The Plant Cell, Vol. 18, 2005–2020, August 2006, www.plantcell.org © 2006 American Society of Plant Biologists

Tobacco Tsip1, a DnaJ-Type Zn Finger Protein, Is Recruited to and Potentiates Tsi1-Mediated Transcriptional Activation [W]

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Tobacco stress-induced1 (Tsi1) is an ethylene-responsive-element binding protein/APETALA2–type transcription factor that plays an important role in both biotic and abiotic stress signaling pathways. We show that Tsi1-interacting protein1 (Tsip1), a DnaJ-type Zn finger protein, interacts with Tsi1 in vitro and in yeast (*Saccharomyces cerevisiae*). The transcript level of *Tsip1* in tobacco (*Nicotiana tabacum*) increased upon treatment with salicylic acid (SA), ethylene, gibberellic acid, NaCl, and virus challenge. Tsip1 appeared to be physically associated with the chloroplast surface but dissociated from it after SA treatment. Tsip1 colocalized and coimmunoprecipitated with Tsi1 in plant cells following SA treatment. Tsip1 expression increased Tsi1-mediated transcription and was able to functionally compensate for loss of the Tsi1 transcriptional activation domain through a direct interaction with Tsi1. Transgenic plants simultaneously coexpressing *Tsi1* and *Tsip1* displayed stronger pathogen resistance and salt tolerance than did transgenic plants expressing either *Tsi1* or *Tsip1* alone. Concurrent with this, the expression of a subset of stress-related genes was induced in a cooperative manner in *Tsi1*/*Tsip1* transgenic plants. These results together implied that Tsi1 recruits Tsip1 to the promoters of stress-related genes to potentiate Tsi1-mediated transcriptional activation.

INTRODUCTION

Plant abiotic and biotic stresses cause major losses in crop productivity. There are multiple stress response signaling pathways in plants (Knight and Knight, 2001), and there is significant crosstalk among pathways activated in response to different stresses (Durrant et al., 2000; Chen et al., 2002). Ultimately, stress response signals are transduced into the nucleus, leading to changes in gene expression. To date, a number of genes have been identified using cDNA microarray analysis that are coordinately regulated by different defense/stress signals (Seki et al., 2001). Stress response–related transcriptional activation leads to de novo synthesis of a variety of defense-related proteins and protective secondary metabolites.

A common phenomenon in the plant stress/defense response is the targeting of certain transcription factors by different signaling pathways. For instance, cAMP response element binding protein, a mammalian transcription factor, is phosphorylated and activated by protein kinases that are regulated by multiple pathways (Hill and Treisman, 1995). The transcriptional activation complex Jun/Fos modulates the transcriptional activity of members of the steroid receptor family (Chinenov and Kerppola, 2001), And SUPPRESSOR OF OVEREXPRESSION OF CO1, a MADS box transcription factor, integrates vernalization and gibberellin signals during flowering in *Arabidopsis thaliana* (Moon et al., 2003).

There are several lines of evidence that protein–protein interactions, which play an important role in signal transduction (Pawson and Scott, 1997), also play an important role in the plant stress/defense response. Using the yeast two-hybrid screen, three proteins involved in the elicitor-induced defense response in tomato (*Solanum lycopersicum*), Pto-interacting protein4 (Pti4), Pti5, and Pti6, have been identified, each of which contains an ethylene-responsive-element binding protein/APETALA2 (EREBP/AP2) domain (Zhou et al., 1997). TGA2, a member of the TGA family of transcription factors, is activated by NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) and is SA responsive (Fan and Dong, 2002). *Arabidopsis* NPR1 is also a cofactor for the reduced form of TGA1, a basic domain/leucine zipper transcription factor (Després et al., 2003). Finally, signaling by *Arabidopsis* cryptochrome1 (CRY1) involves a direct interaction with constitutive photomorphogenic protein1 (COP1). Together, CRY1 and COP1 are negative regulators of photomorphogenesis (Yang et al., 2001).

Tobacco stress-induced1 (*Tsi1*), which encodes an EREBP/AP2-type transcription factor, was isolated from tobacco (*Nicotiana tabacum*) and is regulated by salt, ethephon, and salicylic acid (SA). Overexpression of *Tsi1* in transgenic tobacco plants increased their resistance to pathogen invasion and osmotic stress (Park et al., 2001). These effects correlated with the ability of Tsi1 to bind to both GCC and drought-responsive element/C-repeat (DRE/CRT) motifs in promoters, demonstrating that biotic and abiotic stress pathways can activate a single EREBP/

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[W] Online version contains Web-only data.
Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.106.043158.

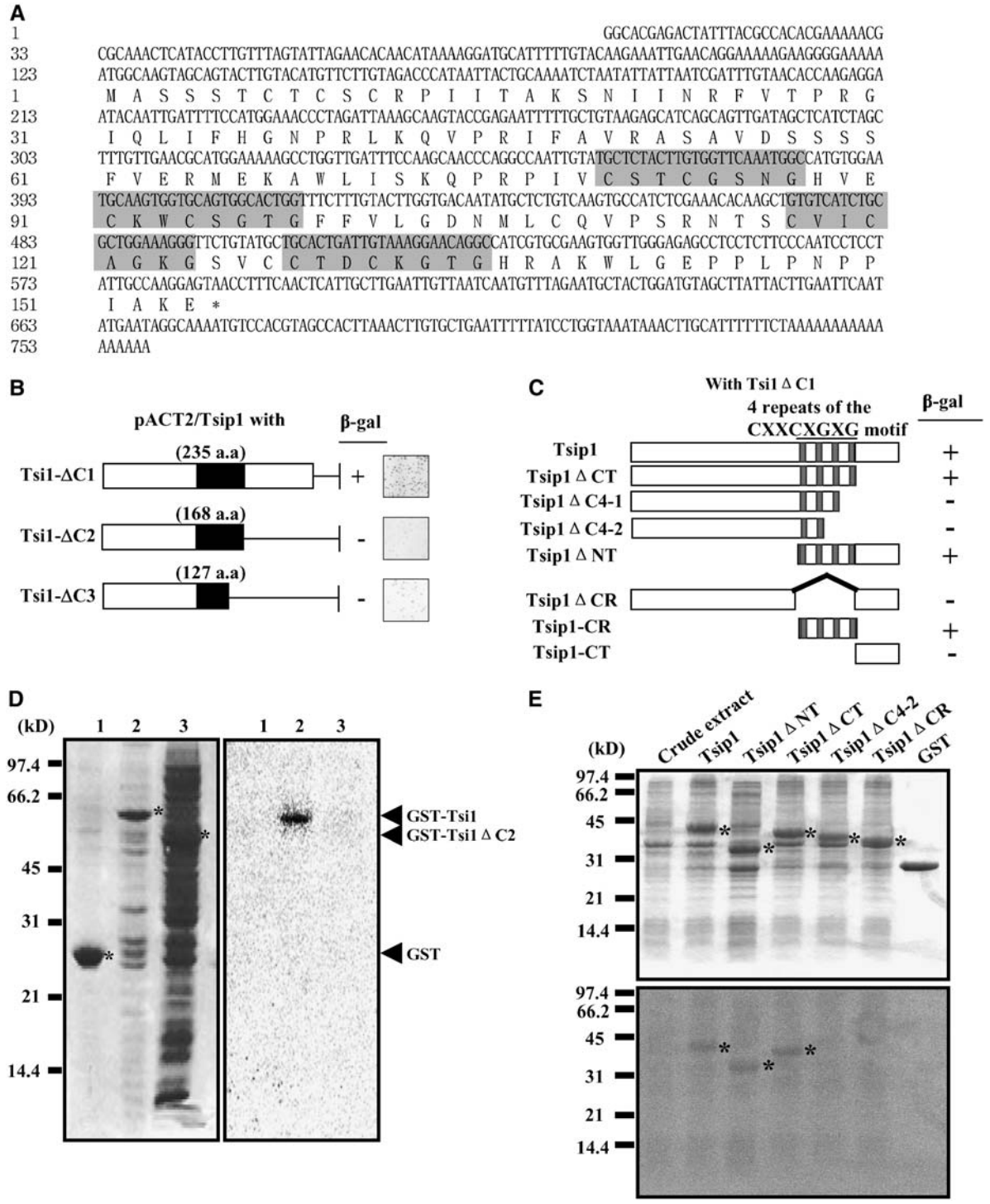

Figure 1. Interaction of Tsip1 and Tsi1.

(A) Nucleotide and predicted amino acid sequences of the tobacco *Tsip1* cDNA clone. Gray boxes indicate the four Cys-rich (CXXCXGXG) repeats.
(B) The C-terminal region of Tsi1 is important for interaction with Tsip1. Schematic representation of the Tsi1 deletion constructs used in the assay. The black box indicates the Tsi1 DNA binding domain. A colony-lift filter assay was performed to monitor protein–protein interactions between Tsip1 and the indicated Tsi1 deletion mutants (small squares to the right of the protein schematics). aa, amino acids.
(C) The CXXCXGXG region of Tsip1 is necessary and sufficient for specific interaction with Tsi1. Schematic representation of the various Tsip1 deletion mutants tested. The shaded boxes represent the CXXCXGXG motifs. β-Gal activity was determined using the filter lift assay. +, interaction; –, no interaction.

AP2-type transcription factor (Park et al., 2001). Moreover, ectopic expression of *Tsi1* in transgenic hot pepper (*Capsicum annuum*) plants increased their resistance to various pathogens, including viruses, bacteria, and oomycetes (Shin et al., 2002). The mechanism(s) by which Tsi1-mediated signaling leads to enhanced protection against plant stresses is not known.

To further investigate the molecular components involved in *Tsi1*-mediated signal transduction, we isolated Tsi1-interacting proteins using the yeast two-hybrid system. Molecular and cellular analysis showed that Tsi1 and Tsi1-interacting protein1 (Tsip1) interacted with each other and cooperatively enhanced transcriptional activation by Tsi1. Moreover, overexpression of Tsi1 and Tsip1 acted in a cooperative manner to confer increased resistance to salt and pathogen stresses in transgenic tobacco plants. These results reveal a putative mechanism by which Tsi1 and Tsip1 coordinately regulate biotic and abiotic stress–related gene expression in plants.

RESULTS

Isolation of Tsip1, a DnaJ-Type Zn Finger Protein

It was previously reported that the C-terminal region of Tsi1 plays an important role in transcriptional activation (Park et al., 2001). We deleted the 15 C-terminal amino acids of Tsi1 to abolish its transcriptional activation activity and used this C-terminal deletion mutant (Tsi1ΔC1) to screen a tobacco cDNA library for Tsi1-interacting proteins using the yeast two-hybrid assay. Of 10^6 transformants screened, 49 colonies were obtained on plates lacking Leu, Trp, and His, but only two also exhibited β-galactosidase (β-gal) activity. Sequence analysis revealed that the ~800-bp cDNA inserts in these two clones were identical, indicating that a single cDNA had been isolated. The putative gene was termed *Tsip1*.

As shown in Figure 1A, the cDNA of *Tsip1* was 758 bp in length and contained an open reading frame encoding 154 amino acids. The deduced amino acid sequence of Tsip1 showed high similarity to that of an *Arabidopsis* protein of unknown function (GenBank accession number AC006585; At2g24860) but did not share significant sequence identity with any other known proteins. Sequence analysis identified a region of the protein that contained four repeated motifs similar to the Cys-rich Zn finger motif (CXXCXGXG) found in some members of the DnaJ class of molecular chaperones (Kelly, 1998).

Tsip1 Interacts Directly with Tsi1 in Yeast and in Vitro

To identify the domains of Tsi1 and Tsip1 responsible for mediating their interaction with each other, we constructed a set of Tsi1 and Tsip1 deletion mutants. An expression plasmid encoding the yeast (*Saccharomyces cerevisiae*) GAL4 activation domain fused to *Tsip1* was cotransformed with expression plasmids encoding the GAL4 DNA binding domain fused to various deletion mutants of *Tsi1*. Coexpression of Tsip1 with Tsi1ΔC1, but not with any of the other deletion mutants tested, resulted in transcriptional activation (Figure 1B), indicating that the C-terminal 15 amino acids of Tsi1 were not required for binding to Tsip1. Further deletion of the C terminus of Tsi1 (Tsi1-ΔC2 and Tsi1-ΔC3) resulted in loss of transcriptional activation (Figure 1B), indicating that these deleted portions in the C terminus of Tsi1 played an important role in the interaction with Tsip1.

Tsip1ΔCT, a mutant of Tsip1 that lacked the C-terminal region but contained an intact set of CXXCXGXG motifs, and Tsip1-CR, a deletion mutant that contained only the four CXXCXGXG motifs, interacted with Tsi1 (Figure 1C). Deletion of one to four of the CXXCXGXG motifs in Tsip1 (Tsip1ΔC4-1, Tsip1ΔC4-2, Tsip1ΔCR, and Tsip1-CT) disrupted the interaction with Tsi1 (Figure 1C). These results indicated that an intact set of four CXXCXGXG motifs in Tsip1 was both necessary and sufficient for its ability to interact with Tsi1.

To test whether the observed interactions between the various deletion mutants of Tsi1 and Tsip1 in yeast could be recapitulated in vitro, we performed a far western gel overlay assay. In vitro–translated full-length Tsip1 bound to glutathione *S*-transferase (GST)-Tsi1, whereas there was no significant interaction with GST alone or with GST-Tsi1-ΔC2 (Figure 1D). Furthermore, in vitro–translated full-length Tsi1 interacted with GST-Tsip1, GST-Tsip1ΔNT, and GST-Tsip1ΔCT but not to GST alone or the deletion mutants Tsip1ΔC4-2 and Tisp1ΔCR (Figure 1E). Thus, the results of the in vitro far western gel analysis were consistent with those obtained in the yeast two-hybrid screen and indicate that the C-terminal region of Tsi1 directly interacts with Tsip1 and that the interaction is mediated by the CXXCXGXG motifs of Tsip1.

Expression of *Tsip1* in Response to Various Stresses

It was previously reported that *Tsi1* in tobacco plants increased strongly upon salt treatment (Park et al., 2001). We were interested in whether *Tsip1* expression was regulated in a similar manner. We found that *Tsip1* expression was induced in tobacco plants within 1 h of NaCl treatment (Figure 2A). By contrast, abscisic acid (ABA) treatment did not appear to effect the expression of *Tsip1* (Figure 2A). We investigated the effect of several other stress stimuli on *Tsip1* expression and found that application of the plant hormones SA, ethephon, and gibberellic

Figure 1. (continued).

(D) Interaction of Tsi1 with Tsip1 in vitro. Left panel: GST, GST-Tsi1, and GST- Tsi1ΔC2 were expressed in *Escherichia coli* BL21 (DE) cells. Proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. Asterisks indicate overexpressed GST, GST-Tsi1, or GST- Tsi1ΔC2. Right panel: far western gel overlay assay using in vitro–translated ^{35}S-Met–labeled Tsip1 as a probe. Lane 1, purified GST; lane 2, the insoluble fraction of GST-Tsi1–expressing cells; lane 3, the insoluble fraction of GST-Tsi1ΔC2–expressing cells.

(E) The CXXCXGXG motifs of Tsip1 are required for the binding of Tsip1 to Tsi1 in vitro. Top panel: 10 μg of the insoluble fractions of cells expressing GST-Tsip1 and the indicated GST-Tsip1 deletion mutants were separated by SDS-PAGE and stained with Coomassie blue. Asterisks indicate overexpressed GST-Tsip1 and the GST-Tsip1 deletion mutants. A far western gel overlay assay was performed using in vitro–translated ^{35}S-Met–labeled Tsi1 (bottom panel) as a probe.

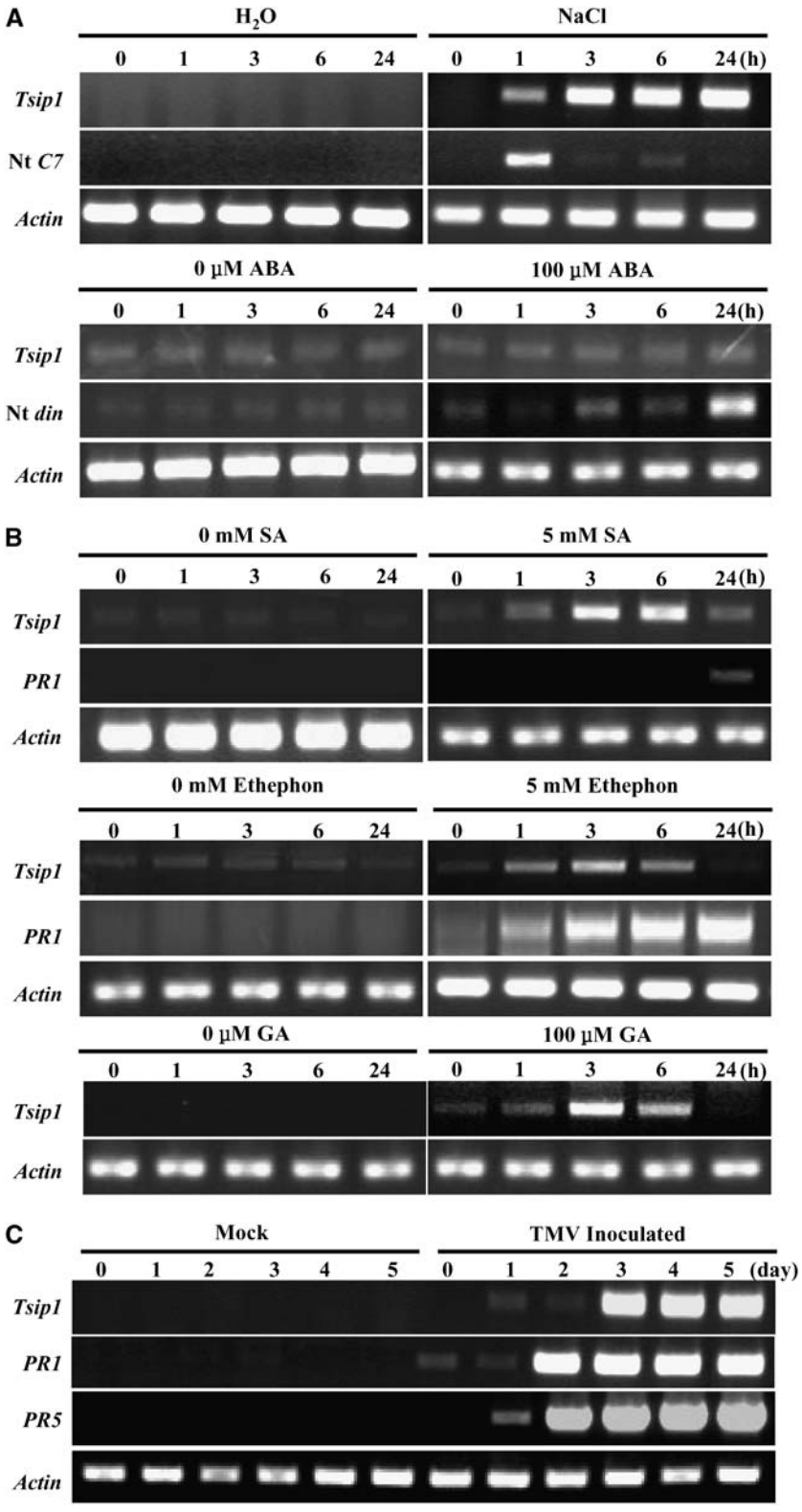

Figure 2. *Tsip1* Gene Expression Patterns Determined by RT-PCR Analysis.

(A) Induction of *Tsip1* expression in tobacco plants by treatment with NaCl or ABA. To confirm the efficacy of treatment, primers specific for Nt *din*, which is induced in response to ABA, and Nt *C7*, which is induced in response to NaCl (Tamura et al., 2003), were also used.
(B) Time course of *Tsip1* expression following treatment with SA, ethephon, or GA. *PR1* was used as a positive control for SA and ethephon treatment.
(C) RT-PCR analysis of *Tsip1*, *PR1*, *PR5*, and *Actin* in TMV- and mock-inoculated tobacco leaves.

acid (GA) to tobacco plants strongly increased the expression of *Tsip1* within 3 h of treatment initiation (Figure 2B).

We also examined the expression of *Tsip1* in *N. tabacum* cv Samsun NN, which are resistant to *Tobacco mosaic virus* (TMV), following exposure to TMV-P_0 (Figure 2C). Mock-inoculated plants served as a control for an irrelevant wounding response due to virus inoculation. Expression of *Tsip1* in mock-inoculated leaves was almost undetectable under the conditions of the assay. By contrast, after inoculation with TMV-P_0, *Tsip1* transcripts began to accumulate 1 d postinoculation, and the level of transcription had increased strongly by 3 d postinoculation.

Tsip1 Is Associated with Chloroplasts

To determine the distribution pattern of Tsip1, green fluorescent protein (GFP) fused to Tsip1 (Tsip1-GFP) was expressed in *Arabidopsis* protoplasts. We found that Tsip1-GFP localized to discreet, punctuate structures in the chloroplasts, whereas in the control protoplasts (smGFP), GFP was distributed uniformly throughout the cytoplasm. Figure 3A also shows the location of Tsi1-GFP, which was found predominantly in the protoplast nucleus.

To investigate whether the Tsip1 protein was associated with membranes or not, crude lysates were prepared from protoplasts transfected with plasmids encoding Tsip1 fused to the hemagglutinin epitope (Tsip1-2XHA) and then fractionated into membrane and soluble fractions by ultracentrifugation. Tsip1-2XHA was found mainly in the soluble fraction, not in the membranous fraction of *Arabidopsis* mesophyll protoplasts (Figure 3B), indicating that Tsip1 is not a chloroplast integral membrane protein.

To further characterize the association of Tsip1 with chloroplasts, *Arabidopsis* protoplasts were transfected with *Tsip1-2XHA* or an N-terminal fusion of the ribulose-1,5-bisphosphate carboxylase small subunit (RbcS) to GFP (*rbcN-GFP*) as a marker for the chloroplast stroma (Bauer et al., 2000; Lee et al., 2002). Intact chloroplasts were subjected to a protease protection assay, and isolated proteins were assayed for the presence of Tsip1-2XHA and rbcN-GFP by protein gel blot analysis using anti-HA and anti-GFP antibodies, respectively (Figure 3C). Tsip1-2XHA was undetectable when chloroplasts were incubated with thermolysin, in the absence or presence of Triton X-100, indicating that Tsip1-2XHA was located on the outside surface of the chloroplast. By contrast, rbcN-GFP was digested by thermolysin only when detergent was added, consistent with the fact that this protein is located inside the chloroplast, and thus protected from protease digestion.

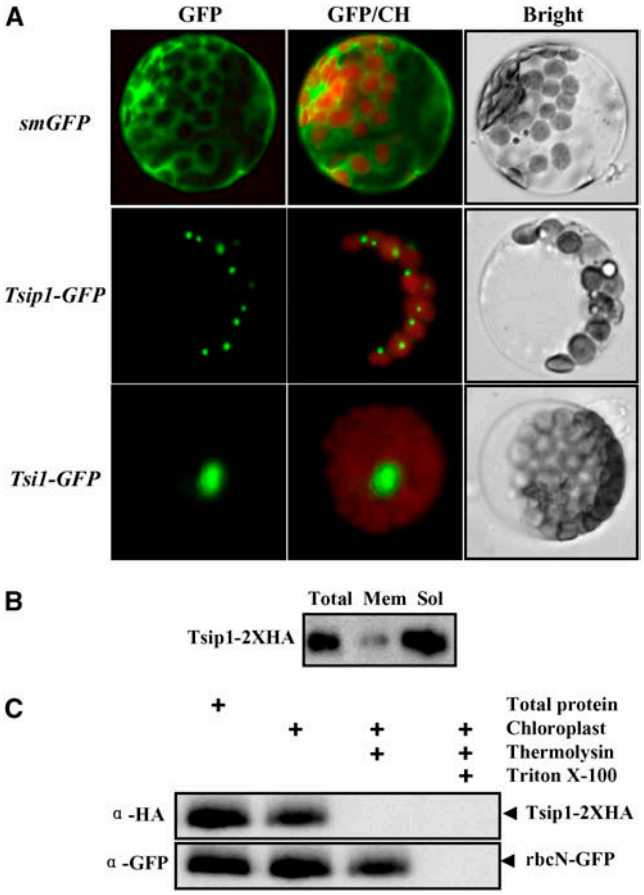

Figure 3. Subcellular Localization of *Tsip1*.

(A) Tsip1-GFP expression in *Arabidopsis* protoplasts. Bright, bright-field image; GFP/CH, overlay of GFP (green) and chlorophyll (red) images; SmGFP, soluble modified GFP; Tsip1-GFP or Tsi1-GFP, Tsip1 or Tsi1 fused in frame to the 5′-end of GFP.

(B) Protein gel blot analysis of Tsip1-2XHA. Total protein was isolated from transfected protoplasts and partitioned into membranous (Mem) and soluble (Sol) fractions. Thirty micrograms of each fraction was separated by SDS-PAGE, and protein gel blot analysis was performed using a polyclonal anti-HA antibody.

(C) Tsip1 localizes to the surface of chloroplasts. Intact chloroplasts were isolated from protoplasts cotransfected with *Tsip1-2XHA* or *rbcN-GFP*. The protease thermolysin was added to the isolated chloroplasts in the presence or absence of Triton X-100, which functioned to dissolve the chloroplast membrane. Fifty micrograms of protein were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were then probed with antisera against HA and GFP. The arrowheads indicate the positions of Tsip1-2XHA and rbcN-GFP.

Tsip1 Translocates into the Cytoplasm after SA Treatment

The observed localization of Tsip1 on the outside surface of chloroplasts was contradictory to the observed direct interaction between Tsip1 and Tsi1 in plant cells. Recently, there have been reports that the localization of proteins in plant cells changes in response to certain signaling molecules (Hwang and Sheen, 2001). To determine whether Tsip1 localization was affected by stress-related stimuli, protoplasts were transfected with *Tsip1-GFP* and then treated with 1 mM SA (Figure 4A). Tsip1-GFP colocalized exclusively with chloroplasts prior to SA treatment, but at 3 h after SA treatment, we began to observe the accumulation of Tsip1-GFP in the protoplast cytoplasm (Figure 4A).

To confirm that Tsip1 relocalized upon SA treatment, *Arabidopsis* protoplasts were transfected with plasmids encoding Tsip1 and two Tsip1 deletion mutants (*Tsip1-2XHA*, *Tsip1ΔNT-2XHA*, and *Tsip1ΔC4-2-2XHA*) and were then either untreated or treated for 12 h with SA. Protoplasts were then fractionated by

ultracentrifugation into chloroplasts and cytosol, and proteins were examined by protein gel blot analysis using anti-HA antibodies (Figure 4B). When protoplasts expressing Tsip1-2XHA were treated with SA, Tsip1-2XHA was detected mainly in the cytosolic fraction (Figure 4B), indicating that Tsip1 was redirected from the outside surface of the chloroplast to the cytoplasm. Tsip1ΔNT was detected exclusively in the cytosolic fraction of chloroplasts, independently of SA treatment (Figure 4B), suggesting that the N terminus of Tsip1 contains an important signal for the localization to the outer chloroplast surface. By contrast, Tsip1ΔC4-2-2XHA behaved similarly to full-length Tsip1, indicating that the C-terminal region of Tsip1 does not play a role in the association of Tsip1 with the outer chloroplast surface or in the redistribution of Tsip1 into the cytoplasm following SA treatment (Figure 4B).

Colocalization of Tsip1 and Tsi1 in the Nucleus of *Arabidopsis* Protoplasts

Because Tsip1 relocated from the outer chloroplast surface to the cytoplasm after SA treatment (Figure 4B), we were interested in whether Tsip1 associated with Tsi1 following SA treatment.

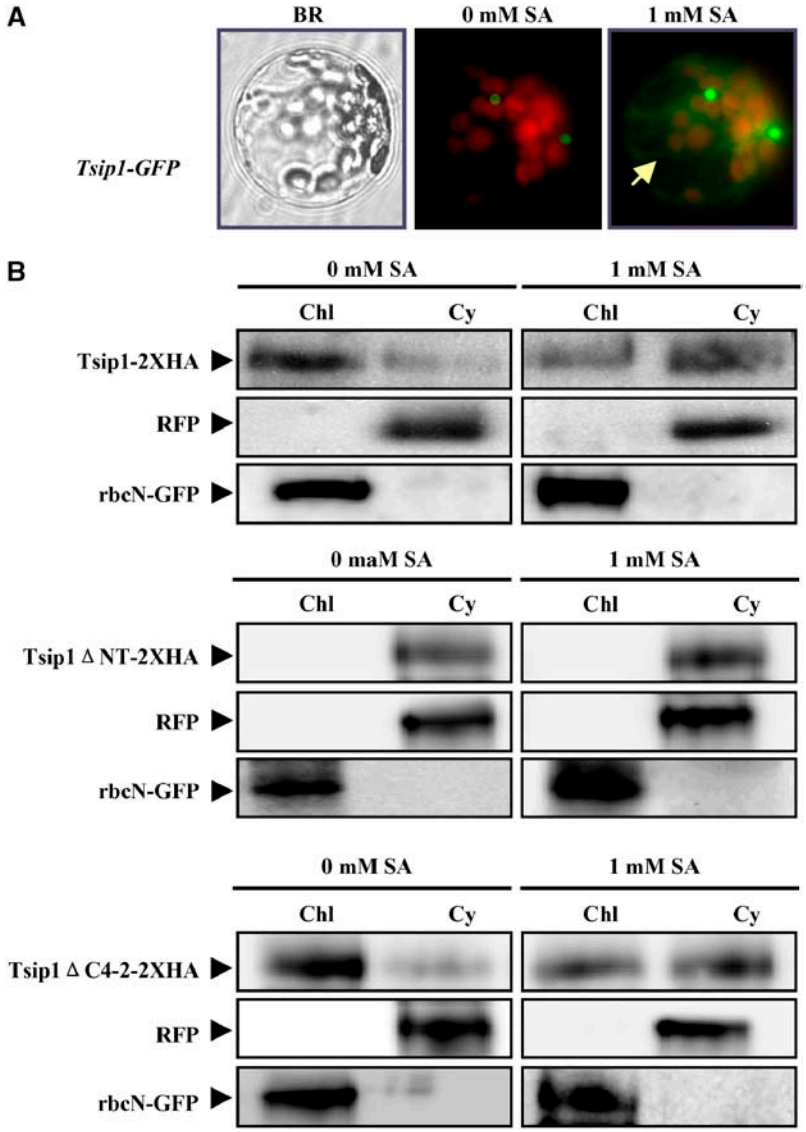

Figure 4. Effect of SA on the Localization of Tsip1.

(A) Protoplasts transformed with *Tsip1-GFP* were incubated with 1 mM SA at room temperature and examined by fluorescence microscopy 1 to 3 h later. The arrow indicates the diffusion of Tsip1-GFP from chloroplasts to the cytoplasm.

(B) Tsip1 relocalizes upon SA treatment. Cytosolic (Cy) and chloroplast (Chl) fractions of protoplasts were prepared by ultracentrifugation. RFP and rbcN-GFP were used as cytosolic and chloroplast markers, respectively. Fifty micrograms of protein were separated by SDS-PAGE, and protein gel blot analysis was performed using anti-HA, anti-RFP, or anti-GFP antibodies.

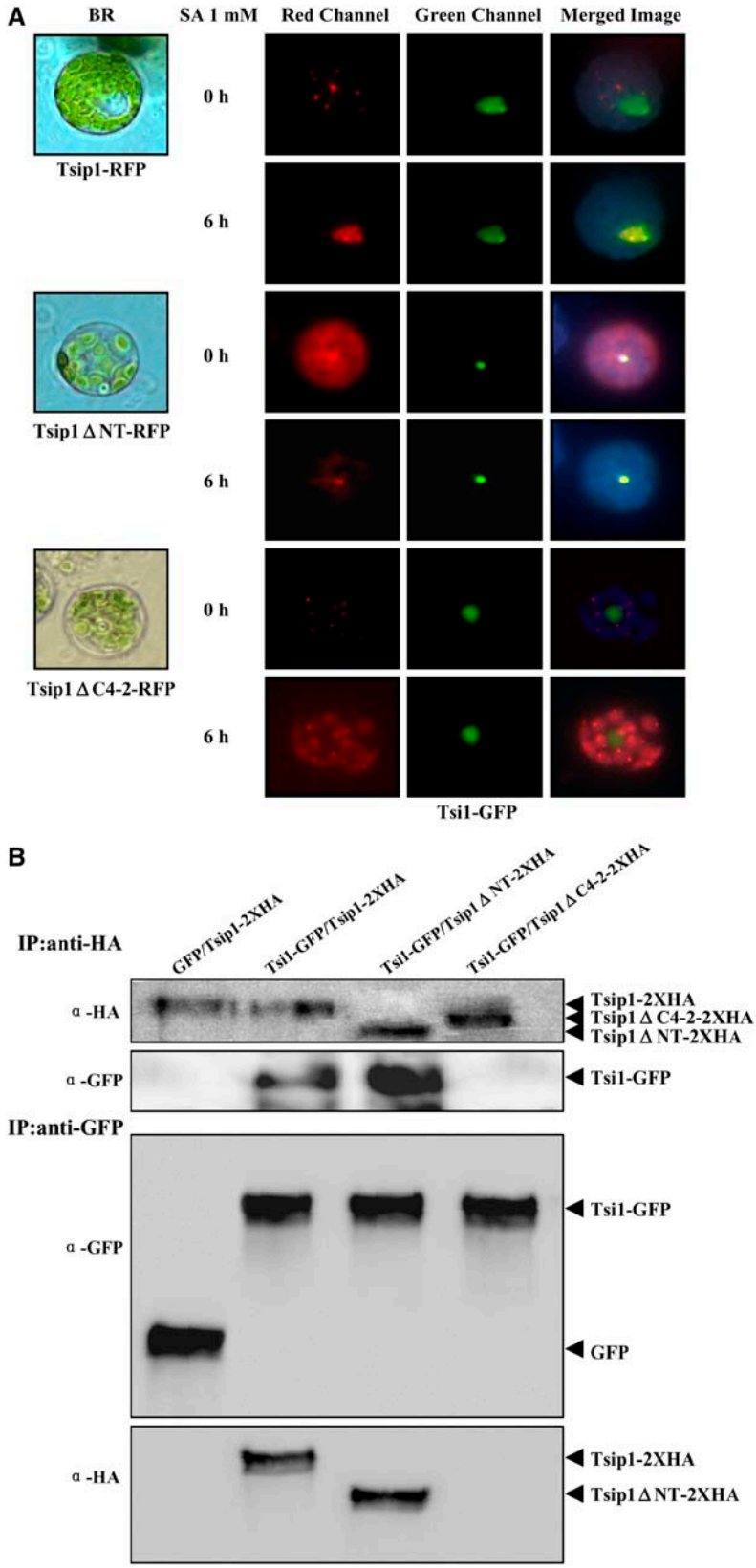

Figure 5. Tsi1 Colocalizes with Tsip1 and Tsip1ΔNT but Not with Tsip1ΔC4-2 in the Nuclei of *Arabidopsis* Protoplasts.

Arabidopsis protoplasts were transfected with plasmids encoding fusion proteins of Tsip1 and various Tsip1 deletion mutants fused to the red fluorescent protein (RFP), Tsip1-RFP, Tsip1ΔC4-2-RFP, and Tsip1ΔNT-RFP, along with *Tsi1-GFP* and then treated with SA. Fluorescence was monitored before and 6 h after SA treatment (Figure 5A). Prior to SA treatment, Tsip1-RFP was located in the chloroplasts, and Tsi1-GFP was in the nucleus of the protoplasts. However, the two proteins clearly colocalized in the nucleus following SA treatment (Figure 5A). Note that coexpression of Tsi1-GFP did not affect the association of Tsip1-RFP with the chloroplasts in the absence of SA treatment. Tsip1ΔNT-RFP did not associate with the chloroplasts but was found throughout the nucleus and the cytoplasm prior to SA treatment (Figure 5A), perhaps due to the presence of Tsi1-GFP. Tsip1ΔNT was able to bind to Tsi1 (Figure 1); thus, it is possible that Tsi1-GFP bound to Tsip1ΔNT-RFP even in the absence of SA and translocated Tsip1ΔNT-RFP into the nucleus. Following SA treatment, the residual Tsip1ΔNT-RFP that was observed in the cytoplasm disappeared, as shown in the merged image (Figure 5A). This may be due to the contribution of newly synthesized Tsi1 following SA stimulation. Tsip1ΔC4-2-RFP localized to the chloroplasts before SA treatment (Figure 5A). However, Tsip1ΔC4-2-RFP failed to translocate to the nucleus after SA treatment and appeared to remain in the cytoplasm and/or around the chloroplasts (Figure 5A). We speculate that this was due to the inability of Tsi1 to bind to Tsip1ΔC4-2 (Figure 1). It thus appeared that the redistribution of Tsip1 from the cytoplasm to the nucleus required the presence of Tsi1 in the cytoplasm and binding of Tsip1 to Tsi1, whereas the association of Tsip1 with the chloroplast outer surface and its relocalization into the cytoplasm are independent of Tsi1.

To determine whether Tsip1 interacted with Tsi1 in planta, *Tsip1-2XHA* and *Tsi1-GFP* constructs were cotransfected into *Arabidopsis* protoplasts (Figure 5B). After SA treatment, plant cell extracts were immunoprecipitated with anti-HA antiserum or anti-GFP antiserum, and the immunoprecipitates were immunoblotted with anti-GFP or anti-HA antibodies. Tsi1-GFP was present in anti-HA immunoprecipitates prepared from protoplasts expressing Tsip1-2XHA and Tsip1ΔNT-2XHA but not Tsip1ΔC4-2-2XHA (Figure 5B). In the reciprocal analysis, Tsip1-2XHA and Tsip1ΔNT-2XHA, but not Tsip1ΔC4-2-2XHA, were present in anti-GFP immunoprecipitates prepared from protoplasts containing Tsi1-GFP (Figure 5B). The immunoprecipitation results were consistent with those in yeast and in the in vitro binding experiments, in that the C-terminal region with CXXCXGXG motifs, not the N-terminal region, of Tsip1 was important for its association with Tsi1. Together with the localization data (Figure 5A), these results implied that Tsi1 interacts with Tsip1 in the cytoplasm and that Tsip1/Tsi1 protein complexes accumulate in the nucleus of plant cells in response to stress signals.

Tsip1 Enhances Tsi1-Mediated Transcriptional Activation

Because Tsip1 colocalized with Tsi1 following SA treatment, we were interested in whether ectopic expression of Tsip1 in plants affected the transcriptional activation activity of Tsi1. To examine this, we analyzed GAL1UAS-containing promoter-driven expression and activation of the *lacZ* reporter gene in yeast. GAL1UAS-driven *lacZ* activity was undetectable in the presence of the GAL4 DNA binding domain (GAL4DB) (Figure 6A), and expression of Tsip1 alone failed to increase *lacZ* reporter activity above the basal level (Tsip1, Figure 6A), indicating that Tsip1 did not recognize or bind to the GAL1UAS *cis*-regulatory region. Expression of GAL4DB fused to Tsi1 (DBTsi1) resulted in activation of *lacZ* expression (Figure 6A), consistent with Tsi1 functioning as a transcription factor with a transcription activation domain. Deletion of the C-terminal 15 amino acids of Tsi1 (DBTsi1ΔC1) resulted in the loss of *lacZ* reporter activity, as expected based on previous data showing that this region of Tsi1 played a critical role in transcriptional activation (Figure 6A). When we coexpressed Tsip1 and DBTsi1ΔC1 in yeast cells, a substantial level of *lacZ* activity was restored (Figure 6A), indicating that Tsip1 contained a transcription activation domain(s). Furthermore, coexpression of DBTsi1 and Tsip1 led to a higher level of *lacZ* activity than did expression of DBTsi1 alone ($P < 0.01$, $n = 20$) (Figure 6A), indicating that Tsip1 cooperates with Tsi1 to enhance Tsi1-mediated transcriptional activation, most likely through a direct interaction with Tsi1.

We previously showed that Tsi1 binds specifically to GCC and DRE/CRT sequences found in the promoter regions of certain genes, and that in vitro, binding of Tsi1 to the GCC box is stronger than that of Tsi1 binding to the DRE/CRT box in vitro (Park et al., 2001). We were interested in whether Tsip1 enhanced Tsi1-mediated transcriptional activation by interacting with Tsi1 that was directly bound to either GCC- or DRE/CRT-containing promoter sequences (Figure 6B). When we examined transcriptional activation driven by tandemly repeated GCC-containing promoter, we detected a basal level of β-gal activity in yeast transformants expressing Tsi1ΔC1 alone, while expression of Tsi1ΔC1 and Tsip1 (Tsi1ΔC1/Tsip1) resulted in a significant increase in β-gal activity (Figure 6B). Similar to the previous results, coexpression of Tsip1 and Tsi1 enhanced β-gal activity more than expression of Tsi1 alone ($P < 0.02$, $n = 20$). We observed similar trends in transcriptional activation driven by tandemly repeated DRE/CRT-containing promoter sequence (Figure 6B).

To determine whether Tsip1 enhanced Tsi1-mediated transcription in plant cells, we performed transient expression experiments in protoplasts isolated from the leaves of *Arabidopsis* plants that were either untransformed or transformed with *Tsip1-2XHA* using the constructs depicted in Figures 7A and 7B. In

Figure 5. (continued).

(A) Subcellular localization of Tsip1-RFP, Tsip1ΔNT-RFP, or Tsip1ΔC4-2-RFP in protoplasts in the presence of cotransfected *Tsi1-GFP*. Overlay of green and red images results in orange/yellow signals in regions of coalignment of the fluorescent signals.

(B) In vivo interaction of Tsip1 with Tsi1. *Arabidopsis* protoplasts were cotransfected with *GFP* and *Tsip1-2XHA*, *Tsi1-GFP* and *Tsip1-2XHA*, *Tsi1-GFP* and *Tsip1ΔNT-2XHA*, or *Tsi1-GFP* and *Tsip1ΔC4-2-2XHA*. After SA treatment for 6 h, immunoprecipitation was performed using anti-HA or anti-GFP antibodies from 1 mg of protoplast extract, followed by immunoblot analysis with the anti-HA (top) or anti-GFP (bottom) antibody.

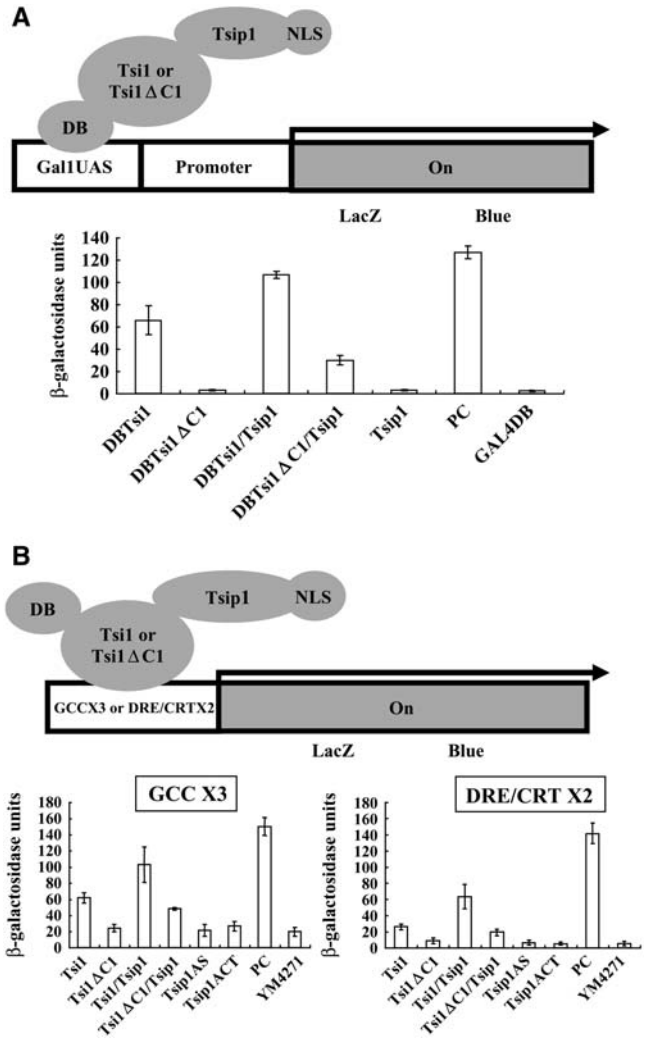

Figure 6. Interaction of Tsip1 with Tsi1 Enhances the Transcriptional Activity of Tsi1 in Yeast.

(A) Schematic representation of transcriptional activation mediated by GAL4 binding to the Gal1 upstream activation sequence Gal1UAS. Twenty independent colonies transfected with each of the indicated constructs were tested for β-gal activity. Data represent the means and SE of assays on all 20 colonies.

(B) Yeast transformants carrying *lacZ* reporter genes under the control of promoters containing a triple repeat of the GCC box or a double repeat of the DRE/CRT box were examined for β-gal activity in the presence of Tsip1 and Tsi1 or Tsi1ΔC1.

A liquid culture assay using *o*-nitrophenyl-β-D-galactopyranoside as a substrate was performed to evaluate transcriptional activity in each of the transformants. The data represent the means and SE of 20 independent colonies for each construct: Tsip1AS, *Tsip1* cloned into pAS2-1; Tsip1ACT, *Tsip1* cloned into pACT2 (Clontech); PC, positive control. PC represents the binding of p53 to three tandem repeats of the consensus p53 binding site. Transformants expressing GalDB or AD-Tsip1 did not display β-gal activity.

addition to plasmids encoding Tsi1 or Tsi1ΔC1, protoplasts were cotransfected with a plasmid encoding the β-glucuronidase (GUS) reporter gene fused to the triple GCC sequence or a double DRE/CRT box. GUS activity in protoplasts increased following SA treatment (Figures 7A and 7B). In addition, expression of either Tsi1 or Tsi1ΔC1 led to higher GUS activity in *Tsip1* protoplasts than in untransformed protoplasts when either of the promoter constructs was tested (GCC or DRE/CRT box; $P < 0.004$, $n = 5$).

The functional analysis of the interaction of Tsi1 and Tsip1 in both yeast and plant cells was strong evidence that Tsip1 interacted directly with Tsi1 and that the two proteins functioned as a transcriptional coactivator for Tsi1-mediated transcriptional activation of the target genes whose promoters contain GCC and/or DRE/CRT box sequences

Tsip1 and Tsi1 Functionally Cooperate to Regulate a Subset of Stress-Related Genes

To understand the biological function of Tsip1 in planta, several different types of transgenic tobacco plants were generated: plants expressing *Tsip1*, *Tsip1ΔC4-2*, *Tsip1*-RNA interference (RNAi), *Tsi1*-RNAi, and both *Tsip1* and *Tsi1* (*Tsip1* × *Tsi1*). *Tsip1* × *Tsi1* transgenic plants were created by crossing *Tsip1* lines 2, 3, and 9 with *Tsi1* line 4. We previously showed that in *Tsi1* line 4 plants, there was an induction of several *PR* genes and an enhanced resistance to pathogens (Park et al., 2001). The expression of *Tsip1*, *Tsi1*, and a variety of stress-related genes was monitored in several independent lines of the various transgenic plants by RT-PCR. Expression levels of the various genes in each of the different transgenic lines was directly compared with control plants, which were transformed with the pMBP2 empty vector (Han et al., 1999) (Figure 8A). *PR1*, *PR2*, *PR5*, *SAR8.2*, *LIPID TRANSFER PROTEIN* (*LTP*), and *HIN1* mRNA levels were higher in *Tsip1* transgenic plants compared with control plants (Figure 8A). By contrast, none of the stress response genes examined were induced in *Tsip1ΔC4-2* transgenic plants (Figure 8A). There were no differences in the expression levels of *Tsi1*, *PR3*, *PR4*, *HSR203J*, and Nt *C7* (Kim et al., 2003; Tamura et al., 2003) in *Tsip1* transgenic plants compared with control plants (Figure 8A), indicating that Tsip1 regulated a subset of stress-related genes. We also observed that the target genes of Tsi1 represented a subset of Tsip1 target genes, suggesting that Tsip1 may have a broader specificity range than Tsi1.

In transgenic plants coexpressing *Tsip1* and *Tsi1*, the same set of *PR* genes that were induced in *Tsi1* or *Tsip1* transgenic plants was also strongly expressed (Figure 8A). In addition, expression of a subset of these genes, *PR4*, *SAR8.2*, and *LTP*, appeared to be higher in *Tsip1* × *Tsi1* transgenic plants compared with either of the single expressors (Figure 8A), suggesting that there was functional cooperation between Tsi1 and Tsip1 in the transcriptional activation of certain common targets. To confirm this result, the levels of mRNA of *PR4*, *SAR8.2*, and *LTP* in *Tsi1*, *Tsip1*, and *Tsip1* × *Tsi1* transgenic plants were examined using quantitative real-time PCR (Figures 8B to 8D). The results confirmed that the levels of *PR4*, *SAR8.2*, and *LTP* mRNA transcripts were higher in *Tsip1* × *Tsi1* plants compared with either *Tsi1* ($P < 0.04$, $n = 6$) or *Tsip1* plants ($P < 0.005$, $n = 6$). Induction of expression of

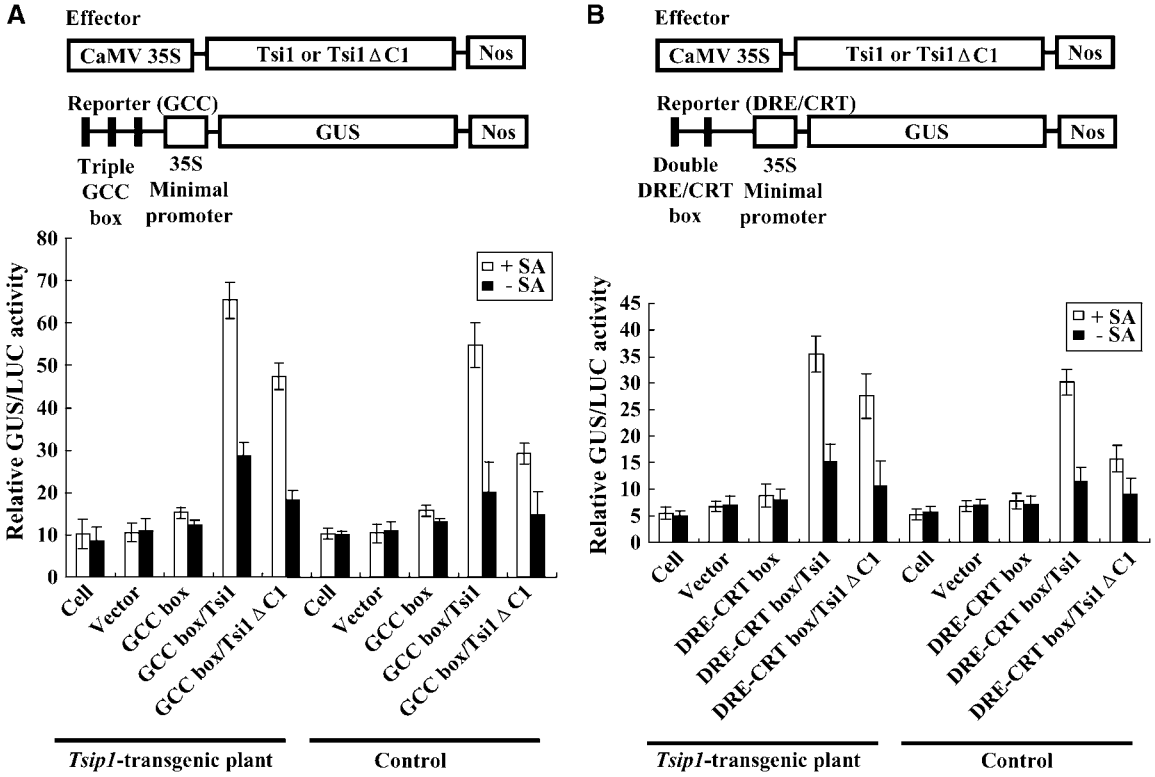

Figure 7. Tsip1 Enhances the Transcriptional Activity of Tsi1 in Planta.

(A) Transactivation of the triple GCC-box-*GUS* reporter gene by Tsi1 in *Arabidopsis* protoplasts in the presence or absence of Tsip1.
(B) Transactivation of the double DRE/CRT box-*GUS* reporter gene by Tsi1 in *Arabidopsis* protoplasts in the presence or absence of Tsip1.
The effector and reporter constructs used in the cotransfection experiments are represented schematically in the top panels. Nos, the polyadenylation signal of the nopaline synthase gene. Effector, reporter, and 35S-LUC constructs were transfected into *Arabidopsis* protoplasts prepared from wild-type or *Tsip1* transgenic plants. Experiments were performed in the presence (white squares) or the absence (black squares) of 1 mM SA. Ten micrograms of protein was used for the estimation of GUS and LUC activities. Data represent the means and SE from five independent experiments. CaMV, *Cauliflower mosaic virus.*

Tsi1 and Tsip1 target genes was abolished in *Tsi1*-RNAi and *Tsip1*-RNAi transgenic plants, even in the presence of SA (Figure 8A). Thus, depletion of endogenous Tsi1 and Tsip1 abolished the expression of all stress-related genes examined, indicating that both Tsi1 and Tsip1 are necessary for the activation of their target genes.

Increased Resistance to Salt and Pathogen Stresses in Tsip1- and Tsi1-Overexpressing Plants

Overexpression of Tsi1 in transgenic tobacco plants led to an increased tolerance to salt stress and pathogen infection (Park et al., 2001), prompting us to examine whether *Tsip1* and *Tsip1* × *Tsi1* transgenic tobacco plants also had increased resistance to salt and pathogen stresses. Leaf discs of transgenic tobacco plants (*Tsip1* × *Tsi1*, *Tsip1*, *Tsi1*, *Tsip1*Δ*C4-2*, *Tsip1*-RNAi, *Tsi1*-RNAi, and pMBP2 vector control) were exposed to a solution of NaCl and then their chlorophyll content was measured (Figure 9A). The leaf discs of Tsip1- or Tsi1-overexpressing plants following exposure to NaCl contained a higher amount of chlorophyll than those of control plants (TC1 and TC2), indicating a moderate level of tolerance to salt stress. Based on chlorophyll content, *Tsip1* × *Tsi1* transgenic plants exhibited a greater level of salt tolerance than either *Tsi1* ($P < 0.005$, $n = 20$) or *Tsip1* ($P < 0.004$, $n = 20$) transgenic plants (Figure 9A). By contrast, the level of salt tolerance of *Tsip1*Δ*C4-2* transgenic plants was similar to that of control plants. In *Tsip1*-RNAi or *Tsi1*-RNAi transgenic plants, salt susceptibility appeared to be higher compared with that of pMBP2-transformed or wild-type plants ($P < 0.05$, $n = 20$). When we tested the susceptibility of *Tsip1*-RNAi plants to a range of concentrations of NaCl, Tsip1-RNAi plants exhibited a slight but consistently higher sensitivity to salt stress than control plants (see Supplemental Figure 1A online; $P < 0.003$, $n = 4$). The phenotypes for salt stress tolerance correlated well with the observed cooperative function between Tsip1 and Tsi1 in previous experiments.

To determine whether overexpression of *Tsip1* and *Tsi1* also conferred enhanced resistance to pathogens, transgenic plants were inoculated with *Pseudomonas syringae* pv *tabaci*, a virulent bacterial pathogen (Figure 9B), and growth inhibition of the bacterial pathogen on inoculated leaves of *Tsip1* × *Tsi1*, *Tsip1*, and *Tsi1* plants was observed and compared with that of control,

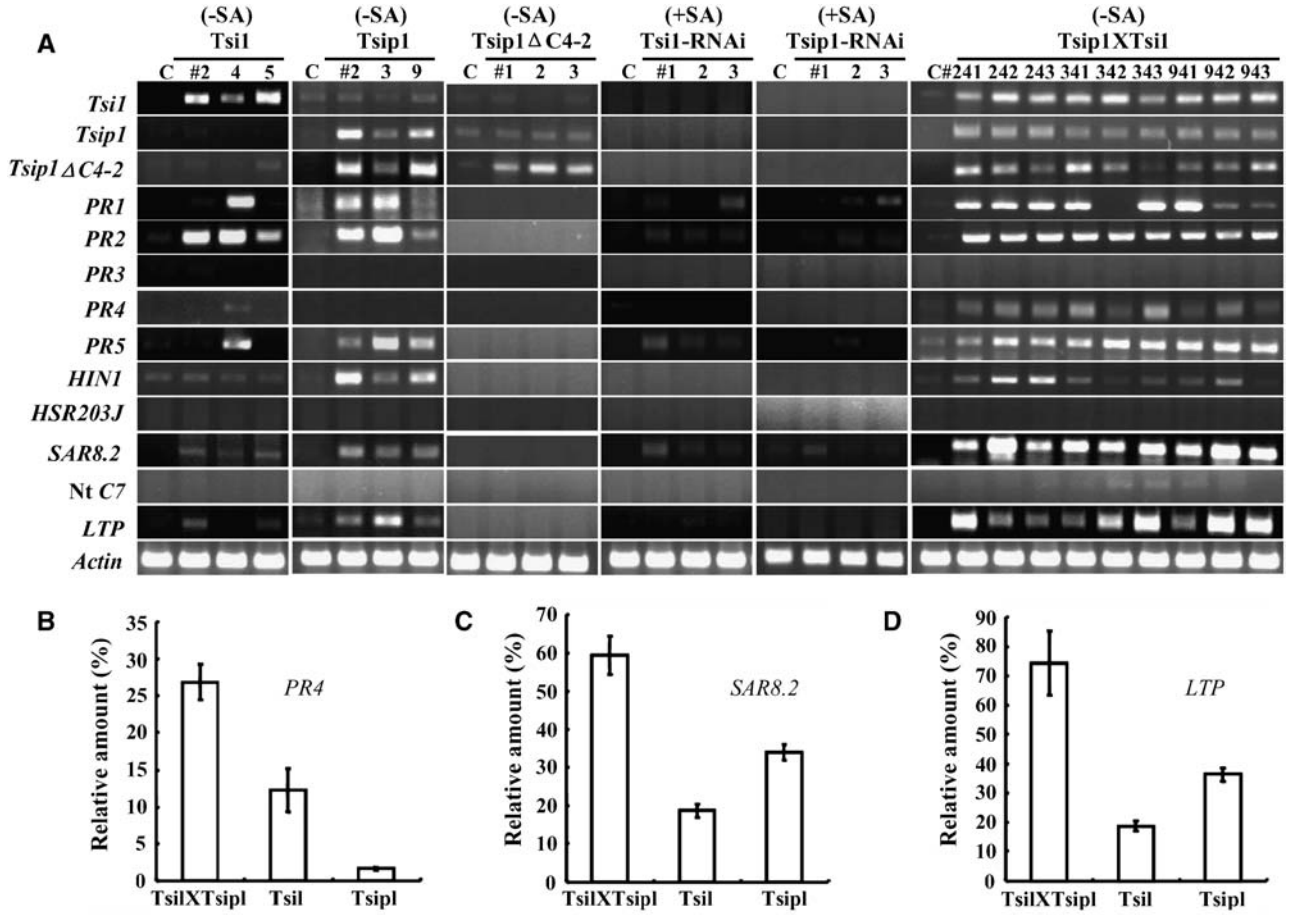

Figure 8. *Tsip1* Overexpression Increases the Expression of *PR* Genes, and Downregulation of *Tsip1* Abolishes the Induction of *PR* Genes by SA Treatment.

(A) Expression of pathogenesis-related genes in *Tsip1* × *Tsi1*, *Tsip1*, *Tsi1*, *Tsip1*Δ*C4-2*, *Tsi1*-RNAi, and *Tsip1*-RNAi transgenic tobacco plants. Primer sets specific for the 3′ untranslated regions of *Tsip1* or *Tsi1* were used for detecting endogenously expressed *Tsip1* or *Tsi1* in *Tsip1*-RNAi or *Tsi1*-RNAi transgenic plants. *PR* gene expression was monitored in SA-treated *Tsi1*-RNAi and *Tsip1*-RNAi transgenic plants. The numbers indicate independent lines of transgenic T1 or F1 plants. The ''C'' indicates a pMBP2 vector-tranformed T1 plant.

(B) to **(D)** Real-time quantitative RT-PCR analysis of *PR4* **(B)**, *SAR8.2* **(C)**, and *LTP* **(D)** expression in *Tsip1* × *Tsi1*, *Tsi1*, and *Tsip1* transgenic tobacco plants. Data were normalized to the level of *Actin* transcripts (set as 100%) and represent the means ± SE of six independent experiments.

Tsi1-RNAi, *Tsip1*-RNAi, and *Tsip1*Δ*C4-2* plants. Seven days after inoculation, there was a fourfold to fivefold inhibition of bacterial growth on *Tsip1* × *Tsi1* plants compared with that on pMBP2-transformed or wild-type plants ($P < 0.01$, $n = 35$), a twofold inhibition compared with *Tsi1* plants ($P < 0.05$, $n = 35$), and a twofold to threefold inhibition compared with *Tsip1* plants ($P < 0.03$, $n = 35$). SA-treated *Tsi1*-RNAi and *Tsip1*-RNAi plants exhibited an increased susceptibility to bacterial pathogen growth compared with control plants ($P < 0.05$, $n = 35$) (Figure 9B), consistent with the loss of SA-induced expression of stress-related genes in *Tsip1*-RNAi or *Tsi1*-RNAi plants (Figure 8A). When we performed a time course of susceptibility of *Tsip1*-RNAi plants to *P. s. tabaci*, we detected a subtle increase in susceptibility of *Tsip1*-RNAi plants at 2 ($P < 0.03$, $n = 3$), 3 ($P < 0.04$, $n = 3$), and 4 d ($P < 0.05$, $n = 3$) after bacterial inoculation compared with control plants (see Supplemental Figure 1B online). Although the apparent functional cooperativeness between Tsip1 and Tsi1 was not as apparent as in the response to salt stress, it appeared that Tsip1 and Tsi1 together conferred higher resistance to bacterial growth than either one of them alone.

In summary, we demonstrated functional cooperativeness between Tsip1 and Tsi1, not only in their ability to activate target gene expression, but also in their ability to confer resistance to salt stress and pathogen attack.

DISCUSSION

Here, we demonstrate that Tsip1, a DnaJ-type Zn finger protein, interacts directly with Tsi1. The binding of Tsip1 to Tsi1 was shown in a yeast two-hybrid assay, in an in vitro far western gel overlay assay, and in plant cells by coimmunoprecipitation analysis. Tsip1 has four CXXCXGXG motifs, originally identified

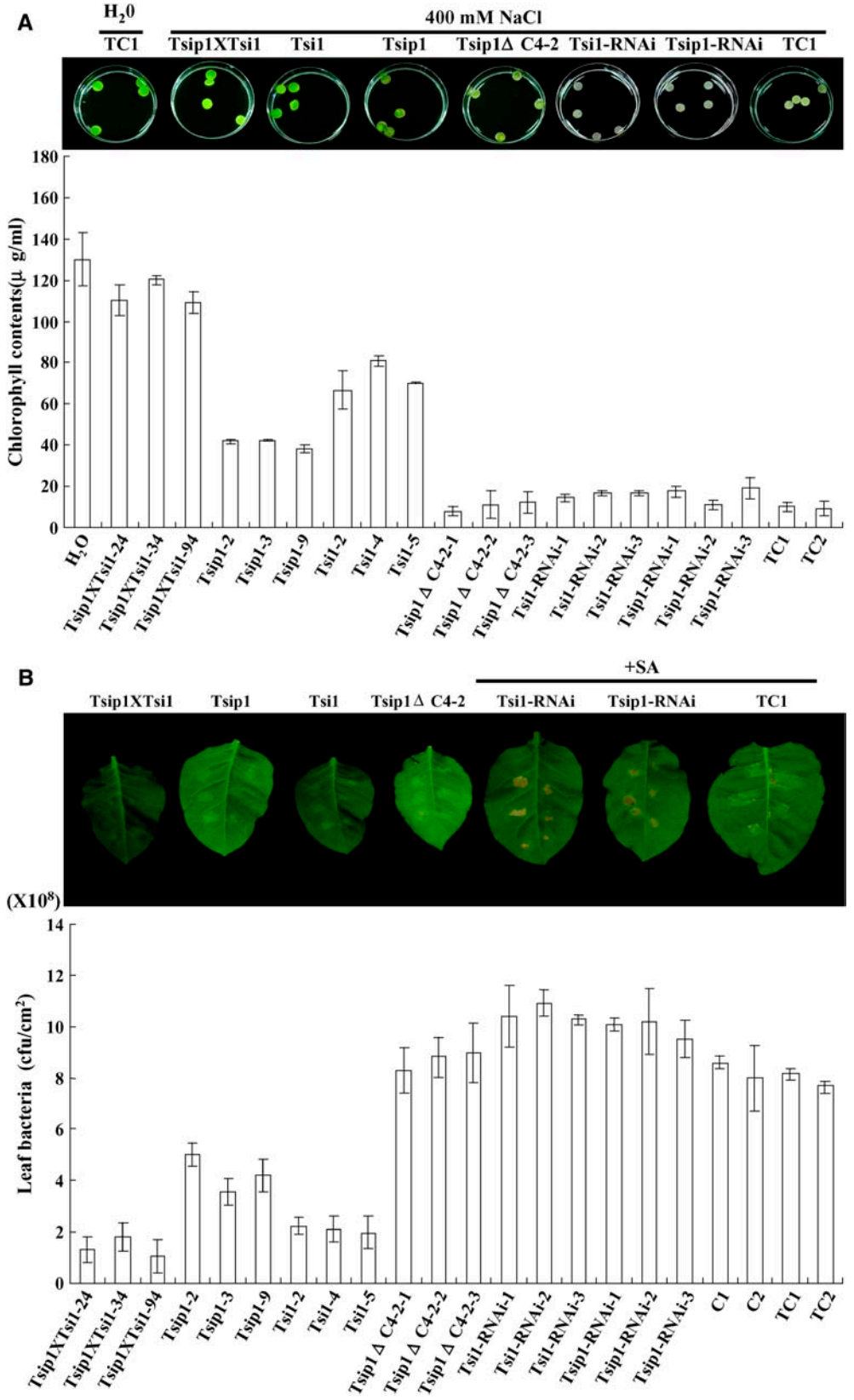

Figure 9. Transgenic Plants Simultaneously Expressing *Tsip1* and *Tsi1* Display Enhanced Salt Tolerance and Resistance to *P. s. tabaci.*

in bacterial DnaJ, that form two Zn finger structures that are believed to be involved in protein–protein interactions (Banecki et al., 1996; Szabo et al., 1996). Consistent with this, these motifs in Tsip1 were both necessary and sufficient for binding to Tsi1. The predicted amino acid sequence of Tsip1 lacks the N-terminal J domain that defines the DnaJ class of molecular chaperones and the large C terminus shared by most DnaJ proteins (Kelly, 1998). Thus, it appears that Tsip1 defines a new class of small Cys-rich proteins in plants.

The C-terminal region of Tsi1, which contains the DNA binding domain, was important for its interaction with Tsip1. The ethylene-responsive transcription factor contains a DNA binding domain, and it has been suggested that this domain forms an amphipathic helix that is sufficient for mediating the interaction of the ethylene-responsive transcription factor with its binding partners (Büttner and Singh, 1997). We found that the interaction between Tsip1 and Tsi1 required not only the DNA binding domain, but additional regions of the C-terminal region of Tsi1 as well. These results imply that the Tsi1 C terminus comprises a functionally important domain involved in the in vivo activity of Tsi1. It also raises the intriguing possibility that Tsip1 binds to Tsi1 differently than other proteins bind to Tsi1.

It has previously been shown that expression of *Tsi1* is induced by treatment with high salt, ethylene, and SA (Park et al., 2001). Compared with the expression of *Tsi1*, that of *Tsip1* was remarkably similar upon exposure of plants to the same stimuli. However, despite this similarity, *Tsi1* mRNA transcripts do not accumulate after treatment with GA, which is an effective elicitor of *Tsip1* expression, indicating that *Tsi1* and *Tsip1* are governed by both common and distinct regulatory mechanisms in the plant cell.

The subcellular localization of Tsip1 was examined, and we found that in protoplasts, Tsip1-GFP was present in a punctuate pattern of distribution and associated with the outer surface of chloroplasts. We were able to identify a putative transit peptide sequence for targeting proteins to the stroma of chloroplasts in the N terminus of Tsip1 using computer programs for predicting subcellular localization signals called PSORT (http://psort.nibb.ac.jp) and ChloroP (http://www.cbs.dtu.dk/services/ChloroP). A deletion of the mutant of Tsip1 that lacked the N-terminal domain failed to associate with chloroplasts, indicating that Tsip1 might be actively targeted to the stroma of the chloroplasts. However, Tsip1, but not RbcS, a stromal marker protein, was degraded following treatment of chloroplasts with thermolysin in the absence of membrane-disrupting detergents, indicating that Tsip1 associates with the outer surface of chloroplasts, rather than residing in the stroma. Transit peptide sequences for targeting proteins to the inside of chloroplasts are not well conserved in terms of sequence and length, and as a result, chloroplast targeting sequences are difficult to predict (Richly and Leister, 2004). The putative transit peptide in the N terminus of Tsip1 might be used to mediate binding to protein components required for import into the stroma of chloroplasts. Further translocation of Tsip1 into the stroma of chloroplasts, however, might be largely blocked due to some unique features in the putative transit peptide sequence of Tsip1. We still cannot exclude the possibility that some portion of Tsip1 might still be able to relocalize in the stroma of chloroplasts. Additional studies will be required to clarify this issue.

Upon treatment of protoplasts with SA, Tsip1 dissociated from the outer surface of the chloroplasts and diffused into the cytoplasm. When Tsi1 was exogenously expressed in the protoplast and the protoplasts were then treated with SA, Tsip1 relocated to the nucleus. A deletion mutant of Tsip1 that was unable to bind to Tsi1, Tsip1ΔC4-2, failed to translocate into the nucleus after SA treatment, although it could diffuse into the cytoplasm from the outer surface of the chloroplasts. Tsi1 was shown to bind to Tsip1 in an in vitro far western assay, in the yeast two-hybrid assay, and in plant cells. Since Tsip1 does not contain a putative nuclear localization sequence, we hypothesize that cytoplasmic Tsi1 binds to Tsip1, and the protein complex is translocated into the nucleus. Tsip1 failed to translocate to the nucleus in the absence of SA treatment, even when Tsi1 was overexpressed, indicating that Tsip1 is constitutively located on the outer surface of the chloroplasts.

Protein relocalization in response to various cell signals has been reported for several proteins. The *Arabidopsis* His-containing phosphotransfer protein shuttles from the cytoplasm to the nucleus in response to cytokinin treatment (Hwang and Sheen, 2001). The nuclear-cytoplasmic shuttling of REPRESSION OF SHOOT GROWTH is inhibited by leptomycin B treatment (Igarashi et al., 2001). NPR1 is reduced from an oligomer to a monomer and translocates from the cytoplasm to the nucleus after treatment with 2,6-dichloroisonicotinic acid (Mou et al., 2003). Our results demonstrate subcellular localization changes in a chloroplast-associated protein in response to SA.

Figure 9. (continued).

(A) Analysis of the salt-induced senescence of *Tsip1* × *Tsi1*, *Tsip1*, *Tsip1ΔC4-2*, *Tsi1*-RNAi, and *Tsip1*-RNAi transgenic plants. Leaf discs from transgenic plants carrying the *Tsip1* gene in the sense orientation and pMBP2 vector-transformed transgenic plants (TC1 and TC2) were floated in 400 mM NaCl solution for 4 d under continuous white light at 25°C, and chlorophyll content (mg/g fresh weight) was measured. As a control, leaf discs of pMBP2 vector-transformed transgenic plants were floated in water. The photograph shows typical leaf discs (10-mm diameter) under each condition. The first bar of the graph represents the control leaf discs without NaCl treatment, and all others are values measured for leaf discs treated with 400 mM NaCl. Data represent the means and SE of five independent experiments of four leaf discs each.

(B) Analysis of resistance to the bacterial pathogen *P. s. tabaci* in wild-type (C1 and C2), pMBP2 vector-transformed transgenic plants (TC1 and TC2), and *Tsip* × *Tsi1*, *Tsip1*, *Tsip1ΔC4-2*, *Tsi1*-RNAi, and *Tsip1*-RNAi transgenic plants. Disease symptoms caused by *P. s. tabaci* are shown in the photographs taken 7 d after bacterial inoculation. Fully expanded leaves of 8-week-old tobacco plants were inoculated with 10^7 colony-forming units/mL of *P. s. tabaci*. Seven days later, discs (10-mm diameter) were cut from infected leaves and the bacterial titer determined. C1 and C2 or TC1 and TC2 indicate wild-type tobacco or pMBP2 vector-transformed transgenic plants, respectively. All transgenic plants used were independent tobacco T1 or F1 lines. Data represent the means and SE of at least seven experiments using five plants per line and five discs from each plant.

The results of our analysis of transcriptional activation by Tsi1 in yeast indicated that Tsip1 functioned as an enhancer of Tsi1-mediated transcriptional activation of GCC- or DRE/CRT-containing promoters through a direct interaction with Tsi1. In addition, Tsip1 compensated for the loss of the C-terminal 15 amino acids of Tsi1, which are essential for its transcriptional activation activity. A similar functional role of Tsip1 was shown in a second, independent system, using transient expression analysis in *Arabidopsis* leaf protoplasts. Taken together, the results indicate that one of the functions of Tsip1 is in the Tsi1-mediated transcriptional activation of target genes.

The GCC box is a DNA sequence element present in the upstream regulatory sequences of many ethylene-responsive genes. It is also found in the promoters of several *PR* genes, such as *PR1*, β-*1,3-glucanase* (*PR2*), *osmotin* (*PR5*), and *SAR8.2* (Ohme-Takagi and Shinshi, 1995; Zhou et al., 1997; Goodman and Song, 2002). Expression of the gene encoding lipid transfer protein (*LTP*) increases after exposure to salinity, ABA, and biotic stresses (Trevino and O'Connell, 1998; Park et al., 2002). Expression of all of these stress-related genes was induced in *Tsip1*-overexpressing transgenic plants in the absence of any stimulus, similar to that seen in *Tsi1*-overexpressing transgenic plants. In addition, knockdown expression of *Tsi1* and *Tsip1* by the process of RNAi abolished the induction of most of these *PR* genes following SA treatment. Interestingly, the set of Tsip1 target genes appeared to be broader than that of Tsi1, suggesting that Tsip1 may be involved in transcriptional activation by other defense-related transcription factors.

Transgenic tobacco plants expressing both *Tsip1* and *Tsi1*, or *Tsi1* or *Tsip1* alone, exhibited increased tolerance in high salt conditions and were resistant to bacterial infection. Moreover, the tolerance of *Tsip1* × *Tsi1* transgenic plants was stronger than *Tsip1* or *Tsi1* transgenic plants. Consistent with this, we demonstrated that simultaneous overexpression of Tsip1 and Tsi1 in transgenic plants resulted in enhanced expression of *PR4*, *SAR 8.2*, and *LTP*. These results correlated well with the effects of Tsip1 overexpression on Tsi1-mediated transcriptional activation in both yeast and plants.

In summary, we present evidence that Tsip1 is a putative transcriptional coactivator that potentiates Tsi1-mediated transcriptional activation of stress-responsive genes. Additional studies will be needed to uncover the mechanism by which Tsip1 enhances Tsi1-mediated transcriptional activation of stress-related target genes.

METHODS

Plant Material, Chemical Treatments, and Virus Inoculation

Tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Col-0) plants were grown as described previously (Clough and Bent, 1998; Park et al., 2001). For chemical treatments, tobacco plants were sprayed with 100 μM ABA, 5 mM SA, 100 μM GA, or 5 mM ethephon, and samples were collected at the indicated time points thereafter. For NaCl treatment, the roots of tobacco plants were soaked in 300 mM NaCl. Tobacco leaf tissue (*N. tabacum* cv Samsun NN) was inoculated with TMV (strain P_0) as described previously (Shin et al., 2003).

Yeast Two-Hybrid Screening

Tsi1 deletion mutants were generated by PCR and subcloned into the vector pAS2-1 (Clontech) (Park et al., 2001). A tobacco leaf cDNA library was constructed using the activation domain expression vector pACT2 (Clontech). Plasmids were cotransformed into *Saccharomyces cerevisiae* strain Y190 according to the manufacturer's instructions (Clontech).

DNA and RNA Analysis

RNA gel blot analyses were performed as previously described (Shin et al., 2003). For RT-PCR analysis, cDNA was synthesized from DNase I–treated total RNA using MMLV-reverse transcriptase (Promega) and oligo(dT) as a primer. Twenty-five cycles of PCR using *Taq* DNA polymerase (Bioneer) (94°C for 3 min; 25 cycles of 94°C for 1 min, 57°C for 45 s, and 72°C for 2 min; 72°C for 7 min) were performed to amplify *Tsi1*, *Tsip1*, *Tsip1ΔC4-2*, the 3′ untranslated regions of *Tsip1* and *Tsi1*, endogenous *Tsip1*, *PR1*, *PR2*, *PR3*, *PR4*, *PR5*, *SAR8.2*, *HIN1*, *HSR203J*, Nt *C7*, Nt *din*, Nt *LTP*, and *Actin* (see Supplemental Table 1 online). Real-time quantitative RT-PCR was performed on a Bio-Rad iCycler using the fluorescent SYBR green method. *PR4*, *SAR8.2*, and *LTP* expression was normalized to *Actin* expression according to the following formula: $2^{-\Delta Ct}$; $\Delta Ct = Ct_{PR4,\ SAR8.2\ or\ LTP} - Ct_{Actin}$.

Protein–Protein Interaction Assay

The various deletion constructs used in the Tsi1–Tsip1 interaction assay were generated by PCR. *Tsip1ΔCT*, *Tsip1ΔC4-1*, *Tsip1ΔC4-2*, *Tsi1*, and *Tsi1ΔC1* were amplified using the synthetic oligonucleotide primers Tsip1dCT, Tsip1dCR1, and Tsip1dCR2 in combination with Tsip1S, Tsi13HS and Tsi1AS, or Tsi13HS and Tsi1dC1, respectively (see Supplemental Table 2 online). The *Tsip1ΔNT* deletion construct was amplified using the primers Tsip1dNS and Tsip1Nonstop (see Supplemental Table 2 online). The *Tsip1ΔCR* deletion construct was generated by inverse PCR using the primers Tsip1CTS and Tsip1NTAS (see Supplemental Table 2 online). All PCR products were subcloned into pACT2, pAS2-1, or pGEX-3X (Amersham Pharmacia Biotech). All the Tsi1- and Tsip1-derived proteins were expressed in *Escherichia coli* BL21 (DE3) cells. A far western get overlay assay was performed using radiolabeled Tsi1 or Tsip1 as a probe according to the method of Braun et al. (1997).

Transactivation Activity Assay

Tsi1, *Tsi1ΔC1*, or *Tsip1* deletion mutants were generated using the pBridge vector (Clontech) (Park et al., 2001). A triple repeat of the GCC box sequence and a double repeat of the DRE/CRT box sequence were generated by PCR using the synthetic oligomers EXhGCC3XS and SalGFP3, and DRE/CRTS and DRE/CRTAS, respectively (see Supplemental Table 2 online), and then subcloned into the multicloning site of pLacZi (Clontech). The resultant plasmids were transformed into *S. cerevisiae* Y187 or YM4271. A liquid culture assay using *o*-nitrophenyl β-D-galactopyranoside as a substrate was performed to determine transcriptional activation. For transactivation experiments in *Arabidopsis* protoplasts, reporter plasmids were constructed in which the three GCC box repeats or two DRE/CRT box repeats were placed upstream of the minimal (−42 to +8) TATA box of the cauliflower mosaic virus 35S promoter; this fragment was then fused to the coding region of the GUS gene. Effector plasmids were constructed by inserting *Tsi1*, *Tsi1ΔC1*, or *Tsip1* coding regions into pCAMBIA2300, generating a fusion protein containing a double hemagglutinin (HA) epitope tag (YPYDVPDYA). All plasmids were introduced by polyethylene glycol–mediated DNA transfection as described previously (Jin et al., 2001). A reporter plasmid encoding 35S-luciferase (LUC) was cotransfected along with the effector and reporter plasmids to control for variation in transformation efficiency

and cell viability. GUS activity was measured as previously described (Jefferson et al., 1986). LUC assays were performed as previously described (Luehrsen et al., 1992). The GUS activity in each sample was expressed relative to its LUC activity.

Polyethylene Glycol–Mediated Protoplast Transformation and Protein Gel Blot Analysis

For analysis of in vivo targeting of Tsi1, Tsip1, Tsip1ΔNT, and Tsip1ΔC4-2, the corresponding cDNAs were generated by PCR using the primers Tsi13HS and Tsi1Nonstop, Tsip1S and Tsip1Nonstop, Tsip1dNS and Tsip1Nonstop, or Tsip1S and Tsip1dCR2, respectively (see Supplemental Table 2 online). *Tsip1-GFP*, *Tsip1-RFP*, *Tsi1-GFP*, *Tsip1ΔNT-RFP*, and *Tsip1ΔC4-2-RFP* fusion proteins were generated by fusing each of the cDNAs (absent a termination codon) in frame with the N terminus of the GFP coding region or the RFP coding region. The constructs were introduced into *Arabidopsis* protoplasts prepared from whole seedlings by the polyethylene glycol–mediated transformation procedure (Jin et al., 2001). Protein expression was monitored as previously described (Lee et al., 2002). To obtain whole cell extracts, protoplasts were harvested as previously described (Jung et al., 2002). Whole cell extracts were fractionated into soluble and membrane fractions by ultracentrifugation at 100,000*g* for 1 h. Fractions were probed for the presence of Tsip1-GFP, Tsi1-GFP, Tsip1-2XHA, Tsip1ΔC4-2-2XHA, or Tsip1ΔNT-2XHA by protein gel blot analysis (Shin et al., 2003) using a polyclonal anti-GFP or anti-HA antibody (Clontech) followed by immunoblotting with the corresponding primary antibody (anti-HA, 1:500 dilution; anti-GFP, 1:500 dilution; anti-RFP, 1:500) (Clontech).

Protease Protection Assays

For preparation of intact chloroplasts, Tsip1-transfected protoplasts were resuspended as previously described (Lee et al., 2002). Intact chloroplasts were lysed, and extracts were prepared. The protease protection assay was performed as described (Giegé et al., 2003).

Immunoprecipitation

Nucleotide sequences encoding a double HA epitope were ligated into the *Sac*I site of a modified pCAMBIA2300 vector (CAMBIA) containing a 35S promoter and the Nos terminator. *Tsip1*, *Tsip1ΔC4-2*, or *Tsip1ΔNT* cDNAs were fused upstream of the HA epitope. Immunoprecipitation experiments were performed as previously described (Crofts et al., 1998).

Analysis of Transgenic Plants Overexpressing *Tsip1* and *Tsi1*, and Generation of RNAi Constructs

cDNAs corresponding to *Tsip1* and *Tsip1ΔC4-2* were subcloned into the binary vector pMBP2 (Hoekema et al., 1983; Clough and Bent, 1998; Han et al., 1999), and the resultant plasmids were transformed into tobacco (*N. tabacum* cv Samsun NN) or *Arabidopsis*. To generate *Tsip1* and *Tsi1* RNAi constructs, a 350-bp fragment of *Tsip1* or *Tsi1* was amplified from full-length *Tsip1* or *Tsi1* cDNAs by PCR using the primers Tsip1iS and Tsip1iAS, and Tsi1iS and Tsi1iAS, respectively. The amplified fragments were subcloned into the plasmids pHANIBAL and pART27 as previously described (see Supplemental Table 2 online; Wesley et al., 2001). To generate *Tsi1* × *Tsip1* plants, flowers of a *Tsi1* parent were emasculated 2 d before anthesis and dusted with pollen from a *Tsip1* parent 2 or 3 d later. To examine the salt tolerance of transgenic plants, leaf discs 1 cm in diameter were cut and floated in Murashige and Skoog medium containing 400 mM NaCl for 4 d (Park et al., 2001). Chlorophyll content was measured as described (Park et al., 2001). To test for resistance to bacterial pathogens, *P. syringae* pv *tabaci* strains were grown in King's medium B (Martin et al., 1993) and infiltrated into transgenic plants by the method of Thilmony et al. (1995).

Accession Number

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AC118129.

Supplemental Data

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

Supplemental Figure 1. *Tsip1*-RNAi Plants Are More Vulnerable to Salt and Pathogen Stresses Than Vector Control Plants.

Supplemental Table 1. Sequences of Primers Used in RT-PCR.

Supplemental Table 2. Sequences of Primers Used for PCR-Based Cloning.

ACKNOWLEDGMENTS

This work was supported by a grant from the Korea Science and Engineering Foundation (KOSEF) to the Plant Signaling Network Research Center (R11-2003-008-02001-0) to K.-H.P. B.-K.H. was supported by a postdoctoral fellowship from KOSEF, and graduate student scholarships were provided by the Korean BK21 program.

Received April 8, 2006; revised April 8, 2006; accepted June 20, 2006; published July 14, 2006.

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