

1 **Insight in the quorum sensing-driven lifestyle of the non-pathogenic *Agrobacterium***
2 ***tumefaciens* 6N2 and the interactions with the yeast *Meyerozyma guilliermondii***

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28 AGROBACTERIA

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31 **Highlights**

32 The avirulent *A. tumefaciens* 6N2 has two replicons and a complex QS architecture

33 The profile of QS-regulated proteins is modified in dual cultures with *Pa. laurentii*

34 The bacterial QS activity alters the proteome of the yeast *Pa. laurentii*

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49 Abstract

50 *Agrobacterium tumefaciens* is considered a prominent phytopathogen, though most isolates
51 are nonpathogenic. Agrobacteria can inhabit plant tissues interacting with other
52 microorganisms. Yeasts are likewise part of these communities. We analyzed the quorum
53 sensing (QS) systems of *A. tumefaciens* strain 6N2, and its relevance for the interaction
54 with the yeast *Meyerozyma guilliermondii*, both sugarcane endophytes. We show that strain
55 6N2 is nonpathogenic, produces OHC8-HSL, OHC10-HSL, OC12-HSL and OHC12-HSL
56 as QS signals, and possesses a complex QS architecture, with one truncated, two complete
57 systems, and three additional QS-signal receptors. A proteomic approach showed
58 differences in QS-regulated proteins between pure (64 proteins) and dual (33 proteins)
59 cultures. Seven proteins were consistently regulated by quorum sensing in pure and dual
60 cultures. *M. guilliermondii* proteins influenced by QS activity were also evaluated. Several
61 up- and down- regulated proteins differed depending on the bacterial QS. These results
62 show the importance of the QS regulation in the bacteria-yeast interactions.

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74 **Introduction**

75 *Agrobacterium tumefaciens* is an alpha-proteobacterium of the Rhizobiaceae family,
76 considered as one of the most important plant pathogens, which produces characteristic
77 crown galls on numerous dicotyledoneous plants [1]. Its pathogenicity is related to the
78 transfer of a piece of DNA, the T-DNA, from its oncogenic Ti plasmid, to the plant cell.
79 However, in nature, most agrobacterial strains are devoid of a Ti plasmid, and are in
80 consequence avirulent commensals [2]. The conjugation of Ti plasmid depends partially on
81 a quorum sensing (QS) -regulated process [3]. QS is a cell–cell communication system that
82 coalesces gene expression with the bacterial cell concentration [4]. It relies upon the
83 production by LuxI homolog enzymes of signal molecules, termed autoinducers, whose
84 concentration theoretically mimics that of the producing bacteria [5]. QS signals are
85 perceived by a complementary LuxR homolog receptor protein when signals, hence cells,
86 reach a threshold concentration [5]. Once the sensor binds the signal, it becomes activated
87 and modifies the expression of QS-target genes. The model *A. fabrum* (formerly *A.*
88 *tumefaciens*) strain C58 possesses a LuxI/LuxR-type QS system that utilizes 3-oxo-*N*-
89 octanoyl-homoserine lactone (3OC8-HSL) as QS signal [6]. 3OC8-HSL, a member of the
90 acyl homoserine lactone (AHL) family, the most characterized QS molecules in
91 proteobacteria, is synthesized by the TraI enzyme, and is bound by the TraR receptor. The
92 3OC8-HSL-TraR complex activates the transcription of genes involved in the conjugative
93 transfer of the Ti plasmid [7]. Although largely characterized in the strain C58 and other
94 pathogenic strains, little is known about QS systems in commensal agrobacteria.

95 Though mostly considered a soil inhabitant, it is now clear that agrobacteria can
96 also colonize the inner plant tissues, living as endophytes in stems, fruits and roots [8,9]. To

97 date, their interactions with the host and other microorganisms in those particular niches
98 remains poorly evaluated. Noteworthy, yeasts are also part of these complexes
99 communities. Ascomycetous and Basidiomycetous yeasts have been identified as
100 endophytes, including *Candida*, *Rhodotorula*, *Cryptococcus*, *Hanseniaspora*,
101 *Debaryomyces* and *Metschnikowia*. It is expectable that these unicellular fungi interact with
102 bacteria, including agrobacteria, in the endophytic polymicrobial communities. Their role
103 in QS mediated interactions is unknown, even if a capacity to inactivate AHLs was
104 demonstrated in several species [10].

105 During a previous survey of the endophytic microbiota of sugarcane (*Saccharum*
106 *officinarum* L.), we isolated the yeast *Meyerozyma guilliermondii* strain 6N and *A.*
107 *tumefaciens* strain 6N2 from the same node section, suggesting that these two
108 microorganisms can co-occupy this niche and, in consequence, interact with each other
109 [11,12]. In contrast to other species, this *M. guilliermondii* isolate show a very weak
110 capacity to inactivate AHLs [10].

111 Information on the influence of the QS regulatory mechanisms on the interkingdom
112 interactions remains scarce. Especially, little is known about how the QS regulation of a
113 microorganism can affect the physiology of a second microorganism. In this report, we
114 describe the complex architecture of the *A. tumefaciens* 6N2 QS system, responsible for the
115 production of several AHLs. We performed proteomic analyses to characterize the QS
116 regulation in this strain, and unveil how it is influenced in a dual culture with *M.*
117 *guilliermondii* 6N and how this second microorganism is affected by the bacterial QS
118 activity.

119

120 **Results**

121 **Strain 6N2 is a bona fide *Agrobacterium tumefaciens* isolate producing several AHLs**

122 Strain 6N2 showed a 16S rDNA sequence highly similar to those of the
123 *Agrobacterium/Rhizobium* group (Genbank accession number MG062741). The sugarcane
124 plant utilized in its isolation presented no symptoms of tumor formation, suggesting the
125 non-pathogenicity of this isolate. This was confirmed with *A. thaliana* and tomato plants,
126 which did not develop the characteristic tumors after inoculation with 6N2 strain (Fig. 1).

127 The fragmentation of molecules obtained from culture extracts confirmed the production of
128 AHLs by strain 6N2, according to the characteristic $[M+H]^+$ of 102 m/z (Fig. 2). The
129 determination of parent ions showed 4 molecules of $[M+H]^+$ 244.4, 272.5, 298.6 and 300.6
130 m/z (Fig. 2), attributed to *N*-3-hydroxy-octanoyl-homoserine lactone (OHC8-HSL), *N*-3-
131 hydroxy-decanoyl-homoserine lactone (OHC10-HSL), *N*-3-oxo-dodecanoyl-homoserine
132 lactone (OC12-HSL) and *N*-3-hydroxy-dodecanoyl-homoserine lactone (OHC12-HSL),
133 respectively (Suppl. Fig. 1).

134

135 **Genomic characterization of *A. tumefaciens* 6N2**

136 Genome sequencing of strain 6N2 revealed 2 replicons of 2,913,790 bp and 2,168,919 bp
137 (Fig. 3A and B). The second replicon was assumed to be a linear chromosome considering
138 the cumulative GC skew that suggested a replication origin at the center of the sequence
139 (data not shown), and the identification of a *telA* ortholog (AT6N2_L1435), coding for
140 TelA protelomerase. Genome annotation produced 3,013 and 2,074 CDS in the circular and
141 the linear chromosome, respectively (Fig. 3). No traces of Ti or At plasmids were detected.
142 Prophage *I6-3* genes (coordinates: 282,851-343,277) were detected in the circular
143 chromosome; several incomplete prophages (RHEph01, RcCronus, XcP1, SH2026Stx1 and
144 Stx2a_F451) were predicted in the circular and linear chromosome (data not shown). Type

145 IV (T4SS) and VI (T6SS) secretion systems were identified in the linear chromosome (Fig.
146 3B). Genomic islands were predicted in both chromosomes (Fig. 3A and B), and a probable
147 integrative and conjugative element (ICE) in the linear chromosome (coordinates: 712,734-
148 940,892) (Fig. 3B).

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150 **Identification of quorum sensing systems in *A. tumefaciens* 6N2**

151 Strain 6N2 genomic sequence showed the absence of a QS system comparable to the
152 TraI/TraR QS system of *A. fabrum* strain C58 [3]. A more complex architecture was
153 identified in the linear chromosome (Fig. 3B and Fig. 4). A first system, here named QS1
154 (coordinates 1,189,496-1,191,920) was composed of *luxR* orthologues AT6N2_L1344 and
155 AT6N2_L1347, one overlapped by the last 4 bp of the *luxI* ortholog AT6N2_L1345.
156 Considering this R-IR topology, similar to *A. fabacearum* strain P4 QS system [13], genes
157 were named accordingly *cinR*, *cinI* and *cinX*.

158 A second QS system, named QS2 (coordinates 793,262-794,901), was found in the linear
159 chromosome transcribed in the same direction as QS1 (Fig. 3B and Fig. 4). With a R-I
160 topology, QS2 was composed of the *luxI* and a *luxR* orthologues *traI2* (AT6N2_L0888) and
161 *traR2* (AT6N2_L0889), respectively. A truncated system, here named tQS (coordinates
162 762,651-763,813) was also found in the linear chromosome and in the opposite direction to
163 QS1 and QS2 (Fig. 3B and Fig. 4). tQS, composed of a *luxR* (AT6N2_L0841) and a
164 truncated *luxI* (AT6N2_L0840) orthologues, was probably originated from a partial
165 duplication and inversion of QS1. Indeed, *luxR* and *cinX* showed 90% identity (641/711);
166 *luxI* and *cinI* showed 92% identity (420/456). With 456 nucleotides, this *luxI* is
167 significantly shorter than *cinI* (765 nucleotides). tQS genes were named accordingly as
168 *cinX_t* and *cinI_t* (Fig. 3B and Fig. 4). Three *luxR* orthologues were identified in the circular

169 chromosome (Fig. 3A). AT6N2_C1772 (coordinates 1,401,123-1,400,383) was named *rhiR*
170 for its homology with *A. radiobacter rhiR* and *A. fabrum C58 Atu0707*. AT6N2_C2737
171 (coordinates 2,231,916-2,232,653) was named *solR* for its homology with *A. radiobacter*
172 *solR* and *A. fabrum C58 Atu2727*. AT6N2_C3352 (coordinates 2,749,807-2,750,523) was
173 named *atxR* due to its homology with *A. fabrum C58 atxR* (Atu2285) (Fig. 3A). The
174 analysis of the putative aminoacid sequences of AtxR, SolR and RhiR showed the
175 characteristics domains for DNA and autoinducer binding (data not shown).

176 A search in *Agrobacterium* genomes allowed the identification of strains with similar
177 topologies in the QS systems. *A. tumefaciens* strain 5A, *A. fabacearum* P4, *A. deltaense*
178 strains RV3 and NCPPB1641 and *A. radiobacter* strain DSM30147 exhibit QS systems
179 similar to QS1 (R-IR topology). Synteny throughout 16,200 bp upstream QS1 is highly
180 conserved among these strains (Suppl. Fig. 2A). QS2 topology (R-I) was detected in *A.*
181 *tumefaciens* strains S2, S33, *Agrobacterium* sp. strain SUL3 and *A. arsenijevicei* strain
182 KFB330, but with no conservation of synteny (data not shown). Homologues of *atxR*
183 (Suppl. Fig. 2B), *solR* (Suppl. Fig. 2C) and *rhiR* (Suppl. Fig. 2D) were identified in all
184 these strains, including strain C58, with synteny highly conserved, encompassing 235,500
185 bp, 785,000 bp, and 98,000 bp, respectively.

186 A multiple alignment of aminoacid sequences of LuxI orthologues showed identities higher
187 between strain 6N2 CinI and proteins with the same R-IR topology (Suppl. Fig. 3A).
188 Orthologues with R-I topology like 6N2 TraI2 presented less similarity among them. CinR,
189 CinX, CinXt, AtxR, SolR and RhiR also showed high similarities with orthologues sharing
190 the topology and synteny (Suppl. Fig. 3B). Similar to TraI2, low similarities were found
191 among 6N2 TraR2 and orthologues with the R-I topology. To note, all the 6N2 LuxI and
192 LuxR orthologues showed low similarities with *A. fabrum C58 TraI* and *TraR*.

193

194 **Modulation of *A. tumefaciens* strain 6N2 proteome by QS**

195 Quorum quenching strategy with pME6863 [14] was successful for the attenuation of the *A.*
196 *tumefaciens* 6N2 (see Suppl. Figure 4). At late exponential growth phase, no growth
197 differences were found between *A. tumefaciens* 6N2 carrying the empty control vector
198 pME6000 and *A. tumefaciens* 6N2 (pME6863). Both strains attained cell densities of ~ 1.5
199 10^9 CFU ml⁻¹ (data not shown). A total of 2,637 proteins were identified in extracts from
200 single cultures of *A. tumefaciens* 6N2 (pME6000) and *A. tumefaciens* 6N2 (pME6863).
201 Considering a $p \leq 0.05$ and a FC ≥ 1.5 , the attenuation of the QS activity altered the relative
202 abundances of 64 proteins in single cultures of *A. tumefaciens* strain 6N2 (6N2^{QSPR} group),
203 coded in the circular (37) and the linear (27) chromosome (Fig. 5A and Table 1). Thirty-
204 three were more abundant in *A. tumefaciens* strains 6N2 (pME6000) in comparison with *A.*
205 *tumefaciens* strain 6N2 (pME6863), indicating an upregulation by QS (6N2^{QSPR_{up}}
206 subgroup); 31 in 6N2^{QSPR} group were less abundant in *A. tumefaciens* 6N2 (pME6000),
207 indicating a downregulation by QS (6N2^{QSPR_{dw}} subgroup) (Fig. 5A and Table 1).
208 6N2^{QSPR} proteins were classified in eggNOG, mainly in Energy production and conversion
209 (4), and Amino acid transport and metabolism (8); 14 were classified as Function unknown
210 (Suppl. Fig. 5A and 5B). To gain insight into the influence of QS on *A. tumefaciens* strain
211 6N2 physiology, the ontology of 6N2^{QSPR} group proteins were analyzed (Suppl. Fig. 6). In
212 the Biological Process (BP) ontology of 6N2^{QSPR} group (Suppl. Fig. 6A and 6B), most were
213 classified in Biosynthesis (GO:0009058), Cell organization and biogenesis (GO:0016043),
214 Metabolism (GO:0008152), Transport (GO:0006810), and Nucleobase, nucleoside,
215 nucleotide and nucleic acid metabolism (GO:0006139). The Cellular Component (CC)
216 ontology (Suppl. Fig. 6C and 6D) showed most of the proteins classified in Cell

217 (GO:0005623), and Intracellular (GO:0005622). In the Molecular Function (MF) ontology
218 (Suppl. Fig. 6E and 6F), most of the $6N2^{QSPR}$ group proteins were in Binding
219 (GO:0005488), Catalytic activity (GO:0003824), Hydrolase activity (GO:0016787) and
220 Transferase activity (GO:0016740).

221 Regulatory and signaling proteins were identified in the $6N2^{QSPR}$ group: CinX regulatory
222 protein (AT6N2_L1344), LacI-type regulator (AT6N2_C1926), and GntR-type
223 (AT6N2_C0879) transcriptional regulators in $6N2^{QSPR}_{up}$ subgroup; two sensor histidine
224 kinases (AT6N2_C3453 and AT6N2_C3125), and YebC-like regulator (AT6N2_L1564) in
225 $6N2^{QSPR}_{dw}$. Several proteins in $6N2^{QSPR}$ group were related to transport of small molecules
226 or ions: a mechanosensitive ion channel protein (AT6N2_C0650), a DMT family
227 transporter (AT6N2_C0483), an ABC transporter permease (AT6N2_C3519), a multidrug
228 efflux RND transporter permease subunit (AT6N2_C3101) and an ABC transporter
229 substrate-binding protein (AT6N2_L1359) in $6N2^{QSPR}_{up}$ subgroup; a transporter substrate-
230 binding domain-containing protein (AT6N2_C3262), a dicarboxylate/amino acid:cation
231 symporter (AT6N2_L0298), an ABC transporter substrate-binding protein
232 (AT6N2_L0602) and an ABC transporter ATP-binding protein/permease (AT6N2_L1331)
233 in $6N2^{QSPR}_{dw}$.

234 Several proteins in $6N2^{QSPR}_{up}$ can be highlighted. Orthologues of AT6N2_L0856 (pilus
235 assembly protein), AT6N2_L0857 (Conjugal Transfer Protein D), and AT6N2_L2010
236 (Mobilization Protein C) are related to the QS-regulated transfer of pTi and pAt in strain
237 C58, and of pAt in strain P4 through a type IV secretion system. In addition to CinX, RhiR
238 (AT6N2_C1772) was the only protein from the complex $6N2$ QS system identified in the
239 proteomic analysis. RhiR was over accumulated ($p < 0.05$) when the QS system was
240 attenuated with a FC=1.49, just below the arbitrary limit established in this work.

241

242 **The yeast *M. guilliermondii* 6N alters the QS regulation in *A. tumefaciens* 6N2**

243 At late exponential growth phase, cell densities of both *A. tumefaciens* 6N2 (pME6000) and
244 *A. tumefaciens* 6N2 (pME6863) were one log unit lower than in pure cultures ($\sim 3.6 \cdot 10^8$
245 CFU ml⁻¹), with no differences between the two strains (data not shown). A total ion
246 current normalization based exclusively on bacterial proteins were applied to allow a
247 comparison between pure and dual cultures.

248 A notable reduction in QS-regulated proteins was determined in dual culture with *M.*
249 *guilliermondii* 6N. Only 33 proteins (6N2^{QSCO} group) were influenced by the QS activity,
250 which were coded in the circular (19) and the linear (14) chromosome (Table 1). In
251 6N2^{QSCO}, 22 were more abundant in strain 6N2 (pME6000), indicating an upregulation by
252 QS (6N2^{QSCO}_{up} subgroup) in co-culture. Eleven proteins of 6N2^{QSCO} were less abundant,
253 indicating a downregulation (6N2^{QSCO}_{dw} subgroup) in dual culture (Fig. 5A and Table 1).

254 6N2^{QSCO} proteins were mainly classified (Suppl. Fig. 5A and 5B) in Transcription (3) and
255 Function unknown (12). In BP ontology (Suppl. Fig. 6A and 6B), most were classified in
256 Biosynthesis (GO:0009058), and Metabolism (GO:0008152). In CC ontology (Suppl. Fig.
257 6C and 6D), the majority were classified in Cell (GO:0005623) and Plasma membrane
258 (GO:0005886). The MF ontology of 6N2^{QSCO} (Suppl. Fig. 6E and 6F) showed most
259 classified in Binding (GO:0005488), Catalytic activity (GO:0003824), and Hydrolase
260 activity (GO:0016787).

261 In the 6N2^{QSCO} group, 4 were regulatory proteins or related to signal transduction: CinX
262 (AT6N2_L1344), ArsR family transcriptional factor (AT6N2_C3363) and Xre family
263 transcriptional factor (AT6N2_L0663) in the 6N2^{QSCO}_{up} subgroup; a Response regulator
264 PleD (AT6N2_C1017) in 6N2^{QSCO}_{dw}. Four were related to transport of nutrients: a

265 component of a metal ABC transporter permease (AT6N2_C1510) and a component of a
266 zinc ABC transporter (AT6N2_C0769) in 6N2^{QSCO}_{up} subgroup; a sugar ABC transporter
267 ATP-binding protein (AT6N2_L0645) and an ABC transporter substrate-binding protein
268 (AT6N2_L1359) in 6N2^{QSCO}_{dw}. Phage proteins were identified in the 6N2^{QSCO}_{dw} subgroup:
269 a major capsid protein (AT6N2_C0409), an ATP-binding protein (AT6N2_C0382) and a
270 DNA polymerase III subunit beta (AT6N2_C0386), all part of the predicted prophage *16-3*.
271 The comparison between the different subgroups showed only 7 common proteins between
272 6N2^{QSPR}_{up} and 6N2^{QSCO}_{up} (Fig. 8A): Nucleotidyltransferase (AT6N2_L0014), Hypothetical
273 Protein (AT6N2_L0851), Pilus assembly protein (AT6N2_L0856), Conjugal Transfer
274 Protein D (AT6N2_L0857), CinX (AT6N2_L1344), TauD/TfdA family dioxygenase
275 (AT6N2_L1355) and ABC transporter substrate-binding protein (AT6N2_L1359). No
276 common proteins were found in the comparison between 6N2^{QSPR}_{dw} and 6N2^{QSCO}_{dw} (Fig.
277 8A). Similar to single cultures, CinX and RhiR were the only components of the 6N2 QS
278 system identified, though in co-culture RhiR was not supported statistically ($p > 0.05$).

279

280 **The QS activity of *A. tumefaciens* 6N2 modifies the proteome of *M. guilliermondii* 6N**

281 The yeast *M. guilliermondii* 6N reached a cell density of $\sim 1.2 \cdot 10^8$ CFU ml⁻¹ in pure
282 culture, one log unit higher in comparison to dual cultures with *A. tumefaciens* 6N2
283 (pME6000) ($3.2 \cdot 10^7$ CFU ml⁻¹) and *A. tumefaciens* 6N2 (pME6863) ($4.6 \cdot 10^7$ CFU ml⁻¹).
284 Similar to *A. tumefaciens* 6N2, a total ion current normalization based exclusively on yeast
285 proteins were applied to allow a comparison between pure and dual cultures.

286 The comparison of the *M. guilliermondii* proteomes between pure and dual cultures,
287 showed 287 proteins (Table 2) whose abundances were modified by *A. tumefaciens*
288 (pME6000) (Y6N^{QS+} group): 141 upregulated (Y6N^{QS+}_{up} subgroup) and 146 downregulated

289 (Y6N^{QS+}_{dw} subgroup). On the other hand, 275 proteins (Table 2) were modified by *A.*
290 *tumefaciens* (pME6863) (Y6N^{QS-} group): 131 up-accumulated (Y6N^{QS-}_{up} subgroup) and
291 144 down-accumulated (Y6N^{QS-}_{dw} subgroup) (Figure 5B). To note, 98 proteins were
292 common among Y6N^{QS+}_{up} and Y6N^{QS-}_{up} subgroups; 86 were common among Y6N^{QS+}_{dw}
293 and Y6N^{QS-}_{dw} (Figure 5B and Table 2). These 184 (98+86) common proteins were then
294 attributed to the presence of the bacterium, independently of the agrobacterial QS activity,
295 and in consequence no longer considered in this report. In comparison to the pure culture,
296 among the fungal proteins increased due to strain 6N2 QS activity, 43 were identified in
297 Y6N^{QS+}_{up} subgroup and 33 were in Y6N^{QS-}_{up}. The categories of each subgroup in eggNOG
298 were dissimilar (Suppl. Fig. 7A). For instance, several categories were more numerous in
299 Y6N^{QS+}_{up}, including RNA Processing and modification, Energy production and conversion,
300 Amino acid transport and metabolism, Lipid transport and metabolism, Posttranslational
301 modification, protein turnover, chaperones, Secondary metabolites biosynthesis, transport
302 and catabolism, and Intracellular trafficking, secretion, and vesicular transport. In
303 Y6N^{QS+}_{dw}, Translation, ribosomal structure and biogenesis was more numerous (Figure
304 7A). Biological Process (BP), Cellular Component (CC) and Molecular Function (MF)
305 ontologies showed differences among Y6N^{QS+}_{up} and Y6N^{QS-}_{up}. These dissimilarities were
306 more notorious in Biosynthesis (GO:0009058), Catabolism (GO:0009056), Metabolism
307 (GO:0008152) and Protein metabolism (GO:0019538) of BP ontology; Cell (GO:0005623)
308 and Intracellular (GO:0005622) of CC ontology; and Binding (GO:0005488), Catalytic
309 activity (GO:0003824), Nucleic acid binding (GO:0003676), Nucleotide binding
310 (GO:0000166) and Transporter activity (GO:0005215) of MF ontology (Suppl. Figures 8A,
311 8C and 8D). In Y6N^{QS+}_{up} subgroup, it is to highlight the identification of E3 ubiquitin-
312 protein ligase (A5DGJ2), E2 ubiquitin-conjugating enzyme (A5DL67), protein kinase

313 domain-containing protein (A5DE57) and RAS-domain containing protein (A5DKQ9). In
314 Y6N^{QS-up}, Vacuolar proton pump subunit B (A5DEC0), Vacuolar protein sorting-associated
315 protein (A5DHU0), V-type proton ATPase subunit (A5DLL8) and Phosphoenolpyruvate
316 carboxykinase (A5DD88). The same observation was made in the comparison of proteins
317 down-regulated by the agrobacterial QS activity in Y6N^{QS+_{dw}} and Y6N^{QS-_{dw}}. Data from
318 eggNOG (Suppl. Fig. 7B) showed, for instance, Y6N^{QS+_{dw}} proteins more numerous in
319 categories that include RNA Processing and modification, Coenzyme transport and
320 metabolism, and Transcription. Proteins in Posttranslational modification, protein turnover,
321 chaperones were more numerous in Y6N^{QS-_{dw}} than in Y6N^{QS+_{dw}}.
322 Biological Process (BP), Cellular Component (CC) and Molecular Function (MF)
323 ontologies of Y6N^{QS+_{dw}} and Y6N^{QS-_{dw}} also presented differences in the values of proteins
324 assigned to each category (Suppl. Fig. 8B, 8D and 8F). Main differences were in Cell
325 communication, Cell cycle, Cell organization and biogenesis, Organelle organization and
326 biogenesis, Protein metabolism, Cell, Intracellular, Catalytic activity and Transferase
327 activity, among others.

328

329 **Discussion**

330 Strain 6N2 belongs to the group of avirulent and commensal agrobacteria. This strain was
331 obtained from sugarcane, which is in contrast to dicots **is** not susceptible to crown gall
332 formation [15]. An At plasmid is also absent in its genome, indicating that strain 6N2 is a
333 plasmid-less agrobacterium. Possibly, this particular niche, with no selective pressure to
334 maintain extrachromosomal replicons, had molded the 6N2 genome [16].

335 In comparison with strain C58 [3], strain 6N2 produces four AHLs, and two AHL
336 synthases are encoded in its linear chromosome. One of this molecule, 3OHC8-HSL, has

337 also been reported in the non-pathogenic strain P4 [13], which similarly harbors CinI coded
338 in a QS system with the same R-IR topology as 6N2 QS1. It is then plausible that 6N2 CinI
339 is also involved in 3OHC8-HSL production. It is tempting to assign the synthesis of
340 3OHC10, 3OHC12-HSL and 3OC12-HSL to 6N2 TraI2. It has to be considered that an
341 enzyme can be involved in the production of more than one AHL [17]. To date, the only
342 *Agrobacterium* LuxI homolog characterized with a QS2 architecture is *A. vitis* AvsI,
343 involved in the production of multiple long chain-AHLs [18]. AtxR, SolR and RhiR are
344 also present in strain C58 [19], though their role in QS have not been evaluated. AviR, a
345 SolR homolog, is a key regulator of the pathogenesis in *A. vitis* [20,21]. A number of three
346 LuxR orphans (i.e., LuxR homologs unpaired to LuxI homologs) in *A. tumefaciens* 6N2 is
347 comparable to some of the strains mentioned in this manuscript (2 LuxR orphans in *A.*
348 *arsenijevicii* KFB330; 3 in *A. tumefaciens* S2, *A. tumefaciens* S33, *A. radiobacter*
349 DSM30147 and *A. fabrum* C58; 4 in SUL3, *A. fabacearum* P4 and *A. deltaense* RV3; 5 in
350 *A. tumefaciens* 5A; 6 in *A. deltaense* NCPPB1641).

351 The lack of similar mechanisms to 6N2 QS1 in linear chromosomes of other agrobacteria,
352 could be associated to the plasticity of these replicons. Also tQS is in a regions of genome
353 plasticity and predicted in an ICE element. This fact could be related to the truncated nature
354 of *cinI_t*. It is plausible that this truncated QS system is a remnant of a duplication and
355 inversion event, without activity. Additionally, the putative CinI_t is only 151 residues long,
356 meaning a lack of 103 residues in the *N*-terminus in comparison to CinI. However, a
357 truncated *luxI* homolog in *Methylobacterium extorquens* AM1 [22], controls the AM1 QS
358 systems.

359 Considering that agrobacterial QS systems are usually involved in plasmid conjugation
360 [13,23,24], their localization in the linear chromosome of strain 6N2 open the question

361 about their functions. Several 6N2 proteins regulated by QS are related to conjugative
362 functions: Pilus assembly protein, Conjugal Transfer Protein D and Mobilization Protein C.
363 It is plausible that the 6N2 QS activity is involved in the conjugation of other genetic
364 elements, though they could also be remnants of an integration event of a conjugative
365 plasmid. The C58 linear chromosome also harbors homologs suggested to participate in the
366 mobilization of part of the chromosome [25]. To note, a mobile element of 228,159 bp
367 (coordinates 712,734-940,892), encompassing tQS, QS2 and the T4SS is predicted in the
368 linear chromosome. It is possible that 6N2 QS systems also modify the bacterial
369 metabolism, considering the proteins under the influence of the QS activity, related to
370 energy production and conversion, amino acid transport and metabolism, transport of ions
371 and small molecules. It remains to be elucidated whether this regulation is exerted directly
372 through the LuxR homolog(s), or through other regulatory proteins found regulated by the
373 6N2 QS activity.

374 The finding that *M. guilliermondii* 6N alters the bacterial proteome is not surprising. It is
375 now clear that the co-cultivation of different species activates gene clusters otherwise
376 silenced, and vice versa, a process driven by chemical and physical interactions [26]. Most
377 astonishing is the modification of the 6N2 QS regulation in co-culture with the yeast:
378 several proteins remain regulated by QS independently of *M. guilliermondii* 6N, but others
379 are affected by the yeast. The 7 common proteins in 6N2^{QSCO}_{up} and 6N2^{QSPR}_{up} could be
380 attributed to a direct QS regulation, while the others could be indirect or susceptible of
381 modification by an environmental factor like the presence of the yeast. To note, three of
382 these common proteins (Hypothetical protein AT6N2_L0851, Pilus assembly protein
383 AT6N2_L0856 and Conjugal Transfer Protein D AT6N2_L0857) are coded between QS2
384 and tQS. As mentioned before (see Modulation of *A. tumefaciens* strain 6N2 proteome by

385 QS), the Pilus assembly protein and the Conjugal Transfer Protein D have been related to
386 the conjugal transfer of pAt in *A. tumefaciens* strain P4. Other three of them (Autoinducer
387 binding domain-containing protein CinX AT6N2_L1344, TauD/TfdA family dioxygenase
388 AT6N2_L1355 and ABC transporter substrate-binding protein AT6N2_L1359) are coded
389 close to QS1.

390 This accompanying microorganism could degrade, metabolize or modify the QS signals
391 modulating in consequence the QS activity [27]. However, it is unlikely that the
392 modification in the 6N2 QS regulation can be attributed to a fungal inactivation of QS
393 signals. Even though QQ is prevalent in yeasts, *M. guilliermondii* 6N exhibits only a weak
394 capacity for inactivating AHLs [10]. Probably other mechanisms take part in the *M.*
395 *guilliermondii* 6N-*A. tumefaciens* 6N2 interactions and the subsequent alteration of the QS
396 regulation, as described in oral biofilms, where cell-cell contacts and production or
397 depletion of metabolites intervene in the establishment of microbial communities [28].
398 Indeed, some QS-regulated proteins are related to the transport and metabolism of ions and
399 metabolites, as mentioned above. A recent report presented a model showing how an
400 environmental cue, through dedicated regulators, act on QS signals or signal receptors
401 modulating the gene expression [29]. Particular attention deserve the prophage 16-3
402 proteins identified in 6N2^{QSCO}_{dw} subgroup, since this is in concordance with the
403 “piggyback-the-winner” theory, which predicts a lysogenic switching at high cell densities
404 [30]. The relationship between QS and lysogeny has been proven for the induction of the
405 lytic cycle of Φ H2O [31]. Our proteomic results suggest that, in addition to QS, other
406 environmental factors, like the simultaneous presence of other microorganism, could
407 influence the phage cycle.

408 First described in the *Pseudomonas aeruginosa-Candida albicans* interactions, it is now
409 clear that QS molecules not only influence the physiology of the signaling microorganism
410 but also that of surroundings microorganisms [32,33]. In this work, we describe for the first
411 time the alteration of a yeast proteome by the bacterial QS activity. It is probable that the
412 AHLs, absent or strongly diminished in the co-culture with *A. tumefaciens* (pME6863),
413 have a direct effect on the yeast. Although no AHL receptor has been described in
414 eukaryotic cells, these molecules can interact with biological membranes modifying the
415 dipole potential [34]. An indirect mechanism is also possible for this modification of the
416 fungal proteome: a QS-mediated alteration of the bacterial physiology could modify the
417 profile of metabolites in the culture medium, altering the fungal proteome. Both direct and
418 indirect mechanisms are not mutually exclusive. Beyond the mechanism that modulates the
419 yeast proteome, it is to note that relevant events are being modified. For instance, a RAS
420 domain-containing protein (A5DKQ9), an E3 ubiquitin-protein ligase (A5DL67) and an E2
421 ubiquitin-conjugating enzyme (A5DL67) in Y6N^{QS+}_{up} subgroup, and an USP domain-
422 containing protein (A5DNC6) and protein FYV10 (A5DFE2) in Y6N^{QS+}_{dw} suggest an up-
423 regulation of an ubiquitylation process, less prevalent when the QS activity is attenuated
424 [35,36]. In agreement, protein metabolism is one of the main terms in BP ontology showing
425 differences between Y6N^{QS+}_{up} and Y6N^{QS-}_{up}. In contrast, a vacuolar proton pump subunit B
426 (A5DEC0), a vacuolar protein sorting-associated protein (A5DHU0) and a V-type proton
427 ATPase subunit (A5DLL8) identified in Y6N^{QS-}_{up}, together with the the GO term “vacuole”
428 in CC ontology put the focus in this organelle, key compartment in the fungal cell [37].
429 Though focused in an *in vitro* description, our results indicate the importance of the *in*
430 *planta* characterization of the *A. tumefaciens* 6N2 QS system, for evaluating its ecological
431 and physiological relevance, including its role in growth and survival. The complete

432 elucidation of the mechanism beneath the *A. tumefaciens* 6N2-*M. guilliermondii* 6N
433 interactions requires the consideration of the QS-influenced proteins, those guided by the
434 presence of the second microorganism and, importantly, also those whose abundances are
435 constant. However, results presented in this report allow a first insight to the complexity of
436 the interactions between these two microorganisms.

437

438 **Materials and methods**

439 **Microorganisms and growth conditions.** *A. tumefaciens* 6N2 and *M. guilliermondii* 6N
440 were cultured at 30 °C in nutrient broth (NB) (peptone 5 g L⁻¹; yeast extract 3 g L⁻¹).
441 *Escherichia coli* DH5 α harboring plasmids pME6000 [38] or pME6863 [14] were cultured
442 in Luria Bertani broth at 37 °C. When required, media were supplemented with agar, 15 g
443 L⁻¹, ampicillin 100 μ g mL⁻¹, tetracycline 15 μ g mL⁻¹ or cycloheximide 50 μ g mL⁻¹.

444

445 **AHL identification.** Five hundred mL of NB broth were inoculated with an overnight
446 culture of *A. tumefaciens* 6N2, and incubated aerobically at 30 °C for 24 h until late
447 exponential growth phase. Supernatants were extracted twice with acidified ethyl acetate
448 [39]. Concentrated extracts were analyzed by UPLC/ESI MS/MS (Waters Acquity UPLC-
449 TQD) with an Acquity HSS C18 (2.1 mm \times 50 mm; 1.8 μ m) at 20 °C with a flow of 0.6 mL
450 min⁻¹ and a gradient of 10% acetonitrile with 0.1% formic acid to 100% acetonitrile with
451 0.1% formic acid in 5 min as mobile phase. AHL identifications were performed by
452 comparison of fragmentation patterns with those of commercial AHLs [39].

453

454 **Genomic sequencing and annotation.** *A. tumefaciens* 6N2 genomic DNA was extracted
455 from a 10 mL overnight culture. Genome sequence was obtained utilizing single-molecule

456 real-time sequencing technology (Pacific Biosciences) (see Supp. Materials for details).
457 Annotation was performed with the MicroScope platform [40] and BASys [41]. For the
458 identification of QS genes, BLAST searches were performed on strain 6N2 genome
459 utilizing as query the *traI* and *traR* of *A. fabrum* C58 and related microorganisms (see
460 Supp. Materials for details). Sequences were deposited in Genbank under accession
461 numbers CP072308 and CP072309.

462

463 **Attenuation of QS activity.** A quorum quenching (QQ) strategy was developed, using the
464 vector pME6863 [14] that allows the constitutive expression of the *Bacillus* spp. AiiA
465 lactonase. The vector was conjugated from DH5 α (pME6863) into strain 6N2 in a
466 triparental mating with *E. coli* DH5 α (pRK2013) [42] on LB agar plates for 24 h at 30 °C.
467 pME6000 [38] was independently conjugated as negative control. Transconjugants were
468 selected on LB agar supplemented with ampicillin and tetracycline. To confirm the QQ
469 strategy, organic extracts were analyzed by RP-TLC using *A. tumefaciens* NT1 (pZLR4) as
470 bioreporter strain [43,44].

471

472 **Pathogenicity assays.** Crown gall tumor formation was assessed on tomato and
473 *Arabidopsis thaliana* plants. *A. tumefaciens* strain 6N2 was cultured on NB agar for 48 h,
474 cells were aseptically scraped off and resuspended in sterile water at a final density of 10⁷
475 CFU mL⁻¹. Cell suspension was inoculated in 4-cm cuts between the first and second node
476 on the stems of young tomato plants. *A. thaliana* was inoculated below the first node.
477 Plants were incubated 2 weeks at 25 °C under 16 h illumination and inspected for the
478 apparition of tumors. *A. fabrum* C58 and sterile water were utilized as positive and negative
479 controls.

480

481 **Preparation of protein extracts and proteomic analysis.** Two hundred and fifty mL
482 flasks containing 20 mL of NB broth were inoculated at an initial concentration of $\sim 10^7$
483 CFU mL⁻¹ for *A. tumefaciens* 6N2 (pME6000) or *A. tumefaciens* 6N2 (pME6863), and $\sim 10^6$
484 CFU mL⁻¹ for *M. guilliermondii* 6N. Dual cultures of *A. tumefaciens* 6N2 (pME6000) plus
485 the yeast, and *A. tumefaciens* 6N2 (pME6863) plus the yeast, were prepared with those cell
486 densities. Flasks were incubated aerobically at 30 °C for 24 h until late exponential growth
487 phase. Protein extracts were obtained using the YPX extraction kit (EXPEDEON), and
488 concentrations were determined with the QuantiPro BCA (SigmaAldrich). Three
489 independent samples were analyzed for each pure or mixed culture. Protein samples were
490 trypsin digested and peptide mixtures were analyzed by a Q-Exactive mass spectrometer
491 coupled to an Easy-nLC system (both from Thermo Scientific). All MS/MS data were
492 processed with Proteome Discoverer 2.1 (Thermo Scientific) coupled to an in-house
493 Mascot search server (Matrix Science, Boston, MA; version 2.5.1). Proteins showing a fold
494 change (FC) ≥ 1.5 and an ANOVA $p \leq 0.05$ were considered as differentially accumulated
495 (see Supp. Materials for details). Complete datasets are available in the ProteomeXchange
496 Consortium via the PRIDE [45] partner repository with the identifier PXD025730.

497

498 **Conflict of interest**

499 None of the authors have any type of conflict of interest.

500

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509

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682

683 Figure 1. Pathogenesis tests on model plants. *A. tumefaciens* 6N2 did not develop the
684 characteristic tumors on tomato (B) and *A. thaliana* (D) plants. A and C show the
685 corresponding controls with *A. fabrum* C58.

686

687 Figure 2. Mass spectrometric identification of AHLs produced by *A. tumefaciens* 6N2. The
688 analysis of supernatant extracts showed the presence of molecules of $[M+H]^+$ 244.4 (A),
689 272.5 (B), 298.6 (C) and 300.6 (D) m/z compatible with OHC8-HSL, OHC10-HSL, OC12-
690 HSL and OHC12-HSL, respectively. In all the cases, the fragmentation produced a
691 characteristic $[M+H]^+$ of 102 m/z. See structures in Suppl. Fig. 1.

692

693 Figure 3. Circular representation of *A. tumefaciens* 6N2 genome. In the circular (A)
694 chromosome, from outer to inner are represented CDS in each strand (blue), GC skew
695 (green and red), GC content (black), genomic islands (blue) and the luxR orthologs *rhiR*,

696 *atxR* and *solR* (red, clockwise sense). In the linear (B) chromosome, are represented CDS in
697 each strand (blue), GC skew (green and red), GC content (black), genomic islands predicted
698 with Islanviewer and ICEs predicted with ICEfinder (blue and green), T4SS and T6SS (red
699 and cyan), and the tQS, QS2 and QS1 (red, clockwise sense). Black triangles indicate the
700 extreme of the linear chromosome.

701

702 Figure 4. Architecture and topology of *A. tumefaciens* 6N2 QS systems based on AHL
703 signals. In the linear chromosome, QS1 composed of *cinR*, *cinI* and *cinX*, and QS2
704 composed of *traI2* and *traR2* were identified. A truncated tQS system composed of *cinX*
705 and a truncated version of *cinI* apparently arose from a partial duplication and inversion of
706 QS1. Respective coordinates are shown. Figure was prepared with SimpleSinteny software.

707

708 Figure 5. Summary of proteomic analysis of *A. tumefaciens* 6N2 and *M. guilliermondii* 6N.
709 Venn diagrams shows bacterial (A) and yeast subgroups of proteins. In 6N2, 7 common
710 AHL-based QS-regulated proteins were found in the 6N2^{QSPR_{up}} and 6N2^{QSCO_{up}} subgroups.
711 In the yeast, 184 differentially accumulated proteins (98+86) were attributed to the
712 presence of the bacterium, with independence of the QS activity. Venn diagram was
713 prepared with Venny web program (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>).

714

715 Suppl. Figure 1. Acyl homoserine lactones produced by *A. tumefaciens* 6N2. UPLC/ESI
716 MS/MS analysis allowed the identification of *N*-3-hydroxy-octanoyl-homoserine lactone
717 (OHC8-HSL), *N*-3-hydroxy-decanoyl-homoserine lactone (OHC10-HSL), *N*-3-oxo-
718 dodecanoyl-homoserine lactone (OC12-HSL) and *N*-3-hydroxy-dodecanoyl-homoserine
719 lactone (OHC12-HSL).

720

721 Suppl. Figure 2. Conservation of synteny around the *A. tumefaciens* 6N2 QS systems
722 related to AHLs. Synteny upstream QS1 (A) spans 16200 bp and is shared by the
723 *Agrobacterium* strains 5A, P4, RV3, NCCPB1641 and DSM30147. Syntenies around *atxR*
724 (B), *solR* (C) and *rhiR* (D) span 235500 bp, 785000 bp, and 98000 bp, respectively, and can
725 be identified in all the strains analyzed. Due to the length of the sequences, only part of the
726 synteny is shown. Respective 6N2 coordinates are shown. Analysis were performed with
727 SimpleSinteny software.

728

729 Suppl. Figure 3A. Similarities among *Agrobacterium* LuxI orthologs. 6N2 orthologs are
730 indicated with the corresponding protein name. Orthologs of other strains are indicated with
731 the corresponding Genbank accession number. Identity matrix constructed with BioEdit
732 was visualized as a heatmap with MORPHEUS. Identity values are indicated in each
733 square. Low identities are represented with orange-red shades; high identities are with
734 green shades; medium identities are in yellow (see scale bar).

735

736 Suppl. Figure 3B. Similarities among *Agrobacterium* LuxR orthologs. 6N2 orthologs are
737 indicated with the corresponding protein name. Orthologs of other strains are indicated with
738 the corresponding Genbank accession number. Identity matrix constructed with BioEdit
739 was visualized as a heatmap with MORPHEUS. Identity values are indicated in each
740 square. Low identities are represented with orange-red shades; high identities are with
741 green shades; medium identities are in yellow (see scale bar).

742

743 Suppl. Figure 4. Attenuation of QS activity in *A. tumefaciens* 6N2. Concentrated extracts of
744 *A. tumefaciens* 6N2 (pME6000) and *A. tumefaciens* 6N2 (pME6863) were analyzed by RP-
745 TLC utilizing MeOH:H₂O (6:4) as mobile phase. 3OHC8-HSL (4 pmol), 3OHC10-HSL
746 (0.25 nmol) and 3OHC12-HSL (2.5 nmol) were utilized as standards (Stds).

747

748 Suppl. Figure 5. Functional classification of *A. tumefaciens* 6N2 QS regulated proteins.
749 6N2^{QSPR_{up}} and 6N2^{QSCO_{up}} subgroups are compared in (A). 6N2^{QSPR_{dw}} and 6N2^{QSCO_{dw}}
750 subgroups are compared in (B). In each figure, full bars correspond to pure cultures and
751 dashed bars correspond to cocultures. eggNOG database was utilized for the analysis. C,
752 Energy production and conversion; E, Amino acid transport and metabolism; F, Nucleotide
753 transport and metabolism; G, Carbohydrate transport and metabolism; H, Coenzyme
754 transport and metabolism; I, Lipid transport and metabolism; J, Translation, ribosomal
755 structure and biogenesis; K, Transcription; L, Replication, recombination and repair; O,
756 Posttranslational modification, protein turnover, chaperones; P, Inorganic ion transport and
757 metabolism; Q, Secondary metabolites biosynthesis, transport and catabolism; S, Function
758 unknown; T, Signal transduction mechanism; U, Intracellular trafficking, secretion, and
759 vesicular transport; NA, not assigned.

760

761 Suppl. Figure 6. Ontology analysis of differentially accumulated proteins of *A. tumefaciens*
762 6N2. Figures show the number of bacterial proteins regulated by the QS activity, associated
763 to the respective GO terms. BP (upper), CC (middle) and MF (lower) ontologies of
764 bacterial proteins are shown. In each figure, full bars correspond to pure cultures and
765 dashed bars correspond to cocultures. 6N2^{QSPR_{up}} and 6N2^{QSCO_{up}} are depicted in green (A).
766 6N2^{QSPR_{dw}} and 6N2^{QSCO_{dw}} are depicted in red (B).

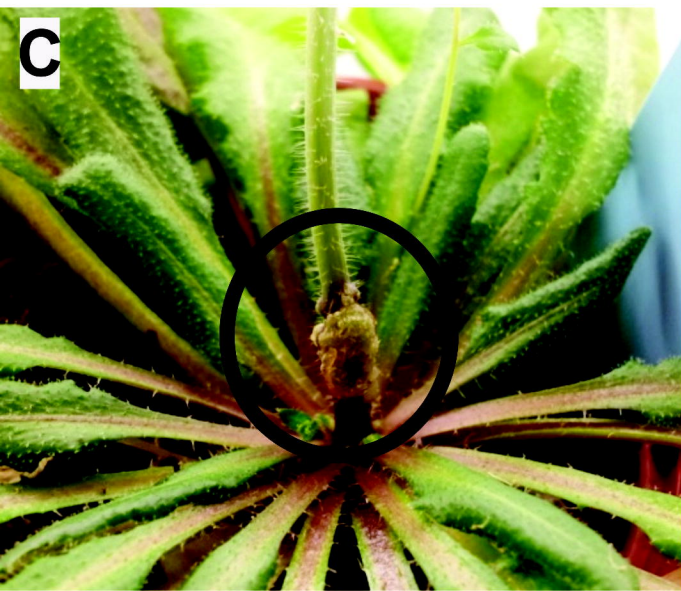
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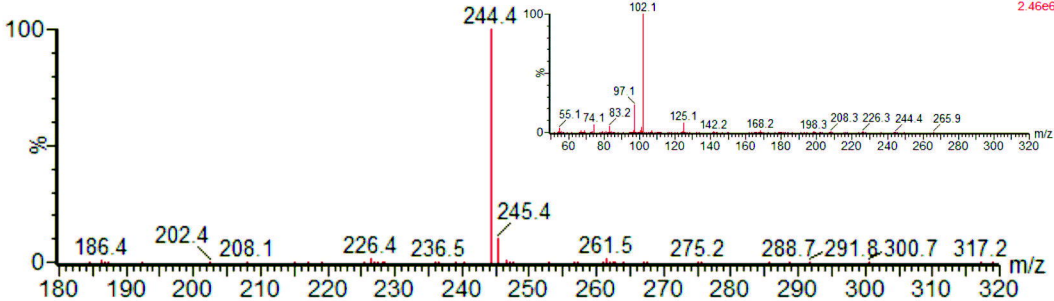
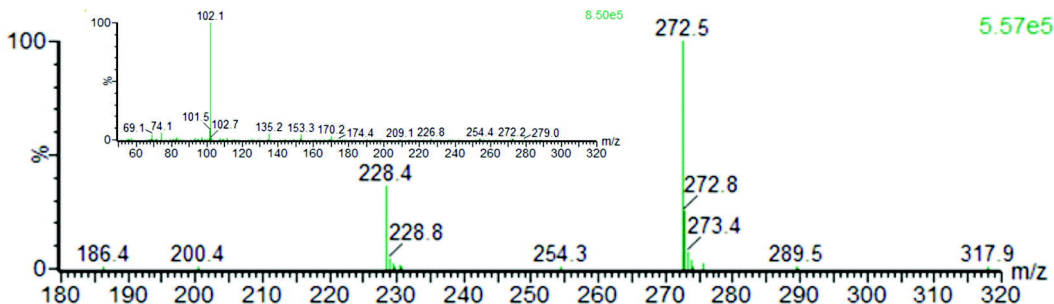
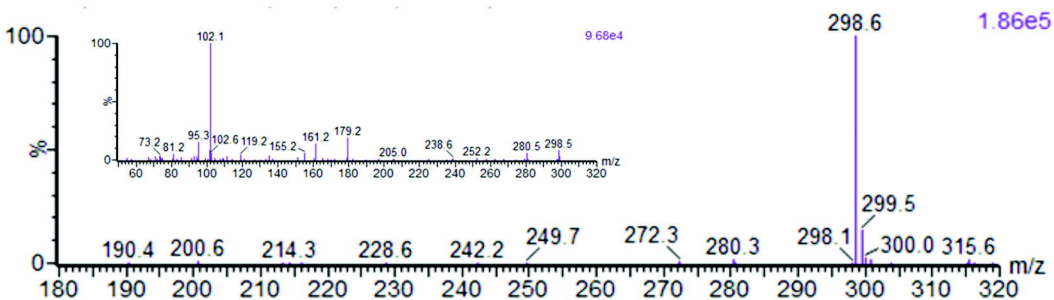
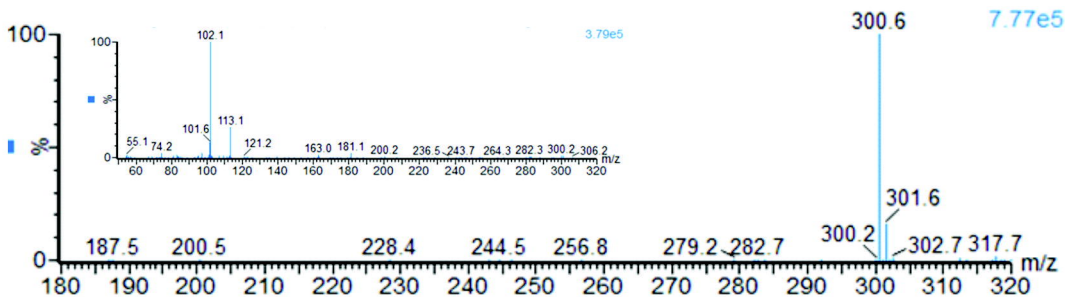
768 Suppl. Figure 7. Functional classification of *M. guilliermondii* 6N proteins regulated by
769 6N2 QS activity. Y6N^{QS+}_{up} and Y6N^{QS-}_{up} subgroups are compared in (A). Y6N^{QS+}_{dw} and
770 Y6N^{QS-}_{dw} subgroups are compared in (B). In each figure, full bars correspond to cocultures
771 with *A. tumefaciens* 6N2 (pME6000) and dashed bars correspond to cocultures with *A.*
772 *tumefaciens* 6N2 (pME6863). eggNOG database was utilized for the analysis. A, RNA
773 Processing and modification; B, Chromatin structure and dynamics; C, Energy production
774 and conversion; D, Cell cycle control, cell division, chromosome partitioning; E, Amino
775 acid transport and metabolism; F, Nucleotide transport and metabolism; G, Carbohydrate
776 transport and metabolism; H, Coenzyme transport and metabolism; I, Lipid transport and
777 metabolism; J, Translation, ribosomal structure and biogenesis; K, Transcription; L,
778 Replication, recombination and repair; M, Cell wall/membrane/envelope biogenesis; O,
779 Posttranslational modification, protein turnover, chaperones; P, Inorganic ion transport and
780 metabolism; Q, Secondary metabolites biosynthesis, transport and catabolism; S, Function
781 unknown; T, Signal transduction mechanism; U, Intracellular trafficking, secretion, and
782 vesicular transport; Y, Nuclear structure; Z, Cytoskeleton; NA, not assigned.

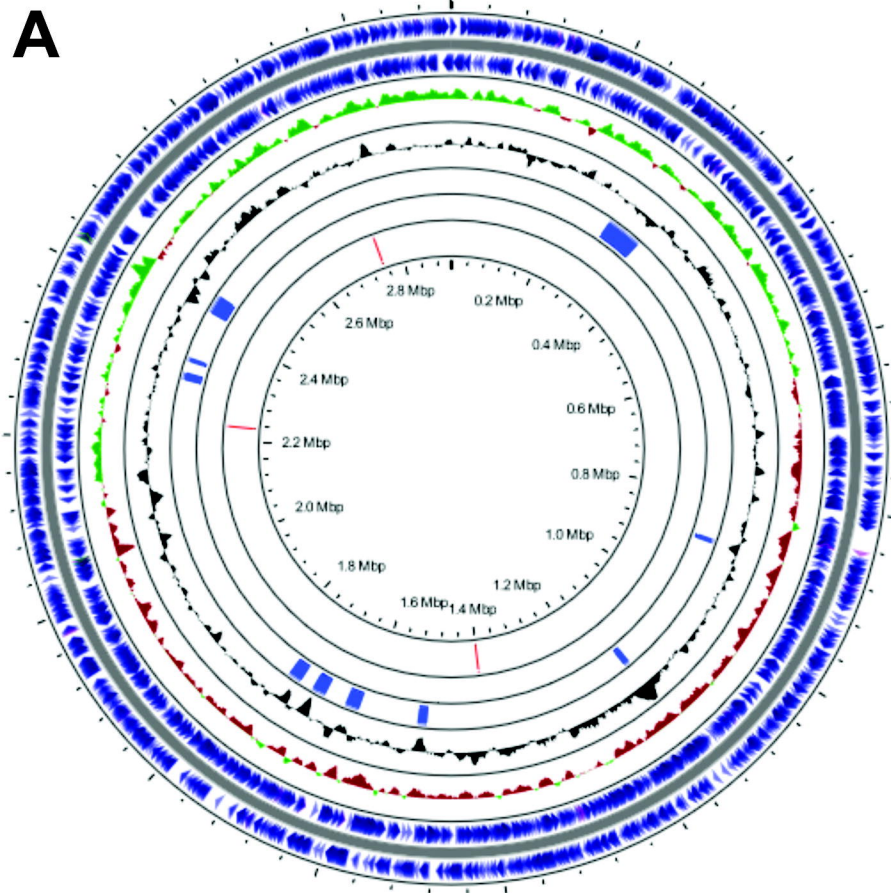
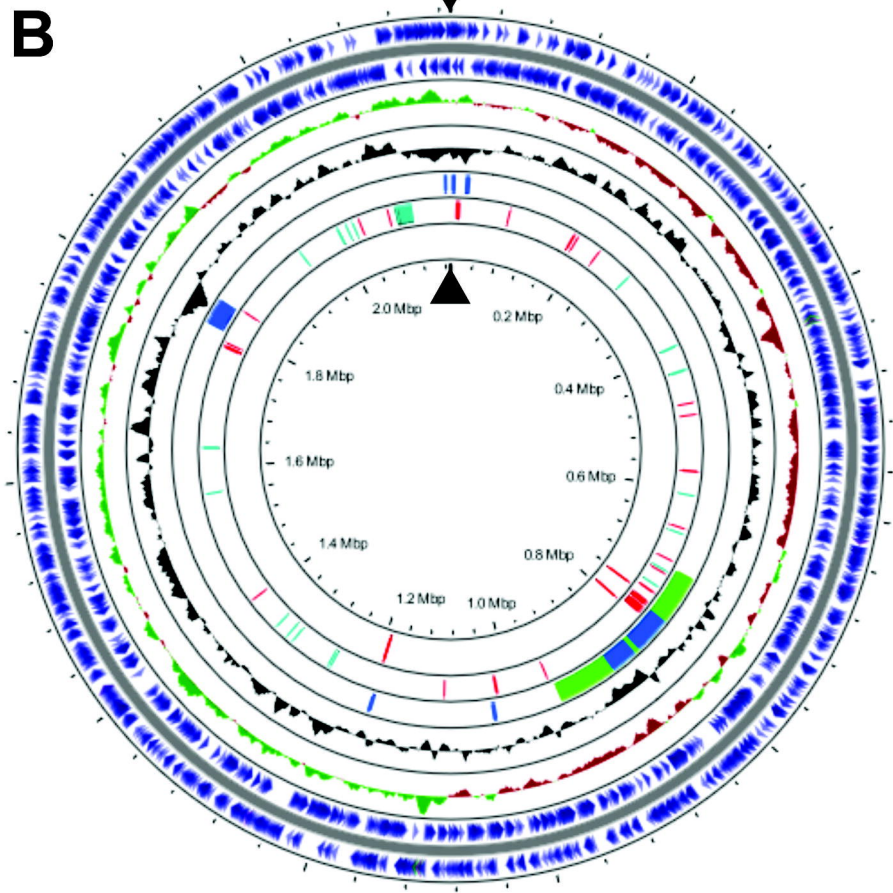
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784 Suppl. Figure 8. Ontology analysis of differentially accumulated proteins of *M.*
785 *guilliermondii* 6N. Figures show the number of yeast proteins influenced by the *A.*
786 *tumefaciens* 6N2 QS activity, associated to the respective GO terms. BP (upper), CC
787 (middle) and MF (lower) ontologies of yeast proteins are shown. In each figure, full bars
788 correspond to cocultures with *A. tumefaciens* 6N2 (pME6000) and dashed bars correspond
789 to cocultures with *A. tumefaciens* 6N2 (pME6863). Y6N^{QS+}_{up} and Y6N^{QS-}_{up} subgroups are
790 depicted in brown (A). Y6N^{QS+}_{dw} and Y6N^{QS-}_{dw} subgroups are depicted in blue (B).

791



A**B****C****D**

A**B**

1189642



1192066

*cinX**cinI**cinR*

CC

762797



763959

*cinI_t**cinX_t*

793408



795011

*traI2**traR2*

