Tobacco ZFT1, a transcriptional repressor with a Cys₂/His₂ type zinc finger motif that functions in spermine-signaling pathway[★]

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Received 29 March 2005; accepted in revised form 27 June 2005

Key words: signal transduction, spermine, tobacco, *Tobacco mosaic virus*, transcription factor, zinc finger protein

Abstract

We previously proposed that a spermine (Spm)-mediated signal transduction pathway is involved in the hypersensitive response induced by *Tobacco mosaic virus* (TMV) in tobacco plants. To identify regulatory component(s) of this pathway, we surveyed a tobacco cDNA library and found that the *ZFT1* gene, which encodes a Cys₂/His₂ type zinc-finger protein, is Spm-responsive. *ZFT1* was not induced by two other polyamines, putrescine and spermidine, or by salicylic acid (SA), jasmonic acid or ethylene. Furthermore, *ZFT1* was upregulated in TMV-inoculated tobacco plants in an *N* gene-dependent manner. Notably, induction of *ZFT1* by Spm and by TMV infection was unimpaired in *NahG*-transgenic tobacco plants, indicating that cross-talk with an SA signaling pathway is not involved in this response. Within the Spm-signaling pathway, we found that *ZFT1* functioned downstream of both mitochondrial dysfunction and mitogen-activated protein kinase activation. The ZFT1 protein has two zinc finger motifs and shows a high degree of similarity to ZPT2-3 in petunia and SCOF1 in soybean. However, unlike the latter two proteins, ZFT1 binds to the EP1S sequence and functions as a transcription repressor. Moreover, interestingly, *ZFT1* overexpression rendered tobacco plants more tolerant to TMV. Based on the results presented here, we propose that ZFT1 functions as a transcription repressor in a Spm signaling pathway, thereby accelerating necrotic local region formation in tobacco leaves.

Introduction

In higher plants, polyamines (PAs), particularly putrescine (Put), spermidine (Spd) and spermine (Spm), are implicated in cell growth, cell division

[★] Accession number: *ZFT1* (AB186899).

and development (Kumar *et al.*, 1997; Malmberg *et al.*, 1998). In addition, these basic, low molecular weight compounds are purported to have adaptive/defensive roles in response to (a)biotic stresses (Bouchereau *et al.*, 1999; Walters 2003a). Of the many research reports concerning the role of PAs in plant pathogenesis, we paid special attention to one in which Yamakawa *et al.* (1998) identified Spm as an endogenous inducer of pathogenesis-related (PR) proteins during the *Tobacco mosaic virus* (TMV)-induced hypersensitive response (HR). The same authors showed that exogenously applied Spm induced the expression of PR proteins and enhanced TMV tolerance in host plants and that two tobacco peroxidase genes are responsive to exogenously applied Spm (Hiraga *et al.*, 2000a, b). Based on that information, we hypothesized that PAs may function as signaling molecules in certain processes and possibly mediate some of the defense signals to pathogen attacks. Initially, we assessed whether PAs modulate phosphorylation/dephosphorylation events, and found that of the PAs tested, Spm specifically activated two mitogen-activated protein kinases (MAPKs), i.e. salicylic acid (SA)-induced protein kinase (SIPK, Zhang and Klessig, 1997) and wound-induced protein kinase (WIPK, Seo *et al.*, 1995), with this effect being mediated through mitochondrial dysfunction (Takahashi *et al.*, 2003). Previously, we identified the *HIN1* (harpin-induced 1) gene and a subset of HR marker genes, including *HSR203J* (Pontier *et al.*, 1994), to be Spm-responsive (Takahashi *et al.*, 2004a, b). On the basis of biochemical and molecular biological data, we proposed an Spm-signaling pathway in tobacco whereby in the HR provoked by TMV infection, the synthesis of PAs is enhanced and the end product, Spm, accumulates in the apoplastic space. Due to its cellular toxicity, Spm might be immediately catabolized by amine oxidase and/or polyamine oxidase, with the resultant hydrogen peroxide serving as a downstream signal transducer. In agreement with this hypothesis, Walters (2003b) proposed the involvement of Spm in signaling the HR to pathogen infection, and pointed out the importance of PA catabolism in such processes.

The Cys₂/His₂-type zinc finger protein family, also called the TFIIIA-type finger protein family, is one of the best characterized groups of eukaryotic transcription factors (TFs). Plant proteins of this family have one to four finger

motif(s) within each molecule (Takatsuji, 1999). Within this family, two-fingered proteins constitute the major class, with examples including the ZPT2 subfamily in petunia, WZF1 in wheat (Sakamoto *et al.*, 1993; Sugano *et al.*, 2003; van der Krol *et al.*, 1999), soybean SCOF-1, which is involved in cold tolerance (Kim *et al.*, 2001), Arabidopsis STZ/ZAT10, which plays a role in salt tolerance (Lippuner *et al.*, 1996) and drought tolerance (Sakamoto *et al.*, 2004), and petunia ZPT2-3, overexpression of which confers drought tolerance (Sugano *et al.*, 2003). Almost all two-fingered members so far reported have been implicated in the regulation of gene activity during various abiotic stresses and during the development of vegetative and floral organs (Takatsuji, 1999). Takatsuji and his colleagues have extensively investigated DNA interactions of the plant zinc finger proteins of this class (Takatsuji *et al.*, 1994; Takatsuji and Matsumoto, 1996; Kubo *et al.*, 1998; Yoshioka *et al.*, 2001). They observed that the Cys₂His₂-type zinc-finger proteins contain the conserved QALGGH sequence in their zinc finger regions, and that those residues are crucial for DNA binding (Kubo *et al.*, 1998).

Here, we report the identification of a TF, *ZFT1*, which is involved in the Spm signaling pathway in tobacco plants. This novel tobacco gene, which encodes a zinc finger protein (ZFT1) with two Cys₂/His₂-type motifs, is responsive to Spm and is induced during the TMV-triggered HR. Importantly, within the Spm signaling pathway, *ZFT1* is positioned downstream of a mitochondrial malfunction event and SIPK/WIPK activation. Moreover, transgenic tobacco plants overexpressing *ZFT1* showed enhanced tolerance to TMV infection. On the basis of this combined data, we discuss a role for *ZFT1* in Spm-mediated signaling in tobacco.

Materials and methods

Plant materials and treatment

Tobacco (*Nicotiana tabacum* L. cv. Xanthi nc and *NahG* transgenic) plants were incubated in a growth chamber at 25 °C with a 14 h light/10 h dark photocycle. *NahG* transgenic tobacco seeds were provided by Syngenta Co. Six- to eight-week-old plants were used for all experiments. For

chemical treatment, leaf discs from healthy tobacco plants were floated on half-strength MS solution (pH 7.0) and incubated for 12 h at 25 °C to minimize the wounding effect. Where necessary, chemicals were added to media and further incubated for the indicated periods. For TMV inoculation, approximately 8-week-old mature detached leaves from tobacco plants were rub-inoculated with or without TMV (10 µg/ml) in 10 mM phosphate buffer (pH 7.0) using carborundum (mesh size 600) unless otherwise described. After incubating at 30 °C for 48 h under continuous light, the temperature was down-shifted to 20 °C to initiate the HR.

RNA gel blot analysis

Total RNA was isolated according to the method of Nagy *et al.* (1988). Aliquots (10 µg each) were separated by electrophoresis on formaldehyde-1.0 % (w/v) agarose gels, and blotted onto Hybond N+ membranes (Amersham Biosciences) in 20x SSC. Hybridization was performed as described previously (Berberich *et al.*, 1999).

Construction of GFP fusion plasmid and microscopic observation

The coding region of *ZFT1* was amplified by PCR with a pair of primers, 5'-AATCTCGAGATGGCACTTGAAACT-3' and 5'-GTCGTACCTTCCCGAATAATTC-3'.

The fragments subcloned into pCR4 Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA) were subjected to direct sequencing. The verified insert digested with *XhoI* and *KpnI* was subcloned into the corresponding sites of the pGFP-2 vector (provided by Dr. N.-H. Chua), to make pZFT1-GFP-2. The pGFP vectors were introduced into *C. communis* leaves by particle bombardment. The leaves were then incubated for more than 6 h at 22 °C in darkness, after which the epidermal layers on the bombarded side were peeled off and observed with a fluorescence microscope (Axioplan 2; Zeiss), as described previously (Yang *et al.*, 2003).

Preparation of protein extracts and protein kinase activity assay

Preparation of protein extracts from tobacco leaves and an in-gel kinase activity assay were

performed as described previously (Takahashi *et al.*, 2003).

Assay for transcription activity

The reporter construct cloned into pUC19 vector has been described previously (Hiratsu *et al.*, 2002). The effector plasmids were constructed as follows: The coding regions of *ZFT1* were amplified by PCR with the appropriate primer pairs; a common forward primer, 5'-TTCCCGGGTATGGCACTTGAAACT-3', a reverse primer for the full-length *ZFT1*, 5'-AAAGTCGACCTATTCCCGAATAA-3' and a reverse primer for *ZFT1ΔC*, 5'-AGTGTGCGACGACACCCTCTGATGA-3'. The PCR products were cloned into pCR4 Blunt-TOPO vector (Invitrogen) and confirmed by direct sequencing. The correct inserts digested with *SmaI* and *SalI* were subcloned into the respective sites of the CaMV35S-GAL4DB plasmid (Ohta *et al.*, 2001), resulting in CaMV35S-GAL4DB-*ZFT1* and CaMV35S-GAL4DB-*ZFT1ΔC*. Analysis of transient expression in *Arabidopsis* leaves after particle bombardment was performed as described previously (Ohta *et al.*, 2001). In brief, 1.6 µg of reporter construct and 1.2 µg of effector construct were used for each bombardment. To normalize the efficiency of bombardment, 0.8 µg of the reference plasmid pPTRL, which included a luciferase gene from *Renilla* under the control of the CaMV 35S promoter, was also co-bombarded. Relative luciferase activity was obtained using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and luminescence reader (Lumat LB9507; Berthold Japan, Tokyo) as described (Hiratsu *et al.*, 2002).

Electrophoretic mobility shift assay EMSA

An enterokinase site was introduced in both sides of the open reading frame (ORF) of *ZFT1* by PCR and then annealed in-frame into the pET-46 Ek/LIC vector (Novagen, Darmstadt, Germany). *Escherichia coli* strain BL21(DE3) cells harboring the above pET-46 Ek/LIC construct were treated with 0.5 mM IPTG and further incubated at 25 °C for 8 h. The harvested cells were resuspended in the extraction buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 20% glycerol, and 2 mM phenylmethylsulfonyl fluoride), and

disrupted by sonication (Branson Sonifer 250, Danbury, CT, USA). Cell debris was eliminated by centrifugation (15,000 rpm for 20 min) and the clear lysate was used for binding assays. The DNA binding reaction was carried out in 25 mM Hepes-KOH pH 7.6, 40 mM KCl, 0.1% Nonidet P-40, 0.01 mM ZnCl₂, 10 µg/ml BSA, and 0.1 mM dithiothreitol. EMSA was performed with 10,000 cpm ³²P-end-labeled probe (EPIS, 5'-TTGACAGTGTCACTTGACAGTGTGTCAC-3' × 2) and 1 µl of the protein solution (100-fold diluted solution of the above clear lysate) per reaction. After incubation at room temperature for 25 min, the mixtures were subjected to electrophoresis in an 8% polyacrylamide gel with 0.5×TBE buffer, as described previously (Takatsuji *et al.*, 1992).

Cloning of NtMEK2 cDNA and generation of a constitutively active NtMEK2 construct

NtMEK2 cDNA (Accession number AF325168) was amplified by PCR, as described (Takahashi *et al.*, 2004b), and its product was cloned into the pCR4 Blunt-TOPO vector (Invitrogen) and confirmed by direct sequencing. NtMEK2^{DD}, an active mutant of NtMEK2, was generated by substituting two conserved residues, Thr at position 227 and Ser at position 233, located between the kinase subdomains VII and VIII, with Asp residues. The mutant was generated by following a procedure described in a previous report (Yang *et al.*, 2001).

Agrobacterium-mediated transient expression assay

Agrobacterium-mediated transient expression assays were performed as described by Yang *et al.* (2001). Corresponding fragments, encompassing the ORF regions of NtMEK2 and the constitutive active mutant NtMEK2^{DD}, were inserted into the *Sma*I-*Sac*I sites of the pBI121 binary vector (Clontech, Palo Alto, CA, USA). The ORFs of NtMEK2 and NtMEK2^{DD} were placed under the control of the Ω sequence from TMV (Gallie *et al.*, 1987). *A. tumefaciens* strains of LBA4404 cells carrying the above constructs were grown overnight in YEP medium containing 100 µg/ml streptomycin, 50 µg/ml kanamycin, and 100 µM acetosyringone. Cells were collected by centrifugation (4000 × g), resuspended to an OD₆₀₀ value

of 0.8 with MS medium (pH 5.9) containing 100 µM acetosyringone, and infiltrated into fully expanded tobacco leaves. Leaf samples were collected at defined intervals, frozen in liquid nitrogen, and stored at -80 °C until use.

Silencing of WIPK and SIPK genes in N. benthamiana by VIGS using PVX

VIGS protocols were performed as described previously (Sharma *et al.*, 2003; Takahashi *et al.*, 2003). *N. benthamiana* plants displayed mild mosaic symptoms three weeks after inoculation of leaves with the transcripts. The third or fourth leaf above the inoculated one, where the silencing is most consistently established, was used for further analysis.

RT-PCR

RT-PCR was performed as described (Takahashi *et al.*, 2003). Constitutively expressed *rbcS* cDNA was additionally amplified by PCR using the following primer pairs (forward, 5'-CCTCTGCA-GCAGTTGCCACC-3'; reverse, 5'-CCTGTGGG-TATGCCTTCTTC-3') and this served as an internal control for the assays.

Transgenic plants

To generate the CaMV35S::*ZFT1* construct, the *ZFT1*-encoded sequence was amplified by PCR with the primer pair, 5'-AATGGATCCATGGC-ACTTGAAACT-3' and 5'-AAAGAGCTCCTA-TTCCCCGAATAA-3', and was subcloned into the pCR4 Blunt-TOPO vector. The sequence-verified *ZFT1* fragment was digested with *Bam*HI and *Sac*I and inserted into the *Bam*HI-*Sac*I site of a binary vector pBI121. The 35S::*ZFT1* recombinant and the control pBI121 vector were introduced into *A. tumefaciens* LBA4404, and transgenic tobacco plants were obtained by a leaf disc transformation method (Yang *et al.*, 2003). Leaves detached from the transgenic plants grown on soil for 5 weeks were rub-inoculated with TMV (25 µg/ml). After incubating at 20 °C for 6 d under continuous light, the diameters of the necrotic lesions were measured. The significance of differences in lesion size between control and transgenic plants was assessed with a *t*-test by standard software.

Results

ZFT1 is an Spm-responsive clone

As mentioned above, we have previously observed that exogenously applied Spm activates two MAP-Ks in a process involving mitochondrial malfunction, leading us to propose a Spm-mediated signaling pathway in tobacco (Takahashi *et al.*, 2003). To identify downstream target genes within this pathway, we performed differential hybridization and isolated the *HIN1* gene as an Spm-responsive clone (Takahashi *et al.*, 2004a). We also reported that a subset of the TMV-induced HR marker genes, including *HSR203J*, were downstream targets of this pathway (Takahashi *et al.*, 2004b). Here, our specific aim was to identify intermediate regulatory components, such as TFs, within the Spm signaling pathway. Extensive screening with usage of differential hybridization of Spm-responsive genes (Takahashi *et al.*, 2004a) led to the identification of *ZFT1* encoding a putative TF of the zinc finger-type (see below). The response of *ZFT1* to Spm treatment is both time- and dose-dependent (Figure 1A, B). Notably, of three polyamines tested, the response of *ZFT1* was specific to Spm (Figure 1B). It is well established that SA is a key player in defense against phytopathogens (Klessig and Malamy, 1994; Chen *et al.*, 1995). Thus, to determine whether SA is involved in Spm-induced *ZFT1* expression, the effect of Spm application on *ZFT1* expression was investigated in *NahG*-transgenic tobacco plants. Induction of *ZFT1* in *NahG*-transgenic plants was similar to that observed in wild type tobacco plants (Figure 1C), indicating that *ZFT1* expression induced by Spm is independent of SA signaling.

ZFT1 is a component of the Spm signaling pathway

The events that occur upstream of activation of the two MAPKs in the Spm signaling pathway are (i) generation of reactive oxygen species (ROS), (ii) calcium influx, (iii) mitochondrial dysfunction, and (iv) Spm catabolism by amine oxidase(s) and/or polyamine oxidase(s) (Takahashi *et al.*, 2003, 2004b). In view of this, here we addressed the following points: first, whether *ZFT1* induction by Spm is associated with the same Spm signaling pathway described above; second, if so, where is it positioned within this pathway?

Pretreatment with antioxidants, specifically 1 mM flavone and 2 mM *N*-acetyl cysteine, suppressed Spm-induced *ZFT1* expression (Figure 2A). Pretreatment with the calcium channel blockers, La^{3+} and Gd^{3+} (both at 0.5 mM), attenuated Spm-induced *ZFT1* expression (Figure 2B). An uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; 0.1 mM) mimicked the effect of Spm on *ZFT1* gene expression (Figure 2C). Upregulation of the *ZFT1* gene by Spm was blocked by an inhibitor of mitochondrial PT pore opening, bongrekic acid (BK, 50 μM) (Figure 2D). Pre-treatment of tobacco leaf discs with both amino guanidine (AG, 2 mM) and MDL72527 (MDL, 10 mM), which are potent inhibitors of copper amine oxidase(s) and polyamine oxidase(s), respectively, suppressed the induction of *ZFT1* by Spm (Figure 2E). MDL suppressed the activation of the 46 kDa-phosphorylation activity and upregulation of *WIPK* by Spm less efficiently compared to AG (Takahashi *et al.*, 2004b). This may be the reason why Spm-induced *ZFT1* expression was only weakly suppressed by MDL-pretreatment. The pattern of *ZFT1* expression, in response to the

Figure 1. *ZFT1* gene is specifically upregulated by Spm. (A) Time-course analysis of the accumulation of *ZFT1* transcripts in tobacco leaves after Spm application. Tobacco leaf discs were floated on half-strength MS medium (pH 7.0), and treated with water (Cont) or 0.5 mM Spm for the indicated periods. (B) Specificity of SPM on *ZFT1* expression. Tobacco leaf discs were floated onto half-strength MS media containing Put, Spd and Spm at a concentration of either 0.2 or 0.5 mM and incubated for 12 h. (C) Spm-specific *ZFT1* expression is unimpaired in *NahG*-transgenic tobacco plants. The *NahG*-transgenic tobacco leaves were treated with 0.5 mM Spm or water as a control (Cont). *EF1α* gene served as a loading control.

various inhibitors described above, was similar to that observed for other downstream targets like *HIN1* and *HSR203J*, indicating that *ZFT1* induction by Spm also occurs downstream of mitochondrial malfunction in the pathway. Next, we addressed whether Spm-induced *ZFT1* expression depends on SIPK/WIPK activation. Introduction of the constitutively active form of NtMEK2 (NtMEK2^{DD}), the upstream kinase of SIPK and

Figure 2. Generation of reactive oxygen species, calcium influx, mitochondrial dysfunction and Spm decomposition are upstream events of *ZFT1* induction in the Spm signaling pathway. (A) Antioxidants (flavone at 1 mM and *N*-acetyl cysteine at 2 mM) were added to tobacco leaf discs 1 h before addition of Spm, and further incubated for 12 h. (B) Instead of antioxidants as in A, calcium channel blockers (La³⁺ at 0.5 mM and Gd³⁺ at 0.5 mM) were added for 1 h and followed by the Spm addition. (C) Leaf discs were treated with an uncoupler (0.1 mM CCCP) or an equal volume of DMSO as a control, and incubated for indicated periods. (D) Mitochondrial PT pore-opening blocker, bongkreikic acid (BK at 50 μ M) was applied for 1 h prior to Spm (0.5 mM) treatment and further incubated for 12 h. (E) Effects of the inhibitors for amine oxidase (AO) and polyamine oxidase (PAO) on Spm-induced *ZFT1* expression. Leaf discs were pretreated with aminoguanidine (AG, 2 mM), inhibitor for AO, or MDL72527 (MDL, 10 mM), inhibitor for PAO, for 1 h, then followed by Spm treatment. Tobacco *EF1α* gene served as a loading control.

WIPK, into tobacco leaves using an *Agrobacterium*-infiltration method (Yang *et al.*, 2001) resulted in upregulation of *ZFT1* expression (Figure 3A). Moreover, we did a loss-of-function experiment, in which the effect of potato virus X (PVX)-mediated silencing of *SIPK* and *WIPK* in *Nicotiana benthamiana* on Spm-induced *ZFT1* expression was evaluated. The phosphorylation activity of a 46 kDa protein and upregulation of *WIPK* transcripts by Spm treatment were partially blocked in the silenced *N. benthamiana* plants (data not shown, see Takahashi *et al.*, 2004b). In those plants Spm-induced *ZFT1* expression was partially blocked (Figure 3B). Thus, both gain-of-function and loss-of-function experiments on the MAPK cascade components revealed that Spm-induced *ZFT1* expression occurs downstream of SIPK/WIPK activation in the pathway.

ZFT1 is upregulated during TMV-elicited HR

Having concluded that Spm is one of the signal transmitters during TMV-induced HR (Takahashi

Figure 3. *ZFT1* induction occurs downstream of SIPK/WIPK activation in the Spm signaling pathway. (A) Gain-of-function experiment. Authentic form and a constitutively active form of the kinase upstream of SIPK/WIPK were introduced into tobacco leaves and *ZFT1* expression was assessed. (B) Loss-of-function experiment. Effect of *SIPK*/*WIPK*-silencing on Spm-induced expression of *ZFT1* gene was investigated. Control or *SIPK*/*WIPK*-silenced leaf discs were treated for 6 h with water (Cont) or 0.5 mM Spm. Total RNA (10 μ g each) was separated on a formaldehyde-1% agarose gel and transferred onto membranes. Blots were hybridized as described in Figure 1.

et al., 2003), we next examined whether *ZFT1* transcripts accumulate during TMV-induced HR. To this end, tobacco leaves carrying the resistance *N* gene were mock- or TMV-inoculated and subjected to RNA blot hybridization. Time-course analysis revealed that *ZFT1* induction during TMV-induced HR started at 3 h and was sustained for at least 24 h (Figure 4A). Next, we addressed whether *ZFT1* expression induced by TMV inoculation depends on the *N* gene. *ZFT1* induction was observed in *N* gene-carrying tobacco but not in an *n* gene-carrying strain, demonstrating that *ZFT1* induction occurs in an incompatible host-virus interaction but not in a compatible one (Figure 4B). SA, jasmonic acid (JA) and ethylene (ET) are known to be involved in the defense response of dicotyledonous plants to pathogens (Dong, 1998). Thus, we investigated whether these compounds affect the steady-state levels of *ZFT1* transcripts. Notably, none of these compounds affected *ZFT1* expression (Figure 4C), while accumulation of *ZFT1* transcripts was observed by Spm treatment under similar conditions. Thus, we again investigated putative SA involvement in TMV-elicited *ZFT1* induction using *NahG*-transgenic tobacco plants. Induction

of *ZFT1* was similar in the transgenic plants and wild type tobacco plants (Figure 4D), suggesting that *ZFT1* upregulation during TMV-triggered HR does not involve cross-talk with an SA signaling pathway.

ZFT1 is a member of the Cys₂/His₂-type zinc finger protein family

The gene product of *ZFT1*, termed ZFT1, is a Cys₂/His₂-type zinc finger protein composed of 253 amino acids (calculated molecular mass, 27.0 kDa), and shows a high degree of similarity to ZPT2-3 (79.1%), SCOF-1 (56.0%), Mspt2-1 (53.9%), STZ/ZAT10 (48.4%), ZPT2-2 (46.0%) and WZF1 (41.5%), all of which belong to the two-fingered protein family (Figure 5). ZFT1 shares a common sequence, QALGGH, within a putative DNA-contacting surface of each finger with these family members (Takatsuji, 1999). The molecule also contains a putative transcription repression domain, the so-called EAR domain (Ohta *et al.*, 2001), close to its carboxy distal end.

Since ZFT1 contains a putative nuclear localization signal (NLS) sequence (KGKRSKR) proximal to the amino terminus, we tested its

Figure 4. *ZFT1* gene expression is upregulated during TMV-triggered HR. (A) *ZFT1* expression was monitored during TMV-induced HR. Mock- and TMV-inoculated leaves of *N* gene-tobacco were harvested at indicated time intervals. (B) Leaves from tobacco plants carrying the TMV resistance gene, *N* gene, and its allelic *n* gene were inoculated with TMV or water as control (Mock), and further incubated for indicated periods. Total RNA was extracted and subjected to northern hybridization. (C) Effect of jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) on *ZFT1* expression. Tobacco leaf discs were floated onto half-strength MS medium, and treated with water (Cont), JA (50 μ M), SA (50 μ M), Spm (0.3 mM) and ethylene (ET, 10 μ l/l). ET gas was injected into the gas-tight glass vessel using a gas-tight syringe. Leaf discs were incubated for 1, 2 and 3 days before total RNA was extracted and subjected to northern hybridization analysis. The blots were re-probed with *PR1a* gene, proteinase inhibitor class II gene and ornithine decarboxylase gene to ensure that chemical treatments were effective (data not shown). (D) *ZFT1* expression during TMV-induced-HR is unaffected in *NahG*-transgenic tobacco plants. Leaves from the non-transgenic (WT) and *NahG*-transgenic tobacco plants were mock- and TMV-inoculated, and further incubated for 6 h and 9 h at 20°C. Total RNA extracted from the above leaf samples was subjected to northern hybridization analysis. *EF1α* gene served as a loading control.

Figure 5. ZFT1 belongs to a zinc finger protein family with Cys₂His₂-type motifs. (A) Alignment of the amino acid sequence of ZFT1 with those of the related plant zinc-finger proteins. ZFT1 (this study), ZPT2-2 (petunia, accession number D26084; Yoshioka *et al.*, 2001), ZPT2-3 (petunia, D26086; Sugano *et al.*, 2003), SCOF-1 (soybean, U68763; Kim *et al.*, 2001), Mszpt2-1 (alfalfa, Y18788, Frugier *et al.*, 2000), STZ/ZAT10 (*A. thaliana*, X95573; Lippuner *et al.*, 1996). Gaps marked by dashes (-) are introduced for maximizing alignment. Identical and conserved amino acids are highlighted with black and gray backgrounds, respectively. Nuclear-localization signal (NLS), EAR domain and two zinc finger motifs are marked with solid and broken thick bars, respectively. (B) Phylogenetic relationship between ZFT1 and its related zinc-finger proteins. AZF1 (AB030731), AZF2 (AB030730), AZF3 (AB030732), ZAT1 (X98669, Meissner and Michael, 1997), ZAT11 (X98672, Meissner and Michael, 1997), ZAT12 (X98674, Meissner and Michael, 1997, Iida *et al.*, 2000), ZPT2-7 (AB006602), ZPT2-8 (AB006603), ZPT2-9 (AB006604), ZPT2-10 (AB006597), ZPT2-11 (AB006598), ZPT2-12 (AB006599), ZPT2-13 (AB006600), and ZPT2-14 (AB006601).

subcellular localization by generating a ZFT1-GFP fusion construct. The construct was delivered by particle bombardment into leaves of *Commelina communis* grown in their natural habitat. The resultant pattern of GFP fluorescence confirmed that ZFT1 localizes to the nucleus (Figure 6).

Notably, ZPT2-2 binds to the conserved *cis*-element (EP1S) of the *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) gene, a potential binding site for proteins of the EPF family, whereas two other two-fingered protein family members, ZPT2-3 and SCOF-1, which are phylogenetically closer to ZFT1, do not bind it. Using (His)₆-fusion form of ZFT1, an electrophoretic mobility shift assay (EMSA) was performed. Unexpectedly, the intact ZFT1 protein exhibited DNA binding activity to the EP1S sequence and its binding complex was competed out by doses of 25- to 50-fold non-radiolabelled EP1S fragment (Figure 7).

Next, we evaluated the effect of ZFT1 on transcription. As expected owing to the presence of the EAR domain, full-length ZFT1 was able to repress reporter gene expression (Figure 8).

However, once the EAR domain was eliminated from ZFT1, this repression activity was almost lost (Figure 8). We conclude that ZFT1 is a transcription repressor and that almost all of its repression activity originates from the EAR domain.

Constitutive expression of ZFT1 confers TMV tolerance to tobacco plants

To further investigate the function of ZFT1, ZFT1-overexpressing tobacco plants were generated. Four control transgenic lines and ten ZFT1-overexpressing lines were randomly selected. There were no apparent gross morphological differences between ZFT1 transgenic plants, wild type plants and control transgenics. Although constitutive expression of ZFT1 was confirmed in the transgenics (Figure 9A), we noted that the steady-state level of ZFT1 transcripts was rather low compared to normal CaMV 35S promoter-driven overexpressed plant genes. Five weeks after transferring the transgenics to soil in pots, the 5th leaf from the top of each transgenic plant was detached with a

Figure 6. ZFT1 localizes to the nucleus. The region covering the open reading frame of *ZFT1* was amplified by PCR and sequence-verified, then subcloned into the *Xba*I and *Kpn*I sites of the pGFP2 vector (a gift from Dr. N.-H. Chua), resulting in pZFT1-GFP2. This recombinant plasmid and pGFP2 were delivered into *C. communis* leaves by particle bombardment. After 12 h incubation at 23 °C under darkness, the epidermal layers peeled off were subjected to microscopic observation. The upper and lower columns show pGFP2- and pZFT1-GFP2-delivered leaves, respectively. Differential interference contrast (DIC, left) and GFP derived-fluorescence (Fluorescence, right) images were observed using a fluorescence microscope. White bar corresponds to 10 μm.

Figure 7. Electrophoretic mobility shift assay (EMSA). ZFT1 protein was produced as (His)₆ fusion form in *E. coli* BL21(DE3) cells, and was used for EMSA. The probe sequence is EP1S (5'- TTGACAGTGTCACTTGACAGTGT-CAC-3'×2). The positions of the DNA-protein binding complex (BC) and free-form (F) are marked with arrowheads. To confirm the specificity of binding, non-radiolabelled probe fragments were added to the binding reaction mixtures at 25- and 50-fold excess.

sharp scalpel and rub-inoculated with TMV solution, as previously described (Yamakawa *et al.*, 1998). The inoculated leaves were floated on water and incubated at 20 °C for 5 days, following which the diameter of 20 local necrotic regions was measured for each leaf and photographed (Figure 9B, C). Interestingly, nine of ten *ZFT1* transgenic lines, with the exception being line No. 3, had more or less smaller sizes of local lesions, suggesting a higher tolerance to TMV than control transgenics (Figure 9B, C, and data not shown). In the transgenic lines, the expression of *NtRdRP* (RNA-dependent RNA polymerase, accession number AJ011576), *HSR515*, *HMGR*, *HSR203J* (Takahashi *et al.*, 2004b) and two *PR* genes (*PR1a* and *PR2*) was monitored. No correlation to the expressional behavior of *ZFT1* was observed (Figure 9A). In contrast, *ZFT1* transgenic line No. 3 accumulated the highest level of *ZFT1*

Figure 8. ZFT1 is a transcriptional repressor. (A) Schematic representation of the reporter and effector constructs. To normalize the efficiency of bombardment to *A. thaliana* rosette leaves, the reference plasmid pPTRL was also used. (B) Assay of transcription activity of ZFT1. The relative luciferase activity (LUC/RLUC) was conducted four times in triplicate. Each activity is presented as a relative value compared to the one obtained with the control effector (pUC19), which is set as 1. In ZFT1ΔC, the C-distal portion (amino acid positions from 197 to 253) including the EAR domain of ZFT1 was deleted.

Figure 9. Overexpression of *ZFT1* in tobacco plants enhances tolerance to TMV. Arabic numerals indicate the transgenic lines. (A) Northern blot analysis of *ZFT1*, *NtRdRP*, *HSR515*, *HMGR*, *HSR203J*, *PR1a* and *PR2* transcript levels in *ZFT1*-overexpressed tobacco plants. *EF1α* gene served as a loading control. (B) Diameters of TMV-induced local necrotic regions in control-transgenic (Cont) and *ZFT1*-overexpressing (*35S::ZFT1*) plants were measured at 5 days post TMV infection. The *t*-test demonstrated that the difference in the diameters of two non-transgenic plants was not significant, but that between the control transgenic plants and *ZFT1*-overexpressing plants was significant at $P < 0.001$. The experiments were repeated three times with reproducible results. (C) Representative pictures of TMV-induced local necrotic regions appearing in the leaves of the control vector (pBI121)-transgenic (Cont) and *ZFT1*-overexpressing (*35S::ZFT1*) tobacco plants 5 days after TMV inoculation. The lower picture shows a higher magnification view of the squared region from the upper photograph.

transcripts, but showed a similar TMV sensitivity as non-transgenic and control transgenic tobacco plants (data not shown). One possible explanation could be that suppression on translational level occurs due to an extremely high amount of mRNA. But at the moment this is only speculative and we did not elucidate on the basic cause for this observation any further.

Discussion

Over recent years, we have investigated the role of Spm signaling during viral pathogen-triggered HR. Here, we identified a transcription factor in

tobacco, *ZFT1*, which is implicated in Spm signaling. This factor is classified into the Cys₂/His₂-type zinc finger protein family and has transcriptional repressor activity. We discuss the role of *ZFT1* in the defense against TMV as well as the broader physiological significance of Spm signaling in defense against pathogen attack.

ZFT1 is up-regulated by exogenously applied Spm and during TMV-induced HR

All known plant genes whose products belong to the two-fingered protein family have been reported to be induced by various abiotic stresses including cold, wounding, UV-B irradiation and heavy

metals (Takatsuji, 1999). Furthermore, overexpression of members of this gene family confers enhanced tolerance to low temperature (Kim *et al.*, 2001), dehydration (Sugano *et al.*, 2003), high salt and drought (Lippuner *et al.*, 1996; Sakamoto *et al.*, 2004) to plants or yeast cells, confirming their involvement in abiotic stress responses. Currently, Kim *et al.* (2004) reported that the hot pepper *CAZFP1* gene, belonging to this gene family, functions as a pathogen-induced early-defense gene. Here, we demonstrate that tobacco *ZFT1*, a novel member of this family, is induced by exogenously applied Spm and also during TMV-induced HR. This is the second report showing that a member of this gene family is induced by a biotic stress. Accumulation of *ZFT1* transcripts was also induced by abiotic stresses, including low temperature and wounding (Uehara *et al.*, unpublished data). In support of these data, upregulation of the *Arabidopsis thaliana* *STZ* gene during *Cucumber mosaic virus* Yellow strain-induced HR was shown by microarray analysis (Marathe *et al.*, 2004). Therefore, it is highly possible that the Cys₂His₂-type zinc finger genes play roles not only in abiotic stress responses, but also in biotic stress responses. We should mention that, in agreement with our findings on *ZFT1*, the *STZ* gene, but not the *AZF1*, *AZF2* and *AZF3* genes, is also upregulated by exogenous Spm at a concentration of 0.1 mM (Berberich *et al.*, unpublished data). This suggests that a similar Spm signaling pathway exists also in *A. thaliana*, with *STZ* participating in that pathway. Very recently, Yi *et al.* (2004) reported that hot pepper CaPF1, an ERF (ethylene response factor)/AP2-type transcription factor, is involved in that plant's response to pathogens and to freezing. The above *CAZFP1* gene was also induced by abiotic stresses such as wounding, salt stress and low temperature (Kim *et al.*, 2004). Thus, accumulating evidence suggests that some TFs play dual roles in responding to abiotic and biotic stress in plants. It is, therefore, of particular relevance to clarify the events upstream/downstream of *ZFT1* induction upon exposure to low temperature (wounding treatment) and upon TMV infection.

ZFT1 is a transcription repressor

Cellular localization study indicated that *ZFT1* is targeted to the nucleus (Figure 6), consistent with

a transcription factor function. Several TFIIIA-type zinc finger plant proteins include an EAR motif repression domain (Ohta *et al.*, 2001). This motif is also present in *ZFT1* and, in fact, the protein was here proven to be an active repressor of transcription (Figure 8). Its truncated version, lacking the carboxy-terminal region including the EAR motif, was practically devoid of repression activity. With the exception of SCOF-1, which is a transcription activator (Kim *et al.*, 2001), all other known TFIIIA-type zinc finger proteins, including *ZFT1*, are transcription repressors (Ohta *et al.*, 2001; Sugano *et al.*, 2003; Sakamoto *et al.*, 2004). *ZFT1* is phylogenically closer to petunia *ZPT2-3* and soybean SCOF-1 (Figure 5B), neither of which show any DNA binding affinity. Based on this knowledge, we initially doubted whether *ZFT1* had any DNA binding activity. However, to our surprise we found that the protein bound to the EPIS sequence (Figure 7), suggesting that *ZFT1* acts as a transcription repressor through binding to the EPIS-like *cis*-element of as yet unidentified target gene(s).

NMR analysis on the single QALGGH zinc finger domain from the *A. thaliana* SUPERMAN protein revealed that residues S (−1), Q (+2), and A (+3) of the α helix could act as base determinants in the interaction of SUPERMAN with the DNA, and recognize the third, second, and first base of the DNA triplet, respectively (Isernia *et al.*, 2003). This recognition hypothesis was supported in the two fingered protein family from petunia (Yoshioka *et al.*, 2001). Those residues were conserved in the domains of *ZFT1* and *ZPT2-3* and the two zinc finger motif sequences are totally identical. Based on these structural data, DNA binding of *ZFT1* as well as of *ZPT2-3* should be expected. It is still a puzzle why *ZFT1* bound to DNA and *ZPT2-3* did not.

Role of ZFT1 in an Spm signaling pathway in tobacco plants

The present work supports the idea that *ZFT1* expression during TMV-induced HR seems to be mediated through Spm, one of the viral pathogen-induced defense signal transducers (Yamakawa *et al.*, 1998). The phenotype observed in *ZFT1*-overexpressing tobacco plants supports this idea, since constitutive expression of *ZFT1* was found to enhance TMV tolerance. To obtain more solid

evidence for our model, transgenic tobacco plant with silenced spermine synthase gene(s) may be useful. In relation to our transgenic work, it should be noted that overexpression of the *CAZFP1* gene, a possible hot pepper counterpart gene of *ZFT1*, in *Arabidopsis* plants enhanced the resistance against infection by a bacterial pathogen (Kim *et al.*, 2004).

Our previous work indicated that events downstream of Spm-triggered mitochondrial dysfunction branched into two pathways. One is dependent on SIPK/WIPK activation and the possible targets are *HSR203J*, *HMGR*, *HSR201* and *HSR515*, while the other pathway is independent of SIPK/WIPK activation and involves *HIN1* (Takahashi *et al.*, 2004b). Spm-induced *ZFT1* expression depends on the activation of MAPKs (Figure 3). In *ZFT1*-overexpressors, the expression of the two *PRs*, *HSR203J*, *HMGR*, *NtRdRP* and *HSR515* genes was unaffected (Figure 9A). This result presents two possibilities: first, another TF(s) may be involved in regulating their expression, second, the downstream pathway of SIPK/WIPK activation branches again, at least in two. Moreover, given that *ZFT1* is a transcription repressor, its most likely target gene(s) are those which are down-regulated both by Spm application and during TMV-induced HR. Identification of these genes is of great interest, not least because at some of them are likely to play a role in facilitating HR-associated cell death. Such experiments are underway using a SuperSAGE approach (Matsumura *et al.*, 2003).

The most pertinent question now is whether the Spm signaling pathway is specific to the defense against viral pathogens or whether it is also activated against whole phytopathogens, if their relationships to host plants are incompatible. Furthermore, whether the pathway contributes to abiotic stress response as well as to biotic stress response is an upcoming issue that warrants investigation.

Acknowledgements

We are grateful to Dr. H. Takatsuji (National Institute of Agrobiological Sciences) for providing a preprint before publication. We also thank to Drs. D. C. Baulcombe (Sainsbury Laboratory, John Innes Centre) and N.-H. Chua (Rockefeller

Univ.) for providing VIGS vectors and GFP plasmids. Aventis Pharma. Co. Ltd (USA) and Syngenta Corporation (Wilmington, DE, USA) are thanked for providing MDL72527 and NahG10 tobacco seeds, respectively. YT was supported by a Research Fellowship for Young Scientists from the Japan Society for the Promotion of Science. The manuscript was edited by Dr. Patrick Hughes of Bioedit.

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