Resistance of *Malus domestica* Fruit to *Botrytis cinerea* Depends on Endogenous Ethylene Biosynthesis

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ABSTRACT

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The plant hormone ethylene regulates fruit ripening, other developmental processes, and a subset of defense responses. Here, we show that 1-aminocyclopropane-1-carboxylic acid synthase (ACS)-silenced apple (*Malus domestica*) fruit that express a sense construct of ACS were more susceptible to *Botrytis cinerea* than untransformed apple, demonstrating that ethylene strengthens fruit resistance to *B. cinerea* infection. Because ethylene response factors (ERFs) are known to contribute to resistance

against *B. cinerea* via the ethylene-signaling pathway, we cloned four *ERF* cDNAs from fruit of *M. domestica*: *MdERF3*, -4, -5, and -6. Expression of all four *MdERF* mRNAs was ethylene dependent and induced by wounding or by *B. cinerea* infection. *B. cinerea* infection suppressed rapid induction of wound-related *MdERF* expression. *MdERF3* was the only mRNA induced by wounding and *B. cinerea* infection in ACS-suppressed apple fruit, although its induction was reduced compared with wild-type apple. Promoter regions of all four *MdERF* genes were cloned and putative *cis*-elements were identified in each promoter. Transient expression of *MdERF3* in tobacco increased expression of the GCC-box containing gene *chitinase 48*.

The plant hormone ethylene is involved in many plant developmental processes such as fruit ripening, seed germination, senescence, abscission, and epinasty. Ethylene is also induced by biotic and abiotic stimuli, including wounding (74). Ethylene response factors (ERFs) are transcription factors (TFs) that affect various environmental responses and developmental processes. They act downstream of plant hormones to regulate ethylene, jasmonate, abscisic acid, and the cytokinin-signaling pathways. ERFs mediate plant development (52,70) and control environmental stress responses to cold, dehydration, and salinity (8,23,46,54,57,59,64,66,75–78).

AP2/ERF proteins are members of the second-largest superfamily of TFs in *Arabidopsis thaliana* (56). This superfamily was believed to be plant specific but the AP2/ERF domain is also present in the cyanobacterium *Trichodesmium erythraeum*, the ciliate *Tetrahymena thermophila*, and the viruses *Enterobacteria phage Rb49* and *Bacteriophage Felix 01* (40). The 122 *Arabidopsis* and 139 rice *ERF* genes have been classified into 12 and 15 subfamilies, respectively, based on structural similarities of AP2/ERF domain sequences and conserved motifs outside the DNA-binding domain (46). *ERF* genes encode one AP2/ERF domain composed of three β -sheets and one α -helix; the β -sheets interact with the GCC-box motif in the target DNA (3). Although the functions of a few *A. thaliana* ERFs, here referred to as

AtERFs, have been analyzed, information about the function of most ERF subfamilies is limited.

Ethylene activates different sets of target genes depending on the specific developmental, abiotic, or biotic signal. The post-translationally regulated TF AtEIN3 indirectly induces defense-related genes via transcriptional activation of AtERF1. In turn, AtERF1 interacts with the GCC-box motif in promoter regions of ethylene-regulated defense genes in *Arabidopsis* (63). On the other hand, AtEIN3 directly binds to and activates ethylene-induced ripening genes (63).

Botrytis cinerea, the causal agent of gray mold and other diseases, has more than 200 host plants, including apple. Ethylene contributes to foliar resistance of *A. thaliana* to *B. cinerea*. AtEIN2, a membrane-bound central regulator of ethylene signaling, acts upstream of AtEIN3 and is required for resistance of *A. thaliana* to *B. cinerea* (4,69). Constitutive overexpression of AtERF1 in *A. thaliana* activates expression of pathogenesis-related proteins and enhances resistance to necrotrophic pathogens, including *B. cinerea* (7).

The objective of this study was to evaluate the potential contribution of ethylene to resistance of apple fruit to *B. cinerea*. Post-harvest ethylene production can be manipulated in climacteric fruit for long-term storage, making it important to understand the effects of ethylene on fruit defenses. We used a transgenic apple line co-suppressed for expression of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) (16) to study the influence of ethylene on pathogen defense. To determine the regulatory mechanism of ethylene-induced partial resistance in apple fruit, we cloned *B. cinerea*-inducible *ERF* cDNAs and tested the expression of a GCC-box containing chitinase as a function of transient *MdERF3* expression in *Nicotiana benthamiana*.

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MATERIALS AND METHODS

Biological materials and treatments. Apple (*Malus domestica*) 'Jonagold' fruits and leaves were obtained from Davis Farm, a commercial orchard in Corvallis, OR, for the sole purpose of cloning *ERF* coding and promoter sequences. To discover and clone pathogen-responsive *MdERFs*, three replicate fruit were subjected to one of three treatments: (i) propylene treatment, in which apple fruits in 19.8-liter jars were exposed to the humidified ethylene analogue propylene at 500 ppm, supplied at a flow rate of 45 to 60 ml/min for 1 or 3 h (2); (ii) *B. cinerea* treatment, consisting of 12 wounds inoculated with 1,000 *B. cinerea* conidia per site and incubated in a moisture chamber for 6 h, 1 day, or 3 days; or (iii) control, consisting of no treatment. Pooled fruit samples from each treatment were frozen and stored at -80°C .

The transgenic 'Greensleeves' (GS) apple line 130Y, co-suppressed for expression of *ACS* (16), was chosen because fruit ethylene concentrations in this line were very low at the stage when untransformed fruit start to lose chlorophyll. Nontransformed GS and transgenic 130Y fruit were harvested at the preclimacteric stage from an experimental orchard in Davis, CA, in 2005, 2006, and 2007. One biological replicate of pooled samples, containing three fruit each, was collected for gene expression studies by semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) every 3 days after harvest in 2006 and by quantitative RT-PCR at 3, 6, 24, and 72 h after *B. cinerea* inoculation after 2 weeks of storage in 2007. Tobacco (*N. benthamiana*) plants were grown in a greenhouse.

Fungal inoculation. *B. cinerea* strain B05.10 was cultured on potato dextrose agar (2). Fruit were punctured along the equator with the tip of a syringe and inoculated with a conidial suspension of *B. cinerea* strain B05.10 (1,000 conidia/inoculation) in water or mock inoculated with water as described (2). Diameters of visible lesions were measured daily using a caliper.

Ethylene measurement. The ethylene concentration of individual fruit was measured daily for 1 week using a gas chromatograph equipped with a flame-ionization detector (Hach-Carle series 400 AGC, Loveland, CO) as described (2).

Cloning of *MdERFs* and chitinase cDNA and promoter sequences. Total RNA was extracted from three pooled apple fruit at each indicated time as described (6). Total RNA from each time point was combined: 1.5 μg from each of two propylene treatments and 1.0 μg from each of three *B. cinerea* treatments for a total of 3 μg per treatment type. A first-strand cDNA synthesis kit (GE Healthcare, Piscataway, NJ) was used.

For initial cloning of *MdERFs*, degenerate primers (Supplementary Table S1) were designed to amplify the AP2/ERF domain based on sequences of *A. thaliana*, *Lycopersicon esculentum*, *M. domestica*, *Oryza sativa*, *Vitis amurensis*, and *Cucumis sativus* from GenBank using the CODEHOP algorithm (bioinformatics.weizmann.ac.il/blocks/codehop.html). All *MdERF* PCR products had the same size of 142 bp after amplification of cDNA using touchdown PCR. Amplification conditions were 1 cycle at 94°C for 3 min; 7 cycles at 94°C for 30 s, 63 to 57°C for 1 min (1°C decrease per cycle), and 72°C for 1 min; 28 cycles at 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min; followed by a final extension at 72°C for 7 min.

Amplification products were fractionated by electrophoresis in 2% NuSieve GTG agarose gels (BMA, Rockland, ME). The most intense band was excised, placed in a dialysis bag, and electroeluted from the gel using a horizontal electrophoresis chamber. Eluted DNA was extracted with phenol/chloroform and precipitated using ethanol and sodium acetate (58).

Purified PCR products were ligated using a pGEM-T Easy Vector (Promega Corp., Madison, WI) and transformed into competent *Escherichia coli* strain JM109. Recombinant plasmid DNA containing PCR products was purified using a Qiaprep spin

miniprep kit (Qiagen, Valencia, CA) and inserts were sequenced. In total, 16, 42, and 56 clones were sequenced for control, propylene-treated, and *B. cinerea*-infected samples, respectively. DNA was sequenced using the T7 forward and SP6 reverse primer on an Applied Biosystems (Carlsbad, CA) Big Dye Terminator chemistry at Gene Gateway (Hayward, CA) or at the Center for Gene Research and Biotechnology, Oregon State University (Corvallis).

Expressed sequence clones of AP2/ERF domains that appeared more than twice following *B. cinerea* infection or propylene treatment were used to design primers for rapid amplification of cDNA ends (RACE). Both 3'- and 5'-end cDNA clones were obtained by RACE using the FirstChoice RLM-RACE kit (Ambion, Austin, TX) with gene-specific outer and inner primers designed from each expressed sequence clone (Supplementary Table S1). Nested PCR was performed with gene-specific outer and inner primers. The 5'-end of chitinase was obtained using gene-specific outer and inner primers based on a partial chitinase sequence (accession number AF494397). Cloning and sequencing was as described above. For confirmation, full-length cDNAs were amplified by PCR and sequenced. Open reading frame (ORF) was determined using the ORF finder program of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/projects/gorf/>).

Phylogenetic analyses. ERF cDNA sequences were entered into the program BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html) for conversion into deduced amino acid sequences. Amino acid sequences from apple and *Arabidopsis* were aligned using Clustal W. This alignment was converted back to nucleotide sequences using the program BioEdit. The aligned nucleotide sequences were used to generate a parsimony tree using PHYLIP (22). Specifically, SEQBOOT was used to generate 100 bootstrapped data sets. DNAPARS was used to generate trees from the 100 data sets. Finally, CONSENSE was used to generate the majority-rule consensus tree containing bootstrap values. The numbering system for *AtERF* genes is based on a recent classification (46).

Semiquantitative RT-PCR and quantitative RT-PCR. Total RNA (5 μg) was extracted from one biological tissue sample from three pooled apple fruit from *ACS*-silenced line 130Y (16) or from untransformed GS as described (6). Potential genomic DNA contaminants were removed by digesting total RNA with FPLC-pure RNase-free DNase (Amersham Bioscience, Piscataway, NJ) for 10 min at 37°C . The enzyme was inactivated at 65°C for 10 min. Apple cDNAs were synthesized with oligo-dT using a first-strand cDNA synthesis kit (GE Healthcare). Semiquantitative RT-PCR was done to check the expression of disease-related genes such as lipoxygenase (*LOX*) and polygalacturonase inhibitor protein (*PGIP*) and *ACS1* expression in transgenic and nontransgenic fruit but quantitative RT-PCR was used to study *MdERF* expression in transgenic and nontransgenic apple. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (CN494000) was used as an internal control (17) for equal loading and for constitutive expression in both semiquantitative and quantitative RT-PCR.

Quantitative RT-PCR primers and TaqMan probes were designed using Primer Express software (Applied Biosystems, Foster City, CA). Nonconserved regions of AP2/ERF domains in both *Arabidopsis* and apple were used to distinguish each *ERF* gene (46); sequence comparisons to *AtERF* genes increased specificity for individual *MdERF* genes (i.e., unique sequences present in apple but not in *Arabidopsis* were used). The reporter dyes VIC and FAM were used for *GAPDH* and *MdERF*, respectively, at the 5' end. The nonfluorescent quencher MGB was used at the 3' end. Quantitative RT-PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Standard thermal conditions were used: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for

60 s. Fluorescent signals were collected during the annealing and extension cycle. Cycle threshold (Ct) values were determined using autothreshold. Relative expression (Δ Ct) of each cDNA sample was obtained by normalizing to the *GAPDH* transcript frequency. Relative mRNA expression was derived using $2^{-\Delta\Delta\text{Ct}}$, where $\Delta\Delta\text{Ct}$ represents the ΔCt of a gene of interest minus the ΔCt at 0 h for each genotype (34). All values at 0 h were set to 1. All samples were loaded in one 96-well plate for simultaneous comparison of all four *MdERF* genes and for testing amplification efficiencies of target and reference genes.

Isolation of promoter regions. Genomic DNA was extracted from Jonagold apple leaves (31). The 5'-upstream region of each *MdERF* gene was obtained using the Universal Genome Walker Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions with gene-specific primers. PCR-amplified DNA products were cloned and sequenced as described above. To verify each promoter sequence, a promoter-specific forward primer was designed for the predicted promoter region in combination with a reverse primer located in the ORF of the respective gene.

Promoter sequences were analyzed using the Signal Scan and Homology Search options of the database PLACE (<http://www.dna.affrc.go.jp/PLACE/>). TATA boxes were found using the neural network promoter prediction program from the Berkeley *Drosophila* genome project (http://www.fruitfly.org/seq_tools/promoter.html).

Transient expression of *MdERF3* in *N. benthamiana*. Binary plasmid with (pDU08.0313) or without (pDU99.2215) *MdERF3* (21) was transferred to competent *Agrobacterium tumefaciens* strain EHA101 by heat shock. This vector contains an ampicillin resistance selectable marker gene and the entire coding region of *MdERF3* between the CaMV35S promoter and the octopine synthase terminator cassette. *Agrobacterium*-mediated transient assays in *N. benthamiana* leaves were as described (35), with the following modifications. The concentration of the *Agrobacterium* suspension was adjusted to an optical density at 600 nm of 0.5 before infiltration. The *Agrobacterium* suspension was injected into the leaves using a 3-ml disposal syringe without a needle. Same-size circles (radius = 1 cm) were drawn on leaves using a marker pen, and *Agrobacterium* suspension was injected until each circle were filled. Leaf color changed in the injected area, which allowed us to distinguish between injected and uninjected areas. The same leaf was inoculated at different sites with *MdERF3*-containing or empty vector. The experiment consisted of three biological replicates. Leaves were collected 1 day after infiltration. Intact leaves without infiltration were used as a control to determine effects of *Agrobacterium* infiltration on gene expression.

Total RNA (3 μ g) was extracted from tobacco (*N. benthamiana*) leaves using a phenol-based method (42). RNA was converted to cDNA using random hexamers for quantitative PCR analysis. Expression of *MdERF3*, *Chn48*, and 18S rRNA in *N. benthamiana* was analyzed using absolute quantification as recommended by Applied Biosystems (Foster City, CA). Mean values of three technical replicates of quantitative RT-PCR reactions of each sample were normalized to 18S rRNA as an internal control. Standard curves for rRNA were generated using salmon sperm DNA with primers and probe for eukaryotic 18S rRNA as the endogenous control (Applied Biosystems, Foster City, CA).

Statistical analyses. Data are presented as means $\pm$ standard error. Area under disease progress curve (28) was used to determine the statistical significance of differences in lesion diameter after *B. cinerea* inoculation. The Mann-Whitney U test was used to determine significant differences in lesion diameter after *B. cinerea* inoculation and in *MdERF* expression (SPSS, Chicago). Transient expression in *N. benthamiana* was analyzed using the Relative Expression Software Tool, considering three biological by three technical replicates (51). The significance threshold was $P = 0.05$.

RESULTS

Effects of ACS silencing on ethylene production and on susceptibility of apple fruit to *B. cinerea*. Suppression of ACS in transgenic apple of line 130Y (16) inhibited endogenous ethylene biosynthesis in intact fruit (Fig. 1) and increased their susceptibility to *B. cinerea* relative to untransformed GS apple (Fig. 2). ACS-suppressed apple fruit lack the climacteric rise in ethylene biosynthesis (16). The magnitude of the genotype-dependent difference in ethylene biosynthesis was smaller immediately after harvest (Fig. 2A) than after incubation at room temperature for 2 weeks, during the climacteric peak (Fig. 2C). Whereas, right after harvest, ethylene production was <7-fold higher in untransformed than in ACS-suppressed apple fruit 7 days post inoculation, this difference in ethylene production was $\approx$ 60-fold higher at the climacteric peak.

Ripening-related differences in ethylene biosynthesis and susceptibility to *B. cinerea* were correlated. The relative increase in lesion expansion between freshly harvested transgenic and untransformed apple fruit was not significant (Fig. 2B). However, the difference in lesion expansion between ACS-silenced and untransformed apple fruit became statistically significant when fruit were incubated at room temperature for 14 days prior to inoculation with *B. cinerea* (Fig. 2D). This trend was observed during three consecutive years (data not shown).

Cloning of fruit-expressed *MdERF* cDNAs. To investigate the role of *ERF* genes in resistance of apple fruit to *B. cinerea*, *MdERF* genes were cloned from Jonagold apple fruit using RT-PCR with degenerate primers (Supplementary Figure S1A) designed to amplify AP2/ERF domains. The corresponding expressed sequence clones from initial PCR with degenerate primers were too short to be classified by similarity to previously defined *ERF* subfamilies (46). Based on similarities of amino acid sequences, expressed sequence clones were classified into four defined groups which were different from previously defined subfamilies (46). Groups 1, 2, and 3 were identified in samples from fruit infected with *B. cinerea* or treated with the ethylene analog propylene. Except for group 1, expressed sequence clones were also identified in control fruit. Although the number of clones was limited, the two expressed sequence clones of group 4 were distinct because their amino acid sequences were not conserved around the third β -sheet.

Three full-length and one partial *MdERF* cDNAs were cloned from fruit tissue by RACE using primers designed from partial cDNA sequences (Fig. 3A; Supplementary Figure S1B). Each *MdERF* cDNA encodes a single AP2/ERF domain containing a predicted DNA-binding site. Similar to *AtERF* genes (46), each *MdERF* gene has conserved motifs of unknown function outside the AP2/ERF domain.

Fig. 1. Ethylene production in untransformed intact apple 'Greensleeves' (GS) and in 1-aminocyclopropane-1-carboxylic acid synthase (ACS)-suppressed fruit (130Y) after harvest. Dates indicate days after the harvest date, which was 11 August 2006.

Phylogenetic comparison of the four *MdERF* coding sequences with all known *Arabidopsis* genes revealed clustering of *MdERF4* and *MdERF5* with *AtERF* genes from subfamilies 1 and 2a (46), respectively, while *MdERF3* and *MdERF6* were members of subfamily 10 (Fig. 3B). *AtERF109*, annotated as a member of subfamily 10 (46), did not cluster with subfamily 10 in our phylogenetic comparisons and, therefore, is not present in the figure. Detailed functional analysis of *AtERF* genes from these three subfamilies is lacking.

To check for the presence of introns, full-length ERFs were amplified from genomic DNA. Genomic amplification of *MdERF4* (accession number JF412351) and *MdERF5* (accession number JF412352) yielded no evidence of introns (data not shown), indicating that the gene structure has been conserved between apple and *Arabidopsis*. However, apple homologs of subfamily 10 were of particular interest because members of this subfamily contain introns in *Arabidopsis* (46). PCR was performed using genomic DNA to amplify *MdERF3* (accession number JF412350) sequences surrounding the predicted intron site based on *Arabidopsis* subfamily 10. Amplification of genomic DNA with *MdERF3*-specific primers yielded a product that was 500 bp larger than expected from the cDNA sequence. This product was sequenced and investigated for the presence of an intron. The Plant GDB GeneSeqer program of the Plant Genome Database (<http://www.plantgdb.org/>) identified an intron. Because the amplified sequence was not identical to *MdERF3*, this gene was named *MdERF6* (accession number JF412353). The *MdERF6*

sequence lacked a stop codon. Our inability to amplify the corresponding region of *MdERF3* using Taq polymerase without proofreading may indicate the presence of a large intron.

mRNA expression of *MdERF*. Semiquantitative RT-PCR of *GAPDH* and *ACS1* showed that *ACS1* was expressed in untransformed but not in *ACS*-silenced apple (Supplementary Figure S2). *ACS1* expression was strongly induced 3 h after wounding. *ACS1* mRNA was barely detectable 6 and 24 h after wounding but increased after 72 h. *ACS1* was gradually induced during *B. cinerea* invasion, peaking 72 h after inoculation.

Quantitative RT-PCR showed that expression of all four *MdERF* genes was reduced in *ACS*-silenced relative to untransformed apple (Fig. 4). Expression of *MdERF4*, -5, and -6 was suppressed in transgenic fruit. In contrast, *MdERF3*, in particular, was induced, albeit to a reduced degree, in *ACS*-silenced apple. Therefore, wound- and pathogen-induced expression of *MdERF3* is only partially dependent on ethylene biosynthesis.

Expression of *MdERF4*, -5, and -6 in untransformed GS apple fruit was highly induced 3 h after wounding, mimicking *ACS1* expression. In contrast, all four *MdERF* genes and *ACS1* were expressed at a low level 3 and 6 h after inoculation of untransformed GS fruit with *B. cinerea*, suggesting that this pathogen suppresses the rapid wound-related induction of ethylene biosynthesis and signaling.

***MdERF* promoter sequence analysis.** Partial cloning of *MdERF3*, -4, -5, and -6 promoter regions revealed 706, 778, 877, and 583 bp of upstream sequences, respectively. All four pro-

Fig. 2. Dependence of apple fruit resistance to *Botrytis cinerea* on endogenous ethylene production. Apple fruit were tested **A** and **B**, immediately after harvest or **C** and **D**, after incubation for 2 weeks at room temperature. Ethylene production during **A** and **C**, *B. cinerea* infection and **B** and **D**, lesion expansion were measured in pathogen-challenged 1-aminocyclopropane-1-carboxylic acid synthase (*ACS*)-silenced (130Y) and untransformed apple fruit 'Greensleeves' (GS) at each time. Vertical bars show $\pm$ standard error of 10 replications. Ethylene production was clearly suppressed in 130Y compared with GS both immediately after harvest and 2 weeks after harvest. The area under disease progress curve showed significant differences in lesion diameter 2 weeks after harvest ($P < 0.001$) but not 1 day after harvest ($P = 0.1874$).

moter regions were analyzed for putative *cis*-acting regulatory elements (26). Three criteria were used to select *cis*-elements: (i) motifs were located on the plus strand upstream of the predicted transcriptional start site; (ii) motifs were at least five nucleotides long; and (iii) motifs related to plant hormone, pathogen, or stress responses were chosen (Table 1; Supplementary Figure S4). A summary of the motifs found in promoter regions of *MdERF3*, -4, -5, and -6 is shown in Table 1.

Fig. 3. Identification of *Malus domestica* ethylene response factor (*MdERF*) genes expressed in apple fruit ‘Jonagold’. **A**, Diagrams of predicted amino acid sequences of *MdERFs* belonging to subfamilies 1, 2a, and 10. Subfamilies shown in parentheses and conserved motifs (CMs) are based on classifications of genomic *ERF* sequences from rice and *Arabidopsis* (46). A.A. = amino acids. *MdERF6* shows a partial coding sequence. **B**, Three clades of a phylogenetic comparison of 122 *AtERF* and four *MdERF* genes. Clades of the most parsimonious tree are shown together with percentages of 100 bootstrap replicates.

Consensus sequences of *cis*-acting elements related to hormonal, abiotic, or biotic stress responses were found in all four promoters. The AtEIN3-binding site (63), an important *cis*-element related to ethylene signaling, was found in promoters of *MdERF3*,

-5, and -6 (Supplementary Figure S3). Upstream sequences of *MdERF5* and -6 contained an additional ethylene-responsive element (ERE). TGA, a bZIP family TF (18), and MYB TF-binding sites were common features of all four *MdERF* genes.

Fig. 4. *Malus domestica* ethylene response factor (*MdERF*) expression after wounding or pathogen challenge. Apple fruit tissues were wounded or wounded and inoculated with *Botrytis cinerea* after 14 days of storage. Total RNA was extracted 0, 3, 6, 24, and 72 h after onset of treatment from three pooled apple fruit. *MdERF* expression was measured by quantitative reverse-transcription polymerase chain reaction, normalized to *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) expression, and shown as fold change relative to 0 h. Log₂ scale was used. Genotype-dependent differences in *MdERF* expression were significant (Mann-Whitney U test, $P < 0.001$). Significant treatment-dependent differences were observed when comparing combined data 3 and 6 h after onset of treatment (Mann-Whitney U test, $P = 0.006$).

Uniquely, three BELL-type homeodomain TF-binding sites were identified in the *MdERF3* promoter. WRKY TF-binding site W-boxes and a wound-activating region are exclusive to *MdERF4*. All *MdERF* genes contained drought-responsive *cis*-elements but different genes contained distinct motifs. Although *MdERF4* and -5 had ABA-responsive elements, *MdERF3* and -6 had dehydration-responsive elements.

Activation of chitinase mRNA expression by *MdERF3*. *MdERF3* was the only one of four *MdERF* genes induced in ACS-silenced apple by wounding and *B. cinerea* infection (Fig. 4). Therefore, the effect of *MdERF3* on expression of the downstream gene *Chn48* was tested in *N. benthamiana* after transient expression of *MdERF3* using *Agrobacterium* infiltration. One day after *Agrobacterium* infiltration, *MdERF3* mRNA expression was clearly elevated relative to the empty vector control (Table 2). In parallel, mRNA expression of the chitinase gene *Chn48* (60) significantly ($P = 0.016$) increased in leaves that expressed *MdERF3* relative to leaves that were treated with the empty vector control. An increase in *Chn48* expression was also observed after *Agrobacterium* infiltration with an empty vector construct relative to unchallenged leaves, suggesting that this chitinase gene responds to either wounding or *Agrobacterium* spp. We did not infiltrate without *Agrobacterium* spp. and, therefore, cannot distinguish between these two possibilities.

DISCUSSION

Ethylene limits expansion of lesions caused by *B. cinerea* in ripening apple fruit. Two lines of evidence illustrate that ethylene is necessary for induction of defenses against *B. cinerea* in apple fruit. First, ACS-suppressed apple fruit were more susceptible to *B. cinerea* (Fig. 2). Second, susceptibility increased in ACS-suppressed apple fruit with fruit ripening. ACS-suppressed fruit were slightly more susceptible to *B. cinerea* than untransformed apple immediately after harvest but this difference in susceptibility was much larger and statistically significant 14 days after harvest. Fruit did not produce ethylene immediately after harvest but ethylene biosynthesis reached its climacteric peak 14 days after harvest. Thus, activation of ethylene biosynthesis prior to inoculation is necessary to induce resistance against *B. cinerea*, implicating ripening-related ethylene production in priming of defense responses against *B. cinerea* (14). Our findings are compatible with ethylene's enhancement of resistance to *B. cinerea* in *Arabidopsis* spp. (7,69). Although genetic modification of the ethylene signal transduction pathway has not yet been accomplished in apple, results analogous to *Arabidopsis* were obtained using genetic suppression of ethylene biosynthesis.

The hypothesis that ethylene induces a defense against *B. cinerea* in *Pyrus communis* (pear) fruit has been tested previously

TABLE 1. Putative *cis*-acting elements in *MdERF* promoters^a

<i>cis</i> -element name	<i>cis</i> -element sequence	MdERF3	MdERF4	MdERF5	MdERF6	Reference ^b
ABA responsive element (ABRE)	ACGTG	...	-205	-99	...	47,61,67
		...	-175	...	...	...
		...	-92	...	...	...
Auxin and brassinosteroid response element	TGTCTC	...	...	-102	...	25,71
OsBIHD, a rice BELL homeodomain transcription factor, binding site	TGTC A	-288	...	...	...	38
		-251	...	...	...	...
		-68	...	...	...	...
Cytokinin enhanced protein-binding site	TATTAG	-680	...	...	...	24
C repeat/dehydration response element (CRT/DRE), low-temperature responsive element (LTRE)	(A/G)(C/T)CGAC	-358	...	-356	...	9,19,29,53,62
Ethylene responsive element (ERE)	A(A/T)TTCAAA	...	...	-721	-437	27,45,55,68
	GAAAAA	...	-541	...	-168	50
GT-1 <i>cis</i> -element		...	-503	...	...	...
		...	-451	...	...	...
		...	-97	...	...	...
		-29	-32	-31	-32	...
		...	...	-82	...	18,30
TATA box	TGACG	...	...	...	...	1
TGA1a	(A/T)AACCA	-568	-245	-775	-583	...
MYB recognition site	ACC(A/T)(A/T)CC	-303	-75	...	...	36,39
		...	...	...	-389	...
		...	-159	...	...	12,44,77,80
W-box, salicylic-acid-induced WRKY-binding site	TTGAC	...	-126	...	...	...

^a Sizes of cloned *MdERF3*, -4, -5, and -6 promoter regions were 706, 778, 877, and 583 bp, respectively. Signal Scan Search options of the PLACE program were used to identify *cis*-elements. Numbers indicate locations of motifs relative to the predicted transcription start site at +1.

^b References are original reports of *cis*-elements or subsequent notes of other genes or other plant species.

TABLE 2. Quantitative polymerase chain reaction results after *Agrobacterium*-mediated transient expression of *MdERF3* in tobacco leaves^a

Treatment	MdERF3	Chn48 ^b	rRNA	ERF/rRNA	Chn48/rRNA
Vector with <i>MdERF3</i>	3.11	124.89	1.67	1.86	74.78
	35.86	228.86	19.66	1.82	11.64
	13.32	83.95	33.88	0.39	2.48
Vector without <i>MdERF3</i>	0.23	25.58	5.40	0.04	4.74
	4.58	196.78	44.50	0.10	4.42
	0.61	42.53	32.38	0.02	1.31
Unchallenged control	0.41	1.07	36.10	0.01	0.03
	0.51	1.63	57.97	0.01	0.03
	0.63	3.79	32.04	0.02	0.12

^a Each value is the mean of three technical replicates. Each line in each treatment represents a biological replicate.

^b Relative expression levels of endogenous *Chn48* mRNA in response to different treatments. Cycle threshold (Ct) values were determined using autothreshold. Relative expression (Δ Ct) of each cDNA sample was obtained by normalizing to the *18S ribosomal RNA* frequency. Statistical analysis using the Relative Expression Software Tool algorithm (51) revealed a significant difference in *Chn48* expression between *MdERF3*-transfected samples and empty vector controls ($P = 0.016$).

using exogenous application of chemicals to accelerate or inhibit ethylene biosynthesis, because no transgenic or mutant pear fruit with defects in ethylene production or signal transduction were available (2). Although effects of ethylene on disease resistance of pear fruit were small, inhibition of ethylene through binding of 1-methylcyclopropene (1-MCP) to the ethylene receptor in pear fruit resulted in a statistically significant increase in susceptibility to *B. cinerea* (2). By comparison, data presented here on endogenous suppression of ethylene biosynthesis in apple fruit showed a stronger and clearer increase in susceptibility to *B. cinerea* than that caused by exogenous manipulation of ethylene action in pear fruit. In contrast to plants, cultured *B. cinerea* produces ethylene in a 2-keto-4-methylbutyric acid-dependent manner (11,15). Ethylene production in ACS-silenced fruit was higher during *B. cinerea* infection (Fig. 2) than during fruit ripening (Fig. 1), suggesting that the fungus may produce ethylene in vivo. Previous comparison of 1-MCP- and aminoethoxyvinylglycine-sensitive ACC and ethylene production during *B. cinerea* infection of pear fruit provides evidence for microbial ethylene production (2). Therefore, it is an intriguing possibility that ethylene produced by *B. cinerea* triggers defense responses in apple fruit, although the contribution of oligosaccharide elicitors cannot be ruled out (10). If so, fungal ethylene production might be a disadvantage for infecting *B. cinerea*.

The expression of several ethylene- and defense-related genes during fruit ripening was tested by semiquantitative RT-PCR. Only the expression of *ACS1* correlated with climacteric ethylene production in apple fruit (Supplementary Figure S4). *PGIP* mRNA was expressed in healthy apple fruit, as previously reported (79). Although *PGIP* is regulated independently of ethylene, it is strongly induced in response to *B. cinerea* infection (79).

Regulation of *MdERF* expression. *MdERF3* was the only gene observed by quantitative RT-PCR to be induced by *B.*

cinerea infection in ACS-silenced apple fruit (Fig. 4). Interestingly, the sequences of *MdERF3* and *MdERF6* are very closely related (Fig. 3A and B) but the response of *MdERF6* to *B. cinerea* was entirely ethylene dependent, suggesting that important pathogen-responsive regulatory sequence elements may be lacking in the *MdERF6* promoter or that biological replication was not sufficient to detect differences in the response of *MdERF6*.

In contrast to *MdERF3*, expression of *MdERF4*, -5, and -6 appeared to be ethylene dependent. Therefore, regulation of *MdERF3* is similar to *MdERF1*, the expression of which increases with apple fruit ripening (73). *MdERF1* expression was not completely suppressed by 1-MCP treatment during fruit ripening, suggesting ethylene-independent regulation of this gene (73).

The transient, wound-induced expression of *MdERF4*, -5, and -6 genes and *ACS1* appeared to be suppressed in *B. cinerea*-challenged apple fruit. Unlike nonpathogenic bacterial species, *Erwinia betavascularum* also suppresses wound-related ethylene production in *Solanum tuberosum* (potato) (33). It remains to be determined whether suppression of wound-related ethylene production is required for pathogenicity. Transcription of all *MdERF* genes and *ACS1* appeared to increase 3 days after wounding of apple fruit. This might be a response to wounding, ripening, placement of apple fruit into a closed moisture chamber, or accumulation of ethylene in the chamber. A flow-through system allowing for exchange of air inside the box would have eliminated the latter possibility.

Putative cis-elements in *MdERF* promoters. Although we did not conduct functional analysis of promoter regions, the putative TF-binding sites identified here are indicative of the differential *MdERF* expression observed. Cis-elements are typically located within 1 kb upstream of the translation start site in *Arabidopsis* (41). The four *MdERF* promoter sequences analyzed were ~500 to 900 bp long, too short for complete description of *MdERF* promoters.

AtEIN3 binds to and activates expression of *AtERF1* in response to ethylene signaling in *Arabidopsis* (63). Promoter sequences of *MdERF3*, -5, and -6 contain putative EIN3-binding sites. Although the *MdERF4* gene did not contain a putative EIN3-binding site in its predicted promoter region, such a site was found between the predicted transcriptional initiation site and the translational start codon ATG (data not shown).

Two types of EREs exist. The A(A/T)TTCAAA motif is found in promoters of the fruit-specific *L. esculentum* (tomato) gene *E4* (45) and senescence-related carnation gene *GST1* (27). The second ERE is the GCC-box but this box is not present in promoters of fruit ripening or flower petal senescence genes (49). Ethylene may regulate *MdERF5* and -6 during fruit ripening, because both genes contain A(A/T)TTCAAA motifs. GCC-boxes in the promoter of *NtERF3* (*N. tabacum*) are apparently needed for its autorepression (48). There was no evidence for GCC-boxes among the limited promoter sequence information for any of the four *MdERF* genes.

Three *BIHD* cis-elements known to bind to the BELL-type homeobox protein (named after the distinctive Bell domain) from *O. sativa* (rice) (38) were found exclusively in the *MdERF3* promoter. In contrast to the function of this gene family in animal development (5), none of the homeodomain (HD)-containing TFs from plants exert homeotic effects. The HD superfamily is classified into six groups based on HD size, location, and association with other domains (5). *BIHD* is an important cis-element activated by *B. cinerea*. The *OVEREXPRESSION OF CATIONIC PEROXIDASE 3* (*OC3*) mutant with a defect in a homeodomain-containing gene enhances jasmonate-dependent resistance to the necrotrophic pathogens *B. cinerea* and *Plectosphaerella cucumerina*, while interactions with biotrophic pathogens such as the oomycete *Hyaloperonospora parasitica* and the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 remain unaffected (13). *OC3* is expressed at a very low level in healthy plants but

Fig. 5. Proposed role of ethylene during *Botrytis cinerea* infection of apple fruit. The model depicts a portion of the signal cascade that activates defense responses and fruit ripening. Ethylene was produced by the fruit and may be produced by *B. cinerea*. Homeodomain transcription factors (HD) may activate expression of *Malus domestica ethylene response factor 3* (*MdERF3*). *MdERF3* may bind to the GCC box and activate expression of defense-related genes such as *chitinase*.

its expression further decreases during *Plectosphaerella cucumerina* infection (13). The *OC3* gene is different from other classes of homeodomain genes such as the knotted-related homeobox (*KNOTTED1*) and the homeodomain associated to a leucine zipper (*HD-Zip*) (13). The HD-Zip type homeobox gene *H52* in *L. esculentum* (tomato) limits spread of programmed cell death (43). In addition, *Arabidopsis* and *Petroselinum crispum* (parsley) homeodomains associated with finger domain (PHD-type homeodomain) proteins bind to and regulate *PR2* expression (32), suggesting that HD-containing TFs play an important role in induced resistance to plant pathogens.

***MdERF3* regulates chitinase expression.** To identify potential target genes containing *cis*-elements important for their induced expression, *MdERF3* was transiently expressed in tobacco and chitinase *Chn48* expression was measured by quantitative RT-PCR. *Chn48* is known to be induced by ethylene; its promoter contains GCC-boxes, a motif necessary for transcriptional activation of pathogenesis-related genes regulated by ethylene (60). Expression of the chitinase *Chn48* significantly increased after transient expression of *MdERF3* (Table 2). These data show that the chitinase promoter is a possible target of *MdERF3*. Transgenic tobacco plants expressing endochitinase from *Trichoderma harzianum* are highly resistant to *B. cinerea* (37). Moreover, transgenic *Fragaria* × *ananassa* (strawberry) plants expressing the chitinase gene *ch5* from *Phaseolus vulgaris* are also highly resistant to *B. cinerea* but not to the anthracnose pathogen *Colletotrichum acutatum* (72). Therefore, it is possible that ethylene-dependent induction of chitinase in apple fruit could contribute to inhibiting growth of *B. cinerea* (Fig. 5).

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Erratum

Corrections were made to Figure 3B on page 1315. Changes to this article were made on November 2, 2011.