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Induced ligno-suberin vascular coating and tyramine-derived hydroxycinnamic acid amides restrict *Ralstonia solanacearum* colonization in resistant tomato

Short title: A pathogen-induced ligno-suberin vascular coating

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2 **Summary**

3

4 • Tomato varieties resistant to the bacterial wilt pathogen *Ralstonia solanacearum* have
5 the ability to restrict bacterial movement in the plant. Inducible vascular cell wall
6 reinforcements seem to play a key role in confining *R. solanacearum* into the xylem
7 vasculature of resistant tomato. However, the type of compounds involved in such
8 vascular physico-chemical barriers remain understudied, while being a key component
9 of resistance.

10 • Here we use a combination of histological and live-imaging techniques, together with
11 spectroscopy and gene expression analysis to understand the nature of *R.*
12 *solanacearum*-induced formation of vascular coatings in resistant tomato.

13 • We describe that resistant tomato specifically responds to infection by assembling a
14 vascular structural barrier formed by a ligno-suberin coating and tyramine-derived
15 hydroxycinnamic acid amides. Further, we show that overexpressing genes of the
16 ligno-suberin pathway in a commercial susceptible variety of tomato restricts *R.*
17 *solanacearum* movement inside the plant and slows disease progression, enhancing
18 resistance to the pathogen.

19 • We propose that the induced barrier in resistant plants does not only restrict the
20 movement of the pathogen, but may also prevent cell wall degradation by the pathogen
21 and confer anti-microbial properties, effectively contributing to resistance.

22

23 **Key words:**

24

25 Bacterial wilt, Feruloyltyramine, HCAAs, Lignin, *Ralstonia solanacearum*, Suberin,
26 Tomato, Vascular coating

28 **Introduction**

29

30 In natural environments plants are constantly exposed to diverse microbiota, including
31 pathogenic organisms. In addition to pre-existing structural cell barriers that act as a first
32 line of defense (Serrano *et al.*, 2014; Falter *et al.*, 2015), pathogen perception results in
33 activation of a complex, multi-layered immune system in plants (Jones and Dangl, 2006).
34 As part of the suite of inducible defenses, *de novo* formation of physico-chemical barriers
35 prevents pathogen colonization and spread inside the plant. Despite their importance, the
36 exact composition of these barriers, as well as the mechanisms that lead to their formation
37 in the plant upon pathogen invasion remain largely unknown.

38

39 The interaction between the soil-borne bacterial wilt pathogen *Ralstonia solanacearum* and
40 tomato offers a paradigmatic scenario to study inducible physico-chemical barriers, because
41 of its agro-economic impact, and the well-developed genetic and molecular tools available
42 in both organisms. *R. solanacearum* enters the root system through wounds or at the points
43 of emergence of lateral roots, where the epidermal and endodermal barriers may be
44 compromised (Vasse *et al.*, 1995; Álvarez *et al.*, 2010; Ursache *et al.*, 2021). After entering
45 the root, the bacterium moves centripetally towards the vasculature and once it reaches the
46 xylem, it multiplies and spreads vertically within the vessels and horizontally to other
47 vessels and the surrounding tissues (Digonnet *et al.*, 2012).

48

49 The xylem tissue is a major battleground for the interaction between vascular wilt
50 pathogens and their hosts, where the outcome of the infection is at stake (Yadeta and
51 Thomma, 2013). To prevent the spread of pathogenic propagules, the xylem vasculature of
52 resistant plants undergoes intense structural and metabolic modifications, such as
53 reinforcing the walls of xylem vessels, pit membranes and surrounding xylem parenchyma
54 cells in response to pathogens (Street *et al.*, 1986; Benhamou, 1995). This prevents
55 pathogen colonization of the surrounding parenchyma cells, nearby vessels and inter-
56 cellular spaces through degeneration of the vessel pit membranes or cell walls by the
57 pathogen (Nakaho *et al.*, 2000; Digonnet *et al.*, 2012; Liu *et al.*, 2005; Pérez-Donoso *et al.*,
58 2010; Lowe-Power *et al.*, 2018). This vascular confinement is an effective strategy

59 commonly found among plants resistant to vascular wilt pathogens such as *R.*
60 *solanacearum*, which otherwise spread systemically and eventually kill the plant
61 (McGarvey *et al.*, 1999; Vasse *et al.*, 2000; Potter *et al.*, 2011; Caldwell *et al.*, 2017;
62 Scorticchini, 2020; Kashyap *et al.*, 2021).

63

64 Among the various tomato germplasms, the cultivar Hawaii 7996 (H7996) is the most
65 effective natural source of resistance against *R. solanacearum* (Nakaho *et al.*, 2004;
66 Grimault *et al.*, 1994). In this cultivar, resistance to *R. solanacearum* is a complex
67 polygenic trait (2000; Thoquet *et al.*, 1996; Mangin *et al.*, 1999; Wang *et al.*, 2013). Our
68 previous study identified the bottlenecks through which H7996 is able to limit *R.*
69 *solanacearum* spread *in planta* (Planas-Marquès *et al.*, 2019), namely: i) root colonization,
70 ii) vertical movement from roots to shoots, iii) circular invasion of the vascular bundle and
71 iv) radial apoplastic spread from the vessels into the cortex. Vascular cell wall
72 reinforcements seem to play a key role in confining *R. solanacearum* into the xylem
73 vascular bundles of resistant tomato H7996. Ultra-microscopic studies in resistant tomato
74 showed that the pit membranes, as well as xylem vessel walls and parenchyma cells form a
75 conspicuously thick coating in the form of an electron dense amorphous layer, as part of the
76 defense response against *R. solanacearum* (Nakaho *et al.*, 2000; Kim *et al.*, 2016;).

77

78 Among the polymers constituting vascular coating structures, lignin is the most typically
79 found, constituting an integral part of the secondary cell wall of the xylem vasculature.
80 Lignin is well documented as a common structural defense against vascular wilt pathogens
81 (Novo *et al.*, 2017; Zeiss *et al.*, 2019; Kashyap *et al.*, 2021) and it is also emerging as an
82 important inducible defense component in other diseases/pests affecting the vasculature
83 (Jhu *et al.*, 2021; Joo *et al.*, 2021) Suberin has also been reported to be deposited in vascular
84 coatings as a defense response (Kashyap *et al.*, 2021), although the mechanisms regulating
85 its synthesis, spatio-temporal dynamics and inducibility remain elusive. Interestingly, root
86 microbiota has been recently shown to shape suberin deposits in the plant, highlighting its
87 central role in plant-microbe interactions (Salas-González *et al.*, 2021). Suberin is a
88 polyester containing long and very long chain fatty acids and derivatives and also some
89 aromatics, mainly ferulic acid. Cells that accumulate suberin also accumulate lignin, whose

90 deposition has been described to precede that of suberin in phellem cells (Lulai and Corsini,
91 1998). This lignin is also known as a lignin-like polymer, which consists of
92 hydroxycinnamates and monolignols (Graça, 2015). The ligno-suberin heteropolymer
93 formed by the lignin-like polymer and suberin has been also referred to as the
94 poly(aromatic) and poly(aliphatic) domains of suberin, respectively. Commonly, suberized
95 cell walls also comprise soluble phenolic compounds, which share biosynthetic pathways
96 with suberin and lignin (Bernards, 2002).

97

98 Ferulic acid present in the suberin and lignin-like fractions is proposed to link both
99 polymers (Graça, 2010) and its continuous production has been demonstrated essential for
100 suberin deposition (Andersen *et al.*, 2021). Ferulic acid amides, such as feruloyltyramine
101 and feruloyloctopamine, have been described as structural components of the lignin-like
102 polymer and in the phenolic soluble fraction of suberizing wound-healing potato tuber
103 (Negrel *et al.*, 1996; Razem and Bernards, 2002). Ferulic acid amides belong to the
104 Hydroxycinnamic acid amide (HCAA) family, which present antimicrobial activity and are
105 considered biomarkers during plant-pathogen interactions (Zeiss *et al.*, 2021). However, the
106 precise role of HCAs in plant defense remains to be elucidated (Macoy *et al.*, 2015).
107 Besides their direct antimicrobial activity as soluble phenols, HCAs have also been
108 proposed to cross-link to cell wall structural polymers during infection, potentially
109 contributing towards the formation of a phenolic barrier that can make the cell wall resilient
110 to pathogenic degradation (Zeiss *et al.*, 2021).

111

112 In the present study, we conducted a detailed investigation of the inducibility, structure and
113 composition of the xylem vascular wall reinforcements that restrict *R. solanacearum*
114 colonization in resistant tomato. Using a combination of histological and live-imaging
115 techniques, together with spectroscopy and gene expression analysis we provide important
116 new insights into the pathogen-induced formation of vascular coatings. In particular, we
117 show that a ligno-suberin vascular coating and tyramine-derived HCAs contribute to
118 restriction of *R. solanacearum* in resistant tomato. In addition, we demonstrate that genes in
119 the ligno-suberin-associated pathways can be explored to engineer resistance against *R.*
120 *solanacearum* into commercial susceptible varieties of tomato.

121

122 **Materials and Methods**123 **Plant materials and growth conditions**

124 Tomato (*Solanum lycopersicum*) varieties Marmande, Hawaii 7996 (H7996) and
125 Moneymaker (wild-type and 35S::THT 1-3, generated by Campos *et al.*, (2014)), were
126 used. Plants were grown in controlled growth chambers at 60% humidity, 12 h day/night
127 and 27°C (LED lighting) or 25°C (fluorescent lighting).

128

129 ***Ralstonia solanacearum* strains and growth conditions**

130 *R. solanacearum* GMI1000 strain (Phylotype I, race 1 biovar 3) was used, including
131 luminescent and fluorescent reporter strains of *R. solanacearum* GMI1000 described in
132 (Cruz *et al.*, 2014; Planas-Marquès *et al.*, 2019).

133

134 **Cloning and stable transformation in tomato**

135

136 For generation of the 35S::FHT-HA construct the *FHT* (Solyc03g097500) coding sequence
137 was amplified from tomato H7996 cDNA using the forward and reverse primers
138 part7FHTF1 and part7FHTHAR1, respectively (Table S1). The amplified product was
139 cloned into the pJET1.2/blunt cloning vector using CloneJet PCR cloning kit
140 (Thermofisher) and then digested by *Sma*I and *Bam*HI. The digested products were purified
141 using NZYGelpure (Nzytech) followed by ligation into the pART7 and later to pART27
142 vector (Gleave, 1992). pART27 containing 35S::FHT-HA was transformed into Marmande.
143 For this, the construct was transformed into *Agrobacterium tumefaciens* strain C58C1.
144 Cotyledon explant preparation, selection, and regeneration followed the methods described
145 by (Mazier *et al.*, 2011). Transformants were selected on kanamycin-containing medium.
146 Accumulation of FHT-HA protein was assayed by immunoblot with a monoclonal HA
147 antibody (GenScript).

148

149 **Bacterial inoculation in plants**

150 Four to five week-old tomato plants were inoculated through roots with *R. solanacearum*
151 using the soil drenching method with a to 1x10⁷ CFU ml⁻¹ suspension of bacteria as

152 described in (Planas-Marquès *et al.*, 2018). Inoculated plants were kept in a growth
153 chamber at 27°C. For tomato leaf infiltration, plants were vacuum-infiltrated by
154 submerging the whole aerial part in a $\sim 10^5$ CFU ml⁻¹ bacterial suspension as described in
155 Planas-Marquès *et al.*, (2018). For inoculation directly onto the stem vasculature, 10 µl (5
156 µl at a time) of 10⁵ CFU ml⁻¹ bacterial suspension was placed at the node of the petiole and
157 pin-inoculated using a sterile needle (30G \times ½", BD Microlance, Becton Dickinson).

158

159 **R. solanacearum** pathogenicity assays and quantification of bacterial growth *in planta*
160 Infected plants were scored for wilting symptoms using a scale from 0 to 4: 0=healthy plant
161 with no wilt, 1=25%, 2=50%, 3=75%, and 4=100% of the canopy wilted as described by
162 Planas-Marquès *et al.*, (2019). The relative light units per second (RLU·s⁻¹) readings were
163 converted to CFU·g⁻¹ tissue as described in Planas-Marquès *et al.*, (2019). For bacterial
164 colonization assays using GFP reporter strain, transverse stem cross-sections were made at
165 the inoculation point and at a distance of 0.5 cm, 1 cm, 2 cm and 3 cm in both upward and
166 downward direction, using a sterile razor blade. Quantification of mean green fluorescence
167 was done using ImageJ software (Planas-Marquès *et al.*, 2019). For leaf *in planta*
168 multiplication assays, 3 leaf discs of 0.8 cm² were homogenized in 200 µl of sterile distilled
169 water. CFU cm⁻² leaf tissue were calculated after dilution plating of samples with
170 appropriate selection antibiotics and CFU counting 24 hours later.

171

172 **Histological methods**

173 Thin (approximately 150 µm) transverse cross-sections were obtained with a sterile razor
174 blade from a 1.5 cm area of the taproot-to-hypocotyl transition zone located immediately
175 below the soil line (Fig. S1a). Inoculated plants were sectioned when bacterial colonization
176 level reached 10⁵ CFU g⁻¹ taproot-to-hypocotyl transition zone tissue. This corresponded to
177 4 dpi in Marmande and 9 dpi in H7996, at which stage only H7996 sections showed a
178 localized browning at one xylem pole indicative of infection. Sections were kept in 70 %
179 ethanol at room temperature for 5 days and examined using fluorescence microscopy using
180 a Leica DM6B-Z microscope under UV illumination (340-380 nm excitation and 410-450
181 nm barrier filters). Autofluorescence emitted from phenolic deposits was recorded using a
182 Leica-DFC9000GT-VSC07341 camera and the signal was pseudo-colored green. Sections

were also stained with Phloroglucinol-HCl for the detection of lignin and observed under bright field (Pomar *et al.*, 2004). Photographs were taken with a DP71 Olympus digital camera. Cross-sections were also observed under UV with a Leica-DM6B-Z microscope (340-380 nm excitation and 410-450 nm barrier filters). To detect the autofluorescent blue-to-green pH-dependent color conversion of wall-bound ferulic acid cross-sections were first mounted in 70% ethanol (neutral pH) and then in 1N KOH (pH above 10) adapting the protocol from (Harris and Trethewey, 2010; Carnachan and Harris, 2000; Donaldson and Williams, 2018). A Leica DM6B-Z microscope was used to observe autofluorescence (340-380 nm excitation and 410-450 nm barrier filters). Images were recorded using a Leica MC190-HD-0518131623 digital camera. To visualize suberin aliphatics, sections were treated with 5 % Sudan IV, dissolved in 70 % ethanol and illuminated with UV light. These sections were subsequently treated with 1N KOH to detect ferulic acid as described above. For both ferulic acid and suberin, the HC PL APO or HC PL FLUOTAR objectives of the Leica DM6B-Z microscope were used and images were captured using a Leica MC190-HD-0518131623 color digital camera. The UV autofluorescence signal from xylem vessel walls and surrounding layers was measured using the LAS X Leica software and changes in ferulate accumulation were quantified using ImageJ software by selecting the area of xylem vessel walls showing autofluorescence. Quantifications of fluorescence intensity were normalized per μm of region of interest (ROI), which corresponded to a particular area of the vascular bundles, where main vessels concentrate (represented in Fig. 1b). Basically the line is drawn at each of the 4 corners in the whole image and then the fluorescence is normalized by the length of the lines.

205

206 **2D-NMR**

207 The samples of a pool of 15 tomato plants (taproot-to-hypocotyl region), water treated or
208 having a bacterial load of 10^5 CFU.g^{-1} were milled and extracted sequentially with water,
209 80% ethanol, and with acetone, by sonicating in an ultrasonic bath during 30 min each time,
210 centrifuging and eliminating the supernatant. Then, lignin/suberin fraction was
211 enzymatically isolated by hydrolyzing the carbohydrates fraction with Cellulysin
212 (Calbiochem), as previously described (Rico *et al.* 2014). Approximately 20 mg of
213 enzymatic lignin/suberin preparation was dissolved in 0.6 mL of DMSO-*d*₆. Heteronuclear

214 single quantum coherence (HSQC) spectra were acquired on a Bruker AVANCE III 500
215 MHz spectrometer equipped with a 5 mm TCI cryoprobe, using the experimental
216 conditions previously described (Rico *et al.*, 2014). HSQC cross-signals were assigned and
217 quantified as described (Rencoret *et al.*, 2018; del Río *et al.*, 2018; Mahmoud *et al.*, 2020).
218 In the aromatic region, the correlation signals of G₂ and S_{2,6} were used to estimate the
219 content of the respective G- and S-lignin units. The C_α/H_α signals of the β-O-4' ethers (A_α),
220 phenylcoumarans (B_α), and resinols (C_α) in the linkages region were used to estimate their
221 relative abundances, whereas the C_γ/H_γ signal was used in the case of cinnamyl alcohol
222 end-units (I_γ).

223

224 **RNA extraction, cDNA synthesis and quantitative RT-PCR analysis**

225 Taproot-to-hypocotyl transition zone sections of ~ 0.5 mm thickness were obtained and the
226 xylem vascular tissues (vascular bundles and surrounding parenchyma cells) were collected
227 and kept in liquid nitrogen. Each sample comprised taproot-to-hypocotyl transition zone
228 xylem tissues of 6 plants. RNA was extracted using the Maxwell RSC Plant RNA Kit
229 (Promega). cDNA was synthesized from 2 µg RNA using High Capacity cDNA Reverse
230 Transcription Kit (Applied Biosystems, USA). cDNA amplification and analysis was
231 performed using the LightCycler 480 System (Roche). The Elongation Factor 1 alpha
232 housekeeping gene (*eEF1 α, Solyc06g005060*) was used as a reference. All reactions were
233 run in triplicate for each biological replicates. Melting curves and relative quantification of
234 target genes were determined using the software LightCycler V1.5 (Roche). The level of
235 expression relative to the reference gene was calculated using the formula $2^{-\Delta CT}$, where
236 $\Delta CT = (CT \text{ RNA target} - CT \text{ reference RNA})$.

237

238 **Statistical analysis**

239 Statistical analyses were performed using Statgraphics software. All statistical tests are
240 indicated in the respective figure legends.

241

242 **Results:**

243

244 **Resistant H7996 tomato restricts *R. solanacearum* colonization and induces a vascular
245 coating response involving wall-bound phenolics**

246

247 In order to understand the mechanisms underscoring restriction of *R. solanacearum* spread
248 in resistant tomato varieties we used the resistant variety Hawaii 7996 (H7996) and
249 compared it to the susceptible cultivar Marmande. In our assay conditions, most Marmande
250 plants were wilted 10 days after inoculation with *R. solanacearum* GMI1000, while H7996
251 plants remained largely asymptomatic (Fig. 1a, S2a and (Planas-Marquès *et al.*, 2019)).
252 Accordingly, bacterial loads in the taproot-to-hypocotyl region were drastically reduced in
253 H7996 compared to Marmande, confirming the remarkable bacterial restriction ability of
254 this cultivar (Fig. S1b and (Planas-Marquès *et al.*, 2019)).

255

256 To identify defense-associated anatomical and physico-chemical modifications in H7996
257 after infection with *R. solanacearum* compared to Marmande we performed histochemical,
258 spectroscopic and gene expression analysis. For this, plants were infected with a 10^7 *R.*
259 *solanacearum* solution or mock through their roots using the soil-drench method and then
260 we collected tissue containing 10^5 CFU g⁻¹ tissue of bacteria at the taproot-to-hypocotyl
261 transition area, located approximately 1 cm below ground (Fig. S1a). Marmande reached
262 10^5 CFU g⁻¹ tissue at around 4 dpi, while the resistant H7996 took approximately 9 days to
263 do so (Fig. S2). We have previously observed that the root-to-hypocotyl area constitutes a
264 key bottleneck for bacterial progression inside the plant (Zuluaga *et al.*, 2015; Puigvert *et*
265 *al.*, 2017; Planas-Marquès *et al.*, 2019), being thus an ideal target zone for analysis of
266 structural defense responses.

267

268 We first analyzed ultraviolet (UV) autofluorescence of transverse cross-sections of the
269 taproot-to-hypocotyl region, indicative of phenolic compounds (Donaldson, 2020). To
270 focus on cell wall-deposited phenolic compounds, soluble phenolic compounds were
271 removed with ethanol prior to observation as reported (Pouzoulet *et al.*, 2013; Araujo *et al.*,
272 2014). Infection with *R. solanacearum* induced a strong UV signal emitted from the walls
273 of the vessels, and also from surrounding xylem parenchyma cells and tracheids in resistant
274 H7996 (Figs. 1b, c). This enhanced autofluorescence was not observed in the susceptible

275 variety Marmande nor in mock-treated samples (Fig. 1b, c). In tissues outside the vascular
276 area, inoculation resulted in a decrease of autofluorescence in both susceptible and resistant
277 tomato lines.

278

279 **Spectroscopic analysis reveals *R. solanacearum*-induced deposition of suberin and**
280 **accumulation of tyramine-derived amides in resistant H7996 tomato and lignin**
281 **structural modifications in susceptible Marmande tomato**

282

283 In order to decipher the composition of the cell wall-deposited compounds we used nuclear
284 magnetic resonance (2D-HSQ NMR), one of the most powerful tools for plant cell wall
285 structural analysis providing information on the composition and linkages in lignin/suberin
286 polymers (Ralph and Landucci, 2010; Correia *et al.*, 2020). 2D-HSQC spectra of infected
287 or mock-treated taproot-to-hypocotyl transition zones of H7996 and Marmande tomato
288 plants were obtained and the main lignin and suberin substructures identified are shown in
289 Fig. 2, while the chemical shifts of the assigned cross-signals are detailed in Table S2.
290 Importantly, the aliphatic region of the 2D-HSQC spectra revealed that H7996 infected
291 plants were more enriched in poly-aliphatic structures characteristic of suberin (magenta-
292 colored signals), compared to its mock control (Fig 2a). Related to this, an olefinic cross-
293 signal of unsaturated fatty acid structures (UF, δ_C/δ_H 129.4/5.31), typical of suberin, was
294 also found to be increased in the HSQC spectrum of the infected H7996 tomato. A rough
295 estimate based on the integration of lignin and suberin HSQC signals, revealed that the
296 suberin/lignin ratio in *R. solanacearum*-infected H7996 plants was doubled compared to
297 mock-treated plants, evidencing an increase in suberin deposition as a consequence of the
298 bacterial infection. Interestingly, signals compatible with feruloylamides (FAm₇; δ_C/δ_H
299 138.6/7.31) and with tyramine-derived amides (Ty in orange; δ_C/δ_H 129.3/6.92, 114.8/6.64,
300 40.5/3.29 and 34.2/2.62) were exclusively found in the spectrum of infected H7996 plants,
301 suggesting the presence of feruloyltyramine exclusively in these samples (Fig. 2a). Since
302 tyramines have been found as structural components co-occurring with suberin (Bernards *et*
303 *al.*, 1995; Bernards and Lewis, 1998), which generates physically and chemically resistant
304 barriers (He and Ding, 2020), our results substantiate the hypothesis of suberin as an
305 important defense element against *R. solanacearum* infection in resistant tomato plants. On

306 the contrary, the 2D-HSQC spectra from the Marmande variety did not display notable
307 variations between mock and infected plants in the signals corresponding to suberin,
308 tyramine-related structures nor feruloylamides (Fig 2a).

309

310 Interestingly, 2D-HSQC NMR spectra also revealed significant structural modifications in
311 the composition of lignin and the distribution of linkages in tomato plants after infection.
312 Lignins with lower S/G ratios are more branched (condensed) and recalcitrant towards
313 pathogen attack (Iiyama *et al.*, 2020). Therefore, lignin in inoculated H7996, with an S/G
314 ratio of 1.0 should be, *a priori*, more resistant than the lignin in inoculated Marmande plants
315 (S/G ratio of 1.5). 2D-HSQC analysis revealed that the infection of susceptible Marmande
316 plants resulted in an increase of the S/G ratio and a clear reduction of all major lignin
317 linkages (β -O-4', β -5' and β - β '; reduction in roughly 9%, 43% and 46%, respectively),
318 evidencing that a lignin depolymerization process took place (Fig. 2a). In contrast, infected
319 H7996 tomato displayed a slight decrease of the S/G ratio, and only β -O-4' linkages (the
320 easiest to degrade in the lignin polymer) were significantly reduced, while the β -5' and β -
321 β ' were not so affected as in the case of Marmande plants. In this context, the major
322 reduction in lignin linkages observed in Marmande after infection could explain, at least in
323 part, its higher susceptibility to the pathogen.

324

325 **Histochemical analysis reveals the formation of structural vascular coatings
326 containing suberin and ferulate/feruloylamine in resistant H7996 tomato in response
327 to *R. solanacearum* infection**

328

329 To confirm our spectroscopic data, we histochemically analyzed taproot-to-hypocotyl
330 transition zone samples of mock and infected H7996 and Marmande tomato plants.
331 Observation of Phloroglucinol-HCl stained sections under brightfield microscopy (Wiesner
332 staining) (Pomar *et al.*, 2002; Pradhan Mitra and Loqué, 2014), showed that mock and
333 infected H9776 (resistant) as well as mock Marmande (susceptible) samples showed a red-
334 purple color characteristic of the reaction of phloroglucinol-HCl in vessels and fibers,
335 indicative of lignin (Fig. 3a). In contrast, infected Marmande sections exhibited reduced
336 phloroglucinol-HCl staining, suggesting a change in composition of xylem lignin upon

337 infection (Fig. 3a). This observation is in agreement with the structural changes specifically
338 detected in the lignin structure of infected Marmande plants by 2D-HSQC NMR (Fig. 2a),
339 which suggest lignin depolymerization and may partly underscore the high susceptibility of
340 this tomato variety.

341

342 UV illumination of phloroglucinol-HCl-stained samples allows quenching the
343 autofluorescence from lignin and hence detect residual cell wall autofluorescence, which
344 has been associated with suberin deposits (Baayen and Elgersma, 1985; Rioux *et al.*, 1998;
345 Pouzoulet *et al.*, 2013). Under these conditions, the increased autofluorescence observed in
346 the vascular coating regions of infected H7996 tomato plants was not quenched in
347 phloroglucinol-HCl stained samples (Fig. 3a, b). A more detailed observation revealed that
348 this non-quenched autofluorescence was localized in specific regions compatible with (i)
349 intervessel and vessel-parenchyma pit membranes or pit chamber walls and (ii) parenchyma
350 coatings with fluorescent signals enriched in intracellular spaces (Fig 3c).

351

352 Next, we analyzed whether the pathogen-induced coating of vessels observed in H7996
353 correlated also with an increase in ferulates, a major suberin component. We performed
354 KOH treatment of plant tissues, which specifically shifts the UV fluorescence of
355 ferulate/feruloyl amide to green, allowing its detection (Carnachan and Harris, 2000; Harris
356 and Trethewey, 2010; Donaldson and Williams, 2018). UV autofluorescence of vascular
357 coatings in response to *R. solanacearum* infection in resistant H7996 shifted from blue to a
358 strong green color upon treatment with alkali (1N KOH) (Fig. S3a). This indicated that the
359 *R. solanacearum*-induced xylem vasculature feruloylation was specific to resistant H7996,
360 as the fainter blue autofluorescence observed in mock-treated resistant H7996 or
361 susceptible Marmande tissues did not change to green at high pH in either early (Fig. S3a,
362 b) or late (Fig. S3c) stages of infection.

363

364 To corroborate that the ferulate/feruloyl amide accumulation in infected H7996 tomato was
365 related with vascular suberization, we combined the ferulate-specific UV-alkali treatment
366 described above with Sudan IV staining, which binds aliphatic components of suberin to
367 produce a reddish-brown coloration. This revealed suberization in the taproot-to-hypocotyl

368 area of *R. solanacearum*-infected H7996 plants, xylem vessel walls as well as the layers of
369 vessels, parenchyma cells and tracheids in the immediate vicinity (Fig. 4). In the periphery
370 of suberized cells, a green signal from UV-alkali was observed (Fig. 4), which may indicate
371 ferulate/feruloyl amide deposition indicative of a preceding stage towards suberization in
372 this cell layer. In comparison, no positive Sudan IV or UV-alkali staining was detected in
373 infected Marmande or mock-treated tomato plants. Together, suberized and feruloylated
374 layers of parenchyma cells, vessels and tracheids might form a “suberization zone” creating
375 a strong physico-chemical barrier to limit *R. solanacearum* spread from the colonized
376 xylem vessel lumen.

377

378 ***R. solanacearum* infection activates the biosynthesis of aliphatic suberin precursors
379 and feruloyl amide, and aliphatic esterification of ferulic acid in the vasculature of
380 resistant H7996**

381

382 Since a differential accumulation of suberin-compatible compounds was specifically
383 observed in infected H7996, we surmised that genes related to suberin and feruloyl amide
384 synthesis, as well as ferulic acid esterification to aliphatics may be upregulated in resistant
385 tomato in response to *R. solanacearum* invasion. To test this hypothesis, we analyzed: i)
386 expression of genes in the phenylpropanoid and suberin biosynthesis pathways, which
387 provide the necessary precursors for the ligno-suberin heteropolymer; ii) the feruloyl
388 transferase FHT (ASFT/HHT in *Arabidopsis*), which is involved in the formation of
389 ferulate esters of fatty acyl compounds necessary to form suberin and soluble waxes
390 (Molina *et al.*, 2009; Gou *et al.*, 2009; Serra *et al.*, 2010); and iii) N-hydroxycinnamoyl
391 transferases (THT), which are involved in the synthesis of HCAAs such as
392 feruloyltyramine, which is found on the lignin-like polymer and in the soluble phenolic
393 fraction of some suberized tissues (Negrel *et al.*, 1993; Schmidt *et al.*, 1999).

394

395 Quantitative RT-PCR from xylem vascular tissue obtained from the taproot-to-hypocotyl
396 zone in *R. solanacearum*- or mock-treated H7996 and Marmande plants showed specific
397 upregulation of all genes analyzed from the suberin biosynthetic pathway in H7996 infected
398 plants compared to the mock controls (Fig. 5, S4). These included essential suberin

399 biosynthesis genes such as *CYP86A1* and *CYP86B1* (fatty acid oxidation), *FAR* (primary
400 alcohol generation), *KCSs* (fatty acid elongases) and *GPAT5* (acylglycerol formation). In
401 addition, feruloyl transferase *FHT* (*ASFT/HHT* in *Arabidopsis*), was also strongly
402 upregulated in infected H7996 plants (Fig. 5 and S5b). Regarding *THT*, in tomato we
403 identified five putative homologs (Fig S6a), all induced by infection in the vascular tissue
404 of H7996 (Fig. 5 and S6b). Among them, *SlTHT1-3* showed the strongest upregulation in
405 H7996 after infection (Fig. 5 and S6b). In comparison, *R. solanacearum* infection had only
406 a modest effect on genes related to phenylpropanoid pathway as only upregulation was
407 detected in the first enzyme of the pathway (PAL) (Fig. 5 and S7).

408

409 Together, these data indicate that upregulation of genes involved in the formation of
410 aliphatic suberin precursors, ferulic acid esterification to aliphatics (FHT) and production of
411 HCAAs, such as feruloyltyramine (THT), constitute a very specific response of H7996
412 plants that takes place in the vasculature upon *R. solanacearum* infection. Further, these
413 data are in agreement with NMR data of infected H7996, which showed a specific increase
414 in insoluble fatty acid structures typical of suberin as well as the appearance of signals from
415 structural tyramine-derived amides and feruloylamides (Fig. 2a).

416

417 **Overexpression of *SlTHT1-3* in a susceptible tomato cultivar confers resistance to *R.***
418 ***solanacearum***

419

420 Based on our results, we set to determine whether overexpressing genes involved in ferulic
421 acid esterification to suberin aliphatics and feruloylamine biosynthesis, such as *SlFHT* and
422 *SlTHT1-3*, respectively, would increase resistance against *R. solanacearum* in a susceptible
423 tomato background. First, we obtained transgenic tomato lines stably overexpressing
424 *SlFHT* on a susceptible Marmande background (Fig. S8). Under normal growth conditions
425 these lines are morphologically undistinguishable from wild-type, although they display a
426 subtle increase in fresh weight (Fig. S9). We analyzed symptom progression and bacterial
427 colonization. *SlFHT* overexpression lines showed a slight delay in disease progression (Fig.
428 6a) and moderately milder symptoms. The taproot-to-hypocotyl of *SlFHT* overexpressors

429 displayed a slight reduction in bacterial loads after soil-soak inoculation in comparison to
430 Wt tomato (Fig. 6b).

431

432 Regarding *S/THT1-3*, the corresponding tomato overexpressing line was readily available
433 on a Moneymaker background (Campos *et al.*, 2014). This line has been shown to
434 overaccumulate soluble HCAA such as feruloyltyramine and also the hormone salicylic
435 acid (SA) upon infection with *Pseudomonas syringae* pv. tomato (Campos *et al.*, 2014). It
436 is worth noting that tomato plants overexpressing *S/THT1-3* display a slight decrease in
437 fresh weight compared to wild-type plants, although with the naked eye they appear
438 undistinguishable (Fig. S10). As expected, the Moneymaker tomato cultivar showed similar
439 susceptibility to *R. solanacearum* as Marmande (Fig. 7a, b). In contrast, overexpression of
440 *S/THT1-3* resulted in a dramatic increase of resistance against *R. solanacearum*, with
441 disease progressing remarkably slower in this line compared to wild type (Fig. 7a, b).
442 Importantly, bacterial loads were significantly lower in the taproot-to-hypocotyl and
443 hypocotyl of the *S/THT1-3* overexpressor after soil inoculation in comparison to Wt tomato
444 (Fig. 7c). Similarly, direct leaf inoculation also showed severe bacterial growth restriction
445 in the *THT1-3* overexpressing line (Fig. S11a). Further, we monitored the colonization
446 patterns of a *R. solanacearum* GFP reporter strain after stem inoculation of the *S/THT1-3*
447 overexpressing line compared to Wt. In transverse stem cross-sections of 6 dpi plants,
448 bacteria stayed confined near the inoculation point in the 35S::*S/THT1-3* line whereas they
449 spread unrestrictedly in susceptible wild type stems from the inoculation point and at least 3
450 cm up and downwards (Fig. 7d, e and S11b).

451

452 Concomitant with the observed restriction of *R. solanacearum* colonization, an increase in
453 autofluorescence around the vasculature was observed in the *S/THT1-3* overexpressor (Fig.
454 8a). At similar bacterial loads, Wt did not display such enhanced vascular fluorescence.
455 Phloroglucinol-HCl staining did not quench the paravascular autofluorescence in *S/THT1-3*
456 (Fig. 8a, d), indicating that similar to what was previously observed for H7996, the
457 observed increase in wall-bound phenolic deposits did not only correspond to lignin. To
458 gain a deeper insight into the composition of the *R. solanacearum*-induced vascular
459 deposits in *S/THT1-3* overexpressing plants we performed combined Sudan IV-alkali

460 staining (Fig. 8b, c, e). Treatment with alkali resulted in a clear blue-to-green shift of UV
461 autofluorescence around xylem vessels occurring specifically in the *StTHT1-3*
462 overexpressor upon infection, which reveals the presence of ferulates/feruloylamides as
463 part of the observed vascular deposits. In contrast, no positive Sudan IV staining was
464 detected, indicating that a canonical suberin polyester does not seem to be part of vascular
465 coatings in *StTHT1-3* overexpressing plants. Since Sudan IV only stains specific moieties
466 of the complex suberin heteropolymer, we cannot rule out that the observed vascular
467 deposits in *StTHT1-3* are formed by a non-canonical ligno-suberin heteropolymer that does
468 not react with Sudan IV. Further investigation will be needed in order to ascertain the exact
469 nature of the *R. solanacearum*-induced vascular deposits in the *StTHT1-3* overexpressor. In
470 conclusion, our data clearly show that *StTHT1-3* ectopic expression provides a very
471 effective resistance mechanism against *R. solanacearum* -potentially mediated by
472 accumulation of elevated amounts of HCAAs such as feruloyltyramine-, which drastically
473 restricts vascular colonization, preventing bacterial spread and blocking the onset of
474 disease.

475

476 Discussion

477 **Ligno-suberin deposits in vascular cell walls and feruloyltyramine accumulation acts 478 as a resistance mechanism restricting *R. solanacearum* colonization in resistant tomato**

479

480 In our study, resistant tomato (H7996) was observed to react aggressively to *R.*
481 *solanacearum* infection by reinforcing the walls of vessels and the surrounding parenchyma
482 cells with UV autofluorescent phenolic deposits (Fig. 1). An increase in autofluorescence
483 had been previously reported in another resistant tomato variety, LS-89, although its
484 composition was not precisely defined (Ishihara *et al.*, 2012). Histochemical analysis of
485 vascular coatings in resistant tomato upon *R. solanacearum* infection showed that suberin-
486 associated autofluorescence was prominent in the vasculature, in line with previous reports
487 using TEM that showed thickening of the pit membranes accumulating electron-dense
488 material in tomato plants resistant to *R. solanacearum* (Nahako *et al.*, 2000 and 2004). The
489 suberin nature of these coatings was further supported by the positive Sudan IV staining of
490 vessels and surrounding parenchyma cells of H7996 taproot-to-hypocotyl transition zone

491 upon infection (Fig. 4). These results are in agreement with coatings detected in tomato
492 plants resistant to *Verticillium albo-atrum* (Robb *et al.*, 1991), where suberin and lignin
493 were both deposited in intercellular spaces between parenchyma cells adjoining a xylem
494 vessel or infusing and occluding pit membranes coatings (Robb *et al.*, 1991; Street *et al.*,
495 1996). Besides, inhibition of the phenylpropanoid pathway inhibited the formation of both
496 lignin and suberin coatings (Street *et al.*, 1996), in agreement with the ferulic acid
497 requirement to correctly deposit suberin (Andersen *et al.*, 2021) and reinforcing our
498 observations of the presence of a ferulate/feruloylamine-derived polymer detected in H7996
499 *R. solanacearum* (Fig. 4). In line with this, NMR data of resistant H7996 tomato vascular
500 tissue revealed the presence of tyramine-derived amides and feruloylamides incorporated
501 into the cell wall and also an enrichment in poly-aliphatic structures characteristic of
502 suberin (Fig. 2) (Graça, 2015; Legay *et al.*, 2016; Figueiredo *et al.*, 2020).

503

504 Beyond histochemistry and spectroscopic signature detections, further evidence supporting
505 the nature of these ligno-suberin coatings as responsible of the resistance observed in
506 H7796 to *R. solanacearum* was unequivocally provided transcriptionally using gene
507 markers. Tissues undergoing suberization have to go through a complex reprogramming
508 involving a network of metabolic pathways, in order to produce the precursors of the
509 polymer and their polymerization into the matrix (Lashbrooke *et al.*, 2016). Transcriptional
510 reprogramming associated to suberin biosynthesis was clearly observed in the taproot-to-
511 hypocotyl transition zone vascular tissue of resistant H7996 tomato upon infection with *R.*
512 *solanacearum* (Fig. 5). Interestingly, PAL, which showed modest upregulation in resistant
513 H7996, had been previously defined as a rate-limiting enzyme of phenylpropanoid pathway
514 (Faragher and Brohier, 1984; Howles *et al.*, 1996). Considering this, the observed
515 upregulation could provide more tyramine and feruloyl-CoA, which together with the
516 upregulation of *THT* would be in agreement with the increased presence of
517 feruloyltyramine detected by 2D-HSQC NMR (Figure 2a).

518

519 2D-HSQC NMR also revealed differences in the composition and structure of lignin
520 between resistant and susceptible tomato cultivars after infection (Fig. 2). The amounts and
521 the level of lignin of a particular tissue affect wall strength, degradability and pathogen

522 resistance (Cho *et al.*, 2012; Mnich *et al.*, 2020). However, its role in
523 resistance/susceptibility responses is not fully understood. Part of the challenge lies in the
524 fact that its composition seems to be less static than what was previously established. A
525 large variety of lignin-like polymers may co-exist in plants depending on the developmental
526 or environmental context. This becomes particularly relevant in plant-pathogen
527 interactions, where a large variety of compounds linked to lignin differentially accumulate
528 upon infection (Cho *et al.*, 2012; Zeiss *et al.*, 2019). The observed lignin structural
529 differences after infection indicate that i) under basal conditions the two tomato varieties
530 display differences in the composition and structure of lignin and ii) *R. solanacearum*
531 infection affects very differently the lignin fraction in the two varieties: resistant tomato
532 shows only a slight decrease in the S/G ratio that may be linked to an accumulation of the
533 ligno-suberin heteropolymer, while susceptible Marmande undergoes pronounced
534 depolymerization that correlates with a decrease in ferulate/feruloylamine (Fig. 3A).
535 Although *R. solanacearum* has not been shown to be able to specifically depolymerize
536 lignin, the pathogen secretes enzymes that can degrade cell wall polysaccharides and could
537 participate in the observed Marmande stem collapse phenotype (Fig. 1A). In resistant
538 H7996, on the other hand, vascular ligno-suberin-containing coatings would allow to create
539 a hydrophobic barrier to prevent enzymes from accessing the cell wall substrates and at the
540 same time create reinforcements, contributing to resistance to the pathogen. The fact that
541 these reinforcements are rich in tyramine/feruloyltyramine, may further reinforce the
542 structural barrier, providing rigidity and hampering cell wall digestibility by the pathogen's
543 hydrolytic enzymes (Macoy *et al.*, 2015; Zeiss *et al.*, 2020). In addition to that, resistant
544 H7996 may have evolved yet undiscovered mechanisms that directly prevent lignin
545 degradation by the pathogen.

546

547 Overall, our data indicate that vascular coating with wall-bound ligno-suberized
548 compounds may restrict horizontal spread of the bacterium (Fig 1). In comparison,
549 susceptible tomato is either not able to induce such vascular coating upon *R. solanacearum*
550 infection or induces a very weak response (Figs. 1, 3), potentially predisposing its vascular
551 walls to disruption by the pathogen's cell wall degrading enzymes. Considering that both
552 varieties seem to possess the metabolic components to build such barriers, the difference in

553 response may be a direct effect of the differential transcriptional activation of the pathway
554 in vascular tissue of H7996 compared to Marmande. The fact that varieties with moderate
555 resistance to *R. solanacearum* show intermediate restriction of colonization (Planas-
556 Marquès et al., 2019), indicate that the formation of these barriers may be a quantitative
557 trait. However, this also opens the possibility that the differential transcriptional activation
558 of the ligno-suberin pathway observed in resistant tomato may have evolved as an effective
559 mechanism to execute defense responses triggered by activation of an immune receptor
560 upon *R. solanacearum* recognition. Very few immune receptors involved in perception of
561 vascular wilt pathogens have been identified so far, and the mechanisms involved in
562 translating this recognition into effective defense responses in the vasculature remain vastly
563 unknown. Considering that the xylem is a dead tissue, it is expected that the surrounding
564 parenchyma cells will have a pivotal role in perception of the pathogen as well as the
565 signaling leading to the synthesis and wall-binding of the metabolites involved in vascular
566 coating structures, such as the one described here. In fact, xylem parenchyma cells have
567 been shown to synthesize vascular coating components in response to the wilt pathogen *V.*
568 *albo-atrum* (Street et al., 1986). However, how suberin is synthesized and deposited in the
569 xylem is still poorly defined. Exciting research currently ongoing in this area will certainly
570 help understanding the origin and transport of ligno-suberin components to form inducible
571 vascular deposits in response to pathogens. This will also help determining the exact point
572 of perception of the pathogen (at a cell type or tissular level). Identification of pathogen-
573 inducible pathways specifically occurring in resistant varieties such as the one presented
574 here open new avenues of research to shed light on this biologically and agronomically
575 relevant question.

576

577 Based on the above observations, we propose the following model (Fig. 9). When reaching
578 the xylem vessels of resistant H7996, *R. solanacearum* multiplies and tries to invade the
579 surrounding healthy vessels and parenchyma cells by degradation of the xylem pit
580 membranes and walls. Resistant tomato plants respond to *R. solanacearum* vascular
581 invasion depositing feruloyltyramine and other HCAA-tyramine derived compounds, and
582 suberin. These deposits would block the pit membrane access and serve as coatings of the
583 vessel walls and parenchyma cells present in the immediate vicinity of colonized vessels,

584 compartmentalizing the infection. These ligno-suberized layers together form a “zone of
585 ligno-suberization” creating a strong physico-chemical barrier to limit *R. solanacearum*
586 spread.

587

588 **Engineering tomato resistance against *R. solanacearum* by inducing the tyramine-
589 HCAA pathway**

590

591 Considering the observed accumulation of ligno-suberin and cell wall-linked
592 feruloyltyramine in resistant H7996 tomato in response to *R. solanacearum* infection, we
593 sought to understand the implications of overexpressing genes involved in the synthesis of
594 these compounds in susceptible tomato cultivars upon *R. solanacearum* infection. We
595 focused on *FHT* and *THT* and because their corresponding transcripts are upregulated in the
596 xylem vasculature of resistant tomato upon *R. solanacearum* infection (Fig. 5) and they are
597 the enzymes related with the synthesis of suberin ferulates and ether linked
598 feruloyltyramine, respectively.

599

600 *SfFHT* overexpression had a small effect on the responses of susceptible tomato against *R.*
601 *solanacearum*, showing a slight delay in wilting symptoms together with a slight decrease
602 of bacterial loads in the plant (Fig. 6). The fact that increasing the levels of FHT in
603 Marmande does only result in a marginal increase in resistance might be linked to a
604 shortfall of aliphatic precursors in this variety (Fig. 5), which constrain a subsequent
605 increase in suberin synthesis. In contrast, transgenic tomato overexpressing *SiTHT1-3* on a
606 susceptible background was highly resistant to *R. solanacearum* (Fig. 7). Importantly, this
607 transgenic line was previously shown to accumulate elevated amounts of soluble HCAs
608 such as feruloyltyramine and also SA upon infection with the bacteria *Pseudomonas*
609 *syringae* pv. *tomato* (*Pto*) and to slightly but significantly restrict bacterial growth (Campos
610 *et al.*, 2014). Since SA does not seem to play a major role in defense responses against *R.*
611 *solanacearum* (Hirsch *et al.*, 2002; Hernández-Blanco *et al.*, 2007; Hanemian *et al.*, 2016),
612 accumulation of this hormone in *SiTHT1-3* overexpressing line may not be the major
613 underlying cause of the observed increase in resistance in this line. Alternatively, enhanced
614 production of tyramine-derived HCAs may constitute an important defense strategy

615 against *R. solanacearum*. Feruloyltyramines exhibit antimicrobial activity (Fattorusso *et al.*,
616 1999; Novo *et al.*, 2017) and that they can be involved in plant priming or an adaptive
617 strategy where plants are in a physiological state with improved defensive capacity (Zeiss
618 *et al.*, 2021). These tyramine-derived HCAAs overproduced in *S/THT1-3* overexpressing
619 plants may interfere with *R. solanacearum* colonization by becoming incorporated into the
620 vascular and perivascular cell walls, providing a stronger cross-linking and restricting the
621 movement of the pathogen inside the plant (Figure 8) but also partly by remaining soluble
622 and acting as direct antimicrobial agents against the pathogen. *R. solanacearum* possesses a
623 hydroxycinnamic acid degradation pathway and it has been shown that mutants that cannot
624 degrade hydroxycinnamic acids are less virulent on tomato (Lowe-Power *et al.*, 2015;
625 Zhang *et al.*, 2019), which clearly underscores the importance of HCAAs in the arms race
626 taking place in this pathosystem. Considering that the ligno-suberin pathway and HCAAs
627 are well-conserved across the plant kingdom (Philippe *et al.*, 2020; Kashyap *et al.*, 2021;
628 Zeiss *et al.*, 2021), these findings open the possibility to engineer disease resistance in other
629 *R. solanacearum* hosts by manipulating these pathways. Interestingly, ligno-suberin
630 deposits and accumulation of HCAAs have been reported in response to drought (Macoy *et*
631 *al.*, 2015; Zhang *et al.*, 2020). Therefore, engineering these pathways could have a double
632 impact both on biotic and abiotic stress responses, improving plant performance in the field
633 under adverse conditions.

634

635 In conclusion, we have provided evidence of the formation of a “ligno-suberization zone”
636 enriched in ether-linked feruloyltyramine and possibly related amides as an effective
637 strategy to confine *R. solanacaerum* into infected vessels of resistant tomato plants,
638 preventing horizontal spread of the pathogen into healthy tissues and delaying disease
639 symptoms. Resistance against *R. solanacearum* can be attained in susceptible tomato
640 background by stably overexpressing *THT*, potentially contributing In the future, it will be
641 interesting to investigate the contribution of HCAAs and suberin to resistance against the
642 pathogen, the mechanisms whereby *R. solanacearum* perception leads to the formation of a
643 ligno-suberin coatings around the vasculature in resistant tomato varieties. Increasing the
644 spatio-temporal resolution of the tomato-*R. solanacearum* interaction will be instrumental
645 to reach a deeper insight into structural resistance mechanisms. Also, since vascular

646 confinement has been reported in different plant species as a means of resistance against
647 various vascular wilt pathogens (De Ascensao and Dubery, 2000; Martín *et al.*, 2008; Xu *et*
648 *al.*, 2011; Sabella *et al.*, 2018), the level of conservation of vascular ligno-suberin
649 deposition and HCAAs as a constituent of vascular coatings and part of a resistance
650 mechanism remains to be determined.

651

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Author Contribution

AK designed and performed experiments, interpreted data and wrote the manuscript.

ALJ-J performed the experiments required for the second submission of the manuscript.

WZ performed experiments.

MC performed experiments, interpreted data and reviewed the manuscript.

SS conducted preliminary spectroscopy experiments and reviewed the manuscript.

JR isolated the lignin/suberin fractions and conducted the 2D-HSQC NMR analysis,
including data interpretation.

AG isolated the lignin/suberin fractions and conducted the 2D-HSQC NMR analysis,
including data interpretation.

AL conducted preliminary spectroscopy experiments and reviewed the manuscript.

OS conducted histopathology staining experiments, interpreted data and reviewed the manuscript.

MF interpreted data and reviewed the manuscript.

MV designed experiments, interpreted data and reviewed the manuscript.

NSC conceptualized the research, designed experiments, interpreted data and wrote the manuscript.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supplemental data:

Table S1: List of primers used in this study.

Table S2: Assignments of the correlation signals in the 2D HSQC spectra.

Figure S1: Tissue used for analysis and bacterial dynamics.

Figure S2: H7996 plants show mild symptoms upon challenge inoculation of *R. solanacearum*.

Figure S3: Vascular coating response to *R. solanacearum* infection with wall bound phenolics.

Figure S4: Expression of suberin biosynthetic genes in xylem vasculature of taproots upon infection of *R. solanacearum*.

Figure S5: Phylogeny of Feruloyl transferase (FHT) orthologues in different plant species and expression of the putative tomato FHT ortholog in response to *Ralstonia solanacearum* infection.

Figure S6: Phylogeny of tyramine hydroxycinnamoyl transferase (THT) orthologues in different plant species and expression of the tomato THT gene family members in response to *R. solanacearum* infection.

Figure S7: Expression of phenylpropanoid pathway genes in xylem vasculature of taproots upon invasion of *R. solanacearum*.

Figure S8: Immunoblot of $35S::S1FHT-HA$ in independent Marmande tomato lines expressing $35S::S1FHT-HA$ (Marmande).

Figure S9: Fresh weight of $35S::S1FHT-HA$ plants.

Figure S10: Fresh weight of $35S::S1THT1-3$ plants.

Figure S11: Overexpression of *SiTHT1-3* in tomato results in restricted colonization by *R. solanacearum*.

Figure Legends

Figure 1: Resistant H7996 tomato restricts *Ralstonia solanacearum* colonization and induces a vascular coating response with wall bound phenolics. Susceptible (Marmande) and resistant (H7996), 5-week old tomato plants were inoculated through roots by soil-soak with $\sim 1 \times 10^7$ colony forming units (CFU)/ml of *R. solanacearum* GMI1000 and incubated at 28°C. **(a)** At 12 days post-inoculation (dpi) most Marmande plants showed severe wilting symptoms, whereas H7996 remained mostly symptomless. **(b)** Cross-sections of the taproot-to-hypocotyl area containing 10^5 CFU g⁻¹ of *R. solanacearum* were analyzed by ultraviolet (UV) microscopy. To focus on cell wall-deposited phenolic compounds, soluble phenolic compounds were removed with ethanol prior to observation. A strong autofluorescence signal emitted from the walls of vessels and surrounding parenchyma cells in infected H7996 plants compared to Marmande or the mock controls can be observed. Fluorescence signal in white was green colored. Images from a representative experiment out of 3 with $n=5$ plants per cultivar. Scale bar = 500 μ m. **(c)** The UV auto-fluorescence signal in (b) was measured using the Leica Application Suite X (LAS X) software. A representative region of interest (ROI) is highlighted in (b) and corresponded to a line traversing the selected vascular bundles. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. Different letters indicate statistically significant differences ($\alpha=0.05$, Fisher's least significant difference test).

Figure 2: Feruloylamides, tyramine-derived amides and suberin-compatible compounds are specifically enriched in resistant H7996 tomato after infection with *R. solanacearum*. **(a)** Two dimensional heteronuclear single-quantum correlation–nuclear magnetic resonance (2D-HSQC NMR) spectra of enzymatically isolated lignin/suberin fractions from mock-treated and *R. solanacearum*-infected taproots (containing 10^5 CFU g⁻¹ taproot-to-hypocotyl transition tissue) of H7996 and Marmande tomato plants. The

experiment was performed twice with similar results. **(b)** Main lignin/suberin structures identified: β -O-4' alkyl aryl ethers (A), β -5' fenylcoumarans (B), β - β ' resinols (C), cinnamyl alcohols end-groups (I), feruloylamides (FAm), tyramine-derived amides (Ty), guaiacyl lignin units (G), syringyl lignin units (S), as well as unassigned aliphatic signals from suberin. The structures and contours of the HSQC signals are color coded to aid interpretation. ^1H and ^{13}C NMR chemical shifts of the assigned signals are detailed in Table S2. To detect FAm₇ signal, the spectrum scaled-up to 2-fold ($\times 2$) intensity. The abundances of the main lignin linkages (A, B and C) and cinnamyl alcohol end-groups (I) are referred to as a percentage of the total lignin units (S + G = 100%).

Figure 3: Resistant H7996 tomato shows vascular autofluorescence not-quenched with phloroglucinol and susceptible Marmande shows a decrease in phloroglucinol-HCl lignin signal. Susceptible (Marmande) and resistant (H7996) 5-week-old tomato plants were root-inoculated with a *R. solanacearum* GMI1000 strain at a concentration of $\sim 1 \times 10^7$ CFU/ml or water mock. **(a)** Cross-sections of the taproot-to-hypocotyl area containing 10^5 CFU g⁻¹ of *R. solanacearum* were stained with phloroglucinol-HCl and observed under UV to visualize other autofluorescent compounds different from lignin (not quenched with phloroglucinol-HCl) (right) and under brightfield to visualize lignin deposition (left). In infected H7996 strong UV autofluorescence could be observed in the walls of xylem vessels surrounding xylem parenchyma cells and tracheids, indicating reinforcement of walls of vascular tissue with phenolics formed *de novo* upon infection. In infected Marmande the red phloroglucinol stain was reduced especially in the intervessel areas. **(b)** The UV auto-fluorescence signal in (a) was measured using the LAS X Leica software after the Phloroglucinol-HCl treatment. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. **(c)** Detailed observation of infected H7996 xylem after the Phloroglucinol-HCl treatment shows the strong UV fluorescence concentrated in specific areas possibly corresponding to intervessel and vessel-parenchyma bordered pit membranes and/or pit chambers (yellow and white arrows, respectively). Fluorescence was also observed in parenchyma cells, specially enriched at intercellular cell corners (green arrow). (b) correspond to a representative experiment out of 3 each with n=6

plants per variety. Different letters indicate statistically significant differences ($\alpha=0.05$, Fisher's least significant difference test). (a) and (c) were representative images. Scale bars = 100 μm in (a, left), 500 μm in (a, right) and 50 μm in (c).

Figure 4: Resistant H7996 tomato shows cell wall ferulate/feruloylamide and suberin deposition in restricted zones of vascular tissue upon *R. solanacearum* infection. Susceptible Marmande or resistant H7996 tomato plants were soil-inoculated with a $\sim 1 \times 10^7$ CFU/ml suspension of *Ralstonia solanacearum* GMI1000 or mock-inoculated with water and incubated at 28°C. Cross-sections were obtained from taproot-to-hypocotyl transition tissue containing 10^5 CFU g^{-1} of *R. solanacearum*. Sections were stained with Sudan IV to visualize suberin aliphatics and subsequently treated with 1N KOH (pH above 10) to visualize ferulic acid bound to cell wall. Sudan IV positive staining (reddish-brown coloration) was observed around xylem vessels specifically in infected H7996, indicating accumulation of suberin aliphatics. Accumulation of ferulic acid bound to cell wall (blue-green coloration) appears also specifically in infected H7996 resistant tomato, surrounding Sudan IV-stained areas. White arrowheads indicate the sites of accumulation of ferulates and aliphatic compounds. Representative images from one experiment out of three with $n=6$ plants each were taken. Scale bar= 100 μm .

Figure 5: Genes of the ligno-suberin heteropolymer biosynthesis pathway are specifically induced in the xylem vasculature of resistant H7996 tomato upon *R. solanacearum* infection. The levels of expression of genes belonging to metabolic pathways relevant for suberin, lignin and feruloyltyramine and related amides biosynthesis were analyzed by quantitative PCR (qPCR) of taproot vascular tissue in infected or mock-treated H7996 or Marmande tomato plants. Plants containing an *R. solanacearum* inoculum of 10^5 CFU g^{-1} were selected and xylem vascular tissue from the taproot-to-hypocotyl transition zone, comprising of metaxylems and surrounding parenchyma cells was collected for RNA extraction and cDNA synthesis. In parallel, xylem tissue was collected from mock plants. Heatmaps show \log_2 fold change RTA (relative transcript abundance) values of infected vs. mock for Marmande (left) and Hawaii (right). The tomato gene encoding for the alpha-subunit of the translation elongation factor 1 (*SleEF1 α*) was used as endogenous

reference. Three biological replicates ($n=3$) were used, and taproots of 6 plants were used in each replicate. All the original qPCR results can be found in Figs. S3, S4, S5 and S6. The scheme represents the phenylpropanoid and suberin biosynthesis pathways providing lignin-like and suberin precursors for the ligno-suberin heteropolymer. Abbreviations: PAL: Phenylalanine ammonia-lyase; C4H: Cinnamate-4-hydroxylase; C3H: Coumarate 3-hydroxylase; 4CL: 4-Coumarate-CoA ligase; HCT: Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; COMT: Caffeic acid 3-O-methyltransferase; CCoAOMT: Caffeoyl CoA 3-O-methyltransferase; CYP86A1 and CYP86B1: cytochrome P450 fatty acid ω -hydroxylases; KCS1/2: 3-ketoacyl-CoA synthase; FAR 1/3/4: Fatty acyl-CoA reductase; GPAT5: glycerol-3-phosphate acyltransferase 5; THT: Tyramine hydroxycinnamoyl transferase; TyDC: Tyrosine decarboxylase; FHT: feruloyl transferase. The question mark (?) denotes a hypothetical reaction.

Figure 6: Overexpression of *SIFHT-HA* in susceptible tomato slightly restricts colonization by *R. solanacearum*. (a, b) A pathogenicity assay was performed comparing Wt and 3 independent $35S::SIFHT-HA$ Marmande tomato lines (a, c and d) after infection with *R. solanacearum* GMI1000 lux reporter strain. Five-week-old plants were soil-soak inoculated with $\sim 1 \times 10^7$ CFU/ml or mock and grown at 28°C. (a) Wilting progress was monitored by rating plants daily on a 0 to 4 disease index scale where 0 = healthy and 4 = 100% wilted. Plotted values correspond to means \pm standard error of 24 independent plants ($n=24$) from a representative experiment out of a total of 3. Asterisks indicate statistically significant differences between Wt and each of the $35S::FHT-HA$ analyzed using a paired Student's t-test (* $p < 0.05$). (b) The level of *R. solanacearum* colonization in the taproot and hypocotyl was calculated as colony forming units per gram of fresh taproot tissue ($CFU \cdot g^{-1}$) at 12 dpi. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. Data presented are of a representative experiment out of a total of 3 experiments. In (a) a Kruskall-Wallis test at each day post infection (dpi) was conducted to examine differences in disease index among different genotypes. Significant differences among genotypes were confirmed by applying a pairwise Wilcoxon test. Asterisks

indicate statistically significant differences between wild type and *35S::FHT-HA* tomato lines in a (* corresponds to a p-value of $p < 0.05$).

Figure 7: Overexpression of *SITHT1-3* in susceptible tomato confers resistance to *R. solanacearum*. **(a, b)** A pathogenicity assay was performed comparing Wt and *35S::SITHT1-3* tomato lines (Moneymaker background) after infection with *R. solanacearum* lux reporter GMI1000 strain. Five-week-old plants were soil-soak inoculated with $\sim 1 \times 10^7$ CFU/ml and grown at 28°C. **(a)** Wilting progress was monitored by rating plants daily on a 0 to 4 disease index scale where 0 = healthy and 4 = 100% wilted. Plotted values correspond to means \pm standard error of 24 independent plants ($n=24$) from a representative experiment out of a total of 3. Asterisks indicate statistically significant differences between Wt and *35S::SITHT1-3* using a Kruskall-Wallis test at each day post infection (dpi) was conducted to examine differences in disease index among different genotypes. Significant differences among genotypes were confirmed by applying a pairwise Wilcox test". (* corresponds to a p-value of $p < 0.05$ and *** to $p < 0.0001$). **(b)** Pictures were taken 12 days post-infection. Wt plants were arranged according to the degree of symptom severity (from 4 to 0). **(c)** Transgenic *35S::SITHT1-3* tomato significantly restricted *R. solanacearum* colonization in both the taproot-to-hypocotyl transition zone and hypocotyl compared to Wt. Five-week-old tomato plants were root-inoculated with a *R. solanacearum* GMI1000 luciferase reporter strain at a concentration of $\sim 1 \times 10^7$ CFU/ml or water mock. The level of *in planta* colonization by *R. solanacearum* was calculated as colony forming units per gram of fresh taproot tissue ($\text{CFU} \cdot \text{g}^{-1}$) at 12 dpi. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. Box-and-whisker plots show data from a representative experiment out of 3 ($n = 14$ to 16). (*** corresponds to a p-value of $p < 0.0001$) **(d)** Transverse stem cross-sections of Wt and transgenic *35S::SITHT1-3* tomato lines were imaged under a confocal microscope 6 days after infection with a *R. solanacearum* GMI1000 green fluorescent protein (GFP) reporter strain. *R. solanacearum* at a concentration of 10^5 CFU ml^{-1} was injected directly into the xylem vasculature of the first internode thorough the petiole. Orange arrow points the site of inoculation. Representative images of *R. solanacearum* colonization progress at the point

of inoculation are shown. Scale bar= 2 mm. **(e)** Mean green fluorescence of the GFP signal emitted from *R. solanacearum* at cross-sections obtained as described in (d) at the point of inoculation (0), below the point of inoculation (-0.5 cm) and above the point of inoculation (+0.5 cm) was measured using ImageJ. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. Data from a representative experiment out of a total of 3, with $n=5$ plants per condition. In (a) a Kruskall-Wallis test at each day post infection (dpi) was conducted to examine differences in disease index among different genotypes. Significant differences among genotypes were confirmed by applying a pairwise Wilcox test. Asterisks indicate statistically significant differences between wild type and *35S::SITHT1-3* tomato lines in (a) (* corresponds to a p-value of $p < 0.05$ and ** to $p < 0.001$).

Figure 8: Overexpression of *SITHT1-3* in susceptible tomato results in vascular autofluorescence not-quenched with phloroglucinol and cell wall ferulate/feruloylamide deposition in restricted zones of vascular tissue upon *R. solanacearum* infection. Susceptible Moneymaker (Wt) or resistant *35S::SITHT1-3* overexpressing tomato plants were soil-inoculated with a $\sim 1 \times 10^7$ CFU/ml suspension of *R. solanacearum* GMI1000 lux reporter strain. Cross-sections were obtained from taproot-to-hypocotyl of both genotypes tissue containing 10^5 CFU g^{-1} of *R. solanacearum*. **(a)** Cross-sections were stained with phloroglucinol-HCl and observed under brightfield to visualize lignin deposition (left) and under UV to visualize other auto-fluorescent compounds different from lignin (not quenched with phloroglucinol- HCl) (right). **(b)** Combined Sudan IV+KOH treatment showed no positive suberin aliphatic signal in *SITHT1-3*, but a significant increase in ferulate/feruloylamide accumulation upon infection. **(c)** Close-ups (10X) of the vascular bundles of Wt and *35S::SITHT1-3* infected plants pointed with a white arrow in (b) are also shown. Images from a representative experiment out of 3 with $n=6$ plants per cultivar. **(d)** Quantification of UV fluorescence after phloroglucinol-HCl staining as shown in (a, right) were performed with LAS X software by selecting the vascular areas surrounding main vessels with strong localized fluorescence or green signal. **(e)** Quantification of UV green fluorescence from ferulate deposits after

Sudan IV+KOH staining as shown in (a, right) were performed with LAS X software by selecting the vascular areas surrounding main vessels with strong localized fluorescence or green signal. Data in (d) and (e) are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. Different letters indicate statistically significant differences ($\alpha=0.05$, Fisher's least significant difference test). Scale bar = 500 μm .

Figure 9: Schematic representation of the vascular ligno-suberization process potentially taking place in infected vessels of resistant H7996 tomato upon *R. solanacearum* infection. Colonization of the vasculature by *R. solanacearum* in resistant tomato plants induces a ligno-suberization process in the walls of the infected vessel (V) and of the adjacent tracheids (T) and parenchyma cells (XP) (red). The lignin-like polymer accompanying suberin would be enriched in structural feruloyltyramine and related amides. The signal of structural ferulic acid (ester or as amide) would extend to the walls of peripheral parenchyma cells, vessels and tracheids (green), indicating a stage preceding suberization or a final layered pattern, still to be resolved. Together, the red and green areas, would form a “zone of ligno-suberization” (black dashed line) potentially creating a physico-chemical barrier to limit *R. solanacearum* spread from the colonized xylem vessel lumen.

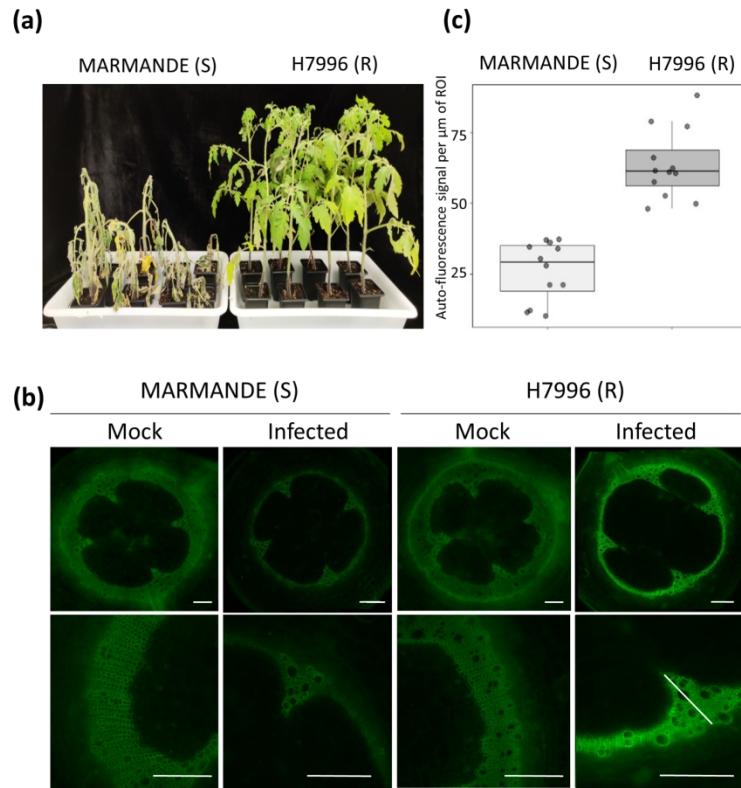


FIGURE 1

Figure 1: Resistant H7996 tomato restricts *Ralstonia solanacearum* colonization and induces a vascular coating response with wall bound phenolics. Susceptible (Marmande) and resistant (H7996), 5-week old tomato plants were inoculated through roots by soil-soak with $\sim 1 \times 10^7$ colony forming units (CFU)/ml of *R. solanacearum* GMI1000 and incubated at 28°C. (a) At 12 days post-inoculation (dpi) most Marmande plants showed severe wilting symptoms, whereas H7996 remained mostly symptomless. (b) Cross-sections of the taproot-to-hypocotyl area containing 105 CFU g⁻¹ of *R. solanacearum* were analyzed by ultraviolet (UV) microscopy. To focus on cell wall-deposited phenolic compounds, soluble phenolic compounds were removed with ethanol prior to observation. A strong autofluorescence signal emitted from the walls of vessels and surrounding parenchyma cells in infected H7996 plants compared to Marmande or the mock controls can be observed. Fluorescence signal in white was green colored. Images from a representative experiment out of 3 with $n=5$ plants per cultivar. Scale bar = 500 μm . (c) The UV auto-fluorescence signal in (b) was measured using the Leica Application Suite X (LAS X) software. A representative region of interest (ROI) is highlighted in (b) and corresponded to a line traversing the selected vascular bundles. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate

second quartile, median and third quartile. Different letters indicate statistically significant differences ($\alpha=0.05$, Fisher's least significant difference test).

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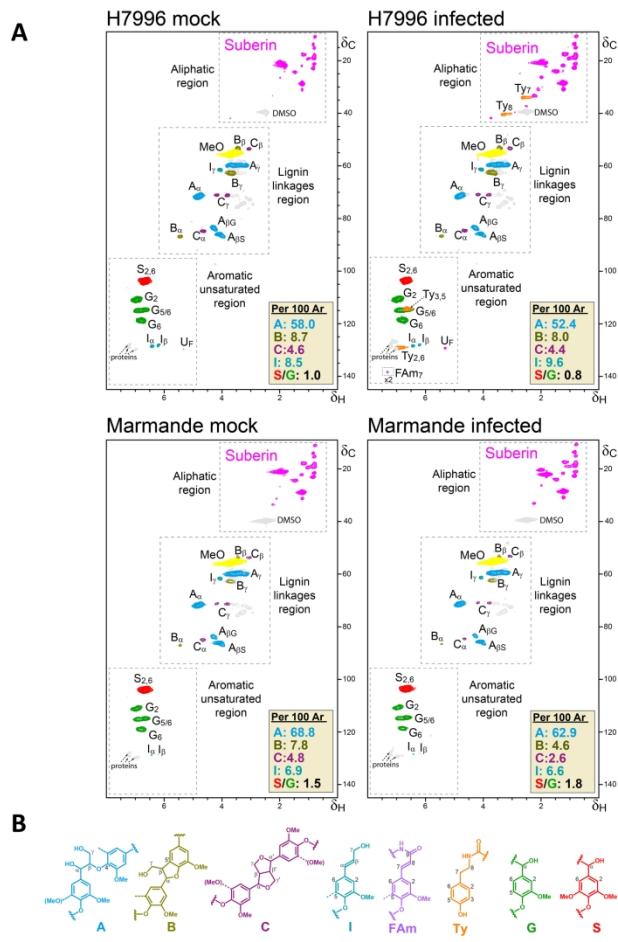


Figure 2: Feruloylamides, tyramine-derived amides and suberin-compatible compounds are specifically enriched in resistant H7996 tomato after infection with *R. solanacearum*. (a) 2D-HSQC NMR spectra of enzymatically isolated lignin/suberin fractions from mock-treated and *R. solanacearum*-infected taproots (containing 105 CFU g⁻¹ taproot-to-hypocotyl transition tissue) of H7996 and Marmande tomato plants. The experiment was performed twice with similar results. (b) Main lignin/suberin structures identified: β -O-4' alkyl aryl ethers (A), β -5' fencylcoumarans (B), β - β ' resinsols (C), cinnamyl alcohols end-groups (D), feruloylamides (FAm), tyramine-derived amides (Ty), guaiacyl lignin units (G), syringyl lignin units (S), as well as unassigned aliphatic signals from suberin. The structures and contours of the HSQC signals are color coded to aid interpretation. 1H and 13C NMR chemical shifts of the assigned signals are detailed in Table S1. To detect FAm7 signal, the spectrum scaled-up to 2-fold ($\times 2$) intensity. The abundances of the main lignin linkages (A, B and C) and cinnamyl alcohol end-groups (D) are referred to as a percentage of the total lignin units (S + G = 100%).

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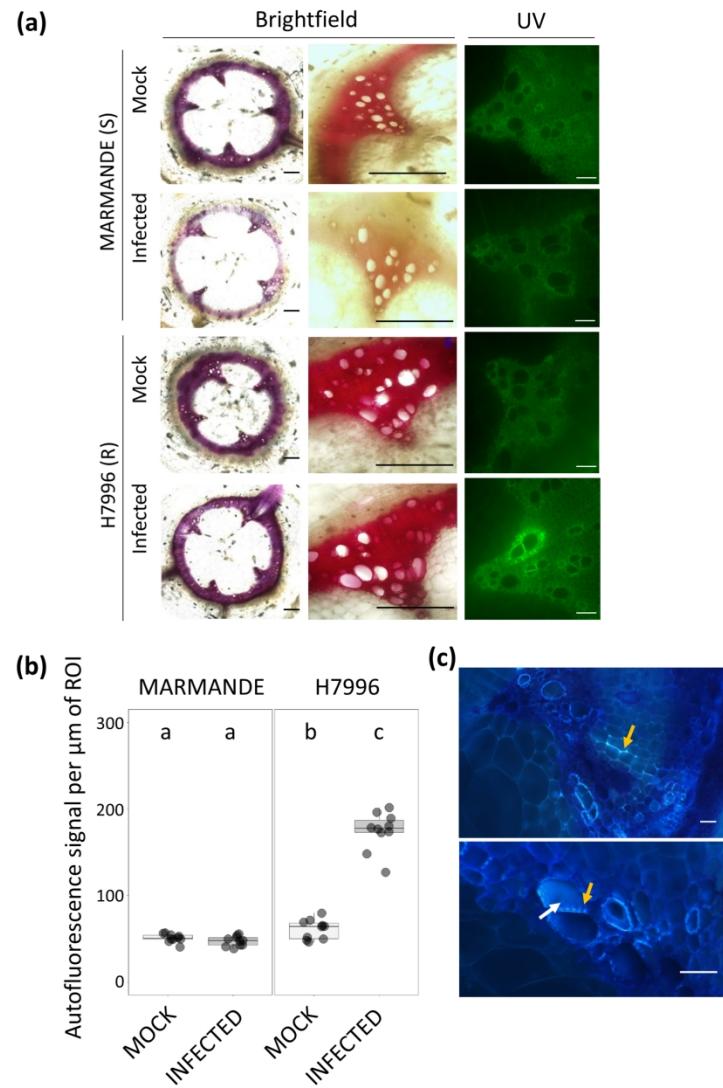


FIGURE 3

Figure 3: Resistant H7996 tomato shows vascular autofluorescence not-quenched with phloroglucinol and susceptible Marmande shows a decrease in phloroglucinol-HCl lignin signal. Susceptible (Marmande) and resistant (H7996) 5-week-old tomato plants were root-inoculated with a *R. solanacearum* GMI1000 strain at a concentration of $\sim 1 \times 10^7$ CFU/ml or water mock. (a) Cross-sections of the taproot-to-hypocotyl area containing 105 CFU g⁻¹ of *R. solanacearum* were stained with phloroglucinol-HCl and observed under UV to visualize other autofluorescent compounds different from lignin (not quenched with phloroglucinol-HCl) (right) and under brightfield to visualize lignin deposition (left). In infected H7996 strong UV autofluorescence could be observed in the walls of xylem vessels surrounding xylem parenchyma cells and tracheids, indicating reinforcement of walls of vascular tissue with phenolics formed *de novo* upon infection. In infected Marmande the red phloroglucinol stain was reduced especially in the intervessel areas. (b) The UV auto-fluorescence signal in (a) was measured using the LAS X Leica software after the Phloroglucinol-HCl treatment. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. (c) Detailed observation of infected H7996 xylem after the Phloroglucinol-HCl treatment shows the strong UV fluorescence

concentrated in specific areas possibly corresponding to intervessel and vessel-parenchyma bordered pit membranes and/or pit chambers (yellow and white arrows, respectively). Fluorescence was also observed in parenchyma cells, specially enriched at intercellular cell corners (green arrow). (b) correspond to a representative experiment out of 3 each with $n=6$ plants per variety. Different letters indicate statistically significant differences ($\alpha=0.05$, Fisher's least significant difference test). (a) and (c) were representative images. Scale bars = 100 μm in (a, left), 500 μm in (a, right) and 50 μm in (c).

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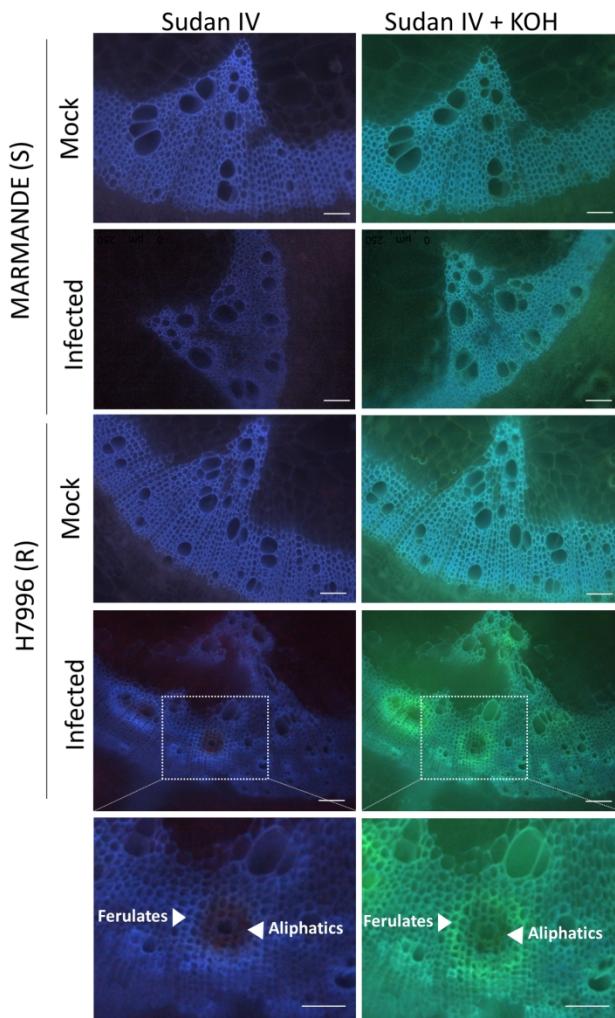


FIGURE 4

Figure 4: Resistant H7996 tomato shows cell wall ferulate/feruloylamide and suberin deposition in restricted zones of vascular tissue upon *R. solanacearum* infection. Susceptible Marmande or resistant H7996 tomato plants were soil-inoculated with a $\sim 1 \times 10^7$ CFU/ml suspension of *Ralstonia solanacearum* GMI1000 or mock-inoculated with water and incubated at 28°C. Cross-sections were obtained from taproot-to-hypocotyl transition tissue containing 105 CFU g⁻¹ of *R. solanacearum*. Sections were stained with Sudan IV to visualize suberin aliphatics and subsequently treated with 1N KOH (pH above 10) to visualize ferulic acid bound to cell wall. Sudan IV positive staining (reddish-brown coloration) was observed around xylem vessels specifically in infected H7996, indicating accumulation of suberin aliphatics. Accumulation of ferulic acid bound to cell wall (blue-green coloration) appears also specifically in infected H7996 resistant tomato, surrounding Sudan IV-stained areas. White arrowheads indicate the sites of accumulation of ferulates and aliphatic compounds. Representative images from one experiment out of three with $n=6$ plants each were taken. Scale bar= 100 μ m.

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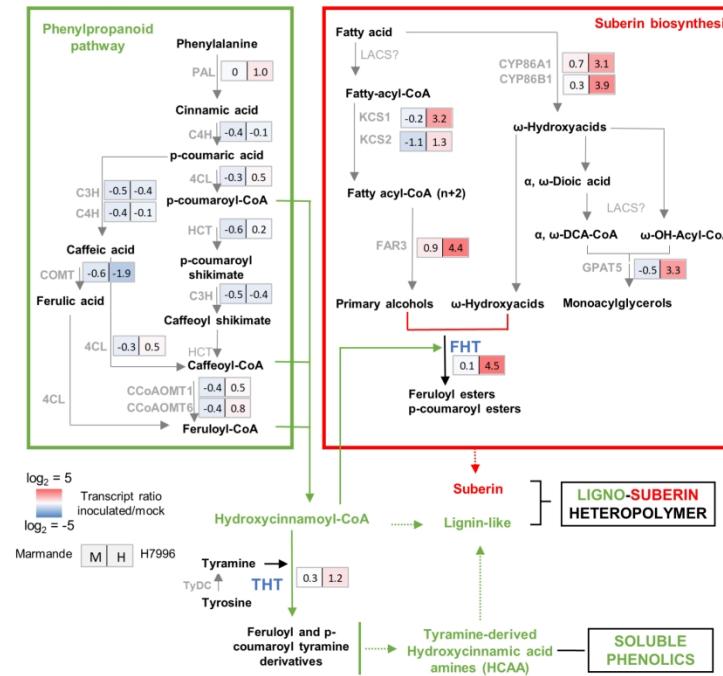


FIGURE 5

Figure 5: Genes of the ligno-suberin heteropolymer biosynthesis pathway are specifically induced in the xylem vasculature of resistant H7996 tomato upon *R. solanacearum*. The levels of expression of genes belonging to metabolic pathways relevant for suberin, lignin and feruloyltyramine and related amides biosynthesis were analyzed by qPCR of taproot vascular tissue in infected or mock-treated H7996 or Marmande tomato plants. Plants containing an *R. solanacearum* inoculum of 105 CFU g⁻¹ were selected and xylem vascular tissue from the taproot-to-hypocotyl transition zone, comprising of metaxylems and surrounding parenchyma cells was collected for RNA extraction and cDNA synthesis. In parallel, xylem tissue was collected from mock plants. Heatmaps show log₂ fold change RTA (relative transcript abundance) values of infected vs. mock for Marmande (left) and Hawaii (right). The tomato gene encoding for the alpha-subunit of the translation elongation factor 1 (SleEF1 α) was used as endogenous reference. Three biological replicates (n=3) were used, and taproots of 6 plants were used in each replicate. All the original qPCR results can be found in Figs. S3, S4, S5 and S6. The scheme represents the phenylpropanoid and suberin biosynthesis pathways providing lignin-like and suberin precursors for the ligno-suberin heteropolymer. Abbreviations: PAL: Phenylalanine ammonia-lyase; C4H: Cinnamate-4-hydroxylase; C3H: Coumarate 3-

hydroxylase; 4CL: 4-Coumarate–CoA ligase; HCT: Hydroxycinnamoyl–CoA shikimate/quinate hydroxycinnamoyl transferase; COMT: Caffeic acid 3-O-methyltransferase; CCoAOMT: Caffeoyl CoA 3-O-methyltransferase; CYP86A1 and CYP86B1: cytochrome P450 fatty acid ω -hydroxylases; KCS1/2: 3-ketoacyl–CoA synthase; FAR 1/3/4: Fatty acyl–CoA reductase; GPAT5: glycerol-3-phosphate acyltransferase 5; THT: Tyramine hydroxycinnamoyl transferase; TyDC: Tyrosine decarboxylase; FHT: feruloyl transferase. The question mark (?) denotes a hypothetical reaction.

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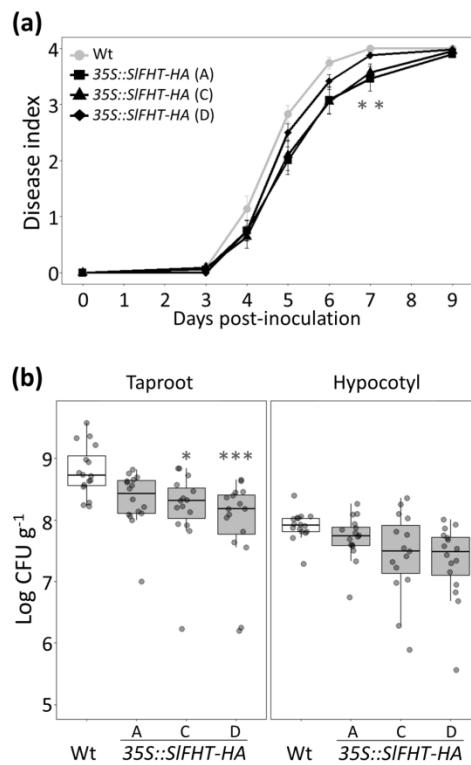


FIGURE 6

Figure 6: Overexpression of SIFHT-HA in susceptible tomato slightly restricts colonization by *R. solanacearum*. (a, b) A pathogenicity assay was performed comparing Wt and 3 independent 35S::SIFHT-HA Marmande tomato lines (a, c and d) after infection with *R. solanacearum* GMI1000 lux reporter strain. Five-week-old plants were soil-soak inoculated with $\sim 1 \times 10^7$ CFU/ml or mock and grown at 28°C. (a) Wilting progress was monitored by rating plants daily on a 0 to 4 disease index scale where 0 = healthy and 4 = 100% wilted. Plotted values correspond to means \pm standard error of 24 independent plants ($n=24$) from a representative experiment out of a total of 3. Asterisks indicate statistically significant differences between Wt and each of the 35S::FHT-HA analyzed using a paired Student's t-test (* $p < 0.05$). (b) The level of *R. solanacearum* colonization in the taproot and hypocotyl was calculated as colony forming units per gram of fresh taproot tissue (CFU·g⁻¹) at 12 dpi. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. Data presented are of a representative experiment out of a total of 3 experiments. In (a) a Kruskall-Wallis test at each day post infection (dpi) was conducted to examine differences in disease index among different genotypes. Significant differences among genotypes were confirmed by applying a pairwise

Wilcox test. Asterisks indicate statistically significant differences between wild type and 35S::FHT-HA tomato lines in a (* corresponds to a p-value of $p < 0.05$).

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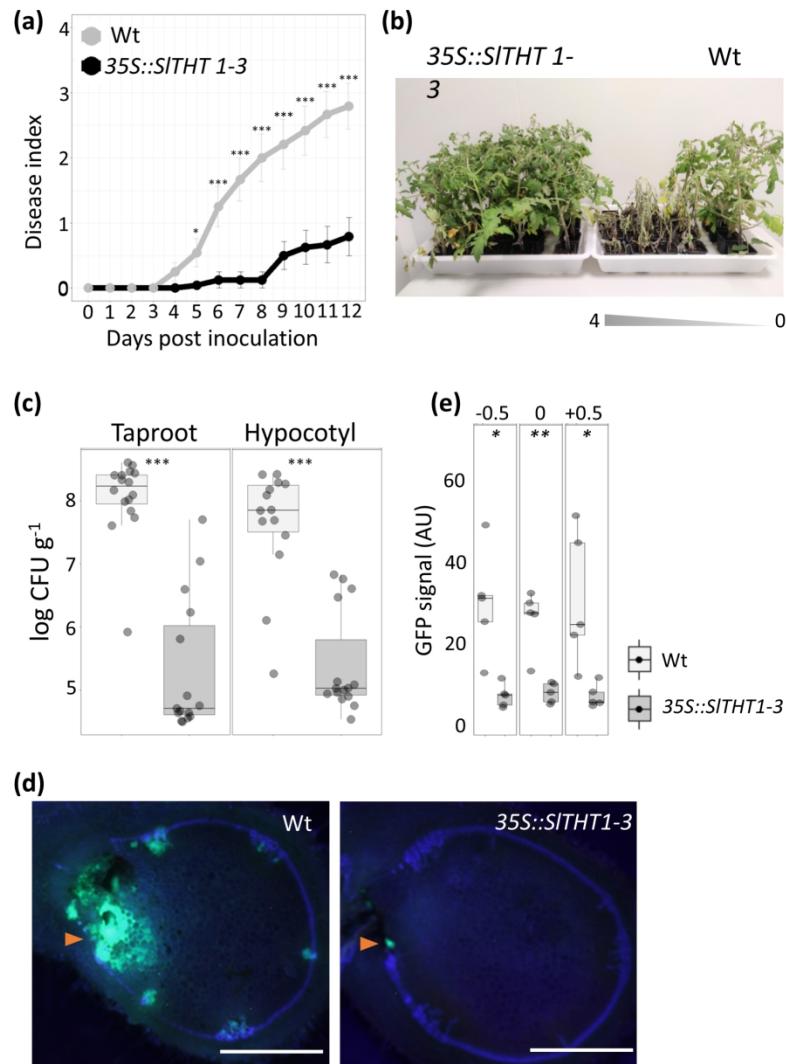


FIGURE 7

Figure 7: Overexpression of SITHT1-3 in susceptible tomato confers resistance to *R. solanacearum*. (a, b) A pathogenicity assay was performed comparing Wt and *35S::SITHT1-3* tomato lines (Moneymaker background) after infection with *R. solanacearum* lux reporter GMI1000 strain. Five-week-old plants were soil-soak inoculated with $\sim 1 \times 10^7$ CFU/ml and grown at 28°C. (a) Wilting progress was monitored by rating plants daily on a 0 to 4 disease index scale where 0 = healthy and 4 = 100% wilted. Plotted values correspond to means \pm standard error of 24 independent plants ($n=24$) from a representative experiment out of a total of 3. Asterisks indicate statistically significant differences between Wt and *35S::SITHT1-3* using a Kruskall-Wallis test at each day post infection (dpi) was conducted to examine differences in disease index among different genotypes. Significant differences among genotypes were confirmed by applying a pairwise Wilcoxon test". (* corresponds to a p-value of $p < 0.05$ and *** to $p < 0.0001$). (b) Pictures were taken 12 days post-infection. Wt plants were arranged according to the degree of symptom severity (from 4 to 0). (c) Transgenic *35S::SITHT1-3* tomato significantly restricted *R. solanacearum* colonization in both the taproot-to-hypocotyl transition zone and hypocotyl compared to Wt. Five-week-old tomato plants were root-inoculated with a *R. solanacearum* GMI1000 luciferase reporter strain at a concentration of $\sim 1 \times 10^7$ CFU/ml

or water mock. The level of in planta colonization by *R. solanacearum* was calculated as colony forming units per gram of fresh taproot tissue (CFU·g⁻¹) at 12 dpi. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. Box-and-whisker plots show data from a representative experiment out of 3 (n = 14 to 16). (** corresponds to a p-value of p < 0.0001) (d) Transverse stem cross-sections of Wt and transgenic 35S::SITHT1-3 tomato lines were imaged under a confocal microscope 6 days after infection with a *R. solanacearum* GMI1000 green fluorescent protein (GFP) reporter strain. *R. solanacearum* at a concentration of 105 CFU ml⁻¹ was injected directly into the xylem vasculature of the first internode thorough the petiole. Orange arrow points the site of inoculation. Representative images of *R. solanacearum* colonization progress at the point of inoculation are shown. Scale bar= 2 mm. (e) Mean green fluorescence of the GFP signal emitted from *R. solanacearum* at cross-sections obtained as described in (d) at the point of inoculation (0), below the point of inoculation (-0.5 cm) and above the point of inoculation (+0.5 cm) was measured using ImageJ. Data are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. Data from a representative experiment out of a total of 3, with n=5 plants per condition. In (a) a Kruskall-Wallis test at each day post infection

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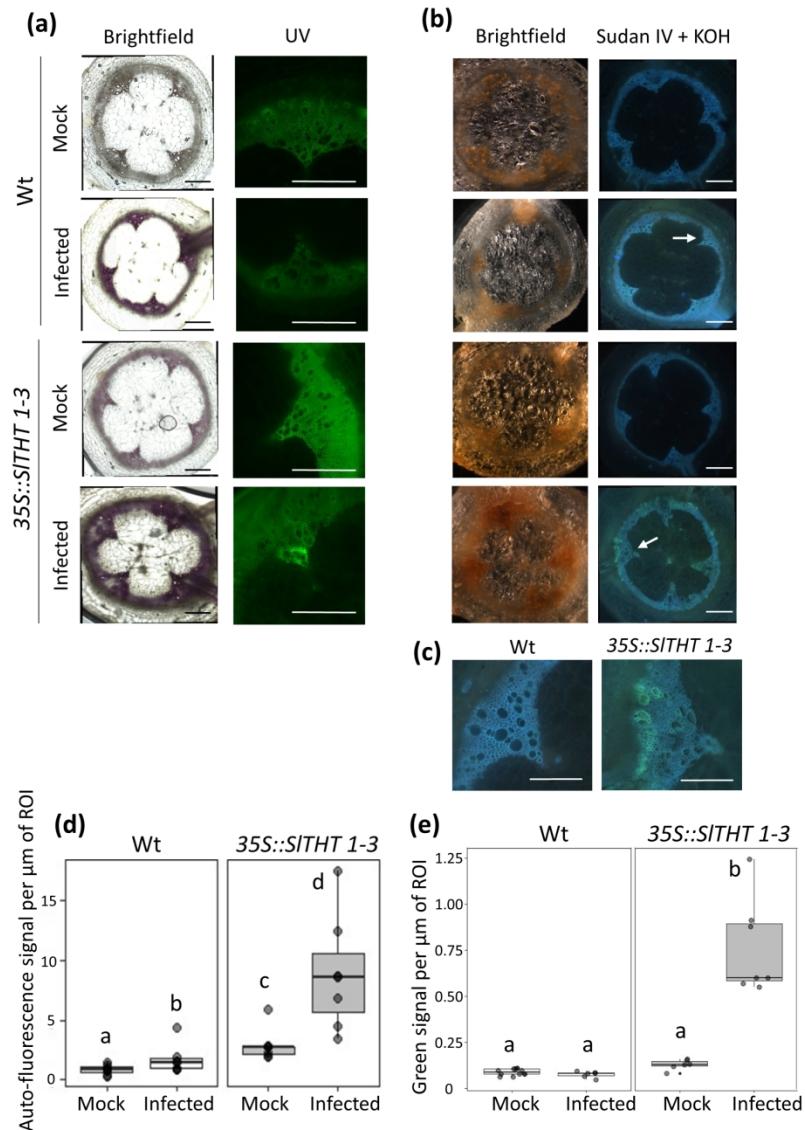


FIGURE 8

Figure 8: Overexpression of SITHT1-3 in susceptible tomato results in vascular autofluorescence not quenched with phloroglucinol and cell wall ferulate/feruloylamide deposition in restricted zones of vascular tissue upon *R. solanacearum* infection. Susceptible Moneymaker (Wt) or resistant 35S::SITHT1-3 overexpressing tomato plants were soil-inoculated with a $\sim 1 \times 10^7$ CFU/ml suspension of *R. solanacearum* GMI1000 lux reporter strain. Cross-sections were obtained from taproot-to-hypocotyl of both genotypes tissue containing 105 CFU g $^{-1}$ of *R. solanacearum*. (a) Cross-sections were stained with phloroglucinol-HCl and observed under brightfield to visualize lignin deposition (left) and under UV to visualize other auto-fluorescent compounds different from lignin (not quenched with phloroglucinol- HCl) (right). (b) Combined Sudan IV+KOH treatment showed no positive suberin aliphatic signal in SITHT1-3, but a significant increase in ferulate/feruloylamide accumulation upon infection. (c) Close-ups (10X) of the vascular bundles of Wt and 35S::SITHT1-3 infected plants pointed with a white arrow in (b) are also shown. Images from a representative experiment out of 3 with n=6 plants per cultivar. (d) Quantification of UV fluorescence after phloroglucinol-HCl staining as shown in (a, right) were performed with LAS X software by selecting the vascular areas surrounding main vessels with strong localized fluorescence or green signal. (e)

Quantification of UV green fluorescence from ferulate deposits after Sudan IV+KOH staining as shown in (a, right) were performed with LAS X software by selecting the vascular areas surrounding main vessels with strong localized fluorescence or green signal. Data in (d) and (e) are represented with box and whiskers plots: whiskers indicate variability outside the upper and lower quartiles and boxes indicate second quartile, median and third quartile. Different letters indicate statistically significant differences ($\alpha=0.05$, Fisher's least significant difference test). Scale bar = 500 μ m.

190x275mm (283 x 283 DPI)

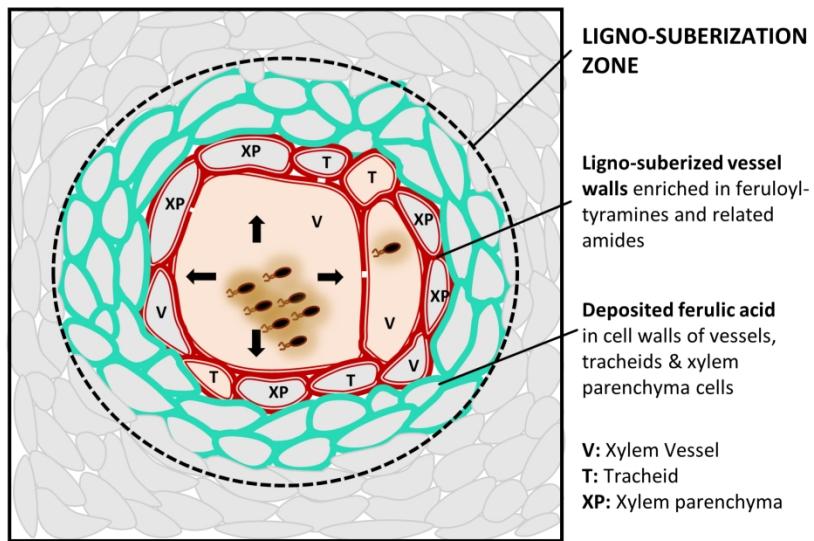


FIGURE 9

Figure 9: Schematic representation of the vascular ligno-suberization process potentially taking place in infected vessels of resistant H7996 tomato upon *R. solanacearum* infection. Colonization of the vasculature by *R. solanacearum* in resistant tomato plants induces a ligno-suberization process in the walls of the infected vessel (V) and of the adjacent tracheids (T) and parenchyma cells (XP) (red). The lignin-like polymer accompanying suberin would be enriched in structural feruloyltyramine and related amides. The signal of structural ferulic acid (ester or as amide) would extend to the walls of peripheral parenchyma cells, vessels and tracheids (green), indicating a stage preceding suberization or a final layered pattern, still to be resolved. Together, the red and green areas, would form a “zone of ligno-suberization” (black dashed line) potentially creating a physico-chemical barrier to limit *R. solanacearum* spread from the colonized xylem vessel lumen.

190x275mm (283 x 283 DPI)