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1 **New insights on HcPTR2A and HcPTR2B, two high affinity peptide transporters from the**  
2 **ectomycorrhizal model fungus *Hebeloma cylindrosporum***

3

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13

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17

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26 **Abstract**

27 Unlike plants which mainly rely on the use of inorganic nitrogen sources like ammonium and nitrate,  
28 soil born microorganisms like the ectomycorrhizal fungus *Hebeloma cylindrosporum* can as well take  
29 up organic N sources like amino acids and peptides from the soil and exploit them as nitrogen and  
30 carbon sources. Two PTR-like peptide transporters have previously been identified and functionally  
31 expressed in the heterologous yeast. This study addresses the function and substrates of HcPTR2A  
32 and HcPTR2B transporters in detail by analysing the transport kinetics in *Xenopus laevis* oocytes.  
33 While both transporters mediated high affinity di- and tri-peptide transport, HcPTR2A also showed  
34 low affinity transport of several amino acids, mostly hydrophobic amino acids with large side chains.

35

36 **Keywords:** ectomycorrhiza, peptide transporter, , gene expression, heterologous expression.

37

38 **Declarations**

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47

## 48        **1. Introduction**

49 Nitrogen (N) is one of the most limiting factors for plant growth even though it is among the most  
50 abundant elements on Earth (Vance 2001). The efficiency and ability to use such limiting nutrients is  
51 an important component of plant productivity (McKane et al. 2002). N forms available in the soil are  
52 largely dependent on different environmental factors like the climate, soil type, acidification and  
53 nitrification processes, but also on vegetation and management, such as fertilization. When the soil  
54 is relatively acidic, cold or poorly aerated, ammonium ( $\text{NH}_4^+$ ) will remain the predominant N-form  
55 of plants, but under conditions favouring nitrification, the conversion to nitrate ( $\text{NO}_3^-$ ) will follow  
56 ammonification (Marschner 2012). In forest soils, N is present either in its main inorganic forms  
57  $\text{NH}_4^+$  and  $\text{NO}_3^-$ , or in organic compounds like amino acids, peptides and proteins (Marschner 2012;  
58 Chalot and Brun 1998).

59 Plants generally acquire N in its inorganic forms, followed by an intracellular reduction into  $\text{NH}_4^+$ ,  
60 assimilation into glutamine and subsequently other amino acids and organic compounds. Since  
61 reduction of  $\text{NO}_3^-$  to  $\text{NH}_4^+$  is associated with a high energetic demand, the direct uptake and usage of  
62  $\text{NH}_4^+$  is often favourable. Nevertheless, adaptation of plants under conditions of high N competition  
63 or low inorganic N availability has selected plants with diverse mechanisms of acquiring N from their  
64 surroundings (Zak et al. 1990; Kaye and Hart 1997; Aerts and Chapin 2000). In addition to using the  
65 inorganic N sources, some plants cooperate with ectomycorrhizal fungi to indirectly access the large  
66 pool of organic N in the soil. These fungi use high affinity peptide transporters to absorb amino acids  
67 and peptides from the soil.

68 Peptide transporters can be categorized into three super-families: (i) the ATP-binding cassette (ABC  
69 family); (ii) the oligopeptide (OPT) transporter family; (iii) and the peptide transporter (PTR/POT)  
70 family (Steiner et al. 1995; Reddy et al. 2012). OPT and PTR/POT families are mainly involved in  
71 the transport of peptides in fungi (Dunkel et al. 2013). Some PTRs are capable of transporting other  
72 nitrogen-containing substrates like nitrate (Chiang et al. 2004). In the ectomycorrhizal fungus (EMf)  
73 *Hebeloma cylindrosporum*, two putative di- and tripeptide transporters, HcPTR2A and HcPTR2B

74 were previously isolated (Benjdia et al. 2006). The functionality of both transporters was shown by  
75 complementation of yeast mutants. Closer characterization by uptake studies with  $^3\text{[H]}$  – labelled  
76 dipeptides could only be performed for HcPTR2A, while a more detailed functional analysis of  
77 HcPTR2B was still lacking. It is worthwhile to note that a dipeptide transporter was characterized in  
78 another mycorrhizal association type, namely in an arbuscular mycorrhizal fungus (Belmondo et al.  
79 2014).

80 In this study, we investigated and compared the transport characteristics of HcPTR2A and HcPTR2B  
81 in detail by functional expression in *Xenopus laevis* oocytes and confirmed high affinity peptide  
82 transport for both transporters. We identified a low affinity amino acid transport activity of HcPTR2A  
83 as well as the voltage-dependent substrate affinities and transport kinetics for several peptides for  
84 both transporters. Moreover, differential expression patterns of these two transporters in the presence  
85 of the host plant argue for different specific roles in plant nutrition via the ectomycorrhizal  
86 association.

87

## 88 **2. Material and methods**

### 89 *2.1 Cloning of HcPTR2A and HcPTR2B into pOO2 and synthesis of cRNA*

90 For expression in *X. laevis* oocytes, the genes coding the HcPTR transporters were transferred from  
91 the yeast expression vector into the *X. laevis* expression vector pOO2 (Ludewig et al. 2002). Coding  
92 regions of the genes came from the cDNA library in Montpellier (*HcPTR2A* and *HcPTR2B*) and were  
93 inserted into the yeast expression vector pFL61 (Lambilliotte et al. 2004). For isolation of the genes,  
94 the vector was restricted with *NotI* and then ends blunted with T4-polymerase (Fermentas, Vilnius,  
95 Lithuania) following the manufacturer's instructions. pOO2 was blunted with *SmaI* and  
96 dephosphorylated to prevent re-ligation. Ligation was done as described by Sambrook et al. (1998)  
97 and the orientation was checked by PCR using a gene and vector specific primer pair.

98 For the synthesis of cRNA, 10  $\mu\text{g}$  of the cDNA containing pOO2 vectors were linearized by digestion  
99 with *MluI* and linearization was verified by gel electrophoresis. The linearized and phenol chloroform

100 cleaned plasmid was used as a template for cRNA synthesis using the “SP6 mMACHINE  
101 mMACHINE Kit” (Life Technologies GmbH, Darmstadt; Germany) following the manufacturer’s  
102 instructions. Quality of the cRNA was checked by RNA gel electrophoresis and cRNA concentrations  
103 were determined photometrically.

## 104

### 105 *2.2 Preparation of *Xenopus laevis* oocytes and microinjection of cRNA*

106 Oocytes were surgically removed from anesthetized *X. laevis*. Oocytes were defolliculated by  
107 collagenase treatment (Ca<sup>2+</sup>-free ND96 with 0,2% collagenase; ND96: 96 mM NaCl, 2 mM KCl,  
108 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 2.5 mM sodium pyruvate, 5 mM HEPES adjusted to pH of 7.4 by  
109 NaOH). After five washing steps with Ca<sup>2+</sup>-free ND96 supplemented with 1 mg ml<sup>-1</sup> BSA solution,  
110 oocytes were manually separated for development stages V and VI (Dumont 1972). Before  
111 microinjection of cRNA, the freshly prepared oocytes were stored overnight at 4 °C.

112 Oocytes were microinjected with 50 nl of 1 µg µl<sup>-1</sup> cRNA solutions with the help of a glass  
113 micropipette and a micromanipulator (Drummond Scientific, Broomall, PA). Control oocytes were  
114 injected with 50 nl H<sub>2</sub>O. Before recordings, the injected oocytes were kept at 16 °C for three to five  
115 days in ND96 solution. The buffer was changed every day depending on the oocytes and solution  
116 conditions. Pipettes for injections and recordings were pulled using a Narishige PP-830 puller  
117 (Tokyo, Japan) or L/M-3P-A puller (List Medical Electronic, Darmstadt, Germany), respectively.

### 118

### 119 *2.3 Measurements of steady-state inward currents across the oocyte plasma membrane*

120 Micropipettes pulled from 1.5 mm thin wall borosilicate glass GB150TF10 (Science Products GmbH,  
121 Hofheim, Germany) were used for recordings. Pipettes were filled with 3 M KCl and connected to  
122 the headstage using a chloride silver wire. Pipette resistance was 1.5 - 3.5 MΩ in ND96 solution.  
123 Two-electrode voltage-clamp (TEVC) was done using a Dagan TEVC-200A amplifier (Dagan Corp,  
124 Minneapolis, USA). Currents were recorded via a LM12 interface (Dagan Corp.) using Clampex  
125 5.5.1 (Axon Instruments Inc., Union City, CA).

126 For measuring the steady-state inward currents, the oocyte plasma membrane was held at -30 mV,  
127 and membrane currents were measured after stepping from the holding potential ( $V_h$ ) to test potentials  
128 ( $V_m$ ) between -140 mV and +40 mV in 20 mV increments. Each voltage pulse was applied for 250 ms.  
129 The currents were filtered at 50 Hz. Peptides and amino acid substrates were supplied by perfusion  
130 of the bath in various concentrations indicated in the figures.

131

#### 132 2.4 RNA extraction and quantitative reverse transcription-PCR

133 Growth conditions of *H. cylindrosporium* mycelium alone and in indirect contact with young seedling  
134 roots of *Pinus pinaster* mimicking the symbiotic interface for 48 h were performed as described  
135 (Becquer et al. 2017; Torres-Aqino et al. 2017). Briefly, fungal mycelia, grown for three weeks in  
136 liquid cultures (N6), and pine seedlings, grown from seeds, cultivated separately in liquid nutritive  
137 medium N1 for two months and finally washed ( $\text{CaSO}_4$  0,2 mM, pH 5.5), were incubated together  
138 for 48 h in an interaction medium ( $\text{MgSO}_4$  0,2 mM,  $\text{CaCl}_2$  0,5 mM, MES 5 mM, TRIS 5 mM; pH  
139 5.5). One fungal mycelium was incubated with three pine seedling roots (indirect contact) or in the  
140 same conditions alone (three repetitions). RNA extraction from these fungal mycelia was performed  
141 using the Spectrum™ Plant Total RNA Kit (Sigma), RNA was treated by DNase (Deoxyribonuclease  
142 I, Amplification Grade, Invitrogen), and cDNA synthesis was performed by retrotranscription  
143 (SuperScript™ III Reverse Transcriptase, Invitrogen).

144 RNA from *P. pinaster-Laccaria bicolor* ectomycorrhiza, harvested three and 28 days post-infection,  
145 were gently provided by Pr F. Canavos (University of Malaga, Spain). Synthesis of these  
146 ectomycorrhizas and RNA extraction are described in Flores-Monterroso et al. (2013). PCR reactions  
147 and data analysis were performed as described in Courty et al. (2009) with 3 biological replicates.  
148 Primers used for control (*HcTub* *H. cylindrosporium*  $\alpha$ -tubulin, GenBank accession number  
149 CK993737 (Tatry et al. 2009) and *LbEf-3* *L. bicolor* elongation factor (F:  
150 CTCATCCTTCCTGCTCTTCTTCACG and R: CGGTGGCTTTTGTAAGTGAATCACG)) or for  
151 analysis of HcPTR2 (HcPTR2A-F (TCGTTTCCCTCTCCACTGAC) and HcPTR2A-R

152 (GCTCGTCCTCTTTGGCATC); HcPTR2B-F (GCGTTACTCAGCACGACCT) and HcPTR2B-R  
153 (ATCAGCGGCAATCACGAC)) and of LbPTR2 (LbPTR2A-F (GGCTTGCGGAGTGTTCTG) and  
154 LbPTR2A-R (TCTTCTTGAGGGCGGTGA); LbPTR2B-F (TCCTTGTCGCTGGTCTCG) and  
155 LbPTR2B-R (CGTTTTGGTGCCTTGGTG)) had an efficiency ranging between 90 and 110%.

156

### 157 2.5 Bioinformatic analysis

158 Transmembrane domain analysis was performed using the TMHMM server  
159 (<http://www.cbs.dtu.dk/services/TMHMM/>). The peptide transporter sequences  
160 from *H. cylindrosporum* were obtained from the JGI project  
161 (<https://genome.jgi.doe.gov/Hebcy2/Hebcy2.home.html>). An additional set of 54 peptide transporter  
162 proteins from fungi and plants were obtained from the NCBI database or the JGI Mycocosm project  
163 (<https://genome.jgi.doe.gov/mycocosm/home>). Protein sequences were aligned using the ClustalW  
164 and their evolutionary relationship was inferred using the maximum-likelihood algorithm with  
165 bootstrap (1000 replicates). Evolutionary analyses were carried out in MEGA7 (Kumar et al. 2016).  
166 3D protein structures of HcPTR2A and 2B have been predicted using Swiss Model  
167 (<https://swissmodel.expasy.org/>; Waterhouse et al. 2018).

168

## 169 3. Results

### 170 3.1 PTR genes of *Hebeloma cylindrosporum* and predicted proteins

171 Initially, HcPTR2A and HcPTR2B had been identified by a functional yeast complementation  
172 screening of a cDNA library as peptide transporters (Benjdia et al. 2006). Sequencing of the  
173 *H. cylindrosporum* genome among other EMf (Kohler et al. 2015) allowed detailed analyses of these  
174 PTR genes and gene products. The sequences of *HcPTR2A* and *HcPTR2B* are randomly distributed  
175 in the genome (scaffold 3 and 22, respectively) whereas in other EMf, e.g. *Suillus luteus*, duplicated  
176 genes are on the same scaffold (Table S1). The length of the genomic nucleotide sequences for  
177 *HcPTR2A* and *HcPTR2B* are of 2,486 bp and 2,452 bp, respectively (Table S1). The coding exon



178 sequences for these *PTR* genes were confirmed by EST alignment and cDNA sequencing; they are  
179 interrupted by short introns with a mean length of 55 and 58 bp, respectively. The actual number of  
180 introns is 13 for *HcPTR2A* and 11 for *HcPTR2B* (Table S1, Figure S1), which is similar to *PTR*  
181 transporters from other basidiomycetes, which normally show between 9 and 14 introns. The deduced  
182 protein sequences of *HcPTR2A* and *HcPTR2B* genes showed a length typical for fungal dipeptide  
183 transporters (591 aa and 603 aa, respectively) (Table S1). The THMM and Swissmodel predicted  
184 proteins with 10 and 11 transmembrane domains, for *HcPTR2A* and *HcPTR2B*, respectively (Figure  
185 S2). The sequence identity between the two proteins is 42.3%. A phylogenetic classification of the  
186 two *HcPTR* transporters is given in Figure 1.

187

### 188 *3.2 Current/voltage (I/V) relationships at saturating substrate concentrations for HcPTR2A*

189 To study the biochemical properties of *HcPTR2A* and *HcPTR2B*, four peptides exhibiting different  
190 properties were chosen. Two of them, LeuLeu and LeuLeuLeu, had been already tested in  
191 *Saccharomyces cerevisiae* (Benjdia et al. 2006). Previous uptake experiments with radiolabelled  
192  $^3\text{[H]LeuLeu}$  showed no significant uptake by *HcPTR2B* (Benjdia et al. 2006). Here, we expressed  
193 both transporters in *X. laevis* oocytes and measured inward currents induced by different peptides. At  
194 neutral pH, only negligible small ionic currents were elicited by the peptides, but substantial ionic  
195 inward currents of up to 0.5  $\mu\text{A}$  were elicited at acidic external pH 4.5. *I/V* relationships at saturating  
196 substrate concentrations were normalized to the maximum induced steady-state current for each  
197 oocyte and are given in Figure 2 for both transporters, *HcPTR2A* and *HcPTR2B*, for different  
198 peptides. Only inward currents were observed, in accordance with the fact that the (uncharged)  
199 substrates were applied externally and uptake was in form of peptide co-transport with  $\text{H}^+$ , driven by  
200 the negative membrane potential. At positive potentials, the currents asymptotically approached zero,  
201 but no outward currents were observed. For comparison, water-injected oocytes were exposed to the  
202 same peptides, but no ionic currents were elicited, clearly showing that the recorded currents were  
203 carried by the respective heterologously expressed transporters.

204

### 205 *3.3 Kinetics of HcPTR2A and HcPTR2B mediated peptide transport*

206 Next, we measured the concentration-dependent transport rates. According to the screening results in  
207 yeast, several substrates were tested for their ability for transport competition (Benjdia et al. 2006).  
208 HcPTR2A as well as HcPTR2B were both capable of transporting the LeuLeu, as well as the  
209 LeuLeuLeu peptide, over a broad range of concentrations (Figure 3). The  $K_m$  of HcPTR2A and  
210 HcPTR2B for the uncharged di- and tripeptides LeuLeu and LeuLeuLeu, and for the zwitterionic  
211 peptide LysAsp were in a similar range, around 0.5-0.8  $\mu\text{M}$  and 1.5-4  $\mu\text{M}$ , respectively (Table 1,  
212 Figure 3).

213 For the neutral dipeptide GlyAsn, containing the small glycine and polar asparagine, HcPTR2A and  
214 HcPTR2B had a much lower affinity with a  $K_m$  in the range of 91  $\mu\text{M}$  and 33.7  $\mu\text{M}$ , respectively. For  
215 the dipeptides LysAsp and LeuLeu, and the tripeptide LeuLeuLeu, the transport saturation of both  
216 transporters was already reached at substrate concentrations below 30  $\mu\text{M}$ . In contrast, for GlyAsn,  
217 significantly higher concentrations were needed to saturate the transport of HcPTR2A and HcPTR2B,  
218 in a range above 300  $\mu\text{M}$  and 100  $\mu\text{M}$ , respectively (Table 1, Figure 3).

219 In all cases, when the currents induced from -40 mV to -140 mV were sufficiently large, we quantified  
220 the affinity for each membrane potential. For HcPTR2A, these calculations could be done for all  
221 peptides, while for HcPTR2B, only the currents induced with LysAsp and LeuLeu were sufficiently  
222 large for this analysis. Interestingly, the concentration of peptides, at which the transport was half-  
223 maximal saturated ( $K_m$ ) increased with more negative membrane potentials (Figure 4), suggesting  
224 that the  $K_m$  was voltage-dependent. This was irrespective of the type of peptide tested and suggests  
225 that both transporters had higher affinity to their substrates at less negative voltages.

226

### 227 *3.4 HcPTR2A mediates low affinity transport of large hydrophobic amino acids*

228 Since all four tested peptides elicited large ionic currents in HcPTR2A, we further tested whether this  
229 transporter additionally mediated transport of individual amino acids. The large hydrophobic amino

230 acids Leu, Phe and Met elicited the strongest currents and induced substantial inward currents at  
231 millimolar concentrations (Figure 5A). Transport rates were measured over a broad range of  
232 concentrations and revealed that the transport saturated with low affinities ( $K_m = 3.37, 4.75$  and  
233  $3.52$  mM for Leu, Phe and Met, respectively) (Figure 5B). Non-injected control oocytes did not show  
234 amino acid-induced currents, confirming that the amino acid transport was carried by HcPTR2A.  
235 However, only few of the 20 proteinogenic amino acids were transported by HcPTR2A at a substrate  
236 concentration of 1 mM. In addition to Leu, Phe and Met, the large neutral amino acid Tyr induced  
237 substantial currents (Figure 5C). Smaller currents were elicited by the small hydrophobic amino acids  
238 Val and Ile, as well as the polar Asn and the charged Arg and Glu (Figure 5C).

239

### 240 *3.5 HcPTR2B was down-regulated in the presence of a plant partner*

241 Expression of both peptide transporters was quantified on the transcriptional level in fungal mycelia  
242 alone compared to mycelia incubated for 48 h in the presence of young pine seedling roots (Figure  
243 6). The expression of *HcPTR2A* was similar in free-living mycelium and in mycelium in indirect  
244 contact with *P. pinaster* roots under same culture conditions. Interestingly, *HcPTR2B* was  
245 significantly down-regulated in presence of its plant host in the conditions of the device mimicking  
246 the interaction space already after 48 h arguing for a tight regulation at early stages of symbiotic  
247 signalling between ectomycorrhizal fungus and roots of the host plant. In addition, expression of both  
248 peptide transporters was quantified on the transcriptional level in *P. pinaster-Laccaria bicolor*  
249 ectomycorrhizal roots, harvested three and 28 days post-infection. *LbPTR2A* was only expressed in  
250 functional ectomycorrhizal roots (28 dpi). *LbPTR2B* was significantly down-regulated 8,5 times in  
251 28 dpi ectomycorrhizal roots compared to 3 dpi ectomycorrhizal roots (normalized expressed of  
252  $0.68 \pm 0.16$  versus  $5.77 \pm 0.38$ ).

253

## 254 **4. Discussion**

255 Previously, two di- and tripeptide transporters (HcPTR2A and HcPTR2B) from *H. cylindrosporium*  
256 were isolated and partially characterized (Benjdia et al. 2006). For PTR2A, a  $K_m$  value of 1.46  $\mu\text{M}$   
257 for LeuLeu was estimated from radiolabelled dipeptide uptake experiments in the *S. cerevisiae*  
258 mutant LR2 expressing *HcPTR2A*. The transport of other substrates was inferred from competition  
259 experiments (Benjdia et al. 2006). However, uptake experiments for PTR2B with  $^3\text{[H]}$ -LeuLeu in  
260 *S. cerevisiae* previously showed a very high standard deviation so that no  $K_m$  value could be estimated  
261 in yeast. To gain a better understanding regarding the transport properties and the substrate  
262 specificities of HcPTR2A and HcPTR2B, both genes were expressed in *X. laevis* oocytes. Both  
263 transporters elicited significant inward currents when oocytes were perfused with a peptide-  
264 containing solution at acidic pH, in line with their function as proton co-transporters. Similarly to  
265 RiPTR2, a peptide transporter of the arbuscular mycorrhizal fungus *Rhizophagus irregularis*  
266 (Belmondo et al. 2014), HcPTR2A showed a wide substrate spectrum with very high affinities for  
267 LysAsp, LeuLeu and LeuLeuLeu. GlyAsn was transported with a significant lower affinity compared  
268 to the other peptides. Similar as in the heterologous host yeast, HcPTR2A had a lower affinity for  
269 peptides starting with a Gly. These results correspond to what has been observed in the uptake  
270 experiments of HcPTR2A in *S. cerevisiae* (Benjdia et al. 2006) and confirm the very high affinity of  
271 HcPTR2A (1.46  $\mu\text{M}$  for  $^3\text{[H]}$ -LeuLeu in *S. cerevisiae* uptakes and 0.73  $\mu\text{M}$  for LeuLeu at -140 mV  
272 in oocytes). The slight difference can be due to the fact that in uptake experiments the membrane  
273 potential is not tightly controlled as in the voltage-clamp experiments and will be an average of all *S.*  
274 *cerevisiae* cells. Whereas the resting membrane potential of plants is between -120 mV and -180 mV  
275 (Marschner 2012) the fungal membrane potential is expected even more negative, it can be down to  
276 -300 mV (Slayman 1985). Interestingly, the  $K_m$  value for HcPTR2A increased with more negative  
277 membrane potentials. A robust inward current was observed using GlyAsn as substrate. Further we  
278 observed solid inward currents when large, mainly hydrophobic, amino acids like Leu, Phe and Met  
279 were supplied in millimolar concentrations. The transport of these three amino acids was saturable as  
280 well but only reached half maximal transport rates at high concentrations of  $\sim 3 - 5$  mM. Therefore,

281 it might be questioned whether this transport is physiologically relevant but affinity might also be  
282 voltage-dependent as shown for other substrates.

283 The second peptide transporter HcPTR2B as well showed a high affinity for LysAsp, LeuLeu and  
284 LeuLeuLeu. HcPTR2B showed a lower  $K_m$  for GlyAsn (33.7  $\mu\text{M}$ ) when compared to HcPTR2A.  
285 However, transport affinities were not as high as observed for HcPTR2A in response to LeuLeu and  
286 LeuLeuLeu. In contrast, the affinity to LysAsp and GlyAsn seemed to be higher.

287 These results indicate that both transporters are high affinity di- and tripeptide transporters, but have  
288 differences in their substrate specificity. Both peptide transporters show high affinity levels that have  
289 never been observed before for any plant or fungal peptide transporter from the PTR family. In *X.*  
290 *laevis* experiments, a  $K_m$  value of 33  $\mu\text{M}$  for LeuLeu could be estimated for a fungal peptide  
291 transporter, originally identified from an *Arabidopsis thaliana* cDNA library (Steiner et al. 1994) but  
292 then turned out to be a fungal contamination (Chiang et al. 2004). However, the  $K_m$  value of fPTR2  
293 does not increase with a more negative membrane potential as it could be shown for both  
294 *H. cylindrosporum* peptide transporters. Yeast uptake experiments with this transporter resulted in a  
295  $K_m$  value of 14  $\mu\text{M}$  for  $^3\text{H}$ -LeuLeu (Steiner et al. 1994) demonstrating the difference between both  
296 heterologous expression systems explained by a series of possible differences in e.g. cytosolic and/or  
297 membrane conditions. However, the results observed for *H. cylindrosporum* peptide transporters  
298 expressed in yeast and *X. laevis* show similar characteristics.

299 Other characterised peptide transporters derived from plants like *Vicia faba* PTR1 ( $K_m$  20 mM;  
300 Miranda et al. 2003), *A. thaliana* PTR1 ( $K_m$  56  $\mu\text{M}$  for AlaAla to 416  $\mu\text{M}$  for AlaAsp; Dietrich et al.  
301 2004a) or *A. thaliana* PTR2 ( $K_m$  52  $\mu\text{M}$  for LeuLeu to 1,832  $\mu\text{M}$  for AlaAsp; Chiang et al. 2004)  
302 show lower substrate affinities. If also not all possible substrates were tested, the broad substrate  
303 specificity of peptide transporters could be shown. This seems to be necessary as Dietrich et al.  
304 (2004b) demonstrated that from the 400 dipeptides from degraded Rubisco only seven were present  
305 more than six times in the intact protein, but most of them exist only one or two times (Dietrich et al.  
306 2004a).

307 Keeping the high substrate affinity of both *H. cylindrosporium* peptide transporters in mind the  
308 question could be addressed if there is a reason for this. Therefore, one has to take a look at the  
309 original habitat of *H. cylindrosporium* and *P. pinaster*. It is divided into two habitats, the “dune”  
310 habitat where the percentage of organic matter ranges from 0.1% to 0.5% and the “forest” habitat  
311 with up to 3% of organic matter (Guidot et al. 2002). This very low percentage of organic matter  
312 could be an explainable reason for the high affinities of the peptide transporters.

313 It is worth to note that the transport rate of HcPTR2B is much lower than that observed for HcPTR2A.  
314 Our results on HcPTR2A and 2B expression are in concordance with Plett et al. (2015) who studied  
315 on arrays the expression level of *Laccaria bicolor* genes in the mycelium grown on medium  
316 containing agar and in ectomycorrhizal roots of *Populus trichocarpa*-*L. bicolor* grown for 2, 4, 6 and  
317 12 weeks after contact. No expression was detected for protID668820 (homolog of HcPTR2A)  
318 whatever the condition was. For protein ID301981 (homologous of HcPTR2B) the expression was  
319 significantly reduced (from 6 to 30 times) in ectomycorrhizal roots whatever the time of contact  
320 between the fungus and the roots. In addition, we also that expression of LbPTR2 was significantly  
321 reduced in functional ectomycorrhizal roots of *P. pinaster*-*L. bicolor*. Here, we show even a strong  
322 reduction of expression after only two days of indirect contact of the fungus with roots from young  
323 pine seedlings arguing for an involvement in early signalling. Why should this peptide transporter be  
324 so down-regulated? Might it be that both transporters are differentially localized, HcPTR2A perhaps  
325 more in external hyphae exploring the soil, HcPTR2B more at the symbiotic interface, within the  
326 Hartig net? In this case, it would be evident that plant factors are down-regulating expression of  
327 *HcPTR2B* to avoid possible competition for peptides. One could suggest conserved roles for both  
328 PTRs during evolution in ectomycorrhizal fungi. Previous analysis on the expression of both *HcPTRs*  
329 in free living mycelium (Avolio et al. 2012) highlighted the fact that HcPTR2B was expressed under  
330 all investigated conditions. So we could propose a role in a “basic” uptake of di- and tripeptides with  
331 a high affinity in free living mycelium. Whereas *HcPTR2A*, that has a higher affinity, would be

332 involved in both free-living mycelium and mycorrhizal conditions. These results suggest that *H.*  
333 *cylindrosporium* can perfectly adapt to conditions of a high competition for nutrient resources.

334

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338

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