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Rapid systemic accumulation of transcripts encoding a tobacco WRKY transcription factor upon wounding

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Abstract An immediate-early, transiently activated wound-responsive gene was identified in tobacco by fluorescent differential display screening. The full-length cDNA encodes a polypeptide of 356 amino acids with a relative molecular mass of 39,082 Da. The deduced amino acid sequence shows two characteristic features; a leucine-zipper motif found in the more N-terminal region and a WRKY domain containing a zinc-finger motif located in the central region. The gene was designated as *wizz* (*w*ound-*i*nduced leucine *z*ipper *z*inc finger). Northern analysis showed that upon wounding *wizz* transcripts were locally and systemically accumulated within 10 min, reached a maximum level by 30 min, and decreased thereafter to the basal level. Analyses of a WIZZ-GFP fusion protein clearly indicated that WIZZ is a nuclear factor. WIZZ specifically binds to sequences containing two TTGAC core motifs that are separated by a spacer of appropriate length. The binding activity was dependent on bivalent cations, most probably zinc. In transient reporter assays, however, WIZZ did not show transactivation activity in tobacco suspension cells, suggesting that it functions together with other components. The results indicate that WIZZ is a new transcription factor which participates in early stages of the wound response.

Key words Tobacco · Wounding · WRKY family · Transcription factor · Fluorescent differential display

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Introduction

Plants have excellent defense mechanisms to protect themselves from diverse environmental stimuli. Wounding caused by mechanical injury, insect predation or pathogen infection is one of the most crucial stresses. To cope with such wound stress, plants begin to express a subset of wound-responsive genes not only in damaged tissues (local response), but also in unwounded tissues (systemic response). Their products have various functions, including (1) the hydrolysis of microbial cell wall components by PR-2 (a β-1,3-glucanase) and PR-3 (a chitinase), (2) phenylpropanoid synthesis by phenylalanine ammonia-lyase and chalcone synthase, (3) reinforcement of the plant cell wall by extensin and peroxidase, (4) ethylene synthesis by 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase, and (5) interference with the digestive system of herbivores by means of proteinase inhibitors (PIs) (Green and Ryan 1972; Mauch et al. 1988; Hahlbrock and Scheel 1989; Kende 1993; Showalter 1993). Expression of the PI genes, in particular, has been widely used as an indicator of the wound-response pathway, although the transcripts only begin to accumulate several hours to a day after wounding.

In recent years, there has been growing interest in the molecular events that occur at very early stages of the wound response, and several immediate-early response genes that are induced within an hour after wounding have been identified. Transcripts of the tobacco *wipk* gene, which encodes a mitogen-activated protein (MAP) kinase homolog, begin to accumulate locally and systemically in leaves as early as 1 min after wounding (Seo et al. 1995). Expression of the tobacco genes *ERF3* and tomato *Twi1*, which encode, respectively, an ethylene-responsive transcription factor and a glucosyl transferase, is induced within 30 min after wounding (O'Donnell et al. 1998; Suzuki et al. 1998). Such immediate-early response genes provide valuable tools for understanding signal transduction in early wound-response pathways.

In addition, the involvement of novel genes encoding polypeptides with no strong similarity to any established gene products has been reported. *Arabidopsis ATHCOR1* gene transcripts are induced by methyl jasmonate and wounding within 30 min. ATHCOR1 has a potential ATP-/GTP-binding site, but its function remains unclear (Benedetti et al. 1998). We previously identified the *ked* gene, which codes for a highly polar polypeptide in tobacco plants, by fluorescent differential display (FDD); systemic expression of this gene becomes evident within 10 min after wounding.

To cast further light on the early stage of the wound response, we have conducted additional FDD screens. In the present paper we report the isolation and characterization of a novel wound-responsive gene, *wizz*, which encodes a new transcription factor of the WRKY family with a zinc-finger motif.

Materials and methods

Plant materials and wound treatments

Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown in a greenhouse at 23 °C under a 14/10 h light/dark photocycle. Mature leaves were wounded by cutting with a pair of scissors. The wounded and upper unwounded leaves were harvested after appropriate time intervals, immediately frozen in liquid nitrogen and stored at −80 °C. Tobacco suspension cultures (*N. tabacum* cv. Bright Yellow 2) were maintained by subculture in Murashige-Skoog medium with 30 g/l sucrose, 0.37 g/l KH_2PO_4, 1 mg/l thiamine hydrochloride and 0.2 mg/l 2,4-dichlorophenoxyacetic acid, at 23 °C in the dark, and cells from the 7th day of subculture were employed for transactivation analyses.

Fluorescent differential display and cDNA cloning

The fluorescent differential display method and following 5′-RACE were performed as described previously (Hara et al. 2000). The C10 cDNA fragment was originally obtained by PCR with the primers 5′-GTTTTTTTTTTTTTTTVC-3′ (where V is a mixture of A, C and G) and 5′-TCGGCGATAG-3′. To amplify missing 5′ ends, 5′-RACE was carried out using the specific primer 5′-TTCCTGAAATGGCAGCAGCAAGTG-3′. The almost full-length cDNA was amplified by RT-PCR with the specific primers 5′-TACACCTGAAGAAGGATTTTAAGC-3′ and 5′-GTTGGAAAACATTGAACAATTGTC-3′, and cloned into the pGEM-T Easy vector (Promega), to give pGEM-C10P. The nucleotide sequence was determined by the dideoxy chain-termination method (ABI PRISM Dye Terminator). Editing of DNA sequences and amino acid sequences were carried out using the GeneWorks software (IntelliGenetics), and sequence comparisons were made using non-redundant databases and the BLAST program on network servers (Altschul et al. 1990).

Northern analysis

Total cellular RNA was isolated by the aurin tricarboxylic acid (ATA) method (Gonzalez et al. 1980) and used for northern analysis. Total RNA samples were size-fractionated by electrophoresis on formaldehyde/1% agarose gels, and transferred to nylon membranes (Hybond-N, Amersham). After crosslinking by irradiation with UV, the membranes were subjected to hybridization in a solution containing 1 mM EDTA, 0.5% SDS, 50 mM TRIS-HCl (pH 7.5), 1 × Denhardt's, 3 × SSC, 50% formamide, 10% dextran sulfate and 0.1 mg/ml denatured salmon sperm at 42 °C for 16 h. The cDNA probes were labeled with [α-^{32}P]dCTP by a random labeling method (BcaBEST Labeling Kit, Takara Shuzo). After washing twice in a solution containing 2×SSC and 0.1% SDS for 20 min at 62 °C, and once in a solution containing 0.5×SSC and 0.1% SDS for 20 min at 62 °C, the membranes were autoradiographed with x-ray film. Signals were also visualized with a bioimage analyzer (BAS-2000, Fuji). The cDNA for *wipk* was provided by Y. Ohashi and those for *PI-II* and *actin* were prepared by PCR in our laboratory.

Plasmid construction

pGEM-C10P was digested with *Eco*RI and the *wizz* cDNA fragment was subcloned into the *Eco*RI site of the pGEX-4T-1 expression vector (Pharmacia) to create pGEX-WIZZ, encoding the GST-WIZZ fusion protein. *E. coli* DH5α cells carrying pGEX-4T-1 or pGEX-WIZZ were cultured in LB broth and treated with 1 mM IPTG for induction of GST or GST-WIZZ synthesis. The harvested cells were disrupted by sonication, and centrifuged. Clear lysates were used for gel-shift assays.

The *wizz* ORF, flanked by *Xba*I (upstream of the start codon) and *Bam*HI (just upstream of the stop codon) sites, was obtained by PCR amplification and verified by direct sequencing. This *Xba*I-*Bam*HI fragment was inserted into the *Xba*I/*Bam*HI site of the vector pGFP-2 (provided by N.-H. Chua and P. Spielhofer), resulting in pWIZZ-GFP2, which encodes WIZZ fused to the N terminus of GFP.

The promoter-less reporter plasmid PL-LUC was created by total deletion of the cauliflower mosaic virus (CaMV) 35S promoter by *Hin*dIII digestion of the 221-luc+ vector (provided by K. Hiratsuka), which carries the 35S promoter, the *luciferase* (*LUC*) gene, and the *nos* terminator. To generate the plasmid −46-LUC plasmid, a minimal 35S promoter, truncated to position −46, was amplified by PCR with incorporation of *Hin*dIII and *Bgl*II sites at the 5′ end and an *Nco*I site at the 3′ end, respectively. The resulting *Hin*dIII-*Nco*I fragment was inserted into the *Hin*dIII/*Nco*I site of 221-luc+. Three copies of the BS65 sequence (de Pater et al. 1996) were placed in the *Hin*dIII/*Bgl*II site of −46-LUC to create the 3BS-46-LUC plasmid. The effector plasmids 35S-WIZZ and 35S-anti-WIZZ were constructed as follows. The *Not*I fragment derived from pGEM-WIZZ (see above) was cloned into the *Not*I site of pBlueScript II SK (+) (Stratagene) in both orientations. Then the respective *Xba*I-*Sac*I fragments were subcloned into *Xba*I/*Sac*I site of the pBI221 vector (Clontech).

Gel mobility shift assays

Gel shift assays were performed essentially as described earlier (Kusano et al. 1995). The IMU sequence is the mutated derivative of a motif I, which contains a G-box-related core sequence (Salinas et al. 1992). The BS65 sequence is the optimal binding site for the *Arabidopsis* WRKY protein ZAP1 (de Pater et al. 1996). The AS1 sequence is the *as-1* element found in the CaMV 35S promoter (Lam et al. 1989). Monomeric double-stranded probes (depicted in Fig. 6a) were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labeled DNA probe (100 fmol) was mixed with *E. coli* crude extract (3 μg protein each), and competitors or EDTA as appropriate, in a 15-μl reaction volume. After a 15-min incubation at 30 °C, the sample was subjected to electrophoresis.

Particle bombardment

BY-2 cells were spread on MS agar medium and the liquid medium was allowed to evaporate. In addition, a 3 cm square fragment of scaly onion leaf fragment was placed on moist paper in a Petri dish. Particle bombardment was performed with the PDS-1000, according to the manufacturer's instructions (Bio-Rad), using gold particles (1.0 μm diameter) coated with plasmids according to the manufacturer's protocol. For transactivation analysis, an appropriate pair of reporter (0.67 μg) and effector (0.67 μg) plasmids was

introduced by bombardment, together with the reference pRTL2-GUS vector (provided by J. Carrington) (0.33 μg) carrying two copies of the 35S promoter and a translation leader enhancer sequence (Restrepo et al. 1990). The plated cells or the onion leaf were placed at a distance of 6 cm or 9 cm from the stopping screen and bombarded twice per sample under a slight vacuum (28 in. of mercury) using a helium pressure of 1100 psi to accelerate the macrocarrier. Bombarded cells and the leaf were kept in the dark for 16 h at 23 °C prior to analysis.

Transactivation assay

Luciferase and GUS activities were measured using a luciferase assay system (PicaGene; Toyo) and a GUS assay system (AURORA GUS; ICN Pharmaceuticals) with a luminometer (Lumat LB9507; Berthold).

Results

Identification of a novel gene that responds to wounding in a rapid and transient manner

We previously identified eight genes that are activated in early stages of the wound response by fluorescent differential display (FDD) screening (Hara et al. 2000). Extended FDD screening identified a new clone (C10), 284 bp long, whose cognate transcript accumulated in a similar manner to those of *ked* and *wipk* (Seo et al. 1995; Hara et al. 2000). Responsiveness to wounding was confirmed by RNA blot analysis (Fig. 1).

WIZZ, a new zinc-finger protein of the WRKY family

The nearly full-length cDNA of C10, obtained by 5′-RACE and following RT-PCR, was found to be 1251 bp long and to contain an ORF encoding a protein of 356 amino acids in length, with a predicted relative molecular mass of 39,082 Da (Fig. 2). Within the 5′ untranslated region, two in-frame stop codons are found,

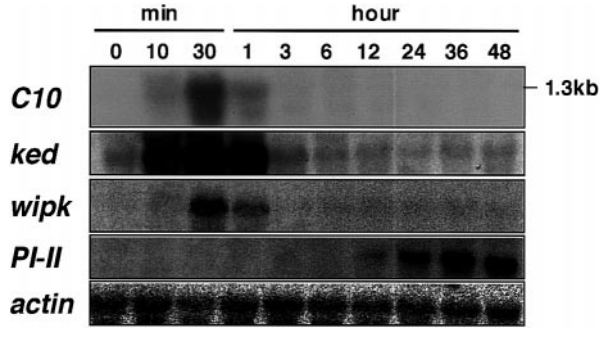

Fig. 1 Rapid and transient accumulation of the C10 transcript upon wounding. Healthy leaves were detached and wounded by cutting into pieces with a pair of scissors, and then floated on water. The wounded leaves were harvested at the indicated time points. Blots containing 35 μg of RNA per lane were subjected to hybridization with the indicated probes

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ATTAATTACA CCTGAAGAAG GATTTTAAGC TTTTTGGATT TGTTGGAATT TTATTAAGAA     60

GAAATTAAAG CAATGGAATT CACAAGTTTG GTTGATACTT CCTTGGATTT GAGTTTTAGA    120
            M  E  F   T  S  L   V  D  T  S   L  D  L   S  F  R

CCTCTTCCAG TTCTTGATAA AGTGCTGAAA CAAGAAGTTC AGAGTAATTT CACTGGATTG    180
P  L  P  V   L  D  K   V  L  K   Q  E  V  Q   S  N  F   T  G  L

AGCAGAGACA ATATGCTGGT GAAAGATGAG GCAGGTGATT TGTTGGAGGA ACTGAACAGA    240
S  R  D  N   M  L  V   K  D  E   A  G  D  L   L  E  E   L  N  R

GTGAGCAGTG AAAACAAGAA ACTAACAGAG ATGCTCACAG TGGTGTGTGA AAATTACAAT    300
V  S  S  E   N  K  K   L  T  E   M  L  T  V   V  C  E   N  Y  N

GCATTAAGAA ACCAACTAAT GGAGTATATG AACAACCAGA ATAATGGTGT AGTAGATGAT    360
A  L  R  N   Q  L  M   E  Y  M   N  N  Q  N   N  G  V   V  D  D

AGTGCTGGAT CAAGGAAAAG AAAAGCTGAA AATATCTCCA ATCCCAACAA CAACAACAAC    420
S  A  G  S   R  K  R   K  A  E   N  I  S  N   P  N  N   N  N  N

AACAAAAACA ACAACTTGGA TATTGTTTGT GGACGTTTAT CAGAAAGCAG TTCAAGTGAT    480
N  K  N  N   N  L  D   I  V  C   G  R  L  S   E  S  S   S  S  D

GAAGAGTCTT GTTGCAAGAA ACCTAGAGAA GAGCACATAA AAACTAAGGT TTCTGTCGTT    540
E  E  S  C   C  K  K   P  R  E   E  H  I  K   T  K  V   S  V  V

TCTATGAGGA CAGAAGCATC TGATACCTCT CTTATTGTAA AGGATGGTTA TCAATGGAGG    600
S  M  R  T   E  A  S   D  T  S   L  I  V  K   D  G  Y   Q  W  R

AAATATGGTC AGAAAGTAAC TAGAGACAAT CCTTCTCCAA GAGCTTACTT CAGGTGCTCT    660
K  Y  G  Q   K  V  T   R  D  N   P  S  P  R   A  Y  F   R  C  S

TTTGCTCCTG GCTGCCCCGT CAAGAAAAAG GTGCAAAGAA GCATAGAAGA TCAGTCAGTT    720
F  A  P  G   C  P  V   K  K  K   V  Q  R  S   I  E  D   Q  S  V

GTGGTGGCAA CATATGAAGG AGAGCATAAC CATCCAGTAA ACCCTTCAAA ACCAGAGGCT    780
V  V  A  T   Y  E  G   E  H  N   H  P  V  N   P  S  K   P  E  A

GCTGCTGGTA CTGCTACTTC CACCGGCAGC CGTTTAAATG TGAGAACTAT TGGGGGTACT    840
A  A  G  T   A  T  S   T  G  S   R  L  N  V   R  T  I   G  G  T

ACAGCTTCAG TCCCTTGCTC TACCACTCTC AATTCATCAG GACCAACCAT TACTCTCGAT    900
T  A  S  V   P  C  S   T  T  L   N  S  S  G   P  T  I   T  L  D

CTTACTGAAC CTACAACAGT AGCAAAAGGC GATATCATGA AGATGAGTAG CAGTATTAGT    960
L  T  E  P   T  T  V   A  K  G   D  I  M  K   M  S  S   S  I  S

CCTACAGGTG GCAGTAGCCA AAGAACAACA GAAGGTGATC ACTATAGTAG GCCAGAGTTT   1020
P  T  G  G   S  S  Q   R  T  T   E  G  D  H   Y  S  R   P  E  F

CAACAGTTCT TGATAGAGCA AATGGCTTCT TCATTGACTA AAGATCCAAG TTTCAAAGCA   1080
Q  Q  F  L   I  E  Q   M  A  S   S  L  T  K   D  P  S   F  K  A

GCACTTGCTG CCGCCATTTC AGGAAAAATT CTCCAACATA ATAATCAAAC AAGTAGATGG   1140
A  L  A  A   A  I  S   G  K  I   L  Q  H  N   N  Q  T   S  R  W

TAAACAAAAG TCCTGCACAC CAGTCAATTT CTATTTATGG ATAGGCTAGA CAATTGTTCA   1200
*

ATGTTTTCCA ACATAGACTC ATTTGGAAAG TTCAGCAAAA AAAAAAAAAA A            1251
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Fig. 2 DNA sequence of the *wizz* cDNA, together with the deduced amino acid sequence. The nucleic acids are presented on the *top line* and the derived one-letter amino acid sequence is shown *below* it. The stop codon is indicated by the *asterisk*. The hydrophobic residues in the putative leucine-zipper motif are *doubly underlined*. The WRKY domain is shown in *italics*. The two cysteines and two histidines in the zinc-finger motif are *circled*. The original C10 clone obtained by FDD screening is *underlined*. The nucleotide sequence has been deposited in the GenBank, EMBL and DDBJ databases under the Accession No. AB028022 (wizz cDNA)

suggesting that the cDNA contains the entire coding region. The inferred amino acid sequence has two striking characteristics: it contains a conserved domain, the WRKY domain – so called after the sequence (N)-**WRKY**GQK-(C) in the parsley protein WRKYs (Rushton et al. 1996), in its central region and a heptad leucine repeat in the N-terminal region (Fig. 3a). Thus the gene was designated as *wizz* (*w*ound-*i*nduced leucine-*z*ipper and *z*inc-finger protein). The WRKY domain is shared by SPF1 from sweet potato (Ishiguro and Nakamura 1994), ABFs from oat (Rushton et al. 1995),

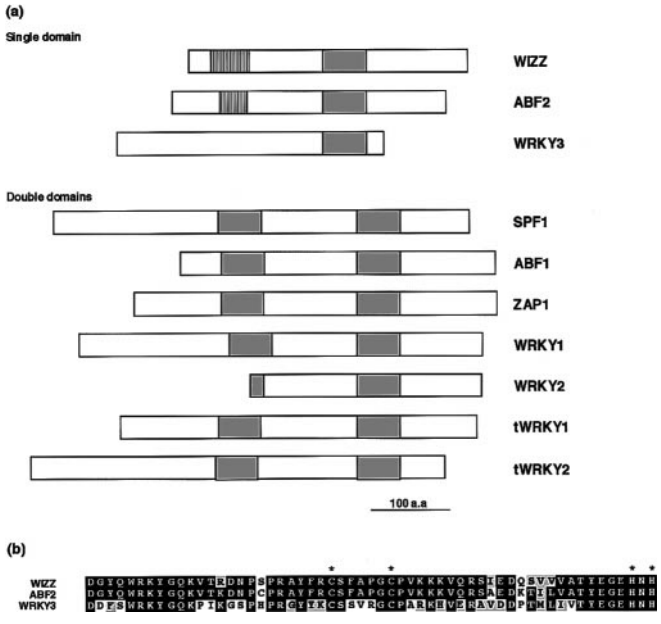

Fig. 3a, b Structural features of WIZZ. **a** Schematic representation of WIZZ and other proteins of the WRKY. The WRKY domains are *shaded* and the leucine-zipper regions are *striped*. The *scale bar* represents a length of 100 residues. **b** Alignment of WRKY domains from WIZZ, ABF2 and WRKY3. Identical and similar residues are highlighted in *black* and *gray*, respectively. The two cysteines and two histidines of the putative zinc finger are indicated by *asterisks*

WRKYs from parsley (Rushton et al. 1996), ZAP1 from *Arabidopsis* (de Pater et al. 1996), and tWRKYs from tobacco (Wang et al. 1998). These WRKY proteins are classified into two groups, a single-domain group and a two-domain type. WIZZ, ABF2 and WRKY3 proteins belong to the former, whereas SPF1, ABF1, ZAP1, WRKY1, WRKY2, tWRKY1 and tWRKY2 fall into the latter class (Fig. 3a). The WRKY domain contains a zinc-finger motif consisting of two cysteines and two histidines. A sequence alignment of WRKY domains among WIZZ, ABF2 and WRKY3 is presented in Fig. 3b. ABF2, but not WRKY3, also has a leucine-zipper motif in the distal region close to the N-terminus. As shown in the schematic illustration, WIZZ resembles ABF2 in structure (Fig. 3a).

Rapid and systemic accumulation of *wizz* transcripts upon wounding

Wound-responsive transcripts often accumulate not only locally but also systemically following wounding (Seo et al. 1995; Hara et al. 2000). To determine whether this is also the case for the *wizz* gene, Northern analysis was performed using total RNAs prepared from wounded and unwounded leaves (Fig. 4). When the full-length *wizz* cDNA was used as a probe, at least three transcripts of different sizes were detected. Judging from its molecular size, we consider that the middle-sized transcript to correspond to the *wizz* cDNA. All of them show both a local and systemic response upon wounding.

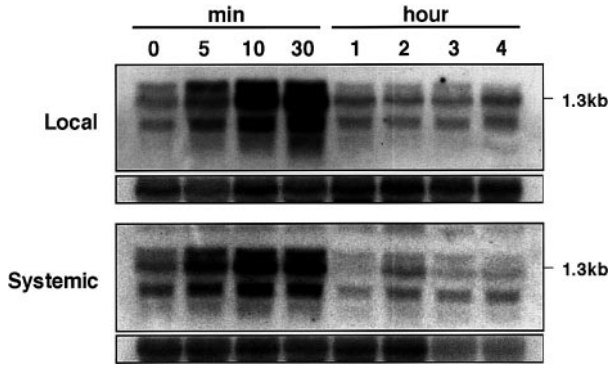

Fig. 4 Local and systemic induction of *wizz* expression by wounding. Healthy leaves were wounded by cutting. The wounded (Local) and upper unwounded (Systemic) leaves were harvested at the indicated time points. The blot, containing 40 μg of RNA per lane, was subjected to hybridization with the full-length *wizz* cDNA (*upper panel*) or an actin probe (*lower panel*)

Nuclear localization of the WIZZ protein

PSORT analysis (Nakai and Kanehisa 1992) indicated a high likelihood of nuclear localization for the WIZZ protein. To confirm this, we constructed a CaMV 35S::WIZZ-GFP gene fusion product that could be used in transient assays. After biolistic bombardment of onion epidermis with a CaMV 35S::GFP control construct (pGFP-2), GFP signals were observed in both the cytoplasm and the nucleus (Haseloff et al. 1997) (Fig. 5). In contrast, the WIZZ-GFP fusion protein was found

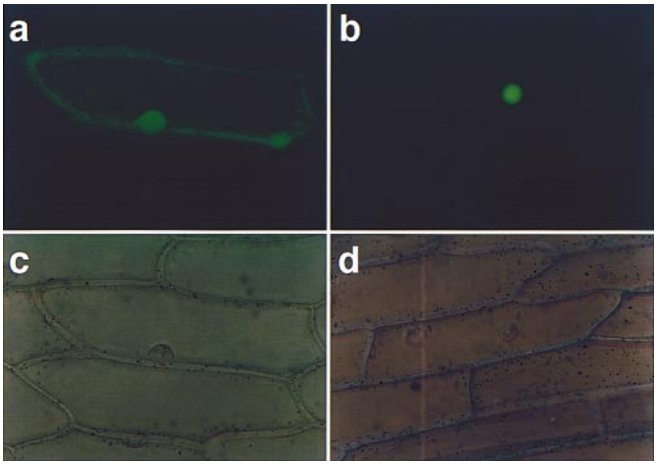

Fig. 5a–d Nuclear localization of the WIZZ-GFP fusion protein in onion cells. **a, c** Onion epidermis cells transfected with GFP-expressing plasmids. **b, d** Onion epidermis cells transfected with plasmids expressing a WIZZ-GFP fusion protein. Green fluorescence under UV light excitation (**a** and **b**) or corresponding differential interference contrast images (**c** and **d**) are shown at the same magnification

exclusively in the nucleus, indicating that WIZZ is a nuclear factor (Fig. 5).

DNA-binding specificity of WIZZ

Since WRKY proteins have been reported to bind DNA, we investigated whether WIZZ does so by a gel mobility shift assay using an *E. coli* crude extract containing WIZZ fused to GST. As in the previous report on ZAP1 (de Pater et al. 1996), three probes, BS65, IMU and AS1, containing two TGAC sites with variable neighboring nucleotides, were primarily tested (Fig. 6a). The GST-WIZZ fusion protein efficiently bound to the BS65 and the IMU probes, but not to AS1. Binding to BS65 and IMU was dramatically reduced by addition of EDTA or the corresponding unlabeled competitor probes. When both TGAC core sequences in BS65 were mutated, the fusion proteins were found not to bind, indicating that the core TGAC motif is important (Fig. 6b). The three probes tested all had two TGAC core sequences, but differed in the spacer and flanking regions. To clarify the DNA binding specificity of WIZZ, further gel mobility shift assays were performed using modified probes. Thus, the distance between the two core TGAC sites was varied (BS65s4–BS65s8). The results showed that the GST-WIZZ protein preferred the BS65s5, BS65s6 and BS65s8 sequences to BS65s4 and BS65s7. When the two TGAC motifs, CTGACG and ATGACG, in AS1 were changed to TTGACC and TTGACT, respectively, to yield AS1m, the WIZZ protein was found to be capable of binding this probe (Fig. 6c). To examine whether or not both TGAC motifs in the BS65 probe are required for WIZZ binding, each core site was appropriately mutated (to BS65tm and BS65mt). Signal intensities for each binding complex were considerably decreased in comparison with the intact BS65 (Fig. 6d).

Lack of transactivation activity of WIZZ

To test for transactivation by WIZZ, a fusion construct encoding the GAL4 DNA binding domain and WIZZ in-frame was created and introduced into yeast cells carrying two reporter plasmids consisting of the GAL4 binding sequence upstream of the *lacZ* and *HIS3* genes, respectively. No transactivation activity of WIZZ was detected in this yeast system (data not shown). Transactivation activity of WIZZ was also assayed using

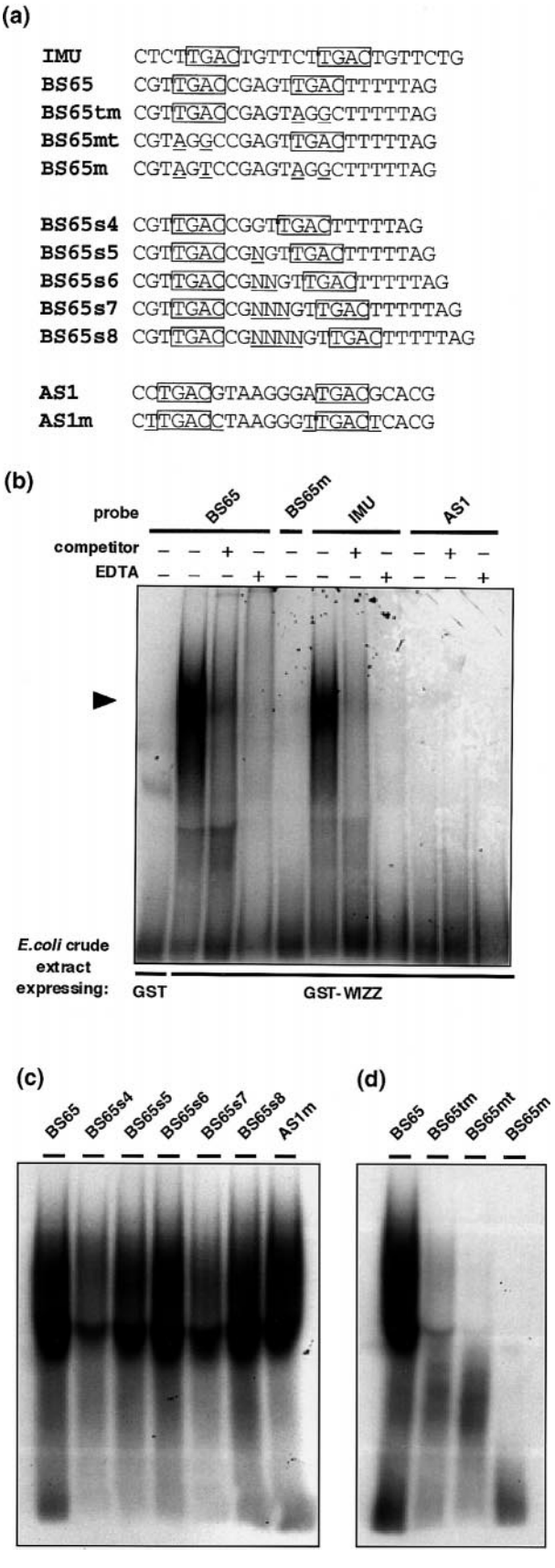

Fig. 6a–d Gel mobility shift assays. **a** Nucleotide sequences of DNA probes; TGAC motifs are *boxed*. Modifications of the BS65 and the AS1 probes are *underlined*. N indicates any base (A, C, G, or T). **b** Effects of a competitor and a chelator on the DNA-binding activity of WIZZ. Gel shift assays were performed with (+) or without (–) unlabeled competitor BS65, IMU, or AS1 in 100-fold molar excess or in the presence of 50 mM EDTA. DNA binding complexes of WIZZ are indicated by the *arrowhead*. **c** Effects of probe modification on the DNA binding activity of WIZZ. **d** Effects of varying the number of TGAC core sites in the BS65 probe

tobacco BY-2 cells as follows. Three reporter plasmids (PL-LUC, –46-LUC and 3BS-46-LUC) and two effector plasmids (35S-WIZZ and 35S-anti-WIZZ) were constructed and transfected into tobacco suspension culture cells (Fig. 7). Co-transfection of the 3BS-46-LUC reporter with the WIZZ binding sites and the 35S-WIZZ effector plasmid failed to reveal transactivation by WIZZ. Similarly, co-transfection with the 35S-anti-WIZZ effector plasmid did not affect the reporter activity in the presence or the absence of the WIZZ binding sites, indicating that WIZZ alone cannot activate gene expression.

Discussion

The present FDD screen of immediate-early wound-responsive genes resulted in the isolation of a gene encoding a new WRKY protein, designated as *wizz*. Several genes for members of the WRKY protein family have recently been identified in potato (Ishiguro and Nakamura 1994), wild oat (Rushton et al. 1995), parsley (Rushton et al. 1996), *Arabidopsis* (de Pater et al. 1996), and tobacco (Wang et al. 1998) (Fig. 3a). Furthermore, dozens of similar genes have been found in the *Arabidopsis* genome. In tobacco plants, at least seven *WRKY* genes are present including *wizz*, five previously reported homologs (Wang et al. 1998; Accession Nos. AB022693, AB020590, AB020023), and another member that responds to TMV infection which we ourselves isolated recently (unpublished data).

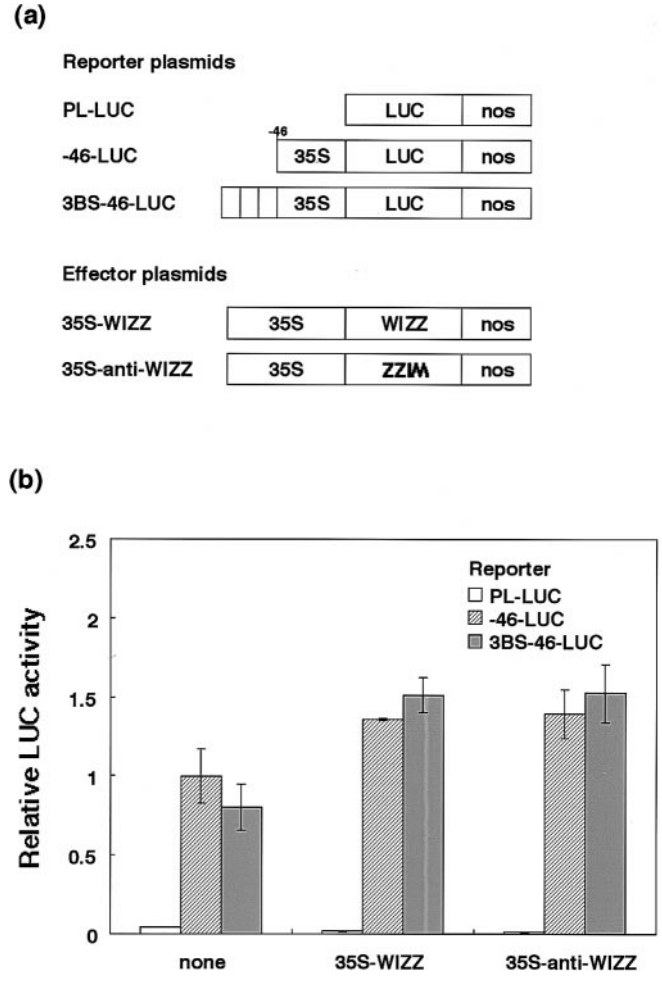

Fig. 7a, b Transactivation analysis of WIZZ in BY-2 cells. **a** Schematic representation of reporter and effector plasmids. **b** Transcriptional activity. The reporter and effector plasmids were introduced into BY-2 cells by particle bombardment, together with the reference vector to monitor the transformation efficiency. Reporter activity is normalized with respect to the reference activity. The reporter activity with the −46-LUC plasmid alone was set to 1. Three independent experiments were carried out. The *vertical bars* represent ±SE ($n=3$)

The WRKY protein family is divided into two groups based on the number of WRKY domains present. The WIZZ protein has a single WRKY domain, as do ABF2 from wild oat and WRKY3 from parsley, and a leucine zipper motif in the N-terminal region, like ABF2. However, it does not contain the PVQ repeats found in WRKY3. The conserved WRKY domain of WIZZ is more closely related to that of ABF2 than WRKY3, suggesting that the WRKY subfamily with a single domain may be further subdivided on the basis of characteristic motifs, a leucine zipper or PVQ repeats.

RNA blot analysis with probes containing the conserved WRKY domain showed that at least three kinds of transcripts were locally and systemically induced by wounding. The transcripts of large size were not detected by the C10 probe, which lacked the conserved sequence encoding the WRKY domain, suggesting that they were derived from the another member of the WRKY family. The small-sized transcripts may correspond to derivatives of the *wizz* transcript that arise by degradation or alternative splicing. Their patterns of accumulation were very similar, indicating control by a common regulatory system. In parsley cells, two *WRKY* transcripts are rapidly induced by elicitor treatment (Rushton et al. 1996). Of these, the *WRKY1* gene was recently reported to be expressed in response not only to elicitors but also to mechanical stress (Gus-Mayer et al. 1998). Further study should shed light on the control of gene expression during the early stages of various defense responses, especially systemic responses to wounding.

The results of the gel mobility shift assays reported here showed that WIZZ, like *Arabidopsis* ZAP1, binds to both BS65 and IMU probes, and that such binding is abolished by the metal-ion chelator EDTA. This indicates that specific WIZZ binding to DNA is dependent on bivalent cations, most probably zinc. The probes employed contain two common TGAC core sequences, modification of which completely inhibited binding activity. Also the number of nucleotides between the two core TGAC motifs greatly affected binding. For example, probes with 5, 6 and 8 spacer nucleotides are bound equally well, whereas those with 4 and 7 are not. However, WIZZ did not bind the AS1 probe, which also contains two TGAC sites with an 8-nucleotide spacer, suggesting that the flanking region and/or the length of the spacer between the two TGAC sites is important for

recognition by WIZZ. Indeed, WIZZ did show binding to AS1 m, in which the nucleotides flanking the TGAC sites in the AS1 probe have been modified. These observations suggest that WIZZ binds specifically to sequences containing two TTGAC(T/C) motifs separated by a spacer of appropriate length. This assumption explains the binding specificity of the WRKY family proteins, despite their common TGAC recognition sequence. Although WIZZ could bind to probes with a single TGAC site (BS65tm and BS65mt), the binding activity was synergistically increased if further TGAC sites were present nearby. This is consistent with observations based on the mutational analysis of parsley *WRKY1* promoter: multiple W-Box elements containing TTGAC(C/T) motifs generated a synergistic effect on gene expression (Eulgem et al. 1999). This suggests that protein-protein interaction, probably homologous dimerization through the leucine zipper motif, may be critical for binding. Recently, a number of studies have pointed to the importance of protein-protein interactions for the WRKY family. In tobacco plants, DNA binding activity induced by TMV infection (TDBA12) could be abolished with a protein dissociating agent, sodium deoxycholate (Yang et al. 1999). The binding activity of potato PBF-1, which binds the elicitor-responsive element in the *PR-10a* promoter, was induced by elicitor and wound treatment and also proved to be sensitive to sodium deoxycholate (Després et al. 1995). Since this element of the promoter contains two TGAC sites, PBF-1 is assumed to be a member of the WRKY family. Moreover, DNA sequences bound by WRKY family members contain multiple TGAC core sites. For example, BS65, the optimal binding sequence for ZAP1, contains two TGAC sites (de Pater et al. 1996), and Box 2, a conserved element in the promoter of α-amylase genes, contains three TGAC sites recognized by ABFs (Rushton et al. 1995).

Among putative WRKY protein family members, only *Arabidopsis* ZAP1 and parsley WRKY1 have so far been shown to act as transcriptional activators in yeast and plant cells (de Pater et al. 1996; Eulgem et al. 1999). In the present study, WIZZ did not transcriptionally activate reporter genes, consistent with a lack of putative activation domains, such as a proline-rich domain. This suggests that a co-activator, which may heterologously interact with WIZZ, is necessary for transcriptional activation. We cannot, however, eliminate the possibility that WIZZ might be activated by post-translational modification, since both TDBA12 and the binding activity of PBF-1 were affected by alkaline phosphatase treatment, suggesting the involvement of protein phosphorylation.

The TGAC core motif was originally identified as an elicitor-responsive element in parsley *PR1-1*, a member of the PR-10 class of genes (Meier et al. 1991). Later this sequence was also found in the elicitor-responsive element of the maize *PRms* gene (Raventos et al. 1995) and in a tobacco gene for basic class I chitinase (Fukuda and Shinshi 1994; Fukuda 1997), so that it may be conserved in both dicot and monocot plants. Further analyses have revealed that this sequence is located in the promoter regions of various wound-response-associated genes encoding tobacco osmotin (Nelson et al. 1992), chitinase (van Buuren et al. 1992; Ohme-Takagi et al. 1998), basic β-1,3-glucanases (Linthorst et al. 1990), PI-II (Balandin et al. 1995) and basic PR-1 (Eyal et al. 1992). Moreover, elicitor- and wound-responsive regions of the tobacco osmotin gene contain two TTGACC sequences (Raghothama et al. 1993), and DNA binding activity was also found to be induced by TMV infection (Wang et al. 1998; Yang et al. 1999). Such a broad but defined distribution of the TGAC sequence suggests that it is a common *cis*-acting element and that WRKY family members function as *trans*-acting factors during general defense responses. In fact, accumulation of WRKY1 transcripts in parsley and DNA binding activity of potato PBF-1 were induced not only by a treatment with elicitor but also by mechanical stimulation (Després et al. 1995; Gus-Mayer et al. 1998). Understanding the complex interactions of the WRKY family should facilitate the elucidation of the general mechanisms underlying activation of defense-related gene expression in plants.

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