Ethylene-Responsive AP2/ERF Transcription Factor MACD1 Participates in Phytotoxin-Triggered Programmed Cell Death

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Submitted 1 November 2012. Accepted 11 April 2013.

To investigate plant programmed cell death (PCD), we developed the model system using phytotoxin AAL, which is produced by necrotrophic pathogen *Alternaria alternata* f. sp. *lycopersici*, and AAL-sensitive *Nicotiana umbratica*. We previously reported that ethylene (ET) signaling plays a pivotal role in AAL-triggered cell death (ACD). However, downstream signaling of ET to ACD remains unclear. Here, we show that the modulator of AAL cell death 1 (MACD1), which is an APETALA2/ET response factor (ERF) transcription factor, participates in ACD and acts downstream of ET signaling during ACD. MACD1 is a transcriptional activator and MACD1 overexpression plants showed earlier ACD induction than control plants, suggesting that MACD1 positively regulates factors affecting cell death. To investigate the role of MACD1 in PCD, we used *Arabidopsis thaliana* and a structural analog of AAL, fumonisin B1 (FB1). FB1-triggered cell death was compromised in ET signaling and *erf102* mutants. The *loh2* mutants showed sensitivity to AAL, and the *loh2-1/erf102* double mutant compromised ACD, indicating that ERF102 also participates in ACD. To investigate the PCD-associated genes regulated by ERF102, we compared our microarray data using ERF102 overexpression plants with the database of upregulated genes by AAL treatment in *loh2* mutants, and found genes under the control of ERF102 in ACD.

Programmed cell death (PCD) is essential for plant development and immunity. Localized PCD is associated with hypersensitive response (HR), which is a constituent of a successful plant innate immune response. Rapid and localized PCD, known as HR cell death, is induced at the infection site to prevent growth and spread of pathogens into healthy tissues (Greenberg 1997). This response is initiated by direct or in-

direct recognition of a pathogen avirulence (*Avr*) factor by a plant resistance (*R*) gene product and is controlled by multiple signal transduction pathways (Moffett 2009). Some pathogen-associated molecular patterns, such as INF1 elicitor (Kamoun et al. 2003), xylanase (Bailey et al. 1990), and AvrXa21 (Lee et al. 2009), also induce HR cell death. The HR cell death signaling pathway has been studied genetically and biochemically but the signaling pathway of plant PCD is not fully understood.

Host-selective toxins (HST) are effectors produced by some necrotrophic pathogenic fungi that typically confer the ability to cause disease (Markham and Hille 2001; Walton 1996). Plant cells exposed to phytotoxins produced by necrotrophic pathogens induce cell death associated with morphological markers indicative of PCD (Gilchrist 1998; Greenberg and Yao 2004). Victorin, a phytotoxin produced by *Cochliobolus victoriae*, triggers PCD in oat cultivars carrying the dominant *Vb* allele (Wolpert et al. 1985). Victorin also induces cell death in *Arabidopsis thaliana* carrying *LOV1*, which is a gene that confers disease susceptibility and, paradoxically, is a member of the coiled-coil nucleotide-binding site leucine-rich repeat *R* family (Lorang et al. 2007). *Thioredoxin-h5* (*TRX-h5*) is also genetically required for victorin sensitivity in *Arabidopsis thaliana* (Sweat and Wolpert 2007). Victorin was recently shown to bind to *TRX-h5* and inhibit its activity (Lorang et al. 2012). *LOV1* guards the inhibition of *TRX-h5* by victorin and elicits a resistance-like response, including cell death (Lorang et al. 2012). AAL is a phytotoxin produced by *Alternaria alternata* f. sp. *lycopersici* (Wang et al. 1996) that causes disease in its sensitive natural host tomato and some *Nicotiana* spp. lacking the *Alternaria stem canker gene 1* (*Asc-1*) (Brandwagt et al. 2000, 2001).

Some phytotoxin-induced PCD requires an ethylene (ET)-dependent signaling pathway. ET inhibitors aminooxyacetic acid (inhibitor of ET biosynthesis) and silver thiosulfate (inhibitor of ET action) also give marked protection against victorin-induced leaf symptoms in oat leaves (Navarre and Wolpert 1999). Slight attenuation of *LOV1*-mediated victorin sensitivity occurs in the *Arabidopsis thaliana ein2* mutant (Lorang et al. 2007). The *never ripe* mutants of tomato, which affect ET perception, show less cell death symptom response to AAL than wild-type tomato (Moore et al. 1999; Zhang et al. 2011). Co-treatment of *asc/asc* leaflets with AAL and ET inhibitors markedly reduces cell death symptoms (Moussatos et al. 1994). AAL-triggered cell death (ACD) in AAL-sensitive *N. umbratica* requires the ET dependent signaling pathway (Mase et al. 2012).

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*The e-Xtra logo stands for “electronic extra” and indicates that 10 supplementary figures, four supplementary tables, one supplementary dataset, and supplementary materials and methods are published online. Also, Figures 1, 3, 4, 5, 7, and 8 appear in color online.

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These studies indicate that the ET-dependent signal pathway contributes to phytotoxin-induced PCD. However, downstream of ET-dependent signaling in PCD is not well understood. Identification of ET downstream components will help us understand how ET signaling participates in plant PCD.

ET-insensitive 3 (EIN3) is a plant-specific nuclear transcription factor that initiates downstream transcriptional cascades for ET responses in *A. thaliana* (Alonso et al. 2003; Solano et al. 1998; Yanagisawa et al. 2003). EIN3 is a short-lived protein that is degraded by a ubiquitin/26S proteasome pathway in the absence of ET. Genetic and biochemical studies showed that EIN3 proteolysis is mediated by F-box proteins EIN3-binding F-box 1 and 2 (EBF1 and EBF2) (Gagne et al. 2004; Guo and Ecker 2003; Potuschak et al. 2003). ET-insensitive 2 (EIN2) is required to degrade EBF1 and EBF2 to accumulate EIN3 and ET-insensitive-like1 (EIL1) (An et al. 2010). Stabilized EIN3 and EIL activate the expression of targets, such as *ET response factor 1* (*ERF1*) transcription factor, which initiates a transcriptional cascade that results in activation and repression of hundreds of genes (Solano et al. 1998). ERF1 belongs to the AP2/ERF family of transcription factors. The AP2/ERF family is only present in the plant kingdom and is characterized by the presence of a highly conserved DNA-binding domain called the AP2 domain (Riechmann et al. 1998). The AP2/ERF family can be divided into five subfamilies from the number and sequences of their AP2 domains (Sakuma et al. 2002). The ERF subfamily has a single AP2 domain and shows DNA-binding activity. Some ERF proteins bind specifically to the GCC-box (AGCCGCC), which is an ET-responsive element. The binding of ERF proteins to the GCC-box regulates ET-responsive transcription (Fujimoto et al. 2000; Ohme-Takagi and Shinshi 1995).

In a previous study, we developed a model system using AAL and *N. umbratica* to analyze plant PCD (Mase et al. 2012). AAL is an HST and is a sphinganine-analog mycotoxin (SAM). SAM structurally resembles sphinganine, an intermediate of sphingolipid biosynthesis (Wang et al. 1996). AAL causes PCD in its sensitive plants by inhibition of ceramide biosynthesis (Gilchrist 1997; Spassieva et al. 2002; Wang et al. 1996). We showed that mitogen-activated protein kinase-mediated ET production is required for ACD in *N. umbratica* (Mase et al. 2012), and that plants with silenced ET signaling components showed depression of ACD, suggesting that ET signaling has pivotal roles in ACD. In this study, we investigated key factors downstream of ET signaling pathways in ACD. Here, we report that the modulator of AAL cell death 1 (*MACD1*), which is an AP2/ERF transcription factor, participates in ACD. *A. thaliana* *MACD1* ortholog *ERF102* also has an important role in the structural analog of AAL, fumonisin B1 (FB1)-triggered cell death (FCD), as well as ACD, in an AAL-sensitive *Arabidopsis* mutant. Also, we identified genes that act downstream of *ERF102* in ACD by using microarray analysis.

RESULTS

Virus-induced gene silencing (VIGS) of *MACD1* compromised ACD.

To investigate unknown downstream components of ET-signaling pathways in ACD, we performed VIGS analysis based on the *Tobacco rattle virus* (TRV) vector in *N. umbratica* (Ratcliff et al. 2001). First, to identify the *MACD* genes, we knocked down ET-responsive *N. tabacum* *ERF* homologs in *N. umbratica*, which were reported as *ET-responsive element-binding protein-1* (*EREBP-1*), *EREBP-2*, and *EREBP-4* (*NtERF1*, *NtERF2*, and *NtERF4*) (Ohme-Takagi and Shinshi 1995) and *NtERF5* (Fischer and Dröge-Laser 2004). We isolated cDNA fragments of *NuERF1* (AB747101), *NuERF2* (AB747102),

NuERF4 (AB747103), and *NuERF5* (AB747104) from the *N. umbratica* cDNA library by using polymerase chain reaction (PCR) with primer design based on the *NtERF* sequences. To avoid off-target effects by VIGS, the fragments, except the AP2/ERF domain, were used for the VIGS vector (Supplementary Fig. S1A). The expression level of *NuERF* was markedly reduced in each TRV:*NuERF*-infected plant compared with the TRV control plants. ACD was compromised in *NuERF4*-silenced plants but not in other *NuERF*-silenced plants (Fig. 1A). The mRNA levels of *NuERF* genes *NuERF1*, *NuERF2*, and *NuERF5* showed no noteworthy differences between TRV:*NuERF4* plants and TRV control plants, suggesting that *NuERF4* silencing does not affect the expression levels of at least three *NuERF* genes. To quantify cell death, we measured the ion leakage of AAL-treated plants (Fig. 1B). AAL-induced ion leakage was suppressed in *NuERF4*-silenced plants. We designated *NuERF4* as *MACD1* from these results.

MACD1 acts downstream of ET signaling in ACD.

We examined whether *MACD1* expression is influenced by ET during ACD. *NuERF1*, the *NtERF1* homolog, was used as a positive control of the ET-inducible gene in *N. umbratica* (Fig. 2A, left). The gene *tobacco EIN3-like* (*TEIL*) and its related genes have been implicated in ET signaling (Hibi et al. 2007; Kosugi and Ohashi 2000; Rieu et al. 2003). We previously showed that ACD is compromised in *TEIL*-silenced leaves (Mase et al. 2012). ET-induced *NuERF1* expression was suppressed in TRV:*TEIL* (Fig. 2A, right). *MACD1* was induced by ET treatment (Fig. 2B), indicating that *MACD1* is also an ET-inducible gene.

We checked the expression profiles of *MACD1* in ACD. *MACD1* peaked at 36 h after AAL treatment of *N. umbratica* (Fig. 2C). ET accumulated in ACD at 24 to 48 h after AAL treatment (Mase et al. 2012); possibly, *MACD1* expression is induced by ET in response to AAL. To confirm this possibility, we examined *MACD1* expression in *TEIL*-silenced plants. The expression level of *MACD1* by AAL was suppressed in TRV:*TEIL* plants (Fig. 2D), indicating that *MACD1* is downstream of ET signaling during ACD.

MACD1 positively regulates factors participating in cell death development.

The predicted protein of *MACD1* does not contain the ERF-associated amphiphilic repression (EAR) motif (L/FDLNL/F [x] P), which acts as a repression domain and converts transcriptional activators into strong repressors when fused with DNA-binding proteins (Ohta et al. 2001). To determine the transactivation activity of *MACD1* in plants, we used a yeast GAL4 system-modified and *Agrobacterium*-mediated transient assay system (Ishihama et al. 2011). The β -glucuronidase (*GUS*) reporter gene containing an intron to avoid expression in *Agrobacterium* was fused under the control of a synthetic promoter consisting of the minimal *Cauliflower mosaic virus* 35S promoter and the GAL4 upstream activation sequence (GAL4UAS) (Fig. 3A). The GAL4 DNA binding domain (GAL4DBD) that was fused to the activation domain of herpes simplex virus VP16 (GAL4DBD-VP16) was used as a positive control to activate the GAL4UAS-*GUS* reporter gene (Fig. 3A and B). We replaced VP16 with *MACD1* and eventually constructed GAL4DBD-*MACD1* chimera DNA (Fig. 3A). If *MACD1* is a transcriptional activator, the *GUS* reporter gene is induced by co-expression of GAL4DBD-*MACD1* and reporter construct in *N. benthamiana* plants. GAL4DBD-*MACD1* showed higher *GUS* activities compared with GAL4DBD (Fig. 3B), indicating that *MACD1* is a positive regulator of transcription.

Some ERF transcription factors most likely function by binding to cognate GCC-box *cis*-elements in the promoter region

of target genes (Fujimoto et al. 2000; Ohme-Takagi and Shinshi 1995). To examine the binding activity of MACD1 with GCC-box, we modified the *Agrobacterium*-mediated transient assay system described above. We replaced GAL4UAS with GCC or mutated GCC (mGCC) elements in reporter plasmids (Fig. 3C). If MACD1 has the binding activity with GCC-box, GUS activity is detected by MACD1 and reporter co-expression. MACD1 was transiently expressed by using *Agrobacterium tumefaciens* infiltration (agroinfiltration) in *N. benthamiana*

plants. Transient MACD1 expression raised the activity of the co-transfected synthetic promoter with GCC elements but not MACD1 with mutated GCC (mGCC) (Fig. 3C and D), suggesting that MACD1 binds to the GCC-box.

To investigate the subcellular localization of MACD1, MACD1 was fused to green fluorescent protein (GFP) in the N terminal or C terminal, because it is possible that GFP position has an effect on the subcellular localization. MACD1-GFP and GFP-MACD1 were expressed transiently in *N. benthamiana* by agroinfiltration. Stain 4',6-diamidino-2-phenylindole (DAPI) was used to indicate nuclear localization (Fig. 3E). MACD1-GFP or GFP-MACD1 was found in the nucleus. These results show that MACD1 has features of the AP2/ERF family.

To examine the function of MACD1 in ACD, *N. umbratica* leaves were inoculated with *A. tumefaciens* containing MACD1 or GUS as a control. Necrotic lesions were not observed in MACD1 overexpression (OE) plants and GUS OE control plants (Fig. 4A to C), indicating that MACD1 OE is not sufficient to induce cell death. *N. umbratica* leaves were treated with AAL at 24 h after agroinfiltration, and ACD emerged more rapidly in MACD1 OE plants than in GUS OE plants (Fig. 4D). AAL-induced ion leakage increased in MACD1 OE plants (Fig. 4E). These results suggest that MACD1 positively regulates factors participating in cell death development.

ERF102 participates in FCD cell death in *Arabidopsis thaliana*.

To further investigate ET signaling in plant PCD, we used a model system using FB1 and *Arabidopsis thaliana*. FB1 is an AAL structural analog, is a SAM, and induces PCD by disturbance in ceramide biosynthesis (Gilchrist 1997; Wang et al. 1996). ET signaling appears to participate in FCD in *A. thaliana* protoplasts (Asai et al. 2000). Using *A. thaliana* instead of *N. umbratica* for the analysis has the following advantages: we can use the completed *A. thaliana* genome sequence (*Arabidopsis* Genome Initiative 2000), well-developed mutants lines, and well-established microarray analysis. First, to examine whether the ET signal pathway is required for FCD in *A. thaliana*, *etr1-1* (Chang et al. 1993) and *ein3* (Solano et al. 1998) mutants and ET receptor mutants and ET signaling mutants, respectively, were treated with FB1. Reduction of FCD and FB1-induced ion leakage were observed in *etr1-1* and *ein3* mutants (Fig. 5A and B), indicating that the ET signaling pathway has important roles in FCD.

Fig. 1. Effects of silencing *NuERF* genes on AAL-triggered cell death (ACD). **A**, Silencing *NuERF4* compromised ACD in *Nicotiana umbratica*. **B**, ACD quantified by ion leakage from AAL-treated leaves. Data are means $\pm$ standard deviations from at least three independent experiments. Data underwent a Student's *t* test: ** and * indicate $P < 0.01$ and 0.05 , respectively, versus *Tobacco rattle virus* (TRV) control plants.

Fig. 2. Transcript accumulation of modulator of AAL cell death 1 (*MACD1*) in response to ethylene (ET) and AAL. mRNA accumulation of **A**, *NuERF1* and **B**, *MACD1* after ET treatment in *Tobacco rattle virus* (TRV) control plants or TRV:*tobacco EIN3-like* (*TEIL*) plants. *EF-1 α* was used as an internal standard. Data are means $\pm$ standard deviations (SD) from three experiments. Expression profiles of *MACD1* after AAL treatment in **C**, *Nicotiana umbratica* and **D**, *MACD1* after AAL treatment in TRV control plants or TRV:*TEIL* plants, *EF-1 α* was used as an internal standard. Data are means $\pm$ SD from three experiments.

To explore the MACD1 homologs in *A. thaliana*, multiple alignment analyses were done using amino acid sequences of the AP2/ERF domain in *A. thaliana*, *N. umbratica*, *N. tabacum*, *Solanum tuberosum*, and *S. lycopersicum* (Supplementary Fig. S2). NuERF4 encoded a protein that was similar to ERF102 (previously called ERF5; At5g47230), ERF103 (previously called ERF6; At4g17490), ERF104 (At5g61600), and ERF105 (At5g51190), which are classified into *A. thaliana* ERF group IX subfamily (Nakano et al. 2006). *A. thaliana* T-DNA insertion mutants for ERF102 (*erf102*; SALK_076967) and ERF103 (*erf103*; SALK_087356) were obtained from the SALK T-DNA collection (Supplementary Fig. S3).

To examine the effects of *erf102* and *erf103* mutation on FCD, we treated *erf102* and *erf103* mutants with FB1. FCD was significantly reduced in the *erf102* mutant (Fig. 5C).

ERF104 interacts with MPK6 and is a vital regulator of basal immunity (Bethke et al. 2009). In our study, *erf104* mutant showed normal FCD development (Fig. 5C). FB1-induced ion leakage was also significantly reduced in the *erf102* mutant but scarcely in *erf103* mutant (Fig. 5D). We examined whether ERF102 rescues the FCD depression phenotype in the *erf102* mutant. Expression of *ERF102* from the 35S promoter complemented the *erf102*-mediated suppression of FCD (Supplementary Fig. S4). These results suggest that ERF102 participates in FCD and is an MACD1 ortholog in *A. thaliana*.

ERF102 (ERF5) and ERF103 (ERF6) are known to have redundant roles in plant defense responses against various pathogens (Moffat et al. 2012; Son et al. 2012). To further investigate the roles of ERF102 and ERF103 in FCD, we tested the *erf102/erf103* double mutant (Moffat et al. 2012). The

Fig. 3. Characterization of modulator of AAL cell death 1 (MACD1). **A**, Schematic diagram of effector, reporter, and reference plasmids used in transient assays to estimate MACD1 transcriptional activity. **B**, Transactivation of the β -glucuronidase (*GUS*) gene by GAL4DBD-MACD1 in *Nicotiana benthamiana* leaves. Total proteins were extracted from leaves coinfiltrated with *Agrobacterium*-containing reporter plasmids, effector plasmids, or reference plasmids. *GUS* activity was normalized to luciferase (*LUC*) activity. **C**, Schematic diagram of effector, reporter, and reference plasmids used in transient assays for evaluation of MACD1 GCC-box-mediated gene expression. **D**, MACD1 can transactivate GCC-box-mediated gene expression in *N. benthamiana* leaves. Total proteins were extracted from leaves coinfiltrated with *Agrobacterium*-containing reporter plasmids, effector plasmids, or reference plasmids. *GUS* activity was normalized to *LUC* activity. **E**, Subcellular localization of MACD1 in *N. benthamiana* epidermal cells. *N. benthamiana* leaves were transformed with MACD1-green fluorescent protein (GFP) and GFP-MACD1 by agroinfiltration. DAPI = 4',6-diamidino-2-phenylindole; DIC = differential interference contrast; bars = 20 μ m.

erf102/erf103 double mutant showed lower conductivity than the *erf102* single mutant (Fig. 5E). Although the *erf103* mutant did not compromise FCD clearly, it is possible that ERF103 also contributes to FCD induction.

ERF102 acts downstream of ET signaling during FCD.

To examine whether ERF102 acts downstream of ET signaling during FCD, we checked the expression profiles of *ERF102*. *ERF1* (At3g23240) was used as a positive control for

the ET-inducible gene (Fig. 6A, left). ET-dependent induction of *ERF1* was suppressed in *etr1-1* and *ein3* mutants (Fig. 6A, middle, right). *ERF102* was also an ET-inducible gene (Fig. 6B). The expressions of *ERF1* and *ERF102* were induced by FB1 treatment (Fig. 6C, left and D, left). However, FB1-inducible expression of *ERF1* and *ERF102* was suppressed in *etr1-1* and *ein3* mutants (Fig. 6C, middle, right and 6D, middle, right). These results indicate that *ERF102* acts downstream of ET signaling during FCD.

ERF102 positively regulates factors participating in cell death development.

We examined ERF102 as an AP2/ERF family transcription factor by using the method shown in Figure 3. MACD1 was a transcriptional activator, showed GCC-box-mediated GUS activity and nuclear localization (Supplementary Fig. S5).

To examine the function of ERF102 in PCD, we produced ERF102 OE plants. The expression of ERF102 increased in ERF102 OE plants (Supplementary Fig. 6B). ERF102 OE lines showed no apparent phenotypic variation compared with Columbia (Col-0) *Arabidopsis* wild-type plants. Necrosis by FB1 was more severe in ERF102 OE plants than in Col-0 control plants. Taken together, ERF102 positively regulates genes participating in cell death induction.

We showed that FCD is more severe in ERF102 OE plants than in Col-0 control plants. Mutual antagonism between jasmonic acid (JA)/ET and salicylic acid (SA) signaling is well known (Glazebrook 2005; Verhage et al. 2010). UV-C-inducible SA-responsive *pathogenesis-related-1* (*PR-1*) is compromised in ERF102 (ERF5) or ERF103 (ERF6) OE plants (Moffat et al. 2012). It is possible that suppression of SA signaling by ERF102 OE is important for the FCD induction. To investigate this possibility, we co-treated ERF102 OE plants with FB1 and SA. We confirmed that *PR-1* is induced by the spray treatment of 1 mM SA (Supplementary Fig. S7A). Col-0, ERF102 OE-1, and ERF102 OE-5 plants were treated with FB1 3 h after SA treatment. FCD was normally induced and ERF102 OE-5 showed severe FCD in the presence of SA. Asai and associates (2000) showed that SA signaling is required for FCD in *Arabidopsis* protoplasts. These data suggest that the FCD phenotype does not depend on the suppression of SA signaling by ERF102 OE.

ERF102 participates in ACD.

AAL disrupts sphingolipid metabolism and subsequent PCD in its sensitive plants (Gilchrist 1997; Spassieva et al. 2002; Wang et al. 1996). Although the exact mechanisms of cell death activation and execution remain elusive, the presence of *Asc*, which is a homolog of the yeast *longevity assurance gene* (*LAG1*), prevents perturbations in sphingolipid metabolism and cell death (Brandwagt et al. 2000). Gechev and associates (2004) established that a knockout of *Asc* homolog *lag one homologue 2* (*loh2*) in *A. thaliana* ecotype Wassilewskija plants was markedly more sensitive to AAL.

To further examine the participation of ERF102 in phyto-toxin-induced cell death, we obtained *loh2* (At3g19260) mutants in Col-0 from the SALK T-DNA collection (Egusa et al. 2013). Each mutant had T-DNA inserted into an exon or an intron of *LOH2* and was named *loh2-1* (SALK_018608C) (Egusa et al. 2013; Ternes et al. 2011) and *loh2-2* (SALK_024192C), respectively (Supplementary Fig. S8A). AAL (1 μ M) induced cell death in *loh2* mutants. The *loh2* mutants showed high sensitivity to AAL, and were treated with low-concentration AAL (0.02 μ M) in the following experiments. To evaluate the participation of ERF102 in ACD, we generated the *loh2-1/erf102* double mutant (Fig. 7A and B), which showed reduced ACD (Fig. 7C and D). In addition, *ERF102* expression was

Fig. 4. AAL-triggered cell death (ACD) emerges more rapidly in modulator of AAL cell death 1 (MACD1) overexpression (OE) plants. **A**, Effects of MACD1 OE by agroinfiltration in *Nicotiana umbratica*. **B**, Transient expression of MACD1 protein in *N. umbratica* leaves. Proteins were analyzed by immunoblotting using anti-hemagglutinin (HA) antibody. Protein loads were monitored by Coomassie Brilliant Blue (CBB) staining of the bands corresponding to ribulose-1,5-bisphosphate carboxylase large subunit (RBCL). Asterisk indicates the pertinent band of MACD1. **C**, Cell death shown in **A** quantified by ion leakage from infiltrated-leaves. Data are means $\pm$ standard deviations (SD) from at least three independent experiments. **D**, Effects of MACD1 OE on ACD. Leaves were treated with AAL at 24 h after agroinfiltration. **E**, ACD shown in **D** quantified by ion leakage from AAL-treated leaves. Data are means $\pm$ SD from at least three independent experiments: ** and * indicate $P < 0.01$ and 0.05 , respectively, versus β -glucuronidase (*GUS*) OE control plants.

Fig. 5. Ethylene signaling mutants and *erf102*, *erf102/103* double mutants compromise fumonisin B1 (FB1)-triggered cell death (FCD). **A** and **B**, Effects of knockouts *ETR1-1* and *EIN3*. FCD shown in **A** was quantified by ion leakage from FB1-treated leaves in **B**. **C** and **D**, Effects of knockouts *ERF102*, *ERF103*, and *ERF104*. FCD shown in **C** was quantified by ion leakage from FB1-treated leaves in **D**. **E**, Effects of knockouts *ERF102*, *ERF103*, and *ERF102/103* on FCD. Data are means $\pm$ standard deviations from at least three independent experiments. Data underwent a Student's *t* test: ** and * indicate $P < 0.01$ and 0.05 , respectively, versus Col-0 control plants.

Fig. 6. Transcript accumulation of *ERF102* in response to ethylene (ET) and fumonisin B1 (FB1). Expression levels of **A**, *ERF1* and **B**, *ERF102* after ET treatment in Col-0 (left), *etr1-1* (middle), and *ein3* (right). *Actin* was used as an internal standard. Data are means $\pm$ standard deviations (SD) from three experiments. Expression profiles of **C**, *ERF1* and **D**, *ERF102* after FB1 treatment in Col-0 (left), *etr1-1* (middle), and *ein3* (right). *Actin* was used as an internal standard. Data are means $\pm$ SD from three experiments.

induced by AAL treatment (Fig. 7E). Taken together, ERF102 participates in *A. thaliana* ACD.

Microarray analysis for ERF102 downstream targets.

To gain insight into genes regulated by ERF102, we profiled gene expression in ERF102 OE plants by using microarray analysis. ERF102 OE plants showed 274 upregulated and 374 downregulated genes (at least threefold changes; false discovery rate, $P < 0.05$) (Supplementary Tables S1 and S2). Functional grouping based on the *Arabidopsis* Information Resource database annotation showed that various genes are upregulated or downregulated in ERF102 OE plants (Supplementary Fig. S9). To explore genes participating in PCD under control of ERF102, our microarray data were compared with microarray data for *loh2* mutants treated with AAL (Gechev et al. 2004). ERF102 is a transcriptional activator and is induced by AAL. We focused on upregulated genes in both ERF102 OE plants and AAL-treated *loh2* mutants (Fig. 8A). Thirty-four genes were selected as candidates for ERF102-regulated genes participating in PCD (Table 1). We examined the expression levels

of these 34 genes in *loh2-1* and *loh2-1/erf102* double mutants at 24 and 48 h after AAL-treatment by using quantitative reverse-transcriptase (qRT)-PCR analysis. Genes *GLUTATHIONE S-TRANSFERASE TAU 2* (*AtGSTU2*) (*At2g29480*), *GLUTATHIONE S-TRANSFERASE TAU 1* (*AtGSTU1*) (*At2g29490*), *glycosyl hydrolase 17* (*GH17*) (*At4g16260*), and *GH3.5*, *WES1* (*At4g27260*) stood out as possibly regulated by ERF102, because they were downregulated in the *loh2-1/erf102* double mutant (Fig. 8B).

DISCUSSION

In this study, we identified MACD1 as the modulator of ACD in *N. umbratica*. MACD1 was a transcriptional activator, induced GCC-box-mediated GUS activity, and localized in the nucleus (Fig. 3), indicating that MACD1 has the features of an AP2/ERF transcription factor. MACD1 OE did not induce cell death in *N. umbratica* (Fig. 4A–C), indicating that MACD1 OE is not sufficient to induce cell death. Previously, we reported that ET signaling has important roles in ACD but ET treatment

Fig. 7. *loh2-1/erf102* double mutant compromises ACD. Expression levels of **A**, *ERF102* and **B**, *LOH2* in Col-0 plants, *erf102* and *loh2-1* mutants, and *loh2-1/erf102* double mutant. *Actin* was used as an internal standard. Data are means $\pm$ standard deviations (SD) from three experiments. **C**, Effects of knockout *ERF102* on ACD in *loh2-1* mutant. **D**, ACD quantified by ion leakage from AAL-treated leaves. Data are means $\pm$ SD from at least three independent experiments. Data underwent a Student's *t* test: ** and * indicate $P < 0.01$ and 0.05 , respectively, versus *loh2-1* mutant. **E**, Expression profiles of *ERF102* in response to AAL in *loh2-1* mutant (left) and Col-0 plants (right). *Actin* was used as an internal standard. Data are means $\pm$ SD from three experiments.

Fig. 8. Upregulated genes in ERF102 overexpression (OE) plants and AAL-treated plants. **A**, Comparison of upregulated genes in ERF102 OE plants to upregulated genes in response to AAL (obtained in microarray experiments by Gechev and associates [2004]). The Venn diagram indicates the number of genes with >3 -fold (upregulated) expression in ERF102 OE plants or AAL-treated plants compared with wild-type or mock-treated plants, respectively. **B**, Transcript accumulation of *At2g29480*, *At2g29490*, *At4g16260*, and *At4g27260* in response to AAL in *loh2-1* and *loh2-1/erf102* double mutants. *Actin* was used as an internal standard. Data are means $\pm$ standard deviations from three experiments. Data underwent a Student's *t* test: ** and * indicate $P < 0.01$ and 0.05 , respectively, versus the *loh2-1* mutant.

itself does not induce cell death and increase ion leakage (Mase et al. 2012). Taken together, the ET signaling pathway seems to be required for full induction of PCD. In this study, the necrotic symptom of ACD was more severe in MACD1 OE plants (Fig. 4D and E). MACD1 was a transcriptional activator (Fig. 3A and B). These results suggest that MACD1 positively regulates factors participating in cell death progression. As an example of cell death induced by ERF OE, NbCD1 OE induces HR-like cell death in *N. benthamiana* (Nasir et al. 2005). NtERF3 and ERF that contain the EAR motif from both tobacco (*N. benthamiana*) and rice also induce HR-like cell death when overproduced in

tobacco plants (Ogata et al. 2012). NbCD1 and NtERF3 showed transcriptional repressor activity through their EAR motif, which was necessary for NbCD1 and NtERF3 to cause HR-like cell death, suggesting that NbCD1 and NtERF3 negatively regulate the negative regulator of cell death.

Multiple alignment analyses showed that *MACD1* homologs in *A. thaliana* are classified into *A. thaliana* ERF group IX (Nakano et al. 2006). To evaluate the participation of MACD1 homologs in toxin-induced PCD in *A. thaliana*, *erf102*, *erf103*, and *erf104* mutants were treated with FB1 (Fig. 5C and D). The *erf102* mutant showed significantly reduced FCD; there-

Table 1. Genes upregulated in ERF102 overexpression (OE) plants and AAL-treated plants^a

AGI	Description	ERF OE		AAL 7		AAL 24		AAL 48		AAL 72	
		FC	P value	FC	P value	FC	P value	FC	P value	FC	P value
Defense											
AT5G44430	PDF1.2c (plant defensin 1.2c)	140	4.95E-09	...	...	...	...	4.3	1.82E-06	11.1	2E-09
AT5G44420	PDF1.2 (plant defensin 1.2)	50.4	1.79E-08	...	...	...	...	8.47	7.96E-09	23.8	1.87E-10
AT5G22570	WRKY38	5.79	0.001805	...	...	4.32	1.9E-06	8.76	6.67E-09	4.76	6.75E-07
AT2G26020	PDF1.2b (plant defensin 1.2b)	144	2.59E-10	...	...	...	...	4.98	4.4E-07	13.5	9.04E-10
AT2G26010	PDF1.3 (plant defensin 1.3)	97.1	6.05E-14	...	...	...	...	3.87	5.55E-06	7.82	1.29E-08
AT1G06160	AP2/ERF 59 DNA binding/transcription activator/transcription factor (ORA59)	5.21	1.02E-05	...	...	...	...	4.1	3.35E-06	3.84	6.98E-06
AT3G49110	PRXCA (PEROXIDASE CA)	10.7	0.000685	...	...	...	...	4.28	3.01E-06	12	2.24E-09
Development											
AT4G27260	GH3.5, WES1	9.12	0.002481	...	...	...	...	2.42	1E-03	...	...
AT3G44750	HDA3 (HISTONE DEACETYLASE 3)	3.13	2.94E-07	...	...	...	...	3.86	5.88E-06	...	...
AT3G16360	AHP4 (HPT PHOSPHOTRANSMITTER 4)	3.61	0.000622	3.25	0.003678	...	...	...	...	...	...
AT2G44790	UCC2 (UCLACYANIN 2)	5.72	0.0003	...	...	...	...	14.9	1.06E-09	7.72	1.78E-08
Metabolism											
AT5G61160	AACT1 (anthocyanin 5-aromatic acyltransferase 1)	6.42	1.53E-06	...	...	...	...	...	...	3.05	8.97E-05
AT3G43190	SUS4 (sucrose synthase 4)	4.24	0.000655	...	...	...	...	4.36	1.79E-06	6.95	3.03E-08
AT1G56650	PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT 1)	3.7	0.000219	...	...	...	...	12.4	1.39E-09	...	...
Senescence											
AT5G01600	ATFER1 (ferric iron binding/iron ion binding)	3.07	2.23E-09	...	...	...	...	5.68	1.36E-07	6.08	7.84E-08
Stress											
AT4G08770	Peroxidase, putative	3.83	0.004602	...	...	...	...	6.47	4.8E-08	33.1	1.07E-10
AT3G09270	ATGSTU8 (GLUTATHIONE S-TRANSFERASE TAU 8)	3.77	0.002656	...	...	4.57	1.11E-06	...	...	...	...
AT2G29490	ATGSTU1 (GLUTATHIONE S-TRANSFERASE TAU 1)	3.96	1.55E-08	...	...	...	...	11.8	1.56E-09	11.8	1.55E-09
AT4G16260	Glycosyl hydrolase 17	3.96	0.002085	...	...	...	...	18.8	3.24E-10	31.5	1.15E-10
Transporter											
AT1G58340	ZF14 (antiporter/ drug transporter/ transporter)	6.55	0.005817	...	...	...	...	11.5	2E-09	35.8	1.2E-10
Unknown											
AT5G44480	DUR (DEFECTIVE UGE IN ROOT)	3.44	0.001349	...	...	3.71	1.42E-05	19.6	2.94E-10	39.1	8.76E-11
AT5G22791	Unknown protein	5.46	3.17E-06	...	...	...	...	6.53	4.97E-08	...	...
AT5G05600	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	4.07	3.35E-05	4.4	1.45E-06	...	...	...	...	...	...
AT4G15970	Unknown protein	6.13	0.000721	...	...	...	...	...	...	3.31	3.23E-05
AT3G55970	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	10.7	0.001212	...	...	...	...	36.7	9.26E-11	65.1	5.31E-11
AT3G49620	DIN11 (DARK INDUCIBLE 11); iron ion binding/oxidoreductase (DIN11)	6.85	1.3E-09	...	...	3.88	0.000441	65.4	5.96E-11	8.44	1.38E-08
AT3G23550	MATE efflux family protein	3.44	3.87E-06	...	...	...	...	...	...	...	...
AT2G43870	Polygalacturonase, putative/pectinase, putative	3.53	3.66E-05	...	...	...	...	4.22	0.000188	...	...
AT2G43590	Chitinase, putative	3.2	8.78E-09	...	...	...	...	...	...	5.24	3.69E-07
AT2G43580	Chitinase, putative	3.46	8.61E-06	...	...	...	...	4.82	6.02E-07	10.7	2.43E-09
AT2G39030	GCN5-related N-acetyltransferase (GNAT) family protein	41.3	3.64E-06	...	...	...	...	18.3	3.48E-10	14.9	6.42E-10
AT2G37870	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	3.25	1.24E-05	...	...	...	...	3.36	4.13E-05	...	...
AT2G29480	ATGSTU2 (GLUTATHIONE S-TRANSFERASE TAU 2)	7.43	0.001261	...	...	3.01	9.97E-05	51.4	6.43E-11	87.9	4.41E-11
AT1G73325	trypsin and protease inhibitor family protein/Kunitz family protein	7.41	1.22E-08	3.66	0.004237	...	...	...	...	...	...
AT1G26450	β-1,3-Glucanase-related	3.24	4.64E-05	...	...	...	...	...	...	3.34	6.74E-05
AT1G15870	Mitochondrial glycoprotein family protein/MAM33 family protein	4	0.001935	...	...	...	...	4.23	2.81E-06	3.28	5.81E-05
AT1G09470	Unknown protein	3.47	0.005066	...	...	...	...	4.81	1.1E-06	...	...
AT3G49840	Unknown protein	7.13	5.6E-08	...	...	...	...	7.34	1.96E-08	...	...

^a ERF = ethylene response factor, FC = fold change

fore, we think ERF102 is a MACD1 ortholog in *A. thaliana*. However, the AP2/ERF superfamily in *A. thaliana* contains 122 members (Nakano et al. 2006). Possibly, some ERF are regulated by the same stimuli and bind to the same promoter element; therefore, a high level of functional redundancy may be expected. This notion is supported by Moffat and associates (2012), who found that ERF5 (ERF102) and ERF6 (ERF103) have redundant roles in JA/ET-mediated defense against *Botrytis cinerea* in *A. thaliana*; although no marked difference in susceptibility to *B. cinerea* was observed in either the *erf5* or *erf6* mutant, the *erf5/erf6* double mutant showed a marked increase in susceptibility. For this functional redundancy, the roles of ERF102 and ERF103 in FCD were investigated using the *erf102/erf103* double mutant (Moffat et al. 2012). The *erf102/erf103* double mutant showed lower conductivity than the *erf102* single mutant (Fig. 5E), suggesting that ERF103 also contributes FCD induction.

ERF102 OE plants showed activation of some defense-related genes, such as *PDF1.2* genes and *chitinase*. Our microarray data agreed with Moffat and associates (2012), who showed that JA-mediated defense-responsive genes, such as *PDF1.2* genes, are upregulated in ERF5 (ERF102) OE plants and ERF6 (ERF103) OE plants. They also showed that ERF5 and ERF6 act as positive regulators of JA-mediated defense against *B. cinerea* and as negative regulators of UV-C-induced *PR-1* expression, which is an SA-inducible gene. The mutual antagonism between the JA/ET and SA signaling pathways is well-established (Glazebrook 2005; Verhage et al. 2010). However, Son and associates (2012) showed that ERF5 (ERF102) appears to negatively regulate plant defense against the necrotrophic fungal pathogen *Alternaria brassicicola* and positively regulate SA signaling and plant defense against bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Although the results of these studies conflict, they suggest that ERF102 participates not only in the induction of PCD but also in response to biotic stress. Now, many examples exist of *Arabidopsis thaliana* IX ERF OE modulating plant defense. ERF1 (At3g23240) was suggested to act as an integrator of JA- and ET-dependent pathways in *A. thaliana* (Lorenzo et al. 2003). ERF1 OE activates the expression of several defense-related genes, including *PDF1.2* and *basic chitinase* (*ChiB*) (Lorenzo et al. 2003; Solano et al. 1998), and confers resistance to several fungi, including *B. cinerea* (Berrocal-Lobo et al. 2002). ORA59 OE (At1g06160) activates the transcription of *PDF1.2* and increases resistance against the fungus *B. cinerea* (Pré et al. 2008). AtERF2 OE (At5g47220) (Brown et al. 2003; McGrath et al. 2005) or AtERF14 OE (At1g04370) (Oñate-Sánchez et al. 2007) also causes high levels of *PDF1.2* and *ChiB* gene expression in transgenic *A. thaliana*. AtERF2 OE plants show resistance to *Fusarium oxysporum* (McGrath et al. 2005). Overexpression of several ERF modifies defense gene expression and resistance to various pathogens but little has been reported on the participation of ERF in PCD induction. Therefore, we think our results provide new insights into the function of ERF in plant PCD.

Microarray analysis showed that ERF102 OE affects the expressions of various genes but showed no clear trend of specific pathways being affected. To further investigate genes regulated by ERF102 in ACD, our microarray data were compared with microarray data for *loh2* mutants treated with AAL (Gechev et al. 2004) (Fig. 8A). We selected 34 upregulated genes in both ERF102 OE plants and AAL-treated plants (Table 1) and investigated the expression profiles of 34 genes by using the microarray databases. As a result, we found that most of the 34 genes are upregulated by JA or ET/JA treatment (Supplementary Fig. S10A), suggesting that ERF102 OE mimics the JA signaling pathway. Exogenous JA treatment has a pro-

motional effect on the susceptibility to *Alternaria alternata* f. sp. *lycopersici* in tomato (Egusa et al. 2009). We also treated *N. umbratica* leaves with JA and inoculated *A. alternata* f. sp. *lycopersici*. The disease symptoms and fungal biomass were developed compared with control plants. Our data suggest that AAL does not only induce PCD in host plants but also promotes susceptibility to *A. alternata* f. sp. *lycopersici* by manipulating host plant JA signaling via ERF102.

Four genes were identified as putative targets of ERF102 by using qRT-PCR analysis (Fig. 8B). ERF102 induced GCC-box-mediated GUS activity. We examined whether a GCC-box is found in 2-kb upstream regions of four genes. We found a GCC-box (AGCCGCC) in only the upstream region of *At4g16260*; possibly, *At4g16260* is directly regulated by ERF102 and has a role in plant PCD. *At4g16260 GH17*, which is a member of the PR-2 protein groups, encodes β -1,3-endoglucanase (Doxey et al. 2007; Mahalingam et al. 2003). *GH17* is highly induced by pathogen stress, such as by *A. brassicicola*, *B. cinerea*, *Erysiphe orontii*, and *Phytophthora infestans* (Doxey et al. 2007). Proteomics analysis shows that the protein level of GH17 increases in response to *A. brassicicola* (Mukherjee et al. 2010). *Arabidopsis thaliana* GH17 T-DNA mutants show increased susceptibility to cyst nematode *Heterodera schachtii* and GH17 OE plants show reduced nematode susceptibility, suggesting a potential role of host GH17 in the defense response against *H. schachtii* infection (Hamamouch et al. 2012). These observations indicate that GH17 mainly has a role in plant defense response. Little knowledge exists of GH17 affecting plant PCD. Pepper (*Capsicum annuum*) PR-10 protein was identified as a leucine-rich repeat protein1 (LRR1)-interacting partner (Choi et al. 2012). Not only does PR-10 confer resistance to pathogens but transient expression of PR-10 also triggers HR cell death in pepper and *N. benthamiana* leaves, which are amplified by LRR1 coexpression as a positive regulator. Possibly, some PR proteins induced by pathogen attack also contribute to PCD induction.

The results of this study show a role for MACD1 and ERF102 in phytoalexin-induced cell death: they regulate genes participating in cell death development. We identified the genes under control of ERF102 during ACD in *A. thaliana*. Future studies will focus on the key target genes of ERF102 responsible for plant PCD.

MATERIALS AND METHODS

Plant growth conditions.

N. umbratica and *N. benthamiana* plants were grown in environmentally controlled growth cabinets under a 16-h photoperiod and an 8-h dark period at 23°C. *A. thaliana* plants were grown in environmentally controlled growth cabinets under a 10-h photoperiod and a 14-h dark period at 23°C.

Mutants and T-DNA insertion lines.

The *erf102* (SALK_076967), *erf103* (SALK_087356), *loh2-1* (SALK_018608C), and *loh2-2* (SALK_024192C) mutants of *A. thaliana* (ecotype, Columbia-0) were obtained from the *Arabidopsis* Biological Resource Center (ABRC); *ein3* (Roman et al. 1995) and *etr1-1* mutants (Chang et al. 1993) were obtained from the ABRC; the *erf104* mutant (Bethke et al. 2009) was provided by J. Lee (University of Cologne); and the *erf102/erf103* double mutant (Moffat et al. 2012) was provided by M. R. Knight (Durham University).

VIGS.

We amplified cDNA fragments of each gene from the *N. umbratica* cDNA library as templates using primers listed in Supplementary Table S3, and the DNA fragments were ligated

into pTV00 vector as described by Ratcliff and associates (2001). The constructs containing these inserts were designated pTRV00:NuERF1 (TRV:NuERF1), pTRV00:NuERF2 (TRV:NuERF2), pTV00:NuERF4 (TRV:NuERF4), and pTV00:NuERF5 (TRV:NuERF5). pTV00:EIL (TRV:EIL) and pTV00:EIL5 (TRV:EIL5) were described by Mase and associates (2012). pTV00 that contained no inserts was used as a control. For VIGS assay as described by Asai and associates (2010), pBINTRA6 or pTV00 and its derivatives were introduced into *Agrobacterium* strain GV3101 by electroporation (Bio-Rad, Hercules, CA, U.S.A.). After an additional 3 to 4 weeks, plants were analyzed by treatment with AAL.

***Agrobacterium tumefaciens*-mediated transient expression (agroinfiltration) in *N. umbratica*.**

The following primers were used to amplify cDNA fragments of *MACD1* from the *N. umbratica* cDNA library. Restriction sites were added to the 5' ends of the forward and reverse primers for cloning into the pGreen binary vector (Hellens et al. 2000): *MACD1*-F-*Cl*aI (5'-CCATCGATATGGC ATCACCACAAGAGAATTGCAC-3') and *MACD1*-R-*B*amHI (5'-CGGATCCTCATATCATGACAAGCTGAGAATAGGC-3'). The binary plasmids were transformed into *Agrobacterium* sp. strain GV3101. Agroinfiltration was done as described by Asai and associates (2008).

RT-PCR.

Total RNA from *N. umbratica* and *Arabidopsis thaliana* leaves was prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's procedure. Reverse transcription was done by using ReverTra Ace -a-, and qRT-PCR analysis was done by using the STEPONEPLUS REAL-TIME PCR system (Applied Biosystems, Foster City, CA, U.S.A.) with POWER SYBR GREEN PCR MASTER MIX (Applied Biosystems). *EF-1 α* and *actin* were used as a control in *N. umbratica* and *A. thaliana*, respectively. The gene-specific primers of each sequence are listed in Supplementary Table S4.

Toxin treatment.

AAL treatment: AAL (1 μ M) or AAL (0.02 μ M) was infiltrated into *N. umbratica* or *A. thaliana* leaves, respectively, by using a needleless 1-ml syringe. FB1 treatment: FB1 (5 μ M) was infiltrated into *A. thaliana* leaves by using a needleless 1-ml syringe.

Measurements of cell death.

Cell death was measured as electrolyte leakage from toxin-treated leaves as described by Asai and associates (2010). For a conductivity test using *N. umbratica*, five discs per leaf (5 mm in diameter) were obtained from the treated leaf. For *Arabidopsis*, six discs (5 mm in diameter) obtained from all phyto-toxin-treated leaves were used. These leaf discs were immersed in 10 ml of ultrapure water for 4 h at room temperature with gentle shaking. The conductivity of the solution was measured by using a pH/COND METER D-54 (HORIBA, Kyoto, Japan).

Assays of transcriptional activity in *N. benthamiana*.

For reporter plasmids, six tandem repeats of GAL4 upstream activation sequence 5'-CGGAGTACTGTCCTCCG-3' were fused upstream of the minimal 35S promoter (246 bp) sequence and were cloned into pGreen binary vector (Hellens et al. 2000). Then, the cDNA of the GUS reporter gene containing one intron was cloned behind the synthetic promoter. For effector plasmids, C-terminal cDNA fragments of *MACD1* (amino acid positions 205 to 273), *ERF102* (amino acid posi-

tions 81 to 170), or the *VP16* activation domain (amino acid positions 413 to 490) were fused downstream of the GAL4 DNA binding domain (amino acid positions 1 to 147) and were cloned into pGreen under the control of the 35S promoter. For reference plasmids, the cDNA fragment of luciferase (*LUC*) containing one intron was cloned into pGreen under the control of the 35S promoter. Transient assays were done using the agroinfiltration method described by Ishihama and associates (2011).

GFP fusion constructs for expression in *N. benthamiana*.

For subcellular localization analysis, cDNA fragments of *MACD1* and *ERF102* were cloned into pGWB405 and pGWB406, respectively, which fused GFP to the C terminus and N terminus, respectively, of the protein (Nakagawa et al. 2007). Each plasmid was transformed into *Agrobacterium* strain GV3101. Infiltration of *Agrobacterium* was done at an optical density at 600 nm of 0.5.

Transient expression to evaluate GCC-box-mediated GUS activity in *N. benthamiana*.

For a reporter gene construct, a 29-bp region containing the GCC box sequence from the *A. thaliana* *HLS1* gene (5'-ATGA GTTAACGCAGACATAGCCGCCATTT-3') was multimerized four times. For reporter plasmids, four tandem repeats of the 29-bp region containing the mutated GCC box (mGCC) sequence (5'-ATGAGTTAACGCAGACATAACCACCATTT-3') were fused upstream of the minimal 35S promoter (-46) and were cloned into pGreen. The cDNA of the GUS reporter gene containing one intron was then cloned behind the synthetic promoter. For effector plasmids, full-length *MACD1* or *ERF102* was cloned into pGreen under the control of the 35S promoter. For reference plasmids, the cDNA fragment of *LUC* containing one intron was cloned into pGreen under the control of the 35S promoter. Transient assays were done using the agroinfiltration method described by Ishihama and associates (2011).

Microscopy.

Epidermal cell layers of *N. benthamiana* leaves were assayed for fluorescence 48 h after agroinfiltration. Cells were visualized using a fluorescence microscope (AXIO IMAGER M1; Carl Zeiss, Oberkochen, Germany) with fluorescent filters: filter set 38HE (excitation BP 470/40, beam splitter FT 395, emission BP 525/50) for GFP and filter set 49 (excitation G 365, beam splitter FT 395, emission BP 445/50) for DAPI. Images were collected by using a CCD camera (AXIO CAM HRC; Carl Zeiss).

Microarray analysis.

Three-week-old wild-type and *ERF102* OE leaves were used for microarray analysis that included replicated experiments 1, 2, 3, and 4. Total RNA was prepared from these leaves using TRIZOL reagent (Invitrogen) according to the manufacturer's procedure. The two-color microarray experiments were done using the Agilent *A. thaliana* OLIGO DNA MICROARRAY with a capacity of 44,000 (ver. 4; Agilent Technologies, Santa Clara, CA, U.S.A.) according to the manufacturer's 60-mer oligo microarray processing protocol. Total RNA (200 ng) from each plant sample was used to prepare Cy3-labeled or Cy5-labeled cRNA, using a LOW INPUT QUICK AMP LABELING KIT (Agilent Technologies). Hybridized and washed arrays were scanned at maximum laser intensity in the Cy3 channel using an Agilent Technologies MICROARRAY SCANNER (Agilent Technologies). Normalization was done using Linear and LOWESS normalization. After normalization, genes scored as present in all genotypes underwent further analysis. Upregulated or downregulated genes with greater than threefold

changes in expression ratio (wild-type plants versus ERF102 OE plants) were selected in the experiments.

Data and statistical analysis.

At least three repetitions with individual biological sample sets were used for statistical treatment of the data results. The data are expressed as mean values; error bars indicate the standard error. The data underwent a Student's *t* test to evaluate their significances of difference.

ACKNOWLEDGMENTS

We thank D. C. Baulcombe for the pTV00 vector, P. Mullineaux and R. Hellens for the pGreen vector, T. Nakagawa for the pGWB vectors, J. Lee for the *erf104* mutant, M. R. Knight for the *erf102/erf103* mutant, the Leaf Tobacco Research Center, Japan Tobacco for *N. umbratica* seed, and M. Egusa for technical assistance. This work was supported by a Grant-in-Aid for Scientific Research (B) (20380028, 23380025) from the Japan Society for the Promotion of Science and Grant-in-Aid for Japan Society for the Promotion of Science Fellows (10J05393).

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