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Dissecting rhizobacteria-induced systemic resistance in tomato against *Meloidogyne incognita* - The first step using molecular tools.

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Dissecting rhizobacteria-induced systemic resistance in tomato against *Meloidogyne incognita* - The first step using molecular tools.

Two rhizobacteria with known ability to induce systemic resistance against different soilborne pathogens were studied in split-root experiments for their ability to induce systemic resistance against the root-knot nematode *Meloidogyne incognita* in tomato. In tomato plants treated with the bacteria strains, *Rhizobium etli* G12 or *Bacillus sphaericus* B43, penetrated significantly less juveniles and also reduced the multiplication of the penetrated females after treatment with G12. Results of split-root experiments showed that the penetration of the nematodes was reduced in the responder root, which was the non-bacterial inoculated root, thus confirmed the occurrence of induced systemic resistance (ISR). The bacterial treatment did not affect the root development of tomato plants.

The observed induced systemic resistance was further characterized at the molecular level by subtractive suppressive hybridization using RNA of bacteria treated, bacteria and nematode treated, and untreated tomato plants. The goal was to isolate and characterize plant genes, which were differentially expressed following rhizobacteria-induced systemic resistance.

To obtain only plant RNA without bacteria or nematode RNA contamination a specific split-root set-up was designed. The bacteria as well as the nematodes were applied to the inducer root of the tomato plants and the RNA was extracted from the responder root, which was the non-inoculated root. That ISR was present in the responder root of this set-up was verified by a biocontrol assay, in which nematodes were inoculated at the responder root in a parallel inoculation experiment.

The subtractive suppressive hybridization resulted in the isolation and characterization of 24 potential differentially expressed genes of the induced plants. To confirm the differential expression, different molecular biological analysis methods were used. Northern blotting combined with chemiluminescent or radioactive detection, were not sensitive enough to detect the very small differences in the transcription profile of induced and non-induced tomato plants. The semiquantitative reverse transcriptase PCR showed that the two different bacteria strains induced different reactions in tomato plants. After a G12 treatment the gene coding for the polygalacturonase isoenzyme 1 beta subunit was down-regulated. A B43 treatment or a combined B43 and nematode application up-regulated the gene coding for the phenylalanine ammonia-lyase enzyme (PAL5). These findings exemplify the complexity of rhizobacteria-induced systemic resistance against *Meloidogyne incognita*.

Analyse der Rhizosphärebakterien-induzierten systemischen Resistenz in Tomate gegen *Meloidogyne incognita* – Der erste Schritt molekulare Methoden zu nutzen.

Zwei Rhizosphärebakterien mit der bereits bekannten Fähigkeit, systemisch Resistenz gegen verschiedene bodenbürtige Krankheitserreger induzieren zu können, wurden in 'split-root' Experimenten auf ihre Fähigkeit untersucht, systemisch Resistenz gegen den Wurzelgallennematoden in Tomaten zu induzieren. In mit den Bakterien, *Rhizobium etli* G12 oder *Bacillus sphaericus* B43, behandelten Tomaten drangen signifikant weniger Nematodenlarven ein und die Vermehrung der eingedrungenen Weibchen nach einer G12 Behandlung war ebenso reduziert. Die Ergebnisse der 'split-root' Experimente zeigten, dass die Eindringung der Nematoden in der 'Responder-Wurzel', also der Wurzel, die nicht mit Bakterien behandelt wurden, verringert war. Das bestätigte eine systemische Resistenzinduktion. Das Wurzelwachstum der Tomaten wurde durch Bakterienbehandlungen nicht beeinflusst.

Diese induzierte systemische Resistenz wurde auf molekularer Ebene mit Hilfe von subtraktiver suppressiver Hybridisierung anhand der RNS Bakterien behandelter, Bakterien und Nematoden behandelter und unbehandelter Tomaten weiter charakterisiert. Ziel war es, Pflanzengene zu isolieren und charakterisieren, die nach einer Rhizosphärebakterien-induzierten systemischen Resistenz differentiell exprimiert waren.

Um nur pflanzliche RNS, ohne Verunreinigungen durch Bakterien- oder Nematoden-RNS zu erhalten, wurde ein spezieller 'split-root' Aufbau gewählt. Dabei wurden sowohl die Bakterien als auch die Nematoden auf die 'Induzierer-Wurzel' der Tomaten appliziert und anschließend die pflanzliche RNS aus der 'Responder-Wurzel' extrahiert. Das Auftreten von induzierter systemischer Resistenz in den 'Responder-Wurzeln' dieses 'split-root' Aufbaus konnte anhand einer Kontrolluntersuchung, in der Nematoden auf die 'Responder-Wurzeln in einem parallel inokulierten Experiment appliziert wurden, bestätigt werden.

Eine subtraktive suppressive Hybridisierung brachte 24 potentiell differentiell exprimierte Gene aus induzierten Pflanzen hervor. Um diese potentiell differentiellen Gene zu bestätigen, wurden verschiedene molekularbiologische Methoden genutzt. Northern Blotting mit chemiluminesent oder radioaktivem Nachweis waren nicht sensitiv genug, um die sehr geringen Unterschiede im Transkriptenprofil einer induzierten und nicht-induzierten Tomatenpflanze festzustellen. Die semiquantitative reverse-Transkriptase PCR zeigte, dass die beiden unterschiedlichen Bakterienstämme verschiedene Reaktionen in den Tomatenpflanzen verursachen. Nach einer G12 Behandlung war das Gen, das für die beta-Untereinheit des Polygalakturonase Isoenzyms 1 kodiert, herunterreguliert. Sowohl eine alleinige B43 Behandlung als auch einer kombinierte Applikation von B43 und Nematoden regulierten das Gen, welches das Enzym Phenylalaninammoniumlyase (PAL5) kodiert, herauf. Diese Forschungsergebnisse veranschaulichen die Komplexität der Rhizosphärebakterien-induzierten systemischen Resistenz gegen *Meloidogyne incognita*.

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Abbreviations

cDNA	complementary deoxyribonucleic acid
CDP-Star	alkaline phosphatase substrate© (DIG- Northern Starter Kit, Roche)
DEPC	di-ethyl-pyro-carbonate
DIG	digoxigenin
DMF	di-methyl-formamide
DNA	deoxyribonucleic acid
EDTA	ethylen-diamin-tetra-acetate
IPTG	iso-propyl-β-D-thio-galactopyranosid
JA	jasmonic acid
LB	lysogeny broth
LSD	Fisher's least significant different procedure
MB	methyl bromide
MOPS	3-(4-morpholino) propan-sulfon acid
mRNA	messenger ribonucleic acid, poly A+ RNA
NaOH	sodium hydroxide
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
PG	polygalacturonase
RNA	ribonucleic acid
RT	room temperature
SA	salicylic acid
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
SSH	subtractive suppressive hybridization
TAE buffer (50 x)	2 M tris, 0.5 M EDTA (pH 8.0 with acetic acid)
TE buffer	10 mM tris-HCl, 1 mM EDTA (pH 8.0 with acetic acid)
TSB	tryptic soya broth
UBI	ubiquitin
X-Gal	5-brome-4-chlor-3-indoxyl-β-D-galactopyranosid

1 Introduction

1.1 Soil Ecosystem and Plant Production

The soil is the basis for plant production. It provides essential resources for plant growth, such as mineral nutrients, water and anchorage. To produce food in a sustainable agricultural system, it is essential to protect the soil from degradation. Each year, an additional 20 million ha of agricultural land becomes too degraded for crop production, or is lost to urban sprawl. The degradation and destruction of agricultural land takes place through water and wind erosion, salinization, loss of organic matter and urban sprawl. As a result, the soil resource for plant production is progressively more limited. This increases the pressure on the available arable land. Over the next 30 years, the demand for food in developing countries is expected to double. Therefore, new land will be farmed, but much of it will be marginal and even more susceptible to degradation (United Nations 2000).

The soil is the habitat and refuge for thousands of species of animals and microorganisms e.g. bacteria, fungi, protozoa, earthworms, termites, and many more. This soil biota contributes to many critical ecosystem functions as soil formation, organic matter decomposition, nutrient availability and carbon sequestration, as well as greenhouse gas emission, nitrogen fixation, plant nutrient uptake, and bioremediation of degraded and contaminated soils through detoxification of contaminants and restoration of soil physical, chemical and biological properties and processes (Abawi and Widmer 2000, Wang *et al.* 2007, Patra *et al.* 2007, Qadir *et al.* 2006, Dzantor 2007). Soil organisms also influence water infiltration and runoff as well as moisture retention through effects on soil structure and composition and indirectly on plant growth and soil cover. Even water quality ultimately depends on the composition of the microbial soil community (Ibekwe *et al.* 2007).

The soil biota can also suppress or induce plant diseases and pests. It can increase or reduce agricultural productivity depending on the presence of specific organisms and the effects of their different activities. In the United States, the pre-harvest crop losses to pests including arthropods, weeds, diseases, and nematodes, were estimated to be about 37 % of the maximum potential yield (Pimentel *et al.* 1993).

However, farming practices alter biotic and abiotic soil conditions, so that the total number of organisms, the diversity of species, the activity of the individual organisms and the aggregate

functions of soil biota are substantially altered, either positively or negatively. When soil conditions are altered so that the overall soil community that buffers the ecosystem is influenced negatively, soilborne pests and pathogens proliferate and cause tremendous yield losses. To ensure long term sustainable, effective land use management is essential (FAO 2003, Abawi and Widmer 2000).

Control of soilborne pathogens is especially difficult, as the whole rhizosphere has to be treated for efficient control, which is economically unacceptable (Buchenauer 1998). Even in developed countries, there are problems in controlling soilborne pests, particularly plant pathogenic nematodes. The only way they can be effectively managed is by soil fumigation, nematizides, the use of resistant varieties, seed dressing, or long-interval crop rotations (Oerke and Dehne 1994). One approach to biological control of soilborne pathogens, such as plant parasitic nematodes, is targeted management of the already available antagonistic potential of the soil ecosystem (Sikora 1992).

1.2 Antagonistic Potential in Soil

The term antagonistic potential of soil stands for the capacity of a soil ecosystem to prevent or reduce the spread of a deleterious organism by natural biotic factors (Sikora 1992). In agro-ecosystems, agricultural practices such as tillage, crop rotations, fertilization, and chemical plant protection substances affect the composition of the biotic soil community. In these cases, the antagonistic potential of a soil is the result of the activity of those antagonists that survive and are stimulated by these treatments thereby limiting the spread and reproduction of deleterious organisms, and additionally protecting crops from pests and diseases. The antagonistic soil biotic community can be composed of bacteria, fungi, arbuscular mycorrhizal fungi, and predatory nematodes, protozoa, and insects. Especially in the rhizosphere, the part of the soil around a plant's roots and the direct surrounding soil, is intensely colonized by microorganisms which can be neutral, beneficial or harmful to the developing plant (Chet *et al.* 1990).

1.3 Plant-Microbe Interaction in the Rhizosphere

Hiltner (1904) created the expression 'rhizosphere'. He underlined the dependence of plants on beneficial soil bacteria as well as the attracting effects of root exudates on pathogenic organisms. The interactions between plant root exudates and soil microbes and nematodes in the rhizosphere was recently reviewed by Bais *et al.* (2006).

In turn, the release of organic substances from the plant influences the composition and number of microorganisms in the rhizosphere, either directly or indirectly (Rovira and Davey 1974, Hale *et al.* 1978). However, these root exudates are not the only substances that promote bacterial distribution on the rhizoplane and in the rhizosphere. Discarded root material, like the cells of the root cap and epidermis or root hairs, as well as the diffusion of substances out of wounded root cell also are regarded as factors influencing the microbial community (Curl 1982, Curl and Truelove 1986).

1.4 Impact of the Microbial Colonization on the Plant

Rhizosphere colonization is of importance to the bacteria as well as to the plant. The main factor in this context is the inhibition or control of pathogens by naturally occurring microorganisms. Basically this natural 'biocontrol' activity is based on the ability of specific microorganisms to reduce disease of the plant by antibiosis, competition for nutrients and space, parasitism, hypovirulence or cross-protection (Bloemberg and Lugtenberg 2001, Chet *et al.* 1990, Liu *et al.* 1995,Sequeira 1984). Aside from this, there is also the possibility that rhizosphere microorganisms stimulate and systemically enhance the resistance of plants against pathogens (Van Loon *et al.* 1998).

1.5 Induced Resistance

Kloepper *et al.* (1992a) defined certain terms, which had been introduced at a NATO Advanced Research Workshop on biocontrol:

1. *Induced disease resistance* is the process of active resistance, dependent on the host's barriers, activated by biotic or abiotic inducing agents.
2. *Local induced resistance* occurs when the protection from disease is limited to the plant region treated with the inducing agent.
3. *Systemic induced resistance* occurs when the plant is protected systemically upon application of an inducing agent to a single part of the plant.
4. The *signal* is a translocatable factor that conditions the host to respond in a resistant manner.
5. The *trigger* is an event or process leading to production of the signal.

One year later, Schönbeck *et al.* (1993) proposed the term *induced resistance* to describe mechanisms which enhance the resistance of a susceptible plant via exogenous stimuli, but without alteration of the genome by breeding. As opposed to conventional chemical or biological practices, in this process, the substances responsible for the induced resistance simply enhance the already existing resistance of plants against pathogens but do not show

any direct toxicity towards the pathogen. They listed the following criteria needed for induced resistance to exist, the first three items are regarded as essential:

- Protection of the plant is based upon an enhancement of the plant's own resistance mechanisms
- A time interval between induction by the inducers and inoculation of the disease.
- Inhibition of the induction with specific blocking substances.
- Unspecific protective activity.
- Besides local also systemic protection.
- Lack of a dose-impact relation, which is typical with a fungicide application.

Unspecific protection implies the occurrence of induced resistance in different plant species caused by the same inducing agent and effectiveness against various pathogens. It occurs in plants after pathogen infestation, contact with chemicals or with rhizosphere bacteria (Gilpatrick and Weintraub 1952, Ross and Bozarth 1960, Edgington *et al.* 1961, Kessmann *et al.* 1994, Wei *et al.* 1991, Liu *et al.* 1995). Induced resistance can be differentiated from cross protection and antagonism as it involves processes that take place inside the plant, generating the resistance already in the plant against a broad range of diseases and parasites. This phenomenon had already been observed at the beginning of the last century (Chester 1933), however, it had not been analyzed until the mid 1970s (Bochow *et al.* 2001). A review that focuses on the mechanisms underlying induced resistance in plants was published in 1983 by Sequeira.

The plants react to infestation by a pathogen with cell death, which can lead to a local hypersensitive reaction of single cells or even to necrotic lesions. This can ultimately lead to systemic acquired resistance (SAR) against viruses, bacteria and fungal pathogens in non-infected tissues (Sticher *et al.* 1997). This reaction has also been reported in response to a chemical treatment (Kessmann *et al.* 1994).

An accumulation of salicylic acid (SA) was only seen after pathogen contact (Friedrich *et al.* 1996). However, SA is essential for the SAR transduction pathway (Ryals *et al.* 1996). In chemically induced-SAR, the chemical compound seems to mimic SA and activates the SAR signal transduction pathway downstream of SA accumulation (Conrath *et al.* 2001, Friedrich *et al.* 1996). The different chemicals 2,6-dichloroisonicotinic acid and its methyl ester both referred to as INA, were the first synthetic compounds shown to activate SAR, and to provide broad-spectrum disease resistance (Métraux *et al.* 1991). The synthetic chemical

benzo(l,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) was also a potent SAR activator, which provides protection in the field against a broad spectrum of diseases in a variety of crops (Friedrich *et al.* 1996, Lawton et al. 1996).

An associated and coordinated expression of a set of so-called SAR genes and pathogenesis related genes (PR genes) is reported in response to pathogen infestation and chemical treatment. The synthesis of so-called SAR proteins after resistance induction was studied in tobacco and *Arabidopsis* (Ryals *et al.* 1996, Ward *et al.* 1991). PR proteins were also studied and their enzymatic activities identified (Stintzi *et al.* 1993, Van Loon and Van Strien 1999). Van Loon and Van Strien (1999) assumed that the accumulation of these proteins plays an important role. Although SAR is often effective against a variety of bacterial, viral as well as fungal pathogens, an antibacterial or antiviral activity has not been shown for any PR protein, nor have they been reported to enhance resistance to plant pathogens, even when over expressed in transgenic plants (Ryals *et al.* 1996, Thulke and Conrath 1998).

Potentially, additional mechanisms may be involved in the process of SAR. Reinforced cell walls were found in systemic induced tissue of cucumber and considered a local defence response (Hammerschmidt and Kuc 1982a). This could be seen as a physical protection system against pathogens in the early defence reaction (Bradley *et al.* 1992). Alternatively, this may also be regarded as a ‘sensitized state’ of the plant or a so-called ‘primed’ state (Hammerschmidt and Kuc 1982a). This demonstrated an enhanced ability of the induced tissue to activate cellular defence responses. Obviously, this can only be detected after pathogen challenge (Conrath *et al.* 2001).

Structural changes and material deposits in tomato plant cells were also observed, to different extents, after rhizobacteria induced systemic resistance against *Fusarium oxysporum* f.sp. *lycopersici* (Mwangi 2002). Treatment with *Pseudomonas fluorescens* strain T58 caused callose deposits on cell walls, whereas *P. putida* strain 53 and *Bacillus sphaericus* B43 treatments induced a build-up of occluding material in the lumina of xylem vessels (Mwangi 2002).

1.5.1 Rhizosphere Bacteria as Inducer of Systemic Resistance

Several research groups have identified strains of plant growth-promoting bacteria which colonized the rhizosphere of a plant and are able to induce systemic resistance (Van Peer *et al.*

1991, Wei *et al.* 1991, Kloepper *et al.* 1992a). This was studied in detail in several plant species (Pieterse *et al.* 1996b). The appearance of ISR-plants was not distinguishable from SAR-plants. The phenotypic characteristics of both ISR- and SAR-plants are:

- the enhanced capacity to defend themselves against many, but not all types of pathogens,
- the development of resistance not only in the area of primary infection, but also in distal, non-inoculated tissue i.e. a systemic activity (Ryals *et al.* 1996),
- a more rapid and effective activation of cellular defence responses - 'priming' - in reaction to pathogen infection (Conrath *et al.* 2001).

Reports on the elicitors and the mechanisms underlying the ISR are quite diverse, and occasionally contradictory (Buchenauer 1998). Some *Pseudomonas* species induced an accumulation of PR proteins, others did not (Maurhofer *et al.* 1994, De Meyer and Höfte 1997).

Differences in the transduction mechanisms underlying these inducing pathways were studied. Although controversial reports exist on the necessity of salicylic acid (SA) in the signal cascade reviewed by Dempsey *et al.* (1999), the fact that SA has an important role in the induction of systemic resistance is not doubted. There is clear evidence of a correlation between the non-accumulation of SA in transgenic tobacco and in *Arabidopsis* plants and the lack of establishment of ISR (Gaffney *et al.* 1993, Delaney *et al.* 1994).

SA plays a dual role in inducing resistance in plants. It can directly induce the activation of a defence gene or it can enhance the activation of multiple defence genes. Furthermore, depending on the concentration of SA applied, different defence genes were activated. At high concentrations of externally applied SA, the PAL gene was activated (Thulke and Conrath 1998). PAL is an enzyme of the phenylpropanoid biosynthesis pathway and its activity is characteristically stimulated by microbial infection leading to the synthesis of lignin-like, wall-bound phenolic material and phenylpropanoid-derived phytoalexin antibiotics (Jones 1984). These defence responses can also be induced by elicitors present in fungal cell walls and culture filtrates (Edwards *et al.* 1985).

The signal transduction pathway of a plant's reaction to pathogens, herbivores or pathogenic rhizobacteria seems to be strictly coordinated. Bostock *et al.* (2001) and Pieterse *et al.* (1998, 2001) suggested models of the potential transduction pathways of inducing signals. These

observations resulted mainly from experiments on the model system: *Arabidopsis* with *Pseudomonas fluorescens* strains inducing resistance against fungal or bacterial pathogens (Pieterse *et al.* 1996a). In experiments with different *Arabidopsis* genotypes, Pieterse *et al.* (1998) revealed that the rhizobacteria mediated ISR requires jasmonic acid (JA) and ethylene, and not necessarily SA, but similar to SAR, the resistance is dependent on NPR1.

Their results were mainly observed on necrotic plant pathogens and in above-ground plant material such as leaves and stems. Only a few studies looked at the root system of plants (Hasky-Günther 1996, Hallmann *et al.* 1998, Reitz *et al.* 2001, Mwangi M. *et al.* 2002a, Alabouvette *et al.* 2003, Schäfer *et al.* 2005), eventhough the root is severely affected by several pathogens and pests. Recently, a publication on the differential gene regulation in leaves and roots of *Coffea arabica* after SAR induction showed that there is a significant difference in gene-response within theses two plant organs (De Nardi *et al.* 2006).

A *Rhizobium etli* strain G12, formerly known as *Agrobacterium radiobacter* was demonstrated to induce resistance against the potato cyst nematode *Globodera pallida,* and the root-knot nematode *Meloidogyne incognita,* but not against the tomato wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici* (Hasky-Günther and Sikora 1995, Reitz 1999, Hauschild *et al.* 2001). It was shown to grow endophytically (Hallmann *et al.* 2001). A second rhizobacterium, *Bacillus sphaericus* strain B43, has ability to induce systemic resistance against all three soilborne pathogens tested in split-root trials (Hauschild *et al.* 2001). Neither strain G12 nor B43 induced an accumulation of PAL or known PR proteins such as glucanases, chitinases or peroxidases. Increased lignification or other physical change of the cell wall were also not observed in induced potato or tomato plants (Hasky-Günther 1996, Mwangi 2002). These results show that neither strain induces a classical SAR-response. This effect was not expected, as the induced resistance to nematode damage was mainly based on reduced nematode penetration, as opposed a reduction in reproduction after penetration. It can therefore be assumed that nematode attraction to the host or host-recognition by nematodes are substantially affected by bacterial ISR reactions of the plant.

The present study focuses on the interactions of tomato, *Lycopersicon esculentum* and two bacteria strains, *Rhizobium etli* strain G12 and *Bacillus sphaericus* strain B43, and the plant based mechanisms of systemic induced resistance against the root knot nematode *Meloidogyne incognita*.

1.6 The root-knot nematode, *Meloidogyne incognita*

Root-knot nematodes belong to the family *Heteroderidae* and the subfamily *Meloidogyninae*, with over 90 species described so far. Only four species are of economic importance to vegetable production, *Meloidogyne incognita, M. javanica, M. arenaria* and *M. hapla* (Sikora and Fernández 2005). There is no doubt that they are the most economically important species (Ferraz and Brown 2002).

As a sedentary endoparasite they depend on the availability of roots for survival, which they invade as second-stage juveniles. They feed on three to six giant cells that are induced by substances secreted by the nematodes themselves. The common name, root-knot nematode relates to the galls formed as the nematode grows and parthenogenetically reproduces in the root. The life-cycle of *Meloidogyne* spp. is completed in three to six weeks depending on the species (Sikora and Fernández 2005).

Because of their short life-cycle and their broad host range, with over 2000 host plants identified to date, there are few alternative possibilities available for effective control of the root knot nematode.

1.6.1 Nematode Management

Globally, plant-parasitic nematodes cause 10 to 20 percent of annual crop yield losses. These losses are however unevenly distributed between crop and location (Oerke and Dehne 2004). In many fields, intensive cultivation is only possible thanks to the application of highly toxic pesticides to control nematodes. In developing countries, where farmers cannot afford these expensive chemicals, even total crop losses are not uncommon. However, the use of such pesticides has significant detrimental side-effects on the environment (Ferraz and Brown 2002). One of the most effective pesticides used to manage soilborne pathogens is methyl bromide, which was listed as one of the ozone depleting substances under the Montreal Protocol Agreement in 1992. In consequence, the fumigant's production has been stopped in 2005 in industrialized countries and will phase out in 2015 in non-industrialized or so called Article 5 countries (UNEP 1999). Nevertheless, methyl bromide is a broad-spectrum fumigant and cannot be replaced by one single product. A wide variety of alternative control treatments have been proposed, but none of them are as effective and economical (Taylor 2001). To obtain an equal control level for nematodes and other pests and diseases, three or more pesticides need to be used before planting e.g. telone, chloropicin, and herbicides (Sikora

2002).

Alternatively, non-chemical methods including host resistance, organic amendments, crop rotation, soil solarization, biofumigation, and cultural practices have been used to control soilborne pests. These methods are compatible with an integrated pest management approach, where multiple tactics are used to maintain damage from pests below an economic threshold, while minimizing the impact to beneficial organisms (Chellemi 2002).

For effective control of nematodes, a combination of several management approaches is essential (Sikora *et al.* 2005):

- Hygiene: exclusion, quarantine
- Cultural and physical methods: crop rotation, heat treatment, biofumigation
- Biological: biological control with antagonistic microbes, resistant cultivars, resistant root-stocks, antagonistic and trap crops
- Pesticides: fumigants, non-fumigant nematizides

Control with biological methods encompass a great deal of alternatives and offers a broad spectrum of strategies for the control of soilborne pests and diseases, such as the root-knot nematode, *Meloidogyne incognita*.

1.7 Aims of Investigations

As outlined above in 1.5.1 plant health promoting or plant growth promoting bacteria have been shown to be effective in reducing root-knot nematodes on tomato. The plant's response to these rhizobacteria that induce systemic resistance against root-knot nematodes is not yet understood. Therefore this study aims to dissect the molecular basis of the rhizobacteria ISR against the root-knot nematode *M. incognita*. As the ISR against root-knot nematodes is not associated with an obvious morphological change in the plant (Mwangi 2002),

1) a reliable test system for induced plants had to be generated;
2) a method to study the different gene regulation had to be developed, and
3) the differential gene regulation had to be compared to known changes in the gene expression of plants upon either resistance induction by rhizobacteria and/or pathogen attack.

2 Materials and Methods

2.1 Bioassays - Split-Root

2.1.1 Tomato (*Lycopersicon esculentum* Mill.)

Tomato seeds *Lycopersicon esculentum* Mill. cv. "Hellfrucht Frühstamm", were sown in small pots containing seedling substrate (peat, Klasmann-Deilmann GmbH, Geeste-Groß Hesepe, Germany). Seeds were left to germinate under greenhouse conditions at a temperature of 22 °C ± 4 °C, relative humidity of 60-70 % and additional light of about 3000 Lux. After two to three weeks, when the seedlings had two differentiated leaves, the smaller plant in each pot was removed. The selected tomato plants were then transplanted into 10 cm diameter pots containing potting soil (Spezialmischung, Klasmann-Deilmann GmbH) and kept under greenhouse conditions until three differentiated leaves had developed. All plants were supplied with nutrients and water as necessary and depending on weather conditions.

For split-root experiments, tomato plants were cut 0.5 to 1.5 cm above the soil surface and the shoot split lengthwise for about 8 to 10 cm. Additionally, the lowest leaves were cut off to reduce the evaporation area. Three square plastic pots with an edge length of 11 cm were used for each split-root chamber. Two pots were filled with a 1:1 mixture of field soil and sand, and placed side by side. Two parallel holes were cut into the bottom of the third pot, so that only a small bridge remained in the middle. This pot was placed above the two pots filled with potting mixture, and was used to stabilize the rootless shoot. Each half of the split shoot was pushed through one opening in the bottom of the top pot and inserted into the soil in one of the bottom pots. Fourteen days later, the tomato stems had re-developed roots and were ready for experimental use.

2.1.2 Nematode Inoculum

A population of race 3 of the root-knot nematode *Meloidogyne incognita* (Kofoid & White) Chitwood (race 3) is permanently maintained on the susceptible tomato cultivar "Furore" in the greenhouse at 27 °C ±5 °C in a box filled with sandy loam was used in all treatments. Nematode eggs were extracted from heavily infested tomato roots using the extraction technique described by Hussey and Barker (1973) with minor modifications. The roots were washed free of soil under running tap water. Roots were cut into ~1 cm pieces before being

macerated in a Warring blender at high speed for 20 sec. The macerated roots were then transferred into a 1 l glass bottle filled with sodium hypochlorite at a final concentration of 1.5 % (v/v) active hypochlorite. For efficient extraction of the nematode eggs, the suspension was shaken vigorously for 3 min. To collect the eggs, the suspension was washed with tap water through 4 nested sieves of 250, 100, 45 and 25 µm, respectively. The eggs were washed from the 25 µm sieve into a 300 ml Erlenmeyer flask with tap water.

To promote the development of eggs and the hatching of second stage juveniles (J2), the nematode eggs/water suspension was kept in darkness at 24 °C and areated with an aquarium pump. After approx. 10 days, 30 % of the J2 had hatched. With a modified Baermann technique J2 were separated from eggs over a 24 h period (Oostenbrink 1960).

In all experiments, plants were inoculated with nematodes by drenching the soil surface around the base of the stem with 5 ml tap water containing 1000 J2. Control plants were given 5 ml tap water.

2.1.3 Rhizosphere Bacteria

Bacillus sphaericus B43 and *Rhizobium etli* G12 (Table 1) were stored at -80 °C in cryo vials (Cryobank^{TM}, Mast Diagnostica, Reinfeld). For production of bacterial inoculum, pellets containing bacteria from the cryo vials were transferred onto agar plates. *B. sphaericus* B43 cultures were grown on Tryptone Soya Agar (TSA) for 24 h and *R. etli* G12 cultures were grown on Kings' B medium supplemented with agar for 36 h both at 28 °C (King *et al.* 1954). From these pre-cultures, a loop-full of bacteria was transferred to liquid TSB for *Bacillus* and Kings' B for *Rhizobium.* The bacteria were cultured at 28 °C while shaking at 100 rpm for 24 h for *Bacillus* or 36 h for *Rhizobium*. To collect the cells, the culture broth was centrifuged at 5000 x g for 20 min at 6 °C. The resulting pellet was re-suspended in sterile water and the concentration was adjusted to an optical cell density of 2 at 560 nm (OD_{560}), which represented approximately 1.8×10^{10} cfu ml^{-1} of *B. sphaericus* B43 and 1.2×10^{10} cfu ml^{-1} of *R. etli* G12 (Hasky-Günther 1996). In all experiments, plants were inoculated with bacteria by drenching the soil surface with 5 ml of the cell suspension. Control plants were treated with the same volume of tap water.

Table 1: Names and origins of the bacteria isolates used in this study.

Isolate	Origin	Effective against	Reference
Bacillus sphaericus B43	Germany, potato	*Globodera pallida* on potato	Racke and Sikora 1992, Hasky-Günther K. 1998, Reitz M. 1999
		Fusarium oxysporum f.sp. *lycopersici* on tomato	Mwangi M. 2002, Mwangi M. *et al.* 2002a
		Meloidogyne incognita on tomato	Hauschild R. *et al.* 2004, Schäfer K. *et al.* 2006
Rhizobium etli G12	Germany, potato	*Globodera pallida* on potato	Racke and Sikora 1992, Hasky-Günther K. 1998, Reitz M. 1999, Hallmann J. *et al.* 2001,
		Meloidogyne incognita on tomato	Hauschild R. *et al.* 2004, Schäfer K. et al. 2006

2.1.4 Test for Induced Systemic Resistance

The fourteen-days-old tomato plants that had been planted in a split-root chamber composed of three pots were used for this experiment (Fig. 1). Each split root stem, which had re-developed roots in one of the two bottom pots was treated individually. At day 0, roots in the left bottom pot (inducer root) were inoculated with 5 ml bacteria suspension, instead the roots of control plants were treated with 5 ml water. In the experiments aimed at evaluating plant response to nematode penetration, roots in both bottom pots were inoculated with 1000 J2 nematodes in 5 ml water on day 3 (Fig. 1A), while only the responder roots of control plants received 1000 J2 in 5 ml water. For studies on the response of the plant to bacteria treatment, the same set-up was used with the difference that at day 3 only the responder roots of the bacteria treated plant were inoculated with the nematodes (Fig. 1B).

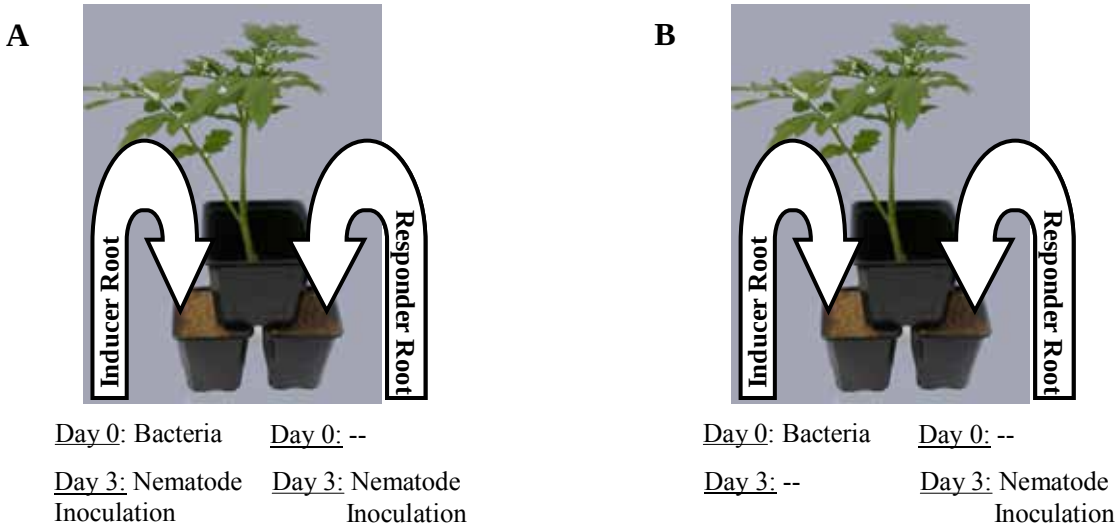


Fig. 1: Split-Root set-up to test for rhizobacteria induced systemic resistance against nematodes in tomato. **A**: Response to nematode penetration; **B**: Response to bacteria treatment

Tomato plants were fertilized with 0.2 % Flory 3 solution (15 % N, 10 % P_20_5, 15 % K_20, 2 % MgO), adjusted to provide optimum plant growth. Flowers were removed as soon as they appeared.

2.1.4.1 Induced Systemic Resistance and Nematode Penetration

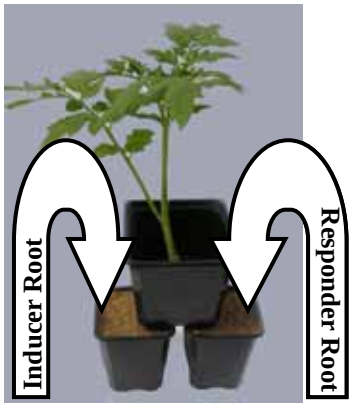
Three weeks after bacteria inoculation (Day 3), nematode penetration was evaluated. The roots in each pot were carefully washed with tap water, their weight and that of the shoot were taken. Nematodes in the inoculated roots were stained inside the plant tissue with acid fuchsine and subsequently counted using a dissecting microscope (Byrd *et al.* 1983, Sikora and Schuster 2000). The experiment was repeated once.

2.1.4.2 Long Term Effect of Induced Systemic Resistance on Nematode Reproduction

Eight weeks after inoculation with bacteria, the nematode reproduction was evaluated. Plants were harvested, roots washed free of soil, and shoot and root fresh weights taken. The eggmasses of female nematodes in the treated roots were stained with Phloxin B (Fig. 1). The gelatinous matrices holding the eggs are readily stained pink to red and can be observed and counted in water (Holbrook *et al.* 1983, O'Bannon *et al.* 1982, Sikora and Schuster 2000). Number of galls, egg masses and eggs per eggmass were counted. This experiment was repeated once.

2.1.5 Harvesting Tomato Roots for RNA Extraction

Plants were treated as described in 2.1.4., however, to construct a substraction library out of the harvested plant RNA, a contamination of the plant RNA with bacterial or nematode RNA had to be prevented. Therefore, both bacteria and nematodes were inoculated onto the same root (inducer root) while the responder root was not treated (Fig. 2). These responder roots were then used for RNA extraction. The root samples were taken 3 and 6 days after bacteria inoculation.



Day 0: Bacteria Day 0: --

Day 3: Nematode Inoculation Day 3: Root Harvest for RNA

Day 6: Root Harvest for RNA

Fig. 2: Split root set-up of tomatoes for RNA extraction

The abbreviations and description of the harvested root samples taken at day 3 and 6 are shown in Table 2. Tomato roots were carefully washed under running tap water and separated from the shoot. Roots were immediately dried with paper tissue, weighed, frozen in liquid nitrogen and stored at -80 °C for later use.

Table 2: Description of the harvested root samples from day 3 and day 6, used for RNA extraction

Sample	Description
U3	root of untreated plants, 3 days after bacteria inoculation
B3	root of *Bacillus sphaericus* B43 treated plants, 3 days after bacteria inoculation
R3	root of *Rhizobium etli* G12 treated plants, 3 days after bacteria inoculation
U6	root of untreated plants, 6 days after bacteria inoculation
BM6	root of *Bacillus sphaericus* B43 and *Meloidogyne incognita* treated plants, 6 days after bacteria and 3 days after nematode inoculation
RM6	root of *Rhizobium etli* G12 and *Meloidogyne incognita* treated plants, 6 days after bacteria and 3 days after nematode inoculation

2.2 Molecular Investigations

The mechanisms underlying the rhizobacteria induced systemic resistance in tomato against the root-knot nematode *Meloidogyne incognita* are still unknown. Therefore an attempt was made to identify the differentially regulated genes in response to rhizobacteria induction using molecular biological tools as described in the following chapter.

2.2.1 Materials

2.2.1.1 List of Bacteria, Vectors and DNA Fragments used for Control Hybridization

Material	Source
Escherichia coli XL1-blue strain	Stratagene
pCR® 2.1-TOPO®	Invitrogen (Fig. 4)
pUC9	New England Biolabs (appendix)
pBluescript SK +	Stratagene (appendix)
Chitinase	potato, 925 bp, provided in pUC9, *
Glucanase	potato, 1195 bp, provided in pUC9, *
Glucose-6-Phosphat-Dehydrogenase	potato, 1536 bp, provided in pBSK+, * (E.C. 1.1.1.49)

*Kindly provided by Mrs. Prof. Dr. A. von Schaewen of the University of Münster, Germany

2.2.1.2 Chemicals, Radioisotopes and other Materials

Chemicals were obtained from AppliChem (Darmstadt), Biomol (Hamburg), Difco (Heidelberg), Merck VWR International (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg), and Sigma-Aldrich (München), if possible with the same quality standard 'per analysis'. Radioactive labelled [^{32}P] dCTP was supplied by Hartmann Analytic GmbH (Braunschweig) with a starting activity of 370 MBq/ml.

2.2.1.3 Enzymes, Markers and Ladders

DNA restriction and modifying enzymes, markers and DNA or RNA ladders were supplied by Fermentas Biosciences (St. Leon-Rot), with the exception of reverse transcriptase, which was supplied by Invitrogen (Karlsruhe) and a DNAse I from Sigma-Aldrich (München).

2.2.1.4 Membranes, Films and Phosphor Imager

For the detection by autoradiography, RNA-transfer was performed with a not charged, neutral Hybond-N membrane from Amersham Biosciences. The radioactive labelled membranes were exposed to a *Phosphor Imager* system, which is a quantitative imaging device from Molecular Dynamics (Amersham Biosciences). The phosphor imager plate was scanned on a Typhoon Scanner with a resolution of 100 dpi and subsequently quantified by

analyzing the digitalized images using *ImageQuant* software program, version 5.2 of Molecular Dynamics, Sunnyvale, CA, USA. For quantification of signal intensity, two calculations the 'volume' and the 'sum above background' were made.

The 'volume' or the integrated intensity of all pixels in the spot excluding the background of the object was quantified. To calculate the volume, *ImageQuant* substracts the background value from the intensity of each pixel in the object and then adds the value.

The 'sum above background' or the integrated intensity of all the pixels in the spot excluding the background also was calculated. In this case *ImageQuant* adds only the pixel values in the object that are above the background.

To detect chemiluminescent, RNA-transfer was done using a positively charged nylon membrane (Roche). To visualize the hybridization results, a lumi film for chemiluminescent detection (Roche) was used and developed with photo chemicals (Kodak).

2.2.1.5 Kits

Total RNA and mRNA were extracted with the NucleoSpin® RNA Plant NucleoSpin and NucleoTrap® mRNA Kit from Macherey-Nagel (Düren), respectively. Subtractive suppressive hybridization was conducted with the Clontech PCR-Select^{TM} cDNA Subtraction Kit (BD Biosciences Clontech, Heidelberg).

For the isolation of mRNA for DNA/mRNA-hybridization, the Oligotex® mRNA Mini Kit was used (Qiagen, Hilden). Nucleic acids were eluted with NucleoSpin® Extract II (Macherey-Nagel, Düren) or the Agarose Gel DNA Extraction Kit (Roche Diagnostics GmbH, Mannheim). Plasmids were extracted with QIAprep Spin® Miniprep Kit (Qiagen, Hilden).

Ligation and transformation of DNA was performed with the Rapid DNA Ligation and Transformation Kit (Fermentas, #K1432). The DIG-labelling was conducted with the DIG Northern Starter Kit and the DIG Wash and Block Buffer Set (Roche Diagnostics GmbH, Mannheim).

For the production of ^{32}P-labelled clones, the HexaLabel^{TM} DNA Labelling Kit (Fermentas,

St. Leon-Rot) was used. For reverse transcription of the RT-PCR, the Superscript™ II Reverse Transcriptase (Invitrogen, Karlsruhe) was used.

2.2.1.6 Database and Software

Homology analyses on nucleotide or protein bases were conducted by comparing generated data sets with the databases available through the National Center for Biotechnological Information (*www.ncbi.nlm.nih.gov/*). Primers were constructed using the freeware software *Primer 3*. The PCR products of a RT-PCR reaction were quantified by analyzing the digitalized images of agarose gels using the *ImageQuant* software program as described above.

2.2.1.7 Media, Antibiotics, Buffers and Solutions

Media, antibiotic stock solutions, buffers and solutions for molecular biological applications were prepared according to Sambrook *et al.* (1989). The water used in all preparations and solutions was ultra pure, additionally autoclaved or, for RNA-applications, treated with DEPC and subsequently autoclaved.

2.2.2 Methods

2.2.2.1 RNA Extraction

The Hot-Phenol-Method as described by Ditzer (2003) was used in this case. Washed, non-inoculated roots of day 3 and day 6 plants (Table 2) were pulverized with a mortar and pestle under liquid nitrogen. Approximately 400 mg frozen powder was transferred to a 2 ml reaction tube with an ice-cold spatula. The powder was treated with 500 µl of 80 °C hot extraction buffer and 80 °C hot phenol per tube. After vortexing for 30 sec, 500 µl chloroform/isoamyl alcohols (24:1) was added, the tube capped and shaken vigorously. The tube was then centrifuged at 16000 x g for 5 min (Eppendorf Centrifuge 5415D). The top phase was collected, an equal volume of 4 M lithium chloride was added, mixed vigorously and kept on ice in a cold chamber or fridge at 0 °C over night.

After centrifugation at 4 °C at 16000 x g for 20 min, the pellet was dissolved in 400 µl water. The RNA was precipitated with 0.1 volume 3 M sodium acetate pH 5.0 and 2 volumes ethanol (absolute) at -70 °C for 2 hours. The RNA was pelleted by centrifugation for 30 min at 16000 x g and 4 °C. The pellet was washed with 70 % (v/v) ethanol, air dried and finally

dissolved in 25 to 50 µl water, depending on the concentrations required.

The concentration of the RNA in each sample was determined prior to polyA+ RNA purification, cDNA-synthesis or RNA/RNA- or RNA/DNA-hybridization, by measuring the optical density at 260 nm (OD_{260}) in a spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech). An optical density of 1 unit at 260 nm corresponding to 40 µg of RNA per ml ($A_{260} = 1 \Rightarrow 40$ µg/ml).

One µl of RNA solution was then dissolved in 500 µl water. The water, in which the RNA was later diluted was used to calibrate the spectrophotometer. Prior to each use, the cuvettes were washed with ethanol and water.

Additionally, the quality of the RNA was cotrolled on a 1 % agarose gel. When the ribosomal bands were visible, the RNA quality was sufficient for the mRNA-extraction, cDNA-production, subtractive suppressive hybridization (SSH), and RNA/RNA- or DNA/RNA-hybridization. RNA samples were stored at -70 °C until further use.

2.2.2.2 Agarose Gelelectrophoresis

From each RNA sample 1 µg was diluted with sterile water to which 0.1 µg ml^{-1} and 6 x Loading Dye (Fermentas) was added in a 1 x final concentration. The solutions were mixed by pipetting up and down and then loaded onto a 1 % agarose gel The RNA was separated at 100 mA constant amperage. Subsequently, the gel was stained with ethidium bromide solution (1 mg l^{-1} ethidium bromide in 1 x TAE-buffer) for 15 min and destained for 10 min in 1 x TAE-buffer. The ethidium bromide is incorporated into the steric structure of the nucleic acids, so that they are visible under UV light (Fig. 3).

Fig. 3: Ribosomal RNA from tomato root samples
U3: untreated at day 3, B3: *B. sphaericus* B43 treated at day 3, G3: *R. etli* G12 treated at day 3, U6: untreated at day 6, B6: B43 and *M. incognita* treated at day 6, G6: G12 and *M. incognita* treated at day 6, C: Control (water with LoadingDye, no RNA sample)

The same procedure was used for separting DNA. To the total volume of 25 µl PCR product 5 µl of Loading Buffer (1 ml Glycerol, 845 µl dd water, 10 µl 6 x Loading Dye) were added and subsequently applied to a 1.5-2 % agarose gel. After the gel run at 120 mA constant amperage, the DNA was stained/destained as described above for RNA and the fragments rated by size. To quantify the amount of the separeted DNA fragments on the gel, the gel was scanned with the Typhoon Scanner. The digitalized image was subsequently used for quantification with the *Image Quant* Software from Molecular Dynamics, Sunnyvale, CA, USA.

2.2.2.3 PolyA+ RNA Extraction

After quantification of the starting RNA, the concentration was adjusted to 250 µg RNA in 250 µl RNAse-free water. The polyA+ RNA was then extracted according to the manufacturer's manual for spin-columns (Qiagen, Oligotex mRNA Mini Kit #70022). Bound polyA+ RNA was eluted twice with 20 µl of 10 mM tris-HCl, pH 8. The resulting end volume of 40 µl was brought down to 20 µl with an EPPENDORF concentrator 5301.

2.2.2.4 Differentially Expressed Genes by Subtractive Suppressive Hybridization

Subtractive suppressive hybridization (SSH) is used to compare two populations of mRNA/polyA+ RNA and to select sequence fragments of genes that are expressed in one population but not in the other. First, both mRNA populations were converted into cDNA. The cDNA derived from untreated plants was used as reference and as "driver" in the following SSH-steps. The pooled cDNA of bacteria treated plants, which in addition to transcripts of constitutively expressed genes contained the specific, differentially expressed transcripts, was used as "tester" in the following SSH-steps.

With the Clontech PCR-Select^{TM} cDNA subtraction kit these initial steps were conducted according to manufacturer's instructions (BD Biosciences Clontech, 2002, #K1804-1). Two independent SSH were conducted, first the cDNA of U3 (untreated, day 3) plants served as "driver" and the pooled B3 and R3 (rhizobacteria induced, day 3) cDNA served as "tester". In the second SSH, cDNA of U6 (untreated, day 6) plants served as "driver" and the pooled BM6 with RM6 (rhizobacteria with nematode induced, day 6) served as "tester".

In the following SSH-steps, differentially expressed genes were further enriched, according to manufacturer's instructions. Finally, the resulting PCR products were ligated in a pcR 2.1 - plasmid vector and transformed into competent *E. coli.* After blue/white-selection, the

bacteria were grown in liquid LB-media supplemented with 50 µg/ml ampicillin. For extented storage at -80 °C, glycerol bacterial suspensions were produced with 20 % (v/v) glycerol and 80 % (v/v) of grown bacteria in LB-media with ampicillin.

2.2.2.5 Plasmid-DNA Isolation of *E. coli*

To screen the cloned cDNA fragments the plasmids were isolated from individually transformed bacteria, which were grown overnight in 2-4 ml liquid LB-medium containing antibiotics at 37 °C on a shaker. To pellet the bacteria cells, 2 ml of the growth medium were centrifuged twice at 4 °C and 1500 x g in a reaction tube. First the supernatant was discarded and the pellet was re-suspended in 200 µl of lysis buffer (50 mM tris pH 7.4 with 2 M HCl, 10 mM EDTA, 100 µg/ml RNAse A, stored at 4 °C) for 2 min at RT. EDTA binds Mg^{2+} ions necessary for DNAses and thus inactivates them. Then 200 µl of the second solution (200 mM sodium-hydroxide, 1 % (w/v) SDS) was added and carefully inverted and was incubated at RT for a maximum of 5 min. The SDS of the second solution destroys membranes and denatures proteins. With sodium hydroxide, a high pH level is achieved to convert the bacterial chromosomal DNA into single strands and hydrolyses the attached proteins. After adding 200 µl of solution 3 (3 M potassium-acetate pH 5.5 with acetic acid) the solution was mixed and kept on ice for 10 min. The potassium acetate in solution 3 neutralizes the solution so that the chromosomal single strand DNA and the proteins aggregate but the circular plasmid-DNA re-natures. After centrifugation for 20 min at 4 °C and 1500 x g, the circular plasmid-DNA remained in the supernatant, whereas the chromosomal DNA together with the denatured proteins were precipated. The supernatant was carefully transfered and mixed with 0.7 volume isopropanol. After centrifugation for 15 min at 4 °C and 1500 x g, the plasmid-DNA was precipated. The isopropanol was discarded and the pellet was washed with 300 µl of 70 % (v/v) ethanol, centrifuged for 5 min at 4 °C and 1500 x g and the supernatant was discarded. The plasmid-DNA pellet was air dried for 5-15 min at RT and dissolved in 30 µl TE-buffer.

The QIAprep Spin® Miniprep Kit (Qiagen, Hilden) was used according to the manufacturer's instruction`s for subsequent sequencing.

2.2.2.6 Restriction Enzyme Digestion

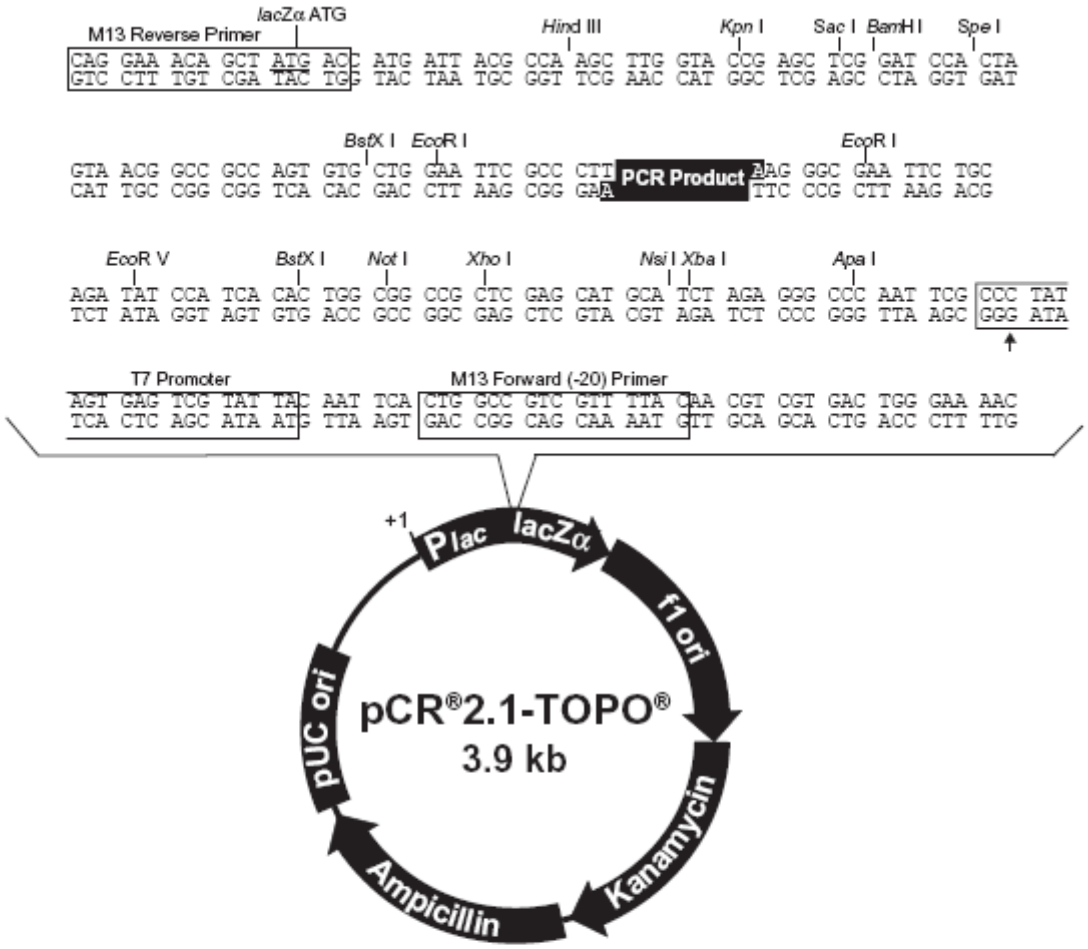
The fragments cloned into the vector pCR® 2.1-® (Fig. 4, marked: PCR Product, Invitrogen) are flanked by the *Eco*RI restriction sites. Hence the inserted DNA-fragment was cut with the restriction enzyme *Eco*R1 (Fermentas) according to Sambrook *et al.* (1989). For further

analysis, these inserts were digested with *Bam*HI, *Hind*III, *Xba*I and *Xho*I restriction enzymes (Fermentas) either in a combination of two, with *Eco*RI or alone.

The reaction mix was mixed in a reaction tube and incubated at 37 °C for 1-2 h (Table 3). The reaction was stopped by cooling the tube on ice for 5 min. For longer storage the tubes were stored at -20 °C. To visualize the DNA fragments, they were added onto a 1% or 1.5% agarose gel with a DNA size marker λ/PstI 24 (Fermentas) and stained with ethidium bromide as described in 2.2.2.2. The length of each fragment was rated on the basis of the DNA size marker and a map of each clone was generated. Later the inserts of interest were isolated from the gel and subsequently sequenced.

Table 3: Basic composition of the reaction approaches for digestion of DNA.

1 µg	**DNA**
x µl	**dd water**
0.1 x	**final volume 10 x reaction buffer**
5 U	**restriction enzyme 1**
5 U	**restriction enzyme 2 (optional)**



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Fig. 4: Map of plasmid vector pCR® 2.1-TOPO® (Invitrogen)

2.2.2.7 Elution of DNA-Fragments out of an Agarose Gel

PCR preparations, cut PCR products and restriction enzyme digestion of plasmids were separated from undesired fragments on an agarose gel. The fragment of interest was cut out of the gel and purified using the NucleoSpin® Extract II kit from Macherey-Nagel (Düren) or the Agarose Gel DNA Extraction Kit (Roche Diagnostics GmbH, Mannheim).

2.2.2.8 Sequencing

The plasmids with the cloned cDNA of interest were sent for sequencing to MWG Biotech AG (Ebersberg). The resulting forward and reverse sequences trimmed of vector sequences are shown in the appendix. To identify redundancies, each sequence was searched against the entire database of sequences using a local BLAST. The virtual translations of the assembled sequences were analyzed by BLASTx to identify homologous sequences in the GeneBank database (Benson *et al.* 1998) and the available accession numbers of the genes are listed in Table 7.

2.2.2.9 RNA/RNA-Hybridization for DIG Chemiluminescence Detection and DNA/RNA-Hybridization for Autoradiography

To verify the potential differentially expressed clones of the SSH, total-RNA or mRNA was hybridized to labelled probes of these genes. To do so, mRNA or total RNA was fractionated by gel electrophoresis. The DIG labelling is a non-radioactive method to prepare probes for hybridization to targeting nucleic acids (RNA) on a RNA blot. DIG-labelled RNA can detect rare mRNAs in nanogram amounts of total RNA. From a DNA template, these labelled RNA probes are generated by *in vitro* transcription (DIG Application Manual for Filter Hybridization, Roche Diagnostics GmbH, 2000).

2.2.2.9.1 Preparing a DIG-labelled RNA-Probe

The potential differentially expressed DNA is cloned into a multiple cloning site of a transcription vector between promoters for different RNA polymerases (e.g. T7 RNA polymerase). Then the vector is linearized at a unique site near the insert so that the RNA polymerase can transcribe the insert DNA into an antisense RNA copy in the presence of a mixture of ribonucleotides including DIG-UTP (DIG Application Manual for Filter Hybridization, Roche Diagnostics GmbH, 2000). The standard method was used to prepare the DNA template and 1 µg of this linearized plasmid DNA was used for transcriptional labelling with the T7 RNA polymerase according to the manufacturer's recommendations.

To add the correct amount of probe to the hybridization, the amount of DIG-labelled probe produced in the labelling reaction was determined. With the direct detection method, a series of dilutions of the labelled probe was compared with a known concentration of DIG-labelled control nucleic acid (Table 4). This was performed according to the manufacturer's instructions. The detection of the labelled RNA was performed with CDP-Star and a Lumi-Film chemiluminescent detection film (Roche). The exposure time varied from 15 min to overnight exposure.

Table 4: Serial dilutions of labelled probes and control probes and description of dilution buffer and control used.

Tube	RNA (µl)	From Tube#	RNA Dilution Buffer (µl)	Overall Dilution (from tube R1)	Final Concentration
R1*		-		none	10 ng/µl
R2	2	R1	18	1:10	1 ng/µl
R3	2	R2	198	1:1000	10 pg/µl
R4	15	R3	35	1:3300	3 pg/µl
R5	5	R3	45	$1:10^4$	1 pg/µl
R6	5	R4	45	$1:3.3x10^4$	0.3 pg/µl
R7	5	R5	45	$1:10^5$	0.1 pg/µl
R8	5	R6	45	$1:3.3x10^5$	0.03 pg/µl
R9	5	R7	45	$1:10^6$	0.01 pg/µl
R10	0	-	50	-	0

*working solution of labeled probe or control RNA
RNA Dilution Buffer Mixture (5:3:2) of DEPC treated water : 20 x SSC : formaldehyde
Nucleic acid control: DIG-labelled actin control RNA, 10 ng/µl (DIG Northern Starter Kit)

2.2.2.10 Ligation and Transformation

Before the clones could be labelled with DIG, a test labelling was performed with cDNA of a cytosolic glucanase-6-phosphate-dehydrogenase (1536 bp, source *Solanum tuberosum*, see 2.2.1.1). This fragment was provided in a pBSK + plasmid. Additional test hybridizations were performed with a cDNA fragment of a potato chitinase and glucanase (data not shown). These DNA fragments were provided in a pUC9 plasmid without a T7 promoter binding site. As the T7 promoter is essential for an *in vitro* transcriptional RNA DIG-labelling, the cDNA fragments were cut out of the pUC9 plasmid with the restriction enzyme *Eco* RI (Fermentas) and subsequently ligated into a pBSK plasmid, which was dephosphorylated with shrimp

alkaline phosphatase (1 unit SAP / 1 picomol DNA-5'). The pBSK plasmid was obtained after plasmid isolation with the "MiniPrepSpinColumn"(Qiagen) of *E. coli* XL1-blue cells (Stratagene). The ligation and the transformation was performed with the "Rapid DNA Ligation and Transformation Kit" (Fermentas) according to the manufacturer's protocol.

2.2.2.11 RNA-Agarose Gelelectrophoresis for RNA Blots

For RNA/RNA or RNA/DNA-blotting the RNA or mRNA was separated by size on a denaturing agarose gel (Table 5). The formaldehyde inhibits the development of secondary structures of RNA, which would make a later hybridization step more complicated.

The buffers, solutions and gel composition for RNA/RNA hybridization were prepared according to the manufacturer's instructions (DIG Application Manual for Filter Hybridization, Roche Diagnostics GmbH, 2000). For DNA/RNA hybridization, the buffers, solutions and gels were prepared according to Sambrook *et al.* (1989).

Table 5: Composition and running condition of a formaldehyde gel for RNA/RNA or DNA/RNA hybridization.

RNA/RNA		**DNA/RNA**	
10 x MOPS, pH 7.0	200 mM MOPS buffer 50 mM sodium acetate 20 mM EDTA adjusted to pH 7.0 with NaOH	**5 x MOPS**	
Loading Buffer	250 µl formamide (always fresh) 83 µl 37 % formaldehyde 50 µl 10 x MOPS 10 µl 2.5 % [w/v] bromphenol-blue-solution 57 µl DEPC treated water	**RNA Sample Buffer**	500 µl formamide (always fresh) 175 µl 37 % formaldehyde 100 µl 5 x MOPS 0.2 µl 10 % [w/v] bromphenol-blue-solution
Running Buffer	1 x MOPS	**Running Buffer**	200 ml/l 5 x MOPS 80 ml/l 37 % formaldehyde
1 % Agarose Gel	2.5 g agarose	**1.2 % Agarose Gel**	1.8 g agarose
	21 ml 10 x MOPS 174.2 ml water 11.2 ml 37 % formaldehyde		30 ml 5 x MOPS 93 ml water 27 ml 37 % formaldehyde
Gel Running Condition	120 V, 10 min then 60 V 150 min	**Gel Running Condition**	70-80 mA, 120-180 min

For gel preparation, agarose was initially melted in the microwave with MOPS and water, and subsequently cooled down to about 50 °C. The formaldehyde was added under the extractor hood.

In preparation for DNA/RNA hybridization, all samples were adjusted to the same volume with water, supplemented with 50 % RNA sample buffer. After denaturing at 65 °C for 30 min, all samples were loaded on the agarose gel and separated.

2.2.2.11.1 Transfer of RNA onto Nylon Membrane and Subsequent Fixing

After electrophoresis, the RNA was blotted onto a nylon membrane, which was positively charged for DIG-hybridization (Roche) or a neutral Hybond-N for autoradiography (Amersham Biosciences) via capillary transfer.

The gel was placed onto two layers of *Whatman* filter paper, the ends of which extended into sufficient 20 x SSC. The transfer was performed according to Sambrook *et al.* (1989) and the set-up is shown in Fig. 5.

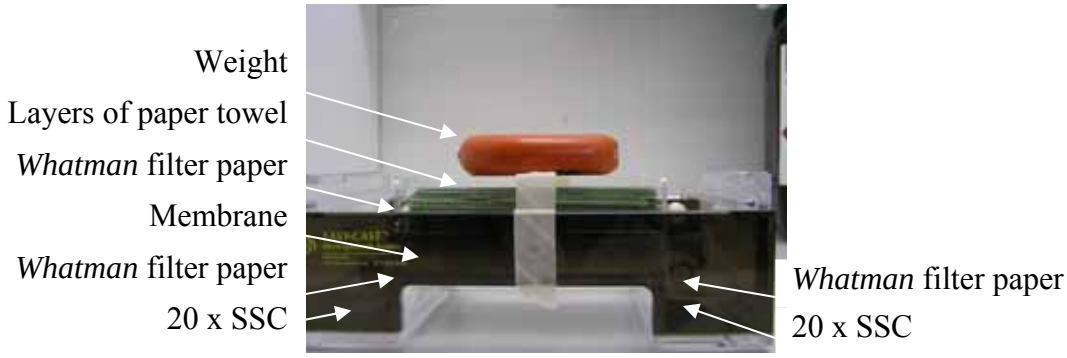


Fig. 5: RNA transfer onto a membrane via capillary power.

After the overnight transfer, the RNA on the membrane was fixed by UV-cross linking (0.6 J/cm², LKB-Transilluminator). In case of autoradiography, the membrane was additionally baked at 80 °C for 2 h to fix RNA. Until further use, the membranes for DIG-hybridization were stored between two sheets of *Whatman* filter paper in a sealed bag at 4 °C or at RT for the membrane used in autoradiography.

2.2.2.11.2 Prehybridizing of RNA Blot and Hybridizing with DIG-labelled RNA Probe

Prior to hybridization, the blot was prehybridized with DIG EasyHub according to the manufacturer's instructions, at 68 °C in a hybridization bag (Roche). For hybridization, the

required amount of the labelled RNA probe was denatured and added after cooling to the required amount of pre-warmed DIG EasyHub hybridization buffer. The pre-hybridization buffer was discarded and the hybridization solution was added to the blot. The hybridization was performed at 68 °C overnight. 24 h later a Low Stringency Buffer (2 x SSC, containing 0.1 % SDS) was used to wash the membrane at RT twice. A High Stringency Buffer (0.1 x SSC, containing 0.1 % SDS) was then added at 68 °C and the membrane was rinsed twice.

2.2.2.11.3 Chemiluminescent Method for Detection of DIG-labelled RNA Probes

For visualization of the probe-target hybrids, the chemiluminescent method with the CDP-star substrate was used (Roche). First, the membrane was washed with 100 ml washing buffer at RT for 2 min and blocked with 100 ml blocking solution at RT for 30 min (DIG Wash and Buffer Set, Roche). Then the probe target-hybrids were localized with the anti-DIG solution, using 20 ml antibody solution, for 30 min while shaking. The membrane was washed twice with 100 ml portions of washing buffer while shaking for 15 min to rinse off unbound antibodies. The membrane was equilibrated for 3 min in 20 ml detection buffer. Then the DIG on the blot was detected with the CDP-star chemiluminescent substrate. For each 100 cm^2 of membrane, 20-30 drops were applied, drop-by-drop, evenly over the entire surface. After 5 min of incubation, the membrane was exposed to the Lumi-film for 5 min to overnight, depending on the strength of the signal. The film was then developed according to the manufacturer's instructions (Kodak, Roche).

2.2.2.11.4 Establishing a ^{32}P-dCTP-labelled DNA Probe

The radioactive labelling reaction was performed according to the manufacturer's instructions with the HexaLabel™ DNA Labelling Kit (Fermentas). At first, the short hexanucleotides are used as random-primers annealing to the denatured DNA as either purified PCR product or restriction fragment. These were extended in the 3'-direction by Klenow-Polymerase up to 80-200 bp, using ^{32}P-labeled dCTP (Feinberg and Vogelstein 1983). To remove non-incorporated nucleotides, gel size exclusion chromatography was performed. To do so, a GF/C-*Whatman* filter piece was put in a 1 ml syringe and the corpus was filled without any air bubbles with 1 ml Sephadex G-50-column material in 1 x TE. The reaction mix was added onto the column and eluted with 100 µl 1 x TE in 10 fractions. After measuring with the scintillation counter, the fractions that contained the first radioactivity peak were combined and used as a probe. The DNA probe was denatured at 95 °C for 5 min directly before adding to the RNA blot for hybridizing.

2.2.2.11.5 Hybridizing a RNA Blot with a ^{32}P-dCTP-labelled DNA Probe

The backed membrane was pre-hybridized with hybridizing buffer for 3-5 h at 42 °C in a shaking water bath (Table 6). Then the denatured radioactively labelled probe was added in fresh hybridizing buffer. The hybridization was performed overnight. The next day, the membrane was rinsed 3-4 times for 20-30 min at 42 °C, until the washing solution was no longer radioactive and the membrane dried on filter paper. The dried membrane was then fixed onto a dry filter paper and covered with wrapping foil. This was put in a lead box and covered with a phosphor imager shield of equal size. The exposure time depended on the intensity of the expected signal and radioactivity. The phosphor imager shield was then scanned with the Typhoon Scanner, which translated the radioactive signals into a digital image of the membrane. This image was used for further analysis.

Table 6: Solutions used for radioactive hybridization

100 x Denhardt's	2 % [w/v] BSA Fraction V 2 % [w/v] Ficoll 400 2 % [w/v] PVP 360,000
Herring Sperm-DNA, sonicated (Biomol)	10 mg/ml desolved in 1 x TE for 2-4 h using a magnetic stirrer and sheared by pipetting the solution up and down, several times; aliquots stored at -20 °C; before use, DNA denatured for 5 min at 95 °C
Hybridizing Buffer	50 ml formamide 25 ml 20 x SSC 1 ml 100 x Denhardt's 1 ml 0.1 M Pipes, pH 6.8 500 µl denatured Herring Sperm-DNA
Washing Solution	0.1 % [w/v] SDS 2 x SSC

2.2.2.12 Semi Quantitative Reverse Transcriptase Polymerase Chain Reaction

The reverse transcriptase PCR is a semiquantitative method for selective detection of mRNAs in an mRNA-pool. The DNA-free mRNAs were transcribed with reverse transcriptase into cDNA. Then the RNA was digested and the single-stranded cDNA was used as a matrix for the PCR-amplification with its specific primers.

2.2.2.12.1 Preparation of RNA for qRT-PCR

Prior to a sensitive application such as qRT-PCR, the RNA samples were treated with DNAse I. According to the manufacturer's manual a ten-fold dilution of total RNA was treated with Amplification Grade DNAse I (Sigma-Aldrich). For this, 1 µl of 10 x Reaction Buffer and 1 µl of Amplification Grade DNAse I (1 unit/µl) was briefly added to 1 µg of RNA sample solved in 8 µl water. This mix was incubated at RT for 15 min. Then 1 µl of EDTA, the Stop Solution was added to bind calcium and magnesium ions and to inactivate the DNAse I. After denaturing at 70 °C for 10 min, the mixture was chilled on ice.

2.2.2.12.2 First-Strand cDNA Synthesis with Superscript ™ II Reverse Transcriptase

The synthesis of the first strand cDNA was performed according to the manufacturer's instructions (Invitrogen). 1 µg RNA was treated with DNAse I to make finally a volume of 11 µl, to this 1 µl Oligo(dT)$_{12-18}$ (500 µg/ml) and 1 µl dNTP Mix (10 mM each) were added. Then the RNA was denatured at 65 °C for 5 min and quickly chilled on ice. After a short centrifugation, 4 µl 5 x First-Strand Buffer and 2 µl 0.1 M DTT were added. This mixture was gently vortexed and kept at 42 °C for 2 min prior to adding 1 µl (200 units) of Superscript ™ II Reverse Transcriptase and mixing by gently pipetting the liquid up and down. To synthesise cDNA, this mixture was incubated at 70 °C for 50 min and then cooled to 4 °C. The cDNA was stored at -20 °C.

2.2.2.12.3 Polymerase Chain Reaction

The PCR reactions were performed using 1 µl of the prepared cDNA as template. The 10 x PCR Buffer was added in a 1 x final concentration together with 1.5 mM of $MgCl_2$ and 0.2 mM of each dNTP. Then 1.0 µM of each primer was added and finally 1 u μl^{-1} of Taq DNA Polymerase was supplied. All ingredients were added on ice into a thin walled PCR tube and the reaction volume was finally adjusted to 25 µl with water.

The ingredients were mixed by gently pipetting the liquid up and down. The whole mixture was then briefly centrifuged to collect the liquid at the bottom of the tube. The tube was then

placed in the thermocycler (PTC-200 Gradient Cycler DNA Engine, MJ Research Peltier) and a PCR program was performed with the following profile:

Initial denaturizing:	94 °C	2 min	
Denaturizing:	94 °C	30 sec	
Annealing:	55 °C	30 sec	20-35 cycles
Elongation:	72 °C	30 sec	
Final Elongation:	72 °C	5 min	
Storage:	4 °C	till stopped	

The cycle numbers were adjusted, depending on the amount of PCR product produced during the reaction.

2.2.2.12.4 Primers for qRT-PCR

Based on the sequences of the potential differential expressed genes (Table 7), the primers were constructed with *Primer 3*, a freeware-software, and chosen depending on the following criteria:

- As hydrophobic bridges are developed between G and C, the end of the primers should contain at least one dGTP or dCTP.
- The primers should not form dimers with themselves or the opposite primers.
- The annealing temperature of both primers of one PCR reaction should be nearly the same, around 60-65 °C.
- The GC-contents should be around 50 %.

The accession numbers for the genes, on which the primer sequences were based, are also listed. All primers were ordered at Sigma Genosys.

Table 7: Clone number, corresponding gene with accession code, sequences of primer pairs and size of PCR product in bp are listed.

	Gene Accession code	Clone No.	Name of gene	Short cut Primer	Sequence from 5´to 3´	size in bp
Internal standard	**AY725475**		**Malate Dehydrogenase**	MDH_for MDH_rev	CAC GGA ATT CAT CAC CAC AG ACC AGC TGG AAT GCC ATA AG	187
	X58253		**Ubiquitin**	UBI_for UBI_rev	CAG GCC CAA TAA CGA AGA AA AGA TCT GCA TCT TCG TCT GGA	142
Clones	**M98466**	**1-3**	**Polygalacturonase Isoenzyme 1 beta subunit**	PG_for PG_rev	ATC AGT GTT GGG TCG AAA CG AAT CAG CAA TTG CCC AAG TC	371
	AF092655	**1-6 and 1-7**	**Putative High Affinity Nitrate Transporter NRT2;1 mRNA**	PNT_for PNT_rev	GTC AAG GGA ACG GAA GAA CA ACA CCT TCG TGG ATT CAA GG	338
	BT013152	**1-9**	**Hypothetical Protein**	HP_for HP_rev	CCT CCC ACG AGA CAG TGT AA TCC CAC TTT CAA CTT CAC ACA	156
	AAK14060.1	**2-1**	**Major Latex-like Protein (primer on clone sequence)**	MLP_for MLP_rev	GGG CAG GTA GAC CAC ATC AT GTG TGT GGA TCT GGC ACA TT	325
	X13497	**2-7**	**Putative Intergenic Region of Potato Wound-induced Genes WIN1 & WIN2 (primer on clone sequence)**	PIG_for PIG_rev	CCC ACA AAC ACT GCA GCT TA GCT TCA ACG GCC TCA CTT AG	396
	P26600	**2-B1**	**Phenylalanine Amonia-lyase PAL5**	PAL_for PAL_rev	GCT GAG CAA CAC AAC CAA GA CTG TCC ACA ACT CGA AGC AA	269

2.2.3 Quantification of Expression Levels

When the digital image of PCR products separated on an agarose gel were available, the amount of these products were calculated. For a quantification of the signal intensity with the *Image Quant* Software, the same calculation was used as with the quantification of the signal intensity of an exsiccated phosphor imager plate (see 2.2.1.4). Additionally, the intensity of produced PCR product of one primer pair was subsequently related to the second primer pair of the qPCR. The primers of the 'housekeeping gene', a gene that is unchanged by any of the treatments in it`s expression level, was used as an 'internal' standard and normalized. The expression levels of the other primer pairs were related to the normalized expression level of the housekeeping gene.

2.2.4 Statistic Evaluation

The data of the bioassay was statistically analyzed with the statistic programs SPSS 11.0 for Windows or Stat Graphics plus for Windows. Fisher's least significant different (LSD) test was used to test for significant differences between means.

3 Results

3.1 Bioassay - Split Root

To dissect the molecular basis of the rhizobacteria mediated induced systemic resistance, a reliable system had to be used to obtain roots for RNA extraction of induced plants. These induced plants do not obviously change their morphology. Reference plants were inoculated simultaneously and these were evaluated for the influence of the rhizobacteria induced systemic resistance on the penetration of and the long-term effect on the reproduction of *Meloidogyne incognita* juveniles. The following chapter deals with the results of the evaluation of these simultaneously inoculated plants.

3.1.1 Induced Systemic Resistance and Nematode Penetration

Whether rhizosphere bacteria, alone in inducer roots, or only together with *M. incognita* in inducer roots are able to systemically induce a resistance effect on the penetration of *M. incognita* in the responder root was evaluated in split-root experiments. The tomato plants were inoculated with either *Bacillus sphaericus* B43 or *Rhizobium etli* G12 on one root half (inducer root, Fig. 1A and B) and three days later both root halves (Fig. 1A) or only the responder root, (Fig. 1B) were infested with *M. incognita.* In each treatment, the control plants and untreated roots received the same volume of water as necessary for inoculation. Three weeks after nematode infestation, nematode penetration was evaluated.

Both inducer and responder roots were inoculated with nematodes to elucidate whether or not the bacteria alone, or only together with the nematode induced a systemic effect on the nematode penetration in the responder root and if the bacteria had a direct effect on nematode penetration. A significantly reduction in nematode penetration could be observed in the responder root of bacteria treated plants and also in the inducer root, where both bacteria and nematodes were inoculated. Compared to the number of nematodes that penetrated the responder roots of control plants, where 56 juveniles/g fresh root penetrated, only 18 juveniles/g fresh root penetrated responder roots of plants inoculated with both *B. sphaericus* B43 and *M. incognita* and only 16 juveniles/g fresh root penetrated the responder roots of plants treated with both *R. etli* G12 and *M. incognita*. This corresponds to a reduction in *M. incognita* penetration of 68 % when roots were directly inoculated with both *B. sphaericus* B43 and *M. incognita*, and a reduction of 71 % when roots were directly treated with both *R. etli* G12 and *M. incognita*. In inducer roots of plants inoculated with both *B. sphaericus*

B43 and nematodes, 21 juveniles penetrated, whereas in inducer roots with *R. etli* G12 and *M. incognita* inoculation, 10 juveniles penetrated. Data shown in Fig. 6 are relative to the root fresh weight, which did not differ significantly between the different treatments (data not shown).

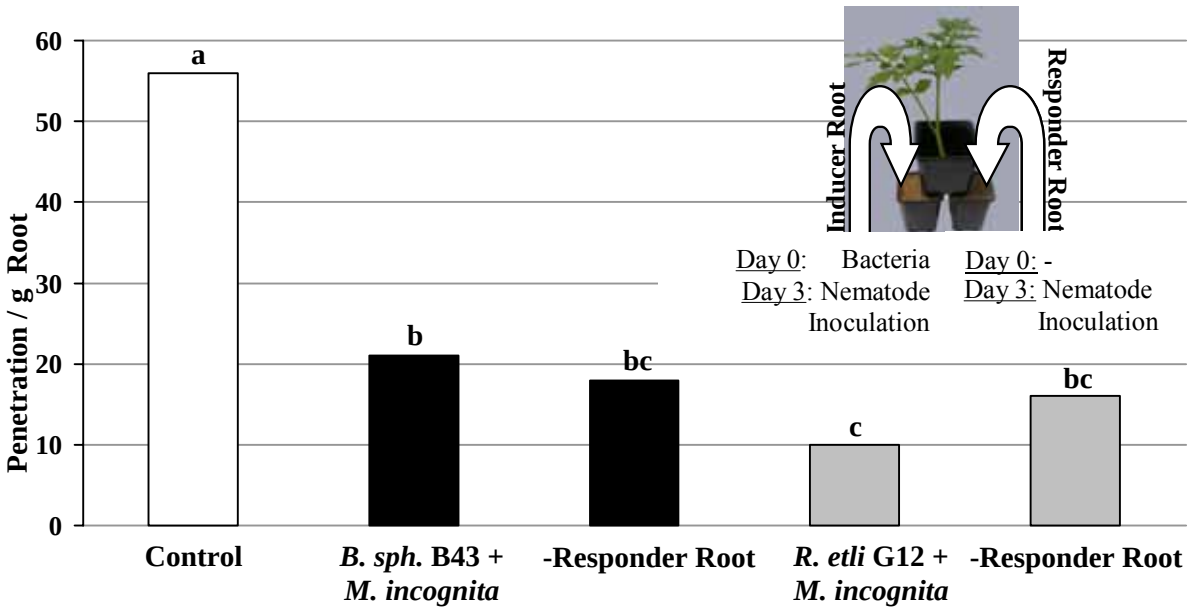


Fig. 6: Direct and indirect effect of inoculation with either *Bacillus sphaericus* B43 or *Rhizobium etli* G12 and *Meloidogyne incognita* on the number of *M. incognita* juveniles that penetrated per gram fresh root in tomato roots, 18 days post bacteria inoculation.
Means with the same letter are not significantly different based on Fisher's least significant difference (LSD) procedure (P< 0.05, n=10).

Another split-root experiment was conducted to study the systemic induced effect of the bacteria treatment on nematode penetration in the responder root (Fig. 7). For this, the inducer root was inoculated with bacteria and only the responder root was infested with nematodes. No significant difference could be observed in root fresh weight between the different treatments (data not shown). When comparing *M. incognita* juvenile penetration rates in responder roots of treated plants and control plants, significant differences were observed. In the control plants 56 juveniles/g fresh root penetrated, compared to 22 juveniles/g fresh root in *Bacillus* treated plants and 12 juveniles/g fresh root in *Rhizobium* treated plants. This corresponds with a reduced infestation of 61 % by the *B. sphaericus* B43 treatment, and of 79 % by the *R. etli* G12 treatment, compared to that in control plants. This clearly illustrated that both bacteria strains alone were able to induce systemic resistance to the root-knot nematode *M. incognita* in tomato.

When the split root experiment was repeated with double the numbers of repetitions, similar results were observed. In relation to the root fresh weight, which did not differ significantly between treatments (data not shown), both bacteria strains induced systemic resistance to the

root-knot nematode (Fig. 8). No significant difference could be observed between both bacteria treatments. In the *M. incognita* control plants, 50 juveniles/g fresh root penetrated, whereas only 24 and 15 juveniles/g fresh root penetrated the responder roots of *B. sphaericus* B43 and *R. etli* G12 treated plants, respectively. This corresponds to a 52 % and 70 % reduction of *M. incognita* penetration in the responder roots of plants where the inducer roots had been treated with *B. sphaericus* B43 or *R. etli* G12, respectively. This demonstrated that the systemic induced reduction of nematode penetration was reproducible. The range of control was stable at around 23 and 14 penetrated juveniles per g root, which corresponded to a reduction in penetration of *M. incognita* of 55 % and 75 % with *B. sphaericus* B43 or *R. etli* G12, respectively.

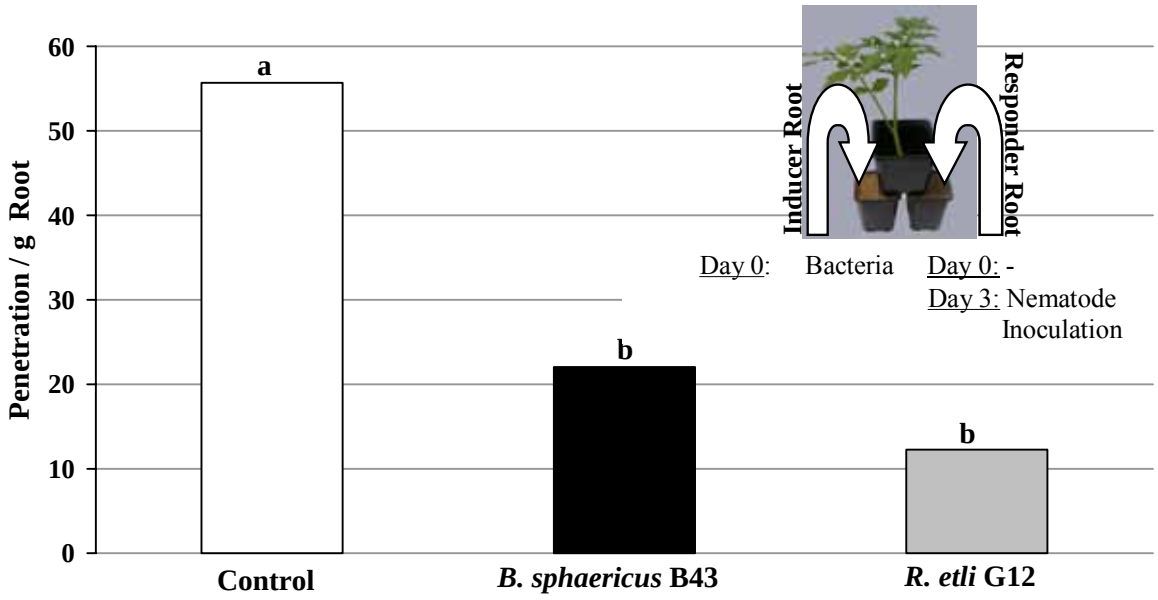


Fig. 7: Induced systemic effect of *Bacillus sphaericus* B43 and *Rhizobium etli* G12 inoculation on the penetration of *Meloidogyne incognita* J2 in the responder roots of tomato in a split-root system, 18 days post bacteria inoculation per gram fresh root (n=10).

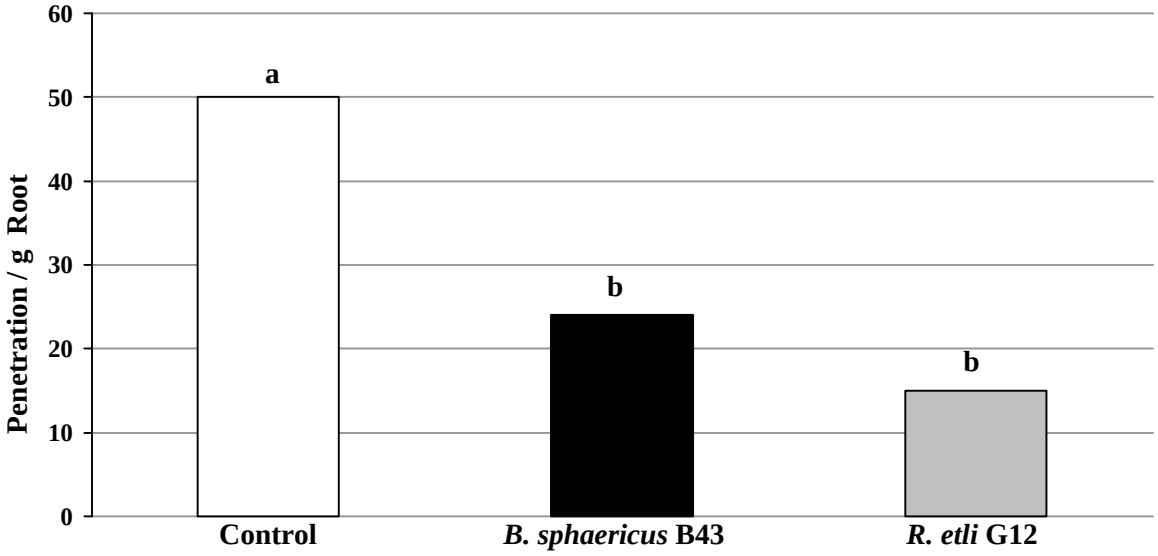


Fig. 8: Induced systemic effect of *Bacillus sphaericus* B43 or *Rhizobium etli* G12 inoculation on number of penetrated *Meloidogyne incognita* J2 in responder roots; 18 days post bacteria inoculation per gram fresh root (n=20).

Means with the same letter are not significantly different based on Fisher's least significant difference (LSD) procedure (P< 0.05).

3.1.2 Long Term Effect of Induced Systemic Resistance on Nematode Reproduction

In a split root experiment of identical set-up as described earlier, the long-term systemic effect of the two bacteria strains on *M. incognita* reproduction was evaluated. Eight weeks after inoculation with bacteria, galls and eggmasses per g fresh root and eggs per eggmass were evaluated. The root fresh weight was never significantly different between any of the treatments (data not shown).

Some treatments were infested with nematodes on both sides (inducer and responder sides) of the split root experiment, as shown in Fig. 1A. This was conducted to determine the direct and the indirect effects of the bacteria on the nematode reproduction in tomato roots and the effect of a combined inoculation in the inducer roots on nematode reproduction in the responder root. Compared to 25 females /g fresh root in the control plants, a combined *B. sphaericus* B43 and *M. incognita* treatment did not result in any significant effect on the female development, although less females were observed. A significant reduction in females /g fresh root was observed in the inducer roots where *R. etli* G12 and *M. incognita* were inoculated together. Here 14 females /g fresh root developed. In the responder roots of both nematode bacteria combination treatments, no significant reduction in females /g fresh root was noted (Fig. 9), although less females were counted than in control plants. In the control plants of this long term split root experiment, significantly less females developed compared to the “short term” split root experiments. However, the range of 14 to 20 developed females per g fresh root in bacteria treated plants remained stable compared to split root experiments evaluated 18 days after bacteria treatment (Fig. 6, Fig. 7, Fig. 8).

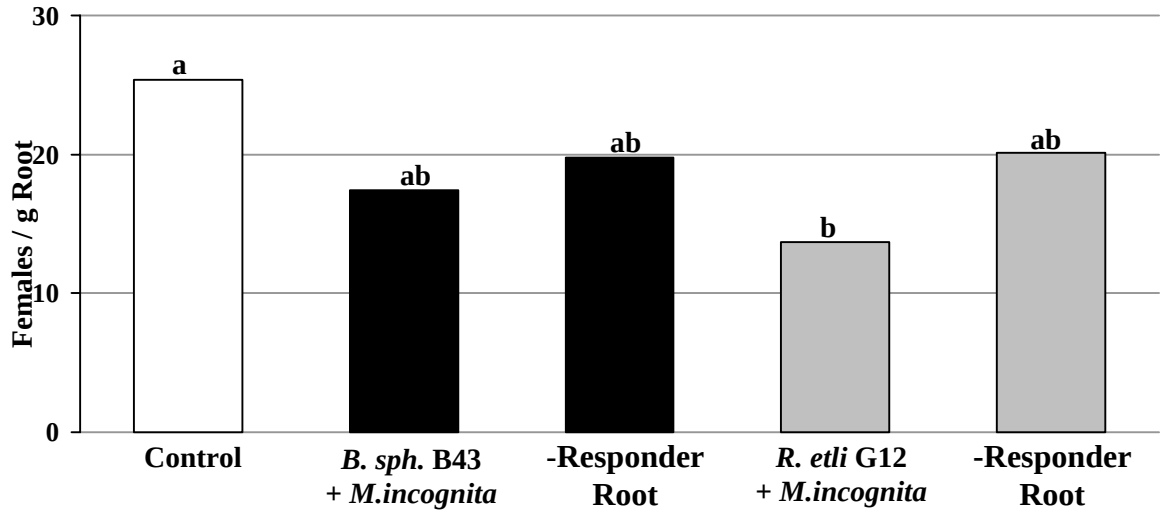


Fig. 9: Effect of inoculations with both *Bacillus sphaericus* B43 or *Rhizobium etli* G12 and *Meloidogyne incognita* on *M. incognita* female number compared to *M. incognita* infested control plants in split root set-ups.
Means with the same letter are not significantly different based on Fisher's least significant difference (LSD) procedure (P< 0.05, n=6).

The production of eggs per female egg mass was not significantly influenced by a combined bacterial and nematode treatment, neither directly nor indirectly (Fig. 10).

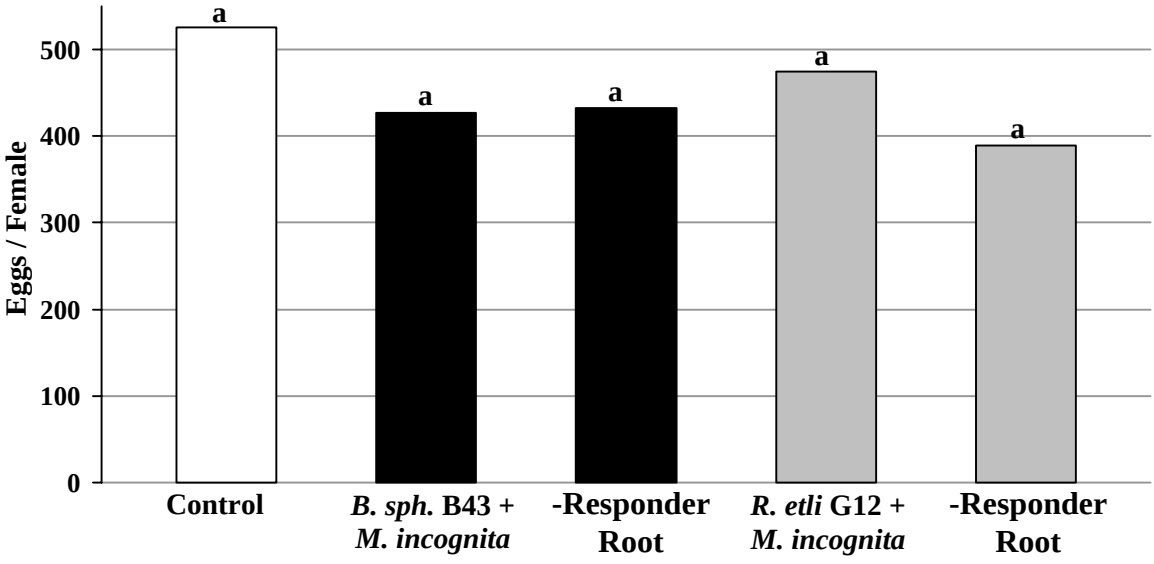


Fig. 10: Effect of inoculations with both *Bacillus sphaericus* B43 or *Rhizobium etli* G12 and *Meloidogyne incognita* on *M. incognita* females, in a split root set-up.
Means with the same letter are not significantly different based on Fisher's least significant difference (LSD) procedure (P< 0.05, n=6).

Another split-root experiment was conducted to elucidate the long term systemic induction effects of the bacteria treatments on nematode fecundity, therefore the inducer roots were treated with the bacteria and only the responder roots were infested with nematodes as described earlier.

No significant effect of the bacterial treatments was observed on female development per g fresh root compared with the control (data not shown). However, the production of eggs per female egg mass was significantly reduced in one bacteria treatment. Compared to the 526 eggs per female that developed in the roots of control plants, the 393 eggs per female that were produced in the responder roots of *B. sphaericus* B43 treated plants were not statistically different from the control nor from the *Rhizobium* treatment. However, there was a significant reduction in the quantity of eggs produced per female in the responder root of *R. etli* G12 treated plants. 337 eggs per female in the *Rhizobium* treated plants were counted (Fig. 11), which corresponds to 36 % fewer eggs per female compared to the control plants.

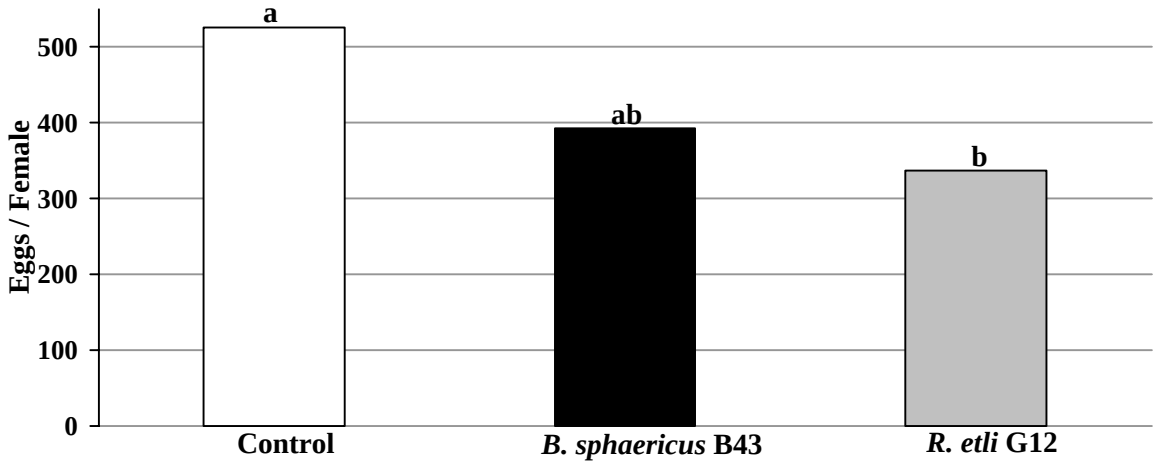


Fig. 11: Effect of *Bacillus sphaericus* B43 or *Rhizobium etli* G12 inoculation on the production of eggs per *Meloidogyne incognita* female.
Means with the same letter are not significantly different based on Fisher's least significant difference (LSD) procedure (P< 0.05, n=6).

In the same experiments, the influence of a combined bacterial and nematode treatment was studied on the number of nematode galls produced relative to the root fresh weight. In control plants 34 galls/g fresh root were counted. In the inducer root of the combined treatment with *B. sphaericus* B43 and *M. incognita*, a direct effect of the bacteria on the nematodes was observed, which lead to a significantly reduced number of galls/g fresh root compared to the control (31 % less galls). In the responder roots of the same treatment, 24 % less galls/g fresh root were produced, though this difference was not significantly different from the control. Neither was a direct effect of *R. etli* G12 on nematode reproduction observed, which would significantly reduce the number of *M. incognita* galls in the inducer roots, nor an indirect effect, which would lead to a significant reduction of galls in the responder roots (Fig. 12).

In treatments, where the systemic effect of the bacteria was studied, no significant difference could be observed in the responder root of the two bacteria treatments compared to the control neither for the root fresh weight (data not shown) nor for galls/g fresh root (Fig. 13).

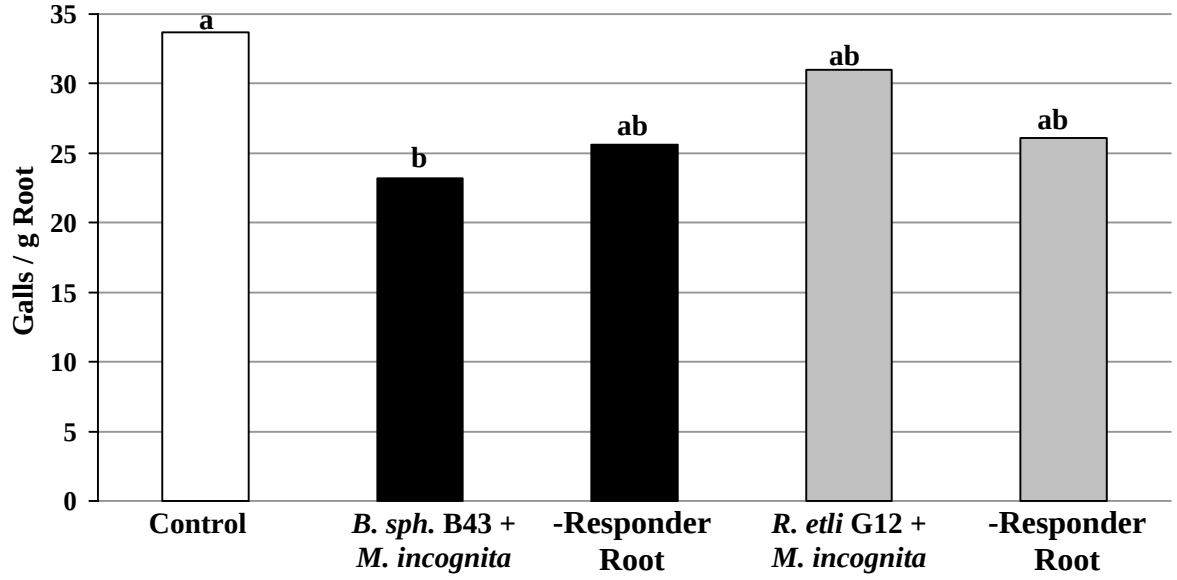


Fig. 12: Effect of bacteria and *Meloidogyne incognita* inoculations on gall numbers per gram fresh root, in split root set-ups.
Means with the same letter are not significantly different based on Fisher's least significant difference (LSD) procedure (P< 0.05, n=6).

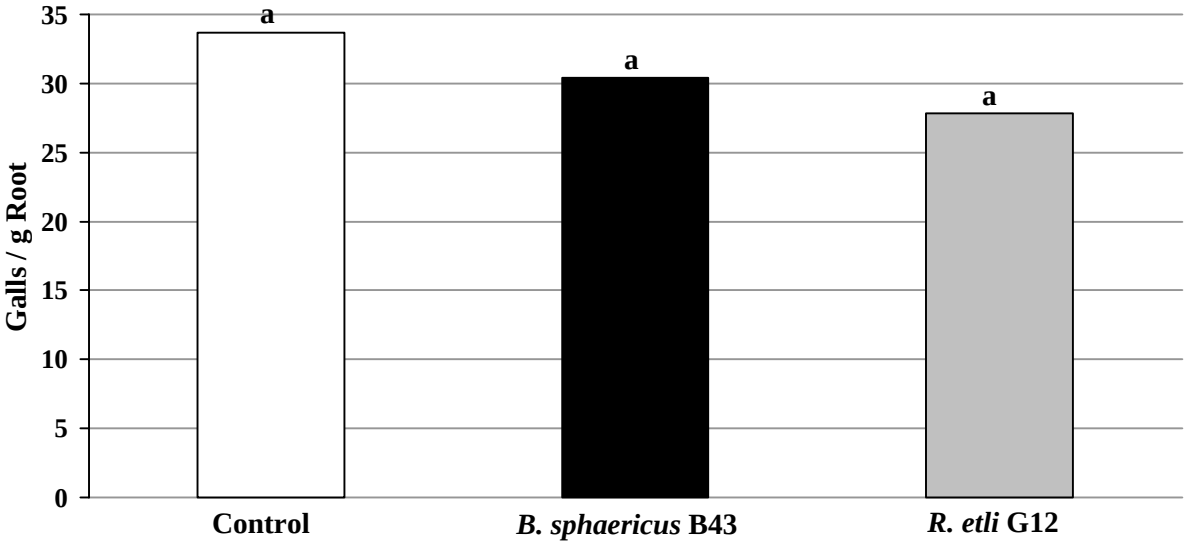


Fig. 13: Induced systemic effect of inoculations with either *Bacillus sphaericus* B43 or *Rhizobium etli* G12 on the number of galls produced per gram fresh root, in split root set-ups.
Means with the same letter are not significantly different based on Fisher's least significant difference (LSD) procedure (P< 0.05, n=6).

3.1.3 Harvesting Tomato Roots for RNA Extraction

In a split-root experiment inoculated according to Fig. 2, the roots were harvested three days after bacteria inoculation. There was no significantly difference observed in root fresh weight between the roots of bacteria treated plants compared to untreated plants (Fig. 14A). Six days after bacteria inoculation, which was three days after nematode inoculation, roots from bacteria and nematode treated plants and from control plants were harvested. Between the

different bacteria with nematode treatments, no significant difference could be observed in root fresh weight compared to the control plants (Fig. 14B).

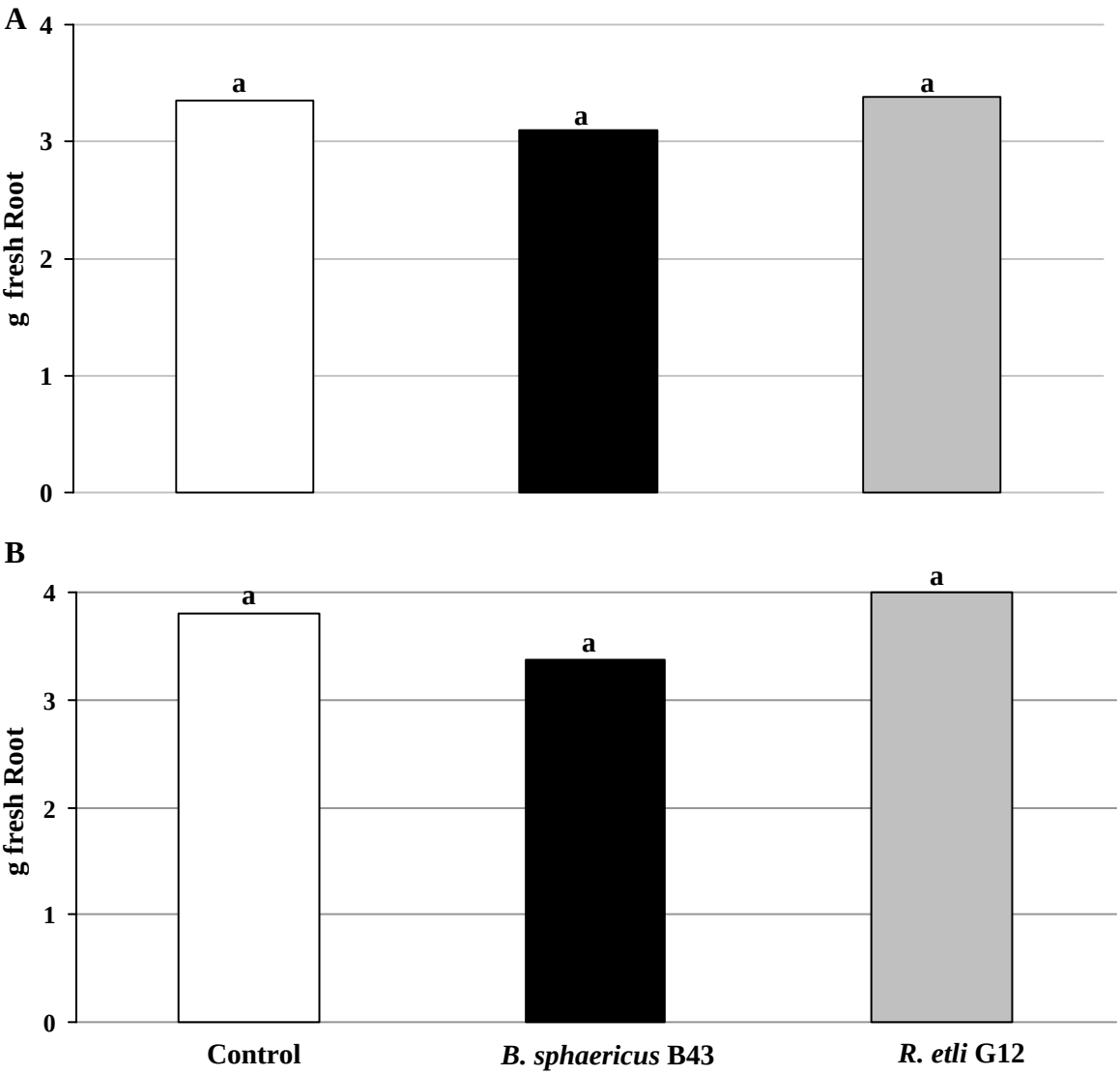


Fig. 14: Root fresh weight in gram of untreated control roots (white column) and of responder roots of *Bacillus sphaericus* B43 or of *Rhizobium etli* G12 treated tomato plants from split root set-ups
A: 3 days after bacteria inoculation. **B:** 6 days after bacteria inoculation (i.e. 3 days after nematode inoculation)

Means with the same letter are not significantly different based on Fisher's least significant difference (LSD) procedure ($P < 0.05$, $n=8$).

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3.2 Molecular Investigations

The previous experiments in the bioassay proved that the rhizobacteria, *Bacillus sphaericus* B43 and *Rhizobium etli* G12 induced a systemic resistance to *Meloidogyne incognita* juvenile penetration in tomato plants. The RNA from the roots of these plants was harvested three and six days after bacteria inoculation (Table 2) and used for the molecular studies regarding the differential gene regulation following rhizobacteria treatment.

3.2.1 Screening for Differentially Expressed Genes by Subtractive Suppressive Hybridization

With subtractive suppressive hybridization (SSH), two populations of mRNA/polyA+ RNA were compared in such a way that genes that were expressed in one population rhizobacteria alone or rhizobacteria with nematode induced plants, but not in the other untreated plants can be purified. After the blue/white selection, 24 potentially differentially expressed gene fragments were isolated. Of these 24 fragments, 14 fragments are believed to be present in the first independent SSH, in the 3 day post treatment plants (untreated : rhizobacteria induced), the other 10 clones came from the second independent SSH, which was performed on 6 day post treatment plants (untreated : rhizobacteria with nematode induced plants).

3.2.2 Restriction Enzyme Digestion

The fragments isolated from the subtractive suppressive hybridization (SSH) were cloned in a pCR 2.1 TOPO plasmid. With *Eco*RI, the inserted DNA fragment was isolated from the plasmid and further characterized by restriction site analysis. To do so, the inserts were digested with five restriction enzymes and visualized on an agarose gel in order to classify them according to Sambrook *et al.* (1989). The sizes of the resulting DNA fragments were estimated.

Based on the results of the restriction fragment length comparison with *Bam*HI, *Hind*III, *Xba*I and *Xho*I or the combinations with *Eco*RI, the fragments were mapped and resulted in 24 unique fragments. Of the first 14 SSH clones, ten clones were sent for sequencing, consisting of eight unique clones, plus one clone from a two-clone group with the same insert and one clone of a four-clone group with the same insert. Of the second set of SSH clones with 11 clones, nine clones were sent for sequencing, as two had the same insert. In total 19 SSH clones were sent for sequencing.

3.2.3 Correlation of the Sequences

The resulting sequences were screened for homologies by *Blast* analysis with known sequences in the *NCBI* database. Nine sequences of the clones were identified to be originating from plant (Table 8).

Table 8: Clone Correlation in Order of Sequence Length

Clone No.	Length bp	Correlation Blast x at *NCBI*, 02/08/2006
1-B1-M13	900	Probably Vector
1-B1-T7	957	Probably Vector
1-8-M13	802	Probably Vector
1-8-T7	20	Probably Vector
1-2-M13	658	None
1-1-T7	517	None
1-5-M13	372	*Pisum sativum* PSI Light-Harvesting Antenna Chlorophyll a/b-Binding Protein (lhcA-P4) mRNA; *Arabidopsis thaliana* mRNA for Mitochondrial half-ABC Transporter (*STA*1 Gene)
1-9-M13	320	*Lycopersicon esculentum* Clone 134156F, mRNA Sequence;
1-9-T7	320	*Lycopersicon esculentum* Clone 134156F, mRNA Sequence;
1-6-M13	306	Putative High-Affinity Nitrate Transporter (*Lycopersicon esculentum*),
1-7-M13	271	Putative High-Affinity Nitrate Transporter (*Lycopersicon esculentum*) Same as clone 1-6-M13
1-4-T7	266	*Solanum demissum* Chromosome 5 Clone PGEC407 Map MAP_LOC, Complete Sequence
1-4-M13	266	*Solanum demissum* Chromosome 5 Clone PGEC407 Map MAP_LOC, Complete Sequence
1-3-T7	103	*Lycopersicon esculentum* Polygalacturonase Isoenzyme 1 beta Subunit Gene; Tomato Polygalacturonase Isoenzyme 1 beta Subunit mRNA
2-5-M13	587	Probably Vector
2-5-T7	946	Probably Vector
2-B1-M13	750	*Lycopersicon esculentum* Phenylalanine Ammonia-Lyase (*PAL*5) Gene
2-B1-T7	564	*Lycopersicon esculentum* Phenylalanine Ammonia-Lyase (*PAL*5) Gene
2-2-M13	950	Probably Vector
2-2-T7	120	Probably Vector
2-6-M13	449	None
2-6-T7	469	None
2-7-M13	788	*Solanum demissum* Chromosome 11 BAC PGEC513 Genomic Sequence; *Solanum bulbocastanum* Chromosome 8 Clone 177O13; Potato Wound-Induced Genes *WIN*1 and *WIN*2; *Lycopersicon pimpinellifolium* 9DC Resistance Gene Cluster; *Lycopersicon esculentum* Chromosome 0 Clone BB-BA4 Map MAP_LOC
2-7-T7	15	None
2-1-M13	680	*Lycopersicon esculentum* LE-ACO4 mRNA for 1-Aminocyclopropane-1-Carboxylate Oxidase
2-1-T7	64	None

Number code of clones: 1-. First SSH, plants of day 3; 2-. Second SSH, plants of day 6 post bacteria treatment

Of these, three showed homology with pathogen response related genes, one to a transporter gene and three others to genomic DNA of *Lycopersicon* or *Solanum* or *Pisum.* Six clones

resulted from the first SSH with the 3 days post treatment plants (untreated : rhizobacteria induced) and three clones from the second SSH with the 6 days post treatment plants (untreated : rhizobacteria with nematode induced plants).

3.2.4 Expression Analysis of the Cloned Gene Fragments

To verify the differential expression of the cloned gene fragments, various expression analysis studies were performed using RNA of untreated and bacteria treated plants harvested at day 3 and day 6, and by northern analysis using different detection methods (Table 2).

3.2.4.1 Analysis of Differential Expression of Cloned Gene Fragments by RNA/RNA-Hybridization for DIG Chemiluminescence Detection

The clones were transcribed *in vitro* into DIG-labelled RNA as this detection is considered to be more sensitive than a DNA/RNA-hybridization.

The result of a DIG-labelled DNA fragment and hybridization with total-RNA of two different tomato tissues is shown in Fig. 15.

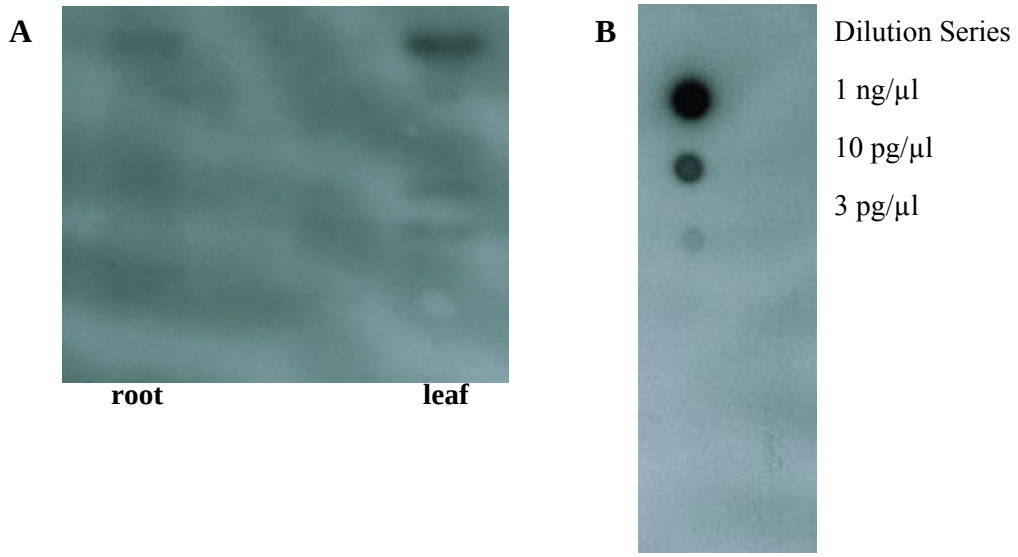

Fig. 15: (**A**) Visualized results of the hybridization test with a DIG-labelled Glucose-6-phosphate-dehydrogenase (*G_6PDH*) and total RNA of untreated tomato root at the left and leaf at the right. (**B**) Dilution series of DIG-labelled *G_6PDH* (E.C. 1.1.1.49).

As a test, a Glucose-6-phosphate-dehydrogenase gene fragment of potato (E.C. 1.1.1.49) was labelled with DIG. Therefore, the DNA fragment for *G6PDH* was ligated into a *PBSK*-plasmid, making it possible for the T_7-RNA-polymerase to attach and perform the transcriptional labelling. To test the detection limit, a dot experiment was performed according to the manufacturer's instructions. The visible three dots on the test dilution series represented an inadequate amount of labelled RNA probe. On the northern blots with RNA of tomato root and leaf, the *G_6PH*, was detected as slightly visible bands in both cases. There

was a higher expression of *G_6PH* detected in the tomato leaf than in the root. As the amount of RNA, which was transferred onto the blot, was not quantified, it is possible that not enough RNA had been cross-linked to the blot.

Limited by either the labelling method or the inadequate amount of RNA, the chemiluminescent method was not practical for the detection of the SSH-clones or to verify whether or not they were differentially expressed.

3.2.4.2 Analysis of Differential Expression of Cloned Gene Fragments by DNA/RNA-Hybridization for Autoradiography Detection

A second approach to verify the potential differential expressed SSH clones was performed. Here the clones were radioactive-labelled and then hybridized with the fractionated total-RNA or polyA+ RNA of day 3 and day 6 plants (Table 2).

The detection of the radioactive-labelled clones was performed as described in the materials and methods chapter. The blots were sequentially hybridized with the different probes. At first, a polygalacturonase (PG) probe was used which was obtained from a PCR product that had been amplified using the PG-forw and -rev primer pair (Table 7) from the polygalcturonase cDNA fragment shown in the appendix. Additionally, a phenylalanine ammonia-lyase (PAL) probe was used, which was also obtained from a PCR product, that had been amplified with a PAL-forw and -rev primer pair (Table 7) from the PAL cDNA fragment shown in the appendix. As a reference for RNA loading, an ubiquitin fragment (UBI) was hybridized in the end, which was then amplified with the UBI-for and -rev primer pair (Table 7). Ubiquitin is a common housekeeping gene that acts as an internal standard in an RNA Blot (Northern Blot) or RT-PCR (Goncalves *et al.* 2005, Oka *et al*. 2001, Zhu and Altmann 2005).

The hybridization of the RNA blots with the different DNA probes showed transcripts of these genes in tomato roots, both in bacteria treated and untreated plants (Fig. 16). The expression level of the polygalcturonase gene transcripts (PG) was at the detection limit so that variations in the expression level between the different treatments were difficult to detect. There seemed to be a lower expression level of PG with a *R. etli* G12 treatment at day 3 and day 6. The expression of the reference transcript, ubiquitin (UBI) was nearly equal to that of *R. etli* G12 treated plants of day 3 and untreated plants. From day 6 plants with the same treatment, the UBI transcript expression was only slightly higher while the PG transcript expression seemed to be lower compared to the day 3 plants. A hybridization with labelled

PAL probes revealed only minor differences. At day 3, the *B. sphaericus* B43 treated plants seemed to express relatively higher amounts of PAL transcripts than an equally the same aged, untreated plant. However, the reference transcript expression in B43 treated plants was also higher compared to the untreated plants, so that the effect could not be clearly detected. Three days later, at day 6, the PAL transcript seemed to be expressed more strongly in *B. sphaericus* B43 and *R. etli* G12 treated plants, compared to the control. The UBI transcript expression, as the reference, of both treatments was nearly the same.

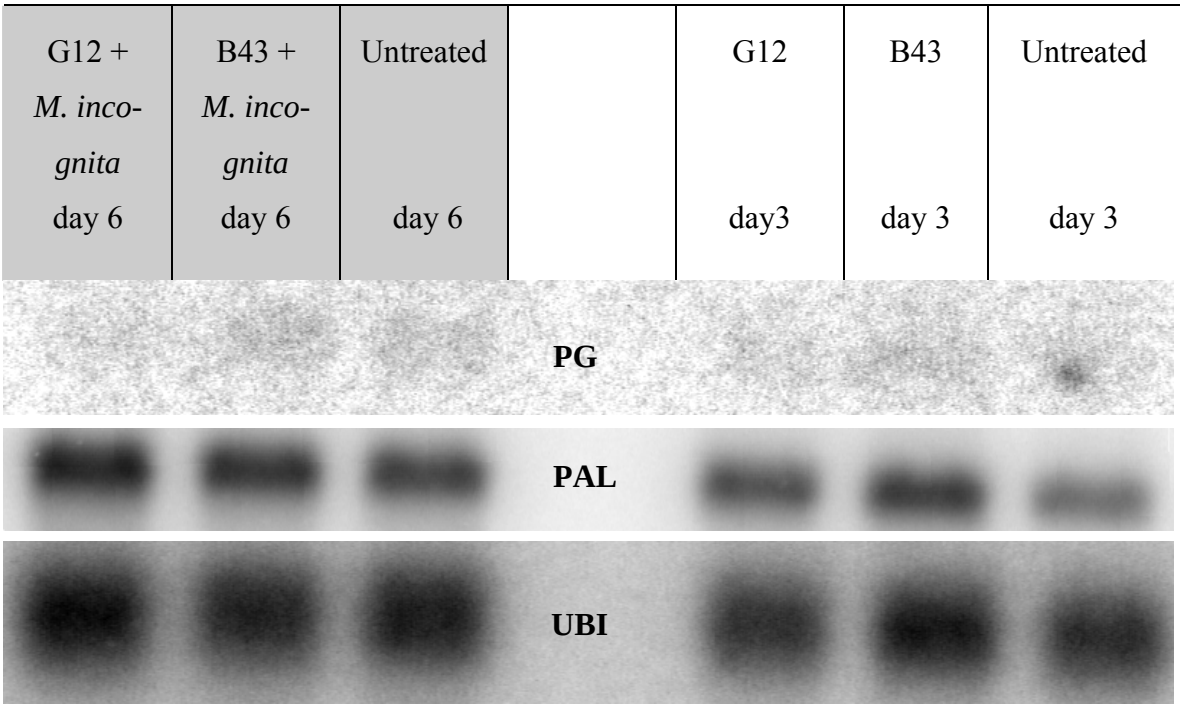

Fig. 16: DNA/RNA Blots with mRNA produced from 250 µg total RNA of 9 individual plants per treatment, hybridized with labelled PG, PAL and UBI fragments. The phosphor image was exposed to the blot for 5 days.

An identical repetition is shown in Fig. 17. All probes were expressed in all treatments at both day 3 and 6. The transcripts of the PG fragment were difficult to detect. The differences between the treatments were even less obvious. There seemed to be a lower expression at day 3 with a G12 treatment compared to the untreated plant. At day 6, no clear differences were observed.

At day 3, the PAL transcript expression was higher in the B43 treated plant than in the untreated plant of the same day, and the reference expression in the B43 treated plant seemed to be even lower than in the control plant. The PAL expression with G12 treated plants of day 3 was slightly lower than in B43 treated plant, but compared to the untreated plant, it was higher. The UBI transcript expression as reference of the G12 treatment was lower than of the

untreated plant. At day 6, no clear difference was observed between the bacteria treatments and the control.

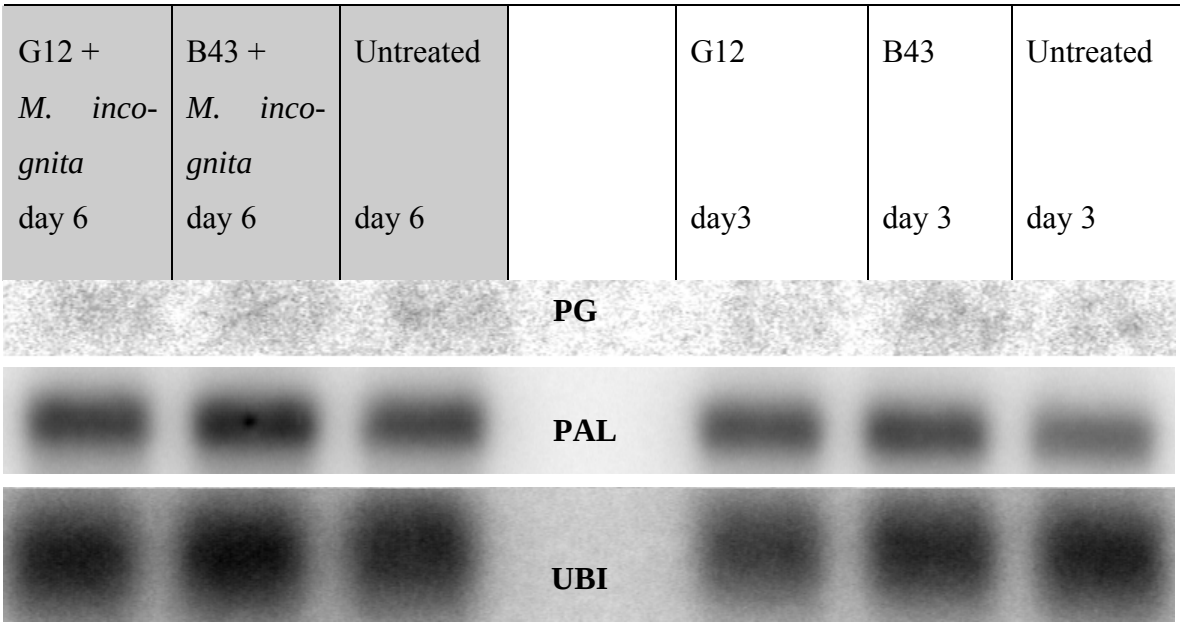

Fig. 17: DNA/RNA Blots with mRNA produced from 250 µg total RNA of 9 individual plants per treatment, hybridized with labelled PG, PAL and UBI fragments. The phosphor image was exposed to the blot for 5 days.

As the results of both DNA/RNA blots were either contradictory or not conclusive, a third method was pursued to determine the different transcript expression levels of the tested gene fragments.

3.2.4.3 Analysis of Differential Expression of Cloned Gene Fragments by Semiquantitative RT-PCR

A sensitive and relatively fast method to determine gene expression levels is qRT-PCR. After transcribing RNA into single strand cDNA, and constructing efficient primer pairs, the most difficult aspect was to obtain quantitative results. Many sources of variations exist, including template concentration and amplification efficiency. Therefore the gene of interest is co-amplified together with an adequate internal control, so that it can be quantified relative to the internal control. Known as semi quantitative RT-PCR (Gause and Adamovicz 1995).

The RNA of individual plants was transcribed into single strand cDNA and used as a template for the semiquantitative RT-PCR. This was the same RNA used for the mRNA extraction and the RNA Blot analysis. Then the adequate cycle number was determined for a co-amplification of the primer pairs of the internal control and the genes of interest.

First, the proper cycle number to be used for qRT-PCR for each primer pair was determined.

3 Results

The amplification of both the gene of interest and internal control must be performed in the linear range before reaction components become limiting. Therefore, the templates were amplified at three different PCR cycle numbers first with the primer pair for the indicated gene. After the PCR reaction was performed, the PCR products were analyzed by agarose gel electrophoresis (Fig. 18).

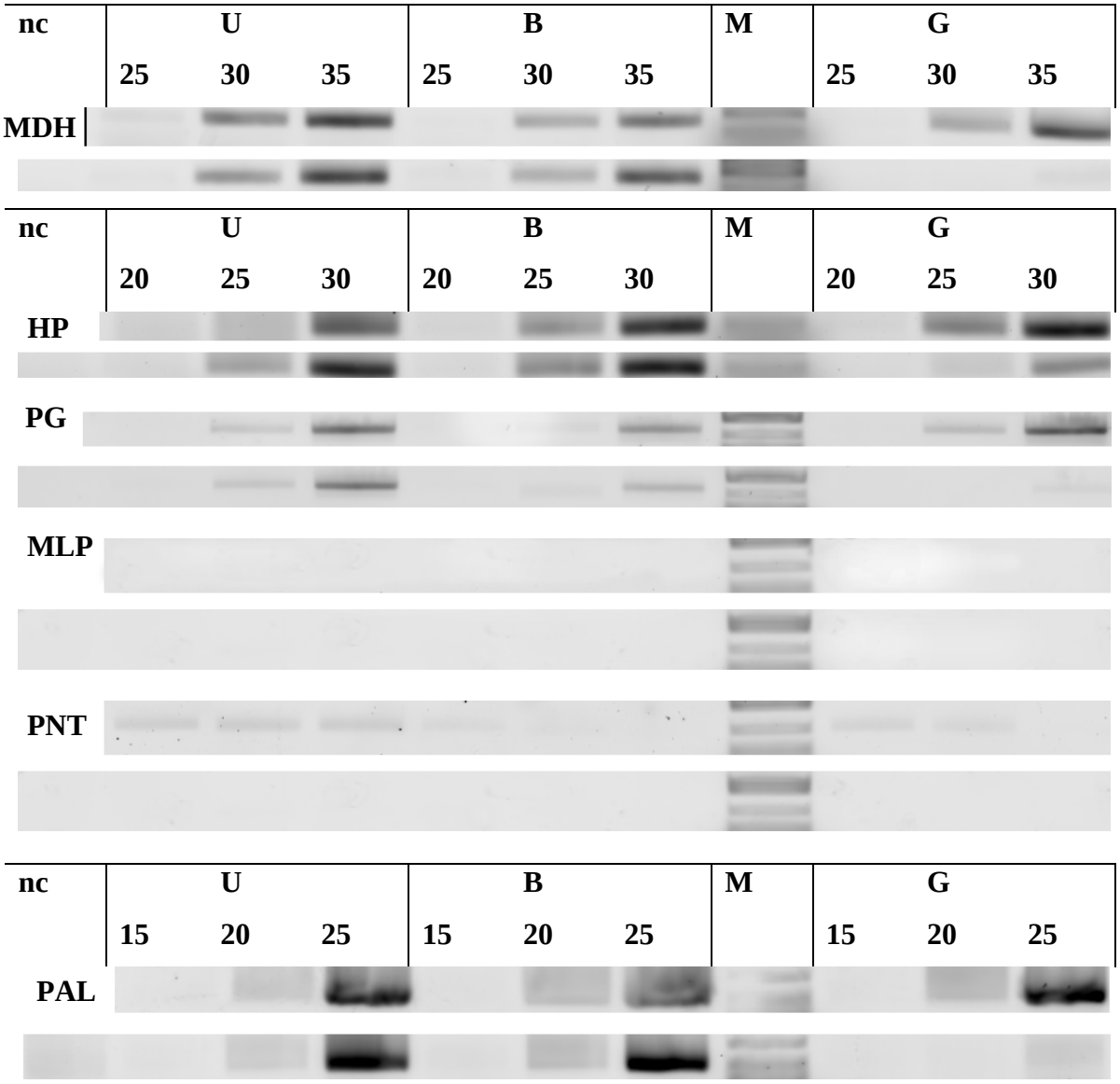

Fig. 18: Scanned gels with the PCR products of the listed primer pairs at the listed cycle numbers. Upper gel slice shows cDNA of day 3 plants, lower slice of day 6 plants.

U: cDNA of control, untreated plants; B: cDNA of *B. sphaericus* B43 treated plants; G: cDNA of *R. etli* G12 treated plants; nc: negative control, PCR Master mix without cDNA

The cycle number was chosen so that the PCR product of each primer pair was amplified in the linear range. For the MDH primer this was determined at to be 30 cycles, for HP and PG, 25 cycles were sufficient. The primer pairs of MLP and PNT did not amplify any product in detectable amounts. The PAL primer pair amplified at around 20 cycles in the linear range.

3 Results

The MDH gene had to be used as internal standard, which means it should be co-amplified together with the primer pairs of the target genes in the linear range. However, most primer pairs were amplified in a linear range in at least 5 cycles less than the MDH.

As MDH did not amplify in the linear range of the gene of interests, another internal standard was chosen. A primer pair for ubiquitin (UBI), which had also been used as reference in the northern blotting, was created and tested at the same cycle numbers as PAL, since PAL was amplified in the linear range with the smallest cycle numbers compared to the other primers. The results of the cycle number test for UBI are shown in Fig. 19.

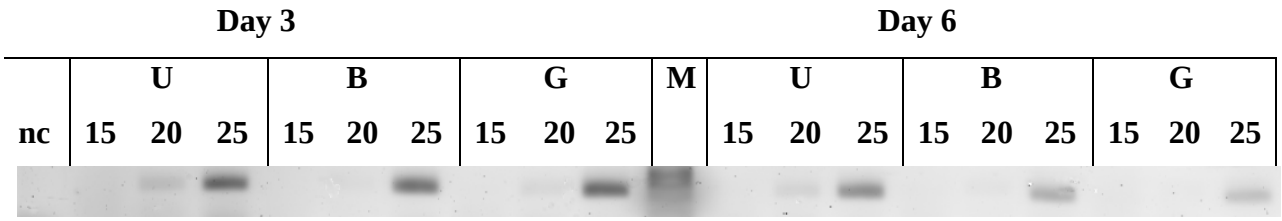

Fig. 19: Results of three different cycle numbers tested for optimal results with cDNA of each treatment, amplified with an ubiquitin primer pair. Left of the molecular weight marker (M) are shown cDNA of day 3 plants and right of M, cDNA of day 6 plants.

U: cDNA of control, untreated plants; B: cDNA of *B. sphaericus* B43 treated plants; G: cDNA of *R. etli* G12 treated plants; nc: negative control, PCR Master mix without cDNA; M: molecular weight marker (λ/PstI 24, Fermentas)

The linear range for UBI amplification was between 20 and 25 cycles, and therefore suitable for a co-amplification with the transcripts of the genes to be tested.

Next, a first co-amplification was performed with the primer pair UBI as internal standard and the primer pair PG to find the best cycle number for a co-amplification. The cDNA of day 3 plants was used. At two different cycle numbers, 30 and 35 cycles, the co-amplification was performed. No detectable amount of PG PCR product was produced at 30 cycles (Fig. 20). Only at 35 cycles was a PCR product visible. There was clearly more PCR product produced with a B43 treatment and less with a G12 treatment. The amount of produced PCR products was quantified with the *Image Quant* Software. The PG transcript PCR products were normalized for the UBI fragments PCR products expression levels. The expression level of the PCR product of the untreated control plant was classified as 100 % expression, assuming that it had been unchanged of any treatment. The transcript expression level of fragments resulting from bacteria treated plants was shown as percentages of the untreated expression level. The PG transcript expression of the B43 treated plant was up-regulated by 174 % and that of the G12 treated plant was down-regulated by 64 % respectively, compared to the

untreated.

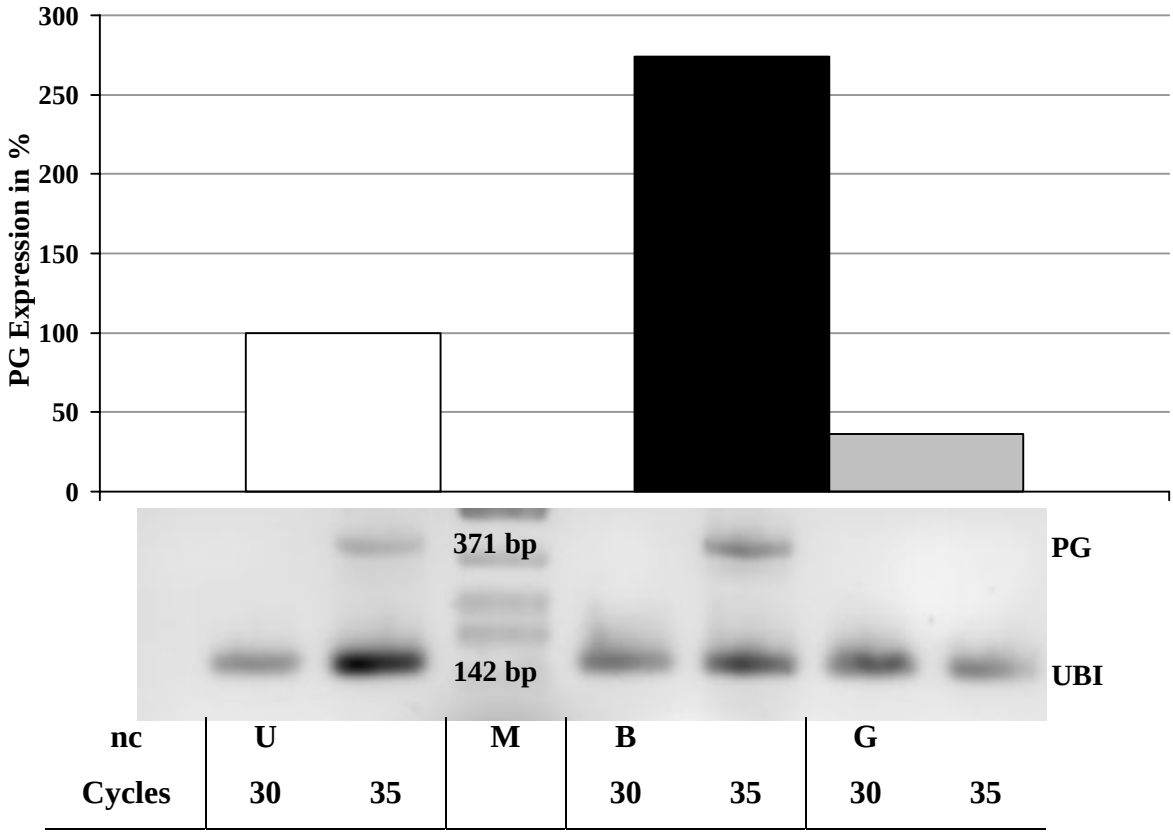

Fig. 20: Graphic of the quantified and normalized expression levels of PG at 35 cycles, underneath the visualized co-amplification of UBI and PG primer pairs with 1 µl cDNA of day 3 plants at 30 and 35 cycles.

cDNA of U: untreated; B: *Bacillus sphaericus* B43 treated; G: *Rhizobium etli* G12 treated plant; nc: negative control, master mix without cDNA; M: molecular weight marker (λ/PstI 24, Fermentas)

This PCR set-up was repeated and after scanning the gel, the amount of the PCR products was again quantified with the *Image Quant* Software. The amount of the PCR product of the untreated control plants was classified as 100 % expression, assuming that it had been unchanged of any treatment and referred to as 'normal'. The expression levels of bacteria treated plants were shown as percentages of the untreated expression (Fig. 21). There were clearly less transcripts of the PG quantified in both plant sets of plants treated with *R. etli* G12. In the first plant set, the G12 treated plant produced 23 % and in the second plant set 57 % of the PG transcripts compared to untreated plants.

The *B. sphaericus* B43 treatment did not show a clear effect on the expression level of PG transcripts. With the first plant set clearly less transcripts were observed, but in the second plant set, slightly more were observed.

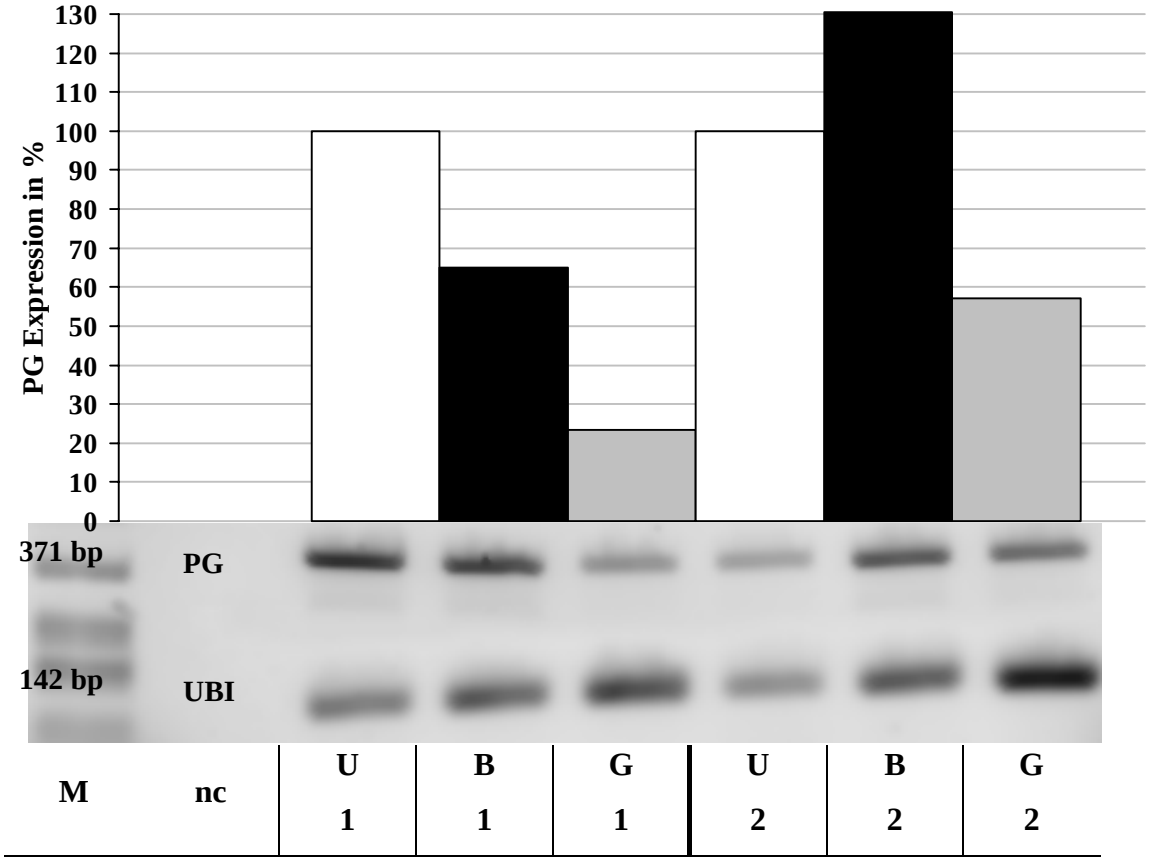

Fig. 21: Graphics of normalized quantification and visualized co-amplification of PG (upper line) and UBI (lower line) with cDNA templates of two day 3 plant sets (1, 2).

cDNA of U: untreated; B: *Bacillus sphaericus* B43 treated; G: *Rhizobium etli* G12 treated plant; M: molecular weight marker (λ/PstI 24, Fermentas); nc: negative control, master mix without cDNA

A third plant set of day 3 plants was taken to produce cDNA. Then the cDNAs of all three plant-sets of day 3 plants were used with the same set-up as for the two plant sets earlier for a co-amplification of UBI and PG primer pairs. The visualized result of these PCR reactions and their quantification is shown in Fig. 22. Less transcripts of the PG gene was observed for all G12 treated plants of all three plant sets, all reproducible at around 50 % compared to untreated plants. The amount of produced PG transcripts in B43 treated plants was not stable. Two plants showed less whereas one plant (2) showed about 50 % more than in untreated plants.

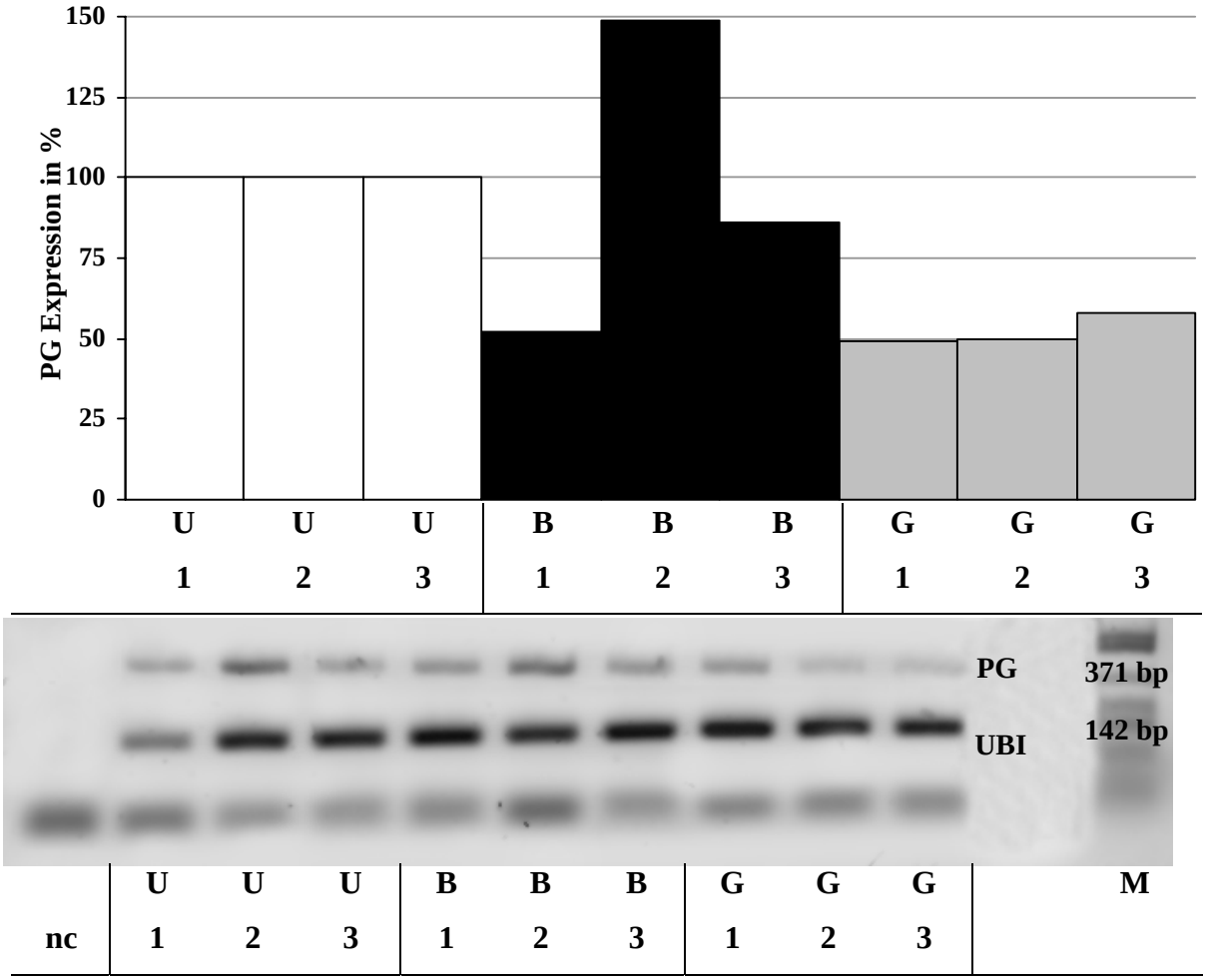

Fig. 22: Grouped graphics of normalized expression level quantification and visualized co-amplification of PG (upper line) and UBI (lower line) with cDNA templates of three day 3 plant sets (1, 2, 3).
cDNA of U: untreated; B: *Bacillus sphaericus* B43 treated; G: *Rhizobium etli* G12 treated plant; M: molecular weight marker (λ/PstI 24, Fermentas); nc: negative control, master mix without cDNA

In a second trial, RNA of 6 or 9 individual plants was pooled for each treatment to obtain more total transcripts. After reverse transcription, the cDNA was controlled on an agarose gel for quality and amount. Then the cDNA was used for qRT-PCR with the same cycle number as with the cDNA of individual plants in earlier experiments.

The visualized results of the PCRs with cDNA templates of day 3 and day 6 plants at 28 and 25 cycles are shown in Fig. 23 and Fig. 24, respectively. The graphics represent the normalized expression levels of PG transcripts under the assumption that untreated plants of day 3 show 100 % non-changed expression of PG transcripts. There were less PG transcripts observed in B43 treated day 3 plants and more in G12 treated day 3 plants. The greater and lesser transcription level of bacteria treated day 3 plants was about 30 % more or less than that in the untreated plants. The transcription level of bacteria treated day 6 plants showed only

minor changes compared to untreated plants of the same day.

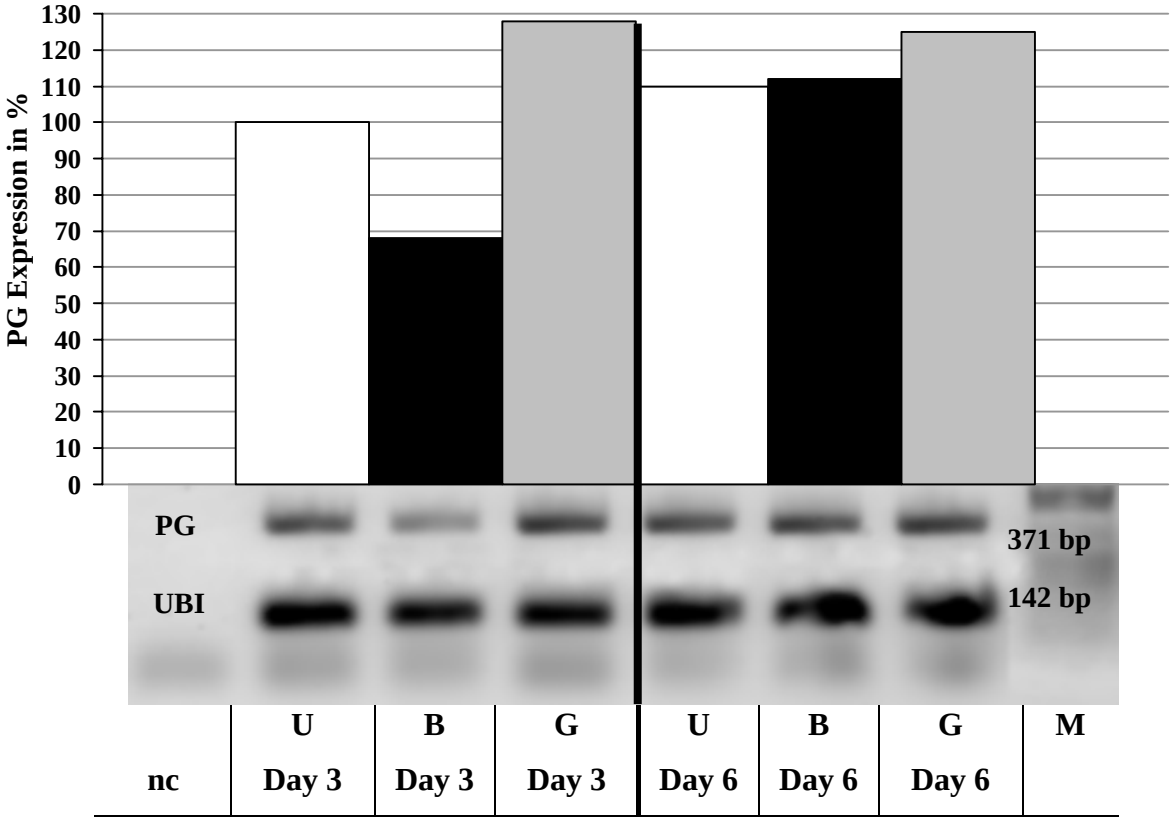

Fig. 23: Graphics of normalized expression level of PG and visualized co-amplification of PG (upper line) and UBI (lower line) with cDNA templates of day 3 plants and day 6 plants with 28 cycles.

cDNA of U: untreated; B: *Bacillus sphaericus* B43 treated; G: *Rhizobium etli* G12 treated plant; M: molecular weight marker (λ/PstI 24, Fermentas); nc: negative control, master mix without cDNA

These PCR reactions were repeated, but only with 25 cycles (Fig. 24). After normalization the bacteria treated day 3 plants showed a lower PG transcription level than untreated plants. B43 treated day 3 plants showed about 60 % and G12 treated day 3 plants about 80 % less expression of PG transcripts compared to untreated plants of the same day. About 60 % decreased expression of PG transcripts in untreated day 6 plants was noted, whereas the bacteria treated day 6 plants showed more, up to about 20 % more, compared to untreated day 3 plants.

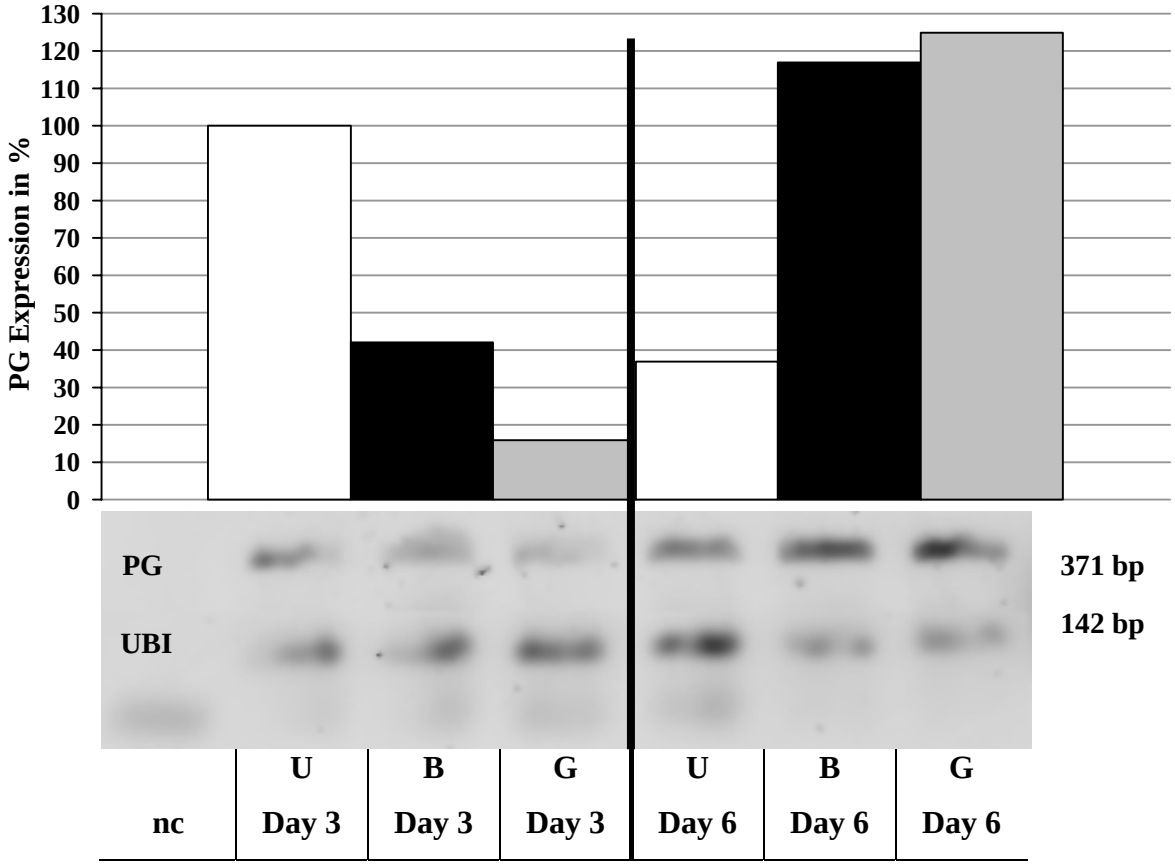

Fig. 24: Graphics of normalized expression level of PG and visualized co-amplification of PG (upper line) and UBI (lower line) with cDNA templates of day 3 plants and day 6 plants with 25 cycles.

cDNA of U: untreated; B: *Bacillus sphaericus* B43 treated; G: *Rhizobium etli* G12 treated plant; M: molecular weight marker (λ/PstI 24, Fermentas); nc: negative control, master mix without cDNA

The PCR-set-up as described earlier was also used for co-amplifications of primers for the PAL gene with the UBI primers and PNT primers with UBI primers. A B43 treatment resulted in a higher amount of detected PAL transcripts in day 3 plant with about 30 % and day 6 plants with about 50 % (Fig. 25). A G12 treatment did not change the expression level of PAL transcripts in both day-sets.

The PNT transcript was about 60 % less detectable in B43 treated day 3 plants and 33 % more three days later (Fig. 26). There were slightly differences in the expression level of PNT transcripts in both day sets with the cDNA of G12 treated plants.

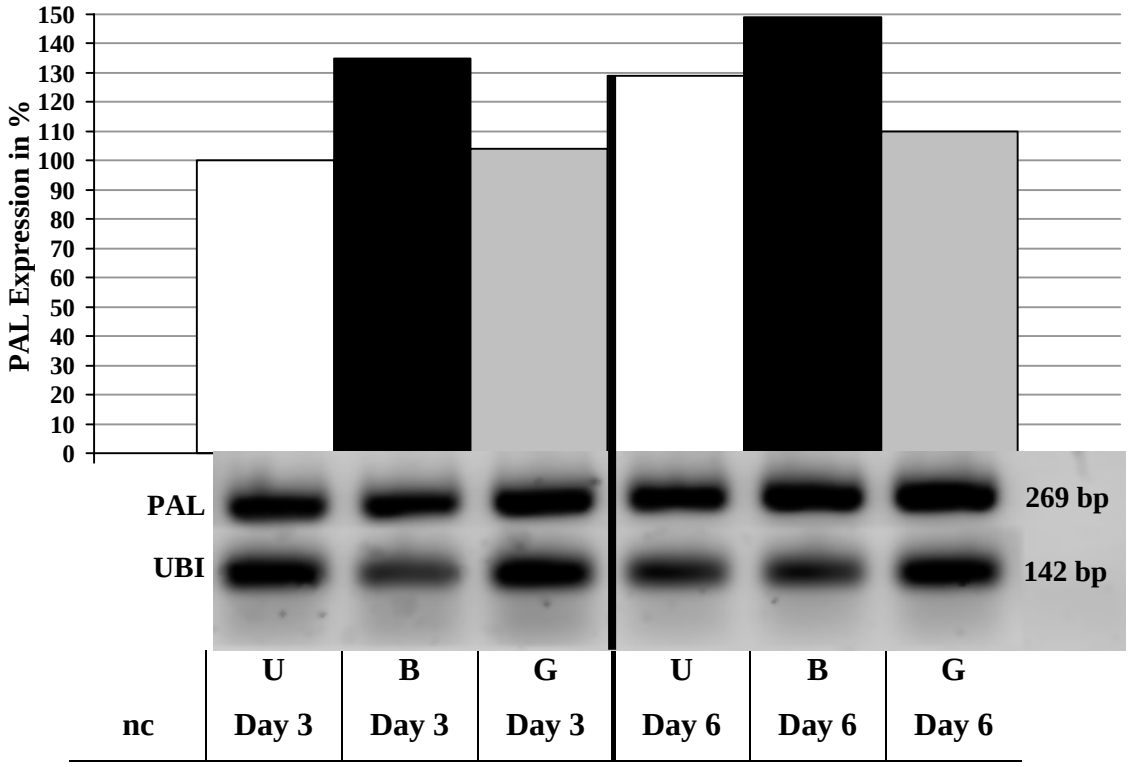

Fig. 25: Visualized co-amplification of PAL (upper line) and UBI (lower line) with cDNA templates of day 3 plants and day 6 plants with 25 cycles.

cDNA of U: untreated; B: *Bacillus sphaericus* B43 treated; G: *Rhizobium etli* G12 treated plant; M: molecular weight marker (λ/PstI 24, Fermentas); nc: negative control, master mix without cDNA

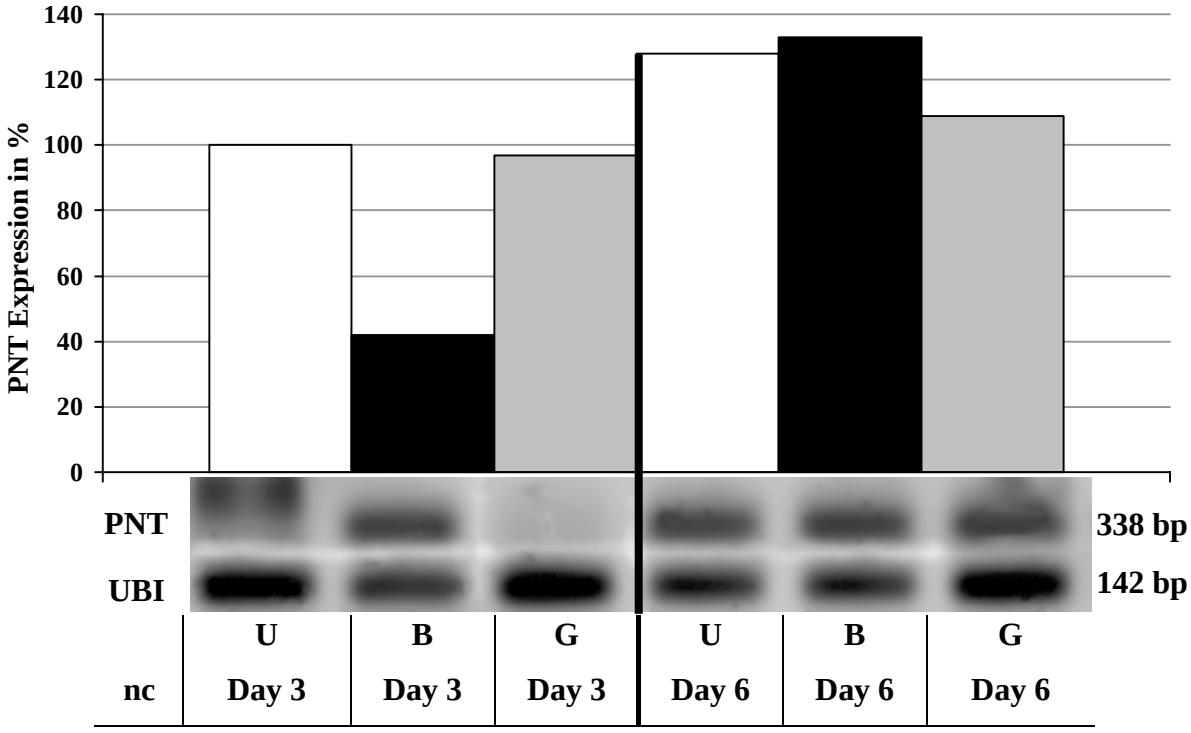

Fig. 26: Visualized co-amplification of PNT (upper line) and UBI (lower line) with cDNA templates of day 3 plants and day 6 plants with 25 cycles.

cDNA of U: untreated; B: *Bacillus sphaericus* B43 treated; G: *Rhizobium etli* G12 treated plant; M: molecular weight marker (λ/PstI 24, Fermentas); nc: negative control, master mix without cDNA

The co-amplifications with the other primer pairs listed in Table 7 did not give any detectable differential transcript expression or there were no co-amplification products detectable (data not shown).

The various studies undertaken to verify a differential expression of the cloned gene fragments, finally showed that:

- Only with qRT-PCR were differential transcript levels clearly and reproducibly detectable.
- In the cDNA of *Rhizobium etli* G12 treated tomato plants harvested at day 3, transcripts of the PG gene fragment were reproducibly detected in lower amounts compared to untreated plants of the harvest day.
- Therefore it was concluded, that the PG gene fragments is obtained from *Rhizobium etli* G12 treated tomato plants harvested at day 3.
- The transcripts of the PAL gene were reproducibly detected in higher amounts in *Bacillus sphaericus* B43 treated tomato plants harvested at day 3 and day 6.
- Therefore PAL could not be clearly assigned to either a day 3 or a day 6 B43 treated tomato plant.

4 Discussion

Knowledge on understanding the plant's reaction to inoculation with rhizobacteria and how this *in planta* reaction induces systemic resistance to nematodes is not yet fully understood. However, it could help improve the use of rhizobacteria for biological control or even to improve breeding for resistance, as the plant genes responsible for induced resistance could be identified and possibly used to protect the plant permanently.

The aims of these investigations were to extend the knowledge of this *in planta* reaction, which results in a systemic resistance induction by rhizobacteria against nematodes. Two rhizobacteria *Bacillus sphaericus* B43 and *Rhizobium etli* G12 were used. Both have already shown an ability to induce systemic resistance against different soil borne pathogens. In 1995, Hasky-Günther and Sikora first reported that these two bacteria strains induced systemic resistance to the potato cyst nematode, *Globodera pallida*, in potato. In later studies with tomato plants, the strain *B. sphaericus* B43 was shown to also induce systemic resistance to the tomato wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici* and the root knot-nematode *Meloidogyne incognita* (Mwangi 2002, Hauschild *et al.* 2000). *R. etli* G12, when tested on tomato, did not induce systemic resistance against the tomato wilt pathogen (Hauschild *et al.* 2001), but did induce resistance against the root-knot nematode (Hauschild *et al.* 2004).

To study differential gene expression following systemic resistance induction in tomato, a reliable system to prove that resistance had been induced in experimental plants had to first be designed, as no morphological changes were observed in such plants in previous studies (Mwangi 2002). Next, convenient methods had to be tested for the detection of the transcripts of the differential gene expressions during rhizobacteria induced systemic resistance.

4.1 Bioassay – Split-Root

Split-root experiments were conducted to confirm that the roots later used for differential gene expression analysis were obtained from tomato plants where rhizobacteria had in fact induced systemic resistance to the root-knot nematode *M. incognita*. For this, tomato plants were grown in a modified split-root system (Dorhout *et al.* 1988, Fuchs *et al.* 1997), with a split detached stem of tomato shoots re-rooted in separated pots as shown in Fig. 1A, B and Fig. 2 (Mwangi 2002).

4 Discussion

In the trial, two different rhizobacteria strains were choosen as inducers of systemic resistance, both with known potential to control nematodes. It is known, that rhizobacteria can have a beneficial impact on a plant's health by directly or indirectly affecting soil borne pathogens (Cook and Baker 1983). The bacteria produce antibiotics and siderophores, which antagonize phytopathogenic fungi and bacteria (Davison 1988). Another mechanism by which rhizosphere bacteria can protect plants against soilborne pathogens is through competition for space and nutrients available in the rhizosphere or even by colonizing the same biological niche as the pathogen through endophytic growth inside the plant (De Leij *et al.* 1995, Handelsman and Stabb 1996). An endorhiza niche is advantageous for both organisms, with bacteria being protected within plant tissues and therefor encountering less competition with other rhizosphere microorganisms. In addition they receive sufficient nutrients and are less exposed to environmental stress. In some cases the plant is supplied with additional nutrients by the bacteria and simultaneously protected from pathogens, which would establish in the same niche as the bacteria (Hallmann *et al.* 1997). However, the latter were the main interrelationship, then the levels of control would depend on the density of bacterial colonization of the root system and the localization of the bacteria related to the nematode feeding site. This was not observed in experiments using potatoes (Hasky-Günther *et al.* 1998). Instead, low levels of endophytic bacteria colonization resulted in a high level of biocontrol (Hallmann et al. 2001).

Most rhizobacteria which have been screened for biological control of *Colletotrichum orbiculare* on cucumber, induced systemic resistance and colonized external and internal root tissues (Kloepper *et al.* 1992b). This phenomenon was already familiar, but was not well studied. Since the beginning of the twentieth century, researchers have known that some type of induced protection from infection by fungi, bacteria and viruses exists in plants (Chester 1933). A systemic protection after a localized virus infection on *Dianthus barbatus* L. was described by Gilpatrick and Weintraub in 1952 and a similar effect was observed on tobacco by Ross and Bozarth in 1960. This effect was confirmed with other viruses on several different plants (Loebenstein 1963). Similar systemic resistance effects were reported by Kuc and his co-workers in cucumber with different organisms (Hammerschmidt *et al.* 1976, Kuc and Hammerschmidt 1978, Staub and Kuc 1980, Hammerschmidt and Kuc 1982a, Hammerschmidt *et al.* 1982b).

Root colonizing bacteria were tested for biological control activity towards fungal, bacterial,

and viral pathogens, and termed *PGPR* (plant growth-promoting bacteria) as, aside from conferring resistance to pathogens onto the treated plants, a growth promoting effect was also observed (Kloepper and Schroth 1979). Most reports on *PGPR* strains indicated that the indirect effect of a change in the microbial community in the rhizosphere was responsible for increased plant growth (Kloepper and Schroth 1981). Root-colonizing bacteria also reduced infestation of nematodes. As health improvement was the main beneficial effect of these bacteria, Sikora (1988) renamed them plant health promoting rhizobacteria (*PHPR*). Seed treatment with *Pseudomonas fluorescens* was used to suppress early root infection of sugar beets by *Heterodera schachtii* (Oostendorp and Sikora 1989). Two different *PHPR*-bacteria, *Bacillus sphaericus* B43 and *Agrobacterium radiobacter* G12, which was later renamed *Rhizobium etli* G12 after 16S-rRNA sequencing (Hasky-Günther *et al.* 1998), reduced infestation of potato roots by the potato cyst nematode *Globodera pallida* in greenhouse and field trials (Racke and Sikora 1992).

The mode-of action of these two strains was investigated using standard split-root tests with intact plant roots to detect induced systemic resistance against *G. pallida* juvenile penetration in the responder root of potato (Hasky-Günther and Sikora 1995, Hasky-Günther 1996, Hasky-Günther *et al.* 1998). The induction of systemic resistance to nematode penetration caused by these bacteria was confirmed by tests with the potato cyst nematode, *G. pallida* and the root knot nematode, *M. incognita* on potato and tomato plants, respectively, where the bacteria significantly reduced penetration rates of potato cyst nematode juveniles in potato in repeated trials and of root knot nematode juveniles in tomato (Hasky-Günther 1996, Hasky-Günther *et al.* 1998, Hauschild *et al.* 2000, Mahdy 2002, Schäfer *et al.* 2006).

The inducer of the observed systemic resistance was further investigated with heat-killed cells and culture filtrates of both bacteria strains. While heat-killed cells of both bacteria could induce systemic resistance, the culture filtrate of *R. etli* G12 alone was unable to do so (Hasky-Günther *et al.* 1998). The inducing agent was later identified in the outer cell wall membrane of *R. etli* G12. The oligosaccharides of the core region of lipopolysaccharides were the main trigger of the systemic resistance induced by that bacterium (Reitz *et al.* 2002). For *B. sphaericus* B43, the inducer has yet to be identified. These reports gave an impression of how complex systemic resistance induction is, and how different two distinct rhiobacteria strains react with the host, though similar in their abbility to induce a systemic resistance against pathogens. An overview of their similarities and differences was published in 1998 by

Hallmann *et al.*. These observations rised the question of how this systemic induced resistance was established in the plant.

Several reports on the molecular basis underlying rhizobacteria induced systemic resistance in a model with *Arabidopsis thaliana* and the *Pseudomonas fluorescence* strain WCS417r showed that ISR was independent of endogenous salicylic acid accumulation and PR gene activation (Pieterse *et al.* 1996a), later reviewed by Bakker *et al.* (2007). Known PR proteins were also not associated with the rhizobacteria mediated ISR by *R. etli* G12 and *B. sphaericus* B43 in potato towards *G. pallida* (Hasky-Günther 1996). However, she observed a novel protein band (38 kDA) on a 1D protein gel electrophoresis with plants that had been inoculated with *R. etli* G12. This novel protein was not associated with known PR proteins as proven by Western blotting. In plants inoculated with *B. sphaericus* B43, the protein pattern did not differ from untreated plants.

In the present study, the molecular basis for induced systemic resistance was investigated using RNA isolated from tomato plants in which resistance to nematode penetration had been induced. To obtain nearly pure plant RNA, free of bacteria or nematode contamination, for later RNA extraction, both the inducer bacteria and the nematodes were inoculated to the same root half in a split-root system and only untreated roots were harvested for RNA extraction. A split-root experiment where both root halves had been inoculated with the nematodes also was conducted to: a) check if the rhizobacteria have a direct effect on nematode penetration and/or development and b) see if nematode penetration in inducer roots changes the effect in the responder roots. Hasky-Günther (1996) found no significant differences between nematode penetration into responder roots of split-rooted potatoes from treatments where either both the inducer and the responder roots were inoculated with nematodes and bacteria was applied only to the inducer roots, or where nematodes were only inoculated onto the responder side and bacteria only on the inducer side. These results agree with those obtained in this study with tomato. Nematode penetration rates were similar in both responder and inducer roots, when both sides were inoculated with nematodes and the inducer roots were additionally treated with bacteria. These rates did not differ from rates of nematode penetration in responder roots, when only the inducer side was treated with bacteria, and nematodes only inoculated onto the responder roots. Hence, the RNA obtained from responder roots of tomato plants grown in a split-root system could serve as a model for the rhizobacteria induced systemic resistance.

Interestingly in the 'long term' experiment, the level of developed *Meloidogyne* females remained strikingly stable at around 20 in the roots of bacterial treated plants compared with the number of penetrated females in plants harvested after two weeks. After eight weeks in control plants only about 25 females were detected, compared to about 50 females after two weeks. Therefore, the biological control effect of the two tested bacteria remained stable, although the females in the control plants did not develop as well as before. This may have been due to specific problems that occurred during their development. To date, no compareable observation has been reported.

Additionally, it still had to be proven that resistance to *M. incognita* penetration really was systemically induced in the tomato plants used for RNA extraction. This was determined in a parallel running experiment with split-rooted tomato plants, where the inducer side was inoculated with bacteria and the responder side was infested with *M. incognita*. Hasky-Günther (1996) showed that the systemic induction of resistance against *G. pallida* juvenile penetration in the responder root of potato reduced penetration 58 % when plants were inoculated with *B. sphaericus* B43 and 55 % with *Rhizobium etli* G12. Similar degrees of induced resistance to the penetration of *M. incognita* juveniles were observed in tomato plants in the present study, with reductions of 61 % and 79 % in penetration by B43 and G12 respectively.

In addition to the observed reduction of *G. pallida* penetration following bacterial inoculation in experiments with potato, nematode reproduction was also reported to have been significantly reduced (Racke and Sikora 1992). However, in the present study this was only observed in *R. etli* G12 treated plants, where the numbers of eggs per female were significantly reduced, compared to the control plants.

For RNA extraction the roots of tomato plants were harvested from the responder side of a split-root experiment at two dates. The first harvest was at 3 days after bacterial inoculation to study the rhizobacteria induced genes and the second at 6 days after bacterial inoculation to study the differential gene regulation upon bacteria and pathogen challenge. Neither at 3 nor at 6 days after bacterial inoculation could improved root growth be observed. The experimental plants of the ISR control experiments were harvested three or eight weeks after bacteria inoculation to observe either the effect of bacterial inoculation on the penetration rate of the nematodes or on the nematode fecundity. However, no increase in root weight was

observed upon bacterial treatment. This correlates with the observations made by Racke and Sikora (1992) in greenhouse and field trials on potato without any increasing effect on the root growth.

4.2 Molecular Investigations

A requirement for understanding rhizobacteria induced systemic resistance against root-knot-nematode is the identification and functional characterization of genes encoding proteins important in the bacteria-plant-nematode interaction. To achieve this, the subtractive suppressive hybridization (SSH) technique exploited in this study has proved useful in a wide range of analyses of plant responses to abiotic and biotic stresses (Diatchenko *et al.* 1996), including changes in gene expression in different plant-microbe interactions (Beyer *et al.* 2001, 2002, Requena *et al.* 2002, Wulf *et al.* 2003, Brechenmacher *et al.* 2004, Weidmann *et al.* 2004). One of the main advantages is that it allows detection of low abundance, differentially accumulated mRNA (von Stein *et al.* 1997), which may characterize early responses of root tissues to microbial cells. Insect feeding induced differential expression of *Beta vulgaris* root genes and their regulation by defence associated signals was studied using SSH (Puthoff and Smigocki 2007). Only recently has the same technique been applied to the identification of genes expressed after application of the non-pathogenic bacterium *Pseudomonas fluorescens* Bk3 to the phyllosphere of the apple scab susceptible cultivar *Malus domestica* cv. Holsteiner Cox (Kurkcuoglu *et al.* 2007).

In the present study using SSH, only 24 potential differentialy expressed genes were obtained. One reason for the low number could have been that the efficiency of the technique is largely influenced by the purity and the complexity of the DNA samples (Viebahn 2005). In previous studies using SSH, more fragments within a wider range of differentialy regulated genes were obtained. Puthoff and Smigocki (2007) identified more than 150 differentialy regulated genes with SSH following insect feeding on both susceptible and moderately resistant sugar beet lines. In contrast to von Stein *et al.* (1997), who screened a SSH-library of 1,444 clones with differential expression.

Nine of the 24 obtained gene fragments in the present study showed a correlation to plant sequences contained in the gene databank on the *NCBI*-homepage with a BLASTx analysis. They were tested to verify whether or not they were really differentially expressed.

At first, a digoxigenin-based detection in northern blots was chosen. The non-radioactive DIG

labelling method is based on a steroid isolated from *Digitalis* plants. The digoxigenin is linked to the C-5 position of uridine nucleotides via a spacer arm containing eleven carbon atoms. A dioxigenin-label can be added by random primed labelling, nick translation, PCR, 3'-end tailing/tailing, or *in vitro* transcription. Detection is based on high affinity *Fab* fragments coupled to alkaline phosphatase. There are different possibilities for visualization. In the most sensitive visualization method, the alkaline phosphatase conjugate generates light with the chemiluminescent substrate (Leitch and Heslop-Harrison 1994, Sambrook and Russel 2001). The last, most sensitive detection method was choosen, but proved not to be practicable as even the positive control, a glucose-6-phosphate-dehydrogenase fragment from potato (G6PDH, E.C. 1.1.1.49, Graeve *et al.* 1994) was not sufficiently labelled as tested in a dilution series. Gene expression studies in many plant species are troublesome because of phenolic compounds, carbohydrates, and other secondary metabolites in plant tissues. Nucleic acid isolation procedures require vigorous homogenization and disruption of the cells. Under these conditions, cell compounds compartmentalized within the organelles and cytoplasm is released from the ruptured cells and may become oxidized and react irreversibly with nucleic acids. Either this or it is possible that even minimal phenolic residues in nucleic acids prevent the interaction between digoxigenin and the alkaline phosphatase-antibody conjugate. The phenolic compounds form covalently linked quinones in oxidized form, which avidly bind nucleic acids (Loomis 1974, Levi *et al.* 1992). Furthermore, the problems with digoxigenin-based detection in northern blots may derive from minor residues of secondary metabolites bound to plant RNA (Jaakola *et al.* 2001).

A second approach with a radioactive-based detection in northern blots was performed. All tested ^{32}P-labelled-DNA probes were expressed in tomato roots of both treated and untreated plants. The comparison of different expression levels with the Phosphor Imager system was very difficult as the expression level of the polygalacturonase beta subunit gene fragment was very low. In earlier studies, the beta subunit could not be detected by northern blot analysis in root, leaf, and flower tissue after an overnight exposure, but at low levels after an exposure time of six days (Zheng *et al.* 1994). The radiocative labelling is thought to be more robust against possible disturbing residues in the nucleic acids. No non-radioactive methods of labelling are as robust or produce as sensitive a probe as ^{32}P (Harris 1991). In a study comparing the detection of a virus infection using both ^{32}P- or DIG-labelled probes, the ^{32}P-labelled RNA proved 30-fold more sensitive than a digoxigenin-labelled RNA (Lemaire *et al.* 1995). The ^{32}P-labelled probes detected the virus at 25-fold higher dilutions than DIG-labelled

probes (Hsu *et al.* 2000).

If problems with northern blotting occur, the qRT-PCR method is often used for gene expression analysis, which was also done here. The method is very sensitive but has also been criticized since it may allow false positives (Hengen 1995). The qRT-PCR results led to the suggestion, that the gene fragment encoding a polygalacturonase isoenzyme 1 beta subunit (*Lycopersicon esculentum)* was differentially expressed. The PG trancripts were repeataedly observed to have accumulated to lesser degrees in day 3 plants that had been treated with *R. etli* G12, compared to untreated plants. Therefore it was concluded that the gene fragment PG was originally obtained from a *R. etli* G12 treated day 3 plant. An advantage of this method is that transcribing RNA into cDNA solves the problem with the potential (phenolic) residues. These residues, if present, do not disturb the enzyme activity during transcription of RNA to cDNA (Jaakola *et al.* 2001).

To compare the expression levels in the qRT-PCR reactions, an adequate internal standard is essential. The expression levels of the target genes are normalized against an internal standard expression level. Here, the gene coding for ubiquitin was chosen, as it is a common housekeeping gene and often used as internal standard in qRT-PCR reactions (Oka *et al.* 2001, Zhu and Altmann 2005).

The gene encoding the polygalacturonase (*PG*) enzyme has been shown to be differentially expressed. It exists in three isoforms called PG1, PG2A and PG2B (DellaPenna *et al.* 1996). PG1 is a complex between either PG2A or PG2B, which are catalytic subunits, and two catalytic inactive proteins called beta-subunits (Knegt *et al.* 1988). It is suggested that the beta-subunits interact with the catalytic subunits and with the plant pectin so that the catalytic properties of the enzyme are altered (DellaPenna *et al.* 1996). The enzyme hydrolyzes the pectin of the cell wall. During fruit ripening the activity of polygalacturonase increases and is responsible for cell wall polyuonide degradation in ripening tomato fruit (Giovannoni *et al.* 1989). It has been shown that during fruit-ripening, beta subunit mRNA levels decreased whereas *PG* enzyme activity increased rapidly (Zheng *et al.* 1992). An analogous situation was reported upon wounding by a herbivore, where PG enzyme activity was highest when the beta subunit was at its lowest levels (Bergey *et al.* 1999). Based on these observations, a regulatory activity of the beta subunit was proposed, which had already been proposed by (Zheng *et al.* 1992). In extensive studies on transgenic tomatoes expressing a beta subunit

antisense gene, it was shown that the PG2 protein alone is responsible for pectin solubilization and depolymerization *in vivo* during fruit ripening, whereas the beta subunit protein is not required for PG2 activity *in vivo*. The reduction of beta subunit polygalacturonase expression affects the pectin metabolism in fruit by limiting the extent of pectin solubilization and depolymerization that can occur during ripening. The functional way is not yet fully understood, the interaction could occur directly between the beta subunit and PG2 or indirectly by the interaction of the beta subunit with the pectic substrate (Watson *et al.* 1994).

The lower expression of the beta subunit gene in the root of the *R. etli* G12 treated day 3 plant suggests that more solid cell walls are produced in these plants, in response to the bacteria treatement. The less beta subunit polygalacturonase is accessible in plant tissues, the less pectin is likely to be solubilized and depolymerised, the stronger the cell walls will be. This could result in a plant tissue that is more resistant to pathogen attack, as for example penetration by a nematode. Upon fungal pathogen attack, a difference in the degree of pectin methylation was related to the reaction of tomato cultivars to *Pseudomonas syringa*e p.v. *tomato* (Venkatesh 2002). Venkatsh suggested that high branching and a high degree of methylesterification, especially in a blockwise pattern makes polysaccharides less easily degradable by pathogen enzymes. In potato stem tissue, a higher percentage of methylated and branched pectins was reported in correlation with resistance against *Erwinia carotovora* subsp. *atroseptica* (Mc Millan *et al.* 1993, Marty *et al.* 1997).

The pectin is situated in the middle lamella of a cell and in the primary cell walls. In host-pathogen interactions, pectin acts as a defence barrier against the invasion of microorganisms. The resistance of primary cell walls to enzyme digestion has been suggested to be the result of the formation of calcium bridges between pectin chains (Ferguson 1984). Pathogens, such as nematodes, that penetrate the cell walls and move through the middle lamella, salivate pectolytic enzymes to facilitate penetration (Hoffmann *et al.* 1994).

On the other hand, Rao *et al.* (1982) indicated that pectins of plant cell walls play a critical role in adherence of bacterial cells to the host surface. With rheological studies it was demonstrated that pectins interact with bacterial lipopolysaccharides (Grolms 1996, Venkatesh 2002). This may be a problem when the pathogen is bacterial, but when the pathogen-antagonist is bacterial, increased adherence to host surface would be a sought after characteristic.

4 Discussion

Living and heat killed cells of the Gram-negative rhizobacterium *R. etli* strain G12 systemically induced resistance to infection by the potato cyst nematode *Globodera pallida* in potato roots. Results of split-root experiments clearly showed that lipopolysaccharides (LPS) of *R. etli* G12 acted as the inducing agent of systemic resistance in potato roots (Reitz *et al.* 2000). LPS are ubiquitous components of the outer membrane of all Gram-negative bacteria. They are comprised of three regions, lipid A, the core region and a highly heterogeneous region comprised of repeating units of oligosaccharides (O-chain). LPS is involved in the specific recognition process between plant pathogenic bacteria and their hosts (Denny 1995). The very conserved core region and lipid-A structure, and the highly variable O-antigen sugar composition is believed to play a key role in plant/microbe interactions (Wolpert and Albersheim 1976, Priefer 1989). Reitz *et al.* (2002) showed, that the oligosaccharides of the core-region of *R. etli* G12 were the main trigger of systemic resistance in potato roots towards *G. pallida* infection. This strongly corelated to other findings where the LPS of Gram-negative rhizobacteria seems to also be an important inducing agent of disease resistance towards soilborne pathogens. Purified LPS of *P. fluorescens* strain WCS417r triggered systemic resistance towards *Fusarium* wilt in carnation, in radish and in *Arabidopsis* (Van Peer *et al.* 1991, Leeman *et al.* 1995, Van Wees *et al.* 1997). To act as an inducing agent, the sugar components of the bacterial LPS must be released from the bacterial surface and then bind to a receptor molecule on the plant root surface. This receptor molecule is still not identified.

The qRT-PCR results also led to the suggestion that the (*PAL5*) gene encoding a phenylalanine ammonia-lyase (*Lycopersicon esculentum)* was differentially expressed. This was observed in *B. sphaericus* B43 treated plants. However, it could not be proven from which harvest day the gene fragment was obtained. The qRT-PCR analysis could also not confirm that the gene fragment encoding a putative high affinity nitrate transporter was differentially expressed. All other tested primer pairs did not give interpretable results with the semiquantitative qRT-PCR reaction.

In parsley culture cells, a strong correlation was found between the ability to trigger ISR and the capacity to enhance phenylalanine ammonia-lyase (*PAL*) gene activation induced by elicitor treatment (Katz *et al.* 1998, Thulke and Conrath 1998). Something similar could be happening in *B. sphaericus* B43 treated tomato plants, where the transcripts of the phenylalanine ammonia-lyase (*PAL5*) gene (*Lycopersicon esculentum)* were expressed at

levels 30-50 % higher than in control plants, three and six days after bacteria inoculation, based on qRT-PCR. Thulke and Conrath (1998) and Conrath *et al.* (2001) observed that the *PAL* augmentation is only seen after a pre-incubation period and expressed upon pathogen attack, which could explain the 20 % increase in expression three days after pathogen challenge in my observations. Based on the observation that the *PAL* gene activation increased in proportion to the length of BTH (benzo (1,2,3) thiadiazole-7-carbothioic acis S-methyl ester (acibenzolar-S-methyl)) pre-treatment, it was assumed that the plant acquired a resistance activator, in a time-dependent process, which induces the synthesis of one or more signal transduction components that shift the cells to an alerted state when needed. Some of these factors might activate certain defence genes, such as those encoding anionic peroxidase (POX), whereas others may act together with elicitor-inducible signalling components, leading to an augmented elicitation of certain other defence responses, such as *PAL* gene activation (Katz *et al.* 1998).

In the early eighties, an increased *PAL* mRNA synthesis was seen as an early event in the defence response leading to an accumulation if phenylpropanoid-derived phytoalexins (Edwards *et al.* 1985). *PAL* catalyzes the first reaction in the biosynthesis of L-phenylalanine, which is the first step for the biosynthesis of the phenylpropanoid skeleton in higher plants (Jones 1984). Its activity is increased upon microbial infection, leading to the synthesis of lignin-like, wall-bound phenolic material and phenylpropanoid-derived phytoalexin antibiotics (Jones 1984, Hahlbrock and Grisebach 1979). A significant change in cell structure and an accumulation of occluding material in the lumina of xylem vessels was reported upon treatment of tomato plants with *B. sphaericus* B43 (Mwangi 2002). Whether or not the increased *PAL* activity is the initial activator for the production of the occluding material is not clear but it could be speculated that this is in fact so. The change in cell structure as described by Mwangi (2002) in reaction to a *Bacillus* treatment could also explain higher *PAL* activity in my studies.

The differences in the activation of the genes coding for a *PG* beta subunit enzyme after a *R. etli* G12 treatment and for the *PAL 5* enzyme after a *B. sphaericus* B43 treatment as seen in this study could at least partially explain the differences detected in earlier protein-biochemical studies between *B. sphaericus* B43 and a *R. etli* G12 treatments (Reitz 1999), and their different potential in controlling soilborne pathogens as shown by Hauschild *et al.* (2001).

5 Summary

The ability of two rhizobacteria, *Bacillus sphaericus* B43 and *Rhizobium etli* G12 to induce systemic resistance against the root-knot nematode *Meloidogyne incognita* on tomato was confirmed. This was manifested in a reduced number of penetrated juveniles in the root, as well as a reduced multiplication of the penetrated females after a *R. etli* G12 treatment.

The systemic effect was studied in a split-root system, where one root was inoculated with the bacteria, later refered to as the inducer root. After a time period of three days the nematodes were either applied to both roots or only to the non-bacterial inoculated root, this was called the responder root. Both bacteria strains significantly reduced nematode penetration, not only in the inducer, but also in the responder root, verifying a systemic resistance effect.

The systemic effect of the bacterial treatment remained stable over a periode of eight weeks, whereas the development of the penetrated females in the untreated control plants was not stable. The number of females that developed in the roots of untreated plants over an 8 week periode was less than the number that had penetrated the roots of plants harvested three weeks after nematode inoculation.

The bacterial treatment did not improve the root growth of tomato plant. Neither after a short exposure time of three or six days, nor after a longer post-inoculation exposure time of two or eight weeks.

The systemic effect of a combined bacterial and nematode application to the inducer root resulted in an equal reduction of nematode penetration in the responder root as a single bacterial treatment of the inducer root. This confirmed that the observed resistance to nematode penetration was due to a systemic induced defence mechanism, and not due to a direct antagonistic relationship between nematode and bacteria. Hence, the RNA obtained from responder roots of tomato plants grown in a spilt-root system with a combined bacterial an nematode application to the inducer root, could serve as a model for the rhizobacteria induced systemic resistance.

Different molecular methods were tested for their potential to prove the obtained gene fragments of a subtractive suppressive hybridization to be differentially expressed in induced

plants. The northern blotting with two different detection methods, one chemiluminescence and one radioactive, were not sensitive enough to detect the very small differences in the transcript profile of induced and non-induced tomato plants.

The semiquantitative reverse transcriptase PCR was suitiable to detect the differences in the transcript profile of the differential gene expression after a bacterial or after a combined bacterial and nematode application.

The molecular analysis of the plant's response to the bacteria application showed that the two different bacteria strains also induced different responses of the plant. This was related to a differential expression of the *PG* gene coding for the polygalacturonase isoenzyme 1 beta subunit after a *R. etli* G12 treatment. The difference in the expression level of the *PAL 5* gene coding for a phenylalanine ammonia-lyase enzyme after a *B. sphaericus* B43 treatment was not obvious. The gene was up regulated three days after the bacteria application as well as after an additional pathogen challenge with *M. incognita* three days later.

5.1 Conclusions and Future Aspects

1. The rhizosphere bacteria *Bacillus sphaericus* B43 and *Rhizobium etli* G12 were able to induce systemic resistance against the root-knot nematode *Meloidogyne incognita* on tomato.
2. The results obtained in this study and others demonstrated, that the molecular basis of an induced systemic resistance is not yet totally understood.
3. The experimental designs and the proven validity of the methods used in this study are a good foundation for further analysis.
4. The two rhizosphere bacteria were only tested for their potential to induce systemic resistance against *M. incognita* on one tomato cultivar. In future studies it would be of interest to see, if the reaction of the plant to a bacterial treatment and a pathogen challenge is cultivar dependent.
5. The more information is available on rhizobacteria induced systemic resistance, the more efficiently such bacteria could be used to protect plants against nematodes.
6. To date, few genetically modified products had been of real advantage to agriculture and the environment or the consumer. Based on the present and on further studies it is imaginable that plants could be genetically modified so as to readily and stably express their own genes known to cause resistance. These genes would be identified through their

increased or decreased activity after induced resistance in plants following a bacterial treatement alone or in combination with a pathogen challenge.

7. During the cultivation of these genetically modified plants, the application of pesticides could be reduced or even omitted. This would be of advantage to the environment and to the consumer. The environment would not be disturbed by pests and the consumer would not have to worry about contamination or harmful residues. Although the acceptance of GMOs is seen very critically, especially in Europe, it is imaginable, that consumers could be sensitized to the large potential of introduced resistance and the probable lower environmental risk involved, compared to pesticide applications. However, this potentially decreased risk has to first be proven in the field.
8. In the present thesis, the study of the molecular basis of rhizobacteria mediated induced systemic resistance was nearly analogous to that of Pieterse *et al.* (1996a). However, in this study, tomato (*Lycopersicon esculentum*) was used instead of the model plant *Arabidopsis thaliana*, and the inducing rhizobacteria used were the already well studied *B. sphaericus* strain B43 and *R. etli* strain G12 instead of the *Pseudomonas fluorescence* strain WCS417r used by Pieterse *et al.* (1996a). Tomato was choosen in my studies as it is a better host for the root-knot nematode *Meloidogyne incognita* than *Arabidobsis*, and additionally, tomato is an important crop plant of global interest. However, to understand the molecular basis of the rhizobacteria mediated induced systemic resistance, an *Arabidopsis* based study could be advantageously. One of the major problems in the present study was the non-availability of a complete database of the *Lycopersicon* genome. Most of the sequenced potential differentially regulated genes of the rhizobacteria treated and also of the additional *M. incognita* treated tomato plants were correlated to either *Arabidopsis* or have an unknown function, because of lack of information on *Lycopersicon* genes. It is imaginable that with a *Lycopersicon* gene database similar to the *Arabidopsis* database, a higher number of gene fragments could have been identified and could probably have been correlated to either a rhizobacteria induction or an induction upon parasite infection.
9. In the future, an *Arabidobsis* based approach would be helpful to study the differential gene regulation upon rhizobacteria induced systemic resistance to nematodes.

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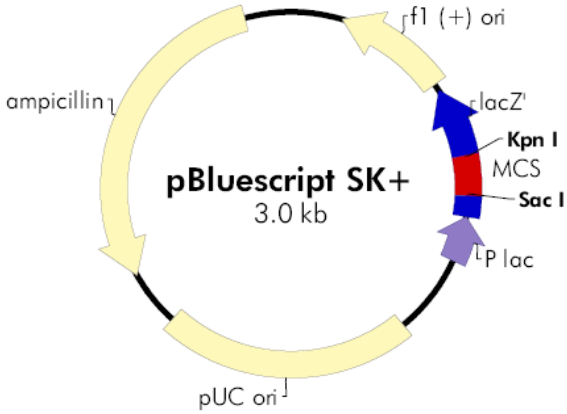
Appendix

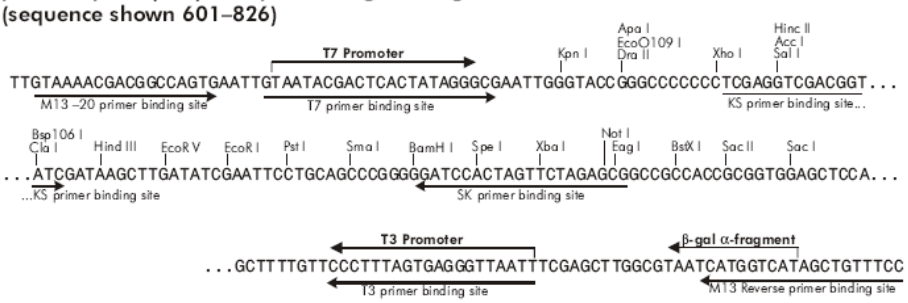
pUC9 (New England Biolabs)

pBluescript SK + (Stratagene)

f1 (+) origin 138–444
β-galactosidase α-fragment 463–816
multiple cloning site 653–760
lac promoter 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833



pBluescript SK (+/–) Multiple Cloning Site Region
(sequence shown 601–826)



Appendix

Sequences of Clones for qRT-PCR Primer Construction

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1-3-T7 18..947 of trace file

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1-7-T7 92..144 of trace file

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

Appendix

1-9-_M13_rev__-29__ 28..628 of trace file

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1-9-T7 36..805 of trace file

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2-1-_M13_rev__-29__ 26..734 of trace file

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2-1-T7 30..140 of trace file

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2-7-_M13_rev__-29__ 25..842 of trace file

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2-7-T7 32..66 of trace file

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Appendix

2-B1-_M13_rev__-29__ 26..804 of trace file

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Appendix

Sequences of Clones without Similarities to *Lycopersicon*, not used in this study

1-1-_M13_rev__-29__ 26..134 of trace file

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1-2-T7 34..119 of trace file

GGATATCTGCAGAATTCGCCCTTAGCGTGGTCGCGGCCGAGGTACAAGCTTATTTTTTTTTTTTTTTTTTTTT
TTTTTTTTTT

1-4-_M13_rev__-29__ 25..570 of trace file

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AGTTAGATGTCCAACAAATTCCAAACTGAACCAAAATGCAACCCAACAATATCTGATAGTCAAACACCTGAGTCT
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GAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCAATTCGCCCTATAGTGAG
TCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGC
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CGCAGCCTGAATGGCGAATG

1-4-T7 36..452 of trace file

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Appendix

1-5-_M13_rev__-29__ 25..938 of trace file

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AGCAAAAACAATAATCTTTTTGGGATTCCAAGAGCCCACGGAAATACCATCATCCTGCAAATCTCCATTTGAAGT
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TCGAGCCCTATAGTGAGTCGTATTAGAAGGGCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATG
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GGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAG
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CTGATAGACGGTT

1-5-T7 670..669 of trace file

1-8-_M13_rev__-29__ 26..888 of trace file

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GACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGA
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CTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAAAG

1-8-T7 31..875 of trace file

TGGATATCTGCAGAATTCGCCCTTACCTCGGCCGCGACCACGCTAAGGGCGAATTCCAGCACACTGGCGGCCGTT
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CCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGT
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GTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGT
CGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCG
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Appendix

1-B1-_M13_rev__-29__ 25..925 of trace file

CGGCCGCCAGTGTGCTGGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCA
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GGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTT
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1-B1-T7 17..974 of trace file

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2-2-_M13_rev__-29__ 25..975 of trace file

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2-2-T7 128..248 of trace file

TAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAA
GCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTG

2-3-_M13_rev__-29__ 59..58 of trace file

2-3-T7 318..317 of trace file

Appendix

2-5-_M13_rev__-29__ 26..613 of trace file

CGGCCGCCAGTGTGATGGATATCTGCAGAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTT
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GCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCC
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2-5-T7 28..974 of trace file

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CCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAG
GCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTC
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2-6-_M13_rev__-29__ 25..893 of trace file

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ACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGC
GCCCTAGCGCCCGCTCCTTTCGCTTCTTCCTTCCTTCTCGCCA

2-6-T7 30..752 of trace file

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CAGTAAAACTGGACATAAACCACACACAAGAAAATAGCATATACTTACAAGAGTTGGATCCTGTATACCTATTGG
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CGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAAC
TCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAAC

2-8-_M13_rev__-29__ 316..315 of trace file

2-8-T7 2705..2704 of trace file

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