Dissection of Bacterial Wilt on *Medicago truncatula* Revealed Two Type III Secretion System Effectors Acting on Root Infection Process and Disease Development

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[R. solanacearum](https://www.plantphysiol.org/doi/10.1104/pp.109.141523) is the causal agent of the devastating bacterial wilt disease, which colonizes susceptible *Medicago truncatula* via the intact root tip. Infection involves four steps: appearance of root tip symptoms, root tip cortical cell invasion, vessel colonization, and foliar wilting. We examined this pathosystem by in vitro inoculation of intact roots of susceptible or resistant *M. truncatula* with the pathogenic strain GMI1000. The infection process was type III secretion system dependent and required two type III effectors, Gala7 and AvrA, which were shown to be involved at different stages of infection. Both effectors were involved in development of root tip symptoms, and Gala7 was the main determinant for bacterial invasion of cortical cells. Vessel invasion depended on the host genetic background and was never observed in the resistant line. The invasion of the root tip vasculature in the susceptible line caused foliar wilting. The *avrA* mutant showed reduced aggressiveness in all steps of the infection process, suggesting a global role in *R. solanacearum* pathogenicity. The roles of these two effectors in subsequent stages were studied using an assay that bypassed the penetration step; with this assay, the *avrA* mutant showed no effect compared with the GMI1000 strain, indicating that AvrA is important in early stages of infection. However, later disease symptoms were reduced in the *gala7* mutant, indicating a key role in later stages of infection.

Very few phytopathogenic bacteria colonize intact plant roots, even if the local soil provides a favorable environment for bacterial growth. Penetration seems to be the limiting step, as most bacteria need natural openings or wounds to invade their hosts and there are few opportunities in roots (Loria et al., 2003). *Ralstonia solanacearum*, a gram-negative soil-borne pathogen responsible for bacterial wilt, naturally invades plants through axils of secondary roots. *R. solanacearum* has to attach to the root, find nutrients, multiply, migrate into plant tissues, and penetrate the xylem in order to cause disease (Vasse, 1995). It has a wide and still-expanding host range that includes several hundred susceptible species in at least 50 different plant families, which include economically important hosts such as potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), peanut (*Arachis hypogaea*), pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), and tobacco (*Nicotiana tabacum*; Hayward, 1991). The high incidence of plant mortality and the lack of effective control methods make *R. solanacearum* one of the world’s most destructive plant pathogens (Prior et al., 1998). This bacterium is a model organism extensively studied by genetic, molecular, and biochemical techniques in order to develop strategies for the control of bacterial wilt.

*R. solanacearum* virulence responds to several environmental stimuli and involves the production of multiple virulence factors (Schell, 2000; Genin and Boucher, 2002). For example, Exopolysaccharide I (EPS I), a large nitrogen-rich acidic exopolysaccharide (Orgambide et al., 1991), is thought to be an important virulence factor. Produced in large amounts by the bacteria, EPS I enhances the speed and extent of stem colonization (Saile et al., 1997) and is presumed to cause wilting by restricting water flow through xylem vessels (Garg et al., 2000). In addition to EPS I, *R. solanacearum* secretes plant cell wall-degrading enzymes through the type II secretion system (T2SS). Pectinolytic enzymes fragment pectin into oligomers, which act as substrates for the bacterial growth (Tans-Kersten et al., 1998); the breakdown of pectin...
encompass virulence by facilitating movements through pectin-rich regions such as vascular bundles (Gonzalez and Allen, 2003). Cellulytic enzymes also facilitate invasion of roots and/or penetration of xylem vessels by degrading cellulosic glucans in the cell wall (Liu et al., 2005).

Like many other pathogenic bacteria, R. solanacearum possesses a type III secretion system (T3SS), encoded by the hypersensitive response and pathogenicity (hrp) genes. In contrast to T2SS, which secretes degrading enzymes, the T3SS allows the injection of effector proteins directly into the plant cell through the hrp pili. The T3SS is essential for pathogenicity, since R. solanacearum hrp mutants cannot induce disease in many susceptible plants such as tobacco, tomato, petunia (Petunia hybrida), and Medicago (Vasse et al., 2000; Kanda et al., 2003; Zolobowska and Van Gijsegem, 2006; Vailleau et al., 2007). All effectors are presumed to facilitate virulence, but on some host cultivars they can be recognized by a cognate resistance protein, thereby triggering host defenses (Stavrinides et al., 2008). Thus, when the cognate resistance protein is present in the host plant, the effectors act as avirulence factors. This recognition response is associated with effector-triggered immunity, which results in a fast and intense plant defense response known as the hypersensitive response (HR).

The HR is characterized by localized programmed cell death that helps to limit the infection to the area of initial contact (Jones and Dangl, 2006). In R. solanacearum strain GMI1000, 74 T3SS effectors (T3Es) have been identified (Poueymiro and Genin, 2009). To date, three of them have been reported to act as avirulence factors: AevA is responsible for eliciting an HR in tobacco (Carney and Denny, 1990; Poueymiro et al., 2009); PopP1 acts as a host-specificity determinant toward tobacco (Poueymiro et al., 2009) and petunia (Lavie et al., 2002); and PopP2 interacts with the Arabidopsis (Arabidopsis thaliana) RESISTANT TO RALSTONIA SOLANACEARUM1-R resistance protein (Deslandes et al., 2003).

Some T3Es are virulence factors: they promote pathogenic functions of the bacteria and disrupt defense responses of the host immune system. Several mechanisms by which T3Es suppress plant defense have been described, including the inhibition of plant signaling components and the alteration of host transcription or posttranscriptional processes. Some T3Es are proteases that can degrade defense components through the host ubiquitination machinery and the 26S proteasome (Block et al., 2008). To date, only a few effectors from R. solanacearum have been shown to have pathogenicity functions. Gala T3Es are the best characterized; they have an F-box and a Leu-rich repeat domain and interact with ASK (for Arabidopsis SKP1-like) proteins in Arabidopsis (Angot et al., 2006). F-box and ASK proteins are part of ubiquitin E3 ligase complexes that attach ubiquitin to specific proteins, which are subsequently degraded by the 26S proteasome.

This work aims to study the sequence of events related to the T3SS-dependent infection process of R. solanacearum on intact plant roots. Many previous studies of plant-R. solanacearum interaction involved bacteria delivered directly into the vascular system through artificially wounded roots, in order to increase penetration potential (Deslandes et al., 1998; Vailleau et al., 2007). However, these inoculation procedures bypassed normal infection routes and potential resistance mechanisms. This study focuses on the early steps of the R. solanacearum infection process on intact roots. In previous work, the R. solanacearum strain GMI1000 was shown to infect the model legume plant Medicago truncatula (Vailleau et al., 2007) in an in vitro wounded root system, and susceptible (Jemalong A17) and resistant (F83005.5) lines were identified. In this work, the in vitro inoculation procedure was adapted for intact M. truncatula roots, thereby enabling us to study the sequence of events related to the T3SS-dependent infection process. Screening of loss-of-function T3E mutants in the GMI1000 strain on intact roots of the susceptible M. truncatula line led to the identification of two virulence effectors, Gala7 and AvrA. We investigated the role of these effectors in various stages of the infection process.

RESULTS

The Interaction between R. solanacearum and M. truncatula Leads to T3SS-Dependent Root Symptoms

In order to study the infection process of R. solanacearum in M. truncatula, we used an in vitro inoculation procedure of intact roots adapted from Vailleau et al. (2007). It enabled us to observe root symptoms resulting from the interaction between R. solanacearum GMI1000 strain and either susceptible (A17) or resistant (F83005.5) lines of M. truncatula. The first root symptoms observed, in both susceptible and resistant lines, were an arrest of root hair elongation 18 h post inoculation (hpi) and a substantial reduction in root growth (Student’s t test, \( P = 2e-16 \)), with no growth occurring after 24 hpi.

Viability of the epidermis and root hairs was monitored by fluorescein diacetate (FDA), which stains living cells (Fig. 1, A, D, and G). The epidermis of the mock inoculation control was viable, as evidenced by green fluorescence. Also, root hair initiation and elongation occurred (Fig. 1, A–C). After inoculation with GMI1000, a loss of fluorescence was first observed at 24 hpi and was complete at 72 hpi, indicating a loss of viability of the root tip epidermis (the root tip was considered the first 2–3 mm of the root; Fig. 1G). In addition, no root hair initiation or elongation was detected, and root tip browning and swelling were observed (Fig. 1, H and I). Root growth arrest, root tip swelling, browning, and epidermis loss of viability will be subsequently referred to “root symptoms.”

Despite the similarity in the root symptoms between resistant and susceptible lines, foliar symptoms were only observed in the susceptible line (A17). The chlo-
rosis and wilting of leaves and cotyledons led to the death of an average of 50% of A17 plants when the experiment was terminated 28 d post inoculation (dpi).

Infection with *R. solanacearum* strains carrying mutations in the regulatory *hrpB* gene (GMI1525) or in the structural T3SS gene *hrcS* (GMI1402) resulted in the absence of root growth arrest at 24 hpi, an intact root tip epidermis at 72 hpi, no swelling and browning at 7 dpi (Fig. 1, D–F), and no wilting of the shoots up to 28 dpi. Root and foliar symptoms, therefore, were dependent on a functional T3SS. Bacterial mutants defective in EPS or T2SS biosynthesis were altered in their ability to induce foliar symptoms but induced root symptoms similar to those caused by GMI1000 (data not shown). Therefore, root symptoms were specifically dependent on T3SS.

**The Infection of M. truncatula by R. solanacearum Strain GMI1000 Involves Two Distinct Root Colonization Steps**

The bacterial distribution and the internal structure of the root tip were observed at 3 and 7 dpi on the susceptible line using semithin resin sections (Fig. 2). After mock inoculation, the root tip was typically organized (Fig. 2A; Groot et al., 2004). At 3 dpi with GMI1000, bacteria adhered to the epidermis surface (Fig. 2C) and started to colonize the inner root tip, where they were detected in cortical cells and extracellular spaces (Fig. 2D). The root tip (the first 2–3 mm of the root) was the only penetration site detected, with no preferential point of entry in that area. Colonization through axes of lateral roots was never observed. Concomitantly, the root tip structure was altered: the epidermal layer exhibited severe cell damage (as illustrated by the FDA test), enlarged cortical cells were highly vacuolated, supernumerary vessels were differentiated, and the disorganized meristic zone showed unconventional vacuolated cells (Fig. 2). At 7 dpi, the cortex was heavily colonized, showing enlarged cells containing numerous bacteria. *R. solanacearum* started to penetrate and proliferate in the vessels of the susceptible line (Fig. 2, E and F) but not in the resistant line.

In order to quantify the differential bacteria colonization, infections were conducted using a *R. solanacearum* strain transformed with a constitutive GUS construct (GMI1559; Etchebar et al., 1998). The GUS activity was revealed in planta, and the observations were performed with a stereomicroscope. At 14 dpi, bacteria had crossed the epidermis and colonized the cortical root tip cells of 45% of the F83005.5 plants and 85% of the A17 plants. Bacteria were never observed in the vessels of F83005.5, whereas 55% of the A17 plants had their vessels colonized at 14 dpi.

Thus, two stages in the infection process were clearly identified. The first one, common to the susceptible and the resistant lines, was the bacterial colonization of the root tip cortical cells. Interestingly, a restriction of bacterial propagation was found in the resistant line F83005.5, indicating the possible onset of resistance. The second stage, specific to the susceptible line, was the colonization of the vascular bundles, leading to plant death.

**The Resistant F83005.5 Line Showed a Higher Cell Wall Autofluorescence and Earlier Endodermis Lignification Than the Susceptible Line A17 after Inoculation with R. solanacearum GMI1000**

To identify potential cell wall modifications in response to infection, cytological studies were performed on transverse root tip sections of the susceptible and resistant lines after pathogen infection.
Autofluorescence of the inner root tip and callose, suberin, and lignin deposits were observed 3 and 7 dpi in both A17 and F83005.5 lines after GMI1000 (Fig. 3) or mock (data not shown) inoculation. Autofluorescence of the cell wall of the cortical cells was observed only after inoculation (Fig. 3). Sections with (Fig. 3A) or without (Fig. 3B) cell wall autofluorescence were observed in both resistant and susceptible roots; however, at 7 dpi, roots of the resistant line showed cell wall autofluorescence at a significantly higher proportion than was observed in the susceptible line (frequency test, $P = 0.027$).

Callose and suberin accumulations were observed equally in susceptible and resistant lines (data not shown). Lignin deposits were observed in the endodermis of the two lines only after inoculation with the GMI1000 strain (Fig. 3). Sections with (Fig. 3C) or without (Fig. 3D) cell wall autofluorescence were observed in both resistant and susceptible roots; however, at 7 dpi, roots of the resistant line showed cell wall autofluorescence at a significantly higher proportion than was observed in the susceptible line (frequency test, $P = 0.027$).

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Root Symptom Development in the Susceptible A17 Line Depends on Two T3Es

As seen previously, root symptoms were specifically dependent on the presence of the T3SS. To identify bacterial molecules involved in the development of these root symptoms in the susceptible line, we used FDA staining to screen a collection of 67 loss-of-function T3E mutants in the GMI1000 background (Supplemental Fig. S1; Poueymiro et al., 2009). Because the analysis of root tip epidermis viability revealed intermediate phenotypes, we established a scale, ranking from a score of 0 (intact root tip epidermis after mock or T3SS-defective control inoculation) to 4 (complete loss of viability of the root tip epidermis as observed after inoculation with GMI1000). The screening revealed two mutants that were partially impaired in their capacity to induce epidermal cell death, gala7 and avrA. Inoculation of A17 with gala7 and avrA mutants led to median scores of 3 and 3.5, respectively (Fig. 4). Root growth arrest at 24 hpi and root tip swelling were reduced after infection with the two mutant strains compared with GMI1000 but were more pronounced than after mock or T3SS-defective control inoculation.

In order to uncover a potential additive effect of the two effectors on the root symptom, an avrA and gala7 double mutant strain was generated. The FDA test...
revealed a median score of 2, showing an additive effect on epidermal cell death (Wilcoxon rank sum test, \(P = 2.10^{-2}\); Fig. 4). Root growth measurements showed high variability, and no additive effect could be demonstrated.

Among the effector mutants showing a minor effect (Supplemental Fig. S1), two of them were selected: GRS118 (GMI1000 \(\Delta\)RSp0846-RSp0847) and GRS230 (GMI1000 \(\Delta\)RSp0842; Cunnac et al., 2004). Two triple mutants were constructed with \(avrA\) and \(gala7\). They were both assayed with the FDA test, and no significant difference could be observed with the double mutant. Thus, in our conditions and from among this group of effectors, \(avrA\) and \(gala7\) seem to be the main determinants of root symptom development. However, it is likely that other effectors have lesser individual effects.

**Gala7 and AvrA T3Es Have Distinct Roles in the Two-Step Root Colonization Process of Susceptible \(M.\ truncatula\)**

After observing the involvement of \(avrA\) and Gala7 in root symptom development after \(R.\ solanacearum\) inoculation, we characterized their roles in root bacterial colonization and the development of foliar symptoms. At 14 dpi, the gala7 mutant was never detected in the root tip cortex and therefore did not colonize the vascular system (Fig. 5), nor did it induce wilting of the A17 plant (Fig. 6). This indicated that Gala7 was necessary for the bacterial penetration into the root of the susceptible \(M.\ truncatula\) line.

At 14 dpi, the \(avrA\) mutant strain was found in the root tip cortex of 18% of the plants and in the vascular system of 4% of the plants, in comparison with GMI1000, which was found in the root tip cortex of 85% of inoculated plants and in the vascular system of 55% (Fig. 5). The plant wilting rate was also 5 times lower following inoculation with \(avrA\) compared with GMI1000 (Fig. 6). At 28 dpi, unwilted plants did not show any sign of infection. Therefore, the \(avrA\) mutant was not delayed in the induction of wilting symptoms but showed a reduced aggressiveness in comparison with GMI1000 strain. All wilted plants inoculated with the \(avrA\) mutant showed root growth arrest similar to that found after GMI1000 inoculation. Therefore, AvrA appears to be not essential for the root penetration of the susceptible \(M.\ truncatula\) line, but it does play a positive role in this process.

In conclusion, the effectors Gala7 and AvrA are involved in the appearance of root symptoms and also play roles in root colonization and disease development. Gala7 was necessary for bacterial penetration of

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**Figure 3.** Autofluorescence and lignin deposition during infection. Transverse root tip section after inoculation with GMI1000. A and C, Natural autofluorescence observed under UV light at 7 dpi. A, Root showing no cell wall autofluorescence. C, Root revealing cortical cell wall autofluorescence (arrow). B and D, Lignin deposits are highlighted in purple after phloroglucinol staining. B, Root showing no endodermis lignification. D, Root with endodermis lignification (arrow). Bars = 70 mm. E, Percentage of roots of A17 (susceptible cultivar) or F83005.5 (resistant cultivar), for which at least one section showed autofluorescence or lignification at different time points after infection. [See online article for color version of this figure.]

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**Figure 4.** Mutations in two bacterial effectors alter the development of root symptoms. Box plot of root tip epidermal viability scored 3 d after inoculation with GMI1000 or the indicated effector mutants. Score 0 was attributed to intact epidermis, as seen with mock or T3SS-defective mutant controls, and score 4 corresponded to a fully altered epidermis observed with GMI1000. Three biological repeats included 10 individual plants per repeat. The median is indicated by a dark vertical line, and the gray box encompasses 1 SD. Dashed lines flanked by thin vertical lines indicate the distribution of all data points. An outlier is indicated as a small circle. [See online article for color version of this figure.]
root cells, whereas AvrA was partially required for root colonization.

**AvrA and Gala7 Are Differentially Involved in Bacterial Propagation in Vessels and Wilting Symptom Development**

After showing the involvement of the two effectors in root penetration, it was interesting to assay their role in the subsequent steps of vascular propagation and wilting development. Therefore, we used an inoculation procedure that bypassed root penetration using root tips that were cut just before the bacterial inoculation (Vailleau et al., 2007). Using this procedure, 100% death of susceptible plants was observed at 14 dpi with either GMI1000 or the \( \text{avrA} \) mutant. Only 40% of plants died after inoculation with the \( \text{gala7} \) mutant, and a similar death rate was observed with the double mutant strain, which showed 30% of wilted plants (Fig. 7). This suggested that AvrA is not necessary for bacterial propagation in the vessels and wilting development, whereas Gala7 is involved in those steps.

**DISCUSSION**

In order to study the root infection process of the devastating pathogen *R. solanacearum*, we developed an in vitro inoculation procedure using intact *M. truncatula* roots. The root tip was identified as the key site for T3SS-dependent root symptom development and root penetration of *R. solanacearum*. The pathosystem consisted of susceptible and resistant *M. truncatula* lines infected with the *R. solanacearum* strain GMI1000. Examination of this pathosystem allowed the dissection of sequential stages of pathogen infection and the identification of two effectors important in this process.

**Root Symptom Development in Response to Infection Is Genetically Controlled by the Bacteria**

The first step in the infection (Fig. 8) was the development of root symptoms, which included root growth arrest, root tip swelling and browning, and epidermal cell death. These root symptoms were not dependent on the plant genetic background, as they were common to both susceptible and resistant lines. They were also observed in other *M. truncatula* lines infected with *R. solanacearum*, four resistant (TN7.22, TN3.17, TN6.17, and DZA315.16) and three susceptible (TN1.21, TN9.1, and TN6.2) lines. They appeared to be the first step in bacterial infection, but they were not always associated with the wilting of the plant. The progression of early steps of the infection processes is not always associated with resistance, as described in other systems (Dita et al., 2007). Indeed, specific recognition leading to defense mechanisms may initiate after penetration of the pathogen into the host tissues (Freytag et al., 1994).

*R. solanacearum* infection processes in intact roots have been described in tomato (Vasse, 1995) and petunia (Zolobowska and Van Gijsegem, 2006). No tomato root symptoms were mentioned. However, in petunia, Zolobowska and Van Gijsegem (2006) ob-
served swelling of the root tip, reduced elongation of main and lateral roots, inhibition of lateral root development, and formation of root lateral structures. Similarly, we found *M. truncatula* root growth arrest, which could result from the disruption of the root meristem structure that was observed. At the same time, internal cell maturation may still occur, as illustrated by the enlarged vacuolated cells and supernumerous mature vessels normally encountered farther from the root tip (Fig. 2B). However, root alterations may not be a specific virulence mechanism but may just be a consequence of infection.

Root tip epidermal cell death, caused by effectors secreted by the bacteria, could be beneficial to *R. solanacearum*. It could lead to the release of specific attractant compounds, as chemotaxis has been described to play a role in fitness and virulence (Yao and Allen, 2006). The release of nutrients may also contribute to the ability of the bacteria to grow and therefore participate in the quorum-sensing mechanism (Von Bodman et al., 2003). Root tip epidermal cell death could also be directly linked to the invasion of the bacteria. The cell death could be induced before bacterial penetration, or bacteria could invade still intact cells as described for the bacterium *Streptomyces scabies* in potato (Loria et al., 2003).

The specific requirement of a functional T3SS for root symptom development led us to test a panel of T3E mutants for their ability to cause epidermal cell death. This screen resulted in the identification of two effectors: Gala7 and AvrA. These two effectors were involved in epidermal cell death and had an additive effect. Other effectors may be involved to a lesser extent, as the root tip epidermis was still partially altered after infection with the double mutant strain. The two effectors were also involved in root growth arrest, but no significant additive effect could be measured due to the variability of root growth. These effectors were both demonstrated to be translocated into plant cells by the T3SS (Angot et al., 2006; Poueymiro et al., 2009), so they may modulate host pathways, for example, acting on the proteasome pathway in the case of Gala7 (Angot et al., 2006).

### R. solanacearum, a Pathogenic Bacterium Able to Penetrate *M. truncatula* through Intact Root Tip Epidermis

The second step in the infection process (Fig. 8) was the bacterial invasion of root tip cortical cells. The root tip epidermal cell death, caused by effectors secreted by the bacteria, could be beneficial to *R. solanacearum*. It could lead to the release of specific attractant compounds, as chemotaxis has been described to play a role in fitness and virulence (Yao and Allen, 2006). The release of nutrients may also contribute to the ability of the bacteria to grow and therefore participate in the quorum-sensing mechanism (Von Bodman et al., 2003). Root tip epidermal cell death could also be directly linked to the invasion of the bacteria. The cell death could be induced before bacterial penetration, or bacteria could invade still intact cells as described for the bacterium *Streptomyces scabies* in potato (Loria et al., 2003).

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Figure 7. Bypassing the root penetration step shows a requirement for Gala7 at later stages of infection. Percentage of wilted A17 plants after inoculation on cut root tips. Inoculation with GMI1000 (squares) and avrA mutant (circles) led to 100% wilted plants at 14 dpi. The gala7 mutant (diamonds) and double mutant (triangles) inoculations showed a lower rate of wilted plants (40% and 30%, respectively). No statistical difference could be detected comparing these two last mutants. Values are means of three biological repeats (10 individual plants per repeat), and error bars represent the confidence interval at 95%.

Figure 8. Dissection of the *R. solanacearum* GMI1000 T3SS-dependent infection process on *M. truncatula*. Step 1, Root symptom development. Root growth arrest, root tip swelling, and epidermal cell death were common to resistant F83005.5 and susceptible A17 lines. A cumulative effect of AvrA and Gala7 on epidermal cell death was seen. Step 2, Bacterial cortex colonization. Bacterial invasion of the cortex was observed in the susceptible and resistant lines, but to a lesser extent in the resistant line. The gala7 mutant was never detected in the cortex of the A17 line, and the avrA mutant presence was strongly reduced compared with GMI1000. Step 3, Bacterial vessel colonization. Plant genetic background was predominant, as the GMI1000 strain was never detected in the vessels of the resistant F83005.5 line. Invasion of the avrA mutant into the A17 line was strongly reduced compared with GMI1000. Step 4, Wilting symptom development on wounded roots. Inoculation on A17 wounded roots revealed no difference in wilting symptom development after inoculation with the avrA mutant or GMI1000. Inoculation with the gala7 mutant revealed a strong reduction of wilting symptoms. Symbols are as follows: X, root symptoms; C, cortex; V, vessels; gray oval, bacteria.
tip was the only zone where we found that the bacteria could penetrate into *M. truncatula* through intact root tissue and not through induced or natural wounds, like axils of secondary roots on tomato (Vasse, 1995). To date, *Streptomyces* species and *Erwinia chrysanthemi* were the only pathogenic bacteria described as being able to penetrate intact root tissues (Nieves-Brun, 1985; Loria et al., 2003).

Cortical cell invasions occurred in both *M. truncatula* lines but were reduced in the resistant line. The cell wall is the primary interface for pathogen interactions and therefore is an important site of potential defense mechanisms, such as cell wall deposition and strengthening (Cano-Delgado et al., 2003). Autofluorescent phenolic compounds are mainly implicated in cell wall strengthening; an autofluorescence assay, therefore, allows a general view of potential defense mechanisms (Carver et al., 1998; Torregrosa et al., 2004). Stronger autofluorescence was detected in F83005.5 cortical cell walls and could be linked with the reduced bacterial penetration in the root.

At this stage, the bacterial effectors were the major determinants of infection, as the *gala7* mutant was never detected in cortical cells of line A17 and *avrA* mutant presence was strongly reduced. Our inoculation procedure highlights a new role for *Gala7* as a determinant of bacterial penetration.

**M. truncatula** Defenses Can Prevent Root Tip Vessel Invasion, Providing a Key Resistance Mechanism against *R. solanacearum*

The third step in the infection process (Fig. 8) was bacterial propagation in the vascular tissue. The *avrA* mutant was detected in vessels of A17 plants, but at a much lower rate than GMI1000, showing bacterial determination at that stage. Plant genetic background was also important at that stage, as GMI1000 was never detected in the vascular tissue of F83005.5 plants. As seen previously, we detected autofluorescent cell wall cortical cells, suggesting the presence of phenolic compounds. Therefore, we assayed for the presence of lignin, a phenylpropanoid known to strengthen the cell wall and inhibit further pathogen ingress (Hijwegen, 1963; Cano-Delgado et al., 2003). Earlier lignification of the F83005.5 root tip endodermis was observed and could constitute a barrier preventing bacterial propagation in the vessels.

This third stage was tightly linked to the next step (wilting development; Fig. 8). No wilting was observed when bacteria did not reach the vessels. This suggests that foliar symptom development depends on the bacterium’s ability to penetrate the root tip vasculature in *M. truncatula*, which was not described before in other systems. Indeed, bacteria could be detected in the vascular system of resistant tomato plants, but at a low level that does not induce plant wilting (Grimault et al., 1995).

The *avrA* mutant strain was able to reach the same stages as GMI1000, but to a lesser extent at each step; therefore, it had a reduced pathogenicity on *M. truncatula*. Although the *AvrA* gene is widely distributed in *R. solanacearum*, disruption of *avrA* in GMI1000 strain did not lead to a reduced pathogenicity on susceptible hosts such as tomato and Arabidopsis (Poueymiro et al., 2009). This report shows that *avrA* is required during the infection process as an important determinant of disease development in *M. truncatula*. It is often hypothesized that avirulence genes may have been conserved through evolution because the encoded protein may promote disease in another pathosystem (Stavrinides et al., 2008). The demonstration of a role for *avrA* in disease development on *M. truncatula* supports this hypothesis.

**AvrA and Gala7, Different Effects in Steps of the Infection Process**

The *gala7* mutant never colonized intact roots and therefore never induced foliar wilting, whereas the *avrA* mutant showed reduced aggressiveness. Therefore, we wondered if those two effectors were also involved in the virulence of *R. solanacearum* after xylem invasion and wilting symptom development. Invasion of the bacteria was made possible by cutting the root tip at the time of inoculation. In this condition, the *avrA* mutation did not affect disease development. In contrast, the *gala7* mutation led to a reduction of wilting symptoms. These results suggested that the two effectors have different roles in *R. solanacearum* virulence, with *AvrA* only involved in the penetration steps.

The effects of the two T3Es have been studied on both root symptom development and disease development of leaves of the susceptible line, and two different mechanisms were revealed. The involvement of the two T3Es differed between early and later stages in infection: they had additive effects on root tip epidermal cell death, whereas disease development in vascular tissues involved *gala7* but not *avrA*. Root penetration likely requires a large number of molecules, consistent with the fact that the development of root symptoms involves a nonspecific mechanism, observed on both resistant and susceptible lines and, at least partially, on petunia (Zolobowska and Van Gijssegem, 2006). Bacterial colonization and disease development seemed to require host-specific signaling pathways and therefore likely require highly specific bacterial effectors.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

The wild-type strain GMI1000 of *Ralstonia solanacearum* and *hrp* mutant derivatives (GMI1525, a *hrpR* regulatory mutant, and GMI1402, a *lrc5* T3SS structural mutant) were described previously (Cumac et al., 2004). Mutant strains carrying disruptions of the global virulence regulator *phcA* (GMI1610; Genin et al., 2005) and the T2SS (*gspD gspH* deletion mutant strain GRS465) were also used. GMI1559 is a GMI1000 derivative carrying a constitutively expressed GUS (uidA) fusion, and GMI1560 is a *hrpC* mutant also carrying this.
uidA reporter fusion (Vailleau et al., 2007). A collection of 67 T3E disruption mutants generated in GMI1000 (Cunnac et al., 2004; Occhialini et al., 2005) was used for screening with the Medicago truncatula root symptom assay. Conditions for routine culture of R. solanacearum were as described (Cunnac et al., 2004). When required, antibiotics were used at the following concentrations: gentamycin (Gm; 10 μg mL⁻¹), spectinomycin (Spec; 40 μg mL⁻¹), and tetracycline (10 μg mL⁻¹).

Construction of an avrA-gala7 Double Mutant Strain

An avrA deletion mutant strain was generated by inserting the interposition carrying a spectinomycin resistance gene (Prentki and Krisch, 1984) in a single EcoRI restriction site engineered between upstream and downstream DNA fragments flanking the avrA coding sequence. These fragments were PCR amplified using the following primers: 5'-AAAGCTTCTCGACCTG-GCACACCG-3' and 5'-GAATTCTCTACATCTCAGTTCA-3' (upstream fragment) and 5'-GAATTCGTGAATCCGCCGCGCCTC-3' and 5'-TCT-AGATGCTTCCGGCGACCC-3' (downstream fragment). This avrA deletion was introduced into GMI1000 by natural transformation as described previously (Cunnac et al., 2004). As expected, the resulting strain, GMI1758, was unable to induce HR on tobacco (Nicotiana tabacum; Poueymiro et al., 2009). In order to generate a double mutant strain carrying mutations in both avrA and gala7, total genomic DNA from strain GRS138 (a gala7 disruption mutant; Angot et al., 2006) was used to transform strain GMI1758. The resulting strain, GRS469, therefore carried both avrA: (Spec') and gala7: (Gm') mutations. The structure of the deleted loci was checked by DNA gel-blot analysis. For complementation analyses of the gala7 and avrA mutations, plasmids pNP221 (Angot et al., 2006) and pSC222 (Poueymiro et al., 2009) were used, respectively.

Plant Material and Inoculation Procedure

Seeds were surface sterilized, vernalized, and germinated according to Boisson-Dernier et al. (2001). Twenty-four-hour germinating seeds were grown on Fahraeus medium (Fahraeus, 1957) covered with filter paper in square petri dishes at 25°C (day/night, 12 h). Roots were kept in the dark with a mask on the petri dish made of craft paper and aluminum foil. Inoculation was performed as described previously (Vailleau et al., 2007). Bacterial concentration was set at 10⁷ colony-forming units mL⁻¹ for the inoculation on cut root tips and on intact root tips. For the inoculation on cut root tips, 1 cm was excised from the root tip right before applying the bacteria. Mock treatment was carried out using the same procedure with water. Inoculation with the TSSS-defective mutant GMI1000 (GMI1402) is referred to herein as the TSSS-defective control condition. Each experiment consisted of three biological replicates.

Macroscopic Infection Process Studies

Roots were measured every 24 h up to 15 dpi with 10 plants per treatment. Root tip browning and deformation were observed using a stereomicroscope (Leica MZ75 equipped with a CCD camera [Leica DFC320]).

GUS staining was performed on 10 plants per sample as described by Jefferson (1987). Wilting symptoms were evaluated during 14 d for the cut root tip condition and 28 d for the intact root condition (10 plants per treatment). Plant death was defined as full chlorosis of cotyledons and leaves.

Microscopic Infection Process Experiments

The FDA viability test was performed as described previously (Heslop-Harrison and Heslop-Harrison, 1970). Ten plants were observed per biological repeat. For semithin sections, fresh material was fixed in 0.1 m sodium cacodylate and 2.5% glutaraldehyde, dehydrated twice for 1 h each time in 20%, 40%, 60%, 75%, 80%, 90%, and 100% ethanol, progressively infiltrated in medium-grade LR white resin (48 h in 1.3 LR white resin and 2.3 ethanol, 48 h in 1.2 LR white resin and 1.2 ethanol, 48 h in 2.3 LR white resin and 1.3 ethanol, and 48 h in pure LR white resin), and enucleated for resin polymerization for 12 h at 65°C. Sections of 1 μm were made with Ultracut E (Reichert-Jung) and stained during 30 s at 65°C with 0.1% toluidine blue in 2.5% Na₂CO₃ at pH 11. Three roots were sectioned per biological replicate.

Root tip swelling and cell wall modifications were assayed on fresh material included in 5% low-melting agar (agarose type 1: Sigma) and sectioned with a vibratom (Leica VT 1000S). Phloroglucinol (hydrochloric solution; VWR Prolabo) coloration (10 min) was used to detect lignin (violet) and suberin (pink) deposits; callose detection was performed using 0.1% aniline blue in 0.15% KPO₄, pH 12.4 (10 min), adapted from Pérez-Del-Luque et al. (2007). Seven plants were sectioned for each biological replicate. All images were obtained using an inverted microscope (Leica DMIREB), and images were acquired with a CCD camera (color cooled view; Photonic Science). For the FDA test, fluorescence was obtained using excitation range 450 to 490 nm, dichroic mirror 510 nm, and long-pass emission filter 515 nm. For the autofluorescence test and callose detection, excitation range 340 to 380 nm, dichroic mirror 400 nm, and long-pass emission filter 425 nm were used.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. A17 root tip epidermal viability scored after inoculation with GMI1000 and all tested T3E mutants at 3 dpi.

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