Tomato RAV Transcription Factor Is a Pivotal Modulator Involved in the AP2/EREBP-Mediated Defense Pathway

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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/VPI (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 SIERF5 promoter. Virus-induced gene silencing of SIEF5 and SIRAV2 in AtCBF1 transgenic and BW-resistant cultivar Hawaii 7966 plants gave rise to plants with enhanced susceptibility to BW. Constitutive overexpression of SIRAV2 in transgenic tomato plants induced the expression of SIERF5 and PR5 genes and increased BW tolerance, while knockdown of expression of SIRAV2 inhibited SIERF5 and PR5 gene expression under pathogen infection and significantly decreased BW tolerance. In addition, transgenic tomato overexpressing SIERF5 also accumulated higher levels of PR5 transcripts and displayed better tolerance to pathogen than wild-type plants. From these results, we conclude that SIERFs may act as intermediate transcription factors between AtCBF1 and PR genes via SIRAV 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. Introgenesis 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-
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 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 C/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-Ottesen 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 (thraumatin-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 1. 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 | Description | Unigene No. | Corresponding Arabidopsis Gene | Ratio<sup>a</sup> | SGN Database: Tomato WGS Scaffolds (2.30) | cis-Acting Elements within the 2-kb Promoter |
|------------|-----------|-------------|-------------|-------------------------------|----------------|-------------------------------------------|---------------------------------------------|
| cLEY14E7   | BE449751  | Protein phosphatase 2C-like protein | SGN-U573715 | At2g25070 | 10.41 ± 2.41 | SL2.30sc04948 | CRT/DRE – – – – |
| C6SR473    | CK574973  | Cys protease (PR7) | SGN-U580215 | At4g32940 | 6.49 ± 0.51 | SL2.30sc03665 | GCC 0 0 5 1 |
| cLEX4M16   | AW219536  | Perooxidase (PR9) | SGN-U581153 | At5g05340 | 6.22 ± 3.96 | SL2.30sc04828 | GCCGCC 0 0 6 0 |
| LEC5R05G01 | CK468708  | PR10 protein | SGN-U578441 | At1g24020 | 5.90 ± 1.49 | SL2.30sc03685 | RAV1A 0 0 3 0 |
| cLEX11D13  | AW621284  | Zinc transporter protein ZIP1 | SGN-U583586 | At1g05300 | 5.87 ± 1.78 | SL2.30sc04135 | RAV1B 5 0 1 1 |
| cLEX8A20   | AW220124  | Dehydrin homolog C17 | SGN-U581375 | At1g20450 | 4.60 ± 1.55 | SL2.30sc04135 | – – – – |
| LEEC101F05 | CK725213  | Acidic endochitinase precursor (PR3) | SGN-U566861 | At5g24090 | 3.80 ± 1.71 | SL2.30sc03902 | – – – – |
| SF471      | CK574994  | Acidic 26-kD endochitinase precursor (PR3) | SGN-U581507 | At3g12500 | 3.65 ± 0.05 | SL2.30sc03665 | – – – – |
| cLEX12C2   | AW621528  | Syntaxin-related protein Ni-syr1 | SGN-U584182 | At3g11820 | 3.53 ± 0.54 | SL2.30sc04133 | – – – – |
| cLEW12A21  | BF096513  | Catalase isozyme 1 | SGN-U578839 | At4g35090 | 3.48 ± 0.03 | SL2.30sc05380 | – – – – |
| cLEW19G18  | BF097084  | PTTN-like protein | SGN-U566184 | At3g19420 | 3.43 ± 0.00 | SL2.30sc03665 | – – – – |
| cLEW27E20  | BF098457  | Senescence-associated protein-related | SGN-U578016 | At5g20700 | 3.21 ± 0.12 | SL2.30sc03731 | – – – – |
| cLEX5K5    | AW219630  | DnaJ-like heat shock protein | SGN-U589575 | At4g13830 | 3.17 ± 1.20 | SL2.30sc03902 | – – – – |
| cLEW8A6    | AW980043  | 1-Acylglycerol-3-phosphate acyltransferase | SGN-U573500 | At1g51260 | 3.00 ± 0.67 | SL2.30sc03876 | – – – – |
| cLEX2M12   | AW219010  | Ubiquitin family protein | SGN-U567499 | At2g30100 | 2.71 ± 0.26 | SL2.30sc04474 | – – – – |
| cLEX2M14   | AW219011  | Eukaryotic translation initiation factor 5A-1 | SGN-U578904 | At1g13950 | 2.65 ± 0.62 | SL2.30sc03701 | – – – – |
| SF146      | CK574987  | Gly-rich protein | SGN-U5131109 | At2g05440 | 2.59 ± 0.55 | SL2.30sc03852 | – – – – |
| cLEW22K5   | BF097441  | Microsomal signal peptidease 25-kD subunit | SGN-U577878 | At2g39960 | 2.58 ± 0.18 | SL2.30sc06557 | – – – – |
| Rs-Ck-1-G3 | CK715671  | Microsomal α-6-desaturase | SGN-U574778 | At3g12102 | 2.52 ± 0.60 | SL2.30sc04057 | – – – – |
| LERCD04N18 | CK715495  | Unknown protein | SGN-U582639 | At3g03870 | 2.52 ± 0.20 | SL2.30sc04199 | – – – – |
| cLEW19L9   | BF097167  | COPI homolog | SGN-U579490 | At2g32950 | 2.21 ± 0.24 | SL2.30sc04607 | – – – – |
| LEC010D07  | CK720580  | Formate dehydrogenase | SGN-U579280 | At5g14780 | 2.19 ± 0.33 | SL2.30sc03665 | – – – – |
| LERCD04N21 | CK715497  | Cys protease (PR7) | SGN-U578421 | At1g47128 | 2.16 ± 0.47 | SL2.30sc05611 | – – – – |
| SF847      | AY257487  | PR-5 | SGN-U578836 | At4g11650 | 2.11 ± 0.39 | SL2.30sc03923 | – – – – |
| cLEW26O13  | BF098337  | DnaJ-like heat shock protein | SGN-U579998 | At3g44110 | 2.02 ± 0.01 | SL2.30sc03604 | – – – – |
| SF547      | CK664757  | β-1,3-Glucanase (PR2) | SGN-U581016 | At3g52720 | 1.29 ± 0.11 | SL2.30sc05010 | – – – – |

<sup>a</sup>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. <sup>b</sup>En dash (–), promoter sequence in SGN database is unavailable.
transgenic plant 5915 as BW-resistant and -susceptible control, respectively. Four \( \textit{AtCBF1} \) 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 \( \textit{AtCBF1} \) gene driven by the abscisic acid (ABA)-inducible \( \textit{ABRC1} \) 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 \( \textit{Ralstonia} \) (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 \( \textit{Ralstonia} \) 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 \( \textit{Ralstonia} \) 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 \( \textit{AtCBF1} \) 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 \( \textit{AtCBF1} \) 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 \( \textit{AtCBF1} \) 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 \( \textit{AtCBF1} \) in transgenic tomato enhances BW resistance by systemic suppression of internal bacterial multiplication via the activation of PR proteins.

Tolerance to \( \textit{Ralstonia} \) Infection Is Not Affected by Exogenous GA\(_3\) Treatment in Transgenic Plants

Constitutive expression of \( \textit{AtCBF1} \) 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 \( \textit{AtCBF} \) 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 \( \textit{AtCBF1} \) 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 \( \textit{PR2} \) (\( \beta\)-1,3-glucanase), \( \textit{PR5}, \textit{PR9}, \) and \( \textit{PR10} \), were up-regulated by 1.29-, 2.11-, 6.22-, and 5.90-fold, respectively, in \( \textit{AtCBF1} \) transgenic plants compared with wild-type tomato plants, as shown in Table I. Although the ratio of \( \textit{PR2} \) is only 1.29-fold, the mRNA density showed it significantly accumulated in \( \textit{AtCBF1} \) transgenic plants (Fig. 2). Northern-blot results showed that exogenous ap-
GA3 treatment, alternate day in a greenhouse with a 16/8-h photoperiod (daylight of about 120 m\(^2\) s\(^{-1}\)). Each value is the mean ± SD (n = 5). Experiments were performed in triplicate.

Probes used to hybridize total RNA were \(^{32}\)P-labeled, and AtCBF1 transgenic T1 plants overexpressing AtCBF1 partially to almost completely restored by GA3 treatment. After transgenic plants were partially to almost completely restored by GA3 treatment. After growth, which was verified by RNA 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.

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 levels 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 GA3 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 \(\mu\)mol m\(^{-2}\) s\(^{-1}\); 26°C ± 2°C; night temperature of 22°C ± 2°C). For GA3 treatment, AtCBF1 transgenic and wild-type plants were sprayed with 5 mg L\(^{-1}\) GA3 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 + GA3 | C5 + GA3 | C15 + GA3 | C21 + GA3 |
|-----------|-----------|----|-----|-----|----------------|--------|----------|----------|
| Control   | FN 21.6 ± 4.1 | 6.0 ± 1.6 | 7.2 ± 1.6 | 1.6 ± 1.1 | 26.6 ± 1.1 | 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 |

RAV modulates the AP2/EREBP-mediated defense pathway

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.

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Figure 2. Northern-blot analyses of PR genes in transgenic tomato plants. Total RNA (10 \(\mu\)g) 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 \(\beta\)-Tubulin cDNA fragments. Equal loading in each lane was verified by RNA 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.
using semiquantitative reverse transcription (RT)-PCR. In addition, we analyzed the expression patterns of newly identified ERFs and RAVs, such as SIERF5 and SlRAV2, and some tomato orthologs of putative Arabidopsis CBF1-regulated AP2/EREBP (Zhang et al., 2004b), such as SIRAP2.1 and SIRAP2.6-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 Arabidopsis protoplasts, we performed RT-PCR with degenerate primers (Supplemental Table S2). Among them, SlRAV2 was the major transcript up-regulated in AtCBF1 transgenic tomato. The mRNA transcripts of AP2/EREBP family genes (i.e. SIERF5, Pti4, Pti5, Pti6, SIRAP2.1, SIRAP2.6-like, SRAV2, and SICBF1) exhibited a moderate to strong increase in AtCBF1 transgenic tomato plants (C5, C15, and C21) as compared with the wild type (Fig. 3).

**SIERF5 and Pti6 Interact with the GCC Box**

To investigate whether SIERF5 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 SIERF5 cDNA, or Pti6 (positive control; Gu et al., 2002) driven by the CaMV35S promoter (Fig. 4A). The pB1221 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/ GLU relative activity). At coexpression of 35S:Pti6 or 35S:SIERF5 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 SIERF5 and Pti6 but not AtCBF1 function as activators of GCC box-mediated transcription.

**SIERF2 Interacts with a Promoter of SIERF5**

SIERF5 and SIERAV2 contain one AP2 domain and belong to the ERF and RAV subfamily of AP2/EREBP proteins, respectively. Presumably, SIERF5 and SIERAV2, like other well-identified AP2/EREBPs, act as transcription factors to regulate gene expression in the nucleus. To verify this assumption, full-length SIERF5 and SIERAV2 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 SIERF5 and SIERAV2 are localized in the nucleus (Supplemental Fig. S3).

The promoter sequences of SIERF5 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 SIERF5 and Pti6 (Supplemental Fig. S2). To verify whether SIERAV proteins play the part of transacting factors binding to the SIERF5 promoter, we performed in vivo transactivation assays with a...
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 (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.
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. A1CBF1 transgenic or H7996 to-mato 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 A1CBF1 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 SIERF5- and SIRAV2-silenced plants exhibited relatively higher levels of bacterial density compared with TRV-only control plants. These results indicated that silencing SIERF5 and SIRAV2 had indeed decreased the resistance of tomato to BW.

Generation and Characterization of SIRAV2RNAi Knockdown as Well as 35S:SlRAV2 and 35S:SlERF5 Transgenic Tomato Plants

To further investigate the functions of SIRAV2 and SIERF5 in pathogen resistance, transgenic tomato plants with knockdown expression of SIRAV2 or over-expression of SIRAV2 or SIERF5 were generated. Transgenic plants with lower insertion (one to two copies) of transgene were selected for further study.

Figure 5. SIRAV2 interacts with the promoter of SIERF5. 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 SIERF5 promoter sequence (776 bp) fused to the firefly luciferase gene Luc. In the effector plasmids, SIRAV2 and Arabidopsis CBF1 genes were driven under the control of the CaMV35S promoter. Nos and i35 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 SIRAV2 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 so. Transient transformation without the effector plasmid (ERF5 or mini35p) was used as a control.

Figure 6. Silencing of SIERF5 and SIRAV2 using TRV-based vector. BW-resistant tomato variety H7996 and A1CBF1 transgenic plants (CBF1) were infected with mixtures of Agrobacterium transformed with pTRV1 and pTRV2 (TRV) or pTRV2 carrying SIERF5 (TRV2-ERF5) or SIRAV2 (TRV2-RAV2) fragments. A, Semi quantitative 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.
Semiquantitative RT-PCR was performed to analyze the mRNA levels in these transgenic plants, including independent lines of SIRAV2 knockdown (RAV2RNAi), 35S:SIRAV2 (RAV2Tr2, -5, and -8), and 35S: SIERF5 (ERF5Tr1 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 SIRAV2 and SIERF5 transgenes with a 35S or nos terminator were expressed only in transgenic plants (Fig. 7A). In addition, SIERF5 and its downstream gene, SIRP5, were not only abundantly expressed in SIERF5 transgenic plants but also highly accumulated in SIRAV2 transgenic plants (Fig. 7A).

We used RT-PCR to examine changes in mRNA levels of SIRAV2 and SIERF5 in pathogen-infected RAV2Tr and RAV2RNAi transgenic tomato. The transcription of SIRAV2 and SIERF5 was up-regulated by Ralstonia infection in wild-type plants (Fig. 7B). The level of SIERF5 and SIRAV2 mRNA transcripts was high in RAV2Tr lines as compared with the wild type under normal conditions but absent in the RAV2RNAi line even after treatment with the pathogen. Taken together, our results support the notion that SIRAV2 may be a key factor regulating SIERF5 gene expression. Hence, the SIRAV2 and SIERF5 transgenic plants were further evaluated for resistance to pathogen infection.

**Constitutive Expression of SIERF5 and SIRAV2 in Tomato Confers Tolerance, While Knockdown of SIRAV2 Expression Causes Hypersensitivity to BW**

SIRAV2- and SIERF5-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 SIRAV2 in tomato promoted plant growth and development (Fig. 8A, top panel). However, how SIRAV2 and SIERF5 participate in tomato growth and development remains to be further investigated. SIRAV2 and SIERF5 transgenic tomato plants were then subjected to Ralstonia challenge to verify their functions in the defense mechanism. The RAV2RNAi 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 SIRAV2 or SIERF5 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 RAV2RNAi, 19% for RAV2Tr, and 37% for ERF5Tr lines. Similarly, the chlorophyll content remained higher in RAV2Tr and ERF5Tr transgenic plants in comparison with the RAV2RNAi knockdown lines and wild-type plants after pathogen infection. The differences in PSII efficiency and chlorophyll content between wild-type plants and transgenic RAV2Tr and ERF5Tr tomato under pathogen treatment were statistically significant (P < 0.01, Student’s t test). Overall, these findings indicated that SIERF5 and SIRAV2 play crucial roles in the basal defense of tomato plants against BW and that SIRAV2 may be a key regulator involved in plant defense.

**DISCUSSION**

CBF genes have been considered “master switches” that increase freezing tolerance in Arabidopsis plants.

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

AtCBF1 transgenic plants (CBFIox) and BW-resistant tomato variety H7996 were infected with mixtures of Agrobacterium transformed with pTRV1 and pTRV2 (TRV-only control) or pTRV2 carrying SIRAV2 (SIRAV2) or SIERF5 (SIERF5) fragments. BW-susceptible tomato varieties L390, 5915 (the background of CBFIox), and TRV-infected CBFIox 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 ± so. 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).
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 Pit4 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 GCCNC 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 SIRAV2 promoter, leading to up-regulated expression of SIRAV2; this in turn elevates the expression of other ERFs (e.g.
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 SIRAV2 gene expression (Supplemental Fig. S7). Furthermore, there are several RAV1A elements presented in the promoters of SIERF5 and Pt6 (Supplemental Fig. S2; Supplemental Table S4), and SIRAV2 can transactivate SIERF5 gene expression (Fig. 5). In addition, overexpression of SIERF5 increases PR5 gene expression, while overexpression of SIRAV2 enhances both the expression of SIERF5 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).

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 SICBF1, SIRAV2, and SIERF5 was induced by pathogen infection (Fig. 7; Supplemental Fig. S5), and SIRP5 transcripts accumulated to high levels in all of the AtCBF1, SIRAV2, and SIERF5 transgenic tomato plants (Figs. 2
AtCBF1 is involved in the regulation of subsets of SA- and JA/ethylene-dependent pathogenesis, and 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 RAVIB element (CACCTG), as reported previously (Kagaya et al., 1999). Interestingly, we did not find a RAVIB element in the promoter region of tomato AP2/EREBP. The existence of novel RAVIB-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 CL5915-93D1-1-0-3 (5915) was provided by AVRDC-The World Vegetable Center, in Taichung, 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**

Transgenic 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 Psso4 (race 1, biovar 3; suspension A₉₀₀ = 0.6, about 2 x 10⁸ 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 p35S:SlERF5 and p35S: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 pH2CG2 (for primers, see Supplemental Table S2), respectively, both of which contain Hpt. For knockdown expression in tomato, the binary vector pSI2RAV2RNAi was constructed by inserting a SlRAV2 N-terminal region (amino acids 27-65) into pH7GW1WG2, 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 (p35S:SlERF5, p35S:SlRAV2, and pSlRAV2RNAi). 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, PR5 (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 p77Blue (R) vector as probes and labeled with [α-³²P]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 other plant species.
stress-treated wild-type tomato plants. AicBF1 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 fluorometer (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 644 and 647 nm. Chlorophyll content was calculated with use of the following equation: total chlorophyll content = 7.04 A₆₄₅ + 20.27 A₆₄₇.

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 SCRBF1 (AY497989), SIERF5 (AY599315), Pti6 (U89255), Pti6 (U89256), SIRAV2 (U89257), SIRAV2 (EU164417), SIRAP2.1 (AK246512), SIRAP2.6-like (EU164442), 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, 3 μL of each primer (Supplemental Table S1). PCR was conducted in a final volume of 25 μL containing cDNA reverse transcribed from 30 ng of total RNA, 3 μL of each primer (Supplemental Table S1). PCR was conducted in a final volume of 25 μL containing cDNA reverse transcribed from 30 ng of total RNA, 3 μL of each primer (Supplemental Table S1). PCR was conducted in a final volume of 25 μL containing cDNA reverse transcribed from 30 ng of total RNA, 3 μL of each primer (Supplemental Table S1). PCR was conducted in a final volume of 25 μL containing cDNA reverse transcribed from 30 ng of total RNA, 3 μL of each primer (Supplemental Table S1). PCR was conducted in a final volume of 25 μL containing cDNA reverse transcribed from 30 ng of total RNA, 3 μL of each primer (Supplemental Table S1). PCR was conducted in a final volume of 25 μL containing cDNA reverse transcribed from 30 ng of total RNA, 3 μL of each primer (Supplemental Table S1).

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 SIERF5 (EU164418) and Pti6 (EU164419) promoter sequences and to obtain the full-length SIRAV2 (EU164447) gene by use of specific primers (Supplemental Table S2). Two micrograms of tomato genomic DNA was digested with HindIII and self-ligated as the template for inverse PCR. The following amplification program was used: one cycle of 95°C for 5 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.

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 SIERF5 promoter (−776 to +23); Supplemental Fig. S2A) and fused to the luc gene. For effectors plasmids, the luc gene in pJD301 was replaced by the coding regions of AicCBF1, SIERF5, Pti6, and SIRAV2. The pBl221 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 micromoles of reporter plasmid and 5 μg of effector plasmid or control plasmid (pUC18) were cotransfected into 4 × 10⁶ protoplasts with 10 μg of internal control plasmid pBl221. The transfected cells were incubated for 20 h at 22°C under light, harvested by centrifugation at 100g 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 (Liu 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). SIRAV2 and SIERF5 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-infected tomato including H7996 and AicCBF1 transgenic plants, were inoculated with Ralstonia (2 × 10⁶ 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 × 10⁶ 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 AicCBF1 protein.

Supplemental Figure S2. Promoter sequences of SIERF5 and Pti6.

Supplemental Figure S3. Subcellular localization of SIERF5 (EYFP:: SIERF5) and SIRAV2 (EYFP:SIRAV2) in Arabidopsis protoplasts.

Supplemental Figure S4. Photosynthesis efficiency and chlorophyll content of 35S:SIRAV2, SIRAV2::RNAi, and 35S:SIERF5 transgenic lines under pathogen infection.

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

Supplemental Figure S6. Genome sequence of SIRAV2.

Supplemental Figure S7. CBF1 activates the reporter gene driven by the SIRAV2 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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