Avirulence protein Avr1b

from *Phytophthora sojae*

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List of abbreviations

A	Ampere
aa	Amino acids
Abs	Absorbance
AP	Alkaline phosphatase
APS	Ammoniumperoxodisulfate
ATP	Adenosine triphosphate
Avr	Avirulence
BAC	Bacterial artificial chromosome
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CDPK	Calcium-dependent protein kinase
C. fulvum	*Cladosporium fulvum*
4CL	4-coumarate:CoA ligase
CoA	Coenzyme A
CTAB	Hexadecyl trimethyl ammonium bromide
cv.	Cultivar
D6aH	3,9-dihydroxypterocarpan 6a-hydroxylase
DEPC	Diethylpyrocarbonate
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide-triphosphate (dATP, dGTP, dCTP, dTTP)
DTT	1,4-Dithiothreitol
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl)-tetraacetic acid
ESI-Q-TOF	Electrospray-ionization-quadrupole-time of flight
EST	Expressed sequence tag
FBS	Fetal bovine serum
Fig.	Figure
FPLC	Fast performance liquid chromatography
f. sp.	Forma specialis
fw	Fresh weight
GBP	Glucan binding protein
GFP	Green fluorescent protein
Gm	*Glycine max*

G. max	*Glycine max*
6xHis-tag	6 Histidine residues
h	Hour
H. parasitica	*Hyaloperonospora parasitica*
hpi	Hours post infection
HPLC	High performance liquid chromatography
HR	Hypersensitive response
Hrp genes	Hypersensitive response and pathogenicity genes
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-galactopyranoside
kb	Kilobase
K_D	Dissoziation constant
kD	Kilodalton
LB medium	Luria Bertani medium
LC	Liquid chromatography
LRR	Leucine-rich repeat
LZ	Leucine zipper
M	Molarity
mA	Milli ampere
MAPK	Mitogen-activated protein kinase
Mbp	Mega base pairs
Me-Ja	Methyl jasmonate
min	Minute
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MW	Molecular weight
β-NADPH	β-Nicotinamide adenine dinucleotide phosphate
NBS	Nucleotide-binding site
NBT	Nitro blue tetrazolium
Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical density
ORF	Open reading frame
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated molecular patterns
PAS	Protein A sepharose
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
pi	post infection
PR protein	“Pathogenesis-related“ protein
P. sojae	*Phytophthora sojae*

pth	Pathogenesis
pv.	Pathovar
PVP	Polyvinyl pyrrolidone
R	Resistance
RE	Raw elicitor
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RP	Reversed phase
Rps	Resistance to *Phytophthora sojae*
rpm	Revolutions per minute
RPP	Recognition of *Peronospora parasitica*
Rt	Room temperature
RT	Reverse transcription
SA	Salicylic acid
SAM	Starvation associated messenger
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	Second
Sf9	*Spodoptera frugiperda*-insect cell line
spp.	Species
SSTE	Sodium chloride-SDS-Tris-EDTA
TAE	Tris-acetate-EDTA buffer
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Trishydroxymethyl-2-aminomethane
Tween 20	Polyoxyethylene sorbitan monooleate
TTSS	Type three secretion system
U	Unit (Enyzme units, µmol/minute)
UV	Ultraviolet
V	Volt
vol	Volume
v/v	Volume/volume
w/v	Weight/volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YFP	Yellow fluorescent protein
YTH	Yeast-two-hybrid

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1 Introduction

1.1 Plant resistance

Plants are exposed to a wide range of microorganisms such as viruses, bacteria, fungi, as well as nematodes, damaging insects and oomycetes. Yet most plants normally resist attack by potential pathogens because they possess effective defence mechanisms.
Plant disease resistance can be defined as the ability of the plant to prevent or restrict pathogen growth and proliferation (Benhamou, 1996). The molecular events constituting critical steps of plant-pathogen interactions can be divided into three steps (Ebel, 1998):

1. Generation and recognition of signal compounds
2. Inter- and intracellular signal conversion and transduction
3. Activation of signal-specific responses in target cells

Disease resistance processes in plants are diverse. Resistance may occur at the sub-species or varietal level (race/cultivar-specific resistance mediated by resistance and avirulence genes, specific resistance) or at the species or genus level (non-host or basic resistance).
In specific resistance, plant perception of elicitors produced by avirulent pathogens, leads to an induction of a rapid and localized programmed cell death response termed hypersensitive response (HR). In this case, invaded cells or tissues are sacrificed to prevent spreading of the pathogen. Disease resistance genes (*R* genes) are thought to encode specific receptors that interact directly or indirectly with pathogen elicitors expressed by avirulence genes (*Avr* genes) and initiate signal transduction pathways leading to HR and expression of disease resistance (Baker *et al.*, 1997). The race-specific resistance was first formulated as the “gene-for-gene” hypothesis for the resistance of flax to rust (*Melampsora lini*) (Flor, 1956; Flor, 1971).
Basic or non-host resistance is less understood in comparison to specific resistance. Many biochemical and cellular components of the specific response also occur in the absence of specific *R* genes as part of the basic resistance response of plants (Kamoun *et al.*, 1999). Basic resistance is characterized by the formation of physical barriers such as thick cell-walls and cutin layers or production of toxic compounds like phenols and saponines (Osbourne, 1996). Pathogen recognition arises through general elicitors, also known as pathogen-associated molecular patterns (PAMPs) (Nürnberger and Brunner, 2002).

Elicitors of plant defence responses fall into two categories depending on their source: exogenous and endogenous compounds. Exogenous elicitors can be considered the primary signals in plant-pathogen interactions. They originate in the pathogen or aggressor itself, appear to have a limited mobility within the plant tissues and evoke a response in cells in the immediate vicinity to the

pathogen. Endogenous elicitors are of plant origin and arise as a result of the interaction with the aggressor (Ebel and Cosio, 1994). Much progress has been made in characterizing elicitors (Boller, 1995; Hahn, 1996; Ebel and Scheel, 1997) which are believed to interact with host plant receptors with high specificity and sensitivity. The receptors transduce the elicitor signal into cellular reactions resulting in the activation of plant defence (Ebel and Mithöfer, 1998; Nürnberger and Scheel, 2001).
Examples of general elicitors from fungi and oomycetes are the elicitins from the species *Phytophthora* and *Pythium* (Kamoun *et al.*, 1993; Kamoun *et al.*,1994; Kamoun *et al.*, 1997; Qutob *et al.*, 2003) and β-glucans from the cell walls of oomycetes (Boller, 1995).

1.2 Signal transduction

Following pathogen recognition, a subsequent intracellular signal transduction occurs. The activation of plant defence response genes involves the transduction of elicitor signals from the site of primary perception at the cell surface to the nucleus where transcription of specific genes is initiated. It is conceivable that different signal transduction pathways exist in different plants and for different types of response. Even in the same plant, different elicitors might activate different signal transduction chains (Ebel and Mithöfer, 1998).

Responses that appear to be consequences of elicitor perception and are possibly related to signal transduction include depolarization of the plasma membrane, increase in the cytosolic Ca^{2+} concentration, alkalinization of the extracellular space, protein phosphorylation, and production of reactive oxygen species (Fig. 1). In addition to these defences, systemic acquired resistance (SAR), which increases the plant's resistance systemically to subsequent pathogen attack, is activated in many plants and can be induced by certain elicitors (Enyedi *et al.*, 1992; Klessig and Malamy, 1994; Ryals *et al*., 1996). Compounds that have been associated with signaling events in plant defence as part of the SAR are salicylate, jasmonate and ethylene.

The effect of *Phytophthora sojae* derived β-glucan elicitors on the plasma membrane potential has been investigated. β-Glucans with different degrees of polymerization transiently depolarized the plasma membrane of soybean roots (Mithöfer *et al*., 2005).
Changes in ion fluxes (Cl^- and K^+ efflux, Ca^{2+} influx, alkalinization of cell culture growth medium) constitute an early inducible response, occurring within minutes after elicitor application in many plant tissues (Bach *et al.,* 1993; Felix *et al*., 1993; Nürnberger *et al*., 1994a; Mithöfer *et al*., 2005), and are a prerequisite for the activation of a variety of defence-related genes (Ebel and Scheel, 1997).
Protein kinases, mitogen-activated protein kinases (MAPKs), calcium-dependent protein kinases (CDPKs), and protein phosphatases might represent further elements of elicitor signal transduction, converting receptor signals into cell-specific responses. Changes in the level of phosporylation of cellular proteins have been observed upon elicitor treatment of a variety of cell cultures (Boller, 1995; Ebel and Scheel, 1997; Daxberger *et al*., unpublished).
The production of reactive oxygen species (ROS) such as $^{\bullet}O_2^-$, $HO^{\bullet}$ and H_2O_2, is a rapid reaction of

plants to infection or elicitor treatment and may originate from two distinct subcellular sources (Allan and Fluhr, 1997). The oxidative burst can lead to the cross-linking of cell wall proteins rendering plant cell walls more resistant to attack by fungal enzymes (Bradley *et al*., 1992). The ROS may serve as second messengers for the activation of genes expressing protective proteins including genes for glutathione S-transferase, glutathione peroxidase and polyubiquitin, as well as enzymes involved in scavenging of ROS (Lamb and Dixon, 1997) or they may be toxic to pathogens. Generation of ROS is a characteristic feature of the HR (Ebel and Scheel, 1997; Lamb and Dixon, 1997) but there is still no conclusive evidence that ROS directly cause plant cell death. The connection between ROS production and phytoalexin synthesis has been explored in different plants such as bean, pea, soybean, tobacco and alfalfa (Mehdy, 1994; Lamb and Dixon, 1997). In some of the systems, a tight correlation between ROS and phytoalexin production has been reported (Jabs *et al*., 1997), whereas in others ROS-independent pathways may exist (Levine *et al*., 1994; Mithöfer *et al*., 1997).

SAR requires the signal molecule salicylic acid (SA) and is associated with accumulation of pathogenesis-related (PR) proteins, which are thought to contribute to resistance. Much progress has been made recently in elucidating the mechanism of SAR. The detection of increased SA levels in neighbouring leaves and in the phloem led many researchers to believe that SA could be the systemic signal for SAR. Further studies suggested that signaling might occur through the conversion of SA to the volatile compound methyl salicylate, which could induce resistance not only in the uninfected parts of the same plant but also in neighbouring plants (Shulaev *et al*., 1997).

Jasmonic acid and its methyl ester, methyl jasmonate (Me-Ja) have been implicated as wounding-activated endogenous signals that may alert undamaged tissues of neighboring plants of a pest attack (Farmer and Ryan, 1992). Addition of jasmonate or Me-Ja to soybean cell suspension cultures increased the activity of the enzyme phenylalanine ammonia-lyase (PAL) which is part of the phenylpropanoid pathway (Gundlach *et al*., 1992; Fliegmann *et al*., 2003). They also stimulated the accumulation of a variety of secondary metabolites in cultures of a wide range of plant species (Gundlach *et al*., 1992). Jasmonates have been shown to stimulate the production of flavonoids. Thus exogenous jasmonate induced the accumulation of genistein, a minor isoflavonoid in the spectrum of soybean defence compounds (Morris *et al*., 1991). However jasmonates may not always be involved in signaling for plant defence, they also promote stomatal closure, accelerate leaf senesce in oats and barley (Ueda and Kato, 1980; Weidhase *et al*., 1987) and alter gene expression by rapidly inducing the synthesis of new proteins (Curtis, 1984; Weidhase *et al*., 1987).

Ethylene is a gaseous plant hormone that regulates many physiological processes in plants (Matoo and Suttle, 1991). It induces several PR proteins and may also be involved in the strengthening of the cell wall following a pathogen or pest attack (Enyedi *et al.*, 1992).

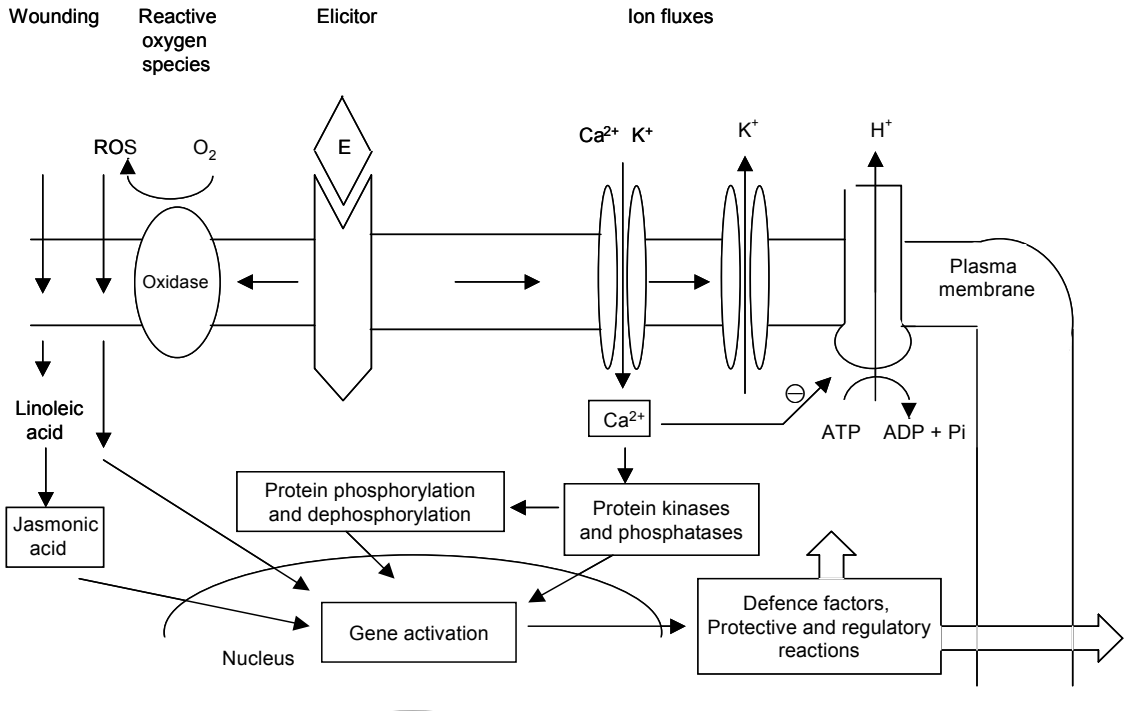


Fig. 1 Simplified model for the activation of defence responses in soybean by elicitors. Figure taken from Ebel and Mithöfer (1998), and modified according to Mithöfer *et al*. (2005).

1.3 The interaction between soybean and *Phytophthora sojae*

1.3.1 Soybean (*Glycine max* L.)

Soybean (*Glycine max* L.) was cultivated in China since 3000 BC and was introduced to Europe in the 18th and to the United States in the 19th century. After the second world war, the United States became the world's leading soybean producing nation, nowadays followed by Brazil, Argentina, China, India and Paraguay. According to the American Soybean Association, the United States produced 66,78 million metric tons of soybean in the year 2004.

Taxonomically soybean belongs to the family Fabaceae of the order Fabales (Strasburger, 2002). Soybean is a bushy, annual, summer legume 30 to 150 cm tall. The pods, stems and leaves are covered with fine brown or gray pubescence. The leaves are trifoliate (sometimes with 5 leaflets). The pods contain between one and five seeds. The small self fertile flowers are either white or purple. The plant is not frost resistant so it must be grown after spring frosts and have time for the seeds to mature before the first fall frost. Many selections or cultivars are bred for production under different day length or other environmental conditions. They range in maturity from 75 to 200 days. Soybean seeds have a high (38-45%) protein and high (20%) oil contents. The beans can be processed in a variety of ways. Common forms include soy meal (used as animal feed), soy flour, soy milk, tofu, and soybean oil. Soybeans are also the primary ingredient involved in the production of soy sauce.

1.3.2 Plant pathogenic oomycetes

Fungi and oomycetes are the two most important groups of eukaryotic plant pathogens. Fungi form a separate kingdom and are evolutionary related to animals. Oomycetes are related to heterokont, biflagellate, golden-brown algae (Fig. 2) and should be included in the kingdom Chromista (Erwin and Ribeiro, 1996) or in the Stramenopiles (Van der Peer and De Wachter, 1997). Fundamental differences in physiology, biochemistry and genetics between fungi and oomycetes exist as shown e.g. by comparison of the genes encoding the small ribosomal subunit, actin and tubulin (Kamoun *et al.*, 1999; Tyler, 2001; Latijnhouwers *et al*., 2003).

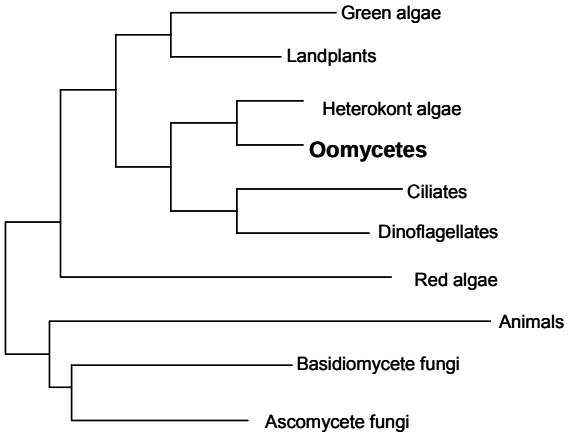


Fig. 2 Phylogenetic tree showing the evolutionary relationships between the major eukaryotic groups. Figure taken from Kamoun *et al*. (1999), adapted from Van de Peer and De Wachter (1997).

Plant-pathogenic oomycetes include over 60 species of *Phytophthora*, numerous genera of the biotrophic downy mildews (such as *Peronospora* and *Bremia*), and more than 100 species of the genus *Pythium*. Oomycetes are responsible for economically and environmentally devastating epidemics, including the 19th century Irish potato famine (*Phytophthora infestans*) which caused starvation and death of over 1 million people in Ireland and a similar number of irish citizens emigrating to the United States and elsewhere (Gregory, 1983).

The International Potato Centre has estimated that worldwide losses in potato production caused by late blight exceeds three billion dollar annually. Another equally devastating species of the genus *Phytophthora* is *Phytophthora ramorum*, responsible of the current sudden oak death epidemic in the United States and throughout Europe (Kamoun, 2000).
The name of *Phytophthora* is derived from the Greek that means plant (*phyto*) destroyer (*phthora*). *Phytophthora* species attack an extraordinarily wide range of agriculturally and ornamentally important plants. All 60 species are destructive pathogens, causing rots of roots, stems, leaves and fruits. Some species such as *P. cinamomi* and *P. cactorum* attack hundreds of different plant host

species, others such as *P. infestans* or *P. sojae* (syn. *Phytophthora megasperma* f. sp. *glycinea*) have narrow host ranges.

Phytophthora species are characterized by hyphal swellings, sporangia, biflagellate zoospores, chlamidospores and the sex organs antheridia, oogonia and oospores. The thallus as in fungi, is called mycelium, from the Greek: *mykes* (fungus) and *lium* (after epithelium). It is composed of hyaline, branched, coenocytic filaments. *Phytophthora* species do not synthesize sterols but require an exogenous source of β-hydroxy sterols for sporulation. *Phytophthora* and other members of the family Pythiaceae such as *Pythium* are resistant to polyene antibiotics such as pimaricin, a characteristic correlated with a requirement for an exogenous source of β-hydroxy sterols (Erwin and Ribeiro, 1996).

The major stages in life cycle of most root-infecting oomycete species of *Pythium* and *Phytophthora* are similar. The life cycle of a typical *Pythium* sp. is shown in Fig. 3. It consists of two cycles that are usually stimulated by environmental conditions. The asexual cycle is characterized by the production of sporangia. Sporangia may germinate either directly in liquid or on a surface to produce a germ tube or may differentiate by a process of cytoplasmic cleavage to form uninucleate, biflagellate zoospores (indirect germination). The zoosporangia of *Phytophthora* species are formed in an aqueous medium. The released zoospores swim in water in search of host tissues where they settle and cyst. The cyst germinates by developing a germ tube that may penetrate the host directly or via an appresorium. The hyphae ramify through the plant using nutrients acquired from it, developing a mycelium, from which sporulation occurs on the dying seedling and the disease cycle is repeated. The sexual cycle generates thick walled oospores adapted for over-wintering and survival under difficult environmental conditions. Oosporogenesis involves the production of a female oogonium and a male antheridium that grows towards and fuses the oogonium. Fertilization occurs through the emptying of some of the contents of the antheridium into the oogonium, leading to the development of an oospore. The oospore can produce single or multiple germ tubes. These germ tubes can then form sporangia thereby recapitulating the asexual cycle of the pathogen (Van West *et al.*, 2003).

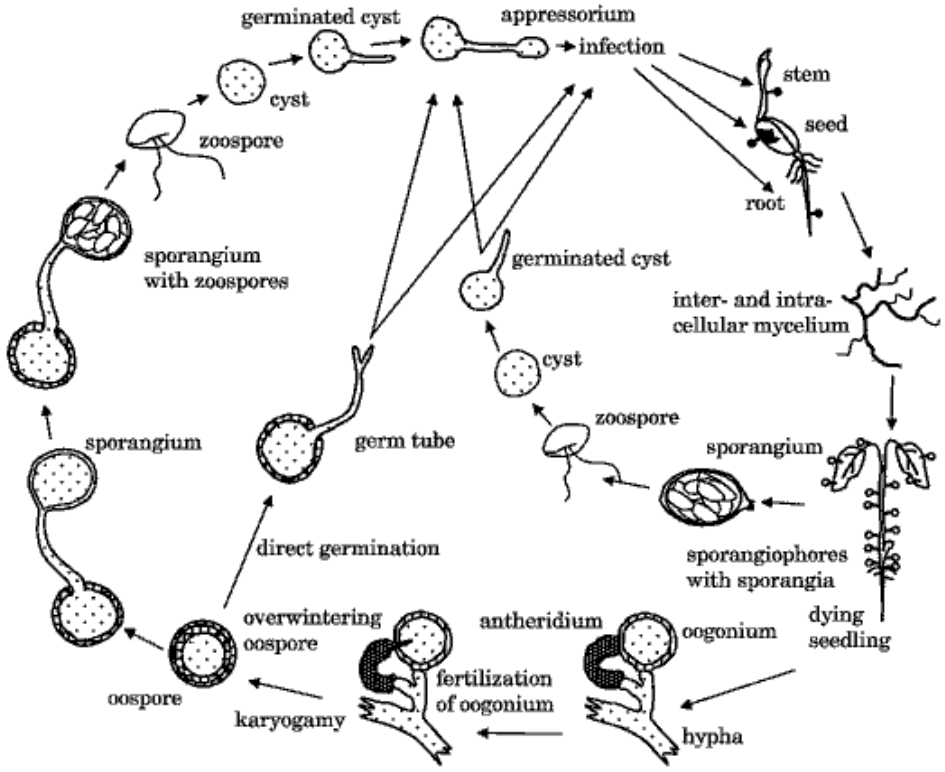


Fig. 3 Life cycle of a typical root-infecting *Pythium* species. Figure taken from Van West *et al*. (2003).

1.3.3 *Phytophthora sojae*

Phytophthora sojae is an hemibiotrophic oomycete that exhibits aggressive, race-specific pathogenicity to soybean and lupines, but causes few or no symptoms on other hosts. *P. sojae* is diploid, homothallic, preferentially self-mating (Erwin and Ribeiro, 1996) and has a genome size of 62 Mbp (Mao and Tyler, 1991). There are four major genotypes of *P. sojae*, based on restriction fragment length polymorphism (RFLP) analyses. The most common genotype (genotype I) includes the most common race (race 1) (Förster *et al*., 1994). Race 1 isolates express avirulence genes for all known *Rps* resistance genes, except *Rps7*.

P. sojae is attracted by chemotaxis to the isoflavones daidzein and genistein which are present in soybean seeds and released by the roots (Tyler *et al*., 1996; Tyler, 2002). This soil-borne pathogen produces free-swimming, unicellular zoospores at temperatures between 25 and 30°C, particularly under wet conditions. Upon reaching the root surface, the zoospores attach and transform into an adhesive cyst that germinates to produce a hypha that penetrates the plant. Soybean cultivars that carry an effective resistance gene to an attacking *P. sojae* strain carrying an avirulence gene, rapidly develop a hypersensitive response (HR) within hours after zoospore attachment. As a consequence of the resistance response, the oomycete growth is limited to the cortex and stele of lateral roots and

remains confined to the vicinity of the infection site (Beagle-Ristaino and Rissler, 1983; Enkerli *et al*., 1997). In susceptible cultivars, *P. sojae* infects lateral roots and progresses into tap roots and hypocotyls, without occurrence of an early HR. An initial biotrophic phase of about 12 h is followed by a necrotrophic, spreading growth mode causing severe, large necrotic lesions at about 24 h (Ward, 1990; Enkerli *et al.*, 1997).

1.3.4 The infection response of soybean

P. sojae interacts with soybean in a race/cultivar-specific manner, depending on corresponding *R* and *Avr* genes, as well as in a broad host resistance type. How these mechanisms contribute to plant defence is not yet clear (Tyler, 2002). Information about how soybean responds to infection has come primarily from studies of basic defence responses induced either by β-glucan elicitors from *P. sojae* or by avirulent strains of the bacterial pathogen *Pseudomonas syringae* pv*. glycinea.*

A wide variety of *Phytophthora*-derived elicitors have been identified, including carbohydrates, proteins, and small molecules (Hahn, 1996; Ebel, 1998) triggering defence responses on both, susceptible and resistant varieties of host plants. Cell wall preparations of varying degrees of complexity have been reported to be effective elicitors of defence responses in many *Phytophthora*-host interactions including *P. sojae* - soybean (Keen *et al.,* 1983), *P. infestans* - potato (Preisig and Kuc, 1985), and *P. parasitica* - tobacco (Bottin *et al*., 1994). The interaction of cell wall-derived β-glucans from *P. sojae* with soybean has been extensively characterized (Ebel, 1998; Hahn, 1996). The hyphal wall of *P. sojae* contains 1,6-linked and 1,3-branched β-glucan polysaccharides. These are released both naturally during the early stages of germination of fungal cysts and in response to β-1,3-glucanases produced by the plant (Yoshikawa *et al*., 1981; Takeuchi *et al*., 1990; Waldmüller *et al*., 1992; Okinaka *et al*., 1995). The released β-glucans interact with a binding site localized in the plasma membrane of soybean (Schmidt and Ebel, 1987; Cosio *et al.*, 1988; Cosio *et al*, 1996). The β-glucan-binding protein (GBP) of soybean has been identified (Cosio *et al*., 1992; Mithöfer *et al*., 1996; Umemoto *et al*., 1997) and shown to contain two different activities (Fliegmann *et al*., 2004). As part of the plasma membrane-localized pathogen receptor complex, it binds the cell wall elicitor, triggering the activation of defence responses (Mithöfer *et al*., 2000). Additionally, the GBP is able to hydrolyze β-1,3-glucans, present in the cell walls of the pathogen (Fliegmann *et al*., 2004; Fliegmann *et al*., 2005). After receptor-dependent recognition of β-glucans, a rapid activation of genes encoding enzymes in the phenylpropanoid pathway is induced, leading to biosynthesis of the phytoalexin glyceollin (Ebel and Grisebach, 1988). The β-glucan elicitor also induces an oxidative burst, a transient enhancement of the cytosolic Ca^{2+} concentration, a MAP kinase activation and a depolarization of the plasma membrane (Mithöfer *et al*., 1997; Mithöfer *et al*., 1999; Ebel and Mithöfer, 1998; Mithöfer *et al*., 2005).

Studies with *Pseudomonas syringae* pv*. glycinea* indicate that hydrogen peroxide accumulation triggers the cross-linking and thus strengthening of cell-wall structural proteins; this cross-linking occurs prior to the activation of defence-related genes in soybean (Brisson *et al*., 1994). Hydrogen

peroxide also stimulates an influx of calcium ions that activates a programmed cell death (Levine *et al*., 1996). Soybean has also been reported to produce chitinase, which can degrade fungal walls (Yeboah *et al*., 1998), polygalacturonase-inhibiting protein with activity against certain fungal endo-polygalacturonases (Favaron *et al.*, 1994) and an array of proteins functionally homologous to the pathogenesis-related (PR) proteins described in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* (Carr *et al*., 1985; Cao *et al*., 1994).

1.3.5 The phytoalexin pathway and glyceollin production in soybean

Phytoalexin production is one of a number of inducible plant defence reactions conferring disease resistance against microbial infections in plants. Soybean tissues produce and accumulate glyceollins (isoflavonoid phytoalexins) after either inoculation with the soybean pathogen *P. sojae,* or treatment with the β-glucan elicitor isolated from oomycete cell walls (Ayers *et al*., 1976a, b; Ebel *et al*., 1986; Ebel and Grisebach, 1988). In principle, all organs from soybean are able to produce glyceollins, e.g. roots, hypocotyls, cotyledons and leaves (Bhattacharyya and Ward, 1986a, b) as well as cell suspension cultures (Ebel *et al*., 1976). Among the isomers, glyceollin I occurs in higher amounts (Burden and Bailey, 1975) accompanied by the isomers II, III and V.

The glyceollin biosynthetic pathway consists of a series of reactions catalyzed by enzymes of general phenylpropanoid metabolism and flavonoid/isoflavonoid biosynthesis as well as of enzymes specifically involved in later steps of pterocarpan phytoalexin biosynthesis (Ebel, 1986). The enzymes of general phenylpropanoid metabolism (phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase and 4-coumarate:CoA Ligase), acetyl-CoA carboxylase and enzymes of common terpenoid pathway are involved in providing the substrates, 4-coumaroyl-CoA, malonyl-CoA, and dimethylallylpyrophosphate for pterocarpan phytoalexin biosynthesis. The reaction linking the general phenylpropanoid metabolism with the flavonoid/isoflavonoid biosynthesis is catalyzed by chalcone synthase, using 4-coumaroyl-CoA and malonyl-CoA as substrates. The central reaction of isoflavonoid biosynthesis involves a 2,3-aryl migration with a flavanone as substrate. Two enzymatic reactions utilizing pterocarpanoid substrates have been identified in soybean cell-free extracts. The 3,9-dihydroxypterocarpan 6a-hydroxylase (D6aH) converts 3,9-dihydroxypterocarpan stereospecifically to 3,6a(S),9-trihydroxypterocarpan (glycinol) in the presence of NADPH and dioxygen (Hagmann *et al*., 1984). The dimethylallylpyrophosphate 3,6a,9-trihydroxypterocarpan dimethylallyltransferase (PRT) catalyzes the formation of the 4- as well as the 2-dimethylallyltrihydroxypterocarpan intermediates in the biosynthesis of the various glyceollin isomers (Zähringer *et al.*, 1981).

1.4 Resistance genes in hosts and avirulence genes in pathogens

Resistance or susceptibility is attributed to a complex exchange of signaling cues between the pathogen and the host resulting either in colonization and disease (compatible interaction), or in recognition of the invading pathogen by the host leading to a successful defence response (incompatible interaction). In the system soybean - *P. sojae*, the primary determinants of compatibility are pathogen avirulence (*Avr*) and host resistance (*Rps*, resistance to *Phytophthora sojae*) genes.

The molecular basis by which plants carrying a resistance (*R*) gene specifically recognize pathogens carrying a corresponding *Avr* gene and activate defence is still largely unknown. Many *R* genes have been cloned (Ji *et al*., 1998; Young, 2000; Dangl and Jones, 2001; Martin *et al*., 2003).

A characteristic of many R proteins is the reiterated leucine-rich repeat (LRR) motif, which contains leucine and other hydrophobic amino acids at regular intervals. Most R proteins that contain LRR motifs, also possess a central nucleotide-binding site (NBS). Some NBS-LRR R proteins posses a putative leucine zipper (LZ) or coiled-coil sequence between the N terminus and the NBS domains. LZs are well known for their roles in homo and hetero-dimerization of eukaryotic transcription factors. Other NBS-LRR R proteins contain a large N-terminal domain called the Toll/interleukin-1/resistance domain, which have been shown to contribute to the immune response (Agrios, 1997). Several of the NBS-LRR proteins have a likely or, in some cases, proven intracellular location (Boyes *et al*., 1998; Young, 2000).

More than fourteen single dominant *R* genes against *P. sojae* have been identified in soybean (Anderson and Buzell, 1992; Buzell and Anderson, 1992). Six genes are clustered at the *Rps1* locus and three at the *Rps3* locus. Several *Rps* genes are used commercially to protect soybean against *P. sojae*, however, races of the pathogen able to infect resistant cultivars have arisen, limiting the usefulness of *Rps* genes (Schmitthener *et al*., 1994; Kaitany *et al*., 2001).

Many *Avr* genes have been cloned to date, mostly from bacteria. Cloned *Avr* genes from fungi include *Avr4*, *Avr9* and *Ecp2* from *Cladosporium fulvum* (Lauge and De Witt, 1998), *NIP1* from *Rhynchosporium secalis* (Rohe *et al*., 1995), *AvrL567* genes from *Melampsora lini* (Dodds *et al*., 2004; Catanzariti *et al*., 2006), *AvrPi-ta* (Orbach *et al*., 2000), *Avr-CO39* (Farman and Leong, 1998) and two species-specific *Avr* genes PWL1 and PWL2 (Kang *et al*., 1995) from *Magnaporthe grisea.* Cloned *Avr* genes from viruses include the coat protein of potato virus X (PVX) (Bendahmane *et al*., 1995) and the coat protein of turnip crinkle virus (TCV) (Zhao *et al*., 2000). Cloned *Avr* genes from oomycetes include *ATR13* from *Hyaloperonospora parasitica* (Allen *et al.*, 2004), *ATR1*^{NdWsB} from *H. parasitica* (Rehmany *et al*., 2005), *Avr3a* from *P. infestans* (Armstrong *et al*., 2005) as well as the race-specific avirulence gene *Avr1b* from *P. sojae* (Shan *et al*., 2004).

Genetic crosses among different races of *P. sojae* provided evidence for eight single dominant avirulence genes, *Avr1a, Avr1b, Avr1d, Avr1k, Avr3a, Avr4, Avr5* and *Avr6*. Genetic mapping has shown that some of the avirulence genes of *P. sojae* are arranged in pairs. For example *Avr1b* and *Avr1k* are inseparable in genetic crosses (Whisson *et al*., 1995).

Direct interactions between pathogen Avr proteins and plant R proteins have been demonstrated in a few cases. One example is the interaction of the tomato *Pto R* gene product with the product of the

AvrPto gene from the bacterium *Pseudomonas syringae* pv. *tomato*, which was demonstrated using the yeast-two-hybrid (YTH) system (Scofield *et al*., 1996; Tang *et al*., 1996). Another example is the interaction of the rice *Pi-ta R* gene product with the product of the *AvrPi-ta* gene from the fungus *Magnaporthe grisea*, which was demonstrated using the YTH and *in vitro* systems (Jia *et al*., 2000). However direct interaction seems to be the exception, leading to the suggestion of the "guard hypothesis" (Van der Biezen and Jones, 1998; Van der Hoorn *et al.*, 2002). The "guard hypothesis" proposes that in host-pathogen relationships no direct physical interaction between *R* and *Avr* gene products occurs and that perception of Avr proteins by their matching *R* gene products is indirect. Indirect perception implies that a third component is required. This third component could be the virulence target of an Avr protein. Binding of the Avr protein to its virulence target is proposed to be perceived by the matching R protein, which is "guarding" the virulence target. An intriguing aspect of the "guard hypothesis" is that the *Avr* gene product causes avirulence of the pathogen through interaction with its virulence target in the plant (Luderer and Joosten, 2001; Chang *et al*., 2004). Indirect Avr-R protein interaction was recently proposed for the tomato-*Cladosporium* system where Avr2 binds and inhibits the extracellular cysteine protease Rcr3 and the Rcr3-Avr2 complex subsequently enables the Cf-2 protein to activate an HR in tomato (Rooney *et al*., 2005).

1.5 The role of avirulence genes in pathogens

It has been reported that many bacterial avirulence gene products are secreted into the host cell using a molecular syringe, the conserved type III secretion system (TTSS) encoded by a group of hypersensitive response and pathogenicity (*hrp*) genes (Lahaye and Bonas, 2001). Additionally, this TTSS is found in both plant and animal pathogens for the export, secretion and often delivery of specific proteinaceous effector molecules (virulence or pathogenicity factors) directly or indirectly into host cells (Gabriel, 1999).

The idea that host-pathogen cross-talk is shaped due to evolutionary forces implies that avirulence genes should provide a selective advantage to the bacterium in susceptible plants. Indeed many bacterial avirulence genes have been shown to play a role in virulence (Kjemptrup *et al*., 2000; White *et al*., 2000). This suggests that avirulence genes in oomycetes may also play a role in virulence (Gabriel, 1999; Bonas and Van den Ackerveken, 1997). Although no TTSS has been identified yet in oomycetes it is possible that specialized systems exist for delivering avirulence proteins into host cells to manipulate defence pathways.

Avr proteins might contribute to pathogen virulence through interaction with specific plant proteins, so-called virulence targets. Interaction of Avr proteins with virulence targets that are, for example, involved in host metabolism or in plant defence, could result in enhanced nutrient availability for the pathogen or a suppression of defence responses, respectively (Van der Biezen and Jones, 1998).

Evidence is accumulating that most *avr* genes are or once were pathogenicity genes found in biotrophic pathogens. *Avr* genes may exist because of horizontal gene transfer, gene duplication or mutation (Gabriel, 1999).

One example of horizontal gene transfer may be *avrBs3*, a gene isolated from *Xanthomonas campestris* pv. vesicatoria strains. The gene resides on a self-mobilizing plasmid and was proved to

be unnecessary for pathogenicity (Bonas *et al*., 1989). Horizontal gene transfer between species is thought to occur to a greater extent than generally assumed (Shapiro, 1999).
Duplication is known for members of the *avrBs3/pthA* gene family. All members of this family present 62 bp terminal inverted repeats. The terminal 38 bp of these inverted repeats are highly similar to the 38 bp consensus terminal sequence of the Tn3 family of transposons. It is therefore possible that these genes can, or once could transpose (De Feyter *et al*., 1993).
Another event might be the loss of a particular wild plant species as host due to mutation and adaptation to new plant species, loosing functional but unnecessary genes that once assisted in pathogenicity on plant species. Evidence for non-functional relics of *Avr* genes have been reported for *Pseudomonas* and *Xanthomonas* (Kobayashi *et al*., 1990; Yang *et al*., 1996).

1.6 *Avr1b* from *P. sojae*

Map-based cloning and sequencing of *Avr1b* from *P. sojae* and the identification of two genes, *Avr1b*-1 and *Avr1b*-2, within the locus for Avr1b were performed in the group of B.M. Tyler (Shan *et al*., 2004). *Avr1b*-1 is the first genetically defined avirulence gene cloned from an oomycete and was localized to a single 60 kb bacterial artificial chromosome (BAC) clone by fine-structure genetic mapping. It was localized within the 60 kb region by identification of an mRNA expressed in a race-specific and infection-specific manner. *Avr1b*-2 was genetically mapped to the same BAC contig as *Avr1b*-1, and was shown to control the accumulation of *Avr1b*-1 mRNA. The *Avr1k* gene, required for avirulence on soybean cultivars containing the resistance gene *Rps1k*, was mapped to the same interval as *Avr1b*-1. In some isolates of *P. sojae* which are virulent on Rps1b-containing cultivars, such as P7081 (race 25, Avr1b$^-$, Avr1k$^-$) and P7076 (race 19, Avr1b$^-$, Avr1k$^-$), the *Avr1b*-1 gene has numerous substitution mutations indicative of strong divergent selection. In other isolates, such as P6497 (race 2, Avr1b$^-$, Avr1k$^+$) there are no substitutions in *Avr1b*-1, but *Avr1b*-1 mRNA does not accumulate (Shan *et al*., 2004).
Avr1b-1 encodes a small, hydrophilic protein of 117 amino acids, rich in α-helices, and containing a putative secretion signal of 21 amino acids at the N-terminus. Avr1b-1 is unusual for a secreted protein since it lacks any disulfide bonds. It shows limited sequence homology to the avirulence protein Avr3a from *P. infestans* (Armstrong *et al*., 2005).
Since *Avr1b*-1 appears to encode a secreted protein, it was proposed that the Avr1b-1 protein might act as a specific elicitor on soybean plants containing the *Rps*1b resistance gene. In support of this hypothesis, recombinant Avr1b-1 protein obtained from the culture medium of the yeast *Pichia pastoris* was infiltrated into soybean leaves and triggered a hypersensitive response observed as a severe necrosis in the leaves of *Rps*1b cultivars two to three days after infiltration. From day four onward this response spread to the mid and upper regions of the plant, beginning not with the nearest uninfiltrated but with the uppermost leaves and moving downward through the plant (Shan *et al*., 2004).

1.7 Objectives

In recent years, a multitude of gene sequences from oomycetes have been published in data bases, providing the possibility of identifying components that regulate vital processes in the pathogen such as sporulation, spore germination and virulence. *Avr1b*-1 was the first avirulence gene from an oomycete (*P. sojae*) to be cloned (Shan *et al*., 2004) and during the last two years other avirulence genes from different oomycetes e.g. *Avr3a* from *P. infestans*, *ATR13* and *ATR1*^{NdWsB} from *H. parasitica* have also been cloned (Armstrong *et al*., 2005; Allen *et al*., 2004; Rehmany *et al*., 2005). The function and properties of the proteins these avirulence genes encode, are not fully understood, therefore further research in the race-specific interaction between soybean and *P. sojae* needs to be performed.

After isolation of the avirulence gene *Avr1b*-1 from *P. sojae* (Shan *et al*., 2004), the properties, function and localization of the encoded protein needed to be investigated in infected soybean plants. Therefore the first objective of this work was the establishment of a reliable transgenic system for heterologous expression of *Avr1b*-1. Subsequently, it was necessary to produce Avr1b-1 protein in sufficient amount to enable testing its biological activity in soybean seedlings and also to raise an anti-Avr1b-antiserum for analysing the presence of Avr1b-1 after infection.

2 Material

2.1 Antibody

Anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate. (Sigma, Munich)

2.2 Plant material

Soybean cultivars Harosoy (*rps*), Harosoy 1372 (*Rps1b*) and Williams L77-1863 (*Rps1b*) were obtained from T. Anderson, Agriculture and Agri-Food, Canada.

2.3 Microorganisms

Phytophthora sojae

Phytophthora sojae races 1 ($Avr1b^+$, $Avr1k^+$, laboratory culture collection) and 2 ($Avr1b^-$, $Avr1k^+$, strain P6497 obtained from B.M. Tyler, Virginia Polytechnic Institute and State University, USA) were routinely grown on Lima bean agar plates at 25°C in the dark.

Escherichia coli

BL21(DE3) (Novagen, Darmstadt)
Genotype: F^-ompT *hsd*S_B (r_B-m_B-)*gal dcm* (DE3)

DH5α (Hanahan, 1983)
Genotype: F^- φ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_k-,m_k+) *pho*A *sup*E44 λ^- *thi gyr*A96 *rel*A1

XL10-Gold (Epicurean ultracompetent cells, Stratagene, Amsterdam)
Genotype: Kan: Tet^RΔ(*mcrA*) *183* Δ(*mcrCB-hsdsMR-mrr*) *173 endA1supE44 thi-1 recA gyrA96 relA1 lac* Hte [F' *proAB lacI*^{q} ZΔ*M15* Tn10 (Tet^R) Tn5 (Kan^R) Amy]^a

DH10Bac (Invitrogen, Karlsruhe)
Genotype: F^- *mcr*A Δ(*mmr-hsd*RMS-*mcr*BC) φ80d*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G /bMON14272 /pMON7124

Sf9 insect cells

Insect cells derived from the fall armyworm *Spodoptera frugiperda* ovarian cells (Invitrogen, Karlsruhe).

2.4 Elicitor

The raw elicitor, obtained from cell walls of *P. sojae*, was prepared through partial acid hydrolysis with trifluoroacetic acid (Hahn *et al.*, 1981; Sharp *et al.*, 1984b).

2.5 Chemicals

Common chemicals used in the laboratory were provided by the companies Roth (Karlsruhe), Sigma (Munich), Serva (Heidelberg) and Merck (Darmstadt).

List of chemicals:

α-D(+)-Glucose monohydrate	Roth (Karlsruhe)
Agar-agar	Roth (Karlsruhe)
Agarose peqGold universal	peqLab (Erlangen)
$AgNO_3$	Sigma (Munich)
Ampicilin sodium salt	Roth (Karlsruhe)
Antimycin A	Fluka (Sigma-Munich)
APS	Roth (Karlsruhe)
Bacto-peptone	Difco (Becton Dickinson, Heidelberg)
Bacto-tryptone	Difco (Becton Dickinson, Heidelberg)
Bacto-yeast-extract	Difco (Becton Dickinson, Heidelberg)
BCIP	Roth (Karlsruhe)
Bradford reagent	BIO-RAD (Munich)
Bromophenol blue	Roth (Karlsruhe)
BSA	Serva (Heidelberg)
$Ca(NO_3)_2.4H_2O$	Merck (Darmstadt)
Complete (Protease inhibitor cocktail tablets)	Roche (Penzberg)
Coomassie brillant blue R	Sigma (Munich)
CTAB	Sigma (Munich)
Cytochrome C	Sigma (Munich)
DNA peqGOLD markers	peqLab (Erlangen)
DMSO	Sigma (Munich)
DEPC	Roth (Karlsruhe)
DTT	Roth (Karlsruhe)
Cellfectin reagent	Invitrogen (Karlsruhe)
EDTA-disodium	Serva (Heidelberg)
EGTA	Roth (Karlsruhe)

Ethidiumbromide 1% solution	Roth (Karlsruhe)
Gelatine	Sigma (Munich)
Gentamycin	Invitrogen (Karlsruhe)
Glucose	Roth (Karlsruhe)
Glyceollin as standard substance	Available in the laboratory
Glycerine	Roth (Karlsruhe)
Glycine	Roth (Karlsruhe)
4-Methylamino phenol	Sigma (Munich)
4-Nitrophenylphospate disodium salt hexahydrate	Roth (Karlsruhe)
IPL41 insect cells medium	Invitrogen (Karlsruhe)
IPTG	Bio Vectra (St. Leon-Rot)
Kanamicin	Roth (Karlsruhe)
L-Arginine	AppliChem (Darmstadt)
Lima bean agar	Difco (Becton Dickinson, Heidelberg)
L-Malate	Fluka (Sigma-Munich)
β-Mercaptoethanol	Roth (Karlsruhe)
$MnCl_2.2H_2O$	Merck (Darmstadt)
β-NADPH	Sigma (Munich)
NBT	Roth (Karlsruhe)
Ni-NTA agarose	Qiagen (Hilden)
Ni-NTA-AP-conjugate	Qiagen (Hilden)
Orange G	Sigma-Aldrich (Steinheim)
Phenol/chloroform/isoamylalcohol (25:24:1)	Roth (Karlsruhe)
PMSF	Sigma (Munich)
Ponceau S	Sigma (Munich)
Prestained protein markers	New England Biolabs (Frankfurt)
Protein A Sepharose CL-4B	Pharmacia Biotech (Freiburg)
PVP-40	Sigma (Munich)
Random primers	Invitrogen (Karlsruhe)
SDS	BIO-RAD (Munich)
SF900 II insect cells medium	Invitrogen (Karlsruhe)
Slim fast	Commercially available
β-Sitosterol	Fluka (Sigma-Munich)
Sodium hypochloride solution (12% Cl)	Roth (Karlsruhe)
Spermidine	Sigma (Munich)
TEMED	Roth (Karlsruhe)
Tetracycline (HCl)	Sigma (Munich)
Trifast-reagent	peqLab (Erlangen)
Tris	Roth (Karlsruhe)
Tween 20	Roth (Karlsruhe)
Urea	Roth (Karlsruhe)

X-Gal	Roth (Karlsruhe)

2.6 Enzymes for molecular biology

*Bam*H I (10 U/µl)	MBI Fermentas (St.Leon-Rot)
*Eco*R I (10 U/µl)	MBI Fermentas (St.Leon-Rot)
Nco I (10 U/µl)	New England Biolabs (Frankfurt)
Pau I (10 U/µl)	MBI Fermentas (St.Leon-Rot)
Sac I (20 U/µl)	New England Biolabs (Frankfurt)
Xba I (10 U/µl)	MBI Fermentas (St.Leon-Rot)
Pst I (10 U/µl)	MBI Fermentas (St.Leon-Rot)
Spe I (10 U/µl)	New England Biolabs (Frankfurt)
Sph I (5 U/µl)	New England Biolabs (Frankfurt)
M-MLV Reverse transcriptase (200 U/µl)	Invitrogen (Karlsruhe)
Pfu polymerase (3 U/µl)	Promega (Mannheim)
RNasin Plus (Ribonuclease inhibitor, 40 U/µl)	Promega (Mannheim)
RQ1 RNase free DNase I (1 U/µl)	Promega (Mannheim)
T4-DNA ligase (2 U/µl)	MBI Fermentas (St.Leon-Rot)
Taq DNA polymerase (5 U/µl)	Qbiogene (Heidelberg)
RNase (10 µg/µl)	Available in the laboratory

2.7 Vectors

pET26b	Novagen (Darmstadt)
pGEM-T	Promega (Mannheim)
pFastBac1	Invitrogen (Karlsruhe)

2.8 Plasmids

pTZ18-Avr1b containing the full length-cDNA encoding Avr1b, was obtained from B.M. Tyler, Virginia Polytechnic Institute and State University, USA.

pET26b-Avr1b containing the full length-cDNA encoding Avr1b, including a periplasmic localization sequence (*pelB*) at the N-terminus and a His_6-sequence at the C-terminus was prepared by D. Becker (Botanical Institute, Munich).

pQE-30/*Gm*4CL1, pQE-30/*Gm*4CL2, pQE-31/*Gm*4CL3, and pQE-30/*Gm*4CL4 (Lindermayr *et al*., 2002) were provided by Dr. M.V. Silber (Botanical Institute, Munich).

2.9 Oligonucleotides

	Sequence
Avr1b_PauI-1	5´-TATGCGCGCCACCATGCGTCTATCTTTTG-3´
Avr1b_PauI-2	5´ TATGCGCGCCACCATGGCAACTGAGTACTC-3´
T7-promoter	5´-TAATACGACTCACTATAGGG-3´
Avr1b-Sac I	5´-GC[GTCGAC]CATGCGTCTATCTTTTGTGC-3´
Avr1b-Not I	5´-TA[GCGGCCGC]TCTGATACCGGTGAAA-3´
Avr1b_for	5´-CCAAGTATCACGAACCATGCG-3´
Avr1b_rev	5´-TGCGATTTCGGCGAGACC-3´
ActA_for	5´-TCATGGTCGGCATGGACCA-3´
ActA_rev	5´-GGCCGTGGTCGTGAACGAG-3´
GmTubB2_for	5´-GTGACTTGAACCATCTGATCTCAGC-3´
GmTubB2_rev	5´- GTTGAAGCCATCCTCAAGCCAG-3´
Cyp93a1_for	5´-GCAAGAAAAACCTTCCACCAAGT-3´
Cyp93a1_rev	5´-AAACGCGAAAAGGAAGTCTTGGG-3´
4CL3_for	5´-ACGGTAGCTGCTTCTCTTGATGC-3´
4CL3_rev	5´-GTCGTCGACGGTCACAACCTT-3´
SP6	5´-ATTTAGGTGACACTATAGAATAC-3´

2.10 Kits for molecular biology

NucleoSpin Extract II kit	Macherey-Nagel (Düren)
BAC-TO-BAC Baculovirus Expression System	Invitrogen (Karlsruhe)
RNeasy Plant Mini Kit	Qiagen (Hilden)

2.11 Instruments

Autoclave SANOclav	Wolf (Geislingen)
Centrifuge Minifuge 5415D	Eppendorf (Hamburg)
Centrifuge 3K18 with rotors Nr. 11133 and Nr. 12154-H	Sigma (Osterode am Harz)
Centrifuge RC-5B Plus, with rotors: SS-34, GS-3, GSA	Sorvall (Hanau)
C10 column and AC10 adaptor for chromatography	Pharmacia Biotech (Freiburg)
Culture flasks T-25, T-75 and T-150	CellStar (Frickenhausen)
Dialysis bags, MCWO 8 000-10 000, vol/cm 3,27 ml	Roth (Karlsruhe)
Electrophoresis apparatus, Bio-Rad minigel	BIO-RAD (Munich)
Evaporator Rotavapor	Büchi (Flawil, Switzerland)
Filters Millex-HV 4 mm	Millipore (Eschborn)
FPLC, with pumps P-500, Superloop, LKV UV-MII, conductivity monitor, controller LCC-501 Plus, fraction collector	Pharmacia Biotech (Freiburg)

HPLC 2690 with Photodiode array detector 474	Waters (Eschborn)
Microscope Axiovert 25, objectives: CP-Akromat 10x/0,2 Ph1; LD-Achrostigmat 20x/0,30 Ph1; LD-Achrostigmat 40x/0,55 Ph2	Zeiss (Jena)
L8-55M ultracentrifuge with rotor: SW28	Beckman (Munich)
Nitrocellulose transfer membranes	Pall Gellman Sciences (VWR International, Ismaning)
Ni-NTA superflow matrix	Qiagen (Hilden)
Optima^{TM} TLX ultracentrifuge with rotor TLA 100.4	Beckman (Munich)
PCR Thermocycler UNOII	Biometra (Göttingen)
pH-meter Calimatic	Knick (Berlin)
Peristaltic pump miniplus 2	Gilson (Villiers le Bel, France)
Growth chamber	Weiss Umwelttechnik (Reiskirchen-Lindenstruth)
Reacti-Therm heating/stirring module	Pierce (Bonn)
RP-column Nova Pak C_{18}, 4,6x150 mm; 4 µm	Waters (Eschborn)
Sonifier (Model 250/450)	Branson Ultrasonics Corporation (USA)
Speedvac	Bachofer (Reutlingen)
Spectrophotometer Ultrospec 3000	Pharmacia Biotech (Freiburg)
Sterile bench	Ehret (Emmendingen)
Vermiculite	Deutsche Vermiculite Dämmstoff GmbH (Sprockhövel)
Western blot LKB-multi Drive XL instrument	Pharmacia Biotech (Freiburg)

2.12 Software and internet resources

Millenium^{32} software	Waters (Eschborn)
Sigma Plot 8.0	Jandel Scientific (Erkrath)
ISIS Draw 2.4	Silicon Graphics (Munich)
Advanced BLAST search	NCBI (Bethesda, USA)
MultAlin MultipleSequenceAlignment	INRA (Toulouse, France)

2.13 Media, buffers and other solutions

Lima bean agar

2,3% (w/v) Lima bean agar, autoclaved.

LB medium

1% (w/v) bacto-tryptone, 0,5% (w/v) bacto-yeast-extract, 1% (w/v) NaCl. For plates 1,5% (w/v) agar-agar. Adjusted to pH 7,5; autoclaved. Antibiotics added prior to use.

SOC^{+++} medium

2% (w/v) bacto-tryptone, 0,5% (w/v) bacto-yeast-extract, 10 mM NaCl, 2,5 mM KCl, adjusted to pH 7,0; autoclaved. 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose added prior to use. Store at -20°C.

Modified Erwin synthetic medium

D(+)-Sucrose	15 g/l
L-Asparagin	2 g/l
$FeSO_4.7H_2O$	1 mg/l
$MgSO_4.7H_2O$	0,2 g/l
$CaCl_2.2H_2O$	0,01 g/l
Thiamine-HCl	1 mg/l
K_2HPO_4	1,9 g/l
KH_2PO_4	1,04 g/l
$ZnSO_4.7H_2O$	4,39 mg/l
$CuSO_4.5H_2O$	79 µg/l
$Na_2MoO_4.2H_2O$	53 µg/l
$MnCl_2.2H_2O$	70 µg/l
β-Sitosterol	0,05 g/l
$CaCO_3$	6,25 g/l (not soluble)

Buffers for insect cell fractionation

1x buffer N: 10 mM Tris-HCl (pH 7,0), 150 mM NaCl, 5 mM $MgCl_2$, 40 mM KCl

1x PBS buffer: 40 mM Na_2HPO_4, 8 mM NaH_2PO_4, 150 mM NaCl, pH 7,4

Buffer C: 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 7,0)

Buffer for harvesting of *E. coli* cells

Sonication buffer: 50 mM Tris-HCl (pH 8,0), 200 mM NaCl, 15 mM EDTA, 100 µM PMSF

Buffers for protein purification under denaturing conditions

Buffer A: 0,1 M NaH_2PO_4, 0,01 M Tris-HCl, 8 M urea, pH 8,0

Wash buffer: 0,1 M NaH_2PO_4, 0,01 M Tris-HCl, 8 M urea, pH 7,0

Elution buffer I: 0,1 M NaH_2PO_4, 0,01 M Tris-HCl, 8 M urea, pH 5,9

Elution buffer II: 0,1 M NaH_2PO_4, 0,01 M Tris-HCl, 8 M urea, pH 4,5

Dialysis buffer: Tris-HCl (pH 7,5), 1 mM EDTA, 0,5 M arginine, 20% glycerol

Buffers for protein refolding

Buffer X: 7 M urea, 0,5 M NaCl, 20% (v/v) glycerol, 20 mM Tris-HCl (pH 7,4), 0,5 mM PMSF

Buffer 1A: 0,5 M NaCl, 20% (v/v) glycerol, 20 mM Tris-HCl, 0,5 mM PMSF. Adjusted to pH 7,4 and degased before use on the FPLC.

Buffer 1B: 0,5 M NaCl; 20% (v/v) glycerol, 20 mM Tris-HCl, 0,5 mM PMSF, 7 M urea. Adjusted to pH 7,4 and degased before use.

Buffer 2A: 50 mM NaH_2PO_4, 300 mM NaCl. Adjusted to pH 7,0 and degased before use on the FPLC.

Buffer 2B: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole. Adjusted to pH 7,0 and degased before use.

Equilibration buffer: 50 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl

Buffer for production of anti-Avr1b-antiserum

1x PBS buffer: 40 mM Na_2HPO_4, 8 mM NaH_2PO_4, 150 mM NaCl, pH 7,4

Buffers for purification of anti-Avr1b-antiserum with protein A sepharose

Binding buffer: 50 mM Tris-HCl, pH 7,0, Rt

Elution buffer: 0,1 M glycine pH 3,0, Rt

Solutions for SDS-PAGE

1x SDS loading buffer: 0,05 M Tris-HCl (pH 6,8), 4% (w/v) SDS, 2 mM DTT, 20% (v/v) glycerin, 0,02 mM PMSF, 0,02 mM aminocaproic acid, $5x10^{-3}$ µg/ml leupeptin, $5x10^{-3}$ µg/ml aprotinin, 0,01% (w/v) bromophenol blue

3x SDS loading buffer: 0,15 M Tris-HCl (pH 6,8), 12% (w/v) SDS, 6 mM DTT, 20% (v/v) glycerin, 0,02 mM PMSF, 0,02 mM aminocaproic acid, $5x10^{-3}$ µg/ml leupeptin, $5x10^{-3}$ µg/ml aprotinin, 0,01% (w/v) bromophenol blue

5x SDS loading buffer: 0,25 M Tris-HCl (pH 6,8), 20% (w/v) SDS, 10 mM DTT, 20% (v/v) glycerin, 0,02 mM PMSF, 0,02 mM aminocaproic acid, $5x10^{-3}$ µg/ml leupeptin, $5x10^{-3}$ µg/ml aprotinin, 0,01% (w/v) bromophenol blue

Stacking gel: 6% (v/v) acrylamide, 0,1291 M Tris-HCl (pH 6,8), 0,1% (w/v) SDS, 0,08% APS, 0,4% TEMED

Separating gel: 17,4% (v/v) acrylamide, 0,369 M Tris-HCl (pH 8,8), 0,1% (w/v) SDS, 0,065% APS, 0,1% TEMED

Running buffer: 192 mM glycine, 25 mM Tris-HCl (pH 8,3), 0,1% (w/v) SDS

Solutions for silver staining

Fixative solution: 40% ethanol, 10% acetic acid

Wash solution: 30% ethanol

Thiosulfate reagent: 0,02% sodium thiosulfate

Silver nitrate reagent: 0,2% silver nitrate, 0,02% formaldehyde (37%)

Developing solution: 3% sodium carbonate, 0,05% formaldehyde (37%), 0,0005% sodium thiosulfate

Stop reagent: 0,5% glycine

Solutions for Coomassie staining

Coomassie solution: 0,1% (w/v) Coomasie Brilliant Blue R-250; 40% (v/v) methanol; 10% (v/v) acetic acid glacial

Destaining solution: 40% (v/v) methanol; 10% (v/v) acetic acid glacial

Solutions for semi-dry Western blotting

Anode buffer I: 300 mM Tris, 20% (v/v) methanol

Anode buffer II: 25 mM Tris, 20% (v/v) methanol

Cathode buffer: 40 mM aminocaproic acid, 0,01% (w/v) SDS, 20% (v/v) methanol

Ponceau S solution: 1% (w/v) Ponceau S solution in 2% (v/v) acetic acid glacial

Solutions for detection with anti-Avr1b-antiserum

TBS buffer: 150 mM NaCl, 1 mM $MgCl_2$, 50 mM Tris-HCl, pH 7,8

TBS-Tween buffer: TBS + 0,05% (v/v) Tween 20

G-Net: 0,25% gelatine, 0,15 M NaCl, 5 mM EDTA (pH 8,0), 0,05% Triton X 100

NBT: 100 mg/ml in 70% (v/v) DMF

BCIP: 50 mg/ml in 100% (v/v) DMF

AP buffer: 33 µl NBT and 33 µl BCIP in 10 ml 100 mM Tris-HCl (pH 9,5), 100 mM NaCl, 5 mM $MgCl_2$

Buffers for immunoprecipitation

Denaturing buffer: 7 M urea, 0,5 M NaCl, 20% glycerol, 20 mM Tris-HCl (pH 7,5), 1x protease inhibitor cocktail

Extraction buffer I: 30 mM $MgCl_2$, 5 mM EDTA, 100 mM NaCl, 50 mM Tris-HCl (pH 7,5), 1x protease inhibitor cocktail

Dilution buffer: 1,25% Triton X 100, 190 mM NaCl, 6 mM EDTA, 60 mM Tris-HCl (pH 7,5), 1x protease inhibitor cocktail

Suspension of PAS: 0,05 mg PAS in 0,45 ml extraction buffer I

Buffers for agarose gel electrophoresis

1x Agarose loading buffer: 30% (v/v) glycerin, 1xTAE, 2,5 mg/ml orange G

1x TAE buffer: 40 mM Tris, 2 mM Na-EDTA, 0,11% (v/v) acetic acid

Solutions for minipreparation

Solution I: 50 mM D-glucose, 25 mM Tris-HCl (pH 8,0), 10 mM EDTA, autoclaved

Solution II: 1% (w/v) SDS, 0,2 N NaOH (should be prepared fresh each time)

Solution III: 3 M potassium-acetate pH 5,5

Solution IV: 15 mM Tris-HCl (pH 8,0), 10 mM EDTA, 100 µg/ml RNase A (should be prepared fresh each time)

TE buffer: 10 mM Tris-HCl (pH 8,0), 1 mM EDTA

Buffers for PCR, colony PCR and RT-PCR

10x *Pfu*-polymerase buffer: 200 mM Tris-HCl (pH 8,8), 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1,0% Triton X 100, 1 mg/ml BSA, 20 mM $MgSO_4$

PCR solution: 10 µl solution contains 10 ng template DNA, 0,6 units *Pfu*-polymerase, 0,2 mM dNTPs, 10 pmol of each specific forward and reverse strand primers in 1x *Pfu*-polymerase buffer

10x *Taq*-DNA-polymerase buffer: 100 mM Tris-HCl (pH 9,0), 2 mg/ml BSA, 15 mM $MgCl_2$, 500 mM KCl, 1,0% Triton X 100

Colony PCR solution: 8 µl solution contains 0,25 units of *Taq*-DNA-polymerase, 0,2 mM dNTPs, 8 pmol of each specific forward and reverse strand primers, 2,5 mM $MgCl_2$ in 1x *Taq*-DNA-polymerase buffer

5x First-strand-buffer: 15 mM $MgCl_2$, 250 mM Tris-HCl (pH 8,3), 375 mM KCl

RT-PCR solution I: 20 µl contains 200 units of M-MLV reverse transcriptase, 80 units of RNasin RNase inhibitor, 80 ng random primers, 1 mM dNTPs, 10 mM DTT in 1x first-strand-buffer

RT-PCR solution II: 10 µl contains 0,25 units of *Taq*-DNA-polymerase, 10 pmol of each specific forward and reverse primers, 2,5 mM $MgCl_2$ in 1x *Taq*-DNA-polymerase buffer. In the case of *4CL3*, 2 pmol of each specific forward and reverse primers and 1,5 mM $MgCl_2$ were used.

Buffer for DNase treatment

DNase treatment solution: 400 µl contains 40 units RNasin RNase inhibitor, 1 unit DNase/µg RNA in 1x DNase buffer

10x DNase buffer: 400 mM Tris-HCl (pH 8,0), 100 mM $MgSO_4$, 10 mM $CaCl_2$

Solution for A-tailing procedure

A-tailing solution: 5 µl solution contains 0,5 µl 2 mM dATP, 2,5 units of *Taq*-DNA-polymerase, 2,5 mM $MgCl_2$ in 1x *Taq*-DNA polymerase buffer

Buffers for ligation

10x Ligase buffer: 300 mM Tris-HCl (pH 7,8), 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP

Ligation solution: 5 solution contains DNA vector, insert, 1 unit T4-DNA-ligase in 1x ligase buffer

Buffer for subcellular fractionation of *P. sojae* mycelium

Grinding buffer: 20 mM HEPES/KOH (pH 7,5), 5 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 250 mM sucrose, 2 mM PMSF, 2 mM DTT

Lysis buffer: 50 mM Tris-HCl (pH 7,5), 250 mM sucrose, 5 mM EDTA, 2 mM DTT, 2 mM PMSF, 2 µg/ml leupeptin

AB buffer: 10 mM Tris-HCl (pH 7,0), 150 mM NaCl, 5 mM $MgCl_2$

Buffer for subcellular fractionation of infected soybean seedlings

Extraction buffer II: 50 mM Tris-HCl (pH 7,5), 5 mM EDTA, 30 mM $MgCl_2$, 0,5 mM PMSF

Solutions for RNA isolation from *P. sojae* mycelium

RNA extraction buffer: 2% (w/v) CTAB, 2% (w/v) PVP, 100 mM Tris-HCl (pH 8,0), 2 M NaCl, 25 mM EDTA, 0,25 g/l spermidine, autoclaved. 2% (v/v) β-mercaptoethanol added prior to use.

SSTE: 1 M NaCl, 0,5% (w/v) SDS, 10 mM Tris-HCl (pH 8,0), 1 mM EDTA, autoclaved.

DEPC-water: 0,1% (v/v) DEPC in distilled water, stirred overnight in a fume-hood, autoclaved.

3 Methods

3.1 Soybean cultures

Soybean seeds were sterilized in 10% sodium hypochloride solution for 10 min and washed several times with distilled water. Afterwards the seeds were planted in autoclaved vermiculite and poured with autoclaved water. After growing 3 to 12 days in a growth chamber with a 16 h (26°C) day and a 8 h (22°C) night period at 65% relative humidity, seedlings were harvested and the vermiculite removed.

3.2 *Phytophthora sojae* cultures

3.2.1 Growth on solid medium

Phytophthora sojae races 1 and 2 were grown on lima bean agar plates. For inoculation of new plates, approximately 1 cm^2 of the previous culture was transferred to a fresh plate with the mycelium being in contact to the agar. Plates were incubated for 7 days at 24°C in the dark when used for maintaining the line or 12 days when used for inoculation of liquid medium or for production of zoospores. Cultures on solid medium can be stored up to 8 weeks at 4°C.

3.2.2 Growth on liquid medium

Growth of *P. sojae* in liquid medium was performed as described by Erwin and Katznelson (1961), Keen (1975), Ayers *et al.* (1976, IX), Antelo (1998). 400 ml of modified Erwin synthetic medium (2.13) was autoclaved in a 1,8 l Fernbach flask. One third of a 12 day-old culture plate was used for inoculation of the medium. Incubation was carried out at 24°C in the dark without stirring. After one week the flask was shaken gently in order to break slightly the mycelium for stimulating further growth. The culture was incubated for 3 weeks if used for hypocotyl assays, or 2 to 6 weeks if used for RNA extraction.

3.3 Insect cell culture

3.3.1 Revival of frozen insect cells

Frozen *Sf9* insect cells were thawed by rapid agitation in a 37°C water bath. As soon as the frozen medium with the cells was thawed, the vial was removed from the water bath and immersed in 70% ethanol at Rt. Cells were then transferred to a cold (4°C) pre-wetted T-25 culture flask containing 5 ml IPL41 + 5% FBS -medium and incubated for 30 min at 28°C for cell attachment. Afterwards the

medium was removed and replaced with 5 ml of fresh medium. After overnight incubation at 28°C, the medium was removed again and replaced with 5 ml of fresh medium, incubated for at least 7 days at 28°C while changing the medium every 72 h.

3.3.2 Maintenance of the insect cell culture

After incubation for at least 7 days (3.3.1) the medium was replaced with 5 ml of fresh IPL41 + 5% FBS medium. Using a pipette the medium was drawn up in a side to side motion removing the cells. Once the cells have been removed they were diluted 1:2 (i.e. 50% cells were transferred to a new T-25 culture flask) in IPL41 + 5% FBS medium. After the cells have been transferred 3 to 4 times, they were diluted 1:10 into a new T-25 flask and incubated for 4 days at 28°C in the dark.

3.3.3 Transfection of insect cells with recombinant bacmid DNA

For each transfection, solution A (5 µl of mini-prep bacmid DNA in 100 µl SF-900 II SFM) was combined with solution B (6 µl cellfectin lipid reagent in 100 µl SF-900 II SFM) producing a lipid-DNA complex, mixed gently and incubated in 1,5 ml reaction tubes for 30 min at Rt. Afterwards 0,8 ml of SF-900 II SFM medium was added to each reaction tube containing the lipid-DNA complexes. In parallel $9x10^5$ insect cells in IPL41 + 5% FBS medium were placed in several 35-mm wells each and allowed to attach to the bottom of the wells for 1 h at 28°C. The IPL41 + 5% FBS medium was then removed and the cells were covered with around 1 ml of the lipid-DNA complexes from the reaction tubes in order to incorporate the bacmid DNA. The cells were incubated for 4 h at 28°C and the SF-900 II SFM medium with the lipid-DNA complexes (transfection mixtures) was removed and replaced with 2,5 ml of fresh SF-900 II SFM medium. Then the cells were incubated for another 72 h at 28°C and the cell culture medium was transferred to a 15 ml falcon tube and centrifuged (500xg, Rt, 5 min). The baculovirus-containing supernatant (P1) was transferred to a new 15 ml falcon tube and stored at 4°C.

3.3.4 Infection of insect cells with recombinant baculovirus particles

Insect cells ($2x10^6$) in IPL41 + 5% FBS medium were incubated in a T-25 culture flask for 30 min at 28°C. After attachment of the cells to the flask the IPL41 + 5% FBS medium was removed and replaced with 2,3 ml SF-900 II SFM medium containing 0,3 ml baculovirus particles (P1). The cells were incubated for 1 h at 28°C. Afterwards 2,7 ml SF-900 II SFM medium were added and the cells were incubated for another 48 h at 28°C. After the incubation the cell culture medium was centrifuged (500xg, Rt, 5 min). The baculovirus-containing supernatant was transferred to a 15 ml falcon tube and stored at 4°C. This stock of baculovirus particles (P2) was used for amplification.

3.3.5 Amplification of recombinant baculovirus particles

Insect cells ($4x10^6$) in IPL41 + 5% FBS medium were transferred from a T-25 to a T-75 culture flask and incubated for 30 min at 28°C. After attachment of the cells to the flask the IPL41 + 5% FBS medium was removed and replaced with 2 ml SF-900 II SFM medium containing 10,5 µl baculovirus particles (P2) and the cells were incubated for 1 h at 28°C. Afterwards 8 ml of fresh SF-900 II SFM medium were added and the cells were incubated for another 48 h at 28°C. After incubation the cell culture medium was centrifuged (500xg, Rt, 5 min). The supernatant was transferred to a 15 ml falcon tube and stored at 4°C. This amplified stock of baculovirus particles (P3) was used for further infection experiments.

3.3.6 Insect cell fractionation

Insect cells ($8x10^6$) in IPL41 + 5% FBS medium were incubated in a T-150 culture flask for 30 min at 28°C. After attachment of the cells to the flask the IPL41 + 5% FBS medium was removed and replaced with 8 ml SF-900 II SFM medium containing 400 µl baculovirus particles (P3). The cells were incubated for 1 h at 28°C. Another 10 ml of SF-900 II SFM medium were added and the cells incubated for 120 h at 28°C. After incubation the cell culture medium was transferred in 15 ml falcon tubes for centrifugation (500xg, Rt, 5 min). A fraction (1 ml) of the supernatant ("culture medium" fraction) was precipitated with TCA (3.8.2) before SDS-PAGE and the rest was dialyzed against 50 mM NaH_2PO_4 (2x1 h followed by 1x16 h, 4°C). This "culture medium" fraction was either frozen and stored at -80°C or used immediately for biotests.

The infected insect cells attached to the flask were used for further fractionation as follows:

In the first case, cells were washed with 5 ml PBS, centrifuged (500xg, Rt, 5min), preswollen in 1 ml buffer C and lysed by homogenization for 10 min on ice. 1 ml 2x buffer N was added to reach a final concentration of 1x buffer N and centrifuged for 5 min at 300xg (4°C), obtaining a pellet and a supernatant (S1). The pellet was resuspended in 1 ml 0,2 M sucrose and the nuclei was pelleted through a 2 ml 1,6 M sucrose cushion (in 1x buffer N) for 45 min at 44 000xg, 4°C. The pellet was washed with 1% Triton X 100 in 1x buffer N, centrifuged (300xg, 4°C, 5 min) and the pellet resuspended in 300 µl 1x buffer N ("nuclei" fraction).

The supernatant (S1) was centrifuged again (300xg, 4°C, 5 min) and the pellet obtained after centrifugation was discarded. Following addition of 20 µl 0,5 M EDTA, the supernatant was centrifuged (10 000xg, 4°C, 10 min). The pellet obtained after centrifugation contains heavy membranes and was resuspended in 300 µl 1x buffer N ("heavy membrane" fraction). The supernatant was centrifuged (150 000xg, 4°C, 1 h) and the pellet obtained was resuspended in 100 µl 1x buffer N ("light membrane" fraction). The supernatant was also collected ("cytosolic" fraction).

In the second case infected insect cells were washed with 5 ml PBS, centrifuged (500xg, Rt, 5 min), preswollen in 1 ml buffer C and lysed by homogenization for 10 min on ice. One ml of 2x buffer N was added to reach a final concentration of 1x buffer N and centrifuged (29 700xg, 4°C, 10 min). The

pellet (“crude membrane” fraction) obtained after centrifugation was resuspended in 500 µl 1x buffer N. The supernatant was also collected (“soluble protein” fraction).
Measurement of protein concentration (3.8.1) was performed with all fractions obtained except of the “culture medium” fraction and 30 µg of protein were precipitated with 1 ml methanol (3.8.2) before SDS-PAGE.

3.4 Expression of *Avr1b* in *E. coli* cells

3.4.1 Growth conditions

Glycerol cultures of BL21(DE3)pET26b and BL21(DE3)pET26b-Avr1b were thawed at Rt and transferred to LB agar plates containing 30 µg/ml kanamicin with a sterile tip and incubated overnight at 37°C. The procedure described below was performed for each of the two cultures: the next day 5 ml LB medium containing 30 µg/ml kanamicin were inoculated with a single colony from the LB agar plate and incubated overnight at 37°C in a rotary shaker (180 rpm). The next day 49 ml LB medium containing 30 µg/ml kanamicin were added to 1 ml of the overnight culture and incubated for 60-90 min at 37°C (180 rpm). After incubation the OD_{600} was determined by diluting 200 µl of the culture with 800 µl LB medium (OD_{600} should be in the range between 0,5 and 1,0). A 1 ml aliquot was stored (-80°C) for further SDS-PAGE (“non-induced control”). For induction, IPTG was added to the culture to a final concentration of 1 mM. The culture was incubated for 2 h at 37°C (180 rpm). After induction and just prior to harvest another 1 ml aliquot was stored (-80°C) for further SDS-PAGE (“induced control”). The OD_{600} was determined by diluting 100 µl of the culture with 900 µl of LB medium (OD_{600} should be in the range between 0,8 and 1,0). Cells with an OD_{600} of 1,0 from the non induced and the induced control were centrifuged (5 000xg, Rt, 30 sec) and the pellets stored at -80°C. Prior to electrophoresis, the pellets were thawed and resuspended in 40 µl 1x SDS loading buffer (2.13). 10 µl were applied to SDS-PAGE.

3.4.2 Harvest

After induction with IPTG (3.4.1), the rest of the two cultures was centrifuged (2 500xg, 4°C, 5 min) and the pellets resuspended in 5 ml sonication buffer (2.13). Sonication on ice took place 2 times for 5 min, at 32 watt and 50% duty. Subsequently the cultures were centrifuged (15 500xg, 4°C, 10 min) and the pellets stored at -20°C for further SDS-PAGE. The pellet from the BL21(DE3)pET26b-Avr1b culture was also used for further Avr1b purification under denaturing conditions or for protein refolding as described below.
For SDS-PAGE the pellets were resuspended in 5 ml of a solution containing 100 mM Tris and 10 mM EDTA and kept in 100, 500 and 1 000 µl aliquots. One of the 100 µl aliquot from each culture was centrifuged (10 000xg, 4°C, 5 min), the pellet resuspended in 100 µl water and the protein concentration measured, 20 µg of protein was used for SDS-PAGE.
For purification of Avr1b under denaturing conditions, the pellet was resuspended in 4 ml buffer A (2.13), centrifuged (29 700xg, 4°C, 15 min) and the supernatant (4 ml lysate) was further used

(3.5.1). For purification of Avr1b after refolding, the pellet was resuspended in 8 ml buffer X (2.13). In case of large scale procedure, 40 ml of lysate were used (3.5.2).

3.5 Purification of Avr1b

Protein purification was performed according to the Qiagen protocol.

3.5.1 Protein purification under denaturing conditions by batch chromatography

A suspension (0,8 ml) of 50% Ni-NTA agarose beads in 30% ethanol was slowly poured into a 12 ml column. When the agarose matrix was settled (0,4 ml bed volume) the column was equilibrated with 2x10 ml of distilled water and then with 2x4 ml of buffer A (2.13). The lysate (4 ml) (3.4.2) was applied on the column and the flow through collected. Afterwards 2x4 ml of wash buffer (2.13) were added and the flow through collected (fractions W1 and W2). Bound proteins were eluted first by addition of 4x0,5 ml elution buffer I (2.13) (elution samples EI1, EI2, EI3, EI4 were collected) and second with 4x0,5 ml elution buffer II (2.13) (elution samples EII1, EII2, EII3, EII4 were collected). 5 µl of 3x SDS loading buffer were added to 15 µl of each sample. Samples were heated for 5 min at 100°C and loaded on a gel for SDS-PAGE. The rest of the elution fractions EI2, EI3 and EI4 were combined and dialyzed against dialysis buffer (2.13) (4x1 h followed by 1x16 h) before being used in a biotest.

3.5.2 Protein refolding by FPLC

A suspension (6 ml) of 50% Ni-NTA superflow resin in 30% ethanol was slowly poured into a C10 column avoiding air bubbles. When the resin was settled (3 ml bed volume), the top adapter was inserted and adjusted to the top of the bed. The column was adapted to the FPLC system and equilibrated with 5 bed volumes of buffer 1B (15 ml; 2.13) at a flow rate of 0,5 ml/min. The lysate (40 ml; 3.4.2) was injected in a loop leading to the Ni-NTA column and the flow through collected (sample “0”). For protein refolding a linear 7-1 M urea gradient in buffer 1A (2.13) was used. The renaturation was performed over a period of 90 min. Two 22,5 ml samples were collected sample “I” (0-45 min) and sample “II” (45-90 min), respectively. After renaturation the column was equilibrated with 15 ml equilibration buffer and a 15 ml fraction was collected (sample “III”). In order to elute the protein a three step imidazole gradient was performed with buffers 2A and 2B over a period of 40 minutes (table 1) and twenty 1 ml fractions were collected (E1-E20). After the imidazole gradient, the column was equilibrated with buffer 2B during 40 min and the last 20 ml fraction was collected (fraction “IV”). Each sample (400 µl) was precipitated with 1,6 ml methanol (3.8.2) before SDS-PAGE. The rest of the samples were used for measurement of protein concentration (3.8.1). Fractions with protein concentrations of more than 20 µg/ml were combined and either stored in 5% glycerol at -80°C for further production of anti-Avr1b-antiserum or used immediately for biotests.

Table 1. Three step elution program of 6xHis-tagged Avr1b protein with imidazole

Time (min)	Buffer 2A (%)	Buffer 2B (%)	Final concentration of imidazole (mM)
0	80	20	50
10	80	20	50
12	28	72	180
20	28	72	180
22	0	100	250
40	0	100	250

3.6 Production of anti-Avr1b-antiserum

Protein refolding (3.5.2) was repeated 8 times in order to collect sufficient amounts of protein and the fractions were combined. The protein concentration was measured and the sample lyophilised. Afterwards 2 mg Avr1b was resuspended in 2 ml 1x PBS (2.13) and aliquoted in 0,2 ml fractions. Anti-Avr1b-antiserum was raised in rabbits at SEQLab.

3.7 Purification of anti-Avr1b-antiserum with protein A sepharose CL-4B by batch chromatography

Protein A sepharose CL-4B (0,2 g) was suspended in 1 ml distilled water and washed with 30 ml distilled water added in several aliquots for 15 min on a sintered glass filter. A slurry was prepared using binding buffer (2.13) in a ratio of 75% settled slurry to 25% buffer and de-gased. The slurry was poured into the column avoiding air bubbles. The column was filled immediately with binding buffer (2.13) and connected to a peristaltic pump. The bottom outlet of the column was opened and the pump set to a 0,5 ml/min flow rate. The column was equilibrated with 5 ml binding buffer, then 1 ml anti-Avr1b-antiserum (approximately 90 mg/ml) was added and finally washed with 5 ml binding buffer. Anti-Avr1b-antiserum was eluted from the column with 3 ml elution buffer (2.13). Aliquots were collected (E1 to E6) in 1,5 ml reaction tubes containing 25 µl 1 M Tris-HCl (pH 9,0) and the protein concentrations were measured. Samples E2 to E5 being highest concentrated were combined, measured again (0,98 mg/ml) and stored in 0,3 ml aliquots at -80°C for further Western blot analyses.

3.8 Basic methods for protein biochemistry

3.8.1 Protein estimation

Protein concentration was determined with the method described by Bradford (1976) using the BioRad protein assay reagent. Five µl of each sample were diluted with 795 µl distilled water and 200 µl of the reagent, mixed and the absorbance was measured at 595 nm after 5 min incubation.

3.8.2 Protein precipitation

Samples were either incubated overnight with 3 volumes of cold methanol at -80°C or for 1 h on ice with 1/10 vol 100% TCA (final concentration of TCA 10%). Methanol-precipitated proteins were centrifuged (29 000xg, 4°C, 15 min) and the pellets resuspended in 20 µl 1x SDS loading buffer (2.13). TCA-precipitated proteins were also centrifuged (14 000xg, 4°C, 10 min), the pellets were washed two times with 100 µl acetone each (14 000xg, 4°C, 5 min), dried for 30 min in a speed vac and resuspended in 20 µl 1x SDS loading buffer (2.13).

3.8.3 SDS-PAGE

For protein separation by SDS-PAGE, the method of Laemmli (1970) was used. Gels were poured to a size of 7x11 cm and 1 mm thickness in a BioRad minigel apparatus. Samples dissolved in 1x SDS loading buffer were incubated for 5 min at 100°C, centrifuged for 1 min at Rt and then loaded with a Hamilton syringe in the gel slots. SDS-PAGE was performed at 30 mA/gel (Rt). Commercially available mixtures of protein molecular markers were used as reference.

3.8.4 Silver staining

After SDS-PAGE, some gels were incubated in fixative solution (2.13) for at least 1 h, washed 2x20 min in wash solution (2.13) and 1x20 min in deionised water. Then the gels were incubated with thiosulfate reagent (2.13) for 1 min and washed 3x20 sec with deionised water. Subsequently they were incubated with silver nitrate reagent (2.13) for 20 min and washed 3x20 sec with deionised water. Gels were developed by incubation for 3x10 min with developing solution (2.13). The reaction was stopped by incubation with stop reagent for 5 min and 3x10 min with deionised water. Finally gels were dried between cellulose-acetate sheets and stored at Rt.

3.8.5 Coomassie staining

After SDS-PAGE, some gels were soaked in Coomassie solution (2.13) for 2 h at Rt and destained with destaining solution (2.13) until the background disappeared. After washing with distilled water, the gels were dried between cellulose-acetate sheets and stored at Rt.

3.8.6 Semi-dry Western blotting

For Western blot analysis, proteins were transferred after SDS-PAGE to a nitrocellulose membrane (40 mA/membrane; 90 min). 11 layers of gel-blotting paper were cut (8,5 cm x 5,5 cm) and 4 of them soaked with anode buffer I (2.13), 3 of them with anode buffer II (2.13) and 4 of them with cathode buffer (2.13). The nitrocellulose membrane was soaked with anode buffer II. The order of the gel-blotting layers, the membrane and the gel is shown in Fig. 4.

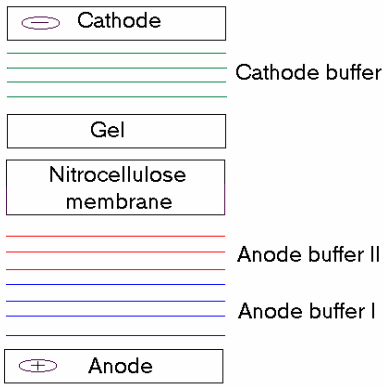


Fig. 4 Schematic drawing of the order of the different layers for Western blotting.

After the protein transfer, the membrane was soaked with Ponceau S solution by gentle agitation for 2 min and then washed with water until bands appeared.

3.8.7 Detection with anti-Avr1b-antiserum

The Western blot membrane was incubated 3 times for 20 min at Rt in G-Net (2.13) and was then incubated overnight at 4°C with a dilution of anti-Avr1b-antiserum (1/750 to 1/20 000) in G-Net. Following the antibody treatment (13 h), the membrane was washed in TBS-Tween buffer (2.13) for 10 min, and 2 times in TBS buffer (2.13) for 10 min at Rt. Afterwards the membrane was incubated for 1 h at Rt in a 1/20 000 dilution of the second antibody (anti-rabbit IgG alkaline phosphatase conjugate; 2.1) in G-Net. The membrane was washed for 10 min in TBS-Tween buffer (2.13) and then 2 times for 10 min in TBS buffer at Rt. Finally the membrane was stained with 10 ml AP buffer (2.13) until bands appeared. The reaction was stopped by rinsing the membrane in water twice.

3.8.8 Protein analysis by mass spectrometry

Protein analysis by mass spectrometry was performed in the groups of Prof. Dr. Lutz Eichacker (Botanical Institute, LMU Munich) and Prof. Dr. Dieter Oesterhelt (Max-Planck Institute, Martiensried), respectively.

In the first case, recombinant Avr1b obtained from a soluble protein fraction of *Sf9* insect cells (300 µg) was precipitated with 1 ml cold methanol (3.8.2) prior to SDS-PAGE. After electrophoresis the band corresponding to Avr1b (15,8 kDA) was excised with a scalpel, stored in a 1,5 ml reaction tube and used for identification by MS/MS on an ESI-Q-TOF instrument.
In the second case, an apoplastic fluid preparation (around 250 µg) obtained from soybean cv. Harosoy seedlings infected with *P. sojae* race 1 zoospores was immunoprecipitated followed by SDS-PAGE. After electrophoresis two bands were excised with a scalpel under sterile conditions, one around 17 kDa and an adjacent one at 24 kDa. The bands were stored in 1,5 ml reaction tubes and used for identification by LC-MS/MS on an ESI-Q-TOF instrument.

3.8.9 Immunoprecipitation with protein A sepharose CL-4B

3.8.9.1 Immunoprecipitation of urea-denatured proteins

Soluble protein preparations (10 µg) were denatured with 10 µl denaturing buffer (2.13) and precipitated with 1 ml cold methanol (3.8.2) overnight. Samples were centrifuged (14 000xg, 4°C, 15 min) and the pellet resuspended in 300 µl extraction buffer I. Following the addition of 5 µl anti-Avr1b-antiserum, the samples were incubated at least 12 h at 4°C by end-over-end mixing. A suspension of protein A sepharose (PAS) (50 µl) was added and samples were incubated for 2 h at Rt by end-over-end mixing. The beads were pelleted (16 100xg, Rt, 2 min) and resuspended in 1x SDS loading buffer (2.13) containing 50 mM DTT before SDS-PAGE. The supernatants were collected and precipitated with 1 ml cold methanol (3.8.2) before SDS-PAGE.
As positive control 10 µg soluble proteins from infected insect cells were denatured with 10 µl denaturing buffer and precipitated overnight with 1 ml cold methanol (3.8.2). Sample was centrifuged (14 000xg, 4°C, 15 min) and resuspended in 1x SDS loading buffer before SDS-PAGE.

3.8.9.2 Immunoprecipitation of SDS-denatured proteins

Protein fractions were diluted in extraction buffer I (2.13) and denatured by adding SDS to a final concentration of 1% and boiling in a water bath (100°C) for 4 min. Four volumes of ice-cold dilution buffer I (2.13) and anti-Avr1b-antiserum (5 µl in the case of recombinant proteins or 50 µl in the case of protein fractions from infected soybean plants) were added. Samples were incubated at least 12 h at 4°C by end-over-end mixing. A suspension of PAS (2.13) (50 µl in the case of recombinant proteins or 300 µl in the case of protein fractions from infected soybean plants) was added and the samples were incubated for 2 h at Rt by end-over-end mixing. The beads were collected (16 100xg, Rt, 2 min) and resuspended in 1x SDS loading buffer (2.13) containing 50 mM DTT before SDS-PAGE. The supernatant of the recombinant protein fraction was collected and precipitated with 1 ml cold methanol (3.8.2) before SDS-PAGE.
As positive controls recombinant proteins were either diluted in extraction buffer I (2.13), denatured by adding SDS to a final concentration of 1% and boiled in a water bath (100°C) for 4 min before

dilution in 5x SDS loading buffer for SDS-PAGE, or diluted directly in 5x SDS loading buffer for SDS-PAGE.

3.9 Methods in molecular biology

3.9.1 Agarose gel electrophoresis

Agarose (0,8% or 1,2%) in 1x TAE buffer (2.13) was boiled, poured into a mould and allowed to harden. Upon hardening the agarose formed a matrix with a density determined by the concentration of agarose. The electrophoresis tank was filled with 1x TAE buffer, the gel mounted on the tank and 1,5 µl ethidium bromide was added to the buffer. DNA samples were mixed with 0,5 µl agarose loading buffer before electrophoresis (Rt, 30 mA/gel). Commercially available mixtures of DNA markers were used as reference.

3.9.2 Small scale plasmid preparation (minipreparation)

Alkaline lysis of cells was performed according to Birnboim and Doly (1979). An overnight culture of transformed *E. coli* cells (1,0 ml) was centrifuged in 1,5 ml reaction tubes (15 000xg, Rt, 2 min). The supernatant was discarded and the pellet centrifuged again (15 000xg, Rt, 2 min) to remove the remaining liquid. The pellet was incubated at -20°C for 10 min, resuspended in 50 µl solution I (2.13) by whirling and then incubated at Rt for 5 min. Solution II (100 µl) was added to the cells and incubated at Rt for 5 min. Afterwards 75 µl of solution III (2.13) was added, mixed immediately and incubated for 5 min on ice. The insoluble material (cell walls, genomic DNA, protein) was precipitated by centrifugation (29 000xg, 15°C, 3 min) and the supernatant (approximately 200 µl) transferred to a new reaction tube and extracted with 200 µl phenol/chloroform/ isoamylalcohol (25:24:1; v:v:v). After brief whirling and centrifugation (15 000xg, Rt, 5 min), the supernatant was again transferred to a new reaction tube, 500 µl ethanol added, incubated at Rt for 10 min and centrifuged (29 000xg, 15°C, 5 min). Plasmid-DNA was colleted in this step. The pellet was then washed with 200 µl 75% ethanol, dried and dissolved in 50 µl TE buffer. The plasmid-DNA was stored at -20°C.

3.9.3 DNA purification from agarose gels

DNA extraction was performed using the MinElute Gel Extraction or the NucleoSpin Extract II kit according to manufacturer instructions.

3.9.4 DNA restriction assay

For DNA restriction of DNA-plasmids and PCR fragments, 10 µl of solution contained approximately 1 unit restriction enzyme/µg DNA, incubation buffer and a variable amount of DNA. In the case of a plasmid-minipreparation DNA was treated with 0,5 µl RNase A. The restriction assay was monitored by agarose gel electrophoresis.

3.9.5 DNA precipitation

For preparative purposes, restricted DNA was precipitated by one of the following methods:

3.9.5.1 DNA precipitation by sodium acetate and ethanol

DNA was mixed with 1/10 vol 3 M sodium acetate (pH 5,2) and 3 vol ethanol. Samples were mixed, incubated at -80°C for 30 min, centrifuged (29 700xg, 4°C, 5 min) and washed with 75% ethanol (29 700xg, 4°C, 2 min). The pellets were dried at 37°C and redissolved in 30 µl TE buffer (2.13).

3.9.5.2 DNA precipitation by sodium acetate and isopropanol

The DNA solution (100 µl) was incubated at 37°C overnight. Phenol/chloroform (100 µl; 50:50; v:v) was added and centrifuged (15 400xg, Rt, 5 min). Sodium acetate (3 M; 11 µl) and 58 µl of isopropanol were added, mixed, incubated for 30 min at Rt, centrifuged (16 100xg, Rt, 10 min) and washed with 75% ethanol (29 700xg, 4°C, 2 min). The pellet was dried at 37°C and redissolved in 15 µl distilled, sterile water.

3.9.6 Polymerase chain reaction (PCR)

PCR was used in order to amplify DNA from different sources. Plasmid DNA, cDNA or DNA from bacterial colonies, picked directly from agar plates, have been used as templates for the reaction. The oligonucleotides used for PCR were commercially available and provided lyophilized. They were dissolved in water to 1 nmol/µl. From this stock solution 50 pmol/µl working solutions were prepared. PCR solutions (2.13) (10 µl) were used for each PCR.

3.9.7 Colony PCR

Colony PCR was used to detect the presence of a recombinant vector with the desired insert after ligation and transformation. A bacterial colony from an agar plate was slightly touched with a sterile pipette tip and transferred into a 200 µl PCR tube by touching the bottom with the tip. “Colony PCR solution” (8 µl) was added to each tube.

3.9.8 DNase treatment of RNA

RNA (10-20 µg) was incubated in DNAse treatment solution (total volume 400 µl) at 37°C for 30 min. RNA was extracted two times with 400 µl phenol/chloroform/ isoamylalcohol (25:24:1; v:v:v) and one time with 400 µl chloroform. After centrifugation (23 000xg, 4°C, 10 min) RNA was precipitated overnight with 1 ml ethanol and 40 µl sodium acetate at -20°C, collected again (29 000xg, 4°C, 25 min) and washed with 70% ethanol (29 000xg, 4°C, 20 min). The pellet was resuspended in 15 µl sterile water.

3.9.9 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was used to monitor gene expression in *P. sojae* and in infected and uninfected soybean plants. DNase-treated RNA (2 µg) was diluted with sterile water to a final volume of 8,6 µl and heated at 65°C for 5 min and then at 90°C for 3 min for denaturation. The RT was performed with the denaturated RNA in RT-PCR solution I (total volume 20 µl) under the following conditions: 25°C for 15 min; 37°C for 90 min; 90°C for 5 min. RT reaction (2 µl) was used for PCR in RT-PCR solution II (total volume 10 µl).

3.9.10 A-tailing procedure

Thermostable DNA polymerases with proofreading activity such as the *Pfu*-DNA polymerase generate blunt-end fragments during PCR amplification. The ends of the PCR fragments were modified by the A-tailing procedure and the fragments ligated into a pGEM-T easy vector. Using this method only a single insert was expected to be ligated into the vector as opposed to multiple insertions that can occur with blunt-ended cloning.
For A-tailing, 2 µl purified DNA were incubated in A-tailing solution (final volume 5 µl) for 30 min at 70°C.

3.9.11 Ligation of DNA fragments

For ligation a T4-DNA ligase from MBI Fermentas was used and the ligation reaction performed according to manufacturer instructions. Usually 25 ng of vector DNA were ligated to an adequate amount of insert which was calculated using the following formula:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The ligation was performed in a final volume of 5 µl ligation solution (2.13) incubated overnight at 4°C.

3.9.12 DNA purification and sequencing

After minipreparation (3.9.2) DNA was purified before sequencing. RNase (1 µl) was added to 20 µl plasmid DNA and incubated 1 h at 37°C. A solution of 0,5 M EDTA (0,5 µl) and 30 µl sterile water were added and extraction of DNA was performed with phenol/chloroform/isoamylalcohol (25:24:1, v:v:v). After centrifugation (16 100xg, Rt, 15 min), the upper phase containing the DNA was mixed with 5,5 µl 3 M sodium acetate and 30 µl isopropanol and incubated 30 min at Rt. DNA was precipitated by centrifugation (16 100xg, Rt, 15 min), washed with 75% ethanol (16 100xg, Rt, 5 min) and resuspended in 10 µl water. For DNA sequencing, performed in the group of Prof. Dr. Herrmann

(Botanical Institute, LMU Munich), 500 ng DNA and 5 pmol sequencing primer were added to 8 µl distilled water.

3.9.13 Transformation of *E. coli* DH5α cells

Electroporation-competent *E. coli* DH5α cells (laboratory collection) were thawed on ice. DNA (50 ng) was added to 40 µl of cells, placed on an electroporation cuvette and transformed with an electrical impulse in the BioRad Gene Pulser at 2,5 KV, 25 µF, and 200 Ω. Immediately after the electroporation, 460 µl SOC^{+++} medium (42°C) were added, mixed and the cells transferred to a 1,5 ml reaction tube and incubated 1 h at 37°C and 180 rpm. The mixture was placed on LB agar plates supplemented with 100 µg/ml ampicilin. Plates were incubated overnight at 37°C.

3.9.14 Transformation of XL10-Gold ultracompetent *E. coli* cells

E. coli XL10-Gold ultracompetent cells (Stratagene) were thawed on ice and then mixed gently by hand. Cell aliquots (40 µl) were placed onto precooled 15 ml falcon tubes. β-Mercaptoethanol (1,6 µl) was added to the cells, gently mixed and incubated on ice for 10 min, swirling them every 2 min. DNA (1 µl) was added to each falcon tube and swirled gently, incubated on ice for 30 min, heat-pulsed for 30 sec in a 42°C water bath and then incubated on ice for 2 min. SOC^{+++} Medium (460 µl) (42°C) was added to each sample and incubated for 1 h at 37°C and 250 rpm. The transformed cells (25 µl) were plated on LB agar plates supplemented with 100 µg/ml ampicilin. Plates were incubated overnight at 37°C.

3.9.15 Transposition of a recombinant donor plasmid into DH10Bac *E. coli* cells

E. coli DH10Bac cells (Invitrogene) were thawed on ice. An aliquot of 50 µl cells was dispensed into a precooled 15 ml polypropylene tube. The recombinant donor plasmid (1 µl) was added and mixed by tapping the side of the tube. The mixture was incubated for 30 min at 30°C, heat-shocked in a 42°C water bath for 45 sec, and cooled on ice for 2 min. SOC^{+++} Medium (450 µl) was added and the mixture shaken for 4 h in an incubator at 37°C and 180 rpm. The cells were serially diluted using SOC^{+++} medium to 10^{-1} and 10^{-2} and 200 µl of each dilution were placed on LB agar plates containing 50 µg/ml kanamicin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml X-gal and 40 µg/ml IPTG. Plates were incubated for at least 24 h at 37°C.

3.9.16 Isolation of recombinant bacmid DNA (minipreparation of bacmid DNA)

Four white *E. coli* DH10Bac colonies containing recombinant bacmid DNA were selected from agar plates for isolation of the bacmid DNA and plated on new agar plates for verifying their coloration by incubating overnight at 37°C. One single colony showing a white phenotype on plates containing X-gal and IPTG was inoculated into 2 ml LB medium supplemented with 50 µg/ml kanamicin, 7 µg/ml gentamicin and 10 µg/ml tetracycline, and after incubation overnight, 1,5 ml of the culture was

centrifuged (15 000xg, Rt, 1 min). The supernatant was discarded and the pellet resuspended in 0,3 ml solution IV (2.13) by pipetting up and down. Solution II (2.13) (0,3 ml) was added and mixed gently. The mixture was incubated at Rt for 5 min and 0,3 ml of solution III (2.13) were added slowly. A thick, white precipitate of *E. coli* genomic DNA and protein was formed. The sample was placed on ice for 10 min and then centrifuged (15 000xg, Rt, 10 min). The supernatant was transferred to another tube containing 0,8 ml isopropanol, mixed by inverting the tube a few times and placed on ice for 10 min. The sample was centrifuged (15 000xg, Rt, 15 min), the supernatant removed and the pellet washed with 0,5 ml 75% ethanol, centrifuged (15 000xg, Rt, 5 min), dried for 10 min at RT and dissolved in 40 µl TE buffer (2.13). The DNA was stored at -20°C.

3.10 Subcellular fractionation of *P. sojae* mycelium

Three week-old *P. sojae* mycelium grown in Erwin synthetic medium (2.13) was used for subcellular fractionation. After adding 69,7 g ammonium sulfate to 100 ml medium, precipitated proteins were collected by centrifugation (23 000xg, 30 min, 4°C). The pellet was redissolved in 2 ml lysis buffer (2.13) and dialyzed against a buffer containing 25 mM Tris-HCl (pH 7,5) and 1 mM EDTA (2x1 h followed by 1x16 h, 4°C) to obtain a “culture medium” fraction.

In parallel, mycelium was placed on a flat surface, washed with cold tap water and finally with distilled water in order to remove agar and calcium carbonate. The mycelium was collected on a 300 µm nylon mesh, washed again with distilled water and either frozen and stored at -80°C or processed immediately. Mycelium (40 g) was homogenized in 120 ml grinding buffer (2.13) in a mortar. The homogenate was filtered through a 300 µm nylon mesh and centrifuged (2 000xg, 4°C, 15 min) to obtain a pellet and a supernatant. The pellet containing cellular fragments and nuclei was resuspended in 3 ml AB buffer (2.13) (“cell debris” fraction) and the supernatant centrifuged again (10 000xg, 4°C, 10 min). This pellet containing mitochondria and peroxisomes was also resuspended in 2 ml AB buffer (“mitochondria” fraction) and the supernatant was centrifuged (100 000xg, 4°C, 3 h). This time the pellet containing microsomes and endoplasmic reticulum was resuspended 1 ml AB buffer (“microsomal” fraction). The supernatant containing soluble proteins (“soluble protein” fraction) was also collected. Protein concentration (3.8.1) was measured in all fractions and 50 µg of protein were precipitated with 1 ml methanol (3.8.2) before SDS-PAGE.

3.11 Hypocotyl assay

The hypocotyl assay was performed according to Albersheim and Valent (1978). Twelve day-old soybean plants were removed from the vermiculite and rinsed in tap water. The plants were mounted on horizontal glass rods by piercing the hypocotyls 5 mm below the cotyledons. Two plants were mounted on each glass rod and the plants were suspended with their roots in water. A small piece of a 3 week-old *P. sojae* mycelium was placed in a vertical slit wound of about 1 cm length. Control plants were also wounded but not inoculated with mycelium. After incubation in a closed chamber for 5 days at 26°C and 100% humidity, the plants were removed from the glass rods and a 2 cm

segment from the wounded hypocotyl area was excised from each plant, frozen in liquid nitrogen, and stored at -80°C for further RNA extraction.

3.12 Zoospore infection assay

Lima bean agar plates (20x10 ml) containing 12 day-old *P. sojae* mycelium were washed 8 times with 30 ml distilled, autoclaved water for 30 min. Each plate was covered with 10 ml distilled, autoclaved water and incubated overnight at 18°C in the darkness. The next day the water solutions containing the *P. sojae* zoospores were collected in a total of ten 50 ml falcon tubes and centrifuged (2 000xg, Rt, 10 min). The upper 16 ml of each tube were discarded and the pelleted zoospores resuspended in the remaining 4 ml. The resuspended zoospores were combined (40 ml in total) and counted with a microscope. The concentration was around 30 000 zoospores/ml. Groups of 15 unwounded, 3 day-old soybean seedlings of cultivars Harosoy, Harosoy 1372 or Williams L77-1863 were dip-inoculated with a zoospore suspension of *P. sojae* containing approximately 10 000 zoospores per seedling as described by Hahn *et al*. (1985). Unwounded, 3 day-old, uninfected seedlings were placed in distilled water and used as control. Following incubation for different periods of time in the darkness (26°C;100% humidity), root segments were excised 1 cm above ("up"), 1 cm around the infection site ("ring") and from the root tip ("tip") as shown in Fig. 5. Root segments were frozen in liquid nitrogen and stored at -80°C.

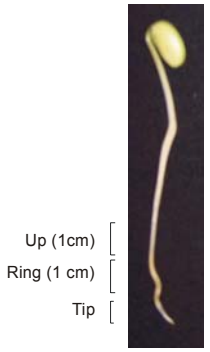


Fig. 5 Picture of an infected soybean seedling. "Up" area, "ring" area, and "tip" are indicated.

3.13 Subcellular fractionation of infected soybean seedlings

Subcellular fractionation of infected soybean seedlings was performed according to De Wit and Spikman (1982). Unwounded, 16x 3 day-old soybean seedlings of the cultivars Harosoy and Williams L77-1863 were dip-inoculated with zoospores of *P. sojae* race 1 (Avr1b). Unwounded, 3 day-old, uninfected seedlings were placed in distilled water and used as control. Root segments from uninfected plants (0 h) and infected plants (24 and 48 h after infection) were excised from the ring at the water-air interface.

Altogether 16 root segments were immersed in 800 µl 10 mM $NaPO_4$ and the apoplastic fluid was extracted under vacuum (Rt; 90 min) followed by centrifugation (10 000xg, Rt, 15 min). The supernatant was collected ("apoplastic fluid" fraction) and the roots were homogenized on a pre-cooled mortar containing 1 ml extraction buffer II (2.13) and centrifuged (4 000xg, Rt, 20 min). The pellet was discarded and the supernatant centrifuged again (26 000xg, Rt, 30 min). This time the pellet was resuspended in 400 µl extraction buffer II ("microsomal" fraction) and also collected like the supernatant ("soluble protein" fraction).

3.14 RNA isolation

3.14.1 RNA isolation from *P. sojae* mycelium

Mycelium (2 to 6 week-old) was placed on a flat surface and washed with tap water and finally with distilled water in order to get rid of agar and calcium carbonate. The mycelium was collected on a 300 µm nylon mesh, weighted and cut into pieces.
Total RNA was isolated according to the method of Chang *et al.* (1993) with minor modifications. Mycelium (3 g) was ground with liquid nitrogen, added to 12 ml of pre-warmed RNA extraction buffer (65°C; 2.13) and mixed by inverting the falcon tube. The mixture was extracted two times with 12 ml chloroform/isoamyl alcohol (24:1; v:v) and separation of the phases was achieved by centrifugation (5 000xg, 20°C, 30 min). The aqueous phase was collected in Corex tubes, and the RNA precipitated by adding one third vol 8 M LiCl and incubated overnight at 4°C. After centrifugation (10 000xg, 4°C, 20 min) the pellet was dissolved in 500 µl of SSTE (2.13) and extracted one more time with chloroform/isoamyl alcohol (24:1; v:v). RNA was precipitated by adding two volumes of ethanol and incubated for 1 h at -80°C. After centrifugation (28 000xg, 4°C, 20 min), the pellet was dried and resuspended in 50-150 µl DEPC-water (2.13).

3.14.2 RNA isolation from *P. sojae* zoospores

A 20 ml zoospore suspension (30 000 zoospores/ml) was centrifuged (2 000xg, 4°C, 10 min), the supernatant was discarded and the pellet homogenized on ice with 1 ml trifast-reagent. The homogenized sample was incubated for 5 min at Rt to permit complete dissociation of nucleoprotein complexes. Chloroform (0,2 ml) was added and shaken vigorously for 15 sec before incubation for 2 min at Rt. After centrifugation (11 000xg, 4°C, 15 min), the mixture separated into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. RNA remained exclusively in the aqueous phase and was transferred to a fresh tube, mixed with 500 µl isopropanol and incubated for 10 min at Rt for RNA precipitation. The sample was centrifuged (11 000xg, 4°C, 10 min) and the pellet washed with 1 ml 70% ethanol (5 800xg, 4°C, 5 min). Finally the pellet was dried 5 min at Rt and dissolved in 30 µl sterile water. RNA was quantified and treated with DNase before RT-PCR. The experiment was performed twice.

3.14.3 RNA isolation from soybean plants

RNA was isolated from 0,1 mg soybean seedlings using the RNeasy Plant Mini Kit from Qiagen. After extraction, RNA was resuspended in 100 µl of sterile water.

3.15 Determination of glyceollin in soybean roots

Unwounded, 3 day-old soybean seedlings were incubated in 300 µl of a testing solution (e.g. protein preparations containing Avr1b or raw elicitor) and 10 mg/ml gentamicin in 1,5 ml reaction tubes. The reaction tubes were placed into a plastic box at 100% humidity and incubated for 22 h in the dark at 26°C. Root segments close to the infection site (“ring”) were excised, weighted, frozen in liquid nitrogen, placed in a pre-cooled mortar with about 1 g sand and mixed by adding liquid nitrogen to obtain a crude homogenate. The crude homogenate was centrifuged (200xg, 4°C, 5 min), and the supernatant placed into a potter, homogenized with methanol (5 ml per gram of fresh weight), and centrifuged (200xg, 4°C, 5 min). The supernatant was evaporated and the residue dissolved in 400 µl methanol. The sample was filtered through Millex-HV filter before analysis by high performance liquid chromatography (HPLC). The HPLC system was equipped with a C_{18} reversed phase column. Elution from the column was accomplished with a gradient shown in table 2. The injection volume was 20 µl and the flow rate 1 ml/min. Eluting compounds were monitored at 284 nm and the glyceollins were identified and quantified by using a glyceollin standard.

Table 2. Gradient elution for HPLC analyses

Time (min)	%Methanol	%Water
0	40	60
2	40	60
12	75	25
15	100	0
17	100	0
18	40	60
20	40	60

4 Results

After isolation of the first avirulence gene (*Avr1b*) from an oomycete (*P. sojae*), the properties and functions of the encoded protein needed to be investigated. Therefore it was necessary to establish a transgenic system for the production of recombinant Avr1b. Since the yeast *Pichia pastoris* was the organism of choice of Shan *et al*. (2004), in this approach the production of recombinant Avr1b was established in *E. coli* and in *Sf9* insect cells. According to the program "PeptideMass" the proprotein Avr1b synthesized in *Sf9* insect cells has an expected molecular weight of 15 800 or 13 500 after cleavage of the putative signal peptide. The His_6-fusion protein synthesized in *E. coli* has an expected molecular weight of 17 500 or 15 300 after cleavage of the putative signal peptide.

4.1 Production of recombinant Avr1b in bacteria

Recombinant proteins synthesized in *E. coli* cells can be produced in a soluble form, but in many cases, especially at high expression levels, they aggregate and form insoluble "inclusion bodies". These inclusion bodies contain the highly enriched recombinant protein and can be isolated by solid/liquid separation. The formation of inclusion bodies is influenced by the nature of the protein, by the host cell and by the expression level resulting from the vector of choice and the growth and induction conditions. Inclusion bodies limit the utility of standard purification procedures requiring the protein's native, soluble form.

Different methods have been published describing the refolding of insoluble proteins (Marston and Hartley, 1990; Kurucz *et al.*, 1995; Rudolph and Lilie,1996; Mukhopadhyay, 1997). Most protocols describe the isolation of inclusion bodies by centrifugation followed by solubilization under denaturing conditions. The protein is then dialyzed or diluted into a non-denaturing buffer where refolding occurs. Because every protein possesses unique folding properties, the optimal refolding protocol for any given protein must be empirically determined.

4.1.1 Synthesis of Avr1b in *E. coli* cells

In a previous project in our group, D. Becker started the heterologous expression of *Avr1b* in *E. coli* cells (D. Becker, personal communication). An *Avr1b* full-length clone, inserted into the vector pTZ18, was obtained from B.M. Tyler (Shan *et al.*, 2004). The gene was amplified by PCR with a *Pfu*-DNA-Polymerase. The fragment was digested with the restriction enzymes *Bsp*68 I and *Sal* I, ligated into a pET26b vector and transformed into *E. coli* XL-10 Gold cells. Avr1b was recovered only in form of inclusion bodies and purified under denaturing conditions for further biotests.

In this study, *E. coli* BL21(DE3) containing the pET26b-Avr1b expression plasmid was used. The plasmid pET26b served as a negative control (3.4). Avr1b was recovered in form of inclusion bodies (Fig. 6) as shown by D. Becker and subsequently purified both under native and under denaturing

conditions. With the purified protein it was possible to perform biotests and to raise an anti-Avr1b-antiserum.

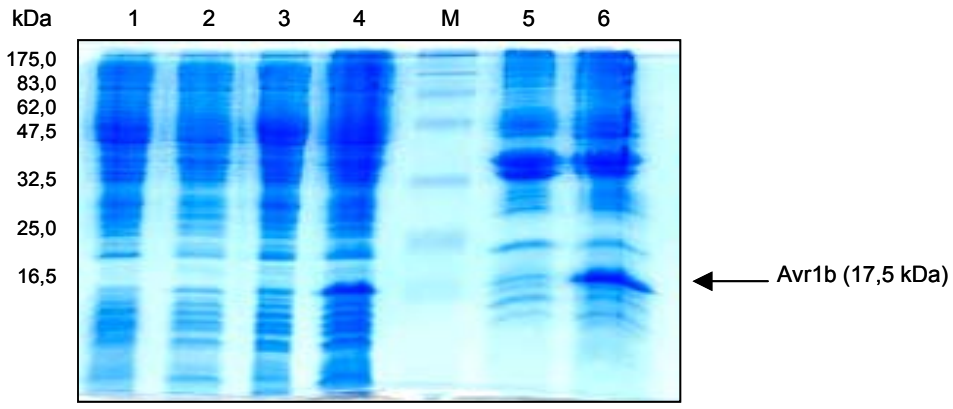


Fig. 6 SDS-PAGE analysis of Avr1b production in *E. coli* cells. Crude protein extracts from cells with an optical density of 0.25 (lanes 1-4) or inclusion body preparations (lanes 5-6) were applied to a 17,4% SDS gel and visualized by Coomassie staining. M) protein markers (kDa), 1) non-induced control cells containing the pET26b vector, 2) non-induced cells harboring the pET26b-Avr1b vector, 3) induced control cells containing the pET26b vector, 4) induced cells harboring the pET26b-Avr1b vector, 5) inclusion body preparation from BL21(DE3)pET26b, 6) inclusion body preparation from BL21(DE3)pET26b-Avr1b.

4.1.2 Purification of Avr1b

Purification of 6xHis-tagged proteins using nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography can be performed under native or under denaturing conditions. Proteins in inclusion bodies are solubilized with detergents or denaturants such as 6 M guanidinium hydrochloride or 8 M urea prior to purification.

The recombinant Avr1b protein produced in bacteria was synthesized as fusion protein with a 6xHis affinity tag located at its carboxy terminus. This tag is able to bind to Ni-NTA attached to a matrix at pH 7,4 or pH 8,0. Proteins lacking the affinity tag are removed from the matrix by applying stringent washing conditions achieved by lowering the pH or adding imidazole at concentrations up to 50 mM. The histidine residues in the 6xHis tag have a pKa of approximately 6,0 and will become protonated if the pH is reduced (pH 4,5-5,9). Under these conditions the 6xHis-tagged protein can no longer bind to the nickel ions and will dissociate from the Ni-NTA resin. Similarly, if the imidazole concentration is increased from 50 mM to 180-250 mM, the 6xHis-tagged protein will also dissociate because they can no longer compete with imidazole for binding sites on the Ni-NTA resin (Fig. 7). If larger amounts of proteins have to be purified or if purification has to be performed using fast performance liquid chromatography (FPLC), a Ni-NTA superflow column has to be used due to its physical stability at high pressures and flow rates.

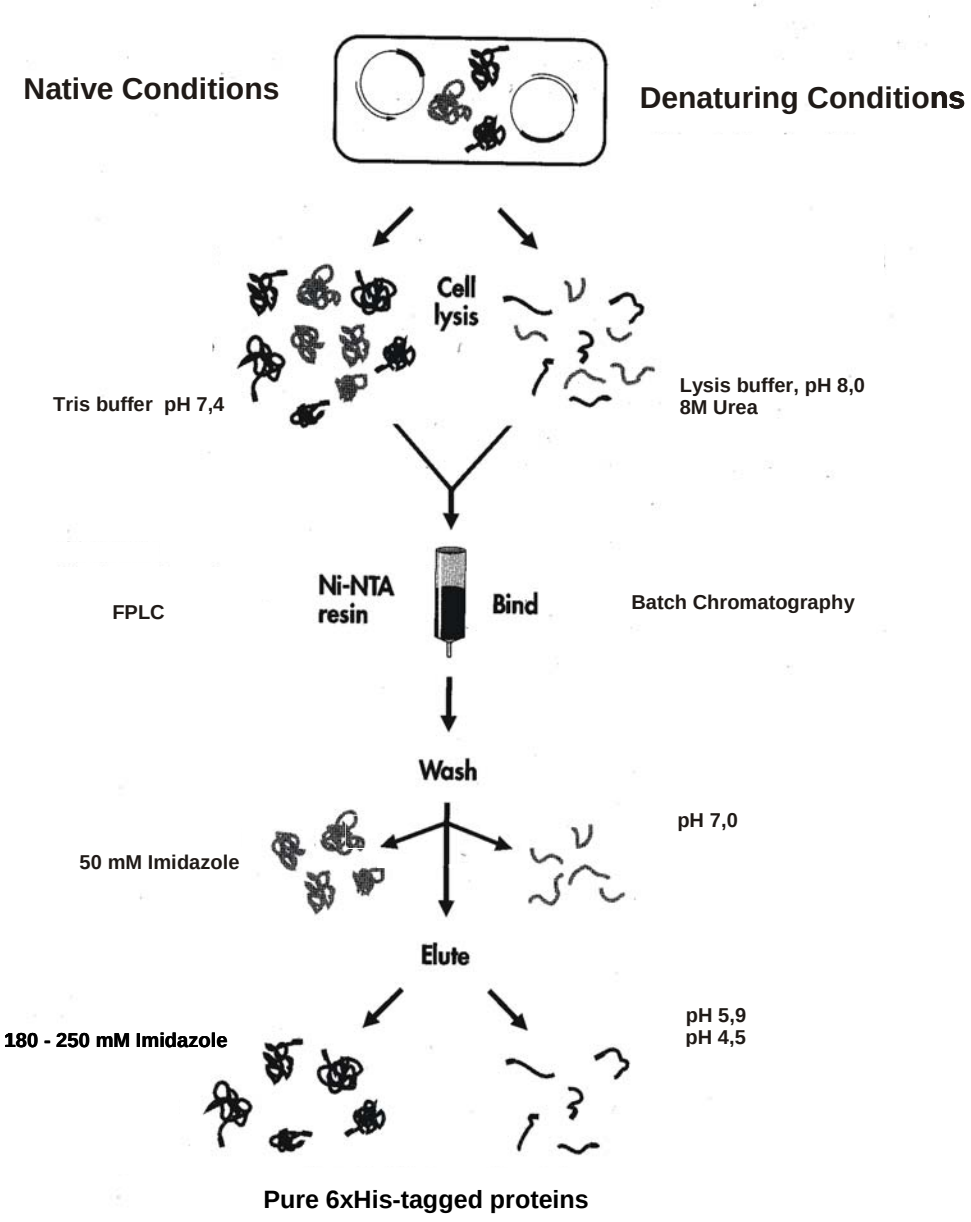


Fig. 7 Purification of 6xHis-tagged proteins under native or denaturing conditions using the QIAexpress System from Qiagen. Figure taken from the manual “QIA Expressionist” from Qiagen and modified.

4.1.2.1 Protein purification under denaturing conditions

Avr1b (6xHis-tagged) was obtained as a recombinant protein (3.4.2) and purified on a 0,4 ml Ni-NTA agarose matrix (batch purification, 3.5.1).

Lysate (4 ml) was applied to the Ni-NTA column. The His_6-fusion epitope from Avr1b was bound to the Ni-NTA agarose matrix under denaturing conditions (8 M urea) at pH 8,0. By lowering the pH to 7,0 unspecifically bound proteins were washed out of the matrix. By reducing the pH to 5,9 the 6xHis-tagged Avr1b protein dissociated from the Ni-NTA resin as shown in Fig. 7.

During batch purification the flow through fraction, washing and eluate fractions were collected and aliquots were subsequently applied to a 17,4% polyacrylamide gel. As shown in Fig. 8, the early eluate fractions contained purified Avr1b. The fractions EI2, EI3 and EI4 were collected, combined and dialyzed for further biological tests.

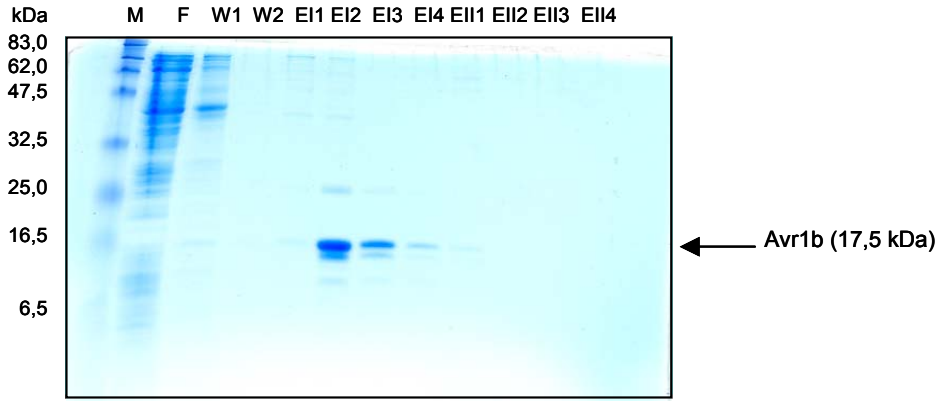


Fig. 8 SDS-PAGE (17,4%) analysis of different fractions collected after purification of Avr1b under denaturing conditions. Proteins were visualized by Coomasie staining. M) protein markers (kDa), F) flow through, W1) washing fraction 1, W2) washing fraction 2, EI1- EI4) fractions eluted with buffer I (pH 5,9), EII1-EII4) fractions eluted with buffer II (pH 4,5).

4.1.2.2 Protein refolding

As shown in 4.1.2.1 it was possible to purify Avr1b from inclusion body fractions under denaturing conditions. Nevertheless it would be more favorable to obtain renatured protein preparations or even to purify Avr1b under native conditions in order to use it in biotests or to produce an anti-Avr1b-antiserum. For these reasons, protein refolding was performed using the Qiagen protocol with the help of the Ni-NTA superflow matrix and the FPLC device as described in 3.5.2.

Lysate (40 ml) was applied to the matrix and renatured in a 7 to 1 M urea gradient. After presumptive refolding, unspecifically bound proteins were removed by applying 50 mM imidazole. To elute Avr1b, a three step imidazole gradient was performed (50, 180, 250 mM imidazole, Fig. 9).

Fig. 9A shows the elution profile obtained during purification. As observed in Figs. 9B and 9C the eluate fractions 10-17 contained purified Avr1b. These fractions were collected, combined and stored at -80°C, or used immediately for biotests (4.5.1.2). This purification procedure was performed eight times and resulted in 2 mg recombinant Avr1b from a total of 1,8 l of induced bacterial cultures.

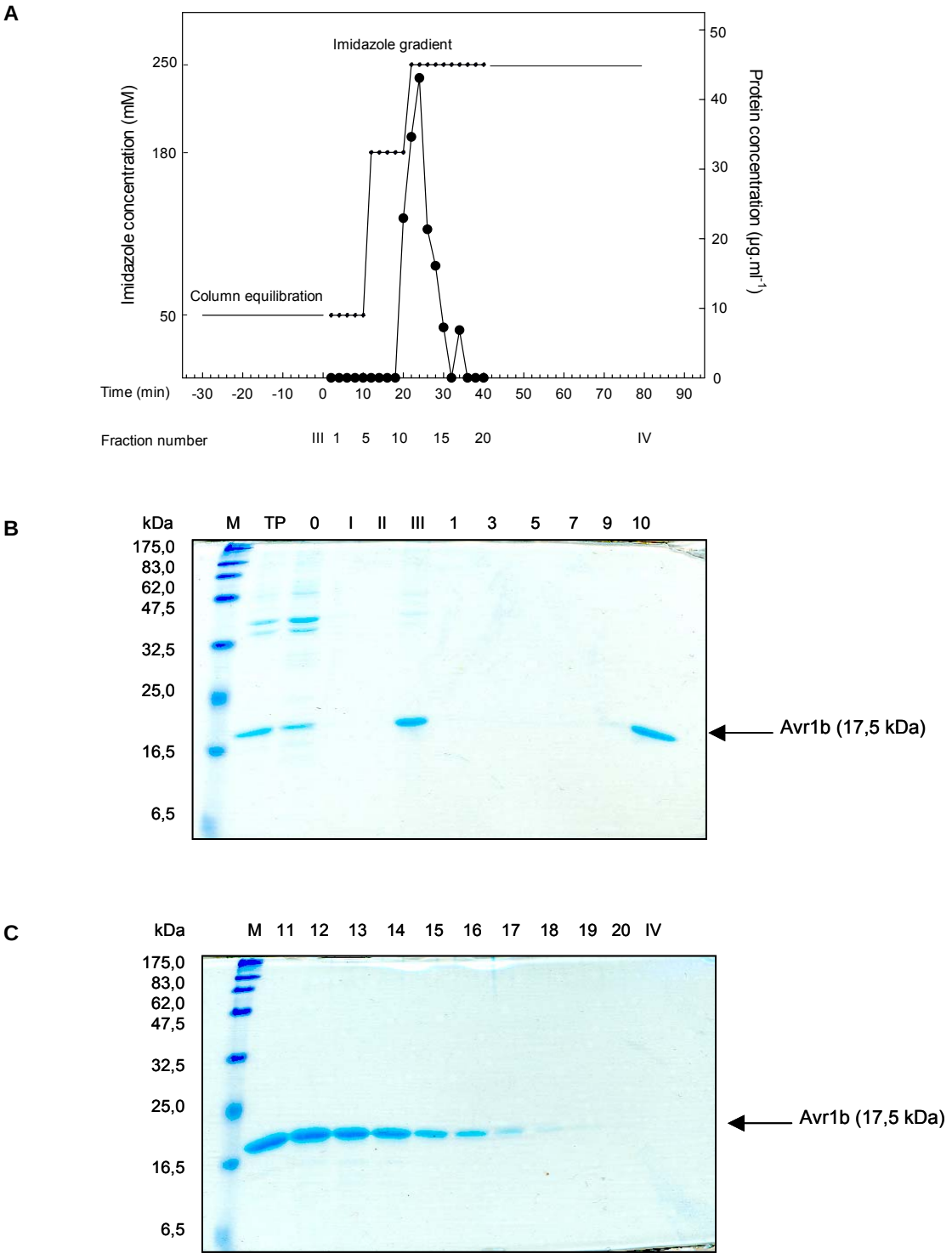


Fig. 9 FPLC purification on a Ni-NTA superflow matrix. 6xHis-tagged Avr1b was purified from lysate (40 ml) derived from 250 ml of an induced *E. coli* culture on 5 ml of Ni-NTA superflow matrix at a flow rate of 0,5 ml/min. (A) Elution profile of the column. (B) Coomassie-stained SDS-PAGE (17,4%), M) protein markers (kDa), TP) Total protein fraction (inclusion bodies) before application onto the column, 0) flow through fraction during sample injection, I) flow through fraction during 7-4 M urea gradient, II) flow through fraction during 4-1 M urea gradient, III) flow through fraction during column equilibration with 50 mM imidazole, 1-10) eluate fractions. (C) Coomassie-stained SDS-PAGE (17,4%). M) protein markers (kDa), 11-20, IV) eluate fractions.

4.2 Production of recombinant Avr1b in *Sf9* insect cells

As an alternative approach, recombinant Avr1b was produced in baculovirus-infected insect cells. For the generation of recombinant, transfection-competent baculovirus DNA, Avr1b was first cloned into the transfer vector pFastBac1, flanked by Tn7 elements. This recombinant donor plasmid was then transposed into *E. coli* DH10Bac cells which contained the bacmid with a mini-*att*Tn7 target and a helper plasmid. The mini-Tn7 element of the pFastbac1 donor plasmid is able to transpose to the mini-*att*Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. After induction with IPTG, the cells with the recombinant bacmid growing on plates containing X-Gal were identified by the white color of its colonies resulting from disruption of the lacZ gene after the transposition. High molecular weight mini-prep DNA was performed on transformed *E. coli* clones containing the recombinant bacmid, which was then used to transfect insect cells. Baculovirus particles harvested from the transfected cells were used to infect fresh *Sf9* insect cells for subsequent synthesis of Avr1b (Fig. 10).

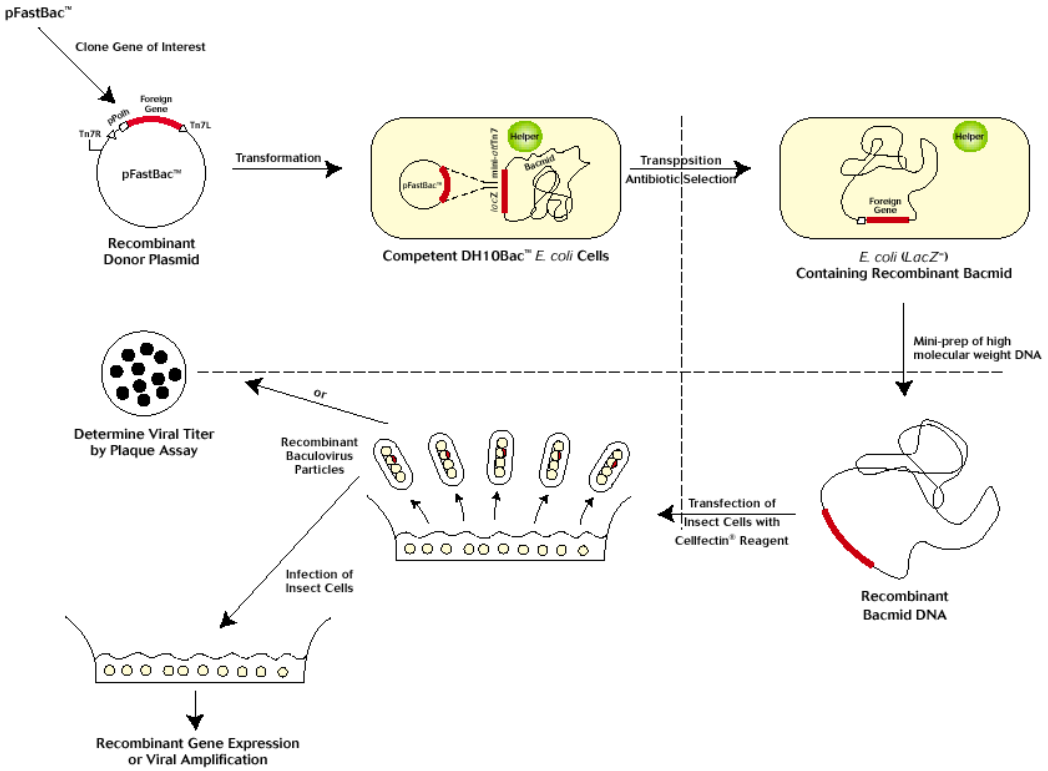


Fig. 10 Generation of recombinant baculoviruses and gene expression. Figure taken from the manual “Bac-to-Bac Baculovirus Expression System” from Invitrogen.

4.2.1 Generation of the expression constructs

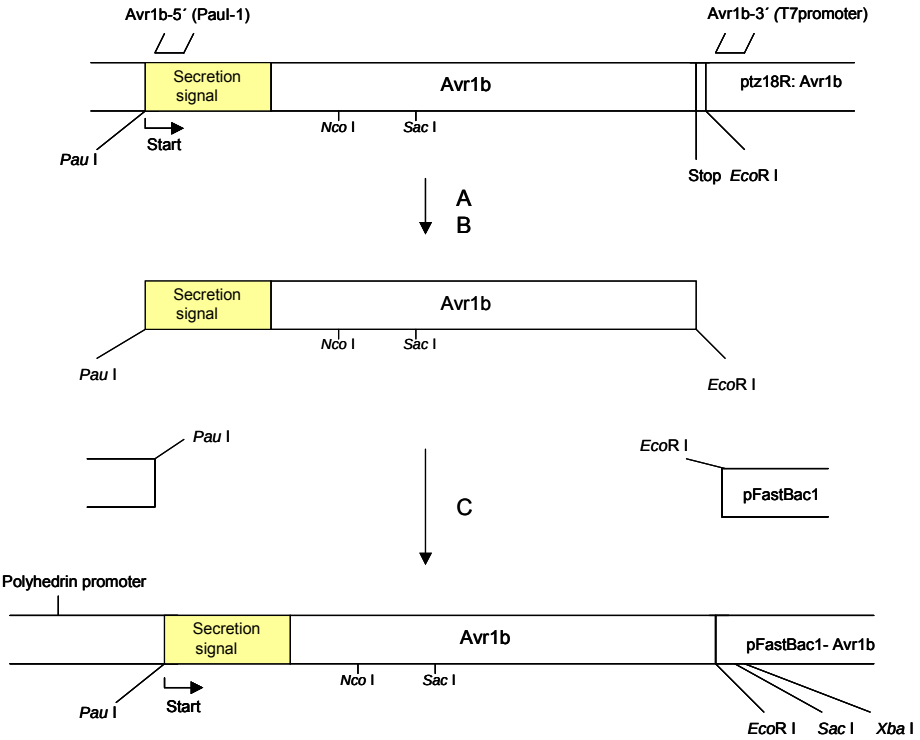
Genes encoding Avr1b with or without the predicted secretion signal were amplified in the vector pTZ18-Avr1b. Separate amplifications by PCR including the oligonucleotides Avr1b_PauI-1 or Avr1b_PauI-2 and T7-promoter were performed using a *Pfu*-DNA-Polymerase. The resulting 427 and 370 bp gene fragments contained the complete open reading frame of *Avr1b* or the sequence lacking the secretion signal, respectively. Both fragments were digested with the restriction enzymes *Eco*R I and *Pau* I. Subsequently they were purified from an agarose gel (3.9.3).

A pFastBac1 donor plasmid was transformed into DH5α *E. coli* cells. After a minipreparation (3.9.2) the plasmid was digested with the restriction enzymes *Eco*R I and *Pau* I in order to ensure that the insertion of the gene fragments will be unidirectional.

These gene fragments encoding Avr1b with or without the predicted secretion signal were ligated into the pFastBac1 vector (3.9.11) and then transformed again into XL10-Gold ultracompetent *E. coli* cells (3.9.14; Fig. 11).

To verify the correct insertion into the donor plasmids, restriction analyses with different restriction enzyme pairs were performed after minipreparation of the plasmid as illustrated in Fig.12. The figure shows clearly that the ligation of the gene fragments into the pFastBac1 plasmid was successful. The bands of the restriction fragments present in the agarose gel correspond with the calculated sizes.

I



II

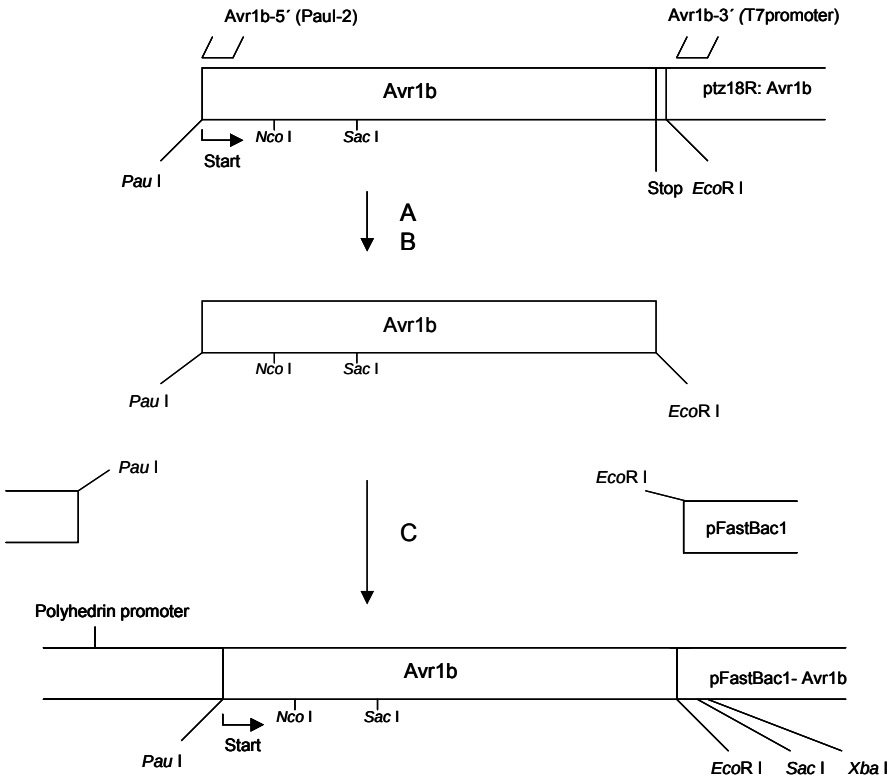


Fig. 11 Cloning procedure of pFastBac1-Avr1b with the putative signal peptide (I) or without the putative signal peptide (II). A) Pfu-PCR with Avr1b_PauI-1 or Avr1b_PauI-2 oligonucleotides and T7 promoter, B) Restriction with the enzymes *Eco*R I and *Pau* I, C) Ligation into the pFastBac1 vector.

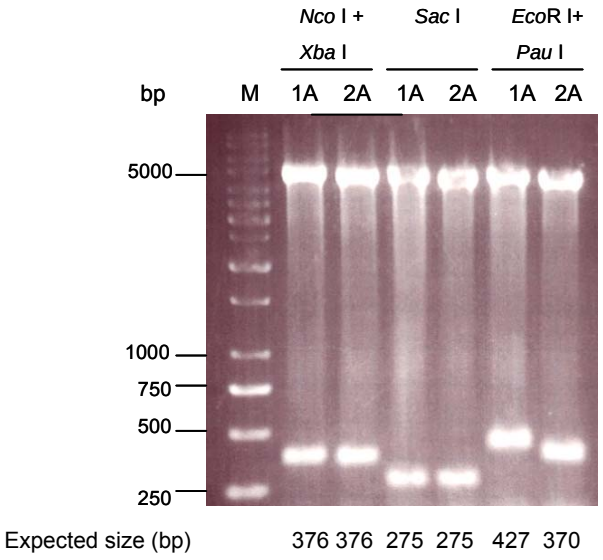


Fig. 12 Restriction analysis performed with *Nco* I and *Xba* I, *Sac* I, *Eco*R I and *Pau* I on pFastBac1-Avr1b(1) (harbors *Avr1b* with the secretion signal, sample 1A) and on pFastBac1-Avr1b(2) (harbors *Avr1b* without the secretion signal, sample 2A). 1,2% agarose gel. M) molecular size markers (bp).

4.2.2 Synthesis of Avr1b in *Sf9* insect cells

Once the *Avr1b* gene had been cloned into the pFastBac1 vector and after the pFastBac1-Avr1b recombinant donor plasmids had been transferred into XL10 ultracompetent *E. coli* cells they were transposed into DH10Bac *E. coli* cells to obtain recombinant *Avr1b* bacmids. These *Avr1b* bacmids were transfected into insect cells (3.3.3). The baculovirus particles (P1) harvested from the transfected cells were used to infect fresh *Sf9* insect cells for subsequent synthesis of Avr1b. As shown in Fig.13, the recombinant Avr1b protein was found in nuclei, in membrane protein and in soluble protein fractions from *Sf9* insect cells infected with baculovirus particles (P1) containing the *Avr1b* bacmid B1 (harboring *Avr1b* with the secretion signal), but not from insect cells infected with baculovirus particles (P1) containing the *Avr1b* bacmid B2 (*Avr1b* without the secretion signal).

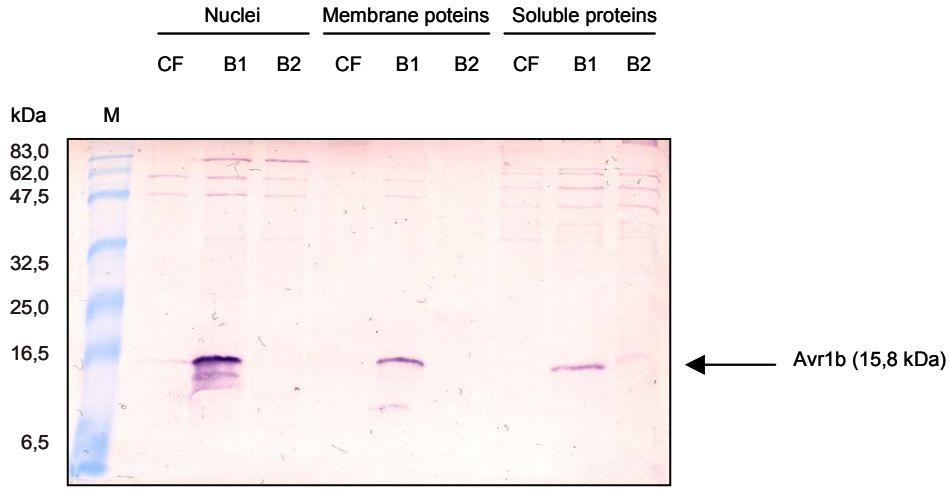
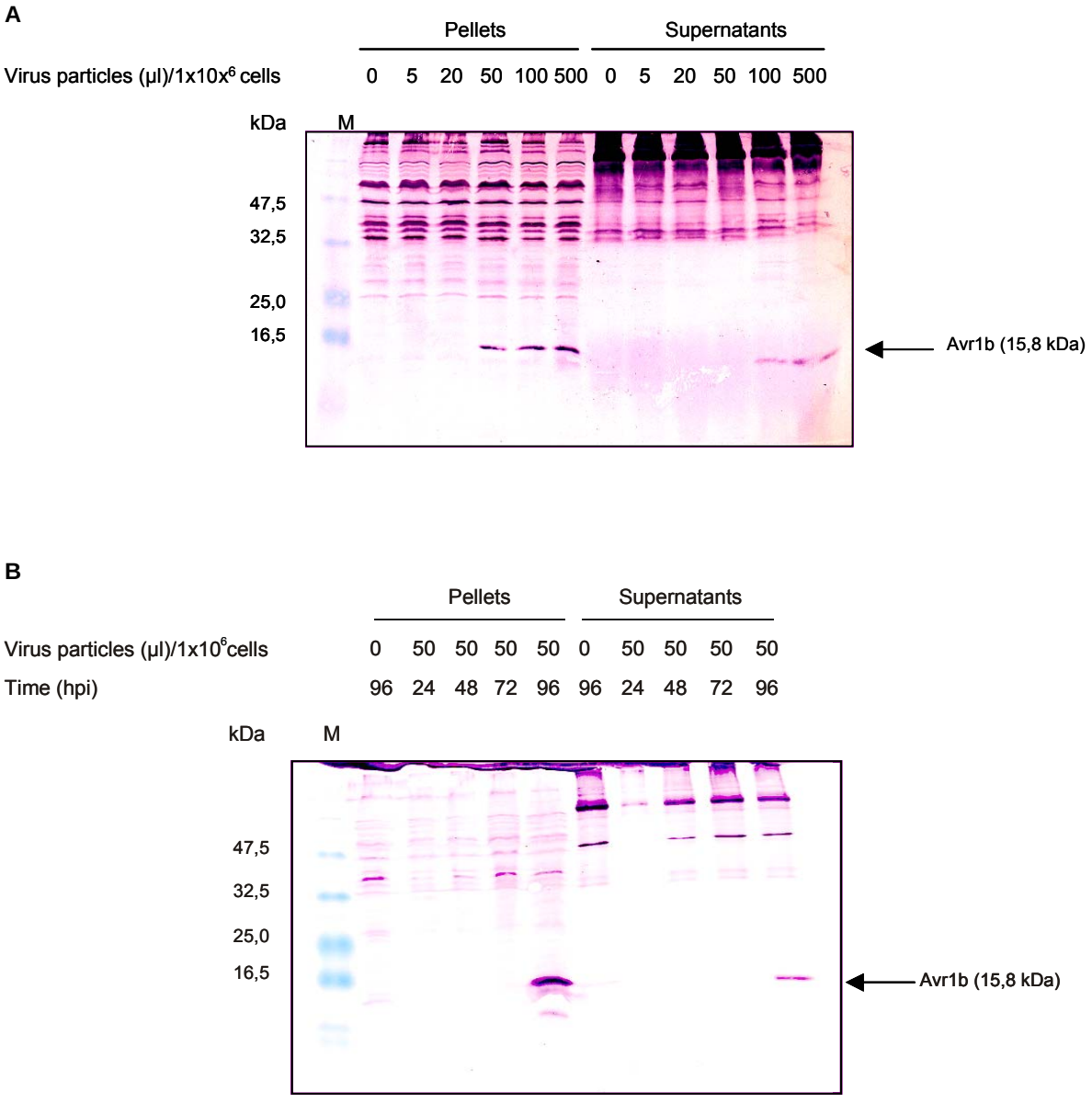


Fig. 13 Proof of synthesis of Avr1b in *Sf9* insect cells. M) protein markers (kDa), CF) Cellfectin-treated insect cells (negative control), B1) insect cells infected with baculovirus-particles derived from bacmid B1 (harboring *Avr1b* including the secretion signal), B2) insect cells infected with baculovirus-particles derived from bacmid B2 (harboring *Avr1b* without the secretion signal). Proteins (30 µg each) were separated by SDS-PAGE (17,4%), transferred onto nitrocellulose membranes and probed with anti-Avr1b-antiserum (1/750 dilution; 3.8.7).

For maximal protein synthesis, it was essential to determine the optimal baculovirus titer for infection and the time necessary for protein recovery. *Sf9* insect cells were first infected with different concentrations of baculovirus particles (5-500 µl P3 baculovirus particles/1x10^6 cells). In an immunoblot analysis (Fig. 14A), it was examined that 50 µl P3 baculovirus particles/1x10^6 cells were sufficient. Using this baculovirus titer, insect cells were infected and harvested in a time course experiment between 24-144 h post-infection (pi). Sufficient abundance of protein expression occurred at 120 hpi (Fig. 14B and 14C). Using these conditions, a subcellular fractionation (3.3.6) of insect cells, infected with P3 baculovirus particles derived from the bacmid B1 that harbors *Avr1b* including the secretion signal, was performed. As a negative control, wild type virus particles were used (2,5 µl/1x10^6 cells). As shown in Fig. 14D, Avr1b did not fractionate into a specific subcellular

fraction but was detectable in culture medium, nuclei, heavy membranes, light membranes and in the cytosol from infected insect cells. Since the fractionation didn't result in a single Avr1b containing fraction, a second, less complex subcellular fractionation obtaining only culture medium, crude membranes and soluble proteins (3.3.6, last paragraph) was performed in additional experiments, where the soluble protein fraction was used as a positive control in Western blot analyses. Figs. 14E and 14D show that Avr1b was present in all fractions of infected insect cells.



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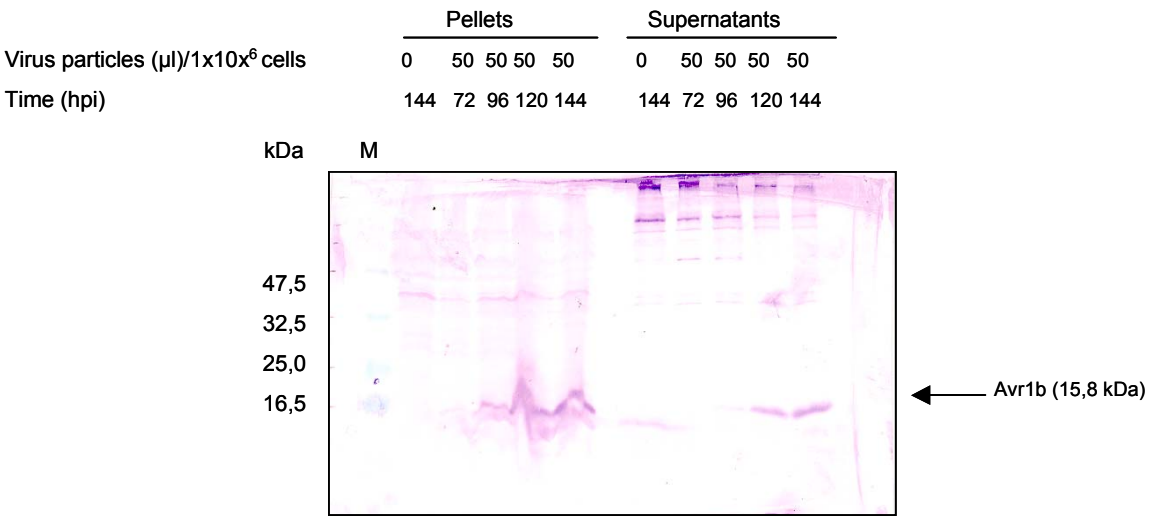
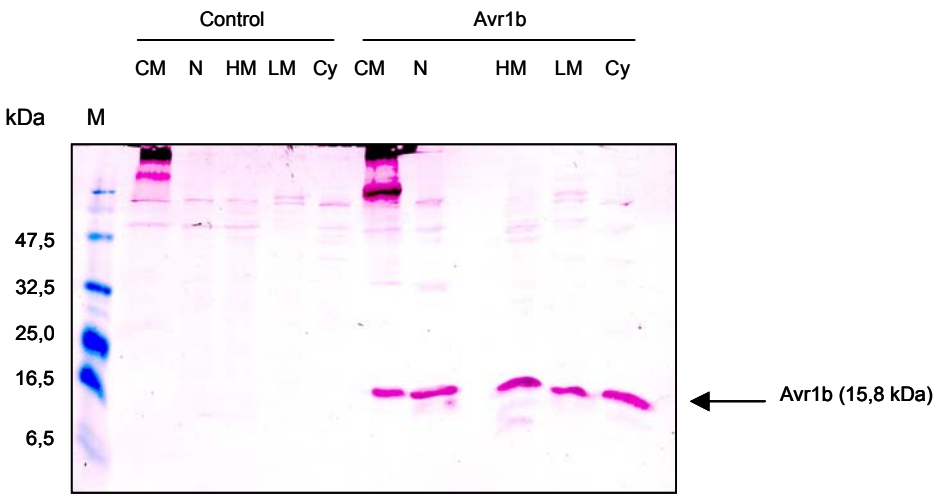

Pellets
Supernatants
Virus particles (µl)/1x10x6 cells
0 50 50 50 50 0 50 50 50 50
Time (hpi)
144 72 96 120 144 144 72 96 120 144
kDa
M
47,5
32,5
25,0
16,5
Avr1b (15,8 kDa)

D


Control
Avr1b
CM N HM LM Cy CM N HM LM Cy
kDa
M
47,5
32,5
25,0
16,5
6,5
Avr1b (15,8 kDa)

E

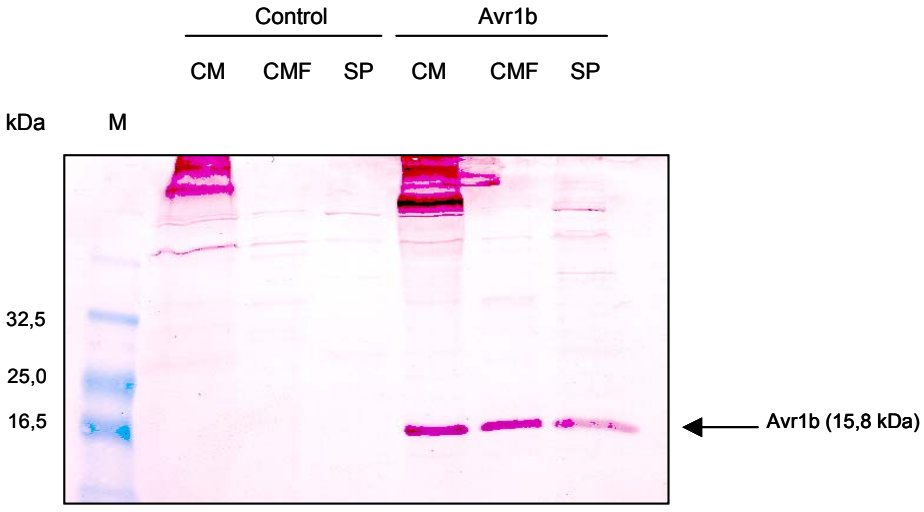
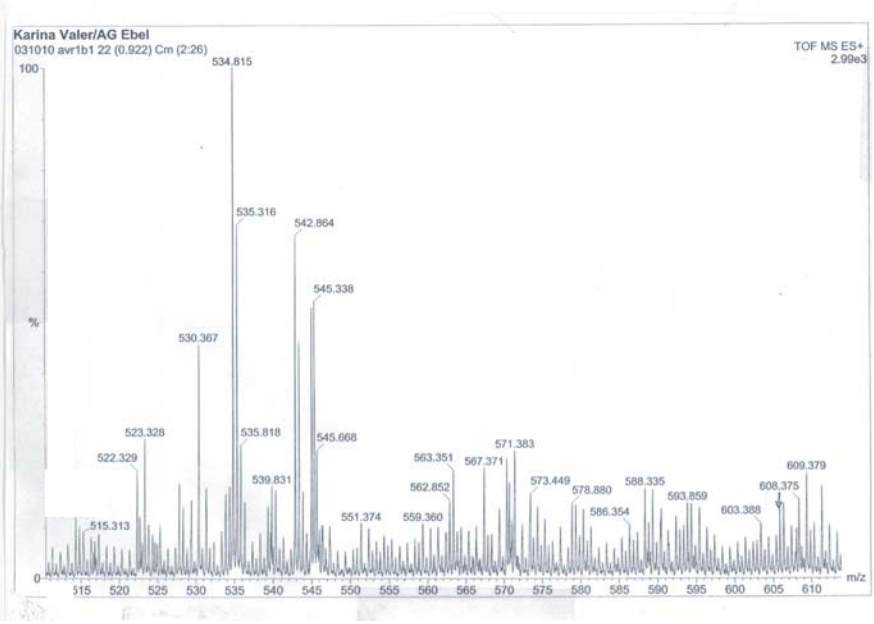

Control
Avr1b
CM CMF SP CM CMF SP
kDa
M
32,5
25,0
16,5
Avr1b (15,8 kDa)

Fig. 14 Synthesis of Avr1b in *Sf9* insect cells. (A) Insect cells were infected with different concentrations of baculovirus particles (5-500 µl P3 baculovirus particles/1x10^6 cells) for optimizing protein production. (B) Insect cells were infected with 50 µl P3 baculovirus particles/1x10^6 cells and harvested in a time course experiment between 24 and 96 hpi. (C) Insect cells were infected with 50 µl P3 baculovirus particles/1x10^6 cells and harvested in a time course experiment between 72 and 144 hpi. (D and E) Subcellular fractionations of infected insect cells at 120 h pi. Control) insect cells infected with 2,5 µl/1x10^6 wild type virus particles, Avr1b) insect cells infected with 50 µl/1x10^6 P3 baculovirus particles (derived from the bacmid that harbors Avr1b including the secretion signal). CM) culture medium, N) nuclei, HM) heavy membranes, LM) light membranes, Cy) cytosol, CMF) crude membrane fraction, SP) soluble protein fraction. M) protein markers (kDa). Proteins (30 µg each) were separated by SDS-PAGE (17,4%), transferred onto nitrocellulose membranes and probed with anti-Avr1b-antiserum (1/750 dilution).

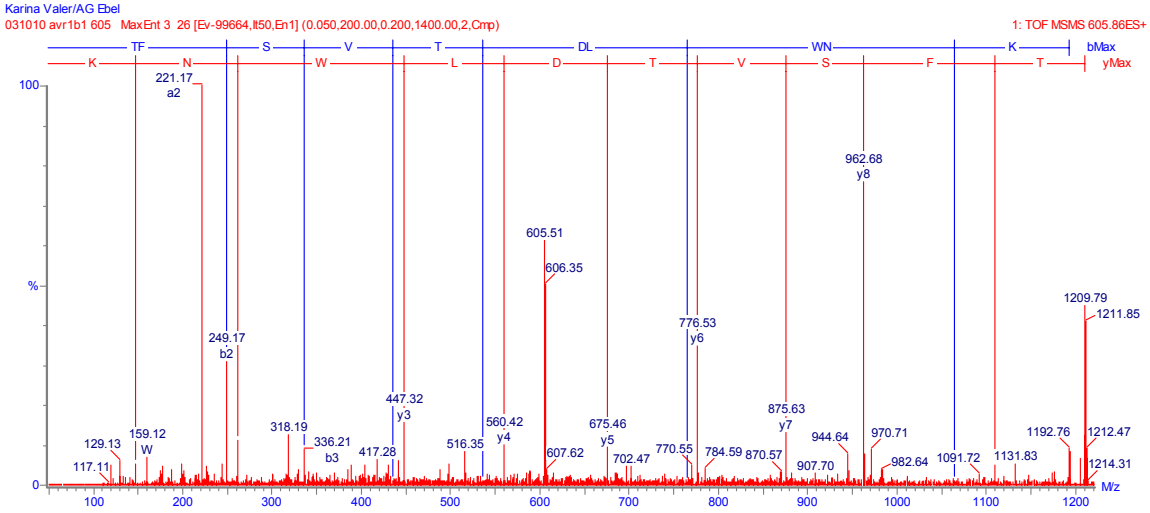
4.2.3 Protein analysis by mass spectrometry

To verify that Avr1b was present in the soluble protein preparations obtained from *Sf9* insect cells, it was analysed by mass spectrometry (3.8.8). The band corresponding to Avr1b (15,8 kDa) was identified by Western blotting and the respective band was excised from another gel lane and subjected to MS analysis. One tryptic peptide **(TFSVTDLWNK)** from Avr1b was found after MS analysis. This result indicates that Avr1b is present in soluble protein preparations from *Sf9* insect cells. The MS spectrum is shown in Fig. 15A, the MS/MS spectrum obtained from **TFSVTDLWNK** in Fig. 15B and the Avr1b sequence with the matching peptide in Fig. 15C.

A



B



C

```
          10        20        30        40        50        60
MRLSFVLSLVVAIGYVVTCNATEYSDETNIAMVESPDLVRRSLRNGDIAGGRFLRAHEED

          70        80        90       100       110       120
DAGERTFSVTDLWNKVAAKKLAKAMLADPSKEQKAYEKWAKKGYSLDKIKNWLAIADPKQ

         130       138
KGKYDRIYNGYTFHRYQS
```

Fig. 15 Protein analysis by mass spectrometry. (A) MS spectrum of a soluble protein preparation from infected insect cells using a ESI-Q-TOF mass spectrometer. The y-axis shows the ion count of the peptides, the x-axis the mass to charge ratio. (B) MS/MS spectrum of the peptide TFSVTDLWNK from Avr1b (calculated molecular weight: 1209,60. In the MS spectrum the peptide has a mass to charge ratio of 605,8). (C) Amino acid sequence of Avr1b. The peptide identified by MS/MS is shown in grey.

4.3 Generation and purification of a polyclonal anti-Avr1b-antiserum

In order to detect the Avr1b protein in soybean - *P. sojae* interactions, it was necessary to have a specific and purified antiserum raised against Avr1b. For this reason recombinant Avr1b obtained from *E. coli* cells was used to immunize rabbits (3.6). The polyclonal antiserum obtained was purified and tested by immunoblot analyses.

4.3.1 Testing of the recipient rabbits

Recombinant Avr1b obtained from *E. coli* cells and refolded using a 7 to 1 M urea gradient on a Ni-NTA column (4.1.2.2) was used to raise an anti-Avr1b-antiserum (SeqLab, Göttingen). Pre-serum obtained from eight rabbits was screened in Western blot analyses for cross reacting antibodies using different protein preparations obtained from *E. coli* cells, *Sf9* insect cells, soybean and *P. sojae* race 1. The pre-serum from one of the rabbits did not show cross reaction with protein preparations in the area corresponding to 15,8 kDa where Avr1b was expected (Data not shown). The purified recombinant Avr1b (2 mg) was used to immunize this rabbit. The anti-Avr1b-antiserum obtained after 6 weeks was also tested in an immunoblot analysis. Avr1b was recognized by the antiserum, however there was a high level of background in the Western blot membrane (Data not shown).

4.3.2 Purification of the antiserum

To decrease the background in immunoblot membranes, it was necessary to purify the anti-Avr1b-antiserum. Many techniques have been developed to purify IgG molecules. Two of them were tested in the present study using protein G sepharose (without success, not shown) and protein A sepharose. The principle of this technique relies on the strong binding of protein A to sites in the second and third constant regions of the Fc portion of the immunoglobulin heavy chain (Deisenhofer, 1981). Each IgG molecule contains two binding sites for protein A. Because protein A itself has four potential sites for binding to IgG (Sjödahl, 1977), it is possible to form multimeric complexes. Protein A coupled to a solid support by cyanogen bromide is supplied by several manufacturers. Each ml of swollen gel can bind approximately 10-20 mg of IgG (equivalent to 1-2 ml of antiserum).The hydrophobic interactions, by which antibodies bind to protein A can be disrupted at low pH, which most antibodies can withstand transiently (Sambrook *et al.*, 1989).

After purification (3.6.6), the eluate fractions E2-E5 were combined and the purified anti-Avr1b-antiserum was tested in an immunoblot analysis using 15 µg of soluble protein preparations from *Sf9* insect cells containing Avr1b (4.2.2). Different dilutions of the purified antiserum were used for membrane development. As shown in Fig. 16, the background in the membrane was significantly reduced when using the purified anti-Avr1b-antiserum. This purified antiserum was used for further immunoblot analyses.

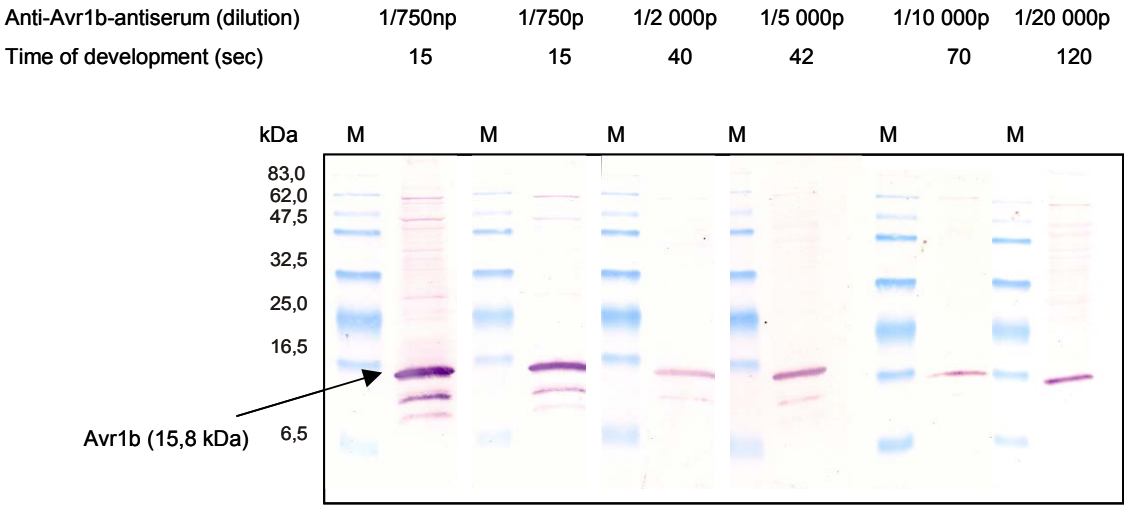


Fig. 16 Purification of anti-Avr1b-antiserum with protein A sepharose using a pH gradient. For each lane, 15 µg of soluble protein preparation obtained from Sf9 insect cells containing Avr1b were separated by SDS-PAGE (17,4%), transferred onto nitrocellulose membranes and probed with non purified (np) and purified (p) anti-Avr1b-antiserum. M) protein markers (kDa).

4.3.3 Evaluation of the antiserum

Once the anti-Avr1b-antiserum had been purified with protein A Sepharose (4.3.2) and proved to detect Avr1b in 15 µg of soluble proteins obtained from *Sf9* insect cells even at dilutions of 1/20 000, it was necessary to select optimised dilution conditions. A 1/1 000 dilution was chosen to avoid a background on the membrane. The diluted antiserum was tested against different dilutions of soluble proteins obtained from *Sf9* insect cells (4.2.2). Fig. 17 shows that the antiserum was able to detect as little as 0,5 µg of the protein preparation containing Avr1b. However the detection limit may vary when testing different protein preparations such as apoplastic fluid, microsomes and soluble proteins obtained from infected soybean seedlings or recombinant Avr1b obtained from *E. coli* cells.

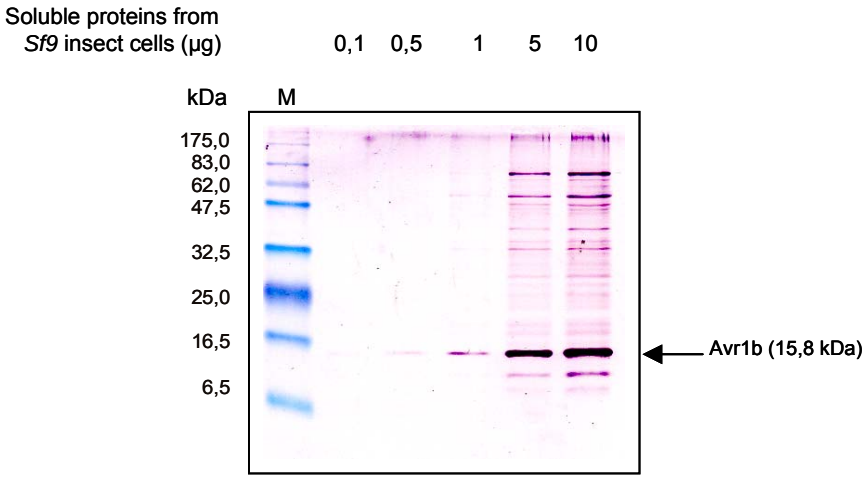


Fig. 17 Detection limit of soluble proteins from *Sf9* insect cells containing Avr1b. M) protein markers (kDa). Different amounts of soluble proteins from *Sf9* insect cells (0,1-10 µg) were separated by SDS-PAGE (17,4%), transferred onto nitrocellulose membranes and probed with purified anti-Avr1b-antiserum (1/1 000 dilution).

4.3.4 Immunoprecipitation

Since the anti-Avr1b-antiserum was not highly sensitive, immunoprecipitation tests were performed in order to detect very low amounts of protein.

4.3.4.1 Immunoprecipitation of urea-denatured protein preparations from *Sf9* insect cells

The anti-Avr1b-antiserum was raised against the recombinant Avr1b protein obtained from *E. coli* cells and refolded under a 7 to 1 M urea gradient (4.1.2.2). It is possible that the protein refolding did not work properly. In this case the antiserum would react preferentially with the denatured Avr1b protein. For this reason, an immunoprecipitation test was performed using urea-denatured Avr1b protein. The fraction used for the test was 10 µg of a soluble protein preparation from *Sf9* insect cells (4.2.2). The immunoprecipitation protocol is described in 3.8.9.1. It was not possible to detect a band corresponding to Avr1b at 15,8 kDa, neither in the pellet nor in the supernatant fractions (Data not shown). Avr1b was found only in the positive control (soluble protein preparation of infected *Sf9* insect cells). Since no washing steps were performed, Avr1b should have been present in either of the fractions. Therefore, an alternative protocol using SDS-denatured protein preparations was performed.

4.3.4.2 Immunoprecipitation of SDS-denatured protein preparation from *E. coli* cells

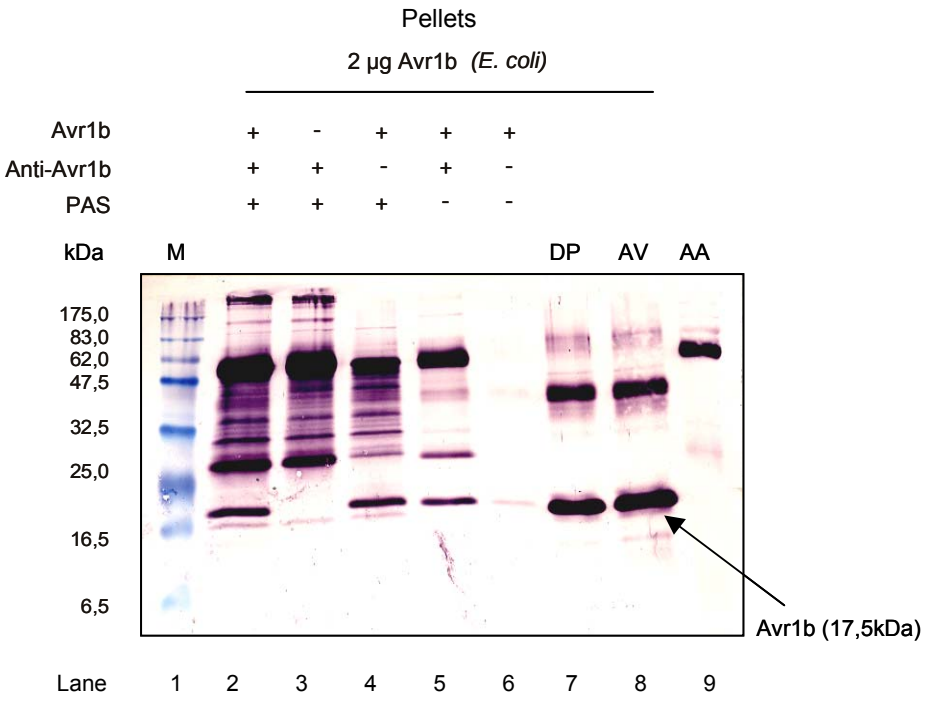
For immunoprecipitation 2 µg of recombinant Avr1b obtained from *E. coli* cells and refolded using a 7 to 1 M urea gradient on a Ni-NTA column (4.1.2.2) was used.
The sample was denatured with SDS followed by immunoprecipitation with anti-Avr1b-antiserum and PAS (3.8.9.2). Three positive controls were used: recombinant Avr1b denatured with SDS and resuspended in 5x SDS loading buffer (DP control), recombinant Avr1b diluted in 5x SDS loading buffer (AV control) and anti-Avr1b-antiserum diluted in 1x SDS loading buffer (AA control). Internal negative controls were also used: no Avr1b, no anti-Avr1b-antiserum or no PAS in the immunoprecipitation.
A band corresponding to Avr1b appears in the positive controls (lines 7 and 8; Figs. 18A and 18B). A band around 47,5 kDa corresponding to the anti-Avr1b-antiserum appears in lane 9 (Figs. 18A and 18B). When there is no Avr1b in the immunoprecipitation, as expected no band corresponding to Avr1b appears in the membranes (lane 3; Figs. 18A and 18B).
When Avr1b is immunoprecipitated with anti-Avr1b-antiserum and PAS, a band corresponding to Avr1b (17,5 kDa) appears in the pellet as expected (line 2, Fig. 18A). However, an Avr1b band also appears in the supernatant (line 2, Fig. 18B), indicating that part of the complex Avr1b - anti-Avr1b-antiserum did not bind to the beads of PAS, and as consequence remains in the supernatant. It is

probable that the amount of PAS used in the immunoprecipitation was not high enough to bind to the complex. In lane 4 (Fig. 18A), there is a band corresponding to Avr1b at 17,5 kDa in the pellet, this result was unexpected and it indicates that Avr1b was able to precipitate without the help of the anti-Avr1b-antiserum, probably the protein was bound to the beads of PAS and therefore was collected in the centrifugation step. In lane 5 (Fig 18), where Avr1b is treated only with the anti-Avr1b-antiserum, an unexpected result is observed. A band corresponding to Avr1b is present in the pellet and also in the supernatant, probably the amount of the complex Avr1b - anti-Avr1b-antiserum was enough for precipitation in the absence of PAS. When anti-Avr1b-antiserum and PAS are missing (line 6; Fig. 18) only a thin band corresponding to Avr1b appears in the pellet (Fig. 18A), and a strong band appears in the supernatant (Fig. 18B), indicating that Avr1b is not able to precipitate alone.

Although it was possible to precipitate the complex Avr1b - anti-Avr1b-antiserum - PAS (line 2, Fig. 18A), the immunoprecipitation method still presents some problems. For example, Avr1b precipitates in the absence of anti-Avr1b-antiserum (line 4, Fig 18A) or part of the complex Avr1b – anti-Avr1b-antiserum remains in the supernatant (line 2, Fig. 18B). However when using enough amount of purified Avr1b (2 µg from *E. coli*), Avr1b can be immunoprecipitated.

The prospects of using immunoprecipitation for the detection of Avr1b in soybean tissues after infection with *P. sojae* (chapter 4.5.7) could be favorable when the Avr1b level is relatively high.

A



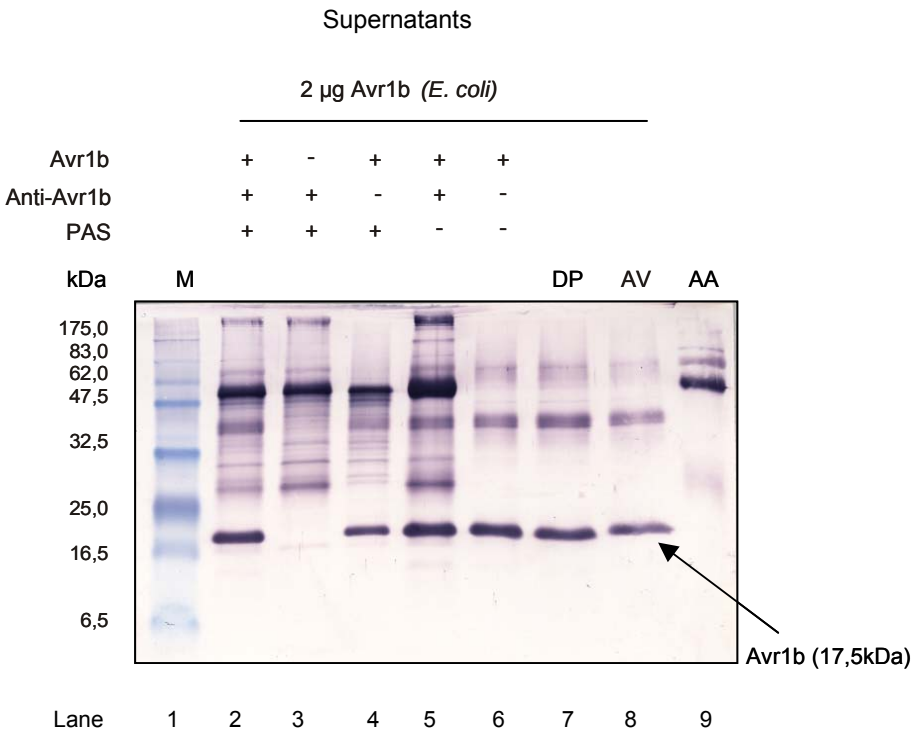


Fig. 18 Immunoprecipitation of purified Avr1b (Avr1b obtained from *E. coli* cells and refolded using a 7 to 1 M urea gradient). (A) Pellets. (B) Supernatants. Symbols denote (+) presence or (-) absence of a compound. M) protein markers (kDa), DP) 2 µg Avr1b denatured with SDS and redissolved in 5x SDS loading buffer (positive control), AV) 2 µg Avr1b diluted in 5x SDS loading buffer (positive control), AA) 2,5 µl anti-Avr1b-antiserum diluted in 1x SDS loading buffer (positive control). Proteins were separated by SDS-PAGE (17,4%), transferred onto nitrocellulose membranes and probed with purified anti-Avr1b-antiserum (1/1 000 dilution).

4.4 Detection of Avr1b in *P. sojae*

To confirm the expression of Avr1b in *P. sojae*, two different approaches have been used. In order to detect the Avr1b protein, *P. sojae* mycelium was fractionated followed by an immunoblot analysis. Furthermore for detecting the *Avr1b*-mRNA in *P. sojae* mycelium, its zoospores and in infected soybean seedlings, RNA was extracted from the tissues followed by RT-PCR analyses.

4.4.1 Fractionation of mycelial material

The subcellular fractionation of *P. sojae* race 1 mycelium (3.10) resulted in four fractions: cell debris, mitochondria, soluble proteins and microsomes. Since Avr1b contains a putative secretion signal, the culture medium was also analysed by Western blotting. Fifty µg of the protein fractions were used for the test. The experiment was performed four times. As shown in Fig. 19, there is no band corresponding to Avr1b (15,8 kDa) in none of the fractions, except of the positive control in lane 6

(soluble protein preparation of *Sf9* insect cells containing Avr1b). Alternative methods with BSA, Tween 20 or milk in the blocking solution were also tested with identical results (data not shown).

Since the molecular weight of recombinant Avr1b is dependent of the organism expressing it (Fig. 19B, lines 3 and 4), it cannot be decided whether protein bands at around 16,5 kDa in Fig. 19A (lines 2, 3 and 5) might be related to Avr1b. To verify that these bands do not correspond to Avr1b, microsomes from *P. sojae* race 2 (avr1b; here the protein is not produced) and race 1 (Avr1b) were compared in a second Western blot analysis. Fig. 19B shows that the pattern of the bands in the crucial 16,5 kDa region of the extracts from both *P. sojae* races are exactly the same (lanes 1 and 2) excluding the possibility of Avr1b synthesis in race 1. These results indicate that Avr1b is not produced in *P. sojae* cultures.

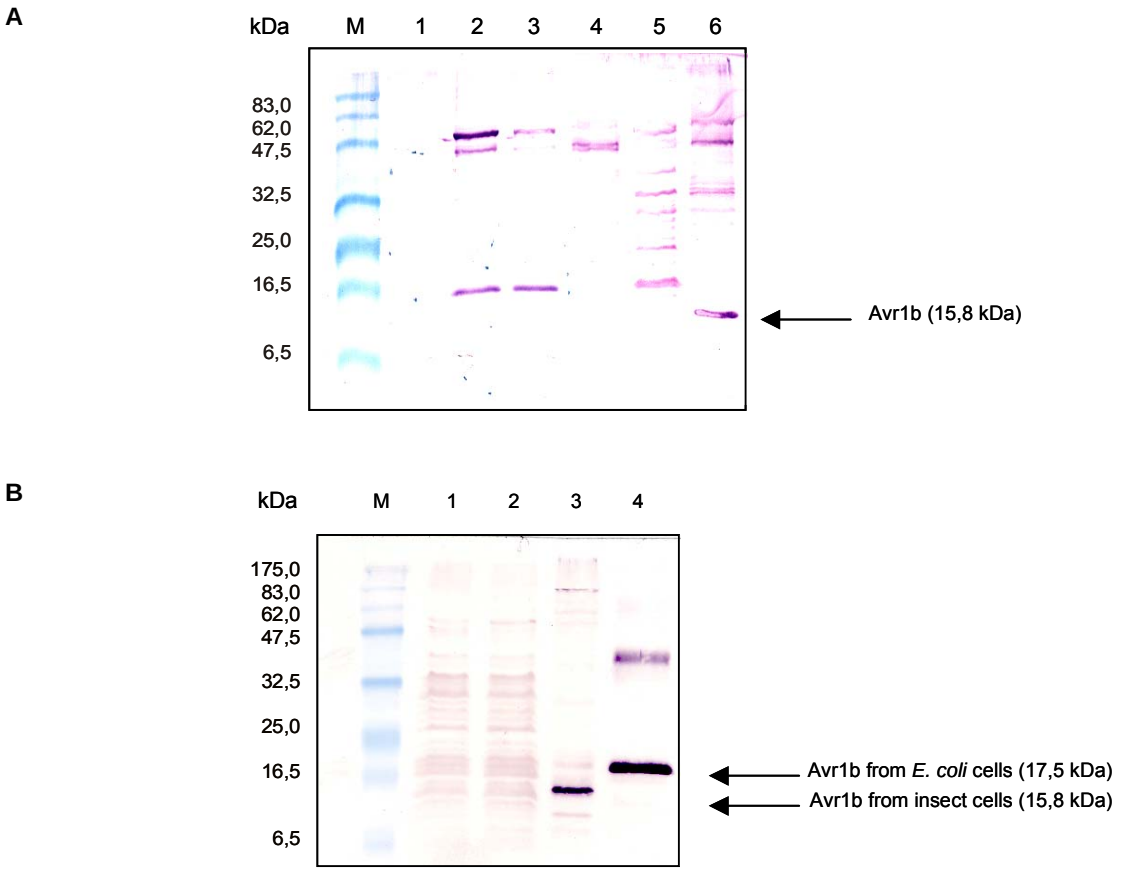


Fig. 19 (A) Subcellular fractionation of a mycelium derived from *P. sojae* race 1. M) protein marker (kDa), 1) culture medium, 2) cell debris, 3) mitochondria, 4) soluble proteins, 5) microsomes, 6) positive control: Avr1b obtained from a soluble protein preparation of infected *Sf9* insect cells. Proteins (50 µg for lanes 1-5; 15 µg for lane 6) were separated by SDS-PAGE (17,4%), transferred onto nitrocellulose membranes and probed with anti-Avr1b-antiserum (1/750 dilution). (B) Immunoblot analysis performed with microsomes from *P. sojae* race 2 (lane 1) and race 1 (lane 2, 20 µg each), 3) 10 µg of a soluble protein preparation from *Sf9* insect cells containing Avr1b (positive control), 4) 2 µg recombinant Avr1b obtained from *E. coli* cells (His_6-fusion protein, positive control). M) protein markers (kDa). Proteins were separated by SDS-PAGE (17,4%), transferred onto nitrocellulose membranes and probed with purified anti-Avr1b-antiserum (1/1 000 dilution).

4.4.2 Analysis of transcript levels in *P. sojae* and in infected soybean roots

Once it had been proved that the Avr1b protein is not expressed in the vegetative mycelium of *P. sojae* race 1 (4.4.1) it was necessary to verify that the *Avr1b* gene was also not transcribed in the mycelium before plant infection. The gene encoding the mRNA is in fact one of two genes required for the Avr1b phenotype and for clarity the gene will be referred to from here on as *Avr1b*-1.

An analysis of transcript levels in *P. sojae* race 1 mycelium (Fig. 20A), its zoospores and in susceptible soybean roots being infected by zoospores of *P. sojae* race 1 (Fig. 20B) was performed.

As observed in Fig. 20, the *Avr1b*-1 gene was not transcribed neither in the zoospores (Fig. 20B, line 3) nor in the mycelium of *P. sojae* race 1 at different growth stages (Fig. 20A), confirming the results obtained in 4.4.1 and by Shan *et al.* (2004). The constitutively expressed *P. sojae Act*A gene was used to check the amounts of RNA loaded on the gels (Figs. 20A and 20B).

The *Avr1b*-1 gene is induced in soybean during infection by *P. sojae* (Fig. 20B, line 2) which suggests that Avr1b might play a role not only in avirulence but also in virulence as it has been demonstrated earlier for many bacterial avirulence proteins (Gabriel, 1999; Bonas and Van den Ackerveken, 1997). The experiment was performed twice.

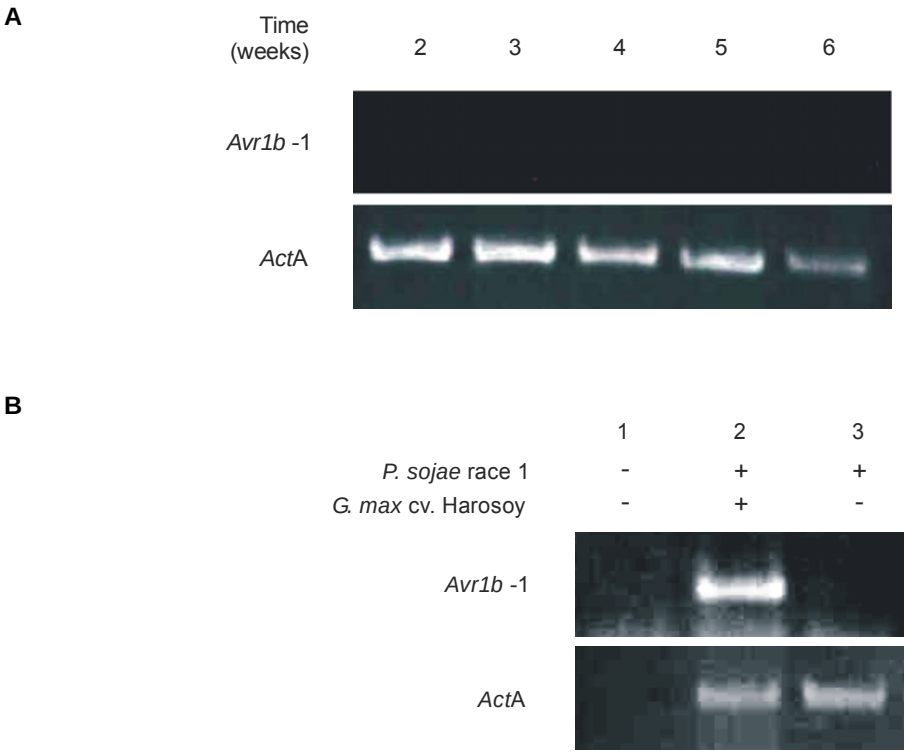


Fig. 20 Semi quantitative RT-PCR of *P. sojae Avr1b*-1 and *Act*A genes obtained from the *P. sojae* race 1 mycelium at different growth stages (A), from its zoospores (B, lane 3) and from soybean cv. Harosoy roots after infection with zoospores of *P. sojae* race 1 (B, lane 2). Lane 1 contains the result of a RT-PCR control assay using water.

4.5 Biological tests

Soybean tissues respond to β-glucan elicitors present in *P. sojae*. This response is the basis of different biological assays that have been developed. The first assay developed by Ayers *et al*. (1976, IX) was a modification of the cotyledon assay used by Frank and Paxton (1971). Briefly, a section, approximately 1 mm thick and 6 mm diameter, is cut from the lower surface of cotyledons obtained from 8 day old soybean seedlings, a 100 µl drop of elicitor solution is placed on the wound, and incubation takes place for 20 h. Production of glyceollins can be detected photometrically at 285 nm (Albersheim and Valent, 1978). This assay has been repeated many times by people in our laboratory. In this work, three types of alternative biotests were performed. Unwounded, 3 day-old soybean seedlings were exposed as described in section 3.15, to protein preparations obtained either from *E. coli* or from *Sf9* insect cells. Glyceollins were isolated, detected photometrically at 285 nm and quantified by high performance liquid chromatography (HPLC).
Furthermore, hypocotyls of 9-12 day-old soybean plants were inoculated with small pieces of *P. sojae* mycelium as described by Albersheim and Valent (1978) (3.11). RNA obtained from infected tissues was isolated and *Avr1b*-1 transcripts were detected by RT-PCR.
In a third biotest, soybean seedlings were infected with zoospores from *P. sojae* (Hahn *et al.*, 1985) (3.12) prior to either Western blotting or RT-PCR.

4.5.1 Biotests on soybean using different protein preparations

4.5.1.1 Biotests on soybean using recombinant protein preparations obtained from *E. coli* cells

The biological activity of Avr1b obtained as a recombinant protein from *E. coli* and purified both under denaturing conditions and after protein refolding (4.1) was tested. For this purpose, unwounded seedlings of soybean cv. Harosoy 1372 (*Rps1b*) were treated with 300 µl of purified Avr1b protein preparations as described in 3.15. In a first test, the fractions El-2, El-3 and El-4 obtained after denaturing purification (4.1.2.1) were combined and dialyzed against dialysis buffer (3.5.1). The biotest was performed using dilutions of Avr1b (0,15; 1,5 and 12 µg/ml) as well as water and dialysis buffer as negative controls. As a positive control for the biotests, the seedlings were treated with 200 µg/ml raw elicitor resulting in an increase of glyceollin concentration to 355 nmol/g fresh weight (fw) in contrast to 55 nmol/g fw and 185 nmol/g fw for water- and dialysis buffer-treated control seedlings, respectively.
In a second test, the fractions 9-14 obtained after a protein refolding (4.1.2.2) were combined and the biotest was performed using dilutions of Avr1b (14 and 53 µg/ml). Here, water and buffer 2B (3.5.2) served as negative controls. As a positive control for the biotests, the seedlings were treated again with 200 µg/ml raw elicitor resulting in an increase of glyceollin concentration to 137 nmol/g fw in contrast to 23 nmol/g fw and 0 nmol/g fw for water- and buffer 2B-treated control seedlings, respectively.

The raw elicitor obtained from cell walls of *P. sojae* (2.4*)* contains a β-glucan fraction which has been shown to elicit phytoalexin production in soybean (Ayers *et al*., 1976a; Albersheim and Valent, 1978; Hahn *et al*., 1985; Ebel *et al*., 1986). The application of Avr1b to the soybean seedlings did not result in an increase of glyceollins above the levels monitored for the negative controls.

4.5.1.2 Biotests on soybean using recombinant protein preparations obtained from *Sf9* insect cells

Activation of defence responses in several plants depends on the presence of extracellular Ca^{2+}. Omission of Ca^{2+} from the culture medium blocked the defence-related phytoalexin formation in soybean (Stäb and Ebel, 1987). Depletion of Ca^{2+} also prevented elicitor-mediated gene activation at the level of transcript accumulation (Nürnberger *et al*., 1994a) and transcript synthesis (Ebel and Scheel, 1992). Werner and Hohl (1990) described the positive influence of bivalent cations like Ca^{2+} and Mg^{2+} on the accumulation and secretion of isoflavonoids in elicitor-induced soybean seedlings.
To test the biological activity of Avr1b obtained as a recombinant protein from infected *Sf9* insect cells, unwounded seedlings of soybean cv. Harosoy 1372 (*Rps1b*) were exposed (3.15) to Avr1b-containing culture medium, soluble protein fraction and a crude membrane fraction obtained from infected *Sf9* insect cells (4.4.2).
Each test was performed using 1 mg/ml protein extract containing Avr1b in the presence or absence of 10 mM $MgCl_2$. Raw elicitor (200 µg/ml) was used as positive control, water and buffer N as negative controls. The experiment was performed twice. Production of glyceollin after exposure to raw elicitor was set as 100% (Fig. 21) and the other fractions were compared to this value.
Seedlings treated with raw elicitor and Mg^{2+} resulted in an increase of glyceollin concentration (710 nmol/g fw; 169%) in contrast to 420 nmol/g fw (100%) for raw elicitor treated seedlings. This effect has also been described previously by T. Waldmüller (personal communication).
Seedlings treated with recombinant Avr1b preparations obtained from infected *Sf9* insect cells with or without Mg^{2+} as well as with control preparations (4.2.2) with or without Mg^{2+} induced similar concentrations of glyceollin (around 300 nmol/g fw; 70%) (Fig. 21).
Since the glyceollin concentration in seedlings treated with and without Avr1b was similar, it can be deduced that the biological activities measured are not due to Avr1b but probably due to plant recognition of other pathogen elicitors or due to unknown factors.

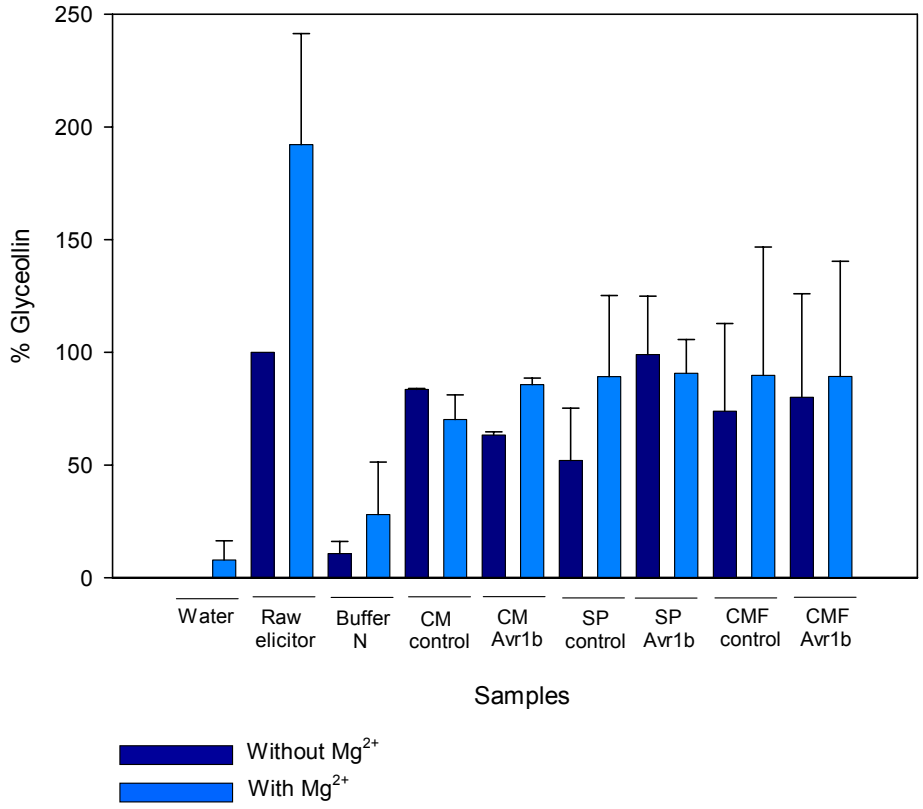


Fig. 21 Quantification of glyceollin in soybean seedlings treated with Avr1b-containing protein fractions obtained from *Sf9* insect cells infected with P3 baculovirus particles and with control protein preparations obtained from *Sf9* insect cells infected with wild type virus particles (containing no Avr1b). CM) culture medium, SP) soluble protein fraction, CMF) crude membrane fraction.

4.5.2 Infection of soybean hypocotyls with mycelium of *P. sojae*

Even though *P. sojae* infects roots, many researchers use hypocotyl infection assays for reasons of convenience and reproducibility. Here, hypocotyls of 9 to 12 day-old seedlings of the soybean cultivars Harosoy (*rps1b*) and Williams L77-1863 (*Rps1b*) were wounded and inoculated with small pieces of *P. sojae* mycelium of either race 1 (*Avr1b*-1) or race 2 (*avr1b*) (Albersheim and Valent, 1978; 3.11). Five days after inoculation, pictures of the seedlings were taken. As illustrated in Fig. 22, resistance was observed only when the soybean cv. Williams L77-1863 (*Rps1b*) was inoculated with *P. sojae* race 1 (*Avr1b*-1) accompanied with local cell death in the region of infection due to a hypersensitive response of the plant. When either the *Avr* gene or the *Rps* gene was missing soybean was not able to recognize *P. sojae* resulting in plant susceptibility and eventually plant death (Fig. 23). The experiment was performed three times.

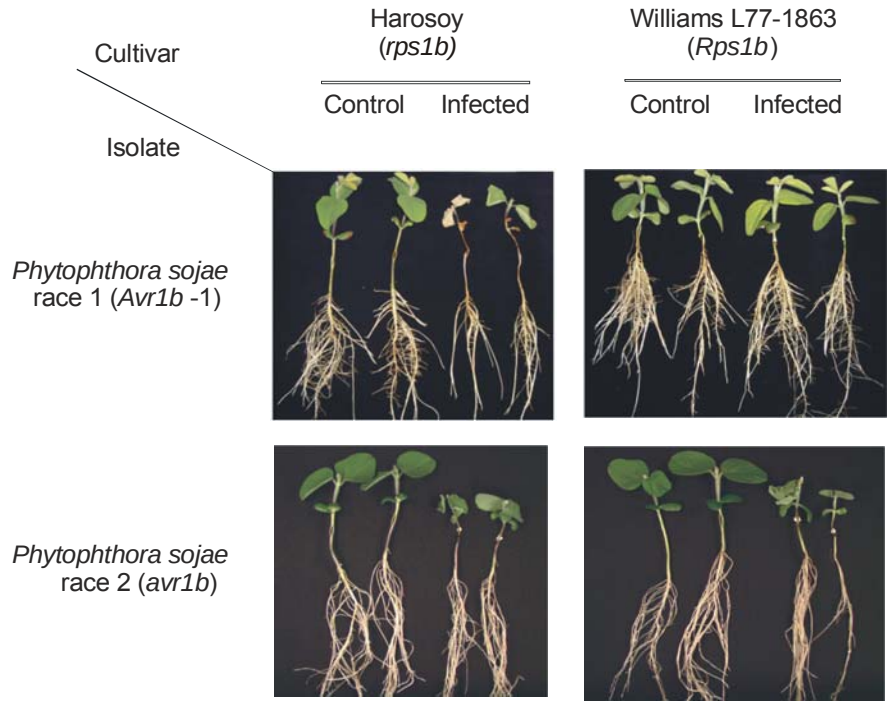


Fig. 22 Tests on soybean hypocotyls. Hypocotyls of 9 to 12 day-old soybean seedlings of the cultivars Harosoy and Williams L77-1863 were wounded and inoculated with small pieces of *P. sojae* mycelium of either race 1 (*Avr1b*-1) or race 2 (*avr1b*). Control) uninfected soybean seedlings.

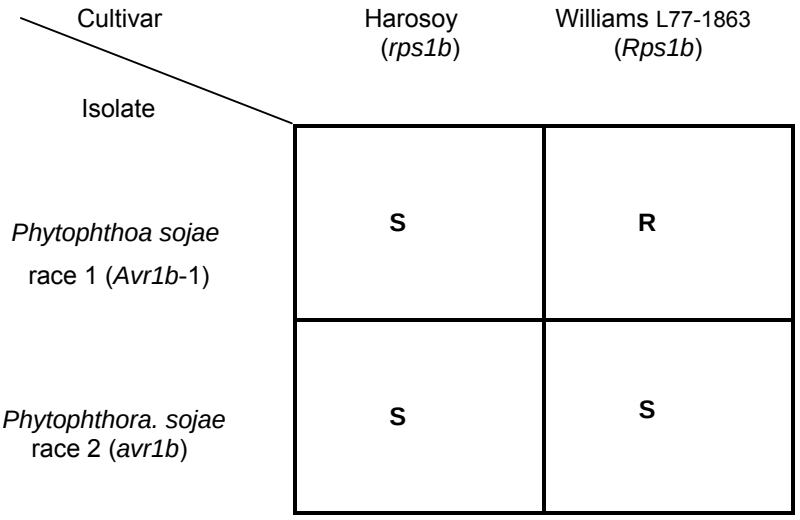


Fig. 23 Gene-for-gene effects, observed after the interaction of different *P. sojae* races with soybean cultivars. R) resistant plants (incompatible interaction), S) susceptible plants (compatible interaction).

4.5.2.1 Analysis of transcript levels of *P. sojae Avr1b*-1, actin *Act*A and soybean tubulin *tub*B2 in soybean hypocotyls inoculated with *P. sojae* mycelium

Five days after infection of soybean hypocotyls with *P. sojae* mycelium (4.5.2), the hypocotyl segments around the infection sites were excised for isolation of total RNA (3.14.3) and subsequent RT-PCR analyses (3.9.9). Transcription of *P. sojae Avr1b*-1 and of the constitutively expressed actin *Act*A genes were tested in both the compatible cv. Harosoy - *P. sojae* race 1 and the incompatible cv. Williams L77-1863 - *P. sojae* race 1 interaction. Amplification of the soybean tubulin *tub*B2 gene was used as control to verify the integrity of soybean mRNAs.

High transcript levels of *Act*A were detected in susceptible soybean hypocotyls of cv. Harosoy infected with race 1 (*Avr1b*-1) or race 2 (*avr1b*), and Williams L77-1863 infected with race 2 (Fig. 24). In the incompatible interaction between soybean cv. Williams L77-1863 and *P. sojae* race 1, the *Act*A transcript level was low. *Avr1b*-1 transcripts were found after infection with *P. sojae* race 1 (Fig. 24A), but not with *P. sojae* race 2 (Fig. 24B). *Avr1b*-1 transcript levels were higher in tissues of susceptible than of resistant soybean cultivars (Fig. 24A) reflecting probably the restriction of pathogen growth in the incompatible interaction, as suggested by low *Act*A transcript levels (Fig. 24A). The experiment was performed twice.

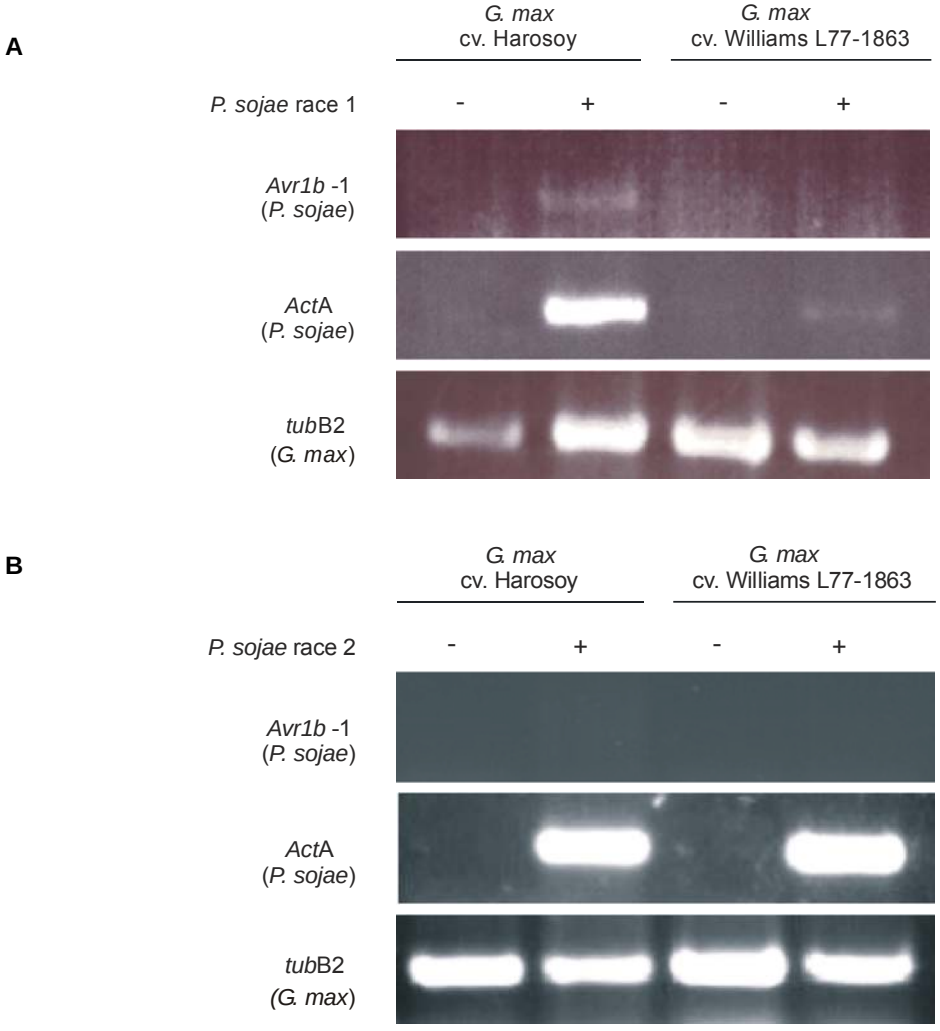


Fig. 24 Analyses of transcript levels in soybean hypocotyls by semi quantitative RT-PCR. Transcript levels of *P. sojae Avr1b*-1, *P. sojae* actin *Act*A and soybean tubulin *tub*B2 genes were monitored using gene-specific primers in the compatible soybean cv. Harosoy - *P. sojae* race 1 and the incompatible soybean cv. Williams L77-1863 - *P. sojae* race 1 interactions (A), as well as in the compatible soybean cv. Harosoy or cv. Williams L77-1863 - *P. sojae* race 2 interaction (B). Symbols denote: (+) soybean infected with either race 1 or race 2 as indicated; (-) uninfected soybean.

4.5.3 Expression levels of *P. sojae Avr1b*-1, actin *Act*A and soybean tubulin *tub*B2 in different segments of soybean roots infected with *P. sojae* race 1 zoospores

In an earlier work (Hahn *et al*., 1985) on the spatial course of hyphae growth and glyceollin I accumulation in infected soybean seedling roots, two *P. sojae* races and a single soybean cultivar were used. The results showed large differences between resistant and susceptible plants. The incompatible interaction was characterized by extensive colonization of the root cortex by the oomycete at the infection site concomitantly with high glyceollin I levels. No hyphae were observed in sections with detectable phytoalexin levels. In contrast, the compatible interaction was characterized by extensive, unimpeded oomycete colonization of the root stele, with lesser growth in the rest of the root. Only small amounts of glyceollin I were detected in whole root extracts during the first 14 h after infection. By 28 h after infection the situation was reversed with the roots from the compatible interaction containing more glyceollin I than the roots from the incompatible interaction (Hahn *et al.*, 1985).

Levels of mRNA and activity levels of various enzymes involved in glyceollin formation in soybean roots increased significantly shortly after inoculation with *P. sojae* zoospores of an avirulent race compared with a virulent race when the same soybean cultivar was used throughout the experiment (Bonhoff *et al*., 1986a, b; Habereder *et al*., 1989). Conversely, there is no such detailed information available on the time course of *Avr1b*-1 mRNA expression in the *P. sojae* - soybean interaction (Shan *et al*., 2004), nor has the Avr1b-1 protein been analysed in infected soybean plants.

In this study, mRNA expression of *P. sojae Avr1b*-1 and actin *Act*A genes was studied in the compatible soybean cv. Harosoy - *P. sojae* race 1 and in the incompatible soybean cv. Williams L77-1863 - *P. sojae* race 1 interaction. By 24 h after infection of roots with zoospores, *Avr1b*-1 and *Act*A mRNA were detected mainly in the basal soybean root segments (“tip” and “ring”; Fig. 5 in 3.12) covered by zoospore suspension (Figs. 25A and 25 B). No mRNA expression was detected in the upper root segments (“up”; Fig. 5 in 3.12) not covered by zoospore suspension (Figs. 25A and 25B). *Avr1b*-1 mRNA was expressed stronger in the compatible interaction (Fig. 25A) with soybean cv. Harosoy in comparison to the incompatible interaction (Fig. 25B) with soybean cv. Williams L77-1863. The experiment was repeated twice.

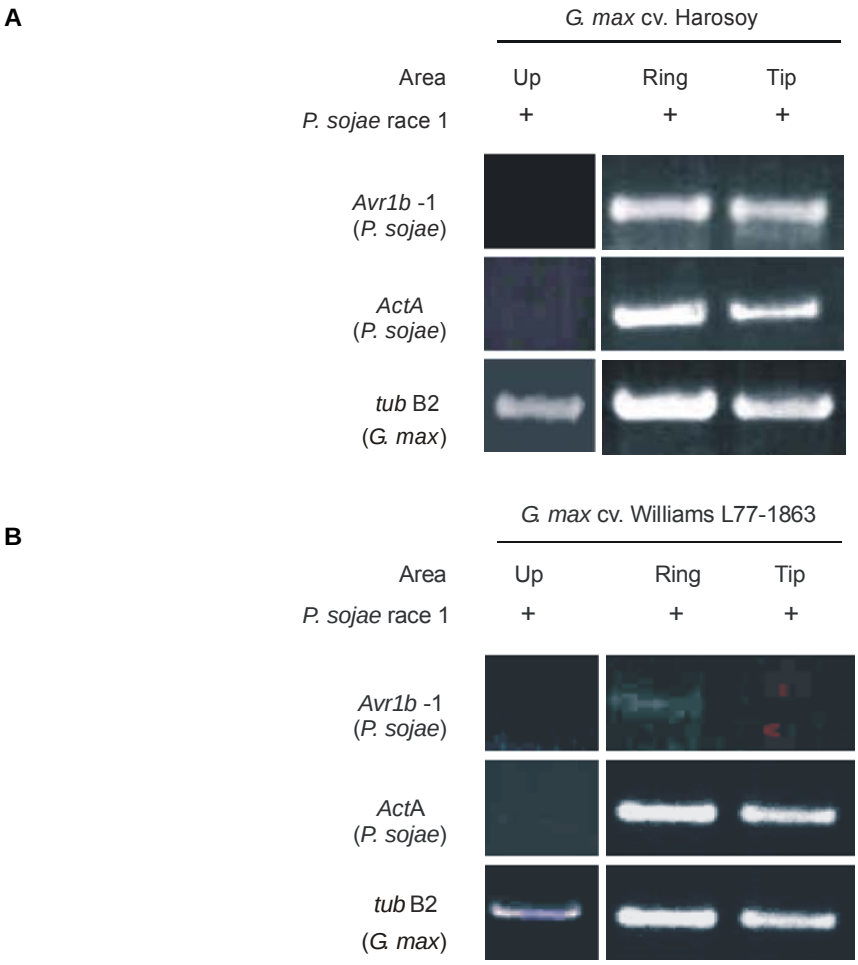


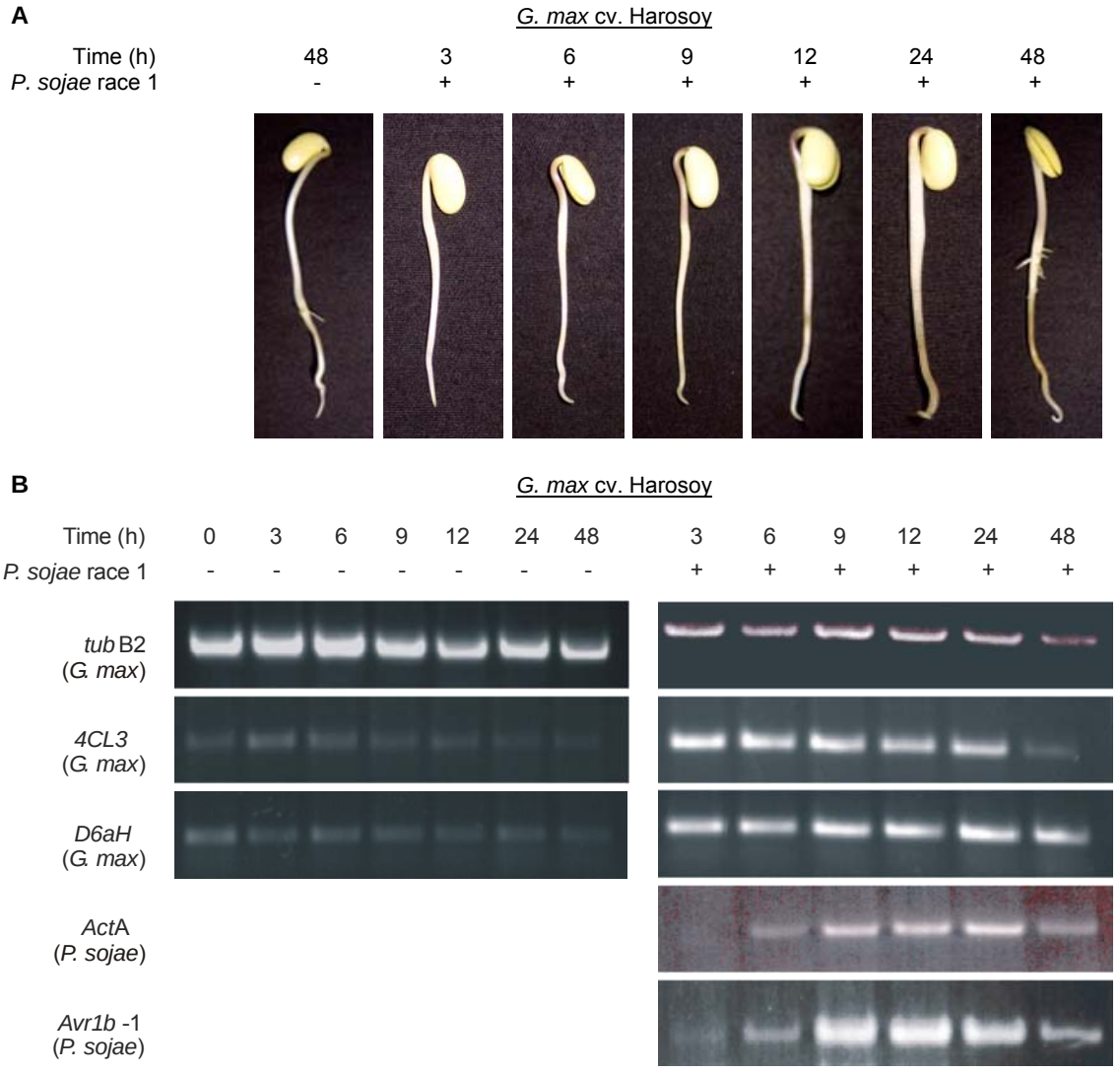
Fig. 25 Spatial course of transcript accumulation in infected soybean roots. Roots of unwounded, 3 day-old soybean seedlings of cv. Harosoy (A) and cv. Williams L77-1863 (B) were dip-inoculated with zoospores of *P. sojae* race 1. After 24 h of inoculation, root segments were excised from the ring at the water-air interface ("ring"), above this ring ("up") and below this ring ("tip") according to Fig. 5 (3.12). Transcript abundance was analysed by RT-PCR using gene-specific primers in the compatible cv. Harosoy - *P. sojae* race 1 (A) and the incompatible cv. Williams L77-1863 - *P. sojae* race 1 (B) interaction.

4.5.4 Time course of mRNA expression in compatible and incompatible *P. sojae* - soybean interactions

Besides *Avr1b*-1, also genes encoding two enzymes of phytoalexin biosynthesis in soybean were chosen to monitor time courses of infection-induced changes of mRNA levels of defence-related metabolic processes in the host plant. One of the genes is coding for 4-coumarate:Coenzyme A ligase (4CL3) (Lindermayr *et al*., 2002), a central enzyme in general phenylpropanoid metabolism involved in the synthesis of one of the phytoalexin precursors. The second gene is coding for 3,9-dihydroxypterocarpan 6a-hydroxylase (D6aH) (Schopfer *et al*., 1998), a hydroxylase specifically involved in a late step of glyceollin biosynthesis (Ebel, 1998). In both interactions, the compatible between soybean cv. Harosoy - *P. sojae* race 1 and the incompatible between soybean cv. Williams

L77-1863 - *P. sojae* race 1, *4CL3* and *D6aH* mRNA levels were enhanced by 3 h after infection. The high mRNA levels were maintained for more than 24 h (4CL3) or even for 48 h (D6aH) (Figs. 26B and 26D, right panel). Low mRNA levels for the two enzymes were observed in uninfected control roots which did not change during the period of the experiment (Figs. 26B and 26D, left panel). The pattern of *Avr1b*-1 mRNA expression appeared to be constitutive throughout the infection and correlated with the amount of pathogen present. By 3 h after infection *Avr1b*-1 and *Act*A mRNAs were barely detectable in either of the interactions. By 6 h low levels were found, subsequently increasing in both types of interaction and remaining high more than 24 h after infection (Figs. 26B and 26D). The ratio of the relative expression levels between the constitutively expressed *Act*A and the *Avr1b*-1 mRNA indicated that *Avr1b*-1 mRNA accumulated to higher amounts in the compatible interaction (Fig. 26B) when compared to the incompatible interaction (Fig. 26D).

The compatible interaction was characterized by a slight browning at the infection site of the roots 8 to 12 h after infection (Fig. 26A). The incompatible interaction was characterized by a darkening at the infection site of the roots 5 h after infection, later identifiable as a hypersensitive ring necrosis (Fig. 26C). The water-treated control seedlings rarely showed any discoloration or tissue reaction at the inoculation sites. The experiments were repeated between two and five times.



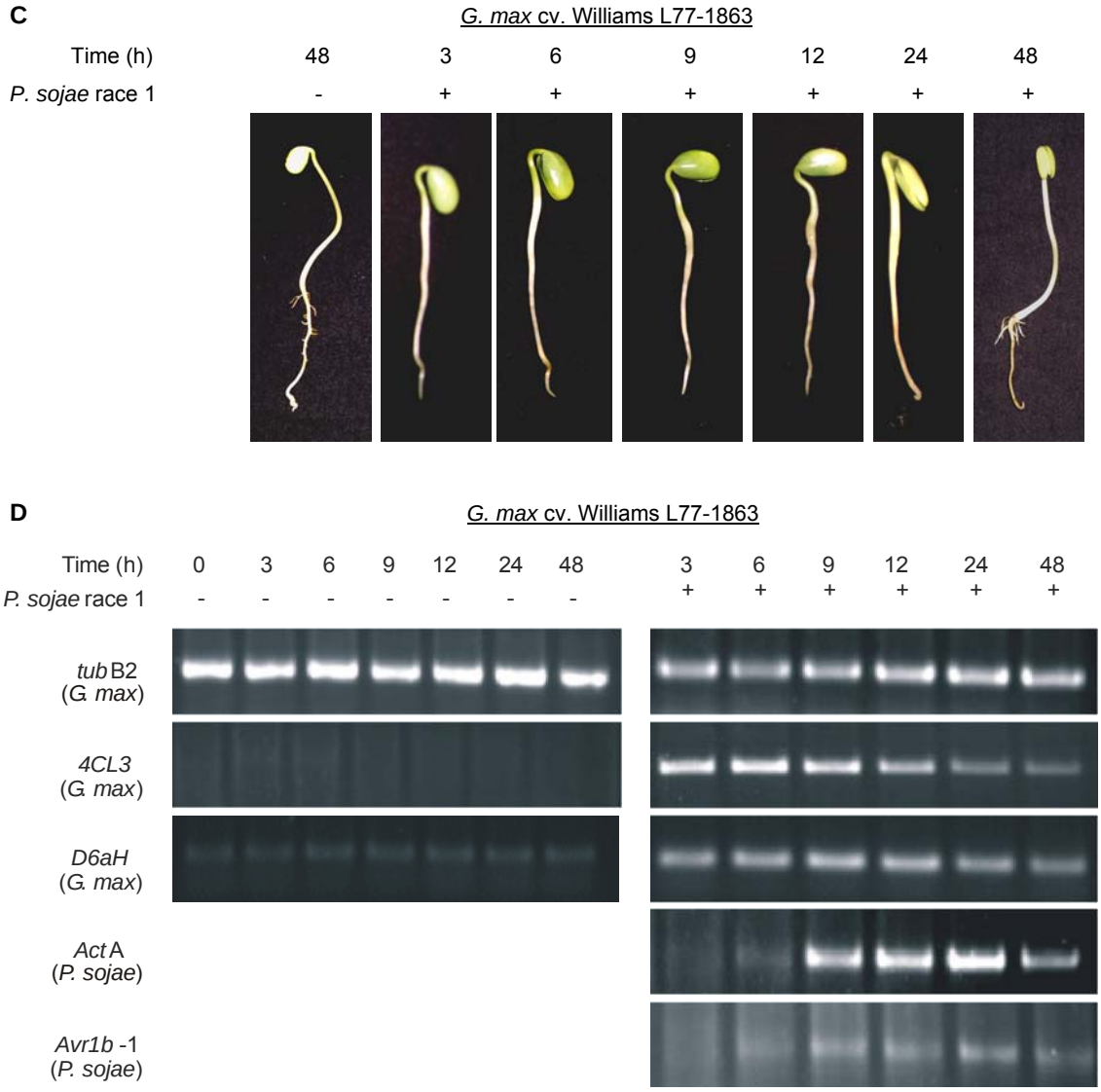


Fig. 26 Time course of mRNA expression in the compatible *P. sojae* race 1 - soybean cv. Harosoy (A and B) and the incompatible *P. sojae* race 1 - soybean cv. Williams L77-1863 interaction (C and D). Roots of unwounded, 3 day-old soybean seedlings were dip-inoculated with zoospores of *P. sojae* race 1 and transcript levels were analysed by RT-PCR using gene-specific primers. After the indicated time of inoculation (h), pictures of the seedlings were taken (A, C) and root segments at the infection site ("ring", 3.12) were excised for isolation of total RNA and RT-PCR analyses (B, D). Transcript levels of the following genes were monitored: tubulin B2 (*tub*B2), isoenzyme 3 of 4-coumarate:CoA ligase (*4CL3*) and dihydroxypterocarpan 6a-hydroxylase (*D6aH*) of soybean; actin A (*Act*A) and avirulence gene 1b-1 (*Avr1b*-1) of *P. sojae*. Symbols denote: (+) roots inoculated with *P. sojae* race 1; (-) roots of untreated control plants.

4.5.5 Analysis of transcript levels of *P. sojae Avr1b*-1, actin *Act*A and soybean tubulin *tub*B2 in soybean plants infected with *P. sojae* race 2 zoospores

To verify that *Avr1b*-1 was not present in the soybean cv. Harosoy - *P. sojae* race 2 and in the cv. Williams L77-1863 - *P. sojae* race 2 compatible interaction, soybean seedlings were infected with the pathogen as described in section 3.12. At 24 h after infection, segments around the infection sites were excised for isolation of total RNA and RT-PCR analyses. As expected and also demonstrated in Fig 24B, *Avr1b*-1 was not detectable in any of the interactions with *P. sojae* race 2 (*avr1b*) (Fig. 27). The experiment was performed twice.

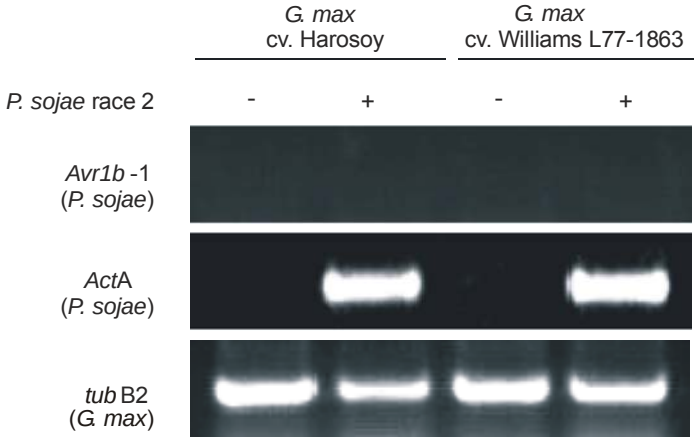


Fig. 27 mRNA expression in the compatible soybean cv. Harosoy - *P. sojae* race 2 and the cv. Williams L77-1863 - *P. sojae* race 2 interaction. Roots of unwounded, 3 day-old soybean seedlings were dip-inoculated with zoospores of *P. sojae* race 2 and transcript levels analysed by RT-PCR using gene-specific primers. At 24 h after inoculation, root segments at the infection site ("ring") were excised for isolation of total RNA. Transcript levels of the following genes were monitored: *P. sojae Avr1b*-1, *P. sojae* actin *Act*A and soybean tubulin *tub*B2. Symbols denote: (+) roots infected with *P. sojae* race 2; (-) roots of untreated control plants.

4.5.6 *Avr1b*-1 DNA sequencing

In order to verify the correct DNA sequence of *Avr1b*-1 found after different analyses of transcript levels in soybean after infection with *P. sojae* race 1, DNA sequencing was performed as described in section 3.9.12.

After a time course test of mRNA expression by RT-PCR in the compatible interaction between soybean cv. Harosoy and *P. sojae* race 1 (section 4.5.4), the band corresponding to *Avr1b*-1 (430 bp) was excised from the agarose gel, purified (3.9.3), modified using A-tailing procedure (3.9.10) and ligated into a pGEM-T vector (3.9.11). After colony PCR (3.9.7) two clones (clone #10 and #11) were found to carry the *Avr1b*-1 gene. After DNA sequencing a deduced amino acid alignment of *Avr1b*-1 (accession number AF449622), *Avr1b*-1 (clone #10) and *Avr1b*-1 (clone #11) was generated using

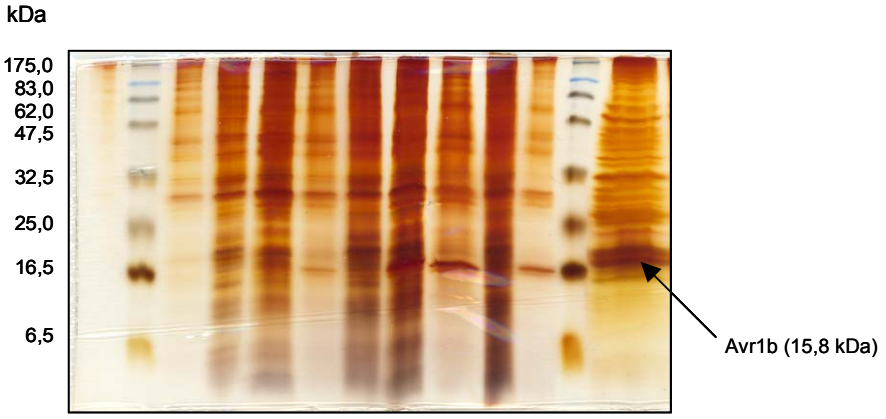
MultAlin and edited using BioEdit (9.4). The deduced amino acid sequences from clones 10 and 11 were identical to the deduced amino acid sequence of the *Avr1b*-1 gene chosen for the alignment except at positions 254 and 335 (9.4). These results demonstrate that the band appearing at 430 bp in the analyses of transcript levels corresponds to *Avr1b*-1.

4.5.7 Subcellular fractionation of infected soybean plants

One of the aims of this work was to localize Avr1b in infected soybean plants. Soybean seedlings of cv. Williams L77-1863 were infected with *P. sojae* race 1 zoospores (incompatible interaction) and root segments were excised 24 and 48 h after infection from the ring at the water-air interface for subcellular fractionation (3.13). Out of the three fractions obtained (apoplastic fluid, microsomes and soluble proteins), in each case 15 µg of protein were used for SDS-PAGE and subsequent silver staining or Western blotting. As shown in Figs. 28A and 28B there is no band corresponding to Avr1b (around 15,8 kDa) in any of the samples, probably due to the low amount of protein loaded on the gels, with exception of the positive control (10 µg soluble protein preparation of *Sf9* insect cells containing Avr1b).

A

Sample	M	AF	Mi	SP	AF	Mi	SP	AF	Mi	SP	M	+Co
Time (h)		0	0	0	24	24	24	48	48	48		
P. sojae race 1		-	-	-	+	+	+	+	+	+		



B

Sample	M	AF	Mi	SP	AF	Mi	SP	AF	Mi	SP	+Co
Time (h)		0	0	0	24	24	24	48	48	48	
P. sojae race 1		-	-	-	+	+	+	+	+	+	

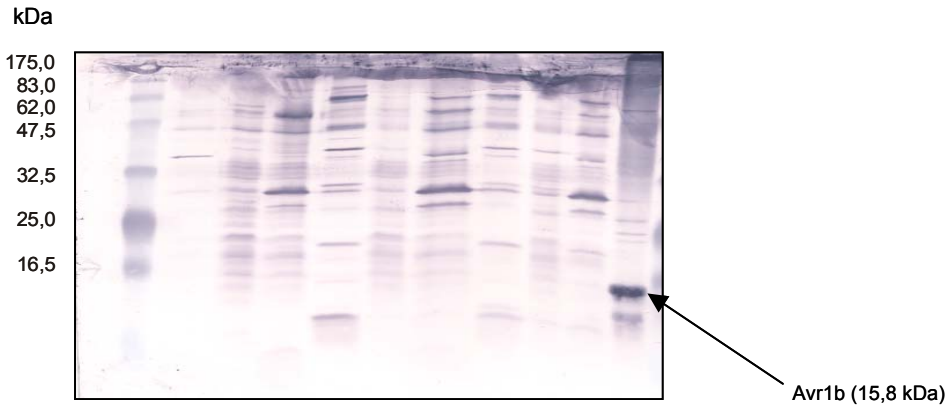


Fig. 28 Subcellular fractionation of soybean seedlings cv. Williams L77-1863 infected with *P. sojae* race 1 zoospores (incompatible interaction). M) protein markers (kDa), AF) apoplastic fluid, Mi) microsomes, SP) soluble proteins. +Co) soluble protein preparation of *Sf9* insect cells containing Avr1b. (A) Silver-stained SDS gel, (B) Western blot analysis. Symbols denote: (+) roots infected with *P. sojae* race 1; (-) roots of untreated control plants. Detection was performed with anti-Avr1b-antiserum (1/1 000 dilution).

Since it was not possible to observe Avr1b, neither in the silver-stained gel nor in the Western blot membrane, another test was performed. This time using 100 µg protein of the three fractions obtained from soybean cv. Harosoy roots infected with *P. sojae* race 1 zoospores (compatible interaction). The results showed no band corresponding to Avr1b in either of the three fractions (Data not shown). The amount of Avr1b present in the fractions was probably still too low, so that it couldn't be detected by the anti-Avr1b-antiserum. The detection limit of anti-Avr1b-antiserum, as determined in section 4.3.3, was approximately 3% of total protein preparation (0,5 µg Avr1b in 15 µg of a soluble protein preparation from infected *Sf9* insect cells).

4.5.7.1 Immunoprecipitation of SDS-denatured protein fractions from infected soybean plants

In order to detect small amounts of protein (e.g. less than 1% of the total protein analysed), an immunoprecipitation test was performed on infected soybean cv. Harosoy, using 150 µg of protein from the apoplastic fluid, 150 µg of the protein from the microsomes and 1 mg of the soluble protein preparation. As shown in Fig. 29 there is no band in the range of 15,8 kDa in the microsomes and soluble proteins and thus no detection of Avr1b was obvious. In the apoplastic fluid fractions, two protein signals with apparent molecular weights of 17 and 24 kDa, respectively, appeared 24 h after infection.

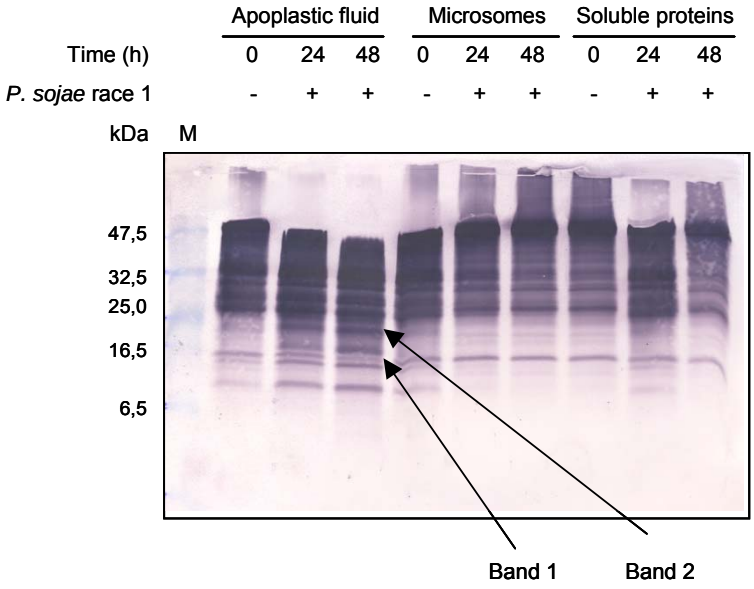


Fig. 29 Western blot analysis performed on immunoprecipitated protein fractions from soybean cv. Harosoy roots infected with *P. sojae* race 1 zoospores (compatible interaction). M) protein markers (kDa). Detection was performed with purified anti-Avr1b-antiserum (1/1 000 dilution).

To characterize the relationship of these proteins (17 and 24 kDa from the apoplastic fluid; Fig. 29) to Avr1b, another immunoprecipitation test was performed, this time using 280 µg apoplastic fluid (24 h after infection). Apoplastic fluid from uninfected seedlings (200 µg; 0 h) was used as negative control. Samples were immunoprecipitated (3.8.9.2) followed by SDS-PAGE analysis (Data not shown). The two bands corresponding to 17 and 24 kDa were excised under sterile conditions from both the infected fraction and the negative control. Bands were analysed by mass spectrometry (LC-MS/MS).

4.5.7.2 Protein analysis by mass spectrometry

After protein analysis by mass spectrometry, tryptic peptides from Avr1b were not detected in fractions from infected plants. This indicates that Avr1b is either not present at all in infected soybean seedlings or only in traces.

Interestingly, two tryptic peptides from soybean, **ALVTDADNVIPK** and **GDAEPNQDELK**, corresponding to a PR-10-like protein were found in infected (after 24 h) soybean seedlings (16,5 kDa band) but not in control seedlings (0 h).

The amino acid sequence of the PR-10-like protein was compared with other amino acid sequences in the data base for similarities. Two starvation-associated message proteins from soybean (*Glycine max, Gm*) *Gm*SAM22 and *Gm*SAM22-like as well as one open reading frame from soybean (*Gm*ORF) showed high sequence homology to the PR-10-like protein. An amino acid alignment of *Gm*PR10-like, *Gm*SAM22, *Gm*SAM22-like, *Gm*ORF and of the two tryptic peptides was generated using MultAlin and edited using BioEdit (Fig. 30).

*Gm*PR10-like shares 92% identity to the soybean *SAM22* gene. PR-10 genes have been reported from a number of species including soybean, alfalfa, potato, lily and rice (Crowell *et al*., 1992; Graham *et al*., 2003; Chou *et al*., 2004). The starvation-associated message proteins are induced by developmental cues like senescence or by different stresses like fungal elicitors. Southern analysis indicates that multiple copies of sequences related to SAM22 exist in the soybean genome (Crowell *et al*., 1992).

The isolation of a PR-10-like protein in the apoplastic fluid of infected soybean cv. Harosoy was an unexpected result.

```
                  10        20        30        40        50        60        70        80
             ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Pep                            ALVTDADNVIPK
GmPR10like   MGVFTFEDEINSPVAPATLYKALVTDADNVIPKALDSFKSVENVEGNGGPGTIKKITFLEDGETKFVLHKIESI------
GmSAM22      MGVFTFEDEINSPVAPATLYKALVTDADNVIPKALDSFKSVENVEGNGGPGTIKKITFLEDGETKFVLHKIESIDEANLG
GmSAM22like  ----TFEDEFNSPVAPATLYKALVTDADNVIPKALDSFKSVVNVEGNGGPGTIKKITFLEDGETKFVLHKIESIDEANLG
GmORF        MGIFTFEDETTSPVAPATLYKALVTDADNVIPKAVEAFRSVENLEGNGGPGTIKKITFVEDGESKFVLHKIESVDEANLG

                  90       100       110       120       130       140       150
             ....|....|....|....|....|....|....|....|....|....|....|....|....|...
Pep                                                  GDAEPNQDELK
GmPR10like   ------------------------ARHGGGSAGKLTVKYETKGDAEPNQDELKTGKAKADALFKAIEAYLLAHPDYN
GmSAM22      YSYSVVGGAALPDTAEKITFDSKLVAGPNGGSAGKLTVKYETKGDAEPNQDELKTGKAKADALFKAIEAYLLAHPDYN
GmSAM22like  YSYSVVGGAALPDTAEKITFDSKLVA-------------------------------------------------
GmORF        YSYSVVGGVGLPDTVEKITFECKLAAGANGGSAGKLTVKYQTKGDAQPNPDDLKIGKVKSDALFKAVEAYLLANPHYN
```

Fig. 30 Comparison of amino acid sequences of a soybean pathogenesis-related protein (*Gm*PR10-like, accession number AAM94617), a soybean starvation-associated message protein (*Gm*SAM22, accession number X60043), a soybean *Gm*SAM22-like protein (accession number CAC18803) and a soybean open reading frame (*Gm*ORF; accession number CAA42647). The alignment was generated using MultAlin and edited using BioEdit. Consensus amino acid residues are boxed in grey. Gaps (shown as dashes) were introduced to emphasize the similarities between the proteins. Pep) Peptides of extracts from infected soybean seedlings identified by LC-MS/MS.

5 Discussion

5.1 Expression of Avr1b in *E. coli* and *Sf9* insect cells

After isolation of *Avr1b* from *P. sojae*, the properties and functions of the encoded protein needed to be investigated. Shan *et al.* (2004) produced Avr1b in the yeast, *Pichia pastoris*, and showed that the protein triggered a hypersensitive response when infiltrated into soybean leaves, however this expression system was not sufficiently reliable (B. Tyler, personal communication). For this reason, two different expression systems were established in the present work by using *E. coli* and *Sf9* insect cells. Avr1b obtained after heterologous synthesis in either of the systems showed no biological activity on soybean roots. This lack of activity might be attributed to incorrect post-translational modifications or folding, mislocalization or to the absence of additional plant factors required for the delivery or maturation of the protein in the heterologous expression system. Problems in finding an adequate expression system are not uncommon. This problem has been experienced for example in the case of FLS2. Flagellin sensitive 2 is a receptor in *Arabidopsis thaliana* that recognises and binds the elicitor-active epitope flg22 from the bacterium flagellum (Felix *et al*., 1999). Attempts to produce FLS2 in cells from other kingdoms such as *E. coli*, *Pichia pastoris* and human embryonic kidney cells did not lead to the accumulation of a functional flg22 binding site. The functionality of the FLS2 receptor was tested successfully only by heterologous production in tomato (*Lycopersicon esculentum*) (Chinchilla *et al*., 2006).

5.2 *Avr1b* expression in compatible and incompatible *P. sojae* - soybean interactions

Many investigations have shown highly dynamic changes in gene expression of plant hosts and microbial aggressors after infection. In a model for plant-oomycete interactions, the *Arabidopsis*-downy mildew pathosystem, several host resistance (*R*) genes were isolated and host responses to infection by *Hyaloperonospora parasitica* were characterized (Holub, 2001; Slusarenko and Schlaich, 2003). To describe the “interaction transcriptome” in plant-pathogen interactions (Birch and Kamoun, 2000), patterns of overall transcriptional profiles of soybean and *P. sojae* at the infection interface were analysed (Moy *et al.*, 2004). In these studies, it was confirmed that soybean genes encoding enzymes of phytoalexin biosynthesis, among others, were strongly upregulated with highest expression levels occurring 24 h after infection. Furthermore, the number of pathogen genes expressed during infection reached a maximum at 24 h. It was concluded that in the soybean - *P. sojae* interaction the pathogen transits from biotrophy to necrotrophy between 12 and 24 h after infection (Moy *et al.*, 2004). It was reported that a considerable number of upregulated genes of the pathogen was probably involved in virulence or avirulence such as proteinases, glucanases, elicitins and others (Moy *et al*., 2004).

A gene which has been proposed to control race-specificity between soybean and *P. sojae*, and which was not included in the analyses mentioned above, is *Avr1b*-1. *Avr1b*-1, the first avirulence gene from an oomycete to be cloned, is expressed in a race- and infection-specific manner (Shan *et al*., 2004). For the comparison of the expression of this avirulence gene with early induced plant defences, two genes encoding phytoalexin biosynthesis enzymes *4CL3* and *D6aH* (Ebel and Grisebach, 1988; Lindermayr *et al.*, 2002; Schopfer *et al.*, 1998) were selected in this work. The results showed that the expression of the defence-related genes, *4CL3* and *D6aH*, was strongly enhanced at the earliest time point of 3 h (4.5.4). The high mRNA levels were maintained for more than 24 h (4CL3) or even for 48 h (D6aH). Conversely, expression of *Avr1b*-1 appeared to be constitutive throughout the infection and to correlate with the amount of pathogen present. Three hours after inoculation, *Avr1b*-1 mRNA might have escaped detection because of the low number of hyphae present at the site of root infection. It could also be possible that expression of the gene started several hours later than expression of the phytoalexin biosynthesis genes. Expression of *P. sojae Avr1b*-1 and actin genes 24 h after infection was detected mainly in the basal soybean root segments covered by the zoospore suspension (Figs. 25A and 25 B). No mRNA expression was detected in the upper root segments not covered by zoospore suspension (Figs. 25A and 25B). The time course of *Avr1b*-1 expression was similar in the compatible and incompatible interaction starting at 3 h post infection. The amount of *Avr1b*-1 mRNA was higher than the amount of *Act*A mRNA used as an internal control in the compatible interaction. In contrast, in the incompatible interaction the amount of *Avr1b*-1 mRNA was lower than the amount of *Act*A mRNA (Figs. 26B and 26D).
A reason for the difference in the patterns of *Avr1b*-1 and defence-related gene expression could be that Avr1b-1 is not required during the initial but during the intermediate biotrophic phase of *P. sojae* until host cell death occurs. In this case, other factors mediating initial recognition of the pathogen by the host plant with subsequent induction of genes involved in early defence responses like those encoding enzymes of phytoalexin production would be required. Because defence responses occur in both compatible and incompatible interactions, factors for initial pathogen recognition might be race non-specific and might be present at the onset of infection, such as some of the general elicitors localized in the cell wall of the pathogen including the oligo-β-glucoside motif of the β-glucan structural polysaccharide (Albersheim and Valent, 1978; Ebel, 1998; Sharp *et al*., 1984a, b). β-Glucan elicitors induce rapid K^+ and Ca^{2+} fluxes leading to depolarization of the plasma membrane potential of soybean root cells within a few minutes after application of the stimulus (Mithöfer *et al.*, 2005). These changes may represent early signaling events initiating several inducible defence responses (Ebel and Grisebach, 1988; Lindermayr *et al*., 2002; Mohr and Cahill, 2001). Therefore resistance or susceptibility of soybean towards *P. sojae* may depend on more than one factor.

In order to find whether the relative timing of expression of defence-related genes, such as phytoalexin biosynthesis genes, and of Avr1b, is more common and might also apply to other defence genes, further experiments should be performed. One candidate gene could be that encoding the PR-10-like protein.

A more favorable way to compare compatible and incompatible interactions in a specific plant - pathogen system is to choose one plant cultivar and two pathogen races. In the work of Hahn *et al*. (1985), a detailed analysis of a single soybean cultivar showed marked differences in the dynamics and sites of accumulation of glyceollin following infection of roots with zoospores of a virulent (compatible interaction) and an avirulent race (incompatible interaction). The use of two separate cultivars of soybean, although differing in resistance to *P. sojae*, can complicate the direct comparison of changes in terms of host specificity (Habereder *et al*., 1989). In this study (section 4.5), two different soybean cultivars and two *P. sojae* races were choosen to compare compatible and incompatible interactions. In order to simplify the experiments and to obtain clearer differences when comparing these two types of interaction, it should have been preferable to choose only one soybean cultivar, Williams L77-1863 and races 1 and 2 of *P. sojae* in the time course of mRNA expression (4.5.4). However race 2 of *P. sojae* does not encode for Avr1b. The better choice to study the function and properties of Avr1b would then be to use two near isogenic lines of soybean and race 1 (*Avr1b*) of *P. sojae.*

5.3 Localization and recognition of Avr proteins

It is known that bacterial plant pathogens secrete effector proteins like avirulence (Avr) proteins into the host plant cytoplasm via the type III secretion system (TTSS) (Van der Ackerveken *et al*., 1996; Lahaye and Bonas, 2001). Inside the plant cell, effector proteins can be recognized directly or indirectly by the corresponding resistance (R) proteins. How eukaryotic pathogens of plants such as fungi and oomycetes are detected by plant cells is not fully understood yet.

The interaction between effector proteins from tomato and the fungus *Cladosporium fulvum* appears to be extracellular. Tomato resistance proteins (Cf proteins) recognize fungal Avr peptides secreted into the leaf apoplast during infection. A direct interaction of Cf proteins with their corresponding Avr proteins has not been demonstrated and the molecular mechanism of Avr protein perception is not known yet. Cf proteins lack an obvious signaling domain, suggesting that defence response activation is mediated through interactions with other partners (De Wit *et al*., 2002; Rivas and Thomas, 2005).

In the plant-pathogen system rice-*Magnaporthe grisea*, a direct interaction between the R Pi-Ta protein and the Avr Pi-Ta protein was demonstrated using the yeast two hybrid (YTH) and *in vitro* systems suggesting that the Avr protein enters the plant cell (Jia *et al*., 2000).

Rust, mildew fungi and oomycetes including *Phytophthora* species form specialized feeding structures called haustoria that penetrate the host plant cell wall but remain separated from the host cytoplasm by a double membrane. *Avr* genes (including *Avr1b*-1, Shan *et al*., 2004) from some of these haustorium-forming eukaryotic pathogens have been cloned (Allen *et al*., 2004; Armstrong *et al*., 2005; Rehmany *et al*., 2005) and proved to encode small proteins with N-terminal secretion signals. In flax rust fungi, it has been shown that *Avr* gene expression occurs in haustoria and their products are recognized inside plant cells (Dodds *et al*., 2004; Catanzariti *et al*., 2006). Although it has not been demonstrated this could also be the case for some oomycetes.

Because engineering of genes in pathogenic microorganisms is not available or difficult to achieve, *Agrobacterium tumefaciens*-mediated or biolistic transient expression analyses of the cloned avirulence genes have been used to confirm indirectly avirulence functions of the cloned genes by inducing HR in host plants (Allen *et al*., 2004; Rehmany *et al*., 2005; Armstrong *et al*., 2005). Expression of truncated forms of some of these genes, e.g. *ATR13* from *H. parasitica* (formerly named *Peronospora parasitica*) (Allen *et al*., 2004) and *Avr3a* from *P. infestans* (Armstrong *et al*., 2005) lacking the secretion signal also induced HR. These data have been interpreted as indication that the two avirulence proteins are detected in the host cytoplasm, which would be consistent with the presumed cytoplasmic localization of the corresponding R proteins (RPP13 and R3a, respectively).

Here it was shown that expression of *Avr1b*-1 occurs only in infected plants, but not in *P. sojae* zoospores or in the mycelium of race 1 at different growth stages (4.4). To localize Avr1b, immunoblot and mass spectrometric analyses were performed on infected soybean plants (4.5.7). Avr1b-1 could not be detected either in the microsomes or in the soluble protein fraction from infected soybean seedlings in immunoblot analyses (Fig. 29). However two adjacent bands at the range of 17 kDa and 24 KDa were observed in the apoplastic fluid fraction of infected, but not in control plants. These two bands were analysed by mass spectrometry. Tryptic peptides from Avr1b-1 were not detected in fractions from infected plants, indicating that Avr1b-1 is either not present at all in infected soybean seedlings 24 h after infection or only in traces and cannot be detected by the experimental techniques used in this work. Thus both methods failed to localize Avr1b-1 in infected plants.

5.4 Biological tests

5.4.1 Biotests on soybean using different protein preparations

In order to defend themselves against microorganisms, among other strategies, plants synthesize phytoalexins restricting the growth of a wide variety of pathogens by recognizing structural components of the cell walls of fungi and oomycetes. Since these components are essential for the survival of the microorganisms they cannot evade detection by altering the structure of these components (Albersheim and Valent, 1978). Soybean tissues accumulate the phytoalexin glyceollin in response either to infection with *P. sojae* or to β-glucan elicitors present in *P. sojae* cultures (Ayers *et al*., 1976a, b; Albersheim and Valent, 1978; Hahn *et al*., 1985; Ebel *et al*., 1986).

To know if glyceollin also accumulates in soybean after treatment with Avr1b-1 (obtained either from *E. coli* or *Sf9* insect cells), biological tests were performed here. Soybean seedlings were treated with the recombinant protein and glyceollin was measured as a component of the soybean defence mechanism.

The results showed that Avr1b-1 obtained as recombinant protein from *E. coli* (4.1.1) or *Sf9* insect cells (4.2.2) had no biological activity on soybean seedlings 24 h after inoculation when assayed at concentrations of approximately 10 µg/ml (Avr1b purified from *E. coli* cells) or 1mg/ml (*Sf9* insect cell

fractions containing Avr1b) (4.5.1). Probably Avr1b-1 concentration was not high enough to induce a measurable response in inoculated plants. Another explanation could be that due to the presence of the signal peptide, Avr1b-1 may not interact with other proteins which could be required for recognition. This is the case for ATR1^{NdWsB} from *H. parasitica* (Rehmany *et al*., 2005) which cannot be recognized by the host plant when its signal peptide is present. In contrast, the ATR13 protein from *H. parasitica*, can be recognized equally with or without its signal peptide (Allen *et al*., 2004) indicating that the signal peptide does not physically interfere with ATR13 recognition. Here, Avr1b-1 lacking the putative signal peptide was not produced as recombinant protein and thus the possible interference of the putative signal peptide with Avr1b-1 recognition has not been analysed.

The major problem in performing biotests was the reproducibility. Since glyceollin concentrations varied in the reproduced experiments, an internal positive control (raw elicitor) as well as negative controls (water and different buffers) were also used in the biotests in order to compare the different experiments. Another important task was to avoid microbial contamination during the experiments, which was problematic since it was not possible to keep a sterile environment in the phytochamber. In order to avoid undesired contamination, seeds were desinfected before being planted on autoclaved vermiculite and only sterile water was used for watering the plants. During the biotests, unwounded seedlings were treated by putting them in reaction tubes containing antibiotics (10 mg/ml gentamicin) together with the different protein preparations. The tubes were placed in a sterile plastic box with some sterile water for keeping a humid environment.

5.4.2 Biotests on soybean hypocotyls using mycelium of *P. sojae*

Wounded hypocotyls inoculated with mycelia or zoospore suspensions of *P. sojae* have been used in previous studies on inducible glyceollin and lignin accumulation, and of the hypersensitive response (HR) in the soybean - *P. sojae* interaction. The results showed large differences between susceptible and resistant plants. Incompatible responses of soybean hypocotyls included the formation of dark lesions, HR and pathogen restriction. Compatible interactions produced light coloured, water-soaked lesions and sporulation and spread of the pathogen (Ayers *et al*. 1976a; Moesta *et al*., 1983; Mohr and Cahill, 2001).

Here, resistance was observed only when the soybean cv. Williams L77-1863 (*Rps1b*) was inoculated with *P. sojae* race 1 (*Avr1b*-1). This incompatible interaction was characterized by HR surrounded by healthy tissue and pathogen restriction. Compatible interactions were observed on cv. Harosoy (*rps1b*) after infection with *P. sojae* races 1 (*Avr1b*-1) or 2 (*avr1b*) and on cv. Williams L77-1863 (*Rps1*b) after infection with *P. sojae* race 2 (*avr1b*) resulting in dark brown lesions and stop of plant growth. Pathogen spread was characterized by growth of hyphae in the plant as described before (Mohr and Cahill, 2001). These results are in accordance with the gene-for-gene hypothesis (Flor, 1956; Flor, 1971) which proposes that resistance occurs only when the R protein from the plant recognizes the Avr protein from the pathogen. When either the R or the Avr protein is missing, the plant is not able to recognize the pathogen resulting in plant susceptibility and eventually plant death.

Analyses of transcript levels revealed expression of *Act*A and *Avr1b*-1 when the susceptible soybean cv. Harosoy (*rps1b*) was infected with *P. sojae* race 1 (*Avr1b*-1). The resistant soybean cv. Williams L77-1863 (*Rps1b*) was able to restrict the growth of *P. sojae* shown by the low transcript level of the constitutive *Act*A gene of *P. sojae* (Fig. 24A).
A clear difference between the hypocotyl and the zoospore assay is that in the hypocotyl test, *P. sojae* race 1 mycelium does not cause a heavy infection on plants even 5 days after infection as demonstrated by the low transcript levels of *Act*A and *Avr1b*-1 in soybean (Fig 24A). In contrast, in the zoospore test, the zoospores of *P. sojae* race 1 infect soybean roots starting 3 hpi with high transcript levels of both *Act*A and *Avr1b*-1 in the compatible, but relatively low *Avr1b*-1 level in the incompatible interaction (Figs. 25 and 26).

5.5 The RXLR motif present in different oomycetes

Cloning of six highly divergent alleles of ATR1^{NdWsB} from eight downy mildew isolates, of avirulence genes from *P. sojae* (*Avr1b*-1) and *P. infestans* (*Avr3a*) and of numerous other oomycete genes has shown that the proteins they encode share a highly conserved motif ("RXLR") (Fig. 31; Rehmany *et al.,* 2005; Armstrong *et al*., 2004). The position of the RXLR residues in the "in planta-induced" IPI-O proteins from *P. infestans* is interesting because it overlaps the RGD tripeptide cell adhesion motif (Senchou *et al*., 2004). The RXLR motif is also found in more than forty diverse secreted *P. infestans* proteins (S. Kamoun, unpublished data) and more than hundred predicted secreted proteins from each of the genome sequences of *P. sojae* and *P. ramorum* with weak sequence similarity to Avr1b-1 (B.M. Tyler, unpublished data). No cysteine-rich proteins from oomycetes have been found that carry the RXLR motif. Therefore, proteins carrying the RXLR motif differ from cysteine-rich class proteins implicated in fungus-plant and oomycete-plant interactions (Templeton *et al*., 1994).
The RXLR motif shares some similarities with the recently described host-targeting signal, which is conserved in numerous proteins from malaria parasites (*Plasmodium* species) and required for translocation of the proteins into the host cell (Hiller *et al*., 2004; Marti *et al*., 2004). Since ATR1^{NdWsB} and Avr3a have been detected in the cytoplasm of host plant cells, Rehmany *et al*. (2004) proposed that the RXLR motif may also play a role in translocating secreted oomycete proteins into the host plant cells. However this hypothesis has not yet been tested experimentally in the soybean - *P. sojae* interaction.

In this work, the recombinant Avr1b-1 protein from *P. sojae* obtained after heterologous synthesis in *E. coli* or *Sf9* insect cells showed no biological activity in the soybean cv. Harosoy 1372 (*Rps1b*). Since the soybean seedlings were incubated with protein preparations containing Avr1b-1 in reaction tubes for 24 h, it is conceivable that the protein was not properly delivered inside the plant and as a result no defence reaction was seen (no glyceollin induction, 4.5.1).

In the case of bacteria, effector proteins are delivered inside the host plant cell via a type III secretion system (TTSS) and they are recognized by the corresponding R proteins (Lahaye and Bonas, 2001). However such a mechanism has not been found in oomycetes, and as a consequence the mode of

translocation of the oomycete avirulence proteins inside the host plants is not fully understood yet. If like the case of *Plasmodium* species, the RXLR motif plays a role in protein translocation, avirulence genes such as *Avr1b*-1 (containing point mutations inside the RXLR motif or not) should be fused to a gene encoding a green or yellow reporter fluorescent protein (GFP and YFP). If the motif is really required for translocating Avr1b-1, the reporters will accumulate inside the soybean cells. The advantage of this technique might reside in the sensitivity for *in situ* detection and thus localization of the target protein.

The identification of further oomycete proteins that enter the plant cell, their functions and protein-uptake mechanisms will be an interesting area for development and for understanding the basis of plant pathogenesis. These insights could provide novel control methods through genetics or targeted chemicals to control this important and destructive class of plant disease-causing organisms (Ellis *et al*., 2006).

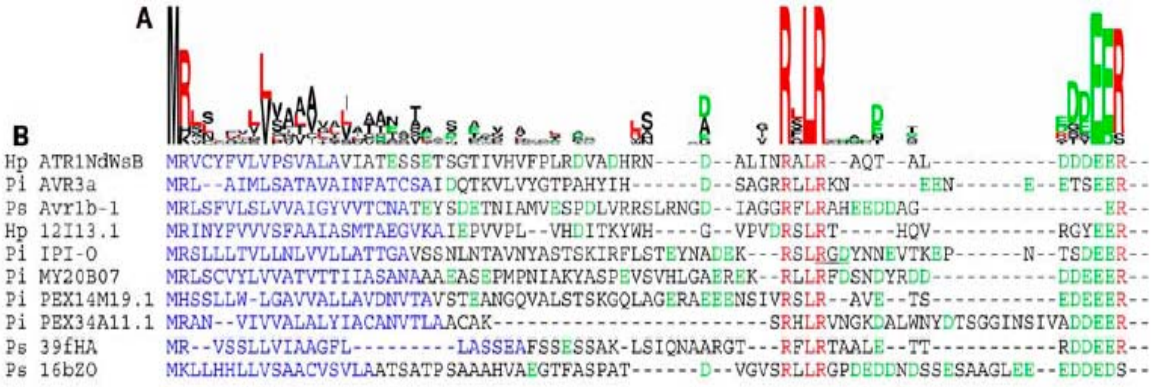


Fig. 31 The RXLR motif. (A) Graphic representation of the sequence alignment shown below in B. The height of each amino acid symbol indicates its frequency at that position. The amino acids leucine (L) and arginine (R) (red) and acidic amino acids (green) are highlighted. (B) Alignment of the N-terminal regions of predicted protein sequences of ATR1NdWsB from *H. parasitica* (Hp) isolate Emoy2, AVR3a (Armstrong *et al*., 2005) from *P. infestans* (Pi), Avr1b-1 (Shan *et al*., 2004) from *P. sojae* (Ps), secreted Pi proteins MY-20-B-07 (Kamoun *et al*., 1999), IPI-O1 and IPI-O2 (Pieterse *et al*., 1994) (IPI-O proteins are identical in this region), a predicted Hp secreted protein from BAC sequence 12I13, putative Pi PEXs from BACs 14M19 (GenBank accession number AC146943) and 34A11 (GenBank accession number AC147544), and secreted Ps proteins 3-9f-HA and 1-6b-ZO (Qutob *et al*., 2002). Dashes indicate gaps in the alignment. Predicted signal peptides (blue), the RXLR motif (red), acidic amino acids (green) and the RGD tripeptide motif (underlined) are highlighted. (Figure taken from Rehmany *et al*., 2005)

5.6 Pathogenesis related proteins found in infected soybean seedlings

Pathogenesis related (PR) proteins were first detected in tobacco cultivars reacting hypersensitively to tobacco mosaic virus (Van Loon and Van Kammen, 1970) and later also in various plant species on infection with viruses, viroids, fungi, bacteria and oomycetes as well as during specific

developmental stages of plants like senescence (Van Loon, 1985). They have in common low molecular weights, extreme isoelectric points and high resistance to endogenous plant proteinases. Although the biological and biochemical functions of many PR proteins remain unknown, some have been shown to possess antimicrobial activity. *In vitro* studies of chitinases (PR-3 class) and β-1,3-glucanases (PR-2 class) have shown that these proteins can inhibit fungal growth (Mauch *et al*., 1988; Sela-Buurlage *et al*., 1993) presumably by hydrolytic degradation of fungal cell walls.

The PR-10 class of proteins, first identified as a major pollen allergen (BetV-1) from white birch, is induced following pathogen attack in a wide variety of plant species such as parsley (Somssich *et al*., 1986), potato (Matton and Brisson, 1989), pea (Barral and Clark, 1991), soybean (Crowell *et al*., 1992), asparagus (Warner *et al*., 1994), sorghum (Lo *et al*., 1999) and rice (McGee *et al*., 2001).

In soybean, transcripts of *GmPR10* were induced in both compatible and incompatible soybean-*Pseudomonas syringae* pv. *glycinea* interactions (Chou *et al*., 2004). PR-10 proteins show structural similarity to ginseng ribonuclease and therefore ribonuclease activity has been proposed. In fact, a PR-10-like protein from white lupin roots was shown to possess ribonuclease activity (Bantignies *et al*., 2000).

Accumulation of the PR protein “SAM22” (starvation associated messenger) has been reported to occur in young leaves after wounding, by transpiration-mediated uptake of salicylic acid, methyl viologen, hydrogen peroxide, sodium phosphate or after treatment with fungal elicitor. SAM22 also accumulates in the roots of very young soybean seedlings and cotyledonous tissue (Crowell and Amasino, 1991; Crowell *et al*., 1992). PR-10 proteins have also been found on needle tissues of white pine after wounding or after infection with the fungus *Cronartium ribicola* (Liu *et al*., 2003).

Here, in an attempt to localize Avr1b-1 via an indirect approach (immunoprecipitation) in soybean seedlings infected with *P. sojae*, a PR protein was fortuitously found (4.7.2). The susceptible soybean cv. Harosoy (*rps*) was infected with zoospores of *P. sojae* race 1 (*Avr1*b-1). Twenty-four hours after infection the apoplastic fluid was immunoprecipitated before electrophoresis, immunoblot and mass spectrometric analyses. Instead of the expected Avr1b-1 in the apoplastic fluid fraction, a *Gm*PR10-like protein with a relative molecular mass similar to Avr1b-1 (15,8 kDa) was found. This was an unexpected and interesting result and it demonstrates that 24 h after infection, the susceptible cv. Harosoy (*rps*) responds to the pathogen attack by activating its basic defence responses. The multicomponent defence response of soybean plants in response to a *P. sojae* infection involves not only glyceollin (Ayers *et al*., 1976a, b; Albersheim and Valent, 1978; Hahn *et al*., 1985; Ebel *et al*., 1986) and lignin- like compounds (Mohr and Cahill, 2001), but also PR proteins as shown here and before (Crowell and Amasino, 1991; Crowell *et al*., 1992). Furthermore, an amino acid alignment showed that the PR-10-like protein shares high sequence homology to another PR protein, the *Gm*SAM22 (Fig. 30).

6 Outlook

Avr1b-1 was demonstrated to be expressed in a race- and infection-specific manner, but its putative role in virulence still remains unclear. Attempts to prove the biological activity of Avr1b-1 in soybean were not successful. In order to better understand the interaction of Avr1b-1 with the resistance protein of soybean, Rps1b, and to clarify the function of the signal peptide of Avr1b-1, further experiments need to be performed.

One possibility would be a cobombardment of *Rps1b*-containing soybean plants with two vectors, the one expressing *Avr1b*-1 (with and without the signal peptide) and the other expressing a green fluorescent protein (GFP). After recognition of Avr1b-1 by Rps1b, a hypersensitive response (resistance) will be observed simultaneously with a reduction of fluorescence due to cell death. Recognition of Avr1b-1 with a N-terminal signal peptide would demonstrate that the signal peptide does not interfere with binding of Rps1b or other target proteins.

Once *Rps1b* of soybean will be cloned, coinfiltration of *Nicotiana benthamiana* leaves with an *Agrobacterium tumefaciens* strain, carrying a construct expressing *Rps1b* and another strain expressing *Avr1b*-1 (with and without the signal peptide) would also be possible. Resistance may occur when Rps1b binds Avr1b-1 and the resulting hypersensitive response could be observed as a necrotic area in the infiltrated leaves. This experiment would only be successful if in the host plant soybean, the mechanism of action involves a direct interaction between Avr1b-1 and Rps1b. Direct interactions between pathogen Avr proteins and plant R proteins have been demonstrated only in a few cases (Scofield *et al*., 1996; Tang *et al*., 1996; Jia *et al*., 2000).

Another possibility would be the transformation of *Avr1b*-1 in *P. sojae* (*avr1b*) strains virulent on soybean. Soybean plants containing Rps1b will be able to recognize Avr1b-1 after infection with the transformed *P. sojae* (*Avr1b*-1) strains leading to hypersensitive response and eventually plant cell death.

In order to localize Avr1b-1, a tagged version of the protein (Avr1b-1 tagged to a GFP protein) could be expressed in *Phytophthora.* Localization studies and purification of the avirulence protein might be possible in soybean seedlings infected with the pathogen.

7 Summary

After the isolation of *Avr1b*-1 from *P. sojae* (Shan *et al*., 2004), it was necessary to investigate the properties, functions and localization of the encoded protein in infected soybean plants. Thus Avr1b-1 containing a N-terminal putative signal peptide was produced as recombinant protein in *E. coli* and in *Sf9* insect cells. A 6xHis-tagged Avr1b-1 was produced only in form of insoluble inclusion bodies in *E. coli* cells. The protein was then purified under denaturing conditions or by protein refolding in order to test its biological activity in soybean plants and to raise an anti-Avr1b-antiserum. The antiserum was purified and used to detect Avr1b-1 in *P. sojae* mycelium and in infected soybean tissues by Western blotting. It was also used to co-precipitate Avr1b-1 from different soybean fractions in immunoprecipitation tests with subsequent mass spectrometric and Western blot analyses for localization of the protein. Only in apoplastic fluid fractions from infected soybean seedlings a band corresponding to the molecular weight of Avr1b-1 was detected in immunoblot analyses. Analysing this band by mass spectrometry did not lead to the identification of the protein, indicating that Avr1b-1 is either not present at all in infected soybean seedlings 24 h after infection or is present only in traces.

In *Sf9* insect cells, Avr1b-1 containing the putative signal peptide was produced as recombinant protein. Avr1b-1 without the signal peptide was not produced so that it was not possible to study the role of the signal peptide in further experiments. Western blot and MS analyses of a soluble protein fraction obtained after fractionation of *Sf9* insect cells showed a clear band of Avr1b-1 at 15,8 kDa and was used as a positive control in further experiments.

To study basic defence responses of soybean upon infection with *P. sojae* and the race-specific interaction between the plant and its pathogen, Avr1b-1 was used as elicitor. For determining basic defence responses, glyceollin accumulation was measured in soybean seedlings inoculated with recombinant Avr1b-1 obtained after heterologous synthesis in *E. coli* or in *Sf9* insect cells. Avr1b-1 did not induce accumulation of glyceollin in soybean seedlings 24 h after inoculation.

For elucidating the race-specific interaction, soybean seedlings were infected with mycelium or with zoospores from *P. sojae* race 1, and total RNA of soybean was analysed by RT-PCR. Expression of *P. sojae Avr1b*-1 and actin mRNA 24 h after infection was detected mainly in the basal but not in the upper soybean root segments. The time course of *Avr1b*-1 expression was similar in the compatible and incompatible interaction. The amount of *Avr1b*-1 mRNA was higher than the amount of *Act*A mRNA used as an internal control in the compatible interaction. In contrast, in the incompatible interaction the amount of *Avr1b*-1 mRNA was lower than the amount of *Act*A mRNA.

Comparing expression of *Avr1b*-1 with early induced plant defences like expression of two enzymes (4CL3, D6aH) for phytoalexin biosynthesis, the mRNAs of the defence-related genes *4CL3* and *D6aH* were strongly enhanced 3 h after infection. Expression of *Avr1b*-1 mRNA was barely detectable at 3 h but was enhanced 6 h after infection. A reason for the difference in the patterns of *Avr1b*-1 and defence-related gene expression could be that *Avr1b*-1 is not required during the initial but during the intermediate biotrophic phase of *P. sojae* until host cell death occurs.

To investigate if Avr1b-1 is produced in *P. sojae* before or after plant infection, the mycelium of the oomycete was fractionated before infection, followed by immunoblot analyses. Furthermore, total RNA from *P. sojae* race 1 mycelium, zoospores and from soybean seedlings infected with *P. sojae* race 1 zoospores was extracted followed by RT-PCR. The results showed that Avr1b-1 is expressed only after infection of the plant.

8 References

Agrios, G.N. (1997) Plant Pathology. Elsevier Academic Press. 4th edition.

Albersheim, P. and Valent, B. (1978) Host-pathogen interactions in plants. Plants, when exposed to oligosaccharides of fungal origin, defend themselves by accumulating antibiotics. J. Cell Biol. 78, 627-643.

Allan, A.C. and Fluhr, R. (1997) Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. Plant Cell 9, 1559-1572.

Allen, R.L., Bittner-Eddy, P.D., Grenville-Briggs, L.J., Meitz, J.C., Rehmany, A.P., Rose, L.E. and Beynon, J.L. (2004) Host-parasite coevolutionary conflict between *Arabidopsis* and downy mildew. Science 306, 1957-1960.

Anderson, T.R. and Buzell, R.I. (1992) Inheritance and linkage of the *Rps7* gene for resistance to *Phytophthora* rot of soybean. Plant Dis. 76, 958-959.

Antelo, L., Cosio, E.G., Hertkorn, N. and Ebel, J. (1998) Partial purification of a GTP-insensitive (1→3)-β-glucan synthase from *Phytophthora sojae*. FEBS Lett. 433, 191-195.

Armstrong, M.R., Whisson, S.C., Pritchard, L., Bos, J.I.B., Venter, E., Avrova, A.O., Rehmany, A.P., Böhme, U., Brooks, K., Cherevach, I., Hamlin, N., White, B., Fraser, A., Lord, A., Quail, M.A., Churcher, C., Hall, N., Berriman, M., Huang, S., Kamoun, S., Beynon, J.L. and Birch, P.R.J. (2005) An ancestral oomycete locus contains late blight avirulence gene *Avr3a*, encoding a protein that is recognized in the host cytoplasm. Proc. Natl. Acad. Sci. USA 102 (21), 7766-7771.

Ayers, A.R., Ebel, J., Finelli, F., Berger, N. and Albersheim, P. (1976a) Host-pathogen interactions. IX. Quantitative assays of elicitor activity and characterization of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. Plant Physiol. 57, 751-759.

Ayers, A.R., Ebel, J., Valent, P. and Albersheim, P. (1976b) 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.

Bac-to-Bac Baculovirus Expression System manual. Invitrogen (Karlsruhe)

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.

Baker, B., Zambryski, P., Staskawicz, B. and Dinesh-Kumar, S.P. (1997) Signaling in plant-microbe interactions. Science 276, 726-733.

Bantignies, B., Seguin, J., Muzac, I., Dedaldechamp, F. and Gulick, P. (2000) Direct evidence for ribonucleolytic activity of a PR-10-like protein from white lupin roots. Plant Mol. Biol. 42, 871-881.

Barral, D.H.P. and Clark, J.A. (1991) Proteins arising during the late stages of embryogenesis in *Pisum sativum*. Planta 184, 14-23.

Beagle-Ristaino, J.E. and Rissler, J.F. (1983) Histopathology of susceptible and resistant soybean roots inoculated with zoospores of *Phytophthora megasperma* f. sp. *glycinea*. Phytopathol. 73, 590-595.

Bendahmane, A., Kohn, B.A., Dedi, C. and Baulcombe, D.C. (1995) The coat protein of potato virus X is a strain-specific elicitor of *Rx1*-mediated virus resistance in potato. Plant J. 8, 933-941.

Benhamou, N. (1996) Elicitor-induced plant defence pathways. Trends. Plant. Sci. 1, 233-240.

Bhattacharyya, M.K. and Ward, E.W.B. (1986a) Expression of gene-specific and age-related resistance and the accumulation of glyceollin in soybean leaves infected with *Phytophthora megasperma* f. sp. *glycinea.* Physiol. Mol. Plant Pathol. 29, 105-113.

Bhattacharyya, M.K. and Ward, E.W.B. (1986b) Resistance, susceptibility and accumulation of glyceollin I-III in soybean organs inoculated with *Phytophthora megasperma* f. sp. *glycinea*. Physiol. Mol. Plant Pathol. 29, 227-237.

Birch, P.R.J. and Kamoun, S. (2000) Studying interaction transcriptoms: Coordinated analyses of gene expression during plant-microorganism interactions. In New Technologies for Life Sciences: A Trends Guide. Wood, R. (Editor). Elsevier Science, London. 77-82.

Birnboim H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acid. Res. 7, 1513-1523.

Boller, T. (1995) Chemoperception of microbial signals in plant cells. Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 189-214.

Bonas, U., Stall, R.E. and Staskawicz, B. (1989) Genetic and structural characterization of the avirulence gene, *avrBs3* from *Xanthomonas campestris* pv. vesicatoria. Mol. Gen. Gen. 218, 127-136.

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 diseases? Plant J. 12 (1) 1-7.

Bonhoff, A., Loyal, R., Ebel, J. and Grisebach, H. (1986a) Race:cultivar-specific induction of enzymes related to phytoalexin biosynthesis in soybean roots following infection with *Phytophthora megasperma* f. sp. *glycinea*. Arch. Biochem. Biophys. 246, 149-154.

Bonhoff, A., Loyal, R., Feller, K., Ebel J. and Grisebach, H. (1986b) Further investigations of race:cultivar-specific induction of enzymes related to phytoalexin biosynthesis in soybean roots following infection with *Phytophthora megasperma* f. sp. *glycinea*. Biological Chemistry Hoppe-Seyler 367, 797-802.

Bottin, A., Veronesi, C., Pontier, D., Esquerre-Tugaye, M.T., Blein, J.P., Russterucci, C. and Ricci, P. (1994) Differential responses of tobacco cells to elicitors from two *Phytophthora* species. Plant Physiol. Biochem. 32 (3), 373-378.

Boyes, D.C., Nam, J. and Dangl, J.L. (1998) The *Arabidopsis thaliana* RPM1 disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. Proc. Natl. Acad. Sci. USA 95, 15849-15854.

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

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

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

Burden, R.S. and Bailey, J.A. (1975) Structure of the phytoalexin from soybean. Phytochem. 14, 1389-1390.

Buzzell, R.I. and Anderson, T.R. (1992) Inheritance and race reaction of a new soybean *Rps1* allele. Plant Dis. 76, 600-601.

Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X. (1994) Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell 6, 1583-1592.

Carr, J.P., Dixon, D.C. and Klessig, D.F. (1985) Synthesis of pathogenesis-related proteins in tobacco is regulated at the level of mRNA accumulation and occurs on membrane-bound polysomes. Proc. Natl. Acad. Sci. USA 82, 7999-8003.

Catanzariti, A.-M., Dodds, P.N., Lawrence, G.J., Ayliffe, M.A. and Ellis, J.G. (2006) Haustorially expressed secreted proteins from flax rust are highly enriched for avirulence elicitors. Plant Cell 18, 243-256.

Chang, S., Puryear, J. and Cairney, J. (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. 11, 113-116.

Chang, J.H., Goel, A.K., Grant, S.R. and Dangl, J.L. (2004) Wake of the flood: ascribing functions to the wave of type III effector proteins of phytopathogenic bacteria. Curr. Opin. Microbiol. 7, 11-18.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T. and Felix, G. (2006) The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell 18, 465-476

Chou, W.M., Shigaki, T., Dammann, C., Liu, Y.Q. and Bhattacharyya, M.K. (2004) Inhibition of phosphoinositide-specific phospholipase C results in the induction of pathogenesis-related genes in soybean. Plant Biol. 6, 664-672.

Cosio, E.G., Pöpperl, H, Schmidt, W.E. and Ebel, J. (1988) High-affinity binding of fungal β-glucan fragments to soybean (*Glycine max* L.) microsomal fractions and protoplasts. Eur. J. Biochem. 175, 309-315.

Cosio, E.G., Frey, T. and Ebel, J. (1992) Identification of a high-affinity binding protein for a hepta-β-glucoside phytoalexin elicitor in soybean. Eur. J. Biochem. 204, 1115-1123.

Cosio, E.G., Feger, M., Miller, C.J., Antelo, L. and Ebel, J. (1996) High-affinity binding of glucan elicitors to cell membranes of species of the plant family Fabaceae. Planta 200, 92-99.

Crowell, D.N. and Amasino, R.M (1991) Induction of specific mRNAs in cultured soybean cells during cytokinin or auxin starvation. Plant Physiol. 95, 711-715.

Crowell, D.N., John, M.E., Russel, D. and Amasino, R.M. (1992) Characterization of a stress-induced, developmentally regulated gene family from soybean. Plant Mol. Biol. 18, 459-466.

Curtis, R.W. (1984) Abscission-inducing properties of methyl jasmonate, ABA, and ABA-methyl ester and their interactions with ethepon, $AgNO_3$, and malformin. J. Plant Growth Regul. 3, 157-168.

Dangl, J.L. and Jones, J.D.G. (2001) Plant pathogens and integrated defence responses to infection. Nature 411, 826-833.

De Feyter, R., Yang, Y. and Gabriel, D.W. (1993) Gene-for-gene interactions between cotton R genes and *Xanthomonas campestris* pv. malvacearum avr genes. Mol. Plant-Microbe Interact. 6, 225-237.

Deisenhofer, J. (1981) Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9 and 2.8-A resolution. Biochem. 20 (9), 2361-2370.

De Wit, P.J.G.M. and Spikman, G. (1982) Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions of *Cladosporium fulvum* and tomato. Physiol. Plant Pathol. 21, 1-11.

De Wit, P.J.G.M., Brandwagt, B.F., Van den Burg, H.A., Cai, X., Van der Hoorn, R.A.L., De Jong, C.F., Van't Klooster, J., De Kock, M.J.D., Kruijt, M., Lindhout, W.H., Luderer, R., Takken, F.L.W., Westerink, N., Vervoort, J.J.M. and Joosten, M.H.A.J. (2002) The molecular basis of co-evolution between *Cladosporium fulvum* and tomato. Antonie van Leeuwenhoek 81, 409-412.

Dodds, P.N., Lawrence, G.J., Catanzariti, A.-M., Ayliffe, M.A. and Ellis, J.G (2004) The *Melampsora lini AvrL567* avirulence genes are expressed in haustoria and their products are recognized inside plant cells. Plant Cell 16, 755-768.

Ebel, J., Ayers, A. and Albersheim, P. (1976) Response of suspension-cultured soybean cells to the elicitor isolated from *Phytophthora megasperma* var. *sojae*, a fungal pathogen of soybeans. Plant Physiol. 57, 775-779.

Ebel, J. (1986) Phytoalexin synthesis: the biochemical analysis of the induction process. Annu. Rev. Phytopathol. 24, 235-264.

Ebel, J., Grisebach, H., Bonhoff, A., Grab, D., Hoffman, C., Kochs, G., Mieth, H., Schmidt, W. and Stäb, M. (1986) Phytoalexin synthesis in soybean following infection of roots with *Phytophthora megasperma* or treatment of cell cultures with fungal elicitor. Recognition in microbe-plant symbiotic and pathogenic interactions. NATO ASI series, series H: cell biology. Springer-Verlag 4, 345-361.

Ebel, J. and Grisebach, H. (1988) Defence strategies of soybean against the fungus *Phytophthora megasperma* f. sp. *glycinea*: a molecular analysis. Trends Biochem. Sci. 1, 23-27.

Ebel, J. and Scheel, D. (1992) Elicitor recognition and signal transduction. Plant gene research. Genes involved in plant defence. Boller, T. and Meins, F. (Editors). Springer, Berlin, Heidelberg, New York. 8, 183-205.

Ebel, J. and Cosio, E.G. (1994) Elicitors of plant defence responses. Int. Rev. Cytol. 148, 1-36.

Ebel, J. and Scheel, D. (1997) Signals in host-parasite interactions. The Mycota V Part A. Plant Relationships. Carroll, G.C. and Tudzynski, P. (Editors). Berlin, Heidelberg: Springer-Verlag. 85-105.

Ebel, J. (1998) Oligoglucoside elicitor-mediated activation of plant defence. BioEssays 20, 569-576.

Ebel, J. and Mithöfer, A. (1998) Early events in the elicitation of plant defence. Planta 206, 335-348.

Ellis, J., Catanzariti, A.M. and Dodds, P. (2006) The problem of how fungal and oomycete avirulence proteins enter plant cells. Trends. Plant Sci. 11, 61-63.

Enkerli, K., Hahn, M.G. and Mims, C.W. (1997) Ultrastructure of compatible and incompatible interactions of soybean roots infected with the plant pathogenic oomycete *Phytophthora sojae*. Can. J. Bot. 75, 1494-1508.

Enyedi, A.J., Yalpani, N., Silverman, P. and Raskin, I. (1992) Signal molecules in systemic plant resistance to pathogens and pests. Cell 70, 879-886.

Erwin, D.C. and Katznelson, H. (1961) Studies on the nutrition of *Phytophthora cryptogea*. Can. J. Microbiol. 7, 15-25.

Erwin, D.C. and Ribeiro, O.K. (1996) *Phytophthora* Diseases Worldwide. APS PRESS. USA.

Farman, M.L. and Leong, S.A. (1998) Chromosome walking to the *Avr*1-*CO39* avirulence gene of *Magnaporthe grisea*: Discrepancy between the physical and genetic maps. Genetics 150, 1049-1058.

Farmer, E.E. and Ryan, C.A (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 4, 129-134.

Favaron, F., D'Ovidio, R., Porceddu, E. and Alghisi, P. (1994) Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. Planta 195, 80-87.

Felix, G., Regenas, M. and Boller, T. (1993) Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state. Plant J. 4, 307-316.

Felix, G., Duran, J.D., Volko, S. and Boller, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. 18, 265-276.

Fliegmann, J., Schüller, G., Boland, W., Ebel, J. and Mithöfer, A. (2003) The role of octadecanoids and functional mimics in soybean defence responses. Biol. Chem. 384, 437-446.

Fliegmann, J., Mithöfer, A., Wanner, G. and Ebel, J. (2004) An ancient enzyme domain hidden in the putative β-glucan elicitor receptor of soybean may play an active part in the perception of pathogen-associated molecular patterns during broad host resistance. J. Biol. Chem. 279 (2), 1132-1140.

Fliegmann, J., Montel, E., Djulić, A., Cottaz, S., Driguez, H. and Ebel, J. (2005) Catalytic properties of the bifunctional soybean β-glucan-binding protein, a member of family 81 glycoside hydrolases. FEBS Lett. 579, 6647-6642.

Flor, H.H. (1956) The complementary genetic systems in flax and flax rust. Adv. Genet. 8, 29-54.

Flor, H.H. (1971) Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9, 275-296.

Förster, H., Tyler, B.M. and Coffey, M.D. (1994) *Phytophthora sojae* races have arisen by clonal evolution and by rare outcrosses. Mol. Plant-Microbe Interact. 7, 780-791.

Frank, J. and Paxton, J. (1971) An inducer of soybean phytoalexin from *Phytophthora megasperma* var. *sojae*. Phytopathol. 61, 954-958.

Gabriel, D.W. (1999) Why do pathogens carry avirulence genes? Physiol. Mol. Plant Pathol. 55, 205-214.

Graham, M.Y., Weidner, J., Wheeler, K., Pelow, M.J. and Graham, T.L. (2003) Induced expression of pathogenesis-related protein genes in soybean by wounding and the *Phytophthora sojae* cell wall glucan elicitor. Physiol. Mol. Plant Pathol. 63, 141-149.

Gregory, P.H. (1983) Some major epidemics caused by *Phytophthora*. *Phytophthora*: Its biology, taxonomy, ecology and pathology. Erwin, D.C., Bartniki-Garcia, S. and Tsao, P.H. (Editors). St Paul, USA: American Phytopathological Society. 271-278.

Gundlach, H., Müller, M.J., Kutchan, T.M. and Zenk, M.H. (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. USA 89, 2389-2393.

Habereder, H., Schröder, G. and Ebel, J. (1989) Rapid induction of phenylalanine ammonia-lyase and chalcone synthase mRNAs during fungus infection of soybean (*Glycine max* L.) roots or elicitor treatment of soybean cell cultures at the onset of phytoalexin synthesis. Planta 177, 58-65.

Hagmann, M.L., Heller, W. and Grisebach, H. (1984) Induction of phytoalexin synthesis in soybean. Stereospecific 3,9-dihydroxypterocarpan 6a-hydroxylase from elicior-induced soybean cell cultures. Eur. J. Biochem. 142, 127-131.

Hahn M.G., Darvill, A.G. and Albersheim, P. (1981) Host-pathogen interactions XIX. The endogenous elicitor, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. Plant Physiol. 68, 1161-1169.

Hahn, M.G., Bonhoff, A. and Grisebach, H. (1985) Quantitative localization of the phytoalexin glyceollin I in relation to fungal hyphae in soybean roots infected with *Phytophthora megasperma* f. sp. *glycinea*. Plant Physiol. 77, 591-601.

Hahn, M.G. (1996) Microbial elicitors and their receptors in plants. Annu. Rev. Phytopathol. 34, 387-412.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 136, 557-580.

Hiller, N.L., Bhattacharjee, S., Van Ooij, C., Liolios, K., Harrison, T., Lopez-Estraño, C. and Haldar, K. (2004) A host-targeting signal in virulence proteins reveals a secretome in malarial infection. Science 306, 1934-1937.

Holub, E.B. (2001) The arms race is ancient history in *Arabidopsis*, the wildflower. Nat. Rev. Genet. 2, 516-527.

Jabs, T., Tschöpe, M., Colling, C., Hahlbrock, K. and Scheel, D. (1997) Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defence gene activation and phytoalexin synthesis in parsley. Proc. Natl. Acad. Sci. USA 94, 4800-4805.

Ji, C., Smith-Becker, J. and Keen, N.T. (1998) Genetics of plant-pathogen interactions. Curr. Opin. Biotechnol. 9, 202-207.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P. and Valent, B. (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J. 19, 4004-4014.

Judelson, H.S. (1997) The genetics and biology of *Phytophthora infestans*: modern approaches to a historical challenge. Fungal Genet. Biol. 22, 65-76.

Kaitany, R.C., Hart, L.P. and Safir, G.R. (2001) Virulence composition of *Phytophthora sojae* in Michigan. Plant Dis. 85, 1103-1106.

Kang, S., Sweigard, J.A. and Valent, B. (1995) The PWL host specificity gene family in the blast fungus *Magnaporthe grisea*. Mol. Plant-Microbe Interact. 8, 939-948.

Kamoun, S., Young, M., Glascock, C.B. and Tyler, B.M. (1993) Extracellular protein elicitors from *Phytophthora*: host-specificity and induction of resistance to bacterial and fungal phytopathogens. Mol. Plant-Microbe Interact. 6 (1), 15-25.

Kamoun, S., Young, M., Förster, H., Coffey, M.D. and Tyler, B.M. (1994) Potential role of elicitins in the interaction between *Phytophthora* species and tobacco. Appl. Environ. Microbiol. 60, 1593-1598.

Kamoun, S., Van West, P., De Jong, A.J., De Groot, K.E., Vleeshouwers, V.G.A.A. and Govers, F. (1997) A gene encoding a protein elicitor of *Phytophthora infestans* is down regulated during infection of potato. Mol. Plant-Microbe Interact. 10 (1), 13-20.

Kamoun, S., Hraber, P., Sobral, B., Nuss, D. and Govers, F. (1999) Initial assessment of gene diversity for the oomycete pathogen *Phytophthora infestans* based on expressed sequences. Fungal Genet. Biol. 28, 94-106.

Kamoun, S., Huitema, E. and Vleeshouwers, V.G.A.A. (1999) Resistance to oomycetes: a general role for the hypersensitive response. Trends Plant Sci. 4, 196-200.

Kamoun, S. (2000) *Phytophthora* in “Fungal Pathology” Kronstad, J. (Editor). Kluwer Academic publishers. 237-265.

Keen, N.T. (1975) Specific elicitors of plant phytoalexin production: Determinants of race specificity in pathogens? Science 187, 74-75.

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.

Kjemptrup, S., Nimchuk, Z. and Dangl, J.L. (2000) Effector proteins of phytopathogenic bacteria: bifunctional signals in virulence and host recognition. Curr. Opin. Microbiol. 3, 73-78.

Klessig, D.F. and Malamy, J. (1994) The salicylic acid signal in plants. Plant Mol. Biol. 26, 1439-1458.

Kobayashi, D.Y., Tamaki, S.J., Trollinger, D.J., Gold, S. and Keen, N.T. (1990) A gene from *Pseudomonas syringae* pv. glycinea with homology to avirulence gene D from *P.s*. pv. tomato but devoid of the avirulence phenotype. Mol. Plant-Microbe Interact. 3, 1103-1111.

Kurucz, I., Titus, J.A., Jost, C.R. and Segal, D.M. (1995) Correct disulfide pairing and efficient refolding of detergent-solubilized single-chain Fv proteins from bacterial inclusion bodies. Mol. Immunol. 32 (17-18): 1443-1452.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lahaye, T. and Bonas, U. (2001) Molecular secrets of bacterial type III effector proteins. Trends Plant Sci. 6 (10), 479-485.

Lamb, C. and Dixon, R.A. (1997) The oxidative burst in plant-disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 251-275.

Latinhouwers, M., De Wit, P. and Govers, F. (2003) Oomycetes and fungi: similar weaponry to attack plants. Trends Microbiol. 11 (10), 462-469.

Lauge, R. and De Witt, P.J.G.M. (1998) Fungal avirulence genes: Structure and possible functions. Fungal Genet. Biol. 24, 285-297.

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.

Levine, A., Pennell, R.I., Alvarez, M.E., Palmer, R. and Lamb, C. (1996) Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr. Biol. 6, 427-437.

Lindermayr, C., Möllers, B., Fliegmann, J., Uhlmann, A., Lottspeich, F., Meimberg, H. and Ebel, J. (2002) Divergent members of a soybean (*Glycine max* L.) 4-coumarate:coenzyme A ligase gene family. Primary structures, catalytic properties, and differential expression. Eur. J. Biochem. 269, 1304-1315.

Liu, J.J., Ekramoddoullah, A.K.M. and Yu, X. (2003) Differential expression of multiple PR10 proteins in western white pine following wounding, fungal infection and cold-hardening. Physiologia Plantarum 119, 544-553.

Lo, C.S.-C., Hipskind, J.D. and Nicholson, R.L. (1999) cDNA cloning of a sorghum pathogenesis-related protein (PR-10) and differential expression of defence-related genes following inoculation with *Cochliobolus heterostrophus* or *Colletotrichum sublineolum*. Mol. Plant-Microbe Interact. 12, 479-489.

Luderer, R. and Joosten, M.H.A.J. (2001) Avirulence proteins of plant pathogens: determinants of victory and defeat. Mol. Plant Pathol. 2 (6), 355-364.

Mao, Y. and Tyler, B.M. (1991) Genome organization of *Phytophthora megasperma* f.sp. *glycinea*. Exp. Mycol. 15, 283-291.

Marston, F.A. and Hartley, D.L. (1990) Solubilization of protein aggretates. Meth. Enzymol. 182, 264-276.

Marti, M., Good, R.T., Rug, M., Knuepfer, E. and Cowman, A.F. (2004) Targeting malaria virulence and remodeling proteins to the erythrocyte. Sience 306, 1930-1933.

Martin, G.B., Bogdanove, A.J. and Sessa, G. (2003) Understanding the functions of plant disease resistance proteins. Annu. Rev. Plant Biol. 54, 23-61.

Matoo, A.K. and Suttle, J.c. (1991) The plant hormone ethylene. CRC Press, Boca Raton, Fl.

Matton, D.P. and Brisson, N. (1989) Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. Mol. Plant-Microbe Interact. 2, 325-331.

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.

McGee, J.D., Hamer, J.E. and Hodges, T.K. (2001) Characterization of a PR-10 pathogenesis-related gene family induced in rice during infection with *Magnaporthe grisea*. Mol. Plant-Microbe Interact. 14 (7), 877-886.

Mehdi, M.C. (1994) Active oxygen species in plant defence against pathogens. Plant Physiol. 105, 467-472.

Mithöfer, A., Lottspeich, F. and Ebel, J. (1996) One-step purification of the β-glucan elicitor-binding protein from soybean (*Glycine max* L.) roots and characterization of an anti-peptide antiserum. FEBS Lett. 381, 203-207.

Mithöfer, A., Daxberger, A., Fromhold-Treu, D. and Ebel, J. (1997) Involvement of an NAD(P)H oxidase in the elicitor-inducible oxidative burst of soybean. Phytochem. 45, 1101-1107.

Mithöfer, A. Ebel, J., Arvind, A.B., Boller, T. and Neuhaus-Url, G. (1999) Transgenic aequorin monitors cytosolic calcium transients in soybean cells challenged with β-glucan or chitin elicitors. Planta 207, 566-574.

Mithöfer, A., Fliegmann, J., Neuhas-Url, G., Schwarz, H. and Ebel, J. (2000) The hepta-β-glucoside elicitor-binding proteins from legumes represent a putative receptor familiy. Biol. Chem. 381, 705-713.

Mithöfer, A., Ebel, J. and Felle, H.H. (2005) Cation fluxes cause plasma membrane depolarization involved in β-glucan elicitor signaling in soybean roots. Mol. Plant-Microbe Interact. 18, 983-990.

Moesta, P., Hahn, M.G. and Grisebach, H. (1983) Development of a radioimmunoassay for the soybean phytoalexin glyceollin I. Plant Physiol. 73, 233-237.

Mohr, P.G. and Cahill, D.M. (2001) Relative roles of glyceollin, lignin and the hypersensitive response and the influence of ABA in compatible and incompatible interactions of soybeans with *Phytophthora sojae*. Physiol. Mol. Plant Pathol. 58, 31-41.

Morris, P.F., Savard, M.E. and Ward, E.W.B. (1991) Identification and accumulation of isoflavonoids and isoflavone glucosides in soybean leaves and hypocotyls in resistance responses to *Phytophthora megasperma* f.sp. *glycinea*. Physiol. Mol. Plant Pathol. 39, 229-244.

Moy, P., Qutob, D., Chapman, B.P., Atkinson, I. and Gijzen, M. (2004) Patterns of gene expression upon infection of soybean plants by *Phytophthora sojae*. Mol. Plant-Microbe Interact. 17, 1051-1062.

Mukhopadhyay, A. (1997) Inclusion bodies and purification of proteins in biologically active forms. Adv. Biochem. Eng. Biotechnol. 56, 61-109.

Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W.R., Hahlbrock, K. and Schell, D. (1994a) High affinity binding of a fungal oligopeptide elicitor to parsley plasma mebrane triggers multiple defence responses. Cell 78, 449-460.

Nürnberger, T. and Scheel, D. (2001) Signal transmission in the plant immune response. Trends Plant Sci. 6, 372-379.

Nürnberger, T. and Brunner, F. (2002) Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. Curr. Opin. Plant Biol. 5, 318-324.

Okinaka, Y., Mimori, K., Takeo, K., Kitamura, S., Takeuchi, Y., Yamaoka, N. and Yoshikawa, M. (1995) A structural model for the mechanisms of elicitor release from fungal cell walls by plant β-1,3-endoglucanase. Plant Physiol. 109, 839-845.

Orbach, M.J., Farrall, L., Sweigard, J.A., Chumley, F.G. and Valent, B. (2000) A telomeric avirulence gene determines efficacy for the rice blast resistance gene *Pi-ta*. Plant Cell 12, 2019-2032.

Osbourne, A. (1996) Saponins and plant defence- a soap story. Trends Plant. Sci. 1, 4-9.

pGEM-T and pGEM-T Easy vector Systems technical manual. Promega (Mannheim)

Pieterse, C.M.J., Van West, P., Verbakel, H.M., Brasse, P.W.H.M., Van den Berg-Velthuis, G.C.M. and Govers, F. (1994). Structure and genomic organization of the ipiB and ipiO gene clusters of *Phytophthora infestans*. Gene 138, 67–77.

Preisig, C.L. and Kuc, J.A (1985) Arachidonic acid-related elicitors of the hypersensitive response in potato and enhancement of their activites by glucans from *Phytophthora infestans*. Arch. Biochem. Biophys. 236, 379-389.

QIA Expressionist manual. Qiagen (Hilden)

Qutob, D., Kamoun, S. and Gijzen, M. (2002) Expression of a *Phytophthora sojae* necrosis-inducing protein occurs during transition from biotrophy to necrotrophy. Plant J. 32, 361-373.

Qutob, D., Huitema, E., Gijzen, M. and Kamoun, S. (2003) Variation in structure and activity among elicitins from *Phytophthora sojae*. Mol. Plant Pathol. 4 (2), 119-124.

Rehmany, A.P., Gordon, A., Rose, L.E., Allen, R.L., Armstrong, M.R., Whisson, S.C., Kamoun, S., Tyler, B.M., Birch, P.R.J. and Beynon, J.L. (2005) Differential recognition of highly divergent downy mildew avirulence gene alleles by *RPP1* resistance genes from two *Arabidopsis* lines. Plant Cell 17, 1839-1850.

Rivas, S. and Thomas, C.M. (2005) Molecular interactions between tomato and the leaf mold pathogen *Cladosporium fulvum*. Annu. Rev. Phytopathol. 43, 395-436.

Rohe, M., Gierlich, A., Hermann, H., Hahn, M., Schmidt, B., Rosahl, S. and Knogge, W. (1995) The race-specific elicitor, NIP1, from the barley pathogen, *Rhyncosporium secalis*, determines avirulence on host plants of the Rsr1 resistance genotype. EMBO J. 14, 4168-4177.

Rooney, H.C.E., Van't Klooster, J.W., Van der Hoorn, R.A.L., Joosten, M.H.A.J, Jones, J.D.G. and De Wit, P.J.G.M. (2005) Cladosporium Avr2 inhibits tomato Rcr3 protease required for *Cf-2*-dependent disease resistance. Science 308, 1783-1786.

Rudolph, R. and Lilie, H. (1996) In vitro folding of inclusion body proteins. FASEB J. 10, 49-56.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. (1996) Systemic acquired resistance. Plant Cell 8, 1809-1819.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning. Cold Spring Harbor Laboratory Press. 2nd edition.

Schmidt W.E. and Ebel, J. (1987) Specific binding of a fungal phytoalexin elicitor to membrane fractions from soybean *Glycine max*. Proc. Natl. Acad. Sci. USA 84, 4117-4121.

Schmitthenner, A.F., Hobe, M. and Bhat, R.G. (1994) *Phytophthora sojae* races in Ohio over a 10-year interval. Plant Dis. 78, 269-276.

Schopfer, C.R., Kochs, G., Lottspeich, F. and Ebel, J. (1998) Molecular characterization and functional expression of dihydroxypterocarpan 6a hydroxylase, an enzyme specific for pterocarpanoid phytoalexin biosynthesis in soybean (*Glycine max* L.). FEBS Lett. 432, 182-186.

Schopfer, C.R. and Ebel, J. (1998) Identification of elicitor-induced cytochrome P450s of soybean (*Glycine max* L.) using differential display of mRNA. Mol. Gen. Genet. 258, 315-322.

Scofield, S.R., Tobias, C.M., Rathjen, J.P., Chang, J.H., Lavelle, D.T., Michelmore, R.W. and Staskawicz, B.J. (1996) Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. Science 274, 2063-2065.

Sela-Buurlage, M.B., Ponstein, A.S., Bres-Vloemans, S.A., Melchers. L.S., Van den Elzen, P.J.M. and Cornelissen, B.J.C. (1993) Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol. 101, 857-863.

Senchou, V., Weide, R., Carrasco, A., Bouyssou, H., Pont-Lezica, R., Govers, F. and Canut, H. (2004) High affinity recognition of a *Phytophthora* protein by *Arabidopsis* via an RGD motif. Cell. Mol. Life Sci. 61, 502-509.

Shan, W., Cao, M., Leung, D. and Tyler, B.M. (2004) The *Avr1b* locus of *Phytophthora sojae* encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene *Rps*1b. Mol. Plant-Microbe Interact. 17, 394-403.

Shapiro, J.A. (1999) Views about evolution are evolving. ASM News. 65, 201-207.

Sharp, J.K., McNeil, M. and Albersheim, P. (1984a) The primary structures of one elicitor-active and seven elicitor-inactive hexa(β-D-glucopyranoside)-D-glucitols isolated from the mycelial walls of *Phytophthora megasperma* f.sp. *glycinea*. J. Biol. Chem. 259, 11321-11336.

Sharp, J.K., Valent, B. and Albersheim, P. (1984b) Purification and partial characterization of a β-glucan fragment that elicits phytoalexin accumulation in soybean. J. Biol. Chem. 259, 11312-11320.

Shulaev, V., Silverman, P. and Raskin, I. (1997) Airborne signaling by methyl salicylate in plant pathogen resistance. Nature 385, 718–721.

Sjödahl, J. (1977) Repetitive sequences in protein A from *Staphylococcus aureus*: arrangement of five regions within the protein, four of them highly homologous and Fc-binding. Eur. J. Biochem. 73, 343-351.

Slusarenko, A.J. and Schlaich, N.L. (2003) Downy mildew of *Arabidopsis thaliana* caused by *Hyaloperonospora parasitica* (formerly *Peronospora parasitica*). Mol. Plant Pathol. 4, 159-170.

Somssich, I.E., Schmelzer, E., Bollman, J. and Hahlbrock, K. (1986) Rapid activation by fungal elicitor encoding "pathogenesis-related" proteins in cultured parsley cells. Proc. Natl. Acad. Sci. USA 83, 2427-2430.

Stäb, M.R. and Ebel, J. (1987) Effects of Ca^{2+} on phytoalexin induction by fungal elicitor in soybean cells. Arch. Biochem. Biophys. 257, 416-423.

Strasburger, E. (2002) Lehrbuch der Botanik für Hochschulen. Spektrum Akademischer Verlag Heidelberg, Berlin. Revision by: Sitte, P., Weiler, E.W., Kadereit, J.W., Bresinsky, A. and Körner, C. 35^{th} edition.

Takeuchi, Y., Yoshikawa, M., Takeba, G., Tanaka, K., Shibata, D. and Horino, O. (1990) Molecular cloning and ethylene induction of mRNA encoding a phytoalexin elicitor-releasing factor, β-1,3-endoglucanase, in soybean. Plant Physiol. 93, 673-682.

Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y. and Martin, G.B. (1996) Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. Science 274, 2060-2063.

Templeton, M.D., Rikkerink, E.H.A. and Beever, R.E. (1994) Small, cysteine-rich proteins and recognition in fungal-plant interactions. Mol. Plant-Microbe Interact. 7, 320-325.

Tyler, B.M., Wu, M.H., Wang, J.M., Cheung, W.W.S. and Morris, P.F. (1996) Chemotactic preferences and strain variation in the response of *Phytophthora sojae* zoospores to host isoflavones. Appl. Environ. Microbiol. 662, 2811-2817.

Tyler, B.M. (2001) Genetics and genomics of the oomycete-host interface. Trends Genet. 17 (11), 611-614.

Tyler, B.M. (2002) Molecular basis of recognition between *Phytophthora* pathogens and their hosts. Annu. Rev. Phytopathol. 40 (1), 137-167.

Ueda, J. and Kato, J. (1980) Isolation and identification of a senescence-promoting substance in wormwood (*Artemisia absinthium* L.) Plant Physiol. 66, 246-249.

Umemoto, N., Kakitani, M., Iwamatsu, A., Yoshikawa, M., Yamaoka, N. and Ishida, I. (1997) The structure and function of a soybean beta-glucan-elicitor-binding protein. Proc. Natl. Acad. Sci. USA 94, 1029-1034.

Van der Ackerveken, G., Marois, E. and Bonas, U. (1996) Recognition of the bacterial avirulence protein AvrBs3 occurs inside the host plant cell. Cell 87, 1307-1316.

Van der Biezen, E.A. and Jones, J.D.G. (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem. Sci. 23, 454-456.

Van der Hoorn, R.A.L., De Wit, P.J.G.M. and Joosten, M.H.A.J. (2002) Balancing selection favors guarding resistance proteins. Trends Plant Sci. 7, 67-71.

Van der Peer, Y. and De Wachter, R. (1997) Evolutionary relationships among the eukaryotic crown taxa taking into account site-to-site rate variation in 18S rRNA. J. Mol. Evol. 45, 619-630.

Van Loon, L.C. and Van Kammen, A. (1970) Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. “Samsun” and “Samsun NN”. II. Changes in protein constitution after infection with tobacco mosaic virus. Virology 40, 199-211.

Van Loon, L.C. (1985) Pathogenesis-related proteins. Plant Mol. Biol. 4, 111-116.

Van West, P., Appiah, A.A. and Gow, N.A.R. (2003) Advances in research on oomycete root pathogens. Phys. Mol. Plant Pathol. 62, 99-113.

Waldmüller, T., Cosio, E.G., Grisebach, H. and Ebel, J. (1992) Release of highly elicitor-active glucans by germinating zoospores of *P. megasperma* f. sp*. glycinea.* Planta 188, 498-505.

Ward, E.W.B. (1990) The interaction of soya beans with *Phytophthora megasperma* f.sp. *glycinea*: pathogenicity. In Biological Control of Soil-Borne Plant Pathogens. Hornby, D. (Editor). Wallingford: CAB International. 311-327.

Warner, S.A., Gill, A. and Draper, J. (1994) The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid biosynthesis. Plant J. 6, 31-43.

Weidhase, R.A., Kramell, H.-M., Lehman, J., Liebisch, H.-W., Lerbs, W. and Parthier, B. (1987) Methyljasmonate-induced changes in the polypeptide pattern of senescing barley leaf segments. Plant Sci. 51, 177-186.

Werner, C. and Hohl, H.R. (1990) The effects of Ca^{2+} and Mg^{2+} on accumulation and secretion of isoflavonoids by soybean roots. Plant Science 72, 181-191.

Whisson, S.C., Drenth, A., Maclean, D.J. and Irwin, J.A.G. (1995) *Phytophthora sojae* avirulence genes, RAPD and RFLP markers used to construct a detailed genetic linkage map. Mol. Plant-Microbe Interact. 8, 988-995.

White, F.F., Yang, B. and Johnson, L.B. (2000) Prospects for understanding avirulence gene function. Curr. Opin. Plant Biol. 3, 291-298.

Yang, A., Yuan, Q. and Gabriel, D.W. (1996) Watersoaking functions of XcmH1005 are redundantly encoded by members of a *Xanthomonas* avirulence/pathogenicity gene family. Mol. Plant-Microbe Interact. 9, 105-113.

Yeboah, N.A., Arahira, M., Nong, V.H., Zhang, D., Kadokura, K., Watanabe, A and Fukazawa, C. (1998) A class III acidic endochitinase is specifically expressed in the developing seeds of soybean (*Glycine max* (L.) Merr.) Plant Mol. Biol. 36, 407-415.

Yoshikawa, M., Matama, M. and Masago, H. (1981) Release of a soluble phytoalexin elicitor from mycelial walls of *Phytophthora megasperma* var. *sojae* by soybean tissues. Plant Physiol. 67, 1032-1035.

Young, N.D. (2000) The genetic architecture of resistance. Curr. Opin. Plant Biol. 3, 285-290.

Zähringer, U., Schaller, E. and Grisebach, H. (1981) Induction of phytoalexin synthesis in soybean. Structure and reactions of naturally occurring and enzymatically prepared prenylated pterocarpans from elicitor-treated cotyledons and cell cultures of soybean. Z. Natursforsch. C: Biosci. 36c, 234-241.

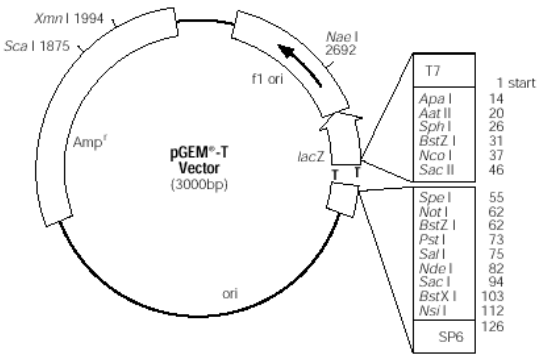
Zhao, Y.I., Del Grosso, L., Yigit, E., Dempsey, D.A., Klessig, D.F. and Wobbe, K.K. (2000) The amino terminus of the coat protein of *Turnip crinkle virus* is the Avr factor recognized by resistant *Arabidopsis*. Mol. Plant-Microbe Interact. 13, 1015-1018.

9 Appendix

9.1 Restriction maps

9.1.1 Map and restriction endonuclease sites for the PGEM-T vector

A



B

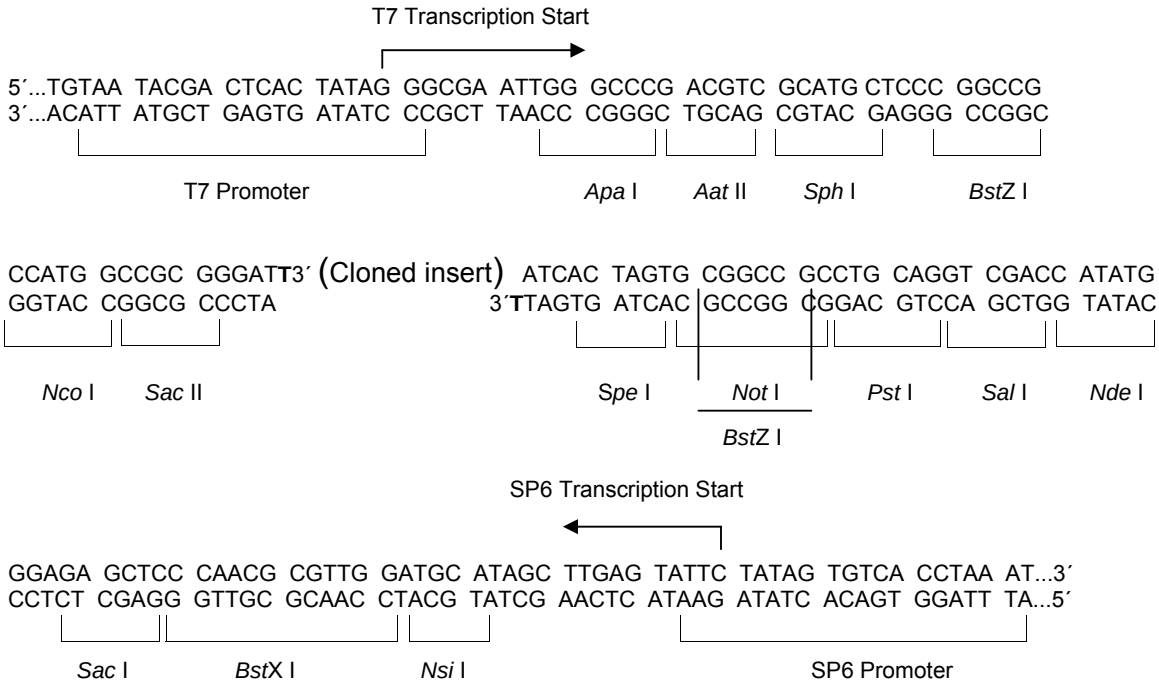
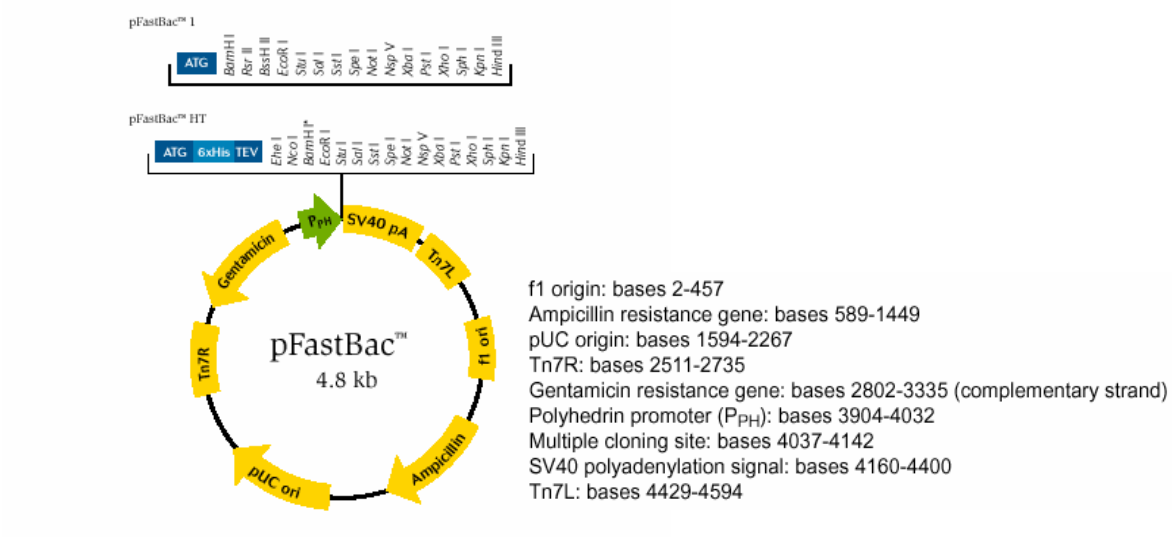


Fig. 32 (A) pGEM-T vector circle map and sequence reference points. (B) Promoter and multiple cloning sequence of the pGEM-T vector. The top strand of the sequence shown corresponds to the RNA synthesised by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesised by SP6 RNA polymerase. Taken from the manual “pGEM-T and pGEM-T easy vector systems” from Promega.

9.1.2 Map and restriction endonuclease sites for the pFASTBAC1 vector

A



B

polyhedrin promoter +1

5´- AAATAAGTA TTTTACTGTT TTCGTAACAG TTTTGTAATA AAAAAACCTA

suggested sequencing primer

5´-TATTCC GGATTATTCA TACC-3´→

TAAATATTCC GGATTATTCA TACCGTCCCA CCATCGGGCG CGGATCCCGG TCCGAAGCGCGC

BamH I *Rsr* II *Bss*H II

GGAATTCA AAGGCCTACG TCGACGAGCT CACTAGTCGC GGCCGCTTTC GAATCTAGAG

*Eco*R I *Stu* I *Sal* I *Sst* I *Spe* I *Not* I *Nsp* V *Xba* I

CCTGCAGTCT CGAGGCATGC GGTACCAAGCTT GTCGAGAAGT AC**TAG**AGGAT

↑Stop codon

Pst I *Xho* I *Sph* I *Kpn* I *Hind* III

CA**TAA**TCAG-3´

↑Stop codon

Fig. 33 (A) pFastBac1 vector circle map and sequence reference points. (B) Promoter and multiple cloning sequence of the pFastBac1 vector. Taken from the manual “Bac-to-Bac Baculovirus Expression System” from Invitrogen.

9.1.3 pTZ18R:Avr1b partial restriction map

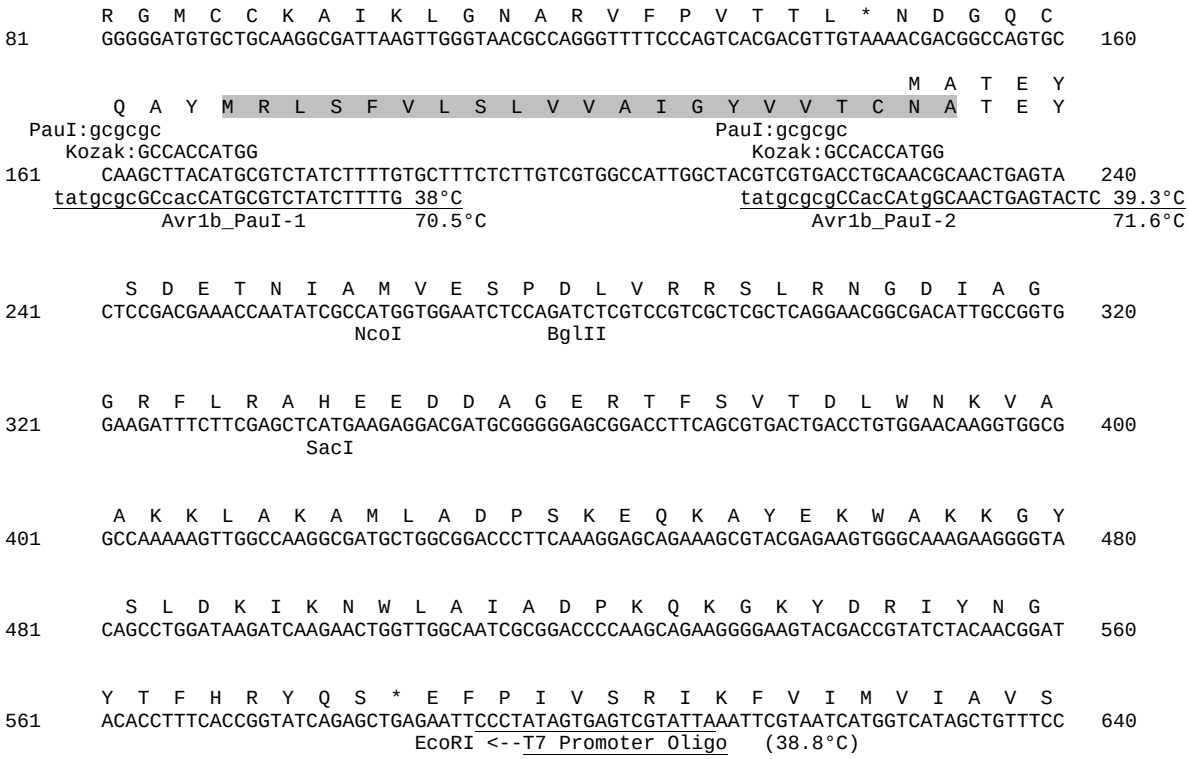


Fig. 34 Partial restriction map of pTZ18R:Avr1b. The signal peptide is shaded in grey. Underlined are the amino acids used for the primer generation.

9.2 Amino acid alignment of *Avr1b, PauI-1 and PauI-2*

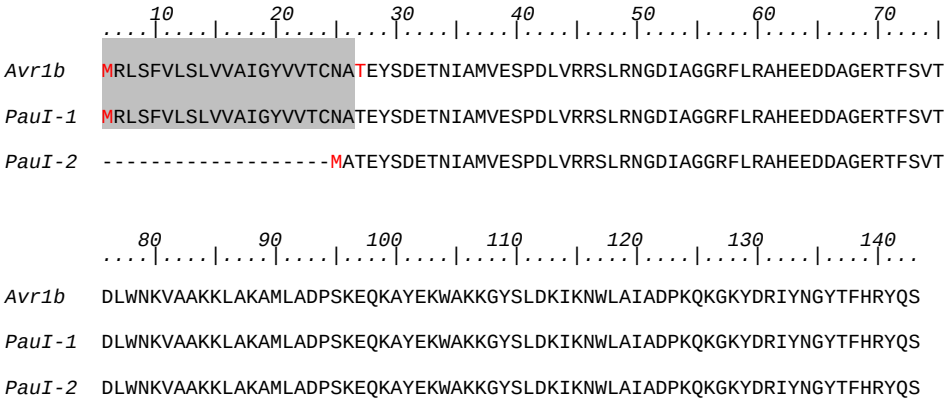


Fig. 35 Amino acid alignment of *Avr1b, PauI-1 and PauI-2.* Amino acids shaded in grey represent the putative signal peptide.

9.3 Amino acid sequence of Avr1b from *E. coli* pET26b

```
          10         20         30         40         50         60
MKYLLPTAAAGLLLLAAQPAMATEYSDETNIAMVESPDLVRRSLRNGDIAGGRFLRAHEE

          70         80         90         100        110        120
DDAGERTFSVTDLWNKVAAKKLAKAMLADPSKEQKAYEKWAKKGYSLDKIKNWLAIADPK

          130        140        150  154
QKGKYDRIYNGYTFHLYQSVDKLAAALEHHHHHH
```

Fig. 36 Amino acid sequence of Avr1b from *E. coli* pET26b containing a periplasmatic localization signal (kursiv); a His_6-fusionepitope and a spacer (in dots).

9.4 Deduced amino acid alignment of *Avr1b*-1 (accession number AF449622), *Avr1b*-1 (clone #10) and *Avr1b*-1 (clone #11)

```
                  10        20        30        40        50        60        70        80
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF449622                  ATGCGTCTATCTTTTGTGCTTTCTCTTGTCGTGGCCATTGGCTACGTCGTGACCTGCAACGCAA
                            M  R  L  S  F  V  L  S  L  V  V  A  I  G  Y  V  V  T  C  N  A
clone #10 CCAAGTATCACGAACCATGCGTCTATCTTTTGTGCTTTCTCTTGTCGTGGCCATTGGCTACGTCGTGACCTGCAACGCAA
                          .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
clone #11 CCAAGTATCACGAACCATGCGTCTATCTTTTGTGCTTTCTCTTGTCGTGGCCATTGGCTACGTCGTGACCTGCAACGCAA
                            .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

                  90       100       110       120       130       140       150       160
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AF449622  CTGAGTACTCCGACGAAACCAATATCGCCATGGTGGAATCTCCAGATCTCGTCCGTCGCTCGCTCAGGAACGGCGACATT
          T  E  Y  S  D  E  T  N  I  A  M  V  E  S  P  D  L  V  R  R  S  L  R  N  G  D  I
clone #10 CTGAGTACTCCGACGAAACCAATATCGCCATGGTGGAATCTCCAGATCTCGTCCGTCGCTCGCTCAGGAACGGCGACATT
          .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
clone #11 CTGAGTACTCCGACGAAACCAATATCGCCATGGTGGAATCTCCAGATCTCGTCCGTCGCTCGCTCAGGAACGGCGACATT
          .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .
```

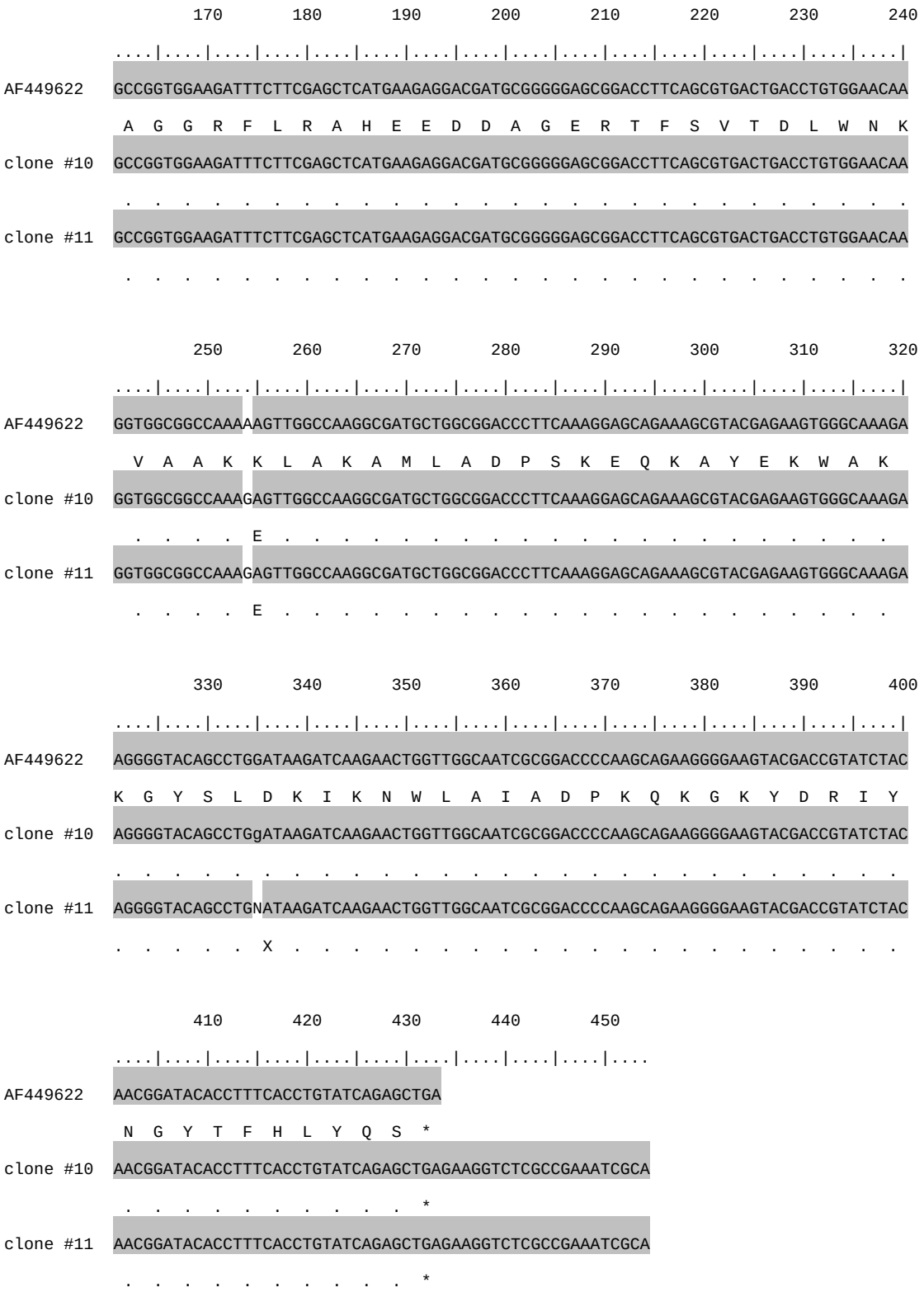


Fig. 37 Deduced amino acid alignment of *Avr1b*-1 (accession number AF449622), *Avr1b*-1 (clone #10) and *Avr1b*-1 (clone #11) see section 4.5.6. The alignment was generated using MultAlin and edited using BioEdit. Consensus amino acid residues are boxed in grey.

9.5 PCR programs

ptz18R: Avr1b: 94°C, 3' -> [94°C, 30'' -> 40°C, 30''-> 72°C, 1']$_{20}$ -> [94°C, 30'' -> 60°C, 30''-> 72°C, 1']$_{15}$ ->72°C, 10' -> 4°C
Primers: T7 promoter and Avr1b_PauI-1 or Avr1b_PauI-2
Expected size: 427 bp

*P. sojae-Act*A: 94°C, 3' -> [94°C, 30'' -> 61°C, 1' -> 72°C, 30'']$_{35}$ -> 72°C, 10' -> 4°C
Primers: Ps_ActA_for and Ps_ActA_rev
Expected size: 450 bp

P. sojae-Avr1b: 94°C, 3' -> [94°C, 30'' -> 61°C, 1' -> 72°C, 45'']$_{35}$ -> 72°C, 10' -> 4°C
Primers: Avr1b_for and Avr1b_rev
Expected size: 430 bp

Soybean *Tub*B2: 94°C, 3' -> [94°C, 30'' -> 61°C, 1' -> 72°C, 45'']$_{35}$ -> 72°C, 10' -> 4°C
Primers: GmTubB2_for and GmTubB2_rev
Expected size: 414 bp

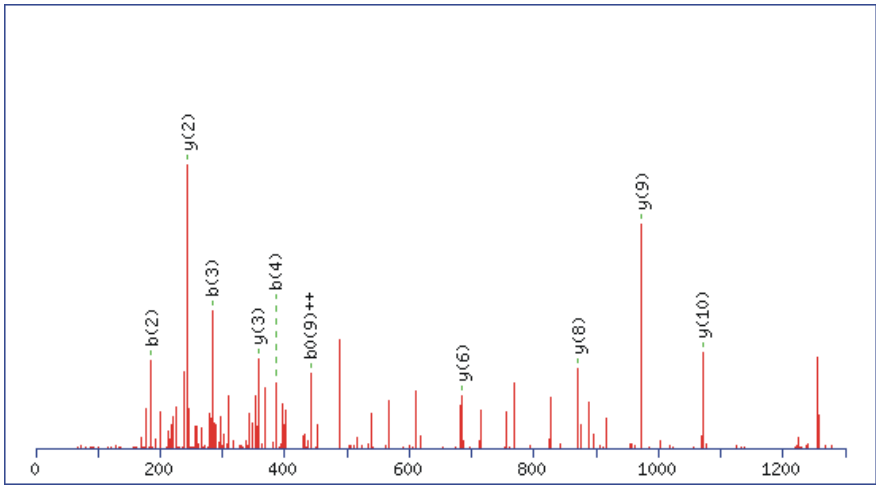
Soybean *Cyp93a1*: 94°C, 3' -> [94°C, 30'' -> 61°C, 1' -> 72°C, 45'']$_{21}$ -> 72°C, 10' -> 4°C
Primers: Cyp93a1_for and Cyp93a1_rev
Expected size: 278 bp

Soybean *4CL3*: 94°C, 3' -> [94°C, 30'' -> 61°C, 1' -> 72°C, 45'']$_{24}$ -> 72°C, 10' -> 4°C
Primers: Gm4CL3_for and Gm4CL3_rev
Expected size: 520 bp

PCR on bacteria: 94°C, 4' -> [94°C, 30'' -> 61°C, 1' -> 72°C, 45'']$_{35}$ -> 72°C, 10' -> 4°C
Primers: Avr1b_for and Avr1b_rev
Expected size: 430 bp

9.6 Identification of PR-10-like protein by mass spectrometry

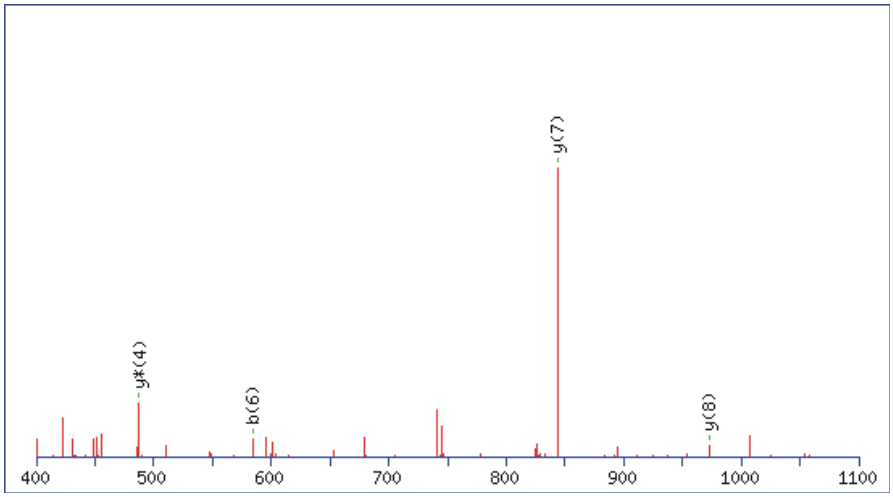
A



B

#	b	b^{++}	b*	b*$^{++}$	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y*$^{++}$	y^{0}	y^{0++}	#
1	72.04	36.53					A							12
2	185.13	93.07					L	1184.65	592.83	1167.63	584.32	1166.64	583.82	11
3	284.20	142.60					V	1071.57	536.29	1054.54	527.77	1053.56	527.28	10
4	385.24	193.13			367.23	184.12	T	972.50	486.75	955.47	478.24	954.49	477.75	9
5	500.27	250.64			482.26	241.63	D	871.45	436.23	854.43	427.72	853.44	427.22	8
6	571.31	286.16			553.30	277.15	A	756.43	378.72	739.40	370.20	738.41	369.71	7
7	686.34	343.67			668.32	334.67	D	685.39	343.20	668.36	334.68	667.38	334.19	6
8	800.38	400.69	783.35	392.18	782.37	391.69	N	570.36	285.68	553.33	277.17			5
9	899.45	450.23	882.42	441.71	881.44	441.22	V	456.32	228.66	439.29	220.15			4
10	1012.53	506.77	995.50	498.26	994.52	497.76	I	357.25	179.13	340.22	170.62			3
11	1109.58	555.30	1092.56	546.78	1091.57	546.29	P	244.17	122.59	227.14	114.07			2
12							K	147.11	74.06	130.09	65.55			1

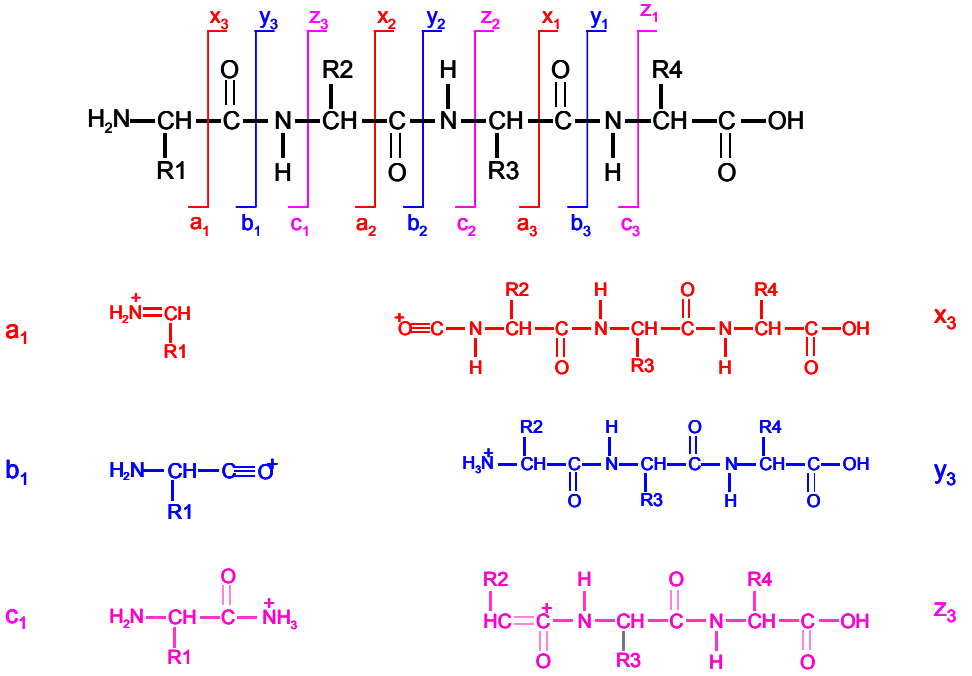
C



D

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	58.029	29.52					G							11
2	173.06	87.03			155.05	78.03	D	1158.5	579.8	1141.5	571.3	1140.5	570.8	10
3	244.09	122.6			226.08	113.5	A	1043.5	522.3	1026.5	513.7	1025.5	513.2	9
4	373.14	187.1			355.12	178.1	E	972.46	486.7	955.44	478.2	954.45	477.7	8
5	470.19	235.6			452.18	226.6	P	843.42	422.2	826.39	413.7	825.41	413.2	7
6	584.23	292.6	567.2	284.1	566.22	283.6	N	746.37	373.7	729.34	365.2	728.36	364.7	6
7	712.29	356.6	695.26	348.1	694.28	347.6	Q	632.32	316.7	615.3	308.2	614.31	307.7	5
8	827.32	414.2	810.29	405.6	809.31	405.2	D	504.27	252.6	487.24	244.1	486.26	243.6	4
9	956.36	478.7	939.33	470.2	938.35	469.7	E	389.24	195.1	372.21	186.6	371.23	186.1	3
10	1069.4	535.2	1052.4	526.7	1051.4	526.2	L	260.2	130.6	243.17	122.1			2
11							K	147.11	74.06	130.09	65.55			1

E



F

Ion type	Ion mass
b	[N] + [M]
b^{++}	(b+H)/2
b*	b - NH_3
b^0	b - H_2O
y	[C] + [M] + H_2
y^{++}	(y+H)/2
y*	y - NH3
y^0	y - H_2O

Fig. 38 Identification of PR-10-like protein by mass spectrometry. (A) MS/MS fragmentation of the peptide **ALVTDADNVIPK** (4.5.7.2). (B) List of “b” and “y” ions obtained after fragmentation of **ALVTDADNVIPK**. Numbers shaded in grey represent the fragments found in the MS/MS spectrum. (C) MS/MS fragmentation of the peptide **GDAEPNQDELK** (4.5.7.2). (D) List of “b” and “y” ions obtained after fragmentation of **GDAEPNQDELK**. Numbers shaded in grey represent the fragments found in the MS/MS spectrum. (E) Peptide ion fragmentation nomenclature. The spine of a peptide contains three types of bond: C-C, C-N and N-C. Any of these may be broken and the ions resulting from the breakage are named a, b, c, x, y and z. MS fragmentation of peptides primarily occurs at the amide bond (-CO-NH-) between two amino acid residues. The most commonly observed fragmentation ions are “b” and “y” ions. “b” ions are sequence-specific fragment ions derived from the N-terminus and “y” ions are from the C-terminus. (F) Formulae to calculate fragment ion masses. [N] is the mass of the N-terminal group, [C] is the mass of the C-terminal group, [M] is mass of the sum of the neutral amino acid residues masses. Peaks seen for ions which have lost ammonia (-17 Da) are donoted b* and y*; peaks seen for ions which have lost water (-18 Da) are denoted b^0 and y^0.

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Erklärung

Hiermit erkläre ich, daß die vorliegende Doktorarbeit selbstständig und nur mit den angegebenen Hilfsmitteln angefertigt wurde. Ebenfalls versichre ich, dass ich zuvor nirgendwo anders promoviert habe bzw. nirgendwo anders mit einer Promotion angefangen habe.

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