Plant Molecular Biology **55:** 825–834, 2004.

Tomato stress-responsive factor TSRF1 interacts with ethylene responsive element GCC box and regulates pathogen resistance to *Ralstonia solanacearum*

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Received 7 June 2004; accepted in revised form 13 August 2004

Key words: ethylene signaling, GCC box, *PR* gene expression, resistance to *Ralstonia solanacearum*, transient expression, TSRF1

Abstract

Ethylene responsive factors (ERFs) are important in regulating plant pathogen resistance, abiotic stress tolerance and plant development. Recent studies have greatly enlarged the ERF protein family and revealed more important roles of ERFs in plants. Here, we report our finding of a tomato ERF protein TSRF1, which is transcriptionally up-regulated by ethylene, salicylic acid, or *Ralstonia solanacearum* strain BJ1057 infection. Biochemical analysis indicates that TSRF1 specifically interacts *in vitro* with the GCC box, an element present in the promoters of many *pathogenesis-related* (*PR*) genes. Further investigation evidences that TSRF1 activates *in vivo* the expression of reporter β-glucuronidase gene controlled by GCC box. More importantly, overexpressing *TSRF1* in tobacco and tomato constitutively activates the expression of *PR* genes, and subsequently enhancing transgenic plant resistance to the bacterial wilt caused by *Ralstonia solanacearum* strain BJ1057. Therefore our investigation not only extends the functions of ERF proteins in plant resistance to *R. solanacearum*, but also provides further clues to understanding the mechanism of host regulatory proteins in response to the infection of pathogens.

Introduction

To survive environmental conditions, such as drought, high salt, extreme temperatures and pathogen attack that greatly affect plant development and productivity, plants have developed an elaborate signaling network that perceives these signals and modulates the expression of specific resistance genes. In these defense response pathways, transcription factors play important roles in controlling the expression of specific stress-related genes (reviewed in Xiong and Zhu, 2003; Guo and Ecker, 2004). Recent studies on defense signaling pathways reveal that ethylene, jasmonic acid (JA) and salicylic acid (SA) play a dominant role in regulating the induced defenses against microbial pathogens (Dong, 1998). These pathways can either positively or negatively interact with each other depending on the type of pathogen (Berrocal-Lobo *et al.*, 2002; Gu *et al.*, 2002; Lorenzo *et al.*, 2003; Guo and Ecker, 2004), but how these defense response pathways communicate with each other is unclear.

The ethylene responsive factors (ERFs), which contain a highly conserved DNA binding domain known as ERF domain, were first identified in tobacco as members involved in ethylene-related pathogen resistance (Ohme-Takagi and Shinshi, 1995). In plants, ethylene signal is perceived by a family of receptors consisting of five members: ETR1, ETR2, EIN4, ERS1 and ERS2 (Solano *et al*., 1998). Downstream of the ethylene receptors is CTR1. In the absence of ethylene, ethylene receptors activate CTR1, which negatively regulates the downstream ethylene response pathway (Kieber *et al*., 1993). Binding of ethylene inactivates the receptors, resulting in deactivation of CTR1 (Hua and Meyerowitz, 1998), which allows EIN2 to function as a positive regulator of the ethylene pathway. In this cascade, the EIN2 protein activates EIN3 and EILs, subsequently activating ERF proteins, such as ERF1 (Solano *et al*., 1998) to regulate the expression of genes involved in the response to ethylene (reviewed in Wang *et al*., 2002). Accumulating evidences indicate that ERF proteins modulate the expression of many *pathogenesis-related* (*PR*) genes through interaction with GCC box present in their promoters (Ohme-Takagi and Shinshi, 1995; Sessa *et al*., 1995; Shinshi *et al*., 1995; Chakravarthy *et al*., 2003; Wang *et al*., 2004). More importantly, the recent investigations reveal that ERF proteins are identified to bind to different *cis*-acting elements (Chakravarthy *et al*., 2003). For example, the tomato Pti4-6 (Zhou *et al*., 1997; Gu *et al*., 2000), LeERF1-4 (Tournier *et al*., 2003), *Arabidopsis* ERF1 (Solano *et al*., 1998), AtERFs (Fujimoto *et al*., 2000), tobacco ERFs (Ohme-Takagi and Shinshi, 1995), and periwinkle ORCA1-3 (Menke *et al*., 1999; van der Fits and Memelink, 2000, 2001) bind to the GCC box (Eyal *et al*., 1993; Ohme-Takagi and Shinshi, 1995; Gu *et al*., 2002). Other subfamilies of ERF proteins, such as CBF1 (Stockinger *et al*., 1997) and DREB1A/2A (Liu *et al*., 1998), interact with a dehydration-responsive element (DRE) that is involved in drought, salt and cold stress. More interestingly, a tobacco ERF protein Tsi1 (Park *et al*., 2001) and a tomato ERF protein JERF3 (Wang *et al*., 2004) could bind to both GCC box and DRE sequences and function in pathogen resistance and salt tolerance. These results strongly suggest that the ERF proteins modulate multiple aspects of plant development and stress responses by interacting with one or more *cis*-acting elements or other transcription factors (Buttner and Singh, 1997). Accumulating evidences indicate that ERF proteins regulate pathogen resistance of *Pseudomonas syringae pv tomato* (Gu *et al*., 2000; Park *et al*., 2001), however, whether and how ERF proteins regulate plant response to *Ralstonia solanacearum* is less understood.

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most devastating soil-borne diseases of plants worldwide and affects many important crop species. *R. solanacearum* has been investigated both biochemically and genetically and recognized as a model system for the analysis of pathogenicity (Deslandes *et al*., 2002, 2003). Since completion of the genome sequencing of *R. solanacearum* (Salanoubat *et al*., 2002), the identification of host proteins regulating resistance to *R. solanacearum* has become a key point in dissecting molecular determinants governing pathogenicity. Some members have been shown to regulate plant *R. solanacearum* response, such as the *Arabidopsis RRS1-R* gene (Deslandes *et al*., 2002) and *Brassica oleracea SRK* (Pastuglia *et al*., 1997); however, the mechanism of plant resistance to *R. solanacearum* is still poorly understood. Here, we report a novel ERF protein, designated *t*omato *s*tress *r*esponsive *f*actor 1 (TSRF1), interacts with the GCC box *in vitro* and activates the expression of reporter gene *in vivo* in transient expression assay and *PR* genes in transgenic *TSRF1* plants, subsequently enhancing the plant resistance to *R. solanacearum* strain BJ1057, a race 1 strain isolated from tomato.

Materials and methods

Plant material and growth conditions

All plants were grown in growth chambers at 25 °C with a 16-h-light/dark cycle (except mentioned in the text). 4-week-old tomato (*Lycopersicon esculentum* cv Lichun) and 6-week-old tobacco (*Nicotiana tabacum* cv Gexin1) were used for Northern blot. Ethylene treatment was performed with 2 ml 40% ethephon and 1 g $NaHCO_3$ dissolving in 200 ml H_2O (in such condition ethephon will liberate ethylene gas), the control and treated tomato plants were placed in a sealed plexiglass chamber before leaf tissue was harvested. For SA treatment, tomato

plants were sprayed with 0.5 mM SA in water (control plants were sprayed with water). For detecting the expression of *TSRF1* and downstream genes in transgenic tobacco or tomato plants, leaves from normally growing 4–5-week-old plants were used.

Screening with yeast one-hybrid

The construction of reporter plasmids and tomato cDNA library, and the screening procedure with yeast one-hybrid are done as described by Wang *et al.* (2004).

RNA transcription analysis

Total RNA was extracted from leaves. Twenty micrograms of total RNA was separated on a 1.5% formaldehyde–agarose gel, and transferred onto nylon membranes. Using [α-^{32}P] dCTP labeled 3′ flanking sequence of *TSRF1* as probe; the membrane was hybridized as the standard procedure of Northern blot. The hybridized membrane was exposed to Imaging Screen-K and visualized with the Molecular Imager® FX (Bio-Rad). The probes for downstream genes expression were cloned by reverse transcriptional polymerase chain reaction (RT-PCR). The tobacco *PR* genes *prb-1b* (*PR1*) (Eyal *et al.*, 1992), *GLA* (*PR2*) (Sperisen *et al.*, 1991), and *CHN50* (*PR3*) (Fukuda *et al.*, 1991) were cloned as described by Wang *et al.* (2004). These primers were used to clone tomato *PR* genes: 5′-CGTTAGAGATTTCTGGCCTC -3′ and 5′-CTCACTAGTGAGTGAAGAAG-3′ for *GluB* (*PR2*) (van Kan *et al.*, 1992); 5′-GAGGAGCACT TTGTGCATC-3′ and 5′-CAAAAGACCTCTG ATTGCC–3′ for *Chi9* (*PR3*) (Danhash *et al.*, 1993).

Binding assay in vitro

The *TSRF1* full encoding cDNA was cloned in frame into *Nde*I–*Hind*III sites of the pET28a vector (Novagen, Madison, USA), yielding pET28-TSRF1. The primers used for amplifying this fragment were *TSRF1-5′* (5′-GGATCCATATGGAG GTTATTGAAGCAATACCG-3′) and *TSRF1-3′* (5′-AAAAAAGCTTTTAGAGCAATCATCGTC TACGTGAC-3′). The pET28-TSRF1 construct was transformed into BL21. Expression and purification of recombinant TSRF1 were done with a commercial kit following the manufacturer's introduction (Invitrogen, Karlsruhe, USA). The wild-type GCC box sequence AGTGCCAAAAG CCGCCACACCCCT and mutant GCC box sequence AGTGCCAAAATCCACTACACCCC-T (mutated oligonucleotides are highlighted with underline) were synthesized and end labeled with [α-^{32}P] dATP after annealing and purification. The assay mixtures contained recombinant TSRF1 (1.2 μg of protein), 2 ng of binding probe (8×10^4 cpm), 2 μg of salmon DNA, 20 mM Hepes–KOH, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 10% glycerol and 0.5 mM DTT in a 10 μl reaction volume. The binding reaction, gel separation and signal recording are done as described by Wang *et al.* (2004).

β-glucuronidase *transient assay* in vivo

For constructing the reporter vector, the four times repeated GCC box or mGCC box sequences were inserted into the upstream of the minimal TATA box (−46 to +10) to replace the cauliflower mosaic virus (CaMV) 35S promoter in pBI121 (Clontech, Palo Alto, CA). For constructing the effector vector, the β-glucuronidase (*GUS*) gene in pBI121 was replaced with the full encoding region of TSRF1, yielding the construct pTSRF1. Transient assay was performed on the 10-day-old wild type tobacco seedlings with bombardment using a Bio-Rad PDS-1000/He machine. About 500 ng of tungsten particles (Bio-Rad) were coated with 1.5 μg of reporter plasmid and 1.5 μg of effector plasmid. After bombardment, the samples were incubated for 48 h at 25 °C with a 16-h-light/dark cycle and then harvested for GUS activity quantification.

Generation of transgenic tomato and tobacco

The above vector pTSRF1 was then introduced into tomato (Lichun) or tobacco (Gexin 1) by *Agrobacterium*-mediated transformation as described by Martin *et al.* (1993) and Hoekema *et al.* (1983).

Plant infection with Ralstonia solanacearum *strain BJ1057*

For Northern blot, disease inoculation was done as by Berrocal-Lobo *et al.* (2002). Plants were dipped for 6 min in a bacterial suspension in 10 mM

$MgCl_2$ containing 0.1% silwett L-77 (Lehle Seeds, TX, USA), and then placed at 28 °C and covered to maintain high humidity. Mock inoculations were performed with 10 mM $MgCl_2$ containing same amount of silwett L-77. The infected plants were harvested at the indicated days.

For disease resistance studies, the inoculation on leaves of tobacco and tomato (infiltration with syringe) and the determination of the bacterial number of *Ralstonia solanacearum* strain BJ1057 was done following the methods for *Pseudomonas syringae* pv *tomato* as described by Thilmony *et al.* (1995). The *Ralstonia solanacearum* strain BJ1057 was cultured as described by He *et al.* (1983). Overnight culture of *R. solanacearum* on nutrient agar medium was re-suspended at the indicated concentration in 10 mM $MgCl_2$, and infiltrated into fully developed leaves with syringe. Approximately 40 μl of inoculum was infiltrated per panel, forming an infiltrated area of 15×15 mm. The identical treatments were verified by counting the bacterial numbers at "0" time point to confirm the bacteria numbers between wild type and transgenic infected leaves are near the same. The inoculated plants were maintained at 28 °C with a 16-h-light/dark cycle. Four days post inoculation (DPI), the bacterial growth was measured by macerating five leaf discs of 1-cm^2 from the inoculated tissue of each sample in 10 mM $MgCl_2$, plating the serial dilutions on nutrient agar plates, and counting the colony-forming units (cfu). The experiments were repeated 3 times for each sample. Images were taken 4 DPI. For root inoculation, 6-week-old tobacco seedlings with ~5 leaves were inoculated with 10^8 cfu/ml *Ralstonia solanacearum* described as by Deslandes *et al.* (2002), and the number of infected leaves was investigated every 5 days and plants with 3/4 leaves infected were classified to be decayed.

Results

Isolation of TSRF1

In order to clone the regulatory proteins that interact with *cis*-acting element GCC box, 1.2×10^6 yeast transformants were screened with the method of yeast one hybrid from tomato expression cDNA library with a four time repeated GCC box present in the promoter (−74–−51 bp) of tomato *NP24* (Jia and Martin, 1999) as bait, and 14 positive clones were isolated. Of one positive clone with full length cDNA, designated *tomato stress responsive factor 1* (*TSRF1*, NCBI access number AF494201) encodes a putative protein containing 240 amino acids with a predicted molecular mass of 26.9 ku. NCBI blast revealed that the deduced amino acid sequence contains a conserved ERF DNA binding domain, suggesting that TSRF1 is a member of ERF proteins. Database analysis suggested that the deduced amino acid sequence of TSRF1 contains a basic region that might function as a nuclear localization signal (NLS) and an acidic region that might act as transcriptional activation domain (Figure 1A), and of all the deduced domains, only the ERF domain was conserved between TSRF1 and other ERF proteins. Further analysis indicated that TSRF1 shows a low similarity to ERF1 (52.8%), Pti4 (31.0%), Tsi1 (20.1%) and DREB2A (20.5%) in whole putative protein sequences, but a very high similarity with ERF1 (91.5%), Pti4 (72.4%), Tsi1 (61.4%), and DREB2A (60.3%) in their ERF domain regions (Figure 1B), suggesting that the *TSRF1* cDNA encodes a novel member of ERF proteins. According to the most recent phylogenetic overview, TSRF1 belongs to the B-3 subfamily of ERF proteins, and some of the B-3 subfamily members have been evidenced to regulate plant disease resistance (Gutterson and Reuber, 2004).

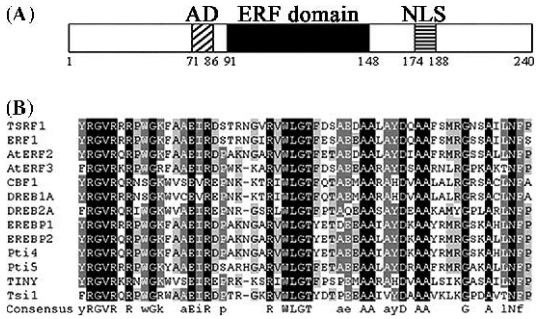

Figure 1. Sequence analysis and expression of *TSRF1*. (A). Scheme of the predicted TSRF1 (access number AAN32899) protein. TSRF1 contains an ERF domain (black box), a putative nuclear localization signal (dashed box), and a putative activation domain (hatched box). Numbers below show the position of each domain. (B). Comparison of deduced amino acid sequence of TSRF1 and those of other ERF proteins in ERF DNA binding domain. The shaded boxes indicate the percentage of sequences at that position with the same amino acid identity (dark gray, 100%; medium gray, 75%; light gray, 50%).

Expression pattern of TSRF1

First, we tested the tissue specific expression of *TSRF1* in leaves, stems, flower and roots under normal growth conditions. Our results indicated that the expression of *TSRF1* was very weak in these mentioned tissues, which was almost undetectable (data not shown), suggesting that TSRF1 might be an inducible protein. Then, we analyzed the expression of TSRF1 responsive to ethylene, and salicylic acid. Comparing to the control treatments, which could not induce the expression of *TSRF1* (data not shown) the expression of *TSRF1* was up-regulated by ethylene or salicylic acid treatments. The transcript of *TSRF1* would accumulate at 3 h, and peak at 7 h after treatment with ethylene. Similar to ethylene, *TSRF1* transcripts were observed at 3 h and peak at 5 h after treatment with SA (Figure 2). The tomato *PR2* gene *GluB* was used as a positive control for ethylene and SA treatments (van Kan *et al*., 1995; Gu *et al*., 2000). In our assays the transcript of *GluB* was accumulated as the expression of *TSRF1* in response to ethylene or SA (Figure 2). Also we checked the expression of *TSRF1* in response to 0.1 mM methyl jasmonic acid (MeJA), unfortunately, we did not detect the obvious expression of *TSRF1* (data not shown), indicating that TSRF1 may be a responsive component of the ethylene- and SA-signaling pathways. Furthermore, *TSRF1* transcripts were also observed 1 DPI and increased until 5 DPI with 10^5 cfu/ml *R. solanacearum*, but did not obviously accumulated within the observation period with mock inoculation (Figure 2), suggesting a potential regulatory role of *TSRF1* in plant resistance to *R. solanacearum*.

Interaction of TSRF1 with the GCC box in vitro *and* in vivo

To test the binding affinity of TSRF1 to GCC box *in vitro*, TSRF1 fusion protein was expressed in pET28a and purified with the affinity column. The binding ability of recombinant TSRF1 to synthesized GCC box and the mutant GCC box was tested by using an electrophoresis mobility shift assay. As shown in Figure 3A, the recombinant TSRF1 protein could bind to GCC box, but not

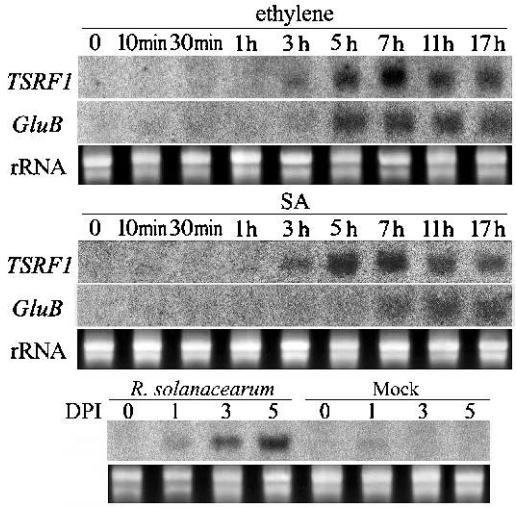

Figure 2. Expression of the *TSRF1* in response to ethylene, salicylic acid or *R. solanacearum* infection. Each lane was loaded with 20 μg of total RNA from tomato plants that had been treated with ethylene, salicylic acid or *R. solanacearum* infection. The numbers above each lane indicate the time of treatment. The RNA gel blots were hybridized with 3′ flanking sequence of *TSRF1* cDNA as probe. The tomato *PR* gene *GluB* was used as positive control for ethylene and SA treatments. Ribosomal RNA (ethidium bromide staining) was used as loading control.

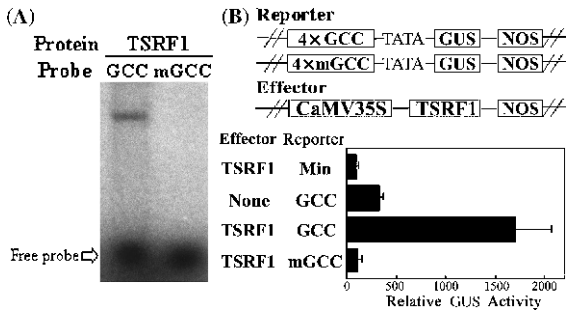

Figure 3. Interaction of TSRF1 with GCC box *in vitro* and *in vivo*. (A). Electrophoretic mobility shift assay showing sequence-specific binding of TSRF1 to GCC box, or mutant GCC box (mGCC box). (B). Transient assay of the interaction between TSRF1 and GCC box *in vivo* with bombardment. Strategy for analyzing interaction between TSRF1 and *cis*-acting element GCC box (upper panel) or mGCC box and quantification of the induced GUS activity (lower panel). The data for GUS activity quantification are average deviated from triplicate samples and three independent experiments, compared to the control of Min (as 100). Min indicates the minimal TATA promoter fused with GUS; GCC and mGCC indicate that the GCC box and mGCC box are inserted upstream of Min as positive reporters respectively; TSRF1 as a positive effector indicates the full length of *TSRF1* gene was driven by 35S promoter; None as the negative control of effector indicates no effector plasmid was added. Error bars indicate $\pm$SD.

mGCC box, suggesting the interaction of TSRF1 with GCC-box *in vitro*.

Then the transcriptional activity of TSRF1 based on GCC box is further tested *in vivo* by transient expression experiment using tobacco seedlings. As shown in Figure 3B, constitutive expression of *TSRF1* strongly activated the expression of GCC box-driven reporter. The GUS activity driven by GCC box was about 17 fold of the control (Min), while the activity driven by mutant GCC box (mGCC box) has no obvious difference with the control, supporting that TSRF1 positively regulates the expression of GCC box regulated genes *in vivo*.

Overexpressing TSRF1 *constitutively activates expression of GCC box-containing genes and enhances resistance to* R. solanacearum *strain BJ1057 in both transgenic tobacco and tomato plants*

Many *PR* genes, such as *PR1*, *PR2*, *PR3*, and *PR5*, have been characterized to have the GCC box sequence in their promoters, and to enhance plant resistance to pathogens (Ohme-Takagi, 1995; Park *et al.*, 2001). To determine whether TSRF1 could induce the expression of GCC box-containing genes in plants, we developed tobacco and tomato transgenic plants that constitutively express *TSRF1* from 35S promoter and got 8 tomato lines (designated as OTM) and 7 tobacco lines (designated as OTB) that stably express *TSRF1* with a copy of insertion. By Northern blot we tested the expression level of *GluB* (*PR2*), *Chi9* (*PR3*) in tomato, and *prb-1b* (*PR1*), *GLA* (*PR2*), and *CHN50* (*PR3*) in tobacco transgenic plants respectively. Results showed that constitutive expression of *TSRF1* in tobacco (Figure 4A) and tomato (Figure 4B) significantly activated the expression of above *PR* genes compared to the transcript level in wild type plants.

Then, using the above transgenic plants, we investigated the role of *TSRF1* in regulating plant resistance to *R. solanacearum* strain BJ1057 that belongs to race 1 from tomato, which is virulent on both tomato and tobacco. Because of the characterization of soil-borne diseases, we first used 10^8 cfu/ml *R. solanacearum* strain BJ1057 to inoculate the roots (Deslandes *et al.*, 2002) of 6-week-old OTB (overexpressing *TSRF1* in tobacco) and WTB (wild type tobacco) plants. The WTB plants

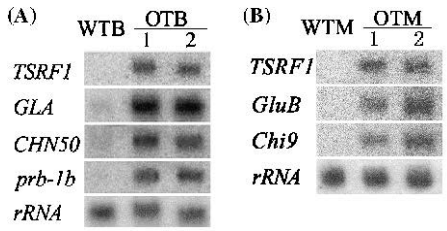

Figure 4. Constitutive expression of *TSRF1* activates expression of *PR* genes in tobacco (A) and tomato (B) plants under normal growth conditions. 18S rRNA probe was used as loading control. WTB, wild type of tobacco; OTB, overexpressing *TSRF1* in tobacco; WTM, wild type of tomato; OTM, overexpressing *TSRF1* in tomato; the numbers following each transgenic plant indicate the different transgenic lines.

showed susceptible phenotype, most of them began to wilt on most of the leaves 10–12 DPI, and then the whole plant wilted 18–20 DPI, while the most of the OTB plants (83.9%) were resistant to the infection (Figure 5A). At this stage, the number of wilted leaves was investigated and plants with 3/4 leaves wilted were classified as being decayed. Our results indicated that the WTB plants had a much heavier infection (57.1% decayed) than the OTB plants (less than 16.1% decayed) at 20 DPI (Figure 5A). Based on this observation, we then investigated the resistance of TSRF1 to *R. solanacearum* strain BJ1057 in 6-week-old transgenic

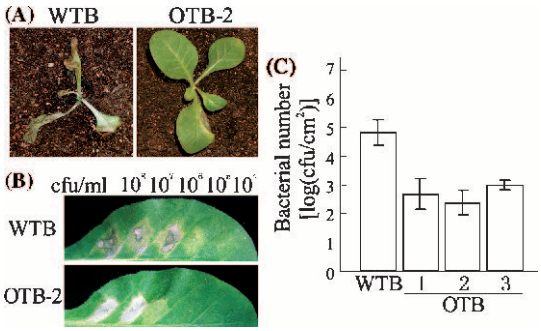

Figure 5. Overexpression of *TSRF1* modulates resistance to *R. solanacearum* strain BJ1057 in tobacco. (A). Symptoms for decayed tobacco plants 20 DPI with root inoculation of 10^8 cfu/ml *R. solanacearum* strain BJ1057. The showing result is the representation of the transgenic tobacco lines tested with similar results. 70–100 plants for each lines were tested. (B). Symptoms for wild type and OTB tobacco leaves 4 DPI with *R. solanacearum* strain BJ1057 at regions between the lateral veins (at orientation of left to right). The showing result is the representation of the transgenic lines tested with similar results. (C) *R. solanacearum* strain BJ1057 growth in tobacco leaves 4 DPI with 10^6 cfu/ml *R. solanacearum* strain BJ1057. The data are average derived from triplicate samples of 10–20 plants and three independent experiments. Error bars indicate ±SE.

tobacco by inoculating WTB and OTB plants with infiltration of 10^4 and 0^8 cfu/ml *R. solanacearum* strain BJ1057 using a syringe between 2 lateral veins. Results indicated that WTB plants developed water-soaked lesions and a chlorotic edge around the infected sites on leaves 4 DPI; this occurred at inoculation concentrations of 10^8 and 10^6 cfu/ml; chlorosis at the inoculated sites occurred at inoculation concentrations of 10^5 and 10^4 cfu/ml. By contrast, OTB plants showed a resistant response at inoculation concentrations of 10^8 and 10^6 cfu/ml and no symptom observed at inoculation concentrations of 10^5 and 10^4 cfu/ml (Figure 5B). Four DPI with 10^6 cfu/ml *R. solanacearum* strain BJ1057, the bacterial number of *R. solanacearum* in OTB plant was 70–280 fold less than that in WTB plant (Figure 5C).

Similarly, the function of TSRF1 against *R. solanacearum* strain BJ1057 was further confirmed by observing the defense response in wild type tomato (WTM) and transgenic tomato overexpressing *TSRF1* (OTM). About 10^3 cfu/ml *R. solanacearum* strain BJ1057 was infiltrated by syringe between 2 lateral veins. WTM plants infected with *R. solanacearum* strain BJ1057 developed water-soaked lesions on leaves 4 DPI. By contrast, the OTM plants showed much weaker symptom (Figure 6A). Compared to the WTM plants, the bacterial number of *R. solanacearum* was reduced 60–200 fold at infected sites in OTM plant (Figure 6B). Therefore, we conclude that TSRF1 has a positive regulatory role in conferring resistance to *R. solanacearum* strain BJ1057.

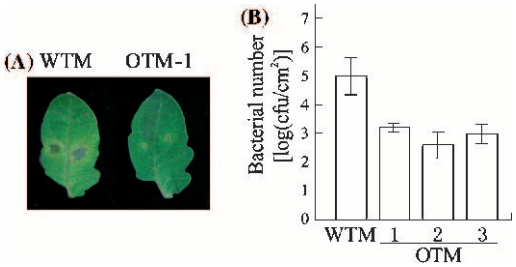

Figure 6. Overexpression of *TSRF1* modulates resistance to *R. solanacearum* strain BJ1057 in tomato. (A). Symptoms for wild type tomato and OTM 4 DPI with 10^3 cfu/ml of *R. solanacearum* strain BJ1057. The showing result is the representation of the transgenic lines tested with similar results. (B). *R. solanacearum* strain BJ1057 growth in tomato leaves 4 DPI with 10^3 cfu/ml *R. solanacearum* strain BJ1057. The data are average derived from triplicate samples with 10–20 plants and three independent experiments. Error bars indicate ± SE.

Discussion

The isolation of regulatory proteins that interact with GCC box is crucial for revealing the regulatory mechanism of gene expression that responds to pathogen infection. In this paper we isolated and identified a transcription factor gene, *TSRF1*, using ethylene responsive GCC box as bait in yeast one-hybrid system. Based on the analysis of predicted protein sequence, TSRF1 protein belongs to ERF proteins, and contains an acidic activation domain in C-terminal and a nuclear localization signal in its N-terminal region. Analysis with transient expression and molecular detection indicate that TSRF1 activates the expression of downstream genes, such as *GUS in vivo* and *PR* genes in transgenic *TSRF1* tobacco and tomato. Biochemical analysis revealed that TSRF1 interacts with GCC box *in vitro*, which is very similar to the characterization of tobacco Tsi1 (Park *et al*., 2001) and tomato Pti4 (Gu *et al*., 2002), but their protein sequence similarity is less than 31%, indicating that TSRF1 is a novel ERF protein as a transcriptional activator through the interaction with GCC box. Because of the sequence difference, it also might indicate that the TSRF1 will have distinct regulation in various physiological responses.

Evidences indicate that ERF proteins are involved in biotic and abiotic stress responses, depending on the interaction with different *cis*-acting elements. For instance, *Arabidopsis* CBF1 (Stockinger *et al*., 1997) and DREB1A/2A (Liu *et al*., 1998) interact with a DRE and modulate plant abiotic stress response. Maize ABI4 is suggested to interact with an abscisic acid (ABA) responsive element coupled element 1 and regulate the plant sensitivity to ABA and glucose (Niu *et al*., 2002). More interestingly, a tobacco ERF protein Tsi1, which could bind to the GCC box and DRE, was also found to function in pathogen and salt resistance (Park *et al*., 2001). However, most of the identified ERF proteins are reported to interact with the GCC box or GCC box-like elements, suggesting their close relationship with plant pathogen resistance. In tobacco, four ERF proteins (ERF1-4) have been identified as GCC box binding proteins (Ohme-Takagi and Shinshi, 1995). ERF2 and ERF4 were evidenced to be transcriptional activators (Ohta *et al*.,

2000), while ERF3 functions as a repressor. In *Arabidopsis*, ERF1, AtERF1, AtERF2, and AtERF5 bind the GCC box and act as transcriptional activators. However, AtERF3 and AtERF4 are transcriptional repressors (Solano *et al*., 1998; Fujimoto *et al*., 2000). In tomato, Pti4-6 and LeERF1 interact with the GCC box and regulate the expression of *PR* genes (Zhou *et al*., 1997; He *et al*., 2001; Gu *et al*., 2002; Tournier *et al*., 2003). Furthermore, an ERF protein ORCA3 was reported to specifically bind and activate gene expression via a GCC box like sequence JA- and elicitor-responsive element in the promoters of JA-response genes (van der Fits and Memelink, 2001). Although these ERF proteins have the similar characterizations of binding activity with the GCC box, they display different regulation roles in plants. For example, expressing Pti4 in *Arabidopsis* activated the expression of SA or ethylene/JA mediated *PR* genes and enhanced the resistance of transgenic plants to *Pseudomonas syringae* tomato and *Erysiphe orontii* (Gu *et al*., 2002), while ERF1 integrates ethylene and JA pathways in plant defense (Lorenzo *et al*., 2003). In this paper, we demonstrated that the expression of *TSRF1* was characteristically expressed responsive to ethylene, SA, but not MeJA, suggesting that the *TSRF1* gene is regulated by certain components of the defense signaling pathways, which is further confirmed by the inoculation with bacterium pathogen. Based on the previous facts (Park *et al*., 2001; Gu *et al*, 2002; Wang *et al*., 2004) and the above analysis, we speculate that synergistic activation of different signaling pathways might be converged to the interaction of GCC box and TSRF1 or other ERF proteins. Then TSRF1 exerts effect on the defense responses through regulating relative *PR* genes expression and/or enhancing the plant tolerance to various pathogens. This hypothesis has been further evidenced in this report. The regulation of TSRF1 in plant bacterial pathogen resistance in transgenic tomato and tobacco suggests that TSRF1 might integrate ethylene and SA signaling pathways in resistance to bacterial wilt caused by *R. solanacearum*, which is one of the most devastating soil-borne diseases of plants. This pathogen resistance is significantly distinct from the functional regulation of Tsi1 (Park *et al*., 2001), Pti5 (He *et al*., 2001) and Pti4 (Gu *et al*., 2000, 2002; Chakravarthy *et al*., 2003), although our novel ERF protein TSRF1 also functions in the regulating pathogen resistance of *Pseudomonas syringae* pv *tomato* (unpublished data). Therefore, the regulation of TSRF1 provides new evidence for understanding the mechanism of plant resistance to *R. solanacearum* strain BJ1057.

As we discussed in the introduction, GCC box is an ethylene responsive element (Ohme-Takagi and Shinshi, 1995), and ethylene signal is perceived by a family of receptors and conducted by CTR1, EIN2 and EIN3 in tandem to activate ERF proteins, such as ERF1 (reviewed in Wang *et al*., 2002), thereby regulating the expression of genes involved in the response to ethylene (Chakravarthy *et al*., 2003). TSRF1 belongs to the ERF proteins showing distinct regulation in resistance to *R. solanacearum* through activation of the expression of downstream *PR* genes that are responsive to ethylene and SA; this indicates that our novel identified TSRF1, similar to ERF1 (Solano *et al*., 1998) might be positioned downstream of the ethylene signaling pathway. For the limited studies on *R. solanacearum*, it still needs further investigations to reveal the underlying mechanism for the ERF protein TSRF1 regulating plant resistance to the bacterial wilt. The enhanced resistance to *R. solanacearum* by expressing TSRF1 may be caused by global expression of *PR* and related genes. It also may be derived from the other transcription factors or the interactions of TSRF1 with other unknown plant pathogen resistance members. Anyway, this study has extended the functions of ERF proteins and may provide new clues to understanding the mechanism of plant *R. solanacearum* resistance.

Acknowledgements

We thank Dr Daowen Wang for his helpful discussion about our results, and Dr Daoxin Xie and Dr Qiang Liu for their help for the construction of GCC box bait and tomato cDNA library. This work was supported by Special Foundation of Transgenic Plants in China (JY03A23-02), the Major State Basic Research program of China (G1999011704 and 2003CB114307), the National Science Foundation of China (to R.F.H) and the National Grand Scientific Engineering Program of China.

References

Berrocal-Lobo, M., Molina, A. and Solano, R. 2002. Constitutive expression of ETHYLENE- RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J. 29: 23–32.

Buttner, M. and Singh, K.B. 1997. *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. Proc. Natl. Acad. Sci. USA 94: 5961–5966.

Chakravarthy, S., Tuori, R.P., D'Ascenzo, M.D., Fobert, P.R., Despres, C. and Martin, G.B. 2003. The tomato transcription factor Pti4 regulates defense-related gene expression via GCC box and non-GCC box *cis* elements. Plant Cell 15: 3033–3050.

Danhash, N., Wagemakers, C.A.M., van Kan, J.A.L. and de Wit, P.J.G.M. 1993. Molecular characterization of four *chitinase* cDNAs obtained from *Cladosporium fulvum*-infected tomato. Plant Mol. Biol. 22: 1017–1029.

Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S. and Marco, Y. 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proc. Natl. Acad. Sci. USA 100: 8024–8029.

Deslandes, L., Olivier, J., Theulieres, F., Hirsch, J., Feng, D.X., Bittner-Eddy, P., Beynon, J. and Marco, Y. 2002. Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive *RRS1-R* gene, a member of a novel family of resistance genes. Proc. Natl. Acad. Sci. USA 99: 2404–2409.

Dong, X. 1998. SA, JA, ethylene and disease resistance in plants. Curr. Opin. Plant Biol. 1: 316–323.

Eyal, Y., Meller, Y., Lev-Yadum, S. and Fluhr, R. 1993. A basic-type *PR-1* promoter directs ethylene responsiveness, vascular and abscission zone-specific expression. Plant J. 4: 225–234.

Eyal, Y., Sagee, O. and Fluhr, R. 1992. Dark-induced accumulation of a basic pathogenesis-related (PR-1) transcript and a light requirement for its induction by ethylene. Plant Mol. Biol. 19: 589–599.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H. and Ohme-Takagi, M. 2000. Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell 12: 393–404.

Fukuda, Y., Ohme, M. and Shinshi, H. 1991. Gene structure and expression of a tobacco endochitinase gene in suspension-cultured tobacco cells. Plant Mol. Biol. 16: 1–10.

Gu, Y.-Q., Wildermuth, M.C., Chakravarthy, S., Loh, Y.T., Yang, C., He, X., Han, Y. and Martin, G.B. 2002. Tomato transcription factors pti4, pti5, and pti6 activate defense responses when expressed in *Arabidopsis*. Plant Cell 14: 817–831.

Gu, Y.-Q., Yang, C., Thara, V.K., Zhou, J. and Martin, G.B. 2000. Pti4 is induced by ethylene and salicylic acid, and its product is phosphorylated by the Pto kinase. Plant Cell 12: 771–785.

Guo, H. and Ecker, J.R. 2004. The ethylene signaling pathway: new insights. Curr. Opin. Plant Biol. 7: 40–49.

Gutterson, N. and Reuber, T.L. 2004. Regulation of disease resistance pathways by AP2/ERF transcription factors. Curr. Opin. Plant Biol. 7: 465–471.

He, L.Y., Sequeira, L. and Kelman, A. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Dis. 12: 1357–1361.

He, P., Warren, R.F., Zhao, T., Shan, L., Zhu, L., Tang, X. and Zhou, J.M. 2001. Overexpression of Pti5 in tomato potentiates pathogen-induced defense gene expression and enhances disease resistance to *Pseudomonas syringae* pv tomato. Mol. Plant Microbe. Interact. 14: 1453–1457.

Hoekema, A., Hirsch, P., Hooykaas, P.J.J. and Schilperoort, R. 1983. A binary plant vector strategy based on separation of virand T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179–180.

Hua, J. and Meyerowitz, E.M. 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell 94: 261–271.

Jia, Y. and Martin, G.B. 1999. Rapid transcript accumulation of pathogenesis-related genes during an incompatible interaction in bacterial speck disease-resistant tomato plants. Plant Mol. Biol. 40: 455–465.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A. and Ecker, J.R. 1993. CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the raf family of protein kinases. Cell 72: 427–441.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10: 1391–1406.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J. and Solano, R. 2003. ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell 15: 165–178.

Martin, G.B., Brommonschenkel, S., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D. and Tanksley, S.D. 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262: 1432–1436.

Menke, F.L., Champion, A., Kijne, J.W. and Memelink, J. 1999. A novel jasmonate- and elicitor-responsive element in the periwinkle secondary metabolite biosynthetic gene Str interacts with a jasmonate- and elicitor-inducible AP2-domain transcription factor, ORCA2. EMBO J. 18: 4455–4463.

Niu, X., Helentjaris, T. and Bate, N.J. 2002. Maize ABI4 binds coupling element1 in abscisic acid and sugar response genes. Plant Cell 14: 2565–2575.

Ohme-Takagi, M. and Shinshi, H. 1995. Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell 7: 173–182.

Ohta, M., Ohme-Takagi, M. and Shinshi, H. 2000. Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions. Plant J. 22: 29–38.

Park, J.M., Park, C.J., Lee, S.B., Ham, B.K., Shin, R. and Paek, K.H. 2001. Overexpression of the tobacco *Tsi1* gene encoding an EREBP/AP2–type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. Plant Cell 13: 1035–1046.

Pastuglia, M., Roby, D., Dumas, C. and Cock, J.M. 1997. Rapid induction by wounding and bacterial infection of an S gene family receptor-like kinase gene in *Brassica oleracea*. Plant Cell 9: 49–60.

Salanoubat, M., Genin, S., Artiguenave, F., Gouzy, J., Mangenot, S., Arlat, M., Billault, A., Brottier, P., Camus, J.C., Cattolico, L., Chandler, M., Choisne, N., Claudel-Renard, C., Cunnac, S., Demange, N., Gaspin, C., Lavie, M., Moisan, A., Robert, C., Saurin, W., Schiex, T., Siguier, P., Thebault, P., Whalen, M., Wincker, P., Levy, M., Weissenbach, J. and Boucher, C.A. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 414: 497–502.

Sessa, G., Meller, Y. and Fluhr, R. 1995. A GCC element and a G-box motif participate in ethylene-induced expression of the *PRB-1b* gene. Plant Mol. Biol. 28: 145–153.

Shinshi, H., Usami, S. and Ohme-Takagi, M. 1995. Identification of an ethylene-responsive region in the promoter of a tobacco class I *chitinase* gene. Plant Mol. Biol. 27: 923–932.

Solano, R., Stepanova, A., Chao, Q. and Ecker, J.R. 1998. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. 12: 3703–3714.

Sperisen, C., Ryals, J. and Meins, F. 1991. Comparison of cloned genes provides evidence for intergenomic exchange of DNA in the evolution of a tobacco glucan endo-1,3-beta-glucosidase gene family. Proc. Natl. Acad. Sci. U.S.A. 88: 1820–1824.

Stockinger, E.J., Gilmour, A.J. and Thomashow, M.F. 1997. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. USA 94: 1035–1040.

Thilmony, R.L., Chen, Z., Bressan, R.A. and Martin, G.B. 1995. Expression of the tomato *Pto* gene in tobacco enhances resistance to *Pseudomonas syringae* pv *tabaci* expressing *avrPto*. Plant Cell 7: 1529–1536.

Tournier, B., Sanchez-Ballesta, M.T., Jones, B., Pesquet, E., Regad, F., Latche, A., Pech, J.C. and Bouzayen, M. 2003. New members of the tomato ERF family show specific expression pattern and diverse DNA-binding capacity to the GCC box element. FEBS Lett. 550: 149–154.

van der Fits, L. and Memelink, J. 2000. ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science 289: 295–297.

van der Fits, L. and Memelink, J. 2001. The jasmonate-inducible AP2/ERF-domain transcription factor ORCA3 activates gene expression via interaction with a jasmonate-responsive promoter element. Plant J. 25: 43–53.

van Kan, J.A., Cozijnsen, T., Danhash, N. and de Wit, P.J. 1995. Induction of tomato stress protein mRNAs by ethephon, 2,6-dichloroisonicotinic acid and salicylate. Plant Mol. Biol. 27: 1205–1213.

van Kan, J.A., Joosten, M.H., Wagemakers, C.A., van den Berg-Velthuis, G.C. and de Wit, P.J. 1992. Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. Plant Mol. Biol. 20: 513–527.

Wang, H., Huang, Z., Chen, Q., Zhang, Z., Zhang, H., Wu, Y., Huang, D. and Huang, R. 2004. Ectopic overexpression of tomato *JERF3* in tobacco activates downstream gene expression and enhances salt tolerance. Plant Mol. Biol. (in press)

Wang, K.L., Li, H. and Ecker, J.R. 2002. Ethylene biosynthesis and signaling networks. Plant Cell 14 (Suppl): S131–S151.

Xiong, L. and Zhu, J.K. 2003. Regulation of abscisic acid biosynthesis. Plant Physiol. 133: 29–36.

Zhou, J., Tang, X. and Martin, G.B. 1997. The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. EMBO J. 16: 3207–3218.