

# DEAR1, a transcriptional repressor of DREB protein that mediates plant defense and freezing stress responses in Arabidopsis

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**Abstract** Plants have evolved intricate mechanisms to respond and adapt to a wide variety of biotic and abiotic stresses in their environment. The Arabidopsis *DEAR1* (DREB and EAR motif protein 1; At3g50260) gene encodes a protein containing significant homology to the DREB1/CBF (dehydration-responsive element binding protein 1/C-repeat binding factor) domain and the EAR (ethylene response factor-associated amphiphilic repression) motif. We show here that *DEAR1* mRNA accumulates in response to both pathogen infection and cold

treatment. Transgenic Arabidopsis overexpressing *DEAR1* (*DEAR1ox*) showed a dwarf phenotype and lesion-like cell death, together with constitutive expression of *PR* genes and accumulation of salicylic acid. *DEAR1ox* also showed more limited *P. syringae* pathogen growth compared to wild-type, consistent with an activated defense phenotype. In addition, transient expression experiments revealed that the *DEAR1* protein represses DRE/CRT (dehydration-responsive element/C-repeat)-dependent transcription, which is regulated by low temperature. Furthermore, the induction of DREB1/CBF family genes by cold treatment was suppressed in *DEAR1ox*, leading to a reduction in freezing tolerance. These results suggest that *DEAR1* has

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an upstream regulatory role in mediating crosstalk between signaling pathways for biotic and abiotic stress responses.

**Keywords** Arabidopsis · DREB1/CBF family · Biotic stress · Abiotic stress · Salicylic acid · EAR motif

## Introduction

Plants undergo continuous exposure to various stresses in their natural environment. In order to survive such a wide variety of stress conditions, ranging from biotic pathogen infection to abiotic low temperature, plants have evolved intricate mechanisms to respond and adapt to these stresses at the molecular, cellular and whole-plant level.

Exposure to freezing temperatures is an abiotic stress that has a huge impact on the survivability and distribution of living organisms. Many temperate plants have the potential to increase their freezing tolerances after a prior exposure to nonfreezing low temperatures in a process known as cold acclimation (Guy 1990; Hughes and Dunn 1996; Browse and Xin 2001; Agarwal et al. 2007). At the molecular level, specific proteins are induced in response to low temperature that help cells cope with cold and freezing stress (Thomashow 1999; Knight et al. 1999; Tähtiharju and Palva 2001; Gong et al. 2002; Hsieh et al. 2002). These induced proteins include those involved in the metabolism of carbohydrates, lipids, and phenylpropanoids, those functioning as antioxidants, molecular chaperones and antifreeze proteins, and many others with a presumed function in tolerance to cellular dehydration caused by extracellular freezing (Guy 1990; Thomashow 1999; Mohapatra et al. 1989). In most cases, proteins involved in cold acclimation are transcriptionally regulated in response to low temperature. The timing of stress-responsive gene expression is regulated by a combination of transcriptional

factors and cis-acting elements in stress-inducible promoters. The DRE/CRT (dehydration-responsive element/C-repeat) sequence contains an A/GCCGAC motif and is a major cis-acting element in cold-inducible genes such as *RD29A* and *COR15A* (Yamaguchi-Shinozaki and Shinozaki 1994; Stockinger et al. 1997). DRE/CRT-binding proteins DREB1/CBF (DRE binding protein 1/C-repeat binding factor) and DREB2 contain the conserved DNA-binding domain found in ERF (ethylene response factor) and AP2 (APETALA2) family proteins. These proteins bind specifically to the DRE/CRT element within gene promoters to activate transcription of the respective gene. In contrast to that for *DREB2*, expression of *DREB1/CBF* genes is induced by cold, but not by dehydration and high-salinity stresses. Moreover, Arabidopsis plants overexpressing *DREB1B/CBF1* and *DREB1A/CBF3* show a high tolerance to freezing stress. DREB1/CBF thus has a prominent role in cold-responsive gene expression in Arabidopsis.

Plants also respond to biotic stress such as pathogen infection by activating a defense mechanism known as plant immunity. One of the most efficient and immediate resistance reactions against pathogen attack in plants is the hypersensitive response (HR), which leads to rapid local cell death at the site of pathogen entry that restricts growth and spread of the pathogen (Heath 2000a, b; Lam 2001). Several *PR* (pathogenesis-related) genes are also induced during HR, and most of these corresponding proteins have been shown to possess antimicrobial activity in vitro or an ability to enhance disease resistance when overexpressed in transgenic plants (Ryals et al. 1996). The control of this HR-mediated cell death in response to pathogen attack seems to involve the concerted action of several signaling molecules. Salicylic acid (SA) has emerged as a key signaling component for activating both HR and *PR* gene expression. Indeed, SA levels increase in conjunction with the activation of *PR* gene expression and disease resistance in many plant species (Malamy et al. 1990; Métraux et al. 1990; Ukniss et al. 1993). Ethylene and jasmonate have also been implicated as an important signal during the plant defense response (Penninckx et al. 1996; Turner et al. 2002; Lorrain et al. 2003), and are produced in response to pathogen infection. Several lines of evidence suggest that there is crosstalk between the SA- and ethylene and/or jasmonate-dependent defense pathways (Felton and Korth 2000; Feys and Parker 2000; Turner et al. 2002).

Transcriptional repressors are emerging as central regulators of development and stress responses in different organisms. By suppressing defense responses and keeping developmental programs under control they are thought to prevent excessive waste of resources and the activation of programmed cell death caused by metabolic imbalances or runaway response pathways (Cowell 1994; Thiel et al.

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2004; Eulgem 2005; Kazan 2006). In plants, the EAR (ERF-associated amphiphilic repression)-motif was first identified in the C-terminal region of class II AP2/ERFs and Cys2/His2-type zinc-finger proteins (Ohta et al. 2001). The EAR motif-containing transcription factor is thought to play an important role in regulating the defense and abiotic stress responses (Kazan 2006). Indeed, the EAR motif was able to convert a transcriptional activator into a strong repressor, and the repressive activity of the EAR motif repression domain was dominant over both intra- and intermolecular activities (Hiratsu et al. 2003).

We report here the identification and characterization of *DEAR1*, which encodes an EAR motif protein and acts as a transcriptional repressor of DREB1/CBF protein. Transgenic plants overexpressing *DEAR1* showed a cell death phenotype, increased resistance to pathogen infection and reduced freezing tolerance. Taken together, the data indicates that *DEAR1* functions to mediate crosstalk between signaling pathways for biotic and abiotic stress responses.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis thaliana* plants were grown at 22°C. Wild-type plants were *A. thaliana* Col-0 ecotype. For germination, seeds were surface sterilized and placed on Murashige and Skoog medium (Wako, Japan) supplemented with 20 g l<sup>-1</sup> sucrose. After an overnight 4°C treatment for stratification, seeds were grown at 22°C and 50% relative humidity under a 16/8 light/dark cycle.

### Molecular cloning of *DEAR1*

Full-length cDNA fragments of *DEAR1* gene (At3g50260) were obtained from RIKEN RBC and sequenced. Transgenic plants overexpressing *DEAR1* alone (*35S::DEAR1*) were produced according to the methods described by Ichikawa et al. (2006). The overexpressor construct for *DEAR1ox* transgenic plants (*35S::DEAR1::GFP*) was generated by cloning the *DEAR1* cDNA fragment into pGWB5 (Saito et al. 1999) through the pENTR vector according to the Gateway instruction manual (Invitrogen, Carlsbad, CA). The resulting construct contains *DEAR1* fused to the C-terminal end of a synthetic *GFP* gene (Chiu et al. 1996) under the control of the CaMV 35S promoter.

### Infection with *Pseudomonas syringae* pv. *tomato* DC3000

The virulent bacterial leaf pathogen *P. syringae* pv. *tomato* DC3000, which causes bacterial speck disease, was grown

overnight at 28°C in NYGB liquid medium [0.8% (w/v) nutrient broth, 0.2% (w/v) yeast extract, 0.2% (w/v) K<sub>2</sub>HPO<sub>4</sub>, 0.05% (w/v) KH<sub>2</sub>PO<sub>4</sub>, 0.5% (w/v) glucose, 0.025% (w/v) MgSO<sub>4</sub>]. Bacterial cells were collected by centrifugation and resuspended in 10 mM MgCl<sub>2</sub> to a final density of 10<sup>5</sup> colony-forming units (cfu) ml<sup>-1</sup>. For the inoculation of plants by leaf dip, the surfactant Silwet-77 (Bio Medical Science) was added to a final concentration of 0.01%. Leaf discs were bored from the infiltrated area, ground in 10 mM MgCl<sub>2</sub>, and serially diluted to measure bacterial numbers (Tsutsui et al. 2008). For each sample, eight leaf discs were pooled five times per data point.

For gene expression analyses, the concentration of *P. syringae* was adjusted to 10<sup>7</sup> cfu ml<sup>-1</sup> and the suspension used to infiltrate leaves of 3-week-old plants. Infected leaves were harvested from each line at the time points indicated.

### Trypan Blue staining

Leaves were submerged in lactic acid–phenol–Trypan Blue solution [2.5 mg ml<sup>-1</sup> Trypan Blue, 25% (w/v) lactic acid, 23% water-saturated phenol, 25% glycerol and H<sub>2</sub>O] at 70°C, slow-release vacuum infiltrated for 2 min, and then re-infiltrated. Leaves were then heated over boiling water for 2 min and cooled for 1 h before replacing the lactic acid–phenol–Trypan Blue solution with a chloral hydrate solution (25 g in 10 ml H<sub>2</sub>O) for destaining.

### Gene expression analysis

Total RNA was isolated from wild-type Col and *35S::DEAR1* transgenic plant leaf tissue with the RNeasy Plant RNA isolation kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from total RNA with reverse transcriptase using 1 µg total RNA and an oligo (dT) primer (RNA PCR kit, TAKARA SHUZO, Shiga, Japan). PCR amplification was then performed using *Taq* DNA polymerase (BioLabs). Gene-specific primers were designed to specifically amplify DNA fragments of the *DEAR1*, *DEAR2*, *DEAR3*, *DEAR4*, *DEAR5*, *DEAR6*, *EF1α*, *SAG12*, *SAG13*, *PR1*, *PR2*, *PR3*, *PR5* and *PDF1.2* cDNAs. The primers used are listed in Supplementary Table S2. mRNA of *EF1α* was also measured as a control (Tsutsui et al. 2006).

The amount of template cDNA required and the number of PCR cycles necessary were determined in preliminary experiments to ensure that amplification occurred in the linear range and allowed accurate quantification of the amplified products by Southern blot hybridization analysis. The amplified DNA products from each reaction were separated on a 1.2% (w/v) agarose gel, transferred to a nylon membrane (Hybond-N<sup>+</sup>, Amersham Pharmacia

Biotech, Buckinghamshire, UK), and hybridized with  $^{32}\text{P}$ -labeled cDNA fragments at 65°C. The filter was washed twice with 2×SSC containing 0.1% SDS at 65°C for 15 min and examined by autoradiography.

#### SA measurement

Salicylic acid was extracted from 0.2 g 4-week-old leaves. Each sample was extracted four times with 1.5 ml methanol. A 5  $\mu\text{l}$  aliquot of 1 mg  $\text{ml}^{-1}$  m-hydroxybenzoic acid was added to the extract as an internal standard. The solution was dried in an evaporator and the residue was dissolved in 150  $\mu\text{l}$  methanol and then 600  $\mu\text{l}$  1 mM KOH was added. Lipophilic substances were removed by extraction with chloroform twice. The aqueous phase was transferred to a new tube, and then 10  $\mu\text{l}$  phosphoric acid and 500  $\mu\text{l}$  ethyl acetate were added. The solution was mixed and centrifuged at 17,000  $g$  for 5 min. The supernatant was transferred to a new tube and the aqueous phase extracted again with ethyl acetate. After centrifugation, the supernatant was dried out and the residue was dissolved in 50% methanol and analyzed by HPLC. SA was detected with a fluorescent detector set at  $\text{Ex} = 295 \text{ nm}$  and  $\text{Em} = 370 \text{ nm}$ . The mobile phase was 20 mM sodium acetate containing 20% methanol (Morita-Yamamuro et al. 2005).

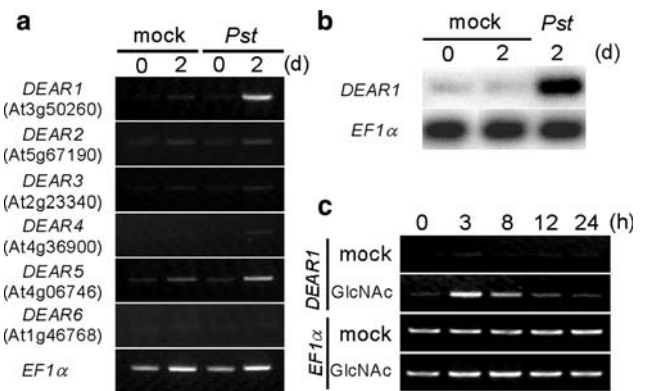
#### Transient expression experiments

The *DREx3::TATA::GUS* reporter plasmid, which contains a *RD29A* minimal TATA promoter with three tandem copies fused to the *GUS* gene, was used as described previously (Liu et al. 1998). Two effector plasmids were used in the transient transactivation experiments; *DREB1A*, *35S- $\Omega$ -DREB1A* (described previously; Liu et al. 1998) and *DEAR1*, *35S-DEAR1*, which contains *DEAR1* coding regions cloned into *pBI221*.

Isolation of Arabidopsis T87 cell protoplasts, polyethylene glycol-mediated DNA transfection, and analysis of reporter activities were performed as described previously (Sato et al. 2004).

#### Freezing stress tolerance analysis

Non-acclimated 2-week-old plants were placed in a programmable freezer at  $-1^\circ\text{C}$  and frozen by ice-seeding. After 1 h at  $-1^\circ\text{C}$ , plants were cooled at  $2.4^\circ\text{C}/\text{h}$  to various subzero temperatures (equilibrium freezing treatment). Plants were removed from the freezer at specific temperature points and thawed overnight at  $4^\circ\text{C}$  in the dark. Plants were then incubated at  $22^\circ\text{C}$  under constant light for 2 days, after which survival of the plants was assessed.



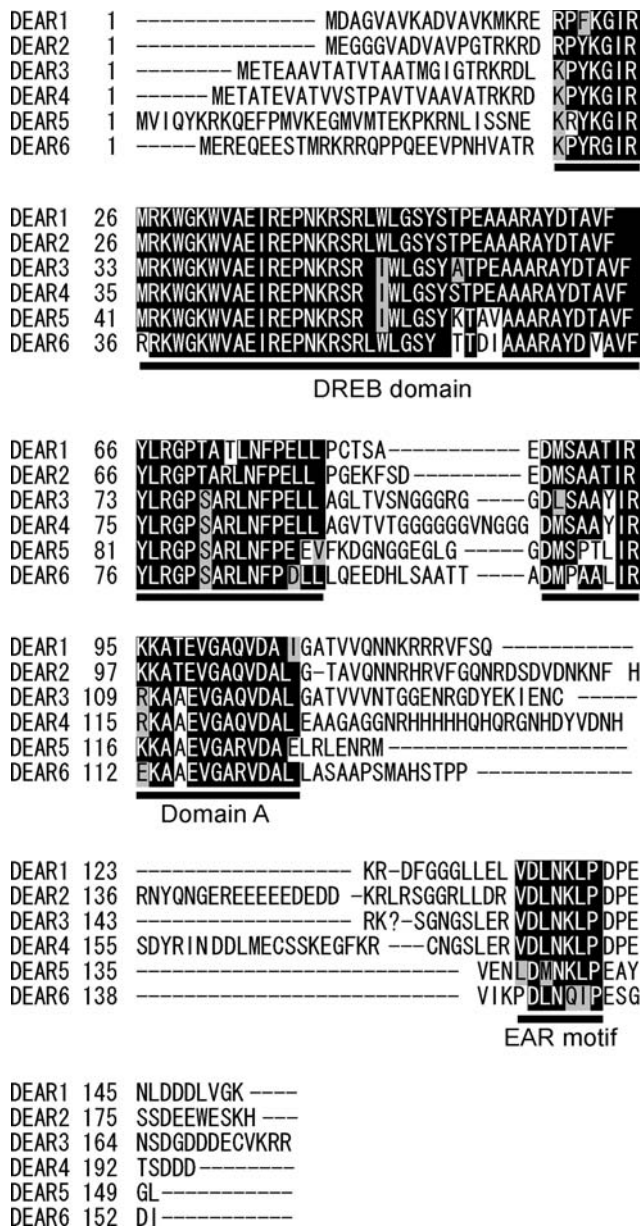
**Fig. 1** Transcriptional activity of genes encoding DEAR1 family proteins in response to pathogen and elicitor treatment. **a** RT-PCR analysis for pathogen inducibility. Three-week-old leaves were inoculated with 10 mM  $\text{MgCl}_2$  (mock) or with virulent bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). At 2 days after inoculation, leaf discs were collected and RNA extracted. Expression of genes encoding DEAR proteins was determined by RT-PCR. *EF1 $\alpha$*  mRNA was also examined as a control. Lanes: 0 Before-infection, 2 2-day-infection. **b** RT-PCR and Southern blot hybridization analysis. Experimental conditions were identical to those in **a**. Lanes: 0 Before-infection, 2 2-day-infection. **c** RT-PCR analysis for elicitor inducibility. RT-PCR analysis was performed to examine *DEAR1* gene expression in 2-week-old plants treated with distilled water (mock) and 10  $\mu\text{g}/\text{ml}$  chitin oligosaccharide (GlcNAc; 8-mer). *EF1 $\alpha$*  mRNA was also examined as a control at 0, 3, 8, 12 and 24 h after treatment

## Results

*DEAR1*, a member of the DREB/CBF family, is induced by pathogen infection

DREB/CBF proteins belong to the AP2/ERF domain-containing protein superfamily and are well known to be involved in regulating abiotic stress responses (Shinozaki and Yamaguchi-Shinozaki 1996). Genome-wide transcriptional analysis of Arabidopsis previously revealed that the *At3g50260* gene is up-regulated by pathogen infection (Thilmony et al. 2006). As this gene encodes an unknown protein belonging to the *DREB/CBF* family, we chose to investigate this gene further. RT-PCR revealed that *At3g50260* is transcriptionally induced 2 h after inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (Fig. 1a), which was confirmed by RT-PCR/Southern analysis (Fig. 1b). The deduced amino acid sequence for *At3g50260* contains both a DREB domain and the EAR motif (Fig. 2), which is essential for repressive activity of the DREB/CBF proteins. This gene was therefore designated as *DEAR1* (*DREB and EAR motif protein 1*).

There are five *DEAR1* homologues within the Arabidopsis genome that also contain sequences with significant homology to the DREB domain and EAR motif. These genes have been designated as *DEAR2* (*At5g67190*),



**Fig. 2** Amino acid sequence alignment of proteins similar to DEAR1. *Black shading* Identical residues, *gray shading* conservative substitution between DEAR1 and other proteins. Numbers on the left refer to amino acid positions in the deduced DEAR1 and other proteins. These proteins contain a DREB domain, an EAR motif and a highly conserved domain (A) that is yet to be characterized

DEAR3 (At2g23340), DEAR4 (At4g36900), DEAR5 (At4g06746) and DEAR6 (At1g 46768), and share 60.2, 53.9, 48.3, 42.9, and 42.1% identity with DEAR1, respectively [Fig. 2; electronic supplemental material (ESM), Fig. S1]. These proteins have all been classified into the same DREB/CBF family subgroup (Nakano et al. 2006a). Within this subgroup, there is strong conservation of amino acid residues comprising the DREB domain and EAR motif; however, DEAR1 contains deletions of

residues more weakly conserved among the subgroup members. The alignments also revealed an additional conserved domain (Domain A), although the function of this domain is yet to be identified. The other five genes in this subgroup (DEAR2–DEAR6) were not transcriptionally induced by pathogen infection (Fig. 1a), indicating that the biotic response is specific to DEAR1. The DEAR5 gene might be pathogen inducible, since it was detectable after infection.

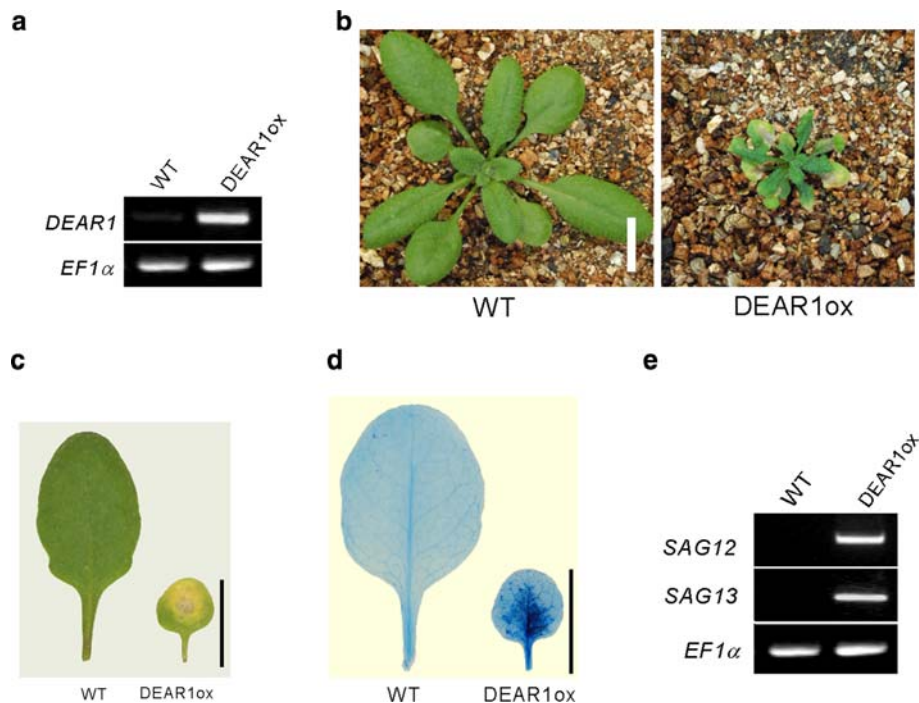
Chitin and chitin oligomers have been shown to function as an elicitor signal in plant immunity (Shibuya and Minami 2001). To investigate the response of DEAR1 to such biotic elicitors, leaves of plants were sprayed once with 10 µg/ml chitin oligosaccharide (8-mer) (Fig. 1c). The DEAR1 transcript was induced in the leaves within 3 h of the spray treatment and then decreased gradually to original levels by 24 h. These data confirm that the DEAR1 gene is transcriptionally regulated as part of the response to biotic stress.

### Overexpression of DEAR1 results in a cell death phenotype

To clarify the function of DEAR1, two independent transgenic Arabidopsis lines were generated that contained either DEAR1 alone (35S::DEAR1) or DEAR1 with GFP fused to the C-term (35S::DEAR1::GFP) under the control of the strong constitutive CaMV 35S promoter. Both independent lines showed a dwarf phenotype with lesion-like cell death on rosette leaves, cauline leaves and stems of 4-week-old plants (ESM, Fig. S2); cell death started as small black- or brown colored lesions. In both independent lines, leaf senescence and chlorophyll breakdown also occurred in younger leaves compared to wild-type. One of these lines (35S::DEAR1::GFP) was thus chosen for subsequent analyses, and is hereafter referred to as “DEAR1ox”.

RT-PCR analysis confirmed that the DEAR1 transcript is accumulated in DEAR1ox rosette leaves (Fig. 3a). The DEAR1ox showed a dwarf phenotype with lesion-like cell death on rosette leaves, cauline leaves and stem of 3-week-old plants (Fig. 3b, c) with strong staining of leaves by Trypan Blue (Fig. 3d), which demonstrates that a high concentration of dead cells occurs around the visible lesion areas.

To characterize the DEAR1ox phenotype at the molecular level, we first monitored the expression of molecular markers for cell death and SA-dependent defense response. Several genes are known to accumulate during cell death in plants, including SAG12 (At5g45890) encoding a cysteine protease, and SAG13 (At2g29350) encoding a short-chain alcohol dehydrogenase (Lohman et al. 1994). Both the SAG12 and SAG13 transcripts accumulated to high levels



**Fig. 3** Characterization of the DEAR1ox transgenic line. **a** Overexpression of *DEAR1* gene in DEAR1ox plants. Total RNA from 4-week-old vegetative shoot tissue of wild-type (WT) and DEAR1ox was analyzed by RT-PCR. *EF1α* was used as a control. **b** Morphology of 4-week-old wild-type Col-0 (WT) and DEAR1ox plants. Dwarfism and cell death phenotypes seen in the DEAR1ox plants were seen to a higher extent when grown on soil (these photos) compared to in a Petri dish (see Fig. 5d). *Bar* 1 cm. **c** Comparison of wild-type (*left*)

and DEAR1ox (*right*) leaf from 4-week-old plants, showing the cell death phenotype of DEAR1ox. *Bar* 1 cm. **d** Trypan Blue staining of leaves from 4-week-old wild-type (*left*) and DEAR1ox (*right*) plants. Intense staining indicates cell death. *Bar* 1 cm. **e** Expression of cell death marker genes, *SAG12* and *SAG13*. Total RNA from 4-week-old vegetative shoot tissue of wild-type (WT) and DEAR1ox was analyzed by RT-PCR. *EF1α* was used as a control

in rosette leaves of DEAR1ox compared to that in wild-type (Fig. 3e), indicating that cell death is promoted in DEAR1ox at the molecular level. Epi-fluorescence imaging of DEAR1ox revealed that the DEAR1-GFP protein was localized exclusively to nuclei (ESM, Fig. S2c), consistent with the proposed function of DEAR1 as a transcriptional factor.

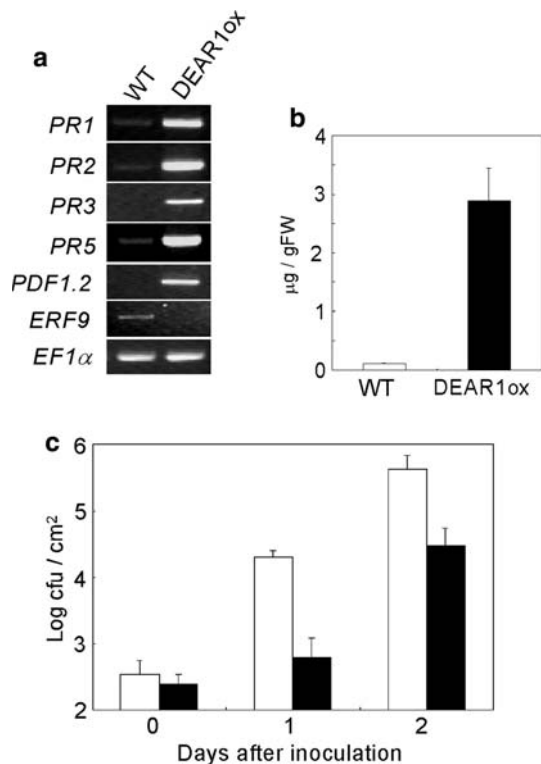
#### Up-regulation of SA-dependent PR genes and accumulation of SA content in DEAR1ox

Interaction with avirulent pathogens results in HR, which leads to cell death and senescence in plant tissues. Following pathogen infection, transcriptional induction of PR proteins is correlated tightly with HR (Shah 2003). To determine whether the cell death observed in DEAR1ox represents an HR-like phenotype, we analyzed expression of three PR genes by RT-PCR. As shown in Fig. 4a, mRNA for *PR1* (At2g14610), *PR2* (At3g57260;  $\beta$ -1,3-glucanase) and *PR5* (At1g75040; osmotin) were greatly increased in DEAR1ox rosette leaves. These PR genes are known to be controlled by SA, and thus it is likely that the SA-dependent HR defense pathway was activated in

DEAR1ox. To clarify the role of SA in the HR-like cell death phenotype, we directly measured endogenous levels of SA by HPLC. In 3-week-old plants, endogenous SA levels were 30-fold higher in DEAR1ox than in wild-type (Fig. 4b), consistent with activation of the SA-dependent cell death pathway in DEAR1ox.

#### Overexpression of DEAR1 results in resistance to pathogen infection

Plants containing an activated cell death pathway usually show resistance to some compatible plant–pathogen interactions (Lorrain et al. 2003). The possibility therefore existed that a basal resistance to pathogens is constitutively activated in DEAR1ox. In order to investigate this, 3-week-old DEARox and wild-type plants were tested for disease tolerance to pathogen growth by challenging with the bacterial strain *P. syringae* pv. *tomato* DC3000. A marked increase in bacterial numbers was detected on wild-type leaves at 1 day after inoculation (Fig. 4c), resulting in disease symptoms such as cell death and chlorophyll loss by day 2 (data not shown). In contrast, bacterial growth was limited on DEAR1ox compared to that on wild-type,



**Fig. 4** Activation of defense response in DEAR1ox. **a** Expression of defense marker genes, *PR1*, *PR2*, *PR3*, *PR5*, *PDF1.2* and *ERF9*. Total RNA from 4-week-old vegetative shoot tissue of wild-type (WT) and DEAR1ox was analyzed by RT-PCR. *EF1α* was used as a control. **b** Comparison of endogenous SA in wild-type (white bar) and the DEAR1ox (black bar) plants. Leaves from 4-week-old plants were harvested, extracted, and analyzed by HPLC. **c** Analysis of bacterial growth (*P. syringae* DC3000) on wild-type (white bar) and DEAR1ox (black bar) plants. Error bars (b, c) Standard error of three replicates. Each experiment was repeated twice with similar results

indicating that the DEAR1ox does show a basal resistance phenotype. Taken together with the transcriptional induction of *PR* genes (Fig. 4a) and high accumulation of endogenous SA (Fig. 4b), these results demonstrate that the SA-dependent defense pathway is activated in the DEAR1ox, which results in the HR-like cell death phenotype.

#### Overexpression of DEAR1 promotes ethylene-inducible gene transcription

The *PDF1.2* (At5g44420) and *PR3* (At3g12500) genes, which encode plant defensin and basic chitinase, respectively, are also induced during the defense response to pathogen attack (Penninckx et al. 1998). Similar to what has been observed for other *PR* genes, *PDF1.2* and *PR3* were transcriptionally activated in DEAR1ox (Fig. 4a). Both *PDF1.2* and *PR3* are known to be induced by jasmonate and/or ethylene, implying that *DEAR1* is also associated with the jasmonate and/or ethylene signaling

pathways. This is consistent with a previous report that the *DEAR1* gene is cooperatively regulated by both jasmonate and ethylene (referred to as *CEJ1*; Nakano et al. 2006b).

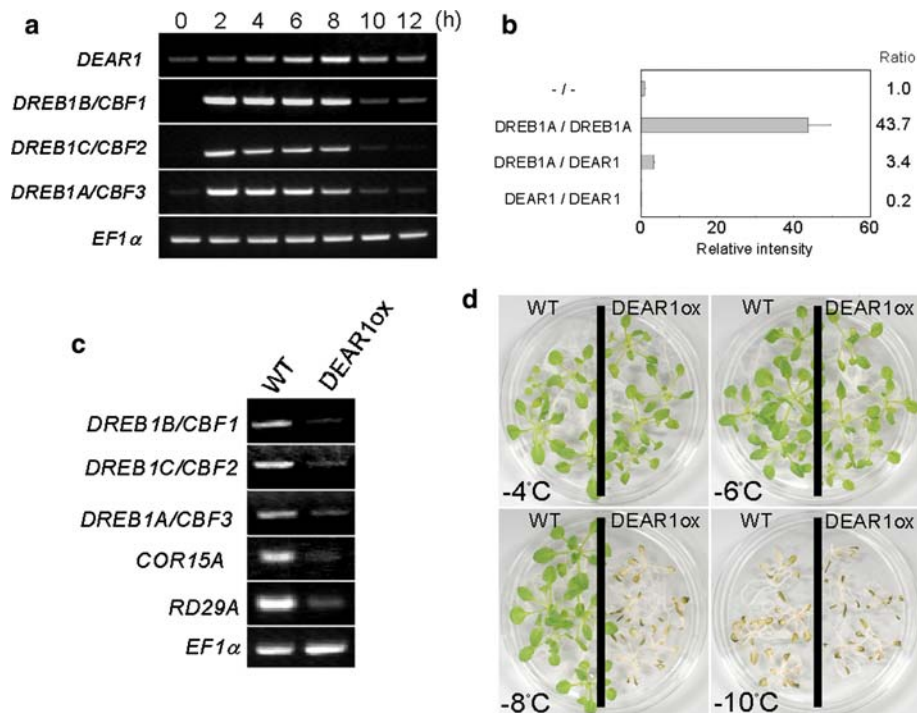
*ERF1* (At3g23240) is an early ethylene responsive protein that acts as a transcriptional factor to regulate a large number of genes in response to both jasmonate and ethylene (Lorenzo et al. 2003). *ERF1* and other *ERF* family members bind to the GCC box in gene promoters and induce the expression of downstream genes, including *PR3* and *PDF1.2* genes. It is therefore possible that transcriptional induction of *PR3* and *PDF1.2* genes in DEAR1ox was due to activation of *ERF1* or other *ERFs*. However, DNA microarray profiling of gene expression in DEAR1ox showed no increase in transcription of *ERF1* and other *ERFs* compared to that in wild-type (ESM, Table S1). On the other hand, *ERF9* transcription was strongly repressed in DEAR1ox (Fig. 4a; ESM, Table S1).

The *ERF9* gene is unique within the ERF family as it contains an EAR motif in its protein sequence, which likely functions as a transcriptional repressor, and a DRE motif in the promoter region, which is known to be bound by DREB proteins (Huang and Liu 2006). Given these characteristics, it is likely that increased DEAR1 protein levels in DEAR1ox acts to repress *ERF9* transcription, which then leads to induction of *PDF1.2* (and *PR3*) due to removal of the transcriptional repression imposed by *ERF9*.

#### Cold induced DEAR1 suppresses genes of the DREB1/CBF family

In addition to the observed biotic response, transcription of the *DEAR1* gene was also induced by abiotic stress treatment. RT-PCR analysis showed that the *DEAR1* transcript gradually accumulated in wild-type plants during the first 8 h of cold treatment, after which transcript levels gradually decreased (Fig. 5a). Increased gene transcripts for *DREB1B/CBF1*, *DREB1C/CBF2* and *DREB1A/CBF3* were also observed specifically between 2 and 8 h of cold treatment.

The DEAR1 protein contains a DREB/CBF domain and EAR motif, and is therefore predicted to function as a transcriptional repressor of genes containing the cis-acting DRE/CRT element within their promoter. To evaluate whether the DEAR1 protein is indeed capable of repressing DRE/CRT-dependent transcription, functional promoter experiments were carried out using protoplasts prepared from Arabidopsis T87 suspension-cultured cells (Fig. 5b). Protoplasts were cotransfected with effector plasmids, consisting of either DREB1A or DEAR1 cDNA under control of a CaMV 35S promoter, and with a reporter plasmid, consisting of a GUS reporter gene fused to trimeric 71-bp fragments containing the DRE/CRT motif



**Fig. 5** Cold inducibility and freezing tolerance of *DEAR1*. **a** Induction of *DEAR1* and the *DREB1/CBF* family by cold treatment. Three-week-old wild-type plants were incubated at 4°C for up to 12 h, and vegetative shoot tissue was harvested at the time points indicated. Total RNA was then prepared and expression of *DEAR1*, *DREB1B/CBF1*, *DREB1C/CBF2* and *DREB1A/CBF3* was analyzed by RT-PCR. *EF1α* was used as a control. **b** Repression of the *RD29A* promoter by *DEAR1*. The reporter gene was transfected with each effector plasmid or the empty vector (*-/-*) as a negative control. To normalize for transfection efficiency, the control CaMV promoter-luciferase (LUC) plasmid was cotransfected in each experiment. *Bars* Standard error of three replicates. Ratio values indicate the fold-difference of reporter activities compared to that obtained with the

control vector (*-/-*). **c** Suppression of cold-related genes in *DEAR1ox*. Expression of *DREB1/CBF* family, *DREB1B/CBF1*, *DREB1C/CBF2* and *DREB1A/CBF3*, *COR15A* and *RD29A* was analyzed by RT-PCR. Total RNA from 4-week-old vegetative shoot tissue of wild-type (WT) and *DEAR1ox* after 6-h cold treatment was used. *EF1α* was used as a control. **d** Reduction of freezing tolerance in *DEAR1ox*. Two-week-old wild-type (*left*) and *DEAR1ox* (*right*) plants exposed to -4°C (control), -6°C, -8°C and -10°C treatments. The cooling rate (2.4°C/h) remained constant for each temperature treatment. Once the indicator temperature was reached, plants were transferred to 4°C overnight and then placed under normal growth conditions for 2 days prior to being photographed

derived from the *RD29A* gene promoter. Expression of the *DREB1A* protein in protoplasts resulted in activation of *GUS* reporter gene expression, consistent with previous data that *DREB1A* protein functions as transcriptional activator of the *RD29A* gene (*DREB1A/DREB1A* in Fig. 5b). In contrast, expression of *DEAR1* protein alone did not activate the *GUS* reporter gene (*DEAR1/DEAR1*). However, expression of the *DEAR1* protein did repress the induction of the reporter construct by *DREB1A* (*DREB1A/DEAR1*), indicating that the *DEAR1* protein functions as an active repressor of *DRE/CRT*-dependent transcription. These in vitro results were also confirmed in planta by RT-PCR analysis (Fig. 5c). In wild-type plants, expression of *COR15A* and *RD29A* genes was induced by cold treatment; however, this induction was suppressed in *DEAR1ox* (Fig. 5c). Interestingly, cold-dependent *DREB1/CBF* gene induction was also not observed in *DEAR1ox*, suggesting that either *DEAR1* or another feedback mechanism is

involved in suppressing this expression when there is a block on the pathway.

#### Overexpression of *DEAR1* reduces freezing tolerance

*Arabidopsis* plants overexpressing *DREB1B/CBF1*, *DREB1A/CBF3* or *COR15A* show a high tolerance to freezing stress (Liu et al. 1998). The suppression of these genes in the *DEAR1ox* therefore suggested the possibility of reduced freezing tolerance. Indeed, in whole-plant survival tests, a reduced tolerance to freezing stress was observed in the *DEAR1ox* plants (Fig. 5d). No difference in plant survival was detected between the overexpressor and wild-type plants following exposure to equilibrium freezing to -6°C; however, the *DEAR1ox* was unable to survive equilibrium freezing at -8°C, in contrast to wild-type plants, which could survive the -8°C treatment, but not the -10°C treatment.



**Discussion**

Sustained activation of plant biotic and abiotic stress responses is a metabolically expensive process, and therefore plants have evolved to keep such responses under tight control during normal growth and development. One key method of controlling stress-related gene expression is to use molecular brakes or repressors. Active repressors contain an intrinsic repression domain and often exert their repressor activity by chromatin modification or by interacting with activators of gene expression. Recently, important roles for EAR-repressors in modulating plant defense and stress responses have been revealed (Kazan 2006). To clarify the mechanisms that control crosstalk between plant biotic and abiotic stress responses, we have focused on the DEAR1 protein, which contains both a DREB domain and an EAR motif.

In vitro promoter analysis revealed that the DEAR1 protein represses transcription of the DRE/CRT motif-containing gene, *RD29A*. Overexpression of *DEAR1* also suppressed expression of *ERF9*, *RD29A* and *COR15A*, all of which have DRE/CRT motifs in their promoter regions (ESM, Table S1). These data demonstrate that the DEAR1 protein functions as an EAR-repressor of DRE/CRT-containing genes in plant cells. Consistent with this role, *DEAR1ox* plants overexpressing *DEAR1* gene displayed a reduced tolerance to freezing stress. Analysis of a large-scale public microarray database (“Electronic Fluorescent Pictograph”; <http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>) showed that cold temperature was the only abiotic stress inducer of *DEAR1* gene expression, and that this gene does not respond to osmotic, salt, drought, oxidative, UV-B, wounding or heat stress (ESM, Fig. S3).

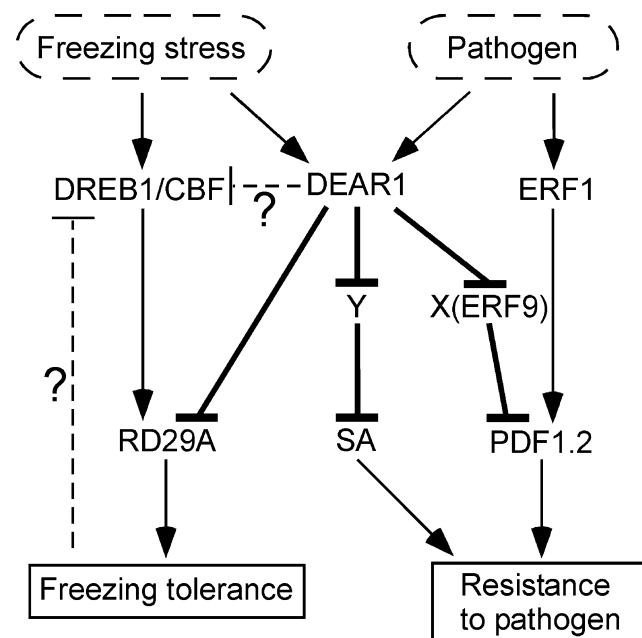
Although much insight has been gained into the molecular mechanisms involved in either biotic or abiotic stress responses, our understanding of convergence points between these two stress signaling pathways remains limited. Several proteins have recently been identified as promising candidates for common players involved in crosstalk between stress signaling pathways, including transcription factors and protein kinases (Fujita et al. 2006).

The AP2 transcription factor family, found only in plants, includes several genes that encode proteins involved in the regulation of disease resistance pathways. These proteins are members of the ERF subfamily, which have only a single DNA-binding domain and are distinct from members of the DREB1/CBF subfamily. On the other hand, DEAR1 differs from typical ERF proteins in that it also contains a DREB domain and belongs to the DREB1/CBF protein subfamily.

Consistent with the presence of the ERF domain, DEAR1 was also implicated in the defense response.

*DEAR1* gene expression was induced by pathogen infection, and overexpression of DEAR1 increased resistance to infection by *P. syringae*. An increased accumulation of SA was also observed in the DEAR1 overexpressor. This was associated with transcriptional induction of *PR* genes, which are known to be regulated by SA. Induction of *PDF1.2* and *PR3* gene expression, which are known to be regulated by ethylene and jasmonate, was also observed. Among the six members of the DEAR family, only *DEAR1* gene expression was stimulated by *P. syringae* infection. Analysis of the Electronic Fluorescent Pictograph microarray database revealed that *DEAR1* is also induced by infection with *Botrytis cinerea* as well as *P. syringae* (ESM, Fig. S4). In general, plant defense to *P. syringae* infection is activated by the SA-mediated pathway, whereas the ethylene/jasmonate-mediated pathway is associated with defense against *B. cinerea* infection. Together with the gene expression patterns observed in the DEAR1 overexpressor, this suggests that DEAR1 functions as a negative transcriptional regulator of both SA- and ethylene/jasmonate-mediated defense pathways, leading to a broad-range resistance to pathogens.

Based on its role in both freezing tolerance and pathogen resistance, we propose the following model for DEAR1 function in plant stress responses (Fig. 6). When plants are exposed to cold stress, expression of the *DREB1/CBF* gene family is immediately stimulated and the translation products enhance *RD29A* (and *COR15A*) gene expression, leading to tolerance to freezing temperature.



**Fig. 6** Proposed model for the role of DEAR1 in freezing tolerance and defense resistance. (→) and (⊥) indicate promotion and repression, respectively

The DEAR1 protein, which is also induced by cold stress, acts as a negative transcriptional regulator to suppress transcription of *RD29A* and *COR15A* (Fig. 5c), implying that DEAR1 functions to keep such responses under tight control during normal growth and development. The DEAR1ox line also resulted in repression of *DREB1/CBF* gene expression (Fig. 5a). Since there are no DRE motifs within their promoters, DEAR1 is not likely to bind and transcriptionally repress the *DREB1/CBF* genes. However, unknown regulatory pathways such as indirect or feedback repression of the *DREB1/CBF* gene can be expected.

On the other hand, when plants are exposed to pathogens, immune pathways such as ethylene and jasmonate signaling that are mediated by transcriptional activation of *ERF1* are activated. This leads to increased pathogen resistance through PDF1.2 (and PR3) protein accumulation. The DEAR1 transcriptional repressor is also induced by pathogen infection, and may be acting to induce *PDF1.2* gene expression by negatively interacting with a repressor of *PDF1.2* (predicted factor X). We propose the *ERF9* gene as a candidate for this predicted factor X based on the following: (1) proteins of the ERF family have the ability to bind to and control the GCC box-containing *PDF1.2* gene; (2) among the ERF family, only the *ERF9* gene has the DRE/CRT motif in the promoter region (ESM, Table S1), which can act as a target for DEAR1; and (3) among members of the ERF family, only *ERF9* gene expression was suppressed in the *DEAR1* overexpressor. Further experimental characterization of *ERF9* is required to confirm this proposed role. The DEAR1 protein is likely to function in a similar manner in the alternative immune pathway mediated by SA. Increased pathogen resistance and overaccumulation of SA was observed in the *DEAR1* overexpressor, indicating that DEAR1 may suppress transcription of a factor (predicted factor Y) that acts negatively upstream of the SA pathway.

We have shown that the DEAR1 transcriptional repressor plays an upstream regulatory role in both freezing tolerance and response to pathogen infection. This dual role strongly indicates that DEAR1 functions to mediate the crosstalk between the abiotic and biotic stress signaling pathways in plants and is a key component within the complex molecular network of stress adaptation.

To confirm DEAR1 functions, a mutant deficient in DEAR1 would be informative. However, we have not observed any phenotypic characters in the *dear1* mutant. Further experiments will be needed.

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