Research Note

Overexpression of *Pti5* in Tomato Potentiates Pathogen-Induced Defense Gene Expression and Enhances Disease Resistance to *Pseudomonas syringae* pv. *tomato*

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The tomato *Pti5* gene encodes a pathogen-inducible ethylene response element-binding protein-like transcription factor that interacts with the disease resistance gene product Pto. Overexpression of *Pti5* or *Pti5*-VP16, a translational fusion with a constitutive transcriptional activation domain, in tomato enhanced resistance to *Pseudomonas syringae* pv. *tomato*. Constitutive expression of *Pti5* or *Pti5*-VP16 did not affect the basal level of pathogenesis-related gene expression, but it accelerated pathogen-induced expression of *GluB* and *Catalase*. The results demonstrate a positive role of *Pti5* in defense gene regulation and disease resistance and suggest that a pathogen-activated posttranscriptional regulatory step is necessary for the pathogen induction of the defense gene expression.

Additional keywords: bacterial infection, GCC box, general resistance, transgenic plants.

The AP2/EREBP (ethylene response element-binding protein) class of transcription factors carries a plant-unique DNA-binding domain that consists of three β -sheets (Allen et al. 1998; Ohme-Takagi and Shinshi 1995). The AP2/EREBP superfamily can be classified into the AP2 and EREBP subfamilies based on sequence similarities and the number of DNA-binding domains (Riechmann and Meyerowitz 1998). All known AP2 subfamily members are involved in plant development, whereas the EREBP subfamily members are likely to be involved in responses to biotic and abiotic stresses (Riechmann and Meyerowitz 1998). Previously, we isolated three tomato genes, *Pti4*, *Pti5*, and *Pti6*, that encode proteins interacting with the Pto kinase, a resistance gene product that mediates resistance to *Pseudomonas syringae* pv. *tomato* (*avrPto*) (Zhou et al. 1997). The three Pti proteins belong to the EREBP family of transcription factors and bind in vitro to

the GCC box. Moreover, the *Pti5* gene is expressed specifically during biotic stresses but not abiotic or hormonal stresses, suggesting a specific role of *Pti5* in plant defense against pathogens (Thara et al. 1999). However, genetic evidence for a role of these proteins in disease resistance has not been provided. Here we report that the overexpression of *Pti5* resulted in accelerated expression of several defense-related genes in tomato plants during pathogen infection. In addition, the *Pti5*-overexpressing plants displayed increased resistance to *P. syringae* pv. *tomato*.

To test whether increased expression of *Pti5* leads to elevated defense gene expression and enhanced disease resistance, we constructed transgenic tomato plants that constitutively express the *Pti5* gene under the control of the *Cauliflower mosaic virus* 35S promoter. The full-length *Pti5* cDNA (Zhou et al. 1997) was inserted between the *Xba*I and *Sac*I sites of pBI121 (Clontech, Palo Alto, CA, U.S.A.). *Pti5* carries a small acidic domain at the amino terminus, but a role of this domain in transcriptional activation has not been demonstrated. We sought to enhance the transcriptional activation activity of *Pti5* by adding the acidic domain (amino acids 393 to 490) of *Human herpes simplex virus* protein VP16 to the carboxyl terminus of *Pti5*. The VP16 acidic activation domain acts constitutively when fused to DNA-binding domains and functions in fungi, animal, and plants. The *Human herpes simplex virus* VP16 activation domain was polymerase chain reaction (PCR)-amplified by using pBinTetVP16 (Weinmann et al. 1994) as a template and primers 5'-ACACACGCGCAGACTGTCTGA-3' and 5'-AACTCG-AGCTACCCACCGTACTCGTCAA-3'. The PCR product was inserted between the *Sal*I and *Kpn*I sites of pBluescript (SK-) (Stratagene, La Jolla, CA, U.S.A.). *Pti5* was PCR-amplified by using the pBluescript::Pti5 plasmid as a template and the T3 primer and 5'-CTGTGCGACGCACATCTTGATTCAAAT-ACATC-3'. The *Pti5* PCR product was digested with *Sal*I and *Eco*RI and inserted into the same sites of the pBluescript plasmid containing the VP16 fragment, resulting in a translational fusion of *Pti5* and the VP16 activation domain. The chimeric DNA was inserted between the *Xba*I and *Sac*I sites of pBI121 to form the 35S::Pti5-VP16 construct. The two con-

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structs were introduced into *Agrobacterium tumefaciens* strain LBA4404 and transformed into tomato cultivar RioGrande PtoS (lacking *Pto*) as described (Joao and Brown 1993).

Twelve 35S::*Pti5* (R plants) and 19 35S::*Pti5*-VP16 (W plants) primary (T1) transgenic plants were generated and confirmed by Southern blot analysis. All transgenic plants were morphologically indistinguishable from nontransgenic PtoS plants. The transgene expression was examined by RNA gel blot analysis. Three 35S::*Pti5* lines and seven 35S::*Pti5*-

VP16 lines that express transgenes at a high level were tested for disease resistance to *P. syringae* pv. *tomato* in the second (T2) generation by inoculating a virulent *P. syringae* pv. *tomato* strain that does not carry the *avrPto* gene at 2×10^4 CFU/ml. Two 35S::*Pti5* lines (R13 and R17) and five 35S::*Pti5*-VP16 lines (W2, W3, W4, W9, and W12) showed reduced disease lesions and leaf bacterial numbers compared with the nontransgenic PtoS plants. The remaining three transgenic lines (R16, W1, and W6) showed similar susceptibility compared with the nontransgenic PtoS plants as measured by disease symptoms and leaf bacterial numbers. Disease symptoms in the nontransgenic PtoS plant and two representative transgenic lines are shown in Figure 1A. Leaf bacterial numbers of two 35S::*Pti5* (R13 and R17) and three 35S::*Pti5*-VP16 (W2, W4, and W9) lines that were tested repeatedly for bacterial growth assays are shown in Figure 1B. Four days after inoculation, the bacterial numbers were approximately six- to tenfold lower in the 35S::*Pti5*-VP16 plants and approximately three- to fivefold lower in the 35S::*Pti5* plants than in nontransgenic PtoS plants. Although the resistance to compatible bacteria in these transgenic plants is significantly weaker compared with the *Pto*-*avrPto* resistance that reduces bacterial numbers by 100- to 1,000-fold (data not shown), a *t* test showed that the differences (between transgenic and nontransgenic plants) at 4 days were significant ($P < 0.01$). The *avrPto* gene confers a virulence effect in plants lacking *Pto*. We tested whether the *Pti5*-mediated resistance was affected by the presence of *avrPto* in the bacterium. The five transgenic lines supported significantly less bacterial growth when an *avrPto* strain was used (Fig. 1B). Although it appeared that the 35S::*Pti5*-VP16 plants were marginally more resistant than the 35S::*Pti5* plants, the difference is statistically insignificant.

Following the completion of our manuscript, Park and colleagues (2001) reported that overexpression of a tobacco EREBP homolog, *Tsi1*, enhanced resistance to a virulent strain of *P. syringae* pv. *tabaci* in tobacco plants. Similar to our results, the overexpression of *Tsi1* reduced bacterial numbers by two- to eightfold. Slightly enhanced resistance to *P. syringae* pv. *tomato* was also observed in *Arabidopsis* plants constitutively expressing the *Pti4* transgene (Y. Gu and G. Martin, *personal communication*).

We have suggested that *Pti5* plays a role in *Pto*-mediated disease resistance because of its ability to interact with *Pto* and the increased *Pti5* RNA expression when the *Pto* pathway is activated (Thara et al. 1999; Zhou et al. 1997). However, expression of *Pti5* antisense RNA failed to alter the gene-for-gene resistance (data not shown). Although antisense results are often inconclusive, it has been suggested that *Pto* normally activates a general resistance pathway that is targeted by bacterial virulence factors such as *AvrPto* (Van der Biezen and Jones 1998). According to this model, an leucine-rich-repeat (LRR) protein is capable of sensing such a virulence attempt upon *Pto*-*AvrPto* interaction and activates gene-for-gene resistance through a distinct pathway. The *Pti5* overexpression results appear to be consistent with a role of *Pti5* in general resistance.

Recent evidence suggests that some EREBP subfamily members are transcription activators, while others are repressors (Fujimoto et al. 2000). We examined the expression of a number of pathogenesis-related (PR) genes in T2 plants of five transgenic lines that showed enhanced resistance to *P. syringae* pv. *tomato*. In the absence of pathogen inoculation,

Fig. 1. Overexpression of *Pti5* enhances resistance to *Pseudomonas syringae* pv. *tomato*. **A**, Reduced disease symptoms in transgenic plants. Shown are the nontransgenic line (PtoS), a 35S::*Pti5* line (R13), and a 35S::*Pti5*-VP16 line (W4) that were inoculated with *P. syringae* pv. *tomato* bacteria at a concentration of 2×10^4 CFU/ml, and the development of disease lesions was photographed 1 week after inoculation. Of the ten transgenic lines tested, seven showed similar results. **B**, Reduced bacterial growth in the transgenic plants. Plants were vacuum-infiltrated with *P. syringae* pv. *tomato* (left) or *P. syringae* pv. *tomato* containing *avrPto* (right), and leaf bacterial numbers were determined at 0 and 4 days after inoculation, respectively. Error bars show standard errors. Similar results were obtained for PtoS, W2, W4, W9, R13, and R17 lines in repeated experiments. Only 5- to 6-week-old plants, carefully grown in greenhouse (to avoid nonspecific induction of defense genes by stresses) and without any sign of stress, were chosen for pathogen inoculation. All T2 plants were individually examined for the presence and expression of the transgenes by Southern and Northern blot analyses. Bacteria were prepared and vacuum-infiltrated into plants at a concentration of 2×10^4 CFU/ml as described (Thara et al. 1999).

Fig. 2. Overexpression of *Pti5* potentiates pathogen-induced defense gene expression. **A**, RNA blot analysis of the pathogen-induced *GluB* and *Catalase* expression. Leaf tissues were collected at the indicated times after *Pseudomonas syringae* pv. *tomato* inoculation and probed with *GluB* or *Catalase* cDNA probes. PtoS-1 and PtoS-2 indicate two individual nontransgenic control plants from a single experiment. The R13 and R17 lines carry the 35S::*Pti5* transgene, whereas the W2 and W4 lines carry the 35S::*Pti5*-VP16 transgene. W2-1 and W2-2 are two siblings of the W2 line. **B**, Relative induction of *GluB* and *Catalase* transcripts in control and transgenic plants. The signal on the X-ray film was quantitated by densitometry, normalized to amounts of total RNA quantified by ethidium bromide staining loaded in the gel, and plotted. **C**, Induction of *GluB* in the W9 line by *P. syringae* pv. *tomato*. Five-week-old plants were vacuum-infiltrated with *P. syringae* pv. *tomato* strain T1 at a concentration of 10^6 CFU/ml. Leaf tissue was collected either before or after the inoculation at the indicated time points. RNA extraction and RNA gel blot analysis were done as described (Goldsbrough et al. 1990). Probes used were *Pti5* (Zhou et al. 1997), *GluB* (van Kan et al. 1992), *Osmotin* (Jia and Martin 1999), and a cDNA clone that encodes a catalase (Xiao et al. 2001).

the transgenic lines and nontransgenic lines showed identical transcript levels for all PR genes tested. However, when inoculated with *P. syringae* pv. *tomato*, the transgenic lines R13, R17, W2, W4, and W9 accumulated more *GluB* transcripts than did the nontransgenic PtoS plants at 3, 6, and 20 h after inoculation (Fig. 2A and C). The 35S::*Pti5* and 35S::*Pti5*-VP16 plants accumulated two- to threefold and four- to elevenfold more *GluB* transcripts as measured by band intensity normalized to the loadings, respectively, than the nontransgenic plants at 6 h after inoculation. A stronger induction at 3 h was detected in W2, W4, and W9 lines but not R13 and R17 lines. Similar results were obtained in three independent experiments. The RNA blots were also probed with *Osmotin*. Only a marginal increase of *Osmotin* transcripts was detected in W4 and W9 lines (data not shown). We have isolated a cDNA encoding a catalase whose transcripts constitutively accumulate in 35S::*Pto* transgenic plants (Xiao et al. 2001). The *Catalase* transcripts are also induced by *P. syringae* pv. *tomato* in nontransgenic plants. Northern blot analysis showed that lines R17, W2, and W4 displayed stronger and earlier induction of the *Catalase* gene after bacterial inoculation (Fig. 2; W9 was not included in this experiment). The earliest induction in the transgenic lines was observed 1.5 to 3 h after bacterial inoculation compared with 6 h in the nontransgenic PtoS plants. At 20 h after inoculation, the *Catalase* transcript levels were two- to fourfold higher in W4 and W2 lines compared with the nontransgenic line (Fig. 2B). The R17 line accumulated roughly twice as much *Catalase* transcripts as the nontransgenic plants between 1.5 and 6 h. The R13 line showed a marginal increase over the nontransgenic control plants at 3 and 6 h after inoculation. For both *Catalase* and *GluB* transcripts, the overall induction was stronger in the 35S::*Pti5*-VP16 (W) lines than the 35S::*Pti5* (R) lines. The results suggest that *Pti5* functions as a transcription activator for *GluB*, *Osmotin*, and *Catalase*. Although it remains undetermined whether *Catalase* carries a GCC box in the promoter, *GluB* and *Osmotin* are known to carry the GCC box in their promoters that may be recognized by *Pti5* (Jia and Martin 1999). Interestingly, *PR1a1* and *PR1b1* both contain a GCC box in the promoters but were not affected by the transgenes (data not shown), suggesting that *Pti5* may affect only a subset of GCC box-containing genes.

Pti5 transcripts accumulate specifically during pathogen infection (Thara et al. 1999). The enhancement of disease resistance by *Pti5* overexpression demonstrates the significance of the pathogen-induced transcription of *Pti5*. However, the expression of *Pti5* enhances defense gene expression only in the presence of pathogen infection. This is reminiscent of *Arabidopsis NPR1*, which when ectopically expressed in plants, results in a stronger PR gene expression in response to bacterial infection (Cao et al. 1998).

The requirement of pathogen infection for defense gene expression in 35S::*Pti5* and 35S::*Pti5*-VP16 plants is surprising, because this is very different from the regulation of ethylene-inducible genes by *Arabidopsis ERF1*. It appears that this regulation is achieved by activating the transcription of the *ERF1* and *AtERF* genes, presumably through EIN3 (Solano et al. 1998). Pathogen infection may regulate the activity of the *Pti5* protein. It has been shown that *Pti4* can be phosphorylated in vitro, and the phosphorylation enhances the binding

affinity of *Pti4* to the GCC box (Gu et al. 2000). It remains to be determined if the pathogen-induced defense gene expression involves the phosphorylation of *Pti5*.

Alternatively, the activation of the defense gene expression may require an additional protein whose expression or function depends on the pathogen infection. An excellent candidate is the WRKY class of transcription factors that specifically recognizes the W box in many PR genes (Rushton and Somssich 1998). It has been shown in parsley that the *WRKY1* transcripts are rapidly induced by pathogen infections but are not detected in healthy tissue. A composite organization of the GCC box and the W box is known for several PR genes (Rushton and Somssich 1998). In fact, the tomato *Osmotin* gene promoter consists of at least four W boxes (J.-M. Zhou, unpublished data) and two GCC boxes (Jia and Martin 1999).

In summary, our results provide the first evidence that *Pti5* functions positively in defense gene regulation and disease resistance. It is intriguing that, in addition to the increased *Pti5* expression, a pathogen-induced signaling mechanism is needed for the induction of the defense gene expression. It will be important to determine if the phosphorylation of *Pti5* or the function of a second protein is responsible for this pathogen inducibility.

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