EARLY RESPONSIVE TO DEHYDRATION 15, a Negative Regulator of Abscisic Acid Responses in Arabidopsis[1]

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EARLY RESPONSIVE TO DEHYDRATION 15 (*ERD15*) is rapidly induced in response to various abiotic and biotic stress stimuli in Arabidopsis (*Arabidopsis thaliana*). Modulation of ERD15 levels by overexpression or RNAi silencing altered the responsiveness of the transgenic plants to the phytohormone abscisic acid (ABA). Overexpression of *ERD15* reduced the ABA sensitivity of Arabidopsis manifested in decreased drought tolerance and in impaired ability of the plants to increase their freezing tolerance in response to this hormone. In contrast, RNAi silencing of *ERD15* resulted in plants that were hypersensitive to ABA and showed improved tolerance to both drought and freezing, as well as impaired seed germination in the presence of ABA. The modulation of ERD15 levels not only affected abiotic stress tolerance but also disease resistance: *ERD15* overexpression plants showed improved resistance to the bacterial necrotroph *Erwinia carotovora* subsp. *carotovora* accompanied with enhanced induction of marker genes for systemic acquired resistance. We propose that ERD15 is a novel mediator of stress-related ABA signaling in Arabidopsis.

Rapid adaptation to changing environmental conditions is essential for plant survival and development of tolerances to both abiotic and biotic stresses. Such tolerance can be achieved by distinct metabolic and physiological adjustments mediated by different plant hormones often specific to a certain type of stress. The phytohormone abscisic acid (ABA) has a wide range of essential functions in plant growth and development, including promotion of seed maturation and dormancy as well as inhibition of seed germination (Finkelstein and Gibson, 2002). During vegetative growth, ABA is a central regulator of plant adaptation to environmental stresses, such as drought and high salinity (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006), and plays a crucial role in the regulation of transpirational water loss (Leung and Giraudat, 1998; Schroeder et al., 2001).

Although several components of ABA signaling have been identified, there is still lack of knowledge of how ABA is perceived and the signal transduced partly due to the complexity and redundancy of such signal networks. Mutants affecting ABA responsiveness have defined components of the ABA-signaling pathway, and one class of important players seems to be the type 2C protein phosphatases ABI1 and ABI2 (Leung et al., 1997; Gosti et al., 1999) as well as PP2CA (Tähtiharju and Palva 2001; Yoshida et al., 2005) that appear to act as negative regulators of ABA responses (Merlot et al., 2001; Tähtiharju and Palva, 2001). The dominant-negative alleles of *ABI1* and *ABI2*, *abi1-1* and *abi2-1*, confer ABA insensitivity during vegetative growth as well as in seed germination and cause defects in plant responses to drought stress (Leung et al., 1997). Other known regulators of ABA responsiveness include PKS3, a Ser/Thr protein kinase, a global negative regulator of ABA responses that has been shown to interact with ABI2 (Guo et al., 2002). The farnesyl transferase ERA1 (Cutler et al., 1996) and inositol phosphatase FRY1 (Xiong et al., 2001b) are also negative regulators of ABA responses.

ABA responsiveness of many of the abiotic stress-inducible genes is conferred by the conserved cis-regulatory ABRE sequence (ABA-responsive element), the binding site for the basic-domain Leu zipper-class transcription factors, AREBs (ABRE-binding proteins), or ABFs (ABRE-binding factors; for review, see Yamaguchi-Shinozaki and Shinozaki, 2005). ABA-dependent phosphorylation is required to activate these transcription factors and consequently the expression of ABRE-containing genes (Yamaguchi-Shinozaki and Shinozaki, 2005, 2006).

Recent studies have suggested that part of the regulation of ABA responses takes place posttranscriptionally (Kuhn and Schroeder, 2003). The Arabidopsis (*Arabidopsis thaliana*) mutants *supersensitive to ABA and drought1* (*sad1*) and *hyponastic leaves1* (*hyl1*) plants show altered response to ABA: Both are hypersensitive to this phytohormone in inhibition of seed germination and show reduced stomatal closure in response to stress. SAD1 is homologous to eukaryotic RNA-binding

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proteins, while *HYL1* encodes a nuclear-localized protein that specifically binds double-stranded RNA (Lu and Fedoroff, 2000; Xiong et al., 2001a). Also, a recent study shows that the mRNA-destabilizing activity of a poly(A)-specific endonuclease, poly(A)-specific ribonuclease (AtPARN), is crucial for proper ABA, salicylic acid (SA), and abiotic stress responses (Nishimura et al., 2005).

Besides its central role in controlling responses to abiotic stress stimuli, recent studies suggest that ABA also influences biotic stress responses and may interfere with signaling that is regulated by the more "traditional" hormones of pathogen defense: SA, jasmonic acid (JA), and ethylene (ET; for review, see Mauch-Mani and Mauch, 2005). Exogenous ABA has been shown to suppress basal as well as JA- and ET-activated transcription of defense genes, whereas ABA-deficient mutants showed a corresponding increase (Anderson et al., 2004). ABA treatment prior to infection increased the susceptibility of Arabidopsis to avirulent *Pseudomonas syringae* pv *tomato*, suggesting that ABA interferes with SA-dependent defense responses (Mohr and Cahill, 2003). In both studies, ABA-deficient mutant plants were less susceptible to the pathogen, indicating that decreased ABA levels appear to improve either JA- or SA-dependent defenses (Mohr and Cahill, 2003; Anderson et al., 2004). On the other hand, the β-amino-butyric acid-primed accumulation of callose and following resistance to the necrotrophic pathogens *Alternaria brassicicola* and *Plectosphaerella cucumerina* has been shown to be dependent on ABA (Ton and Mauch-Mani, 2004; Mauch-Mani and Mauch, 2005).

Here, we report that EARLY RESPONSIVE TO DEHYDRATION 15 (ERD15), a small, acidic protein with no known function, is one of the key negative regulators of ABA responses in plants. *ERD15* was originally described as a rapidly drought-responsive gene in Arabidopsis (Kiyosue et al., 1994). In this study, we show that alteration of *ERD15* expression modulates ABA responsiveness in Arabidopsis. We present evidence showing that the ABA sensitivity of *ERD15* overexpression plants is reduced, while RNAi silencing of *ERD15* results in hypersensitivity to ABA observed both in seed germination and as enhanced drought and freezing tolerance. We also show that *ERD15* is induced by pathogen attack and that overexpression of this gene enhances SA-dependent pathogen defense and plant resistance to *Erwinia carotovora*. Our results indicate that ERD15 mediates cross talk between abiotic and biotic stress responses.

RESULTS

The Arabidopsis *ERD15* Gene Is Rapidly Induced by Both Biotic and Abiotic Factors

To identify early signaling components of plant defense, we isolated Arabidopsis genes rapidly induced in response to *E. carotovora* elicitors using suppressive subtractive hybridization (Brader et al., 2001). One of these genes was *ERD15*, previously characterized as *EARLY RESPONSIVE TO DEHYDRATION 15* (Kiyosue et al., 1994). Subsequent characterization of *ERD15* expression pattern showed that, in addition to pathogen elicitors and dehydration, the gene was also rapidly induced after *E. carotovora* infection, as well as by SA, ABA, and wounding (Fig. 1A). Interestingly, *ERD15* was not responsive to methyl jasmonate (MeJA; Fig. 1A), even though *E. carotovora* is a pathogen that can trigger both SA- and JA-dependent defense signaling in Arabidopsis (Kariola et al., 2003; Li et al., 2004; Kariola et al., 2005). This broad responsiveness of *ERD15* to different types of environmental cues could suggest that it is a component of both biotic and abiotic stress responses in Arabidopsis.

Generation and Characterization of Transgenic *ERD15* Plants

To explore the possible role of ERD15 in plant defense and stress tolerance, we generated Arabidopsis Columbia (Col-0) lines harboring overexpression or RNAi constructs of *ERD15*. The effect of the transgenes on *ERD15* transcript accumulation was assessed by gel-blot hybridization using a gene-specific RNA probe for this gene. Two overexpression lines with increased and two RNAi lines with clearly decreased expression of *ERD15* were employed for further studies (Fig. 1B). *ERD15* overexpression but not silencing resulted in some morphological differences from the wild type with more narrow leaves (Fig. 1C). The phenotype of the transgenic lines was further confirmed by determining ERD15 protein levels in the plants after drought exposure. The difference in protein amounts was evident: *ERD15* overexpressor lines accumulated more ERD15 protein when compared with the control, whereas in RNAi-silenced lines hardly any protein could be detected (Fig. 1C). The *ERD15* expression in the transgenic lines was also characterized following exposure to either biotic (*E. carotovora*) or abiotic (drought) stress. The rapid but transient induction of *ERD15* in response to both types of stimuli was clearly evident in the vector control, whereas the overexpression plants showed a constitutive high level accumulation of the *ERD15* transcript. In contrast, in the RNAi-silenced plants, *ERD15* expression was almost completely abolished even when induced by either biotic or abiotic stress (Fig. 1D).

Overexpression of *ERD15* Sensitizes Plants to Drought

Drought stress rapidly induces *ERD15* as shown above (Fig. 1D; Kiyosue et al., 1994). This suggested that the corresponding protein could be involved in abiotic stress adaptation and prompted us to test whether the drought tolerance of the *ERD15* transgenic plants was altered. To assess the drought tolerance phenotype of the transgenic plants, we transferred *ERD15* overexpression, *ERD15* RNAi, and control

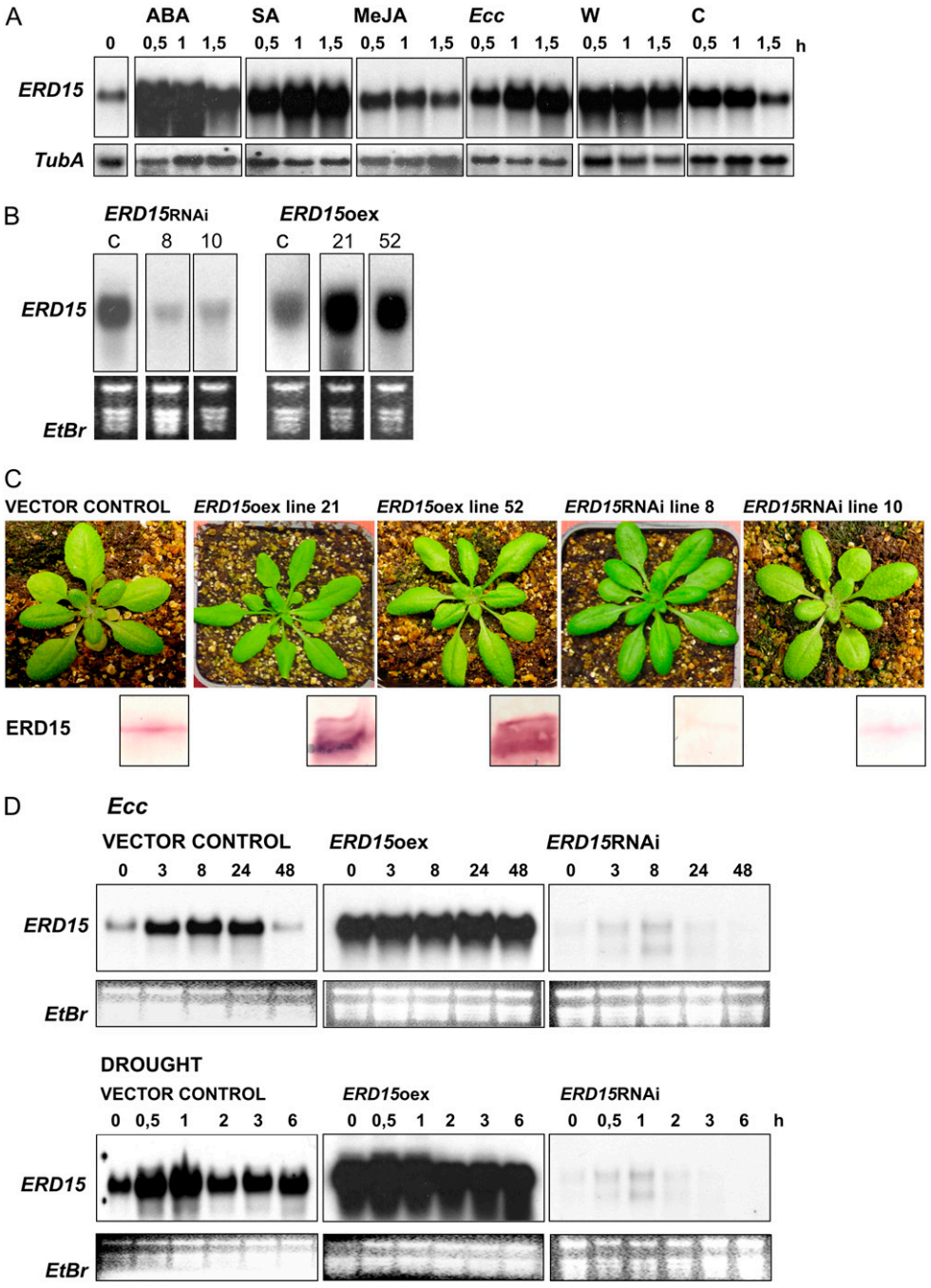

Figure 1. Induction of the *ERD15* gene and characterization of transgenic lines. A, Wild-type Arabidopsis was treated with ABA, SA, MeJA, *E. carotovora* (*Ecc*), wounding (W), and with deionized water as control treatment (C). Local samples were collected 0, 0.5, 1, and 1.5 h after the treatment. The samples were analyzed in RNA gel-blot hybridization with gene-specific RNA probe for *ERD15*. Equal loading of RNA samples was checked using a probe for the constitutively expressed α-tubulin (*TubA*) gene. B, The transgenic Arabidopsis lines generated carrying *ERD15* RNAi and overexpression (oex) constructs. Samples for evaluating the efficacy of RNAi silencing of *ERD15* as well as the vector control sample (C) were collected 1.5 h after treating the plants with pathogen elicitor preparation. Evaluation of the efficiency of overexpression was done from nontreated samples. All samples were analyzed by RNA gel-blot hybridization with gene-specific RNA probe for *ERD15*. As a control for equal loading, ethidium bromide (EtBr) staining of RNA is shown at the bottom. C, Phenotypes of Arabidopsis Col-0 vector control, *ERD15* oex (lines 21 and 52), and *ERD15* RNAi-silenced (lines 8 and 10) plants. Level of ERD15 protein after 4 h of drought stress is shown for each line. D, Induction of *ERD15* in wild-type, *ERD15* oex, and *ERD15* RNAi-silenced plants after biotic (*E. carotovora* = *Ecc*) and abiotic (drought) stress. Local leaf samples were collected from *ERD15* oex, *ERD15* RNAi-silenced, and vector control plants 0, 3, 8, 24, and 48 h after inoculating the plants with *Ecc* and 0, 0.5, 1, 2, 3, and 6 h after exposing the plants to drought stress. All samples were analyzed by RNA gel-blot hybridization with gene-specific RNA probe for *ERD15*. As a control for equal loading, EtBr staining of RNA is shown at the bottom.

plants to lower humidity conditions and left them without watering. After 2 weeks of drought stress, the phenotypic difference between the plants was striking and surprising: The majority (72%) of *ERD15* overexpression plants were dead, whereas a significant fraction of vector control plants were still alive (Fig. 2, A and B). Moreover, only 14% of the plants with RNAi-silenced *ERD15* were dead, and the survivors appeared healthier than the controls (Fig. 2, A and B). The altered drought tolerance seen after modulation of ERD15 levels strongly indicates that this gene has a role in abiotic stress adaptation in Arabidopsis.

Silencing of *ERD15* Increases Plant Freezing Tolerance

The altered drought tolerance of the transgenic *ERD15* plants and the inducibility of the gene with different abiotic stress stimuli (Fig. 1A) suggested that the transgenic plants might show altered tolerance to related abiotic stresses, such as freezing. To test this possibility, the plants were exposed to freezing temperatures and the survival was assessed. The difference in tolerance between the transgenic lines was evident immediately after the temperature was returned back to 22°C. Most of the *ERD15* overexpressors as well as vector control plants appeared to have lost their turgor, whereas *ERD15* RNAi plants looked healthy and turgid (data not shown). When the survival was assessed 7 d after exposure to the freeze-thaw cycle, the difference between the lines was clear: Most of the *ERD15* RNAi plants had survived without any or with only minor damage, and only a small fraction (11%) of the plants were killed (Fig. 2, C and D). In contrast, the majority of the *ERD15* overexpression plants had suffered severe frost damage, and most (84%) of these plants were killed. They appeared even more freezing sensitive than the vector control plants, of which 53% were dead (Fig. 2, C and D).

Freezing tolerance of many temperate plant species, including Arabidopsis, is increased by exposure to low, nonfreezing temperatures, a phenomenon called cold acclimation (Guy, 1990). We characterized whether the modulation of ERD15 levels, besides altering the basal freezing tolerance, also had an impact on the capability of the plants to cold acclimate. Interestingly, all the plants, including *ERD15* overexpression plants that showed decreased frost survival without cold acclimation, were capable of (+4°C; 2 d) low-temperature acclimation (data not shown). Taken together, these data argue that, while the modulation of ERD15 expression does not interfere with the ability of the plants to cold acclimate, in nonacclimated plants high-level expression of *ERD15* is detrimental to the basal freezing tolerance of Arabidopsis.

Overexpression of *ERD15* Impairs Development of Freezing Tolerance

The marked effect on drought and freezing tolerance caused by altered *ERD15* expression suggested

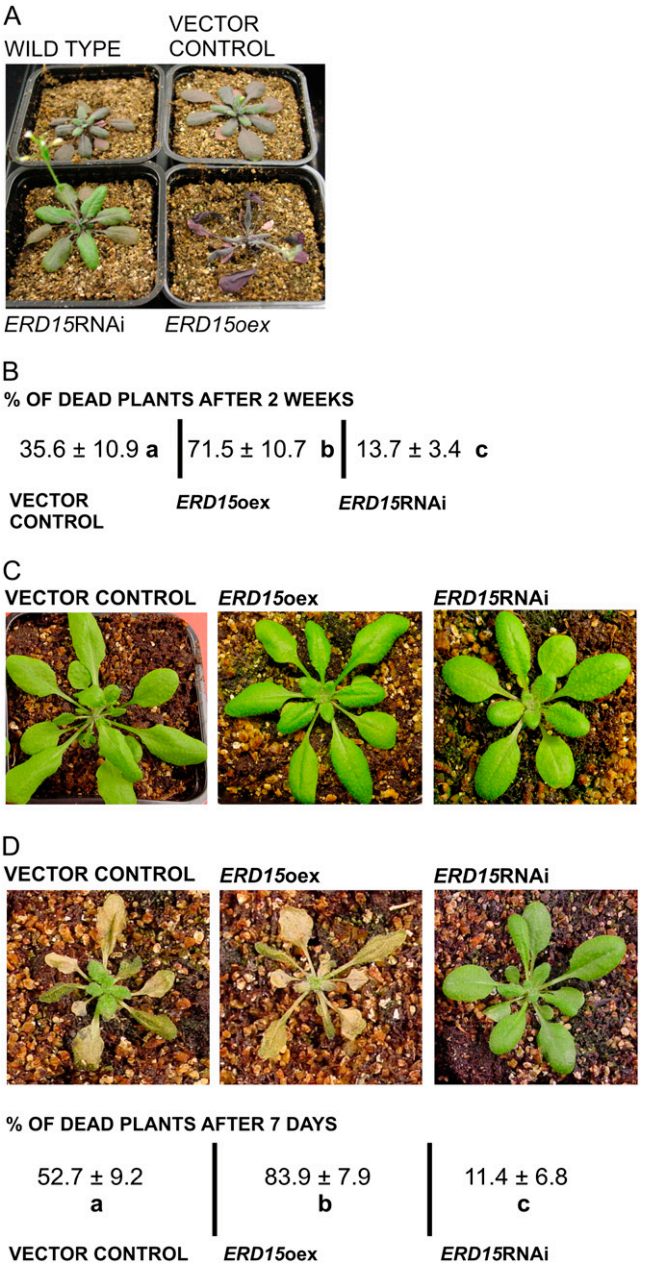

Figure 2. Modulation of *ERD15* expression affects drought and freezing tolerance of the transgenic plants. A, Drought tolerance of *ERD15* RNAi, *ERD15* overexpression (oex), wild-type, and vector control plants was tested by keeping them under 50% humidity for 2 weeks without watering. B, Percentage of dead plants after 2 weeks is shown. The values represent the average of three replicates ±SD. Different letters indicate significant differences ($P < 0.05$) calculated with one-way ANOVA followed by Tukey's HSD test. C and D, The freezing tolerance of vector control, *ERD15* oex, and *ERD15* RNAi plants was tested in a freezing survival experiment. The plants were photographed before freezing survival (C) and 7 d after the temperature was returned to normal (D). Percentage of dead plants after 7 d is shown. The values represent the average of three replicates ±SD. Different letters indicate significant differences ($P < 0.05$) calculated with one-way ANOVA followed by Tukey's HSD test.

that ABA, a central hormone in drought signaling, might be involved. In addition to low temperature, ABA can also induce the development of freezing tolerance in various higher plants, including potato (*Solanum tuberosum*; Chen and Gusta, 1983) and Arabidopsis (Lång et al., 1989; Mäntylä et al., 1995). To elucidate the effect of ERD15 on ABA-induced freezing tolerance, we compared the tolerance of axenically grown *ERD15* overexpression, *ERD15* RNAi, and control plants induced by 60 μM exogenous ABA. The freezing tolerance of the plants was determined 1 and 3 d after ABA treatment by measuring electrolyte leakage (EL_{50}) after exposure to freezing temperatures (Fig. 3A). Nonacclimated *ERD15* overexpression plants appeared more susceptible to freezing than control plants. Although *ERD15* transgenic lines were still responsive to exogenous ABA, the freezing tolerance achieved in *ERD15*-overexpressing plants was

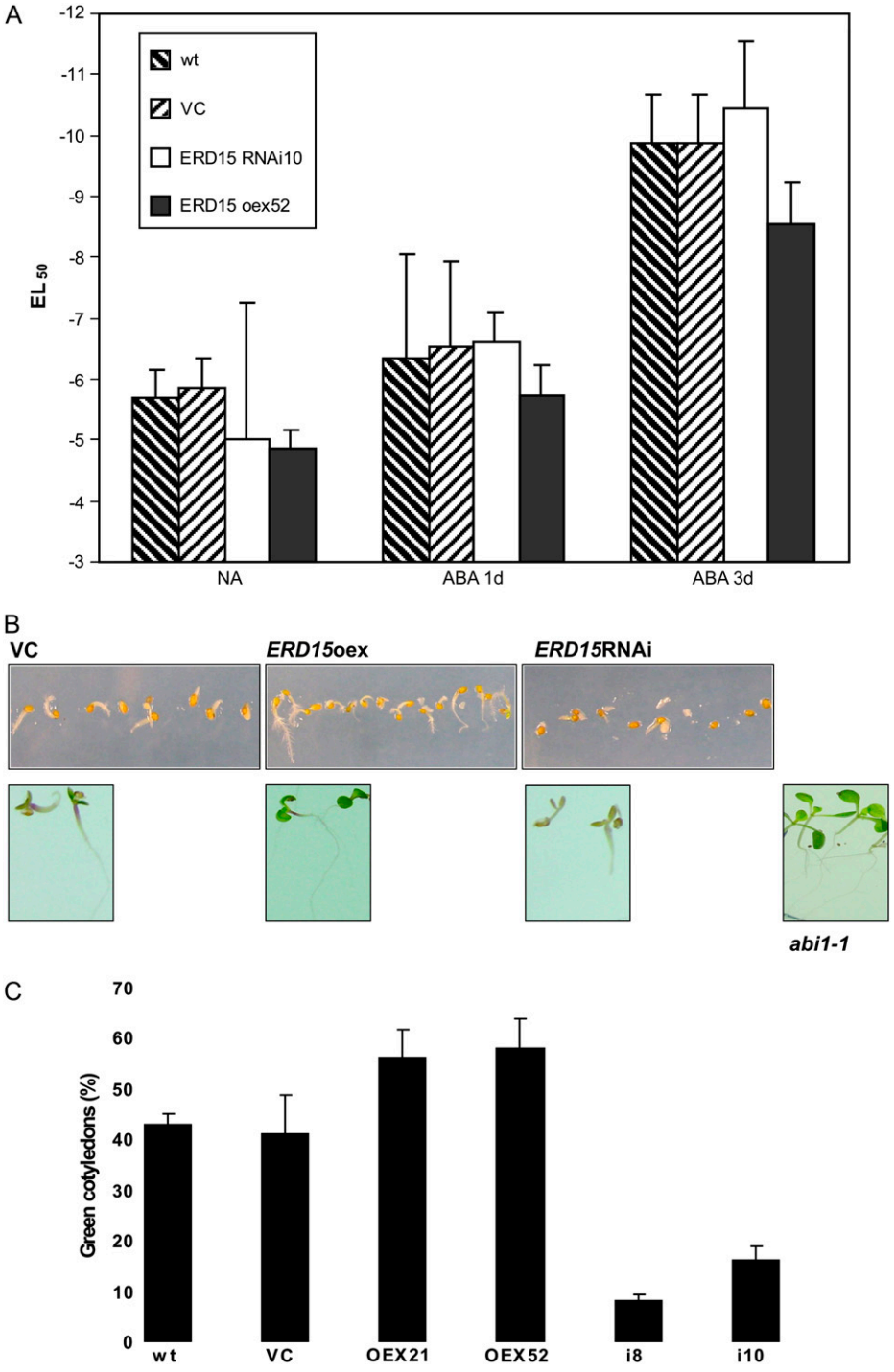

Figure 3. Modulation of *ERD15* expression alters development of freezing tolerance and seed germination in the presence of ABA. A, Freezing tolerance (EL_{50}) was measured by an electrolyte leakage assay from axenically grown wild-type Col-0, vector control, *ERD15* RNAi-silenced, and *ERD15* overexpression (oex) plants before (NA = nonacclimated) and 1 and 3 d after treatment with 60 μM ABA. The values represent the average of three replicates ±SD calculated by Probit analysis. At −10°C, *ERD15* oex plants had a significantly higher ion leakage than wild-type, vector control, and RNAi plants calculated with one-way ANOVA followed by Tukey's HSD test ($P < 0.05$). B, Seeds of *ERD15* oex, *ERD15* RNAi, and vector control were germinated on MS + 2 μM ABA plates. Germination is shown after five (top row) and 10 (bottom row) d. For comparison, the germination of *abi1-1* is shown after 10 d. C, Percentage (±SE) of green cotyledons 10 d after germination with 2 μM ABA is shown for two *ERD15* oex and two *ERD15* RNAi-silenced lines and wild-type and vector control plants. In all cases, similar results were obtained from three independent experiments.

significantly lower than in control or *ERD15*-silenced lines (Fig. 3A).

ERD15 RNAi Plants Are Hypersensitive to ABA during Seed Germination

To explore the spectrum of ABA-controlled processes that were affected by modulating *ERD15* expression, we elucidated whether the response was specific to stress tolerance in the vegetative parts of the plant or whether it applied also to ABA-regulated processes at other stages of development. Inhibition of seed germination is one of the processes controlled by ABA, and a number of Arabidopsis mutants affecting seed germination due to altered sensitivity to this hormone, such as ABA-insensitive *abi1-1* and *abi2-1* as well as ABA-hypersensitive *abh1* (*abscisic acid hypersensitive1*), have been characterized.

Germination of the seeds of *ERD15* transgenic and control plants was similar in the absence of ABA (data not shown). However, when ABA was added, seeds from *ERD15* RNAi-silenced plants germinated poorly and only 10% were able to produce green cotyledons (Fig. 3, B and C). In contrast, seeds of *ERD15* overexpression plants exhibited clearly improved seed germination during the first week when compared to control plants, and most of them (approximately 60%) produced green cotyledons 10 d after germination (Fig. 3, B and C). This could be an indication of altered ABA sensitivity; RNAi silencing of *ERD15* sensitizes the seeds to exogenous ABA, whereas the overexpression of this gene seems to reduce sensitivity to exogenous ABA in germination.

ERD15 Modulates ABA-Induced Gene Expression

ABA regulates the expression of numerous plant genes involved in plant responses to abiotic environmental stresses, especially those involved in drought response (Yamaguchi-Shinozaki and Shinozaki, 2005). To correlate the ABA-related abiotic stress phenotypes of the transgenic *ERD15* plants with corresponding gene expression, we exposed *ERD15* overexpression, *ERD15* RNAi-silenced, and vector control plants to drought stress, and followed the accumulation of transcripts of two ABA-responsive genes, *RAB18* (Lång and Palva, 1992) and *LTI78* (Nordin et al., 1991, 1993). In *ERD15* overexpression plants, the drought-induced expression of *RAB18* was reduced when compared with vector control and *ERD15* RNAi-silenced plants. Similar reduction of *LTI78* expression was observed in *ERD15* overexpression plants (Fig. 4A). To further explore if the altered inducibility of these genes was due to impaired ABA sensing, the transgenic plants were exposed to ABA and we checked ABA-induced transcript accumulation of *RAB18* and *LTI78*. Similar to drought treatment, expression of these marker genes was reduced in plants overexpressing *ERD15*. These expression data support the notion that ERD15 interferes with ABA signaling in Arabidopsis and indicate that modulation of ERD15 levels has an impact on ABA responsiveness of the plants (Fig. 4B).

To correlate the changes seen in abiotic stress tolerance with possible alterations in endogenous hormone levels, we measured the accumulation of ABA in drought-stressed *ERD15* transgenic lines and control plants. Interestingly, the basal ABA level was slightly higher in *ERD15* overexpression plants already under nonstressed conditions when compared with control and *ERD15* RNAi plants (Fig. 4C). This difference was accentuated in drought-stressed plants. After 3 h of drought exposure, the ABA level in *ERD15* overexpression plants was almost 2-fold when compared to the control and *ERD15* RNAi plants (Fig. 4C). These results, together with the drought-tolerance phenotype, inhibition of seed germination, and gene expression data, indicate that alterations of ABA levels cannot explain the observed phenotypes and argue that *ERD15* controls ABA sensitivity of Arabidopsis.

Drought Induction of *ERD15* Is Abolished in *abi1-1* and *abi2-1* Plants

Our results suggest that ERD15 is involved in ABA signaling and could be a negative regulator of several ABA-controlled processes. Interestingly, *ERD15* itself is induced by ABA as well as by drought (Fig. 1, A and D; Kiyosue et al., 1994). To explore the interaction of ERD15 with other regulators of ABA responses, we determined the expression of *ERD15* in ABA-insensitive mutants. To this aim, we drought stressed wild-type Landsberg *erecta* (LE) and ABA-insensitive *abi1-1* and *abi2-1* mutant plants and characterized *ERD15* transcript accumulation. In addition, we employed a double loss-of-function mutant of ABI1 and ABI2, *abi1-1R5 abi2-1R1*, which has hardly any detectable activity of these two phosphatases (Merlot et al., 2001; Fig. 5). In both wild-type plants and in the loss-of-function double mutant *abi1-1R5 abi2-1R1*, *ERD15* was rapidly induced in response to drought. In contrast, in *abi1-1* and *abi2-1* mutant plants, this gene was already upregulated in the untreated controls, and no induction but rather a decrease in *ERD15* expression was evident after drought exposure (Fig. 5). The observed *ERD15* expression in the ABA-insensitive *abi1-1* and *abi2-1* mutants further strengthen the notion that this gene is involved in ABA signaling in Arabidopsis.

ERD15 Promotes Resistance to *E. carotovora*

The inducibility of *ERD15* by a pathogen and pathogen-derived elicitors (Fig. 1), as well as the recent studies suggesting involvement of ABA in the pathogen response of plants (Mohr and Cahill, 2003; Anderson et al., 2004; Mauch-Mani and Mauch, 2005), prompted us to investigate the role of ERD15 in resistance to pathogens. To assess the possible contribution of ERD15 to plant defense, the transgenic lines as well as control plants were locally inoculated with *E. carotovora*, and symptom development as well as

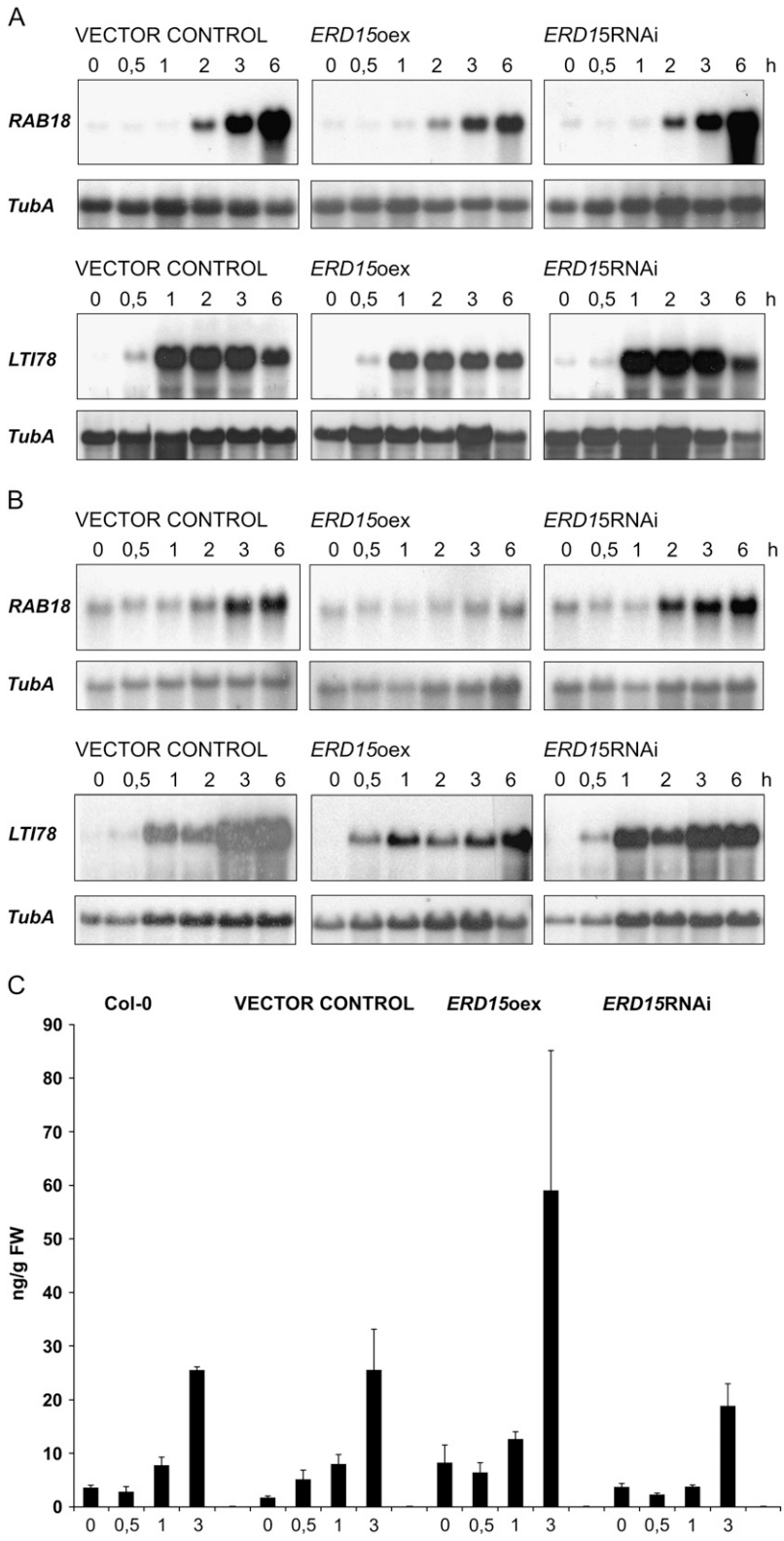

Figure 4. Overexpression of *ERD15* decreases expression of drought-inducible genes but increases stress-induced ABA accumulation. A and B, Local leaf samples were collected from *ERD15* overexpression (oex), *ERD15* RNAi-silenced, and vector control plants 0, 0.5, 1, 2, 3, and 6 h after exposing the plants to drought stress (A) and treating the plants with 100 μM ABA (B). Total RNA was extracted and analyzed by RNA gel-blot hybridization with gene-specific probe for *RAB18* and *LTI78*. Equal loading of RNA samples was checked using a probe for the constitutively expressed α-tubulin (*TubA*) gene. C. Accumulation of ABA was determined in *ERD15* oex, *ERD15* RNAi-silenced, wild-type (Col-0), and vector control plants 0, 0.5, 1, 2, and 3 h after exposing the plants to drought stress. FW, Fresh weight. In both cases, similar results were obtained from two independent experiments.

bacterial growth were followed. *ERD15* overexpression plants displayed enhanced resistance to this pathogen: The majority of the inoculated leaves showed no or minor symptom development after 24 h (Fig. 6A), and a clear reduction was seen in the pathogen growth (Fig. 6B). In contrast, in both vector control and *ERD15* RNAi plants, the disease symptoms spread rapidly and the inoculated leaves were almost completely macerated after 24 h (Fig. 6A). Also, the bacterial growth was clearly improved when compared to

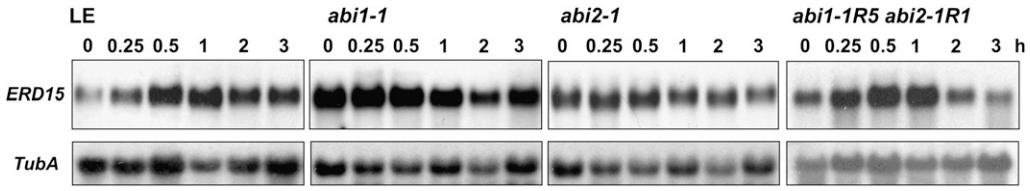

Figure 5. The basal expression level of *ERD15* is increased in *abi1-1* and *abi2-1* mutants. Wild-type (LE), *abi1-1*, *abi2-1*, and *abi1-1R5 abi2-1R1* plants were exposed to drought stress, and local leaf samples were collected 0, 0.25, 0.5, 1, 2, and 3 h after this. Total RNA was extracted and analyzed by RNA gel-blot hybridization with gene-specific probe for *ERD15*. As a control for equal loading, the samples were probed with the constitutively expressed α-tubulin (*TubA*) gene. In both cases, similar results were obtained from three independent experiments.

ERD15 overexpression plants (Fig. 6B). These results demonstrate that overexpression of *ERD15* promotes plant resistance against *E. carotovora*.

The Expression of SAR Marker Genes Is Enhanced in *ERD15* Overexpression Plants

To explore the cause for the dramatic improvement in plant resistance to *E. carotovora* in *ERD15* overexpression lines, we elucidated the role of different defense pathways in this resistance. Enhanced resistance to *E. carotovora* in Arabidopsis can be generated either by induction of JA/ET-mediated (Vidal et al., 1998; Norman-Setterblad et al., 2000; Kariola et al., 2003) or SA-mediated defenses (Palva et al., 1994; Kariola et al., 2003; Li et al., 2004).

To distinguish between these possibilities, we explored the effect of *ERD15* levels on expression of defense pathway-specific marker genes following induction of defense responses. To avoid possible problems due to differences in the progress of infection, we used SA and MeJA in addition to pathogen inoculation

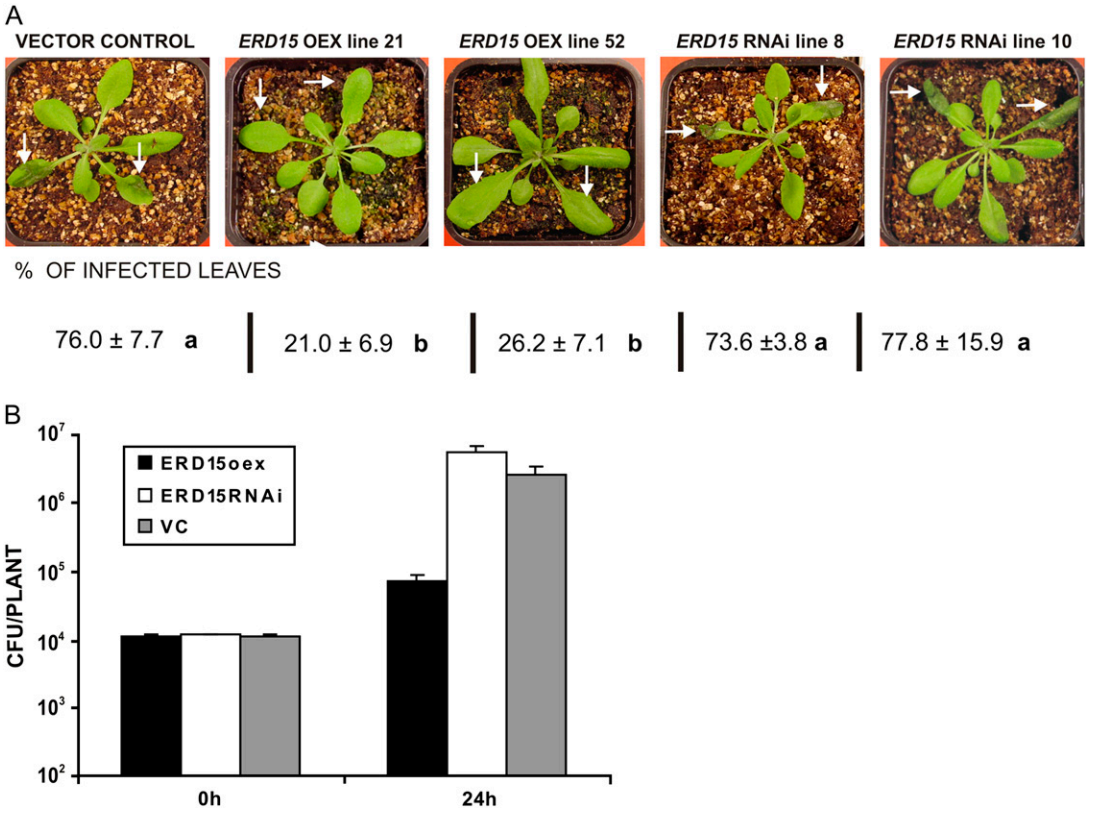

Figure 6. *ERD15* overexpression (oex) plants are resistant to *E. carotovora* infection. A, Two to three leaves of Arabidopsis *ERD15* oex, RNAi-silenced, and vector control lines were inoculated by infiltration with *E. carotovora*. Infiltrated leaves are indicated with arrows. *ERD15* oex, RNAi-silenced, and vector control plants 24 h after bacterial inoculation are shown. Percentage of infected leaves 24 h after inoculation is shown. Different letters indicate significant differences ($P < 0.05$) calculated with one-way ANOVA followed by Tukey's HSD test. B, Growth of *E. carotovora* in planta 0 and 24 h after the inoculation. Colony forming units of four to six plants were determined from the time points in three independent experiments. The values represent the average of four replicates ±SE.

of the plants. First, we monitored expression of *PDF1.2*, a JA/ET-responsive gene (Penninckx et al., 1996), and found that the induction was both delayed and decreased in *ERD15* overexpression plants in response to both MeJA and *E. carotovora* when compared with *ERD15* RNAi and vector control plants (Fig. 7A).

Expression of the *PR2* gene is associated with the SA-mediated systemic acquired resistance (SAR) response (Nawrath and Metraux, 1999). In *ERD15* overexpression plants, the SA-induced expression of *PR2* was clearly up-regulated after 24 and 48 h when compared with vector control and *ERD15* RNAi plants (Fig. 7B). The enhanced SA-mediated defense in *ERD15* overexpression plants was also evident after the challenge with *E. carotovora*: *PR2* induction was clearly faster in these plants—strong induction was evident already at 8 h (Fig. 7B). The results show that the improved disease resistance of the *ERD15* overexpression plants is correlated with enhanced expression of the SAR marker *PR2* and suggest that it could be due to improved induction of SA-dependent defenses. However, the increased SAR response does not seem to be a result of altered SA or JA production, since there was no detectable change in the basal levels of these hormones in the transgenic *ERD15* plants (data not shown).

Insensitivity to ABA Enhances Resistance to *E. carotovora* in Arabidopsis

The altered sensitivity to ABA and pronounced differences in resistance to *E. carotovora* in *ERD15* overexpression and RNAi-silenced plants prompted us to elucidate the contribution of ABA to the resistance of Arabidopsis against this pathogen. To assess this, wild-type LE plants, along with the ABA-insensitive mutants *abi1-1* and *abi2-1*, were inoculated with

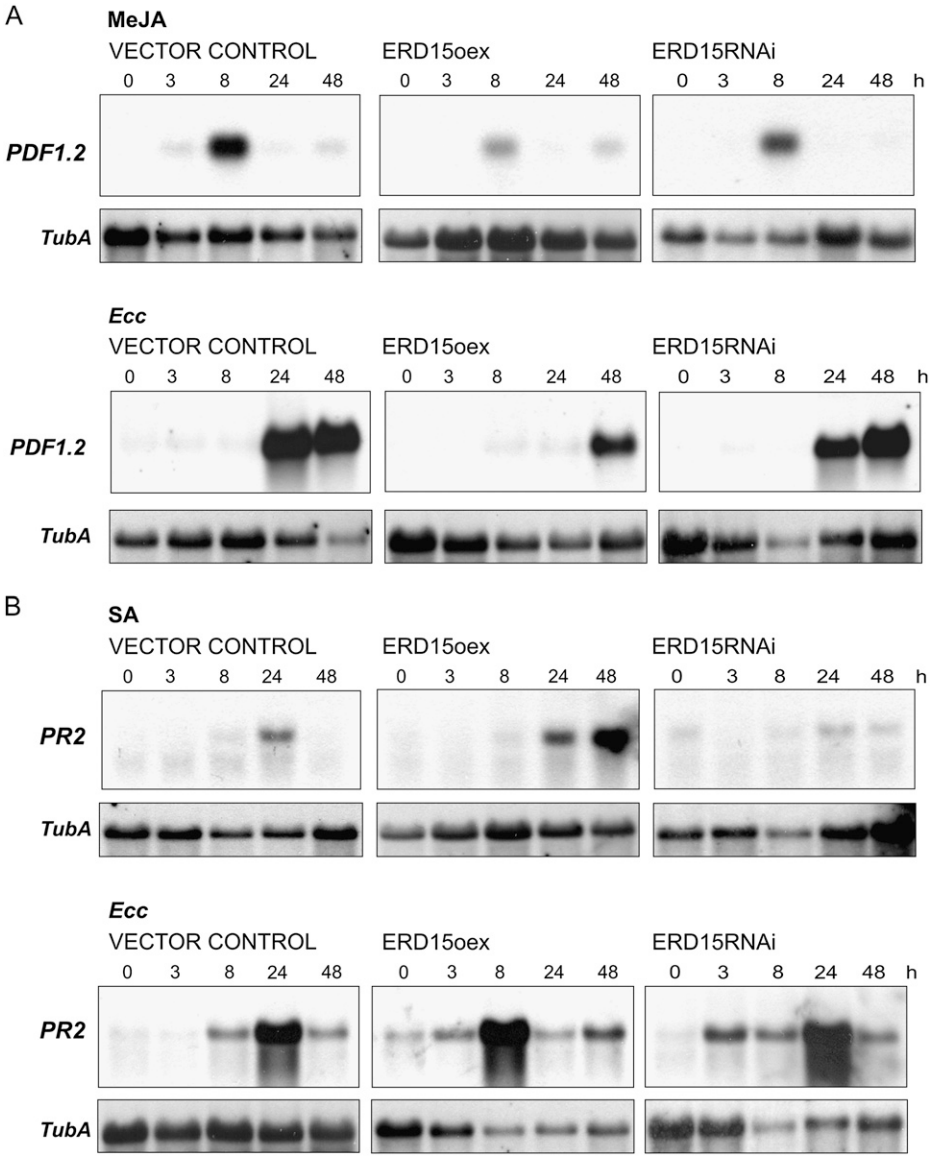

Figure 7. The expression of SAR marker genes is enhanced in *ERD15* overexpression (oex) plants. Local leaf samples were collected from *ERD15* oex, *ERD15* RNAi-silenced, and vector control plants 0, 3, 8, 24, and 48 h after inoculation with *E. carotovora* and treatments with SA and MeJA. Total RNA was extracted and analyzed by RNA gel-blot hybridization with gene-specific probe for *PDF1.2* after treatment with MeJA and after inoculation with *E. carotovora* (*Ecc*; A) and with *PR2* after treatment with SA and inoculation with *Ecc* (B). Equal loading of RNA samples was checked using a probe for the constitutively expressed α-tubulin (*TubA*) gene. In both cases, similar results were obtained from three independent experiments.

E. carotovora and symptom development was followed. Already 24 h after inoculation with the pathogen, the difference in resistance between the plant lines was obvious (Fig. 8A). In LE plants the maceration had proceeded considerably, whereas most *abi1-1* and *abi2-1* plants showed clearly reduced symptom development (Fig. 8A). The decreased maceration in *abi1-1* and *abi2-1* plants was accompanied with a distinct reduction in the pathogen growth (Fig. 8B).

Earlier studies on the role of ABA in pathogen resistance indicate that depletion of this phytohormone enhances SA-mediated defense responses (Audenaert et al., 2002; Thaler and Bostock, 2004). To assess this, we employed the ABA-insensitive mutants *abi1-1* and

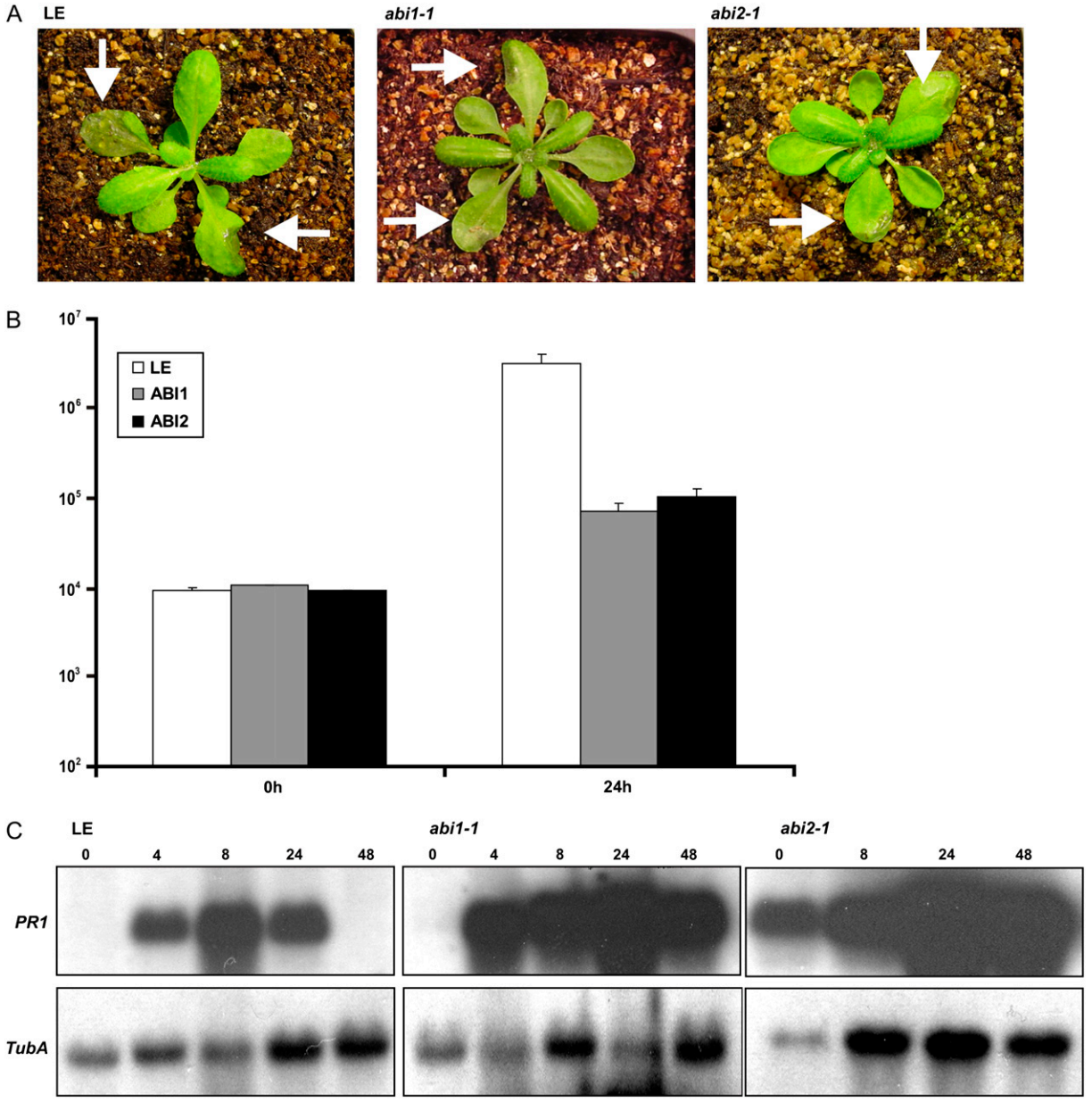

Figure 8. Insensitivity to ABA enhances SA-dependent defense gene expression and improves resistance to *E. carotovora*. A, Wild-type (LE), *abi1-1*, and *abi2-1* mutants were inoculated by infiltration with *E. carotovora* and shown 24 h after infiltration. Infiltrated leaves are indicated with arrows. B, Growth of *E. carotovora* in planta 0 and 24 h after the inoculation. Colony forming units of four to six plants were determined from the time points in two independent experiments. The values represent the average of four replicates ±SE. C, Local leaf samples were collected from wild-type (LE), *abi1-1*, and *abi2-1* mutants 0, 4 (LE and *abi1-1*), 8, 24, and 48 h after treatment with SA. Total RNA was extracted and analyzed by RNA gel-blot hybridization with gene-specific probe for *PR1*. Equal loading of RNA samples was checked using a probe for the constitutively expressed α-tubulin (*TubA*) gene. In both cases, similar results were obtained from two independent experiments.

abi2-1 and characterized the SA-induced expression of *PR1*, a marker for SA-dependent defenses. The expression of this gene was clearly up-regulated in both ABA-insensitive mutants when compared with wild-type LE (Fig. 8C). The expression of *PR1* was already slightly up-regulated in the nontreated mutant samples and rapidly induced by SA to much higher levels than in the wild type (Fig. 8C). These results further support the hypothesis that ABA affects defense signaling of Arabidopsis and indicate that insensitivity to this phytohormone contributes to the resistance against *E. carotovora*.

DISCUSSION

ERD15 is rapidly but transiently induced in response to various stress factors and stress-related hormones, such as dehydration (Kiyosue et al., 1994), ABA, wounding, SA, the plant pathogen *E. carotovora* (Fig. 1), salt, and low temperature (data not shown) in Arabidopsis. Rapid response to such a wide variety of abiotic and biotic factors suggests a significant role for this gene in mediating plant stress responses. However, the actual function of ERD15 has been an enigma since it was first characterized (Kiyosue et al., 1994). In this study, we provide evidence that ERD15 controls ABA-mediated stress responses in Arabidopsis and propose that ERD15 is a novel, negative regulator of ABA signal transduction related to these processes.

Our results demonstrate that overexpression of *ERD15* decreases tolerance of the transgenic plants to stresses that involve ABA signaling: drought and freezing. Accordingly, silencing of *ERD15* resulted in improved drought as well as freezing tolerance of the plants. Overexpression of *ERD15* was also accompanied by decreased expression of the ABA-responsive genes *RAB18* and *LTI78* (Fig. 4B). Our results are best explained by altered responsiveness to ABA due to modulation of ERD15 levels. Overexpression of *ERD15* results in reduced sensitivity to ABA, while silencing of the gene results in ABA hypersensitivity. The altered responsiveness of *ERD15* transgenic plants was also observed in seed germination in the presence of ABA: Silencing of *ERD15* resulted in hypersensitivity to this phytohormone, whereas the seeds of overexpression plants demonstrated reduction of sensitivity. Furthermore, overexpression of *ERD15* resulted in increased accumulation of ABA, a phenotype observed with other ABA-insensitive mutants (Lång and Palva, 1992; Mäntylä et al., 1995; Verslues and Bray, 2006; Fig. 4C). Interestingly, besides abiotic stress, the modulation of *ERD15* expression had an impact on the biotic stress tolerance of Arabidopsis as well: Overexpression of this gene enhanced the induction of SAR response and resistance to the pathogen *E. carotovora*.

The altered ABA sensitivity of transgenic *ERD15* plants can be explained as a result of changed expression of a negative regulator of ABA responses (Fig. 9). ABA is the central hormone mediating drought responses and overexpression of *ERD15* decreased the drought and freezing tolerance of the plants, a likely consequence of enhanced activity of a negative

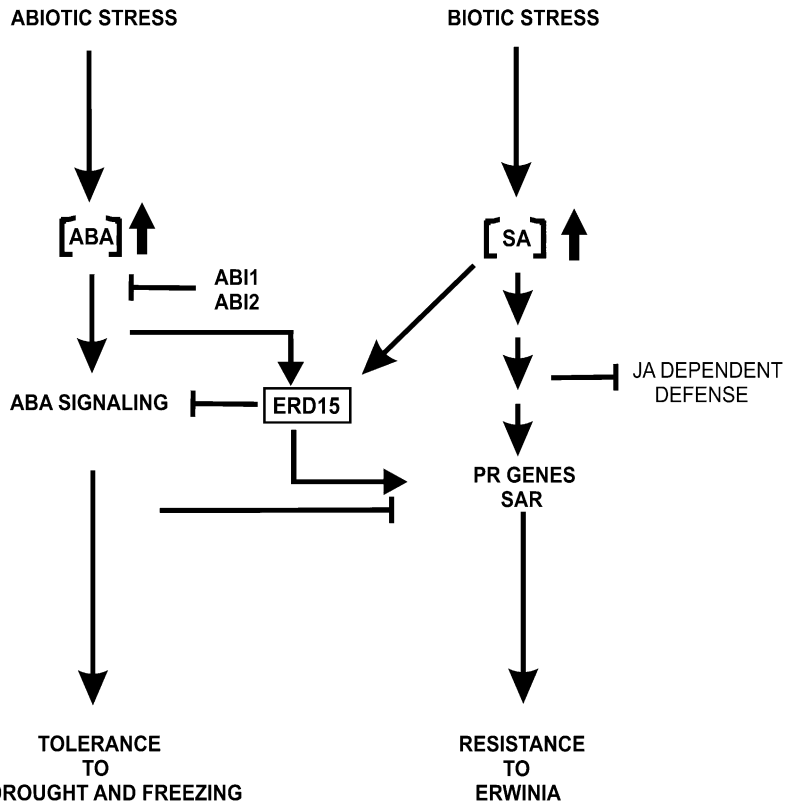

Figure 9. Hypothetical model presenting the role of ERD15 in ABA responses. The activation of ABA-signaling pathway by abiotic stress leads to drought and freezing tolerance. ERD15 negatively regulates the transduction of ABA signal, possibly downstream of the protein phosphatases ABI1 and ABI2. The negative effect of ERD15 on ABA signaling enhances SA-dependent defense seen as improved induction of *PR* genes and leading to enhanced resistance to the pathogen *E. carotovora*. Simultaneously, the enhanced SAR response down-regulates JA-dependent defense responses.

regulator. Freezing is closely related to drought stress since it involves cellular dehydration (Thomashow, 1999). The fact that neither overexpression nor silencing of *ERD15* had an effect on the capability of the plants to improve their freezing tolerance in response to low temperature underlines the ABA-specific role of ERD15. We propose that the enhanced activity of a negative regulator, ERD15, confers the observed reduction in ABA sensitivity. It is possible that the plant tries to compensate this reduced ABA sensitivity by producing more ABA. Lack of feedback can explain the moderately increased basal endogenous as well as the increased stress-induced ABA level in *ERD15* overexpression plants in comparison to controls (Fig. 4C). A similar, feedback regulation-related increase in ABA levels has previously been observed in the ABA-insensitive *abi1-1* and *abi2-1* mutants (Lång and Palva, 1992; Verslues and Bray, 2006).

The impaired seed germination of *ERD15* RNAi-silenced plants in the presence of ABA supports increased sensitivity to this hormone as a consequence of silencing *ERD15*. Also, the improved drought and freezing tolerance of *ERD15* RNAi-silenced plants can be interpreted as a result of more efficient response to cellular dehydration stress after removal of a negative regulator. The altered expression of *ERD15* in the ABA-insensitive *abi1-1* and *abi2-1* mutants further strengthens the role of this gene in ABA-mediated processes. However, ERD15 does not seem to be under the control of ABI1 and ABI2 alone, since the loss-of-function double mutant *abi1-1R5 abi2-1R1* has similar expression of this gene as the wild-type plants. We suggest that ERD15 has a role as a negative regulator in the early stages of ABA signaling controlling stress tolerance and affecting seed germination (Fig. 9). This is supported by the rapid induction of *ERD15* by both ABA and stress.

Not only does ERD15 control abiotic stress tolerance, but it also has a clear impact on biotic stress responses as demonstrated by the improved resistance of *ERD15* overexpression plants to the plant pathogen *E. carotovora*. Consequently, it seems that the insensitivity or slow response to the ABA signal is not necessarily bad for the plant stress responses but could improve disease resistance. We hypothesize that the enhanced resistance of the overexpression plants could be due to the observed reduction in ABA sensitivity (Fig. 9). This is clearly different from previous studies where ABA deficiency, not insensitivity, has been proposed as the basis for the decreased pathogen susceptibility (Mauch-Mani and Mauch, 2005).

Until recently, the main focus of ABA research has been in its role in abiotic stress responses, and, thus, the role of this hormone in plant-pathogen interactions still poses many questions (Mauch-Mani and Mauch, 2005). Mohr and Cahill (2003) demonstrated that when Arabidopsis was either drought stressed or treated with ABA prior to infection with an avirulent strain of *P. syringae* pv *tomato*, the outcome was necrosis and chlorosis, symptoms of a susceptible interaction. Related studies with the ABA-deficient tomato mutant *sitiens* indicate that depletion of ABA enhances the resistance of these mutant plants against the fungal pathogen *Botrytis cinerea*, and the susceptibility of the *sitiens* plants can be restored by application of ABA (Audenaert et al., 2002).

Both Audenaert et al. (2002) and Thaler and Bostock (2004) showed that depletion of ABA enhanced SA-dependent defense responses and suggest an antagonistic effect of ABA on SA-mediated defense. SA-dependent defenses have been shown to be effective against *E. carotovora* (Li et al., 2004; Kariola et al., 2005), and, indeed, the SAR response was enhanced in Arabidopsis plants overexpressing *ERD15*, evidenced by enhanced induction of SAR markers as well as resistance to *E. carotovora*. Alterations in the hormone levels do not seem to be causing these differences, since basal contents of SA or JA are not altered in these transgenic lines (data not shown). We propose that the reduced ABA sensitivity of *ERD15* overexpression plants has a positive impact on the SA-dependent defense responses (Fig. 9). This is further supported by the improved resistance the ABA-insensitive *abi1-1* and *abi2-1* mutant plants display to *E. carotovora*, also accompanied by a stronger SAR response (Fig. 8). Interestingly, recently characterized AtPARN, involved in mRNA degradation, could be a common component for ABA- and SA-signaling pathways: It has a prominent role not only in ABA- but also in SA-mediated stress responses in Arabidopsis (Nishimura et al., 2005).

An antagonism has also been reported between ABA and JA signaling: Anderson et al. (2004) demonstrated that exogenous ABA down-regulated JA- or ET-dependent defense genes. This could in turn improve SA-dependent defenses since the mutual antagonism between SA and JA signaling is well established (Petersen et al., 2000; Kunkel and Brooks, 2002; Li et al., 2004, 2006; Glazebrook, 2005). This antagonism may also explain the observed down-regulation of the JA/ET-dependent *PDF1.2* gene by the enhanced SAR response in plants overexpressing *ERD15*.

How is ERD15 able to modulate ABA responses? Recently, ERD15 was described to have a PAM2 motif that enables the interaction with the C terminus of poly(A)-binding proteins (PABP; Albrecht and Lengauer, 2004; Kozlov et al., 2004), an interaction demonstrated in a yeast two-hybrid assay by Wang and Grumet (2004). PABPs are important in the regulation of translation and mRNA stability since they bind to the poly(A) tails of mRNAs before these are taken to the translational machinery (Belostotsky, 2003; Albrecht and Lengauer, 2004). Several mutations in genes encoding proteins involved in RNA metabolism, such as the mRNA cap-binding *ABH1*, have been shown to affect ABA sensitivity in Arabidopsis (Hugouvieux et al., 2001; Kuhn and Schroeder, 2003). A recent study by Razem et al. (2006) characterized the RNA-binding protein FCA as a receptor for ABA, which further strengthens the prominent role of posttranscriptional regulation in ABA signal transduction. ERD15 with its

PABP-binding ability combined with the effect it has on ABA sensing fits this category well, and future studies including microarray analysis should further clarify the role of ERD15 in plant stress responses. (A microarray analysis of ABA-induced gene expression in *ERD15* overexpression plants compared to control plants has been performed and the data can be found in the Web pages of NASC Affymetrix http://affymetrix.arabidopsis.info/, experiment reference no. NASCARRAYS-321.)

Based on our results, we suggest that ERD15 is a negative regulator of the early stages of stress-related ABA signaling in Arabidopsis (Fig. 9). It prevents the plants from responding too fast after the onset of abiotic stress, possibly by acting as a capacitor attenuating the ABA response: Only after input of sufficient stimuli is the capacitor overflown and the downstream response triggered. This system would ensure that the plant responds only when it becomes essential to invest assets in stress adaptation. It would be a waste of resources to activate a large-scale response before it is certain that the stress prevails and adaptation is necessary. Heil (2002) introduced a similar concept for biotic stress. Elucidating the mechanistic role of ERD15 in detail and identifying the possible translational partners and the specific transcripts this protein regulates will be of great interest for future studies and give new insights into plant ABA signaling.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotypes Col-0 and LE and mutant plants derived from LE were used in all experiments. Seeds were germinated on Murashige and Skoog (MS) medium (Sigma-Aldrich) plates and seedlings transferred either to soil or to MS in 12-well plates (Cellstar; Greiner Bio-One) after 1 week. Plants were grown in 1:1 peat:vermiculite (Finnpeat B2; Kekkilä Oyj) with a 12-h-light period at 22°C. Four- to 5-week-old plants were used for experiments.

Generation of Transgenic Plants

A 790-bp full-length fragment for *ERD15* was cloned from a cDNA library of Arabidopsis plants treated with CF by PCR using the following primer pair: 5′-GACATATTTATCAACTTGATCAACTTGAG-3′ and 5′-CGGAATTCAACTCTAGTTCTCATTTCTCTTC-3′. The full-length PCR fragment was digested with *Xba*I and *Eco*RI, cloned into a pBluescript II SK vector (Stratagene), and sequenced to verify the sequence. The plasmid harboring the full-length fragment for *ERD15*-designed pBluescript-*ERD15* was digested with *Xba*I and *Eco*RI, and then subcloned into the corresponding sites of the binary vector pCP60, which is derived from pBIN19 containing the 35S promoter of *Cauliflower mosaic virus*, multiple cloning sites, and NOS, resulting in the overexpression construct S-pCP60-*ERD15*, with the 35S promoter directing expression in the sense orientation of the full-length *ERD15*.

A 511-bp DNA fragment was obtained using the *ERD15* full-length fragment as template with the primer pair 5′-CGGAATTCTCAGCGAGGCTGGTGGATG-3′ and 5′-AGGGAGCTCTGAGAATGGCGATGGTATCAGGA-3′, digested with *Eco*RI and *Sac*I, and then cloned into the *Eco*RI-*Sac*I sites of pBluescript-*ERD15*. Since this fragment is in antisense orientation, the vector was called pBluescript-*ERD15*-loop. The *Xba*I-*Sac*I fragment from the loop construct was cloned into pCP60, resulting in RNAi construct A-pCP60-*ERD15*. The fidelity of all constructs was confirmed by restriction and sequence analysis. Arabidopsis transformation was performed as described previously (Clough and Bent, 1998). Transgenic progeny lines with single insertion loci were selected on MS plates containing kanamycin and carried to homozygosity. The empty vector pCP60 was used to generate transgenic control plants in a similar manner.

Production of Polyclonal Anti-ERD15 Serum

Polyclonal antibodies against ERD15 were raised by immunizing a rabbit four times subcutaneously at 21-d intervals. Before the immunization, a preimmune blood sample was taken. For the primary immunization, 300 μg of purified ERD15 protein (received from Jack Leo and Adrian Goldman) emulsified with complete Freund's adjuvant (MP Biomedicals) was used. In subsequent boosters, 300 μg of ERD15 and incomplete Freund's adjuvant were used. Serum was collected 1 week after the last immunization. Specificities of the preimmune serum and the anti-ERD15 serum were determined by western blotting (1:100–1:50,000 dilutions) using anti-rabbit IgGs conjugated with alkaline phosphatase (Promega) secondary antibodies.

Protein Extraction and Western-Blot Analyses

Protein extraction was done as described by Lång et al. (1989). Ten micrograms of protein extract was loaded to 12 5% SDS-PAGE gels. SDS-PAGE and western blotting were done according to standard protocols. Anti-ERD15 serum was diluted 1:1,000 and used for immunodetection of ERD15. A goat-anti-rabbit antibody conjugated with alkaline phosphatase was diluted 1:10,000 and used as secondary antibody. Detection was made using NBT/BCIP as substrate. ERD15 was detected from drought-stressed samples.

Pathogen Strains and Plant Stress Treatments

Erwinia carotovora subsp. *carotovora* strain SCC1 (Rantakari et al., 2001) was propagated in Luria medium (Miller, 1972) at 28°C. An overnight culture was centrifuged for 7 min (6,500*g*), the pellet resuspended in 1 mL of 0.9% NaCl, and diluted to the appropriate concentration. The plants were infected by infiltrating *E. carotovora* subsp. *carotovora* SCC1 culture (approximately 10^4–10^5 cfu/plant) with a needleless syringe. The plants were infected at approximately 200 μmol m^{-2} s^{-1} photon flux density at approximately 80% humidity in a growth chamber with a 12-h-light period.

MeJA was applied to the plants as 100 μM and SA as 5 mM both by pipetting 5- × 5-μL droplets on the leaves. ABA was added by spraying as 100 μM solution (soil-grown plants) and by pipetting to MS media to final concentration of 60 μM (axenically grown plants). Wounding was done by pressing two leaves per plant with forceps. Salt was added by infiltrating 0.9% NaCl solution to two leaves per plant. Plants were exposed to drought stress by cutting off leaves and leaving them to dry on Whatman 3 paper for different periods of time for gene expression and determination of ABA. To see the drought phenotype, the plants were put to growth chamber with 50% humidity and left without watering for 2 weeks.

Assessment of Freezing Tolerance

To determine the degree of freezing tolerance, two methods were used. In freezing survival test, 3-week-old soil-grown plants were placed at −2°C in a phytotron for 1 h, after which freezing of the plants was initiated by spraying the plants with ice cold tap water. The plants were kept at −2°C for additional 4 h. The temperature was then decreased by 2°C per hour until it reached −10°C and kept there for 20 h. The temperature was allowed to return slowly to 22°C during 20 h. The plants were moved to normal growth conditions and assessed visually after 7 d.

In the electrolyte leakage test (Sukumaran and Weiser, 1972), axenically grown plants were harvested without roots and wrapped in moist Miracloth (Calbiochem). Plants were placed in test tubes in a controlled freezing bath. Extracellular freezing was initiated at −1.5°C by touching the samples with a frosted wire. After a 1.5-h equilibrium period, the temperature of the bath was decreased by 2°C per hour. Samples were taken at 1°C or 2°C intervals and thawed on ice overnight. Leaking electrolytes were extracted with deionized water (20 mL) by shaking for 1 h at room temperature and the conductivity was measured. The samples were then frozen in liquid nitrogen, reextracted with the original solution by shaking for 1 h at room temperature, and the conductivity was measured again. Plants showing leakage of 50% (EL_{50}) or more were considered dead and EL_{50} values were calculated by Probit analysis with SPSS 10 (SPSS).

RNA Gel-Blot Analyses

Isolation of total RNA, labeling of DNA probes with digoxigenin (DIG), and RNA gel-blot analysis was performed as described previously (Kariola et al., 2003), and the membranes were hybridized with PCR-labeled gene-specific DNA or RNA DIG probes. DIG labeling of RNA, hybridization, and detection were done according to the manufacturer's instruction (Roche, Basel). A 790-bp cDNA fragment cloned to pCR2.1 (Invitrogen) was used as a template for an *ERD15* (At2g41430)-specific RNA probe synthesized with T7 RNA-polymerase (Promega). DNA probes were amplified by PCR from the cDNA of *PR1* (At2g14610; Uknes et al., 1992) and *PR2* (At3g57260; Chen et al., 1995). *PDF1.2* (At5g44420) and *GST1* (At1g02930) probes were obtained from the Arabidopsis Biological Resource Center (GenBank accession nos. T04323 and N37195).

Quantification of Plant Hormones

Drought-stressed leaves (80–150 mg) were frozen and ground in liquid nitrogen, and ABA, SA, and JA were quantified with the vapor-phase extraction method described by Schmelz et al. (2003) using 40 ng of $^{13}C_1$-SA, 20 ng of dihydrojasmonic acid (Montesano et al., 2005), and 10 ng of D_6-ABA from Icon Isotopes as internal standard for each sample. GC-MS analysis was performed on a Trace-DSQ from Thermo as described previously (Montesano et al., 2005).

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