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ORIGINAL PAPER

Arabidopsis DAL1 and DAL2, two RING finger proteins homologous to *Drosophila* DIAP1, are involved in regulation of programmed cell death

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Abstract Programmed cell death (PCD) is a precise, genetically controlled cellular process with important roles in plant growth, development, and response to biotic and abiotic stress. However, the genetic mechanisms that control PCD in plants are unclear. Two Arabidopsis genes, *DAL1* and *DAL2* (for *Drosophila* DIAP1 like 1 and 2), encoding RING finger proteins with homology to DIAP1 were identified, and a series of experiments were performed to elucidate their roles in the regulation of PCD and disease resistance. Expression of *DAL1* and *DAL2* genes was induced in *Arabidopsis* plants after inoculation with virulent and avirulent strains of *Pseudomonas syrinage* pv. *tomato* (*Pst*) DC3000 or after infiltration with fumonisin B1 (FB1). Plants with mutations in the *DAL1* and *DAL2* genes displayed more severe disease after inoculation with an avirulent strain of *Pst* DC3000, but they showed similar disease severity as the wild-type plant after inoculation with a virulent strain of *Pst* DC3000. Significant accumulations of reactive oxygen species (ROS) and increased cell death were observed in the *dal1* and *dal2* mutant plants after inoculation with the avirulent strain of *Pst* DC3000. The *dal* mutant plants underwent extensive PCD upon infiltration of FB1 and displayed higher levels of ROS accumulation, callose deposition, and autofluorescence than the wild-type plants. Our data suggest that DAL1 and DAL2 may act as negative regulators of PCD in Arabidopsis.

Keywords Programmed cell death · RING finger protein · DAL1 and DAL2 · Fumonisin B1 · Disease resistance

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Introduction

Within all living organisms, some cells commit suicide to achieve and maintain homeostasis during normal development, environmental stress, or pathogen attack. This functionally conserved and gene-directed cell suicide is known as programmed cell death (PCD). Programmed cell death is a ubiquitous feature of all living cells, from prokaryotes to eukaryotes (Beers and McDowell 2001; Golstein et al. 2003; Lawen 2003; Lam 2004; Madeo et al. 2004). In plants, one of the most familiar forms of PCD is associated with pathogen attack. Normal plant–pathogen interactions depend on the classical gene-for-gene resistance model that requires an avirulence gene in the pathogen and a corresponding resistance gene in the plant (Jones and Dangl 2006). A rapid, localized cell death known as hypersensitive response (HR), which results in the formation of necrotic lesions around the infection sites, is the usual characteristic of an incompatible interaction between host plants and pathogens (Goodman and Novacky 1994; Lam et al. 2001). Morphologically and biochemically, the HR that occurs in response to infection from avirulent pathogens shares most features of PCD (Mur et al. 2008; Greenberg and Yao 2004; Lam 2004).

Extensive studies on the physiological, biochemical, and molecular basis of HR-PCD during plant–pathogen interactions suggest that it is controlled by endogenous signaling cascades (Torres and Dangl 2005; Delledonne 2005; Mur et al. 2008; Jones and Dangl 2006) initiated by reactive oxygen species (ROS), nitric oxide, salicylic acid (SA), ethylene, and oxylipins (Jabs et al. 1996; Rate et al. 1999; Epple et al. 2003; Torres and Dangl 2005; Torres et al. 2005; Delledonne 2005; Brodersen et al. 2005; Mur et al. 2006; Bouchez et al. 2007). On the other hand, PCD is a precise, genetically controlled cellular process that can be regulated by a number of genes. Much of our understanding on the genetic and molecular basis for the control and execution of plant PCD comes from the analysis of lesion mimic mutants that exhibit spontaneous HR-like PCD in the absence of a pathogen. Dozens of such mutants have been isolated, including *accelerated cell death* (*acd*) and *lesion simulating disease* (*lsd*) in Arabidopsis, which are unable to control the rate and extent of lesions causing chlorosis in large areas (Lorrain et al. 2003). Some of the individual genes that have been identified in these mutants provide new insights into the mechanism of PCD control in plants.

The Arabidopsis protein LSD1 is a negative regulator of PCD that modulates cellular ROS status and requires PAD4 and EDS1, two components of the SA-dependent defense signaling pathway (Dietrich et al. 1997; Jabs et al. 1996; Rusterucci et al. 2001; Aviv et al. 2002). In addition, LSD1 antagonistically interacts with bZIP10, a positive regulator of PCD (Kaminaka et al. 2006). The characterization of ACD5 and ACD11 as a ceramide kinase and a sphingosine transfer protein, respectively, suggests important roles for sphingolipids in regulating PCD (Greenberg et al. 2000; Brodersen et al. 2002; Vailleau et al. 2002; Liang et al. 2003; Raffaele et al. 2006, 2008; Shi et al. 2007; Yang et al. 2007; Wang et al. 2008; Reina-Pinto et al. 2009). Other factors have also been demonstrated to modulate PCD in plants. Such PCD-regulating factors include tomato MAPKKKα (del Pozo et al. 2004), phospholipid hydroperoxide glutathione peroxidase (Chen et al. 2004), Arabidopsis PLP2 (La Camera et al. 2009), BAP1 and BAP2 (Yang et al. 2007), FMO1 and NUDT7 (Bartsch et al. 2006), ACD2 (Mach et al. 2001; Yao and Greenberg 2006), and copine proteins (Yang et al. 2006).

Many of the signaling mechanisms of PCD are conserved among eukaryotes, including animals and plants. Some of the genes that control PCD are functionally conserved across wide evolutionary distances (Higashi et al. 2005; Williams and Dickman 2008). For example, the mammal Bax-induced cell death inhibitor BI-1 has homologues in plants, including Arabidopsis, rice, and barley (Lacomme and Santa Cruz 1999; Sanchez et al. 2000; Kawai-Yamada et al. 2001, 2004; Yu et al. 2002; Chae et al. 2003; Matsumura et al. 2003; Huckelhoven et al. 2003; Watanabe and Lam 2006; Eichmann et al. 2004). Similarly, animal apoptotic regulators such as human Bcl-2 and Bcl-xl, as well as nematode CED-9, can either induce or suppress cell death in transgenic plants (Dickman et al. 2001; Lincoln et al. 2002; Xu et al. 2004). In *Drosophila melanogaster*, expression of *DIAP1* can suppress apoptosis, and the loss of DIAP1 function results in early embryonic death resulting from apoptosis (Wang et al. 1999; Goyal et al. 2000; Lisi et al. 2000). In this study, we identified two Arabidopsis genes, *DAL1* and *DAL2* (for the *Drosophila* inhibitor of apoptosis like), encoding RING finger proteins with homology to DIAP1. Mutations in *DAL1* and *DAL2* results in a significant accumulation of superoxide anions, causing PCD after inoculation with an avirulent strain of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Similarly, both *dal1* and *dal2* mutants exhibit accelerated PCD progression upon treatment with fumonisin B1, a toxin produced by the necrotrophic fungal plant pathogen, *Fusarium moniliforme*. Our data suggest that DAL1 and DAL2 may act as negative regulators of PCD in Arabidopsis.

Materials and methods

Plant materials and growth

Seeds of *Arabidopsis thaliana* wild-type [ecotype Columbia-0 (Col-0)] and mutant lines were soaked in water at 4°C for 2 days to break dormancy. Afterward, the seeds were sown in pots with a mixture of perlite:vermiculite:plant ash (1:6:2). All plants were grown under fluorescent light at 150 μE m^{-2} s^{-1} at 22 ± 3°C with 60% relative humidity and long-day conditions (16-h light/8-h dark cycle).

All T-DNA insertion lines on the Col-0 background were obtained from the Arabidopsis Biological Resource Center at Ohio State University. The *dal1* mutants (SALK_002099 for *dal1-1* allele and SALK_063571 for *dal1-2* allele) were obtained from the SALK population, whereas the *dal2* mutants (SALK_024744 for *dal2-1* allele and WiscDsLox383C6 for *dal2-2* allele) were obtained from the SALK and Wisconsin populations, respectively. PCR genotyping was performed to identify homozygous plants using gene-specific primers and T-DNA primers (Lba1, TGG TTC ACG TAG TGG GCC ATC G for SALK lines and JL202, CAT TTT ATA ATA ACG CTG CGG ACA TCT AC-3 for Wisconsin line). The gene-specific primers used for identification of homozygous lines were as follows: SALK_002099-LP, GAA TCG AGC AAA CAC CTC ATG and SALK_002099-RP, GCT GCC ATG TATA ACA GGC TG for SALK_002099 line; CS853707-LP, AAT ATT AGG ATT CAT CCC GCG, and CS853707-RP,

GCC AAC ATC TCC TGA TAC TGC for WiscDsLox383C6 line; SALK_024744-LP, GCG TTG GGT TAT CTT AAA GGC, and SALK_024744-RP, CAG CAG CAC ATA TGA CCA CAC for SALK_024774 line; SALK_063571-LP, CCC CTT TCG AAA TTC TCC TC, and SALK_063571-RP, ATT ACA TGG ACA CGG CTT GTC for SALK_063571 line. The expression levels of *DAL1* and *DAL2* genes in homozygous plants were analyzed by reverse transcription polymerase chain reaction (RT-PCR) using gene-specific primers DAL1-1F (ATG ATT CCT TGG GGT GGA GTT AC)/DAL2-1R (TCA GTG ACG ATA TGT CTT AAC C) and DAL2-1F (CAA GTA AGA GTG ACT TCA GAG TC)/DAL2-1R (GAA TGT TTC TGA GGC CTG AGT TG), respectively.

Assays for disease phenotype and treatment with fumonisin B1

Pst strains DC3000 and DC3000-AvrRPM1 were grown in liquid King B medium (10 g L^{-1} peptone, 1.5 g L^{-1} K_2HPO_4, 15 g L^{-1} glycerol, 5 mM $MgSO_4$, pH 7.0) with rifampicin (25 μg mL^{-1}) at 28°C for 8–12 h in a refrigerated shaker. Bacteria were harvested by centrifugation at 2,500×*g* for 10 min and resuspended in sterile 10 mM $MgCl_2$ solution. Bacterial inocula were then adjusted to the desired concentrations. Inoculations were performed on the leaves of 4-week-old plants by infiltration with suspensions of *Pst* in 10 mM $MgCl_2$ using a 1 mL needleless syringe. Mock inoculation was done by infiltrating similar amounts of 10 mM $MgCl_2$ solution. All inocula were injected on both sides of the abaxial surface of the leaves. To determine bacterial growth, ten leaves were collected at different times after inoculation, and leaf discs of the same size were made using a hole puncher. The bacterial titer per leaf area was determined as described previously (Mengiste et al. 2003).

The mature leaves of 4-week-old wild-type and mutant plants grown in soil were infiltrated with approximately 20 μL of 10 μM fumonisin B1 (FB1, Sigma) in 10 mM $MgSO_4$ solution, or 10 mM $MgSO_4$ solution alone for mock treatment, using a needleless syringe. The infiltrated plants were kept in a controlled growth chamber for up to 4 days, and lesion formation was observed.

Cell death assays

Cell death was detected using Evans blue staining as described by Wright et al. (2000) with minor modifications. Detached leaves were completely submerged in a 0.1% w/v Evans blue solution and subjected to three 5 min cycles of vacuum followed by 20 min incubation under vacuum. The leaves were then washed with water and cleared by boiling for 2 min in alcoholic lactophenol (95% ethanol:lactophenol, 2:1). They were rinsed in 50% ethanol and then in distilled water. Cell death in infiltrated leaves was also assayed by measuring electrolyte leakage from leaf discs obtained at different time points following FB1 treatment. Up to 3–4 leaf discs (6 mm diameter) were floated on 4 mL of distilled water for 3 h at room temperature. After incubation, the conductivity of the bathing solution was measured using a DDS-IIAT-type conductivity meter. The leaf samples in the bathing solutions were then boiled for 5 min, and volumes of the bathing solution were brought up to the initial volumes, followed by measurement of total conductivity. The percentage of electrolyte leakage attributable to the FB1 treatment was calculated as 100% × (conductivity of the test samples)/(conductivity after boiling). Measurements for each time point were performed at least in triplicate.

RT-PCR analysis of gene expression

Leaf samples were collected at different time points after inoculation with *P. syringae* pv. *tomato* DC3000 or treatment with FB1. Total RNA was extracted using TRIZOL reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Gene expression was analyzed by RT-PCR using gene-specific primers. Actin1 was used as an internal control. One microgram of total RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Shanghai, China), and the synthesized first strand cDNAs were used as templates in the PCR. PCR was carried out using Taq DNA polymerase (Takara, Dalian, China) and the following cycle settings: 94°C for 15 s, 55–62°C for 30 s, and then 72°C for 30–50 s. The gene-specific primers used were as follows: DAL1-rt-1F, GTC AAG GTT GTA CAA ATA TGC CT; DAL1-rt-2R, TTC GTC TCC GAC AAA GTG GAC A; DAL2-rt-2F, GAG TAA GAC ACG TTG AGC GTG T; DAL2-rt-2R, AGC ATG GTG TGC AGC AGC ACA; PR1-1F, TCG TCT TTG TAG CTC TTG TAG GTG; PR1-1R, TAG ATT CTC GTA ATC TCA GCT CT; Actin1-1F, GGC GAT GAA GCT CAA TCC AAA CG; and Actin1-1R, GGT CAC GAC CAG CAA GAT CAA GAC G. Each experiment was done at least twice using independent samples.

Detection of callose deposition, autofluorescence, and reactive oxygen species

Callose deposition and autofluorescence were detected according to Dietrich et al. (1994) with slight modifications. The detached leaves were fixed in an autofluorescence-fixing solution (10% formaldehyde, 5% acetic acid, and 45% ethanol). The samples were then cleared in alcoholic lactophenol, rinsed with distilled water to remove the lactophenol, and stained overnight with aniline blue (0.01% aniline blue

powder in 150 mM K_2HPO_4, pH 9.5). The samples were equilibrated in 50% glycerol before mounting onto slides. Aniline blue staining for callose deposition was visualized under a Leica TCS SPS stereo-fluorescence microscope using a blue fluorescent protein filter set (excitation 405 nm, emission 420/90 nm, and dichroic 420 nm), whereas autofluorescence was visualized using the enhanced green fluorescent protein filter set (excitation 488 nm, emission 500/60 nm, and dichroic 495 nm). Hydrogen peroxide was detected by an in situ histochemical staining procedure using 3,3′-diaminobenzidine (DAB) (Thordal-Christensen et al. 1997). The detached leaves were placed in a solution containing 1 mg mL^{-1} DAB (pH 5.5) for 2 h at room temperature, boiled in 95% ethanol for 2 min, and stored in distilled water. Hydrogen peroxide produces a reddish-brown coloration in the DAB staining method. Staining for the presence of superoxide (O_2^-) employed the nitroblue tetrazolium (NBT) uptake method, which was performed as described (Doke 1983). The detached leaves were immersed for 30 min in 5 mL NBT staining solution (1 mg mL^{-1} NBT in 10 mM NaN_3 and 10 mM phosphate buffer). After staining, the leaves were cleared in 96% boiling ethanol and then analyzed.

Statistical analysis

All experiments were repeated independently twice, and the data obtained were statistically analyzed using Student's *t* tests.

Results

Characterization of DAL1 and DAL2 in Arabidopsis

DIAP1 is a functional anti-apoptotic protein in *D. melanogaster* (Vaux and Silke 2005). To determine whether higher plants also have DIAP1-like proteins, homologous searches against the Arabidopsis genome sequence database were performed using DIAP1 as the query. These searches identified two genes, *At1g63900* and *At1g59560*, encoding putative proteins that show similarities to DIAP1 with *P* values at e−8. These two proteins were designated as DAL1 (At1g63900) and DAL2 (At1g59560) for *Arabidopsis thaliana* DIAP1-like proteins. DAL1 is annotated as a 343 amino acid protein, supported by three full-length cDNAs (AK176213, AK176755 and AY128352); DAL2 is predicted as a 338 amino acid protein, supported by two full-length cDNAs (AK228076 and AY089055) (Fig. 1a). Similar searches also identified two putative DAL proteins (Os03g24500 and Os07g45350) in rice genomes. Both DAL1 and DAL2 contain a RING domain at the C-terminus with six conserved cysteine residues (Fig. 1a). Aside from the conserved RING domain, no other conserved domains were present in the DAL proteins. Phylogenetic tree analysis revealed that DAL1 and DAL2 shared approximately 17% amino acid identity with DIAP1 (Fig. 1b). These indicate that DAL1 and DAL2 represent a group of RING finger proteins with structural and possibly functional homology to DIAP1 in *Drosophila*.

Expression of *DAL1* and *DAL2* in response to Pst infection

The expression patterns of *DAL1* and *DAL2* genes in wild-type plants were first analyzed after inoculation with virulent and avirulent strains of *Pst* DC3000. In the mock-inoculated plants, no significant expression of *DAL1* and *DAL2* genes was observed, indicating that the basal expression of these two genes is very low in healthy plants grown under normal conditions (Fig. 2). Expression of *DAL1* was significantly upregulated at 18 hours post-inoculation (hpi), whereas expression of *DAL2* was also upregulated at 36 hpi (Fig. 2a). Similarly, expression of *DAL1* was dramatically induced after inoculation with the avirulent strain of *Pst* DC3000, whereas expression of *DAL2* was upregulated only slightly (Fig. 2a). These indicate that the expression of *DAL* genes was induced through different patterns in response to virulent and avirulent strains of *Pst* DC3000.

Identification of *dal* mutant lines

To determine the functions of *DAL1* and *DAL2*, T-DNA knockout mutants were identified and characterized for these two genes. The mutants SALK_002099 and SALK_063571, containing T-DNA insertions in the eighth intron and in the third exon of the *DAL1* gene, respectively, were designated as *dal1-1* and *dal1-2* (Fig. 2b). The mutants SALK_024744 and WiscDsLox383C6, containing T-DNA insertions in the eighth intron and in the first exon of the *DAL2* gene, respectively, were designated as *dal2-1* and *dal2-2* (Fig. 2b). PCR-based genotyping was performed to screen for the homozygous plants of these T-DNA insertion lines using gene-specific primers and T-DNA primers. Analysis using RT-PCR revealed no significant expression of *DAL1* or *DAL2* genes in the homozygous *dal1* and *dal2* plants using primers for the full-length cDNAs of the *DAL1* and *DAL2* genes (Fig. 2c). These results indicate that the isolated *dal1* and *dal2* lines are null mutants of the *DAL1* and *DAL2* genes.

Disease phenotypes of *dal1* and *dal2* mutants against *Pst*

To explore the possible involvement of *DAL1* and *DAL2* genes in defense response, the disease phenotypes of the

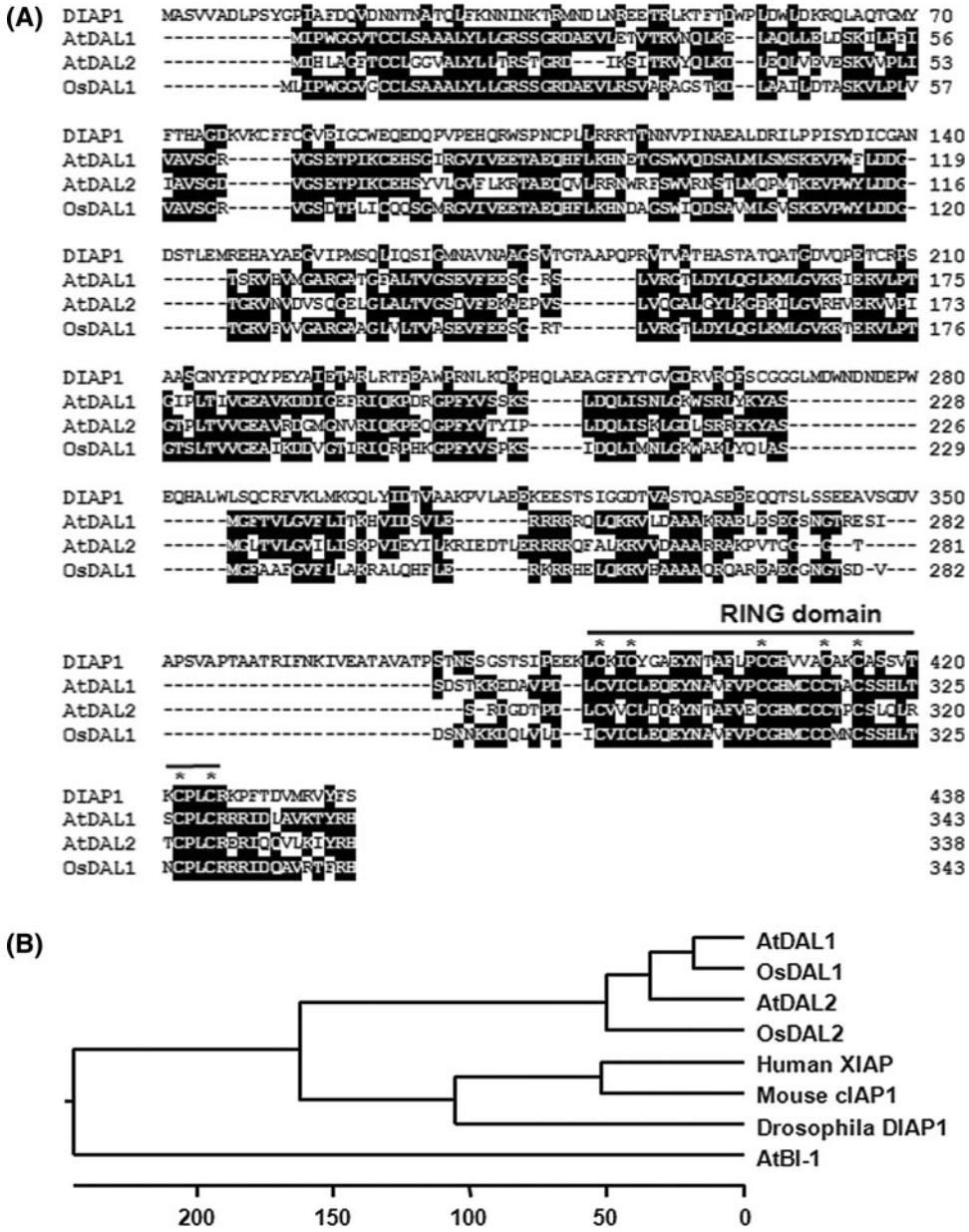

Fig. 1 Arabidopsis DAL1 and DAL2 are RING finger proteins with sequence homology to Drosophila DIAP1. **a** Sequence alignment. The conserved cysteine residues are indicated by *asterisks*, and the putative RING domain is indicated by a *bold line*. **b** Phylogenetic tree

dal1 and *dal2* plants were analyzed and compared with the wild type after inoculation with a virulent strain of *Pst* DC3000. All tested genotypes (*dal1*, *dal2*, and wild type) showed chlorosis visible 4 days after inoculation which developed progressively on the infected leaves (Fig. 3a). The severity of chlorosis in the *dal1* and *dal2* plants was similar to that in the wild-type plants. The measurement of bacterial titers in the inoculated plants further demonstrated significant differences in bacterial growth among the leaves from *dal1*, *dal2,* and wild-type plants (Fig. 3a). These results indicate that mutations in the *DAL1* and *DAL2* genes do not alter the defense response against a virulent strain of *Pst* DC3000.

Next, the disease phenotypes of the *dal1* and *dal2* plants were analyzed against an avirulent *Pst* DC3000 strain carrying the avirulence gene *AvrRpm1*. When a bacterial suspension of $OD_{600} = 0.002$ was infiltrated into the wild-type plants, only very slight chlorosis was observed (Fig. 3b). In contrast, very severe symptoms, resulting in extensive chlorotic lesions, were observed on the inoculated leaves of the *dal1* and *dal2* plants (Fig. 3b). Similarly, the *dal1* and *dal2* plants supported much more bacterial growth in the inoculated leaves, resulting in an average 10- to 50-fold increase in the bacterial population compared with the wild-type plants (Fig. 3b). The bacterial population in the inoculated leaves of wild-type plants was 8.1×10^3 cfu cm^{-2} at 2 dpi and 3.3×10^4 cfu cm^{-2} at 4 dpi. In contrast, bacterial populations in the inoculated leaves were 9.8×10^4 cfu cm^{-2} in *dal1-1* and 12.1×10^4 cfu cm^{-2} in *dal1-2* at 2 dpi. These counts rose to 1.5×10^6 cfu cm^{-2} in

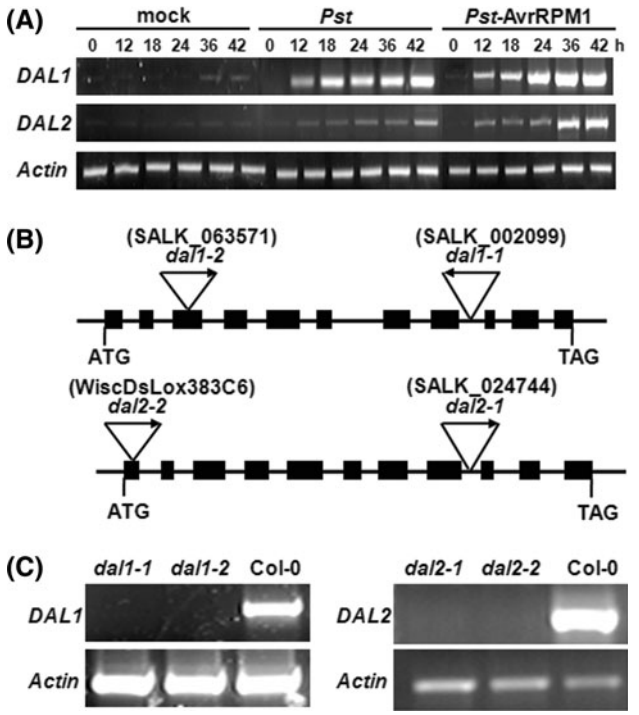

Fig. 2 Expression of *DAL1* and *DAL2* genes in response to *Pst* infection and characterization of *dal* mutant lines. **a** Expression of *DAL1* and *DAL2* in response to infection by virulent and avirulent strains of *Pst* DC3000. Four-week-old plants were inoculated by infiltration with *Pst* DC3000 wild-type or AvrRPM1 strains ($OD_{600} = 0.002$). Leaf samples were collected at the indicated times after inoculation for RNA isolation. Expression of *DAL1* and *DAL2* genes was analyzed by RT-PCR using gene-specific primers. The actin gene was used as internal control. **b** Exon/intron organization of the *DAL1* and *DAL2* genes and the T-DNA insertion lines for *dal1* and *dal2* mutants. *Closed boxes* represent exons, and *bold lines* represent introns. The positions of the T-DNA insertions, the initiating ATG, and stop codon (TAG) are indicated. **c** RT-PCR analysis of the expression of the *DAL1* and *DAL2* genes in the mutant plants. Leaf samples were collected from 4-week-old plants, and total RNA was used for RT-PCR. The experiments were repeated independently twice with similar results

dal1-1 and 1.8×10^6 cfu cm^{-2} in *dal1-2* plants by 4 dpi. The bacterial populations in the inoculated leaves of *dal2-1* and *dal2-2* plants were also higher than in the wild types, measuring 7.1×10^4 and 8.5×10^4 cfu cm^{-2} at 2 dpi and 1.1×10^6 and 1.3×10^6 cfu cm^{-2} at 4 dpi, respectively (Fig. 3b). These results indicate that mutations in the *DAL1* and *DAL2* genes result in susceptibility to avirulent strains of *Pst* DC3000.

PCD and ROS accumulation in *dal1* and *dal2* plants after infection with *Pst*

The wild-type Arabidopsis plant normally responds to infection by an avirulent strain of *Pst* DC3000 with HR and generation of ROS. The PCD levels and the accumulation of ROS were therefore analyzed in wild-type and *dal1*/*dal2*

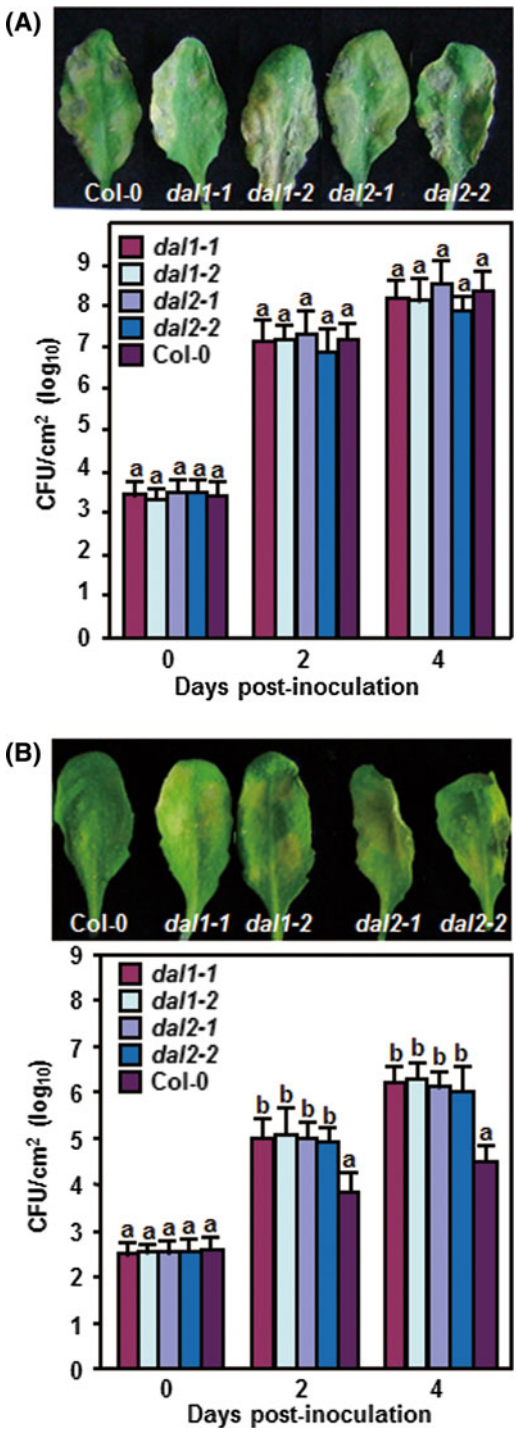

Fig. 3 The disease phenotypes of the *dal1* and *dal2* plants after infection by virulent and avirulent strains of *Pst* DC3000. **a** Representative leaves showing disease symptoms (*top*) and bacterial growth (*bottom*) 4 dpi after infiltration with a bacterial suspension ($OD_{600} = 0.004$) of *Pst* DC3000. **b** Representative leaves showing disease symptoms (*top*) and bacterial growth (*bottom*) 2 dpi after infiltration with a bacterial suspension ($OD_{600} = 0.002$) of *Pst*DC3000-AvrRpm1. The experiments were repeated independently twice, and the data presented are the means and standard errors from three independent experiments. *Different letters above the columns* indicate significant differences at $p < 0.05$ level by *t* test

plants in response to *Pst* DC3000-AvrRpm1. The results of the Evans blue staining indicate that cell death levels were very low and that only a few cells died in the wild-type plants (Fig. 4a). In contrast, the *dal1* and *dal2* plants showed higher levels of cell death compared with the wild-type plants (Fig. 4a). However, the cell death levels varied to some extent among the *dal1* and *dal2* mutant plants; the *dal2-1* plants had severe cell death, whereas the *dal1-1* and *dal2-2* plants responded with moderate cell death. This was probably due to the different T-DNA locations among these mutants. The accumulation of superoxide anions (O_2^-) was tested by staining with NBT (Doke 1983). In wild-type plants, no significant accumulation of O_2^- was observed at 2 dpi (Fig. 4b). In contrast, significant accumulations of O_2^- were detected in the leaves of the *dal1* and *dal2* plants at 2 dpi (Fig. 4b).

Extensive PCD induced by FB1 in *dal1* and *dal2* plants

We further studied whether the *dal1* and *dal2* plants showed altered responses to FB1, a fungal toxin that triggers PCD in plants (Asai et al. 2000; Stone et al. 2000). Reportedly, infiltration with FB1 can trigger both necrotic lesion formation and characteristic PCD responses in Arabidopsis leaves (Stone et al. 2000). When a 10 μM FB1 solution was infiltrated into the mature leaves of 4-week-old soil-grown Arabidopsis wild-type plants under controlled environmental conditions, lesions appeared on the leaves within 2–3 dpi (Fig. 5a). At 4 dpi, infiltration with FB1 resulted in a greater number of necrotic lesions in all the *dal1* and *dal2* plants compared with the wild-type (Fig. 5a). Evans blue staining confirmed that cell death progression was accelerated in the *dal1* and *dal2* plants (Fig. 5b). Relatively high levels of cell death were observed in the *dal1-1*, *dal1-2*, and *dal2-1* plants compared with the wild-type plants 48 h after infiltration (Fig. 5b). The progression of cell death was further examined by quantifying ion leakage in the wild-type, hemizygous, and homozygous *dal1* and *dal2* plants. The hemizygous and wild-type *dal1* and *dal2* plants exhibited similar cell death

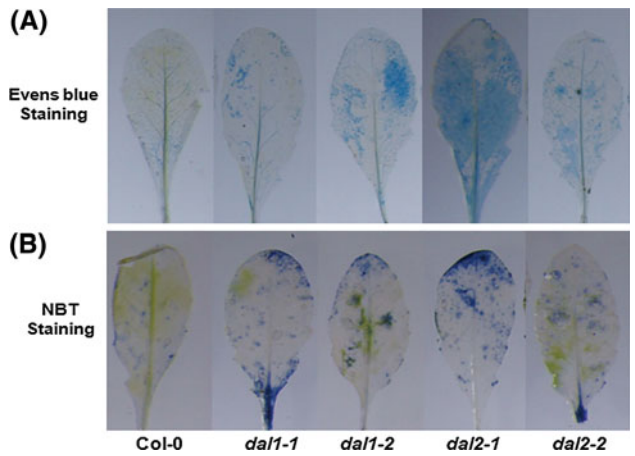

Fig. 4 Cell death and ROS accumulation in *dal1* and *dal2* plants after infection with virulent and avirulent strains of *Pst* DC3000. **a** and **b** Four-week-old plants were inoculated by infiltration with *Pst* DC3000-AvrRpm1 ($OD_{600} = 0.002$), and leaf samples were taken at 3 dpi. Representative leaves showing cell death (**a**) and accumulation of superoxide anion (**b**) detected by Evans Blue and NBT staining, respectively. The experiments were repeated twice with similar results

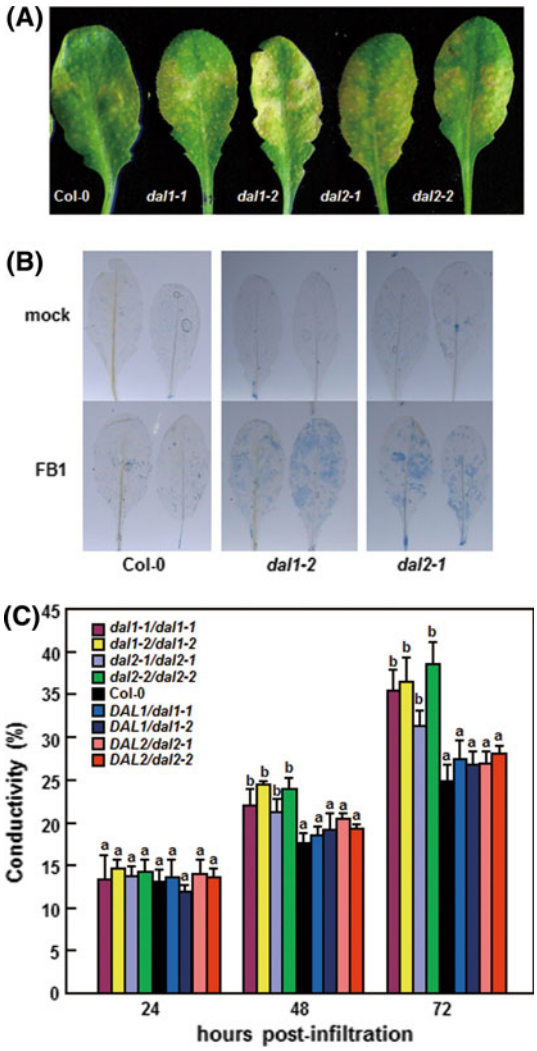

Fig. 5 Responses of the *dal1* and *dal2* plants to fumonisin B1. The leaves of the 4-week-old wild-type and *dal1* and *dal2* mutant plants were infiltrated with 10 μM FB1 in 10 mM $MgSO_4$ or with 10 mM $MgSO_4$ as mock treatment. **a** Representative leaves showing necrotic symptoms 4 days after FB1 infiltration. **b** Representative leaves showing cell death, as revealed by Evans blue staining, 2 days after infiltration. **c** Cell death progression measured by ion leakage. The experiments were repeated independently twice, and the data presented are the means and standard errors from three independent experiments. *Different letters above the columns* indicate significant differences at $p < 0.05$ level by *t* test

progression in response to FB1 infiltration (Fig. 5c). However, the cell death levels in the homozygous *dal1* and *dal2* plants were much higher in response to FB1 (Fig. 5c).

We then analyzed whether the expression of *DAL* genes is induced in wild-type plants in response to FBI. Analysis using RT-PCR revealed that 18 h after FB1 infiltration, the expression of *DAL1* and *DAL2* genes was dramatically upregulated, whereas no significant expression was observed in the mock-treated mutant plants (Fig. 6a). In addition, the defense-related *PR-1* gene exhibited similar upregulation in mutant plants after FB1 treatment (Fig. 6a).

The ROS production in the wild-type and *dal* plants after infiltration of FB1 was then analyzed and compared. In mock-inoculated plants, no significant accumulation of H_2O_2 or O_2^- was detected in either the wild-type or *dal* plants (data not shown). At 2 days following FBI infiltration, however, significantly greater accumulations of O_2^- and H_2O_2 were detected in the leaves of the *dal1* and *dal2* plants compared with those of the wild-type plants (Fig. 6b, c). Microscopic observations revealed increased callose deposition and autofluorescence in the FB1-infiltrated leaves of the *dal1* and *dal2* plants compared with that of the wild types (Fig. 7). These results indicate that mutations in the *DAL1* and *DAL2* genes result in increased ROS accumulation and enhanced cell death in response to FB1.

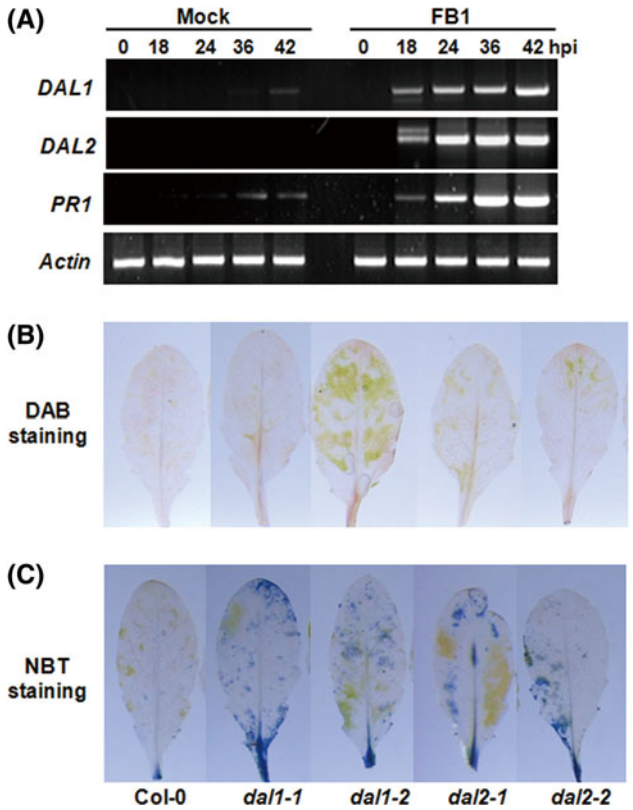

Fig. 6 Expression of *DAL* and *PR-1* genes and accumulation of ROS in response to FB1. Four-week-old wild-type plants were infiltrated with 10 μM FB1 in 10 mM $MgSO_4$ or with 10 mM $MgSO_4$ as mock treatment. Leaf samples were collected at the indicated times for analysis. Expression of the *DAL* and *PR-1* genes was analyzed by RT-PCR using gene-specific primers with actin as the internal control. Accumulation of H_2O_2 (**b**) and superoxide anion (**c**) in leaves was detected by DAB and NBT staining, respectively. The experiments were repeated independently twice with similar results

Discussion

Programmed cell death is a genetically controlled cellular process induced in response to pathogen infection, and a number of genes have been identified that are negative or positive regulators of PCD. Compared with animal studies, however, our understanding of the genetic basis and molecular biology of PCD in plants is rudimentary. In this study, two Arabidopsis RING finger proteins, DAL1 and DAL2, were identified, and these proteins were proven as negative regulators (response inhibitors) of PCD in Arabidopsis. Mutations in *DAL1* and *DAL2* genes result in extensive PCD in response to both avirulent bacteria and the chemical toxin FB1.

Based on their amino acid sequences and conserved domain organizations, the DAL1 and DAL2 proteins belong to the RING family whose members have diverse biological functions in plant growth and development, hormone signaling, and stress responses (Stone et al. 2005). The DAL1 and DAL2 proteins contain a conserved RING domain at their C-terminus and show moderate sequence homology with DIAP1 in fruit flies (Wang et al. 1999; Goyal et al. 2000; Lisi et al. 2000). In addition to the typical RING domain, a BIR domain is present in DIAP1 (Vaux and Silke 2005). However, no further conserved domains were identified in the DAL1 and DAL2 proteins. Our observation that mutations in the *DAL1* and *DAL2* genes resulted in enhanced cell death in response to an avirulent pathogen and low doses of FB1 mirrors results in *Drosophila,* where the loss of DIAP1 function results in early embryonic death from massive apoptosis (Wang et al. 1999; Goyal et al. 2000; Lisi et al. 2000). Most RING finger proteins have enzymatic activities that catalyze reactions within the ubiquitination/26S proteasome protein degradation system (Stone et al. 2005). DIAP1 has E3 ubiquitin ligase activity, and the RING domain is critical for its biological activity and regulation of PCD (Goyal et al. 2000; Lisi et al. 2000). Indeed, the Arabidopsis RING1, which possesses E3 ubiquitin ligase activity in vitro, is also involved in PCD (Lin et al. 2008). However, the biochemical activity and putative function of the RING domain in DAL proteins remain undetermined.

Programmed cell death and disease resistance are often intricately linked in higher plants. In Arabidopsis, most of

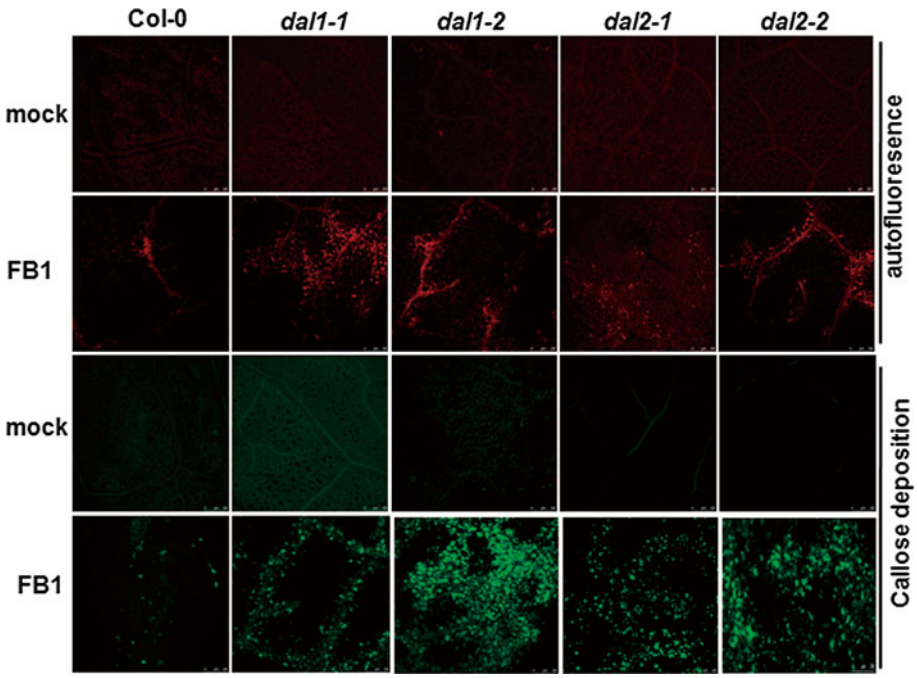

Fig. 7 Callose deposition and autofluorescence in the *dal1* and *dal2* plants in response to FB1. The leaves of 4-week-old plants were infiltrated with 10 μM FB1 in 10 mM $MgSO_4$ or with 10 mM $MgSO_4$ as mock treatment. They were detached at 72 hpi and analyzed for callose deposition and autofluorescence. The experiments were repeated independently twice with similar results

the lesions mimic mutants like *acd* and *lsd* show spontaneous, uncontrolled PCD manifesting as large areas of chlorosis in the leaves (Lorrain et al. 2003). In this study, spontaneous lesions were not observed on the leaves of the *dal* plants grown under normal conditions. This phenotype is similar to Arabidopsis *bap1*/*bap2* mutants and *RING1*-silenced plants in that the loss of BAP1/BAP2 and RING1 function is associated with accelerated HR-mediated cell death induced by exogenous stimuli, but this does not cause spontaneous cell death (Yang et al. 2007; Lin et al. 2008). The *dal* plants also exhibited elevated stress responses to an avirulent strain of *Pst* DC3000 that resulted in extensive PCD. Compared with the wild-type plants, the *dal1* and *dal2* plants supported higher levels of bacterial growth after inoculation with *Pst* DC3000, indicating that both *DAL1* and *DAL2* genes may also function in *Rpm1*-mediated disease resistance. In this sense, *DAL1* and *DAL2* genes are similar to *BAP* and *BON* genes that act both as direct repressors of PCD and as regulators of the activity of specific *R* genes, resulting in enhanced disease resistance against a variety of virulent pathogens (Yang and Hua 2004; Yang et al. 2007). Further research to clarify the function of *DAL* genes in *R* gene-mediated disease resistance is clearly warranted.

During incompatible interactions between plants and pathogens, cell death in HR is often associated with the development of plant disease resistance, resulting in the halting of pathogen growth in plant tissues. However, cell death can also be uncoupled from the resistance response. In *defense no death 1* (*dnd1*) Arabidopsis mutants, for example, resistance to *Pst* occurs without HR-mediated cell death (Yu et al. 1998). In this study, the high levels of PCD and bacterial growth observed among the *dal1* and *dal2* mutant plants after inoculation with an avirulent strain of *Pst* DC3000 indicate that DAL1 and DAL2 have distinct functions in regulating PCD and disease resistance, a negative role in PCD, and a positive role in *Rpm1*-mediated resistance. Thus, DAL1 and DAL2 seemingly act as modulators to maintain the balance between the extents of PCD and disease resistance against avirulent pathogens.

Interestingly, mutations in the *DAL1* and *DAL2* genes did not affect the response of the corresponding mutant plants against a virulent strain of *Pst* DC3000 (Fig. 3). A similar phenotype was also observed in the Arabidopsis *bap2* mutant plants, which showed the same disease phenotype against a virulent strain of *Pst* DC3000 as wild-type plants (Yang et al. 2007). Therefore, extensive PCD in *dal* plants may likely be unrelated to the defense response against virulent pathogens. This phenomenon was further confirmed by studies on Arabidopsis *dnd1*/*dnd2* and *bap2* mutants. The *dnd1* and *dnd2* plants are unable to undergo HR-mediated cell death but retain their disease resistance against avirulent pathogens in a gene-for-gene manner (Yu et al. 1998; Clough et al. 2000; Jurkowski et al. 2004). Meanwhile, the PCD in *bap2* plants is not associated with an altered defense response against virulent pathogens, including *Pst* DC3000 and virulent strains of *Hyaloperonospora parasitica* (Yang et al. 2007). Therefore, in some cases, HR-mediated cell death is not strictly required for disease resistance against virulent pathogens. Furthermore, overexpression of plant negative regulators (e.g., BI-1 and

BAP1/2) inhibits PCD induced by various biotic and abiotic stimuli, as well as confers enhanced disease resistance against different pathogens (Yang et al. 2007; Kawai-Yamada et al. 2001 Matsumura et al. 2003; Huckelhoven et al. 2003). Determining whether the overexpression of *DAL1* and *DAL2* increases disease resistance is interesting. If this is the case, DAL1 and DAL2 can be promising targets for the generation of disease-resistant transgenic plants.

In Arabidopsis, FB1-induced HR-like PCD response shared many of the features of PCD elicited by an avirulent bacterial pathogen, including increased callose deposition and increased autofluorescence (Stone et al. 2000). This FB1-induced PCD depended on multiple signaling pathways, including jasmonate, ethylene, SA, and ROS accumulation (Asai et al. 2000; Stone et al. 2000; Watanabe and Lam 2006; Shi et al. 2007). In this study, FB1 infiltration induced the expression of *DAL1* and *DAL2* genes in the leaves of the wild-type plants and triggered extensive PCD in the leaves of the *dal* plants (Figs. 5, 6). This extensive PCD was revealed by significant necrotic lesions, increased numbers of dead cells, and greater ion leakage from the leaves of the *dal* plants (Figs. 5, 6). In addition, increased callose deposition and autofluorescence were observed in the *dal* plants upon FB1 treatment (Figs. 6, 7). This is consistent with observations in *atbi1* mutants, where mutant plants exhibited an accelerated PCD phenotype associated with enhanced levels of callose deposition and autofluorescence after treatment with FB1 (Watanabe and Lam 2006). In this study, relatively higher levels of H_2O_2 and O_2^- were detected in the FB1-infiltrated leaves of the *dal* plants compared with wild-type plants (Fig. 6), suggesting that DAL1 and DAL2 may function upstream of ROS generation in the PCD pathways.

In summary, the results presented in this study demonstrate that the Arabidopsis proteins DAL1 and DAL2, two RING domain-containing proteins with homology to DIAP1 in fruit flies, regulate PCD. Mutations in *DAL1* and *DAL2* genes resulted in extensive PCD in response to an avirulent strain of *Pst* DC3000 and following exposure to the toxin FB1, indicating that both DAL1 and DAL2 proteins may act as negative regulators of PCD in Arabidopsis. As RING finger proteins, the DAL1 and DAL2 proteins may be E3 ubiquitin ligases within the ubiquitin/26S proteasome system. They may possibly target other positive regulators of PCD for degradation, thereby reducing the threshold for PCD. Further studies on the biochemical activities of DAL1 and DAL2 can provide new insights into our understanding of the functions of DAL1 and DAL2 in PCD.

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