

1 **Revealing the secrets beneath grapevine and *Plasmopara viticola* early**
2 **communication: a picture of host and pathogen proteomes**

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4 Running title: Grapevine and *P. viticola* proteomes communication

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6 Joana Figueiredo^{1,*}, Rita B. Santos¹, Leonor Guerra-Guimarães^{2,3}, Céline C. Leclercq⁴, Jenny
7 Renaut⁴, Lisete Sousa⁵, Andreia Figueiredo¹

8 1 Grapevine Pathogen Systems Lab, BioISI – Biosystems & Integrative Sciences Institute, Plant
9 Biology Department, Faculty of Sciences, University of Lisbon, 1749-016 Lisboa, Portugal

10 2 Centro de Investigação das Ferrugens do Cafeeiro, Instituto Superior de Agronomia,
11 Universidade de Lisboa, 2784-505 Oeiras, Portugal

12 3 Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia,
13 Universidade de Lisboa, 1349-017 Lisboa, Portugal

14 4 Luxembourg Institute of Science and Technology, L-4362 Esch-sur-Alzette, Luxembourg

15 5 Departamento de Estatística e Investigação Operacional, Centro de Estatística e Aplicações,
16 Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal

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18 * Correspondence: jffigueiredo@fc.ul.pt

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21 **Abstract:**

22 Plant apoplast is the first hub of plant-pathogen communication where pathogen effectors are
23 recognized by plant defensive proteins and cell receptors and several signal transduction
24 pathways are activated. As a result of this first contact, the host triggers a defence response that
25 involves the modulation of several extra and intracellular proteins. In grapevine-pathogen
26 interactions, little is known about the communication between cells and apoplast. Also, the role
27 of apoplastic proteins in response to pathogens still remains a blackbox. In this study we focused
28 on the first 6 hours after *Plasmopara viticola* inoculation to evaluate grapevine proteome
29 modulation in the apoplastic fluid (APF) and whole leaf tissue. *Plasmopara viticola* proteome
30 was also assessed enabling a deeper understanding of plant and pathogen communication. Our
31 results showed that oomycete recognition, plant cell wall modifications, ROS signalling and
32 disruption of oomycete structures are triggered in Regent after *P. viticola* inoculation. Our
33 results highlight a strict relation between the apoplastic pathways modulated and the proteins
34 identified in the whole leaf proteome. On the other hand, *P. viticola* proteins related to
35 growth/morphogenesis and virulence mechanisms were the most predominant. This pioneer
36 study highlights the early dynamics of extra and intracellular communication in grapevine
37 defence activation that leads to the successful establishment of an incompatible interaction.

38

39 **Keywords (3-10):** *Vitis vinifera*, apoplastic fluid, cell compartments, secretomes, oomycete,
40 proteomics

41

42 **1. Introduction**

43 As sessile organisms, plants have developed mechanisms to rapidly adapt to environmental
44 changes and pathogen attack. To overcome pathogen challenges, plants must quickly recognize
45 the invaders and mount a successful defence strategy. This chess game between the plant and
46 pathogen is illustrated by the “zig zag model”, coined by Jones and Dangl in 2006¹. In this model,
47 the plants first recognize the pathogen-associated molecular patterns (PAMPs) through pattern
48 recognition receptors (PRR) in the apoplast, leading to a PAMP-triggered immunity (PTI). The
49 apoplast is extremely relevant for plant defence since is where plant and pathogen first meet
50 and where recognition begins. Pathogen recognition culminates in the activation of plant
51 defence responses including the induction of defence genes, production of reactive oxygen
52 species (ROS) and deposition of callose. In a second phase, effector-triggered susceptibility or
53 ETS, the pathogen overcomes the plant first response by deploying effectors that increase
54 pathogen virulence, like Crinklers and RxLR effectors¹. In an incompatible interaction, the plant
55 recognizes the pathogen effectors through R-proteins. The interaction between plant R-proteins
56 and pathogen effectors results in an effector-triggered immunity (ETI), that ultimately results in
57 a hypersensitive cell death response (HR) at the pathogen entry site¹. This interaction implies a
58 tight communication between host and pathogen with the traffic of plant proteins and pathogen
59 effector proteins between the apoplast and the intercellular space. While still misgraded, the
60 study of plant apoplast is of extreme importance in plant-pathogen interactions so to identify
61 proteins with a key role in plant defence strategies and better understand their interaction with
62 pathogen molecules. Apoplast proteome was characterized for few plant models, constitutively

63 or under abiotic/biotic stress, as for example, grapevine^{2,3}, poplar⁴, tobacco⁵, cowpea⁶, rice⁷,
64 coffee^{8,9} and Arabidopsis¹⁰. However, few studies focus on uncovering apoplast proteome
65 modulation considering plant-pathogen interactions and even less when considering woody
66 crop plants, such as grapevine and obligatory biotrophic oomycetes, as the downy mildew
67 etiological agent, *Plasmopara viticola*.

68 Grapevine (*Vitis vinifera* L.), is one of the major crops grown in temperate climates, however is
69 highly susceptible to downy mildew, caused by *P. viticola* ((Berk. and Curt.) Berl. & de Toni)¹¹.
70 In Europe, *P. viticola* infection leads to heavy crop losses and disease management for downy
71 mildew relies on the massive use of pesticides in susceptible varieties in each growing season.
72 This practice is against the demands of the European Union guidelines for pesticide reduction
73 and sustainable viticulture (Directive 2009/128/EC), so the search for more plant- and
74 environment-friendly solutions is imperative.

75 In a modern viticulture context, the development of grapevine crossing lines, in breeding
76 programs, is a very well established and accepted strategy to fight against the excessive use of
77 pesticides. These crossing lines are the result of the introgression of pathogen resistant genes,
78 present in Asian and American *Vitis* species, with genes related to the good quality of grapes for
79 wine production, present in susceptible grapevine cultivars. The result is a cultivar that present
80 desired characteristics for wine producers at the same time that resists more to pathogen attack.
81 'Regent' is a successful example of breeding for resistance and harbours RPV3.1 resistance to *P.*
82 *viticola* loci¹². Several studies have been performed in 'Regent'-*P. viticola* interaction with the
83 aim to better understand the molecular mechanisms and the key molecules that are responsible
84 to the well-known tolerance that this crossing line has against *P. viticola*¹³⁻¹⁷.

85 In a climate change scenario, viticulture will face new emerging diseases as well as several
86 outbreaks of the established diseases, such as downy mildew. Thus, a comprehensive
87 knowledge on the grapevine strategies to overcome pathogens, mainly in cultivars with some
88 resistance level, as well as the evolution of pathogen infection mechanisms is paramount to
89 tackle this challenge. Thus, in the present study, we have focused on the early communication
90 between grapevine and *P. viticola* and assessed, for the first time, grapevine apoplast proteome
91 modulation and *P. viticola* proteome and secretomes. We have focused on the first 6 hours post
92 inoculation (hpi), as the events occurring in this time-point were previously shown to be crucial
93 for the outcome of the interaction. We have also highlighted extra and intra-cellular
94 communication pathways by comparing the proteome modulation in the apoplast and in the
95 whole leaf. Up to our knowledge this is the first time where grapevine apoplast and whole leaf
96 proteome communication is revealed during host-pathogen interaction and also the first *P.*
97 *viticola* proteome sequencing. Our results elucidate the interaction between grapevine and *P.*
98 *viticola* proteins taking place in the apoplast and how the plant and pathogen proteomes evolve
99 at the first stages of infection.

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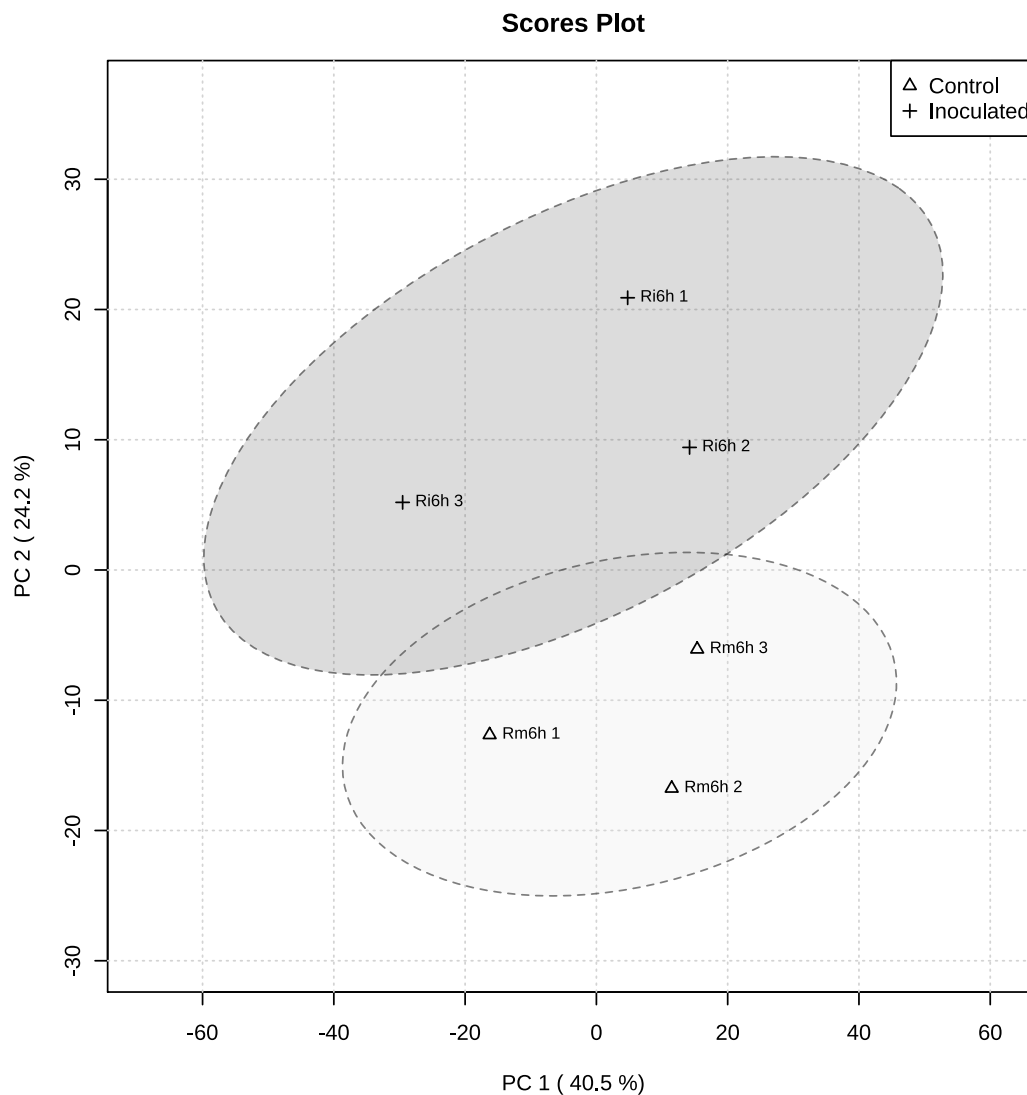
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102 2. Results

103 2.1 Early 'Regent' APF proteome modulation under *P. viticola* infection

104 The impact of *P. viticola* infection in the modulation of APF proteome in 'Regent' leaves was
105 analysed at 6 hours post inoculation (hpi). By a principal components analysis (PCA), a clear
106 distinction between the proteome of inoculated and mock-inoculated (control) samples was
107 obtained (Fig.1). The distribution of the biological replicates within the PCA scores plot indicates
108 the absence of unwanted variation in the dataset, increasing the confidence in the
109 reproducibility of the differential accumulation analysis.

110



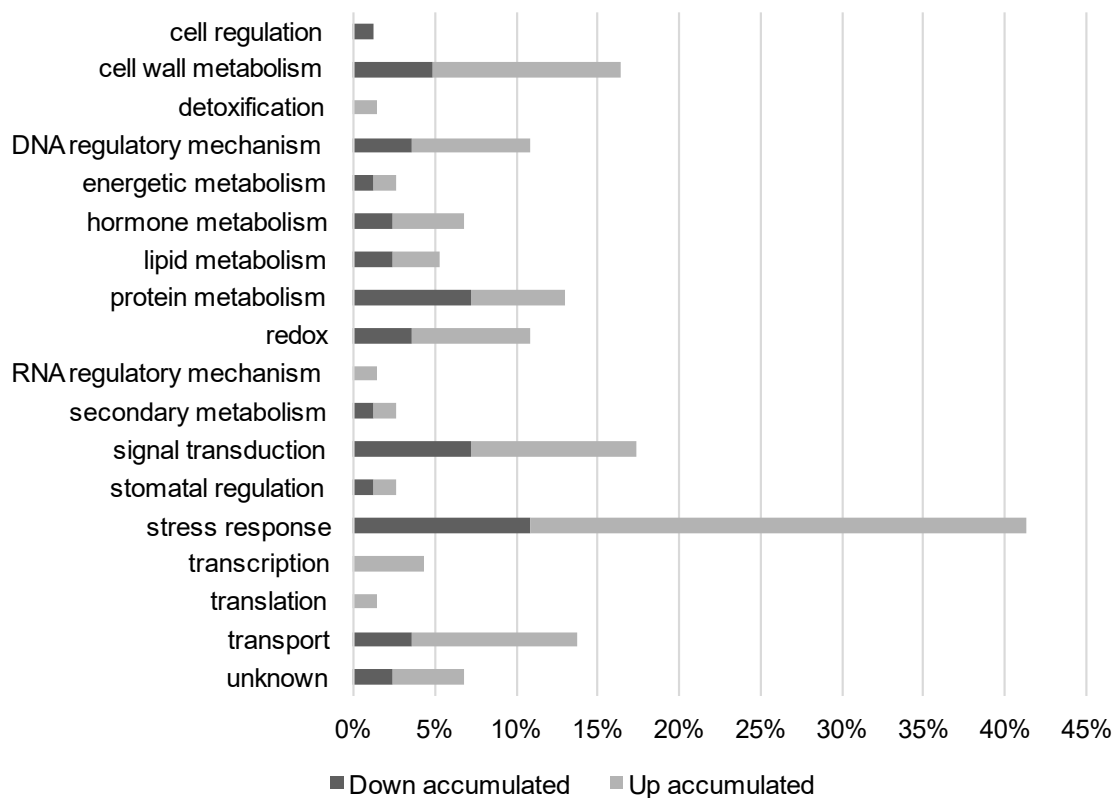
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112 **Fig.1.** Principal component analysis of the differential protein profiles in *V. vinifera* cv. 'Regent' at 6 hours
113 post-inoculation with *P. viticola*. The plot shows principal component 1 (PC1) on X axis and principal
114 component 2 (PC2) on Y axis, together they explain 64.7% of protein abundance variability. Ri: 'Regent'
115 inoculated samples; Rm: 'Regent' mock-inoculated samples.

116

117 When comparing APF inoculated samples with mock-inoculated samples, a hundred and
118 eighteen proteins were differentially accumulated (DAPs; 74 up accumulated and 44 down
119 accumulated). These proteins are mainly related to stress response, signal transduction, cell wall
120 metabolism, transport and protein metabolism (Fig.2). At 6hpi, *P. viticola* infection leads to an
121 increase in the presence of plant stress response proteins, like glucan endo-1,3- β -glucosidases,
122 disease resistance proteins RPV1-like and RUN1-like [associated to Resistance to *P. viticola* (RPV)
123 loci and Resistance to *Uncinula necator* (RUN) loci], receptor-like protein kinase FERONIA and
124 GDSL esterases/lipases. An up accumulation of LRR kinases related to signal transduction occurs.
125 ROS-related proteins, like peroxidase 4-like and xanthine dehydrogenase 1-like isoform X1, were
126 also detected in 'Regent' APF after oomycete challenged.

127



128

129 **Fig.2.** Biological process annotation of the differentially accumulated 'Regent' APF proteins, 6 hours post
130 inoculation with *P. viticola*. Dark grey bars: percentage of down accumulated proteins; Light grey bars:
131 percentage of up accumulated proteins.

132

133 Several proteins involved in 'Regent' defence mechanism were modulated at 6hpi (Table 1).
134 Indeed, apoplastic proteins related to oomycete perception, that may lead to the activation of
135 several defence signalling pathways, were found to be accumulated (Table 1). Apoplastic
136 proteins associated with the remodelling of plant cell wall were also identified as well as proteins
137 associated with auxin signalling and its regulation in response to *P. viticola* infection (Table 1).
138 Moreover, several proteins associated to ROS production and signalling were modulated at this
139 early time-point of infection (Table 1). Lastly, plant proteins involved in the disruption of
140 oomycete structures were also identified (Table 1).

141

142 **Table 1.** ‘Regent’ APF proteins up accumulated at 6hpi with *P. viticola*, involved in key cellular pathways.

NCBI accession	NCBI description	Biological process	log ₂ (FC)
Modulation of plant physical barriers			
XP_002281842.1	ABC transporter G family member 32 (ABCG32)	stress response	19,00
XP_002263127.1	fatty acyl-CoA reductase 3-like (FAR3)	lipid metabolism	18,83
NP_001268091.1	pectinesterase/pectinesterase inhibitor PPE8B-like (PPE8B)	cell wall modification	15,93
XP_002277293.4	pectinesterase	cell wall metabolism	-1,80
Modulation of plant plasma membrane proteins in response to infection			
XP_019071787.1	disease resistance protein RPV1-like isoform X1 (RPV1)	stress response	19,12
XP_019073586.1	disease resistance protein RUN1-like isoform X1 (RUN1)	stress response	17,99
XP_010644327.1	disease resistance protein At4g27190-like	stress response	4,69
XP_002280315.3	probable disease resistance protein At1g61300	stress response	18,99
XP_019077695.1	probable disease resistance protein At1g61300	stress response	4,47
XP_010654733.1	probable disease resistance protein At5g63020	stress response	21,94
XP_010645387.1	TMV resistance protein N	stress response	21,70
XP_019078946.1	TMV resistance protein N	stress response	17,22
XP_019075299.1	leucine-rich repeat receptor protein kinase MSP1-like	signal transduction	16,43
XP_002267269.1	probable leucine-rich repeat receptor-like protein kinase At1g35710	signal transduction	16,39
XP_002282474.2	serine-threonine protein kinase, plant-type, putative	signal transduction	18,49
XP_010660578.1	receptor-like protein kinase FERONIA (FERONIA)	stress response	18,38
XP_010664467.1	ATPase 9, plasma membrane-type	transport	19,39
XP_002279498.1	putative calcium-transporting ATPase 13, plasma membrane-type (Ca ²⁺ -ATPase)	transport	15,64
XP_002283826.1	protein unc-13 homolog	stomatal regulation	18,55
XP_010647591.2	ankyrin repeat-containing protein ITN1-like isoform X6 (ITN1)	stress response	16,63
Activation of auxin signalling			
XP_002274153.1	protein kinase PINOID	hormone signalling	22,45
XP_002277611.1	protein NDL2	hormone signalling	16,72
XP_002265864.1	ubiquitin-NEDD8-like protein RUB2	protein metabolism	16,62
Regulation of ROS during plant defence response			
XP_002269918.1	peroxidase 4-like	redox	21,05
XP_002274392.1	purple acid phosphatase (PAP)	energetic metabolism	20,55
XP_002285473.1	xanthine dehydrogenase 1-like isoform X1 (XDH1)	redox	16,51
Disruption of oomycete structures			
XP_010664681.1	glucan endo-1,3-beta-D-glucosidase	stress response	19,85
XP_002283647.1	glucan endo-1,3-beta-glucosidase	stress response	15,09
XP_002268991.2	GDSL esterase/lipase (GELP)	stress response	15,72
XP_002276525.1	GDSL esterase/lipase At4g01130 (GELP)	stress response	6,00

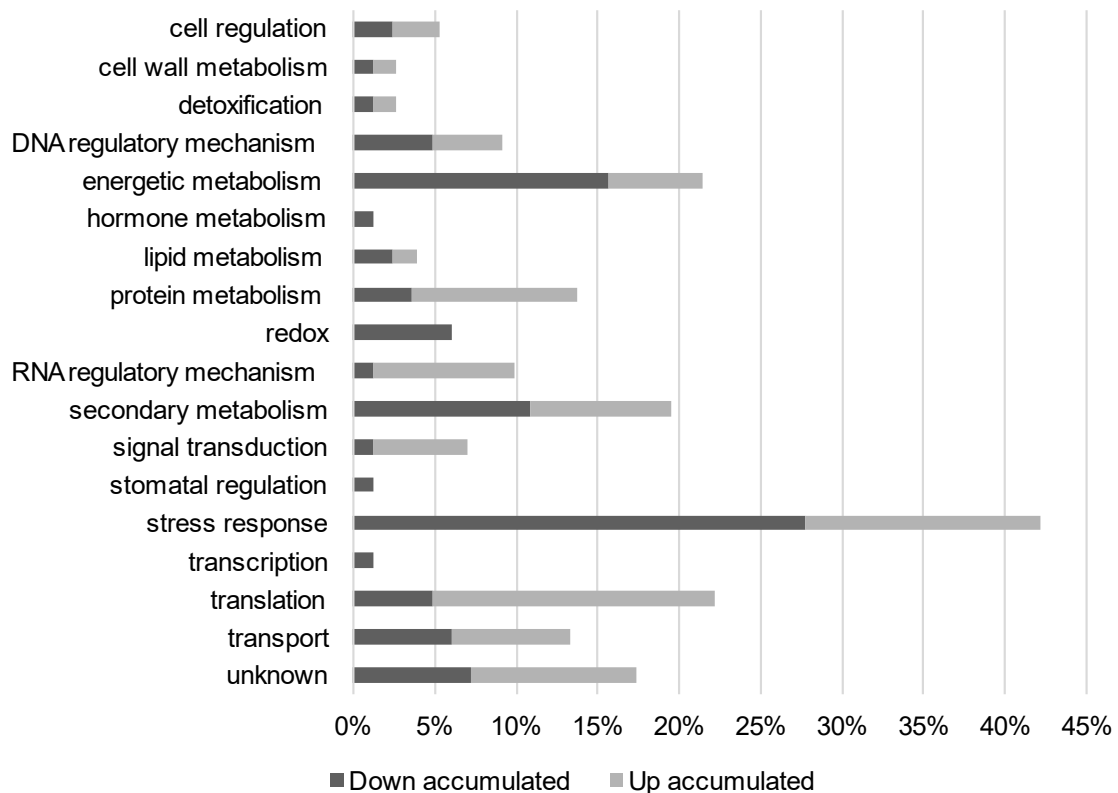
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145 2.2 'Regent' whole leaf proteome modulation at 6h post *P. viticola* infection

146 Whole leaf proteome of 'Regent' during *P. viticola* infection time-course was obtained in 2020
147 ¹³. Raw data deposited on the Pride database of the 6hpi was re-analysed following the same
148 pipeline that was implemented for the APF proteome analysis. Hundred and fifty-two DAPs were
149 identified. Of those, 69 proteins were up accumulated and 83 were down accumulated. These
150 proteins were mainly related to stress response, energy and secondary metabolisms and
151 translation (Fig.3). At 6hpi, *P. viticola* infection induces a modulation in the abundance of several
152 stress-related proteins, like heat shock proteins, cysteine proteinases and glucanases. In
153 addition, a great number of photosynthesis-related proteins are down accumulated in 'Regent'
154 leaves in response to infection. Translation and signal transduction-related proteins, like
155 ribosomal proteins and serine/threonine protein kinase (RSTK), respectively, were up
156 accumulated after *P. viticola* infection.

157



158

159 **Fig.3.** Biological process annotation of the differentially accumulated 'Regent' whole leaf proteins, 6 hours
160 post inoculation with *P. viticola*. Dark grey bars: percentage of down accumulated proteins; Light grey
161 bars: percentage of up accumulated proteins.

162

163 The functional annotation of several of the proteins identified in whole leaf are closely related
164 with the pathways that were found to be modulated in APF proteins (Table 2). Indeed, we have
165 identified whole leaf proteins involved in the modulation of plant physical barriers and activation
166 of plant defence signalling through plasma membrane receptors, regulation of ROS levels, and

167 disruption of oomycete structures (Table 2). Also, proteins associated with calcium signalling,
 168 and intracellular trafficking vesicles were modulated in the whole leaf context (Table 2).

169

170 **Table 2.** Regent' whole proteins up accumulated at 6hpi with *P. viticola*, involved in key cellular pathways.

NCBI accession	NCBI description	Biological process	log ₂ (FC)
Modulation of plant physical barriers and plasma membrane receptors in response to infection			
CAN83165.1	pectinesterase inhibitor 9 (PMEI9)	cell wall metabolism	2,39
CBI32865.3	alpha-L-arabinofuranosidase 1 (ASD1)	cell wall metabolism	-18,97
XP_002267434.3	serine/threonine protein kinase (RSTK)	signal transduction	6,90
XP_002265462.3	serine/threonine-protein kinase pakA (RSTK)	signal transduction	17,31
Regulation of ROS during plant defence response			
CBI31928.3	peroxisome biogenesis protein 19-2-like (PEX19-2)	protein metabolism	19,28
CBI32544.3	protein TIC 62, chloroplastic isoform X1	transport	18,74
CAN66554.1	succinate dehydrogenase assembly factor 4, mitochondrial (SDHAF4)	energetic metabolism	17,63
XP_002283860.1	15.7 kDa heat shock protein, peroxisomal	stress response	20,89
CAN67665.1	17.3 kDa class II heat shock protein-like	stress response	6,61
XP_003634522.1	peroxidase 12	redox	-21,36
XP_002285652.2	peroxidase A2-like	redox	-8,08
XP_010647098.1	polyphenol oxidase, chloroplastic-like	redox	-20,80
CBM39273.1	18.2 kDa class I heat shock protein	stress response	-20,66
CBI30632.3	28 kDa heat- and acid-stable phosphoprotein	stress response	-19,75
CBM39216.1	class I heat shock protein	stress response	-21,56
CBI23075.3	small heat shock protein, chloroplastic	stress response	-21,87
Disruption of oomycete structures			
CBI32343.3	endo-1,3;1,4-beta-D-glucanase-like	stress response	-21,33
CBI26171.3	endo-1,3;1,4-beta-D-glucanase-like isoform X3	stress response	-19,29
CAN71820.1	glucan endo-1,3-beta-glucosidase	stress response	-20,49
CBI36040.3	profilin 1	cell regulation	20,96
Modulation of calcium signalling			
CBI15387.3	calcium sensing receptor, chloroplastic	stomatal regulation	-19,74
CBI24493.3	CDGSH iron-sulfur domain-containing protein NEET	secondary metabolism	-7,88
Increase of protein trafficking			
CBI28256.3	putative clathrin assembly protein At5g35200	stress response	21,36

171

172 **2.3 *P. viticola* proteome during infection establishment**

173 We have sequenced for the first time the *P. viticola* proteome, obtained from grapevine leaves
 174 apoplast at 6hpi. Sixty proteins were identified being mainly involved in two biological
 175 processes: growth/morphogenesis (e.g. cell division cycle 5 and β -glucan synthesis-associated
 176 SKN1) and virulence (RxLR proteins and serine protease trypsin's). Proteins involved in signalling
 177 processes like agc kinase (ACG), serine threonine kinase and small GTP-binding Rab28 were also
 178 identified (Table 3).

179

180

181 **Table 3.** *P. viticola* proteins, identified in ‘Regent’ apoplast after 6hpi, involved in virulence and
182 growth/morphogenesis mechanisms.

Protein name	Protein code (INRA Database)
Proteins involved in virulence mechanisms	
Coproporphyrinogen III oxidase (CPOX)	PVIT_0003600.T1
RxLR-like protein (RxLR)	PVIT_0014146.T1
RxLR-like protein (RxLR)	PVIT_0014142.T1
Serine protease trypsin	PVIT_0011817.T1
Serine protease trypsin	PVIT_0011837.T1
Serine threonine kinase	PVIT_0018302.T1
Tetratricopeptide repeat 26	PVIT_0013696.T1
TKL kinase (TKL)	PVIT_0016228.T1
Proteins involved in growth/morphogenesis mechanisms	
Beta-glucan synthesis-associated SKN1 (SKN1)	PVIT_0022780.T1
CAMKK kinase (CaMK)	PVIT_0013015.T1
Cell division cycle 5 (CDC5)	PVIT_0005546.T1
CMGC CDK kinase (CDK)	PVIT_0011642.T1
CMGC MAPK kinase (MAPK)	PVIT_0009065.T1
FAD synthase-like	PVIT_0001050.T1
Serine threonine kinase	PVIT_0018302.T1
Proteins involved in both mechanisms	
AGC kinase (ACG)	PVIT_0009891.T1
Calpain-like protease	PVIT_0019872.T1
Small GTP-binding Rab28	PVIT_0005551.T1

183

184 **3. Discussion**

185 **3.1 *P. viticola* leads to a broad modulation of ‘Regent’ APF and whole leaf proteomes**

186 During grapevine-*P. viticola* interaction, the apoplast compartment is the first hub where plant
187 and pathogen secretomes meet. Several proteins are crucial for the outcome of the interaction,
188 both from the host or pathogen sides. In the apoplast, processes involving pathogen recognition
189 through membrane receptors that activate signal transduction pathways for expression of host
190 defence-associated genes or proteins that directly communicate with pathogen molecules
191 inhibiting infection progress are essential. Considering the whole leaf tissue, trafficking of
192 several proteins to respond to the plant defence requirements must be activated as well as
193 processes that lead to a broad activation of defence-related mechanisms. Thus, communication
194 between the apoplast and the host intracellular organelles is essential for a concerted and quick
195 defence response against the pathogen. Moreover, during the interaction, *P. viticola* develops
196 its infection structures, namely hyphae culminating in plant cell invasion and development of
197 haustorium for feeding. In the first hours of interaction, host and pathogen communications are
198 expected to be very dynamic and to define the outcome of the interaction.

199

200 **3.1.1 The dual battle at the gate: host strengthens its physical barriers while the pathogen**
201 **triggers plant cell wall degradation**

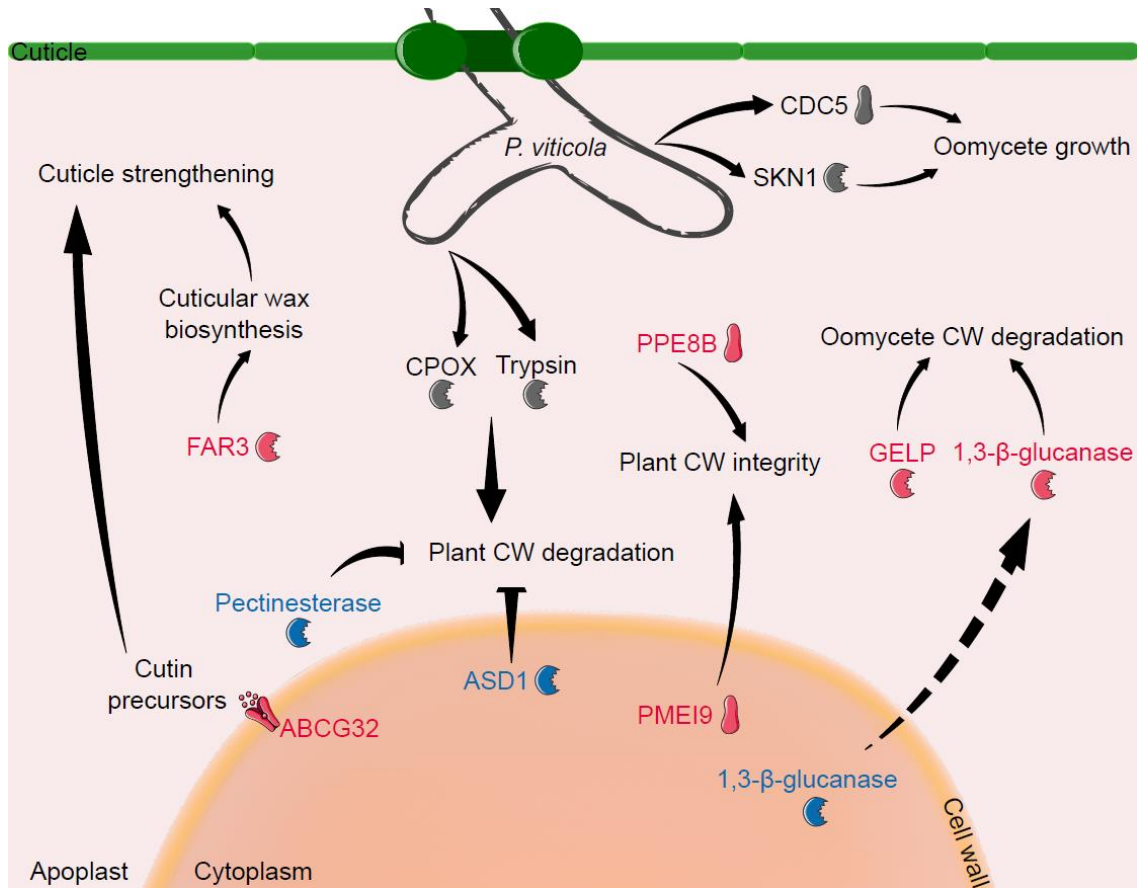
202 The cuticle is a barrier coating the outer surface of epidermal cells of organs of the aerial parts
203 of the plants. It protects against water loss and various abiotic and biotic stresses¹⁸. In ‘Regent’
204 APF, two cuticle-related proteins were found to be up accumulated after *P. viticola* infection,
205 the ABC transporter G family member 32 (ABCG32) and the fatty acyl-CoA reductase 3-like
206 protein (FAR3), (Table 1; Fig.4). These ABC transporters have been frequently shown to be
207 involved in pathogen response, surface lipid deposition and transport of the phytohormones
208 auxin and abscisic acid^{19,20}. In Arabidopsis, the ABCG32 was reported to be involved in cuticle
209 formation, most likely by exporting cutin precursors from the epidermal cell²¹. The fatty acyl-
210 CoA reductase 3-like protein is involved in cuticular wax biosynthesis²². In incompatible
211 grapevine-*P. viticola* interaction, such as the one that occurs in ‘Regent’, a higher abundance of
212 these proteins leads to the hypothesis that the host activates processes to promote the
213 strengthening the cuticular barrier in order restrain pathogen penetration (Fig.4).

214 Also, plants have developed a system for sensing pathogens and monitoring the cell wall
215 integrity, upon which they activate defence responses that lead to a dynamic cell wall
216 remodelling required to prevent pathogen progression. Plant cell wall-associated proteins were
217 found to be differentially modulated in ‘Regent’ APF in response to *P. viticola*. A down
218 accumulation of a pectinesterase, a protein involved in plant cell wall degradation as well as an
219 up accumulation of pectinesterase/pectinesterase inhibitor PPE8B-like protein (PPE8B), (Table
220 1; Fig.4). This protein is involved in plant cell wall reconstruction and, in cotton, genes encoding
221 this type of protein are specifically up regulated in plant resistant variety upon *Aspergillus*
222 *tubingensis* infection²³.

223 In the whole leaf proteome of ‘Regent’, the abundance of pectinesterase inhibitor 9 (PMEI9)
224 increased, a protein involved in resistance to pathogens²⁴, and a decrease in the accumulation
225 of alpha-L-arabinofuranosidase 1, a protein involved in cell wall degradation²⁵, was detected
226 (Table 2; Fig.4). The whole leaf and apoplast proteomes are modulated as a defence strategy to
227 prevent cell wall degradation, maintaining its integrity and thus inhibiting the entry of the
228 oomycete. On the other hand, proteins involved in plant cell wall degradation were found in *P.*
229 *viticola* proteome (Table 3; Fig.4), namely: coproporphyrinogen III oxidase, which is a peroxidase
230 with the ability to degrade lignin, one of the components of plant cell walls²⁶; trypsin, a serine
231 proteases family, also identified in the secretomes of several fungus²⁷, and linked to
232 pathogenicity against plant hosts²⁸. In *P. viticola*, the two identified trypsin were predicted to
233 be apoplastic effectors and so it is expected a direct interaction with plant molecules for cell wall
234 degradation.

235 During plant-pathogen interactions, plant cell wall is a dynamic structure that functions as a
236 barrier that pathogens need to breach to colonize the plant tissue. Biotrophic pathogens, like *P.*
237 *viticola*, require a localized and controlled degradation of the cell wall to keep the host cells alive
238 during feeding. The regulation of the abundance of these cell wall-related proteins in the
239 apoplast and whole leaf of ‘Regent’ suggests an adaptation of the grapevine proteome to
240 prevent cell wall disruption while *P. viticola* secretes proteins that degrade de cell wall to invade
241 the plant cell.

242



243

244 **Fig.4.** Triggering of host cell wall degradation by *P. viticola* through CPOX and trypsin proteins secretion
 245 while in the host several proteins associated to the strengthening of the physical barriers (cuticle – eg
 246 ABCG32, FAR3; cell wall - eg PME19, PPE8B) are positively modulated. At the host also a negative
 247 modulation of proteins involved in cell wall degradation is promoted, moreover, proteins as 1,3-β-
 248 glucanases are translocated to the APF for pathogen cell wall degradation. Proteins represented in red
 249 are positively modulated, proteins represented in blue are negatively modulated (compared to mock-
 250 inoculated control). Pathogen proteins are represented in grey. 1,3-β-glucanase – includes endo-1,3;1,4-
 251 beta-D-glucanase-like, endo-1,3;1,4-beta-D-glucanase-like isoform X3 and glucan endo-1,3-beta-
 252 glucosidase; ABCG32 - ABC transporter G family member 32; ASD1 - alpha-L-arabinofuranosidase 1; CDC5
 253 - cell division cycle 5; CPOX - coproporphyrinogen III oxidase; CW - cell wall; GELP - GDSL esterase/lipase;
 254 FAR3 - fatty acyl-CoA reductase 3-like protein; PME19 - pectinesterase inhibitor 9; PPE8B -
 255 pectinesterase/pectinesterase inhibitor PPE8B-like; SKN1 - beta-glucan synthesis-associated SKN1.

256

257 3.1.2 *P. viticola* recognition and signalling events are established as soon as 6hpi

258 The first layer of plant defence relies on the recognition of conserved microbe-associated
 259 molecular patterns (MAMPs) by the so-called pattern recognition receptors (PRRs). PRRs are
 260 generally plasma membrane receptors which are often coupled to intracellular kinase domains
 261 ²⁹. The second layer of plant immunity depends on the ability of the plant to recognize the
 262 pathogen effectors, like RxLRs, by disease resistance proteins (R) and trigger a robust resistance
 263 response ³⁰. Several states such as oxidative burst, cell wall strengthening, induction of defence
 264 gene expression, and rapid cell death at the site of infection (hypersensitive response) occur in
 265 downstream cellular events leading to the establishment of an incompatible interaction ³¹.

266 These local hypersensitive responses can trigger long-lasting systemic responses (systemic
267 acquired resistance (SAR)) that prime the plant for resistance against a broad spectrum of
268 pathogens^{32,33}.

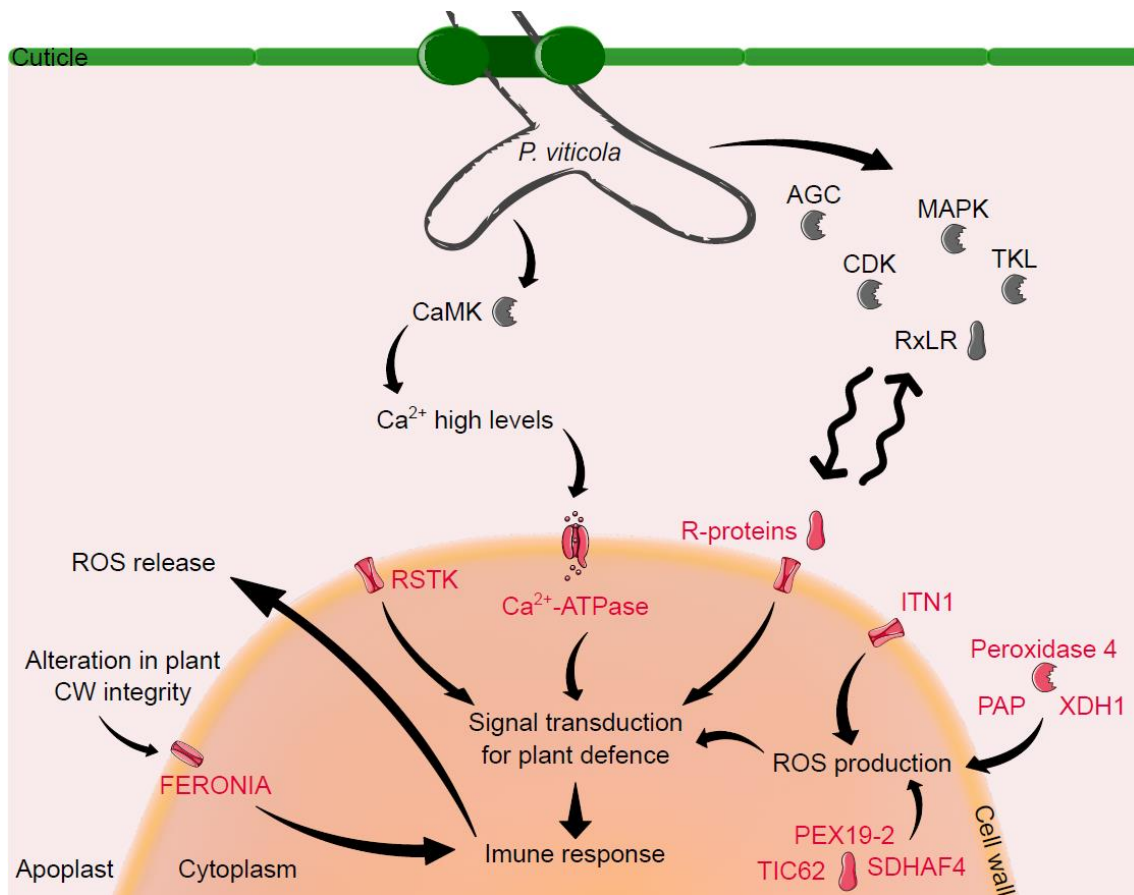
269 We have identified several virulence-related proteins that are secreted by *P. viticola* or that are
270 present in oomycete infection structures (Table 3; Fig.5). RxLR effectors were detected in *P.*
271 *viticola* proteome as soon as 6hpi and were predicted to be secreted to the apoplast. These
272 proteins are key players in virulence for downy mildew species³⁴ since they are known to defeat
273 plant immune responses through many routes, which include reprogramming host gene
274 expression, altering RNA metabolism, and binding to host proteins involved in signalling³⁵. On
275 the host side, eight R-proteins were up-accumulated in the APF after *P. viticola* infection (Table
276 1; Fig.5), including RPV1-like isoform X1 and RUN1-like isoform X1, which confer resistance to
277 multiple downy and powdery mildews, respectively, by promoting cell death^{36–38}. Also, an up
278 accumulation of several RSTK was observed both in APF (Table 1) and in whole leaf proteome
279 (Table 2). These receptors are involved in a wide array of processes ranging from developmental
280 regulation to disease resistance, including activation of signal transduction for plant defence
281 response initiation^{39,40}. One of the identified receptors in APF is the FERONIA (Fig.5). FERONIA
282 is a plant recognition receptor kinase which plays a significant role in plant immune system. In
283 *Catharanthus roseus*, FERONIA acts as a sensor of cell wall integrity challenged by the host-
284 pathogen interaction and further triggers downstream immune responses in the host cell. In
285 response to changes in the cell wall, FERONIA induces the release of ROS (reviewed in⁴¹).
286 Furthermore, in spinach response to infection with the oomycete *Peronospora effusa*, an up-
287 regulation of the genes encoding for the FERONIA and a LRR receptor-like RSTK was observed⁴².
288 These results highlight the importance of these receptors in the plant cell membrane and in the
289 first line of defence in plant strategies to stop oomycete proliferation.

290 Moreover, an up accumulation of a H⁺-ATPase (ATPase 9) was also detected in 'Regent' APF at
291 6hpi. Plasma membrane H⁺-ATPases maintain low cytoplasmic concentrations of H⁺ and are
292 dynamically regulated by biotic and abiotic events, being involved in signalling mechanisms
293 during plant-pathogen interaction (reviewed in⁴³). In response to internal and/or external cues,
294 the trafficking of these plant pumps, to and from the plasma membrane, is also highly regulated.
295 Indeed, we have identified an up accumulation of a protein involved in H⁺-ATPases translocation
296 for the plasma membrane, the protein unc-13 homolog (Table 1). In Arabidopsis, a protein unc-
297 13 homolog (PATROL1) controls the translocation of a H⁺-ATPase to the plasma membrane
298 during stomata opening⁴⁴. PATROL1 resides in the endosome and moves to and from the plasma
299 membrane in response to environmental stimuli. When the stomata open, intracellular vesicles
300 incorporate the plasma membrane and PATROL1 may be carried with them to tether the H⁺-
301 ATPase into the plasma membrane⁴⁴. Modifications of the H⁺-ATPases concentration in the
302 plasma membrane act as an alternative mean to control stomata opening⁴⁵ and in the case of
303 grapevine-*P. viticola* interaction, the stomata are the entry sites of the oomycete. Despite the
304 role of these ATPases in grapevine remains to be elucidated, an up accumulation of ATPase 9
305 and protein unc-13 homolog could suggest its involvement in stomata opening regulation during
306 'Regent'-*P. viticola* interaction.

307 During 'Regent' response to *P. viticola* infection, the up accumulation of another
308 transmembrane protein (TMP), ankyrin repeat-containing protein ITN1-like isoform X6 (ITN1),

309 was also detected (Table 1; Fig.5). Plant TMPs are essential for normal cellular homeostasis,
 310 nutrient exchange, and responses to environmental cues⁴⁶. ITN1 has been implicated in diverse
 311 cellular processes such as signal transduction and, in Arabidopsis, this protein was proposed to
 312 be related with abscisic acid signalling pathway and promotion of ROS production⁴⁷.

313



314

315 **Fig.5.** First communication events between host and pathogen: pathogen effectors are recognized by host
 316 R proteins leading to a broader remodulation of proteins related to ROS production, signal transduction
 317 and immune response establishment. Also, *P. viticola* is able to perceive host molecules activating
 318 signalling transduction pathways. Proteins represented in red are positively modulated, proteins
 319 represented in blue are negatively modulated (compared to mock-inoculated control). Pathogen proteins
 320 are represented in grey. AGC - AGC kinase; Ca²⁺-ATPase - putative calcium-transporting ATPase 13; CaMK
 321 - CAMKK kinase; CDK - CMGC CDK kinase; CW - cell wall; FERONIA - receptor-like protein kinase FERONIA;
 322 ITN1 - ankyrin repeat-containing protein ITN1-like isoform X6; MAPK - CMGC MAPK kinase; PAP - purple
 323 acid phosphatase; PEX19-2 - peroxisome biogenesis protein 19-2-like; RSTK - serine/threonine protein
 324 kinase; ROS – reactive oxygen species; RxLR - RxLR-like proteins; R-proteins – includes RPV1-like isoform
 325 X1 and RUN1-like isoform X1 and RSTKs; SDHAF4 - succinate dehydrogenase assembly factor 4; TIC62 -
 326 protein TIC 62; TKL - TKL kinase; XDH1 - xanthine dehydrogenase 1.

327

328 3.1.3 Auxin signalling plays an important role in apoplast

329 Auxin is a plant growth hormone that plays a role in many aspects of growth and development,
 330 and its signalling have been also highly associated to plant stress responses^{48,49}. Several auxin

331 signalling-related proteins were up accumulated in APF after infection (Table 1): PINOID (a RSTK
332 involved in the regulation of auxin signalling, being a positive regulator of cellular auxin efflux
333 ⁴⁸); N-MYC DOWNREGULATED-LIKE2 (NDL2) protein (involved in auxin signalling through the
334 positive regulation of auxin transporter protein 1 (AUX1) a carrier protein responsible for
335 proton-driven auxin influx ⁵⁰); and NEDD8 (neural precursor cell expressed developmentally
336 down-regulated 8), a post-translational modifiers of cullin proteins ⁵¹. Cullin1 is one of the
337 proteins that forms the “SKP1 cullin F-box” (SCF) ubiquitin ligase complex, that is involved in
338 auxin signalling pathway ⁵². Auxin homeostasis could be perturbed by stress-induced changes
339 that affects the auxin efflux carriers and that modify the apoplastic pH disturbing the auxin
340 uptake and distribution ^{53,54}. Indeed, in plant-pathogen interactions, auxin was shown to play a
341 dual role both in regulating plant defence and in bacterial virulence ⁵⁵. It was shown that some
342 pathogens may promote host auxin accumulation which leads to the suppression of salicylic acid
343 (SA)-mediated host defences. On the other side, host auxin accumulation also modulated
344 expression of particular pathogen virulence genes ⁵⁵. Despite the fact that none of the *P. viticola*
345 effectors that we have found to be present in the APF was previously related to host auxin
346 metabolism modulation, we may hypothesize that the modulation of auxin-related host proteins
347 detected may reflect pathogen induced reprogramming. In fact, *P. viticola* being a biotroph,
348 modulation of auxin metabolism leading to a depletion of SA-host defenses could be an infection
349 strategy.

350

351 **3.1.4 Apoplastic ROS signalling in grapevine immunity**

352 ROS play an important role in pathogen resistance by directly strengthening host cell walls via
353 cross-linking of glycoproteins, promoting lipid peroxidation and activation of ROS-signalling
354 networks ⁵⁶⁻⁵⁸. We have previously shown that ROS accumulation occurs in ‘Regent’ at 6hpi with
355 *P. viticola* ^{13,59}. In the APF proteome, proteins involved in ROS production, like peroxidase 4-like
356 and purple acid phosphatase (PAP), are up-accumulated in response to the infection (Table 1;
357 Fig.5), which is in accordance with our previous results. In Arabidopsis, PAP5 is induced in the
358 early stages (6hpi) of *Pseudomonas syringae* infection and is involved in ROS generation ⁶⁰.
359 Apoplastic ROS are very important in plant development and responses to stress conditions,
360 being involved in the activation of signal transduction from extracellular spaces to the cell
361 interior and may directly eliminate invading pathogens (reviewed in ⁶¹⁻⁶³). Small amounts of ROS
362 lead to expression of stress-responsible genes as a plant resistance mechanism. However, high
363 levels of ROS during a long period of time could culminate in damage of plant molecules and
364 consequently in cell death. A strict regulation of ROS levels is thus important to induce a
365 resistance response by the plant without promoting cell injury. In ‘Regent’ APF after infection
366 with *P. viticola*, an up accumulation of xanthine dehydrogenase 1 (XDH1)-like isoform X1 was
367 identified (Table 1; Fig.5). In Arabidopsis, this protein has dual and opposing roles in the ROS
368 metabolism, contributing to H₂O₂ production in epidermal cells to fight pathogen haustoria and
369 producing uric acid to scavenge chloroplast H₂O₂ in mesophyll cells to minimize oxidative
370 damage ⁶⁴. In grapevine, the role of this protein was not been elucidated yet. However, in our
371 results, an up accumulation of this ROS-related protein in ‘Regent’ APF after infection was
372 detected, suggesting a possible involvement of XDH1 in plant defence mechanism through ROS
373 metabolism regulation.

374 In whole leaf proteome, an up accumulation of ROS-related proteins such as peroxisome
375 biogenesis protein 19-2-like (PEX19-2) ^{13,65,66}, protein TIC 62 (TIC62) ⁶⁷ and succinate
376 dehydrogenase assembly factor 4 (SDHAF4) ⁶⁸ was observed at the same time that proteins like
377 peroxidases and polyphenol oxidase ⁶⁹ were less accumulated after infection (Table 2; Fig.5).
378 Also, several heat shock proteins with different abundance levels in infected leaves, when
379 compared to non-infected leaves, were identified (Table 2; ⁷⁰). As we already mentioned, the
380 regulation of ROS metabolism during plant-pathogen interaction presents a complex dynamic.
381 ROS are generated to overcome the infection but at the same time a tight regulation of high ROS
382 levels is needed to protect the plant from oxidative stress (reviewed in ⁶¹⁻⁶³). This regulation is
383 clearly evident in 'Regent' whole leaf and APF through the presence of several ROS-related
384 proteins at different abundance levels, highlighting the importance of ROS in the 'Regent'
385 defence mechanism against *P. viticola* as previously reported ^{13,59}.

386

387 **3.1.5 Calcium related signalling is prevalent in the APF at 6hpi**

388 In grapevine-*P. viticola* interaction, the increase in calcium leaf concentration in response to
389 infection was already reported ⁷¹. In 'Regent' APF proteome we have found an up accumulation
390 of a putative calcium-transporting ATPase 13 (Ca²⁺-ATPase), (Table 1; Fig.5). Ca²⁺-ATPases play
391 critical roles in sensing calcium fluctuations and relaying downstream signals by activating
392 definitive targets, thus modulating corresponding metabolic pathways (reviewed in ⁷²). We have
393 also detected an up accumulation of PINOID (Table 1). In Arabidopsis, PINOID activity is
394 regulated by several calcium binding proteins, like a calmodulin-like protein, and the binding of
395 these proteins to PINOID is enhanced by calcium ⁷³.

396 In *P. viticola* proteome, we have detected a Ca²⁺/calmodulin-dependent protein kinase (CaMK)
397 that responds to high levels of calcium ⁷⁴, (Table 3; Fig.5). In pathogens, CaMKs are involved in
398 several pathogenicity-related cellular mechanism. For example, in *C. albicans*, CaMKs functions
399 in cell wall integrity and cellular redox regulation ⁷⁵; in *Neurospora*, a genus of Ascomycete fungi,
400 CaMKs are related to growth and development of the pathogens ⁷⁶; and in *M. oryzae*, conidial
401 germination and appressorial formation were delayed and virulence was attenuated in mutants
402 of a CaMK ⁷⁷. However, in *P. viticola*, up to our knowledge, there is no information about the
403 role of these type of kinases in oomycete development and/or pathogenicity.

404 At the same time, in 'Regent' whole leaf proteome, calcium-related proteins, like calcium
405 sensing receptor ⁷⁸ and CDGSH iron-sulfur domain-containing protein NEET ⁷⁹, are less
406 accumulated after infection (Table 2).

407 These results suggest that, at such an early stage of the infection such as 6hpi, this calcium-
408 associated response and regulation, as consequence of high levels of calcium in the infection
409 site, is only taking place in the apoplast, which is the first contact point between plant and
410 pathogen. As such, this regulation of the abundance of proteins associated with calcium
411 metabolism in the leaf tissue is not evident.

412

413

414 **3.1.6 Activation of enzymes to disrupt oomycete structures**

415 One of the plant defence mechanisms during pathogen infection is the secretion of proteins
416 involved in the degradation of pathogen structures to inhibit its growth and thus stop its
417 proliferation. Oomycete cell walls consist mainly of β -1,3-glucans, β -1,6-glucans and cellulose
418 rather than chitin, essential constituent of fungal cell walls ⁸⁰.

419 In grapevine APF after *P. viticola* infection an up accumulation of two glucan endo-1,3-beta-D-
420 glucosidases and two GDSL esterase/lipases (GELPs) was observed (Table 1; Fig.4). The first are
421 involved in the degradation of the polysaccharides of the pathogen cell wall and the GELPs
422 possess lipase and antimicrobial activities that directly disrupt pathogen spore integrity ⁸¹. In
423 contrast, in whole leaf proteome, proteins like endo-1,3;1,4-beta-D-glucanase-like, endo-
424 1,3;1,4-beta-D-glucanase-like isoform X3 and glucan endo-1,3-beta-glucosidase, are less
425 abundant after infection when compared to the non-infected leaves (Table 2; Fig.4). These
426 results suggest that a demobilization of these proteins from the inside of the cell to the APF
427 might be occurring in response to *P. viticola* infection. The accumulation of these proteins in
428 'Regent' APF leads to a disruption of oomycete structures as defence mechanism to inhibit the
429 infection progress.

430 Moreover, a protein with antifungal activity, profilin 1, was found to be accumulated in 'Regent'
431 whole leaf after *P. viticola* infection (Table 2). In Arabidopsis, this protein showed significant
432 intracellular accumulation and cell-binding affinity for fungal cells, being capable to penetrate
433 the fungal cell wall and membrane and act as inhibitor of fungal growth through ROS generation
434 ⁸².

435

436 **3.1.7 The importance of protein trafficking during grapevine-*P. viticola* interaction**

437 During plant-pathogen interaction, protein trafficking is very important for plant cells to quickly
438 respond to pathogen infection. This trafficking occurs through the secretory and endocytic
439 pathways that involves a complex set of proteins associated to vesicle formation, transport,
440 docking, and fusion with the respective target membrane (reviewed in ⁸³). Clathrin-mediated
441 endocytosis is the best-known mechanism of endocytosis in plants and involves the generation
442 of small vesicles surrounded by a coat of clathrin and other associated proteins. During
443 interaction of 'Regent' with the oomycete *P. viticola*, we observed an accumulation of clathrin
444 assembly protein in the whole leaf proteome at the first hours of infection (Table 2). Indeed, the
445 increase of proteins involved in the generation of trafficking vesicles, like clathrin assembly
446 protein, reinforces our hypothesis of a relocation of specific proteins within the cell and to the
447 APF as a plant defence mechanism. We have already mentioned the presence of the protein
448 unc-13 homolog in the APF, which is responsible for the translocation of H⁺-ATPases to the
449 plasma membrane ⁴⁴ and we have also proposed the translocation of glucanases from the inside
450 of the cell to the APF in response to the infection. These results highlight the massive molecular
451 reprogramming that occurs when a plant is exposed to an environmental stress like pathogen
452 infection.

453 Even for the pathogen, the molecular trafficking that occurs within the cells is very important
454 for its growth and pathogenicity. In *P. viticola*, we have identified the small GTP-binding Rab28

455 (Ras homologue from brain), (Table 3). Rab proteins, which constitute the largest family of
456 monomeric GTPases, are small proteins involved in many biological processes (reviewed in ⁸⁴).
457 Members of this family participate in cell regulation, growth, morphogenesis, cell division, and
458 virulence. Also, they are known as master regulators of intracellular bidirectional vesicle
459 transport and, consequently, they localize in ER, vesicles, and multivesicular bodies, as well as
460 in early and late endosomes ⁸⁵. Rabs have been implicated in regulating vesicle motility through
461 interaction with both microtubules and actin filaments of the cytoskeleton ⁸⁶. In fungi, Rab
462 participates in the secretion of metabolites and lytic enzymes and, in *Fusarium graminearum*,
463 Rab GTPases are essential for membrane trafficking-dependent growth and pathogenicity ⁸⁷.
464 However, in *P. viticola* the specific function of Rab proteins was not yet elucidated.

465

466 **3.1.8 Host and pathogen proteases as hubs during the interaction**

467 Proteases are enzymes that catalyse the breakdown of proteins into smaller polypeptides or
468 single amino acids and play important roles in numerous biochemical processes. Pathogens
469 produce a variety of proteases to degrade host tissue or to disrupt or modify host defence to
470 create suitable conditions for successful colonization (reviewed in ^{88,89}). In *P. viticola*, a calpain-
471 like protease was detected (Table 3) and a possible role in pathogenicity is suggested as previous
472 studies in *M. oryzae* point out that calpains play multiple roles in conidiation, sexual
473 reproduction, cell wall integrity and pathogenicity ⁹⁰. In *Saccharomyces cerevisiae* a calpain is
474 also required for alkaline adaptation and sporulation ⁹¹. Moreover, two trypsins and serine
475 proteases involved in pathogenicity ²⁸ were identified in *P. viticola* proteome during grapevine
476 infection (Table 3).

477 Host subtilisin-like protease SBT5.3, was found more accumulated in the APF after infection. This
478 accumulation is in accordance with the previously published studies on subtilisin-like proteases
479 in grapevine-*P. viticola* interaction. A 10-fold change in SBT5.3 gene expression was observed
480 also at 6hpi together with the increase of expression of several subtilase genes in 'Regent' ¹⁵.
481 Previously, it has also been hypothesized that subtilases play a crucial role in the establishment
482 of the incompatible interaction between 'Regent' and *P. viticola*. In Arabidopsis, the subtilase
483 SBT3.3 was shown to accumulate in the extracellular matrix after infection and to initiate a
484 downstream immune signalling process ⁹².

485 Our results highlight that both host and pathogen proteases are essential on the first contact
486 hours and might play an important role in pathogen recognition and in the overcome of host
487 defences.

488

489 **3.1.9 *P. viticola* proteome at 6hpi reflects actively regulated processes leading to** 490 **infection development**

491 In *P. viticola*, several kinases are putatively involved in the infection mechanism were also found
492 in the APF at 6hpi (Table 3). We have detected, in the *P. viticola* proteome, a AGC kinase (cAMP-
493 dependent, cGMP-dependent and protein kinase C), (Table 3; Fig.5). This protein family
494 embraces a collection of Ser/Thr kinases that mediate a large number of cellular processes, such
495 as cell growth, response to environmental stresses, and host immunity ⁹³⁻⁹⁶. A study using kinase

496 inhibitors showed that kinase C protein plays a key role in the signal transduction mechanisms
497 during maintenance of motility of the zoospores, essential during their migration to the stomata
498 ⁹⁷. This evidence suggested that AGC kinases might be important pathogenic factors during
499 pathogen infection, leading us to hypothesize that the *P. viticola* AGC kinase might be also
500 relevant for the pathogenicity of this oomycete.

501 We have also detected a CDK (cyclin-dependent kinase) in *P. viticola* proteome that might be
502 participating in pathogen infection mechanisms through cell polarization for the germ tube
503 formation (Table 3; Fig.5). Indeed, several CDK members have been reported to be
504 pathogenicity-related. In phytopathogenic fungus *U. maydis*, a CDK that is essential for growth
505 and maintenance of cell polarity in this pathogen was identified. *cdk5ts* mutants showed to be
506 drastically less virulent, probably because of the involvement of this protein in the induction of
507 the polar growth required for the infection process ⁹⁸. In *P. viticola*, cell polarity guides the
508 emergence and the growth of the germ tube during the infection mechanism, namely the
509 penetration into the stomatal cavity ^{99,100}.

510 A MAPK (Mitogen-Activated Protein Kinase) has also been identified in the *P. viticola* proteome
511 during the interaction with 'Regent' leaves (Table 3; Fig.5). MAPK cascades are very important
512 in numerous cellular mechanisms in pathogens, regulating infection-related morphogenesis, cell
513 wall remodelling, and high osmolarity stress response (reviewed in ^{101,102}). For example, in
514 *Peronophythora litchii*, the oomycete pathogen causing litchi downy blight disease, a mutation
515 in PIMAPK10, a MAPKP, led to a reduced mycelial growth rate, less sporulation and weakened
516 pathogenicity, indicating an important function of MAPK signal pathway in oomycete
517 pathogenicity ¹⁰³. In *Phytophthora sojae*, another oomycete, the authors showed that the MAPK
518 PsSAK1 controls zoospore development and that it is necessary for pathogenicity ¹⁰⁴. In *M.*
519 *oryzae*, initiation of appressorium formation is controlled by the cyclic AMP-protein kinase A
520 pathway and the appressorium development and invasive growth is regulated by the Pmk1
521 MAPK pathway ^{105,106}.

522 Tyrosine kinase-like (TKL) was also identified in *P. viticola* proteome after 6 hours of infection
523 (Table 3; Fig.5). The TKL family is present in most eukaryotes and participates in many biological
524 processes, however, information about its role in oomycetes is still scarce. In *P. guiyangense*,
525 TKLs were up-regulated at early infection stages and silencing of TKLs led to reduced mycelia
526 growth, zoospore production and alteration of stress responses. Also, silencing of TKLs resulted
527 in a reduced virulence of *P. guiyangense* ¹⁰⁷, suggesting a key role of these kinases in pathogen
528 infection strategies. In *P. infestans* kinome prediction 139 TKLs were identified, however their
529 function is still unknown ¹⁰⁸.

530 Tetratricopeptide repeat (TPR)- and SEL1-containing proteins were also identified in *P. viticola*
531 proteome (Table 3). These have been reported to be directly related to virulence-associated
532 functions in bacterial pathogens ^{109–111}. In *Francisella tularensis*, a bacterial pathogen, a TPR-
533 containing protein is a membrane-associated protein that is required for intracellular replication
534 of the microbe, *in vivo* virulence, and heat stress tolerance ¹¹². In *P. infestans*, TPR was predicted
535 as one of the most common domains within proteins ¹¹³. However, up to our knowledge, the
536 role of TPR-containing proteins in oomycete/fungus pathogenicity is still unknown. Regarding
537 SEL1-containing proteins, in *Candida albicans*, a human fungal pathogen, these proteins are
538 capable of shaping host immune response and the severity of fungal systemic infection and were

539 suggested as a novel fungus-derived pattern-associated molecular pattern (PAMP)¹¹⁴. However,
540 up to our knowledge, there is no information about pathogen Sel1-containing proteins in plant-
541 oomycete interaction.

542 Growth-related proteins were also detected in the *P. viticola* proteome sequencing (Table 3;
543 Fig.4). These types of proteins are essential for the development of the pathogen structures
544 during infection and besides they are not directly related to pathogenicity, their presence is an
545 indicator of the pathogen growth and thus the progression of the infection in the host. In *P.*
546 *viticola*, we have identified the SKN1 protein that is required for synthesis of the β -glucans, the
547 major components of oomycete cell walls¹¹⁵. We have also identified the cell division cycle 5
548 (Cdc5) protein, a highly conserved nucleic acid binding protein among eukaryotes that plays
549 critical roles in development, however, in pathogens this protein is still poorly characterized¹¹⁶.

550

551 **4. Conclusions**

552 Plants have developed several defence mechanisms to rapidly respond to pathogen attack. A
553 fast recognition of the pathogen structures and activation of a defence response is primordial
554 for the establishment of the incompatible interaction. Apoplast dynamics becomes an essential
555 part of the battle between plants and pathogens. Here, we reveal for the first time the
556 communication between grapevine extracellular and intracellular spaces at the first hours of
557 interaction with *P. viticola* as well as the pathogen strategies to overcome grapevine defence.
558 Our results highlight several defence mechanisms that are first activated and modulated in
559 grapevine apoplast during infection, namely leading to plant cell wall plasticity to prevent
560 disruption and disturbance of oomycete structures. Several proteins were also identified in the
561 whole leaf proteome of 'Regent' that are closely related with the mechanisms involved in the
562 apoplast modulation during infection, highlighting a tight communication between the APF and
563 cell interior. Moreover, we have shown that *P. viticola* proteome is enriched in virulence-related
564 proteins as a strategy to defeat the plant defence response and in growth-related proteins to
565 develop the infection structures of the *P. viticola*.

566 The analysis of the plant and oomycete proteins involved in the first hours of the interaction
567 between grapevine and *P. viticola* revealed that both sides are modulating their offense and
568 defence strategies very early on. This reinforces the hypothesis that host-pathogen cross-talk in
569 the first hours of interaction is highly dynamic and processes such as ETI, ETS, PTI and PTS may
570 occur simultaneously.

571

572 **5. Materials and Methods**

573 **5.1 Plant material and inoculation experiments**

574 The tolerant *V. vinifera* cv. 'Regent' (VIVC number 4572) was used in this study. Wood cuttings
575 from 'Regent' were obtained at the Portuguese Grapevine Germplasm Bank (¹¹⁷; INIAV – Dois
576 Portos, Portugal) and grown in 2.5 L pots in universal substrate under controlled conditions in a
577 climate chamber at natural day/night rhythm, relative humidity 60% and a photosynthetic
578 photon flux density of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

579 For plant inoculation, downy mildew symptomatic leaves were harvested at the Portuguese
580 Grapevine Germplasm Bank, sprayed with water and incubated overnight at 22°C in the dark to
581 enhance sporulation. Next day, *P. viticola* sporangia were collected and their vitality was
582 checked by microscopy. Inocula was propagated in the laboratory using the susceptible Müller-
583 Thurgau.

584 For the experimental assay, 'Regent' abaxial leaf surface was sprayed with an inoculum solution
585 containing 3.5×10^5 sporangia/mL. Mock-inoculations with water were also made and used as
586 control. After inoculation, plants were kept in a greenhouse under high humidity conditions and
587 25°C. The third to fifth fully expanded leaves beneath the shoot apex were harvest at 6 hours
588 post-inoculation for apoplastic fluid extraction.

589

590 **5.2 Apoplastic fluid extraction**

591 Apoplastic fluid and total soluble protein extraction was performed as described in ³. Briefly, 5
592 volumes of 0.1 M ammonium acetate in methanol were used to precipitate the proteins
593 overnight at -20°C. Samples were centrifuged at 4000 *g*, during 30 min at -10°C. Recovered
594 pellets were washed (once with 0.1 M ammonium acetate in 100% methanol, twice with 80%
595 (v/v) acetone and twice with 70% (v/v) ethanol), dried and resuspended in 0.03 M Tris-HCl buffer
596 (pH 8.8) solution containing, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS ³. For cytoplasmic content
597 contamination control, malate dehydrogenase assay was used according to ³.

598

599 **5.3 MS-Based protein identification**

600 Separation and MS-based identification of proteins was performed as described in ³. For
601 identification of *V. vinifera* and *P. viticola* proteins, the genome assembly of *Vitis*12X database
602 (GCF-000003745.3; 41 208 sequences, July 2009) and *Plasmopara viticola* genome database
603 (INRA-PV221 isolate; 15 960 sequences, April 2018), respectively, were used via Mascot Daemon
604 (v.2.6.0. Matrix Science), imported to Progenesis QIP and matched to peptide spectra. The
605 Mascot research parameters were: a peptide tolerance of 20 ppm, a fragment mass tolerance
606 of 0.3 Da, carbamidomethylation of cysteine as fixed modification and oxidation of methionine,
607 N-terminal protein acetylation and tryptophan to kynurenine as variable modifications. Only the
608 proteins identified with a significance MASCOT-calculated threshold P-value < 0.05, at least two
609 significant peptides per proteins and one unique peptide per proteins were accepted.

610 For identification of *P. viticola* proteins present in apoplast of 'Regent' leaves inoculated with
611 the oomycete, only the sequenced proteins that fulfilled the following two criteria at the same
612 time were considered: be present in at least 2 of the biological replicates in the inoculated
613 samples; and present in only 1 biological replicate or totally absent in control samples.
614 Functional information about *P. viticola* proteins was obtained from *P. viticola* genome
615 database. Protein secretion and effector function were predicted using TargetP 2.0
616 (<https://services.healthtech.dtu.dk/service.php?TargetP-2.0>; ¹¹⁸) and EffectorP
617 (<http://effectorp.csiro.au/>; ¹¹⁹), respectively.

618

619 **5.4 Whole leaf proteome data**

620 For 'Regent' whole leaf proteome analysis, 6hpi with *P.viticola*, an already published dataset
621 deposited on the ProteomeXchange Consortium via the PRIDE partner repository with the
622 identifier PXD021613 was used ¹³.

623

624 **5.5 Statistical analysis of APF and whole leaf proteomes**

625 Principal Component Analysis (PCA) of 'Regent' APF proteome at 6hpi with *P. viticola* was
626 performed using the program MetaboAnalyst 5.0 (<http://www.metaboanalyst.ca/>, ¹²⁰).

627 For the statistical analysis and consequent identification of differentially accumulated proteins,
628 both whole leaf and APF proteomes of 'Regent' leaves inoculated with *P. viticola* (6hpi) were
629 matched to the respective control samples, allowing a comparative analysis between datasets.
630 This was done by applying Rank Products (RP), a powerful statistical test designed for identifying
631 differentially expressed genes in microarrays experiments ¹²¹, nevertheless, it also provides a
632 simple and straightforward tool to determine the significance of observed changes in other
633 omics data. RP procedure makes weak assumptions about the data and provides a strong
634 performance with very small data sets, allowing for the control of the false discovery rate (FDR)
635 and familywise error rate (probability of a type I error) in the multiple testing situation.

636

637 **5.5 Subcellular location prediction of APF DAPs**

638 For the APF DAPs from grapevine, a subcellular localization prediction was performed using
639 SignalP 5.0, TargetP 2.0 and SecretomeP 2.0 servers (<http://www.cbs.dtu.dk/services/>, ¹²²⁻¹²⁴),
640 ApoplastP (<http://apoplastp.csiro.au/>, ¹²⁵), BUSCA (<http://busca.biocomp.unibo.it/>, ¹²⁶), PredSL
641 (<http://aias.biol.uoa.gr/PredSL/>, ¹²⁷), Mercator ([https://www.plabipd.de/portal/mercator-](https://www.plabipd.de/portal/mercator-sequence-annotation)
642 [sequence-annotation](https://www.plabipd.de/portal/mercator-sequence-annotation), ¹²⁸) and Blast2GO (version 5.2.5, <https://www.blast2go.com/>, ¹²⁹). The
643 default parameters were used for all the programs.

644 Based on the subcellular localization prediction, the APF proteins were grouped in 4 different
645 classes according to the following criteria (as described in ³): (1) proteins with a predicted signal
646 peptide (SP) by SignalP (Class I); (2) proteins predicted to be secreted through classical secretory
647 pathways but, by other software than SignalP 5.0 (Class II); (3) proteins predicted to be secreted
648 by unconventional secretory pathways (USP) based on SecretomeP (Class III), and proteins with
649 no predicted secretion (Class IV). Only the proteins belonging to the Class I, II and III were
650 considered for further functional analysis.

651

652 **5.6 APF and whole leaf proteome data functional analysis**

653 Functional annotation based on Gene Ontology annotation (Biological Process) using Blast2GO
654 software was performed.

655

656 **Data Availability:** The mass spectrometry proteomics data have been deposited to the
657 ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier
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659

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666

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670

671 **Author Contributions:** J.F. and A.F. conceived the study; J.F., R.B.S, L.G.G. and A.F. were
672 responsible for the plant material and performed the APF extraction method; J.F. prepared the
673 samples for nanoLS-MS/MS analysis; C.C.L. and J.R. performed the proteome profiling by
674 nanoLC-MS/MS; L.S. performed the statistical analysis; J.F. and A.F. analysed the data and wrote
675 the manuscript. All authors have read and agreed to the published version of the manuscript.

676

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