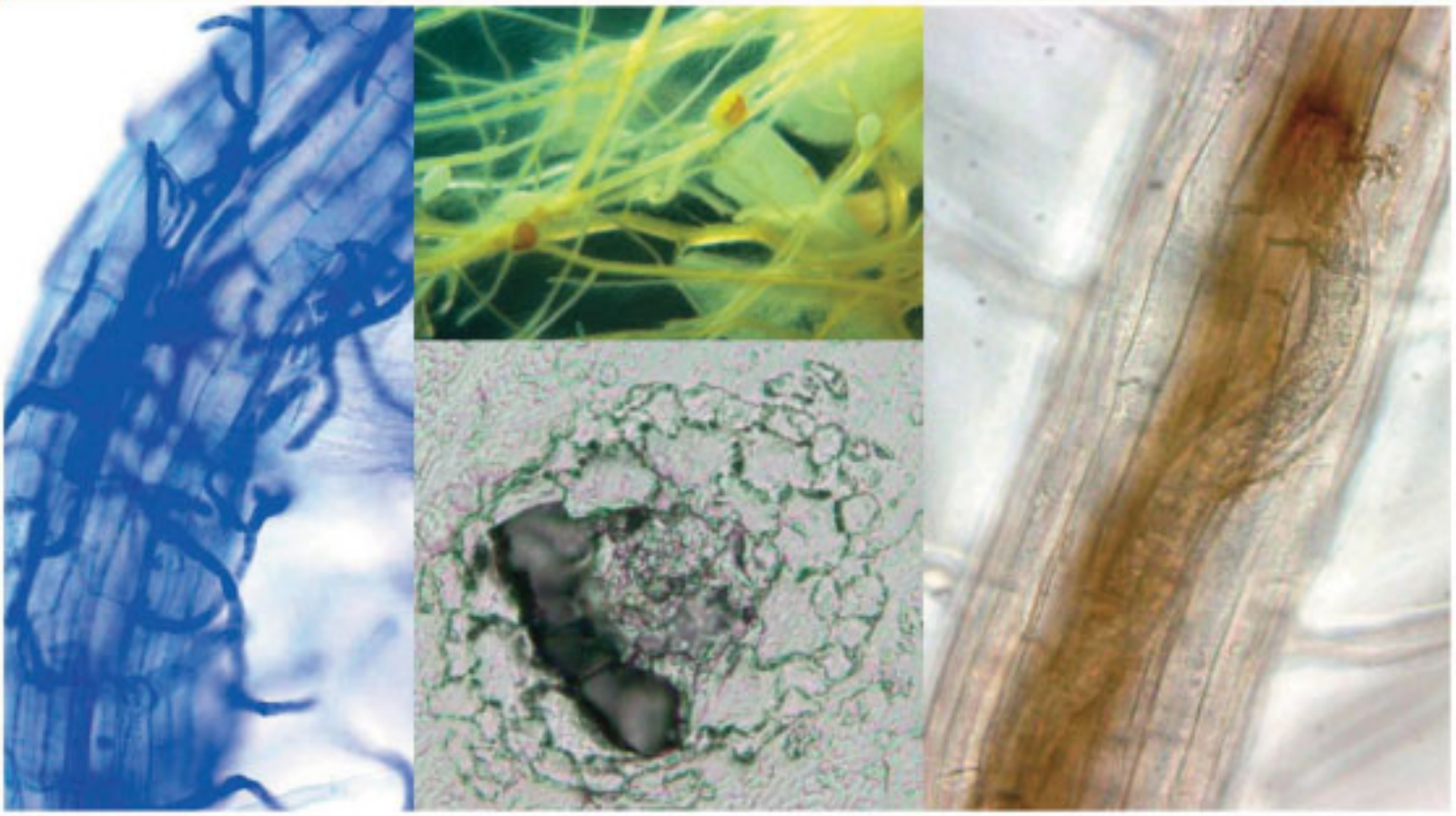
Katrin Knecht

Molecular mechanisms of the *Hs1*$^{pro-1}$-mediated nematode (*Heterodera schachtii*) resistance and its potential for genetic engineering of plant disease resistance



Cuvillier Verlag Göttingen
Internationaler wissenschaftlicher Fachverlag

Aus dem Institut für Phytopathologie
der Christian-Albrechts-Universität zu Kiel

Molecular mechanisms of the *Hs1*$^{pro-1}$-mediated nematode (*Heterodera schachtii*) resistance and its potential for genetic engineering of plant disease resistance

Dissertation
zur Erlangung des Doktorgrades
der Agrar- und Ernährungswissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von
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aus Preetz

Kiel, 2009

Dekan: Prof. Dr. Uwe Latacz-Lohmann
1. Berichterstatter: Prof. Dr. Daguang Cai
2. Berichterstatter: Prof. Dr. Christian Jung
Tag der mündlichen Prüfung: 30.April 2009

Gedruckt mit Genehmigung der Christian-Albrechts-Universität zu Kiel

Bibliografische Information der Deutschen Nationalbibliothek
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über http://dnb.ddb.de abrufbar.
1. Aufl. - Göttingen : Cuvillier, 2009
Zugl.: Kiel, Univ., Diss., 2009

978-3-86955-081-7

Nonnenstieg 8, 37075 Göttingen
Telefon: 0551-54724-0
Telefax: 0551-54724-21
www.cuvillier.de

1. Auflage, 2009
Gedruckt auf säurefreiem Papier

978-3-86955-081-7

List of Abbreviations

AFLP	Amplified fragment length polymorphism
ATP	Adeneosine triphosphate
Avr	Avirulence
BA	6-benzylaminopurine
bp	Base pairs
BCN	Beet cyst nematode
BSA	Bovine serum albumin
°C	Celsius
CC	Coiled coil
CDPKs	Calmodulin (CaM)-like domain protein kinases
CIM	Callus induction medium
COI1	Coronatine insensitive 1
CTAB	Cetyltrimethylammoniumbromid
2,4-D	2, 4-dichlorophenoxyacetic acid
DAB	3,3`-Diaminobenzidine
dATP	2´-Desoxyadenosin-5´-triphosphate
dCTP	2´-Desoxycytidin-5´-triphosphae
DD	Differential display
DNA	Deoxyribonucleic acid
dNTP	2´-Desoxynucleotide-5´-triphosphate
Dpi	Days post inoculation
dsRNA	double-stranded RNA
E. coli	Escherichia coli
EDR1	Enhanced disease resistance 1
EDS1	Enhanced Disease Susceptibility1
EIN2	Ethylene-insensitive 2
et al.	et alii
eLRR	Extracellular LRR
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
GLPs	Germin-like proteins

GUS	β-glucuronidase
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
IMR	Intermotif region
ILs	Interleukins
ISR	Induced systemic resistance
JA	Jasmonic acid
kbp	Kilo base pairs
Km	Kanamycin
KO	Knockout
LB-medium	Luria-Bertani-medium
LRR	Leucine rich repeat
LZ	Leucine zipper
$MAMP_S$	Microbial-associated molecular patterns
M	Mol
MS	Murashige and Skoog
NAA	Naphthaline acetic acid
NBS	Nucleotide binding site
NCBI	National Centre for Biotechnology Information
NDR1	Non-race specific Disease Resistance
NHO1	NON HOST RESISTANCE 1
NLR	NOD-like receptor
nlRGAs	NBS-LRR-RGAs
NO	Nitric oxide
NPR1	NON-EXPRESSOR OF PR1
OA	Oxalic acid
OxO	Oxalate oxidase
PAD4	Phytoalexin Deficient4
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDF	Plant defensin
PGIP	Polygalacturonaseinhibiting protein

PGPR	Plant growth-promoting rhizobacteria
pH	potentia hydrogenii
PIs	Proteinase inhibitors
PR	Pathogenisis related
PRRs	Pathogen recognition proteins
PTI	PAMP-triggered immunity
RGA	Resistance gene analog
R-gene	Resistance gene
RLK	Receptor-like protein kinase
RLP	Receptor-like proteins
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Rounds per minute
R-protein	Resistance protein
PRRs	Pattern recognition receptors
RIM	Root inducing medium
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SA	Salicylic acid
SAGE	Serial analysis of gene expression
SAR	Systemic acquired resistance
SCN	Soybean cyst nematode
SEM	Shoot elongation medium
SGT1	Suppressor of the G2 allele of skp1
SIM	Shoot induction medium
ssp.	subspecies
STS	Silver thiosulphate
TDFs	Transcript-derived fragments
TE	Tris-EDTA-buffer
TIR	Toll-Interleukin-1 receptor
TLR	Toll-like receptor
TM	Transmembrane domain

u	Unit
v/v	Volume per volume
WA	Water agar
w/v	Weight per volume
X-Gluc	5-bromo-4-chloro-3-indolyl β-D-glucuronide

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Chapter I: Introduction

1 Plant pathogen resistance

1.1 General mechanisms of plant pathogen resistance

Plants are haunted by various diseases caused by phytopathogenic fungi, bacteria, viruses, insects and nematodes. In agriculture, severe damage is especially caused by rust and mildew fungi, *Fusarium* spp., Barley yellow dwarf virus (BYDV) as well as cyst and root knot nematode species. Rice is mainly attacked by *Xanthomonas, Fusarium* spp. and *Magnaporthe* causing bacterial blight, root rot and stem rot disease. The lack of genetic diversity within the genomes of cultivated crop species as well as changes in cultivation techniques such as large-scale cropping of genetically uniform plants, reduced crop rotation and the expansion of crops into less suitable regions, resulted in an increasing susceptibility to different pests. For crops, the total global actual loss due to pests varies between about 26% in soybean and more than 40% in potato production (Table 1).

Table 1: Overall summary of the loss potential and the actual losses due to fungal and bacterial pathogens, viruses, animal pests and weeds in wheat, rice, maize, potatoes, soybean and cotton, in 2001-03. According to Oerke et al. 2006.

	Crop losses due to in %									
	Pathogens		Viruses		Animal pests		Weeds		Total	
	Potential	Actual	Potential	Actual	Potential	Actual	Potential	Actual	Potential	Actual
Wheat	15.6	10.2	2.5	2.4	8.7	7.9	23	7.7	49.8	28.2
Rice	13.5	10.8	1.7	1.4	24.7	15.1	37.1	10.2	77.0	37.4
Maize	9.4	8.5	2.9	2.7	15.9	9.6	40.3	10.5	68.5	31.2
Potatoes	21.2	14.5	8.1	6.6	15.3	10.9	30.2	8.3	74.9	40.3
Soybeans	11	8.9	1.4	1.2	10.7	8.8	37	7.5	60	26.3
Cotton	8.5	7.2	0.8	0.7	36.8	12.3	35	8.6	82	28.8

One of the most important ways of protecting plants against harmful organisms and of improving agricultural production is the use of plant protection products. The use of pesticides has increased dramatically since the early 1960s. Even though pesticides may provide a certain control level, their use may also involve risks and hazards for humans, animals and the environment. Despite crop protection, about 32%, 29% and 40% of attainable

maize, cotton and potatoe production is still lost to pests (Table 1). Therefore, breeding for disease resistance in plants is a promising alternative for controlling plant diseases.

To counter pathogen attacks plants have evolved sophisticated and multi-faceted defense mechanisms. In essence, two branches of the plant immune system do exist. The older, basal MAMP-/PAMP-triggered immunity (PTI) (Figure 1), that is reminiscent of innate immunity in vertebrates, uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs) (Figure 1). The second one, the effector-triggered immunity (ETI) (Figure 1) relying on resistance (R) proteins confers a pathogen-specific resistance that is often associated with a form of programmed cell death around the infection site termed the hypersensitive response (HR). PTI activates a MAP kinase signaling cascade and an extensive transcriptional reprogramming leading to downstream defense responses as production of reactive oxygen species, accumulation of phenolics, production of phytoalexins, papilla formation, induction of PR genes and callose deposition to reinforce the cell wall at sites of infection (Chisholm et al. 2006; Truman et al. 2007; Zipfel et al. 2004).
The best characterized szenario complementing our understanding of the plant response to PAMPs relates to flagellin, the major protein of flagella which is recognized by a receptor like protein kinase (RLK) from *Arabidopsis thaliana*, FLS2, carrying extracellular leucinerich repeats (LRRs), a transmembrane domain (TM) and a cytoplasmic serine/threonine protein kinase domain (Gomez-Gomez and Boller 2002). Other examples of MAMPs include lipopolysaccharides, fungal chitin, oomycete Pep-13 or heptaglucosides. Immune responses induced by the interaction of bacterial flagellin (elicitor) with the plasmamembrane-localized FLS2 receptor restrict the growth of the virulent *Pseudomonas syringae* pv. tomato strain DC3000, whereas *fls2* mutant plants are more susceptible to bacterial infection (Nürnberger et al. 2006). Sheen and colleagues identified a complete MAP kinase cascade and WRKY transcription factors that function downstream of flg22 perception (Kovtun et al. 2000; Tena et al. 2001). Even though this signaling machinery was identified in response to a bacterial PAMP, activation of defenses by WRKY overexpression decreased symptoms caused by both bacteria and fungi, indicating that the resistance mechanisms induced are not specific to bacteria (Asai et al. 2002). Interestingly, PAMP perception in animals is also predominantly mediated by pattern recognition receptors carrying extracellular LRR domains (Toll-like receptors, TLR) (Nürnberger et al. 2004).

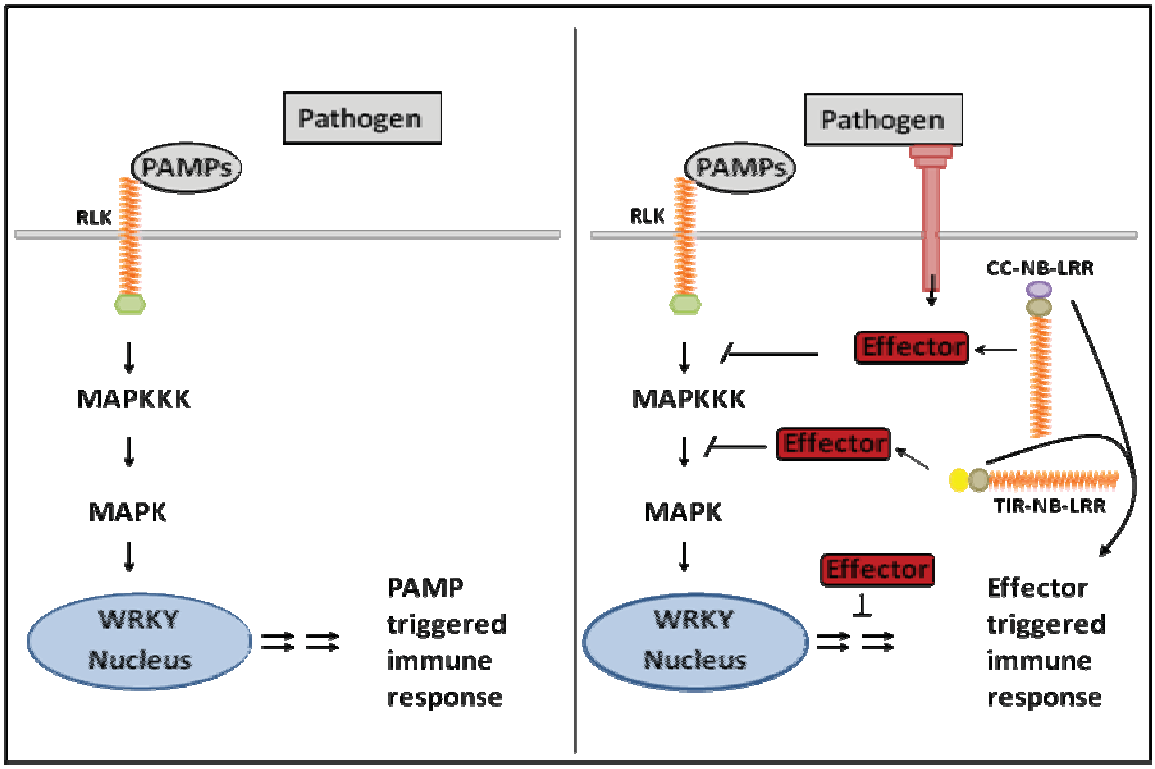

Figure 1: Simplified model for the evolution of resistance in plants according to Chisholm et al. 2006 and Bent 2007. Left to right, recognition of pathogen-associated molecular patterns (PAMPs) by extracellular receptor-like kinases (RLKs) triggers basal immunity, which requires signaling through MAP kinase cascades and transcriptional reprogramming mediated by plant WRKY transcription factors. Effector proteins target multiple host proteins and suppress basal immune responses. Plant R- proteins (CC-NB-LRR and TIR-NB-LRR) recognize effector activity and restore resistance through effector-triggered immune responses. kinase, LRR, TIR, NB, CC.

1.2 Plant susceptibility

Pathogens can overcome basal immune systems and colonize the plant successfully by the delivery of effector proteins into the plant cell, which can interfere directly with components of PTI or lead to changes in the transcritption of PTI genes (Li et al. 2005; Thilmony et al. 2006) resulting in effector-triggered susceptibility (ETS) (Murray et al. 2007; Truman et al. 2006) (Figure 1). Effectors, such as toxins and effector proteins, are virulence factors that interact with the host. Thus, the *P. syringae* effectors AvrPto and AvrRpt2 inhibit defense responses elicited by PAMP recognition (Hauck et al. 2003; Kim et al. 2005b). Several effector proteins from *P. syringae* pathovars are known to inhibit the HR localized to infection sites (Nomura et al. 2006). Fungal pathogens deliver their effectors; most of them are small proteins of unknown function containing a signal for secretion, via a specialized infection structure, the haustorium into the plant intercellular space (apoplast). Cyst nematodes secrete their parasitism proteins that often function in syncytium induction and

maintenance, through a stylet into the cytoplasma (Fuller et al. 2008). The secretions of interest originate from three pharyngeal (oesophageal) glands, one located dorsally and two subventrally (Lilley et al. 2005).

1.3 R-gene mediated resistance

Once the pathogen succeeded in suppressing the insufficient basal defenses, plants evolve R-proteins which directly or indirectly interact in a specific manner with microbial effector proteins and thereby trigger plant immune responses. This is referred to as ETI and is synonymous to pathogen race-plant cultivar-specific host resistance or gene-for-gene resistance (Jones et al. 2004, 2006) (Figure 1). The recognized effector is termed an avirulence (Avr) protein. Pathogens evolve further and suppress ETI, which again results in new R-gene specificities so that ETI can be triggered again (Jones et al. 2004, 2006).
To date numerous R-genes have been cloned which confer resistance to several classes of pathogens, including viruses, bacteria, fungi, oomycetes, insects, and even nematodes. R-gene products can be categorized into two main classes based on conserved structural features (Dangl et al. 2001; Chisholm et al. 2006). The largest class of R-proteins possessess, in addition to a leucine-rich repeat (LRR) domain implicated in signal perception, a central nucleotide binding site (NBS) domain shared by plant disease R-proteins, mammalian NLR (NOD-like receptor or CATERPILLER) proteins, and animal apoptotic proteins, such as mammalian Apaf-1 and C. elegans CED-4 (Chisholm et al. 2006; DeYoung and Innes 2006; Jones and Dangl 2006; Ting et al. 2005). There is considerable evidence that plant and animal innate immune systems are conserved as a consequence of convergent evolution suggesting that common signaling events are the basis of defense cascades (Palma et al. 2007; Afza et al. 2008). In mammals, two families of soluble pathogen recognition proteins (PRRs) called NOD1 and NOD2 are intracellular sensors of pathogenicity that recognize molecules derived from pathogens as well as from the host itself (Inohara et al. 2002; Ryan et al. 2007). The NBS-LRR class of R-proteins is further subdivided into coiled-coil (CC) NB-LRR and Toll-interleukin-1 receptor (TIR) NB-LRR according to their amino-terminal domain (Burch-Smith et al. 2007).

LRR domains are located at the carboxy termini of plant NBS-LRR R-proteins and are composed of tandem LRRs, thought to be involved in effector binding and maintenance of regulatory functions (DeYoung et al. 2006). The NBS domain (also called the NB, NB-ARC, Nod or NACHT domain) contains blocks of sequence that are conserved in both plant and

animal proteins. Those include the nucleotide-binding kinase 1a or P-loop and kinase 2 motifs (also called Walker's A and B boxes) and the kinase 3a motif, as well as several blocks of conserved motifs of unknown function (RNBS-A, RNBS-C, GLPL, RNBS-D and MHD) (Traut et al.1994; Aravind et al. 1999). ATP binding coordionnating by the histidine residue of the MHD motif is necessary for signaling in plant NBS-LRR R-proteins because binding of ATP initiates a conformational change in plant NBS-LRR proteins, resulting in their activation. The amino-terminal domain also seems to mediate the physical association between R-proteins and pathogen effector targets, at least for those R-proteins that use an indirect recognition mechanism.

A second major class of R-genes encodes extracellular LRR (eLRR) proteins. Three subclasses of eLRRs have been classified according to their domain structures (Fritz Laylin et al. 2005). These subclasses include RLP (receptor-like proteins; extracellular LRR and TM domain, RLK (extracellular LRR, TM domain, and cytoplasmic kinase) and PGIP (polygalacturonaseinhibiting protein; cell wall LRR). RLPs, for example, are represented by the tomato *Cf* genes, which confer resistance to infection by the biotrophic leaf-mold pathogen *C. fulvum* carrying the elicitors Avr2, Avr4, and Avr9 (Jones et al. 1994). The nematode resistance gene $Hs1^{pro-1}$ from sugar beet (Cai et al. 1997) encodes for a protein that forms a LRR-TM structure as well.

Many R-genes are located in clusters that comprise several copies of homologous R-gene sequences arising from a single gene family (simple clusters) or colocalized R-gene sequences derived from two or more unrelated families (complex clusters). Intergenic unequal crossover has the potential to place R-genes in new structural contexts that may alter expression, whereas intragenic mispairing generates chimeric genes that may encode novel functions. In the absence of pathogen pressure, recombination and transposon activity at R-gene clusters are expected to be inhibited presumably by chromatin modification. This is also described by the Birth and Death Model (Michelmore and Meyers 1998). Although a very limited number of R-proteins are functionally characterized in detail there is now evidence that plants use both direct and indirect mechanisms of pathogen detection (DeYoung et al. 2006).

Although there is evidence that some plant NBS-LRR R-proteins have been under diversifying selection, the direct detection hypothesis for pathogen recognition fails to explain how a relatively limited number of plant R-proteins can specifically recognize the vast diversity of potential pathogens and their effectors. Not only this apparent disparity but also the lack of substantial evidence for direct Avr-R-protein interaction led to the 'guard hypothesis' (Van der Biezen and Jones 1998), which proposes that the Avr-protein induces a

change in a host protein that is normally recruited by the pathogen via its Avr-protein to establish a successful infection, and that this change sensed by the R-protein (guard) leads to the activation of the R-protein and subsequent defense signaling (Dangl and Jones et al. 2001; Bent and Mackey 2007; van der Hoorn 2008). For instance, in rice, Xa-21 protein requires XB3 a ubiquitin ligase that is phosphorylated by Xa-21 for complete *Xa-21*-mediated disease resistance (Wang et al. 2006). In Arabidopsis, the host protein RIN4 is structurally modified by Avr elicitors AvrRpm1 or AvrB from the bacterial pathogen *P. syringae* which in turn leads to the activation of *RPM1*-mediated resistance (Axtell and Staskawicz 2003; Mackey et al. 2002; Kim et al. 2005b; Coaker et al. 2005; Day et al. 2005). This model may provide a good explanation for resistance response networks triggered by other R-genes which for example has been proven for the $Hs1^{pro-1}$ -mediated nematode resistance. Additional support for the guard hypothesis comes from the tomato protein *Prf* involved in the indirect detection of *P. syringae* effectors AvrPto and AvrPtoB (Tang et al. 1999; Xiao et al. 2003).

As it is generally known, a large number of sequences with similarity to R-genes exist in plant genomes, which are referred to as RGAs (resistance gene analogs, Leister et al. 1996). The common motifs within the NBS domain are sufficient for PCR amplification of resistance gene analogs (RGAs) from a wide variety of plant species using degenerated primers, for example from soybean (Kanazin et al. 1996), potato (Leister et al. 1996), lettuce (Meyers et al. 1999), cereals (Pan et al. 2000), sugar beet (Tian et al. 2004), rape (Tanhuanpaa 2004) and cotton (He et al. 2004). The cloned RGAs have been found to cluster in plant genomes and some are located in close genetic distance to known resistance loci thus suggesting their possible role in disease resistance response in plants (Kanazin et al. 1996; Collins et al. 1998; Aarts et al. 1998; Ashfield et al. 2003; Radwan et al. 2005). RGAs not only provide a source for new R-gene species, but also represent candidates for putative interacting partners for functional R-genes according to the guard hypothesis described above. Van Hoorn (2008) describes an improved system of plant-pathogen interactions, the Decoy Model. In the absence of a functional R-gene, natural selection is expected to drive the guardee to decrease its binding affinity to the effector. However, in the presence of a functional R-gene, natural selection is expected to favor guardees with improved interaction with an effector to enhance pathogen perception. These two conflicting selection pressures result in an evolutionarily unstable situation that could be relaxed upon the evolution of a host protein, termed here "decoy," that specializes in perception of the effector by the R-protein but itself has no function either in the development of disease or resistance.

1.4 Non-host resistance

Potentially phytopathogenic microorganisms incapable of infecting any cultivar of a given plant species are referred to as heterologous pathogens, while plants that are resistant to all isolates of a given pathogen species are called non-host plants (heterologous plant–microbe interaction; basic incompatibility) (Nürnberger et al. 2005). Preformed physical or chemical barriers (passive defense mechanisms) at the plant surface, such as wax layers, cell walls, antimicrobial compounds and other secondary metabolites are the first obstacle a pathogen faces before invading the plant. The second obstacle is the inducible plant defense response (active defense mechanisms), such as de novo synthesis of phytoalexins, antimicrobial reactive oxygen species or several signaling components as well as localized reinforcement of the plant cell wall and programmed cell death (Thordal-Christensen et al. 2003; Nürnberger et al. 2005).

Mysore (2004) proposes that non-host resistance against bacteria, fungi and oomycetes can be classified into two types. During type I non-host resistance no visible symptoms occur and multiplication and penetration of the pathogen into the plant cell is completely abolished. In contrast, within the type II non-host resistance, that is always associated with a HR and is phenotypically more similar to an incompatible gene-for-gene interaction, an elicitor is recognized by the plant and a defense reaction is activated.

Non-host resistance in Arabidopsis against the non-adapted barley pathogen, *B. graminis* f. sp. *hordei* (Bgh) normally involves the rapid production of cell wall appositions (physical barriers) and antimicrobial metabolites at the site of pathogen entry, but no HR. Arabidopsis penetration mutants PEN1 (syntaxin) (Collins et al. 2003), PEN2 (peroxisomal glucosyl hydrolase) (Lipka et al. 2005) and PEN3 (plasma membrane ABC transporter) (Stein et al. 2006) are partially compromised in this response suggesting that cell wall structures play an important role as physical barriers. Syntaxins belong to the superfamily of SNARE (soluble N-ethylmaleimide- sensitive fusion protein attachment protein receptor) proteins representing key mediators of membrane fusion events in yeast and animal cells (Nürnberger et al. 2005). Even though significant similarities exist between non-host and gene-for-gene resistance such as HR, production of reactive oxygen species (ROS), lignification and ubiquitin ligase-associated protein SGT1, there are also differences between the two. Recent studies suggest that non-host cell death requires caspase-like activity (Christopher-Kozjan and Heath 2003). In these experiments, two caspase inhibitors significantly impaired cell death kinetics exclusively in several non-host combinations, but not in incompatible host interactions. Resistance conferred by single dominant R-genes is specific to a particular pathogen race that

can express the corresponding Avr-gene(s). Pathogen Avr-genes can be easily mutated or eliminated and hence protection conferred by R-genes is not durable. By contrast, non-host resistance can be more durable (Mysore et al. 2004).

2 Plant resistance responses

2.1 Early recognition events

When a plant and a pathogen come into contact, close interaction occur, whereby the plant is able to recognize the invading pathogen and to initiate defenses, while successful pathogens cause disease by suppressing host defense (Hammond-Kosack et al. 1997). In the simplest interaction plants contain dominant R-genes that specifically recognize the corresponding Avr-gene within the pathogen in a direct or indirect manner. Specific recognition results in the induction of signaling cascades and defense gene expression. The activation of plant defense leads to immediate responses at the point of infection that include protein phosphorylation, ion fluxes across the plasma membrane, ROS production, nitric oxide (NO), in some cases a HR and accumulation of phenolic compounds (Garcia Brugger et al. 2006; McDowell et al. 2003). The activation of MAPK families but also other PKs like CDPKs (calmodulin (CaM)-like domain protein kinases) is one of the earliest induced events after elicitor perception (Garcia Brugger et al. 2006), but also serves to mediate interaction between pathways (Rojo et al. 2003).
Next to local tissue responses, there are also systemic responses as the synthesis of pathogenesis-related (PR) proteins, accumulation of phytohormones, and cell wall strengthening, that prime uninfected parts of the plant against potential pathogen attack (systemic acquired resistance, SAR) in a long lasting and effective manner.

2.2 Signaling components

Both PTI and ETI are controlled by a complex signaling network that includes three major endogenous signals, the hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (De Vos et al. 2005; Bodenhausen et al. 2007). The ET and JA-dependent defense responses seem to be activated by necrotrophic pathogens such as *Alternaria*, *Botrytis*, *Septoria*, *Phytium*, *Erwinia*, *Plectosphaerella*, whereas the SA-dependent response is triggered by viruses like tobacco mosaic virus (TMV) and biotrophic bacteria and fungi such as *Pseudomonas*, *Peronospora*, *Erisyphe* and nematodes (Thomma et al. 1998, 2002; Rojo et al. 2003) (Figure 2). Most of the studies indicate that ET or JA and SA responses inhibit each other suggesting that events of cross-talk among the pathways exist (Spoel et al. 2003;

Pieterse et al. 2001). But also cases of synergistic interactions between SA and JA or ET in defense responses to pathogens have been reported (Rojo et al. 2003).

Elicitor perception is often followed rapidly by a Ca^{2+} influx and intracellular Ca^{2+} signaling as well as anion effluxes that initiate plasma membrane depolarization which, in turn, activates voltage-dependent Ca^{2+} channels (Romeis et al. 2001; Sanders et al. 2002). Modifications of plasma membrane potential allow signal integration triggering events as oxidative burst and MAPK activation (Ward et al. 1995; Garcia Brugger et al. 2006). ROS, highly reactive and toxic oxygen species, such as superoxide anion (O_2 •–), hydroperoxyl radical (HO_2 •), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•) are produced by plant cells because of the enhanced enzymatic activities of plasma-membrane-bound NADPH oxidases, cell-wallbound peroxidases and oxidases in the apoplast (Laloi et al. 2004). In a wide range of incompatible plant–pathogen interactions a biphasic ROS production has been observed, with a first phase peaking after 20 min and a second phase occurring 4 to 6 h later (Lamb and Dixon 1997; Laloi et al. 2004). ROS are thought to be general cell death effectors; they play an important role in modification of the cellular redox state, activation of MAPK as well as in reinforcing plant cell walls via oxidative cross-linking and increasing lignifications (Kawasaki et al. 2006; Laloi et al. 2004). In plants, the redox state regulates NPR1 (NON-EXPRESSOR OF PR1), an essential regulator of SAR (Figure 2). NPR1 accumulates in the cytosol as an inactive oligomer maintained by disulfide bridges. During a SAR response, its reduction releases monomeric units that accumulates in the nucleus and interact with the reduced TGA1 (TGACG-sequence-specific binding-protein1) transcription factor which activates the SA-dependent defense gene expression (Mou et al. 2003; Laloi et al. 2004). In a simplified model, two different R-gene-mediated signaling pathways have been described in *Arabidopsis thaliana* (Hammond-Kosack et al. 2003). The first one involves the TIR-NBS-LRR type of R-genes (e.g. *RPP1* and *RPP5*) and requires *EDS1* (Enhanced Disease Susceptibility) and *PAD4* (Phytoalexin Deficient) function to attain full resistance. The second one involves the CC-NBS- LRR type of R-genes (e.g. *RPM1* and *RPS2*) and requires functional *NDR1* (Non-race specific Disease Resistance), *RAR1* and *SGT1* (Figure 2).

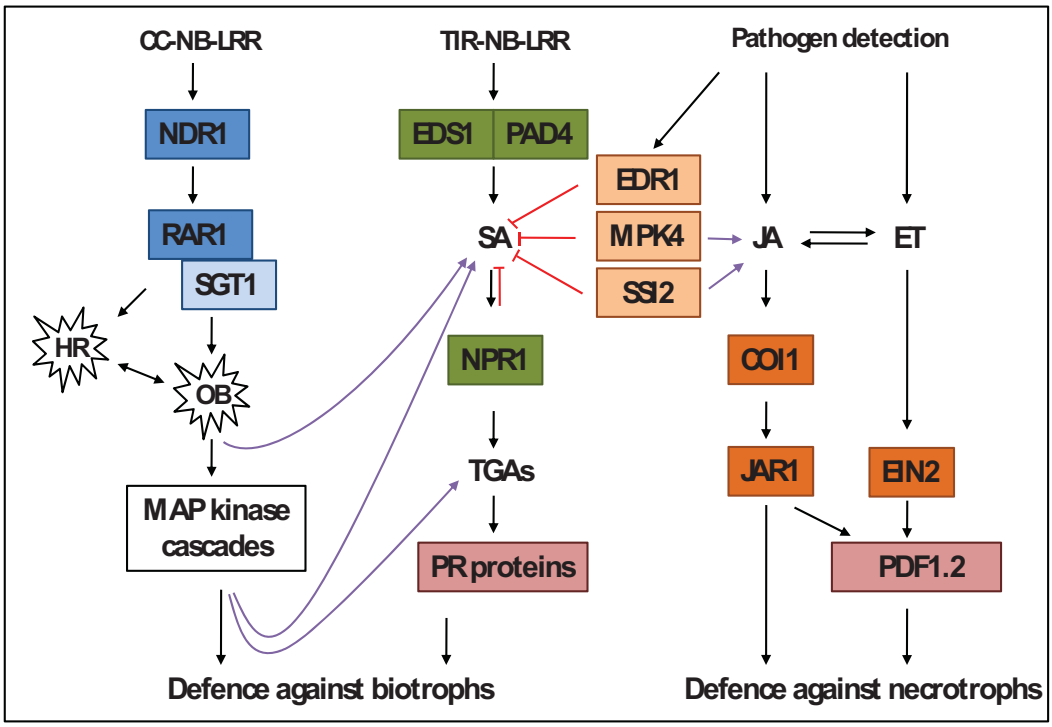

Figure 2: Simplified model of the signaling networks controlling defense responses according to Hammond-Kosack et al. 2003. Three main signaling cascades for resistance against biotrophic and necrotrophic pathogens are shown. COI1, coronatine insensitive 1; EDR1, enhanced disease resistance 1; EIN2, ethylene-insensitive 2; NDR1, non-race specific disease resistance 1; PAD4, phytoalexin-deficient 4; PDF1.2, plant defensin 1.2; RAR1, required for Mla-dependent resistance 1; SGT1, suppressor of G2 allele of SKP1; EDS1, enhanced disease susceptibility 1; NPR1, non-expressor of PR1; SSI2, fatty acid biosynthesis 2; JAR1, jasmonate resistance 1; SA, salicylic acid; JA, jasmonic acid; ET, ethylene; HR, hypersensitive response; OB, oxidative burst; MAPK, mitogen-activated protein kinase.

2.3 Defense related proteins

In the end, microbe- or elicitor-induced signal transduction pathways lead to the production of antimicrobial metabolites (phytoalexins), PR-proteins such as glucanases, chitinases, defensins and enzymes of oxidative stress protection. PR-proteins have been defined as a set of extracellular proteins encoded by the host plant, but induced by various types of pathogens and were originally divided into 5 groups (Pieterse et al. 2001). PR-1 has been extensively used as a marker for SA-mediated SAR defense, PR-5 proteins are thaumatin like proteins, the pathogen-inducible *PR-3* gene encodes a basic chitinase, and *PR-4* a hevein-like protein (Pieterse et al. 1998, 1999). PDF1.2 and PDF2.3 are members of the group of plant defensins with antimicrobial activities but their expression level is not influenced by SA. The SA-dependent defense signaling pathway regulates the expression of acidic PR-proteins such as PR-1, PR-2, and PR-5 whereas the ET/JA-dependent signaling pathway regulates the expression of basic PR-proteins such as PR-3, PR-4, and PDF1.2 (Penninckx et al. 1998; Thomma et al. 1998; Pieterse and van Loon 1999). Recent evidence indicates that in Arabidopsis SA-dependent expression of PR-1, PR-2 and PR-5 is required for increased

protection against biotrophic pathogens, while SA-independent but JA dependent induction of the plant defensin gene PDF1.2 is associated with the induced resistance against necrotrophic pathogens (Hammond-Kosack et al. 2003; Figure 2). PR and PDF proteins could be used commonly as marker genes for SAR/ISR (Induced systemic resistance) and provide therefore a promising tool for characterizing signal transduction pathways in plant resistance.

3 Nematode resistance in plants

3.1 Agricultural importance of plant pathogenic nematodes

Root-knot nematodes of *Meloidogyne* spp. and cyst nematodes of the genera *Heterodera* and *Globodera* are both obligate sedentary endoparasites and devastating pathogens of major crops worldwide. The estimated worldwide losses due to plant parasitic nematodes are about $125 billion US dollars world wide annually (Chitwood 2003). Agronomically important species of cyst nematodes, mainly active in temperate regions of the world, are *G.rostochiensis* and *G.pallida* on potato and *H.glycines* on soybean. In addition, more than 80% of the Chenopodiaceae and Brassicaceae species are hosts of *H. schachtii* (Steele 1965) including economically important crops like sugar beet (*Beta vulgaris*), spinach (*Spinacea oleracea*) radish (*Raphanus sativus*) and rape seed (*Brassica napus*). Today *H. schachtii* is spread over 40 other sugar beet-growing countries throughout the world (McCarter et al. 2008).

Root-knot and cyst nematodes completely penetrate main and lateral roots in the elongation or root hair zones of a susceptible plant as motile infective second-stage juveniles (J2) which hatch in the soil from eggs contained within a protective cyst (cyst nematodes) or egg sac (root-knot nematodes). They penetrate the plant cell walls using their robust stylet. However, before the stylet penetrates, cell walls are degraded by a number of enzymes released from the nematodes subventral glands. These include β -1, 4-endoglucanases (cellulases) (Gao et al. 2001), a pectate lyase (Doyle and Lambert 2002) and an expansin (Qin et al. 2004). J2s migrate within the root cortex towards the vascular cylinder and induce remarkable changes in a number of host cells to establish highly metabolically active feeding cells sustaining the nematode throughout its life cycle (syncytium for cyst nematodes and giant cell for root-knot nematodes) (Davis et al. 2004, 2008; Fuller et al. 2008). After three additional molts, adult males emerge from the root and are attracted to the females where fertilization occurs. At maturity, the females of cyst nematodes die, and their body is transformed into a light brown cyst where eggs and juveniles survive and remain dormant until root exudates stimulate

juveniles to hatch and emerge from the cyst. By contrast, eggs of Meloidogyne are released on the root surface in a protective gelatinous matrix.
Chemical control of the beet cyst nematode is more or less restricted; most of the nematicides are withdrawn from the market due to high costs and environmental risks. Another way to control nematodes is the use of rotations with non host plants including wheat, barley, corn, beans, and alfalfa as well as nematode-resistant radish and mustard. These so called trap crops can be used to reduce the soil population of *H. schachtii*, but usually a rotation of three to five years is required, which is often not economically practical. In this context, resistant sugar beet cultivars are the most promising alternative to control *H. schachtii*. So far, no agronomically desirable nematode resistant cultivar is available.

3.2 Nematode resistance genes

In the last years, several nematode R-genes have been cloned from plants, the most of them from several species of crop wild relatives. The first nematode R-gene to be cloned was *Hs1*$^{pro\text{-}1}$ from sugar beet, which confers resistance against the sugar beet cyst nematode *H. schachtii* (Cai et al. 1997). The encoded protein has no homology to known R-proteins and in addition it has an unusual structure. However, other cloned nematode R-genes closely resemble known plant R-genes in their domain structure. Four of these genes, *Mi-1*, *Hero, Gpa2* and *Gro1-4*, all cloned from tomato or potato relatives, fall into the NBS-LRR class of R-genes (Williamson et al. 1996, 2006) (Figure 3).

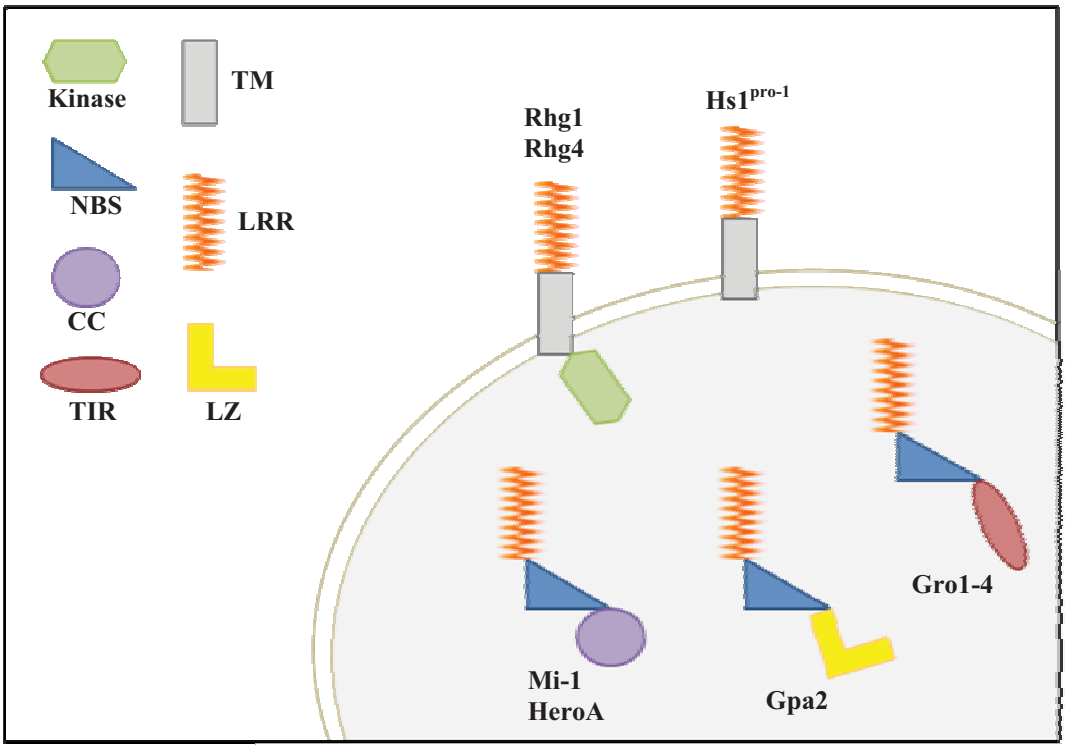

Figure 3: Structure of plant nematode resistance gene products. Cloned nematode resistance genes encode for proteins with conserved motifs; a leucin zipper (LZ), a nucleotide binding side (NBS), a leucine rich repeat region (LRR), a protein kinase (kinase), a transmembrane domain (TM), coiled coil (CC), a toll- interleukin receptor-like domain (TIR). Modified according to Willliamson et al. 2006.

The tomato genes *Mi-1* and *Hero,* originated from the wild relatives *Lycopersicon pimpinellifolium* and *L.peruvianum,* confer broad-spectrum resistance to several root-knot nematode species (Milligan et al. 1998; Vos et al. 1998) and to several pathotypes of the potato cyst nematodes *G. rostochiensis* and *G. pallida* (Ernst et al. 2002), respectively. The *Mi* resistance was first transferred into commercial tomato cultivars in the 1950s (Gilbert et al. 1956). *Mi* also confers resistance to two totally unrelated parasites, the potato aphid *Macrosiphum euphorbiae* and the white fly, *Bemisia tabaci* (Rossi et al. 1998; Nombela et al. 2003), whereas the potato genes *Gpa2* and *Gro1-4* mediate resistance to a narrow range of pathotypes of the potato cyst nematode *G.pallida* (van der Vossen et al. 2000; Paal et al. 2004). *Mi*, *Gpa2* and *Hero* all belong to the cytoplasmically located NBS-LRR class of R-genes that does not contain an N-terminal TIR domain, whereas *Gro1-4* encodes a member of the TIR-NBS-LRR subclass of R-proteins (Williamson et al. 2006) (Figure 3). *Gpa2* is highly similar in predicted amino acid sequence to the *Rx* gene, which confers resistance to potato virus X (Bendahmane et al. 1999, 2002); both genes are in the same gene cluster on chromosome 12 in potato with a high degree of homology and seem to have a common

ancestor, although they confer resistance to unrelated pathogens (Rouppe van der Voort et al. 1999; van der Vossen et al. 2000). In contrast, *Gro1* and *Hero* share little sequence similarity although both genes trigger resistance against pathotype Ro1 of *G.rostochiensis* (Grube et al. 2000). Recently a putative root-knot nematode R-gene (*CaMi*) was isolated from resistant pepper *Capsicum annuum* by a degenerated primer based PCR approach (Chen et al. 2007). *Rhg1* and *Rhg4* cloned from soybean and conferring resistance to *H.glycines* both encode proteins with extracellular LRRs, a TM and a cytosolic serine–threonine kinase domain (Meksem et al. 2001; Ruben et al. 2006) (Figure 3). So far, little is known about the action mode of the cloned nematode R-genes. It is generally believed that these genes recognize nematode effectors triggering specific signaling pathways that lead to resistance response (Williamson et al. 2006). More agronomically important nematode R-genes are likely to be cloned in the near future, including the *H1* gene that confers resistance to *G.rostochiensis* in potato (Bakker et al. 2004) and the *Me* gene of pepper for resistance to *Meloidogyne* species (Djian-Caporalino et al. 2007). The wheat nematode R-genes *Cre1* and *Cre3* co-localize with clusters of NBS-LRR genes, but functional confirmation has not been completed (Ayliffe et al. 2004; Fuller et al. 2008).

The complete resistance against *H. schachtii* mediated by $Hs1^{pro-1}$ was found in *B. Procumbens,* a species of the section *Procumbentes* and was transferred into sugar beet by conventional breeding methods resulting among others in an euploid (2n=18) line carrying a translocation from the wild beet chromosome 1 encompassing $Hs1^{pro-1}$ (Cai et al. 2003). Finally, a YAC based cloning approach led to the isolation of the $Hs1^{pro-1}$ gene (Cai et al. 1997). The predicted mature protein of $Hs1^{pro-1}$ of 282 amino acids forms a LRR-TM structure suggesting that $Hs1^{pro-1}$ is located at the plasma membrane and functions as a receptor recognising putative elicitors released from nematodes (Cai et al. 2003) (Figure 3). Hs1 promoter analysis showed that the transcript of $Hs1^{pro-1}$ was specifically induced after nematode infection (Thurau et al. 2003).

Recently, an extended version of the $Hs1^{pro-1}$ (DQ148271) protein which includes an additional 176 amino acid N-terminal extension has been reported by McLean et al. 2007, which confers resistance to *H. glycines* the soybean cyst nematode additionally confirming the role of $Hs1^{pro-1}$ in nematode resistance. In Arabidopsis, which is also a host for *H. schachtii*, a series of sequence homologues of $Hs1^{pro-1}$ have been detected, but none of these confers resistance and only the wild beet $Hs1^{pro-1}$ variant was able to efficiently protect Arabidopsis upon transformation (Zhang et al. 2008; Cai et al. 1997, 2003). However, as no complete resistance could be observed by transgenic sugar beet plants so far, it is proposed

that a second gene may be required for the resistance expression (Schutle et al. 2006). A putative cation transporter gene linked to *Hs1*$^{pro-1}$ (Oberschmidt et al. 2003) as well as a gene encoding a protein with homology to phosphatidylinositol-specific phospholipase X-box domain from the food-borne human pathogen *Listeria monocytogenes* were isolated from nematode-resistant sugar beet by cDNA-AFLP fingerprinting (Samuelian et al. 2004). Both genes were suggested to be used for inducing cyst nematode resistance in plants.
However, focussing the search on NBS-LRR type of RGAs cZR-1, cZR-3, cZR-7 and cZR-9 were identified from a nematode resistant line. However, the molecular mechanisms underlying the *Hs1*$^{pro-1}$ mediated nematode resistance remains largely unknown.

3.3 Nematode resistance response

Resistance to nematodes in plants is generally characterized by a delayed response occurring several days after initiation of the feeding systems resulting in disintegration of established feeding structures and stagnation of nematodes (Williamson et al.1996, 2006; Cai et al. 1997). Another principle is the failure of nematodes to produce functional feeding sites based on a HR of the root tissue, which leads to the death of the nematode (Grundler et al. 1997). For the resistance mediated by *Hero*, the response seems to be initiated after a normal feeding site induction and leads to necrotic cells surrounding the syncytium resulting to its degradation (Sobczak et al. 2005). Resistance to root-knot nematodes of the genus *Meloidogyne* is characterized by a more rapid localized cell death and the generation of ROS resulting in a disruption of feeding site establishment and death of the nematode (Paulson and Webster 1972; Melillo et al. 2006).
It is hypothesized that *Mi* is capable of recognizing two or more elicitors or that *Mi* recognizes a plant product modified by different pest effectors (Williamson et al. 2006). A recessive mutation in tomato, termed *rme1* for ‘resistance to *Meloidogyne* species’ that is unlinked to *Mi*, completely and specifically abolishes *Mi*-mediated resistance (Martinez de Ilarduya et al. 2003, 2004). According to the guard model, the authors suggested that the gene product of *Rme1* acting upstream of *Mi* may be the target for the different nematode, aphid and whitefly Avr effectors, and *Mi* detects the changes in *Rme1* resulting from these interactions (Martinez de Ilarduya et al. 2004; Fuller et al. 2008). A third type of feeding cell disruption could be observed in sugar beet (*B. vulgaris*) conferring resistance to the beet cyst nematode *H. schachtii*. Syncytia do not develop regularly suffering due to formation of specific membrane aggregations filling large parts of the syncytium, consequently causing the degradation of syncytia and the death of nematode juveniles (Holtmann et al. 2000; Cai et al. 2003).

4 Genetic engineering

4.1 Natural resistance mechanisms

Although scientists gained many substantial insights into innate resistance mechanisms in plants several gaps still exist in the developed models of the defense signal transduction networks and the knowledge of the plants own defense machinery is relatively limited. However, useful applications to reduce the ongoing pathogen pressure have already been developed and others will follow in the near future.

In many cases, a single R-gene can provide complete resistance to one or more strains of a pathogen and therefore represents an attractive tool for disease control. R-genes are used in conventional resistance breeding programs as well as in transgenic approaches to achieve an efficient reduction of pathogen growth without collateral damage to the plant or adverse environmental effects. The *Bs2* gene confers durable resistance to bacterial spot disease caused by the bacterium *Xanthomonas campestris* in pepper and works effectively in tomato after transformation (Tai et al. 1999). Also the tomato *Ve1* and *Ve2* genes can provide resistance to different *Verticillium* species and are functional in potato when expressed as transgenes (Gurr et al. 2005). So far intraspecific transfer of nematode resistance genes by transgenic techniques has been successful, but there has been limited success in transferring these genes to new species. *Mi*-mediated root-knot nematode resistance transferred from tomato to tobacco is not functional anymore (Williamson et al. 2006) and transfer of the tomato *Hero* gene into potato did not result in resistance to potato cyst nematodes (Sobczak et al. 2005). More promising results were achieved when *Mi* was transferred in aubergine (Goggin et al. 2006). There are, however, some potential problems with this approach. Resistances, which rely on gene-for-gene relationships, can easily be overcome by co-evolving pathogens, and therefore do not provide broad-spectrum resistance or durability (McDowell et al. 2003). This is the case for nematode resistance genes used in a transgenic approach as well, however durability of R-genes to sedentary plant nematodes has been generally high (Williamson et al. 2006).

Oneway to overcome this problem is the use of multiple R-genes (pyramiding) (Jones et al. 2001); another way to achieve broad-spectrum resistance is the manipulation of different components of pathogen induced signaling pathways so-called master switch genes, such as kinases and transcription factors that activate the entire arsenal of defense responses. The Arabidopsis *NPR1* gene regulates defense gene transcription in SA-mediated resistance and its overexpression in Arabidopsis and rice leads to enhanced resistance to diverse pathogens

(Cao et al. 1998; Chern et al. 2001). PR-genes that might increase the level of pre-formed barriers against pathogen invasion and genes that are involved in the biosynthesis of hormones such as SA, JA and ET are also promising candidates for engineering increased disease resistance (Gurr et al. 2005). However, the manipulation of such master switch genes is not free from collateral effects, thus plants overexpressing a defense pathway component show reduced yield or plant vigour and increased susceptibility to other pathogens because of existing antagonism between the different defense pathways (Stuiver et al. 2001; McDowell et al. 2003).

The last resort providing useful tools for genetic engineering of pathogen resistance is the huge group of effector proteins. The overexpression of antipathogenic proteins might be the oldest but also the most wideley used strategy to engineer pathogen resistance in plants. Compared to the pathway-modulating approach described above this strategy is much more specific, the putative negative impact yield or the interference with other defense pathways is absent (Gurr et al. 2005). Most fungal and bacteria pathogens produce a diverse range of compounds playing a crucial role as key factors in the infection process and are often designated as pathogenicity factors.Thus strategies of interfering with pathogenesis by neutralizing those compounds can be designed. For example, overexpression of oxalate oxidase or oxalate decarboxylase leads to a significant high level of resistance against *S. sclerotiorum* by breaking down oxalic acid that serves as an important pathogenicity factor of several fungal pathogens (*S. sclerotiorum, S. rolfsii, R. solani*). Expression of a plant or bacterial chitinase, which is capable of degrading one major cell wall component (chitin) of most filamentous fungi, in transgenic tobacco was shown to enhance the resistance of plants to *R. solani* (Melchers et al. 2000). Researchers at Zeneca MOGEN were the first to demonstrate that the constitutive co-expression of tobacco chitinase and β-1,3-glucanase genes in tomato plants confers higher levels of resistance to fungal pathogens (i.e. *Fusarium oxysporum*) than either gene alone.

It remains a challenge to find out whether chitinases/glucanases or oxalate oxidases could have an impact on resistance to other phytopathogens such as nematodes.

4.2 Toxins and RNAi

Particularly for engeneering nematode resistance in plants, tools relying on anti-feeding strategies are a promising approach. The effective use of anti-nematode genes that cause disrupture of feeding cells to generate resistance relies on their targeted expression or non-

phytotoxicity to the host (Cai et al. 2003). Important classes of proteins with putative anti-nematode activity are lectins and Bt crystal proteins (Fuller et al. 2008).
Alternatively, plant proteinase inhibitors (PIs), toxic to the nematode but non-phytotoxic, harbor a great potential as anti-nematode effectors (Koritsas and Atkinson 1994; Lilley et al. 1996, 1997; Urwin et al. 1997, 1998). PIs expressed in plants reduce the rate of insect development for example, cowpea trypsin inhibitor (CpTI) on lepidopteran insect *Heliothis virens* in tobacco plants (Hilder et al. 1987), potato serine inhibitor PI (PIN2) on *Spodoptera exiguia* in tobacco (Johnson et al. 1989) and as a transgene in wheat on the cereal cyst nematode *Heterodera avenae* (Vishnudasan et al. 2005) as well as the sweet potato serine PI, sporamin on *H. schachtii* in transgenic sugar beet hairy roots (Cai et al. 2003). In the first demonstration of a transgenic technology working against root-knot and cyst nematodes, the modified rice cystatin (Oc-I) was expressed in transgenic Arabidopsis plants and uptake of the cystatin was correlated with loss of nematode cysteine proteinase activity (Urwin et al. 1997, 2003).
Many genes encoding products that are secreted from plant parasitic nematodes via the stylet into the cytoplasm of the plant cell have been identified, providing a rich source of candidate avirulence and pathogenicity genes. Stylet secretions are generally necessary for parasitism (Hussey 1989; Davis et al. 2000, 2004). The first demonstration of RNA interference (RNAi) in plant-parasitic nematodes was done by Urwin et al. 2002 and Atkinson et al. 2003 who developed a procedure that results in the uptake of double-stranded RNA (dsRNA) molecules by J2s of cyst nematodes. Developments in the application of RNAi for gene silencing in plant-parasitic nematodes have recently culminated in the demonstration that plants expressing dsRNA targeting a nematode gene display resistance to infection (Gheysen & Vanholme 2007; Lilley et al. 2007; Fuller et al. 2008). Huang et al. (2006) determined a gene 16D10 encoding a 13-amino-acid peptide that is secreted from the gland cells of *M. incognita* being responsible for the regulation of feeding cell phenotype and possibly interacts with a SCARECROW-like plant transcription factor (Huang et al. 2006). The 16D10-RNAi plants demonstrated similar strong resistance to the different *Meloidogyne* species *M. incognita*, *M. javanica*, *M. arenaria* and *M. hapla*, suggesting a fundamental and essential role of the 16D10 peptide in plant parasitism by root-knot nematodes.

5 Outline of the thesis

The $Hs1^{pro-1}$ gene conferring resistance to the beet cyst nematode *H. schachtii* was cloned from nematode resistant sugar beet (Cai et al. 1997). The $Hs1^{pro-1}$ gene product shows no

homology to known R-proteins and has an unusual structure, probably representing a new resistance mechanism that remains largely unknown. Therefore, an overall goal of this dissertation is to elucidate molecular mechanisms governing the *Hs1*$^{pro-1}$ mediated nematode resistance. In total, this dissertation consists of 6 chapters.

Chapter I: The general introduction presents the current knowledge about plant resistance response to pathogens and provides an insight into nematode resistance as well;

Chapter II: Presentation that *BvGLP-1* encoding for a germin-like protein regulates the *Hs1*$^{pro-1}$ mediated nematode (*Heterodera schachtii* Schm.) resistance by its oxalate oxidase activity in sugar beet (*Beta vulgaris* L.) and *Arabidopsis thaliana;*

Chapter III: Presentation that overexpression of *BvGLP-1* encoding a germin-like protein from sugar beet in Arabidopsis leads to resistance against phytopathogenic fungi (*Rhizoctonia solani* and *Verticillium longisporum*), but does not affect the beneficial interaction with the growth-promoting endophyte *Piriformospora indica;*

Chapter IV: Demonstration that two NBS-LRR carrying resistance gene analogs (RGAs) are involved in the *Hs1*$^{pro-1}$-mediated nematode (*Heterodera schachtii* Schm.) resistance in sugar beet (*Beta vulgaris* L.);

Chapter V: Demonstration of a two-step protocol for improving shoot regeneration frequency from hypocotyl explants of oilseed rape (*Brassica napus* L.) and its application for Agrobacterium-mediated transformation and

Chapter VI: The general discussion includes a summary of main findings in this thesis and concluding remarks.

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Chapter II

The gene *BvGLP-1* encoding for a germin-like protein regulates the *Hs1*$^{pro-1}$-mediated nematode (*Heterodera schachtii* Schm.) resistance by its oxalate oxidase activity in sugar beet (*Beta vulgaris* L.) and *Arabidopsis thaliana*

(In submission)

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Key words: *Hs1*$^{pro-1}$, nematode resistance, Oxalate oxidase-like germin, ROS, signal transduction, transcript profiling

1 Abstract

Germins and germin-like proteins (GLPs) constitute a large and highly diverse family of plant proteins and are involved in many developmental stages and stress-related processes. The *Hs1*$^{pro-1}$ locus confers resistance to the beet cyst nematode *Heterodera schachtii* in sugar beet (*Beta vulgaris*). The gene *Hs1*$^{pro-1}$ had been cloned, but the resistance mechanism remains obscure. To understand the molecular mechanisms governing the *Hs1*$^{pro-1}$-mediated nematode resistance, transcript profiling was conducted on both of sugar beet with the cDNA-AFLP analysis and Arabidopsis with the ATH1 GeneChip hybridization experiments. This approach has identified the gene *BvGLP-1* from sugar beet. The gene encodes for an oxalate oxidase-like germin protein that is highly upregulated in the resistant, but not in the susceptible sugar beet in response to nematode infection. Also, a homolog gene (*GLP3*) of *BvGLP-1* was identified from the ATH1 GeneChip, which represents the highest upregulated gene in the *Hs1*$^{pro-1}$ transgenic Arabidopsis genome. These data strongly suggest an active role of *BvGLP-1* in the *Hs1*$^{pro-1}$ mediated resistance. For functional analysis, we transferred *BvGLP-1* into sugar beet roots and Arabidopsis plants and challenged the transgenic roots and plants with the beet cyst nematode. Our data demonstrate that expression of *BvGLP-1* in nematode feeding cells (syncytia) of both sugar beet roots and Arabidopsis plants was sufficient to initiate nematode resistance while knockout of the homolog gene of *BvGLP-1* in Arabidopsis significantly increased in plant susceptibility to nematode infection. Moreover, we demonstrate that *BvGLP-1* exhibits oxalate oxidase activity generating hydrogen peroxide (H_2O_2) in plant cells and regulates the expression of the pathogenesis-related proteins in which PR-1 to PR-4 and PDF1.2 were enhanced and PDF2.1 was downregulated. These data suggest that *BvGLP-1* plays a central role in regulating plant nematode resistance, representing a promising candidate gene for genetic engineering of plant nematode resistance. A possible signal transduction pathway is discussed.

2 Introduction

Plants have evolved multi-faceted defense mechanisms to counter pathogen attacks. In addition to static defenses the PAMP-triggered innate immunity (PTI) stymies many would-be pathogens while an effector-triggered immunity (ETI) relying on resistance (R) proteins confers a pathogen-specific resistance that is often associated with a form of programmed cell death around the infection site termed the hypersensitive response (HR). Recent genome research revealed that each plant genome encodes hundreds of R-proteins (Meyers et al. 2003; Monosi et al. 2004).

A set of resistance (R) genes has been functionally cloned from diverse plant species for resistance against various pathogens (DeYoung and Innes 2006). A major class of the cloned R-genes belongs to the NBS-LRR gene family which carries a variable N-terminal domain, followed by a putative nucleotide-binding site (NBS) and a C-terminal tandem array of LRR motifs. These features share high homology to proteins that function in animal innate immunity and apoptosis implicating a conserved mechanism of cell death programmes in plants and animals (Saraste et al. 1990; Li et al. 1997; van der Biezen and Jones 1998; Aravind et al. 1999).

Great efforts have been made worldwide towards understanding mechanisms of R-gene mediated plant resistance. The oxidative burst, a rapid and transient production of huge amounts of reactive oxygen species (ROS) is one of the earliest events in association with the plant HR within the attacked cells (Lamb et al. 1997; Apel et al. 2004; Melillo et al. 2006). Nitric oxide (NO_x), a redox-active molecule with a critical role in the activation of mammalian defense responses also functions as an important signal in plant resistance response (Thomma et al. 2001; Hammond-Kosack and Parker 2003). Responding to pathogen infection, salicylic acid (SA) accumulates in the plant tissue and proved to be a necessary signal molecule for the induction of systemic acquired resistance (SAR) which heightens defenses in noninoculated tissues against a broad spectrum of pathogens (Ryals et al. 1996). However, the balance and cooperation between ROS, NO_x and SA produced early in the plant resistance response seem to play an imperative role in the expression of the HR (Klessig et al. 2000; Delledonne et al. 2001; Chandok et al. 2003). Consequently, accumulation of defense related gene transcripts and production of antimicrobial compounds follow these initial events in the attacked cells. Also, cellular responses rapidly occur upon pathogen perception, which include, for instance, alteration of membrane potentials, calcium influx, increase in lipoxygenase activity, activation of protein kinase, alkalinisation of the extracellular space,

formation of cell wall appositions at the site of attempted penetration often followed by collapse of lignin formation (Mehdy 1994).

In the last years, several nematode R-genes have been cloned. They include *Hs1*$^{pro-1}$ from sugar beet against *Heterodera schachtii* (Cai et al. 1997), *Gpa2* and *Gro1* from potato against *Globodera rostochiensis* and *G. pallida* and (Van der Vossen et al. 2000; Paal et al. 2004), *Hero* and *Mi* from tomato against *Globodera* spp. and *Meloidogyne* spp., respectively (Milligan et al. 1998; Ernst et al. 2002). Recently, two major genes *Rhg4* and *Rhg1* conferring resistance to soybean cyst nematode (SCN) in soybean have been reported (Meksem et al. 2005; Ruben et al. 2006). So far, little is known about the action mode of the cloned nematode R-genes. It is generally believed that these genes recognize nematode effectors triggering specific signaling pathways that lead to resistance responses (Williamson et al. 2006). The most of these effector molecules are believed to be secreted via the nematode stylet directly into the cytoplasm of the plant cell. Stylet secretions are generally necessary for parasitism (Davis et al. 2004). The molecular nature of the nematode secretions remains largely unknown.

Germin and GLPs belong to a subgroup of plant cupin proteins. They are water-soluble, protease-resistant, heat stable and SDS-tolerant glycoproteins (Lane 1994; Woo et al. 2000), which often assemble into homohexameric complexes in vivo (Zhang et al. 1995; Vallelian et al. 1998; Christensen et al. 2004). Diverse reports have been made that germin and GLPs may be involved in plant defense responses (Thompson et al. 1995; Zhang et al. 1995; Hurkman et al. 1996; Zaghmout et al. 1997; Wei et al. 1998; Liang et al. 2001; Ramputh et al. 2002; Hu et al. 2003; Livingstone et al. 2005). Several members of the germin family contain enzymes with oxalate oxidase (OxO) activities which catalyze the degradation of oxalic acid (OA) to produce carbon dioxide and hydrogen peroxide (Chiriboga 1966; Lane et al. 1993). Because germin and GLPs accumulate in epidermal cells after pathogen attack (Wei et al. 1998), they are generally considered to have a structural role in stressed leaves by e.g. serving as cross-linking substrates for cell wall reinforcement. But, inoculation of wheat leaves with *Blumeria graminis* f.sp. *tritici* induced the germin gene expression (Schweizer et al. 1999), which is however accompanied by an increase of the germin oligomer and the OxO activity. Similarly, the OxO activity was observed to be corresponding to an accumulation of germin isoforms in powdery mildew infected barley leaves (Dumas et al. 1995). Thus, a role of germin and GLPs in plant defense response has been proposed mainly based on the capacity of the OxO to produce H_2O_2, a reactive oxygen species. Through the generation of H_2O_2, OxO or OxO-like proteins may catalyse cross-linking of plant cell wall proteins in papillae at the infection site

and lignification for the reinforcement of the cell wall (Olson and Varner 1993; Thordal-Christensen et al. 1997; Wei et al. 1998). On the other hand, they may act as a secondary messenger activating HR (Levine et al. 1994) which is known to be orchestrated by H_2O_2 production during oxidative burst (Lane 1994; Zhou et al. 1998). In addition, as a variety of fungal pathogens secrete OA as a pathogenicity factor into host cells (Livingstone et al. 2005); degradation of oxalate in plant cells was considered a major action mode of germin and GLPs in plant defense to fungal pathogens. Waetzig et al. (1999) proposed that both plasma membrane-bound and extracellular enzymes may involve the generation of H_2O_2 which is connected with the incompatible plant-pathogen interaction of *A. thaliana* against *Heterodera glycines*.

The *Hs1*$^{pro-1}$ locus was transferred from the wild beet, *Beta procumbens* into the sugar beet. The cloned *Hs1*$^{pro-1}$ gene has no homology to known R-proteins and an unusual structure probably representing a new resistance mechanism (Cai et al. 2003). The gene product was predicted to span the cytoplasm membrane and function as a receptor interacting with nematode effectors (Cai et al. 1997). Overexpression of the gene in sugar beet hairy roots and Arabidopsis plants resulted in significant reduction of developed female nematodes (Cai et al. 1997, 2003). Recently, McLean et al. reported (2007) that a 5′-extended *Hs1*$^{pro-1}$ sequence showed significantly enhanced resistance of soybean against SCN after its transfer into the soybean. As no complete resistance could be observed in transgenic sugar beet plants so far, it is proposed that a second gene may be required for the resistance expression (Schulte et al. 2006).

The cDNA-AFLP and Genechips based transcript profiling proved to be efficient tools for understanding plant-nematode interaction, especially for a compatible plant-nematode interaction. Affymetrix GeneChips have been used for studying the transcriptome of syncytia induced in soybean roots by *H. glycines* (Ithal et al. 2007b; Klink et al. 2007b), the transcriptome of feeding sites induced by *H. schachtii* and *H. glycines* in Arabidopsis roots (Puthoff et al. 2003) as well as of *Meloidogyne incognita* galls on Arabidopsis roots (Jammes et al. 2005). In addition, genes encoding secretory proteins were cloned from the esophageal gland cell with cDNA libraries leading to identification of several genes crucial for parasitism (Gao et al. 2001, 2003; Wang et al. 2001). Using the cDNA-AFLP and the differential cDNA-display techniques, several candidate genes for being involved in the *Hs1*$^{pro-1}$ mediated resistance were also identified (Oberschmidt 2000; Samuelian et al. 2004). The action mode of these genes is so far not clear.

Here, we report identification of the gene *BvGLP-1* that encodes for a germin-like oxalate oxidase from sugar beet. Also, a homolog gene (*GLP3*) of *BvGLP-1* was identified from the ATH1 GeneChip, which represents the highest upregulated gene in the $Hs1^{pro-1}$ transgenic Arabidopsis. We demonstrate that expression of the gene in nematode feeding cells of both beet roots and Arabidopsis plants showed significant anti-nematode effect whereas knockout of the homolog gene of *BvGLP-1* in Arabidopsis drastically increased plant susceptibility to nematode infection.

In addition, we demonstrate that *BvGLP-1* is functional as an oxalate oxidase and expression of the gene resulted in generation of H_2O_2 and regulates the expression of pathogenesis-related proteins in plant cells. These data suggest that *BvGLP-1* plays a central role in regulating plant nematode resistance, representing therefore a promising candidate gene for genetic engineering of plant nematode resistance. A possible signal transduction pathway is discussed.

3 Materials and Methods

3.1 Plant material

The susceptible sugar beet line 93161p and the nematode resistant line A906001 (B883) were kindly provided by the breeding company Strube-Dieckmann GmbH & Co. KG (Söllingen, Germany). Line A906001 carries a translocation from wild beet *Beta procumbens* housing the nematode resistance gene $Hs1^{pro-1}$ (Jung et al. 1992) while line 93161p used as a susceptible sugar beet control does not carry any wild beet chromatin.
The Arabidopsis ecotype C24 used in this study was purchased from Lehle Seeds, Round Rock, USA. It is highly susceptibel to *H. schachtii* (Sijmons et al. 1991).

3.2 cDNA-AFLP analysis

cDNA-AFLP was performed with cDNA of both susceptible and resistant sugar beet roots as described by Vos et al. (1995) and Bachem et al. (1996). The plants were grown in the greenhouse, from which half were infected with 2000 J2 larvae of the nematode *H. schachtii* each. Three, seven and 14 days after infection, plant roots were harvested for RNA preparation. Total RNA was extracted from the roots following the trizol protocol (Invitrogen, Karlsruhe, Germany). cDNA was synthesized using the Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturers` instruction. Synthesized cDNA (100–500 ng) was digested with 2 U *Eco*RI/*Mse*I restriction endonucleases in 1 x RL-buffer (1 x OPA+ buffer, 10 mg/ml BSA, 25 mM DTT).
Five pM adapter corresponding to the rare cutter (*Eco*RI, *Pst*I) and 50 pM adapter corresponding to the frequent cutter (*Mse*I) were ligated to the restricted fragments using 1 U T4 ligase (GE Healthcare, Chalfont St. Giles, UK). Ligated substrate (5–10 ng) was used as a template for preamplification. The reaction was performed in 23 cycles with 50 ng of the corresponding primers having no or one selective nucleotide with AmpliTaq DNA polymerase (Applied Biosystems, Darmstadt, Germany). Amplification with two selective nucleotides at the 3`-end of the corresponding primers was performed. For each reaction one primer was radioactively labelled by phosphorylating the 5` end with γ-(33P)-dATP by polynucleotide kinase. A standard touchdown program was performed for 31 cycles with the following profile: a 30 s DNA denaturation step at 94°C, a 30 s annealing step and a 60 s extension step at 72°C. The annealing temperature of the first cycle was 65°C, subsequently reduced each cycle with 0.7°C for the next 12 cycles, and was continued at 56°C for the remaining 18 cycles. The transcripts were visualized on a 6% (w/v) polyacrylamide gel at 60

W. The gels were dried on Whatman paper (Whatman, Kent, UK) at 70°C for one hour and exposed to Kodak Biomax film (Fermentas, St. Leon-Rot, Germany) for two to three days.

3.3 Cloning, sequencing and sequence analysis

Elution of the DNA from the polyacrylamide was performed according to Sambrook et al. (1998). The DNA was reamplified with the corresponding primers and retrieved from 1% (w/v) agarose gel with Sephaglas kit (GE Healthcare). Reamplified PCR products were ligated in pGEMTeasy vector (Promega, Madison, WI, USA).
Transformation of plasmids was performed in *E. coli* DH10B (Invitrogen) through electroporation. For this purpose 0.2 cm Gene Pulsar® Cuvettes with a Gene Pulser System II (Bio-Rad Laboratories GmbH, München, Germany) were used. Electroporation was performed at 2.5 kV/cm, 25 µF and 200 Ω. Bacteria cells were recovered with SOC (2% (w/v) Tryptone Pepton; 0,5% (w/v) Yeast extract; 10 mM $NaCl_2$; 2,5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM Glucose) at 37°C for one hour. Subsequently transformed bacteria colonies were grown on selective LB medium. Recombinant plasmids were isolated from bacterial clones with the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) following the manufacturers` instruction. Sequencing of positive inserts was performed on an ABI373 upgrade automated sequencer. DNA sequences were analysed with the Laser Gene software package (DNASTAR, Madison, WI, USA). DNA and protein sequences were compared with nucleotide and protein sequences databases at the National Centre for Biotechnology Information (NCBI).

3.4 Full-length cDNA isolation

The full-length cDNA clone for the *BvGLP-1* gene has been isolated from a yeast two hybrid cDNA library by PCR constructed from mRNA isolated from nematode infected roots of the sugar beet translocation line A906001. 500 seedlings have been infected with 300 J2 *H. schachtii* each and total RNA was isolated at 16 h, 48 h, one week, two weeks, three weeks and four weeks dpi from roots following the trizol protocol (Invitrogen), after which the mRNA fraction has been isolated using the Ologotex mRNA kit (Qiagen). The library has been constructed using the Clontech Matchmaker kit (Clontech, Mountain View, USA). The cDNA was cloned into the pGAD10 vector and transformed into *E. coli* DH5α resulting in approx. 25% clones containing an insert larger than 0.5 kb. Nested primers have been designed using the TDF as a template (5R13: GAACTGAATAGCTGGGTTTGCACC; 3R13:GTAATGTTGGTACAACCCCGGCTAC; 5NR13:

CCATTCAAACTAACAAACGCGGTAG; 3NR13: GGCTACCGCGTTTGTTAGTTTGAATG). Primers located on the opposite side were based on the cDNA library vector sequence. Fragments containing the entire coding region for the *BvGLP-1* gene, together with parts of their 3` and 5` UTRs have been amplified using a proof reading polymerase and were subcloned into the pGEMTeasy vector (Promega). Eight independent colonies have been sequenced for each construct. No sequence differences have been observed within these clones.

3.5 Transformation of Arabidopsis and sugar beet with *A. tumefaciens*/*A. rhizogenes*

3.5.1 Plasmid constructs and agrobacterial cultures

BvGLP-1 was cloned as an *Eco*RI-fragment into the binary vector pAM194 (KWS Saat AG, Einbeck, Germany) under the transcriptional control of the 35S promoter resulting in the plant expression construct pAM194-BvGLP-1 as well as an *Xba*I-fragment into the binary vector pBIN121 under the transcriptional control of the $Hs1^{pro1}$ promoter (Thurau et al. 2003) resulting in the plant expression construct pBIN121-BvGLP-1, respectively. The recombinant binary vectors were transformed into *A. tumefaciens* strain GV3101 (Koncz and Schell 1986) for *A. thaliana* root transformation as well into *A. rhizogenes* strain AR15834 (Kifle et al. 1999) for sugar beet transformation by using electroporation (Gene Pulser System II, Bio-Rad). The transformed Agrobacterium cells were grown on 2YT medium containing 50 µg/ml kanamycin and 100 µg/ml rifampycin (*A. rhizogenes*) and 50 µg/ml kanamycin and 50 µg/ml gentamycin (*A. tumefaciems*) overnight. A single overnight colony was used to inoculate 150 ml 2YT medium without antibiotics, grown to an OD600 of ~0.4-0.6, centrifuged at 4000 x g, 4°C for 10 min and was then resuspended in 15 ml 2YT medium ready for transformation of sugar beet leaf stalks. For transformation of *A. thaliana* root explants a single overnight colony was used to inoculate 5 ml 2YT liquid culture medium with 50 µg/ml kanamycin and 50 µg/ml gentamycin and was grown at 28°C, 210 rpm for 24 h. The overnight culture was used to inoculate 50 ml 2YT medium without antibiotics. Cells were harvested by centrifugation for 10 min at 4°C, 2900 x g and then resuspended in 50 ml B5 medium (Gamborg et al. 1968). The washing step was repeated. 1 ml was transferred into 20 ml fresh B5 medium ready for transformation.

3.5.2 Sugar beet hairy roots transformation

Leaf stalks of the susceptible sugar beet line 93161p were used for sugar beet hairy root transformation. Leaf stalks were sterilized by submergence in 5% (w/v) $CaCl_2O_2$ for 10 min,

followed by treatment with 70% (v/v) ethanol for 5 min. After washing with sterile water, the sterilized leaf stalks were cut into pieces of 2 cm length and incubated with *A. rhizogenes* for 10 min. The infected explants were soaked on sterilized filter paper before cultivation on solid ½ B5 medium. After two days of co-cultivation in the dark, explants were transferred to ½ B5 medium containing 400 µg/ml cefotaxime to eliminate *A. rhizogenes*, and incubated in a plant growth chamber (26°C, 16/8 h light/dark photoperiod). Single hairy roots 1 cm in size were excised and sub-cultured on ½ B5 medium containing 150 mg/l cefotaxime under the same conditions.

3.5.3 *Arabidopsis thaliana* root transformation

Transgenic Arabidopsis plants were generated by using the root transformation protocol (Valvekens et al. 1988). C24 Arabidopsis seeds were surface sterilized for 5 min in 70% (v/v) ethanol, in 5% (w/v) $CaCl_2O_2$ containing 0.05% (v/v) Tween 20 for 10 min, and rinsed four times with sterile distilled water. Sterile seeds were transferred into 500 ml Erlenmeyer flasks containing 100 ml liquid B5 medium and germinated for nine days by gently shaking (22°C, 16/8 h light/dark photoperiod). Roots were cut into small pieces of about 0.5 cm and transferred to a nylon membrane (100 Mikron, Hydro-Bios, Altenholz, Germany) lying on solid CIM medium for a preconditioning time of 72 h. After preconditioning the root explants were incubated with the prepared *A. tumefaciens* culture for 2 min. The infected explants were soaked on sterile filter paper before co-cultivation on fresh CIM medium for 48 h. After co-cultivation the explants were washed four times with liquid B5 medium and transferred to SIM medium. Single green shoots were excised and transferred to SEM medium for shoot elongation and then to RIM medium to form roots. Rooted plants were transferred to soil to set seeds.

3.6 DNA, Southern hybridization and PCR

Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as described by Rogers and Bendich (1985). 14 days old Arabidopsis plants and 21 days old hairy root cultures were harvested for isolation of genomic DNA. PCR was carried out in a total volume of 20 µl containing 50 ng of template DNA, 10 mM PCR buffer (pH 8.3), 2 mM $MgCl_2$, each dNTP at 0.5 mM, each primer at 10 pmol, and 1 U of Taq DNA polymerase (Invitrogen) in a thermocycler (Biometra, Göttingen, Germany) under the following conditions: 5 min at 94°C, 60 s at 94°C, 60 s at 55 °C, 1 min 30 s at 72°C for 34 cycles,

followed by 10 min at 72°C. PCR products were separated on a 1% (w/v) agarose gel and analyzed with the software Quantity One (Bio-Rad Laboratories GmbH).
For Southern hybridization experiments the extracted DNA was digested with two restriction enzymes (*Eco*RI, *Xho*I), electrophoretically separated on 0.75% (w/v) agarose gels and blotted on Hybond-N+ membrane (GE Healthcare) by capillary diffusion overnight using 0.25 M NaOH/1.5 M NaCl as blotting solution. Southern blots were hybridized with 32P-labelled DNA probe (Feinberg and Vogelstein 1983) at 60°C, washed twice (0.5 x SSC; 0.2% (w/v) SDS) for 30 min and exposed at -70°C for 48 h. The DNA probe for Southern-hybridization was generated by PCR with *BvGLP-1* gene specific primers CTCCTAGCCTCTTGTAATTCTAGC and GAATGGAAACAAGCAACATATGATATC using plasmid DNA as template. PCR fragments were separated on a 1.3% (w/v) low melting agarose gel ready for radio-labelling.

3.7 RNA isolation, RT-PCR and qRT-PCR

Semi-quantitative RT-PCR was used to assay the expression patterns of the genes of interest. 14 days old transgenic as well as control Arabidopsis plants and 21 days old hairy root cultures of sugar beet were used for RNA extraction following the trizol protocol (Gibco BRL Life Technologies). The total RNA was treated with RNAse-free DNAse (Fermentas) for 60 min at 37°C. Synthesis of cDNA was carried out using the Superscript III First-Strand Synthesis System (Invitrogen) according to the instructions of the manufacturer. The semi-quantitative PCR amplification was performed in 50 µl reactions consisting of 2.5 µl 10 ng/µl cDNA, 5 µl 10 x buffer, 0.5 µl 10 mM dNTPs, 5 µl each of 10 pmol/µl primer, 2.5 U Taq polymerase (Invitrogen) and 31.5 µl H_20 under the PCR programme: 25 x (94°C 50 s, 54°C 1 min and 72°C 1 min), followed by 10 min at 72°C. Amplicons were separated on a 1% (w/v) agarose gel and analyzed with the software Quantity One (Bio-Rad Laboratories GmbH). The housekeeping *ubiquitin* gene served as a control and the mRNA levels for each cDNA probe were normalized to the *ubiquitin* message RNA level (ACTCTCACCGGAAAGACAATC and TGACGTTGTCGATGGTGTCAG for Arabidopsis; ACTCTCACCGGAAAG ACAATC and TGACGTTGTCGATGGTGTCAG for sugar beet). The real-time PCR was performed using the ABI7300 Detection System (Applied Biosystems, Foster City, USA). The SYBR QPCR Supermix (Invitrogen) was used for PCR reactions according to the manufacturers´ instruction in a final volume of 20 µl. The ABI7300 was programmed to 95°C 10 min, 35 x (95°C 30 s, 55°C 40 s, 72°C 45 s), 72°C 10 min followed by a melting curve programme (55 to 95°C in increasing steps of 0.5°C). All reactions were repeated at least twice. The mRNA

levels for each cDNA probe were normalized with respect to the *ubiquitin* message level. Fold induction values were calculated with the ΔCP equaltion of Pfaffl (2001) and related to the mRNA level of the target genes in wild type roots, which were defined as 1.0.
The following primers were used in the described gene expression experiments:

Target	Accession no.	Primer 5′ - 3′	Reference
PDF1.2	AT5G44420	ATGGCTAAGTTTGCTTCCA TTAACATGGGACGTAACAGATAC	Huffaker et al. 2007
PDF2.1	AT2G02120	GATGGGTCCAGTCACGGTC TTCAAGAACACACTAAACACGC	Thomma et al. 1998
PDF2.3	AT2G02130	CACACACAACTGTGCAAACG CGGAAACACACAAACCAATG	Thomma et al. 1998
PR1	AT2G14610	GGAGCTACGCAGAACAACTA AGTATGGCTTCTCGTTCACA	Huffaker et al. 2007
PR2	AT3G57260	CTACAGAGATGGTGTCA AGCTGAAGTAAGGGTAG	Uknes et al. 1992
PR3	AT3G12500	TGGATGGGCTACAGCACC CTAAATAGCAGCTTCGAGGAGG	Thomma et al. 1998
PR4	AT3G04720	CACATTCTCTTCCTCGTGTTC TAGTTAGCTCCGGTACAAGTG	Thomma et al. 1998
SGT1b	AT4G11260	ATGGCCAAGGAATTAGCAGA CGGTTTGGCAGGTGCTGCAGGA	Tör et al. 2002
NPR1	AT1G64280.1	TCAACCATAGGAATCCGAGGG CCGACGACGATGAGAGAGTTTACG	this study
EDS1	AT3G48090	ACCAGATCATGGTCAGCC TGTCCTGTGAACACTATCTGTTTTCTACT	Feys et al. 2001

3.8 Nematode infection experiments

H. schachtii was propagated on in vitro stock cultures of mustard (*Sinapis alba* cv. Albatros) roots grown on 0.2 x Knop medium supplemented with 2% (w/v) sucrose and 0,8% (w/v) Daishin agar under sterile conditions.
Fully developed cysts were harvested from the roots onto 50 µm gauze. Hatching of juveniles was stimulated by soaking cysts in 3 mM $ZnCl_2$ for eight to ten days. The larvae were harvested with 10 µm gauze, surface-sterilized, washed four times in sterile water, resuspended in 0.2% (w/v) Gelrite (Duchefa, Harlem, Netherlands) and used directly for inoculation experiments. Inoculation of hairy roots and Arabidopsis plants with nematodes was performed in vitro according to the protocol described by Sijmons et al. (1991). Sugar beet hairy roots one cm long and one week old Arabidopsis seedlings were transferred to Petri dishes and 6-well plates containing 0.2 x Knop medium under sterile conditions. Sterile infective *H. schachtii* juveniles were added to each Petri dish with one root tip and to each Arabidosis plant in a single well of 6-well plates. The number of developed females was

determined four weeks after infection under a stereomicroscope (Stemi SV 11, Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

3.9 OXO enzyme activity tests

Oxalic acid wilting assay:

Seven days old wild type and transgenic Arabidopsis plants were transferred from agar plates into 4 mM oxalic acid solution at pH 4 (adjusted with HCl) for cultivation. Wild type plants in 4 mM oxalic acid solution at pH 4 (adjusted with HCl) and transgenic Arabidopsis plants without oxalic acid served as a control. The whole test was performed in a plant growth chamber at 24°C under a 16/8 h light/dark photoperiod. The plants were visually scored in respect of wilting symptoms every one hour after incubation.

DAB staining:

To visualize H_2O_2 in situ, 3.3'-diaminobenzidine (DAB) staining was performed on ten days old transgenic Arabidopsis seedlings inoculated or not with *H. schachtii*. The Arabidopsis wildtype C24 served as control. The seedlings were placed in 0.5 mg/ml DAB-HCL, pH 3.8 covered only roots and incubated in the dark for at least 30 min. They were then transferred into a growth chamber under light for 60 min until brown precipitate was observed. Samples were fixed with a solution of 4:1 (v/v) ethanol/chloroform with 0.15% (w/v) trichloroacetic acid for 24 h and then placed in 10 mM citrate buffer for microscopy. The samples were stored in 96% (v/v) ethanol. DAB stained seedlings were fixed in 4% paraformaldehyde at 4°C over night, dehydrated through an ethanol series, and then embedded in Technovit 7100 resin following the manufacturers' instruction (Heraeus Kulzer GmbH, Wehrheim, Germany) The samples were sectioned (10 µm thickness) with a glass knife on a rotation microtome (Ultracut UCT; Leica, Wetzlar, Germany), dried on glass slides and used for microscopic observations (Zeiss).

3.10 ATH1-microarray analysis

For ATH1-microarray analysis, Arabidopsis with the C24 genetic background were used. They are transgenic line pAM194-15 carrying $Hs1^{pro\text{-}1}$ and line pAM194-V transformed with the empty pAM194 vector. Arabidopsis seedlings were grown on Petri dishes containing 0.2 x Knop medium under sterile conditions. Seven days old seedlings were inoculated with 200 sterile infective nematode juveniles each plant/Petri dish. Roots were harvest three days, seven days and 14 days after inoculation, pooled and frozen for use. Total RNA was extracted from the Arabidopsis roots following the trizol protocol (Gibco BRL Life Technologies). The

total RNA was linearly amplified and biotinylated using the One-Cycle target-labeling kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturers´ instruction. Labeling, hybridization, and detection on the ATH1 Arabidopsis Chip (Affymetrix) were performed at the Affymetrix Platform at the Universitätsklinikum Tübingen and UKSH, Kiel, Germany, as described by Mueller et al. (2008). Briefly, 15 µg of labeled and fragmented copy RNA was hybridized to Arabidopsis ATH1 GeneChip arrays (Affymetrix). Arrays were scanned using the GCS3000 GeneChip scanner (Affymetrix) and GCOS 1.3 software. The Affymetrix GeneChip software MAS 5.0 was used for calculation of expression ratios of genes and generation of report files (Journot-Catalino et al. 2006). For statistical data analysis, three independent probe replicates were performed for generation of data files. The resulting data were imported into Genespring 7.1 (Agilent Technologies, Böblingen, Germany) for normalization and probe summarization (Wu et al. 2003). Genes with an at least twofold increase or decrease in average expression ratio were chosen for further analysis. Functions of differentially expressed transcripts were annotated using the NetAFFX analysis center. The data discussed in this publication have been deposited at the National Center for Biotechnology Information Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.-gov/geo/).

3.11 Analysis of *A. thaliana* mutant

A. thaliana GLP3 (AT5G20630) exhibits 43% amino sequence identity to *BvGLP-1* (E-value 1e-43). The knockout (ko) Arabidopsis mutant of AT5G20630 was obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, OH, USA), which contains a T-DNA insertion in AT5G20630 (SAIL 620_D04). After successive selfing, four homozygous ko lines were selected by PCR using the T-DNA left border-specific primer (TAGCATCTGAATTTCATAACCAATCTCGATACAC) in combination with *GLP3*-specific primers 620_D04 f (GGTCAGGGTTCTTGCAAGAGTAAC) and 620_D04 r (CTAAAATCACAAGAAGCCCATGC). Knockout of the *GLP3* transcripts in the ko line was confirmed by RT-PCR with the same *GLP3*-specific primers as described above. The ko line was designated as *glp3.*

4 Results

4.1 Cloning of differential expressed fragments from resistant beets by use of cDNA-AFLP expression profiling

Because so far no microarray/chip is available for the sugar beet genome, we applied cDNA-AFLP technology for generation of cDNA-ALFP expression patterns from nematode resistant (A906001) and susceptible (93161p) sugar beet lines upon nematode infection. In total, 156 AFLP- primer combinations (132 for *Eco*RI/*Mse*I and 24 for *Pst*I/*Mse*I) have been tested. Each primer pair generated 50 -100 Transcript-Derived Fragments (TDFs) in the size range of 20-500 bp. In total, approximately 10,000 TDFs were generated. By comparison, 66 fragments were identified only in the nematode resistant material, from which twelve were specific for nematode infection.

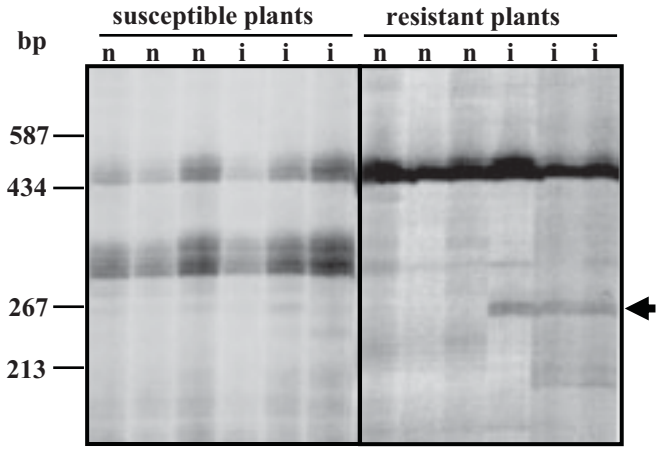

Figure1: cDNA-AFLP autoradiograph representing the TDF expression pattern from 3 susceptible (sugar beet line 93161p) and 3 resistant (sugar beet line A906001) sugar beet plants subjected to nematode inoculation or not. The arrow labeled band corresponds to TDF_Car differentially expressed in the inoculated resistant material. n, not inoculated; i, inoculated.

Thus, these twelve TDFs were chosen as candidates for resistance-specific transcripts for further analysis. To get sequence information, the 12 TDFs expressed only in the resistant plans were excised from the gels, re-PCRed, subcloned into the pGEMTeasy vector and sequenced. To verify the cloning and sequencing of the correct fragments, the same cDNA-AFLP primers were used in a PCR reaction with the pGEMTeasy clones as template. The resulting products were loaded on a polyacrylamid gel next to the cDNA-AFLP reactions from which the fragments had originally been isolated. In this way, all twelve fragments have been verified (data not shown). To confirm the expression pattern of the TDFs, primers were designed from sequence information of the twelve TDFs and RT-PCR analysis was performed

with mRNA from the infected as well as non-infected resistant (A906001) and susceptible (93161p) plants. Of twelve TDFs, one fragment, TDF_Car was specifically induced to express in roots of resistant plants upon nematode infection (Figure 1). Therefore, TDF_Car was selected for further analysis. The remaining TDFs were either not expressed or were expressed in both inoculated and non-inoculated susceptible and resistant plants.

4.2 Isolation and characterization of full-length cDNA sequence of TDF_Car

The full-length cDNA clone for TDF_Car was isolated by PCR from a yeast two hybrid cDNA library made from RNA of the infected resistant roots. This approach led to the isolation of two overlapping fragments spanning the entire coding region together with 40 bp of its 5` UTR and 173 bp of its 3` UTR. Consequently, the cloned 837 bp full-length cDNA corresponding to TDF_Car contains a single open reading frame of 624 bp encoding for a 208 aa polypeptide. A blast homology search revealed high homology to several oxalate oxidase-like germin ESTs of *Beta vulgaris* (AAG36665.1, E-value 2e-109; AAG36667.1, E-value 1e-89; AAG36666.1, E-value 3e-90) and genes of diverse plant species including, Arabidopsis, maize, rice, mustard, barley and pine as well as wheat (Figure 2). Therefore, it is referred to as *BvGLP-(Wag)1* (accession number: AY243465), renamed *BvGLP-1* in this paper.

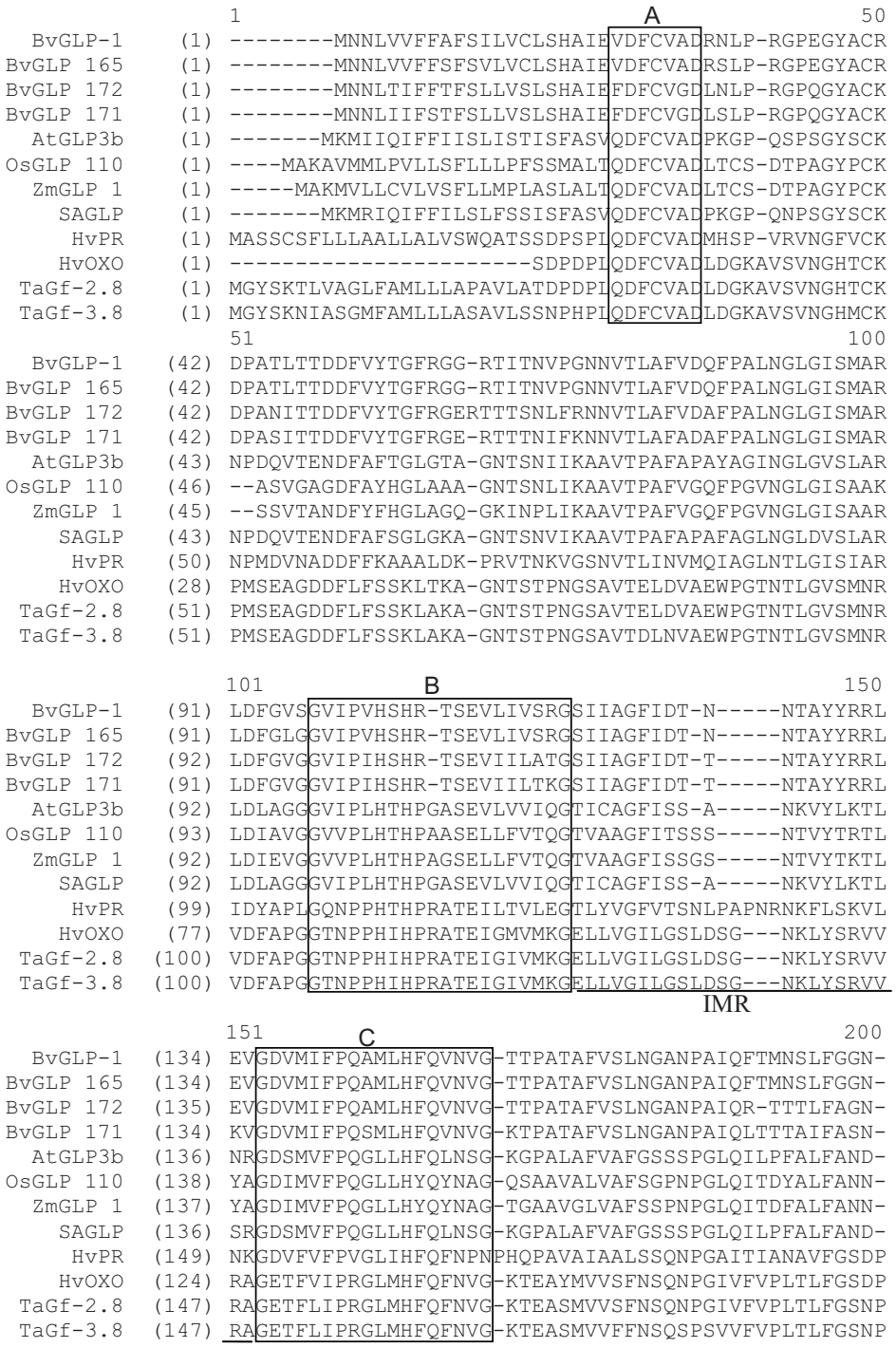

Figure 2: Comparative multiple sequence alignement of partial *BvGLP-1* nucleotide sequence with sequences of 11 germin and GLPs from sugar beet, Arabidopsis, maize, rice, mustard, barley, pine and wheat. The GenBank accession numbers of the sequences analyzed are: for GLPs: *BvGLP165*, AAG36665.1; *BvGLP*172, AAG36667.1; *BvGLP*171, AAG36666.1; *AtGLP3b*, U75195; *OsGLP110*, AB015593; *ZmGLP1*, AY394010; *SaGLP*, X84786; *HvPR*, X93171; *HvOXO*, L15737; and for true germins:*TaGf-2.8*, M63223; *TaGf-3.8*, M63224. The three conserved boxes A, B and C are framed. The inter motif region is underlined.

Germin and GLPs belong to a subgroup of cupin proteins exhibiting characteristic domains. Sequence alignment analysis reveals that *BvGLP-1* has three conserved subdomains of cupin proteins, the boxes A, B and C (Figure 2). Box A consists of conserved amino acids of QDFCVAD, but in *BvGLP-1* the first residue is V instead of Q. While Box B has the conserved motif of G--P-H-HPRATEXXXX-G, Box C has the consensus GXXHFQ-N-G, in which X corresponds to hydrophobic amino acids. In Box B and C, three histidine residues known to be involved in metal binding were found in *BvGLP-1* (Figure 2). In addition, Box B and C are separated by an intermotif region (Figure 2), which proved to be required for formation of a six-stranded beta barrel structure (Woo et al. 2000). The expression profile of *BvGLP-1* was investigated in roots of resistant and susceptible plants in response to nematode infection (Figure 3).

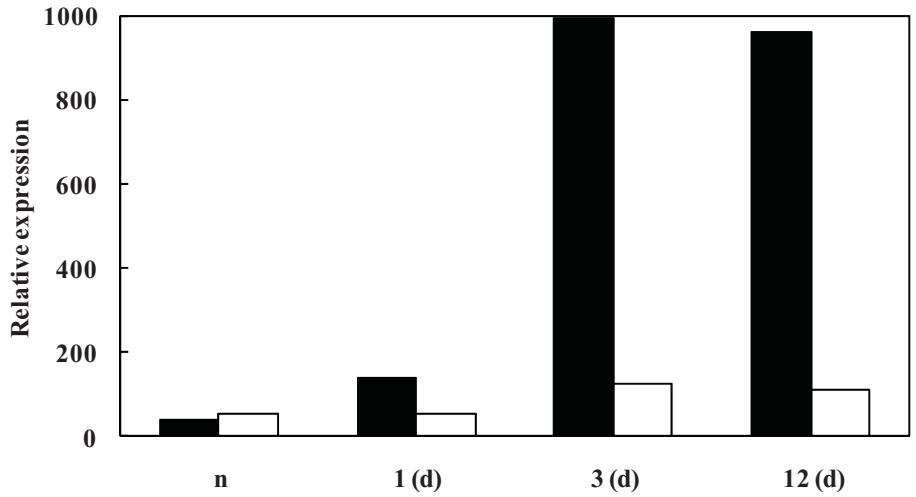

Figure 3: Expression profiling of *BvGLP-1* in resistant and susceptible sugar beet as investigated by qRT-PCR. Plants were inoculated with *H. schachtii* J2 juveniles and roots were harvested 1, 3 and 12 days after inoculation. Non infected roots (n) served as a control. Black columns represent the resistant sugar beet line A906001 and white columns the susceptible sugar beet line 93161p. The expression of the signals was calculated on the basis of a normalised data output as a relative ratio between the expression of the target gene and a constitutive control (ubiquitin10) according to the assumption that the expression of the ubiquitin 10 gene is constitutive for all plant assays and dates of observation.

qRT-PCR showed that the expression of the gene was upregulated in resistant plants one day after infection (Figure 3) while no upregulation was observed in susceptible sugar beet upon infection. Three days after nematode infection the transcript level in resistant plants was about ten times higher than in susceptible plants, although a slight increase in the transcript was also visible in susceptible plants upon nematode infection. Even seven days after inoculation, the gene expression still maintained at a higher level in resistant plants when compared to the susceptible plants (Figure 3).

4.3 Functional analysis of *BvGLP-1* in transgenic sugar beet hairy roots and *Arabidopsis thaliana* plants

The potential of *BvGLP-1* in activation of nematode resistance was investigated in both transgenic sugar beet hairy roots and Arabidopsis plants, respectively. To this end, two plant expression constructs were generated (Figure 4A). In the construct pAM194-BvGLP-1, the full length cDNA *BvGLP-1* was driven by the 35S promoter for a constitutive gene expression while in the construct pBIN121-BvGLP-1 the sequence was under the control of the $Hs1^{pro-1}$ promoter for a nematode feeding site-specific gene expression (Figure 4A). For transformation into sugar beet hairy roots, both gene constructs were introduced into the susceptible sugar beet line 93161p. In total, 45 independent transgenic hairy roots for pAM194-BvGLP-1 and 36 for pBIN121-BvGLP-1 were obtained by PCR analysis with gene-specific primer (data not shown). The transgenic roots harbour one to three copies of the transgene as revealed by Southern blot analysis (data not shown) but no obvious differences with respect to growth rate and morphology of the hairy roots among the different transgenic events were observed. The 15 transgenic roots carrying a single copy of the transgene were chosen for further analysis.

Similarly, both gene constructs were transformed into the *A. thaliana* ecotype C24 by *A. tumefaciens* mediated transformation. From four transformation events 131 T0 transformants with pBIN121-BvGLP-1 were obtained, while only 30 transformants with pAM194-BvGLP-1 could be selected. The generation rate with pBIN121-BvGLP-1 was about ten times higher than that with pAM194-BvGLP-1. Obviously, a strong overexpression of *BvGLP-1* resulted in H_2O_2 stress, from which the plant regeneration suffered. The presence and the copy number as well as the expression of transgenes were determined by Southern blot analysis and PCR (Figure 4B, C). As shown in Figure 4C, a gene-specific PCR fragment of 780 bp in size was given in the selected transformants but not in the *A. thaliana* C24 control. In addition, Southern analysis of four independent transformants for each gene construct revealed that the transgene integrated in Arabidopsis varied from one to three copies (Figure 4B). Consequently, eight independent Arabidopsis transformants carrying a single copy of the transgene for each construct were selected to propagate to T4 generation, from which eight homozygous lines of each gene construct were obtained.

The transcript levels of the transgenes were determined by semi-quantitative RT-PCR using *BvGLP-1* gene-specific primers (Figure 5). As expected, specific transcripts of 780 bp in size were detected in all transgenic plants, but an enhanced expression of the transgene responsive to nematode infection was given only in the plants transformed with the pBIN121-BvGLP-1.

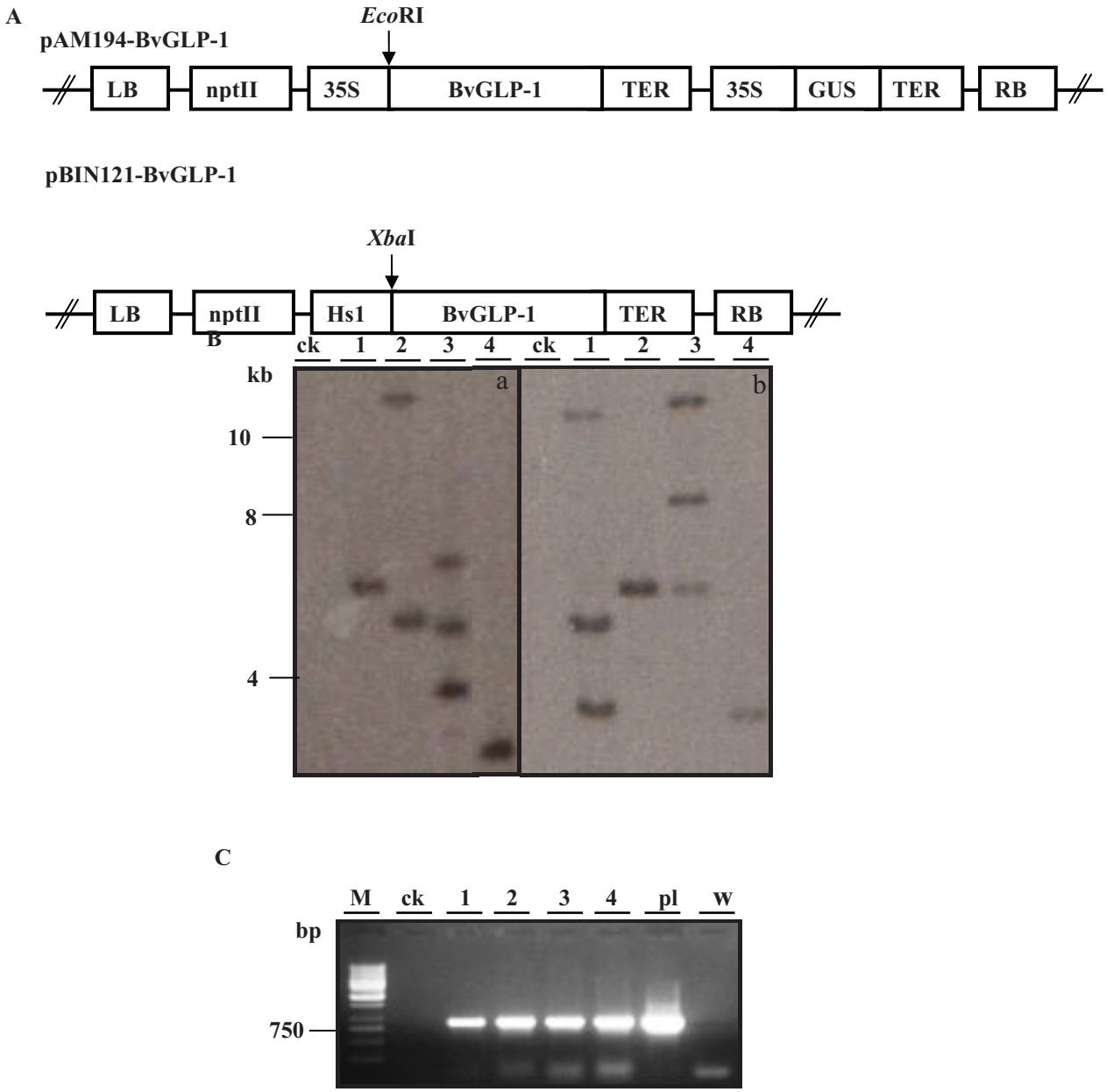

Figure 4: Molecular characterization of *BvGLP-1* transgenic *A. thaliana* transformands by Southern blot analysis and genomic PCR. **A**: The *Eco*RI recognition site within the pAM194-BvGLP1 construct and the *Xba*I recognition site within the pBIN121-BvGLP-1 construct. **B**: Determination of copy numbers of the integrated *BvGLP-1* gene. Genomic DNA was digested with two restriction enzymes (*Eco*RI, *Xho*I), electrophoretically separated on 0.75% (w/v) agarose gels, blotted on Hybond-N+ membrane and hybridized with *BvGLP-1*. ck: *A. thaliana* C24 as a control; 1-4: *BvGLP-1* transgenic *A. thaliana*, a: pAM194-BvGLP1; b: pBIN121-BvGLP-1. Molecular length markers are indicated at left in kb. Exposure time: 48 h. **C**: Representative PCR analysis of *BvGLP-1* transgenic *A. thaliana* plants, using *BvGLP-1* gene-specific primers. ck: *A. thaliana* C24 as a control; 1-4: *BvGLP-1* transgenic *A. thaliana*; pl: plasmid DNA pAM194-BvGLP-1 as positive control; w: water control.

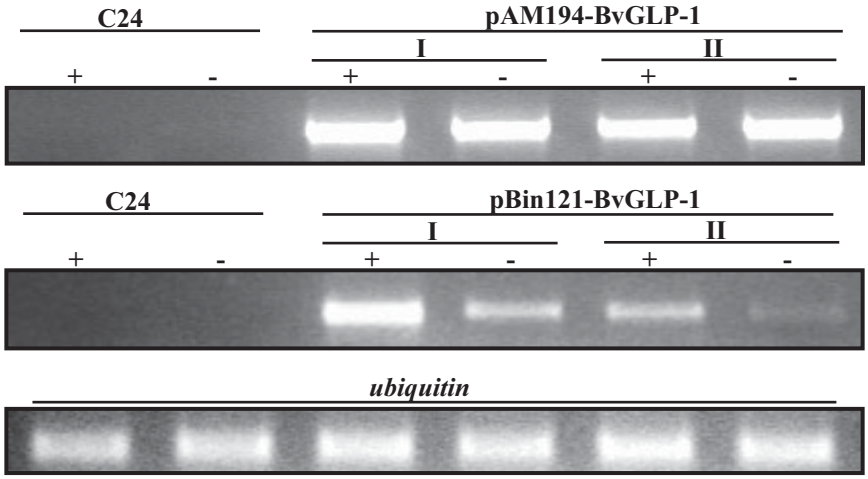

Figure 5: Expression of *BvGLP-1* gene in transgenic *A. thaliana* determined by semiquantitative RT-PCR with 2 independent *BvGLP-1* transgenic *A. thaliana* lines for both constructs pAM194-BvGLP1 and pBIN121-BvGLP-1 (I, II) inoculated or not with *H. schachtii*. *A. thaliana* wild type C24 plants served as control. The *BvGLP-1* transcript is present in all transgenic lines and is differentially expressed in pBIN121-BvGLP-1 transgenic *A. thaliana* after nematode inoculation. The mRNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level.

4.4 Nematode inoculation experiments

Twenty independent transgenic hairy root cultures transformed with pAM194-BvGLP-1 and 18 for pBIN121-BvGLP-1 were subjected to a nematode resistance assay, in which the susceptible sugar beet roots transformed with the empty vector served as a control. Each nematode infection experiment was performed with 150 infective nematode juveniles and tested with seven repetitions. Four weeks after inoculation, developed nematode females were scored under the stereomicroscope. The results of nematode resistance assays are summarized in Figure 6.

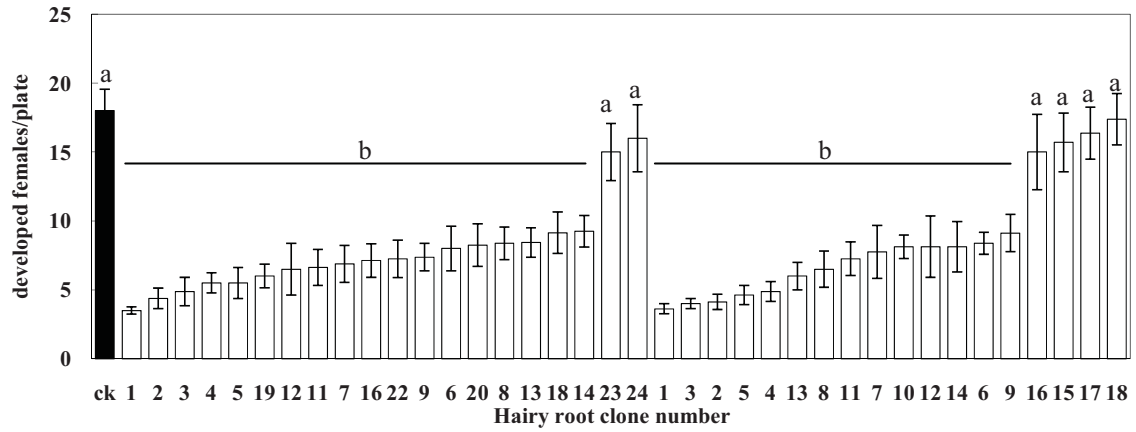

Figure 6: Results of nematode resistance assays with hairy roots transformed with pAM194-BvGLP-1 (left) and pBIN121-BvGLP-1 (right). Hairy root clones obtained by transformation with the empty vector served as the susceptible control (ck). Each clone was analysed in 8 independent inoculation assays. The average number of developed females is presented as a bar, and the standard deviation as a blank ended line. Significantly different means are indicated by different letters calculated with t test at $P < 0.05$.

On the susceptible control roots, 10 to 20% of the applied J2 nematode juveniles developed into females resulting in an average number of developed females of 18± 2 (mean±STD, n=35). The number of developed females among transgenic hairy roots was relatively variable, ranging from three to 21. However, in 18 out of 20 pAM194-BvGLP-1, and 14 out of 18 pBIN121-BvGLP-1-transgenic root cultures, the number of developed females was significantly lower than that in the susceptible controls (Figure 6). On average, the pAM194-BvGLP-1-transgenic roots have 7±2 (mean±STD, n=160) females each culture and pBIN121-BvGLP-1-transgenic roots have 5±2 (mean±STD, n=144) developed females each root culture. By comparison, no difference between two gene constructs in respect to their efficiency in inhibition of female nematodes was given (Figure 6).

A similar experiment was performed with eight transgenic Arabidopsis lines selected for each gene construct. Eight individual plants per line were cultivated on 6-well agar plates and seven days old seedlings were each inoculated with 150 infective nematode juveniles. Plants transformed with the empty vector served as a susceptible control. 28 days after inoculation, females on each plant were counted as well as documented under a stereomicroscope. The results were summarized in Figure 7.

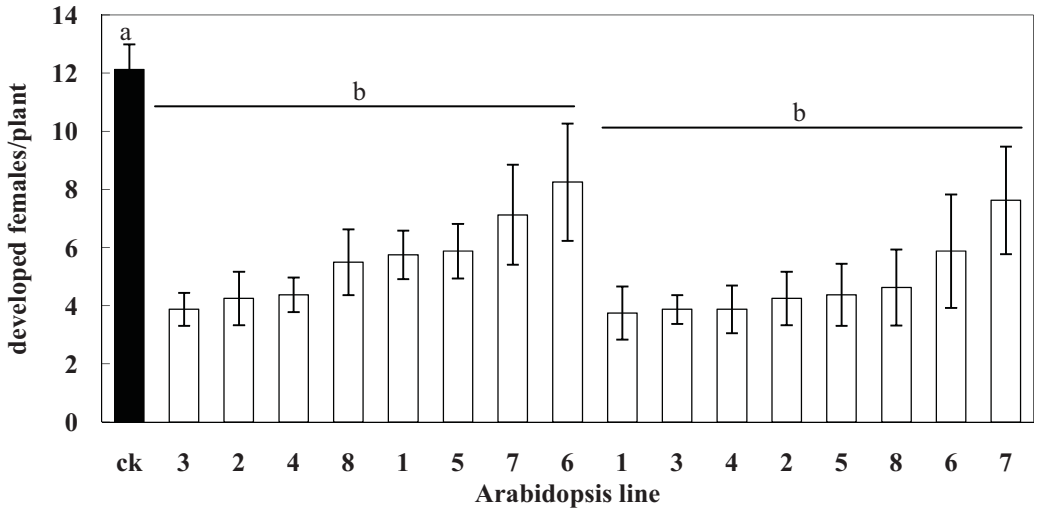

Figure 7: Results of nematode resistance assays with *A. thaliana* plants transformed with pAM194-BvGLP1 (left) and pBIN121-BvGLP-1 (right). *A. thaliana* plants obtained by transformation with the empty vector served as the susceptible control (ck). The average number of developed females is presented as a bar, and the standard deviation as a blank ended line from 8 individual plants for each line. Significantly different means are indicated by different letters calculated with t test at $P < 0.05$.

On average, 12±4 (mean±STD, n=36) females developed on the control plants, which approx. corresponds to 10 to 15% of inoculated J2. Although the female number among transgenic lines varied from two to 15, a significant reduction in developed females on transgenic lines carrying both gene constructs was defined as revealed by statistical analysis (Figure 7). Respectively, 5±3 (mean±STD, n=64) and 4±2 (mean±STD, n=64) females were counted on pAM194-BvGLP-1 and on pBIN121-BvGLP-1 transgenic plants, which are significantly lower than that on the control plants 12±4 (mean±STD, n=36). By comparison, two different constructs did not show significant difference in suppression of female nematode development similar to the observation made by sugar beet hairy roots. Taking together, these data demonstrate that both the constitutive and feeding cell-specific expression of *BvGLP-1* in plants interferes with nematode infection leading to a significant reduction of developed females. This is consistent with our previous observation made by the $Hs1^{pro-1}$ mediated nematode resistance in sugar beet.

4.5 $Hs1^{pro-1}$ regulates the expression of GLP genes in Arabidopsis

To identify the $Hs1^{pro1}$-regulated genes in transgenic Arabidopsis, we used the Affymetrix Arabidopsis ATH1 GeneChip, which contains >22.500 probe sets representing 24.000 genes. For this, $Hs1^{pro-1}$ was transformed into *A. thaliana* ecotype C24 under the transcriptional control of the 35S promoter by use of Agrobacteium-mediated transformation. In total, 78

independent transgenic *A. thaliana* expressing *Hs1*$^{pro-1}$ were selected and subsequently subjected to nematode infection experiments. Of these 65 plants showed a reduction of developed female nematodes to some degree and 12 could be selected, which showed a significant reduction of developed females, varied from 4±3 to 10±2 (mean±STD, n=22), compared to plants transformed with the empty binary vector pAM194 (pAM-V, 16±4, mean±STD, n=22) (Figure 8). Moreover, the same incompatible reactions to nematode infection could be observed in the transgenic *A. thaliana* as those in sugar beet (Figure 9). The infective J2 juveniles penetrate the roots eliciting a strong necrotic reaction around invasion and feeding sites. The nematodes cannot develop further because they are unable to induce the formation of syncytia in the vascular cylinder.

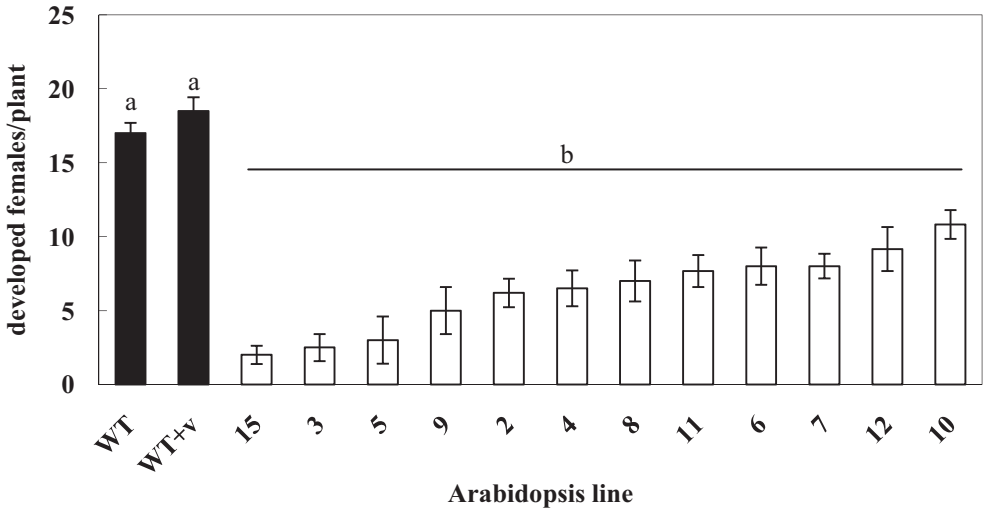

Figure 8: Results of nematode resistance assays with *A. thaliana* plants transformed with pAM194-Hs1^{pro-1}. *A .thaliana* C24 wild type plants and *A. thaliana* plants obtained by transformation with the empty vector served as the susceptible control (WT; WT+v). The average number of developed females is presented as a bar, and the standard deviation as a blank ended line from 8 individual plants for each line. Significantly different means are indicated by different letters calculated with t test at $P < 0.05$.

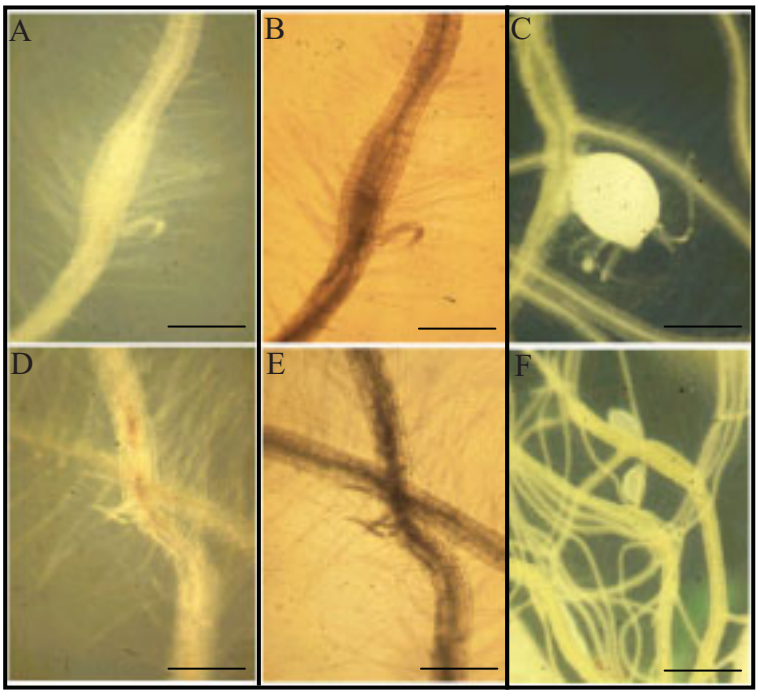

Figure 9: Microscopic observation of *Hs1*$^{pro-1}$ transgenic *A. thaliana* roots two days, four days and three weeks after nematode infection compared to *A. thaliana* roots transformed with the empty binary vector pAM194. Normal nematode development on susceptible control roots two and four days after infection (A, B) and developed nematode females three weeks after infection (C). Incompatible reactions to nematode infection on resistant transgenic *A. thaliana* roots: a strong necrotic reaction around the feeding sites 2 and 4 days after infection (D, E) and translucent females three weeks after infection (F). The bar equals 350µm in A, B, D, E and 300µm in C, F.

The *Hs1*$^{pro-1}$ homozygous transgenic line pAM15 and the Arabidopsis C24 transformed with the empty vector pAM-V were chosen for ATH1 GeneChip analysis. Roots of 20 seedlings were harvest three, seven and 14 days after nematode infection to create RNA pools for pAM15 and pAM-V respectively. This procedure was repeated two times for generation of two biologically independent pools of RNA.

To determine the *Hs1*$^{pro-1}$ -regulated genes in Arabidopsis, we compared the GeneChip data between pAM15 and pAM-V. As a result, about 66% of total Arabidopsis transcripts on the ATH1 GeneChip were detectable with both samples. In total 711 transcripts were differentially expressed sequence tags between pAM15 and pAM-V from which the transcript change fold varied from +43 to –50. Genes with 2.5 change folds ($P < 0.01$) were considered significantly regulated. When this threshold was applied in the *Hs1*$^{pro-1}$ transgenic Arabidopsis 217 transcripts were up- and 492 transcripts downregulated. About 65% of the differentially expressed transcripts are genes with defined functions (Table 1). We identified three germin-like genes in the upregulated genes. They are AT5G20630/ 246004_at (*GLP-3*),

AT5G38910/249476_at (*GLP9*) and AT5G39100/249495_at (*GLP6*) and all showed a substantial transcript exchange fold of 21, 7.5 and 6.7, respectively (Table 1). Because AT5G20630/ 246004_at for *GLP3* showed the highest transcript change fold and an amino acid identity of 43% to *BvGLP-1* as revealed by BLAST search (blastx), it was chosen for further analysis.

To validate the microarray data, we performed qRT-PCR analysis for AT5G20630 *GLP-3*. The qRT-PCR results showed that the gene was highly upregulated in $Hs1^{pro-1}$ transgenic Arabidopsis and the transcript increased slightly in both of wild type Arabidopsis C24 and Col-0 three days after nematode infection. These results support the validity of the Arabidopsis $Hs1^{pro-1}$-regulated transcriptome from the ATH1 GeneChip analysis and indicate that $Hs1^{pro-1}$ regulates the expression of GLP genes in transgenic Arabidopsis.

Table 1: A part of differentially expressed (>2.5 change folds) gene transcripts with defined functions originated from ATH1 GeneChip analysis with *Hs1*$^{pro-1}$ homozygous transgenic Arabidopsis line pAM15 and the Arabidopsis C24 transformed with the empty vector pAM-V.

Gene Titel	Change Fold	Gene Name
245226_at	43.72	germination protein-related
246004_at	21.01	germin-like protein (GLP3)
264073_at	20.12	pseudogene, hypothetical protein
247991_at	15.87	expansin, putative (EXP14)
263809_at	15.84	GDSL-motif lipase/hydrolase family protein
253352_at	15.14	pathogenesis-related protein, putative
265486_at	14.59	expressed protein
248372_at	13.78	expressed protein
254678_at	11.99	hypothetical protein
267364_at	9.973	expressed protein
263262_at	9.648	tumor susceptibility protein-related
251472_at	9.151	RWP-RK domain-containing protein
250067_at	8.551	hypothetical protein
260753_at	7.776	zinc finger (C3HC4-type RING finger) family protein
249174_at	7.622	expressed protein
249476_at	7.493	germin-like protein (GLP9)
249539_at	7.167	methyltransferase-related
254833_s_at	7.012	copper amine oxidase family protein
260869_at	6.886	acyl-(acyl-carrier-protein) desaturase
249495_at	6.707	germin-like protein (GLP6)
265817_at	6.612	histone H1-3 (HIS1-3)
262954_at	6.478	rubredoxin family protein
260851_at	5.933	nodulin MtN21 family protein

4.6 Functional analysis of Arabidopsis plants knocked out in locus of AT5G20630

To define the potential of *GLP3* in regulation of nematode resistance, we tested *glp3*-ko Arabidopsis mutant plants with the Col-0 genetic background. Because Arabidopsis Col-0 plants are more tolerant to *H. schachtii* infection, we expected an enhanced susceptibility of *glp3*-ko mutant plants. The mutant knocked out in locus AT5G20630, SAIL 620_D04 was identified from the Arabidopsis Biological Resource Center (Ohio State University, OH, USA), which carries a T-DNA insertion in the locus AT5G20630. Homozygous *glp3*-ko lines were selected by PCR analysis using the T-DNA-LB-specific primer and the locus-specific primers 620_D04 f and 620_D04 r. The *glp3*-ko lines do not show obvious morphological alteration in comparison with the wild type (Figure 10B). To confirm that homozygous *glp3*-ko plants did not process a functional *GLP3* transcript, RT-PCR analysis was performed with RNA of 14 days old seedlings of the *glp3*-ko and wild type plants (WT) using the same *GLP3*-specific primers as described above. As indicated in Figure 10A, no *GLP3* transcript could be detected in *glp3*-ko plants whereas WT RNA shows a 346 bp band corresponding to *GLP3* mRNA. Therefore, a complete knockout for this gene has been obtained.

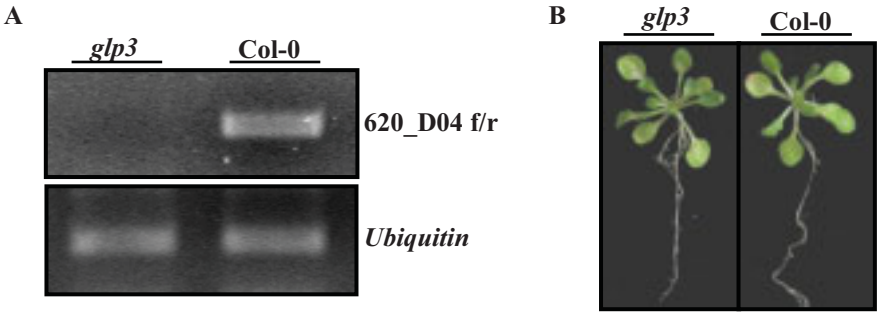

Figure 10: Characterization of *A. thaliana glp3*-ko plants. **A**: RT-PCR analysis was performed with RNA of 14 days old seedlings of the *glp3*-ko and wild type plants (Col-0) using GLP3-specific primers. The mRNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level. **B:** No morphological differences were observed between *glp3*-ko and wild type plants Col-0.

The *glp3*-ko plants were subjected to a nematode inoculation experiment, in which wild type plants were used as a control. Each plant was inoculated with approx. 150 infective nematode juveniles and monitored after three weeks. The experiment was repeated three times with 20 individuals. The results were summarized in Figure 11. As indicated in Figure 11, on *glp3*-ko plants 6 cysts each plant developed and this number is significantly higher than on wild type Col-0 plants which exhibit 2 to 3 cysts each plant on average. Therefore we conclude that knockout of *GLP3* in Arabidopsis Col-0 plants resulted in super-hypersensitivity of Col-0

plants to *H. schachtii* infection, strongly supporting an active role of GLP proteins in plant nematode resistance, in general.

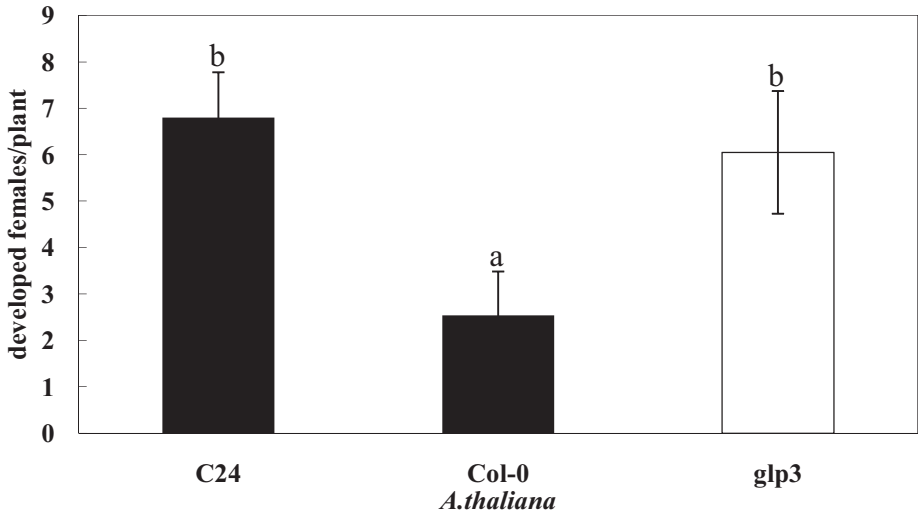

Figure 11: Results of nematode resistance assays with *A. thaliana glp3*-ko plants. *A. thaliana* wild type plants C24 and Col-0 served as the control. The average number of developed females is presented as a bar, and the standard deviation as a blank ended line from 8 individual plants for each line. Significantly different means are indicated by different letters calculated with t test at $P < 0.05$.

4.7 Determination of oxalate oxidase activity of *BvGLP-1*

To determine OxO activity of *BvGLP-1*, we performed an oxalic acid wilting test with transgenic Arabidopsis plants transformed with pAM194-BvGLP-1 in comparison with control plants transformed with the empty vector. OA is highly toxic to most plants, and incubation of plants in oxalic acid solution results in a rapid plant cell death and consequently plant tissues became wilting (Thompson et al. 1995). However, OxO degrades OA into CO_2 and H_2O_2, therefore transgenic plants expressing *BvGLP-1* with oxalate oxidase activity are expected to survive in OA solution. Following this, we placed seven days old transgenic Arabidopsis seedlings in a set of solutions with OA concentrations from 4 to 20 mM, and control plants into water and in a solution with 4 mM OA (Figure 12), respectively. The plants were visually scored each hour after infection and documented. We observed that seedlings of the control plants suffered severely from 4 mM OA, showing drastically wilting six hours after incubation. In contrast, transgenic seedlings grew regularly in the 4 mM OA solution without any obvious phenotypic alteration, and further survived in a solution with 8 mM OA over an extended 24 hours (Figure 12). The results, confirmed with four independent transgenic lines with three repetitions indicate that overexpression of *BvGLP-1* in plants

resulted in an increased OxO activity in plant cells, which detoxified OA in transgenic plants, consequently.

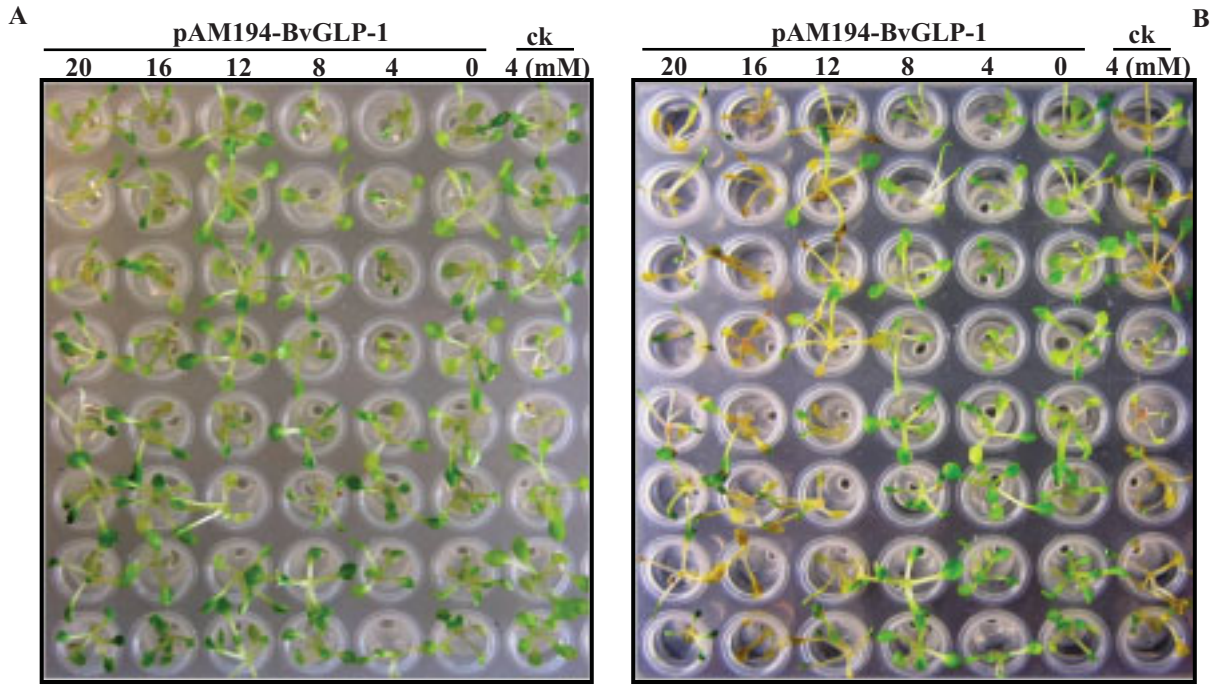

Figure 12: OA wilting assay with seven days old pAM194-BvGLP-1 transgenic *A. thaliana* seedlings. pAM194-BvGLP-1 transgenic *A. thaliana* seedlings without OA and *A. thaliana* seedlings obtained by transformation with the empty vector (ck) in 4 mM OA served as controls. In the presence of *BvGLP-1* with its OxO activity OA is degraded into CO_2 and H_2O_2 and transgenic plants expressing *BvGLP-1* survive. **A**: 1 hour after incubation; **B**: 24 hours after incubation.

For confirmation, we performed in situ (DAB) staining experiments to visualized H_2O_2, generated in transgenic plants with and without nematode infection in comparison with the control. In the presence of H_2O_2, DAB will be oxidized resulting in a specific brown-colored polymer, which intensity correlates with the amount of H_2O_2 and therefore can be qualitatively assessed under microscope. As expected, DAB-staining occurred strongly in the roots of transgenic plants in which the transgene is driven by 35S promoter with as well as without nematode infection (Figure 13A, B), whereas no or only very weak background staining was obvious in the control roots (Figure 13C, D). These data are in consistent with the results of the wilting tests, providing the evidence that the expression of *BvGLP-1* increases OxO activity in plant cells.

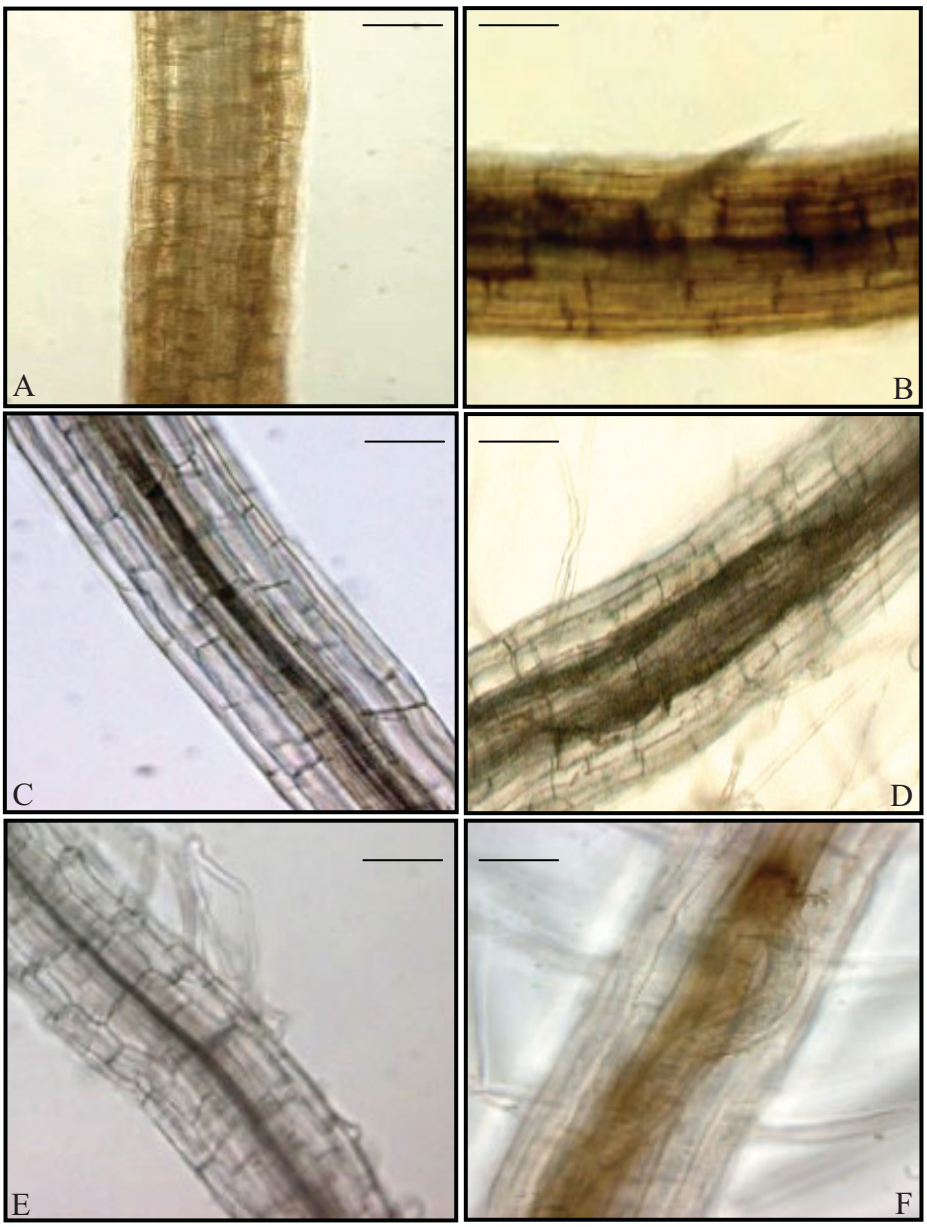

Figure 13: In-vivo DAB (3.3-diaminobenzidine) staining was performed on *BvGLP-1* transgenic Arabidopsis seedlings with and without nematode infection. Arabidopsis wild type C24 plants served as control. In the presence of H_2O_2, DAB will be oxidized resulting in a specific brown-colored polymer. **A, C, E**: without nematode inoculation; **B, D, F**: with nematode inoculation. DAB-staining occurred strongly in the roots of transgenic plants in which the transgene is driven by 35S promoter with as well as without nematode infection (**A,B**); A dominant staining signal in nematode feeding structure and especially around nematode penetration site occurred in pBIN121-BvGLP-1 transgenic roots (**F**); no or only very weak background staining was obvious in C24 control roots (**C,D**) as well as in the non-inoculated pBIN121-BvGLP-1 transgenic roots (**E**). The bar equals 250µm.

Furthermore, we analysed transgenic plants transformed with pBIN121-BvGLP-1 in which the transgene is under the transcriptional control of $Hs1^{pro-1}$ promoter. As shown in Figure 13, we detected dominant staining signal in nematode feeding structure and especially around

nematode penetration site of transgenic roots (Figure 13F), but not of control roots. In addition, we conducted a microtome sectioning experiment with DAB stained roots and observed that the H_2O_2 staining signal was restricted mainly in the developed syncytium (Figure 14A). These results demonstrate that a syncytium-specific expression of *BvGLP-1* resulted in an increase in the H_2O_2 level in the syncytium, locally. Therefore, we conclude that *BvGLP-1* is an OxO and *BvGLP-1* regulates nematode resistance via its OxO activity by adjusting H_2O_2 levels in plant cells attached.

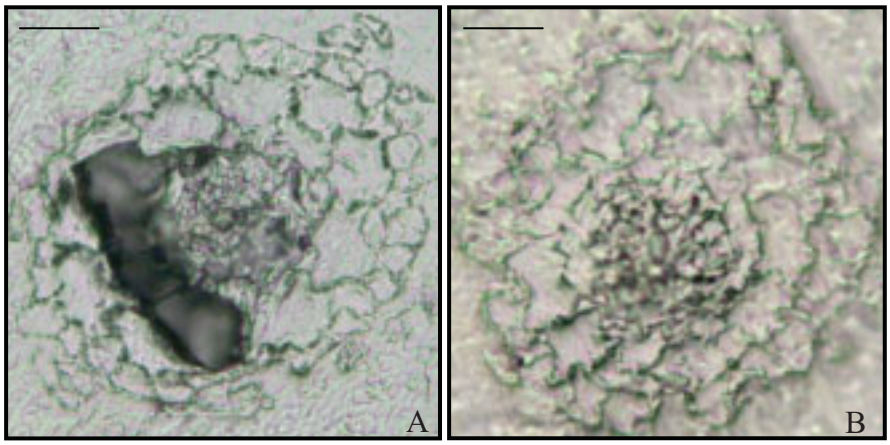

Figure 14: Microtom sections of DAB stained pBIN121-BvGLP-1 transgenic roots. The H_2O_2 staining signal was restricted mainly in the developed syncytium (**A**), whereas no DAB staining signal was present in the non inoculated control (**B**). The bar equals 250µm.

4.8 *BvGLP-1* activates plant resistance response

Well known is that H_2O_2 can act as a secondary messenger activating plant resistance responses. To define whether *BvGLP-1* involves the signaling for activation of plant defense mechanisms, we analysed the transcript levels of a set of key regulators as well as defense-related proteins in transgenic Arabidopsis plants and compared them with those in control plants by semi-quantitative RT-PCR (Figure 15, Figure 16). Included are genes, *NPR1* and *EDS1*which are key regulators for resistance responses triggered by TIR-NBS-LRR-R-proteins as well as *SGT1* which is critically positioned in resistance responses mediated by non-TIR-(CC) NBS-LRR-R- proteins (Hammond-Kosack et al. 2003), and genes for PR- and PDF proteins. Four and two independent transgenic lines were analysed, respectively. Unexpected, no significant change in the transcript levels of *NPR1* and *EDS1* as well as *SGT1* was detectable in transgenic plants when compared to the control (Figure 15). However, abundant transcripts for the *PR-1* to *PR-4* genes were detectable in the transgenic plants but not in the control. While the expression of *PDF1.2* was strongly upregulated in transgenic plants, the expression of *PDF2.3* and *PDF2.2* was not changed and the expression of *PDF2.1*

was in contrast downregulated (Figure 16). Thus, we conclude that *BvGLP-1* may play an active role in activation of expression of defense related proteins in downstream to a signaling pathway leading to resistance response.

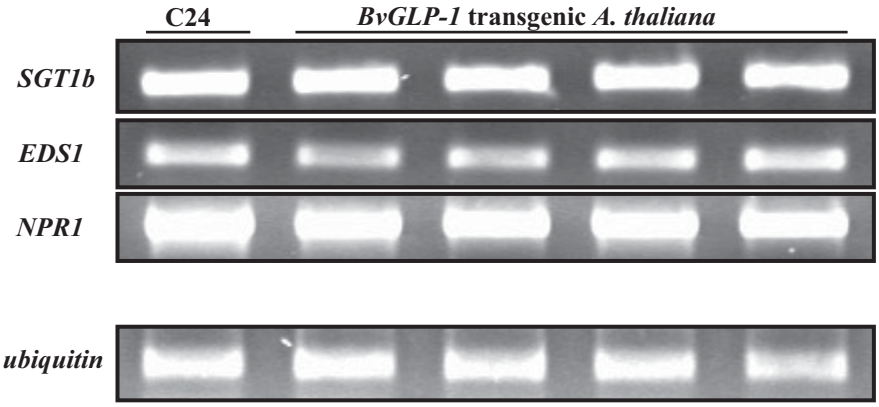

Figure 15: Expression of *BvGLP-1* activated defense related genes in transgenic Arabidopsis. *EDS1, SGT1b, NPR1* gene expression levels were determined by semiquantitative RT-PCR with four independent *BvGLP-1* transgenic Arabidopsis lines and Arabidopsis wild type C24 plants served as control. The mRNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level.

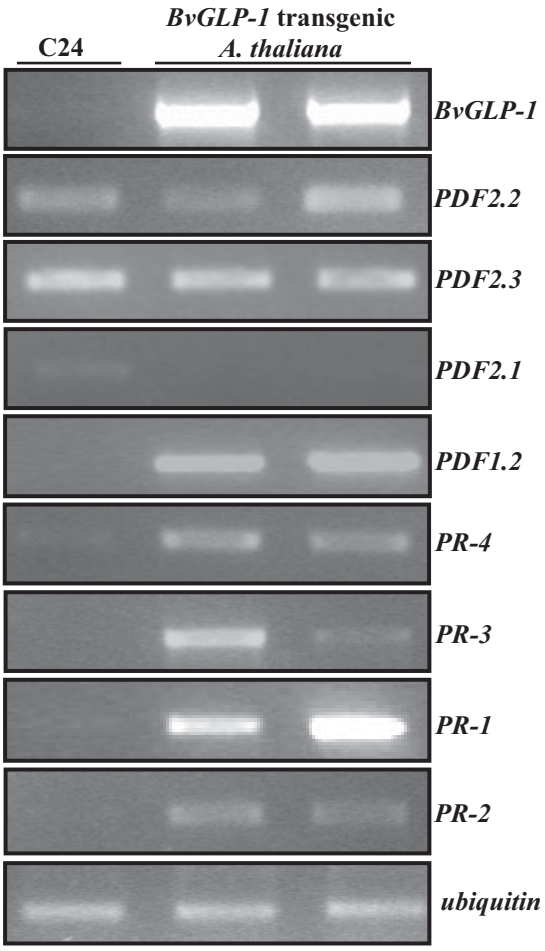

Figure 16: Expression of *BvGLP-1* activated defense related genes in transgenic Arabidopsis. PR and PDF gene expression levels were determined by semiquantitative RT-PCR with 2 independent *BvGLP-1* transgenic Arabidopsis lines and Arabidopsis wild type C24 plants served as control. The *BvGLP-1* transcript is present in all transgenic lines (first lane) and the housekeeping *ubiquitin* gene served as a control (last lane). The mRNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level.

5 Discussion

5.1 *BvGLP-1* is involved in activation of nematode resistance

Transcript profiling techniques and mutant analysis have been successfully applied for identification of a wide array of genes which are involved in both signaling pathways and plant resistance responses. This approach allows the molecular dissection of the signaling networks of various resistance reactions as well as the identification of global regulators governing resistance responses, thus offering the possibility for elucidation of the mechanism underlying and genetic engineering of plant disease resistance (Stuiver et al. 2001). Because the GeneChip for sugar beet is not yet available, we applied the cDNA-AFLP technique to isolate genes from sugar beet genome, which are involved in the resistance responses. Similar to that reported by Samuelian et al. (2004) we obtained from more than 10.000 TDFs only a small number of resistance specific fragments. Of these only one, *BvGLP-1* showed a clear responsive expression upon nematode infection in resistant roots. Instead, ATH1 GeneChip experiments revealed more than 700 transcripts which are differentially expressed between the $Hs1^{pro-1}$ transgenic and the control Arabidopsis plants. Thus, the relatively small number of resistance-specific nematode-induced TDFs out of our cDNA-ALFP experiment reflects the limit of the cDNA-AFLP technique for detection of such time- and tissue-specific expression of genes involved in resistance response. In addition, the majority of genes involved in the resistance pathways may be expressed at very low levels and this expression is locally regulated in feeding cells. Consequently, a number of such genes and especially of syncytium-specific transcripts might have been beyond the detection level of the cDNA-AFLP technique performed in this study and therefore overlooked (Samuelian et al. 2004).

The gene *BvGLP-1* deserved our further attention because of following reasons: (1) *BvGLP-1* is an oxalate oxidase-like germin homolog. The role of germin or GLPs in plant defense has been functionally assessed recently. Transient expression of a pathogen-induced germin in epidermal wheat and barley cells reduced the penetration efficiency of *Blumeria graminis* on transformed cells therefore being more resistant (Schweizer et al. 1999a; Christensen et al. 2004). Soybean, tobacco, sunflower and rapeseed plants transformed with a wheat germin showed enhanced resistance to *Sclerotinia sclerotiorum* (Zaghmout et al. 1997; Donaldson et al. 2001; Hu et al. 2003; Dong et al. 2008) whereas poplar expressing the same gene was more resistant to *Sclerotinia musiva* (Liang et al. 2001). Plant defense related proteins were also induced when a barley germin was introduced into sunflower (Hu et al. 2003) and

furthermore, expression of a wheat germin gene in corn increased the resistance against insect predation (Ramputh et al. 2002); (2) as revealed by transcript profiling experiments, the gene *BvGLP-1* coding for a germin-like oxalate oxidase is transcribed in both resistant and susceptible plants at a relatively low level even without nematode attack. But, its expression is highly upregulated upon nematode infection in the resistant plants, suggesting its active role in nematode resistance response. A constitutive expression of the gene at a low level in both resistant and susceptible plants may generally imply its contribution to the plant basal resistance.

The potential of the gene in activation of nematode resistance was investigated by functional analysis in transgenic sugar beet hairy roots and Arabidopsis plants. Both sugar beet hairy roots and Arabidopsis plants have been widely used as reliable test systems for studying host-parasite interaction, and especially for investigation of a certain gene upon nematode attack (Urwin et al. 1995, 1997; Cai et al. 1997; Kifle et al. 1999, Cai et al. 2003, Samuelian et al. 2004). Even though a high variability in the number of developed females among the transgenic hairy roots as well as Arabidopsis plants was observed, the majority of transgenic sugar beet hairy roots and Arabidopsis plants showed a significant anti-nematode effect: The number of developed female nematodes was significantly reduced and the regular development of nematodes into females was inhibited when compared to the controls. The same resistance mechanism has been described in resistant sugar beet plants (Cai et al. 1997). Moreover, a feeding cell-specific expression of the gene driven by the $Hs1^{pro-1}$ promoter was sufficient to activate nematode resistance in both sugar beet roots and Arabidopsis plants, providing an indubitable role of the gene in activation of nematode resistance.

5.2 *BvGLP-1* is a functional oxalate oxidase

Sequence analysis of *BvGLP-1* based on the database provides the first indication that *BvGLP-1* may represent a member of a germin-like OxO. Amino acid alignment analysis revealed high homology to various plant GLPs with OxO activitiy, in which all crucial residues and subdomains (Woo et al. 2000) characteristic of a GLP-like OxO were conserved, implying its enzymatic nature. Germin and GLPs belong to a subgroup of plant cupin proteins, in which several members contain enzymes with OxO activities which catalyze the degradation of OA to produce carbon dioxide and hydrogen peroxide (Chiriboga 1966; Lane et al. 1993). Inoculation of wheat leaves with *Blumeria graminis* f.sp. *tritici* induced the germin gene expression (Schweizer et al. 1999), accompanied by an increase of the OxO

activity. Similarly, the OxO activity was observed to be corresponding to an accumulation of germin isoforms in powdery mildew infected barley leaves (Dumas et al. 1995). Thus, a role of germin and GLPs in plant defense response has been proposed mainly based on the capacity of the oxalate oxidase to produce H_2O_2, a reactive oxygen species.
The Arabidopsis genome contains at least twelve GLP genes (Carter and Thornburg 1999; Carter et al. 1998), similar to barley (Druka et al. 2002; Wu et al. 2000) and rice (Membré and Bernier 1998). The role of the enzymes encoded by GLPs in dicots is still being debated. It is therefore of great interest to find out the functional relationship and divergence between/among various subgroups. In the wilting experiment, transgenic Arabidopsis plants expressing the gene were able to survive in a culture solution with a relatively higher concentration of OA than the control plants. This can be explained by a higher OxO activity due to the overexpression of the gene in plant cells, which let to detoxification by degradation of OA into $C0_2$ and H_2O_2 (Thompson et al. 1995). In consistence, strong DAB staining signals were observed either in whole transgenic roots when the 35S promoter was applied or majorly restricted in feeding cells when the gene expression was driven by the $Hs1^{pro-1}$-promoter. This is in consistence with the observation reported previously that the $Hs1^{pro-1}$-promoter activates a nematode-induced and syncytium-specific gene expression as demonstrated with GUS reporter gene (Thurau et al. 2004). The H_20_2 production can occur in cells which are mechanically destroyed by the nematodes or cells incorporated into or directly adjacent to the syncytium. It is unlikely that the detected H_20_2 staining signal is a consequence of mechanical damage, because no or very weak signals were visible in the control cells although they were also penetrated by nematodes. When *BvGLP-1* was overexpressed e.g. in case with the 35S promoter a lower regeneration rate as well as stagnation of the growth of both transgenic beet roots and Arabidopsis plants were observed, which obviously suffered from an excessive hydrogen peroxide stress produced by *BvGLP-1*.

5.3 *BvGLP-1* represents a key regulator of the $Hs1^{pro-1}$-mediated nematode resistance

Arabidopsis is susceptible to *H. schachtii* and no natural resistance has been identified, so far. A homologous sequence of $Hs1^{pro-1}$ (At2g4000) is present in the Aarbidopsis genome with more than 45% amino acid identity, but the sequence was not able to trigger resistance even when the sequence was overexpressed in Arabidopsis plants (data not shown). In contrast, the $Hs1^{pro-1}$ seems to be able to activate a similar resistance mechanism in Arabidopsis plants as that observed in sugar beet. Transgenic plants expressing $Hs1^{pro-1}$ showed a significant reduction in the number of developed females. A small fraction of females developed into

cysts, but they were not filled with eggs. Evidently, strong necroses occurred around the nematode penetration sites on the *Hs1*$^{pro-1}$ transgenic roots as revealed by microscopic observation. Thus, it is reasonable to conclude that *Hs1*$^{pro-1}$ initiates HR in or around nematode feeding cells, which restricts expansion of syncytium finally leading to its stagnation (Waetzig et al. 1997, Holtmann 2000). The resistance phenotype of *A. thaliana* expressing *Hs1*$^{pro-1}$ was stably inherited and transmitted to several generations (data not shown). Similarly, McLean et al. reported (2007) an enhanced resistance of soybean against SCN after transfer of a 5′-extended *Hs1*$^{pro-1}$ sequence into soybean, but also with a high variability in the cyst number of each plant. A relatively high variability in the number of females in transgenic Arabidopsis and soybean plants may reflect the "epistatic" effects caused by different genetic backgrounds. It is worth noting that so far no complete resistance could be observed either in transgenic sugar beet plants or in Arabidopsis and soybean, therefore a second house-specific "switch gene" was proposed to be crucial for a stable resistance expression (Schulte et al. 2006).

Our working hypothesis on the resistance observed in the *Hs1*$^{pro-1}$ transgenic Arabidopsis involves a resistance mechanism conserved between sugar beet and Arabidopsis including e.g. signaling components and the signal transduction. Following this, we generated transcript profiles for the *Hs1*$^{pro-1}$ transgenic plants using the Arabidopsis ATH1 GeneChip. Our results demonstrated that a set of genes were regulated by *Hs1*$^{pro-1}$, including 217 transcripts up- and 492 transcripts downregulated. About 65% are genes with defined functions (data in preparation). Strikingly, three GLPs, (*GLP3, 6*, and *9*) were identified in the most upregulated first ten genes. Although the biological function of these genes remains largely unknown, their expression was reported to be related to stress or defense response (Carter et al. 1998). There are several lines of evidence suggesting that *GLP3* may represent a homolog sequence of *BvGLP-1* in Arabidopsis: (1) *GLP3* shares the highest homology to *BvGLP-1* of 42% at the amino acid level; (2) it gave the highest transcript change fold by transcript profiling analysis with the *Hs1*$^{pro-1}$ transgenic Arabidopsis; (3) the sequence indicates a potential function as an oxalate oxidase and (4) *glp3*-ko Arabidopsis Col-0 plants showed a significantly enhanced susceptibility to nematode infection as compared to wild type. Therefore, we conclude that *Hs1*$^{pro-1}$ activates the expression of *BvGLP-1* and *GLP3* in both sugar beet and Arabidopsis, respectively, which consequently lead to resistance response.

5.4 A possible action mode of *BvGLP-1*

The resistance enhancing effect could partially be uncoupled from its OxO activity. It may play a structural role in attached roots by e.g. serving as cross-linking substrate for cell wall reinforcement. Germin and GLPs were demonstrated to accumulate in epidermal cells after pathogen attack (Schweizer et al. 1999; Wei et al. 1998). Due to its OxO activity, *BvGLP-1* can be considered to involve nematode resistance response at different levels: First, activation of *BvGLP-1* may lead to degradation of OA in attached cells, a causal agent produced by many fungal pathogens (Hollowell et al. 2001; Zou et al. 2007). OA aids the pathogen in infection through a number of proposed routes, like reducing the pH in its environment, increasing polygalacturonase activity and sequestering calcium ions from calcium pectate (Punja et al. 1985; Stone and Armentrout 1985; Zou et al. 2007). OA suppresses the oxidative burst (Cessna et al. 2000) and serves as an elicitor signaling a PCD that is required for pathogenicity as well as for disease development of *S. sclerotiorum*, a necrotrophic ascomycete fungus; second, through the generation of H_2O_2, *BvGLP-1* may catalyse cross-linking of plant cell wall proteins at the infection site and lignification for the reinforcement of the cell wall (Olson and Varner 1993; Thordal-Christensen et al. 1997; Wei et al. 1998), and third, activation of *BvGLP-1* leads to the oxidative burst. The H_2O_2 produced can act as a signaling molecule by inducing a HR that finally leads to resistance response (Levine et al. 1994: Zhou et al. 1998).
The rapid generation of ROS, the oxidative burst, is the first plant reaction in response to an attack by avirulent or virulent pathogens. In incompatible interactions between avirulent pathogens and resistant plants this non-specific weak and transient ROS production is followed by a second burst with massive and prolonged ROS accumulation finally inducing the HR (Bestwick et al. 1997; Melillo et al. 2006). A possible involvement of H_2O_2 was demonstrated by Waetzig et al. (1999). They proposed that both plasma membrane-bound and extracellular enzymes may take part in the generation of H_2O_2 which is directly connected to the defense response of *A. thaliana* against *H. glycines*.

To test whether Arabidopsis plants overexpressing *BvGLP-1* induce a specific pathway leading to activation of defense genes, we analyzed the expression of genes, *NPR1* and *EDS1* as well as *SGT1*, and a set of PR and PDF genes, representative for different signaling pathways (Thomma et al. 1998; Hammond-Kosack et al. 2003). When *BvGLP-1* was overexpressed in Arabidopsis, none of *NPR1* and *EDS1* as well as *SGT1* was found to be regulated in transgenic plants. Therefore, we conclude that *BvGLP-1* does not participate in

the early signaling events in activation of the *Hs1*$^{pro-1}$ mediated resistance. In contrast, transgenic plants expressing the gene exhibited enhanced transcripts for *PR-1, PR-2, PR-3, PR-4* and for *PDF1.2* as well. Hence, we conclude that *BvGLP-1* expression in Arabidopsis leads to the activation of both the SA dependent as well as the pathogen-induced ET/JA-dependent pathways, upregulating genes encoding proteins with known antimicrobial properties (e.g. *PR-2*, *PR-3, PR-4* and *PDF1.2*). These data strongly suggest that *BvGLP-1* may involve the second oxidative burst in the *Hs1*$^{pro-1}$ mediated incompatible interaction, thus representing a major "switch" signaling component of defense mechanisms by directly regulating the expression of defense-related genes. In support of this, we found recently that overexpression of *BvGLP-1* in Arabidopsis leads to a broad resistance against the phytopathogenic fungi *Rhizoctonia solani* and *Verticillium longisporum* (Knecht et al. submitted).

6 Acknowledgments

This work was financially supported by the Deutsche Forschungsgemeinschaft (grants No. CA 220/2-3, SFB617- A19) and the EU (FAIR6-CT08-4235).

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Chapter III

Overexpression of *BvGLP-1* encoding a germin-like protein from sugar beet in Arabidopsis leads to resistance against phytopathogenic fungi (*Rhizoctonia solani* and *Verticillium longisporum*), but does not affect the beneficial interaction with the growth-promoting endophyte *Piriformospora indica*

(Submitted to MPMI)

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Key words: Oxalate oxidase-like germin, ROS, *Rhizoctonia solani*, *Verticillium longisporum, Piriformospora indica,* disease resistance.

1 Abstract

BvGLP-1 from sugar beet (*Beta vulgaris*) regulates the $Hs1^{pro-1}$-mediated nematode (*Heterodera schachtii*) resistance. Because *BvGLP-1* functions as an active oxalate oxidase producing hydrogen peroxide in plant cells, we assume that the gene can also be involved in plant resistance against fungal phytopathogens. We transferred *BvGLP-1* into Arabidopsis and challenged the transgenic plants with the pathogenic fungi, *Verticillium longisporum* and *Rhizoctonia solani*, as well as with the beneficial endophytic fungus *Piriformospora indica*. Expression of *BvGLP-1* in Arabidopsis results in significant resistance to the two pathogens, but does not affect the beneficial interaction induced by *P. indica*. However, in older plants on soil, the antifungal activity of *BvGLP-1* restricts growth of *P. indica* and thus abates the benefits for the plants to some extent, without causing any harm. Molecular analysis demonstrated that expression of *BvGLP-1* in Arabidopsis activates the expression of the pathogenesis-related proteins PR-1 to PR-4 and PDF1.2, but not of PDF2.3. In contrast, the PDF2.1 mRNA level was down-regulated. These data suggest a central role of *BvGLP-1* in regulating plant defence responses following a specific signaling route that diverges from that induced by the beneficial fungus *P. indica*.

2 Introduction

Plant genomes encode a subgroup of cupins called germin and germin-like proteins (GLPs), water soluble, protease resistant, heat stable and SDS-tolerant glycoproteins (Lane 1994, Woo et al. 2000), which assemble into homohexameric complexes *in vivo* (Zhang et al. 1995; Vallelian et al. 1998; Christensen et al. 2004). Several members of the germin family contain enzymes with oxalate oxidase (OxO) activities which catalyze the degradation of oxalic acid (OA) to produce carbon dioxide and hydrogen peroxide (Chiriboga 1966; Lane et al. 1993). OxO have been proposed to play an important role in several aspects of plant development or stress tolerance, and especially received considerable attention for their possible contribution to plant defense against pathogens. Through the generation of H_2O_2, OxO or OxO-like proteins may catalyse cross-linking of plant cell wall proteins in papillae at the infection site and lignifications for the reinforcement of the cell wall (Olson and Varner 1993; Thordal-Christensen et al. 1997; Wei et al. 1998). On the other hand, OxO may act as a signaling molecule by inducing a hypersensitive response (HR) in a direct or indirect manner (Lane 1994; Zhou et al. 1998) which is known to be orchestrated by H_2O_2 production during oxidative burst (Levine et al. 1994).

A wide variety of fungal pathogens produce OA in the plant cells following infection (Bateman and Beer 1965; Stone and Armentrout 1985; Bennett and Hindal 1989; Ritschkoff et al. 1995; Liang et al. 2001; Hollowell et al. 2001; Zou et al. 2007). OA aids the pathogen in infection through a number of proposed routes, like reducing the pH in its environment, increasing polygalacturonase activity and sequestering calcium ions from calcium pectate (Punja et al. 1985; Stone and Armentrout 1985; Zou et al. 2007). Also OA suppresses the oxidative burst (Cessna et al. 2000) and disturbes the guard cell function (Guimaraes and Stotz 2004). Kim et al. (2008) demonstrate that OA acts as an elicitor and a signaling molecule for inducing a programmed cell death (PCD) that is required for pathogenicity as well as for disease development of *Sclerotinia sclerotiorum*, a necrotrophic ascomycete fungus. Activation of OxO leads to the degradation of OA and this may represent an important aspect of plant resistance mechanism against fungal pathogens.

Transgenic technology has been used for demonstration of the function of OxO in enhancing plant resistance to various pathogens. Transgenic oilseed rape expressing a barley OxO gene can degrade exogenously applied OA (Thompson et al. 1995). Soybean, tobacco, sunflower and rapeseed plants transformed with a wheat OxO gene showed enhanced resistance to *S. sclerotiorum*, by OA detoxification (Zaghmout et al. 1997; Donaldson et al. 2001; Hu et al.

2003; Dong et al. 2008) whereas poplar expressing the same gene was more resistant to *S. musiva* (Liang et al. 2001). In sunflower overexpression of a barley OxO gene induced plant defense related proteins (Hu et al. 2003) suggesting an active role of OxO in plant defense reactions. Similarly, epidermal wheat and barley cells transiently overexpressing GLP genes are more resistant against powdery mildew *Blumeria graminis* (Schweizer et al. 1999; Christensen et al. 2004), and furthermore, expression of a wheat OxO gene in corn increased the resistance against insect predation (Ramputh et al. 2002).

Recently, we cloned the gene *BvGLP-1* from the $Hs1^{pro-1}$ nematode (*Heterodera schachtii*) resistant sugar beet (*Beta vulgaris*). *BvGLP-1* is a GLP exhibiting OxO activity which plays a central role in the $Hs1^{pro-1}$–mediated nematode (*H. schachtii*) resistance in sugar beet (Knecht et al., in preparation). Because the *BvGLP-1* is an active OxO producing H_20_2 in plant cells, we assume that the gene could also be involved in plant resistance mechanisms against fungal phytopathogens.

The *Rhizoctonia solani* complex and *Verticillium* spp. represent two economically important groups of soil-borne pathogens with a great diversity of host plants. *R. solani* infects primarily roots and stems (Keijer 1996; Weinhold and Sinclair 1996). Within the cortex, the rapid hyphal growth ultimately results in the collapse of the infected plants whereas *Verticillium* spp. invades the vascular system of the host plants through the root and systemically spreads by conidia during the vegetation period (Zeise and Tiedemann 2002). Both *R. solani* and *Verticillium* spp. infect Arabidopsis. The endophytic fungus *Piriformospora indica* colonizes roots of many plant species including Arabidopsi*s* resulting in an increase in the biomass of roots and shoots (Sahay and Varma 1999; Varma et al. 1999; Rai et al. 2001; Peskan-Berghöfer et al. 2004). The beneficial fungus promotes nutrient uptake, allows plants to survive under water and salt stress, confers resistance to toxins, heavy metal ions and pathogenic organisms as well as stimulates seed production (Oelmüller et al. 2004, 2005; Peskan-Berghöfer et al. 2004; Pham et al. 2004; Sahay and Varma 1999; Shahollari et al. 2005, 2007; Varma et al. 1999, 2001; Verma et al. 1998; Waller et al. 2005).

In response to microbes, two distinct types of resistance are induced systemically throughout the plant: systemic acquired resistance (SAR, Ross 1961b) and induced systemic resistance (ISR, Kloepper et al. 1992). While SAR is induced by an infection with a necrotizing pathogen, ISR follows the colonization of the rhizosphere with selected non-pathogenic plant growth-promoting rhizobacteria (PGPR, Pieterse et al. 1996). PGPR are present in large numbers on the root surface and protect plants from pathogen infection through induction of systemic resistance, but without provoking any symptoms themselves. Specific recognition

between protective ISR-inducing rhizobacteria and the plant activates the signaling cascade leading to ISR. The downstream signaling events in the rhizobacteria-mediated ISR pathway differ from those in the pathogen-induced SAR pathway. ISR is mainly regulated by the jasmonic acid (JA) and ethylene (ET) signaling routes (Van Baarlen et al. 2007; Glazebrook 2005). JA induces the expression of genes encoding defense related proteins, such as thionins (Epple et al. 1995) and proteinase inhibitors (Farmer et al. 1992), whereas ethylene activates several members of the *PR* gene superfamily (Potter et al. 1993). Both regulators are involved in the activation of genes encoding plant defensins (Penninckx et al. 1996) and enzymes for phytoalexin biosynthesis (Gundlach et al. 1992). SAR is characterized by an early increase in salicylic acid (SA; Malamy et al. 1990) and the concomitant activation of *PR-1* gene expression (Ward et al. 1991) whereas plants expressing ISR did not, confirming that ISR and SAR are controlled by distinct signaling pathways that diverge in their requirement for SA. PR-1 has been extensively used as a marker for SA-mediated SAR defense, whereas the expression level of PDF1.2 and PDF2.3 is not influenced by SA (Pieterse et al. 1998, 1999).

Here we demonstrate that expression of *BvGLP-1* confers resistance to two fungal pathogens, *R. solani* and *V. longisporum*, but does not affect the beneficial interaction induced by *P. indica* in transgenic Arabidopsis even though the antifungal activity of *BvGLP-1* seems to restrict growth of *P. indica* in older plants without causing any harm. Furthermore, *BvGLP-1* activates expression of a set of defense-related proteins in Arabidopsis. These data suggest that *BvGLP-1* establishes plant resistance following a signaling route diverging from that induced by *P. indica*.

3 Material and Methods

3.1 Plant material and fungal strains

Arabidopsis thaliana C24 (Lehle Seeds, Round Rock, USA) was used for generation of transgenic plants. The *Rhizoctonia solani* strain AG2-1, kindly provided by Dr. Jianrong Guo (Institut für Phytopathologie, Kiel), was grown on potato dextrose agar for seven days at 24°C with a 12 h photoperiod according to Keijer et al. (1996). The *Verticillium longisporum* isolate 43, kindly provided by Dr. Elke Diederichsen (Institut für Biologie, Angewandte Genetik, FU Berlin, Germany), was cultured on potato dextrose agar (PDA) for 14 days in the dark and further cultivated every two to three weeks according to Eynck (2007).

3.2 Generation of transgenic *A. thaliana* plants

BvGLP-1 was cloned into the binary vector pAM194 (KWS Saat AG, Einbeck, Germany) under the transcriptional control of the 35S promoter resulting in the plant expression construct pAM194-BvGLP-1. The recombinant binary vector was transformed into *A. tumefaciens* strain GV3101 (Koncz and Schell 1986). Transgenic Arabidopsis plants were generated by using the root transformation protocol (Valvekens et al. 1988). Transgenic plants were propagated under selective condition to the T3 generation. The homozygous phenotype was selected by kanamycin resistance. The presence of the transgene was confirmed by GUS assay, transgene-specific PCR amplification and Southern hybridization.

3.3 Infection assay of Arabidopsis plants with Rhizoctonia on agar plates

Transgenic as well as wild type Arabidopsis seeds were sterilized with 5% $CaCl_2O_2$ for 10 min and then in 70% ethanol for further 5 min. After washing with sterile water, seeds were germinated on 0.2 x Knop medium for 5 days in a plant growth chamber (22°C, 16/8h light/dark photoperiod). Seedlings were transferred to new 0.2 x Knop agar plates for infection experiments in which the non-transgenic Arabidopsis seedlings served as a control. The seedlings were incubated with 1 cm diameter mycelium plaques from seven days old potato dextrose *R. solani* culture. The plates were incubated in the growth chamber (22°C, 16/8h light/dark photoperiod). Three, six, nine and 12 days after inoculation, the plant survival ratio was scored.

3.4 Infection assay of Arabidopsis plants with Rhizoctonia in soil

Sterile soil was infected by placing a 1 cm diameter disc of mycelium isolated from seven days old potato dextrose broth culture of *R. solani* at 1 cm depth in each pot 10 days before planting. Arabidopsis seeds were sterilized as described before and grown at 22°C with a 16/8 h light/dark photoperiod on agar plates containing 0.2 x Knop medium for seven days. One week old Arabidopsis seedlings were transferred to infected pots, and grown in a growth chamber at 22°C with a 16/8h light/dark photoperiod. Based on the symptoms, scored seven, 14 and 21 days after planting, plants were classified either to healthy (no or scarce disease symptoms) or diseased (severe wilting or dead plants) groups.

3.5 Infection assay of Arabidopsis plants with Verticillium in soil

Arabidopsis plants were cultivated in soil in small pots (diameter 35 mm) under short day conditions (8h/16h light/dark photoperiod) for 14 days. Infection with *V. longisporum* was conducted using 1 ml of conidia (4 x 10^7 conidia ml^{-1}) obtained from liquid CZAPEK-DOX medium. Plants were weekly scored (7, 14, 21, 28 dpi) based on the developed disease symptoms and classified into nine classes (Zeise 1992) with following modifications: 1, no symptoms; 2, slight symptoms on oldest leaf (yellowing, black veins); 3, slight symptoms on next younger leaves; 4, about 50% of leaves show symptoms; 5, >50% of leaves show symptoms; 6, up to 50% of leaves are dead; 7, >50% of leaves are dead; 8, only the apical meristem is still alive; 9, plants are dead.

3.6 Cocultivation experiments with *P. indica*

Arabidopsis seeds were surface sterilized and placed on petri dishes containing MS nutrient medium (Murashige and Skoog 1962). The plates were incubated for seven days at 22°C under continuous illumination (100 µmol m^{-2} s^{-1}). *P. indica* was cultured as described previously (Peškan-Berghöfer et al. 2004). After inoculation with the fungus the plates were kept at room temperature in the dark for one to two weeks. Nine days old *A. thaliana* seedlings were transferred to nylon discs (mesh size 70 µm) placed on top of a modified PNM culture medium (Peškan-Berghöfer et al. 2004). One seedling was used per Petri dish. After 24 h, fungal plugs of 5 mm in diameter were placed at a distance of 1 cm from the roots. Plates were then incubated at 22°C under continuous illumination from the side. Plants were assayed 4, 6, 8, 10, 12, 14 days after co-cultivation. The experiments on soil were followed the protocol described by Peškan-Berghöfer et al. (2004). Arabidopsis seedlings were

germinated on MS medium before transfer to sterile soil mixed with the fungus (0.6 %, w/w). Cultivation occurred in multi-trays with Aracon tubes in a plant growth chamber at 22°C under continuous illumination and long day conditions. For the exposure of Arabidopsis seedlings to a loan of *P. indica*, the fungus was inoculated in liquid KM media until an OD_{650} of 0.5 and 3.5 ml of this suspension was distributed on top of a petri dish containing 20 ml of the modified PNM culture medium. The plates were then incubated at 22°C under continuous illumination (80 µmol m^{-2} sec^{-1}) for 72 h. During this time, the fungal hyphae started to develop a loan. Nine days old Arabidopsis seedlings were then transferred to the loan.

3.7 Determination of the degree of root colonization

Roots of two months old plants co-cultivated with *P. indica* were used for the determination of colonization. The colonized (and control) roots were removed from the soil, intensively rinsed with an excess of sterile water (50 ml each) to remove the soil and the loosely attached fungal hyphae, and were then frozen in liquid nitrogen for RNA or DNA extraction. *P. indica* was monitored with a primer pair for the translation elongation factor 1 (*Pitef1*; Bütehorn et al. 2000): ACCGTCTTGGGGTTGTATCC and TCGTCGCTGTCAACAAGATG. The degree of the root colonization was determined based on the amount of DNA or RNA of the fungus relative to the *actin* DNA or RNA from the plant.

3.8 Staining assays and light microscopy observations

Lactophenol blue as well as safranin red /anillin blue staining were used to analyse host-pathogen interaction (Perl-Treves et al. 2004). To monitor fungal association with Arabidopsis roots, seedlings were removed from agar plates and washed under running tap water for 2 min to remove non-associated hyphae. The lactophenol blue staining was carried out in small petri dishes with gentle shaking, and seedlings were cleared using hot 10% (w/v) KOH for 10 min. The seedlings were subsequently stained using lactophenol blue solution for 5 min and destained with water for at least 20 min until no more blue colour came out. In addition, seedlings were immersed for 1 to 2 min in 0.25% (w/v) safranin red, then for 1 to 2 min in aniline blue (25% (w/v) lactic acid, 50% (w/v) glycerol, 0.5% (w/v) aniline blue), followed by 1 min of destaining in the same solution without dye. Stained seedlings were examined and documented under the bright field microscope (Zeiss Jena, Germany).

3.9 Semi-quantitative and real-time PCR

Semi-quantitative and real-time RT PCR were used to assay the expression patterns of the genes of interest. Transgenic as well as control Arabidopsis roots were removed from the soil or plates, rinsed 12 times with an excess of sterile water (50 ml each) and frozen in liquid nitrogen for RNA or DNA extraction. Extraction of total RNA from Arabidopsis roots was performed following the trizol protocol (Gibco BRL Life Technologies). The total RNA was treated with RNAse-free DNAse (Fermentas, St. Leon-Rot, Germany) for 60 min at 37°C. Synthesis of cDNA was carried out using a Superscript III First-Strand Synthesis System (Invitrogen, Karlsruhe, Germany) according to the instructions of the manufacturer. The semi-quantitative PCR amplification was performed in 50 µl reactions consisting of 2.5 µl 10 ng/µl cDNA, 5 µl 10 x buffer, 0.5 µl 10 mM dNTPs, 5 µl each of 10 pmol/µl primer, 2.5 U of Taq polymerase (Invitrogen) and 31.5 µl H_20 under the PCR programme: 94°C for 50 s, 54°C for 1 min and 72°C for 1 min for 25 cycles, followed by 10 min at 72°C. Amplicons, separated on a 1% (w/v) agarose gel were visualised under UV-light. The housekeeping *ubiquitin* gene served as a control and the mRNA levels for each cDNA probe were normalized to the *ubiquitin* message RNA level (ACTCTCACCGGAAAGACAATC and TGACGTTGTCGATGGTGTCAG). The real-time PCR was performed using the iCycler iQ Detection System (Bio-Rad). The iQ SYBR Supermix (Bio-Rad) was used for PCR reactions according to the manufacturer´s instructions in a final volume of 20 µl. The iCycler was programmed to 95°C 2 min, 35 x (95°C 30 s, 55°C 40 s, 72°C 45 s), 72°C 10 min followed by a melting curve programme (55 – 95°C in increasing steps of 0.5°C). All reactions were repeated at least twice. The mRNA levels for each cDNA probe were normalized with respect to the *actin* message level. Fold induction values were calculated with the ΔΔCP equaltion of Pfaffl (2001) and related to the mRNA level of the target genes in wild type roots, which were defined as 1.0.

The following primers were used in the described gene expression experiments:

Primer	Primer sequence 5`-3`
PR-1f/r	GGAGCTACGCAGAACAACTA/ AGTATGGCTTCTCGTTCACA
PR-2 f/r	CTACAGAGATGGTGTCA/ AGCTGAAGTAAGGGTAG
PR-3 f/r	TGGATGGGCTACAGCACC/ CTAAATAGCAGCTTCGAGGAGG
PR-4 f/r	GGCCGGACAACAATGCGGTCGTCAAGG/ CAAGCATGTTTCTGGAATCAGGCTGCC
PDF1.2 f/r	ATGGCTAAGTTTGCTTCCA/ TTAACATGGGACGTAACAGATAC
PDF2.1 f/r	GATGGGTCCAGTCACGGTC/ TTCAAGAACACACTAAACACGC
PDF2.3 f/r	CACACACAACTGTGCAAACG/ CGGAAACACACAAACCAATG
BvGLP-1 f/r	CTCCTAGCCTCTTGTAATTCTAGC/ GAATGGAAACAAGCAACATATGATATC

3.10 PCR and Southern analysis

Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as described by Rogers and Bendich (1985). PCR was carried out in a total volume of 20 µl containing 50 ng of template DNA, 10 mM PCR buffer (pH 8.3), 2 mM $MgCl_2$, each dNTP at 0.5 mM, each primer at 10 pmol, and 1 U of Taq DNA polymerase (Invitrogen) in a thermocycler (Biometra, Göttingen, Germany) under the following conditions: 5 min at 94°C, 60 s at 94°C, 60 s at 55 °C, 1 min 30 s at 72°C for 34 cycles, followed by 10 min at 72°C. PCR products were separated on a 1% (w/v) agarose gel and analyzed with the software Quantity One (Bio-Rad Laboratories GmbH, München, Germany). For Southern hybridization experiments the extracted DNA was digested with two restriction enzymes (*Eco*RI, *Xho*I), electrophoretically separated on 0.75% (w/v) agarose gels and blotted on Hybond-N^+ membrane (Amersham) by capillary diffusion overnight using 0.25 M NaOH/1.5 M NaCl as blotting solution. Southern blots were hybridized with ^{32}P-labelled DNA probe (Feinberg and Vogelstein 1983) at 60°C, washed twice (0.5 x SSC; 0.2 % (w/v) SDS) for 30 min and exposed at -70°C for 48 h. The DNA probe for Southern hybridization was generated by PCR with *BvGLP-1* gene specific primers CTCCTAGCCTCTTGTAATTCTAGC and GAATGGAAACAAGCAACATATGATATC using plasmid DNA as template. PCR fragments were separated on a 1.3% (w/v) low melting agarose gel before radio-labelling.

4 Results

4.1 Transgenic Arabidopsis plants expressing *BvGLP-1*

Twenty two independent transgenic plants expressing *BvGLP-1* under the control of the 35S promoter were generated and propagated to the T3 generation. Southern hybridization using *BvGLP-1* as a probe revealed one to three copies of the transgene integrated in the Arabidopsis genome. No differences in growth rate and morphology between transgenic and wild type Arabidopsis plants were observed. Two transgenic plants carrying a single copy of the transgene were chosen for further propagation to generate homozygous transgenic lines. The presence of the transgene was also verified by PCR with gene-specific primers. Each homozygous line showed a PCR fragment of 780 bp in size, as expected, whereas no PCR amplification was detected in the control. Furthermore, RT-PCR with *BvGLP-1* specific primers demonstrated that the transgene is expressed in all selected homozygous lines (data not shown).

4.2 Infection experiments of Arabidopsis plants with *R. solani* and *P. indica* on agar plates

BvGLP-1 is implicated to be involved in defense responses by producing H_2O_2 in the extracellular space. To check its role in pathogenic and beneficial plant-fungus interactions, we infected the transgenic Arabidopsis plants with *R. solani* (AG 2-1) and *P. indica* on agar plates in which the wild type C24 plants served as a control. For infection with *R. solani*, wild-type and transgenic plants were transferred to agar plates, inoculated with fungal mycelium and co-cultivated in a growth chamber.

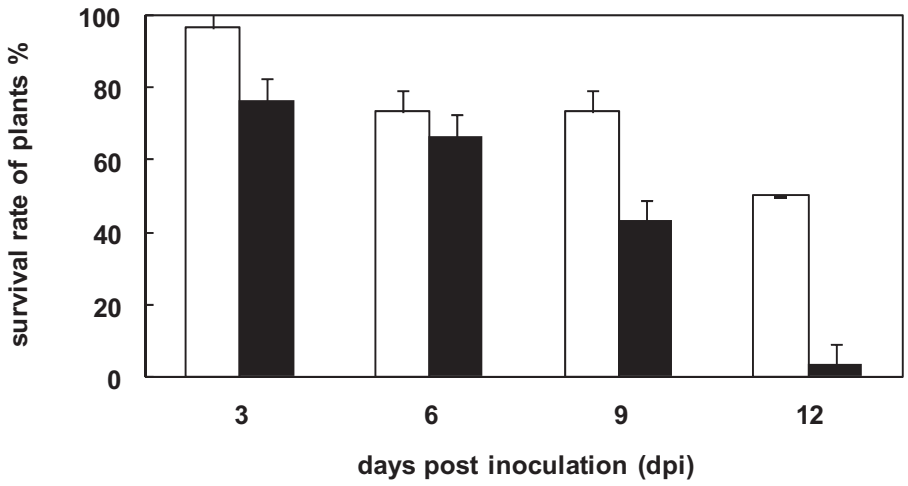

Figure 1: Survival rate of Arabidopsis plants in agar test. The plants were infected with *Rhizoctonia solani* AG2-1. Seven days old seedlings of *A. thaliana* were transfered to agar plates for infection and the plants survival rate was scored 3, 6, 9 and 12 days post inoculation (Black columns: Arabidopsis wildtype C24 and white columns: *BvGLP-1* transgenic Arabidopsis). Bars represent standard errors based on three independent experiments with ten seedlings each.

Three days after infection wild type C24 plants showed clear disease symptoms and 23 ±3 % (n=30) of them suffered from the infection (Figure 1), while only a few transgenic plants (4±1%) showed visible symptoms. Twelve days after infection wild type plants suffered seriously from the fungal infection; they showed strong disease symptoms and 97 ± 3% (n=30) of the infected plants died (Figure 1, Figure 5 A). In contrast, more than 50 ± 3% (n=30) of the transgenic plants were still healthy and grew regularly. These data demonstrated that the expression of *BvGLP-1* in Arabidopsis interferes with *R. solani* infection giving rise to resistance against the pathogen.

A similar experiment was performed with *P. indica*. Wild type and transgenic plants were transferred to agar plates with a low density fungal loan (Figure 2). After ten days of co-cultivation, we observed a 24 ± 4% (n = 60 plates) increase in the fresh weight of wild type seedlings co-cultivated with *P. indica* relative to the uncolonized control. A similar result was obtained for the two transgenic Arabidopsis lines (27 ± 5% and 23 ± 3%, respectively; n = 60 plates) (Figure 3). We conclude that processes leading to *P. indica*-mediated growth promotion of Arabidopsis seedlings on agar plates are independent of *BvGLP-1* and that higher OxO levels do not prevent the beneficial effects for the seedlings.

Figure 2: Co-cultivation of wild type and transgenic Arabidopsis seedlings with *Piriformospora indica* for ten days. *A. thaliana* seedlings were transferred to nylon discs placed on top of a modified PNM culture medium. One seedling was used per Petri dish.

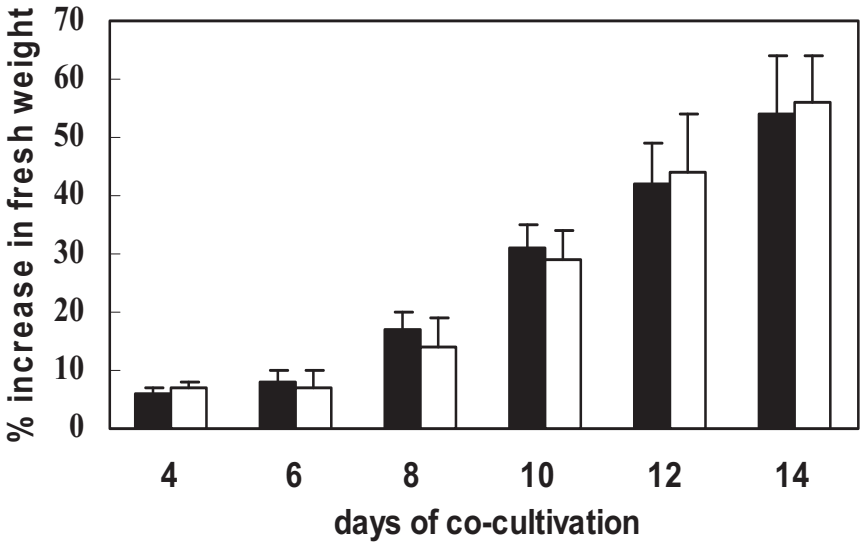

Figure 3: Fresh weight of wild-type and transgenic Arabidopsis seedlings which were co-cultivated with *Piriformospora indica*. The fresh weight of *P. indica*–colonized seedlings at $t = 0$ was set as 100% and all other values are expressed relative to it. Black columns, wild-type plants, white columns average of the two independent *BvGLP-1* transgenic Arabidopsis lines, which did not differ significantly from each other. Bars represent standard errors, based on five independent experiments with 60 seedlings each.

4.3 Infection experiments of Arabidopsis plants with *R. solani* and *V. longisporum* in soil

To verify the data obtained from the agar plate experiments we assessed the resistance of the *BvGLP-1*–expressing Arabidopsis plants against *R. solani* in soil. For the *R. solani* infection assay, wild type and transgenic Arabidopsis plants were directly transferred to pots filled with *R. solani*-infected soil and co-cultivated in a growth chamber in which uninfected plants served as a control. Seven days after co-cultivation, clear differences between wild type and transgenic plants were obvious (Figure 4): strong symptoms appeared on most leaf surfaces of wild type plants and more than 40% ± 3% of them died after *R. solani* infection. In contrast, transgenic plants grew regularly even though slight disease symptoms were visible on a few leaves (Figure 4, Figure 5B). Twenty one days after co-cultivation 87% ± 3% of the wild type plants died after fungal infection while more than 76% ± 3% of transgenic plants survived (Figure 4). These data are consistant with the results obtained from the agar-plate assays and demonstrate that the expression of *BvGLP-1* in Arabidopsis confers resistance against *R. solani* infection.

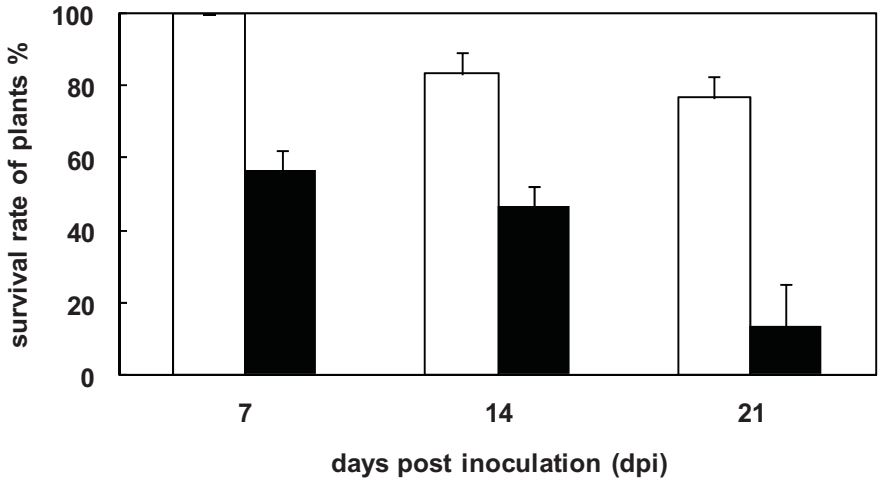

Figure 4: Survival rate of Arabidopsis plants infected with *Rhizoctonia solani* AG2-1 in soil. Seven days old seedlings of *A. thaliana* were transfered to soil for infection. The plant survival rate was scored 7, 14 and 21 days post inoculation (Black columns: Arabidopsis wild type C24, white columns: *BvGLP-1* transgenic Arabidopsis). Bars represent standard errors, based on three independent experiments with ten seedlings each.

A similar experiment was performed with *V. longisporum*. Wild type and transgenic Arabidopsis seedlings were grown in small pots filled with soil for 14 days before infection. The infected plants were weekly scored and classified into the categories 1-9, based on the development of the disease symptom (Table 1).

Table 1: Disease scores on *A. thaliana* plants inoculated with *V. longisporum* in soil. Assessment for scoring disease symptoms induced by *Verticillum* sp. was performed according to Zeise (1992): **1**, no symptoms; **2**, slight symptoms on oldest leaf (yellowing, black veins); **3**, slight symptoms on next younger leaves; **4**, about 50% of leaves show symptoms; **5**, >50% of leaves show symptoms; **6**, up to 50% of leaves are dead; **7**, >50% of leaves are dead; **8**, only the apical meristem is still alive; **9**, plants are dead. Disease scores were made based on 3 independent experiments with 10 seedlings each.

	Total no. of seedlings	Disease scores			
		Days post inoculation (dpi)			
		7	14	21	28
Non-infected C24	30	1	1	1-2	1-2
Infected C24	30	1	2	4-5	5-7
Non-infected *BvGLP-1*	30	1	1	1-2	1-2
Infected *BvGLP-1*	30	1	1	1-2	2

Clear differences in the development of the disease symptoms were observed between transgenic and wild type plants (Table 1, Figure 5 B). Fourteen days after infection the first disease symptoms appeared on wild type plants, in form of chlorosis and dark-coloured veins mainly on older leaves, but not yet on transgenic leaves, on which the first slight disease symptoms were only visible 21 days after infection (Table 1; Figure 5 B). In contrast, 21 days after infection wild type plants suffered from fungal infection and more than 50% of the leaves showed severe disease symptoms (Table 1). Twenty eight days after infection more than 50% of the leaves of wild type plants were dead, while only slight disease symptoms were detectable on the oldest leaves of the transgenic plants (Table 1). These data suggest that the expression of *BvGLP-1* in Arabidopsis significantly reduces the susceptibility of the plants to *V. longisporum* infection.

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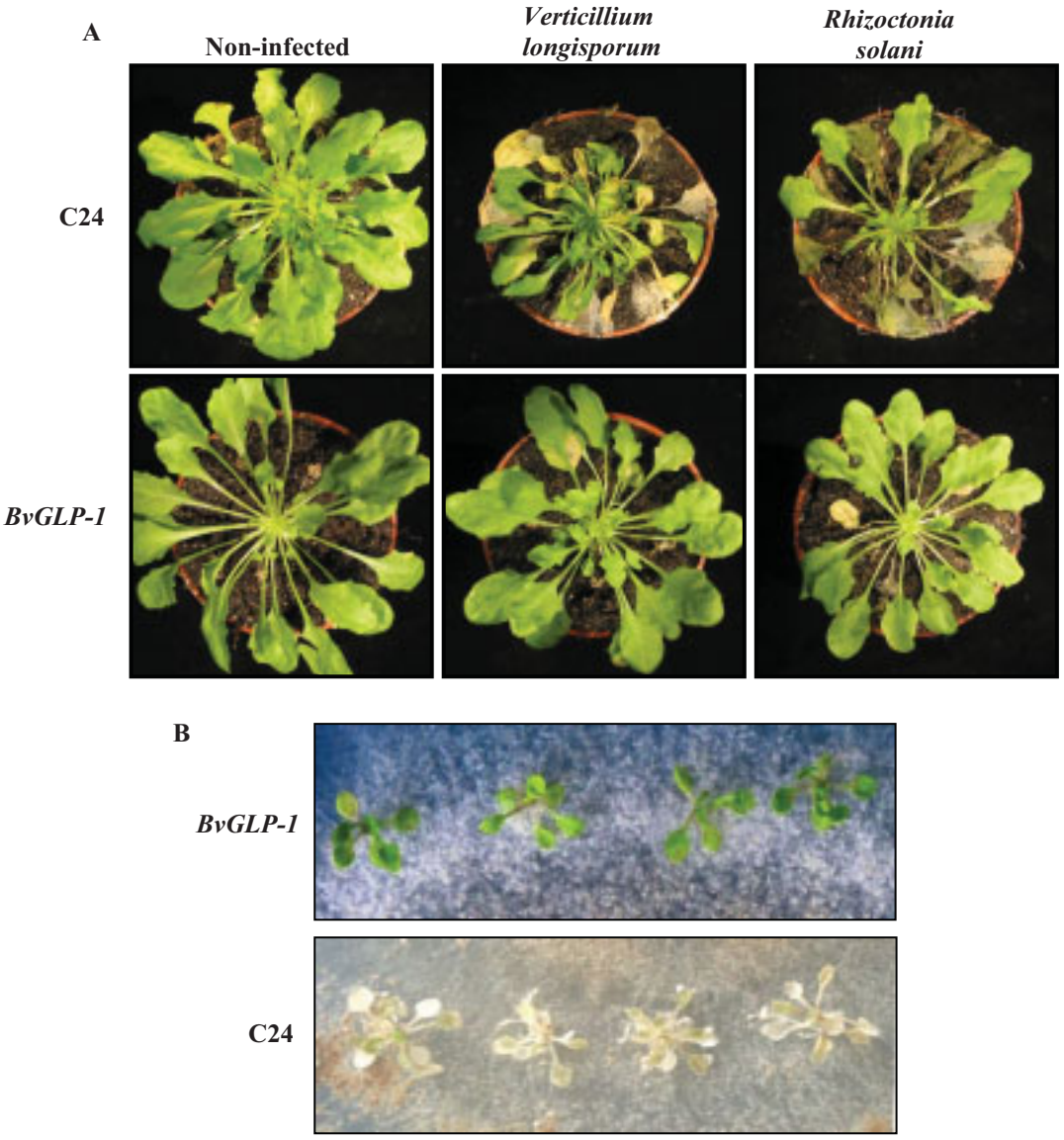

Figure 5: Examples of visual comparison between infected transgenic *A. thaliana* expressing *BvGLP-1* and wild type C24 plants in soil and on agar plates, respectively. **A**: Arabidopsis wild type C24 and *BvGLP-1*-transgenic Arabidopsis plants infected with *V. longisporum* and *R. solani* in soil 21 days post inoculation and **B**: Arabidopsis wild type C24 and *BvGLP-1*-transgenic Arabidopsis plants infected with *R. solani* on agar plates 12 days post inoculation. Non-infected plants served as control.

4.4 Microscopic observations of Arabidopsis roots infected with the phytopahogenic fungi

For microscopic observation of the *R. solani* infection process, we stained the infected roots with lactophenol blue and safranin red/anillin blue solution 72 h post inoculation. The aniline blue/lactophenol blue stained fungal hyphae, whereas safranin red stained the root meristems. Washing the root system under running tap water removed most of the unattached mycelium.

As shown in Figure 6, dramatically reduced amounts of hyphae were visible on transgenic roots compared to wild type roots, suggesting a higher penetration rate of the fungus into the wild type (Figure 6 A-C). In accordance, apart from the lower number of infection cushions, the size of the lesions on the transgenic leaves was also reduced when compared to the wild type plants (Figure 6 E, F). In conclusion, the microscopic observations support the finding from the infection experiments that *BvGLP-1* inhibits fungal infections on Arabidopsis plants.

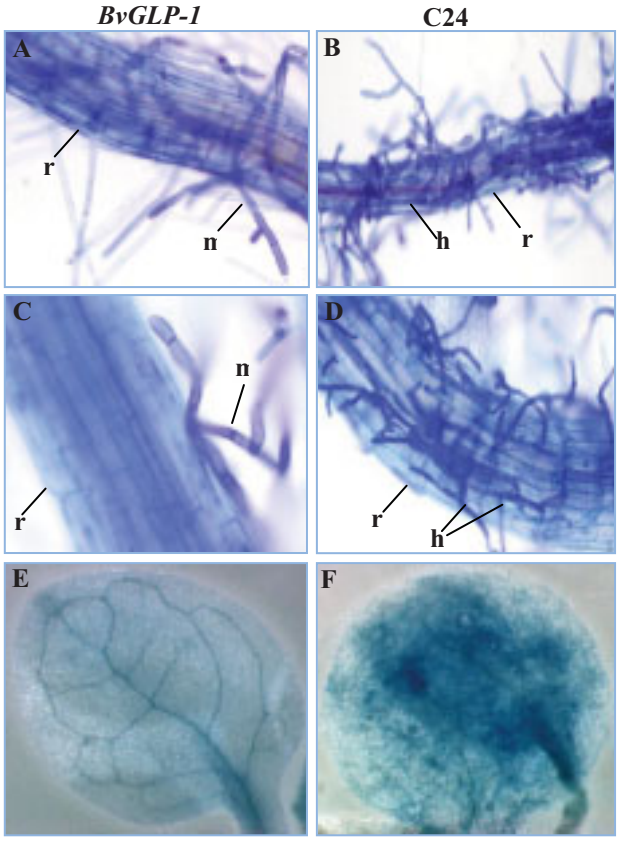

Figure 6: Light microscopic observation of *BvGLP-1* transgenic and wild type Arabidopsis plants infected with *R. solani* after washing and staining with lactophenol blue and safranin red/anillin blue solution 72 h post inoculation. **A** and **B**: safranin red/anillin blue staining, **C-F**: lactophenol blue staining; **A-D**: roots stained; **E** and **F**: leaves stained; **A** and **C**: *R. solani* growing in an undirected manner on the root surface of *BvGLP-1* transgenic Arabidopsis without firm attachment; **B** and **D**: Attachment and directed-growth of *R. solani* hyphae over the root of Arabidopsis wild type C24 and formation of dome-like infection cushions. **E**: intact leaf of *BvGLP-1* transgenic Arabidopsis; **F**: *R. solani* overgrowing Arabidopsis wild type C24 leading to leaf and tissue maceration; r = root; m = mycelium; h = hyphae.

4.5 Analysis of the transcript levels of selected defense related genes in transgenic plants

PR proteins are involved in fungal pathogenesis as well as plant resistance to fungal infection. To define whether the resistance observed in this study is correlated with the activation or increased expression of plant defense-related genes, we analysed the *PR* and *PDF* transcript

levels in the transgenic Arabidopsis plants and compared them with those in wild type plants by semi-quantitative RT-PCR. As expected, all transgenic plants exhibited substantial levels of *BvGLP-1* transcripts (Figure 7, panel 1). Even without pathogen attack, abundant transcript levels for the *PR1-4* genes were detectable in the transgenic plants, but not in wild type plants, whereas no difference in the transcript levels for *PDF2.*3 was visible. Furthermore, while the expression of *PDF1.2* was strongly upregulated in transgenic plants, the expression *PDF2.1* was drastically down-regulated when compared to the wild type (Figure 7, panel 6 and 8). Thus, we conclude that *BvGLP-1* may play an active role in regulating plant defense mechanisms against different pathogens by activation of plant defense-related genes.

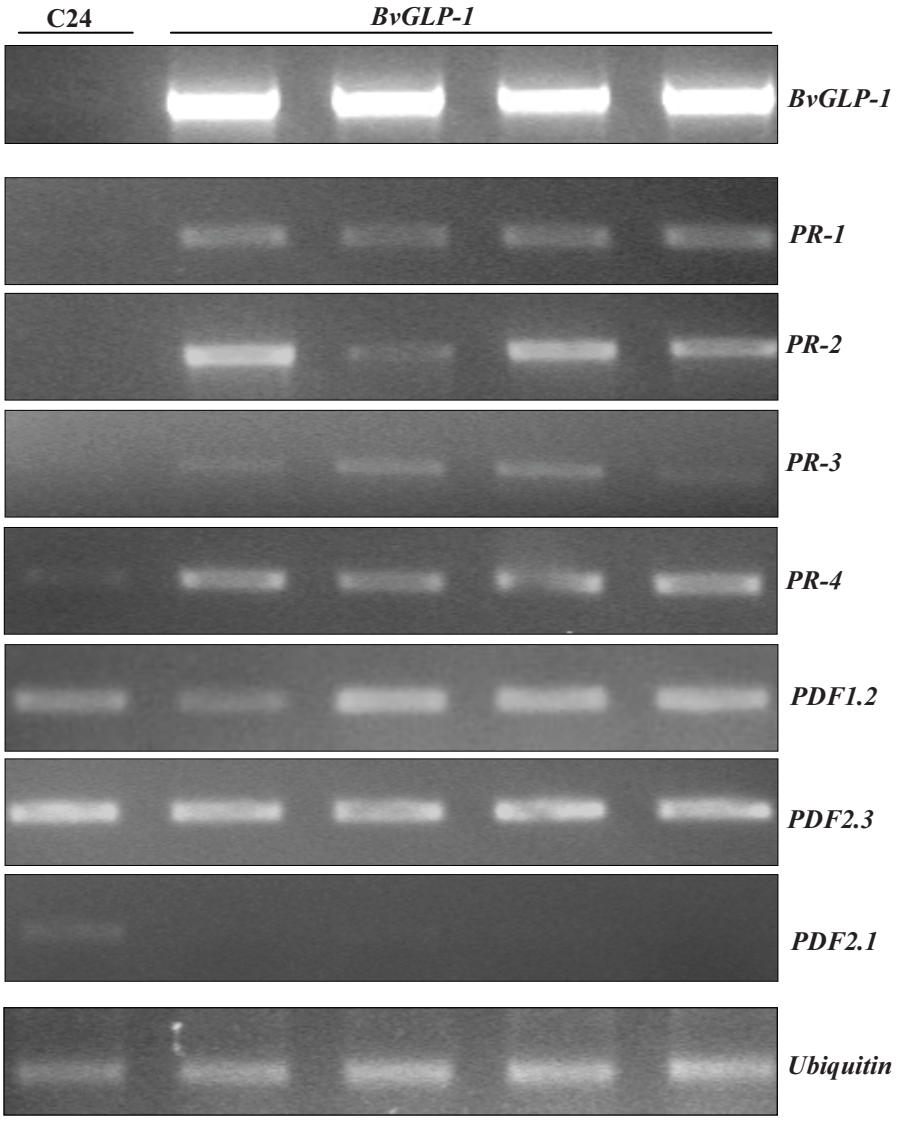

Figure 7: Expression of *BvGLP-1* activated defense related genes in transgenic Arabidopsis. *PR* and *PDF* gene expression levels were determined by semi-quantitative PCR with 4 independent *BvGLP-1*-transgenic Arabidopsis lines and Arabidopsis wild type C24 plants served as control. The *BvGLP-1* transcript is present in all transgenic lines (first lane) and the housekeeping *ubiquitin* gene served as a control (last lane). The mRNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level.

4.6 Co-cultivation of Arabidopsis with the growth promoting fungus *P. indica* in soil

To define the role of *BvGLP-1* in a beneficial plant-fungus interaction, we performed a whole life co-cultivation assay of Arabidopsis with *P. indica* in soil. To this end, we transferred Arabidopsis plants co-cultivated with or without the fungus from agar plate to pots. A growth promotion for the transgenic plants co-cultivated with the fungus was still observed over a period of 30 days, and this promotion was comparable to the wild type (Figure 8). However, while the wild type plants responded to the fungus also during later phases, flowered approximately two weeks earlier than the uncolonized control and produced more seeds, the beneficial effects for the transgenic plants became less. We observed only a slight, but not significant increase in the fresh weight before the plants set flowers, the flowering time was only a few days earlier and the seed yield was increased by only 5 ± 2% compared to the uncolonized transgenic plants. Thus, at the end of the life, the presence of *P. indica* does no longer confer significant benefits to the plants, although the fungus did not cause harm to the plant.

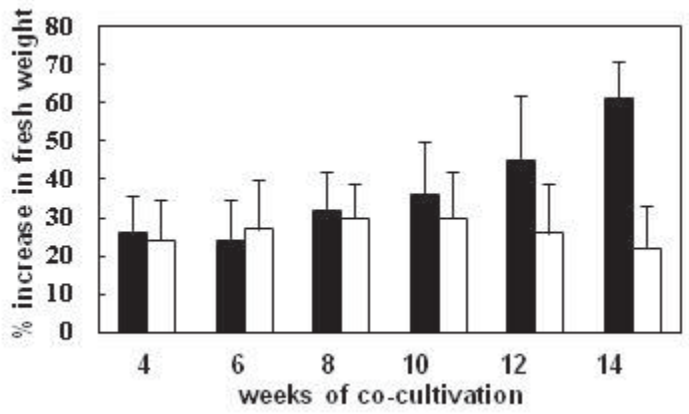

Figure 8: Fresh weight of wild-type and transgenic Arabidopsis plants in pots which were co-cultivated with *Piriformospora indica.* The fresh weight of *P. indica* colonized plants at $t = 0$ was set as 100% and all other values are expressed relative to it. Bars represent standard errors, based on two independent experiments with 60 plants each.

Because root colonization is a critical parameter for the beneficial interaction between *P. indica* and Arabidopsis, we checked the degree of root colonization of the transgenic plants and compared it with the wild type. We extracted DNA and RNA from the roots of 14 day- and two months old plants co-cultivated with *P. indica*. No significant difference in the degree of root colonization was detected for wild type and transgenic plants co-cultivated with the fungus for 14 days, but we observed a significant reduction in the amount of fungal DNA and RNA in the two months old transgenic plants when compared to the wild type (Figure 9). In two independent experiments with 30 plants each, root colonization was

reduced by more than 60% compared to the wild type. This indicates that *BvGLP-1* restricts root colonization during later growth phases, which subsequently leads to a decrease in the benefits to the plant.

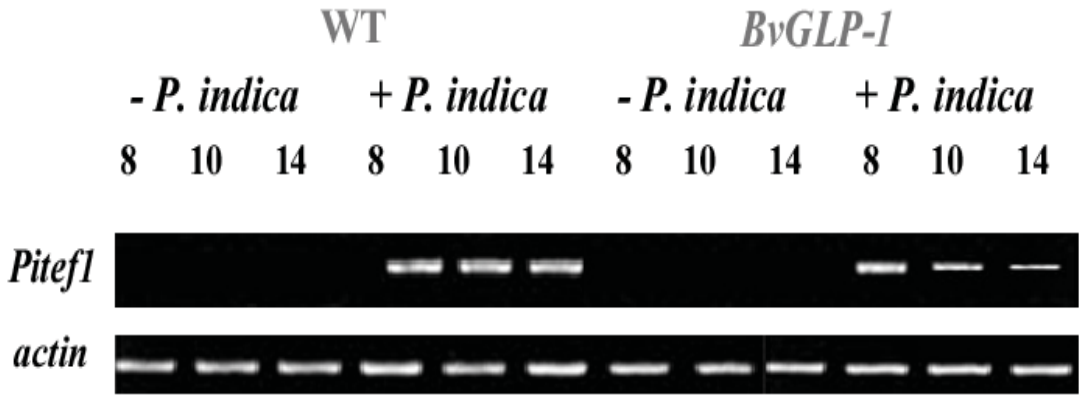

Figure 9: Colonization of the roots of 2 month-old wild-type and transgenic Arabidopsis plants grown in the presence of *P. indica*. The transcript levels of the fungal translation elongation factor 1 mRNA (*cPitef1*) and genomic DNA (*gPitef1*) in the roots of colonized Arabidopsis seedlings was compared to the amount of the plant *actin* nucleic acids. RNA and/or DNA were isolated from the roots of the transgenic lines and the wild-type (wt). After reverse transcription *cPitef1* and *actin* were amplified (left lanes). For the right three lanes, genomic DNA was amplified with the same primers. To obtain quantitative data, real time PCR was performed. The *actin* mRNA normalized *Pitef1* transcript levels of the different lines are expressed.

5 Discussion

GLP genes are members of large multigene families exhibiting diverse patterns of expression (Bernier and Berna 2001). The Arabidopsis genome contains at least six *GLP* genes (Carter and Thornburg 1999; Carter et al. 1998), similar to barley (Druka et al. 2002; Wu et al. 2000) and rice (Membré and Bernier 1998). In sugar beet, *BvGLP-1* is induced in response to nematode infection in nematode resistance sugar beet. Here, we demonstrate that the same protein confers also a broad resistance to *R. solani* and *V. longisporum* when transferred into Arabidopsis. The exact mechanism by which GLPs with OxO activities confer enhanced resistance to these pathogens is unclear.

Signaling pathways required for plant defense responses are complex, and even members from the same gene family that are induced by a single pathogen may require different signal molecules or combinations of signaling pathways for their expression (Ferrari et al. 2003). SA, JA and ET are hormones involved in the regulation of resistance against different pathogens. SA is a key regulator of pathogen-induced SAR (Gaffney et al. 1993), whereas JA and ET regulate a largely distinct set of genes and are required for ISR (Pieterse et al. 2001). Both types of induced resistance are effective against a broad spectrum of pathogens and several lines of evidence demonstrate cross talk between the pathways (Glazebrook 2001). It appears that defense genes that are activated against necrotrophic fungi are regulated primarily by the ET and JA signal transduction pathways, whereas biotrophic pathogens are countered more efficiently by SA-controlled defense mechanisms (Thomma et al. 1998, 1999). To test whether Arabidopsis plants overexpressing *BvGLP-1* induce defense genes from SAR or other defense pathways, we analyzed the expression of *PR* and *PDF* genes, representative for two different signaling pathways (Thomma et al. 1998). In Arabidopsis, induction of *PR-1*, *PR-2*, and *PR-5* follows an SA-dependent pathway, whereas the induction of the plant defensin *PDF1.2*, the basic chitinase gene *PR-3*, and the hevein-like protein gene *PR-4* depends on a pathway involving at least JA as a signal molecule (Li et al. 2004). When *BvGLP-1* was overexpressed in Arabidopsis, transgenic plants exhibited enhanced transcript levels for *PR-1-4* and *PDF1.2*. Hence, *BvGLP-1* expression in Arabidopsis leads to the activation of both the SA- und JA/ET-dependent pathways, since the SA-dependent *PR-1* and *PR-2* genes and the JA/ET-dependent *PR-3, PR-4* and *PDF1.2* genes are upregulated in the transgenic lines. Since activation of the respective genes in the transgenic lines occurs even in the absence of the pathogens, the plants appear to be better protected. This strongly suggests that *BvGLP-1* may be functional as a key signaling component of plant general defense

mechanisms by specifically regulating the expression of plant defense-related genes prior to pathogen attack.

Since OA appears to be a virulence factor of various pathogens (Bateman and Beer 1965; Maxwell and Bateman 1968; Stone and Armentrout 1985; Godoy et al. 1990), it is reasonable to speculate that the degradation of fungal OA may represent an important aspect of the *BvGLP-1*-mediated resistance against both fungal pathogens. OA may aid the pathogen in infection, by reducing the pH in their environment, increasing polygalacturonase activity and sequestering calcium ions from calcium pectate (Zou et al. 2007). OA also suppresses the oxidative burst (Cessna et al. 2000) and disturbs the guard cell function (Guimaraes and Stotz 2004). Kim et al. (2008) demonstrate that OA acts as an elicitor inducing a programmed cell death (PCD) that is required for pathogenicity as well as for disease development of *Sclerotinia sclerotiorum*, a necrotrophic ascomycete fungus. Similarly, over-expression of an oxalate decarboxylase from *Collybia velutipes*, an OA-degrading enzyme, was found to confer resistance to infection caused by the OA-producing fungus *Sclerotinia sclerotiorum* in transgenic tobacco and tomato (Kesarwani et al. 2000). Further analysis of the role of OA produced by the two fungi in their pathogenicities may shine more light on the function of *BvGLP-1* in plant defense.

Through the generation of H_2O_2, *BvGLP-1* may also be involved in catalysing cross-linking of plant cell wall proteins in papillae at the infection site and lignifications for the reinforcement of the cell (Olson and Varner 1993; Thordal-Christensen et al. 1997) which consequently leads to the protection of the cell against fungal penetration. We found in our microscopic observations a drastically reduced penetration rate of *R. solani* hyphae into transgenic roots expressing *BvGLP-1* when compared to the wild type. Wei et al. (1998) originally postulated that the powdery mildew-inducible epidermal-specific barley *HvGER4* may be involved in the generation of H_2O_2 which is required for crosslinking reactions during papillae formation. Subsequently, it was demonstrated that *HvGER4* expressed in Arabidopsis possessed superoxide dismutase activity (Christensen et al. 2004; Zimmermann et al. 2006). Our results showed that the transgenic plants were not completely protected against infection by *R. solani and V. longisporum*, since they became infected and necrotic after a longer period of infection. It has been reported that overexpression of PR proteins such as chitinases (Broglie et al. 1991, Datta et al. 1999) or a ribosome-inactivating protein (Maddaloni et al. 1997) resulted in enhanced resistance to *R. solani*, primarily through a delay in the development of disease symptoms. However, the observed delay of the disease may give the transgenic plants enough time to induce other defense mechanisms to ward off the pathogen systemically.

Because of a broad resistance to various pathogens, *BvGLP-1* provides a promising candidate gene for genetic engineering for improving crop resistance to different pathogens.
Most interestingly, the presence of *BvGLP-1* does not primarily affect the beneficial interaction between *P. indica* and Arabidopsis. This clearly indicates that different signaling processes are required for the activation of the resistance against the two tested necrotrophs and the beneficial interaction with *P. indica*. With this respect, it appears that Arabidopsis distinguishes between foes and friends, since signaling processes leading to the beneficial interaction in the plants are not affected by the defense machinery controlling the growth of the necrotrophs. Also the growth of *P. indica* is not inhibited during the first weeks of co-cultivation. However, in long-term co-cultivation experiments, the activation of defense responses such as PR gene expression in Arabidopsis plants expressing *BvGLP-1* appears to have an impact on growth and development of the beneficial fungus as well, since root colonization by *P. indica* is significantly reduced in older transgenic plants on soil. Thus, long-term harmony between the two symbionts is diminished to some extent if the plants restrict hyphal growth due to the activation of defense responses. It is also conceivable that H_2O_2 production by the transgenic plant restricts growth of *P. indica*. Since the transgenic seedlings and young adult plants carrying *BvGLP-1* respond to *P. indica* like the wild type, activation of signaling pathways leading to the beneficial interaction is not prevented in the presence of defense gene activation. It is worth noting that in spite of the reduced growth promotion during later stages of the interaction, we did not observe any harm to the plants, and the biomass and seed production was not lower for plants co-cultivated with *P. indica* when compared to the uncolonized control. In this context, the transgenic Arabidopsis plants expressing *BvGLP-1* offer an interesting material for studying beneficial plant-fungus interactions.

6 Acknowledgments

This work was financially supported by the Deutsche Forschungsgemeinschaft (grants No. SFB167- A19 and SFB604 - A7) and the Bundesministerium für Bildung und Forschung, Germany (grant No. 03152 31B).

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Chapter IV

Two NBS-LRR carrying resistance gene analogs (RGAs) are involved in the *Hs1*$^{pro-1}$-mediated nematode (*Heterodera schachtii* Schm.) resistance in sugar beet (*Beta vulgaris* L.)

(Manuscript in preparation)

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Key words: *Hs1*$^{pro-1}$, NBS-LRR, R-gene, nematode resistance, resistance gene analogs, signal transduction

1 Abstract

Most of plant disease resistance genes encode for proteins that share highly conserved motifs including nucleotide binding sites and leucine-rich repeats (NBS-LRR). By using degenerated primer based on the NBS/LRR-sequences conserved among *Mi*, *Gpa2*, *Cre3* and *Prf* three expressed RGAs had been identified from the nematode resistance sugar beet line A906001, that carries the $Hs1^{pro-1}$ gene. The RGAs, cZR-3, cZR-7 and cZR-9 all belong to the CC-NBS-LRR resistance protein family and share high sequential and structural homology to a set of recently cloned resistance proteins, suggesting their potential role in nematode resistance. To investigate this, we generated transgenic *Arabidopsis thaliana* plants expressing each of the RGAs, respectively and challenged the transgenic plants with *Heterodera schachtii*. Here we demonstrate that expression of cZR-3 or cZR-7 in Arabidopsis plants showed a strong anti-nematode effect resulting in a significant reduction of the number of females developed on transgenic plants when compared to the control plants. In support of this, we tested *rpm1*-ko Arabidopsis mutant plants in which the transcript of *RPM1,* a cZR-3 homolog gene is completely abolished. As indicated by nematode resistance tests, the *rpm1*-ko plants showed a significant enhanced susceptibility to nematode infection compared to the wild type plants. These results strongly support an active role of both RGAs in nematode resistance. By contrast, no significant anti-nematode effect could be observed on transgenic plants expressing cZR-9, on which females were able to develop regularly as on the control plants. We demonstrate that the expression of cZR-3 or cZR-7 in Arabidopsis strongly elevates the transcript levels of *RAR1* and *SGT1* but not of *NPR1* and *EDS1* and also upregulates the expression of PR proteins PR-1, -2, -4, -5, but not of PDF1.2, PDF2.2 and PDF2.3. Therefore, we conclude that cZR-3 and 7 may be involved in the $Hs1^{pro-1}$ mediated nematode resistance following a signaling route specific for CC-NBS-LRR resistance proteins, which finally leads to enhanced expression of defence related proteins. Thus, interference of PR- proteins may represent an important aspect of the $Hs1^{pro-1}$ mediated nematode resistance in sugar beet. A possible functional mode is discussed.

2 Introduction

During the evolution, plants have evolved an efficient immune system against various pathogens, which can be represented by a four-phased model (Jones and Dangl 2006). This model comprises: (1) microbial/pathogen-associated molecular patterns (MAMPs/ PAMPs) are recognized by plant pattern recognition receptors resulting in PAMP-triggered immunity (PTI); (2) pathogens evolved specialized abilities to suppress the primary defences (PTI) e.g. by secreting effector proteins into the plant cell resulting in effector-triggered susceptibility (ETS) (Nürnberger et al. 2004; Chisholm et al. 2006); (3) once the effector is directly or indirectly recognized by a plant resistance protein, the effector-triggered immunity (ETI) became activated and (4) pathogen isolates are selected that have lost the effectors and therefore gained new ones to overcome resistance again. The effector-triggered immunity (ETI) relying on resistance (R) proteins confers a pathogen-specific resistance that is often associated with a form of programmed cell death around the infection site termed the hypersensitive response (HR).

A range of disease resistance genes (R-genes) have been cloned from various plant species, which are characterized by conserved domains in their predicted protein structures. The majority of cloned R-genes fall into the nucleotide- binding site/leucine-rich repeat (NBS-LRR)-containing gene family. NBS-LRR gene products are characterized by a variable N-terminal of 200 amino acids, followed by a putative NBS domain consisting of Ploop/ kinase-1a, kinase-2, and kinase-3a motifs and by a more variable tandem array of approximately 10-40 short LRR motifs (Traut 1994; Jones and Jones 1997). NBS-LRR proteins can be further subdivided into TIR and non-TIR proteins based on the presence or absence of an amino-terminal (N-terminal) TIR domain (Perker et al. 1997). These features share high homology to the proteins that function in animal innate immunity and apoptosis implicating a conserved mechanism of cell death programmes in plants and animals (Saraste et al. 1990; Li et al. 1997; van der Biezen and Jones 1998, Aravind et al. 1999).

Plant NBS-LRR-R-proteins act through a network of signaling pathways and induce a series of plant defense responses including the activation of an oxidative burst, neutralization of reactive oxygen species, production of antimicrobial metabolites and the programmed cell death (Van Baarlen et al. 2007; Glazebrook et al. 2005). So far, only several key regulators involved in R-gene activated pathways have been described. They include e.g. *RAR1* (required for Mla12 resistance), *NPR1* (nonexpresser of PR genes), *EDS1* and *PAD4* (lipase-like proteins) as well as *SGT1* (suppressor of the G2 allele of skp1). Genetic studies showed

that CC-NBS-LRR-R-genes operate plant resistance responses dependent on *RAR1* and *SGT1*, whereas *NPR1*, *EDS1* and *PAD4* are required by resistance genes that belong to the TIR-NBS-LRR-R-gene class (Hammond-Kosack et al. 2003).

Several nematode resistance genes have recently been isolated from plants, all conferring resistance against sedentary nematodes including *Cre3* (Lagudah et al. 1997), *Gpa2* (Van der Vossen et al. 2000), *Gro1* (Paal et al. 2004), *Hero* (Milligan et al. 1998), *Mi-1* (Ernst et al. 2002) and $Hs1^{pro-1}$ (Cai et al. 1997), which was the first nematode resistance gene to be cloned. Recently, two major genes *Rhg4* and *Rhg1* conferring resistance to the soybean cyst nematode (SCN) in soybean have been reported (Meksem et al. 2005; Ruben et al. 2006). By comparison, $Hs1^{pro-1}$ has no homology to known R-proteins and is therefore designated as a member of a new class of resistance genes. The gene product was predicted to span the cytoplasm membrane and function as a receptor interacting with nematode effectors (Cai et al. 1997). As no complete resistance could be observed by transgenic sugar beet plants so far, it is proposed that a second gene may be involved in the resistance expression (Schulte et al. 2006).

Recent genome research revealed that each plant genome encodes hundreds of R-proteins (Meyers et al. 2003; Monosi et al. 2004). Degenerated primer-based PCR amplification has been successfully used for isolation of resistance gene candidates (RGCs) or resistance gene analogues (RGAs) from a wide variety of plant species (Leister et al. 1996; Timmerman-Vaughan et al. 2000; Pan et al. 2000; Bai et al. 2002; Tian et al. 2004; Calenge et al. 2005). RGAs have also been identified from the sugar beet genome and several of these were found to be associated with resistance to various pathogens (Hunger et al. 2003; Tian et al. 2004; Lein et al. 2007). In our previous project, we had cloned a set of expressed NBS-LRR-containing RGAs including 4 full-length cDNAs from a nematode resistant sugar beet. Their function in nematode resistance response still remains unsolved.

The *Arabidopsis thaliana* ecotype C24 is a host of the beet cyst nematode *Heterodera schachtii*, and therefore has been intensively used for studying plant-parasite interaction (Sijmons et al. 1991). Here we report that expression of cZR-3 or cZR-7 in Arabidopsis plants gave rise to a strong anti-nematode effect whereas knockout of a homologue gene of cZR-3 in Arabidopsis enhanced susceptibility to nematode infection. In addition, we demonstrate that cZR-3 as well as cZR-7 upregulates the expression of *RAR1*, *SGT1* and of *PR-1, -2, -4, -5*. Thus we conclude that cZR-3 as well as cZR-7 are involved in the $Hs1^{pro-1}$ mediated nematode resistance following a *RAR1*/*SGT1* dependent signaling route that is specific for

CC-NBS-LRR R- proteins, and that the interference of PR-proteins may represent an important aspect of the *Hs1*$^{pro-1}$ mediated nematode resistance in plants.

3 Material and Methods

3.1 Plant material

Arabidopsis thaliana C24 and Col-0 (Lehle Seeds, Round Rock, USA) were used for generation of transgenic plants and the mutant analysis, respectively.

3.2 Generation of transgenic plants

3.2.1 Plasmid constructs and Agrobacteria cultures

The full-length cDNAs of cZR-3, cZR-7 and cZR-9 were subcloned as an *XhoI*-fragment into the *Xho*I site of the binary vector pAM194 (KWS Saat AG, Einbeck, Germany) under the control of the 35S promoter resulting in the plant expression constructs pAM194-cZR-3, pAM194-cZR-7 and pAM194-cZR-9. The recombinant binary vectors were transformed into *A. tumefaciens* strain GV3101 (Koncz and Schell 1986) for *A. thaliana* transformation by using electroporation (Gene Pulser System II, Bio-Rad, Hercules, USA). Electroporation was performed at 2.5 kV/cm, 25 µF and 200 Ohm. Bacteria cells were recovered with SOC (2% (w/v) Tryptone Pepton; 0.5% (w/v) Yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$; 20 mM Glucose) at 37°C for 1 hour. The transformed Agrobacterium cells were grown on 2YT medium containing 50 ppm kanamycin and 50 ppm gentamycin overnight. For transformation of *Arabidopsis thaliana* root explants a single overnight colony was used to inoculate 5 ml 2YT liquid culture medium with 50 ppm kanamycin and 50 ppm gentamycin and was grown at 28°C, 210 rpm for 18–24 h. The overnight culture was used to inoculate 50 ml 2YT medium without antibiotics. Cells were harvested by centrifugation for 10 min at 4°C, 2900 x g and then resuspended in 50 ml B5 medium (Gamborg B5 Micro and Macro elements 3.18 g/l; Gamborg B5 vitamins 0.112 g/l; Glucose 2% (w/v)). The washing step was repeated. 1 ml was transferred into 20 ml fresh B5 medium ready for transformation.

3.2.2 *Arabidopsis thaliana* root transformation

Transgenic Arabidopsis plants were generated by using the root transformation protocol (Valvekens et al. 1988). C24 Arabidopsis seeds were surface sterilized for 5 min in 70% (v/v) ethanol, in 5% (w/v) NaClO containing 0.05% (v/v) Tween 20 for 10 min, and rinsed four times with sterile distilled water. Sterile seeds were transferred into 500 ml Erlenmeyer flasks containing 100 ml liquid B5 medium and germinated for nine days by gently shaking (22°C, 16/8 h light/dark photoperiod). Roots were cut into small pieces of about 0.5 cm and transferred to a nylon membrane (100 Mikron, Hydro-Bios, Altenholz, Germany) lying on

solid CIM medium for a preconditioning time of 72 h. After preconditioning the root explants were incubated with the prepared *A. tumefaciens* culture for 2 min. The infected explants were soaked on sterile filter paper before co-cultivation on fresh CIM medium for 48 h. After co-cultivation the explants were washed four times with liquid B5 medium and transferred to SIM medium. Single green shoots were excised and transferred to SEM medium for shoot elongation and then to RIM medium to form roots. Rooted plants were transferred to soil to set seeds.

3.3 PCR and Southern analysis

Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as described by Rogers and Bendich (1985). Kanamycin-resistant *A. thaliana* plants were harvested for isolation of genomic DNA after seven days. PCR was carried out in a total volume of 20 µl containing 50 ng of template DNA, 10 mM PCR buffer (pH 8.3), 2 mM $MgCl_2$, each dNTP at 0.5 mM, each primer at 10 pmol, and 1 U of Taq DNA polymerase (Invitrogen, Karlsruhe, Germany) in a thermocycler (Biometra, Göttingen, Germany) under the following conditions: 5 min at 94°C, 60 s at 94°C, 60 s at 55 °C, 1 min 30 s at 72°C for 34 cycles, followed by 10 min at 72°C. PCR products were separated on a 1% (w/v) agarose gel and analyzed with the software Quantity One (Bio-Rad Laboratories GmbH, München, Germany). For Southern hybridization experiments the extracted DNA was digested with two restriction enzymes (*EcoR*I, *Xho*I), electrophoretically separated on 0,75% (w/v) agarose gels and blotted on Hybond-N+membrane (GE Healthcare, Chalfont St. Giles, UK) by capillary diffusion overnight using 0.25 M NaOH/1.5 M NaCl as blotting solution. Southern blots were hybridized with 32P-labelled DNA probe (Feinberg and Vogelstein 1983) at 60°C, washed twice (0.5 x SSC; 0.2 % w/v SDS) for 30 min and exposed at -70°C for 48 h. The DNA probe for Southern-hybridization was generated by PCR with gene specific primers GGGTAAAACTGCTCTTGC and AAGCCCTCTTTCTCCATC for cZR-7(f/r), AGTTATTGATAGGGCTATGG and ATACTTGAGGCAGTCAGG for cZR-3 (f/r) as well as TGGGAAGACAACATTGGC and ACCTTTTTGGCAGGAATC for cZR-9 (f/r) using plasmid DNA as template. PCR fragments were separated on a 1.3% (w/v) low melting agarose gel before radio-labelling.

3.4 Semi-quantitative RT-PCR and qRT-PCR

Semi-quantitative RT PCR was used to assay the expression patterns of the genes of interest. Transgenic as well as control Arabidopsis were removed from the plates, rinsed 12 times with

an excess of sterile water (50 ml each) and frozen in liquid nitrogen for RNA extraction. Extraction of total RNA was performed following the trizol protocol (Invitrogen). The total RNA was treated with RNAse-free DNAse (Fermentas, St. Leon-Rot, Germany) for 60 min at 37°C. Synthesis of cDNA was carried out using a Superscript III First-Strand Synthesis System (Invitrogen) according to the instructions of the manufacturer. The semi-quantitative PCR amplification was performed in 50 µl reactions consisting of 2.5µl 10ng/µl cDNA, 5µl 10 x buffer, 0.5µl 10 mM dNTPs, 5 µl each of 10 pmol/µl primer, 2.5 U of Taq polymerase (Invitrogen) and 31.5 µl H_20 under the PCR programme: 94°C for 50 s, 54°C for 1min and 72°C for 1min for 25 cycles, followed by 10 min at 72°C. Amplicons, separated on a 1% (w/v) agarose gel were visualised under UV-light. The housekeeping *ubiquitin* gene served as a control and the mRNA levels for each cDNA probe were normalized to the *ubiquitin* message RNA level (ACTCTCACCGGAAAGACAATC and TGACGTTGTCGATGGTGTCAG for Arabidopsis).

The real-time PCR was performed using the ABI7300 Detection System (Applied Biosystems, Foster City, USA). The SYBR QPCR Supermix (Invitrogen) was used for PCR reactions according to the manufacturer´s instructions in a final volume of 20 µl. The ABI7300 was programmed to 95°C 10 min, 35 x (95°C 30 s, 55°C 40 s, 72°C 45 s), 72°C 10 min followed by a melting curve programme (55 to 95°C in increasing steps of 0.5°C). All reactions were repeated at least twice. The mRNA levels for each cDNA probe were normalized with respect to the *ubiquitin* message level (ACTCTCACCGGAAAG ACAATC and TGACGTTGTCGATGGTGTCAG for sugar beet). Fold induction values were calculated with the ΔCP equaltion of Pfaffl (2001) and related to the mRNA level of the target genes in wild type roots, which were defined as 1.0.

The following primers were used in the described gene expression experiments:

Target	Accession no.	Primer 5′ - 3′	Reference
PDF1.2	AT5G44420	ATGGCTAAGTTTGCTTCCA TTAACATGGGACGTAACAGATAC	Huffaker et al. 2007
PDF2.1	AT2G02120	GATGGGTCCAGTCACGGTC TTCAAGAACACACTAAACACGC	Thomma et al. 1998
PDF2.3	AT2G02130	CACACACAACTGTGCAAACG CGGAAACACACAAACCAATG	Thomma et al. 1998
PR1	AT2G14610	GGAGCTACGCAGAACAACTA AGTATGGCTTCTCGTTCACA	Huffaker et al. 2007
PR2	AT3G57260	CTACAGAGATGGTGTCA AGCTGAAGTAAGGGTAG	Uknes et al. 1992
PR4	AT3G04720	GGCCGGACAACAATGCGGTCGTCAAGG CAAGCATGTTTCTGGAATCAGGCTGCC	Thomma et al. 1998
PR5	AT1G75040	CACATTCTCTTCCTCGTGTTC TAGTTAGCTCCGGTACAAGTG	Thomma et al. 1998
RAR1	AT5G51700	ATGACGACAATCCTCAAGG CTTGATCTGTTCTTTGGGTTGGG	this study
SGT1b	AT4G11260	ATGGCCAAGGAATTAGCAGA CGGTTTGGCAGGTGCTGCAGGA	Tör et al. 2002
NPR1	AT1G64280.1	TCAACCATAGGAATCCGAGGG CCGACGACGATGAGAGAGTTTACG	this study
EDS1	AT3G48090	ACCAGATCATGGTCAGCC TGTCCTGTGAACACTATCTGTTTTCTACT	Feys et al. 2001
PAD4	AT3G52430	ATACGTTGCTATACCGGC GGTTGAATGGCCGGTTATCA	Feys et al. 2001

3.5 Nematode infection experiments

Heterodera schachtii was propagated on in vitro stock cultures of mustard (*Sinapis alba* cv.Albatros) roots grown on 0.2 x Knop medium supplemented with 2% sucrose and 0.8% Daishin agar under sterile conditions. Fully developed cysts were harvested from the roots onto 50 µm gauze. Hatching of juveniles was stimulated by soaking cysts in 3 mM $ZnCl_2$ for 8 - 10 days. The larvae were harvested with 10 µm gauze, surface-sterilized, washed four times in sterile water, resuspended in 0.2% Gelrite (w/v) (Duchefa, Harlem, Netherlands) and used directly for inoculation experiments. Inoculation of *A. thaliana* plants with nematodes was performed *in vitro* according to the protocol described by Sijmons et al. (1991). One-week old Arabidopsis seedlings were transferred to 6-well plates containing 0.2 x Knop medium under sterile conditions. Two hundred sterile infective juveniles of the beet cyst nematode were added to each single *A. thaliana* plant on 6-well plates. The number of developed females was determined 4 weeks after infection under a stereomicroscope (Stemi SV 11, Zeiss, Germany).

3.6 *A. thaliana* mutants

The database BLAST search (blastx) revealed that the cZR-3 encoded protein is 30% identical (E-value 5e-46) to *RPM1* from Arabidopsis (AT3G07040).The knockout Arabidopsis mutants of AT3G07040 were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, OH, USA), which contain a T-DNA insertion in AT3G07040 (*RPM1*, CS8637). After successive selfing, 4 homozygous ko lines were selected by PCR using the T-DNA-left border-specific primer (TGGTTCACGTAGTGGGCCATCG) in combination with *RPM-1*-specific primers RPM1-f (TCCACGGTTATCGTAGCTGCGC) and RPM-r (CCGTCCGATGAGCTTTCCCTT). Knockout of the transcript in the ko line was confirmed by RT-PCR with the same gene-specific primers as described above.

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4 Results

4.1 Sequence, structure and transcript analysis of 3 sugar beet RGAs

In our previous work, three full-length sugar beet non-TIR RGAs, cZR,-3, -7 and -9 had been identified from the nematode resistant sugar beet line A906001. The RGAs share strong sequential and structural similarity to several recently cloned NBS-LRR R-genes including e.g. *Mi* (6.5e-75, rootknot nematode R-protein; Milligan et al. 1998), *Gpa2* (5.1e-31, cyst nematode R-protein; van der Vossen et al. 2000), *Rx* (1.4e-18, viral R-protein; Bendahmane et al. 1999), *I2C1* (9.8e-38, vascular wilt disease R-protein; Ori et al. 1997) and as well as *Prf* and *RPM1* (1.0e-25 and 1.9e-42, *P. syringae* R-proteins; Grant et al. 1995; Salmeron et al. 1996). Therefore, we assumed that these genes may play crucial role in the $Hs1^{pro-1}$-mediated resistance.

To get a view of transcript profiles of three RGAs, we performed a qRT-PCR analysis on resistant plants upon nematode infection and compared them with those of susceptible plants.

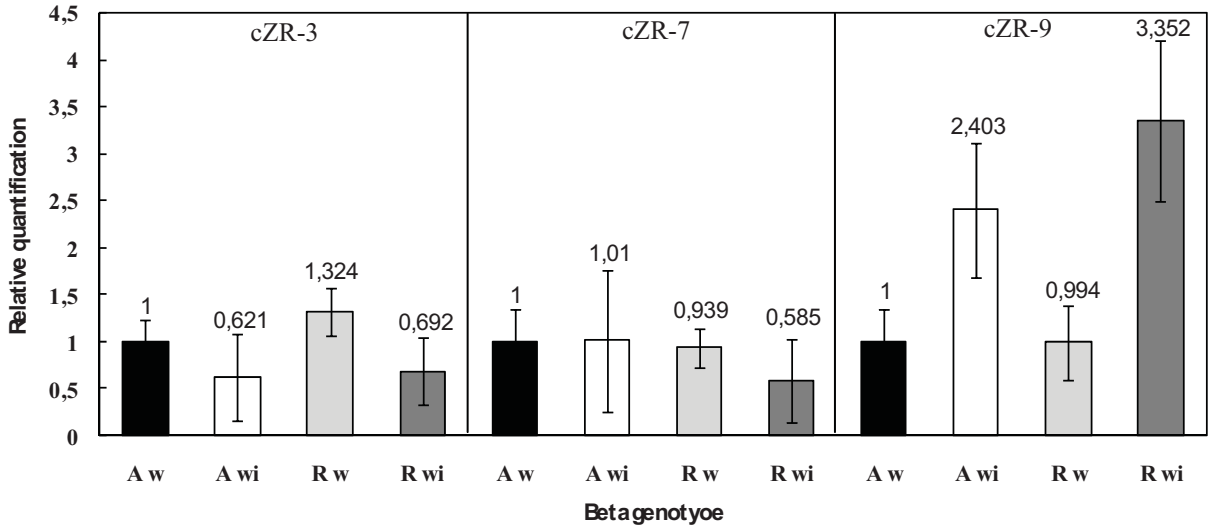


Figure 1: Expression profiling of cZR-3, cZR-7 and cZR-9 in roots of nematode resistant sugar beet line A906001 and of susceptible sugar beet line 93161p investigated by qRT-PCR. Nematode resistant plants (R) as well as susceptible plants (A) were inoculated with *H. schachtii* J2 juveniles and roots were harvested 12 days after inoculation (Rwi; Awi). Non-infected resistant (Rw) and susceptible (Aw) plants served as a control. The housekeeping *ubiquitin* gene served as a control and the mRNA levels for each cDNA probe were normalized to the *ubiquitin* message RNA level. Bars represent standard errors based on 3 independent experiments with 10 plants each.

As shown in Figure 1 substantial levels of transcripts of cZR-3, 7 and 9 could be detected in both resistant and susceptible plants. However, in response to nematode attack, no significant change in transcript levels of cZR-3 and cZR-7 was observed in both resistant and susceptible

plants. A strong transcriptional upregulation of cZR-9 was observed in both resistant and susceptible plants. These results indicate that cZR-3, 7, 9 are constitutively expressed in plants in consistence with most of plant R-proteins and RGAs but expression of cZR-9 is additionally enhanced in response to nematode attack.

4.2 Generation of transgenic Arabidopsis plants expressing each of the RGAs

To define the possible role of the RGAs in nematode resistance, we transferred them into Arabidopsis plants and test them for nematode resistance. Each of the RGAs was subcloned as a *Xho*I fragment into the binary vector pAM194 resulting in three plant expression constructs pAM194-cZR-3, pAM194-cZR-7, pAM194-cZR-9 (Figure 2A).

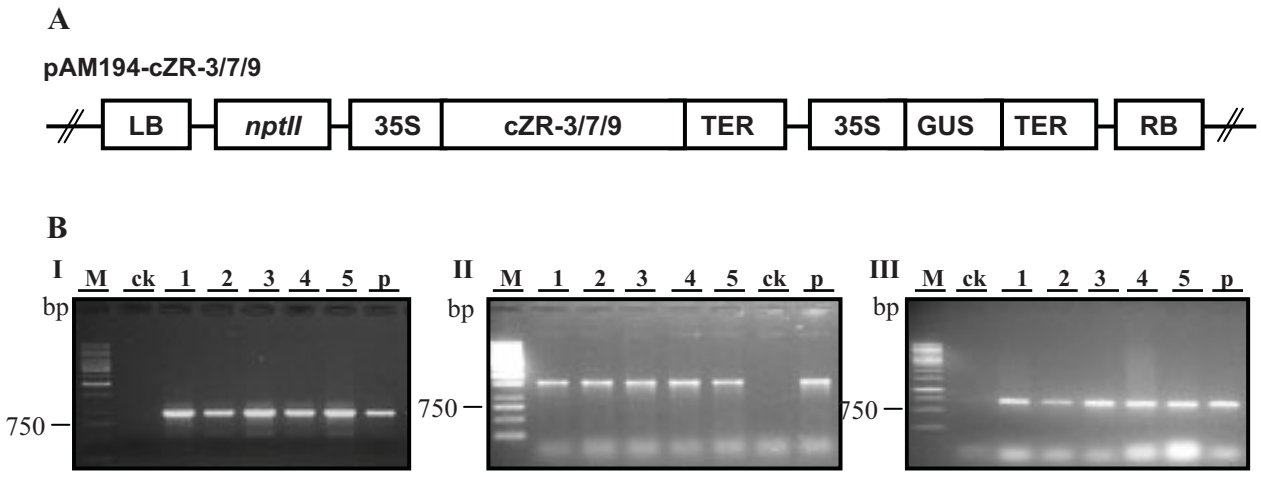


Figure 2: Molecular characterization of cZR-3, cZR-7 and cZR-9 transgenic *A. thaliana* plants by genomic PCR. **A**: Map of plant expression constructs pAM194-cZR-3, pAM194-cZR-7 and pAM194-cZR-9. LB and RB: left and right borders*; nptII*: neomycin-phosphotransferase gene giving resistance to kanamycine; GUS: GUS gene with intron. **B**: Determination of the transgene in *A. thaliana* plants transformed with pAM194-cZR-3, pAM194-cZR-7, pAM194-cZR-9 by genomic PCR. **I**: PCR amplifications with cZR-3 gene specific primers cZR-3f/r , **II**: PCR amplifications with cZR-7 gene specific primers cZR-7f/r, **III**: PCR amplifications with cZR-9 gene specific primers cZR-9f/r. M: 1 kb marker, cK: negative control pAM194 transformed *A. thaliana*, lines 1-5: transgenic *A. thaliana* plants harbouring pAM194-cZR-3, pAM194-cZR-7 and pAM194-cZR-9, p: positive control plasmid DNA harbouring the respective genes.

Subsequently, all three gene constructs were transformed into the *Arabidopsis thaliana* ecotype C24 by *A. tumefaciens* mediated transformation. In total, 45 independent transformants (T0) were obtained for pAM194-cZR-3, 95 for pAM194-cZR-7 and 58 for pAM194-cZR-9 (Table 1). The presence and the copy number of the transgenes were determined by PCR (Figure 2B) as well as by Southern blot analysis with 18 randomly selected transformands of each gene construct (data not shown). The number of the transgene varied from one to four, but most of the transformands harbor only one (data not shown). The expression of the transgene in transgenic Arabidopsis was also confirmed by RT-PCR using gene-specific primers. As demonstrated in Figure 3, RT-PCR fragments of the transgene cZR-

3, 7 and 9 were detected in transgenic plants carrying respective gene constructs, but not in the control plant transformed with the empty vector.

Table 1: Results of the *A. thaliana* transformation experiments with pAM194-cZR-3, pAM194-cZR-7 and pAM194-cZR-9. Transgenic *A. thaliana* families were selected for nematode resistance.

RGA	Selectable marker	Binary vector	No of T0 plants regenerated	No of T1 plants selected	No of T2 families	No of T3 families
cZR-3	Kan	pAM194	45	5	5	4
cZR-7	Kan	pAM194	95	46	8	8
cZR-9	Kan	pAM194	58	78	12	8

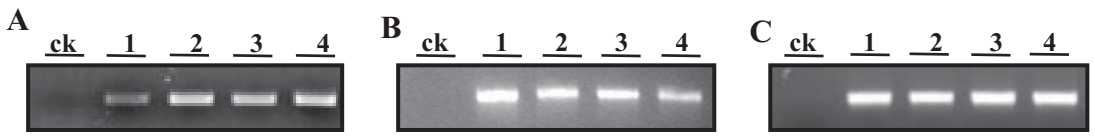


Figure 3: Expression of cZR-3, cZR-7 and cZR-9 in transgenic Arabidopsis determined by RT-PCR with 4 independent transgenic Arabidopsis lines for each of the three gene constructs. **A**: PCR amplifications with cZR-3 gene specific primers cZR-3f/r, **B**: PCR amplifications with cZR-7 gene specific primers cZR-7f/r, **C**: PCR amplifications with cZR-9 gene specific primers cZR-9f/r. Arabidopsis plants transformed with the empty vector served as control (ck). The cZR-3, cZR-7 and cZR-9 transcripts are present in all transgenic lines transformed with the respective genes. The mRNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level.

4.3 Nematode resistance tests with transgenic Arabidopsis plants

For determination the transgene effect on nematode development, the T1 progenies from the 18 randomly selected T0 transformands of each gene construct were subjected to nematode resistance tests, in which the Arabidopsis plants transformed with the empty vector served as a control. For this, transgenic seeds (T1) were geminated on agar plates carrying 50 µg/ml kanamycin for selection of transgenic plants (Figure 4). The surviving seedlings were transferred to 6-well agar plates for nematode infection experiments (Figure 6A), in which each plant was inoculated with 200 infective nematode juveniles and repeated three times each with 24 individual plants.

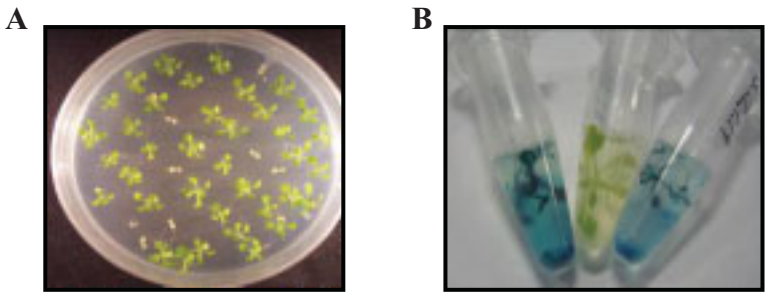
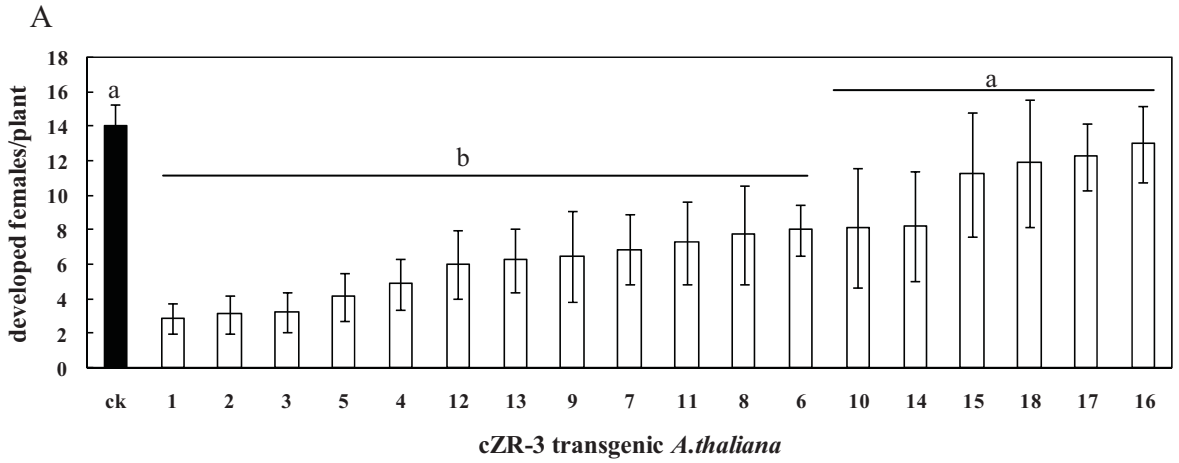


Figure 4: Selection of transgenic *A. thaliana* plants on kanamycin containing medium (**A**); green healthy plants were used for nematode inoculation experiments. Selection of transgenic *A. thaliana* plants by histochemical GUS staining (**B**).

Four weeks after inoculation, females on each plant were counted as well as documented under a stereomicroscope. The results were summarized in Figure 5. The number of developed females on12 cZR-3 transgenic plants varies between 3±1 and 8±2 (mean±STD, n=72), on 14 cZR-7 transgenic plants between 3±1 to 9±3 (mean±STD, n=20) which are significantly less than those counted on the control plants (Figure 5A, B). On cZR-9 transgenic plants the number of developed females varies between 8±4 and 14±2 (mean±STD, n=72), thus no significant reduction of developed females was given on the transgenic plants expressing cZR-9 when compared them to the control plants (Figure 5C). The development of nematodes on cZR-3 or cZR-7 transgenic plants was observed under the microscope and documented weekly. On control plants developed females could be easily counted under the microscope 4-5 weeks after nematode infection, whereas the majority of females were not able to develop regularly and remained smaller and translucent on transgenic plants (Figure 6B-E).



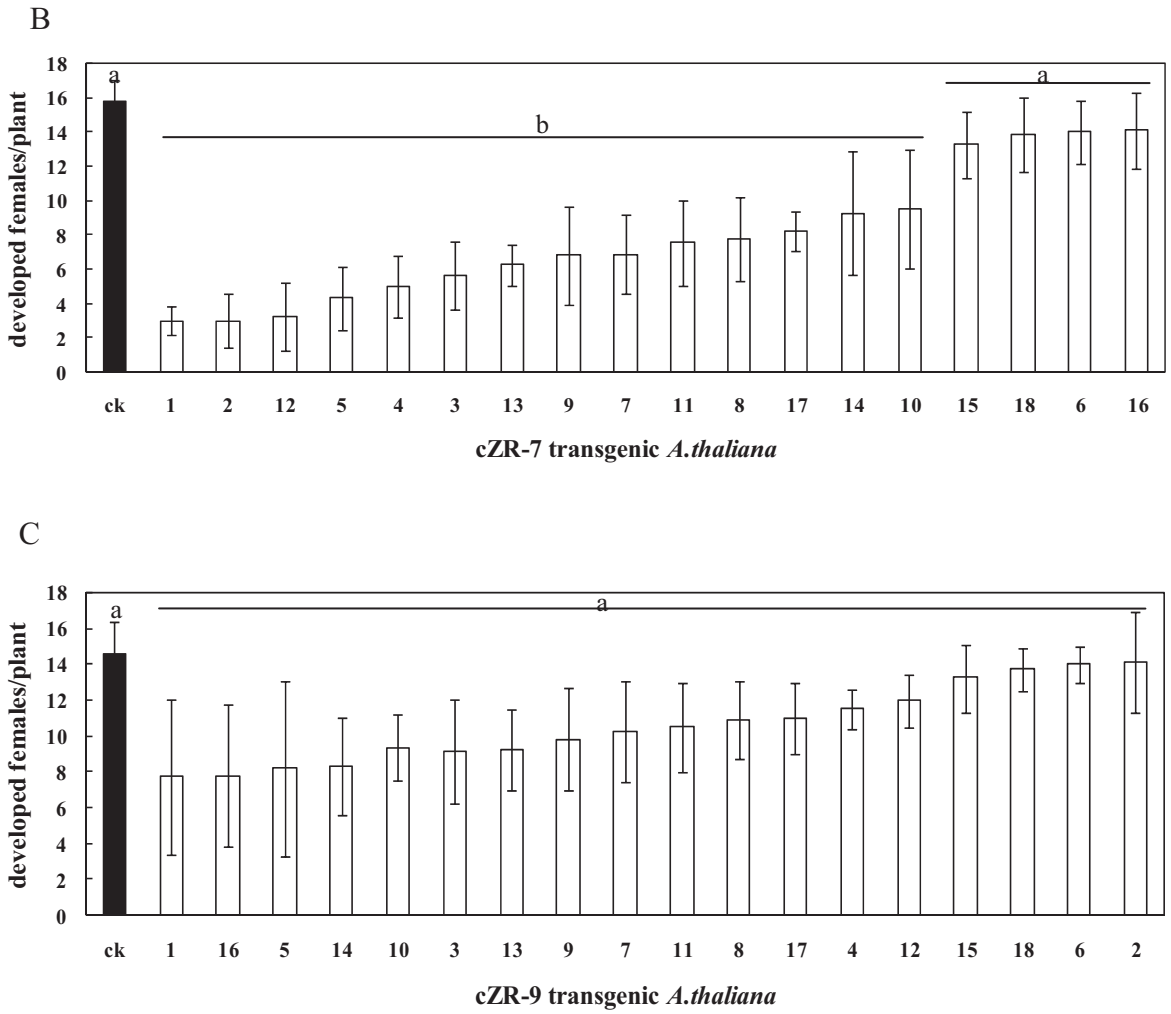


Figure 5: Results of nematode resistance assays with transgenic *A. thaliana* plants transformed with pAM194-cZR-3 (A), pAM194-cZR-7 (B) and pAM194-cZR-9 (C). *A. thaliana* plants obtained by transformation with the empty vector pAM194 served as the susceptible control (ck). The average number of developed females is presented as a bar, and the standard deviation as a line from 24 individual plants for each line. Significantly different means are indicated by different letters (a and b) calculated with additive t test at $P < 0.05$.

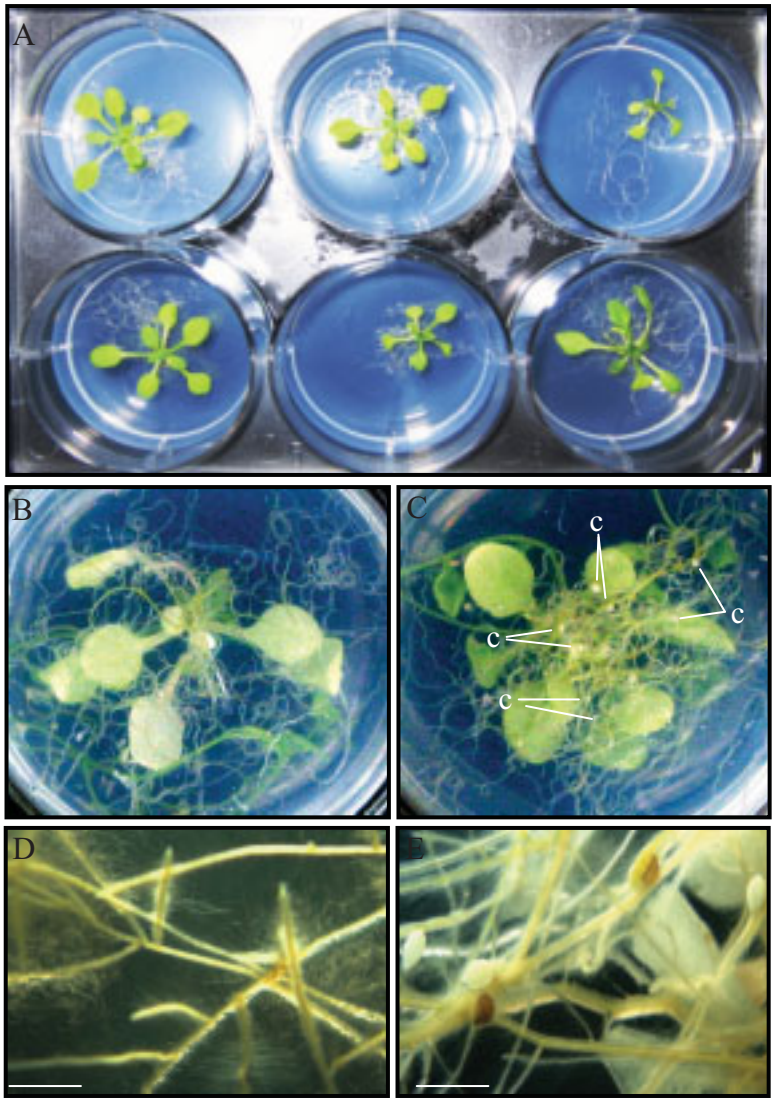


Figure 6: Visual comparison of transgenic *A. thaliana* plants and susceptible control plants in nematode resistance tests. **A**: Transgenic *A. thaliana* plants on 6-well plates one week after inoculation; **B, D**: Developed females could be easily counted under the microscope 4 weeks after nematode infection on control plants; **C, E**: Females were not able to develop regularly and no cysts were formed on transgenic plants. c: developed females. The bar equals 500µm.

By comparison, no significant difference between cZR-3- and cZR-7 in respect of inhibitory efficiency on female development in transgenic plants was observed. This observation differs from the results made by Tian (2003) with transgenic sugar beet hairy roots, in which cZR-3 gave a significant stronger effect than cZR-7 and cZR-9. To determine the stability and inheritability of the resistance mediated by cZR-3 and cZR-7 in transgenic Arabidopsis, we have chosen the four best events out of nematode resistance tests and successively propagated them to T4 generation resulting in 4 homozygous lines for cZR-3 and cZR-7, respectively. Subsequently, 24 individuals of each line were analysed by a second nematode resistance test performed as described above. Similar to the results obtained from the nematode test with T1

plants (Figure 7). All selected T4 lines of each gene construct showed significant resistance to nematode infection compared to the control.

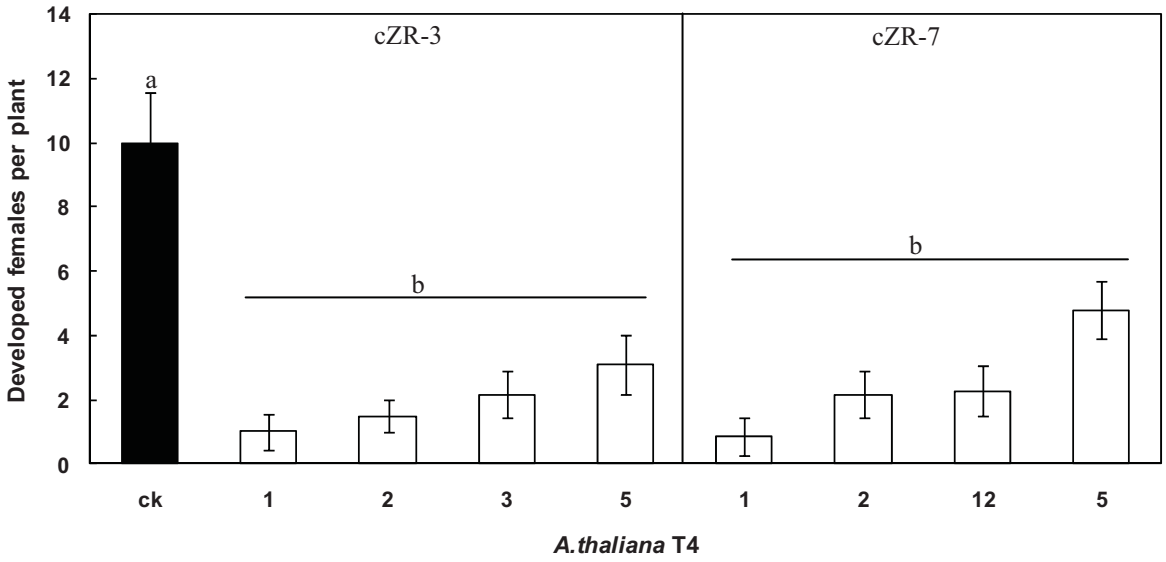


Figure 7: Results of nematode resistance test with selected transgenic *A. thaliana* T4 plants expressing cZR-3 and cZR-7. *A. thaliana* plants obtained by transformation with the empty vector pAM194 served as the susceptible control (ck). The average number of developed females is presented as a bar, and the standard deviation as a line from 24 individual plants for each line. Significantly different means are indicated by different letters (a and b) calculated with additive t test at $P < 0.05$.

4.4 Functional analysis of *A. thaliana* mutant plants

To define an active role of cZR-3 in nematode resistance response, we analysed Arabidopsis knockout (ko) mutant plants with the Col-0 genetic background, in which *RPM1* a cZR-3 homologue sequence is abolished. The ko Arabidopsis mutant plants of AT3G07040 were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, OH, USA) and contains a T-DNA insertion in AT3G07040 (*RPM1*, CS8637). Because Arabidopsis Col-0 plants are more tolerant to *H. schachtii* infection when compared to C24, we expected an enhanced susceptibility of *rpm1*-ko mutant plants. The mutant plants do not show any obvious morphological alterations in comparison with Col-0 wild type plants. Homozygous *rpm1*-ko lines were selected by PCR analysis using the T-DNA-LB-specific primer and locus-specific primers. As indicated in Figure 8, no *RPM1* transcript was detected in *rpm1*-ko plants while Col-0 wild type plants show a band of 247 bp in size.

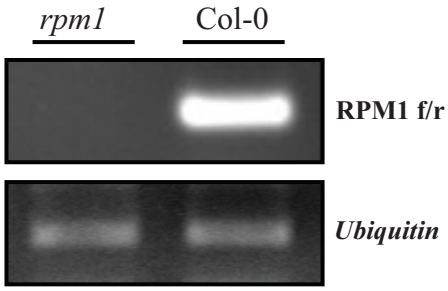


Figure 8: Characterization of *A. thaliana rpm1*-ko plants by RT-PCR analysis. RT-PCR was performed with RNA of 14 days old seedlings of the *rpm1*-ko and wild-type plants (Col-0) using RPM1-specific primers. The mRNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level.

For nematode inoculation, seedlings were geminated on 6-well agar plates, each containing 4 Arabidopsis mutant seedlings and 2 control plants. The plates were incubated in a growth chamber (22°C, 16/8h light/dark photoperiod) for seven days and were subsequently inoculated with approximately 200 infective nematode juveniles. Four weeks post inoculation, the number of developed female nematodes was scored. These experiments were repeated three times with eight individual plants. As indicated in Figure 9, *rpm1* ko-mutant plants exhibited a clear hypersusceptibility to *H. schachtii* with seven females developed per plant. By comparison, wild type Col-0 plants exhibited one to three females per plant (Figure 9). This result strongly suggests an active role of cZR-3 homologue sequence *RPM1* in Arabidopsis defence response to nematode infection, generally.

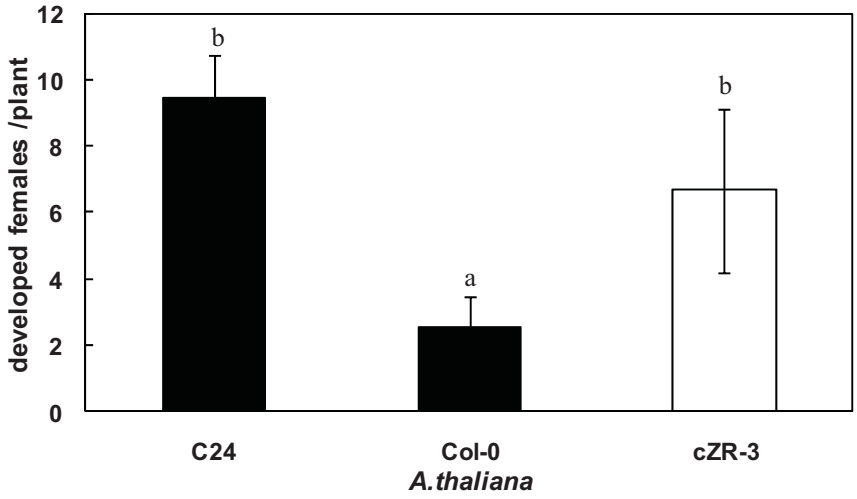


Figure 9: Results of nematode resistance test with *A. thaliana rpm1*-ko plants. *A. thaliana* wild type plants C24 and Col-0 served as the control. The average number of developed females is presented as a bar, and the standard deviation as a line from 8 individual plants for each line. Significantly different means are indicated by different letters calculated with t test at $P < 0.05$.

4.5 Determination of RGA-mediated signaling pathways

To define whether expression of cZR-3 and cZR-7 activates a specific signaling pathway we analysed the transcript levels of five key genes determining distinct defense pathways in transgenic Arabidopsis plants and compared them with those in wild type plants by semi-quantitative RT-PCR (Figure 10). Included are *NPR1* and *EDS1*, determinants for the TIR-NBS-LRR-R-protein activated signaling pathway and *RAR1*as well as *SGT1*, which are both positioned in the non-TIR-(CC)-NBS-LRR-R-protein mediated resistance response (Hammond-Kosack et al. 2003). Three independent transgenic lines for each gene construct were employed for the analysis. As expected, transgenic lines exhibited substantially elevated levels of cZR-3 and cZR-7 transcripts, respectively. While an enhanced expression of *RAR1* as well as *SGT1* occurred in all transgenic plants as compared to the control, no clear change in *NPR1* and *EDS1* transcript levels was obvious (Figure 10). These data strongly suggest that expression of cZR-3 and cZR-7 activates a *RAR1*/*SGT1* dependent signaling pathway in plants, which is well-known specific for non-TIR-(CC)-NBS-LRR-R-protein mediated resistance response. This matches the predicted structure and function of all three RGAs since they are all members of a non-TIR-(CC)-NBS-LRR-R-gene family.

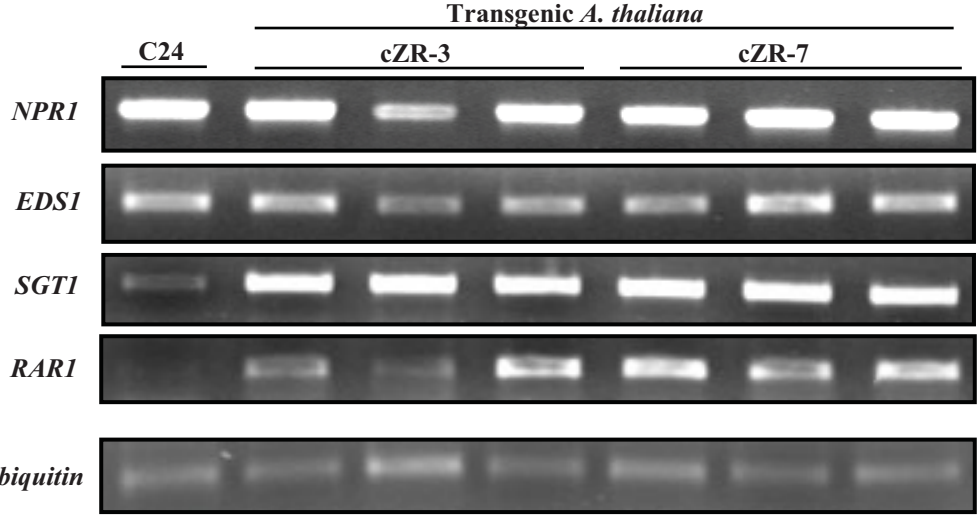


Figure 10: Expression of key genes of specific defence pathways in cZR-3 and cZR-7 transgenic Arabidopsis. *EDS1*, *SGT1*, *NPR1* and *RAR1* gene expression levels were determined by semiquantitative RT-PCR with 3 independent transgenic Arabidopsis lines for each gene construct and Arabidopsis wild type C24 plants served as control. The mRNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level.

PR-proteins and plant defensins (PDFs) are involved in plant defense responses to different pathogens, but very little is known about their role in plant nematode resistance. To explore whether cZR-3 and cZR-7 activate signal transduction pathways regulating the expression of

defense related genes, we determined transcript levels of a set of defense-related proteins (PR and PDF proteins) in transgenic Arabidopsis plants by semi-quantitative RT-PCR and compared them with those in control plants. An enhanced transcriptional activity was given for the *PR-1, PR-2, PR-4* and *PR-5* genes in the transgenic plants (Figure 11). No clear change in the transcript levels for *PDF1.2, PDF2.2* and *PDF 2.3* genes was visible (Figure 11). Thus, we conclude that expression of cZR-3 and cZR-7 in Arabidopsis plants activates a *RAR1/SGT1* dependent signaling pathway leading to enhanced expression of PR-proteins. This may represent an important aspect of the $Hs1^{pro-1}$ mediated nematode resistance.

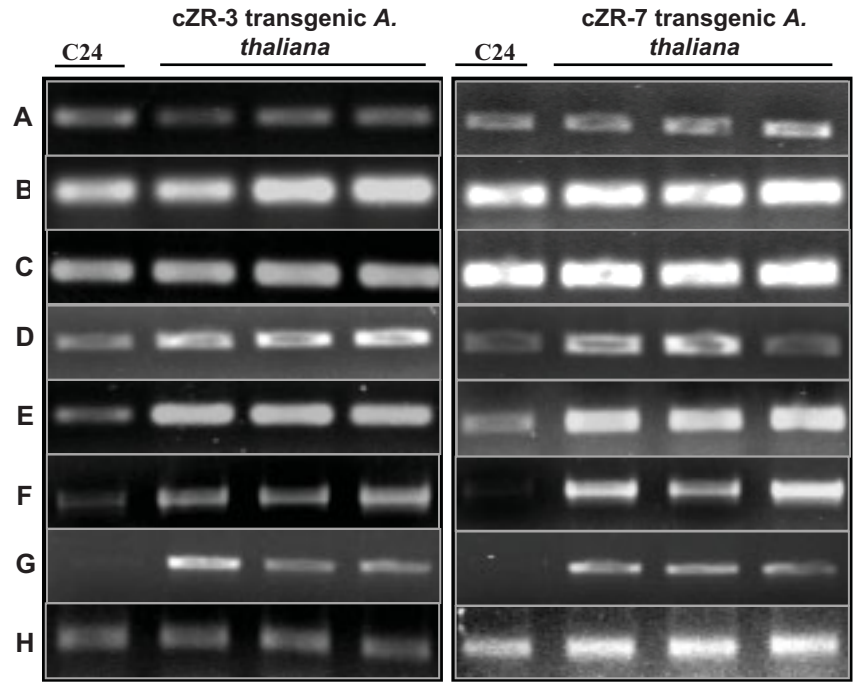


Figure 11: Expression of cZR-3 and cZR-7 activated defence related genes in transgenic Arabidopsis. PR and PDF gene expression levels were determined by semiquantitative RT-PCR with 3 independent transgenic Arabidopsis lines for each gene construct and Arabidopsis wild type C24 plants served as control. **A**: PCR amplifications with *PDF1.2*, **B**: PCR amplifications with *PDF2.2,* **C**; PCR amplifications with *PDF2.3,* **D**: PCR amplifications with *PR-1*, **E**: PCR amplifications with *PR-2*, **F**: PCR amplifications with *PR-4*, **G**: PCR amplifications with *PR-5*, **H**: PCR amplifications with *ubiquitin*. The mRNA levels for each cDNA probe were normalized with the *ubiquitin* mRNA level.

5 Discussion

Due to the limitations of working with the crop plant sugar beet we used Arabidopsis as a model in this study to investigate the interaction between beet cyst nematode and RGAs isolated from sugar beet. As no complete resistance could be observed either in *Hs1*$^{pro-1}$ transgenic sugar beet plants or Arabidopsis plants so far, a second gene is proposed to be essential for the resistance (Schulte et al. 2006). In addition, the fact that *Hs1*$^{pro-1}$ exhibits an unusual structure with no homology to known R-proteins and the NBS-LRR domain proved to be essential for recognition of the pathogen and activation of resistance response provokes us to search for a NBS-LRR carrying gene, which may be involved in both recognition and activation of the signaling pathway in the *Hs1*$^{pro-1}$ mediated resistance. Also, evidence was given from investigations on *Pto* resistant plants. It has been demonstrated that *Pto* implicitly requires *Prf* for an active defense response upon recognition of AvrPto by *Pto* as the NBS-LRR domain of *Prf* is indispensable to both recognition of elicitor and activation of the downstream signaling pathway of *Pto*. Thereby, *Prf* is suggested as "guardee" of *Pto* (Dangl and Jones 2001).

Accordingly, NBS-LRR containing RGAs were identified from the nematode resistant sugar beet genome (Tian et al. 2004). Unexpectedly, all RGAs belong to the non-TIR-NBS-LRR R-protein family and were characterized by a potential coiled-coil structure, an NB-ARC domain and a tandem array of approximately 19-29 short LRR motifs. There are several indications that the full length RGAs cZR-3, cZR-7 and cZR-9 may be involved in nematode resistance: (1) they are constitutively expressed in resistant as well as susceptible plants as revealed by transcript profiling analysis, except for cZR-9 that shows an enhanced expression by nematode attck; (2) the homology search (http://www.ncbi.nlm.nih.gov/blast/) showed significant similarities of the predicted proteins to several NBS-LRR R-proteins identified from different species including nematode R-protein *Mi*, *Gpa2 and Hero* (Milligan et al. 1998; van der Vossen et al. 2000; Ernst et al. 2002) as well as *P. syringae* resistance proteins *Prf* and *RPM1* (Salmeron et al. 1996; Grant et al. 1995) and (3) preliminary experiments have shown that transgenic sugar beet hairy roots overexpressing those RGAs hampered female development.

We found that the expression of cZR-3 or cZR-7 in Arabidopsis plants gave rise to a significant anti-nematode effect: the number of developed females was drastically reduced and the regular development of nematodes was hampered as that observed in the resistant sugar beet. By contrast, the *rpm1* ko-mutant Arabidosis plants were becoming

hypersusceptibel to nematode infection. Most importantly, the resistance was stabile inherited to T4 generation. This agrees with the results made by (Tian 2003) that the two RGAs were able to confer nematode resistance in transgeic sugar beet hairy roots. Although the expression of cZR-9 seems to be strongly upregulated by nematode attack in both resistant as well as susceptible sugar beet plants, no significant nematode inhibitory effect could be observed on transgenic plants expressing cZR-9. Thus, a partial resistance in cZR-9-transgenic sugar beet roots observed by Tian (2003) may be a true reflection of the "in vitro" nature of the hairy roots.

Most importantly, we found that cZR-3 as well as cZR-7 confers nematode resistance in transgenic Arabidopsis via activation of a specifc signaling pathway. Generally, signaling pathways required for plant defense responses are complex, and even members from the same gene family that are induced by a single pathogen may require different signal molecules or combinations of signaling pathways for their expression (Ferrari et al. 2003). Recenly, several plant defense key regulators involved in distinct pathways have been identified, thus offering us the opportunity to molecularly determine/dissect respective signaling pathways activated by cZR-3 and cZR-7 as well. These include *RAR1* (required for Mla12 resistance), *NPR1* (Nonexpresser of PR gene 1), *EDS1* as well as *SGT1* (suppressor of the G2 allele of skp1). Remarkably, we found that the expression of cZR-3 or cZR-7 in transgenic Arabidopsis plants strongly enhanced the expression of *RAR1* and *SGT1*, but not of *NPR1* and EDS1, clearly suggesting that both RGAs activate a *RAR1/SGT1* dependent signaling route in Arabidopsis, which is essential for disease resistance triggered by a wide range of non-TIR-NBS-LRR R-proteins (Hammond-Kosack et al. 2003).

It is worth noting that cZR-3 as well as cZR-7 consequently upregulate the expression of PR-proteins via the *RAR1/SGT1* dependent signaling route in Arabidopsis plants as indicated by RT-PCR analysis. While transcript levels of *PDF1.2*, *PDF2.2* and *PDF2.3* were not changed; transcriptional activities of all analysed PR-genes were strongly enhanced. Because a set of NBS-LRR R-genes upregulates genes encoding proteins with antimicrobial properties including *PR-1, PR-2, PR-4, PR-5*, it is therefore tempted to speculate that the interference of PR-proteins may represent an important aspect of the $Hs1^{pro-1}$-mediated nematode resistance in sugar beet. In Arabidopsis, induction of *PR-1, PR-2,* and *PR-5* follows a SA-dependent pathway, whereas the induction of the plant defensin *PDF1.2* and *PR-4* depends on a pathway involving at least JA as a signal molecule (Li et al. 2004).

Several scenarios are considerable to explain why expression of both RGAs in Arabidopsis can induce nematode resistance response. First, cZR-3 and cZR-7 may represent close

homolog sequences of the second nematode R-gene that resides in the translocation of the resistant line A906001 (Tian et al. 2004). Overexpression of such homologous genes triggers resistance response even though they are functionally cryptic in respect to conferring nematode resistance in sugar beet, normaly. Thus, a complete cloning of the translocation and analysis of translocation mutants recently generated by radio-irradiation will shed light on it; alternatively, cZR-3 and cZR-7 may represent RGAs evolved during the evolution in the sugar beet genome. They served as reservoir of variation for resistance specificities during the evolution. These RGAs harbour the ability to activate plant defence mechanisms when they are overexpressed in plants. Third, it is most likely that cZR-3 and cZR-7 may play an active role in regulating the *Hs1*$^{pro-1}$-mediated nematode resistance. Increasing evidence supports the guard hypothesis that NBS-LRR-containing R-proteins "guard" and protect the host defence machinery from pathogens that manipulate it. Following this, the sugar beet RGAs, cZR-3 and cZR-7 may function as host targets/components in facilitating the *Hs1*$^{pro-1}$-mediated resistance response. In this case, overexpression of cZR-3 or cZR-7 will activate a specific signal cascade finally leading to resistance response. Thus, a great challenge remains to explore the possible interactions among *Hs1*$^{pro-1}$, cZR-3, cZR-7 and other components as well as the possible impact of these interactions on nematode resistance response.

6 Acknowledgments

This work was financially supported by the Deutsche Forschungsgemeinschaft (grants No. JU 205/12-1, SFB617-A19).

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Chapter V

A two-step protocol for improving shoot regeneration frequency from hypocotyl explants of oilseed rape (*Brassica napus* L.) and its application for *Agrobacterium*-mediated transformation

(In press, Biologica Plantarum))

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Keywords: Oilseed rape (*Brassica napus* L.), Shoot regeneration frequency, Two-step protocol, *Agrobacterium*-mediated transformation, Plant tissue culture

Abbreviations: BA: 6-benzylaminopurine; CIM: Callus induction medium; CTAB: cetyl trimethyl ammonium bromide; 2.4-D: 2, 4-dichlorophenoxyacetic acid; GUS: β-glucuronidase; MS: Murashige and Skoog; NAA: Naphthaline acetic acid; SIM: Shoot induction medium; STS: Silver thiosulphate; X-Gluc: 5-bromo-4-chloro-3-indolyl β-D-glucuronide.

1 Abstract

A two-step shoot regeneration protocol from hypocotyl explants of oilseed rape was established and applied to Agrobacterium-mediated transformation with a semi-winter-type of oilseed rape (*Brassica napus* L.) cultivar. An optimal medium for shoot regeneration from hypocotyl explants was determined based on MS medium supplemented with different concentration combinations in BA (2-5 mg dm^{-3}) and NAA (0.05~0.15 mg dm^{-3}). The maximum shoot regeneration frequency (13%) was obtained on the MS medium supplemented with 4 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA. But, the shoot regeneration frequency could increase upto 24.45% when 20 μM STS was added into the medium. Based on this result, a two-step protocol for an efficient shoot regeneration from oilseed rape hypocotyl explants was established, in which the hypocotyl explants were first put on CIM medium including 2, 4-D (0.5 to 1.5 mg dm^{-3}) for 3 -7 days pre-cultivation to induce embryogenesis and then transferred onto SIM medium (4 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA with STS) for shoot regeneration via organogenesis. In this way, the shoot appearance time was 7 days earlier and the shoot regeneration frequency dramatically increased (up to 96.67%), compared to those observed by use of one-step protocol. To explore the potential of the two-step protocol for generation of transgenic oilseed rape plants, it was applied for Agrobacterium-mediated transformation experiments with the gene cZR-3, a nematode resistance gene candidate. About 14% of plants generated proved to be cZR-3-transgenic as confirmed by GUS assays, by cZR-3-specific PCR and by Southern-blot hybridization experiments as well.

2 Introduction

Brassica is the most economically important genus in the Cruciferae. It includes vegetable crops, oilseed crops, forage crops and some condiment crops, such as mustard. Oilseed rape (*Brassica napus* L.) produces highly valuable oil for human nutrition as well as for biofuels, representing an important crop in *Brassica*. Many *Brassica* species occupy pivotal potential for plant basic research due to its close genetic relation to the model plant *Arabidopsis thaliana* (The Arabidopsis Genome Initiative 2000). Oilseed rape is therefore getting more and more importance also as a model crop for genome and functional genome analysis. For instance, more than 70% gene transformation experiments reported in *Brassica* so far were either for identification or for functional analysis of novel genes (Sparrow et al. 2004).
The Agrobacterium-mediated transformation had been widely applied for generation of transgenic plants in various *Brassica* species including oilseed rape (Cardoza and Stewart 2003; Lee et al. 2004; Cho et al. 2008). An efficient transformation system for oilseed rape strongly relies on shoot regeneration frequency (Akasaka-Kennedy et al. 2005), which is still an obstacle to be overcome. Generally, there are two strategies successfully leading to shoot regeneration via plant tissue culture: one is via somatic embryogenesis and the other one is via organogenesis (Hanson and Wright 1999). The organogenesis system enables to generate organs, especially shoots directly from the tip of explants while the somatic embryogenesis system generates first embryos from the explants via callus formation and then shoots from the embryos. The proliferating somatic embryos proved to be suitable targets for Agrobacterium-mediated transformation because the origin of proliferating embryogenic tissues is at or near the surface of the older embryos and thus accessible to DNA delivery. The organogenesis system has been frequently applied for shoot regeneration from many *Brassica* species. In case of oilseed rape, various explants of can be used for shoot regeneration via organogenesis. They include cotyledon (Narasimhulu and Chopra 1988; Tang et al. 2003), hypocotyl (Khehra and Mathias 1992; Tang et al. 2003; Jonoubi et al. 2005), flowering internodes (Klimaszewska and Keller 1985; Tang et al. 2003), stem sections (O'Neill et al. 1996), immature cotyledons (Turgut et al. 1998), leaves (Akasaka-Kennedy et al. 2005) and as well thin cell layer (Shu and Loh 1991, Ghnaya et al. 2008).
Cotyledon explants of oilseed rape were most frequently used for shoot regeneration because they are easier to regenerate (Tang et al. 2003). The transformation efficiency of cotyledon explants by Agrobacterium-mediated transformation is but extremely low. Mukhopadhyay et al. (1992) reported that 2 chimetric transformed shoots could be obtained from more than

10,000 cotyledons treated by Agrobacterium. Besides, the transformation efficiency was strongly genotype-dependent (Sparrow et al. 2004). In contrast, hypocotyl explants of oilseed rape are easily prepared and as well transformed by Agrobacterium- mediated transformation. But they often suffered from poor shoot regeneration frequency (Mukhopadhyay et al. 1992). In this paper, we report a two-step protocol for shoot regeneration from hypocotyl explants of oilseed rape, which can significantly improve shoot regeneration frequency from hypocotyls explants. Its potential for generation of transgenic plants by Agrobacterium-mediated transformation is demonstrated.

3 Materials and methods

3.1 Plant materials

Semi-winter type oilseed rape (*B. napus* L.) cultivar, Zheshuang 758, kindly provided by the Zhejiang Academy of Agricultural Science, PR China was used for shoot regeneration and Agrobacterium-mediated transformation experiments.

3.2 Explant preparation

The sterile hypocotyl segments (5-10 mm) from germinated 5 to 6 days old seedlings were used as explants for shoot regeneration and transformation. Seeds were first surface-sterilized in 70% ethanol for 0.5 min and then submerged in 10% calcium hypochlorite including 2 drops of Tween20 for 25 min, and then rinsed 4 to 5 times with sterilized distilled H_2O. The seeds were sowed in 150 cm^3 triangular flask containing 50 cm^3 half-length MS (Murashige and Skoog, 1962) phytohormone-free germination medium supplemented with 20 g dm^{-3} sucrose and 8 g dm^{-3} agar for solidification (pH 5.8). Fifteen seeds were sowed in each triangular flask.

3.3 Plant tissue culture

The basal medium for this experiment was MS medium supplemented with 30 mg dm^{-3}, and solidified with 8 g dm^{-3} agar (pH 5.8). Phytohormones, MS basal salts and antibiotics were purchased from Sigma (Germany). All media were sterilized with sucrose and agar by autoclaving at 120°C for 20 min. Phytohormone, STS and antibiotics were filter-sterilized by passing through a 0.2 μm syringe filter and added to the cooled (70°C) autoclaved media before pouring into 90 mm sterile Petri-dishes..

3.4 Shoot regeneration protocol

Shoots were regenerated by use of one- and two-step protocol, respectively. By one-step protocol, hypocotyl segments were cultured only on the MS medium supplemented with different combination concentration of BA (2~5 mg dm^{-3}) and NAA (0.05~0.15 mg dm^{-3}) with or without 20 μM STS (Table 1). By two step protocol, hypocotyl segments were first pre-cultured on callus induction medium (CIM) complemented with 2.4-D (0.5~1.5 mg dm^{-3}) for 3 or 7 days and then transferred onto the shoot induction medium (SIM) with 4 mg dm^{-3} BA and 0.10 mg dm^{-3} NAA in combination with or without 20 μM STS solution (Table 2) as well. In all cases, ten explants were placed in one 90 mm Petri-dish and at least forty explants

were prepared. Cultures were incubated in a culture room at 25±1°C under a lighting regime of 16h/8h (light/dark; light intensity, 100 μmol m^{-2} s^{-1}). After 3-4 weeks of culture, the adventitious shoots that formed on the explants were counted. Shoot regeneration frequency (number of explants with shoots / total number of explants×100%) was averaged from 3 to 4 replicates. SPSS 11.0 software (SPSS Inc. USA) was used statistically for data analysis.

3.5 Plant transformation

For plant transformation, a recombinant binary vector pAMcZR-3 was used, which contains the full-length cDNA of cZR-3 (Tian et al. 2004), a sugar beet resistance gene candidate (accession number: DQ907613). In addition, the binary vector carries the GUS reporter gene and a selectable marker, neomycinphosphotranferase gene (*nptII*) for kanamycin resistance. The plasmid DNA of pAMcZR-3 was transformed into Agrobacterium strain GV3101 by electroporation (Shen and Forde, 1989). Ten mm hypocotyl segments were directly immersed in bacterial suspension (O.D.600 0.1~0.2) with gentle shaking for 10 min. The segments were subsequently blotted on sterile filter paper and transferred in the 90 mm Petri-dish containing fresh CIM to co-culture with bacteria for 2 days. Twenty hypocotyl segments were inoculated in each Petri-dish. After 2 days co-cultivation the segments were washed 3 times with liquid CIM containing 500 mg dm^{-3} carbenicillin to kill the excessive Agrobacterium on the segments. The segments blotted on the sterilized filter paper and placed on the CIM containing the antibiotic to inhibit the growth of Agrobacterium for 5 days. After it, the segments were transferred on the new SIM containing 500 mg dm^{-3} carbenicillin and 50 mg dm^{-3} kanamycin for selection till the putative green shoots appeared. The whole segments including green shoots were transferred onto solid half-strength MS medium combined with 10 g dm^{-3} sucrose, 9 g dm^{-3} agar and antibiotics for selection for shoot elongation. After shoot elongation for 3 weeks the green shoots were transferred on the same shoot elongation medium to develop the roots. The plantlets were transferred to soil in greenhouse and vernalized at 4°C for 40 days. The plants were isolated with plastic bags during the flowering time.

3.6 Histochemical GUS assays

For histochemical GUS assays, 10 cm^3 X-Gluc solution including 50 mM Na_3PO_4 buffer (pH 7.0), 0.2 mg cm^{-3} X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) and two drops of Triton-100 was added in the Petri-dish which contained the putative transgenic and negative control leaves. The leaves were covered with X-Gluc solution and incubated at 37°C for 16 h.

After it, the leaves were cleared using 70% ethanol to fade the chlorophyll. The GUS staining signals were evaluated under a stereomicroscope (Stemi SV 11, Zeiss, Germany).

3.7 PCR and Southern analysis

Genomic DNA from young leaf tissue was extracted using CTAB method as described by Rogers and Bendich (1985). The gene-specific primers used for amplification are: cZR-3A: 5'- *AGTTATTGATAGGGCTATGG* -3' and cZR-3 B: 5'- *ATACTTGAAGCAGTCAGG*-3' resulting in a fragment (cZR3-A/B) of 410 bp in size (Lein et al., 2007). PCR was performed as follows: DNA denaturation at 94°C for 3 min, followed by 35 cycles of 1 min at 94°C, 1 min at 53°C and 1.3 min at 72°C. A final extension cycle was at 72°C for 10 min. The PCR products were visualized by electrophoresis on a 1.2% agarose gel stained with ethidium bromide. For Southern blot analysis, 50 ng genomic DNA of oilseed rape leaves was restricted by restriction enzymes at 37°C for 5 hours. The digested DNA was fractioned on 0.75 % agarose gel. DNA was transferred onto a Hybond-N+ membrane (GE Healthcare, USA) by capillary diffusion blotting with 0.25M NaOH/1.5 M NaCl blotting solution, overnight. Southern blots were hybridized using ^{32}P-labelled cZR-3 A/B DNA fragment as probe (Feinberg and Vogelstein 1983) at 62°C overnight. The blots were washed twice with 0.5xSSC, 0.2% w/v SDS for 30 min and together with the film exposed at –70°C for 48 h.

4 Results

4.1 Optimization of shoot regeneration medium from hypocotyl explants

To set up a two-step protocol for shoot regeneration from oilseed rape explants, the optimal shoot regeneration medium was first determined with one-step regeneration protocol (Tang et al. 2003). The MS medium containing was supplemented with different concentration combinations of BA (2 to 5 mg dm^{-3}) and NAA (0.05 to 0.15 mg dm^{-3}) (Table 1). The shoot regeneration frequencies from different media were calculated and compared (Table 1). Typically, explants began to be swollen after being cultured in the regeneration medium for 7 days and first shoots could be observed after 20 days. Callus formation from the tip of hypocotyl explants was observed in all BA and NAA concentration combinations. Although adventitious shoots could be observed from the cut-end of hypocotyl explants in most of cases 30 days after cultivation, frequencies of shoot regeneration from different media varied from 0% to 13% (Table 1). The maximal shoot regeneration frequency (13%) was given on the medium supplemented with 4 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA, it was therefore used as a basal medium for establishment of two-step shoot regeneration protocol further. In addition, the shoot regeneration frequency increased in all media when 20 μM STS was added (Table 1).

Table 1: Effect of BA and NAA concentration combinations on the shoot regeneration frequency from hypocotyl explants on SIM with one-step protocol after cultivation 30 d. Values represent the mean of three replicates. Means ± SE followed by the same letter indicate no significant difference (Duncan's multiple range test, Pα0.05).

BA (mg dm^{-3})	NAA (mg dm^{-3})	Shoot regeneration frequency (%)	
		-STS	+STS
2	0.05	1.11 ± 1.11^{a}	16.67 ± 3.33^{bcd}
3	0.05	7.22 ± 0.28^{abc}	24.45 ± 2.22^{d}
4	0.05	3.53 ± 0.23^{ab}	16.67 ± 3.33^{bcd}
5	0.05	10.42 ± 2.08^{abc}	6.67 ± 6.67^{abc}
2	0.1	6.67 ± 1.67^{abc}	19.45 ± 6.41^{cd}
3	0.1	2.22 ± 1.11^{a}	20.00 ± 3.85^{cd}
4	0.1	13.00 ± 1.00^{abcd}	24.45 ± 2.22^{d}
5	0.1	0.00^{a}	13.33 ± 1.82^{abcd}
2	0.15	7.78 ± 1.92^{abc}	15.56 ± 2.94^{bcd}
3	0.15	1.67 ± 1.67^{a}	8.89 ± 0.88^{abc}
4	0.15	6.67 ± 2.31^{abc}	10.00 ± 3.35^{abc}
5	0.15	0.00^{a}	0.00^{a}

4.2 Improvement of shoot regeneration frequency from hypocotyl explants by use of a two-step protocol

To increase shoot regeneration frequency from oilseed rape hypocotyl explants, we applied a two-step protocol for shoot regeneration, in which two media CIM and SIM were involved. Hypocotyl explants were first pre-cultured on a CIM Medium containing 2,4-D (0.5 to 1.5 mg dm^{-3}) to induce callus formation for 3 or 7 days and then transferred on a SIM medium with 4 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA for shoot regeneration. As shown in Table 2, three days pre-culture of hypocotyls explants on all CIM media tested (varied 2.4-D concentration and with/without STS) did not give rise to improvement of shoot regeneration frequency (Table 2) whereas 7 days pre-culture of hypocotyls explants on CIM media resulted in a great increase in shoot regeneration frequency ranging from 21.67% to 96.67% (Table 2). Strikingly, an extremely high frequency of shoot regeneration was given in the case when hypocotyl explants have been 7 days pre-cultured on a CIM containing 2.4-D in a higher concentration of 1.5 mg dm^{-3} and subsequently cultured on a SIM supplemented with 20 µM STS (Table 2).

Table 2: Effect of 2.4-D concentration, pre-cultivation time and supplement of STS on the shoot regeneration frequency from hypocotyl explants on MS medium containing 4 mg dm^{-3} BA and 0.01 mg dm^{-3} NAA. Values represent the mean of three replicates. Means ± SE, followed by the by the same letter are not significantly different (Duncan's multiple range test, Pα0.05).

2.4-D concentration (mg dm^{-3})	Pre-cultivation for 3days		Pre-cultivation for 7days	
	- STS	+ STS	- STS	+ STS
0.5	6.67 ± 0.77^{ab}	40.00 ± 17.32^{d}	26.67 ± 5.77^{cd}	57.33 ± 11.01^{e}
1.0	0.00 ± 0.00^{a}	22.33 ± 6.80^{bc}	0.00 ± 0.00^{a}	71.33 ± 14.43^{e}
1.5	3.33 ± 0.77^{a}	21.67 ± 2.89^{bc}	10.00 ± 0.32^{ab}	96.67 ± 5.77^{f}

4.3 Improvement of shoot regeneration frequency from hypocotyl explants by addition of STS

STS, silver thiosulphate is an ethylene inhibitor in plant tissue culture. To test its effect on shoot regeneration from hypocotyl explants of oilseed rape 20 μM STS was added into different SIM media and the shoot regeneration frequencies were scored and compared with those from without addition of STS. As shown in Table 1 and 2 the shoot regeneration frequencies dramatically increased by addition of STS either by use of one or by use of two-step regeneration protocol. In case of one-step protocol, the shoot regeneration frequency was improved from 1.11% to 16.67% on the medium with 2 mg dm^{-3} BA and 0.05 mg dm^{-3} NAA. The shoot regeneration frequency could further increase up to 24.45% when the medium was supplemented by 4 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA in combination (Table 1). Strikingly, an extremely high frequency of shoot regeneration (96.67%) was given by use of the two-step protocol when hypocotyl explants have been 7 days pre-cultured on a CIM containing a higher 2.4-D concentration of 1.5 mg dm^{-3} and subsequently cultured on the SIM supplemented with 20 μM STS (Table 2).

Furthermore, the shoot regeneration from hypocotyl explants was significantly quickened by two-step protocol in combination with addition of 20μM STS in the SIM medium. As shown in Figure 1A, the explants started to be swollen 5 days after transferring on the SIM, callus appeared clearly around the cut end of hypocotyls and potential shoots emerged about 10 days after the transferring on the SIM. In average, the shoot emerging time was 7 days earlier compared to that observed by use of one-step protocol.

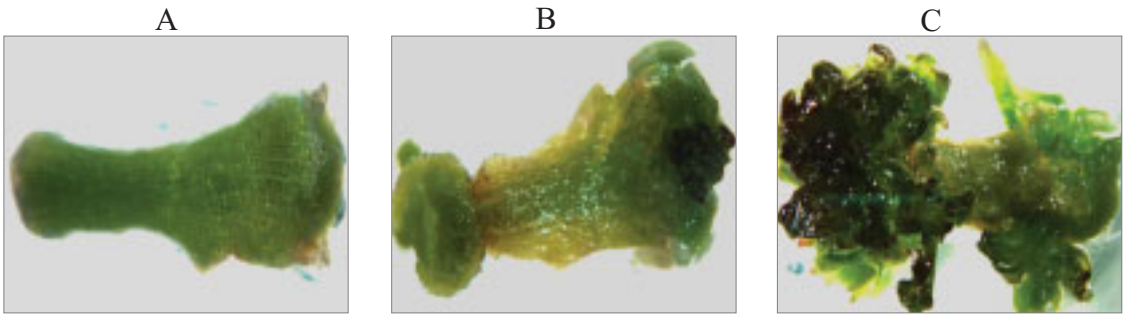

Figure 1: Development of shoots regenerated from hypocotyl explants 5 days (A), 10 days (B) and 20 days (C) after cultivation on SIM following the two-step protocol. The hypocotyl explants were first pre-cultured for 3 days on the CIM medium with 1.0 mg dm^{-3} 2,4-D. The SIM was supplemented with 4 mg m^{-3} BA, 0.10 mg dm^{-3} NAA and 20 μM STS.

4.4 Application of two-step regeneration protocol for generation of transgenic oilseed rape plants

The two-step regeneration protocol was applied for generation of oilseed rape transgenic plants with Agrobacterium-mediated transformation (Fig. 2). About 200 fresh hypocotyl segments of oilseed rape cultivar, Zheshuang 758, were incubated with 100ml Agrobacterium suspension cells carrying pAMcZR-3. Two days after co-cultivation on CIM, the segments were subject to being cultivated for shoot regeneration following the two-step protocol as described. In total, 270 shoots were regenerated, from which 43 putative transgenic plants were obtained.

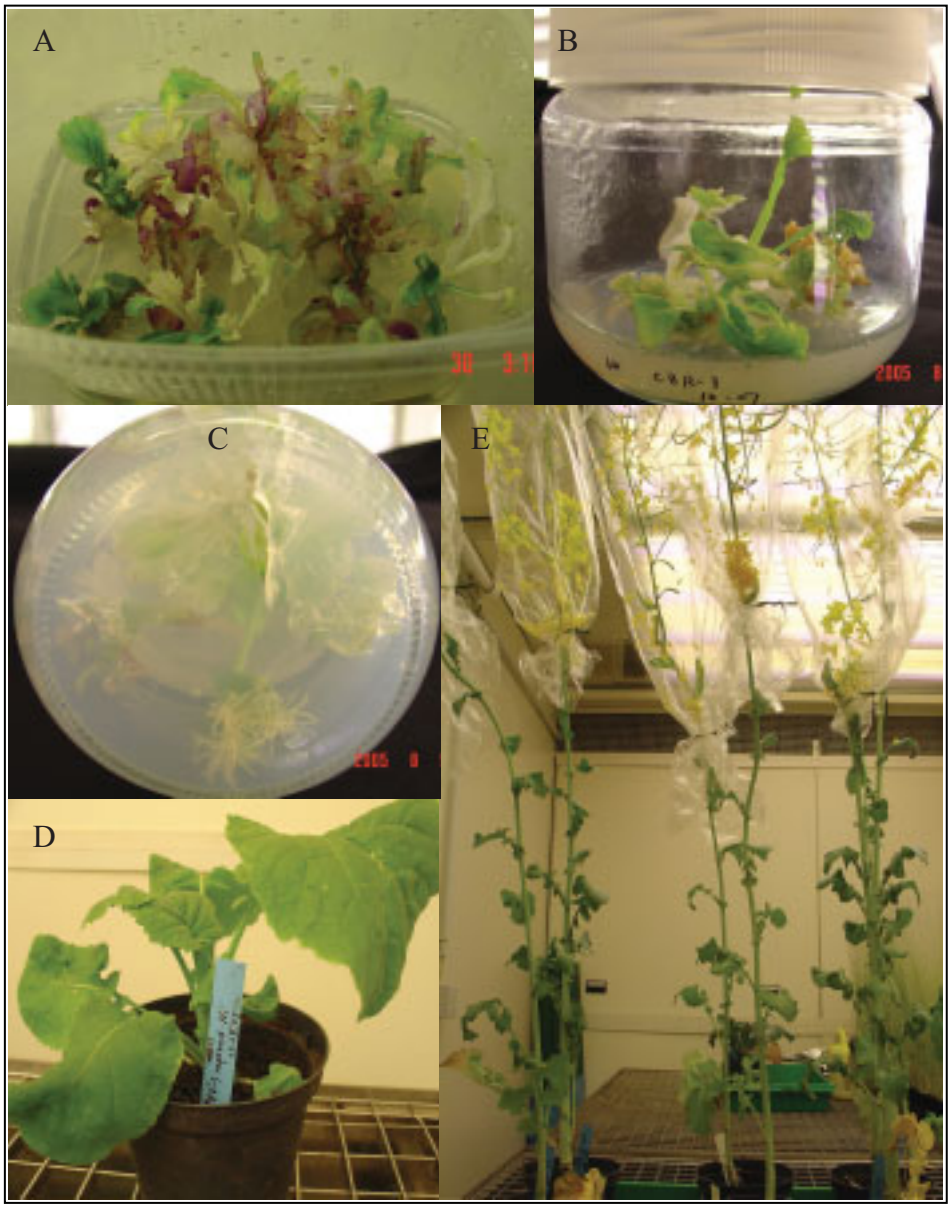

Figure 2: Generation of transgenic plants from hypocotyl explants of semi-winter *B. napus* type cultivar cv. *Zheshuang758*. (**A**) Shoots regenerated from hypocotyl explants on SIM containing 500 mg dm^{-3} carbenicillin and 50 mg dm^{-3} kanamycin sulphate, (**B**) The elongated shoots on SIM, (**C**) Roots developed in half-strength MS medium containing 500 mg dm^{-3} carbenicillin and 50 mg dm^{-3} kanamycin sulphate, (**D**) the transgenic plantlet transferred in the soil, (**E**) Propagation of transgenic oilseed rape plants in a growth chamber (25°C /16°C, day/night).

To verify the transgenic nature, all of 43 putative transgenic plants were subject to being analysed by histochemical GUS assays, by cZR-3-specific PCR and as well by Southern-blot hybridization experiments using a cZR-3-specific probe. As a result, 6 of 43 plants (about 14 %) proved to be cZR-3-transgenic, giving a strong GUS staining, the cZR-3-specific PCR amplification and the cZR-3-specific Southern-hybridization as shown in Figure 3, 4 and 5.

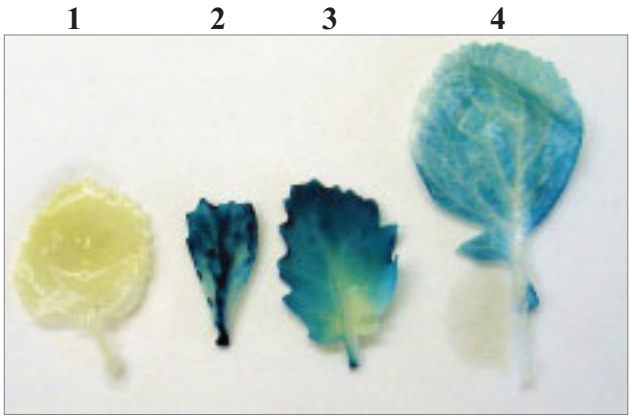

Figure 3: Histochemical GUS assays with putative transgenic (2, 3, 4) leaves and negative control (1). The GUS-staining (blue) were evaluated under a stereomicroscope (Stemi SV 11, Zeiss, Germany).

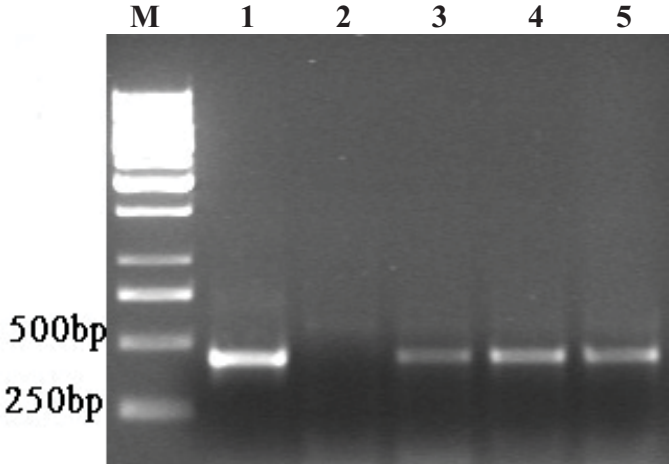

Figure 4: PCR analysis with GUS positive transgenic plants. cZR-3-specific amplification of 410 bp in size was given. M: molecular size marker (1kb ladder), Lane1: positive control with a recombinant plasmid DNA, Lane 2: negative control (untransformed); Lane 3, 4 and 5: GUS-positive plants.

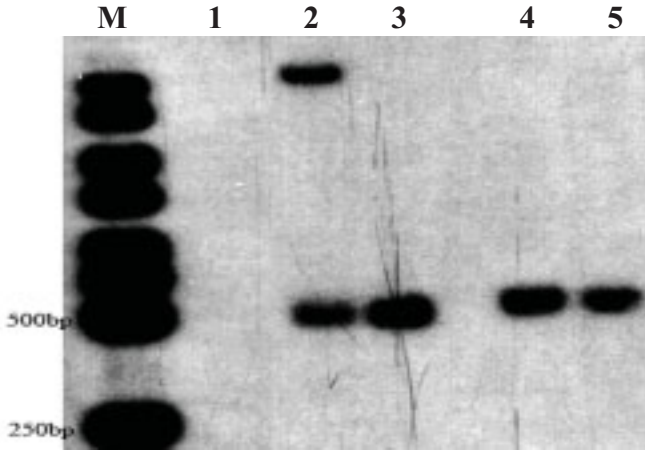

Figure 5: An autoradiogram of the Southern blot hybridization. *M:* molecular size marker 1 kb ladder; *lane 1*: negative control (wild type); *lane 2, 3, 4, 5*: GUS and PCR positive plants. The cZR-3A/B was used as a probe.

5 Discussion

In this study, we reported a two step shoot regeneration protocol for oilseed rape (*B. napus* L.) and demonstrated that the shoot regeneration frequency from hypocotyl explants of oilseed rape can be greatly improved by using the two-step protocol. The maximal shoot regeneration frequency reached 96.67%, and the shoot appearance was significantly quickened. The duration of pre-cultivation on CIM and the supplementation with STS were, in addition to 2.4-D concentration, two main factors significantly influencing shoot regeneration frequency. This result is in coincidence with observation made by Tang et al. (1999). We observed that the 2.4-D concentration in medium had promoting effect on the callus quantity, but the callus quality was influenced obviously by the duration of pre-cultivation on CIM (data not shown). Application of 2.4-D into the CIM had been reported to induced excessive callus growth during the subsequent shoot regeneration phase consequently leading to increase in the frequency of gene multiple-copy by Agrobacterium-mediated transformation (Cardoza and Stewart 2003)

An important finding in this study is that supplement of STS into SIM medium gave rise to a dramatic increase of shoot regeneration frequency from hypocotyls explants of oilseed rape. A similar observation had been reported by several groups in different regeneration systems, e.g. with cotyledonary explants of *B. campestris* (Chi et al.1991), hypocotyls explants of *B. juncea* (Pua and Chi 1993), cotyledon, leaves and thin cell layer explants of *B. napus* (Tang et al. 2003; Akasaka et al. 2005; Ben Ghnaya et al. 2008). STS, silver ion, is a potential ethylene inhibitor and is considered to suppress shoot morphogenesis in vitro. In consistence with the observation by Khan et al. (2003), we observed that most of transformed shoots were becoming necrosis on the medium that was not supplemented by STS (data not shown). The fact that an extremely high frequency of shoot regeneration could be archived when hypocotyl explants had been 7 days pre-cultured on a CIM containing 2.4-D in a higher concentration of 1.5 mg dm^{-3} and subsequently cultured on a SIM supplemented with 20 µM STS (Table 2) strongly suggests a synergic effect of three factors on the shoot regeneration from hypocotyl explants of oilseed rape. The mechanism underlying is unknown.

We demonstrated that the two step regeneration protocol can be successfully used for Agrobacterium-based transformation for generation of transgenic plants from a semi-winter type oilseed rape (*B. napus* L.) cultivar, Zheshuang 758. About 14% regenerated plants by use of this protocol proved to be transgenic, demonstrating its potential in this aspect. However, comparing to a high shoot regeneration frequency with the two step protocol, the transformation efficiency obtained in this study was still low. Only 2.2% (6 plants from 270

shoots) regenerated shoots were transgenic. We found that explants could be regenerated in the selection medium containing 50 mg l^{-1} kanamycin (Fig. 6A), but most of these failed to develop when shoots were subjected to elongation on shoot elongation medium containing 100 mg l^{-1} kanamycin. Obviously, they were not able to develop roots and therefore becoming vitrified (Fig. 6B). Even though the Agrobacterium-mediated transformation has been applied for rapeseed breeding and gene function analysis since 1987 (Fry et al. 1987), most of transformation experiments on *B. napus* had been restricted to spring cultivars and to a very few genotypes as well (Cardoza and Stewart 2003). Improvement of transformation efficiency for *B. napus* still remains a great challenge.

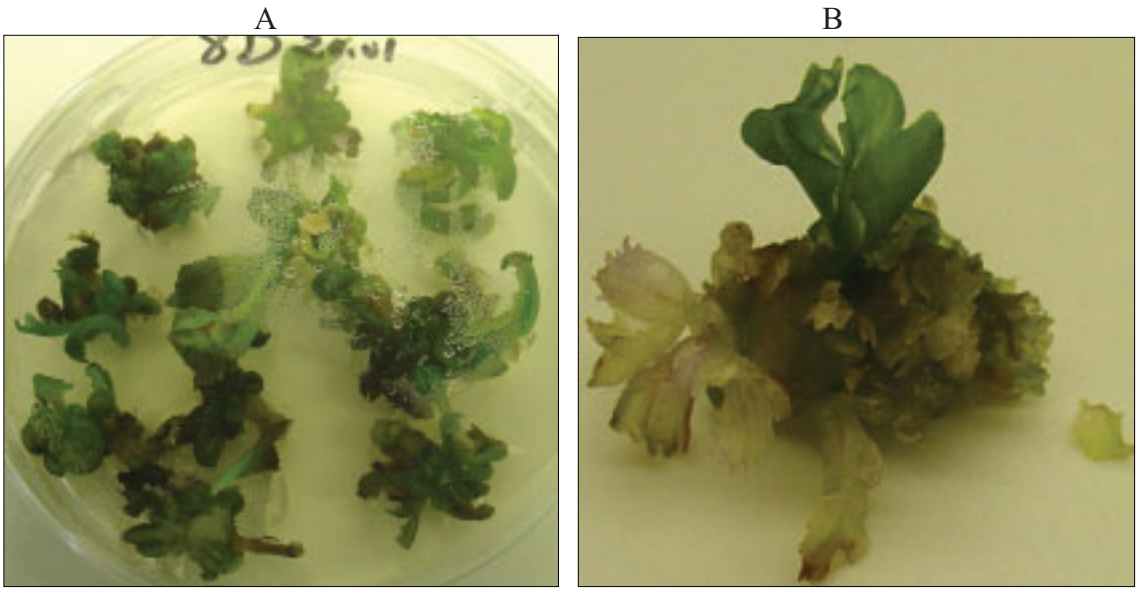

Figure 6: High frequency of shoot regeneration from oilseed rape hypocotyl explants for *Agrobacterium* mediated transformation. (A) Regenerated shoots after cultivation 25 days on the SIM containing 500 mg dm^{-3} carbenicillin and 50 mg dm^{-3} kanamycin sulphate, (B) The vitrificated shoots after cultivation 30days in the shoot elongation medium containing 500 mg dm^{-3} carbenicillin and 100 mg dm^{-3} kanamycin sulfate.

6 Acknowledgements

The research was financially supported by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, Zhejiang province, P R China and the German research foundation DFG (grant No. SFB617-A19). Authors thank the German Academic Exchange Service (DAAD) for providing a short visiting scholarship.

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Chapter VI: General discussion

1 *BvGLP-1* represents a new class of oxalate oxidase-like genes and plays a role in plant resistance

Plant genomes encode a subgroup of cupins called germin and germin like proteins (GLPs), water-soluble, protease-resistant, heat stable and SDS-tolerant glycoproteins (Lane 1994; Woo et al. 2000). Here, we analyzed the nematode inducible germin like gene *BvGLP-1* which possesses an oxalate oxidase (OxO) activity and in this way might be responsible for the production of a H_2O_2 burst and/or transmission of a signal leading to resistance response. Several members of the GLP multigene family have been suggested to exhibit an OxO enzyme activity, which catalyses the degradation of oxalic acid (OA) to produce carbon dioxide and hydrogen peroxide (Chiriboga 1966; Lane et al. 1993). Through the generation of H_2O_2, OxO or OxO-like proteins may catalyse cross-linking of plant cell wall proteins at the infection site and lignifications for the reinforcement of the cell wall (Olson and Varner 1993; Thordal-Christensen et al. 1997; Wei et al. 1998). On the other hand, OxO may act as a signaling molecule involving in plant hypersensitive responses (Lane 1994; Zhou et al. 1998) which is known to be orchestrated by H_2O_2 production during oxidative burst (Levine et al. 1994). Thus, a role of germin and GLPs in plant defense response has been proposed mainly based on the capacity of the OxO to produce H_2O_2. It is very unlikely that all GLPs exhibit an enzyme activity or are related to resistance response since we know that in plants with small genomes such as Arabidopsis and rice more than 30 different GLPs exist (Dunwell et al. 2008).

However, the nematode responsive character of *BvGLP-1* was the first strong indication for its functionality in nematode resistance. As revealed by transcript profiling experiments, the gene *BvGLP-1* is transcribed in both of resistant and susceptible plants at a relatively lower level even without nematode attack. But, its expression is highly up-regulated upon nematode infection in the resistant plant, suggesting its active role in nematode resistance.

Transgenic technology has been used for demonstration of the function of OxO in enhancing plant resistance to various pathogens. Oilseed rape (*Brassica napus*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), and sunflower (*Helianthus annuus*) transformed with a wheat OxO gene demonstrated increased resistance to *S.sclerotiorum* by OA detoxification (Thompson et al. 1995; Donaldson et al. 2001; Hu et a.l. 2003) and a similar strategy was used to provide disease resistance in poplar (Liang et al. 2001), peanut (Livingstone et al. 2005), tomato (Walz et al. 2007) and American chestnut (Welch et al. 2007). There are also

reports of improved resistance to European corn borer (*Ostrinia nubilalis*) in maize (*Zea mays*) expressing a wheat OxO (Ramputh et al. 2002).
The potential of the gene in activation of nematode resistance was investigated by functional analysis in transgenic sugar beet hairy roots and Arabidopsis plants, respectively. Using the transgenic approach, *BvGLP-1* was found to encode an active OxO that activates the *PR-1*-dependent signaling pathway in transgenic beet roots and Arabidopsis plants leading to a strong anti-nematode effect. Finally, we could demonstrate that an enhanced expression of *BvGLP-1* in feeding cells resulted in a rapid local generation of hydrogen peroxide (H_2O_2) indicating that OxO activity of *BvGLP-1* is correlated to its resistance effect. Another possible role, a structural function of *BvGLP-1* in cell wall reinforcement cannot be excluded, but the propability is limited due to its confirmed enzyme activity function.

In addition, a wide variety of fungal pathogens insert OA into the plant cells following the infection (Bateman and Beer 1965; Stone and Armentrout 1985; Bennett and Hindal 1989; Ritschkoff et al. 1995; Liang et al. 2001; Hollowell et al. 2001; Zou et al. 2007). OA aids the pathogen in infection through a number of proposed routes, like pH reduction in its environment, increasing polygalacturonase activity and sequestering calcium ions from calcium pectate (Punja et al. 1985; Stone and Armentrout 1985; Zou et al. 2007). In addition OA suppresses the oxidative burst (Cessna et al. 2000) and disturbes the guard cell function (Guimaraes and Stotz 2004). OxO is found to provide a high level of quantitative resistance to fungal pathogens of wheat (Faris et al. 1999) and one GLP is speculated to be responsible for producing the penetration-associated H_2O_2 in barley and wheat after fungal attack (Wei et al. 1998; Schweizer et al. 1999; Christensen et al. 2004). This let us assume that H_2O_2 production by *BvGLP-1* might be generally important for non race specific resistance, including basal resistance. The resistance enhancing effect of overexpressed *BvGLP-1* against the fungal species *Verticillium longisporum* and *Rhizoctonia solani* confirms our hypothesis. *R.solani* and *V.longisporum* are thought to produce OA, the substrate of OxO, as a pathogenicity factor. However there is no indication for OA production by plant parasitic nematodes, so far. The explanation may well have to do with the fact that oxalate might be formed for example from L-ascorbic acid as suggested by Lou et al. (2006) since it is required for another enzymatic reaction.

2 NBS-LRR containing RGAs are involved in the *Hs1*$^{pro-1}$-mediated resistance

The degenerated primer based approach using common motifs within the NBS domain that are highly conserved is sufficient for PCR amplification of resistance gene analogs (RGAs) from a wide variety of plant species, for example soybean (Kanazin et al. 1996; Yu et al. 1996), potato (Leister et al. 1996), lettuce (Meyers et al. 1999), cereals (Pan et al. 2000), sugar beet (Tian et al. 2004), rape (Tanhuanpaa 2004) and cotton (He et al. 2004). RGAs are useful in physical mapping and as gene candidates in positional cloning (Zhang et al. 2008). However, R-gene families are large multigene families and are abundant in plants. To date, 800 RGAs have been obtained via PCR amplification from nearly 20 plant species, for example, ca. 200 RGAs are known in Arabidopsis and ca. 500 non-TIR/NBS/LRR R-genes in rice (Shen et al. 2002; Meyers et al. 2003). The most cloned RGAs are part of clusters of related sequences and are co-localized with resistance loci facilitating the identification of R-genes suggesting their possible role in disease resistance responses in plants (Lopez et al. 2003; Graham et al. 2000). Thus, the *Rp1-D* rust resistance gene from maize was positively identified by the RGA PIC20 (Collins et al. 1998; 1999). Some clusters have been identified as regions where resistance to a pathogen was previously mapped (Decroocq et al. 2005) and cosegregation between RGAs and major genes or quantitative trait loci (QTL) involved in disease resistance has been frequently reported (Pflieger et al. 2001; Hunger et al. 2003). This indicates that components of qualitative and quantitative resistance to pathogens may be controlled by structurally and functionally similar genes or may have evolved from common ancestors. Within a cluster of RGAs, there may be more than one gene conferring resistance to different isolates of a specific pathogen or to biologically diverse pathogen taxa (Cooley et al. 2000). For instance, the *Xa21* locus in rice consists of at least eight sequences spanning 230 kb (Chen et al. 2002; Yang et al. 2002), the *M* locus of flax contains 15 or more sequences spread over a distance of less than 1 Mb (Ellis et al. 1995; Anderson et al. 1997), and the *Dm3* cluster of lettuce consists of at least 24 non-TIR NBS-LRR sequences that span approximately 3.5 Mb (Meyers et al. 1998; Shen et al. 2002).

As has been suggested, most RGAs do not encode a functional product and exist for relatively short periods of time probably serving as reservoirs of potential variation. Published models for the generation of novel R-gene specificities propose recombination involved in alterations in the gene copy number, gene conversion, and unequal crossing over for the generation of duplicated arrays of resistance genes as the primary mechanisms in generating haplotype

diversity (Shepherd and Mayo 1972; Pryor et al. 1987; Richter et al. 1995; Hammond-Kosack and Jones 1997). Incidentally, a novel RGA confers recognition of a pathogen and becomes a R-gene when it confers resistance. As described above, RGAs closely linked to R-genes may be relatively easy to find, but the identification of a functional R-gene within a complex multigene family is much more difficult and time consuming. Definitive proof that these sequences are R-genes requires transgenic complementation.

As no complete nematode resistance could be observed by *Hs1*$^{pro-1}$ transgenic sugar beet plants, it is proposed that a second gene may be required for the resistance (Cai et al. 2003; Schutle et al. 2006). Genetic complementation experiments in *Arabidopsis thaliana* proved that cZR-3 and cZR-7 are able to trigger a *Hs1*$^{pro-1}$ -independent and complete resistance reaction. These results strongly support an active role of both RGAs in nematode resistance. By contrast, no significant anti-nematode effect could be observed on transgenic plants expressing cZR-9, on which females were able to develop regularly as on the control plants. Within the nematode resistance experiments on transgenic plants a large number of the J2 juveniles failed to complete the penetration process and stagnated during penetration. Similar to the *Hs1*$^{pro-1}$-mediated resistance reaction, strong necroses around the feeding sites and disruption of the feeding structure resulting in programmed cell death were also characteristic for cZR-3 (Tian 2003). We hypothesized that cZR-3 and cZR-7 may function in concert with *Hs1*$^{pro-1}$ to activate disease resistance demonstrating that resistance activity conferred by R-genes is not only determined by the gene itself but also largely dependent on the genetic background of the host carrying the gene. This is also suggested for *RPW8.1* and *RPW8.2* conferring resistance to a wide range of powdery mildew diseases of *A. thaliana. RPW8* may interact with NBS-LRR R-proteins to initiate resistance responses (Xiao et al. 2001), possibly as targets for pathogen virulence factors that are guarded by NBS-LRR R-proteins as has been proposed for *Pto* (Xiao et al. 2003).

The identification of RGAs enlarges the use of both conventional and transgenic approaches to increase disease resistance of plants. They can be used directly as molecular markers in breeding and/or provide a good starting point for map-based cloning of potential R-genes in the corresponding clusters. The PCR based approach using degenerated primer also has some limitations. Thus, since the primers used to identify RGAs are made from a highly conserved domain, they are unlikely to seperate a functional gene from its nonfunctional paralog (Graham et al. 2000). Dissection of such complex loci will be aided by the development of oligonucleotide primers specific to individual RGA sequences.

The ratio of TIR and non-TIR/coiled-coil RGAs can vary among different plant species. Thus, Kanazin et al. (1996) in soybean and Martinez Zamora et al. (2004) in strawberry identified mainly TIR-subfamily sequences, but Donald et al. (2002), using a similar PCR strategy with degenerate primers in grapevine, found a high proportion of non-TIR sequences. Tian et al. (2004) assumed that TIR-type NBS-LRR sequences are compeletly lost in sugar beet and that this could also be the case for other dicot species. In contrast non-TIR-type RGA sequences are absent in apricot genomes (Soriano et al. 2005).

3 A possible function model for the *Hs1*$^{pro-1}$-mediated resistance response

Gaining experimental support in recent years, the guard hypothesis describes the indirect recognition of pathogen elicitors by NBS-LRR R-genes through their effects on "guarded" proteins (Van der Biezen and Jones 1998; Dangl and Jones 2001; Jones and Dangl 2006). Having this hypothesis in mind we suggest a model explaining the role of *Hs1*$^{pro-1}$ and cZR-3/cZR-7 in nematode resistance that is mainly based on results of this thesis and previously described models (Tian et al. 2004; Cai et al. 2003).

Hs1^{pro-1} encodes a plasma membrane protein with an extensive leucine-rich region, a transmembrane spanning domain and a short hydrophobic c-terminal domain. Therefore, it could be speculated that *Hs1*$^{pro-1}$ resides in the plasma membrane as a receptor with its N-terminus towards the extracellular space (Cai et al. 1997). Nematode compounds injected via the stylet or released from cuticular ducts of gland cells may contain molecules which are recognised by *Hs1*$^{pro-1}$. In this regards, the leucine rich region may be involved in signal perception and the positively charged C-terminal region of *Hs1*$^{pro-1}$ located in the cytosol may be responsible for the activation of signal transduction pathways leading to nematode resistance responses (Cai et al. 2003). Expression of the extracellular LRR domain of *Xa21* solely in rice plants confers partial resistance to six races of *X. oryzae*, which suggests that the LRR domain of *Xa21* is involved in pathogen recognition (Song et al. 1995).

In contrast to *Hs1*$^{pro-1}$, cZR-3 and cZR-7 like recently cloned nematode R-genes including *Mi* (Milligan et al. 1998), *Gpa2* (Van der Vossen et al. 2000) and *Hero* (Ernst et al. 2002), belong to the non-TIR-NBS-LRR class of R-proteins lacking a signal sequence. This suggests that recognition of the nematode by the host would occur within the cytoplasm. Although nematodes are extracellular pathogens, they are thought to inject the secretions into the cytoplasm of living plant cells to initiate the development of feeding cells (Williamson and Hussey 1996; Davis et al. 2000, 2004).). Therefore, cZR-3 may function as a co-receptor

with *Hs1*$^{pro-1}$ together recognizing the Avr products released by nematodes into the cytoplasm initiating signal transduction that finally leads to resistance response (Figure 4).
The *Cf-9* (Jones et al. 1994) and *Cf-2* (Dixon et al. 1996) loci of tomato are composed of arrays of five or more related genes; while the *Pto* (Martin et al. 1993) locus of tomato contains five *Pto* homologous genes and an NBS-LRR gene, *Prf*, which is essential for *Pto*-mediated resistance. *Cf*-like and *Pto*-like encoded products such as *Hs1*$^{pro-1}$ may at least constitute a two-component receptor system resembling the transmembrane LRR kinase *Xa21* (Baker et al. 1997), which is essential for recognizing *H. schachtii* effectors leading to initiation of defense responses. This activation is followed by similar physiological changes: the production of an oxidative burst and expression of several PR-proteins.

Sequence analysis of *BvGLP-1* showed that three conserved domains, germin/GLP boxes A, B and C as well as the RGD-like tripeptide motif are present in *BvGLP-1*. Amino acid alignment analysis of BvGLP-1 revealed a high homology to various plant GLPs with OxO activitiy, in which all crucial residues and sub-domains (Woo et al. 2000) characteristic of a GLP-like OxO were conserved, implying its enzymatic nature. In animal cells, the RGD motif is important for protein-protein interactions (Kim et al. 2004), therefore we suggest that these conserved residues also constitute the signal transduction activity for *BvGLP-1* in nematode resistance. Remarkably, *BvGLP-1* contains a putative auxin binding site named Box A that may reinforce the role of *BvGLP-1* as a signal transducer in the *Hs1*$^{pro-1}$ initiated resistance reaction (Figure 4).
It has been shown that the *Hs1*$^{pro-1}$ orthologous Arabidopsis proteins AtHSPROl/2 are implicated in plant pathogen resistance by interacting with AKINbc subunits of SnRKl (SNF1-related protein kinase) heterotrimeric complex through the GBD domain and that metabolic enzymes such as ACC (1-aminocyclopropane-1-carboxylate), an auxin dependent ethylene producer are downstream targets of SnRK1 complexes (Gissot et al. 2006). Moreover, it is already proven that auxin plays a prominent role in the early stages of syncytium development (Goverse et al. 2000) in susceptible plants which let us suggest that *BvGLP-1* could act as an alternative target for auxin in resistant plants. When *BvGLP-1* was overexpressed in Arabidopsis, neither *NPR1* and *EDS1* nor *SGT1* was found to be regulated in transgenic plants, therefore, we conclude that *BvGLP-1* does not participate in the early signaling events in activation of the *Hs1*$^{pro-1}$ mediated resistance.
In plants and animals alike LRR receptors are believed to transduce most signals through the activation of co-receptors or accessory proteins (Dievart et al. 2004). For instance, in rice, Xa-

21 protein requires XB3 for complete *Xa-21*-mediated disease resistance (Wang et al. 2006). Plant RLPs (receptor like proteins) perceive signals through their extracellular domain and propagate the signal through the intracellular domain (Afzal et al. 2008). The gene product of *Rme1* remains unidentified, but the authors speculated that it may be a protein kinase acting either upstream of *Mi-1* or at the same early stage of the transduction pathway raising the possibility that *Rme1* may be the target for the different nematode, aphid and whitefly Avr effectors, and *Mi-1* detects the changes in *Rme1* resulting from these interactions (Martinez de Ilarduya et al. 2004; Fuller et al. 2008).

The function of both $Hs1^{pro-1}$ and cZR-3/cZR-7 in terms of nematode resistance should be verified in whole sugar beet plants and it remains a great challenge to explore the possible direct interactions among $Hs1^{pro-1}$, cZR-3/cZR-7 and other signaling components physically. Future approaches should aim at determining all interacting components and their role and position in the $Hs1^{pro-1}$-mediated resistance response using forward (transgenic plants) and reverse genetic experimental tools (mutant plants, RNAi) to confirm this model.

4 A possible signaling pathway leading to the $Hs1^{pro-1}$-mediated resistance response

In a simplified model, two different R-gene-mediated signaling pathways have been described in *Arabidopsis thaliana* at present (Hammond-Kosack et al. 2003). The first one involves the TIR-NBS-LRR type of R-genes (e.g. *RPP1* and *RPP5*) and requires *EDS1* (Enhanced Disease Susceptibility) and *PAD4* (Phytoalexin Deficient) function to attain full resistance; and the second one involves the CC-NBS-LRR type of R-genes (e.g. *RPM1* and *RPS2*) and requires functional *NDR1* (Non-race specific Disease Resistance), *RAR1* and *SGT1* (suppressor of the G2 allele of skp1). It is known that *RAR1*and *SGT1* are required for the function and steady state accumulation of a subset of R-genes in monocot and dicot plant species; thus they are involved in *Mi-1.2*, *MLA1* and *MLA6* as well as in *Xa-21* mediated resistance (Azevedo et al. 2002; Liu et al. 2002; Wang et al. 2006). Therefore we selected several key defense signaling components representing different signaling pathways as well as a set of PR and PDF genes. In Arabidopsis, induction of antimicrobial protein genes *PR-1, PR-2,* and *PR-5* follows a SA-dependent pathway, whereas the induction of the plant defensin *PDF1.2* depends on a pathway involving at least JA as a signal molecule (Pieterse 1999).

Our data demonstrates that cZR-3 (as well as cZR-7) requires *RAR1* and *SGT1*, whereas its function is independent of *NPR1* and *EDS1* indicating that cZR-3 (cZR-7) induces a defense pathway previously described for non-TIR-(CC) NBS-LRR R-genes. In contrast, all investigated defense signaling components were not changed after overexpressing of *BvGLP-*

1 suggesting that *BvGLP-1* may act at a further position in the signal transduction pathway in a more general manner. However, the expression pattern of all investigated defense related genes was more or less the same in all transgenic plants. *PDF 1.2, PDF 2.2* and *PDF 2.3* were not changed and all PR-gene expression levels are enhanced after overexpression of cZR-3 (cZR-7) as well as *BvGLP-1*. H_2O_2 produced by OxO members of the germin and GLP family may act as a messenger for the initiation of other defense-related genes and secondary metabolites may accumulate through H_2O_2-signaling pathways (Dunwell et al. 2008; Lou et al. 2006). With regard to our results, cZR-3 is speculated to activate a SAR pathway which functions in a SA-dependent manner and results in the production of PR-proteins.

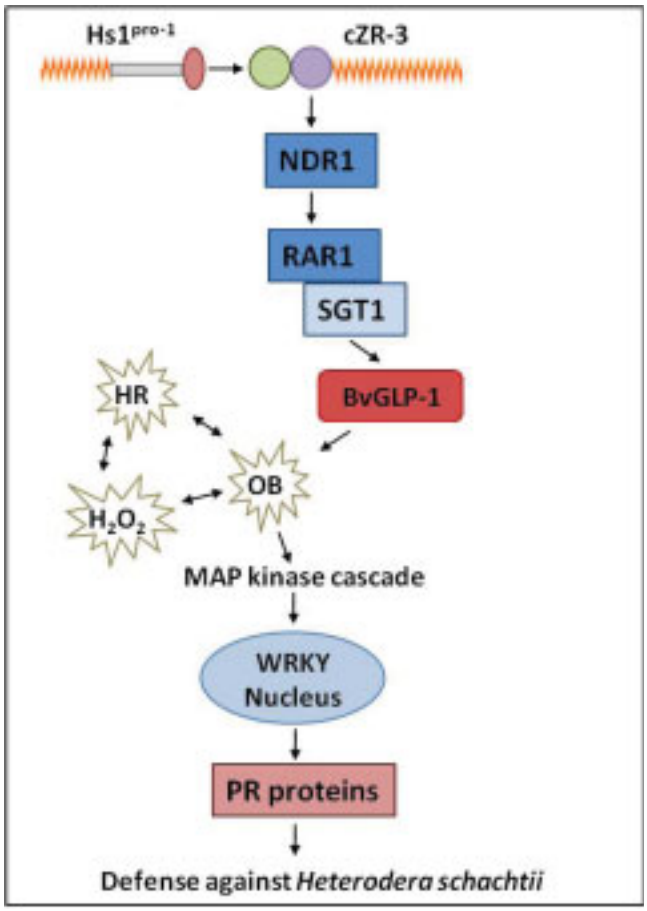

Figure 4: A putative signaling pathway leading to the *Hs1*$^{pro-1}$ -mediated nematode resistance response. cZR-3 requires *RAR1* and *SGT1*, whereas *BvGLP-1* may act at a further position in the signal transduction pathway in a more general manner. H_2O_2 produced by *BvGLP-1* may act as a messenger for the initiation of other defense-related genes. HR, hypersensitive response; OB, oxidative burst; WRKY, Zn finger-type plant transcription factors, MAPK, mitogen activated protein kinase; NDR1, non-race specific disease resistance 1; RAR1, required for Mla-dependent resistance 1; SGT1, suppressor of G2 allele of SKP1. transmembrane domain, coiled-coil, NBS domain, LRR.

Other important components of plant defense may be proteins of mitogen-activated protein kinases (MAPK) that were also found in transcript profiling experiments with nematode resistant sugar beets by using suppressive subtractive hybridisation (SSH) technique indicating their possible role in the *Hs1*$^{pro-1}$ mediated resistance (D.Cai, unpublished results).

5 Practical relevance

5.1 The potential of RGAs and BvGLP-1 in genetic engineering

Cyst nematodes are devasting plant pathogens responsible for high yield losses. Nematicides are available but often not desired because of enviromantal and human health risks. In this context resistant varieties provide the most potential opportunity to prevent plants against nematode attack. Breeding a new variety requires considerable time and effort, but it is an

essential process not only because many disease causing organisms exhibit increasing insensitivity to pesticides.

Our currently used food crops were firstly domesticated from wild species about 10.000 years. During this period strong selection pressure on the genetic diversity found in the wild species would have resulted in a reduction of genetic variation in domesticated plants (Tanksley et al. 1997). The limited genetic diversity of crops makes them more vulnerable to disease and insect epidemics. Thus, the breeder's task has been to find new resistance sources and these are often found in poorly adapted more or less related wild species. Not only by simplifying the access to secondary/third genepools of wild relatives plant biotechnology will aid in the search and application for/of new R-genes.

Plant biotechnology has become a tool for crop improvement by facilitating the efficient introgression of genes into adapted genotypes and the cloning of R-genes (Michelmore 1995). Plant transformation therefore offers an efficient means to introduce specific R-genes into crop species without the introduction of deleterious 'background' genes as is sometimes the case with classical backcrossing. Techniques exist for the delivery of isolated or modified single genes into almost all cultivated species however, depending on the plant species, transformation frequencies can be low and only a few may have appropriate transgene expression levels (Pink et al. 1999; Punja et al. 2001). Genetic engineering may also allow transfer across species that are difficult or impossible to cross sexually; however the transformation of single R-genes between closely related species is morel likely to be successful than between more distantly related species (Michelmore et al. 2003). The techniques used to develop transgenic plants have improved dramatically in the last decade allowing the development of new disease resistant crops, some of which are commercially available (Dempsey et al. 1998). However, the transfer of R-genes from model to crop plants as well as between distantly related crops could be limited by a phenomenon termed 'restricted taxonomic functionality' (RTF) that might reflect an inability of the R-protein to interact with signal transduction components in the heterologous host (Michelmore et al. 2003). The lack of evidence for direct R-Avr interactions led to the development of the 'guard hypothesis' by Van der Biezen and Jones that changed our view on R-gene-mediated defense which previously had been explained by the classical receptor-ligand model (Flor et al. 1971). The divergence or absence of an appropriate guardee or the inability of the R-protein to interact with downstream signaling components lacking in a heterologous host may explain the RTF of R-genes.

There is now a variety of genes from diverse sources that might be used to enhance disease resistance. These include cloned R-genes, specific signaling components like hydrogen peroxide (H_2O_2), SA, genes with antimicrobial activity such as PR-proteins, antifungal proteins (osmotin- and thaumatin-like), antimicrobial peptides (thionins, defensins, lectin), and phytoalexins as well as gene products that can enhance the structural defenses in the plant such as peroxidase and lignin. The identification of global regulators of resistance response, 'master switches', offers the possibility to engineer disease resistance (Stuiver et al. 2001). Due to the variety of genes it is increasingly important to gain insight into the pathways used to induce their synthesis and the steps and mechanisms by which resistance is activated. In this way new opportunities to improve existing methods and to create novel strategies for engineering durable, broad-spectrum disease resistance in plants evolve. This study provides novel insight into the $Hs1^{pro-1}$ -mediated nematode resistance response and leads to a better understading of the function of different components involved in the resistance response such as RGAs and OxO. Thus first steps for their use in genetic engineering of sugar beet and rapeseed with respect to reduction of nematode infection can be deduced.
The creation of durable resistance is of particular interest; in this context the deployment of genetic engineering approaches based on expression of two or more R-genes in a specific crop should provide more effective disease control than the single-gene strategy (Punja et al. 2001). It is possible to pyramid R-genes by supertransformation or sexual crosses with each gene effective against different pathotypes or against different pathogens. But also for achieving complete resistance, gene pyramiding would be a useful tool in heterologous hosts lacking the whole mechanistic network for complete resistance. In our study the simultaneous deployment of $Hs1^{pro-1}$ and a RGA sequence involved in the same nematode resistance pathway led to enhanced resistance response compared to single gene constructs (D.Cai, unpublished results).

The use of molecular markers and MAS techniques have facilitated identification, mapping, and transfer of many disease R-genes and QTLs in many crop species (Foolad et al. 2007). MAS can reduce the costs, can increase the precision and efficiency of selection and breeding and can facilitate the identification of genes or QTLs within wild species and their transfer into cultivated species. Numerous molecular markers linked to disease R-genes have been reported. RGAs are excellent molecular markers for resistance and resistance diversity. Candidate genes involved in both recognition (e.g. RGAs) and general plant defense mechanisms (e.g. OxO genes) were used as either PCR-based markers or restriction fragment

length polymorphism (RFLP) markers that included RGAs or putative plant defense genes from rice, barley, and maize as described by Dunwell et al. 2008.

Of particular interest would also be the establishment of transgenic plants with broad-spectrum disease resistance especially for pathogens with a wide host range, such as *Rhizoctonia solani* and *Botrytis cinerea*, for which only few sources of resistance in crops are available (Punja et al. 2001). Overexpression of the *NPR1* gene, which regulates the SA-mediated signaling leading to SAR, in transgenic Arabidopsis increased the level of PR-proteins during infection and enhanced resistance to *Peronospora parasitica* (Cao et al. 1998). Engineering pathogen resistance by overexpression of antipathogenic proteins is much more specific than the pathway modulating approach. The impact on yield or the interference/antagonism with other defense pathways is limited or absent (Stuiver et al. 2001). Chitinases and PIs have also a great potential in conferring enhanced resistance to other phytopathogens and also additive resistance effects of effector proteins have been described (Broglie et al. 1991, Urwin et al. 2003) demonstrating the effector based strategy as the most effective.
The activation of general defense responses in transgenic plants would provide protection against viral, bacterial and fungal pathogens in addition to nematodes. Here we describe the analysis of *BvGLP-1* which exhibits an OxO activity resulting in increased levels of H_2O_2. OxO led to the reduction of nematode development and fungal infection in transgenic plants indicating that *BvGLP-1* enzyme activity results in the activation of general defense pathways which is useful for engineering broad spectrum disease resistance. However, there are a number of cases where transgene products such as thionins, RIP, peroxidases and H_2O_2 generating enzymes expressed at high levels induce plant cell damage or had other undesirable effects on plant growth, development, and crop yield.

5.2 Importance of specific inducible promoters

Constitutive promoters have been used to achieve high expression levels throughout most tissues of the plant. The CaMV35S promoter has been widely used in many biotechnological applications. However, it is not ideal as a promoter for delivering antinematode effectors because it is down-regulated in syncytial feeding cells induced by cyst nematodes (Goddijn et al. 1993). If only specific tissues need to be protected or if the antimicrobial compounds need to be expressed at certain targeted sites in the cell to minimise sideeffects, specific promoters are needed. In this respect, the toxic protein may be better deployed through inducible

expression approaches by use of leaf-specific promoters (Stahl et al. 2004), storage root-specific promoters (Oltmanns et al. 2006), wound-inducible promoters (Dimmer et al. 2004) or nematode-inducible promoters (Thurau et al. 2003).

Having these problems in mind we applied the H_2O_2 generating gene *BvGLP-1* under the control of both promoters the constitutive 35S and the nematode-inducible syncytium-restricted Hs1 promoter. As expected, the transgenic plants expressing OxO constitutively suffered from physiological stress resulting in a decreased number of transformants and we suggest that OxO expression level in completely regenerated transgenic plants must be relatively low otherwise the plants could not be regenerated. The nematode-inducible promoter restricted the *BvGLP-1* transgene expression to pathogen infection sites, limited the cost of resistance and provided a restricted and therefore enhanced resistance level without phytotoxicity to the host plant.

An alternative strategy for engineering broad-spectrum resistance is based on the coordinate expression of an R-gene and a corresponding Avr transgene, controlled by a pathogen-inducible promoter (McDowell et al. 2003) as well as on synthetic promoters by combining pathogen-inducible cis-regulatory elements, the GCC-like elements (Ohme-Tagaki et al. 2000) and the W boxes (Rushton et al. 1998; Eulgem et al. 2000). An ideal promoter would respond rapidly to a wide variety of pathogens and must be inactive under disease free conditions. Particularly for cyst nematodes the disruption of feeding cells is an attractive target for transgenic resistance which would restrict nematode development (Atkinson et al. 2003). Most economically important plant parasitic nematode species are root parasites. Several root tissue-specific promoters have been identified and provide considerable potential for a range of biotechnological applications (Atkinson et al. 2003): the TUB-1 promoter (Breviario et al. 2000), RPL16A promoter (Williams et al. 1995) and the serine/threonine kinase (ARSK1) promoter (Hwang et al. 1995). Nematodes activate enzymatic processes within the feeding cells, which eventually result in the change of activity of transcription factors that bind to the $Hs1^{pro-1}$-promoter elements enhancing the $Hs1^{pro-1}$ transcription supporting that an enhanced expression of $Hs1^{pro-1}$ in feeding cells is required for initiation of resistance (Cai et al. 2003). The $Hs1^{pro-1}$-promoter has a great potential for engineering nematode resistance in plants consequently leading to death of the nematode juveniles shortly after initiation of the feeding structure (Thurau et al. 2003). This study is the first proof of its practical application in the whole plant; in this case *A. thaliana*, also indicating that the mechanisms are conserved in sugar beet and Arabidopsis.

6 Conclusions and Outlook

The current work provides insight in the *Hs1*$^{pro-1}$ -mediated nematode resistance but future research will require the fine tuning of engineered gene expression to prevent pathogen infection. Moreover, the combination of different R-genes with different resistance mechanisms in one plant will offer the opportunity to breed varieties with a more durable and broader resistance. With respect to the protection ability of hydrolytic enzymes, signaling components or enzymes for phytoalexin synthesis in particular against pathogens that have been typically difficult to control, the regulated expression in order to prevent destruction of the plant becomes more and more important. Probably, the challenge for the future is to develop the use of associated components representing different steps in the resistance response, receptors, signaling molecules and effectors for engineering disease resistance.

It has become evident that the introduction of a single transgene is not sufficient to achieve durable and broad-spectrum disease resistance. Numerous natural R-genes and RGAs, including those identified in the current study for nematode resistance, are available for immediate deployment, but how these R-genes/RGAs are deployed in a crop determines both the durability and effectiveness of resistance response. The goal is to keep more than one R-gene and all additional components within the cultivated plant, since it is known that R-genes do not function properly in heterologous hosts, possibly because the guarded protein is absent. Transgenic approaches to achieve durable disease resistance require a supply of cloned R-genes in their naturally occurring signaling network. Thus, it is essential to clarify the different steps within the signaling pathway even thought this task is complicated.

In order to gain more insight into the complex plant resistance network global gene expression strategies are necessary for the rapid identification of candidate genes. Such approaches include differential display (DD), serial analysis of gene expression (SAGE), cDNA-amplified fragment length polymorphism (cDNA AFLP) and gene chips. Using these differential transcriptional profiling methods will allow gaining more insight into biological changes during plant response to pathogen infection.
Additionally, pathogen genomics become more and more important. In this context, whole genome high-throughput sequencing initiatives will enable scientists to better understand pathogens infectious lifestyle and genome structure. Gaining insights into pathogens evolutionary potential would help to predict the durability of R-gene-based disease control. In

particular, identification of parasitism genes secreted by the nematode into host cells and also including nematode Avr-genes, is a challenge for the future. This will help to determine the appropriate host targets. The identification of a nematode Avr-gene interacting with the *Hs1*$^{pro-1}$ gene will provide the confirmation of its receptor function within the incompatible interaction. Moreover, characterized nematode Avr/parasitism genes will lead to the development of new control strategies for example host-derived RNA interference (RNAi).

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Summary

The beet cyst nematode *Heterodera schachtii* Schm. is an obligate sedentary endoparasite causing great losses in agriculture. The nematode is mainly active in temperate regions of the world attacking most of the Chenopodiaceae and Brassicaceae species including sugar beet (*Beta vulgaris*), spinach (*Spinacea oleracea*) and oilseed rape (*Brassica napus*). Cyst nematodes can completely penetrate the roots of susceptible plants intracellularly as motile infective second-stage juveniles (J2) and induce changes in a number of host cells to form highly metabolically active feeding cells sustaining the nematode throughout its life cycle. Nematicides are available but often not allowed because of their environmental and mammalian toxicity. In this context resistant varieties provide the most economically and environmental friendly opportunity to prevent plants from nematode attack. Furthermore, sugar beet suffers heavily from diseases like powdery mildew (*Erysiphe betae*), *Cercospora* leaf spot (*Cercospora beticola*), *Rhizoctonia* root and crown rot (*Rhizoctonia solani*) and Rhizomania (BNYVV).

The $Hs1^{pro-1}$ locus confers resistance to the beet cyst nematode *H. schachtii* in sugar beet (*Beta vulgaris*). The gene $Hs1^{pro-1}$ had been cloned from the resistant sugar beet line, but the resistance mechanism still remains obscure.

By use of transcript profiling strategy, the gene *BvGLP-1* was identified from the sugar beet genome. It encodes for an oxalate oxidase-like germin protein and is highly upregulated in resistant, but not in susceptible sugar beet in response to nematode infection. For functional analysis, we transferred *BvGLP-1* into sugar beet roots and Arabidopsis plants and challenged them with the beet cyst nematode. While the expression of *BvGLP-1* in nematode feeding cells (syncytia) of both sugar beet roots and Arabidopsis plants was sufficient to initiate nematode resistance, knockout of the homolog gene of *BvGLP-1* in Arabidopsis significantly increase plant susceptibility to nematode infection. In addition, we found that *BvGLP-1* functions as an oxalate oxidase generating hydrogen peroxide (H_2O_2) in plant cells and regulate the expression of pathogenesis-related proteins suggesting that *BvGLP-1* plays a central role in regulating plant nematode resistance.

Of great interest is the establishment of transgenic plants with resistance against a broad-spectrum of pathogens, especially these with a wide host range such as *Rhizoctonia solani*. To check the potential of *BvGLP-1* in conferring resistance against fungal phytopathogens, we analyzed *BvGLP-1* in infection assays with *V. longisporum* and *R. solani* as well as with the beneficial endophytic fungus *Piriformospora indica*. As a result, the

expression of *BvGLP-1* in Arabidopsis resulted in significant resistance to the two fungal pathogens, but does not affect the beneficial interaction induced by *P. indica*. Thus, we conclude that *BvGLP-1* regulates plant defense responses following a specific signaling route that diverges from that induced by the beneficial fungus *P. indica*.

In addition, three RGA sequences, cZR-3, cZR-7 and cZR-9 were investigated in respect of their potential for initiating resistance in transgenic Arabidopsis. The three RGAs all belong to the CC-NBS-LRR resistance protein family and share high sequence and structure similarity to a set of recently cloned resistance proteins, suggesting their potential role in nematode resistance. Transgenic *A. thaliana* plants expressing each of the RGAs were challenged with *H. schachtii*. We found that transgenic Arabidopsis expressing cZR-3 or cZR-7, respectively, showed a significant anti-nematode effect compared to the control plants whereas knockout of a homolog gene of cZR-3 in Arabidopsis drastically increased the susceptibility to nematode infection. These results strongly suggest an active role of both RGAs in nematode resistance. In addition, the expression of cZR-3 and cZR-7 in Arabidopsis elevates the transcript levels of *RAR1* and *SGT1* but not of *NPR1* and *EDS1* and consequently upregulates the expression of a set of PR proteins. Therefore, we conclude that cZR-3 and 7 are involved in the $Hs1^{pro-1}$ mediated nematode resistance following a signaling route specific for CC-NBS-LRR resistance proteins. It is worth speculating that the interference of PR proteins may represent an important aspect of the mechanism underlying the $Hs1^{pro-1}$ mediated nematode resistance.

An efficient transformation protocol for oilseed rape (*Brassica napus* L.) was also established in this study. It is a two-step shoot regeneration protocol from hypocotyl explants of oilseed rape, thus providing efficient tools to transfer the gene of interest into the oilseed rape genome by Agrobacterium-mediated transformation in the future.

Taken together, the results obtained from this study provide a deep insight into the molecular mechanism of the $Hs1^{pro-1}$ mediated nematode resistance but also novel strategies for genetic engineering of plant disease resistance e.g. by use of various natural resistance mechanisms.

Zusammenfassung

Der Zuckerrübenzystennematode *Heterodera schachtii* Schm. führt als obligat sedentärer Endoparasit zu erheblichen Ertragsverlusten in der Landwirtschaft. *H. schachtii* tritt hauptsächlich in gemäßigten Klimazonen auf und befällt verschiedene Arten der *Chenopodiaceae* und *Brassicaceae*, z.B. auch wichtige Kulturpflanzen wie die Zuckerrübe (*Beta vulgaris*), Spinat (*Spinacea oleracea*), und Raps (*Brassica napus*). Zystennematoden dringen als infektiöse Larven des zweiten Entwicklungsstadiums in ihre Wirtspflanzen ein und richten vor allem durch die intrazelluläre Wanderung zum Zentralzylinder, wo sie ein metabolisch hoch aktives Nährzellensytem induzieren, erheblichen Schaden an (Davis et al. 2004; Fuller et al. 2008). Nematizide Wirkstoffe sind zwar verfügbar, aber aufgrund ihrer umweltschädigenden Wirkung und Toxzizität für Säugetiere häufig verboten.
Daher bietet der Anbau resistenter Sorten eine ökonomisch lohnende sowie umweltfreundliche Alternative Kulturpflanzen vor parasitären Nematoden zu schützen. Neben dem Rübenzystennematoden spielen auch andere Erreger im Zuckerrübenanbau eine wichtige Rolle, wie der Rübenmehltau (*Erysiphe betae*), die Blattfleckenkrankheit (*Cercospora beticola*), Rizomania und die Wurzel- und Kronenfäule, die durch den pilzlichen Erreger *Rizoctonia solani* hervorgerufen wird.
Der $Hs1^{pro-1}$ Locus vermittelt Resistenz gegenüber dem Rübenzystennematoden *H. schachtii* in der Zuckerrübe (*Beta vulgaris*) (Cai et al. 1997). Das $Hs1^{pro-1}$ Gen wurde zwar aus resistenten Zuckerrüben kloniert, aber die zugrunde liegenden Resistenzmechanismen bleiben ungeklärt.

Durch einen *transcript profiling* Ansatz konnte das *BvGLP-1* Gen im Zuckerrübengenom identifiziert werden. *BvGLP-1* kodiert für ein funktionales Oxalatoxidase-ähnliches Germinprotein, das in resistenten, aber nicht in anfälligen Zuckerrübenpflanzen nach Nematodenbefall stark hoch reguliert ist. Für die funktionale Analyse wurde das *BvGLP-1* Gen in Zuckerrüben *hairy roots* und Arabidopsis Pflanzen eingebracht und die transgenen Wurzeln/Pflanzen in Nematodenresistenztests untersucht. Während die Expresssion des *BvGLP-1* Gens in Syncytien in transgenen Zuckerrübenwurzeln so wie auch in transgenen Arabidopsispflanzen eine Nematodenresistenz hervorrief, führte das *knockout* eines *BvGLP-1* homologen Gens im Arabidopsisgenom zu einer erhöhten Anfälligkeit der Pflanzen gegenüber Nematoden. Desweiteren wurde bewiesen, dass das *BvGLP-1* Gen als funktionale Oxalatoxidase in pflanzlichen Zellen agiert und dort Wasserstoffperoxid (H_2O_2) generiert. Außerdem reguliert *BvGLP-1* die Expression der Pathogen-assoziierten Gene (PR-

Gene). Dies unterstreicht, dass *BvGLP-1* eine zentrale Rolle in der pflanzlichen Nematodenresistenz spielt.

Die Erstellung und Etablierung transgener Pflanzen mit einer Resistenz gegenüber einem weiten Pathogenspektrum, besonders aber gegenüber Pathogenen mit einem weiten Wirtspflanzenkreis, wie z.B. *Rhizoctonia solani* ist von großem Interessse. Um festzustellen, ob *BvGLP-1* Resistenz gegenüber pilzlichen Pathogenen verleihen könnte, haben wir die *BvGLP-1* transgenen Pflanzen in Infektionsexperimenten mit den pflanzenpathogenen Pilzen *V. longisporum* und *R. solani*, sowie mit dem wachstumsfördernden endophytischen Pilz *Piriformospora indica* analysiert. Die Expression des *BvGLP-1* Gens in Arabidopsis resultierte in einer signifikanten Resistenz gegenüber den beiden pilzlichen Pathogenen, beeinflusste aber nicht die nützliche Interaktion mit *P. indica*. Folglich reguliert *BvGLP-1* planzliche Resistenzantworten, die auf einem spezifischen Signaltransduktionsweg basieren, der sich jedoch von einem durch den wachstumsfördernden endophytischen Pilz *P. indica* induzierten Signalweg unterscheidet.

Ein weiteres Ziel dieser Arbeit war es, drei unterschiedliche RGA Sequenzen (cZR-3, cZR-7 und cZR-9), im Hinblick auf ihre Fähigkeit Nematodenresistenz in Arabidopsis zu vermitteln, funktional zu charakterisieren. Alle drei RGA Sequenzen gehören zu der CC-NBS-LRR Resistenzproteinfamilie und weisen eine hohe Sequenz- und Strukturhomologie zu schon bekannten Resistenzproteinen auf, was eine Funktion in der Nematodenresistenz vermuten lässt. Um die Rolle der drei RGA Sequenzen in der Nematodenresistenz festzustellen, haben wir transgene *A. thaliana* Pflanzen erzeugt, die die RGAs exprimieren und diese in Nematodenresistenztests untersucht. Wir konnten zeigen, dass transgene Arabidopsispflanzen, die cZR-3 und cZR-7 exprimieren, einen signifikanten, den Nematodenbefall reduzierenden Effekt hervorriefen, wohingegen die Unterdrückung eines cZR-3 homologen Gens in Arabidopsis zu einer signifikant ansteigenden Anfälligkeit der Pflanzen gegenüber Nematoden führte. Diese Ergebnisse unterstützen eine aktive Rolle beider RGAs in der Nematodenresistenz. Ebenso führte die Expression von cZR-3 und cZR-7 in Arabidopsis zu einem erhöhten Transkriptniveau von *RAR1* und *SGT1* aber nicht von *NPR1* und *EDS1* und damit zu einer verstärkten Expression von PR-Genen. Daraus folgern wir, dass cZR-3 und cZR-7 in der $Hs1^{pro-1}$ vermittelten Nematodenresistenz einen für CC-NBS-LRR Resistenzproteine spezifischen Signalweg regulieren, der schließlich in der erhöhten Expression von PR-Proteinen endet. PR-Proteinen stellen scheinbar einen wichtigen Aspekt der $Hs1^{pro-1}$vermittelten Nematodenresistenz dar.

Ein zweiphasiges Sprossregenerationsprotokoll für Rapshypokotylexplantate wurde ebenfalls in dieser Arbeit etabliert und bietet zukünftig ein hilfreiches Werkzeug für die *Agrobakterium tumefaciens* vermittelte Transformation von Targetgenen in Raps (*Brassica napus* L.).

Die in dieser Arbeit erzielten Ergebnisse liefern einen tiefen Einblick in die molekularen Mechanismen der $Hs1^{pro-1}$ vermittelten Nematodenresistenz und neue Strategien zur gentechnischen Erzeugung von Krankheitsresistenz z.B. durch die Nutzung natürlicher Resistenzmechanismen auf.

Appendix

```
                1                                                    50
BvGLP-1     (1) ---------MNNLVVFFAFSILVCLSHAIEVDFCVADRNLPR--GPEGYA
   GLP3     (1) --------MKMIIQIFFIISLISTISFASVQDFCVADPKGPQ--SPSGYS
   GLP6     (1) --------------------------------------------------
   GLP9     (1) MTIKSLSFLAALSLFALTLPLVIASDPSPLQDFCVGVNTPADGVFVNGKF

                51                                                   100
BvGLP-1    (40) CRDPATLTTDDFVYTGFRGGRTITNVPGNNVTLAFVDQFPALNGLGISMA
   GLP3    (41) CKNPDQVTENDFAFTGLGTAGNTSNIIKAAVTPAFAPAYAGINGLGVSLA
   GLP6     (1) ----------------------------------------MGISLV
   GLP9    (51) CKDPRIVFADDFFFSSLNRPGNTNNAVGSNVTTVNVNNLGGLNTLGISLV

                101                                                  150
BvGLP-1    (90) RLDFGVSGVIPVHSHR-TSEVLIVSRGSIIAGFIDTN---NTAYYRRLEV
   GLP3    (91) RLDLAGGGVIPLHTHPGASEVLVVIQGTICAGFISSA---NKVYLKTLNR
   GLP6     (7) RIDYAPYGQNPPHTHPRATEILVLIEGTLYVGFVSSNQDNNRLFAKVLYP
   GLP9   (101) RIDYAPNGQNPPHTHPRATEILVVQQGTLLVGFISSNQDGNRLFAKTLNV

                151                                                  200
BvGLP-1  (136) GDVMIFPQAMLHFQVNVGTTPATAFVSLNGANPAIQFTMNSLFGG--NLP
   GLP3  (138) GDSMVFPQGLLHFQLNSGKGPALAFVAFGSSSPGLQILPFALFAN--DLP
   GLP6   (57) GDVFVFPIGMIHFQVNIGKTPAVAFAGLSSQNAGVITIADTVFGSTPPIN
   GLP9  (151) GDVFVFPEGLIHFQFNLGGTPAVAIAALSSQNAGVITIANTIFGSKPDVD

                201                    226
BvGLP-1  (184) ADIAQQITLLSNAEVMRMKRAFGTA-
   GLP3  (186) SELVEATTFLSDAEVKKLKGVLGGTN
   GLP6  (107) PDILAQAFQLDVNIVEDLEAKFRN--
   GLP9  (201) PNVLARAFQMDVNAVRNLQARF----
```

Figure I: Comparative multiple sequence alignement of BvGLP-1 amino acid sequence with sequences of 3 Arabidopsis GLPs GLP3 (AT5G20630), GLP6 (AT5G39100), GLP9 (AT5G38910) which are upregulated in *Hs1*$^{pro-1}$ transgenic Arabidopsis as identified by ATH1 GeneChips.

Table I: Results of nematode resistance tests with cZR-3 transgenic *A. thaliana* T1 families. 24 individual plants were tested with two repitions.

A. thaliana T1	mean (n=24)	mean (n=24)	mean (n=24)
1	3,4	2,8	2,7
2	2,8	3,7	2,5
3	7,6	5,3	4,1
4	4,9	5,6	4,5
5	5,8	4,3	2,8
6	15	13,3	13,7
7	6	8,4	6,2
8	5,4	6,5	11,3
9	6,8	7	6,6
10	9	8	11,5
11	9,2	7,9	5,5
12	2,9	3,8	3,0
13	6,5	6,3	5,9
14	9,5	8,2	10,0
15	10,9	16,9	11,9
16	15,3	17,3	9,7
17	9	7,8	7,8
18	15,3	9,4	16,9
ck	15,5	17,9	13,9

Table II: Results of nematode resistance tests with cZR-7 transgenic *A. thaliana* T1 families. 24 individual plants were tested with two repitions.

A. thaliana T1	mean (n=24)	mean (n=24)	mean (n=24)
1	2,4	3	3,2
2	3	4	2,3
3	2,8	4,2	2,7
4	5,1	4,5	5,0
5	6,2	4,1	2,0
6	10,1	7,8	6,1
7	5,8	6,5	8,3
8	6,8	8,5	7,9
9	6	7,5	5,9
10	9,5	8,4	6,4
11	6	7,8	7,9
12	8	5,4	4,6
13	7,2	5,4	6,1
14	9,5	7,5	7,6
15	10,9	13,2	9,6
16	13,8	12,4	12,7
17	12	10,9	13,8
18	10,2	13,4	11,9
ck	16,2	13,8	12

Table III: Results of nematode resistance tests with cZR-9 transgenic *A. thaliana* T1 families. 24 individual plants were tested with two repitions.

A. thaliana T1	mean (n=24)	mean (n=24)	mean (n=24)
1	6,6	6,1	10,5
2	12,8	15,4	14,1
3	9	10,5	7,95
4	10,4	11,2	12,8
5	8,2	10,5	5,8
6	16	12,5	13,4
7	9,25	10,2	11,3
8	8,8	12,4	11,5
9	9,56	11,3	8,5
10	9,5	7,9	10,6
11	12,4	9,59	9,5
12	10	12,5	13,5
13	8,5	11,87	7,3
14	7,9	10,2	6,8
15	12,1	14,8	12,7
16	6,2	9,05	8,1
17	12,9	9,15	10,9
18	15	12,8	13,4
ck	16,4	13,9	13,3

Table IV: Results of nematode resistance tests with cZR-3, cZR-7 and cZR-9 transgenic *A. thaliana* T4 plants. 24 individual plants were tested for each gene constructs with two repitions.

A. thaliana T4		mean (n=24)	mean (n=24)	mean (n=24)
cZR-3	1	0,5	0,75	1,7
	2	0,9	1,75	1,8
	3	1,2	3,5	1,7
	5	4,2	2,9	2,1
	ck	8,9	10,2	13,7
cZR-7	1	0,4	1,2	0,8
	2	1	2,3	3,1
	12	2	1,9	2,9
	5	3,2	5,2	5,9
	ck	10	8,4	8,5
cZR-9	1	6	8,4	6,2
	16	6,2	9,3	6,9
	5	9	6,5	7,2
	14	8	7,8	9,0
	ck	11,2	8,9	9,8

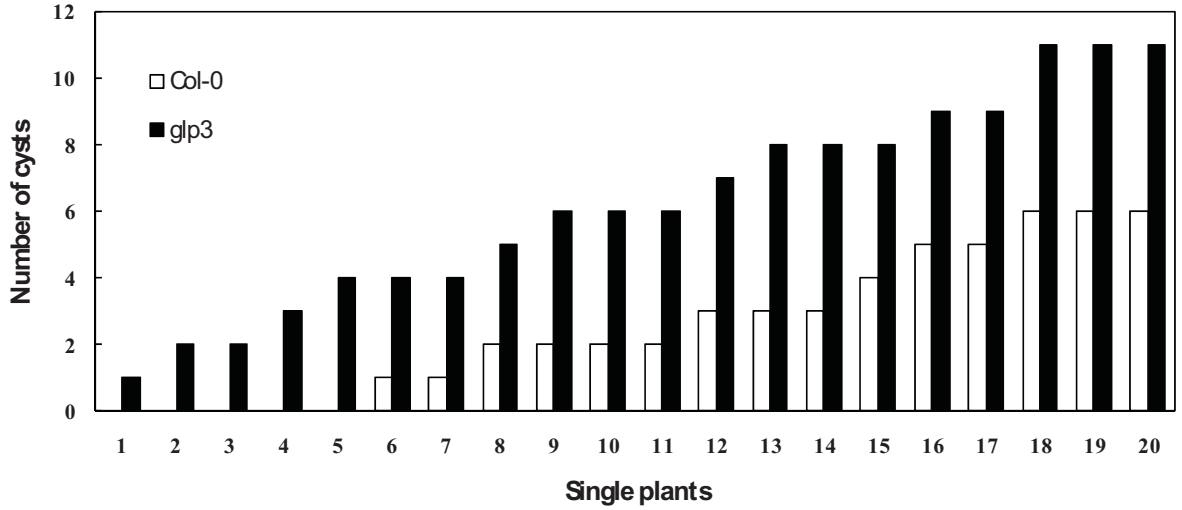

Figure I: Partial results of nematode resistance tests with *glp3*-ko mutant plants in comparison to Col-0 wild type plants. 20 individual plants were tested with two repitions.

Table V: Results of nematode resistance tests with pAM194-BvGLP-1transgenic sugar beet hairy roots. 20 independent clones were tested with seven respitions.

Clone nr	1	2	3	4	5	6	7	8
ck	16	15	14	17	18	21	23	20
1	3	4	3	4	3	4	3	4
2	3	6	4	3	4	7	5	3
3	4	5	7	5	3	7	4	4
4	3	5	4	5	8	9	4	6
5	3	4	5	7	8	9	5	3
6	6	7	6	8	9	9	11	8
7	13	3	7	3	6	8	6	7
8	11	9	8	9	9	7	5	9
9	6	7	7	7	7	8	12	5
11	6	8	5	3	12	6	5	8
12	6	8	5	3	11	7	5	8
13	6	11	9	7	8	7	11	9
14	11	7	10	5	7	9	16	9
16	12	6	7	6	6	5	9	6
18	12	8	9		10	13	3	9
19	6	9	9	7	8	7	11	9
20	12	7	4	8	7	9	9	10
22	12	6	7	6	6	6	9	6
23	23	12	19	10	18	9	15	14
24	15	21	12	13	18	16	17	16

Table VI: Results of nematode resistance tests with pBIN-BvGLP-1 transgenic sugar beet hairy roots. 18 independent clones were tested with seven respitions.

Clone nr	1	2	3	4	5	6	7	8
ck	16	15	14	17	18	21	23	20
1	3	5	3	4	3	4	3	4
2	3	6	4	3	4	5	5	3
3	3	3	4	5	4	5	4	4
4	4	5	7	5	3	7	4	4
5	3	4	3	7	6	4	5	5
6	6	7	8	8	9	10	11	8
7	13	3	7	3	5	11	9	11
8	11	6	8	5	5	3	5	9
9	12	5	7	9	12	7	12	9
10	11	7	8	5	8	9	8	9
11	7	8	9	9	9	9	4	3
12	6	8	17	3	12	6	5	8
13	6	6	4	3	5	7	8	9
14	7	8	5	5	5	12	8	15
15	12	16	17	18	11	18	19	18
16	14	8	14	18	19	20	19	8
17	18	16	14	16	14	17	17	16
18	19	20	16	20	19	18	15	9

Table VII: Results of nematode resistance tests with pAM194-BvGLP-1 transgenic *A. thaliana plants*. Eight independent lines were tested with eight individuals each.

A. thaliana	1	2	3	4	5	6	7	8
ck	11	13	12	14	10	15	11	11
1	5	6	7	6	7	8	3	4
2	3	2	3	4	7	4	4	7
3	3	4	3	3	3	5	6	4
4	3	4	5	4	4	4	7	4
5	3	4	5	6	7	9	7	6
6	12	9	4	9	4	7	11	10
7	11	7	12	5	6	4	8	4
8	5	6	2	6	6	5	4	10

Table VIII: Results of nematode resistance tests with pAM194-BvGLP-1 transgenic *A. thaliana* plants. Eight independent lines were tested with eight individuals each.

A. thaliana	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
ck	11	13	12	14	10	15	11	11
1	2	3	3	6	7	2	3	4
2	3	2	3	4	7	4	4	7
3	3	4	4	3	3	4	6	4
4	3	2	5	4	4	2	7	4
5	3	2	5	2	7	3	7	6
6	12	2	4	6	5	2	6	10
7	11	7	12	5	8	7	8	3
8	5	2	2	3	6	5	4	10

Danksagung

Ein erster und besonderer Dank geht an Prof. Dr. Daguang Cai für seine Aufwendungen und Anstrengungen für diese Arbeit. Herr Prof. Cai stand mir immer mit vielen Tipps und Ideen zur Seite. Ohne seine Bereitwilligkeit zur Diskussion und die informativen fachlichen Beratungen und Gespräche hätte diese Arbeit nie entstehen können. Dafür danke ich ihm sehr.

Auch Herrn Prof. Dr. Christian Jung danke ich für die Betreuung und Unterstützung während der ersten 2 Jahre am Institut für Pflanzenzüchtung.
Mein Dank gilt allen ehemaligen und aktuellen Mitarbeitern des Instituts für Pflanzenzüchtung, die mir mit Rat und Tat zur Seite standen. Besonders viel verdanke ich Jessica Endrigkeit, Daniela Schulte, Gina Capistrano und Tina Lange. Viele nette aber auch schwierige Stunden haben wir zusammen verbracht und gemeistert. Ich hoffe, wir bleiben in Kontakt.
Für die sehr wertvolle technische Unterstützung während meiner Arbeit möchte ich mich herzlich bei Irene Pauselius und Inga Rix bedanken. Ihr habt mir doch einiges an Arbeit abgenommen. Desweiteren bei Bärbel Wohnsen, Monika Bruisch, Erwin Danklefsen für die Unterstützung bei allen anfallenden Arbeiten im Gewächshaus, bei Hilke Jensen für die Hilfe im Labor (nicht zu vergessen die netten Gespräche) und bei Antje Jakobeit, dafür das sie immer mindestens ein offenes Ohr hatte.
Ebenfalls bedanken möchte ich mich bei Prof. Dr. Joseph-Alexander Verreet und allen Mitarbeitern des Instituts für Phytopathologie für die herzliche Aufnahme unserer Arbeitsgruppe.
Ein besonderer Dank geht an die gesamte Abteilung Molekulare Phytopathologie, an Elisabeth Petersen, Friederike Bernsdorff, Sabrina Butze, Katharina Peetz, Helga Ladehoff, Claudia Häder, Dr. Wanzhi Ye, Dr. Tim Thurau und Jan Menkhaus für die Unterstützung, die nette Zusammenarbeit und die guten Wünsche in den letzten Wochen. Einer verdient hier besondere Erwähnung: Jan, vielen lieben Dank für die notwendige Ablenkung und die aufmunternden Gespräche, trotz allem hatten wir immer viel zu Lachen.
Viel lernen konnte ich bei Dr. Christine Desel im Bereich der Mikroskopie und dem Nachweis von reaktiven Sauerstoffspezies. Vielen Dank für deine Zeit und Geduld. Prof. Dr. Gui-Xiang Tang möchte ich herzlich für die fruchtbare Zusammenarbeit im Bereich der Rapstransformation danken. Mein Dank gilt ebenfalls Prof. Dr. Ralf Oelmüller für die

unterstützenden Arbeiten zu *Piriformospora indica* und Prof. Dr. Kai Wun Yeh für die sehr interessanten und lehrreichen Erfahrungen in Taipei.
Für die Durchsicht dieser Arbeit möchte ich mich bei Jens Aumann und Jan Menkhaus bedanken.
Sicherlich konnte auch diese Arbeit nicht ohne finanzielle Unterstützung erledigt werden, dafür danke ich der DFG, dem BMBF und dem Land Schleswig-Holstein.
Der letzte aber bei weitem der wichtigste Dank gilt meiner Familie.Vielen Dank Ingo für deine guten Kontakte und deine Bemühungen diese auch zu vermitteln (Danke Oliver für die gute Korrektur dieser Arbeit). Arne, Gerda und Wolfgang, euch danke ich sehr für die unendliche Geduld, Kraft und Liebe. Ihr habt immer, auch wenn ich es selber nicht mehr tat, an mich und diese Arbeit geglaubt.

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