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Additional Information

Auxin growth hormone drives Tomato Spotted Wilt Virus (TSWV) resistance through epigenetic regulation of Auxin Response Factor *ARF8* expression in tomato

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Abstract

Tomato spotted wilt virus (TSWV) causes severe losses of tomato crops worldwide. To cope dynamically with such a threat, plants deploy strategies acting at the molecular and the epigenetic levels. Upon TSWV infection, tomato symptomatology is modulated in a specific-genotype manner. Regarding contrasting genotypes, hypermethylation pattern of a short sequence within the Auxin Response Factor (*ARF8*) promoter coupled to an enhanced expression of *miRNA167a* impaired negatively the *ARF8* gene expression and were closely associated to a decreased level of the auxin hormone. This constitutes a deliberate attempt of TSWV to disrupt plant growth and auxin network promoting their spread in sensitive cultivars. Epigenetic regulation through the level of cytosine methylation and the *miR167a-ARF8* module are part of a complex network modulating auxin-triggered synthesis and shaping tomato responses to TSWV. Furthermore, *miR167a-ARF8* regulatory module could be applied in tomato-resistance breeding programs.

Key words: Tomato Spotted Wilt Virus (TSWV)~~TSWV~~, Nsm, Auxin Response Factor *ARF8, miR167a,* epigenetic regulation, auxin, tomato

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1. Introduction

Tomato spotted wilt virus (TSWV) is the type member of the *Orthotospovirus* genus, belonging to the family *Tospoviridae* and order Bunyavirales (Nilon et al. 2021). TSWV is an ubiquitous virus, worldwide dispersed through thrips transmission, leading to severe threats of agronomic and horticultural crops (Oliver and Whitfield 2016). TSWV genome is organized in three single-stranded RNAs encoding proteins required for virus replication, infection and particle assembly (Tripathi et al., 2015). One of the known mechanisms of natural plant resistance to virus infection is the hypersensitive response (HR) (Kombrink and Schmelzer 2001). Within *Tospoviruses*, HR-based resistance relays on dominant genes corresponding to Sw-5 and Tsw, in tomato and pepper, respectively (Dianese et al. 2011). This reaction consists of a rapid death of cells bordering the viral infection leading to a restricted viral cell to cell movement and thereby limiting subsequent spread of the virus to all parts of the plant (De Oliveira et al. 2016). HR corresponds to local lesions surrounding the viral entry site. Concomitantly, the TSWV avirulent (Avr) determinant of Sw-5b-mediated resistance is the movement protein (NSm) (Hallwass et al. 2014; Olaya et al. 2020).

Plant-based immunity against viral infections is implemented in a broader system that includes a dual feature of genome stability maintenance and genome plasticity. Dynamic changes in the host epigenome is operating via effects on the levels of cytosine methylation and may be part of a common and ancient defense in plants (Alvarez et al. 2010; Zhu et al. 2016). Epigenetic marks are associated to pathogens as diverse as viruses (Rodríguez-Negrete et al. 2013), bacteria (Sha et al. 2005) and viroids as well (Martinez et al. 2014; Castellano et al. 2015; Castellano et al. 2016). Upon viral challenge, host DNA transcriptional reprograming acts through chromatin modification and remodeling and cytosine methylation (Alvarez et al. 2010). It seems that hypermethylation occurs widely across the whole genome and hypomethylation concerns resistance-related genes (Peng and Zhang 2009).~~ DNA methylation at repeat regions of the promoter was reported to increase the expression of defense genes (Yang et al. 2013; Yang et al. 2016)~~. Host-DNA methylation patterns, including coding and non-coding regions, concern either CG or non-

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CG methylation as CHG and CHH (H represents A, C, or T). De novo DNA methylation is catalyzed by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DMR2) through RNA-directed DNA METHYLATION (RdDM) (Matzke and Mosher, 2014). The symmetrical CG and CHG methylation are maintained by DNA METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively. The asymmetric CHH context requires de novo continuous maintenance and relies on DNA METHYLTRANSFERASE 2 (DMR2) during the replication process (Sigman and Slotkin, 2016).

Apart from alteration in host cytosine methylation, miRNA active contribution to antiviral defense is well established. They act through RNA-mediated gene expression regulation and constitutes an aspect of epigenetic modulation (Holoch and Moazed 2015). MicroRNAs are endogenous non-coding RNAs of 20–24 nucleotides. They are processed by Dicer-like (DCL) proteins from imperfectly paired hairpin precursor RNAs, and act in suppression of target gene expression through sequence-specific cleavage or translational inhibition (Holoch and Moaze 2015). miRNAs contribute to the regulation of plant development, and basal resistance against pathogens stress responses (Ruiz-Ferrer and Voinnet, 2009). Among 872 miRNAs identified in plants, *miRNA167a* was reported to target auxin response factors (*ARF8*) (Zouine et al. 2014).

To counteract pathogens, epigenetic marks have relevant role in shaping plant hormones signaling pathways. Pathogens often interfere with host physiology and lead to the display of an altered phenotype. Among growth promoting phytohormones, auxin might be a plant response regulator to environmental stresses (Jain and Khurana 2009; Van Ha et al. 2013). Auxin can be altered through a complex network triggering resistance or susceptibility during disease development (Kazan and Manners 2009). The active forms of auxin or indole-3-acetic acid (IAA) correspond to the free forms and related compounds, the remaining inactivated forms being conjugated mainly to amino acids and sugars in tissues (Ludwig-Müller 2011). Auxin signaling pathway starts with the hormone perception and binding to a complex corresponding to an auxin/indole-3-acetic acid (Aux/IAA) protein and an Fbox transport inhibitor response1/auxin signaling F-box (TIR1/AFB) protein (Dharmasiri et al. 2005; Salehin et al. 2015). This leads to the ubiquitination of Aux/IAA proteins by TIR1/AFBs and their degradation by the 26S

proteasome. Subsequently, occurs the de-repression of auxin response factors (ARFs), previously sequestered by Aux/IAAs through heterodimerization or oligomerization. Once released, ARFs specifically bind to conserved Auxin Response Element (AuxRE) present in the promoters of Auxin-regulated genes and to control transcription level associated to plant responses to auxin (Gray et al. 2001; Salehin et al. 2015). ARF gene family has been identified in several plants as *Arabidopsis* (Liscum and Reed 2002) and tomato (Wu et al. 2011; Zouine et al. 2014). Viral proteins impeding the ARF activity and manipulate the auxin signaling, support a strategy undertaken by pathogens to invade plant hosts (Zhang et al. 2020). Previous reports pointed out that auxin signaling cascades is manipulating by Rice Dwarf Virus to enhance disease development (Jin et al. 2016). Similarly, TMV interacts with Aux/IAA proteins (Collum et al. 2016).

This work is performed to address the potential involvement of the phytohormone auxin in tomato response to TSWV infection. Toward this aim, ~~we challenged~~ tomato cultivars were challenged with a Mediterranean TSWV strain. Visual phenotype scoring jointed to virus accumulation level allowed the identification of two tomato groups with contrasting degree of adaptation to TSWV infection. In the frame of auxin signaling, ~~we profiled comparative~~ differential expression between *miRNA167a* and its gene target *ARF8* in infected cultivars, were compared. Results were associated to the epigenetic status of a short sequence located in *ARF8* promoter. ~~Our f~~Findings support TSWV-induced DNA methylation changes that contribute to *ARF8* down regulation, which in turn, leads to auxin disruption signaling and tomato susceptibility towards TSWV.

2. Materials and Methods

2.1. Plant material

~~In this study, we used four tomato cultivars corresponding to California (Cali), Cœur de bœuf (Cbf), Merveille des Marchés (MdM) and Heinz. Plants were growing in an environmentally controlled green house at 25°C/18°C, day/night and a 16-h light/8-h dark cycle with 40–50% relative humidity~~.

Seeds from four tomato cultivars corresponding to California (Cali), Cœur de bœuf (Cbf), Merveille des Marchés (MdM) and Heinz were sterilized with a 95% ethanol, 0.1%

tween solution and sown into the pots with 500 mm height and filled with a mixture of plant compost (40%), peat moss (40%), sands (20%) and enriched with a fertilizer NPK 20-20-20 (pH 6.8, Terranum). Pots were bottom water-irrigated every 3 days. Plants were growing in an environmentally controlled greenhouse, that has relative humidity of 40–50%, 8/16h dark/light (100 μmol/m^2.s) with day/night temperature of 25°C/18°C during the experimental period

2.2. TSWV inoculation

Viral inoculation was performed using a TSWV strain (LL-N05, accession number: FM163373.1), kindly provided by the Institute of Molecular and Cellular Plant Biology of Valencia (Spain). TSWV viral strain was maintained *via* lyophilized infected leaves of *Nicotiana*. 100 mg of TSWV-infected *Nicotiana benthamiana* leaf tissue was grinded in an extraction buffer (0.01M sodium phosphate buffer (pH 7.0) with 0.4% β-mercaptoethanol in a final dilution 1:10 (w/v) and mixed with an abrasive (carborandum). The infectious viral inoculum was used to challenge mechanically three leaf-stage tomato cultivars, by rubbing the upper of the leaves. The inoculated cultivars were maintained in a greenhouse to monitor for symptom expression. Visual scoring was performed at ~~10, 20 and 30days~~10-, 20- and 30-days post inoculation (dpi). Infected leaves of inoculated tomato plants were systematically harvested and stored at -80°C until use. We used three biological replicates for each cultivar. Mock plants correspond to a set of three uninfected plants.

2.3. Dot-blot hybridization

The efficiency of mechanical inoculation of TSWV to tomato cultivars was assessed by viral detection on treated leaves, one-week post inoculation, using dot-blot hybridization. Sap extraction was carried out as previously described (Sánchez-Navarro et al. 1996) with minor modifications. A random primed-digoxygenin spanning the TSWV CP-gene was used under high-stringency hybridization conditions (60°C). The labelling reaction and immunological revelation were performed according to the manufacturer's instructions (Boehringer-Roche Mannheim, Germany).

2.4. Total RNA extraction

Total RNAs were extracted from tomato leaf tissues using TRIZOL Reagent (Trizol RNA stabilization solution, Invitrogen; Life Technologies, Carlsbad, CA, USA) according

to the manufacturer's instructions. Total RNA was quantified by ND-1000 spectrophotometer (Nanodrop Technologies, USA).

2.5. TSWV quantification by RT-qPCR

First strand cDNA was synthesized from 2 µg of total RNA with oligo (dT) and MMLV reverse transcriptase (200U/µl, Invitrogen) according to the manufacturer's instructions. An ABI A Prism 7500 sequence detection system (Applied Biosystems, USA) was used for quantitative real-time PCR (qPCR). Reactions were performed in a final volume of 25 µl under the following thermal profile: 50°C for 2 min, 95°C for 2 min, followed by 39 cycles each consisting of 95°C for 15 sec and 60°C for 1 min, followed by melting at temperature between 65-95°C with an increment of 0.5 °C for 10 sec. TSWV quantification was performed using TSWV NSm primers (Forward : 5´-GAATCAAATTTAGCCTGTGAC-3´ and Reverse : 5´-GACGTTGTATCCAGAAGG-3´) (Aramburu et al. 2010). The tomato β-actin gene was used as an internal reference gene (Løvdal and Lillo 2009).

Reactions were carried out in 96-well optical reaction plates (Applied Biosystems, USA). The reaction mixture included 2ul of 20-fold dilution of cDNA, each primer at 2.5mM concentration and 12.5 ul of IGreen PCR master Mix-Rox (BIOMATIK, USA). Relative quantification was performed by applying the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) Accordingly, data correspond to the fold change in gene expression normalized to the endogenous reference gene (β-actin) and relative to the calibrator (untreated control).

~~to calculate the changes in gene transcript as a relative fold difference between an experiment and calibrator sample~~.

2.6. *ARF8-miRNA 167a* module quantitative real time PCR analysis

For *ARF8* expression gene analysis, total RNAs were isolated and qRT-PCR was performed as described above, using primers corresponding to Forward: TGGGAAAGGAAGAGGCTGAA and Reverse: GCGATCCAAGAGATGGCATT (Feng et al. 2011). For *miR167a* purification was carried out using NucleoSpin® miRNA–Separation of small and large RNA (Mechery & Nagel, USA). *miR167a* stem loop RT primer (5'GGGTCCGAGGTATTCGCACTGGATACGACGAGATC3') and primers sequences (Forward: 5'CCTGATGAAGCTGCCAGCAT3' and Reverse

5’GTGCAGGGTCCGAGG3’) were designed based on sequences retrieved from miRNA Database (http://www.mirbase.org). First strand cDNA synthesis was conducted using the stem loop primer according to ReverseAid cDNA synthesis kit manufacturer’s instructions (Thermo, scientific). *miR167a*-specific forward primer and universal reverse primer were used for qPCR amplification according to the protocol previously described. Reactions were performed using technical triplicates. Relative quantification was performed by applying the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

~~Analyses of qPCR results were performed with Data AssistTM v3.0 Software (Applied Biosystems, USA). The output data were analyzed using two-way ANOVA with times and cultivars as the two predictor variables. Differences at Tukey’s test HSD (P=0.05) were considered statistically significant. Only the comparisons with *P*<0.05 were regarded as showing differential expression. A signal correlation was performed to visualize the correlation of the expression of *miR167a* and *ARF8* gene during viral infection based on Pearson’s correlation.~~

Total genomic DNA was extracted from the leaves of each TSWV inoculated tomato cultivar (Dellaporta et al. 1983) with an additional step corresponding to Proteinase K treatment (20mg/ml) (Scientific Thermofisher). 500ng of DNA was treated with sodium bisulfite (Martinez et al., 2014) using the Methylation Kit (Zymo, USA). Modified DNA was amplified by PCR using Taq DNA polymerase (Promega). Primers were determined using the MethPrimer software (http://www.urogene.org/methprimer/) (Li and Dahiya 2002). Primer sequences and positions, regarding ARF promoter are the following: Forward: 5’AATGATTAGTGAAGGATGGGTAG3’, position -605 bp and Reverse: 5’TCTTTATACCCC ACCCGAC3’, position -366 bp. The amplicons were purified from agarose gel and cloned using the pGEM-TEasy vector systems (Promega). Three clones were selected for sequencing analysis for each mock and TSWV inoculated cultivar DNA. Sequencing was performed in both strands with an automated DNA sequencer ABI Prism DNA-377 apparatus (Applied Biosystems, Paris, France

2.8 Auxin purification and quantification

Fresh tomato leaves and stems were used for auxin purification according to Pan et al., 2010. Quantification was performed using a liquid chromatography system coupled

with UV detector (Agilent 1100 series HPLC system). Samples were manually injected at a

volume of 20µl into a C18 inverter a stationary phase column (Zorbax SB-C18, Agilent Technologies, Palo Alto, CA, USA). The mobile phase consists of 60% ®/ 40% H2O acetic acid (0.6%). The flow rate is 0.8ml/min during the whole separation. Endogenous auxin was detected by matching the peak retention times obtained from the sample to the peak retention times obtained from the IAA standard (Sigma Aldrich, USA) at 274nm. Endogenous auxin concentration was calculated as following: Sample concentration= (Sample area X Standard concentration)/ (Standard area)

2.9 Statistical analysis

Analyses of gene expression were performed with DataAssist TM v3.0 Software (Applied Biosystems, USA). Data were analyzed using two-way ANOVA with times and varieties as the two predictor variables. Number of asterisks *; **; *** and **** indicates that differences at Tukey's test HSD are considered as statistically significant at $p < 0.05$; $p < 0.01$; $p < 0.001$ and $p < 0.0001$, respectively. For real-time PCR experiments, three independent biological replicates and three technical replicates for each cultivar were analyzed. Analyses were performed using GraphPad Software (version 8.0, CA, USA). The relationship between the expression of *miR167a* and *ARF8* was evaluated by the Pearson's correlation coefficients using R software (R Core Team, Vienna, Austria, 2020). For epigenetic analysis, DNA sequences were aligned, compared to each other to calculate the percentages of cytosine methylation using www.cymate.og online softaware.

3. Results

3.1. Tomato phenotyping for TSWV infection

TSWV inoculation of a set of 4 tomato cultivars was mechanically performed. The efficiency of viral inoculation was assessed by sap extraction of infected leaves followed by Dot-blot hybridization using a digoxigenin-labeled TSWV CP gene as a probe (Fig. S1). ~~Visual recording of symptom expression was meticulously carried~~ TSWV-infected cultivars were monitored for the appearance of visual symptoms during ~~different stages corresponding to~~ 10, 20 and 30 dpi stages. Evaluation of the level of ~~tomato resistance~~ TSWV incidence revealed that tomato cultivars were exhibiting a variety of symptoms

allowing their clustering into two major groups. The first group, containing Cbf, MdM and Heinz cultivars, showed systemic viral infection as soon as 10 dpi. These sensitive plants displayed foliar necrosis, ~~accompanied~~ associated to~~by~~ stunting and dwarfing ~~and associated to a growth stop~~ (Fig. S2a and S2b). The Heinz cultivar, although clustered within sensitive group, exhibited general mosaic and slower growth (Fig. S2c). The second group, consisting of the cultivar Cali, exhibited ~~local manifestations, some of which developed~~slightly chlorotic spots and ~~into~~ necrotic lesions which are characteristics of a local hypersensitive reaction (HR). Meanwhile, the Cali cultivar displayed normal growth and green symptomless emerging leaves, indicating a recovery phenotype (Fig. S3).

3.2. TSWV accumulation

To validate phenotypic assortment of tomato cultivars into tolerant and sensitive groups, viral diagnosis was undertaken by qRT-PCR targeting Nsm gene to quantify TSWV accumulation in systemic infected leaves. Correlating with its lack of symptomatology within the newly emerging leaves, Cali genotype contained barely TSWV amounts, with a drastic decreased level of the virus at the 30 dpi stage. Conversely, the remaining cultivars, displayed an increasing level of TSWV, starting already from the 20 dpi stage. This significant viral accumulation argued for their susceptibility to TSWV infection (Fig. 1)

3.3. *miR167a-ARF8* module is differentially expressed in tomato contrasting groups

miR167a is a conserved miRNA, present in a wide range of plants and seems to be part of the plant immunity (Khraiwesh et al. 2012). It might modulate the auxin signaling pathway (Wang et al. 2015) through targeting the auxin response factors *ARF6, ARF8* and the resistance genes *IAA-Ala 3* (IAR3) (Wang et al., 2015; Wu et al., 2006). Based on these data, we explored the role of *miR167a* in the tomato response to TSWV infection, via the regulation of the *ARF8* gene expression and in association with of the auxin signal disruption. We performed qRT-PCR assay to examine the expression pattern of the *miR167a-ARF8* module in the TSWV- susceptible and resistant groups and in healthy cultivars as well (Fig. 2). The results showed that *miR167a* was upregulated in the tolerant Cali cultivar at 10 dpi, before reducing drastically at the end of the infection stage (30 dpi).

By contrast, *miR167a* expression was significantly increased upon TSWV infection in the susceptible cultivars (Fig. 2a). Meanwhile, healthy susceptible cultivars showed a lowered *miR167a* expression compared to the infected ones. Inversely, Cali healthy cultivar displayed an increased expression profile when compared to the infected Cali genotype (Fig. 2b). Worth to notice that at 30 dpi, *ARF8* transcript accumulation was not significantly different between TSWV-infected and healthy Heinz cultivars.

Knowing that miRNAs repress their target gene expression at transcriptional or translational level, transcripts accumulation of *ARF8* gene, which is the target of *miR167a*, was explored in both sensitive and tolerant cultivars and in healthy cultivars (Fig. 3). *ARF8* mRNA accumulation was clearly increased in the tolerant Cali TSWV infected plants through all the infection stages whereas the induced ARF8 expression was much lowered in the susceptible genotypes (Fig. 3a). At 30 dpi, the Heinz cultivar accumulated significantly more *ARF8* transcripts than the remaining sensitive cultivars. Concomitantly, the expression profile of *ARF8* in Cbf and MdM healthy cultivars pointed to an enhanced expression during the latest stages of TSWV infection (20 and 30 dpi), while Heinz cultivars showed no significant changes at these time points. The tolerant Cali TSWV-infected and healthy cultivars exhibited opposite trends when compared to sensitive cultivars (Fig. 3b).

Using pairwise Pearson correlation, the correlation coefficient (r) between *miR167a* and *ARF8* differential expression was performed. Based on their relative and differential expression, it is relevant to notice a negative correlation between *ARF8* gene and *miR167a* expression in both groups of tomato cultivars whether tolerant or not to TSWV. Analysis revealed a significant negative correlation validating negative regulation of the target *ARF8* by *miRNA167a*. Further analysis pointed to a strong significant and negative correlation mainly at 20, 30 dpi with corresponding r values of -0.998 and -0976 respectively (Fig. 4). The *miR167a-ARF8* module is likely involved in the tomato immunity regulatory pathway in response to TSWV infection, operating at the latest experimental stage of viral infection (30 dpi).

3.4. TSWV mediated dynamic changes in the DNA methylation of the *ARF8* promoter

~~Epigenetic regulation mediated by DNA methylation has been previously reported as dynamic response linked to plant immunity (Dowen et al. 2012).~~

To determine whether *ARF8* downregulation in the susceptible tomato cultivars is associated with the alterations of cytosine methylation (mCs) within the promoter, we focused on a short 239 bp region, located upstream start codon *ARF8* gene. At 30 dpi time point, genomic DNA of mock cultivars and TSWV-infected cultivars belonging to both contrasting groups was bisulfite converted, amplified by PCR and cloned before submitted for sequencing. Multiple clone sequences alignments revealed that the targeted sequence is a differentially methylated region (DMR) (Fig. 5).

Regarding the mock cultivars, they showed an average mCs percentage, corresponding to 47% (Cbf), 17% (MdM), 30% (Heinz) and 31% (Cali) (Fig. 5a). Conversely, upon TSWV infection, susceptible cultivars displayed enhanced mCs reaching 92% (Cbf), 70% (MdM) and 58% (Heinz). Meanwhile, the tolerant group consisting of the Cali cultivar exhibited a slighter mCs increase (34%) (Fig. 5a). Deeper analysis pointed that methylation of cytosine bases was observed in the context of symmetric CG and CHG, and asymmetric CHH (where H = A, C, or T) (Fig. 5b). Considering mock cultivars, the distribution of CHH was 29% (Cali); 14% (Heinz); 18% (MdM) and 15% (Cbf). During TSWV infection, CHH sites show a dual trend of being actively hypermethylated within the sensitive cultivars (59% for Heinz; 73% for MdM and 89% for Cbf) while maintaining a constant average methylation status within the tolerant one (34%).

TSWV infectivity is correlated to the alteration of the dynamic methylation status, promoting cytosine hypermethylation mainly in the symmetric CHH motifs, providing thus a link between epigenetic regulation and TSWV infectivity.

3.5. TSWV disturbs the phytohormone auxin accumulation in tomato

Auxin spatio-temporal production is intimately connected to plant responsiveness to environmental stresses. It is striking that Auxin presumed role in modulating tomato growth facing viral stress remains not completely elucidated. In this context, Auxin accumulation was analyzed in contrasting tomato TSWV infected cultivars and mock plants at 30 dpi stage (Fig. 6). Results outlined that auxin concentration is modulated according to the infectious status of the cultivar, in a genotypic manner. 30 days upon

TSWV infection, auxin concentration increased two folds in the Cali cultivar and showed an obvious reduction in the susceptible cultivars.

4. Discussion

It was proven that in response to biotic and abiotic stresses, physiological traits (Rowland et al., 2005, Goharrizi et al., 2020a), biochemical characteristics (Milošević et al., 2003, Goharrizi et al., 2020b), genes expression (Catto et al., 2021; Nazari et al., 2021, Kiarash et al., 2018) and proteomic profile (Badillo-Vargas et al., 2012; Goharrizi et al., 2019) of different plants change significantly.

Plant responses to viral invasion are extremely complex and involve changes at the transcriptomic, epigenetic and phytohormonal levels to ensure growth and survival. Key actors and pathways supporting plant tolerance or susceptibility to pathogens need to be explored, in a context of global climate changing and perpetual virus emerging. Plants are constantly constrained by viral stress in their immediate environment, impacting negatively their growth and development. Responsiveness to pathogen invasion most likely includes a cross-talk between transcription factors and plant growth regulators. To gain more insights in the involvement of the auxin in TSWV tomato responses, the transcriptional level of auxin-responsive genes was determined and correlated to epigenetic status of the auxin-response gene *ARF8*.

Pathogens cause~~s~~ abnormal host plant development. Tomato cultivars challenged with TSWV exhibited wide range of symptoms that can notably be clustered in two main groups. The first set of cultivars showed a systemic infection associated to wilting, stunting and growth slow or nanism and was consequently assigned to a sensitive group. Conversely, the Cali tolerant cultivar displays local lesion, normal growth and symptomless emerging leaves. Tolerance to viral infection acts through HR induction, associated with rapid cell death on inoculated leaves. Regarding tomato-based TSWV resistance, the viral Nsm protein is the Avr determinant of the Sw-5b gene (Peiro et al. 2014; Hallwass et al. 2014). Despite of their diversity in *Solanum*, the orthologous proteins “R” are recognized by the Nsm protein and triggers HR subsequent reaction (Huang et al. 2018). As we do not have at our disposal an infectious TSWV clone and because of the fact that the thrips vectors are hard to handle, we performed mechanical transmission to

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challenge tomato cultivars with TSWV. Plant inoculation with viral sap extract was an efficient methodology that allowed identification of two plant clusters with contrasting behavior. To provide further evidence of such assignment we conducted a qRT-PCR tests targeting the viral Nsm gene (Jain and Khurana 2009; Jameson and Clarke 2015). that to validate the presence of the virus.

Following viral invasion, developmental and growth defects suggest that viruses disrupt plant hormone signaling and impart susceptibility to their host (Jameson and Clarke 2002; Pan et al. 2020). Among phytohormones, auxin coordinates defense responsive genes through a regulatory and functional network (Rosas-Diaz et al., 2018; Spaepen and Vanderleyden, 2011). In fact, auxin plays positive or negative roles in plant defense against pathogen infection depending on host specie and genotype (Spaepen and Vanderleyden 2011). In *Arabidopsis*, auxin displays dual roles through enhancing resistance against necrotic pathogens while promoting *Pseudomonas syringae* infectivity (Qi et al. 2012). Conjointly, viruses regulate the auxin pathway to ensure their accumulation and spread (Jin et al., 2016).

In tomato, ARFs genes act as key regulators of auxin network during plant pathogens invasion. They are altered by viral infection supporting that the auxin pathway is connected to the plant defense response via ARF gene regulation (Bouzroud et al. 2018). During viral infection, the viral P26 protein of TMV was reported to interact with Aux/IAA proteins to modulate a big set of ARFs genes (Padmanabhan et al. 2008). In tomato, 22 different ARFs were identified and characterized (Zouine et al. 2014). Plant miRNAs and ARFs mediate concomitantly the auxin signaling and the consequent symptom expression (Islam et al. 2018). Previous reports gave further evidence that plant pathogens might interplay with *ARF8-miR167a* module expression to adjust the host immunity (Zhao et al. 2020). Accordingly, we found that during TSWV challenge, the increased transcription level of *ARF8* was negatively and significantly correlated with the down-regulation of *miRNA167a*. Within the Cali tolerant genotype, *miR167a* repression, coupled to enhanced *ARF8* was associated to normal growth, compared to mock inoculated tomatoes. Restrained *miR167a* expression contributes to *ARF8* up-regulation which enhances both shoot and leaf development (Bouzroud et al. 2018). Regarding sensitive genotypes, the *ARF8-miR167a* module behaved oppositely impairing their growth and development. Our

results suggest that the auxin pathway is connected to the plant defense response via ARF gene regulation against TSWV.

Stress-induced epigenome modification are undertaken by both plants and pathogens. Plants display DNA genome methylation as a rapid-effective response to pathogen attack or as a process contributing to the increase of their pool of benefit genes as well (Yaish 2013; Martínez-Pérez et al. 2017; Tirnaz and Batley 2019). Repression genes through hypermethylation limit cells demanding and provides more energy to the plants to cope with pathogens. By contrast, hypomethylation of resistance genetic resources allow genes to be activated and expressed. Pathogens are also following a similar trend by inducing changes in the methylation status of plant genes to ensure their multiplication and spread (Boyko and Kovalchuk 2008; Boyko and Kovalchuk 2011). Following viral invasion, plant epigenome modification has been well established. In the frame of different chromatin context and epigenetic-related gene modulation, we explored the potential alteration in the dynamics of DNA methylation in the *ARF8* promoter region. At 30 dpi, analysis of DNA methylation of a short sequence located in the *ARF8* promoter revealed divergent DNA methylation patterns within contrasting TSWV-response cultivars. A significant methylation increase in the asymmetric CHH within the tolerant tomato cultivar was observed. Despite of these changes, an overall stable epigenetic status was noticed. Oppositely, when considering the sensitive cultivar set, a dynamic methylation occurred in asymmetric CHH and symmetric CG/CHG context, as well. Methylation in the asymmetric CHH is reported to vary according to distinct pathogens whereas the symmetric CG and CHG context always displays the same changes (Bartels et al. 2018). Current evidence supports that virus's mediate DNA methylation changes in the host genome. Potyviruses performed the decrease of cytosine methylation in auxin biosynthesis genes (Yang et al. 2013). Similarly, Geminiviruses decrease DNA methylation of the promoter region of a gene regulator involved in the acid salicylic pathway (Yang et al., 2013; Zhang et al., 2011). These results strongly suggested that in addition to HR-mediated resistance and *miR167a*-silencing process, DNA methylation pathways is recruited for enhancing plant defenses. The extent and effectiveness of epigenetic regulation is intimately dependent on the degree of viral adaptation to the host.

When facing stressful conditions, plant viruses can enhance their virulence through interference with the IAA production and transport pathway (Robert-Seilaniantz et al. 2007). TMV displays an ability to interact with phloem-expressed Aux/IAA proteins, gaining thus an advantage in phloem loading and systemic spread (Collum et al. 2016). Similarly, it has been reported that a high auxin concentration can directly decrease systemic replication of the White Clover Mosaic Virus (Clarke et al. 2000). Increased concentrations of IAA results to β-1,3-glucanase repression, engendering callose deposition which reduces size exclusion of plasmodesma. Consequently, viral movement from cell to cell is drastically slowed down leading to a decreased infectivity (Iglesias and Meins 2000). To seek if changes at the transcriptional level of *ARF8* reflected changes in auxin production, we monitored auxin concentrations trough the TSWV infection. The auxin profile clearly differed between both contrasting cultivar sets indicating that this phytohormone might display a regulatory role in defense response against virus infection. Significant decreases in auxin concentrations in TSWV susceptible tomato plants were recorded when compared to the Cali tolerant cultivar. Thus, our results indicated that viral infection causes repression of the *ARF8* transcription, which ultimately leads to the inhibition of the auxin.

At low auxin concentrations, Aux/IAA proteins are more stable and downregulate ARF transcription factors through direct interaction with their domains III and IV (Guilfoyle and Hagen 2007). At the opposite, when the auxin concentration becomes higher, auxin binds Aux/IAA repressors resulting in their subsequent ubiquitination by SCFTIR1/AFB E3 ligase and proteolysis by the 26S proteasome (Gray et al. 2001). This degradation leads to ARF derepressing and release, allowing transcription of auxin response genes. In plants, ARF transcription factors are the key effectors that orchestrate the auxin signaling pathways. Aux/IAA proteins seem to be master repression regulators through binding and control of ARFs that are reported to act critically for plants normal growth and development. Even so, ARFs role in plant defense remains so far poorly studied. In rice, *OsARF17* overexpression enhanced a wide range viral resistance, while *OsARF17*-mutant plants exhibited more pronounced symptoms (Zhang et al., 2020). Upon Rice Dwarf Virus (RDV) infection, tolerant rice genotype actively enhances an auxin-ARFs mediated regulating network (Jin et al., 2016). Also, Rice black streak dwarf virus

(RBSDV) infection alters the auxin dynamic network through repressing auxin-related genes (Zhang and Friml 2020). In line with these reports, instances of auxin disruption during Tomato leaf curl New Delhi Virus (ToLCNDV) infection are outlined (Vinutha et al. 2020). Viral AC4 protein interacts with host factors leading to low auxin biosynthesis and viral spread. Furthermore, pre-treatment with a foliar exogenous auxin contributes to recovery of infected plants and better counter defense responses (Vinutha et al. 2020; Qin et al. 2020).

5. Conclusion

Overall, our data demonstrated that *miR167a* and epigenetic marks act as a negative regulator in tomato immunity to enable TSWV infection via auxin dynamic disturb. Based on the substantial change in the *ARF8* profile expression associated to auxin concentration perturbation, our findings support that auxin signaling is manipulated by plant pathogens to optimize their own replication and spread. Susceptibility to TSWV is ensured through the joint and complementary action of *miR167a* and cytosines methylation. The outcome is the *ARF8* gene down regulation, accounting for the tomato growth defect while enhancing virus's ability to move through plasmodesmata and to engender systemic infection.

Reprograming auxin signaling pathways relies thus on transcriptional plasticity of hormone-related genes through epigenetic status. Auxin mediated a fine balance between host defense promoting and beneficial growth responses.

Authors contributions: **SW** and **FG** conceived and designed the experiments. **SW** performed the experiments, analyzed the data and wrote the first draft of the paper. **FG** supervised this work and wrote the final paper. **FAH** contributed to the experiments and reviewed the paper. **HF** obtained funding acquisition and provided critical suggestions. All authors have read and approved the final manuscript.

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Figures

Fig.1

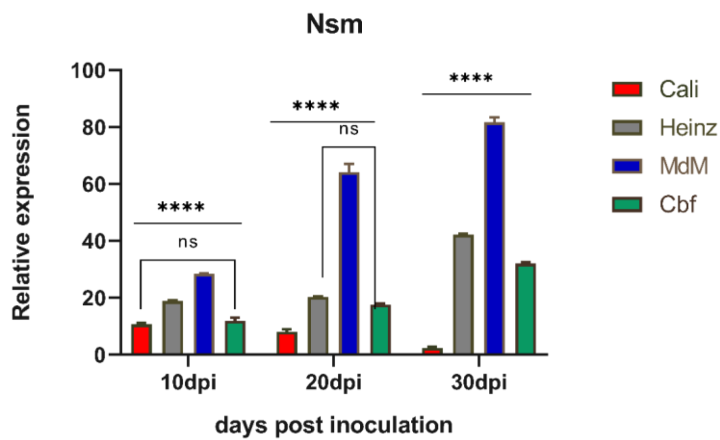

Fig. 1. QRT-PCR estimation of relative TSWV amounts in tomato cultivars at 10, 20 and 30 dpi. Error bars represents standard deviation. The number of ~~A~~asterisks indicates the level of significant differences as determined by Tukey's test ~~(**, *P*<0.01); ns: no significant difference~~; dpi: days post inoculation.

Fig.2

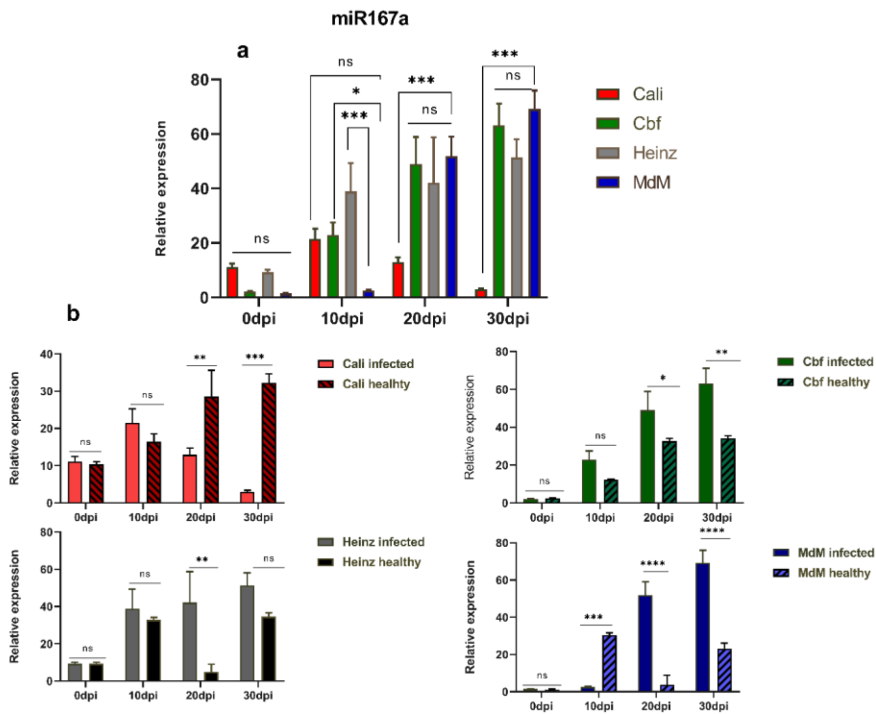

Fig. 2. Relative expression of *miR167a*. (a) TSWV-infected cultivars; (b) TSWV-infected and healthy cultivars. Total RNA was purified from tomato leaves of plants exposed to viral stress at 0 dpi, 10 dpi, 20 dpi and 30 dpi. Error bars represents standard deviation. The number of ~~A~~asterisks indicates the level of significant differences as determined by Tukey's test~~ (**, $P<0.01$); ns: no significant difference~~; dpi: days post inoculation.

Fig.3

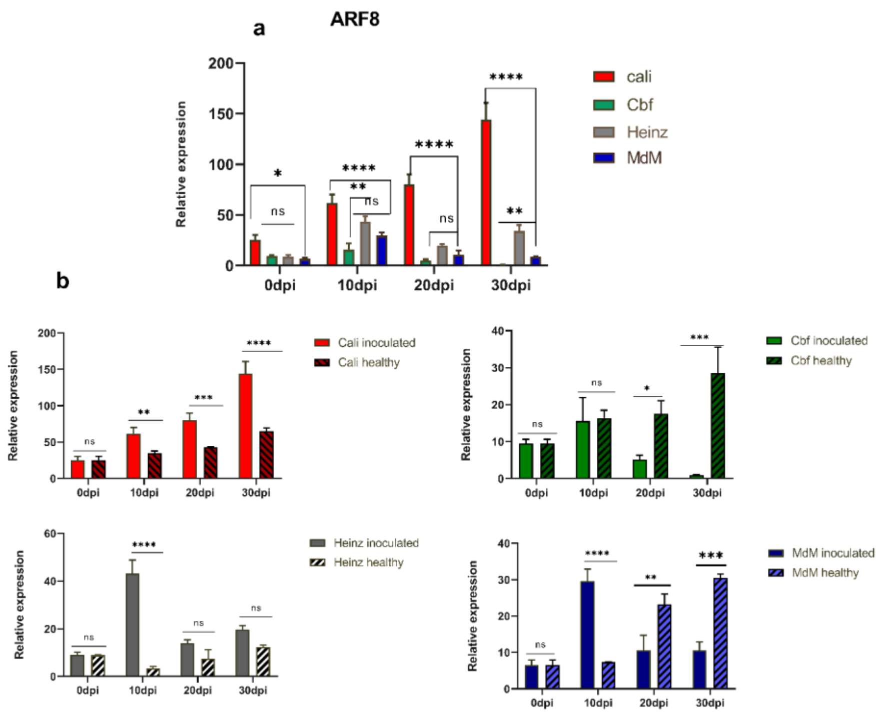

Fig. 3. Relative expression of the target *ARF8* gene. (a) TSWV-infected cultivars; (b) TSWV-infected and healthy cultivars. Total RNA was purified from tomato leaves of plants exposed to viral stress at 0 dpi, 10dpi, 20 dpi and 30 dpi. Error bars represents standard deviation. The number of ~~A~~asterisks indicates the level of significant differences as determined by Tukey's test ~~(**, *P*<0.01); ns no significant difference~~; dpi: days post inoculation.

Fig.4

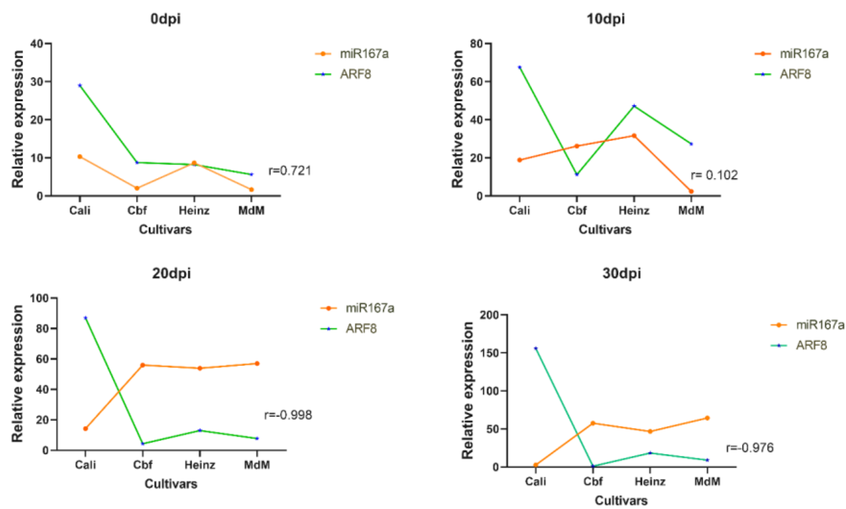

Fig. 4. Correlation between *ARF8* and *miRNA167a* expression at four time points of the experiment. Pearson's correlations test between *ARF8* and *miR167a* differential expression at 0 dpi, 10 dpi, 20 dpi, 30 dpi. Pearson correlation and the significance level $p<0.05$ were calculated for each time point.

Fig.5

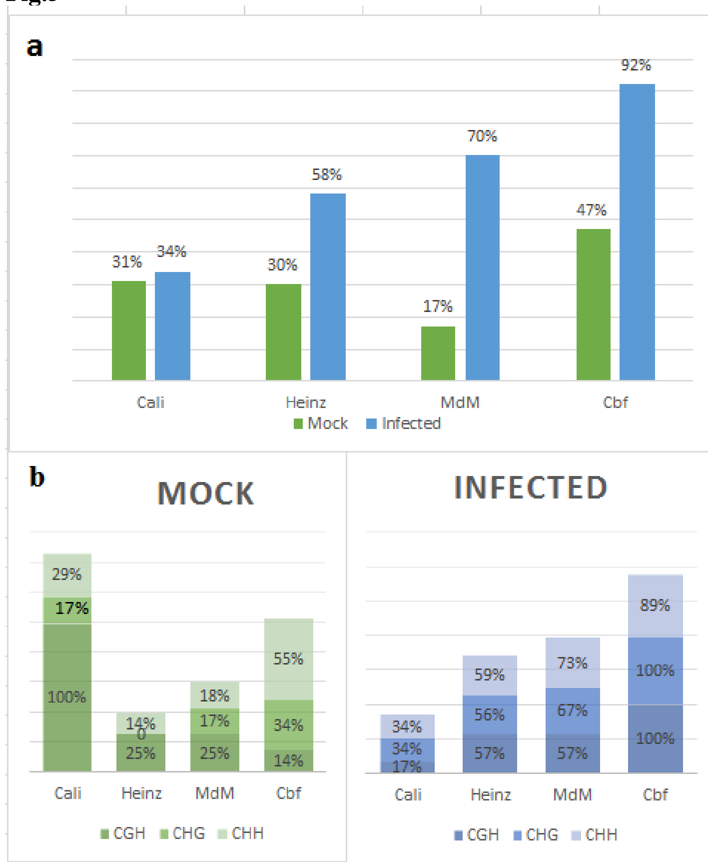

Fig. 5: The epigenetic status of methylation within the target sequence of the *ARF8* promoter at 30 dpi. (a) Average level of mCs in the mock and the TSWV-infected tomato cultivars; (b) Pattern distribution of symmetric CG, CHG and asymmetric CHH among mCs.

Fig.6

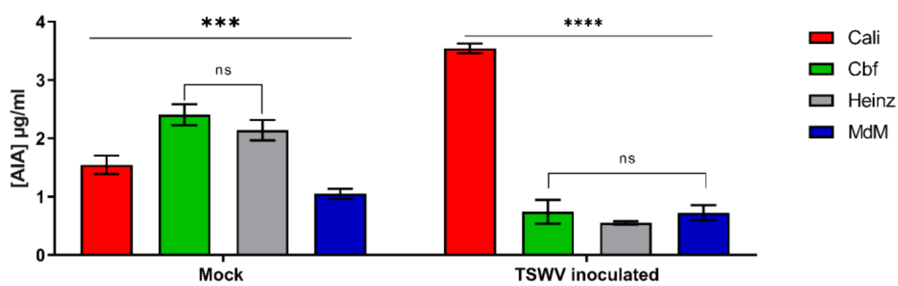

Fig. 6. Auxin content in TSWV infected and mock cultivars. The error bars indicate the standard error between the three replicates performed. Error bars represents standard deviation. The number of ~~A~~asterisks indicates the level of significant differences as determined by Tukey's test~~ (**, *P*<0.01); ns: no significant difference~~; dpi: days post inoculation.