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A cotton cDNA (*GaPR-10*) encoding a pathogenesis-related 10 protein with in vitro ribonuclease activity☆

Xiang-Jun Zhou, Shan Lu, Yan-Hua Xu, Jia-Wei Wang, Xiao-Ya Chen *

National Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, PR China

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Abstract

A cDNA, *GaPR-10*, encoding a pathogenesis-related class 10 protein, was isolated from *Gossypium arboreum*, a diploid cotton species. The predicted protein of 159 amino acids contains a conserved domain (K-A-X-E-X-Y-L) in the C-terminal helix. The glycine-rich P-loop structure found in Betv1 [1] is variable in GaPR-10. The bacterially expressed GaPR-10 exhibited ribonuclease activity in vitro. Substitution of Glu^{148} with Lys, and Tyr^{150} with Phe, respectively, remarkably decreased the activity, suggesting that both residues in the C-terminus play a major role in catalyzing RNA degradation. Substitution of Gly^{51} with Ala, Lys^{55} with Asn (both in the P-loop region), and Glu^{96} with Lys, affected the activity by 50–60%, indicating that these amino acid residues might be related, but not essential to RNase activity. RNA blot analysis detected a certain level of *GaPR-10* transcripts in roots of untreated seedlings, and the transcript level was elevated after the seedlings were treated with *Verticillium dahliae* elicitors. In *G. arboreum* suspension cells, induction of *GaPR-10* transcription by the fungal elicitor was gradual and prolonged, and the transcription was also inducible by jasmonate, but not by salicylic acid and 1-aminocyclopropane-1-carboxylate.

Keywords: *Gossypium arboreum*; *GaPR-10*; Site-directed mutagenesis; Ribonuclease; *Verticillium dahliae* elicitation

1. Introduction

Plants are induced to synthesize a class of proteins when they are exposed to pathogens or environmental stresses, these proteins are termed pathogenesis-related (PR) proteins [2,3]. Accumulation of PR proteins represents a component of plant defense responses, and has been shown to correlate with disease resistance in plants [4]. Five families (PR-1 to PR-5) of these proteins were first described and characterized in tobacco leaves infected by tobacco mosaic virus [5], and were found in many other higher plants afterwards [6]. Additional six families (PR-6 to PR-11) were categorized when the definition of PR proteins was extended to all novel proteins that were produced in plants during defense responses [7]. In a more recent classification, plant defensins, thionins, and lipid-transfer proteins were included in this superfamily as PR-12, PR-13, PR-14, respectively, and altogether 14 families of plant PR proteins were recognized [8].

PR-10 family consists of primarily acidic proteins with low molecular weight of 16–19 kDa. Members of this family have been identified in a variety of angiosperms, including the dicot parsley [9], pea [10], bean [11], soybean [12] and potato [13], as well as the monocot asparagus [14], lily [15] and rice [16]. Comparative analysis of the PR-10 amino acid sequences revealed their similarity with a major birch pollen allergen Betv1 [17] and a ginseng ribonuclease [18]. A few members of the family were reported to possess RNase activity in vitro [19], however, no structural homology was found to any other RNases in the Protein Data Bank. According to X-ray and NMR data, structure of Betv1 contains a seven-stranded anti-parallel β-sheet that wraps around a 25 residue-long C-terminal α-helix,

☆ The nucleotide sequence reported in this paper has been submitted to the EMBL/GenBank Database under the accession number AF416652.

* Corresponding author. Tel: +86-21-64042090; fax: +86-21-64042385

E-mail address: xychen@iris.sipp.ac.cn (X.-Y. Chen).

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forming a 30 Å-long forked cavity that penetrates the entire protein. In the β-strands II and III of Betv1, there is a glycine-rich loop, which shows similarity to the P-loop motif found in many nucleotide binding proteins [1]. Moreover, the conserved amino acid residues Glu^{97}, Glu^{149} and Tyr^{151} (of Betv1) are assumed to be involved in the catalytic reaction [20].

A complex expression pattern of PR-10s has been reported, including the typically activated transcription by pathogens or related factors, and the developmentally regulated expression with tissue-specificity. For instance, Betv1 proteins are constitutively expressed in birch pollen, and a subset of *Betv1* genes are transcriptionally activated in leaves and suspension cultured cells by bacterial or fungal elicitation [21]. In addition, PR-10s of bean [22], soybean [12] and white lupin [19] show a constitutive expression in certain vegetative organs, particularly in roots, of seedlings or mature plants.

Fungal elicitation could activate a range of plant defense related genes and finally lead to a series of defense reactions, including biosynthesis of phytoalexins and accumulation of PR proteins [9]. Cotton plants accumulate gossypol and related sesquiterpene aldehydes which possess fungistatic properties and insecticidal activities. Previous investigations showed that genes encoding enzymes in the gossypol pathway were induced at transcription level by fungal or bacterial elicitation [23,24,27]. Cotton defense reactions are not restricted to phytoalexin biosynthesis. Here we report a PR-10 cDNA isolated from the elicitor-treated suspension cells of *Gossypium arboreum* L. After expression in *Escherichia coli*, the protein exhibited RNase activity in vitro. Site-directed mutagenesis of five amino acid residues indicated that Glu^{148} and Tyr^{150} were critical to the catalytic reaction. Transcription of this gene could be induced by the fungal elicitor, and by the signaling molecule jasmonate (JA).

2. Materials and methods

2.1. Plant material

Seeds of *G. arboreum* L. cv. Qingyangxiaozi were obtained from Jiangsu Agricultural Academy, China. Plants were grown in the greenhouse, under 25/22 °C with 16/8 h (L/D) photoperiod. Tissues were harvested by amputation into liquid nitrogen and then stored at −70 °C before analysis. The yeast RNA was purchased from Sigma (St. Louis, MO).

2.2. cDNA clone isolation and sequence analysis

According to an EST sequence (GenBank/EMBL accession No. AA659995) from the *G. hirsutum*-*Verticillium dahliae* interaction Lambda Zap Expression cDNA library, primers SPR1 (TGAAGGCCTTCCATACCAATAT) and APR1 (ATTCAAAGGAAGCTGTATCTTTAG) were designed. The primers were used to screen a cDNA library of *G. arboreum* suspension cultured cells treated with fungal elicitors [25], using a PCR-mediated method [26].

2.3. Treatment of plants with elicitors and chemicals

V. dahliae Kleb strain VD-8 was cultured in potato dextrose broth and the fungal elicitor was prepared as previously described [23]. For elicitation, the two-week-old *G. arboreum* seedlings were grown in vermiculite, and then transferred to a 1/2 MS liquid medium containing the fungal elicitor (applied at a final concentration of 1 μg sucrose equivalent per ml medium) for 24 h. Elicitation of *G. arboreum* suspension cultured cells by the *V. dahliae* elicitor was performed as previously described [23], and the cells were also treated with 5 mM salicylic acid (SA), 45 μM JA, and 1 μM 1-aminocyclopropane-1-carboxylate (ACC), respectively, or with sterile water as control.

2.4. RNA analyses

Total RNA was prepared from *G. arboreum* tissues or suspension cultured cells by a cold-phenol method [28]. The RNA (10 μg per lane) was separated on 1.0% denature agarose gel. Ethidium bromide was included for monitoring equal amount of RNA loaded. The RNA was then transferred onto a Hybond-XL membrane (Amersham Pharmacia, Sweden). For probe preparation, the *GaPR-10* cDNA was digested with *Bam*HI and *Xho*I, and the released fragment of 733 bp was ^{32}P-labeled by using a random primer labeling system (Promega, Madison, WI). After hybridization overnight, the blot was washed twice at room temperature in 2 × SSC, 0.1% SDS for 15 min and twice in 0.2 × SSC, 0.1% SDS at 55 °C for 15 min, and exposed to X-ray film for 24 h.

2.5. Expression of GaPR-10 in E. coli

The cDNA of *GaPR-10* was amplified with primers T7 (Stratagene, La Jolla, CA), and COTPR4 (TGGATCCGATGGGTGTTGTGAGTTATG), in which a *Bam*HI site (underlined) was introduced. The PCR product was digested with *Bam*HI and *Xho*I, and subcloned into a pET-32b(+) vector. *E. coli* BL21(DE3) was transformed with the construct and the cells were grown in LB with ampicillin at 37 °C. Isopropyl-1- thio-β-D-galactopyranoside (IPTG) was added to the cultures when OD_{600} reached 0.6. The cells were further cultured for 3 h and collected by centrifugation at 5000 rpm for 5 min. The protein product was purified with the Ni-NTA resin (QIAGEN,

Valenicia, CA) according to the manufacture's manual. To remove the N-terminal His-tag fragment, the fusion protein was digested with enterokinase (EC 3.4.21.9) (Sigma) at 37 °C for 6 h, to digest the cleavable site of DDDK. Proteins were examined by SDS-PAGE [29].

2.6. Site-directed mutagenesis of GaPR-10

Five primers were designed to convert Gly^{51} to Ala^{51} (CAATACTTGCAGGACTAGCA), Lys^{55} to Asn^{55} (GTATTGTAAATATCACCTTTGTTG), Glu^{96} to Lys^{96} (GATTTTCTTAAGCTTGTCCC), Glu^{148} to Lys^{148} (GATAAGCTTTAATAGCCTTG) and Tyr^{150} to Phe^{150} (GCCAAAAGAAAAGCTTCAAT), respectively (mismatched nucleotides were underlined). Site-directed mutagenesis was performed by double PCR amplifications with *Pfu* DNA polymerase (Promega) as described in Ref. [30]. The PCR products were inserted into pGEM-T vector (Promega), and verified by DNA sequencing. The mutant genes were expressed in *E. coli* BL21(DE3) as described above.

2.7. Assay of ribonuclease activity

RNase activity was determined according to Barna et al. [31]. The assay was conducted by incubating the enzyme with yeast total RNA at 56 °C for 30 min, the reaction was terminated by adding 1 volume of 4 M LiCl to the mixture, which was then stored at 4 °C for 3 h before centrifugation at $12\,000 \times g$ for 15 min. Supernatant was diluted and absorbance at 260 nm was measured. One unit of enzyme activity was defined as the amount of protein causing an increase in absorption of 1.0 after 30 min of incubation.

3. Results and discussion

3.1. Isolation and analysis of G. arboreum PR-10 cDNA

V. dahliae is a soil-born fungus causing wilting disease of cotton and a number of other crops. An EST obtained from a *G. hirsutum–V. dahliae* interaction library showed high sequence identities with plant PR-10 proteins. Based on this, a clone was isolated from a *G. arboreum* cDNA library, which was constructed from suspension cultured cells treated with *V. dahliae* elicitors. The clone contains an insert of 806 bp, including a complete open reading frame (ORF) of 480 bp, which encodes a peptide of 159 amino acid residues, with a predicted molecular mass of 17.3 kDa and an isoelectric point of 4.95. The protein was likely to be cytoplasmic as no signal peptide sequence was detected. A blast search showed that the deduced protein was most similar to plant PR-10 proteins, as expected. Comparison of the amino acid sequences revealed that it had sequence identities of 52.8, 44.9, 38.1, 31.4 and 30.4% with PR-10 proteins of *Betula pendula* [17], *Phaseous vulgaris* [11], *Petroselinum crispum* [9], *Sorghum bicolor* [4] and *Asparagus officinalis* [14], respectively, among which the *P. crispum* protein was suggested to be the 'type member' of the family [7]. Therefore, the protein encoded by this *G. arboreum* mRNA belongs to the PR-10 family, and the cDNA clone was designated as *GaPR-10*. Fig. 1 shows that a number of conserved amino acid residues are also found in GaPR-10, and a consensus sequence (K-A-X-E-X-Y-L) in the C-terminal helix [1,16] is completely conserved. In Betv1, there is a P-loop structure (G-X-G-G-X-G) that might be an RNA binding site, and the conserved Lys^{55}, two residues after the P-loop, may serve a similar function [1]. In GaPR-10, this motif was changed to GDASPGSIVK.

A Southern analysis indicated that there are likely several (3–4) copies of *GaPR-10* in the diploid genome of *G. arboreum* (data not shown).

3.2. RNase activity of bacterially expressed GaPR-10

Expression of GaPR-10 as a fusion protein with a cleavable His-tag at the N-terminus in *E. coli* showed that, a protein band of about 37 kDa appeared after induction with IPTG (Fig. 2A), which was in good agreement with calculated size of the fusion protein.

When incubated with yeast total RNA, the fusion protein isolated by affinity column did not show any detectable RNase activity. After cleavage of N-termianl His-Tag fragment with enterokinase (Fig. 2B), the GaPR-10 exhibited an RNase activity of about 221 U/mg protein (Fig. 3). This activity is close to that of Betv1, but significantly lower than that of pancreatic RNaseA [32]. The digested protein was also applied to the culture medium of *V. dahliae* to detect its anti-fungal activities in vitro. After up to two days of culturing, GaPR-10 did not show any inhibition on spore germination and hyphal growth of *V. dahliae* (data not shown).

To investigate the functional importance of the conserved amino acid residues related to RNase activities, we conducted site-directed mutagenesis on GaPR-10. The mutational strategy involved in the following replacement: G51A, K55N, E96K, E148K and Y150F. These amino acids were chosen because they were either in the region corresponding to the P-loop of Betv1 (Gly^{51} and Lys^{55}), or proposed to be involved in the catalytic reaction (Glu^{96}, Glu^{148}, and Tyr^{150}). Nucleotide sequencing confirmed that only desired mutations had been introduced.

After bacterial expression and enterokinase digestion (Fig. 2), the five mutants displayed significantly lower RNase activities than the wild type (Fig. 3). Activities of the G51A and K55N mutant proteins were around 84 and 104 U/mg protein, decreased by 50–60%. Therefore,

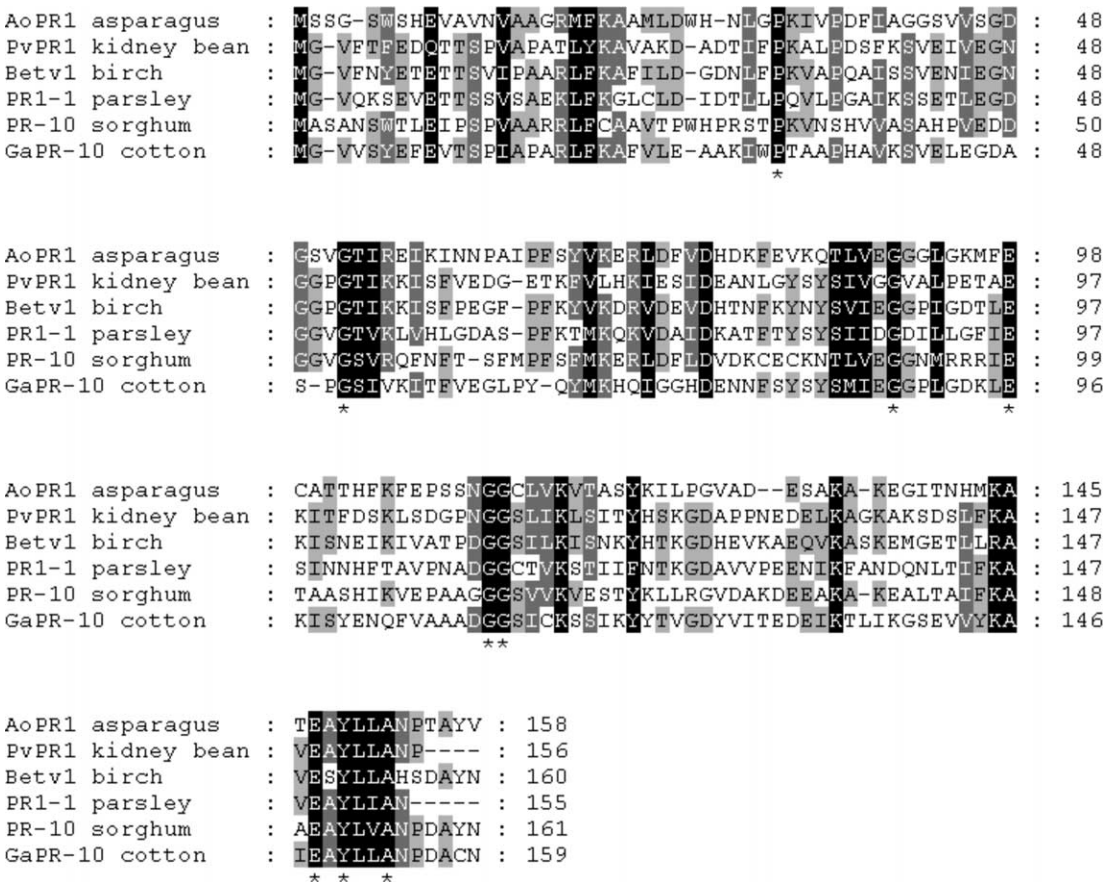

Fig. 1. Alignment of the amino acid sequences of GaPR-10 and other PR-10 proteins from different plants. The plant sources and GenBank accession numbers of the sequences are shown as follows: AoPR1 (*Asparagus officinalis*), Q05736; Betv1 (*Betula pendula*), S05376; PR10 (*Sorghum bicolor*), U60764; PvPR1 (*Phaseous vulgaris*), P25985; PR1-1 (*Petroselinum crispum*), P19417. Asterisks indicate strictly conserved amino acid residues of the PR-10 family.

the two substitutions in the P-loop region did not completely abolish the RNase activity of GaPR-10. The P-loop motif detected in Betv1 has been found in many nucleotide binding proteins such as ATP- and GTP-binding proteins [33] and plant resistance proteins [34]. However, this motif is variable among different PR-10 members, and is atypical in GaPR-10 (Fig. 1). Thus the function of this putative motif needs further investigation.

The E96K mutant also showed a 55% decrease of the RNase activity. When the mutagenesis was performed on a conserved domain in the C-terminal helix, the resultant E148K and Y150F proteins lost most of their activities. It has been suggested, on the basis of X-ray and NMR structure of Betv1, that Glu^{149} and Tyr^{151} are located at two opposing sides of the long C-terminal helix of the molecule while the position of Glu^{97} is at a rather large distance at the N-terminus of β-strand VI, and the side chains of Glu^{97}, Glu^{149} and Tyr^{151} have functional groups presumably involved in the catalytic reaction [1,20]. Our data presented here provide experimental evidence to indicate an essential role of the hydroxyl group of Tyr^{150} and the carboxyl group of Glu^{148} for catalysis, whereas Glu^{96} is not as important as the two amino acid residues in the C-terminus, at least for the *GaPR-10*. It is likely that the C-terminal α-helix conserved in PR-10 proteins plays a major role in ribonucleic acid degradation, and this function of RNase has been retained during evolution of plant PR-10 proteins.

3.3. Expression pattern of GaPR-10

The tissue-specific expression of *GaPR-10* in *G. arboreum* seedlings and plants was examined by RNA gel blot analyses, which demonstrated that *GaPR-10* transcripts were present at a low level in roots of the seedlings, whereas in hypocotyls, cotyledons, leaves, flowers, and stems the transcripts were undetectable (Fig. 4A). In order to investigate if the *GaPR-10* expression could be induced by elicitation, *G. arboreum* seedlings were treated with an elicitor-preparation of *V. dahliae*. *GaPR-10* transcription level in roots increased 2–3-fold at 24 h post-elicitation, and the transcripts also appeared in hypocotyls in the same treatment. However,

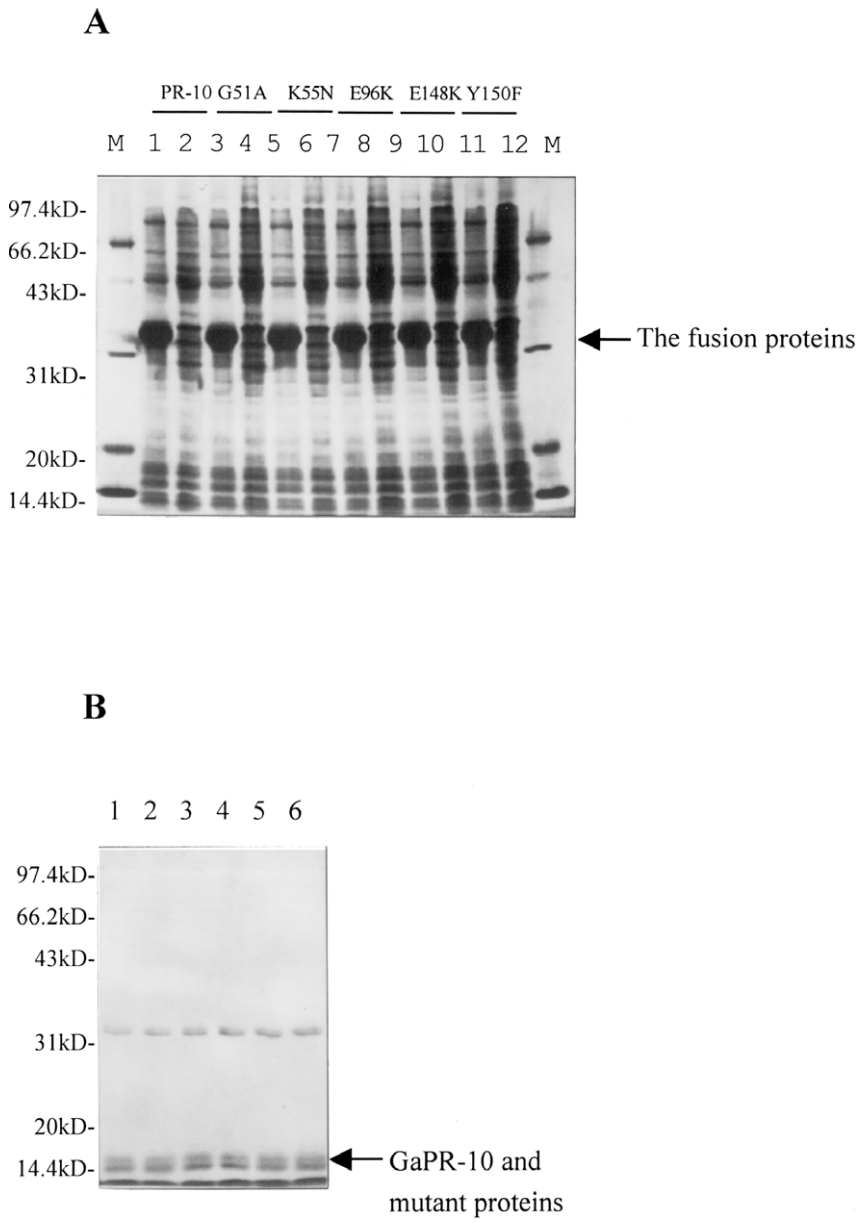

Fig. 2. SDS-PAGE analysis of the wild type and mutant GaPR-10 proteins produced in *E. coli.* (A) Bacterial proteins with (odd numbers) or without (even numbers) 1 mM IPTG induction; 1–2: wild type; 3–4: G51A, 5–6: K55N, 7–8: E96K, 9–10: E148K, 11–12: Y150F, M: protein mass markers. (B) Affinity purified proteins digested with enterokinase; 1–6: wild type, G51A, K55N, E96K, E148K and Y150F, respectively. The GaPR-10 protein product, after digestion, was estimated to be approximately 17 kDa, and the cleaved His-tag was slightly smaller.

the transcripts were still undetectable in cotyledons after elicitation (Fig. 4B).

The induced expression pattern of *GaPR-10* gene was further examined in *G. arboreum* suspension cultured cells. The *GaPR-10* transcripts began to accumulate after application of the *V. dahliae* elicitor to cultured cells, and reached the peak level at 12 h post-elicitation. Then the level was kept high during subsequent 24 h (Fig. 5A). JA and SA are signaling molecules believed to be involved in wounding responses and disease resistance of plants, and ACC is a biosynthetic precursor of ethylene. In *G. arboreum* suspension cells, when JA was added, a clear induction of *GaPR-10* was indeed observed, however, the induction by SA or ACC was insignificant (Fig. 5B). In rice, *PR10* mRNA was induced by JA, SA and H_2O_2, but ethylene and abscisic acid failed to induce its expression [16].

As an important means for regulating gene expression, plants alter the levels of RNase activities in response to a variety of exogenous stimuli, such as pathogens [31]. Our results show that *GaPR-10* was weakly expressed in cotton roots, suggesting that it is one of the pre-infectional repertoires of the cotton defense mechanism. Furthermore, much higher level of

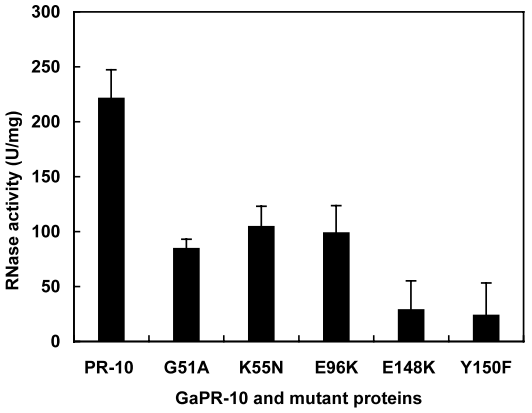

Fig. 3. RNase activity analysis of GaPR-10 and mutant proteins. The activity was determined by OD_{260} of nucleotides after incubation with 1 μg GaPR-10 or mutant proteins at 56 °C for 30 min.

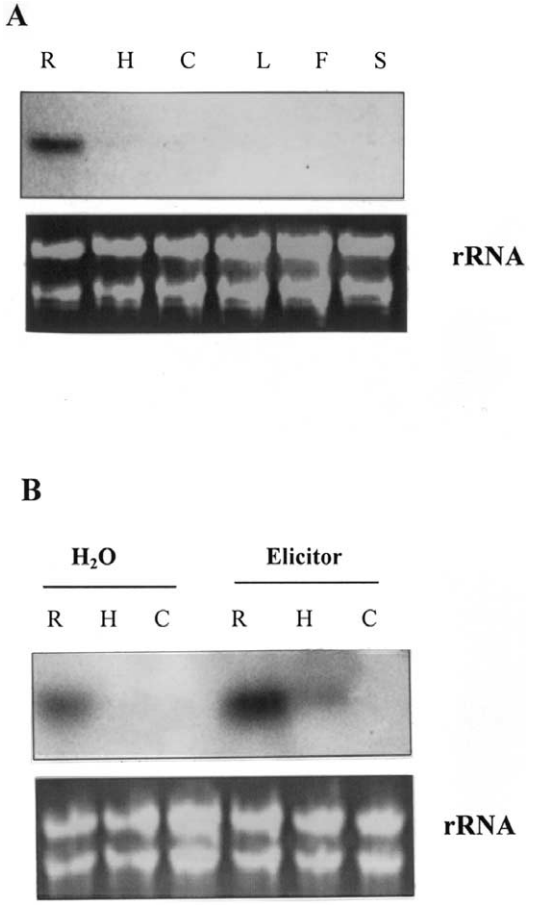

Fig. 4. Expression pattern of *GaPR-10* in two week-old seedlings and plants of *G. arboreum*. (A) Tissue specific expression; R: roots; H: hypocotyls; C: cotyledons; L: leaves; F: flowers; S: stems. (B) Expression of *GaPR-10* at 24 h post-elicitation with the *V. dahliae* elicitor, R: root; H: hypocotyls; C: cotyledon.

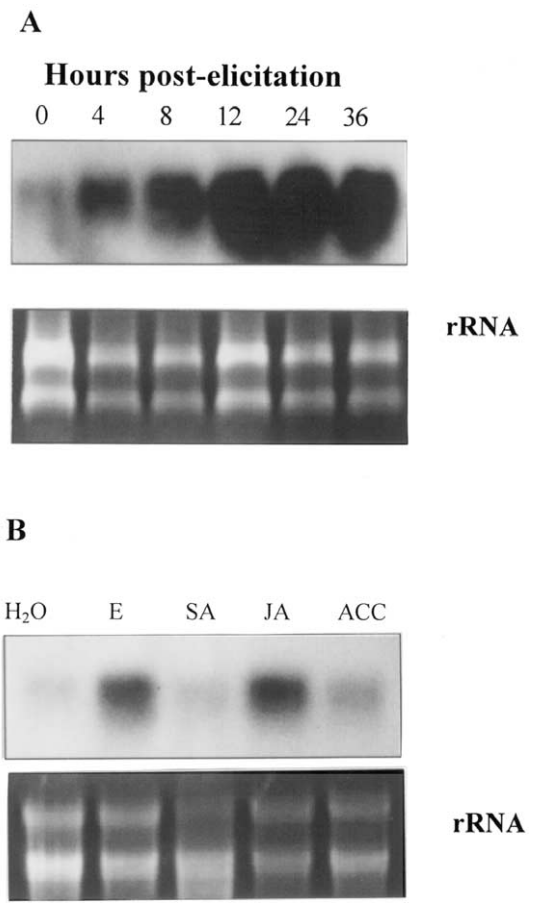

Fig. 5. *GaPR-10* expression pattern in suspension cultured cells of *G. arboreum*. (A) Time course expression of *GaPR10* in elicitor-treated suspension cultured cells. (B) Expression of *GaPR-10* suspension cultured cells 24 h after treatment with SA, JA, ACC and the *V. dahliae* elicitor (E), respectively.

expression after elicitation indicates that it might be involved in activated responses of plants to pathogens. It has been postulated that these intracellular PR proteins, once they are induced upon interaction of the pathogen with cells at the infectious sites, may degrade cellular RNAs and thus contribute to the hypersensitive reaction [35]. On the other hand, PR-10 proteins may function to selectively degrade mRNA species induced during stress or pathogen attack, such a mechanism would allow the plants to return to normal physiological status [22]. If this is the case, a slower rate of induction of PR-10 proteins than certain defense genes may be expected. For cotton, (+)-δ-cadinene synthase (CAD1) is a key enzyme for biosynthesis of gossypol and related sesquiterpene phytoalexins, which are also toxic to plant cells. Our previous investigation showed that, in *G. arboreum* suspension cells, the *CAD1-C* gene was rapidly induced by elicitation, and the transcript reached the maximal level around 4–8 h after elicitation, then declined subsequently [23,25]. The relatively fast induction of *CAD1* was also observed in present investigation (data not shown), while the *GaPR-10* gene showed a gradual induction and a prolonged elevation of its transcript level in the same elicited cells (Fig. 5A). Prolonged induction of PR-10 was also found in sorghum mesocotyls [4]. This induction pattern, to-

gether with the fact that PR-10 genes are also constitutively expressed in certain organs, seems to favor the later hypothesis.

Acknowledgements

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