Running head: RAV modulates the AP2/EREBP-mediated defense pathway

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Tomato RAV transcription factor is a pivotal modulator involved in the AP2/EREBP-mediated defense pathway

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ABSTRACT

_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 overexpressing _Arabidopsis CBF1_ (AtCBF1) that 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 (VIGS) of SlERF5 and SlRAV2 in AtCBF1 transgenic and BW-resistant cultivar H7996 plants gave rise to plants with enhanced susceptibility to BW. Constitutive overexpression of SlRAV2 in transgenic tomato plants induced expression of SlERF5 and PR5 genes and increased BW tolerance, while knockdown 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.
INTRODUCTION

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 tomato crop; the resulting disease, bacterial wilt (BW) can be devastating and difficult to control by conventional approaches. Introgession 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 resistances 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 has 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 (ROS) during the oxidative burst, and induction of hypersensitive cell death (hypersensitive response, HR)
(Gomez-Gomez, 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 beta-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 upregulated 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 plant defense response to pathogen infection (Zhang et al., 2004a). Recently, AP2/EREBP (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 the CRT (C-repeat)/DRE (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 4 subfamilies: AP2, DREB (dehydration response element binding protein), ERF, and RAV (related to ABI3/VP1). The members of the ERF subfamily, which include tobacco ERF1-4, Arabidopsis ERF1-5, ORA59, tomato Pti4-6, tomato ERF1-4, and 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; Pre 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.,
In addition, rice TERF1, barley HvRAF and tomato TSRF1 are involved in regulation of both biotic and abiotic stress tolerance (Jung et al., 2007; Gao et al., 2008). These findings strongly suggest that 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 (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 are tolerant to various abiotic stresses such as chilling, oxidative stress, high salt, and water deficit (Hsieh et al., 2002a; Hsieh et al., 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 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; Hsieh et al., 2002b). To identify the genes that were differentially expressed in AtCBF1 transgenic tomato plants, we now used subtractive hybridization and home-made microarray systems (Liu et al., 2006). Expression was increased by at least 2-fold for 25 genes in AtCBF1 transgenic plants compared to 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 (ribonuclease-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 upregulation 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 cultivar “Hawaii 7996” (H7996) has displayed stable resistance against various R. solanacearum strains (Grimault et al., 1995). In this study, we used tomato cultivar H7996 and the background of the AtCBF1 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 (1-2 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 ABRC1 promoter (line AC3) for pathogen infection (Lee et al., 2003). Similar to the BW-resistant tomato cultivar H7996, the transgenic lines (C5, C15, C21 and C22) did not show any signs of wilting at 7 days 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 \textit{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 ($\geq 10^7$ CFU/g 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 to that in wild-type and in 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) and 40\% of AC3 plants wilted on 7 dpi, and all had withered on 35 dpi. We thus conclude that constitutive expression of \textit{AtCBF1} in transgenic tomato enhances BW resistance by systemic suppression of internal bacterial multiplication via activation of PR proteins.

\textbf{Tolerance to \textit{Ralstonia} infection is not affected by exogenous GA$_3$ treatment in}
Constitutive expression of AtCBF1 in tomato resulted in a dwarf phenotype that was alleviated by application of gibberellin 3 (GA3) (Hsieh et al., 2002a; Hsieh et al., 2002b). To test whether GA3 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 GA3 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 for 1.29, 2.11, 6.22 and 5.90 folds, respectively, in AtCBF1 transgenic compared with wild-type tomato plants as shown in Table I. Although the ratio of PR2 is only 1.29 folds, the mRNA density showed significantly accumulated in AtCBF1 transgenic plants (Fig. 2). Northern blot result showed that exogenous application of GA3 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 GA3. Thus, we conclude that the expression of PR genes in AtCBF1 transgenic plants results from overexpression of AtCBF1 by a GA3-independent pathway.

Wild-type and AtCBF1 transgenic tomato plants were infected with high cell density of Ralstonia and allowed to grow under controlled conditions. In the absence of GA3 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 partial to almost complete restored by GA3 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 GA3 treatment. More importantly, no significant to little difference in the fruit yield between non-infected wild-type plants and
Ralstonia-infected GA$_3$-treated AtCBF1 transgenic lines were found (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 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 binds to 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 the CRT/DRE but not the GCC box *in vitro*.

**Several AP2/EREBP family genes are upregulated in AtCBF1 transgenic tomato**

According to the obtained EMSA results, AtCBF1 specifically interacts with the CRT/DRE rather than the GCC box. We therefore hypothesized that AtCBF1 overexpression in tomato regulates PR genes through either an indirect pathway or through 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 using semi-quantitative 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 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 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 2 RAV genes, designated SIRAV1 and SIRAV2, that expressed in AtCBF1 transgenic tomato plants. Afterwards, the full-length RAV genes were obtained by using RACE or the genome walking method (primers shown in Supplemental Table S2). Among them, SIRAV2 was the major transcript upregulated in AtCBF1 transgenic tomato plants. The mRNA transcripts of AP2/EREBP family genes, i.e. SIERF5, Pti4, Pti5, Pti6, SIRAP2.1, SIRAP2.6-like, SIRAV2, 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 luciferase (Luc) reporter gene driven by a CaMV35S (cauliflower mosaic virus 35S) 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 pBI221 plasmid containing the β-glucuronidase (GUS) gene driven by the CaMV35S promoter was used as an internal control. Plasmids were cotransfected into protoplasts, 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 SIERF5 and Pti6 but not AtCBF1 function as activators of GCC box-mediated transcription.

**SlRAV2 Interacts with Promoter of SIERF5**

SIERF5 and SlRAV2 contain one AP2 domain and belong to the ERF and RAV subfamily of AP2/EREBP protein, respectively. Presumably, SIERF5 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 SIERF5 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 SIERF5 and SlRAV2 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 numbers: EU164418 and EU164419, respectively). Several RAV1A elements (CAACA) are present, but neither sequences for a CRT/DRE nor a GCC box could be seen in the promoter regions of SIERF5 and Pti6 (Supplemental Fig. S2). To verify whether SIRAV 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 SIERF5 promoter (776 bp). As a control, Luc driven by the CaMV35S minimal promoter (mini35S) was employed. The effector plasmids were SIRAV2 or AtCBF1 CDS driven by the CaMV35S promoter (Fig. 5A). Methyl jasmonate (MJ) that acts as a global regulator of defense responses (Reymond and Farmer, 1998) was applied for mimic the pathogen or elicitor treatment. Coexpression of the SIERF5 promoter (ERF5p) with 35S:SIRAV2 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 of that of control (ERF5p reporter only) in the presence of MJ (Fig. 5B). By contrast, cotransfection of SIERF5 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 SIERF5 promoter interacts with SIRAV2 but not with AtCBF1, and that MJ enhances the transactivation of SIERF5 and SIRAV2. Therefore, SIRAV2 and SIERF5/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 tomatoes, thus enhancing tolerance to Ralstonia infection.

**Virus-induced gene silencing (VIGS) of SIERF5 and SIRAV2 attenuates the defense against BW in tomato**

The Tobacco rattle virus (TRV) based-VIGS approach is an efficient silencing system to study the function of candidate genes responsible for certain disease resistance and their signaling pathway(s) (Brigneti et al., 2004; Chen et al., 2009). To find out whether SIERF5 and SIRAV2 are involved in the BW-defense mechanism in both natural cultivar H7996 and AtCBF1 transgenic tomato plants, we performed experiments silencing these AP2/EREBP transcription factors. A mixture of
Agrobacterium cultures containing TRV1 and TRV2-X (X = partial cDNA sequence of SlERF5 or SlRAV2, primers shown in 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 semi-quantitative 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 Ubi RNA served as an internal control for RNA quality. The Ubi 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 pre-infected with TRV-ERF5 and TRV-RAV2, respectively, displayed a severe wilt phenotype after inoculation with Ralstonia (Fig. 6B). Furthermore, we carried out bacterial titers assay for gene-silenced plants at 5 dpi (Table III). Here, tomato cultivar 5915, the background of AtCBF1 transgenic plants, and the susceptible cultivar 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 10⁹ CFU/g fresh weight at both stem bases and mid-stems, Table III). The stem base and mid-stem 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 *SIRAV2-RNAi* knockdown as well as *35S:SIRAV2* and *35S:SIERF5* transgenic tomato plants

To further investigate the function of *SIRAV2* and *SIERF5* in pathogen resistance, transgenic tomato plants with knockdown expression of *SIRAV2* or overexpression of *SIRAV2* or *SIERF5* were generated. Transgenic plants with lower insertion (1-2 copies) of transgene were selected for further study. Semi-quantitative 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 level of *Hpt* and *Actin* were used as transgenic and internal controls, respectively. The foreign transcripts of *SIRAV2* and *SIERF5* transgenes with *35S* or *nos* terminator were expressed only in transgenic plants (Fig. 7A). In addition, *SIERF5* and its downstream gene, *SIPR5*, 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 level of *SIRAV2* and *SIERF5* in pathogen-infected *RAV2Tr* and *RAV2RNAi* transgenic tomato. The transcription of *SIRAV2* and *SIERF5* was upregulated 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 *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, upper 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 RAV2RNAi knockdown line already presented a severely wilted phenotype at 5 dpi, while the wild-type wilted at 7-9 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 $F_v/F_m$ was on average 75% in wild-type (5915) plants, whereas transgenic lines showed a reduction of 88% for RAV2RNAi, 19% for RAV2Tr, and 37% for *ERF5Tr* lines, respectively. 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 *SlERF5* and *SlRAV2* play crucial roles in the basal defense of tomato plants against bacterial wilt and that *SlRAV2* may be a key regulator involved in plant defense.
DISCUSSION

CBF genes have been considered “master switches” which increase freezing tolerance in Arabidopsis plants via activation of COR genes (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999). Its tomato orthologs CBF1 and CBF2 are upregulated 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 upregulated by pathogen infection (Supplemental Fig. S5). However, it still remained unclear whether CBF regulons directly participate in biotic stress response. In the present 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 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 suppress internal pathogen multiplication rather than 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; Zhang et al., 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 tomato stress-responsive factor (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; Zhang et al., 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 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 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 response to biotic and abiotic stresses (Sakuma et al., 2002; Nakano et al., 2006). The members of different subfamily 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 direct activate PRs 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 affinity 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 upregulated expression of SlRAV2; this in turn elevate
other ERFs (e.g., 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 2 or 3 CRT/DRE elements present in the promoter region of rice RAV genes, i.e., Os01g04800 (-1895 and -2371 from ATG), Os05g47650 (-373 and -2032) and Os07g17230 (-683, -1188 and -2256). Completion of the tomato genome sequencing project (the Sol Genomics Network) may reveal more information to find out 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 trans-activate SlRAV2 gene expression (Supplemental Fig. S7). Furthermore, there are several RAV1A elements presented in the promoters of SlERF5 and Pti6 (Supplemental Fig. S2, Table S4), and SlRAV2 can trans-activate 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; Zhang et al., 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, 7 and 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 and co-workers reported that MAPK-, JA/ethylene- and SA-related defense signaling pathways are involved in the resistance to tomato to bacterial wilt (Chen et al., 2009). 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 the present 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 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, the *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 which are related to 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 more in 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 previously reported (Kagaya et al., 1999). Interestingly, we did not find an 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; Hsieh et al., 2002b; Lee et al., 2003). In the present report, we observed that overexpression of either AtCBF1, SlERF5 or SlRAV2 in tomato plants conferred 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.
EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Tomato cultivar CL5915-93D1-1-0-3 (5915) was provided by AVRDC - The World Vegetable Center, 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 MS basal medium at 26°C with a 16/8 hr photoperiod at 120 μmol m⁻² s⁻¹.

Resistance scoring of transgenic tomato plants

Tomato plants were inoculated with *Ralstonia* as described (Chen et al., 2009). The resistance of transgenic tomato plants to bacterial wilt was evaluated as described previously (Lin et al., 2004; Chen et al., 2009). *Ralstonia solanacearum* strain Pss4 (race 1, biovar 3) (suspension A₆₀₀ = 0.6, about 2 x 10⁸ CFU ml⁻¹) was used as the inoculum. Additionally, for *R. solanacearum* colonization experiments, ten 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 a 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*-mediated tomato transformation were carried out as described (Hsieh
et al., 2002a; Hsieh et al., 2002b; Lee et al., 2003). For constitutive overexpression in tomato, constructs p35S:SIERF5 and p35S:SLRAV2 were prepared by inserting the SIERF5 and SLRAV2 coding sequence between the CaMV35S promoter and the nos or the 35S terminator in pCAMBIA1390/35S (Hsiao et al., 2007) and pH2GW7 (primers are shown in Supplemental Table S2), respectively, both of which contain the hygromycin phosphotransferase gene (Hpt). For knockdown expression in tomato, the binary vector pSLRAV2-RNAi was constructed by inserting a SLRAV2 N-terminal region (aa 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 kanamycin (pCAMBIA2301/35S:AtCBF1) or 20 mg/l hygromycin (p35S:SIERF5, p35S:SLRAV2, and pSLRAV2-RNAi). All transgenic plants were analyzed by Southern and northern blot hybridization or RT-PCR, respectively, as described previously (Hsieh et al., 2002a; Hsieh et al., 2002b). The following probes were used for northern blot hybridization; tomato \( \beta \)-tubulin, PR2 (\( \beta \)-1,3-glucanase, accession number: CK664757), PR5-like (accession number: AY257487), PR9-like (peroxidase, accession number: AW219536) and PR10-like (ribonuclease-like, accession number: CK468708). cDNA fragments were excised from pT7Blue (R) vector as probes and labeled with [\( \alpha \)-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 stress-treated
wild-type tomato plants. *AtCBF1* transgenic tomato and control plant RNA was 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, Effeltrich, Germany). 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, Carlsbad, CA, USA) according to the manufacturer’s instructions. Reverse transcription was conducted as described by the manufacturer (Promega, Madison, WI, USA). 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, Taiwan), 0.2 mM of each dNTP, 2 units of Taq DNA polymerase (Violet) and 100 pmol of each primer (Supplemental Table S1). The following amplification program was used: 1 cycle of 95°C for 3 min, 25-30 cycles of 95°C for 25 s, 58°C for 30 s, and 72°C for 1 min, and then 1 cycle of 72°C for 7 min. The RT-PCR products were resolved in 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, Palo Alto, CA, USA). In addition to genome walking, inverse PCR was used to extend the SlERF5 (EU164418) and Pti6 (EU164419) promoter sequence and to obtain full-length Sirav2 (EU164417) 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 iPCR. The following amplification program was used for the first PCR of genome walking and iPCR: 1 cycle of 95°C for 1 min, 7 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, then 1 cycle of 67°C for 7 min. The program for the second PCR of genome walking and iPCR was 1 cycle of 95°C for 1 min, 5 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, then 1 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 sequence (Fig. 4B) were multimerized four times and placed upstream of the 35S minimal promoter and SlERF5 promoter (-776 to +23; Supplemental Fig. S2A) and fused to the firefly luciferase (Luc) gene. For effector plasmids, the Luc gene in pJD301 was replaced by the coding region of AtCBF1, SlERF5, Pti6 and Sirav2. The pBI221 plasmid containing the GUS gene driven by the CaMV35S promoter was used as an internal control for transactivation assay.
Arabidopsis 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 μg of reporter plasmid and 5 μg of effector plasmid or control plasmid (pUC18) were co-transfected into 4×10^4 protoplasts with 10 μg 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).

Tobacco Rattle Virus-based VIGS Assay

VIGS vectors (pTRV1, 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 and RAV2-VIGS-R; ERF5-VIGS-F and 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 semi-quantitative RT-PCR using specific primers (ERF5-RT-F and ERF5-RT-R; RAV2-RT-F and RAV2-RT-R; UBI3-F and 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 solanacearum* (2 × 10^8 CFU ml⁻¹) by root drenching. Five days later, 1-cm sections from the mid-stem 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 L390 susceptible control. For the assay of each gene, 10-12 plants were used in each experiment, and three independent experiments were performed. Pair-wise comparisons were made between wild-type (WT) (or TRV-infected plant in H7996) and silenced plants with Student’s *t*-test method.

**ACKNOWLEDGMENTS**

We wish to thank AVRDC-The World Vegetable Center for technical assistance and providing biological materials. This research was supported by a grant from Academia Sinica and grant NSC-93-2317-B-001-012 from the National Science Council of the Republic of China. We thank Dr. Choun-Sea Lin (Agricultural Biotechnology Research Center, Academia Sinica) and Ms. Fu-Hui Wu for technical support in protoplast isolation and transfection, and Ms. Chia-Hui Liao for tomato transformation. We thank Drs. Su-Chiung Fang and Heiko Kuhn for helpful discussions and manuscript editing.

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N,N-Dimethylformamide. Plant Physiol 65: 478-479


Supplemental Data

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

Figure S2. Promoter sequences of SlERF5 and Pti6.

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

Figure S4. Photosynthesis efficiency and chlorophyll content of 35S:SlRAV2, SlRAV2RNAi and 35S:SlERF5 transgenic lines under pathogen infection.

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

Figure S6. Genome sequence of SlRAV2.

Figure S7. CBF1 activate the reporter gene driven by the SlRAV2 promoter.

Table S1. Oligonucleotides used for RT-PCR.

Table S2. Oligonucleotides used for genome walking, inverse PCR and vector constructions.
**Table S3.** Prediction of the CBF1-binding elements in the promoter region of the *Arabidopsis* AP2/ERF genes.

**Table S4.** Prediction of the AP2/ERFs binding elements in the promoter region of Tomato AP2/ERFs.
<table>
<thead>
<tr>
<th>RAVIA Ware ID</th>
<th>RAVIA term</th>
<th>Scaffolds (2.30)</th>
<th>SGA number</th>
<th>Description</th>
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<tbody>
<tr>
<td>At4g11650</td>
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<td>BF097167</td>
<td>0</td>
<td>cLEW19L9</td>
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<td>cLEX2M14</td>
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<td>cLEX5K5</td>
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<tr>
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<td>SGN3</td>
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<td>0</td>
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</table>

**Table 1.** Protein phosphatase 2C interacting motif of transcription factor.
Table II. The pathogen tolerance of transgenic tomato plants is not affected by exogenous GA3 treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WT</th>
<th>C5</th>
<th>C15</th>
<th>C21</th>
<th>WT+GA3</th>
<th>C5+GA3</th>
<th>C15+GA3</th>
<th>C21+GA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN</td>
<td>21.6 ± 4.1</td>
<td>6.0 ± 1.6</td>
<td>7.2 ± 1.6</td>
<td>1.6 ± 1.1</td>
<td>26.6 ± 4.1</td>
<td>24.8 ± 3.6</td>
<td>22.4 ± 3.2</td>
<td>17.4 ± 5.8</td>
</tr>
<tr>
<td>Control SN</td>
<td>48.7 ± 9.2</td>
<td>8.4 ± 2.7</td>
<td>6.8 ± 1.3</td>
<td>2.4 ± 0.9</td>
<td>43.7 ± 9.2</td>
<td>25.4 ± 3.0</td>
<td>22.6 ± 2.6</td>
<td>29.6 ± 14.8</td>
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<tr>
<td>FW</td>
<td>132.4 ± 7.1</td>
<td>80.6 ± 5.1</td>
<td>106.8 ± 9.2</td>
<td>85.0 ± 3.9</td>
<td>147.4 ± 7.1</td>
<td>133.4 ± 13.8</td>
<td>138.8 ± 13.6</td>
<td>127.6 ± 8.7</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>FW</th>
<th></th>
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<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>FN</td>
<td>0 ± 0</td>
<td>9.8 ± 1.4</td>
<td>10.8 ± 1.6</td>
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<td>0 ± 0</td>
<td>25.8 ± 3.7</td>
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<tr>
<td>Ralstonia SN</td>
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<td>14.6 ± 3.2</td>
<td>20.8 ± 7.2</td>
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<tr>
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<td>121.4 ± 4.0</td>
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</table>

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 ± standard deviation (n = 5 individual plants). Wild-type and AtCBF1 transgenic plants were grown in pots with peat moss and watered every alternate day in greenhouse with a 16/8 hr photoperiod (Day light about 120 μmol m⁻² s⁻¹, 26°C ± 2°C; night temperature, 22°C ± 2°C). For GA₃ treatment, AtCBF1 transgenic and wild-type plants were sprayed with 5 mg L⁻¹ GA₃ three times in a 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 the fresh weight, and calculated the fruit and seed numbers.
Table III. Assessment of Ralstonia solanacearum density in silenced tomato plants.

<table>
<thead>
<tr>
<th>Tomato plants</th>
<th>Silenced gene</th>
<th>Sample no.</th>
<th>Stembases + Log (CFU g⁻¹ plant tissue)</th>
<th>Mid-stems + Log (CFU g⁻¹ plant tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5915</td>
<td>WT</td>
<td>-</td>
<td>30</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>CBF1OX</td>
<td>TRV</td>
<td>36</td>
<td>20</td>
<td>4.6 ± 0.6b</td>
</tr>
<tr>
<td>CBF1OX</td>
<td>SIRAV2</td>
<td>35</td>
<td>27</td>
<td>8.7 ± 1.0a</td>
</tr>
<tr>
<td>CBF1OX</td>
<td>SIERF5</td>
<td>36</td>
<td>25</td>
<td>8.0 ± 0.8a</td>
</tr>
<tr>
<td>H7996</td>
<td>WT</td>
<td>TRV</td>
<td>36</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L390</td>
<td>WT</td>
<td>-</td>
<td>6</td>
<td>10.7 ± 0.2</td>
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</table>

AtCBF1 transgenic plants (CBF1OX) 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 fragments (SIERF5). BW-susceptible tomato varieties L390, 5915 (the background of CBF1OX), and TRV-infected CBF1OX and H7996 plants were treated with Ralstonia. The bacterial titer inside the test plants was measured in stem bases and mid-stems at 5 dpi. Number of total assayed plants and positively detected plants (+) were indicated. Each value is the mean ± standard deviation. Pair-wise comparisons were made between wild-type (WT in 5915) (or TRV-infected plant in H7996) and silenced plants with Student’s t-test method (a, P < 0.01; b, P < 0.05).
Figure 1A
Figure 1B
Figure 1. *AtCBF1* transgenic plants exhibit enhanced resistance to *Ralstonia solanacearum*. A, Test plants were inoculated with *Ralstonia* and then kept at 28°C with a photoperiod of 16 h. A photograph was taken at 7 days post-inoculation (dpi). The test plants comprised wild-type plants (susceptible variety 5915, the genetic background of transformants), a bacterial wilt-resistant control variety (H7996), a control transgenic line (*AtCBF1* driven by ABA-inducible *ABRC1* promoter), and T2 transgenic plants continuous 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 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.
Figure 2
Figure 2. Northern blot analyses of PR genes in transgenic tomato plants. Total RNA (10 μg) was extracted from wild-type (WT) and transgenic T1 plants overexpressing AtCBF1 (C5, C15, C21). Probes used to hybridize total RNA were 32P-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.
**Figure 3.** RT-PCR analysis of *SIERF* 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 *SICBF1*, *SIERF5*, *Pti4*, *Pti5*, *Pti6*, *SIRAP2.1*, *SIRAP2.6*-like (*SIRAP2.6L*), *SIRAV2*, *actin1*, *AtCBF1* transgene (*AtCBF1-nos*) and *NptII* transcripts were amplified by RT-PCR and analyzed on a gel stained with ethidium bromide.
A

Reporter plasmid

mini35S

GCC mini35S

mGCC mini35S

Effector plasmid

ERF5

Pti6

CBF1

Internal control

pBl221

B

• mini35S (35m)

| GGA | TCC | AGA | CCA | AGA | CCT | CTC | CTA | TAA | AGG | AAG | TTC | ATT | TCA | TGT | CTC | AGG | GA
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
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| AAG | CTT | GAT | CAT | GAG | TCC | TCT | TGG | TCA | TGT | CTC | AGG | GA

Figure 4AB
Figure 4C
Figure 4. SIERFs activate the reporter genes driven by CaMV35S minimal promoter containing vicinal GCC boxes. A, Schematic diagram 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 SIERF5 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 box, respectively. C, Transactivation of the Luc reporter gene by AtCBF1, Pti6 and SIERF5 in Arabidopsis protoplasts. Different effectors were co-transfected with the reporter and internal control plasmid (pBI221). The data represent means of 3 independent transient transformations. Error bars indicate standard deviations. Transient transformations without the effector plasmid were used as a control.
A

**Reporter plasmid**
- mini35S
- ERF5p
- SIERF5 promoter
- Luciferase
- nos

**Effector plasmid**
- RAV2
- CaMV35S promoter
- SIRAV2
- t35
- CBF1
- CaMV35S promoter
- AtCBF1
- nos

**Internal control**
- pBI221
- CaMV35S promoter
- GUS
- nos

*Figure 5A*
Figure 5B
**Figure 5.** *SIRAV2* interacts with the promoter of *SIERF5*. A, Schematic diagram 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 *t35* denote the terminator of *nopalin e 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 methyl jasmonate. The data represent means of 3 independent transient transformations. Error bars indicate standard deviations. Transient transformation without the effector plasmid (*ERF5p* and *mini35p*) was used as a control.
**Figure 6**

A

| Cycles |  |  |  |  |  |  |  |
|--------|---|---|---|---|---|---|
| 20     | 24 | 27 | 30 | 35 | 40 | NC |

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| Cycles |  |  |  |  |  |  |  |
|--------|---|---|---|---|---|---|
| 20     | 24 | 27 | 30 | 35 | 40 | NC |

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B

TRV  TRV-ERF5  TRV-RAV2

[Image of plant growth comparison]
• **Figure 6.** Silencing of *SIERF5* and *SIRAV2* 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 *SIERF5* (TRV2-ERF5) or *SIRAV2* fragments (TRV2-RAV2). 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.
Figure 7A
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Figure 7B
Figure 7. Analysis of 35S:SIRAV2, SIRAV2RNAi and 35S:SIERF5 transgenic tomato lines. A, Endogenous and transgenic mRNA transcript levels of SIRAV2, SIERF5 and SIPR5 genes in SIERF5 overexpression (ERF5Tr1 and ERF5Tr5), SIRAV2 overexpression (RAV2Tr2, RAV2Tr5 and RAV2Tr8) and SIRAV2 knockdown (RAV2RNAi) tomato plants. B, Expression of SIRAV2 and SIERF5 in wild-type, RAV2Tr and RAV2RNAi tomato lines under Ralstonia infection for 12 h. mRNA levels of the indicated genes in pathogen-treated (pathogen) and non-treated (mock) plants were determined by semi-quantitative RT-PCR. SIRAV2-T35S and SIERF5-Tnos show transgenic expression amplified by the specific forward primers (SIRAV2-F and SIERF5-F) and the terminator reverse primers (35T-R and nos3'R, Table S1). SIRNAilF-T35S show pRAV2-RNAi fragment amplified by 35T-R reverse primer and a forward primer (RNAil-F) located in the intron of the RNAi vector pH7GWIWG2. Hpt and Actin expression levels were analyzed as a transgenic and quantification control, respectively.
Figure 8AB

A

Before Infection

5 dpi

9 dpi

B

Days after infection

Percentage of wilted plants (%)
C

Before Infection

WT
ERF5Tr1
ERF5Tr5

7 dpi

D

Percentage of wilted plants (%) vs. Days after infection

Figure 8CD
**Figure 8.** Comparison of 35S:SIRAV2, SIRAV2RNAi and 35S:SIERF5 transgenic lines with the wild type under pathogen infection. A, Wild-type plants (cultivar 5915, WT), T2 SIRAV2 knockdown (SIRAV2RNAi), and overexpression lines (RAV2Tr2, RAV2Tr5 and RAV2Tr8) were inoculated with *Ralstonia*. The photograph was taken at 0, 5 and 9 dpi. B, Percentage of wilted plants was calculated at 7, 14, 21, 28 and 35 dpi. C, Wild-type plants (5915, WT) and SIERF5 T2 overexpression lines (ERF5Tr1 and ERF5Tr5) were inoculated with *R. solanacearum* and then kept at 28°C with a photoperiod of 16 h. The photograph was taken at 0 and 7 dpi. D, Percentage of wilted plants was measured. Each value represents the mean ± standard deviation (*n* = 10 individual plants) of three independent experiments. *, *P* < 0.01 (Student’s t-test).
DREB family such as CBF1

Bind to CRT/DRE

RAV family, such as RAV2

Bind to RAV1A

ERF family, such as ERF5, Pti4, Pti5, Pti6, RAP2.1 and RAP2.6

Bind to GCC box

Pathogenesis-related (PR) genes, such as PR2, PR5, PR9, PR10

Pathogen resistance

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