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Citrus Responses to *Xylella fastidiosa* Infection, the Causal Agent of Citrus Variegated Chlorosis

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ABSTRACT

Among citrus diseases, citrus variegated chlorosis is one of the most important in Brazil. It causes annual losses of US$100 million to the citrus industry with chemical control of the vectors, pruning and roguing of diseased trees. The production of severely infected trees is heavily affected since they end up with reduced fruit size with hard rind, and not useful for industry or direct consume. The disease is caused by *Xylella fastidiosa*, a Gram-negative bacterium that lives inside the xylem vessels. First identified as a pathogen of vine, this bacterium has its pathogenicity associated with the colonization of the vessels, forming a dense biofilm that blocks the flow of the sap, generating a strong water deficit in the upper part of the affected plants. The tree physiology is heavily affected with a misbalance in different processes of the susceptible plants. The spectrum of susceptibility/resistance varies widely among the citrus group and genetic factors seems to be associated with the resistance in particular species. The understanding of the resistance mechanisms in citrus is deeper and points to an active response mediated by recognition elements that signalize the presence of the pathogen leading to the production of defense weapons that kill the bacterium. This chapter reports the complex responses associated with the presence of *X. fastidiosa* both at the physiological and genetic levels that allowed a better understanding of this host-pathogen interaction in a search for resistant varieties with increased agronomical value.

Keywords: CVC, gene expression, mandarin, resistance, sweet orange

CONTENTS

CITRUS VARIEGATED CHLOROSIS

Origin, etiology and symptoms

Citrus variegated chlorosis (CVC) was firstly related in 1987 in both Triangulo Mineiro region of Minas Gerais State, and Northern and Northwestern regions of São Paulo State, Brazil (Rossetti *et al.* 1990). The disease was associated with the xylem–limited bacterium *Xylella fastidiosa*, and the Koch's postulate was full filled by independent researchers groups (Chang *et al.* 1993; Hartung *et al.* 1994). Since the bacterium can affect different hosts, the CVC strain has been tentatively reclassified as *X. fastidiosa* subsp. *pauca* (Schaad *et al.* 2004; Schuenzel *et al.* 2005). The symptoms of CVC can be observed in leaves, fruits, branches and the whole tree, and include foliar wilt which become more evident in young leaves as a water deficit symptom (**Fig. 1A**). Typical irregular chlorosis evolve in mature and old leaves, recognized by interveinal yellowing on the upper side of leaf and corresponding brownish gum-like material on over side (**Fig. 1B**). Zinc- and iron-like deficiency can be frequently observed in the affected leaves (**Fig. 1C**). Later on, the brown spots may coalesce and the leaves drop. Stunted trees show twig dieback, fruits of reduced size and hardened becoming unsuitable for the juice industry as well as for the fresh fruit market (**Fig. 1D, 1E**).

Spread and geographic occurrence

First reported in 1987, the CVC spread over all citrus production regions of Brazil, reaching in 2005 almost 43% of the 200 million sweet orange trees growing in São Paulo State (www.fundecitrus.com. br). The geographical distribu-

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Invited Review

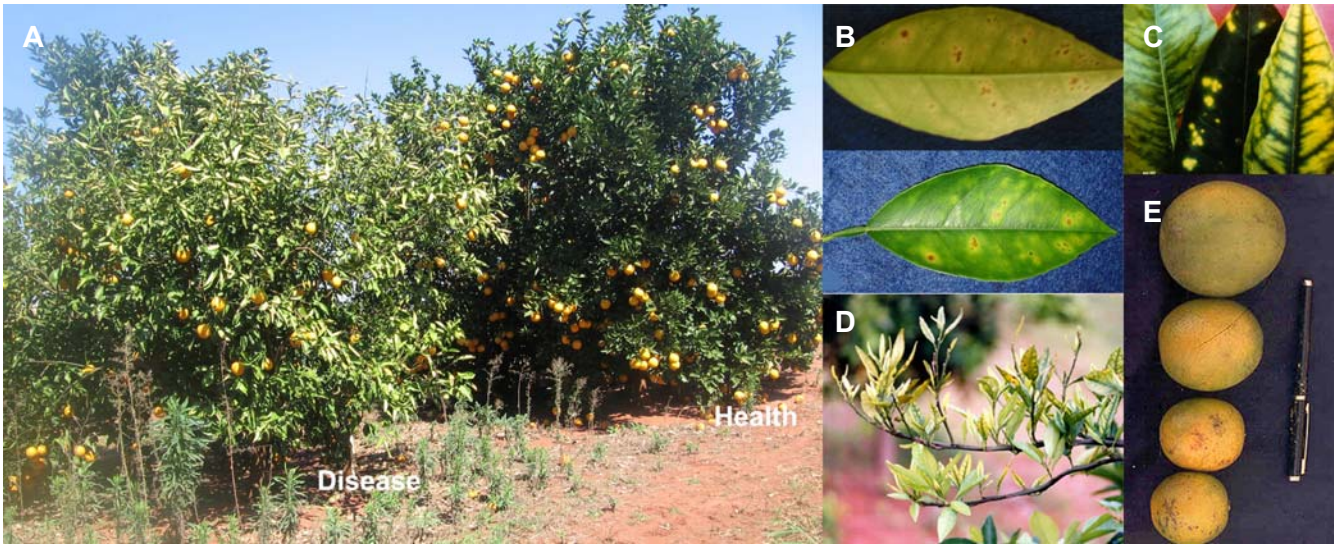

Fig. 1 General aspects of citrus variegated chlorosis symptoms. (**A**) Diseased and healthy trees. (**B**) Leaves symptoms (interveinal yellowing on the upper side (adaxial surface) and corresponding brownish on the under side, i.e. abaxial surface). (**C**) Zinc and iron associated deficiency. (**D**) Leaves drop and dieback; (**E**) Reduction of fruit size.

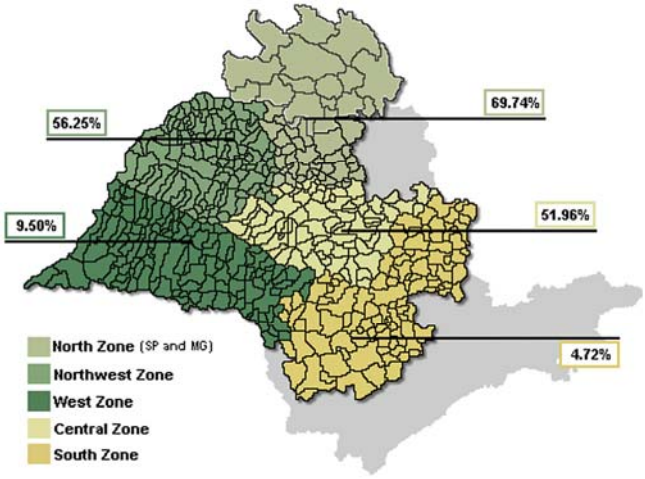

Fig. 2 Map of São Paulo State showing the CVC incidence in different geographic regions. (Source: http://www.fundecitrus.com.br)

tion of CVC in the main citrus growing areas in Brazil is uneven and may be associated with temperature and water availability throughout the year. The most severely affected North and Northwestern regions in São Paulo State are characterized by high temperature and uneven rainfall, while the South and West regions with mild temperature and a well distributed rainfall are less affected by CVC (**Fig. 2**).

In the beginning of the outbreak, the most significant spreading of CVC throughout the Sao Paulo orchards had probably occurred by the *Xylella*-infected nursery materials (buds and young trees). However, after 2002 a nursery program in which the plants are grown under a vector protected system became mandatory. As consequence, currently the spreading of CVC through orchards has been attributed exclusively to several species of xylem-feeder sharpshooters. At least 11 species of sharpshooters (Cicadellinae) are known to be involved in the transmission of *X. fastidiosa* from plant to plant. Four of them (*Acrogonia citrina*, *Bucephalogonia xanthophis*, *Dilobopterus costalimai*, and *Oncometopia facialis*) are the most important for the disease spread once these species can be frequently found in a high number in citrus disease trees (Parra *et al.* 2005).

CVC has been reported in Argentina (Brlansky *et al.* 1991), Paraguay (Segnana *et al.* 1998), and recently in Costa Rica (Aguilar *et al.* 2005). In Argentina the citrus disease called 'pecocita' was firstly reported before CVC in Brazil, but despite the fact that both diseases have the same symptoms, it was only lately that *X. fastidiosa* was associated to 'pecocita' (Beltran *et al.* 2004). Therefore CVC is an emergent disease, which seems to be spreading slowly over the American continent.

Natural plant hosts

Under natural conditions in Brazil CVC is essentially a disease of sweet orange trees (*C. sinensis* L. Osb), the most cultivated citrus species in the world. But there is a complete spectrum from resistance to susceptibility to CVC within the genus *Citrus*. Analyses of more than 200 accessions of sweet orange failed to detect any resistant or tolerant variety to *X. fastidiosa*. Some mandarins (Carvalhais, Emperor, Wilking, and Tankan) are also high sensitive. Sour orange (*C. aurantium* L.), tangelos (Page, Swanee, and Willians), and tangors (Dweet, Hansen, Ortanique, Temple, and Umatilla) are also susceptible to CVC. On the other hand, varieties of mandarins (*C. reticulata*), acid lime (*C. aurantiifolia*), lemon (*C. limon*), grapefruit (*C. paradisi*), pummelo (*C. grandis*), and tangor (*C. sinensis x C. reticulata*), kumquats, and *Poncirus trifoliata* present high tolerance and resistance to the disease (Laranjeira *et al.* 1998; Coletta-Filho *et al.* 2007).

Information regarding other non-citrus natural hosts for *X. fastidiosa* of CVC is rare and controversial. Under natural conditions, *X. fastidiosa* of CVC was unevenly found in 10 out of 23 species of weed plants sampled in two groves affected by CVC. However, the bacterium titer in those weeds is probably below the leafhoppers acquisition threshold, consequently, unimportant to the epidemiology of the disease (Lopes *et al.* 2003). Under greenhouse and artificial inoculation the information about non-citrus host for citrus–*X. fastidiosa* is also controversial. Li *et al.* (2001, 2002) showed that the citrus-*X. fastidiosa* is able to colonize grape and coffee plants and incite symptoms of Pierce's disease and coffee leaf scorch, respectively. On the other hand, under greenhouse and artificial infection, no successful long-term infection of coffee plants by the citrus-*X. fastidiosa* strain was obtained and consequently no disease symptoms were developed (Almeida *et al.* 2008). These last results match those of Prado *et al.* (2008), indicating that the used citrus-*X. fastidiosa* isolates are unable to establish in coffee plants.

Disease management and economic damage

In fact, CVC strongly affects sweet orange production mainly in the North, Northwest, and Central areas of the

São Paulo State, Brazil, leading to important economic losses. The growers have undertaken several control measures including pruning of affected branches in weakly or mildly infected trees and eradication of highly diseased plants to remove the inoculum, spraying of insecticides to reduce the population of transmission vectors (sharpshooters) and using healthy nursery trees (Coletta-Filho *et al.* 2000). Bové and Ayres (2007) estimated in 10% the reduction of fruits production as consequence of CVC in whole São Paulo State that results in a lost of US$ 125 million, according to the authors.

Physiological and genetic aspects

Although the understanding of the genome-level mechanisms underlying plant-phytopathogen interactions has greatly advanced over the last years, the physiological characteristics that have been affected by the infection processes are still not well understood. In particular, the study of the interaction between *X. fastidiosa* and its hosts is even more difficult since the development of the disease is very slow, which may affect the monitoring of the pathogen during the infection process as well as investigations regarding the plant-host physiology.

Utilizing a pathosystem that involves the infection of citrus and grapevine plants with *X. fastidiosa*, we can systematically dissect the defense response by the characterization of appropriate defense-related mechanisms of those plants, however important factors such as the concentration and specific content of the xylem fluid (amino acids and sugars) may differentially affect growth, aggregation, and biofilm formation of the *X. fastidiosa* according to the plant host (Bi *et al.* 2007).

Regardless of the limitations found in the study of this pathosystem, considerable effort has been made to elucidate the physiological modifications that occur in citrus plants infected by *X. fastidiosa*, which includes traits like gas exchange, sucrose, starch and reducing sugar contents (Gomes *et al.* 2003a), abscisic acid and indole-3-acetic acid contents (Gomes *et al.* 2003b) and nitrogen metabolism (Purcino *et al.* 2007).

Changes in photosynthesis found in citrus plants infected with *X. fastidiosa* (Gomes *et al.* 2003b) are also detected in symptomless leaf tissues, indicating that *X. fastidiosa* causes damage to the plant before symptoms become visible (Machado *et al.* 1994; Ribeiro *et al.* 2003). Interestingly, in grapevine, leaf-scorch symptoms in Pierce's disease can occur even with low concentrations of the bacterium (Gambetta *et al.* 2007). However, it has also been suggested that the extent of vessel blockage by bacterial colonization is highly likely to be a crucial variable in symptom expression (Newman *et al.* 2003). To determine in citrus how the process is affected by *X. fastidiosa* before visible symptoms become apparent, Ribeiro *et al.* (2003) measured leaf gas exchanges, chlorophyll a fluorescence and photosynthetic oxygen evolution and concluded that the lower photosynthetic rates in the leaves were caused by low stomatal conductance, biochemical injuries to the photosynthetic machinery (which might be caused by bacterial toxins) and an increase in alternative electron sinks.

The higher vulnerability of CVC-affected plants to water stress may be a consequence of the decrease in the xylem hydraulic conductivity causing stomatal closure, which are associated to the blockage of these vessels by *X. fastidiosa* (Machado *et al.* 1994, 2007). Indeed, the extracellular polysaccharides produced by *X. fastidiosa* in the xylem, and in which the bacterium is found, may contribute to the adhesion between the bacterial colonies and the vessels, then causing the occlusion of the xylem (Habermann *et al.* 2003).

Although the mechanisms of virulence of *X. fastidiosa* are not entirely understood, including how they contribute to the development of CVC, the occurrence of xylem-limited *X. fastidiosa* biofilm leading to vessel occlusion and subsequent water deficits (reduced water conductance) has been found to be especially critical to cause the CVC symptoms (Hopkins 1989).

A lower hydraulic conductivity to the CVC-affected fruits is a key factor in the reducing of the size and weight of citrus fruits (Gazzola *et al.* 1991) as well as in modifying internal compounds concentration like sugars (Laranjeira and Palazzo 1999).

Induced water deficits resulted in an accelerated symptom development of CVC as compared to citrus plants not subjected to drought (Gomes *et al.* 2003a). The use of irrigation decreased the negative effects of CVC, but it did not prevent disease establishment in sweet orange plants inoculated with *X. fastidiosa* (Machado *et al.* 2007). Actually, even healthy citrus plants subjected to water stress show a decrease in the activation and total activity of the Rubisco enzyme, i.e., the carboxylation efficiency (Vu and Yelenosky 1988).

Indeed, most of the studies on physiology of citrus plants with CVC are largely based on correlative evidence with PD of grapevine, a disease that also shows clear association with water deficits symptoms. In grapevine, impaired hydraulic conductance, low leaf water potential and turgor and higher stomatal resistance were associated with PD symptoms (Goodwin *et al.* 1988). However, by comparing grapevines exposed to water deficits, stem inoculation with *X. fastidiosa*, and combinations of both to evaluate whether symptoms of PD were a consequence of water deficits, Thorne *et al.* (2006) found that factors other than water deficits may be involved in producing the symptoms of PD.

Recently, Purcino *et al.* (2007) demonstrated by proteomics approaches that the metabolism of N of citrus plants is highly affected by CVC, which could be demonstrated by a higher activity of glutamine synthetase and protease and a high concentration of the polyamine putrescine in diseased plants, which might be a consequence of photorespiration or proteolysis, typical components identified during a regular senescence process. In addition, a clear modification in the protein pattern between healthy and diseased citrus plants was found. However, it remains unclear if the most of the modifications in the N metabolism are a direct response to the pathogen or a consequence of the water stress. Susceptible citrus plants that are colonized by *X. fastidiosa* show typical water deficit symptoms (decreases of photosynthesis, transpiration, stomatal conductance, and water potential), as compared to leaves of healthy plants (Machado *et al.* 2004).

Also, a severe decrease in the content of certain nutrients is found in leaves of symptomatic trees infected with *X. fastidiosa*, which may be caused by absorption from the bacteria that are inside the tissues (Hopkins 1989; Chang and Donaldson 1993). Moreover, throughout the genome of *X. fastidiosa*, various putative transporters related to substrates such as organic acids, sugars, amino acids, inorganic ions like phosphates and nitrates, peptides and vitamins were identified, which could confer the bacteria a capacity to compete for nutrients from the sap.

A study conducted to investigate whether the disease directly affects the levels of the abscisic acid and auxin, in citrus trees, found no correlation between the presence of the bacteria and a disturbance in the hormones concentration in the leaves (Gomes *et al.* 2003a).

It has been clearly shown that *X. fastidiosa* influences the citrus physiology, especially by affecting hydraulic conductance, N metabolism as well as the photosynthesis rates, however it remains not well understood how they interact with each other and which additional plant pathways may be affected in the interaction with the pathogen.

How the dramatic changes in the citrus physiology caused by CVC could be explained by the genetic modulation in gene expression remained unclear until the more recent studies focusing the sequencing of ESTs from citrus infected with *X. fastidiosa*.

Differential expression represents an interesting way of detecting changes in the behavior of organisms in face of different conditions/treatments. A huge EST sequencing project was carried in Brazil focusing the identification of ex-

pressed sequences of citrus and related genera that are important for disease resistance, water stress, fruit quality, among others. In one of these analyses, the focus was on CVC, and therefore, the authors evaluated the different genes expressed in CVC diseased sweet orange plants. In this analysis, two cDNAs libraries were constructed from non-inoculated and CVC-diseased sweet orange leaves. A total of 15,944 sequences were obtained and clusterized forming 4,066 contigs (consensi sequences), from which 37 were identified with significant variation in expression. In this subset, 21 were up-regulated and 16 were down-regulated in plants with CVC (de Souza *et al.* 2007a). Analysis of the main functional categories of the down-regulated genes in CVC-diseased plants revealed that they are primarily associated to metabolism, protein modification, energy and transport facilitation. As expected, transcripts related to photosynthesis were down-regulated in plants with CVC. The authors point that this may occur as a consequence of disorders that occur in the photosynthetic apparatus of CVC symptomatic plants. Expression of genes involved in secondary metabolism and cell wall structure were also down-regulated indicating that this plant may in fact be weaker and more susceptible to the attack of the pathogen.

On the other hand, they showed that the majority of the up-regulated transcripts in plants with CVC were associated to metabolism and defense response. One such gene is involved in cell wall structure and together with the down-regulated genes, reveals that changes in the cell wall are really taking place probably as a result of the infection. The other processes that seem to be altered in diseased plants are oxidative stress and detoxification, synthesis of secondary metabolites, ions uptake, and water stress.

CVC induces water and nutritional stresses that are part of the physiological changes observed in the plants. The evaluation of the differentially expressed genes showed that the responses of the plant seem to be much more related to responses to the damages caused by the disease development. It reflects very well the physiological changes observed in the plant with CVC, especially in the case of photosynthesis.

Regardless of the damage caused by CVC, this disease does not kill affected plants. Since there should be adaptation mechanisms that keep the plants alive, the up-regulation of genes related to reorganization of cell walls, ions transport and water stress in plants with CVC may be part of the process.

The genetics of CVC resistance in *C. reticulata*

Unlike for other diseases such as citrus canker or huanglongbing, there are sources of resistance to CVC in the citrus group. A complete spectrum of tolerance or resistance to CVC can be found among the citrus genera and their relatives. Tests for the presence of *X. fastidiosa* in plants grown in areas containing high inoculum sources with different genotypes of mandarins (*C. reticulata* Blanco), limes (*C. aurantifolia* L.), lemons (*C. limon* L. Burm. f.), grapefruits (*C. paradisi* Macf.), pummelos (*C. grandis* L. Osb.), and tangors (*C. sinensis* x *C. reticulata*) were all negative (Laranjeira *et al.* 1998). Moreover, experiments using different genotypes of mandarins failed to reproduce the CVC symptoms even though some genotypes tested positive for the presence of *X. fastidiosa* (Jaimes *et al.* 2002).

Although much is known about the disease, little information is available on the resistance to CVC. Within the tangors, the cv. 'Murcott' (an important variety both for fresh fruit market and industry) is resistant to *X. fastidiosa* (Laranjeira *et al.* 1998). Focusing this resistance of *C. reticulata* and its hybrids, our group initiated a breeding program for CVC resistance in 1997 by crossing 'Pera', sweet orange, the main sweet orange cultivar grown in Brazil, and the 'Murcott' tangor. A 'Pera'-'Murcott' hybrid population was selected (Oliveira *et al.* 2007) and utilized for evaluating the multiplication of *X. fastidiosa* and the reproduction of CVC symptoms. The spectrum of responses to the presence of *X. fastidiosa* was wide within these hybrids, which were classified as susceptible, tolerant, and resistant to CVC (Coletta-Filho *et al.* 2007). Since CVC symptoms is a result of the blockage of the xylem vessels by the bacterial colonization, one of the possibilities to explain this broad spectrum of phenotypes observed in relation to CVC resistance is the diameter of the xylem vessels, which could be larger in mandarins than in sweet orange plants. To test this hypothesis, these authors did histological sections and measured the diameter of the vessels from tangor 'Murcott' and sweet orange and their hybrids. The conclusion of the authors was that there was no difference between the susceptible and resistant hybrids that could explain the resistance. Therefore, it suggests that other mechanisms must exist in order to explain the resistance to *X. fastidiosa*.

Table 1 PCR and recovery of *X. fastidiosa* from artificially inoculated plants of 'Pera' sweet orange and Ponkan mandarin.

Plant inoculation	PCR results[1]			*X. fastidiosa* recovery[2]
	Weeks after inoculation			
	2	4	8	
Ponkan 1	+	+	+	-
Ponkan 2	-	+	+	-
Ponkan 3	+	+	+	-
Ponkan 4	+	+	+	-
Ponkan mock	-	-	-	-
Pera 1	-	+	+	+
Pera 2	-	+	+	+
Pera 3	+	+	+	+
Pera 4	+	+	+	+
Pera mock	-	-	-	-

[1]*Xylella*-specific amplification products from plant inoculated samples collected at two, four and eight weeks after inoculation: (+) positive amplification; (-) negative amplification

[2]Recovery of *Xylella fastidiosa*, from plant inoculated tissues, on PW medium at 11 weeks after inoculation: P - successful bacteria recovery; N - unsuccessful bacteria recovery.

Among the mandarins, 'Ponkan' is the predominant variety in Brazil. It is considered resistant to CVC since it presents no symptom or economical damage as a result of the bacterial infection. Therefore, this species has been included in citrus breeding programs focusing resistance to CVC and used as a source for the identification of genes with potential for a possible production of transgenic resistant plants. In 'Ponkan' mandarins the bacterium is still detected by PCR at 30 and 60 days after inoculation but recovery of the microorganism is not attained (**Table 1**). It seems that the bacteria start colonizing the plant but fail and die inside the vessels (de Souza *et al.* 2007a). To study the changes in gene expression that could lead to the resistance, EST libraries were recently produced just like what was done for the sensitive sweet orange infected with *X. fastidiosa* (de Souza *et al.* 2007a, 2007b; de Souza *et al.* unpublished data) by using non-inoculated mandarin plants and infected plants at 30 and 60 days after inoculation. These libraries were compared *in silico* for identifying the genes differentially expressed which could help the understanding of the genetic responses leading to resistance to CVC. In this work, analyses were performed in a universe of more than 25,000 sequences (available in GenBank, accession numbers from EY758170 to EY783598) that were assembled and evaluated using bioinformatics tools. Emphasis was given to the induced genes at 30 and 60 days after inoculation compared to the non-inoculated control. The evaluations of the libraries showed an induction of different sets of genes at these time-points (http://www.centrodecitricultura.br/~alessandra/cr_xf/) and revealed a probable multifactor anti-pathogen response involving perception, signal transduction and activation of defense-related genes. In the first time-point, various different genes involved in recognition and signal transduction were found.

For recognition, a gene encoding a NBS-LRR-like disease resistance protein was identified. This type of disease

resistance protein is normally involved in responses in which avirulence (Avr) proteins from the pathogens are injected into the plant cells. Since no genes for Avr proteins or type III secretion apparatus were found in the genome of *X. fastidiosa* (Simpson *et al.* 2000), this resistance protein could be involved in the recognition of a still unknown cytosolic elicitor. In addition, plants can perceive general elicittors 'non self' denoted PAMPs (pathogen-associated molecular patterns). This is the basis for the basal or non-host resistance, where general elicitors derived from conserved structures required for pathogen function are recognized in a non-specific manner by PRRs (pattern recognition receptors) and activate signaling pathways leading to gene expression and, consequently, resistance (Ingle *et al.* 2006). *X. fastidiosa* shows several molecules that can function as PAMPs including exopolyssacharides, lipopolyssacharides, adhesins and exoenzymes that are able to degrade the plant cell wall (Osiro *et al.* 2004; Fedatto *et al.* 2006; Wulff *et al.* 2006; Roper *et al.* 2007a). These exoenzymes are considered to be pathogenicity factors since they allow the systemic movement of the bacterium inside the xylem vessels (Newman *et al.* 2003). The expression of an endoglucanase (engXCA) has already been detected in *X. fastidiosa* growing in biofilm (de Souza *et al.* 2004). This kind of growth occurs inside the xylem vessels, resulting in their blockage in susceptible plants, like sweet orange, inducing typical development of CVC symptoms. It has been reported that cell wall degrading enzymes are ubiquitous virulence factors among plant pathogens (Boudart *et al.* 2003; Poinssot *et al.* 2003), and their enzymatic degradation products can also function as non-specific elicitors, since they are known to induce immune responses in plants (Fagard *et al.* 2007).

Resistance mechanisms induced by PRR-recognition of general elicitors and R-protein-mediated recognition of specific elicitors represents plant innate immune systems. Both share similar signal transduction pathways, including changes in cytoplasm calcium levels, ROS production and mitogen-activated protein kinase (MAPK) cascades (Jones and Takemoto 2004). 'Ponkan' showed up-regulation of one MAPK and an ethylene-related transcription suggesting an involvement of signal transduction related to these pathways in presence of *X. fastidiosa*. This activation will possibly lead to an amplification of the signal by a crosstalk of regulatory pathways could be controlling different cellular processes in mandarin - *X. fastidiosa* interaction. This crosstalk involves JA, ethylene, and SA pathways (de Souza *et al.* unpublished data).

At 30 days after infection with *X. fastidiosa*, genes involved with oxidative burst and antimicrobial activity were identified. Among these genes, cytochrome P450 encoding genes were up-regulated in 'Ponkan'. Interestingly, it mediates a wide range of oxidative reactions involved with biosynthesis of phenylpropanoids, terpenes, alkaloids, and common defense agents (Whitbred and Schuler 2000) and also, a P450-encoding gene was down-regulated in sweet orange diseased plants, which could show the involvement of such genes in CVC disease/resistance. To reinforce the importance of P450 in the resistance, genes encoding this protein were also up-regulated at 60 days after inoculation, when the bacterial population is declining. Other genes related to biosynthesis of secondary metabolites were also found making it evident the active response against the infection.

Comparison of the responses of the susceptible *C. sinensis* and the resistant *C. reticulata*

The group at the Centro de Citricultura Sylvio Moreira also compared the *C. sinensis* and *C. reticulata* responses at 30 days after inoculation (de Souza *et al.* 2007b). The genes with higher expression in the resistant *C. reticulata* encode NBS-LRR, S-adenosyl-L-methionine:salycilic acid methyltransferase, lipoxygenase, Fe-superoxide dismutase, cytochrome P450, and DnaJ. Another work comparing these two species identified specific chaperones belonging to the HSP70 family only in 'Ponkan' at 30 and 60 days after infection, csHSP70-3 and csHSP70-1. The increase in expression of these genes could be related to the oxidative stress response in 'Ponkan' (Fietto *et al.* 2007). Specific PR proteins were also identified as being differentially expressed in *C. reticulata* at 30 days after infection compared with *C. sinensis*, indicating their participation in the resistance of this organism (Campos *et al.* 2007).

Like observed from stems of PD resistant and susceptible sibling genotypes of grape infected with *X. fastidiosa* (Lin *et al.* 2007), signal transduction and defense-related response genes were up-regulated in *C. reticulata*. The results obtained in this comparison together with the evaluation of the expression pattern in the time course of infection of *C. reticulata* reinforce the idea that there is an active mechanism occurring in response to the presence of *X. fastidiosa* in the resistant plants. This mechanism involves perception, signal transduction, and activation of different pathways that may interact in order to develop resistance to the pathogen.

On the other hand, the genes with higher expression in sweet orange compared to mandarin at 30 days after inoculation belonged to the functional category of putative genes related to energy, which includes mainly genes associated with photosynthesis (de Souza *et al.* 2007b). These results in fact corroborate the previous findings obtained for sweet orange infected with *X. fastidiosa* that showed alterations in the expression pattern of photosynthesis related genes even without any symptom (de Souza *et al.* 2007a).

Hypothetical model of mandarin resistance response against *Xylella fastidiosa*

Since the resistance is not a result of the caliber of the vessels, there should be active defense responses leading to the resistance of *C. reticulata*. Intriguingly, the host perception of the intruder must be occurring in a tissue where the cells are dead. However, living cells that constitute the primary xylem are present inside the secondary xylem and could be responsible for the perception of the signals.

As described above, initially gene expression after inoculation with *X. fastidiosa* is associated mainly with recognition and signalization. Activation of the plant defense responses, in the interaction with *X. fastidiosa* (30 days after inoculation), is slow compared to other plant-pathogen interactions (Gibly *et al*. 2004). It probably occurs because *X. fastidiosa* is inoculated directly into the xylem vessels, which is basically constituted of dead cells and therefore, as mentioned before, the perception may occur in the few living cells present in the xylem, resulting in a response delay. Another possibility is that the bacteria adhere to xylem vessels, multiply, form biofilm, and then the cell wall degrading enzymes are produced allowing movement through the xylem pit membranes (Newman *et al.* 2003; Roper *et al.* 2007b). These enzymes and their degradation products probably act as non-specific elicitors which could be recognized by surface PRRs (pattern recognition receptors) and trigger a basal resistance response through the perception of multiple and distinct PAMPs. In the response to fungi, cell wall degradation is a very important factor that triggers the defense responses in plants. Sensor proteins present in the cell wall perceive the changes in this structure and induce the expression of genes involved in the defense of the plant (Vorwek *et al.* 2004). Since the colonization of *X. fastidiosa* involves cell wall degradation, it is possible that this defense mechanism is activated in mandarin in a way that is similar to what is observed in the interaction of plant with fungi.

Later on there is an increase in the number of genes associated to the expression of resistance-related genes, mainly involved in oxidative stress, P450 and biosynthesis of phenolic compounds. The whole process seems to lead to the decline in the bacterial population up to its disappearance. A defense response model of mandarins against *X. fastidiosa* is shown in **Fig. 3**.

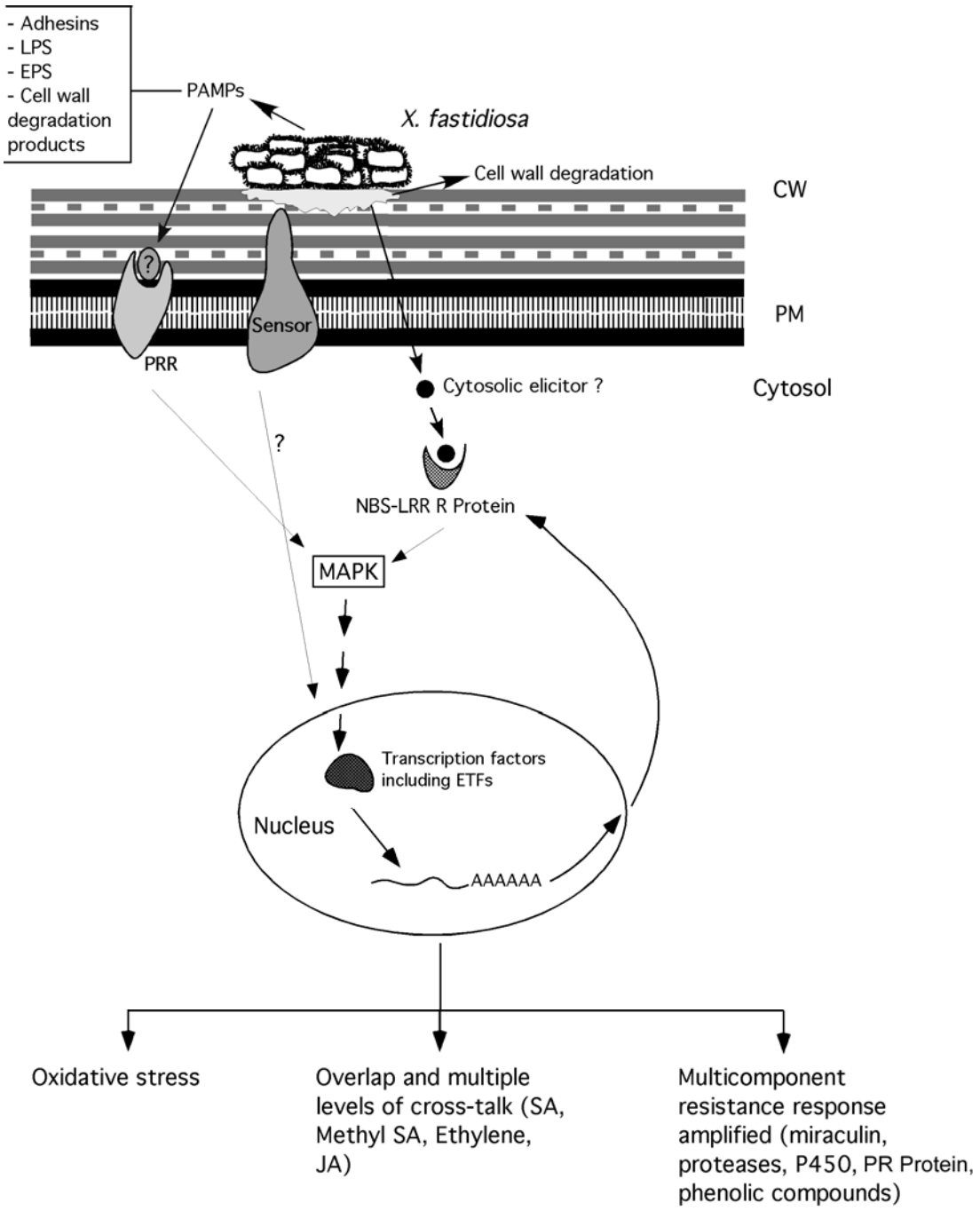

Fig. 3 Hypothetical model of mandarin defense response induced by *Xylella fastidiosa*. At 30 days after inoculation with *X. fastidiosa*, cell-wall degradation products, LPS, EPS, and adhesins could function as non-specific elicitors. They may be recognized by surface PRRs (pattern recognition receptors) and trigger a basal resistance response throughout the perception of multiple and distinct PAMPs. This could activate a MAPK kinase cascade leading to the expression of genes involved in different defense responses. In addition, the expression of the *NBS-LRR R* gene could be induced by a putative cytosolic elicitor, leading to a signal transduction that may induce its own synthesis. Plant cells can sense and respond to changes in the mechanical properties of walls. Since *X. fastidiosa* degrades the cell wall, stability of the cell wall moiety could be sensed by proteins present in this structure, which ultimately leads to defense responses in a way that resembles the defense against fungi. A crosstalk among regulatory pathways appears to be involved, with the participation of SA, Methyl SA, Ethylene, and JA. At 60 days after inoculation, some other genes involved in resistance are activated, leading to a multicomponent resistance response amplified that impairs the multiplication of the bacterium in the plant, avoiding the symptoms and consequently the disease. SA (salicylic acid), JA (jasmonic acid), ETF (ethylene-related transcription factor), LPS (lipopolysaccharides), EPS (exopolysaccharides), PAMPs (pathogen-associated molecular patterns).

However, some questions arise from the model. Is there really a cytosolic elicitor involved in the plant resistance response? If so, how is it injected into the plant cell? Moreover, how is the interaction between this elicitor and the resistance protein that showed up-regulation? These are open questions so far and may be interesting subjects for future works.

CONCLUSION

Even though CVC is a major threaten for the citriculture especially in Latin America, because of the absence of effective control measures, good perspectives for its control in the future make the scenario not so dramatic. Genetic enhancement through the production of hybrids is an alternative since there are sources of resistance inside the Citrus group. The understanding of the molecular mechanisms that lead to this resistance also allow the identification of good candidates for the production of genetic modified plants

that could be resistant to CVC. Since the work done so far shows a very complex network activated in response to the presence of the bacterium in the vessels of the resistant *C. reticulata*, the identification of key elements in this response in an essential step towards the production of these genetically modified plants. It remains to be investigated if one or few of the genes identified in the resistant plants are sufficient for generating resistance when over-expressed in the susceptible host.

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ADDITIONAL ELECTRONIC MATERIAL

A table listing all the induced genes in *Citrus reticulata* at 30 and 60 days after inoculation with *X. fastidiosa* is available at http://www.centrodecitricultura.br/~alessandra/cr_xf/

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