GmEREBP1 Is a Transcription Factor Activating Defense Genes in Soybean and *Arabidopsis*

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Ethylene-responsive element-binding proteins (EREBPs) are plant-specific transcription factors, many of which have been linked to plant defense responses. Conserved EREBP domains bind to the GCC box, a promoter element found in pathogenesis-related (PR) genes. We previously identified an EREBP gene from soybean (*GmEREBP1*) whose transcript abundance decreased in soybean cyst-nematode-infected roots of a susceptible cultivar, whereas it increased in abundance in infected roots of a resistant cultivar. Here, we report further characterization of this gene. Transient expression analyses showed that *GmEREBP1* is localized to the plant nucleus and functions as a transcriptional activator in soybean leaves. Transgenic soybean plants expressing *GmEREBP1* activated the expression of the ethylene (ET)-responsive gene *PR2* and the ET- and jasmonic acid (JA)-responsive gene *PR3*, and the salicylic acid (SA)-responsive gene *PR1* but not the SA-responsive *PR5*. Similarly, transgenic *Arabidopsis* plants expressing *GmEREBP1* showed elevated mRNA abundance of the ET-regulated gene *PDF1.2* but not the ET-regulated *GST2*, and the SA-regulated gene *PR1* but not the SA-regulated *PR2* and *PR5*. Transgenic soybean and *Arabidopsis* plants inoculated with cyst nematodes did not display a significantly altered susceptibility to nematode infection. These results collectively show that *GmEREBP1* functions as a trans-acting inducer of defense gene expression in both soybean and *Arabidopsis* and mediates the expression of both ET- and JA- and SA-regulated defense-related genes in these plant species.

Additional keywords: *cis* elements, ethylene-insensitive soybean mutant *etr1-1*, GFP, GUS, protoplasts, quantitative real-time reverse-transcription PCR.

Plants have the ability to defend themselves against pathogens by activating defense responses to constrain pathogen infection (Feys and Parker 2000; Glazebrook 2001, 2005; McDowell and Dangl 2000; Thomma et al. 2001). Effective induction of defense responses usually requires pathogen recognition followed by a network of signal transduction processes, resulting in the rapid activation of defense gene expression (Bostock 2005; Rojo et al. 2003). A number of classes of regulatory proteins and transcription factors are known to play important

roles in relaying the pathogen-initiated signals to downstream components for the activation of plant defense responses (Singh et al. 2002). Among these, ethylene-responsive element-binding protein (EREBP) transcription factors (Nakano et al. 2006; Riechmann and Meyerowitz 1998) are implicated as key regulators of plant defense responses (Gutterson and Reuber 2004; Ohme-Takagi et al. 2000). Conserved EREBP domains bind to the GCC box, a promoter element found in many pathogenesis-related (PR) genes. The GCC box is necessary and sufficient for ethylene (ET) regulation of plant genes (Stepanova and Ecker 2000).

We previously showed that the messenger (m)RNA abundance of an *Arabidopsis* EREBP gene (*AtEBP*) decreased during successful sugar beet cyst nematode infection (Hermesmeier et al. 2000). The downregulation of *AtEBP* in cyst-nematode-infected *Arabidopsis* roots prompted us to examine the possible role of EREBP genes in the economically very important cyst nematode–soybean (*Glycine max*) interaction.

The soybean cyst nematode, *Heterodera glycines*, is a sedentary biotrophic endoparasite of roots that causes extensive damage to soybean worldwide (Wrather et al. 2001a,b). Infective larvae of *H. glycines* enter host roots and migrate intracellularly within the cortical tissue to the vascular cylinder. Larvae then initiate localized reorganization of the host's cell morphology and physiology, resulting in the formation of specialized feeding sites called syncytia (Jones 1981). *H. glycines* nematodes feed exclusively from their syncytia as they develop into adult males or females. They reproduce sexually and, once fertilized, the female produces up to several hundred eggs that, for the most part, are retained within the nematode uterus. After the female's death, her body develops into a protective cyst around the eggs, giving these nematodes their name.

In our efforts to elucidate the roles of EREBPs in cyst nematode–plant interactions, we identified the soybean EREBP gene *GmEREBP1* and determined that its transcript abundance decreased in soybean cyst-nematode-infected roots of a cyst-nematode-susceptible soybean cultivar, whereas it increased in abundance in infected roots of a resistant cultivar (Mazarei et al. 2002). These results suggested that *GmEREBP1* may be part of a plant defense response and that cyst nematode infection may actively suppress EREBP expression in the susceptible interaction as a means to avoid plant defense responses.

In the present study, we report further characterization of *GmEREBP1*, including protein localization and regulatory activity, as well as the analysis of transgenic soybean and *Arabidopsis* plants expressing *GmEREBP1*. Our results show that *GmEREBP1* functions as a transcription factor in soybean and *Arabidopsis*, inducing expression of defense-related genes.

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RESULTS

GmEREBP1 localizes to the nucleus.

Analysis of the amino acid sequence of GmEREBP1 revealed that this protein contains a canonical nuclear localization signal (Mazarei et al. 2002). To examine the subcellular localization of GmEREBP1, its coding region was fused translationally with the green fluorescent protein (GFP) and β -glucuronidase (GUS) reporter genes and placed under the transcriptional control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. The resulting fusion product is large enough (117 kDa) to prevent passive diffusion into nuclei (Grebenok et al. 1997). The 35S::GmEREBP1-GFP-GUS fusion construct was introduced into onion epidermal cells and into *Arabidopsis* protoplasts. Transient expression of GmEREBP1-GFP-GUS translational fusions showed that the GmEREBP1 protein was targeted to the plant nucleus (Fig. 1). In contrast, when 35S::GFP-GUS was expressed, GFP fluorescence was distributed throughout the cell cytoplasm (data not shown).

GmEREBP1 is a transcriptional activator.

GmEREBP1 has been demonstrated to bind to the GCC box and to not bind to a mutated version of the GCC box in vitro (Mazarei et al. 2002). The ability of the GmEREBP1 to regulate transcription in plant cells was tested using transient expression assays. Soybean leaves were cotransformed with i) a reporter plasmid containing a GUS gene under the control of a minimal promoter containing a GCC box and ii) an effector plasmid consisting of the *GmEREBP1* complementary (c)DNA under the control of the CaMV 35S promoter. It has been shown that the binding affinity of EREBPs to GCC boxes can vary depending on the nucleotide sequences flanking the GCC box (Gu et al. 2002); therefore, we used two GCC box sequences, one from the tobacco β -1,3-endoglucanase promoter (Ohme-Takagi and Shinshi 1995) and one from the bean basic chitinase promoter (Broglie et al. 1989) in these experiments (Fig. 2A). Cotransformation of either reporter plasmid (i.e., containing either the tobacco or the bean GCC::GUS constructs) with the effector plasmid (35S::GmEREBP1) resulted in a statistically significant 3.5- to 4-fold increase in GUS activity (Fig. 2B and C). The extent of trans-activation was slightly higher for the tobacco GCC box se-

Fig. 1. Nuclear localization of *Glycine max* ethylene-responsive element-binding protein 1 (GmEREBP1) in plant cells. A, GmEREBP1-green fluorescent protein (GFP)- β -glucuronidase fusion construct was transiently expressed in A, B, and C, onion epidermal cells and D, E, and F, *Arabidopsis* protoplasts under the control of *Cauliflower mosaic virus* 35S promoter after delivery of vector DNA. Cells were analyzed for GFP fluorescence by fluorescence microscopy under bright-field or dark-field. Images A and D were taken using bright-field. Images B and E were taken using dark-field. C, overlay of images A and B. F, overlay of images D and E. Arrowheads indicate location of nuclei.

quence than for the bean GCC box sequence (Fig. 2C). Collectively, these results indicate that GmEREBP1 acts as an activator of transcription, which could be shown with two distinct GCC boxes.

Expression of *GmEREBP1* in soybean induces expression of defense-related genes.

In order to analyze whether GmEREBP1 regulates expression of defense-related genes in soybean and which genes are regulated, the expression of different classes of defense-related genes was examined in transgenic soybean plants expressing *GmEREBP1*. To generate transgenic plants, the recombinant binary vector containing the *GmEREBP1* cDNA under the control of the CaMV 35S promoter and the *bar* (phosphinothricin acetyltransferase) gene as selectable marker for resistance to Basta herbicide was introduced into the soybean cv. Thorne using *Agrobacterium*-mediated transformation (Paz et al. 2004). Five independent transformants (R0) were generated. Progenies (R1) of three independent transformants, S1, S2, and S3, showed a 3:1 segregation ratio for resistance and susceptibility to the Basta selection (Paz et al. 2004). Polymerase chain reaction (PCR) was used to confirm the presence of the 35S-*GmEREBP1* and *bar* transgenes in the genome of the R1 plants developed from the independent transgenic lines S1, S2, and S3. For initial screening, RNA slot blot hybridizations were used to assess *GmEREBP1* mRNA levels in transgenic soybean plants. Because *GmEREBP1* mRNA is barely detectable in RNA blots derived from wild-type soybean leaves (Mazarei et al. 2002), it was possible to assess transgene expression levels in slot blots of RNA from these organs. These analyses revealed that the *GmEREBP1* transcript was readily detectable in total RNA from leaves of transgenic soybean plants transformed with the 35S::*GmEREBP1* construct (Fig. 3A), whereas the *GmEREBP1* transcript was almost undetectable in total RNA from leaves of untransformed soybean plants (wild-type plants) (Fig. 3A). Furthermore, judging from the intensity of the hybridization signals, elevated *GmEREBP1* mRNA levels were most pronounced in soybean plants developed from the transgenic S3 line (Fig. 3A). Overexpression of *GmEREBP1* was not accompanied by obvious morphological or physiological plant phenotype changes.

Progenies (R2) of those transgenic R1 lines that showed 100% resistance to the Basta selection (i.e., homozygous lines) were used in the following experiments. In order to analyze expression of a panel of defense-related genes in transgenic soybean plants over-expressing *GmEREBP1*, we used quantitative real-time reverse-transcription (qRT)-PCR. In these assays, we first quantified the mRNA level of the *GmEREBP1* transgene in roots and shoots of the transgenic plants to confirm *GmEREBP1* overexpression in these organs. These measurements were in agreement with the RNA slot blot analyses showing elevated *GmEREBP1* mRNA levels in the transgenic plants (Fig. 3B). After establishing the *GmEREBP1* expression levels, we quantified mRNA steady-state levels of pathogenesis-related (PR) genes *PR1*, *PR2* (β -1,3-endoglucanase), *PR3* (basic chitinase), and *PR5* (thaumatin-like) (Table 1). Expression of *GmEREBP1* in transgenic soybean plants caused an increase in the steady-state levels of the *PR1*, *PR2*, and *PR3* transcripts in both roots and shoots when compared with wild-type plants (Fig. 3C; data of shoots not shown). No apparent change in the abundance of the *PR5* transcript was observed in the transgenic soybean plants compared with wild-type plants in either roots or shoots (Fig. 3C).

Expression of *GmEREBP1* in *Arabidopsis* induces expression of defense-related genes.

GmEREBP1 originally was isolated from soybean because its homologue in *Arabidopsis* (*AtEBP*) was shown to be downregu-

lated in sugar beet cyst nematode *H. schachtii*-infected *Arabidopsis* roots (Hermsmeier et al. 2000; Mazarei et al. 2002). In order to analyze whether GmEREBP1 regulates transcription of defense-related genes in *Arabidopsis* as well, the expression of different classes of defense-related genes was examined in transgenic *Arabidopsis* plants expressing *GmEREBP1*. To generate transgenic plants, the recombinant binary vector containing the *GmEREBP1* cDNA under the control of the CaMV 35S promoter and neomycin phosphotransferase (*nptII*) gene as selectable marker for resistance to kanamycin was introduced into the *Arabidopsis* ecotype Columbia using *Agrobacterium*-mediated transformation (Clough and Bent 1998). Fifteen independent

transformants (T0) were identified and maintained. Progenies (T1) of four independent transformants, A1 through A4, that showed a 3:1 segregation ratio for resistance and susceptibility to the kanamycin selection (Clough and Bent 1998) were chosen for further analysis. PCR analyses were used to confirm the presence of the *GmEREBP1* and *nptII* transgenes in the genome of the T1 plants generated from the independent transgenic lines A1 through A4. Slot blots of leaf RNA samples were used to assess *GmEREBP1* mRNA levels in transgenic *Arabidopsis* plants. The *GmEREBP1* transcript was evident in transgenic plants containing the 35S::*GmEREBP1* construct compared with untransformed plants (wild-type plants) (Fig. 4A). Furthermore,

Fig. 2. Transcriptional activation effects of *Glycine max* ethylene-responsive element-binding protein 1 (GmEREBP1) in soybean leaves. **A**, Schematic diagram of the reporter and the effector constructs used in co-bombardment experiments. The reporter constructs contain the tobacco or bean GCC box fused to the minimal TATA box (–42 to +8) from *Cauliflower mosaic virus* (CaMV) 35S promoter, the β -glucuronidase (GUS) reporter gene, and the nos terminator. The effector construct contains the CaMV 35S promoter, the GmEREBP1 cDNA, and the nos terminator. **B**, Histochemical GUS activity as a result of activation of the tobacco GCC-GUS reporter construct by GmEREBP1. The reporter plasmid was co-bombarded with the effector plasmid or the control plasmid pUC19. The internal reference plasmid used to normalize for transfection efficiency contains the CaMV 35S promoter fused to the luciferase (LUC) reporter gene. **C**, Fluorometric assay for GUS activity of the tobacco or bean GCC-GUS reporter construct co-expressed with GmEREBP1. GUS/LUC represents the ratio between the co-expressed GUS and LUC activities. Each bar represents the mean value of GUS activity obtained from three independent experiments with the standard errors of the mean noted. Significant GUS activity changes (indicated by asterisks) were determined statistically by paired *t* test ($P < 0.05$).

Fig. 3. Overexpression of *Glycine max* ethylene-responsive element-binding protein 1 (*GmEREBP1*) in soybean. **A**, RNA slot blot analysis of transgenic soybean plants. Blots of total RNA were obtained from leaves of wild-type and individual transgenic plants from the R1 generation of the independent lines S1, S2, and S3. Blots were hybridized with a probe derived from *GmEREBP1* and standardized using an actin probe. **B**, Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis of *GmEREBP1* messenger (m)RNA steady-state levels. *GmEREBP1* mRNA accumulation in root and shoot tissues of wild-type and transgenic plants from the R2 generation of the independent lines S1, S2, and S3. Each bar represents the mean of three independent experiments with the standard errors of the noted mean. **C**, Expression analysis of the soybean pathogenesis-related (*GmPR*) genes using qRT-PCR. mRNA accumulation of the *GmPR* genes in root tissue of the transgenic plants shown in B relative to the wild type (set to 1.0). Each bar represents the mean of three independent experiments with the standard errors of the mean noted. Mean values significantly different from 1.0 are indicated by asterisks as determined by paired *t* test ($P < 0.05$).

the intensity of the hybridization signals indicated that *GmEREBP1* expression was highest in *Arabidopsis* plants from the A4 line (Fig. 4A). As observed in transgenic soybean plants, overexpression of *GmEREBP1* did not produce visibly altered phenotypes.

Progenies (T2) of those independent lines that showed 100% resistance to the kanamycin selection (i.e., homozygous lines) were used in all of the following experiments. We used qRT-PCR to quantify the mRNA level of the *GmEREBP1* transgene as well as the *Arabidopsis* defense-related genes *PR1*, *PR2*, *PR3*, *PR5*, *PDF1.2* (plant defensin protein), and *GST2* (glutathione *S*-transferase) (Table 1) in roots and shoots of the transgenic *Arabidopsis* plants (Fig. 4B and C). Assessment of the transcript abundance of these defense-related genes in transgenic *Arabidopsis* plants showed that *PR1*, *PR3*, and *PDF1.2*, but not *PR2*, *PR5*, and *GST2* were induced in roots and shoots of *GmEREBP1*-expressing transgenic lines when compared with wild-type plants (Fig. 4C; data of shoots not shown). These results demonstrate that *GmEREBP1* also is functional in *Arabidopsis*.

Expression characteristics of soybean *PR* genes.

Although expression characteristics of *Arabidopsis* *PR* genes are well documented, this information is not available for the soybean *PR* genes assayed in the analyses of the transgenic soybean plants described above. Therefore, in order to establish the expression characteristics of soybean *PR1*, *PR2*, *PR3*, and *PR5* genes, we used qRT-PCR to analyze the effects of salicylic acid (SA), ET, and jasmonic acid (JA) on their mRNA steady-state levels in roots and shoots of wild-type soybean. Results of these experiments are shown in Figure 5. *PR1* mRNA abundance was significantly elevated in shoots but not in roots following SA treatment, whereas treatment with an ET precursor (1-aminocyclopropane-1-carboxylic acid [ACC]) and JA did not cause a significant change in the *PR1* mRNA steady-state level in either tissue. *PR2* mRNA abundance was significantly elevated in both shoots and roots following ACC treatment but was not altered by JA and SA treatments. *PR3* mRNA abundance was significantly increased by ET and JA in shoots and roots but was unaffected by SA. Treatment with ACC, JA, or SA produced a trend of decreased *PR5* mRNA steady-state levels, which was statistically significant only in SA-treated shoots.

The effect of ET on *PR*-gene expression was investigated further in the ET-insensitive soybean mutant *etr1-1* (Hoffman et al. 1999). mRNA expression of the *PR2* and *PR3* genes (as measured by qRT-PCR) was lower in this soybean mutant when compared with the isogenic wild-type parent line (Fig. 6), supporting our results that both genes were upregulated by ET. As was predicted from our results from ACC treatments, the expression of *PR1* and *PR5* genes was not changed in the ET-insensitive soybean mutant (Fig. 6).

Effect of *GmEREBP1* expression on cyst nematode infection.

Expression of *GmEREBP1* in transgenic soybean and *Arabidopsis* resulted in elevated mRNA abundance of a subset of defense-related genes (Figs. 3 and 4). This observation raised the possibility that defense responses are activated in these plants and that susceptibility to certain pathogens is decreased. To address this issue, transgenic soybean and *Arabidopsis* plants expressing *GmEREBP1* at varying levels, as assayed on the mRNA level, were inoculated with the soybean cyst nematode *H. glycines* (for soybean plants) or the sugar beet cyst nematode *H. schachtii* (for *Arabidopsis* plants) and the nematode reproductive success was assessed by the number of females (cysts) per root system and the number of eggs per female. Re-

sults of these experiments showed that only the transgenic soybean plants originating from the S3 line (i.e., the line that exhibited the highest *GmEREBP1* mRNA abundance) were significantly altered in their susceptibility, which was observable using both the number of females (cysts) per root and the number of eggs per female parameters. However, these soybean plants were more susceptible than untransformed control plants, which was statistically significant in only one of the three experiments (Fig. 7). No transgenic *Arabidopsis* lines showed statistically significant or otherwise consistent changes in susceptibility to cyst nematode infection when compared with wild-type, untransformed plants (data not shown).

DISCUSSION

We previously reported identification of the soybean *EREBP* gene *GmEREBP1*, whose transcript abundance decreased in soybean cyst-nematode-infected roots of a susceptible cultivar, whereas it increased in abundance in infected roots of a resistant cultivar (Mazarei et al. 2002). These findings gave rise to the hypothesis that *GmEREBP1* plays a functional role in defense against cyst nematode infection and that its expression is suppressed in a compatible cyst nematode-plant interaction. This article describes an in-depth characterization of this gene and its protein product and provides insights into the legitimacy of this hypothesis.

Nuclear localization and transcription activation by *GmEREBP1*.

As expected for transcription factors, *GmEREBP1* was localized to the nucleus, which could be shown in cells of both monocotyledonous (onion) and dicotyledonous (*Arabidopsis*) plant species. *GmEREBP1* contains a predicted nuclear localization signal (NLS) (Mazarei et al. 2002), and it is most likely that this NLS is responsible for the targeting of *GmEREBP1* to

the nucleus. This finding is critical because it corroborates the predicted function as a transcription factor and our previous results showing that *GmEREBP1* interacts with the GCC box, a promoter element found in many ET-regulated genes, thereby suggesting that *GmEREBP1* controls the expression of a subset of GCC box-containing genes.

Characterizations of other EREBPs showed that the *Arabidopsis* EREBP AtEBP, in addition to a GCC box, also interacted with a basic leucine-zipper transcription factor involved in the expression of plant defense genes (Buttner and Singh 1997) and that the rice EREBP OsEBP-89 interacted with an MYC transcription factor (Zhu et al. 2003), indicating that EREBPs act in concert with other transcription factors to regulate gene expression. Furthermore, a tomato EREBP, Pti4, regulated defense gene expression by binding to a non-GCC box *cis*-element, in addition to binding to the GCC box (Chakravarthy et al. 2003). In other words, known EREBPs exhibit considerable diversity in their modes of action. It remains to be seen whether *GmEREBP1* interacts with transcription factors or also binds non-GCC box promoter elements.

The in vitro interaction of *GmEREBP1* with a GCC box alone (Mazarei et al. 2002) did not prove functionality as a transcriptional activator. In tobacco, at least four different EREBPs, ERF1 through ERF4, have been identified (Ohme-Takagi and Shinshi 1995) and, while ERF2 and ERF4 enhance GCC box-mediated transcription, ERF3 functions as a repressor (Ohta et al. 2000, 2001). Similarly, differential regulatory activity of the *Arabidopsis* EREBPs AtERF1 through AtERF5 also was demonstrated: AtERF1, AtERF2, and AtERF5 act as transcriptional activators for GCC box-dependent transcription, whereas AtERF3 and AtERF4 act as transcriptional repressors (Fujimoto et al. 2000). Recently, it has been shown that NbCD1, a *Nicotiana benthamiana* EREBP, acts as a transcriptional repressor for GCC box-dependent transcription and that the transcriptional repressor activity is necessary for NbCD1 to confer plant cell

Table 1. Sequences of the gene-specific primer pairs used in quantitative real-time reverse-transcription polymerase chain reaction experiments

Plant, gene ^a	GenBank accession number	Forward and reverse primers (amplicon size [bp])
Soybean		
<i>GmEREBP1</i>	AF357211	5'-TTCATCACCGTCGTCCTCGTGTCCA-3' (70) 5'-CCAGGCTCTTCCTCCTCTTGTCCCT-3'
<i>UBI3</i>	D28123	5'-GTGTAATGTTGGATGTGTTCC-3' (107) 5'-ACACAATTGAGTTCAACACAAACCG-3'
<i>PR1</i>	BU577813	5'-AACTATGCTCCCCCTGGCAACTATATTG-3' (76) 5'-TCTGAAGTGAGTCTTCTACATCGAAACAA-3'
<i>PR2</i>	M37753	5'-TGAAATAAGGGCCACGAGTCCAAATG-3' (103) 5'-ATGGTACATGCAGACTCAAGAATGCAGAT-3'
<i>PR3</i>	AF202731	5'-AACTACAATTACGGGCAAGCTGGCAA-3' (139) 5'-TTGATGGCTTGTTCCTGTGCAGT-3'
<i>PR5</i>	BU765509	5'-GCGCTTGCTCCGCTTTCAACT-3' (89) 5'-CTTGGAATAGACGGTGGGCTTGC-3'
<i>Arabidopsis</i>		
<i>18S</i>	X16077	5'-GACAGACTGAGAGCTCTTCTTGA-3' (123) 5'-ACGTAGCTAGTTAGCAGGCTGAG-3'
<i>PR1</i>	NM-127025	5'-GCTCTGTAGGTGCTCTTGTCTTCCCT-3' (75) 5'-CTGGTTGTGAACCCTTAGATAATCTTGTGG-3'
<i>PR2</i>	NM-115586	5'-CAATCTCCCTTGCTCGTGAATCTTACCC-3' (109) 5'-CGTTATCAACAGTGGACTGGGCGG-3'
<i>PR3</i>	NM-112085	5'-TTAACGGCCTCCTCGAAGCTGCTATT-3' (136) 5'-CGCAACATAAACAGTGAAACATCATTGGAA-3'
<i>PR5</i>	NM-106161	5'-CAAGAACGCTTGCCCTGACGCTTA-3' (72) 5'-GCTCCGGTACAAGTGAAGGTGCTCGTT-3'
<i>PDF1.2</i>	NM-123809	5'-CAAGTGGGACATGGTCAGGGGTT-3' (115) 5'-CACTTGTGTGCTGGGAAGACATAGTTGC-3'
<i>GST2</i>	NM-116486	5'-CCAGTTCCTCGAGAAGGTTCACTGAGAA-3' (111) 5'-GAAATTGGGCAATGAGAAAGCCGCTT-3'

^a Soybean: *GmEREBP1* (Glycine max ethylene-responsive element-binding protein 1), *UBI3* (ubiquitin-3), *PR1* (acidic pathogenesis-related 1), *PR2* (basic β-1,3-endoglucanase), *PR3* (basic chitinase), and *PR5* (thaumatin-like). *Arabidopsis*: *18S* (18S ribosomal RNA), *PDF1.2* (plant defensin protein), and *GST2* (glutathione S-transferase).

death defense responses (Nasir et al. 2005). These studies show that different EREBPs play different roles in controlling expression of GCC box-containing genes (Fujimoto et al. 2000; Hao et al. 1998; Ohme-Takagi et al. 2000). Our results established that GmEREBP1 acts as an activator of transcription.

Activation of ET- and JA-regulated genes by GmEREBP1.

Overexpression of *GmEREBP1* in soybean induced the expression of *PR2* and *PR3* genes. These soybean genes were

shown by us to be inducible by ET (*PR2*) or ET as well as JA (*PR3*). ET responsiveness of *PR2* and *PR3* also can be inferred by our observations that expression of these genes was shown to be decreased in the ET-insensitive soybean mutant *etr1-1* impaired in ET signaling (Hoffman et al. 1999).

Due to the lack of information regarding the promoter sequence of the soybean *PR2* gene (Takeuchi et al. 1990), the presence of GCC box in the promoter and its possible role in the induction of this gene by GmEREBP1 could not be deter-

Fig. 4. Overexpression of *Glycine max* ethylene-responsive element-binding protein 1 (*GmEREBP1*) in *Arabidopsis*. **A**, RNA slot blot analysis of transgenic *Arabidopsis* plants. Blots of total RNA were obtained from leaves of wild-type and individual transgenic plants from T1 generation of the independent lines A1, A2, A3, and A4. Blots were hybridized with a probe derived from *GmEREBP1* and standardized using an actin probe. **B**, Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) analysis of *GmEREBP1* messenger (m)RNA steady-state levels. *GmEREBP1* mRNA accumulation in root and shoot tissues of wild-type and transgenic plants from T2 generation of the independent lines A1, A2, A3, and A4. Each bar represents the mean of three independent experiments with the standard errors of the noted mean. **C**, Expression analysis of the *Arabidopsis* defense-related genes using qRT-PCR. mRNA accumulation of the defense-related genes in root tissue of the transgenic plants shown in B relative to the wild type (set to 1.0). Each bar represents the mean of three independent experiments with the standard errors of the mean noted. Mean values significantly different from 1.0 are indicated by asterisks as determined by paired *t* test ($P < 0.05$).

mined. However, an 11.3-kb genomic fragment containing the 2.4-kb soybean *PR3* gene was fully sequenced (Gijzen et al. 2001), and we found a GCC-like element (AGCAGCC) (Tournier et al. 2003; Wu et al. 2002) 134 nucleotides upstream of the predicted translation start site in the 1,160-bp genomic fragment directly upstream of the *PR3* coding region using the plant *cis*-acting elements (PLACE) database (Higo et al. 1998). Interestingly, we also found five JA-responsive elements (TGACG) (Brown et al. 2003; Rouster et al. 1997) within this region, effectively explaining the JA responsiveness of *PR3* observed by us. The regulation of ET- and JA-responsive genes by GmEREBP1 also was observable in *Arabidopsis*, where constitutive expression of *GmEREBP1* induced the expression of the ET-responsive *PR3* and the ET- and JA-responsive *PDF1.2* (Penninckx et al. 1998; Samac et al. 1990). Both these genes contain GCC boxes in their respective promoters (Manners et al. 1998; Samac et al. 1990), which likely is the cause for the observed induction by GmEREBP1.

EREBPs are important components of the ET signal transduction pathway (Alonso and Stepanova 2004; Guo and Ecker 2004; Solano et al. 1998; Wang et al. 2002). In *Arabidopsis*, the ET signal is perceived by a family of receptors and transduced sequentially by CTR1, EIN2, and EIN3/EILs. The nuclear proteins EIN3/EILs induce transcription of *EREBP* genes such as *ERF1* and *AtEBP*, and EREBPs, in turn, regulate the expression of GCC box-containing genes among other regulatory functions mentioned above (Guo and Ecker 2004; Ogawa et al. 2005; Solano et al. 1998). GmEREBP1, similar to ERF1 and AtEBP (Ogawa et al. 2005; Solano et al. 1998), shows activation of ET-responsive GCC box-containing genes, suggesting a downstream position of GmEREBP1 in the ET signal transduction pathway.

Activation of SA-regulated genes by GmEREBP1.

Expression of *GmEREBP1* in soybean increased the expression of *PR1* but not *PR5*, which both were shown to be SA-responsive genes in the present study. Similarly, the expression of *GmEREBP1* in *Arabidopsis* enhanced the expression level of the SA-responsive gene *PR1* but not *PR2* and *PR5* (Dong et al. 1991; Rogers and Ausubel 1997; Uknes et al. 1992; Ward et al. 1991). It is not known whether GmEREBP1 binds *cis* elements of SA-regulated *PR* genes directly or indirectly by interacting with protein factors that are involved in SA-regulated *PR* gene expression. Interestingly, the *Arabidopsis* EREBP AtEBP was identified because of its interaction with an *ocs* element-binding protein, a basic leucine-zipper transcription factor (Buttner and Singh 1997), which binds specifically to the SA-responsive elements in the promoter of *PR1* genes (Despres et al. 2000; Zhang et al. 1999). Thus, it is possible that GmEREBP1 interacts with other transcription factors to activate a subset of SA-regulated genes. Activation of SA-regulated genes by constitutive expression of some *EREBP* genes has been reported and possible roles for EREBPs in mediating cross-talk between the SA and ET and JA signaling pathways have been proposed (Gu et al. 2000, 2002; Guo et al. 2004; Ogawa et al. 2005; Park et al. 2001).

Effect of *GmEREBP1* expression on cyst nematode infection.

To date, overexpression of EREBP genes in transgenic plants has been conducted to assay effects on bacterial, fungal, and viral pathogens (Berrocal-Lobo and Molina 2004; Berrocal-Lobo et al. 2002; Fischer and Droge-Laser 2004; Gu et al. 2002; Guo et al. 2004; He et al. 2001; Lee et al. 2004, 2005; McGrath et al. 2005; Park et al. 2001; Shin et al. 2002; Tang et al. 2005; Yi et al. 2004; Zhang et al. 2004). Here, we further extend insights into the effect of EREBP expression on plant defense

responses against cyst nematodes. We previously showed that mRNA levels of the *Arabidopsis* EREBP *AtEBP* were down-regulated locally in syncytia of the sugar beet cyst nematode (Hermesmeier et al. 2000). Further, we previously showed that *GmEREBP1* mRNA levels were decreased in a susceptible soybean cultivar following cyst nematode infection but were increased in a resistant cultivar following infection (Mazarei et al. 2002). These results suggested that GmEREBP1 may be involved in cyst nematode resistance and that cyst nematode infection of a susceptible cultivar may actively suppress EREBP expression as a means to avoid plant defense responses. We tested whether EREBP-mediated defenses had effects on nematode infection by constitutively expressing GmEREBP1 in transgenic soybean and *Arabidopsis*, which would negate cyst-nematode-mediated transcriptional downregulation. As described above, in these experiments, we observed activation of several ET- and

Fig. 5. Expression analysis using quantitative real-time reverse-transcription polymerase chain reaction of soybean pathogenesis-related (*GmPR*) genes in soybean plants subjected to treatments with plant hormones. Messenger (m)RNA accumulation of the *GmPR* genes in root and shoot tissues of soybean plants treated with 1 mM 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene (ET)-producing chemical, 100 μ M jasmonic acid (JA), or 2 mM salicylic acid (SA) relative to mRNA levels in corresponding untreated control root or shoot tissues (set to 1.0). Each bar represents the mean of two independent experiments with the standard errors of the mean noted. Mean values significantly different from 1.0 are indicated by asterisks as determined by paired *t* test ($P < 0.05$).

JA- and SA-regulated defense-related genes; however, constitutive expression of *GmEREBP1* had no uniform effect on plant susceptibility to cyst nematodes.

Infection of a plant by a pathogen induces a variety of defense responses that imply the action of several signaling molecules, including SA, ET, and JA (Baker et al. 1997; Rojo et al. 2003). Depending on the pathogen, different pathways are triggered, leading to the activation of defined sets of target genes (Glazebrook 2005). For example, resistance to the necrotrophic fungus *Botrytis cinerea* utilizes ET and JA signaling networks and, accordingly, overexpression of the *Arabidopsis* EREBP *ERF1* (a gene that is a downstream component of both the ET and JA signaling pathways and that functions as a key integrator of ET and JA defense responses) (Lorenzo et al. 2003) enhanced resistance to this fungus (Berrocal-Lobo et al. 2002). In contrast, resistance to the biotrophic bacterium *Pseudomonas syringae* utilizes SA signaling and overexpression of the same EREBP, *ERF1* (i.e., the gene that is not a regulator of SA defense responses), enhanced susceptibility to this bacterium (Berrocal-Lobo et al. 2002). These results show that overactivation of ET signaling (at least by *ERF1* overexpression) has different effects in different pathosystems. In other words, positive and negative interactions can be established depending on the type of pathogen assessed. This notion is reflected in a number of recent publications showing that overexpression of EREBP genes leads to constitutive expression of defense-related genes, nevertheless resulting in enhanced resistance, enhanced susceptibility, or no change to pathogen attack (Berrocal-Lobo and Molina, 2004; Berrocal-Lobo et al. 2002; Gu et al. 2002; Guo et al. 2004; Lee et al. 2004, 2005; McGrath et al. 2005; Park et al. 2001; Shin et al. 2002; Tang et al. 2005; Yi et al. 2004; Zhang et al. 2004). It also has been shown that overexpression of EREBP genes enhanced resistance to certain pathogens without leading to constitutive expression of defense-related genes (Fischer and Droge-Laser 2004; He et al. 2001).

Because *GmEREBP1* mRNA is upregulated in a resistant soybean cultivar infected by avirulent cyst nematodes and because expression of this gene produces elevated mRNA levels for most PR genes tested, it appears that *GmEREBP1* may be activated in a defense response. In our transgenic lines, however, *GmEREBP1* overexpression apparently did not trigger a plant response conferring effective defense against the infecting cyst nematode. One of several possible explanations could be that *GmEREBP1* influences the response pathways of both ET and SA. These plant-signaling molecules elicit dramatically different susceptibility phenotypes. Our experiments using SA-insensitive and SA-overproducing *Arabidopsis* mutants

showed that SA signaling has an inhibitory effect on cyst nematode infection (M. J. E. Wubben and T. J. Baum, *unpublished results*). Similarly, ET signaling modulates susceptibility to cyst nematodes in *Arabidopsis* (Goverse et al. 2000; Wubben et al. 2001, 2004) and soybean (M. Mazarei and T. J. Baum, *unpublished results*)—however, in the opposite direction as SA, because increased ET signaling leads to increased susceptibility and decreased ET signaling frequently causes decreased susceptibility. Because *GmEREBP1* overexpression activates both the SA and ET pathways, a counterplay of two competing pathways may be reflected in the susceptibility of transgenic lines overexpressing *GmEREBP1*.

Fig. 7. *Heterodera glycines* susceptibility of wild-type and transgenic soybean plants overexpressing *Glycine max* ethylene-responsive element-binding protein 1 (*GmEREBP1*). Susceptibility to *H. glycines* of wild-type and transgenic plants (R2 generation of line S3) was measured as number of females and number of eggs per female in three independent experiments (numbers 1, 2, and 3). Each bar represents the mean of 15 individual plants with the standard errors of the mean noted. The asterisk denotes the mean value significantly different from the wild type as determined by paired *t* test ($P < 0.05$).

Fig. 6. Expression of soybean pathogenesis-related (*GmPR*) genes in the ethylene-insensitive soybean mutant *etr1-1* as determined by quantitative real-time reverse-transcription polymerase chain reaction. Messenger (m)RNA accumulation of *GmPR* genes in root and shoot tissues of the soybean mutant is shown relative to the corresponding wild-type root or shoot tissues (set to 1.0). Each bar represents the mean of three independent experiments with the standard errors of the mean noted. Mean values significantly different from 1.0 are indicated by asterisks as determined by paired *t* test ($P < 0.05$).

Finally, other EREBPs are reported to interact with proteins that function in pathogen-induced gene expression, such as transcription factors, a resistance gene product, a nitrilase-like protein, a mitogen-activated protein kinase, a ubiquitin-conjugated enzyme, and an acyl-CoA-binding protein (Buttner and Singh 1997; Cheong et al. 2003; Koyama et al. 2003; Li and Chye 2004; Xu et al. 1998; Zhou et al. 1997; Zhu et al. 2003). These facts underscore that the biological significance of EREBPs in planta are divergent and manifold. Although constitutive expression of *GmEREBP1* did not show resistance to cyst nematodes, it still may affect susceptibility to other pathogens. Although we have not examined this possibility yet, it appears likely that these transgenic plants overexpressing *GmEREBP1* may show elevated levels of disease resistance to other pathogens due to constitutive expression of a number of PR genes. Although the functions of many PR proteins have not yet been defined, some PR proteins, such as PR2 and PR3 (i.e., the genes that are upregulated in the *GmEREBP1* overexpressing plants), are hydrolytic enzymes that have been shown to degrade fungal cell walls and to inhibit fungal growth both in vitro and in vivo (Broglie et al. 1991; Saikia et al. 2005; Sela-Buurlage et al. 1993; Tohidfar et al. 2005; Zhu et al. 1994). Among the genes upregulated in *GmEREBP1* overexpressing plants is *PDF1.2*, which encodes the antifungal defensin protein (Penninckx et al. 1996).

In closing, *GmEREBP1* has a role in regulating plant defenses, because our work discovered that *GmEREBP1* mediates the expression of PR genes that are regulated by both ET and JA or by SA. These discoveries held true in both a homologous (soybean) and a heterologous (*Arabidopsis*) background, which shows a functional conservation of EREBP regulatory mechanisms in these two plant species. However, these regulatory functions had no consistent effect on plant susceptibility against cyst nematodes in the experimental system chosen in this study.

MATERIALS AND METHODS

Subcellular localization.

The cDNA coding region of *GmEREBP1* (Mazarei et al. 2002) was cloned into the *HindIII* and *SalI* sites of pRJG23 vector to create an in frame translational fusion to the GFP and GUS reporter genes and expressed under the control of the CaMV 35S promoter (Grebek et al. 1997). The plasmid construct (35S::GmEREBP1-GFP-GUS) was isolated by using a QIAfilter Plasmid Maxi Kit (Qiagen, Valencia, CA, U.S.A.). Transient assays were performed using onion epidermal layers and *Arabidopsis* protoplasts.

Transfection of onion cells. Young onion epidermal layers were placed inside up on Murashige and Skoog (MS)-containing medium according to Varagona and associates (1992) and solidified with 0.6% agar. Plasmid DNA (3 µg) was precipitated onto 1.5 mg of 1.6-µm gold particles (BioRad, Carlsbad, CA, U.S.A.) using 2.5 M CaCl₂ (Sigma-Aldrich, St. Louis) and 100 mM spermidine (Sigma-Aldrich). The onion layers were bombarded with DNA-coated particles using a Biolistic PDS-1000/He system (Du Pont, Wilmington, DE, U.S.A.). Each epidermal layer was bombarded with 0.5 mg of gold (equivalent to 1 µg of DNA) at a pressure of 1,100 psi. Bombarded onion layers were maintained on the plates and incubated at 25°C in darkness for 18 to 24 h.

Transfection of Arabidopsis protoplasts. *Arabidopsis* suspension cells were cultivated in 50 ml of modified Linsmaier and Skoog (LS) medium (per liter: 4.73 g of LS salt [Caisson Laboratories, Rexburg, ID, U.S.A.], 20 g of sucrose, 590 mg of morpholineethanesulfonic acid (MES), 50 µl of kinetin [stock 1 mg/ml], and 1 ml of naphthalene acetic acid [stock 1

mg/ml], pH 5.7) at room temperature on an orbital shaker (125 rpm) and subcultured weekly. Protoplasts were generated by digestion of 4- to 5-day-old suspension cells with 0.15% macerozyme R-10 (Yakult Honsha, Tokyo) and 0.31% cellulase Onozuka RS (Yakult Honsha). Transfection basically followed procedures described elsewhere (Sheen 2001) with minor modifications; 100-µl protoplasts (approximately 2×10^4 cells) were incubated with 30 µg of plasmid DNA and 0.4 ml of polyethylene glycol (PEG) solution (40% wt/vol PEG 4000, 0.4 M mannitol, and 1 M CaCl₂) on ice for 20 min with gentle shaking every 4 min. The protoplasts then were removed, washed in 5 ml of W5 solution (0.4 M mannitol, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose, and 1.5 mM MES, pH 5.7) and collected by centrifugation at 500 rpm for 10 min. The cells were resuspended in 1.5 ml of modified LS medium supplemented with 0.4 M mannitol and incubated in six-well Falcon culture plates (BD Biosciences, San Jose, CA, U.S.A.) at 25°C in darkness for 18 to 24 h.

Onion and *Arabidopsis* cells were observed using a Zeiss Axiovert 100 microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY, U.S.A.). GFP expression was monitored with a Piston GFP filter (Chroma, Rockingham, VT, U.S.A.). Pictures were taken at $\times 20$ (onion) or $\times 60$ (*Arabidopsis*) with a Zeiss AxioCam MRc5 digital camera and processed with Zeiss Axiovision software (Carl Zeiss MicroImaging Inc.) and Adobe Photoshop.

Transcriptional activity assays.

Two types of reporter construct were generated. The tobacco reporter construct contained two GCC boxes in tandem based on the GCC box sequence from the tobacco β -1,3-endoglucanase gene (Ohme-Takagi and Shinshi 1995) as follows: 5'-CAT AAGAGCCGCCACTAAATAAGACCGATCAAATAAGAGC CGCCAT-3'. The bean reporter construct contained three GCC boxes in tandem based on the GCC box sequence from the bean basic chitinase gene (Broglie et al. 1989) as follows: 5'-CGCT TGGGAAGCCGCCGGGTGGGCCCCGCA-3'. The tobacco and bean GCC fragments were prepared by synthesizing both strands with *EcoRI-HindIII* and *XhoI* sites at the 5' and 3' ends, respectively. The individual fragments were inserted into the *EcoRI* and *XhoI* sites of pBSII to yield tobacco GCC and bean GCC, respectively. The minimal TATA box (-42 to +8) from the CaMV 35S promoter was synthesized with *SalI* and *BamHI* sites at the 5' and 3' ends, respectively, and inserted into pBI101 vector (Clontech, Palo Alto, CA, U.S.A.) at the 5' end of the GUS reporter gene and then was fused with the *HindIII* and *XhoI* GCC fragment to yield respective constructs tobacco GCC-TATA-GUS and bean GCC-TATA-GUS. To generate the effector construct, the GUS gene in the pBI221 vector (Clontech) was replaced by the coding region of *GmEREBP1* (Mazarei et al. 2002) under the control of the CaMV 35S promoter to yield plasmid construct 35S-GmEREBP1. The plasmid constructs were isolated by using a QIAfilter Plasmid Maxi Kit (Qiagen).

Transient assays were performed by particle gun bombardment method using soybean leaves. Uniform-sized primary leaves were cut from 2- to 3-week-old soil-grown soybean plants (discussed below) and placed on 1% agar in petri dishes, and one leaf was used for each bombardment. The reporter plasmid was mixed with the effector plasmid or blank plasmid (empty vector with no insert was used as control) at a 1:1 ratio. The firefly luciferase (LUC) gene under the control of the CaMV 35S promoter was used as a reference. In cotransformation assays, a total of 10 µg of DNA was used for each bombardment: 4 µg of reporter plasmid, 4 µg of effector plasmid, and 2 µg of reference plasmid. The DNA mixture was precipitated onto 3 mg of 1.6-µm gold particles (BioRad) using 2.5 M CaCl₂

(Sigma-Aldrich) and 100 mM spermidine (Sigma-Aldrich). For each bombardment, 0.5 mg of particles was used. The soybean leaves were bombarded with DNA-coated particles using a PDS-1000/He system (Du Pont) at a pressure of 1,100 psi. Bombarded leaves were maintained on the agar plates for 24 h before assays for GUS and LUC activities. GUS expression was determined by histochemical and fluorometric assays (Jefferson, 1987) as previously described (Mazarei et al. 2004). LUC activity was measured using a Promega Luciferase Assay System Kit (Promega Corp., Madison, WI, U.S.A.) following the manufacturer's instructions. Protein concentration was determined using a Pierce BCA-200 Protein Assay Kit (Pierce, Rockford, IL, U.S.A.). GUS and LUC activities were determined in the same extract. GUS expression was reported as a ratio between co-expressed GUS activity (picomoles of 4-methylumbelliferone [4-MU] per milligram of protein per minute) and LUC activity (relative light units [RLUs] per microgram of protein per second).

Transgenic soybean and *Arabidopsis* plants.

Soybean transformation. The GUS gene in the pBI221 vector (Clontech) was replaced by the coding region of *GmEREBP1* (Mazarei et al. 2002) under the control of the CaMV 35S promoter. Then, the resulting cassette was cloned into the multiple cloning site of the binary vector pZY101 (Paz et al. 2004; Zeng et al. 2004). *Agrobacterium tumefaciens* EHA101 was transformed with the binary construct by the three-parental mating (Ditta et al. 1980) with *Escherichia coli* HB101 containing the pRK2013 helper plasmid (Clontech). The binary construct was introduced into soybean cv. Thorne using *A. tumefaciens*-mediated transformation by the Iowa State University Plant Transformation facility (Ames, U.S.A.) (Paz et al. 2004).

Arabidopsis transformation. The coding region of *GmEREBP1* (Mazarei et al. 2002) was cloned into the multiple cloning site of the binary vector pKYLX71 (Schardl et al. 1987) under the control of the CaMV 35S promoter. *A. tumefaciens* C58 was transformed with the binary construct by the three-parental mating (Ditta et al. 1980) with *E. coli* HB101 containing the pRK2013 helper plasmid (Clontech). The transgene was introduced into *Arabidopsis* (wild-type Columbia) using *Agrobacterium tumefaciens*-mediated transformation following the floral dip method (Clough and Bent 1998).

Plant cultivation.

Transgenic soybean. Soybean transformants (R0) were selected for Basta resistance (Paz et al. 2004) and self-pollinated in greenhouse. The progenies (R1) of these transformants were observed for Basta selection and 3:1 (Basta-resistant/Basta-susceptible) segregating lines were selected and self pollinated in greenhouse. Progenies (R2) of the individual R1 plants were observed for Basta selection and those that showed 100% resistance to Basta were selected (homozygous lines). Soybean plants were grown in soil (Sunshine Universal Mix; Consumer Supply, Storm Lake, IA, U.S.A.) at 26°C.

Transgenic Arabidopsis. *Arabidopsis* seed from transformed plants (T0) were harvested and sowed on MS medium (Invitrogen, Carlsbad, CA, U.S.A.) containing kanamycin (Kan) at 50 µg/ml (Fisher, Fair Lawn, NJ, U.S.A.). Primary transformants (T1) were selected and self pollinated in the greenhouse. The progenies of these primary transformants were observed on selective medium and 3:1 (Kan-resistant/Kan-susceptible) segregating lines were selected and transformed to a greenhouse for self pollination. Progenies (T2) of the individual T1 plants were observed on selective medium and those that showed 100% resistance to Kan were selected (homozygous lines). *Arabidopsis* plants were grown in soil (Sunshine Professional Growing Mix, Bellevue, WA, U.S.A.) at 22°C.

Chemical treatments of soybean plants.

Soybean cv. Thorne plants were grown in soil (Sunshine Universal Mix, Consumer Supply) at 26°C. Treatments were performed on 2-week-old plants. The root systems of whole plants were washed gently with water to remove soil and then the plants were used for each treatment. In all, 10 plants per treatment were used. The whole plants were incubated for 48 h in 100 µM JA (Sigma-Aldrich), 2 mM SA (Sigma-Aldrich), and 1 mM ACC (Sigma-Aldrich), an ET-producing chemical. Control plants were incubated in water alone. Root and shoot tissues were harvested, frozen in liquid nitrogen, and kept at -80°C until use.

RNA slot blot analyses.

Transgenic soybean and *Arabidopsis* plants were grown in soil (discussed above). Three to five individual plants from each transgenic line were chosen for analysis. Total RNA was isolated from leaves of 3-week-old plants ground under liquid nitrogen as described by Pawlowski and associates (1994). RNA samples containing 10 µg of RNA were denatured for 15 min at 65°C and spotted on the nylon membranes (S&S Nytran Plus; Schleicher & Schuell, Keene, NH, U.S.A.) using a "Minifold II" slot blot system (Schleicher & Schuell). RNA was fixed to the membranes in a FB-UVXL-1000 crosslinker (Fisher Scientific, Pittsburgh).

GmEREBP1 insert in the binary vector was amplified by PCR, gel-purified using a Qiaex II Kit (Qiagen), radiolabeled via PCR, and then used as a probe in RNA slot blot analyses. Hybridizations were carried out at 42°C in a hybridization buffer composed of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 5× Denhardt's solution (Sambrook et al. 1989), herring-sperm DNA at 0.1 mg/ml, and labeled gene probe at 3×10^6 cpm/ml. The hybridized blots were washed three times for 15 min in 0.1× SSC/0.1% SDS at 65°C. Bound radiolabeled probes were imaged with a Molecular Dynamics Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.), which allowed quantification of hybridized probe by using the ImageQuant software (Molecular Dynamics).

qRT-PCR.

In order to conduct qRT-PCR assays (Bustin 2002), gene-specific primers to the 3' region of *GmEREBP1* and the defense-related genes *UBI3* and *18S* were designed (Table 1). These primers amplified a single product for each corresponding gene (Table 1), as confirmed by the melting temperature of the amplicons and gel electrophoresis. Total RNA was isolated from the respective frozen tissues using RNeasy columns (Qiagen) following the manufacturer's instructions. Total RNA (5 µg) was denatured in the presence of an oligo-d(T) primer, cooled on ice, and divided evenly into two reverse-transcription reactions. One of these reactions was used as the reverse-transcription (RT) control (no reverse transcriptase was added), and the other reaction was used for later qRT-PCR, which was conducted in triplicate. The following additions were made to each reaction: 4 µl of 5× first-strand buffer (Gibco BRL, Rockville, MD, U.S.A.), 2 µl of 0.1 M dithiothreitol, 1 µl of 10 mM dNTP mix, and 1 µl of Superscript II Reverse Transcriptase (Gibco BRL). In the RT control reaction, water was substituted for Superscript II. Each 20-µl reaction was incubated at 42°C for 1 h. First-strand cDNA was diluted (equivalent to 10 ng of total RNA/PCR reaction) and placed in each PCR reaction. PCR was conducted on the iCycler (BioRad, Hercules, CA, U.S.A.) with a 96-well reaction block in the presence of SYBR Green as previously described (Mazarei et al. 2003). Threshold cycles (C_t) were determined using iCycler (BioRad) software for all treatments. In order to

quantify relative mRNA concentrations, a standard curve was prepared for each individual gene. For this purpose, a threefold dilution series of a total of six dilutions was prepared from RNA sample (which was known to contain detectable mRNA amounts), and each dilution was subjected to qRT-PCR analyses in triplicate using the corresponding specific primers. Obtained C_t values were used by the iCycler software package to plot a standard curve that allowed the quantification of mRNA in other RNA samples relative to the RNA sample used to prepare the standard curve. mRNA starting quantity values obtained from the soybean RNA samples were normalized using *UBI3* mRNA starting quantity values, whereas those obtained from the *Arabidopsis* RNA samples were normalized using *18S* RNA starting quantity values. These relative mRNA quantities of RNA samples are presented as “relative expression” values in the figures. For quality assurance purposes, only qRT-PCR assays that resulted in standard curves with the following parameters (Bustin 2002), as calculated by the iCycler software, were considered: i) linear standard curve throughout the measured area, ii) standard curve slope between -3.5 and -3.2 , and iii) R^2 value above 0.99 .

Use of the *18S* RNA, which is not poly-adenylated, as an expression standard for quantitative PCR analyses using oligo-d(T)-primed cDNA is a viable approach because we observed that the *18S* PCR primers amplified the correct single product of 123 bp (expected size) as confirmed by the melting temperature of the amplicon and gel electrophoresis. This single product was observed only in the RT-positive reaction (i.e., when reverse transcriptase was added) and not in the RT-negative reaction (i.e., no reverse transcriptase was added). These observations clearly indicate successful cDNA synthesis with the oligo-d(T) primer in the RT positive reaction. This successful reverse transcription of the *18S* RNA using an oligo-d(T) primer is due to the fact that the mature *18S* RNA has A-rich sequence regions at the 3' end, which can bind an oligo-d(T) primer. Also, repeated independent reverse transcription of the same RNA extracts produced the same levels of *18S* cDNA in our hands and independent reverse transcription of different RNA samples produced comparable *18S* cDNA concentrations, which further documents the robustness of this approach.

Nematode infection.

Soybean inoculation. *H. glycines* (field population race 3) was propagated in greenhouse cultures with soybean (cv. Kenwood 94) as host. Assessment of the transgenic soybean plants for their susceptibility to the *H. glycines* infection was conducted under greenhouse conditions, utilizing a water bath set to 26°C to control temperature fluctuations. Individual plants were grown in Conetainers (Ray Leach Cone-Tainer Nursery, Canby, OR, U.S.A.) containing a 2:1 sand/soil mixture and arranged in a random block design within plastic 5-gallon buckets (partially filled with sand), which were placed inside the water bath. Initially, two to three soybean seed were planted in each of 15 Conetainers placed within each bucket. Young seedlings then were thinned to one plant per Conetainer. The 10 to 12-day-old plants were inoculated individually with approximately 3,000 eggs of *H. glycines*. Four weeks post inoculation, cysts from the root system of each plant were collected and counted with a Zeiss Stemi SV11 dissecting microscope (Carl Zeiss MicroImaging Inc.). The cysts then were crushed and eggs were collected and counted with a Zeiss Axiovert 100 microscope (Carl Zeiss MicroImaging Inc.).

Arabidopsis inoculation. *H. schachtii* was propagated in greenhouse cultures with sugar beet (cv. Monohi) as host. Assessment of the transgenic *Arabidopsis* plants for their susceptibility to the *H. schachtii* infection was conducted under aseptic conditions. Surface-sterilized second-stage juveniles (J2) of

H. schachtii were prepared as previously described (Baum et al. 2000). *Arabidopsis* seed were surface sterilized with 2.6% sodium hypochlorite for 5 min, washed three times with sterile distilled water, then planted aseptically, one seed per well, in 12-well Falcon culture plates (BD Biosciences) containing modified Knop medium (Sijmons et al. 1991) solidified with 0.8% Daishin agar (Brunschwig Chemie BV, Amsterdam, The Netherlands). Plants were grown at 26°C with a 12-h photoperiod of approximately 1,500 Lux provided by fluorescent light bulbs. The 10- to 12-day-old plants were inoculated individually with approximately 500 surface-sterilized J2. At 15 days post inoculation, nematode females developing on the root system of each plant were counted with a Zeiss Stemi SV11 dissecting microscope (Carl Zeiss MicroImaging Inc.).

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LITERATURE CITED

- Alonso, J. M., and Stepanova, A. N. 2004. The ethylene signaling pathway. *Science* 306:1513-1515.
- Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S. P. 1997. Signaling in plant-microbe interactions. *Science* 276:728-733.
- Baum, T. J., Wubben, M. J. E., II, Hardy, K. A., Su, H., and Rodermel, S. R. 2000. A screen for *Arabidopsis thaliana* mutants with altered susceptibility to *Heterodera schachtii*. *J. Nematol.* 32:166-173.
- Berrocal-Lobo, M., and Molina, A. 2004. Ethylene response factor 1 mediates *Arabidopsis* resistance to the soilborne fungus *Fusarium oxysporum*. *Mol. Plant-Microbe Interact.* 17:763-770.
- Berrocal-Lobo, M., Molina, A., and Solano, R. 2002. Constitutive expression of *ETHYLENE-RESPONSE-FACTOR1* in *Arabidopsis* confers resistance to several necrotrophic fungi. *Plant J.* 29:23-32.
- Bostock, R. M. 2005. Signal crosstalk and induced resistance: Straddling the line between cost and benefit. *Annu. Rev. Phytopathol.* 43:545-580.
- Brogli, K. E., Biddle, P., Cressman, R., and Brogli, R. 1989. Functional analysis of DNA sequences responsible for ethylene regulation of a bean chitinase gene in transgenic tobacco. *Plant Cell* 1:599-607.
- Brogli, K. E., Chet, I., Holiday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C. J., and Brogli, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* 254:1194-1197.
- Brown, R. L., Kazan, K., McGrath, K. C., Maclean, D. J., and Manners, J. M. 2003. A role for the GCC-box in jasmonate-mediated activation of the *PDF1.2* gene of *Arabidopsis*. *Plant Physiol.* 132:1020-1032.
- Bustin, S. A. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): Trends and problems. *J. Mol. Endocrinol.* 29:23-39.
- Buttner, M., and Singh, K. B. 1997. *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 94:5961-5966.
- Chakravarthy, S., Tuori, R. P., D'Ascenzo, M. D., Fobert, P. R., Despres, C., and Martin, G. B. 2003. The tomato transcription factor Pti4 regulates defense-related gene expression via GCC box and non-GCC box cis elements. *Plant Cell* 15:3033-3050.
- Cheong, Y. H., Moon, B. C., Kim, J. K., Kim, C. Y., Kim, M. C., Kim, I. H., Park, C. Y., Kim, J. C., Park, B. O., Koo, S. C., Yoon, H. W., Chung, W. S., Lim, C. O., Lee, S. Y., and Cho, M. J. 2003. BWMK1, a rice mitogen-activated protein kinase, locates in the nucleus and mediates pathogenesis-related gene expression by activation of a transcription factor. *Plant Physiol.* 132:1961-1972.
- Clough, S. J., and Bent, A. F. 1998. Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16:735-743.
- Despres, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P. R. 2000. The *Arabidopsis* NPR1/NM1 protein enhances the DNA binding activity of

- a subgroup of the TGA family of bZIP transcription factors. *Plant Cell* 12:279-290.
- Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. 1980. Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. *Proc. Natl. Acad. Sci. U.S.A.* 77:7347-7351.
- Dong, X., Mindrinos, M., Davis, K. R., and Ausubel, F. M. 1991. Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. *Plant Cell* 3:61-72.
- Feys, B. J., and Parker, J. E. 2000. Interplay of signaling pathways in plant disease resistance. *Trends Genet.* 16:449-455.
- Fischer, U., and Droge-Laser, W. 2004. Overexpression of *NtERF5*, a new member of the tobacco ethylene response transcription factor family enhances resistance to tobacco mosaic virus. *Mol. Plant-Microbe Interact.* 17:1162-1171.
- Fujimoto, S. Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. 2000. *Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *Plant Cell* 12:393-404.
- Gijzen, M., Kuflu, K., Qutob, D., and Chernys, J. T. 2001. A class I chitinase from soybean seed coat. *J. Exp. Bot.* 52:2283-2289.
- Glazebrook, J. 2001. Genes controlling expression of defense responses in *Arabidopsis*-2001 status. *Curr. Opin. Plant Biol.* 4:301-308.
- Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43:205-227.
- Goverse, A., Overmars, H., Engelbertink, J., Schots, A., Bakkar, J., and Helder, J. 2000. Both induction and morphogenesis of cyst nematode feeding cells are mediated by auxin. *Mol. Plant-Microbe Interact.* 13:1121-1129.
- Grebenok, R. J., Pierson, E., Lambert, G. M., Gong, F. C., Afonso, C. L., Haldeman-Cahill, R., Carrington, J. C., and Galbraith, D. W. 1997. Green-fluorescent protein fusions for efficient characterization of nuclear targeting. *Plant J.* 11:573-586.
- Gu, Y. Q., Yang, C., Thara, V. K., Zhou, J., and Martin, G. B. 2000. *Pti4* is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. *Plant Cell* 12:771-785.
- Gu, Y. Q., Wildermuth, M. C., Chakravarthy, S., Loh, Y. T., Yang, C., He, X., Han, Y., and Martin, G. B. 2002. Tomato transcription factors *Pti4*, *Pti5*, and *Pti6* activates defense responses when expressed in *Arabidopsis*. *Plant Cell* 14:817-831.
- Guo, H., and Ecker, J. R. 2004. The ethylene signaling pathway: New insights. *Curr. Opin. Plant Biol.* 7:40-49.
- Guo, Z. J., Chen, X. J., Wu, X. L., Ling, J. Q., and Xu, P. 2004. overexpression of the AP2/EREBP transcription factor OPBP1 enhances disease resistance and salt tolerance in tobacco. *Plant Mol. Biol.* 55:607-618.
- Gutterson, N., and Reuber, T. L. 2004. Regulation of disease resistance pathways by AP2/ERF transcription factors. *Curr. Opin. Plant Biol.* 7:465-471.
- Hao, D., Ohme-Takagi, M., and Sarai, A. 1998. Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. *J. Biol. Chem.* 273:26857-26861.
- He, P., Warren, R. F., Zhao, T., Shan, L., Zhu, L., Tang, X., and Zhou, J. M. 2001. Overexpression of *Pti5* in tomato potentiates pathogen-induced defense gene expression and enhances disease resistance to *Pseudomonas syringae* pv. *tomato*. *Mol. Plant-Microbe Interact.* 14:1453-1457.
- Hermesmeier, D., Hart, J. K., Byzova, M., Rodermel, S. R., and Baum, T. J. 2000. Changes in mRNA abundances within *Heterodera schachtii*-infected roots of *Arabidopsis thaliana*. *Mol. Plant-Microbe Interact.* 13:309-315.
- Higo, K., Ugawa, T., Iwamoto, M., and Higo, H. 1998. PLACE: A database of plant *cis*-acting regulatory DNA elements. *Nucleic Acids Res.* 26:358-359.
- Hoffman, T., Schmidt, J. S., Zheng, X., and Bent, A. F. 1999. Isolation of ethylene-insensitive soybean mutants that are altered in pathogen susceptibility and gene-for-gene disease resistance. *Plant Physiol.* 119:935-949.
- Jefferson, R. A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.* 5:387-405.
- Jones, M. G. K. 1981. Host cell responses to endoparasitic nematode attack: Structure and function of giant cells and syncytia. *Ann. Appl. Biol.* 97:353-372.
- Koyama, T., Okada, T., Kitajima, S., Ohme-Takagi, M., Shinshi, H., and Sato, F. 2003. Isolation of tobacco ubiquitin-conjugating enzyme cDNA in a yeast two-hybrid system with tobacco ERF3 as bait and its characterization of specific interaction. *J. Exp. Bot.* 54:1175-1181.
- Lee, J. H., Hong, J. P., Oh, S. K., Lee, S., Choi, D., and Kim, W. T. 2004. The ethylene-responsive factor like protein 1 (CaERFLP1) of hot pepper (*Capsium annuum* L.) interacts *in vitro* with both GCC and DRE/CRT sequences with different binding affinities: Possible biological roles of CaERFLP1 in response to pathogen infection and high salinity conditions in transgenic tobacco plants. *Plant Mol. Biol.* 55:61-81.
- Lee, J. H., Kim, D. M., Lee, J. H., Kim, J., Bang, J. W., Kim, W. T., and Pai, H. S. 2005. Functional characterization of NtCEF1, an AP2/EREBP-type transcriptional activator highly expressed in tobacco callus. *Planta* 222:211-224.
- Li, H. Y., and Chye, M. L. 2004. *Arabidopsis* acyl-CoA-binding protein ACBP2 interacts with an ethylene-responsive element-binding protein, AtEBP, via its ankyrin repeats. *Plant Mol. Biol.* 54:233-243.
- Lorenzo, O., Piqueras, R., Sanchez-Serrano, J. J., and Solano, R. 2003. ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* 15:165-178.
- Manners, J. M., Penninckx, I. A., Vermaere, K., Kazan, K., Brown, R. L., Morgan, A., Maclean, D. J., Curtis, M. D., Cammue, B. P., and Broekaert, W. F. 1998. The promoter of the plant defensin gene *PDF1.2* from *Arabidopsis* is systematically activated by fungal pathogens and responds to methyl jasmonate but not to salicylic acid. *Plant Mol. Biol.* 38:1071-1080.
- Mazarei, M., Puthoff, D. P., Hart, J. K., Rodermel, S. R., and Baum, T. J. 2002. Identification and characterization of a soybean ethylene-responsive element binding protein gene whose mRNA expression changes during soybean cyst nematode infection. *Mol. Plant-Microbe Interact.* 15:577-586.
- Mazarei, M., Lennon, K. L., Puthoff, D. P., Rodermel, S. R., and Baum, T. J. 2003. Expression of an *Arabidopsis* phosphoglycerate mutase homologue is localized to apical meristems, regulated by hormones, and induced by sedentary plant-parasitic nematodes. *Plant Mol. Biol.* 53:513-530.
- Mazarei, M., Lennon, K. L., Puthoff, D. P., Rodermel, S. R., and Baum, T. J. 2004. Homologous soybean and *Arabidopsis* genes share responsiveness to cyst nematode infection. *Mol. Plant Pathol.* 5:409-423.
- McDowell, J. M., and Dangel, J. L. 2000. Signal transduction in the plant immune response. *Trends Biochem. Sci.* 25:79-82.
- McGrath, K. C., Dombrecht, B., Manners, J. M., Schenk, P. M., Edgar, C. I., Maclean, D. J., Scheible, W. R., Udvardi, M. K., and Kazan, K. 2005. Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genomic-wide screen of *Arabidopsis* transcription factor gene expression. *Plant Physiol.* 139:949-959.
- Nakano, T., Suzuki, K., Fujimura, T., and Shinshi, H. 2006. Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiol.* 140:411-432.
- Nasir, K. H., Takahashi, Y., Ito, A., Saitoh, H., Matsumura, H., Kanzaki, H., Shimizu, T., Ito, M., Fujisawa, S., Sharma, P. C., Ohme-Takagi, M., Kamoun, S., and Terauchi, R. 2005. High-throughput in planta expression screening identifies a class II ethylene-responsive element binding factor-like protein that regulates plant cell death and non-host resistance. *Plant J.* 43:491-505.
- Ogawa, T., Pan, L., Kawai-Yamada, M., Yu, L. H., Yamamura, S., Koyama, T., Kitajima, S., Ohme-Takagi, M., Sato, F., and Uchimiya, H. 2005. Functional analysis of *Arabidopsis* ethylene-responsive element binding protein conferring resistance to Bax and abiotic stress-induced plant cell death. *Plant Physiol.* 138:1436-1445.
- Ohme-Takagi, M., and Shinshi, H. 1995. Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell* 7:173-182.
- Ohme-Takagi, M., Suzuki, K., and Shinshi, H. 2000. Regulation of ethylene-induced transcription of defense genes. *Plant Cell Physiol.* 41:1187-1192.
- Ohta, M., Ohme-Takagi, M., and Shinshi, H. 2000. Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions. *Plant J.* 22:29-38.
- Ohta, M., Matsui, K., Hiratsu, K., Shinshi, H., and Ohme-Takagi, M. 2001. Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* 13:1959-1968.
- Park, J. M., Park, C., Lee, S., Ham, B., Shin, R., and Paek, K. 2001. Overexpression of the tobacco *Tsi1* gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *Plant Cell* 13:1035-1046.
- Pawlowski, K., Kunze, R., De Vries, S., and Bisseling, T. 1994. Isolation of total, poly(A) and polysomal RNA from plant tissues. Pages D5/1-D5/13 in: *Plant Molecular Biology Manual*. S. B. Gelvin and R. A. Schilperoort, eds. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Paz, M. M., Shou, H., Guo, Z., Zhang, Z., Banerjee, A. K., and Wang, K. 2004. Assessment of conditions affecting *Agrobacterium*-mediated soybean transformation using the cotyledonary node explant. *Euphytica* 136:167-179.

- Penninckx, I. A. M. A., Eggermont, K., Terras, F. R. G., Thomma, B. P. H. J., De Samblanx, G. W., Buchala, A., Mettraux, J. P., Manners, J. M., and Broekaert, W. F. 1996. Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell* 8:2309-2323.
- Penninckx, I. A. M. A., Thomma, B. P. H. J., Buchala, A., Mettraux, J. P., and Broekaert, W. F. 1998. Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *Plant Cell* 10:2103-2113.
- Riechmann, J. L., and Meyerowitz, E. M. 1998. The AP2/EREBP family of plant transcription factors. *Biol. Chem.* 379:633-646.
- Rogers, E. E., and Ausubel, F. M. 1997. *Arabidopsis* enhanced disease susceptibility mutants exhibit enhanced susceptibility to several bacterial pathogens and alterations in *PR-1* gene expression. *Plant Cell* 9:305-316.
- Rojo, E., Solano, R., and Sanchez-Serrano, J. J. 2003. Interactions between signaling compounds involved in plant defense. *J. Plant Growth Regul.* 22:82-98.
- Rouster, J., Leah, R., Mundy, J., and Mills, C. V. 1997. Identification of a methyl jasmonate-responsive region in the promoter of a lipoxygenase 1 gene expressed in barley grain. *Plant J.* 11:513-523.
- Saikia, R., Singh, B. P., Kumar, R., and Arora, D. K. 2005. Detection of pathogenesis-related proteins-chitinase and beta-1,3-glucanase in induced chickpea. *Curr. Sci.* 89:659-663.
- Samac, D. A., Hironaka, C. M., Yallaly, P. E., and Shah, D. M. 1990. Isolation and characterization of the genes encoding basic and acidic chitinase in *Arabidopsis thaliana*. *Plant Physiol.* 93:907-914.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. A. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Schardl, C., Byrd, A. D., Benzion, G. B., Altschuler, M. A., Hildebrand, D. F., and Hunt, A. G. 1987. Design and construction of a versatile system for the expression of foreign genes in plants. *Gene* 61:1-11.
- Sela-Buurlage, M., Ponstein, A. S., Bres-Vloemans, S. A., Melchers, L. S., Van den Elzen, P. J. M., and Cornelissen, B. J. C. 1993. Only specific tobacco (*Nicotiana tabacum*) chitinase and β -1,3-glucanases exhibit antifungal activity. *Plant Physiol.* 101:857-863.
- Sheen, J. 2001. Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. *Plant Physiol.* 127:1466-1475.
- Shin, R., Park, J. M., An, J. M., and Paek, K. H. 2002. Ectopic expression of *Ts1l* in transgenic hot pepper plants enhances host resistance to viral, bacterial, and oomycete pathogens. *Mol. Plant-Microbe Interact.* 15:983-989.
- Sijmons, P. C., Grundler, F. M. W., Von Mende, N., Burrows, P. R., and Wyss, U. 1991. *Arabidopsis thaliana* as a new model host for plant-parasitic nematodes. *Plant J.* 1:245-254.
- Singh, K. B., Foley, R. C., and Onate-Sanchez, L. 2002. Transcription factors in plant defense and stress responses. *Curr. Opin. Plant Biol.* 5:430-436.
- Solano, R., Stepanova, A., Chao, Q., and Ecker, J. R. 1998. Nuclear events in ethylene signaling: A transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev.* 12:3703-3714.
- Stepanova, A. N., and Ecker, J. R. 2000. Ethylene signaling: From mutants to molecules. *Curr. Opin. Plant Biol.* 3:353-360.
- Takeuchi, Y., Yoshikawa, M., Takeba, G., Tanaka, K., Shibata, D., and Horino, O. 1990. Molecular cloning and ethylene induction of mRNA encoding a phytoalexin elicitor-releasing factor, β -1,3-endoglucanase, in soybean. *Plant Physiol.* 93:673-682.
- Tang, W., Charles, T. M., and Newton, R. J. 2005. Overexpression of the pepper transcription factor CaPF1 in transgenic Virginia pine (*Pinus virginiana* Mill.) confers multiple stress tolerance and enhances organ growth. *Plant Mol. Biol.* 59:603-617.
- Thomma, B. P. H. J., Penninckx, I. A. M. A., Broekaert, W. F., and Cammue, B. P. A. 2001. The complexity of disease signaling in *Arabidopsis*. *Curr. Opin. Immunol.* 13:63-68.
- Tohidfar, M., Mohammadi, M., and Ghareyazie, B. 2005. *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum*) using a heterologous bean chitinase gene. *Plant Cell Tiss. Organ Cult.* 83:83-96.
- Tournier, B., Sanchez-Ballesta, M. T., Jones, B., Pesquet, E., Regad, F., Latche, A., Pech, J. C., and Bouzayen, M. 2003. New members of the tomato ERF family show specific expression pattern and diverse DNA-binding capacity to the GCC box element. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 550:149-154.
- Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. 1992. Acquired resistance in *Arabidopsis*. *Plant Cell* 4:645-656.
- Varagona, M. J., Schmidt, R. J., and Raikhel, N. V. 1992. Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opaque-2. *Plant Cell* 4:1213-1227.
- Wang, K. L. C., Li, H., and Ecker, J. R. 2002. Ethylene biosynthesis and signaling networks. *Plant Cell* 14(Suppl.):S131-S151.
- Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., and Ryals, J. A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3:1085-1094.
- Wrather, J. A., Anderson, T. R., Arsyad, D. M., Tan, Y., Ploper, L. D., Porta-Puglia, A., Ram, H. H., and Yorinori, J. T. 2001a. Soybean disease loss estimates for the top ten soybean-producing countries in 1998. *Can. J. Plant Pathol.* 23:115-121.
- Wrather, J. A., Stienstra, W. C., and Koenning, S. R. 2001b. Soybean disease loss estimates for the United States from 1996 to 1998. *Can. J. Plant Pathol.* 23:122-131.
- Wu, K., Tian, L., Hollingworth, J., Brown, D. C. W., and Miki, B. 2002. Functional analysis of tomato *Pti4* in *Arabidopsis*. *Plant Physiol.* 128:30-37.
- Wubben, M. J. E., II, Su, H., Rodermer, S. R., and Baum, T. J. 2001. Susceptibility to the sugar beet cyst nematode is modulated by ethylene signal transduction in *Arabidopsis thaliana*. *Mol. Plant-Microbe Interact.* 14:1206-1212.
- Wubben, M. J. E., II, Rodermer, S. R., and Baum, T. J. 2004. Mutation of a UDP-glucose-4-epimerase alters nematode susceptibility and ethylene responses in *Arabidopsis* roots. *Plant J.* 40:712-724.
- Xu, P., Narasimhan, M. L., Samson, T., Coca, M. A., Huh, G. H., Zhou, J., Martin, G. B., Hasegawa, P. M., and Bressan, R. A. 1998. A nitrilase-like protein interacts with GCC box DNA-binding proteins involved in ethylene and defense responses. *Plant Physiol.* 118:867-874.
- Yi, S. Y., Kim, J. H., Joung, Y. H., Lee, S., Kim, W. T., Yu, S. H., and Choi, D. 2004. The pepper transcription factor *CaPF1* confers pathogen and freezing tolerance in *Arabidopsis*. *Plant Physiol.* 136:2862-2874.
- Zeng, P., Vadrnais, D. A., Zhang, Z., and Polacco, J. C. 2004. Refined glutofosinate selection in *Agrobacterium*-mediated transformation of soybean (*Glycine max* (L) Merrill). *Plant Cell Rep.* 22:478-482.
- Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. 1999. Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. *Proc. Natl. Acad. Sci. U.S.A.* 96:6523-6528.
- Zhang, H., Zhang, D., Chen, J., Yang, Y., Huang, Z., Huang, D., Wang, X. C., and Huang, R. 2004. Tomato stress-responsive factor TSRF1 interacts with ethylene responsive element GCC box and regulates pathogen resistance to *Ralstonia solanacearum*. *Plant Mol. Biol.* 55:825-834.
- Zhou, J., Tang, X., and Martin, G. B. 1997. The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:3207-3218.
- Zhu, Y., Cai, X. L., Wang, Z. Y., and Hong, M. M. 2003. An interaction between MYC protein and an EREBP protein is involved in transcriptional regulation of the rice *Wx* gene. *J. Biol. Chem.* 278:47803-47811.
- Zhu, Q., Maher, E. A., Masoud, S., Dixon, R. A., and Lamb, C. J. 1994. Enhanced protection against fungal attack by constitutive co-expression of chitinase and glucanase in transgenic tobacco. *Bio/Technology* 12:807-812.