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1 **Comparative proteomics of two citrus varieties in response to infection by**
2 **the fungus *Alternaria alternata***

3 Milena Santos Dória¹; Meg Silva Guedes¹; Edson Mario de Andrade Silva¹; Tahise
4 Magalhães de Oliveira¹; Carlos Priminho Pirovani¹; Katia Cristina Kupper²; Marinês
5 Bastianel²; Fabienne Micheli^{1,3,*}.

6
7 ¹ Centro de Biotecnologia e Genética (CBG), Universidade Estadual de Santa Cruz (UESC),
8 BA, Brazil.

9 ² Centro de Citricultura “Sylvio Moreira” – Instituto Agrônomo de Campinas (IAC) SP,
10 Brasil.

11 ³ CIRAD, UMR AGAP, F-34398 Montpellier, France

12 *Corresponding author: Fabienne Micheli. E-mail: fabienne.micheli@cirad.fr

13

14 **Short title:** Proteomics of citrus–*Alternaria alternata* interaction

15 **Abbreviations:** 2-DE: bi-dimensional electrophoresis; ABS: *Alternaria* brown spot; hai:
16 hours after inoculation; APX: ascorbate peroxidase; GST: glutathione S-transferase; PCA:
17 principal component analysis; ROS: reactive oxygen species; SOD: superoxide dismutase.

18

19 **Abstract**

20 *Alternaria* brown spot (ABS) is a disease caused by the necrotrophic fungus *Alternaria*
21 *alternata*, which induces necrotic lesions on fruits and young leaves due to the production of
22 the host-specific ACT toxin by the fungus. To better understand the citrus–*A. alternata*
23 interaction and to identify putative resistance proteins, as well as the receptor of the ACT
24 toxin, citrus plants susceptible (‘Minneola’ mandarin) and resistant (‘Clemenules’ tangor) to

25 *A. alternata*, infected or not (control) with the pathogen were analyzed by proteomics. Protein
26 changes were observed between citrus genotypes after infection, and 150 candidate proteins
27 were obtained. A general scheme of the metabolic processes involved in susceptible and
28 resistant citrus–*A. alternata* interactions was designed. Susceptible plants presented a high
29 level of proteins involved in stress response at the final stages of the infection, whereas
30 resistant plants presented high level of ROS proteins, metabolic proteins, and proteins
31 involved in the immune system process. Proteins like ferredoxin and cyclophilin are specific
32 to the susceptible variety and may be good candidates as fungal effector-interacting proteins.
33 This is the first citrus–*A. alternata* proteomics analysis, which has allowed a better
34 understanding of the molecular bases of the citrus response to ABS disease.

35

36 **Keywords:** ‘Minneola’, ‘Clemenules’, mass spectrometry.

37

38 **1. Introduction**

39 Citrus cultivation is one of the pillars of the Brazilian agricultural economy; about 60% of the
40 orange juice consumed in the world comes from Brazilian orchards [1]. In 2013, Brazil
41 exported about 1 million tons of orange juice representing about 434 million dollars [1]. In
42 compensation, the production of fruits *in natura* destined for exportation is still low; in 2013,
43 Brazil only exported about 24,000 tons of citrus *in natura* (including mandarins and oranges),
44 representing about 10 million dollars [1]. The segment of agribusiness related to fruit
45 production for *in natura* consumption could be greatly improved by investing in the
46 production of fruits with aggregate characteristics in relation to quality such as intense reddish
47 orange coloration, seedlessness, balanced sweetness/acidity, ease of peeling, and absence of
48 spots on skin, particularly in the case of mandarins [2].

49 Despite the prominent position of Brazil regarding the citrus culture, orchards suffer the
50 influence of biotic and abiotic factors limiting the production and, in some cases, causing
51 severe damage responsible for the destruction of entire plantations [3]. Among the biotic
52 stresses, Alternaria brown spot (ABS)—a disease caused by the tangerine pathotype of the
53 necrotrophic fungus *Alternaria alternata* (Fr.) Keissl.—highly prejudices Brazilian mandarin
54 orchards [4]. ABS induces necrotic lesions (small brown or black spots) on fruits and young
55 leaves, defoliation, and fruit drop in susceptible citrus genotypes [5, 6]. These symptoms are
56 due to the production of the ACT toxin by the fungus: this host-specific toxin is released
57 during the germination of the conidia, rapidly affecting the plasma membrane integrity of the
58 cells in the susceptible hosts [5, 6]. Indirect evidence suggests the existence of a toxin
59 receptor in susceptible citrus genotypes [6-8].

60 Recently, it has been shown that the inheritance of ABS resistance is controlled by a
61 single recessive locus (*ABSr*) located near the centromere of the chromosome III [8-10]. The
62 fine mapping of this region allowed the identification of possible candidate resistance genes
63 [6]. As a complementary approach to identifying the possible receptor to the ACT toxin as
64 well as to confirm the resistance proteins, but also to understand the overall cascades involved
65 in citrus plant resistance versus susceptibility to *A. alternata*, a proteomics approach was
66 used. Proteomics provides information regarding protein expression during the plant–
67 pathogen interaction such as the identification of which proteins are translated during the
68 infection process, as well as identification of the signaling mechanisms triggered by the plant
69 when subjected to infection [11]. Here, we report the first proteomic analysis of the
70 interaction between citrus and *A. alternata*, with the aim of a better understanding of the
71 molecular bases of the citrus response to ABS disease.

72

73 **2. Materials and Methods**

74 **2.1 Plant materials**

75 The experiments were conducted using ‘Clemenules’ (*Citrus clementina* Hort. ex Tan) and
76 ‘Minneola’ (*Citrus paradisi* Macf. x *Citrus tangerina* Tan) citrus plants that are resistant and
77 susceptible to *Alternaria alternata*, respectively [8]. Plant material cultivation and inoculation
78 procedures were performed in the Centro de Citricultura “Sylvio Moreira” – IAC
79 (Cordeirópolis, SP, Brazil). Citrus plants (clones) were grown in a greenhouse for 2 months;
80 at this stage the plantlets were pruned to induce new shoots. Finally, the inoculation was
81 performed in 3-month-old plants.

82 **2.2 Obtaining and inoculation of *Alternaria alternata***

83 The inoculation was performed with the *A. alternata* mandarin pathotype AT4303 previously
84 obtained from plants severely infected [12, 13]. For the collection of *A. alternata*, 10 ml of
85 sterile water was added to the colonies of the fungus. The conidia were separated from the
86 mycelium by dismantling and then filtered using sterile gauze. The suspension was
87 centrifuged twice for 20 min at 12,000 g. The concentration of the conidia in the suspension
88 was determined by counting under a light microscope and a hemocytometer. The suspension
89 was adjusted to 1×10^4 conidia/ml. One drop of suspension was incubated on a microscope
90 slide for 18 h in a humid chamber to check the conidia viability. Then the mycelium of the
91 fungus was cultivated in potato-dextrose-agar medium for 7 days at 26°C and photoperiod of
92 12:12 h. The inoculation of the plants with *A. alternata* was made in young leaves, spraying 5
93 ml of the spore solution (10^5 spores/ml) on each leaf [14]. Control plants were submitted to
94 inoculation using distilled water instead of spore suspension. Five inoculated and 5 control
95 plants were used in our experiment. From them, leaves were harvested 6 h after inoculation
96 (hai) and 12 hai according to previous experiments describing the earlier steps of fungus

97 infection [13]. For each genotype, for each plant (inoculated and control) and at each
98 harvesting time, 5 leaves were collected (1 sample = 5 leaves from 1 plant). The samples from
99 the 5 plants (inoculated vs. control) were pooled for further experiments. Pooling samples
100 before molecular analysis has the advantage of reducing the variation caused by biological
101 replication and sample handling [15]. Samples were frozen in liquid nitrogen, lyophilized, and
102 kept at room temperature until use.

103 **2.3 Protein extraction**

104 Inoculated and control samples from each harvesting time were macerated in liquid nitrogen
105 until a fine powder was obtained. Proteins were extracted from 0.4 g of powder as previously
106 described [16]. Briefly, this procedure was based on the use of phenol and dense SDS, as well
107 as successive washings in acetone/TCA mixture associated with sonication steps. The proteins
108 were dissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 5 mM
109 tributylphosphine, and 0.5% IPG buffer) and stored at -20°C until used. Proteins from each
110 extract were quantified using a 2-D Quant Kit (GE Healthcare - Brazil) according to the
111 manufacturer's recommendations.

112 **2.4 2-DE approach**

113 Proteins (500 µg) were resuspended in the rehydration buffer (described above) containing
114 DTT (50 mmol.L-1) and 0.5% ampholytes pH 3-10 NL (nonlinear; Amersham Biosciences).
115 Protein samples were applied on 13 cm gel strips with 3-10NL immobilized pH gradient
116 (IPG-immobilized pH gradient) and then submitted to an EthanIPGphor III Isoelectric Focal
117 Unit. The second dimension was performed on 12.5% polyacrylamide gel in a Hoefer SE 600
118 Ruby vertical electrophoresis system (Amersham Bioscience). After electrophoresis, the
119 polyacrylamide gels were placed in fixation buffer (40% ethanol and 10% acetic acid) for 20
120 min, and then stained for 7 days with colloidal Coomassie blue (8% ammonium sulfate, 0.8%

121 phosphoric acid, 0.08% G-250 Coomassie blue, and 20% methanol) [17]. Discoloration was
122 obtained by transferring and keeping the gels in distilled water under gentle agitation until
123 removal of the nonspecific staining. Then the gels were kept in acetic acid at 7% until
124 analysis. For each treatment three gels were obtained (experimental replicates).

125 **2.5 Image acquisition and statistical analysis**

126 The gels were scanned with a LabScanner (Amersham Bioscience), and the images analyzed
127 with an ImageMaster 2D Platinum 7.0 (GE Healthcare) to identify and quantify each spot,
128 considering their area and intensity. Each data spot was transformed into a volume value
129 (volume%) by the software; this volume corresponded to protein accumulation. In addition,
130 for each treatment, a reference gel of the experimental triplicates was established. By
131 comparison between treatments, the software allowed the identification of exclusive spots
132 (i.e., present only in one treatment) as well as the relative and possibly differential spots
133 between treatments (corresponding to differentially expressed proteins). PCA of differentially
134 expressed proteins was conducted using the “vegan” package in R version 3.1.0. PCA results
135 were represented as graphs, showing a variation among individuals (treatments), considering
136 the variables (proteins) that exhibited the highest expression located in the same area of the
137 graph.

138 **2.6 Hierarchical clustering analysis**

139 Clustering was performed using the Heatmap function of the Complex-Heatmap library in the
140 Bioconductor package in the R program [17]. The matrix was built with normalized, log-
141 transformed ratio values for each spot obtained by the analysis with the ImageMaster 2D
142 Platinum 7.0 software. Euclidean distance was used to calculate the distance or dissimilarity
143 between conditions, and the Complete Link plugin of Cluster 3.0 was used for clustering.

144 **2.7 Mass spectrometry and protein identification**

145 The exclusive and differential spots selected for protein identification by mass spectrometry
146 were excised from the 2-D gel and subjected to tryptic digestion as previously described [18].
147 The sample containing the proteolytic digestion was fractionated by phase-reversed
148 chromatography on nanoAcquity UPLC coupled to a Q-ToF micro mass spectrometer
149 (Waters) as previously described [19]. The obtained spectra were analyzed using the
150 ProteinLynx v2.3 software and compared with the NCBI non-redundant database, setting the
151 taxonomy parameter to green plants, and with the SWISSPROT database setting the
152 parameter to *Citrus sinensis*. The protein identification was obtained using the MASCOT
153 MS/MS IonSearch (www.matrixscience.com) with the following parameters: trypsin enzyme
154 digestion, 1-cleavage site lost, cysteinecarbamidomethylation (Cys) as fixed modification and
155 as methionine oxidation (Met) modification, error tolerance of 30 ppm, tolerance for mass
156 error equal to 0.3 Da, and 0.1 Da for the error of the fragmented ions. The protein
157 categorizations according to their ontology and biological functions were performed using
158 BLAST2GO (www.blast2go.com).

159 **2.8 Interaction network analysis**

160 The protein–protein interaction network was carried out using the publicly available program
161 STRING 9.05 (<http://string-db.org/>). The protein list was compared with the *Arabidopsis*
162 *thaliana* STRING database that included the physical and functional relationships of protein
163 molecules supported by associations derived from 8 pieces of evidence: neighborhood in the
164 genome, gene fusions, co-occurrence across genome, co-expression,
165 experimental/biochemical data, databases (associated in curated databases), text mining (co-
166 mentioned in PubMed abstracts) and homology. The following parameters were used: no
167 more than 50 interactions and a high confidence score (0.700). The matrices of networks

168 extracted from STRING were submitted to a merge in Cytoscape software (2.8.3). The
169 interaction file extracted from Cytoscape was used for centrality analysis (betweenness and
170 degree) in the Igraph package in R. The betweenness analysis was done with the Betweenness
171 function (parameters: rede, v = V [rede], directed = FALSE, weights = NULL, nobigint =
172 TRUE, normalized = FALSE), and degree analysis was done with the Degree function
173 (parameters: rede, v = V [rede], loops = TRUE, normalized = FALSE).

174

175 **3. Results**

176 **3.1 Analysis of protein profile in response to fungus infection and functional** 177 **classification**

178 The protein profile of both varieties is presented in Fig. 1. In the ‘Minneola’ variety
179 (susceptible to *A. alternata*), a total of 450 spots in the non-infected sample (MNI) were found
180 (Fig. 1A), whereas 350 and 457 spots were observed in the ‘Minneola’ variety 6 hai (MI6AI)
181 (Fig. 1B) and 12 hai (MI12AI) (Fig. 1C), respectively. A total of 211 spots had a differential
182 accumulation level between the three conditions (Additional File 1: Table S1). A total of 365
183 spots (Fig. 1D) was detected on the non-infected ‘Clemenules’ (CNI) 2-D gel, whereas 455
184 and 420 spots were detected in the ‘Clemenules’ infected - 6 hai (CI6AI) and in the
185 ‘Clemenules’ infected - 12 hai (CI12AI) 2-D gels, respectively (Fig. 1D and 1F). The
186 treatments were compared and 150 spots with a significant differential expression level ($P \leq$
187 0.005) were found. From them, 102 were identified through mass spectrometry (Additional
188 File 1: Table S2). The identified proteins were separated according to the biological processes
189 in which they are involved (Fig. 2). The proteins of the ‘Minneola’ variety were distributed in
190 6 categories (Fig. 2A) and those of the ‘Clemenules’ variety in 9 categories according to
191 biological processes (Fig. 2B). Six categories were common to both varieties: metabolic

192 process (33% e 26%), cellular process (26% e 26%), single-organism process (23% e 22%),
193 response to stress (10% e 11%), biological regulation (5% e 5%), and localization (3% e 2%).
194 Three categories were exclusive to ‘Clemenules’: developmental process (2%), detoxification
195 (5%) and immune system process (1%). Most of the proteins distributed in the categories
196 mentioned above were upregulated for both ‘Clemenules’ and ‘Minneola’ (Fig. 2C). Eighty-
197 four percent of all the proteins identified as being involved with the metabolic process were
198 upregulated, and 81% of proteins related to response to stress presented an increase of
199 regulation. As well as these two biological processes, the proteins related to other processes
200 increased the level of regulation.

201

202 **3.2 Metabolic process**

203 In general, for ‘Minneola’, 122 proteins related to metabolic process were identified. Among
204 them, 41 are common to the three conditions and have differential expression level, 26 are
205 exclusive to ‘Minneola’ 6 hai, and 55 are exclusive to ‘Minneola’ 12 hai. Among the proteins,
206 NADP-dependent D-sorbitol-6-phosphate dehydrogenase-like (spot 82), caffeic acid 3-O-
207 methyltransferase-like (spot 239), probable NADPH dehydrogenase (quinone) FQR1-like 1
208 (spot 247), proteasome subunit alpha type-4 (spot 254), and protein USF (spot 52) were
209 significantly up-regulated on the ‘Minneola’ 12 hai. The proteins 40S ribosomal S12 (spot 8),
210 glutamate-1-semialdehyde chloroplastic (spot 98), aspartate aminotransferase glutamate (spot
211 115), and malate dehydrogenase (spot 238) were regulated mainly on the treatment infected
212 after 12 hai. A total of 65 proteins involved with cell metabolism was identified in
213 ‘Clemenules.’ Among them, 32 were differentially expressed between the three conditions, 8
214 were exclusive to ‘Clemenules’ 6 hai and 25 were exclusive to ‘Clemenules’ 12 hai. Of the
215 differentially expressed proteins, 49 were significantly up-regulated and 16 were down-
216 regulated. The expression of protein fructose-bisphosphate (spot 410) was significantly down-

217 regulated because the level of expression reduced comparing ‘Clemenules’ control and
218 ‘Clemenules’ 12 hai. Malate dehydrogenase (spot 235) was up-regulated in ‘Clemenules’ 6
219 hai but presented a reduction in ‘Clemenules’ 12 hai. Peroxiredoxin (spot 124) decreased
220 from control to ‘Clemenules’ 6 hai and increased again in ‘Clemenules’ 12 hai. Superoxide
221 dismutase (SOD) protein (spot 120; Additional File 1: Table S2) showed increased expression
222 in both infection conditions.

223

224 **3.3 Cellular process**

225 The ‘Minneola’ variety presented 96 proteins related to cellular process. Among them, 32
226 were differentially expressed, 17 were exclusive to ‘Minneola’ 6 hai treatment, and 47 were
227 exclusive to ‘Minneola’ 12 hai. In this category, 78 proteins were significantly up-regulated
228 and 18 were down-regulated. Among the up-regulated proteins, cyclophilin (spot 171) had an
229 expression level that doubled in the two infection conditions (‘Minneola’ 6 hai and
230 ‘Minneola’ 12 hai) compared to ‘Minneola’ control. Phosphomannomutase (spot 253) and
231 oxygen-evolving enhancer (spot 255) proteins maintained a constant level of expression
232 between the control and infected conditions after 6 hai and increased after 12 hai (1X to 253
233 and 2X to 255, respectively). ‘Clemenules’ variety showed 65 proteins related to this process
234 (we can highlight the spots 37, 92, 110, 124, 139, 187, 300, 409, 410, and others). In this case,
235 36 were common to all conditions and presented differential accumulation level. Eight were
236 exclusive to ‘Clemenules’ 6 hai, and 21 were exclusive to the ‘Clemenules’ 12 hai treatment.
237 Of the total of these proteins identified, 52 were up-regulated and 13 were down-regulated.

238

239 **3.4 Response to stress**

240 Forty-eight proteins related to defense and stress responses were identified in this work. In
241 ‘Minneola’ 35 proteins were identified, and 29 were identified in ‘Clemenules.’ Most of these

242 proteins were up-regulated for both varieties. ‘Minneola’ presented 8 proteins with difference
243 in the accumulation, 8 were exclusive to ‘Minneola’ 6 hai treatment, and 19 were exclusive to
244 ‘Minneola’ 12 hai. Among these proteins, we can cite that T23e23.17 (spot 576) was
245 exclusive to the infected sample, that ferredoxin (spot 84) increased the accumulation 6 hai
246 and 12 hai in ‘Minneola’, and that peroxiredoxin (spot 317) was identified only in ‘Minneola
247 12 hai. Another protein exclusive to ‘Minneola’ 12 hai was the universal stress A-like protein
248 (spot 330). In ‘Clemenules’, of the 29 proteins identified, 19 presented differential
249 accumulation, 2 were exclusive to ‘Clemenules’ 6 hai, and 8 were exclusive to ‘Clemenules’
250 12 hai. A heat shock protein 90 (spot 471) was down-regulated in infected sample and
251 glutathione S-transferase (GST) DHAR2 (spot 439; Table 2) was up-regulated on
252 ‘Clemenules’ 6 hai but was not identified in ‘Clemenules’ 12 hai.

253

254 **3.5 Biological regulation**

255 The proteins related to this function were identified in ‘Minneola’ and in ‘Clemenules’. In
256 ‘Minneola’, only the infected samples presented proteins involved with this function. Four
257 proteins were exclusive to ‘Minneola’ 6 hai and 14 were exclusive to ‘Minneola’ 12 hai. For
258 ‘Clemenules’ 13 proteins related to this process were identified, and 7 presented differential
259 accumulation (spots 92, 139, 300, 314, 316, 379 and 407); of these, the 20 kDa chaperonin
260 (spot 139; Table 2) protein increased the accumulation in ‘Clemenules’ 6 hai but was reduced
261 in ‘Clemenules’ 12 hai, the 26S protease regulatory (spot 316; Additional File 1: Table S2)
262 protein presented a reduction on the accumulation in ‘Clemenules’ 6 hai but increased in
263 ‘Clemenules’ 12 hai, and the GTP-binding nuclear protein (spot 407; Table 2) doubled the
264 accumulation in ‘Clemenules’ 6 hai but was repressed in ‘Clemenules’ 12 hai.

265

266 **3.6 Detoxification**

267 Proteins related to the detoxification process were detected and identified in the susceptible
268 and resistant varieties. Of the proteins identified in ‘Clemenules’, 10 presented differential
269 accumulation and only 2 proteins were unique to the ‘Clemenules’ 12 hai. Here SOD (spot
270 120), 2-Cys peroxiredoxin (spots 119, 124, and 236 are isoforms), and peroxidase (spot 237)
271 can be cited. Of all the proteins related to this process identified in this work, only one
272 presented reduction on the accumulation.

273

274 **3.7 PCA and hierarchical clustering analysis**

275 To understand which proteins (variables) contribute to the variation in the three conditions
276 (control, 6 hai, and 12 hai) for the 2 varieties, PCA was performed. PCA indicated that the
277 three conditions for ‘Minneola’ and ‘Clemenules’ can be discriminated against each other
278 according to differentially expressed proteins. The first principal component (PC1) of
279 ‘Minneola’ explained 66.7% of the total variation and discriminated the biological samples on
280 the basis of infection of fungus, whereas PC2 explained 33.33% of the variation (Additional
281 File 2: Fig. S1A). According to the score plot for PC1 and PC2, the ‘Minneola’ 6 hai
282 treatment presented more discrepant behavior than the control and ‘Minneola’ 12 hai. Overall,
283 the ‘Minneola’ PCA demonstrated difference in protein expression for the three conditions.
284 To clarify which of the proteins contributed to the discrimination, variable importance plots
285 were produced based on the loading scores for PC1 and PC2 (Additional File 2: Fig. S1B). In
286 ‘Clemenules,’ PC1 represented 52.2% of the total variation between the conditions and PC2
287 represented 47.8% of variation (Additional File 2: Fig. S1C). The variables that presented a
288 contribution to this variation were also plotted according loading scores for PC1 and PC2
289 (Additional File 2: Fig. S1D). According to the classification of relevant variables, those with
290 the highest influence on the variability between control and infected samples for ‘Mineola’
291 (Table 1) and ‘Clemenules (Table 2), respectively, were selected (Additional File 2: Fig. S2).

292 The proteins with the greatest influence on the variability of the conditions were submitted to
293 a hierarchical grouping to show the pattern of protein expression in our diversity treatments
294 (Fig. 3). It was possible to observe the separation of the proteins into 8 clusters for ‘Mineola’
295 (Fig. 3A) and 11 different clusters for ‘Clemenules’ (Fig. 3B), according to the level of
296 expression in each condition.

297

298 **3.8 Protein–protein interaction network (interactomics)**

299 Proteins are in constant interaction in a living cell. They do not perform their functions alone
300 but integrate a series of networks and protein–protein interactions that are fundamental in
301 almost all cellular processes (Pallas and García 2011). To understand how *A. alternata*
302 interacts with susceptible and resistant plant proteins to affect cell functions, the proteins
303 identified as differentially expressed were analyzed by searching the String database and the
304 protein–protein interaction network. The abbreviations of the specific protein names related to
305 *A. thaliana* in the network for ‘Minneola’ are shown in Additional File 1, Table S3, and the
306 relation between proteins of ‘Clemenules’ and orthologous proteins in *A. thaliana* are shown
307 in Table S4. The networks were used to determine the importance of proteins based on their
308 centrality according to betweenness and degree information.

309 **4. Discussion**

310 Plants are constantly attacked by microorganisms and insects, but only a portion can
311 successfully invade a plant and establish a relationship with the host and exploit it as an
312 energy source. Thus, the occurrence of the disease in the host is not a common result, but an
313 exception [20], because a plant has defense mechanisms capable of neutralizing the harmful
314 effects of microorganisms [21]. Proteins are directly related to these defense responses,
315 because they are responsible for catalyzing reactions at the metabolic level. For this reason,

316 the change in the accumulation of proteins in stress situations can be determinant of the
317 tolerance of a plant to the stress that was submitted [22].

318 In this study, proteins differentially expressed in citrus plants infected with *A.*
319 *alternata* were identified using a 2-DE approach. Comprehensive analysis of these proteins
320 will help to elucidate the molecular mechanism involved in the interaction between plants
321 infected with this fungus and understand how these plants respond to infection considering the
322 resistance and susceptibility. Here we discuss the main responses that occur in plants with
323 pathogen infection according to the proteins that most affected the separation of the
324 conditions established in this work. The proteomic analysis allows the understanding of how a
325 plant responds to the presence of a microorganism in real time, allowing identification of the
326 defense mechanisms and the signaling that occurs when a plant is affected by some stress
327 situation. The information contained in the genome of each organism defines the potential
328 contribution for each cellular function, but the expression of the proteome represents the
329 contributions of the present moment in which the plant is submitted to stress [23]. The
330 proteomic analysis of ‘Minneola’ versus ‘Clemenules’ interactions with *A. alternata* allowed
331 the establishment of a general scheme of biological processes for each interaction through a
332 time course (Fig. 4).

333

334 **4.1 Proteins related to metabolic and cellular processes**

335 Metabolic processes are generally affected in plants subjected to stress, including infection by
336 microorganisms. Several pathways of energy production, including tricarboxylic acid (TCA),
337 oxidative phosphorylation, and glycolysis, are active in almost all organisms and are
338 responsible for the storage, release and production of this energy [24]. Many enzymes are
339 directly involved with these pathways. In this study, some enzymes related to energy
340 production were identified. Malate dehydrogenase, for example, is an enzyme that participates

341 in the TCA cycle and has the ability to convert malate to oxaloacetate reversibly [25]. Here,
342 this protein was down-regulated in the susceptible ‘Minneola’ (spot 238; Additional File 1:
343 Table S1), but up-regulated in the resistant ‘Clemenules’ (spot 235; Additional File 1: Table
344 S2). Malate dehydrogenase is a protein very susceptible to oxidative stress [26], so the
345 decrease in the regulation of this protein, as we can see here with the susceptible variety, can
346 be a defense response against infection [27], because the reduction of this protein can
347 decrease the production of reactive oxygen species (ROS). In a study with resistant *Brassica*
348 *oleracea* plants susceptible to *Xanthomonas campestris* pv. *Campestre*, this protein was only
349 identified in the resistant variety [28]. In another study carried out with plants susceptible to
350 *Candidatus Liberibacter asiaticus*, this protein also showed a reduction in the level of
351 expression in the symptomatic plants [29], which is the same as the results found in our work.
352 Another protein classified according to metabolic processes was RuBisCo (1,5-bisphosphate
353 carboxylase oxygenase), which is the most abundant protein in plants and is responsible for
354 the fixation of carbon dioxide in its organic form [30]. In the present work, this protein was
355 identified as up-regulated in both susceptible (Spots 304 and 306) and resistant (Spot 639)
356 varieties. In the study conducted by Villet et al. (2016) [28] the opposite happened: a
357 decrease was observed in the regulation of this protein in resistant plants, which reflected a
358 strategy of resistance of plants. In several other studies involving plant–pathogen
359 relationships, this protein also showed a decrease in the regulation in infected plants [29, 31-
360 33]. The reduction in the regulation of these proteins can affect photosynthesis during the
361 infection process, but it is not clear if this reduction is a defense response of the plant in
362 relation to the development of the fungus, because the fungus will be deprived of the
363 necessary nutrients for its development making this event favorable for the host [34]. In this
364 work, however, the increase in the regulation of RuBisCo in both resistant and susceptible
365 plants might be a defense response of the plant to infection because *A. alternata* is a

366 necrotrophic fungi and the decrease of metabolism and possible death of the plant would
367 benefit the development of the microorganism [35]. In this work, an ATP synthase was
368 identified in the resistant variety (spot 566; Table 2) and considered as up-regulated because it
369 was exclusive to the infected sample. This protein is very important to the process of ATP
370 production. It is responsible for catalyzing the phosphorylation of ADP through an
371 electrochemical gradient of protons generated by the electron transport chain [36]. Another
372 protein related to cell metabolism identified here was carbonic anhydrase, which has the
373 ability to catalyze the conversion reaction of carbon dioxide and water into carbonic acid
374 (H_2CO_3) [29]. Carbonic anhydrase was exclusive to the ‘Minneola’ variety (Spot 57). We
375 observed a significant reduction in the accumulation of this protein in infected samples. In
376 stress situations, the regulation of some global proteins can be altered, because cellular
377 “machinery” will be focused on the regulation of proteins related to stress responses [37]. The
378 PCA indicated that all of the above-mentioned results contributed highly to discrimination
379 between the different conditions to which the plants were submitted (Additional File 2: Fig.
380 S2).

381

382 **4.2 Proteins related to stress-defense response**

383 The defense responses of a plant to stress are a dynamic process and depend on the intensity
384 and duration of the process [22]. In this work, although the two varieties used presented
385 different characteristics in relation to the susceptibility to the fungus, both the resistant and the
386 susceptible varieties presented defense responses. Of the proteins that most influenced the
387 separation of the individuals, the majority presented an increase in their regulation.
388 Ferredoxin is an important protein related to stress response. This protein was identified only
389 in the ‘Minneola’ variety and presented up-regulation in ‘Minneola’ 6 hai and 12 hai. This
390 protein has the ability to delay the action of the microorganism’s effectors used to develop the

391 disease through the recognition of these effectors by the host cells [38]. Independent lines of
392 transgenic rice plants carrying the gene corresponding to ferredoxin showed enhanced
393 resistance to *Xanthomonas oryzae* pv. *Oryzae* [39]. In our work, the increased expression of
394 the ferredoxin protein can play a similar role in the ‘Minneola’ variety, once the disease
395 MMA in susceptible plants develops considering the relation between the microorganism’s
396 effector and the plant. Cyclophilin was identified only in the susceptible variety and showed
397 an increase in its regulation mainly in ‘Minneola’ 12 hai. Cyclophilin is involved in several
398 molecular processes such as protein folding, signal transduction during plant development,
399 and stress response [40]. Evidence shows that this protein has the ability to recognize
400 pathogen-effecting molecules [41]. A protein that can be highlighted as important to the stress
401 response process is the elongation factor (E1). E1 is a multifunctional protein that is present in
402 several cell compartments [40] and participates in processes such as protein synthesis and
403 RNA processing, as well as inducing immunity from and resistance to pathogenic bacteria. In
404 addition, it is associated with programmed cell death control [39]. In our study, this protein
405 was also identified only in the ‘Minneola’ variety (Spot 292; Table 1) and presented up-
406 regulation because it was exclusively identified in the ‘Minneola’ 12 hai sample. In another
407 study, the abundance of this protein was higher in the variety resistant to infection by
408 *Xanthomonas campestris* pv. *Campestris* [28]. Much research has been carried out to
409 investigate the defense mechanisms adopted against fungi [21, 42, 43]. A very specific
410 mechanism that can be highlighted is the action of chitinase, which has the ability to cleave
411 the chitin that is the main component of the cell wall of fungi [44]. Chitinase (spot 205) was
412 identified in our work and showed increased accumulation mainly in the treatment 12 hai. The
413 increase of the gene expression level corresponding to this protein has already been observed
414 in citrus plants resistant to the fungus *A. alternata* [13]. In a study carried out with citrus
415 plants susceptible to the *Citrus tristeza virus*, this protein also presented up-accumulated [32],

416 which is the same as the result found in the present work. The fine mapping of some citrus
417 genome regions has allowed the identification of possible candidate resistance genes to *A.*
418 *alternata*. Among these genes, cysteine protease was identified [6]. The protein corresponding
419 to this gene is considered an autophagic protein essential for autophagic cell death and
420 pathogenicity of fungi [45]. In the present work, cysteine protease was identified and
421 presented an increased level of expression in the susceptible and resistant varieties. Some
422 proteins homologous to cysteine protease can control cell differentiation and pathogenicity. In
423 some cases, the effect of this protein can be controlled by the production of ROS, which can
424 control the autophagy and can regulate the activity of this protein [46]. ROS also play a key
425 role in defense responses against microorganisms. In this case, many proteins are involved in
426 redox regulation to try to reduce the damage caused [47]. Four proteins related to oxidative
427 stress significantly influenced the separation of individuals according to the PCA (Additional
428 File 2; Fig. S2); they are GST, ascorbate peroxidase (APX), Cys peroxiredoxin, and
429 superoxide Mn dismutase (SOD). Regarding APX (spot 546; Table 1), this protein was
430 detected only in the susceptible variety and showed an increased regulation. This enzyme
431 plays a crucial role in the degradation of hydrogen peroxide in plant cells, because the ability
432 to eliminate ROS and reduce harmful effects on these macromolecules seems to represent an
433 important trait of stress tolerance [48]. GST was identified in both susceptible (spot 352;
434 Table 1) and resistant (spot 439; Table 2) varieties. This protein was up-regulated in
435 ‘Minneola’ and down-regulated in ‘Clemenules’. This enzyme is responsible for removing the
436 ROS active in the maintenance of homeostasis in cotton plants infected with the necrotrophic
437 fungus *Rhizoctonia solani*, because five GSTs were identified [43]. In another work, this
438 protein was identified in the susceptible and resistant varieties and decreased its accumulation
439 in both [27, 28]. Cys peroxiredoxin (spot 434; Table 2) and SOD (spot 427; Table 2) had a
440 greater influence on the separation of ‘Clemenules’ individuals and increased their expression

441 level. SOD is the enzyme that constitutes the first line of ROS combat and has the capacity to
442 catalyze the dismutation of superoxide, producing oxygen and hydrogen peroxide [49]. Most
443 of the proteins related to oxidative stress in this work were identified mainly in the resistant
444 variety, and this is one of the main results of our work, because the performance of these
445 detoxifying enzymes may reflect a resistance strategy and because this resistance to the
446 fungus may be associated with both the loss of the fungal toxin target and the detoxification
447 process [50] as happened in this work. The two varieties studied here, both susceptible and
448 resistant, presented stress and defense responses. Indirect evidence shows that susceptible
449 plants have the toxin target receptor [7]. Even being susceptible to this pathogen, a plant
450 responds to defend itself or facilitates the development of pathogenicity of the fungus,
451 because recent studies have indicated that the reduction of the damage caused by ROS is
452 essential for the pathogenicity [51].

453

454 **4.3 Proteins related to regulation and localization**

455 Proteins involved in the regulation were identified with a not too high frequency (5% for
456 ‘Minneola’ and ‘Clemenules’, (Fig. 2A and 2B, respectively). This category was mainly
457 represented by proteins involved with transcription translation factors such as the protein
458 transcription factor BTF3 (spot 334; Table 1) identified exclusively in ‘Minneola’ and the
459 protein eukaryotic translation initiation factor 5A-2 (eIF) (spot 92; Table 2) that was detected
460 in ‘Clemenules’ and was down-accumulated in infected samples. The regulatory proteins are
461 very important to the regulation of gene expression. The BTF3 identified in this work, for
462 example, was identified originally as a basal transcription factor responsible for initiating
463 transcription, forming a stable complex with the enzyme RNA polymerase [52]. This protein
464 exerts an important and distinct role on rice growth and development [53] and is involved in
465 regulating the localization of proteins during translation [54]. Here this protein was up-

466 regulated in infected samples. On the other hand, the eIF identified in ‘Clemenules’ is also
467 biologically important to growth and development in plants. The main function of this protein
468 is related to translation initiation because these factors interact with the ribosomal proteins
469 [55]. A very important fact is that these components of the eukaryotic translation initiation
470 complex are related to genes of recessive resistance against plant viruses. These factors were
471 essential determinants in the outcome of RNA virus infections, especially those belonging to
472 the *Potyvirus* family [56]. In relation to proteins involved with cell localization, an example
473 that was identified in the two varieties and influenced the separation of individuals according
474 to PCA was porin proteins (spot 376 in ‘Minneola and spot 481 in ‘Clemenules’). Porins are
475 proteins that compose the selective diffusion pores present in cell membranes and confer
476 permeability to the membrane allowing the passage of certain molecules. These proteins are
477 found in all eukaryotic organisms [57]. It was discovered that a mutant porin has some drastic
478 effects on agrobacterium-mediated tumorigenesis [58]. In this study, this protein presented
479 up-regulation in susceptible and resistant plants to *A. alternata* because it was identified only
480 in infected plants after 12 hai, but the accumulation was higher in resistant plants.

481 **5. Conclusion**

482 The ‘Minneola’ (susceptible) and ‘Clemenules’ (resistant) citrus varieties presented distinct
483 metabolic responses to the infection by the fungus *A. alternata* (Fig. 4). Susceptible plants
484 presented an increase of proteins involved in stress response, ROS detoxification, and
485 regulation during the interaction and a reduction of protein expression involved in metabolic
486 and cellular processes. Some ‘Minneola’ exclusive proteins such as ferredoxin and
487 cyclophilin may be good candidates for proteins interacting with *A. alternaria* protein
488 effectors. In the resistant plant, ROS detoxification process was also observed with a higher
489 number of proteins involved than in the susceptible variety; proteins such as glutathione S-

490 transferase, APX, Cys peroxiredoxin, and SOD were mainly involved. Specific proteins
491 related to immune system processes were observed. Inverse to the susceptible variety, the
492 resistant plants showed an increase in the regulation of proteins related to photosynthesis and
493 other proteins involved in metabolism maintenance.

494

495 **Declarations**

496 *Availability of data and materials*

497 The data sets supporting the results of this article are included within the article and its
498 additional files.

499

500 *Competing interests*

501 The authors declare that the research was conducted in the absence of any commercial or
502 financial relationships that could be construed as a potential conflict of interest.

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510

511 *Authors' Contributions*

512 MSD conducted all the experiments. MSG and TMO contributed to proteomic analyses.
513 EMAS made the cluster analysis. KCK and MB were responsible for plant and fungus
514 production and plant inoculation. MSD and FM analyzed the data and wrote the manuscript.

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520 **6. References**

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694

695 **Tables**696 **Table 1.** Differentially expressed proteins with highest influence on the variability between control and infected samples of ‘Minneola’.

Spot ^a	Accession	Species	Protein ID	Fold Change ^b 6h / 12h	PCA ^c		PPI network	
					Influence PC1 (%)	Influence PC2 (%)	Centrality ^e	Arabidopsis ID
17	gi 985465608	<i>Citrus sinensis</i>	Bis (5'-adenosyl)-triphosphatase-like	0.6 / 0.5	-6%	-	-	FHIT
33	XP_006430087.1	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10012459mg	0.3 / 0.5	-8%	3%	B	At5g06290
35	ADQ74414.1	<i>Anisodus tanguticus</i>	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (plastid)	np / 0.6	-12%	9%	HB	RBCL
52	gi 568830768	<i>Citrus sinensis</i>	Protein usf	0.2 / 0.4	-18%	-	-	AT2G32520
57	gi 568832193	<i>Citrus sinensis</i>	Carbonic anhydrase 2-like isoform X1	0.2 / 0.5	-12%	3%	B	CA1
70	orange1.1g024380m	<i>Citrus sinensis</i>	Legume lectin domain (Lectin_legb)	np / 1.2	-12%	31%	-	Not found
80	EEF43857.1	<i>Ricinus communis</i>	Lactoylglutathione lyase, putative	0.6 / 0.4	-5%	-2%	B	GLX1
81	gi 568826116	<i>Citrus sinensis</i>	Putative lactoylglutathione lyase	0.5 / 0.4	-11%	-2%	B	GLX1
84	gi 568825162	<i>Citrus sinensis</i>	Ferredoxin--NADP reductase, leaf-type isozyme, chloroplastic	np / 0.3	-3%	-	-	Not found
98	gi 568880700	<i>Citrus sinensis</i>	Glutamate-1-semialdehyde 2,1-aminomutase 2, chloroplastic	0.6 / 0.4	-4%	-	-	GSA2
101	XP_006476982.1	<i>Citrus sinensis</i>	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) / Triosephosphate dehydrogenase	0.6 / 0.4	-27%	-17%	H	GAPC1
132	XP_006492541.1	<i>Citrus sinensis</i>	Adenosylhomocysteinase / sahase	0.7 / 0.6	-6%	-5%	B	HOG1
135	XP_006492541.1	<i>Citrus sinensis</i>	Adenosylhomocysteinase / sahase	np / 1.1	-6%	13%	B	HOG1
140	XP_006481907.1	<i>Citrus sinensis</i>	Phosphopyruvate hydratase / Enolase	0.5 / 0.2	-7%	-4%	HB	LOS2
145	orange1.1g009075m	<i>Citrus sinensis</i>	Polygalacturonase-like protein	np / 0.1	-18%	-	-	Not found
146	ACP43315.1	<i>Citrus maxima</i>	20S proteasome beta subunit 5	0.8 / 0.7	-6%	-6%	H	AT3G26340
148	XP_006492150.1	<i>Citrus sinensis</i>	Chaperonin CPN6/0-2, mitochondrial	0.2 / 0.3	-37%	4%	HB	HSP60
155	XP_006485108.1	<i>Citrus sinensis</i>	ATP-dependent Clp protease ATP-binding subunit clpa homolog CD4B, chloroplastic	np / 0.1	-19%	1%	B	CLPC1
161	BAI63297.1	<i>Citrus jambhiri</i>	Thaumatococin-like protein	1.1 / 0.7	-	-6%	-	AT2G28790

168	XP_012070412.1	<i>Jatropha curcas</i>	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	0.3 / np	-16%	-8%	C	ATMS1
171	gi 512125698	<i>Citrus sinensis</i>	Chain A, Structure Of A Cyclophilin From Citrus Sinensis (cscyp) In Complex With Cycloporin A	1.7 / 1.9	7%	-	-	Not found
173	AAL35658.1	<i>Flacourtia jangomas</i>	Ribulose 1,5-bisphosphate carboxylase, partial (chloroplast)	1.4 / 1.5	39%	3%	HB	RBCL
180	XP_006491863.1	<i>Citrus sinensis</i>	Copper transport protein cch	np / 1	4%	-2%	C	At1g66240
181	XP_006468538.1	<i>Citrus sinensis</i>	Thioredoxin	np / 1	4%	-3%	C	TO1
191	gi 568870196	<i>Citrus sinensis</i>	Osmotin-like protein	np / 1.7	5%	-	-	Not found
222	gi 568846849	<i>Citrus sinensis</i>	Hsp70-Hsp90 organizing protein 3-like	2.0 / 2.3	6%	2%	C	Hop3
234	gi 568839727	<i>Citrus sinensis</i>	Glyceraldehyde-3-phosphate dehydrogenase GAPB, chloroplastic	1.2 / 1.8	3%	5%	HB	GAPB
238	gi 568864588	<i>Citrus sinensis</i>	Malate dehydrogenase, mitochondrial	0.8 / 0.6	-13%	-15%	C	mMDH2
239	gi 568883665	<i>Citrus sinensis</i>	Caffeic acid 3-O-methyltransferase-like	np / 1.5	-5%	22%	C	OMT1
252	gi 568854455	<i>Citrus sinensis</i>	Uncharacterized protein At5g02240-like	0.8 / 0.4	-5%	-5%	C	AT2G37660
253	gi 568875954	<i>Citrus sinensis</i>	Phosphomannomutase	1.2 / 2.7	3%	9%	C	PMM
254	gi 568879954	<i>Citrus sinensis</i>	Proteasome subunit alpha type-4	0.9 / 3.9	6%	20%	H	PAC1
255	gi 568863109	<i>Citrus sinensis</i>	Oxygen-evolving enhancer protein 2, chloroplastic	1.3 / 3.2	6%	12%	C	PSBP-1
•273	gi 568875954	<i>Citrus sinensis</i>	Phosphomannomutase	∞	3%	9%	C	PMM
•275	gi 568873338	<i>Citrus sinensis</i>	3-ketoacyl-coa thiolase 2, peroxisomal	∞	2%	8%	C	PKT3
•292	gi 568869888	<i>Citrus sinensis</i>	Elongation factor 1-alpha	∞	5%	16%	C	AT1G07920
•298	gi 568840591	<i>Citrus sinensis</i>	Glutaredoxin-C4	∞	2%	7%	C	AT5G20500
•300	gi 568832597	<i>Citrus sinensis</i>	10 kda chaperonin-like	∞	4%	12%	C	CPN10
•304	gi 568857400	<i>Citrus sinensis</i>	Ribulose bisphosphate carboxylase small chain, chloroplastic-like	∞	4%	13%	C	RBCS2B
•306	gi 568857400	<i>Citrus sinensis</i>	Ribulose bisphosphate carboxylase small chain, chloroplastic-like	∞	8%	23%	C	RBCS2B
•318	gi 568864066	<i>Citrus sinensis</i>	Glycine cleavage system H protein, mitochondrial	∞	2%	8%	C	AT1G32470
•334	gi 568823453	<i>Citrus sinensis</i>	Transcription factor BTF3 homolog 4	∞	2%	7%	H	BTF3
•341	gi 568863109	<i>Citrus sinensis</i>	Oxygen-evolving enhancer protein 2, chloroplastic	∞	2%	7%	C	PSBP-1

• 344	gi 568863109	<i>Citrus sinensis</i>	Oxygen-evolving enhancer protein 2, chloroplastic	∞	2%	7%	C	PSBP-1
•352	gi 568852489	<i>Citrus sinensis</i>	Glutathione S-transferase F6	∞	-	8%	-	GSTF6
•371	gi 568879954	<i>Citrus sinensis</i>	Proteasome subunit alpha type-4	∞	2%	8%	H	PAC1
•376	gi 568857970	<i>Citrus sinensis</i>	Mitochondrial outer membrane protein porin of 34 kda	∞	-	8%	-	VDAC1
•385	gi 568825219	<i>Citrus sinensis</i>	Proteasome subunit alpha type-7	∞	2%	7%	H	PAD2
•387	gi 568881387	<i>Citrus sinensis</i>	Tropinone reductase homolog At5g06060-like	∞	-	8%	-	AT5G06060
•389	gi 568831451	<i>Citrus sinensis</i>	Prohibitin-3, mitochondrial	∞	2%	8%	C	PHB3
•421	gi 568845013	<i>Citrus sinensis</i>	UDP-glucose 4-epimerase GEPI48-like	∞	2%	7%	C	UGE5
•450	gi 568826102	<i>Citrus sinensis</i>	Aminomethyltransferase, mitochondrial	∞	2%	8%	C	AT1G11860
•453	gi 568832251	<i>Citrus sinensis</i>	Peptidyl-prolyl cis-trans isomerase CYP38, chloroplastic isoform X1	∞	5%	15%	C	CYP38
•462	gi 568873338	<i>Citrus sinensis</i>	3-ketoacyl-coa thiolase 2, peroxisomal	∞	2%	8%	C	PKT3
•463	gi 568838553	<i>Citrus sinensis</i>	Cysteine proteinase RD21a	∞	4%	14%	C	RD21A
o546	gi 300837175	<i>Citrus limon</i>	Ascorbate peroxidase	∞	-	8%	-	APX1
o572	XP_015901577.1	<i>Citrus sinensis</i>	Mlp-like protein 423-related	∞	6%	-13%	-	MLP423
o575	XP_012080320.1	<i>Citrus sinensis</i>	Translation initiation factor 5a-related	∞		-7%	C	ELF5A-1
o576	gi 9369404	<i>Arabidopsis thaliana</i>	T23e23.17	∞	3%	-7%	-	MLP423
o599	gi 568872179	<i>Citrus sinensis</i>	Endo-1,3;1,4-beta-D-glucanase-lik	∞	-	-7%	-	AT3G23600
o601	XP_006474693.1	<i>Citrus sinensis</i>	20 kda chaperonin, chloroplastic	∞	3%	-7%	C	CPN20
o603	gi 568838521	<i>Citrus sinensis</i>	MFP1 attachment factor 1-like isoform X1	∞	-	-7%	-	WPP2
o604	gi 227937349	<i>Citrus maxima</i>	20S proteasome beta subunit 5	∞	3%	-6%	H	AT3G26340
o613	gi 568822583	<i>Citrus sinensis</i>	Acid phosphatase1	∞	3%	-8%	-	AT5G51260
o623	orange1.1g024615m	<i>Citrus sinensis</i>	L-ascorbate peroxidase 2, cytosolic	∞	3%	-7%	-	Not found
o629	gi 567892199	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10032366mg	∞	5%	-10%	-	Not found
o633	XP_006481991.1	<i>Citrus sinensis</i>	Nascent polypeptide associated complex alpha subunit-related	∞	3%	-6%	H	AT3G12390

o639	gi 1026057598	<i>Capsicum annuum</i>	Prohibitin-3, mitochondrial-like	∞	3%	-7%	B	AT2G30050
o657	gi 568826827	<i>Citrus sinensis</i>	30S ribosomal protein S5, chloroplastic	∞	3%	-6%	C	MOD1
o685	gi 567861648	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10028594mg	∞	3%	-6%	HB	FBA2
o643	gi 568826827	<i>Citrus sinensis</i>	30S ribosomal protein S5, chloroplastic	∞	3%	-6%	HB	EMB3113
o686	gi 225449016	<i>Vitis vinifera</i>	Adenosine kinase 2	∞	3%	-7%	B	ADK2
o687	gi 567919464	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10008562mg	∞	8%	-17%	C	mMDH2
o689	gi 568864779	<i>Citrus sinensis</i>	Protein ASPARTIC PROTEASE IN GUARD CELL 1	∞	4%	-8%	-	ASPG1
o690	gi 568862533	<i>Citrus sinensis</i>	Aspartic proteinase CDR1-like	∞	-	-7%	-	CDR1
o703	XP_006471634.1	<i>Citrus sinensis</i>	Pectinacetylerase family protein	∞	6%	-12%	-	AT4G19420

697 ^a • Exclusives Spots from Infected Samples 6 hai; o Exclusives Spots from Infected Samples 12 hai.

698 ^b Fold change (infected sample volume/control normalized volume): underexpressed proteins have values between 0 to 1, while overexpressed proteins have values from 1 to ∞. np = protein not
699 found in gel.

700 ^c Variable importance for the PCA of differentially expressed protein.

701 ^d B: bottleneck; H: hub; C: common; HB: hub-bottleneck.

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710 **Table 2.** Differentially expressed proteins with highest influence on the variability between control and infected samples of ‘Clemenules’.

Spot ^a	Accession	Species	Protein ID	Fold change ^b	PCA ^c		PPI network	
					Influence PC1 (%)	Influence PC2 (%)	Centrality ^d	Arabidopsis ID
30	XP_008359031.1	<i>Malus domestica</i>	PREDICTED: proteasome subunit beta type-4-like	3 / 1.9	3%	-5%	H	PBG1
31	gi 283131283	<i>Citrus jambhiri</i>	Thaumatococcus-like protein	2.9 / 1.3	-	-6%	-	AT2G28790
42	gi 568856629	<i>Citrus sinensis</i>	Fructose-bisphosphate aldolase cytoplasmic isozyme	2.2 / 3.1	7%	2%	B	FBA6
45	XP_006441425.1	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10020931mg	2.7 / 2.7	20%	-3%	C	AT5G08300
46	XP_006489733.1	<i>Citrus sinensis</i>	Malate dehydrogenase	1.8 / 2.0	3%	1%	C	c-NAD-MDH2
92	XP_008443857.1	<i>Cucumis melo</i>	Eukaryotic translation initiation factor 5A-2	0.5 / 0.5	-5%	4%	B	ELF5A-1
124	XP_006481573.1	<i>Citrus sinensis</i>	2-Cys peroxiredoxin BAS1, chloroplastic	0.6 / 1.0	-4%	14%	B	At5g06290
139	XP_006474693.1	<i>Citrus sinensis</i>	20 kda chaperonin, chloroplastic	2.6 / 1.1	-1%	-3%	C	CPN20
151	XP_008457647.1	<i>Cucumis melo</i>	Triosephosphate isomerase, chloroplastic	0.6 / 0.8	-5%	7%	H	TIM
166	XP_006483853.1	<i>Citrus sinensis</i>	Carbonic anhydrase 2 isoform X1	0.3 / 0.7	-3%	4%	-	BCA4
175	XP_006450842.1	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10009194mg	4.2 / 4.3	56%	-14%	C	AT5G06740
187	KDO84609.1	<i>Citrus sinensis</i>	Hypothetical protein CISIN_1g023620mg	0.5 / 0.7	-10%	12%	HB	PAF2
224	XP_006477460.1	<i>Citrus sinensis</i>	Malate dehydrogenase, chloroplastic	0.2 / 0.3	-10%	7%	C	MDH
235	XP_017638623.1	<i>Gossypium arboreum</i>	Malate dehydrogenase, mitochondrial-like	1.8 / 1.1	-2%	-5%	C	mMDH2
261	BAU51794.1	<i>Citrus depressa</i>	O-methyltransferase	0.6 / 0.8	-5%	6%	B	OMT1
300	XP_010102294.1	<i>Morus notabilis</i>	GDP-mannose 3,5-epimerase 1	0.6 / 0.6	-4%	4%	C	SWP
303	XP_017976224.1	<i>Theobroma cacao</i>	Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic isoform X1	0.4 / 0.6	-9%	10%	HB	RCA
319	XP_017976224.1	<i>Theobroma cacao</i>	Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic isoform X1	0.6 / 0.8	-7%	13%	HB	RCA
375	XP_006424516.1	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10028085mg	0.6 / 0.6	-15%	15%	HB	CPN60A
379	ADM35974.1	<i>Citrus limon</i>	Protein disulfide isomerase	0.7 / 0.6	-28%	21%	B	PDIL1-1

389	XP_006492570.1	<i>Citrus sinensis</i>	Stromal 70 kda heat shock-related protein, chloroplastic isoform X2	0.6 / 0.6	-23%	24%	HB	cpHsc70-2
407	XP_007013338.1	<i>Theobroma cacao</i>	GTP-binding nuclear protein Ran-3	1.8 / np	-4%	-3%	C	RAN3
409	KDO62916.1	<i>Citrus sinensis</i>	Hypothetical protein CISIN_1g018871mg	3.6 / np	-4%	-6%	-	AT2G38380
410	XP_006481883.1	<i>Citrus sinensis</i>	Fructose-bisphosphate aldolase cytoplasmic isozyme	3.3 / np	-13%	-23%	B	FBA6
413	XP_014506143.1	<i>Vigna radiata var. radiata</i>	18.5 kda class I heat shock protein-like	2.5 / np	-3%	-2%	-	AT1G07400
418	XP_006476377.1	<i>Citrus sinensis</i>	Nucleoside diphosphate kinase III, chloroplastic/mitochondrial-like	2.9 / np	-9%	-13%	H	AT4G23900
427	ADB10839.1	<i>Citrus japonica</i>	Manganese superoxide dismutase	2.5 / np	-5%	-6%	C	MSD1
433	KHN28540.1	<i>Glycine soja</i>	Oxygen-evolving enhancer protein 2, chloroplastic	0.6 / np	-4%	1%	C	PSBP-1
434	XP_006481573.1	<i>Citrus sinensis</i>	2-Cys peroxiredoxin BAS1, chloroplastic	2.0/ np	-4%	-3%	B	At5g06290
439	XP_006486019.1	<i>Citrus sinensis</i>	Glutathione S-transferase DHAR2-like	1.6 / np	-5%	-3%	C	DHAR2
446	EOY23718.1	<i>Theobroma cacao</i>	Nascent polypeptide-associated complex subunit alpha-like protein 2	5.3 / np	-18%	-62%	C	NACA2
459	XP_002277301.1	<i>Vitis vinifera</i>	Elongation factor tub, chloroplastic	0.6 / np	-12%	2%	H	RABE1b
460	XP_006464261.1	<i>Citrus sinensis</i>	Bifunctional aspartate aminotransferase and glutamate/aspartate-prephenate aminotransferase isoform X1	0.4 / np	-6%	2%	B	AAT
462	XP_006487727.1	<i>Citrus sinensis</i>	Rubisco accumulation factor 1, chloroplastic	0.4 / np	-35%	9%	C	AT3G04550
471	XP_010528013.1	<i>Tarenaya hassleriana</i>	Heat shock protein 90-1-like isoform X1	0.6 / np	-17%	3%	HB	CR88
• 474	gij568866092	<i>Citrus sinensis</i>	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic isoform X2	∞	14%	9%	HB	RCA
•480	gij568863336	<i>Citrus sinensis</i>	ATP-dependent Clp protease ATP-binding subunit clpa homolog CD4B, chloroplastic	∞	3%	2%	C	CLPC1
•481	gij568824081	<i>Citrus sinensis</i>	Mitochondrial outer membrane protein porin 2-like	∞	18%	11%	-	VDAC2
•485	gij568846150	<i>Citrus sinensis</i>	28 kda ribonucleoprotein, chloroplastic-like	∞	4%	3%	C	RBP31
•487	gij568838553	<i>Citrus sinensis</i>	Cysteine proteinase RD21a	∞	4%	3%	B	RD21A
•488	gij460398169	<i>Solanum lycopersicum</i>	Nucleoside diphosphate kinase 3-like	∞	11%	7%	H	AT4G23900
•494	gij568855988	<i>Citrus sinensis</i>	2-Cys peroxiredoxin BAS1, chloroplastic	∞	5%	3%	B	At5g06290
•495	gij641845205	<i>Citrus sinensis</i>	Hypothetical protein CISIN_1g0270582mg, partial	∞	3%	3%	C	AT3G08740
•501	gij567919984	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10009198mg	∞	7%	5%	C	LHCB3

•506	gi 945081	<i>Petunia x hybrida</i>	P21	∞	13%	8%	-	AT3G45310
•508	gi 568843850	<i>Citrus sinensis</i>	NADH-cytochrome b5 reductase-like protein	∞	22%	14%	C	AT5G20080
•509	gi 567917652	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10009184mg	∞	6%	4%	C	AT5G06740
•513	gi 567861290	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10028904mg	∞	4%	3%	-	AT4G39230
•515	gi 567919326	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10008909mg	∞	9%	6%	H	ATARCA
•517	gi 567917574	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10008097mg	∞	4%	-	-	GAMMA-VPE
•518	gi 567899352	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10020519mg	∞	7%	5%	C	OASB
•519	gi 359807616	<i>Glycine max</i>	Uncharacterized protein LOC100817577	∞	3%	2%	C	MAB1
•521	gi 116787631	<i>Picea sitchensis</i>	Unknown	∞	5%	3%	B	PRK
•525	gi 1012089035	<i>Arachis duranensis</i>	Glyceraldehyde-3-phosphate dehydrogenase GAPC2, cytosolic	∞	8%	5%	H	GAPC2
•537	gi 15232888	<i>Arabidopsis thaliana</i>	6-phosphogluconate dehydrogenase, decarboxylating 3 []	∞	3%	2%	C	AT3G02360
•538	gi 359329064	<i>Citrus medica</i>	Ribulose bisphosphate carboxylase large subunit, partial (chloroplast)	∞	10%	6%	B	RBCL
•550	gi 470110155	<i>Fragaria vesca subsp. vesca</i>	Endoplasmic homolog	∞	8%	5%	C	SHD
•552	gi 15232963	<i>Arabidopsis thaliana</i>	Ribonuclease and inhibitor rraa/Dimethylmenaquinone methyltransferase	∞	3%	-	-	AT3G02770
•563	gi 116788974	<i>Picea sitchensis</i>	Unknown	∞	4%	3%	H	AT1G45000
•566	XP_006468800.1	<i>Citrus sinensis</i>	ATP synthase subunit beta, mitochondrial	∞	4%	3%	B	AT5G08680
○569	XP_006480857.1	<i>Citrus sinensis</i>	Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic	∞	-1%	-11%	HB	RCA
○580	XP_002530746.1	<i>Ricinus communis</i>	Probable small nuclear ribonucleoprotein F	∞	-1%	-10%	B	RUXF
○585	XP_002530746.1	<i>Ricinus communis</i>	Probable small nuclear ribonucleoprotein F	∞	-1%	-12%	B	RUXF
○587	XP_006475838.1	<i>Citrus sinensis</i>	Uncharacterized protein LOC102628231 isoform X1	∞	-1%	-8%	C	AT2G41475
○605	XP_006466586.1	<i>Citrus sinensis</i>	Caffeoyl-coa O-methyltransferase-like	∞	-1%	-7%	C	CCoAOMT1
○608	XP_006467509.1	<i>Citrus sinensis</i>	Chlorophyll a-b binding protein of LHCII type 1	∞	-1%	-9%	C	CAB3
○615	XP_006469979.1	<i>Citrus sinensis</i>	Prohibitin-3, mitochondrial	∞	-1%	-3%	C	PHB3
○616	AAM65657.1	<i>Arabidopsis thaliana</i>	Actin 4	∞	-	-4%	-	ACT4

o626	XP_006473275.1	<i>Citrus sinensis</i>	Cysteine proteinase RD21a	∞	-1%	-12%	B	RD21A
o639	XP_006488046.1	<i>Citrus sinensis</i>	Rubisco large subunit-binding protein subunit alpha	∞	-1%	-5%	HB	CPN60A
o641	XP_006439784.1	<i>Citrus clementina</i>	Hypothetical protein CICLE_v10019638mg	∞	-1%	-16%	B	ALDH2B4
o658	XP_006488102.1	<i>Citrus sinensis</i>	Protein disulfide isomerase-like 1-4 isoform X1	∞	-1%	-12%	C	PDIL1-4

711 ^a • Exclusives Spots from Infected Samples 6 hai; o Exclusives Spots from Infected Samples 12 hai.

712 ^b Fold change (infected sample volume/control normalized volume): underexpressed proteins have values between 0 to 1, while overexpressed proteins have values from 1 to ∞ . np = protein not
713 found in gel.

714 ^c Variable importance for the PCA of differentially expressed protein.

715 ^d B: bottleneck; H: hub; C: common; HB: hub-bottleneck.

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718 **Figure legends**

719 **Figure 1. 2-DE analysis of ‘Minneola’ (susceptible) and ‘Clemenules’ (resistant)**
720 **variety proteins. A, B and C.** ‘Mineola’ variety. **D, E and F.** ‘Clemenules’ variety. **A and**
721 **D.** Control condition. **B and E.** 6 hai. **C and F.** 12 hai. The proteins indicated by the
722 arrows were differentially expressed under the applied treatment. hai: hour after infection.

723

724 **Figure 2. Distribution of the differentially expressed proteins. A.** According to
725 biological processes for 'Minneola' variety. **B.** According to biological processes for
726 ‘Clemenules’ variety. **C.** According to the expression level for ‘Minneola’ and
727 ‘Clemenules’ analyzed together.

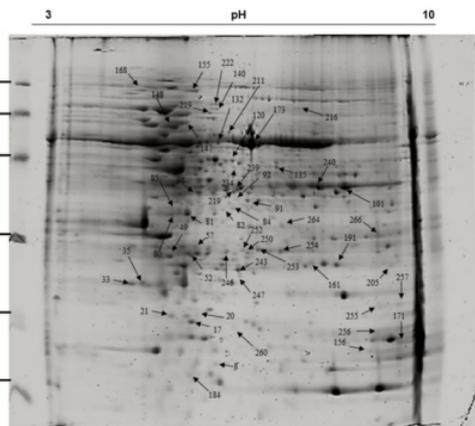
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729 **Figure 3. Bi-directional hierarchical clustering of differential expression of proteins.**
730 **A.** ‘Minneola’ variety proteins. **B.** ‘Clemenules’ variety proteins.

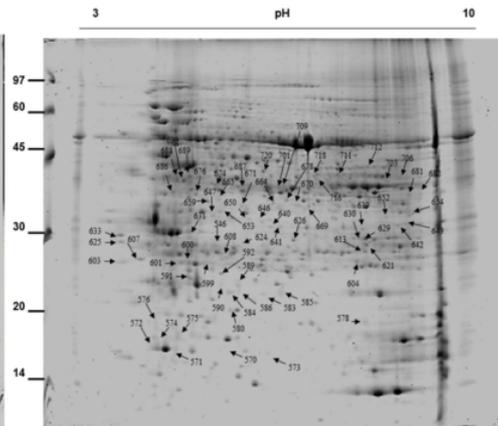
731

732 **Figure 4. General scheme of biological processes involved in mandarin varieties**
733 **infected by *Alternaria alternata*. A.** ‘Minneola’ variety. **B.** ‘Clemenules’ variety. **B:**
734 bottleneck; **C:** common; **H: hub**; hai: hour after infection; **HB:** hub-bottleneck.

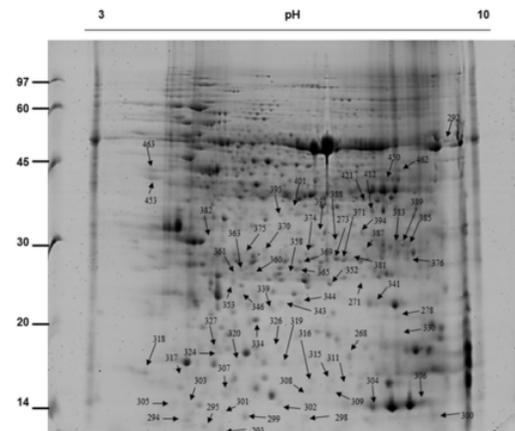
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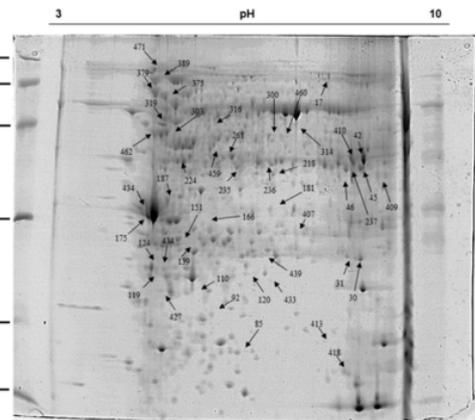
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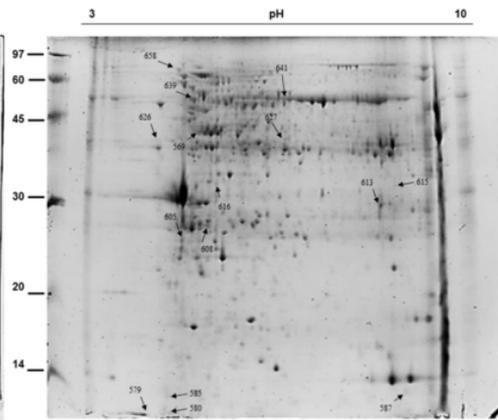
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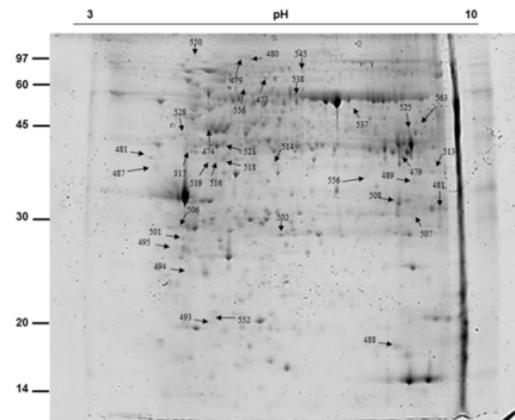
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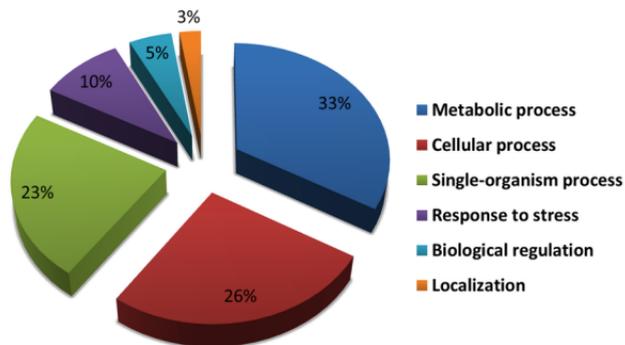
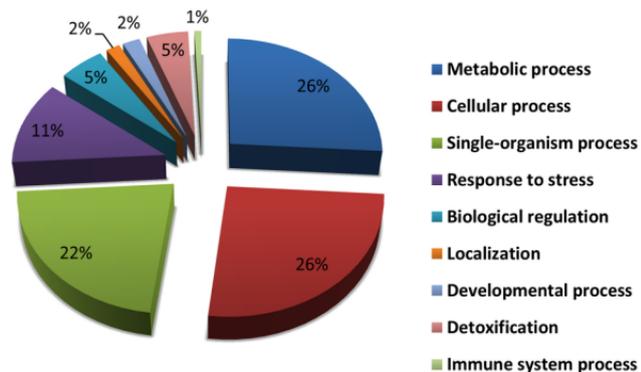
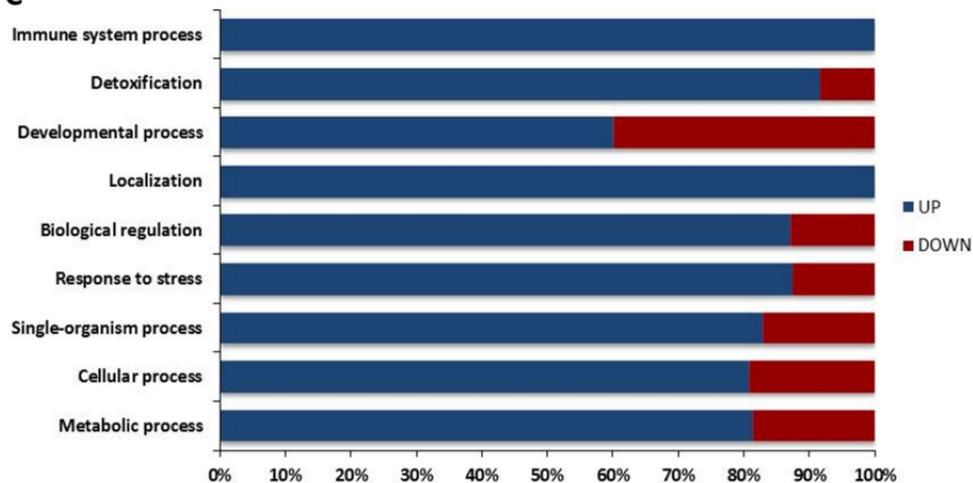
D



E



F

A**B****C**

Minneola (susceptible)

Clemenules (resistant)

Key proteins (centrality)

Control

6 hai

12 hai

Control

6 hai

12 hai

Key proteins (centrality)

Carbonic Anhydrase (B)
 Ribulose-1,5-bisphosphate carboxylase (HB)
 Malate dehydrogenase, mitochondrial (C)
 Phosphomannomutase (C)
 Oxygen-evolving enhancer protein 2 (C)
 Ferredoxin--NADP reductase (C)
 Elongation factor 1-alpha (C)
 Hsp70-Hsp90 organizing protein 3-like (C)
 Chitinase (C)
 Ascorbate peroxidase (C)
 Thioredoxin (C)
 Transcription factor BTF3 homolog 4 (H)

Metabolic and cellular processes

Stress response

ROS detoxification

Proteins related to regulation

Metabolic and cellular processes

Immune system process

ROS detoxification

Proteins related to regulation

ATP synthase (B)
 Malate dehydrogenase (C)
 Fructose-bisphosphate aldolase(B)
 Ribulose-1,5-bisphosphate carboxylase (HB)
 Nucleoside diphosphate kinase III (H)
 4-hydroxy-3-methylbutyl diphosphate synthase (C)
 Cysteine protease like (C)
 Cys-peroxiredoxin (B)
 Manganese superoxide dismutase (C)
 GTP-binding nuclear protein Ran-3 (C)
 O-methyltransferase (B)