Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Physiological and Molecular Plant Pathology 68 (2006) 41–50
PMPP
www.elsevier.com/locate/pmpp

Structural changes of homogalacturonan, rhamnogalacturonan I and arabinogalactan protein in xylem cell walls of tomato genotypes in reaction to *Ralstonia solanacearum*

Kerstin Wydra*, Hina Beri

Institute of Plant Diseases and Plant Protection, University of Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany

Accepted 9 June 2006

Abstract

Healthy and *Ralstonia solanacearum*-inoculated tomato genotypes susceptible or resistant to bacterial wilt including recombinant inbred lines (RILs) deriving from a cross between the resistant genotype Hawaii7996 and the susceptible Wva700 were compared for symptom and bacterial population development, and for the composition and structure of pectic polysaccharides and arabinogalactan proteins (AGPs) of xylem cell walls by immunological staining of tissue prints. Constitutive differences were observed between resistant and susceptible RILs, with a higher degree of methyl-esterification of homogalacturonan (HG) detected by antibody JIM7 in the resistant plants. After inoculation, decreased methyl-esterification of HG indicated by stronger labeling with antibody JIM5 was observed in all susceptible genotypes and in five of eleven resistant genotypes, with a clear increase in the non-blockwise de-esterification pattern of HG (LM7) only in the susceptible lines, indicating the mode of action of the pectinmethylesterase of *R. solanacearum*. In the susceptible lines infection generally leads to increased branching of rhamnogalacturonan I indicated by the detection of arabinan (LM6) and galactan (LM5) side chains, and of arabinogalactan protein (LM2), while only few of the resistant genotypes reacted with changes in these epitopes. All the resistant, symptomless genotypes contained relatively high pathogen populations in stems. A clear relation between cell wall composition and degree of latent infection of resistant genotypes was not found.
© 2006 Elsevier Ltd. All rights reserved.

Keywords: Bacterial wilt; Basic resistance; Immuno-tissue print; Plant cell wall structure; *Ralstonia solanacearum*; Resistance mechanisms; Tomato (*Lycopersicon esculentum*)

1. Introduction

Bacterial wilt caused by *Ralstonia solanacearum* ranks as one of the world's most important phytopathogenic bacteria prevalent in tropical and subtropical regions of the world, but is also a threat in temperate climates [1,2]. *R. solanacearum* [3,4] is a soilborne, Gram-negative, aerobic, motile rod that naturally infects roots [5,6], and causes bacterial wilt in more than 450 crop species, among them tomato as one of the economically most important hosts [7].

Owing to the lack of reliable means of control, it is difficult to restrict the disease, once it appears [8]. Only a holistic approach combining multiple measures in an integrated disease management strategy can reduce the losses [9,10]. Among the control strategies, breeding for resistance is one of the main approaches to control the disease, but although this has led to good levels of site-specific resistance, breakdown of resistance is frequently observed in tomato cultivars grown under the heat stress of the lowland humid tropics, away from the areas, where the resistant line was developed [11,12]. The variability of the pathogen and the continuous evolution of new and more virulent strains also play a role in breakdown of resistance [2,13,14], and, therefore, for most solanaceous crops only tolerance to the disease could be achieved on a regional level under conditions not too hot or wet [10].

R. solanacearum is known to latently infect and colonize host plant genotypes without causing wilt symptoms. These genotypes may be classified as resistant, hindering growth

*Corresponding author. Fax: +49 511 7623015.
E-mail address: wydra@ipp.uni-hannover.de (K. Wydra).

0885-5765/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.pmpp.2006.06.001

and/or development of the pathogen, or tolerant, able to endure the presence of the pathogen in high numbers without development of disease symptoms and damage [15]. Resistance to bacterial wilt has been defined in the past as a high percentage of plant survival under a certain infection pressure. However, latent colonization of *R. solanacearum* without obvious wilting symptoms has been reported in some resistant cultivars of tomato [6,9,16–19]. Thus, resistant plants are often partially colonized by the pathogen and show reduced damage by the disease [19].

In tomato, resistance against bacterial wilt has been reported to be polygenic, with numerous QTL involved in resistance, including markers spanning a large region of chromosome 6 [20,21]. Since the number of identified QTL is high and they are linked to undesired characteristics, their transfer into one cultivar has been unsuccessful. Some highly resistant tomato breeding lines such as Hawaii7996, which are only susceptible to very few strains, do not produce large fruits [22, Wydra, unpublished]. Thus, some limited knowledge exists on the genetic background of resistance to bacterial wilt, but the physiology and histology of host response to infection and biochemical factors involved in resistance still remain largely unknown [10].

Preformed resistance mechanisms usually include structural, morphological and chemical factors such as quality of cuticle (thickness, quantity and quality of waxes), and cell wall characteristics (thickness and composition) acting as barriers and providing resistance against potential invaders, while chemical compounds such as phytoanticipins may be directly toxic, or indirectly after transformation [23–26]. On the other hand, after the penetration of the pathogen, induced processes are initiated. A complex signaling network involving cytosolic Ca^{2+} and H^{+} ions, reactive oxygen intermediates (oxidative burst), jasmonate, salicylic acid and ethylene triggers the induction of defense mechanisms [27]. Lignification and the production of other structural barriers in cell walls, e.g. the formation of calcium bridges between pectin chains, were observed in many plant species following attempted infection by pathogenic organisms [28,29].

As the skin of the plant cell, the wall participates in adhesion, cell–cell signaling, numerous growth and differentiation processes and defense. The wall of enlarging plant cells is composed of approximately 30% cellulose, 30% hemicellulose, and 35% pectin, with perhaps 1–5% structural protein on a dry weight basis. Pectin forms a gel phase in which the cellulose–hemicellulose network is embedded. Pectic polysaccharides are the most soluble of the wall polysaccharides and constitute a heterogeneous group. Some pectins have a relatively simple primary structure such as homogalacturonan (HG), a linear polymer of $(1\rightarrow4)$ α galacturonic acid esterified to various degrees with methyl, acetyl, and some unidentified groups [30,31]. Rhamnogalactouronan I (RGI), another pectic polysaccharide, is composed of repeating subunits of $(1\rightarrow2)$ α-L-rhamnosyl-$(1\rightarrow2)$-α-D-galacturonyl disaccharides, and is highly diverse in structure and composition, with side chains of variable length of arabinan, galactan and arabinogalactan. The size of RGI is reported to range from 500 to 2000 kDa [32]. Pectins are subject to a number of modifications that alter their conformation and linkage in the wall, and this could explain the changes in pectins with the onset of resistance mechanisms [33]. HG derived oligogalacturonides generated by pectinolytic cleavage are involved in signaling processes during development and in defense responses to plant pathogens [34–36].

Another important component of the extracellular matrix in cell wall architecture are arabinogalactan-proteins (AGPs). AGPs are highly glycosylated cell surface proteins which play a role in plant growth and development [8]. They form a large and diverse group of macromolecules in plants and are described as extracellular proteoglycans composed of a hydroxyproline polypeptide backbone to which branched 1,3:1,6 galactan chains are attached by *O*-glycosidic bonds. The galactan is substituted by arabinose residues and minor amounts of glucose, uronic acids, xylose and rhamnose, and membrane-associated glycoproteins [37–39]. At the tissue level, AGPs are—among other tissues—especially abundant in the xylem. In this tissue they are associated with, and hypothesized to function in secondary cell wall thickening and programmed cell death of xylem cells in order to allow for water transport [40,41]. AGPs belong to the hydroxyproline-rich glycoproteins, which were reported to be often involved in resistance reactions of the plant [41].

Alterations in cell wall components such as pectic polysaccharides or AGPs as well as lignification and metabolic alterations of cells at infection sites were suggested by various authors to play a role in pathogen defense and, specifically, in basic resistance [29,41–44]. Cell wall thickening is a common feature of plant resistance mechanisms and was reported for tomatoes affected by vascular wilt disorders due to fungal infection [29,45]. AGPs might also contribute to cell wall strengthening by association with other cell surface molecules or with one another. For example LeAGP-1 was reported to interact with pectin by clusters of basic amino acid residues or by Ca^{2+}-mediated binding. However, it remains doubtful whether these bindings are strong enough to play a role in xylem cell wall strengthening and therefore in induced resistance [8,41].

Isolation of the cell wall polysaccharides from tomato stems yielded a fraction containing almost all polysaccharides present and few other components [46]. Tomato pectins were revealed to possess long branches, the existence of which was not known previously [47]. The neutral components of pectin belong to the most variable biological molecules and were therefore chosen for further studies on their involvement in the resistance reaction. HGs differing in the degree of methyl-esterification extracted from potato genotypes were reported to be related to resistance to *Erwinia carotovora* ssp. *carotovora* [33].

Electronmicroscopic studies on *R. solanacearum* development in stems showed that the limitation of bacterial spread associated with the resistance of tomato to bacterial wilt was mainly attributed to an induced, non-specific, physical barrier [48]. These studies indicated that the resistance does not arise from an inability of the bacteria to invade the roots, but rather from a limitation of their spread from the collar to the mid-stem. Electron-dense materials of unknown origin accumulated in or around pit cavities in parenchyma cells next to vessels with bacteria, and in vessels with bacteria [49]. Plugging the vessels by bacterial mass [48] and complete occlusion of vessels by bacteria, 'gums' and tyloses [51] have been considered to be the cause of wilting. It was suggested that the coating material observed as resistance reaction of vessels cannot be degraded by the bacteria and limits vascular colonization [52]. First indications on the biochemical nature and origin of this material involved in defense against *R. solanacearum* were only recently described and indicated a possible role of pectic cell wall polysaccharides of variable structure and composition and of AGP in the resistance reaction [46].

The increased awareness of the complexity and dynamic nature of the pectic network has been largely due to the recent development of appropriate tools such as antibodies highly specific to epitopes of HG and RGI, which serve to determine the structural complexity of the cell wall and to dissect this complexity at the cell biological level. The recently developed antibodies to defined pectic antigens and epitopes are important probes for the study of function and organization of plant cell walls [42,53–59]. Using six antibodies against epitopes present in pectic polysaccharides and AGP, we studied the modification of the pectic cell wall components of tomato genotypes after infection by tissue printing. The imprint is formed by soluble molecules released at the surface that bind irreversibly to the membrane, which is subsequently probed with antibodies. Modifications in pectic epitopes and AGPs of stem cell walls were studied in relation to symptom development and latent infection by *R. solanacearum* in resistant and susceptible tomato genotypes.

2. Materials and methods

2.1. Plant material

Fifteen tomato genotypes differing in resistance to bacterial wilt were received from the Asian Vegetable Research and Development center (AVRDC), Taiwan: Hawaii7996, CLN2123C, CLN1-3-13, CLN4-22-4, CLN1-1-12, CLN1-5-12, BL333, NHG13, NHG60, NHG162, NHG140 as resistant genotypes, and Wva700, L390, NHG3 and NHG167 as susceptible genotypes. Genotype King Kong2 [moderately resistant [9] was obtained from KnownYou Company, Taiwan. The NHG-lines are part of about 180 recombinant inbred lines (RILs) deriving from a cross between the susceptible genotype Wva700 and the resistant Hawaii7996. Seeds were sown in a greenhouse (20 °C day/night temperature, 14 h of light per day/30 K Lux, 70% RH) and transplanted after 4 weeks to individual pots with 330 g of soil (Fruhstorfer Erde, Type P, with 150 mg/L N, 150 mg/L P_2O_5, and 250 mg/L K_2O).

2.2. Reaction of tomato genotypes to bacterial wilt

Bacterial inoculum was produced from the fluidal, highly virulent *R. solanacearum* strain To-udk2 (race 1, biovar 3) obtained from Thailand (N. Thaveechai, Kasetsart University, Bangkok) by streaking a single colony on NGA agar medium (0.3% beef extract, 0.5% Bacto peptone, 0.25% D-glucose, 1.5% agar) and incubating at 30 °C for 48 h. Cells were harvested from agar plates and a suspension with an optical density of 0.06 at 600 nm wavelength, corresponding to about 7.8×10^7 colony-forming units per milliliter (CFU/mL) was prepared.

Ten 4-week-old plants per genotype were inoculated by soil drenching with 33 mL of bacterial suspension per pot, corresponding to about 10^7 CFU/g of soil, around the base of the plants directly after transplanting. After inoculation plants were kept in a climate chamber with 30/27 °C day/night temperature, 14 h light, and 30 K Lux, 85% RH). Pots were watered after inoculation up to the soil field capacity without producing a surplus.

Symptom development was evaluated daily as incidence—proportion of dead plants at the evaluation date out of the total number of plants in the treatment—over a period of 40 days after inoculation. The mean wilt incidence of each genotype was calculated and used to determine the area under wilt incidence progress curve (AUWiPC) of genotypes based on evaluation dates according to the following formula [60,61]: $\mathrm{AUDPC} = \sum [(xi + xi{-}1)/2](ti - ti{-}1)$, with xi and $xi{-}1$ as wilt incidence at time ti and $ti{-}1$, respectively; ti and $ti{-}1$ are consecutive evaluation dates; and $ti - ti{-}1$ was equal to 1.

2.3. Quantification of bacteria in stems

Bacterial numbers were quantified in stems of the resistant and moderately resistant genotypes as described by Li and Jan [62]. Detection in susceptible genotypes was not possible due to early death of plants. Three symptomless plants per resistant genotype were randomly harvested 4 weeks after inoculation. The mid-stem pieces (5–10 g) were surface-sterilized by submerging in 70% ethanol for less than 1 min, rinsed in sterile water, and macerated by adding about 20 mL sterile, distilled water. The macerate was filtered through cheesecloth and centrifuged for 10 min at 7000*g*. The pellet was re-suspended in 1 mL sterile, distilled water. Suspensions were serially, tenfold diluted and 100 μL from at least four dilution levels were plated in duplicates on triphenyl tetrazolium chloride (TTC) medium: 20 g Bacto peptone, 5 g glucose, 1 g casamino acids, 15 g Bacto agar and 1000 mL H_2O; after autoclaving, 10 mL of filter-sterilized solution of 0.5% (w/v) of 2, 3,

5-TTC (SERVA, Germany) were added [86]. Typical bacterial colonies were counted after 48 h of incubation at 30 °C and calculated as colony forming units per gram of fresh matter (CFU/g). Identity of colonies was confirmed by NCM-ELISA and PCR [63].

2.4. Tissue printing

For tissue printing the cut surface of mid-stems from two plants of each healthy and inoculated treatments 5 days after inoculation was firmly and evenly pressed onto a nitrocellulose membrane (ELISA Kit, Biorad, Germany) for approximately 15–20 s [64,65]. The tissue printing was repeated three times with newly grown plants, and in each trial prints were performed in duplicate for each antibody, to test the repeatability of the method. Each stem material was tested with six antibodies specific for epitopes of the pectic polysaccharides HG and rhamnogalacturonan I (RGI), and for arabinogalactan protein (AGP): JIM5 (low methyl-esterification grade 31–40% of HG), JIM7 (‘high’ methylesterification grade 15–80%), LM7 (non-blockwise de-esterification of HG), LM5 [(1→4)-β-D-galactan side chains of RGI], LM6 [(1→5)-α-L-arabinan side chains of RGI] and LM2 (AGP) (PlantProbes, Leeds, UK).

After drying the prints at room temperature, the membrane was blocked by incubation with phosphate buffered saline (PBS) containing 5% milk powder (MPBS, pH 7.2) for 1 h prior to incubation in the primary antibodies diluted 1/10 in MPBS for 1.5 h. After washing extensively under running tap water and for 10 min in PBS containing 0.1% (v/v) Tween 20 (PBST), membranes were incubated in the secondary antibody (anti-rat horseradish peroxidase conjugate, Sigma) diluted 1/1000 in MPBS for 1.5 h. Membranes were washed again as described above and developed in substrate solution [25 mL deionized water, 5 mL methanol containing 10 mg/mL 4-chloro-1-naphthol, 30 μL 6% (v/v) H_2O_2] until a clear color reaction developed. All steps were performed at room temperature. The membranes were evaluated visually on an illuminating table by grading the color intensity of stained stem imprints in seven categories: ‘–’ no staining, ‘(+)’ slight staining visible, ‘+’ staining of vessels, ‘(+ +)’ clear staining of vessels, ‘+ +’ clear staining of vessels and vascular cambium, ‘(+ + +)’ staining of vessels, vascular cambium and piths, ‘+ + +’ strong staining of the whole stem imprint including pith. Data of the two replicates and the three repetitions were compared and their repeatability confirmed. Representative results are given.

2.5. Statistical methods

Data were processed using analysis of variance in SAS (SAS System for Windows V8, Release 8.02 TS Level 02M0; 1999–2001, Institute Inc., Cary, USA). For all analyses a significance level of $P = 0.05$ or lower was used, as indicated. The bacterial counts on media, expressed as CFU/g fresh weight of stem material were log-transformed and analyzed using parametric analysis procedures in SAS. A lack of growth on plates of all replications was plotted on the log scale as one, which gives 0 CFU g^{-1}. Tukey’s studentized range (TSR) test ($P = 0.05$) was used within parametric analysis of variance (ANOVA) as incorporated in SAS version 8.02 to compare AUWiPC data of the susceptible genotypes and bacterial numbers in stems of the resistant and moderately resistant genotypes.

3. Results

3.1. Symptom development in tomato genotypes

The 16 tomato genotypes were classified into two significantly different groups, i.e. resistant and susceptible, on the basis of the area under the wilt incidence progress curve (AUWiPC) after inoculation with *R. solanacearum* strain To-udk2. Among the RILs, NHG3 and NHG167 were classified as susceptible with AUWiPC $102.2 \pm 3.7a$ and $100.7 \pm 3.5a$, respectively, with similar AUWiPC as the susceptible standard genotypes L390 and Wva700 with $98.1 \pm 4.6a$ and $99.5 \pm 3.3a$, respectively. First symptoms appeared in susceptible genotypes 5 days after inoculation, and plants were severely attacked 10 days after inoculation, resulting in plant death. Genotypes Hawaii7996, CLN2123C, CLN1-3-13, CLN4-22-4, CLN1-1-12, CLN1-5-12, BL333 and the RILs NHG60, NHG140, NHG13 and NHG162 did not show any wilt symptoms and were grouped as resistant. King Kong2 was identified as moderately resistant in former trials [11].

3.2. Latent bacterial multiplication

R. solanacearum was detected in the mid-stem regions of symptomless plants of all the resistant genotypes four weeks after inoculation, with bacterial numbers between 885 and 2.9×10^7 colony forming units (CFU)/g stem. The bacterial population was significantly higher in genotypes NHG60, NHG140 and King Kong2 than in CLN1-3-13 and CLN2123C, and in CLN4-22-4 than in CLN2123C ($P = 0.0001$) (Fig. 1). Among the resistant genotypes, CLN2123C showed the lowest bacterial density in the stem. Among the RILs, bacterial numbers ranged from 2.3×10^4 to 2.9×10^7 CFU/g stem, with the lowest level in NHG162.

3.3. Characterization of pectic polysaccharides by immunochemical stem tissue printing

Inoculated and non-inoculated plants of all genotypes were characterized for the composition of their pectic cell wall polysaccharides in stems by stem imprints on nitrocellulose, stained by monoclonal antibodies specific to epitopes of pectic polysaccharides or AGP: JIM5 for low-esterification grade of HG, JIM7 for high esterification grade of HG, LM7 for non-blockwise methylde-esterification of HG, and by antibodies LM5 and LM6 specific to

(1→4)-β-galactan and (1→5)-α-arabinan epitopes, respectively, in side chains of RGI, and LM2, specific for AGP.

Comparing the constitutive cell wall composition of resistant and susceptible RILs, tissue prints of the resistant lines showed higher degrees of esterification of HG than the susceptible lines NHG3 and NHG167, indicated by more intense labeling with JIM7 (Table 1). Comparing all genotypes, the observed constitutive differences in epitopes of pectic polysaccharides were not clearly related to the degree of resistance of genotypes, and, generally, differences in cell wall composition between healthy resistant and susceptible genotypes were only minor.

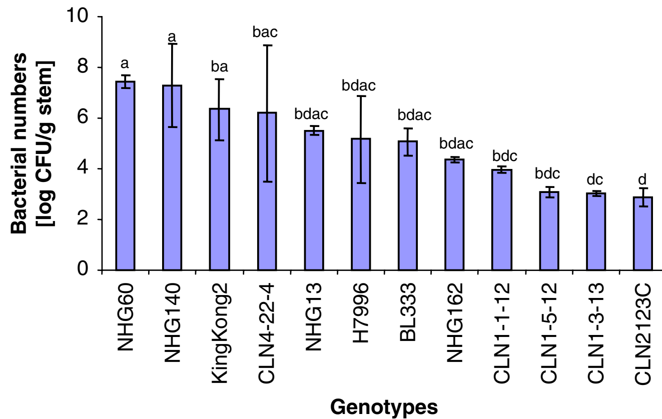

Fig. 1. Bacterial numbers in mid-stems of asymptomatic plants of tomato genotypes NHG60, NHG140, CLN4-22-4, NHG13, Hawaii7996, BL333, NHG162, CLN1-1-12, CLN1-5-12, CLN1-3-13 and CLN2123C (resistant to *R. solanacearum*) and King Kong2 (moderately resistant) at 4 weeks after inoculation with *R. solanacearum* To-udk2 by soil drenching. Data are means of 4 plants ±SE. Letters indicate significant differences among genotypes.

After inoculation major changes in staining with all antibodies were observed in the susceptible genotypes, with most prominent increases in the de-esterification of HG indicated by increased staining with JIM5—corresponding to a lower staining with JIM7 specific for high methylesterification-, and in the non-blockwise de-esterification pattern of HG (LM7) (Table 1), as shown for the susceptible genotype L390 in Fig. 2. Increases were also observed in arabinan (LM6) and galactan (LM5) side chains of RG I and in AGP (LM2). The changes could clearly be located in xylem vessel walls (Fig. 3). Among the susceptible genotypes, the de-esterification of HG was strongest in NHG3. Genotype L390 showed greatest changes in all tested cell wall components, while another susceptible genotype, Wva700, only reacted to inoculation with increases in low-esterified HG and in non-blockwise de-esterification of HG, but no changes in the side chain composition of RGI nor in AGP were observed. Among the eleven resistant genotypes, four showed increases in low-esterified HG, two in arabinan, three in galactan side chains of RGI, and two in AGP after inoculation. Genotypes CLN2123C, CLN1-5-12, CLN1-1-12, BL333, H7996, CLN4-22-4 and NHG140 did not react with changes in side chains of RGI, and among them, BL333,

Table 1
Characterization of pectic polysaccharides in xylem vessels of resistant and susceptible tomato genotypes before and after inoculation with *R. solanacearum* strain To-udk2 by immuno-tissue printing

Genotypes[a]	Reaction to *Rs*[b]	JIM5 low ester-HG		JIM7 high ester-HG		LM7 n-blockwise HG		LM5 galactan-RGI		LM6 arabinan-RGI		LM2 AGP	
		H[c]	I	H	I	H	I	H	I	H	I	H	I
CLN2123C	R	+[d]	++	+++	+++	–	–	–	–	+	+	+	(++)
CLN1-3-13	R	+	++	+++	+++	–	–	–	++	+	+	+	++
CLN1-5-12	R	+	(++)	+++	+++	–	(+)	+	+	+	+	+	+
CLN1-1-12	R	+	++	+++	+++	–	(+)	+	+	+	+	+	+
NHG162	R	+	+	+++	+++	–	–	(+)	(+)	+	++	+	+
BL333	R	+	+	++	++	–	–	+	+	+	+	+	+
H7996	R	+	++	+++	+++	–	–	+	+	+	+	+	+
NHG13	R	(++)	++	(+++)	+++	–	(+)	+	(+++)	(+)	++	+	++
CLN4-22-4	R	+	+	+++	+++	–	–	+	+	+	+	+	+
KingKong2	MR	+	+	+++	+++	–	–	–	+	+	(++)	++	++
NHG140	R	++	++	(+++)	+++	–	(+)	+	+	+	+	+	+
NHG60	R	++	++	+++	(+++)	–	–	+	(++)	+	++	++	++
L390	S	+	++	+++	++	–	++	+	++	+	++	+	++
Wva700	S	+	++	+++	+++	–	(+)	+	+	+	+	+	+
NHG167	S	(++)	+++	++	++	(+)	++	+	(++)	+	(++)	+	(+++)
NHG3	S	+	+++	++	++	–	+	+	++	+	+	+	++

Nitrocellulose membranes with imprints were probed with antibodies specific to low esterification (JIM5), high esterification (JIM7) or non-blockwise de-esterification patterns (LM7) of HG, arabinan (LM6) and galactan (LM5) side chains of RGI and arabinogalactan protein (LM2).

[a]Trial was repeated three times, table shows representative results.

[b]Reaction to *R. solanacearum*: R = resistant, MR = moderately resistant, S = susceptible according to their wilt incidence calculated as AUWiPC; resistant and moderately resistant genotypes are arranged in order of increasing latent infection in midstems, susceptible genotypes in order of increasing AUWiPC.

[c]H = healthy plants, I = *R. solanacearum*-inoculated plants at 5dpi.

[d]Color intensity was evaluated in seven categories: –, (+), +, (++), ++, (+++), ++.

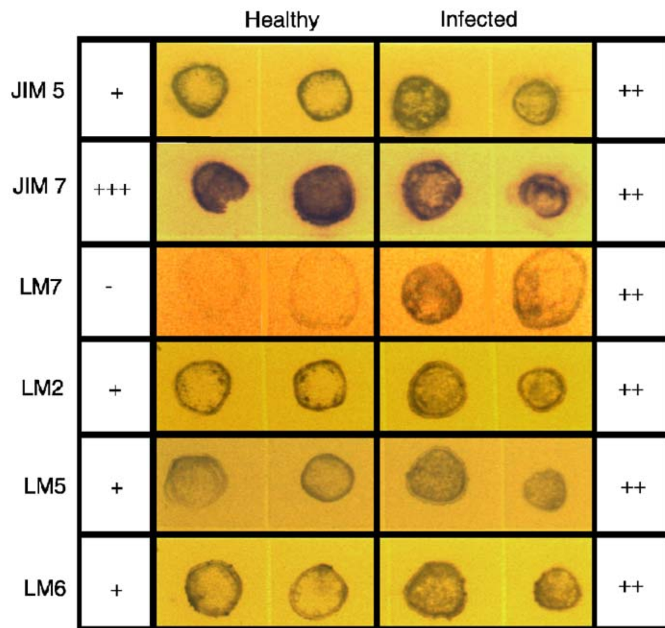

Fig. 2. Stem tissue prints of healthy and *R. solanacearum*-infected plants of the susceptible tomato genotype L390 stained for structural composition of plant cell wall polymers with antibodies JIM5 for low-esterification grade of homogalacturonan (HG), JIM7 for high esterification grade of HG, LM7 for non-blockwise methylde-esterification of HG, LM5 and LM6 specific to $(1\rightarrow4)$-β-galactan and $(1\rightarrow5)$-α-arabinan epitopes, respectively, in side chains of RGI, and LM2, specific for arabinogalactan protein (AGP). Infection caused increases in Jim5, LM7, LM2, LM5 and LM6, and decreases in JIM7, indicating a non-blockwise de-esterification of HGs, possibly by pectin methylesterase of *R. solanacearum*, and a plant reaction in form of increased branching of RGI and production of AGP in susceptible and some resistant genotypes.

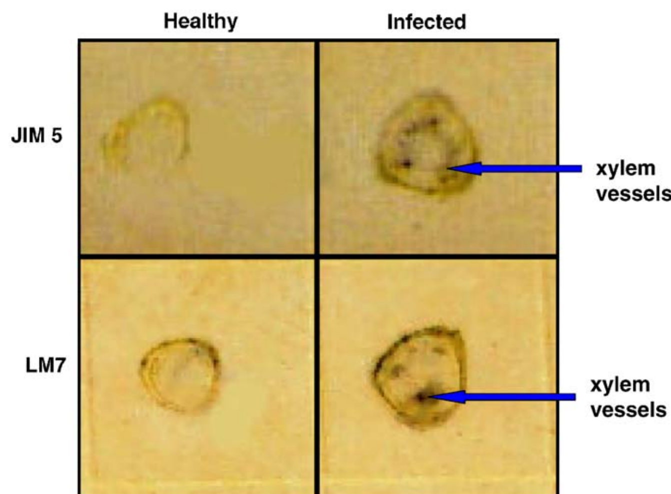

Fig. 3. Stem tissue prints of healthy and *R. solanacearum*-infected plants of the susceptible tomato genotype L390 showing degradation of homogalacturonan (HG) in xylem vessels after infection. Antibody JIM5 stains HG of low-esterification grade, and LM7 non-blockwise methylde-esterification of homogalacturonan (HG). Arrows indicate stained vessels.

CLN4-22-4, CLN1-5-12 and NHG140 showed no reaction to inoculation in all the tested cell wall components. The moderately resistant genotype King Kong2 reacted to inoculation with increased branching of RGI in form of galactan and, less, arabinan side chains, but no changes in esterification of HG occurred.

Evaluating changes in cell wall components of the resistant and susceptible genotype groups after inoculation, strong effects of inoculation and high differences in reaction between resistant and susceptible genotypes were observed in the increase in non-blockwise de-esterification pattern of HG (Fig. 4), the increase in low esterification degree of HG, and, generally, in detection of AGP in susceptible genotypes, in decreasing order of prominence, while a more intense staining for galactan side chains of RGI constituted a minor difference between the genotype groups.

Comparing latent bacterial populations in resistant genotypes and cell wall characteristics, only slight tendencies for lower bacterial numbers in stems with higher

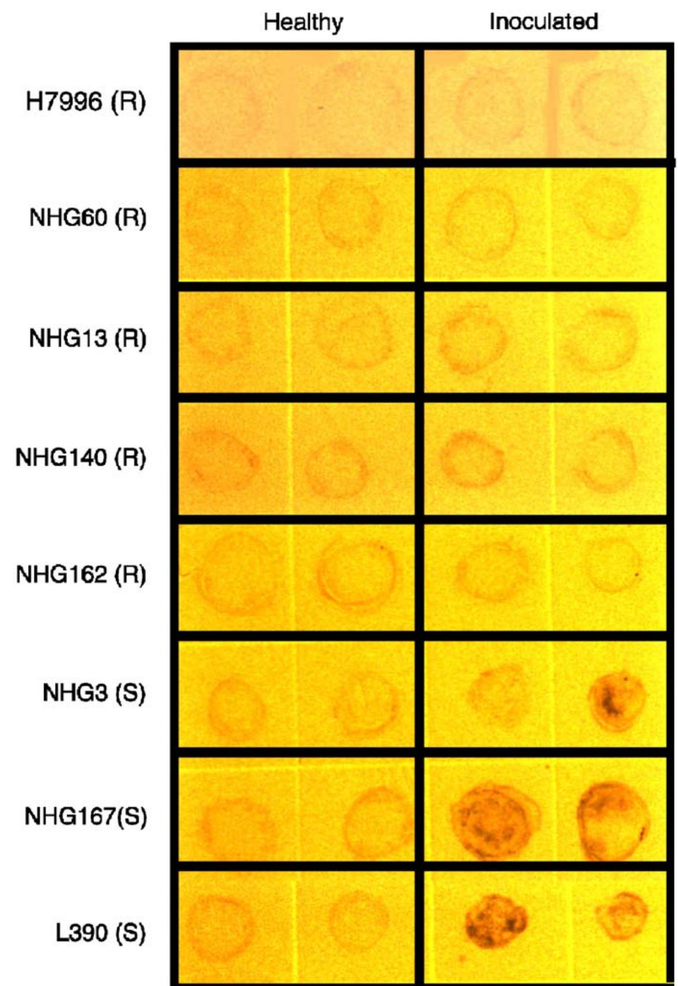

Fig. 4. Stem tissue prints of healthy and *R. solanacearum*-inoculated plants of tomato genotypes Hawaii7996, NHG60, NHG13, NHG140, NHG162 (resistant to *R. solanacearum*) and NHG3, NHG167 and L390 (susceptible) stained with antibody LM7, specific for the non-blockwise methylde-esterification of homogalacturonan (HG). Strong staining of inoculated treatments was only observed in susceptible genotypes, indicating the de-esterification of HG in a non-blockwise mode by pectin methylesterase of *R. solanacearum*.

methyl-esterification of HG and less branched RGI, but no clear relation between bacterial population and cell wall composition were observed (Fig. 1, Table 1).

4. Discussion

The 16 tomato genotypes were classified as resistant and susceptible after inoculation with *R. solanacearum* strain To-udk2. In all the resistant, completely asymptomatic genotypes relatively high populations of bacteria were detected in stems, though with significant differences among genotypes, with the lowest bacterial concentration in genotype CLN2123C. Grimault et al. [18] and Vasse et al. [52] reported a decrease of bacterial density in midstems compared to the collar, and Grimault et al. [17] correlated the bacterial population in midstems to the degree of resistance. On the other hand, an additional restriction of bacterial invasion and/or multiplication at the root level might play a role as resistance mechanism [9,52].

Several solanaceous species, such as tobacco (*Nicotiana tabacum* L.), potato (*Solanum tuberosum* L.) and also tomato are known to be symptomless carriers of the pathogen. Latent populations in some resistant groundnut cultivars were reported to affect root proliferation and tolerance to drought [62,66]. Thus, latent infection appears to be a common trait in bacterial wilt pathogenesis [67]. The different reaction types of plants to latent infections can be related to resistance levels, and, thus, genotype CLN 2123C might have valuable characteristics to be included in breeding programs. Nevertheless, genotypes have to be tested against various, differing strains of *R. solanacearum*, which was not the scope of the present study.

Analysing the cell wall structure and composition, constitutive differences were observed between susceptible and resistant RILs in the structure of HG, with an HG of higher degree of esterification in the resistant than in the susceptible genotypes. Similar observations were reported when extracted pectic polysaccharides of the healthy resistant and susceptible genotypes H7996 and L390 were biochemically analyzed, where a significantly higher degree of methyl-esterification was observed in the resistant genotype [46]. Also the immunodot-blot analysis of these extracted polysaccharides in our former studies confirmed differences in the structure of HG, with a homogeneous de-esterification pattern of HG detected in stems of genotype L390, in contrast to a more blockwise pattern in Hawaii7996, while both genotypes contained highly branched RGI with arabinose and galactose side chains. Though, these differences between the two genotypes were not equally reflected in the present immuno-tissue print analysis, which might be due to the different methodological approach. In the pectic network of the cell wall, the epitopes of HG may not always be easily accessible to the antibodies, and, thus, an extraction of polysaccharides may reveal other features than an in situ immuno-tissue print staining which can only detect the soluble fraction of those polysaccharides, which are transferred to the membrane. Our observations need to be confirmed with further resistant and susceptible RILs.

The range of pectic polysaccharides demonstrated by the presented immuno-tissue prints of stems of tomato is typical for the presence of highly branched pectins. Also in roots the chemically determined degree of methylation of extracted HGs was by a factor of six higher in polysaccharides from genotype Hawaii7996 than from L390 [46], which may contribute to the observed lower population density of *R. solanacearum* in roots of the resistant genotypes [9]. High branching and high degree of methyl-esterification, especially in a blockwise pattern, is suggested to make polysaccharides less easily degradable by pathogen enzymes. A difference in the degree of pectin methylation determined biochemically was also related to the reaction of tomato cultivars to *Pseudomonas syringae* pv. *tomato* [68], and, in potato stem tissue, the higher percentage of methylated and branched pectins has been reported to correlate with resistance against *E. carotovora* subsp. *atroseptica* [33,69].

To date little is known about the resistance mechanisms of tomato to bacterial wilt. Chellemi et al. [70] suggested that the amino and organic acids present in the xylem fluid of tomato plants may be determinants for resistance against bacterial wilt disease. But, since pathogen multiplication was equally well in the xylem sap collected from healthy Hawaii7996 and a susceptible cultivar, constitutive xylem fluid compounds seem not to play a role in resistance [71]. Also phenols and ascorbic acid present in high amount in roots and stems were suggested to be involved in resistance of tomato to *R. solanacearum* [72]. Proteins bands identified as PPO1 (polyphenol oxidase), PPO12 and PPO9 in analyzed extracts of roots of resistant cultivars, and the bands PPO2, PPO4, PPO5 and PPO7 in moderately resistant ones seemed not to be related to resistance at root level [73]. Thus, it may be speculated, that the activation of the secondary plant metabolic pathways for phenolic compounds in reaction to an infection may result in changes on plant cell wall level.

The increase in staining with all the antibodies except JIM7 in susceptible genotypes after inoculation is suggested to be at least partly due to pathogen action. Thus, increased labeling for low-esterified HG (JIM5) and the non-blockwise de-esterification of HG (LM7) indicates for the first time the possible mode of action of the cell wall degrading enzyme of *R. solanacearum* pectinmethylesterase (PME), which was shown to be involved in the pathogenesis of bacterial wilt [74]. HGs with low, non-blockwise esterification possess an increased capacity to form gels, which may act as reservoirs of water and nutrients for the bacteria, and, through a possible synergistic interaction of pathogen- and plant-derived poylsaccharides [75,76] enhance the occlusion of vessels. Thus, pectins with these properties might contribute to a fast establishment of the disease in a susceptible genotype. Further changes in the physico-chemical properties of the cell wall can be due to the increased capacity of the modified HG to form

calcium-mediated gels with distinct properties in terms of porosity and elasticity [59].

The detection of JIM7-stained epitopes to a similar level before and after inoculation does not contradict the observation of increases in low esterification detected by JIM5, since JIM7 detects a wide range of esterification grades of HG from 15% to 80%. The increase in low-esterified HG also in most of the resistant genotypes may as well indicate the action of bacterial enzymes, produced by the latent *R. solanacearum* population which is present in stems partly in high numbers.

The de-esterification of HG by PME is the prerequisite for the subsequent action of polygalacturonases PehA, PehB and PehC of *R. solanacearum*, which are not able to degrade highly methylated pectins [74]. This is the first step in breaking down the pectin-containing pit membranes, that separate adjacent xylem vessels and, thus, contributes to bacterial spread and colonization. Additionally, the enzymes lead to a release of elicitor-active oligogalacturonides, so that the plant cell may recognize pathogens indirectly via the pectin-derived oligogalacturonide fragments [77–79]. Tomato plants produce active oxygen species after elicitation with oligogalacturonides, and protease inhibitors after elicitation with dimers and trimers of galacturonic acid. Generally, also phytoalexin accumulation [80] or release of proteinase inhibitor-inducing factor [81] and formation of necrosis [82] are known as defense mechanisms elicited by oligogalacturonides. Evidence for the participation of oligogalacturonides in active plant defense was reported by Cervone et al. [83], whereas the mechanisms by which the fragments activate defense responses, remain unknown [77].

Further reactions to inoculation manifested in increases in AGP and in branching of RGI, mostly in form of side chains of galactan. These changes were preferably observed in susceptible genotypes, but also some of the resistant genotypes reacted with increases in AGP and galactan or arabinan side chains of RGI. It is suggested that these conformational changes play a role in the microscopically often observed cell wall thickening process [10,52]. Deposition of new wall material with different composition or changes in branching patterns can strengthen the wall and provide resistance to the action of pectic enzymes, making the cell wall less accessible to degradation by the pathogen. Thus, our observations provide indications that the electron-dense material of unknown biochemical nature forming deposits at cell walls and vascular coating after infection [17] may be at least partly composed of AGP and highly branched RGI. To date, only tyloses in xylem vessels into which the pathogen was observed to migrate, and the deposition of unknown electron-dense material had been described as resistance reaction in tomato xylem vessels [84]. The increases in thickness and electron density of pit membranes and the development of a more conspicuous electron-dense layer near the pits and along vessel walls might therefore be caused by changes or increased production of RGI and AGP. Also in histochemical studies on pepper, the degradation of plant cell wall material and, at the same time, deposition of new wall material was suggested to indicate a resistance mechanism in the resistant genotype [85]. Nevertheless, it cannot be ruled out, that the more intense binding of LM5 and LM6 may not be caused by an increased production or changed structure of the RGI, but be due to better epitope accessibility for the antibodies at lower and non-blockwise esterification of HG in the susceptible genotypes, and, thus, due to conformational changes in the structure of HG [59].

Though some tendencies for a relation of cell wall compositions to the degree of latent infection in resistant genotypes were observed, a clear relationship between cell wall compounds and bacterial numbers was not observed. This does not rule out that a correlation may exist, since the bacterial quantifications and the cell wall structure analysis were not performed on the same plants, and the presented data are means from several plants. Additionally, our method allowed only characterization of soluble polysaccharide fractions.

The changes in the cell wall composition in the susceptible genotypes observed after infection were generally not observed in the same intensity in the resistant genotypes. Therefore it is suggested, that *R. solanacearum* PME de-esterifies the highly esterified HG in a non-blockwise manner, making the physico-chemical properties of the cell wall more suitable to pathogen multiplication. On the other hand, de-esterification of HG with subsequent production of pectic fragments, changes in the branching pattern of RGI and possible increases in production of AGP are suggested to be involved in the resistance reaction of the plant. Histochemical methods based on immunofluorescence microscopy will reveal more detailed information on the role of these cell wall components in the interaction.

References

[1] Buddenhagen I, Kelman A. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Ann Rev Phytopathol 1964;2:203–30.

[2] Elphinstone JG. The current bacterial wilt situation: a global view. In: Allen C, Prior P, Hayward AC, editors. Bacterial wilt disease and the *Ralstonia solanacearum* species complex. St. Paul, Minnesota: APS Press; 2005. p. 9–28.

[3] Yabuuchi E, Kosako Y, Yano I, Hotta H, Nishiuchi Y. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus pseudomonas homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol Immunol 1992;36:1251–75.

[4] Yabuuchi E, Kosako Y, Yano I, Hotta H, Nishiuchi Y. Transfer of two *Burkholderia* and an *alcaligenes* species to *Ralstonia* gen. Nov.: proposal of *Ralstonia picketti* (Ralston, Palleroni and Doudoroff 1973) comb. Nov., *Ralstonia solanacearum* (Smith 1896) comb. Nov. and *Ralstonia eutropha* (Davis 1969) comb. Nov Microbiol Immunol 1995;39:897–904.

[5] Genin S, Boucher C. *Ralstonia solanacearum*: secrets of a major pathogen unveiled by analysis of its genome. Mol Plant Pathol 2002;3:111–8.

[6] Hayward AC. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Ann Rev Phytopathol 1991;29:65–87.

[7] Swanson JK, Yao J, Tans-Kersten J, Allen C. Behavior of *Ralstonia solanacearum* race 3 biovar 2 during latent and active infection of geranium. Phytopathology 2005;95:136–43.

[8] Gao M, Showalter AM. Immunolocalization of LeAGP-1, a modulator arabinogalactan protein, reveals its developmentally regulated expression in tomato. Planta 2000;210:865–74.

[9] Dannon E, Wydra K. Interaction between silicon amendment, bacterial wilt development and phenotype of *Ralstonia solanacearum* in tomato genotypes. Physiol Mol Plant Pathol 2004;64:233–43.

[10] Denny TP. Plant pathogenic *Ralstonia* species. In: Gnanamanickam SS, editor. Plant associated bacteria. Netherlands: Springer; 2006: p. 573–644.

[11] Hanson P, Wang JF, Liardo O, Hanudin Mah SY, Hartman GL, Lin YC, et al. Variable reaction of tomato lines to bacterial wilt evaluated at several locations in Southeast Asia. Hort Sci 1996;31:143–6.

[12] Hayward AC. *Ralstonia solanacearum*. In: Lederberg J, editor. Encyclopedia of microbiology. vol. 4. San Diego, California: Academic Press; 2000. p. 32–42.

[13] Cook D, Barlow E, Sequeira L. Genetic diversity of *Pseudomonas solanacearum*: detection of restriction fragment length polymorphisms with DNA probes that specify virulence and the hypersensitive response. Mol Plant Microbe Interact 1989;2:113–21.

[14] Elphinstone JG, Hennessy J, Wilson JK, Stead DE. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. Bull OEPP/EPPO 1996;26:663–78.

[15] Parlevliet J. Components of resistance that reduce the rate of epidemic development. Ann Rev Phytopathol 1979;17:203–22.

[16] Grimault V, Prior P. Bacterial wilt resistance in tomato associated with tolerance of vascular tissues to *Pseudomonas solanacearum*. Plant Pathol 1993;42:589–94.

[17] Grimault V, Prior P. Invasiveness of *Pseudomonas solanacearum* in tomato, eggplant and pepper: a comparative study. Eur J Plant Pathol 1994;100:259–67.

[18] Grimault V, Prior P, Anais G. A monogenic dominant resistance of tomato to bacterial wilt in Hawaii7996 is associated with plant colonization by *Pseudomonas solanacearum*. J Phytopathol 1995;143: 349–52.

[19] Prior P, Bart S, Darrasse A, Anais G. Resistance to bacterial wilt in tomato as discerned by spread of *Pseudomonas* (*Burkholderia) solanacearum* in the stem tissues. Plant Pathol 1996;4:720–6.

[20] Lindhout P, Bai Y, Bonnema G, Van der Hulst R. QTLs for tomato powdery mildew resistance (*Oidium lycopersici*) in *Lycopersicon parviflorum* G1.1601 co-localize with two qualitative powdery mildew resistance genes. Mol Plant Microbe Interact 2003;16:169–76.

[21] Mangin B, Olivier J, Thoquet P, Grimsley H. Temporal and multiple qantitative trait loci analyses of resistance to bacterial wilt in tomato permit the resolution of linked loci. Genetics 1999;151:1165–72.

[22] Jaunet TX, Wang JF. Variation in genotype and aggressiveness of *Ralstonia solanacearum* race 1 isolated from tomato in Taiwan. Phytopathology 1999;89:320–7.

[23] Baker B, Zambuyski P, Staskawicz B, Dineshkumar SP. Signaling in plant microbe interactions. Science 1997;276:726–33.

[24] Keen N. Mechanisms of pest resistance in plants. In: Traynor PL, Westwood JH, editors, Proceedings of a workshop: ecological effects of pest resistance genes in managed ecosystems, Bethesda, Maryland, January 31–February 3 1999. Organized by Information System for Biotechnology 〈http://www.isb.vt.edu/proceedings99/proceedings.keen.html〉

[25] Knogge W. Elicitors and suppressors of the resistance response. In: Hartleb H, Heitefuß R, Hoppe HH, editors. Resistance of crop plants against fungi. Jena, Germany: Gustav Fischer; 1997. p. 159–82.

[26] Schlösser E. Preformed structural and chemical barriers. In: Hartleb H, Heitefuss R, Hoppe HH, editors. Resistance of crop plants against fungi. Jena, Germany: Gustav Fischer; 1997. p. 84–99.

[27] Bostock RM. Signal crosstalk and induced resistance: straddling the line between cost and benefit. Ann Rev Phytopathol 2005;43:545–80.

[28] Ferguson J. Calcium in plant senescence and fruit ripening. Plant Cell Environ 1984;7:477–89.

[29] Sticher L, Mauch-Mani B, Metraux JP. Systemic acquired resistance. Ann Rev Phytopathol 1997;35:235–70.

[30] Kim J, Carpita N. Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. Plant Physiol 1992;98:646–53.

[31] McCann M, Shi J, Roberts K, Carpita N. Changes in pectin structure and localization during the growth of unadapted and NaCl- adapted tobacco cells. Plant J 1994;5:773–85.

[32] Talbott L, Ray P. Changes in molecular size of previously deposited and newly synthesized pea cell wall matrix polysaccharides. Effects of auxin and turgor. Plant Physiol 1992;98:369–79.

[33] McMillan GP, Hedley D, Fyffe L, Perombelon MCM. Potato resistance to soft-rot erwinias is related to cell wall pectin esterification. Physiol Mol Plant Pathol 1993;42:279–89.

[34] Huxham I, Jarvis M, Dover C, Johnson D, Knox J. Electron-energy-loss spectroscopic imaging of calcium and nitrogen in the cell walls of apple fruits. Planta 1999;208:438–43.

[35] Moerschbacher BM, Wietholter N, Graessner B, Mierau M, Mort AJ. Differences in the methyl ester distribution of homogalacturonans from near-isogenic wheat lines resistant and susceptible to the wheat stem rust fungus. Mol Plant Microbe Interact 2003;16: 945–52.

[36] Samaj J, Ensikat S, Baluska F, Knox J, Barthlott W, Volkmann D. Immunogold localization of plant surface arabinogalactan-proteins using glycerol liquid substitution and scanning electron microscopy. J Micro Sci Oxf 1999;193:150–7.

[37] Bacic A, Moody SF, Clarke AE. Structural analysis of secreted root slime from maize (*Zea mays* L.). Plant Physiol 1986;80: 771–7.

[38] Fincher GB, Stone BA, Clarke AE. Arabinogalactan proteins: structure, biosynthesis and function. Ann Rev Plant Physiol 1983;34: 47–70.

[39] Pennell RI, Knox JP, Scofield GN, Selvendran RR, Roberts K. A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. J Cell Biol 1989;108:1967–77.

[40] Ding L, Zhu JK. A role of arabinogalactan proteins in root epidermal cell expansion. Planta 1997;203:289–94.

[41] Showalter AM. Arabinogalactan-proteins: structure, expression and function. Cell Mol Life Sci 2001;58:1399–417.

[42] Knox JP, Willats WGT, Clausen MH. Synthetic methyl hexagalacturonate hapten inhibitors of anti-homogalacturonan antibodies LM7, JIM5 and JIM7. Carbohydr Res 2003;338:1797–800.

[43] Van Loon LC, Bakker PAHM, Pieterse CMJ. Systemic resistance induced by rhizosphere bacteria. Ann Rev Phytopathol 1998;36: 453–83.

[44] Wojtaszek P. Oxidative burst: an early plant response to pathogen infection. Biochem J 1997;322:681–92.

[45] Robb J, Powell DA, Street PFS. Vascular coating: a barrier to colonization by the pathogen in *Verticillium* wilt of tomato. Can J Bot 1989;67:600–7.

[46] Wydra K, Beri H, Schacht T. Polygalacturonase-inhibiting protein (PGIP) and structure and composition of cell wall polysaccharides of tomato in relation to resistance to *Ralstonia solanacearum*. In: Gananamanickam S, editor. Emerging trends in plant microbe interactions. India: Centre for Advanced Studies in Botany, University of Madras; 2005. p. 217–23.

[47] Round AN, Ring GS, Morris VJ. Unexpected branching in pectin observed by atomic force microscopy. Carbohydr Res 1997;303: 251–3.

[48] Vasse J, Frey P, Trigalet A. Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum*. Mol Plant Microbe Interact 1995;8:241–51.

[49] Nakaho K, Hibino H, Miyagawa H. Possible mechanisms limiting movement of *Ralstonia solanacearum* in resistant tomato tissues. J Phytopathol 2000;148:181–90.

[51] Wallis FM, Truter SJ. Histopathology of tomato plants infected with *Pseudomonas solanacearum*, with emphasis on ultrastructure. Physiol Plant Pathol 1978;13:307–17.

[52] Vasse J, Danoun S, Trigalet A. Microscopic studies of root infection in resistant tomato cultivar Hawaii7996. In: Allen C, Prior P, Hayward AC, editors. Bacterial wilt disease and the *Ralstonia solanacearum* species complex. St. Paul, Minnesota: APS Press; 2005. p. 285–91.

[53] Freshour G, Clay R, Albersheim P, Hahn M. Developmental and tissue-specific structural alterations of the cell wall polysaccharides of *Arabidopsis thaliana* roots. Plant Physiol 1996;110:1413–29.

[54] Knox JP. Intriguing, complex and everywhere: getting to grips with arabinogalactan proteins. Trends Plant Sci 1999;4:123–5.

[55] Knox JP, McCartney L. Regulation of pectic polysaccharide domains in relation to cell development and cell properties in the pea testa. J Exp Bot 2002;53:707–13.

[56] McCabe P, Valentine T, Pennell R. Soluble signals from cells identified at the cell wall established a developmental pathway in carrot. Plant Cell 1997;9:2225–41.

[57] Willats WGT, Gilmartin PM, Mikkelsen JD, Knox JP. Cell wall antibodies without immunization: generation and use of de-esterified homogalacturonan block-specific antibodies from a naïve phage display library. Plant J 1999;18:57–65.

[58] Willats WGT, McCartney L, Knox JP. Pectin cell biology: complexity in context. In: Visser J, Voragen AGJ, editors. Advances in pectins and pectinases research. Netherlands: Kluwer Academic Publishers; 2003. p. 147–57.

[59] Willats WGT, Orfila C, Limberg C, Buchholt HC, Van Alebeek GJWM, Voragen AGJ, et al. Modulation of the degree and pattern of methyl esterification of pectic homogalacturonan in plant cell walls. J Biol Chem 2001;276:19404–13.

[60] Jeger MJ, Viljanen-Rollinson SLH. The use of the area under the disease-progress curve (AUDPC) to assess quantitative disease resistance in crop cultivars. Theor Appl Genet 2001;102:32–40.

[61] Shaner G, Finney RE. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 1977;67:1051–6.

[62] Li WR, Jan YJ. Inoculation techniques for groundnut bacterial wilt. Oil Crops China 1984;2:77–81.

[63] Leykun Z. Latent infection of 'resistant' tomato genotypes with *Ralstonia solanacearum* and the viable but non-culturable state of the pathogen in tomato tissue. MSc thesis, University of Hannover, 2003.

[64] Jones L, Seymour GB, Knox JP. Localization of pectic galactan using a monoclonal antibody specific for to $(1\rightarrow4)$-β-D-galactan. Plant Physiol 1997;113:1405–12.

[65] McCartney L, Ormerod AP, Gidley MJ, Knox JP. Temporal and spatial regulation of pectic $(1\rightarrow4)$-β-D-galactan in cell walls of developing pea cotyledons: implications for mechanical properties. Plant J 2000;22:105–13.

[66] Liao BS, Shan ZH, Duan NX, Lei YJ, Tan Y, Li D, et al. Relationship between latent infection and groundnut bacterial wilt resistance. In: Prior Ph, Allen C, Elphinstone J, editors. Bacterial wilt disease. Molecular and ecological aspects. Reports of the Second International Bacterial Symposium held in Gossier, Guadeloupe, France, 22–27 Jun 1997. Berlin: Springer; 1998. p. 89–98.

[67] Prior P, Grimault V, Schmitt J. Resistance to bacterial wilt (*Pseudomonas solanacearum*) in tomato: recent status and prospects. In: Hayward AC, Hartman GL, editors. Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. Wallingford: CAB International; 1994. p. 209–23.

[68] Venkatesh B. Characterization of bacterial lipopolysaccharides (*Pseudomonas syringae* pv. *tomato* and *Pseudomonas syringae* pv. *apii*) and pectins of tomato and celery plants (*Lycopersicon esculentum* and *Apium graveolens*) regarding their possible role in host/pathogen interaction. PhD thesis, University of Göttingen, Germany, 2002. 〈http://webdoc.sub.gwdg.de/diss/2002/venkatesh/venkatesh.pdf〉

[69] Marty P, Jouan B, Bertheau Y, Vian B, Goldberg R. Charge density in stem cell walls of S*olanum tuberosum* genotypes and susceptibilty to blackleg. Phytochemistry 1997;44:1435–41.

[70] Chellemi DO, Anderson PC, Brodbeck B, Dankers W, Rhoads FM. Correlation of chemical profiles of xylem fluid of tomato to resistance to bacterial wilt. In: Prior Ph, Allen C, Elphinstone J, editors. Bacterial wilt disease. Molecular and ecological aspects. Reports of the Second International Bacterial Symposium held in Gossier, Guadeloupe, France, 22–27 Jun 1997. Berlin: Springer; 1998. p. 225–32.

[71] McGarvey JA, Schell MA, Denny TP. Spatial-temporal and quantitative analysis of growth and EPS I production by *Ralstonia solanacearum* in resistant and susceptible tomato cultivars. Phytopathology 1999;89:1233–9.

[72] Kumar PGS, Rajan S, Peter KV. Role of phenols and ascorbic acid in imparting rersistance to bacterial wilt in tomato. In: Third International Bacterial Wilt Symposium, February 4–8, 2002, abstract 〈http://ibws.nexenservices.com/RSA%20Programm/Thursday_poster.htm〉

[73] Rajan S, Bose A. A biochemical marker for screening of bacterial wilt resistance in tomato. In: Third International Bacterial wilt Symposium, February 4–8, 2002, abstract 〈http://ibws.nexenservices.com/RSA%20Programm/Thursday_poster.htm〉

[74] Allen C, Tans-Kersten J, Yanfeen G. *Ralstonia solanacearum* pectin methylesterase is required for growth on methylated pectin but not for bacterial wilt virulence. Appl Environ Microbiol 1998;64:4918–23.

[75] Rudolph K. Thirty-five years of phytobacteriology research with special emphasis on pathogenicity of *Pseudomonas syingae*. In: De Boer, editor. Plant pathogenic bacteria. Dordrecht: Kluwer Academic Publishers; 2001. p. 109–17.

[76] Wydra K, Rudolph K. A model for mechanisms of resistance and susceptibility on a molecular level in plant–microbe-interactions. In: Lemattre M, Freigoun S, Rudolph K, Swings JG, editors. Plant pathogenic bacteria. Paris: INRA; 1994. p. 653–8.

[77] D'Ovidio R, Raiola A, Capodicasa C, Devoto A, Pontiggia D, Roberti S, et al. Characterization of the complex locus of bean encoding polygalacturonase-inhibiting proteins reveals subfunctionalization for defence against fungi and insects. Plant Physiol 2004;135:1–12.

[78] Esquerre-Tugaye MT, Boudart G, Dumas B. Cell wall degrading enzymes, inhibitory proteins, and oligosaccharides participate in the molecular dialogue between plants and pathogens. Plant Physiol Biochem 2000;38:157–63.

[79] Ridley BL, O'Neill MA, Mohnen D. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Photochemistry 2001;57:929–67.

[80] Davis KR, Lyon GD, Darvill AG, Albersheim P. Host–pathogen interactions XXV. Endopolygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. Plant Physiol 1984;74:52–60.

[81] Bishop PD, Makus JD, Pearce G, Ryan AC. Proteinase inhibitor-inducing factor activity in tomato leaves resides in oligosaccharides enzymically released from cell walls. PNAS 1981;78:3536–40.

[82] Cervone F, De Lorenzo G, Degrà L, Salvi G. Elicitation of necrosis in *Vigna unguiculata* Walp. by homogeneous *Aspergillus niger* endo-polygalacturonase and by α-D-galacturonate oligomers. Plant Physiol 1987;85:626–30.

[83] Cervone F, Hahn MG, De Lorenzo G, Darvill A, Albersheim P. Host pathogen interactions XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol 1989;90:542–8.

[84] Grimault V, Prior P, Schmit J, Gelei B. Comparative histology of resistant and susceptible tomato cultivars infected by *Pseudomonas solanacearum*. Physiol Mol Plant Pathol 1994;4:105–23.

[85] Rahman MA, Abdullah H, Vanhaecke M. Histopathology of susceptible and resistant *Capsicum annuum* cultivars infected with *Ralstonia solanacearum*. J Phytopathol 1999;147:129–40.

[86] Kelman A. The relationship of pathogenicity in Pseudomonas solanacearum to colony appearance on tetrazolium medium. Phytopathology 1954;44:693–5.