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1 **Involvement of structurally distinct cupuassu chitinases and osmotin in plant resistance**
2 **to the fungus *Moniliophthora perniciosa***

3

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20

21 **Abstract:**

22 The cupuassu tree (*Theobroma grandiflorum*) is a crop of great economic importance to
23 Brazil, mainly for its pulp and seeds, which are used in food industry. However, cupuassu
24 fruit production is threatened by witches' broom disease caused by the fungus *Moniliophthora*
25 *perniciosa*. As elements of its defense mechanisms, the plant can produce and accumulate

26 pathogenesis-related (PR) proteins such as chitinases and osmotins. Here, we identified three
27 PR proteins from cupuassu (TgPR3, TgPR5 and TgPR8) from cupuassu-*M. perniciosa* RNA-
28 seq data. TgPR3 and TgPR8 corresponded to chitinases, and TgPR5 to osmotin; they are
29 phylogenetically related to cacao and to Arabidopsis PR sequences involved in biotic and
30 abiotic stress. The TgPR proteins' tridimensional structure was obtained through homology
31 modeling, and molecular docking with chitin and chitosan showed that the TgPR proteins can
32 interact with both cell wall molecules and presented a higher affinity for chitosan. TgPR gene
33 expression was analyzed by RT-qPCR on resistant and susceptible cupuassu genotypes
34 infected by *M. perniciosa* at 8, 24, 48 and 72 hours after infection (hai). The TgPR genes
35 showed higher expression in resistant plants compared to the susceptible ones, mainly for
36 TgPR5 at 8 and 24 hai, while the expression was lower in the susceptible cupuassu plants. To
37 our knowledge, this is the first *in silico* and *in vitro* reports of cupuassu PR protein. The data
38 suggested that TgPRs could be involved in recognizing mechanisms of the plant's innate
39 immune system through chitin receptors. Our results also suggest a putative role of
40 chitinase/chitosanase for the TgPR5/osmotin.

41

42 **Keywords:** Pathogenesis-related proteins, molecular docking, witches' broom disease, gene
43 expression, chitin, chitosan

44

45 **1. Introduction**

46 The cupuassu (*Theobroma grandiflorum* [Willd. Ex Spreng.] Schum.) belongs to the
47 Malvaceae family; it is native to the Amazon region and is economically important to Brazil,
48 where it is cultivated [1, 2]. Cupuassu pulp and seeds are used in the cosmetics and food
49 industries, mainly for candy, ice cream, beverage and *cupulate* (a product similar to
50 chocolate) production [3]. *Cupulate* is an interesting alternative to chocolate production for an

51 actual worldwide economic situation in which the production of cocoa (*Theobroma cacao*,
52 Malvaceae family) beans cannot supply the increased worldwide demand for chocolate [4, 5].
53 However, both cocoa and cupuassu cultures are affected by pests and pathogens that cause
54 damage, loss of production and even plant death. Among them, witches' broom disease,
55 caused by the fungus *Moniliophthora perniciosa*, is the main phytopathological problem for
56 cupuassu cultures [6].

57 Because they are attacked by a wide variety of pathogens, plants have developed
58 pattern-recognition receptors to recognize molecular patterns associated with pathogens
59 (PAMPs), which lead, among other outcome, to the production and accumulation of
60 pathogenesis-related proteins (PR proteins), which are considered crucial to plant defense
61 mechanisms [7-9]. The current classification divides PR proteins into 17 classes (PR1–PR17)
62 depending on their biological activity, physicochemical properties and/or sequence homology
63 [10, 11]. The PR-3, PR-4, PR-8 and PR-11 families correspond to different types of chitinases
64 (EC 3.2.1.14). Among them, the PR-3 proteins include glycoside hydrolase 19 (GH19)
65 chitinases, and the PR-8 family corresponds to glycoside hydrolase 18 (GH18) chitinases. The
66 PR-5 family classification corresponds to thaumatin-like protein (TLPs), whose biological
67 function in plants has yet to be established [12]. Among TLPs, the osmotins are
68 multifunctional proteins that may participate alongside chitinases in protein complexes
69 involved in plant defense against pathogens [8, 13]. GH18, GH19 and osmotin show
70 antifungal activity through cell wall hydrolysis or plasma membrane permeabilization [12,
71 14]; in the case of PR-5, it has been proposed that the existence of specific target receptors on
72 the membrane leads to fungus sensitivity or resistance [15]. The GH18 chitinases show a
73 characteristic barrel structure [16], while GH19 chitinases are composed of a high
74 concentration of α -helices and loops [14]. The TLP proteins have three well-characterized
75 domains: domain I is composed of β sheets and loops called a β -sandwich, domain II is

76 composed of α -helices and domain III is composed of two β -sheets [17]. Because chitin is
77 present in the fungus cell wall and not in plant cells, it is considered an ideal PAMP and as
78 one of the first barriers in the plant-pathogen battlefield [18, 19]. Chitin and chitosan, its
79 deacetylated derivative, have been heavily studied, and their role in plant-pathogen
80 interaction has received considerable attention, mainly because they are considered useful for
81 biotechnological applications aimed at reducing fungus activity and/or development [19-21].
82 *Moniliophthora perniciosa*'s chitin metabolism, including associated enzymes such as chitin
83 synthases and chitinases, has previously been studied [22-25].

84 Molecular studies of cupuassu related to plant-pathogen interaction are still poorly
85 developed, and few works are related to resistance to witches' broom disease [26-28].
86 Moreover, unlike cocoa, the cupuassu genome has not been sequenced yet, and molecular
87 analyses focused on genetics and genomics of resistance are only based on recent
88 transcriptomic data [26, 28]. Here, we selected and analyzed three PR genes from cupuassu
89 (TgPR3, TgPR5 and TgPR8) with putative chitinase or chitinase-associated functions as well
90 as anti-fungal activity. We showed, *in silico*, the TgPR proteins' structural aspects and
91 potential activity in relation to chitin and chitosan ligands, and, *in vitro*, the contrasting
92 expression levels of the *TgPR* genes in resistant and susceptible cupuassu plants inoculated
93 with *M. perniciosa*. Their expression profiles could be related to phylogenetic analysis using
94 homologous PR sequences from *T. cacao* and *A. thaliana*, which are involved in response to
95 biotic and abiotic stress. To our knowledge, this is the first PR protein report on cupuassu-*M.*
96 *perniciosa* interaction. The overall data data suggest that TgPRs could be involved in
97 recognizing mechanisms of the plant's innate immune system through chitin receptors. Our
98 results also suggest a putative role of chitinase/chitosanase for the TgPR5/osmotin.

99

100 2. Methods

101 **2.1 Identification and characterization of *TgPR* sequences**

102 The PR sequences were selected from a previously obtained RNA-Seq database of cupuassu
103 [28]. Open reading frame (ORF) detection was performed using the ORFinder software
104 (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The proteins were analyzed with BLASTp
105 against the Conserved Domain Database (CDD;
106 <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) for conserved domain identification,
107 protein sequence length checking and TgPR protein classification. Signal peptide was
108 identified using the SignalP 4.1 software [29], while the molecular weight and isoelectric
109 point were obtained using the Compute pI/Mw program [30]. The subcellular locations were
110 predicted using the YLoc-HighRes Plants tool [31].

111

112 **2.2 Phylogeny**

113 Cacao and Arabidopsis nucleotide sequences that are homologous to TgPRs were obtained
114 using local alignment (BLAST) from CocoaGenDB [32] and TAIR
115 (<https://www.arabidopsis.org>), respectively. The global multiple alignment of all sequences
116 was made using ClustalW available with MEGAX software [33]. The MEGAX software was
117 used to construct a rooted phylogenetic tree using the maximum likelihood method with 1,000
118 bootstrap sampling steps, the Tamura-Nei model and the G+I substitution method.
119 Phylogenetic trees were visualized and edited using the FigTree v1.4.4 tool
120 (<http://tree.bio.ed.ac.uk/software/figtree/>).

121

122 **2.3 Homology modeling of TgPRs and molecular docking with chitin and chitosan**

123 The tri-dimensional (3D) structure of the TgPR proteins was obtained using the Swiss-Model
124 server [34]. The crystal structures of homologous proteins available from the Protein Data
125 Bank (PDB; <https://www.rcsb.org/>) were used as templates to build the structural models of

126 TgPR (Supplementary material 1). The stereochemical quality of the models was analyzed
127 with ANOLEA [35] and Procheck [36]. Water molecules were removed from and polar
128 hydrogens were added to the TgPR protein models, before calculation of their Gasteiger
129 charges; the results were exported in PDBQT format. The active sites were identified based
130 on TgPR protein alignment with PR proteins from other species (Supplementary materials 2
131 to 4), using the ClustalW tool [37]. The amino acid residues from the TgPR active sites of the
132 proteins as well as the grid box defining the docking region were marked using AutoDock
133 Tools v1.5.6 [38]. For docking, chitin (CID: 444514) and chitosan (CID: 71853) were used as
134 ligands; their structures were downloaded in SMILES format. Using the MarvinSketch
135 15.7.13.0 tool, the ligands were converted into 3D structures, checked for conformation and
136 saved in mol2 format. The Kollman charges were calculated using AutoDockTools v1.5.6,
137 and the results were exported in PDBQT format. The docking was done using the AutoDock
138 Vina software [39] with default parameters. The results were visualized in PyMOL v1.7.4
139 [40] and Discovery Studio 4.5.

140

141 **2.4 Plant material**

142 *TgPR* gene expression was analyzed in adult plants obtained from clonal cuttings of cupuassu
143 genotypes C174 (resistant to witches' broom disease) and C1074 (susceptible to witches'
144 broom disease), grown in the experimental station of CEPLAC (Belém, Pará, Brazil) [41].
145 Cupuassu apical meristems were inoculated with *M. perniciosa* using the droplet method (30
146 μL of suspension containing $1 \times 10^5 \text{ ml}^{-1}$ basidiospores) [42]. After inoculation, the stem apex
147 region was wrapped in a plastic bag (a water-saturated environment) to improve basidiospore
148 germination and plant infection by the fungus [43]. The control plants were submitted to the
149 same procedure, but the basidiospore suspension was replaced by distilled water. The apical
150 meristems of the inoculated C174 and C1074 genotypes were harvested 8, 24, 48 and 72

151 hours after inoculation (hai), while non-inoculated ones (control) were harvested at 8 hai
152 Three plants (biological replicates) were used for each genotype at each harvesting time. The
153 harvested samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

154

155 **2.5 Reverse transcription quantitative PCR (qPCR) analysis**

156 Each biological replicate (individual plant) was macerated in liquid nitrogen, and 3 mg of the
157 macerate was used to extract its total RNA using the RNAqueous® kit according to the
158 manufacturer's recommendations (Ambion). Total RNA (15 μl) was treated with the DNase I
159 kit according to the manufacturer's recommendations (Invitrogen). The cDNA was
160 synthesized using the RevertAid First Strand cDNA Synthesis Kit, a cDNA synthesis kit,
161 according to the manufacturer's recommendations (ThermoScientific). The cDNA was
162 quantified on a GeneQuant Pro Spectrophotometer UV/Vis Reader (Amersham). For qPCR,
163 the *TgPR* primers were designed with the Primer 3 plus tool [44] using previously described
164 parameters [45], and checked for dimer and hairpin formation using the Oligoanalyzer
165 (<https://www.idtdna.com/calc/analyzer>) (Supplementary material 5). Three reference genes
166 (acyl carrier protein/ACP, malate dehydrogenase/MDH and tubulin/TUB; Supplementary
167 material 5) previously identified in cupuassu [27] were analyzed for stability in three pooled
168 samples of each cupuassu genotype: i) a non-inoculated pool sample (3 plants); ii) a pool of 8
169 and 24 hai samples (6 plants); and iii) a pool of 48 and 72 hai samples (6 plants). Quantitative
170 PCR was performed in an Eppendorf thermocycler Realplex4. The reaction was made in a
171 final volume of 10 μl containing 5 μl of Taq READYMIX SYBR Green, 0.1 μl of ROX
172 (fluorescence signal normalizer), 0.5 μl of primers (forward and reverse, at a concentration of
173 10 mM each) and 4.4 μl of cDNA 1/20 (230 ng). The cycling conditions were 95°C for 2 min
174 followed by 45 cycles of 95°C for 15 s, 55°C for 15 s and 60°C for 45 s, followed by a
175 dissociation curve step. The fluorescence data and primer efficiency were analyzed using

176 MINER 4.0 [46]. The gene-expression stability was calculated using NormFinder [47], which
177 determined the best combination of the reference genes ACP and MDH for relative expression
178 calculation (Supplementary material 6). The gene expression was analyzed in each biological
179 replicate (1 biological replicate = 1 plant) for each genotype (inoculated vs. non-inoculated) at
180 each harvesting time, using the qPCR cycling conditions described above and three technical
181 replicates. The relative expression was obtained using the REST-2009 software [48], which
182 considers the primer efficiency as well as the average ACP and MDH values for calculation.

183

184 **3. Results**

185 **3.1 Identification and characterization of *TgPR* sequences**

186 Analysis of the cupuassu RNA-seq allowed the selection of three sequences – codified as
187 C106, C68 and C356 – and showed homology with chitinases [28]. Sequence C106 showed
188 homology with chitinases from the GH19 family, which are involved in pathogenesis and are
189 related to the PR3 class (Table 1). For this reason, sequence C106 was re-named as *T.*
190 *grandiflorum* pathogenesis-related protein 3 (TgPR3). The *TgPR3* ORF was 966 bp in length
191 and encoded for a 322 aa protein with a molecular weight and an isoelectric point of 35.5 kDa
192 and 6.64, respectively (Table 1). TgPR3 contained a signal peptide (26 aa) responsible for
193 extracellular addressing (86.48% of probability). The protein contained 17 and three predicted
194 phosphorylation and glycosylation sites, respectively (Table 1). The C68 sequence showed
195 homology with the thaumatin like protein (TLP-P) family, which belongs to the pathogenesis-
196 related protein 5 (PR5) class and includes chitinases and osmotins. The C68 sequence was
197 renamed *T. grandiflorum* pathogenesis-related protein 5 (TgPR5) (Table 1). The TgPR5's
198 ORF was 672 bp in length, and it encoded a 224 aa protein with a molecular weight of 24.2
199 kDa and an isoelectric point of 6.68 (Table 1). The protein contained a 23 aa signal peptide
200 responsible for extracellular addressing (99.7% probability). TgPR5 contains one predicted

201 glycosylation and eight predicted phosphorylation sites (Table 1). The protein encoded by the
202 C356 sequence was classified as a chitinase from the GH18 family and as hevine enzyme,
203 which are chitinases belonging to the class of pathogenesis-related protein 8 (PR8). This
204 sequence was renamed *T. grandiflorum* pathogenesis-related protein 8 (TgPR8). The TgPR8
205 ORF was 927 bp in length, and it encoded for a 309 aa protein with a molecular weight of
206 33.3 kDa and a pI of 4.27 (Table 1). TgPR8 contains a signal peptide (29 aa), which
207 potentially addresses the protein to the extracellular (52.2% probability) or the vacuolar
208 (34.5% probability) compartments. The TgPR8 protein showed one and 13 glycosylation and
209 phosphorylation sites, respectively (Table 1).

210

211 **3.2 Phylogeny**

212 **BLAST made from *TgPR* sequences allowed the identification of 11 and 14 homologous**
213 **sequences of TgPR3, 30 and 28 of TgPR5 and 14 and one of TgPR8 in *T. cacao* and *A.***
214 ***thaliana*, respectively (Fig. 1). TgPR3 showed a high homology with *T. cacao* sequences**
215 **Tc04_t029180 and Tc06_t000490 and with *A. thaliana* sequences AT3G16920.1 and**
216 **AT1G05850.1 (Fig. 1A). TgPR5 was clustered with the sequences Tc00_t060970,**
217 **Tc03_t026960, Tc03_t026980, Tc03_t026990, Tc03_t027000, Tc03_t027010 and**
218 **Tc03_t027030 from *T. cacao* and with AT4G11650 (OSM34) from *A. thaliana* (Fig. 1B).**
219 **TgPR8 was clustered with the sequences Tc01_t032120, Tc03_t017760, Tc03_t017780,**
220 **Tc03_t017790, Tc10_t015260 and Tc10_t015330 from *T. cacao* as well as with AT5G24090**
221 **from *A. thaliana* (Fig. 1C).**

222

223 **3.3 Homology modeling and validation of TgPR's 3D structure**

224 The TgPR3 protein's 3D structure (Fig. 2A) was obtained through homology modeling with
225 the GH19 chitinase (PDB: 4TX7) from *Vigna unguiculata* (39% of identity and 91% of

226 coverage; Supplementary material 1). The TgPR3 protein 3D model showed that 87% of the
227 amino acid residues was in the most favored regions, 11.8% was in the additional allowed
228 regions, 0.6% was in the generously allowed regions and 0.6% was in the disallowed regions
229 (Supplementary material 7A). The TgPR3 model contained 12 helices, 26 β -turns, eight γ -
230 turns and three disulfide bonds (Fig. 2A, Supplementary material 8). The alignment of TgPR3
231 with other GH19 chitinases from other species (Supplementary material 2) allowed the
232 identification of TgPR3's catalytic site containing the amino acid residues Lys₇₀, Glu₉₂ and
233 Tyr₁₂₅ (Fig. 2A). The TgPR5 protein's 3D structure (Fig. 2B) was obtained by homology
234 modeling with an osmotin (PDB: 4L2J) from *Calotropis porcine* (75% of identity and 100%
235 of coverage; Supplementary material 1). The TgPR5 protein 3D model showed 88.5% of the
236 amino acid residues in the most favored regions, 11.1% in the generously allowed regions and
237 0.4% in the allowed regions, and it had no amino acid residues in disallowed regions
238 (Supplementary material 7B). The TgPR5 model contained 14 β -sheets, five helices, 25 β -
239 turns, two γ -turns, one bulge, five hairpins and seven disulfide bonds (Fig. 2B, Supplementary
240 material 8). The alignment of TgPR5 protein with thaumatococcal proteins from other species
241 (Supplementary material 3) allowed the identification of putative amino acid residues from
242 the active cleft: Arg₄₃, Glu₈₃, Asp₉₆ and Asp₁₀₁ (Fig. 2B). The TgPR8 protein's 3D structure
243 (Fig. 2C) was obtained by homology modeling with hevamine A (PDB: 1LLO) from *Hevea*
244 *brasiliensis* (64% of identity and 99% of coverage; Supplementary material 1). The TgPR8
245 protein's 3D structure showed 78.8% of the amino acid residues in the most favored regions,
246 18.9% in the additional allowed regions, 0.9% in the generously allowed regions and 1.4% in
247 disallowed regions (Supplementary material 7C). The TgPR8 model contained a barrel shape
248 with nine β -sheets, 15 helices, three disulfide bonds, 16 β -turns, one γ -turn, four bulges, one
249 hairpin, six α - β units and one PSI loop (Fig. 2C, Supplementary material 8). The alignment of
250 TgPR8 with GH18 chitinases from other species (Supplementary material 4) allowed the

251 identification of motifs and conserved domains, including the predicted active site containing
252 the amino acid residues Tyr₆, Phe₃₂, Asp₁₂₅, Glu₁₂₇, Gln₁₅₅, Gln₁₇₈, Tyr₁₈₀ and Trp₂₅₃ (Fig. 2C).

253

254 **3.4 Molecular docking between TgPRs and chitin or chitosan**

255 The molecular docking of TgPR3 with chitin had an affinity energy of -6.4 kcal/mol
256 (Supplementary material 9). It showed conventional hydrogen bond interactions with amino

257 acid residues Ser₉₂, Gly₉₄, Glu₁₁₂ and Tyr₂₂₅, and carbon hydrogen bonds with amino acid
258 residues Lys₉₀ and Pro₂₁₇ (Fig. 3). The molecular docking of TgPR3 with chitosan had an

259 affinity energy of -7.4 kcal/mol (Supplementary material 9). It showed conventional
260 hydrogen bond interactions with amino acid residues Lys₉₀, Ser₉₂, Asp₁₁₈, Tyr₁₁₉, Tyr₁₄₅,

261 Tyr₁₄₈, Arg₁₈₂, Leu₂₂₄, Tyr₂₂₅ and Asp₂₃₆; carbon hydrogen bonds with amino acid residues
262 Thr₉₁, Tyr₁₀₉ and Arg₁₁₁; and alkyl interactions with the amino acid residue Pro₁₄₃ (Fig. 3).

263 The molecular docking of TgPR5 with chitin had an affinity energy of -6.5 kcal/mol
264 (Supplementary material 9) and showed conventional hydrogen bonds with the amino acid

265 residues Thr₄₀, Glu₈₃, Gln₈₈, Asp₉₆ and Pro₁₇₉; one carbon hydrogen bond with the amino acid
266 residue Asp₁₈₂; and one pi-sigma interaction with the amino acid residue Tyr₁₇₆ (Fig. 3). The

267 molecular docking of TgPR5 with chitosan had an affinity energy of -6.8 kcal/mol
268 (Supplementary material 9) with conventional hydrogen bonds in the amino acid residues

269 Thr₄₀, Ala₄₂, Tyr₇₄, Gly₇₅, Glu₈₃, Tyr₈₄, Asp₉₆, Asp₁₀₁, Tyr₁₇₆ and Asp₁₈₂ and one carbon
270 hydrogen bond in the amino acid residue Cys₁₅₄ (Fig. 3). The molecular docking of TgPR8

271 with chitin had an affinity energy of -7.0 kcal/mol (Supplementary material 9) and showed
272 conventional hydrogen bonds with the amino acid residues Gln₉, Ala₈₁, Glu₁₂₇, Gln₁₅₅, Tyr₁₈₀

273 and Tyr₂₅₇ (Fig. 3). The docking of TgPR8 with chitosan had an affinity energy of -7.3
274 kcal/mol (Supplementary material 9) and showed conventional hydrogen bonds with the

275 amino acid residues Gln₉, Ser₄₃, Met₄₄, Asn₄₅, Leu₄₆, Gln₁₅₅, Asn₁₈₁, Asn₁₈₂, Ala₂₂₁, Trp₂₅₃ and
276 Tyr₂₅₇, and carbon hydrogen bonds with the amino acid residues Gln₁₇₈ and Tyr₁₈₀ (Fig. 3).

277

278 **3.5 Analysis of *TgPR* gene expression**

279 The primers PR3, PR5, PR8, ACP and MDH showed efficiency from 91% (MDH on the
280 C1074 genotype) to 102% (P3 on both the C174 and C1074 genotypes; Supplementary
281 material 10). For both genotypes and for all of the harvesting points, the PCR amplification
282 occurred at the same and unique melting temperature for each gene **and a unique band was**
283 **visible on electrophoresis agarose gel**, showing that only the corresponding gene was
284 amplified (Supplementary material 11). The relative expression of the *TgPR* genes was lower
285 in the susceptible genotype at all of the harvesting times, as compared to the resistant
286 genotype (Figs. 4A and B). The *TgPR3* gene showed two to three times higher expression in
287 the resistant genotype than in the susceptible one (Fig. 4B). Similarly, the *TgPR8* gene
288 showed two to four times higher expression in the resistant plants compared to the susceptible
289 ones (Fig. 4B). For both the *TgPR3* and *TgPR8* genes, the expression through the time course
290 disease slightly increased from 8 to 72 hai in the resistant plants (Fig. 4B). The *TgPR5* gene
291 showed a very high expression at 8 and 24 hai in the resistant genotype (about 25 of relative
292 expression; Fig. 4A), which decreased at 48 and 72 hai (about 2 of relative expression; Fig.
293 4B). Except for *TgPR5* 8 hai, the relative expression of the *TgPR* genes in the susceptible
294 genotype was almost constant (Fig. 4B).

295

296 **4. Discussion**

297 **4.1 TgPRs are structurally distinct and show a high energy affinity for chitosan**

298 The TgPR3 and TgPR5 proteins showed similar isoelectric points and share the same cellular
299 addressing (extracellular; Table 1), whereas the TgPR8 protein shows a more acidic

300 isoelectric point. Although the predictions for TgPR8 suggested extracellular and vacuolar
301 addressing (Table 1), type GH18 chitinases are found in the apoplast [49]. Although GH19
302 (PR-3) chitinases are composed of α -helices and loops, they are highly thermostable [50].
303 GH19 are divided into loopful and loopless classes, where loopful chitinases have loops at
304 both ends of the substrate-binding site, while loopless chitinases have a non-end loop
305 structure, which is commonly found in bacteria [51]. The TgPR3 protein showed homology
306 with the loopful GH19 class because of the presence of the loops near the substrate-binding
307 site (Fig. 2A). Most GH18 proteins (PR-8) correspond to chitinases, although some have been
308 reported to have xylanase-inhibiting activity or to have lost their chitinase activity [16]. As
309 chitinases, the GH18 proteins have the same activity as GH19 but are extremely distinct, both
310 structurally and evolutionarily [52]. One pattern that defines the chitinase activity in GH18 is
311 the presence of a DXDXE motif [53]; the TgPR8 showed the DFDIE motif at positions 123–
312 127 of the sequence, corroborating its classification and potential chitinase activity
313 (Supplementary material 8). Osmotin antifungal activity is associated with the presence of the
314 acidic cleft region [15, 54], which in TgPR5 protein is composed of the residues Glu₈₃, Asp₉₆
315 and Asp₁₀₁ (Figure 1B). *In vitro* studies have shown that barley seeds' GH19 chitinase can
316 efficiently catalyze tetrameric N-acetylglucosamine in dimers but that most GH19 chitinases
317 prefer larger substrates [55]. These data corroborate our docking results, where the TgPR3
318 protein had an affinity of -6.4 kcal/mol with the chitin molecule (the smaller one) and -7.4
319 kcal/mol with the chitosan molecule (the larger one), showing that TgPR3 also has a higher
320 affinity for larger substrates (Supplementary material 9, Fig. 3). TgPR8 protein has similar
321 affinity energy for chitin and chitosan ligands (-7.0 kcal/mol and -7.3 kcal/mol, respectively;
322 Supplementary material 9). The docking results reinforced the putative TgPR8 protein
323 chitinase activity, with an interaction within the DFDIE motif, in which it is essential for the
324 chitinase activity of this protein class [53]. Although structurally different and having

325 different catalytic mechanisms [52], the proteins TgPR3 and TgPR8 have a very close affinity
326 energy for the chitosan molecule. This may be related to their activity type, as both are
327 glycoside hydrolases [56]. The TgPR5 protein had an affinity energy of -6.5 kcal/mol with
328 the chitin molecule and -6.8 kcal/mol with the chitosan molecule (Supplementary material 9;
329 Fig. 3). These results show that TgPR5 can bind chitin molecules and may have potential
330 chitinase and antifungal activity.

331

332 **4.2 TgPRs are homologous to other plant PR proteins involved in biotic and abiotic** 333 **stress**

334 TgPR3 was clustered with Tc06_t000490 and Tc04_t029180 (Fig. 1), which showed a high
335 constitutive expression across treatments, Tc06_t000490 being up-regulated during cacao-
336 *Phytophthora palmivora* interaction [57]. The AT3G16920 from *A. thaliana* that also
337 clustered with TgPR3 (Fig. 1) is involved in response to mechanical stress [58] as well as to
338 heat, salt and drought stress [59]. AT1G05850 is related to cellulose biosynthesis [60] and
339 also to response to high temperatures, salt and drought stress [59]. Sequences Tc00_t060970,
340 Tc03_t026990, Tc03_t027000, Tc03_t027010 and Tc03_t027030, which were clustered with
341 TgPR5 (Fig. 1), showed increased expression levels during cacao interaction with the
342 pathogenic fungi *P. palmivora* and *Colletotrichum theobromicola* [57]. The *A. thaliana*
343 AT4G11650 sequence showed an increased expression with *Agrobacterium tumefaciens* at 24
344 hai [61]. This gene is also regulated by the ethylene and jasmonic acid–signalization pathway
345 [62]. Transgenic Arabidopsis plants knocked-out for the transcription factor WRKY33 –
346 which is mediated by ethylene and the jasmonic acid signalization pathway – showed a
347 susceptibility pattern to *Botrytis cinerea* and a reduction of AT4G11650 expression level,
348 while the wild-type plants showed increased AT4G11650 expression in response to the
349 pathogen [62]. The *A. thaliana* sequence AT5G24090, homologous to TgPR8 (Fig. 1),

350 showed an expression pattern specific to stress response; however, no expression was
351 observed during the plant development stage [63]. In *T. cacao*, the Tc03_g017760 gene
352 homologous with TgPR8 (Fig. 1) showed an increased expression level when the plant was
353 inoculated by *P. palmivora* or *Colletotrichum theobromicola* [57]. The PR gene's expression
354 in response to biotic and abiotic stress could be related to the phytohormone pathways that
355 regulate their expression. This could be related to the literature data about *M. perniciosa*,
356 which modifies the metabolism of *T. cacao* during compatible interactions through the
357 phytohormone pathway [64].

358

359 **4.3 TgPR genes were highly expressed in the cupuassu genotype resistant to *M.*** 360 ***perniciosa***

361 The relative expression of *TgPR3*, *TgPR5* and *TgPR8* was highly significant in the resistant
362 genotype infected by *M. perniciosa*, at all of the harvesting points, but mainly at 8 and 24 hai
363 for *TgPR5* (Fig. 3A). In the *T. cacao*–*M. perniciosa* interaction, which is closely related to
364 and also deeper studied than the cupuassu-*M. perniciosa* pathosystem, the basidiospore
365 germination and the beginning of the germination tube in the plant tissues were observed at 6
366 hai [65], a time period that is coherent with the high and early expression of the TgPRs (Figs.
367 3A and B). The very high expression observed for TgPR5 since 8 hai would corroborate other
368 reports, in which the PR5 was overexpressed in resistant plant genotypes after pathogen
369 infection [17, 66-68]. The osmotin's function and mode of action are still unclear, and
370 different functions have been shown, such as in inhibiting beta-glucanase or xylanase [17,
371 69]. Here, we propose that TgPR5 may act as chitinase due to its strong *in silico* interaction
372 with chitin and chitosan, as well as its extracellular localization (Fig. 2, Table 1). **Thus, we the**
373 **resistant cupuassu genotype may have been able to recognize *M. perniciosa* through its**
374 **innate immune system – probably through effectors [70] – and to generate a defense response**

375 against the pathogen from the beginning of the infection process (Fig. 5), which explains why
376 the C174 resistant genotype does not present symptoms or disease features in the field [71]. In
377 the susceptible cupuassu genotype, the TgPR genes showed reduced expression at all of the
378 harvesting times (Fig. 3) showing that the pathogen can bypass the innate immune system of
379 cupuassu and cause virulence. In *T. cacao*–*M. perniciosa* interaction, the fungus can interfere
380 with the host’s metabolism and alter its gene expression [64]. The reduction of plant chitinase
381 expression levels during the cupuassu–*M. perniciosa* interaction can be highly beneficial to
382 the pathogen, which can grow and colonize the plant tissues (Fig. 5). Moreover, the reduction
383 of cell wall degradation by chitinases (i.e. cell wall fragments) reduces the recognition by the
384 plant’s innate immune system through chitin receptors [72, 73] (Fig. 5). In contrast, increased
385 plant chitinase expression and secretion can cause serious damage to the pathogen, allowing
386 the plant to recognize the pathogen, and triggering defense mechanisms and the plant’s
387 resistance [73] (Fig. 5).

388

389 5. Conclusion

390 Here, we have shown the first *in silico* and *in vitro* analysis of pathogenesis-related proteins
391 from *T. grandiflorum*. We show that TgPR3 and TgPR8 are putative chitinases with distinct
392 structures, and that TgPR5 is a putative osmotin, all with affinity for chitin and chitosan
393 molecules. These results suggest a new role for TgPR5/osmotin as chitinase/chitosanase in
394 plant–pathogen interactions. TgPR expression highly increased in the resistant cupuassu
395 genotype infected by *M. perniciosa* (mainly TgPR5 at the first infection times) and was low in
396 the susceptible one. These results could be related to the phylogenetic analysis of the *TgPR*
397 genes showing that *T. grandiflorum* sequences clustered with cacao and Arabidopsis *PR*
398 sequences are involved in response to biotic and abiotic stress, some through phytohormone-

399 **signaling pathways.** These observations suggest that TgPRs could be involved in recognizing
400 mechanisms of a plant's innate immune system through chitin receptors.

401

402 **Availability of supporting data**

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

405

406 **Abbreviations:** 3D: tridimensional; PAMP: molecular patterns associated with pathogens;
407 PR: pathogenesis related; TLP: thaumatin-like protein

408

409 **Competing interest**

410 No conflicts of interest to declare.

411

412 **Authors' contribution**

413 RJSS was responsible for the execution of all the experimental steps. RJSS, LHM and FM
414 analyzed and discussed the data. RJSS and FM wrote the manuscript. KPG and RMA were
415 responsible for plant material production and inoculation with *M. pernicioso*. RMA, LHM,
416 FM and KPG were responsible for the conception and design of the experiments. FM was
417 responsible for the financial support of the research. FM and LHM were responsible for the
418 advising of RJSS.

419

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428

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640

641 **Tables**

642

643 **Table 1.** TgPR gene and protein sequence characteristics.

Name	Gene data			Protein data						
	Identification*	mRNA * (bp)	ORF size (bp)	Protein size (aa)	Signal peptide (aa)	Molecular weight (kDa)	pI	Subcellular localization (probability)	Phosphorylation sites	Glycosylation sites
TgPR3	C106	1239	966	322	26	35.5	6.64	Extracellular (86.48%)	S ₃₇ S ₄₁ S ₁₁₄ S ₁₂₃ S ₁₉₃ T ₉₁ T ₉₉ T ₁₈₅ T ₂₀₅ T ₂₁₉ T ₂₄₀ Y ₆₂ Y ₁₁₉ Y ₁₂₄ Y ₁₂₆ Y ₁₄₅ Y ₁₆₇	N ₂₀ N ₁₇₁ N ₂₀₇
TgPR5	C68	950	672	224	23	24.2	6.68	Extracellular (99.7%)	S ₁₁₈ S ₁₃₉ S ₁₆₁ S ₁₇₆ T ₁₄₃ T ₂₅₆ Y ₁₃₇ Y ₂₃₉	N ₁₆₃
TgPR8	C356	1111	927	309	29	33.3	4.27	Extracellular (52.2%) Vacuolar (34.5%)	S ₇₇ S ₉₅ S ₁₄₇ S ₂₆₆ T ₁₅ T ₄₁ T ₈₈ Y ₆₆ Y ₉₉ Y ₁₃₄ Y ₁₄₉ Y ₁₆₂ Y ₂₂₇	N ₁₄₅

644 * Data from RNA-seq as described by [28].

645 **Figure legends**

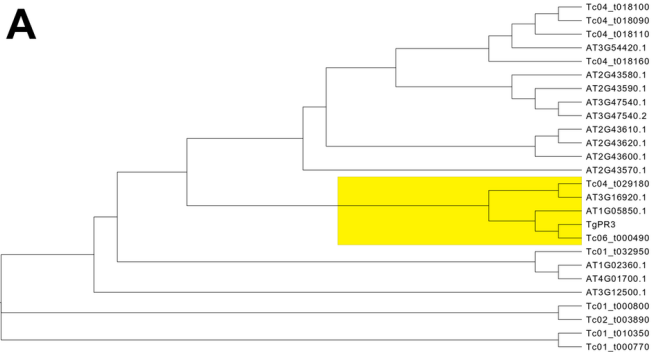
646 **Figure 1.** Phylogenetic tree obtained with the *TgPR* nucleotide sequences and their
647 homologues from *T. cacao* and *A. thaliana*. **A.** Phylogenetic tree from *TgPR3*. **B.**
648 Phylogenetic tree from *TgPR5*. **C.** Phylogenetic tree from *TgPR8*. Sequence names
649 beginning with Tc and AT correspond to *T. cacao* and *A. thaliana*, respectively. The
650 yellow square highlights the *TgPR*'s closest phylogenetic branches.

651 **Figure 2.** The 3D structure of *TgPR* proteins. **A.** *TgPR3*'s 3D structure, with the amino
652 acid residues Lys70, Glu92, and Tyr125 of the active site highlighted. **B.** *TgPR5*'s 3D
653 structure with the amino acid residues Arg43, Glu83, Asp96 and Asp101 of the active
654 site highlighted. **C.** *TgPR8*'s 3D structure with the amino acid residues Tyr6, Phe32,
655 Asp125, Glu127, Gln155, Gln178, Tyr180 and Trp253 of the active site highlighted.

656 **Figure 3.** Representations (2D and 3D) of docking between *TgPRs* and chitin/chitosan.

657 **Figure 4.** Relative expression of *TgPR* genes in resistant (R) and susceptible (S)
658 cupuassu genotypes 8, 24, 48 and 72 hours after inoculation (hai) with *Moniliophthora*
659 *perniciosa*. **A.** Full-scale representation. **B.** Reduced scale for the best visualization of
660 lower relative expression.

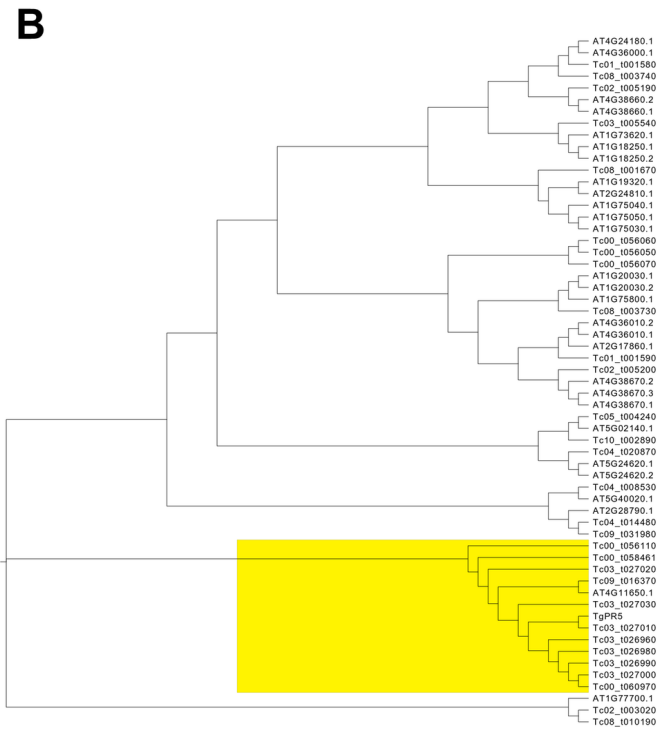
661 **Figure 5.** Putative scheme of the early steps (6–72 hai) of the interactions between *T.*
662 *grandiflorum* plants and the hemibiotrophic fungus *M. perniciosa*. **A.** Resistant
663 interaction. **B.** Susceptible interaction. PTI: PAMP-triggered immunity; ETI: effector-
664 triggered immunity; PRR: pattern-recognition receptors; PR: pathogenesis-related
665 protein; PCD: programmed cell death.



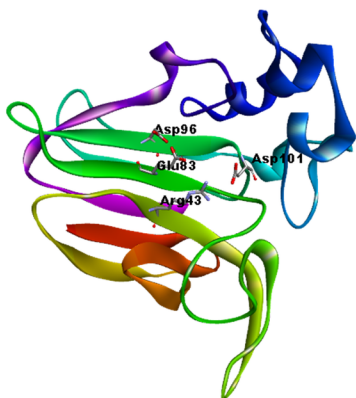
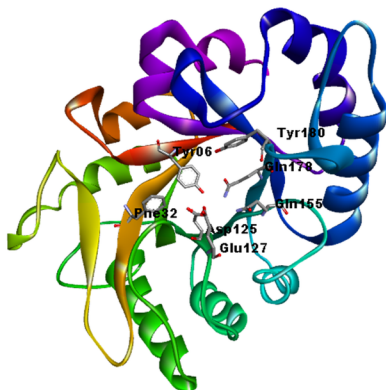
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0.7



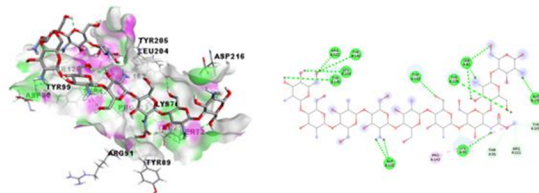
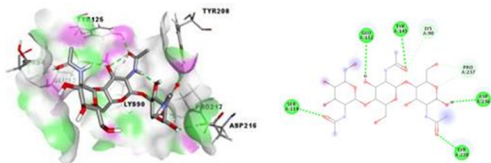
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A**TgPR3****B****TgPR5****C****TgPR8**

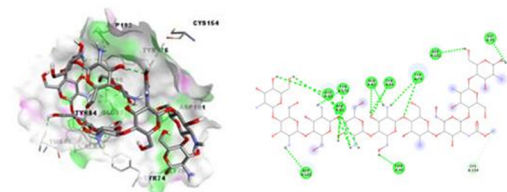
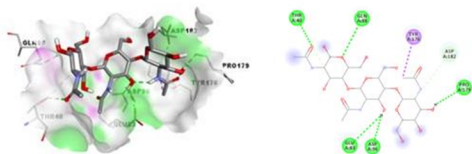
Chitin

Chitosan

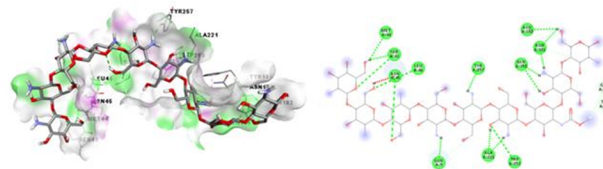
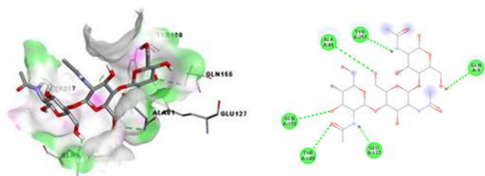
TgPR3



TgPR5



TgPR8



H-Bonds

Donor

Acceptor

Interactions

Conventional Hydrogen Bond

Carbon Hydrogen Bond

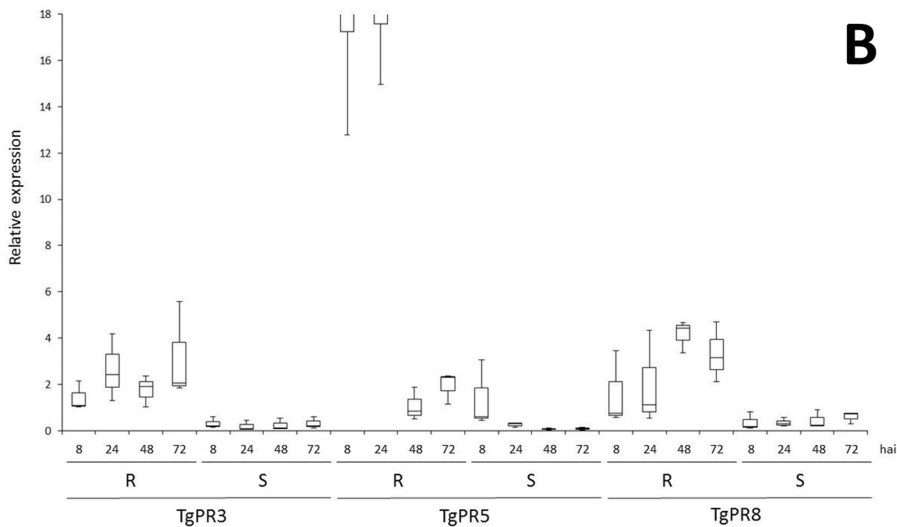
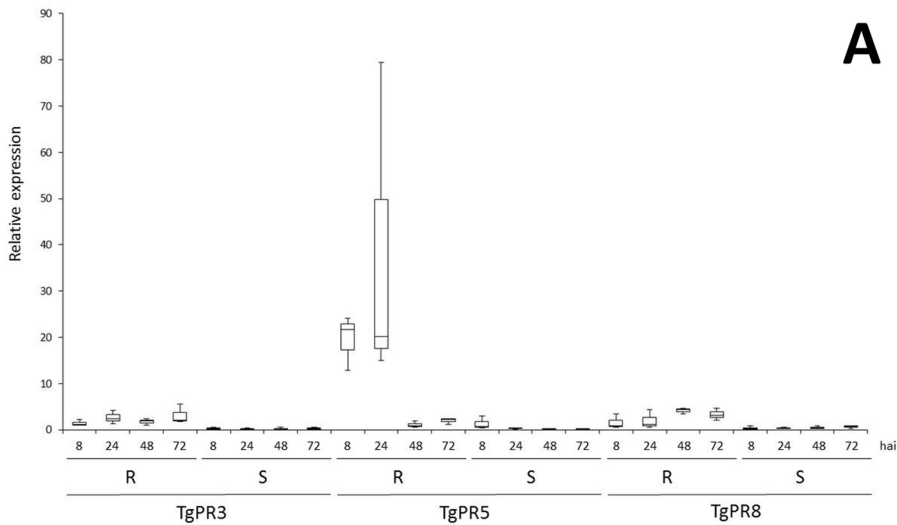
Pi-Alkyl

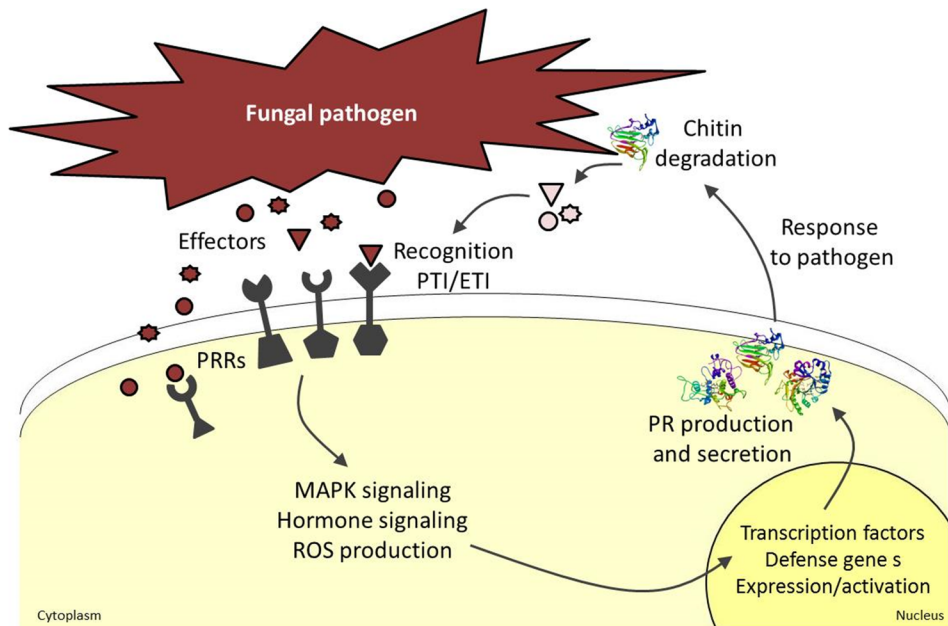
Unfavorable Donor-Donor

Pi-Sigma

Donor

Acceptor



A**B**