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GENETICS AND GENOMICS

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# Detection of the putative *cis*-region involved in the induction by a *Pyricularia oryzae* elicitor of the promoter of a gene encoding phenylalanine ammonia-lyase in rice

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**Abstract** A rice PAL (phenylalanine ammonia-lyase) gene sequence (*rPAL-P5*), which is highly similar to and likely the same as a previously described rice *ZB8PAL* gene, including the 5′-upstream and exon I coding regions of *PAL*, was isolated using PCR amplification. The expression of several *PALs*, including *rPAL-P5*, was strongly induced following inoculation with *Pyricularia oryzae* or treatment with a *P. oryzae* elicitor. To identify the promoter region induced by the *P. oryzae* elicitor, we constructed and subsequently transformed *rPAL-P5* promoter deletion series into rice calli using particle bombardment. Results from both elicitor-inducible reporter gene and gel mobility shift assays demonstrated that the sequence −349 to −256 of the *rPAL-P5* promoter includes a *cis*-element involved in the induction of *P. oryzae*.

**Keywords** Phenylalanine ammonia-lyase · *cis*-Element · Elicitor · Rice calli · Promoter

**Abbreviations** *CTAB:* Cetyltrimethylammonium bromide · *2,4-D:* 2,4-Dichlorophenoxyacetic acid · *GUS:* β-Glucuronidase · *4-MU:* 4-Methylumbelliferone · *4-MUG:* 4-Methylumbelliferyl glucuronide · *NOS:* Nopaline synthase · *PAL:* Phenylalanine ammonia-lyase

## Introduction

Secondary metabolites such as phytoalexins, lignins and flavonoids produced by the phenylpropanoid pathway may help protect higher plants from pathogen attack (Dixon and Pavia 1995). Phenylalanine ammonia-lyase (PAL) is a key enzyme in this pathway, catalyzing the first committed step in phenylpropanoid biosynthesis, namely, the deamination of phenylalanine to yield *trans*-cinnamic acid and $NH_4^+$ (Bate et al. 1994; Howles et al. 1996).

PAL genes are induced by pathogen infection and specific elicitor molecules (Hughes et al. 1989; Sharan et al. 1998). The promoter sequences responsible for this induction have been identified in some genes, including parsley *PAL-1*, pea *PSPAL-1*, carrot *gDcPAL1* and others (Silva et al. 1993; Yamada et al. 1994; Takeda et al. 2002). Many of these *cis*-elements share a conserved AC-rich element, the mutation or deletion of which can result in a reduction or loss of induced activation following pathogen attack (Lois et al. 1989; Seki et al. 1997, 1999; Murakami et al. 1997).

Relatively few studies have dealt with monocot PAL genes. To date, only *PALs* in rice (Minami et al. 1989; Minami and Tanaka 1993; Zhu et al. 1995), wheat (Liao et al. 1996) and barley (Kervinen et al. 1998) have been cloned, and only rice *ZB8PAL* and barley *hpal2*, *-3*,*-4*, *-6* have been shown to be induced following pathogen infection. No further analysis of these promoters has been reported.

We report here the induction of the rice *rPAL-P5* gene following *Pyricularia oryzae* infection or elicitor treatment and identify a region of the promoter involved in this induction.

## Materials and methods

### Plant material, pathogen and vectors

Seeds of rice (*Oryza sativa* L. cv. Aizhixu) and fungal strain of *Pyricularia oryzae* cv. 131 were kindly provided by Dr. Peng Youliang (China Agricultural University). Seedlings of *O. sativa* L. cv. Aizhixu were grown in a heated greenhouse (28–32°C). The induction and culture of rice calli were performed as previously described by Yuan et al. (2002). T-vector pUCm-T was purchased from the Shanghai Sangon Company. The plant transformation

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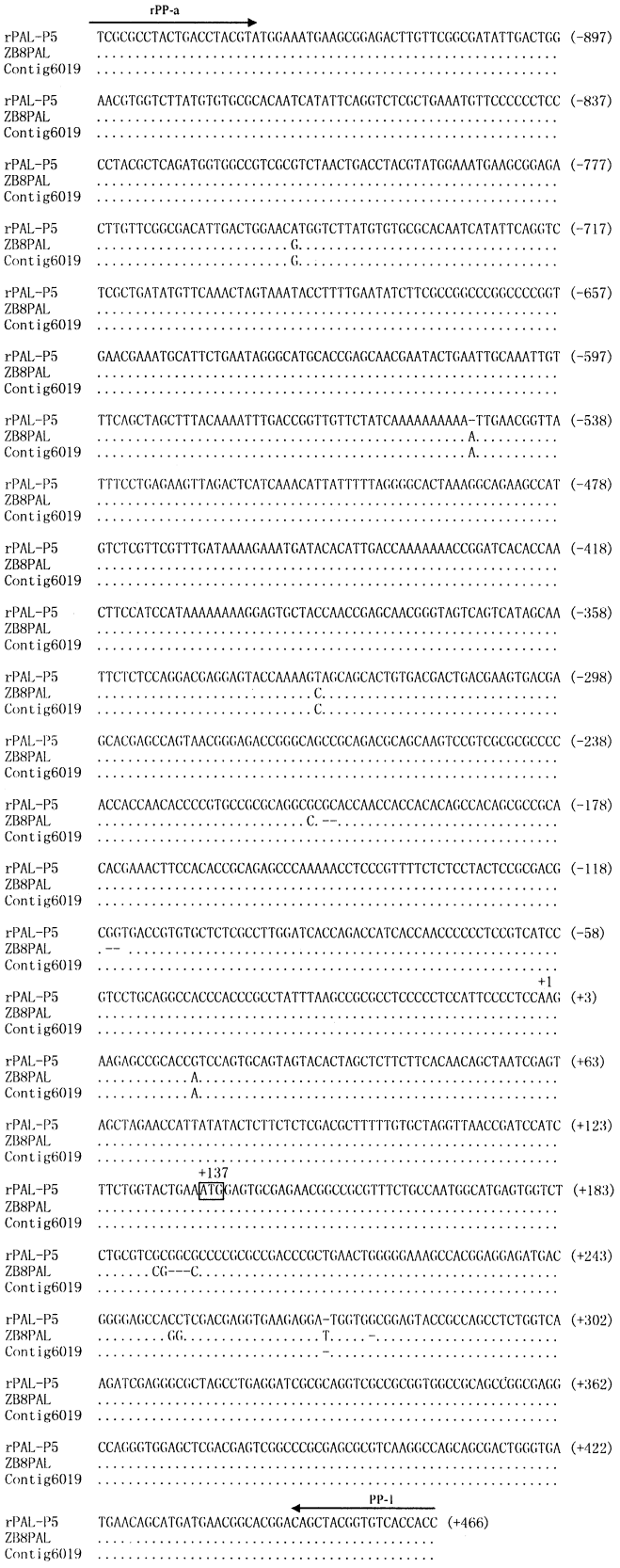


**Fig. 1** Comparison of *rPAl-P5*, *ZB8PAL* and contig6019 nucleotide sequences. The sequence of *rPAL-P5* is shown on the *top line*. Bases in *ZB8PAL* and contig6019 common to all three nucleotides are denoted with *dots*, with only the variable bases indicated for the former two sequences. Contig6019 is from the rice genome sequences of HuaDa, China. The transcription start site "*A*" in the *shaded area* is at the +1 position. The codon representing translational initiation is indicated by a *rectangle*, and the "A" of ATG is at the +137 position. *Arrow* Primers (rPP-a and PP-1) for *rPAL-P5*

vectors pCambia1381Xa (hygromycin R, *GUS-NOS*) and pCambia1301 (hygromycin R, *35S-GUS-NOS*) were kindly provided by Dr. Richard Jefferson.

## Primers and PCR for *rPAL-P5* and 5′-truncated promoters

Based on the nucleotide sequence of *ZB8PAL*, the *rPAL-P5* DNA fragment (position −956 to +466) containing the putative promoter and a part of *PAL* exon I was generated using primers rPP-a (5′) (−956: 5′-TCGCGCCTACTGACCTACGTAT-3′) and PP-1 (3′) (+447 to +466: 5′-GGTGGTGACACCGTAGCTGT-3′) (Fig. 1). The 5′ truncated promoter segments from *rPAL-P5* were obtained using rPP-a, rPP-b (−646: 5′-CATTCTGAATAGGGCATGCA-3′), rPP-c (−424: 5′-CACACCAACTTCCATCCATA-3′), rPP-d (−264: 5′-AGACGCAGCAAGTCCGTCGC-3′), rPP-e (−45: 5′-ACCCACCCGCCTATTTAAGC-3′) and PP-a (3′) (+126 to +145: 5′-AAGCTTGCACTCCATTTCAGTACCAG-3′ with a terminal *Hin*dIII site) (Fig. 3). The transcriptional start site was at +1, and the "A" residue of the translational start site ATG was at +137.

Rice genomic DNA was extracted from 1 g of leaves of 1-month old plants by the CTAB method (Doyle et al. 1990). PCR parameters were 35 cycles of pre-denaturing at 94°C for 120 s, denaturing at 94°C for 60 s, annealing at 60°C for 70 s, enzymatic reaction at 72°C for 90 s. The samples were then kept at 72°C for 10 min.

## Elicitation of rice calli

After *P. oryzae* had been cultured on solid oat-dextrose medium at 28°C in the dark for 7–8 days, the mycelium was washed with 4 ml water from the solid medium, transferred to 200 ml oat liquid medium and incubated at 28°C in the dark for 4–5 days. The mycelium was then harvested by centrifugation at 10,000 *g* for 5 min, washed several times with water, homogenized in 20 ml water using a glass homogenizer and then centrifuged at 10,000 *g* for 5 min. The supernatant was autoclaved and stored at −70°C.

For the elicitation of rice calli, approximately 1 g of rice calli was treated at 25°C for 1, 3, 6 and 12 h in the dark with a five-fold diluted crude elicitor solution, or with water as a control. The treated rice calli were frozen in liquid nitrogen at each time point and stored at −70°C until used for RNA extraction.

## RNA isolation and Northern blot hybridization

At each time point, total RNA was isolated from rice calli treated with crude elicitor using the RNeasy Plant Mini kit (QIAGEN, Valencia, Calif.) and then electrophoresed through a 1.2% denaturing agarose gel (10 $\mu$g per lane). A 136-bp specific untranslated sequence of *rPAL-P5* was used as a probe, and Northern blot hybridization was carried out according to the Bio-Rad Zeta-GT membrane manual (Bio-Rad, Hercules, Calif.).

## Construction of chimeric genes, transformation and selection of rice calli

The *rPAL-P5* promoter (−956 to +145) and its several 5′ truncated promoter segments obtained by PCR were cloned into the pUCm-T vector and sequenced by the Sangon Company. These fragments were then excised and ligated to *GUS* in pCambia1381Xa (Fig. 3). All open reading frames (ORFs) of *rPP-GUS* chimeric genes were

identified by DNA sequencing. The series of *rPP-GUS* chimeric genes were then transformed into rice calli by particle bombardment, selected on N6 medium containing 50 mg/l hygromycin (Zheng et al. 1997; Yuan et al. 2002) and then PCR-amplified (data not shown).

### Fluorometric GUS assay of elicitor-treated transgenic rice calli

Transgenic calli were cut into small pieces and cultured in N6 liquid medium containing 2 mg/l 2,4-D and 50 mg/l hygromycin. The calli were treated for 8 h with a five-fold diluted crude elicitor solution or water with gentle agitation at 100 rpm, at 25°C in the dark. The GUS activity of rice calli, determined using 4-MUG according to the procedure of Jefferson (1987), is presented as 4-MU pmol/mg per protein per minute.

### Preparation of nuclear proteins

Rice calli treated with elicitor or water for 0, 1, 1.5 and 2 h were harvested individually. Nuclear proteins were extracted as described by Nagao (1993) with some modifications: (1) the concentration of protease inhibitors in all buffers was doubled; (2) the supernatant was separated from the pellet produced by 45% saturation with ammonium sulfate, then continued to be brought to 85% saturation. These precipitated proteins were collected by centrifugation at 14,000 rpm for 10 min. The Bradford Bio-Rad Protein Assay kit was used for the determination of nuclear protein. Aliquots of extract were stored at −70°C until used.

### Gel mobility shift assays

Two DNA fragments, RP-a (−424 to −326) and RP-b (−349 to −256), were amplified by PCR and cloned into the pBS vector to construct the recombination plasmids pQRP-a and pQRP-b, respectively. The primers used for RP-a were rPPc (5′-CACACCAACTTCCATCCATA-3′) and PP2D-3 (5′-GGGAAGCTTCTACTTTTGGTACTCCTC-3′, containing a terminal *Hin*dIII site), while the primers used for RP-b were PP2D-5 (5′-GGGAAGCTTGACGAGGAGTACCAAAAGT-3′, containing a terminal *Hin*dIII site) and dp-1r (5′-TTGCTGCGTCTGCGGCTGCC-3′). RP-a and RP-b were then excised from pQRP-a and pQRP-b by *Hin*dIII digestion, and 60 ng DNA of each was radiolabeled at the terminus using $\alpha$-[$^{32}$P]-dCTP and Klenow DNA polymerase. The labeled DNA fragments were precipitated with $NH_4OAc$ and ethanol at −70°C for 1.5 h.

Gel mobility shift assays were performed using the protocol described by Nagao et al. (1993). Unlabeled RP-b fragments and unlabeled unrelated DNA fragments were used as DNA competitors or non-competitors, respectively. The protein/DNA complexes were analyzed by electrophoresis through a 5% native polyacrylamide gel (29:1 cross-linking ratio) in 1× TBE buffer. Following electrophoresis, the gel was dried and autoradiographed.

## Results

### Cloning of *rPAL-P5* gene

A specific DNA fragment was amplified from rice genomic DNA with the PCR primers rPP-a and PP-1. Sequencing showed that this fragment contained a gene (named *rPAL-P5*) which was 99.2% similar to the rice *ZB8PAL* sequence (Zhu et al. 1995) and included 956 bp of promoter sequence, a 136-bp 5′ untranslated region and 330 bp of exon I. Furthermore, *rPAL-P5* displayed significant sequence homology (99.7%) to the DNA sequence contig6019 of the rice genome from 5,987 to 5,032 (Fig. 1). The fact that *rPAL-P5* and *ZB8PAL* shared significant sequence homology and that they were both located at the same site (Contig6019) in the rice genome indicated that *rPAL-P5* and *ZB8PAL* were the same gene but that each contained a few different bases, probably as a result of varied rice breeds. Furthermore, blasting in the rice genome indicated that the promoter and 5′ untranslated region of *rPAL-P5* were specific and present only as a single copy in the rice genomic DNA.

### Induction of *rPAL-P5* gene by the elicitor

Consistent with other reports, the induction of *PAL* genes was detected in rice plants infected with *P. oryzae* or in rice calli treated with elicitor. Following transcription, PAL enzyme activity increased markedly and reached a maximum 8 h following the initiation of elicitor treatment in rice calli (data not shown). In an effort to analyze the specific induction of *rPAL-P5* by elicitor, we treated rice calli with elicitor and subsequently carried out Northern blot RNA analysis using the 136-bp *rPAL-P5* untranslated sequence as the probe (Fig. 2). *rPAL-P5* transcripts were present 1 h after induction and accumulated to a

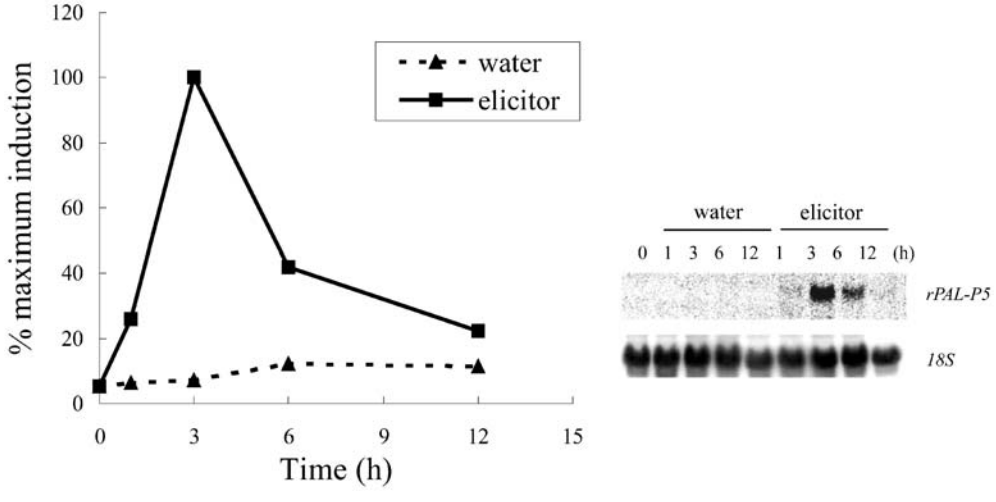


**Fig. 2** Induction of *rPAL-P5* by the *Pyricularia oryzae* elicitor. *rPAL-P5*, a specific DNA fragment representing 136 bp of an untranslated sequence in *rPAL-P5*, was used as a probe; *18S*, part of a cDNA derived from 18S RNA was used as a probe

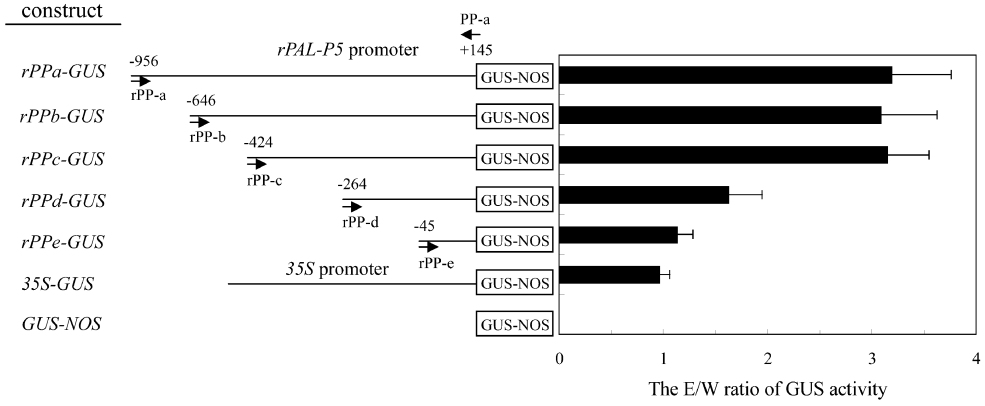


**Fig. 3** Effects of the *P. oryzae* elicitor on the expression of *rPP-GUS* chimeric genes. A schematic representation of the various chimeric constructs used is shown on the *left*. All of these were inserted into pCambia1381Xa 5′ to *GUS-NOS*. The E/W ratios reflecting GUS enzyme activity due to different chimeric genes in the transgenic rice calli are shown on the *right*. *E* Treatment with elicitor for 8 h, *W* treatment with water for 8 h. Ratios were from at least three independent transgenic clones. *Error bars* Standard deviation. GUS activity was detected using a fluorometric assay

maximum level—about 20-fold that of the control—3 h post-induction. By 6 h, *rPAL-P5* mRNA levels were lower than 50% that observed at 3 h, and by 12 h, they were nearly identical to that of the controls.

## Elicitor induction of *rPP-GUS* chimeric genes in rice calli

In an effort to analyze the presence of a functional *cis*-acting region associated with the induction by pathogen elicitor, we constructed chimeric genes containing the *rPAL-P5* promoter or 5′ promoter deletions of *rPAL-P5*, as shown in Fig. 3, and introduced these into rice calli using particle bombardment. PCR confirmed the presence of chimeric *rPP-GUS* genes in the transformed calli (data not shown).

The basal expression levels of GUS activity differed greatly amongst the different clones, which may be due to variable copy numbers being introduced into the latter using the particle bombardment method. The GUS activity ratios between elicitor and water treatments (E/W ratio) for the same *rPP-GUS* chimeric gene were similar. Consequently, the effects of elicitor on the expression of different *rPP-GUS* chimeric genes were arrived at by calculating the E/W ratios of GUS activity, as shown Fig. 3. Since PAL activity in rice calli reached a maximum 8 h following elicitor treatment (data not shown), we elected to examine the GUS activity of transgenic calli at this time point. A construct lacking a promoter (*GUS-NOS*) was used as a negative control, and the *35S-GUS-NOS* construct was used as positive control. The negative control showed no GUS activity, while the E/W ratio for the positive control construct was approximately 1. The E/W ratios of transgenic calli with *rPPa-GUS*, *rPPb-GUS* or *rPPc-GUS* were about 3, indicating that the elicitor induced gene expression threefold. These results suggest that the −956 to −424 region is not involved in induction. While the E/W ratios of *rPPd-GUS* and *rPPe-GUS* were only about 1.6 and 1.1, respectively, which suggests that the absence of the −424 to −264 or −264 to −45 regions could cause a reduction in the induction of the *rPAL-P5* promoter and that the −424 to −264 region plays a greater role in decreasing the induction than the −264 to −45 region, it is suggested that the −424 to −264 region is more important for the elicitor-induced response of *rPAL-P5*.

## Elicitor-induced binding assay of the *cis*-region of the *rPAL-P5* promoter

Although two regions (−424 to −264 and −264 to −45) were associated with the induction of the *rPAL-P5* promoter, the −424 to −264 region seemed to have a greater effect. In an effort to further characterize this region, we broke down these regions into two fragments—RP-a (−424 to −326) and RP-b (−349 to −256)—and analyzed protein binding using a gel mobility shift assay. As shown in Fig. 4A, complexes were only formed when the RP-b fragment was mixed with elicitor-treated nuclear extracts, with maximum binding appearing 2 h following elicitor treatment (Fig. 4B). In addition, the complexes were enhanced by increasing the levels elicitor-treated nuclear extracts and inhibited by a 25- or 50-fold excess of competitor (unlabeled RP-b fragments) but not by non-competitors (unlabeled unrelated DNA fragments) (Fig. 4C). These findings reflect the presence of binding sites for elicitor-induced nuclear factors within the −349 to −256 region, which is a *cis*-region involved in the activation of the *rPAL-P5* promoter by the *P. oryzae* elicitor.

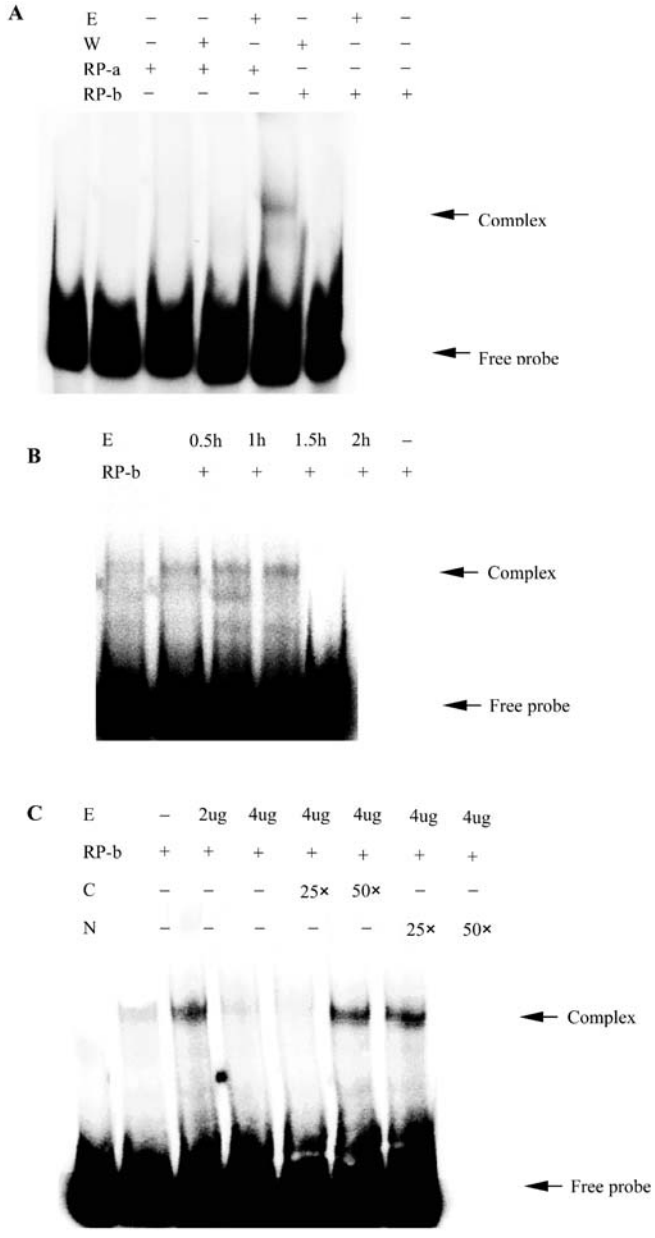


**Fig. 4A–C** Binding of nuclear extracts to DNA fragments of the *rPAL-P5* promoter. *E* Nuclear extracts from elicitor-treated rice calli, *W* nuclear extracts from water-treated rice calli, *RP-a* –424 to –326 fragment, *RP-b* –349 to –256 fragment, *C* specific competitors, *N* nonspecific competitors. The amount of labeled probe used was 1 ng for each lane. **A** The amount of nuclear extract used from rice calli treated with E or W for 2 h was 2 μg for each lane, **B** the amount of nuclear extract used for the different time points was 2 μg for each lane, **C** competitors were unlabeled RP-b fragments

## Discussion

Analysis of 5′ deleted promoters suggested that the –424 to -265 region is important for the elicitor induction of *rPAL-P5* (Fig. 3). Further, gel mobility shift assays showed that the –349 to -256 region of the *rPAL-P5* promoter could form specific DNA-protein complexes with nuclear extracts from rice calli treated with *P. oryza*e crude elicitor, indicating that the –349 to –256 region might contain *cis*-elements responsible for elicitor induction (Fig.4). AC-rich elements have been reported to be important for pathogen or elicitor induction of *PALs* and other genes of the phenylpropanoid pathway (such as *CHS* and *4CL*) (Douglas et al. 1987; Seki et al. 1999) in several dicotyledonous plants, but this has not yet been reported for monocotyledonous plants. We failed to find putative AC-rich elements in the *cis*-region (–349 to –256) of the *rPAL-P5* promoter, suggesting that *cis*-elements different from the AC-rich sequence might be present in this region.

The identification of *PAL* regulatory proteins associated with pathogen activation is very important for furthering our understanding of the regulatory mechanisms that *PALs* play in terms of plant defense responses. To date, only a few plant regulatory proteins have been reported. In parsley, the BPF-1 (BoxP-binding Factor) protein, which binds specifically to BoxP of the *PcPAL* promoter, has been cloned, and it was found that the BPF-1 gene is activated by elicitor and co-expressed with *PAL* at infection sites (Silva et al. 1993). Sugimoto et al. (2000) found that tobacco NtMYB2 can interact with BoxL of the *PAL* promoter, that the gene encoding NtMYB2 is activated by pathogen and elicitor and that the constitutive expression of *NtMYB2* stimulates the expression of *PAL* in uninfected tobacco plants. In addition, the MAPK (mitogen-activated protein kinase) cascade reaction (NtMEK2-SIPK/WIPK) was reported to be associated with the expression of *PALs* and probably takes part in the regulation of defense-related genes, including *PALs*, in plants (Yang et al. 2001). The regulation of *PALs* in plants appears to be quite complicated and much remains unknown, especially in the case of monocotyledonous plants. We are presently trying to isolate and identify the regulatory factor of *rPAL-P5* that is activated following pathogen infection in rice plant.

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