Deciphering the resistance mechanism of tomato plants against whitefly-mediated *Tomato curly stunt virus* infection through UHPLC-MS based metabolomics approaches.

L Rossouw, NE Madala, F Tugizimana, PA Steenkamp, LL Esterhuizen*, IA Dubery*

Department of Biochemistry, University of Johannesburg, Auckland Park, 2006, South Africa

Supplementary Information

S1: Extended Experimental Procedures

Supplementary Figures

Figure S1.1: Diagram illustrating the experimental design employed for this study.

Figure S1.2: Dot blot analysis of viral DNA accumulation in resistant (RT) and susceptible (S) control plants, as well as RT and S plants subjected to whitefly-mediated ToCSV infection (WF+Vir) on days 8, 15, 25 and 35.

S 1. Experimental Procedures

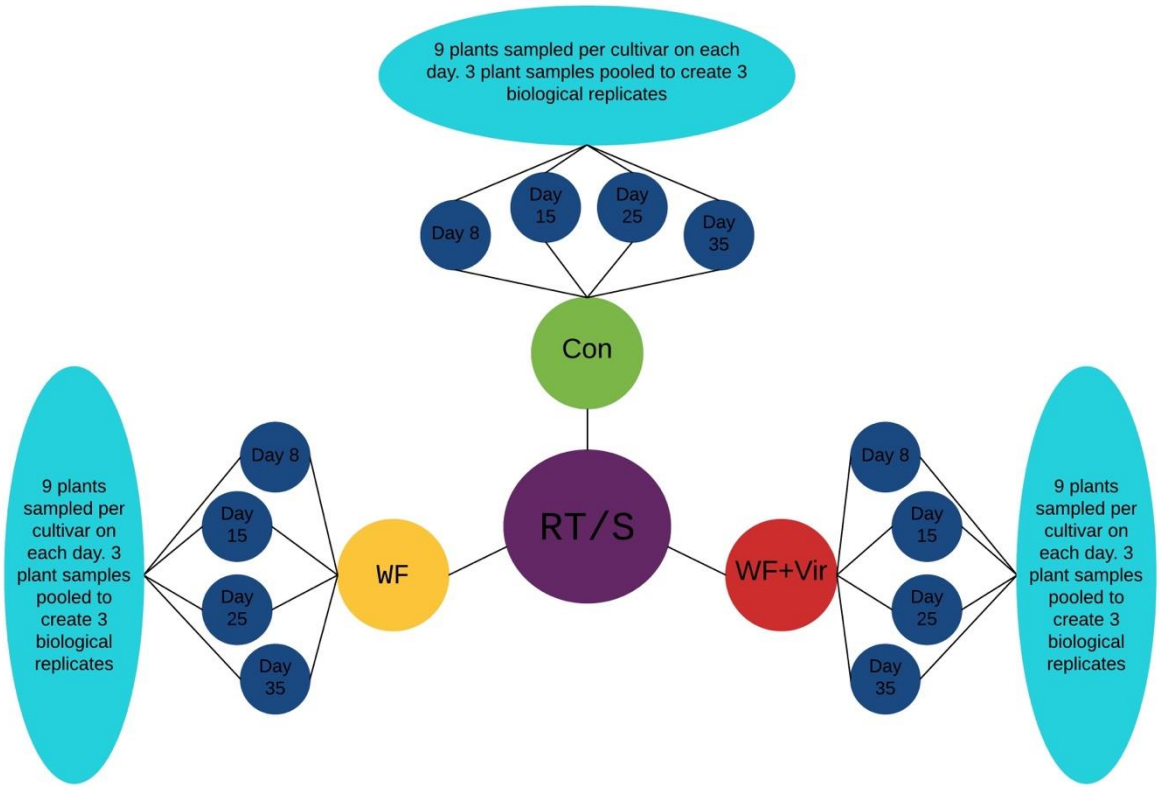

Figure S1.1: *Diagram illustrating the experimental design employed for this study. The centre indicating the two cultivars used, radiating from that the three treatments each cultivar were subjected to and radiating from that each day on which samples were taken (with day 1 being the day the treatment started) (Rossouw, 2016).*

S 1.1 Plant material

Seed of two inbred isogenic tomato lines were obtained from TomaTech Ltd. (Rehovot, Israel). One line, 906-4, is susceptible to TYLCV infection (hereafter S) and the other, 902, is resistant to TYLCV infection (hereafter RT). Both lines originated from a breeding program aimed at introgressing TYLCV resistance genes from *Solanum habrochaites* (accessions LA386 and LA1777) into domestic susceptible *S. lycopersicum*. Seeds from another susceptible tomato cultivar, Rooikhaki, were obtained from Sakata Vegenetics (Lanseria, South Africa).

All seeds were pre-treated in order to control seed borne pathogens by sterilisation in a 2.4% bleach (sodium hypochlorite) solution for 20 min. Subsequently, the seeds were rinsed in distilled water and incubated in a water bath at 30 °C for 25 min and 50 °C bath for 10 min. Finally, the seeds were placed in 10% tri-sodium phosphate buffer pH 8 for 15 min and allowed to dry.

The seeds were sown into a styrofoam seedling tray containing seedling mix (consisting of 1 part 8 mm pine bark, 1 part peat moss, 100 g superphosphate and 200 g lime added per 1 m^3, the mix is steam sterilised prior to use (Sakata, Vegenetics). Three duplicate trays were sown, one for each of the three different treatments: (i) control (Con), (ii) whitefly (WF) only and (iii) viruliferous whitefly (WF+Vir) and covered with vermiculite. The seeds were watered and the tray covered with a black plastic bag. The covered tray was incubated in a greenhouse at 25 °C for three days. After three days the seedlings were uncovered and allowed to grow in a greenhouse at 25 °C.

All sampled plants were treated identically, being allowed to grow under the same conditions throughout the experiment, receiving the same nutrients and being subjected to identical pesticide treatments. Con plants (treatment i) received no treatment other than that stated here, which all plants in the trial received. WF plants (treatment ii) were treated the same as the Con plants, except they were subjected to mock-inoculation by non-viruliferous whitefly (*B. tabaci* MEAM1). WF+Vir plants (treatment iii) were treated the same as the Con plants except they were subjected to whitefly mediated *Tomato curly stunt virus* (ToCSV) inoculation. After allowing whitefly (non-viruliferous on WF plants and viruliferous on WF+Vir plants) four days to feed on the plants, they were killed using pesticides.

Pesticide treatment lasted three days and was started after plants were treated. On the first day, all the treated seedlings (treatment 1, ii and iii) were drenched with Kohinor (Imidacloprid) and sprayed with a mixture of Nomolt (Teflubenzuron), Genesis 100 EC (pyriproxyfen), Actara (Thiamethoxam) and Wetcit (Borax, orange oil) (ratio of 2:5:1:3). On the second day, the seedlings were drenched with a mixture of Previcure (Propamokarb HCL) and Breakthru (ratio of 5:1) and sprayed with the same mixture as the previous day. On the third day, the seedlings were again drenched with Kohinor (Imidacloprid) and sprayed with a mixture of Kocide (copper hydroxide), Dithane (Mancoseb), Biomectin and Topaz (Penconazole) (ratio of 4:4:1.2:0.45). After pesticide treatment the seedlings were transplanted in an insect proof greenhouse and grown hydroponically through spring and early summer employing standard pest and disease control spray schemes (insecticides, miticides and fungicides).

S 1.2 Whitefly colony

Whitefly colonies consisted of *B. tabaci* of the Middle East-Asia Minor 1 (MEAM1) species (previously called biotype B) originating from nymphs that were collected on *S. lycopersicum* in Trichardtsdal, South Africa. The specific *B. tabaci* cryptic species was determined using the mitochondria cytochrome oxidase marker (mtCOI) (Esterhuizen, 2012). Insects were reared in a growth chamber (14 h light / 10 h night photoperiod, 26 ± 2 °C, 50% relative humidity) on healthy cotton and tomato (cv. Rooikhaki) plants.

S 1.3 Preparation of ToCSV infected plant material as source for virus acquisition

S 1.3.1 Virus culture

Agrobacterium tumefaciens strain C58C1 transformed with an agroinfectious clone of ToCSV (Esterhuizen, 2012) were cultured on LB-agar plates containing 50 µg/mL kanamycin and 50 µg/mL rifampicin at 28 °C for two days.

S 1.3.2 Agroinoculation

The viral cultures were used to agroinoculate eighteen day old seedlings of tomato cv. Rooikhaki via the agro-prick method. Agroinoculation of the same seedlings was repeated one week later. Twenty eight to thirty days after the first agroinoculation, samples were taken of the youngest fully expanded leaves of all agroinoculated plants to confirm infection.

S 1.3.3 DNA isolation

DNA of each plant was isolated as follows: 150 mg of plant material was ground to a fine powder using liquid nitrogen and a glass rod; where after 500 µL of extraction buffer (100 mM Tris-HCl pH 8.0 (Merck), 50 mM EDTA pH 8, 500 mM NaCl, 10 mM β-mercaptoethanol, 1% SDS was added. The mixture was incubated at 65 °C for 30 min then 150 µL 5 M potassium acetate was added and the mixture placed on ice for 10 min. The tubes were then centrifuged at 13 000 x*g* (4 °C) for 10 min and the supernatant transferred to a new tube containing 400 µL of 100% ice cold isopropanol and 250 µL 5 M NaCl. The supernatant/isopropanol/NaCl mixture was then incubated at -20 °C overnight. The next day the samples were centrifuged at 13 000 x*g* for 10 min and the supernatant discarded. The pellets were washed twice with 70% ethanol and centrifuged at 13 000 x*g* for 5 min. The supernatants were discarded and the pellets allowed to air dry after which they were reconstituted in 50 µL TE buffer and stored at -20 °C until further use.

S 1.3.4 PCR to confirm infection

To confirm begomovirus infection, viral DNA was amplified by PCR using universal begomovirus primers, TY1(+): 5'-GCCCATGTA(T/C)CG(A/G)AAGCC-3' and TY2(−): 5'-GG(A/G)TTAGA(A/G)GCATG(A/C)G-TAC-3'. The primer pair amplifies a 580 bp DNA fragment comprising the V1 gene (coat protein, CP) from the DNA-A component of begomoviruses. PCR was performed using KAPA Taq DNA polymerase using an Eppendorf thermal cycler. Each PCR was carried out in 25 µL volumes and contained a final concentration of 1 X KAPA Taq buffer A containing 2 mM $MgSO_4$, 0.2 mM dNTPs, 0.2 µM of each primer, 1U KAPA Taq DNA polymerase and 2-3 µL of total DNA extracted from infected plant material. The cycling parameters were as follows: initial denaturation at 94 °C for 2 min, then 35 cycles of denaturation at 94 °C for 20 sec, annealing at 55 °C for 30 sec and elongation at 72 °C for 40 sec, followed by a final elongation step at 72 °C for 10 min. Amplified fragments were separated on a 1% agarose gel stained with 1 µg/mL ethidium bromide and visualized under long-wave UV light.

S 1.3.5. Virus acquisition

Rooikhaki tomato plants which tested positive for ToCSV infection via PCR were placed into cages and non-viruliferous whitefly transferred onto them from cotton leaves. The whiteflies were allowed a three day acquisition access period (AAP). Viral accumulation dot blot - To evaluate viral DNA accumulation throughout the study dot blot analysis was performed. A DIG-labelled probe was synthesised using PCR digoxygenin (DIG) Probe Synthesis kit following the manufacturer's instructions. The probe was synthesised using an abutting primer set, *Xho*-F 5'-GTCTCGAGGTTGTGAAGGCCCATGTAAGATCCAG-3' and *Xho*-R 5'-GTCTCGAGGGACATCAGGGCTTCTATACATTCTG-3'. The primers bind within the coat protein (CP) gene of ToCSV and amplifies the full-length viral genome (~2,7 kb) (Esterhuizen, 2012). The PCR was performed with ExSel high fidelity DNA polymerase using an Eppendorf thermal cycler. Each PCR was carried out in 25 µl volumes and contained a final concentration of 1 X reaction buffer containing 2 mM $MgSO_4$, 0.2 mM dNTPs, 0.2 µM of each primer, 0.08 U ExSel DNA polymerase and 2-3 µL of total DNA extracted from infected plant material. The cycling conditions were as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 20 sec, annealing at 68 °C for 30 sec, extension at 72 °C for 3 min, followed by a final elongation step at 72 °C for 15 min. DNA samples (8 µL each) of isolated plant DNA from each plant were spotted onto positively charged nylon membranes. DNA was then crosslinked to the membrane using UV light. Hybridisation was performed according to the manufacturer's instructions using the DIG-labelled probe and chemiluminescent detection. Membranes were pre-hybridised in DIG-easy-Hyb solution (42 ^{o}C, 30 min agitation). Followed by hybridisation overnight at 42 ^{o}C in fresh DIG-easy-Hyb solution containing 20 ng/mL denatured DIG-probe. Chemiluminescence detection was then carried out using a DIG Luminescence detection kit according to the manufacturer's instructions with DIG-antibody and CSPD chemiluminescent substrate.

S 1.3.5. Viral accumulation

Upon whitefly-mediated inoculation of ToCSV, the **S** line were susceptible and developed symptoms typical of ToCSV infection (upper leaf yellowing, reduction in leaflet area, upward curling margins and moderate to severely stunted internodes) compared to non-inoculated controls. The **RT** line remained symptomless and continued to develop normally, with flowering and fruit setting appearing similar to non-inoculated controls. Dot blots were used to evaluate the accumulation of viral DNA in the **WF+Vir** plants of each cultivar throughout the study.

Figure S1.*2* shows dot blots of viral DNA present in **RT** and **S** control plants and in **RT** and **S WF+Vir** plants on days 8, 15, 25 and 35. Viral DNA accumulation is seen to increase (dots become slightly darker and slightly larger) from day 8 to 15 in **RT WF+Vir** plants. After day 15 virus titer appears to remain approximately the same up to day 25, after which it decreases, as dots again become lighter. As expected, viral DNA is seen to increase steadily in **S** plants from day 8 to 15, as the dots are seen to become darker and larger. After day 15 the virus titer appears to increase slightly up to day 25, where after it remains approximately the same. The decrease in virus titer observed in **RT** plants after day 25 is not surprising, as similar results have been seen for TYLCV DNA

accumulation in the same cultivars used in this study (**RT** and **S**). Upon whitefly-mediated inoculation with TYLCV, the **RT** line remained symptomless and contained barely detectable amounts of virus, while the **S** line presents typical disease symptoms and contained large amounts of virus. This indicates that an antiviral host defense mechanism operates in the RT line, limiting viral accumulation and spread within the plant.

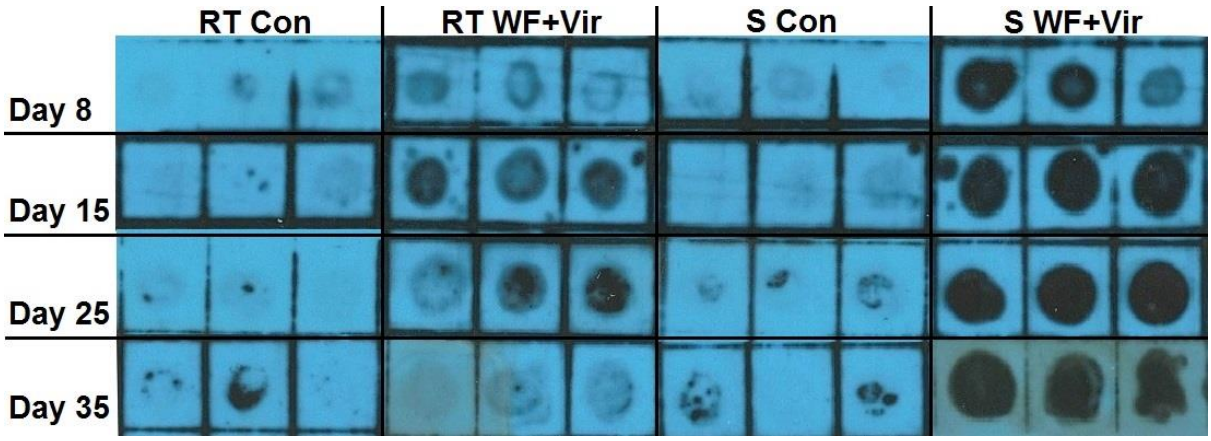

Figure S1.2: *Dot blot analysis of viral DNA accumulation in resistant (RT) and susceptible (S) control plants, as well as RT and S plants subjected to whitefly-mediated ToCSV infection (WF+Vir) on days 8, 15, 25 and 35.*

References

Esterhuizen, L.L., A study of the South African *Tomato curly stunt virus* pathosystem: epidemiology, molecular diversity and resistance. PhD thesis, Department of Biochemistry, University of Johannesburg, South Africa, **2012,** pp. 1-241.

Rossouw, L.T., Metabolite profiling of defence-related secondary metabolites in *Solanum lycopersicum*, in response to whitefly mediated begomovirus infection. MSc dissertation, Department of Biochemistry, University of Johannesburg, South Africa, **2016,** pp.1-165.

Vidavsky, F., Czosnek, H., Tomato breeding lines resistant and tolerant to *Tomato yellow leaf curl virus* issued from *Lycopersicon hirsutum*. Phytopathology **1998**, *88*, 910–914.