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A new cell wall located N-rich protein is strongly induced during the hypersensitive response in *Glycine max* L.

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Abstract

Soybean (*Glycine max* (L.) Merill, cv. Williams 82) plants and cell cultures respond to avirulent pathogens with a hypersensitive reaction. After inoculation of soybean with *Pseudomonas syringae* pv. *glycinea*, carrying the avirulence gene *avrA*, or zoospores from the fungus *Phytophthora sojae* Race 1, a resistance-gene-dependent cell death programme is activated. A new gene was identified by differential display of mRNAs that is specifically activated during the early phase of incompatible pathogen–soybean interactions but does not respond to compatible pathogens. The gene is strongly induced within 2 h after addition of *P. sojae* zoospores. A similar kinetic pattern was observed for *P. syringae* (*avrA*) inoculated soybean cell cultures. The gene encodes a deduced protein of 368 amino acids with a very high content of asparagine and was therefore termed N-rich protein (NRP). The protein is composed of two distinct domains, of which only the C-terminal domain has striking homology to proteins of unknown function from other plants. An antibody raised against the recombinant NRP recognizes a protein of 42 kDa. The protein is located in the cell wall as indicated by cell fractionation studies. Comparison of the genomic DNA-sequence with the cDNA, identified two introns within the open reading frame. The *NRP*-gene is not directly induced by salicylic acid or hydrogen peroxide, indicating a distinct and specific signal transduction pathway which is only activated during programmed cell death. The *NRP*-gene appears to be a new marker in soybean activated early in plant disease resistance.

Abbreviations: HR – hypersensitive reaction; NRP – N-rich protein; Ps 1 – *Phytophthora sojae* Race 1; Psg – *Pseudomonas syringae* pv. *glycinea*; Psg(*avrA*) – Psg with avirulence gene *avrA*; SA – salicylic acid.

Introduction

Plants often recognize microbial pathogens via a specific interaction system. In the soybean cultivar Williams 82, the *Rpg2*-resistance gene interacts with the avirulence gene *avrA* from the bacterium *Pseudomonas syringae* pv. *glycinea* (Keen and Buzzel, 1991) according to the classical gene-for-gene interaction model (Flor, 1971). Generally, Psg is a pathogen of soybean causing bacterial blight disease (Daft and Leben, 1972). However recognition of Psg(*avrA*) by soybean plants results in strong hypersensitive response (HR), a form of programmed cell death (pcd) in plants leading to resistance against microbial attack. Resistance-gene dependent signalling is therefore essential for the plant to restrict a pathogen and remain healthy. The pcd in plants is often accompanied by nuclear DNA-fragmentation down to nucleosomal size (Wang et al., 1996). Cytological studies of cells, undergoing the HR, indicate cell shrinkage and cytoplasmatic compartmentation of the cellular content (Levine et al., 1996). In contrast to animal cells, regulator proteins for plant pcd have not been identified. However, salicylic acid (SA) is often necessary for the plant cell to execute the cell death programme. Metabolic depletion of SA in cells by expressing

a bacterial gene for salicylate hydroxylase (*nahG*) results in a loss of pcd in several plant–pathogen interactions (Delaney et al., 1994). Along the same line some *Arabidopsis* mutants with a spontaneous pcd can be blocked by removing SA through expression of the *nahG*-gene (Weymann et al., 1995). The molecular mechanism for SA-control of pcd is still unknown.

Marco et al. (1990) performed an extensive screening in tobacco for genes which are specifically induced during HR. They fall into two classes, one being exclusively up-regulated in pcd (Pontier et al., 1994), whereas genes from the other class are only preferentially induced in a HR (Czernic et al., 1996). In *Arabidopsis*, a gene coding for an aromatic alcohol dehydrogenase (eli-3, Somssich et al., 1996) was activated in a R-gene dependent manner (Kiedrowski et al., 1992). Beside R-gene specific gene induction, the activation of protein kinases is commonly observed in plant pcd (Romeis et al., 1999; Zhang and Klessig, 1999). However, the same kinases are often stimulated by several signals, including wounding and elicitors (Hirt, 1997). Similarly the production of reactive oxygen species is specifically stimulated during the HR and can thus be classified as a R-gene dependent plant response (Orlandi et al., 1992; Hammond-Kosack and Jones, 1996). However, numerous other signals are also known to activate the oxidative burst making it very difficult to draw unambiguous conclusions from the activation of this plant defense response (Wojtaszek, 1997).

The aim of this work was to identify new genes with induction patterns strictly restricted to signals generated during an incompatible plant–microbe interaction. These genes are potentially of interest as marker genes for pcd and may also be involved in the execution of the cell death programme. Here we report on the cloning and characterization of a novel gene encoding a protein with unusual properties, which is not induced by disease causing pathogens but is specifically upregulated in the HR.

Materials and methods

Biological material

Suspension cultured cells of soybean (*Glycine max* cv. Williams 82) were grown in the dark at 26 °C in MS medium and subcultured every 7 days by 1 : 6 dilution in fresh medium as described (Levine et al., 1994).

Soybean plants of the same cultivar were grown by germinating surface-sterilized seeds (3% sodium hypochloride, 5 min) in Petri dishes at 26 °C in the dark for 2 days. Seedlings were planted into potting soil and grown in a growth chamber at 26 °C for 2 weeks with a daily light period of 16 h.

Pseudomonas syringae pv. *glycinea* (Psg) race 4, harbouring the plasmid pLAFR1 carrying or lacking the *avrA* avirulence gene (Keen and Buzzell, 1991), were grown overnight in King's B medium (20 g of protease peptone, 10 ml of glycerol, 2.25 g of K_2HPO_4, and 1.5 g of $MgSO_4 \cdot 7H_2O\,l^{-1}$, pH 7.2) containing 25 $\mu g\,ml^{-1}$ streptomycin. The bacteria were collected by centrifugation, resuspended in sterile water and used for inoculum at a final concentration of 3×10^7 cfu ml^{-1}.

Phytophthora soja race 1 (Ps1) was grown in Petri dishes on lima bean agar (Difco, Detroit, Michigan) (Eye et al., 1978). Nine days after subculturing the production of zoospores was induced by washing the cultures five times with sterile bidestilled water for 30 min at 18 °C in the dark. The cultures were left for 16 h at 18 °C in the dark covered with 5 ml of sterile distilled water to release zoospores. Zoospores were collected, counted by using a Fuchs-Rosenthal hematocytometer and used for inoculum at a concentration of 10^5 zoospores ml^{-1}.

Treatment of soybean cell cultures and plants

Three days after subculturing, soybean suspension cultures were inoculated with Psg or zoospores of Ps1. Where applicable, SA (50 μM) or other substances were added to the cells. Aliquots of soybean cells were harvested at designated times after treatment by filtration and directly frozen in liquid nitrogen. The cell death was monitored by staining cells of the remaining culture with Evans blue (Levine et al., 1994). For infection of soybean plants with Psg, leaves were infiltrated with a bacterial suspension or with water as a control. At designated time points leaves were excised and frozen in liquid nitrogen. For infection of soybean plants with Ps1 zoospores, leaves were cut off the plants, placed in Petri dishes on wet paper towels and treated with drops of zoospores on the leaf surface. At designated times, leaf tissue under the drops was harvested and frozen in liquid nitrogen. All experiments were repeated at least two times with similar results.

Construction and screening of the cDNA library

Total RNA was isolated from three day old soybean cell cultures either inoculated with Psg, Ps1 zoospores

or treated with various substances as described for Northern blot analysis. Poly(A)$^+$ mRNA was further purified from total RNA by a magnetic separation technique using Dynabeads paramagnetic particles according to the manufacturer's protocol (Dynal, Hamburg,Germany). A cDNA library was synthesized from 5 μg of mRNA using the λ-Uni-Zap kit (Stratagene, La Jolla, CA, USA), ligated into the ZAP express vector and packaged into λ phages with the Gigagold system (Stratagene). The primary library contained about 4.5×10^6 plaque-forming units. The library was amplified and aliquots were stored at both 4 °C and −70 °C. The screening procedure using the 280 bp long differential display PCR fragment for the NRP was performed as described by the manufacturer (Stratagene). Five independent cDNA clones were obtained.

Sequencing and computer analysis

The cDNA clones were sequenced with the thermo sequenase fluorescence labelled primer cycle sequencing kit (Amersham) and products separated on an automatic sequencer (Licor, MWG, Ebersberg, Germany). DNA homology searches against the GenBank database were performed with the blast tools (Altschul et al., 1990). Protein sequences were aligned with the clustalX program and analysed by using the expasy tools (www.expasy.ch/tools).

DNA Blot Analysis

Genomic DNA from soybean cells was isolated as described by Taylor et al. (1993). Restricted DNA was separated on a 0.7% agarose gel, transferred to a nylon membrane (Nytran$^+$, Schleicher und Schuell, Dassel, Germany), and hybridized with the ^{32}P-labelled NRP cDNA as described for the Northern blot analysis.

RNA blot analysis

Total RNA was isolated from plant material by the guanidinium-thiocyanate/acidic phenol method as described (Chomczynski and Sacchi, 1987). RNA (10 μg) was separated on a denaturating 1.1% agarose gel (Ausubel et al., 1994), transferred to a positively charged nylon membrane (Nytran$^+$, Schleicher und Schuell, Dassel, Germany) and cross linked to the membrane using a UV crosslinker (model RPN 2500, Amersham). To verify equal RNA loading the gel was stained with ethidium bromide and photographed under UV-light (312 nm) prior to blotting. Hybridization experiments were performed at 62 °C according to the protocol of Church and Gilbert (1984) but using 0.25 M $NaHPO_4$ instead of 0.5 M. The *NRP* cDNA probe was labelled with ^{32}P-dCTP using the HighPrime Kit (La Roche, Mannheim, Germany). Blots were exposed to X-ray films (Kodak Biomax MS) at −70 °C.

Expression of the NRP cDNA in E. coli *and the generation of antibodies*

The full length *NRP* cDNA insert was cloned into the pQE30 expression vector (Qiagen, Hilden, Germany) and transformed into *E. coli* XL-1. Expression of the 6×His-tagged fusion protein was carried out in 100 ml scale by induction of the bacteria with 0.5 mM isopropylthio-β-galactoside overnight at 30 °C. The fusion protein was purified under denaturating conditions on nitrilotriacetic acid–agarose according to the Qiagen protocol. After extensive dialysis of the purified protein against PBS buffer, the protein was used to immunize two rabbits (4 injections with 150 μg protein each time; BioGenes, Berlin, Germany).

Protein extraction

A crude protein extract from soybean cells was obtained by boiling the cells in an SDS-containing buffer (24% glycerol, 3% SDS, 125 mM Tris/Cl pH 6.8, 1.4 M β-mercaptoethanol) followed by vortexing the cells with glass particles. After brief centrifugation the supernatant was transferred to a new eppendorf tube and stored at room temperature. Soluble proteins were extracted from soybean cells by homogenization in ice-cold 50 mM Tris–buffer (pH 8.0) containing 1 mM EDTA, 14 mM β-mercaptoethanol and 0.5 mM PMSF. Cell fragments were separated from soluble proteins by centrifugation and the protein extract was stored on ice. Proteins from the nuclei were isolated according to Armstrong et al. (1992). Suspension cultured cells (1 g) were ground to a fine powder under liquid nitrogen and resuspended in 2.2 volumes (v/w) of nuclear isolation buffer B (70% (v/v) glycerol, 20 mM Tris/Cl pH 7.8, 5 mM $MgCl_2$, 5 mM KCl, 250 mM sucrose). The homogenate was strained through nylon meshes of 60 and 20 μm, respectively. The filtrate was centrifuged for 1 h at 3500 × g at 4 °C. The supernatant was decanted and the pellet was resuspended in 1.5 volumes (v/w) buffer B

and centrifuged as above for 40 min. The pellet was then resuspended in 500 µl of high salt buffer (20 mM Tris/Cl pH 7.8, 5 mM $MgCl_2$, 0.5 M NaCl), incubated on ice for 15 min with slow agitation and centrifuged for 20 min at 20 000 × *g* at 4 °C. The supernatant containing the nuclear proteins were stored in aliquotes at −70 °C until use. Cell wall proteins were isolated with the method described by Kauss and Bowles (1976). One hundred mg of soybean cells were snap frozen in liquid nitrogen and homogenized in 200 µl of ice-cold 0.6 M sucrose/0.01 M potassium phosphate, pH 6 using a teflon potter. After adding 650 µl of ice-cold 0.3 M sucrose/0.01 M potassium phosphate, pH 6, the cell walls were sedimented at 350 × *g* for 10 min. The pellet was washed four times with 650 µl of 0.5% (w/v) SDS and four times with 650 µl of water and finally stored in water at −70 °C.

Western blot analysis

Approximately 10 µg of protein per lane was separated on a 10% SDS-PAGE and blottet on a PVDF membrane (Immobilon-P, Millipore). Immunological detection of the NRP was performed by incubating the membrane with the polyclonal antibody (1 : 1000). The primary antibody cross-reaction was detected by an alkaline phosphatase-conjugated secondary antibody against rabbit IgG from goat (1 : 4000, Bio-Rad) and visualized with NBT/BCIP.

Glycoproteins were detected by using a concanavalin A (50 µg ml^{-1}) horseradish peroxidase (50 µgml^{-1}) complex. The horseradish peroxidase activity was detected by colour reaction with 0.3 mg ml^{-1} 4-chloro-1-naphthol and 0.03% (v/v) H_2O_2 as substrate.

Results

Isolation of a gene for a new asparagine-rich protein (NRP)

By differential display of mRNA we isolated a 280 bp gene fragment induced during the inoculation of soybean cell cultures with an incompatible race of *Pseudomonas syringae* pv. *glycinea* (Psg(*avrA*)) (Seehaus and Tenhaken, 1998). A soybean cDNA library was screened to obtain a corresponding full length cDNA clone. The identified 1450 bp cDNA contained the differential display fragment near the 3′ untranslated region (Figure 1). The open reading frame of 1107 bp encodes a deduced polypeptide of 368 amino acids with a predicted molecular weight of 41.5 kDa. The protein obviously has two domains, which is further described in more detail in Figure 2. The N-terminal domain A is very asparagine-rich (24%) and has several hypothetical N-glycosylation and N-myristolation sites (according to PROSITE analysis, Bairoch et al., 1997). Because of its extremely high asparagine content we termed it N-rich protein (NRP). Hydropathy profile analysis of the protein indicates its highly hydrophilic nature with a complete lack of membrane-spanning domains (Figure 2B). The A-domain is significantly more hydrophilic than the C-terminal B-domain. Comparison of the deduced amino acid sequence of the N-rich protein (NRP) to databases using the BLAST tools (Altschul et al., 1990) revealed strong homology to proteins of unknown function in *Citrus paradisi* (McKendree et al., unpublished) and *Daucus carota* (Schrader et al., unpublished). The soybean NRP shared 50% over all amino acid identity (151 out of 305 residues) to the hypothetical protein from grapefruit and to the so called B2-protein from carrot (Figure 2A), mostly in the B-domain of the proteins. For the B-domain (155 amino acids) at the C-terminus of the NRP, the sequences of soybean and carrot are 84% identical and >90% homologous taking conserved exchanges into account. Very similar numbers were obtained for the grapefruit protein. The strikingly high homology within the B-domain suggests the separation of the NRP into the two domains. This notion is further underlined by the different hydrophobicities in each domain (Figure 2B). The N-rich N-terminus is very diverse between the proteins from *Citrus* and soybean. Presumably, only a partial cDNA clone from carrot was sequenced. A RNA blot with carrot mRNA detects a 1.4 kbp mRNA, similar to that detected with the soybean *NRP*-gene as a probe (data not shown). Unfortunately, neither protein has a known function, but the B2-protein from carrot is induced during redifferentiation of cell cultures after the withdrawal of auxins (Schrader et al., 1997).

Genomic organization of the NRP-*gene*

To estimate the number of *NRP* genes in the soybean genome, chromosomal DNA was digested with *Bam*HI, *Cla*I and *Xba*I and hybridized to the *NRP* cDNA. The radioactively labelled full length cDNA detected a 7.5 kb *Cla*I fragment, a 5.5 kb *Xba*I fragment and two *Bam*HI fragments of about 6 kb and 3.2 kb

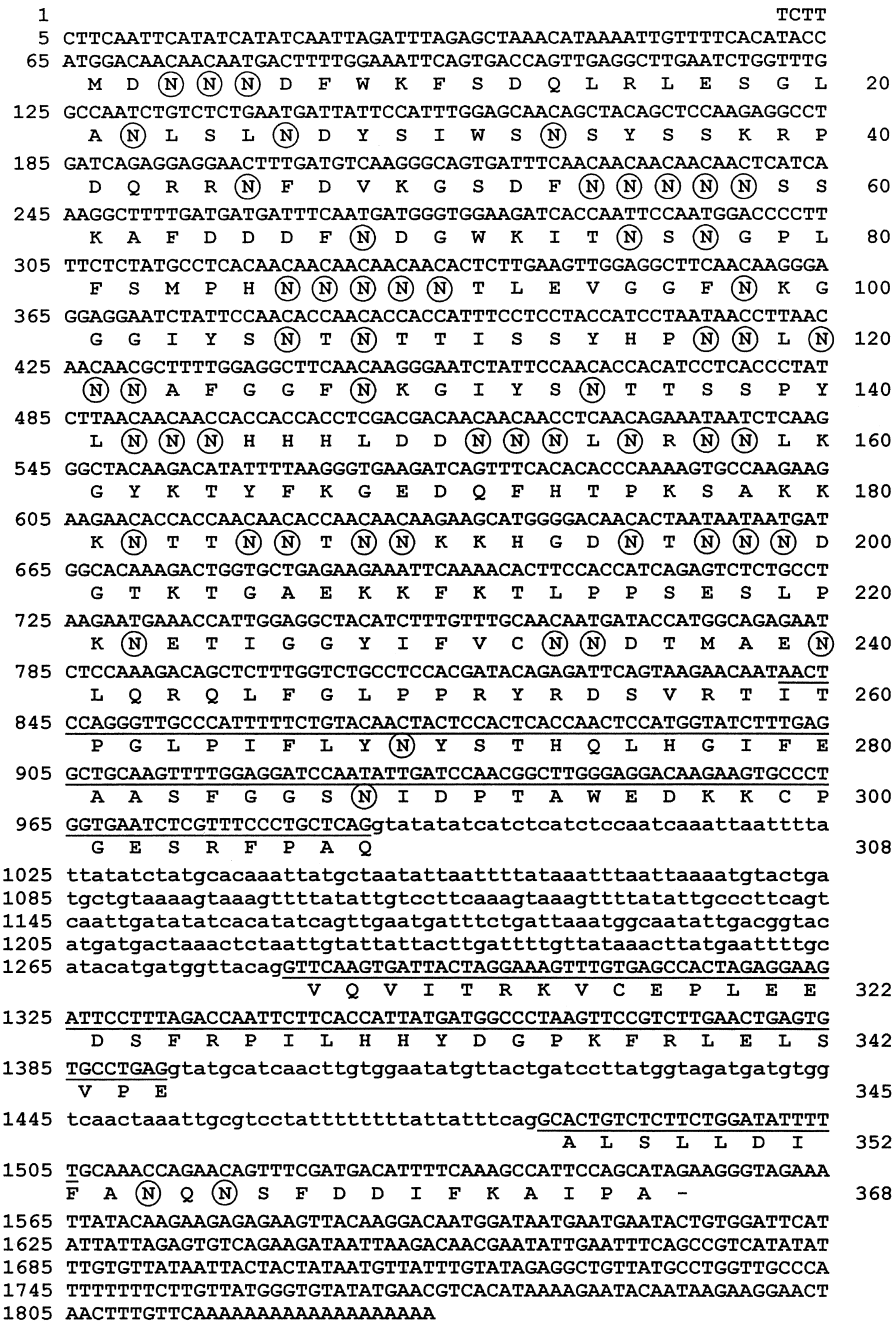

Figure 1. Nucleotide sequence and deduced amino acid sequence of the *NRP*-full length clone. The underlined region indicates the original dd 1/51-fragment. After sequencing the *NRP*-cDNA we have isolated the genomic sequence of the *NRP*-gene by PCR. The open reading frame (capital letters) of the *NRP*-gene is interrupted by two small introns (small letters), which are both located in the dd 1/51 fragment. The numbers on the left side refer to the nucleotide sequence, those on the right side to the amino acid sequence. The amino acid Asn (N) is encircled.

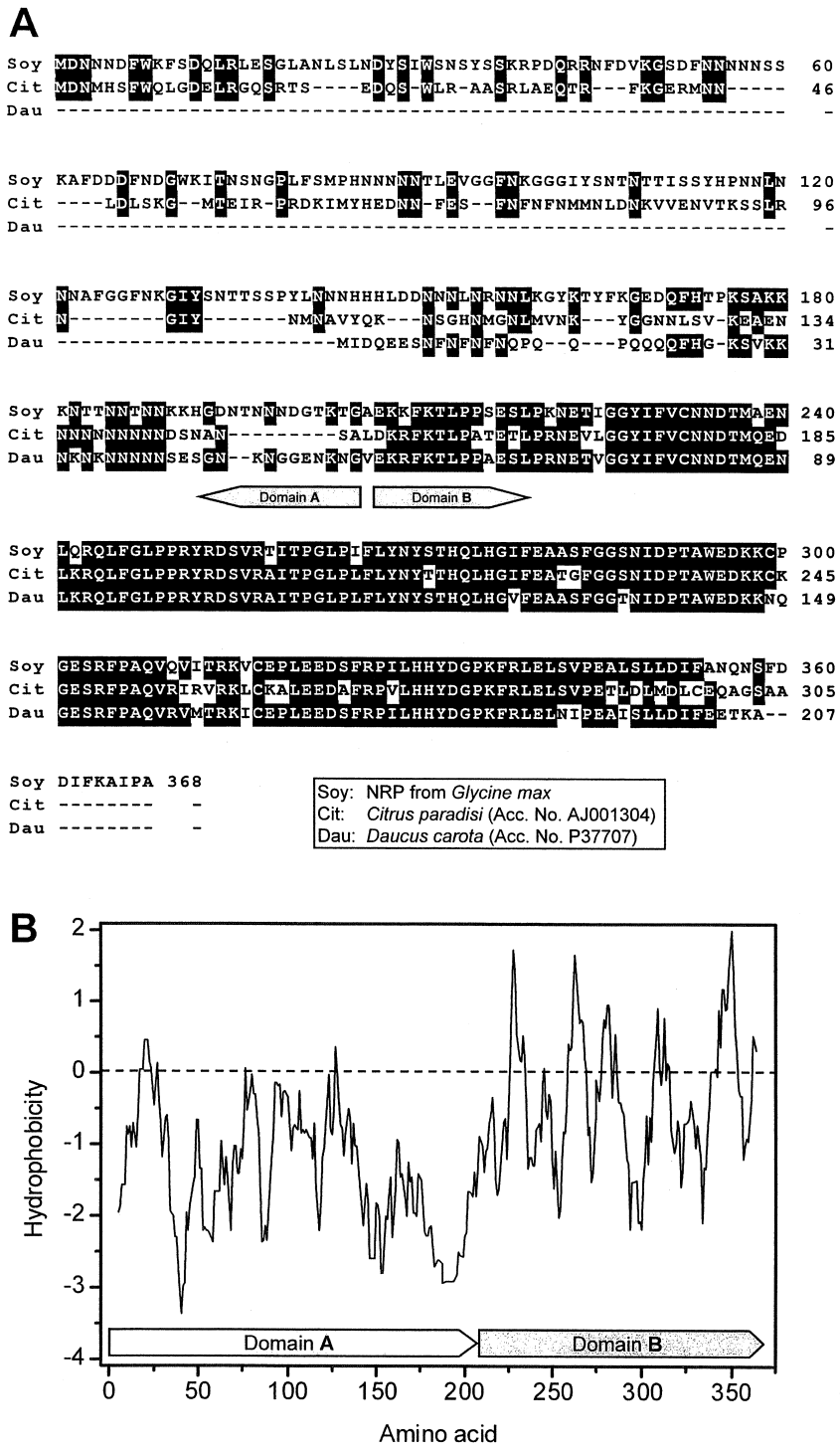

Figure 2. (A) Comparison of the amino acid sequence of the NRP-protein from soybean with proteins from *Citrus paradisi* and *Daucus carota*. Identical amino acids are shown inverted. The numbers at the right represent the positions of the last amino acid residue shown for each sequence. The carrot sequence is likely not full length. The arrows at pos. 210 (NRP) indicate the border between the divers N-terminal domain A and the highly conserved C-terminal domain B. (B) Hydropathy plot for the amino acid sequence of the *NRP* calculated according to the method of Kyte and Doolittle (1982) with a window size of 9 amino acids. Similar to the data from the multiple alignment in A, the hydrophobicity analysis suggests a separation of the protein into two domains (arrows).

*Xba*I fragment and two *Bam*HI fragments of about 6 kb and 3.2 kb in the Southern-blot (data not shown). *Bam*HI cleaves the *NRP* cDNA internally. These results suggest that the *NRP*-gene is a single copy gene without other closely related sequences in soybean. The coding region is interrupted by two introns of 293 and 89 bp length (Figure 1). Though the junction splice sites are within the consensus for introns found in *Arabidopsis* (Brown et al., 1996), two commonly used computer programs Grail-1.3 (http://compbio.ornl.gov/Grail-1.3-bin) and GenScan (http://genome.dkfz-heidelberg.de/cgi-bin/GENSCAN/genscan.cgi) for exon/intron structure prediction failed to predict the introns. However, the BMC-finder program (http://dot.imgen.bmc.tmc.edu:9331/gene-finder/gf.html) correctly forecasts both introns in the soybean *NRP*-gene. Surprisingly, both introns are located within the highly conserved B-domain and not between the junction of the A- and B-domain.

Expression of the NRP *gene*

As the results of the differential display analysis suggest that the *NRP*-gene is induced during infection of soybean cell cultures with Psg, we characterized this induction in more details. Challenging of Williams 82 cell cultures with Psg(*avrA*) results in a hypersensitive cell death whereas infection with Psg lacking the *avrA* gene does not (Levine et al., 1994). We inoculated soybean cell cultures with both the compatible and the incompatible Psg and investigated the changes in *NRP*-gene expression during infection by RNA blot analysis. The labelled cDNA-probe hybridized to an mRNA of about 1400 nt length, estimated by comparison to a RNA size standard (data not shown) and thus recognizes an mRNA of the predicted size. The *NRP*-gene although expressed in control cells, is clearly induced upon Psg(*avrA*) infection 8 h after addition of bacteria (Figure 3A). In the soybean cell culture addition of physiological concentrations of salicylic acid accelerates the cell death program (Shirasu et al., 1997; Ludwig and Tenhaken, 2000). However, SA (100 μM) alone did not induce the *NRP*-gene (see Figure 6B and data not shown). We therefore assessed whether the induction of the *NRP*-gene is correlated with the accelerated cell death. Addition of 50 μM salicylic acid during Psg(*avrA*) infection caused an earlier accumulation of the *NRP*-mRNA in comparison to cells treated only with the bacteria. Thus the accumulation of the *NRP*-mRNA reaches its maximum level by 3 h after challenge (Figure 3A) as compared to 8 h in the absence of SA. Noteworthy is the fact that the *NRP*-gene is specifically induced by incompatible Psg(*avrA*) causing programmed cell death. Inoculation however with compatible Psg neither results in cell death nor in an induction of the *NRP*-gene (Figure 3A).

The induction of the *NRP*-gene after Psg(*avrA*) inoculation is not limited to the soybean cell culture system but occurs also in leaves of the same soybean cultivar. Infiltration of soybean leaves with Psg(*avrA*) bacteria results in *NRP*-mRNA accumulation within 2 h after treatment and mRNA remains elevated for the next 24 h (Figure 3B). The first HR symptoms are macroscopically visible on the leaf 12–15 h after inoculation (data not shown). Thus, the *NRP*-gene induction clearly precedes the plant HR-response and is activated during the establishment of pcd. In contrast, no gene induction is observed in water-treated controls or Psg-infected leaves (Figure 3B). We then asked whether the *NRP*-gene is induced during the HR caused by other microbial pathogens and thus reflects a more general marker of pcd in soybean. We therefore investigated the *NRP*-gene expression after inoculation of soybean cell cultures and leaves with zoospores of the oomycete *Phytophthora sojae* (Ps). The *avr1k* avirulence gene of Ps (race 1) is recognized by the resistance gene *Rps1-k* in the soybean cultivar Williams 82 and therefore infection results in hypersensitive cell death (Bhat et al., 1992; Judelson, 1996). Treatment of soybean cell cultures as well as leaves with Ps (race 1) causes a strong accumulation of the *NRP*-mRNA within 2 h after inoculation (Figures 3C,D). At this time, most zoospores of Ps have germinated and the first begin to adhere to the soybean cells (data not shown). Under our conditions appressorium formation and penetration of fungal hyphae starts approximately 4 h after inoculation. The *NRP*-gene induction occurs before the penetration of the first fungal hyphae indicating a specific and very early signal exchange in this incompatible plant–pathogen interaction.

We also tested several elicitors of defence responses for their ability to induce the *NRP*-gene. Treatment of soybean cells with reduced glutathione (GSH), a pathogen mimic elicitor in soybean (Wingate et al., 1988), or the heavy metal $CuSO_4$ results in a moderate increase of the corresponding mRNA 3–5 h after treatment (Figure 4). H_2O_2 (1 mM) does not stimulate the *NRP*-gene expression. The induction of the *NRP*-gene and a H_2O_2- and elicitor-responsive glutathione-S-transferase gene (GST) (Levine et al., 1994) was further investigated. Increasing concentrations of H_2O_2 only slightly induce the *NRP*-gene but

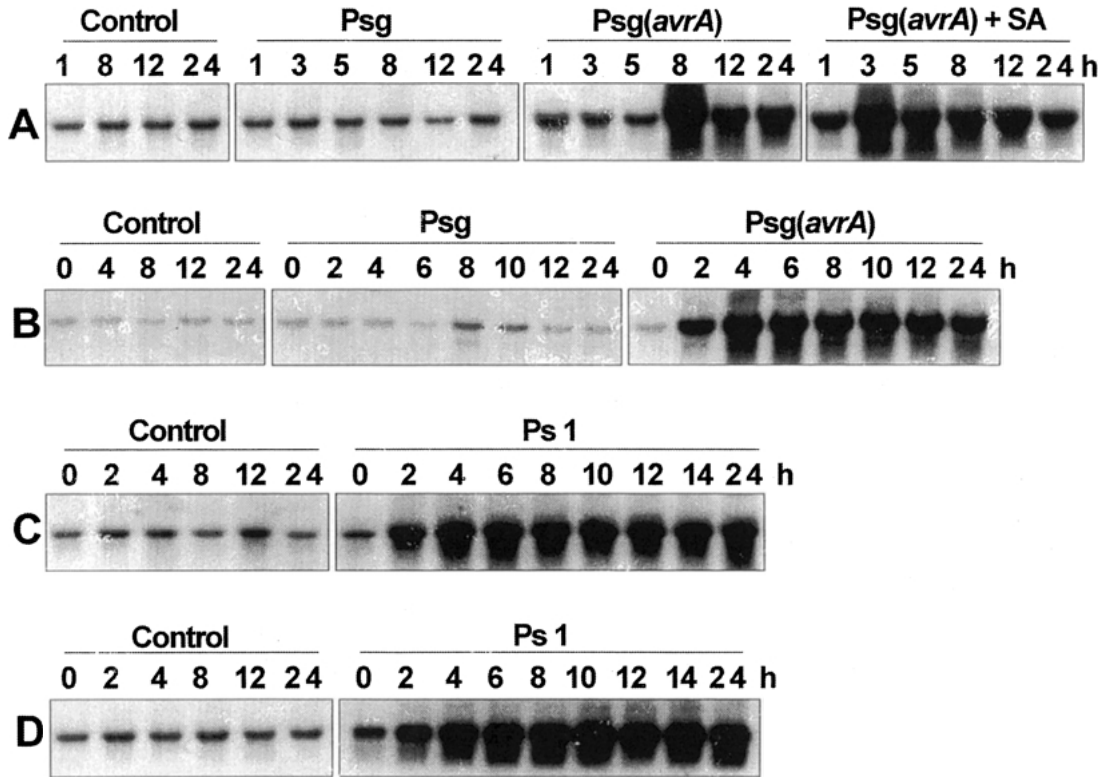

Figure 3. RNA blot analysis of the *NRP* gene expression during incompatible interactions of soybean with *Pseudomonas syringae* (Psg) (A,B) or *Phytophthora sojae* (Ps) zoospores (C,D). Soybean cell cultures (A) or leaves from soybean plants (B) were inoculated with 3×10^7 cfu ml^{-1} of incompatible Psg(*avrA*) bacteria to induce a HR. In the cell culture SA (50 μM) accelerates the cell death program as well as the *NRP* induction (Psg(*avrA*) + SA). Compatible Psg bacteria (Psg) lacking the *avrA* gene did not cause any induction of the *NRP* compared to the untreated control. The *NRP* was also induced by inoculation of soybean cell cultures (C) or soybean leaves (D) with 10^5 zoospores ml^{-1} of *Phytophthora sojae* race 1 (Ps1). RNA was extracted at indicated time points (hours) and assayed for accumulation of the *NRP*-mRNA by RNA blot analysis.

strongly induce the GST-gene at 2 mM H_2O_2. Addition of higher concentrations (0.2–0.5 mM) of glutathione to soybean cell cultures clearly induce the *NRP*-gene (Figure 5) whereas a cell wall elicitor preparation from *Phytophthora sojae* cause only a weak induction of the gene. Using the same conditions the GST-gene is strongly induced by glutathione and to a lesser extent by the glucan elicitor (Figure 5).

Neither SA (100 μM), nor the synthetic inducers of systemic acquired resistance, 2,6-dichloro-isonicotinic acid (100 μM) or benzothiadiazole (100 μM, Bion®) did induce the NRP-gene expression (data not shown). To confirm that the cultured soybean cells respond to SA-treatment (50 μM) we have probed the RNA blot in Figure 6A with a PR-gene from bean (*PvPR2*) which belongs to a highly conserved class of proteins (Walter et al., 1990). SA as expected transiently induces the *PR*-gene. In contrast, the *NRP*-gene is not induced by SA as shown in Figure 6B.

Interestingly, cycloheximide (1 μg ml^{-1}) caused a rapid induction of the *NRP*-gene expression comparable to the inoculation of soybean cells with HR-causing bacteria. Though cycloheximide is a potent inhibitor of protein biosynthesis, it induces apoptosis in animal cells (Wertz and Hanley, 1996) and causes cell death in cultured soybean cells (data not shown). Therefore, the specific induction of the *NRP*-gene during programmed cell death is consistent with the data obtained by cycloheximide treatment.

Induction of the NRP-protein

In addition to *NRP*-gene expression we also investigated the accumulation of the corresponding protein

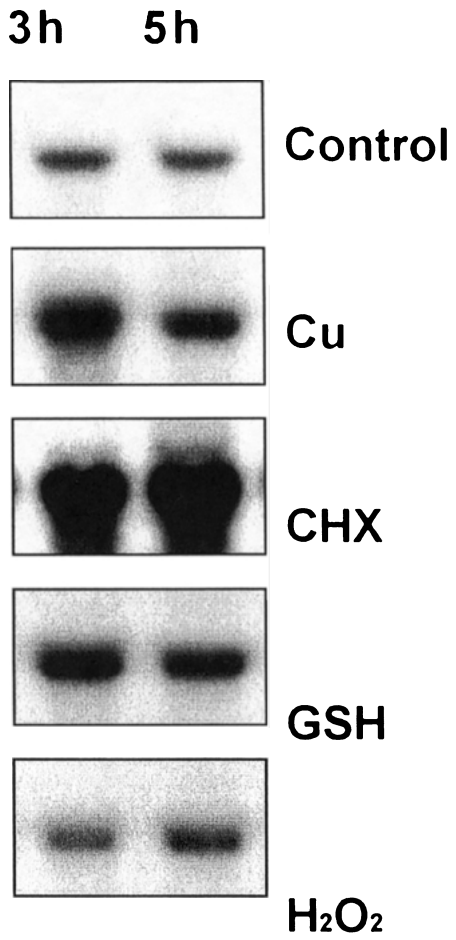

Figure 4. Effect of various stress inducers on the *NRP*-gene expression. Soybean cell cultures were treated with 100 μM $CuSO_4$ (Cu), 0.5 μg ml^{-1} cycloheximide (CHX), 200 μM reduced glutathione (GSH) or 1 mM H_2O_2. All compounds except H_2O_2 cause an accumulation of the *NRP*-mRNA 3 to 5 h after treatment in comparison to the untreated control. A gene induction comparable to the HR-response (see Figure 3A) was only observed with cycloheximide.

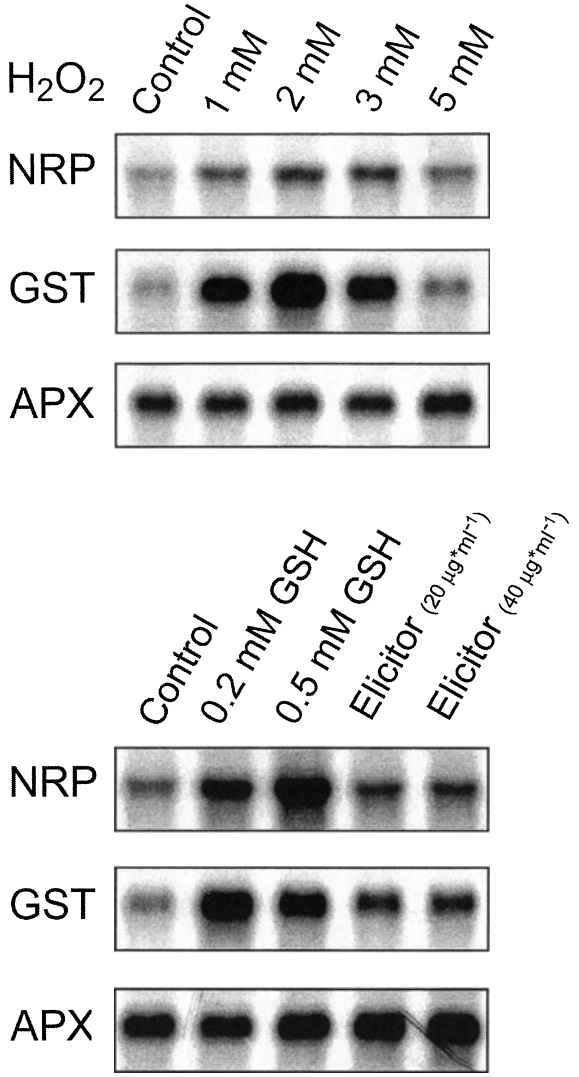

Figure 5. The NRP-gene and a GST gene respond differently to H_2O_2 and elicitors. Soybean cell cultures were treated with H_2O_2, glutathione or a glucan elicitor from *P. sojae* at the indicated concentrations. RNA was isolated from cells collected 5 h after treatment. Three identical RNA-blots were prepared and hybridized with the *NRP*-gene, a GST gene, or a gene coding for cytosolic ascorbate peroxidase (APX), respectively. The APX-gene serves as a loading control and is expressed at constant levels in soybean cell cultures (our unpublished observations).

in soybean after Psg(*avrA*) infection. Since the function of NRP is unknown, it was necessary to obtain a specific antiserum against the protein. The soybean NRP was expressed as a 6×His-tagged fusion protein in *E. coli* and used to immunize rabbits. In protein blots the obtained antibody specifically binds to a 42 kDa protein that accumulates in cells undergoing pcd (Figure 6D). The enhanced accumulation of NRP is obvious 8 h after inoculation with Psg(*avrA*). The NRP-protein accumulates in detectably larger amounts after the peak of mRNA accumulation (Figure 6C). The lack of synchrony between mRNA accumulation (2–3 h after inoculation) compared to the late appearance of the NRP-protein (10–12 h after inoculation) points to a long lag-phase for protein biosynthesis in pathogen treated soybean cells. This time lag between mRNA and protein accumulation

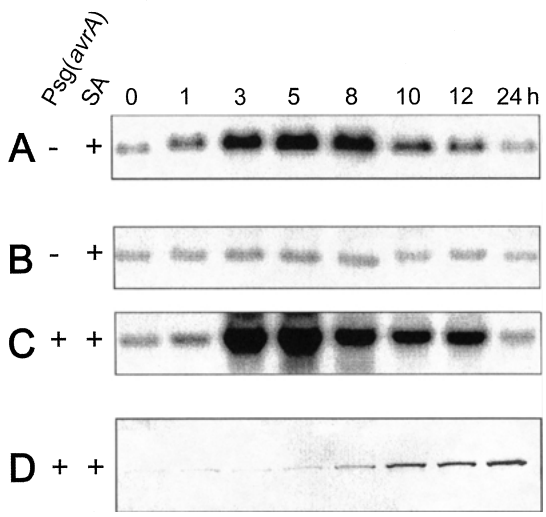

Figure 6. Gene induction of the NRP-mRNA in comparison to the NRP-protein during the HR caused by Psg(*avrA*) infection. Soybean cell cultures were treated with 50 μM SA as controls (A,B) or inoculated with 3×10^7 cfu ml^{-1} Psg(*avrA*) (C,D) in the presence of SA to induce an accelerated HR. At indicated timepoints (hours) cells were harvested and used either for protein extraction or for mRNA extraction. (A) The RNA blot was hybridized with a SA-responsive *PR*-gene (*PvPR2*, Walter et al. 1990) to demonstrate that the cells respond to SA-treatment. (B,C) The RNA blots were hybridized with the ^{32}P-labeled *NRP* cDNA probe. The *NRP*-gene expression is not induced by SA (B) but in the HR (C) caused by inoculation of the cell culture with Psg(*avrA*). (D) Proteins (10 μg per lane) were subjected to protein blot analysis with subsequent incubation with a polyclonal antibody (1 : 1000 dilution) raised against the NRP.

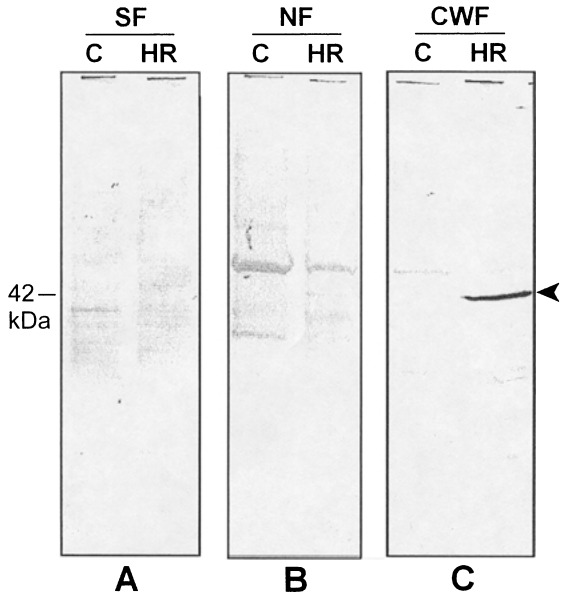

Figure 7. Immunolocalization of the NRP in the soybean cell wall. Soybean cell cultures were left untreated as control (C) or inoculated with 3×10^7 cfu ml^{-1} Psg(*avrA*) in the presence of 50 μM SA (HR) for 24 h. Cells were used to isolate soluble proteins (SF, panel A), proteins from the nuclei (NF, panel B) or from the cell walls (CWF, panel C). Each protein fraction was analysed on protein blots by detection with the polyclonal NRP antibody. Ovalbumin (43 kDa) was used as a standard. The detected NRP is marked by an arrow head.

results from a transient block of protein biosynthesis in pathogen inoculated cells caused by the transient repression of the mRNA for a catalytic ribosomal protein (rpL2) (Ludwig and Tenhaken, 2001).

Localization of the NRP

The protein blot shown in Figure 6D represents the SDS-extractable proteins of the soybean cell culture. Surprisingly, the polyclonal antibody did not react with a protein band at 42 kDa in soluble protein extracts (data not shown), suggesting that NRP is tightly bound to a cellular insoluble structure. We therefore assessed whether the NRP is bound to cell structures such as the cell wall or is located within the nucleus. We isolated cell wall proteins as well as proteins from the nucleus and used them for protein blot studies. As the NRP signal is very weak in control cells (see Figure 6, time point 0 h) we compared untreated controls with Psg(*avrA*) infected cells in which NRP is readily visible (Figure 6). The NRP-antibody clearly detects a reactive band in the cell wall fraction (Figure 7), but not in the cytosolic or the nuclear fraction. The detected protein band has the predicted molecular mass of approximately 42 kDa and is only visible in cell extracts undergoing pcd as expected. Duplicate membranes were probed with the lectin concanavalin A to test a possible glycosylation interaction between NRP and the lectin. However, none of the signals corresponded to the predicted molecular weight of 42 kDa and was specific for the HR-sample (data not shown).

Discussion

A number of plant defense genes have been shown to be activated rapidly during plant–pathogen interactions and often preferentially and earlier during incompatible interactions (Kombrink and Somssich, 1995). Using the method of mRNA differential display, a new

gene was isolated from soybean which was rapidly induced during HR and which encoded a cell wall located protein. The *NRP* gene shows a moderate constitutive expression in unstressed plants in every tissue investigated, independent of the tissue age (Figure 3 and data not shown). During an incompatible interaction of soybean cell cultures as well as in plants inoculated with Psg(*avrA*) we demonstrated a rapid induction of the *NRP* gene expression. This induction occurred in soybean in response to two HR-inducing pathogens namely *Pseudomonas syringae* pv. *glycinea* carrying the *avrA* avirulence gene and *Phytophthora sojae* race 1 (Figure 3). Salicylic acid has been shown to accelerate the cell death programme in soybean cell cultures infected with Psg(*avrA*) (Shirasu et al., 1997; Ludwig and Tenhaken 2000). The induction of the *NRP* occurred about 5 h earlier in SA treated cells and thus correlated with the accelerated HR. In addition, inoculation of soybean with isogenic compatible Psg did not result in cell death (Levine et al., 1994) nor the accumulation of the *NRP*-mRNA (Figures 3A,B). H_2O_2 from the oxidative burst has been suggested to be a key regulator of HR cell death (Alvarez et al., 1998; Levine et al., 1994). But gene expression of *NRP* was not affected by direct application of a 1 mM H_2O_2 pulse (Figure 4) or H_2O_2 constantly produced over a several hour time period by the glucose/glucose oxidase system (data not shown). However, in a dose-response experiment for H_2O_2-induced gene expression a small induction of the *NRP*-gene occurred after a 2–3 mM H_2O_2 pulse. These concentrations cause cell death in soybean cell cultures some hours later (Levine et al., 1994). Similar data for cell death were obtained for the experiment shown in Figure 5. Recently, it has been shown that the production of ROS is not sufficient and may not even be necessary for the establishment of the HR (Dorey et al., 1999; Glazener et al., 1996; Yano et al., 1999). It is intriguing to see that the *NRP*-gene is strongly induced by a pathogen triggered cell death programme but is not responsive to cell death caused by excessive H_2O_2. This remains to be clarified in future studies.

The results in this paper strongly suggest that the induction of the *NRP* is closely linked to the genetically defined pcd, induced by incompatible pathogens. Marker genes for pcd in plants, which do not respond to compatible pathogens, are very rare. The *Arabidopsis* Eli-3 gene is induced earlier and for a longer period of time in incompatible interactions with *Pseudomonas*. However, compatible bacteria still induce the Eli-3-mRNA to comparable amounts at later time points after inoculation (Kiedrowski et al., 1992). A similar observation was made by Czernic et al. (1996) for the tobacco genes *hsr201* and *hsr515*. Again incompatible bacteria preferentially induce both genes but similar expression levels were obtained by inoculation with compatible bacteria some hours later. The Eli-3 gene from *Arabidopsis* (aromatic alcohol dehydrogenase, Somssich et al., 1996) as well as the *hsr515* gene from tobacco (cytochrome-P-450 monooxygenase, Czernic et al., 1996) are likely involved in the phenylpropanoid metabolism and thus may reflect a part of a more general plant defence strategy, e.g. providing substrates for oxidative cross-linking of phenolic compounds to reinforce the cell wall (Brisson et al., 1994). Another tobacco gene (*hsr203J*) has been shown to be specifically induced by incompatible pathogens and at early time points (Marco et al., 1990). A much lower induction by compatible strains occurred only at later time points 12–24 h after inoculation (Pontier et al., 1994). In summary, plant genes whose induction is absolutely specific for pcd in plants have not been identified yet but the tobacco *hsr203J* gene as well as the soybean *NRP*-gene most closely match these criteria. Searches in databases revealed homology to proteins from *Citrus paradisi* and *Daucus carota* (Figure 2), but both without any known function. The so-called B2-protein from carrot was induced during the somatic embryogenesis of cell cultures after the withdrawal of the phytohormone auxin (Schrader et al., 1997). Thus the *NRP* homologue from carrot seems to have a function in cell differentiation. It has been suggested that cell cycle, cell differentiation and cell death are closely linked and that the withdrawal of extracellular signals as well as their appearence can lead to apoptosis instead of cell differentiation or proliferation (Logeman et al., 1995; Williams and Smith, 1993). Differentiation e.g. leading to tracheary elements involves pcd (Groover et al., 1997) consistent with the proposed specific induction of the *NRP*-gene in pcd. Therefore one possible role for the *NRP* is a participation in the signalling pathway leading to HR.

An alternative function of the NRP protein might be a role in modifying the plant cell wall. This is suggested by the localization of the protein in the cell wall (Figure 7). Local reinforcement of the plant cell wall by cross-linking hydroxy-prolin-rich glycoproteins or prolin-rich proteins have been demonstrated in several systems (Brisson et al., 1994; Otte and Barz, 1996). Though currently there is no direct proof that the NRP-protein functions as a cell wall structural protein, support for such a model comes from transgenic *Arabidopsis* plants expressing the soybean *NRP*-gene

under the control of the CaMV35S-promoter. Several independent lines showed a moderate to high expression of the gene as analysed by RNA blot hybridization. However, we failed to detect the NRP in soluble protein extracts from these CaMV35S :: *NRP* plants (data not shown) using the same procedure which successfully detects the protein in soybean extracts (Figure 7).

The NRP has a highly hydrophilic nature and completely lacks transmembrane domains. One striking feature of the protein is its unusually high content in the amino acid asparagine. The A-domain has one of the highest N-amounts (24%) of any protein known in the databases (Swiss-Prot). The average N-amount in proteins is 4.44% based on 80 000 sequences in the current release of Swiss-Prot 38 (http://www.expasy.ch/cgi-bin/list?relnotes.txt). Asparagine and glutamine are known to be rapidly deaminated dependent on the pH, the ionic strength and the temperature of the surrounding medium (Robinson and Robinson, 1991). Since deamination involves a change in charge, the protein would become more unstable due to possible disruptions of its molecular structure. Thus deamination of proteins is discussed to serve as a molecular clock for biological processes by shortening the life time of a protein. The Western blot (Figure 7) however, suggests that NRP is relatively stable. Though the mRNA amount for NRP returned already to control levels 10 to 12 h after bacterial inoculation the protein signal is strongest at the 24 h time point.

Protein motifs with repeats of three or more N-residues are also found in yeast (*Saccharomyces cerevisiae*) proteins. They include zinc finger containing transcripional activators (O'Hara et al., 1988; Peterson and Herskowitz, 1992; Pearson et al., unpublished) and a superfamily of glutathion-S-transferases (Coschigano and Magasanik, 1991). In *Plasmodium reichenowi* a surface antigen was found to be very N-rich, containing 62 repeats of N-A-N-P (Lal and Goldman, 1991). The biological role for the N-richness of these proteins remains unclear.

The location in the plant cell wall, the first space for a pathogen to pass, the unusual amino acid composition and the possibility to be post-translationally modified suggests that NRP is an important protein.

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