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Candidate genes in coffee (*Coffea arabica* L.) leaves associated with rust (*Hemileia vastatrix* Berk. & Br) stress

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Background. Coffee leaf rust (CLR) caused by *Hemileia vastatrix* Berk. & Br, is one of the most threatening diseases for *Coffea arabica* L. It is hypothesized that host tolerance to CLR relies on non-race-specific resistance genes.

Methods. This study evaluated gene expression in leaves of two susceptible coffee cultivars (one inbred and one F_1 hybrid) under different stress conditions: rust control (fungicide and untreated) and fruit thinning (thinned and un-thinned) treatments. RNA-seq analysis focused on the association of differentially expressed genes (DEGs) with CLR and associated the effect of the most significant genes into the phenotype, using regression and prediction statistical models.

Results. Gene expression and gene ontology (GO) analysis allowed identification of 100 genes associated with quantitative traits. From these, 88 were correlated with rust incidence, rust severity, and rust sporulation. The expression of genes coding for pathogenesis-related proteins increased positively with rust incidence in the inbred, while genes involved in homoeostasis and broader cell wall structuring processes were upregulated in the F_1 hybrid. The enriched gene functions and associations revealed that a possible hypersensitive response (HR) in the inbred and a systemic acquired resistance (SAR) in the F_1 hybrid were involved in the tolerance mechanisms to CLR stress. This is the first study to demonstrate the specific interactions between CLR and host at a molecular level, useful for identifying control targets for breeding perennial species.

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Introduction

Pathogenic fungi represent a significant constraint to coffee production. The most significant disease that affects the leaves (and fruits) of susceptible cultivars is coffee leaf rust (CLR) caused by *Hemileia vastatrix* Berk. et Br. (McCook 2006; Talhinhas et al. 2016). Because CLR coevolved with *Coffea* in Africa, it has been able to evolve at least 53 different physiological races to overcome race-specific resistance genes in different coffee cultivars developed by the plant (Rodrigues et al. 1975).

The complex host-pathogen compatible interactions have been described by a gene-for-gene model (Flor 1942) governed by nine major (NBS-LRR) R-loci (S_H1-S_H9) (Bettencourt & Rodrigues 1988; Silva et al. 2002). One of the major goals of coffee breeding programs around the world is to achieve durable combinations of *R* alleles in order to prevent the compatible reaction of the CLR races with the host. To date, breeding programs have favored the use of gene pyramiding in order to achieve durable resistance. However, this strategy has its limits since more new races are emerging, which overcome deployed race-specific resistance in the field (Talhinhas et al. 2016). The value of non-race-specific resistance genes is recognized because of their durability and protection against multiple pathogens or races (Herrera P. et al. 2009). However, this partial resistance is not well understood in coffee (Romero et al. 2010).

Plant stress responses are normally mediated by the production of reactive oxygen species (ROS) which are controlled in the plant by reduction and oxidation processes (Redox) (Noctor 2006). The interaction of Redox intermediates with other molecules such as lipid derivatives, plant hormones like ethylene, jasmonic acid (JA), and salicylic acid (SA), together with nitric oxide (NO), regulate plant homeostasis and morphogenesis (Serrano et al. 2015). In coffee, ROS activated signals have been detected in both CLR compatible (qualitative resistance, host, or gene-for-gene) and incompatible (quantitative resistance or non-host) interactions (Diniz et al. 2012; Fernandez et al. 2012). However, the identification of specific genes and pathways involved in the hypersensitive response (HR) or systemic acquired resistance (SAR) in coffee CLR tolerance, remain unknown.

Homologues of leucine-rich repeat receptor kinases (LRR-RKs) have a central role in different plants (including coffee) in the perception of environmental conditions or stresses. They contribute to transduce signals to plant hormones and histones to enhance signal perception and induce transcription of genes involved in resistance mechanisms. Over time, the networking induces systemic acquired resistance (SAR) in more resistant cultivars.

Our previous findings showed that the management of the coffee plant in on-farm conditions, altered CLR infection in a genotype-dependent manner (Echeverria-Beirute et al. 2017). In this study we took advantage of this same field experiment to identify differentially expressed quantitative resistance genes attributable to cultivar and treatment interactions, in order to find candidate genes for future validation studies. Given challenges in developing experimental populations of perennial crops, the identification of genes for quantitative resistance to CLR in coffee leaves could be useful to better understand pathogenesis mechanisms and pathways. Knowledge of the pathogenesis could have implications for future selection strategies and breeding

40 programs, while candidate genes could serve as editing targets to prevent the evolving pathogen
41 to overcome all available resistance.

42 Materials & Methods

43 Experimental design

44 The experiment was established as previously reported by (Echeverria-Beirute et al. 2017). Briefly,
45 the treatments involved two CLR susceptible adult coffee cultivars (*Coffea arabica* L.): an inbred
46 (Red Catuai 44, F_8 originated from 'Caturra' x 'Mundo Novo') and a hybrid (H3, F_1 of 'Caturra'
47 x 'Ethiopian 531'). The cultivars were subjected to fruit thinning (0% or 50% after self-pollination)
48 and rust control (with or without cyproconazole and epoxiconazole spray application). The
49 experimental design was a split-split-split plot, summarized in Table 1. The control (C) treatment
50 did not have the rust control (R) or fruit thinning (T) treatments, and represented the most stressful
51 condition for the plants in this study.
52

53 RNA isolation and transcriptome analysis

54 Two young leaf samples were collected from the top canopy of each plant and bulked according
55 to their repetition and treatment. Leaf sampling was done in the experimental plot once during the
56 highest infection phase of rust disease and fruit harvest (November), between 9:00 and 11:00 a.m.
57 of a drizzle-cool day. Each bulked sample from 10 total plants (20 leaves) was immediately placed
58 in liquid nitrogen inside a foam cooler. The samples were later transported inside an insemination
59 tank and stored in a -80°C freezer at the Centro de Investigaciones en Biotecnología (CIB)
60 laboratory of the Instituto Tecnológico de Costa Rica (ITCR, Cartago, Costa Rica) until their use
61 in the laboratory. For RNA extraction, the frozen tissue was quickly ground in a mortar and
62 homogenized in liquid nitrogen. Approximately one hundred milligrams of each ground sample
63 was suspended in the extraction buffer supplied in the PureLink® RNA Mini Kit
64 (LifeTechnologies Inc.) in a 1.6 µl microcentrifuge tube. The extraction of the RNA was performed
65 according to the manufacturer's protocol. In each extraction process, RNA concentration and
66 contamination were analyzed at 260 and 280 nm on a DeNovix DS-11 Spectrophotometer. Quality
67 analysis was assessed following electrophoresis on a 1.5% agarose gel. Dehydration and
68 stabilization of the RNA samples for long term storage and normal temperature transportation were
69 done using the RNAstable® solution (Biomatrica Inc.). The dehydration followed the
70 manufacturer's protocol. Briefly, 100 µg of the RNA sample was mixed with 20 µl of RNAstable®
71 solution and later slowly dried in a SpeedVac Concentrator (Thermo® Savant DNA 110) for one
72 hour at ambient temperature. The dehydration process was performed in the Laboratorio de
73 Biotecnología en Ciencias Agrarias of the Universidad Nacional (UNA, Heredia, Costa Rica).
74

75 cDNA library synthesis

76 cDNA library synthesis was performed at the Institute of Biotechnology at Cornell University by
77 Polar Genomics LLC (Ithaca, NY). Strand-specific RNA-seq library construction was carried out
78 using their own developed protocol compatible with the TrueSeq Stranded Total RNA Library

79 preparation kit (Illumina®), based on (Zhong et al. 2011). All cDNA libraries obtained from each
80 RNA sample treatment were size selected by AMPure XP Beads and then PCR amplified using
81 Illumina primers. The library quality determination was done using an Agilent 2100 Bioanalyzer
82 (Agilent Technologies, Santa Clara, California, USA).
83
84 **RNA-seq analysis**
85 Sequencing of the libraries was conducted with the Illumina HiSeq2500 (Illumina®), using a
86 single–end, 101 bp strategy at the Institute of Biotechnology at Cornell University. Quality and
87 quantity of the resulting reads were analyzed with the FastQC Software v.0.11.5 (Andrews 2010).
88 The quality, removal of primer, adapters, and contaminants, were done with Trimmomatic v0.36
89 with default parameter settings (Bolger et al. 2014). Following cDNA library synthesis and
90 sequencing, we obtained high quality sequence reads for 23 samples, representing three biological
91 replications for all treatments with the exception of treatment #7 which only had two replicates
92 (Table 1). RNA-seq analysis was performed using the CLC Genomics Workbench software v.9.5.2
93 (QIAGEN®, Aarhus, Denmark) with the *Coffea canephora* as reference genome (Denoeud et al.
94 2014). The reads were mapped using the following parameters: mismatch cost of 2, insertion and
95 deletion cost of 3, length fraction of 0.8, similarity fraction of 0.8, global alignment = yes, map to
96 intergenic regions = yes, strand specific = both, maximum number of hits for a read = 10,
97 expression value = total counts and use EM estimation = yes. Following mapping of the reads to
98 the annotated *C. canephora* genome, the resulting gene expression (GE) annotation table was used
99 for further analysis. The sequencing depth per annotated gene was calculated as described by
100 (Dugas et al. 2011) considering the total number of bases mapped to a gene (exons only) divided
101 by total gene (exon) length.
102
103 **Differentially expressed genes (DEGs)**
104 To find DEGs gene dispersion analysis was performed in CLC Genomics Workbench v.9.5.2
105 software using the differential expression of RNA-Seq (DESeq2) (Love et al. 2014). The
106 comparison of DEGs within and between the same treatment and cultivars was done in order to
107 find DEGs attributable to treatment and genetic background. Contrast tests considered as limiting
108 conditions included: a Bonferroni correction $p \leq 0.01$, a false discovery rate (FDR) of $p \leq 0.01$, and
109 a fold change (log) cut off of |1| ($\log_2$). Only genes significant in all of these tests and conditions
110 between cultivars and treatments are referred as DEGs. The comparisons between cultivars were
111 done for each treatment as fixed conditions. The comparison between treatments was made by
112 comparing the rust control (R), fruit thinning (T), and both (R+T) treatments, with the no control
113 (C) treatment, within cultivars. Venn Diagrams (Bioinformatics & Evolutionary Genomics, Gent,
114 Belgium, http://bioinformatics.psb.ugent.be/webtools/Venn/) were used to compare and visualize
115 the DEGs according to the experimental conditions to represent shared and unique groups. The
116 DEGs were used to perform gene ontology (GO) analysis using the Singular Enrichment Analysis
117 (SEA) tool in agriGO v2.0 (Tian et al. 2017) according to the *Coffea canephora* annotation
118 (Denoeud et al. 2014). Significant GO terms were found using the default FDR $p \leq 0.05$ cutoff
119 value.

120
121 **Candidate genes associated with the phenotypic traits**
122 From the differentially expressed genes involved in the control versus rust control treatments (C
123 vs R) within each cultivar, a Spearman's correlation and Stepwise regression were performed using
124 the JMP Pro 13.0.0. (SAS Institute Inc. USA) software package. The normalized expression values
125 of the significant DEGs were correlated to each trait previously described in the field by
126 (Echeverria-Beirute et al. 2017): total leaves (TL), overall condition (OC), rust incidence (RI), rust
127 severity (RS), rust sporulation (RE), and total harvest (TH). The correlation analysis was
128 performed using the pairwise estimation method and a significance *p*-value lower than 0.01. The
129 stepwise analysis was performed using the minimum Bayesian information criterion (BIC) as a
130 stopping rule to select the best adjusted coefficient of determination (closest to 1.0) and a FDR *p*-
131 value lower than 0.05. The DEG was set as the independent variable while the trait corresponded
132 to the dependent variable. Any DEG that belonged to a significant GO term, showed significant
133 contribution in the Spearman correlations, and/or was included in the stepwise regression model,
134 was classified as a candidate gene for that trait. Linear regressions using 11 to 12 samples from all
135 treatments for each cultivar were used to model the gene expression (normalized counts) as
136 predictors of the percentage of rust sporulation (RE).

137 # Results
138 **Quality of the leaf transcriptome**
139 A total of 5.75 x 10^8 high-quality reads were obtained from the 23 RNA samples after trimming.
140 Approximately 98% of the sequences were between 100-101 bp length after trimming, with 45.0%
141 GC content, and a phred score showing that 98% of the sequences were higher than 30 (99.9%
142 accuracy in base calling). On average, 82% of the sequences aligned to exons and over 93% of the
143 fragments were uniquely mapped to the diploid *C. canephora* (Denoeud et al. 2014) reference
144 genome. An average of 4,895 annotated genes (21% of the overall 23,057 annotated genes in the
145 *C. canephora* genome) had a sequencing depth higher than 0.5X (Table S1, Supporting
146 information). The trimmed reads for all 23 samples has been deposited at NCBI Short Read
147 Archive under BioProject PRJNA448416, accessions SAMN08832173 - SAMN08832195.
148
149 **Comparing cultivars**
150 Comparing both cultivars whatever the treatment (thinning or chemical applications), 460 DEGs
151 were found to be differentially expressed between the inbred and the hybrid (Table S2 in
152 Supporting information shows 128 DEGs with a sequence depth >0.5X, and with known
153 annotation descriptions). The inbred had 195 genes that increased in expression when compared
154 against the hybrid, while 265 DEGs increased in the hybrid. The DEGs obtained between cultivars,
155 were compared in order to find common DEGs interacting with the treatments (Figure 1). A core
156 set of 62/195 DEGs (32%) in the inbred and 72/265 DEGs (27%) in the hybrid were found
157 significant across rust control treatments (Figure 1). Since almost all DEGs were also found to be

158 associated with treatment effects, we further analyzed the control (C) versus treatment effects (R,
159 T, or R+T).
160
161 **Comparing treatments within cultivars**
162 In order to effectively quantify each treatments effects (R, T, or R+T), the normalized values of
163 the genes within each cultivar were used to find differential gene expression by comparing each
164 genes expression to the control (C). A total of 2,043 unique DEGs were found to be significant
165 within the hybrid and inbred using a FDR and Bonferroni correction of $p \leq 0.01$ (Figure 2). Among
166 the DEGs in the hybrid, the greatest amount was due to the rust control (R) or rust control and fruit
167 thinning (R + T) treatments. In the inbred there was a smaller number of DEGs overall with the
168 exception of fruit thinning (T), more genes were overexpressed in the control (C) treatment.
169
170 **Gene ontology analysis**
171 All DEGs from each cultivar and each treatment comparison were used to find enriched gene
172 ontology (GO) terms (Table S3). The comparison between the untreated control *vs*. rust control
173 fungicide (C vs. R) treatments for both cultivars was highly enriched, showing overall 20 GO terms
174 in the inbred and 35 GO terms in the hybrid. The inbred had a higher number of significantly
175 enriched GO terms in the control (C) treatment (Table S3 - #1), while nearly the opposite occurred
176 in the hybrid, which had a higher number of significantly enriched GO terms in the rust control
177 (R) treatment (Table S3 - #8). When examining the treatment of both rust control and fruit thinning
178 compared against the control treatment (C vs R+T) only the hybrid showed an enrichment in GO
179 terms (Table S3 - #11 and 12).
180
181 **Candidate genes associated with phenotypic traits**
182 The DEGs that were significant when comparing the no control versus rust control (C vs R)
183 treatments were used to find correlations to the quantitative phenotypic traits described in Table 1.
184 A total of 906/2,043 annotated genes were found both differentially expressed and correlated to at
185 least one rust-related traits (i.e. rust incidence (RI), rust severity (RS), rust sporulation (RE)) (data
186 not shown). A total of 144 annotated genes, chosen by stepwise regression analysis, were also
187 enriched in a GO term classification (Table S4). A total of 785 correlations were found between
188 DEGs and traits, but only 88 candidate genes were a) statistically significant (Bonferroni
189 correction and FDR <0.01), b) correlated to RI, RS, and RE, and c) significant in the stepwise
190 regression (Tables S5 and 6).
191 From the 88 DEGs associated with the traits, 24 candidate genes were found differentially
192 expressed in the inbred cultivar (Table S5). These 24 genes exhibited increased expression with
193 an increase of disease-related parameters (RI, RS, and RE). Figure 3 shows six candidate genes
194 regressed to rust sporulation (RE). The predicted functions of the DEGs found in the inbred were
195 related to oxidation and reduction process, transmembrane transportation, and protein regulation
196 in general.
197 In the case of the hybrid, candidate genes expression profiles correlated to phenotypic traits were
198 positive or negatively in the rust control treatment (control was correlated to RI, RS, and RE

199 disease-related parameters; Table S6). Figure 4 shows 9 candidate genes regressed with rust
200 sporulation (RE). Protein kinases, cation transportation and binding, oxidation and reduction
201 processes, and pathogenesis-related processes, were in general enriched under rust control
202 treatments (less RI, RS, and RE) in the hybrid. Transcription regulation and biosynthesis
203 processing were also found to be enriched under no rust controlled treatments in the hybrid.
204 The overall correlation of the phenotype with the transcriptome profiles revealed that the
205 management of CLR disease enriches certain GO terms. From 100 candidate DEGs associated
206 with phenotypic traits, 88 were correlated with rust incidence, rust severity, and rust sporulation
207 in a genotype-management dependent manner (Supporting information 5 and 6).

208 Discussion

209 In a previous study we showed that rust control (cyproconazole and epoxiconazole) successfully
210 controlled rust incidence (RI =5 to 7% respectively for the inbred and the hybrid) whereas without
211 these fungicides RI was significantly higher (21 and 16 % for the inbred and hybrid). The hybrid
212 showed more overall tolerance to CLR than the inbred as well as a delayed appearance of severe
213 CLR symptoms and leaf drop-off. Manual thinning by coffee workers to reduce fruits by 50%
214 (which in fact reduced yield 38%) resulted in between 4 to 6% of lower RI (respectively for the
215 inbred and the hybrid). Similar results by (Toniutti 2017) confirmed that CLR sporulation depends
216 on the physiological status of the coffee plant, which itself depends on agronomic conditions and
217 on hybrid vigour.
218 Here we focused on the transcriptome of the coffee leaf response from these agronomic
219 management strategies to control coffee leaf rust disease (CLR).
220

221 High quality of the leaf transcriptome

222 Approximately 89% of the *C. arabica* sequences obtained through RNA-seq analysis aligned to
223 the *C. canephora* genome which was higher than previous work using a *de novo* transcriptome
224 assembly from *C. arabica* (Ivamoto et al. 2017). However, since *C. arabica* is an allotetraploid
225 derived from hybridization between *C. canephora* and *C. eugenoides*, a subset of the reads are
226 likely from the *C. eugenoides* subgenome that have aligned to the orthologous genes in the *C.*
227 *canephora* subgenome. Because the genome sequence of *C. arabica* wasn´t available at the time
228 we analyzed our data, we had to align our reads to the *C. canephora* genome to identify
229 differentially expressed genes across our samples and treatments.
230 According to our data, the total variation of the normalized gene expression was attributed to the
231 cultivars and the agronomic treatments. Since both cultivars and four different treatments were
232 involved in changes in gene expression, controlling one parameter (i. e. cultivars), led us to a better
233 statistical approach to dissect and quantify differential gene expression (DGE) of the other
234 parameter.
235

236 **Genes differentially expressed between treatment and cultivars**
237 The number of DEGs obtained when comparing cultivars was higher due to treatment interactions
238 within the hybrid (Figure 2). When comparing the treatment effects (intended to reduce disease-
239 related stresses) against the no-treatment negative control (C), a greater number of genes were
240 differentially expressed in the hybrid when treated with fungicide (Figure 2, R and R+T
241 treatments). The presence of a higher number of DEGs that increased expression under
242 management to reduce stress in the hybrid, suggests higher transcriptomic plasticity associated
243 with better homoeostasis as was noted by Bertrand et al. (2011) when they compared an
244 allopolyploid with its two diploids parents. Another reasonable explanation is that the fungicide
245 acted as an elicitor of biotic stress in the hybrid, as shown by (Monteiro et al. 2016) using phosphite
246 products.
247 Contrary to what was observed in the hybrid, the inbred had more DEGs in the treatment with
248 more stress (i.e. the control treatment). The presence of genes upregulated during stress in fewer
249 GO terms, suggests that homozygosity limits the efficiency of plant defense mechanisms which in
250 turn favors the physiological disorder of rust penetration and tissue invasion by the pathogen. As
251 expected from inbreeding, higher fixed and selected alleles by selfing were obtained across the
252 autogamous process, which in this cultivar is over eight generations (Menzel et al. 2015).
253 We used the DEGs identified in the treatments to find enriched gene ontology (GO) terms. It was
254 possible to verify that the hybrid and inbred had different GO terms enriched between treatments.
255 In the case of the inbred, the overexpressed DEGs in the control treatment were related to an
256 increase of disease-related stress. The enriched GO terms were related to carbohydrate,
257 monoxygenase, and heme binding processes, associated with oxidative stress. This contrasts with
258 the hybrid where the enriched GO terms in rust control treatment were related to defense response
259 and apoptosis, which are associated with host-pathogen interactions (Kushalappa et al. 2016),
260 suggesting a possible elicitor effect of the fungicide in this cultivar and/or less disorders caused by
261 the pathogen.
262
263 **Candidate DEGs associated with traits**
264 The disease parameters most related to CLR that were altered in the field were the rust incidence
265 (RI), rust severity (RS), and rust sporulation (RE) (Table 1). The treatments comparing the
266 untreated control (no fungicide sprays) with the rust control (fungicide spray) treatments (C vs.
267 R), showed a higher number of significantly enriched GO terms in both cultivars. Considering that
268 the spray application reduced rust incidence, rust severity, and rust sporulation an average of 12%,
269 3%, and 27% respectively in both cultivars (Echeverria-Beirute et al. 2017), spray application
270 treatments were also useful to reveal variation in gene expression.
271 The overall transcriptome information and candidate gene analysis revealed two different types of
272 defense response. In the case of the inbred, the defense response was highly oriented into
273 carbohydrate metabolism and monooxygenase activity (Figure 2), associated with a hypersensitive
274 response (HR) (Birch et al. 1999; Guidetti-Gonzalez et al. 2007; Moeder et al. 2002). However, in
275 the case of the hybrid, a higher number of defense-related and recovery-related protein synthesis
276 were expressed under stress conditions (*i.e.* control treatments when compared to R or R+T). Such

277 patterns are reported to be related to systemic acquired resistance (SAR) (Florez et al. 2017; Guzzo
278 et al. 2009).
279
280 The increase of gene expression under more stress (*i.e.* no controlled treatment) suggests an early
281 hypersensitive response (HR) induced by the oxidative burst in the inbred. Expression of genes
282 encoding a putative hyoscyamine 6-dioxygenase, a putative stellacyanin, a tyrosine
283 aminotransferase, a putative acidic mammalian chitinase, a putative methyltransferase
284 DDB_G0268948, and other proteins related to oxidoreductase activities increased with RE (Figure
285 3; Supporting information 6). The hyoscyamine 6-dioxygenase (Figure 3E; Supporting
286 information 6) has been found to be expressed early in HR responses of potato under *Phytophthora*
287 *infestans* attack (Birch et al. 1999). The stellacyanins (Supporting information 4 to 6) have stronger
288 oxidation potential than other cupredoxins (Nersissian et al. 1998), which represent more sensitive
289 signaling under ROS accumulation. Tyrosine aminotransferase (Figure 3D) is involved in
290 tocopherol synthesis (Munné-Bosch 2005) and other benzylisoquinoline alkaloids (Lee & Facchini
291 2011) which are natural antioxidants. Chitinase activity (Figure 3C), the presence of a putative
292 anion transporter, and calcium influx, are signatures that an early oxidative burst event associated
293 with the HR was ongoing, as has also been suggested in citrus when examining broad combinations
294 of limiting factors, phenological stages, and tissues (Guidetti-Gonzalez et al. 2007). The putative
295 methyltransferase DDB_G0268948 (Supporting information 5), has also been found highly
296 expressed under Redox activity after *Pseudomonas syringae* pv *tomato* inoculation in a resistant
297 tomato (Balmant et al. 2015) and in *Paulownia* stress tolerance (Dong et al. 2016).
298 The inbred had candidate DEGs related to carbohydrate catabolic process and monooxygenase
299 activity increased under no controlled treatments (Supporting information 3 - #1). Expression of
300 genes encoding proteins such as 1-aminocyclopropane-1-carboxylate (ACC) synthase and oxidase
301 (Figure 3F; Supporting information 5) increased with rust incidence (RI). These proteins are the
302 precursors for ethylene production (Moeder et al. 2002). ROS (especially ozone) activates ethylene
303 production to induce ethylene-mediated cell death, suggesting a hypersensitive response (HR) is
304 initiated in the inbred-CLR interaction mediated by pathogenesis-related proteins (Guerra-
305 Guimarães et al. 2009).
306 In the hybrid, however, one of the candidate genes exhibiting differential expression was the
307 disease resistance gene RGA2 between rust controlled and no controlled treatments (Figure 4A;
308 Supporting information 6). The function of RGA2 is related to signal perception and transduction
309 under a systemic acquired resistance (SAR) interaction. From our findings, the expression of
310 RGA2 increased with less rust incidence (RI) and rust sporulation (RE) in the hybrid. The same
311 pattern was exhibited by an ABC transporter C family member 1 gene (Supporting information 6),
312 which is involved in synthesis and transport of antimicrobial metabolites (Guzzo et al. 2009). The
313 arginine decarboxylase (Supporting information 6) involved in signal perception and transduction
314 was downregulated in the hybrid as well. The putative LRR receptor-like serine/threonine-protein
315 kinase FLS2 (similar to RGA2) was reported by (Florez et al. 2017) in coffee-CLR interaction,
316 which is also related to CLR recognition (Supporting information 6). Overall, these gene

317 expression changes show that active gene regulation was occurring under host-pathogen
318 interactions.
319 The transmembrane transport and protein structure GO categories were highly represented during
320 the CLR biotic stress in the hybrid (Supporting information 3 - #7 and #11). The higher expression
321 of the oligopeptide transporter 4 correlated with rust incidence (RI) and rust sporulation (RE) in
322 the hybrid (Supporting information 6) indicates plant and fungus interactions, as also reported in
323 grape (Balestrini et al. 2017). Genes containing RING finger domains and chitinases (Figure 4C;
324 Supporting information 6), also found by (Guzzo et al. 2009), are in charge of regulating protein
325 degradation and antimicrobial proteins, respectively. A sodium/hydrogen exchanger (Figure 4F)
326 and armadillo/beta-catenin-like repeat C2 calcium/lipid-binding domain (CaLB) protein
327 (Supporting information 6), have been shown to be involved in submergence tolerance in rice
328 (Kottapalli et al. 2006). According to (Li et al. 2018), the armadillo/beta-catenin-like repeat
329 domains are also involved in transcriptional regulation, protein degradation, chromatin
330 remodeling, and cytoskeletal regulation under stress, suggesting that intense regulation and
331 remodeling of the cell wall was occurring under CLR infestation, and may explain the lower rust
332 incidence (RI) and rust sporulation (RE).
333 Growth and developmental proteins such as UDP-glycotransferases, putative uridine/cytidine
334 kinase, and a putative myosin-J heavy chain protein (Figure 4, Supporting information 6), were
335 also correlated with rust sporulation (RE) in the hybrid. Increases in UDP-glycotransferases have
336 been associated with the addition of sugars to plant hormones under ROS cascades, modulating
337 indole-3-butiric acid (IBA) homoeostasis and inducing water stress tolerance (Tognetti et al.
338 2010). The uridine/cytidine kinase (EC 2.7.1.48) is in charge of nucleoside degradation and
339 salvage, which supports active growth (Belmonte et al. 2011). Increase of its expression under
340 biotic stress suggests a defense mechanism to regulate purine and pyrimidine metabolism in order
341 to reduce cell proliferation. The myosin proteins are in charge of the reorganization and
342 polarization of actin filaments inside the cell (Yang et al. 2014). Since at the penetration sites, the
343 first barrier to limit the pathogens growth and infestation is by reordering the cytoskeleton and
344 organelles, an increase in the expression of myosin under stress suggests a cytological defense
345 mechanism to limit CLR penetration and expansion.
346 The protein biosynthesis and maturation process were also affected under CLR disease in the
347 hybrid (Supporting information 3 - #11). Expression of genes encoding a putative small subunit
348 processome component 20 homolog (Figure 4D) and a putative pre-mRNA-processing protein
349 40A (Supporting information 6) increased with decreasing rust incidence (RI) and rust sporulation
350 (RE). The small ribosomal subunit (SSU) processome is a nuclear large ribonucleoprotein (RNP)
351 required for processing the precursors of the 18S small subunit RNA of the ribosome, in charge of
352 rRNA transcription and ribosome assembly (Phipps et al. 2011). The putative pre-mRNA-
353 processing protein 40A interacts with a mediator complex 35 as co-regulators of protein
354 transcription (Dolan & Chapple 2017). If the increase in gene expression for these two proteins
355 resulted in increased protein activity, this suggests that *de novo* biosynthesis of proteins was

356 regulated in the nuclei in response to CLR stress, perhaps to continue cell growth and facilitate the
357 metabolism change in order to mitigate the stress and later recovery (Baena-González 2010).
358 Expression of genes related to anti-oxidation processes were also increased with CLR stress in the
359 hybrid (Supporting information 3 - #11). For example, expression of a peroxiredoxin-2B was
360 found to be correlated with RE (Supporting information 6). The peroxiredoxin-2B, was also
361 reported by (Margaria et al. 2013) in grape under phytoplasma attack, which was one of the first
362 chloroplastic enzymes involved in the response to oxidative stress and recovery to steady-state.
363 Expression of the genes coding for the aldo-keto reductase yakc proteins (Figure 4I; Supporting
364 information 6) increased with decrease of RI, RS, and RE, and this has also been seen in response
365 to drought stress in maize (Zhao et al. 2016). The aldo-keto reductase yakc proteins are activated
366 in an abscisic acid (ABA)-dependent way, revealing that hormone signaling and oxidation-
367 reduction activity were ongoing during CLR attack in the hybrid in a manner consistent with
368 systemic acquired resistance (SAR).

369 Conclusions

370 The overall transcriptome and correlation analyses, suggested that the hybrid had a broader
371 response to the coffee leaf rust (CLR) disease than the inbred. The quantitative expression of genes
372 related to stress under higher rust sporulation in the control treatment (without any management),
373 but also apoptosis and a qualitative response under less stressed treatments, reveal that the hybrid
374 had a greater ability to actively regulate the gene transcription network dependent on management.
375 Even though both cultivars are susceptible to CLR, the hybrid showed a 4%, 1%, and 5%, overall
376 reduction in rust incidence, rust severity, and rust sporulation, respectively, when compared to the
377 inbred (Echeverria-Beirute et al. 2017), which suggests some tolerance level and validates the
378 hypothesis of lower impact of the pathogen in F_1 hybrids due to their higher vigor and better
379 homeostasis. Our present results showed that rust provokes more molecular disorders in the inbred
380 than in the hybrid. All together those findings are important because they show, at the molecular
381 level, the impact of new races of rust capable to overcome the R genes has much more serious
382 physiological consequences in a homozygous plant than in a heterozygous plant. Unfortunately,
383 the Arabica orchard is composed almost exclusively of very homozygous varieties, which
384 amplifies the harmful consequences in case of strong parasitic pressures.
385 CLR is the most devastating disease for this culture. Highly specific complete resistance result has
386 turned out not to be durable (van der Vossen 2015). Developing strategies to improve the durability
387 of rust resistance based on genetic mechanisms combining both partial along with appropriate
388 cultural practices, may be the best way to control this disease in the future. However, the use of
389 fungicides must be increasingly limited. Therefore, we advocate for breeding programs that focus
390 on vigorous and heterozygous plants that can withstand both biotic and abiotic stresses.
391 Further research has to be conducted in order to first validate the set of candidate genes obtained
392 in this project, and later should be expanded to other genotypes, management conditions, and
393 environments. While we showed that the gene expression was affected by the CLR management
394 in two susceptible coffee genotypes in the field, comprehensive pathways and networks that

395 describe how the tolerance is effective as a durable alternative of disease control, remains to be
396 elucidated.

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570
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573

574 # Figure Legends

575 **Figure 1.** Shared DEGs within treatments when compared between cultivars.
576 DEGs are classified according to which treatment is increasing the gene expression in the A) inbred
577 or B) hybrid cultivars. More details in Supporting information 3.
578
579 **Figure 2.** Effect of treatment on DEGs in each cultivar.
580 DEGs are classified according to which treatment (blue color bar) or control (red color bar)
581 resulted in increased gene expression. The comparison between treatments was done by comparing
582 the rust control (R), fruit thinning (T), and both (R+T) treatments, with the no control (C)
583 treatment, within cultivars. Table underneath bars explains major GO categories increasing with
584 each treatment, with some no applicable (N/A) GO terms were found as described in Table S3.
585
586 **Figure 3.** Linear regressions modeling gene expression (GE) as predictors of rust sporulation (RE)
587 in the inbred.
588 The gene expression (GE, total counts) of the candidate gene is used as a predictor of the
589 percentage of rust sporulation (RE). The gene ID corresponds to A) Cc07_g16100, B)
590 Cc04_g09390, C) Cc06_g15430, D) Cc05_g07600, E) Cc05_g10390, and F) Cc05_g02900, as
591 shown in Supporting information 5. Coefficient of determination (R^2), significance level ($p<0.01$),
592 and prediction equation are shown in each graph. Data used to plot the linear regression was done
593 using 11 to 12 samples from all treatments.
594
595 **Figure 4.** Linear regressions modeling gene expression (GE) as predictors of rust sporulation (RE)
596 in the hybrid.
597 The gene expression (GE, total counts) of the candidate gene is used as a predictor of the
598 percentage of rust sporulation (RE). The gene ID corresponds to A) Cc00_g26280, B)
599 Cc01_g17540, C) Cc04_g15950, D) Cc00_g24200, E) Cc09_g09040, F) Cc11_g05270, G)
600 Cc01_g21050, H) Cc00_g30680, and I) Cc02_g36130, as shown in Supporting information 6.
601 Coefficient of determination (R^2), significance level ($p<0.01$), and prediction equation are shown
602 in each graph. Data used to plot the linear regression included 11 to 12 samples from all treatments.

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Figure 1

Shared DEGs within treatments when compared between cultivars.

DEGs are classified according to which treatment is increasing the gene expression in the A) inbred or B) hybrid cultivars. More details in Supporting information 3.

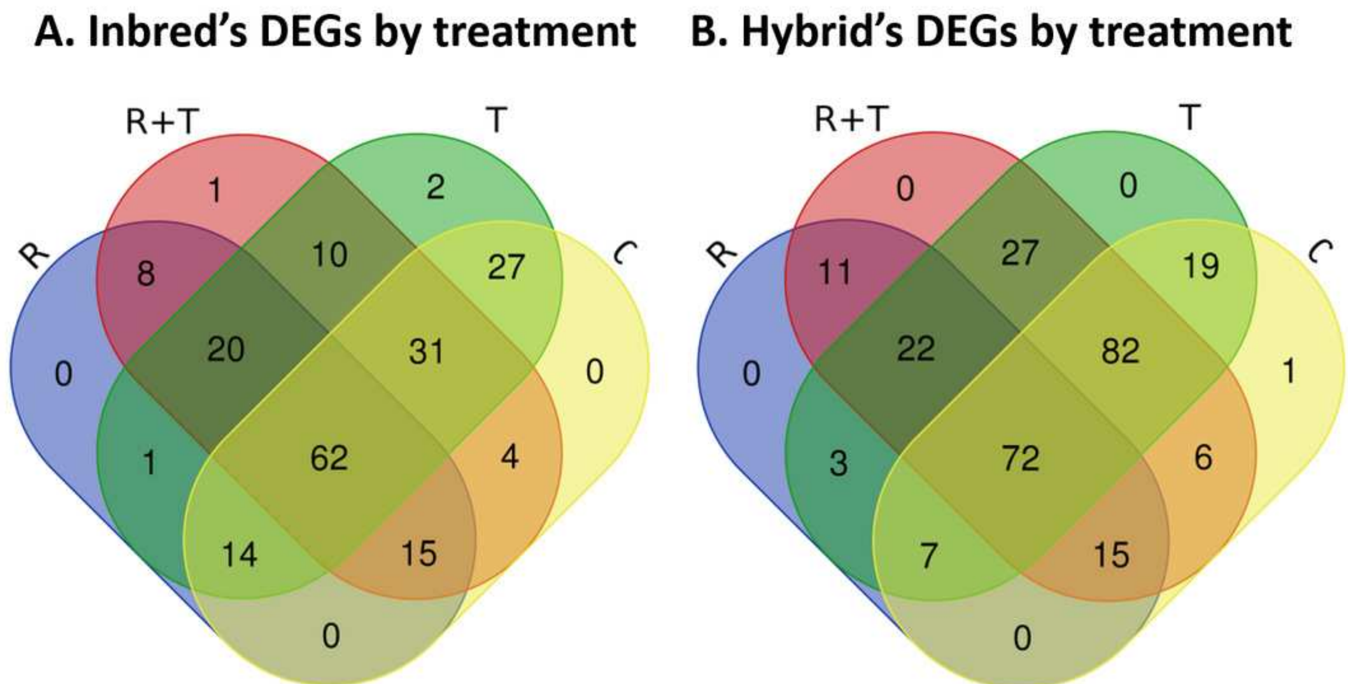

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Figure 2

Effect of treatment on DEGs in each cultivar.

DEGs are classified according to which treatment (blue color bar) or control (red color bar) resulted in increased gene expression. The comparison between treatments was done by comparing the rust control (R), fruit thinning (T), and both (R+T) treatments, with the no control (C) treatment, within cultivars. Table underneath bars explains major GO categories increasing with each treatment, with some no applicable (N/A) GO terms were found as described in Table S3.

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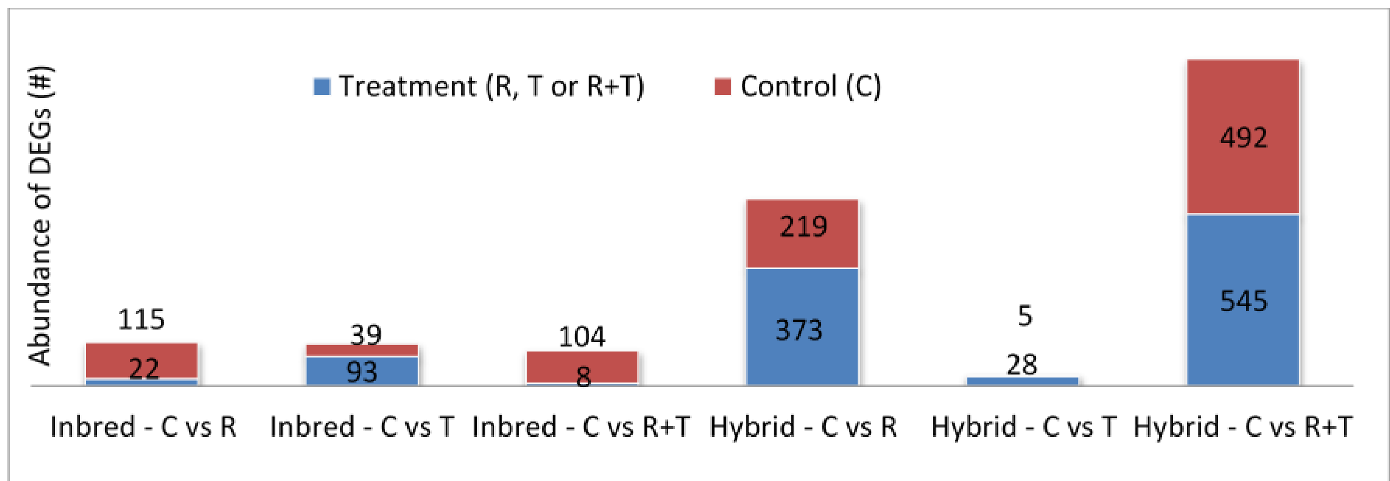

GO terms enriched					
Upregulated in C carbohydrate catabolic process. Mon-ooxygenase activity. heme binding.	**Upregulated in C** N/A	**Upregulated in C** N/A	**Upregulated in C** trans-membrane transport	**Upregulated in C** N/A	**Upregulated in C** translation. oxidation reduction. amino acid transport. ribosome.
Upregulated in R cation binding	**Upregulated in T** N/A	**Upregulated in R + T** N/A	**Upregulated in R** transcription. apoptosis. defense response. others…	**Upregulated in T** N/A	**Upregulated in R + T** microtubule-based process. microtubule motor activity.

Figure 3

Linear regressions modeling gene expression (GE) as predictors of rust sporulation (RE) in the inbred.

The gene expression (GE, total counts) of the candidate gene is used as a predictor of the percentage of rust sporulation (RE). The gene ID corresponds to A) Cc07_g16100, B) Cc04_g09390, C) Cc06_g15430, D) Cc05_g07600, E) Cc05_g10390, and F) Cc05_g02900, as shown in Supporting information 5. Coefficient of determination (R^2), significance level ($p<0.01$), and prediction equation are shown in each graph. Data used to plot the linear regression was done using 11 to 12 samples from all treatments.

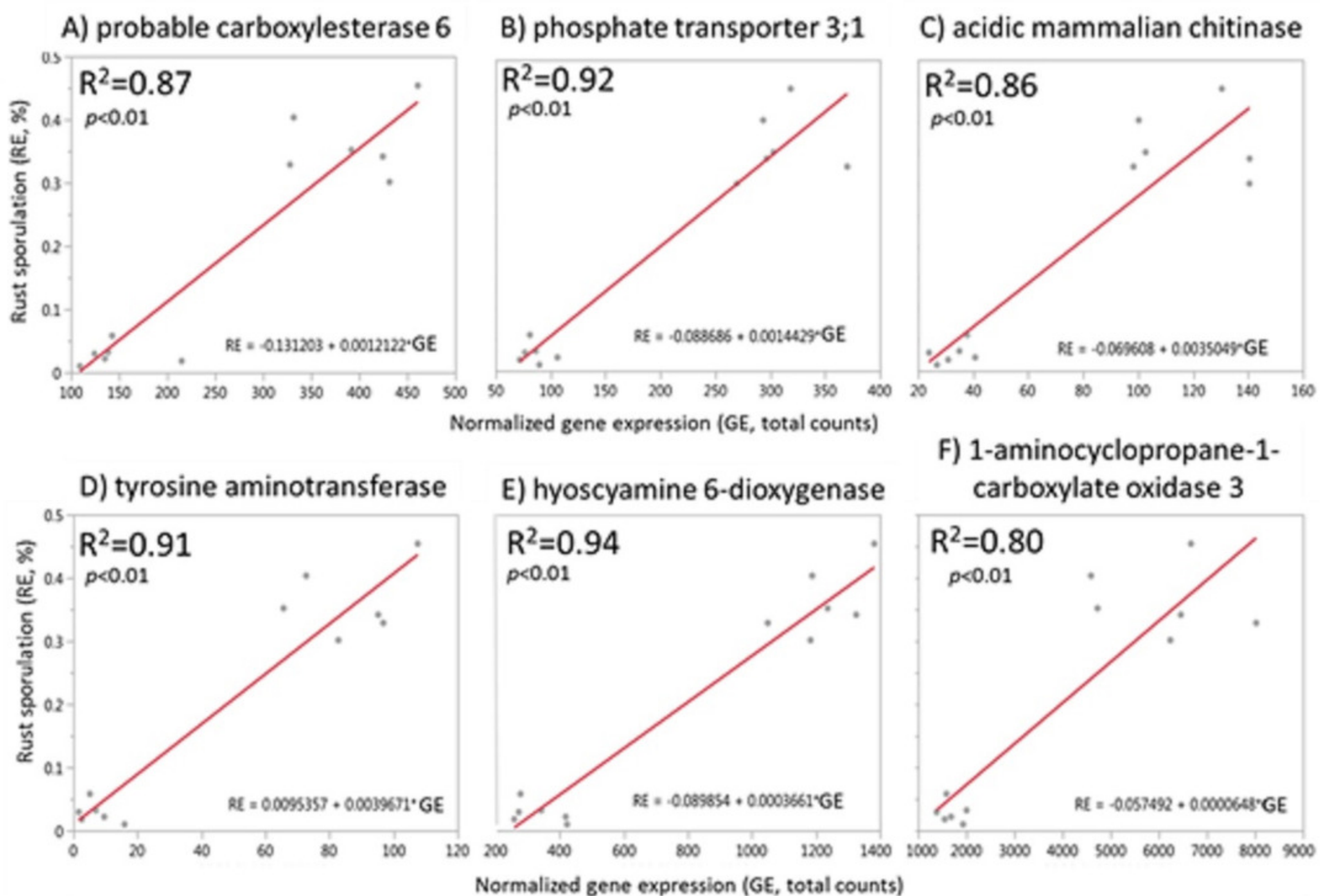

Table 1(on next page)

Table 1 . Overall mean effect of rust control (no or yes) and manual fruit thinning (0% or 50%) on plant performance traits

total leaves (TL), overall condition (OC), rust incidence (RI), rust severity (RS), rust sporulation (RE), and total harvest (TH), in both the inbred and hybrid cultivars. Modified with permission from (Echeverria-Beirute et al. 2017) . Copyright 2018 American Chemical Society.

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1 # Tables

2 **Table 1.** Overall mean effect of rust control (no or yes) and manual fruit thinning (0% or 50%) on plant performance traits: total
3 leaves (TL), overall condition (OC), rust incidence (RI), rust severity (RS), rust sporulation (RE), and total harvest (TH), in both the
4 inbred and hybrid cultivars. Modified with permission from (Echeverria-Beirute et al. 2017). Copyright 2018 American Chemical
5 Society.
6

Treatment (number of biological samples)	Code	Cultivar	Fruit thinning	Rust control	RI	RS	RE	TL	OC	TH
1 (3)	R	Inbred	0%	Yes	7.0% c	0.8% a	3.0% a	15.4 c	2.3 cd	2.9 abc
2 (3)	C	Inbred	0%	No	21.0% f	5.4% c	34.0% e	15.8 bc	2.3 d	2.9 abc
3 (3)	R+T	Inbred	50%	Yes	4.0% ab	0.3% a	2.0% a	17.5 a	2.7 a	1.9 c
4 (3)	T	Inbred	50%	No	18.0% e	4.2% b	34.0% d	16.2 b	2.3 cd	1.8 c
5 (3)	R	Hybrid	0%	Yes	5.0% b	0.7% a	2.0% a	12.8 d	2.3 c	3.8 a
6 (3)	C	Hybrid	0%	No	16.0% e	5.0% c	29.0% c	11.7 e	1.9 e	3.7 ab
7 (2)	R+T	Hybrid	50%	Yes	3.0% a	0.3% a	2.0% a	13.3 d	2.4 b	2.5 bc
8 (3)	T	Hybrid	50%	No	10.0% d	0.7% a	21.0% b	13.2 d	2.3 cd	2.2 abc

7 Letters next to the value represent least significant differences (LSD) at $p \leq 0.05$.

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Figure 4

Linear regressions modeling gene expression (GE) as predictors of rust sporulation (RE) in the hybrid.

The gene expression (GE, total counts) of the candidate gene is used as a predictor of the percentage of rust sporulation (RE). The gene ID corresponds to A) Cc00_g26280, B) Cc01_g17540, C) Cc04_g15950, D) Cc00_g24200, E) Cc09_g09040, F) Cc11_g05270, G) Cc01_g21050, H) Cc00_g30680, and I) Cc02_g36130, as shown in Supporting information 6.

Coefficient of determination (R^2), significance level ($p<0.01$), and prediction equation are shown in each graph. Data used to plot the linear regression included 11 to 12 samples from all treatments.

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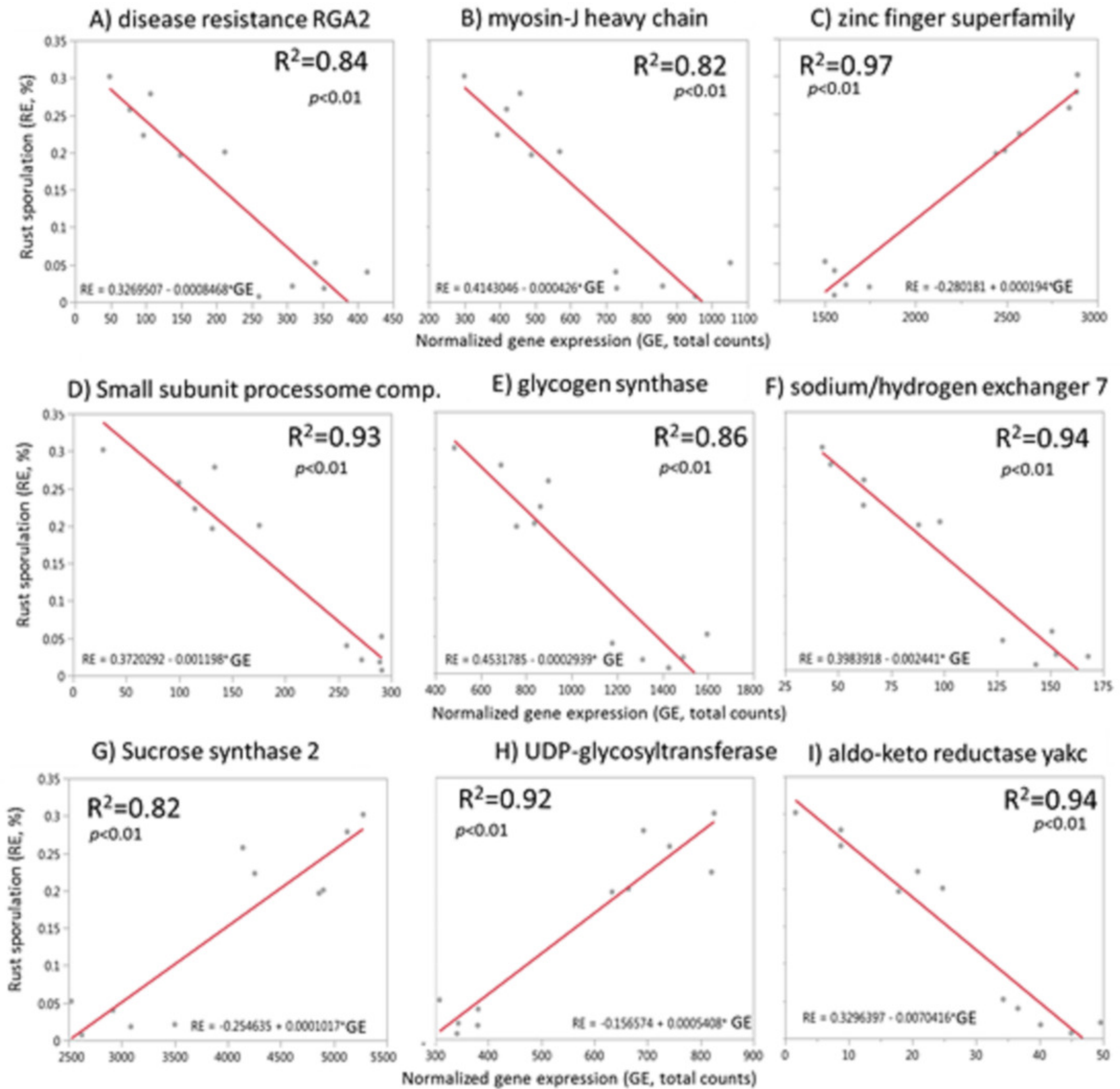
A) disease resistance RGA2
R^2=0.84
p<0.01
RE = 0.3269507 - 0.0008468*GE
B) myosin-J heavy chain
R^2=0.82
p<0.01
RE = 0.4143046 - 0.000426*GE
C) zinc finger superfamily
R^2=0.97
p<0.01
RE = -0.280181 + 0.000194*GE
D) Small subunit processome comp.
R^2=0.93
p<0.01
RE = 0.3720292 - 0.001198*GE
E) glycogen synthase
R^2=0.86
p<0.01
RE = 0.4531785 - 0.0002939*GE
F) sodium/hydrogen exchanger 7
R^2=0.94
p<0.01
RE = 0.3983918 - 0.002441*GE
G) Sucrose synthase 2
R^2=0.82
p<0.01
RE = -0.254635 + 0.0001017*GE
H) UDP-glycosyltransferase
R^2=0.92
p<0.01
RE = -0.156574 + 0.0005408*GE
I) aldo-keto reductase yakc
R^2=0.94
p<0.01
RE = 0.3296397 - 0.0070416*GE
Rust sporulation (RE, %)
Normalized gene expression (GE, total counts)