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Universität Kaiserslautern, FB Biologie, Kaiserslautern, Germany

Cloning, Expression and Characterization of Protein Elicitors from the Soyabean Pathogenic Fungus *Phytophthora sojae*

J. Becker*, S. Nagel* and R. Tenhaken

Authors' address: Universität Kaiserslautern, FB Biologie, Geb. 22, 67653 Kaiserslautern, Germany (correspondence to R. Tenhaken, Tel.: + 49 631 2053040; fax: + 49 631 2052600; e-mail: tenhaken@rhrk.uni-kl.de)

With 6 figures

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Abstract

The oomycete *Phytophthora sojae* is a severe pathogen of soybean. Several resistance genes against races of *P. sojae* exist in soybean but the nature of corresponding avirulence genes is unknown. Clones encoding four different isoforms of a protein elicitor from *P. sojae* (sojein 1–4) belonging to the class of acidic α-elicitins have been isolated. These 98 amino acid proteins show high homology to elicitins from other *Phytophthora* species. The different sojein isoforms were expressed in *Escherichia coli* as His-tagged fusion proteins. Purified sojein as well as recombinant sojein isoforms induce hypersensitive reaction (HR)-like lesions in tobacco but are not active as race-specific elicitors in soybean. However all sojein isoforms induce defence-related genes like those encoding phenylalanine ammonia lyase, glutathione-S-transferase and chalcone synthase in tobacco and soybean plants and cell cultures. It is concluded that sojeins contribute to the induction of defence responses but that they are not involved in race specific recognition of the *P. sojae* races by soybean plants.

Zusammenfassung

Klonierung, Expression und Charactier von Proteinelictoren aus dem Soyabohnenpathogen *Phytophthora sojae*

Der Oomycet *Phytophthora sojae* ist ein ernstes Pathogen der Sojabohne. In der Sojabohne gibt es mehrere Resistenzgene gegen verschiedene Rassen von *P. sojae*, jedoch ist die Natur der korrespondierenden Avirulenzgene unbekannt. Wir haben 4 verschiedene Isoformen eines Protein-Elicitors aus *P. sojae* (Sojein 1–4) kloniert, die zur Klasse der sauren α-Elicitine gehören. Sie kodieren für Proteine mit 98 Aminosäuren und zeigen hohe Homologie zu Elicitinen aus anderen *Phytophthora* Spezies. Aus genomischer DNA und aus revers-transkribierter mRNA wurden die gleichen 4 Isoformen erhalten. Die verschiedenen Sojeine wurden in *Escherichia coli* als His-markierte Fusionproteine exprimiert. Sowohl gereinigtes als auch rekombinantes Sojein induziert HR-ähnliche Läsionen in Tabak. In der Sojabohne sind sie allerdings nicht als rassenspezifische Elicitoren aktiv. Dagegen induzieren alle Sojein-Isoformen Abwehrgene wie die Phenylalanin Ammonium-Lyase, Glutathion-S-Transferase und Chalkonsynthase in Tabak-und Sojabohnenpflanzen und Zellkulturen. Die Sojeine tragen also zur Induktion von Abwehrreaktionen bei, sind aber nicht in die rassenspezifische Erkennung von *P. sojae* durch Sojabohnenpflanzen involviert.

Introduction

The oomycete *Phytophthora sojae* is a severe pathogen of soybean causing great losses in susceptible cultivars (Sinclair and Backman., 1989). Progress in classical breeding has identified more than 14 loci conferring resistance to individual races of *P. sojae*. A growing number of fungal races responding to theses resistance genes has been isolated from infected plants worldwide (Sinclair and Backman., 1989; Foerster et al., 1994). Despite the commercial interest in this plant–pathogen interaction neither a soybean resistance gene nor a *P. sojae* avirulence gene was cloned (Hegstad et al., 1998). Non race-specific elicitors derived from the cell wall of *P. sojae* were shown to be branched β-1,3-1,6-glucans down to the size of heptamers which bind with high affinity to soybean plasma membranes (Cosio et al., 1992). The binding protein was recently cloned by Umemoto et al. (1997). As the glucan elicitor is a part of the typical *P. sojae* cell wall it cannot be involved in race-specific recognition.

Protein elicitors from *Phytophthora* species have been known for a long time. They fall into several subgroups.

*These two authors contributed equally to this work.

The nucleotide sequences for sojein 1–4 were submitted to the EMBL-database under the accession numbers AJ007858, AJ007859, AJ007860, and AJ007861.

The most prominent class are nonglycosylated 10 kDa proteins called elicitins which have a typical length of 98 amino acids in the mature peptide. They have been found in almost all *Phytophthora* species analysed so far (Kamoun et al., 1994) as well as in other oomycetes (Huet et al., 1995). Elicitins were long regarded as avirulence factors as they cause hypersensitive reaction (HR)-like lesions when infiltrated into tobacco (Kamoun et al., 1993). As a consequence elicitins provoke all of the HR-related responses known from the infection of plants with incompatible pathogens. These include the induction of pathogenesis related (PR)-genes and systemic acquired resistance (Bonnet et al., 1996; Keller et al., 1996a, b) as well as the common rapid responses like the oxidative burst and pH-changes in the medium of plant cell cultures (Simon-Plas et al., 1997), activation of MAP-kinases (Zhang et al., 1998) and phytoalexin biosynthesis (Chappell et al., 1997).

The elicitins from *P. sojae* are not well characterized but one full length and one partial sequence of two isoforms were presented by Mao and Tyler (1996). Four elicitin genes have been cloned from *P. sojae* and expressed as His-tagged fusion proteins in *Escherichia coli* to investigate their potential role in race-specific interactions between *P. sojae* race 1 and resistant soybean plants of the cultivar Williams 82, carrying the *P. sojae* resistance gene *Rps* 1 *k*.

Materials and Methods

Biological material

Phytophthora sojae race 1 was kindly obtained from Elmon Schmelzer (Cologne, Germany) and soybean seeds were kindly obtained from R.I. Buzzel (Harrow, Ontario, Canada). Plants were grown in a greenhouse at 25°C with a 16 h daily light period. The soybean cell culture (cv. Williams 82) was maintained as described (Tenhaken and Rübel, 1997). The tobacco BY-2 cell culture was subcultured weekly in MS-medium supplemented with 0.2 g KH_2PO_4 and 0.2 mg 2,4-Di-chlorophenoxy acetic acid per litre. *Phytophthora sojae* was kept on V8-agar plates and subcultured every 2 months. A liquid culture in asparagine-medium (Keen, 1975) was inoculated with a few pieces from the agar plates and kept as still culture at 20–22°C for 6–8 weeks.

Purification of elicitins from the fungal culture filtrate

The culture filtrate of a typically 8-week-old culture of *P. sojae* was lyophilized, resuspended in a small amount of water and subsequently dialysed against 10 mM Na-acetate pH 4.0. The dialysed protein was applied to a SP-sephadex (Pharmacia Biotech, Freiburg, Germany) column equilibrated with the same buffer. The column was washed extensively with start buffer and then with 200 mM NaCl in acetate buffer. Elicitins were eluted from the column with 0.5 M NaCl in acetate buffer.

Cloning of elicitin genes from *P. sojae*

Elicitin genes from *P. sojae* were amplified by polymerase chain reaction (PCR) using the following Primers: 5′ ACAGGATCCACCACGTGCACCTCGTCGCAG 3′ (forward), 5′ AGGGGTACCTTACAGCGACGCGCACGTGGA 3′ (reversed). The underlined sequence corresponds to the elicitin genes, flanked by a restriction enzyme site (BamHI in the Fwd-primer and KpnI in the Rev-primer). The cycle conditions were step1: 94°C 1 min; step 2: 92°C 40 s; 70°C 40 s –0.5°C/cycle, 20 times; step 3: 92°C 40 s, 60°C 40 s + 1 s/cycle, 20 times. The 300 bp fragment was cut with BamHI and KpnI, gel purified and subsequently ligated into pBluescript (Stratagene, Heidelberg, Germany) digested with the same enzymes. The DNA-sequence of individual clones was determined by cycle-sequencing using the Thermosequenase system (Amersham, Freiburg, Germany) and fluorescent labelled primers (MWG-Biotech, Ebersberg, Germany). Reactions were run on a Licor (MWG-Biotech, Ebersberg, Germany) automatic DNA-sequencer.

PolyA(+) RNA was isolated from *P. sojae* mycelium by the RNA-Oligotex kit with oligo(dT)-latex beads (Qiagen, Düsseldorf, Germany), according to the manufacturer. The mRNA was reverse transcribed with Expand reverse transcriptase (Boehringer, Mannheim, Germany) using the sojein-reverse primer. The PCR was performed on the cDNA-template as described for the genomic DNA. Homology searches were performed with the BLAST-analysis tools. Multiple alignment of DNA and protein sequences were performed with the ClustalX program (Thompson et al., 1994) and restyled with the Genedoc-program (Nicholas and Nicholas, 1997) for final printing. The isoelectric points and the theoretical molecular weight was calculated on the expasy-server (HTTP://www.expasy.ch)

Recombinant elicitins

Plasmids coding for individual sojein isoforms (1–4) were cut with BamHI and KpnI and inserts isolated by gel electrophoreses. The 300 bp inserts were ligated into the expression vector pQE30 (Qiagen) and transferred into *E. coli* XL-1. To produce recombinant elicitins a 50 ml culture was grown to an optical density at 600 nm (OD_{600}) ~ 0.6 and then induced with 0.5 mM Isopropyl-, 3-D-1-thiogalactoside (IPTG) for 6 h. Bacteria were collected by centrifugation and frozen in liquid N_2. The recombinant sojeins were purified on NTA-Agarose using the denaturing protocol (Qiagen). Renaturing was achieved by dialysis of the urea-containing protein solution against 10 mM Na-acetate buffer pH 4 with one buffer exchange. The dialysed protein fractions were stored at 4°C.

Western blot analysis

Sojeins were separated on a 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF-membrane. The membrane was blocked with 3% BSA in TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.02% Tween 20), incubated with the primary antibody (anti α-megaspermin (a kind gift from S. Kaufman, Strasbourg), 1 : 1000 dilution), washed four times with TBST and further incubated with a secondary

antibody (anti rabbit IGG coupled with peroxidase, 1 : 10 000 dilution). After the final washing five times with TBST the membrane was incubated with enhanced luminol chemoluminescence reagents and exposed to a X-ray film for 30 s.

Infiltration of plants

Sojein-containing solutions were brought into infiltration buffer (10 mM 2-(N-Morpholino) Ethane-sulfonic acid (MES), pH 6.5) by rapid gel filtration on a PD10-column (Pharmacia) immediately before use. Protein concentrations were routinely determined with the BCA-assay (Pierce, Rockford, IL, USA) as the Bradford reagents gave only very poor results. The concentration was verified by UV-absorption calculated for the pure protein (HTTP://www.expasy.ch). The sojeins were infiltrated into leaves with a 1 ml syringe without needle and the infiltrated area was marked with a pen. At designated time-points leaf discs were punched out and snap frozen in liquid N_2 to isolate total RNA from the plant material.

RNA-isolation and Northern-blot hybridization

Total RNA was isolated from frozen cell culture or leaf disk material with the Tri-Reagent protocol using the salt precipitation modification (Chomczynski and Sacchi, 1987). RNA was separated on denaturing MOPS-formaldehyde gels, transferred to a positively charged nylon membrane (Macherey Nagel, Düren, Germany) and hybridized with ^{32}P-labelled cDNA-probes according to the protocol from Church and Gilbert (1984).

Results

Purification of sojeins and cloning of the genes

Phytophthora sojae (race 1) secretes protein elicitors when grown in liquid medium. In the dialysed medium the 10 kDa class of protein elicitors are dominant which were purified by SP-sephadex chromatography to apparent homogeneity (Fig. 1). A Western blot with antiserum against α-megaspermin, an elicitin from the closely related species *Phytophthora megasperma*, confirmed that the 10 kDa protein is immunologically related to the elicitins (Fig. 4C). During our attempts to clone the gene coding for the *P. sojae* elicitin Mao and Tyler (1996) reported in a brief note on the partial sequence of two *P. sojae* elicitins called sojeins. On the basis of the available sequence, which is highly homologous to other elicitins, the elicitin genes have been cloned using a PCR-assisted strategy from *P. sojae* genomic DNA as well as from reverse transcribed mRNA. Approximately 70 individual clones were sequenced which fall into four distinct classes, called sojein 1–4 (Fig. 2). The same clones were obtained from genomic DNA and from first strand cDNA indicating that all of the genes are transcribed in *P. sojae*. A Southern blot with restricted genomic DNA revealed four to five bands consistent with a small gene family for elicitins in *P. sojae* (data not shown, Mao and Tyler, 1996). The high similarity between the different sojein isoforms results in mature proteins of 98 amino acids which differ only in four positions between the isoforms 1–4. A multiple alignment of the sojeins and some related

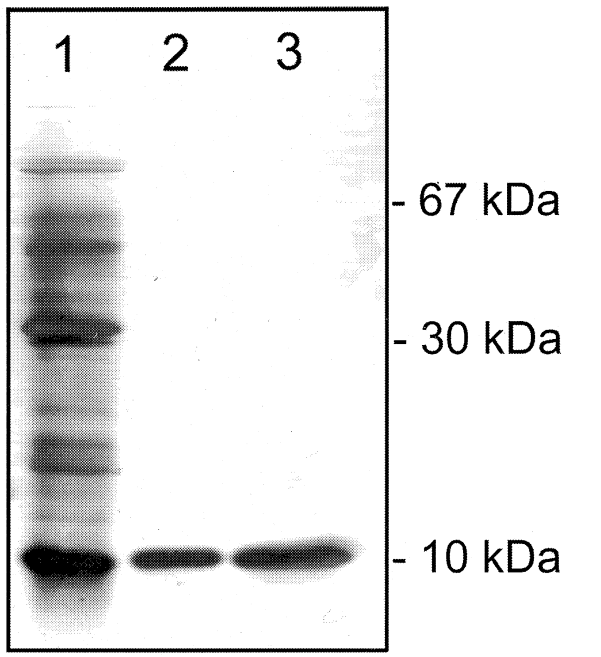

Fig. 1 Purification of a 10 kDa protein elicitor from the culture filtrate of *P. sojae* by chromatography on SP-sephadex. Protein extracts from a dialysed culture filtrate (Lane 1, concentrated approximately 10-fold by lyophilization) and from two purifications (Lane 2 and 3) were separated on a 15% SDS-PAGE and silver-stained to visualize proteins

```
          1                                                          60
Sojein1   ACCACGTGCACCTCGTCGCAGCAGACCGCCGCGTACGTGGCTTTGGTGAGCATCCTCTCG
Sojein2   ............................................................
Sojein3   ............................................................
Sojein4   ............................................................

          61                                                         120
Sojein1   GACTCGTCCTTCAACCAGTGCGCGACGGACTCGGGCTACTCGATGCTCACGGCCACGGCG

Sojein2   ........................G...................................
Sojein3   ............................................................
Sojein4   ........................G...................................

          121                                                        180
Sojein1   CTGCCCACGACGGCGCAGTACAAGCTCATGTGCGCGTCCACGGCGTGCAACACCATGATC
Sojein2   ............................................................
Sojein3   ............................................................
Sojein4   ............................................................

          181                                                        240
Sojein1   ACCAAGATCGTGTCGCTCAACCCGCCCGACTGCGAGCTGACCGTGCCCACGAGCGGCCTG
Sojein2   .AG.........G.....................C.........................
Sojein3   .AG.........G.....................C.........................
Sojein4   ............................................................

          241                                                    297

Sojein1   GTGCTCAACGTCTACTCGTACGCCAACGGCTTCTCGTCCACGTGCGCGTCGCTGTAA
Sojein2   ...................................................
Sojein3   ...................................................
Sojein4   ...................................................
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Fig. 2 DNA sequence comparison. The nucleotide sequences coding for the 98 amino acids of mature sojeins 1–4 are aligned. The sequences are largely identical (represented by dots) except for a few exchanges at position 89, 182, 183, 193 and 216. The simultaneous identical exchange of nucleotides in two sequences suggests a gene duplication of the ancient sojein gene

elicitins from other *Phytophthora* species is shown in Fig. 3A. Most of the amino acid residues are conserved in all elicitins. On the basis of the criteria that the amino acid no. 13 in α-elicitins is a valine all sojeins are clearly α-elicitins (Pernollet et al., 1993). In addition the predicted isoelectric points of sojeins 1–4 are 4.23, 5.7, 4.58 and

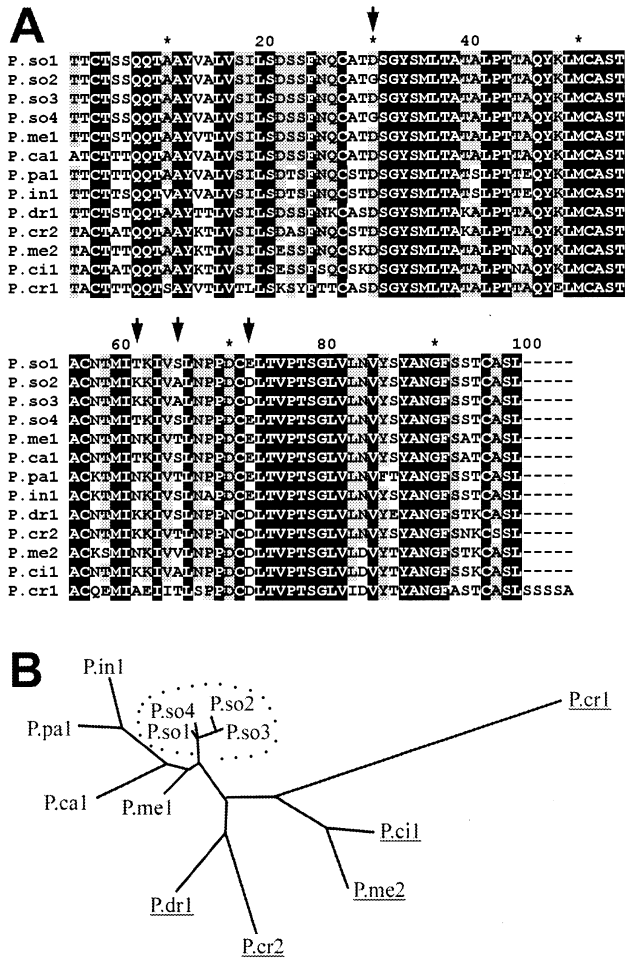

Fig. 3 (A) Multiple alignment of several *Phytophthora* elicitins with the four sojeins from *P. sojae*. Identical residues are printed inversely and grey-shaded residues include conserved amino acids as defined in the strong Blossum30-matrix. The arrows indicate the variable amino acids between the sojein isoforms. The numbers and the * serve as position indicators. (B) The sequences from (A) were used to generate a tree by the neighbour-joining methods and a bootstrap value of 1000. The unrooted tree shows a distinct branch for the β-elicitins (underlined). The acidic α-elicitins fall into several subclasses and the four sojeins represent a distinct new subgroup. (P.me1 = *P. megasperma* [P35698]; P.ca1 = *Phytophthora capsici* [P15571]; P.pa1 = *Phytophthora parasitica* [P41801]; P.in1 = *Phytophthora infestans* [Q01905]; P.dr1 = *Phytophthora drechsleri* [P35697]; P.cr1 = *Phytophthora cryptogea* [P15570]; P.me2 = *P. megasperma* [P35699]; P.ci1 = *Phytophthora cinnamoni* [P15569]; P.cr^2 = *Phytophthora cryptogea* [P41804], corresponding accession numbers are given in [ ])

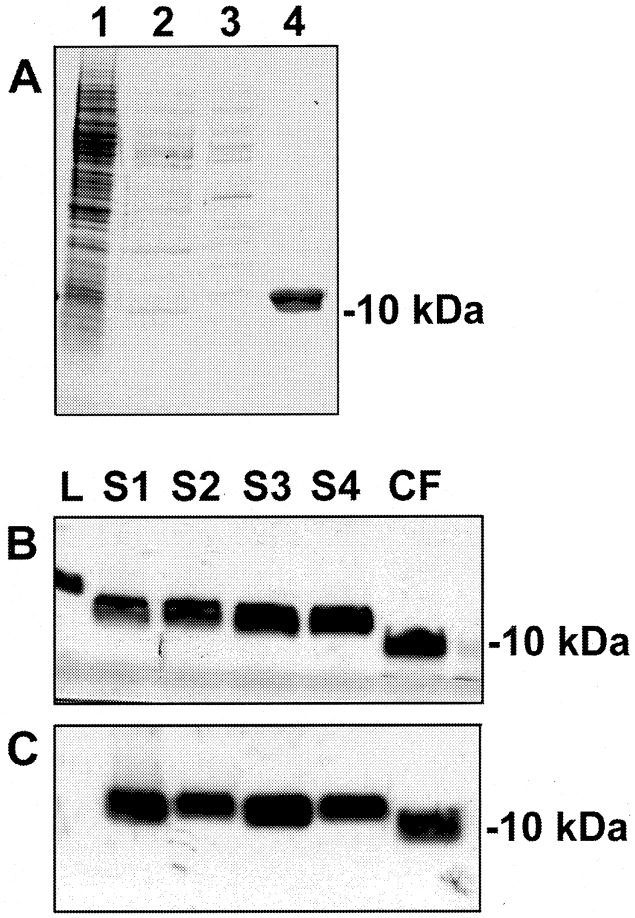

Fig. 4 Purification of recombinant sojein from *E. coli* lysates and Western blot analysis. An *E. coli* culture expressing the sojein as a His-tagged fusion protein was induced with 0.5 mM IPTG for 6 h. The cells were disrupted in lysis buffer and protein extracts applied to a NTA-agarose column. (A) Coomassie-stained SDS-PAGE showing the purification of the recombinant elicitin (here sojein2). Lane 1, *E. coli* lysate, lanes 2 and 3, eluates from the washing procedure, lane 4, purified sojein2. (B) Silver-stained gel with 1.5 μg each of lysozyme (L), recombinant sojein 1–4 (S1 – S4) and purified sojein from the culture filtrate of *P. sojae* (CF). The recombinant sojeins have a higher molecular mass due to their His-tag (sequence is MRGSHHHHHH-sojein). (C) Western blot of a duplicate gel as shown in (B) using an anti α-megaspermin antiserum. The antibodies recognizes all recombinant sojein isoforms as well as the sojein purified from culture filtrate. Lysozyme as a negative control is not detected by the antibodies

4.56, respectively. Thus all sojeins are acidic proteins which is consistent with the class of α-elicitins. A phylogenetic relationship of the aligned elicitins (Fig. 3A) is shown in Fig. 3B as an unrooted tree generated by the neighbour-joining algorithm. It is obvious that all sojeins are very closely related and form a new subgroup within the elicitin family. The β-elicitins are more distantly related to the sojeins and belong to a separate branch. The α-megaspermin is the closest homolog to the sojeins in agreement with the former taxonomy of *P. sojae* as *P. megasperma* f.sp. *glycinea*. An anti α-megaspermin antibody recognizes all four sojein isoforms as shown in Fig. 4C.

Expression of sojeins in *E. coli*

The individual sojein genes were cloned into the *E. coli* protein expression vector pQE30 with an N-terminal (His)6-tag for affinity purification. An example of the expression and purification of a recombinant sojein is shown in Fig. 4. The sojeins were produced in *E. coli* in high concentrations but in an insoluble form, allowing the purification only under denaturing conditions. Attempts to modify the conditions for the expression of the protein in *E. coli* (low temperature, less IPTG, variable induction time) to obtain native soluble protein were unsuccessful. Therefore a renaturing procedure was developed to obtain biologically active elicitins. Dialysis of urea-denatured recombinant sojein against neutral buffers (pH 6–7.5) always led to protein precipitation of

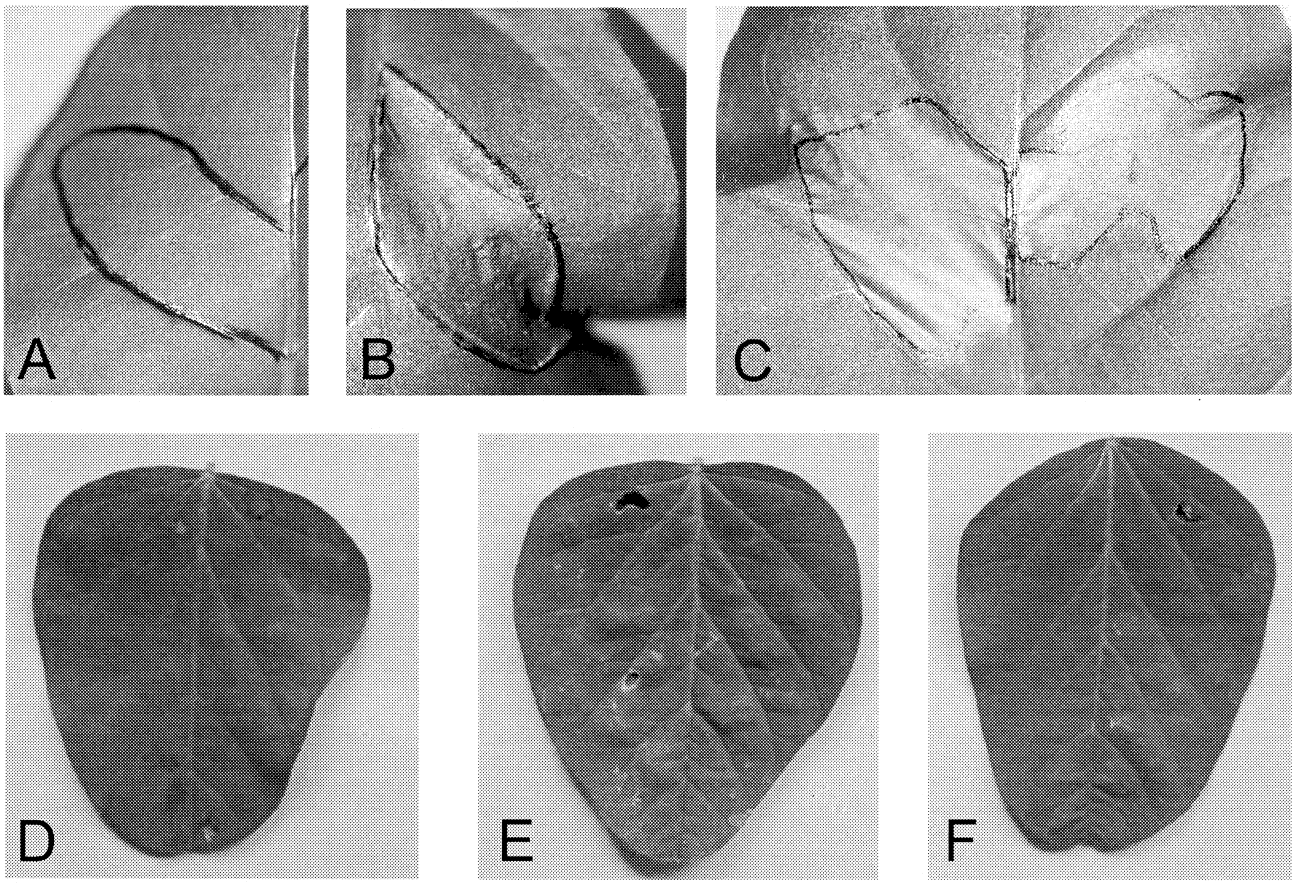

Fig. 5 Necrosis of tobacco leaves infiltrated with purified or recombinant sojein. The infiltrated area was marked with a pen. Buffer control (A), sojein purified from culture filtrate of *P. sojae* (B), 10 μg/ml, recombinant sojein1 (C, left side) or sojein2 (C, right side) (50 μg/ml). The sojein-infiltrated tobacco leaves show a typical necrosis with glossy appearance after 12 h (photos). The infiltrated area later dries out to a brownish dry lesion within 3 days. Infiltrated soybean leaves (D–F) show no visible symptoms in buffer-infiltrated controls (D) or with purified sojein from the culture filtrate (E) or with recombinant sojeins 1 and 2 (F)

the sojein in the dialysis bag. The best results were obtained by using an acidic buffer (pH 4.0) which gave a renatured protein that was biologically active and could be stored at 4°C for a couple of weeks. Only in rare cases after prolonged storage at 4°C was a later precipitation was observed.

Effects of sojeins in tobacco and soybean

To test whether the sojeins are race-specific elicitors in soybean culture filtrate-purified or recombinant sojeins have been infiltrated into leaves of the resistant soybean cultivar Williams 82 (*Rps*-1 *k*). Application of zoospores of *P. sojae* race 1 to roots of young soybean seedlings gave a strong HR with browning and cell death within 6–8 h indicating an incompatible interaction. In contrast, the parental soybean line Williams lacking the *Rps* 1 *k*-gene was heavily infected (A. Ludwig, R. Tenhaken unpublished results). No visible lesions in the infiltrated areas of the plants were observed (Fig. 5D–F). In contrast, infiltration of various sojeins into tobacco leaves gave strong HR-like lesions (Fig. 5A–C). Boiling of the elicitins for 5 min did not diminish the biological activity of the sojeins (data not shown). A digest of the recombinant sojein with proteases (proteinase K, trypsin or pronase), followed by 5 min boiling totally destroyed the elicitor activity of the sojeins confirming the proteinacous nature of the elicitor (data not shown).

Defence gene induction by sojeins

We then asked if the sojein can act as a protein elicitor in soybean and induce defence-related genes. Indeed infiltration of sojein into soybean leaves resulted in the induction of chalcone synthase (Fig. 6C, lower panel), a key enzyme of phytoalexin biosynthesis in this plant. Using a cell culture of the same soybean cultivar Williams 82 it was confirmed that the induction of the chalcone synthase gene by sojeins (Fig. 6C, upper panel). In addition, a glutathion-S-transferase, which is known to respond to the plant oxidative burst (Levine et al., 1994) was also strongly induced (Fig. 6B). Sojeins also induced defence-related genes in tobacco. As an example, the induction of the phenylalanine ammonia lyase gene, a

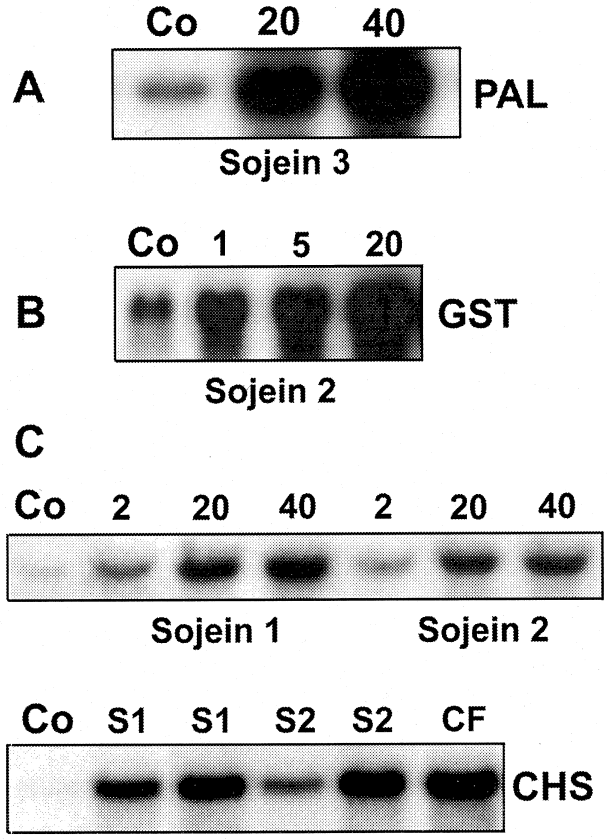

Fig. 6 Northern blots with RNA from tobacco (A) or soybean (B, C) cell cultures or plants. (A) Tobacco BY-2 cell cultures were treated with sojein3 for 6 h and RNA from these cells was electrophoretically separated, transferred to a Nylon membrane and hybridized with a cDNA-probe for phenylalanine ammonia lyase (PAL). (B) Soybean cells were treated with sojein2 for 5 h and the RNA was hybridized with a cDNA-probe for a glutathion-S-transferase (GST). A similar experiment is shown in (C) (upper panel) with sojein1 and 2 using chalcone synthase (CHS) cDNA as a hybridization probe. The lower panel in (C) shows a Northern blot with RNAs from sojein-infiltrated soybean plants. S1 and S2 refer to (100 μg/ml) sojein1 and 2 (two independent plants) and CF to the sojein purified from fungal culture filtrate. The buffer-infiltrated control (Co) shows no significant induction of the CHS-gene. The numbers refer to the sojein concentrations (μg/ml)

key enzyme for the biosynthesis of cell wall phenolics and salicylic acid, by sojein 3 is shown in Fig. 6A.

Using the different recombinant sojeins to test the relative biological activity of the isoforms it was found that sojein 1 and 3 are the most active forms in soybean and tobacco. The differences in the activity of the isoforms are less than three-fold. An exact dose–response curve is however, difficult to generate as the isoforms exhibit a different tendency to precipitate in neutral solutions which cannot easily be followed in infiltrated plant material.

Discussion

In this study, four different elicitin genes from *P. sojae* were cloned and characterized. Two of them were already published by Mao and Tyler (1996) although their sequences have an insertion at amino acid 10 of the mature protein that was never observed in the sequences of the present study. All of the identified sojeins have an acidic isoelectric point and encode valine at position 13, two characteristic properties of α-elicitins (Pernollet et al., 1993). Infiltration of sojein into tobacco leaves causes a strong HR-lesion visible after 12 h (Fig. 5). The infiltrated area appears glossy in the initial stage and dries out to a brownish HR-like lesion within 3 days, indicating that tobacco is able to recognize all known *Phytophthora* elicitins. Numerous studies have suggested the avirulence character of the elicitins in tobacco (e.g. Ricci et al., 1989; Kamoun et al., 1993; Bonnet et al., 1994) but in recent years it became more and more evident that the effect of elicitins to induce programmed cell death is basically restricted to tobacco plants (Kamoun et al., 1997). Some weak symptoms were also reported for radish plants after infiltration with elicitins (Kamoun et al., 1993; Keizer et al., 1998) but were not observed in other solanaceous plants.

Infiltration of sojeins into leaves of a resistant soybean cultivar although not causing the formation of HR-like lesions (Fig. 5E,F) lead to the induction of defence-related genes (Fig. 6B,C). Thus glutathione-S-transferase is strongly induced in soybean cell culture which was recently reported to be synthesized upon occurrence of hydrogen peroxide from the oxidative burst or treatment with other elicitors (Levine et al., 1994). The observation that sojein does not cause programmed cell death in incompatible *P. sojae*–soybean interactions agrees with the recently changed view of elicitins as specific elicitors in tobacco plants.

Recombinant sojeins

Expression of sojeins in *E. coli* as His-tagged fusion proteins (Fig. 4) provides a simple tool to examine the biological function of different sojein isoforms. The main problem with this method still is the necessity to purify the recombinant proteins under denaturing conditions. This implies a renaturing procedure of the protein before it can be used as an elicitor in plants. Using an acidic buffer in the dialysis procedure was superior to neutral phosphate, MES or Tris-buffers which tend to produce insoluble elicitin aggregates after dialysis. This appears to be caused by the formation of incorrect disulphide bridges, which are formed to some degree intermolecularly and not solely intramolecularly thus giving oligo- to polymeric structures. This became evident by SDS-PAGE under nonreducing conditions in which elicitins that had been stored for prolonged times run as a high molecular smear beside the correct 10 kDa monomeric band (data not shown). This view is supported by the fact that precipitated sojein could be redissolved with β-mercapto-ethanol at higher concentrations (100 mM, data not shown).

Function of sojein in the *Phytophthora*–soybean interaction

The function of elicitins in plant–pathogen interactions remains puzzling. In addition to their role as avirulence factors other functions were suggested such as a phospholipase activity or the more recent finding of a lipid/sterol binding activity (Mikes et al., 1997). None of

these functions is unambiguously proven and therefore it remains to be elucidated why a single *P. sojae* isolate would need four very closely related proteins to bind sterols. As these concepts have not been totally worked out, the search for new functions is still worthwhile. It is interesting that ergosterol, the main sterol of higher fungi is a highly potent elicitors in plant systems shown to elicit extracellular alkalinization in tomato cells in the lower nanomolar range (Granado et al., 1995). The failure of the sojeins to induce an HR in soybean cultivars resistant to *P. sojae*, race 1 (Fig. 5), suggests other elicitors from this fungus yet to be identified as signalling compounds for the race-specific recognition of *P. sojae* by resistant soybean cultivars.

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