Elicitor-Induced Defence Reactions in Cell Suspension Cultures of Soybean Cultivars

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Oxidative Burst, PR-Proteins, Phenylpropanoid Metabolism

Suspension cultured soybean (*Glycine max* [L.] Merr.) cells of four cultivars (Wilis, Lumut, Kalmit, Doko RC) were compared for their response to different fungal and bacterial elicitors. Cells were treated either with crude cell wall extracts of the fungal pathogens *Phytophthora sojae* (Pmg-elicitor) and *Rhizoctonia solani* (Riso-elicitor) or with two isolates of the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* (Psg01/02) and a broad spectrum of antimicrobial defence reactions was measured. Cells of all four cultivars showed the same elicitor-induced rapid (H_2O_2 accumulation, alkalinization of the culture medium, peroxidative cross-linking of cell wall proteins) and slow (activation of phenylpropanoid metabolism, accumulation of phenolic compounds, induction of PR-proteins) defence responses. However, the reactivity of the cultivars was not identical in terms of time courses and intensities. Furthermore, the ability of the various elicitors to induce defence responses varied markedly. These differences indicate that (1) cells of the same species but of different cultivars are equipped with the same array of perception systems to recognise various stimuli but (2) the sensitivity of these perception systems or later steps in the signal transduction seem to be stimulated to a different extent in the analysed cultivars.

Introduction

Plants possess a broad spectrum of constitutive and inducible defence reactions against pathogen attack. Induced defence reactions include rapid reactions independent of a *de novo* protein synthesis such as early ion fluxes across the plasma membrane (Bach *et al.,* 1993) and the very rapid transient accumulation of reactive oxygen species (Wojtaszek, 1997) which is often accompanied by reinforcement of cell walls through cross-linking of phenolic compounds (Nicholson and Hammerschmidt, 1992) and of structural cell wall proteins (Bradley *et al.,* 1992; Otte and Barz, 1996). Synthesis of PR-proteins (Van Loon *et al.,* 1994), activation of phenylpropanoid metabolism (Hahlbrock and Scheel, 1989) and accumulation of phytoalexins (Barz, 1997) are prominent slow defence reactions depending on transcription and translation (Kombrink and Somssich, 1995).

A prerequisite for the induction of defence reactions is the recognition of the pathogen by the plant. Molecules released or generated during pathogen attack, so-called elicitors, are thought to act as signals that are perceived by plant cells and activate defence reactions. Among these elicitors are fragments of the fungal cell wall such as glucans, chitins, glycopeptides, glycoproteins and sterols which are supposed to be recognised by receptors on the plant plasma membrane. For some purified elicitors strong evidence for high affinity binding proteins at the plasma membrane has been presented (Nürnberger, 1999). In contrast to fungal pathogens bacterial pathogens, at least in several incompatible interactions of *Pseudomonas syringae* and *Xanthomonas*, seem to be recognised in the cytosol of plant cells. According to a current hypothesis such bacterial elicitors are transferred into the plant cell by a bacterial tunnel-forming

Abbreviations: Aro, crude cell wall extract of *Athelia rolfsii*; cv, cultivar; DPI, diphenyleneiodonium; LMCL, luminol-dependent chemiluminescence; MES, 2-[N-morpholino]ethanesulfonic acid; PAL, phenylalanine ammonia lyase; Pmg, crude cell wall extract of *Phytophthora sojae*; Psg01/02, *Pseudomonas syringae* pv. *glycinea* isolate 01/02; Riso, crude cell wall extract of *Rhizoctonia solani*; RLU, relative light units; hrp, hypersensitive reaction and pathogenicity.

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system encoded by *hrp*-genes (Bonas and van den Ackerveken, 1997).

Plant defence reactions were measured in plant/pathogen systems as well as in the simplified model system of cell suspension culture/elicitor. Most analyses of defence reactions were performed on a few cultivars and elicitors only. Even for the well-examined plant species soybean (*Glycine max* [L.] Merr.) only three cultivars (i. e. Harosoy, Williams, Kent) have extensively been studied (Ebel *et al.*, 1984; Graham and Graham, 1991; Levine *et al.*, 1994). Furthermore most publications deal with a few defence reactions. Thus, little knowledge has been gained about the reactivity of different cultivars towards a set of various stimuli. Therefore in parallel experiments suspension cultured soybean cells of four different cultivars (Wilis, Lumut, Kalmit, Doko RC) were treated with crude cell wall extracts of the fungal pathogens *Phytophthora sojae* and *Rhizoctonia solani* and with two isolates of the bacterial pathogen *Pseudomonas syringae* pv. *glycinea*. As antimicrobial defence reactions the transient accumulation of H_2O_2 and the adherent alkalinization of the culture medium, the peroxidative cross-linking of cell wall proteins, the activation of phenylpropanoid metabolism and its branched pathways as well as the induction of PR-proteins were analysed. All cultivars showed the same range of defence reactions but time courses and intensities differed significantly between the cultivars and the elicitors.

Materials and Methods

Chemicals and antisera

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), laminarin, diphenyleneiodonium (DPI) and 4-hydroxybenzoic acid hydrazide were purchased from Sigma (Munich, Germany), carboxymethylated-chitin-RBV from Loewe Chemicals (Sauerlach, Germany), hydrogen peroxide (30% solution) from Fluka (Buchs, Switzerland). The anti-p33-serum, a polyclonal antiserum generated by immunisation of rabbits with p33 PRP from soybean cells (Bradley *et al.*, 1992), was provided by Dr. C. J. Lamb (La Jolla, Calif., USA). All other chemicals were of the highest purity commercially available.

Plant cell cultures

Cell suspension cultures of *Glycine max* (L.) Merr. cv Wilis, Lumut, Kalmit and Doko RC had been previously established in our laboratory by I. Rath and were grown in the dark in modified MS-medium (Murashige and Skoog, 1963; Gamborg *et al.*, 1968). Cell cultures were transferred (2–3 g f.w./40 ml) to fresh medium every 7 d (growth yield approximately 7 g f.w per flask). For the determination of phenylalanine ammonia lyase activity a defined inoculum of cells (1.5–2.0 g f. w./20 ml medium, depending on the cultivar) was allowed to grow for 5 d before use. For the measurements of H_2O_2, of extracellular pH changes as well as for the determination of PR-proteins and of phenolic compounds cells were separated from the medium 5 d after subculture, washed and transferred to assay medium (4% MS, 3 % sucrose, 10 mM MES (2-[N-morpholino]ethanesulfonic acid), pH 5.7), pH-medium (4% MS, 3 % sucrose, pH 6.0) or MS-medium, respectively. Before elicitation cells were equilibrated in the respective medium for 2 ½ h on a rotary shaker (120 rpm) in the light.

Elicitors

From the fungal soybean pathogens *Phytophthora sojae*, *Rhizoctonia solani* and *Athelia rolfsii* heat-soluble cell wall extracts were prepared according to Ayers *et al.* (1976). *P. sojae* race 1 was a kind gift of Prof. Dr. J. Ebel, LMU, Munich, *R. solani* (Nr. 277.81 ex soja) and *A. rolfsii* (Nr. 140.82 AG-1 ex soja) were purchased from the Instituut voor Schimmelculturen, Baarn, The Netherlands.

The freeze-dried wall material was diluted in bidistilled water and autoclaved. If not stated otherwise the final concentration used in the experiments was 100 µg per ml cell suspension.

Two isolates of *Pseudomonas syringae* pv. *glycinea* were collected in Indonesia with the help of Dr. M. Machmud, Research Institute for Food Crop Biotechnology, Bogor, Indonesia, and kindly characterised by Dr. I. Reiff, Münster University. The bacterial cells were treated and prepared for elicitation as described by Orlandi *et al.* (1992). The optical density at 500 nm of the bacterial suspensions used in the experiments was 0.2 if not stated otherwise.

Extracellular pH changes and H_2O_2-production

Extracellular pH changes were measured continuously in the cell suspension cultures after elicitation using an electrode. The H_2O_2 formed in soybean cell suspensions was determined by luminol-dependent chemiluminescence as described by Otte and Barz (1996) except that the measurements were carried out under light. A H_2O_2 standard curve allowed the calculation of relative light units in µM of H_2O_2.

Detection of cell wall proteins

Cell wall proteins were extracted from elicited cells 5 d after subculture. Protein extracts, sodium dodecylsulfate polyacrylamide gel electrophoresis and Western Blot analysis were performed as described earlier (Otte and Barz, 1996).

Preparation of enzyme extracts

Treatment of cells and medium for the preparation of enzyme extracts was identical for chitinase, β-1,3-glucanase and phenylalanine ammonia lyase (PAL) assays, respectively, except for the buffers. For the two PR-proteins the buffer contained 100 mM potassium acetate, pH 5.2, for the determination of PAL-activity 100 mM potassium phosphate, pH 8.0 and 1.4 mM β-mercaptoethanol. Cells were separated from the medium by filtration. Medium and cells were shock frozen in liquid nitrogen and stored at −20 °C until further use.

Crude cell extracts were obtained by homogenizing × g cells with ½ × ml buffer, polyvinyl polypyrrolidone ($^{x}/_{100}$ g) and sand in a mortar. After centrifugation for 15 min at 26.000×*g* the supernatant was desalted on Sephadex G-25 (PD 10, Pharmacia, Freiburg, Germany). Except for the measurements of PAL-activity which were performed immediately after the isolation the enzyme solution was stored at −20 °C. The protein content of enzyme extracts was determined by the method of Bradford (Bradford, 1976; Read and Northcote, 1981) with bovine serum albumin as a standard.

The medium was desalted on Sephadex G-25 (s. above) and also stored at −20 °C until use.

Chitinase and β-1,3-glucanase activities

Chitinase activity was determined according to Wirth and Wolf (1990). Determination of β-1,3-glucanase activity was performed as described by Kombrink and Hahlbrock (1986) except that 4-hydroxybenzoic acid hydrazide was used as a colour reagent to determine the amount of released reducing sugar moieties. As substrate reduced laminarin was used prepared after Boller (1992).

Phenylalanine ammonia lyase activity

Phenylalanine ammonia lyase activity was determined according to Moerschbacher (1988).

600 µl boric acid buffer (0.1 M, pH 8.8) were incubated for 1 min at 40 °C with 300 µl of enzyme extract. The reaction was started with 100 µl of L-phenylalanine solution (60 mM in boric acid buffer, s. above). The extinction was measured for 20 min using an UVIKON 933 Double Beam UV/VIS Spectrophotometer (Kontron Instruments, Neufahrn, Germany) at 290 nm against 10 mM phenylalanine in buffer (s. above). The enzyme activity was calculated by linear regression with the help of a cinnamic acid calibration curve.

Phenolic compounds

For the determination of soluble phenolic compounds according to Swain and Hills (1959) cells were transferred to fresh medium (0.25–0.3 g f.w./6 ml medium) 5 d after subculture in 6-well plates and equilibrated for 2 ½ h before treatment. Directly after elicitation as well as 4 d later 1 ml aliquots of the cell suspension cultures were harvested, centrifuged (16.060 g) and 150 µl of the supernatant were diluted with 800 µl of bidistilled water and incubated with 50 µl Folin-Ciocalteus phenolic reagent. After 3 min 100 µl of a saturated sodium bicarbonate solution (35 mg/100 ml A. bidest.) was added. 60 min later the samples were measured photometrically at 700 nm against water instead of supernatant. For quantitative determinations a calibration curve set up with phloroglucinol (9.5 to 95 nmol) was used.

Results

As part of a comprehensive research program on soybean cultivars (i.e. Wilis, Lumut, Kalmit, Doko RC) well growing cell suspension cultures had also been established. In order to compare these soybean cultivars with regard to the expression of defence reactions we treated the cell sus-

pension cultures of the four cultivars with crude cell wall extracts of *Rhizoctonia solani* (Riso-elicitor) and *Phytophthora sojae* (Pmg-elicitor) as well as with living bacterial cells of two isolates of *Pseudomonas syringae* (Psg01/02).

Oxidative burst

One of the earliest elicitor-induced cell responses is a rapid and transient accumulation of reactive oxygen species (ROS), the so-called oxidative burst. One representative species, namely H_2O_2, was determined by luminol-dependent chemiluminescence. Cells were transferred on day five after subculture to assay medium and then adapted for 2 ½ h before addition of elicitors because cell transfer to a medium with a different osmolarity is a stress factor in itself which induces an oxidative burst. The resulting value in chemiluminescence (20–30 RLU * 10^3 * 10 s^{-1}) is low compared to elicitation (300–4.000 RLU * 10^3 * 10 s^{-1} after addition of Riso-elicitor) but to clearly determine elicitor effects a preincubation of 2 ½ h was necessary. After this time period chemiluminescence of the untreated cell suspension cultures is stable and low (data not shown).

As an example a typical kinetic of the elicitor-induced H_2O_2 accumulation in soybean cell suspension cultures is shown in Fig. 1. It demonstrates

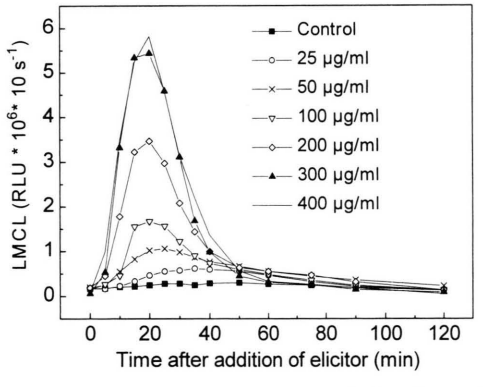

Fig. 1. Elicitor-induced H_2O_2 accumulation in soybean cell suspension culture. Cell suspension cultures of cultivar Wilis (1 g f. w. of cells/20 ml medium) were treated with different concentrations of crude cell wall extracts of *Rhizoctonia solani*. As control an equal volume of sterile bidistilled water was added. H_2O_2 was measured by luminol-dependent chemiluminescence (LMCL) and values are given in relative light units (RLU). Data is a representative measurement of four independent experiments.

a very rapid increase in H_2O_2 starting five min after addition of the fungal elicitor. After 20–40 min maximal values were reached and the reaction was completed around 70–90 min after elicitation. The extent of the elicitor-induced oxidative burst depended on the elicitor concentration, but the concentration did not influence the general time course.

Comparative studies of two cultivars (Wilis, Lumut) showed differences between the maximal values and time courses of H_2O_2 accumulation. In suspension cultures of cv Wilis the bacterial elicitor Psg02 induced a sharp transient increase of H_2O_2 whereas cells of cv Lumut showed a prolonged burst for more than one hour (Fig. 2). Furthermore, the cells of the two soybean cultivars greatly differed in their reactivity towards Psg01 and Psg02. With the bacterial elicitors reactivity of cells from cv Lumut was equally strong for both isolates of *Pseudomonas syringae*, unlike cells from cv Wilis which produced much more H_2O_2 after addition of isolate 02 than isolate 01 (Fig. 3). Heat-killed as well as living bacterial cells led to the release of H_2O_2.

When comparing the maximal amounts of H_2O_2 induced by the fungal elicitors the cell suspension cultures of three cultivars produced significantly less H_2O_2 after application of Pmg-elicitor than after addition of Riso-elicitor (students t-test: 10% significance for cv Kalmit; 2.5% for cv Wilis and Lumut). Only cells of cv Doko RC showed nearly equal values for both elicitors (Fig. 3). The elicitor-induced H_2O_2-maximum for cv Wilis was found to be considerably lower in comparison with the three other cultivars. Though some batches showed higher values.

These results indicate that the four cultivars possess a perception system for both the bacterial and the fungal elicitors used in this study but that the sensitivity of the cells varies depending on the elicitor and cultivar.

The extent of H_2O_2 production is dependent on elicitor concentration but even with high amounts of Pmg-elicitor (400 µg/ml) cells of cv Wilis and Lumut produced less H_2O_2 than after addition of 100 µg/ml Riso-elicitor (data not shown). Thus, it can be assumed that the two fungal elicitors contain different elicitor-active substances.

In several plant and animal systems desensitisation of signal perception after a second elicitor

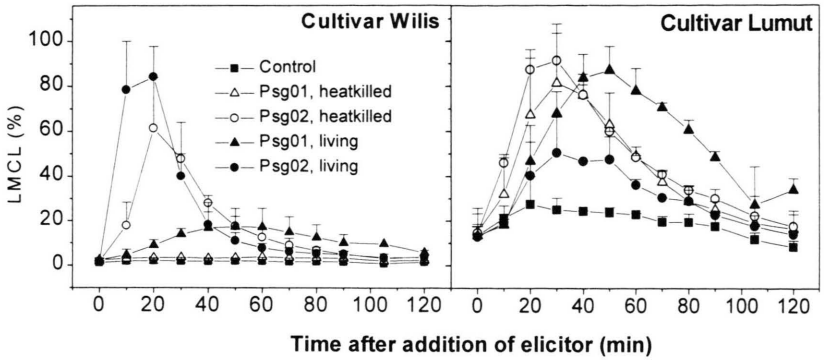

Fig. 2. Kinetics of H_2O_2-formation induced by bacterial elicitors in two soybean cultivars. On day 5 after subculture cells were transferred to assay medium, incubated for 2 ½ h and elicited with bacterial cell suspension (od_{500}=0.2, 50 µl/ml medium) of two *Pseudomonas* ssp. isolates (Psg01, 02). As control an equal amount of MES-buffer was added instead of elicitor. The results are means (± se) of three independent experiments. H_2O_2 was measured by luminol-dependent chemiluminescence (LMCL). The maximal H_2O_2-formation of each cell batch was defined as 100%. ■ control, Δ Psg01 (heat-killed), ○ Psg02 (heat-killed), ▲ Psg01 (living), ● Psg02 (living).

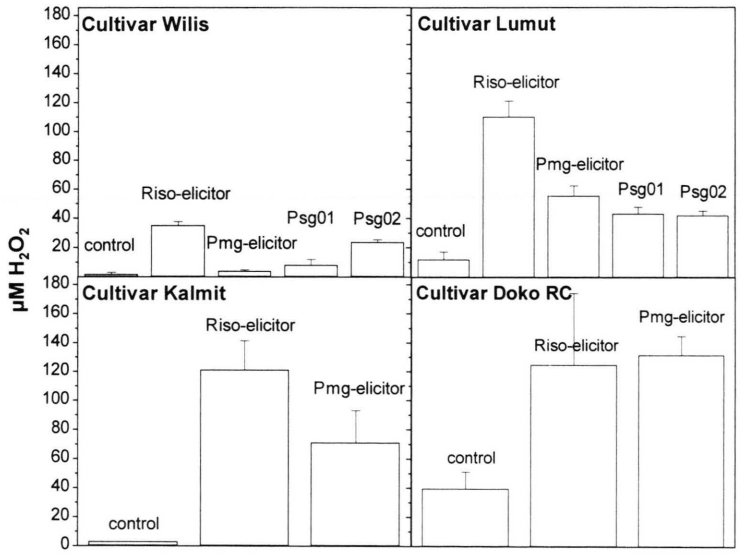

Fig. 3. Induction of H_2O_2 in cell suspension cultures of four soybean cultivars by biotic elicitors. H_2O_2 was measured as luminol-dependent chemiluminescence. The highest H_2O_2 accumulation reached after elicitation is shown. The fungal elicitors were crude cell wall extracts (100 µg/ml medium) of *Rhizoctonia solani* (Riso-elicitor) and *Phytophthora sojae* (Pmg-elicitor), respectively. The bacterial elicitors were two isolates of living cells of *Pseudomonas syringae* pv. *glycinea* (Psg01/02) (50 µl/ml medium, od_{500}= 0.1). As control an equal amount of sterile bidistilled water or MES-buffer (0.5 mM, pH 6.0) was added. Data are means (± se) of three independent experiments.

treatment has been described (Binet *et al.*, 1998). Results on the production of H_2O_2 after two successive treatments with the same (homologous) or a different (heterologous) elicitor are shown in Table I. After a first treatment of cells of cv Wilis and Lumut with Riso- or Pmg-elicitor a second application of the same or another elicitor induced a much lower value of H_2O_2. The cells were completely or partly desensitised. When Riso-elicitor was applied to cells of cv Wilis after a first treatment with Riso- or Pmg-elicitor the luminol-dependent chemiluminescence reached only 6.3 and 15.4%, respectively, of the values obtained by a single treatment with Riso-elicitor (Table I). A similar result was obtained by a 2-fold treatment with Pmg-elicitor. Application of Riso-elicitor and a subsequent treatment with Pmg-elicitor did not lead to a clear desensitisation as demonstrated by the high standard error. Using bacterial elicitor as first stimulus only a slight desensitisation was ob-

Table I. Repeated elicitation of soybean cell suspension cultures. Percentage of the elicitor-induced chemiluminescence stimulated by the second stimulus compared with the first. Cell suspension cultures of cv Lumut and Wilis were treated twice with the same or different elicitor. The maximal chemiluminescence induced by the second stimulus was referred to the values obtained by the first elicitation with this elicitor. Data are means (±se) of at least 4, for cv Lumut of at least 8 independent experiments. Riso-elicitor – cell extracts of *Rhizoctonia solani* (100 µg/ml), Pmg-elicitor – cell extracts of *Phytophthora sojae* (100 µg/ml), Psg02 – bacterial suspension of *Pseudomonas syringae* pv. *glycinea* isolate 02 (OD_{500}=0.2).

Cultivar Wilis	First elicitation	
Repeated elicitation with	Riso-elicitor Percentage of first elicitation [%]	Pmg-elicitor Percentage of first elicitation [%]
Riso-elicitor	6.3 ± 3.0	15.4 ± 4.8
Pmg-elicitor	93.0 ± 28.5	14.2 ± 4.7
Cultivar Lumut		
Repeated elicitation with	Riso-elicitor Percentage of first elicitation [%]	Bacteria (Psg02) Percentage of first elicitation [%]
Riso-elicitor	2.9 ± 1.6	75.0 ± 3.8
Bacteria (Psg02)	0	84.7 ± 7.8

served in cell suspension cultures of cv Lumut if bacterial suspension or Riso-elicitor were applied as second stimulus.

Alkalinization of the culture medium

For measurements of pH-changes induced in the culture medium cells were treated in the same way as for the determination of H_2O_2 except that an unbuffered medium (see Methods) was used. The fungal elicitors induced a transient pH-increase in the culture media of the four cultivars. The pH-values started to rise 5 min after elicitation, reached a maximum after 20–40 min and after approximately 70 min the initial pH-value was obtained again (Fig. 4). As already seen for the oxidative burst the reactivity of the cells of the soybean cultivars to the various elicitor preparations significantly differed. The differences in the intensities of the pH-response between the cultivars corresponded with the results obtained for the measurements of the oxidative burst. Elicitors causing a strong oxidative burst were also capable of inducing a greater transient pH-increase. Thus, the maximal pH-increase stimulated by Riso-elicitor in the cell suspension cultures of cultivars Wilis, Lumut and Doko RC was 0.5–0.6 pH-units. For cell suspension cultures of cv Kalmit the increase was only 0.2 pH-units whereas the effects caused by Pmg-elicitor were generally very small (Fig. 4).

Furthermore, except for the application of Psg02 to cell suspension cultures of cv Wilis bacterial elicitors did not induce a transient alkalinization in cell suspension cultures of cv Wilis and Lumut (data not shown).

In all cultivars the alkalinization was followed by a strong acidification of the medium. Control cells also showed an acidification of the culture medium, however, right after onset of the experiment. This reaction is probably the consequence of the uptake of ions from the culture medium catalysed by the activity of the plasma membrane H^+-ATPase. Especially ammonium nutrition leads to an extracellular increase of protons (Kirkby and Mengel, 1962).

The strong correlation between the elicitor-stimulated oxidative burst and the extracellular pH-changes indicated that there should be a causal relationship between the two reactions. However, further analyses with an inhibitor of the oxidative burst (diphenyleneiodonium, DPI) showed that inhibition of the oxidative burst was much stronger (65 % ± 9.02) than suppression of the alkalinization response (35 % ± 10.82).

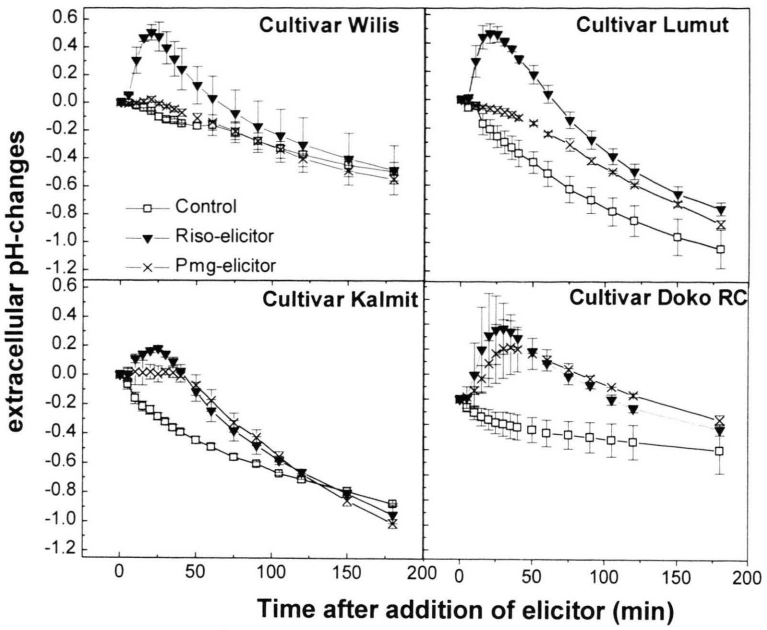

Fig. 4. Induction of extracellular alkalinization in cell suspension cultures of four soybean cultivars by two fungal elicitors. The elicitors are crude cell wall extracts (100 µg/ml medium) of *Rhizoctonia solani* (Riso elicitor) and *Phytophthora sojae* (Pmg-elicitor), respectively. As control an equal amount of sterile bidistilled water was added. Values are referred to the pH at t_0. Data are means (± se) of three independent experiments.

Oxidative cross-linking

Elicitation of soybean cells also resulted in peroxidative cross-linking of two cell wall proteins (p33, proline-rich protein; p100, a putative member of hydroxyproline-rich glycoprotein family) catalysed by peroxidases (Bradley *et al.*, 1992). Kinetics of this insolubilisation process differed between the cultivars. Thirty min after treatment with the fungal elicitors p33 and p100 could no longer be visualized in the extracts of cv Wilis, Lumut and Doko RC. In cv Kalmit the reaction was only completed after 90 min (data not shown).

Phenylpropanoid metabolism

The key enzyme of phenylpropanoid metabolism, phenylalanine ammonia lyase (PAL), was stimulated by Riso-Elicitor as well as by bacteria (Psg02) in cell suspension cultures of the soybean cultivars Wilis, Lumut and Kalmit, repectively. Time courses and total enzyme activities varied between the cultivars and elicitors (Fig. 5). PAL activity of cells from cv Wilis and Kalmit was much higher after addition of Riso-elicitor than after application of bacterial suspension. In contrast, cells of cv Lumut reacted nearly equally strong to both elicitors though the high standard error has to be considered. Thus, as already seen for the rapid defence reactions, time courses and total activities also varied for this slow defence response between cultivars and elicitors.

The activation of PAL in soybean often correlates with the production of isoflavonoid phytoalexins (Börner and Grisebach, 1982). In the three cell suspension cultures used for the determination of PAL activity neither Riso-elicitor nor bacterial suspension induced any increase in chalcone synthase activity, a specific enzyme of flavonoid metabolism, until 24 hours after elicitor treatment. Furthermore, accumulation of glyceollin phytoalexins was also not observed (data not shown).

However, the investigated fungal and bacterial elicitors tested induced an accumulation of soluble phenolics in the culture medium. There was a slightly stronger induction by the fungal elicitors than by the bacterial pathogens (Fig. 6). Relative reactivity against the different elicitors was the same for the three cultivars but the total increase in soluble phenolics when compared to water-treated control was higher in suspension cultures of cv Lumut (160% after addition of Riso-elicitor) than of cv Kalmit and Wilis (120 % each).

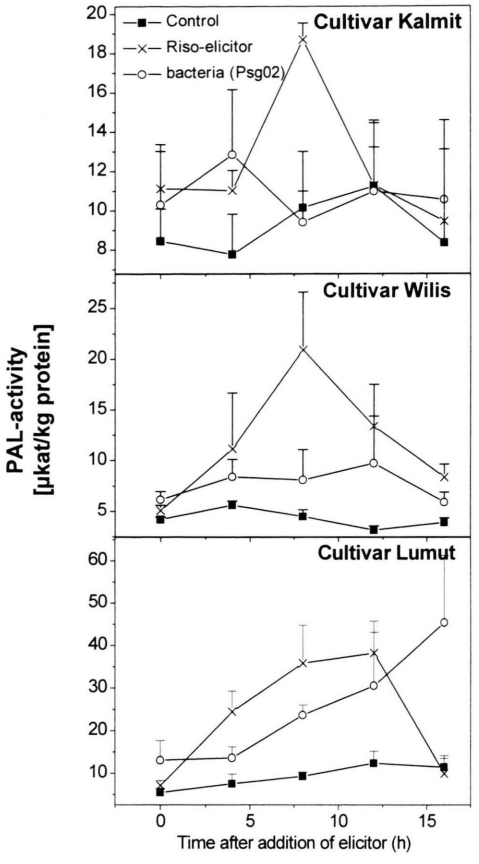

Fig. 5. Induction of phenylalanine ammonia lyase in cell suspension cultures of three soybean cultivars by fungal and bacterial elicitors. Riso-elicitor – crude cell wall extracts of *Rhizoctonia solani* (100 µg/ml medium), Psg02 – bacterial suspension of *Pseudomonas syringae* pv. *glycinea* isolate 02 (od_{500}=0.2, 50 µl/ml medium). As control an equal amount of sterile MES-buffer (0.5 mM, pH 6.0) was added. Data are means (± se) of two independent experiments.

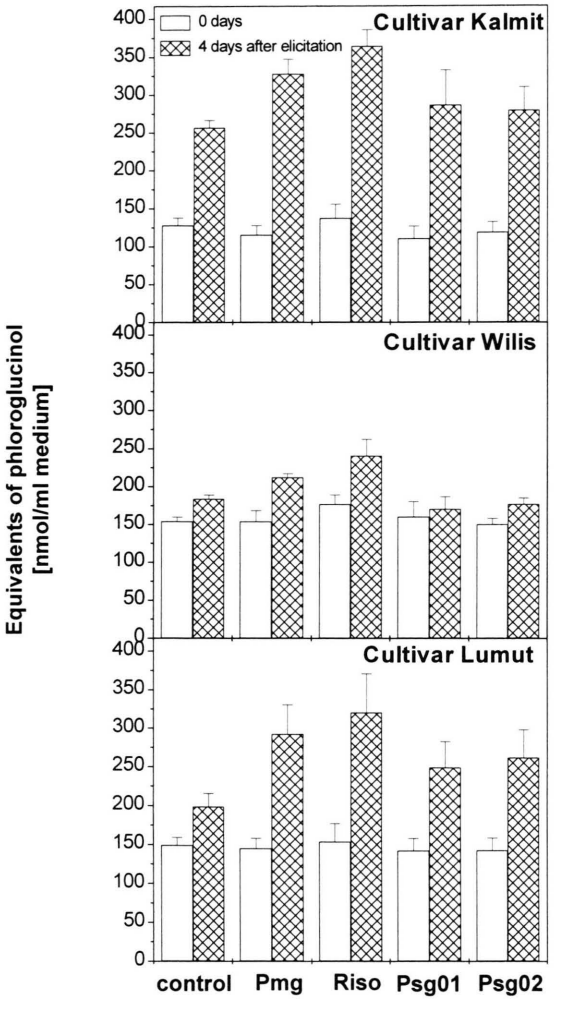

Fig. 6. Accumulation of soluble phenolic compounds in the culture medium of cell suspension cultures of three soybean cultivars by fungal and bacterial elicitors. Phenolic compounds were determined by Folin-Ciocalteous phenol reagent. The fungal elicitors were crude cell wall extracts (100 µg/ml medium) of *Rhizoctonia solani* (Riso-elicitor) and *Phytophthora sojae* (Pmg-elicitor), respectively. The bacterial elicitors were two isolates of living cells of *Pseudomonas syringae* pv. *glycinea* (Psg01/02) (od_{500}=0.2, 50 µl/ml medium). As control an equal amount of sterile bidistilled water or MES-buffer (0.5 mM, pH 6.0) was added. Data are means (± se) of four independent experiments.

PR-proteins

β-1,3-Glucanase and chitinase enzyme activities in soybean cell suspensions cultures of cultivars Wilis, Lumut and Kalmit, respectively, were determined in tissue extracts and in the culture medium following treatment with Riso-elicitor and bacterial suspension (Psg02).

As demonstrated in Fig. 7 both elicitors induced an increase in chitinase activity with similar kinetics in the culture medium and in the cell extracts. In general the enzyme activity per flask was higher in the extracellular compartment than in the cells. In the culture medium of cv Lumut and Wilis the increase was stronger after addition of Riso-elicitor (1.5- and 2-fold, respectively) than after application of bacterial suspensions (1.3-fold). In contrast, in cell suspension cultures of cv Kalmit both elicitors caused

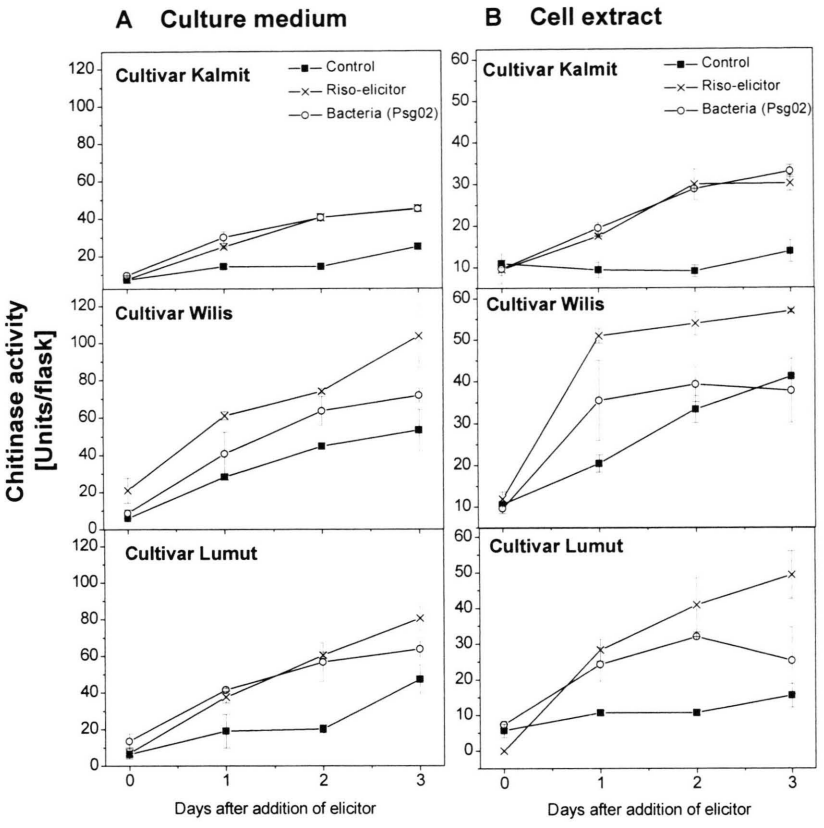

Fig. 7. Induction of chitinase activity in cell suspension cultures of three soybean cultivars by fungal and bacterial elicitors. **A** culture medium, **B** cell extracts. Riso-elicitor – crude cell wall extract of *Rhizoctonia solani* (100 µg/ml medium), Psg02 – bacterial suspension of *Pseudomonas syringae* pv. *glycinea* isolate 02 (od_{500}=0.2, 50 µl/ml medium). As control an equal amount of sterile MES-buffer (0.5 mM, pH 6.0) was added. Data are means (± se) of two independent experiments.

a 2–3-fold augmentation. The highest chitinase activity was generally found more than three days after addition of the elicitors.

In contrast to the elicitor-induced chitinase activity β-1,3-glucanase activity was mainly stimulated in the cells. The relative intensity of β-1,3-glucanase induction by the two elicitors (Fig. 8) was similar to chitinase activity. β-1,3-Glucanase stimulation by bacteria (Psg02) in cell suspension cultures of cv Wilis and Lumut was much less pronounced than by Riso-elicitor, whereas cells of cv Kalmit were nearly equally sensitive to both elicitors.

Discussion

Considerable differences in the elicitor activity of different elicitor preparations as shown in this study have been observed for early or late defence responses in other systems too. Treatment of tobacco cells with pectin lyase and oligogalacturonides demonstrated a differential reactivity with regard to the elicitor-induced alkalinization of the culture medium and to the extracellular accumulation of H_2O_2 (Rouet-Mayer *et al.*, 1997). In soybean cotyledons glucomannans extracted from cell walls of Pmg with soybean β-1,3-endoglucanase were the most active elicitors for phytoalexin synthesis followed by carbohydrates from Pmg whereas chitin and chitosan exhibited a very low activity (Keen *et al.*, 1983). The results indicate that in addition to signal recognition cells possess the ability to finely regulate the signal transduction pathway.

Cellular reactivity in terms of time courses and intensities of defence responses was not only different with regard to the various elicitor-preparations but also varied significantly when cells of the four cultivars Wilis, Lumut, Kalmit and Doko RC (Figs. 2–5, 7, 8) were compared. Cultivar-specific differences are mainly described for compatible and incompatible interactions. For example, soy-

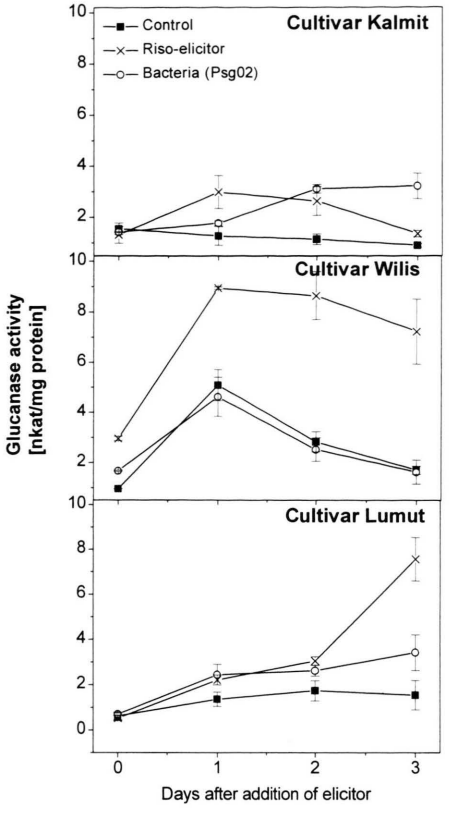

Fig. 8. Induction of β-1,3-glucanase activity in cells of three soybean cultivars by fungal and bacterial elicitors. Riso-elicitor – crude cell wall extract of *Rhizoctonia solani* (100 µg/ml medium), Psg02 – bacterial suspension of *Pseudomonas syringae* pv. *glycinea* isolate 02 (od_{500}= 0.2, 50 µl/ml medium). As control an equal amount of sterile MES-buffer (0.5 mM, pH 6.0) was added. Data are means (± se) of two independent experiments.

bean and chickpea cells show a slower and weaker induction of phenylpropanoid metabolism and of phytoalexins in compatible than in incompatible interactions (Börner and Grisebach, 1982; Daniel *et al.*, 1989).

Such elicitor- and cultivar-specific differences could be due to a differential dose-dependency of the perception system as postulated by Kombrink and Hahlbrock (1986). Support for this hypothesis are measurements of the reactivity of cells of species of the family *Fabaceae* after treatment with the β-glucan-elicitor from *P. sojae* (Cosio *et al.*, 1996). The same quantitative response (accumulation of phytoalexins) in chickpea cell suspension cultures required a 40- to 100-fold amount of elicitor than necessary for the other legumes analysed in that study. Furthermore, according to Bourque *et al.* (1998) the modulated reactivities may be determined by initial transduction steps which activate more or less efficiently a common downstream pathway. According to recent publications the information required for the intensity and time course of defence responses after the recognition of the elicitor could be transferred by Ca^{2+} signals (Goddard *et al.*, 2000).

Rapid release of active oxygen species is a characteristic response of plant cells to elicitor preparations or inoculation with microbial pathogens (Wojtaszek, 1997). This elicitor-induced oxidative burst is responsible for the peroxidative cross-linking of structural cell wall proteins which renders the plant cell wall less digestible to cell wall degrading enzymes (Brisson *et al.*, 1994). In the four soybean cultivars investigated in this study the peroxidative cross-linking of the proline-rich protein p33 and of a putative hydroxyproline-rich glycoprotein occurred after treatment with the fungal elicitors. At first glimpse for cv Kalmit there was no correlation with the elicitor-stimulated oxidative burst because the oxidative burst was induced five minutes after elicitor-treatment (Fig. 1) whereas the insolubilisation was finished only after 90 min (data not shown). The reason for this discrepancy might be that the oxidative burst was measured 2 ½ h after cell transfer. The samples for the insolubilisation were taken on day five after subculture without cell transfer. Thus, in contrast to the cells of the other three cultivars cells of cv Kalmit seem to secrete substances (e. g. antioxidants) which delay the release of H_2O_2 and the peroxidative cross-linking of cell wall proteins. A similar delayed reaction was also obtained with cell suspension cultures of *Echinacea purpurea* (Schelhaas *et al.*, 2000). The maximal H_2O_2 stimulation and the insolubilisation of a putative hydroxyproline-rich cell wall glycoprotein of 200 kDa occurred more than 120 min after elicitation.

The consecutive treatment of cells with the same or another elicitor gave a further hint on the recognition process. The application of the bacterial suspensions as first elicitor did not decrease the induction of H_2O_2 when a second bacterial or fungal stimulus was applied (Table I). In contrast, a subsequent challenge with fungal elicitor led to a partly refractory state (desensitisation). Desensiti-

sation was also observed in tomato and tobacco cells (Felix *et al.*, 1998; Binet *et al.*, 1998). On the other hand, a refractory state could not be shown in cell cultures derived from wild species of tomato after repeated application of chitin (Felix *et al.*, 1998). The reasons for this differential behaviour of plant cells is unknown. In the animal system desensitisation is caused by sequestration or down-regulation of the (β-adrenergic) receptors from the cell surface as well as by covalent modifications (phosphorylation) of the receptors (Sibley and Lefkowitz, 1985; Sibley *et al.*, 1987).

Extracellular alkalinization of the culture medium is an often used marker for the induction of defence responses. Our work shows a close correlation in time courses and intensities between the accumulation of H_2O_2 and the extracellular alkalinization (Figs. 1, 3, 4) but the addition of an inhibitor of the oxidative burst had a stronger decreasing effect on the H_2O_2 induction than on the alkalinization. This indicates that the two reactions are mostly unrelated and the alkalinization may be due to an activation of ion channels and to a transient inhibition of the plasma membrane H^+-ATPase (Pugin and Guern, 1996). This is in accordance with the results obtained by Tenhaken and Rübel (1998).

Bacterial suspensions (Psg02) induced a significantly weaker H_2O_2 accumulation than Riso-elicitor in cell suspension cultures of cv Lumut and Kalmit (Fig. 3) but the height of PAL-activity (Fig. 5) and PR-protein induction (Figs. 7, 8), respectively, was nearly equivalent for both elicitors. It is possible that different elicitor-active substances stimulate both the early and late defence reactions. Two different eliciting compounds have been found in an elicitor-preparation from *Verticillium dahliae* (Davis *et al.*, 1993). Furthermore, these observations indicate that the activation of PR-proteins, of phenylpropanoid metabolism and of the oxidative burst are activated by several at least partly unrelated signal reaction chains. This hypothesis is corroborated by the study of Hein (2000). When analysing a broad spectrum of elicitor-induced defence genes in chickpea cells she showed that there were no two genes activated with the same kinetic.

The induction of phenylpropanoid metabolism in soybean cells and plants often coincides with the accumulation of the isoflavonoid phytoalexins glyceollin I–III. In contrast, in our study during the 24 h of incubation the bacterial (Psg02) and fungal (Riso) elicitors stimulated PAL-activity but did neither lead to an induction of chalcone synthase, the introductory enzyme of flavonoid metabolism, nor of phytoalexins. The unlinked expression of the two enzymes was observed by Ebel *et al.* (1984) as well. In the present work the products of phenylpropanoid metabolism seem to be used for the production of secreted phenolic substances (Fig. 6). These phenolics may have antimicrobial activity (Nicholson and Hammerschmidt, 1992). But they may also reinforce the plant cell wall (Graham and Graham, 1991).

Extensively as in our work several other studies have demonstrated that chitinases mainly accumulate extracellularly and β-1,3-glucanases intracellularly (Vogelsang and Barz, 1990). It is hypothesised that these hydrolytic enzymes function on the one hand as a last line of defence because when the cells collapse the hydrolytic enzymes are released (Mauch and Staehelin, 1989). Furthermore in *in vitro* studies it was shown that they are capable to stop the growth of pathogens (Mauch *et al.*, 1988). On the other, hand β-1,3-glucanases may contribute to a release of endogenous elicitor-active substances from the pathogens' cell walls (Keen and Yoshikawa, 1983). In this work the relative elicitor-induced intensities of β-1,3-glucanase and chitinase-activities were similar but the time courses differed significantly. Thus, the two enzyme classes are probably differentially regulated. Conrads-Strauch *et al.* (1990) revealed a differential activation of β-1,3-glucanases and chitinases in *Brassica campestris* after infection with living or heat-killed avirulent bacteria *of Xanthomonas campestris* pv. *vitians.* Several other publications also found the induction of different isoforms in compatible and incompatible interactions (Yi and Hwang, 1996).

Future work will have to test if the cultivar- and elicitor-specific differences found in the cell suspension cultures also occur in plants and can be correlated with resistance.

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Ayers A. R., Valent J., Ebel J. and Albersheim P. (1976), Host-pathogen interactions X. Fractionation and biological activity of an elicitor isolated from the mycelial walls of *Phytophthora megasperma* var. *sojae*. Plant Physiol. **57**, 760–765.

Bach M., Schnitzler J. P. and Seitz H. U. (1993), Elicitor-induced changes in Ca^{2+} influx, K^+ efflux and 4-hydroxybenzoic acid synthesis in protoplasts of *Daucus carota* L. Plant Physiol. **103**, 407–412.

Barz W. (1997), Phytoalexins. In: Hartleb, H., Heitefuss R. and Hoppe H.-H. (eds), Resistance of Crop Plants against Fungi. Gustav Fischer, Jena, Stuttgart, Lübeck, Ulm, pp. 183–201.

Binet M.-N., Bourque S., Lebrun-Garcia A., Chiltz A. and Pugin A. (1998), Comparison of the effects of cryptogein and oligogalacturonides on tobacco cells and evidence of different forms of desensitization induced by these elicitors. Plant Sci. **137**, 33–41.

Boller T. (1992), Biochemical analysis of chitinases and β-1,3-glucanases. In: Gurr S. J., McPherson M. J. and Bowles D. (eds.), Molecular Plant Pathology. Oxford Univ. Press, New York, pp. 23–30.

Bonas U. and van den Ackerveken G. (1997), Recognition of bacterial avirulence proteins occurs inside the plant cell: a general phenomenon in resistance to bacterial disease? Plant J. **12/1**, 1–7.

Börner H. and Grisebach H. (1982), Enzyme induction in soybean infected by *Phytophthora megasperma* f. sp. *glycinea*. Arch. Biochem. Biophys. **217**, 65–71.

Bourque S., Ponchet M., Binet M.-N., Ricci P., Pugin A. and Lebrun-Garcia A. (1998), Comparison of binding properties and early biological effects of elicitins in tobacco cells. Plant Physiol. **118**, 1317–1326.

Bradford M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254.

Bradley O. J., Kjellbom P. and Lamb C. J. (1992), Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defence response. Cell **70,** 21–30.

Brisson L. F., Tenhaken R. and Lamb C. J. (1994), Function of the oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell **6**, 1703–1712.

Conrads-Strauch J., Dow J. M., Miligan D., Parra R. and Daniels M. J. (1990), Induction of hydrolytic enzymes in *Brassica campestris* in response to pathovars of *Xanthomonas campestris*. Plant Physiol. **93**, 238–243.

Cosio E. G., Feger M., Miller C. J., Antelo L. and Ebel J. (1996), High-affinity binding of a fungal β-glucan elicitor to cell membranes of species of the familiy Fabaceae. Planta **200**, 92–99.

Daniel S., Tiemann K., Wittkampf U., Bless W., Hinderer W. and Barz W. (1990), Elicitor-induced metabolic changes in cell cultures of chickpea (*Cicer arietinum* L.) cultivars resistant and susceptible to *Ascochyta rabiei*. Planta **182**, 270–278.

Davis D., Merida J., Legendre L., Low P. and Heinstein P. (1993), Independent elicitation of the oxidative burst and phytoalexin formation in cultured plant cells. Phytochemistry **32**, 607–611.

Ebel J., Schmidt W. E. and Loyal R. (1984), Phytoalexin synthesis in soybean cells: elicitor induction of phenylalanine ammonia lyase and chalcone synthase mRNAs and correlation with phytoalexin accumulation. Arch. Biochem. Biophys. **232/1**, 240–248.

Felix G., Baureithel K. and Boller T. (1998), Fragments in tomato cells. Plant Physiol. **117**, 643–650.

Gamborg O. L., Miller R. A. and Ojima K. (1968), Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**, 151–158.

Goddard H., Manison N. F. H., Tomos D. and Brownlee C. (2000), Elemental propagation of calcium signals in response-specific patterns determined by environmental stimulus strength. Proc. Natl. Acad. Sci. USA **97/4**, 1932–1937.

Graham M. Y. and Graham T. L. (1991), Rapid accumulation of anionic peroxidases and phenolic polymers in soybean cotyledon tissue following treatment with *Phytophthora megasperma* f. sp. *glycinea* wall glucan. Plant Physiol. **97**, 1445–1455.

Hahlbrock K. and Scheel D. (1989), Physiology and molecular biology of phenylpropanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**, 347–369.

Hein F. (2000), Dissertation, WWU Münster, in press.

Keen N. T. and Yoshikawa M. (1983), β-1,3-Endoglucanase from soybean releases elicitor-active carbohydrates from fungus cell walls. Plant Physiol. **71**, 460–465.

Keen N. T., Yoshikawa M. and Wang M. C. (1983), Phytoalexin elicitor activity of carbohydrates from *Phytophthora megasperma* f. sp. *glycinea* and other sources. Plant Physiol. **71**, 466–471.

Kirkby E. A. and Mengel K. (1962), Ionic balance in different tissues of the tomato plant in relation to nitrate, urea, or ammonium nutrition. Plant Physiol. **72**, 6–14.

Kombrink E. and Hahlbrock K. (1986), Responses of cultured parsley cells to elicitors from phytopathogenic fungi. Timing and dose dependency of elicitor-induced reactions. Plant Physiol. **81**, 216–221.

Kombrink E. and Somssich I. E. (1993), Defence responses of plants to pathogens. Adv. Bot. Res. **21**, 1–34.

Levine A., Tenhaken R., Dixon R. and Lamb C. (1994), H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79**, 583–593.

Mauch F. and Staehelin L. A. (1989), Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell **1**, 447–457.

Mauch F., Mauch-Mani B. and Boller T. (1988), Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. **88**, 936–942.

Moerschbacher B. M. (1988), Ligninbiosynthese und die Resistenz des Weizens gegen Schwarzrost. Dissertation, RWTH Aachen.

Murashige T. and Skoog F. (1963), A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473–497.

Nicholson R. L. and Hammerschmidt R. (1992), Phenolic compounds and their role in disease resistance. Annu. Rev. Phytopathol. **30**, 369–389.

Nürnberger T. (1999), Signal perception in plant pathogen defence. Cell. Mol. Life Sci. **55**, 167–182.

Orlandi E. W., Hutcheson S. W. and Baker C. J. (1992), Early physiological responses associated with race-specific recognition in soybean leaf tissue and cell suspensions treated with *Pseudomonas syringae* pv. *glycinea*. Physiol. Mol. Plant Pathol. **40**, 173–180.

Otte O. and Barz W. (1996), The elicitor-induced oxidative burst in cultured chickpea cells drives the rapid insolubilisation of two structural cell wall proteins. Planta **200**, 238–246.

Pugin A. and Guern J. (1996), Mode of action of elicitors: involvement of plasma membrane functions. C. R. Acad. Sci. Paris, Sciences de la vie/life sciences **319**, 1055–1061.

Read S. M. and Northcote D. H. (1981), Minimization of variation in the response to different proteins of the Coomassie Blue G dye-binding assay for protein. Anal. Biochem. **116**, 53–64.

Rouet-Mayer M.-A., Mathieu Y., Cazalé A.-C., Guern J. and Laurière C. (1997), Extracellular alkalinization and oxidative burst induced by fungal pectin lyase in tobacco cells are not due to the perception of oligogalacturonide fragments. Plant Physiol. Biochem. **35/4**, 321–330.

Schelhaas U., Hüster K., Li W., W. and Barz W. (2000), Elicitor-stimulated events in *Echinacea purpurea* cell suspension cultures. (in preparation)

Sibley R. D. and Lefkowitz R. J. (1985), Molecular mechanisms of receptor desensitization using the β-adrenergic receptor-coupled adenylate cyclase system as a model. Nature **317**, 124–129.

Sibley R. D., Benovic J. L., Caron M. G. and Lefkowitz (1987), Regulation of transmembrane signaling by receptor phosphorylation. Cell **48**, 913–922.

Swain T. and Hills W. E. (1959), The phenolic constituents of *Prunus domestica* I: the quantitative analysis of phenolic constituents. J. Sci. Food Agric. **10**, 63–68.

Tenhaken R. and Rübel C. (1998), Induction of alkalinization and an oxidative burst by low doses of cycloheximide in soybean cells. Planta **206**, 666–672.

Van Loon L. C., Pierpoint W. S., Boller T. and Conjero V. (1994), Recommendations for naming pathogenesis-related proteins. Plant Mol. Biol. Rep. **12/3**, 245–264.

Vogelsang R. and Barz W. (1990), Elicitation of β-1,3-glucanase and chitinase activities in cell suspension cultures of *Ascochyta rabiei* resistant and susceptible cultivars of chickpea (*Cicer arietinum*). Z. Naturforsch. **45c**, 233–239.

Wirth S. J. and Wolf G. A. (1990), Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. J. Microbiol. Methods **12**, 197–205.

Wojtaszek P. (1997), Oxidative burst: an early plant response to pathogen infection. Biochem. J. **322**, 681–692.

Yi S. Y. and Hwang B. K. (1996), Differential induction and accumulation of β-1,3-glucanase and chitinase isoforms in soybean hypocotyls and leaves after compatible and incompatible infection with *Phytophthora megasperma* f.sp. *glycinea*. Physiol. Mol. Plant Pathol. **48**, 179–192.