Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors

Thomas Eulgem, Paul J. Rushton,
Elmon Schmelzer, Klaus Hahlbrock and
Imre E. Somssich¹

Max-Planck-Institut für Züchtungsforschung, Abteilung Biochemie,
Carl-von-Linné Weg 10, D-50829 Köln, Germany

¹Corresponding author
e-mail: somssich@mpiz-koeln.mpg.de

Parsley WRKY proteins comprise a family of plant-specific zinc-finger-type factors implicated in the regulation of genes associated with pathogen defence. *In vitro*, these proteins bind specifically to functionally defined TGAC-containing W box promoter elements within the Pathogenesis-Related Class10 (PR-10) genes. Here we present *in vivo* data demonstrating that WRKY1 is a transcriptional activator mediating fungal elicitor-induced gene expression by binding to W box elements. *In situ* RNA hybridization revealed that the WRKY1 gene is rapidly and locally activated in parsley leaf tissue around fungal infection sites. Transient expression studies in parsley protoplasts showed that a specific arrangement of W box elements in the WRKY1 promoter itself is necessary and sufficient for early activation and that WRKY1 binds to such elements. Our results demonstrate that WRKY transcription factors play an important role in the regulation of early defence-response genes including regulation of WRKY1.

Keywords: *cis*-acting elements/fungal elicitor/
Petroselinum crispum/Phytophthora sojae/zinc-finger
protein

Introduction

Reprogramming of cellular functions in response to external stimuli involves complex changes in gene expression. The activities of a multitude of genes are subject to up- or down-regulation and follow defined temporal programmes. The perception of an external stimulus immediately leads to the activation of primary-response genes. Activation of these genes is mediated by pre-existing signalling components including transcription factors. In some cases, the accumulation of the corresponding mRNA is not only very rapid but also transient in nature. The activation of a subset of these immediate-early genes, encoding signal proteins and transcription factors, is a prerequisite for the subsequent activation of secondary-response genes (Herschman, 1991).

In plants, the regulation of genes responsive to external stimuli, including light and various kinds of environmental stress, is being studied intensively. Our interest focuses on the regulatory mechanisms controlling the defence

response of plants to invading pathogens. The molecular recognition of a pathogen, a process involving ligand–receptor interactions (Nürnberger *et al.*, 1994), is followed by several waves of gene activation (Somssich and Hahlbrock, 1998). Much of the underlying research has been performed using cultured plant cells stimulated with pathogen-derived signal molecules called elicitors. Accumulation of numerous mRNA species can be observed in such cell culture systems as well as locally or systemically in infected plants. The promoters of several genes that are activated by pathogen infection or elicitor treatment have been characterized at the molecular level (Rushton and Somssich, 1998).

Previously, we identified three parsley (*Petroselinum crispum*) DNA-binding proteins, called WRKY1, -2 and -3, implicated in the regulation of the parsley PR1 genes encoding PR-10 class proteins (Rushton *et al.*, 1996). These three WRKY proteins bind specifically *in vitro* to functionally defined elicitor-response elements of the W box type [(T)TGAC(C)], designated W1, W2 and W3, present in PR1 promoters. As with PR1, the mRNAs corresponding to WRKY1 and WRKY3 are induced in cultured parsley cells in response to fungal elicitor treatment. This induction occurs rapidly and transiently and precedes that of PR1 mRNA by ~2 h. Induction of WRKY1 mRNA is most pronounced. Being undetectable in the absence of elicitor, the WRKY1 mRNA level reaches almost 50% of its maximum value within 15 min after application of elicitor to the cells.

WRKY proteins have been identified in a variety of plant species and appear to be restricted to the plant kingdom (Ishiguro and Nakamura, 1994; Rushton *et al.*, 1995; de Pater *et al.*, 1996; Kim *et al.*, 1997; Wang *et al.*, 1998). Most of these proteins were shown to bind *in vitro* to W box-type DNA sequences. One common feature of all WRKY proteins is the occurrence of either two (group 1) or one (group 2) copies of the highly conserved WRKY domain consisting of ~60 amino acids. This domain carries, in addition to a novel type of zinc finger motif (C-X₄₋₅-C-X₂₂₋₂₃-H-X-H) at its C-terminus, the N-terminal sequence WRKYGQK, which is strictly conserved in all WRKY domains. Metal-chelating agents such as EDTA and 1,10-*o*-phenanthroline impair binding of WRKY proteins to their DNA target sequence, demonstrating a requirement for metal ions for binding (Rushton *et al.*, 1995; de Pater *et al.*, 1996). Furthermore, in the case of the sweet potato and *Arabidopsis* WRKY proteins, SPF1 and ZAP1, the major *in vitro* DNA-binding activity has been mapped to regions comprising 163 and 149 amino acids, respectively, each of which carries the more C-terminal one of the two WRKY domains (Ishiguro and Nakamura, 1994; de Pater *et al.*, 1996). Outside of the domains there are no sequence similarities between individual members of this protein family, although they do

Fig. 1. *In situ* localization of WRKY1 mRNA in parsley leaf buds inoculated with zoospores of *P. sojae*. (A and B) Cross-section through an infection site 6 h post-inoculation. (C and D) Cross-section through an infection site 12 h post-inoculation. Infection sites (arrows) were identified under UV-epifluorescent light (A and C). The same sections were hybridized with WRKY1 antisense RNA (B and D). Bar = 100 μ m.

share typical features of transcription factors, e.g. putative transcriptional activation domains and putative nuclear localization signals (NLS). A large number of EST sequences encoding WRKY proteins are present in the database, suggesting great complexity of this gene family.

In this study we report on the cloning of the *WRKY1* and *WRKY3* genes from parsley and present a detailed functional dissection of the *WRKY1* promoter in the homologous system. We show that a novel arrangement of palindromically positioned W boxes functions as an extremely rapid-acting elicitor-response element important for mediating early *WRKY1* expression. In addition, we present an extensive *in vivo* functional characterization of the *WRKY1* protein revealing that it acts as a pathogen-induced transcription factor capable of transactivating via binding to W boxes within the promoters of both its own and the *PR1* target genes. A model is proposed implicating WRKY transcription factors in the regulation of two successive steps in the elicitor-induced gene activation cascade.

Results

Pathogen-induced WRKY1 expression in planta

To study *WRKY1* expression in the true plant–pathogen interaction, young primary parsley leaf buds were infected with *Phytophthora sojae*, the fungal source of the elicitor used to induce defence reactions in leaves and cultured parsley cells. Figure 1 shows the results of *in situ* RNA hybridizations at 6 or 12 h post-inoculation with fungal zoospores. Strong signals were detected for *WRKY1* mRNA locally around the sites of fungal infections in cross-sections 6 h after inoculation (Figure 1A and B). In contrast, little *WRKY1* mRNA above background occurred in tissue areas more distant from the infection site, in other parts of the uninfected leaf bud and in sections 12 h post-inoculation even at the infection site (Figure 1C and

Fig. 2. Structure of *WRKY1* and -3. (A and B) Restriction maps of *WRKY1* (A) and *WRKY3* (B). Restriction sites used for subcloning and for the generation of promoter–GUS constructs are indicated. Exons are represented by grey boxes. The transcription start sites are marked +1. (C) Sequences of the *WRKY1* promoter up to position –175, the 5' untranslated region and the first six codons present in all *WRKY1* promoter–GUS constructs. The 5' end points of promoter deletions are indicated by triangles. A putative GCC box motif is marked by dots and three W boxes are boxed. The transcriptional start site is indicated as a bold letter and the translational start site in underlined bold letters. (D) Comparison of *WRKY1* and *WRKY3* promoter regions carrying palindromes of W box motifs (boxed). Arrows indicate the orientation of individual W boxes.

D). Qualitatively identical results were obtained using either the entire *WRKY1* cDNA or a 3' gene-specific fragment as probe. Thus, pathogen-induced *WRKY1* expression is local, rapid and transient *in planta*.

The parsley WRKY1 and WRKY3 genes

Screening of a genomic parsley library using a mixture of *WRKY1*, -2 and -3 cDNAs as probes resulted in the isolation of several independent genomic clones. One clone each for *WRKY1* and *WRKY3* was further characterized (Figure 2A and B). A 4.8-kb region of *WRKY1* was sequenced and found to carry the entire coding region present on five exons together with 1.8 kb of upstream region (Figure 2A). Sequencing of 6.1 kb of the *WRKY3* region revealed that it contains 4 kb of upstream sequence, the first two exons and a portion of exon 3, but lacks 302 bp of 3' coding region (Figure 2B). DNA-blot analysis with genomic parsley DNA suggested that *WRKY1* and *WRKY3* are single copy genes. In the case of *WRKY1*, we found an allelic RFLP downstream of the gene, which segregated in 10 individual parsley plants and the cell culture as a single locus (data not shown). In contrast to the 989-bp coding sequence of *WRKY3*, which was found to be identical to the *WRKY3* cDNA sequence, the *WRKY1* ORF differed in three positions from the *WRKY1* cDNA (positions 360, 492 and 525 of the cDNA; Rushton *et al.*, 1996). Therefore, additional *WRKY1* cDNA clones were isolated and sequenced in the relevant area. Only clones identical to the published *WRKY1* cDNA or

Fig. 3. 5' deletion analysis of *WRKY1* promoter-GUS constructs in parsley protoplasts. The average elicitor inducibility (fold induction) derived from at least seven independent experiments and the corresponding standard error of each construct are given.

WRKY1 were found. Both classes of cDNA occurred in a ratio of nearly one to one, suggesting the existence of two equally strongly expressed alleles. The transcription start sites of both genes were determined by 5' RACE.

A 175-bp *WRKY1* promoter fragment confers strong elicitor responsiveness

To identify *cis*-acting elements that mediate strong pathogen-induced activation of *WRKY1*, we performed transient expression studies in parsley protoplasts. A series of 5' deletions from a promoter fragment, which were translationally fused to the ORF of the *Escherichia coli* β -glucuronidase gene (*uidA*; GUS reporter), was tested for elicitor-stimulated expression (Figure 3). A 1764-bp fragment showed a 30-fold induction of GUS activity after elicitor treatment. 5' Deletions down to position -1042 had no effect, whereas further deletions to positions -354 and -175 resulted in an increase in inducibility (50-fold induction). However, deletion to -108 caused a drastic reduction in elicitor responsiveness and deletion to -79 abolished it almost completely. Strong elicitor-responsive *cis*-acting elements are therefore present within the proximal 175-bp fragment of the *WRKY1* promoter.

The 175-bp *WRKY1* promoter fragment carries three functional W boxes

Examination of this 175-bp fragment revealed the presence of four previously described core sequences of elicitor-response elements (Rushton and Somssich, 1998): three TGAC repeats, which is the core sequence of W box elements, and one AGCCGCC motif found in GCC box elements (Figure 2C; Ohme-Takagi and Shinshi, 1995). The central W box motif, designated *W_B*, is flanked on both sides by reversely oriented W box motifs, designated *W_A* and *W_C*, such that each pair of W box motifs

Fig. 4. Determination of functional W boxes in the *WRKY1* promoter. (A) Schematic representation of the *WRKY1* promoter region with W boxes *W_A*, *W_B* and *W_C*. The wild-type sequences (*W_{ABC}*) of the W boxes are given in upper-case letters. Base pairs altered by block mutations (*W_{ABC}mut*) are given in lower-case type. The TGAC core motifs and their mutated versions are given in bold letters. (B) Mutational analysis of W boxes within the 175-bp *WRKY1* promoter. (C) Gain-of-function with *W_{BC}*. Trimers of wild-type *W_{BC}* as well as a mutated version inserted upstream of the -46 *CaMV* 35S minimal promoter. (B and C) Average values of elicitor inducibility derived from at least seven independent experiments along with their standard errors are shown at the right.

constitutes a palindrome (Figure 2D). A palindromic arrangement of W box motifs similar to that of *W_B* and *W_C* occurs in the *WRKY3* promoter at an almost identical position (Figure 2D).

To address the question as to whether *W_A*, *W_B* and *W_C* are responsible for the strong elicitor responsiveness of the 175-bp promoter, block mutations were introduced to alter the TGAC core motif to CCTT (Figure 4A). Mutation of each W box individually caused a clear-cut reduction of the promoter's capacity to mediate elicitor responsiveness (Figure 4B, *W_Amut*, *W_Bmut* and *W_Cmut*). In particular, mutations of *W_B* or *W_C* had strong effects. Simultaneous mutations of two W box motifs resulted in further significant decreases (Figure 4B, *W_{AB}mut*, *W_{AC}mut* and *W_{BC}mut*) and mutations of all three motifs abolished elicitor responsiveness almost completely. This applies to the 175-bp as well as the large, nearly 1.8-kb *WRKY1*

Fig. 5. Response to elicitor by a palindromic arrangement of W boxes. (A) Sequences of the multimerized W boxes. Restriction sites used for multimerization are given in lower-case type and the W box motifs in bold letters. The TGAC core motifs are underlined. (B, C and D) Comparison of the curves representing the time courses of relative accumulation of elicitor-induced GUS activity for the three constructs as indicated. Each curve represents the average of at least six independent experiments. The respective 8-h values were set arbitrarily to 100%. Bars indicate standard errors. (E) Representative absolute values for the 8-h time points.

promoter fragments (Figure 4B, W_{ABC}mut), indicating that the elicitor responsiveness is conferred mainly by the three W boxes. Comparison of the fold-induction values for the constructs carrying two mutations (Figure 4B, W_{AB}mut, W_{AC}mut and W_{BC}mut) suggests that W_B, which is directly oriented, is the strongest of the W boxes (8-fold induction with W_{AC}mut). The two reversely oriented W boxes (W_A and W_C) seem to act equally strongly (~3-fold induction with W_{AB}mut or W_{BC}mut). However, mutation of W_A alone had a weaker effect than mutation of W_C alone (Figure 4B, constructs W_Amut and W_Cmut). Because of the more pronounced effect on elicitor responsiveness and the occurrence of a similar W box palindrome in the *WRKY3* promoter (Figure 2D), we focused our further studies on the *WRKY1* W_{BC} palindrome.

In gain-of-function experiments using a trimer of the W_{BC} palindrome combined with a minimal *CaMV* 35S promoter–GUS reporter construct, strong elicitor-induced expression was detected (25-fold induction; Figure 4C). In contrast, expression of a similar construct carrying a trimer of the mutated version of this palindrome remained almost non-responsive. Thus, the W_{BC} palindrome alone is sufficient to direct elicitor-stimulated gene expression and the W box core sequence TGAC is required for functionality.

The W_{BC} palindrome mediates rapid response to elicitor

Elicitor-mediated transcriptional activation of *WRKY1* precedes that of the *PR1* genes, its putative targets, by ~2 h (Rushton et al., 1996). Whether W_{BC} is sufficient to mediate a more rapid elicitor- dependent activation than W boxes from the *PR1* genes, was investigated by comparing the time courses of transient reporter gene expression mediated by the W_{BC} palindrome and by box W2 from the *PR1-1* promoter. The GUS reporter constructs contained a

dimer of W_{BC} (W_{BC}×2) or a tetramer of W2 (W2×4) placed upstream of a minimal *CaMV* 35S promoter. Both constructs therefore contained four copies of the W box core motif TGAC (Figure 5A). As illustrated in Figure 5B, the maximal rate of elicitor-induced GUS accumulation, which coincides with the maximal slopes of the curves, was reached 2 h earlier with W_{BC}×2 (open circles) than with W2×4 (filled triangles). This indicates that W_{BC} reaches its full activating capacity more rapidly than W2.

The importance of the palindromic arrangement to confer more rapid activation was demonstrated in similar experiments using a construct containing four directly repeated copies of W_B (W_B×4), which is one half-site of the W_{BC} palindrome (Figure 5A). The kinetic behaviour observed with this construct was almost identical to that observed with W2×4, and clearly delayed and distinct from that observed with W_{BC}×2 (Figure 5C and D; open squares).

Although two values (4 h with W_{BC}×2 and 6 h with W_B×4) showed some variation, the characteristic kinetic behaviour of each construct, namely the early onset of maximal expression with W_{BC}×2 and a delayed response with W_B×4 or W2×4, was highly reproducible in the individual experiments. Our conclusion that W_{BC} is functionally distinct from directly repeated units of W_B was further supported by a 2-fold higher elicitor inducibility measured after 8 h (Figure 5E). The absolute expression levels measured with the individual reporter constructs were quite different (Figure 5E). Lowest levels were detected with the W_B tetramer, three to four times higher levels with the W_{BC} dimer and 20 times higher levels with the tetramer of W2. This considerable difference in the expression levels observed between the tetramers of W_B and W2 illustrates that the characteristic timing of their action is independent of the overall strength of expression.

Fig. 6. Subcellular localization of WRKY1. Parsley protoplasts transfected with the control construct encoding GFP only (A) or a construct encoding a WRKY1-GFP fusion protein (B). Protoplasts were viewed under blue light (excitation wavelength 450–490 nm). Bar = 25 μ m.

WRKY1 is targeted to the nucleus

To determine the *in vivo* subcellular localization of WRKY1, parsley protoplasts were transfected with an expression construct encoding a WRKY1-GFP fusion protein; a construct encoding GFP alone served as a control. Protoplasts transfected with the control construct showed GFP fluorescence throughout the entire cytoplasm and the nucleus (Figure 6A). In contrast, protoplasts transfected with the construct encoding the WRKY1-GFP fusion protein showed GFP fluorescence exclusively in the nucleus (Figure 6B). Nuclear targeting of the WRKY1-GFP fusion protein was independent of elicitor treatment of the protoplasts (data not shown).

The WRKY1 protein is a transcriptional activator binding specifically to W boxes from the PR1 and WRKY1 promoters

Co-transfection of parsley protoplasts with an expression construct encoding a fusion protein of WRKY1 and the strong transcriptional activation domain of the viral VP16 protein together with the W2 $\times$ 4-GUS-reporter construct yielded high specific GUS activity (Figure 7A, row 1). Omitting the WRKY1-VP16 fusion protein or replacing it with a GAL4-VP16 fusion protein (M.Sprenger and B.Weisshaar, unpublished results) significantly reduced specific GUS activity (Figure 7A, rows 2 and 3). Thus, WRKY1-VP16 caused a clear increase of reporter gene expression. In contrast, very low expression of a reporter gene carrying four copies of the GAL4 binding site UAS_G was measured in the presence or absence of WRKY1-VP16 (Figure 7A, rows 4 and 5). Since we previously demonstrated that WRKY1 can bind directly to W boxes *in vitro* (Rushton *et al.*, 1996), these results strongly suggest direct, sequence-specific *in vivo* binding of WRKY1-VP16 to W2, but could also be based on other types of interaction, such as indirect effects. Almost identical expression levels were detected with W2 $\times$ 4 in the presence or absence of GAL4-VP16 (Figure 7A, rows 2 and 3), indicating that the sequence-specific interactions of WRKY1-VP16 with W2 are based exclusively on its WRKY1 portion and not the VP16 domain. WRKY1 also interacted sequence specifically with box W3 from the *PR1*-2 promoter and with the native 840-bp *PR1*-1 promoter which carries both box W2 and box W1 (data not shown), suggesting that WRKY1 can bind specifically *in vivo* to various W boxes within the promoters of two of its putative target genes.

Interactions of WRKY1 with the W_{BC} palindrome from the *WRKY1* promoter were also analysed. For these experiments, a trimer of W_{BC} (W_{BC} $\times$ 3) was used and strong interactions of WRKY1-VP16 with W_{BC} $\times$ 3 were measured (Figure 7A, row 6). Mutation of the TGAC core motifs in W_{BC} (W_{BC}mut $\times$ 3), which was sufficient to abolish elicitor responsiveness, was also sufficient to abolish interactions of WRKY1-VP16 with W_{BC} $\times$ 3 (Figure 7A, rows 8 and 9). Furthermore, clear interactions between WRKY1 and the native 175-bp *WRKY1* promoter were detected (Figure 7B, rows 1 and 2), which were also abolished by mutations of the W boxes (Figure 7B, row 3). The intensity of WRKY1 interactions with W_{BC} $\times$ 3 was compared with that of WRKY3. The WRKY3-VP16 fusion protein caused a much weaker activation of W_{BC} $\times$ 3, which was nevertheless dependent on intact TGAC core motifs (Figure 7A, rows 10 and 11). These results clearly show that WRKY1 interacts *in vivo* strongly and specifically with W boxes from the *WRKY1* promoter, both in a heterologous as well as in the native promoter context. These interactions are probably based on direct binding.

Additional co-transfection experiments were performed to ascertain whether WRKY1 is capable of activating transcription on its own. Expression of WRKY1 led to a 4-fold increase in expression from the W_{BC} $\times$ 3 construct (Figure 7C, rows 1 and 2), revealing that WRKY1 can act as a transcriptional activator *in vivo*.

The transactivation capabilities of WRKY1 were also tested in yeast. The N- and C-terminal halves of WRKY1 were expressed as fusion proteins with the bacterial LexA DNA-binding domain in the yeast strain EGY48 (Figure 7D). This strain is auxotrophic for the selectable leucine marker (Estojak *et al.*, 1995). Both WRKY1-LexA fusion proteins strongly activated expression of the *LEU2* reporter gene carrying six LexA binding sites within its promoter, as indicated by strong colony growth on leucine-lacking plates (Figure 7D, -Leu). A human p53-LexA fusion protein, a strong activator, served as a positive control. Expression of the LexA DNA-binding domain alone or fused to the isolated N-terminal WRKY domain of WRKY1 was not sufficient for *LEU2* reporter gene activation and leucine-independent growth. All five yeast clones grew equally well on plates containing leucine (Figure 7D, +Leu). The fact that the tested N- and C-terminal parts of the protein activate transcription independently suggests the presence of at least two separate regions mediating transcriptional activation in yeast.

The C-terminal WRKY domain of WRKY1 carries a W box-specific DNA-binding activity

To test whether the two WRKY domains of parsley WRKY1 are capable of binding independently, co-transfection experiments were performed with each of the two domains. A comparison of the WRKY domain sequences from published members of group 1 WRKY proteins revealed high degrees of sequence conservation directly flanking these domains (Figure 8A). The N-terminal domain is followed by 10 highly conserved residues, whereas in the case of the C-terminal domain, 24 conserved amino acids precede this domain and eight conserved residues are present thereafter (Figure 8A).

Assuming that primary structural features required for proper folding of a WRKY domain are conserved among

Fig. 7. Characterization of the WRKY1 DNA-binding abilities (A and B) and transactivation capacity (C) by co-transfection experiments in parsley protoplasts. The effector and reporter constructs used are given on the left. The average specific GUS activities derived from at least seven independent experiments along with their standard errors are represented by the grey bars and error bars and are given in absolute values on the right. All values were normalized using an internal control plasmid encoding luciferase under the control of the constitutive parsley *ubi4-2* promoter. (D) Transactivation by WRKY1 in yeast. Yeast cells expressing the indicated LexA fusion proteins were plated on to media containing (+Leu) or lacking leucine (–Leu) and were assayed for activation of the 6×*lexA* operator–*LEU2* reporter gene, which allows leucine-independent growth. Schematic representations of WRKY1 and the three WRKY1 fragments tested as LexA fusion proteins are given below with the positions of putative transactivation and WRKY domains indicated.

different WRKY proteins, constructs expressing extended WRKY1 N-terminal (NTD; Figure 8A) and C-terminal (CTD, Figure 8A) WRKY domains fused to the transcriptional activation domain of VP16 were tested. No interactions of NTD were observed with W_{BC}×3 (Figure 8B, row 3). In contrast, CTD clearly interacted with this element (Figure 8B, row 2). As with the full-length WRKY1 protein, DNA interactions of CTD were dependent on intact TGAC core motifs of W_{BC} as illustrated by

lack of increased expression with the W_{BC}mut×3 construct (Figure 8B, rows 4 and 5).

Thus, the extended C-terminal WRKY domain (CTD) of WRKY1 appears to be sufficient for DNA binding and for conferring binding specificity for W boxes. However, since the full-length WRKY1–VP16 fusion protein caused four to five times higher levels of reporter gene expression (Figure 7A, row 6), additional parts of the protein must influence its overall DNA-binding abilities.

Fig. 8. Binding specificity of the WRKY domains to W boxes. (A) Comparison of WRKY domain sequences from published group 1 WRKY proteins from wild oat (ABF1; Rushton *et al.*, 1995), tobacco (NtWRKY1 and -2; Wang *et al.*, 1998), sweet potato (SPF1; Ishiguro and Nakamura, 1994), parsley (WRKY1 and -2; Rushton *et al.*, 1996) and *Arabidopsis* (ZAP1; de Pater *et al.*, 1996). Conserved residues are shown in red. The boxed area is conserved among all WRKY domains. Below the sequence alignments those areas from WRKY1 (NTD and CTD) tested in co-transfection experiments are shown. (B) Binding of isolated WRKY domains from WRKY1 to W_{BC}. The tested effector and reporter constructs are given on the left. The normalized average specific GUS activities of at least six independent experiments along with their standard errors are represented by grey bars and error bars and the absolute values given on the right.

Discussion

Parsley WRKY1 is a pathogen-inducible transcriptional activator

Our previous work with cultured parsley cells showed that *WRKY1* expression was rapidly, strongly and transiently induced by a specific fungus-derived elicitor. As reported here, rapid, strong and transient *WRKY1* expression is also observed *in planta*, locally around fungal infection sites. The pathogen-induced *WRKY1* mRNA accumulation coincided spatially with that of *PR1* (Somssich *et al.*, 1988) but differed from *PR1* in its temporal kinetics. *WRKY1* mRNA was strongly induced in infected tissue sections 6 h post-inoculation but was no longer detectable as soon as 6 h later. In contrast, elevated *PR1* mRNA levels remained detectable up to 25 h post-inoculation (Somssich *et al.*, 1988; Reinold and Hahlbrock, 1996).

As demonstrated using a *WRKY1*-GFP chimeric protein, *WRKY1* is targeted exclusively to the cell nucleus. Nuclear localization of *WRKY1*-GFP was independent of elicitor stimulation. Therefore, upon induced synthesis, *WRKY1* must be subject to immediate nuclear import.

Nuclear import can occur by diffusion or by active transport (Görlich and Mattaj, 1996). Due to its predicted molecular mass of 58 kDa, *WRKY1* is unlikely to diffuse freely into the nucleus but probably requires NLS sequences for active transport. *WRKY1* contains several putative NLS sequences, one of which perfectly fits the consensus of the SV40 large T-antigen-type motif (K-R/K-X-R/K; Garcia-Bustos *et al.*, 1991).

Co-transfection experiments indicated that *WRKY1* is capable of activating gene expression *in vivo* via sequence-specific interactions with W box elements present in both the *PR1* and *WRKY1* promoters. These W boxes are the major elicitor-response elements mediating induced expression of *WRKY1* itself and of *PR1* (Rushton *et al.*, 1996). The C-terminal *WRKY* domain was sufficient for *in vivo* sequence-specific DNA recognition whereas the N-terminal *WRKY* domain alone failed to bind W box elements. These results more precisely define the DNA-binding domain. They are in agreement with previous *in vitro* studies with SPF1 (Ishiguro and Nakamura, 1994) and ZAP1 (de Pater *et al.*, 1996), where the major DNA-

Fig. 9. Proposed nuclear events in elicitor-mediated signalling. Simplified model depicting the early activation of *WRKY1* by pre-existing WRKY proteins interacting with a specific arrangement of boxes W_A , W_B and W_C . The initial signal transduction events are described elsewhere (Somssich and Hahlbrock, 1998). WRKY1 directs expression of secondary target genes such as *PRI* by binding to W boxes in their promoters. WRKY1 may also act in an autoregulatory loop by binding to W boxes within its own promoter. Additional element(s), such as the GCC box, and other factors may participate in initiating *WRKY1* expression.

binding activities were mapped to regions including the C-terminal WRKY domains, whereas the N-terminal WRKY domains, including at least 112 additional flanking amino acids on both sides, failed to bind. They are also consistent with the fact that the single WRKY domains of group 2 family members, which also show sequence-specific DNA binding to W boxes, are more similar in sequence to the C-terminal WRKY domain of group 1 proteins than to the N-terminal domain. The biological function of the N-terminal WRKY domain remains to be elucidated. Although our results show that the C-terminal domain suffices for DNA binding, they also indicate that additional parts of the protein must contribute to the overall strength of this binding. The N-terminal domain may increase the overall binding affinity of the protein by making additional contacts with DNA or by interacting with other proteins.

Although weaker than the strong VP16 transactivator, WRKY1 does have considerable *in vivo* transactivating potency. Several functional classes of transcriptional activation domains have been defined (Triezenberg, 1995). Based on primary structure, WRKY1 contains at least seven putative activator domains of the acidic, proline-rich and glutamine-rich type. In yeast, the N-terminal as well as the C-terminal half of the WRKY1 protein showed strong transactivation capacity, suggesting that it contains at least two separate functional domains. The WRKY protein, ZAP1, has also been shown to activate transcription *in vivo* (de Pater *et al.*, 1996). However, the 75-amino-acid region of ZAP1, identified as having transactivator function in yeast, has no sequence similarity to WRKY1 nor to known activation domains present in other transcription factors.

Early pathogen-induced *WRKY1* expression through a specific arrangement of W boxes

Functional dissection of the *WRKY1* promoter revealed that fungal elicitor responsiveness is mediated mainly by

the presence of three closely positioned W boxes within the first 116 bp upstream of the transcription start site. Simultaneous mutation of all three boxes strongly reduced this responsiveness, but some residual activity suggests the existence of at least one additional weak promoter element. Such an element could be the sequence AGCCGCC at positions -134 to -128, which represents one copy of the GCC box known to be bound by members of the EREBP family of transcription factors (Ohme-Takagi and Shinshi, 1995; Zhou *et al.*, 1997). Consistent with an element being located upstream of W_A is the observation that mutation of W_A (at position -116 to -110) in the context of the 175-bp promoter fragment caused a considerably less drastic reduction in elicitor responsiveness than did 5' deletion down to -108. Furthermore, substitution of both Gs within the AGCCGCC motif by Ts resulted in a 2-fold reduction of elicitor responsiveness in the context of the wild-type 175-bp promoter (T.Eulgem and I.E.Somssich, unpublished results).

As was clearly shown by the mutational analysis, the individual contributions of the W boxes to elicitor-induced gene expression were rather low. The overall response of the wild-type promoter cannot be explained simply by additive effects of three independently acting W box elements. Rather, our data suggest that the presence of these multiple sites enables cooperative binding of WRKY factors, thereby generating a synergistic effect on transcription. Cooperative DNA binding, originally described for the yeast transcriptional activator GAL4 (Giniger and Ptashne, 1988), has been observed for several transcription factors including some from plants (Yunes *et al.*, 1998). Cooperative binding of WRKY factors to the *WRKY1* promoter may partly explain the more rapid activation of *WRKY1* transcription compared to that of its target gene *PRI*, since it increases the factor's affinity for DNA, allowing the promoter to become saturated at relatively low activator concentrations (Tanaka, 1996).

As judged by loss-of-function experiments, the W_{BC} palindrome has a very pronounced effect on elicitor responsiveness. Both the native minimal *WRKY1* promoter fragment containing only W_B and W_C (Figure 3, -108 construct) as well as multimers of W_{BC} (Figures 4C and 5E) proved sufficient for strong elicitor-induced expression. As is the case for all W boxes tested, W_{BC} function is dependent on intact TGAC core motifs indicating interactions with WRKY proteins. The detailed kinetic studies revealed that in the case of the W_{BC} palindrome, full elicitor-induced expression is reached earlier than with identical copies of W box elements arranged non-palindromically. Thus, functionally distinct types of W boxes exist. The usefulness of dyad symmetric sequence motifs lies in the ability of monomeric protein factors to contact one half of the dyad substrate, thereby enabling protein dimerization (Lamb and McKnight, 1991). Recruitment of homo- or heterodimers to such sites can result in unique transcriptional activation properties as a consequence of local increased levels of bound factors and/or by juxtaposing different combinations of activation domains (Tanaka, 1996; Ptashne and Gann, 1997). Future work must address the question of whether WRKY proteins can in fact dimerize, and if so, if there is a preference for distinct partners.

Early gene regulatory events involving WRKY transcription factors

Previous results have demonstrated that fungal elicitor-induced transcription of the parsley *PR1-1* and *PR1-2* genes is almost exclusively mediated by W box elements, which are binding sites for WRKY-type proteins (Rushton *et al.*, 1996). The ability of parsley WRKY1 to transactivate *in vivo* via binding to such W box elements lends additional support for a vital role of WRKY transcription factors in regulating certain plant defence responses. Our discovery that elicitor-stimulated transcription of the rapidly activated *WRKY1* gene is itself also mediated mainly by a set of W box elements clearly implicates WRKY proteins in the regulation of this gene as well. The fact that WRKY1 has a relatively high binding affinity for a subset of such W boxes and is capable of transactivating is suggestive of a possible autoregulatory loop involving WRKY1.

Several transcription factors can act as autoregulators in genetic circuits which can be of a simple or complex type (Calkhoven and Ab, 1996). Since no WRKY1 mRNA is detectable in non-stimulated parsley cells, a WRKY1-independent mechanism involving other WRKY proteins must initiate *WRKY1* activation. These factors, which may also include EREBP-type transcription factors, are most likely activated by post-translational modification. Once WRKY1 synthesis is triggered, it activates transcription of *PR1* and could also participate in the regulation of its own gene acting in a positive or negative manner.

WRKY proteins are present in non-stimulated parsley cells and may be activated by phosphorylation, thereby triggering *WRKY1* expression. This would be consistent with recent findings in tobacco, where the enhanced binding affinities of two WRKY proteins to W boxes upon tobacco mosaic virus infection were shown to require formation of a multimeric complex and to be dependent on phosphorylation (Yang *et al.*, 1999). A similar phosphorylation-dependent binding activity, PBF-1, has been detected in association with a functionally important W box-containing promoter region of the potato *PR-10a* gene (Després *et al.*, 1995). Similarly, three tomato EREBPs have been isolated in a yeast two-hybrid screen by their ability to bind to Pto, a protein kinase encoded by the tomato *Pto* gene which confers resistance to a phytopathogenic bacterium (Zhou *et al.*, 1997). Indeed, *in vivo* protein phosphorylation and activation of a specific MAP kinase have also been demonstrated to be part of an early receptor-mediated parsley signal transduction pathway triggered by the fungal elicitor (Dietrich *et al.*, 1990; Ligterink *et al.*, 1997).

A simplified model based on our current knowledge of how elicitor-induced early nuclear events are initiated in parsley is proposed in Figure 9. We expect the *in vivo* situation to be more complex, with WRKY factors interacting directly or indirectly with other regulators and auxiliary proteins to form the multimeric structures required for efficient transcriptional activation, maintenance of expression and subsequent repression of their target genes. WRKY proteins may be involved in all of these processes.

Since all WRKY proteins analysed to date show specific binding to W box sequences, an important question which needs to be tackled in future investigations is how

specificity for certain promoters is accomplished in view of the stereotypic binding preference. Some specificity may be conferred by the different sequences flanking the TGAC core motifs. Distinct clustering of functional W boxes, as in the *WRKY1* promoter, may indicate another level of specificity. Such a defined arrangement of elements may lead to interactions of only selected WRKY proteins, resulting in discrete higher-order protein–DNA complexes. This would then result in changes in the efficacy of the bound WRKY proteins and give rise to distinct transcriptional outputs (Lamb and McKnight, 1991; Tjian and Maniatis, 1994). The large structural diversity of WRKY proteins could enable the assembly of such architecturally distinct three-dimensional nucleoprotein complexes, thereby compensating for the lack of diversity in DNA sequence preference.

Materials and methods

In situ RNA hybridization

³⁵S-labelled riboprobes generated either from the entire WRKY1 cDNA or from a 504-bp fragment of its 3' portion were used for *in situ* RNA–RNA hybridization. Inoculation of parsley seedlings with zoospores of *P.sojae*, tissue treatment and hybridization were performed as previously described (Schmelzer *et al.*, 1989).

Library screenings and structural analysis of WRKY1 and WRKY3

Approximately 1.5×10^6 recombinant plaques of a genomic parsley library (Somssich *et al.*, 1988) were screened for hybridization to a mixture of ³²P-labelled WRKY1, -2 and -3 cDNA probes. Isolated clones were characterized and parts of WRKY1 and -3 were subcloned and sequenced. The transcription start sites were determined by 5' RACE (Frohman *et al.*, 1988). Additional WRKY1 cDNA clones were isolated from a parsley cDNA library (Korfhage *et al.*, 1994) using the WRKY1 cDNA as probe. All cloning and DNA-blotting procedures were performed according to Sambrook *et al.* (1989).

Protoplast transfection experiments

Transient expression analyses were performed with parsley cell culture protoplasts as described previously (van de Löcht *et al.*, 1990). In each assay 2×10^6 protoplasts were transfected with 20 µg of GUS-reporter plasmid. Elicitor treatment was carried out with either 50 µg/ml crude Pmg elicitor (Hahlbrock *et al.*, 1981) or 100 ng/ml synthetic Pep-25 elicitor (Nürnberg *et al.*, 1994). For comparison of the temporal behaviour of various reporter gene constructs, 3×10^6 protoplasts were transfected with 30 µg of GUS-reporter plasmid. Transfected protoplasts were split and one half was treated with elicitor while the other half remained untreated. Aliquots were subsequently harvested and treated as described previously (van de Löcht *et al.*, 1990). In co-transfection assays $0.5\text{--}1 \times 10^6$ protoplasts were transfected with 20 µg of expression plasmid, 5 µg of GUS-reporter plasmid and 5 µg of an internal control plasmid encoding luciferase under the control of the constitutive parsley *ubi4-2* promoter (Feldbrügge *et al.*, 1994). Luciferase expression was used to normalize specific GUS activities. For subcellular localization of WRKY1, 2×10^6 protoplasts were transfected with 20 µg of WRKY1–GFP or GFP expression plasmids. Microscopic analysis of GFP-expressing protoplasts was performed as described by Reichel *et al.* (1996).

Plasmid constructions

WRKY1 promoter–GUS constructs were cloned into pUC9 (van de Löcht *et al.*, 1990). In all cases, the *WRKY1* promoter is fused translationally to the *E.coli* β-glucuronidase (*uidA*) gene. Apart from the *WRKY1* promoter, these constructs carry the 5' untranslated region of the gene and the first six codons. For each construct the relevant *WRKY1* region was amplified by PCR and introduced into a *Bam*HI site in pUC9–GUS. Internal restriction sites (see Figure 2A) were used for the cloning of the –1764, –1224, –1042, –355 and –79 *WRKY1* promoter fragments. For the –732, –175 and –108 promoter constructs, restriction sites were introduced at the 5' end. Site-directed mutagenesis of W boxes within *WRKY1* promoter constructs was achieved using the PCR-based

'megaprimer' method (Landt *et al.*, 1990). For the W_{BC} , W_{BCmut} and W_B multimers, W_{BC} or W_{BCmut} monomers and a W_B dimer with a 5' *Bam*HI site and a 3' *Bgl*III site were made by annealing complementary oligonucleotides. These were 5' phosphorylated, ligated with T4 DNA ligase and then digested with *Bam*HI and *Bgl*III. The resulting multimers had W_{BC} , W_{BCmut} or W_B units in the same orientation. Dimers and trimers of W_{BC} or W_{BCmut} and tetramers of W_B ($2 \times W_B$ dimer) were cloned into the *Bam*HI site of pBluescript II KS (+). Correctly oriented inserts were excised by *Bam*HI–*Spe*I digestion and cloned into the corresponding restriction sites upstream of the –46 *CaMV* 35S minimal promoter of pBT10, a derivative of pBT-2 (Weisshaar *et al.*, 1991; M.Sprenger and B.Weisshaar, unpublished data). A similar approach was used to construct $W_{2 \times 4}$ except that a 5' *Spe*I site and a 3' *Xba*I site were used. After phosphorylation and annealing, the oligonucleotides were inserted between the *Spe*I and *Xba*I sites of pBT10.

The WRKY1–GFP expression construct contains a 490 bp fragment of the *Agrobacterium tumefaciens mas* 2' promoter (kindly provided by Dr S.Rosahl) between the *Sal*I and *Cl*aI sites in pBluescript II KS (+). Downstream from the *mas* 2' transcription start site a DNA cassette encoding the WRKY1–GFP fusion protein was inserted via a *Hind*III restriction site. This cassette contains the WRKY1 cDNA from positions 14 to 1571, which includes the translation start site and the ORF minus the last seven codons. At position 1571, the WRKY1 ORF is fused in-frame via an engineered *Nco*I site with the CAT–GFP ORF and the *CaMV* 35S polyadenylation site derived from pCK–CAT–GFP (Reichel *et al.*, 1996; G.Jach, personal communication). The control plasmid was constructed by cloning a 1.8 kb *Hind*III fragment from pCK–CAT–GFP, carrying the *CaMV* 35S promoter with a duplicated transcriptional enhancer, the tobacco etch virus translational enhancer (TL), the coding region of CAT–GFP and the *CaMV* 35S polyadenylation signal into the *Hind*III site of pBluescript II KS (+). Expression from the *mas* 2' promoter in parsley cells is not influenced by fungal elicitor treatment in contrast to the *CaMV* 35S promoter which is strongly downregulated (I.E.Somssich, unpublished data).

The effector plasmid expressing WRKY1 was made by replacing the *CPRF1* ORF in pBT35S–*CPRF1* (Feldbrügge *et al.*, 1994) with the WRKY1 ORF. A *Nco*I restriction site was introduced by PCR 11 bp upstream of the *WRKY1* ATG and a *Bgl*III site was introduced to replace two in-frame stop codons. The complete coding region was inserted between the *Nco*I and *Bcl*I sites in the expression vector to create the WRKY1 expression construct. The WRKY1–VP16 and WRKY3–VP16 effector constructs were created by replacing the bZIP^{FL} insert from pBT35S–bZIP^{FL} (Feldbrügge *et al.*, 1994) with either the WRKY1 or WRKY3 ORF. In each case an *Asp*718 site was introduced 11 bp upstream of the ATG codon and an *Ngo*MI site downstream to replace the stop codon. The complete ORFs were then inserted between the *Asp*718 and *Age*I sites in the vector to produce WRKY1–VP16 and WRKY3–VP16. In each case the VP16 portion was fused C-terminal to the WRKY portion.

To construct yeast expression vectors encoding WRKY1–LexA fusion proteins, we amplified fragments of the WRKY1 coding region and the human p53 coding region (from codon 1 to codon 144) by PCR and introduced an *Eco*RI site directly upstream and an *Xho*I site directly downstream. These fragments were cloned between the *Eco*RI and *Xho*I sites of the yeast expression vector pEG202 (Gyuris *et al.*, 1993) fusing coding regions in-frame to that of the LexA DNA-binding domain. The correctness of all constructs was verified by restriction analysis and sequencing.

Yeast transactivation assay

Standard yeast manipulations were performed as previously described (Ausubel *et al.*, 1995). *Saccharomyces cerevisiae* EGY48 [*MAT α* *ura3* *trp1* *his3* *lexA* operator–*LEU2* (Estojak *et al.*, 1995)] was transformed with various derivatives of pEG202 (HIS+) encoding LexA fusion proteins. Clones were grown on synthetic dextrose (SD) plates lacking histidine for 3 days at 30°C. Individual colonies were then resuspended in sterile water and streaked on to SD plates lacking histidine, and SD plates lacking histidine and leucine, and incubated at 30°C for 2 days.

Acknowledgements

We thank P.Wernert, I.Rouhara and L.Bollenbach for excellent technical assistance, Drs M.Sprenger and B.Weisshaar (both from our department) for the pBT10 vector and additional vector constructs, Dr S.Rosahl (Halle, Germany) for the construct containing the *mas* 2' promoter and Dr G.Jach (Köln, Germany) for the pCK CAT–GFP construct. Dr B.Weisshaar is also thanked for critical reading of the manuscript.

Note

DDBJ/EMBL/GenBank accession numbers for the genomic parsley *WRKY1* and *WRKY3* sequences are AF121353 and AF121354, respectively.

References

- Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1995) *Short Protocols in Molecular Biology*. John Wiley & Sons, Inc., New York.
- Calkhoven,C.F. and Ab,G. (1996) Multiple steps in the regulation of transcription-factor level and activity. *Biochem. J.*, **317**, 329–342.
- de Pater,S., Greco,V., Pham,K., Memelink,J. and Kijne,J. (1996) Characterization of a zinc-dependent transcriptional activator from *Arabidopsis*. *Nucleic Acids Res.*, **24**, 4624–4631.
- Després,C., Subramaniam,R., Matton,D.P. and Brisson,N. (1995) The activation of the potato *PR-10a* gene requires the phosphorylation of the nuclear factor PBF-1. *Plant Cell*, **7**, 589–598.
- Dietrich,A., Mayer,J.E. and Hahlbrock,K. (1990) Fungal elicitor triggers rapid, transient and specific protein phosphorylation in parsley cell suspension cultures. *J. Biol. Chem.*, **265**, 6360–6368.
- Estojak,J., Brent,R. and Golemis,E.A. (1995) Correlation of two-hybrid affinity data with *in vitro* measurements. *Mol. Cell. Biol.*, **15**, 5820–5829.
- Feldbrügge,M., Sprenger,M., Dinkelbach,M., Yazaki,K., Harter,K. and Weißhaar,B. (1994) Functional analysis of a light-responsive plant bZIP transcriptional regulator. *Plant Cell*, **6**, 1607–1621.
- Frohman,M.A., Dush,M.K. and Martin,G.R. (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl Acad. Sci. USA*, **85**, 8998–9002.
- Garcia-Bustos,J., Heitman,J. and Hall,M. (1991) Nuclear protein localization. *Biochim. Biophys. Acta*, **1071**, 83–101.
- Giniger,E. and Ptashne,M. (1988) Cooperative DNA binding of the yeast transcriptional activator GAL4. *Proc. Natl Acad. Sci. USA*, **85**, 382–386.
- Görlich,D. and Mattaj,I.W. (1996) Nucleocytoplasmic transport. *Science*, **271**, 1513–1518.
- Gyuris,J., Golemis,E., Chetkov,H. and Brent,R. (1993) Cdi1, a human G₁ and S phase protein phosphatase that associates with Cdk2. *Cell*, **75**, 791–803.
- Hahlbrock,K., Lamb,C.J., Purwin,C., Ebel,J., Fautz,E. and Schäfer,E. (1981) Rapid response of suspension-cultured parsley cells to the elicitor from *Phytophthora megasperma* var. *sojae*. Induction of enzymes of general phenylpropanoid metabolism. *Plant Physiol.*, **67**, 768–773.
- Herschman,H.R. (1991) Primary response genes induced by growth factors and tumor promoters. *Annu. Rev. Biochem.*, **60**, 281–319.
- Ishiguro,S. and Nakamura,K. (1994) Characterization of a cDNA encoding a novel DNA-binding protein, SPF1, that recognizes SP8 sequences in the 5' upstream regions of genes coding for sporamin and β -amylase from sweet potato. *Mol. Gen. Genet.*, **244**, 563–571.
- Kim,D.-J., Smith,S.M. and Leaver,C.J. (1997) cDNA encoding a putative SPF1-type DNA-binding protein from cucumber. *Gene*, **185**, 265–269.
- Korfhage,U., Trezzini,G.F., Meier,I., Hahlbrock,K. and Somssich,I.E. (1994) Plant homeodomain protein involved in transcriptional regulation of a pathogen defense-related gene. *Plant Cell*, **6**, 695–708.
- Lamb,P. and McKnight,S.L. (1991) Diversity and specificity in transcriptional regulation: the benefits of heterotypic dimerization. *Trends Biochem. Sci.*, **16**, 417–422.
- Landt,O., Grunert,H.-P. and Hahn,U. (1990) A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene*, **96**, 125–128.
- Ligerink,W., Kroj,T., zur Nieden,U., Hirt,H. and Scheel,D. (1997) Receptor-mediated activation of a MAP kinase in pathogen defense of plants. *Science*, **276**, 2054–2057.
- Nürnberg,T., Nennstiel,D., Jabs,T., Sacks,W., Hahlbrock,K. and Scheel,D. (1994) High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multiple defense responses. *Cell*, **78**, 449–460.
- Ohme-Takagi,M. and Shinshi,H. (1995) Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *Plant Cell*, **7**, 173–182.
- Ptashne,M. and Gann,A. (1997) Transcriptional activation by recruitment. *Nature*, **386**, 569–577.

- Reichel,C., Mathur,J., Eckes,P., Langenkemper,K., Koncz,C., Schell,J., Reiss,B. and Maas,C. (1996) Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. *Proc. Natl Acad. Sci. USA*, **93**, 5888–5893.
- Reinold,S. and Hahlbrock,K. (1996) Biphasic temporal and spatial induction patterns of defense-related mRNAs and proteins in fungus-infected parsley leaves. *Plant Physiol.*, **112**, 131–140.
- Rushton,P.J. and Somssich,I.E. (1998) Transcriptional control of plant genes responsive to pathogens. *Curr. Opin. Plant Biol.*, **1**, 311–315.
- Rushton,P.J., Macdonald,H., Huttly,A.K., Lazarus,C.M. and Hooley,R. (1995) Members of a new family of DNA-binding proteins bind to a conserved *cis*-element in the promoters of α -Amy2 genes. *Plant Mol. Biol.*, **29**, 691–702.
- Rushton,P.J., Torres,J.T., Parniske,M., Wernert,P., Hahlbrock,K. and Somssich,I.E. (1996) Interaction of elicitor-induced DNA binding proteins with elicitor response elements in the promoters of parsley PR1 genes. *EMBO J.*, **15**, 5690–5700.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schmelzer,E., Krüger-Lebus,S. and Hahlbrock,K. (1989) Temporal and spatial patterns of gene expression around sites of attempted fungal infection in parsley leaves. *Plant Cell*, **1**, 993–1001.
- Somssich,I.E. and Hahlbrock,K. (1998) Pathogen defence in plants—a paradigm of biological complexity. *Trends Plant Sci.*, **3**, 86–90.
- Somssich,I.E., Schmelzer,E., Kawalleck,P. and Hahlbrock,K. (1988) Gene structure and *in situ* transcript localization of pathogenesis-related protein 1 in parsley. *Mol. Gen. Genet.*, **213**, 93–98.
- Tanaka,M. (1996) Modulation of promoter occupancy by cooperative DNA binding and activation-domain function is a major determinant of transcriptional regulation by activators *in vivo*. *Proc. Natl Acad. Sci. USA*, **93**, 4311–4315.
- Tjian,R. and Maniatis,T. (1994) Transcriptional activation: a complex puzzle with few easy pieces. *Cell*, **77**, 5–8.
- Trizenberg,S.J. (1995) Structure and function of transcriptional activation domains. *Curr. Opin. Genet. Dev.*, **5**, 190–196.
- van de Löcht,U., Meier,I., Hahlbrock,K. and Somssich,I.E. (1990) A 125 bp promoter fragment is sufficient for strong elicitor-mediated gene activation in parsley. *EMBO J.*, **9**, 2945–2950.
- Wang,Z., Yang,P., Fan,B. and Chen,Z. (1998) An oligo selection procedure for identification of sequence-specific DNA-binding activities associated with plant defense. *Plant J.*, **16**, 515–522.
- Weißhaar,B., Armstrong,G.A., Block,A., da Costa e Silva,O. and Hahlbrock,K. (1991) Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. *EMBO J.*, **10**, 1777–1786.
- Yang,P., Wang,Z., Fan,B., Chen,C. and Chen,Z. (1999) A pathogen- and salicylic acid-induced WRKY DNA-binding activity recognizes the elicitor response element of tobacco class I chitinase gene promoter. *Plant J.*, **18**, 141–149.
- Yunes,J.A., Vettore,A.L., da Silva,M.J., Leite,A. and Arruda,P. (1998) Cooperative DNA binding and sequence discrimination by the Opaque2 bZIP factor. *Plant Cell*, **10**, 1941–1955.
- Zhou,J., Tang,X. and Martin,G.B. (1997) The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis-related genes. *EMBO J.*, **16**, 3207–3218.

Received April 8, 1999; revised and accepted July 15, 1999