Phosphorylation/dephosphorylation response to light stimuli of *Symbiodinium* proteins: specific light-induced dephosphorylation of an HSP-like 75 kDa protein from *S. microadriaticum*

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Background

Some genera of the family Symbiodiniaceae establish mutualistic endosymbioses with various marine invertebrates, with coral being the most important ecologically. Little is known about the biochemical communication of this association and the perception and translation of signals from the environment in the symbiont. However, specific phosphorylation/dephosphorylation processes are fundamental for the transmission of external signals to activate physiological responses. In this work, we searched phosphorylatable proteins in amino acids of Ser, Thr and Tyr from three species of the family Symbiodiniaceae, *Symbiodinium kawagutii*, *Symbiodinium* sp. Mf11 and *Symbiodinium microadriaticum*.

Methods

We used specific antibodies to the phosphorylated aminoacids pSer, pThr and pTyr to identify proteins harboring them in total extracts from three species of *Symbiodinium* in culture. Extractions were carried out on logarithmic phase growing cultures under a 12 h light/dark photoperiod. Various light/dark, nutritional and other stimuli were applied to the cultures prior to the extractions, and proteins were subjected to SDS-PAGE and western immunoblotting. Partial peptide sequencing was carried out by MALDI-TOF on specific protein spots separated by 2D electrophoresis.

Results

At 4 h of the light cycle, several Thr-phosphorylated proteins were consistently detected in the three species suggesting a genus-dependent expression; however, most Ser- and Tyr-phosphorylated proteins were species-specific. Analysis of protein extracts of *S. microadriaticum* cultures demonstrated that the level of phosphorylation of two Thr-phosphorylated proteins with molecular weights of 43 and 75 kDa, responded inversely to a light stimulus. The 43 kDa protein, originally weakly Thr-phosphorylated when the cells were previously adapted to their 12 h dark cycle, underwent an increase in Thr phosphorylation when stimulated for 30 min with light. On the other hand, the 75 kDa protein, which was significantly Thr-phosphorylated in the dark, underwent dephosphorylation in Thr after 30 min of the light stimulus. The phosphorylation response of the 43 kDa protein only occurred in *S. microadriaticum*, whereas the dephosphorylation of the 75 kDa protein occurred in the three species studied suggesting a general response. The 75 kDa protein was separated on 2D gels as two isoforms and the sequenced spots

corresponded to a BiP-like protein of the HSP70 protein family. The presence of differential phosphorylations on these proteins after a light stimulus imply important light-regulated physiological processes in these organisms.

1 **Phosphorylation/dephosphorylation response to light stimuli**

2 **of *Symbiodinium* proteins: specific light-induced**

3 **dephosphorylation of an HSP-like 75 kDa protein from *S.***

4 ***microadriaticum*[§].**

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23 Laboratories of the Caribbean (AMLC) in Mérida, Yucatán, México.

25 **Abstract**

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31 the environment in the symbiont. However, specific phosphorylation/dephosphorylation

32 processes are fundamental for the transmission of external signals to activate physiological

33 responses. In this work, we searched phosphorylatable proteins in amino acids of Ser, Thr and

34 Tyr from three species of the family Symbiodiniaceae, *Symbiodinium kawagutii*, *Symbiodinium*

35 sp. Mf11 and *Symbiodinium microadriaticum*.

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39 proteins harboring them in total extracts from three species of *Symbiodinium* in culture.

40 Extractions were carried out on logarithmic phase growing cultures under a 12 h light/dark

41 photoperiod. Various light/dark, nutritional and other stimuli were applied to the cultures prior to

42 the extractions, and proteins were subjected to SDS-PAGE and western immunoblotting. Partial

43 peptide sequencing was carried out by MALDI-TOF on specific protein spots separated by 2D

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47 At 4 h of the light cycle, several Thr-phosphorylated proteins were consistently detected in the

48 three species suggesting a genus-dependent expression; however, most Ser- and Tyr-

49 phosphorylated proteins were species-specific. Analysis of protein extracts of *S. microadriaticum*

50 cultures demonstrated that the level of phosphorylation of two Thr-phosphorylated proteins with

51 molecular weights of 43 and 75 kDa, responded inversely to a light stimulus. The 43 kDa

52 protein, originally weakly Thr-phosphorylated when the cells were previously adapted to their 12

53 h dark cycle, underwent an increase in Thr phosphorylation when stimulated for 30 min with

54 light. On the other hand, the 75 kDa protein, which was significantly Thr-phosphorylated in the

55 dark, underwent dephosphorylation in Thr after 30 min of the light stimulus. The

56 phosphorylation response of the 43 kDa protein only occurred in *S. microadriaticum*, whereas

57 the dephosphorylation of the 75 kDa protein occurred in the three species studied, suggesting a

58 general response. The 75 kDa protein was separated on 2D gels as two isoforms and the

59 sequenced spots corresponded to a BiP-like protein of the HSP70 protein family. The presence of

60 differential phosphorylations upon light stimulus on these proteins imply important light-

61 regulated physiological processes in these organisms.

62

63 **Introduction**

64

65 The dinoflagellates of the genus *Symbiodinium* are photosynthetic microorganisms that live

66 either freely in the seas or captured within a mutualistic symbiotic relationship in the tissues of

67 some marine invertebrates. In both their free and symbiotic stages, they require fine sensing

68   mechanisms to respond to changing light conditions and other stimuli from the environment that

69   trigger signal-transduction pathways. Such signaling cascades must function with particular sets

70   of receptor, adapter and effector proteins. One particular and fundamental switch to turn on and

71   off signaling within the cell is through exquisitely regulated phosphorylation and

72   dephosphorylation reactions via protein kinases and phosphatases, respectively, on key target

73   molecules. Protein kinases are involved in all cellular functions, and in eukaryotic organisms

74   belong to a superfamily formed by hundreds to thousands of copies. The larger protein kinase

75   repertoire is encoded by flowering plants (600-2500 members; over 1,000 were identified in the

76   *Arabidopsis thaliana* genome), while a smaller number of related genes (only 518 plus 106

77   pseudogenes) were identified in the human genome (The Arabidopsis Genome Initiative 2000;

78   Manning et al., 2001; Lehti-Shiu and Shiu, 2012). In *Symbiodinium* however, the kinase domains

79   are the second most abundant domains in the so far sequenced genomes, with 869 sequences

80   (Pfam PF00069) showing a potential kinase activity (González-Pech et al., 2017; Liu et al.,

81   2018). This abundance highlights the fundamental regulatory functions of the protein kinase

82   superfamily. For example, 177 transcripts from more than 20 serine/threonine-protein kinase

83   families significantly changed their expression levels when *Symbiodinium* sp. (clade F, ITS2)

84   was thermally stressed (Giertz et al., 2017). In another example, the general dephosphorylation

85   of PSII proteins under stress conditions plays an important regulatory role; that is, the

86   dephosphorylation of the D1 and D2 proteins leads to their proteolytic degradation within the

87   PSII repair cycle in response to light stress (Aro and Ohad, 2003; Baena-Gonzales et al., 2004;

88   Rintamaki et al., 1996). Additionally, a rapid dephosphorylation of the proteins of the PSII

89   reaction center seems to be a regulatory reaction to water stress in the photosynthetic membranes

90   of plants (Liu et al., 2009). On the other hand, it is believed that the dephosphorylation of LHCII

91   promotes the association with the PSII to prevent its lateral migration within the thylakoid

92   membrane (Vener, 2007), thus maintaining the LHCII *in situ* for further degradation (Liu et al,

93   2009). These reactions occur through sensing environmental signals by specific receptors at the

94   cell membrane, transducing the signals through adapter/mediator proteins which subsequently

95   turn targets on and off by interaction and/or phosphorylation/dephosphorylation, to finally reach

96   their effectors.

97   Photoreceptors are widely used by organisms to detect and respond to their light environment.

98   Several putative photoreceptors, including phytochromes, cryptochromes, phototropins and

99   rhodopsins, have been identified in the *Symbiodinium* transcript (Xiang et al., 2015).

100   Cryptochromes are receptors for flavoproteins that are sensitive to blue light. They are found in

101   plants, animals, insects, fungi and bacteria (Chaves et al., 2011), and have recently been shown

102   to respond to both blue and red light in *Chlamydomonas reinhardtii* (Beel et al., 2012). Studies

103   in animals and plants suggest that cryptochromes play a critical role in the generation and

104   maintenance of circadian rhythms (Chaves et al., 2011). It is possible that *Symbiodinium*

105   cryptochromes are involved in the circadian control of several light sensitive processes (Jones

106   and Hoegh-Guldberg, 2001; Sorek et al., 2014), especially because blue is the main quality of

107   light that penetrates water column in the oceans. Unfortunately, no detailed information on the

108   possible light-sensing mechanisms and/or consequent signaling cascades in marine

109   dinoflagellates is available.

110   Since there is very little information from the genus *Symbiodinium* regarding proteins that

111   participate in key phosphorylation processes for signal-transduction events that arise from

112   environmental stimuli, we sought to identify phosphorylated proteins from *S. kawagutii*, *S.*

113   *microadriaticum* and *S*. Mf11. We identified a 75 kDa protein that was susceptible of changes in

114   its phosphorylation levels upon a light stimulus. Our findings indicate that light is an important

115   switch that impacts the phosphorylation status of several proteins in *Symbiodinium*. In particular,

116   the 75 kDa protein may be regulated through putative conserved regulatory mechanisms of

117   phosphorylation/dephosphorylation since its presence and light-stimulated dephosphorylation

118   response were found in all three *Symbiodinium* species analyzed.

119

120   **Materials and methods**

121

122   **Antibodies and reagents**

123

124   Polyclonal anti-phosphothreonine (anti-pThr cat. 9381S) and anti- phosphoserine (anti-pSer cat.

125   2261S) antibodies were from Cell Signaling Technology ™, Inc. (Danvers, MA). Monoclonal

126   anti-phosphotyrosine antibodies (anti-pTyr sc-508) were from Santa Cruz Biotechnology (Dallas,

127   TX). Monoclonal anti-actin antibody N350 was from Amersham; this antibody is commercially

128   discontinued but we still maintain an available stock (Villanueva et al., 2014). Alkaline-

129   phosphatase (AP) conjugated polyclonal anti-rabbit IgG and anti-mouse IgG antibodies raised in

130   goat were from Zymed™-Life Technologies (Grand Island, NY). The tripeptides RAD (Arg-

131   Ala-Glu) o RGD (Arg-Gly-Glu) were from CALBIOCHEM®-Millipore (Billerica, MA).

132   Ethylene glycol-bis(2-aminoethylether)-NNN´N´-tetraacetic acid (EGTA), bovine casein

133   hydrolyzate (N-Z-Amine A), and the amino acids, glutamic acid, arginine and glycine, were all

134   from Sigma. Reagents 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium

135   (NBT) were from Promega (Madison, WI).

136

137   *Symbiodinium* **cell cultures**

138

139   Dinoflagellate cultures of *Symbiodinium kawagutii* (referred as *S. kawagutii*) were a kind gift of

140   Dr. Robert K. Trench (University of California at Santa Barbara). *Symbiodinium* sp. Mf11.5b.1

141   (referred as *S*. Mf11), and *Symbiodinium microadriaticum* Subsp. *microadriaticum* (also known

142   as MAC-CassKB8 and from now on referred to as *S*. KB8) originally isolated from the jellyfish

143   *Cassiopea xamachana,* were a kind gift of Dr. Mary Alice Coffroth (State University of New

144   York at Buffalo). These cell lines correspond to clades F, B and A, respectively; they were

145   routinely maintained in our laboratory in ASP-8A medium under photoperiod cycles of 12 h

146   light/dark at 25°C. Light intensity was maintained at 80-120 μmole quanta m^{-2} sec^{-1}.

147

148   **Preparation of total protein extracts for phospho protein screening and responses to stimuli**

149

150   Six-d-old *Symbiodinium* cells in ASP-8A culture medium were collected 4 h after the light phase

151   of the photoperiod initiated, and were concentrated by centrifugation at 1,400 x *g* for 8 min. The

152   cell pellet was suspended with 500 μl of 1X Laemmli buffer (60 mM Tris-HCl, pH 6.8, 1 %

153   (w/v) sodium dodecyl sulfate (SDS), 10 % (v/v) glycerol, 3 % (v/v) β-mercaptoethanol, 0.02 %

154   bromophenol blue) (Laemmli, 1970), supplemented with 0.2 mM NaVO$_3$, 10 mM NaPPi and a

155   cocktail of protease inhibitors (Complete®, Roche, Basel, Switzerland), and placed in screw-cap

156   polypropylene vials with a 0.25 ml total volume of glass beads (465-600 μm diameter)

157   previously cooled. Subsequently, the cells were lysed with vigorous shaking in a MINI-BEAD

158   BEATER™ (Biospec products) at maximum speed for 3 min. Then, the cell lysate was heated at

159   95°C for 5 min, and centrifuged at 16,000 x *g* for 10 min. The supernatant was used for analysis

160 in polyacrylamide gels by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel

161 electrophoresis) and western blot.

162

163 **Protein electrophoresis in SDS-PAGE gels and western blot**

164

165 The protein extracts were separated in discontinuous denaturing gels (Laemmli 1970), of 10 %

166 polyacrylamide in the separation zone [375 mM Tris-HCl, pH 8.8; 10 % acrylamide/bis-

167 acrylamide; 0.1 % SDS; 0.1 % ammonium persulphate (APS); 0.106 % N, N, N, N'-

168 tetramethylethylenediamine (TEMED)], and 4 % polyacrylamide in the stacking zone [125 mM

169 Tris-HCl, pH 6.8; 4 % acrylamide; 0.137 % bisacrylamide; 0.1 % SDS; 0.1 % APS; 0.066 %

170 TEMED], in a Mini-PROTEAN®3 System (Bio-Rad, Hercules, CA). After electrophoresis, the

171 proteins were transferred to PVDF membranes in "friendly buffer" (25 mM Tris-HCl, 192 mM

172 glycine, 10 % isopropanol, Villanueva, 2008) at a constant current of 300 mA for 1 h. The

173 membranes were blocked in a solution of 3 % bovine serum albumin (BSA) in PBS (2.79 mM

174 NaH_2PO_4, 7.197 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.5) for 1 h at 50°C, with gentle agitation.

175 After blocking, the primary antibodies, anti-pThr (1:2,500), anti-pSer (1:500), anti-pTyr (1:500)

176 or anti-actin (1:1,000) diluted in PBS containing 0.01 % Triton X-100 (PBS-T), were added to

177 the membranes and incubated overnight with gentle rocking at room temperature. Then, the

178 membranes were washed five times, 5 min each, in PBS-T, and incubated with the appropriate

179 secondary antibodies (alkaline-phosphatase conjugated anti-rabbit IgG or anti-mouse IgG) at

180 1:2,500 dilution for 2 h at room temperature. Subsequently, the membranes were washed again

181 five times, 5 min with PBS-T. Finally, they were developed with a commercial solution of 5-

182 bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) according to

183 manufacturer (Promega, Madison, WI) in PBS-T for the primary polyclonal antibodies to ensure

184 more astringency and less background. In the case of primary monoclonal antibodies, the final

185 wash was followed by a brief rinse with alkaline developing solution (100 mM Tris-HCl, 150

186 mM NaCl, 1 mM $MgCl_2$, pH 9), and development in the same buffer. Western blot incubations,

187 washing and development were also carried out in PBS-T or TBS-T (20 mM Tris, 150 mM

188 NaCl, 0.01 % TX-100, pH 7.6) with identical results.

189

190 **Two-dimensional gel electrophoresis and partial peptide sequencing**

191

192 Six-d-old cell cultures of *S*. KB8 collected 2 h before the dark phase of the photoperiod, were

193 concentrated by centrifugation and suspended in 50 ml of fresh ASP-8A medium. After a 12 h

194 incubation in the dark, the cells were precipitated by centrifugation at 2,600 x *g*, 5 min, 26°C and

195 suspended in 10 ml of 0.2 mM $NaVO_3$ and 10 mM NaPPi in PBS-T added with a Complete®

196 (Roche, Basel, Switzerland) cocktail of protease inhibitors,

197 taking special care to carry out the whole procedure under dark conditions. The cells were then

198 disrupted with a French press (SLM-AMINCO French® pressure cell press) at a pressure of

199 20,000 psi. Then, the cell lysate was chilled on ice and centrifuged at 100,000 x *g*, 1 h to recover

200 the proteins from the supernatant.

201 Next, the protein pellet was dissolved with seven volumes of PB (2.79 mM NaH_2PO_4, 7.197 mM

202 Na_2HPO_4, pH 7.6) and applied to a column with 1 ml matrix volume of DEAE-sephacel

203 previously equilibrated with 50 ml 0.02 M NaCl in PB. Then, the column was washed with 10 ml

204 of the same buffer and eluted with 2 ml of 0.4 M NaCl in PB. The eluted proteins were

205 precipitated with 8 volumes of cold acetone (at -20°C), and one volume of trichloroacetic acid

206   (TCA) for 1 h at -20°C. Then, the mixture was centrifuged at 5,000 x *g* for 5 min and the protein

207   pellet was washed twice with 2 ml of acetone at -20°C. Afterwards, the proteins were dissolved

208   in BioRad® ReadyPrep buffer (8 M Urea, 2 % CHAPS, 50 mM DTT, 0.2 % w/v ampholytes

209   3/10 BioLite® from BioRad and bromophenol blue), and the solution was used to hydrate a 17

210   cm IPG strip (ReadyStrip pH 4-7, Bio-Rad, Hercules, CA) for 12 h at 18°C. Then, the proteins

211   adsorbed in the strip were separated with a programmed current of 250 V, 20 min, 4,000 V, 2 h

212   and 4,000 V to cover 10,000 V-h in a PROTEAN IEF cell (Bio-Rad, Hercules, CA).

213   Subsequently, the proteins in the strips were first denatured in 1.5 ml of Buffer I® (Bio-Rad; 6M

214   urea, 2 % SDS, 0.375M Tris-HCl pH 8.8, 20 % glycerol and 2 % w/v DTT) for 15 min, and then

215   in 1.5 ml of Buffer II® (Bio-Rad; 6 M urea, 2 % SDS, 0.375 M Tris-HCl pH 8.8, 20 % glycerol

216   added with 2.5 % w/v iodoacetamide) for 15 min. Then, the strip was briefly rinsed in running

217   buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3.) and loaded onto a 10 %

218   polyacrylamide SDS-PAGE gel to separate the proteins in the second dimension. Finally, the

219   proteins were either stained with colloidal coomassie blue, or transferred onto a PVDF

220   membrane for analysis with anti-pThr antibodies. The gel spots corresponding to the anti-pThr

221   immunodetected signal were excised with a pipet tip, placed in a sterile eppendorf tube with 50

222   µL milliQ water, and sent for partial peptide sequencing to the Unit of Proteomics of the Institute

223   of Biotechnology from UNAM in Cuernavaca, Morelos, México. Each spot was also reanalyzed

224   by western blot with the anti-pThr antibodies to ensure their identity. In parallel, an adjacent

225   mock spot corresponding to a major Mr ~ 77 kDa protein was included as negative control for

226   the analysis. The most probable sites of phosphorylation in threonine from the sequences were

227   predicted by the NetPhosK server (Blom et al., 2004).

228

Manuscript to be reviewed

229 **Light treatments on *Symbiodinium* cells adapted to darkness**

230

231 Six-d-old cultures from *S*. KB8, *S*. Mf11 and *S. kawagutii* from three biological replicates

232 collected 2 h before of the dark phase of the photoperiod, were concentrated and suspended in 40

233 ml of fresh ASP-8A medium to reach 0.8-1.4 x 10^6 cells/ml. Equal aliquots of each phylotype

234 were placed in four 15 ml Falcon tubes wrapped with aluminum foil. The cells in the tubes were

235 allowed to adapt in the dark (12 h) during their night cycle. After this period, the cells from one

236 of the tubes were concentrated and broken with glass beads without exposing them to light

237 throughout the process (control), and the other three tubes were released from the aluminum foil

238 to expose them to light. Protein extractions with glass beads were performed at 30, 60 and 240

239 min after light exposure, and the supernatant from the lysate was denatured 1:1 in 2X Laemmli

240 buffer, heated at 95° C, 5 min, and used for the analysis. Equal loads of proteins were adjusted

241 with equal aliquots of cells and also standardized by staining with coomassie blue in 10 %

242 polyacrylamide SDS-PAGE gels. Finally, the extracts were analyzed by western blot which

243 included actin as an additional internal loading control. The bands from the triplicate samples

244 were captured with a ChemiDoc-It2 Imager (UVP-Analytik Jena, Upland, CA, USA) and

245 analyzed by densitometry with the system's VisionWorks LS software, normalized with the

246 internal actin control to obtain an arbitrary level of intensity for each antibody reaction. The

247 results were integrated into graphs displaying the average band intensity of the three biological

248 replicates for each treatment.

249

250 **Treatments of *Symbiodinium* cells with various stimulating agents**

251

252   Six-d-old *S.* KB8 cells from a 1 L culture were collected by centrifugation at 1,400 x *g* for 8 min,

253   and suspended in 10 ml fresh medium, they were then divided into 9 aliquots of 1 ml in 15 ml

254   Falcon tubes. The cells were concentrated again by centrifiugation as above and the pellets were

255   resuspended in: a) 10 ml of fresh ASP-8A medium (control); or b) 10 ml fresh medium

256   supplemented with either 0.1% (w/v) casein, 10 mM glycine, 10 mM arginine, 10 mM glutamic

257   acid, 100 µg/ml of the tripeptides RAD (Arg-Ala-Glu), RGD (Arg-Gly-Glu) (CALBIOCHEM®-

258   Millipore; Billerica, MA), 15 mM EGTA (ethylene glycol-bis (2-aminoentylether)-NNN'N'-tetra

259   acetic acid), or 20 mM $CaCl_2$. All samples were incubated for 30 min at room temperature and

260   after the incubations, the cells were concentrated by centrifugation followed by protein

261   extraction with glass beads using the Bead Beater. Equal loads of proteins were analyzed by

262   protein staining on SDS-PAGE and western blot with anti-pThr antibodies.

263

264   **Results**

265

266   **Anti-pTyr, -pSer and -pThr antibodies immunodetect several phosphoproteins from *S.***

267   ***kawagutii*, *S*. KB8 and *S*. Mf11 total extracts.**

268

269   Protein extracts from the three phylotypes under study were analyzed with the three anti-

270   phosphoaminoacid antibodies by western blot. Anti-pTyr antibodies revealed intense bands with

271   Mr ~ 32 kDa in all three species (asterisk in Fig. 1C, lanes 1-3, respectively, and Fig. S1C),

272   whereas species-specific bands of Mr's ~ 43, 57, 62 and 77 kDa were observed for *S*. KB8 (Fig.

273   1C, lane 1, and Fig. S1C, lane 1); of Mr's ~ 30 and 54 kDa for *S*. Mf11 (Fig. 1C, lane 2, and Fig.

274   S1C, lane 2); and of Mr's ~ 48, 51 and 79 kDa for *S. kawagutii* (Fig. 1C, lane 3, and Fig. S1C,

275   lane 3), indicating that these bands corresponded to proteins phosphorylated on Tyr residues but

276   were not ubiquitous, except for the Mr ~ 32 kDa protein. Anti-pSer antibodies revealed intense

277   bands with Mr ~ 65 kDa in all three species (Fig. 1B, asterisk in lanes 1-3, and Fig. S1B, lanes 1-

278   3), and a weaker Mr ~ 35 kDa band also in the three species analyzed (asterisk in Fig. 1B, lanes

279   1-3, and Fig. S1B, lanes 1-3). A relatively high number of weaker bands both ubiquitous and

280   species-specific, were also detected with these antibodies in the three species analyzed (Fig. 1B,

281   lanes 1-3, and Fig. S1B, lanes 1-3). These results indicated that a considerable number of

282   proteins from all three species were phosphorylated on Ser residues. Finally, anti-pThr antibodies

283   revealed several phospho proteins that were present in all three species analyzed. Here, we

284   observed weak bands of Mr's ~ 43, 46, 50 and 55 kDa (asterisks in Fig. 1C, lanes 1, 2 and 3),

285   and two clear bands with Mr's ~ 75 (double asterisk in Fig. 1A, lanes 1-3, and Fig. S1A, lanes 1-

286   3) and 91 kDa (asterisk in Fig. 1A, lanes 1-3, and Fig. S1A, lanes 1-3). Additionally, the anti-

287   pThr antibodies detected a clear band of Mr ~ 29 kDa present in *S*. KB8 and *S*. Mf11 (Fig. 1A,

288   lanes 1 and 2, and Fig. S1A, lanes 1 and 2) as well as a clear Mr ~ 31 kDa protein band present

289   only in *S. kawagutii* (Fig. 1A, lane 3, and Fig. S1A, lane 3), and a Mr ~ 107 kDa protein band

290   present only in *S*. KB8 (Fig. 1A, lane 1, and Fig. S1A, lane 1). All the proteins found to cross-

291   react with the anti-phospho amino acid antibodies and their corresponding Mr's are shown in

292   Fig. S1. These data indicated: a) the presence of proteins phosphorylated on Thr residues; and b)

293   that there were phosphorylated proteins on the amino acids Tyr, Ser and Thr, in all three

294   *Symbiodinium* species. One protein of Mr ~ 75 kDa, which at this point we named pp75, fulfilled

295   our criteria of being present in all three species and presenting a clear reproducible reaction upon

296   developing by western blot. Therefore, we chose to further analyze this protein for possible

297   changes upon treatment of the cells with light/dark and other stimuli.

298

Some phosphorylated proteins in threonine responded to a light stimulus

300

301 Analysis by western blot with anti-pThr in cells from all three species adapted to darkness during

302 the dark phase of the 12-h photoperiod showed that pp75 maintained a considerable degree of

303 apparent phosphorylation under this condition (Fig. 2A-C, lanes 1; arrow labeled 75).

304 Conversely, when the cells were stimulated with light for 30 min and analyzed by western blot,

305 the intensity of pp75 decreased significantly (Fig. 2A-C, lanes 2; arrow labeled 75). The weak

306 intensity of the band was maintained even after 60 and 240 min of exposure to light (Fig. 2A-C,

307 lanes 3 and 4, respectively; arrow labeled 75). These results indicated that this protein, during the

308 dark phase, is significantly phosphorylated on Thr; however, it dephosphorylates after 30 min of

309 a "shock" of light and remains dephosphorylated for at least 240 min of such light exposure.

310 Interestingly, the opposite behavior was clearly observed for a Mr ~ 43 kDa protein only in *S.*

311 KB8 cells before the light stimulus. This protein was not observed as phosphorylated in Thr

312 when the cells were adapted to darkness (Fig. 2A, lane 1; arrow labeled 43). However, after 30

313 min of light exposure, the anti-pThr antibodies detected an intense band, indicating an enhanced

314 phosphorylated state (Fig. 2A, lane 2; arrow labeled 43), which was maintained even after 60 and

315 240 min of exposure to light (Fig. 2A, lanes 3 and 4, respectively; arrow labeled 43). Species-

316 specific light-stimulated phosphorylation behaviors were observed for a number of proteins

317 immunodetected with anti-pThr antibodies. One Mr ~ 29 kDa protein with similar behavior was

318 detected in *S.* KB8 and *S.* Mf11 (Fig. 2A and B, lanes 1-4; arrow labeled 29), two of Mr's ~ 40

319 and 107 kDa in *S.* KB8 (Fig. 2A, lanes 1-4; arrows labeled 40 and 107, respectively); and finally,

320 one of Mr ~ 31 kDa from *S. kawagutii* (Fig. 2C, lanes 1-4; arrow labeled 31). The

321   dephosphorylation of pp75 on Thr was consistently and reproducibly observed in cells from all

322   three species when the light stimulus was applied in comparison to the cells adapted to darkness

323   before the stimulus. The revealed bands were specific and the whole membrane showed specific

324   reactions of the 75 and 43 kDa bands upon development of the blot (Fig. 2). In both cases, we

325   quantified the response by densitometric analysis of the blots using triplicate biological samples

326   and the intensity of the bands was normalized to the reaction of an anti-actin monoclonal

327   antibody as internal control. The results were normalized, averaged, subjected to ANOVA, and

328   graphed. In all cases, a statistically significant decrease in the phosphorylation level of pp75 in at

329   least one time point after the light treatment was detected for all three species analyzed (Fig. 3A;

330   *S.* KB8, lane 3; *S.* Mf11, lanes 2 and 3; *S. kw*, lane 4). The same analysis was carried out on the

331   43 kDa protein with similar results showing the opposite behavior for *S.* KB8 but not for the

332   other two species (Fig. 3B). Although we cannot assure that they are the same as the 43 kDa

333   protein that responds to light in *S.* KB8, Mr ~ 43 kDa proteins from *S.* Mf11 and *S. kawagutii*

334   cells, also recognized by the anti-pThr antibodies did not change their level of apparent

335   phosphorylation after equivalent times after the light stimulus (Fig. 2B and C, lanes 2-4; Fig. 3B,

336   *S.* Mf11 and *S. kw*, respectively), compared to the control (cells adapted to darkness, Fig. 2B and

337   C, lanes 1; Fig. 3B, *S.* Mf11 and *S. kw*, respectively). This indicated that the Mr ~ 43 kDa protein

338   may have a species-specific behavior and only pp75 fulfilled the criterion of ubiquity in at least

339   all three *Symbiodinium* species analyzed.

340

341   **Threonine-phosphorylated proteins from *S.* KB8 did not show differential phosphorylation**

342   **in response to other stimuli**

343

344   In addition to light, we sought to stimulate *S*. KB8 cells in culture in order to observe possible

345   changes in phosphorylation levels of proteins and relate those changes with other metabolic or

346   signal-transduction pathways. Nevertheless, no changes were detected upon any of the

347   treatments. None of the nutritional stimuli provided by incubation with the amino acids: glycine,

348   arginine, glutamate, or a casein hydrolyzate caused significant changes in the degree of apparent

349   phosphorylation in Thr of any protein including pp75 (Fig. S3, lanes 2, 3, 4 and 5, respectively),

350   compared to the control (Fig. S3, lane 1). In parallel, extracellular matrix stimuli were tested

351   with the tripeptide RGD (Arg-Gly-Asp), which is an inhibitor of the adhesion of integrins to their

352   receptors. These RGD tripeptides did not cause any change on the phosphorylation of the

353   analyzed proteins in *S*. KB8 cells (Fig. S3, lane 6), compared to the control without stimulation

354   (Fig. S3, lane 1). As expected, the non-active tripeptide control RAD (Arg-Ala-Asp), did not

355   cause any significant effect either (Fig. S3, lane 7). Finally, no alterations were observed in the

356   phosphorylation pattern when the $CaCl_2$ concentration was increased in the medium (Fig. S3,

357   lane 8), or when calcium ions were sequestered with EGTA (Fig. S3, lane 9), compared to the

358   control (Fig. S3, lane 1). These results support the role of specific pThr phosphorylation as part

359   of a light-regulated response in *Symbiodinium*.

360

361   **The threonine-phosphorylated 75 kDa protein from *S*. KB8 is a BiP-like protein of the**

362   **HSP70 protein family**

363

364   The light-responding pp75 from *S*. KB8 was enriched through anion exchange column

365   chromatography on DEAE-sephacel and the eluted fraction precipitated with ice-cold acetone-

366   TCA, followed by analysis and separation by two-dimensional gel electrophoresis as described

367   in Materials and Methods. The western blot of the separated proteins immunodetected with anti-

368   PThr antibodies identified two Mr ~ 75 kDa phosho protein spots of pI's ~ 5.4 and 5.6 (Fig. 4A,

369   spots 1 and 2, respectively), indicating the presence of at least two isoforms of pp75. Only a few

370   spots corresponding to the Mr's of the phospho proteins previously immunodetected by the anti-

371   PThr antibodies from *S*. KB8 extracts (Fig. S1A, lane 1) were observed, whereas a complex

372   mixture of proteins was observed in the equivalent coomassie blue-stained 2D gel (Fig. 4B, spots

373   1 and 2). In addition, the excised spots as well as an adjacent mock spot (Fig. 4B, M)

374   corresponding to a major protein of similar Mr, were re-analyzed by western blot with anti-PThr

375   antibodies and only the lanes corresponding to the phospho protein spots were immunostained

376   (Fig. S4, lanes 1 and 2), while the mock spot showed no cross-reaction (Fig. S4, lane 3). Both

377   spots were sent for a partial peptide sequencing service and the identity from either spot matched

378   a BiP-like protein sequence belonging to the HSP70 protein family from the *S. microadriaticum*

379   transcriptome with 100 % identity (Fig. 4C, Spot 1 and 2 sequences in red; Accession No.

380   OLP91134). It is important to note that the reported sequence displayed a putative molecular

381   weight of >200 kDa for the translated product, while pp75 migrates at Mr ~ 75 kDa. Therefore,

382   we analyzed this sequence further by comparing the corresponding genomic and transcriptomic

383   sequences. This comparison revealed the correct start ATG and stop codons that resulted in the

384   correctly translated product of 75 kDa. This corrected sequence is shown in Fig. 4C and it was

385   used for a BLAST analysis against the *S. microadriaticum* transcriptome, which yielded at least

386   other four related sequences encoding HSP70-like proteins (with long enough sequence showing

387   relevant domains; Accession No's. OLQ05431, OLP7913, OLP80221 and OLP86850), and one

388   likely related short sequence (Accession No. OLP81269). Three of these sequences, including

389   pp75, corresponded to BiP-like proteins (Accession No's. OLP91134, OLP86850 and

390   OLP81269). The sequence also contained 6 potential phosphorylated threonine residues (Fig. 4C,

391   green Thr residues). These results indicated that the Mr ~ 75 kDa protein from *S*. KB8 displaying

392   differential phosphorylation on Thr upon a light stimulus was inequivocally identified as a BiP-

393   or HSP70-like protein. Therefore, we renamed the protein as SmicHSP75.

394

395   **Discussion**

396

397   *Symbiodinium* usually lives as endosymbiont in reef hosts (Muscatine and Porter, 1977). Due to

398   its photosynthetic nature, it must rely on fine sensing mechanisms to respond to changing light

399   conditions in its environment, which must also be transduced to molecular signals that become

400   physiological responses. Phosphorylation and dephosphorylation events are major cellular

401   switches that trigger cellular responses upon environmental stimuli and are likely to play an

402   important role in such fine sensing mechanisms (Graves and Krebs, 1999). Since there is very

403   little information regarding proteins that participate in key phosphorylation processes for signal-

404   transduction events from the genus *Symbiodinium*, we sought to identify phosphorylated proteins

405   on the amino acids, threonine, serine and tyrosine from three different *Symbiodinium* species, *S*.

406   *kawagutii*, *S*. KB8 and *S*. Mf11. We further sought to identify a protein that was susceptible of

407   changes in its phosphorylation levels upon various types of stimulus. Several proteins containing

408   phosphorylated amino acids were identified, although there were variations in the three species

409   analyzed. Therefore, we chose to focus our analysis on proteins that fulfilled two criteria. First,

410   that their cross-reaction with the antibody was clear and reproducible. Second, that their presence

411   was not species-specific so that they were observed in all three species analyzed. Our first

412   screening with anti-pThr, anti-pSer and anti-pTyr antibodies, revealed several candidate proteins

413   (Fig. S1), indicating that phosphorylation is an important feature of *Symbiodinium* proteins,

414   which is also consistent with the hypothesis that such proteins could be key players of signaling

415   events. A first attempt revealed only a few bands; however, we found two critical conditions that

416   improved the signal-to-noise ratio significantly: first, when we used PBS-T for development of

417   the incubations with polyclonal primary antibodies but alkaline developing solution for

418   monoclonal primary antibodies (see Materials and Methods), more and stronger bands appeared;

419   second, the use of PVDF instead of nitrocellulose as transfer media increased the band resolution

420   significantly. Therefore, we used these conditions for all subsequent western blot experiments.

421   The proteins detected with anti-pThr antibodies with Mrs ~ 43, 46, 50, 55, 75 and 91 kDa, were

422   present in all three species (Figs. 1A and S1A). A relatively high number of bands both

423   ubiquitous and species-specific were also detected with anti-pSer antibodies (Figs. 1B and S1B),

424   as well as with anti-pTyr antibodies (Figs. 1C and S1C), but none fulfilled the criteria of both

425   clear identification and presence in all three species. Conversely, one protein cross-reactive with

426   anti-pThr antibodies, that of Mr ~ 75 kDa (pp75), that was present in all three species, gave a

427   strong cross-reaction with the anti-pThr antibodies (Fig. 1A), and underwent significant changes

428   in its phosphorylated state upon a light stimulus (Figs. 2A and 3A). This protein displayed at

429   least two isoforms of the same molecular weight (Fig. 4A, spot 1 and 2), as immunodetected

430   with the anti-pThr antibodies likely indicating a differential degree of phosphorylation. This is

431   consistent with the modifications in the phosphorylation level detected upon light exposure of

432   the cells (Fig. 3A). The partial peptide sequencing of this protein allowed its inequivocal

433   identification as a BiP-like protein of the HSP70 protein family (Accession No. OLP91134) from

434   *S. microadriaticum*. We therefore re-named pp75 as SmicHSP75. Since the annotated

435   transcriptomic sequences are uncured raw sequences, and the one corresponding to SmicHSP75

436   yielded a translated product of >200 kDa, we took the full SmicHSP75 genomic sequence from

437   the *S. microadriaticum* genome (Aranda et al., 2016) and used it to obtain the correct amino acid

438   sequence (Fig. 4C). When we used this corrected sequence for BLAST analysis against the

439   transcriptome, we detected at least six isoforms of the protein, as well as a conserved Thr in such

440   sequences (Fig. 4C, asterisk). This conserved Thr has been reported as a target of

441   phosphorylation in *C. reihardtii*, involved in metabolic regulation upon a nutritional stimulus

442   (Díaz-Troya et al., 2011). In addition, we detected six potential phosphorylated threonine

443   residues (Fig. 4C, green Thr residues), which are consistent with the possibility that the

444   identified isoforms could correspond to different combinations of phosphorylations. The

445   subsequent finding that this protein was originally phosphorylated on Thr during the dark phase

446   of the *Symbiodinium* growth cycle, and was dephosphorylated upon light exposure, indicated that

447   light was an important signal that triggered a number of signaling cascades that lead to its

448   dephosphorylation. This also implied the existence of a phosphatase that must be able to rapidly

449   respond to the light stimulus and act upon SmicHSP75. Another protein of Mr ~ 43 kDa present

450   in all three species and cross-reactive with anti-pThr antibodies appeared to undergo a gradual

451   enhanced phosphorylation upon light exposure but this modulatory effect was detected

452   exclusively in *S*. KB8 (Fig. 3B, *S*. KB8). This suggested that it was part of a species-specific

453   process; therefore, our subsequent analyses were performed exclusively on SmicHSP75, which

454   was present in all three species.

455   We do not know which other proteins participate both upstream and downstream of the signaling

456   cascade of SmicHSP75 but the dephosphorylation response also implies the existence of a

457   photoreceptor which transmits the signal to other relay molecules that lead to the action of the

458   yet unknown phosphatase. Similarly, unknown molecules downstream of SmicHSP75 must

459   exist, to transduce the signal into a physiological response. The fact that the protein is highly

460   phosphorylated during the dark phase and a dephosphorylation occurs upon the light stimulus,

461   points towards two other important implications: first, there must be a kinase that phosphorylates

462   the protein at the end of the light phase and during the dark phase of the cycle; and second, there

463   must also be a phosphatase that responds upon the activation of the molecular switch that is

464   triggered after 30 min of a light stimulus. This is also suggestive of a mechanism that puts the

465   cell into a new metabolic (photosynthetic) state after the light is turned on. This may also reflect

466   a light-sensing mechanism occurring under the natural state of the free-living and/or

467   endosymbiotic cells in the water column after sunrise. It has been reported that blue light is most

468   likely the type of radiation that enters the water column (Braun and Smirnov, 1993) and

469   perception mechanisms by chryptochromes are likely to be at play under those conditions. We

470   are currently searching for the companion proteins of SmicHSP75 in this interesting light-

471   modulated interplay.

472   Besides light, other stimuli might also cause changes in phosphorylation on the same protein. We

473   tested a number of such stimuli that included nutritional (casein hydrolyzate and amino acids),

474   extracellular matrix stimulating peptide (RGD), and calcium exposure and depletion (Ca^{2+} and

475   EGTA). In none of the cases significant dephosphorylation of SmicHSP75 or enhanced

476   phosphorylation of the 43 kDa protein were observed (Fig. S3). This indicated that

477   *Symbiodinium* cells are much more sensitive to light than other stimuli, which is also consistent

478   with their photosynthetic nature.

479   Our results represent pioneer studies on proteins that are likely to participate in light modulated

480   signal-transduction events and that have not been studied in these symbiotic photosynthetic

481   microorganisms to date. Future research will focus on the detailed functional characterization of

482   this light-responsive protein target and its companion players in the light-sensing mechanism.

483

484   **Conclusions**

485

486   We identified a 75 kDa protein (SmicHSP75) cross-reactive with anti-pThr antibodies, that was

487   present in the three species of *Symbiodinium*, *S.* KB8 (*S. microadriaticum*), *S. kawagutii* and *S.*

488   Mf11. The protein was significantly phosphorylated at the end of the dark phase of the growth

489   cycle but underwent a significant decrease in its phosphorylated state after 30 min of a light

490   stimulus, and remained hypophosphorylated even after 260 min. This

491   phosphorylation/dephosphorylation behavior indicated that light was an important signal that

492   triggered a number of signaling cascades that are likely to be transient and this protein could be

493   part of a mechanism that warns the cell to switch to a new (photosynthetic) metabolic state. The

494   protein was inequivocally identified as a BiP-like protein of the HSP70 protein family.

495

496   **Acknowledgements**

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498

499   **References**

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590

591   **Figure Legends**

592

593   Figure 1. Immunodetection by western blot of phospho amino acids present in proteins of three

594   species of *Symbiodinium* with anti-pThr (A), anti-pSer (B) and anti-pTyr (C) antibodies. In the

595   figure, the lanes correspond to: molecular weight markers (Mw); protein extracts of

596   *Symbiodinium* KB8 (lanes 1), *Symbiodinium* Mf11 (lanes 2) and *Symbiodinium kawagutii* (lanes

597   3). The asterisks within the figure indicate protein bands immunodetected in all three species and

598   the arrows to the left indicate the relative molecular weights in kDa. pp75 is denoted with a

599   double asterisk at the right of panel A.

600

601   Figure 2. Immunodetection by western blot with anti-pThr antibodies of pp75 from *in vitro*

602   cultures of *Symbiodinium* KB8 (A), *Symbiodinium* Mf11 (B) and *Symbiodinium kawagutii* (C),

603   incubated for 12 h in darkness (lanes 1) and after stimulation with light for 30 (lanes 2), 60 (lanes

604   3) and 240 (lanes 4) min.

605

606   Figure 3. Changes in the phosphorylation level of three independent biological replicates

607   determined by western blot and densitometric analysis with anti-pThr and anti-actin monoclonal

608   antibodies (panel labeled "actin" shown as example) used as internal loading control for

609   normalization. The figure shows the bands corresponding to pp75 (A), and the 43 kDa (B)

610   protein from *in vitro* cultures of *Symbiodinium* KB8 (*S*. KB8), *Symbiodinium* Mf11 (*S*. Mf11)

611   and *Symbiodinium kawagutii* (*S. kw*), after the cells were incubated for 12 h in darkness (lanes 1)

612   and analyzed after light stimulation for 30 (lane 2), 60 (lane 3) and 240 (lane 4) min. The bars in

613   the graphs represent the average of the density for each band normalized relative to the density of

614   the actin band (panel labeled "actin").

615

616   Figure 4. Separation by two-dimensional (2D) gel electrophoresis (IEF + SDS-PAGE) and

617   immunodetection of SmicHSP75 from *S*. KB8 enriched protein extracts for subsequent isolation

618   and partial peptide sequencing. (A) Western blot of the resolved proteins on the 2D gel of

619   DEAE-sephacel enriched extracts incubated after 12 h darkness. The two spots labeled spot 1

620   and spot 2 correspond to two SmicHSP75 isoforms of pI's ~ 5.4 and 5.6, respectively. (B) An

621   equivalent 2D gel stained with coomassie blue where the SmicHSP75 isoforms are located (spot

622   1 and 2, respectively). (C) Multiple alignment of the partial sequences of the peptides obtained

623   from both spots with the HSP70/BiP sequences of *Oryza sativa* (Accession No. Q6Z7B0),

624   *Cryptecodinium cohnii* (Accession No. Q8S4R0), and *Symbiodinium microadriaticum* (*S. mic*;

625   Accession No. OLP91134.). The figure shows the motif of retention in the endoplasmic

626   reticulum (Munro and Pelham, 1987) highlighted in gray and characteristic of the BiP; highlited

627   in green are the most probable sites of phosphorylation in threonine predicted by the NetPhosK

628   server (Blom et al., 2004); in addition, the asterisk indicates a conserved phosphorylatable

629   threonine presumably involved in protein synthesis regulation in *Chlamydomonas reinhardtii*

630   (Diaz-Troya et al., 2011).

631

632   Figure S1. Relative molecular weights of the phospho proteins shown in Figure 1 and

633   corresponding to the immunodetection by western blot with anti-pThr (A), -pSer (B), and -pTyr

634   (C) antibodies in protein extracts of *Symbiodinium* KB8 (lanes 1), *Symbiodinium* MF11 (lanes 2)

635   and *Symbiodinium kawagutii* (lanes 3).

636

637   Figure S2. Western blot analysis of phosphothreonine-containing proteins with either PBS-T or

638   TBS-T. Extracts after dark incubation for 12 h from *Symbiodinium* KB8 (lanes 1), *Symbiodinium*

639   Mf11 (lanes 2) and *Symbiodinium kawagutii* (lanes 3), were analyzed by western blot with anti-

640   pThr antibodies using incubations, washes and development in either PBS-T (A) or TBS-T (B).

641

642   Figure S3. Analysis by western blot of the proteins phosphorylated on threonine from

643   *Symbiodinium* KB8 cells upon various stimuli. Extracts of proteins from cells without

644   stimulation (control, lane 1) or added with: 0.1% (w/v) hydrolyzed casein (lane 2), 10 mM

645   glycine (lane 3), 10 mM arginine (lane 4), 10 mM glutamic acid (lane 5), 100 µg/ml of RGD

646   (lane 6) or RAD (lane 7) tripeptide, 15 mM EGTA (lane 8), or 20 mM $CaCl_2$ (lane 9). The

647   arrows to the right indicate the molecular weights (kDa) of the proteins immunodetected with the

648   anti-pThr antibodies.

649

650   Figure S4. Re-analysis by western blot with anti-pThr antibodies of the two SmicHSP75

651   isoforms from two-diomensional gels. The proteins corresponding to spot 1 (lane 1; labeled "spot

652   1 in Figure 4), spot 2 (lane 2; labeled "spot 2" in Figure 4), and an adjacent major spot (lane 3;

653   labeled "M" in Figure 4) as negative control, excised from the two-dimensional gel of proteins

654   from *Symbiodinium* KB8 cultures previously incubated under 12 h of darkness were analyzed by

655   western blot with anti-pThr antibodies. Only the lanes corresponding to spot 1 (lane 1) and spot 2

656   (lane 2) showed the presence of SmicHSP75.

Figure 1

Immunodetection by western blot of phospho amino acids present in proteins of three species of *Symbiodinium* with anti-pThr, anti-pSer and anti-pTyr antibodies.

Immunodetection by western blot of phospho amino acids present in proteins of three species of *Symbiodinium* with anti-pThr (A), anti-pSer (B) and anti-pTyr (C) antibodies. In the figure, the lanes correspond to: molecular weight markers (Mw); protein extracts of *Symbiodinium* KB8 (lanes 1), *Symbiodinium* Mf11 (lanes 2) and *Symbiodinium kawagutii* (lanes 3). The asterisks within the figure indicate protein bands immunodetected in all three species and the arrows to the left indicate the relative molecular weights in kDa. pp75 is denoted with a double asterisk at the right of panel A.

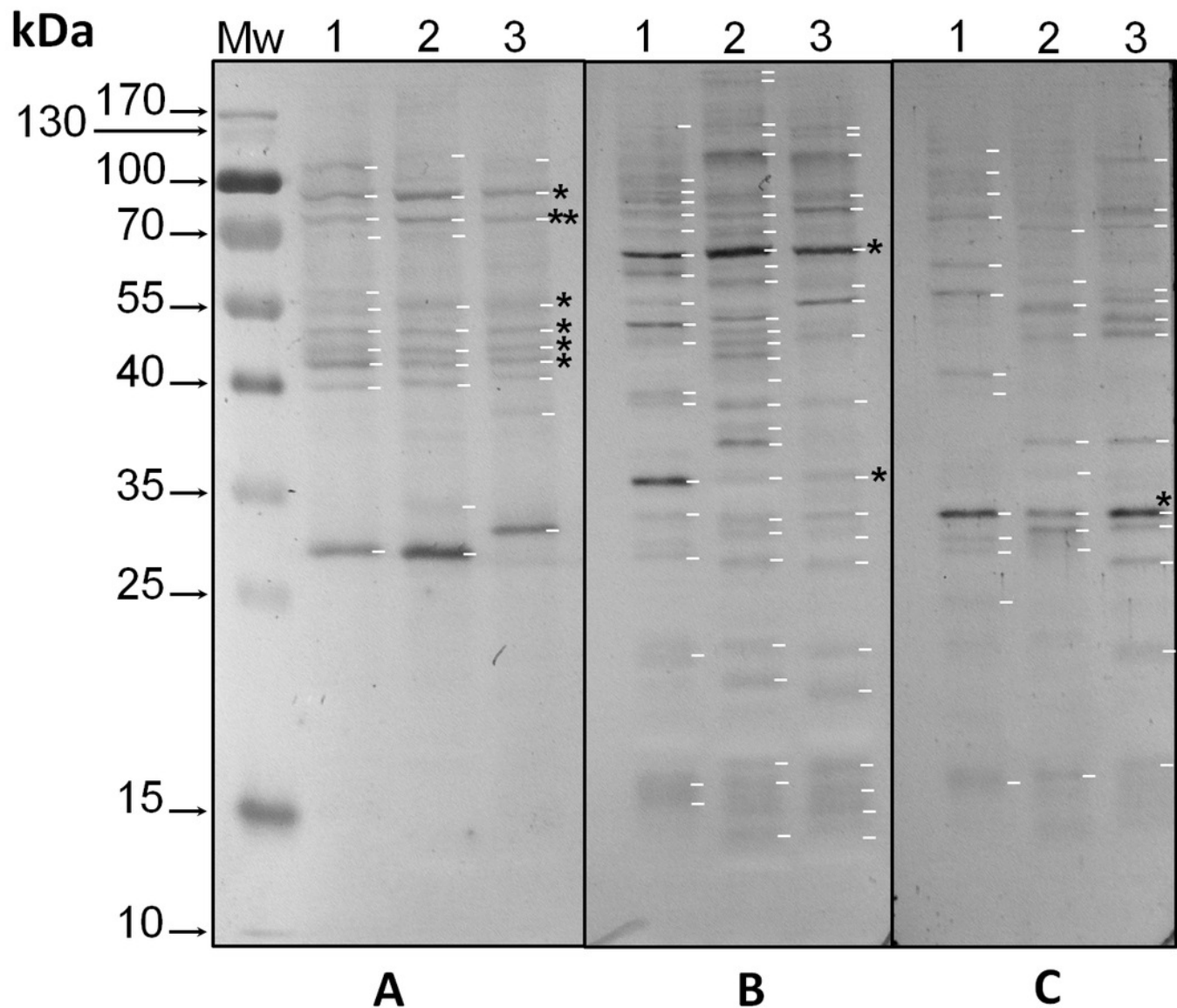



Figure 2

Immunodetection by western blot with anti-pThr antibodies of pp75 from *in vitro* cultures of *Symbiodinium* KB8, *Symbiodinium* Mf11 and *Symbiodinium kawagutii* under dark or light-stimulated conditions.

Immunodetection by western blot with anti-pThr antibodies of pp75 from *in vitro* cultures of *Symbiodinium* KB8 (A), *Symbiodinium* Mf11 (B) and *Symbiodinium kawagutii* (C), incubated for 12 h in darkness (lanes 1) and after stimulation with light for 30 (lanes 2), 60 (lanes 3) and 240 (lanes 4) min.

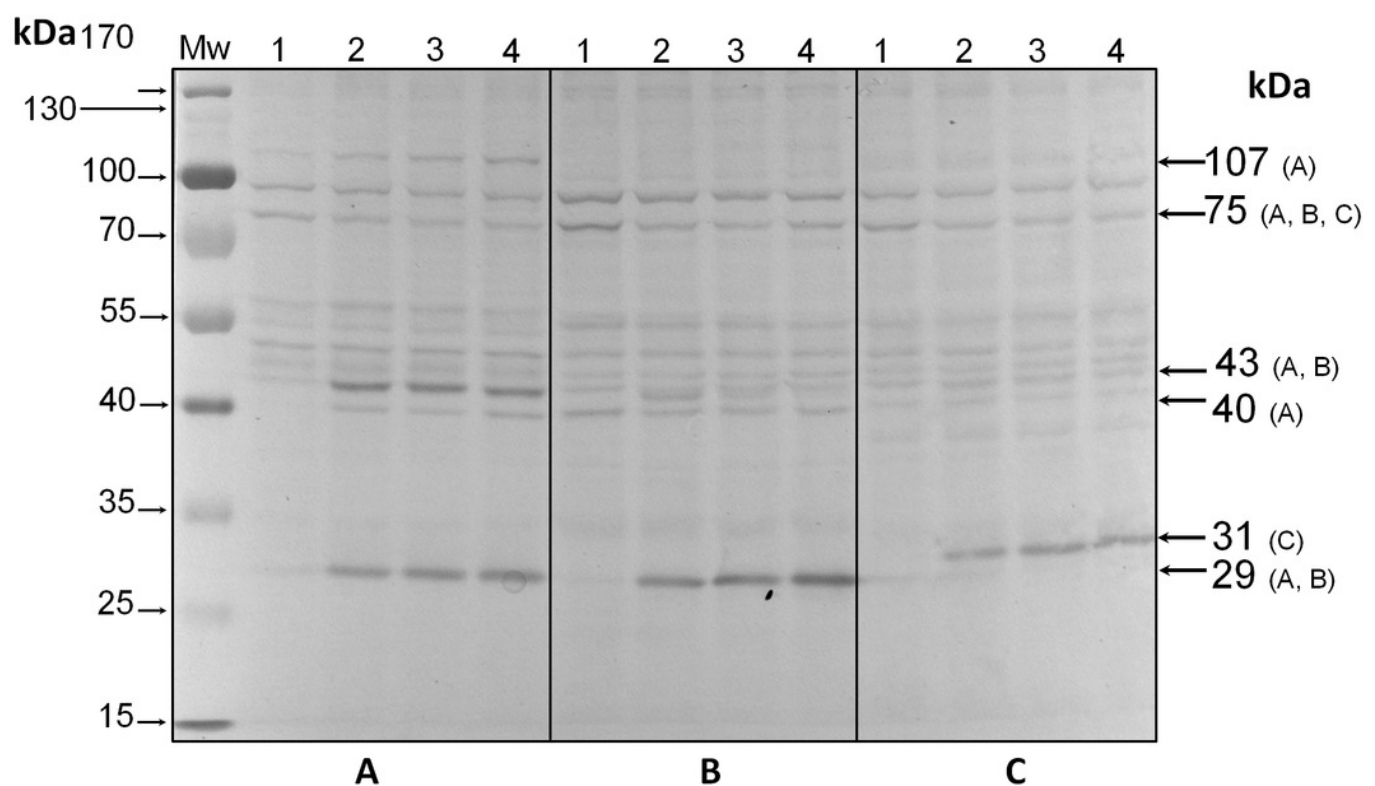
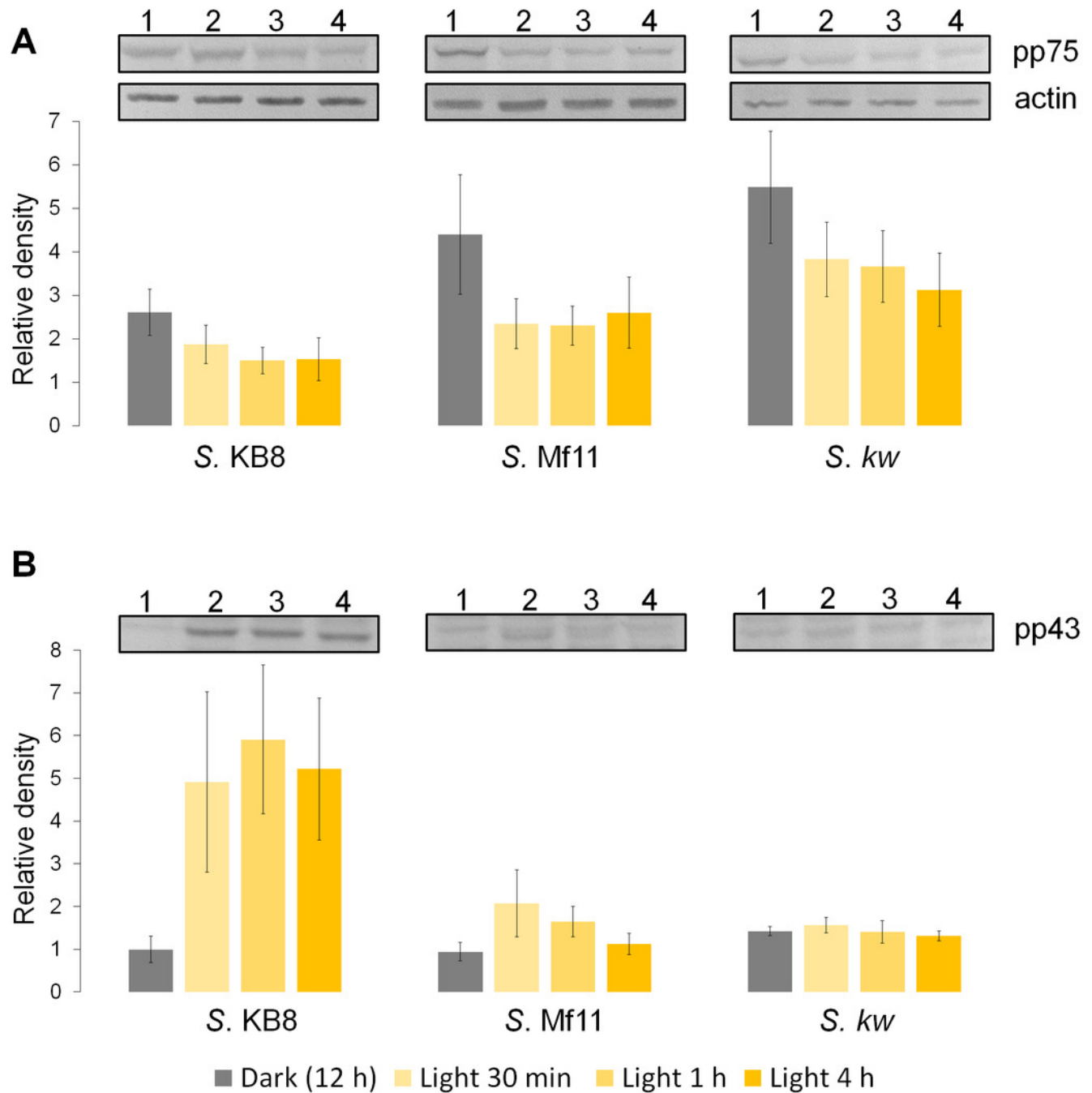



Figure 3

Changes in the phosphorylation level of three independent biological replicates from *in vitro* cultures of *Symbiodinium* KB8, *Symbiodinium* Mf11 and *Symbiodinium kawagutii* determined by western blot and densitometric analysis wi

Changes in the phosphorylation level of three independent biological replicates determined by western blot and densitometric analysis with anti-pThr and anti-actin monoclonal antibodies (panel labeled "actin" shown as example) used as internal loading control for normalization. The figure shows the bands corresponding to pp75 (A), and the 43 kDa (B) protein from *in vitro* cultures of *Symbiodinium* KB8 (*S*. KB8), *Symbiodinium* Mf11 (*S*. Mf11) and *Symbiodinium kawagutii* (*S*. *kw*), after the cells were incubated for 12 h in darkness (lanes 1) and analyzed after light stimulation for 30 (lane 2), 60 (lane 3) and 240 (lane 4) min. The bars in the graphs represent the average of the relative density for each band normalized with the density of the actin band (panel labeled "actin").

A

1 2 3 4   1 2 3 4   1 2 3 4   pp75

actin

Relative density

S. KB8   S. Mf11   S. kw

B

1 2 3 4   1 2 3 4   1 2 3 4   pp43

Relative density

S. KB8   S. Mf11   S. kw

■ Dark (12 h)   ■ Light 30 min   ■ Light 1 h   ■ Light 4 h

Figure 4

Separation by two-dimensional (2D) gel electrophoresis (IEF + SDS-PAGE) and immunodetection of SmicHSP75 from *S*. KB8 enriched protein extracts for subsequent isolation and partial peptide sequencing.

Separation by two-dimensional (2D) gel electrophoresis (IEF + SDS-PAGE) and immunodetection of SmicHSP75 from *S*. KB8 enriched protein extracts for subsequent isolation and partial peptide sequencing. (A) Western blot of the resolved proteins on the 2D gel of DEAE-sephacel enriched extracts incubated after 12 h darkness. The two spots labeled spot 1 and spot 2 correspond to two SmicHSP75 isoforms of pI's ~ 5.4 and 5.6, rspectively. (B) An equivalent 2D gel stained with coomassie blue where the SmicHSP75 isoforms are located (spot 1 and 2, respectively). (C) Multiple alignment of the partial sequences of the peptides obtained from both spots with the HSP70/BiP sequences of *Oryza sativa* (Accession No. Q6Z7B0), *Cryptecodinium cohnii* (Accession No. Q8S4R0), and *Symbiodinium microadriaticum* (*S. mic*; Accession No. OLP91134.). The figure shows the motif of retention in the endoplasmic reticulum (Munro and Pelham, 1987) highlighted in gray and characteristic of the BiP; highlited in green are the most probable sites of phosphorylation in threonine predicted by the NetPhosK server (Blom et al., 2004); in addition, the asterisk indicates a conserved phosphorylatable threonine presumably involved in protein synthesis regulation in *Chlamydomonas reinhardtii* (Diaz-Troya et al., 2011).

kDa

4 ◄─────── IEF (pH) ───────► 7

Spot 1 Spot 2

130 →
100 →
70 →
55 →
40 →
35 →

A

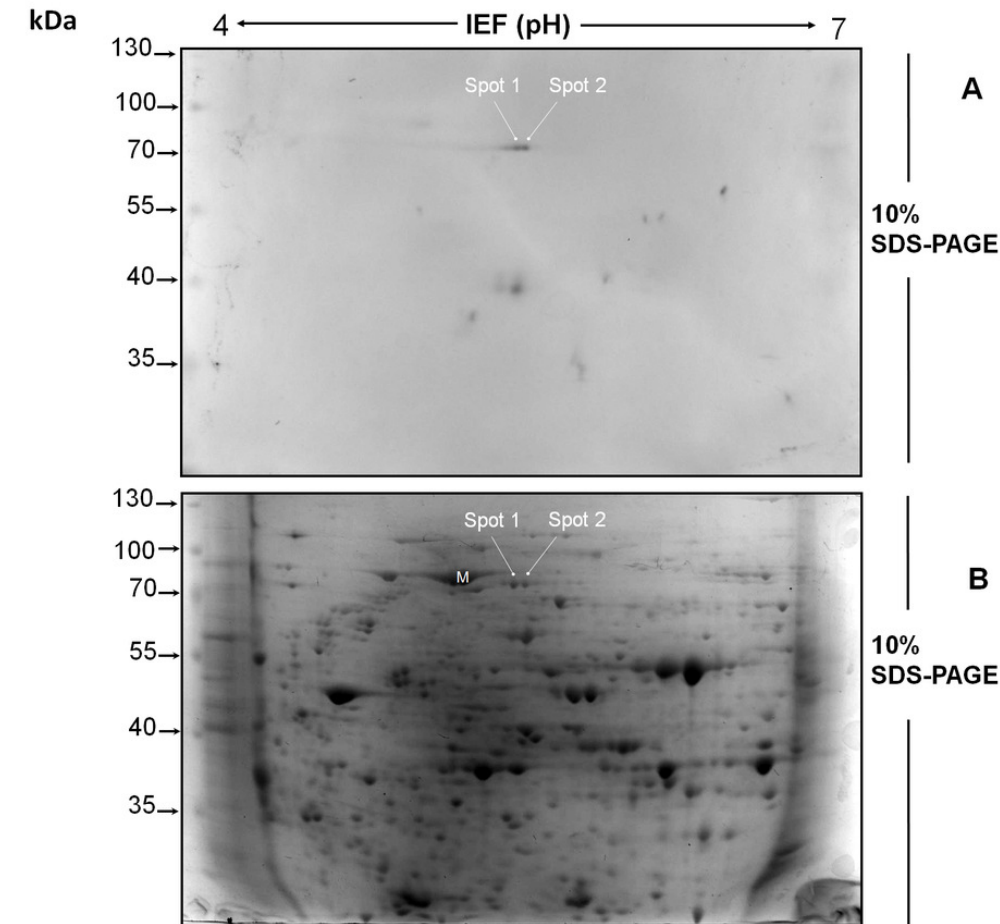
10%
SDS-PAGE

Spot 1 Spot 2

130 →
100 →
70 →
55 →
40 →
35 →

M

B

10%
SDS-PAGE

C

```
O. sativa                                                                MDRVRGCAFLLGVLLAGSLFAFSVAKEETKKL-GTVIGIDLGTTYSCVGVYKNG
C. cohnii                                                       GTSVDVLSFGKLSAWDTAPLFAMARLHWAGIFVLALACVAVAKDDEKKIDGPVIGIDLGTTYSCVGIYKNG
S. mic          MVISHGSRLPVSQRRTHGHEASCQGVCCSQTWHSVRCAQACKAFLVEAEPRVTMWKVAFVTVFALASFAAAKEEDKKIDGPVIG----TTYSCVGINKNG

O. sativa       HVEIIANDQGNRITPSWVAFTDSERLIGEAAKNQAAVNPERTIFDVKRLIGRKFEDKEVQRDMKLVPYKIVNKDGKPYIQVKIKDGENKVFSPEEVSAMI
C. cohnii       RVEIIPNDQGNRITPSYVAFTEDERLIGEAAKNQATINPAQTLFDVKRLIGRRFKDSTVQKDIKLLPFDIVDKNGKPQISVKVK-GETKQMAPEEVSSMV
S. mic          RVEIIPNDQGNRITPSYVAFTDDERLIGEAAKNQATINPTQTLFDVKRLIGRRFKDSTVQKDIKLLPYKIVDKSTKPMIAVKVK-GEEKVMAPEEVSSMV
Spot1           VEIIPNDQGNRITPSYVAFTDDER      NQATINPTQTLFDVK                                     VMAPEEVSSMV
Spot2           VEIIPNDQGNRITPSYVAFTDDER      NQATINPTQTLFDVK                                     VMAPEEVSSMV

O. Sativa       LGKMKETAEAYLGKKINDAVVTVPAYFNDAQRQATKDAGVIAGLNVARIINEPTAAAIAYGLDKKGGEKNILVFDLGGGTFDVSILTIDNGVFEVLATNG
C. cohnii       LTKMKETAENYLGKEVKHAVITVPAYFNDQQRQSTKDAGVIAGLNVLRIINEPTAAAIAYGLDKKA-EKNILVYDLGGGTFDVSLLTIDNGVFEVVATNG
S. mic          LTKMKETAENYLGKEVKHAVTVPAYFNDAQRQSTKDAGTIAGLNVLRIINEPTAAAIAYGLDKKT-EKNILVYDLGGGTFDVSLLTIDNGVFEVVATNG
Spot1           LTK          HAVVTVPAYFNDAQR   DAGTIAGLNVLRIINEPTAAAIAYGLDK
Spot2           LTK                        DAGTIAGLNVLRIINEPTAAAIAYGLDK

O. sativa       DTHLGGEDFDQRIMEYFIKLIKKKYSKDISKDNRALGKLRREAERAKRALSNQHQVRVEIESLFDGTDFSEPLTRARFEELNNDLFRKTMGPVKKAMDDA
C. cohnii       DTHLGGEDFDQRVMQHFMKVFQKKHSKDMSKDKRAIQKLRREVEKAKRALSSTHQARLEIEALFDGVDFSETLTRARFEEINNDLFKNTLGPVKQVIEDS
S. mic          DTHLGGEDFDQRVMQHFMKIFEKKHGKDMSKDKRSIQKLRREVEKTKRALSSTHQARLEIEALYDGTDFSETLTRARFEELKADLFKNTLGPVKQVLDDS
Spot1                                                       LEIEALYDGTDFSETLTR
Spot2                                                       LEIEALYDGTDFSETLTR

O. sativa       GLEKSQIHEIVLVGGSTRIPKVQQLLRDYFEGKEPNKGVNPDEAVAYGAAVQGSILSGEGGDETKDILLLDVAPLTLGIETVGGVMTKLIPRNTVIPTKK
C. cohnii       GLKKTQIDEIVLVGGSTRIPKVQQLIKDFFNGKEPNRGINPDEAVAYGAAVQAGILSGEGGQ---DLLLLDVTPLTLGIETVGGVMTKLINRNTVIPTKK
S. mic          GLKKNQVDEIVLVGGSTRIPKVQQLIKDFFNGKEPNRGINPDEAVAYGAAVQ-------------DVTPLTLGIETVGGVMTKLISRNTVIPTKK
Spot1           NQVDEIVLVGGSTR
Spot2           NQVDEIVLVGGSTR

                                                                                             *
O. sativa       SQVFTTYQDQQTTVSIQVFEGERSMTKDCRLLGKFDLSGIPAAPRGTPQIEVTFEVDANGILNVKAEDKGTGKSEKITITNEKGRLSQEEIDRMVREAEE
C. cohnii       SQTFSTYQDNQPAVNIQVFEGERPMTKDNHLLGKFELGGIPPAPRGQPQIEVTFEIDSNGILNVGAEDKATGKGEKITITNDKGRLTEEQIEKMIKEAEQ
S. mic          SQIFSTYQDNQPAVNIQVFEGERPMTKDNHLLGKFELGGIPPAPRGQPQIEVTFEIDSNGILNVGAEDKGTGKSEKITITNDKGRLTEEQIEKMIREAEE
Spot1           SQIFSTYQDNQPAVNIQVFEGERPMTK

O. sativa       FAEEDKKVKERIDARNQLETYVYNMKNTV---GDKDKLADKLESEEKEKVEEALKEALEWLDENQTAEKEEYEEKLKEVEAVCNPIISAVYQRTGGAPGG
C. cohnii       FADEDKKVKERVDAKNSFDGYIHSMRSATEGSGDNKGLSEKMDEDEKEKILEALKDGQSWLDSNPEGDAEDIKEKHKEVEGICAPIVSK-YYGVGG-GGA
S. mic          FADEDKKVKERVDAKNAFDGYIHSMRSATEGSGDNKGLSEKMDSDEKEKILDALKDGQSWLDSNPEADAEEIKEKHKEVEGICAPIVSK-YYGGGGAGGA
Spot1                       NAFDGYIHSMR                       DGQSWLDSNPEADAEEIKEKHKEVEGICAPIVSK

O. sativa       GADGEGGVDDEHDEL
C. cohnii       GAADEDE-DEAHDEL
S. mic          GGADDDE-EEAHDEL
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