Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes

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PR1 is a pathogenesis-related protein encoded in the parsley genome by a family of three genes (PR1-1, PR1-2 and PR1-3). Loss- and gain-of-function experiments in a transient expression system demonstrated the presence of two fungal elicitor responsive elements in each of the PR1-1 and PR1-2 promoters. These elements, W1, W2 and W3, contain the sequence (T)TGAC(C) and mutations that disrupt this sequence abolish function. Gel shift experiments demonstrated that W1, W2 and W3 are bound specifically by similar nuclear proteins. Three cDNA clones encoding sequence-specific DNA-binding proteins were isolated by South-Western screening and these proteins, designated WRKY1, 2 and 3, also bind specifically to W1, W2 and W3. WRKY1, 2 and 3 are members of the family of sequence-specific DNA-binding proteins, which we call the WRKY family. Treatment of parsley cells with the specific oligopeptide elicitor Pep25 induced a transient and extremely rapid increase in mRNA levels of WRKY1 and 3. WRKY2 mRNA levels in contrast showed a concomitant transient decrease. These rapid changes in WRKY mRNA levels in response to a defined signal molecule suggest that WRKY1, 2 and 3 play a key role in a signal transduction pathway that leads from elicitor perception to PR1 gene activation.

Keywords: parsley/pathogenesis-related protein/plant defence/transcriptional regulation/WRKY proteins

Introduction

The plant defence response towards fungal pathogens consists of pre-existing physical barriers together with a large array of inducible mechanisms. Transcriptional activation of specific defence-related genes is a vital and major part of this response. We have used suspension-cultured parsley cells (Petroselinum crispum) to study the molecular events associated with this complex plant defence response. A defined oligopeptide elicitor molecule (Pep25) is sufficient to initiate a large number of intracellular processes, resulting to a large extent in the same defence response as observed in infected whole-plant tissue (Nürnberger et al., 1994; Hahlbrock et al., 1995). The elicitor is bound specifically by a receptor in the plasma membrane and this leads to large and transient increases in several ion fluxes (Ca²⁺, H⁺, K⁺ and Cl⁻) and in H₂O₂ formation (Scheel et al., 1991; Nürnberger et al., 1994). This is followed by changes in the phosphorylation status of various proteins (Dietrich et al., 1990) and finally by the activation of numerous defence-related genes (Somssich et al., 1989). Studies of the process of transcriptional activation of a number of defence-related genes and gene families (PAL, 4CL, PR1 and PR2) suggest that the molecular mechanisms involved in transcriptional activation differ greatly among them (Hahlbrock et al., 1995). A number of different cis-acting elements have been shown to confer elicitor responsiveness (Meier et al., 1991; Korfhage et al., 1994) illustrating that different combinations of trans-acting factors are responsible for the elicitor mediated activation of different defence-related genes.

Our interest is focused on the cis-acting elements that confer fungal elicitor responsiveness on the parsley PR1 genes and the corresponding trans-acting factors. PR1 is a pathogenesis-related protein of the PR-10 class of intracellular PR proteins and is represented in parsley by a family of three genes (PR1-1, PR1-2 and PR1-3). Previous work demonstrated that the PR1 gene family is regulated at the level of transcription (Somssich et al., 1988). Subsequent studies of the PR1-1 promoter using in vivo footprinting revealed two sites of DNA–protein interaction (Meier et al., 1991). One of these interactions was inducible with elicitor and was detectable within 30 min of treatment. Both sites contain the motif TTGACpPuGTAA, suggesting that this may be an important elicitor response element.

Here we report the characterization of three functionally related, elicitor responsive (T)TGAC(C) elements within the parsley PR1-1 and PR1-2 promoters and three structurally related cognate DNA-binding proteins. We have named the elements W boxes and the proteins WRKY, as deduced from one of the most highly conserved amino acid sequences occurring within this recently discovered family of DNA-binding proteins (Rushton et al., 1995).

Results

Functionally independent W boxes in the PR1-1 and PR1-2 promoters

An 840 bp promoter fragment from PR1-1 was sufficient to confer high level elicitor responsive expression of the reporter gene GUS when analysed in a homologous transient expression system (Figure 1, construct PR1-1). A 5′ deletion to position –273 still resulted in elicitor-mediated expression, although the level was somewhat
present upstream constructs that promoters to constitutive Deletion is located between DNA of the PRI elicitor region (Figure 1, WI between responsive 36 bp almost abolished PR1-'h PRP-lk PR1llg PR -'u PR'-if WI between responsive elements PRI-1 (Meier et al., 1991). Mutated elements are indicated by a white box with a black cross. The 5' end of each promoter relative to its transcriptional start site is shown, together with the positions of the TATA box and translational start site (ATG). The fold elicitor inducibility and standard deviation for each construct is given at the right. Constructs PR1-1h and PR1-1k contain a minimal parsley CHS promoter fragment that alone is not elicitor responsive. A minimum of seven independent transformation experiments were performed with each construct.

Reduced (construct PR1-1a). A further deletion to –226 almost completely abolished expression (PR1-1b). This suggests that at least one cis-acting element required for elicitor responsive expression is present between –273 and –226. This corresponds well with the position of Box W1 between –255 and –220, originally defined by in vivo DNA footprinting studies (Meier et al., 1991). An additional constitutive footprint was also defined by these studies which we now call Box C, and although it appears not to be involved in the elicitor responsiveness of the PRI promoters it does have an effect on basal expression of the PRI genes (unpublished data).

Mutation of the W1 sequence, in the context of the –840 PRI-1 promoter, did not reduce the level of expression (Figure 1, PR1-1c and PR1-1d; Figure 3), suggesting that an additional elicitor responsive element must be present upstream of –273. The location of this additional element was determined by a series of 5′ deletions within constructs where the W1 and C sequences had been mutated. Deletion to –743 had no effect on the activity of the promoter (PR1-1e), whereas a further deletion of 36 bp to –707 abolished expression (PR1-1f). Thus an additional elicitor responsive element, designated Box W2, is located between –743 and –707. The location of Box W2 and the functional importance of both Boxes W1 and W2 is illustrated clearly by construct PR1-1g. Deletion (PR1-1a) or mutation (PR1-1c) of a single W box has little effect on the activity of the promoter. However, mutation of the W1 and W2 sequences alone is sufficient to abolish function. The presence of two independently functioning W boxes was confirmed by fusing the area upstream of Box W1 to a minimal CHS promoter, that alone does not respond to elicitor (PR1-1k) (van de Locht et al., 1990). The resulting construct (PR1-1h) showed high level elicitor responsive expression. W1 and W2 are therefore functionally independent elements required for high level elicitor responsive expression.

Fig. 2. Identification of the elicitor responsive elements Box W1 and Box W3 in the parsley PRI-1-2 promoter. A schematic representation of the parsley PRI-1-2 promoter-GUS translational fusion constructs used for transient expression studies is shown. The symbols are as described in Figure 1 and the position of Box W3 is indicated. Values for fold elicitor inducibility at the right are the average of a minimum of seven independent experiments.

Similar results were also obtained using the PRI-1-2 promoter. A 443 bp promoter fragment was sufficient to confer elicitor responsive expression (Figure 2A, PRI-1-2a). This construct showed very similar levels of elicitor responsive expression to those shown by longer PRI-1-2 promoter constructs (data not shown). A construct containing the –268 promoter was still elicitor responsive although the level was reduced to 5.2-fold (PRI-1-2b). This reduction in level suggests either that there may be other regulatory sequences between Box W1 and Box W3, or that the deletion to –268 is close enough to Box W1 to effect its function. A deletion to –195, in contrast, abolished expression (PR1-1-2c). In a similar manner to PRI-1-1, these experiments indicate the presence of an elicitor responsive element between –268 and –195 and this area within the PRI-1-2 promoter contains Box W1. The PRI-1-2 promoter also contains another independently functioning W box located less far upstream than in the PRI-1-1 promoter. Construct PRI-1-2d shows that mutation of the W1 sequence within the PRI-1-2a construct had little
effect on the level of elicitor inducibility. A similar construct that is further deleted to −371 showed only a very low level of elicitor responsive expression (PRI-1-2e). When the W1 sequence was not mutated, the promoter retained high level expression (PRI-1-2f). These data indicate that the PR1-2 promoter also contains two independently functioning W boxes. One is Box W1 and the other is located between −371 and −443. We call this upstream element Box W3. The functional importance of Boxes W1 and W3 was further confirmed by constructs PRI-2h and PRI-1-2k. Mutation of the W1 and W3 elements within a 656 bp promoter fragment was sufficient to abolish function and also served to further pinpoint the location of Box W3.

**W boxes contain (T)TGAC(C) elements**
The data presented in Figures 1 and 2 suggest that Boxes W1, W2 and W3 are elements necessary for responsiveness of the PR1 promoters to elicitor. A comparison of the W box sequences and the mutations that abolish function suggests that the three W boxes are similar elements. Box W1 contains a TTGACC sequence and a 10 bp block mutation that destroys this sequence also renders Box W1 non-functional (Figure 3, W1 Mut; Figure 1, PRI-1-f). Box W2 also contains a TTGACC sequence and its mutation also abolishes function (Figure 3, W2 Mut a, PRI-1-k). This strongly suggests that Boxes W1 and W2 are similar TTGACC-containing elicitor responsive elements. An additional mutation in Box W2 (Figure 3, W2 Mut b) also abolishes function (data not shown). This mutates the sequence TTCAGCC, that is similar to the TTGACC sequence, and suggests that Box W2 may consist of two similar elements. Box W3 appears slightly different to Boxes W1 and W2. However, it contains two TGAC elements in opposing orientations (Figure 3). A mutation that alters just three bases in Box W3 and destroys both TGAC sequences is sufficient to abolish function (Figure 3, W3 mut; see also below). Boxes W1, W2 and W3 therefore appear to be similar elicitor responsive elements with a TGAC core sequence.

**W boxes alone confer elicitor responsiveness**
The position of Box W3 within the PR1-2 promoter occurs in a region of the promoter where the similarity between the PR1-1 and PR1-2 promoters is very high. Within the region encompassing Box W3 there are only six bases that are dissimilar to the corresponding region in the PR1-1 promoter (Figure 4), and yet PR1-2 contains a functional elicitor responsive element whereas PR1-1 does not. This led us to investigate further the bases within Box W3 that are required for function. Deletion constructs of the PR1-1 and PR1-2 promoters were made with the Box W1 sequence mutated (Figure 4, PRI-1-n and PRI-1-2d). The PR1-2d construct has a functional W3 box and gave high level elicitor responsive expression. The PR1-1n construct contains, in contrast, no functional W box and was almost inactive (Figure 4). Mutation of just three of the dissimilar bases in the PR1-2 construct was sufficient to abolish function of the promoter (Figure 4, PRI-1-2m). The mutation destroyed both of the TGAC elements in Box W3. In addition to this loss-of-function experiment, a gain-of-function experiment was performed by taking the inactive PRI-1-n construct and changing these three bases so that they were the same as in the PR1-2 promoter. This changed the sequence of the PR1-1 promoter to include two TGAC elements and the promoter then showed
high level elicitor responsive expression (Figure 4, PR1-1p). This demonstrates that W boxes act as elicitor response elements within the context of 453 bp of the PR1-1 promoter and that intact TGAC elements constitute an important part of Box W3.

In addition, a tetramer of Box W1 was placed upstream of a minimal CaMV 35S promoter. This construct displayed high level elicitor responsive expression (Figure 5). The level of expression with the Box W1 tetramer after the addition of Pep25 was \(~54%\) of that of the full PR1-1 promoter. The level of induction by Pep25 was 27.2-fold for the PR1-1 promoter and 9.6-fold for the Box W1 tetramer, whereas the minimal CaMV 35S promoter alone showed no elicitor inducibility (Figure 5). This lower fold induction observed with the Box W1 tetramer was largely due to a slightly increased background level of transcription in the absence of Pep25 (data not shown). Taken together these experiments clearly illustrate that W boxes are elicitor response elements and that they alone are sufficient to confer high level elicitor responsiveness.

**Boxes W1, W2 and W3 are bound by similar nuclear proteins**

W boxes from the PR1-1 and PR1-2 promoters appear similar with respect to function and sequence. This led us to investigate the nuclear factors that bind to these W boxes. Gel shift experiments using parsley nuclear proteins and W box oligonucleotides demonstrated that each W box is specifically bound by nuclear protein (Figure 6A and B). Similar patterns of nuclear protein binding are seen with all three W boxes (Figure 6). In each case mutated W boxes (Figure 6) failed to compete for binding (Figure 6A and B). We can therefore correlate mutations in the W boxes (Figure 6) with loss of function (Figures 1, 2 and 4) and the inability to compete for nuclear protein binding (Figure 6).

In contrast to the mutated versions, all of the authentic W boxes cross-competed for binding. This is illustrated in Figure 6B, where binding to Box W3 is competed not only by Box W3 oligonucleotides but also effectively by Box W1 and Box W2 oligonucleotides. Taken together the gel shift experiments suggest that Boxes W1, W2 and W3 are bound by similar nuclear factors and that mutations that abolish function of the W boxes also abolish binding of these factors.

**WRKY, WRKY2 and WRKY3 bind specifically to W boxes**

To isolate cDNA clones encoding proteins that bind specifically to W boxes, a parsley cDNA expression library in Agt11 was screened with a mixture of probes containing concatamers of each of the three W boxes. Four independent positive clones were isolated. The binding specificities of the proteins was established by the use of individual probes. Five probes were used; Box W1, Box W2, Box W3, Box W1 Mut and MRECHS (an unrelated Myb recognition element from the parsley CHS promoter; M. Feldbrügge and B. Weißhaar, personal communication). One of the proteins (NSB1) bound non-specifically to all probes (Figure 7), demonstrating that all probes were capable of being bound. Three clones, called WRKY1, WRKY2 and WRKY3, showed sequence-specific binding.
Each of these proteins bound to Boxes W1, W2 and W3 but not to Box W1 Mut or the unrelated sequence MRECHS (Figure 7). WRKY1, 2 and 3 therefore show binding characteristics similar to those of the nuclear proteins responsible for the sequence-specific binding to W boxes in gel shift experiments. We can now correlate a mutation in Box W1 (Figure 3) with loss of function, the inability of nuclear proteins to bind in gel shift experiments and the inability of WRKY1, 2 and 3 to bind to this element in vitro.

**WRKY1, 2 and 3 are members of the WRKY family of proteins**

Analysis of the WRKY1, 2 and 3 cDNAs revealed that none were full-length. A λzAPII library (Korfhage et al., 1994) was therefore screened with the three cDNA inserts and cDNA clones containing the entire coding regions of WRKY1 and WRKY3 were isolated. Except for the additional nucleotides at each end, the sequences of these two λzAPII clones were identical to the original λgt11 clones. Although an additional WRKY2 cDNA clone was isolated, it was slightly shorter than the original one. The nucleotide and deduced amino acid sequences of the three clones are presented in Figure 8. WRKY1, 2 and 3 are related proteins, belonging to the same family. This family has recently been described (Rushton et al., 1995) and is characterized by a conserved region of 56–58 amino acids. Evidence suggests that this conserved region contains a DNA-binding domain and that it may include a novel type of zinc finger (Rushton et al., 1995). The first four amino acids of the most highly conserved region within the conserved domain are WRKY and we therefore call this family of proteins the WRKY family and the conserved domain that defines the family the WRKY domain.

The WRKY1 cDNA is 1798 bp in length with an open reading frame extending from nucleotides 52 to 1594 (Figure 8A). Both the 5′ and 3′ untranslated regions have in-frame stop codons and there is a poly(A) tail. Two different locations of the poly(A) tail were apparent among the WRKY1 λzAPII cDNA clones, at either 1796 or 1798. The predicted WRKY1 protein has a molecular mass of 58 kDa, contains two WRKY domains and, in addition, possesses a number of features that have been shown to be associated with transcription factors. These include two acidic domains, a basic domain (putative nuclear localization signal), a glutamine-rich domain and a serine/threonine-rich domain. The original λgt11 clone extended from nucleotide 42 to 1403 and contained both WRKY domains.

The WRKY2 partial cDNA is 1208 bp in length and is truncated at the 5′end (Figure 8B). It contains an open reading frame extending to position 888 followed by over 300 bp of 3′ untranslated region. The WRKY2 protein is similar to WRKY1 in that it contains two WRKY domains. However, the first WRKY domain is truncated, containing only the last 19 amino acids. The ability of the truncated WRKY2 protein to bind in a sequence-specific manner suggests that the C-terminal WRKY domain alone is sufficient for binding to W boxes. The WRKY2 protein is quite similar to WRKY1 (40% sequence identity and 57% similarity) and like WRKY1 it contains two acidic domains and a basic domain.

The WRKY3 cDNA is 1286 bp long and encodes a protein of 38 kDa whose only similarity to WRKY1 and WRKY2 at the amino acid sequence level is that it contains a WRKY domain (Figure 8C). WRKY3 does however contain a number of domains that are similar in nature to those present in the WRKY1 and 2 proteins. These include a basic domain, a serine/threonine-rich domain and a glutamine-rich domain. The second half of
Fig. 8. Nucleotide and deduced amino acid sequences of WRKY1, WRKY2 and WRKY3. (A) Nucleotide and deduced amino acid sequences of WRKY1. The two WRKY domains are shown as black boxes. Within the WRKY domain the WRKY sequence is underlined (dots) and the two invariant cysteines and two invariant histidines are also underlined (solid). Glutamine residues within the glutamine-rich domain are underlined, as are acidic domains (dashed), the basic domain (large dots) and the serine/threonine-rich domain (small dots). Stop codons are indicated by stars. The original XgtI clone is indicated by a black line above the first and last bases. (B) Nucleotide and deduced amino acid sequences of the WRKY2 partial cDNA. The WRKY domains and other protein features are as marked in (A). (C) Nucleotide and deduced amino acid sequences of WRKY3. The single WRKY domain and other features of WRKY3 are illustrated as in (A). The original XgtI clones are indicated by a black line above the first and last bases (A). The nucleotide sequences will appear in the GenBank database under the accession numbers U48831 (WRKY1), U58540 (WRKY2) and U56834 (WRKY3).

The glutamine-rich domain, between nucleotides 464 and 523, is notable as it consists entirely of glutamine, proline and valine/leucine/isoleucine residues, mostly in the form of PVQ repeats. The two XgtI clones obtained by Southern screening consisted of nucleotides 24–1191 and 42–1069, each possessing the single WRKY domain.
present that of WRKY1 gene elicitor. The level of WRKY1 mRNA is extremely rapid and transient with the addition of the elicitor Pep25. We have shown by loss- and gain-of-function experiments that WRKY1 mRNA levels are detected in untreated cells at a very low level and reach the maximum in untreated cells. Within 15 min of treatment this level starts to decline and a minimum is reached after ~3 h of treatment. During the first hour of the response of parsley cells to Pep25 WRKY1 mRNA accumulation therefore shows inverse kinetics with respect to WRKY1 and 3. Between 1 and 3 h the mRNA levels of all three WRKYs decline. After this timepoint the level of WRKY2 mRNA begins to rise and appears to continue to rise after the 8 h timepoint until a level similar to that found in untreated cells is reached (data not shown). The level of PR1 mRNA is also shown in Figure 9. It starts to rise after 15~30 min and continues to rise at a steady rate until ~3 h after the addition of Pep25. The level between 3 and 8 h is only slightly less than the maximum. The maximum in WRKY1 and 3 mRNA level is 2 h in advance of that of PR1.

**Discussion**

**W boxes and WRKY proteins**

We have shown by loss- and gain-of-function experiments that WRKY1 mRNA levels are detected in untreated cells at a very low level and reach the maximum in untreated cells. Within 15 min of treatment this level starts to decline and a minimum is reached after ~3 h of treatment. During the first hour of the response of parsley cells to Pep25 WRKY1 mRNA accumulation therefore shows inverse kinetics with respect to WRKY1 and 3. Between 1 and 3 h the mRNA levels of all three WRKYs decline. After this timepoint the level of WRKY2 mRNA begins to rise and appears to continue to rise after the 8 h timepoint until a level similar to that found in untreated cells is reached (data not shown). The level of PR1 mRNA is also shown in Figure 9. It starts to rise after 15~30 min and continues to rise at a steady rate until ~3 h after the addition of Pep25. The level between 3 and 8 h is only slightly less than the maximum. The maximum in WRKY1 and 3 mRNA level is 2 h in advance of that of PR1.

**Pep25 induces rapid and transient changes in WRKY1, 2 and 3 mRNA levels**

The expression patterns of the WRKY1, 2 and 3 genes themselves during the response to Pep25 were established. Figure 9A shows the time courses of changes in WRKY1, 2 and 3 mRNA levels upon treatment with the oligopeptide elicitor Pep25, and Figure 9B the relative levels of mRNA as quantified by a Phospholmager. In parsley cells that had not been treated with Pep25 no WRKY1 mRNA was detected (Figure 9A and B). However, after 15 min of Pep25 treatment WRKY1 mRNA was detected at a level almost 50% of that of the maximum observed during the time course. This indicates that transcription of the WRKY1 gene is an extremely rapid response to fungal elicitor. A maximum in the WRKY1 mRNA level is reached between 45 and 60 min of Pep25 treatment, after which the level declines. After 4 h the level is only ~12% of the maximum value and continues to decline. Similarly, an extremely rapid and transient increase in WRKY3 mRNA level is also observed (Figure 9A and B), suggesting that WRKY1 and WRKY3 are coordinately regulated. The level of WRKY3 mRNA is however less than that of WRKY1 and, unlike WRKY1, WRKY3 mRNA is present in untreated cells at a level up to 25% of that of the maximum. In contrast, the level of WRKY2 mRNA shows a different pattern (Figure 9C). Unlike WRKY1 and 3, which are rapidly up-regulated, WRKY2 is rapidly down-regulated. A very low level of WRKY2 mRNA is present in untreated parsley cells. Within 15 min of treatment this level starts to decline and a minimum is reached after ~3 h of treatment. During the first hour of the response of parsley cells to Pep25 WRKY2 mRNA accumulation therefore shows inverse kinetics with respect to WRKY1 and 3. Between 1 and 3 h the mRNA levels of all three WRKYs decline. After this timepoint the level of WRKY2 mRNA begins to rise and appears to continue to rise after the 8 h timepoint until a level similar to that found in untreated cells is reached (data not shown). The level of PR1 mRNA is also shown in Figure 9. It starts to rise after 15~30 min and continues to rise at a steady rate until ~3 h after the addition of Pep25. The level between 3 and 8 h is only slightly less than the maximum. The maximum in WRKY1 and 3 mRNA level is 2 h in advance of that of PR1-1.
DNA-binding proteins and evidence suggests that each WRKY domain contains a zinc finger (Rushton et al., 1995). When WRKY-like sequences from the best database are included then WRKY proteins have been shown to be present in parsley, wild oat, sweet potato, rice, turnip, cucumber and Arabidopsis. Evidence suggests that WRKY proteins may play a role in a number of important plant processes, including hormonal regulation (Rushton et al., 1995) and carbohydrate-regulated gene expression (Ishiguro and Nakamura, 1994) in addition to defence-related gene regulation.

Figure 10A shows a comparison of the WRKY domains from WRKY1, 2 and 3 with all available WRKY domains present in the GenBank database. The WRKY domain consensus illustrates that there is a group of seven invariant amino acids near the N-terminal end of the WRKY domain (WRKY GQK) and that the cysteines and histidines that may form a zinc finger are also invariant. A comparison of WRKY1, 2 and 3 with ABF1, ABF2 and SPF1 shows that the WRKY family can be divided into two groups (Figure 10B, Rushton et al., 1995). WRKY1, WRKY2, ABF1 and SPF1 are group 1 WRKY proteins possessing two WRKY domains (Figure 10A). The WRKY domains are separated by between 106 and 121 amino acids and between the two are two acidic domains and a basic domain. The remaining WRKY proteins, WRKY3 and ABF2, belong to group 2. Both members of this group have only one WRKY domain and this represents the only major similarity at the amino acid sequence level between the group 1 and group 2 proteins and, indeed, between WRKY3 and ABF2 themselves.

Although many of the WRKY proteins are entirely different in primary amino acid sequence outside the WRKY domain itself, they do share a number of features which suggest that they function as transcription factors. Each WRKY protein contains a basic domain that might function as a nuclear localization signal (Raikhel, 1992), suggesting that they are nuclear proteins. In addition every WRKY protein so far characterized has been isolated by virtue of its sequence-specific DNA-binding and contains at least one domain that could function in vivo as a transcriptional activation domain. Four distinct types of such domains have been identified and classified according to their primary sequence as acidic, proline-, glutamine-, and serine/threonine-rich (Mitchell and Tjian, 1989; Gerber et al., 1994). Possible examples of all of these are present in the six characterized WRKY proteins consistent with the WRKY family being a family of plant transcription factors.

The isolation of four independent clones encoding W box-binding proteins (two independent WRKY3 clones were isolated), all of which were WRKY proteins, is evidence for a WRKY protein–W box interaction. It is not at present clear whether all WRKY proteins can bind to TGAC-containing elements nor whether this type of element is the only type to which WRKY proteins bind. ABF1 and ABF2 both bind specifically to the Box 2 sequence found in the promoters of wheat, wild oat and barley α-Amy2 genes (Lanahan et al., 1992; Rushton et al., 1992). Interestingly, this element contains three TGAC sequences (TTGAC, TGTGAC and TGAC) within 22 bp (Rushton et al., 1992). This further suggests that many WRKY proteins can bind to such elements. In this respect, W boxes and WRKY proteins appear to resemble ACAT elements and bZIP factors (Armstrong et al., 1992). SPF1, however, has been shown to bind to two sequences (SP8a and SP8b) that contain no TGAC elements and bear little similarity to each other (Ishiguro and Nakamura, 1994). It is possible that a number of the WRKY proteins may be able to bind to more than one type of element. Each WRKY domain of the group 1 proteins may contain a zinc finger-like DNA-binding domain (Rushton et al., 1995); however, the C-terminal alone appears to be sufficient for sequence-specific binding (Figures 7 and 8).
consistent with similar observations using truncated versions of SPF1 (Ishiguro and Nakamura, 1994). It is possible that the group 1 WRKY proteins have twin autonomous DNA-binding domains (Rushton et al., 1995), similar to the rice DNA-binding protein GT-2 (Dehesh et al., 1992). In addition to the group 2 WRKY proteins, another family of plant DNA-binding proteins, called the Dof family, that have just one putative zinc finger motif has recently been characterized (Yanagisawa, 1995). It will be of interest to determine the binding specificities of each WRKY domain from group 1 WRKY proteins and to compare this to the group 2 WRKY domains.

The possible roles of WRKY1, 2 and 3 in the response to fungal elicitor

The specific binding of WRKY1, 2 and 3 to functional W boxes suggests that they constitute at least part of a protein complex bound to W boxes in the PRI1 promoters. Additionally, the coordinate induction of WRKY 1 and 3 mRNA is extremely rapid and correlates well with the appearance of the elicitor-inducible in vivo footprint observed previously around Box W1 (Meier et al., 1991). These mRNA accumulation studies using the defined peptide elicitor Pep25 strongly imply that the WRKY genes are components of the stimulus-dependent signal transduction pathway and that the proteins act directly as transcription factors in modulating expression of downstream target genes such as PRI1. The WRKY2 gene, however, displays a different pattern of mRNA accumulation. We can therefore speculate as to the combination of WRKY proteins that may be binding to the W boxes during the course of the elicitor response. Our data suggest that one possible consequence of elicitor addition may be the rapid replacement of WRKY2 with WRKY1 and 3. Whether WRKY1 and 3 bind alone or together to W boxes remains to be determined. The possible role of WRKY2 is less obvious. It is possible that WRKY2 binds to W boxes in the absence of elicitor and it may act as a repressor, being replaced by WRKY1 and 3 upon elicitor induction. Alternatively WRKY2 may not be completely replaced but the combination of WRKY2 with 1 and 3 may lead to transcriptional activation in a similar way to the selective heterodimer formation between CPRF bZIP factors that bind to the parsley CHS promoter (Armstrong et al., 1992).

Interestingly, the relative patterns of mRNA accumulation of WRKY1 and 3 in respect to PRI1 is very similar to that seen with the parsley DNA-binding protein BPF-1 in respect to PAL (da Costa e Silva et al., 1993). BPF-1 mRNA accumulates rapidly and transiently upon the addition of fungal elicitor, the increase being detectable within 30 min, with a maximum being reached at around 2 h, in advance of the maximum in PAL mRNA level. Thus two endpoints of a bifurcated signal transduction pathway initiated by fungal elicitor appear to parallel each other.

Fungal elicitor causes changes in the phosphorylation state of many proteins in suspension-cultured parsley cells (Dietrich et al., 1990) and this can have profound effects on their activity and interaction with other proteins (Stone and Walker, 1995). WRKY1 and 3 (and possibly also WRKY2) have serine/threonine-rich domains that may be the sites for phosphorylation (Tague and Goodman, 1995). Phosphorylation is known to affect the cellular localization, DNA-binding activity, transactivation activity and turnover of transcription factors (Hunter and Karin, 1992; Papavassilou et al., 1992) and this raises the possibility that the activity of WRKY proteins may also be regulated by phosphorylation. A role for a serine/threonine kinase-mediated phosphorylation cascade during a plant–pathogen interaction has recently been shown in tomato, where the serine/threonine kinase Pto that confers resistance to bacterial speck disease specifically phosphorylates another serine/threonine kinase Pti (Zhou et al., 1995). Furthermore, a 30 bp promoter region containing a TGAC sequence in the centre (Matton et al., 1993) was found to be necessary for the activation of the potato PRI10a gene by elicitor (Després et al., 1995). This region is bound specifically by two factors, PBF-1 and PBF-2. The binding and phosphorylation of PBF-1 is induced by elicitor and this phosphorylation is associated with gene activation (Després et al., 1995). The similarity in binding site and kinetics of mRNA accumulation to the results presented here raises the possibility that PBF-1 may be a WRKY protein.

The combined results presented here lead us to suggest that WRKY1, 2 and 3 are transcription factors that play a key role in a signal transduction pathway that leads from elicitor perception to PRI1 gene activation.

Materials and methods

Cell and protoplast cultures

Cell suspension cultures of parsley (Petroselinum crispum) were maintained in modified BS medium as described earlier (Kombrink and Hahlbrock, 1986). Cell cultures 5–6 days after passage were used for isolation of protoplasts according to Dangl et al. (1987). The elicitor was prepared from hyphal cell walls of the fungus, Phythophthora sojae, after the method of Ayers et al. (1976).

Plasmid constructions

All plasmid constructs used in these experiments are translational fusions of various PRI-1 and PRI-2 promoter derivatives to the bacterial glucuronidase (GUS) reporter gene contained in the base vector pUC9 (van de Locht et al., 1990). 5' end-point deletion constructs were generated by Bal31 exonuclease digestion of the target DNA, replenishment of blunt ends by addition of DNA polymerase I (Klenow enzyme) and the addition of HindIII linkers by T4 DNA ligase or by PCR using appropriate in end-point primers. Site-directed mutagenesis of the different promoter elements as described in the text were achieved using the PCR-based ‘megaprimer’ method (Landt et al., 1990). To obtain the W1 tetramer a Box W1 monomer with a 5' BamHI site and a 3' BgIII site was constructed. The monomers were ligated with T4 ligase and then digested with BamHI and BgIII. The resulting multimers, that had all W1 monomers in the same orientation, were gel purified and the tetramers were eluted and ligated into the BamHI site upstream of the 35S CaMV promoter region (~46 to +8) of pBT-10 (M.Sprenger and B.Weissaar, unpublished data), a derivative of pBT-2 (Weißhaar et al., 1991). The correctness of all constructs was verified by sequencing. All plasmids were amplified in the bacterial strain Gm2163 (New England Biolabs, Beverly, USA) and purified either on anion exchange resin columns (QIAGEN) or by the clear-lysatse procedure and twice banded through CsCl gradients.

Transient expression assays

Linearized plasmid DNA (10–20 μg/10^6 protoplasts) was transfected into freshly prepared protoplasts using polyethylene glycol (PEG) as described previously (van de Locht et al., 1990). Each transfection assay of 2×10^6 protoplasts was split, placed in two 3 ml plates and one was treated for 8 h with either fungal elicitor (50 ng/ml) or Pep25 (100 ng/ml). Protoplasts were collected by centrifugation, frozen in liquid nitrogen, crude protein extracts prepared and GUS activity assayed (Jefferson et al., 1987). Bradford assays (Bio-Rad, Hercules, USA) were used for protein determination.
Preparation of nuclear extracts and gel mobility shift assays

Nuclear extracts were prepared as described by Armstrong et al. (1992) either from untreated parsley cells or from cells treated for 3 h with elicitor. Gel mobility shift assays were performed as previously described (da Costa e Silva et al., 1993). Each binding reaction was performed with 100 pg [32P]-labelled probe. 3 µg nuclear protein extract, 0.2–0.4 µg poly (dI:dC) and specific competitor DNA as indicated.

South-Western screening

A random-primed 3441 cDNA library constructed using poly(A)+ RNA prepared from an equal mixture of total RNA samples isolated from parsley cells that had been treated with elicitor for 0.5, 1.5 and 3 h was used (Kawalleck et al., 1992). The expression library was screened as previously described (Rushon et al., 1995) using multimers of the W boxes labelled by nick translation (Weilbahr et al., 1991).

RNA isolation and Northern hybridization

Parsley cells were treated with Pep25 (final concentration 100 ng/ml) for the required time, frozen in liquid nitrogen and then ground to a fine powder under liquid nitrogen. Total RNA was extracted from ~2.5 g of powdered cells using a Total RNA Maxi Kit (Qiagen, Hilden, Germany). Twenty micrograms of total RNA from each timepoint were used for Northern blot hybridization as previously described (Lois et al., 1989). WRKY1, 2 and 3 cDNA fragments were labelled by random priming (Feinberg and Vogelstein, 1984). The relative level of mRNA at each time point was measured by a Phosphorimager using the Storm System Hardware and Image Quant Software (Molecular Dynamics, Krefeld, Germany).

DNA sequencing and sequence analysis

The dideoxy chain termination method (Sanger et al., 1977) was employed and the reactions were performed using a T7 sequencing kit (Pharmacia, Freiburg, Germany). Sequence analysis was performed using the GCG software package (Deveraux et al., 1984).

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References


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