New insights into HcPTR2A and HcPTR2B, two high-affinity peptide transporters from the ectomycorrhizal model fungus *Hebeloma cylindrosporum*

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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

4 Tobias Müller¹, Benjamin Neuhäuser², Uwe Ludewig², Gabriella Houdinet³, Sabine D.
5 Zimmermann³, Pierre Emmanuel Courty⁴ and Daniel Wipf^{1,4}

6

7 ¹ University Bonn, IZMB, Transport in Ectomycorrhiza, 53115 Bonn, Germany

8 ² Institute of Crop Science, Nutritional Crop Physiology, University of Hohenheim, Fruwirthstr. 20,
9 D-70593 Stuttgart, Germany.

10 ³BPMP, Univ Montpellier, CNRS, INRAE, Montpellier SupAgro, Montpellier, France.

11 ⁴ Agroécologie, AgroSup Dijon, CNRS, Université de Bourgogne, INRAE, Université de Bourgogne
12 Franche-Comté, Dijon, France.

13

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18 **Corresponding Author:** daniel.wipf@inrae.fr, Agroécologie, AgroSup Dijon, CNRS, Université de
19 Bourgogne, INRAE, Université de Bourgogne Franche-Comté, 17 rue Sully, 21000 Dijon, France,
20 Tel: + 33 3 80 69 34 52; Fax: + 03 80 69 37 44.

21 ORCID B Neuhäuser: 0000-0002-2391-0598

22 ORCID U Ludewig: 0000-0001-5456-1055

23 ORCID S Zimmermann: 0000-0002-5020-1447

24 ORCID PE Courty : 0000-0003-2789-7818

25 ORCID D Wipf : 0000-0001-7197-5612

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

1. Introduction

Nitrogen (N) is one of the most limiting factors for plant growth even though it is among the most abundant elements on Earth (Vance 2001). The efficiency and ability to use such limiting nutrients is an important component of plant productivity (McKane et al. 2002). N forms available in the soil are largely dependent on different environmental factors like the climate, soil type, acidification and nitrification processes, but also on vegetation and management, such as fertilization. When the soil is relatively acidic, cold or poorly aerated, ammonium (NH_4^+) will remain the predominant N-form of plants, but under conditions favouring nitrification, the conversion to nitrate (NO_3^-) will follow ammonification (Marschner 2012). In forest soils, N is present either in its main inorganic forms NH_4^+ and NO_3^- , or in organic compounds like amino acids, peptides and proteins (Marschner 2012; Chalot and Brun 1998).

Plants generally acquire N in its inorganic forms, followed by an intracellular reduction into NH_4^+ , assimilation into glutamine and subsequently other amino acids and organic compounds. Since reduction of NO_3^- to NH_4^+ is associated with a high energetic demand, the direct uptake and usage of NH_4^+ is often favourable. Nevertheless, adaptation of plants under conditions of high N competition or low inorganic N availability has selected plants with diverse mechanisms of acquiring N from their surroundings (Zak et al. 1990; Kaye and Hart 1997; Aerts and Chapin 2000). In addition to using the inorganic N sources, some plants cooperate with ectomycorrhizal fungi to indirectly access the large pool of organic N in the soil. These fungi use high affinity peptide transporters to absorb amino acids and peptides from the soil.

Peptide transporters can be categorized into three super-families: (i) the ATP-binding cassette (ABC family); (ii) the oligopeptide (OPT) transporter family; (iii) and the peptide transporter (PTR/POT) family (Steiner et al. 1995; Reddy et al. 2012). OPT and PTR/POT families are mainly involved in the transport of peptides in fungi (Dunkel et al. 2013). Some PTRs are capable of transporting other nitrogen-containing substrates like nitrate (Chiang et al. 2004). In the ectomycorrhizal fungus (EMf) *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 μg 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 mMESSAGE
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^{2+} -free ND96 with 0,2% collagenase; ND96: 96 mM NaCl, 2 mM KCl,
108 1 mM MgCl_2 , 1.8 mM CaCl_2 , 2.5 mM sodium pyruvate, 5 mM HEPES adjusted to pH of 7.4 by
109 NaOH). After five washing steps with Ca^{2+} -free ND96 supplemented with 1 mg ml^{-1} 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 $\mu\text{g } \mu\text{l}^{-1}$ 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_2O . 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 M Ω 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. cylindrosporum* 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 (CaSO_4 0,2 mM, pH 5.5), were incubated together
138 for 48 h in an interaction medium (MgSO_4 0,2 mM, 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. cylindrosporum* α -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 (GGCTTGCGGAGTGTCTG) 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[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 μ 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 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 μM and 1.5-4 μM , 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 μM and 33.7 μM , 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 μM . In contrast, for GlyAsn,
217 significantly higher concentrations were needed to saturate the transport of HcPTR2A and HcPTR2B,
218 in a range above 300 μM and 100 μM , 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

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

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

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

4. Discussion

255 Previously, two di- and tripeptide transporters (HcPTR2A and HcPTR2B) from *H. cylindrosporum*
256 were isolated and partially characterized (Benjdia et al. 2006). For PTR2A, a K_m value of 1.46 μM
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 μM for $^3\text{[H]}$ -LeuLeu in *S. cerevisiae* uptakes and 0.73 μM 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 $\sim 3 - 5 \text{ mM}$. 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 μ 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 μ 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 μ M for 3 [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 μ M for AlaAla to 416 μ M for AlaAsp; Dietrich et al.
301 2004a) or *A. thaliana* PTR2 (K_m 52 μ M for LeuLeu to 1,832 μ 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. cylindrosporum* 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. cylindrosporum* 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

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

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6. References

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