1 **Early events of the endophytic symbiotic between *Oryza sativa***
 2 **and *Nostoc punctiforme* involve the SYM pathway**

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19 **Competing interests**

20 The authors declare no competing interests.

21

22 **Abstract**

23 Symbiosis between cyanobacteria and plants is considered pivotal for biological
 24 nitrogen deposition in terrestrial ecosystems. Despite the large knowledge in the
 25 ecology of plant-cyanobacteria symbioses, little is known about the molecular
 26 mechanisms involved in the crosstalk between partners. A SWATH-mass
 27 spectrometry has been used to analyse, at the same time, the differential
 28 proteome of *Oryza sativa* and *Nostoc punctiforme* during the first events of the
 29 symbiosis. *N. punctiforme* activates the expression of thousands of proteins
 30 involved in signal transduction and cell wall remodelling, as well as 11 Nod-like
 31 proteins that might be involved in the synthesis of cyanobacterial-specific Nod
 32 factors. In *O. sativa* the differential protein expression was connected to a
 33 plethora of biological functions including signal transduction, defense-related
 34 proteins, biosynthesis of flavonoids and cell wall modification. *N. punctiforme*
 35 symbiotic inspection of *O. sativa* mutants in the SYM pathway reveals the
 36 involvement of this ancestral symbiotic pathway in the symbiosis between the
 37 cyanobacterium and the plant.

38 1. INTRODUCTION

39 *Nostoc punctiforme* (hereafter *Nostoc*) is one of the most versatile N₂-fixing
 40 cyanobacteria. It occurs as free-living forms or in symbioses with plants from
 41 the four major phylogenetic divisions of terrestrial plants, reflecting a high
 42 diversity and low host specificity in its symbiotic interactions (Svenning et al.,
 43 2005; Adams et al., 2013; Warsham et al., 2018). *Nostoc* provides different
 44 modes of symbiotic associations with their host plants. It grows epiphytically in
 45 specialized compartments of liverworts, hornworts and *Azolla*, meanwhile
 46 colonizes endophytically stem glands of *Gunnera* sp. *Oryza sativa* (hereafter
 47 *Oryza*) and *Triticum vulgare*, providing fixed nitrogen to the plant (Gantar et al.,
 48 1991; Santi et al. 2013; Álvarez et al., 2020). Symbiotic nitrogen fixation with
 49 cereals is central for exploring new sustainable agricultural practices that may
 50 reduce the usage of synthetic fertilizers, whose application results in adverse
 51 environmental consequences (diCenzo et al., 2020). However, despite the
 52 beneficial effects of cyanobacterial nitrogen fixation in terrestrial ecosystems, to
 53 date little is known about the signalling mechanisms, crosstalk between
 54 partners and metabolic adaptations underlying the symbiotic process.

55 Independently of the mode of association, symbiotic interaction of *Nostoc*
 56 with plants comprise an early phase of interaction, which includes chemical
 57 signalling between partners, followed by a later phase where the cyanobacteria
 58 are physically associated with the host and supply nutrients to the plant. In
 59 response to nitrogen limitation, the plant produces signals that trigger
 60 hormogonia differentiation (which are the cyanobacterial infection units) and
 61 others compounds that act as chemoattractants (Meeks and Elhay, 2002;
 62 Nilson et al., 2006). Most of the knowledge on colonization steps and
 63 maintenance of *Nostoc*–plant symbioses are based on the *Nostoc* response to
 64 the liverwort *Blasia*, the hornworts *Anthoceros* and *Phaeoceros*, and the
 65 angiosperm *Gunnera* (Adams et al., 2013). Key cyanobacterial genes required
 66 for symbiosis have been predicted *in silico* (Warshan et al., 2017; 2018) and
 67 experimentally identified by proteomic and transcriptomic analyses (Ekman et
 68 al., 2006; Campbell et al., 2008; Wharshan et al., 2017). They include genes
 69 encoding proteins involved in chemotaxis and motility, oxidative stress
 70 response, transport of phosphate, amino acids and ammonium, and repression

71 of photosynthetic CO₂ fixation. These changes imply that the cyanobacterium
72 shifts from photo-autotrophic to heterotrophic lifestyle, relying of the carbon
73 provided by the host to sustain N₂-fixation (Meeks and Elhai, 2002). Very little is
74 known about the plant changes in response to the cyanobacterium. Expression
75 changes in response to *Nostoc* have been studied by RNA-seq in *Anthoceros*
76 and *Arabidopsis* (Li et al., 2020; Belton et al., 2020). Activation of receptor
77 kinases and genes involved in stress response have been reported.

78 In other plant-microbial endosymbiosis, including the legume-*Rhizobium*,
79 *Parasponia-Rhizobium*, actinorhizal plants-*Frankia* and arbuscular mycorrhizal
80 (AM) fungi (Glomeromycota) symbioses, molecular genetic studies have
81 revealed that signalling pathways of host plants largely overlap (Geurts et al.,
82 2016; Oldroyd, 2013; Radhakrishnan et al., 2020). These signalling pathways
83 comprise a well conserved network in land plants known as the ‘common
84 symbiosis signalling pathway’ (SYM) (Oldroyd, 2013). The SYM pathway
85 encompasses plasma membrane-localized LysM-type and LRR-type receptor
86 kinases, a transcriptional network involving two predicted cation channels,
87 CASTOR and POLLUX, the calcium/calmodulin-dependent protein kinase
88 CCAMK, and CYCLOPS, which is phosphorylated by CCAMK (Parniske, 2008).
89 This pathway is present in *Oryza* and is essential to AM symbiosis (Gutjar et al.,
90 2009). SYM pathway is activated by Nod and mycorrhizal (Myc) factors,
91 produced by rhizobia or AM, respectively (Oldroyd, 2013). They are
92 chitooligosaccharides containing a pentameric chitin backbone and specific acyl
93 groups that give plant selectivity (Oldroyd, 2013). Neither Nod factors nor Myc
94 factors have never been identified in a cyanobacterium. However, *Nostoc* DNA
95 sequences homologous to the rhizobial *nod* genes were identified two decades
96 ago by heterologous hybridization (Rasmussen et al., 1996).

97 In contrast to the extensive knowledge of the signalling mechanisms in
98 *Rhizobium*-legume symbioses, none of the signalling networks involved in
99 *Nostoc* symbioses have been identified. In the present work, we have evaluated
100 changes that occur in *Oryza* and *Nostoc* at an early phase of recognition (1
101 day) and a later phase of contact (7 days), providing a valuable overview of the
102 recognition process. These changes have been determined by sequential
103 window acquisition of all theoretical mass spectra (SWATH-MS) (Huang et al.,

2015; Liu et al., 2019). This method is able to do label-free quantification in an MRM-like manner, providing the expression profile of a protein at proteome scale. We provide, for the first time, simultaneous temporal regulation recognition pathways in both the plant and the cyanobacterium during co-culture. We have identified a total of 2906 proteins, 1397 from *Nostoc* and 1509 from *Oryza*. Analysis of differentially expressed proteins revealed metabolic changes involved in adaptation to symbiosis when both partners were in contact. Analysis of *Nostoc* colonization on different *Oryza* mutants in the SYM pathway reveals the involvement of this common symbiotic pathway in the symbiosis between *Nostoc* and *Oryza*.

2. METHODS

2.1. Organisms and growth conditions

Rice seedlings (*Oryza sativa* L.) var. Puntal (Indica) were used for the proteomic analysis and the colonization inspection. The Nipponbare background was used for lines mutated in POLLUX (lines NC6423 and ND5050, *pollux-2* and *pollux-3*, respectively), in CCAMK (lines NE1115 for *ccamk-1* and NF8513 for *ccamk-2*, respectively) and in CYCLOPS (lines NC2415, and NC2713 for *cyclops-2*, and *cyclops-3*, respectively) (Gutjahr, et al 2008). All of them were kindly provided by Prof. Uta Paszkowski, University of Cambridge.

Rice seedlings were surface-sterilized by washing first with distilled water and then with 0.5% (w/v) calcium hypochlorite for 20 min. Later, the seeds were thoroughly washed with sterilized tap water and kept for germination on a wet filter paper in a container. Rice plants were grown hydroponically in 50-ml conical tubes with BG11₀ (-N) medium (free of combined nitrogen) (Rippka et al. 1979). Experiments were carried out in a growth chamber at 28 °C, 75% relative humidity, 12 h light-12 h dark cycles and light intensity of 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Nostoc punctiforme PCC 73102 (also known as ATCC 29133) was obtained from the Biological Cultures Service of the IBVF, and grown in BG11₀ medium supplemented with 2.5 mM NH₄Cl, 5 mM TES-NaOH buffer (pH 7.5) at 30 °C in continuous light (50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) in shaken liquid cultures (100 rpm) or

on medium solidified with 1% (w/v) Difco agar. In order to avoid hormogonia differentiation, filaments of *Nostoc* were grown to a concentration of 2-3 $\mu\text{g Chl}\cdot\text{ml}^{-1}$ in BG11₀ medium supplemented with 2.5 mM NH₄Cl, 5 mM TES-NaOH buffer (pH 7.5) and 4 mM sucralose (Splitt and Risser 2016). Induction of hormogonia differentiation was carried out by washing and incubated in BG11₀ without sucralose for 16 h.

2.2. Co-cultivation of *Nostoc* and rice plants

Seedlings of rice grown for 7 days were suspended in 50-ml conical tubes with BG11₀ medium. After 4–5 days of acclimation, *Nostoc* inoculants containing hormogonia were added to the solutions at a final concentration of 0.8 $\mu\text{g Chl}\cdot\text{ml}^{-1}$. Co-cultivation was carried out in a growth chamber up to 35 days as described above for rice plants. Cultures of each partners were also grown separately in parallel as controls.

To determine association to plant roots, Chlorophyll a content was measured as in Álvarez et al., 2020, according to Arnon (1949). Root samples were directly taken at different times of co-culture. Chlorophyll was referred to the roots fresh weight.

2.3. Sample preparation for confocal microscopy

Fresh rice roots were cut and washed intensively with tap water. Alternatively, root slices were obtained by excision with a blade. Samples were mounted in a coverslip and examined with a Leica TCS SP2 confocal microscope using HC PL-APO CS $\times 20$ or HCX PLAM-APO $\times 63$ 1.4 NA oil immersion objectives. Cyanobacterial autofluorescence was excited with 488 nm light irradiation from an Argon laser, and fluorescence emission was monitored across windows of 650–700 nm. Root's lignin and suberin autofluorescence was similarly excited with 476 and 488 nm laser irradiation, and fluorescence emission was monitored across windows of 510–533 nm. Z series containing from 90 to 130 frames were stacked and processed with the Image J program (version 1.41).

2.4. Protein extraction and trypsin digestion

164 Total protein extracts were prepared from biological triplicates at 1 day and 7
165 days post inoculation from control *Nostoc* samples (5 ml of culture at 50 µg
166 Chl·ml⁻¹ per replicates), control *Oryza* root samples (300 mg from 15 plants per
167 replicate) and *Nostoc* treated plant roots (300 mg from 15 plants per replicate).
168 Samples were ground to powder in liquid nitrogen and then incubated in lysis
169 buffer (50 mM Tris-Cl pH 7.5, 1 mM PMSF (phenylmethanesulfonylfluoride), 1
170 mM EDTA (ethylenediaminetetraacetic acid), 2% SDS and Complete™ mini
171 EDTA free protease inhibitor cocktail) and centrifuged at 16,000 ×g at 4 °C for
172 15 min. Protein concentrations were determined by Bradford method (Bio-Rad).

173 Protein samples were precipitated by TCA/acetone. Precipitated samples
174 were resuspended in 50 mM ammonium bicarbonate with 0.2% Rapigest
175 (Waters) for protein determination. Protein samples (20 µg) were digested with
176 trypsin as described previously (Vowinckel et al., 2014).

177 **2.5. Proteome analysis by SWATH-MS**

178 The SWATH-MS analysis was performed at the Proteomic Facility of the
179 Institute of Plant Biochemistry and Photosynthesis, Seville, Spain. A data-
180 dependent acquisition (DDA) approach using nano-LC-MS/MS was initially
181 performed to generate the SWATH-MS spectral libraries.

182 Peptide and protein identifications were performed using Protein Pilot
183 software (version 5.0.1, Sciex) with the Paragon algorithm. The search was
184 conducted against Uniprot *Oryza* proteome, Uniprot *Nostoc* proteome or a
185 combined database with Uniprot *Oryza+Nostoc* proteome. Automatically
186 generated reports in ProteinPilot were manually inspected for FDR (false
187 discovery rate) cut-off proteins. Only proteins identified at FDR ≤1% were
188 considered for output and subsequent analysis. Protein-specific peptide and
189 peptides not shared between the two organisms were selected for further
190 analysis. The combined *Oryza+Nostoc* library was used to detect interspecies
191 peptides. Peptides shared by *Oryza* and *Nostoc* were not used in the
192 downstream analysis. As a result of this process, we generated two spectral
193 libraries, one for *Oryza* and one for *Nostoc* containing species-specific peptides.

194 For relative quantification using SWATH analysis, each biological
195 replicate was quantified using three technical replicates and a data-independent

196 acquisition (DIA) method. Each sample (1 µg of protein) was analysed using
197 SWATH-MS acquisition method on the LC-MS equipment with a LC gradient.
198 The method consisted of repeated acquisition cycles of TOF MS/MS scans (230
199 to 1500 m/z, 60 ms acquisition time) of 60 overlapping sequential precursor
200 isolation windows of variable width (1 m/z overlap) covering the 400-1250 m/z
201 mass range from a previous TOF MS scan (400-1250 m/z, 50 ms acquisition
202 time) for each cycle. The total cycle time was 3.7 s. Autocalibration of the
203 equipment and chromatographic conditions were controlled by an injection of a
204 standard of digested β-galactosidase from *Escherichia coli* between the
205 replicates.

206 SWATH MS spectra alignment was performed with the PeakView 2.2
207 (Sciex) software with the MicroApp SWATH 2.0 using the *Oryza* or *Nostoc*
208 spectral libraries generated as described above.

209 After data processing, the processed mrkw files containing protein
210 information from PeakView were loaded into MarkerView (Version 1.2.1.1, AB
211 Sciex) for normalization of protein intensity (total area sums) using the built-in
212 total ion intensity sum plug-in.

213 Mass spectrometry raw proteomic data have been deposited to the
214 ProteomeXchange Consortium via the PRIDE partner repository with the
215 identifier PXD022229.

216 **2.5. Computational methods**

217 Quality of normalised data was analysed using PCA analysis and results were
218 visualized using *ggbiplot* R package. Normalised data were later processed with
219 *limma* R package (Ritchie et al., 2015) to extract changes in protein abundance.
220 We performed the following comparisons between the cyanobacterial samples:
221 *Nostoc* plus *Oryza* after 24 h (*Nostoc_Oryza_24h*) versus *Nostoc* without plant
222 after 24h (*Nostoc_24h*), *Nostoc* plus *Oryza* after 7 days (*Nostoc_Oryza_7days*)
223 versus *Nostoc* without plant after at 7 days (*Nostoc_7days*), *Nostoc* plus *Oryza*
224 after 7 days (*Nostoc_Oryza_7days*) versus *Nostoc* plus *Oryza* after 24 h
225 (*Nostoc_Oryza_24h*) and *Nostoc* without *Oryza* after 7 days (*Nostoc_7days*)
226 versus *Nostoc* without *Oryza* after 24 h (*Nostoc_24h*). The same logic
227 comparisons were followed for the plant samples. A total of 1076 cyanobacterial

228 proteins had a statistically differential abundance in at least one comparison
229 (fold change > 1.5 or < 0.66667, and adjust p-value < 0.05). In the case of the
230 plant, a total of 736 plant proteins fulfilled the same criteria.

231 Clustering analysis was carried out using *mfuzz* R package (Kumar and
232 Futschik, 2007) using a ‘fuzzifier’ of 1.25647 ($m = 1.25647$) for *Nostoc* and
233 1.265078 ($m = 1.265078$) for *Oryza*. Cyanobacterial or plant proteins
234 considered to be in the core of a cluster were selected using a strict confidence
235 threshold of 0.80.

236 3. RESULTS

237 3.1. Roots of *Oryza sativa* sp. Indica are endophytically colonized by 238 *Nostoc punctiforme*

239 Association of *Nostoc* to *Oryza* roots was first studied. A fixed amount of *Nostoc*
240 hormogonia containing $0.8 \mu\text{g Chl}\cdot\text{ml}^{-1}$ was inoculated to hydroponic cultures of
241 *Oryza* plants. Chlorophyll of the roots was measured at different time points
242 ranging from 24 h to 35 days after inoculation (Figure 1A). *Nostoc* hormogonia
243 reached the plant within few hours after inoculation, being strongly attached to
244 the plant roots (Figure 1B and C). The cyanobacterium started growing in the
245 proximity of the roots, increasing the biomass until 15 days. At 35 days post
246 inoculation colonization of the plant was evident, being epidermal cells and
247 radical hairs entirely colonized (Figure 1C). At this time, a significant difference
248 was observed in length and fresh weight of plants treated with *Nostoc* (Figure
249 S1).

250 3.2. Simultaneous quantitative proteomics of *Nostoc* and *Oryza*

251 To assess the proteome coverage of the early steps of recognition of the two
252 partners, 1 day and 7 days of co-culture were selected for the quantitative
253 analysis. Freshly hormogonia from *Nostoc* and *Oryza* seedlings were co-
254 cultured in BG11₀. As controls, cultures of each partners were grown separately
255 in parallel. Total protein extracts from controls and *Nostoc* treated plants (in
256 triplicate) at 1 day and 7 days post inoculation were analysed by SWATH

257 (Figure 2). Among 2906 total identified proteins, 1397 proteins were from
258 *Nostoc* and 1509 proteins belong to *Oryza*.

259 We performed pair-wise comparison to explore the similarities and
260 differences in the corresponding proteomes (Supplementary File 1). As a result,
261 666 and 862 proteins were found to change their abundance at 1 day and 7
262 days post inoculation relative to non-inoculated samples, respectively (p -value $\leq$
263 0.05), fold change ≥ 1.5 or < 0.66667). At 1 day, 159 plant proteins and 507
264 cyanobacterial proteins changed their abundance. After 7 days post-inoculation,
265 321 plant proteins and 541 cyanobacterial proteins had a differential
266 accumulation (Figure 3). Among the 1397 proteins identified in *Nostoc*, 36%
267 and 39% proteins changed their abundance at 1 day and 7 days after post-
268 inoculation, respectively. However, only 10.5% and 21% of identified plant
269 proteins had a differential accumulation after 1 day and 7 days of treatment.
270 The higher number of differentially expressed proteins in *Nostoc* from the very
271 first moment reveals profound changes in the active metabolism of the
272 cyanobiont responding to the plant. In the case of the plant, most of the
273 changes take place at 7 days after co-cultivation.

274 As an initial characterization and check of our experimental design,
275 samples were analysed using principal component analysis (PCA). The PCA
276 analysis showed a good quality data and no batch effect or confounding factors.
277 Samples belonging to the different conditions and times segregated into well
278 separated groups, and biological replicates clustered tightly together (Figure
279 S2). No outlier values were detected, so all the replicates were used for further
280 analyses.

281 In order to understand the timeline of the symbiosis as a whole, we
282 performed pair-wise comparisons that consider not only the *Nostoc* treatment
283 but also the time-course expression of proteins. In order to ascertain groups of
284 proteins with the same regulation, a clustering analysis using *mfuzz* R package
285 was carried out in both organisms (Kumar and Futschik, 2007). We obtained 8
286 clusters in *Nostoc* and 6 clusters in *Oryza* with at least 80% confidence (Figures
287 S3 and S4). Proteins belonging to each cluster can be found at glance at the
288 data files Supplementary File 1. A heat map representation of the proteins in

each cluster illustrates the consistency of the clustering and between individual replicates of the treatments (Figure 4).

3.3. Proteins up-regulated in *Nostoc* and *Oryza* in response to the partner

In *Nostoc*, 178 proteins were significantly induced at 1 day of co-culture (included in cluster N1) and 292 proteins were induced at 7 days of co-culture (included in clusters N2 and N3). From them, 81 proteins were induced both a 1 and 7 days in response to the plant partner. In *Oryza*, a total amount of 104 proteins were significantly induced in the presence of *Nostoc* (included in cluster O1 and O2). Most of the changes in the plant were observed at 7 days post inoculation, being only 16 proteins induced at 24 h. Proteins activated in both organisms can be found at glance at the data files Supplementary File 1 filtering by 1. A group of selected proteins is shown in Table 1.

3.4.1. Signalling

In *Nostoc*, we found a number of proteins highly induced in response to the plant. Some of them had been previously identified in other reports studying *Nostoc*-plant symbioses (Campbel et al., 2008; Warsham et al., 2017; Ekman et al., 2006). For example, we found three proteins (PtxB, PtxD and PtxG) of a system controlling positive phototaxis (Ptx system; Campbell et al., 2015), the response regulator receiver HmpB and two GAF sensor signal transduction histidine kinases (Npun_R1597 and Npun_F2781). Additionally, we found other proteins not described previously, including four response regulator receiver proteins (Npun_F0021, Npun_R0589, Npun_F6378 and Npun_AR130) and a putative cGMP-specific 3',5'-cyclic phosphodiesterase (Npun_R2479). These sensor proteins might be involved in transducing plant signals involved in plant recognition. In *Oryza*, we found a predicted E3 ubiquitin ligase (B8BET8), a SH3 domain-containing protein (B8AKM5), two response regulators (B8AYY5 and B8AG68) and an Importin- α (A2X9V6).

3.4.2. Cell adhesion and cell wall modification

Nostoc showed a strong adherence to the *Oryza* roots only a few hours after co-cultivation (Fig. 1). In accordance to this, we found upregulated *Nostoc*

319 proteins involved in cell adhesion, including two FG-GAP repeat proteins
320 (Npun_R5402 and Npun_R1349). FG-GAP proteins are involved in chemical
321 and physical contact in the *Nostoc*-moss symbiosis (Warsham et al., 2017). In
322 *Oryza*, three fasciclin-like arabinogalactan proteins (FLAs), a subclass of
323 arabinogalactan proteins (AGPs), were significantly activated (A2YWR4,
324 A2XUI8 and A2YTW2). FLAs.

325 Cell walls are deeply involved in the molecular talk between partners
326 during plant-microbe interactions. We found a number of proteins annotated in
327 the category of plant cell-wall modification, significantly induced at 7 days post
328 inoculation. In *Oryza*, a cellulose synthase (A2YRG4), an expansin-like EG45
329 domain-containing protein (A2ZA45), a putative endoglucanase (A2XV47) and
330 an omega-hydroxypalmitate O-feruloyl transferase (A2YA31), involved in the
331 synthesis of the suberin polymer, were significantly induced. In *Nostoc*, they
332 included a pullulanase (1,4-alpha-glucan branching enzyme GlgB,
333 Npun_R0164), a putative β -glucosidase (Npun_F2285) and a pectate lyase
334 (Npun_R5506). The later was also upregulated in the exoproteome of *Nostoc* in
335 contact with *Pleurozium schreberi* (Warsham et al., 2017). The activation of
336 these enzymes might reveal a remodelling of the cell plant wall in the presence
337 of the host, as already reported in the *Medicago*-AM symbiosis (Siciliano et al.,
338 2007).

339 Induction of proteins involved in bacterial peptidoglycan recycling and
340 modulation was also detected in *Nostoc*. We found three N-acetylmuramoyl-L-
341 alanine amidases (AmiC1, Npun_F6436 and Npun_R6032), two N-
342 acetylmuramidases (Npun_R4588 and Npun_F5932), a N-acetylmuramic acid
343 6-phosphate hydrolase (Npun_R6016), a L-alanyl-D-glutamate peptidase
344 (Npun_R4589) and a penicillin amidase (Npun_R3418). Up-regulation of these
345 peptidoglycan-modifying enzymes is indicative of strong physiological changes
346 in the cyanobacterium that might be related to the adaptation to the symbiotic
347 lifestyle.

348 3.4.3. Defense response and oxidative stress

349 Convergence between signalling mechanisms in plant pathogenesis and
350 symbiosis is known. Comparison of the two interactions has revealed that plants
351 appear to detect both pathogenic and symbiotic microbes by a similar set of
352 genes (Zhao et al., 2008). We detected in *Oryza* a number of significantly
353 activated proteins involved in defense response. They included three class III
354 peroxidases (A2Y667, A2YHC0 and A2WZD6), three glutathione S-transferases
355 (A2Z9J9, Q6WSC3 and B8A962), a catalase (B8AGH7) and a Gnk2-
356 homologous domain-containing protein (A2XZM8).

357 In *Nostoc*, a number of proteins involved in response to oxidative stress
358 were significantly induced mostly at 7 days in co-culture with the plant. They
359 included two thioredoxins (Npun_R3962 and Npun_R5376), a thioredoxin
360 reductase (Npun_R0672), three catalases (Npun_F0233, Npun_R4582 and
361 Npun_F5237), a peroxidase (Npun_R5469), a superoxide dismutase
362 (Npun_F1605), a flavorubredoxin (Npun_F4866) and four N-terminal domain
363 (NTD) of the photoactive orange carotenoid (OCP)-like proteins (NTD-like
364 proteins) (Npun_F6242, Npun_F6243, Npun_R5130 and Npun_F5913). NTD-
365 like proteins are involved in photoprotection and response to oxidative stress. It
366 is well known that catalases are essential for the establishment of plant-
367 microbial symbioses (Jamet et al., 2003). In that sense, it is important to note
368 that Npun_R4582 and Npun_F5237 were also activated in the cyanobacterium
369 in symbiosis with bryophytes (Ekman et al., 2006; Warshan et al., 2017).

370 3.4.4. Plant flavonoids and cyanobacterial Nod factors

371 Flavonoids are crucial signalling molecules in the symbiosis between legumes
372 and their nitrogen-fixing symbionts (Liu et al, 2016). In *Oryza*, we found a
373 putative flavonoid 3'-monooxygenase (A2Z5X0) involved in the eriodictiol
374 biosynthesis from naringenin. Naringenin is exuded by some legume roots and
375 also acts as an inducer of *nod* genes in rhizobia (Novak et al., 2002). We have
376 found significantly activated in our proteomic analysis a number of *Nostoc*
377 proteins with homology to Nod factor biosynthetic enzymes from *Rhizobium* and
378 *Frankia*. They included a putative NodB polysaccharide deacetylase
379 (Npun_F3876), four putative NodE beta-ketoacyl synthases (Npun_R0041,
380 Npun_R6590, Npun_F3359 and Npun_F3360) and six proteins with homology

381 to NodO (Npun_R6244, Npun_F1217, Npun_DR001, Npun_R3354,
382 Npun_F3370 and Npun_F4679).

383 **3.5. The *Oryza* SYM pathway is required for *Nostoc* colonization**

384 Nod factors (in *Rhizobium*) and MyC factors (in AM) induce symbiotic
385 responses specifically on roots of the plant hosts through the common
386 symbiosis (SYM) pathway (Streng et al, 2011). In *Oryza*, components of the
387 SYM pathway have been already identified (Gutjahr et al., 2008). In order to
388 know the involvement of the SYM pathway in the *Nostoc-Oryza* symbiosis,
389 homozygous *Oryza* mutant lines with insertions in SYM signalling components
390 upstream and downstream of Ca²⁺ spiking, were analysed in *Nostoc*
391 colonization. They included two predicted cation channels, CASTOR and
392 POLLUX, the calcium/calmodulin-dependent protein kinase CCAMK, and
393 CYCLOPS which is phosphorylated by CCAMK. Thus, *Nostoc* hormogonia were
394 inoculated to plants of the different mutants and colonization was evaluated by
395 means of confocal microscopy. We found that colonization was severely
396 affected in all the mutants tested (Figure 5). Their deficient symbiotic
397 phenotypes are consistent with a crucial role of the common SYM pathway in
398 rice.

399

400 **4. DISCUSSION**

401 In this paper, we present data from the first proteomic analysis of the symbiotic
402 interaction between *Oryza* and *Nostoc*. We have identified and quantified
403 protein changes in both organisms at the same time through a novel analytical
404 pipeline, thus facilitating the study of the *Nostoc-Oryza* symbiosis in the host
405 microenvironment. From the whole proteome of both organisms, we have
406 generated species-specific libraries that might be used as reference libraries to
407 future studies. This strategy overcomes traditional proteomic studies in which
408 proteins of both organisms are extracted and analysed separately.

409 Previous studies of colonization of *Oryza* with *Nostoc* strains showed an
410 early phase of contact that started a few hours of co-culture followed by a

second phase of colonization that lasted 20 days (Nilsson et al., 2002; Álvarez et al 2020). This first phase of contact at early stages is critical for the success of the symbiotic process, since is the period when both organisms exchange signals and react to the presence of the partner. We have a total of 1748 proteins differentially expressed along 7 days when both organisms remain in contact. This high number of proteins provides evidences of the complexity of the pre-symbiotic crosstalk between *Nostoc* and *Oryza* and importance for the fruitful of the symbiotic process.

This work is the first to show the *Oryza* proteomic changes in response the symbiotic interaction with a cyanobacterium. However, we find similarities with other symbiotic interactions. As in the case of *Oryza*-AM symbiosis, the plant activates expression of three FLAs (Gutjahr et al., 2015). FLAs are cell wall structural glycoproteins that play a crucial role in plant development, cell adhesion and signalling (Seifert, 2018; Wu et al., 2020; Ma and Zhao, 2010). Induction of FLA proteins in response to *Nostoc* might positively influencing accommodation of the cyanobacterium and its interaction with the plant at early stages of the symbiotic process.

Traditionally, plant symbiotic and pathogenic microorganisms have been considered to operate with different modes of action. However, they both induce in the plant signalling components which facilitate colonization and contribute to both symbiotic and pathogenic relationships (Zeilinger et al., 2015; Hentschel et al., 2020). We find overexpressed in *Oryza* three type III peroxidases that might cope with the production of hydroxyl radicals in the plant roots (Table 1). Type III peroxidases have been found induced in rice-AM symbiosis (Guilmil et al, 2005; Gutjahr et al., 2008, 2015). Although their role in the symbiotic process is not yet well understood, they are important for plant cell growth by nonenzymatic loosening of the cell wall during AM infection (Gutjahr et al., 2008). Interaction with beneficial microbes activates in the host plant defence mechanisms in order to facilitate the symbiosis. Thus, glutathione S-transferase (GST) enzymes are induced in *Oryza* in response to AM symbiosis to cope with toxic reactive oxygen species (ROS) and lipid hydroperoxides (Gutjahr et al., 2015). Our proteomic analysis revealed the expression in response to *Nostoc* of three GST enzymes. It has been shown recently that *N. punctiforme* induces

the expression of GST enzymes in *Arabidopsis* to reduce toxic ROS and limit the extent of the hypersensitive response (Belton et al., 2020). A protein with a Gnk2-homologous domain was also significantly induced in *Oryza* in response to *Nostoc*. Gnk2 domain, similar to calcium-dependent protein kinases from *Arabidopsis*, is found in protein induced by pathogen infection and treatment with ROS or salicylic acid and are involved in the hypersensitive reaction, which is a typical system of programmed cell death (Miyakawa et al., 2009).

In *Nostoc*, 541 proteins were differentially expressed in the presence of *Oryza*. It is important to note that a number of them have been previously described in other *Nostoc*-plant symbioses, reinforcing the robustness of our study. Hence, we find 45 proteins previously highlighted in a supervised machine learning approach of *Nostoc*-Moss symbiosis (Warshan et al., 2017), 8 proteins identified in a previous transcriptomic analysis in response to *Anthoceros punctatus* (Campbell et al., 2008) and 9 proteins in a proteomic analysis of freshly isolated *Nostoc* from the symbiotic gland tissue of the angiosperm *Gunnera* (Ekman et al., 2006). They are involved in signal reception, adhesion, plant cell wall degradation and response to oxidative stress (some of them highlighted in Table 1). The presence of such high number of coincident proteins in the three different *Nostoc*-plant symbiosis might be indicative of the low host selectivity of this symbiotic cyanobacterium. *N. punctiforme*, isolated more than 30 years ago from the cycad *Macrozamia*, is able to establish both epiphytic and endophytic symbiosis with all four of the major phylogenetic divisions of terrestrial plants (Meeks and Elhai, 2002; Adams et al., 2012). In this line, it is easy to imagine that cyanobacteria, unlike rhizobia, have evolved to sense signals from multiple partners spanning nearly the entire plant kingdom.

Whilst confirming the importance of several characterized proteins and molecular pathways in other *Nostoc*-plant symbioses, this study provides novel evidence to support several new proteins and pathways involved in the pre-symbiotic signalling process. We have found a number of putative Nod-like proteins highly expressed in *Nostoc* in response to the plant, luckily producing lipochitooligosaccharides (LCOs), also known as Nod factors (NFs). They comprise a putative NodB polysaccharide deacetylase, four putative NodE

477 beta-ketoacyl synthases and six proteins with homology to NodO. Reinforcing
 478 our results, it has to be noted that *Nostoc* genes with homology to *nodE* had
 479 been already detected by Southern blot in previous studies (Rasmussen et al.,
 480 1996). *In silico* studies in *Nostoc* have recently identified two ORFs encoding
 481 putative homologs of *Rhizobium* NodB and NodC proteins (Gunawardana,
 482 2019). Rhizobial NFs are induced by flavonoids derived from prospective plant
 483 hosts (Mus et al., 2016). That might be the case in the *Nostoc*-plant
 484 endosymbioses. Induction in *Oryza* of a Flavonoid 3'-monooxygenase involved
 485 in the eriodyctiol biosynthesis from naringenin provides first clues of the same
 486 regulation in our system. Whether nod genes from *Nostoc* are regulated by
 487 plant flavonoids and the role of cyanobacterial Nod factors in the *Nostoc*-plant
 488 symbiosis will be addressed in future studies.

489 NFs play a major role in plant colonization and symbiosis (Mbengue et
 490 al., 2020). They produce modifications of the root hair growth leading the
 491 formation of pre-infection threads, providing the entrance to the plant. However,
 492 this might not be the mode of entry of *Nostoc* into the plant roots. We have
 493 observed that the infection started in the plant body and then extended through
 494 the root hairs, being root tips unaffected (Álvarez et al., 2020). In legume-
 495 rhizobia symbiosis NFs induce at the cellular level depolarization of the
 496 cytoplasmic membrane and calcium spiking response, activating genes of the
 497 SYM pathway (Kosuta et al., 2008). Genes involved in the SYM pathway are
 498 invariantly conserved in all land plant species possessing intracellular
 499 symbionts, implying a recruitment of this signalling pathway, independently of
 500 the nature of the intracellular symbiont (Radhakrishnan et al., 2020). Our results
 501 provide compelling evidence of the requirement of the SYM pathway for *Nostoc*
 502 accommodation into the plant roots. However, further studies need to be
 503 conducted to understand the role of this pathway during endophytic interactions
 504 between *Nostoc* and their host plants.

505 Overall, this study provides an excellent resource to study proteomic
 506 changes in interacting partners without altering the host microenvironment. Our
 507 findings reveal molecular pathways activated at earlier stages of the *Nostoc*-rice
 508 symbiosis, providing information about the signals produced by these beneficial

509 bacteria which can have long-term implications towards sustainably improving
510 agriculture.

511

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516

517 **Contributions**

518 CA and VM designed and performed the experiments. CA, MB and VM curated
519 the data and interpreted the results. CA and VM conceived the project and
520 wrote the manuscript. FPM-H and MB discussed the results and contributed to
521 the writing. All authors read and approved the final manuscript.

522

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778 Figures

779 **Figure 1. Association of *Nostoc* to *Oryza* sp. Indica roots.** A)
780 Cyanobacterial association to rice roots, quantified as μg chlorophyll *a*
781 (Chla)·mg⁻¹ root weight. The values are the means $\pm$ SE. B) Appearance of rice
782 roots inoculated with *Nostoc* at 1 and 7 days post inoculation (dpi). C) Rice
783 roots inoculated with *Nostoc* at 1, 7, and 35 days post inoculation (dpi)
784 visualized by a confocal microscope. Stacks generated from 90 to 130 frames
785 are shown. In green, suberin autofluorescence from the plant cell walls; in
786 magenta, cyanobacterial chlorophyll autofluorescence. Scale bar: 100 μm .

787 **Figure 2. Overview the schematic workflow used for quantitative**
788 **proteomic in *Nostoc* and *Oryza*.** The workflow consists of a first step of
789 sample preparation and data acquisition by LC-MS/MS for generation of
790 species-specific spectral libraries. In a second step, relative quantification of
791 protein changes was made by SWATH-MS analysis. The subsequent data
792 normalization was made with *limma* R package to extract changes in protein
793 abundance.

794 **Figure 3. Global protein expression changes in *Oryza* and *Nostoc* during**
795 **co-culture.** Protein expression was analysed by *limma* R package. The number
796 of differentially expressed proteins is shown in green, for proteins significantly
797 upregulated and in red, for proteins significantly downregulated (fold change >
798 1.5 or < 0.66667, and adjust p-value < 0.05).

799 **Figure 4. Heat map profiles of proteins differentially expressed in *Nostoc***
800 **and *Oryza*.** Differentially expressed proteins were clustered into eight groups in
801 *Nostoc* (N1 to N8), and six groups in *Oryza* (O1 to O6) based on their
802 expression profile and *mfuzz* analysis. The legend shows Z-scores. The values
803 were mean centred, and the colours scaled from -3 to +3 standard deviations.

804 **Figure 5. Symbiotic phenotypes of *Oryza sym* mutants.** Rice roots
805 inoculated with *Nostoc* at 35 days post inoculation (dpi) were visualized by a
806 confocal microscope. Stacks generated from 90 to 130 frames are shown. In
807 green, suberin autofluorescence from the plant cell walls; in magenta,
808 cyanobacterial chlorophyll autofluorescence. Scale bar: 200 μm .

Table 1. Selection of proteins significantly activated in *Oryza* and *Nostoc* at 1 day and 7 days of co-culture

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Organism	Anotation	Locus	FC 1d	P value	FC 7d	P value
Signaling						
<i>Nostoc</i>	PtxB	Npun_F2162	25.51	1.3E-06	3.47	3.4E-02
	PtxD	Npun_F2164	1.26	2.8E-01	1.83	4.5E-03
	PtxG	Npun_F2167	1.68	1.4E-01	2.30	1.8E-02
	HmpB	Npun_F5961	0.47	2.4E-04	2.24	7.9E-05
	GAF sensor	Npun_R1597	18.11	1.7E-07	3.91	4.2E-03
	GAF sensor	Npun_F2781	1.13	6.9E-01	2.32	2.0E-03
	Response regulator receiver	Npun_F0021	4.54	5.5E-05	0.88	7.6E-01
	Response regulator receiver	Npun_R0589	2.56	6.0E-04	2.16	3.9E-03
	Response regulator receiver	Npun_F6378	0.99	9.4E-01	2.50	9.5E-08
	Response regulator receiver	Npun_AR130	1.10	8.1E-01	2.34	1.3E-02
	cGMP-specific 3',5'-cyclic phosphodiesterase	Npun_R2479	5.15	1.9E-24	1.68	1.1E-10
<i>Oryza</i>	E3 ubiquitin ligase	B8BET8	0.66	2.6E-01	3.93	4.5E-05
	SH3 domain-containing protein	B8AKM5	0.70	8.9E-02	2.17	1.4E-04
	Importin- α	A2X9V6	5.29	1.1E-05	17.08	1.9E-10
Symbiosis-related proteins						
<i>Nostoc</i>	Putative NodB	Npun_F3876	1.22	7.6E-01	5.76	2.8E-03
	Putative NodE	Npun_R0041	8.34	6.3E-08	0.49	2.7E-02
	Putative NodE	Npun_R6590	2.13	1.8E-04	2.75	2.1E-06

	Putative NodE	Npun_F3359	1.05	6.3E-01	2.72	2.0E-13
	Putative NodE	Npun_F3360	1.85	6.9E-04	1.98	2.0E-04
	Putative NodO	Npun_R6244	2.53	4.6E-02	5.39	4.5E-04
	Putative NodO	Npun_F1217	0.71	1.2E-03	1.91	9.1E-08
	Putative NodO	Npun_DR001	3.98	4.9E-06	1.25	4.2E-01
	Putative NodO	Npun_R3354	1.65	3.9E-04	0.70	7.2E-03
	Putative NodO	Npun_F3370	1.78	2.1E-06	0.56	1.9E-06
	Putative NodO	Npun_F4679	1.57	3.7E-03	0.57	4.0E-04
Oryza	Flavonoid 3'-monooxygenase	A2Z5X0	0.78	6.4E-01	2.23	3.6E-02
	Ankyrin_REP_REGION domain-containing protein	A2XP52	1.06	9.2E-01	2.20	8.5E-03
Cell adhesion						
Nostoc	FG-GAP	Npun_R5402	3.03	1.3E-03	1.08	8.6E-01
	FG-GAP	Npun_R1349	2.51	1.7E-06	0.61	4.0E-03
Oryza	FL-AGP	A2YWR4	2.05	2.5E-01	3.10	3.6E-02
	FL-AGP	A2XUI8	0.78	4.8E-02	2.00	1.7E-07
	FL-AGP	A2YTW2	0.89	5.6E-01	1.64	1.8E-03
Cell wall modification						
Nostoc	Pullulanase	Npun_R0164	1.18	5.4E-02	2.23	1.5E-11
	β -glucosidase	Npun_F2285	0.8	4.4E-03	1.88	2.2E-10
	Pectate lyase	Npun_R5506	1.31	4.6E-04	1.52	8.0E-07
	N-acetylmuramoyl-L-alanine amidases	Npun_F1845	2.07	7.4E-07	1.16	2.5E-01
	N-acetylmuramoyl-L-alanine amidases	Npun_F6436	1.14	9.1E-03	1.82	4.3E-14

	N-acetylmuramoyl-L-alanine amidases	Npun_R6032	2.85	8.8E-03	1.48	3.3E-01
	N-acetylmuramidases	Npun_R4588	3.26	1.6E-06	17.72	3.0E-15
	N-acetylmuramidases	Npun_F5932	1.49	1.9E-06	2.09	4.5E-12
	N-acetylmuramic acid 6-phosphate hydrolase	Npun_R6016	0.74	9.4E-03	2.08	6.8E-08
	L-alanyl-D-glutamate peptidase	Npun_R4589	1.56	2.9E-01	3.64	1.7E-03
	Penicillin amidase	Npun_R3418	2.9	4.8E-03	1.04	9.4E-01
Oryza	Cellulose synthase	A2YRG4	1.13	8.2E-01	2.28	1.4E-02
	Omega-hydroxypalmitate O-feruloyl transferase	A2YA31	1.16	7.6E-01	2.06	2.4E-02
	Putative endoglucanase	A2XV47	1.12	7.8E-01	2.2	3.7E-03
Defense response and oxidative stress						
Nostoc	OCP N-terminal domain-containing proteins	Npun_F6242	2.23	5.1E-07	13.55	2.7E-19
	OCP N-terminal domain-containing proteins	Npun_F6243	0.63	3.6E-01	10.36	9.3E-06
	OCP N-terminal domain-containing proteins	Npun_R5130	1.39	6.5E-02	5.33	1.7E-11
	OCP N-terminal domain-containing proteins	Npun_F5913	1.52	1.7E-01	4.34	9.2E-06
	Thioredoxin	Npun_R3962	0.95	6.5E-01	2.37	1.2E-09
	Thioredoxin	Npun_R5376	1.00	9.7E-01	1.70	1.3E-10
	Thioredoxin reductase	Npun_R0672	0.95	8.3E-01	1.59	2.6E-02
	Catalase	Npun_F0233	0.48	2.5E-02	3.19	5.9E-04
	Catalase	Npun_R4582	1.09	6.2E-01	2.60	1.5E-07
	Catalase	Npun_F5237	1.38	7.3E-07	2.12	3.9E-15

	Peroxidase	Npun_R5469	2.06	4.4E-06	3.01	1.0E-09
	Superoxide dismutase	Npun_F1605	0.95	6.8E-01	1.62	2.1E-05
Oryza	Class III Peroxidases	A2Y667	1.58	1.9E-03	1.35	2.9E-02
	Class III Peroxidases	A2YHC0	0.84	8.5E-01	5.46	5.5E-03
	Class III Peroxidases	A2WZD6	0.99	9.4E-01	1.53	2.6E-04
	Glutathione S-transferases	A2Z9J9	0.87	9.0E-01	4.44	2.0E-02
	Glutathione S-transferases	Q6WSC3	0.86	6.2E-01	1.78	8.9E-03
	Glutathione S-transferases	B8A962	1.58	2.6E-03	0.67	5.6E-03
	Catalase	B8AGH7	1.08	4.8E-02	1.61	7.3E-15
	Gnk-2-homologous domain	A2XZM8	0.73	5.4E-01	3.75	8.9E-04

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A) *Nostoc*

B) *Oryza*

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Álvarez et al. Figure 4

Álvarez et al. Figure 5