Powdery Mildew Resistance Conferred by Loss of the ENHANCED DISEASE RESISTANCE1 Protein Kinase Is Suppressed by a Missense Mutation in *KEEP ON GOING*, a Regulator of Abscisic Acid Signaling[1][W][OA]

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Loss-of-function mutations in the Arabidopsis (*Arabidopsis thaliana*) *ENHANCED DISEASE RESISTANCE1* (*EDR1*) gene confer enhanced resistance to infection by powdery mildew (*Golovinomyces cichoracearum*). *EDR1* encodes a protein kinase, but its substrates and the pathways regulated by EDR1 are unknown. To identify components of the EDR1 signal transduction pathway(s), we conducted a forward genetic screen for mutations that suppressed *edr1*-mediated disease resistance. Genetic mapping and cloning of one of these suppressor mutations revealed a recessive missense mutation in the *KEEP ON GOING* gene (*KEG*; At5g13530), which we designated *keg-4*. *KEG* encodes a multidomain protein that includes a RING E3 ligase domain, a kinase domain, ankyrin repeats, and HERC2-like repeats. The KEG protein has previously been shown to have ubiquitin ligase activity and to negatively regulate protein levels of the transcription factor ABCISIC ACID INSENSITIVE5. *KEG* mRNA levels were found to be 3-fold higher in *edr1* mutant plants compared to wild type. Loss-of-function mutations in *KEG* are seedling lethal and are hypersensitive to glucose and abscisic acid (ABA). The *keg-4* mutation, in contrast, conferred resistance to 6% glucose and suppressed *edr1*-mediated hypersensitivity to ABA, suggesting that the *keg-4* mutation suppresses ABA signaling by altering KEG function. Several *ABA*-responsive genes were found to be further up-regulated in the *edr1* mutant following ABA treatment, and this up-regulation was suppressed by the *keg-4* mutation. We conclude that *edr1*-mediated resistance to powdery mildew is mediated, in part, by enhanced ABA signaling.

Powdery mildew fungi are obligate biotrophic pathogens that can grow only on living plant tissues. These pathogens must evade or suppress host defenses until their life cycle is complete. A number of Arabidopsis (*Arabidopsis thaliana*) mutants displaying enhanced disease resistance to powdery mildew (in this case, *Golovinomyces cichoracearum*) have been characterized (Frye and Innes, 1998; Vogel and Somerville, 2000; Vogel et al., 2002; Tang et al., 2005a, 2006). These mutants can be grouped into two broad classes based on the presence or absence of mildew-induced lesions. The *enhanced disease resistance1* (*edr1*), *edr2*, and *edr3* mutants typify the former class (Frye and Innes, 1998; Tang et al., 2005a, 2006). In these mutants, fungal growth is inhibited at a very late stage of the infection process and resistance correlates with a more rapid activation of host defenses relative to wild-type plants, including programmed cell death (PCD). The most striking phenotypes caused by the *edr1* mutation, besides powdery mildew-induced lesions, are enhanced drought-induced growth inhibition and enhanced ethylene-induced senescence (Frye et al., 2001; Tang et al., 2005b). The former two phenotypes require an intact salicylic acid (SA) signaling pathway, while the latter does not (Tang et al., 2005b). The general processes of PCD, drought responses, and senescence have all been linked to enhanced sensitivity to abscisic acid (ABA; Beaudoin et al., 2000; Ghassemian et al., 2000; Anderson et al., 2004; Mohr and Cahill, 2007; Xie et al., 2007), suggesting that EDR1 may be also be involved in ABA signaling (Frye et al., 2001).

ABA regulates many important events during both vegetative and reproductive growth of plants. These range from relatively slow effects, such as promotion of seed storage reserve synthesis, acquisition of desiccation tolerance and dormancy, and tolerance to drought, salt, and cold stresses (Leung and Giraudat, 1998), to rapid effects, such as stomatal closure (Leung and Giraudat, 1998; Finkelstein et al., 2002). Cumulative evidence suggests that the cross talk between ABA and SA is important for adaptation of plants to combinations of abiotic and biotic stresses (Kunkel and Brooks, 2002; Mauch-Mani and Mauch, 2005). SA

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inhibits ABA-induced stomatal closure (Rai et al., 1986), leaf abscission (Apte and Laloraya, 1982), and inhibition of seedling growth (Ray, 1986), while ABA increases susceptibility to biotrophic pathogens by counteracting SA-dependent defenses (Mohr and Cahill, 2003; de Torres-Zabala et al., 2007; Mohr and Cahill, 2007). Conversely, ABA-dependent priming of callose biosynthesis promotes enhanced resistance to some necrotrophic pathogens (Ton and Mauch-Mani, 2004).

The complex connections between SA signaling and ABA signaling are also observed during leaf senescence, which shares many physiological events with pathogen-induced defense responses, such as increases in ethylene and SA levels (Ryals et al., 1996; Morris et al., 2000), accumulation of hydrogen peroxide (Levine et al., 1994; Pastori and Del Rio, 1997), and accumulation of transcripts from pathogenesis-related (PR) genes (Hanfrey et al., 1996; Butt et al., 1998; Pontier et al., 1999; Quirino et al., 1999, 2000; Yoshida et al., 2001). ABA is considered a senescence promoter, although evidence for an in vivo role is rather poor compared with ethylene (Nooden and Leopold, 1988; Madhu et al., 1999; Panavas et al., 1999). Several mutations that inhibit defense responses in Arabidopsis also inhibit senescence (Morris et al., 2000). For example, the *pad4* mutation, which enhances disease susceptibility and reduces SA accumulation (Jirage et al., 1999), displays a dramatic delay in PCD during senescence (Morris et al., 2000). Consistent with these observations, SA levels increase approximately 4-fold in senescing Arabidopsis leaves (Morris et al., 2000). Determining cause and effect in these processes is difficult, however, as SA-signaling pathways include positive feedback loops. For example, cell death promotes SA production, but SA production also promotes cell death (Glazebrook, 2005). Accordingly, it has been proposed that high concentrations of SA, such as those generated at the sites of pathogen entry, are required for cell death induction, whereas SA at low levels, detected beyond the margins of the initial infection sites, might lead to cell survival and lesion containment (Alvarez, 2000).

Because loss of EDR1 function leads to enhanced PCD and senescence, it is considered to be a negative regulator of these processes. The EDR1 protein belongs to a small family of protein kinases in Arabidopsis that includes the CTR1 protein (Frye et al., 2001), a negative regulator of ethylene responses (Kieber et al., 1993; Cao et al., 1997). Unlike loss of CTR1 function, however, loss of EDR1 does not activate ethylene-signaling pathways (Frye et al., 2001). The specific function of EDR1 thus remains unknown. To uncover additional genes in the EDR1 kinase pathway or identify other pathways that interact with the EDR1 pathway, we performed a suppressor screen to identify mutations that suppress the *edr1* mutant phenotype. Here we describe one such suppressor mutation, which was found to be a missense mutation in the *KEEP ON GOING* (*KEG*) gene. *KEG* encodes a ubiquitin ligase thought to be involved in ABA signaling (Stone et al., 2006). Interestingly, we found that transgenic overexpression of KEG induces massive cell death in Arabidopsis.

RESULTS

The *supp69* Mutation Blocks *EDR1*-Dependent Resistance to *G. cichoracearum*

Because *edr1* mutant plants show enhanced drought-induced growth inhibition (Tang et al., 2005b), we suspected that the *edr1* mutation might be enhancing sensitivity to ABA. To test this hypothesis, we performed a seed germination assay on varying levels of ABA, which is known to inhibit germination (Finkelstein, 1994). Figure 1 shows that the *edr1* mutant is indeed hypersensitive to exogenous ABA as the percent germination at 3 d of incubation on 0.6 μM ABA was only approximately 15% for *edr1* seeds compared to greater than 50% for wild type. This enhanced ABA sensitivity suggested that we could enrich for suppressor mutants by germinating mutagenized seed on ABA-containing plates.

To enrich for *edr1* suppressor mutants, we screened an ethyl-methane sulfonate-mutagenized *edr1* population on agar plates containing 0.7 μM ABA (60,000 M2 seeds derived from 3,500 M1 *edr1* plants). Approximately 1,000 seedlings were identified that germinated within the first 3 d of incubation, a time period during which very few *edr1* mutant seeds had germinated. Seedlings were transplanted to pots containing Metromix 360. Four to 5 weeks later these plants were inoculated with *G. cichoracearum* and scored for disease responses 8 d postinoculation. Seventy-four mutants displaying visible powder were selected and their response to *G. cichoracearum* retested in the next generation. Among these, 11 mutants were found to be fully susceptible to *G. cichoracearum*, lacking *edr1*-dependent necrotic lesions and allowing abundant development of *G. cichoracearum* conidiophores. Here we describe one mutant, which was designated *supp69*.

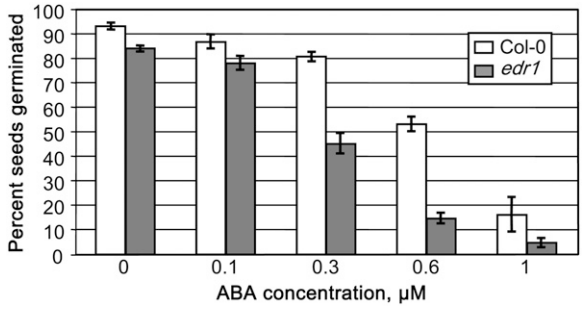

Figure 1. ABA hypersensitivity of the *edr1* mutant. Seeds of either wild-type Col-0 or the *edr1* mutant were planted on one-half-strength Murashige and Skoog agar supplemented with the indicated concentrations of ABA. The percentage of seeds germinating after 3 d is shown.

Characterization of the other mutants is ongoing and will be described elsewhere.

The *supp69* mutant displayed a wild-type Columbia-0 (Col-0)-like phenotype 8 d after infection with *G. cichoracearum* (Fig. 2). No other obvious developmental or morphological phenotypes of *supp69* plants were observed when grown under normal conditions. Complementation tests revealed that *supp69* is not allelic to *pad4* or *npr1* (data not shown), which have been previously shown to suppress the *edr1* phenotype (Tang et al., 2005b). Segregation analysis of a backcross to the *edr1* mutant revealed that susceptibility to *G. cichoracearum* was caused by a single recessive mutation. We therefore proceeded with genetic mapping of the *supp69* mutation and a detailed characterization of the *supp69* mutant phenotype.

The *supp69* Mutation Maps to Chromosome 5

Genetic mapping of the *supp69* mutation was complicated by a lack of *edr1* mutant alleles in Arabidopsis accessions other than Col-0. We therefore crossed the *supp69* mutant (*edr1-supp69*) to the Landsberg *erecta* (L*er*) accession and identified F3 families that were homozygous for the *edr1* mutation and segregating for the *supp69* mutation (see "Materials and Methods"). Twenty-eight F3 families were selected and pooled for mapping purposes. F3 plants were scored for susceptibility to *G. cichoracearum*. Susceptibility segregated in an approximately 1:3 ratio, confirming that *supp69* was caused by a single recessive mutation. DNA was isolated from 629 susceptible F3 plants and scored for microsatellite markers distributed across the Arabidopsis genome. Initially, the *supp69* mutation was mapped to a region between microsatellite markers MYH9 and nga151 on chromosome 5 (Fig. 3). To further localize the mutated gene, we created PCR-based markers at intervals between MYH9 and nga151 using small insertions/deletions that are polymorphic between L*er* and Col-0 (Jander et al., 2002). Fine-mapping localized the mutation to a 126-kb interval covering the 3′ end of bacterial artificial chromosome (BAC) clone T6I14 (GenBank accession AL391710) and the 5′ end of BAC MSH12 (GenBank accession AB006704) defined by one recombinant at each border (Fig. 3). This region harbors 31 loci (from At5g13470–At5g13750). Twenty-one of these were amplified by PCR and sequenced. A single G to A transition mutation was identified in At5g13540, which was recently found to be misannotated, with the full open reading frame encompassing both the At5g13530 and At5g13540 loci (Stone et al., 2006). The combined gene has been named *KEG* for "keep on going" (Stone et al., 2006). The *supp69* mutation was located in the 15th exon of *KEG*, and causes a Gly-to-Ser substitution (G1144S) in the HERC2-like domain of the KEG protein (Fig. 3; Stone et al., 2006).

Loss-of-function *keg* mutants display a strong post-germinative growth arrest shortly after the emergence of the first true leaves (Stone et al., 2006), indicating that KEG is essential for plant development. Because *supp69* plants show normal growth and development, we conclude that the mutation in *KEG* does not cause a complete a loss of function. As three *keg* mutants have been described previously (Stone et al., 2006), the *keg* mutation in *supp69* was designated *keg-4*.

Complementation of the *supp69* Mutation

To confirm that the mutation in At5g13530 was responsible for the suppression of the *edr1* phenotype, we transformed *supp69* plants with a genomic copy of the *KEG* gene under control of its native promoter and tested for restoration of the *edr1* mutant phenotype. Thirteen independent T1 transgenic plants were inoculated with *G. cichoracearum*. All 13 were resistant to *G. cichoracearum* and showed necrotic lesions and almost no conidiation 8 d after infection, demonstrating that the *KEG* genomic construct complemented the *keg-4* mutation (data not shown). The transgene did not cause any growth phenotypes, as all transgenic lines were indistinguishable from wild-type Col-0 plants prior to inoculation.

The *keg-4* Mutation Suppresses the Enhanced Ethylene-Induced Senescence Phenotype of *edr1*

Besides showing enhanced resistance to powdery mildew, *edr1* mutants display an enhanced ethylene-induced senescence phenotype (Frye et al., 2001). To test whether the *keg-4* mutation also suppressed this trait, we exposed plants to ethylene (100 μL L^{-1}) for 3 d. The *supp69* plants showed the same rate of senescence as Col-0 plants in contrast to the enhanced senescence

Figure 2. Suppression of the *edr1* powdery mildew resistant phenotype. Col-0, *edr1*, and *supp69* plants 8 d after powdery mildew infection. Abundant white powder visible on Col-0 and *supp69* indicates asexual sporulation, thus a susceptible response; the lower leaves of the *edr1* mutant display regions of chlorosis and necrosis and are free of visible powder.

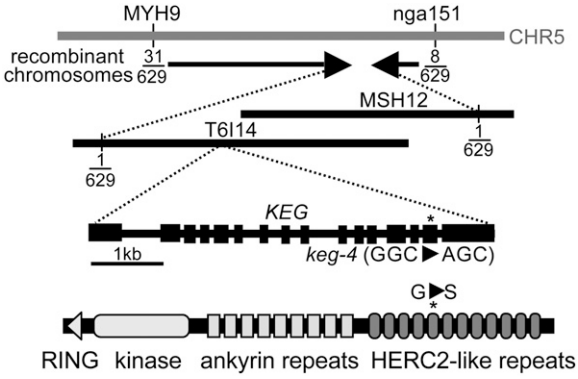

Figure 3. Positional cloning of the *supp69* mutation. The number of recombination events between the indicated markers and the *supp69* mutation over the total number of chromosomes scored is shown. Horizontal lines indicate BAC clones spanning the region to which *keg-4* was mapped. Sequencing of candidate genes in this interval revealed a G to A transition in the *KEG* gene. The genomic structure of the *KEG* gene with exons indicated by black boxes and the position of the *keg-4* mutation are shown. The domain structure of the KEG protein and the location of the Gly to Ser substitution caused by *keg-4* are also shown.

phenotype of *edr1* (Fig. 4A). To determine whether the *keg-4* mutation by itself affected senescence rates, we crossed out the *edr1* mutation by backcrossing to wild-type Col-0 plants and selecting homozygous *EDR1/EDR1 keg-4/keg-4* plants in the F2. Ethylene induced visible chlorosis (yellowing) on the oldest two to three leaves of wild-type Col-0, *supp69*, and *keg-4* plants after 3 d exposure to ethylene. However, in *edr1* mutant plants, chlorosis was observed on much younger leaves and appeared earlier (Fig. 4A). Quantification of chlorophyll levels revealed significant differences between ethylene-treated *edr1* plants and the other three genotypes (Fig. 4B). Consistent with this finding, *edr1* plants also showed a faster rate of senescence under standard short-day growth conditions, which became visibly obvious by 11 weeks of growth (Fig. 4C). We thus conclude that the *keg-4* mutation by itself does not delay senescence in a wild-type background, but fully suppresses the enhanced senescence of the *edr1* mutant.

The *keg*-4 Mutation Suppresses *edr1*-Mediated Drought-Induced Growth Inhibition

We previously reported that *edr1* plants appeared more sensitive than wild-type Col-0 plants to underwatering, often growing slower than wild-type Col-0 plants (Tang et al., 2005b). To further characterize *supp69*, we grew plants under standard growth conditions for 3 weeks and then stopped watering them for 2 weeks. The *edr1* plants were significantly smaller than wild-type Col-0, *supp69*, or *keg-4* plants at the end of the 2-week drought period, although they were the same size at the start (Fig. 5A). Control *edr1* plants grown with the standard watering regime did not significantly differ in size from Col-0, *supp69*, or *keg-4* at 5 weeks (Fig. 5A). To quantify the *edr1*-mediated drought-induced growth phenotype, we weighed the individual plants (fresh weight) grown under standard or drought conditions. Figure 5B shows that *edr1* mutant plants weighed almost the same as the other tested plants when grown under standard conditions, but weighed significantly less when grown under drought conditions. These data indicate that *keg-4* also suppresses the *edr1*-mediated drought sensitivity.

The *keg-4* Mutation Suppresses the ABA Hypersensitivity of *edr1*

The suppression of *edr1*-mediated drought sensitivity suggested that the *keg-4* mutation should also suppress the ABA hypersensitivity of *edr1* mutants. We therefore tested the *supp69* mutant for sensitivity to ABA using the seed germination assay described above. We plated seeds on Murashige and Skoog agar containing 0.7 μM ABA. As described above, this level of ABA inhibited germination of *edr1* seeds more than wild-type seeds, which resulted in noticeably smaller seedlings at 5 d of incubation (Fig. 6). The *supp69* and *keg-4* mutant seedlings were indistinguishable from wild-type plants in this assay, confirming that *keg-4* also suppresses *edr1*-mediated ABA hypersensitivity.

The *supp69* and *keg-4* Mutants Show Lowered Sensitivity to Glc

Null alleles of *KEG4* have been shown previously to confer hypersensitivity to exogenous Glc (Stone et al., 2006), which causes growth arrest of wild-type Arabidopsis seedlings when added to agar at 6% (Zhou et al., 1998; To et al., 2002). Glc influences postgerminative growth of Arabidopsis via its ability to activate ABA biosynthesis genes and consequent activation of ABA-inducible genes (Cheng et al., 2002; Finkelstein and Gibson, 2002), and many Glc-insensitive mutants are also ABA insensitive. We therefore tested whether the *keg-4* mutation affected Glc sensitivity. Significantly, the *keg-4* mutation conferred Glc insensitivity (Fig. 7). After 15 d on Glc-containing media, the *supp69* and *keg-4* seedlings showed only a slight growth inhibition relative to plants grown in the absence of Glc, while wild-type, Col-0, and *edr1* seeds showed almost no growth (Fig. 7). All mutants and wild-type plants grew similarly on plates containing 6% mannitol, a nonmetabolizable sugar, demonstrating that the response was not simply the result of osmotic stress (Fig. 7). There were also no significant differences between plants grown on Murashige and Skoog with 6% Suc (data not shown). These data indicate that the *keg-4* allele confers phenotypes opposite to that of a *keg* loss-of-function mutation (Stone et al., 2006), and further support our conclusion that the *keg-4* mutation inhibits ABA signaling.

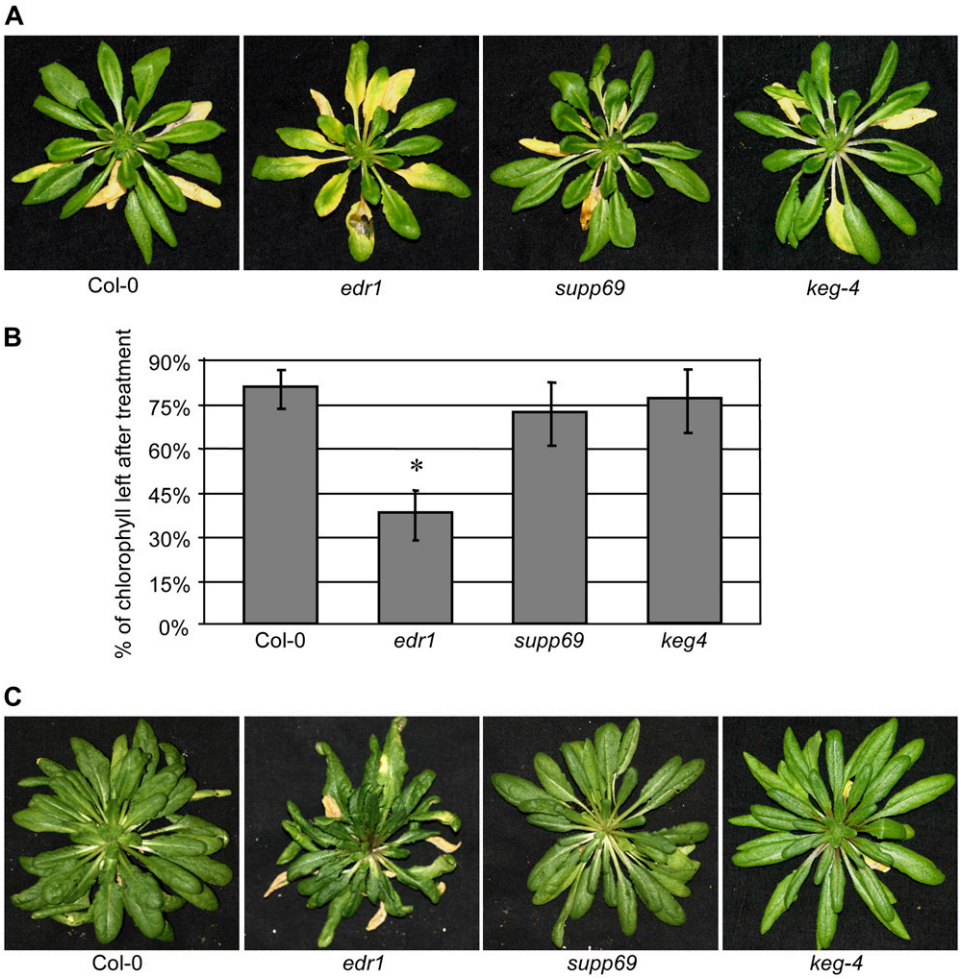

Figure 4. The *keg-4* mutation suppresses enhanced ethylene-induced senescence in the *edr1* mutant. A, Plants were photographed after 3 d of exposure to 100 μL L^{-1} ethylene. B, The chlorophyll content in leaves five to eight (leaf one being the first true leaf) of the plants shown in section A. Bars represent the relative mean and SD of values obtained from four plants in comparison to untreated controls (*, significantly different [$P < 0.001$] by one-way ANOVA with Tukey's multiple-comparison post hoc test). C, The *edr1* mutant shows signs of accelerated senescence at 11 weeks. All plants were grown under standard short-day growth conditions. Similar results were obtained in two additional independent experiments.

The *keg-4* Mutation Suppresses *edr1*-Mediated Changes in Gene Expression

ABA induces the expression of many genes that are important for adaptation to stress. Based on the *edr1* mutant phenotypes observed above and the ability of the *keg-4* mutation to suppress them, we hypothesized that EDR1 might play a role in ABA-induced changes in gene expression. To test this hypothesis, we examined the expression of *RESPONSIVE TO DESSICATION29A* (*RD29A*; At5g52310), a well-characterized ABA-inducible gene (Yamaguchi-Shinozaki and Shinozaki, 2006). In wild-type Col-0, treatment with 100 μM ABA for 3 h induced *RD29A* (Fig. 8A). Induction of this gene was greater in the *edr1* mutant, consistent with *edr1* being hypersensitive to ABA. The *keg-4* mutation fully suppressed the enhanced expression in *edr1*, indicating that *keg-4* suppresses *edr1*-mediated hypersensitivity at the level of gene induction (Fig. 8A).

We also analyzed expression of *ABSCISIC ACID INSENSITIVE5* (*ABI5*; At2g36270) and *MAP KINASE3* (*MPK3*; At3g45640). *ABI5* is a key transcription factor required for the induction of many ABA-responsive genes, and is itself inducible by ABA (Lopez-Molina et al., 2001; Brocard et al., 2002). It is most highly expressed in germinating seeds (Lopez-Molina et al., 2001; Brocard et al., 2002). *MPK3* has been linked to ABA signaling in seedlings because overexpression of *MPK3* increases ABA sensitivity in ABA-induced postgermination growth arrest (Lu et al., 2002). In addition, Arabidopsis plants with guard cell-specific silencing of *MPK3* display a partial insensitivity to ABA-mediated inhibition of stomatal opening (Gudesblat et al., 2007). Although ABA treatment does not activate MPK3 in leaf mesophyll protoplasts (Kovtun et al., 2000), ABA does appear to activate MPK3 in Arabidopsis seedlings (Lu et al., 2002). *ABI5* was highly induced by ABA treatment in both wild-type and *edr1* seedlings (Fig. 8B), indicating that there is a positive feedback loop regulating this gene. Induction appeared to be slightly enhanced in the *edr1* mutant, but this difference was not statistically significant. In contrast, *MPK3* transcript levels were suppressed approximately 2-fold in wild-type plants in response to ABA treatment, while in *edr1* seedlings *MPK3* was induced 2-fold, resulting in a 4-fold difference in *MPK3* transcript levels between wild-type and *edr1* mutant seedlings. This difference was fully suppressed by the *keg-4* mutation.

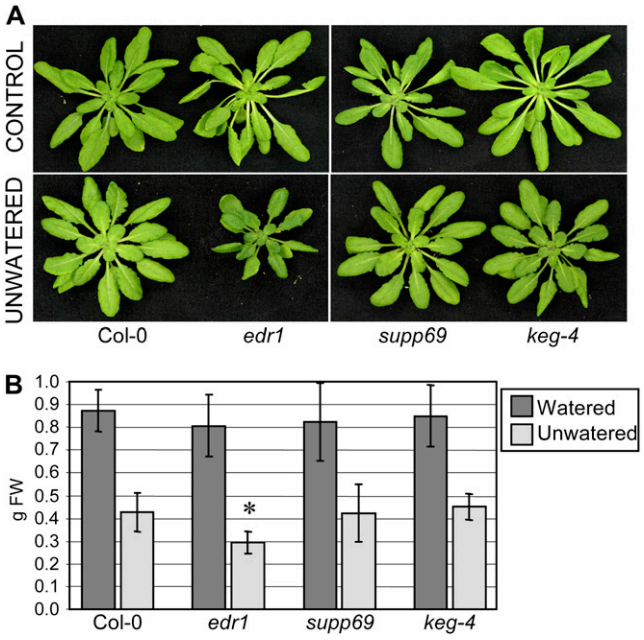

Figure 5. The *keg-4* mutation suppresses *edr1*-mediated drought-induced growth inhibition. A, Plants were grown under standard growth conditions for 3 weeks and then watering was withheld for 2 weeks. B, Quantification of drought-induced growth inhibition. Aerial portions were weighed immediately after removal from soil. Bars represent the mean and SD of values from 10 plants (*, significantly different [$P < 0.001$] from others by one-way ANOVA with Tukey's multiple-comparison post hoc test). The experiment was repeated two additional times with similar results.

We also examined the expression of two powdery mildew-inducible genes, *ETHYLENE-RESPONSIVE BINDING FACTOR1* (*ERF1*; At4g17500) and *XYLOGLUCAN ENDOTRANSGLYCOSYLASE* (*XET*; At5g57560). At 30 h postinoculation (hpi), both genes were induced about 3-fold in wild-type Col-0 plants (Fig. 8C). The basal and induced levels of *ERF1* were higher in the *edr1* mutant, while only the induced levels of *XET1* were higher. The *keg-4* mutation restored the expression of both genes to wild-type levels. Thus the *keg-4* mutation is able to suppress the effect of *edr1*-induced changes in gene expression for both ABA- and pathogen-responsive genes.

Overexpression of *KEG* Leads to Massive Cell Death

To further analyze the impact of *KEG* expression in plant development, we tested the effect of overexpressing the *KEG* gene in transgenic Arabidopsis plants. We constructed transgenic plants expressing the full-length *KEG* cDNA under the control of the constitutive cauliflower mosaic virus 35S promoter (35S::KEG). We were unable to obtain any transformants containing the 35S::*KEG* construct, which suggested that constitutive overexpression of *KEG* in Arabidopsis may be lethal. We therefore constructed transgenic Col-0 plants expressing the *KEG* cDNA under the control of a steroid-inducible promoter (Aoyama and Chua, 1997). We generated 24 transgenic lines containing this construct. All developed large necrotic lesions within 40 h of treatment with 50 μM dexamethasone (DEX; Fig. 9). In contrast, none of the DEX-treated leaves of control plants or ethanol-treated plants containing the DEX::*KEG* transgene showed any visible cell death (data not shown). These data suggest that ectopic overexpression of *KEG* results in cell death and that the level of KEG is tightly controlled.

Analysis of *KEG* Expression

To gain insight into the spatial and temporal pattern of *KEG* expression, we searched the Arabidopsis microarray data available through the Genevestigator Web interface (https://www.genevestigator.ethz.ch/). Microarray analyses showed that *KEG* is expressed in various tissues and organs at all developmental stages and is not specifically induced by any factor. To investigate *KEG* expression more directly, we constructed transgenic plants expressing a *KEG promoter*::*GUS* fusion. A 1,044-bp fragment 5′ to the *KEG* start codon was fused to the GUS reporter gene and the construct was transformed into wild-type Col-0 plants. We obtained a number of *pKEG*::*GUS* transformants and analyzed a total of 24 transgenic lines. Supplemental Figure S1 shows representative GUS staining patterns. GUS staining was observed in all tissues of 8-d-old seedlings (most prominent in the meristem parts), consistent with the microarray data. However, in 7-week-old flowering plants, GUS staining was only observed in the youngest parts of the stem, anthers, and the receptacle of immature siliques. No staining was observed in mature leaves, older parts of the stem, flower parts other than anthers, or mature siliques. These results suggest that the expression of *KEG* may be under developmental regulation and that *KEG* is expressed mainly in the actively growing and dividing cells.

Figure 6. The *keg-4* mutation suppresses *edr1*-mediated ABA hypersensitivity. Plants were germinated on plates containing 0.7 μM ABA and the photo was taken at day 5. The experiment was repeated three additional times with similar results.

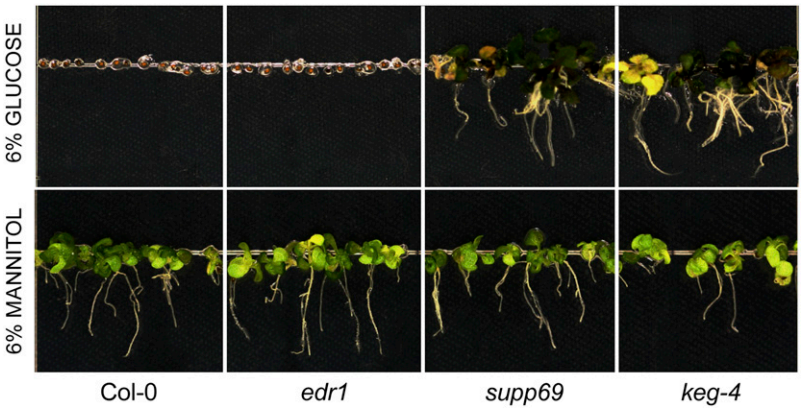

Figure 7. The *keg-4* mutation confers Glc insensitivity. Col-0, *edr1*, *supp69*, and *keg-4* seeds were germinated on solid Murashige and Skoog media with the addition of either 6% Glc or 6% mannitol. The photograph was taken 15 d after seeds germinated on the mannitol plates. The experiment was repeated four additional times with similar results.

To further investigate the expression *KEG* and *EDR1*, we performed quantitative reverse transcription (RT)-PCR analyses. In 1-week-old seedlings, treatment with 100 μM ABA for 3 h induced the expression of both *EDR1* and *KEG* (Supplemental Fig. S2A). The *edr1* mutation did not affect *KEG* transcript levels. We also examined expression of these genes in 5-week-old plants 30 h after powdery mildew treatment (Supplemental Fig. S2B). Both *EDR1* and *KEG* were slightly induced by powdery mildew. The transcript level of *EDR1* was reduced about 2-fold in the *edr1* mutant compared to the wild-type Col-0. Because the *edr1* mutation creates an early stop codon (Frye et al., 2001), this reduction in *edr1* transcript level is likely due to nonsense-mediated decay, a cellular mechanism of mRNA surveillance that detects nonsense mutations and prevents the expression of truncated or erroneous proteins (Chang et al., 2007). Interestingly, the level of *KEG* transcript in 5-week-old plants was about 3-fold greater in *edr1* than wild-type Col-0 plants (Supplemental Fig. S2B), suggesting that that EDR1 negatively regulates *KEG* transcription in rosette leaves.

DISCUSSION

The *edr1* mutant of Arabidopsis displays enhanced resistance to powdery mildew and undergoes more rapid senescence than wild-type plants when exposed to ethylene (Frye and Innes, 1998). In addition, *edr1* mutants display enhanced growth inhibition and spontaneous cell death in response to drought (Tang et al., 2005b), and enhanced cell death mediated by the RPW8 powdery mildew resistance gene (Xiao et al., 2005). All of these phenotypes, except for ethylene-induced senescence, can be suppressed by mutations in the SA signaling pathway (*sid2*, *npr1*, *pad4*, and *eds1*; Frye et al., 2001; Tang et al., 2005b; Xiao et al., 2005). However, *edr1* mutant plants do not show constitutive expression of SA-inducible genes when grown under optimal conditions (Frye and Innes, 1998), thus it has been unclear why loss of EDR1 function leads to these various phenotypes. The data presented above suggest that there may be a mechanistic link between EDR1 function, ABA signaling, and SA enhancement of PCD.

Several lines of evidence point to ABA as a central player in *edr1*-mediated phenotypes. The most direct is the hypersensitivity of *edr1* mutant seeds to ABA-mediated inhibition of germination (Fig. 1). In addition, the enhanced drought-induced growth inhibition of *edr1* plants is consistent with enhanced ABA sensitivity (Fig. 5), as is the enhanced induction of *RD29A* by exogenous ABA (Fig. 8A). Most compelling, however, is the identification of the *keg-4* missense mutation in *KEG*, which suppresses all known *edr1*-mediated phenotypes. Because loss-of-function mutations in *KEG* cause accumulation of the ABI5 transcription factor and ABA hypersensitivity, and because KEG physically associates with ABI5, KEG is believed to be a central regulator of ABA signaling (Stone et al., 2006). Furthermore, loss-of-function mutations in ABI5 substantially suppress the phenotypes caused by loss-of-function mutations in KEG, indicating that a primary role of KEG is regulating ABI5 levels (Stone et al., 2006). The observation that *keg-4* suppresses all known *edr1*-mediated phenotypes, including ethylene-induced senescence, appears to place KEG function upstream of SA signaling because mutations in *SID2*, *NPR1*, *PAD4*, and *EDS1* do not suppress the ethylene induced senescence phenotype of *edr1* (Tang et al., 2005b). Understanding the function of *KEG* and the nature of the *keg-4* mutation thus appears key to understanding how the *edr1* mutation confers its various phenotypes.

The KEG protein is quite large (178 kD) and contains multiple functional domains (Fig. 3). Starting at the N-terminal end, these are the RING (for really interesting new gene) E3 ligase domain, a kinase domain, nine tandem ankyrin repeats, and 12 HERC2-like (for HECT and RCC1 like) repeats. The RING domain of KEG has been shown to have E3 ubiquitin-ligase activity in vitro, and the kinase domain autophosphorylates in vitro (Stone et al., 2006). The ankyrin repeats are required for interaction between KEG and the ABI5 transcription factor, at least in in vitro pull down assays (Stone et al., 2006). The function of the HERC2-like repeats is unknown. The HERC2-like repeats is where

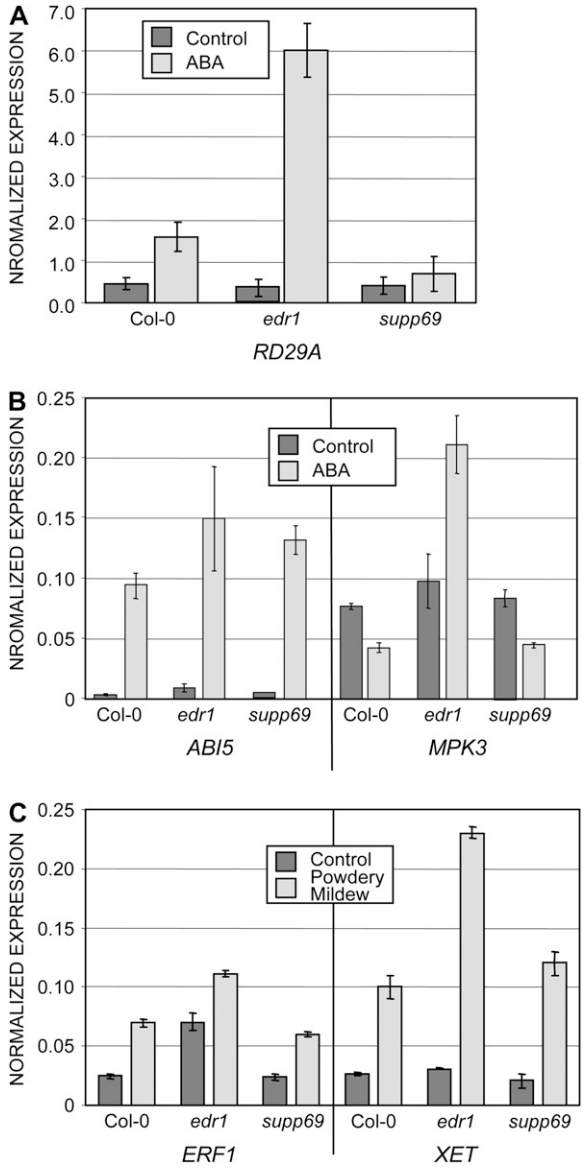

Figure 8. The *keg-4* mutation suppresses *edr1*-mediated changes in gene expression. A, Quantitative RT-PCR analysis of *RD29A* expression in 8-d-old seedlings in response to 100 μM ABA treatment. Total RNA was isolated from whole seedlings 30 h after treatment. B, *ABI5* and *MPK3* expression in 3-d-old seedlings germinated on Murashige and Skoog agar containing 0.7 μM ABA. C, *ERF* and *XET* expression in 5-week-old plants inoculated with powdery mildew. Total RNA was isolated from leaves 30 hpi. All data represent the average and SD of three biological replicates from independent experiments.

the *keg-4* mutation is located, however, suggesting that this domain plays a critical role in KEG function.

The *keg-4* mutation causes a Gly-to-Ser substitution in the fifth HERC2-like repeat (Fig. 3). This Gly residue is highly conserved among the 12 HERC2-like repeats of KEG (Stone et al., 2006). The HERC2-like repeats were first defined in the Arabidopsis KEG protein as a 61 amino acid motif with similarity to the mammalian HERC2 protein, which is a HECT-type ubiquitin E3 ligase (Garcia-Gonzalo and Rosa, 2005; Stone et al., 2006). However, HERC2 contains only a single copy of this motif. The combination of multiple HERC2-like repeats and a RING E3-ligase domain appears to be unique to plants. Single *KEG* homologs have been identified in rice (*Oryza sativa*), *Medicago truncatula*, and *Populus tricocarpa* (Stone et al., 2006), thus KEG appears to be highly conserved among angiosperms.

The phenotypes conferred by the *keg-4* mutation are generally opposite to the phenotypes conferred by loss-of-function mutations in *KEG*. In particular, the *keg-4* mutant is resistant to high levels of exogenous Glc, while *keg* loss-of-function mutants are hypersen-

Figure 9. Overexpression of the *KEG* gene is toxic to plants. Wild-type Col-0 and transgenic T1 *supp69* plants carrying a DEX-inducible wild-type copy of *KEG* were sprayed with 50 μM DEX. The photos show the same plant at time 0 (before spraying) and 7 d after induction for three independent transgenic lines. Note that large necroses develop on all leaves irrespective of their ages.

sitive to Glc (Fig. 7; Stone et al., 2006), suggesting that *keg-4* has reduced sensitivity to ABA rather than hypersensitivity. Consistent with this, *keg-4* suppressed the ABA hypersensitivity of *edr1* (Fig. 6). Also, *keg* loss-of-function mutants undergo a growth arrest shortly after germination, consistent with hypersensitivity to endogenous ABA, while *keg-4* mutants germinate and grow similar to wild-type plants on half-strength Murashige and Skoog agar. One plausible explanation for these observations is that the HERC2-like repeat domain functions to regulate the E3 ligase activity of KEG in response to ABA. In the absence of ABA, KEG presumably keeps ABI5 protein levels low via ubiquitylating ABI5 and thus targeting it for degradation. In the presence of ABA, protein levels of ABI5 increase dramatically, thus ABA must somehow prevent the ubiquitylation of ABI5, presumably by modifying KEG activity (Stone et al., 2006). We speculate that the *keg-4* mutation renders KEG insensitive to ABA by modifying the structure of the HERC2-like repeats, locking KEG in an on position relative to ABI5 ubiquitylation.

One observation that seems inconsistent with the above model is the recessive nature of the *keg-4* mutation. If the *keg-4* mutation causes KEG to constitutively ubiquitylate ABI5 even in the presence of ABA, then one would expect reduced ABI5 levels, and thus reduced responsiveness to ABA even in a heterozygous state. We attempted to assess ABI5 protein levels directly in *edr1* and *keg-4* mutant seedlings, both in the presence and absence of exogenous ABA, using a previously described antibody (Lopez-Molina et al., 2001), but were unable to detect any protein even in wild-type ABA-treated seedlings. It was thus not possible to compare ABI5 protein levels between the various mutant and wild-type plants. Given the recessive nature of *keg-4*, we speculate that the level of mutant KEG protein present in heterozygous plants is insufficient to ubiquitylate all of the ABI5 protein and that there may be a minimum threshold level of ABI5 that the plant must go below to suppress *edr1*-mediated signaling.

The *edr1* mutant displays approximately 3-fold elevated levels of *KEG* mRNA in rosette leaves, both before and after powdery mildew inoculation (Supplemental Fig. S2). One would expect this to lead to increases in KEG protein levels and thus decreases in ABA sensitivity. It is possible, however, that this increase simply reflects an elevated steady-state level of ABA signaling in the *edr1* mutant as exogenous application of ABA induces *KEG* transcript levels about 3-fold in wild-type seedlings (Supplemental Fig. S2). If EDR1 normally functions as a negative regulator of *ABA* signaling, then plants may compensate for loss of EDR1 function by increasing KEG levels. This compensation may allow for normal growth under nonstressed conditions, but under times of abiotic or biotic stress, is insufficient, leading to overactivation of ABA pathways.

KEG overexpression in mature rosettes leads to rapid cell death (Fig. 9). This phenotype cannot be explained by reduction in ABI5 protein levels alone, as ABI5 null mutants are viable (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000). This observation suggests that KEG may have other substrates in addition to ABI5 and/or that KEG overexpression leads to ubiquitylation and degradation of inappropriate substrates. The former hypothesis is supported by the finding that *abi5* mutations do not fully suppress *KEG* loss-of-function mutant phenotypes (Stone et al., 2006). Furthermore, *KEG* loss-of-function mutations partially suppress the ABA insensitivity of *abi5-1* mutants (Stone et al., 2006), which suggests that loss of KEG causes accumulation of an ABA-responsive transcription factor that can compensate for loss of ABI5 function. If KEG regulates the levels of multiple transcription factors, then *keg-4* suppression of *edr1* phenotypes may be a consequence of reducing the levels of these specific transcription factors in addition to ABI5.

Our data indicate that *edr1* plants have enhanced ABA signaling and that this is causally related to enhanced resistance to powdery mildew. This conclusion would seem to be at odds with recent work showing that the hemibiotrophic bacterial pathogen *Pseudomonas syringae* strain DC3000 specifically induces ABA biosynthesis in Arabidopsis to promote virulence and that ABA-insensitive Arabidopsis mutants have enhanced resistance to strain DC3000 (de Torres-Zabala et al., 2007). If our model is correct, one would predict that the *edr1* mutant should have enhanced susceptibility to *P. syringae*, but in fact, *edr1* was originally isolated in a screen for mutants with enhanced resistance to *P. syringae* (Frye and Innes, 1998). Further analysis of the *edr1* mutant phenotype, however, revealed that the enhanced resistance to *P. syringae* was variable, and in nonstressed plants was not reproducible (Frye and Innes, 1998). We speculate that the enhanced responses of *edr1* to abiotic stresses such as drought may indirectly promote resistance to *P. syringae*, and that the *edr1* mutant was isolated in the original screen because it had undergone a stress response prior to inoculation.

The role of ABA in regulating defense responses is still poorly understood and, depending on the pathogen studied, ABA may enhance resistance or enhance susceptibility (Mauch-Mani and Mauch, 2005). For example, both salt stress and exogenous ABA enhance the resistance of barley (*Hordeum vulgare*) to the biotrophic powdery mildew fungus *Blumeria graminis* (Wiese et al., 2004). Likewise, ABA application enhances resistance of Arabidopsis to the oomycete *Pythium irregulare*, while ABA-deficient and ABA-insensitive mutants are more susceptible (Adie et al., 2007). Similarly, ABA application protects Arabidopsis against the necrotrophic fungi *Alternaria brassicicola* and *Plectosphaerella cucumerina*, while the ABA-deficient and ABA-insensitive mutants display enhanced susceptibility to these pathogens (Ton and Mauch-Mani, 2004; Adie et al., 2007). Finally, ABA-deficient and ABA-insensitive Arabidopsis mutants also display enhanced susceptibility to

soilborne bacterium *Ralstonia solanacearum* (Hernandez-Blanco et al., 2007). We are currently testing whether the *abi-5* mutation and other mutations that confer ABA insensitivity or ABA deficiency affect *edr1*-mediated resistance to powdery mildew. We predict that *abi-5* should at least partially suppress *edr1.*

Although the above examples show that ABA can positively regulate resistance against some pathogens, including powdery mildews, the majority of studies have shown that ABA promotes susceptibility to pathogens. Early studies showed that exogenous application of ABA enhanced susceptibility of potato (*Solanum tuberosum*) tubers to *Phytophthora infestans* and *Cladosporium cucumerinum* (Henfling et al., 1980), the susceptibility of soybean (*Glycine max*) to *Phytophthora megasperma* f. sp. *glycinea* (Ward et al., 1989), the susceptibility of rice to rice blast (*Magnaportha grisea*; Matsumoto et al., 1980), and the susceptibility of tobacco (*Nicotiana tabacum*) to blue mold (*Peronospora tabacina*; Salt et al., 1986). More recently, ABA treatment has been shown to increase the susceptibility of Arabidopsis to *Peronospora parasitica* and the avirulent bacterium *P. syringae* pathovar *tomato* strain 1065 (Mohr and Cahill, 2003), but not to the virulent strain DC3000. As described above though, strain DC3000 up-regulates endogenous ABA levels in Arabidopsis (de Torres-Zabala et al., 2007); thus, exogenous ABA may have little effect on this strain. ABA application also enhances the susceptibility of Arabidopsis to *Fusarium oxysporum* (Anderson et al., 2004) and the susceptibility of tomato (*Solanum lycopersicum*) to *Botrytis cineria* (Audenaert et al., 2002). Consistent with this, ABA-deficient Arabidopsis mutants are more resistant to *F. oxysporum* (Anderson et al., 2004) and *B. cinerea* (Adie et al., 2007), and the ABA-deficient *sitiens* mutant of tomato is more resistant to *B. cineria* (Audenaert et al., 2002) and to *P. syringae* (Thaler and Bostock, 2004).

The molecular mechanisms underlying ABA regulation of defense responses are just beginning to be defined. In Arabidopsis, exogenous application of ABA suppresses transcription of defense genes induced by jasmonic acid (JA) and ethylene (Anderson et al., 2004), as well as defenses induced by SA (Yasuda et al., 2008); thus, the enhanced resistance of ABA-deficient mutants could be explained by up-regulation of JA/ethylene- and/or SA-mediated defense pathways. The enhanced resistance of the *sitiens* mutant of tomato also correlated with enhanced SA-dependent defense signaling (Thaler and Bostock, 2004), suggesting that ABA negatively regulates SA-dependent defenses in tomato as it does in Arabidopsis. In light of these data, it is difficult to reconcile how the *edr1* mutant could have both enhanced ABA sensitivity (this study) and enhanced expression of *PR-1* (Frye and Innes, 1998), and why resistance of *edr1* plants to powdery mildew is dependent on SA signaling (Frye et al., 2001).

The regulatory interactions between ABA, JA, and ethylene are clearly complex, as SA signaling is usually considered antagonistic to JA/ethylene signaling (Kunkel and Brooks, 2002; Thaler et al., 2002; Li et al., 2004), yet both signaling pathways are up-regulated in ABA-deficient mutants. To add to this complexity, transcriptional profiling analyses have revealed a large number of genes that respond similarly to exogenous methyl jasmonate and SA application (Schenk et al., 2000), despite their generally antagonistic effects on well-characterized defense genes such as *PR-1* and *PDF1.2*. It may thus be an oversimplification to conclude that ABA antagonizes all JA- and SA-induced defenses, when most studies to date have focused on just a few well-characterized genes (Anderson et al., 2004; Yasuda et al., 2008). It is plausible that ABA acts synergistically with some SA-inducible genes and antagonistically with others, with the former set being particularly important for resistance to powdery mildew.

The challenge in front of us is to determine which ABA-regulated responses contribute to resistance to some pathogens, and which responses contribute to susceptibility to others. This will require careful analyses, including transcriptional profiling of mutants blocked in more defined defense signaling steps than analyzed to date, and these mutants need to be tested against a diverse collection of pathogens.

MATERIALS AND METHODS

ABA Germination Assay

For testing ABA sensitivity (Fig. 1), seeds were sterilized then plated on one-half-strength Murashige and Skoog salts (Sigma-Aldrich) supplemented with varying concentrations of ABA and 0.8% agar. Plates were placed at 4°C for 72 h then transferred to a growth room set to 23°C and a 9 h light (150 mE m^{-2} s^{-1})/15 h dark cycle for 3 d, at which time seeds were scored for germination (a root emerging from the seed coat).

Plant Growth Conditions and Mutant Screening

Ethyl methanesulfonate-mutagenized *edr1* plants (M2 generation) were planted on one-half-strength Murashige and Skoog plates supplemented with 0.7 μM ABA, 1% Suc, and 0.8% agar and grown in growth rooms as described in the previous paragraph. Seedlings germinating by day 3 were transplanted to MetroMix 360 and allowed to grow for 5 weeks in the same growth rooms, at which time they were inoculated with powdery mildew (*Golovinomyces cichoracearum* strain UCSC1). Disease phenotypes were scored 8 d after inoculation. Plants displaying powder and no necrotic lesions were selected and allowed to set seeds. Approximately 60,000 M2 plants derived from 3,500 M1 plants were screened. For liquid cultures, seeds were put into half-strength Murashige and Skoog without agar and shaken continuously at 200 rpm under continuous light.

Powdery Mildew Infections

G. cichoracearum strain UCSC1 was maintained on hypersusceptible Arabidopsis (*Arabidopsis thaliana*) *pad4-2* mutant plants. Plants were inoculated between 4 and 6 weeks of age by gently brushing the leaves of diseased plants and healthy plants together to pass the conidia (asexual spores). The disease phenotype was scored 8 d after inoculation.

Ethylene-Induced Senescence Assay

Five-week-old plants were placed in a sealed chamber containing 100 μL L^{-1} of ethylene for 3 d. Leaves five to eight (leaf one being the oldest true leaf) were

removed and chlorophyll was extracted and measured as previously described (Frye et al., 2001).

Drought Stress Assay

Plants were grown in growth rooms as described above for 3 weeks and then watering was stopped for 2 weeks. The aerial portions of plants were then weighed and photographed. The mean weight from 10 plants of each line was used to represent the growth phenotype.

Genetic and Physical Mapping of *supp69*

Genetic mapping was accomplished using an F2 population derived from a cross between the *supp69* mutant (carrying the *edr1* mutation in the Columbia genotype, Col-0) and L*er*. F2 seeds were planted and inoculated with *G. cichoracearum* as described above and plants displaying an *edr1* phenotype were selected for collection of F3 seeds. Fifty-seven F3 families were planted (12 plants per family) and scored for disease susceptibility to *G. cichoracearum*. Twenty-eight of these families segregated susceptible plants, indicating that they contained the *supp69* mutation. Genomic DNA was isolated from 84 susceptible F3 plants chosen from these 28 F3 families and scored with published microsatellite markers. This initial mapping localized the *supp69* mutation between molecular markers MYH9 and nga151 on chromosome 5. New molecular markers at intervals between these two markers were next developed using the Monsanto Col-0 and L*er* polymorphism database (http://www.arabidopsis.org/Cereon/index.jsp; primer sequences available upon request). We then selected 629 susceptible F3 plants representing 1,258 meioses and scored them for recombination between markers MYH9 and nga151. Ultimately, the *supp69* mutation was localized to BAC clone T6I14. This analysis defined a 126-kb region that cosegregated with the *supp69* mutation.

Sequencing of Candidate Genes

The genetic interval to which the *supp69* mutation was mapped contained 31 loci within a 126-kb region. We amplified 21 of these genes from the *supp69* mutant using the PCR and directly sequenced the PCR products. Once a mutation was identified, sequencing was stopped and the identity of *supp69* confirmed by complementation. All sequencing reactions were performed using BigDye Terminator kits (Applied Biosystems) and separated on an ABI 3730 automated DNA sequencer (Applied Biosystems).

Complementation of *supp69* by *KEG* and Overexpression of *KEG*

The genomic sequence of *KEG* together with its promoter (1,044 bp upstream of ATG) was PCR amplified from BAC T6I14 using primers to create attB end products and inserted into the pDONR207 vector using an Invitrogen BP Clonase kit (Invitrogen). The insert was next recloned into the pGWB19 vector (Nakagawa et al., 2007). All cloning products were checked for proper sequences.

The full-length *KEG* cDNA was amplified by PCR from a plasmid containing a *KEG* cDNA (a kind gift of Judy Callis, University of California, Davis) using primers with attB sites for recombination. The PCR product was introduced into the pDONR207 vector. The resulting clone was sequence verified and inserts recombined into the C-terminal hemagglutinin-tagged, DEX-inducible Gateway destination vector pBAV154 (Vinatzer et al., 2006) using the Invitrogen LR Clonase kit.

Plant Transformation

Plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation with selection on Luria-Bertani plates containing 50 μg mL^{-1} kanamycin sulfate (Sigma). Arabidopsis plants were transformed using the floral-dip method (Clough and Bent, 1998). Transgenic plants were selected either by growing on one-half-strength Murashige and Skoog salts plus 0.8% agar and 50 μg mL^{-1} kanamycin or by spraying 1-week-old seedlings grown in soil with 300 μM BASTA (Finale; Fornam Companies Inc.) five times in 2-d intervals. Transformants were transplanted to soil and allowed to set seeds.

Construction of the KEG Promoter::GUS Reporter and GUS Activity Assays

A 1,044-bp promoter fragment of *KEG* was amplified by PCR from genomic DNA of wild-type Col-0 using primers to create attB end products. The resulting PCR products were then gel purified using the QIAquick Gel extraction kit (Qiagen) and the Invitrogen BP Clonase kit was then used to recombine the products into the Gateway donor vector pDONR207 (Invitrogen). The resulting clones were sequence verified and inserts recombined into the C-terminal GUS-tagged pGWB3 vector (constructed by Tsuyoshi Nakagawa, Shimane University, Izumo, Japan). The clone was also verified by sequencing and transformed into Agrobacterium strain GV3101 by electroporation. Plant transformation was conducted as described above. GUS activity analysis was performed as described (Jefferson et al., 1987) using 8-d-old seedlings and 7-week-old flowering plants.

Quantitative RT-PCR Analysis

For *G. cichoracearum* treatment, plants were grown and inoculated as described above. Leaves were removed from plants at 30 hpi. For ABA treatment seedlings were grown in liquid Murashige and Skoog media for 1 week in a room at 25°C under constant light. The seedlings were exposed to 100 μM ABA for 3 h before RNA was extracted. For checking *ABI5* and *MPK3* expression, seeds were germinated for 72 h on half-strength Murashige and Skoog plates with or without addition of 0.7 μM ABA.

Total RNA was isolated using the Qiagen RNeasy kit and treated with DNase (Invitrogen) to remove DNA contamination. The High Capacity reverse transcriptase kit (Applied Biosystems) was utilized to obtain cDNA, and the samples purified with Qiagen QIAquick PCR purification kit. Quantitative RT-PCR was performed using primers listed in Supplemental Table S1. A tubulin gene (At5g19770) was used as a control for normalizing the amount of cDNA. The Takara SYBR Premix Extaq was used for all quantitative RT-PCR runs and the Mx3000P (Stratagene) protocol was followed.

Statistical Analysis

Statistical significance of observed differences in datasets was determined using one-way ANOVA as implemented in the Analyze-it add in to Microsoft Excel (Analyze-It Software, Ltd.). The Tukey post hoc test was used to identify differences between single treatments when the ANOVA was significant.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. KEG is expressed in all tissues of seedlings but not in mature leaves.

Supplemental Figure S2. EDR1 and KEG are induced by ABA and powdery mildew.

Supplemental Table S1. Oligonucleotides used for quantitative RT-PCR experiments.

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LITERATURE CITED

Adie BA, Perez-Perez J, Perez-Perez MM, Godoy M, Sanchez-Serrano JJ, Schmelz EA, Solano R (2007) ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. Plant Cell **19:** 1665–1681

Alvarez ME (2000) Salicylic acid in the machinery of hypersensitive cell death and disease resistance. Plant Mol Biol **44:** 429–442

Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, Maclean DJ, Ebert PR, Kazan K (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. Plant Cell **16:** 3460–3479

Aoyama T, Chua NH (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J **11:** 605–612

Apte P, Laloraya M (1982) Inhibitory action of phenolic compounds on abscisic acid-induced abscission. J Exp Bot **33:** 826–830

Audenaert K, De Meyer GB, Hofte MM (2002) Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. Plant Physiol **128:** 491–501

Beaudoin N, Serizet C, Gosti F, Giraudat J (2000) Interactions between abscisic acid and ethylene signaling cascades. Plant Cell **12:** 1103–1115

Brocard IM, Lynch TJ, Finkelstein RR (2002) Regulation and role of the Arabidopsis abscisic acid-insensitive 5 gene in abscisic acid, sugar, and stress response. Plant Physiol **129:** 1533–1543

Butt A, Mousley C, Morris K, Beynon J, Can C, Holub E, Greenberg JT, Buchanan-Wollaston V (1998) Differential expression of a senescence-enhanced metallothionein gene in Arabidopsis in response to isolates of *Peronospora parasitica* and *Pseudomonas syringae*. Plant J **16:** 209–221

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

Chang YF, Imam JS, Wilkinson MF (2007) The nonsense-mediated decay RNA surveillance pathway. Annu Rev Biochem **76:** 51–74

Cheng WH, Endo A, Zhou L, Penney J, Chen HC, Arroyo A, Leon P, Nambara E, Asami T, Seo M, et al (2002) A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. Plant Cell **14:** 2723–2743

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

de Torres-Zabala M, Truman W, Bennett MH, Lafforgue G, Mansfield JW, Rodriguez Egea P, Bogre L, Grant M (2007) *Pseudomonas syringae* pv. *tomato* hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. EMBO J **26:** 1434–1443

Finkelstein RR (1994) Maternal effects govern variable dominance of two abscisic acid response mutations in *Arabidopsis thaliana*. Plant Physiol **105:** 1203–1208

Finkelstein RR, Gampala SS, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell (Suppl) **14:** S15–S45

Finkelstein RR, Gibson SI (2002) ABA and sugar interactions regulating development: cross-talk or voices in a crowd? Curr Opin Plant Biol **5:** 26–32

Finkelstein RR, Lynch TJ (2000) The *Arabidopsis* abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell **12:** 599–609

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

Garcia-Gonzalo FR, Rosa JL (2005) The HERC proteins: functional and evolutionary insights. Cell Mol Life Sci **62:** 1826–1838

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, McCourt P (2000) Regulation of abscisic acid signaling by the ethylene response pathway in *Arabidopsis*. Plant Cell **12:** 1117–1126

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol **43:** 205–227

Gudesblat GE, Iusem ND, Morris PC (2007) Guard cell-specific inhibition of Arabidopsis MPK3 expression causes abnormal stomatal responses to abscisic acid and hydrogen peroxide. New Phytol **173:** 713–721

Hanfrey C, Fife M, Buchanan-Wollaston V (1996) Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis-related proteins. Plant Mol Biol **30:** 597–609

Henfling JWDM, Bostock R, Kuc J (1980) Effect of abscisic acid on rishitin and lubimin accumulation and resistance to *Phytophthora infestans* and *Cladosporium cucumerinum* in potato tuber tissue slices. Phytopathology **70:** 1074–1078

Hernandez-Blanco C, Feng DX, Hu J, Sanchez-Vallet A, Deslandes L, Llorente F, Berrocal-Lobo M, Keller H, Barlet X, Sanchez-Rodriguez C, et al (2007) Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall formation enhances disease resistance. Plant Cell **19:** 890–903

Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL (2002) Arabidopsis map-based cloning in the post-genome era. Plant Physiol **129:** 440–450

Jefferson RA, Bevan M, Kavanagh T (1987) The use of the Escherichia coli beta-glucuronidase as a gene fusion marker for studies of gene expression in higher plants. Biochem Soc Trans **15:** 17–18

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, Glazebrook J (1999) *Arabidopsis thaliana* PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. Proc Natl Acad Sci USA **96:** 13583–13588

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

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci USA **1997:** 2940–2945

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Curr Opin Plant Biol **5:** 325–331

Leung J, Giraudat J (1998) Abscisic acid signal transduction. Annu Rev Plant Physiol Plant Mol Biol **49:** 199–222

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79:** 583–593

Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell **16:** 319–331

Lopez-Molina L, Chua NH (2000) A null mutation in a bZIP factor confers ABA-insensitivity in *Arabidopsis thaliana*. Plant Cell Physiol **41:** 541–547

Lopez-Molina L, Mongrand S, Chua NH (2001) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proc Natl Acad Sci USA **98:** 4782–4787

Lu C, Han MH, Guevara-Garcia A, Fedoroff NV (2002) Mitogen-activated protein kinase signaling in postgermination arrest of development by abscisic acid. Proc Natl Acad Sci USA **99:** 15812–15817

Madhu A, Thomas G, Edward N (1999) The roles of abscisic acid and ethylene in the abscission and senescence of cocoa flowers. Plant Growth Regul **27:** 149–155

Matsumoto K, Suzuki Y, Mase S, Watanabe T, Sekizawa Y (1980) On the relationship between plant hormones and rice blast resistance. Ann Phytopathological Soc Jpn **46:** 307–314

Mauch-Mani B, Mauch F (2005) The role of abscisic acid in plant-pathogen interactions. Curr Opin Plant Biol **8:** 409–414

Mohr PG, Cahill DM (2003) Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. *tomato* and *Peronospora parasitica*. Funct Plant Biol **30:** 461–469

Mohr PG, Cahill DM (2007) Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with *Pseudomonas syringae* pv. *tomato*. Funct Integr Genomics **7:** 181–191

Morris K, MacKerness SA, Page T, John CF, Murphy AM, Carr JP, Buchanan-Wollaston V (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. Plant J **23:** 677–685

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng **104:** 34–41

Nooden LD, Leopold AC (1988) Senescence and Aging in Plants. Academic Press, San Diego

Panavas T, Pikula A, Reid PD, Rubinstein B, Walker EL (1999) Identification of senescence-associated genes from daylily petals. Plant Mol Biol **40:** 237–248

Pastori GM, Del Rio LA (1997) Natural senescence of pea leaves (an activated oxygen-mediated function for peroxisomes). Plant Physiol **113:** 411–418

Pontier D, Gan S, Amasino RM, Roby D, Lam E (1999) Markers for hypersensitive response and senescence show distinct patterns of expression. Plant Mol Biol **39:** 1243–1255

Quirino BF, Noh YS, Himelblau E, Amasino RM (2000) Molecular aspects of leaf senescence. Trends Plant Sci **5:** 278–282

Quirino BF, Normanly J, Amasino RM (1999) Diverse range of gene activity during Arabidopsis thaliana leaf senescence includes pathogen-independent induction of defense-related genes. Plant Mol Biol **40:** 267–278

Rai V, Sharma S, Sharma S (1986) Reversal of ABA-induced stomatal closure by phenolic compounds. J Exp Bot **37:** 129–134

Ray S (1986) GA, ABA, phenol interaction and control of growth: phenolic compounds as effective modulators of GA-ABA interaction in radish seedlings. Biol Plant **28:** 361–369

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell **8:** 1809–1819

Salt SD, Tuzun S, Kuc J (1986) Effect of β-inonone and abscisic acid on the growth of tobacco and resistance to blue mold: mimicry of effects of stem infection by *Peronospora tabacina Asam*. Physiol Mol Plant Pathol **28:** 287–297

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM (2000) Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proc Natl Acad Sci USA **97:** 11655–11660

Stone SL, Williams LA, Farmer LM, Vierstra RD, Callis J (2006) KEEP ON GOING, a RING E3 ligase essential for *Arabidopsis* growth and development, is involved in abscisic acid signaling. Plant Cell **18:** 3415–3428

Tang D, Ade J, Frye CA, Innes RW (2005a) Regulation of plant defense responses in Arabidopsis by EDR2, a PH and START domain-containing protein. Plant J **44:** 245–257

Tang D, Ade J, Frye CA, Innes RW (2006) A mutation in the GTP hydrolysis site of Arabidopsis dynamin-related protein 1E confers enhanced cell death in response to powdery mildew infection. Plant J **47:** 75–84

Tang D, Christiansen KM, Innes RW (2005b) Regulation of plant disease resistance, stress responses, cell death, and ethylene signaling in Arabidopsis by the EDR1 protein kinase. Plant Physiol **138:** 1018–1026

Thaler J, Bostock R (2004) Interactions between abscisic-acid-mediated responses and plant resistance to pathogens and insects. Ecology **85:** 48–58

Thaler JS, Fidantsef AL, Bostock RM (2002) Antagonism between jasmonate- and salicylate-mediated induced plant resistance: effects of concentration and timing of elicitation on defense-related proteins, herbivore, and pathogen performance in tomato. J Chem Ecol **28:** 1131–1159

To JP, Reiter WD, Gibson SI (2002) Mobilization of seed storage lipid by Arabidopsis seedlings is retarded in the presence of exogenous sugars. BMC Plant Biol **2:** 4

Ton J, Mauch-Mani B (2004) Beta-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J **38:** 119–130

Vinatzer BA, Teitzel GM, Lee MW, Jelenska J, Hotton S, Fairfax K, Jenrette J, Greenberg JT (2006) The type III effector repertoire of Pseudomonas syringae pv. syringae B728a and its role in survival and disease on host and non-host plants. Mol Microbiol **62:** 26–44

Vogel J, Somerville S (2000) Isolation and characterization of powdery mildew-resistant Arabidopsis mutants. Proc Natl Acad Sci USA **97:** 1897–1902

Vogel JP, Raab TK, Schiff C, Somerville SC (2002) PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis*. Plant Cell **14:** 2095–2106

Ward EW, Cahill DM, Bhattacharyya MK (1989) Abscisic acid suppression of phenylalanine ammonia-lyase activity and mRNA, and resistance of soybeans to *Phytophthora megasperma* f.sp. *glycinea*. Plant Physiol **91:** 23–27

Wiese J, Kranz T, Schubert S (2004) Induction of pathogen resistance in barley by abiotic stress. Plant Biol (Stuttg) **6:** 529–536

Xiao S, Calis O, Patrick E, Zhang G, Charoenwattana P, Muskett P, Parker JE, Turner JG (2005) The atypical resistance gene, RPW8, recruits components of basal defence for powdery mildew resistance in Arabidopsis. Plant J **42:** 95–110

Xie Z, Zhang ZL, Hanzlik S, Cook E, Shen QJ (2007) Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic-acid-inducible WRKY gene. Plant Mol Biol **64:** 293–303

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol **57:** 781–803

Yasuda M, Ishikawa A, Jikumaru Y, Seki M, Umezawa T, Asami T, Maruyama-Nakashita A, Kudo T, Shinozaki K, Yoshida S, et al (2008) Antagonistic interaction between systemic acquired resistance and the abscisic acid-mediated abiotic stress response in *Arabidopsis*. Plant Cell **20:** 1678–1692

Yoshida S, Ito M, Nishida I, Watanabe A (2001) Isolation and RNA gel blot analysis of genes that could serve as potential molecular markers for leaf senescence in Arabidopsis thaliana. Plant Cell Physiol **42:** 170–178

Zhou L, Jang JC, Jones TL, Sheen J (1998) Glucose and ethylene signal transduction crosstalk revealed by an Arabidopsis glucose-insensitive mutant. Proc Natl Acad Sci USA **95:** 10294–10299