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Review

Molecular response of Mexican lime tree to "*Candidatus Phytoplasma aurantifolia*" infection: An overview

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"*Candidatus Phytoplasma aurantifolia*" is the causative agent of witches' broom disease in the Mexican lime tree (*Citrus aurantifolia* L.), and is responsible for major tree losses in Southern Iran and Oman. The pathogen is strictly biotrophic, and thus is completely dependent on living host cells for its survival. The molecular basis of compatibility and disease development in this system is poorly understood. Transcryptomic analysis of the susceptible lime cultivar at the representative symptoms stage showed a number of candidate genes that might be involved in the interaction of Mexican lime trees with "*Ca P. aurantifolia*". These included the genes for modifier of *snc1*, autophagy protein 5, formin, importin B3, transducin, L-asparaginase, glycerophosphoryl diester phosphodiesterase, and RNA polymerase b. In contrast, genes involved in basal metabolism like a proline-rich protein, ubiquitin-protein ligase, phosphatidyl glycerol specific phospholipase C-like, and serine/threonine-protein kinase. Proteomic analysis results reveal proteins that were involved in oxidative stress defense, photosynthesis, metabolism, and the stress response, regulate in infected trees. For the moment these results should help to identify genes that could be targeted to increase plant resistance and inhibit the growth and reproduction of the pathogen.

Key words: *Candidatus phytoplasma aurantifolia*, proteomix, transcryptomix.

INTRODUCTION

Witches' broom disease, which affects Mexican lime trees (*Citrus aurantifolia* L.), is caused by an obligate biotrophic plant pathogen, "*Candidatus Phytoplasma aurantifolia*". Phytoplasmas are prokaryotes that inhabit the phloem and are transmitted by phloem-sucking insects (Cimerman et al., 2006 and Hanboonsong et al., 2002, Zamharir, 2011b). This is a devastating disease that results in significant economic losses. The disease was first reported in the Northern coastal plain of the Sultanate of Oman, and since then, it has extended throughout the region (Bove et al., 1993). The disease has also affected Mexican lime trees in Southern Iran since approximately ten years (Zamharir et al., 2011a and Taheri et al., 2011). In the field, affected trees present with witches' brooms, which are shoot structures that are characterized by their compactness and their very small, pale green leaves. Witches' brooms display many thin secondary shoots with shortened internodes; these shoots develop from axillary buds that normally stay dormant. In the advanced stages of the disease, the leaves become dry and many witches' brooms appear. Finally, the tree collapses within four or five years after infection. The witches' broom structures lack flowers or fruits and normal shoots in infected plants produce fruits

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that are reduced in size (Bove et al., 1988). Interference with hormonal balance by ‘‘*Ca. P. aurantifolia*’’ has also been correlated with a series of morphological changes in infected plants, such as virescence (petals are green), phyllody (transformation of the floral organs in leaves), proliferation (development of leaves from floral organs), formation of witches' brooms from lateral buds, and floral malformations and abortions.

In addition, alterations in flowering and vegetative cycles (flowering in winter and anticipation of vegetative growth), modification of the internodes, and production of small and malformed fruits have been observed in phytoplasma-infected plants (Zamharir et al., 2011a; Taheri et al., 2011; Chang, 1998 and Lee et al., 2000). Our knowledge about the molecular mechanisms that are involved in ‘‘*Ca. P. aurantifolia’’* pathogenicity and the symptoms evoked in host plants is limited. Recent Studies by different molecular methods have been help to identify genes and proteins involved in Mexican lime tree response to ‘‘*Ca. P. aurantifolia*’’ infection (Zamharir et al., 2011a, Taheri et al., 2011).

The present paper identifies a number of candidate genes and proteins that might be involved in the interaction of Mexican lime trees with “*Ca P. aurantifolia*”. These results should help to elucidate the molecular basis of the infection process.

HISTOPATHOLOGY AND METABOLOMIC

The deposition of callous in the sieve plates, accumulation of starch in chloroplasts and disorganization of chloroplasts, alterations in cell wall thickness, and accumulation of polyphenols have been reported in plants infected with phytoplasma (Mardi et al., 2011). Phloem necrosis has also been observed as a symptom of diseases caused by phytoplasma.

Changes in metabolism and secondary metabolites, including reductions in chlorophyll a, chlorophyll b, and in the total chlorophyll content, have been reported (Zamharir et al., 2011a, Taheri et al., 2011). In addition, the content of carotenoids in leaves of lime plants decreases in several weeks after inoculation (Taheri et al., 2011).

TRANSCRYPTOMIC ANALYSIS

Comparative transcriptomic analysis of healthy Mexican lime trees and those infected by “*Ca. P. aurantifolia*” shows transcriptional changes that affected the expression of several genes related to physiological functions that would affect most leaves in infected tissues. Infection with “*Ca. P. aurantifolia*” causes wide-spread gene repression in Mexican lime trees (Zamharir et al., 2011a).

Several genes that were modulated in Mexican lime trees by infection with “*Ca. P. aurantifolia*” were related to defense, cell walls, and response to stress. The expression of autophagy protein 5 was repressed. Autophagy is a survival mechanism that protects cells against unfavourable environmental conditions, such as microbial pathogen infection, oxidative stress, nutrient starvation, and aggregation of damaged proteins (Kwon and Park, 2008).

It has been shown that carbohydrate starvation induces the expression of autophagy genes (Rose et al., 2006) and stimulates the formation of reactive oxidative species (ROS) in plants (Kwon and Park, 2008). It is likely that the accumulation of carbohydrate reduces the expression of autophagy genes in the host and limits the burst of ROS burst (hypersensitivity reaction). These effects might result in reduced host resistance to phytoplasma and create a suitable condition for phytoplasma survival in the host (Zamharir et al., 2011a).

It has been shown a cell wall hydroxyl proline-rich protein transcript was induced in lime response to “*Ca. P. aurantifolia*”. Proline-rich proteins are among the major structural proteins of plant cell walls. Environmental stresses can alter the composition of the plant cell wall markedly (Lamb et al., 1989). The induction of the hydroxyl proline-rich protein might reflect a defense mechanism of Mexican lime tree in response to phytoplasma infection (Zamharir et al., 2011a).

Another induced transcript contained a lysine domain that is found in several enzymes that are involved in degradation of the bacterial cell wall (Bateman et al., 2000). The role of this gene in the response of Mexican lime trees to the pathogens remains to be determined (Zamharir et al., 2011a).

Two of repressed genes were identified as a modifier of *snc1* (*MOS1*). Plant resistance (R) genes encode immune receptors that recognize pathogens directly or indirectly and activate defense responses (Jones and Dang, 2006). The expression levels of R genes have to be regulated tightly due to costs to the fitness of plants that are associated with maintaining R-protein mediated resistance. Recently, it has been reported that MOS1 regulates the expression of SNC1 which encodes a TIR-NB-LRR-type of R protein in Arabidopsis. It has been shown that mos1 mutations reduce the expression of endogenous *snc1*, which results in the repression of constitutive resistance responses that are mediated by *snc1* (Li et al., 2006). It is likely that down-regulation of Mexican lime tree MOS1 in response to the pathogen reflects a reduction in plant resistance responses to phytoplasma infection (Zamharir et al., 2011a).

Lipid-derived molecules act as signals in plant pathogen interactions, and the roles of jasmonic acid and related oxylipins that are produced from membrane-derived fatty acids through beta-oxidation, are particularly important (Shah, 2006). During infection, low level defense responses can be activated in susceptible plants (Lin et al., 2007; Polesani et al., 2008). Therefore, it is likely that well-established “*Ca. P. aurantifolia*” infections

involve the up-regulation of genes that encode components of the lipid metabolism pathway, such as phosphatidyl glycerol specific phospholipase C-like. This enzyme regulates the phosphatidylglycerol content via a phospholipase C-type degradation mechanism (Simockova et al., 2008). Another gene involved in lipid metabolisms, glycerophosphoryl diester phosphodiesterase was repressed during the infection. This enzyme has both phosphoric diester hydrolase and glycerophosphodiester phosphodiesterase activity and is involved in the metabolism of glycerol and lipids (Tommassen et al., 1999).

Among finding transcripts several were related to metabolism. These were genes that encoded ribosomal proteins and enzymes involved in protein degradation. The expression of ubiquitin-protein ligases and a 50 S ribosomal protein L15 were repressed, whereas another 50 S ribosomal protein L15 was induced. This suggests that the infection results in a general induction of protein turnover, which could reflect an adaptive response in the plants to remove misfolded proteins that have accumulated as a result of stress (Zamharir et al., 2011a).

Few modulated genes had signal transduction and/or gene regulation functions. They corresponded two transducin family protein that were repressed by infection and a serine/threonine protein kinases that was induced during infection (Zamharir et al., 2011a). Serine/threonine protein kinases are a group of enzymes that catalyze the phosphorylation of serine or threonine residues in proteins, with ATP or other nucleotides acting as phosphate donors. The phosphorylation of proteins on serine, threonine, or tyrosine residues is an important biochemical mechanism to regulate the activity of enzymes and is used in many cellular processes (Romeis, 2001).

Among down-regulated proteins, some were identified as members of the transducin family and contained WD40 domain. This domain is found in several eukaryotic proteins that with wide variety of functions, which include adaptor/regulatory modules in signal transduction, together with proteins involved in pre-mRNA processing, and cytoskeleton assembly (Lee et al., 2006). It is unclear how these changes contribute to the response of Mexican lime tree to infection (Zamharir et al., 2011a).

PROTEOMIC ANALYSIS

Proteomic analysis shows that some proteins were less abundant in infected lime plants by *Ca. P. aurantifolia* than in healthy plants, and some others proteins were more abundant in infected plants than in healthy plants. These proteins were involved in stress response, metabolism, growth and development, signal transduction, photosynthesis, cell cycle, and cell wall organization (Monavarfeshani at al., 2013).

It has been distinguished oxidative scavenging enzymes is downregulated in Mexican lime trees in response to pathogen ‘‘*Ca. P. aurantifolia*’’ (Taheri et al., 2011). The downregulation of ROS scavenging enzymes has also been reported in resistant rice plants during bacterial leaf blight infection (Kottapalli et al., 2007) and cucumber response to *Pseudoperonospora cubensis* (Li et al., 2011). It is likely that downregulation of these proteins in Mexican lime trees in response to pathogen ‘‘*Ca. P. aurantifolia*’’ contributes to the accumulation of ROS, which in turn induces a hypersensitive response in the plant (Taheri et al., 2011).

The proteomic analysis provided evidence for the downregulation of photosynthetic proteins including two oxygen-evolving enhancer proteins 1, two ribulose-1,5 bisphosphate carboxylase activases and ribulose-1,5 bisphosphate carboxylase/oxygenase (Taheri et al., 2011). These results are consistent with the previous reports that environmental stresses inhibit the expression of genes that encode photosynthetic proteins (Li et al., 2011, Seki et al., 2002 and Wu, et al., 2010). The differential expression and degradation of photosynthetic proteins have also been revealed by the proteomic analysis of the response of mulberry to phytoplasma (Ji, et al., 2009). Scharte et al. have suggested that photosynthesis must be switched off to initiate respiration and other processes that are required for plant defence against pathogen (Scharte et al., 2005).

Pathogenesis-related protein (PR)-10 was identified as one of the upregulated proteins (Taheri et al., 2011). Induction of this protein is consistent with a previous report of the expression of the PR-5 gene in grapevine and *Chrysanthemum carinatum* in response to phytoplasma infection (Margaria and Palmano, 2011; Zhong. and Shen, 2004).

Upregulation of PR-10 gene expression has been demonstrated in a wide variety of plant species after infection by pathogens, including infection of *Capsicum annuum*, *Cronartium ribicola* on *Pinus monticola*, *Pseudomonas syringae* pv. *pisi* on *Vitis vinifera*, *Magnaporthe grisea*, and *Acidovorax avenae* on rice (Taheri et al., 2011). The PR-10 family is one of the most important among 17 groups of PR proteins (Yan, et al., 2008). PR-10 is typically intracellular and it has been reported to have various functions, including antimicrobial activity, *in vitro* ribonuclease activity, and enzymatic activity in plant secondary metabolism (Liu and Ekramoddoullah et al., 2006). These functions implicate PR-10 in plant defence against pathogen attack (Taheri et al., 2011).

In addition miraculinlike proteins and three homologues of it upregulate during infectious process of lime trees by ‘‘*Ca. P. aurantifolia*’’(Taheri et al., 2011). Miraculins are highly glycosylated proteins that belong to a family of protease inhibitors. The specific function of miraculin-like proteins in the stress response has not yet been elucidated. However, upregulation of these proteins has been reported in compatible pathogen–plant interactions, in

including some that are caused by fungi or treatment with methyl jasmonate (MeJA) (Tsukuda et al., 2006) in *Citrus sinensis* leaves infested by the leafhopper *Homalodisca oagulate* (Mozoruk et al., 2006) and in citrus leaves in response to the spotted spider mite *Tetranychus urticae* (Maserti, et al., 2011). The different responses of various isoforms of miraculin-like proteins suggest that a complex regulatory network modulates their expression patterns. The upregulation of miraculin-like might provide insight into the defence mechanism of the Mexican lime tree against the pathogen ‘‘Ca. *P. aurantifolia”* (Taheri et al., 2011).

Several differentially expressed proteins were involved in protein translation and fate. These included the 40S ribosomal protein S12, a copper chaperone and ubiquitin-conjugating enzyme 1 which were upregulated, and three heat shock proteins and a putative GroES chaperonin, which were downregulated. The downregulation of heat shock proteins, which act to maintain the structural and functional integrity of damaged proteins, and upregulation of ubiquitin-conjugating enzyme, which is involved in important cellular mechanisms that target abnormal or short-lived proteins for degradation, could reflect an adaptive response in plants to remove misfolded proteins that have accumulated as a result of phytoplasma infection (Taheri et al., 2011).

Overall, these results suggest that proteomic changes in response to infection by phytoplasmas might support phytoplasma nutrition by promoting alterations in the host's sugar metabolism, cell wall biosynthesis, and expression of defense-related proteins. Regulation of defense-related pathways suggests that defense compounds are induced in interactions with susceptible as well as resistant hosts, with the main differences between the two interactions being the speed and intensity of the response ((Monavarfeshani at al., 2013).

COMPARISON OF TRANSCRIPTOME AND PROTEOME OF THE MEXICAN LIME TREE INFECTED BY PHYTOPLASMA

Comparison of the responses of the transcriptome and proteome of the Mexican lime tree to phytoplasma infection shows that different sets of modulated proteins were identified by these two approaches. Although the expression of ubiquitin-protein ligase was decreased at the mRNA level, we found that the level of ubiquitin-protein ligase protein increased in response to phyto-plasma infection (Zamharir et al., 2011a).

qRT-PCR analysis also showed a similar expression pattern at mRNA and protein levels and there was little correlation between the changes in mRNA and protein expression levels under stress conditions relative to normal conditions (Taheri et al., 2011). This poor correlation between transcriptomic and proteomic results confirmed that mRNA levels do not necessarily correlate with protein levels. Discrepancies between the expression levels of mRNA and those of their corres-ponding proteins have also been shown elsewhere. The lack of correspondence between transcript and protein levels might have been due to the fact that mRNA levels usually peak before protein levels increase. Post-transcriptional and post-translational modifications and different rates of degradation of mRNA and protein could also contribute to the discrepancies (Taheri et al., 2011; Zamharir et al., 2011a).

CONCLUSION

We believe that analysis of the expression of genes, proteins and metabolites involved in the interaction of Mexican lime trees with “*Ca. P. aurantifolia*” allowed several novel genes to be identified from Mexican lime trees, because a significant proportion of the TDFs and proteins are not currently represented in citrus databases. Researches show that infection resulted in the down-regulation of Mexican lime tress transcripts and proteins in all major functional categories. However, certain genes and proteins required for plant pathogen interactions were modulated positively during infection at the symptomatic stage. These results will serve as a basis to address new questions and design new experiments to elucidate the biology of plant-phytoplasma interactions and the associated re-programming of the host metabolism. They might also pave the way to identify genes and proteins that can be targeted to elevate plant resistance or inhibit the growth and reproduction of the pathogen. However, further research is required to elucidate the roles of these genes and proteins in the susceptibility/resistance of Mexican lime tress to “*Ca. P. aurantifolia*”, and to determine how strategies might be developed to incorporate these genes into molecular breeding programs (Taheri et al., 2011 and Zamharir et al., 2011a).

There are several immediate extensions that will increase our understanding of plant response to pathogen and may result in applications enhancing plant resistance. These extensions include using mass spectrometry to identify proteins that remain unidentified from the differentially expressed proteins reported before, examining highly responsive proteins and transcripts such as ascorbate peroxidase 2, Cu/Zn superoxide dismutase, miraculin-like proteins, and annexin p35 in other tissues and different time points and understanding whether the regulated proteins reflect a direct effect of the interaction with the phytoplasma or a secondary effect of the development of symptoms. It is also interesting to determine whether the observed protein and transcripts changes in response to pathogens are reflections of changes in gene and protein expression or post-translational modifications. Future studies are required to understand the role of the regulated genes

and proteins.

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