Biochemical analysis of the inhibition of *Ralstonia solanacearum* polygalacturonases by polygalacturonase-inhibiting proteins (PGIP) from tomato stems and biochemical, histochemical and molecular analysis of the silicon effect in the tomato (*Solanum lycopersicum*) – *Ralstonia solanacearum* interaction

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ZUSAMMENFASSUNG

Bakterielle Welke, verursacht durch *Ralstonia solanacearum*, ist eine der bedeutendsten Bakteriosen in den Tropen und Subtropen. Eine chemische Bekämpfung von *R. solanacearum* ist nahezu unmöglich. Daher spielt die Entwicklung integrierter Bekämpfungsmaßnahmen, unter Einbeziehung von Wirtspflanzenresistenzen und Resistenzinduktion durch verschiedene Induktoren, eine bedeutende Rolle in der Bekämpfung von *R. solanacearum*. Ein Hauptaugenmerk der bisherigen Forschung richtete sich auf die Interaktion zwischen Wirtspflanze und *R. solanacearum*, aber die genauen Resistenzmechanismen sind bis heute weitgehend unbekannt. Aus diesem Gund bestand der erste Teil dieser Arbeit aus der Untersuchung einer Interaktion des Pathogens mit der Modellpflanze Tomate, nämlich der Protein – Protein - Interaktion von Pflanzenzellwand-abbauenden Polygalakturonasen (PGs) von *R. solanacearum* und Proteinen aus der Pflanzenzellwand, den Polygalakturonase-inhibierenden Proteinen (PGIPs). Eine Inhibierung bakterieller PGs durch pflanzliche PGIPs wird in der vorliegenden Arbeit zum ersten Mal beschrieben.

Der zweite Teil der Arbeit befasste sich mit der Untersuchung von möglichen Mechanismen der Siliziumdüngung über Bodenapplikation als Resistenzinduktur in Tomate gegenüber *R. solanacearum*. Biochemisch wurden Peroxidasen (PODs) und Polyphenoloxidasen (PPOs), histochemisch Lignifizierung, Tylosenbildung, Wasserstoffperoxid (H_2O_2) Akkumulation und Calloseablagerung, immunohistochemisch Veränderungen struktureller Komponenten der pektischen Polysaccharide wie Arabinogalaktanprotein (AGP), (1→5)-α-L-Arabinan und nicht-blockweise deesterifizierte pektische Epitope des Homogalakturonans, sowie mit einem molekularen Ansatz die Expression von Genen verschiedener Pflanzenzellwandkomponenten, wie AGP, Extensin and Callosesynthase, aber auch Gene involviert in ‚plant defense signalling pathways', wie non-inducible immunity (NIM), jasmonate ZIM-domain protein1 (JAZ1), ethylene responsive factor1 (ERF1) und coronatine-insensitive1 (COI1) untersucht.

Die Ergebnisse deuten auf eine Rolle von PPO und Tylosenbildung in der Silizium-induzierten Resistenz von Tomate gegenüber *R. solanacearum* und, einen Einfluss von Calloseablagerung, Pflanzenzellwandkomponenten wie AGP, Extensin, Callosesynthase, aber auch der Gene NIM and JAZ1 in der Interaktion von *R. solanacearum* mit Tomate hin.

Ralstonia solanacearum, Protein – Protein - Interaktion, Silizium - induzierte Resistenz

SUMMARY

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most important bacterial diseases in the subtropics and tropics. Chemical control of *R. solanacearum* is nearly impossible, thus integrated approaches, including host plant resistance and resistance induction by various inducers, are promising for a bacterial wilt management system. Many studies focused on the interaction of *R. solanacearum* and its host plants, but resistance mechanisms are not well understood so far. Thus, we conducted the first part of this study in order to elucidate the interaction of the pathogen with the model host plant tomato, investigating the protein-protein interaction of the pathogen's cell wall degrading polygalacturonases (PGs) and plant cell wall proteins, the polygalacturonase-inhibiting proteins (PGIPs). Our results demonstrate for the first time the effect of a PGIP that inhibits bacterial PGs.

In the second part of the study possible mechanisms by which silicon, supplied as soil amendment, induces resistance in tomato to *R. solanacearum* should be identified.

We focussed in biochemical analyses on peroxidase (POD) and polyphenol oxidase (PPO), histochemically on lignifications, tylsosis formation, hydrogen peroxide (H_2O_2) accumulation and callose deposition, immunohistochemically on structural componentes of pectic polysaccharides like arabinogalactan protein (AGP), (1→5)-α-L-arabinan and non-blockwise de-esterified epitopes of homogalacturonan and, with a molecular approach on the expression of genes related to plant cell wall components like AGP, extensin and callose synthase as well as on genes of plant defense signalling pathways like non-inducible immunity (NIM), jasmonate ZIM-domain protein1 (JAZ1), ethylene responsive factor1 (ERF1) and coronatine-insensitive1 (COI1).

Based on the observations, we suggest that PPO and tylosis formation are involved in the silicon-induced resistance of tomato to *R. solanacearum* and, callose deposition, the cell wall related components AGP, extensin, callose synthase, but also genes such as NIM and JAZ1 are involved in the tomato – *R. solanacearum* interaction.

Ralstonia solanacearum, protein – protein - interaction, silicon - induced resistance

CONTENTS

ABBREVIATIONS

$^1O_2$	singlet oxygen
3-OH PAME	3-hydroxypalmitic acid methyl ester
ABA	abscisic acid
ADA	agarose diffusion assay
AFLP	amplified fragment length polymorphism
AG-I	arabinogalactan I
AGP	arabinogalactan-protein
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
APX	ascorbate peroxidase
Ara*f*	arabinofuranosyl
AUDPC	area under disease progress curve
AUD_IPC	area under disease incidence progress curve
AUD_SPC	area under disease severity progress curve
avr	avirulence
AVRDC	Asian Vegetable Research and Development Centre
BABA	*β*-aminobutyric acid
BM	basal medium
BSA	bovine serum albumin
BTB	broad-complex, tramtrack, and bricà–brac
BTH	benzothiadiazole
CallSyn	callose synthase
CAT	catalase
CFU	colony forming units
ChbA	exoglucanase of *Ralstonia solanacearum*
CHCA	α-cyano-4-hydroxyl-cinnamonic acid
COI1	coronatine-insensitive1
DAB	3,3'-diaminobenzidin
ddH_2O	double destilled H_2O
DI	disease incidence
DP	degree of polymerization
dpi	days post inoculation
DS	disease severity
Egl	*β*-1,4-endoglucanase of *Ralstonia solanacearum*
EGTA	ethylene glycol bis(β-aminoethyletherl) tetraacetic acid
EIN2	ethylene insensitive2
endo-PG	endopolygalacturonase
EPS I	exopolysaccharide I
ERF1	ethylene responsive factor1
ET	ethylene
exo-PG	exopolygalacturonase
FACE-PAGE	**f**luorophor-assisted carbohydrate – polyacrylamid – gel electrophoresis
FITC	fluoroisothiocyanat
FW	fresh weight
Galp	β-D-galactopyranosyl

Gal*p*A	galactopyranosyluronic acid
GPI	glycosylphosphatidyl-inositol
GPX	glutathione peroxidase
GSL	glucan synthase-like
h	healthy
H_2O_2	hydrogen peroxide
HG	homogalacturonan
HGK	housekeeping gene
HIC	hydrophobic interaction chromatography
HO	hydroxyl radical
hpi	hours post inoculation
HR	hypersensitive response
HRGPs	hydroxyproline-rich glycoproteins
hrp	hypersensitive response and pathogenicity
i	inoculated
IAA	indole-3-acetic acid
INA	2,6-dichloroisonicotinic acid
IPP	Institue of Plant Diseases and Plant Protection
IPTG	isopropyl-β-D-thiogalactopyranoside
ISR	rhizobacteria-induced systemic resistance
JA	jasmonate
JA-Ile	jasmonyl-isoleucine
JAR1	jasmonate resistant1
JAZ1	jasmonate ZIM-domain protein1
LRR	leucine-rich repeat
MeJA	methyl jasmonate
MES	2-(N-Morpholino)-ethane sulphonic acid
MS	mass spectrometry
mSi	milli Siemens
NGA	nutrient glucose agar
NIM1	noninducible immunity1
NLS	nuclear localization sequence
NO	nitrous oxide
NPR1	nonexpressor of PR-1
ns	non-slimy
O_2	oxygen
O_2^-	superoxide anion
OD	optical density
OGA	oligosaccharide
PAL	phenylalanine ammonia lyase
PBS	phosphate buffered saline
PC	phenotype conversion
PCD	programmed cell death
PehA	endopolygalacturonase of *Ralstonia solanacearum*
PehB	exo-poly-α-D-galacturonosidase of *Ralstonia solanacearum*
PehC	exopolygalacturonase of *Ralstonia solanacearum*
PG	polygalacturonase
PGA	polygalacturonic acid
PGIP	polygalacturonase-inhibiting protein
PGK	phosphoglycerate kinase

PHB	poly-β-hydroxybutyrate
PIPES	piperazine-N,N'-bis(2-ethanesulphonic acid)
PME	pectin methylesterases
Pmr 1-4	powdery mildew resistance 1-4
POD	peroxidase
POZ	poxvirus zinc finger
PPO	polyphenol oxidase
PR	pathogenesis-related
Pst	*Pseudomonas syringae* pv. t*omato*
PTFE	polytetrafluorethylen
PUFAs	polyunsaturated fatty acids
qRT-PCR	quantitative real-time polymerase chain reaction
Rah*p*	rhamnosyl
rat1	*resistant* to *Agrobacterium transformation*
Rf	retention factor
RFLP	restriction fragment length polymorphism
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
RIL	recombinant inbred line
ROS	reactive oxygen species
Rs	*Ralstonia solanacearum*
rs	retarded slimy
RT-PCR	reverse transcriptase polymerase chain reaction
s	slimy
SA	salicylic acid
SAI1	salicylic acid insensitive1
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamid gel electrophoresis
SE	standard error
Si	silicon
SM	synthetic medium
SOD	superoxide dismutase
syn	synonym
T3SS	type III secretion system
TAL	tyrosine ammonia lyase
TE	tomato stem extract
TERF1	tomato responsive factor1
TLC	thin layer chromaotgraphy
TMV	tobacco mosaic virus
TT	tomato cell wall tissue
TTC	2,3,5-triphenyl tetrazolium chloride
TUB	α-tubulin
WT	wild-type
ZIM	Zinc-finger inflorescence meristem

GENERAL INTRODUCTION

Ralstonia solanacearum

Bacterial wilt disease is caused by the aerobic Gram-negative bacterium *R. solanacearum* (formerly *Pseudomonas solanacearum*) (Yabuuchi *et al*., 1992; Yabuuchi *et al*., 1995). This bacterium can be classified in the non-fluorescent rRNA homology group II within the *Proteobacteria* β-subdivision (Oepp/Eppo, 2004). Genomes from all examined *R. solanacearum* strains are composed of at least two independently replicating circular replicons. Genes for basic cellular functions are apparently located on a replicon of 3.8Mb size, whereas many virulence and pathogenicity genes are manifested on a 1.9Mb plasmid (former called megaplasmid) (Boucher *et al*., 1986; Schell, 2000).

Ralstonia solanacearum is distributed worldwide and causes a lethal wilting disease on over 450 different host plant species, including economically important hosts such as tomato, tobacco, potato, peanut and banana (Buddenhagen and Kelman, 1964; Hayward, 1991; Prior *et al*., 1996). No other bacterial disease is comparable in harmfulness concerning the actual number of plants destroyed in major crops such as banana, groundnut, tobacco and tomato each year (Kelman, 1998). The importance of the disease becomes obvious by losses of about 75% of potato or even a total destruction of the harvest of tomato, one of the most susceptible crops towards *R. solanacearum* (Persley *et al.*, 1986; Hayward, 2000; Elphinstone, 2005).

Ralstonia solanacearum isolates were classified into three races by Buddenhagen *et al.* in the year 1962, and two new classes were described by Buddenhagen (1986). This classification was based on the host range of the bacterium. The different biovars of *R. solanacearum* can be distinguished by their ability to utilize and/or oxidize several hexose alcohols and disaccharides (Hayward, 1991). In 1992, a tropical variant of biovar 2 was recognized by Hayward *et al*. (1992). He differentiated biovar 2 in subphenotypes by additional tests. More recent results, obtained from genetic studies such as restriction fragment length polymorphism (RFLP) analysis, propose a segregation of the races into two divisions based on the geographic origin of the pathogen. Division 1 is formed by the Asian strains of race 1 (biovars 3, 4, 5), and division 2 by the South American strains of race 1 (biovar 1), race 2 (biovar 1) and race 3 (biovar 2). (Cook and Sequeira, 1988;

Hayward, 2000; Schell, 2000; Oepp/Eppo, 2004). This classification was suggested by Cook *et al*. in 1989 and similar observations were reported by Gillings and Fahy (1993) and Taghavi *et al*. (1996). More recent investigations showed, that some biovar 2 phenotypes are clustered in division 1, while some isolates of biovar 1 do not fall into the 2nd division. This indicates that there is not as strict correspondence between the biovar and the 16S rRNA division of isolates (Fegan *et al*., 1998; Boudazin *et al*., 1999). PCR-RFLP analysis of the *hrp* gene region (Poussier *et al*., 1999), PCR-RFLP complemented by amplified fragment length polymorphism (AFLP), sequencing of the 16S rRNA gene (Poussier *et al*., 2000a) and phylogenic analysis of the endoglucanase and *hrp*B genes has confirmed the presence of a new group of strains originated in Africa (Poussier *et al*., 2000b).

The symptoms caused by bacterial wilt disease on tomato are formation of adventitious root nodules and foliage discoloration, followed by rapid wilting and death (Buddenhagen and Kelman, 1964). Wilting symptoms predominantly occur on young leaves approximately five days after infection. Wilting of the whole plant follows rapidly under favourable environmental conditions for *R. solanacearum* such as high temperature and a moderate water content of the soil. Under less favourable conditions the development of the disease is less rapid, a stunting of the plant may occurs, and a high number of adventitious roots are produced. In the vascular tissue of the stem a brown discoloration can be observed and by cutting its stem, drops of white or yellowish bacterial ooze may be visible (Oepp/Eppo, 2004).

Ralstonia solanacearum invades plants from the soil through wounds or lateral emerge points and then multiplies in the xylem vessels and spreads through the plants' vascular system (Wallis *et al*,. 1978; Vasse *et al.,* 1995). Due to the fact, that *R. solanacearum*'s journey begins mainly in the soil and ends with a massive infection of the stems, a successful completion of its cycle requires many specialized gene products (Schell, 2000). However, the mechanisms by which *R. solanacearum* produces wilting is still not fully understood (Roberts *et al*., 1988a; Huang and Allen 1997). The primary factor involved in development of wilt symptoms may be the production of extracellular polysaccharide slime (Exopolysaccharide I, EPS I) (Hussain and Kelman, 1958; Schell, 2000). Additionally, *R. solanacearum* secretes several extracellular enzymes. Among them are extracellular plant cell wall-degrading enzymes, such as pectin methylesterase, endoglucanase and three polygalacturonases (Schell, 1987; Roberts *et al*. 1988a; Huang and Allen 1997), but

enzymes that directly hydrolyse pectin, for instance pectate lyase, have never been detected (Schell, 2000). Furthermore, bacterial motility - twitching motility and flagellar motility - is contributing to virulence (Liu *et al*., 2001; Tans-Kersten *et al*., 2001). The expression of the virulence factors is controlled by a complex regulatory cascade that responds to bacterial cell density. Especially for the production of polygalacturonases it is hypothesized that the genes are expressed early in the wilt disease development (Schell, 2000).

Broad geographical distribution and extensive host range of the pathogen exhibits difficulties to establish a unique control strategy. Furthermore, control strategies for bacterial wilt disease in cool temperate regions, where only *R. solanacearum* biovar 2 appears, will differ from control in the lowland tropics, where strains of wide host range are endemic. Due to this fact, no universal solution is present, but only principles that can be applied and adapted in particular situations (Hayward 1991).

The main approach to control *R. solanacearum* is the breeding of resistant cultivars and some success had been achieved for tobacco, peanut (Hayward, 1991) and tomato (Wang *et al*., 2000). But, even though there are good levels of site-specific resistance of tomato, breakdown of resistance has been repeatedly observed in cultivars grown under the heat stress of the lowland humid tropics (Prior *et al*., 1996; Hayward, 2000), and resistance was shown to be unstable, and accompanied by latent infection, for instance in potato (Hayward, 1991; Hayward, 2000).

In some developing countries farming practices such as intercropping, crop rotation and disease avoiding are likely a strategy for control of *R. solanacearum* by reducing soil populations of the pathogen and root-to-root transmission (Hayward, 1991). Additionally, biological control can be seen as a putative control strategy to reduce yield losses caused by bacterial wilt. Agents tested for biological control include antagonistic rhizobacteria and avirulent mutants of *R. solanacearum* (Kempe and Sequeira, 1983; Ciampi-Panno *et al*., 1989; Trigalet and Trigalet-Demery, 1990). Mechanisms that might be involved in biological control depend upon active colonisation of the rhizosphere with antagonistic soil bacteria or bacteriocin- and bacteriophage-producing strains of *R. solanacearum* (Chen and Echandi, 1984), protection by competitive exclusion (McLaughlin and Sequeira, 1988) or induced resistance (Hayward, 1991).

A widespread means to control soilborne diseases caused by soilborne pathogens is the use of soil amendments (Huang and Huang, 1993). Some effect in suppression of the pathogen was obtained by soil amendments such as the so called S-H mixture from Thailand, with a high content of silicon oxide and calcium oxide. Additionally, it was observed in Surinam that bacterial wilt never occurred on the seashell ridges of the plains. Thus, an amendment of sea-shell grit - containing 42% CaO - to the soil is recommended. Both methods achieved good results in suppression of *R. solanacearum* (Hayward, 1991). Investigations of Dannon and Wydra (2004) confirmed a suppressive influence of silicon on bacterial wilt infection of tomato plants. In their experiments, conducted with tomato plants in hydroponic culture, bacterial wilt incidence was reduced by up to 50% compared to plants without silicon treatment. Since an accumulation of silicon was only detected in the roots, the enhanced resistance in tomato stems against *R. solanacearum* is suggested to be caused by induced resistance mechanisms and increased tolerance.

However, especially the chemical control of *R. solanacearum* is extremely difficult or impossible. Reasons are a wide host range, the capacity of survival in various environments such as irrigation water and soil or sheltered places, for instance plant debris in the soil, so that even soil fumigation with chloropicrin or applied antibiotics showed hardly an effect (Hayward, 1991; Oepp/Eppo, 2004).

Based on the above mentioned reasons, only a combination of host plant resistance, and cultural and biological measures seems to be promising for an effective control of *R. solanacearum* (Dannon and Wydra, 2004; Diogo and Wydra, 2007).

Plant resistance

Nonhost resistance is the most common form of disease resistance and can be defined as immunity in an entire plant species to all isolates of a microbial species, and thus affects the host range of a pathogen (Heath, 2000; Nürnberger and Lipka, 2005). In contrast, plants or plant genotypes can exhibit specific resistance towards a pathogen within an otherwise susceptible host species (Heath, 2000). This immunity is triggered upon direct or indirect recognition of the pathogen, for instance in the gene-for-gene resistance, also called *R*-gene-mediated resistance (Flor, 1971; Feys and Parker, 2000). Here, plant disease resistance (*R*) genes initiate active disease responses by recognizing the presence of a corresponding avirulence (*avr*) gene from the pathogen. The disease resistance gene *Pto* in tomato and the *avrPto* gene in *Pseudomonas syringae* pv *tomato* is one example of a gene-for-gene interaction (Ronald *et al.*, 1992) and it was demonstrated that a direct interaction of Pto and AvrPto proteins is required for activation of disease resistance (Scofield *et al.*, 1996; Tang *et al.*, 1996). This resistance is often accompanied by the hypersensitive response (HR), resulting in a fast collapse of infected plant tissue which efficiently halters pathogen ingress at infection sides (Staskawicz *et al.*, 1995; Feys and Parker, 2000.

Determining factor of the failure or success of the colonization of a plant by pathogen is the speed, by which plants are able to mobilize defense mechanisms to restrict the invading pathogen (Ton and Mauch-Mani, 2004). After the pathogen starts colonizing the plant tissue, a race-nonspecific host resistance, the so-called basal resistance, is activated around the sites of pathogen invasion in susceptible plants, limiting the disease severity by slowing down the pathogen ingress, but it is generally to weak to prevent the disease (Ton and Mauch-Mani, 2004; Ton *et al.*, 2005; Hückelhoven, 2007). Important factors of the basal resistance are cell wall-associated defence mechanisms and this resistance seemed to be suppressed by virulent pathogens (Hückelhoven, 2007).

Induced resistance is described as the enhanced effectiveness of basal resistance by specific stimuli experienced by the plant before contact with the pathogen (Ton *et al.*, 2005; Sticher *et al.*, 1997; Pieterse *et al.*, 1998). Typically, induced resistance in plants results in decreased symptom development and pathogen growth compared to non-induced plants (Hammerschmidt, 1999). The classical type of induced resistance is often referred to as systemic acquired resistance (SAR) and is activated after primary infection, typically with a necrotizing pathogen, conferring resistance to a broad range of virulent pathogens,

generally pathogens that colonize the apoplast and multiply within the host for a certain time period before symptoms such as cell death and tissue damage occur. The reaction occurs not only locally in the attacked plant parts but also systemically in distant areas of the plant (Kuc, 1982; Ryals *et al.*, 1996; Sticher *et al.*, 1997; Feys and Parker, 2000; Pieterse *et al.*, 2001; Kunkel and Brooks, 2002).

The signalling pathway controlling SAR requires endogenous accumulation of the stress hormone salicylic acid (SA) at the infection site and systemically in the plant (Gaffney *et al.*, 1993, Mauch-Mani and Métraux, 1998; Nawrath and Métraux, 1999) and an intact defence regulatory protein nonexpressor of PR-1 (NPR1) also described in the literature as noninducible immunity1 (NIM1) or salicylic acid insensitive1 (SAI1) (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997; Bostock, 2005). NPR1 has also been suggested to be involved in the activation of JA and ethylene responses (Pieterse, *et al.*, 1998; Pieterse *et al.*, 2001). The induction of pathogenesis- related proteins (PRs) is commonly observed during *R* gene-*avr* gene interactions and SAR (Hammond-Kosack and Jones, 1996; Van Loon, 1997).

Another signalling pathway is dependent on a functional jasmonate (JA) and ethylene (ET) signalling in plants, which generally confers resistance to necrotrophic organisms or insect herbivory and, both compounds are also involved in the classically termed induced systemic resistance (ISR), that is stimulated after challenging the roots with non-pathogenic plant growth-promoting rhizobacteria (van Loon *et al.*, 1998; Feys and Parker, 2000; Pieterse *et al.*, 2001; Schreiber and Desveaux, 2008). The synergistical induction of several defense related genes, including plant defensins and enzymes involved in phytoalxein biosynthesis, by jasmonate and ethylene in response to different pathogens is known (Xu *et al.*, 1994; Ecker and Davis, 1987; Gundlach *et al.*, 1992; Penninckx *et al.*, 1998).

Nevertheless, the role of ET in plant resistance seems to be somewhat controversy, leading to disease resistance in some cases, or symptom development in other cases (Pieterse *et al.*, 2001; Broekaert *et al.*, 2006). This ethylene insensitivity has been shown to increase susceptibility to pathogens such as *Septoria glycines*, *Rhizoctonia solani*, *Phytium* spp., *Botrytis cinerea*, and *Erwinia carotovora* in various plant species (Knoester *et al.*, 1998; Hoffman *et al.*, 1999; Thomma *et al.*, 1999; Norman-Setterblad *et al.*, 2000). Different classes of proteins have an ET-responsive element (GCC-box) in their promoter regions,

including vacuolar β-1,3 glucanases (PR-2), vacuolar basic-chitinases (PR-3), acidic hevein-like proteins (PR-4) and plant defensins (PDFs; PR-12). Induction of these genes occurs, as described above, synergistically with the JA pathway (reviewed in Broekaert *et al.*, 2006).

Especially for the signalling molecules SA and JA, antagonistic effects have been demonstrated (Pena-Cortés *et al.*, 1993; Doares *et al.*, 1995; Felton *et al.*, 1999), but also synergistic effects between the SA-dependent and JA/ET-dependent pathways are known, and are described especially for JA and ET positive interactions (van Wees *et al.*, 2000; Kunkel and Brooks, 2002; Bostock, 2005). Thus, cross-talk between signalling pathways likely allows the plant to prioritize responses, leading to an optimized activation of plant defense responses (Reymond and Framer, 1998; Feys and Parker, 2000; Pieterse *et al.*, 2001).

Besides the well established plant endogenous signalling molecules SA, JA and ET (Dong, 1998; Pieterse *et al.*, 2001; Thomma *et al.*, 2001), emerging evidence arose in the last decades that additional molecules modulate disease resistance, when exogenously applied. Among them are benzothiadiazole (BTH) (Friedrich *et al.*, 1996; Iriti and Faoro, 2003), β-aminobutyric acid (BABA) (Cohen, 2002; Ton and Mauch-Mani. 2004), oligosaccharides (OGAs) from plant cell walls (Hahn *et al.*, 1981), chitin and chitosan (Barber *et al.*, 1989; Agrawal *et al.*, 2002), phosphates (Gottstein and Kuc, 1989) and the application of silicon, all of which have proven to enhance the resistance reactions in various plant species (Epstein, 1994, Epstein, 1999).

Silicon

Silicon in plant biology

Silicon (Si) is a beneficial nutrient element for plants present in most soils, and the second most abundant element in the earth's crust. A common range for silicon concentrations in the soil solution varies between 0.1 – 0.6 mM, influenced by several biotic and abiotic factors. Silicon occurs as silica (SiO_2) and aluminium silicates, iron or calcium silicate, and it is readily absorbed by the plant in form of silicic acid (H_4SiO_4), resulting from the contact of SiO_2 with water,. Many effects of silicon *in planta* are attributed to the incorporation of solid amorphous silica ($SiO_2 * nH_2O$) into the cell walls (Epstein, 1994; Epstein, 1999). The content varies greatly among species, with a range from 0.1% to 10% silicon in dry weight (Ma and Takahashi, 2002). Plants can be classified into three groups dependent on their silicon accumulation capacity: (**1**) typical silicon-accumulators, with a silicon content of more than 1% silicon and a Si/Ca mol ratio higher than 1, common amog the graminaceoues plants such as rice; (**2**) silicon-intermediate-accumulators with a silicon content of 0.5 – 1% silicon or higher, but with a Si/Ca mol ratio less than 1, with cucumber belonging to this group; and (**3**) classical silicon-non-accumulators with a silicon content less than 0.5%, such as tomato plants. The uptake mode is active for the first group, passive for the second and rejective for the third group (Mitani and Ma, 2005; Ma *et al.*, 2001). Besides a positive effect of silicon nutrition on the sugar content in sugarcanes and on the yield of rice (Savant *et al.*, 1999; Seebold *et al.*, 2000), silicon has been proven to be beneficial in various aspects of plant biology, resulting in increase in photosynthesis, enhanced tolerance to metal toxicity, alleviated biotic and abiotic stress tolerance, reduction of frost damages and improvement of soil fertility, physical soil properties and increased pest and disease resistance (Epstein, 1994; Fawe *et al.*, 1998; Perry and Keeling-Tucker, 1998; Epstein, 1999; Iwasaki *et al.*, 2002a; Dannon and Wydra, 2004; Gao *et al.*, 2004; Liang *et al.*, 2005a,b; Diogo and Wydra, 2007).

Silicon in plant resistance

The investigation of the silicon effect on plant disease resistance was subject of numerous studies in the last decades. Most experiments were performed with silicon-accumulator and silicon-intermediate-accumulator plants investigating plant-fungal interactions. Positive effects on plant resistance by silicon application were also described in *Arabidopsis thaliana* to the powdery mildew fungus *Erysiphe cichoracearum* (Ghanmi *et al.,* 2004), in cucumber with the powdery mildew fungus *Sphaerotheca fuliginea* (Menzies *et al.*, 1991), for various fungal diseases in rice including blast caused by *Magnaporthe grisea*, brown spot caused by *Cochliobolus miyabeanus,* sheath blight caused by *Rhizoctonia solani*, stem rot caused by *Magnaporthe salvinii* and leaf scald caused by *Monographella albescens* (Seebold *et al.,* 2000; Rodrigues *et al.,* 2003; Fauteux *et al.,* 2005) and in wheat for powdery mildew caused by *Blumeria graminis*, septoria leaf blotch caused by *Mycosphaerella graminicola*, leaf spot caused by *Puccinia nodorum* and eyespot caused by *Oculimacula yallundae* (Rodgers-Gray and Shaw, 2004). Silicon amendment showed not only increased resistance towards fungal diseases, but also towards insects, such as a reduced preference, longevity, and production of nymphs of the green-aphids *Schizaphis graminum* on wheat (Basagli *et al.*, 2003).

Only few studies of silicon-non-accumulator plants were conducted so far. An induced resistance by silicon application to tomato was observed against bacterial wilt, caused by *R. solanacearum* (Dannon and Wydra, 2004; Diogo and Wydra, 2007). The mode of action by which silicon induces resistances remains speculative, but for accumulator plants it is assumed that silicon forms a mechanical barrier to fungal penetration, supported by observations in barley, where silicon accumulated in papilla (Carver *et al.*, 1987). Indeed, numerous studies focused on mechanical barriers and showed an involvement of this cell wall strengthening, particularly for silicon-accumulator plants (Epstein, 1994; Kim *et al.,* 2002; Bélanger *et al.,* 2003; Dakora and Nelwamondo, 2003; Fauteux *et al.,* 2005).

However, the reinforcement of the cell wall can not be an universal explanation, considering the induced resistance of silicon in non-accumulator plants (Dannon and Wydra, 2004; Diogo and Wydra, 2007) or the loss of prophylactic effects of silicon in cucumber against powdery mildew after stopping the silicon application (Samuels *et al.*, 1991).

According to the mechanical barrier hypothesis, the irreversibly accumulated silicon should have at least slowed the pathogen spread in the plant tissue, while accumulating at the infection sites, which was not in all studies the case (Chérif *et al.*, 1992b; Fauteux *et al.*, 2005). Interestingly, enhanced activity of flavonoid phytoalexins, peroxidases and polyphenoloxidases, chitinases, and increased accumulation of phenolic compounds in cucumber by silicon fertilization after infection with *Pythium ultimum* and *Sphaerotheca fuliginea* was observed (Samuels *et al.*, 1991; Chérif *et al.*, 1994a; Fawe *et al.*, 1998; Liang *et al.*, 2005b). A higher accumulation of antimicrobial compounds, for instance diterpenoid phytoalexins, was present in rice at infection sites of *Magnaporthe grisea* due to silicon treatment and enhanced activity of peroxidase and elevated PR-1 levels were observed (Rodrigues *et al.*, 2004; Rodrigues *et al.*, 2005). These are indications for a type of silicon-induced resistance in plants as proposed earlier by Kessmann *et al.* (1994) and Schneider and Ullrich (1994).

The accumulation of silicon and early activation of PR proteins are suggested to be key mechanisms of pant resistance mediated by silicon (Dann and Muir, 2002; Liang *et al*., 2005a). Determinants for susceptibility or resistance in plants are not only the presence or absence of expressed genes in the reaction, but also the rapidity and magnitude with which the genetic information is expressed. Thus, a possible involvement of Si in the regulation of plant defense genes was suggested by Chérif *et al*. (1992a). Furthermore, Si is supposed to induce plant defense only in response to infection with pathogens, in order to invest energetic costs only in infected plants (Chérif *et al.*, 1994b; Schneider and Ullrich, 1994).

However, the exact mechanism by which silicon interferes in plant signaling remains still unclear (Fauteux *et al.*, 2005).

CHAPTER 1

Inhibition of endo- and exopolygalacturonases of *Ralstonia solanacearum* by polygalacturonase-inhibiting protein (PGIP) activity in tomato stem extracts

Abstract

Polygalacturonases (PGs) activities of *Ralstonia solanacearum* comparing wild-type and non-virulent phenotype conversion mutant (PC) strains, and their inhibition by polygalacturonase inhibiting proteins (PGIPs) from tomato stems were investigated. In cultures of wild-type strain ToUdk2, slimy (s), retarded slimy (rs) and non-slimy (ns) colonies appeared. The conversion of 's' into 'rs' colony form coincided with begin of PG production. PG activity of the PC strain increased about 5 h earlier, and was up to 35 times higher in media supplemented with diverse tomato stem extracts or polygalacturonic acid compared to the wild-type at 6 hpi, and generally 4 to 8 times higher across test media and time. By chromatography (HIC), FACE - PAGE and MS analyses endo-PG PehA and exo-PGs PehB and PehC were identified. PGs of the PC mutant consisted mainly of endo-PG. The increased PG production after supplementing the medium with tomato cell wall extract was reflected by a higher activity of exo-PGs for both strains. Total PGs and endo-PG and exo-PGs activities were inhibited by PGIPs of tomato stem extracts. PGIP activity was concentration dependent, constitutively present, and not related to resistance nor susceptibility of tomato recombinant inbred lines. For the first time a plant PGIP activity against a bacterial pathogen is reported. Observations indicate that endo- and exo-PG production is governed by a sensitive regulatory network, which, in interaction with PGIP and cell wall degradation products, leads to generation or avoidance of elicitor-active oligomers, and, thus, may contribute to the development of the compatible or incompatible interaction.

1.1 Introduction

Plant cell wall degrading enzymes

Various enzymes are involved in plant cell wall degradation by pathogens (Collmer and Keen, 1986). *Ralstonia solanacearum* secretes an endoglucanase for the degradation of cellulose (Roberts *et al.*, 1988a), while three polygalacturonases are responsible for the hydrolytic degradation of the pectic compounds, major constituents of the primary cell wall and middle lamella and of the pit membranes in vascular tissue (Allen *et al.*, 1993; Huang and Allen 2000). The polygalacturonases secreted by *R. solanacearum* are an endopolygalacturonase (PehA or PglA), an exo-poly-α-D-galacturonosidase (PehB) and an exopolygalacturonase (PehC) (Huang and Allen, 1997; Allen *et al.*, 1991; González and Allen 2003). The enzymes differ in the release of reaction products after digesting polygalacturonate as substrate, with PehA releasing trigalacturonic acid and larger oligomers by cleaving polygalacturonate randomly along the chain, PehB producing digalacturonic acid and PehC generating only monogalacturonic acid (Tans-Kersten *et al.*, 1998). Experiments with site-directed mutants lacking PehA, PehB or both revealed significantly reduced virulence in all mutants, thus indicating that polygalacturonase activity contributes quantitatively to bacterial wilt development (Schell *et al.*, 1988; Huang and Allen 1997)

Though, the exact role of polygalacturonases in the infection process is not clear. Inactivation of cell wall degrading enzymes in *R. solanacearum* deletion mutants revealed that each single enzyme is not essential for a successful infection and disease development, but can contribute – even though to a different extent comparing enzymes - to bacterial virulence (Huang and Allen 2000; González and Allen 2003; Denny, 2006).

PehA is regulated by a two-component regulator (PehSR) at low population densities, which also affects PehB and PehC. Thus, PGs are mainly secreted early in the infection process and therefore discussed rather as beneficial for invasion and spread of *R. solanacearum* (Allen *et al.*, 1997), than as providers of nutrients for the pathogen. This observation was claimed at least for PehC by González and Allen (2003), who demonstrated that degradation products of this exo-PG are not necessarily metabolised by *R. solanacearum*.

However, secretion of cell wall degrading enzymes is also well known for a great number of phytopathogenic pathogens to achieve successful colonization of plant tissue by depolymerization of cell wall components (Bateman and Millar, 1996; Collmer and Keen, 1986). Thus, the importance of pectic enzymes as virulence factors of pathogenic fungi has been demonstrated for two inducible pectate lyases of *Nectria hematococca*, pectin methylesterase of *Botrytis cinerea* and for endopolygalacturonase of *Alternaria citri* and *Botrytis cinerea* (Ten Have *et al.*, 1998; Rogers *et al*. 2000; Isshiki *et al.*, 2001; Valette-Collet *et al.*, 2003; D'Ovidio *et al.*, 2004a).

Polygalacturonase-inhibiting proteins

The degradation of plant cell walls by enzymes can be influenced by the presence of polygalacturonase-inhibiting proteins (PGIPs) - leucine-rich repeat (LRR) proteins, that were shown to specifically inhibit fungal polygalacturonases (Bellincampi *et al*. 2004). Formation of a complex between polygalacturonase and the polygalacturonase-inhibiting protein (PGIP) *in vitro* results in an alteration of the balance between release of elicitor-active oligogalacturonides and depolymerization of this oligogalacturonides to inactive molecules, thus favouring accumulation of elicitor-active components (De Lorenzo *et al*. 1994).

Polygalacturonase-inhibiting proteins are in most cases plant cell wall bound or, to a lesser extent soluble proteins of the extracellular matrix (Cervone *et al.*, 1997; Mattei *et al*. 2001), and widely distributed in different dicotyledonous and monocotyledonous plants (De Lorenzo *et al.*, 2001; Kemp *et al.*, 2003). Generally, their primary structure is characterized by the presence of repeats derived from a 24-amino acid leucine-rich peptide (De Lorenzo *et al.*, 2001) and their inhibitory activity appears to be the result of a complex formation from polygalacturonase and PGIP (Cervone *et al.*, 1987), where the binding of the PGIP to the barrel cleft (Armand *et al.*, 2000) or to the region opposite the substrate binding cleft, leads to a conformational change of the polygalacturonase, followed by a decrease of enzymatic activity (King *et al.*, 2002).

Inhibitory activity of PGIPs towards polygalacturonases not only differ among various plant sources, even from a single plant source differences in activity of polygalacturonases from various fungi or different polygalacturonases from the same fungus were observed,

though varying in strength (De Lorenzo *et al.*, 2001). PGIPs were demonstrated constitutively in uninfected plant tissues (Toubart *et al.*, 1992), but also an increased synthesis was induced by several stimuli, for example mechanical wounding (Yao *et al.*, 1999).

Until now, PGIPs have been shown to be only effective against fungal polygalacturonases, but ineffective against other pectic enzymes or even polygalacturonases of microbial or plant origin (Cervone *et al.*, 1990; Vidhyasekaran 2002). Therefore, the aim of this study was to investigate a possible PGIP activity against the PGs of *R. solanacearum*, and to elucidate the interaction between endo- and exo-PGs and extracts of tomato stems containing PGIPs, deriving from genotypes differing in resistance to *R. solanacearum*.

1.2 Materials and methods

1.2.1 Bacterial cultures and media

Ralstonia solanacearum strain ToUdk2 (race 1, phylotype 1; originated from Thailand) in two colony forms, wild-type (mucoid, virulent) and a spontaneous phenotype conversion (PC) mutant (non-mucoid, non-virulent), were grown on TTC medium [10 g / L Bacto peptone, 1 g / L casamino acid, 5 g / L glucose, 15 g / L agar; 10 mL of a 0.5 % 2,3,5-triphenyl tetrazolium chloride (Sigma, Germany) were sterile filtrated and separately added to the cooled TTC medium after autoclaving (Kelman, 1954)] for 48 h at 30°C. A single colony was used for inoculation of the pre-culture. A modified EG medium, originally described in by Schell *et al.* (1988) for crude protein preparation, was used as liquid pre-culture (24 h at 30°C and 110 rpm) and as basal medium (50 mM sodium-potassium phosphate, pH 7.0, 0.07% $(NH_4)_2SO_4$, 0.03% $MgSO_4$ * $7H_2O$, 0.00003% $ZnSO_4$, 0.00005% $Ca(NO_3)_2$, 0.00002% $MnSO_4$, 0.00003% $FeCl_3$, 0.1% Casamino acid, 0.1% yeast extract, 1% glycerol). An aliquot of 0.9 mL of the pre-culture was transferred to the following media: (a) basal medium, (b) tomato stem extract (TE) medium obtained by homogenisation of 6 g tomato (genotype KingKong2) stem tissue per L medium in basal medium and subsequent filtration, (c) tomato cell wall tissue (TT) medium, the solid fraction from the TE-medium preparation, was added to the basal medium, (d) basal medium supplemented with polygalacturonic acid (PGA, 1% w/v, Na-polygalacturonic acid, Sigma, Germany), and incubated for 24 h on a rotary shaker (110 rpm) at 30°C. The culture fluid was harvested by centrifugation (5,800 x g, 15 min) at 4°C and used for enzyme assays. The bacterial growth rate was monitored by measuring the optical density of the medium at 620 nm with a spectrophotometer and by counting of dilution platings.

E. coli strains expressing either PehB or PehC (pQHBET and pPehC7, respectively; kindly provided by C. Allen) were grown on LB medium (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) for 24 h at 30°C before transferring them into 30 mL King B liquid pre-culture medium (2% Bacto peptone, 0.15% K_2HPO_4, 1.5% glycerol, 0.15% $MgSO_4$*$7H_2O$) and further cultivation for 24 h at 30°C under agitation. Pre-cultures were used to inoculate 1,000 mL King B medium and incubated over night as described above. When necessary, media were supplemented with antibiotic kanamycin (25 µg mL^{-1}) or ampillicin (50 µg mL^{-1}). For induction of over-expression of plasmid qQHBET a

1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, Germany) solution was added to the medium 4 h before harvesting of the cultures. Cultures were centrifuged for 15 min at 5,818 x g at 4°C and the pellet was suspended in 50 mM 2-(N-Morpholino)-ethane sulphonic acid (MES; Carl Roth, Germany) buffer (pH 5.7), subsequently sonicated on ice and centrifuged for 15 min at 9,425 x g at 4°C. Supernatants were used to determine polygalacturonase activity with thin layer chromatography (TLC), as described in 1.2.3.1.

1.2.2 Fungal culture, medium and preparation of enzyme extract

Fusarium oxysporum f.sp. *lycopersici* (IPP reisolate) was grown in synthetic medium (SM) consisting of 0.2 g $MgSO_4*7H_2O$, 0.4 g KH_2PO_4, 0.2 g KCl, 1 g NH_4NO_3, 0.01 g $FeSO_4$, 0.01 g $MnSO_4$ in 1 L demineralised H_2O (Di Pietro and Roncero 1996). Medium was supplemented with 1% (w/v) Na-polygalacturonate from citrus (Sigma, Germany). After incubation for 36 h at 28°C and 150 rpm the mycelium was separated by filtering and the filtrate centrifuged for 10 min at 9,500 x g at 4°C. The supernatant was collected and kept at –20°C until determination of polygalacturonase activity.

1.2.3 Detection of polygalacturonase activity

1.2.3.1 Thin layer chromatography

Thin layer chromatography was performed according to Lojkowska *et al.* (1995). Briefly, bacterial or fungal culture supernatants were incubated with polygalacturonic acid substrate (4% Na-polygalacturonate (Sigma, Germany) in 50 mM MES buffer, pH 5.7) for 1 h at 45°C and 25 µL total sample were applied on Whatmann thin layer chromatography plates (K5 silica gel 150A, 20 x20 cm, layer thickness 250 µm). As solvent solution a 5 : 3 : 2 mixture of n-butanol : water : glacial acetic acid was used, before staining the plates with 96% ethanol, phosphomolybdic acid spray (Sigma, Germany) and concentrated sulphuric acid in a 6:3:1 ratio. On each plate, a standard of mono-, di- and trigalacturonic acid (Sigma, Germany), each in a concentration of 4 µg, was additionally applied. The retention factor (Rf) was calculated.

1.2.3.2 Agarose diffusion assay (ADA)

Based on the method of Dingle *et al*. (1953) PG activity was estimated as described in Schacht (2005). Briefly, bacterial and fungal enzyme extract was applied to wells in agarose gels composed of 50 mM MES buffer pH 5.7, 0.8% agarose and 0.5% Na-polygalacturonate from citrus (Sigma, Germany). After incubation of 48 h at room temperature, plates were developed with 5 M HCl. Appearing halos or rings in the gel around the inoculation well were measured. All tests were conducted in triplicates in the same gel, the control without addition of plant extract was tested in four replicates.

1.2.3.3 Degradation assay

The PG activity was measured in 25 mM citrate buffer (pH 5.0) at 37^{o}C. The PG enzyme activity assay was initiated by the addition of 2% polygalacturonic acid (Sigma) to the culture filtrate containing PGs, to a final concentration of 0.5%. The activity was measured with a spectrophotometer (OD 550 nm; Pharmacia, Sweden) based on the changes in reducing sugars, according to Nelson (1944) and Somogyi (1945). The PG activity is expressed as µmol glucose equivalents released per min at 37°C per mL fraction.

1.2.4 Plant material and inoculation procedure

Tomato plants of genotype King Kong2 (Known-You Seed Co., Taiwan) and tomato recombinant inbreed lines NHG3, NHG13, NHG162, NHG60 (AVRDC, Taiwan) were either cultivated in white peat (Klasmann-Deilmann, Germany) supplemented with 4 g L^{-1} $CaCO_3$ (Roth, Germany) or in substrate (Fruhstorfer Erde, Germany) and kept under greenhouse conditions (20°C with 14 h light per day at 30 K lux and 70% relative humidity). Plants grown in white peat were watered with a nutrient solution composed of 5 mM $Ca(NO_3)_2$, 1.875 mM K_2SO_4, 1.625 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.04 mM H_3BO_3, 0.001 mM $ZnSO_4$, 0.001 mM $CuSO_4$, 0.01 mM $MnSO_4$, 0.00025 mM Na_2MoO_4, 0.05 mM NaCl and 0.1 mM Fe-EDTA.

Four to five week old plants were inoculated with *R. solanacearum* strain ToUdk2 (race 1, phylotype 1; originated from Thailand) directly after transplanting and transferring them into a growth chamber (30°C / 27°C day/night temperature, 85% relative humidity, 30 K

Lux and 14 h light per day). Inoculum suspensions were prepared from two day old bacterial cultures of *R. solanacearum* strain ToUdk2 wild-type grown on TTC medium by adjusting the suspension in demineralised water to an optical density of 0.06 at 620 nm for NHG3, NHG13, NHG162, NHG60 or diluting the suspension by 1:5 for King Kong2, corresponding to approximately $1.13*10^8$ or $2.03*10^7$ CFU per mL, respectively. Per gram substrate 0.1 mL suspension was applied to each plant. Controls were treated with the same quantity of demineralised water. The plants were harvested either 12 hours post inoculation (hpi) or 5 days post inoculation (dpi).

1.2.5 Symptom Evaluation

Symptoms of ten plants per treatment were monitored daily and classified in six classes as disease severity: **0** = healthy plant, **1** = one leaf wilted, **2** = two leaves wilted, **3** = three leaves wilted, **4** = all leaves wilted except the tip of the plant, **5** = whole plant wilted.

The mean of disease scores represents the wilt disease severity (DS). The disease incidence (DI) was recorded daily and calculated as the percentage of dead plants in the total number of plants at the evaluation date. The area under disease progress curve (AUDPC) was calculated on the basis of either wilt disease severity or disease incidence using the following formula (cited after Jeger and Viljanen-Rollinson, 2001):

$$AUDPC = \sum_{i=1}^{n-1} \left[(x_i + x_{i-1})/2 \right] (t_i - t_{i-1})$$

with x_i and x_{i-1} - wilt incidence or disease severity scale, and t_i and t_{i-1} - consecutive evaluation dates (t_i-t_{i-1} is equal to 1 day).

1.2.6 Extraction of plant material for determination of polygalacturonase-inhibiting protein (PGIP) activity

Midstem parts of three plants per treatment of genotypes NHG3, NHG13, NHG162, NHG60 and King Kong2 were homogenized in extraction buffer (10 mM MES, 1 M NaCl, pH 5.7) at a ratio of 4 mL buffer per g fresh weight (FW) and incubated under stirring on ice for 2 h. Subsequently, the slurry was filtered through cheese cloth and centrifuged

(16,600 x g for 15 min at 4°C). Supernatants were used as plant extracts for PGIP activity test.

1.2.7 Extraction and characterization of PG isozymes

1.2.7.1 Isozyme separation by hydrophobic interaction chromatography (HIC)

The crude bacterial extracts were loaded in 0.5 M ammonium acetate buffer (pH 6.0) containing 1.5 M ammonium sulphate in a total volume of 60 mL onto a phenyl sepharose column (5 cm, Ø 0.5 cm, 2 mL gel) (Pharmacia, Sweden), calibrated with the same buffer with a flow rate of 1 mL min^{-1}. Bound protein was eluted by a linear gradient (60 mL) of 1.5 M ammonium sulphate in 0.5 M ammonium acetate buffer (pH 6.0) to 0.5 M ammonium acetate buffer (pH 6.0) or to water. For eluting of remaining proteins, the column was additionally washed with 10 mL water. Fractions of 3 mL were collected and subsequently tested for conductivity (mSi) and PG activity.

1.2.7.2 Fluorophor-assisted carbohydrate – polyacrylamid – gel electrophoresis (FACE-PAGE) for quantification and analysis of liberated carbohydrate fragments

The single PG isozymes deriving from the peaks of the HIC-chromatography were incubated 15 and 120 minutes with polygalacturonic acid (PGA, Sigma, Germany; 1% final concentration). The reaction was stopped by shock frosting the samples. About 15 to 20 µg mL^{-1} total carbohydrate per sample was dried in a Speed-Vac concentrator and dissolved in a mixture of 20 µL sodium-cyanoborohydride (1 M in dimethylsulfoxid) and 20 µL ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid, 0.2 M in acetic acid : water 3 : 17 v/v) and incubated for 16 h at 37°C. Subsequently, the samples were dried again in a Speed-Vac and dissolved in 20 µL sample buffer (62.5 mM Tris-HCl, pH 6.8; 20% glycerol). The samples were analysed on a 40% acrylamide gel (T : C / 18 : 1) and run for 90 min at 300 V on ice, using a BioRad Mini Protean II system. The gels were photographed with a digital camera at 305 nm wavelength and the pictures were processed with Adobe Photoshop CS. As marker a mixture of 0.1 µg mono-, 0.2 µg di-, and 0.4 µg trigalacturonic acid per lane was used. Each unused lane contained 2 µL phenol red (0.25 mg mL^{-1}) and 2 µL bromphenol blue (0.25 mg mL^{-1}) for orientation.

1.2.7.3 Mass spectrometry

N-terminal cleavage products obtained from Coomassie Blue-stained polyacrylamide gels were identified after tryptic digestion in a MALDI-TOF mass spectrometer (Ultraflex I, Bruker Daltonics, Bremen, Germany). Briefly, bands were cut out of the gel, destained using 50% acetonitrile and dried by addition of 100% acetonitrile and incubation in a speed vac system. Trypsin solution (10 ng L^{-1}) was added to the dried gel piece. After overnight incubation at 37°C, peptides were extracted from the gel using 5% trifluoracetic acid (TFA) containing 10% acetonitrile. Extraction solutions were dried and dissolved in 0.2% TFA and 50% acetonitrile. Samples were mixed with 1 µg of α-cyano-4-hydroxyl-cinnamonic acid (CHCA), dissolved in 0.2% TFA and 50% acetonitrile and applied to the MALDI target. MS and MS/MS spectra were collected and all data were analyzed using the Biotools (Bruker Daltonic) and MASCOT (Matrix Science, UK) software packages.

1.2.8 Polygalacturonase-inhibiting protein (PGIP) activity

1.2.8.1 Agarose diffusion assay (ADA)

PGIP activity was determined as described by Dingle *et al.* (1953), modified by Schacht (2005). A mixture of either 10 µL *R. solanacearum* or 25 µL *F. oxysporum* f. sp. *lycopersici* enzyme extract and plant extracts [either 0.5 mg (2 µL), 2 mg (8 µL) or 8 mg (32 µL) FW plant extract] were applied to wells in an agarose gel. As control *R. solanacearum* or *F. oxysporum* f.sp. *lycopersici* enzyme extract without plant extract were applied on each gel. All samples were adjusted to the same quantity in wells (42 µL *R. solanacearum* or 57 µL *F. oxysporum*) with 50 mM MES buffer pH 5.7. After incubation for 48 h at room temperature the plates were developed with 5 M HCl for ten minutes. Diameters of appearing halos or rings in the gel around the inoculation wells were measured as enzyme activity. Brightness of halos were included in the evaluation by multiplying diameter values by a factor deriving from visual estimation of the halo intensity: 3 - control and samples with identical bright white halos, 2.5 - white halo, 2 - whitish halo, 1.5 - slightly white halo, 1- fade halo. All tests were conducted in triplicates in the same gel, the control without plant extract in four replicates, assays were repeated with plant samples from different trials.

1.2.8.2 Calculation of PGIP activity

The relative inhibitory activity [%] of the plant extracts was calculated based on the formula described in Richter (2005):

100 – [(diameter *Rs* enzyme extract + plant extract) / diameter *Rs* control] * 100

1.2.8.3 Degradation assay

One hundred microliters of isozymes derived from the HIC were incubated for 10 min with 50 mg FW, 100 mg FW, and 137.5 mg FW of stem extracts. Reducing sugars were determined as described in 1.2.3.3 degradation assay:

100 – [(red. sugars *Rs* enzyme extract + plant extract) / red. sugars *Rs* control] * 100

1.2.8.4 Characterization of polygalacturonase-inhibiting protein activity

Plant extracts were filtered through 0.2 µm, 0.45 µm, 0.8 µm, 1.2 µm cellulose acetate membrane filters (Minisart; Sartorius, Germany) and 0.45 µm nylon and PTFE membrane filters (Roth, Germany). These filtrates and the supernatants from boiled (10 min) and ammonium sulphate precipitated plant extract were tested for activity with 10 µL *R. solanacearum* enzyme extract in the agarose diffusion assay. The possible effect of buffer quantity was tested by incubation of *R. solanacearum* culture filtrate with different quantities of extraction buffer (10 mM MES, 1 M NaCl, pH 5.7). The activity of the supernatant from a 70% ammonium sulphate precipitation of plant extract was tested against *R. solanacearum* culture filtrate. For control, plant extracts from inoculated plants without addition of bacterial culture filtrate were tested in the ADA test for PG activity.

1.2.9 Other methods

SDS-PAGE was performed according to Laemmli (1970). Total protein content was determined by using Coomassie assay kit (Pierce) for microtiter plates according to the manufacturer's instruction, or by the method of Bradford (1976). Statistical analyses were performed with R (R Development Core Team – R Foundation for Statistical Computing, Vienna, Austria). The program was used for Tuckey test or Welch test at $\alpha = 5\%$.

1.3 Results

1.3.1 Polygalacturonase activity of bacterial and fungal culture

Polygalacturonase (PG) activity of *R. solanacearum* culture filtrates was detected by an agarose diffusion assay optimized in gel thickness, enzyme quantity addition and incubation time and temperature, in order to obtain a maximum halo or ring formation in the polygalacturonic acid (PGA) containing gel indicating the enzymatic activity (data not shown). By thin layer chromatography the formation of monomers, dimers and trimers after digestion of PGA by PGs of culture filtrates of *R. solanacearum* (Rf: 0.45 – 0.47, 0.36 – 0.39 and 0.29 – 0.32, respectively), and of monomers and dimers after addition of culture filtrates of *Fusarium oxysporum* f.sp. *lycopersici* (Rf: 0.46) and *Escherichia coli* pQHBET expressing PehB (Rf: 0.35 – 0.37), respectively was confirmed. *E. coli* BL21(DE3) used for control for non-expression of pectolytic enzymes in *E. coli* as well as *E. coli* pPehC7 showed no reaction products (data not shown).

1.3.2 Multiplication and polygalacturonase activity of *R. solanacearum in vitro*

Population development of the *R. solanacearum* wild-type (WT) and the non-virulent phenotype conversion mutant (PC) strains in media supplemented with tomato stem extract (TE), polygalacturonic acid (PGA) or tomato stem cell wall tissue (TT) were generally similar over the trial period of 30 hours (Fig. 1.1A and 1.1B), while clear differences were observed in the PG activity between the strains and comparing media over 52 hours (Fig. 1.1C and 1.1D).

Three types of colonies were observed in the media, (i) slimy [s] colonies, corresponding to the wild type, (ii) retarded slimy [rs] colonies, with a four days delayed exopolysaccharide (EPS) production, and (iii) non-slimy [ns] colonies. Towards the end of the exponential phase at 6 hpi, the wild type colonies partly converted to the retarded slimy form, in basal medium (BM) + PGA and BM + TT, or to non-slimy colonies in BM and in BM + TE (Table 1.1, Fig. 1.1C). The latter conversion was reversible, and normal type colonies occurred again after about 27 hpi in BM and BM + TE.

PG activity increased to a maximum at 30 hpi in the wild-type strain, with high values in the BM + PGA and BM + TT, followed by lower activities in BM + TE and BM (Fig. 1.1C). The conversion of the slimy bacteria into the rs colony form coincided with the beginning of the PG production (Fig. 1.1C; arrows). In cultures of the PC mutant exclusively PC-type colonies were observed. PG activity of the PC mutant increased about 5 h earlier, was up to 35 times higher in the BM + supplements compared to the wild-type at 6 hpi, and generally 4 to 8 times higher across test media and time (Fig. 1.1D). Maximal PG activity was observed at 30 hpi, with highest values in BM + PGA, followed by BM + TE and BM + TT, and BM medium. The PG activity showed a slight retardation at 24 hpi before reaching the maximum and generally decreased in all media with both strains after 30 hpi.

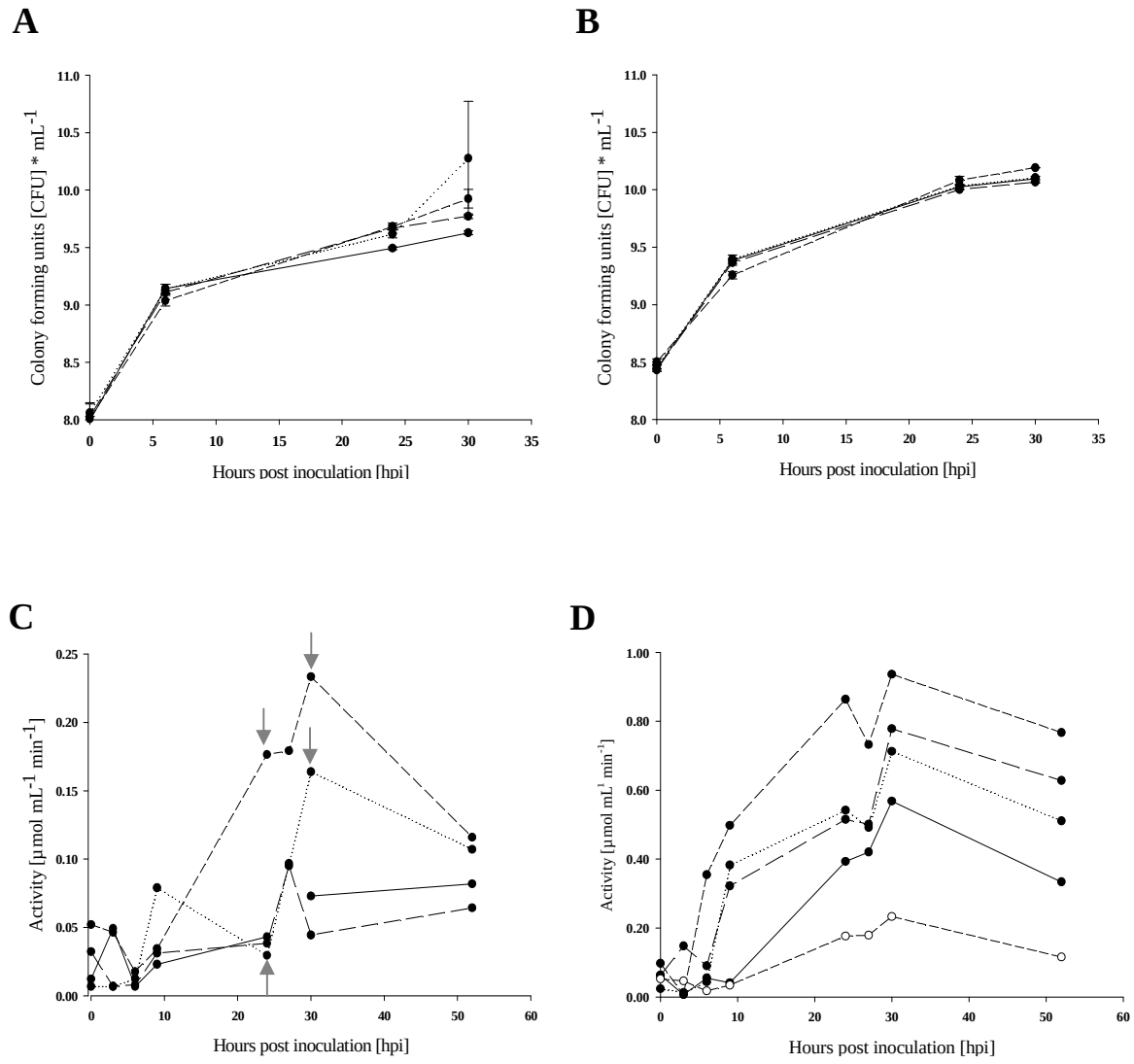

Fig. 1.1: Multiplication and polygalacturonase activity of *R. solanacearum* wild-type (A, C) and phenotype conversion (PC) mutant (B, D) in liquid cultures of basal medium and basal medium supplemented with PGA (1%), tomato cell wall tissue (6 g/L) and tomato stem extract (6 g/L).

Data [colony forming units (log CFU) / ml] are means of two replicates ± SE for the wild-type and the mutant.
Solid line: BM (basal medium); long dashed line: BM + TE (basal medium supplemented with tomato stem extract), short dashed line: BM + PGA (basal medium supplemented with 1% polygalacturonic acid), dotted line: BM + TT (basal medium supplemented with tomato cell wall tissue).
Standard errors in figure A and B are low.
D: line with open symbols represents highest PG activity from the wild-type in BM + PGA of Fig. 1c Arrows in C represent occurrence of retarded slimy colonies. PG activity: difference between sugars in the reaction mixture after one minute incubation and reduced sugars from polygalacturonate after 30 min incubation, in reduced sugars (µmol mL^{-1} min^{-1}).

Table 1.1: Occurrence of *R. solanacearum* colony types (log CFU * mL^{-1}) 24 and 30 hours post inoculation (hpi) in basal medium (BM) and basal medium supplemented with tomato stem extract (TE), tomato cell wall tissue (TT) and polygalacturonic acid (PGA).

Medium	Hours post inoculation (hpi)	*R. solanacearum* colony type		
		Slimy (s)	**Non-slimy (ns)**	**Retarded slimy (rs)**
BM	24	9.5 ± 0.01	7.1 ± 0.04	-
BM + TE	24	9.7 ± 0.05	7.3 ± 0.10	-
BM + TT	24	9.6 ± 0.02	-	7.0 ± 0.00
BM + PGA	24	9.7 ± 0.02	-	7.9 ± 0.04
BM	30	9.6 ± 0.02	-	
BM + TE	30	9.8 ± 0.02	-	
BM + TT	30	10.3 ± 0.40	-	6.2 ± 0.20
BM + PGA	30	9.9 ± 0.08	-	6.5 ± 0.10

Data are means of two replicates ± SE. - : no occurrence of the colony type.
Retarded slimy (rs) colony phenotype was non-slimy for 2 days, before slime production started on TTC medium.
BM: basal medium; BM + TE: basal medium supplemented with tomato extract; BM + PGA: basal medium supplemented with 1% polygalacturonic acid; EG + TT: basal medium supplemented with tomato cell wall tissue.
Retarded colonies are also indicated as arrows in Figure 1.1c.

1.3.3 Isozyme specific polygalacturonase activity in wild-type and mutant

After separation of the crude enzyme extracts of the wild-type and the PC strains by hydrophobic interaction chromatography (HIC), both extracts showed two PG activity peaks indicating two different isozymes (I, II), with a 10 times higher activity of peak I in the mutant than in the wild type strain (Fig. 1.2A and 1.2B).

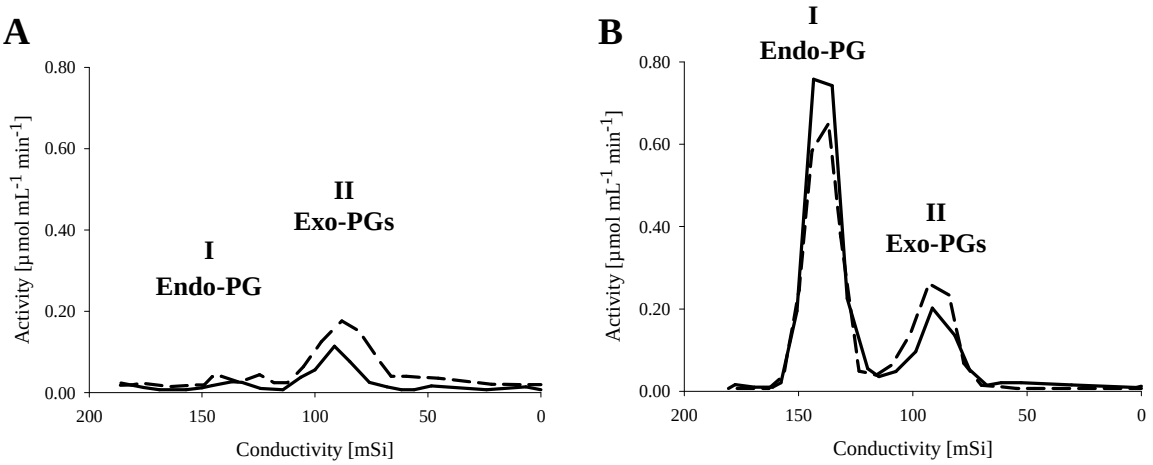

Fig. 1.2: Elution profile from hydrophobic interaction chromatography (HIC) of *R. solanacearum* wild-type (A) and mutant (B) polygalacturonases after cultivation in basal medium (straight line) and basal medium with tomato cell wall tissue (dashed line) (BM + TT) 30 h after incubation.

The extract was loaded in ammonium acetate buffer (0.5 M) containing 1.5 M ammonium sulphate on a 2 mL phenyl sepharose column and eluted with a linear gradient from buffer A (0.5 M ammonium acetate buffer + 1.5 M ammonium sulphate) against buffer B (0.5 M ammonium acetate buffer). PG activity: difference between existing sugars in the reaction mixture after one minute incubation and reduced sugars from polygalacturonate after 30 min incubation period, in reduced sugars (µmol mL^{-1} min^{-1}). Representative results of several repetitions are shown.

1.3.4 Isozyme characterization

1.3.4.1 Cleavage mode

PGs exist as exo-cleaving (EC number 3.2.1.67) or endo-cleaving enzymes (EC number 3.2.1.15). Fluorophor-assisted carbohydrate – polyacrylamid – gel electrophoresis (FACE - PAGE) of HIC peak 1 showed breakdown products ranging from dimers to oligomers with a degree of polymerization (DP) of > 12 at fifteen minutes after incubation with PGA, and degradation to dimers and oligomers with a DP of < 6 after 120 min incubation, revealing a cleavage mode typical for endopolygalacturonases (Fig. 1.3). Cleavage of PGA with HIC peak II resulted in monomer and dimer breakdown products, with an increase in formation of monomers over the incubation time, revealing a cleavage profile typical of an exopolygalacturonase. In the following, peak 1 is referred to as endo-PG and peak 2 as exo-PGs.

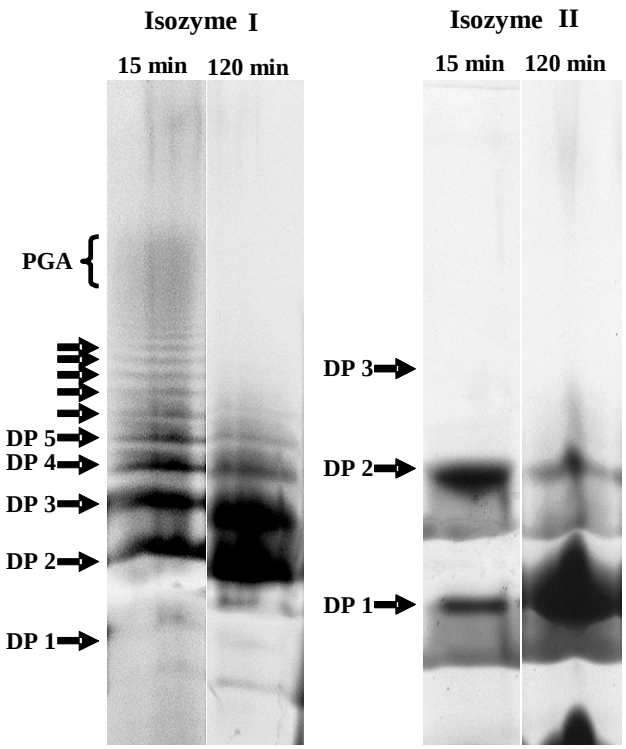

Fig. 1.3: Product analysis from polygalacturonic acid incubated with endo- or exo-polygalacturonase isozymes by fluorophor assisted carbohydrate - polyacrylamid – gel electrophoresis (FACE - PAGE).

Single polygalacturonase isozymes from HIC were incubated with PGA and the reaction was stopped after 15 or 120 min incubation. The liberated cleavage products were derivatized with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and subsequently analyzed by PAGE. DP 1, DP2, and DP 3 were loaded as markers. Oligosaccharides with a higher DP were calculated by rf-values.

1.3.4.2 Mass spectrometry

Presence of *R. solanacearum* endo-PG (PehA) in HIC peak I and exo-PGs PehB and PehC in HIC peak II were confirmed by MS analysis. In HIC peak II five further *R. solanacearum* proteins were detected (Fig. 1.4, Table 1.2).

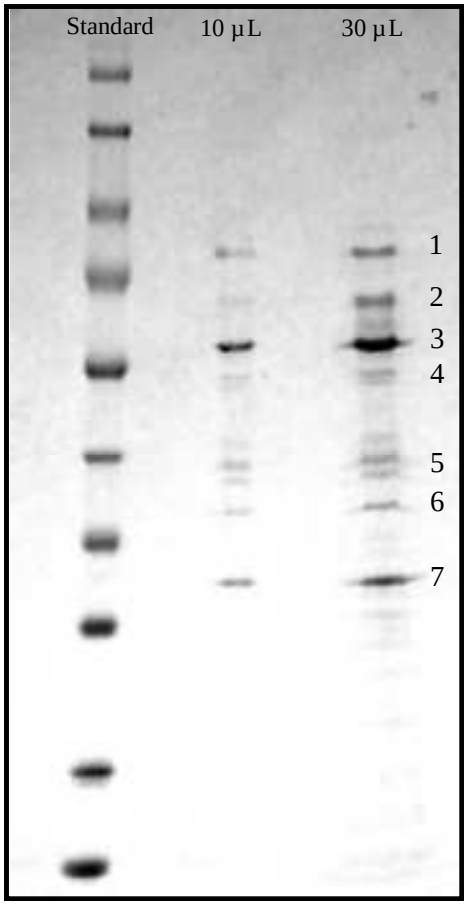

Fig. 1.4: Protein bands of exo-PG fraction obtained by hydrophobic interaction chromatography (HIC).

Ten microliter and 30 µL correspond to 55 µg and 166 µg protein, respectively. No 1 – 7 correspond to MS analysed proteins in Table 2

Table 1.2: Mass spectrometry (MS) analysis of *R. solanacearum* proteins in peak II (exo-PG peak) of hydrophobic interaction chromatography.

Band No	Protein [Accession]	Gene locus[1]
1	**Exo-poly-galacturonisidase (PehB), (signal peptide protein) [Q8XYK2_RALSO]**	**RSc1756**
2	**Exopolygalacturonase (PehC), (polygalacturonase transmembrane protein) [Q8XRJ8_RALSO]**	**RSp0833**
3	Putative bacterial extracellular solute-binding, family 1, Abc transporter protein [Q8XUY2_RALSO]	RSc3051
4	Probable catalyse hydroperoxide hpll oxidoreductase protein [Q8XPQ7_RALSO]	RSp1581
5	Putative extracellular endo-α-1,4 poly-galactosaminidase Or related polysaccharide hydrolase protein [Q8XX75_RALSO]	RSc2241
6	Putative porin signal peptide protein [Q8XV98_RALSO]	RSc2933
7	Porbable transmembrane protein [Q8XX78_RALSO]	RSc2238
	Endopolygalacturonase (PehA, PglA)[2] [PGLR1_RALSO]	**RSp0880**

[1] gene locus according to Salanoubat *et al*. (2002).
[2] Endo-polygalacturonase (PehA) was found in HIC peak 1, independently determined by MS analysis (gel not shown)

1.3.4.3 Stimulation of isozymes by tomato cell wall tissue

The increase of total PG production 30 hpi in medium supplemented with tomato cell wall tissue of 124% (0.07µmol*mL^{-1}*-min^{-1} in BM and 0.16 µmol*mL^{-1}*-min^{-1} in BM + TT) and 25% (0.57 µmol*mL^{-1}*-min^{-1} in BM and 0.71 µmol*mL^{-1}*-min^{-1} in BM + TT) in the wild-type and the PC mutant, respectively (Fig. 1.1C and 1.1D) is reflected by a higher activity of exo-PGs for wild-type and mutant strains, and of endo-PG for only the wild-type strain (Fig. 1.2A and 1.2B). Endo-PG activity of the mutant decreased in BM + TT medium in comparison to the basal medium. Supplementing the basal medium with tomato cell wall tissue led to a 54% (0.11 µmol*mL^{-1}*-min^{-1} in BM and 0.18 µmol*mL^{-1}*-min^{-1} in BM + TT) and 30% (0.20 µmol*mL^{-1}*-min^{-1} in BM and 0.26 µmol*mL^{-1}*-min^{-1} in BM + TT) increase in exo-PG for the WT and the PC strain, respectively.

1.3.5 Polygalacturonase-inhibiting protein (PGIP) activity in tomato stem extracts

Stem extracts of healthy (h) and *R. solanacearum* inoculated (i) plants from tomato genotype King Kong2, moderately resistant to bacterial wilt, showed inhibitory activity on PGs of *R. solanacearum* in the agarose diffusion assay (Fig. 1.5). Inhibition increased with quantities of plant extracts applied, irrespective of the healthy or inoculated status of the extracted plants. The quantity of plant extract applied in the test was standardized on mg FW tomato stem in order to screen possible differences in PGIP accumulation between genotypes, which might be underestimated when adjusting on total protein contents in the extracts.

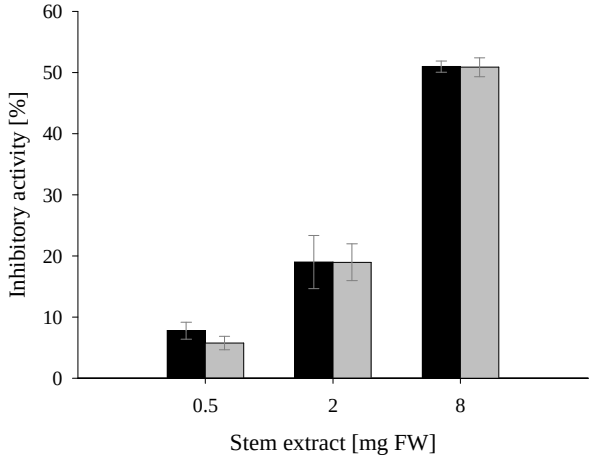

Fig. 1.5: Inhibition of polygalacturonase activity of *R. solanacearum* by plant extracts from tomato stems of genotype King Kong2, healthy or inoculated with *R. solanacearum* strain ToUdk2 at 5dpi, in agarose diffusion assays.

Black columns: healthy plants; grey columns: inoculated plants.
Data are means ± standard error of four independent experiments with three plants per treatment.
Inhibitory activity (%) was calculated according the formula:
100 – [(diameter *Rs* enzyme extract + plant extract) / diameter *Rs* control] * 100

Comparing extracts of four tomato recombinant inbred lines (RILs) differing in resistance, either healthy or *R. solanacearum* inoculated (12 hpi and 5 dpi) [NHG 13 with an area under disease incidence progress curve (AUD_IPC) 0.0 ± 0.0, NHG 162 AUD_IPC 55.0 ± 55.0 (resistant); NHG 3 AUDPC 763.3 ± 140.3, NHG 60 AUD_IPC 230.0 ± 153.1 (susceptible)] for PGIP activity, no differences between genotypes nor an effect of inoculation were observed (Fig. 1.6A and 1.6B). Generally, PGIP activity increased significantly from 12 hpi to 5 dpi across genotypes and treatments with plant age using extract from 8 mg stem fresh weight (FW) in the test.

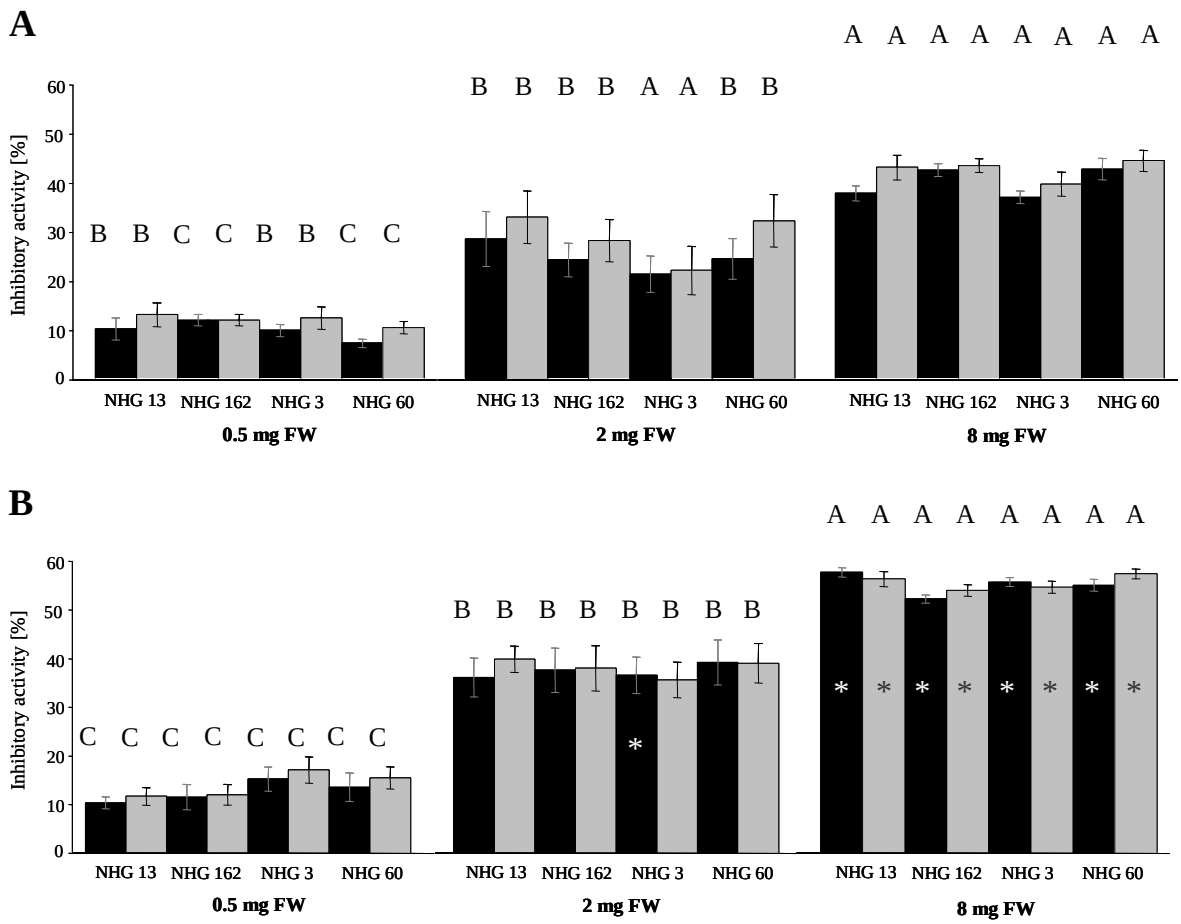

Fig. 1.6: Inhibition of PG of *R. solanacearum* by stem extracts of tomato recombinant inbred lines NHG 3, NHG 60 (susceptible) and NHG 13, NHG 162 (resistant) healthy and inoculated with *R. solanacearum* strain ToUdk2 at 12hpi (A) and 5dpi (B) in agarose diffusion assays.

Black columns: healthy plants; grey columns: inoculated plants.
Data are means ± standard error of two independent experiments with six plants per treatment. Capital letters refer to comparison of the same genotype, time point and treatment (healthy or inoculated) with different quantities of plant extracts. The same letters are not significantly different with Tuckey test at 5%. *: significantly higher inhibitory activity with extract from 5 dpi compared to 12 hpi for the same treatment with Tuckey test at 5%. Inhibitory activity (%) was calculated according to the following formula: 100 – [(diameter *Rs* enzyme extract + plant extract) / diameter *Rs* control] * 100

The total protein content was significantly higher in inoculated, susceptible genotypes NHG3i (24.4 ± 3.3 mg g^{-1} FW) and NHG60i (29.4 ± 5.2 mg g^{-1} FW) than in non-inoculated ones (NHG3h: 9.5 ± 1.9 mg g^{-1} FW, NHG60h: 7.1 ± 1.3 mg g^{-1} FW), and tendenciously higher in genotypes NHG13i and NHG162i (18.5 ± 3.7 mg g^{-1} FW and 15.9 ± 1.3 mg g^{-1} FW, respectively) than in NHG13h and NHG162h (9.3 ± 1.9 mg g^{-1} FW and 8.1 ± 2.4 mg g^{-1} FW, respectively) (data not shown).

1.3.6 Characterization of polygalacturonase-inhibiting protein (PGIP) activity

The inhibitory activity of extracts of healthy and *R. solanacearum*-inoculated tomato RIL genotypes was generally at least four times higher across treatments with *R. solanacearum* than with *F. oxysporum* f. sp. *lycopersici* enzyme extract (Fig. 1.7).

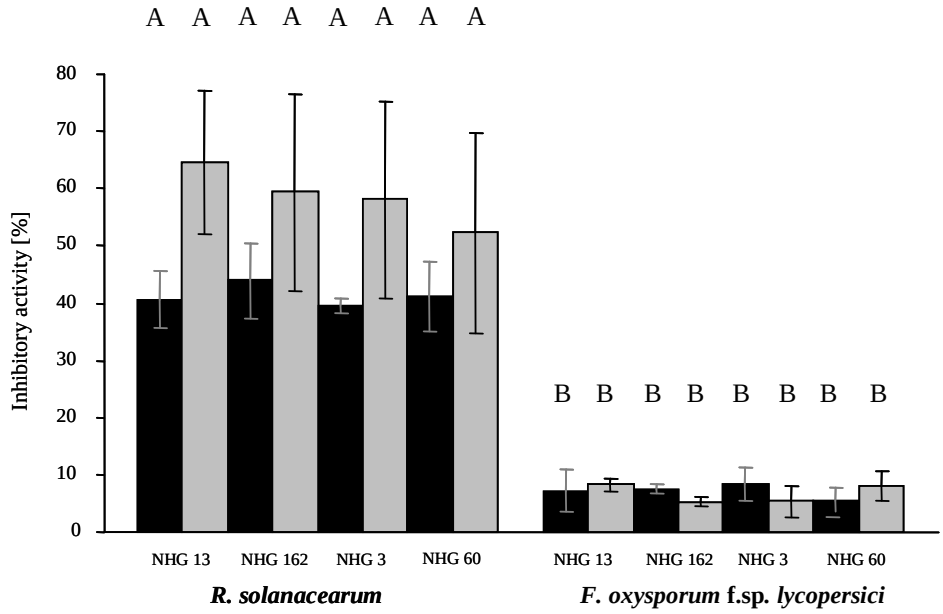

Fig. 1.7: Inhibitory activity (%) of extract from healthy and inoculated tomato recombinant inbred lines NHG 13 and NHG 162 (resistant) and NHG 3 and NHG 60 (susceptible) (2 mg stem FW) to *R. solanacearum* and *F. oxysporum* f.sp. *lycopersici* enzyme extracts.

Evaluation of halo diameter with brightness factor.
Black columns: healthy plants; grey columns: inoculated plants.
Data are means of six plants per treatment for *F. oxysporum* f.sp. *lycopersici* and of six plants per treatment in three replicates for *R. solanacearum* ± standard error. Columns followed by different letters are significantly different with Welch t-test at 5% comparing enzyme extracts from the two pathogens. Brightness factor 3 - control and samples with identically bright white halo; 2.5- white halo; 2- whitish halo; 1.5 - slightly white halo; 1 - fade halo. Inhibitory activity (%) = 100 – [(diameter enzyme extract + plant extract) / diameter control] * 100.

PGIP activity of extracts was not influenced by heat treatment at 100°C for ten minutes, nor by filtering of the plant extracts (filters of pore sizes 0.2 – 1.2 µm; materials: cellulose acetate, nylon or PTFE). PG activity remained unaffected by increasing quantities of MES buffer with 1M NaCl. No PG activity was detected by applying inoculated plant extracts (8 mg FW quantity) to the agarose diffusion assay. Supernatants from ammonium sulphate precipitated plant extracts lost their ability to inhibit *R. solanacearum* PGs (data not shown).

1.3.7 PGIP activity on endo- and exo- polygalacturonases

Stem extract of healthy and *R. solanacearum*-inoculated plants of genotype King Kong2 inhibited endo-PG and exo-PG of *R. solanacearum*, with increasing inhibition with higher quantities of plant extract (Fig. 1.8). An activation of endo-PG, but not of exo-PG was observed after adding the lowest quantity of plant extract.

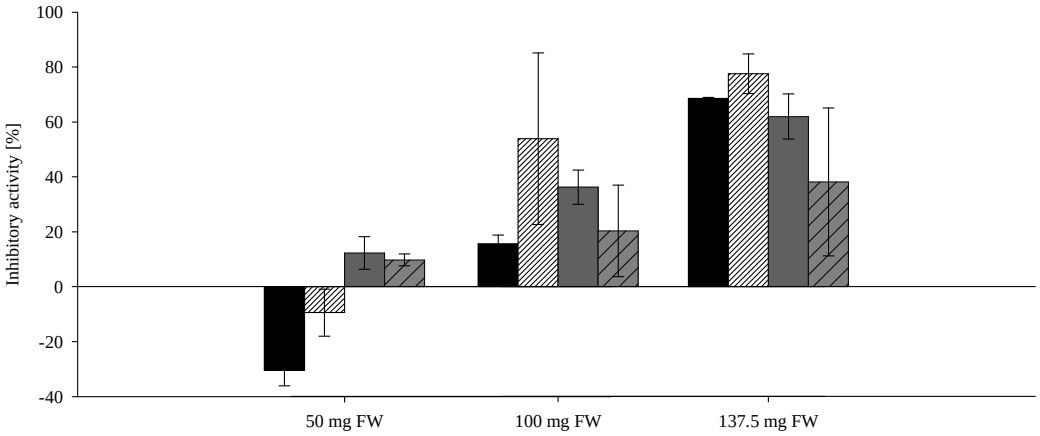

Fig. 1.8: Inhibition of endo-PG and exo-PG of *R. solanacearum* wild type by extracts of tomato genotype King Kong2, healthy and inoculated with *R. solanacearum*, measured by concentration of degradation products.

Data are means ± SE from three extracts of healthy and two extracts of inoculated plants of tomato genotype King Kong2.
Endo-PG (black columns) and exo-PG (grey columns); healthy (filled columns) and inoculated (striped columns).
One hundred microliter of either endo-PG or exo-PG derived from HIC were incubated with extracts of 50 mg FW, 100 mg FW, and 137.5 mg FW tomato stems. For controls the quantity of plant extract was substituted by water.
The inhibitory activity (%) was calculated according to the formula:
100 – [(produced galacturonic acid of *Rs* enzyme extract + plant extract) / produced galacturonic acid *Rs* control] * 100

1.4 Discussion

The activity of PGIP on bacterial PGs was observed for the first time. The PGIP effect in tomato stems was demonstrated *in vitro* on the chromatographically separated endo- and exo-PGs of *R. solanacearum*. PG activity of *R. solanacearum* was characterized.

Though the population development of the wild type and the mutant were similar in all test media, PG activity increased notably after occurrence of colonies of the converted rs and ns form in wild type cultures supplemented with plant cell wall compounds. The conversion of the wild-type to the typical PC colony form normally occurs under stress conditions in a low frequency and is in most cases not reversible (Denny, 2006), though Poussier *et al.* (2003) described a reversion from PC to wild-type colonies *in planta*. Thus, our rs and ns type colonies represent other conversion forms of *R. solanacearum*, different from the typical PC form. In our former studies, a higher percentage of converted colonies were re-isolated from stems of resistant plants at 5 days post inoculation compared to stems of a susceptible genotype, suggesting that the conversion resulted from the interaction with components of the defence reaction of the plant (Diogo, 2005).

In our studies the typical PC mutant produced PGs mainly consisting of endo-PG earlier and at lower population densities than the wild type strain. It remains to be investigated if the PC type is avirulent, because it may be better recognized by the plant due to the action of endo-PGs, which are known to produce oligogalacturonates with a degree of > 10, which are potent elicitors of defence responses in plants (Ryan, 1987). It is known that PC mutants of *R. solanacearum* are unable to wilt host plants and deficient in exopolysaccharide production (Kelman, 1954), but their endo-PG secretion is elevated (Brumbley and Denny, 1990). Also the conversion from slimy to retarded slimy and non-slimy colonies in cultures of the wild type occurring specifically in media supplemented with PGA and tomato cell wall tissue was related to PG production, though this conversion was reversible, since they were not found at later sampling times. Thus, *R. solanacearum* possesses a highly sensitive system to react flexible in production of various virulence factors such as PGs and extracellular polysaccharides to environmental changes, thereby exhibiting various degrees of phenotypic conversion.

R. solanacearum strain ToUdk2 culture filtrate separated by HIC showed two peaks containing endo- or exo-PGs. Further analysis of the two peaks by MS identified all three

polygalacturonases described for this pathogen (Schell, 2000), with endo-PG eluting separately from the two exo-PGs suggesting that the exo-PGs have similar biochemical characteristics. In the wild type strain the exo-PG secretion was higher than the endo-PG secretion and was additionally stimulated by about 50% by tomato cell wall tissue [TT] in the medium, while Schell *et al.* (1988) found the major polygalacturonase of *R. solanacearum* strain AW being endo-active. The detected, though low endo- and exo-PG activity in culture supernatants of the wild-type suggest that PGs are constitutively expressed. A certain level of constitutive or basal synthesis of the inducible PGs is suggested to provide degraded plant cell wall components in the early infection stage, eliciting the resistance signaling cascade, and triggering the defence response of the plant (Alghisi and Favaron, 1995).

In its optimised form, the ADA test revealed suitable for detection and quantification of PG activity, though the quantities of secreted polygalacturonases in culture filtrates of *E. coli* PehC seemed not sufficient for detection in this test.

Also Allen *et al.* (1991) observed an increased PG activity of *R. solanacearum* strain K60 cultures in minimal medium supplemented with intercellular fluids from tobacco leaves, and higher PG activity from *in planta* grown bacteria, but they did not distinguish between the isozymes responsible for the increase. Similar to our observations, García Maceira *et al.* (1997) found that an exo-PG of *Fusarium oxysprum* f. sp. *lycopersici* was stimulated by PGA. Additionally, Kelemu and Collmer (1993) described pectate lyase (PL) isozymes of *Dickeya chrysanthemi* EC16, induced by cell walls of chrysanthemum stem pieces, but not by pectate. Whether PehB or PehC or both exo-PGs of strain ToUdk2 are stimulated needs further investigations. Molecular approaches using microarray technology recently showed that the environmentally induced type III secretion pathway also influences the secretion of exo-poly-α-D-galacturonosidase (PehB) by HrpG (Valls *et al.*, 2006), and an influence of HrpB on exo-PG C (PehC) was suggested (Hikichi *et al*., 2007), indicating that both exo-PGs can be stimulated by plant tissue. Thus, contact with the plant cell wall in *R. solanacearum* strain GMI1000 is suggested to influence the expression of PGs.

The spontaneous mutant strain we used in our study showed typical PC characteristics such as non-mucoid morphology (Poussier *et al*., 2003), and a 10 times increased endo-PG activity compared to the wild-type strain, while the exo-PG activity was similar in both strains cultured in basal medium. But also in the PC strain, a tendenciously increased exo-

PG activity of about 30% was observed after addition of plant tissue. Also Brumbley and Denny (1990) reported a higher activity in PGs without differentiation of endo- and exo-PGs isolated from *R. solanacearum* PC-type and PC-like mutants obtained by Tn5 mutagenesis than the wild-type.

Pathogenicity and virulence of *R. solanacearum* are regulated by an extensive genetic network, the Phc (phenotype conversion) system (Schell, 2000), composed of a LysR-type transcriptional regulator, PhcA (Schell, 1993) and products of an operon, phcBSRQ (Clough *et al.*, 1994; Clough *et al.*, 1997), controlling the levels of active PhcA in response to variations in bacterial cell densities. Activation of several virulence genes, such as EPS biosynthesis, pectin methylesterse (Pme) and β-1,4-endoglucanase (Egl) occurs after a certain cell density threshold is exceeded, while parallely genes coding for motility, pili, endo-PG (PehA), siderophore and Hrp machinery are inhibited at this state (Schell, 2000). The endogenous signal molecule 3-hydroxypalmitic acid methyl ester (3-OH PAME) (Flavier *et al.*, 1997) autoregulates the expression of *phcA*, providing a system for *R. solanacearum* to distinguish between early and late virulence functions, depending on bacterial density (Genin and Boucher, 2002). In our *in vitro* studies, PG production started at about 10^9 cells per mL for the wild type and the PC mutant.

Interestingly, the pectinolytic enzymes are reported not to be coordinately controlled and the exo-PGs PehB and PehC seemed to be separately regulated apart from PehA (Schell, 2000). As mentioned above, the PhcA system controls PehA, but in an indirect way by reducing the function of the PehS/PehR two-component regulatory system, normally positively controlling the expression (Denny, 2006). PehR is activated by a so far unknown plant signal (Tans-Kersten *et al.*, 2004), and the inactivation leads to loss of endo-PG activity, and flagellar and twitching motility. Accordingly, in both strains the exo-PG activity was stimulated by addition of tomato cell wall tissue in the culture medium.

The FACE-PAGE analysis of exo-PG from *R. solanacearum* and thin layer chromatographic analyses showed breakdown products of only dimers and monomers, but not of elicitor active oligomers which range in size from 10 to 13 oligogalacturonides (Ryan, 1987). Thus, by higher expression of exo-PG the pathogen might escape effectively plant defence responses by being late or not recognized by the plant, since potent elicitor active oligomers are not produced or could be rapidly degraded by the action of exo-PG.

We found that endo-PG can be stimulated by low quantities of plant extracts *in vitro*. Also after incubation of PGs of *R. solanacearum* PC mutant with plant extracts in the agarose diffusion assay, an increase in PG activity for some plant extract quantities was observed (data not shown). Similar observations were made by Kemp *et al.* (2004) in a fungal system describing a pH dependent activation of two constitutively expressed endo-PGs from *Aspergillus niger* by PGIP2 from *Phaseolus vulgaris*. The differential increase of exo- and endo-PG activities observed after addition of tomato cell wall tissue or plant extracts, respectively, point at a highly sensitive regulation system of *R. solanacearum* in interaction with the plant.

We demonstrated for the first time that compounds in plant extract actively and specifically inhibited bacterial PGs. It is well described that fungal PGs can be inhibited by polygalacturonase-inhibiting proteins present in the cell wall of numerous plant species (De Lorenzo *et al.*, 2001), but also PGs from the phytophagous insects *Lygus rugulipennis* and *Adelphocoris lineolatus* were inhibited by PGIPs (D'Ovidio *et al.*, 2004b). Thus, also different PGIPs recognized and inhibited specifically various fungal PGs (De Lorenzo *et al.*, 2001). But, an inhibition of bacterial PGs as well as PGs of plant origin, and of other microbial pectic enzymes i.e. pectate lyases could not be successfully demonstrated to date (Cervone *et al.*, 1990; De Lorenzo and Ferrari 2002).

A specific inhibition of PGs by plant extract was observed, with an at least four times higher inhibition of PGs from *R. solanacearum* than from *F. oxysporum* f. sp. *lycopersici* indicating that the plant extracts contain either the same PGIP with variable specificity towards PGs from different origin, or that different isoforms of the protein or different PGIPs are responsible for the inhibition. The PGIP activity can vary depending on the plant species, but different inhibitory activities were also observed by PGIPs from the same plant against PGs from various fungal species, and even for PGs from the same fungus (De Lorenzo and Ferrari, 2002). Thus, the existence of a variety of PGIPs or isoforms of PGIP is probable, since it was found that only few amino acid-replacements of the PGIPs decide over the inhibitory activity towards different PGs (Leckie *et al.*, 1999). The characterization studies on the nature of PGIP suggest that the active principle is a glycoprotein, indicated by its retained activity after heating and its absent activity in extract supernatants after precipitation by 70% ammoniumsulfate.

The analysis of the interaction between *R. solanacearum* – tomato PGIP reveals a constitutive expression of PGIP, without significant differences between extracts from tomato genotypes differing in resistance to *R. solanacearum*, and, generally, comparing healthy and inoculated treatments. On the other hand, some evidence was found that PGIPs in *Phaseolus vulgaris* and apple play a role in the discrimination of resistant and susceptible genotypes (Lafitte *et al.*, 1984; Buza *et al.*, 2004), and more recently the role of PGIPs in plant defence was discussed as part of the plant innate immunity (Federici *et al.*, 2006). To identify possible differences in PGIP accumulation between genotypes or in reaction to infection, we chose to standardize the test by using the same quantities of plant tissue and not to adjust to the same protein concentration. The latter could lead to an underestimation of the effect since the protein level in plants of different resistance reaction and treatment could vary greatly, specifically when also bacterial proteins are prevalent. We observed that the inhibition of polygalacturonases by extracts from *R. solanacearum* inoculated plants was more heterogeneous than by extracts from healthy plants. Additionally, with extract from inoculated plants the inhibition of endo-PG was tendenciously higher than the inhibition of exo-PG. These indications for a possible specifically induced inhibition of endo-PG should be further analysed.

Extract of healthy control and *R. solanacearum* inoculated plants at 12 dpi were more active than from plants at 5 dpi. These results are supported by our quantitative gene expression studies, where an increase of PGIP mRNA in tomato stems was observed in *R. solanacearum* inoculated plants 72 hpi (Ghareeb, 2007). Different PGIP activities were also found in different plant tissue types of various plant species, tissues of different age, specifically in fruits of variable maturity, with higher activities in the immature state compared to the mature state (Abu-Goukh *et al.*, 1983; Johnston *et al.*, 1993; Fisch, 2005). Exploring vegetative plant tissues, PGIP activity increased with seedling age (Salvi *et al.*, 1990).

The observations indicate that endo- and exo-PG production is governed by a highly sensitive and finely regulated network, which, in interaction with PGIP and plant cell wall degradation products, leads to the generation or avoidance of elicitor-active oligomers, and, thus, contributes to the development of a compatible or incompatible interaction. In conclusion, PGIP is suggested to be involved in the resistance reaction of tomato to *R. solanacearum*, though its precise role in the molecular interaction has to be further characterized.

CHAPTER 2

Effect of silicon nutrition and *Ralstonia solanacearum* inoculation on peroxidase and polyphenol oxidase activity in tomato stems

Abstract

Silicon-amendment led to decreased symptom development in three of four tomato recombinant inbred lines (RILs) differing in their resistance to *R. solanacearum*. Investigations of peroxidase (POD) activity of these RILs showed generally no significant differences in the enzymatic profile among genotypes and treatments at 5 days post inoculation (dpi), an evaluation date corresponding to the beginning of wilt symptom development. The experiments were extended with the moderately resistant commercial genotype King Kong2, examining four evaluation dates corresponding to the beginning of symptom development (5dpi), difference in symptom development of silicon- treated and non-treated plants (8 dpi and 12 dpi) and survived plants (26 dpi). Polyphenol oxidase (PPO) measurements were included in this set of experiments.

Differences in POD activity ($\mu mol*min^{-1}*gFW^{-1}$) across treatments at the same evaluation dates could be observed with lower activity in non-inoculated (+Si-Rs) treated plants compared to both inoculated treatments (-Si+Rs and +Si+Rs) at 8 dpi. Comparing the same treatments at different evaluation dates, a significant increase in activity was observed in non-silicon, non-inoculated (-Si-Rs) plants at 26 dpi compared to 5 dpi, and a significant decrease in activity for silicon-treated, non-inoculated (+Si-Rs) plants at 8 dpi compared to 12 and 26 dpi. Differences in specific POD activity (per mg protein) were not observed.

At 8 dpi, PPO units per mg protein were significantly decreased in the silicon-treated inoculated (+Si+Rs) plants compared to the non-treated, non-inoculated (-Si-Rs) controls. Comparing the same treatment at different evaluation dates, PPO activity of non-treated (-Si-Rs) controls was significantly decreased at 8 dpi compared to 5dpi for

units per gram fresh weight and for silicon treated, inoculated (+Si+Rs) plants at 8 dpi compared 5 and 12 dpi for units per gram fresh weight and units per mg total protein.

2.1 Introduction

Characteristics and enzymatic reactions: peroxidases

Peroxidases (PODs) are heme-containing enzymes which catalyze oxireduction between hydrogen peroxide and reductans: $H_2O_2 + AH_2 \rightarrow 2H_2O + A$; Donor + $H_2O_2 \rightarrow$ oxidized donor + $2H_2O$ (Hiraga *et al*., 2001; Blokhina *et al*., 2003).

They are classified into three superfamilies based on their structural and catalytic properties (Welinder, 1991). Guaiacol peroxidase is grouped into class III plant peroxidase (EC 1.11.17), which are secretory plant peroxidases that are located in cells walls or transported into vacuoles (Barceló and Munoz, 2000).

Peroxidases in plant resistance

Peroxidases have been shown to play a role in physiological processes like lignification (Whetten *et al.*, 1998), suberization (Espelie *et al*., 1986), germination under high osmotic conditions (Amaya *et al.*, 1999), but also in the cross-linking of cell wall structural proteins (Fry, 1986), just to name a few.

An involvement of peroxidases in defence strategies of plants to pathogens was indicated early by Lovrekovich *et al.* (1968), later they were classified as pathogen-related (PR) proteins – PR-9- by van Loon *et al*. (1994). Induction of these enzymes was reported after wounding (Roberts *et al.*, 1988b) and after infection with fungi (Harrison *et al*. 1995; Thordal-Christensen *et al*., 1992), viruses (Lagrimini and Rothstein, 1987; Hiraga *et al.*, 2000) and bacteria (Reimers *et al.*, 1992; Rasmussen *et al.*, 1995). Peroxidase are suggested to (a) strengthen the plant cell wall via lignification, suberization, feruloylated polysaccharides and hydroxyproline-rich glycoproteins (Vance *et al.*, 1980; Fry, 1986; Bowles, 1990); (b) elevate production of reactive oxygen species (ROS) as signal mediators and antimicrobial agents (Bolwell *et al.*, 1995; Wojtaszek, 1997; Kawano and Muto, 2000); and (c) increase phytoalexin production (Kristensen *et al.*, 1999).

Characteristics and enzymatic reactions: Polyphenol oxidases

Polyphenol oxidases (PPOs) are located in the thylakoid membranes of chloroplasts and often occur in multiple forms (Mayer and Harel, 1979; Moore and Flurkey, 1995). They are classified into monophenol oxidase (tyrosinase; EC 1.14.18.1) and catechol oxidase (or *o*-diphenol:oxygen oxidoreductase, EC 1.10.3.2), which all have a dinuclear copper complex with histidine ligands at the active site (Mayer and Harel 1979; Mayer, 2006). The primary substrates are phenolic compounds and as enzymatic reaction, oxygen is inserted in a position *ortho*- to an existing hydroxyl group in an aromatic ring (monophenol oxidase activity; Fig. 2.1), usually followed by oxidation of the diphenol to the corresponding quinone (diphenol oxidase activity; Fig. 2.2) (Mayer and Harel, 1979; Yoruk and Marshall, 2003; Siegbahn, 2004; Mayer, 2006). Molecular oxygen is needed in both reactions as co-substrate (Mayer, 2006). Generally, the most prevalent form of PPO activity in higher plants is the diphenolase activity (Yoruk and Marshall, 2003).

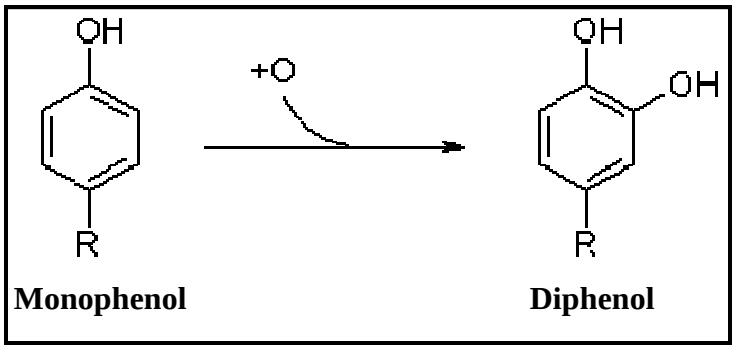

Fig. 2.1: Monophenol oxidase pathway producing the diphenol (from Marshall *et al*., 2000)

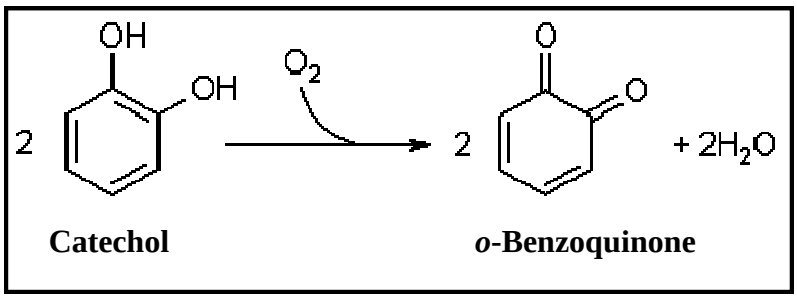

Fig. 2.2: Diphenol oxidase pathway producing the quinones, (from Marshall *et al*., 2000)

The resulting orthoquinones form melanins by polymerization, which are dark insoluble polymers, involved in melanogenesis, a process which is also called enzymatic browning prevalent in fruits and vegetables (Marshall *et al.*, 2000; Mayer, 2006).

Polyphenol oxidase in plant resistance

Besides their involvement in biosynthetic processes like the biosynthesis of betalains (Steiner *et al.*, 1999; Strack *et al.*, 2003), polypenol oxidases are also suggested to play a role in plant resistance to stresses and pathogens (Mayer, 2006). Early observations revealed that polyphenol oxidsase activity increases after infection with virus, bacteria, fungi or mechanical injury (Mayer and Harel, 1979). More recently, over-expression of a potato PPO in tomato plants resulted in an enhanced resistance towards *Pseudomonas syringae* (Li and Steffens, 2002). Additionally, the introduction of antisense PPO cDNA in tomato resulted in down-regulation of all members of the PPO gene family and increased susceptibility to the same pathogen (Thipyapong *et al.*, 2004a). Raj *et al.* (2006) demonstrated an involvement of PPO in the resistance of *Penisetum glaucum* (pearl millet) to *Scerospora graminicola* (downy mildew), where polyphenol oxidase was rapidly induced and showed localized, elevated levels after infection of PPO in resistant genotypes, while an accumulation in the susceptible genotype was not observed.

2.2 Materials and methods

2.2.1 Plant material and inoculation procedure

Tomato plants of genotype King Kong2 (Known-You Seed Co., Taiwan) and tomato recombinant inbreed lines (RILs) NHG13, NHG162, NHG3, NHG60 (Asian Vegetable Research and Development Centre, AVRDC; Taiwan) were cultivated in white peat (Klasmann-Deilmann, Germany) supplemented with 4 g L^{-1} $CaCO_3$ (Roth, Germany) for the non-silicon treatment and 4 g L^{-1} $CaCO_3$ plus 1g / L Aerosil (Degussa, Germany) for the silicon treatment. Plants were kept under greenhouse conditions (20 °C with 14 h light per day at 30 K lux and 70% relative humidity) and watered throughout the whole experiment with a nutrient solution composed of 5 mM $Ca(NO_3)_2$, 1.875 mM K_2SO_4, 1.625 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.04 mM H_3BO_3, 0.001 mM $ZnSO_4$, 0.001 mM $CuSO_4$, 0.01 mM $MnSO_4$, 0.00025 mM Na_2MoO_4, 0.05 mM NaCl and 0.1 mM Fe-EDTA for the non-silicon treatment, and the same solution containing additionally monosilicic acid at a final concentration of 1.4 mM [$Si(OH)_4$] for silicon treatments. Monosilisic acid was obtained after exchange of potassium silicate solution K_2SiO_2 (VWR, Germany) with cation exchangers (20 mL volume, Biorad Laboratories, Germany) (Hochmuth, 1999).

Five week old plants were inoculated with *R. solanacearum* strain ToUdk2 (race 1, phylotype 1; originated from Thailand) directly after transplanting and transferring into a growth chamber (30 °C / 27 °C day/night temperature, 85 % relative humidity, 30 K Lux and 14h light per day). Inoculum suspensions were prepared from two day old bacterial cultures of *R. solanacearum* grown on TTC medium [10 g / L Bacto peptone, 1 g / L casamino acid, 5 g / L glucose, 15 g / L agar; 10 mL of a 0.5% 2,3,5-triphenyl tetrazolium chloride (Sigma, Germany) were sterile filtrated and separately added to the cooled TTC medium after autoclaving (Kelman, 1954)] or on nutrient glucose agar (NGA; 3 g / L beef extract, 5 g / L peptone from casein, 2.5 g / L glucose, 15 g / L agar) by adjusting the suspension in demineralised water to an optical density of 0.06 at 620 nm, diluted 1 : 5, corresponding to approximately $2.03*10^7$ CFU per mL. Per gram substrate, 0.1 mL bacterial suspension was applied to each plant. Controls were treated with the same quantity of demineralised water. Stem samples from recombinant inbred lines were taken 5 days post inoculation (5dpi) and from genotype King Kong 2 additionally at 8 dpi, 12 dpi and 26 dpi. Non-inoculated controls of each treatment were included in all experiments.

2.2.2 Symptom evaluation

Symptoms of ten plants per treatment were monitored daily and classified in six classes as disease severity: **0** = healthy plant, **1** = one leaf wilted, **2** = two leaves wilted, **3** = three leaves wilted, **4** = all leaves wilted except the tip of the plant, **5** = whole plant wilted.

The mean of disease scores represents the wilt disease severity (DS). The disease incidence (DI) was recorded daily and calculated as the percentage of dead plants in the total number of plants at the evaluation date. The area under disease progress curve (AUDPC) was calculated on the basis of either wilt disease severity or disease incidence using the following formula (cited after Jeger and Viljanen-Rollinson, 2001):

$$AUDPC = \sum_{i=1}^{n-1} \left[(x_i + x_{i-1}) / 2 \right] (t_i - t_{i-1})$$

with x_i and x_{i-1} - wilt incidence or disease severity scale, and t_i and t_{i-1} - consecutive evaluation dates (t_i-t_{i-1} is equal to 1 day).

2.2.3 Bacterial quantification in tomato stems

Ralstonia solanacearum was quantified in the stems of symptomless inoculated plants, either with or without silicon amendment. Stem parts were surface sterilized with 70% EtOH for 15 s, subsequently washed with sterile demineralised water and macerated in 3 mL of sterile water. After incubation for 20 min at room temperature the macerate was filtered through cotton and centrifuged at 7000 x g at room temperature. The pellet was re-suspended in 1 mL sterile water, tenfold dilutions were prepared and 100 µL of appropriate dilutions were plated in two replicates on TTC medium (Kelman, 1954), followed by incubation for 48 h at 30 °C. Bacterial colonies were counted and calculated as colony forming units (CFU) per gram of fresh matter, expressed in log CFU / g.

2.2.4 Determination of dry matter

After finalizing symptom evaluation, plants were collected, weighed and dried at 80°C for one week. Dry matter was calculated as:

Dry matter = (Fresh weight of the plant / dry weight of the plant) * 100

2.2.5 Silicon quantification

Total silicon content in stems and roots of tomato genotype King Kong2 of the same samples used for bacterial quantification and enzyme assays at 5, 12 dpi and four weeks after inoculation was determined by spectrophotometry, using the method developed by Novozamsky *et al*. (1984), modified according to Iwasaki *et al.* (2002b).

Stem and root samples were dried at 80°C for at least 3 days and grounded in a swing mill (Sartorius, Germany). For each sample 10 mg material was weighed in an Eppendorf tube and digested with 500 µL of a solution composed of 1M HCl and 2.3 M HF in a ratio of 1 : 2, while shaking overnight. After a centrifugation step at 10,000 x g for 10 min, 20 µL supernatant was added to 250 µL 3.2% H_3BO_3 and incubated overnight with shaking. Then 250 µL color reagent (a 1 : 1 mixture of 0.08 M H_2SO_4 and 20g / L $(NH_4)_6Mo_7O_{24}*4H_2O$) were added and incubated for 30 min at room temperature. Color development occurred after adding 250 µL of a solution composed of 33 g / L tartaric acid and 0.25 mL of 4 g / L ascorbic acid. Samples were measured in micro cuvettes at 811 nm by spectrophotometry (Beckmann DU 640, USA) against a blank containing 20 µL HCl and 2.3 M HF (1 : 2) without plant material. A series of standards with Si concentrations ranging from 0 to 100 ppm was prepared and the silicon content of the samples (mg / g dry weight) was calculated using regression equation of the standard absorbance values.

2.2.6 Enzyme assays

2.2.6.1 Plant extraction

Plant material of silicon non-treated and treated, healthy and *R. solanacearum* inoculated tomato RILs NHG3, NHG13, NHG60 at 5 dpi and for genotype King Kong2 at 5, 8, 12 dpi and 4 weeks after inoculation was used for enzyme assays. Frozen stem parts were macerated in a ratio 1 : 10 (w/v) in 10 mM sodium phosphate buffer (pH 6.0) for 50 s and subsequently centrifuged at 16,600 x g for 15 min at 4°C. Supernatants were used as plant extract for enzyme assays and total protein quantification.

2.2.6.2 Guaiacol peroxidase activity

Guaiacol peroxidase activity was measured as described in Fecht-Christoffers *et al*. (2003) with modifications. The reaction mixture contained 850 µL 20 mM guaiacol (Sigma, Germany) as substrate in 10 mM sodium phosphate buffer (pH 6.0), 50 µL enzyme extract. The reaction was started by adding 100 µL 0.3% H_2O_2 (Merk, Germany) to the reaction mixture. The formation of tetraguaiacol resulting in a linear change of absorbance at 470 nm was monitored for 2 min with a spectrophotometer (Beckmann DU640, USA). A mixture containing substrate and enzyme extract served as blanks for each sample.

Enzyme activity was calculated from the change in absorbance:

$$\text{Activity} = \frac{\Delta OD}{\min} \times \frac{Vt}{Vs} \times \frac{1}{\varepsilon d} \times F = \mu mol * min^{-1} * mL^{-1}$$

With: ΔOD = change of absorbance per minute, Vt = total volume of the assay (mL), Vs = volume of enzyme extract of sample (mL), ε = extinction coefficient; guaiacol: 26.6 $mM^{-1}cm^{-1}$, d = diameter of the cuvette used in the assay, equal to 1, and F = dilution factor.

The specific activity represents the moles converted per unit time per unit mass of enzyme (enzyme activity / actual mass of protein present). Specific activity of peroxidases was calculated as:

$$\text{Specific activity} = \frac{\text{Enzyme activity}}{\text{Total protein content}}$$

2.2.6.3 Polyphenol oxidase activity

As substrate for polyphenol oxidase activity measurement 20 mM catechol (Sigma, Germany) and 1 g / L sodium dodecyl sulphate (SDS; Merk, Germany) in 20 mM sodium phosphate buffer (pH 6.0) were used, and enzyme activity was measured as described in Onyezili and Harris (1993), with modifications. The reaction was initiated by adding 250 µL plant extract to 1.500 µL substrate. After incubation for 20 min at 25°C, absorbance of the samples were measured at 410 nm against a blank of 1.750 µL demineralised H_2O. Each assay set included a plant extract blanks for each sample (250 µL plant extract and 1.500 µL demineralised H_2O) and a substrate blank (1.500 µL substrate and 250 µL demineralised H_2O). One unit of PPO activity was defined as the increase of

0.1 in A_{410} by the enzyme under experimental conditions and expressed as units per g fresh weight tomato stem tissue. The specific activity was calculated as described for guaiacol peroxidase activity.

2.2.7 Total protein content

Total protein contents were determined according to Bradford (1976) with bovine serum albumin (BSA; Sigma, Germany) as standard. A volume of 50 µL plant extract was incubated with 1.450 µL Bradford reagent (100 mg Coomassie brilliant blue G250, 50 mL ethanol absolut, 100 mL o-phosphoric acid, 850 mL demineralised H_2O) and incubated for 20 min at room temperature. Samples were measured spectrophotometrically at 595 nm (Beckmann DU 640, USA) against a blank containing Bradford reagent and 50 µL demineralised H_2O. A standard series was prepared in a range of 0 to 1.000 µg bovine serum albumin (BSA) per mL rising in 100 µg steps, and 50 µL of each standard was incubated with Bradford reagent in triplicate. Total protein concentration was calculated by using regression equation of the standard concentrations and the corresponding absorbance values.

2.2.8 Peroxidase and polyphenol oxidase activity in *Ralstonia solanacearum* liquid cultures

Ralstonia solanacearum strain ToUdk2 was grown on nutrient glucose agar (NGA) for two days before transferring two loops of bacteria into a modified EG medium [0.07% $(NH_4)_2SO_4$, 0.03% $MgSO_4$ * $7H_2O$, 0.00003% $ZnSO_4$, 0.00005% $Ca(NO_3)_2$, 0.00002% $MnSO_4$, 0.00003% $FeCl_3$, 0.1% casamino acid, 0.1% yeast extract, 1% glycerol in 50 mM sodium phosphate buffer pH 7.0; originally described by Schell *et al*. (1988)] as pre-culture. Liquid pre-cultures were incubated at 30°C for 24 hours in a rotary shaker at 110 rpm. An aliquot was then transferred into either EG medium or EG medium supplemented with 6 g / L tomato stem tissue of genotype King Kong2 as main-culture in duplicates and incubated on a rotary shaker (110 rpm) at 30°C. Non-inoculated EG medium and EG medium supplemented with 6 g / L tomato stem tissue were included as controls in the experiment. One millilitre aliquots of each culture fluid was harvested

directly after inoculation of the main-cultures and subsequently at 3, 24 and 27 hours post inoculation (hpi) by centrifugation (5,800 x g, 15 min) at 4°C. Supernatants were used for enzyme assays. Additionally, bacterial growth was monitored by counting dilution platings on TTC medium, based on the method described in bacterial quantification in tomato stems, and was calculated as colony forming units (CFU) per mL bacterial culture.

Peroxidase, polyphenol oxidase and total protein content in the supernatants were determined as described in 'enzyme assays'. As a positive control, activity of both enzymes in plant extract derived from stems of genotype King Kong2 was measured once at 27 hpi.

2.2.9 Statistical analyses

The data were analysed with the statistical software "R" (R Development Core Team – R Foundation for Statistical Computing, Vienna, Austria). For all data, the Wilcoxon rank-sum Test was used and the P values were adjusted with the method of Holm for family-wise error rate correction. A significance level of $p < 0.05$ was used throughout the whole statistical analysis of the data.

2.3 Results

2.3.1 Symptom development and bacterial quantification in tomato stems

Experiments were performed with tomato stems of healthy and *R. solanacearum*-inoculated **recombinant inbred lines** (RILs) NHG13, NHG162 (resistant to bacterial wilt) and NHG3, NHG60 (susceptible to bacterial wilt), supplemented either with (+Si) or without silicon (-Si), at five days post inoculation (dpi), corresponding to first occurrence of wilt symptoms in the susceptible genotype NHG3 (Fig. 2.3C). Silicon amendment retarded and reduced symptom development in genotypes NHG162 and NHG3 (Fig. 2.3B and 2.3C), while no clear effect was observed in genotype NHG60 (Fig. 2.3D). Genotype NHG13 showed generally only few symptoms (Fig. 2.3A).

For investigation of a possible role of peroxidases and polyphenol oxidases in silicon-induced resistance towards *R. solanacearum* in a later state of infection, mid-stem parts of the moderately resistant genotype **King Kong2** were analysed at 5dpi (begin of symptom development), 8dpi and 12dpi (differences in symptom development between treatments) and 26 dpi (survived plants) (Fig. 2.4A and 2.4B). Disease development was retarded and disease severity and incidence were decreased in silicon treated plants, though not significantly due to the typical expression of moderately resistance, leading to death of some and nearly healthy survival of other inoculated plants (Fig. 2.4A and 2.4B, Table 2.2).

Decreased symptom development, expressed in area under disease progress curve based on disease severity (AUD_SPC) and disease incidence (AUD_IPC), was observed for genotypes NHG162 and NHG3, for AUD_SPC for genotype NHG13, but no clear differences occurred for genotype NHG60 (Table 2.1). Additionally, AUD_SPC and AUD_IPC were decreased, though not significantly, in silicon treated plants compared to non-silicon treated plants for genotype King Kong2 (Table 2.2)

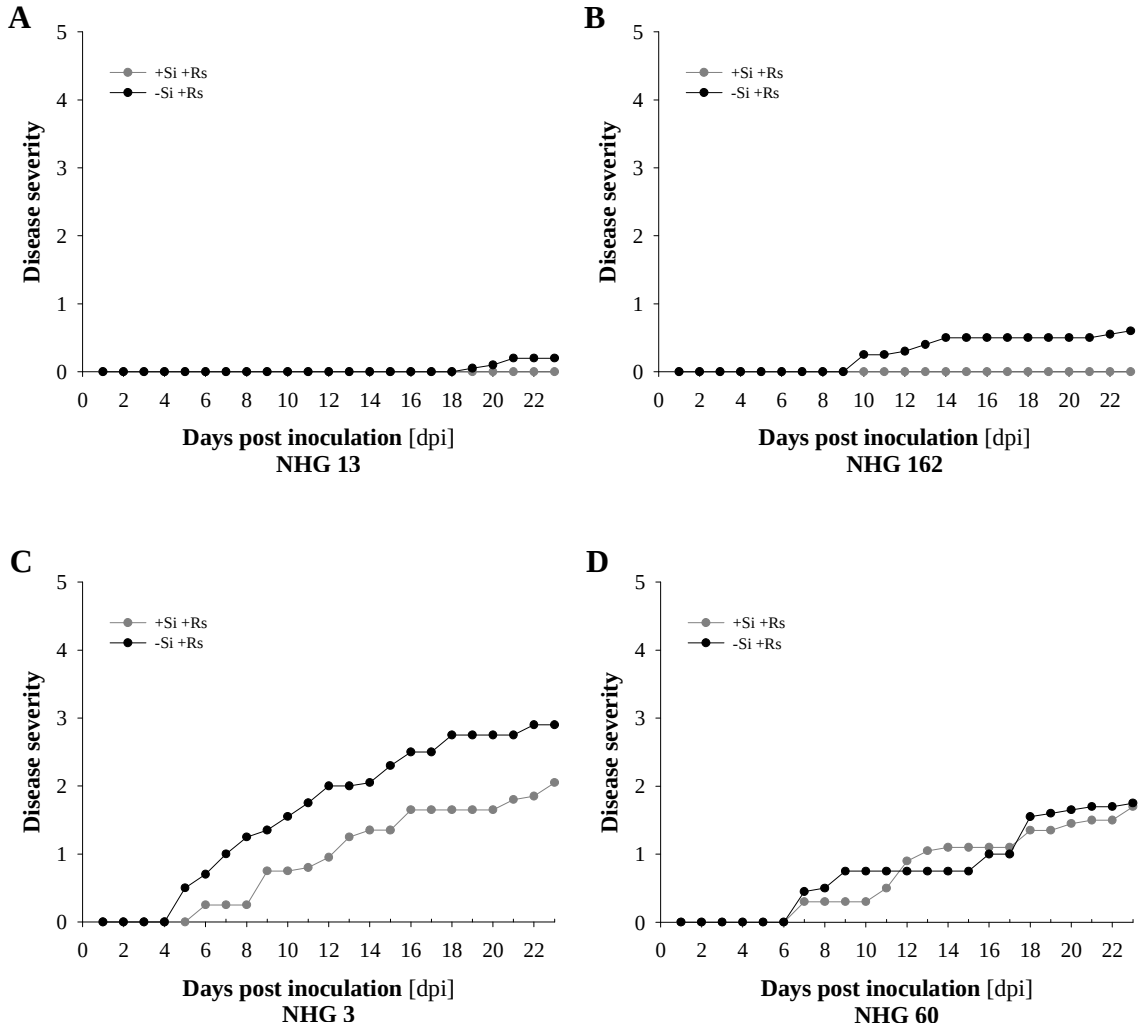

Fig. 2.3: Development of disease severity of recombinant inbred lines (A) NHG13, (B) NHG162 [resistant to bacterial wilt] and (C) NHG3, (D) NHG60 [susceptible to bacterial wilt], inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon, over three weeks.

Data are means of ten plants per inoculated treatment and two biological repetitions.
Bacterial wilt severity was evaluated according to the scale: 0 = no leaf wilted, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, 4 = whole plant wilted except the top, 5 = dead plant.
Disease incidence is the percentage of dead plants at each evaluation date.

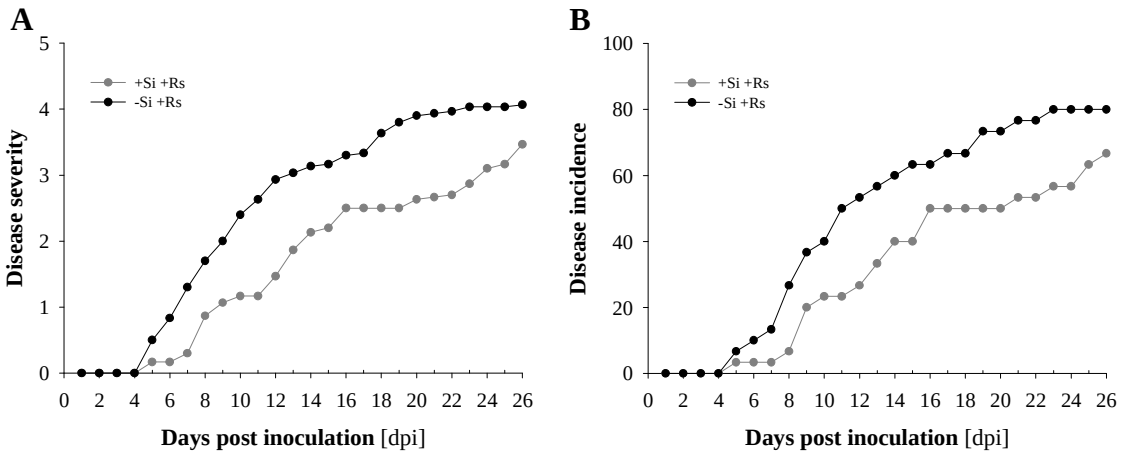

Fig. 2.4: Development of disease severity (A) and disease incidence (B) of tomato genotype King Kong2, inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon over four weeks.

Data are means of three individual experiments with ten plants per treatment.
Bacterial wilt severity was evaluated according to the scale: 0 = no leaf wilted, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, 4 = whole plant wilted except the top, 5 = dead plant.
Disease incidence is the percentage of dead plants at each evaluation date.

Table 2.1: Area under disease progress curve (AUDPC) based on disease severity and disease incidence for recombinant inbred lines NHG13, NHG162 [resistant to bacterial wilt] and NHG3, NHG60 [susceptible to bacterial wilt].

	Recombinant inbred line [RIL]			
Treatment	**NHG13 (res)**	**NHG162 (res)**	**NHG3 (sus)**	**NHG60 (sus)**
$AUD_S PC$				
- silicon	0.65 ± 0.7	7.93 ± 7.9	44.05 ± 10.1	18.21 ± 9.7
+ silicon	0.00 ± 0.0	0.13 ± 0.1	26.98 ± 15.5	17.05 ± 3.2
$AUD_I PC$				
- silicon	0.00 ± 0.0	82.50 ± 82.5	852.50 ± 187.5	345.00 ± 175.0
+ silicon	0.00 ± 0.0	0.00 ± 0.0	462.50 ± 237.5	295.00 ± 25.0

res: resistant; sus: susceptible.
Data are means of two independent experiments ± SE, calculation based on disease severity or disease incidence, respectively. AUDPC over 23 days.

Table 2.2: Area under disease progress curve based on disease severity (AUD_SPC) and disease incidence (AUD_IPC) for tomato genotype King Kong2 inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon.

	King Kong2	
Treatment	**AUD_SPC**	**AUD_IPC**
- silicon	63.38 ± 8.5 a	1190.00 ± 168.6 a
+ silicon	41.35 ± 11.7 a	788.33 ± 229.8 a

Data are means ± SE of three independent trails with three plants per treatments. Calculation based on bacterial wilt severity or disease incidence. Small letters refer to the comparison of treatments for disease severity and disease incidence. Same letters are not significantly different with Wilcoxon rank-sum Test at α = 5%. AUDPC over 26 days.

Bacterial numbers in stems were not significantly different comparing –Si and +Si treatment across genotypes (Table 2.3), except of genotype King Kong2 at 12dpi where no bacteria were detected in silicon treated plants (Table 2.4).

Table 2.3: Bacterial numbers (log CFU / g) in tomato stems of recombinant inbred lines NHG13, NHG162 [resistant to bacterial wilt] and NHG3, NHG60 [susceptible to bacterial wilt], inoculated with *R. solanacearum* strain ToUdk2 with and without silicon nutrition at 5 days post inoculation (dpi).

	Recombinant inbred line [RIL]			
Treatment	**NHG13 (res)**	**NHG162 (res)**	**NHG3 (sus)**	**NHG60 (sus)**
- silicon	3.40 ± 0.9 a	3.98 ± 1.4 a	3.19 ± 1.6 a	2.79 ± 1.4 a
+ silicon	2.52 ± 0.8 a	3.88 ± 0.9 a	3.97 ± 1.4 a	3.00 ± 1.0 a

res: resistant; sus: susceptible.
Data are means ± SE of two independent trails with three plants per treatments. Small letters refer to the comparison of treatments for the same genotype. Similar letters are not significantly different with Wilcoxon rank-sum Test at α = 5%.

Table 2.4: Bacterial numbers (log CFU / g) in tomato mid-stems of genotype King Kong2, inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon at 5, 8, 12 and 26 days post inoculation (dpi).

	Days post inoculation (dpi)			
Treatment	**5**	**8**	**12**	**26**
- silicon	4.19 ± 0.7 aA	5.40 ± 1.1 aA	3.50 ± 1.5 aA	5.01 ± 0.5 aA
+ silicon	3.96 ± 0.7 aA	5.42 ± 1.2 aA	0.00 ± 0.0 aB	5.49 ± 0.9 aA

Data are means ± SE of three independent trails with three plants per treatments.
Small letters refer to the comparison of treatment (-Si and +Si) for the same evaluation date. Capital letters refer to the comparison of the same treatment at different evaluation dates. Same letters are not significantly different with Wilcoxon rank-sum Test at $\alpha = 5\%$.

2.3.2 Plant growth

Shoot fresh matter was not different across treatments for the recombinant inbred lines at 5 dpi and 23 dpi after inoculation and for genotype King Kong2 at all investigation dates, except for genotype NHG60 with increased fresh matter in the +Si+Rs treatments compared to the –Si-Rs control at 5 dpi (Table 2.5, Table 2.6).

Comparing shoot fresh matter of the recombinant inbred lines for the same treatment and evaluation date, silicon treated, *R. solanacearum* inoculated plants of genotype NHG60 showed increased fresh matter compared to NHG3 at 5 dpi. The shoot fresh matter of genotype NHG3 was significantly decreased compared to genotype NHG13 for all treatments and compared to NHG60 in the non-silicon, healthy and *R. solanacearum* inoculated treatments at 23 dpi (Table 2.5).

Table 2.5: Shoot fresh matter (g) of tomato recombinant inbred lines NHG13, NHG162 [resistant to bacterial wilt] and NHG3, NHG60 [susceptible to bacterial wilt] healthy and inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon at 5 days post inoculation (dpi) and 23 dpi.

	Recombinant inbred line [RIL]			
Treatment	**NHG13 (res)**	**NHG162 (res)**	**NHG3 (sus)**	**NHG60 (sus)**
5 dpi				
- Si – Rs	9.06 ± 1.5 aA	10.03 ± 2.4 aA	6.72 ± 1.5 aA	10.13 ± 1.5 bA
- Si + Rs	9.13 ± 2.2 aA	10.30 ± 2.8 aA	7.55 ± 1.7 aA	12.90 ± 3.4 abA
+ Si - Rs	10.73 ± 1.6 aA	10.13 ± 2.7 aA	7.55 ± 1.7 aA	12.12 ± 1.1 abA
+ Si + Rs	11.73 ± 1.4 aAB	8.16 ± 1.3 aAB	8.09 ± 0.6 aB	12.60 ± 1.6 aA
23 dpi				
- Si – Rs	27.77 ± 6.0 aA	19.51 ± 3.2 aAB	13.38 ± 0.9 aB	28.93 ± 3.9 aA
- Si + Rs	31.37 ± 5.0 aA	23.16 ± 3.9 aAB	15.05 ± 1.0 aB	28.16 ± 4.9 aA
+ Si - Rs	32.29 ± 3.4 aA	21.72 ± 5.1 aAB	12.66 ± 1.5 aB	24.91 ± 5.5 aAB
+ Si + Rs	27.09 ± 2.9 aA	26.36 ± 3.3 aAB	19.36 ± 3.1 aB	25.09 ± 5.2 aAB

Si: silicon; Rs: *R. solanacearum*; res: resistant; sus: susceptible.
Data are means ± SE of two independent trails with three plants per treatments, respectively. Small letters refer to the comparison of treatments for the same genotype. Capital letters refer to the comparison of the same treatment across genotypes. Similar letters are not significantly different with Wilcoxon rank-sum Test at $\alpha = 5\%$.

Table 2.6: Shoot fresh matter (g) of genotype King Kong2, healthy and inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon at 5, 8, 12 and 26 days post inoculation (dpi).

	Days post inoculation [dpi]			
Treatment	**5**	**8**	**12**	**26**
- Si – Rs	5.89 ± 0.4 a	6.96 ± 1.0 a	11.55 ± 1.8 a	26.51 ± 3.3 a
- Si + Rs	7.48 ± 0.8 a	7.66 ± 1.1 a	14.41 ± 2.0 a	25.17 ± 3.1 a
+ Si - Rs	6.33 ± 0.6 a	6.52 ± 0.8 a	12.07 ± 4.0 a	29.49 ± 3.0 a
+ Si + Rs	5.78 ± 0.5 a	6.98 ± 1.3 a	14.82 ± 4.2 a	23.47 ± 3.4 a

Si: silicon; Rs: *R. solanacearum*
Data are means ± SE of three independent trails. Small letters refer to the comparison of treatments for the same genotype. Similar letters are not significantly different with Wilcoxon rank-sum Test at $\alpha = 5\%$.

Ralstonia solanacearum inoculated, non-silicon treated plants showed decreased dry matter at 26 days post inocularion compared to all other treatments, though the difference was not statistically significant due to the typical occurrence of death and healthy plants (Table 2.7).

Table 2.7: Shoot dry matter of tomato plants of genotype King Kong2, healthy and inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon at 26 days post inoculation (dpi).

Treatment	**Dry matter**
- silicon – *R. solanacearum*	8.76 ± 0.3 a
- silicon + *R. solanacearum*	5.37 ± 1.7 a
+ silicon – *R. solanacearum*	9.11 ± 0.4 a
+ silicon + *R. solanacearum*	8.14 ± 1.1 a

Same letters are not significantly different comparing the treatments with Wilcoxon rank-sum Test at $\alpha = 5\%$.

2.3.3 Silicon quantification in stems and roots of tomato genotypes

Silicon content was elevated in **roots** of silicon treated compared to non-silicon treated plants of genotype King Kong2, with similar values at 5, 12 and 26 dpi (Table 2.8).

Comparing silicon content in **stems**, plants supplemented with silicon showed slight, significant increases in quantities for non-inoculated and inoculated treatments (+Si-Rs, +Si+Rs) at 5 dpi and for the silicon treated inoculated plants (+Si+Rs) compared to non-silicon treated, inoculated (-Si+Rs) plants at 12 dpi.

Comparing the silicon content in **plant organs** for the same treatment and evaluation date, silicon content in roots was significantly increased for all silicon treated plants at all dates, and generally slightly increased in the roots for all treatments at 12 and 26 dpi.

Table 2.8: Silicon content (mg / g dry matter) in stems and roots of tomato genotype King Kong2, healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon at 5, 12 and 26 days post inoculation (dpi).

	Days post inoculation (dpi)		
Treatment	**5**	**12**	**26**
Stem			
- silicon - *R. solanacearum*	0.26 ± 0.02 bA	0.12 ± 0.03 abB	0.17 ± 0.02 aB
- silicon + *R. solanacearum*	0.28 ± 0.002 bA	0.08 ± 0.003 bB	0.17 ± 0.05 aA
+ silicon - *R. solanacearum*	0.36 ± 0.02 aB	0.16 ± 0.03 abB	0.23 ± 0.04 aB
+ silicon + *R. solanacearum*	0.43 ± 0.04 aB	0.18 ± 0.02 aB	0.32 ± 0.06 aB
Root			
- silicon - *R. solanacearum*	0.23 ± 0.02 bA	0.25 ± 0.04 bA	0.27 ± 0.03 bA
- silicon + *R. solanacearum*	0.30 ± 0.02 bA	0.23 ± 0.04 bA	0.25 ± 0.03 bA
+ silicon - *R. solanacearum*	1.17 ± 0.1 aA	1.10 ± 0.22 aA	1.17 ± 0.13 aA
+ silicon + *R. solanacearum*	1.00 ± 0.05 aA	0.92 ± 0.12 aA	1.19 ± 0.09 aA

Data are means of three plants per treatment of three independent trails ± SE.
Small letters refer to the comparison of treatments at the same sampling date for stems and roots. Capital letters refer to the comparison of plant organs (stems and roots) for the same treatment and sampling date. Same letters are not significantly different with Wilcoxon rank-sum Test at $\alpha = 5\%$. Silicon content was determined in tomato genotype King Kong2, representative for the silicon excluder plant tomato. Other tomato genotypes were included in former studies (Dannon and Wydra, 2004; Huong, 2006; Diogo and Wydra, 2007).

2.3.4 Peroxidase activity in tomato stems

An increase of enzyme activity, though not statistically different, was observed for the inoculated plus silicon (+Si+Rs) treatment in stems of **recombinant inbred lines** NHG162 and NHG60 compared to the control (-Si-Rs) and the single treatments of either Si or Rs (-Si+Rs and +Si-Rs), and in silicon treated plants with and without inoculation (+Si-Rs and +Si+Rs) for NHG3 (Fig. 2.5A). Peroxidase activity was constant across treatments in the resistant line NHG13, and lowest activity was observed in genotype NHG162 for non-silicon treatments of healthy and inoculated (-Si-Rs and –Si+Rs) plants and for silicon treatment in healthy plants (+Si-Rs).

Taking the total protein content in tomato stems into account, the specific peroxidase activity did generally not change across treatments for all genotypes (Fig. 2.5B), though a significant increase in activity was found in the + Si +Rs treatments for NHG60 compared to NHG13

Peroxidase activity ($\mu mol*min^{-1}*gFW^{-1}$) in stems of genotype **King Kong2** differed generally not across treatments at the evaluation dates except of a lower activity in silicon treated, non-inoculated (+Si-Rs) plants compared to both inoculated treatments (-Si+Rs and +Si+Rs) at 8 dpi (Fig. 2.6A). Comparing evaluation dates, a significant increase in activity was observed in non-silicon treated, non-inoculated (-Si-Rs) plants at 26 dpi compared to 5 dpi and a significant decrease in activity for silicon treated, non-inoculated (+Si-Rs) plants at 8 dpi compared to 12 dpi and 26 dpi.

Differences in specific peroxidase activity were not statistically significant (Fig. 2.6B).

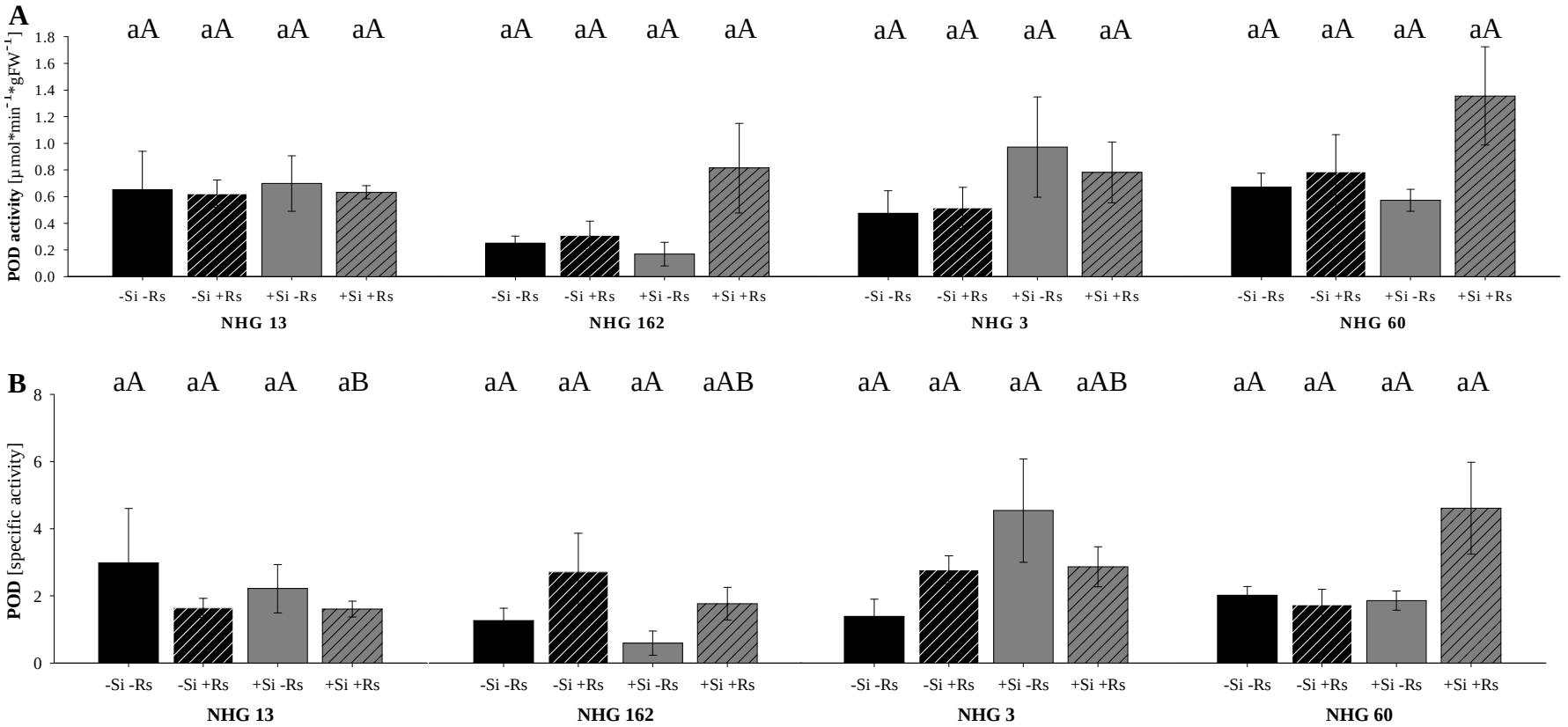

Fig. 2.5: Peroxidase (POD) activity in (A) µmol*min^{-1}*gFW^{-1} and (B) specific POD activity in tomato stems of recombinant inbred lines NHG13, NHG162 [resistant to bacterial wilt] and NHG3 and NHG60 [susceptible to bacterial wilt] healthy and inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon at 5 days post inoculation (dpi).

Data are means of three plants per treatment from two independent trails ± SE. Small letters refer to the comparison of treatments for the same genotype, capital letters to the comparison of the same treatment for the genotypes. Same letters are not significantly different with Wilcoxon rank-sum Test at α = 5%

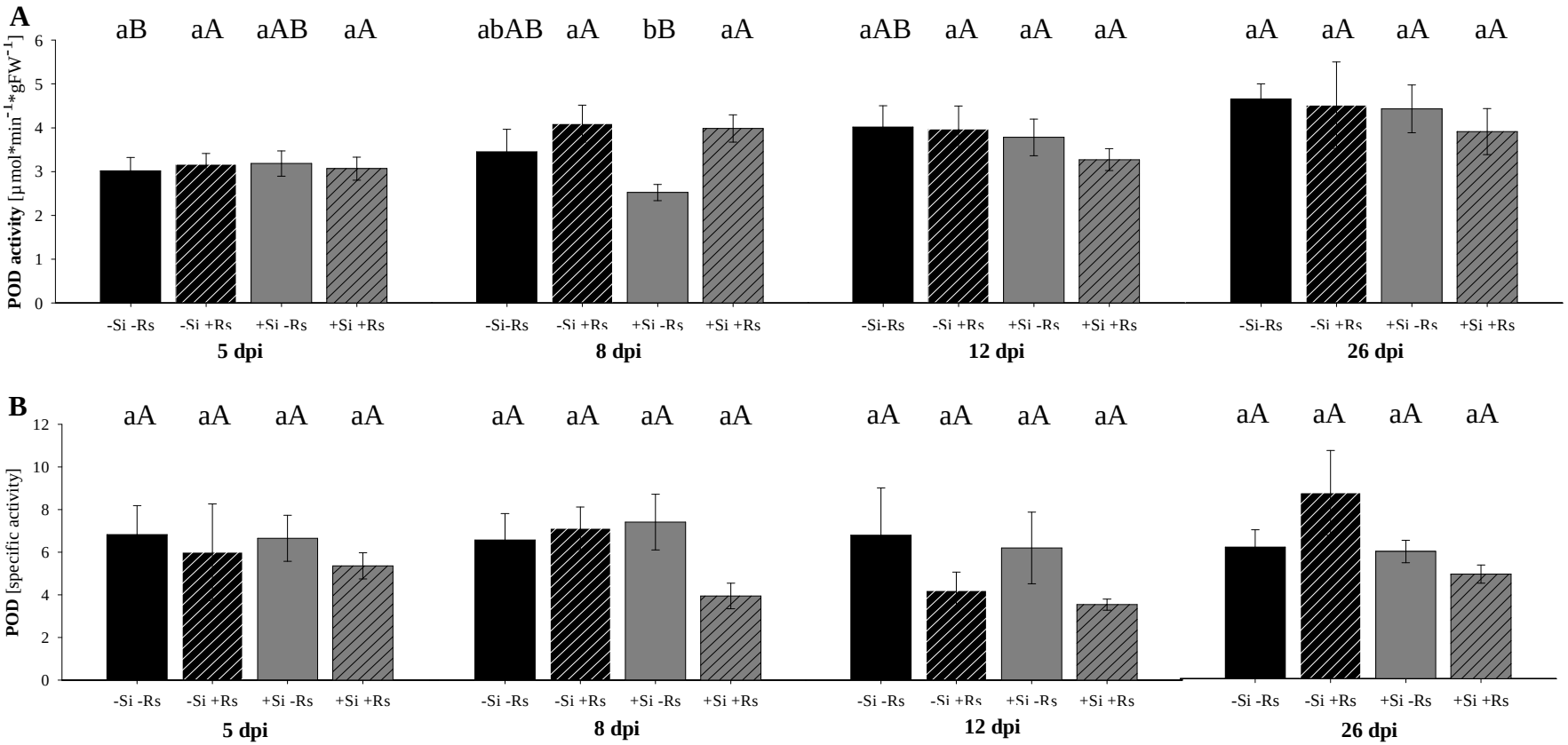

Fig. 2.6: Peroxidase (POD) activity in (A) µmol*min^{-1}*gFW^{-1} and (B) specific POD activity in tomato stems of genotype King Kong2 healthy and inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon at 5, 8, 12 and 26 days post inoculation (dpi).

Data are mans of two independent experiments with three plants per treatment ± SE. Small letters refer to the comparison of treatments at the same evaluation date. Capital letters refer to the comparison of the same treatment at different evaluation dates. Same letters are not significantly different with Wilcoxon rank-sum Test at $\alpha = 5\%$

2.3.5 Polyphenol oxidase activity in tomato stems

Polyphenol oxidase activity was not significantly different in stems of genotype King Kong2 across treatments at the same evaluation dates, except of PPO activity in units per mg protein at 8 dpi, where enzyme activity was significantly decreased in the silicon-treated inoculated (+Si+Rs) plants compared to the non-treated (-Si-Rs) control (Fig. 2.7A and 2.7B).

Comparing the same treatment at different evaluation dates for PPO activity in units per gram fresh weight, non-treated (-Si-Rs) controls were significantly decreased at 8 dpi compared to 5dpi and silicon treated, inoculated (+Si+Rs) plants were significantly decreased at 8 dpi compared 5 and 12 dpi for units per gram fresh weight and units per mg total protein (Fig. 2.7A and 2.7B).

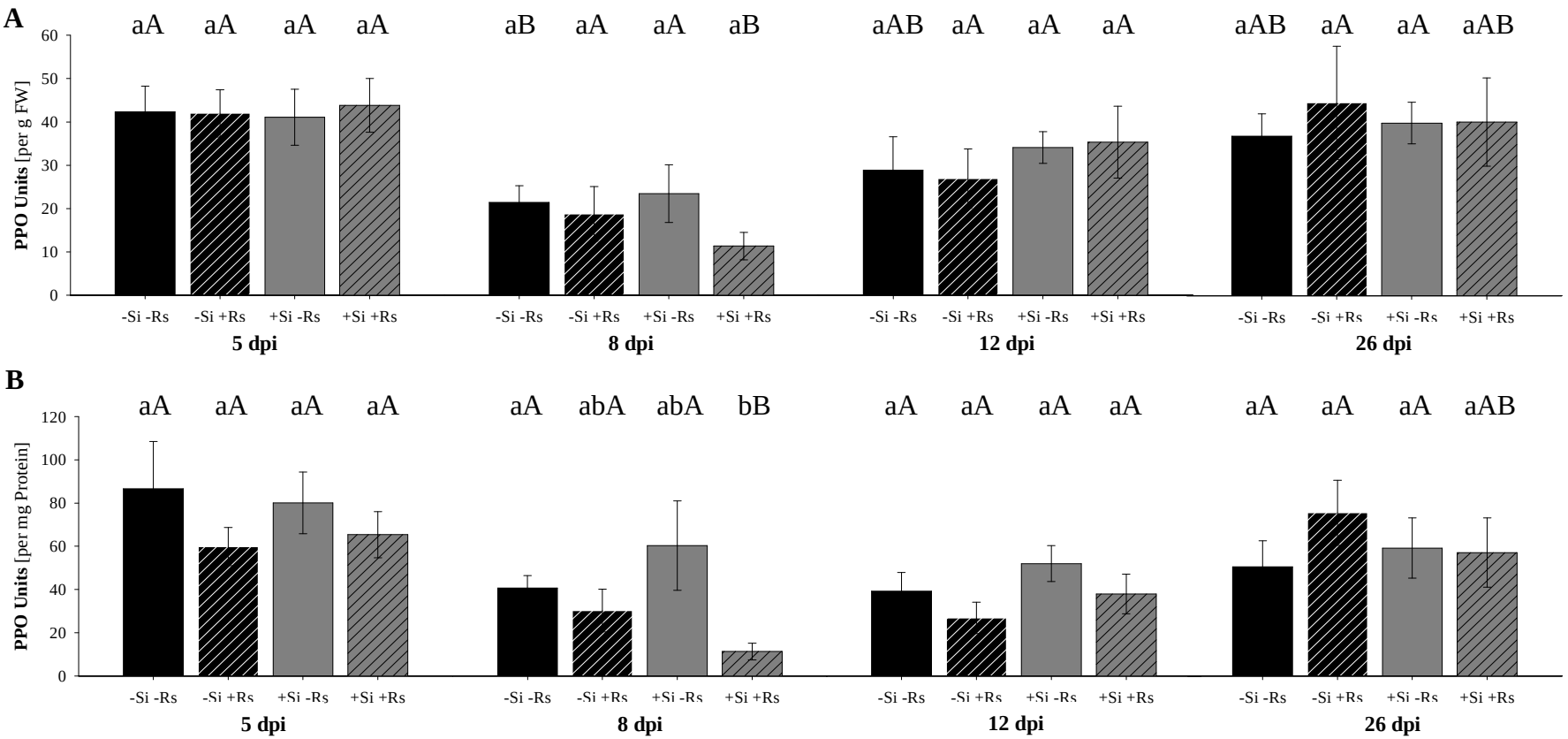

Fig. 2.7: Polyphenol oxidase (PPO) in (A) units per gram fresh weight and (B) units per mg total protein in tomato stems of genotype King Kong2 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon at 5, 8, 12 and 26 days post inoculation (dpi).

Data mare mans of two independent experiments with three plants per treatment ± SE. Small letters refer to the comparison of treatments at the same evaluation date. Capital letters refer to the comparison of the same treatment at different evaluation dates. Same letters are not significant different with Wilcoxon rank-sum Test at α=5%

2.3.6 Peroxidase and polyphenol oxidase activity in bacterial cultures

Neither peroxidase nor polyphenol oxidase activity was observed in supernatants of *R. solanacearum* liquid cultures at 0, 3, 24 and 27 hours post inoculation (hpi) of the main-culture using either guaiacol or catechol as substrates respectively, even when bacterial numbers exceed log CFU of 9 per mL cultures at 24 and 27 hpi, similar to bacterial numbers in highly infected plants expressed in log CFU / g tomato tissue (data not shown). Furthermore, enzymatic activity of peroxidase and polyphenol oxidase was not induced by supplementing the medium with tomato stem tissue. Total protein content in the media started to be detectable at 24 with 0.02 mg / mL and increased to 0.03 mg / mL at 27 hpi in *R. solanacearum* inoculated media, independently of the supplement with tomato stem tissue. Plant extract of tomato cultivar King Kong2 was used as positive control and showed peroxidase and polyphenol oxidase activity (data not shown).

2.4 Discussion

Lower peroxidase (POD) activity was observed for the recombinant inbred lines (RILs), NHG13, NHG162 (both resistant to bacterial wilt) and NHG3, NHG60 (both susceptible to bacterial wilt) compared to the moderately resistant commercial tomato genotype King Kong 2. The RILs originated from crossing of the resistant tomato genotype Hawaii 7996 and the susceptible tomato genotype Wva700 (Balatero *et al.*, 2002) and have a different genetical background than tomato cultivar King Kong2, that may lead to a different enzymatic profile. However, generally no significant differences across treatments, genotypes and evaluation dates for POD activity were observed. Peroxidases have been shown to participate in resistance reactions in plants including the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* in rice, where induction of a cationic peroxidase in xylem vessels in the incompatible interaction was observed (Young *et al.*, 1995). These results were mainly obtained by immunoelectron microscopy using a specific antibody for the peroxidase, which differs to our approach using a biochemical assay with guaiacol, a common substrate for measurement of peroxidase activity (Hiraga *et al.*, 2001). Nevertheless, high induction of total guaiacol-peroxidase activity was observed by Delannoy *et al*. (2003) investigating the incompatible interaction of virulent race 18 of *Xanthomonas campestris* pv *malvacearum* and cotton cotyledons, demonstrating that the guaiacol assay is suitable for the detection of changes in peroxidase activity in plant material. Thus, we conclude that an induction of PODs by *R. solanacearum* or silicon treatment seems not to be likely in tomato, but occurrence of enzymatic activity in all treatments supports the role of PODs in physiological processes like lignin biosynthesis (Campa, 1991), as discussed in chapter 3 (histochemistry).

Common routine assays for the detection of mono- and diphenolase activity are the spectrophotometrical determination of quinone formation at a wavelength near 400 – 500 nm. Sodium dodecyl sulphate (SDS) has been shown to alter the behaviour of polyphenol oxidases from different plant sources with changing pH (Yoruk and Marshall, 2003). These alterations can be due to activation of latent forms of the enzyme as demonstrated for broad bean and peach PPOs (Moore and Flurkey, 1990; Laveda *et al.*, 2000), but also examples of changes in enzymatic activity with regard to pH by SDS which are not related to an activation of latent forms are known (Fraignier *et al.*, 1995; Marques *et al.*, 1995). Polyphenol oxidase activity in tomato stems was not observed in absence of SDS in the

reaction mixture (data not shown), which is similar to observations of Moore and Flurkey (1990) for broad bean polyphenol oxidase, where only low enzymatic activity in absence of SDS was found, suggesting that polyphenol oxidase in tomato stems is present in a latent form, which can be activated by SDS.

Polyphenol oxidase activity can be found in almost all development stages of plants (Yoruk and Marshall, 2003). Thus, the regulation of tomato polyphenol oxidases expression in various tomato plant tissues showed a complex regulation of PPO mRNA in vegetative and reproductive tissues with most abundant PPO transcripts present in tomato leaves and flowers, but also in phloem cells of the stems (Thipyapong *et al.*, 1997), which might explain the observed PPO activity in tomato stems of all treatments in our experiments.

Besides a constitutive or developmentally regulated PPO expression, the enzyme can be induced in wounded tissues as observed for various plants such as apple, potato and tomato (Boss *et al.*, 1995; Thipyapong *et al.*, 1995; Thipyapong and Steffens 1997), indicating that oxidative browning mediated by PPO might be an important defense response against infection or wounding. Additionally, overexpression of a potato polyphenol oxidase gene in transgenic tomato plants resulted in enhanced resistance to *Pseudomonas syringae* pv. *tomato* (Li and Steffens, 2002). We found decreased PPO activity in tomato stems most obvious at 8 dpi but also at 12 dpi, time points where differences in symptom development of non-silicon and silicon amended plants were observed. Generally, PPO activity increased with leaf or plant age in many plants, including tomato (Mayer and Harel, 1979; Mayer, 1987; Felton *et al.*, 1989). The difference of enzymatic activity was significantly decreased for silicon treated inoculated plants compared to non-silicon treated healthy plants at 8 dpi, taking the total protein content of the plants into account. These results indicate that upregulation of PPO in infected plants seems not to contribute to silicon-induced resistance to *R. solanacearum* in tomato plants, and PPO regulation might differ in leaves and stems of tomato plants. Recently, Thipyapong *et al.* (2004b) demonstrated that suppression of PPO activity in transformed tomato plants resulted in improved drought tolerance of the plants relative to non-transformed controls and PPO overexpressing plants. Additionally, they observed a general decrease of PPO activity with increasing age while monitoring leaves from different nodes of the same plant.

Occurrence of polyphenol oxidase seems to be almost universally abundant in animals, plants, fungi and bacteria (Mayer, 2006) and evidence for three different PPO genes in *R. solanacearum* was provided by Hernández-Romero *et al.* (2005). They could detect laccase as dimethoxyphenol oxidase activity in cellular extracts of *R. solanacearum*, catalyzed by a multicopper protein encoded by the RSp1530 locus, suggesting that this gene might be involved in the resistance to phenolic compounds. Furthermore, they found two additional genes with tryosinase-like activities with typical copper-binding sites of PPOs. We could neither detect peroxidase nor polyphenol oxidase activity in supernatants of liquid cultures of *R. solanacearum*, which might be due to different experimental procedures in both studies. Our aim was to demonstrate that *R. solanacearum* does not actively secrete peroxidase and polyphenol oxidase in the medium and that enzyme secretion is not stimulated by plant components, that might interfere with the enzyme assay detecting plant PODs and PPOs. Another possibility for the non-detection of enzymatic activity in culture supernatants of *R. solanacearum* might be the use of different substrates for the enzyme assays. Fungal and plant polyphenol oxidases act on a wide range of mono- and *o*-diphenols as substrates, whereas oxidases derived from animal tissues are relatively specific for tyrosine and dopa, thus, PPOs from various sources have preferences for certain substrates (Mayer and Harel, 1979). For our tests we used the substrates guaiacol and catechol for peroxidase and polyphenol oxidase, respectively, comparable to the ones in the assays with plant extracts. Thus, variations in the enzymatic profile in tomato stems are due to the plants action.

CHAPTER 3

Effect of silicon nutrition on plant cell wall components related to resistance - histochemical observations.

Abstract

Plant call wall components were investigated to elucidate their involvement in silicon-induced resistance of tomato to bacterial wilt disease caused by *R. solanacearum*. Inoculated tomato plants with silicon amendment showed delayed onset and decreased symptom development compared to non-silicon treated plants, even though bacterial numbers in tomato stems were generally similar in both treatments. Increased tylosis formation in inoculated plants of tomato genotype King Kong2 was observed, with highest occurrence of tylosis in silicon-treated, *R. solanacearum* inoculated plants at 8 days post inoculation (dpi). Lignification and hydrogen peroxide (H_2O_2) accumulation in genotype King Kong2 was not altered comparing treatments, except of a reduced to absent H_2O_2 accumulation in highly infected, silicon and non-silicon treated plants. Callose deposition was generally less pronounced (non-silicon treatment) to absent (silicon treatment) in highly infected plants of tomato genotype King Kong2 at 5 dpi, whereas differences in callose accumulation in the susceptible recombinant inbred line (RIL) NHG3 was generally not observed.

3.1 Introduction

Ralstonia solanacearum distribution in planta

Bacterial wilt is caused by the soilborne bacterium *R. solanacearum* which enters the host plants mainly through wounds or lateral roots and subsequently heavily colonizates and multiplies in the vascular tissue of the stems, resulting in a rapid wilting of the plant occurs (Vasse *et al.*, 1995, Denny, 2006). Investigations of the distribution of *R. solanacearum* in the vascular tissues of stems of tomato cultivars with various resistant states towards the pathogen revealed that bacterial spread and multiplication are limited in resistant genotypes compared to susceptible ones (Grimault *et al.*, 1994; Prior *et al.*, 1996). Nakaho *et al.* (2004) examined 11 resistant tomato cultivars with different genetic background and showed that bacterial movement from protoxylem or the primary xylem to other xylem tissues was limited in the resistant cultivars. Thus, restriction of the movement of the pathogen in vascular tissues can lead to an increased resistance towards the pathogen, and therefore, we examined different parameters that have been shown to be involved in resistance reactions to various pathogens, as described in the following.

Lignin

Lignins are complex and heterogeneous, cell wall bound phenolic polymers abundant in almost all land plants (Kubitzki, 1987; Whetten *et al.*, 1998; Rogers and Campbell, 2004) and are principally found in specific tissue types such as the tracheids and vessel elements of the xylem, but also in sclerenchyma, phloem fibers and periderm (Esau, 1977). A main function is the stabilization of the plant, as intra- and inter-molecular glues, to maintain water and mineral transport through the xylem under negative pressure (Rogers and Campbell, 2004), and, additionally, generation of lignin was observed after wounding and in response to pathogen challenge (Vance *et al.*, 1980; Lange *et al.*, 1995). The difficultly degradable liginin provides an important barrier to pathogen ingress (Hammond-Kosack and Jones, 1996) and limits the digestibility of plant matter by herbivores, probably resulting in decreased desirability as food source (Moore and Jung, 2001). Monolignols, monomeric subunits (Fig. 3.1), polymerize to form lignin, whereas three different forms of this phenylpropanoid components can be incorporated into the polymer: *p*-coumaryl

alcohol, coniferyl alcohol or sinapyl alcohol, and the aromatic portions of these phenylpropanoids are described as: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) moieties, respectively (Lewis and Yamoto, 1990; Boerjan *et al.*, 2003; Rogers and Campbell, 2004).

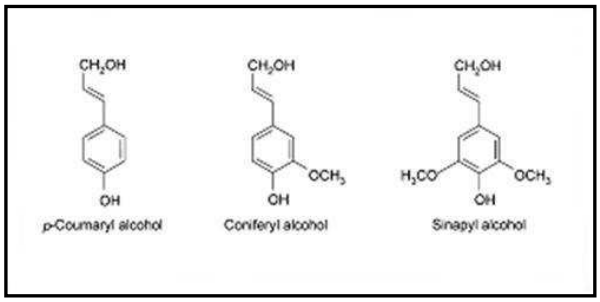

Fig. 3.1: Three different monomeric precursors, that can be incorporated into lignin polymers (Rogers and Campbell, 2004).

A key enzyme in the synthesis of lignin precursors is *l*-phenylalanine ammonia lyase (PAL), which catalyses the conversion of *l*-phenylalanine to form *trans*-cinnamic acid, followed by the dehydroxylation of cinnamic acid to *p*-coumaric and caffeic acids by specific hydroxylase enzymes (Argyropoulos and Menachem, 1997). Alternatively in grasses, tyrosine is deaminated by tyrosine ammonia-lyase (TAL) to form *p*-coumaric acid (Whetten *et al.*, 1998). Further enzymatic activity results in the formation of the precursors coniferyl and sinapyl alcohols. A simplified metabolic pathway of *l*-phenylalanine to lignin precursors is shown in Fig. 3.2 (from Argyropoulos and Menachem, 1997).

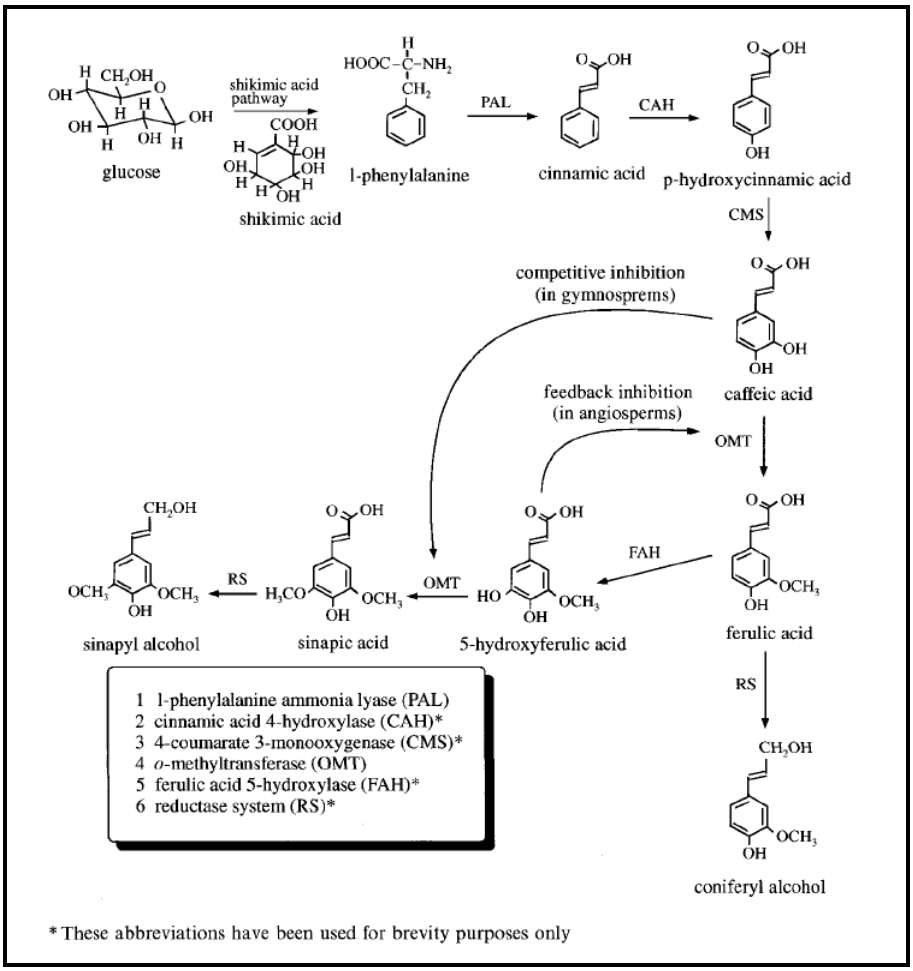

Fig. 3.2: A simplified metabolic pathway of *l*-phenylalanine to lignin precursors (from Argyropoulos and Menachem (1997).

Candidates for the formation of higher-ordered polymers from monolignols are peroxidases (hydrogen peroxide dependent) and laccases (hydrogen peroxide-independent), whereas the hydrogen peroxide independent oxidases can be differentiated in catechol oxidases (polyphenol oxidases) and laccases (diphenol oxidases),which both can be involved in lignification (Whetten *et al.*, 1998).

The dehydrogenation of monomers by peroxidases resulting in resonance stabilized free radicals is shown in Fig. 3.3, followed by the polymerization process, probably with aid of glycoproteins, also called dirigent proteins (Argyropoulos and Menachem, 1997; Lewis, 1999).

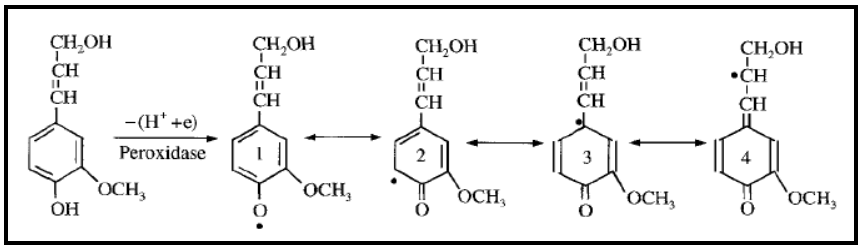

Fig. 3.3: Resonance forms of softwood lignin phenoxy radicals (from Argyropoulos and Menachem, 1997).

Cellulose, hemicelluloses, pectins as well as structural proteins are laid down first to establish the overall architecture of the secondary cell wall, before lignin deposition starts in the cell corners and primary wall, extending to the middle lamella and secondary cell wall regions (Lewis, 1999).

Tylosis

Occurrence of tylsosis have been reported in trees (Rioux *et al.*, 1998; Clérivet *et al.*, 2000), but also in plant species such as cassava (Kpémoua *et al.*, 1996), cotton (Mace, 1978) and in tomato (Bishop and Cooper 1984). They might be part of the normal aging process (Rioux *et al.*, 1998; Canny, 1997) or induced by infections (Rioux *et al.*, 1998). The formation of tylosis after infections or injury has been considered as an active defence mechanism towards vascular diseases, resulting in partial or complete occlusion of xylem vessels with the aim of restricting growth or spread of pathogens (Beckmann, 1987; Rioux *et al.*, 1998; Clérivet *et al.*, 2000; Soukup and Votrubová, 2005). They are formed by material secreted from adjacent xylem parenchyma cells through membranes of half-bordered pit pairs (Rioux *et al.*, 1998). The chemical structure of tylosis and vascular wall coatings was investigated in several studies and is suggested to be mainly composed of pectic or phenolic substances (Clérivet *et al.*, 2000, Soukup and Votrubová, 2005), but also presence of callose or lignin-like molecules (Kpémoua *et al.*, 1996) or suberin (Robb *et al.*, 1991) was reported.

Hydrogen peroxide (H_2O_2) accumulation

Many different processes in plants are controlled by reactive oxygen species (ROS) and they are continuously produced as byproducts of different metabolic pathways (Lamb and Dixon, 1997; Mittler *et al.*, 2004). Hydrogen peroxide (H_2O_2) belongs to the reactive and toxic derivates of oxygen (O_2), additionally to singlet oxygen ($^1O_2$), superoxide anion (O_2^-) and hydroxyl radical ($HO^\cdot$) (Baker and Orlandi, 1995; Mittler, 2002). They participate in the control and regulation of the normal plant metabolism including growth, photosynthesis, respiration and development, in the modification of polyunsaturated fatty acids (PUFAs), DNA, carbohydrates and proteins, additionally they are also involved in programmed cell death (PCD) and in responses to biotic and abiotic stresses (Mittler, 2002; Mittler *et al.*, 2004; Møller *et al.*, 2007). The modulation of ROS in plants requires a large network of genes for either production of ROS or efficient ROS-scavenging mechanisms, due to the high toxicity of the derivates. Major source of the ROS production are chloroplasts, mitochondria, microbodies, peroxisoms and cytosol by the action of for instance NADH oxidases, amine oxidases and cell-wall bound peroxidases (Mittler, 2002). In the scavenging process, different plant enzymes are involved, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), class III plant peroxidases and peroxiredoxin and are found in nearly each subcellular compartment (Mittler, 2002; Apel and Hirt, 2004).

The activation of ROS by biotic stresses plays a central role in the defence against pathogen attack in plants (Mittler, 2002, Apel and Hirt, 2004). Enhanced activity of plasma-membrane bound NADPH oxidases, cell wall-bound peroxidases and amine oxidases in the apoplast leads to production of ROS and the H_2O_2 produced during this process, in combination with the inhibition of ROS detoxifying enzymes by salicylic acid (SA) and nitrous oxide NO, is suggested to activate plant responses like PCD.

Two distinct phases of ROS response in plants can be distinguished in the incompatible interaction of plants with pathogens, demonstrated for *Pseudomonas syringae* pathovars in tobacco suspension cells (Baker and Orlandi, 1995). A rather non-specific response occurred rapidly after inoculation with either compatible or incompatible pathovars, stated as phase I. The second phase (phase II), prolonged compared to phase I, followed 1.5 to 3h after inoculation and appeared to be specific for the incompatible interaction. The reaction is not only initiated by bacterial pathogens, it can also be induced by elicitors such as

fungal polygalacturonases, fungal cell wall extractions and oligogalacturonides of the plant cell wall (Baker and Orlandi, 1995, Lamb and Dixon, 1997).

Callose

Callose, a linear plant β-1,3-glucan, is synthesized by callose synthases during development, for instance as a transitory component of the cell plate in dividing cells or as major component of pollen mother cell walls and pollen tubes (Jacobs *et al.*, 2003) and in response to biotic and abiotic stresses in plants (Verma and Hong, 2001; Østergaard *et al.*, 2002).

One of the earliest defence reactions of plants towards pathogen e.g. the vascular invading *Fusarium oxysporum* f. sp. *lycopersici* in tomato is the deposition of callose (Beckmann *et al.*, 1982; Beckmann, 1987). It is suggested rather as a general defence reaction, but occurrence of papillae or more extensive apposition layers containing callose at the site of fungal contacts, providing a physical barrier to penetration, was observed (Beckmann, 1987, Nishimura *et al.*, 2003). Callose deposition was not only found in fungal-host plant interactions, but also in the reaction to bacterial infections, such as flavanoid accumulation and occurrence of papilla enriched in callose in a resistant cotton genotype inoculated with *Xanthomonas campestris* pv. *malvacearum* (Dai *et al,*.1996), and lignification and suberization associated with callose deposition, that reinforced host barriers in the phloem in the cassava – *Xanthomonas campestris* pv. *manihotis* interaction (Kpémoua *et al.*, 1996).

3.2 Materials and methods

3.2.1 Plant material and inoculation procedure

Tomato plants of genotype King Kong2 (Known-You Seed Co., Taiwan) and tomato recombinant inbreed lines NHG3, NHG13, NHG162, NHG60 (AVRDC, Taiwan) were cultivated in white peat (Klasmann-Deilmann, Germany) supplemented with 4 g L^{-1} $CaCO_3$ (Roth, Germany) for non-silicon treatment and 4 g L^{-1} $CaCO_3$ plus 1g / L Aerosil (Degussa, Germany) in silicon treatments. Plants were kept under greenhouse conditions (20 °C with 14 h light per day at 30 K lux and 70% relative humidity) and watered throughout the whole experiment with a nutrient solution composed of 5 mM $Ca(NO_3)_2$, 1.875 mM K_2SO_4, 1.625 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.04 mM H_3BO_3, 0.001 mM $ZnSO_4$, 0.001 mM $CuSO_4$, 0.01 mM $MnSO_4$, 0.00025 mM Na_2MoO_4, 0.05 mM NaCl and 0.1 mM Fe-EDTA for non-silicon treatment, and the same solution containing additionally monosilicic acid in a final concentration of 1.4 mM [$Si(OH)_4$] for silicon treatments. Monosilisic acid was obtained after exchange of potassium silicate solution K_2SiO_2 (VWR, Germany) with cation exchangers (20 mL volume; Biorad Laboratories, Germany) (Hochmuth, 1999).

Five week old plants were inoculated with *R. solanacearum* strain ToUdk2 (race 1, phylotype 1; originated from Thailand) directly after transplanting and transferred into a growth chamber (30°C / 27°C day/night temperature, 85% relative humidity, 30 K Lux and 14h light per day). Inoculum suspensions were prepared from two day old bacterial cultures of *R. solanacearum* grown on TTC medium [10 g / L Bacto peptone, 1 g / L casamino acid, 5 g / L Glucose, 15 g / L agar; 10 mL of a 0.5 % 2,3,5-triphenyl tetrazolium chloride (Sigma, Germany) were sterile filtrated and separately added to the cooled TTC medium after autoclavating (Kelman, 1954)] or on nutrient glucose agar (NGA; 3 g / L beef extract, 5 g / L peptone from casein, 2.5 g / L glucose, 15 g / L agar) by adjusting the suspension in demineralised water to an optical density of 0.06 at 620 nm and subsequently 1 : 5 diluted, corresponding to approximately $2.03*10^7$ colony forming units (CFU / mL). Per gram substrate 0.1 mL suspension was applied to each plant. Controls were treated with the same quantity of demineralised water. Samples from recombinant inbred lines were harvested 5 days post inoculation (dpi) and genotype King Kong2 additionally at

8 dpi, 12 dpi and four weeks after inoculation. Non-inoculated controls of each treatment were included in all experiments.

3.2.2 Symptom evaluation

Symptoms of ten plants per treatment were monitored daily and classified in six classes as disease severity: **0** = healthy plant, **1** = one leaf wilted, **2** = two leaves wilted, **3** = three leaves wilted, **4** = all leaves wilted except the tip of the plant, **5** = whole plant wilted.
The mean of disease scores represented wilt disease severity (DS), accordingly the wilt incidence (DI) was calculated as the percentage of dead plants to the number of total plants at each evaluation date.
Wilt incidence was recorded daily and calculated as the percentage of dead plants at the evaluation date. The area under disease progress curve (AUDPC) was calculated on the basis of either wilt disease severity or wilt incidence using the following formula (cited after Jeger and Viljanen-Rollinson, 2001):

$$AUDPC = \sum_{i=1}^{n-1} \left[(x_i + x_{i-1}) / 2 \right] (t_i - t_{i-1})$$

with x_i and x_{i-1} - wilt incidence or disease severity scale, and t_i and t_{i-1} - consecutive evaluation dates (t_i-t_{i-1} is equal to 1 day).

3.2.3 Bacterial quantification in tomato stems

R. solanacearum was quantified in stems and partly in roots of symptomless inoculated plants. Stem and root parts were surface sterilized with 70% EtOH for 15 s and 20 s, respectively, subsequently washed with sterile demineralised water and macerated in 3 mL of sterile water. After incubation for 20 min at room temperature the macerate was filtered through cotton and centrifuged at 7000 x g at room temperature. The pellet was re-suspended in 1 mL sterile water, tenfold dilutions were prepared and 100 µL of appropriate dilutions were plated in two replicates on TTC medium, followed by incubation for 48 h at 30°C. Bacterial colonies were counted and calculated as colony forming units per gram of fresh matter (log CFU / g).

3.2.4 Staining of *Ralstonia solanacearum* poly-β-hydroxybutyrate (PHB) *in planta*

Sections from a wilting plant having a wilting score of four were stained with a 1% aqueous solution of Nile Blue A (w/v; Merck, Germany) for 10 min at 55°C, washed with demineralised H_2O and subsequently incubated with 8% acetic acid for 1 min at room temperature to remove excess stain. After an additional washing step, sections were mounted in Citifluor (AF1) antifade (Agar scientific, UK) and observed with Zeiss Axioscope microscope using a filter set (450-490 nm excitation filter, 510 nm dichroic mirror, 520 nm barrier filter) (modified after Denny and Hayward, 2001). The cellular organic reserve material PHB appears as bright orange fluorescence. Sections from a non-infected plant served as control and unstained sections were additionally observed with bright field microscopy.

3.2.5 Histochemical analyses of plant cell wall components

3.2.5.1 General

For the analysis of histochemical changes of plant cell wall constituents related to resistance reactions – lignification, callose accumulation and tylosis – free-hand sections of mid-stem parts of healthy and inoculated (5 dpi, 8 dpi and 12 dpi, if not otherwise indicated) tomato plants of genotype King Kong2, for callose detection additionally genotype NHG3, with and without silicon nutrition, were stained with different dyes as described in the following, and directly observed under a photomicroscope (Axioskop 2 plus, Carl Zeiss, Göttingen, Germany) with bright field, if not otherwise indicated.

For each staining procedure three sections of three individual plants per treatment (healthy, inoculated, silicon, non-silicon) were observed and representative vessels photographed. The experiments were repeated at least twice. For quantification of tylosis, vessels containing clearly blue stained structures from a representative section of each plant were counted and calculated as percent tylosis in relation to the total numbers of vessels per section.

3.2.5.2 Detection of lignin

For detection of lignin the phloroglucinol-HCl method was used (Hamiduzzaman *et al.*, 2005). Sections were decolorized in 96% EtOH before incubation with a solution of 10 g phloroglucinol (Sigma, Germany) in 95 mL 96% EtOH for 3 min at room temperature and subsequently washed with 25% HCl, resulting in red coloration, indicative for lignification of the plant tissue.

3.2.5.3 Detection of H_2O_2 accumulation

H_2O_2 was demonstrated using 3,3'-Diaminobenzidin (DAB) (modified after Orozco-Cardenas and Ryan, 1999). Three plants per treatment were harvested at each time point and experiment, and directly placed with the cut stem surface into approximately 10 mL of 1 mg / mL DAB solution, pH 3.8 (Sigma, Germany). Plants were allowed to take up the solution for 4 hours, before leaflets were discarded and mid-stem parts decolorized in 96% EtOH. Subsequently, hand-sections were cut and directly observed under a photomicroscope (Axioskop 2 plus, Carl Zeiss, Göttingen, Germany).

3.2.5.4 Detection of tylosis

Sections were stained with a solution containing 0.33% safranin (w/v; Merk, Germany) and 0.67% alcian blue (w/v; Carl Rot, Germany), and further 1 : 1 diluted with H_2O dest directly before use (modified after Öhmann, 2006). After incubation for 15 min at room temperature, sections were washed twice with H_2O for 5 min. Red staining of lignified cell walls due to safranin and blue staining of tylosis due to alcian blue was observed.

3.2.5.5 Detection of callose

Sections were decolorized in 96% EtOH before incubation with a solution composed of 0.01% aniline blue (w/v, Serva, Gemany) in 150 mM K_2HPO_4 (pH 9.5) over night (modified after Adam and Sommerville, 1996). Sections were observed under a photomicroscope (AxioPlan 2, Carl Zeiss, Göttingen, Germany) equipped with epifluorescence illumination with Zeiss filterset No 2, excitation: 365 nm, beamsplitter:

395 nm, emission: 420 nm) (Carl, Zeiss, Göttingen, Germany). White coloration indicates callose. Autofluorescence controls – incubated only in 150 mM K_2HPO_4 (pH 9.5) – were made for each plant.

3.2.6 Statistical analyses

The data were analysed with the statistical software “R” (R Development Core Team – R Foundation for Statistical Computing, Vienna, Austria). For all data, Wilcoxon rank-sum test was used and the P values were adjusted with the method of Holm for family-wise error rate correction. A significance level of $p < 0.05$ was used throughout the whole statistical analysis of the data.

3.3 Results

3.3.1 Occurrence of *Ralstonia solanacearum in planta*

The cellular organic reserve compound poly-β-hydroxybutyrate (PHB) present in *R. solanacearum* was observed in vessels of a highly infected plant of genotype King Kong2, indicating the occurrence of high numbers of bacteria in vascular bundles and surrounding plant tissue (Fig. 3.4a-i). The darker coloration of vessel walls of unstained sections observed with bright field illumination was typically observed in highly infected vessels (Fig. 3.4a-c, circles). Sections of a healthy plant showed no yellow staining of PHB (data not shown).

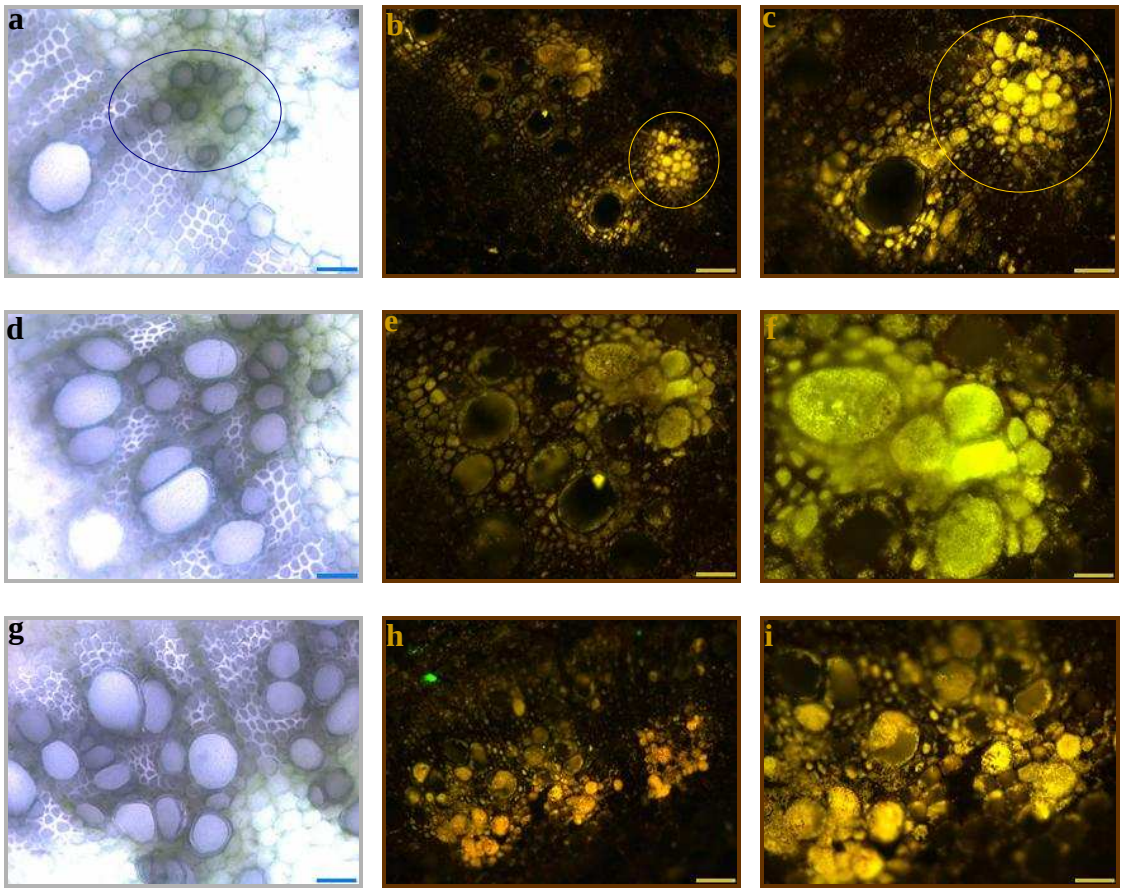

Fig 3.4: Distribution of *R. solanacearum* strain ToUdk2 in vascular bundles of a highly infected tomato plant of tomato genotype King Kong2, stained by PHB.

Pictures a, d, g = bright field illumination; pictures b, c, e, f, h, i = epifluorescence with filter set: 450-490 nm excitation filter, 510 nm dichroic mirror, 520 nm barrier filter
Bar in a, c, d, f, g, i = 50 µm, bar in b, e, h = 100 µm. Circles in pictures a-c: vessel coloration in bright field illumination and occurrence of *R. solanacearum in planta.*

3.3.2 Symptom development and bacterial populations in genotype King Kong2

Symptoms evaluated as disease severity and disease incidence, started earlier in non-silicon treated plants compared to silicon treated plants (Fig. 3.5A and 3.5B) and AUDPC based on disease severity and disease incidence was decreased in silicon treated plants, but, typically for a moderately resistant bacterial wilt infected genotype, without significant differences (Fig. 3.6A and 3.6B).

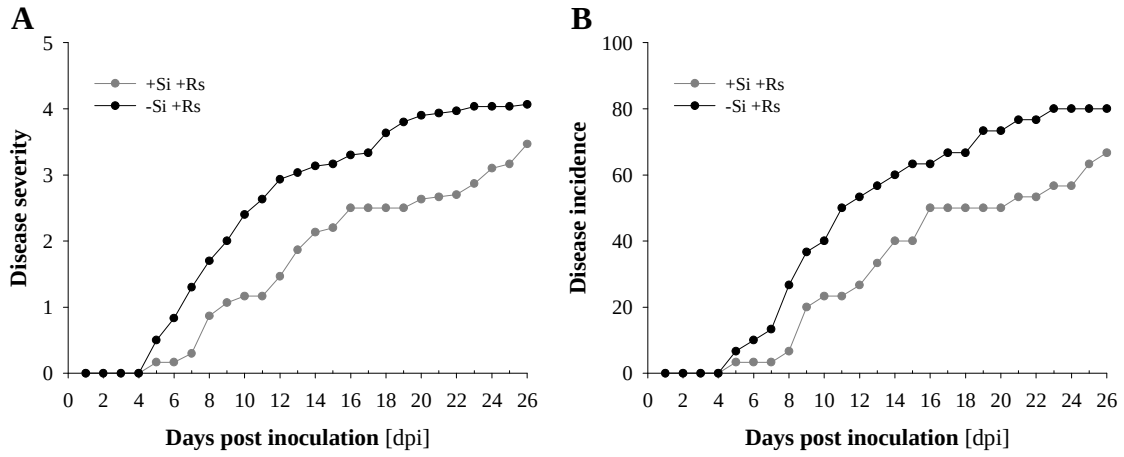

Fig. 3.5: Disease severity (A) and disease incidence (B) of genotype King Kong2 inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon.

Data are means of three individual experiments with ten plants per treatment.
Bacterial wilt severity was evaluated according to the scale: 0 = no leaf wilted, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, 4 = whole plant wilted except the top, 5 = dead plant.
Disease incidence is the percentage of dead plants at each evaluation date.

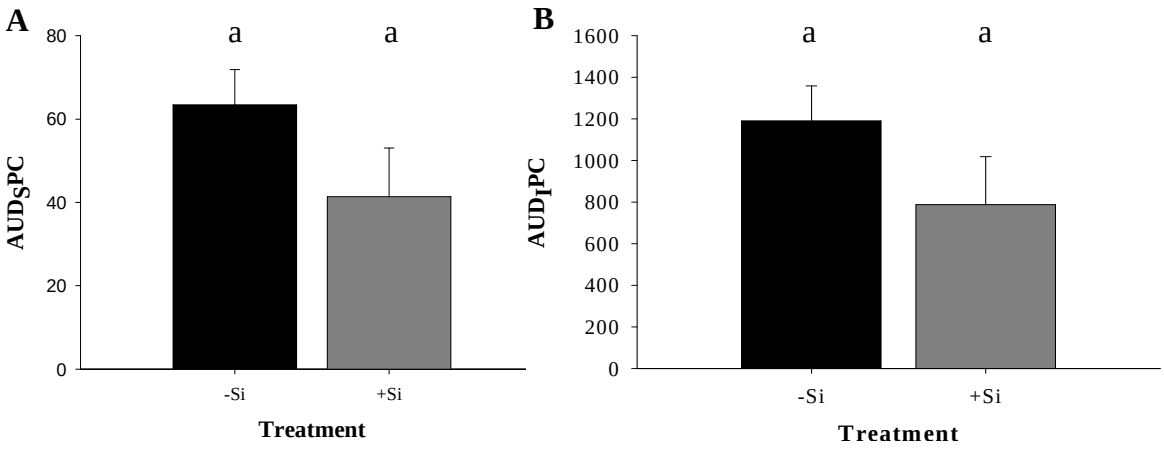

Fig. 3.6: Area under disease progress curve (AUDPC) of tomato genotype King Kong2 inoculated with *R. solanacearum* strain ToUdk2, based on (A) disease severity and (B) disease incidence.

Data are means of three individual experiments with ten plants per treatment ± SE. Same letters are not significantly different with Wilcoxon rank-sum Test at $\alpha = 5\%$.

Bacterial numbers were similar in silicon treated plants compared to non-treated plants at 5 and 8 dpi. At 12 dpi no bacteria were foundin the silicon-treated plants, and number was reduced compared to the earlier sampling dates (Table 3.1).

Table 3.1: Bacterial numbers (log CFU / g) in stems of *R. solanacearum* inoculated tomato genotype King Kong2 amended with and without silicon amendment at several evaluation dates.

	Bacterial numbers [log CFU / g]		
Treatment	**5 dpi**	**8 dpi**	**12 dpi**
-silicon + *R. solanacearum*	4.94 ± 0.6 aA	5.76 ± 0.7 aA	3.50 ± 1.6 aA
+silicon + *R. solanacearum*	5.39 ± 0.5 aA	5.10 ± 0.9 aA	0.00 ± 0.0 aB

Data are means of nine plants per treatment derived of three individual experiments for 5dpi and 8 dpi and six plants per treatment derived of two individual experiments for 12 dpi ± SE. Dpi: days post inoculation; CFU: colony forming units.

Small letters refer to the comparison of treatments at the same evaluation date. Capital letters refer to the comparison for the same treatment at different evaluation dates. Same letters are not significantly different with Wilcoxen rank-sum Test at $\alpha = 5\%$.

3.3.3 Lignification in tomato stems

Lignification was observed in tissue surrounding the vascular bundles in all examined plants of tomato genotype King Kong2 at 8 dpi, but generally no differences in the intensity of stained lignin were observable between treatments (Fig.3.7a-r). No differences occurred comparing low or high bacterial populations in vessels (Fig. 3.7d-i and 3.7m-r). Bacteria were visible as darker masses in single vessels in silicon treated, inoculated plant, where they reached 9.96 log CFU/g (Fig. 3.7p-r), but not visible in highly infected non-silicon treated plants (Fig. 3.7g-i), possibly due to sample and cut selection. At 12 dpi no differences in lignification were observed for all treatments, additionally no bacteria could be reisolated from these inoculated plants at the evaluation date (Fig. 3.8a-l, Table 3.1).

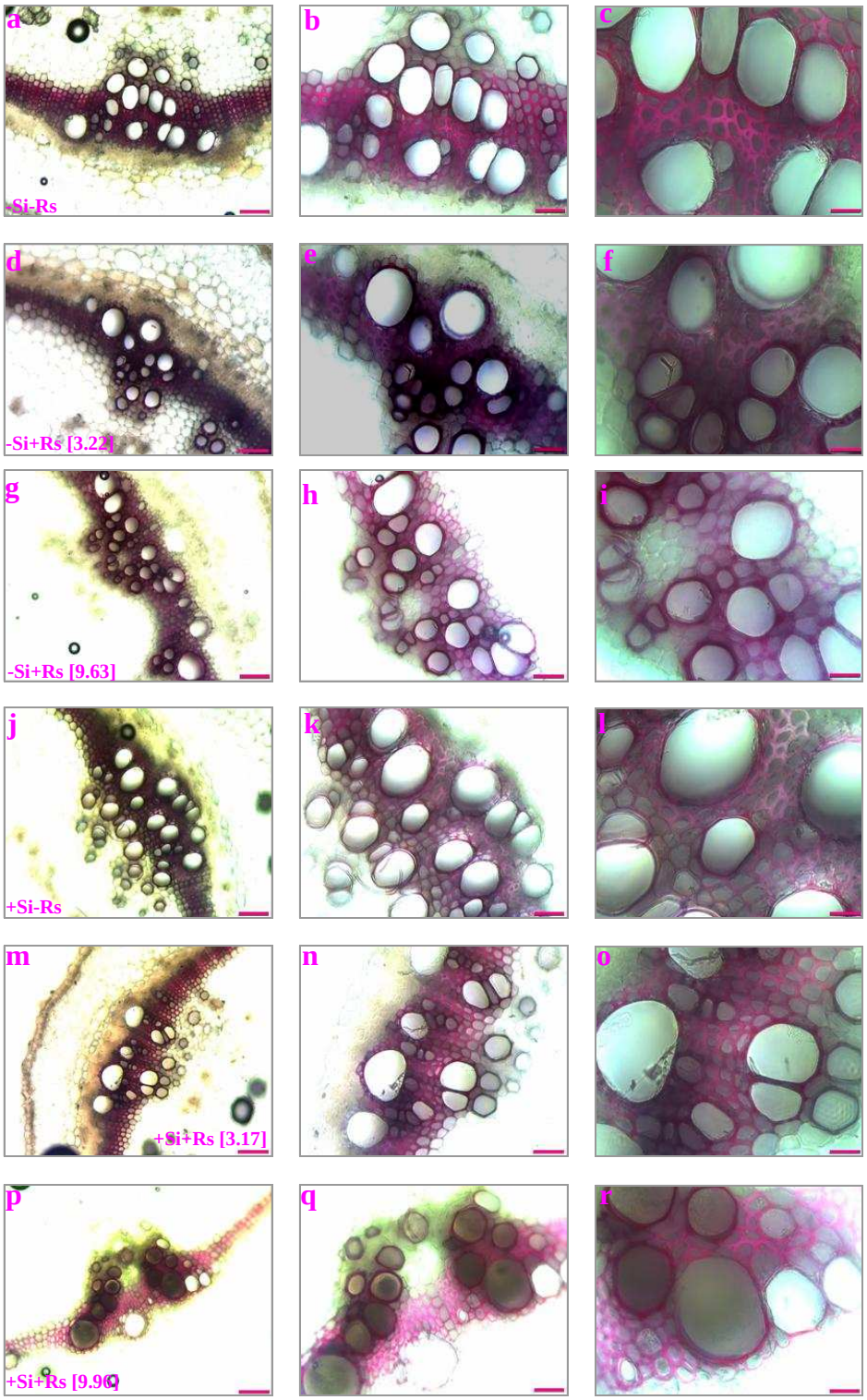

Fig. 3.7: Lignification of vascular bundles of tomato plants of genotype King Kong2, healthy and inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon, 8 days post inoculation, stained with phloroglucinol-HCl, shown in 3 magnifications.

a-c: –Si–Rs; d-f: -Si+Rs (log CFU/g: 3.22); h-i: -Si+Rs highly infected (log CFU/g: 9.63); j-l: +Si–Rs; m-o: +Si+Rs (log CFU/g: 3.17); p-r: +Si+Rs highly infected (log CFU/g: 9.96). Bar in a, d, g, j, m, p = 100 µm; bar in b, e, h, k, n, q = 50 µm; bar in c, f, i, l, o, r = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

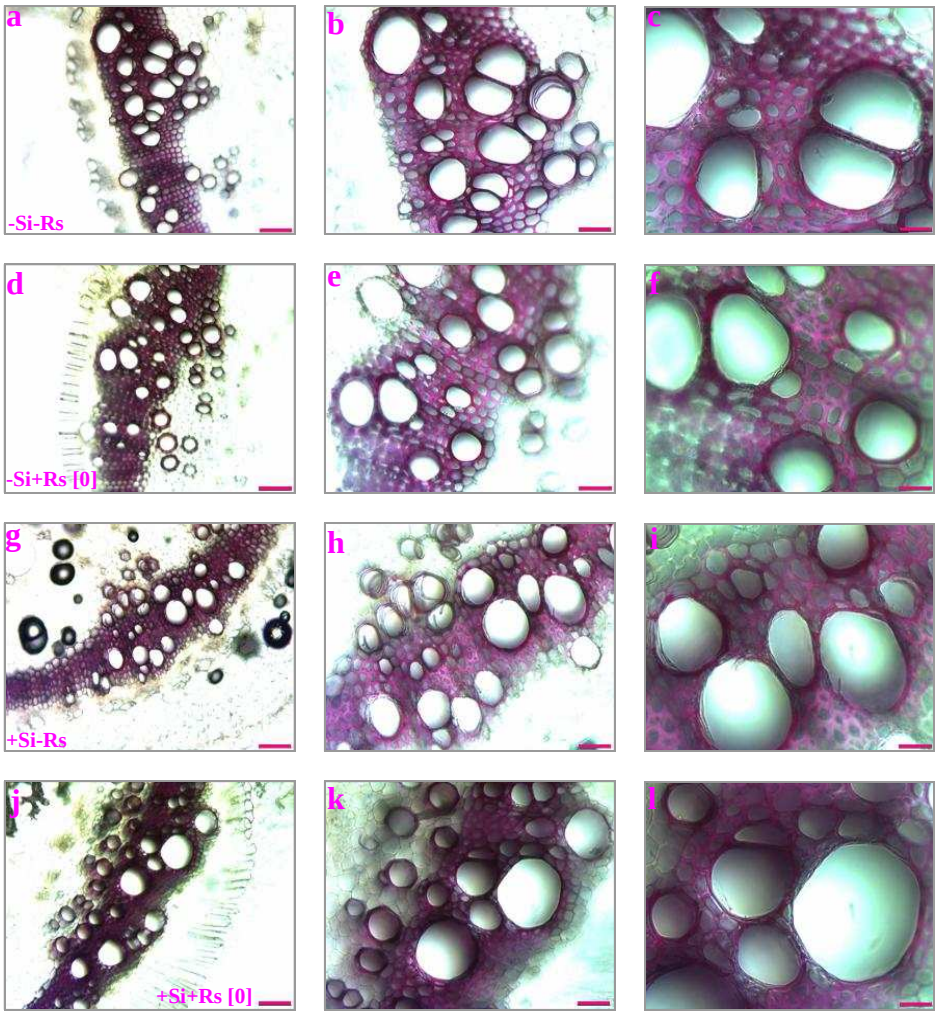

Fig. 3.8: Lignification of vascular bundles of tomato genotype King Kong2, healthy and inoculated with *R. solanacearum* strain ToUdk2 with and without silicon, 12 days post inoculation, stained with phloroglucinol-HCl, shown in 3 magnifications.

a-c –Si–Rs; d-f: -Si+Rs (log CFU/g: 0); g-i: +Si–Rs; j-l: +Si+Rs (log CFU/g: 0); bar in a, d, g, j = 100 µm, bar: b, e, h, k = 50 µm, bar in c, f, i, l = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

3.3.4 Tylosis formation in stems

Vessels with clearly visible, globular-shaped structures and completely filled vessels were counted throughout the experiments (Fig. 3.9).

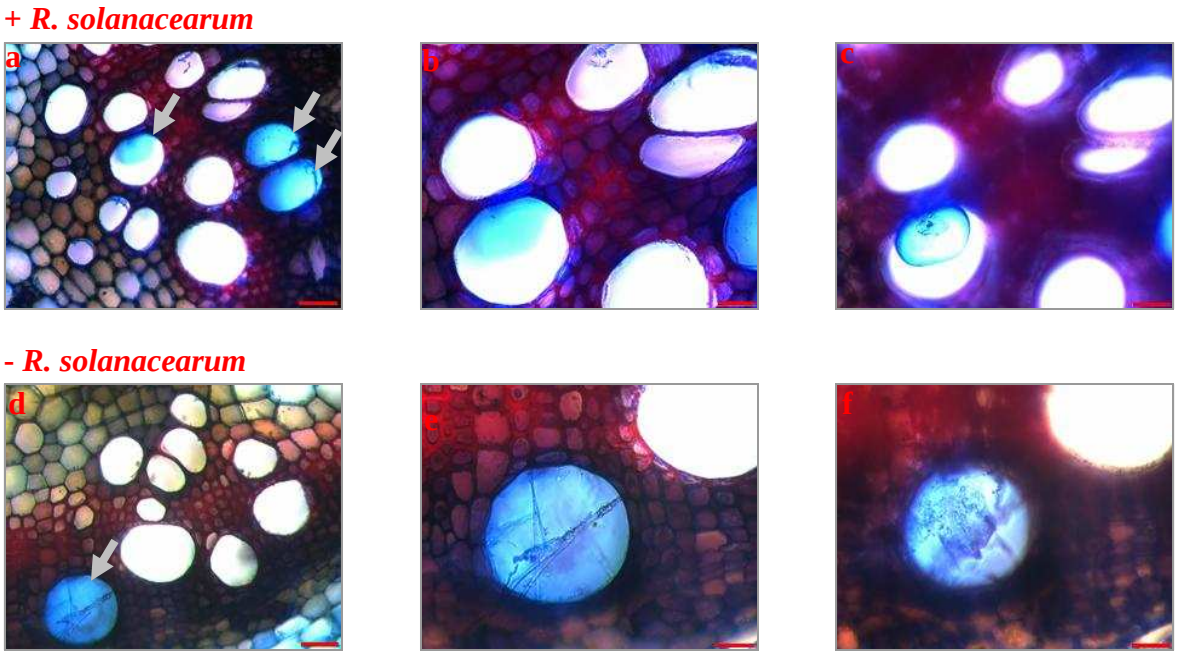

Fig. 3.9: Vessels filled with tylosis of (a-c) silicon amended, *R. solanacearum*-inoculated plant 5 days post inoculation (dpi) and (d-f) non-silicon amended healthy plant at 8 dpi, stained with an Alcian blue / safranin solution, shown in 2 magnifications.
Bar in a, d = 50 µm; bar in b, c, e, f = 20 µm.

Tylosis were found in each treatment, irrespective of inoculation and silicon or non-silicon amenment with a tendency to increased tylosis formation in inoculated plants (Table 3.2).

A significantly increased tylosis formation was found comparing +Si +Rs to +Si–Rs and –Si-Rs treatment at 8 dpi. This effect was also observed as a tendency at 12 dpi, and at 5 dpi in one experiment, where neither symptom development nor bacteria populations in stems after inoculation could be detected (Table 3.2). Comparing the same treatment at different evaluation dates, tylosis were increased and most stable in silicon treated, inoculated (+Si+Rs) plants at 8 and 12 dpi compared to 5 dpi.

Table 3.2: Tylosis formation (%) in stems of tomato genotype King Kong2 healthy and inoculated with *Ralstonia solanacearum* strain ToUdk2 amended with and without silicon at 5 dpi, 8 dpi and 12 dpi and in an experiment without symptom development at 5 dpi.

	Tylosis formation [%]			
Treatment	**5dpi**	**8dpi**	**12dpi**	**5dpi***
-Si –Rs	8.11 ± 1.5 aA	7.45 ± 1.1 bA	5.93 ± 1.9 aA	5.17 ± 0.5
-Si +Rs	9.17 ± 1.5 aA	10.60 ± 1.2 abA	9.88 ± 1.7 aA	10.37± 5.0
+Si –Rs	9.11 ± 1.6 aA	7.11 ± 1.4 bA	7.68 ± 0.9 aA	8.23 ± 2.5
+Si +Rs	9.16 ± 0.7 aA	13.54 ± 1.9 aA	13.01 ± 1.6 aA	13.07 ± 4.5

Data are means ± SE of three experiments for 5 and 8dpi, and two experiments for12dpi, with three plants per treatment. Si: silicon; Rs: *R. solanacearum*

*: experiment without symptom development; data are means ± SE of three plants per treatment

Small letters refer to the comparison of treatments at the same evaluation date. Capital letters refer to the comparison of the same treatment at different evaluation dates. Same letters are not significantly different with Wilcoxen rank-sum Test at α 5%.

3.3.5 Hydrogen peroxide (H_2O_2) accumulation in stems

Hydrogen peroxide (H_2O_2) accumulated in tissue around vascular bundles, but generally, no differences in H_2O_2 accumulation related to silicon nutrition were observed, neither at 5 dpi nor at 8 dpi (Fig. 3.10a-r and Fig. 3.11a-r). In highly infected plants (log CFU / g > 8) H_2O_2- staining was reduced or absent, independent of silicon amendment (Fig. 3.10g-i, 3.10p-r and Fig. 3.11g-i; 3.11p-r).

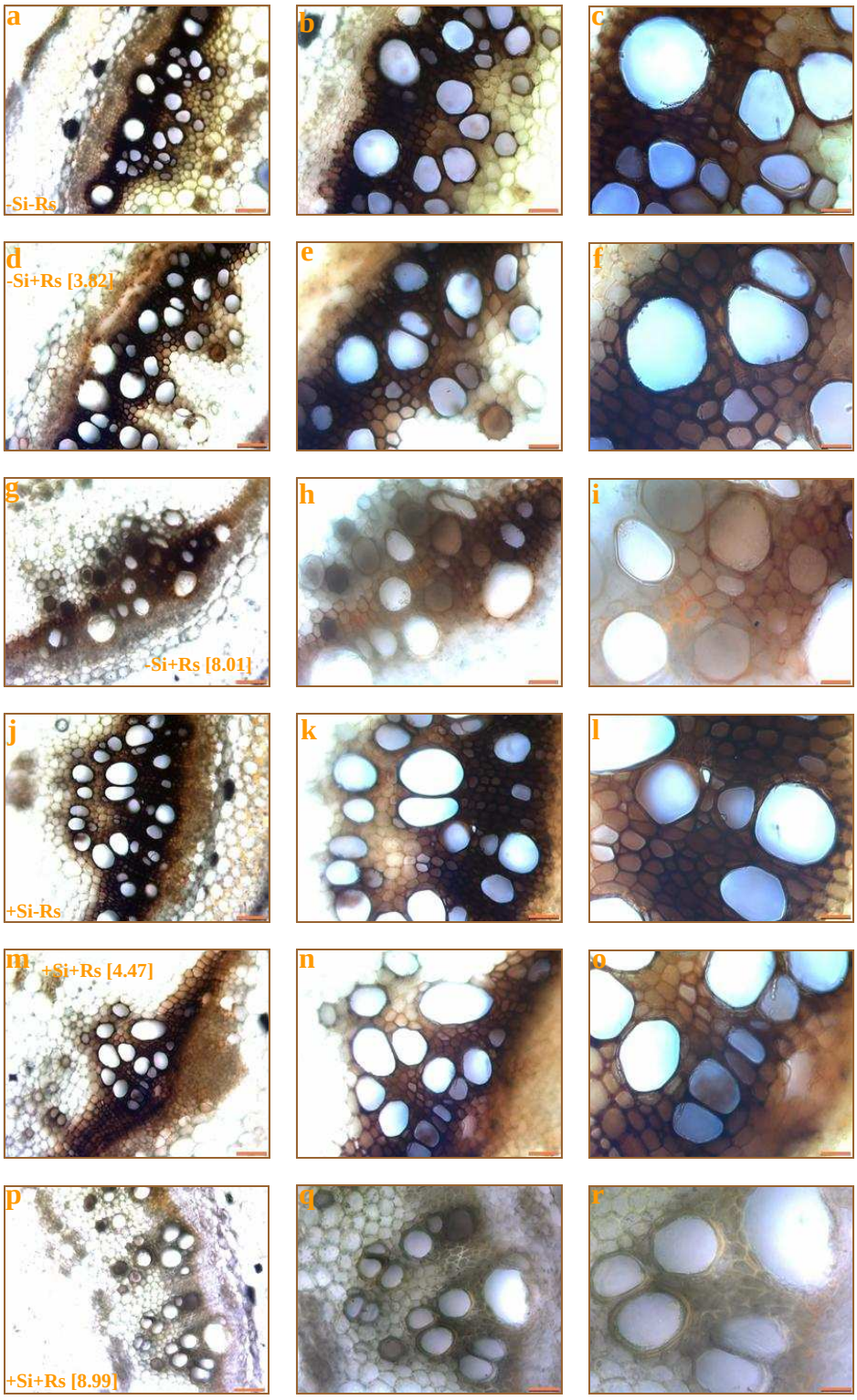

Fig. c10: Hydrogen peroxide (H_2O_2) accumulation in stems of tomato genotype King Kong2, healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon, 5 days post inoculation (dpi), stained with DAB, shown in 3 magnifications.

Pictures are representative for each treatment: a-c: –Si–Rs; d-f: -Si+Rs (log CFU/g: 3.82); h-i: -Si+Rs highly infected (log CFU/g: 8.01), j-l: +Si–Rs; m-o: +Si+Rs (log CFU/g: 4.47); p-r: +Si+Rs highly infected (log CFU/g: 8.99). Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Bar in a, d, g, j, m, p = 100 µm; bar in b, e, h, k, n, q = 50µm; bar in c, f, i, l, o, r = 20µm

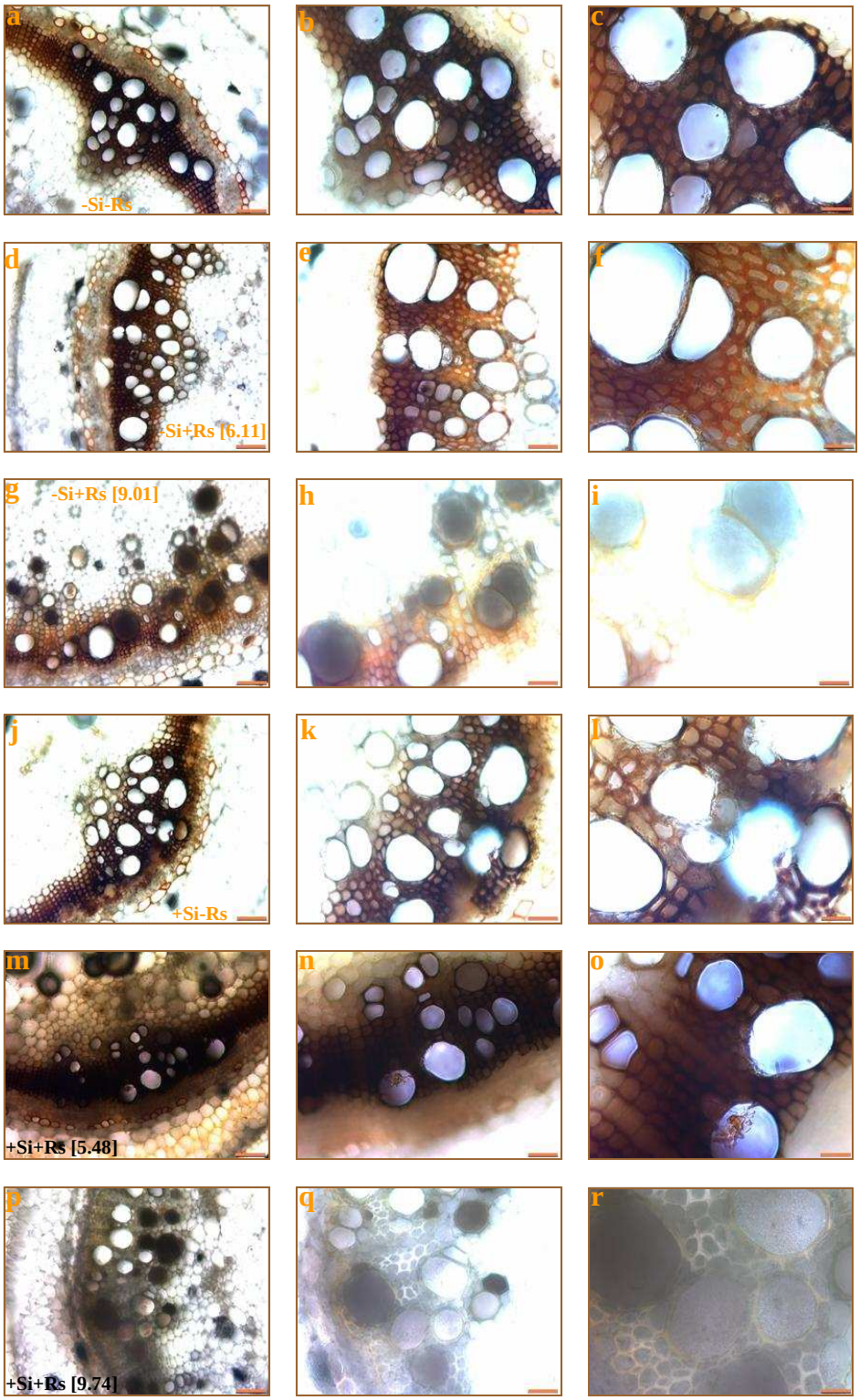

Fig. c11: Hydrogen peroxide (H_2O_2) accumulation in stems of tomato genotype King Kong2, healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon, 8 days post inoculation (dpi), stained with DAB, shown in 3 magnifications.

Pictures are representative for each treatment: a-c: –Si–Rs; d-f: -Si+Rs (log CFU/g: 6.11); h-i: -Si+Rs highly infected (log CFU/g: 9.01), j-l: +Si–Rs; m-o: +Si+Rs (log CFU/g: 5.48); p-r: +Si+Rs highly infected (log CFU/g: 9.74). Additionally, treatment and bacterial numbers [log CFU/g] are given in each first picture representative for the row. Bar in a, d, g, j, m, p = 100 µm; bar in b, e, h, k, n, q = 50µm; bar in c, f, i, l, o, r = 20µm.

3.3.6 Callose deposition: symptom development and bacterial quantification in tomato stems

Symptom development, expressed in AUD_IPC was similar for tomato genotype King Kong2 and RIL NHG3 in non-silicon and silicon treated, inoculated plants. Both genotypes showed reduced symptom development after silicon amendment (Table 3.3).

Table 3.3: Symptom development expressed as area under disease incidence progress curve [AUD_IPC] of tomato genotype King Kong2 and RIL NHG3 in experiments for callose observation.

	Area under disease incidence progress curve [AUD_IPC]	
Treatment	**King Kong2**	**NHG3**
- silicon + *R. solanacearum*	1306.25 ± 221.3	1155.0 ± 60.0
+ silicon + *R. solanacearum*	665.0 ± 45.0	791.25 ± 338.8

Data are means ± SE of two individual experiments.

Bacterial numbers were increased in roots compared to stems across genotypes, treatments and evaluation dates (Table 3.4). Higher bacterial populations were present in stems and roots of non-silicon treated plants of RIL NHG3 at all evaluation dates and for genotype King Kong2 in stems and roots at 8 dpi.

Table 3.4: Bacterial numbers (log CFU / g) in stems and roots of *R. solanacearum*-inoculated tomato genotype King Kong2 and RIL NHG3 amended with and without silicon at 5dpi, 8 dpi and 12 dpi.

		Bacterial numbers [log CFU / g]			
		King Kong2		**NHG3**	
Treatment		**Stem**	**Root**	**Stem**	**Root**
5dpi	**-Si +Rs**	3.24 ± 0.9	3.41 ± 1.5	5.20 ± 1.3	7.03 ± 0.4
	+Si +Rs	6.02 ± 0.6	7.05 ± 0.5	3.47 ± 1.2	6.71 ± 0.5
8dpi	**-Si +Rs**	6.83 ± 1.1	9.14 ± 1.7	4.50 ± 0.2	7.01 ± 0.4
	+Si +Rs	5.20 ± 0.4	5.73 ± 0.5	3.90 ± 0.2	5.26 ± 0.6
12dpi	**-Si +Rs**	0.82 ± 0.8	4.71 ± 2.4	8.61 ± 0.4	9.01 ± 0.4
	+Si +Rs	1.18 ± 1.2	6.06 ± 0.5	3.43 ± 1.8	4.77 ± 2.4

Data are means ± SE of three plants per treatment of two experiments for 5 dpi, and for one experiment for 8 and 12 dpi.

3.3.7 Autofluorescence and callose deposition

Differences in autofluorescence were not observed across genotypes, treatments and evaluation dates (Fig. 3.12a, b, e, f, i, j, m, n, q, r, u, v - Fig. 3.17a, b, e, f, i, j, m, n, q, r, u, v). Callose stained with aniline blue, resulted in white coloration surrounding vascular bundles under UV illumination, most abundant at 12 dpi for both genotypes and all treatments (Fig.3.16c, d, g, h, k, l, o, p, s, t, w, x and Fig. 3.17c, d, g, h, k, l, o, p, s, t, w, x).

In genotype King Kong2, those inoculated plants which harboured higher bacterial numbers (log CF/g > 6) showed decreased staining in non-silicon treated plants and no callose deposition in silicon-treated plants at 5 dpi (Fig 3.12k, l, w, x). Also in genotype NHG3 reduced callose deposition in moderately infected non-silicon treated plant compared to all other treatments was observed (Fig. 3.13g and 3.13h). At 8 dpi, callose staining was nearly absent in both genotypes and all treatments (Fig. 3.14c, d, g, h, k, l, o, p, s, t, w, x and Fig. 3.15c, d, g, h, k, l, o, p, s, t, w, x), and differences between treatments in both genotypes were generally not observed at 8 and 12dpi (Fig. 3.14 c, d, g, h, k, l, o, p, s, t, w, x - Fig. 3.17 c, d, g, h, k, l, o, p, s, t, w, x).

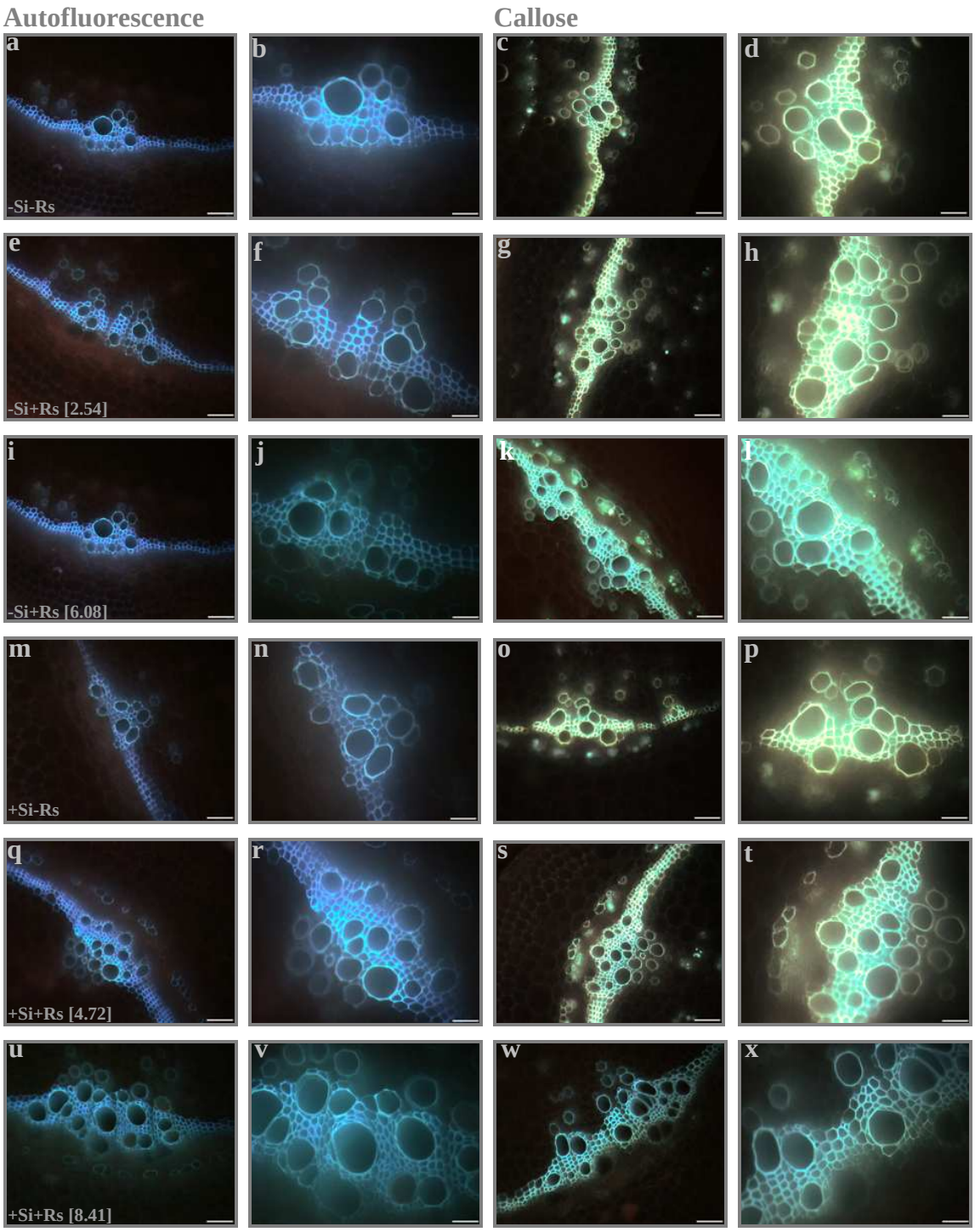

Fig. 3.12: Autofluorescence and callose deposition around vascular bundles in stems of healthy and *R. solanacearum*-inoculated plants of tomato genotype King Kong2 amended with and without silicon 5 days post inoculation (dpi), stained with aniline blue, shown in two magnifications.

a, b: -Si–Rs autofluorescence control (AF); c, d: –Si–Rs callose; e, f: -Si+Rs AF (log CFU/g: 2.45); g, h: -Si+Rs callose; i, j: -Si+Rs AF (log CFU/g: 6.08); k, l: -Si+Rs callose; m, n: +Si–Rs AF; o, p: +Si–Rs callose; q, r: +Si+Rs AF (log CFU/g: 4.72); s, t: +Si+Rs callose; u, v: +Si+AF (log CFU/g: 8.41), w, x: +Si+Rs callose. Bar in a, c, e, g, i, k, m, o, q, s, u, w = 100 µm; bar in b, d, f, h, j, l, n, p, r, t, v, x = 50 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

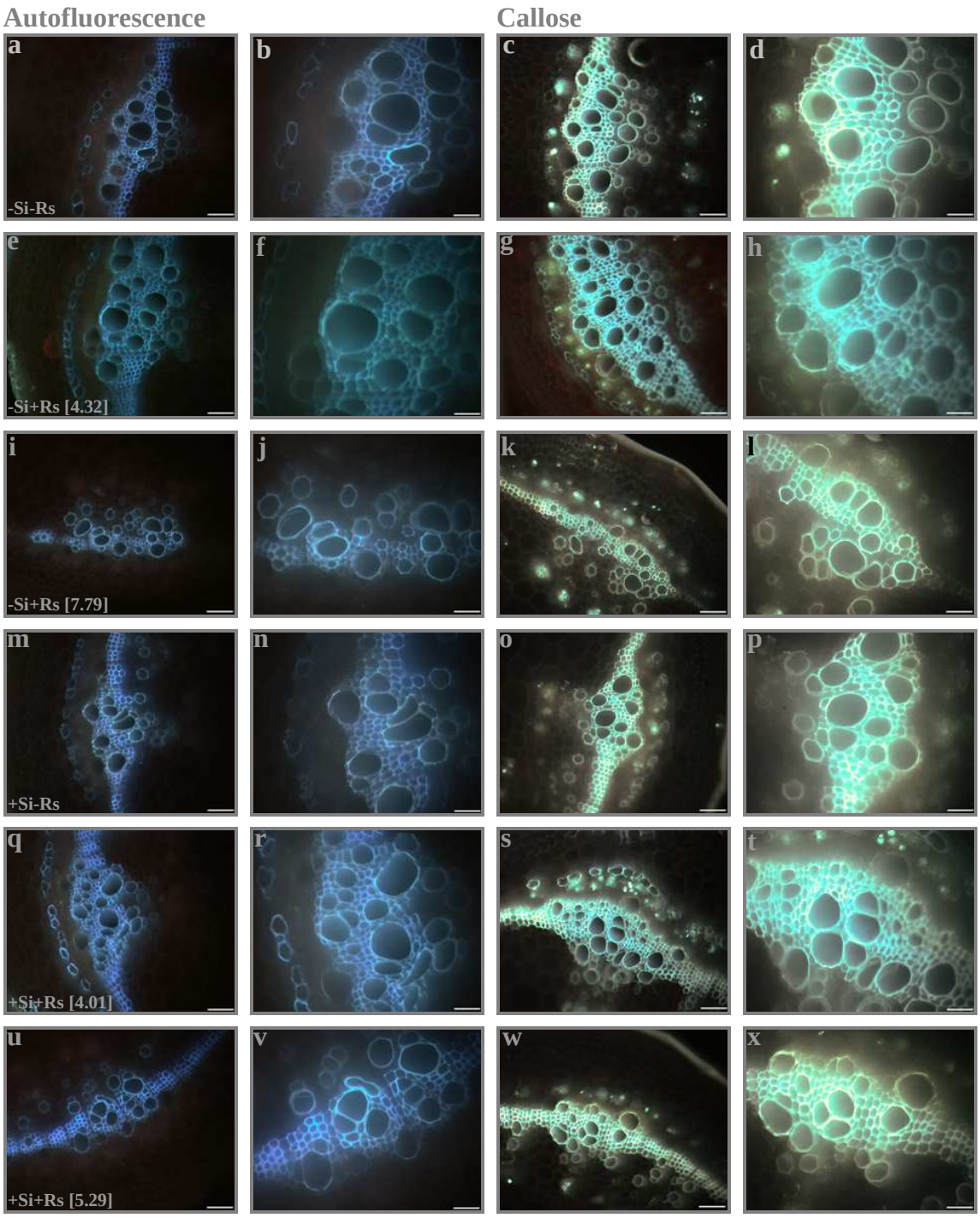

Fig. 3.13: Autofluorescence and callose deposition around vascular bundles in stems of healthy and *R. solanacearum*-inoculated plants of tomato genotype NHG3 amended with and without silicon 5 days post inoculation (dpi), stained with aniline blue, shown in two magnifications.

a, b: -Si–Rs autofluorescence control (AF); c, d: –Si–Rs callose; e, f: -Si+Rs AF (log CFU/g: 4.32); g, h: -Si+Rs callose; i, j: -Si+Rs AF (log CFU/g: 7.79); k, l: -Si+Rs callose; m, n: +Si–Rs AF; o, p: +Si–Rs callose; q, r: +Si+Rs AF (log CFU/g: 4.01); s, t: +Si+Rs callose; u, v: +Si+Rs AF (log CFU/g: 5.29); w, x: +Si +Rs callose. Bar in a, c, e, g, i, k, m, o, q, s, u, w = 100 µm; bar in b, d, f, h, j, l, n, p, r, t, v, x = 50 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

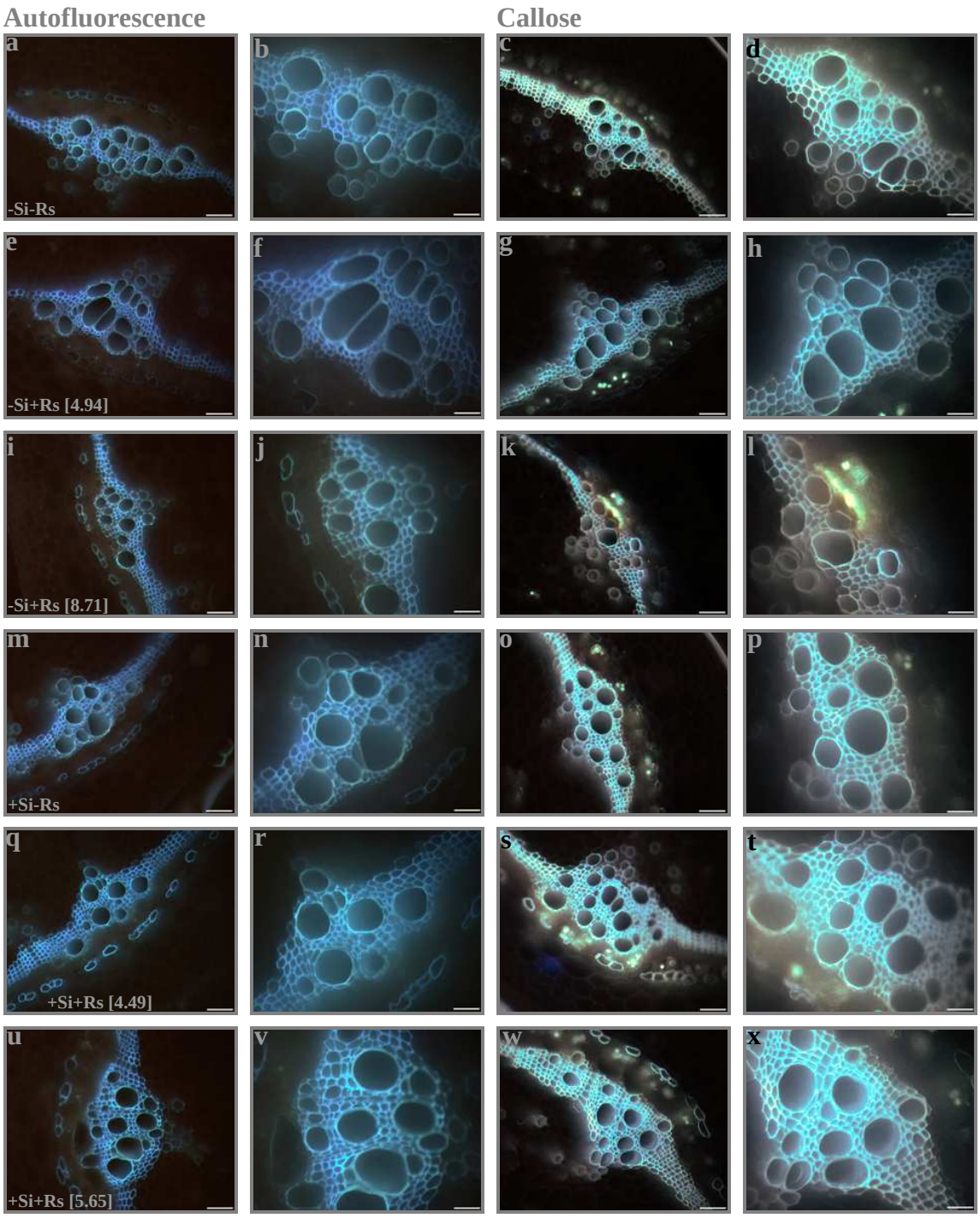

Fig. 3.14: Autofluorescence and callose deposition around vascular bundles in stems of healthy and *R. solanacearum*-inoculated plants of tomato genotype King Kong2 amended with and without silicon 8 days post inoculation (dpi), stained with aniline blue, shown in two magnifications.

a, b: -Si–Rs autofluorescence control (AF); c, d: –Si–Rs callose; e, f: -Si+Rs AF (log CFU/g: 4.94); g, h: -Si+Rs callose; i, j: -Si+Rs AF (log CFU/g: 8.71); k, l: -Si+Rs callose; m, n: +Si–Rs AF; o, p: +Si–Rs callose; q, r: +Si +Rs AF (log CFU/g: 4.49); s, t: +Si+Rs callose; u, v: +Si+Rs AF (log CFU/g: 5.65), w, x: +Si+Rs callose. Bar in a, c, e, g, i, k, m, o, q, s, u, w = 100 µm; bar in b, d, f, h, j, l, n, p, r, t, v, x = 50 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

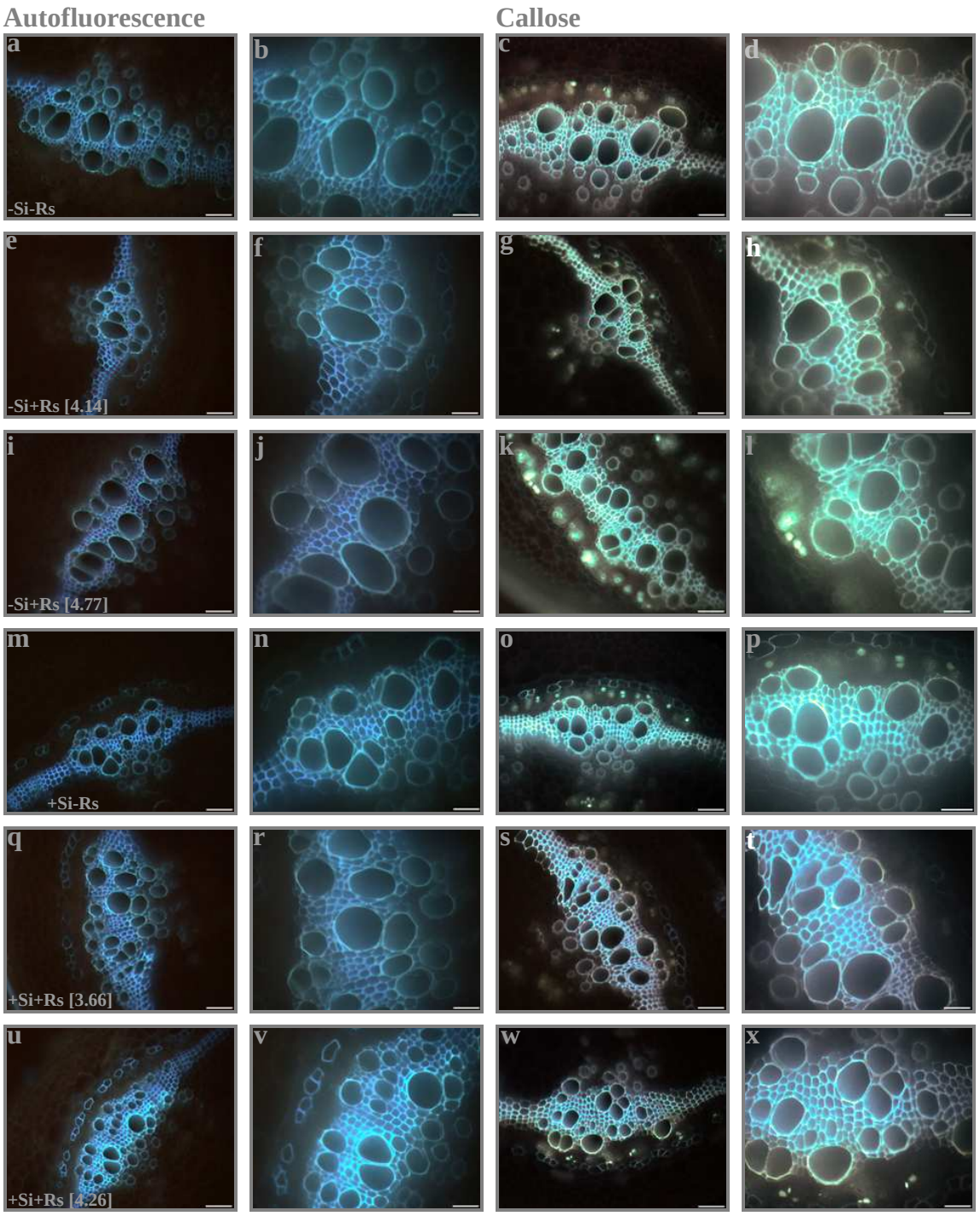

Fig. 3.15: Autofluorescence and callose deposition around vascular bundles in stems of healthy and *R. solanacearum*-inoculated plants of tomato genotype NHG3 amended with and without silicon 8 days post inoculation (dpi), stained with aniline blue, shown in two magnifications.

a, b: -Si –Rs autofluorescence control (AF); c, d: –Si–Rs callose; e, f: -Si +Rs AF (log CFU/g: 4.14); g, h: -Si+Rs callose; i, j: -Si +Rs AF (log CFU/g: 4.77); k, l: -Si+Rs; m, n: +Si–Rs AF; o, p: +Si–Rs callose; q, r: +Si+Rs AF (log CFU/g: 3.66); s, t: +Si+Rs callose; u, v: +Si+Rs AF (log CFU/g: 4.26), w, x: +Si+Rs callose. Bar in a, c, e, g, i, k, m, o, q, s, u, w = 100 µm; bar in b, d, f, h, j, l, n, p, r, t, v, x = 50 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

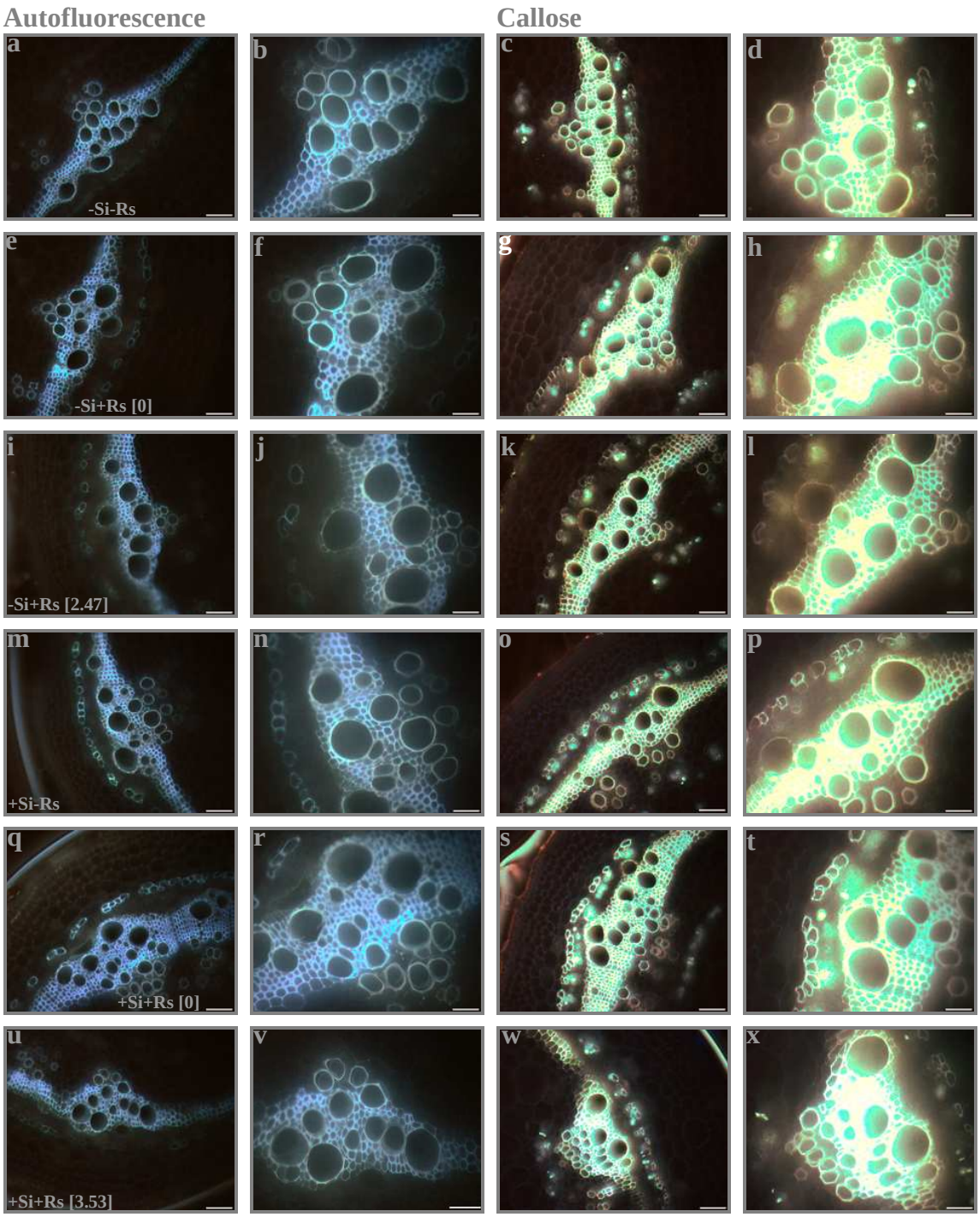

Fig. 3.16: Autofluorescence and callose deposition around vascular bundles in stems of healthy and *R. solanacearum*-inoculated plants of tomato genotype King Kong2 amended with and without silicon 12 days post inoculation (dpi), stained with aniline blue, shown in two magnifications.

a, b: -Si–Rs autofluorescence control (AF); c, d: –Si–Rs callose; e, f: -Si+Rs AF (log CFU/g: 0); g, h: -Si+Rs callose; i, j: -Si+Rs AF (log CFU/g: 2.47); k, l: -Si +Rs callose; m, n: +Si–Rs AF; o, p: +Si–Rs callose; q, r: +Si +Rs AF (log CFU/g: 0); s, t: +Si+Rs callose; u, v: +Si+Rs AF (log CFU: 3.53), w, x: +Si +Rs callose. Bar in a, c, e, g, i, k, m, o, q, s, u, w = 100 µm; bar in b, d, f, h, j, l, n, p, r, t, v, x = 50 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

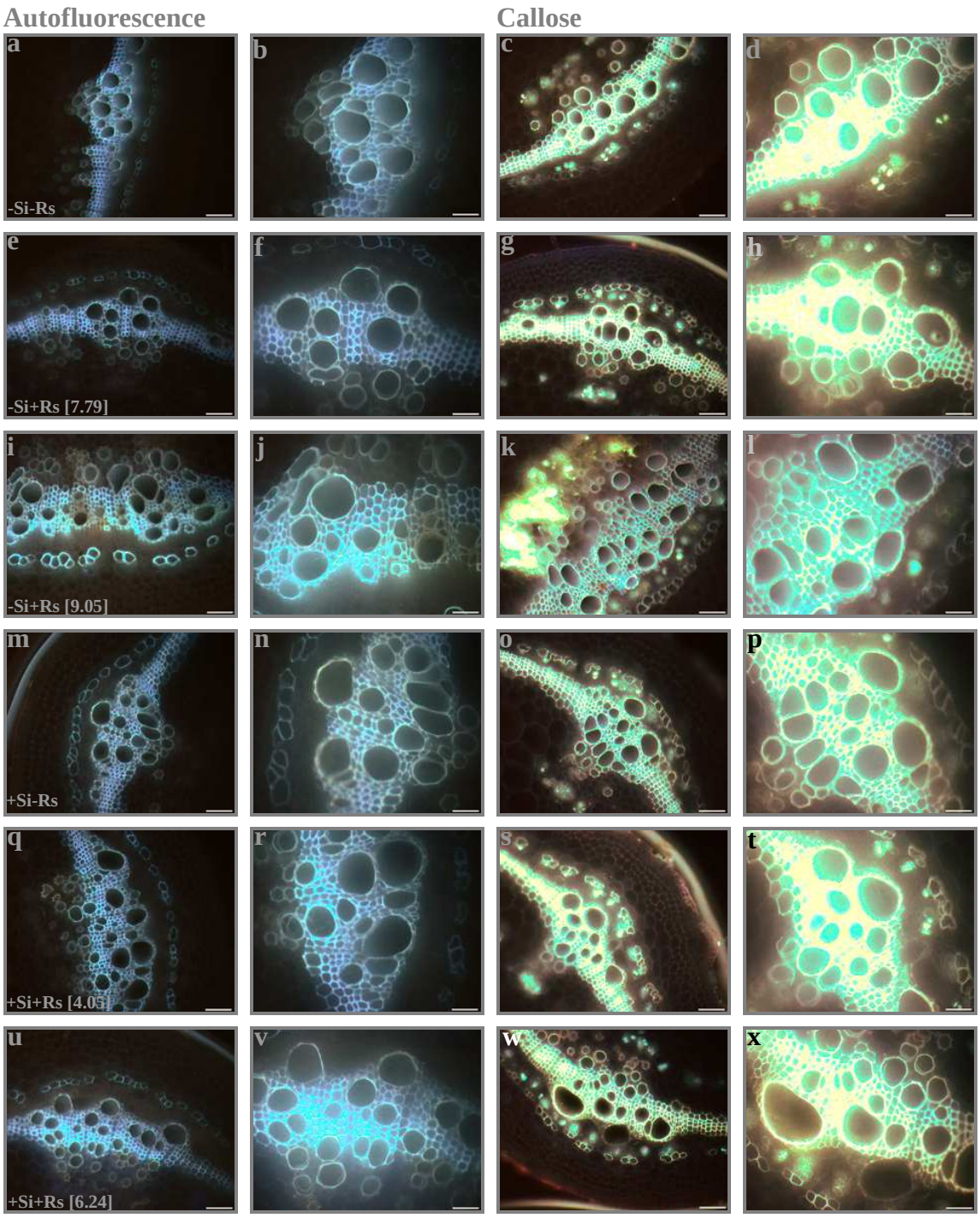

Fig. c17: Autofluorescence and callose deposition around vascular bundles in stems of healthy and *R. solanacearum*-inoculated plants of tomato genotype NHG3 amended with and without silicon 12 days post inoculation (dpi), stained with aniline blue, shown in two magnifications.

a, b: -Si–Rs autofluorescence control (AF); c, d: –Si–Rs callose; e, f: -Si+Rs AF (log CFU/g: 7.79); g, h: -Si+Rs callose; i, j: -Si+Rs AF (log CFU/g: 9.05); k, l: -Si+Rs callose; m, n: +Si–Rs AF; o, p: +Si–Rs callose; q, r: +Si+Rs AF (log CFU/g: 4.05); s, t: +Si+Rs callose; u, v: +Si+Rs AF (log CFU/g: 6.24), w, x: +Si +Rs callose. Bar in a, c, e, g, i, k, m, o, q, s, u, w = 100 µm; bar in b, d, f, h, j, l, n, p, r, t, v, x = 50 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

3.4 Discussion

Staining of sections with alcian blue revealed an extensive blue coloration of structures inside the vessels referred to as tylosis, indicating pectin as the major component (Benes, 1968; Soukup and Votrubová, 2005). No comparable structures were observed after staining with aniline blue indicative for callose (Eschrich and Currier, 1964), phloroglucinol-HCl indicative for aldehydes of cinnamic acids of lignin (Clifford, 1974; Vance *et al.*, 1980; Lewis and Yamamoto, 1990) and toluidine blue (data not shown), indicative for phenols (Ramalingam and Ravindranath, 1970).

The appearance of tylosis in healthy petioles of sunflowers was suggested to be triggered by the frequent embolization of vulnerable vessels, resulting in incompressible tissue in order to maintain the pressure during the water flow of transpiration (Canny, 1997). On the other hand, early investigations revealed vascular occlusions, including tylosis formation, in the plants' reaction towards vascular fungi (Beckmann, 1987), but tylosis were also found in the reaction with bacterial pathogens, for instance Pierce's disease in resistant grape cultivars (Mollenhauer, 1976).

In our study, tylosis were found in all treatments with a general trend to higher tylosis formation in inoculated plants, with greatest extent in silicon-treated, inoculated plants at later pathogen infestation states (8 and 12 dpi), postulating not only a physiological role of tylosis in tomato, but also as active defence mechanism against *R. solanacearum*. It was reported that tylosis were initiated with equal facility in near iso-genic lines of tomato, resistant and susceptible to *Fusarium oxysporum* f.sp. *lycopersici*, in the early infection process (1 and 2 dpi), but tylosis development continued in the resistant host plant, whereas the susceptible host showed a retarded formation of tylosis (Beckmann *et al.*, 1972, Elgersma *et al.*, 1972). In the interaction of tomato with *R. solanacearum*, tylosis were found in stems of the susceptible tomato cultivar Floradel and in the resistant cultivar Caraibo, but appearance and location of tylosis differed in the genotypes (Grimault, *et al.*, 1994). In the resistant cultivar tylosis occurred only in colonized and adjacent vessels, whereas the susceptible cultivar produced tylosis mostly in non-colonized vessels. Additionally, bacterial spread was restricted by tylosis in cultivar Caraibo. Furthermore, the authors stated that tylosis formation is an induced process, since no tylosis occurred in non-inoculated plants. In tomato genotype King Kong2, tylosis were present in inoculated plants, as well as non-inoculated plants but with higher frequency in inoculated plants and

highest in silicon-treated inoculated plants, thus we also consider the reaction related to silicon treatment as induced or primed effect after pathogen challenge in this genotype, whereas priming means a state of enhanced ability to mobilize pathogen- or elicitor-induced cellular defence responses after infection (Conrath *et al.*, 2002).

Interestingly, the same trend was observed in a trial with *R. solanacearum*-inoculated plants of genotype King Kong2, where no bacteria were detected in the stems at 5 dpi and no wilt symptom development occured. These findings suggest an induction of tylosis in stems by a so far unknown component in inoculated plants or an induction through root colonization. A further characterization of the nature of the induction needs further investigations. Additionally, this observation proves that staining is not due to a cross-reaction of the staining solution with colonizing bacterial cells in vessels.

Generally, lignin is classified into two groups: (1) gymnosperm lignins, primarily consisting of guaiacyl subunits [G], polymerized from coniferyl alcohol, and small portions of *p*-hydroxyphenyl units [H] derived from *p*-coumaryl alcohol; and (2) angiosperm lignins, containing both syringyl units [S], polymerized from sinapyl alcohol and guaiacol units [G], with low quantities of *p*-hydroxyphenyl units [H] (Whetten *et al.*, 1998). Staining of tomato stem sections with phloroglucinol-HCl resulted in a purple coloration of vascular bundles, indicative for substituted cinnamaldehyde groups (Vance *et al.*, 1980) for all treatments and at all evaluation dates observed in this study, without differences. Variation in the proportions of the lignin precursors is described within the cell wall, between cell types, and in response to pests and pathogens and wounding (Whetten, *et al.*, 1998), thus the chemical nature of the lignification after wounding or pathogen attack can differ of the normal lignin in the cell wall (Vance *et al.*, 1980; Lewis and Yamamoto, 1990). An additional test specific for syringyl groups is suggested to detect possible changes of the chemical structure of lignins in the interaction of silicon-induced resistance of tomato and *R. solanacearum*.

Safranin, a rather unspecific reagent for lignin staining, gives a red coloration with phenols (Lewis and Yamamoto, 1990), but did not show a homogenous staining pattern among treatments in our experiments, suggesting that phenols are apparently not involved in the resistance reaction of silicon-treated plants to *R. solanacearum*.

We observed hydrogen peroxide (H_2O_2) accumulation in the stems' vascular system of tomato genotype King Kong2, irrespectively of silicon treatment and inoculation with *R. solanacearum*, at 5 and 8 dpi, suggesting that H_2O_2 accumulation is part of physiological processes in this tissue.

With increasing knowledge of the ROS network in plants, ROS have been shown as key feature of the hypersensitive response (HR) and programmed cell death (PCD), responding to pathogen attack in a genetically controlled response (Foyer and Noctor, 2005). The reaction of the plant cells towards ROS is not only depending on the ROS itself; concentration, side of production and the interaction with other stresses, but also on physiological parameters such as developmental stage and prehistory of the cell should be considered (Møller *et al.*, 2007).

The production of active oxygen species in the incompatible plant pathogen interaction is characterized by the occurrence of two distinct phases (Baker and Orlandi, 1995). The first phase is a relatively short-lived and non-specific response, appearing directly after the addition of compatible and incompatible pathogens, and the second phase is relatively long-lived later in the incompatible interaction. Baker *et al.* (1995) demonstrated that high bacterial inoculum levels in suspensions cultures lead to decreased ROS response in the second phase. Reasonable for this was an increase of ROS-scavenging activity induced earlier during the treatments with higher inoculum levels. We also observed a decreased to absent H_2O_2 staining with DAB in highly infected tomato plants. Since *R. solanacearum* as a xylem invading pathogen capable to totally block whole vessels (Nakaho *et al.*, 2000), we rather suggest that blocking of the vessels had lead to a disruption of the water-flow in the vascular bundles and thus, uptake of the dye solution failed in highly infected plants.

Reactive oxygen species, in particular H_2O_2, have been implicated to act as signals that mediate the systemic activation of gene expression in response to a wide range of biotic and abiotic stresses (Mittler, 2002; Laloi *et al.*, 2004). The common occurrence of H_2O_2 in xylem vessels was reported by Brown *et al.* (1998) in healthy tissues of French beans, which is according to our observations that H_2O_2 accumulates in vascular bundles of all treatments of tomato genotype King Kong2. This might be explained by the necessity of H_2O_2 for the oxidative coupling of phenols by peroxidases in lignification (Brown *et al.*, 1998; Apel and Hirt, 2004), and it can additionally act as a signal for further defence responses, as demonstrated for tomato (Orozco-Cardenas *et al.*, 2001). Nevertheless, ROS has been shown to act antimicrobial at least *in vitro*, when exogenous applied (Baker and

Orlandi, 1995) and thus, for some pathogens ROS detoxifying mechanisms were found. The conversion of H_2O_2 to H_2O and O_2 by catalases is found in most aerobic organisms, mostly to reduce high amounts of H_2O_2 in peroxisomes (Baker and Orlandi, 1995). *Ralstonia solanacearum* posses catalase activity, thus having the ability to detoxify H_2O_2 (Valls *et al.*, 2006). It was demonstrated that bacteria can tolerate higher H_2O_2 levels and the primary factor was the bacterial concentration, the higher the bacterial concentration, the faster the reduction of the H_2O_2 to tolerable levels (Baker and Orlandi, 1995). It remains to elucidate if *R. solanacearum* catalase is actively involved in the degradation of H_2O_2 at high bacterial densities in infected tomato xylem vessels.

Callose deposition was observed in vascular tissue of tomato stems in all treatments of tomato genotypes King Kong2 and NHG3, most evident at12 dpi. Also Asselbergh and Höfte (2007) found that callose naturally occurs in vascular tissues of tomato leaves.

The role of callose deposition in papillae formation to block pathogen entry has been accepted for a long time (Aist, 1976). The resistance of lettuce to the oomycete *Plasmopara lactucae-radicis*, based on callose deposition around the haustoria of the fungus (Stanghellini *et al.*, 1993) and an earlier and more pronounced accumulation of callose in *Arabidopsis* plants treated with β-amino-butyric acid (BABA) (Ton and Mauch-Mani, 2004) are supporting evidences for the role of callose deposition in plant resistance. However, in silicon-induced resistance of tomato to *R. solanacearum*, we could not find an induction of callose by silicon, in contrast, we observed less callose deposition in vascular bundles of higher infected, silicon and non-silicon treated plants, most evident in genotype King Kong2 at 5 dpi. Vogel and Somerville (2000) identified *Arabidopsis* mutants with altered growth capabilities of the powdery mildew pathogen *Erysiphe cichoracearum*, designated as powdery mildew resistant 1-4 (*pmr*1-4). The mutant *pmr*4 showed almost complete loss of callose accumulation beneath fungal colonies and after wounding, even at a later infestation state of the fungus, but pollen tubes had abundant callose in this mutant. In contrast to the expectation, the mutant was more resistant to powdery mildew and they suggest, as one possible scenario, that decreased callose accumulation leads to recognition of the fungus and a secondary defence pathway, more effective compared to the wild-type, is activated by the plant. These observations were confirmed by Nishimura *et al.* (2003) in the same mutant and extended analysis revealed that the *pmr4*-based resistance is most likely due to enhanced activation of the SA signal transduction pathway.

In contrast, it is known that bacterial pathogens evolved mechanisms to suppress basal resistance (Hückelhoven, 2007). The bacterial effectors AvrPto, AvrPtoB, AvrE and HopPtoM of *Pseudomonas syringae* are known to suppress callose deposition and the expression of host genes for papilla-associated proteins (Hauck *et al.*, 2003; DebRoy *et al.*, 2004; de Torres *et al.*, 2006). *R. solanacearum* harbours a type III secretion system (T3SS) which secretes effector proteins in the cytoplasm of plant cells (Alfano and Collmer, 2004) and Angot *et al.* (2006) provided evidence that T3SS effectors of *R. solanacearum* contain plant-like F-box domains that might contribute to the virulence on several host plants. It is suggested that effectors can either elicit or repress basal resistance of the plant. Their function as defense suppressors has first been described in *P. syringae* (Alfano and Collmer, 2004), but a possible suppression of callose deposition by bacterial effectors in the *R. solanacearum*-tomato interaction has so far not been described.

Another possibility for the decreased callose deposition might be an enzymatic degradation of callose by β-1,3-glucanase, which hydrolyze β-1,3 glycosidic bonds in linear or branched glucans (van Loon *et al.*, 2006). Among the virulence factors of *R. solanacearum* are six cell wall degrading enzymes: β-1,4-endoglucanase (Egl), exoglucanase (ChbA), endopolygalacturonase (PehA), exopolygalacturonases (PehB and PehC) and pectin methylesterase (Pme) (Denny, 2006), though β-1,3-glucanase has not been reported.

On the other hand, the accumulation of pathogenesis-related (PR) proteins has been described in many plant species as reaction towards pathogen attack (van Loon *et al.*, 2006). *Beta*-1,3- glucanases are classified as PR-2 and overexpression of glucanases have been shown to increase resistance towards different pathogens (van Loon *et al.*, 2006). This effect might be due to possible degradation of microbial cell wall components by the enzymes or by the generation of endogenous signal molecules, functioning as elicitors of further defense mechanisms. Plant-derived callose degradation would not only explain the reduced callose in infected plants at 5 dpi but also the reduced callose in all treatments at 8dpi, indicating rather a physiological host plant alteration rather than resistance reaction at the later time point.

From the data achieved so far it is not obvious whether the reduced or absent callose deposition in infected tomato vascular bundles might be favourable for the pathogen in the infection process or bacterial manifestation in vascular bundles or if it leads to the

activation of an alternative defence mechanism in tomato. This topic is further discussed in chapter 5 (gene expression) and in the general discussion.

Conclusion

Among the analysed histochemical changes due to infection and silicon treatment, only tyloses formation and callose deposition seem to be involved in the interaction.

CHAPTER 4

Immunohistochemical analyses of tomato cell wall structures – effect of silicon nutrition and *Ralstonia solanacearum* inoculation on arabinogalactan protein, (1→5)-α-L-arabinan and non-blockwise de-esterified epitopes of homogalacturonan of tomato recombinant inbred lines

Abstract

Tomato recombinant inbred lines (RILs) NHG3 and NHG60, susceptible to bacterial wilt, and NHG13 and NHG162, resistant, were analysed after silicon amendment and/or *R. solanacearum* inoculation for their stem cell wall structure using antibodies detecting arabinogalactan protein (LM2), (1→5)-α-L-arabinan (LM6) and non-blockwise de-esterified epitopes of homogalacturonan (LM7), monitored by immunofluorescence microscopy at 5 days post inoculation (dpi).

The ***autofluorescence*** of stems was higher tomato recombinant inbred lines NHG60 and NHG13 than in NHG3 and NHG162, irrespective of the treatment.

Intensive staining of vessels and surrounding tissues with LM2, detecting **arabinogalactan protein**, occurred in tomato genotypes NHG60 and NHG13, whereas genotypes NHG3 and NHG162 showed generally staining of only single vessels. Staining was slightly increased in genotype NHG60 for silicon and non-silicon treated, non-inoculated plants (-Si-Rs and +Si-Rs) and for silicon treated healthy and inoculated plants (+Si-Rs and +Si+Rs) in genotype NHG13. No differences in treatments were observed for genotype NHG3 and NHG162.

Staining of ***(1→5)-α-L-arabinan*** in vascular bundles with LM 6 was slightly increased in the non-inoculated treatments (-Si-Rs and +Si-Rs) in genotype NHG3. In genotypes

NHG60 and NHG13 increased staining was observed for both inoculated treatments (-Si+Rs and +Si+Rs) and additionally, for genotype NHG13 for non-inoculated non-silicon treated plants (-Si-Rs). In plants of genotype NHG162 a slightly increased staining was observed for non-silicon treated inoculated plants (-Si+Rs) and silicon treated non-inoculated plants (+Si-Rs).

Staining of ***non-blockwise de-esterified epitopes of homogalacturonan*** with LM7 was homogeneous across treatments in genotypes NHG3 and NHG162, whereas staining of genotype NHG3 was overall weaker compared to genotype NHG162. In genotype NHG60 increased staining was observed for both inoculated treatments (-Si+Rs and +Si+Rs) and was highest for non-inoculated silicon treated plants (+Si-Rs) in genotype NHG13.

4.1 Introduction

The plant cell wall

For many years, the plant cell wall was considered only as rigid inert structure that provides mechanical strength, but the view has changed, and currently it is considered as a dynamic, responsive structure necessary not only for a variety of developmental events, but also involved in relaying information from external stimuli, additionally to the mechanical strengthening of the wall (Rose *et al.*, 2000; Pilling and Höfte, 2003; Humphrey *et al.*, 2007).

This complex, semi-rigid structure surrounds the cytoplasmatic membrane and can be differentiated into three different zones – middle lamella, primary wall and secondary wall. The most external one, the middle lamella, functions mainly as separating panel and consists almost exclusively of pectic substances (Heredia *et al.*, 1995). After differentiation of the middle lamella, deposition of carbohydrates, fundamentally cellulose, forms the primary cell wall during the cell expansion, building up the structural base of the skeleton of the plant (Heredia *et al.*, 1995; Kaczkowski, 2003). The secondary wall is considered to be a supplementary wall with predominately mechanical function, usually connected with lignifications (Heredia *et al.*, 1995; Kaczkowski, 2003). Besides, the cellulose in the primary cell wall, an amorphous phase or matrix with heterogeneous composition, appears and two large groups of the non-cellulose polysaccharides of the matrix can be distinguished, the hemicelluloses and pectic substances (Heredia *et al.*, 1995). Pectins are the only major class of plant polysaccharides which are largely restricted to primary cell walls (Willats *et al.*, 2001a) and occur either soluble as strongly hydrated and gelling fractions present in the outer wall surface or insoluble when linked to cellulose structures (Kaczkowski, 2003).

The most representative pectic polysaccharides of the plant cell wall are homogalacturonan (HG), rhamnogalacturonan I (RG-I), RG-II, arabinan, galactan, arabinogalactan I. The main component, homogalacturonan (HG) consists of a linear chain of 1,4-linked α-D-galactopyranosyluronic acid (Gal*p*A), which is methyl esterified at the carboxyl groups to various degrees (Heredia *et al.*, 1995; Ridley *et al.*, 2001; Kaczkowski, 2003). Characteristic for the RG-I is a backbone of repeating disaccharide [→4) α-D- Gal*p*A-

(1→2) - α-L-Rah*p*-(1→]. The predominant side chains contain linear and branched α-L-arabinofuranosyl (Ara*f*), and/ or β-D-galactopyranosyl (Galp) residues (Rose *et al.*, 2000; Ridley *et al.*, 2001). The RG-II has an 1,4-linked α-D- Gal*p*A backbone with four different oligosaccharide side chains and thus, not structurally related to RG-I. It is present in primary walls predominantly as a dimer that is cross-linked by a 1 : 2 borate-diol ester (Rose *et al.*, 2000; Ridley *et al.*, 2001; Vincken *et al.*, 2003).

It is often assumed that HG, RG-I and RG-II are covalently linked to each other in primary cell walls (Rose *et al.*, 2000; Ridley *et al.*, 2001) and the highly branched RG-I with AG-I and arabinan are often referred to as pectic hairy regions, whereas the HG domains are referred to as smooth regions (Willats *et al.*, 2001a; Vincken *et al.*, 2003).

Pectin methyl esterases (PMEs) are enzymes which remove methyl-ester groups from HG (Willats *et al.*, 2001a). The removal of the methyl groups results in stretches of acidic residues that can associate with other HG chains by calcium cross links, thus controlling the assembly and disassembly of the pectic network, but the degree and pattern of methyl esterification is also important in regulating the cleavage of HG by pectinolytic enzymes (Willats *et al.*, 2001a).

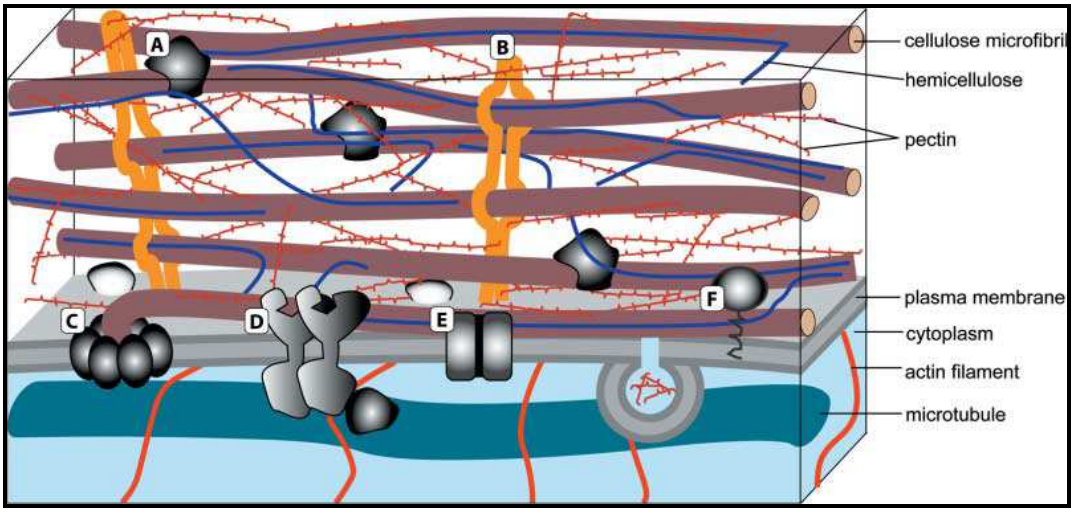

Fig. d0: Model of the cell wall-plasma membrane-cytoskeleton adapted from Humphrey *et al.* (2007).

The model illustrates the main polysaccharide and protein components. The wall consists of cellulose microfibrils, cross-linked by hemicelluloses, and embedded in a pectin matrix as well as numerous protein components such as (A) expansins, (B) extensins and (F) glycosylphosphatidylinositol (GPI)-anchored proteins which are heavily glycosylated and associated with the extensive polysaccharide network. Various plasma membrane proteins such as the (C) cellulose synthase complex, (D) receptor kinases, (E) ion channels, and (F) GPI-anchored proteins interact with the wall matrix as well as with internal cytoplasmic proteins, and the actin and tubulin cytokeleton.

Arabinogalactan-protein (AGP)

Arabinogalactan-proteins (AGPs) belong to the family of highly glycosylated hydroxyproline-rich glycoproteins (HRGPs), probably universally distributed in the plant kingdom (Majewska-Sawka and Nothnagel, 2000; Showalter, 2001). Typical for AGPs is the low protein content of less than 10%, generally rich in hydroxproline (Hyp), alanine, glycine and serine and a high carbohydrate content of more than 90%, where arabinose and galactose as major sugar residues are linked by *O*-gylcosidation to the OH group of hydroxproline or to serine residues in the protein core (Kreuger and van Holst, 1996).

They are classified into two groups depending on their polypeptide backbones as (1) 'classical' AGPs with an N-terminal secretion sequence that is removed from the mature protein, in general a central domain rich in Pro/Hyp and a C-terminal hydrophobic domain, and (2) 'non-classical' AGPs with regions that are atypical of AGPs, like regions rich in Asn or Cys residues additionally to the Pro/Hyp (Du *et al.*, 1996; Majewska-Sawka and Nothnagel, 2000; Gaspar *et al.*, 2001; Showalter, 2001). Additionally, the hydrophobic transmembrane domain at the C terminus of classical AGPs is replaced by a glycosylphosphatidylinositol (GPI) lipid anchor in the mature protein, which seems to be absent in the so far known non-classical AGPs (Schultz *et al.*, 1998; Gaspar *et al.*, 2001). AGPs are widely distributed in organs and plant tissues and they are developmentally regulated, in an organ-specific and tissue specific manner in various degrees, depending on the individual member of the AGP family (Showalter, 2001). By the use of descriptive studies with monoclonal antibodies or β-Yariv reagent the biological role of AGPs has been implicated in many processes of plant growth and development such as cell expansion (Willats and Knox, 1996; Ding and Zhu, 1997), cell differentiation (Penell and Roberts, 1990; Knox *et al.*, 1991), cell proliferation (Serpe and Nothnagel, 1994; Thompson and Knox, 1998) and somatic embryogenesis (Kreuger and van Holst, 1993), but also the involvement of AGPs in plant-microbe interactions are suggested (Seifert and Roberts, 2007). The *Arabidopsis rat1* (*resistant* to *Agrobacterium transformation*) mutant was identified with a T-DNA tag in the promoter region of an AGP gene (Nam *et al.*, 1999) and is suggested to be either involved in the signal transduction that enables *Agrobacterium tumefaciens* to infect the wild-type plants or to be required for binding of the bacterium to the root surface (Gaspar *et al.*, 2004).

4.2 Materials and methods

4.2.1 Plant material and inoculation procedure

Tomato plants of tomato recombinant inbreed lines (RILs) NHG3, NHG13, NHG162, NHG60 (Asian Vegetable Research and Development Centre, AVRDC; Taiwan) were cultivated in peat moss (Klasmann-Deilmann, Germany) supplemented with 4 g L^{-1} $CaCO_3$ (Roth, Germany) for non-silicon treatment and 4 g L^{-1} $CaCO_3$ plus 1g / L Aerosil (Degussa, Germany) for silicon treatment. Plants were kept under greenhouse conditions (20 °C with 14 h light per day at 30 K lux and 70% relative humidity) and watered throughout the whole experiment with a nutrient solution composed of 5 mM $Ca(NO_3)_2$, 1.875 mM K_2SO_4, 1.625 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.04 mM H_3BO_3, 0.001 mM $ZnSO_4$, 0.001 mM $CuSO_4$, 0.01 mM $MnSO_4$, 0.00025 mM $MoNa_2O_4$, 0.05 mM NaCl and 0.1 mM Fe-EDTA for non-silicon treatment, and the same solution containing monosilicic acid in a final concentration of 1.4 mM [$Si(OH)_4$] for silicon treatments. Monosilisic acid was obtained after exchange of potassium silicate solution K_2SiO_2 (VWR, Germany) with cation exchangers (20 mL volume, Biorad Laboratories, Germany) (Hochmuth, 1999).

Five week old plants were inoculated with *R. solanacearum* strain ToUdk2 (race 1, phylotype 1; originated from Thailand) directly after transplanting and transferred into a growth chamber (30 °C / 27 °C day/night temperature, 85 % relative humidity, 30 K Lux and 14h light per day). Inoculum suspensions were prepared from two day old bacterial cultures of *R. solanacearum* grown on TTC medium [10 g / L bacto peptone, 1 g / L casamino acid, 5 g / L glucose, 15 g / L agar; 10 mL of a 0.5 % 2,3,5-triphenyl tetrazolium chloride solution (Sigma, Germany) were sterile filtrated and separately added to the cooled TTC medium after autoclaving (Kelman, 1954)] or on nutrient glucose agar (NGA; 3 g / L beef extract, 5 g / L peptone from casein, 2.5 g / L glucose, 15 g / L agar) by adjusting the suspension in demineralised water to an optical density of 0.06 at 620 nm and subsequently 1:5 diluted, corresponding to approximately to $2.03*10^7$ CFU. Per gram substrate 0.1 mL suspension was applied to each plant. Controls were treated with the same quantity of demineralised water. Samples from recombinant inbred lines were harvested 5 days post inoculation (5dpi). Non-inoculated controls of each treatment were included in all experiments.

4.2.2 Symptom evaluation

Symptoms of ten plants per treatment were monitored daily and classified in six disease severity classes: **0** = healthy plant, **1** = one leaf wilted, **2** = two leaves wilted, **3** = three leaves wilted, **4** = all leaves wilted except the tip of the plant, **5** = whole plant wilted.

The mean of disease scores represented wilt disease severity (DS). The disease incidence (DI) was calculated as the percentage of dead plants to the number of total plants at each evaluation date. Disease incidence was recorded daily.

The area under disease incidence progress curve (AUD_IPC) was calculated on the basis of either wilt disease severity or disease incidence using the following formula (cited after Jeger and Viljanen-Rollinson, 2001):

$$AUDPC = \sum_{i=1}^{n-1} \left[(x_i + x_{i-1}) / 2 \right] (t_i - t_{i-1})$$

with x_i and x_{i-1} - wilt incidence or disease severity scale, and t_i and t_{i-1} - consecutive evaluation dates (t_i-t_{i-1} is equal to 1 day).

4.2.3 Bacterial quantification in tomato stems

R. solanacearum was quantified in the stems and roots of symptomless inoculated plants. Stem and root parts were surface sterilized with 70% EtOH for 15 or 20 s, respectively, and subsequently washed with sterile demineralised water before being macerated in 3 mL of sterile water. After incubation for 20 min at room temperature the macerate was filtered through cotton and centrifuged at 7,000 x g at room temperature. The pellet was re-suspended in 1 mL sterile water, tenfold dilutions were prepared and 100 µL of appropriate dilutions were plated in two replicates on TTC medium (Kelman, 1954), followed by incubation for 48 h at 30°C. Bacterial colonies were counted and calculated as colony forming units per gram of fresh matter (log CFU / g).

4.2.4 Immunohistochemical analyses

To detect changes in the tomato cell wall structure related to silicon nutrion and / or *R. solanacearum* infection, mid-stem parts of tomato recombinant inbred lines (RILs) NHG3 and NHG60 (susceptible to bacterial wilt) and NHG13 and NHG162 (resistant to bacterial wilt) were sampled five days post inoculation (dpi), cut into thin slices < 0.5 mm by free-hand sectioning and subsequently transferred into fixative PIPES buffer (50 mM piperazine-N,N'-bis(2-ethanesulphonic acid) [PIPES]; 5 mM $MgSO_4$; 5 mM ethylene glycol bis(β-aminoethyletherl) tetraacetic acid [EGTA], pH = 6.9) containing 4% paraformaldehyde (Merck, Germany). Sections were kept at 4°C overnight before blocking in phosphate buffered saline (PBS; pH 7.2) (135 mM NaCl, 3 mM KCl, 10 mM $Na_2HPO_4*2H_2O$, 2 mM KH_2PO_4) containing 5% skim milk powder (w/v, Fluka, Switzerland) for 1 h at room temperature was performed. Initial incubation with primary antibodies (LM2, LM6, LM7) received from Plant Probes, c/o P. Knox, University of Leeds, UK (Table 4.1) 1 : 10 diluted in PBS + 5% skim milk powder was for 1 h at room temperature, followed by incubation at 4°C over night. Slices were washed with PBS + Tween 0.1% 3 times for 5 minutes followed by 3 times washing with dH_2O.

Incubation with the secondary antibody anti-rat IgG FITC (fluoroisothiocyanat, green fluorescence) (Sigma, Germany) at 1 : 100 dilution in PBS + 5% skim milk powder was then performed overnight at 4°C. Subsequently, the slices were washed again as described above. Finally, the sections were mounted in Citifluor (AF1) antifade (Agar scientific, UK) on glass slides and observed under a photomicroscope (Axioskop 2 plus, Carl Zeiss, Göttingen, Germany) equipped with epifluorescence illumination with a filter system appropriate for fluoroscein florescence excitation: 450 - 490 nm, beamsplitter: 500 nm, emission: 510 - 576 nm) (Carl Zeiss, Germany). All sets of the experiment included healthy and inoculated as well silicon and non-silicon amended plants in each combination, with 3 plants per treatment, respectively. Control samples of each plant were prepared with Citifluor only to investigate autofluorescence. Also samples with secondary antibody were observed to determine unspecific fluorescence.

Table 4.1: Primary antibodies used for structural analysis of pectic polysaccharides.

Primary antibody	Pectin domain	Epitope detected
LM2	Rhamnogalacturonan I	arabinogalactan-protein (AGP)
LM6	Rhamnogalacturonan I	(1→5)-α-L-arabinan
LM7	Homogalacturonan	non-blockwise de-esterification

4.2.5 Statistical analyses

The data were analysed with the statistical software "R" (R Development Core Team – R Foundation for Statistical Computing, Vienna, Austria). For all data, Wilcoxon rank-sum Test was used and the P values were adjusted with the method of Holm for family-wise error rate correction. A significance level of $p < 0.05$ was used throughout the whole statistical analysis of the data.

4.3 Results

4.3.1 Symptom evaluation and bacterial populations

Wilt symptom development expressed as area under disease progress curve (AUDPC) based on either disease severity or disease incidence, was highest in RILs NHG3 and NHG60. Silicon treatment reduced disease severity by 93% and 6% in NHG3 and NHG60, respectively and by 100% and 98% in NHG13 and NHG162, respectively, and disease incidence by 46% and 43% in NHG3 and NHG60, respectively, and by 0% and 100% in NHG13 and NHG162, respectively (Table 4.2). Non-silicon treated plants of NHG3 showed increased wilt symptom development compared to silicon treated plants for AUD_SPC and AUD_IPC. AUD_IPC for NHG60 was slightly increased in non-silicon treated plants. NHG162 and NHG13 showed less wilt symptom development compared to NHG3 and NHG60, with wilting plants occur mainly in non-silicon treatments.

Table 4.2: Area under disease progress curve (AUDPC) based on disease severity and disease incidence for recombinant inbred lines NHG3, NHG60 [susceptible to bacterial wilt] and NHG13, NHG162 [resistant to bacterial wilt] amended with and without silicon.

	Recombinant inbred line [RIL]			
Treatment	**NHG3 (sus)**	**NHG60 (sus)**	**NHG13 (res)**	**NHG162 (res)**
AUD_SPC				
- silicon	44.05 ± 10.1	18.21 ± 9.7	0.65 ± 0.7	7.93 ± 7.9
+ silicon	26.98 ± 15.5	17.05 ± 3.2	0.00 ± 0.0	0.13 ± 0.1
AUD_IPC				
- silicon	852.50 ± 187.5	345.00 ± 175.0	0.00 ± 0.0	82.50 ± 82.5
+ silicon	462.50 ± 237.5	295.00 ± 25.0	0.00 ± 0.0	0.00 ± 0.0

sus: susceptible, res: resistant.
Data are means ± SE of two independent trails with ten plants per treatments, respectively.

Bacterial populations (expressed in log CFU / g) were similar in the stems across genotypes in silicon and non-silicon treated plants at 5 dpi (Table 4.3). In the roots, increased bacterial populations in non-silicon treatments of NHG3 and NHG162, but similar bacterial numbers in NHG13 and NHG60, compared to silicon treatments, were observed.

Comparing plant organs (stems and roots) for the same genotype and treatment, bacterial populations were increased in the roots of non-silicon treated plants of NHG3, NHG60 and NHG162 and in the roots of silicon treated plants of NHG60 (Table 4.3).

All differences were not significantly different.

Table 4.3: Bacterial populations (log CFU / g) of *R. solanacearum* strain ToUdk2 in tomato stems and roots of recombined inbred lines (RILs) NHG3, NHG60 [susceptible to bacterial wilt] and NHG13, NHG162 [resistant to bacterial wilt] amended with and without silicon at 5 days post inoculation (dpi).

	Recombinant inbred line [RIL]			
Treatment	**NHG3 (sus)**	**NHG60 (sus)**	**NHG13 (res)**	**NHG162 (res)**
Stem				
- silicon	3.19 ± 1.6 aA	2.79 ± 1.4 aA	3.40 ± 0.9 aA	3.98 ± 1.4 aA
+ silicon	3.97 ± 1.4 aA	3.00 ± 1.0 aA	2.52 ± 0.8 aA	3.88 ± 0.9 aA
Root				
- silicon	5.02 ± 1.7 aA	4.14 ± 1.7 aA	3.05 ± 1.4 aA	5.83 ± 1.3 aA
+ silicon	3.64 ± 1.7 aA	4.92 ± 1.0 aA	3.32 ± 1.1 aA	3.89 ± 1.8 aA

sus: susceptible, res: resistant.
Data are means ± SE of two independent trails with three plants per treatments, respectively. Small letters refer to the comparison of treatments (-Si *vs* +Si) for the same plant organ and genotype. Capital letters refer to the comparison of stems and roots for the same genotype and treatment. Similar letters are not significantly different with Wilcoxon rank-sum Test at $\alpha = 5\%$

4.3.2 Immunohistochemical observations

4.3.2.1 Autofluorescence

Generally, genotypes NHG60 and NHG 13 showed increased ***autofluorescence*** compared to NHG3 and NHG162 independent of treatments (Fig. 4.1a, b, c, g, h, i, m, n, o, s, t, u - Fig. 4.2a, b, c, g, h, i, m, n, o, s, t, u).

4.3.2.2 Detection of arabinogalactan-protein with antibody LM2

Arabinogalactan-protein (primary antibody LM2), was detected mainly in vascular bundles of all genotypes (Fig. 4.1d, e, f, j, k, l, p, q, r, v, w, x - Fig. 4.4d, e, f, j, k, l, p, q, r, v, w, x). Intensive staining of vessels and surrounding tissues with LM2 occurred in tomato genotypes NHG60 and NHG13, whereas genotypes NHG3 and NHG162 showed generally staining of single vessels (Fig. 4.1d, e, f, j, k, l, p, q, r, v, w, x - Fig. 4.4d, e, f, j, k, l, p, q, r, v, w, x). Staining was slightly increased in genotype NHG60 for silicon and non-silicon treated, non-inoculated plants (-Si-Rs and +Si-Rs) compared to both inoculated treatment (-Si+Rs and +Si+Rs) (Fig. 4.2d, e, f, j, k, l, p, q, r, v, w, x) and for silicon treated healthy and inoculated plants in genotype NHG13 (+Si-Rs and +Si+Rs) compared to both non-silicon treatments (-Si-Rs and –Si+Rs) (Fig. 4.3d, e, f, j, k, l, p, q, r, v, w, x). No differences in treatments were observed for genotypes NHG3 and NHG162 (Fig. 4.1d, e, f, j, k, l, p, q, r, v, w, x and Fig. 4.4d, e, f, j, k, l, p, q, r, v, w, x).

4.3.2.3 Detection of (1→5)-α-L-arabinan with antibody LM6

Antibody LM6, detecting ***(1→5)-α-L-arabinan*** showed generally staining of whole stem sections, except in some treatments (-Si-Rs in genotype NHG60, -Si-Rs and +Si+Rs in genotype NHG13, +Si+Rs in genotype NHG162), where staining was rather restricted to vascular bundles (Fig. 4.5d, e, f, j, k, l, p, q, r, v, w, x - Fig. 4.8d, e, f, j, k, l, p, q, r, v, w, x).

Staining of (1→5)-α-L-arabinan in vascular bundles was slightly increased in the non-inoculated treatments (-Si-Rs and +Si-Rs) in genotype NHG3 compared to inoculated treatments (Fig. 4.5d, e, f, j, k, l, p, q, r, v, w, x). In genotype NHG60 increased staining was observed for both inoculated treatments (-Si+Rs and +Si+Rs) compared to non-inoculated treatments (Fig. 4.6d, e, f, j, k, l, p, q, r, v, w, x) and in genotype NHG13 for

non-silicon treated, non-inoculated plants and for both inoculated treatments and (-Si-Rs, -Si+Rs, +Si+Rs) compared to silicon treated, non-inoculated plants (Fig. 4.7d, e, f, j, k, l, p, q, r, v, w, x). In plants of genotype NHG162, a slightly increased staining was observed for non-silicon treated, inoculated plants (-Si+Rs) and silicon treated, non-inoculated plants (+Si-Rs) compared to non-silicon treated, non-inoculated and silicon treated, inoculated plants (-Si-Rs and +Si+Rs) (Fig. 4.8d, e, f, j, k, l, p, q, r, v, w, x).

4.3.2.4 Detection of non-blockwise de-esterified pectic epitopes with antibody LM7

Antibody LM7, specific for ***non-blockwise de-esterified pectic epitopes*** showed staining of only vascular bundles for all genotypes and treatments (Fig. 4.9d, e, f, j, k, l, p, q, r, v, w, x - Fig. 4.12d, e, f, j, k, l, p, q, r, v, w, x).

Staining of genotype NHG3 and NHG162 was homogenous across treatments (Fig.4.9d, e, f, j, k, l, p, q, r, v, w, x and Fig. 4.12d, e, f, j, k, l, p, q, r, v, w, x), whereas staining of genotype NHG3 was overall weaker compared to genotype NHG162. In genotype NHG60 increased staining was observed for both inoculated treatments (-Si+Rs and +Si+Rs) compared to non-inoculated treatments (Fig. 4.10d, e, f, j, k, l, p, q, r, v, w, x) and was highest for non-inoculated silicon treated plants (+Si-Rs) in genotype NHG13 (Fig. 4.11d, e, f, j, k, l, p, q, r, v, w, x).

4.3.2.5 Autofluorescence and staining with secondary antibody in genotype NHG3 four weeks after inoculation

Autofluorescence was decreased in plants of NHG3 four weeks after inoculation compared to 5 dpi, except of one highly infected (log CFU/g = 8.71) plant of silicon inoculated treatment, showing extensively autofluorescence (Fig. 4.13a, b, e, f, i, j, m, n, q, r). Sections incubated only with ***secondary antibody*** had identically fluorescence compared to autofluorescence controls (Fig. 4.13c, d, g, h, k, l, o, p, s, t).

Table 4.4 summarizes the increased detection of autofluorescence and antibody labelling.

In figures, representative views of two biological repetitions with three plants per treatment, respectively, are shown.

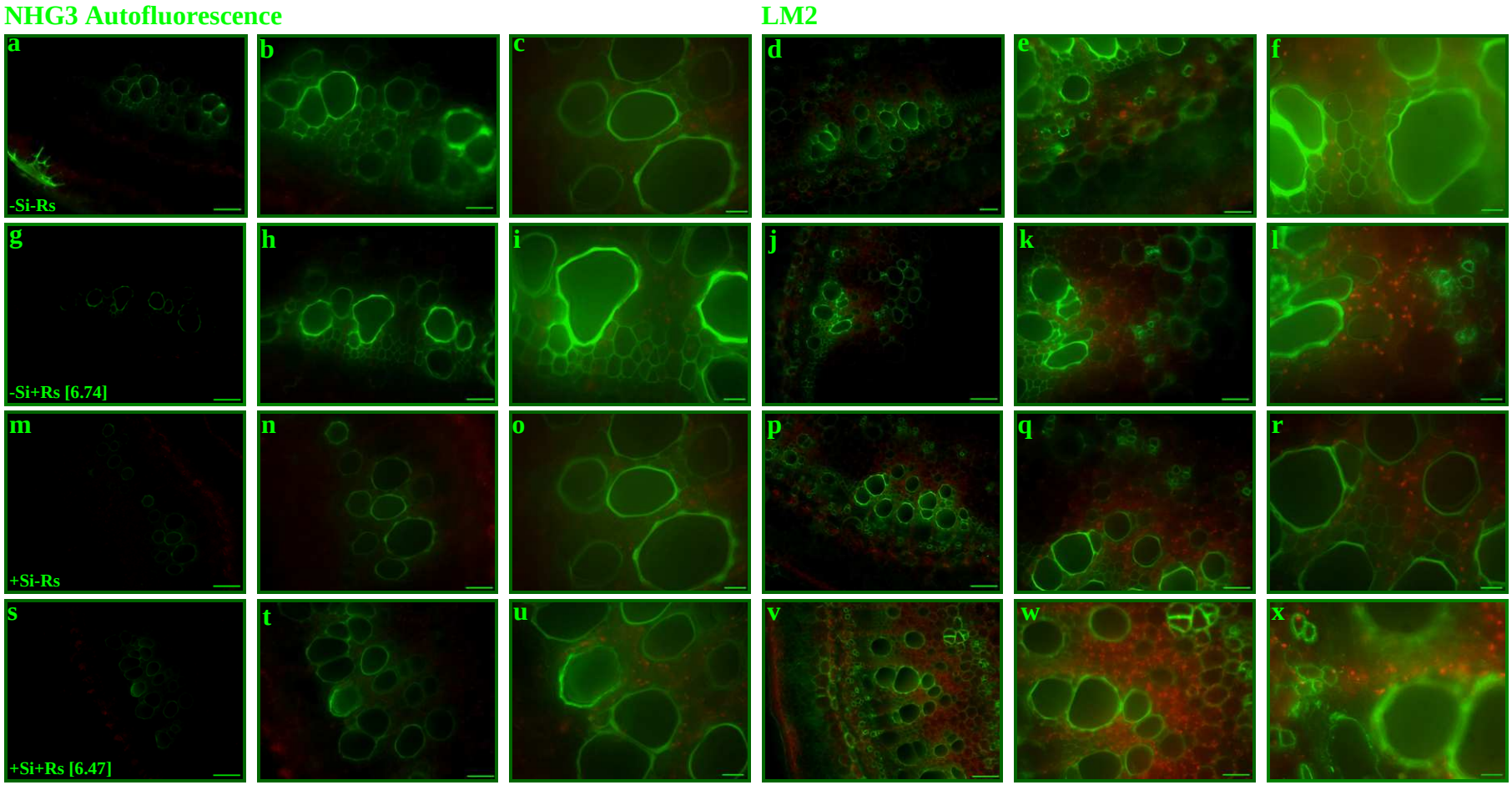

Fig. 4.1: Autofluorescence and probing with antibody LM2, detecting arabinogalactan-protein of tomato genotype NHG3 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon at 5 days post inoculation (dpi).

a-c –Si –Rs autofluorescence (AF); d-f: -Si-Rs LM2; g-i: -Si+Rs AF (log CFU/g: 6.74); j-l: -Si+Rs LM2; m-o: +Si-Rs AF; p-r: +Si-Rs LM2; s-u: +Si+Rs AF (log CFU/g: 6.47); v-x: +Si+Rs LM2. Bar in a, d, g, j, m, p, s, v = 100 µm, bar in b, e, h, k, n, q, t, w = 50 µm, bar in c, f, i, l, o, r, u, x = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

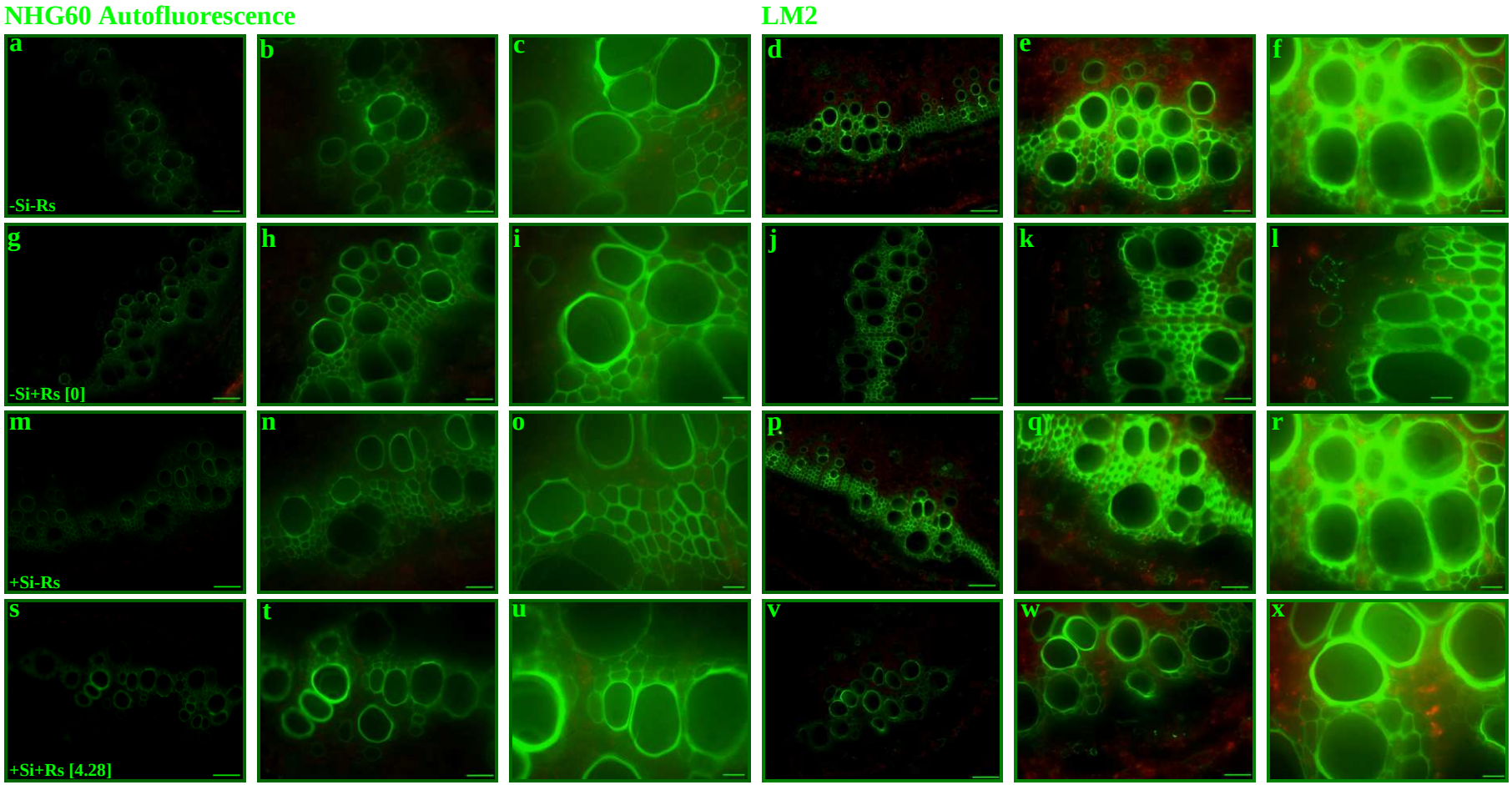

Fig. 4.2: Autofluorescence and probing with antibody LM2, detecting arabinogalactan-protein of tomato genotype NHG60 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-c –Si –Rs autofluorescence (AF); d-f: -Si-Rs LM2; g-i: -Si+Rs AF (log CFU/g: 0); j-l: -Si+Rs LM2; m-o: +Si-Rs AF; p-r: +Si-Rs LM2; s-u: +Si+Rs AF (log CFU/g: 4.28); v-x: +Si+Rs LM2. Bar in a, d, g, j, m, p, s, v = 100 µm, bar in b, e, h, k, n, q, t, w = 50 µm, bar in c, f, i, l, o, r, u, x = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

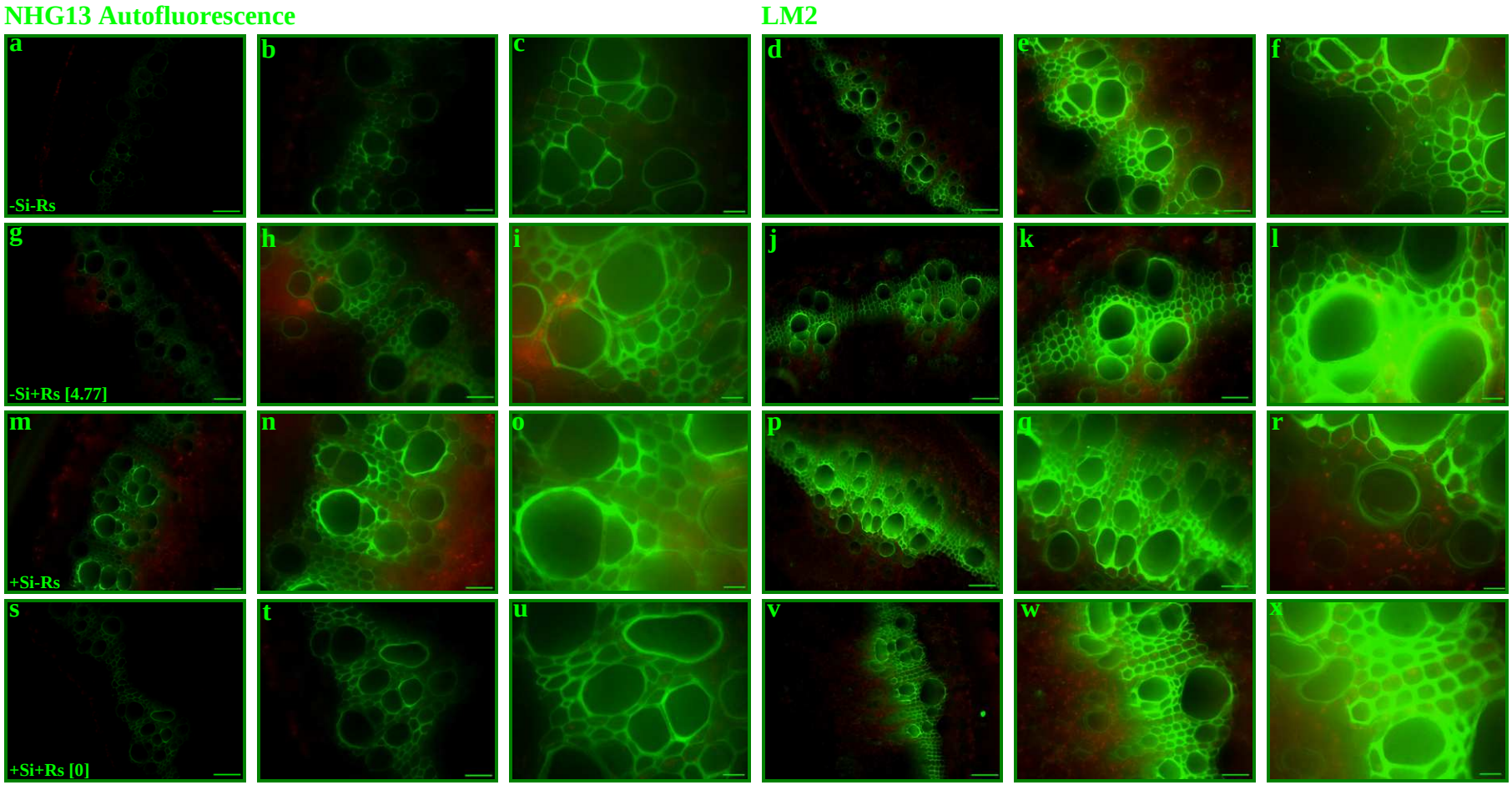

Fig. 4.3: Autofluorescence and probing with antibody LM2, detecting arabinogalactan-protein of tomato genotype NHG13 healthy and inoculated with *R. solanacearum* strain toUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-c –Si –Rs autofluorescence (AF); d-f: -Si-Rs LM2; g-i: -Si+Rs AF (log CFU/g: 4.77); j-l: -Si+Rs LM2; m-o: +Si-Rs AF; p-r: +Si-Rs LM2; s-u: +Si+Rs AF (log CFU/g: 0); v-x: +Si+Rs LM2. Bar in a, d, g, j, m, p, s, v = 100 µm, bar in b, e, h, k, n, q, t, w = 50 µm, bar inc, f, i, l, o, r, u, x = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

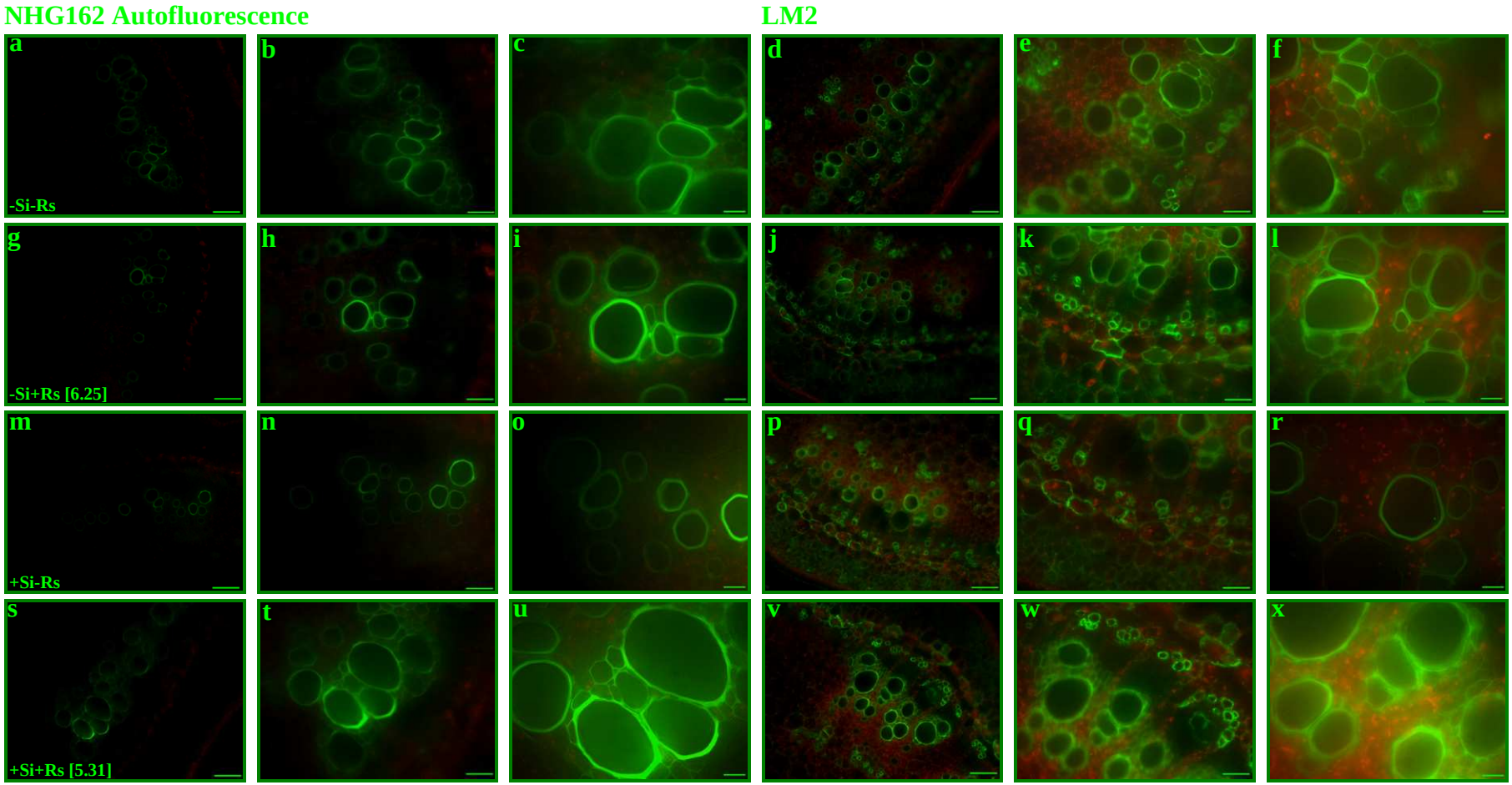

Fig. 4.4: Autofluorescence and probing with antibody LM2, detecting arabinogalactan-protein of tomato genotype NHG162 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-c –Si –Rs autofluorescence (AF); d-f: -Si-Rs LM2; g-i: -Si+Rs AF (log CFU/g: 6.25); j-l: -Si+Rs LM2; m-o: +Si-Rs AF; p-r: +Si-Rs LM2; s-u: +Si+Rs AF (log CFU/g: 5.31); v-x: +Si+Rs LM2.Bar in a, d, g, j, m, p, s, v = 100 µm, bar in b, e, h, k, n, q, t, w = 50 µm, bar in c, f, i, l, o, r, u, x = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

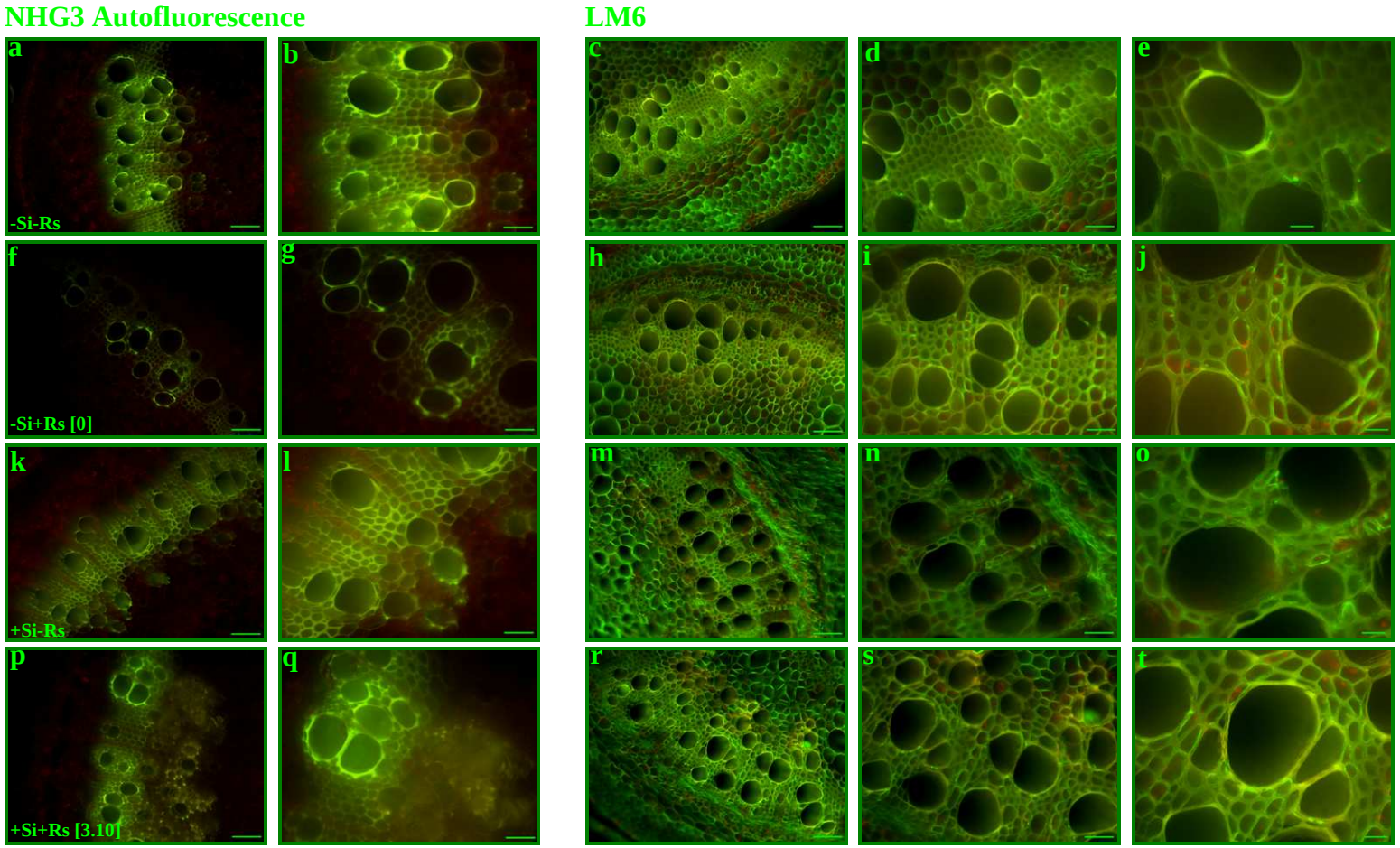

Fig. 4.5: Autofluorescence and probing with antibody LM6, detecting (1→5)-α-L-arabinan of tomato genotype NHG3 and healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-b –Si –Rs autofluorescence (AF); c-e: -Si-Rs LM6; f-g: -Si+Rs AF (log CFU/g: 0); h-j: -Si+Rs LM6; k-l: +Si-Rs AF; m-o: +Si-Rs LM6; p-q: +Si+Rs AF (log CFU/g: 3.10); r-t: +Si+Rs LM6. Bar in a, c, f, h, k, m, p, r = 100 µm, bar in b, d, g, i, l, n, q, s = 50 µm, bar in e, j, o, i, t = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

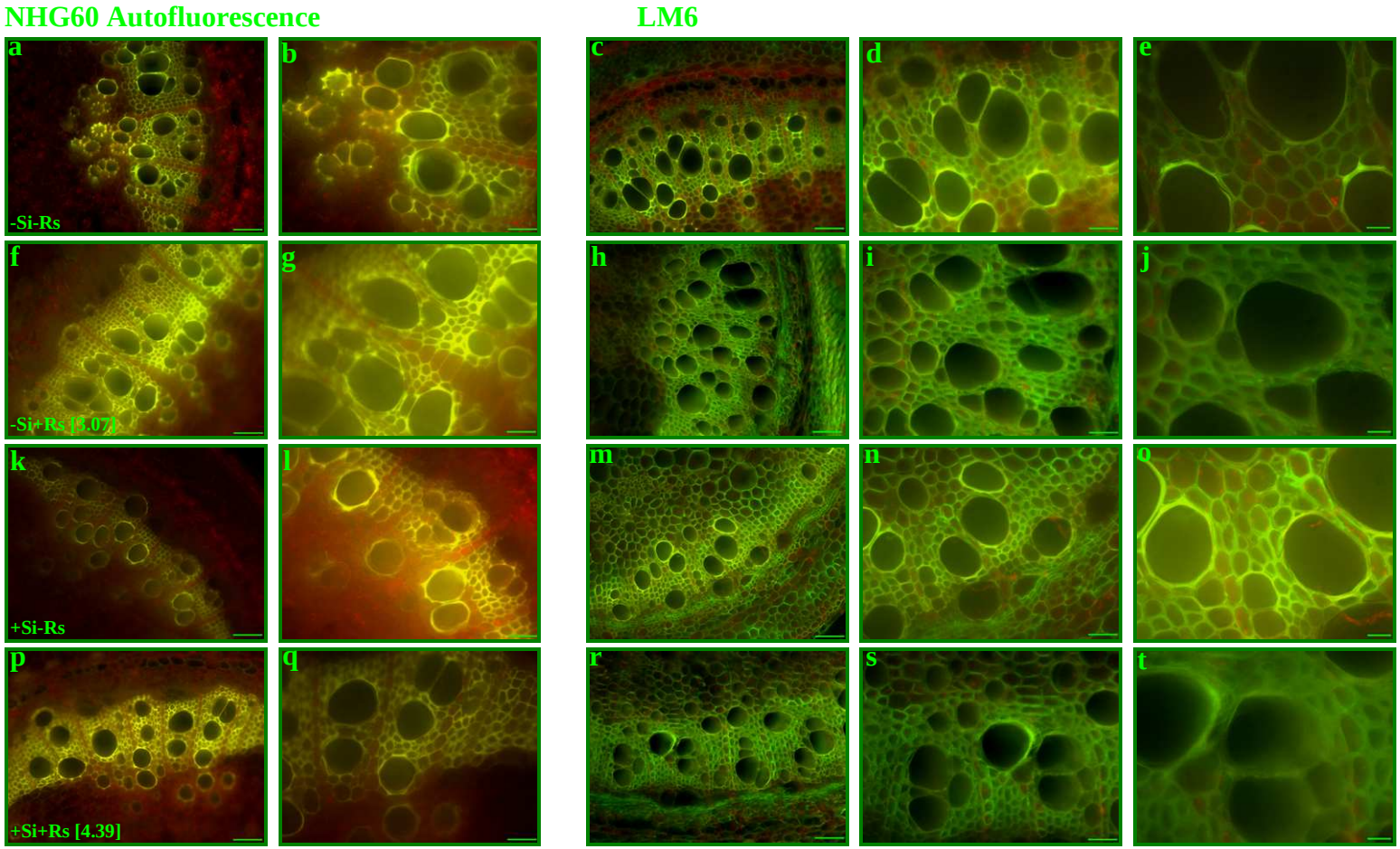

Fig. 4.6: Autofluorescence and probing with antibody LM6, detecting (1→5)-α-L-arabinan of tomato genotype NHG60 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-b –Si –Rs autofluorescence (AF); c-e: -Si-Rs LM6; f-g: -Si+Rs AF (log CFU/g: 3.07); h-j: -Si+Rs LM6; k-l: +Si-Rs AF; m-o: +Si-Rs LM6; p-q: +Si+Rs AF (log CFU/g: 4.39); r-t: +Si+Rs LM6. Bar in a, c, f, h, k, m, p, r = 100 µm, bar in b, d, g, i, l, n, q, s = 50 µm, bar in e, j, o, i, t = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

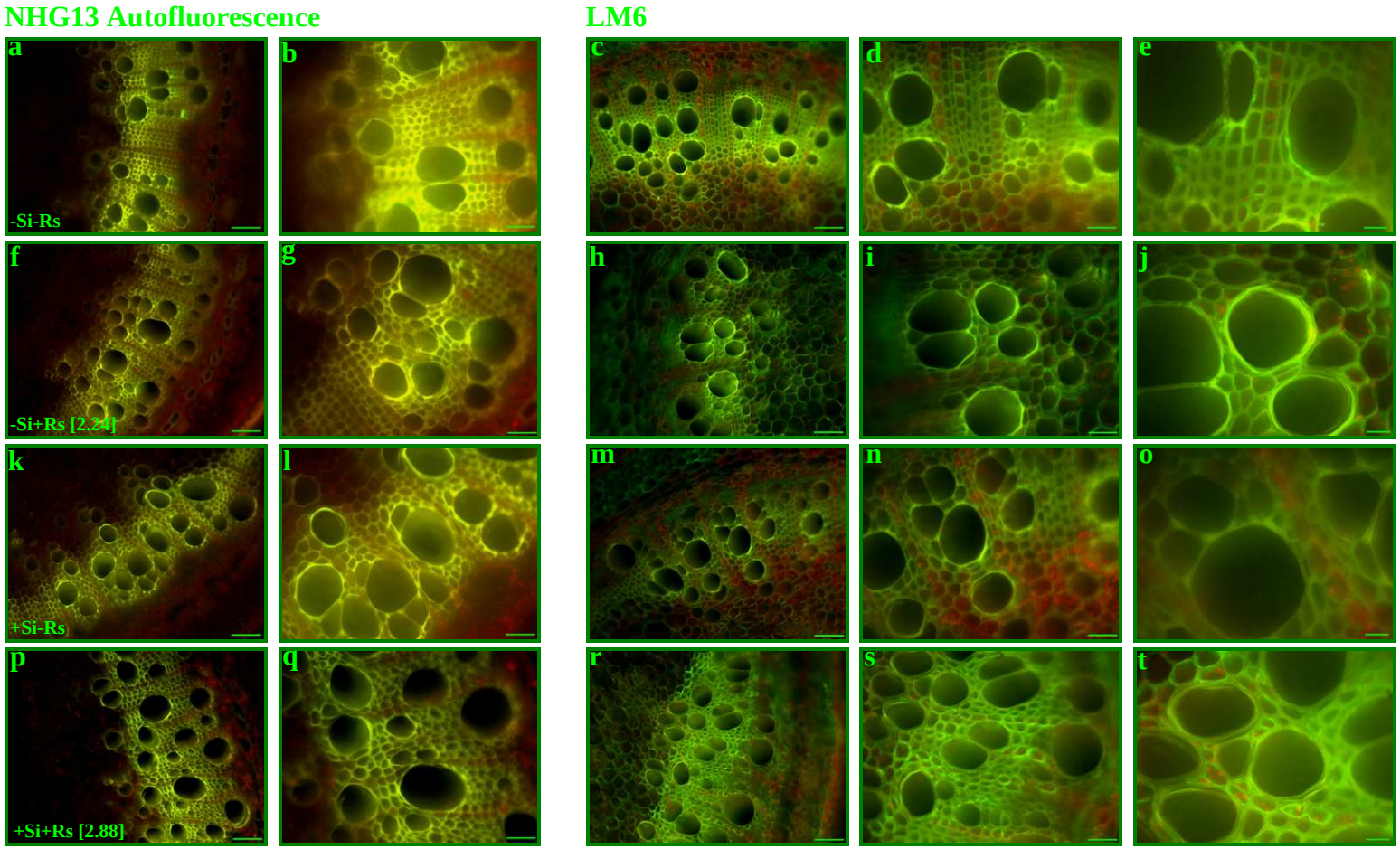

Fig. 4.7: Autofluorescence and probing with antibody LM6, detecting (1→5)-α-L-arabinan of tomato genotype NHG13 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-b –Si –Rs autofluorescence (AF); c-e: -Si-Rs LM6; f-g: -Si+Rs AF (log CFU/g: 2.24); h-j: -Si+Rs LM6; k-l: +Si-Rs AF; m-o: +Si-Rs LM6; p-q: +Si+Rs AF (log CFU/g: 2.88); r-t: +Si+Rs LM6. Bar in a, c, f, h, k, m, p, r = 100 µm, bar in b, d, g, i, l, n, q, s = 50 µm, bar in e, j, o, i, t = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

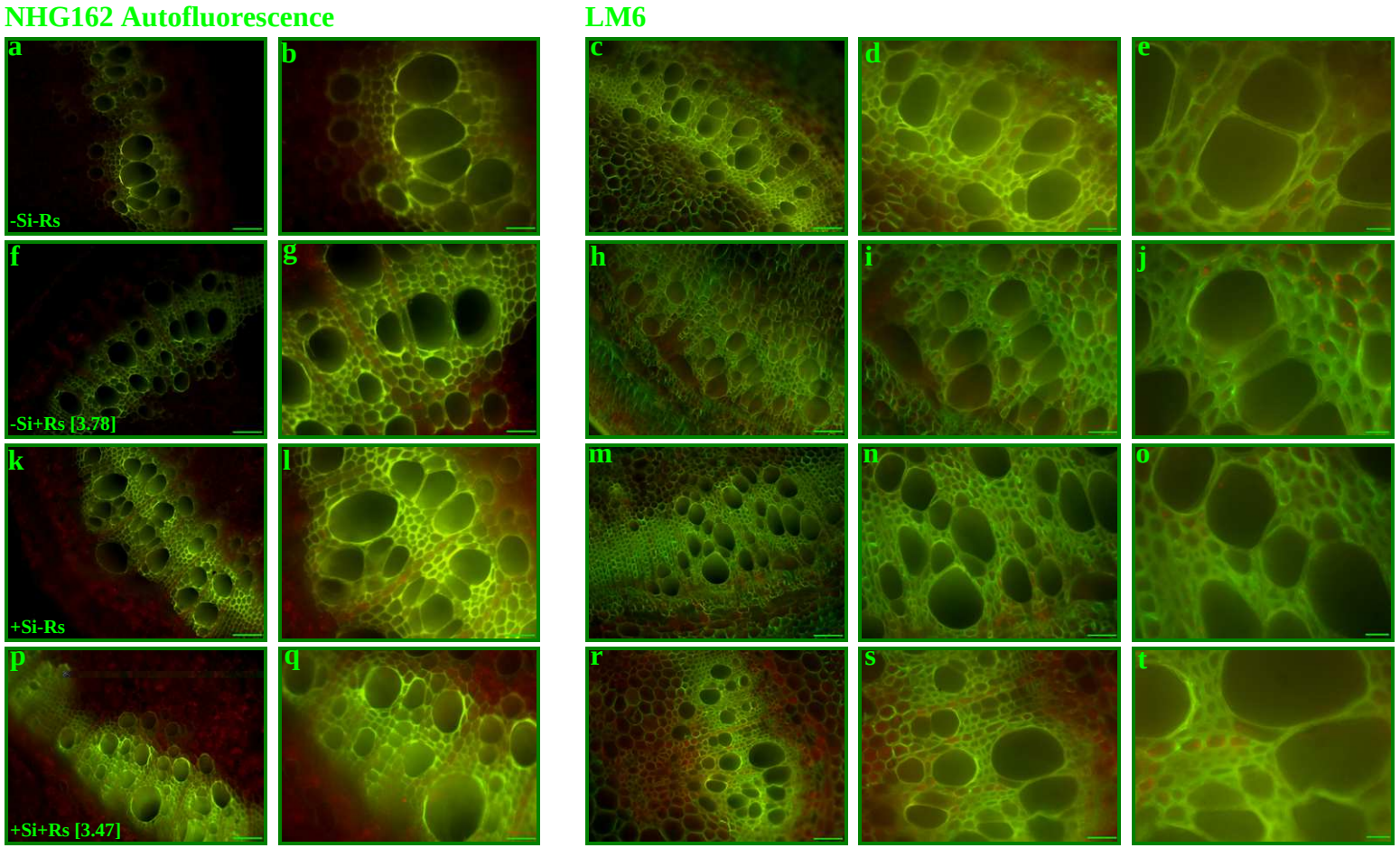

Fig. 4.8: Autofluorescence and probing with antibody LM6, detecting (1→5)-α-L-arabinan of tomato genotype NHG162 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-b –Si –Rs autofluorescence (AF); c-e: -Si-Rs LM6; f-g: -Si+Rs AF (log CFU/g : 3.78); h-j: -Si+Rs LM6; k-l: +Si-Rs AF; m-o: +Si-Rs LM6; p-q: +Si+Rs AF (log CFU/g: 3.47); r-t: +Si+Rs LM6. Bar in a, c, f, h, k, m, p, r = 100 µm, bar in b, d, g, i, l, n, q, s = 50 µm, bar in e, j, o, i, t = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

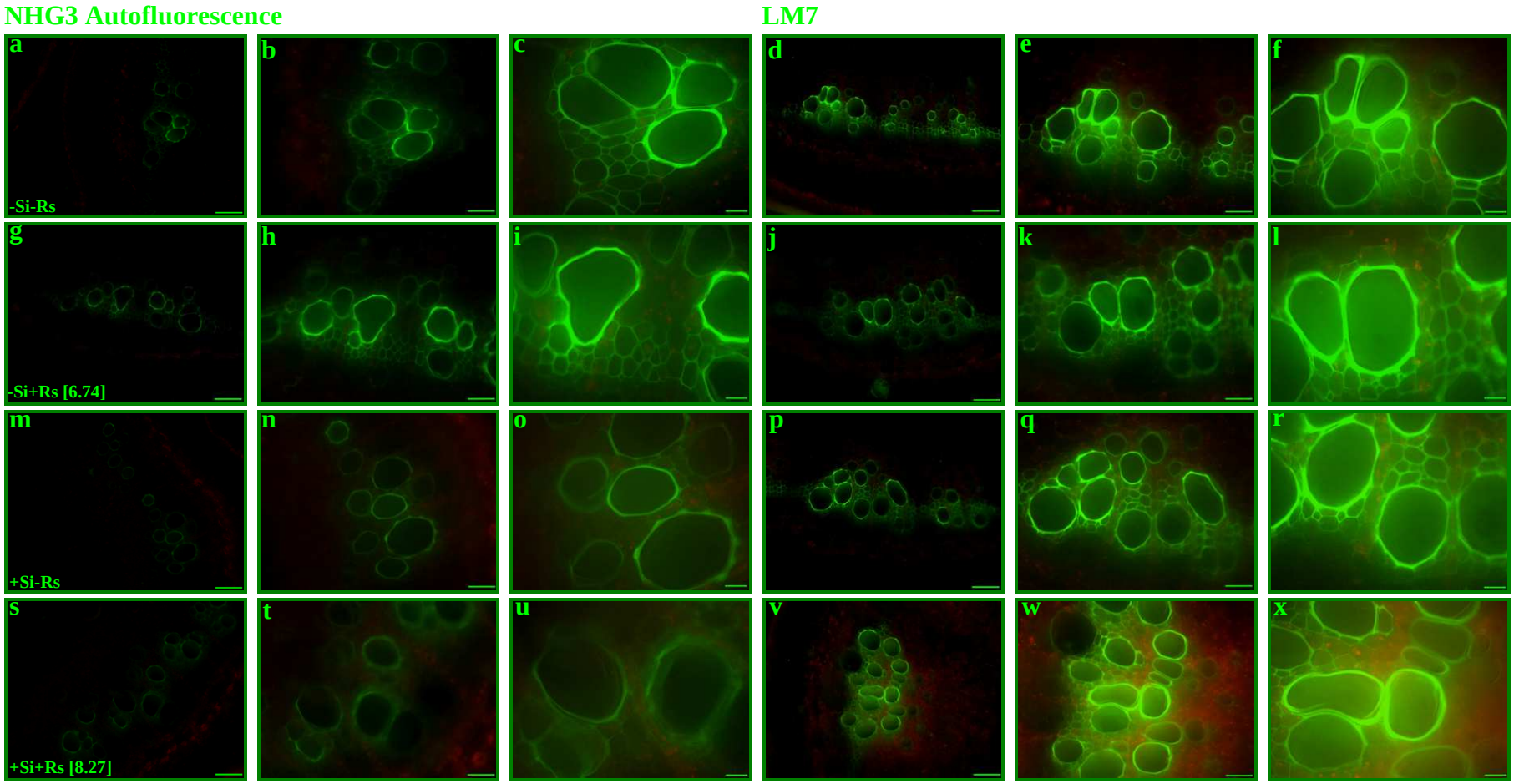

Fig. 4.9: Autofluorescence and probing with antibody LM7, detecting non-blockwise de-esterified epitopes of homogalacturonan of tomato genotype NHG3 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-c –Si –Rs autofluorescence (AF); d-f: -Si-Rs LM7; g-i: -Si+Rs AF (log CFU/g: 6.74); j-l: -Si+Rs LM7; m-o: +Si-Rs AF; p-r: +Si-Rs LM7; s-u: +Si+Rs AF (log CFU/g: 8.27); v-x: +Si+Rs LM7. Bar in a, d, g, j, m, p, s, v = 100 µm, bar in b, e, h, k, n, q, t, w = 50 µm, bar in c, f, i, l, o, r, u, x = 20 µm. Treatment and bacterial numbers log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

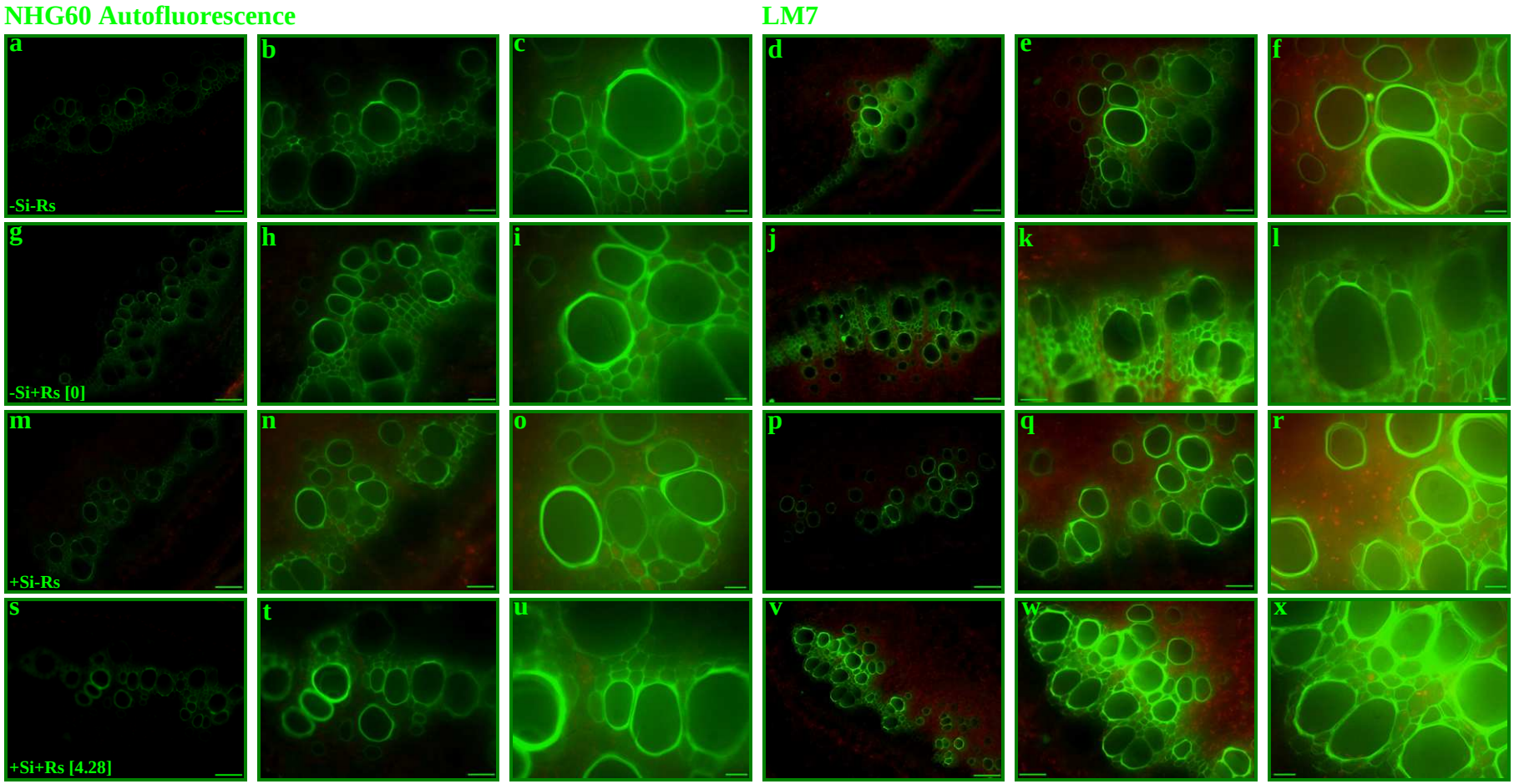

Fig. 4.10: Autofluorescence and probing with antibody LM7, detecting non-blockwise de-esterified epitopes of homogalacturonan of tomato genotype NHG60 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-c –Si –Rs autofluorescence (AF); d-f: -Si-Rs LM7; g-i: -Si+Rs AF (log CFU/g: 0); j-l: -Si+Rs LM7; m-o: +Si-Rs AF; p-r: +Si-Rs LM7; s-u: +Si+Rs AF (log CFU/g: 4.28); v-x: +Si+Rs LM7. Bar in a, d, g, j, m, p, s, v = 100 µm, bar in b, e, h, k, n, q, t, w = 50 µm, bar in c, f, i, l, o, r, u, x = 20 µm. Treatment and bacterial numbers [log 10 CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

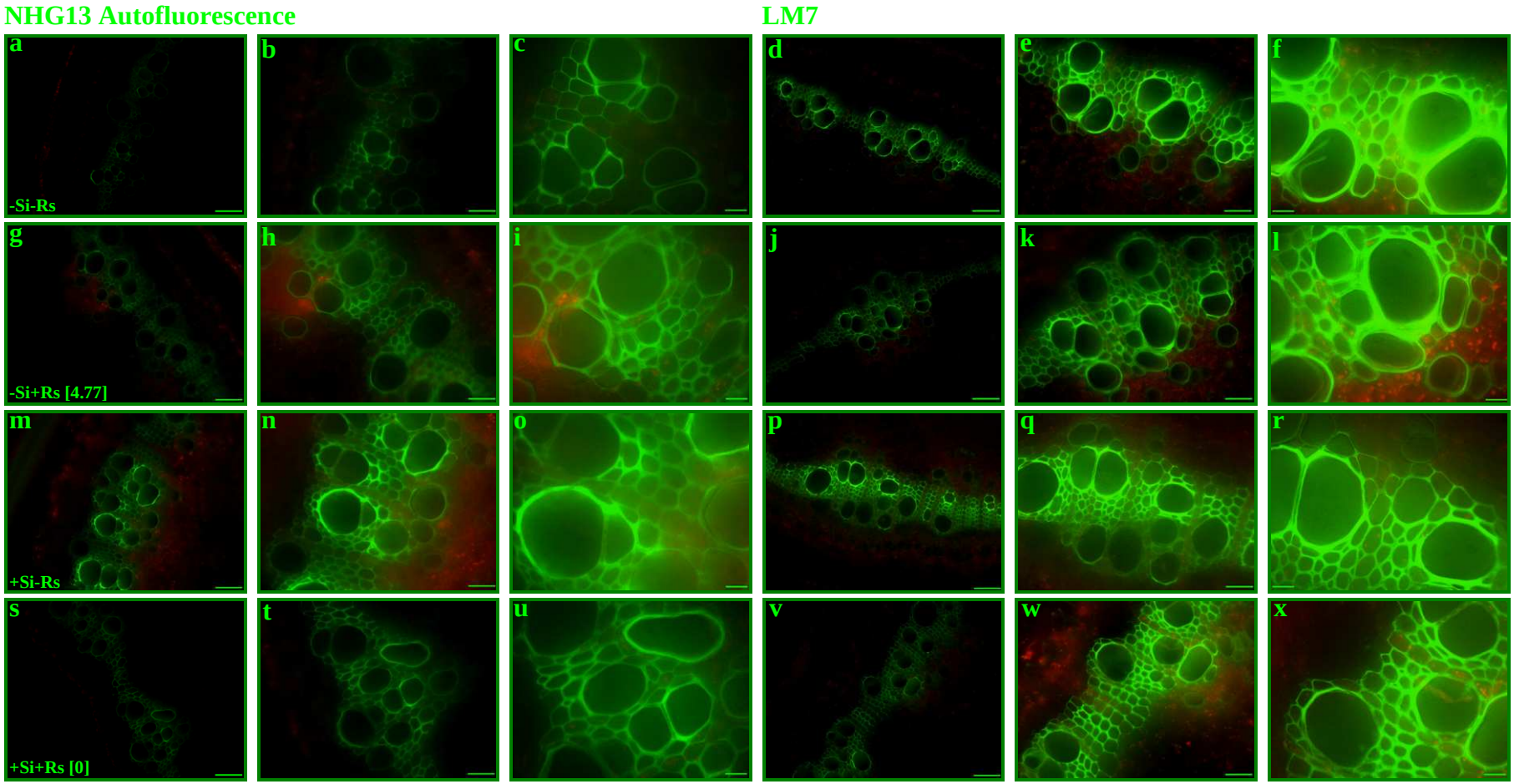

Fig. 4.11: Autofluorescence and probing with antibody LM7, detecting non-blockwise de-esterified epitopes of homogalacturonan of tomato genotype NHG13 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-c –Si –Rs autofluorescence (AF); d-f: -Si-Rs LM7; g-i: -Si+Rs AF (log CFU/g: 4.77); j-l: -Si+Rs LM7; m-o: +Si-Rs AF; p-r: +Si-Rs LM7; s-u: +Si+Rs AF (log CFU/g: 0); v-x: +Si+Rs LM7. Bar in a, d, g, j, m, p, s, v = 100 µm, bar in b, e, h, k, n, q, t, w = 50 µm, bar inc, f, i, l, o, r, u, x = 20 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

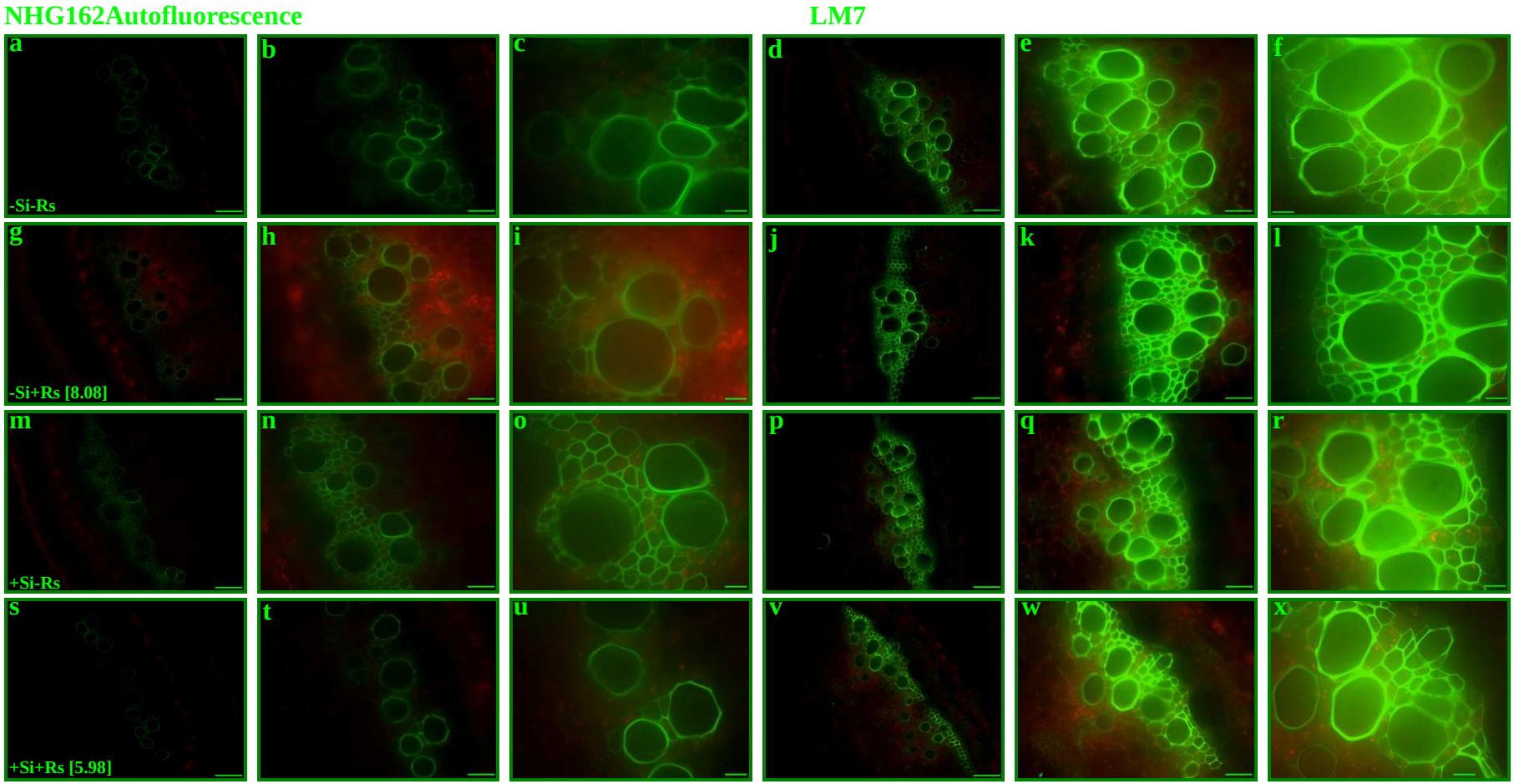

Fig. 4.12: Autofluorescence and probing with antibody LM7, detecting non-blockwise de-esterified epitopes of homogalacturonan of tomato genotype NHG162 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 5 days post inoculation (dpi).

a-c –Si –Rs autofluorescence (AF); d-f: -Si-Rs LM7; g-i: -Si+Rs AF (log CFU/g: 8.08); j-l: -Si+Rs LM7; m-o: +Si-Rs AF; p-r: +Si-Rs LM7; s-u: +Si+Rs AF (log CFU/g: 5.98); v-x: +Si+Rs LM7. Bar in a, d, g, j, m, p, s, v = 100 µm, bar in b, e, h, k, n, q, t, w = 50 µm, bar in c, f, i, l, o, r, u, x = 20 µm. Treatment and bacterial numbers (log CFU /g) are addtionally given in each first picture representative for the row. Pictures are representative for each treatment.

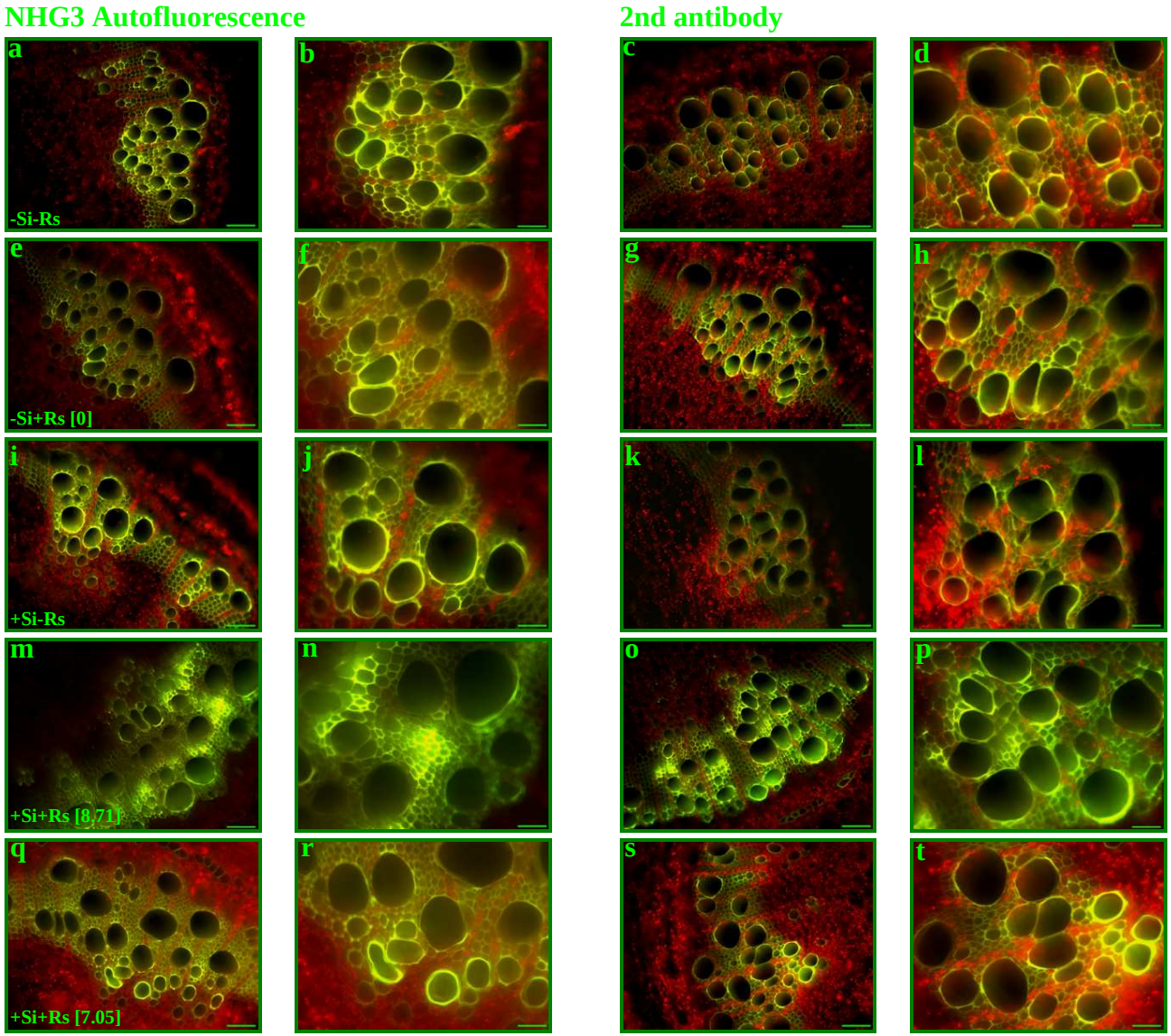

Fig. 4.13: Autofluorescence and probing with secondary antibody of tomato genotype NHG3 healthy and inoculated with *R. solanacearum* strain ToUdk2 amended with and without silicon 4 weeks after inoculation.

a-b –Si –Rs autofluorescence (AF); c-d: -Si-Rs 2nd AB; e-f: -Si+Rs AF (log CFU/g: 0); g-h: -Si+Rs 2nd AB; i-j: +Si-Rs AF; k-l: +Si-Rs 2nd AB; m-n: +Si+Rs AF (log CFU/g: 8.71); o-p: +Si+Rs 2nd AB; q-r: +Si+Rs AF (log CFU/g: 7.05); s-t: +Si+Rs 2nd AB.

Bar in a, c, e, g, I, k, m, o, q, s= 100 µm, bar in b, d, f, h, j, l, n, p, r, t = 50 µm. Treatment and bacterial numbers [log CFU/g] are additionally given in each first picture representative for the row. Pictures are representative for each treatment.

Table 4.4: Summary of increased autoflourescence and antibody labelling of primary antibodies LM2, LM6 and LM7, detecting arabinogalactan protein, (1→5)-α-L-arabinan and non-blockwise de-esterification of homogalacturonan, respectively, for tomato recombinant inbred lines NHG3, NHG60 [susceptible to bacterial wilt] and NHG 13, NHG162 [resistant to bacterial wilt].

Treatment	Autofluorescence				Primary Antibody											
					LM2				LM6				LM7			
	susceptible		resistant		susceptible		resistant		susceptible		resistant		susceptible		resistant	
	NHG 3	NHG 60	NHG 13	NHG 162	NHG 3	NHG 60	NHG 13	NHG 162	NHG 3	NHG 60	NHG 13	NHG 162	NHG 3	NHG 60	NHG 13	NHG 162
- Si – Rs	-	+	+	-	-	+	-	-	+	-	+	-	-	-	-	+
- Si + Rs	-	+	+	-	-	-	-	-	-	+	+	+	-	+	-	+
+ Si - Rs	-	+	+	-	-	+	+	-	+	-	-	+	-	-	+	+
+ Si + Rs	-	+	+	-	-	-	+	-	-	+	+	-	-	+	-	+

+: increased autofluorescence and antibody staining; Si: silicon, Rs: *Ralstonia solanacearum*

4.4 Discussion

Staining of antibodies LM2 and LM7 was generally restricted to vascular bundles and surrounding tissues, whereas antibody LM6 stained the whole sections. Staining of all antibodies occurred in all examined recombinant inbred lines, even though in various degees, irrespectively of treatments. A uniform staining pattern concerning resistance status of the plant, *R. solanacearum* inoculation and silicon treatment was not observed.

Arabinogalactan proteins (AGPs) were detected by immunohistochemistry in vascular bundles of tomato recombinant inbred lines, using the monoclonal antibody LM2, that was raised against rice AGPs recognizing a glucuronic acid-containing epitope, but also detects AGPs that are secreted by suspension cultured carrot *(Daucus carota* L.) cells (Smallwood *et al.*, 1996). Vessels and surrounding tissues of RILs NHG60 and NHG13 was intensively stained, whereas RILs NHG3 and NHG162 showed generally antibodystaining of only vessels. Staining was highest in both non-inoculated treatments of the susceptible RIL NHG60 and in both silicon treatments of the resistant genotype NHG13.

Structural proteins of different plants, including tomato, rich in glycine, proline and hydroxyproline were demonstrated in the xylem, phloem and cambium (Heredia *et al.*, 1995). A role of AGPs, which belong to the hydroxyproline-rich glycoproteins (HRGPs) (Majewska-Sawka and Nothnagel, 2000; Showalter, 2001), is suggested in the vascular formation, because certain AGP epitopes spatially and temporarily correlate with xylem differentiation, at least experimentally demonstrated for *Zinnia elegans* (Motose *et al.*, 2004). Additionally, Liu and Mehdy (2007) observed that AGP31 is a protein localized in the cell wall, and its expression was observed in the vascular bundles throughout the plant. The involvement of AGPs in physiological processes such as vascular formation can explain the observed staining of LM2 in all vascular tissues of all RILs, irrespectively of treatments.

In former studies, a strong fluorescence of antibody LM2 in genotype King Kong2 after infection with *R. solanacearum* in non-silicon treated plants compared to the plants with silicon amendment was detected, (Diogo and Wydra, 2007). Additionally, indications for the involvement of AGPs in response to wounding, particularly the secretion of AGP-containing gums by wounded tissues of some plants were reported (Fincher *et al.*, 1983), and microarray analysis suggested that AGPs respond to biotic and abiotic stress (Schultz

et al., 2002), reduction of special AGP expression was observed not only for NaAGP4 from *Nicotiana alata* after infection of leaves with the fungus *Botrytis cinerea* and after stem-wounding (Gilson *et al.*, 2001), but also for a putative AGP mRNA in tomato leaves and fruits (Pogson and Davis, 1995) and for *Lycopersicon esculentum* AGP-1 (LeAGP-1) in young and old tomato internodes after wounding (Li and Showalter, 1996), respectively.

The observed discrepancy between the staining pattern of the RILs in this study and the pattern observed in the study of Diogo and Wydra (2007) might be at least partly due to the selected genotype, which was King Kong2. Our data indicates that tomato genotypes differ in their plant cell wall structure and react different upon various treatments. Additionally, the investigation points of time differed in the studies. Diogo and Wydra (2007) investigating changes of the plant cell wall structure 20 days after inoculation and in our study we examined the RILs at 5 days post inoculation. This suggests that changes in the plant cell wall structure occur later in the tomato – *R. solanacearum* – interaction.

The distribution of (1→5)-α-L-arabinan was detected with the antibody LM6, an antibody detecting five residues of 1→5-linked α-L-arabinan in the side chains of RG-I (Willats *et al.*, 1998; Willats *et al.*, 2001a) and was hetergenosly across genotypes and treatments. Increased staining was observed in both non-inoculated treatemtens of RIL NHG3, in both inoculated treatments of RIL NHG60, both non-inoculated treatments and silicon treated, inoculated plants of RIL NHG13 and non-silicon treated, inoculated and silicon treated, non-inoculated plants of RIL NHG162, suggesting that the genotypes exhibiting a different distribution of (1→5)-α-L-arabinan and react differently on the treatments. Using the same antibody, sections of genotype King Kong2 showed a strong green fluorescence around some vessel walls and in the xylem parenchyma in silicon treated, inoculated plants, whereas non-silicon treated, inoculated plants showed increased yellow-greenish fluorescence in tissues around vessels after labelling with the antibody LM6 (Diogo and Wydra, 2007). The differences in the staining patterns of both studies, like the differences for AGP, are also might explainable by the selected genotypes and time-points.

Highly branched pectin RG-I is believed to be less easily degradable by pectinolytic enzymes of bacterial pathogens (Marty *et al.*, 1997) but interplay of genotypes, silicon amendment and bacterial wilt infection in tomato needs further investigations.

Staining of RIL NHG3 and NHG162 with antibody LM7, detecting non-blockwise de-esterified epitopes of homogalacturonan, was homogenous across treatments genotype. In RIL NHG60 increased staining was observed for both inoculated treatments and staining was highest in non-inoculated, silicon treated plants for RIL NHG13.

The antibody LM7 was raised against a HG epitope that was produced *in vitro* and selected on the basis of two series of pectins with non-blockwise patterns of methyl-esterification by the action of either an *Aspergillus* PME or by alkaline de-esterification, but does not recognize a pectin series with blockwise de-esterification pattern derived from the action of plant PME (Willats, *et al.*, 2001b). This epitope could be detected *in planta* and suggests that some plant PMEs may have a non-blockwise action pattern (Willats *et al.*, 2001a).

Homogalacturonan appears to be synthesized in the Golgi apparatus and deposited in the plant cell wall in a highly methyl esterified form (O'Neill *et al.*, 1990; Mohnen, 1999). The methyl ester groups are removed from the homogalacturonan by the action of pectin methyl esterases (PMEs), enzymes which are abundant in all species of higher plants tested and are also known to be present in various plant pathogenic fungi and bacteria (Cassab and Varner, 1988). Multigene families of PMEs are known, encoding isoforms of the enzyme with different action patterns and are believed to act either in a block-wise or non-blockwise, also called random fashion on HG (Willats *et al.*, 2001a; Willats *et al.*, 2001b). Plant PMEs are generally regarded to cause blockwise de-esterification of pectin, whereas microbial PMEs typically cause random or non-blockwise de-esterification (Limberg *et al.*, 2000a; Limberg *et al.*, 2000b). The enzymes are most likely developmentally regulated and seem to have different action patterns at different locations within cell walls, resulting in either random or block-wise distribution of methyl esters and in various degrees of methyl esterification (Willats *et al.*, 2001a).

Differences in the non-blockwise methylester distribution of tomato genotype King Kong2 inoculated with *R. solanacearum* in relation to silicon treatment were observed, whereas silicon treated and inoculated plants showed a decreased antibody staining of antibody LM7, compared to non-silicon treated plants (Diogo and Wydra, 2007). A possible explanation for the observed differences in inoculated plants might be partly due to the action of the pathogens pectin methylesterase.

Results from McMillan *et al.*, (1993) indicate that rotting of potato tubers and stems by soft-rot erwinias are closely related to the degree of pectin esterification and potato stem tissue with a higher percentage of methylated and branched pectins correlate with resistance against *Erwinia carotovora* subsp. *atroseptica*, furthermore an additional PME isoform was found in susceptible potato cultivars (Marty *et al.*, 1997). Differences between wheat near-isogenic lines resistant and susceptible to stem rust (*Puccinia graminis* f.sp. *tritici*) are suggested to differ in the pattern of methyl esterification, assuming a non-random and more blockwise distribution of methyl esters in the HGs of susceptible wheat cultivars compared with a presumably more random distribution pattern in the resistant ones (Wiethölter *et al.*, 2003).

However, we could not detect a uniform immunohistochemical pattern concerning resistant or susceptible genotypes or major changes in the pectic polysaccharide structure of stem cell walls comparing silicon and non-silicon treated plants, while using the four tomato RILs. This is contrary to results obtained earlier for the *R. solanacearum* - tomato interaction (Diogo and Wydra, 2007; Wydra and Beri, 2007), but might be explained by the investigation of different tomato genotypes in the studies, which might react differentially in their modulation of the pectic polysaccharides. Another possible explanation might be the different investigation time points used for the examination. Diogo and Wydra (2007) used plants at 20 dpi, whereas we collected our samples earlier, at 5 days post inoculation which might be not enough time for the plant to react with changes in the modulation of pectic components, at least in relation to pathogen infection.

CHAPTER 5

Gene expression study

Abstract

Expression of resistance related genes was investigated in stems of the moderately resistant tomato genotype King Kong2 of non-silicon and silicon treated, *Ralstonia solanacearum* inoculated plants (-Si+Rs and +Si+Rs) and silicon treated, non-inoculated plants (+Si-Rs) compared to non-silicon treated, non-inoculated control plants (-Si-Rs) at 7 days post inoculation (dpi), when bacterial numbers and symptom development were decreased in silicon treated plants.

Ralstonia solanacearum inoculation resulted in a tendenciously downregulation in plants with and without silicon treatment for non-inducible immunity (NIM) and for jasmonate ZIM-domain protein1 (JAZ1), whereas expression of plants of the +Si+Rs treatments were the most downregulated for JAZ1 in the experiment. Expresion of ethylene responsive factor1 (ERF1) was slightly, even though hetergenously increased in *R. solanacearum*-inoculated plants without silicon amendment (-Si+Rs) and basal levels of expression in all treatments were observed for coronatine-insensitive1 (COI1).

For the cell wall related genes callose synthase (CallSyn), arabinogalactan protein (AGP) and extensin, a tendency to downregulation after *Ralstonia solanacearum*-inoculation in silicon treated and non-silicon treated plants was observed.

5.1 Introduction

Former studies revealed that genes related to signalling pathways of plants (Ghareeb, 2007) and changes in the plant cell wall structure (Wydra and Beri, 2006; Diogo and Wydra, 2007, Wydra and Beri, 2007) are involved in the resistance reaction of tomato to *R. solanacearum*. Thus, we conducted this study to investigate various genes described for defense signalling and crosstalk of the pathways, but also genes for cell wall related compounds, upon their involvement in the establishement of silicon-induced resistance of tomato to bacterial wilt.

Interference in signalling pathways: Overview

COI1 inactivates negative regulators of JA-mediated responses
JAZ1 acts as suppressor for JA-mediated response
ERF1 mediates the crosstalk between JA and ET pathways
NIM is required for SAR mediated by SA, but also involved in JA/ET responses

Screens in *Arabidopsis* mutants, which are non-responsive to JA or coronatine led to the identification of mutant alleles of the genes jasmonate resistant1 (JAR1) and **coronatine-insensitive1** (COI1) (Staswick *et al.*, 1992; Feys *et al.*, 1994). COI1 encodes an F-box protein with a series of leucine-rich repeats, which are hypothesized to be involved in targeting proteins for polyubiquitination and degradation (Xie *et al.*, 1998). Indeed, the involvement of COI1 as part of a complex that modulates JA-responsive gene expression was demonstrated (Xu *et al.*, 2002).

Coronatine is a phytotoxin secreted by several strains of *Pseudomonas syringae* and was described as contributor to virulence of this bacterial pathogen (Moore *et al.*, 1998; Bender *et al.*, 1999). The *Arabidopsis coi1* mutants showed methyl jasmonate (MeJA) insensitivity and increased SA-signaling in response to *P. syringae* infection (Feys *et al.*, 1994, Kloek *et al.*, 2001), which supports the hypothesis that the pathogen secretes coronatine to activate the JA-signaling pathway, which interferes with the induction of SA-responses (Reymond and Farmer, 1998; Kloek *et al.*, 2001).

Jasmonate ZIM-domain proteins (JAZ) belong to a larger group of so-called ZIM-domain proteins, named after the putative transcription factor Zinc-finger inflorescence meristem (ZIM) (Nishi *et al.*, 2000). It is established that JAZ proteins are targets for jasmonate-dependent degradation *via* the SKP1, Cullin, F-box protein complex (SCF^{COI1}) – a type of E3 ubiquitin ligase - 26S proteasome pathway (Staswick, 2008). JAZ1 is suggested to interact specifically with COI1 to form a COI1 - JAZ complex, which is promoted by jasmonyl - isoleucine (JA – Ile) (Thines *et al.*, 2007; Staswick, 2008). Thus, JAZ likely acts as repressors of JA-responsive genes, by controlling the transcription factor MYC2, known as key activator of JA-regulated gene expression (Staswick, 2008). A model for the action of JAZ in *Arabidopsis* was proposed by Staswick (2008): While no jasmonate signal is present in the plant, e.g.in a non-stressed state, response genes are maintained in a repressed condition, because JAZ inhibits the transcriptional activity of MYC2. When biosynthesis of JA occurs in response to a stress condition, it is conjugated to JA-Ile, which promotes the interaction of SCF^{COI1} with the JAZ proteins. As a result, JAZ proteins are degraded, leading to the expression of genes *via* MYC2 (Fig.5.1).

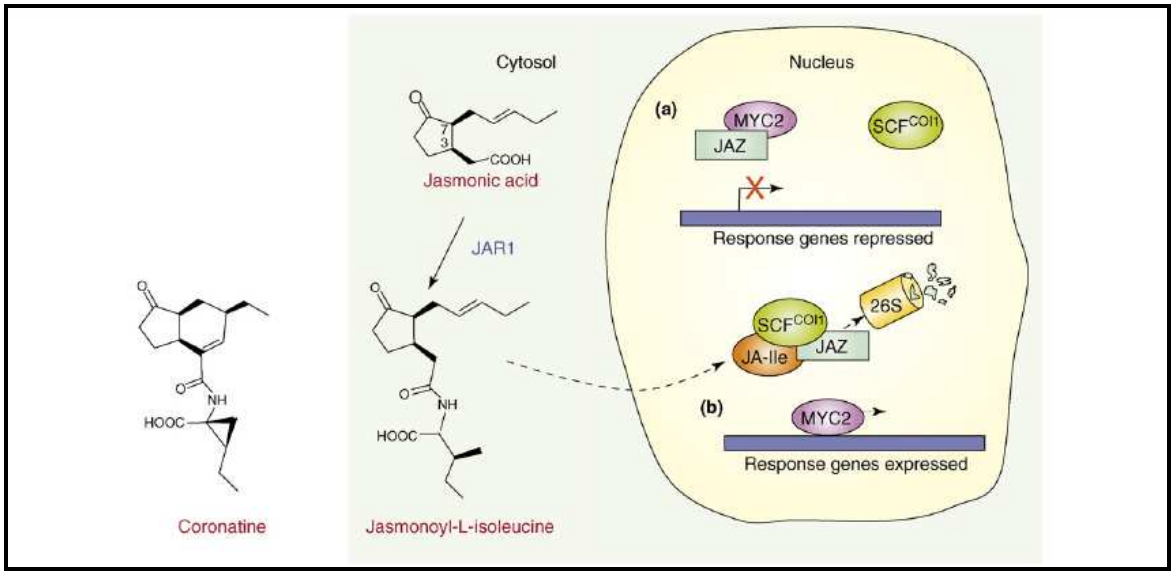

Fig. 5.1: Model for COI1–JAZ jasmonate signaling in *Arabidopsis*.

Jasmonoyl-isoleucine (JA-Ile) promotes SCF^{COI1} interaction with JAZ transcriptional repressors, leading to their ubiquitination and degradation by the 26S proteasome. The MYC2 transcription factor is then free to regulate the expression of genes involved in jasmonate response. The JAR1 conjugating enzyme is localized in the cytosol, so JA-Ile might be synthesized there and translocated to the nucleus where MYC2 and JAZ are located, although other scenarios for proteolysis are possible. The structural relationship between JA-Ile and coronatine is shown. Jasmonic acid (JA) is depicted as the (3R,7S) form (Staswik, 2008).

The **ethylene response factor1 (ERF1)** belongs to a family with a high number of genes in *Arabidopsis* (Lorenzo *et al.*, 2003; Gutterson and Reuber, 2004), and members of this family are also described in tomato, for instance the tomato responsive factor1 (TERF1) (Huang *et al.*, 2004). In *Arabidopsis*, ERF1 appears to mediate the crosstalk between the JA and ET pathways, acting most likely downstream of the intersection between the two pathways and thus, may be an important factor in signal integration, that regulates the expression of pathogen response genes (Lorenzo *et al.*, 2003). The ERF proteins were described in various plant species and bind to the GCC-box present in promotors of several ET-inducible genes, including PR proteins (Broekaert *et al.*, 2006). Previous studies revealed that constitutive expression of ERF1 in transgenic *Arabidopsis* is able to confer resistance to necrotrophic fungi, including *Botrytis cinerea* and *Plectosphaerella cucumerina* (Berrocal-Lobo *et al.*, 2002), supported by transcriptome analysis in Arabidopsis, that ERF1 regulates a high number of defense-related genes (Lorenzo *et al.*, 2003).

The regulatory protein **noninducible immunity1 (NIM1)**, also referred to as nonexpressor of PR-1 (NPR1) or salicylic acid insensitive1 (SAI1) (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997; Bostock, 2005) encodes for a protein with bipartite nuclear localization sequence (NLS) and two potential protein-protein interaction domains: one ankyrin repeat domain and one BTB/POZ (*broad - complex, tramtrack*, and *bricà – brac* / poxvirus, zinc finger) domain (Cao *et al.*, 1997; Aravind and Koonin, 1999; Kinkema *et al.*, 2000). The protein encodes for an important positive regulator in the salicylic acid (SA) -mediated systemic acquired resistance (SAR) (Beckers and Spoel, 2006). *Arabidopsis* plants, overexpressing NPR1, exhibited increased induction of PR genes after pathogen infection, accordingly an enhanced disease resistance to bacterial and oomycete pathogens, dependent on NPR1 dosage (Cao *et al.*, 1998). Additionally, the overexpression of the NPR1 gene from *Arabidopsis* in rice conferred enhanced resistance to bacterial blight caused by *Xanthomonas oryzea* pv. *oryzea* (Chern *et al.*, 2001). Furthermore, NPR1 has also been shown to be involved in the activation of plant defense responses meditated by JA and ethylene (Pieterse *et al.*, 1998; Pieterse *et al.*, 2001).

Plant cell wall

Extensins are a family of highly basic and highly glycosylated hydroxyproline-rich glycoproteins (HRGPs) present in the cell wall of higher plants, particular abundant in dicots. The proteins are characterized by richness in hydroxyproline and serine, containing usually the repeating pentapeptide Ser-Hyp_4; most of the hydroxproline residues are glycosylated with arabinoside chains of one to four units in length (Cassab and Varner, 1988; Showalter, 1993; Bowles, 1990).

Extensins are thought to be preferentially localized in sclerenchyma and cambium cells and also associated with phloem tissues and secondary xylem, but can be found in other tissues as well (Showalter, 1993; Cassab, 1998). The rod-shaped cell wall proteins – extensins - are key components responsible for cell wall rigidification proteins (Humphrey *et al.*, 2007), but also suggested to participate in wound healing, and plant defense by increased deposition and increased extensin cross-linking, that could lead to a more impenetrable cell wall barrier (Showalter *et al.*, 1991; Showalter, 1993).

Various conditions and treatments generally increase the expression of extensins. Among them are development, wounding, fungal and viral infection, fungal and endogenous elicitors, ethylene, red light and heat shock (Showalter, 1993), but extensins might also act directly on certain pathogens by immobilization of the pathogens probably due to agglutination from positively charged extensin molecules with negatively charged surfaces of certain plant pathogens (Showalter *et al.*, 1991; Showalter, 1993).

The glucan synthase-like (*GSL*) genes most likely encodes the **callose synthases** which are located in the plasma membrane (Richmond and Sommerville, 2001; Farrokhi *et al.*, 2006). The enzyme is responsible for the synthesis of the linear plant β-1,3-glucan, callose (Jacobs *et al.*, 2003). Plant callose synthase genes are members of multigene families, with 12 genes in *Arabidopsis* and it is proposed that each gene is responsible for callose synthesis in a different location within the plant (Hong *et al.*, 2001; Verma and Hong, 2001). They are suggested to consist of complexes containing a multiple number of homologous or heterologous of callose synthase subunits (Verma and Hong, 2001; Farrokhi *et al.*, 2006).

Callose was not only reported as major component of pollen tubes (Parre and Geitmann, 2005), but also the accumulation in response to biotic and abiotic stresses in plants was

described (Verma and Hong, 2001; Østergaard *et al.*, 2002). One example is the deposition of callose as early defense response to the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* in tomato, a vascular invading pathogen (Beckmann *et al.*, 1982; Beckmann, 1987).

Arabinogalactan-proteins (AGPs) belong to the family of highly glycosylated hydroxyproline-rich glycoproteins (HRGPs), probably universally distributed in the plant kingdom (Majewska-Sawka and Nothnagel, 2000; Showalter, 2001).

AGPs are widely distributed in organs and plant tissues and they are developmentally regulated, in an organ-specific and tissue specific manner in various degrees, depending on the individual member of the AGP family (Showalter, 2001). By the use of descriptive studies with monoclonal antibodies or β-Yariv reagent the biological role of AGPs has been implicated in many processes of plant growth and development such as cell expansion (Willats and Knox, 1996; Ding and Zhu, 1997), cell differentiation (Penell and Roberts, 1990; Knox *et al.*, 1991), cell proliferation (Serpe and Nothnagel, 1994; Thompson and Knox, 1998) and somatic embryogenesis (Kreuger and van Holst, 1993), but also the involvement of AGPs in plant-microbe interactions are suggested (Seifert and Roberts, 2007). The *Arabidopsis rat1* (*resistant* to *Agrobacterium transformation*) mutant was identified with a T-DNA tag in the promoter region of an AGP gene (Nam *et al.*, 1999) and is suggested to be either involved in the signal transduction that enables *Agrobacterium tumefaciens* to infect the wild-type plants or to be required for binding of the bacterium to the root surface (Gaspar *et al.*, 2004).

5.2 Materials and methods

5.2.1 Plant material and inoculation procedure

Tomato plants of genotype King Kong2 (Known-You Seed Co., Taiwan) were cultivated in white peat (Klasmann-Deilmann, Germany) supplemented with 4 g L^{-1} $CaCO_3$ (Carl Roth, Germany) for non-silicon treatment and 4 g L^{-1} $CaCO_3$ plus 1 g / L Aerosil (Degussa, Germany). Plants were kept under greenhouse conditions (20°C with 14 h light per day at 30 K lux and 70% relative humidity) and watered throughout the whole experiment with a nutrient solution composed of 5 mM $Ca(NO_3)_2$, 1.875 mM K_2SO_4, 1.625 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.04 mM H_3BO_3, 0.001 mM $ZnSO_4$, 0.001 mM $CuSO_4$, 0.01 mM $MnSO_4$, 0.00025 mM Na_2MoO_4, 0.05 mM NaCl and 0.1 mM Fe-EDTA for non-silicon treatment, and the same solution containing additionally monosilicic acid in a final concentration of 1.4 mM [$Si(OH)_4$] for silicon treatments. Monosilisic acid was obtained after exchange of potassium silicate solution K_2SiO_2 (VWR, Germany) with cation exchangers (20 mL volume, Biorad Laboratories, Germany) (Hochmuth, 1999).

Five week old plants were inoculated with *R. solanacearum* strain ToUdk2 (race 1, phylotype 1; originated from Thailand) directly after transplanting and transferring into a growth chamber (30 °C / 27 °C day/night temperature, 85 % relative humidity, 30 K Lux and 14h light per day). Inoculum suspensions were prepared from two day old bacterial cultures of *R. solanacearum* grown on TTC medium [10 g / L bacto peptone, 1 g / L casamino acid, 5 g / L glucose, 15 g / L agar; 10 mL of a 0.5 % 2,3,5-triphenyl tetrazolium chloride (Sigma, Germany) were sterile filtrated and separately added to the cooled TTC medium after autoclaving (Kelman, 1954)] or on nutrient glucose agar (NGA; 3 g / L beef extract, 5 g / L peptone from casein, 2.5 g / L glucose, 15 g / L agar) by adjusting the suspension in demineralised water to an optical density of 0.06 at 620 nm and subsequently 1:5 diluted, corresponding to approximately $2.03*10^7$ CFU per mL. Per gram substrate, 0.1 mL bacterial suspension was applied to each plant. Controls were treated with the same quantity of demineralised water. Samples from genotype King Kong2 were harvested 7 days post inoculation (dpi). Non-inoculated controls of each treatment were included in all experiments.

5.2.2 Symptom evaluation

Symptoms of ten plants per treatment were monitored daily and classified in six classes as disease severity: **0** = healthy plant, **1** = one leaf wilted, **2** = two leaves wilted, **3** = three leaves wilted, **4** = all leaves wilted except the tip of the plant, **5** = whole plant wilted.

The mean of disease scores represents the wilt disease severity (DS). The disease incidence (DI) was recorded daily and calculated as the percentage of dead plants in the total number of plants at the evaluation date. The area under disease progress curve (AUDPC) was calculated on the basis of either wilt disease severity or disease incidence using the following formula (cited after Jeger and Viljanen-Rollinson, 2001):

$$AUDPC = \sum_{i=1}^{n-1} \left[(x_i + x_{i-1}) / 2 \right] (t_i - t_{i-1})$$

with x_i and x_{i-1} - wilt incidence or disease severity scale, and t_i and t_{i-1} - consecutive evaluation dates (t_i-t_{i-1} is equal to 1 day).

5.2.3 Bacterial quantification in tomato stems

Ralstonia solanacearum was quantified in the stems of symptom less inoculated plants, either with or without silicon amendment. Stem pieces were surface sterilized with 70% EtOH for 15 s, subsequently washed with sterile demineralised water and macerated in 3 mL of sterile water. After incubation for 20 min at room temperature, the macerate was filtered through cotton and centrifuged at 7000 x g at room temperature. The pellet was re-suspended in 1 mL sterile water, tenfold dilutions were prepared and 100 µL of appropriate dilutions were plated in two replicates on TTC medium (Kelman, 1954), followed by incubation for 48 h at 30°C. Bacterial colonies were counted and calculated as colony forming units (CFU) per gram of fresh matter, expressed in log CFU / g.

5.2.4 Genomic DNA extraction

Genomic DNA of tomato stems was isolated for primer verification according to the method described by Edwards *et al.* (1991). One hundred mg of tomato stem material was homogenized in 400 µL extraction buffer (200 mM TrisHCl, pH 7.5; 250 mM NaCl; 25 mM EDTA and 0.5% SDS (w/v)) and centrifuged for one minute at 13,000 x g. An

aliquot of 300 µL supernatant was mixed with 300 µL isopropanol, incubated at room temperature for two minutes and subsequently centrifuged for five minutes at 13,000 x g. Pellets were air-dried and dissolved in 100 µL H_2O bidest.

5.2.5 mRNA extraction

Total mRNA from tomato stems for the quantitative real-time PCR (qRT-PCR) experiment was purified according to the method described in McRae (2007) with modifications. Approximately 200 mg tomato stem material was homogenized in liquid nitrogen, then 1 mL of TriZOL (0.4 M ammonium thiocyanate, 0.8 M guanidine thiocyanate, 0.1 M sodium acetate, 38 % (w/v) phenol pH 5.0, 5% (v/v) glycerol) was added to the frozen material and vortexed twice, each for 10 seconds. After incubation for 15 min at room temperature the homogenate was centrifuged at 12,000 x g for 10 min at 4°C and the supernatant mixed with 200 µL 98% chloroform and subsequently incubated at room temperature for 3 min. A centrifugation step at 12,000 x g for 15 min at 4°C resulted in phase separation and approximately 400 µL of the upper phase, containing RNA, was mixed with 250 µL isopropanol and 250 µL high salt solution (0.8 M sodium citrate, 1.2 M sodium chloride) and incubated for 10 min at room temperature. Precipitated RNA was centrifuged for 10 min at 4°C and the pellet washed two times with 1 mL 75% (v/v) EtOH. Finally, the pellet was air-dried and dissolved in 20 µL RNAse free H_2O and stored at -80°C until DNAse treatment and cDNA synthesis. All solutions were prepared with DEPC-H_2O.

5.2.6 Preparation of DNA-free RNA and cDNA synthesis

First, two µg of total RNA was treated with deoxyribonuclease (Fermentas, Germany) to achieve DNA free RNA according to the manufacturer's instruction. Subsequently, one µg RNA was reverse transcribed in 10 µL reaction volume with the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Germany) using the oligo (dT) primer, according to the manufacturer's instructions.

5.2.7 Primer design

Primers for qRT-PCR analysis were designed with Vector NTI v10 software (Invitrogen). The designed primers had to meet the BIO-RAD criteria; primer length 19 - 22 nucleotides, annealing temperature 56 - 60°C, avoiding four or more repeated nucleotides, avoiding more than two C or G nucleotides at the 3`end, GC content 20 - 80%. Primers used in this study are shown in Table 5.1. Identity of the primers was verified by BLAST similarity search (The Gene Index Project).

5.2.8 Primer verification

Reaction mixture for PCR analysis consisted of 25 µL, each containing 0.2 mM dNTPs (Carl Roth, Germany), 1.25 units of Taq polymerase (Fermentas, Germany), 1x PCR Buffer II, 1.5 mM $MgCl_2$ (Fermentas, Germany), and 10 pmol of each primer. One µL of genomic DNA or cDNA as template was added to the PCR mix, to check the primers on DNA and RNA basis, respectively. PCR amplifications were performed in a thermocycler (Whatman, Biometra) programmed for an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56.5°C for 30 s and extension at 72°C for 1 min, and final extension at 72°C for 7 min.

Seven microlitres of the PCR product were mixed with 3 µL of a loading buffer (3.44 mL glycerin, 6.54 mL bidestilled sterile water, bromophenol blue-sodium-salt) and loaded onto a 2% (w/v) agarose gel (Carl Roth, Germany) containing 0.2 µg / mL ethidium bromide (Sigma, Germany). To monitor the correct molecular weight of the PCR products, 2 µL of a Lambda-Pst-marker (140 µL Lambda DNA, 80 U Pst I, 30 µL 10 x restriction buffer, 122 µL ddH_2O) for DNA or 2 µL of a commercial available 100 bp marker (Carl Roth, Germany) for cDNA were loaded onto the gel. The gel was prepared in 1 x Tris-Acetate-EDTA (TAE) buffer and electroseparation was carried out at 80 V for 1 h. The gel was visualized on an UV transilluminator (Spectroline TVL-312A, Fröbel GmbH, Germany).

5.2.9 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

For qRT-PCR analysis cDNA of six extracted plants per treatments obtained from two biological repetitions were used. Each cDNA was measured in three repitions in qRT-PCR. The PCR mixture contained 1 µL cDNA (diluted 3 x), 12.5 µL PerfeCTa™ SYBR® Green SuperMix for iQ™ (Quanta Biosciences) and 1 µL from each gene-specific-primer (10 mM) in 25 µL total volume. PCR was performed using the Real-time Thermocycler Bio-Rad MyiQ. Cycling parameters were the same for primers, except of AGP and PGIP (see below); initial 95°C for 4 min, followed by 40 cycles of 95°C for 30 s, 56.5°C for 30 s, plate read step, 72 °C for 1 min; then product melting curve 55–95°C. Amplification of AGP was performed started with an; initial 95°C for 6 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, plate read step, then product melting curve 55–95°C. In the case of *PGIP* an additional step was performed at 78°C for 30 s before plate read step (Ghareeb, 2007).

5.2.10 Determination of amplification efficiency

Amplification efficiency and correlation coefficient of each primer pair were determined with aid of a standard curve, derived of a tenfold dilution series (undiluted up to 10^{-5}) of pooled cDNAs and the corresponding C_T values, using the iQ5 Optical System Software 2.0 (Bio-Rad, Germany) based on the following formulas:

$$E = 10^{-1/slope}$$

$$\%\,efficiency = (E - 1) * 100\%$$

5.2.11 Data analysis of qRT-PCR

Based on the algorithm according to Vandesompele *et al.* (2002), Real-time PCR data were calculated with the program iQ5 Optical System Software 2.0 (Bio-Rad, Germany) taking amplification efficiency, correlation coefficient and relative expression level into consideration. The two housekeeping genes phosphoglycerate kinase (*PGK*) and tubulin (*TUB*) were used for the normalization of samples and the $2^{-\Delta\Delta CT}$ –method was applied to calculate the relative gene expression. The formula includes the number of cycles needed

for the amplification to reach a fixed threshold in the exponential phase of PCR reaction (*CT*):

$$\Delta C_T = C_T \text{ target gene} - C_T \text{ housekeeping gene}$$
$$\Delta\Delta C_T = C_T \text{ treatment} - C_T \text{ control}$$
$$\text{Ratio} = 2^{-\Delta\Delta CT}$$

This calculation formula assumes that cDNA duplicates in each cycle corresponding to the optimal real time efficiency. Nevertheless, amplification efficiency ranges and efficiency adjusted equations take this in consideration:

$$\text{ratio} = \frac{(\text{E target gene})^{\Delta C_T \text{ target gene (control - treatment)}}}{(\text{E housekeeping gene})^{\Delta C_T \text{ housekeeping gene (control - treatment)}}}$$

Ideally, housekeeping genes are not regulated both in treatment and control, so that the denominator becomes 1 and is of no consequence. As a result, the ratio is only dependent on expression differences of the target gene (Livak and Schmittgen, 2001).

Table 5.1: Primers related to signaling pathways and the plant cell wall, and housekeeping genes investigated in the gene expression study.

Gene name*	Gene description	Accession No	Forward Primer	Reverse Primer	Amplicon seize
AGP	Arabinogalactan protein	X99147	GCTGCACCAACTAAGCCGAAA	TGGAGCGGTTACTGGAGCAA	93
CallSyn	Callose Synthase	SGN-U314083	CTTAAGTGAGGACATATTTGCTGGC	GAAACGATGTCCAAGCCTGTATAAAT	193
COI1	Coronatine-insensitive1	AY423550	CACTGTTTCTTACTGCAAAGGTGTCC	AATCCTCTGTGTGTAACCGCACCTT	182
ERF1	Ethylene responsive factor	AY044236	TCAAGAAGAGGTAACATCCATAGAGAAA	ATTGATAATGCGGCTTGATCATAAG	198
EXTEN	Extensin	X55686	CCTAATTCACATTGGGAACCAAAAC	TGGTGATGAGTAGTAGTAAGTGGGAGG	105
JAZ1	Jasmonate ZIM-domain protein1	EF591123	GCAAATGAAATCATGAAGTTAGCCA	GGCATTGACAGTTTAGGAAGTTCTTG	146
NIM	Noninducible immunity	AY640378	CGACAAGTTTCAGAGACACCTATTGG	CAGCACGTGAATCAGTGATTTGTTT	157
PGIP	Polygalacturonase-inhibiting protein	L26529	CCAATATCTCCGGCCAAATTCC	GCTTCGCAATTGCAGGTGGAAT	114
PGK	**Phosphoglycerate kinase**	**TC191955**	**AGAAGGTAGGACTTGCAGAGAAGATG**	**CATATATTGCTTAGGCGTCATCCAG**	**123**
PME	Pectin methylesterase PME1.9	U50986	TTGGTGATGGAATGTATGCTACGAC	CCTGTAGTATAAATCCTTGGCCGAC	114
PPO A	Polyphenol oxidase A	AJ635324	GCCACATGTACACAGAGTTGGAGAT	TCACCGCGATAGTATCTTCATCTTC	116
TUB	**α-tubulin**	**TC215609**	**CTGAACAACTCATAAGTGGCAAAGAA**	**TACCACCACCAACAGCATTAAAGAC**	**168**

* gene names used in this study
Callose synthase primer derived from SOL Genomics Network database (Mueller *et al.*, 2005).
Housekeeping genes are typed in bold.

5.3 Results

5.3.1 Bacterial quantification and symptom development in tomato stems

Bacterial numbers in tomato stems of the moderately resistant genotype King Kong2 were significantly decreased by 54.3% in silicon treated plants at 7 dpi compared to non-silicon treated plants at 7 dpi (Table 5.2). Stems of the same plants for bacterial quantification served as samples for RNA extraction and subsequent gene expression studies.

Wilt symptom development was retarded and symptom expression lower in silicon-treated plants compared to non-silicon treated plants (Fig. 5.2), with reduction in AUDPC of disease severity and wilt incidence by 34.8 and 33.8%, respectively in silicon treated plants (Table 5.3).

Table 5.2: Bacterial numbers (log CFU/g) in tomato stems of genotype King Kong2 amended with and without silicon at 7 days post inoculation (dpi).

	Bacterial numbers (log CFU/g)
- silicon	5.88 ± 0.80 a
+ silicon	2.69 ± 1.47 b

Data are means ± SE of three independent trials with three plants per treatment. Same letters are not significantly different with Wilcoxon rank-sum Test at $\alpha = 5\%$.

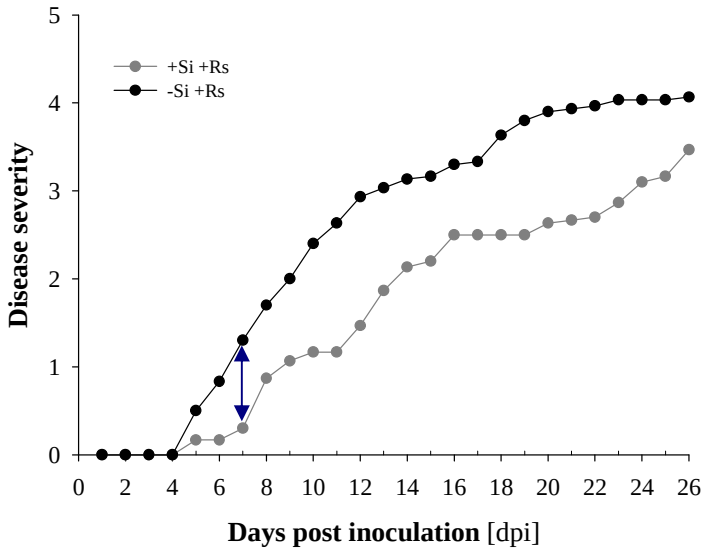

Fig. 5.2: Wilt symptom development expressed in disease severity classesof tomato genotype King Kong2 inoculated with *R. solanacearum* strain ToUdk2, amended with and without silicon.

Data are means of three individual experiments with ten plants per treatment.
Bacterial wilt severity was evaluated according to the scale: 0 = no leaf wilted, 1 = one leaf wilted, 2 = two leaves wilted, 3 = three leaves wilted, 4 = whole plant wilted except the top, 5 = dead plant. Arrow shows difference in disease severity at sampling date.

Table 5.3: Area under disease progress curve (AUDPC) based on disease severity and disease incidence for tomato genotype King Kong2 inoculated with *R. solanacearum* strain ToUdk2.

	$AUD_S PC$	**$AUD_I PC$**
- silicon	63.38 ± 8.5 a	1190.00 ± 168.6 a
+ silicon	41.35 ± 11.7 a	788.33 ± 229.8 a

Data are means ± SE of three individual experiments with ten plants per treatment. Same letters are not significant different with Wilcoxon rank-sum Test at $\alpha = 5\%$.

5.3.2 Primer verification on genomic DNA and RNA basis

All primer pairs described in materials and methods (Table 5.1) showed specific amplification of PCR products using genomic DNA as template (data not shown).

Primers for pectin methylesterase showed no amplification on RNA basis in preliminary reverse transcriptase PCR (RT-PCR) including cDNA of all treatments and were not included in the real-time PCR analysis (data not shown). All other primer pairs gave specific amplification of PCR products using RNA as template (data not shown).

Polyphenol oxidase (PPO A) and polygalacturonase-inhibiting protein (PGIP) showed PCR products in RT-PCR, but had two distinct peaks in melt curve analysis (Fig. 5.3A, 5.3B) after quantitative real-time PCR analysis. Therefore, PPO A and PGIP were not included in the subsequent gene expression study. All remaining genes showed only one peak in melt curve analysis, as demonstrated for the housekeeping genes phosphoglycerate kinase (*PGK*) and α-tubulin (*TUB*) (Fig. 5.3 C, 5.3D)

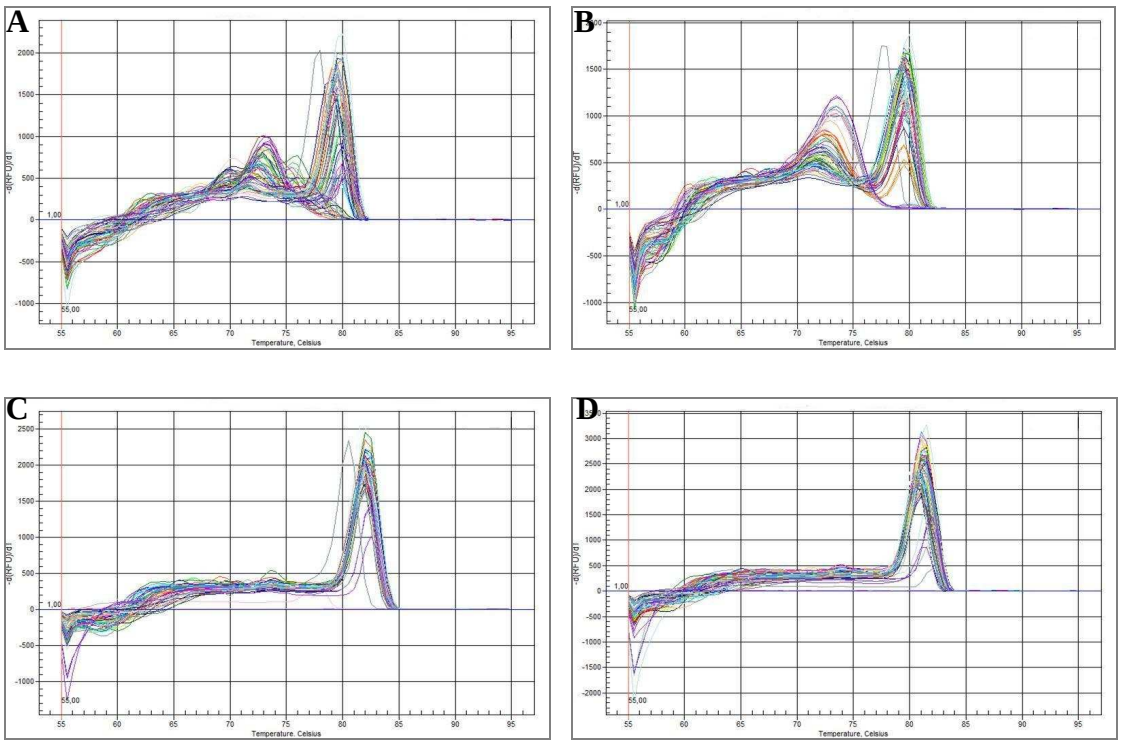

Figure 5.3: Melt curve analysis of two target genes (A) polyphenol oxidase A [PPO A] and (B) polygalacturonase-inhibiting protein [PGIP] and the housekeeping gens (C) phosphoglycerate kinase [*PGK*] and (D) α-tubulin [*TUB*].

5.3.3 Gene expression of signaling pathways related genes: *COI1, ERF1, NIM* and *JAZ1*

Expression of genes of the signaling pathways in silicon treated and/or *R. solanacearum* inoculated plants generally showed basal levels compared to the non-silicon treated, non-inoculated control (-Si-Rs) for COI1 (Fig. 5.4A). In *R. solanacearum*-inoculated plants without silicon amendment (-Si+Rs) the expression of ERF1 was slightly, even though heterogeneously, increased (Fig. 5.4B), while a tendency to downregulation in inoculated plants of both silicon treatments for NIM (Fig. 5.4C) and JAZ1 (Fig.5.4D) was observed. Silicon treatment of inoculated plants led to reduced expression of JAZ1 compared to the control. Silicon treatment alone showed no effect besides a slight reduction in expression of ERF1. No significant differences were observed.

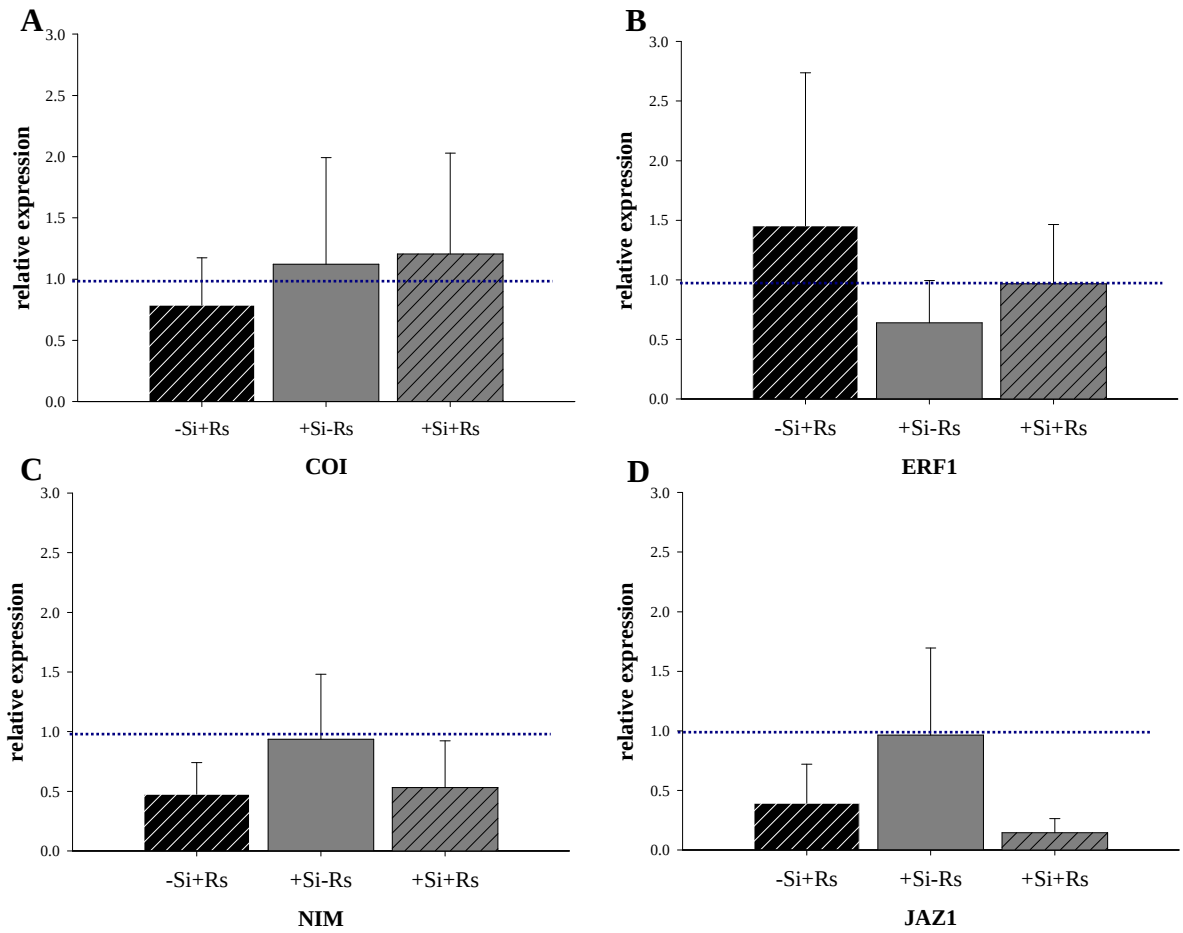

Figure 5.4: Relative expression of signalling pathway related genes (A) coronatine-insensitve1 [COI1], (B) noninducible immunity [NIM], (C) ethylene response factor1 [ERF1] and (D) jasmonate ZIM-domain protein1 [JAZ1] of silicon treated, healthy and inoculated with *R. solanacearum* strain ToUdk2 tomato plants of genotype King Kong2 at 7 days post inoculation (dpi), compared to healthy, non-silicon treated controls.

Data are means ± SE of six plants per treatment, obtained from two independent biological trails.
Relative expression was calculated according to the $2^{-\Delta\Delta CT}$-method, using phosphoglycerate kinase (*PGK*) and α-tubulin (*TUB*) as housekeeping genes. Dotted line shows relative expression of the non-inoculated, non-silicon treated control (-Si-Rs), set as 1.

5.3.4 Gene expression of plant cell wall related genes: *CallSyn, AGP* and *Extensin*

For the cell wall related genes callose synthase (CallSyn), arabinogalactan protein (AGP) and extensin, a trend to downregulation in *R. solanacearum*-inoculated plants irrespective of silicon treatment was observed, while silicon treatment of healthy plants showed no effect (Fig. 5.5A-C). No significant differences were observed.

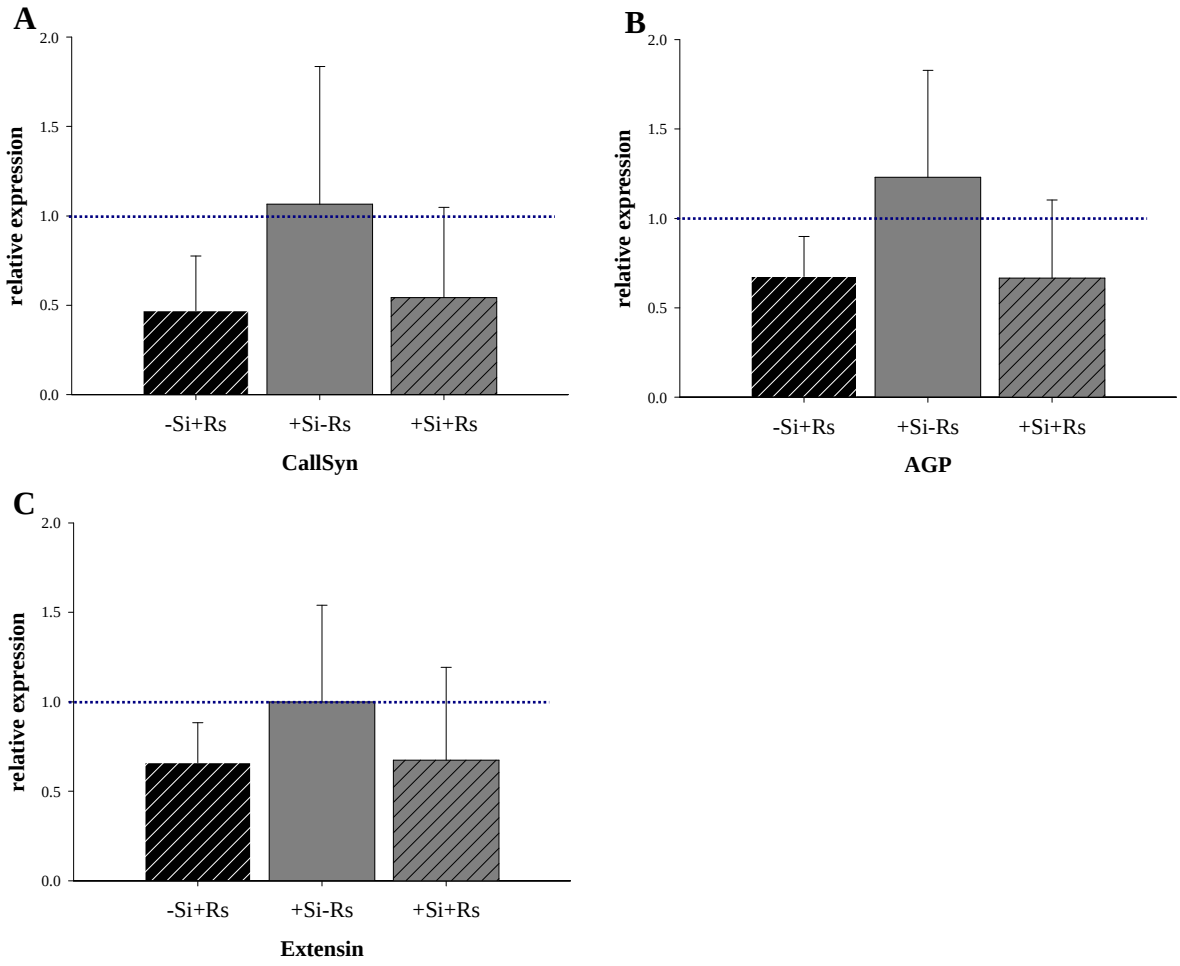

Fig. 5.5: Relative expression of cell wall genes (A) callose synthase [CallSyn], (B) arabinogalactan protein [AGP] and (C) extensin of silicon treated, healthy and inoculated with *R. solanacearum* strain ToUdk2 tomato plants of genotype King Kong2 at 7 days post inoculation (dpi), compared to healthy, non-silicon treated controls.

Data are means ± SE of six plants treatment, obtained from two independent biological trails.
Relative expression was calculated according to the $2^{-\Delta\Delta CT}$-method, using phosphoglycerate kinase (*PGK*) and α-tubulin (*TUB*) as housekeeping genes. Dotted line shows relative expression of the non-inoculated, non-silicon treated control (-Si-Rs), set as 1.

5.4 Discussion

The relative gene expression of the target genes was calculated according to the $2^{-\Delta\Delta CT}$-method, using phosphoglycerate kinase (*PGK*) and α-tubulin (*TUB*) as housekeeping genes (HGKs). Careful selection of appropriate HKGs is necessary because they are used as internal control genes and the expression levels must be constant irrespective of the experimental treatments. In former studies, the expression of some HKG was shown to be significantly different in their expression stability in response to biotic and abiotic stresses (Kim *et al.*, 2003; Volkov *et al.*, 2003; Nicot *et al.*, 2005; Jain *et al.*, 2006), including the commonly used HKG actin in response to silicon treatment in tomato plants (Ghareeb, 2007). For this reason, the well established HKG α-tubulin and phosphoglycerate kinases were chosen as HKG for silicon experiments in tomato (Coker and Davis, 2003; Ghareeb, 2007).

Former studies revealed that reactions related to basal resistance in the plant - *R. solanacearum* interaction, especially in the plant cell wall structure, occur at later infection state of the bacterium, e.g. 5 and 12 dpi (Diogo and Wydra, 2007; Hartmann, 2008). Similar to these results we observed decreased PPO activity and increased tylosis formation, most obvious in silicon treated, *R. solanacearum* inoculated plants at 8 dpi. Therefore, we chose 7 dpi as time point to investigate changes in the defense signaling pathways and plant cell wall related compounds on the molecular level.

Results from the gene expression study indicate that *R. solanacearum*-inoculation influences NIM (syn. NPR1) and JAZ1 expression by downregulation of these genes at the tested time point, 7 dpi, as tendenciously observed for both inoculated treatments.

The expression of NPR1 requires most likely an activation step, because overexpression of NPR1 alone does not result in constitutive PR gene expression when SAR is not induced. Evidences were found that NPR1 is involved in the regulation of SA-mediated gene expression in the nucleus, but more in protein-protein interactions, through interaction with transcription factors (Beckers and Spoel, 2006). It was demonstrated that cellular redox changes - which can occur after induction of SAR, followed by an initial oxidative burst - led to the conversion of NPR1 from an oligomeric to a monomeric state, which moves to the nucleus, leading in turn to the activation of SAR - related gene expression (Mou *et al.*,

2003). Thus, without changes in the SA levels *in planta*, no accumulation of PR proteins occurs. Indeed, NPR1 was previously shown to be constitutively expressed, and induction with SA resulted only in a two-fold increase on transcript level (Cao *et al.*, 1998). This might be one explanation why we could not detect any major changes in the mRNA accumulation of the NIM gene in the tomato - *R. solanacearum* - silicon interaction; it might already exist in the oligomeric form at chose late the investigation time point.

Additionally, it is well documented that *Arabidopsis npr1* mutants exhibit suppressed expression of PR-1 after stimulation with known SAR-inducers like SA or 2,6-dichloroisonicotinic acid (INA) (Cao *et al.*, 1994; Cao *et al.*, 1997). PR-1 and β-1,3-glucanase have been widely used as markers for SA-mediated induced resistance (Uknes *et al.*, 1992; Uknes *et al.*, 1993; Cao *et al.*, 1994). The accumulation of PR-1 was included in a gene expression study related to the silicon - *R. solanacearum* - tomato interaction, and transcript accumulation was increased for all treatments, namely silicon-treated, *R. solanacearum*-inoculated and the combination of both treatments compared to the non-silicon, non-inoculated controls at 12 hpi, but returned to nearly basal transcript levels at 72 hpi (Ghareeb, 2007). These results indicate that SA-mediated induced resistance *via* PR-1 accumulation, and thus accumulation of NPR1, is most likely not involved in the maintenance of the resistance due to silicon at a later pathogenesis and resistance reaction state.

However, NPR1 has also been shown to be involved in the rhizobacteria-induced systemic resistance (ISR), which is not associated with SA, indicating that NIM also takes part in the cross-talk between both pathways (Pieterse *et al.*, 1998). Van Wees *et al.* (2000) demonstrated that increased protection of *Arabidopsis* to *Pseudomonas syringae* pv. *tomato* was dependent on the simultaneous expression of SAR and ISR requiring NPR1, but both pathways do not compete for NPR1, suggesting that the constitutive expression of the protein is sufficient to confer protection by the action of both types of induced resistance in this case. Later, it was suggested that the essential nuclear localization of NPR1 for the SA-mediated SAR seems not to be required for the suppression of JA signaling, suggesting that NPR1 functions in the cytosol in the communication of the SA and JA pathway (Spoel *et al.*, 2003). We could find only basal levels or a tendency to downregulation of NIM in silicon treated and/or *R. solanacearum* inoculated plants.

Additionally, the expression of JAZ1 was shown to be 11 fold upregulated in *R. solanacearum* inoculated, silicon treated plants at 72 hpi (Ghareeb, 2007). However, we found a downregulation of this gene for the same treatment at a later time point of infection (7 dpi) with the bacterial pathogen. It is suggested that JAZ upregulation results from an earlier upregulation of the JA-pathway signalling in the Si-induced resistance of tomato to *R. solanacearum* to prevent possible cellular damages because of strong stimulation of defence related compounds. This might not be necessary in the maintenance of Si-induced resistance at a later pathogenesis state, then leading to downregulation of the gene.

The expression of ERF1 in our study was slightly, but heterogeneously increased in the *R. solanacearum* - inoculated, non-silicon treated plants at the chosen time point, with similar levels for both silicon treatments compared to the healthy, non-silicon treated controls. Whether silicon influences the expression of ERF1 in tomato plants challenged with *R. solanacearum* warrants further explorations.

The stimulation of different ERF genes by disease related compounds, such as ET, JA or SA, and by infection with virulent and avirulent pathogens has been demonstrated (Fujimoto *et al.*, 2000; Gu *et al.*, 2000; Chen *et al.*, 2002; Onate-Sanchez and Singh, 2002; Brown *et al.*, 2003; Chakravarthy *et al.*, 2003; Lorenzo *et al.*, 2003; Guo and Ecker, 2004; McGrath *et al.*, 2005), indicating a significant cross-talk between the signaling pathways and the involvement of ERFs in defense responses in various plant species, albeit dependent on the particular plant-pathogen interaction (Broekaert *et al.*, 2006)

ERF1 confers resistance, when overexpressed in *Arabidopsis* and/or after infection with several pathogens (Berrocal-Lobo *et al.*, 2002; Chen *et al.*, 2002; Onate-Sanchez and Singh, 2002). Furthermore, the expression of ERF1 is dependent on simultaneous activation of both pathways, with requirement of ethylene insensitive2 (EIN2) for JA-dependent induction and COI1 for the ET-dependent induction of ERF1, suggesting that ERF1 is a downstream component of both pathways involved in defense responses in *Arabidopsis* (Lorenzo *et al.*, 2003). Interestingly, the tomato ERF protein *TSRF1* is suggested to integrate ET and osmotic stress pathways (Huang *et al.*, 2004) and was transcriptionally upregulated after ET, and SA treatment and after infection with *R. solanacearum* strain BJ1057. Additionally, transgenic tobacco and tomato plants overexpressing *TSRF1* showed increased resistance to bacterial wilt (Zhang *et al.*, 2004). Nevertheless, increased expression of ERFs can also result in increased susceptibility to

pathogens as demonstrated by the overexpression of the *Arabidopsis* AtERF4, resulting in enhanced susceptibility to *Fusarium oxysporum* (McGrath *et al.*, 2005).

Furthermore, we could not detect major changes in COI1 expression, indicating that regulation of ERF1 at this point of time might be independent of COI1 for the tomato – *R. solanacearum* – silicon interaction, in contrast to the findings in *Arabidopsis*, which additionally was at an earlier point of time after inoculation (Lorenzo *et al.*, 2003).

Enhanced susceptibility towards the pathogens *Alternaria brassicicola* and *Botrytis cinerea* was observed in the *Arabidopsis* JA-response mutant *coi1*, and for *Pectobacterium carotovorum* in the *Arabidopsis* ethylene - (*ein2*) and jasmonate - (*coi1*) insensitive mutants (Thomma *et al.*, 1998; Norman-Setterblad *et al.*, 2000). An increased level of growth of *Pseudomonas syringae* pv. *tomato* in the JA-insensitive *Arabidopsis* mutant *jar1* was described by Pieterse *et al.* (1998), suggesting that JA-dependent responses are involved in resistance reactions against various microbial pathogens. The *Arabidopsis* coi1-20 mutant exhibited resistance to *Pseudomonas syringae,* but did not react significantly concerning resistance to a fungal (*Erisyphe cichoracearum*) and a viral pathogen (cauliflower mosaic virus), indicating that the resistance of this mutant is restricted to pathogenic pseudomonads (Kloek *et al*., 2001). The resistance correlated with a stronger and faster induction of PR-1 expression after infection with *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 compared to wild-type plants, suggesting that SA-mediated defense responses are sensitized in the response to infection by *Pst* DC3000. As mentioned above, we could not detect changes in expression, suggesting that COI1 is not involved in the reaction process at this point of time.

The expression of most of the extensin genes is developmentally regulated, but accumulation of extensins or the insolubilization of extensins, which means the preexisting HRGPs in the cell wall become insolubilized by H_2O_2-mediated oxidative cross-linking, can also be an inducible phenomenon occurring in response to wounding or elicitor treatment (Bradley *et al.*, 1992; José and Puigdomènech, 1993).

First investigations of Showalter *et al.* (1991) showed that extensin mRNA is present in unwounded tomato stems, but their expression in response to wounding differed. Further investigations revealed that both classes, extensin class I and II, are responsive to wound treatment in tomato stems (Showalter *et al.*, 1992). Nevertheless, these findings suggest not

only a role as structural components of the cell wall, but also an additional role in wound healing. The fact that extensin genes are transcriptionally activated in *Arabidopsis* leaves near infection sites of *Xanthomonas campestris* pv. *campestris* or accumulation of extensins occurs after infection, for instance in melon plants infected with the fungus *Colletotrichum lagenarium,* also suggests a role in plant defence (Mazau and Esquerré-Tugayé, 1986; Merkouropoulos and Shirsat, 2003). More recently, the overexpression of an extensin in *Arabidopsis* led to stem thickening and height reduction as well as reduced lesion development after infection with *Pseudomonas syringae* (Roberts and Shirsat, 2006; Wei and Shirsat, 2006). In contrast, we observed a tendency to decreased extensin mRNA accumulation for both inoculated treatments at 7 dpi in our experiments, suggesting rather a downregulation of the gene or suppression of extensin expression by the pathogen, than an active defense mechanism by upregulating the gene at this evaluation date.

The induction of extensins by wounding in tomato is dependent on the tissue. Thus, increased transcripts were observed in wounded stems, but not in wounded leaves (Showalter *et al.,* 1992). The expression of individual HRGPs in one plant species can also vary depending on the stress stimuli as demonstrated for French bean (Corbin *et al.*, 1987; Sauer *et al.,* 1990). Three transcripts of bean HRGPs were induced by a fungal elicitor, wounding or infection with the fungus *Collctotrichum lindemuthianum*, but the transcripts exhibited markedly different patterns of accumulation (Corbin *et al.,* 1987). The induction of a new member of the bean HRGP gene family was investigated by Sauer *et al.*, (1990) and they demonstrated that both, infection with fungal spores of *Colletotrichum lindemuthianum* and treatment with a fungal elicitor preparation, resulted in decreased mRNA levels of this gene, in contrast to wounding which resulted in a rapid induction of the transcript. Thus, it remains to investigate whether various extensins might be involved in the reaction and whether the expression is differently regulated upon silicon treatment and *R. solanacearum* inoculation.

An accumulation of hydroxyproline rich proteins, the class of proteins to which extensins are belonging, upon ET treatment was described in earlier studies (Esquerré-Tugayé *et al.*, 1979; Tagu *et al.*, 1992). Ecker and Davis (1987) analysed the extensin gene expression after ethylene treatment and wounding in carrot roots and found that ethylene induced two mRNAs, whereas wounding led to the accumulation of an additional extensin mRNA, suggesting that the two signals are distinct. The accumulation of tomato extensin mRNA and protein was observed in response to various stimuli including ethylene treatment by

Showalter *et al.*, (1992), but differences in the mRNA accumulation after abscisic acid (ABA) treatment and drought stress could not be observed, suggesting that ethylene mediates extensins wound responses. The expression pattern of extensin differed from the pattern of ERF in our study, but showed similarity to the expression of NIM, involved in SA-mediated responses. In Arabidopsis, application of SA and MeJA to wild-type roots resulted in the accumulation of extensin (*atExt1*) mRNA in the leaves and stems, while levels of this gene in the roots remains unaffected (Merkouropoulos *et al.*, 1999). We found a tendency to downregulation for NIM and extensins and it remains to investigate if decreased SA-responses in inoculated plants might affect cell wall related genes like extensins, callose synthase and AGP, where the same expression patterns were observed (also discussed below).

Additionally, it is proposed that extensins can act as nonspecific agglutinins of microbial pathogens, resulting in the immobilization of the pathogen and thus allow the plant to build up compounds which directly reduce bacterial numbers in the plant (Cassab and Varner, 1988; Wei and Shirsat, 2006). A strong agglutination of avirulent strains of *R. solanacearum*, but only weak agglutination of virulent strains by a potato agglutinin suggesting an important role of these proteins in binding of bacteria to the cell wall that might initiate a recognition process or limit the bacterial spread in the plant (Leach *et al.*, 1982). We could observe reduced bacterial numbers in silicon treated plants, but no increased gene expression of extensin for the same treatment at 7 dpi. It is speculative if a higher gene expression at an earlier time point might have led to a decrease of *R. solanacearum* in tomato stems of silicon treated plants.

As mentioned above, a tendencious downregulation in both inoculated treatments of the two additionally examined cell wall related genes, AGP and callose synthase, was observed.

The expression of AGPs is widely distributed in organs and plant tissues and they are developmentally regulated, in an organ-specific and tissue-specific manner in various degrees depending on the individual member of the AGP family (Showalter, 2001).

Evidence for the participation of AGPs in plant-microbe interactions was provided by Gaspar *et al.* (2004), investigating the *Arabidopsis* rat1 mutant. The mutation in this gene correlates with the down-regulation of AGP17 in the roots, affecting the binding of *Agrobacterium tumefaciens* to the roots. Furthermore they proposed an involvement of

AGP17 in the reduction of SA-mediated responses by *Agrobacterium* in the roots during the infection process. Liu and Mehdy (2007) observed decreased mRNA levels of a non-classical AGP gene (AGP31) of *Arabidopsis* in response to methyl JA (MeJA) treatment, wounding and abscisic acid (ABA) treatment, whereas the MeJA suppression was mainly dependent on the action of coronatine-insensitive (COI).

We observed a trend to downregultation of callose synthase in both *R. solanacearum* inoculated treatments. Callose synthase was reported to be involved in plant defense responses, for instance, a systemically enhanced β-1,3-glucan synthase activity in induced cucumber plants, indicating that the plant can react more rapidly with the production of callose-containing papillae (Schmele and Kauss, 1990). The enzyme has also been reported located at reaction sites during the early stages of papilla deposition in interaction of French bean (*Phaseolus vulgaris* L.) with a hrpA mutant of *Xanthomonas campestris* pv. *vesicatoria* and saprophytic strain of *X. campestris* (Brown *et al.*, 1998).

The expression of 12 Arabidopsis callose synthase genes upon infection with the fungal pathogen *Hyaloperonospora arabidopsis* or treatment with SA was investigated by Dong *et al.* (2008). Five of the twelve genes reacted on both treatments, with strongest reaction for the genes *CalS1* and *CalS12*, whereas treatment with MeJA showed no significant expression for all examined genes. Furthermore, the induction of the *CalS1* and *CalS12* after pathogen challenge and SA was reduced in the *Arabidopsis npr1* mutant, indicating that induction of *CalS1* and *CalS12* by SA and pathogens requires a functional NPR1. But, they also stated that a weak induction of callose synthase genes by pathogens can be independent of NPR1. Additionally, the T-DNA insertional mutant of *cals12* was more resistant to *H. arabidopsis*, but showed reduced callose deposition around haustoria. Similar results were obtained for the *Arabidopsis pmr*4 mutant, which is also defective in callose deposition, in the interaction with the fungal pathogen *Erysiphe cichoracearum* (Vogel and Somerville, 2000; Nishimura *et al.*, 2003), suggesting that decreased callose deposition leads to recognition of the pathogen and more effective defence mechanisms, compared to wild-type plants. The enhanced resistance of *pmr4* is most likely due to the action of the SA signal transduction pathway (Nishimura *et al.*, 2003).

We con not rule out that the observed downregulation in both inoculated treatments for NIM1, JAZ1, extensin, callose synthase and AGP, might be, at least partly, due to the active suppression of these genes by the pathogen, since such mechanisms are described

for bacterial pathogens (reviewed in da Cunha *et al.*, 2007). Thus, the interplay of downregulation of the genes and SA-signalling in the infection process should be investigated in further studies.

Besides findings that pectin methyl esters can vary quantitatively in their degree of esterifications in resistant and susceptible cultivars in different pathosystems (Marty *et al.*, 1997; Boudart *et al.*, 1998), the pattern or qualitative distribution of methyl esterification is suggested to participate in the resistance of wheat near-isogenic lines to the stem rust fungus *Puccinia graminis* f.sp. *tritici* (Wiethölter *et al.*, 2003). Moreover, constitutive differences in the methyl ester distributions in xylem vessel walls of tomato genotypes differing in their resistance status to *R. solanacearum* were observed (Wydra and Beri, 2007). Thus, the degree of esterification and the distribution pattern of pectins presumably influence the depolymerisation of the plant cell wall by pectic enzymes secreted by the pathogens (Chen and Mort 1996; van Alebeek *et al.*, 2002).

The response of pectin methyl esterases (PMEs) can vary in response to biotic and abiotic stresses (Pelloux *et al.*, 2007), including alterations of transcript-levels of a putative pectin methylesterase in *Arabidopsis* after treatment with oligogalacturonides observed in microarray analyses (Moscatiello *et al.*, 2006) and up-regulation of genes in plants infected with phloem feeding insects (Thompson and Goggin, 2006). We could not detect any transcripts of PME on RNA basis, even though cDNA of all treatments used in this study, were included in the RT-PCR analysis, indicating that PME might be part of a physiological process or in the defence response at a different time point.

Conclusions

Ralstonia solanacearum inoculation affects the expression of plant cell wall related genes, such as AGP, extensin and callose synthase, and pathway related genes, such as NIM and JAZ1, since a trend to downregultion of these genes was observed at the time of analysis at 7 dpi, irrespective of silicon treatment.

The expression of ERF1 was slightly, even though heterogeneously, increased in the non-silicon, *R. solanacearum* inoculated treatment, but no differences in the expression of COI1 and NIM across treatments were observed, indicating that rather ERF1 than COI1 and NIM are involved in the reaction at the investigation time point.

GENERAL DISCUSSION

In the interaction of tomato and *R. solanacearum* it is known, that the bacterium is able to invade even highly resistant genotypes, whereas wilt symptoms do not necessarily occur (Grimault and Prior, 1993). The tomato genotype Hawaii7996, considered as one of the most resistant tomato genotypes, showed the highest level of resistance in growth chambers, and a good level of resistance in the field (Scott *et al.*, 1993; Wang *et al.*, 1998). Additionally, the resistance can be influenced by various environmental factors (Hayward, 1991) and can also be strain specific (Danesh and Young, 1994; Jaunet and Wang, 1999). We also observed differences in symptom development after inoculation with *R. solanacearum* strain ToUdk2 of four RILs, having the same genetical background, all derived from a cross between the susceptible genotype Wva700 and the resistant genotype Hawaii7996 (Wydra and Beri, 2006). When treated with monosilic acid and silicon dioxide, the silicon effect was most obvious for NHG3, the most susceptible genotype among the RILs, additionally; *R. solanacearum* strain ToUdk2 was capable to wilt single plants of the resistant genotypes NHG13 and NHG162, generally in the non-silicon treatment. Furthermore, silicon treatment lead to decreased wilting, most obvious in the moderately resistant tomato genotype King Kong2, as previously reported for this genotype (Dannon and Wydra, 2004; Diogo and Wydra, 2007), indicating that silicon amended, moderately resistant genotypes are the most promising candidates for the investigation of this resistance.

The silicon content in healthy and *R. solanacearum* inoculated tomato genotype King Kong2 was in average for three investigation time points for non-silicon treatments in stems 0.18 ± 0.03, for roots 0.26 ± 0.01 mg / g dry matter, and for the silicon treatment in stems 0.28 ± 0.04 and in the roots 1.09 ± 0.04 mg / g dry matter. This is according to other reports for a silicon-non-accumulator plant such as tomato, showing elevated levels of the element in roots of silicon treated plants (Ma *et al.*, 2001; Dannon and Wydra, 2004; Diogo and Wydra, 2007).

Based on the observation that bacterial numbers were decreased in stems of silicon treated plants, while no differences in bacterial numbers in silicon treated and non-treated roots were observed, it is suggested that silicon induces resistance in stems of tomato plants, even though silicon primarily accumulates in the roots of treated plants (Dannon and

Wydra, 2004; Diogo and Wydra, 2007). Earlier investigations support the hypothesis that resistance does not result from a physical barrier to root penetration in resistant genotypes, but to the ability of the plant to restrict the bacterial movement to the stems (Grimault *et al.*, 1994), which might be further promoted by silicon treatment.

However, Huong (2006) and Ghareeb (2007) reported no significant changes of the bacterial populations in stems of the investigated moderately resistant tomato genotype, which is according to our results. Bacterial populations were generally not significantly different between silicon and non-silicon treated stems of the tomato genotypes at different sampling dates, even though a trend to reduction in symptom development of silicon treated plants was generally observed throughout the experiments. A high variability in disease incidence between individual plants is characteristic of bacterial wilt infection, and specifically of moderately resistant genotypes, wilting and healthy plants occur as replicates in one treatment, causing highly variable results with low significance values in bacterial populations and severity classes. Therefore, the observed low significances and the differences of the studies might be explainable by the selection of the analyzed plants.

It is known that resistance induction in plants does not lead the total reduction in symptoms, but to decreased symptom development and pathogen growth compared to non-induced plants (Hammerschmidt, 1999), which is according to our and previous findings in the tomato – silicon - *R. solanacearum* interaction, having delayed wilt symptom development and decreased bacterial populations in silicon treated plants (Dannon and Wydra, 2004; Diogo and Wydra, 2007).

Besides the role of silicon in the formation of mechanical barriers to pathogen penetration (Epstein, 1994), enhanced enzymatic activities in cucumber and tabacco, e.g chitinase, β-1,3-glucanase, peroxidase and polyphenoloxidase, after silicon application in fungal infections were observed (Schneider and Ullrich, 1994), suggesting silicon as an agent which confers induced resistance.

In our study, we investigated different components that can be involved in plant resistance reactions towards pathogens with biochemical, histochemical and immunohistochemical methods and, in the gene expression study, focused mainly on marker genes related to the cross-talk in the main defense-pathways and, genes involved in plant cell wall formation.

Based on the biochemical observations we can conclude that rather polyphenol oxidase than peroxidase is involved in the resistance reaction of tomato towards *R. solanacearum*.

Peroxidases can be induced by pathogen attack (Thordal-Christensen *et al.*, 1992; Young *et al.*, 1995), but also decreased resistance towards powdery mildew in barley epidermal cells after overexpression of one special peroxidase (*Prx7*) was observed (Kristensen *et al.*, 2001), suggesting that this POD might function as a susceptibility factor. Besides the above mentioned induction of PODs in plants, this enzyme catalyzes biochemical processes like lignification (Whetten *et al.*, 1998; Hiraga *et al.*, 2001), an important component that forms a strongly hydrophobic framework, thus acting as a stabilizing agent in the plant to maintain water mineral transport through the xylem under negative pressure (Heredia *et al.*, 1995; Rogers and Campbell, 2004). We could not observe a significant difference in peroxidase activity among the treatments at different investigation dates, which is according to our findings that lignification of xylem vessels was homogenous among treatments and no induction, neither by silicon nor by pathogen infection was observed. This rather indicates a physiological role of peroxidase and not an active defence mechanism in the *R. solanacearum* tomato interaction.

Polyphenol oxidase is suggested to function in the defense of plants to pathogens in different ways. The oxidative polymerization of trichome exudates leads to entrapment of small-bodied insects by glandular trichomes of *Lycopersicon* and *Solanum* species (Kowalski *et al.*, 1992; Yu *et al.*, 1992), an antinutritive effect by covalent modification of proteins by quinones (Felton *et al.*, 1992; Felton *et al.*, 1989;) or a direct effect of the toxic quinones towards pathogens (Mayer and Harel, 1979). We found decreased PPO activity in silicon-amended tomato stems after inoculation with *R. solanacearum* suggesting a role of the enzyme in the resistance reaction. An example for differences in enzyme activity was observed by Carver *et al.* (1998) with a higher increase in PAL activity in oat (*Avena sativa* L.), non-treated with silicon compared to the silicon treated plants. They hypothesized that the increased enzyme activity compensates for the lack of silicon and contributs to the penetration resistance to *Blumeria gramini*. However, it is unknown so far, if the reduction of PPO activity resulted from an active downregulation of PPOs or by an indirect effect, for instance the avoidance of drought stress in these plants. Drought stress in combination with continuous light can lead to induction of PPO accumulation in various tissues including xylem parenchyma in non-transformed and PPO-overexpressing tomato plants. An increased activity, for instance in stem xylem parenchyma, was due to the transcriptional activation of one isozyme (PPO B), but was not obseverd for another isozyme (PPO D). Furthermore, transformed tomato plants with suppressed PPO showed no induction of these genes, but exhibited improved water relations and delayed

photoinhibition and photooxidative damage during drought stress (Thipyapong *et al.*, 2004b).

We chose PPO A for gene expression studies but could not detect differences in gene expression because of the occurrence of an unspecific product, which interfered with the real-time PCR analysis. The polyphenol oxidase gene family in tomato consists of seven members: PPO A, A', B, C, D, E and F (Newmann *et al.*, 1993) and similarities in these genes might have led to the amplification of the unspecific product.

The regulation of PPO F gene expression was investigated by Thipyapong and Steffens (1997), who demonstrated that transcription was not only differentially activated by abiotic and biotic stresses, but also in cell-specific responses to SA, jasmonate and ethylene-signalling pathways. The induction of PPO F by jasmonates was only observed in young leaves, by ethylene only in older leaves and salicylic acid treatment led to induction of the gene in stems and foliage at all development stages. An induction of PPO by wounding in tomato was described earlier by Constable *et al.* (1995), and further investigations showed a clear and strong induction of PPO not only after wounding, but also after methyl jasmonate (MeJA), which is a component, together with closely related jasmonates, of the tomato octadecanoid pathway suggested to transduce wound signals inside the cell (Constable and Ryan, 1998). The inducible PPOs, such as PPO B and PPO F, might serve as suitable candidates for further gene expression studies to confirm an involvement of PPO either indirectly by less drought stress of the plants or the downregulation in the interaction of silicon-induced resistance reaction to *R. solanacearum* in tomato plants.

Even though lignification and H_2O_2 accumulation are described in the literature as resistances factors (Sticher *et al.*, 1997; Hammerschmidt, 1999), we could not find any differences related to *R. solanacearum* infection and/or silicon treatments in vascular bundles of tomato stems, but we found increased tylosis formation in silicon-treated, *R. solanacearum* inoculated plants. The formation of tylosis can result in the partial or complete occlusion of xylem vessels, restricting growth or spread of pathogens, and is considered as an active defense mechanism to vascular diseases (Beckmann, 1987; Rioux *et al.*, 1998; Clérivet *et al.*, 2000; Soukup and Votrubová, 2005). Evidence for the involvement of ET in the xylem occlusion response towards the wilt pathogen *Fusarium oxysporum* f.sp. *lycopersici,* in order to limit further spread through the plants vascular system, was provided by VanderMolen and co-workers (1983). We could detect increased

tylosis formation in tomato plants at a later infestation state of *R. solanacearum* in silicon-treated plants, but results derived from the gene expression study most likely exclude ET as signal in this process. We can not rule out that ET might be involved in the process at an earlier state in the interaction, as well as other plant hormones that could be involved in process, such as auxin and indole-3-acetic acid (IAA) (Beckmann, 1982).

For the establishment and/or maintenance of silicon-induced resistance in tomato, maybe alternative defence pathways are worth to be investigated. Besides the well-established SA and JA/ET signalling pathways in plants (Ryals *et al.*, 1996; Thomma, *et al.*, 2001), emerging evidences were found that alternative signals, such as abscisic acid (ABA), are involved in responses to water and salt stress, but also participate in the plant resistance to pathogen attack (Walton, 1980; Zeevaart and Creelman, 1988; Flors *et al.*, 2005).

ABA is known to induce susceptibility, as demonstrated in *Arabidopsis* where ABA treatment or drought stress led to more susceptible plants towards the pathogens *Pseudomonas syringae* pv. *tomato* and *Peronospora parasitica* (Mohr and Cahill, 2003). Support for the interactions of ABA with other signalling pathways, in particular with SA, was provided by Thaler and Bostock (2004). Data obtained from their biochemical assays in combination with bioassays in the tomato-*P. syringae* pv. *tomato* interaction suggests that elevated ABA interferes with SA-mediated resistance towards the bacterial pathogen, demonstrated by more resistant tomato plants towards *P. syringae* pv. *tomato* in an ABA-deficient mutant background due to a higher levels of SA-mediated responses.

Nevertheless, Ton and Mauch-Mani (2004) demonstrated that primed callose deposition in *Arabidopsis* against necrotrophic pathogens is controlled by an ABA-dependent pathway and reacts independently of SA, JA and ET as defence signal. The ABA-dependent pathway resulted in decreased callose deposition in *Arabidopsis* plants treated with β-amino-butyric acid (BABA) (Ton and Mauch-Mani, 2004). Additionally, ABA was shown to inhibit the transcription of a basic β-1,3-glucanase in tobacco cell cultures (Rezzonico *et al*., 1998). Tobacco mutants which are deficient in β-1,3-glucanase activity were more resistant to tobacco mosaic virus (TMV), due to more callose accumulation around TMV-induced lesions (Beffa *et al.*, 1996). We noticed a tendency to decreased callose deposition in higher infected tomato plants of genotype King Kong2 at 5 dpi in histochemical observations. This might rise the question if the callose degradation is linked to pathogen action for instance by the secretion of β-1,3-glucanases. Among the known

virulence factors of *R. solanacearum* are six cell wall degrading enzymes: β-1,4-endoglucanase (Egl), exoglucanase (ChbA), endopolygalacturonase (PehA), exopolygalacturonases (PehB and PehC) and pectin methylesterase (Pme) (Denny, 2006), but to our knowledge, β-1,3-glucanase was not detected so far. Furthermore, our gene expression study showed a downregulation of callose synthase in inoculated plants (7 dpi) and this indicates that reduced callose deposition in tomato King Kong2 is rather derived from the plant side, than by action of the pathogen.

Recently, Hao *et al.* (2008) demonstrated that callose deposition in sieve plates of the phloem of rice plants contributes to the resistance towards the brown planthopper (*Nilaparvata lugens* Stål) and, that involvement of the callose degrading β-1,3-glucanase might contribute to susceptibility. They demonstrated that callose synthase and β-1,3 glucanase genes were upregulated in resistant and susceptible rice plants, but induction of the callose degrading β-1,3 glucanase genes was stronger in susceptible plants, suggesting that degradation of callose facilitates the insects continuous feeding on the plant. Accumulation of plant β-1,3 glucanase was not investigated in this study, but might serve as candidate for further investigations, together with osmotins, belonging to the thaumatin and thaumatin-like proteins grouped into the PR-5 family (Tuzun and Somanchi, 2006), which were also shown to be regulated by ABA and involved in adaption to osmotic stress (Singh *et al.*, 1987; Singh *et al.*, 1989).

And, interestingly, AtMYC2, which is involved in JAZ1 'signaling', has also been shown as a positive regulator of ABA (Mauch-Mani and Mauch, 2005). There are incidences that JAZ1 is involved in the silicon-induced resistance of tomato to *R. solanacearum* (this study; Ghareeb, 2007).

Even though, significant differences in developmental locations of AGP epitopes between species are proposed (Knox, 1995), the monoclonal AGP antibody JIM13 recognized cells in the epidermis but also in developing xylem in the root apex of carrot (Knox *et al.*, 1991). We also detected AGP with the antbody LM2 in the vascular tissue of the tomato recombinant inbred lines for all treatments, which suggests an involvement of the AGP in physiological processes of the plant. Additionally, we could not observe a uniform distribution pattern of AGP related to the resistance status of the examined tomato RILs, pathogen infection or silicon nutrition at 5 dpi and we even observed a trend to downregulation in the inoculated treatments of King Kong2 in the gene expression study at

7 dpi. The observed differences in the pectic polysaccharide structures, detected by monoclonal antibodies specific for AGP (LM2), arabinan side chains (LM6) and non-blockwise pectin methylester distribution (LM7) in infected tomato genotype King Kong2 inoculated with *R. solanacearum* as described by Diogo and Wydra, (2007), were not so clearly observed for the recombinant inbred lines (RILs) in this study, indicating that the genotypes are reacting differently in their modulation of pectic polysaccharides after infection. Another possible explanation might result from the different investigation time points in both studies. A change in the pectic structure for King Kong2 was observed at 20 days post inoculation (dpi) in the previous study, while we chose 5 dpi in this study, which might be too early for the detection of the modulation of the polysaccharides. Furthermore, we could not detect an increase in AGP transcript accumulation for genotype King Kong2 at 7 dpi with real-time PCR analysis in the gene expression study, suggesting that accumulation of AGPs occurs later in the reaction of tomato plants with *R. solanacearum*.

Normally, investigation time points for molecular analyses are chosen within the first hours after inoculation with the pathogen. For instance in the study of Gaspar *et al.* (2004), where the interaction of Arabidopsis *rat1* mutant and *Agrobacterium tumefaciens* on roots was examined, samples were collected in one hour intervals lasting from 0 to 4 hours post infection. In the interaction of *R. solanacearum* with tomato, the pathogen enters the plant through the root system, but resistance mechanisms are suggested to be activated in the stems (Grimault *et al.*, 1994; Prior *et al*,. 1994). First wilt symptoms generally occured around five days post inoculation and differences between silicon treated and non-silicon treated plants were most pronounced between 8 and 12 dpi. Furthermore, initial molecular investigations of the role of silicon nutrition in the resistance to *R. solanacearum* revealed, that expression of several genes was triggered at 72 hpi, investigating various time points ranging from 0.5 to 72 hpi (Ghareeb, 2007). Thus, we chose 7 dpi as a time point for our gene-expression study to investigate a possible influence of the candidate genes in the establishment of silicon-induced resistance to *R. solanacearum* in tomato.

In the expression studies we investigated different genes mainly related to the cross-talk between the SA, JA or ET pathways, but we could not detect major mRNA accumulation of these genes at 7 dpi, a time point suggested for the establishment of silicon-induced resistance in plants. We can not rule out that these genes might be important for the initial resistance at an earlier time point, as demonstrated for JAZ1 (Ghareeb, 2007), which was

highly upregulated at 72h post inoculation in silicon-treated, *R. solanacearum*-inoculated plants. Furthermore, we found mostly a trend to downregulation of the genes after inoculation with *R. solanacearum,* especially for the JAZ1 in the silicon *R. solanacearum*-inoculated treatment, indicating that these genes might be involved earlier in the resistance process.

In the context of induced resistance it is known that defense responses, when expressed constitutively, carry fitness cost, thus resources are only allocated away from e.g. growth or reproduction when necessary (Bostock, 2005; Walters *et al.*, 2005). The downregulation of genes has been postulated to reduce synthesis of unneeded proteins to conserve energy, suggested then to be invested for enhancement of the cell wall structures (Sheng *et al.*, 1991). Interestingly, all cell wall related genes examined in the gene expression study showed a trend to downregultaion in both inoculated treatments, though histochemical studies at 5 and 20 dpi had clearly demonstrated changes in cell wall structure after inoculation (Beri and Wydra, 2006; Diogo and Wydra, 2007; Wydra and Beri, 2007). This indicates that reinforcement of the plant cell wall, at least for the examined genes, is not a major resistance factor at the observed time point and benefits of the saved costs are invested in a so far unknown mechanism.

The same expression pattern was also observed for the NIM, involved in SA-mediated responses (Beckers and Spoel, 2006), but also in plant defense responses meditated by JA and ethylene (Pieterse, *et al.*, 1998; Pieterse *et al.*, 2001) and for JAZ1, likely acting as repressors of JA-responsive genes (Staswick, 2008). It is speculative if downregulation of these genes also affects the cell wall related genes and warrants further explorations.

Another possibilty might be the suppression or down-regulation of these genes by the pathogen's released virulence factors in order to facilitate host colonization, as previously proposed for the 1,3 β-glucanase and chitinase class II genes in the *R. solanacearum*-tomato-silicon interaction (Ghareeb, 2007). Whether the down-regulation is plant- or pathogen derived warrants further exploration.

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DECLARATION BY CANDIDATE

I, Tanja Schacht, declare that this thesis, entitled **'Biochemical analysis of the inhibition of *Ralstonia solanacearum* polygalacturonases by polygalacturonase-inhibiting proteins (PGIP) from tomato stems and biochemical, histochemical and molecular analysis of the silicon effect in the tomato (*Solanum lycopersicum*) – *Ralstonia solanacearum* interaction'** is an original piece of work conducted by myself and has not been submitted for a degree in any other university.

Hannover, 2009

Tanja Schacht

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