BACTERIAL WILT AND CANKER OF TOMATO: FUNDAMENTALS OF A COMPLEX BIOLOGICAL SYSTEM

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Abstract

Tomato (*Solanum lycopersicum*) is well-known as a model for study of plant-pathogen interactions, since it is a crop of global relevance and susceptible to multiple bacterial, fungal, viral and nematode pathogens. Among bacterial phytopathogens, the actinomycete *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is the causal agent of bacterial wilt and canker of tomato, considered a quarantine disease at international level. The tomato-Cmm interaction has been studied to decipher the pathogenicity mechanisms in Cmm, susceptibility mechanisms in tomato, molecular basis of resistance to Cmm in wild species relative to domesticated tomato, and the level of genetic variability in Cmm. The objective of this review is to discuss recent advances in tomato-Cmm compatible interaction, which can be integrated for application in early diagnosis and biological control of bacterial wilt and canker of tomato. Further study of plant-microorganism interactions is a promising field for improvements in tomato pathogen resistance.

Introduction

The interaction between plant and microorganism is a dynamic and complex biological system. It involves a link between microbial and plant metabolic pathways, which are interconnected and influenced by environmental factors. Interactions of this sort result in one of three scenarios: disease, resistance or benefit. However, little is known about the mechanisms that give rise to each scenario (Heuberger et al. 2014; Boyd et al. 2013; Reinhold-Hurek and Hurek 2011; Abramovitch et al. 2006). Progress in understanding the plant-pathogen interaction, specifically bacterial phytopathogens, has primarily been made with bacteria belonging to the Proteobacteria group (Baltrus et al. 2011; Cai et al. 2011; Mole et al. 2007; Abramovitch et al. 2006; Jones and Dangl 2006; Abramovitch and Martin 2004). Limited information is available on plant-pathogenic actinomycetes such as *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), which causes bacterial wilt and canker of tomato, a plant disease with world quarantine and scientific-economic relevance (Sen et al. 2015; Mansfield et al. 2012; Eichenlaub and Gartemann 2011; Chalupowicz et al. 2010; Hogenhout and Loria 2008).

***Clavibacter michiganensis* subsp. *michiganensis*: pathogenicity, diagnosis and control.**

Measuring the disease in terms of economic and scientific importance, Cmm ranks among the top ten bacterial plant pathogens (Mansfield et al. 2012). Cmm is a plant-pathogenic actinomycete that causes a systemic vascular infection in the tomato, known as bacterial canker, which is spread by way of contaminated seeds and contaminated debris. Cmm penetrates the plants through wounds and natural openings, such as hydathodes and stomata (Ialacci et al. 2016; Tancos et al. 2013; de Leon et al. 2011; Carlton et al. 1998). Symptoms are unilateral wilting, the appearance of moist and corky spots on the stems, known as cankers, and lesions on the surface of the fruit, known as "birds eye lesions". Cmm can produce latent asymptomatic infections and is able to survive in the soil in plant debris (Vega and Romero 2016; Sharabani et al. 2013; Jahr et al. 1999). This results in the risk of the disease affecting the same unit of production in various cycles, as well as for it to rapidly spread and take root in areas considered to be disease-free.

Pathogenicity factors. Studies on the reference strain Cmm NCPPB382 have unearthed an array of pathogenicity mechanisms. The characteristic symptoms of the bacterial canker are tied to the presence of the *celA* (which codes for an endo-β-1-4 glucanase) and *pat-1* (which codes for a serine protease) genes. These genes are located on plasmids pCM1 and pCM2, respectively (Jahr et al. 2000; Dreier et al. 1997; Meletzus et al. 1993). Analysis of the Cmm NCPPB382 genome has revealed a 129-kb region with low GC content, divided into two subregions: the *chp* subregion, containing genes that code for a variety of serine protease enzymes, and the *tomA* subregion, containing genes involved in carbohydrate metabolism, including the gene *tomA*. The product of *tomA* is known as tomatinase (endo-1, 4-beta glycosidase) which is an enzyme that removes the carbohydrate units of α-tomatine, a glycoalkaloid with antifungal activity in the tomato (Gartemann et al. 2008; Kaup et al. 2005). Gartermann et al. (2008) demonstrated the importance of this genomic region in pathogenicity. In fact, a mutant strain of Cmm lacking the 129-kb low GC content region was non-virulent and unable to colonize plant tissue. The evidence suggests that the genes located in this 129-kb genomic region might unleash a signaling cascade that manipulates plant metabolism to make the tomato more hospitable to Cmm (Chalupowicz et al. 2017; Stork et al. 2008; Gartemann et al. 2008; Kaup et al. 2005) (Table 1).

In summary, the location of the pathogenicity factors in Cmm suggests that they can be horizontally transferred, as they are located on plasmids and in one region of the genome.

The pathogenicity genes in Cmm are absent in gram-negative bacteria and uncommon in other plant-pathogenic actinomycetes. Cmm pathogenicity mechanisms include strategies related to host recognition, colonization, and evasion/suppression of defense responses, which require greater study to develop more effective strategies for timely diagnosis and disease control (Francis et al. 2010; Hogenhout and Loria 2008).

Diagnosis. The European and Mediterranean Plant Protection Organization (EPPO) has established a diagnostic protocol for symptomatic and asymptomatic tomato plants and seeds. The protocol describes the symptoms of the disease and the process to isolate Cmm from plant tissue or seeds using growth media for non-selective and semi-selective culture, the subsequent identification of suspicious colonies using serological and molecular methods, and confirmation via pathogenicity tests in tomato seedlings (OEPP/EPPO 2016).

The protocol for Cmm diagnostics in symptomatic tomato plants starts with Cmm extraction from infected tissue (stem, damaged leaves and fruits) with 0.01 M phosphate buffered saline (PBS), continues with Cmm isolation on non-selective culture media – like Yeast Peptone Glucose Agar (YPGA) or yeast-dextrose-calcium carbonate (YDC) – or in combination with semi-selective culture media – SCM, CMM1T or SCMF – which contain antibiotics that inhibit the growth of saprophytes. The bacterial colonies obtained in the culture media, which show the morphology suspicious of Cmm, should be purified by subculture in nutritive media. The suspicious colonies of Cmm should be subjected to identification test, which include tests like indirect immunofluorescence (IF) and polymerase chain reaction (PCR), based on amplification of 268 pb fragment of 16S-23S rRNA intergenic region. However, both techniques show low specificity, it means the tests may detect other microorganisms and provide positive results, therefore confirmatory tests that include bioassays to assess pathogenicity, molecular tests (like real time PCR or genomic fingerprinting), biochemical or physiological test (Biolog system or fatty acid profile) are required. In the case of the diagnosis of latent infections in nursery seedlings without evident symptoms, OEPP/EPPO (2016) proposes a method in which Cmm isolation is carried out in the semi-selective media mentioned above. However, the method has not yet been validated due to the difficulty of sampling a large number of plants to obtain.

Since the Cmm dissemination occurs through infected seed, OEPP/EPPO (2016) has proposed two procedures for detection and identification of Cmm in seeds. Both

procedures should be applied to untreated seeds, but they could be applied to seeds that were subjected to disinfection treatments with HCl or sodium hypochlorite.

Procedure A starts with Cmm extraction from seeds by using 50 mM phosphate buffer (PB), this step may include maceration and low speed centrifugation. Then, the extract is inoculated on semi-selective culture media (CMM1T, SCMF o SCM). The suspicious bacterial colonies should be purified and identified by bioassays to assess pathogenicity, molecular tests (including real time PCR or genomic fingerprinting), biochemical or physiological test (Biolog system or fatty acid profile).

The procedure B allows proliferation of Cmm in a suspension of seeds with 0.1 M phosphate buffered saline (PBS), at room temperature with constant agitation during 3 days. Later, the methodology of IF should be applied. The samples IF positive must be confirmed by PCR. Samples PCR positive should be subjected to bioassays to assess pathogenicity. For this purpose, the seed extract is inoculated into tomato seedlings, the symptomatology should be monitored and Cmm must be re-isolated in non-selective media culture. For subsequent identification of bacterial colonies, the rapid test may be applied. The proposed methodologies for diagnostic of Cmm are robust and reliable, since they require a series of controls at each step, including the use of reference strains (OEPP/EPPO 2016)**.**

Other studies have demonstrated that molecular diagnoses of Cmm by way of polymerase chain reactions (PCR) possess high sensitivity and specificity by detecting and quantifying specific gene fragments. Examples of specific genes are *cytC*, which codes for a ferrodoxin reductase (Cho et al. 2012) and *tomA* which codes for the tomatinase pathogenicity factor (Kokoskova et al. 2010), both genes are located on the genomic pathogenicity island.

Yasuhara-Bell et al. (2013) developed an alternative molecular diagnosis method using detection of the chromosomal gene *micA*, through the implementation of the loop-mediated isothermal amplification (LAMP) technique, which makes it possible to amplify a DNA fragment by way of an enzyme reaction at 65°C. This study showed high sensitivity and specificity in detecting diverse *Cmm* strains, while also stressing the potential of LAMP as a portable molecular diagnosis tool that is easy to implement and interpret.

Molecular diagnosis can be considered an early and accurate diagnosis tool for Cmm, because its high sensitivity makes it possible to detect a low Cmm titer in tissues during early stages of infection when symptoms are not evident (Kokoskova et al. 2010). The high specificity makes it possible to detect and even could differentiate between different

pathogenic strains of Cmm (Cho et al. 2012; Jacques et al., 2012; Kokoskova et al. 2010). It is worth underscoring that molecular diagnosis can be conducted directly on the plant tissue without requiring any preliminary microbiological culture. However, PCR could detect death bacteria. In this regard, given the international quarantine nature of Cmm and potential risk involved for productive sector, we consider it would be appropriate not to reject the results of PCR diagnosis. Because in the scenario of detecting DNA from dead Cmm cells, it is also likely that a fraction of that DNA comes from some viable Cmm cells, capable of proliferating and causing disease in the medium term. In addition, Luo et al. (2008) reported the application of DNA binding dye ethidium monoazide to real time PCR approach that allowed discrimination between viable and death *Cmm* cells. Accordingly, molecular diagnosis of *Cmm* is a tool with lots of potential for the productive sector in terms of its ability to guarantee and certify seed productivity.

Control. The origin of bacterial canker of tomato outbreaks are infected seeds, since Cmm has the ability to infect tomato seeds internally through xylem or superficially via "birds-eye" lesions of fruit (OEPP/EPPO 2016; Tancos et al. 2013). The attempts to reduce the bacterial titter by way of acid treatment on seeds and the application of copper salts on seedlings are very frequent but ineffective practices in the medium- and long-term (Jiang et al. 2016; Hausbeck et al. 2000). Jiang et al. (2016) described that *in planta* conditions such as a low pH and concentrations of $CuSO_4$ provide a conducive environment for Cmm to be viable but non-culturable (VBNC) state; in other words, a state in which the metabolically active Cmm cells are unable to form bacterial colonies on culture media.

The bacterial VBNC state represents a survival mechanism in the face of unfavorable environmental conditions, produced by the defense responses of the plant or by the use of antimicrobial agents, such as $CuSO_4$. When the conditions improve, the bacterial cells can emerge from the VBNC maintaining their phenotypic characteristics. The VBNC state has been described in gram-negative phytopathogens like *Pseudomonas syringae* pv. *syringae*, in which VBNC may be a survival mechanism against the oxidative environment of the apoplast triggered by the host plant as defense responses in its attempt to stop the advance of phytopathogen (Mock et al. 2015; Postnikova et al. 2015). Likewise, the VBNC state can be induced in *Ralstonia solanacearum* by low temperatures (Kong et al. 2014). In *Erwinia amylovora* and *Xanthomonas axonopodis* pv. *citri*, VBNC state is associated with the treatment of their host plants with $CuSO_4$ (del Campo et al. 2009; Ordax et al. 2009).

In this context, the VBNC state in Cmm constitutes a defense mechanism against adverse *in planta* conditions, such as low pH and high concentrations of $CuSO_4$. The surviving Cmm cells that emerge from the VBNC state do maintain and express their pathogenic capacity (Jiang et al. 2016). In light of the above phenomena, the VBNC state in Cmm could: **i)** explain disease cycles recorded in the production units in spite of constant applications of $CuSO_4$, **ii)** presage variations in the pathogenicity levels of Cmm in each disease cycle, since VNBC may act as selecting agent for more virulent strains, **iii)** constitutes a serious limitation on microbiological culture-based diagnosis methods, and **iv)** provide information about the mechanisms of stress tolerance in grampositive phytopathogens.

However, in the international productive sector, the most effective strategies for disease control include adhering to strict cultural practices like removal and disposal of infected plants or plants that are suspected to be infected, implementing quarantines in certain production units, personnel management and training, disinfecting materials and machinery, using certified seeds, administering biological phytosanitary products and constantly monitoring plant health via molecular diagnosis (Jiang et al. 2016; Sharabani et al. 2013; Kokosková et al. 2010).

Additionally, novel control strategies have also been proposed, including the integration of genetic engineering with the use of bacteriophages, viruses that kill Cmm specifically (Witmann et al. 2016).

The ability of bacteriophage to cause cell lysis of a specific bacterial host is carried out by hydrolases enzymes called endolysins, their function is to degrade the peptidoglycan from inside host cell at the end of replication viral cycle, to release viral progeny. New lytic bacteriophages can infect nearby bacterial cells and, therefore, lytic activity can be amplified depending on the amount of bacterial host present. In terms of plant disease control the above mentioned constitutes an advantage over the use of antibiotics (Buttimer et al., 2017; Frampton et al. 2012).

In the light of above, it has been proposed the application of bacteriophages and endolysins as agents for control of plant diseases (Buttimer et al., 2017; Frampton et al. 2012; Schmelcher et al. 2012). At this regard, the bacteriophages CMP1 and CN77 produce active and specific endolysins against Cmm that hydrolyze from outside the unusual peptidoglycan of *Clavibacter michiganensis*, which would allow the external application of endolysins to tomato crop with the aim of control of Cmm without perturbing microbial diversity (Witmann et al. 2010).

However, it is necessary to take into account that the effectiveness of bacteriophage or its endolysins for control of plant disease, under intensive agricultural production, can be influenced by environmental factors, like physicochemical properties of water-soil, the use of fertilizers, pH, temperature (Frampton et al. 2012). As alternative, Witmann et al. (2016) obtained transgenic tomato plants that expressed the *lys* gene of the bacteriophage CMP1, as a preventive tool for Cmm infection. Transgenic tomato plants challenged with Cmm did not show symptoms of the disease; nevertheless, levels of Cmm were detected in leaf and xylem sap. The authors suggest that the increase of level expression of *lys* gene would increase the effectiveness of control.

On the other hand, Balaji and Smart (2012) obtained transgenic plants that constitutively overexpressed *snakin-2* (*SN2*) and *extension-like protein* (*ELP*) genes. Snakin-2 (SN-2) is a cysteine rich peptide, and ELP is a cell-wall hydroxyproline-rich glycoprotein related to plant defense to pathogens and wounding, both genes considered as antimicrobial peptides. The transgenic plants challenged with Cmm displayed delays in the onset of symptoms and a reduction in the degree of disease. Likewise, a significant decrease in the bacterial titer of Cmm was detected as compared to the non-genetically-modified control plants. Balaji and Smart (2012) speculate that SN-2 and ELP protein could help to retard Cmm proliferation and therefore tomato tissue colonization.

The application of antimicrobial peptides (AMP) – found in almost all living organisms – for biological control of plant disease, is a promising tool since their structural and biochemical diversity guarantee different mechanisms to interact with microbial membranes with the subsequent antimicrobial effect. As a result, AMP possesses broad spectra and could show synergic effects among them for biological control of plant disease (Breen et al. 2015).

In both cases, the evidence confirms that the transgenes employed under laboratory *in planta* conditions did display anti-Cmm activity that significantly reduced the disease damage. These strategies could help mitigate productivity losses even in the presence of phytopathogen.

Moreover, some researchers have proposed harnessing the antagonistic activity of certain microorganisms against Cmm, such as *Bacillus subtilis* (Jung et al. 2014), *Pseudomonas* sp. (Paulin et al. 2017; Deng et al. 2015; Lanteigne *et al*., 2012) and *Streptomyces* sp. (Zhang *et al*., 2010). It is important to bear in mind that the aforementioned microorganisms inhabit the rhizosphere of numerous plant species and therefore can play an antagonistic role: **i)** in soil or plant tissue, **ii)** directly against Cmm (by way of secondary

metabolites with antimicrobial activity) or **iii)** indirectly by stimulating the plant to unleash biochemical pathways that activate induced systematic resistance (IRS) against Cmm.

The studies cited contain novel actions with significant agro-biotechnological potential to diagnose, monitor and control Cmm. In terms of diagnosis and monitoring, specifically, the information gathered in these studies could result into the development of epidemiological models for specific situations or influence the design of control strategies of affected areas.

Solanum-Clavibacter **interaction**

A variety of approaches have been used to study the compatible interaction between the tomato and Cmm to elucidate the mechanisms that make the tomato susceptible and Cmm pathogenic. Next we carried out the analysis of them in chronological order with the aim to let the reader know how the information about tomato-Cmm interaction was generated. In Table 1, the below-cited studies are classified according to the findings in the host or in the pathogen.

A study of the tomato transcriptome four days after infection with the reference strain Cmm NCPPB382 revealed differential expression of genes involved in basal defense responses and in producing free radicals. Additionally, genes involved in biosynthesis and the ethylene response genes were induced. The role of ethylene in the compatible interaction between tomato and Cmm was demonstrated by infecting the *Never ripe* (Nr) mutant line of tomatoes, which is not sensitive to ethylene. The Cmm-infected tomatoes Nr mutant significantly delayed the onset of symptoms. As such, the evidence suggests that at an early stage of the infection before symptoms appear, ethylene can make the tomato more susceptible to Cmm (Balaji et al. 2008).

Ethylene acts as a signaling molecule during activation of plant defense against phytopathogens (Thakur and Sohal 2013), like *Arabidopsis-Pseudomonas syringae* (Guan et al. 2015), tobacco-*Phytophthora parasítica* (Wi et al. 2012). Therefore, it is possible that Cmm has taken advantage of the ethylene-mediated signaling in tomato to generate an environment conducive to its development, since ethylene is involved in softening process of plant tissues – like fruit ripening – in this scenario, its wide repertoire of cell-wall-degrading enzymes is more effective for colonization purposes (Broekgaarden et al. 2015; Tancos et al. 2013; Van Loon et al. 2006; Lund et al. 1998).

In order to understand the chain of events in the initial period of infection through transcriptomic approach, the tomato was infected with the following strains: Cmm NCPPB382, Cmm00 (a strain that lacks plasmids), and Cmm27 (a strain that lacks the

genomic pathogenicity island) (Chalupowicz et al. 2010). In each experiment, the expression of plasmid and chromosomal virulence factors in Cmm and the expression of genes related to tomato defense were quantified using qPCR. Gene expression profiles for Cmm NCPB382 revealed the induction of plasmid virulence factors in the first hours of infection, followed by the induction of chromosomal virulence factors and a reduced expression of defense genes in tomato. It should be mentioned; Cmm27 induced the expression of Pathogenesis Related (PR) proteins in tomato, involved in plant defense, and showed a reduction of gene expression levels of plasmid-borne pathogenesis factors. While Cmm00 showed a reduction of gene expression levels of pathogenesis factors located at genomic pathogenicity island. The evidence suggests that the chromosomal virulence factors may participate in suppressing the tomato's defense mechanisms, while also permitting the Cmm population to multiply and to shift from an endophytic to a pathogenic state (Chalupowicz et al. 2010).

With the aim to elucidate the pathogenicity mechanisms, the overall gene expression of Cmm through the DNA microarrays was analyzed under: a) *in vitro* conditions that simulated an infection, which was achieved by incubating the Cmm NCPPB382 strain in a growth medium supplemented with a tomato homogenate, and b) *in planta* conditions ten days after tomato infection. Under *in vitro* conditions, comparing the gene expression pattern of Cmm incubated for a long time period (12 h) and a short time period (12 min) in the supplemented medium with that of the unsupplemented medium, it should be noted that various virulence factors – including extracellular serine proteases encoded in the genomic pathogenicity island, an endocelullase and extracellular serine protease both plasmid-borne – were down-regulated at both periods. In addition, genes involved in carbohydrate metabolism located at *tomA* subregion of genomic pathogenicity island were induced at long time period. It is noteworthy the down regulation of genes involved in biosynthesis of siderophore at both periods, in this sense tomato homogenate could provide iron as assimilable physiological form by Cmm. Therefore, under *in vitro* conditions siderophores would not be required, since addition of tomato homogenate to growth medium simulates the later stages of an infection because the macerated tissue can furnish conditions equivalent to those in a plant with advanced symptomatology. The gene expression profile of Cmm under *in planta* conditions allowed deepening evens more in the late stage of infection. The analysis showed down regulation of extracellular serine proteases, which would confirm their function at early stages of Cmm infection. In addition, an increase in transcript levels of genes involved in biosynthesis of extracellular

polysaccharide (EPS), formation of biofilms – that would facilitate plant colonization process – and encoding a putative perforine, which could delivery effectors in host cells. The evidence suggests that virulence factors, like extracellular serine proteases, could be required at early stage of infection, while other genes – like those located in *tomA* subregion of genomic pathogenicity island, and involved in phosphate and iron metabolism – could be required for utilization of plant-derived soluble nutrients. The above, highlights the type of physiological responses that allow the adaptation of Cmm to conditions of the microenvironment that is established in tomato tissues during late stages of infection (Flügel et al. 2012).

Mass spectrometry was used to study the set of proteins involved in the tomato-Cmm interaction, known as the "interactome" (Savidor et al. 2012). The study revealed that during infection, Cmm expresses multiple types of hydrolytic enzymes, which include serine proteases and glycosyl hydrolases, which jointly make it possible for Cmm to colonize the tissue by degrading the cell wall and plant tissue. At the same time, the tomato generates a response to the Cmm infection by producing phosphatases, kinases, phospholipases, peroxidases and enzymes involved in methionine metabolism. Strikingly, high levels of the ACC oxidase enzyme, which is implicated in the biosynthesis of ethylene, were found, suggesting that ethylene synthesis in the tomato is spurred by the Cmm infection, confirming its role in the development of the disease (Savidor et al. 2012, Balaji et al. 2008).

The analysis of the tomato-Cmm interaction interactome (Savidor et al. 2012) pointed to two proteins with a potential role in the signaling for the pathogenicity mechanisms, which are Vatr1 and Vatr2 (virulence-associated transcriptional regulator). A Cmm strain carrying mutations at *Vatr1* and *Vatr2* genes showed a reduced symptomatology in tomato. Turning off the genes that code for *vatr1* and *vatr2* in Cmm NCPBB382, through targeted mutagenesis, resulted in strains (Δ*vatr1* and Δ*vatr2*) that were less virulent than the Cmm NCPPB383 reference strain (Savidor et al. 2014). The plants infected with the mutant strains displayed lower ethylene levels than those found in the plants infected with the Cmm NCPBB382 strain. Moreover, the transcriptome analysis of the mutant strains revealed low levels of expression for the virulence factors, like *celA* and *patI.* As such, the evidence suggests that *Vatr1* and *Vatr2* genes play a central role in regulating the pathogenicity mechanism in tomato, making tomato tissues more hospitable for Cmm. (Savidor et al. 2014).

To corroborate the role of the genes located in the genomic pathogenicity island in Cmm during infection process, mutant strains missing one of those genes – including the genes coding for serine proteases (*chpC, sbtA*), hydrolytic enzymes (*pgaA, endX/Y*), putative perforin (*perF*), putative sortase (*srtA*) and the transcription regulator *vatr2* – were created. The mutant strains exhibited: i) a significant reduction in the incidence and severity of the symptoms on tomato leaves (spots) as compared to the Cmm NCPBB382 strain; ii) it reduced ethylene levels and iii) comparatively moderate wilting symptoms with respect to the Cmm NCPBB382 strain. It should be mentioned, that *chpC* gene could be involved in suppression of tomato defense responses, since tomato infected with Cmm mutant *chpC* showed induction of PR protein genes as compared with tomato Cmm wild type-infected.
In addition, *perF* gene might be involved in translocation of effectors into host cell, and *srtA* gene could contribute to adhesion to plant surfaces and, therefore, colonization of tomato tissues, like mesophyll. In addition, the evidence suggests that chromosomal virulence factors play a different role in local and systemic colonization of tomato tissue (Chalupowicz et al. 2017).
In broad strokes, the evidence suggests that the tomato is unable to unleash a defense response to counteract colonization by Cmm. On another note, the evidence signals that Cmm can manipulate the tomato's metabolism to evade defense responses and colonize the plant tissue. Furthermore, this ability is driven by genes located on the genomic pathogenicity island.

Genetic diversity in the tomato-*Clavibacter michiganensis* biological system

The molecular building blocks underlying the compatible interaction between the tomato and Cmm are diverse and complex, which is a pretty clear indication of the degree to which Cmm has adapted to the tomato; having said that, it is essential to examine the level of genetic diversity on both sides of the interaction, as genetic diversity directly influences the disease progress.
Outbreaks of Cmm have been reported in Argentina (Wassermann et al. 2017), Italy (Ialacci et al. 2016; Bella et al. 2012), Uruguay (Croce et al. 2016), the U.S. (Tancos et al. 2015; Quesada-Ocampo et al. 2012), Belgium (Zaluga et al. 2013), Serbia (Milijašević-Marčić et al. 2012), Turkey (Baysal et al. 2011), Japan (Kawaguchi et al. 2010), Spain (De León et al. 2009), Mexico (Borboa-Flores et al. 2009; Holguín-Peña et al. 2006), Israel (Kleitman et al. 2008), Iran (Nazari et al. 2007) and Lithuania (Burokiene et al. 2005). As a result, several Cmm strain banks have been established. Cmm′s global presence has

helped to facilitate analysis of genetic diversity via molecular strategies and correlation with certain phenotype properties, including level of virulence.

Analysis of genetic diversity and the temporal and geographic context of each case suggest that Cmm outbreaks that display high levels of genetic variability likely originated from multiple infection sources (Wassermann et al. 2017; Croce et al. 2016; Tancos et al. 2015; Milijašević-Marčić et al. 2012; Baysal et al. 2011; Kleitman et al. 2008). On the other hand, Cmm outbreaks with moderate or low genetic variability likely originated from a single infection source and adapted to survive for such environmental conditions (Ialacci et al. 2016; Bella et al. 2012; Zaluga et al. 2013; De León et al. 2009). Now, the genetic diversity of a plant pathogen is directly related to the agro-ecological environment it inhabits (Stukenbrock and McDonald 2008). In this regard, a Cmm population with high genetic diversity may be able to adapt and respond to the activities inherent to growing tomatoes in a given agro-ecosystem – greenhouse or field – by modifying features such as tolerance/resistance to agrochemicals, colonization of new hosts, level of virulence, and capacity to spread (Gillings and Stokes 2012; Jacques et al. 2012; Lannou 2012; Yim et al. 2012; Stukenbrock and McDonald 2008).

The compatible interaction tomato-Cmm is favored by conditions of agroecosystems – high host density, low genetic diversity in host, tillage and plant disease control activities, fertirrigation, protected agriculture – that enable fast and easy dissemination of diverse Cmm genotypes well adapted to said conditions and resistant to pesticides (Karasov et al. 2014; Stukenbrock and McDonald 2008). In addition, it is necessary to consider that the extremely genetically-diverse Cmm sits in sharp contrast with the genetic erosion of commercial tomato cultivars. The loss of genetic diversity in the tomato, known as domestication syndrome, is the result of over-selection for tomato genotypes that express the phenotype and physiological features of the fruit (shape, size, carotenoid content). Recall that even in places where there are highly productive tomato cultivars, this domestication syndrome has brought on reduced tolerance to biotic and abiotic stress (Tomato genome consortium 2012; Bai and Lindhout 2007).

Considering the tomato-Cmm biological system in the context of genetic diversity, the situation is such that Cmm has the resources to establish a compatible interaction with the tomato plant and is able to adapt to changes in its ecological niche. In other words, Cmm is a plant pathogen that has adapted to the commercial tomato cultivars scattered far and wide across the globe. On the other hand, the host lacks the ability to respond effectively against the Cmm infection as a result of domestication syndrome (Tomato genome

consortium 2012; Bai and Lindhout 2007). The foregoing explains why there are no commercial tomato cultivars with stable and lasting tolerance to diverse Cmm genotypes.

Tomato cultivars do not have the high levels of genetic diversity needed to guarantee tolerance to Cmm. Next-generation sequencing (NGS) in wild tomato species – *S. arcanum, S. habrochaites, S. pennellii* – has revealed genome variability up to 20 times higher than that which is found in commercial cultivars (Aflitos et al. 2014). The genomic information obtained through NGS on commercial cultivars and wild species suggests, moreover, the tomato domestication process have "left a footprint" at the DNA level, it means, a large proportion of tomato genome has been fixed, which encode variants for fruit morphological traits, which could limit conventional genetic improvement through breeding with wild species (Lin et al. 2014).

Genome information derived from the wild species is an invaluable resource that can help shed light on domestication syndrome in the tomato and determine the biochemical mechanisms that support the agrochemical traits of interest (Perez-Fons et al. 2014). Accordingly, a genome and metabolome analysis of *S. pennellii*, a wild species that is highly tolerant to drought, suggests there is a role to be played by metabolic pathways involved in the biosynthesis of lipids that minimize water loss (Bolger et al. 2014; Perez-Fons et al. 2014).

As such, wild species constitute a vital source of genetic variability with extremely desirable characteristics for breeding programs, such as tolerance towards diseases (Adhikari et al. 2017; Hassan et al. 2017; García-Cano et al. 2010), including the bacterial wilt and canker of the tomato, considered the most serious tomato disease (Sen et al. 2013; Kabelka et al. 2002; Van-Heusden et al. 1999). In this regard, identifying the phenotype and genotype traits of interest and understanding their respective molecular basis in wild species is essential to apply them in tomato improvement.

In this sense, using genetic mapping, three genome regions, known as quantitative trait loci (QTL), were identified in *S. arcanum* LA2157 (Van-Heusden et al. 1999) and two QTLs in *S. habrochaites* LA408 (Kabelka et al. 2002) were found, endowing isogenic tomato lines (obtained by crossing the tomato and wild species) with resistance against the Cmm infection. Sandbrink et al. (1995) reported to *S. peruvianum* LA2172 as susceptible species to Cmm infection.

With the aim to identify the proteins involved in resistance to Cmm, an analysis of the proteome of the isogenic tomato lines infected with Cmm and containing the QTLs Rcm 5.1 and Rcm 2 identified in *S. habrochaites* LA408, revealed the role of oxidative

metabolism as a successful defensive measure against *Cmm.* It is also suggested that the QTLs are codominant and can unleash different tolerance mechanisms (Coaker et al. 2004).

In order to deepen the effective defense responses against Cmm in wild species, Lara-Ávila et al. (2012) performed the comparison of the temporal gene expression profiles obtained from the wild species *S. arcanum* LA2157 (known as resistant species), *S habrochaites* LA2128, and *S. peruvianum* LA2172 (known as susceptible species) with those obtained from tomato, it was possible to identify the induction of transcripts in each species various hours following infection, as a result. As expected, no symptoms were observed in *S. arcanum* LA2157, but low levels of symptoms were observed in *S. habrochaites* LA2128 and *S. peruvianum* LA2172 compared to tomato. Examples of differentially expressed genes between four species analyzed are *PBC1* and *SCE1 SUMO E2* genes that participate in the pathway to break down specific proteins by attaching with ubiquitin. The evidence suggests that the wild species can respond to a Cmm infection more quickly and intensely than tomato, and Cmm could evade/manipulate potential defense responses of tomato. All of this makes clear how well Cmm has adapted to commercial tomato cultivars (Lara-Ávila et al. 2012).

In this regard, the role of the *SCE1 SUMO E2* transcript in the tolerant phenotype in *S. peruvianum* LA2172 was demonstrated by way of virus-induced gene silencing (VIGS) of said transcript. When the *S. peruvianum* LA2172 plants with the silenced *SCE1* transcript were infected by Cmm, they displayed more symptomatology than the unsilenced plants, which suggests the *S. peruvianum* LA2172 is tolerant species to Cmm infection and the essential role played by defense pathways dependent on *SCE1* gene (Esparza-Araiza et al. 2015).

In addition to the aforementioned wild species, molecular methods have confirmed the existence of tolerance in other species, such as *S. pimpinellifolium* GI.1554, *S. parviflorum* LA735, *S. parviflorum* LA2072, *S. glandulosum* IVT 63102, and *S. minutum* (Sen et al. 2013). Due to the high level of genome variability in wild species, that it is likely that tolerance mechanisms vary from species to species, and are in turn shaped by multiple metabolic pathways; as a result, it is highly unlikely that Cmm will be able to adapt to resistant wild species, which implies that the Cmm-tolerant trait in the wild species could be stable and lasting.

Prospects

The compatible interaction between Cmm and tomato is an extremely complex and multi-faceted biological system, one of whose facets is the level of genetic variability in each organism. Nevertheless, the cultivated tomato – due to domestication syndrome – lacks the capacity to counteract infection by Cmm, which is a genetically diverse plant pathogen able to manipulate the tomato's metabolism. The foregoing underscores the importance of early diagnosis and disease monitoring strategies, which can complement promising proposals for control, including harnessing microbial diversity and genetic engineering of the tomato. On another note, although the tomato lacks the resources to counteract the Cmm infection, in wild species, a stable and lasting feature of tolerance towards Cmm has been described, due to higher levels of genetic diversity, which could help lay the groundwork for restoring the degraded genetic base of the tomato. There are lots of resources in the tool chest, including genome, transcriptome, proteome and metabolic analyses of both the tomato and of resistant wild species; altogether, these analyses in conjunction with other disciplines could pave the way to improve the tomato through the genetic and metabolic variation of the wild species. Likewise, these analyses also elucidate the molecular mechanisms associated with complex quantitative features – such as disease resistance – and can help develop novel multidisciplinary tools to study complex biological systems. For the time being, although progress has been made in understanding the mechanisms underlying susceptibility and pathogenicity in the tomato-Cmm biological system, our research group would pose the following questions to tackle: What is the *quorum sensing* mechanism in Cmm and how is it related to pathogenicity? Which metabolites could serve as specific markers of the Cmm infection in the tomato? What is the metabolome of Cmm like at different stages of infection in the tomato? How is that Cmm manages to evade/suppress the tomato's defense responses? What is the metabolic difference between compatible and incompatible interactions in Solanum-Clavibacter biological system?

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List of tables

table 1

Solanum-Clavibacter Interaction	'Omic' Technology	Methodology	Contribution	Reference
The host factors	Genomics	qPCR	Identification of tomato wild relatives (*S. pimpinellifolium* GI.1554, *S. parviflorum* LA735, *S. parviflorum* LA2072, *S. glandulosum* IVT 63102, *S. minutum*) with resistance to Cmm.	Sen et al. 2013
		QTL mapping	The QTL Rcm 5 and Rcm 2.1 from *S. habrochaites* LA408 confer resistance to Cmm in tomato	Kabelka et al. 2002
			Three QTL from *S. arcanum* LA2157 confer resistance to Cmm in tomato	Van-Heusden et al. 1999
	Transcriptomics	Virus Induced Gene Silencing (VIGS)	SCE1 gene is essential for resistance phenotype to Cmm in *S. peruvianum* LA2172.	Esparza-Araiza et al. 2015
		cDNA-AFLP	In resistant tomato wild relatives (*S. arcanum* LA2157, *S. peruvianum* LA2172, *S. habrochaites* LA2128) genes involved in plant defense are induced while such genes are repressed in tomato at early stages of Cmm infection.	Lara-Avila et al. 2012
		qRT-PCR	Genomic pathogenicity island in Cmm could inhibit expression of genes involved in defense in tomato, like Pathogenesis Related (PR) proteins.	Chalupowicz et al. 2010
		Microarrays	The phytohormone ethylene contributes to pathogenicity of Cmm in tomato.	Balaji et al. 2008
	Proteomics	LC-MS	The tomato produced a limited and delayed defense response against Cmm infection, mediated by ethylene.	Savidor et al. 2012
		2DE-PAGE	Rcm 5 and Rcm 2.1 loci from *S. habrochaites* LA408 confer resistance against Cmm infection in tomato through different mechanisms.	Coaker et al.2004
The pathogen factors	Genomics	Genome sequencing	Identification of genomic region involved in pathogenicity trait	Gartemann et al. 2008
	Transcriptomics	qRT-PCR	Plasmidic and chromosomic virulence factors from Cmm, showed different function during local and systemic infection of tomato	Chalupowicz et al. 2017
		qPCR RNA-seq	Vatr1 and Vatr2 proteins regulate expression of genes involved in pathogenicity making tomato tissues more hospitable for Cmm.	Savidor et al. 2014
		Microarrays	At early stage of infection virulence factors, like extracellular serine proteases, and genes involved in carbohydrate, phosphate and iron metabolism could be required for accessing and using plant-derived soluble nutrients	Flügel et al. 2012
		qRT-PCR	The expression levels of virulence factors, both genomic and plamisdic-borne, are influenced by themselves, which suggest	Chalupowicz et al. 2010

			the transition of Cmm from endophytic to pathogenic stage.	
	Proteomics	LC-MS	Cmm expresses multiple types of hydrolytic enzymes to colonize tomato plants by degrading the cell wall and plant tissue.	Savidor et al. 2012