Tomato RAV Transcription Factor Is a Pivotal Modulator Involved in the AP2/EREBP-Mediated Defense Pathway[1][W][OA]

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Ralstonia solanacearum is the causal agent of bacterial wilt (BW), one of the most important bacterial diseases worldwide. We used cDNA microarray to survey the gene expression profile in transgenic tomato (*Solanum lycopersicum*) overexpressing Arabidopsis (*Arabidopsis thaliana*) *CBF1* (*AtCBF1*), which confers tolerance to BW. The disease-resistant phenotype is correlated with constitutive expression of the *Related-to-ABI3/VP1* (*RAV*) transcription factor, ethylene-responsive factor (*ERF*) family genes, and several pathogenesis-related (*PR*) genes. Using a transient assay system, we show that tomato RAV2 (SlRAV2) can transactivate the reporter gene driven by the *SlERF5* promoter. Virus-induced gene silencing of *SlERF5* and *SlRAV2* in *AtCBF1* transgenic and BW-resistant cultivar Hawaii 7996 plants gave rise to plants with enhanced susceptibility to BW. Constitutive overexpression of *SlRAV2* in transgenic tomato plants induced the expression of *SlERF5* and *PR5* genes and increased BW tolerance, while knockdown of expression of *SlRAV2* inhibited *SlERF5* and *PR5* gene expression under pathogen infection and significantly decreased BW tolerance. In addition, transgenic tomato overexpressing *SlERF5* also accumulated higher levels of *PR5* transcripts and displayed better tolerance to pathogen than wild-type plants. From these results, we conclude that SlERFs may act as intermediate transcription factors between *AtCBF1* and *PR* genes via SlRAV in tomato, which results in enhanced tolerance to BW.

Tomato (*Solanum lycopersicum*) is the second most consumed vegetable worldwide. The productivity and quality of tomato fruits are often threatened by a broad range of plant diseases caused by fungi, bacteria, nematodes, and arthropods (Deslandes et al., 2002; Hemming et al., 2004). *Ralstonia solanacearum* is one of the most common soil-borne vascular diseases of the tomato crop; the resulting disease, bacterial wilt (BW), can be devastating and difficult to control by conventional approaches. Introgression of traits has played a pivotal role in developing BW-resistant varieties to reduce yield loss; however, only a few of the generated varieties show stable resistance because of the great diversity of pathogen strains (Hai et al., 2008). Genetic engineering is a promising alternative strategy to enhance plant disease resistance to a wide range of pathogens. The validity of this approach has been demonstrated in crops into which a wide array of plant disease resistance genes and pathogen virulence genes have been cloned. Although many genetic engineering programs in major tomato-growing areas worldwide focus on producing BW-tolerant varieties, the genetic network regulating plant tolerance to BW remains poorly understood. However, understanding plant defense mechanisms and responses to pathogens is critical to developing resistant tomato varieties (Robb et al., 2007).

Being sessile in nature, plants use a variety of strategies to protect themselves from pathogen infection. The protection is manifested by a single gene or a group of genes working in coordination to modulate specific defense responses via signal transduction cascades and transcriptional activation of many genes (Zhang et al., 2004a; Wang et al., 2005). The integrated defense systems are reflected in the expression of transcription factors and protein kinases as well as changes in cytosolic calcium fluxes, an increase in reactive oxygen species during the oxidative burst, and induction of hypersensitive cell death (the hyper-

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sensitive response; Gómez-Gómez, 2004; Ryan et al., 2007). The expression of various defense genes also leads to the production of defensive compounds, such as pathogenesis-related (PR) proteins and enzymes involved in the biosynthesis of protective secondary metabolites (Gu et al., 2002). Even though the functions of most PR gene products are unknown, some of these proteins, such as β-1,3-glucanase (PR2) and chitinase (PR3), are known to inhibit fungal growth, and thaumatin-like/osmotin (PR5) has been found to induce apoptosis (He et al., 2001; Gu et al., 2002).

Many PR genes induced during pathogen infection are up-regulated by one or more signaling molecules, such as salicylic acid (SA), ethylene, and jasmonic acid (JA; Koo et al., 2007). Recent evidence indicates that transcription factors play key roles in controlling the expression of PR genes; for instance, ethylene-responsive factor (ERF) proteins activate *PR* genes by binding to the *GCC* box (GCCGCC) of their promoters, thereby regulating the plant defense response to pathogen infection (Zhang et al., 2004a). Recently, AP2/EREBP (for apetala2/ethylene-responsive element-binding protein) proteins were shown to be integrators of biotic and abiotic stress responses through their interaction with cis-acting elements, the *GCC* box, and/or *CRT/DRE* (for C-repeat/dehydration response element; Park et al., 2001; Zhang et al., 2005). These proteins comprise unique transcription factors to the plant lineage and are classified into four subfamilies: AP2, DREB (for dehydration response element-binding protein), ERF, and RAV (for related to ABI3/VP1). The members of the ERF subfamily, which include tobacco (*Nicotiana tabacum*) ERF1 to -4, Arabidopsis (*Arabidopsis thaliana*) ERF1 to -5, ORA59, tomato Pti4 to -6, tomato ERF1 to -4, and tomato stress-responsive factor (TSRF1), have been identified as transcriptional activators that bind to the *GCC* box in response to biotic stresses (Gu et al., 2002; Chakravarthy et al., 2003; Zhang et al., 2007; Pré et al., 2008). Although *AtERF4/7* also regulates genes by interacting with a *GCC* box, it is a transcriptional repressor and thus a negative regulator capable of modulating both biotic and abiotic stress responses (Yang et al., 2005). In addition, rice (*Oryza sativa*) TERF1, barley (*Hordeum vulgare*) HvRAF, and tomato TSRF1 are involved in the regulation of both biotic and abiotic stress tolerance (Jung et al., 2007; Gao et al., 2008). These findings strongly suggest that the induction of PR genes in plants is mediated by different ERF proteins and/or signaling molecules. By contrast, the regulation of PR genes by the subfamily members DREB and RAV in response to biotic stress remains unclear.

CBF/DREB1 (for CRT-binding factor or DRE-binding protein 1) genes, including *CBF1* (*DREB1B*), *CBF2* (*DREB1C*), and *CBF3* (*DREB1A*), are located on Arabidopsis chromosome 4 (Gilmour et al., 1998). The *CBF* family can bind to *CRT/DRE* elements present in the promoters of cold-regulated (*COR*) genes, such as *KIN1*, *COR15a*, *COR47*, and *RD29A*, to induce these genes in response to low temperature and dehydration (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Sakuma et al., 2002). Moreover, overexpression of cDNA encoding CBF3 in transgenic Arabidopsis activated several stress-tolerance genes, thus enhancing the tolerance of plants to drought, freezing, and salt stresses (Liu et al., 1998; Gilmour et al., 2000; Sakuma et al., 2006). Previously, we have reported that transgenic tomato expressing Arabidopsis *CBF1* (*AtCBF1*) cDNA is tolerant to various abiotic stresses such as chilling, oxidative stress, high salt, and water deficit (Hsieh et al., 2002a, 2002b; Lee et al., 2003). Here, we report that *AtCBF1* transgenic tomato plants are tolerant to *Ralstonia* infection in greenhouse experiments and that AtCBF1 modulates the plant defense response against *Ralstonia* by repressing the proliferation of bacteria in vascular tissues. In addition, we have used cDNA microarray to identify downstream defense components that connect AtCBF1 with disease defense response. Our study provides new insights into signaling pathways and defines a possible mechanism of how AtCBF1 directly or indirectly regulates other AP2/EREBP transcription factors, thereby improving tolerance of tomato against *Ralstonia*.

RESULTS

Several Pathogenesis-Related Genes Are Activated in *CBF1* Transgenic Plants

In previous studies, we have demonstrated that constitutive expression of *AtCBF1* in tomato increased tolerance to chilling and water deficit (Hsieh et al., 2002a, 2002b). To identify the genes that were differentially expressed in *AtCBF1* transgenic tomato plants, we now used subtractive hybridization and homemade microarray systems (Liu et al., 2006). Expression was increased by at least 2-fold for 25 genes in *AtCBF1* transgenic plants compared with wild-type plants (Table I). Among those genes, the following were pathogenesis-related genes: *PR3* (chitinase), *PR5* (thaumatin-like protein), *PR7* (endoproteinase), *PR9* (peroxidase), and *PR10* (RNase-like protein). Thus, heterologous expression of *AtCBF1* appears to result in enhanced expression of several *PR* genes.

Progression of BW Is Delayed in Transgenic *AtCBF1* Plants by Systemic Suppression of Bacterial Multiplication

We hypothesized that up-regulation of *PR* genes in *AtCBF1* transgenic tomato may enhance its resistance to *Ralstonia* infection. We observed transgenic plants in the greenhouse to discover which plants were more tolerant to pathogen attack. *Ralstonia* inoculation assay was performed to examine whether overexpression of *AtCBF1* in tomato can enhance pathogen resistance. Tomato natural cv Hawaii 7996 (H7996) has displayed stable resistance against various *R. solanacearum* strains (Grimault et al., 1995). In this study, we used tomato cv H7996 and the background of the *AtCBF1*

Table I. *Putative target genes of heterologous AtCBF1 in transgenic tomato plants*

Sequences of cis-acting elements are as follows: CRT/DRE, CCGAC or RYCGAC (HvCBF); GCC, GCCGCC; RAV1A, CAACA; and RAV1B, CACCTG.

Clone Name	Accession No.	Description	Unigene No.	Corresponding Arabidopsis Gene	Ratio[a]	SGN Database: Tomato WGS Scaffolds (2.30)	cis-Acting Elements within the 2-kb Promoter			
							CRT/DRE	GCC	RAV1A	RAV1B
cLEY14E7	BE449751	Protein phosphatase 2C-like protein	SGN-U573715	At2g25070	10.41 ± 2.41	No hit found	–[b]	–	–	–
C6SR473	CK574973	Cys protease (PR7)	SGN-U580215	At4g32940	6.49 ± 0.51	SL2.30sc04948	1	0	3	0
cLEX4M16	AW219536	Peroxidase (PR9)	SGN-U581155	At5g05340	6.22 ± 3.96	SL2.30sc03665	0	0	5	1
LEC5R05G01	CK468708	PR10 protein	SGN-U578441	At1g24020	5.90 ± 1.49	SL2.30sc04828	0	0	6	0
cLEX11D13	AW621284	Zinc transporter protein ZIP1	SGN-U583586	At1g05300	5.87 ± 1.78	SL2.30sc03685	0	0	3	0
cLEX8A20	AW220124	Dehydrin homolog C17	SGN-U581375	At1g20450	4.60 ± 1.55	SL2.30sc04135	5	0	1	1
LEEC101F05	CK725213	Acidic endochitinase precursor (PR3)	SGN-U566861	At5g24090	3.80 ± 1.71	SL2.30sc03902	1	0	3	0
SF471	CK574994	Acidic 26-kD endochitinase precursor (PR3)	SGN-U581507	At3g12500	3.65 ± 0.05	SL2.30sc03665	1	0	4	0
cLEX12C2	AW621528	Syntaxin-related protein Nt-syr1	SGN-U584182	At3g11820	3.53 ± 0.54	SL2.30sc04133	1	0	5	0
cLEW12A21	BF096513	Catalase isozyme 1	SGN-U578839	At4g35090	3.48 ± 0.03	SL2.30sc05380	1	0	6	0
cLEW19G18	BF097084	PTEN-like protein	SGN-U566184	At3g19420	3.43 ± 0.00	SL2.30sc03665 (gap in promoter)	–	–	–	–
cLEW27E20	BF098457	Senescence-associated protein-related	SGN-U578016	At5g20700	3.21 ± 0.12	SL2.30sc03731	1	0	5	0
cLEX5K5	AW219630	DnaJ-like heat shock protein	SGN-U589575	At4g13830	3.17 ± 1.20	SL2.30sc03902	2	0	1	0
cLEW8A6	AW980043	1-Acylglycerol -3-phosphate acyltransferase	SGN-U575300	At1g51260	3.00 ± 0.67	SL2.30sc03876	0	0	6	0
cLEX2M12	AW219010	Ubiquitin family protein	SGN-U567499	At2g30100	2.71 ± 0.26	SL2.30sc04474	1	0	3	0
cLEX2M14	AW219011	Eukaryotic translation initiation factor 5A-1	SGN-U578904	At1g13950	2.65 ± 0.62	SL2.30sc03701	0	0	3	0
SF146	CK574987	Gly-rich protein	SGN-U313109	At2g05440	2.59 ± 0.55	SL2.30sc03852 (gap, 313 bp checked)	1	0	1	0
cLEW22K5	BF097441	Microsomal signal peptidase 25-kD subunit	SGN-U577878	At2g39960	2.58 ± 0.18	SL2.30sc06557 (gap, 1,534 bp checked)	0	0	2	0
Rs-Ck-1-G3	CK715671	Microsomal ω-6-desaturase	SGN-U574778	At3g12120	2.52 ± 0.60	SL2.30sc04057	2	0	3	0
LERCD04N18	CK715495	Unknown protein	SGN-U582639	At3g03870	2.52 ± 0.20	SL2.30sc04199	0	1	6	0
cLEW19L9	BF097167	COPI homolog	SGN-U579490	At2g32950	2.21 ± 0.24	SL2.30sc04607	4	0	7	0
LEECI01D07	CK720580	Formate dehydrogenase	SGN-U579280	At5g14780	2.19 ± 0.33	SL2.30sc03665	1	0	4	0
LERCD04N21	CK715497	Cys protease (PR7)	SGN-U578421	At1g47128	2.16 ± 0.47	SL2.30sc05611	1	0	3	0
SF847	AY257487	PR-5	SGN-U578836	At4g11650	2.11 ± 0.39	SL2.30sc03923	1	2	4	1
cLEW26O13	BF098337	DnaJ-like heat shock protein	SGN-U579998	At3g44110	2.02 ± 0.01	SL2.30sc03604	0	0	8	0
SF547	CK664757	β-1,3-Glucanase (PR2)	SGN-U581016	At3g57270	1.29 ± 0.11	SL2.30sc05010	0	1	4	1

[a]Ratio = (fluorescence intensity of each cDNA for transgenic plants/fluorescence intensity of each cDNA for wild-type plants) ÷ (fluorescence intensity of ubiquitin for transgenic plants/fluorescence intensity of ubiquitin for wild-type). Each value is the mean ± SD of three independent experiments. [b]En dash (–), promoter sequence in SGN database is unavailable.

transgenic plant 5915 as BW-resistant and -susceptible control, respectively. Four *AtCBF1* constitutive overexpression lines (C5, C15, C21, and C22) with high expression but low insertion (one to two copies) of transgene were selected for further investigation. In parallel, we created transgenic tomato plants with the *AtCBF1* gene driven by the abscisic acid (ABA)-inducible ABRC1 promoter (line AC3) for pathogen infection (Lee et al., 2003). Similar to the BW-resistant tomato cv H7996, the transgenic lines (C5, C15, C21, and C22) did not show any signs of wilting at 7 d post inoculation (dpi) with *Ralstonia* (Fig. 1A). Wild-type (5915) and AC3 plants without ABA treatment were severely wilted at 7 dpi (Fig. 1A). Upon ABA treatment, AC3 plants exhibited enhanced resistance to *Ralstonia* infection (data not shown). To further investigate the nature of the enhanced BW resistance seen in the transgenic lines, we monitored the in planta multiplication of *Ralstonia* after inoculation. The bacterial titers in various tissues of susceptible control plants (5915 and AC3) reached a very high level ($\geq 10^7$ colony-forming units [cfu] g^{-1} fresh tissue) at 7 dpi (Fig. 1B). By contrast, the internal bacterial titers in the transgenic lines and H7996 were much lower than those in 5915 and AC3 except in roots. In addition, the pattern and level of bacterial growth suppression in *AtCBF1* transgenic lines was similar to that in H7996, the BW-resistant variety, with gradually declining levels of bacteria from the roots to the top stems (Fig. 1B).

To reveal the correlation between disease resistance and *AtCBF1* expression, disease progression in H7996 and in transgenic lines exhibiting high levels of BW resistance were compared with that in wild-type and AC3 plants. Less than 20% of the transgenic and H7996 plants wilted during the test period (Fig. 1C), and *AtCBF1* transgenic lines exhibited a disease incidence nearly equivalent to that of H7996 over the test period. By contrast, nearly 50% of the wild-type (5915) plants and 40% of AC3 plants wilted on 7 dpi, and all had withered on 35 dpi. Thus, we conclude that constitutive expression of *AtCBF1* in transgenic tomato enhances BW resistance by systemic suppression of internal bacterial multiplication via the activation of PR proteins.

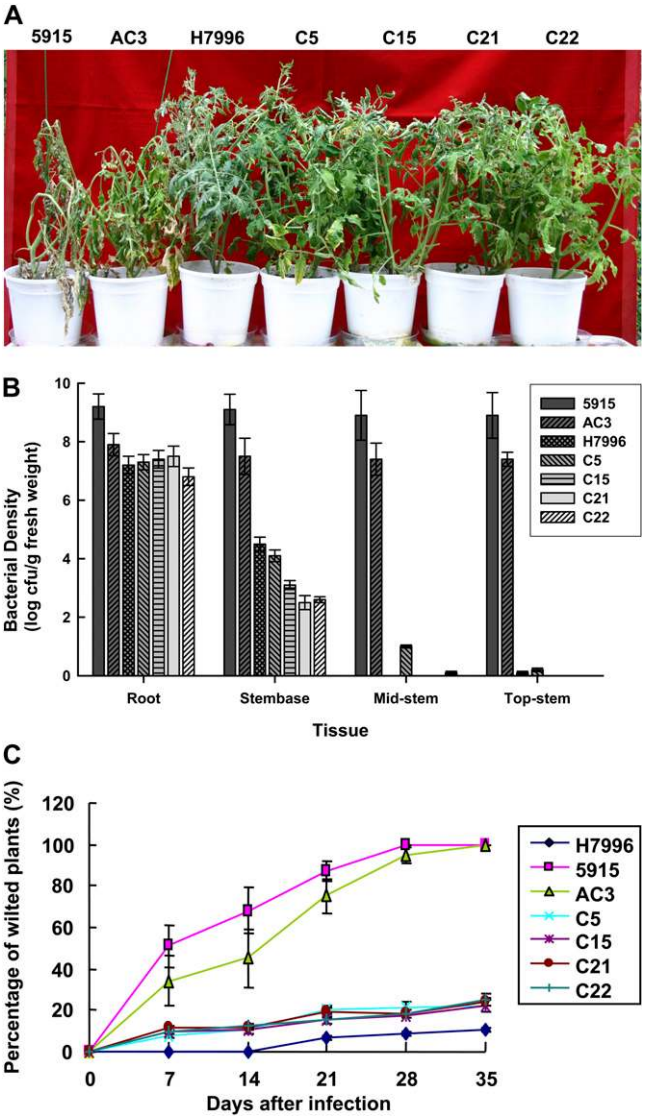

Figure 1. *AtCBF1* transgenic plants exhibit enhanced resistance to *Ralstonia*. A, Test plants were inoculated with *Ralstonia* and then kept at 28°C with a photoperiod of 16 h. The photograph was taken at 7 dpi. The test plants comprised wild-type plants (susceptible variety 5915, the genetic background of transformants), a BW-resistant control variety (H7996), a control transgenic line (AC3; *AtCBF1* driven by an ABA-inducible *ABRC1* promoter), and T2 transgenic plants continuously expressing *AtCBF1* (C5, C15, C21, and C22). B, *Ralstonia* multiplication in transgenic tomato plants was systemically suppressed. The bacterial titer inside the test plants was measured in different tissues at 7 dpi. The data are means of three independent measurements. C, Disease progression of BW was delayed in transgenic tomato plants. The response of plants subjected to BW bioassays was scored as the percentage of wilted plants over time.

Tolerance to *Ralstonia* Infection Is Not Affected by Exogenous GA_3 Treatment in Transgenic Plants

Constitutive expression of *AtCBF1* in tomato resulted in a dwarf phenotype that was alleviated by the application of GA_3 (Hsieh et al., 2002a, 2002b). To test whether GA_3 is a common antagonist of the *AtCBF* network that not only restores the dwarf and late-flowering phenotype but also influences the expression of the *PR* genes, we examined the expression levels of *PR* genes in *AtCBF1* transgenic lines (C5, C15, and C21) and wild-type plants in the presence and absence of GA_3 treatment by northern-blot analyses (Fig. 2). The tested *PR* genes, such as *PR2* (*β-1,3-glucanase*), *PR5*, *PR9*, and *PR10*, were up-regulated by 1.29-, 2.11-, 6.22-, and 5.90-fold, respectively, in *AtCBF1* transgenic plants compared with wild-type tomato plants, as shown in Table I. Although the ratio of *PR2* is only 1.29-fold, the mRNA density showed it significantly accumulated in *AtCBF1* transgenic plants (Fig. 2). Northern-blot results showed that exogenous ap-

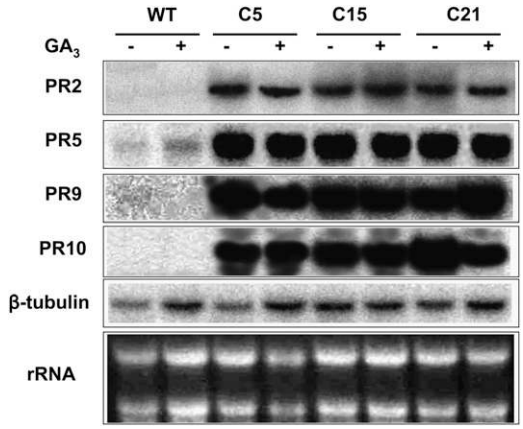

Figure 2. Northern-blot analyses of *PR* genes in transgenic tomato plants. Total RNA (10 μg) was extracted from wild-type plants (WT) and transgenic T1 plants overexpressing *AtCBF1* (C5, C15, and C21). Probes used to hybridize total RNA were ^{32}P-labeled *PR2*, *PR5*, *PR9*, *PR10*, and *β-Tubulin* cDNA fragments. Equal loading in each lane was verified by rRNA detection, which was carried out by ethidium bromide staining of the gel followed by visualization of bands under UV illumination. Experiments were performed in triplicate.

plication of GA_3 did not change the level of expression of *PR* genes in the transgenic plants. The transcripts of these genes were barely detectable in the wild-type plants with or without GA_3. Thus, we conclude that the expression of *PR* genes in *AtCBF1* transgenic plants results from the overexpression of *AtCBF1* by a GA_3-independent pathway.

Wild-type and *AtCBF1* transgenic tomato plants were infected with a high cell density of *Ralstonia* and allowed to grow under controlled conditions. In the absence of GA_3 treatment, the fruit set, seed number, and fresh weight of the *AtCBF1* transgenic lines were severely affected (Table II). However, the defects in fruit set, seed number, and fresh weight of *AtCBF1* transgenic plants were partially to almost completely restored by GA_3 treatment. After *Ralstonia* infection, all of the tested wild-type plants wilted too severely to reach the reproductive stage, while *AtCBF1* transgenic plants survived and reproduced either with or without GA_3 treatment. More importantly, not significant to little differences were found in the fruit yield between noninfected wild-type plants and *Ralstonia*-infected GA_3-treated *AtCBF1* transgenic lines (Table II; $P > 0.05$ in line C5 and C15, Student's t test). These results strongly suggest that the protection of tomato plants from *Ralstonia* infection by overexpression of *AtCBF1* is independent of the restoration of growth to normal levels by exogenous GA_3.

AtCBF1 Binds to *CRT/DRE* But Not to the *GCC* Box

To study the DNA-binding activity of AtCBF1 to the *GCC* box, which is generally present in the promoter region of *PR* genes, we performed electrophoretic mobility shift assay (EMSA) experiments with a purified His-tagged AtCBF1 fusion protein. The results indicated that AtCBF1 recombinant protein binds the *CRT/DRE* sequence but not the *GCC* box and mutated *CRT/DRE* (Supplemental Fig. S1). Binding to this element was sequence specific, as the association was efficiently inhibited by a 10- to 100-fold excess of unlabeled competitive *CRT/DRE* fragment (Supplemental Fig. S1C). From these results, we conclude that AtCBF1 binds competitively to *CRT/DRE* but not to the *GCC* box in vitro.

Several *AP2/EREBP* Family Genes Are Up-Regulated in *AtCBF1* Transgenic Tomato

According to the obtained EMSA results, AtCBF1 specifically interacts with the *CRT/DRE* rather than the *GCC* box. Therefore, we hypothesized that AtCBF1 overexpression in tomato regulates *PR* genes through either an indirect pathway or an accessory protein. To identify potential intermediate modulators involved in the signaling cascade of AtCBF1 contributing to BW tolerance, we examined changes in mRNA level of several well-known *GCC* box-binding ERFs, such as *Pti4*, *Pti5*, and *Pti6*, in *AtCBF1* transgenic tomato by

Table II. *The pathogen tolerance of transgenic tomato plants is not affected by exogenous GA_3 treatment*

Data shown in each column, from top to bottom, are fruit number (FN) per plant, seed number (SN) per fruit, and fresh weight (FW; g) per plant. Each value is the mean ± SD ($n = 5$ individual plants). Wild-type and *AtCBF1* transgenic plants were grown in pots with peat moss and watered every alternate day in a greenhouse with a 16/8-h photoperiod (daylight of about 120 μmol m^{-2} s^{-1}, 26°C ± 2°C; night temperature of 22°C ± 2°C). For GA_3 treatment, *AtCBF1* transgenic and wild-type plants were sprayed with 5 mg L^{-1} GA_3 three times per week (Hsieh et al., 2002a). One-month-old plants were inoculated with *Ralstonia*. Disease progression of BW was defined as wilted plant number divided by total plant number. Three months later, these plants were harvested, weighed for fresh weight, and calculated for fruit and seed numbers.

Treatment	Wild Type	C5	C15	C21	Wild Type + GA_3	C5 + GA_3	C15 + GA_3	C21 + GA_3
Control								
FN	21.6 ± 4.1	6.0 ± 1.6	7.2 ± 1.6	1.6 ± 1.1	26.6 ± 4.1	24.8 ± 3.6	22.4 ± 3.2	17.4 ± 5.8
SN	48.7 ± 9.2	8.4 ± 2.7	6.8 ± 1.3	2.4 ± 0.9	43.7 ± 9.2	25.4 ± 3.0	22.6 ± 2.6	29.6 ± 14.8
FW	132.4 ± 7.1	80.6 ± 5.1	106.8 ± 9.2	85.0 ± 3.9	147.4 ± 7.1	133.4 ± 13.8	138.8 ± 13.6	127.6 ± 8.7
Ralstonia								
FN	0 ± 0	9.8 ± 1.4	10.8 ± 1.6	3.8 ± 1.0	0 ± 0	25.8 ± 3.7	20.6 ± 4.1	13.8 ± 2.6
SN	0 ± 0	8.3 ± 3.2	7.7 ± 1.5	3.8 ± 1.2	0 ± 0	14.6 ± 3.2	20.8 ± 7.2	14.3 ± 5.2
FW	12.3 ± 6.3	112.4 ± 14.8	119.0 ± 15.5	120.0 ± 11.5	13.6 ± 5.3	121.4 ± 4.0	118.2 ± 8.3	113.8 ± 7.2

using semiquantitative reverse transcription (RT)-PCR. In addition, we analyzed the expression patterns of newly identified *ERFs* and *RAVs*, such as *SlERF5* and *SlRAV2*, and some tomato orthologs of putative Arabidopsis CBF1-regulated *AP2/EREBP* (Zhang et al., 2004b), such as *SlRAP2.1* and *SlRAP2.6-like* genes, in *AtCBF1* transgenic tomato. RAV transcription factors belong to a subfamily of the *AP2/EREBP* superfamily (Nakano et al., 2006). In Arabidopsis and in the rice genome, six members of the *RAV* family contain both AP2 and B3 domains (Nakano et al., 2006). However, the exact size of the tomato RAV family still remains unclear. Therefore, to determine the number of RAV genes that are expressed in *AtCBF1* transgenic tomato, we performed RT-PCR with degenerate primers (Supplemental Table S1) designed from the B3 and AP2 domains of AtRAV2 (At1g68840). We identified two *RAV* genes, designated *SlRAV1* and *SlRAV2*, that were expressed in *AtCBF1* transgenic tomato plants. Afterward, the full-length *RAV* genes were obtained by using RACE or the genome walking method (for primers, see Supplemental Table S2). Among them, *SlRAV2* was the major transcript up-regulated in *AtCBF1* transgenic tomato plants. The mRNA transcripts of *AP2/EREBP* family genes (i.e. *SlERF5*, *Pti4*, *Pti5*, *Pti6*, *SlRAP2.1*, *SlRAP2.6-like*, *SlRAV2*, and *SlCBF1*) exhibited a moderate to strong increase in *AtCBF1* transgenic tomato plants (C5, C15, and C21) as compared with the wild type (Fig. 3).

SlERF5 and Pti6 Interact with the *GCC* Box

To investigate whether SlERF5 binds the *GCC* box, an element present in the promoters of *PR* genes, and directly regulates the expression of *PR* genes, we performed transactivation assays with Arabidopsis mesophyll protoplasts. We constructed a series of reporter plasmids with a firefly luciferase (*Luc*) reporter gene driven by a cauliflower mosaic virus *35S* (*CaMV35S*) minimal promoter (*mini35S*), four *GCC* box repeats with a *mini35S* promoter (*GCCmini35S*), four mutant *GCC* box repeats with *mini35S* (*mGCCmini35S*), and effector plasmids with either *AtCBF1* or *SlERF5* cDNA, or *Pti6* (positive control; Gu et al., 2002) driven by the *CaMV35S* promoter (Fig. 4A). The pBI221 plasmid containing the *GUS* gene driven by the *CaMV35S* promoter was used as an internal control. Plasmids were cotransfected into protoplasts and incubated for 20 h, and soluble proteins were extracted to determine transactivation of the reporter gene (*Luc*/*GUS* relative activity). At coexpression of *35S*:*Pti6* or *35S*:*SlERF5* with *GCCmini35S*, transactivation of the reporter gene was increased 3- to 13-fold compared with *mGCCmini35S* or *mini35S*, respectively (Fig. 4C). However, cotransfection of *35S*:*AtCBF1* with *mGCCmini35S* decreased the transactivation of the reporter gene to the basal level, in agreement with the EMSA results. These results indicated that SlERF5 and Pti6 but not AtCBF1 function as activators of GCC box-mediated transcription.

SlRAV2 Interacts with a Promoter of *SlERF5*

SlERF5 and SlRAV2 contain one AP2 domain and belong to the ERF and RAV subfamily of AP2/EREBP proteins, respectively. Presumably, SlERF5 and SlRAV2, like other well-identified AP2/EREBPs, act as transcription factors to regulate gene expression in the nucleus. To verify this assumption, full-length SlERF5 and SlRAV2 coding regions were fused with yellow fluorescent protein (YFP) under the control of the 35S promoter and transiently expressed in Arabidopsis protoplasts. Indeed, we found that SlERF5 and SlRAV2 are localized in the nucleus (Supplemental Fig. S3).

The promoter sequences of *SlERF5* and *Pti6* were identified via the genome walking method and submitted to GenBank (accession nos. EU164418 and EU164419, respectively). Several *RAV1A* elements (CAACA) are present, but neither sequences for *CRT/DRE* nor a *GCC* box could be seen in the promoter regions of *SlERF5* and *Pti6* (Supplemental Fig. S2). To verify whether SlRAV proteins play the part of transacting factors binding to the *SlERF5* promoter, we performed in vivo transactivation assays with a

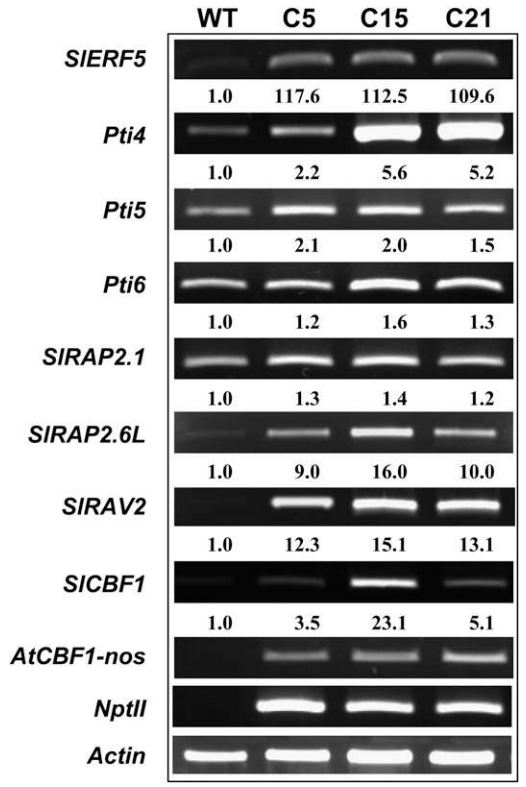

Figure 3. RT-PCR analysis of *SlERF* genes in *AtCBF1* transgenic tomatoes. Total RNA was isolated from *AtCBF1* transgenic tomatoes (C5, C15, and C21) and wild-type (WT) plants and reverse transcribed into cDNA as the templates for RT-PCR. Tomato *SlCBF1*, *SlERF5*, *Pti4*, *Pti5*, *Pti6*, *SlRAP2.1*, *SlRAP2.6-like* (*SlRAP2.6L*), *SlRAV2*, *Actin1*, *AtCBF1* transgene (*AtCBF1-nos*), and *NptII* transcripts were amplified by RT-PCR and analyzed on a gel stained with ethidium bromide.

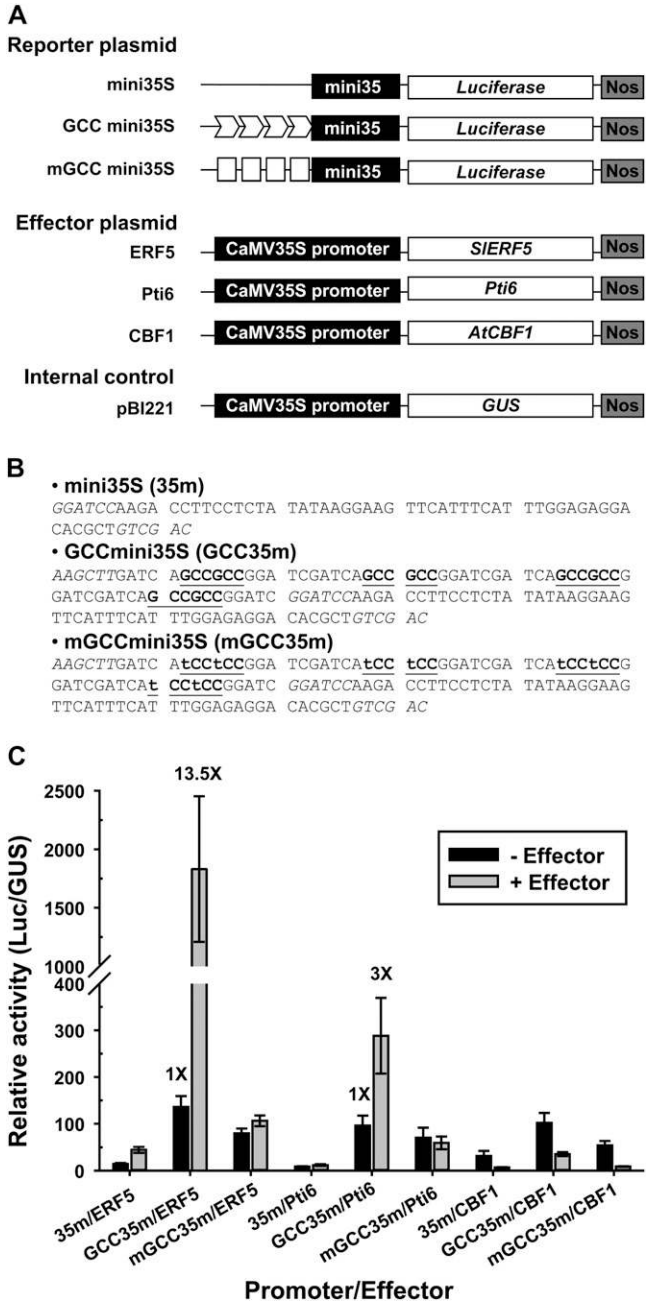

Figure 4. SlERFs activate the reporter genes driven by the *CaMV35S* minimal promoter containing vicinal *GCC* boxes. A, Schematic diagrams of the reporter, effector, and internal control plasmids used in the transient transactivation assay in Arabidopsis leaf protoplasts. The reporter plasmids contain a repeat of four *GCC* or *mGCC* boxes fused to the *CaMV35S* minimal promoter and the firefly luciferase gene *Luc*. In the effector plasmids, Arabidopsis *CBF1*, tomato *Pti6*, and *SlERF5* genes were under the control of a *CaMV35S* promoter. *Nos* denotes the terminator of *nopaline synthase*. The pBI221 vector contains a *CaMV35S* promoter driving GUS as the internal control. B, DNA sequences of the promoter region in the reporter plasmids. Sequences shown in boldface and underlined mark the wild-type and mutant *GCC* boxes, respectively. C, Transactivation of the *Luc* reporter gene by *AtCBF1*, *Pti6*, and *SlERF5* in Arabidopsis protoplasts. Different effectors were cotransfected with the reporter and internal control plasmid (pBI221). The data represent means of three independent transient transformations. Error bars indicate SD. Transient transformations without the effector plasmid were used as a control.

reporter plasmid carrying the *Luc* reporter gene driven by the *SlERF5* promoter (776 bp). As a control, *Luc* driven by the *CaMV35S* minimal promoter (*mini35S*) was employed. The effector plasmids were coding sequence of *SlRAV2* or *AtCBF1* driven by the *CaMV35S* promoter (Fig. 5A). Methyl jasmonate (MJ), which acts as a global regulator of defense responses (Reymond and Farmer, 1998), was applied to mimic the pathogen or elicitor treatment. Coexpression of the *SlERF5* promoter (*ERF5p*) with 35S:*SlRAV2* resulted in an induction of transactivation of the reporter gene 2.6 times higher than the control; this induction even increased further to a level of 4.9 times that of the control (*ERF5p* reporter only) in the presence of MJ (Fig. 5B). By contrast, cotransfection of the *SlERF5* promoter with *35S:AtCBF1* reduced the transactivation of the reporter gene to the basal level, with no effect by MJ on transactivation of the reporter gene. These results indicated that the *SlERF5* promoter interacts with SlRAV2 but not with AtCBF1 and that MJ enhances the transactivation of SlERF5 and SlRAV2. Therefore, SlRAV2 and SlERF5/Pti6 may be intermediate transcription factors acting between *AtCBF1* and *PR* genes. Taken together, we hypothesize that overexpression of *AtCBF1* regulates some *RAV* genes to adjust *ERF* genes that further modulate the expression of *PR* genes in transgenic tomato, thus enhancing tolerance to *Ralstonia* infection.

Virus-Induced Gene Silencing of *SlERF5* and *SlRAV2* Attenuates the Defense against BW in Tomato

The tobacco rattle virus (TRV)-based virus-induced gene silencing (VIGS) approach is an efficient silencing system to study the function of candidate genes responsible for certain disease resistance and their signaling pathways (Brigneti et al., 2004; Chen et al., 2009). To find out whether *SlERF5* and *SlRAV2* are involved in the BW-defense mechanism in both natural cv H7996 and *AtCBF1* transgenic tomato plants, we performed experiments silencing these AP2/EREBP transcription factors. A mixture of *Agrobacterium tumefaciens* cultures containing TRV1 and TRV2-X (X = partial cDNA sequence of *SlERF5* or *SlRAV2*; for primers, see Supplemental Table S2) T-DNA constructs was infiltrated into cotyledons of 10-d-old *AtCBF1* transgenic seedlings as well as into cotyledons of the BW-resistant H7996 and the BW-susceptible varieties 5915 and L390 as controls. Fifteen days post agroinfiltration, total RNA was prepared from leaves and used for semiquantitative RT-PCR analyses of silenced genes, *SlERF5* and *SlRAV2*. In TRV-*ERF5*- and TRV-*RAV2*-infected plants, the transcripts of *SlERF5* and *SlRAV2* were reduced compared with the TRV-only infected control (Fig. 6A). The *Ubiquitin* RNA

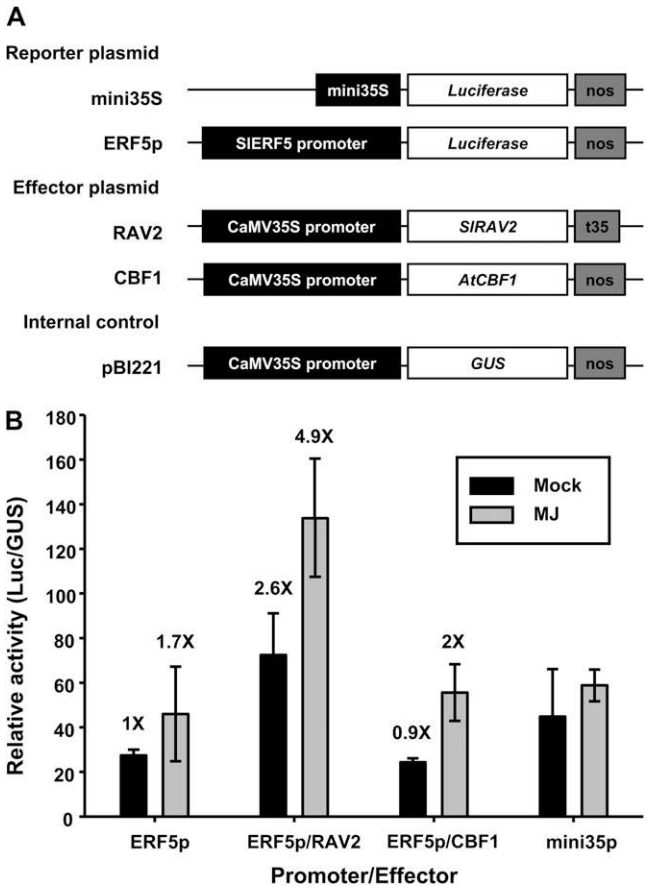

Figure 5. *SlRAV2* interacts with the promoter of *SlERF5*. A, Schematic diagrams of the reporter, effector, and internal control plasmids used in the transient transactivation assay in Arabidopsis leaf protoplasts. The reporter plasmid contains the *CaMV35S* minimal promoter and the *SlERF5* promoter sequence (776 bp) fused to the firefly luciferase gene *Luc*. In the effector plasmids, *SlRAV2* and Arabidopsis *CBF1* genes were driven under the control of the *CaMV35S* promoter. *Nos* and *t35* denote the terminators of *nopaline synthase* and *CaMV35S*, respectively. The pBI221 vector contains a *CaMV35S* promoter driving *GUS* as the internal control. B, Transactivation of the *Luc* reporter gene by *SlRAV2* and *AtCBF1* in Arabidopsis protoplasts. Different effectors were co-transfected with the reporter and internal control plasmid (pBI221). Mock, Methanol; MJ, 30 μM MJ. The data represent means of three independent transient transformations. Error bars indicate SD. Transient transformation without the effector plasmid (*ERF5p* or *mini35p*) was used as a control.

served as an internal control for RNA quality. The *Ubiquitin* transcript levels were similar in silenced plants, TRV-*ERF5* and TRV-*RAV2*, and TRV-only infected plants (Fig. 6A).

Ten days post agroinfiltration, the plants were challenged with *Ralstonia*. Both visual symptom development and internal bacterial density in both the stem base and stem were determined at 5 dpi. All of the TRV-only infected tomato plants showed resistance to *Ralstonia* infection. *AtCBF1* transgenic or H7996 tomato plants preinfected with TRV-*ERF5* or TRV-*RAV2*, respectively, displayed a severe wilt phenotype after inoculation with *Ralstonia* (Fig. 6B). Furthermore, we carried out a bacterial titer assay for gene-silenced plants at 5 dpi (Table III). Here, tomato cv 5915, the background of *AtCBF1* transgenic plants, and the susceptible cv L390 were used as the control to confirm the success of pathogen infection. These cultivars displayed a severe wilt phenotype with a very high bacteria level (mean value was greater than 10^9 cfu g^{-1} fresh weight at both stem bases and midstems; Table III). The stem base and midstem of *SlERF5*- and *SlRAV2*-silenced plants exhibited relatively higher levels of bacterial density compared with TRV-only control plants. These results indicated that silencing *SlERF5* and *SlRAV2* had indeed decreased the resistance of tomato to BW.

Generation and Characterization of *SlRAV2*RNAi Knockdown as Well as *35S:SlRAV2* and *35S:SlERF5* Transgenic Tomato Plants

To further investigate the functions of *SlRAV2* and *SlERF5* in pathogen resistance, transgenic tomato plants with knockdown expression of *SlRAV2* or overexpression of *SlRAV2* or *SlERF5* were generated. Transgenic plants with lower insertion (one to two copies) of transgene were selected for further study.

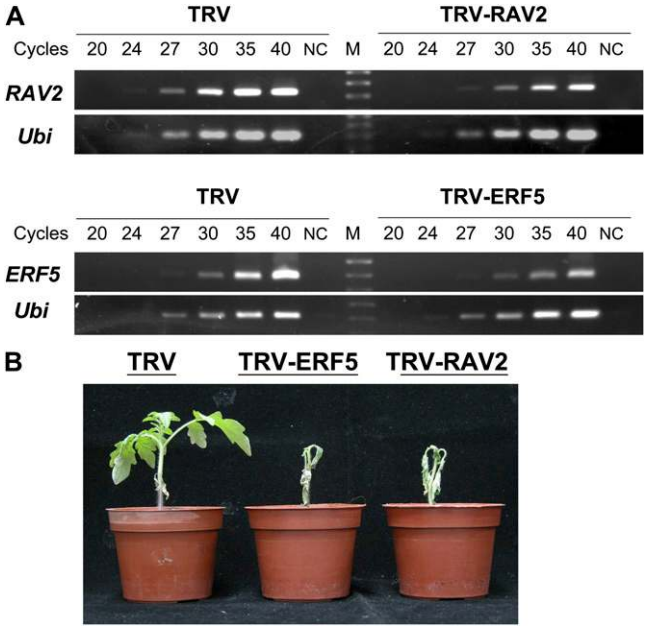

Figure 6. Silencing of *SlERF5* and *SlRAV2* using TRV-based vector. BW-resistant tomato variety H7996 and *AtCBF1* transgenic plants (CBF1) were infected with mixtures of *Agrobacterium* transformed with pTRV1 and pTRV2 (TRV) or pTRV2 carrying *SlERF5* (TRV2-ERF5) or *SlRAV2* (TRV2-RAV2) fragments. A, Semiquantitative RT-PCR analysis showing the effect of VIGS on tomato *ERF5* and *RAV2*. For each sample, six amplification products (following 20, 24, 27, 30, 35, and 40 cycles of PCR) were analyzed. *Ubiquitin* product (*Ubi*) was used as a reference. Lane NC represents the negative control, in which the RT reaction mix without reverse transcriptase was used as a template. Lane M represents a DNA marker. B, TRV-alone, TRV-ERF5-, and TRV-RAV2-infected H7996 plants were treated with *Ralstonia* for 2 weeks.

Table III. *Assessment of Ralstonia density in silenced tomato plants*

AtCBF1 transgenic plants (*CBF1*OX) and BW-resistant tomato variety H7996 were infected with mixtures of *Agrobacterium* transformed with pTRV1 and pTRV2 (TRV-only control) or pTRV2 carrying *SlRAV2* (SlRAV2) or *SlERF5* (SlERF5) fragments. BW-susceptible tomato varieties L390, 5915 (the background of *CBF1*OX), and TRV-infected *CBF1*OX and H7996 plants were treated with *Ralstonia*. The bacterial titer inside the test plants was measured in stem bases and midstems at 5 dpi. The number of total assayed plants and positively detected plants (+) are indicated. Each value is the mean ± SD. Pairwise comparisons were made between wild-type plants (or TRV infected in H7996) and silenced plants with Student's *t* test ([a] $P < 0.01$, [b] $P < 0.05$).

Tomato Plants	Silenced Gene	Sample No.	Stem Bases		Midstems	
			+	Log	+	Log
				cfu g^{-1} *plant tissue*		*cfu g*$^{-1}$ *plant tissue*
5915						
Wild type	–	30	30	9.1 ± 0.3	30	7.9 ± 0.9
*CBF1*OX	TRV	36	20	4.6 ± 0.6[b]	10	3.0 ± 0.5[b]
*CBF1*OX	SlRAV2	35	27	8.7 ± 1.0	19	7.5 ± 0.7
*CBF1*OX	SlERF5	36	25	8.0 ± 0.8[a]	22	6.0 ± 1.2[b]
H7996						
Wild type	TRV	36	22	3.6 ± 0.5	6	1.3 ± 0.4
Wild type	SlRAV2	35	25	5.8 ± 0.6[a]	18	4.3 ± 0.7[a]
Wild type	SlERF5	29	18	3.9 ± 0.7	11	2.4 ± 0.7[b]
L390						
Wild type	–	6	6	10.7 ± 0.2	6	10.1 ± 0.4

Semiquantitative RT-PCR was performed to analyze the mRNA levels in these transgenic plants, including independent lines of *SlRAV2* knockdown (*RAV2*-RNAi), *35S*:*SlRAV2* (*RAV2*Tr2, -5, and -8), and *35S*:*SlERF5* (*ERF5*Tr1 and -Tr5) transformants. The mRNA levels of the hygromycin phosphotransferase gene (*Hpt*) and *Actin* were used as transgenic and internal controls, respectively. The foreign transcripts of *SlRAV2* and *SlERF5* transgenes with a *35S* or *nos* terminator were expressed only in transgenic plants (Fig. 7A). In addition, *SlERF5* and its downstream gene, *SlPR5*, were not only abundantly expressed in *SlERF5* transgenic plants but also highly accumulated in *SlRAV2* transgenic plants (Fig. 7A).

We used RT-PCR to examine changes in mRNA levels of *SlRAV2* and *SlERF5* in pathogen-infected *RAV2*Tr and *RAV2*RNAi transgenic tomato. The transcription of *SlRAV2* and *SlERF5* was up-regulated by *Ralstonia* infection in wild-type plants (Fig. 7B). The level of *SlERF5* and *SlRAV2* mRNA transcripts was high in *RAV2*Tr lines as compared with the wild type under normal conditions but absent in the *RAV2*RNAi line even after treatment with the pathogen. Taken together, our results support the notion that SlRAV2 may be a key factor regulating *SlERF5* gene expression. Hence, the *SlRAV2* and *SlERF5* transgenic plants were further evaluated for resistance to pathogen infection.

Constitutive Expression of *SlERF5* and *SlRAV2* in Tomato Confers Tolerance, While Knockdown of *SlRAV2* Expression Causes Hypersensitivity to BW

SlRAV2- and *SlERF5*-overexpressing transgenic tomato plants exhibited a slightly dwarf phenotype (Fig. 8, A and C) and generated less fruit and seeds under normal conditions, while the knockdown expression of *SlRAV2* in tomato promoted plant growth and development (Fig. 8A, top panel). However, how SlRAV2 and SlERF5 participate in tomato growth and development remains to be further investigated. *SlRAV2* and *SlERF5* transgenic tomato plants were then subjected to *Ralstonia* challenge to verify their functions in the defense mechanism. The *RAV2*RNAi knockdown line already presented a severely wilted phenotype at 5 dpi, while the wild type wilted at 7 to 9 dpi (Fig. 8, A and B). On the other hand, all of the transgenic plants overexpressing either *SlRAV2* or *SlERF5* exhibited more resistance to BW (Fig. 8). When plants were inoculated with *Ralstonia*, both transgenic and wild-type plants showed reduction in PSII efficiency and chlorophyll content (Supplemental Fig. S4). The reduction in maximum photochemical efficiency of PSII in the dark-adapted state was on average 75% in wild-type (5915) plants, whereas transgenic lines showed reductions of 88% for *RAV2*RNAi, 19% for *RAV2*Tr, and 37% for *ERF5*Tr lines. Similarly, the chlorophyll content remained higher in *RAV2*Tr and *ERF5*Tr transgenic plants in comparison with the *RAV2*RNAi knockdown lines and wild-type plants after pathogen infection. The differences in PSII efficiency and chlorophyll content between wild-type plants and transgenic *RAV2*Tr and *ERF5*Tr tomato under pathogen treatment were statistically significant ($P < 0.01$, Student's *t* test). Overall, these findings indicated that *SlERF5* and *SlRAV2* play crucial roles in the basal defense of tomato plants against BW and that SlRAV2 may be a key regulator involved in plant defense.

DISCUSSION

CBF genes have been considered "master switches" that increase freezing tolerance in Arabidopsis plants

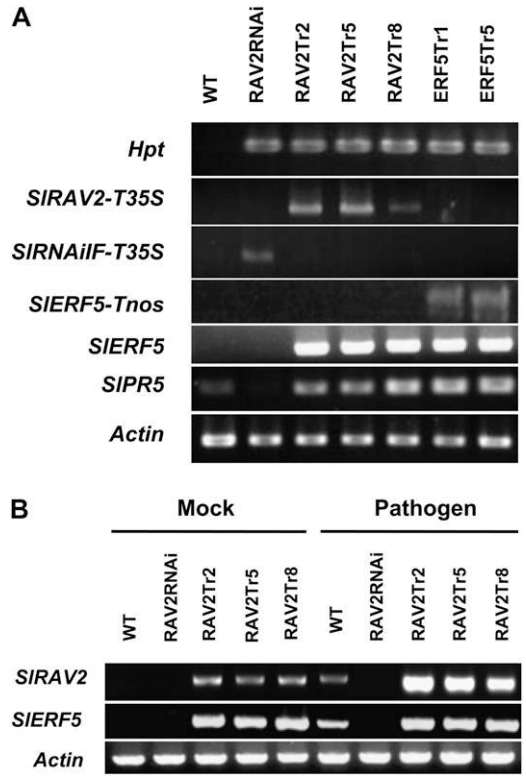

Figure 7. Analysis of *35S:SlRAV2*, *SlRAV2*RNAi, and *35S:SlERF5* transgenic tomato lines. A, Endogenous and transgenic mRNA transcript levels of *SlRAV2*, *SlERF5*, and *SlPR5* genes in *SlERF5* overexpression (*ERF5*Tr1 and *ERF5*Tr5), *SlRAV2* overexpression (*RAV2*Tr2, *RAV2*Tr5, and *RAV2*Tr8), and *SlRAV2* knockdown (*RAV2*RNAi) tomato plants. B, Expression of *SlRAV2* and *SlERF5* in wild-type (WT), *RAV2*Tr, and *RAV2*RNAi tomato lines under *Ralstonia* infection for 12 h. mRNA levels of the indicated genes in pathogen-treated (pathogen) and nontreated (mock) plants were determined by semiquantitative RT-PCR. *SlRAV2-T35S* and *SlERF5-Tnos* show transgenic expression amplified by the specific forward primers (*SlRAV2*-F and *SlERF5*-F) and the terminator reverse primers (*35T*-R and *nos*3′R; Supplemental Table S1). *SlRNAiIF-T35S* show the p*RAV2*RNAi fragment amplified by the *35T*-R reverse primer and a forward primer (RNAiI-F) located in the intron of the RNAi vector pH7GWIWG2. *Hpt* and *Actin* expression levels were analyzed as a transgenic and a quantification control, respectively.

via the activation of *COR* genes (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). Its tomato orthologs *CBF1* and *CBF2* are up-regulated by chilling and drought stress but not by other types of stress, such as high salinity or ABA treatment (Zhang et al., 2004b). In a BW-susceptible tomato variety, 5915, expression of *SlCBF1*, but not *SlCBF2* and *SlCBF3*, was up-regulated by pathogen infection (Supplemental Fig. S5). However, it still remained unclear whether CBF regulons directly participate in the biotic stress response. In this study, we showed that overexpression of *AtCBF1* in tomato leads to the constitutive accumulation of several PR genes (Table I; Fig. 2) and further enhanced tolerance to BW by suppressing the proliferation of *Ralstonia*. Furthermore, the degree and nature of enhanced resistance to BW observed in most of the tested transgenic lines was similar to that in H7996, a natural BW-resistant tomato cultivar (Fig. 1; Table III). Previously, BW resistance in H7996 was found to be related to suppressed internal pathogen multiplication rather than to the efficiency of root invasion or upward movement (Wang et al., 2000).

Accumulating evidence suggests that different ERF transcription factors induce a diverse set of PR genes under biotic and abiotic stresses (Park et al., 2001; Zhang et al., 2005, 2007). In agreement with our observations, overexpression of tomato *Pti4* and Arabidopsis *ERF1* in transgenic Arabidopsis plants led to the constitutive activation of several PR genes, resulting in enhanced tolerance against certain bacterial and fungal pathogens (Gu et al., 2002). Interaction of TSRF1 with the *GCC* box in the promoters of *PR* genes in response to *Ralstonia* infection was demonstrated in tobacco and tomato (Zhang et al., 2004a, 2007). Constitutive expression of tomato *JERF3* in transgenic tobacco activated the expression of PR genes and resulted in enhanced salt tolerance (Wang et al., 2004). In addition, ectopic expression of the pepper (*Capsicum annuum*) pathogen-induced transcription factor CaRAV1 in transgenic Arabidopsis plants induced some PR genes and enhanced the resistance of plants against infection by *Pseudomonas syringae* pv *tomato* strain DC3000 (Sohn et al., 2006). Recently, Endres et al. (2010) reported that tobacco RAV2 is an important factor in the viral suppression of silencing and that the role of RAV2 is to divert host defenses toward responses that interfere with antiviral silencing.

Within the *AP2/EREBP* family, the *AP2* subfamily members are involved in plant development, and some *ERF* subfamily members are likely involved in the responses to biotic and abiotic stresses (Sakuma et al., 2002; Nakano et al., 2006). The members of different subfamilies specifically bind to different cis-acting elements, such as the *CRT/DRE*, the *GCC* box, and/or the *RAV1A/B* elements (Sohn et al., 2006). With respect to the mechanism by which the expression of *PR* genes in *AtCBF1* transgenic tomato plants is regulated, two hypotheses may be proposed. The first hypothesis is that ectopic overexpression of *AtCBF1* directly activates *PR* gene expression. However, the results of EMSA and transactivation assays revealed that AtCBF1 did not interact with the *GCC* box (Fig. 4; Supplemental Fig. S1). Thus, this hypothesis might be excluded. CBF/DREB was found to bind to the common core region of CCGNC of *CRT/DRE* and the *GCC* box with different affinities in vitro (Sakuma et al., 2002). Therefore, we cannot entirely exclude the possibility that a fraction of the heterologous AtCBF1 protein overproduced in tomato plants might partially bind to the *GCC* box in the promoter region of *PR* genes.

The second hypothesis is based on an indirect activation of *PR* genes. We hypothesized that AtCBF1 interacts with *CRT/DRE* elements in the *SlRAV2* promoter, leading to up-regulated expression of *SlRAV2*; this in turn elevates the expression of other *ERF*s (e.g.

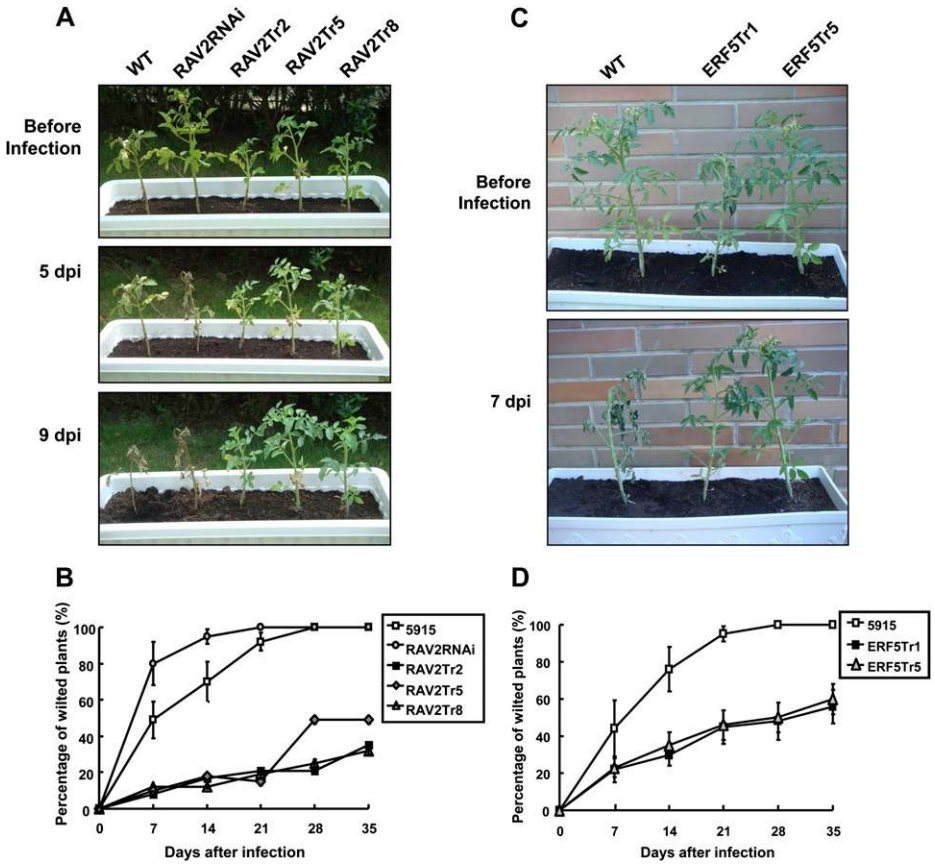

Figure 8. Comparison of *35S:SlRAV2*, *SlRAV2*RNAi, and *35S:SlERF5* transgenic lines with the wild type under pathogen infection. A, Wild-type plants (cv 5915; WT), T2 *SlRAV2* knockdown (*SlRAV2*RNAi), and overexpression lines (*RAV2*Tr2, *RAV2*Tr5, and *RAV2*Tr8) were inoculated with *Ralstonia*. The photographs were taken at 0, 5, and 9 dpi. B, The percentage of wilted plants was calculated at 7, 14, 21, 28, and 35 dpi. C, Wild-type plants (5915) and *SlERF5* T2 overexpression lines (*ERF5*Tr1 and *ERF5*Tr5) were inoculated with *Ralstonia* and then kept at 28°C with a photoperiod of 16 h. The photographs were taken at 0 and 7 dpi. D, The percentage of wilted plants was measured. Each value represents the mean ± SD (n = 10 individual plants) of three independent experiments.

SlERF5). Subsequently, these *ERFs* specifically interact with the *GCC* box in the promoters of *PR* genes, thus enhancing transgenic plant resistance to *Ralstonia* infection (Fig. 9). In Arabidopsis, the promoter regions of some *AP2/ERF* genes contain several *CRT/DRE* elements (Supplemental Table S3); among them, *ERF1*, *ERF2*, *ERF4*, *RAP2.1*, *RAP2.6*, and *RAV1* were identified as cold-inducible downstream genes of the CBF/DREB transcriptional factor (Fowler and Thomashow, 2002; Sharabi-Schwager et al., 2010). In addition, there are two or three *CRT/DRE* elements present in the promoter region of rice *RAV* genes, such as Os01g04800 (−1,895 and −2,371 from ATG), Os05g47650 (−373 and −2,032), and Os07g17230 (−683, −1,188, and −2,256). Completion of the tomato genome sequencing project (the Sol Genomics Network) may reveal more information regarding whether a CBF1-binding site exists in the promoter region of tomato AP2/ERF transcription factors (Supplemental Table S4). We surveyed the cis-acting elements of the *SlRAV2* promoter and found that there is one *CRT/DRE* and one *CRT/DRE-like* element presented (Supplemental Fig. S6). Transactivation assays with Arabidopsis mesophyll protoplasts proved that AtCBF1 can transactivate *SlRAV2* gene expression (Supplemental Fig. S7). Furthermore, there are several *RAV1A* elements presented in the promoters of *SlERF5* and *Pti6* (Supplemental Fig. S2; Supplemental Table S4), and SlRAV2 can transactivate *SlERF5* gene expression (Fig. 5). In addition, overexpression of *SlERF5* increases *PR5* gene expression, while overexpression of *SlRAV2* enhances both the expression of *SlERF5* and its downstream *PR5* in tomato plants (Figs. 7 and 8). Mounting evidence suggests that overexpression of *ERF* genes activates the expression of some *PR* genes, which results in enhanced tolerance to biotic and abiotic stresses (Park et al., 2001; Wang et al., 2004; Zhang et al., 2005, 2007). The VIGS assay and pathogen challenge test in *SlERF5* and *SlRAV2* overexpression and *SlRAV2* knockdown tomato plants performed in our study also verify that SlRAV2 and SlERF5 participate in the enhancement of BW tolerance (Figs. 6–8).

Many *AP2/EREBP* genes have been shown not only to be induced by pathogen infection but also to be regulated by stress-related plant hormones, such as ethylene, JA, and SA (Gutterson and Reuber, 2004). Chen et al. (2009) reported that mitogen-activated protein kinase-, JA/ethylene-, and SA-related defense signaling pathways are involved in the resistance in tomato to BW. Ectopic expression of *CARAV1* in Arabidopsis strongly induced the expression of some *PR* genes regulated by the SA-dependent signaling pathway, such as *PR1*, *PR2*, and *PR5* (Sohn et al., 2006). In this study, endogenous expression of *SlCBF1*, *SlRAV2*, and *SlERF5* was induced by pathogen infection (Fig. 7; Supplemental Fig. S5), and *SlPR5* transcripts accumulated to high levels in all of the *AtCBF1*, *SlRAV2*, and *SlERF5* transgenic tomato plants (Figs. 2

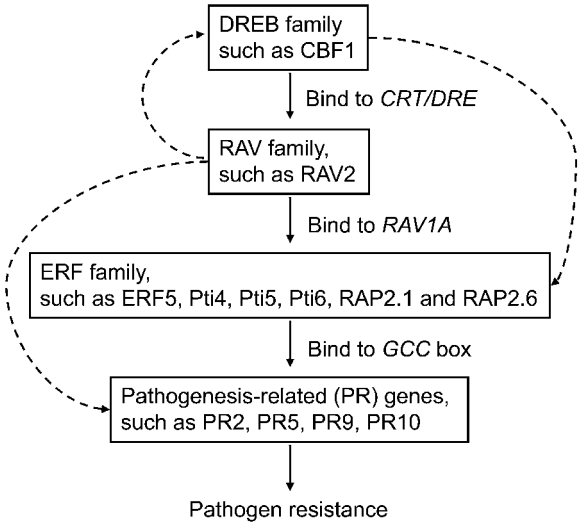

Figure 9. Proposed role of AP2/EREBP superfamily members in the plant defense pathway. The model illustrates the genetic interactions between AP2/EREBP transcription factors in the regulation of *PR* genes, which leads to enhanced tolerance to *Ralstonia*, in *AtCBF1* transgenic tomato plants.

and 7). Therefore, SA may play an important role as an intermediary in the defense mechanism between *AtCBF1*, *SlRAV2*, *SlERF5*, and the *PR* genes. In addition, *SlERF5* was up-regulated by SlRAV2 and enhanced the level of induction by exogenous MJ in the transactivation assay (Fig. 5). As described by Chen et al. (2009), SA- and JA/ethylene-dependent pathways may interact synergistically, rather than antagonistically, in tomato defense mechanisms. JA may also play a regulatory role in the defense mechanism of the CBF-RAV-ERF-PR signaling cascade.

In summary, this study provides evidence that AtCBF1 is involved in the regulation of subsets of *RAV* family, *ERF* family, and *PR* genes that are related to the biotic stress response. Our observations indicate that the RAV2 transcription factor may comprise a key modulator in the plant defense signal pathway (Fig. 9). However, further studies are needed to understand in more detail the mechanism of the RAV2-mediated signaling cascade in plant defense. In addition to the AP2 domain, RAV transcription factors have another DNA-binding domain, the B3 domain, which can recognize the *RAV1B* element (CACCTG), as reported previously (Kagaya et al., 1999). Interestingly, we did not find a *RAV1B* element in the promoter region of tomato *AP2/EREBP*. The existence of novel *RAV1B-like*/B3-binding elements or the participation of post-translational modifications and/or protein-protein interactions in the RAV-mediated defense mechanism need to be further investigated. *AtCBF1* has been introduced into the tomato genome previously, resulting in transgenic plants that were tolerant to four different kinds of stress: chilling, oxidative stress, high salt, and water deficit (Hsieh et al., 2002a, 2002b; Lee et al., 2003). In this report, we observed that overexpression of either *AtCBF1*, *SlERF5*, or *SlRAV2* in tomato plants conferred an enhancement of *Ralstonia* tolerance. These observations indicate that a targeted transgenic approach with a single transgene may be sufficient to enhance plant resistance to several environmental stresses, including abiotic and biotic stresses, and thus may be applied for crop improvement.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Tomato (*Solanum lycopersicum*) cv CL5915-93D_4-1-0-3 (5915) was provided by AVRDC-The World Vegetable Center, in Tainan, Taiwan, and was used as the background line for transformation. Before surface sterilization, seeds were soaked for 1 h at 32°C, treated with 1% (v/v) NaOCl for 10 min, washed several times with sterile water for 5 min, and then germinated on Murashige and Skoog basal medium at 26°C with a 16/8-h photoperiod at 120 μmol m^{-2} s^{-1}.

Resistance Scoring of Transgenic Tomato Plants

Tomato plants were inoculated with *Ralstonia solanacearum* as described (Chen et al., 2009). The resistance of transgenic tomato plants to BW was evaluated as described previously (Lin et al., 2004; Chen et al., 2009). *Ralstonia* strain Pss4 (race 1, biovar 3; suspension A_{600} = 0.6, about 2×10^8 cfu mL^{-1}) was used as the inoculum. Additionally, for *Ralstonia* colonization experiments, 10 plants were randomly harvested from each treatment at each sampling time. Three independent experiments were performed. Plants were uprooted, soil was washed off, and plants were soaked in 70% (v/v) ethanol for 3 to 5 min, rinsed twice in sterile water, and blotted to dryness on paper towels. For BW evaluation, tomato varieties H7996 and an ABA-inducible promoter driving *AtCBF1* in a 5915 variety (AC3) line were used as resistant and susceptible controls, respectively (Wang et al., 2000). H7996, L390, and 5915 seeds were kindly provided by AVRDC.

Vector Construction and Plant Transformation

Construction of the binary vector carrying pCaMBIA2301/*35S:AtCBF1* and *Agrobacterium tumefaciens*-mediated tomato transformation were carried out as described (Hsieh et al., 2002a, 2002b; Lee et al., 2003). For constitutive overexpression in tomato, constructs p*35S:SlERF5* and p*35S:SlRAV2* were prepared by inserting the *SlERF5* and *SlRAV2* coding sequences between the *CaMV35S* promoter and the *nos* or the *35S* terminator in pCAMBIA1390/35S (Hsiao et al., 2007) and pH2GW7 (for primers, see Supplemental Table S2), respectively, both of which contain *Hpt*. For knockdown expression in tomato, the binary vector p*SlRAV2*RNAi was constructed by inserting a *SlRAV2* N-terminal region (amino acids 27–65) into pH7GWIWG2, followed by transformation into tomato plants by the *Agrobacterium*-mediated transformation method.

Molecular Characterization of Transgenic Tomato Plants

Transgenic tomato plants were selected on 100 mg L^{-1} kanamycin (pCAMBIA2301/*35S:AtCBF1*) or 20 mg L^{-1} hygromycin (p*35S:SlERF5*, p*35S:SlRAV2*, and p*SlRAV2*RNAi). All transgenic plants were analyzed by Southern- and northern-blot hybridization or RT-PCR, as described previously (Hsieh et al., 2002a, 2002b). The following probes were used for northern-blot hybridization: tomato *β-tubulin*, *PR2* (β-1,3-glucanase; accession no. CK664757), *PR5-like* (accession no. AY257487), *PR9-like* (peroxidase; accession no. AW219536), and *PR10-like* (RNase-like; accession no. CK468708). cDNA fragments were excised from pT7Blue (R) vector as probes and labeled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983).

Microarray Analysis

We previously constructed a tomato cDNA microarray comprising 12,448 cDNA clones derived from 5,600 tomato root EST clones and 15 libraries from

stress-treated wild-type tomato plants. *AtCBF1* transgenic tomato RNA and control plant RNA were probed. Probe labeling, hybridization, and scanning of the cDNA microarray were performed as described previously (Liu et al., 2006).

Determination of Chlorophyll Fluorescence Values and Chlorophyll Content

Chlorophyll fluorescence values were measured using a pulse-activated modulation fluorimeter (Walz). Chlorophyll content in leaves was determined by extraction with *N*,*N*-dimethylformamide as described (Moran and Porath, 1980). Absorption of the extracts was measured at 664 and 647 nm. Chlorophyll content was calculated with use of the following equation: total chlorophyll content = 7.04 A_{664} + 20.27 A_{647}.

RT-PCR Analysis

Total RNA was isolated from leaves of wild-type and transgenic tomato plants by use of TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RT was conducted as described by the manufacturer (Promega). PCR involved gene-specific primers of *SlCBF1* (AY497899), *SlERF5* (AY559315), *Pti4* (U89255), *Pti5* (U89256), *Pti6* (U89257), *SlRAV2* (EU164417), *SlRAP2.1* (AK246512), *SlRAP2.6-like* (EU164420), and *SlActin1* (U60480; Supplemental Table S1). PCR was conducted in a final volume of 25 μL containing cDNA reverse transcribed from 30 ng of total RNA, 1× Taq buffer (Violet), 0.2 mM of each deoxyribonucleotide triphosphate, 2 units of Taq DNA polymerase (Violet), and 100 pmol of each primer (Supplemental Table S1). The following amplification program was used: one cycle of 95°C for 3 min; 25 to 30 cycles of 95°C for 25 s, 58°C for 30 s, and 72°C for 1 min; and then one cycle of 72°C for 7 min. The RT-PCR products were resolved on a 1% agarose gel and visualized by ethidium bromide staining.

Promoter Isolation

Genomic DNA was extracted from leaves of wild-type tomato plants (Murray and Thompson, 1980). Genome walking was performed as described by the manufacturer (BD GenomeWalker Universal Kit; Clontech). In addition to genome walking, inverse PCR was used to extend the *SlERF5* (EU164418) and *Pti6* (EU164419) promoter sequences and to obtain the full-length *SlRAV2* (EU164417) gene by use of specific primers (Supplemental Table S2). Two micrograms of tomato genomic DNA was digested with *Hin*dIII and self-ligated as the template for inverse PCR. The following amplification program was used for the first PCR of genome walking and inverse PCR: one cycle of 95°C for 1 min; seven cycles of 94°C for 25 s and 72°C for 3 min; 32 cycles of 94°C for 25 s and 67°C for 3 min; and then one cycle of 67°C for 7 min. The program for the second PCR of genome walking and inverse PCR was as follows: one cycle of 95°C for 1 min; five cycles of 94°C for 25 s and 72°C for 3 min; 25 cycles of 94°C for 25 s and 67°C for 3 min; and then one cycle of 67°C for 7 min. The PCR products were ligated into the pGEMT Easy vector (Promega) for DNA sequencing.

Arabidopsis Protoplast Transient Expression and Reporter Gene Activity Assay

For the reporter gene constructs, the *CaMV35S* promoter in pJD301 was replaced by the *35S* minimal promoter from −42 to +8 containing the TATA box. The *GCC* and mutant *GCC* box sequences (Fig. 4B) were multimerized four times and placed upstream of the 35S minimal promoter and the *SlERF5* promoter (−776 to +23; Supplemental Fig. S2A) and fused to the *Luc* gene. For effector plasmids, the *Luc* gene in pJD301 was replaced by the coding regions of *AtCBF1*, *SlERF5*, *Pti6*, and *SlRAV2*. The pBI221 plasmid containing the *GUS* gene driven by the *CaMV35S* promoter was used as an internal control for transactivation assay.

Arabidopsis (*Arabidopsis thaliana*) protoplasts were isolated from 4-week-old leaves and transfected by a modified polyethylene glycol method as described (Abel and Theologis, 1994; Wu et al., 2009). Ten micrograms of reporter plasmid and 5 μg of effector plasmid or control plasmid (pUC18) were cotransfected into 4 × 10^4 protoplasts with 10 μg of internal control plasmid pBI221. The transfected cells were incubated for 20 h at 22°C under light, harvested by centrifugation at 100*g* for 2 min, and then lysed in lysis buffer (Promega). Luciferase activity was measured by use of a luciferase assay kit (Promega) according to the manufacturer's instructions, and GUS activity was determined (Lu et al., 1998).

TRV-Based VIGS Assay

VIGS vectors (pTRV1 and pTRV2) and construction procedures for their derivatives have been described (Liu et al., 2002; Chen et al., 2009). *SlRAV2* and *SlERF5* cDNA fragments (301 and 318 bp, respectively) were obtained by PCR using specific primers (*RAV2*-VIGS-F/*RAV2*-VIGS-R and *ERF5*-VIGS-F/*ERF5*-VIGS-R; Supplemental Table S2) and recombined into pTRV2 to generate pTRV2-RAV2 and pTRV2-ERF5. For the VIGS assay, pTRV1 and pTRV2 and its derivatives (pTRV2-RAV2 and pTRV2-ERF5) were introduced into *Agrobacterium* strain GV3101 by electroporation. BW-resistant tomato variety H7996 and BW-susceptible variety L390 were grown in pots at 24°C in a growth chamber under a 16-h-light/8-h-dark cycle. The TRV inoculation procedure was performed as described (Dinesh-Kumar et al., 2003). The efficiency of VIGS in TRV-only, TRV-ERF5-, and TRV-RAV2-silenced tomato leaves on day 15 post agroinfiltration was examined by semiquantitative RT-PCR using specific primers (ERF5-RT-F/ERF5-RT-R, RAV2-RT-F/RAV2-RT-R, and UBI3-F/UBI3-R). On day 10 post agroinfiltration, TRV-, TRV-ERF-, and TRV-RAV2-infiltrated tomato, including H7996 and *AtCBF1* transgenic plants, were inoculated with *Ralstonia* (2 × 10^8 cfu mL^{-1}) by root drenching. Five days later, 1-cm sections from the midstem and stem base of these *Ralstonia*-inoculated plants were weighed and ground, and then the bacterial density was measured by direct plating. A lower inoculum dose (5 × 10^5 cfu mL^{-1}) was used for the L390 susceptible control. For the assay of each gene, 10 to 12 plants were used in each experiment, and three independent experiments were performed. Pairwise comparisons were made between wild-type (or TRV-infected plant in H7996) and silenced plants with Student's *t* test.

Gene accession numbers of all sequence data from this article can be found in "Materials and Methods" and Supplemental Tables S1 and S4.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. EMSA characterization of the DNA-binding affinity of the recombinant AtCBF1 protein.

Supplemental Figure S2. Promoter sequences of *SlERF5* and *Pti6*.

Supplemental Figure S3. Subcellular localization of SlERF5 (EYFP::SlERF5) and SlRAV2 (EYFP::SlRAV2) in Arabidopsis protoplasts.

Supplemental Figure S4. Photosynthesis efficiency and chlorophyll content of *35S:SlRAV2*, *SlRAV2*RNAi, and *35S:SlERF5* transgenic lines under pathogen infection.

Supplemental Figure S5. Expression of tomato endogenous *CBF* genes under *Ralstonia* infection.

Supplemental Figure S6. Genome sequence of *SlRAV2*.

Supplemental Figure S7. CBF1 activates the reporter gene driven by the *SlRAV2* promoter.

Supplemental Table S1. Oligonucleotides used for RT-PCR.

Supplemental Table S2. Oligonucleotides used for genome walking, inverse PCR, and vector construction.

Supplemental Table S3. Prediction of the CBF1-binding elements in the promoter region of the Arabidopsis *AP2/ERF* genes.

Supplemental Table S4. Prediction of the AP2/ERF-binding elements in the promoter region of tomato AP2/ERFs.

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