UNIVERSIDADE DE SÃO PAULO
ESCOLA SUPERIOR DE AGRICULTURA 'LUIZ DE QUEIROZ'

Laboratório de Genética Molecular de Plantas Cultivadas

ANALYSIS OF PLANT GENE EXPRESSION DURING PASSION FRUIT-*Xanthomonas axonopodis* INTERACTION IMPLICATES LIPOXYGENASE 2 IN HOST DEFENCE

Munhoz, CF[1]; Santos, AA[1]; Arenhart, RA[2]; Santini, L[1]; Monteiro-Vitorello, CB[1]; Vieira, MLC[1]

1 USP/ESALQ, Departamento de Genética, Piracicaba, SP; 2 UFRGS, Departamento de Genética Vegetal, Porto Alegre, RS. E-mail: carlamunhoz@usp.br

INTRODUCTION

Passiflora edulis is the principal species of passionflowers grown worldwide, mainly for juice production and fresh fruit. The bacterial leaf spot induced by *Xanthomonas axonopodis* pv. *passiflorae* (*Xap*) is one of the most severe diseases of the crop, particularly under moist field conditions (Figure 1), causing great losses to producers. The objective of this study was to describe a first analysis of host gene expression in this pathosystem.

Figure 1 A passion fruit orchard infected with *Xap*.

MATERIALS AND METHODS

Two subtractive cDNA libraries were constructed by performing the SSH method (Diatchenko et al., *Proc. Natl. Acad. Sci.*, 1996) from leaf transcripts of the 'IAPAR-123' plant accession. The libraries were enriched for transcripts induced and repressed by *Xap*, 24 h post inoculation, with a highly virulent strain (AP302) or saline solution (mock-inoculated). After sequencing the clones and sequence data processing, the unisequences were annotated using Blast2GO tool (Conesa et al., *Bioinformatics*, 2005) and the PLAZA Platform (Proost et al., *The Plant Cell*, 2009). *A. thaliana* proteins related to defence, resulting from the PLAZA analysis, were evaluated for possible interactions in the STRING tool (Franceschini et al., *NAR*, 2013).

The expression profiles of transcripts involved in most of categories, chiefly related to plant defence mechanisms, were analyzed by qPCR. For this, we used mRNA from leaf collected at early (24 hpi) and late (5 and 9 dpi) stages of interaction. Reactions were performed in a SteOne thermal cycler, in triplicate, using standard conditions. Amplification efficiencies and C_T values were determined using the software LinReg (Ramakers et al., *Neurosci. Lett.*, 2003). Relative changes in gene expression ratios (E) were calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, *Methods*, 2001). The t-test was used to estimate significant changes in relative expression levels (p-value < 0.05).

The passion fruit LOX2 transcript was translated *in silico* and BLASTP results compared with amino acids sequences publicly available in the GenBank database. Amino acid sequences that have shown high similarity (> 50%) to the passion fruit LOX2 were selected to perform a multiple sequence alignment implemented in the ClustalW algorithm of MEGA software v. 5.10 (Tamura et al., *Mol. Biol. Evol.*, 2011), and an estimated evolutionary distance for each alignment was computed.

RESULTS AND DISCUSSION

High quality non-redundant 998-nucleotide sequences were annotated. In accordance with BLASTX results performed by Blast2GO tool, 866 (86.7%) unisequences showed similarity to other plant species' proteins related to different functional categories. The vast majority of the top-hits made by our sequences were to *Populus trichocarpa* (41%), *Ricinus communis* (27%), and *Vitis vinifera* (8%). Sixty-three transcripts were similar to *Arabidopsis thaliana* defence-related proteins identified in the PLAZA platform. *In silico* predicted protein-protein interactions were detected on the basis of the STRING database for 35 of the 63 defence-related proteins, and grouped into five interaction clusters (Figure 2).

At the early stage of interaction, 63 genes selected from Blast2GO annotation were analysed by qPCR. The expression profiles changed in response to the pathogen for 48 of these genes (76.1%), and the differences in expression ratios were low (0.51 to 1.83-fold) but significant (p < 0.05), suggesting the onset of plant transcriptional reprogramming responses to the pathogen.

In late stages of interactions (5 and 9 days post inoculation) when disease-associated symptoms were visible, qPCR analyses were performed for 14 genes selected from both libraries. The expression profiles of all genes were found to be changed by the pathogen, and the differences in expression ratios ranged from 0.19-fold to 500-fold (Figure 3). The gene that responded most strongly to the pathogen attack encodes a lipoxygenase 2 (LOX2). In inoculated plants, its expression was induced 500-fold and 300-fold, 5 and 9 dpi, respectively, compared to controls, suggesting an important role of this gene in passion fruit defence. Moreover, we showed that most of the genes involved in well-known pathogen recognition signaling pathways were repressed by *Xap* and this lends support to the idea that the jasmonic acid signaling pathway fails to be activated during the first hours of interaction.

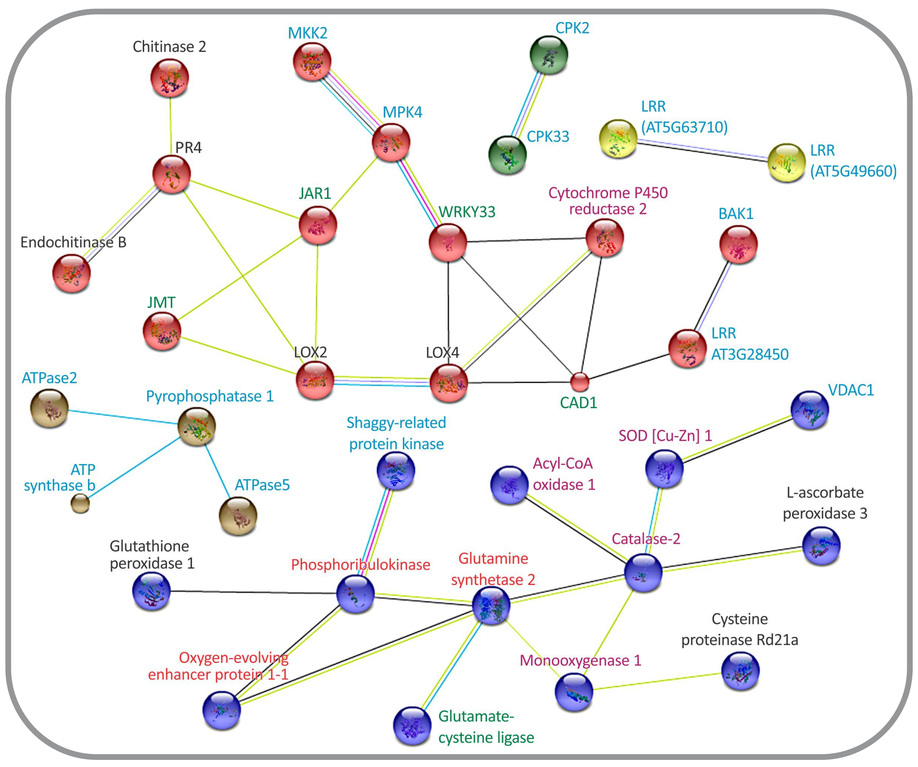

Figure 2 *Arabidopsis thaliana* protein-protein interactions obtained in STRING 9.1 database. Network spheres are proteins and lines represent the existence of evidence types used in predicting the associations as follows: coexpression (black), experimental (purple), database (light blue), text mining (light green), and homology (violet). The colour of the spheres and protein names respectively characterize the interaction clusters (obtained by MLC algorithm using inflation 1) and the categories to which proteins were assigned: intracellular signal transduction and transport (blue), activation of defence genes/ regulation of transcription (green), oxidoreductases (purple), pathogenesis-related proteins (black) and others (red).

The passion fruit partial CDS region encoding LOX2 has 2,279 nucleotides, and the 759 amino acids predicted *in silico* correspond to 84.7% of the complete protein sequence of *A. thaliana*, AtLOX2 (P38418.1). Figure 4 depicts the aligned sequences of eight plant species, including *P. edulis* and *A. thaliana*, as well as the conserved protein residues. The results of pairwise distance analysis showed that the passion fruit LOX2 amino acid sequence is very similar to the lipoxygenases of *Populus trichocarpa* (72%), *Vitis vinifera* (71%), *Prunus mume* (71%), *Ricinus communis* (70%), *Malus domestica* (70%) and *Camellia sinensis* (68%). A lower similarity of 59% was found for passion fruit and AtLOX2.

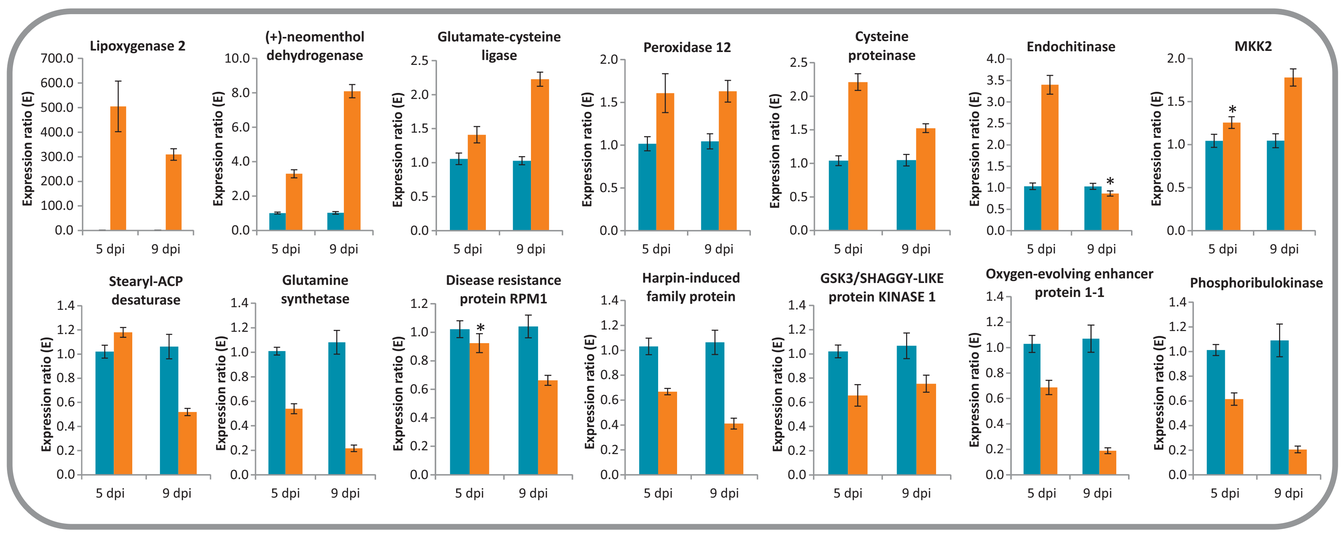

Figure 3 Quantification of the relative expression levels of 14 passion fruit genes in response to *Xap* infection, 5 and 9 days post inoculation (dpi). E: Expression ratio (Mean ± SD; n = 6 biological replicates) between inoculated plants (orange bars) and mock-inoculated plants (blue bars) using the $2^{-\Delta\Delta Ct}$ method. Histone, 60s ribosomal protein and a transcription initiation factor were used as housekeeping genes for normalizing expression signals. *Not significantly different according to t-test (p > 0.05).

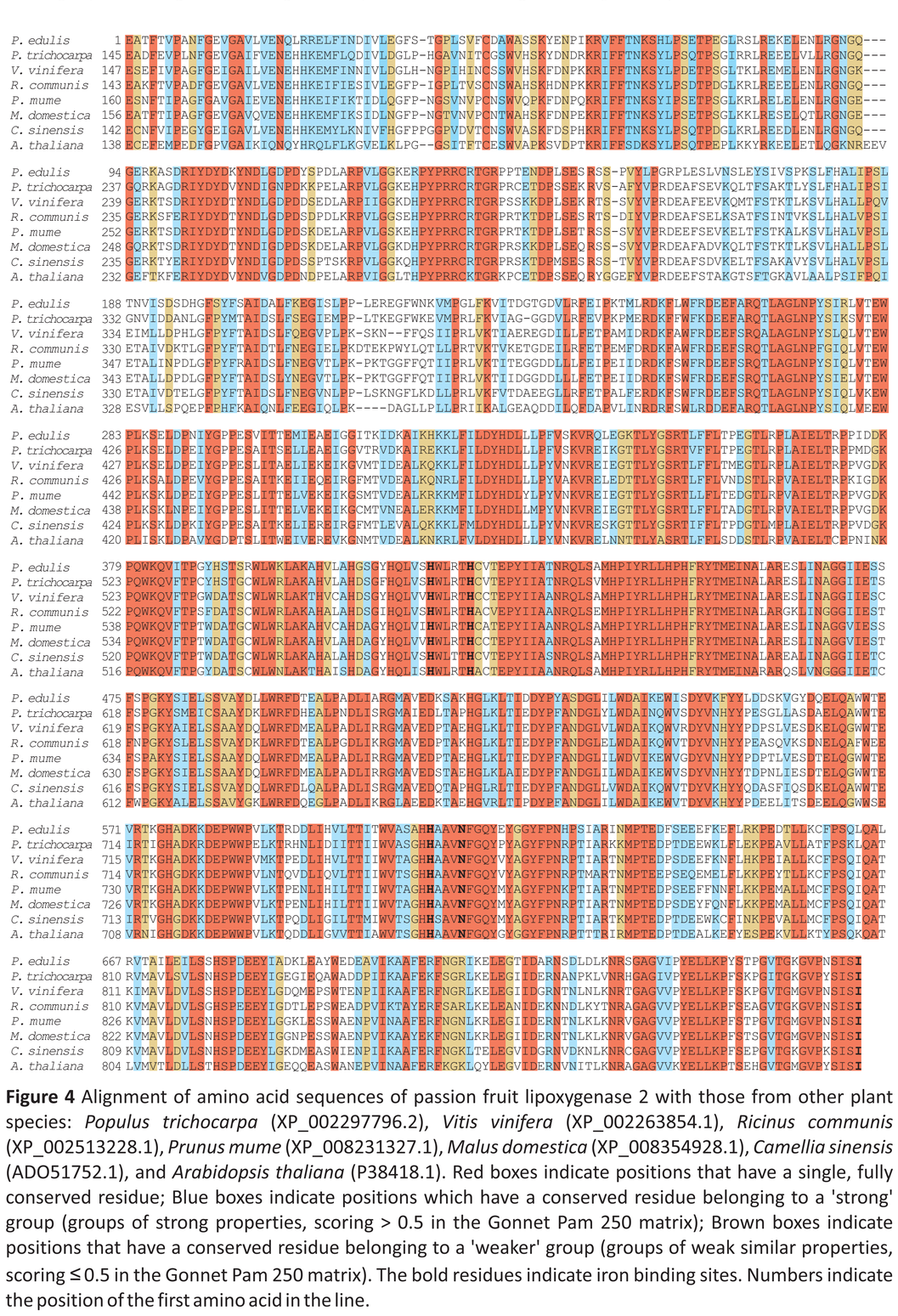

Figure 4 Alignment of amino acid sequences of passion fruit lipoxygenase 2 with those from other plant species: *Populus trichocarpa* (XP_002297796.2), *Vitis vinifera* (XP_002263854.1), *Ricinus communis* (XP_002513228.1), *Prunus mume* (XP_008231327.1), *Malus domestica* (XP_008354928.1), *Camellia sinensis* (ADO51752.1), and *Arabidopsis thaliana* (P38418.1). Red boxes indicate positions that have a single, fully conserved residue; Blue boxes indicate positions which have a conserved residue belonging to a 'strong' group (groups of strong properties, scoring > 0.5 in the Gonnet Pam 250 matrix); Brown boxes indicate positions that have a conserved residue belonging to a 'weaker' group (groups of weak similar properties, scoring ≤ 0.5 in the Gonnet Pam 250 matrix). The bold residues indicate iron binding sites. Numbers indicate the position of the first amino acid in the line.

DEPARTAMENTO DE GENÉTICA, ESALQ/USP - Av. Pádua Dias, 11 CEP 13418-900 - Piracicaba, SP - BRASIL
Fones: (19) 3429-4125; 3429-4126; Fax: (19) 3447-8620 - http://www.genetica.esalq.usp.br - e-mail: mlcvieir@usp.br

Apoio financeiro: CNPq FAPESP