A Circadian Rhythm-Regulated Tomato Gene Is Induced by Arachidonic Acid and *Phythophthora infestans* Infection[1[W]]

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A cDNA clone of unknown function, *DEA*1, was isolated from arachidonic acid-treated tomato (*Solanum lycopersicum*) leaves by differential display PCR. The gene, *DEA*1, is expressed in response to the programmed cell death-inducing arachidonic acid within 8 h following treatment of a tomato leaflet, 16 h prior to the development of visible cell death. *DEA*1 transcript levels were also affected by the late blight pathogen, *Phytophthora infestans*. To gain further insight into the transcriptional regulation of *DEA*1, the promoter region was cloned by inverse PCR and was found to contain putative stress-, signaling-, and circadian-response elements. *DEA*1 is highly expressed in roots, stems, and leaves, but not in flowers. Leaf expression of *DEA*1 is regulated by circadian rhythms during long days with the peak occurring at midday and the low point midway through the dark period. During short days, the rhythm is lost and *DEA*1 expression becomes constitutive. The predicted DEA1 protein has a conserved domain shared by the eight-cysteine motif superfamily of protease inhibitors, α-amylase inhibitors, seed storage proteins, and lipid transfer proteins. A DEA1-green fluorescent protein fusion protein localized to the plasma membrane in protoplasts and plasmolysis experiments, suggesting that the native protein is associated with the plasmalemma in intact cells.

Pathogen infection can induce cells to die in a regulated manner with morphological features similar to apoptosis, or programmed cell death (PCD) as characterized in animals (Wang et al., 1996; Lawen, 2003). PCD occurs in both resistant (e.g. hypersensitive response; Gilchrist, 1998; Greenberg and Yao, 2004) and susceptible interactions (Wang et al., 1996; Gilchrist, 1998). Both bacterial and fungal pathogens can co-opt plant PCD pathways to establish infections and facilitate colonization of the host (Richael and Gilchrist, 1999). For example, transgenic expression of animal antiapoptotic genes in several plant species reduced disease caused by many pathogens (Dickman et al., 2001; Lincoln et al., 2002), and application of tetrapeptide apoptotic inhibitors to plants prevented bacterial infection (Richael et al., 2001).

Pathogen-produced chemicals that trigger host cell death, such as toxins and elicitors, are useful surrogates in the study of disease- and death-related processes (Tyler, 2001; Curtis and Wolpert, 2002; Nuernberger and Brunner, 2002). One such molecule, arachidonic acid (AA), is a polyunsaturated fatty acid that is widely dispersed in the lipids of *Phytophthora infestans* and related oomycetes, and is released from germinating spores during plant infection (Ricker and Bostock, 1992). Treatment of potato (*Solanum tuberosum*) tissue with AA elicits cell death and systemic responses similar to those observed following inoculation with *P. infestans* (Bostock et al., 1981; Bostock et al., 1986). In potato and tomato (*Solanum lycopersicum*) leaves, AA induces local cell death and transcription-dependent systemic responses to *P. infestans* (Coquoz et al., 1995). AA and lipoxygenase-derived hydroperoxides of AA also induce PCD in tomato protoplasts (Knight et al., 2001).

Signaling pathways leading to cell death and other cellular responses do not exist in isolation, but rather as networks of pathways connecting diverse stimuli to plant responses (Bostock, 2005). One stimulus that is emerging as an interacting factor in many biological response networks is the circadian rhythm (Kreps and Simon, 1997; He et al., 2002; Luna et al., 2005). For example, cold-induced gene expression is known to be affected by the phase of the circadian rhythm during which the cold stress is applied (Fowler et al., 2005). Similarly, the Arabidopsis (*Arabidopsis thaliana*) gene *PCC1* is reported to be a convergence point of pathogen resistance and the circadian rhythm (Sauerbrunn and Schlaich, 2004).

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Differential display PCR (DD-PCR) was used in this study to identify transcriptionally activated genes following AA treatment (Liang and Pardee, 1992; Sambrook and Russell, 2001). DD-PCR and a related technique, suppression subtractive hybridization (Diatchenko et al., 1996), have been used to identify a series of transcripts corresponding to death- and disease-activated genes (Birch et al., 1999; Xiao et al., 2001; Bos et al., 2003). The products of some genes that mediate PCD in animals are transcriptionally induced by apoptotic stimuli (Fridman and Lowe, 2003), but analogous genes have not yet been characterized in plants.

In this paper we characterize *DEA*1, a tomato gene that is induced by the pathogen *P. infestans* and its PCD-inducing elicitor, AA. We describe the temporal and spatial expression patterns of this gene as well as its regulation by photoperiod and circadian rhythm. Sequence analysis revealed that DEA1 has an eight-Cys motif (8CM) domain shared by a superfamily of proteinase inhibitors, α-amylase inhibitors, seed storage proteins, and lipid transfer proteins (Marchler-Bauer et al., 2003; Jose-Estanyol et al., 2004). Members of this group are postulated to have a protective function against abiotic and biotic stresses including cold, pathogens, and pests (Molina and Garcia-Olmedo, 1997; Bubier and Schlappi, 2004).

RESULTS

Identification of AA-Induced Genes

AA infiltration of tomato leaves induces cell death that first becomes visible with defined lesion margins at 12 h posttreatment under our experimental conditions. At 500 μM AA, the cell death is confined to small areas surrounding the point of infiltration despite delivery of the AA suspension to the entire leaflet (Fig. 1A). When the entire leaflet is infiltrated with higher concentrations of AA (i.e. 3 mM), the area of cell death spreads from the infiltration point to cover 5% to 10% of the total leaflet area (Fig. 1A). Infiltration with water does not visibly damage the leaf (Fig. 1A). Leaflets treated by brushing AA at 3 mM onto the leaf surface begin to show necrotic patches across the leaf surface at 24 h posttreatment, and lesions cover approximately 10% of the leaf area. Patches of cell death from AA brush treatment are more distributed throughout the leaf surface than with the infiltration treatment (data not shown). Brush application of AA at lower concentrations such as 500 μM does not typically result in visible lesions.

To study the profile of gene expression during AA treatment, DD-PCR (Liang and Pardee, 1992; Sambrook and Russell, 2001) was performed on total RNA extracted from water-infiltrated (control) and AA-infiltrated leaves 24 h after treatment. The DD-PCR reaction was performed separately on two independent samples of AA-infiltrated leaf RNA, and a 440-bp product was consistently observed. The intensity of the band from the AA-treated samples was at least 10-fold greater than the corresponding DD-PCR band from water-treated control RNA (Fig. 1B).

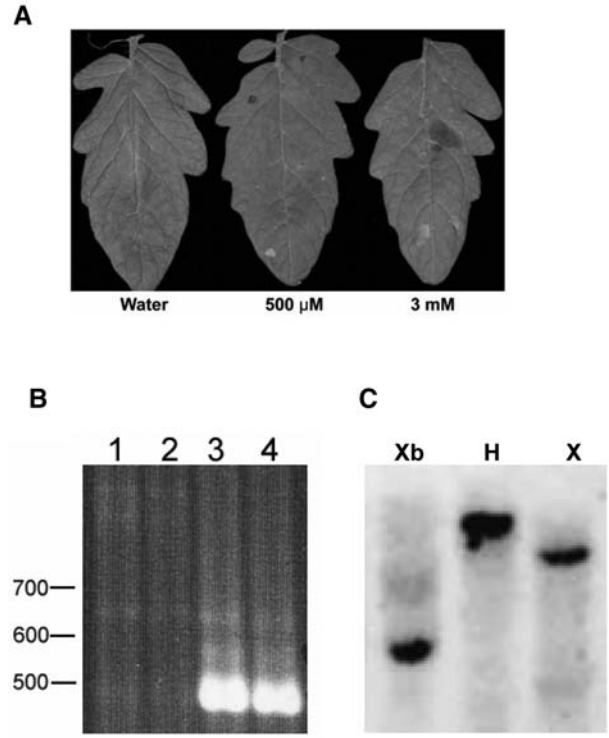

Figure 1. A, Tomato leaflets 24 h following infiltration of the entire leaflet with water or AA at the indicated concentrations. B, DD-PCR gel showing products from control-treated (lanes 1 and 2) and AA-infiltrated (3 mM; lanes 3 and 4) DD-PCR reactions. Band sizes are indicated by the scale to the left in number of base pairs. C, Southern-blot analysis of tomato genomic DNA digested with the restriction enzymes *Xba*I (Xb), *Hin*dIII (H), and *Xho*I (X), and probed with a labeled *DEA*1 fragment.

Isolation of the Full-Length *DEA*1 cDNA and Sequence Analysis

A cDNA library was constructed using total RNA extracted from tomato leaves 24 h after AA treatment. The library was screened using the 440-bp DD-PCR product as a probe to obtain a full-length cDNA representative of this induced transcript. Six positive clones containing cDNA inserts between 700 and 750 bp were isolated and sequenced. The resulting sequences shared an identical open reading frame (ORF) and displayed varying degrees of polyadenylation in the 3′ untranslated region (UTR). One of the clones, *DEA*1, named for being differentially expressed in response to AA, was selected for this study.

The cDNA of *DEA*1 is 729 bp long and contains a 414-bp ORF flanked by a 43-bp 5′ UTR and a 262-bp 3′ UTR, including a poly(A) tail of 23 bp (Supplemental Fig. 1). The deduced amino acid sequence consists of 138 amino acids. The predicted mature protein has a molecular mass of 11.6 kD and a pI of pH 9.1.

Hydropathy analysis of the deduced amino acid sequence using the Genetics Computer Group (GCG) program shows that the DEA1 protein contains extensive hydrophobic regions. The hydropathy plot of the encoded polypeptide reveals a 25-amino acid sequence characteristic of a membrane-spanning cleavable signal sequence at the N terminus (Fig. 2A; Walter et al., 1984). Sequence analysis using the SPScan (GCG) also predicted a secretory signal peptide in DEA1. The predicted cleavage site is located after amino acid 25 between residues A and C (Fig. 2A). The signal sequence is followed by a 25-amino acid Pro-rich region, which, in turn, is followed by a hydrophobic region extending to the C-terminal end (Fig. 2A). The presence of a putative secretory signal sequence combined with long hydrophobic segments is consistent with a possible association with the plasma membrane (Singer, 1990).

To estimate the copy number of the *DEA*1 gene in the tomato genome, Southern-blot hybridization analysis was performed by probing tomato genomic DNA with a 408-bp *Xba*I and *Xho*I fragment from the *DEA*1 cDNA clone, pDEA1. The DNA fragment used as a probe contained the 3′ half of the *DEA*1 ORF plus the 3′ UTR because *DEA*1 contains an *Xba*I site approximately midway through the ORF. A single hybridization band was observed with *Xba*I, *Xho*I, and *Hin*dIII digested genomic DNA (Fig. 1C), suggesting that *DEA*1 is encoded by a single gene per haploid genome.

Sequence Analysis of Coding Region Reveals That DEA1 Belongs to the 8CM Superfamily of Proteinase Inhibitors, α-Amylase Inhibitors, Seed Storage Proteins, and Lipid Transfer Proteins

Searches using tBLASTn returned many closely related matches in a wide range of plant species. The closest match is a gene of unknown function from *Capsicum chinense* (GenBank accession no. AJ879120) that is 68% identical and 81% similar over the length of the entire protein at the amino acid level. A second equally close match is the Pro-rich protein (NtEIG-C29) from tobacco (*Nicotiana tabacum*; GenBank accession no. AB041519). The Arabidopsis gene *EARLI 1* (GenBank Accession no. L43080) and its paralogs are

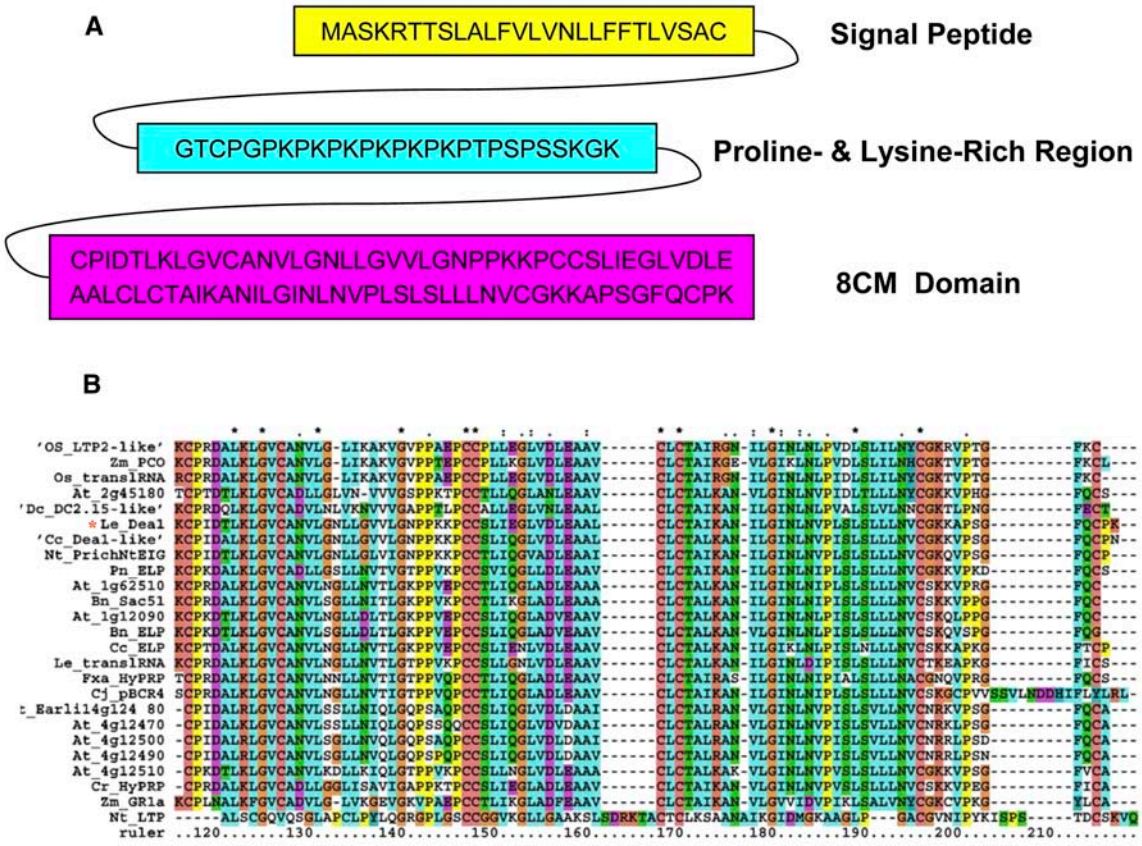

Figure 2. A, Graphical representation of the three domains of *DEA*1. B, Alignment of the C-terminal domain of proteins closely related to *DEA*1. Sites with a consensus are colored as follows: G = orange; *P* = yellow; all other aliphatic residues are colored in blue; C, K, R, and H = pink; D and E = purple; and S, T, M, B, and Q = green. For comparison, a tobacco LTP with confirmed biochemical LTP activity was included (Nt_LTP, BAA03044). Additional accession numbers of proteins not listed in Figure 2: Os_LTP2-like (AF026382), Zm_PCO (AY105972), Os_translRNA (XM_467170), Dc_DC2.15 (AB037109), Le_*DEA*1 (AY509122), Nt_PRichNTIEG-C29 (AB041519), Pn_ELP (D83227), Bn_Sac51 (S68113), Bn_ELP (AF346659), Cc_ELP (AJ879072), Le_TranlRNA (BT013185), Fxa_HyPRP (AF026382), Cj_pBCR4 (AY100448), Cr_HyPRP (X85206), and Zm_GR1 (AB018587).

50% to 65% identical to DEA1 at the amino acid level. A few of the proteins closely related to DEA1 (Fa-HyPRP and Cr-HyPRP) have been termed hybrid Pro-rich proteins (HyPRPs) due to their N-terminal Pro-rich domain that accompanies the Cys-rich C-terminal region creating a protein that is a hybrid of these two domains (Blanco-Portales et al., 2004; Jose-Estanyol et al., 2004). Many other genes in the database share greater than 50% amino acid identity with *DEA*1, but all, like NtEIG-C29, *EARLI 1*, and the HyPRPs, encode proteins of unknown function (Fig. 2B).

Reverse position-specific iterated BLAST, a bioinformatic approach to search for distant homology and potential function, indicated that DEA1 has a domain shared by two superfamilies, pfam00234 (*E*-value 9×10^{-8}) and cd00010 (*E*-value 1×10^{-6}; Marchler-Bauer et al., 2003). These two superfamilies are very similar and possess many of the same members including trypsin protease inhibitors, α-amylase inhibitors, seed storage proteins, and lipid transfer proteins. Recently, this set of proteins has been designated the 8CM superfamily (Jose-Estanyol et al., 2004). DEA1 and its closely related homologs described above constitute a subfamily within the 8CM superfamily. The common feature of proteins within these superfamilies is a series of eight positionally conserved Cys residues (Jose-Estanyol et al., 2004). Although DEA1 contains 10 Cys residues, the C-terminal eight Cys align well with the consensus sequences for the 8CM superfamily (Fig. 2B; Jose-Estanyol et al., 2004). All of the closely matching BLAST hits to *DEA*1 in many plant species represent a small subfamily within the 8CM superfamily, and it is unknown to what degree *DEA*1 and its homologs share the functions of proteins of other subfamilies within the 8CM.

*DEA*1 Is Circadian Rhythm Regulated

*DEA*1 expression levels differed in preliminary elicitor-induction experiments depending on the starting time of the experiment. This led us to examine whether a biological clock was controlling *DEA*1 expression, and samples were collected every 4 h for a 48-h period. Samples taken during the dark were collected with a light-tight tarp over the chamber door to prevent any outside light from reaching the plants during the dark cycle of the photoperiod. Northern-blot analysis of samples taken from plants trained to long days (LDs [16-h light, 8-h dark]) indicated substantial diurnal fluctuations of the *DEA*1 transcript that peaked about midday and decreased during the night cycle (Fig. 3). Expression was often observed to decrease in anticipation of the dark cycle (Figs. 3 and 4), indicating that the response is circadian clock controlled and not simply light responsive. In plants grown in short days (SDs [10-h light, 14-h dark]), expression of *DEA*1 did not exhibit circadian regulation and instead was more constitutively expressed

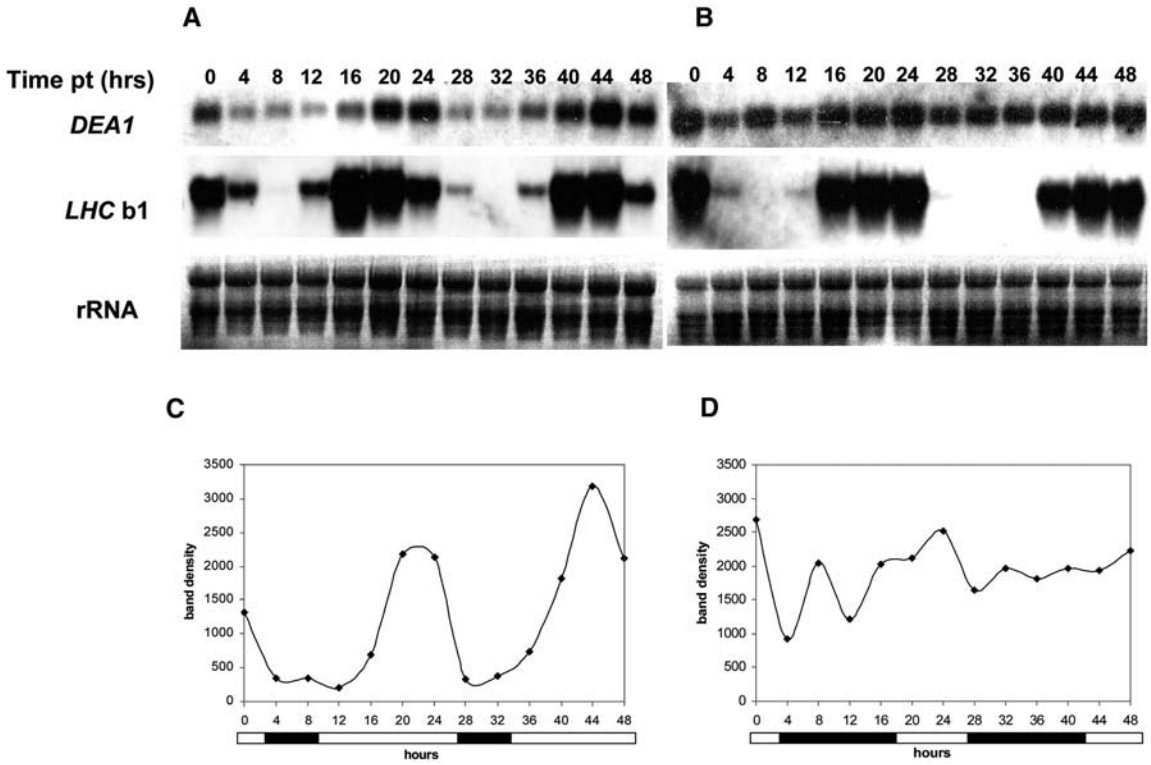

Figure 3. *DEA*1 and *LHC* expression in tomatoes grown under 16-h days (A) and 10-h days (B). C and D, Quantification of *DEA*1 bands from A and B, respectively. Light or darkness at each time point is indicated by the bar at the bottom of the horizontal axis. The data are representative of at least three separate experiments.

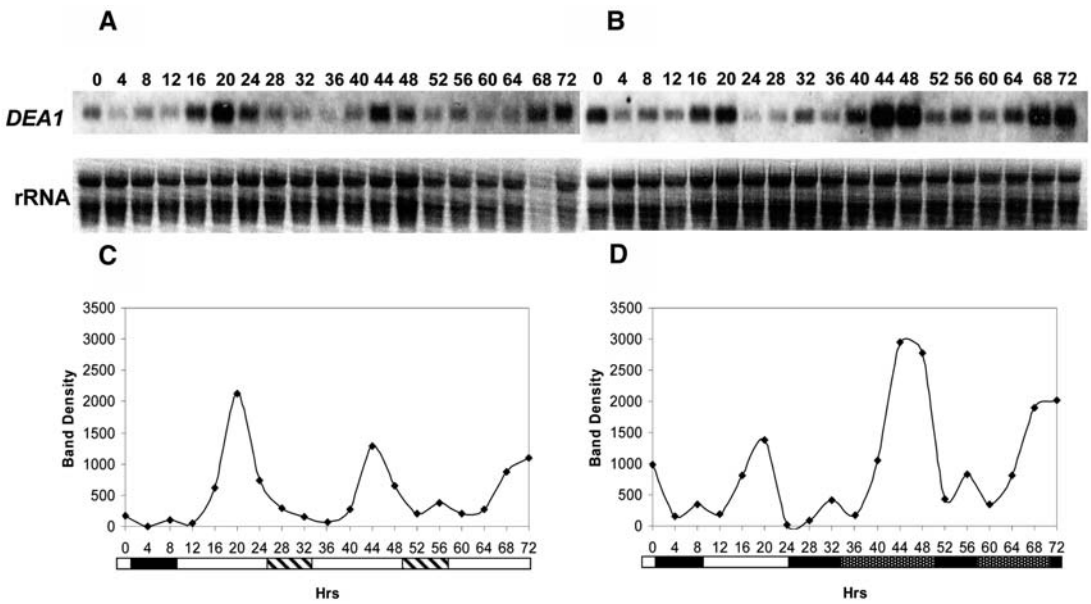

Figure 4. *DEA*1 expression in plants trained to 16 h LDs and transferred to LL conditions (A) and DD conditions (B). C and D, Quantification of *DEA*1 bands from A and B, respectively. Light or darkness at each time point is indicated by the bar at the bottom of the horizontal axis. Hatched regions (C) correspond to time points that would have been dark under normal LD conditions. Stippled regions (D) correspond to time points that would have been light under normal LD conditions. The data are representative of three separate experiments.

near the peak expression level of LD plants. One trial of three is shown in Figure 3B, and no clearly discernable patterns are visible, although the transition from light to dark usually produced a slight decline in *DEA*1 expression. As a control for the maintenance of circadian rhythms, we examined expression of the circadian-regulated light-harvesting protein genes (*LHC*, formerly chlorophyll *a/b*-binding protein genes; Meyer et al., 1989). Figure 3 shows that under both SD and LD conditions, circadian rhythms were maintained for *LHC*. Thus, because *LHC* rhythms were maintained, the loss of circadian regulation for *DEA*1 under SD conditions was not due to a complete abrogation of circadian cycling, but rather was due to a regulatory mechanism more specific to *DEA*1.

The diurnal cycling was maintained even in the absence of night and day oscillations. Plants were grown in LD conditions and sampled for 24 h in LD conditions. At 27 h when the transition from light to dark was supposed to take place, plants were placed in chambers set for either all-light conditions (LL) or all-dark conditions (DD). Thus, plants transferred to LL only experienced darkness during the sampling at the 4- and 8-h time points. The plants transferred to DD experienced darkness at their normal time during the first 24-h cycle of the sampling but did not experience any more light for the duration of the sampling. In both conditions, the *DEA*1 expression cycles were maintained (Fig. 4). This, along with the fact that the expression would often begin to decrease before the onset of darkness, is consistent with a circadian rhythm control of *DEA*1 transcription (Eriksson and Millar, 2003).

*DEA*1 Is Expressed in Many Vegetative Tissues

*DEA*1 is expressed in leaves, stems, and roots, but not in flowers (Fig. 5). Expression was greater in older plants (4-week-old versus 1-week-old seedlings) and was strongest in stems and tap roots in both 4-week-old and 1-week-old plants. In 1-week-old plants, expression was higher in developing true leaves than in the cotyledons. This trend was not observed in the 4-week-old plants where leaves of intermediate age (i.e. recently expanded) expressed more strongly than

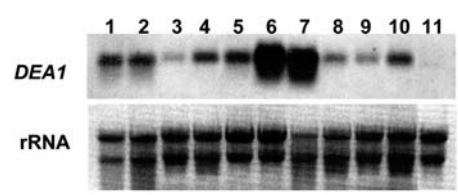

Figure 5. RNA gel-blot analysis of constitutive *DEA*1 expression in different organs of the tomato plant. Tissue was harvested midday from plants of indicated age. Roots from 1-week-old plants (1), hypocotyls from 1-week-old plants (2), cotyledons from 1-week-old plants (3), leaves from 1-week-old plants (4), lateral roots from 4-week-old plants (5), tap roots from 4-week-old plants (6), stems from 4-week-old plants (7), oldest leaf on 4-week-old plants (8), youngest and still expanding leaves from 4-week-old plants (9), third leaf up from bottom on 4-week-old plants (10), and flowers (11).

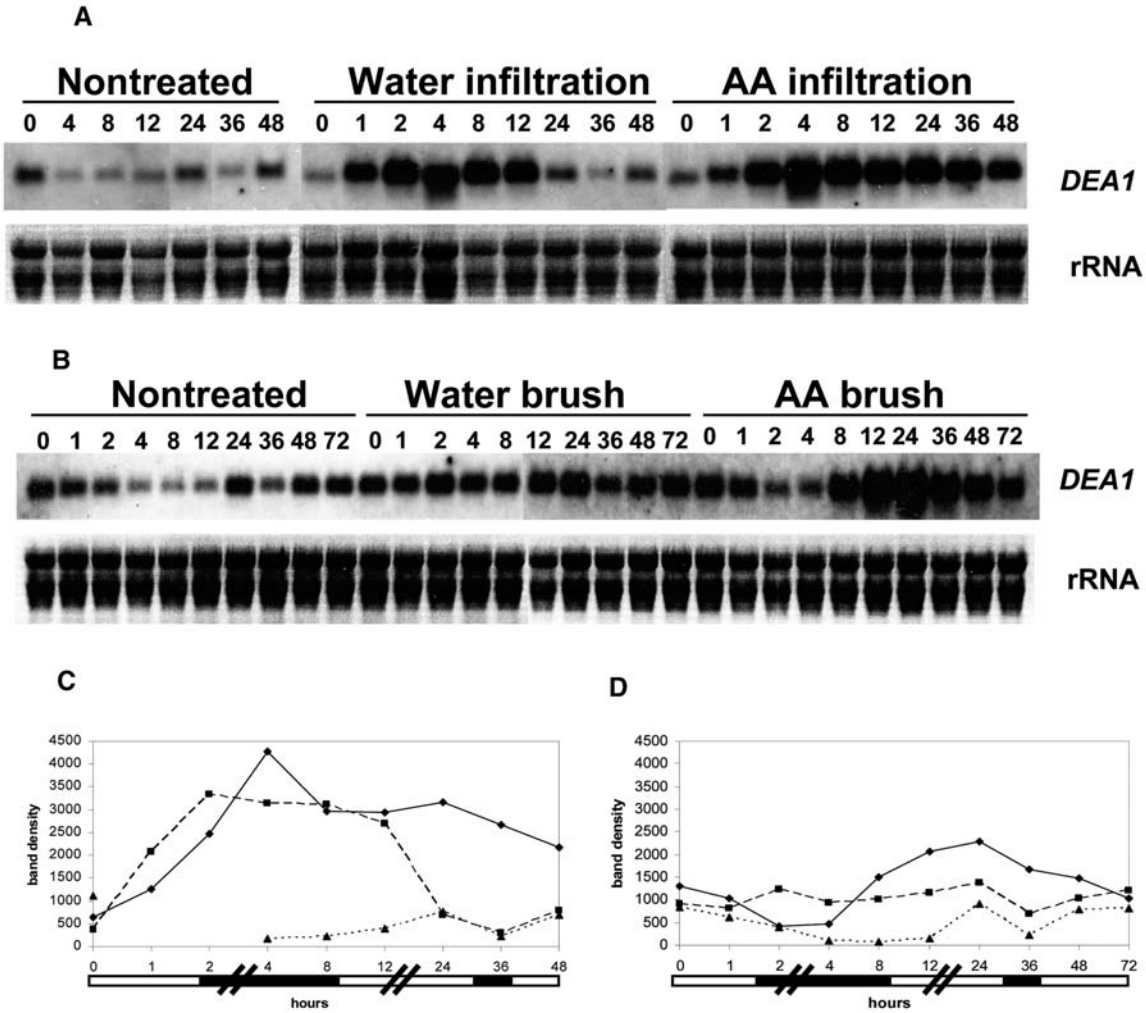

Figure 6. A, *DEA*1 expression in nontreated leaflets or in response to infiltration with water or AA (330 μM). B, *DEA*1 expression in nontreated leaflets or in response to brush application of water or AA (3 mM). C and D, Quantification of *DEA*1 bands from A and B, respectively. Solid lines and diamond symbols indicate AA treated, dashed lines and squares indicate water treated, and dotted lines and triangles indicate nontreated tissue. Light or darkness at each time point is indicated by the bar at the bottom of the horizontal axis. The data are representative of at least two separate experiments.

both the still-developing and the oldest leaves that had been fully expanded for 2 weeks. Flowers were the only tissue in which expression was completely undetectable.

Signaling- and Pathogen-Induced Expression of *DEA*1

The expression of the *DEA*1 gene in AA-treated leaves grown under LD conditions was examined by northern hybridization. Both AA-infiltrated and water-infiltrated treatments induced *DEA*1 in tomato leaf tissue although the induction profile differed for the two treatments (Fig. 6A). This induction occurred rapidly in less than 1 h, and high levels of expression in both AA and control treatments continued through the 12-h postinfiltration time point. *DEA*1 expression in AA- and water-infiltrated leaflets peaked at 25-fold higher than untreated controls 4 h postinfiltration. The 4-h time point occurred during the dark cycle, and the high level of expression indicated that the circadian rhythm regulation could be overridden by these treatments. Expression by AA and water infiltration greatly exceeded normal untreated daylight peaks, typically by a factor of four (Fig. 6A). By 24 h *DEA*1 expression due to water infiltration returned to nontreated levels, while expression in AA-infiltrated tissue remained elevated approximately 3-fold through the 48-h time point (Fig. 6A).

To examine the effects of AA without the background infiltration induction, AA was brushed onto leaflets and a time course was performed and analyzed by northern blot. Water-brush treatments abolished the circadian patterns of *DEA*1, but did not result in *DEA*1 induction (Fig. 6B). However, AA-brush treatment induced *DEA*1 over water-brushed background at 8 h posttreatment, and levels remained elevated over 48 h

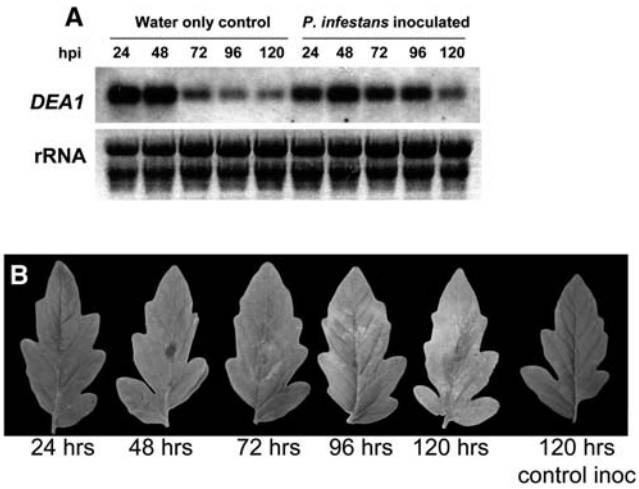

Figure 7. A, Tomato leaf *DEA*1 expression in response to *P. infestans* inoculation. B, Infection progression at each time point. The data are representative of at least two separate experiments.

posttreatment. *DEA*1 expression levels began to diminish at 48 h and decreased further at 72 h posttreatment. Considering the brush and infiltration data together, the effect of *DEA*1 induction by AA is evident.

Expression of *DEA*1 also was examined in response to the pathogen *P. infestans* and to the plant defense-inducing chemicals, acibenzolar-*S*-methyl and jasmonic acid (JA). As was observed for the infiltration experiments, the water control for *P. infestans* also induced *DEA*1 (Fig. 7A). However, 48 h postinoculation, *DEA*1 expression in the water control decreased while expression in the *P. infestans* treatment remained induced in two replicate experiments. This increased expression remained at an elevated level until the leaf was completely colonized by the pathogen at 120 h postinoculation whereupon the level decreased (Fig. 7A). This induction at time points 72 h and 96 h corresponds to points where the pathogen has caused significant cell death on the plant leaf (Fig. 7B).

Controls for both acibenzolar-*S*-methyl and JA treatments also induced *DEA*1 even though treatments were applied by brushing with a cotton-tip applicator instead of infiltration as was AA. Brush controls for both acibenzolar-*S*-methyl and JA both resulted in transient increases in *DEA*1 expression during the first 12 to 24 h posttreatment (data not shown). In contrast to AA, no change in expression patterns was observed for acibenzolar-*S*-methyl and JA when compared to the respective brush control throughout the 72-h time course (data not shown).

Isolation of Genomic Copy of Gene and Promoter Region

Primers were designed to amplify the ORF portion of *DEA*1 and PCR was performed using tomato genomic DNA as a template. The reaction yielded an approximately 400-bp product which, when sequenced, was found to be identical to the cDNA, indicating the absence of introns from *DEA*1 (data not shown).

Two rounds of inverse PCR (IPCR) were performed using the enzymes *Nsp*I and *Sau*3AI to cut tomato genomic DNA. The resulting products were sequenced and compiled to yield 901 bp of sequence from the region 5′ of the cDNA sequence (Supplemental Fig. 1). Transcription factor-binding site prediction was performed using the program Genomatix MatInspector (Cartharius et al., 2005). This analysis returned matches to 70 known promoter motifs with a matrix similarity of 80% or higher. Basic transcriptional binding sites such as the CAAT box and TATA box were identified at positions −51 bp and −45 bp before the predicted transcriptional start site, respectively. Given the apparent circadian and pathogen regulation of *DEA*1, we were particularly interested in motifs known to confer regulation by stress and environmental stimuli, and a subset of the promoter motif prediction results is presented in Table I. Among the stress-response motifs predicted by Genomatix were several abscisic acid (ABA) responding elements including the ABA response element motif, the coupling element-1, and the Sph/RY motif (Hattori et al., 1995; Niu et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2005).

Other stress-response motifs predicted to be in the *DEA*1 promoter include a W-box involved in responses to pathogens, wounding, and senescence (Eulgem et al., 2000) and the Alfin1-response motif involved in response to salt stress (Winicov and Bastola, 1999). Several elements predicted to be in the promoter of *DEA*1 are involved in responses to light in other systems. For example, CCA1 is an element characterized by its involvement in phytochrome-mediated responses to light (Wang et al., 1997). Two light-responsive promoter sites for GT-1 binding are predicted to be in the promoter as well as three GATA transcription factor-binding sites that are often found in the promoters of light- and circadian-regulated plant genes (Gilmartin et al., 1990). A fourth light-responsive promoter motif, an I-box, is also predicted to be in the *DEA*1 promoter and has been implicated in light and circadian regulation in plants (Giuliano et al., 1988).

DEA1 Localizes at the Plasma Membrane

Analysis of the mature DEA1 sequence with nine different membrane protein prediction programs indicated a transmembrane signal peptide and between zero and three transmembrane helices (Supplemental Table II). To test the prediction that DEA1 localizes to the plasma membrane, *Agrobacterium tumefaciens*-mediated transient gene expression was undertaken with a green fluorescent protein (GFP) fusion protein in tobacco (Haseloff and Siemering, 1998). A binary vector was constructed (pDEA1-GFP) in which GFP was fused to the C terminus of DEA1 and driven by the 35S promoter (Supplemental Fig. 2). Construct pBINm-GFP5-ER was used as a GFP-only control (Haseloff et al., 1997). As a positive control for a membrane-

Table I. *Results of MatInspector promoter element prediction*

The various features are listed along with position of the motif (base pair upstream of the predicted transcription start site [strand]), the sequence of the motif (uppercase letters are part of consensus sequence), and notes about the motif (see text for references).

Feature	Position	Motif	Notes
W-box	−72 to −68 (+)	ATGACC	Binding site for WRKY transcription factor. Genes with this element are induced by pathogens, wounding, and senescence.
Alfin1	−31 to −35 (−)	tagGaGGTGgcta	This salt stress-responsive element is found on *MsPRP2*, a protein with similar Pro-rich domain and Cys motif as *DEA*1.
ABA response element	−219 to −211 (+)	caTtatACGTGtaga	Presence of this element along with the coupling region is sufficient for induction by ABA, a hormone involved in seed development and stress response.
CE1	−723 to −719 (−)	CACCG	Coupling element for ABA response element. Involved in ABA induction of genes during seed maturation.
SPH/RY	−871 to −845 (+)	taacCATGCAtaaatt	Responsible for ABA-independent activation of embryogenesis-related proteins. Involved in activation of LEA proteins which are induced in seeds and vegetative tissue by drought and cold.
Circadian clock-associated 1	−674 to −660 (+)	acacaTAAATCTga	Necessary for phytochrome-induced expression of the light-harvesting chlorophyll *a/b* protein gene, Lhcb7*3.
GATA factors	−139 to −123 (−) −387 to −371 (−) −442 to −426 (−)	gcataGATAattattca tagcaGATAaaattttt tatgGATAagcgacga	Present in the promoter of light-induced chlorophyll *a/b*-binding proteins, transcription is activated by light and circadian rhythms.
GT1-box binding factors	−824 to −808 (+) −83 to −93 (−)	ttttatGTTAgTgacgt ggtcatGTAAaTataca	This light-activated transcription factor-binding site is present in promoters of light-activated Rubisco genes.
I-box	−239 to −223 (−)	aaaaTATAAGaaaata	This light-activated transcription factor-binding site is present in promoters of light-activated Rubisco genes.

associated protein, pCPK1-GFP was created by fusing LeCPK1 to GFP (Supplemental Fig. 2; Rutschmann et al., 2002). LeCPK1 is a tomato calcium-dependent protein kinase that is peripherally associated with the plasma membrane (Rutschmann et al., 2002). *A. tumefaciens* cells that harbored the binary vector constructs were pressure infiltrated into leaves to initiate transformation of leaf cells. Figure 8 shows laser scanning-confocal microscope images of *Nicotiana benthamiana* leaf mounts expressing the constructs. The cells transformed with the pDEA1-GFP fusion show a GFP signal that localizes to the cell periphery (Fig. 8B). In contrast, areas infiltrated with pBINm-GFP5-ER display a GFP signal throughout the cell (Fig. 8A).

To determine whether the peripheral localization of DEA1 was to the cell wall or plasma membrane, protoplasts were made from the construct-infiltrated areas of leaf tissue. Protoplasts made from areas infiltrated with pBINm-GFP5-ER displayed GFP throughout the cells, as was observed in cells of leaf disc mounts before protoplast preparation (Fig. 8C). In comparison, the GFP signal appeared to be localized in the plasma membrane in protoplasts derived from areas infiltrated with either pDEA1-GFP or pCPK1-GFP (Fig. 8, D and E).

To assess further the possible membrane localization of DEA1, the GFP signal from cells transformed with pBINm-GFP5-ER and pDEA1-GFP was examined in cells of *N. benthamiana* epidermal peels following plasmolysis in 1 M Suc. Green fluorescence was observed throughout the cells transformed with pBINm-GFP5-ER (Supplemental Fig. 3A), whereas the GFP signal was observed in the cell periphery in pDEA1-GFP-transformed cells. After plasmolysis the fusion protein separated with the plasma membrane rather than the cell wall (Supplemental Fig. 3, B and C). Collectively, the results are consistent with DEA1 localization to the plasma membrane in its native state.

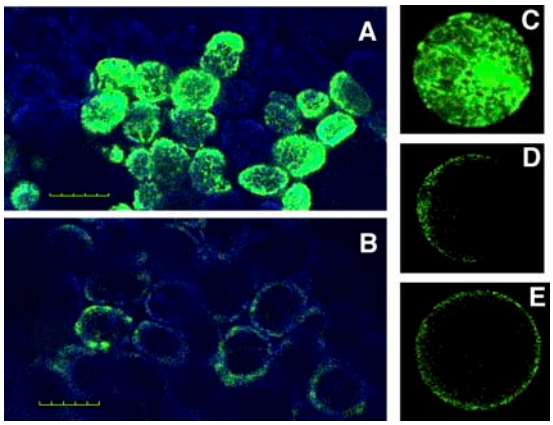

Figure 8. Transient expression of the *DEA*1-GFP fusion protein and GFP in *N. benthamiana* leaves viewed under a laser scanning confocal microscope. Leaves were infiltrated with pBINm-GFP5-ER (A) and pDEA1-GFP (B; GFP shown in green, chlorophyll autofluorescence shown in blue, scale bar = 33 μm). Protoplasts made from *N. benthamiana* tissue infiltrated with pBINm-GFP5-ER (C), pDEA1-GFP (D), and pCPK1-GFP (E), and viewed with laser scanning confocal microscope to show GFP (magnified 400 ×).

DISCUSSION

*DEA*1, a previously unreported tomato gene, was recovered by DD-PCR in response to AA treatment. The gene is expressed very strongly in stems and tap roots and to a lesser extent in leaves. Expression is absent from flowers, and, based on data reported in the Tomato Expression Database, expression of *DEA*1 is likely to be absent from fruits as well as developing seeds (Fei et al., 2004). Brush application of AA induced *DEA*1 expression between time points 4 and 8 h. This rapid induction corresponds to a time point occurring 16 h before the onset of visible cell death. Both AA- and water-infiltration treatments induce *DEA*1 even though water infiltration does not cause any visible damage to the leaf. This water induction indicates that the stress of infiltration alone is enough to induce *DEA*1 above nontreated background. The induction by control infiltration was transient, and after 24 h, *DEA*1 expression in response to AA infiltration was elevated above controls. Brush treatment did not induce *DEA*1 as strongly as infiltration, but nonetheless indicates that AA rapidly induces *DEA*1.

P. infestans also induces *DEA*1 at 72 and 96 h following inoculation. The lag in *DEA*1 induction compared to direct application of AA may indicate that pathogen biomass must accumulate to achieve a threshold-inducing concentration of AA in the leaf tissue. By 120 h after inoculation, *DEA*1 expression had decreased. Moy et al. (2004) report that many genes commonly considered defense genes such as peroxidase and lipoxygenase are down-regulated as disease ensues, and the same pattern of regulation may be occurring for *DEA*1 (Moy et al., 2004). At earlier time points such as 24 and 48 h, *DEA*1 is induced equally in both control and *P. infestans* treatments and may be due to the sensitivity of *DEA*1 to initial perturbations of experimental setup. Other compounds such as JA and acibenzolar-*S*-methyl that induce many pathogen-responding genes failed to induce *DEA*1, indicating that *DEA*1 expression is not regulated by the JA- or salicylic acid-mediated signaling pathways (Glazebrook et al., 2003).

*DEA*1 induction by AA-brush treatment begins between 4 and 8 h posttreatment. This is similar to other AA-induced tomato genes such as LOX (Fidantsef et al., 1999) and P4 (Fidantsef et al., 1999). Transcripts of P4 and LOX begin to increase 4 to 6 h after AA treatment, and the elevated mRNA levels of these two genes remain much longer after induction than *DEA*1, which falls to baseline control levels within 72 h posttreatment.

To identify putative regulatory motifs in the *DEA*1 promoter, we performed IPCR to obtain the sequence of the *DEA*1 5′ promoter region. Analysis of the *DEA*1 promoter region by MatInspector (Genomatix) predicted binding sites for cold-, salt-, pathogen-, and light-regulated transcriptional activators. MatInspector uses a collection of known transcription factor-binding sites to form a sequence matrix for a particular motif. Searching a sequence against the matrix scores allows for identification of motifs that may possess natural sequence variation. All elements identified by promoter analysis have matrix similarities of 80% to 100%. We have shown that biotic and abiotic variables such as pathogen infection and photoperiod regulate *DEA*1. Promoter prediction programs provide hypotheses to test for potential regulatory networks controlling *DEA*1 expression, all of which could be evaluated using this experimental system.

One of the most prominent features of *DEA*1 is its regulation by circadian rhythms during LDs. *DEA*1 is likely to be circadian regulated rather than light regulated because transcription of *DEA*1 decreases in advance of nightfall and rhythms were maintained in both constant light and constant darkness. It is not clear from our experiments whether circadian regulation results from de novo transcript synthesis, transcript turn over, or both. Regulation due to synthesis of *DEA*1 transcript could be due to the presence of several promoter elements found in circadian-regulated photosynthetic genes (Table I). Elements such as CCA1 and GATA may be responsible for the circadian regulation in LD conditions, but the presence of other stress-associated promoter elements may lead to a more complicated regulatory network in SD conditions when other stresses such as cold may be experienced. A soybean (*Glycine max*) gene (*SbPRP*) that is closely related to *DEA*1, was also found to be regulated by circadian rhythms (He et al., 2002). Although *SbPRP* is expressed in different plant organs than *DEA*1 and has a different pattern of induction by signaling molecules, the entire subfamily of proteins comprising *DEA*1 and its homologs may be circadian regulated.

Twenty-eight percent of the circadian-regulated genes in Arabidopsis encode proteins of unknown function, as is the case for *DEA*1 (Harmer et al., 2000). One Arabidopsis gene, *EARLI1*, that is both biochemically uncharacterized and closely related to DEA1, is regulated by circadian rhythms during LDs in a phase similar to *DEA*1 (S. Harmer, personal communication). *EARLI1* was found to be induced by cold and vernalization stimuli, and this induction was synergized by LDs (Wilkosz and Schlappi, 2000). Although the influence of circadian regulation on vernalization induction was not specifically addressed, perhaps the rhythms are responsible for the synergy. *EARLI1* confers partial protection from freezing stress in experiments where the gene was overexpressed in Arabidopsis (Bubier and Schlappi, 2004). We have recently found that *DEA*1 transcript is increased in response to cold and this increase is dependent upon the phase of the circadian rhythm at which the cold stress is applied (P.D. Weyman, Z. Pan, Q. Feng, D.G. Gilchrist, and R.M. Bostock, unpublished data). Overexpression of DEA1 in yeast (*Saccharomyces cerevisiae*) increases the proportion of cells surviving freezing stress. While increasing or knocking down DEA1 expression in tomato did not have an observable phenotype in relation to cold-stress protection, pathogen suscepti-

bility, PCD, or development, the yeast data support a cold-protection function for DEA1.

Although *DEA*1 contains an 8CM domain shared by trypsin inhibitors, α-amylase inhibitors, seed storage proteins, and lipid transfer proteins, assignment of function is still tenuous (Marchler-Bauer et al., 2003; Jose-Estanyol et al., 2004). While a large subset of the 8CM family consists of both putative and biochemically characterized lipid transfer proteins (LTPs), differences in size and in the X residue in the CXC motif may indicate a lack of lipid transfer activity (Douliez et al., 2000). Also missing from DEA1 is a stretch of hydrophilic residues between the CC and CXC motif that is thought to be involved in hydrogen binding with fatty acids (Baud et al., 1993). Other members of the 8CM superfamily share the Cys patterns as well as the hydrophobic residue in the CXC motif but do not form hydrophobic pockets. One such protein, the soybean hydrophobic protein (HPS) is similar in size to DEA1 and has a similar CLC motif, but HPS has a different pattern of Cys bridging that does not allow for the formation of a hydrophobic pocket (Baud et al., 1993). It remains to be determined whether DEA1 binds and transports lipids or is insoluble and of unknown function as HPS.

Localization studies utilizing a DEA1-GFP fusion suggest that DEA1 is associated with the plasma membrane in the protoplast system. These results were extended by following the DEA1-GFP fusion in plasmolysed cells. Using the GCG computer-based prediction programs, we identified a putative secretory signal at the N terminus of DEA1, similar to most other members of this protein family. The presence of a signal sequence is consistent with its processing by the secretory pathway and its localization to the plasma membrane (Singer, 1990). The strong hydrophobic nature of DEA1 may further support a membrane association. Most transmembrane helix prediction programs suggest several transmembrane helices in the DEA1 sequence. The question remains whether DEA1 assumes a globular structure and associates peripherally with the plasmalemma or whether DEA1 is an integral membrane protein. The experimental systems used herein cannot exclude the cell wall as an additional localization site. Also, we cannot exclude the possibility that transient overexpression of DEA1 results in a cellular location different from that of the native protein expressed during normal growth and development or in response to external stresses.

Many proteins in the 8CM superfamily, particularly the LTPs, may play a role in plant response to pathogens because they are transcriptionally induced by pathogen infection, inhibit bacterial and fungal pathogens in vitro, and decrease symptom development in transgenic plants in which they are overexpressed (Molina and Garciaolmedo, 1993; Garcia-Olmedo et al., 1995). Both compatible and incompatible pathogens induce LTPs (Garcia-Olmedo et al., 1995) in situations where cell death is exhibited following inoculation. In addition to pathogens, LTPs and LTP-like genes are induced by environmental stresses such as salt, drought, and cold and the plant hormones that signal these stresses such as ABA (Plant et al., 1991; Torres-Schumann et al., 1992; Ouvrard et al., 1996; Gaudet et al., 2003). Taken together, these observations suggest an adaptive role for members of the 8CM superfamily wherein their presence may mitigate pathogen and environmental stresses on the plant.

The induction of *DEA*1 long before the onset of visible cell death may indicate a role in cell death regulation. Consistent with this notion is the observation that one LTP-like protein, TED4, is known to abrogate PCD by inhibiting a PCD-activating proteosome complex (Endo et al., 2001). The authors propose that TED4 may protect surrounding cells from damage by inhibiting proteolytic activity in differentiating tracheary elements. DEA1 may act similarly to limit damage caused by PCD in response to AA and possibly other PCD-inducing treatments. Further experiments are under way to determine the biochemical activities of DEA1, and define a PCD phenotype, if any, in transgenic plants in which *DEA*1 is overexpressed or diminished by antisense.

MATERIALS AND METHODS

Plants, Pathogens, and Chemicals

Tomato (*Solanum lycopersicum* L. cv New Yorker) plants were grown in Conviron model TC-30 growth chambers at 25°C, 70% relative humidity, with fluorescent and incandescent lights at 150 μE m^{-2} s^{-1}. Plants were used at the three and four leaf stage (approximately 1 month after planting seeds) and were fertilized weekly with Miracle-Gro for tomatoes at the manufacturer's recommended rate. For all time course experiments, an opaque curtain was placed over the entrance of the chamber so that opening the chamber during the dark cycle would not expose the plants to light. A light-emitting diode head lamp with a green filter was used to illuminate the plants for collection during the dark cycle. *Nicotiana benthamiana* plants were grown in the growth chamber under similar conditions as tomato and were used after several large leaves had expanded and flowering had begun. AA was purchased from Sigma-Aldrich and dissolved in ethanol to make a stock solution of 50 mg mL^{-1} that was stored under N_2 gas at −20°C. A working solution was produced by adding the required amount of stock solution to a glass vial, blowing off the ethanol with N_2 gas, adding water to give the desired concentration, and sonicating briefly using a sonicating probe (Bransonic) to ensure emulsion. For DD-PCR, plants were infiltrated with 3 mM AA, while for all time course infiltration experiments plants were treated with 500 μM AA. AA was also brushed on tomato leaves with a saturated cotton-tip applicator at a concentration of 3 mM. The aqueous suspension was used immediately to pressure infiltrate into tomato leaflets using a needleless syringe. JA was made by hydrolysis of methyl jasmonate (a mixture containing 90.6% [w/w] 1R, 2R and 8.1% [w/w] 1R, 2S; Bedoukian Research) according to the procedure of Farmer et al. (1992). JA was dissolved in 1 mL ethanol and then brought up to 200 mL with water. Control (0.5% ethanol) and JA treatments were brushed on leaves using a cotton-tipped applicator. Acibenzolar-*S*-methyl (benzo[1,2,3]thiadiazole-7-carbothioic acid *S*-methyl ester, 50% active ingredient), a gift from Syngenta, was diluted in water to a concentration of 1.2 mM. Control (inert carrier prepared at half the strength of acibenzolar-*S*-methyl) and acibenzolar-*S*-methyl treatments were brushed on leaves using a cotton-tipped applicator. *Phytophthora infestans* (Mont.) de Bary race 1, which yields a compatible interaction on tomato cv New Yorker, was maintained on rye flour agar and grown at 20°C in the dark. Inoculations were performed by collecting sporangia from an agar plate, filtering through cheesecloth, and adjusting sporangia concentration to 1×10^5 sporangia mL^{-1}. Sporangia were then applied as a 10 μL droplet to detached leaflets kept in 10-cm petri plates containing moist filter paper and incubated at room temperature (approximately 25°C).

Recombinant DNA Techniques

Standard DNA techniques were performed as described (Sambrook and Russell, 2001). Plasmid vector pBluescript SK+ (Stratagene) was used for DNA cloning. *Escherichia coli* DH5α was used for all of the DNA transformation experiments. All primers used in this study are presented in Supplemental Table I.

RNA Isolation

Plant tissue was collected at the time points indicated in the text, immersed in liquid nitrogen, and stored at −80°C until needed. Total RNA was extracted according to Verwoerd et al. (1989) with modifications for scale. Briefly, approximately 400 mg of frozen tissue was ground to a fine powder by vortexing in 50-mL tubes with stainless steel ball bearings for 2 to 3 min after liquid nitrogen had evaporated. Stem and root tissue required grinding in liquid nitrogen using a mortar and pestle. The powder then was transferred to 0.5 mL of prewarmed (80°C) extraction buffer (0.1 mM LiCl, 100 mM Tris-Cl pH 8.0, 10 mM EDTA, 1% SDS [w/v]) in a centrifuge tube. An equal volume of Tris-buffered phenol was added, mixed by vortex for 10 s, and incubated at room temperature for 10 min. Chloroform (0.5 mL) was added, and the tubes were vortexed for 30 s. After a 10 min centrifugation at 14,000*g* at room temperature, the aqueous phase was transferred to a clean tube and mixed with one volume of 4 M LiCl. The tubes were incubated on ice for 1 h, and RNA was pelleted by centrifugation at 12,000*g* for 10 min at 4°C. The resulting pellet was resuspended in diethyl pyrocarbonate-treated water and precipitated with ethanol. This pellet was washed with 70% (v/v) ethanol, dried under vacuum, and resuspended in diethyl pyrocarbonate water. RNA was quantified by measuring the A_{260} of 1 μL in a 100-μL cuvette in a spectrophotometer (Becton Dickinson).

DD-PCR

DD-PCR (Liang and Pardee, 1992; Sambrook and Russell, 2001) was performed using total RNA extracted from two independently treated sets of tomato leaves collected 24 h after treatment with AA (3 mM) or water. RNA was extracted as above. Potential chromosomal DNA contamination was removed by DNase I (Promega) treatment. Five micrograms of each total RNA sample was reverse transcribed in a 50-μL reaction volume using 300 units SuperScript II reverse transcriptase (Life Technologies) and 25 pmol dT12VN (TVN, V = A, C, or G; *n* = A, C, T, or G) as primer. PCR amplification was performed using 1 μL cDNA in the presence of TVN and one of each of the three arbitrary 10-mers, DD1 (5′-AAGCCGAAGC-3′), DD2 (5′-CAGGC-TTGTC-3′), and DD3 (5′-TCAGCACGGA-3′). For the control reaction, cDNA was not added. In a total volume of 20 μL, the reaction consisted of 20 pmol TVN primer, 5 pmol 10-mer primer, 50 μM of each of the four dNTPs, and 1 unit of Vent DNA polymerase (New England Biolabs [NEB]). Forty cycles of PCR were performed at 94°C for 30 s, 42°C for 30 s, and 72°C for 40 s. Reaction products were separated on 1.5% (w/v) agarose gels and stained with ethidium bromide. Bands reproducibly induced in the two independent RNA samples were excised, directly cloned after DNA isolation using the QIAEX II kit (Qiagen), sequenced, and used as probes for cDNA library screening.

cDNA Library Construction and Screening

Poly(A+) mRNA was prepared from total RNA extracted from tomato seedlings using Oligo(dT) cellulose (Life Technologies). Complementary DNA was synthesized and cloned into the *Eco*RI-*Xho*I sites of the λZAP vector (Stratagene) and packaged into phages using a Gigapack II, according to the manufacturer's instructions. Approximately 1.5×10^6 pfu were probed with the cloned 440-bp DD-PCR product that was radiolabeled using the Random-Prime kit (Roche). Membranes (Hybond N; Amersham) were hybridized overnight at 65°C in 5× SSC (1× SSC is 0.15 M NaCl and 15 mM sodium citrate, pH 7.2), 5× Denhardt's solution (1× Denhardt's solution is 0.02% [w/v] Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% [w/v] bovine serum albumin), and 0.5% (w/v) SDS, and washed twice with 2× SSC and 0.1% (w/v) SDS at 65°C for 20 min followed by twice with 0.1× SSC and 0.1% (w/v) SDS at 65°C for 20 min. Filters were then exposed to x-ray film (Kodak) with an intensifying screen at −80°C overnight. After secondary screening, positive clones were excised with the helper phage to generate phagemid that contained the cDNA insert. Six clones were sequenced and further analyzed. Sequence analysis revealed that these clones contained identical ORFs with varying amounts of 3′ polyadenylation. One of the clones, designated pDEA1, was arbitrarily chosen for further studies.

Sequence Analysis

Clones were sequenced using the LiCor sequencing system (model 4000 L). Sequences were analyzed using the GCG package (version 10.1). Database searches involved BLAST (Altschul et al., 1997), reverse position-specific iterated BLAST (Marchler-Bauer et al., 2003), and FASTA (Pearson and Lipman, 1988). Sequence alignment was performed using GeneDoc (www.psc.edu/biomed/genedoc).

Southern-Blot Analysis and Genomic Sequence of *DEA*1

Total plant DNA was prepared from tomato leaves by the cetyl-trimethyl-ammonium bromide method (Murray and Thompson, 1980). Ten micrograms of total DNA were digested with restriction enzymes (NEB), separated by electrophoresis on a 1% agarose gel, transferred onto a Hybond N nylon membrane (Amersham Pharmacia Biotech) by capillary blot, and fixed by UV cross-linking according to the manufacturer's instructions. DNA blots were hybridized with a ^{32}P-dCTP-labeled *Xba*I-*Xho*I fragment of pDEA1 that encodes the C-terminal portion of the *DEA*1 protein. Hybridizations were performed as in the cDNA library screening described above.

To amplify the coding region of *DEA*1 from a genomic template, primers 1 and 2 were designed to amplify the 5′ and 3′ ends of the ORF, respectively. The 5′ primer was augmented with a 5′ *Bam*HI site and the 3′ primer contained a 5′ *Eco*RI site. Tomato genomic DNA was extracted using the DNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. PCR was performed in a total volume of 50 μL in a reaction consisting of 50 ng genomic template, 20 pmol each primer, 250 μM of each of the four dNTPs, and buffer, enhancer, and 1 unit of Taq DNA polymerase from Eppendorf. Thirty cycles of PCR were performed at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. The products were purified with the QIAquick PCR purification kit (Qiagen) and sequenced at the University of California, Davis, Division of Biological Sciences Sequencing Center.

IPCR for Promoter Sequencing

Tomato genomic DNA was extracted from leaf tissue with the MasterPure plant leaf DNA kit (Epicentre) according to the kit instructions. A restriction enzyme digestion using 500 ng DNA was performed using *Sau*3AI (NEB) in a total volume of 10 μL according to the company's recommended protocol. After a 6 h digest, the enzyme was heat inactivated by heating at 65°C for 30 min. The restriction fragments were self ligated in 100 μL total volume using 10 Weiss units of ligase (NEB) according to manufacturer's protocol. The reaction was ligated overnight at 14°C, extracted in phenol:chloroform, and ethanol precipitated. The self-ligated fragments were used as template for PCR that was performed as above for genomic copy amplification but with outward primers 3 and 4 and an annealing temperature of 55°C. Products were run on 1% agarose gels, excised and cleaned with the QIAquick PCR purification kit (Qiagen), and PCR was repeated using the nested, inward-oriented primers 5 and 6. The resulting product was purified and sequenced as described above. To obtain more promoter sequence, a second round of IPCR and sequencing was carried out as above using the restriction enzyme *Nsp*I (NEB) and outward primers 7 and 8. The IPCR products were confirmed with PCR using the inward-oriented nested primers 9 and 10, and the IPCR product was sequenced. After the two pieces of promoter sequence had been obtained, the region spanning the promoter region and the adjacent *DEA*1 coding region was amplified by PCR using forward primer 11 located approximately 900 bases upstream of the predicted transcription start site and reverse primer 12 located at the beginning of the *DEA*1 ORF. The PCR yielded band of 950 bp as expected. This PCR product was purified as above and sent for sequencing; the results confirmed the sequence of the promoter obtained through IPCR.

Northern-Blot Analysis

Total RNA (10 μg) was denatured with a glyoxyl buffer and separated on 1% agarose gels according to (Sambrook and Russell, 2001). Equal loading of RNA on lanes was confirmed by visualizing ethidium bromide-stained RNA under UV light. RNA was transferred overnight to Hybond N+ membranes

(Amersham) in 10× SSC and baked in a vacuum oven at 80°C for 2 h (Sambrook and Russell, 2001).

Alkaline phosphatase-labeled probes were prepared from in vitro transcription products of the *DEA*1 gene. The full-length *DEA*1 cDNA was excised from pDEA1 with *Eco*RI and *Xho*I and cloned into similarly cut pBluescriptII-KS. An antisense RNA copy of the *DEA*1 cDNA was generated from the T3 promoter site using the MegaScript T3 kit (Ambion). The plasmid was cut with *Eco*RI before transcription so that the transcripts would only be made from the *DEA*1 cDNA. The RNA was purified and stored at −80°C. Probes were constructed using 100 ng RNA per probe according to the Alkphos kit instructions (Amersham Pharmacia Biotech). *LHCb1* probes were prepared from a gel-purified PCR product created using the cloned *LHCb1* gene as a template. The *LHCb1* ORF was amplified from a tomato cDNA library (see above) using primers 19 and 20. An approximately 550-bp band was obtained and cloned into pGEM-T Easy vector (Promega). The resulting clone was sequenced and confirmed to be identical to the *LHCb1* (GenBank accession no. M14443). This clone was used as a template for PCR using primers 19 and 20, and the resulting product was run on a 1% agarose gel, excised, cleaned (ZymoClean, Zymo Research Corp), and labeled as above for *DEA*1.

Baked blots were rewetted with 10 × SSC and prehybridized in Alkphos hybridization buffer (containing NaCl and Blocking reagent) at 55°C for 1 h. Probe was then added and hybridized overnight. Washing was performed according to Alkphos procedure. Primary washes were performed at 65°C for 10 min each and three sary washes at room temperature were performed for 5 min each. The blots then were developed with CDP-Star chemiluminescent substrate (Sigma-Aldrich) and exposed to film. Densitometry of bands on exposed film was performed using Lab Works analysis software (UVP) and was expressed as the sum of the band pixel values less the background.

*DEA*1-GFP Fusion Vector Construction

The GFP expression vector, pBINm-GFP5-ER, originally constructed for targeting GFP to the endoplasmic reticulum, was obtained from Haseloff and Siemering (1998).

To create a vector expressing a fusion of GFP to the C terminus of DEA1, the *DEA*1 cDNA coding region was amplified from template pDEA1 by PCR using primer 13 that bears a *Bam*HI site on the 5′ end and primer 14 that contains an *Eco*RI site at the 5′ end following the last codon of *DEA*1. PCR was performed using Pfu DNA polymerase (Stratagene) and the resulting product was cloned and sequenced to ensure that no errors were introduced into the sequence. The complete *DEA*1 coding sequence was excised using *Eco*RI and *Bam*HI and ligated into similarly cleaved pBINm-GFP5-ER. The ligated product was in frame with the GFP sequence, and the resulting plasmid was designated pDEA1-GFP.

As a positive control for a membrane-bound protein, the LeCPK1 coding region (Rutschmann et al., 2002) was amplified from a tomato leaf cDNA library using Pfu DNA polymerase and primer 15 that contains an *Xho*I site on the 5′ end, and primer 16 that contains a *Bam*HI site on the 5′ end following the last codon of the LeCPK1 sequence. Due to an internal *Eco*RI site in LeCPK1, the GFP sequence had to be reamplified from pBINm-GFP5-ER using primer 17, which contains a 5′ *Bam*HI site, and primer 18, which contains a 5′ *Xba*I site following the last codon of the GFP sequence. The LeCPK1 and GFP PCR products were cloned separately into pGEM-T Easy vectors (Promega) after addition of adenines to the 3′ ends of the PCR fragment by Taq polymerase. The plasmids were transformed into *E. coli*, and sequenced to ensure no errors were introduced. The LeCPK1 and GFP sequences were cut out of the vectors using *Bam*HI and *Xho*I for LeCPK1 and *Bam*HI and *Xba*I for GFP. The cut fragments were ligated together and into the *Xho*I and *Xba*I sites of the plant transformation vector pCB404 (denoted pCPK1-GFP).

Transient Expression and Confocal Microscopy

The binary plasmid vectors pBINm-GFP5-ER, pDEA1-GFP, and pCPK1-GFP were mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) by electroporation using a Gene Pulser (Bio-Rad), followed by selection on kanamycin- and streptomycin-containing plates. Thirty-hour cultures were grown in Luria-Bertani broth at 28°C with shaking and with 100 mg L^{-1} kanamycin and 50 mg L^{-1} streptomycin. The cells were collected by centrifugation and resuspended in distilled, deionized water (OD600 equals approximately 0.8). Slow steady pressure was applied to infiltrate 0.5 to 0.8 mL of *A. tumefaciens* culture into an interveinal region of the abaxial surface of fully expanded *N. benthamiana* leaves using a 1 mL needleless syringe. Four days after infiltration, leaf discs were sliced from the infiltrated areas, mounted on slides, and imaged with a laser scanning MRC1024 confocal microscope (Bio-Rad) using the 488 nm laser line for excitation, the green emission filter 522 DF 32, and for chlorophyll autofluoresence, emission filter 680 DF 32.

Protoplasts were made by cutting out infiltrated areas on *N. benthamiana* leaves 4 d after infiltration and finely dicing tissue into small pieces in a pH 5.8 solution containing 8.3% (w/v) mannitol, 0.6% (w/v) cellulose, and 0.25% (w/v) macerozyme (Yakult Pharmaceutical), 200 μM sodium phosphate, 1 mM potassium nitrate, 10 mM calcium chloride, 1 mM magnesium sulfate, and 0.1 μM copper sulfate. Samples were incubated overnight in the dark at room temperature. The suspension was then filtered through cheesecloth and floated on top of a 0.65 M Suc solution in a centrifuge tube, centrifuged at 100*g*, and allowed to decelerate gently without breaking. The protoplasts were collected at the interface of the Suc and the protoplasting solution, mounted on glass slides, and viewed with the laser scanning confocal microscope in a similar manner as for the whole tissue mounts.

Plasmolysis of Epidermal Peels

N. benthamiana plants were infiltrated with pBINm-GFP5-ER and pDEA1-GFP as described above, and after 3 d, thin epidermal strips were peeled carefully from infiltrated areas of the abaxial leaf surface. The strips were mounted on glass slides in sterile distilled water, and the same fields were examined under both light and epifluorescent microscopy and photographed using a Nikon Microphot-SA fluorescence microscope (Nikon). Plasmolysis was performed by wicking a series of Suc solutions (0.25–1.0 M) through the mounted tissue (Mellersh and Heath, 2001).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AY509122.

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LITERATURE CITED

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:** 3389–3402

Baud F, Pebay-Peyroula E, Cohen-Addad C, Odani S, Lehmann MS (1993) Crystal structure of hydrophobic protein from soybean: a member of a new cysteine-rich family. J Mol Biol **231:** 877–887

Birch PRJ, Avrova AO, Duncan JM, Lyon GD, Toth RL (1999) Isolation of potato genes that are induced during an early stage of the hypersensitive response to *Phytophthora infestans*. Mol Plant Microbe Interact **12:** 356–361

Blanco-Portales R, Lopez-Raez JA, Bellido ML, Moyano E, Dorado G, Gonzalez-Reyes JA, Caballero JL, Munoz-Blanco J (2004) A strawberry fruit-specific and ripening-related gene codes for a HyPRP protein involved in polyphenol anchoring. Plant Mol Biol **55:** 763–780

Bos JIB, Armstrong M, Whisson SC, Torto TA, Ochwo M, Birch PRJ, Kamoun S (2003) Intraspecific comparative genomics to identify avirulence genes from *Phytophthora*. New Phytol **159:** 63–72

Bostock R (2005) Signal crosstalk and induced resistance: straddling the line between cost and benefit. Annu Rev Phytopathol **43:** 545–580

Bostock RM, Kuc J, Laine RA (1981) Eicosapentaenoic and arachidonic acids from *Phytophthora infestans* elicit fungitoxic sesquiterpenes in the potato. Science **212:** 67–69

Bostock RM, Schaeffer DA, Hammerschmidt R (1986) Comparison of elicitor activities of arachidonic acid, fatty acids and glucans from *Phytopthora infestans* in hypersensitivity expression in potato tuber. Physiol Mol Plant Pathol **29:** 349–360

Bubier J, Schlappi M (2004) Cold induction of EARLI1, a putative

Arabidopsis lipid transfer protein, is light and calcium dependent. Plant Cell Environ **27:** 929–936

Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics **21:** 2933–2942

Coquoz JL, Buchala AJ, Meuwly P, Metraux JP (1995) Arachidonic acid induces local but not systemic synthesis of salicylic acid and confers systemic resistance in potato plants to *Phytophthora infestans* and *Alternaria solani*. Phytopathology **85:** 1219–1224

Curtis MJ, Wolpert TJ (2002) The oat mitochondrial permeability transition and its implication in victorin binding and induced cell death. Plant J **29:** 295–312

Diatchenko L, Lau Y-FC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA **93:** 6025–6030

Dickman MB, Park YK, Oltersdorf T, Li W, Clemente T, French R (2001) Abrogation of disease development in plants expressing animal antiapoptotic genes. Proc Natl Acad Sci USA **98:** 6957–6962

Douliez J-P, Michon T, Elmorjani K, Marion D (2000) Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. J Cereal Sci **32:** 1–20

Endo S, Demura T, Fukuda H (2001) Inhibition of proteasome activity by the TED4 protein in extracellular space: a novel mechanism for protection of living cells from injury caused by dying cells. Plant Cell Physiol **42:** 9–19

Eriksson ME, Millar AJ (2003) The circadian clock: a plant's best friend in a spinning world. Plant Physiol **132:** 732–738

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci **5:** 199–206

Farmer EE, Johnson RR, Ryan CA (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. Plant Physiol **98:** 995–1002

Fei Z, Tang X, Alba RM, White JA, Ronning CM, Martin GB, Tanksley SD, Giovannoni JJ (2004) Comprehensive EST analysis of tomato and comparative genomics of fruit ripening. Plant J **40:** 47–59

Fidantsef AL, Stout MJ, Thaler JS, Duffey SS, Bostock RM (1999) Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, *Lycopersicon esculentum*. Physiol Mol Plant Pathol **54:** 97–114

Fowler SG, Cook D, Thomashow MF (2005) Low temperature induction of Arabidopsis CBF1, 2, and 3 is gated by the circadian clock. Plant Physiol **137:** 961–968

Fridman JS, Lowe SW (2003) Control of apoptosis by p53. Oncogene **22:** 9030–9040

Garcia-Olmedo F, Molina A, Segura A, Moreno M (1995) The defensive role of nonspecific lipid-transfer proteins in plants. Trends Microbiol **3:** 72–74

Gaudet DA, Laroche A, Frick M, Huel R, Puchalski B (2003) Cold induced expression of plant defensin and lipid transfer protein transcripts in winter wheat. Physiol Plant **117:** 195–205

Gilchrist DG (1998) Programmed cell death in plant disease: the purpose and promise of cellular suicide. Annu Rev Phytopathol **36:** 393–414

Gilmartin PM, Sarokin L, Memelink J, Chua N-H (1990) Molecular light switches for plant genes. Plant Cell **2:** 369–378

Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA, Cashmore AR (1988) An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc Natl Acad Sci USA **85:** 7089–7093

Glazebrook J, Chen W, Estes B, Chang H-S, Nawrath C, Metraux J-P, Zhu T, Katagiri F (2003) Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J **34:** 217–228

Greenberg JT, Yao N (2004) The role and regulation of programmed cell death in plant-pathogen interactions. Cell Microbiol **6:** 201–211

Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science **290:** 2110–2113

Haseloff J, Siemering KR (1998) The uses of green fluorescent protein in plants. *In* M Chalfie, S Kain, eds, Green Fluorescent Protein: Properties, Applications, and Protocols. John Wiley & Sons, Chichester, UK, 191–220

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localization of green fluorescent protein are required to mark transgenic Arabidopsis plants brightly. Proc Natl Acad Sci USA **94:** 2122–2127

Hattori T, Terada T, Hamasuna S (1995) Regulation of the Osem gene by abscisic acid and the transcriptional activator VP1: Analysis of cis-acting promoter elements required from regulation by abscisic acid and VP1. Plant J **7:** 913–925

He CY, Zhang JS, Chen SY (2002) A soybean gene encoding a proline-rich protein is regulated by salicylic acid, an endogenous circadian rhythm and by various stresses. Theor Appl Genet **104:** 1125–1131

Hoekema A, Hirsch PR, Hooykass PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of Vir and T region of the Agrobacterium-Tumefaciens Ti plasmid. Nature **303:** 179–180

Jose-Estanyol M, Gomis-Rueth FX, Puigdomenech P (2004) The eight-cysteine motif, a versatile structure in plant proteins. Plant Physiol Biochem **42:** 355–365

Knight VI, Wang H, Lincoln JE, Lulai EC, Gilchrist DG, Bostock RM (2001) Hydroperoxides of fatty acids induce programmed cell death in tomato protoplasts. Physiol Mol Plant Pathol **59:** 277–286

Kreps JA, Simon AE (1997) Environmental and genetic effects on circadian clock-regulated gene expression in Arabidopsis. Plant Cell **9:** 297–304

Lawen A (2003) Apoptosis: an introduction. Bioessays **25:** 888–896

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **257:** 967–971

Lincoln JE, Richael C, Overduin B, Smith K, Bostock R, Gilchrist DG (2002) Expression of the antiapoptotic baculovirus p35 gene in tomato blocks programmed cell death and provides broad-spectrum resistance to disease. Proc Natl Acad Sci USA **99:** 15217–15221

Luna CM, Pastori GM, Driscoll S, Groten K, Bernard S, Foyer CH (2005) Drought controls on H_2O_2 accumulation, catalase (CAT) activity and CAT gene expression in wheat. J Exp Bot **56:** 417–423

Marchler-Bauer A, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S, Hurwitz DI, Jackson JD, Jacobs AR, Lanczycki CJ, et al (2003) CDD: a curated Entrez database of conserved domain alignments. Nucleic Acids Res **31:** 383–387

Mellersh DG, Heath MC (2001) Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration. Plant Cell **13:** 413–424

Meyer H, Thienel U, Piechulla B (1989) Molecular characterization of the diurnal-circadian expression of the chlorophyll A-B-binding proteins in leaves of tomato and other dicotyledonous and monocotyledonous plant species. Planta **180:** 5–15

Molina A, Garcia-Olmedo F (1993) Developmental and pathogen-induced expression of 3 barley genes encoding lipid transfer proteins. Plant J **4:** 983–991

Molina A, Garcia-Olmedo F (1997) Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. Plant J **12:** 669–675

Moy P, Qutob D, Chapman BP, Atkinson I, Gijzen M (2004) Patterns of gene expression upon infection of soybean plants by *Phytophthora sojae*. Mol Plant Microbe Interact **17:** 1051–1062

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res **8:** 4321–4325

Niu X, Helentjaris T, Bate NJ (2002) Maize ABI4 binds coupling element1 in abscisic acid and sugar response genes. Plant Cell **14:** 2565–2575

Nuernberger T, Brunner F (2002) Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. Curr Opin Plant Biol **5:** 318–324

Ouvrard O, Cellier F, Ferrare K, Tousch D, Lamaze T, Dupuis J-M, Casse-Delbart F (1996) Identification and expression of water stress- and abscisic acid-regulated genes in a drought-tolerant sunflower genotype. Plant Mol Biol **31:** 819–829

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA **85:** 2444–2448

Plant AL, Cohen A, Moses MS, Bray EA (1991) Nucleotide sequence and spatial expression pattern of a drought- and abscisic acid-induced gene of tomato. Plant Physiol **97:** 900–906

Richael C, Gilchrist D (1999) The hypersensitive response: a case of hold or fold? Physiol Mol Plant Pathol **55:** 5–12

Richael C, Lincoln JE, Bostock RM, Gilchrist DG (2001) Caspase inhibitors reduce symptom development and limit bacterial proliferation in susceptible plant tissues. Physiol Mol Plant Pathol **59:** 213–221

Ricker KE, Bostock RM (1992) Evidence for release of the elicitor arachidonic acid and its metabolites from sporangia of *Phytophthora infestans* during infection of potato. Physiol Mol Plant Pathol **41:** 61–72

Rutschmann F, Stalder U, Piotrowski M, Oecking C, Schaller A (2002) LeCPK1, a calcium-dependent protein kinase from tomato: plasma membrane targeting and biochemical characterization. Plant Physiol **129:** 156–168

Sambrook J, Russell DW (2001) Molecular Cloning: A Laboratory Manual, Ed 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sauerbrunn N, Schlaich NL (2004) PCC1: a merging point for pathogen defence and circadian signalling in Arabidopsis. Planta **218:** 552–561

Singer SJ (1990) The structure and insertion of integral proteins in membranes. Annu Rev Cell Biol **6:** 247–296

Torres-Schumann S, Godoy JA, Pintor-Toro JA (1992) A probable lipid transfer protein gene is induced by sodium chloride in stems of tomato plants. Plant Mol Biol **18:** 749–757

Tyler BM (2001) Genetics and genomics of the oomycete-host interface. Trends Genet **17:** 611–614

Verwoerd TC, Dekker BM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNA. Nucleic Acids Res **17:** 2362

Walter P, Gilmore R, Blobel G (1984) Protein translocation across the endoplasmic reticulum. Cell **38:** 5–8

Wang H, Li J, Bostock RM, Gilchrist DG (1996) Apoptosis: a functional paradigm for programmed plant cell death induced by a host-selective phytotoxin and invoked during development. Plant Cell **8:** 375–391

Wang Z-Y, Kenigsbuch D, Sun L, Harel E, Ong MS, Tobin EM (1997) A Myb-related transcription factor is involved in the phytochrome regulation of an Arabidopsis Lhcb gene. Plant Cell **9:** 491–507

Wilkosz R, Schlappi M (2000) A gene expression screen identifies EARLI1 as a novel vernalization-responsive gene in *Arabidopsis thaliana.* Plant Mol Biol **44:** 777–787

Winicov I, Bastola DR (1999) Transgenic overexpression of the transcription factor Alfin1 enhances expression of the endogenous MsPRP2 gene in alfalfa and improves salinity tolerance of the plants. Plant Physiol **120:** 473–480

Xiao F, Tang X, Zhou J-M (2001) Expression of 35S:Pto globally activates defense-related genes in tomato plants. Plant Physiol **126:** 1637–1645

Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters. Trends Plant Sci **10:** 88–94