domain of the animal Raf protein; however, this similarity is too low to infer any substrate specificity (Tang and Innes, 2002). To investigate the function of the EDR1 kinase domain, we overexpressed the EDR1 C terminus in the wild-type and *edr1* mutant backgrounds. The EDR1 kinase domain does not complement the *edr1* mutant phenotype. Rather, it negatively affects native EDR1 function, indicating that EDR1 function is dependent upon the N-terminal regulatory region. Alternatively, the EDR1 kinase domain may compete with the EDR1 full-length protein by sequestering substrates in planta. Our results support a model for *edr1*-mediated disease resistance, ethylene-induced senescence, and drought-induced spontaneous lesion and growth phenotypes (Fig. 4). In this model, *E. cichoracearum* and stress both induce SA accumulation, which lead to formation of lesions and inhibition of growth in the absence of EDR1. Thus, the normal function of EDR1 would be to prevent inappropriate initiation of cell death, growth inhibition, and senescence.

MATERIALS AND METHODS

Plants Growth and Inoculation with *Erysiphe cichoracearum*

Arabidopsis (*Arabidopsis thaliana*) seeds were sown in pots filled with vegetable plug mix (Scotts, Marysville, OH). Seeded pots were held at 4°C for 3 d before moving to a growth room maintained at 23°C and 70% relative humidity. Plants were subjected to a 9-h-light/15-h-dark cycle with an average light intensity of 150 $\mu\text{E m}^{-2} \text{s}^{-1}$ at plant level. *E. cichoracearum* was maintained on hyper-susceptible *pad4* plants and passed to fresh plants every 8 to 10 d. To inoculate plants, diseased plants were used to brush healthy 4- to 6-week-old plants to pass asexual spores (conidia) onto the new plants. Disease phenotypes were scored 8 d after inoculation.

Drought Stress Assay

Plants were grown in growth rooms as described above for 3 weeks, then watering was withheld for 2 weeks. The aerial portions of plants were then weighed and photographed. Ten plants of each line were weighed and the mean weight was used to represent the growth phenotype.

Ethylene-Induced Senescence Assay

Six-week-old plants were placed in a sealed chamber containing 100 ppm ethylene for 3 d. The plants were scored and photographed as previously described (Frye et al., 2001).

Double Mutant Analysis

Isolation of *edr1/ein2-1*, *edr1/eds1-1*, *edr1/pad4-2*, and *edr1/npr1(nim1-1)* double mutants has been described previously (Frye et al., 2001). The *edr1/ore9-1*, *edr1/ndr1-3*, *edr1/coi1-1*, and *edr1/sid2-2* double mutants were created by standard genetic crosses. To identify double mutants, we used PCR to amplify the respective genes, which were then sequenced to identify plants that were homozygous for the mutations. All double mutant analyses was conducted on F3 homozygous lines except for *edr1/coi1-1*, which is male sterile for the double mutant. F₂ individuals homozygous for *edr1/coi1-1* were identified and assessed by PCR followed by direct sequencing.

Plant Transformation Constructs

A full-length EDR1 cDNA carried in the pGEM-T Easy vector (Promega, Madison, WI) was used as a PCR template. Primers (5'-AAGGCTCGAGATG-GATGTTGGTGAATGTGAAATTC-3' and 5'-AAGGCTAGACTAATGTTGG-TGTAGGAAGTACAAGC-3') were used to amplify the kinase domain. Restriction sites for *XhoI* and *XbaI* and a start codon ATG were incorporated in the primer. The PCR products were cloned into the pTA7002 vector, which contains a dexamethasone-inducible promoter (Aoyama and Chua, 1997). The clones were verified by sequencing and transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation.

Plant Transformation

Arabidopsis plants were transformed by using a floral dip method (Clough and Bent, 1998). Transgenic plants (T1 generation) were selected by growing on 0.5 × Murashige and Skoog salts plus 0.8% agar and 200 nM dexamethasone. Seedlings were scored 5 d after germination. Five-week-old transgenic plants were sprayed with 50 μM dexamethasone and the phenotype observed for 1 week. To test the effect of the EDR1 kinase domain on powdery mildew disease resistance, transgenic plants were inoculated with *E. cichoracearum* and then immediately sprayed with 50 μM dexamethasone. The phenotype was scored 8 d after inoculation. In the ethylene-induced senescence assay, 5-week-old plants were sprayed with 50 μM dexamethasone and placed in a sealed chamber containing 100 ppm ethylene immediately for 3 d. To prepare protein extract, plant leaves were collected 3 d after spraying with dexamethasone.

Transient Overexpression of the EDR1 Kinase Domain

The toxicity of the EDR1 kinase domain was examined in seedling and adult plants, respectively. Transgenic lines carrying DEX::kdEDR1 were sown on 0.5 × Murashige and Skoog salts plus 0.8% agar and 200 nM dexamethasone. Seedlings were scored 5 d after germination. Five-week-old transgenic plants were sprayed with 50 μM dexamethasone and the phenotype observed for 1 week. To test the effect of the EDR1 kinase domain on powdery mildew disease resistance, transgenic plants were inoculated with *E. cichoracearum* and then immediately sprayed with 50 μM dexamethasone. The phenotype was scored 8 d after inoculation. In the ethylene-induced senescence assay, 5-week-old plants were sprayed with 50 μM dexamethasone and placed in a sealed chamber containing 100 ppm ethylene immediately for 3 d. To prepare protein extract, plant leaves were collected 3 d after spraying with dexamethasone.

Antibody Production

A peptide containing the C-terminal 16 amino acids of EDR1, showing the least similarity to homologs of EDR1 in *Arabidopsis*, was synthesized and conjugated to keyhole limpet hemocyanin (Tang and Innes, 2002). A polyclonal antiserum was raised in rabbits against this synthetic peptide as previously described. (Tang and Innes, 2002). Crude serum was used for immunoblot analyses.

Preparation of Protein Extracts

Preparation of protein extracts was conducted as previously described (Tang and Innes, 2002). Briefly, 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 dithiothreitol, 10 mM NaF, 10 mM Na₃VO₄, 50 mM beta-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 $\mu\text{g}/\text{mL}$ anti-pain, 2 $\mu\text{g}/\text{mL}$ aprotinin, and 2 $\mu\text{g}/\text{mL}$ leupeptin, 10% glycerol). The extract

was centrifuged at 4,000 rpm for 30 min at 4°C, and the supernatant was used as total protein. The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as the standard.

Immunoblotting

Immunoblotting was conducted as described previously (Tang and Innes, 2002). Briefly, protein samples (20 µg total protein per lane) were separated by electrophoresis through a 10% SDS-polyacrylamide gel and protein was transferred to nitrocellulose membranes by wet electroblotting in transfer buffer (10 mM Tris, 192 mM Gly, and 20% methanol). The membrane was blocked using 5% nonfat dry milk in Tris-buffered saline (TBS) buffer (10 mM Tris-HCl, pH7.5, 100 mM NaCl) for 1 h, incubated with anti-EDR1 antisera at a dilution of 1:5,000 for 2 h at room temperature, and then washed three times with TBS buffer. The first two washes contained 0.05% Tween 20. The membrane was then incubated with anti-rabbit IgG antibody at a dilution of 1:10,000 for 1 h at room temperature. After three washes with TBS, the antibody-antigen was visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) following the manufacturer's protocol.

Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

ACKNOWLEDGMENTS

We thank H.G. Nam for providing *ore9-1* mutant seeds, J.G. Turner for providing *coi1-1* mutant seeds, and F.M. Ausubel for providing *sid2-2* seeds, J. Parker for providing F₂ seeds from a cross between *edr1* and *pad4-2* and between *edr1* and *eds1-1*, and the Arabidopsis Biological Resource Center at Ohio State University for providing *ein2-1* seeds. We also thank J. Glazebrook for critical reading of the manuscript.

Received January 31, 2005; revised March 8, 2005; accepted March 8, 2005; published May 13, 2005.

LITERATURE CITED

- Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. *Science* **284**: 2148–2152
- Aoyama T, Chua NH (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J* **11**: 605–612
- Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88**: 57–63
- Century KS, Shapiro AD, Repetti PP, Dahlbeck D, Holub E, Staskawicz BJ (1997) NDR1, a pathogen-induced component required for Arabidopsis disease resistance. *Science* **278**: 1963–1965
- Clarke JD, Volko SM, Ledford H, Ausubel FM, Dong X (2000) Roles of salicylic acid, jasmonic acid, and ethylene in *cpr*-induced resistance in Arabidopsis. *Plant Cell* **12**: 2175–2190
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16**: 735–743
- Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. *Nature* **411**: 826–833
- Dietrich RA, Delaney TP, Uknes SJ, Ward ER, Ryals JA, Dangl JL (1995) Arabidopsis mutants simulating disease resistance response. *Cell* **77**: 565–577
- Falk A, Feys BJ, Frost LN, Jones JD, Daniels MJ, Parker JE (1999) EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. *Proc Natl Acad Sci USA* **96**: 3292–3297
- Frye CA, Innes RW (1998) An Arabidopsis mutant with enhanced resistance to powdery mildew. *Plant Cell* **10**: 947–956
- Frye CA, Tang D, Innes RW (2001) Negative regulation of defense responses in plants by a conserved MAPKK Kinase. *Proc Natl Acad Sci USA* **98**: 373–378
- Geffroy V, Sicard D, de Oliveira JC, Seignac M, Cohen S, Gepts P, Neema C, Langin T, Dron M (1999) Identification of an ancestral resistance gene cluster involved in the coevolution process between *Phaseolus vulgaris* and its fungal pathogen *Colletotrichum lindemuthianum*. *Mol Plant Microbe Interact* **12**: 774–784
- Greenberg JT, Silverman FP, Liang H (2000) Uncoupling salicylic acid-dependent cell death and defense-related responses from disease resistance in the Arabidopsis mutant *acd5*. *Genetics* **156**: 341–350
- Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. *Cell* **115**: 667–677
- Huang Y, Li H, Hutchison CE, Laskey J, Kieber JJ (2003) Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in Arabidopsis. *Plant J* **33**: 221–233
- Ichimura K, Tena G, Henry Y, Zhang S, Hirt H, Ellis BE, Morris PC, Wilson C, Champion A, Innes RW, et al (2002) Mitogen-activated protein kinase cascades in plants. Nomenclature of Arabidopsis MAP kinases (MPKs) and MAP kinase kinases (MKKs). *Trends Plant Sci* **7**: 301–308
- Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. *Cell* **72**: 427–441
- Nawrath C, Metraux JP (1999) Salicylic acid induction-deficient mutants of Arabidopsis express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* **11**: 1393–1404
- Overmyer K, Tuominen H, Kettunen R, Betz C, Langebartels C, Sandermann H Jr, Kangasjarvi J (2000) Ozone-sensitive Arabidopsis *rcd1* mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death. *Plant Cell* **12**: 1849–1862
- Potuschak T, Lechner E, Parmentier Y, Yanagisawa S, Grava S, Koncz C, Genschik P (2003) EIN3-dependent regulation of plant ethylene hormone signaling by two Arabidopsis F box proteins: EBF1 and EBF2. *Cell* **115**: 679–689
- Rao MV, Davis KR (1999) Ozone-induced cell death occurs via two distinct mechanisms in Arabidopsis: the role of salicylic acid. *Plant J* **17**: 603–614
- Rao MV, Hyung-il Lee H, Davis KR (2002) Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. *Plant J* **32**: 447–456
- Rate DN, Cuenca JV, Bowman GR, Guttman DS, Greenberg JT (1999) The gain-of-function Arabidopsis *acd6* mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defenses, and cell growth. *Plant Cell* **11**: 1695–1708
- Reed SI (2003) Ratchets and clocks: the cell cycle, ubiquitylation and protein turnover. *Nat Rev Mol Cell Biol* **4**: 855–864
- Ryals J, Weymann K, Lawton K, Friedrich L, Ellis D, Steiner HY, Johnson J, Delaney TP, Jesse T, Vos P, et al (1997) The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. *Plant Cell* **9**: 425–439
- Stirnberg P, van De Sande K, Leyser HM (2002) *MAX1* and *MAX2* control shoot lateral branching in Arabidopsis. *Development* **129**: 1131–1141
- Tang D, Innes RW (2002) Overexpression of a kinase-deficient form of the *EDR1* gene enhances powdery mildew resistance and ethylene-induced senescence in Arabidopsis. *Plant J* **32**: 975–983
- Wang KL, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. *Plant Cell (Suppl)* **14**: S131–S151
- Woo HR, Chung KM, Park JH, Oh SA, Ahn T, Hong SH, Jang SK, Nam HG (2001) ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. *Plant Cell* **13**: 1779–1790
- Xie DX, Feys BE, James S, Nieto-Rostro M, Turner JG (1998) *COI1*: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science* **280**: 1091–1094
- Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J (1998) PAD4 functions upstream from salicylic acid to control defense responses in Arabidopsis. *Plant Cell* **10**: 1021–1030