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Elicitor-specific induction of one member of the chitinase gene family in *Arachis hypogaea*

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Summary. Chitinases are believed to play an important role in plant defence against bacterial and fungal attack. In peanut (*Arachis hypogaea*) chitinase genes form a small multigene family. Four chitinase cDNAs (*chit 1–4*) were isolated from cultured peanut cells. Expression of individual *chit* genes was assayed by the polymerase chain reaction (PCR) followed by analysis of restriction fragment length polymorphisms (RFLP). UV irradiation, dilution of cell cultures and treatment with *Phytophthora megasperma* (Pmg) elicitor or yeast extract were used to induce expression of *chit* genes. The *chit 3* gene is constitutively expressed at a low level in untreated as well as in treated cultures; the expression of *chit 4* gene is induced by each of the stimuli tested, whereas the *chit 1* gene is activated by cell culture dilution and by yeast extract treatment. The *chit 2* gene is strongly activated by treatment with cell wall components from the fungus *Phytophthora megasperma* but not by the other stimuli. These results indicate that *chit 2* gene expression may be controlled by pathogen-specific regulatory elements.

Key words: Cell culture – *Phytophthora magasperma* elicitor – Signal transduction – Chitinase cDNA – *Arachis hypogaea*

Introduction

Plants possess a large arsenal of constitutive and inducible biochemical defence mechanisms directed against potential pathogens. The assumption that the so-called "pathogenesis-related" (PR) proteins are involved in, and responsible for, defence mechanisms is based mainly on the close correlation between the induction of these proteins and acquired systemic resistance. Plants react to infection with viruses, viroids, fungi, bacteria, but also when senescing, with synthesis of these PR proteins (van Loon 1985).

With few exceptions, the biochemical functions of these, mainly low molecular weight (10–40 kDa) proteins – often located on the cell surface – are unknown. Chitinases belong to the class of PR proteins whose roles are better understood. Chitin, the substrate for this hydrolytic enzyme, is a polymer of N-acetylglucosamine and does not occur in plants. It is however a constituent of fungal cell walls and of the larval style of nematodes, as well as of the exoskeleton of insects. The relative content of chitin in cell walls of different fungi ranges from 0.4% in *Phytophthora* to about 50% in *Fusarium* (Bartnicki-Garcia 1968; 1973). Purified basic chitinases from bean plants cause potent inhibition of the growth of the fungus *Trichoderma viride* in vitro (Schlumbaum et al. 1986). In addition to the direct inhibitory effect of these antifungal hydrolases, they may be involved in more complex plant defence reactions: products released from fungal cell walls by chitinases and β-1,3-glucanases may be recognized by the plant cells as elicitors of defence reactions (Darvill and Albersheim 1984; Mauch et al. 1988). Chitinases also exhibit lysozyme activity (Boller 1985) possibly indicating that these enzymes play a role in antibacterial defence. The possibility that chitinases act in other, as yet unknown, ways on endogeneous, plant-derived substrates cannot be ruled out.

Plant chitinases are usually coded for by a small multigene family. Six proteins have been described in potato (Kombrink et al. 1988), four in maize (Nasser et al. 1988), four in tobacco (Legrand et al. 1987; Payne et al. 1990) and four in bean (Broglie et al. 1986). Chitinases can be induced by a variety of stimuli: infection with pathogens, wounding, ageing, elicitor or ethylene application, abiotic stress (e.g. salt, salicylic acid, UV light) or hormonal treatment (see Boller 1985; 1988). However, little is known about the expression patterns of individual members of this important gene family. In the present work, we determined which of the chitinase genes of *Arachis hypogaea* are induced in cell suspension cultures in response to fungal elicitors, UV light, yeast extract and dilution. In particular we wanted to know whether the different members of the gene family re-

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spond to the same or to different stimuli. We focussed our interest on the analysis of mRNA levels, because the rapid but transient induction of the synthesis of PR proteins correlates with changes in mRNA synthesis (Carr et al. 1985; Somssich et al. 1986; Lawton and Lamb 1987; Hedrich et al. 1988).

In this paper we describe qualitative and quantitative differences in chitinase gene expression and identify a particular chitinase gene that is specifically induced by *Phytophthora megasperma* (Pmg) elicitor and not by abiotic stress.

Materials and methods

Cell suspension culture. Peanut cells (*Arachis hypogaea* L.) were cultivated as described (Rolfs et al. 1981; 1987). Cells were grown on a horizontal shaker in Schenk-Hildebrandt medium (Schenk and Hildebrandt 1972) at 22° C in Erlenmeyer flasks. After 8 days, on reaching the stationary phase, the culture was propagated by diluting 1:5 in fresh medium.

Induction experiments. Cell cultures (50 ml) were induced 7 days after propagation. UV light treatment was performed by irradiating the cells in two open 15 cm diameter petri dishes with 275 nm light from an inspection lamp for 12 min at a distance of 10 cm (Rolfs et al. 1987). Fungus *Phytophthora megasperma* f. sp. glycinea (Pmg) elicitor was prepared from hyphal cell walls according to the method of Ayers and coworkers (1976a). Plant cells were induced with 25 µg Pmg elicitor per ml medium. β-glucan elicitor was prepared by partial hydrolysis of Pmg cell walls with a crude extract containing β-glucanases from soybean cotyledons. After purification the average size of the carbohydrates was between 1000–10000 daltons (R. Tiemann, personal communication). Protein analysis, using the method of Bradford (1976), revealed that the glucan elicitor preparation was free of protein. Cells were treated with 2.5 µg glucan elicitor per ml medium. Yeast extract (DIFCO Laboratories) was used at a final concentration of 1 mg/ml. Dilution of the cell culture was performed by adding 200 ml fresh Schenk-Hildebrandt medium to 50 ml of the cell suspension.

RNA and DNA isolation. Cells were collected by filtration of the cultures. Total RNA was prepared by a modification of the procedure described by Chirgwin and coworkers (1979). The dried cells were ground to a fine powder in liquid N_2, dissolved in 4 ml lysis buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% N-laurylsarcosinate) and carefully shaken for 10 min. After pelleting the cell debris (10 min, 10000 × *g*), the cell extract was layered on a 1.5 ml cushion of 5.7 M CsCl, 50 mM TRIS-HCl pH 8, 5 mM EDTA, 5 mM sodium acetate in a Beckmann SW55 Ti polyallomer tube and the RNA was recovered by centrifugation for 18 h at 35000 rpm at 20° C. The RNA pellet was resuspended in H_2O and precipitated twice with ethanol.

Genomic DNA was isolated from the interphase of the CsCl gradient used for the RNA isolation. After washing with n-butanol, the DNA was air-dried and carefully redissolved in H_2O.

Primer extension analysis. Primer extension reactions were carried out according to Dean et al. (1987). 2 pmoles of primer complementary to RNA sequence were labelled using T4 polynucleotide kinase (BioLabs) with 15 µCi of γ^{32}P-ATP (5000 Ci/mmol, Amersham) in a volume of 10 µl. 0.2 pmoles of the primer were hybridized to 10 µg of total RNA for 3 h at 42° C in 10 µl of 10 mM TRIS-HCl pH 7.9, 250 mM KCl. Reverse transcriptase reactions were carried out in a volume of 50 µl containing 10 µl hybridization mixture, 1 mM dNTPs, 50 mM TRIS-HCl pH 8.2, 6 mM $MgCl_2$, 10 mM DTT, 100 mM NaCl and 12 U AMV reverse transcriptase (Boehringer Mannheim) at 42° C for 1 h.

PCR analysis. Primer extension reactions were performed as described above; however, instead of 0.2 pmol primer, 50 pmol primer labelled with 1 µCi γ^{32}P-ATP were annealed to total RNA by heating for 2 min at 70° C and then cooling to room temperature for 20 min. After ethanol precipitation, primer extension products were used for PCR directly (Saiki et al. 1985). PCR was performed as recommended by the manufacturer of Taq polymerase (BioExcellence). PCR conditions were: 20 cycles of 2 min denaturation at 92° C followed directly by 3 min synthesis at 70° C, i.e. without an annealing step.

PCR products were loaded on a 6% polyacrylamide gel and eluted after autoradiography by phenol-chloroform extraction. The ethanol precipitate was digested in a volume of 100 µl for 3 h with the restriction enzyme *Bcl*I at 50° C. After adding the restriction enzymes *Dde*I and *Hin*fI the incubation was continued overnight at 37° C. The digested PCR products were separated on denaturing 9% polyacrylamide, 7 M urea gels and visualized by autoradiography.

Other techniques. DNA cloning, DNA sequencing, Northern and Southern blot analyses were performed as described by Sambrook et al. (1989).

Results

Induction of chitinase mRNA in peanut (Arachis hypogaea) *cell culture*

In order to study the elicitor-induced accumulation of chitinase mRNAs in peanut cell culture, we used a crude, water-soluble elicitor preparation from *Phytophthora megasperma* (Pmg). Although Pmg is a soybean pathogen, its cell wall components are able to elicit expression of PR proteins in non-host plant cells e.g. parsley (Somssich et al. 1986) and peanut cell culture (Vornam et al. 1988; Hain et al. 1990).

An *Arachis hypogaea* cell culture was treated for 6 h with 25 µg/ml Pmg elicitor, total RNA was isolated and

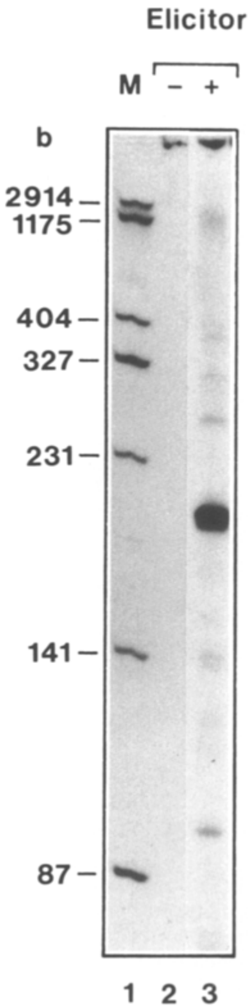

Fig. 1. Primer extension analysis of 10 µg total RNA isolated from Pmg-induced (lane 3) and non-induced (lane 2) *Arachis hypogaea* cells. End-labelled chitinase primer 516 has the following sequence 5′-CAC CAC CCG AAI TIG CTG CAA CA-3′. Products were separated on a 6% polyacrylamide gel containing 7 M urea. Marker (lane 1) was bacteriophage ϕX174 DNA digested with *Taq*I and labelled by a fill-in reaction with ^{32}P-dCTP

analysed for chitinase mRNAs in a primer extension experiment. The primer sequence 516 (5-′CAC CAC CCG AAI TIG CTG CAA CA-3′) was selected based on a comparison of tobacco (Shinshi et al. 1987) and soybean (Broglie et al. 1986) chitinase cDNA sequences. The 23 bases hybridize to the mRNA sequence coding for amino acids 17 to 24 of the mature protein, i.e. at a distance of 186 bases from the 5′ end of the bean cDNA.

The autoradiogram in Fig. 1 demonstrates the drastic increase in chitinase mRNA in elicitor-treated cells (lane 3) in comparison with untreated cells (lane 2). There is no signal detectable in untreated cells, but in elicited cells a strong double band of about 195 and 205 bases is visible, corresponding well with the expected length, as deduced from the 5′ region of a nearly full-length soybean chitinase cDNA clone (Broglie et al. 1986), and with the predicted transcription initiation site of a cloned genomic chitinase gene (Broglie et al. 1989). This result demonstrates that Pmg elicitor induces chitinase mRNA in peanut cells.

Cloning of chitinase cDNA fragments and sequence comparison

In order to obtain accurate sequence data on the different chitinase genes in *Arachis hypogaea*, cDNA clones were isolated by using two oligonucleotide sequences, (synthesized on an Applied Biosystems 380B DNA synthesizer) based on two highly conserved regions in bean and tobacco chitinases. Oligonucleotide 32 (5′-CCI CAI TCC AIG CCI CCI TTG ATG ATG TTC GT-3′) hybridizes to bases 835–866 and oligonucleotide 33 (5′-ATC TCI TTC AAG TCI GCI CTC TGG TTI TGG ATG-3′) is equivalent to bases 697–730 of bean chitinase cDNA pCH18 (Broglie et al. 1986).

For primer extension experiments oligonucleotide 32 was hybridized with 10 µg total RNA of Pmg elicitor-stimulated *Arachis hypogaea* cells and the product was

```
           1                                                                                             90
chit 2     ATCTCGTTCA AGTCGGCGCT CTGGTTGTGG ATGACAGAGC AGAAACCAAA ACCTTCTTGC CACAACGTCA TGGTTGGGAA TTACGTGCCA
chit 1A    .......... .......... .......... .......CA. .AGG.AAC.. G..A..AA.. ..TG.T.... .CACC..A.G A.GGACA...
chit 1B    .......... .......... .......... .......CA. .AGG.AAC.. G..A..AA.. ..TG.T.... .CACC..A.G A.GGACA...
chit 3     .......... .......... .......... ......CCA. .AGG.AAC.. G..A..AA.T ..TG.T..T. .CACC..A.G A.GGACA..G
chit 4     .......... .......... .......... ......CCA. .AGGGAAT.. G..C..G... ...G...... .CACCAATGC A.GGAG....
Sol. tub.  .....A.... .AA.T..T.. ......T... .....CCCC. .ATC...... G.....G... ...G...... .CA....C.G A.GGAAC...
N. tab.    .....A.... ....A..T.. ......T... .....TCCT. .ATC...... .......... ...G.T.... .CA....A.G A.GGCAA...
P. vulg.   .....C.... ....C..C.. ......C... .....C.CA. ..TCC..C.. G.....C.C. ...G...... .CACCTCTCG A.GGACC..C
primer 33  ATCTCITTCA AGTCIGCICT CTGGTTITGG ATG-3'

           91                                                                   170
chit 2     ACAGCATCTG ATAGAGCAGC AAATAGAACC TTAGGGTTTG GGTTGGTTAC GAACATCATC AACGGCGGCC TGGACTGCGG
chit 1A    T.G..CG.G. .C.GG..G.. CGGCC..GT. .CC..A.... .AG..A.C.. .......... .......... ..........
chit 1B    T.G..CG.G. .C.AG..G.. CGGCC..GT. .CC..A.... .AG..A.C.. .......... .......... ..........
chit 3     T.C.ATG... .C..G..G.. CGG.C..GT. .CC..A.... .AG..A.C.. .......... .......... ..........
chit 4     ..C..CA... .CTCG..G.. .GG.C..G.. CC...C.AC. .TG.CA.C.. .......... .......... ..........
Sol. tub.  T.TT.CG... .CC....... C...C..CTG CCT..A..C. .TG.CA.C.. .........T ..T..G.... .A..A..T..
N. tab.    T.GT.TG... .CC.C..... C...C.TCT. CCT..A.... .TG.CA.C.. .......... ..T..T...T ....A..T..
P. vulg.   T.CT.TG.C. .CGTC..C.. CCGCC.GCTT CCC..C.AC. .CACT..G.. .......... .....A.... ....G.....
primer 32                                               3'-TG CTTGTAGTAG TTICCICCGI ACCTIACICC
```

Fig. 2. cDNA sequences of chitinases from *Arachis hypogaea* (*chit 1–4*), *Solanum tuberosum*, *Phaseolus vulgaris* and *Nicotiana tabacum*. The PCR primers used (32 and 33) are shown, the single base pair difference between *chit 1A* and *1B* is indicated

Table 1. Comparison of chitinase cDNA sequences (170 bp) from peanut (*chit 1* to *4*), *Solanum tuberosum* (bases 703–872; Gaynor 1988), *Nicotiana tabacum* (bases 622–791; Shinshi et al. 1987) and *Phaseolus vulgaris* (bases 697–866; Broglie et al. 1986).

	chit 1A	*chit 2*	*chit 3*	*chit 4*
chit 1A	100%	71%	94%	82%
chit 2	71%	100%	69%	71%
chit 3	94%	69%	100%	81%
chit 4	82%	71%	81%	100%
Sol. tub.	72%	68%	72%	72%
N. tab.	75%	72%	75%	71%
P. vulg.	72%	62%	71%	72%

subsequently amplified by 30 cycles of PCR with primers 32 and 33. This procedure resulted in a 170 bp cDNA fragment which was cloned in the pUC 19 vector after digestion with *Hin*dII. Fourteen different clones were sequenced and grouped into five different chitinase sequence classes (*chit 1A*, *chit 1B*, *chit 2*, *chit 3* and *chit 4*; Fig. 2). The cDNA sequences were compared to each other and to known chitinase sequences from other plant species. Two of the *Arachis hypogaea* chitinases (*chit 1A* and *1B*) differ in only one nucleotide (position 104) resulting in a single amino acid alteration. While in *chit 1A* this position codes for lysine it codes for arginine in *chit 1B*. In potato (Gaynor and Unkenholz 1989), tobacco (Shinshi et al. 1987) and in *chit 1A*, *2* and *3* this position codes for arginine whereas in bean (Broglie et al. 1986) a valine and in *chit 4* a serine is coded for at this position. We do not know whether *chit 1A* and *chit 1B* cDNA sequences are derived from different alleles of the same gene or whether the difference is due to misincorporation by reverse transciptase during cDNA synthesis or by Taq polymerase during the amplification of the cDNA.

Nucleotide sequence similarities among the different isolated chitinase cDNAs range from 99.4% between *chit 1A* and *1B* to 69% between *chit 2* and *3* (Table 1). Comparison with potato, tobacco and bean chitinases showed homology of 62% between *chit 2* and bean chitinase and 75% for *chit 1* and *3* in comparison with tobacco chitinase (Table I). These data confirm that the isolated *Arachis hypogaea chit* sequences belong to the chitinase multigene family (Shinshi et al. 1990).

Expression analysis of chit 1-4 *in cell cultures subjected to various treatments*

Arachis hypogaea cell cultures were stimulated either by UV irradiation, or dilution, or by addition of Pmg elicitor, glucan elicitor or yeast extract. From each culture, RNA was prepared 6 h after stimulation. 40 µg of RNA from each sample was used for Northern blot analysis. Hybridization of the filter with a chitinase probe, prepared by PCR from *chit 3* cDNA with primers 32 and 33 in the presence of ^{32}P-dCTP, shows that hardly any

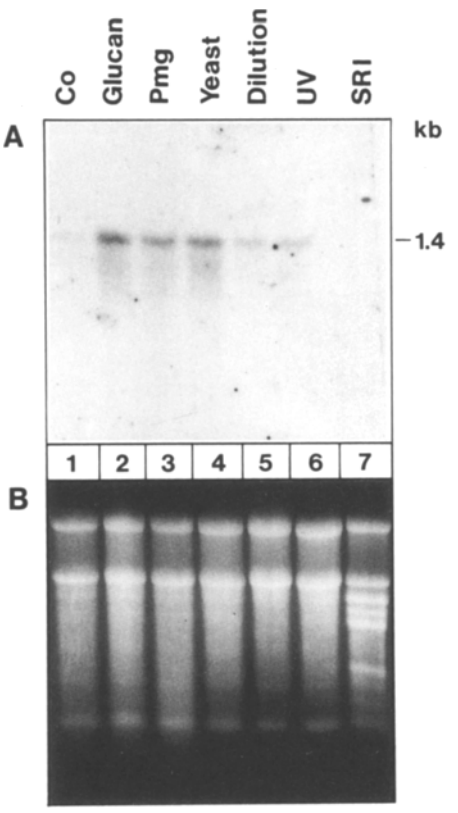

Fig. 3A and B. Northern blot analysis of total RNA isolated from *Arachis hypogaea* cells and separated on a 1.2% agarose, 2.2 M formaldehyde gel. Cultured cells were either not induced (lane 1) or induced for 6 h with glucan elicitor, Pmg elicitor or yeast extract (lane 2 to 4, respectively), or by dilution (lane 5) or UV irradiation (lane 6). Lane 7 was loaded with RNA isolated from tobacco SR1 grown under sterile conditions. **A** The autoradiogram obtained after hybridizing the blot with a ^{32}P-labelled *chit 3* probe under low stringency conditions (last washing step 2 × SSC at 60° C) is shown. **B** shows the ethidium bromide-stained gel to control for RNA quality and quantity

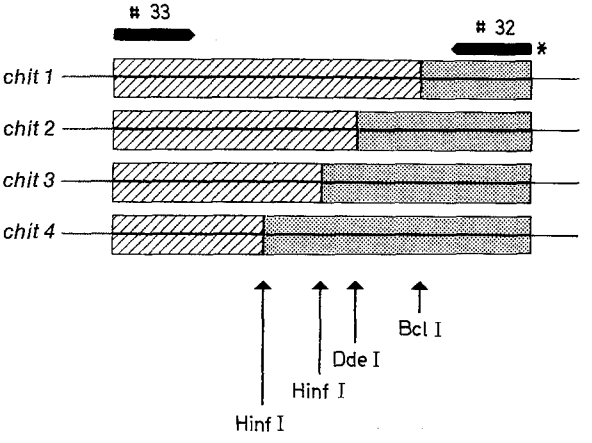

Fig. 4. Scheme explaining the method of analysis of the chitinase gene family. With the primers 32 (end-labelled) and 33, a conserved chitinase sequence was amplified (boxed area). The indicated restriction enzymes can distinguish between the different transcripts (arrows), leading to four different fragment sizes (dotted area)

signal is detectable in untreated cells (Fig. 3, lane 1). The highest level of induction of chitinase transcripts (1.4 kb in length) was achieved by incubating the cells with glucan elicitor, Pmg elicitor or yeast extract (Fig. 3 lanes 2, 3, 4, respectively). A weaker induction was observed after UV irradiation or after dilution (Fig. 3 lane 5, 6). No signal was detectable with RNA from SR1 tobacco plants grown under aseptic conditions (lane 7). Rehybridizing the same blot with a full-length potato chitinase cDNA probe (kindly provided by E. Kombrink) showed the same hybridization pattern as with the peanut probe, except that an additional band of about 1250 bases was visible with the tobacco SR1 control RNA (data not shown).

To determine which of the different chitinase genes was responsible for the 1.4 kb transcript observed after induction with the various stimuli, we made use of a method (Fritz 1989; Becker-André and Hahlbrock 1989; Wang et al. 1989; Fritz et al. submitted) which allows accurate quantification of mRNAs of low abundance. The following technique (Fig. 4) was used to differentiate between homologous transcripts: after extension of primer 32 on mRNA templates, two oligonucleotides, 32 and 33, were used for the amplification of a conserved region of chitinase cDNA sequences. After 20 cycles the reaction was stopped. Fewer than 15 cycles resulted in insufficient amounts of PCR products. After more than 30 cycles, the PCR reaction reached the saturation phase (see Saiki et al. 1988; Syvänen et al. 1988). Minor sequence differences between different chitinase genes result in the presence of different restriction sites in the mixture of PCR products and these can be utilized to discriminate between the different transcripts. Figure 5A shows a PCR reaction with RNA from *Arachis hypogaea* cells after treatment with various inducing agents as starting material. The result of this experiment confirms the pattern of chitinase gene expression obtained in the Northern blot shown in Fig. 3. Again, treatment of the cells with glucan elicitor, Pmg elicitor or yeast extract resulted in the strongest induction of chitinase mRNA visualized by the PCR products (Fig. 5A). Dilution of the cell culture and UV light treatment caused only a slight induction. While with the Northern blot technique almost no chitinase transcripts were detectable in the untreated control culture (Fig. 3, lane 1), the more sensitive PCR method demonstrates that uninduced cells in fact express chitinase transcripts at a low level (Fig. 5A, lane 1).

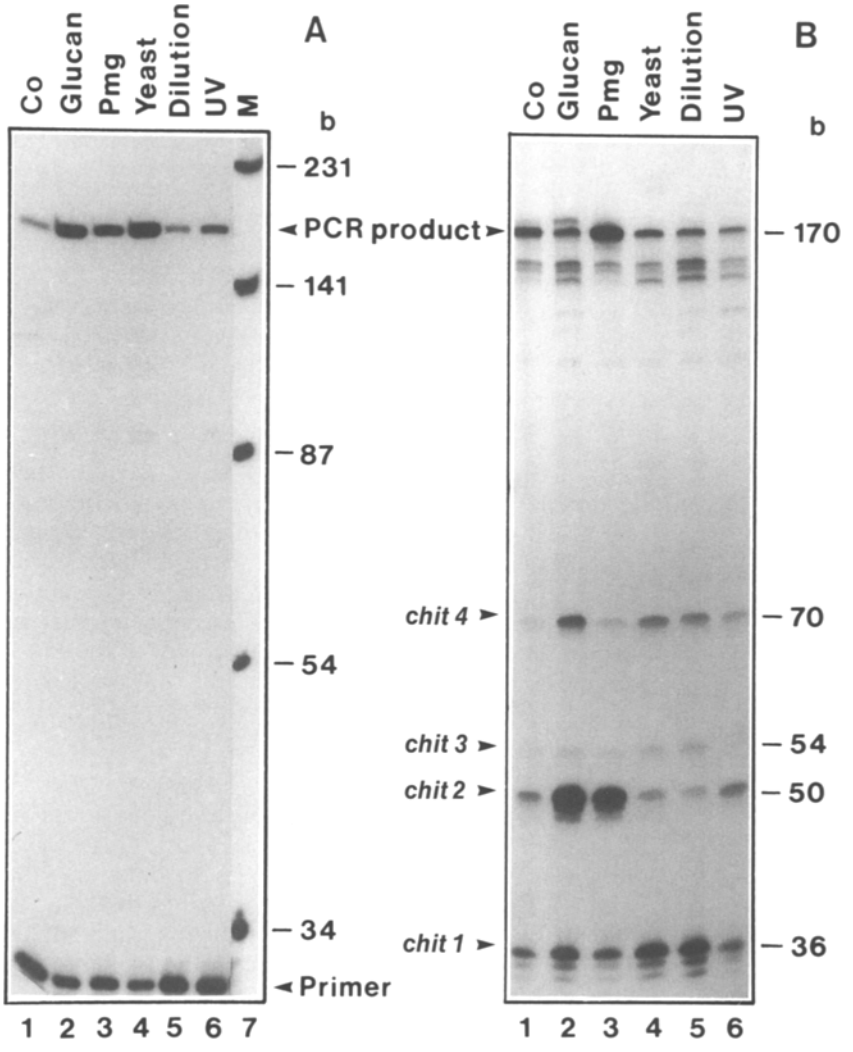

Fig. 5A and B. Analysis of different *chit* transcripts by PCR and RFLP. RNAs were the same as those used for the Northern blot analysis (Fig. 3). **A** After twenty cycles of PCR an aliquot was loaded on a 6% polyacrylamide, 7 M urea denaturing gel. The autoradiogram shows that the expression of chitinase genes is differentially induced. Size markers are *Taq*I fragments of ϕX174 DNA. The bands below the 170 bp band in **B** are most probably PCR byproducts, which are not visible in **A** because of the much shorter exposure time for the autoradiograph. **B** PCR products of different RNAs (see Fig. 5A) were digested by *Bcl*I, *Dde*I, and *Hin*fI and fragments were separated on a 9% polyacrylamide, 7 M urea denaturing gel. Sizes of fragments and the corresponding chitinases are indicated

Table 2. Induction of four peanut chitinase transcripts in response to different stimuli as compared to non-induced control levels. The symbols plus (+), plus/minus (+/−) and minus (−) indicate increased, unaltered and reduced mRNA levels respectively.

	UV-Light	Dilution	Yeast	Pmg.	Glucan
chit 1	+/−	++	++	+/−	+
chit 2	+/−	−	+/−	+++	+++
chit 3	−	+	+	+/−	+
chit 4	++	++	++	++	+++

+++ 8 –12 fold induction
++ 2 – 8 fold induction
+ 1.2– 2 fold induction
+/− 0.8– 1.2 fold induction
− 0.5– 0.8 fold induction

In a second step the PCR products mentioned above were isolated from a polyacrylamide gel and an aliquot was digested with the restriction enzymes *Bcl*I, *Dde*I and *Hin*fI (see Fig. 4). The autoradiogram shown in Fig. 5B represents an example of the RFLP analysis of the PCR products. *chit 1* transcripts result in a 36 bp *Bcl*I fragment, *chit 2* in a 50 bp *Dde*I fragment, and *chit 3* and *4* in 54 and 70 bp *Hin*fI fragments, respectively. This assay did not discriminate between *chit 1A* and *chit 1B* sequences.

The PCR-RFLP data were evaluated by two independent methods: a) X-ray films were scanned with an LKB densitometer and b) radioactive bands were cut out of the gel and the radioactivity was measured in a scintillation counter. The results of three independent induction experiments are summarized in Table 2.

The *chit 3* gene is constitutively expressed at a low level under all conditions tested. Treatment with yeast extract or glucan elicitor (1.6–1.8-fold increase) and by UV light or Pmg elicitor (0.7–0.8-fold of control level) appear to result in slight alterations in transcript levels as compared to untreated control cells. Expression of the *chit 4* gene is induced by all stimuli, i.e., this gene reacts to biotic (especially glucan elicitor) as well as to abiotic stress factors.

The *chit 1* gene is activated 4.2 and 3.1-fold by dilution and by yeast extract, respectively, whereas the other stimuli have only a minor effect.

The most interesting gene is the *chit 2* gene. Its expression was not enhanced by either abiotic stress factors (UV light, dilution) or by yeast extract treatment, but responded very markedly to Pmg and glucan elicitor. The *chit 2* gene not only reacted with the highest induction level (9.5-fold with Pmg and 12-fold with glucan elicitor) but also gave the strongest signals in absolute terms.

The band of 170 bp in Fig. 5 represents undigested PCR products in all six samples. Possibly, this band is caused by hybridisation of two different chitinase cDNA strands forming a heterodimer which contains a mismatch at the restriction site. The formation of undigestable heterodimers may have occurred during the PCR reaction or more probably during phenol extraction after isolation of the PCR products from the polyacrylamide gel.

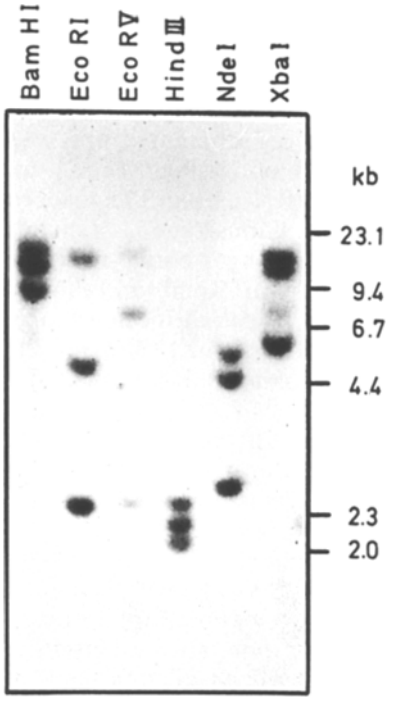

Fig. 6. Southern blot analysis performed with 10 µg *Arachis hypogaea* DNA digested with the enzymes indicated and hybridized with a ^{32}P-labelled *chit 2* probe. The membrane was washed at 60° C with 2 × SSC. The marker was Lambda DNA digested with *Hin*dIII

We also cloned and sequenced bands that were either smaller or larger than 170 bp. However we could not detect any homology to chitinases or other known sequences (except for the primer sequences) for the six cDNA sequences we determined. These bands therefore seem to be byproducts of the PCR reaction.

Genomic Southern blot analysis

Genomic *Arachis hypogaea* DNA, isolated from the cultured cells, was digested with different restriction enzymes, transferred to nylon membrane (Amersham) and hybridized with a ^{32}P-labelled *chit 2* probe. The autoradiogram (Fig. 6) shows three to four bands depending on the restriction enzyme used. This indicates that in our peanut cell culture three to four copies of the *chit 2* gene are present.

Discussion

In this paper we describe the relative levels of expression of four different chitinase genes in a peanut (*Arachis hypogaea*) cell culture. This expression data and the Southern blot analysis (Fig. 6) show that in this cell culture, the genes coding for chitinases form a small multigene family similar to what has been observed in other plant genomes.

Use of a cell culture system for analysis of the chitin-

ase gene family has several advantages. Disease resistance responses in plants are induced in cells that are in direct contact with the invading pathogen. It can therefore be difficult to obtain sufficient tissue for the isolation of protein or mRNA from infected plants. Treatment of suspension culture cells with an elicitor ensures that almost all cells are in contact with the elicitor and may even respond in a synchronous manner, thus making the observations both more reproducible and easier to characterize. Primer extension (Fig. 1) and Northern blot analysis (Fig. 3) showed strong induction of chitinase mRNA after treatment with Pmg, glucan elicitor or yeast extract. UV irradiation and dilution of the culture resulted in a weaker induction. While in Northern blot experiments we see a signal representing the sum of all expressed chitinase mRNAs, the combination of PCR with RFLP analysis allows us to estimate the levels of transcripts from each of the four chitinase genes individually (Fig. 4). In comparison with other methods, which have been used for analysis of other gene families, such as oligonucleotide hybridization with resveratrol synthase mRNA (Lanz et al. 1990) or S1 mapping with chalcone synthase mRNA (Ryder et al. 1987), the method described in this paper is independent of the hybridization properties and specific radioactivity of the probe. Furthermore, in any one sample, several transcripts can be examined qualitatively as well as quantitatively.

The results shown in Fig. 5A and summarized in Table 2 demonstrate that individual chitinase genes are activated independently and can be selectively induced by different environmental stimuli. While expression of *chit 4* is induced by all of the different stimuli tested (up to 8.6-fold by glucan elicitor), the *chit 3* gene appears to be active at a low level with or without induction. It is of course conceivable that *chit 3* requires an unknown inducer, different from those tested in our experiments. Expression of the *chit 1* gene is enhanced significantly (4.2-fold) only by diluting the cell culture or adding yeast extract (3.1-fold). Yeast extract seems to contain substances which are recognized by the plant cell in such a way that the *chit 1* and *4* genes are activated.

The most interesting gene with respect to plant-pathogen interaction is the *chit 2* gene which is induced significantly by Pmg elicitor (9.5-fold) and glucan elicitor (12-fold) as compared with untreated control cells. The *chit 2* gene not only has the highest level of induction but also exhibits the highest level of expression in absolute terms when compared to *chit 1*, *3* and especially *4*. Pathogen races are very specific in their interaction with a host (Kombrink et al. 1988). In contrast fungal elicitors such as Pmg elicitor have no host specificity. Therefore the induction by both Pmg and glucan elicitor presumably mimics a non-host response (Ebel and Grisebach 1988). However since *chit 2* gene expression is only induced in response to Pmg-derived substances, but not other stimuli, *chit 2* may harbor a pathogen-specific promoter. As mentioned above, *chit 4* also is strongly induced by glucan elicitor; however as it is equally well activated by the other stimuli the reaction is less specific.

The question of which component(s) of pathogen cell walls are recognized by the plants is as yet unanswered. The active components of crude Pmg elicitor in soybean are β-glucans (Ayers et al. 1976a, b, Yoshikawa et al. 1983), especially a branched heptaglucan of defined structure (Sharp et al. 1984). However, proteinaceous constituents of Pmg elicitor have also been identified as active eliciting compounds in parsley (Parker et al. 1988). Although the *chit 2* gene was efficiently induced by 25 μg/ml of crude Pmg elicitor, which consists of about 90% carbohydrates, it was even more efficiently induced by 2.5 μg/ml glucan elicitor, which was isolated from the Pmg elicitor by β-glucanase treatment. Thus, it seems likely that, as in the soybean system, smaller glucans trigger the defense reaction in peanut.

To our knowledge, the *chit 2* gene is the first example of a gene selectively induced in response to pathogen elicitors and not by other biotic or abiotic stress factors. Fusing its promotor to a reporter gene like GUS and expressing this construct in transgenic plants should help us to get a more detailed picture of compatible and incompatible plant-pathogen interactions and of systemic effects or induced resistance. It is noteworthy that the *chit 2* cDNA and the deduced protein sequence are less similar to the other peanut chitinase cDNAs (69–71%), than these are to each other (81–94%, Table 1). This feature might also reflect a special role of the *chit 2* protein in plant defense against pathogens.

The results presented here suggest that different signal transduction pathways may lead to the differential induction of individual members of the chitinase gene family.

At this point, we cannot rule out the possibility that the differences found in chitinase mRNA levels are modulated by posttranscriptional regulation mechanisms like splicing, mRNA transport or stability.

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