

The amino acid permease PpAAP1 mediates arginine transport in maritime pine

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13 **The amino acid permease PpAAP1 mediates arginine transport in maritime pine**
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Abstract

Forest trees have access to diverse nitrogenous compounds in the soil such as ammonium, nitrate and amino acids. Recent progress has been made in the identification and characterization of ammonium and nitrate transporters. However, much more limited is our current knowledge of amino acid transport systems despite their relevance to fully understanding nitrogen nutrition in trees.

In the present study, we have identified 10 genes encoding putative amino acid permeases of the AAP family in maritime pine (*Pinus pinaster* Ait.). Four members of this family, PpAAP1, PpAAP2, PpAAP3, and PpAAP4 were phylogenetically related to AtAAP5, involved in arginine transport in *Arabidopsis thaliana*. One of these genes, *PpAAP1*, exhibited enhanced expression levels in maritime pine roots when arginine was externally supplied. PpAAP1 was functionally characterized by complementation of a yeast mutant strain defective in the transport of arginine, allowing yeast to take up [¹⁴C]-arginine with high affinity. Furthermore, PpAAP1 was able to restore the severely affected root uptake of arginine displayed by AtAAP5 T-DNA mutants. Uptake rates of ¹⁵N-labelled arginine were significantly higher in *PpAAP1*-overexpressing plants when compared to wild-type and AtAAP5 mutant plants. Taken together, our results indicate that PpAAP1 is a high-affinity arginine transporter in maritime pine.

Keywords: AAP family, amino acid transport, arginine uptake, organic nitrogen nutrition, root uptake, *Pinus pinaster*

27 Introduction

28

29 Nitrogen uptake is a key process for plant growth and productivity. Nitrogen (N) is available
30 to plants as an inorganic form in soil, such as ammonium and nitrate, and also as organic
31 compounds such as amino acids and peptides (Rentsch et al. 2007). Due to its paramount
32 importance as constituent of essential biomolecules, plants have evolved different ways and
33 mechanisms to incorporate, transport and store N in different organs and tissues (Xu et al.
34 2012).

35 Plant species differ in their preferred N source, absorbing this nutrient by the roots
36 primarily in inorganic and organic forms (Tegeeder and Masclaux-Daubresse, 2018). Conifers
37 from boreal forests have a marked preference for ammonium over nitrate due to their natural
38 distribution in ammonium-rich soils (Öhlund and Näsholm, 2004; Miller and Hawkins, 2007;
39 Cañas et al. 2016). In some forest ecosystems, organic N in the form of amino acids and
40 peptides is also quantitatively important and may represent a major source of N for tree
41 nutrition (Lipson and Näsholm, 2001; Näsholm et al. 2009). However, the rates of amino acid
42 uptake are highly dependent on substrate abundance (Wright, 1962) and their natural
43 concentrations in soils are usually found in the lower micromolar range (Öhlund and Näsholm,
44 2004; Jones et al. 2005). Among them, arginine has the highest nitrogen to carbon ratio, which
45 makes it especially suitable as a source of organic N.

46 The first evidence that amino acids are taken up by plants started to appear at the
47 beginning of the 20th century (Hutchinson and Miller, 1912). Since then, molecular cloning
48 and functional complementation in yeast have revealed that plants possess a large number of
49 different amino acid transporters. In *Arabidopsis thaliana*, *Populus trichocarpa* and *Oryza*
50 *sativa*, at least 100, 134 and 96 genes respectively were annotated as putative amino acid
51 transporters (Yuan et al. 2005; Tuskan et al. 2006; Ouyang et al. 2007; Pratelli and Pilot, 2014).

52 Plant genes encoding putative amino acid transporters can be classified into two major
53 groups: the amino acid transporter family (ATF) and the amino acid polyamine choline (APC)
54 superfamily (Wipf et al. 2002). Most research has been performed on *Arabidopsis* and much
55 less is known about amino acid transport systems in other plant species (Pratelli and Pilot,
56 2014).

57 In *Arabidopsis*, the ATF family, also called amino acid/auxin permease family (AAP),
58 is the largest family consisting of 46 members (Rentsch et al. 2007). The ATFs can be divided
59 into six subfamilies, being the amino acid permease family (AAP) the best characterized with
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1
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3 60 8 members. When examined in heterologous expression systems, *Arabidopsis* AAPs generally
4 display an affinity for neutral and acidic amino acids, with the exceptions of AtAAP3 and
5 61 display an affinity for neutral and acidic amino acids, with the exceptions of AtAAP3 and
6 AtAAP5, which efficiently mediate transport of basic amino acids (Fischer et al. 2002;
7 62 AtAAP5, which efficiently mediate transport of basic amino acids (Fischer et al. 2002;
8 Okumoto et al. 2004). Especially important for long-distance transport of arginine is AtAAP5,
9 63 Okumoto et al. 2004). Especially important for long-distance transport of arginine is AtAAP5,
10 64 which transports arginine and lysine with high affinity (Svennerstam et al. 2008) having a role
11 64 which transports arginine and lysine with high affinity (Svennerstam et al. 2008) having a role
12 65 in the uptake of basic amino acids by roots (Svennerstam et al. 2011). Screening of *Arabidopsis*
13 66 T-DNA insertional mutants showed that *aap5* mutants displayed a clear phenotypic divergence
14 66 T-DNA insertional mutants showed that *aap5* mutants displayed a clear phenotypic divergence
15 67 when high levels of arginine were present in the growth media and the root uptake of basic
16 67 when high levels of arginine were present in the growth media and the root uptake of basic
17 68 amino acids was severely affected (Svennerstam et al. 2008).
18 68 amino acids was severely affected (Svennerstam et al. 2008).

19 69 In conifers, arginine is a key amino acid for N storage and mobilization that constitutes
20 70 a large proportion of the amino acid content in the seed storage proteins (Cañas et al. 2016;
21 70 a large proportion of the amino acid content in the seed storage proteins (Cañas et al. 2016;
22 71 Llebrés et al. 2018). In the soil of boreal forests, amino acids such as arginine represent an
23 71 Llebrés et al. 2018). In the soil of boreal forests, amino acids such as arginine represent an
24 72 important fraction of the total nitrogen that is available for tree nutrition (Inselsbacher and
25 72 important fraction of the total nitrogen that is available for tree nutrition (Inselsbacher and
26 73 Näsholm, 2012). However, in spite of the relevance of arginine for nitrogen nutrition and
27 73 Näsholm, 2012). However, in spite of the relevance of arginine for nitrogen nutrition and
28 74 metabolism no specific transporter for this amino acid has been yet identified in conifers.
29 74 metabolism no specific transporter for this amino acid has been yet identified in conifers.

29 75 In this study, 10 full-length cDNA sequences of putative AAPs have been identified in
30 76 maritime pine using the available information of *P. pinaster* transcriptome (Canales et al. 2014).
31 76 maritime pine using the available information of *P. pinaster* transcriptome (Canales et al. 2014).
32 77 The expression level of one of them, PpAAP1, specifically increased in the roots when
33 77 The expression level of one of them, PpAAP1, specifically increased in the roots when
34 78 maritime pine plantlets were supplied with arginine. We have functionally characterized
35 78 maritime pine plantlets were supplied with arginine. We have functionally characterized
36 79 *PpAAP1* in yeast to determine its kinetics and ability for arginine transport. In addition,
37 79 *PpAAP1* in yeast to determine its kinetics and ability for arginine transport. In addition,
38 80 transgenic *Arabidopsis* overexpressing *PpAAP1* were generated to further study its function
39 80 transgenic *Arabidopsis* overexpressing *PpAAP1* were generated to further study its function
40 81 during plant development with arginine. Altogether, our results suggest that PpAAP1 is an
41 81 during plant development with arginine. Altogether, our results suggest that PpAAP1 is an
42 82 efficient arginine transporter in maritime pine.
43 82 efficient arginine transporter in maritime pine.

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85 **Materials and methods**

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87 *Plant material, transformation and growth conditions*

88 Seeds of *Pinus pinaster* Aiton were imbibed in distilled water for 24 h and grown in vermiculite
89 as a substrate. Seedlings were cultivated in a plant growth chamber at 24 °C and 16 h
90 photoperiod, light intensity of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and watered twice a week with distilled water.
91 For expression analysis, cotyledons, hypocotyls and roots from one-month-old pine seedlings
92 were collected separately, frozen in liquid nitrogen and stored at -80 °C. For hydroponic
93 cultivation, two-week-old seedlings grown in vermiculite were harvested and individually
94 transferred to a 2 L pot with a nutritive solution (Canales et al. 2010) containing (mg/L):
95 Na_2HPO_4 (20), KCl (40), CaCl_2 (40), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20), chelated Fe (5.6), $\text{NaMoO}_4 \cdot 7\text{H}_2\text{O}$
96 (0.001), $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ (0.006), H_3BO_3 (0.09), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.7). Seedlings
97 were grown either without a nitrogen source or supplemented with 2.25 mM of arginine. To
98 avoid contamination the nutrient solutions adjusted to pH 5.8 were changed every day. Root
99 samples were harvested after 1, 4, 8, 24 and 48 hours of treatment with the nutrient solution,
100 frozen in liquid nitrogen and stored at -80 °C until use for expression analysis. Three biological
101 replicates for each time point were harvested.

102 The full-length *PpAAP1* sequence was retrieved from the SustainPine Database v.3.0
103 (Canales et al. 2014). PCR amplification was performed from 100 ng of *P. pinaster* cDNA
104 using *PpAAP1* specific primers listed in Table S1. The PCR product was recombined into
105 pDONR207 (Invitrogen, Germany) and cloned into the destination vector pGWB2, which
106 contains a hygromycin selectable marker gene and the CaMV 35S promoter to drive the
107 expression of the transgene. The construct was verified by sequencing.

108 Seeds of *Arabidopsis thaliana* AtAAP5 T-DNA insertion line (*aap5-1*, SALK-041999,) were
109 obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK). Seeds were
110 grown in soil at 24 °C and 16 h light photoperiod for 8 weeks and the presence of the T-DNA
111 insert was verified by PCR using specific primers previously described by Svennerstam et al.
112 (2008). Homozygous mutants were transformed by the floral dipping method (Clough and Bent,
113 1998) with *Agrobacterium tumefaciens* strain C58C1 containing *PpAAP1* in pGWB2.
114 Transgenic seedlings were selected on half-strength Murashige and Skoog (MS; Murashige
115 and Skoog, 1962) medium containing 20 $\mu\text{g mL}^{-1}$ hygromycin B (Duchefa Biochemie, Haarlem,
116 the Netherlands).

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3 117 For *in vitro* studies, *Arabidopsis* sterile seeds were germinated on plates containing N
4 118 free half-strength MS media with 0.8 % plant agar (Duchefa Biochemie, Haarlem, the
5 119 Netherlands), 0.5 % sucrose, 3 mM potassium nitrate and several concentrations of arginine
6 120 (10 μ M, 50 μ M, 100 μ M or 1 mM). The media was buffered to pH 5.8 with MES (Sigma).
7 121 Plants were grown on vertical plates (to avoid roots penetrating the agar surface) in a growth
8 122 chamber under long day (16 h light/ 8 h dark) at 22 °C and photosynthetic photon flux density
9 123 of 150 μ mol m⁻² s⁻¹. *A. thaliana* wild-type (ecotype Columbia-0) plants were used as controls
10 124 in all experiments described. Root length and biomass accumulation were measured.
11 125

126 *RNA isolation and qPCR analysis*

127 Total RNA was extracted as described by Canales et al. (2012). Samples were ground in liquid
128 nitrogen and 100 mg of each were extracted with 650 μ L extraction buffer containing 3 %
129 cetyltrimethylammonium bromide (CTAB), 100 mM Tris pH 8.0, 2 M NaCl, 2 % PVP-40 and
130 30 mM ethylenediaminetetraacetic acid (EDTA). Residual DNA was removed by a treatment
131 with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) and preparation purified using
132 Nucleospin® Gel and PCR Clean-Up (Macherey-Nagel, Düren, Germany). Total RNA was
133 quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham,
134 USA). cDNA synthesis was performed from 500 ng of total RNA in 10 μ L reaction volume
135 using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) under the following
136 conditions, 25 °C for 5 min, 46 °C for 20 min and 95 °C for 1 min. Real-time PCR was carried
137 out on a CFX384 Real-Time System C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA)
138 using RT-qPCR SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) under the
139 following conditions, 95 °C for 2 min (1 cycle), 95 °C for 1 s and 60 °C for 5 s (40 cycles). A
140 reaction mixture was performed in a total reaction volume of 10 μ L containing primers (0.5 μ M
141 each), cDNA (10 ng), and 5 μ l of SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA,
142 USA). Three biological replicates with three technical replicates were performed per sample
143 type. *Elongation factor alpha 1* (EF1 α) was used as a reference gene. Gene-specific primers
144 used are listed in Table S1.

146 *Immunolocalization of PpAAP1 under nitrogen starvation*

147 One-month-old seedlings were harvested and transferred to a hydroponic nutritive
148 solution containing macronutrients and micronutrients with no source of nitrogen (Canales et
149 al. 2010). After 2 days, root tips were transferred to 4% (v/v) paraformaldehyde and 0.01%

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3 150 (v/v) Triton X-100 in 0.1 M PBS pH 7.4 and vacuum infiltrated. Fixation was then performed
4
5 151 overnight at 4 °C. Root tips were sectioned on a vibratome into 80 µm transverse and 0.5 mm
6
7 152 longitudinal slices. The samples were permeabilized with 0.05% (v/v) Tween-20 in 0.1 M PBS
8
9 153 pH 7.4 for 1 h and blocked with 5% (w/v) bovine serum albumin (BSA) in 0.1 M PBS pH 7.4
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11 154 for 1 h at room temperature (RT). Then, the sections were incubated with primary antibodies
12
13 155 specific for PpAAP1 (1:200) [PpAAP1-5'-H2N-GSIEGIIQDLKSYKPFHTAY-CONH2-3']
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15 156 (Biomedal SL, Seville, Spain) in 0.1 m PBS pH 7.4 containing 1% (w/v) BSA at 4 °C
16
17 157 overnight. A negative control incubated with non-immune serum was also prepared. The
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19 158 samples were rinsed with 0.1 M PBS containing 0.01% (v/v) Triton X-100 and then incubated
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21 159 with 2 µg mL⁻¹ of fluorescent secondary antibodies (CF 488A conjugates, Sigma-Aldrich)
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23 160 (1:100) for 3 h at room temperature in the dark. Finally, the sections were washed twice with
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25 161 0.1 M PBS pH 7.4 and stored in the dark at 2-8 °C. Antibody specificity is shown in Figure S3
26
27 162 using recombinantly produced PpAAP1 according to Cantón et al. (1996).

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29 163 Confocal microscopy was performed using a Leica SP5 HyD confocal microscope
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31 164 equipped with argon, krypton and HeNe lasers and an acousto-optical beam splitter (AOBS)
32
33 165 head system (Leica Microsystems, Bannockburn, IL, USA) capable of spectrally separating the
34
35 166 emissions from green fluorescent protein (GFP) and yellow fluorescent protein (YFP). The
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37 167 488 nm excitation line of an argon ion laser was used to excite GFP and YFP. The confocal
38
39 168 detectors included 2 × HyD and 3 × PMT + 1 for reflection. Line averaging was performed over
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41 169 16 frames for single images. The images were processed using confocal software and
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43 170 Fiji ImageJ software (version 4.1.1).

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45 172 *Yeast growth and transformation*

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47 173 The *Saccharomyces cerevisiae* strain 22Δ8AA is a mutant lacking multiple amino acid uptake
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49 174 systems (Mata gap1-1, put4-1, uga4-1, Δcan1, Δapl1, Δlyp1, Δhip1, Δdip5, ura3-1; Fischer et
50
51 175 al. 2002). The coding sequences corresponding to *PpAAP1* and *AtAAP5* were subcloned in the
52
53 176 yeast expression vector *pDR196* and introduced into yeast strain 22Δ8AA by heat shock
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55 177 according to Dohmen et al. (1991). Colonies harbouring PpAAP1, AtAAP5 and the empty
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57 178 pDR196 vector were selected on a synthetic minimal defined medium without uracil, and
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59 179 containing 0.67 % yeast nitrogen base without amino acids, 2 % glucose, 0.01 % (adenine,
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180 cysteine, leucine, lysine, threonine, tryptophan), 0.005 % (aspartic acid, histidine,
181 isoleucine, methionine, phenylalanine, serine, tyrosine, valine) and 2 % agar. The
182 transformants were grown in liquid URA-free SD minimal medium to reach stationary phase.

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3 183 Cells were pelleted, washed with sterile distilled water and serially diluted (1, 1:10, 1:100 and
4 184 1:1000). A volume of 10 μ l corresponding to each dilution was spotted onto plates. Growth
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6 185 complementation assays were performed in N-free minimal media (0.17 % yeast nitrogen base
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8 186 without amino acids and ammonium sulphate, 2 % glucose and 2 % agar) containing arginine
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10 187 (10 μ M and 0.5 mM), or 0.5 mM lysine, proline or aspartic acid as the sole nitrogen source.
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12 188 AtAAP5 was used as control in this study and empty pDR196 vector as a negative control. For
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14 189 positive control N-free media was supplemented with 0.5 % ammonium sulphate. The growth
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16 190 of two independent yeast colonies was examined after 3 days of culture at 30°C.

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20 192 *Transport measurement of [¹⁴C]-arginine in yeast*

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22 193 PpAAP1 transport activity was determined by measuring the initial uptake rate of [¹⁴C]-
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24 194 arginine. Yeast cells were grown to reach logarithmic phase, harvested at OD₆₀₀ of 0.6, washed,
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26 195 and resuspended in ice-cold buffer (50 mM KH₂PO₄, pH 5.0), to a final OD₆₀₀ of 5. Before the
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28 196 uptake measurements, an aliquot of yeast cells was supplemented with 20 mM glucose and
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30 197 incubated at 30 °C for 5 min. Uptake was initiated by the addition of 100 μ L of radioactive
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32 198 substrate mixture, containing L-[¹⁴C]-arginine (specific activity 274.3 mCi mmol⁻¹, Perkin
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34 199 Elmer, Boston, MA, USA) at several concentrations ranged between 2.5 μ M to 150 μ M.
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36 200 Accumulated counts were measured 30 s, 1 min, 2 min and 4 min after the addition of the
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38 201 radioactive substrate. Radioactivity (count per min) was measured in a scintillation counter
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40 202 (Beckman LS6000IC). Transport measurements were performed by triplicate and the values
41
42 203 were well fitted by a Michaelis-Menten model. For determination of kinetic parameters uptake
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44 204 assays were performed with PpAAP1-pDR196 and an empty vector under the same conditions
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46 205 according to Gietz and Woods (2002). Transport activity was analyzed using Origin (Pro),
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48 206 Version 2018b (Origin Lab Corporation, Northampton, MA, USA) and fitting with non-linear
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50 207 curve fit, growth/sigmoidal category.

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54 209 *Uptake measurement of [¹⁵N]-arginine in Arabidopsis*

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56 210 *Arabidopsis* wild-type, *aap5-1* and two independent homozygous lines (T3 generation plants)
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58 211 overexpressing *PpAAP1* [*35S::PpAAP-1* (L1), *35S::PpAAP-5* (L5); see below] were grown on
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60 212 plates containing 0.8 % agar, 0.5 % sucrose, N-free half-strength MS medium and 3 mM nitrate
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214 under long day (16 h light/ 8 h dark) conditions, 22 °C and photosynthetic photon flux density
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of 150 μ mol m⁻² s⁻¹. After 21 days plants were harvested and washed thoroughly in 0.5 mM
CaCl₂. Roots of the intact plants were then submerged for 2 h in 1 mL of the uptake solution

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3 216 per plant on a shaking table. Uptake solution of 0.5 mM CaCl₂ pH 5.8 included 30 μM dual-
4 217 labelled arginine ([¹³C₆¹⁵N₄]-L-arginine-HCL, ThermoFisher, USA). Three replicate samples,
5 218 each comprising five plants, were incubated for each line. Plants were collected and washed
6 219 three times in 0.5 mM CaCl₂. Roots were excised, dried at 60 °C, weighed and homogenized.
7 220 Finally, samples were analysed for ¹⁵N content at the University of Málaga Research Facility
8 221 (Unit of Atomic Spectrometry) using a flash EA 1112 elemental analyser coupled to a Delta V
9 222 Advantage isotope ratio mass spectrometer (IRMS; Thermo Electronic Corporation).
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17 224 *Determination of ammonium content*

18 225 *Arabidopsis* wild-type, *aap5-1*, *35S::PpAAP-1* (L1) and *35S::PpAAP-5* (L5) plants were
19 226 grown on plates under the same conditions described above. After 21 days, three replicate
20 227 samples comprising five plants each, were harvested and ground in liquid nitrogen. Ammonium
21 228 was extracted with 2% 5-sulfosalicylic acid (SSA) (100 mg FW mL⁻¹). The extracts were
22 229 centrifuged at 13.000 g for 15 min and supernatants were recovered (Ferrario-Méry et al. 1998).
23 230 Ammonium content was determined following the phenol hypochlorite method (Solórzano et
24 231 al. 1969).
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32 233 *Sequence analysis of P. pinaster AAP family*

33 234 Full-length amino acid sequences of plants AAPs were retrieved using PLAZA 3.0 database
34 235 (<http://bioinformatics.psb.ugent.be/plaza/>). Protein alignment was performed with the
35 236 ClustalW2 package. For phylogenetic analyses, the alignments were imported into the
36 237 Molecular Evolutionary Genetics Analyses software (MEGA) version 7.0 (Kumar et al. 2016).
37 238 Phylogenetic analyses were conducted using the neighbour-joining (NJ) method with the
38 239 Poisson correction model for distance computation, the pairwise deletion option and bootstrap
39 240 test with 1000 replicates (Couturier et al. 2010). Names of the species are abbreviated using a
40 241 two-letter code *P. pinaster* (Pp), *A. thaliana* (At), *P. trichocarpa* (Pt) and *O. sativa* (Os).
41 242 Accession numbers are provided as supplementary data in Table S3. Transmembrane domains
42 243 were predicted by CCTOP algorithm (Constrained Consensus TOPology,
43 244 <http://cctop.enzim.ttk.mta.hu>; Dobson et al. 2015). The expression localization data of *PpAAP*
44 245 genes is available at ConGenIE.org (<http://v22.popgenie.org/microdissection/>).
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248 Results

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250 *Identification and phylogenetic analysis of AAP family members in maritime pine*

251 A total of 10 members of the AAP family were identified in the *P. pinaster* database
252 (<http://www.scbi.uma.es/sustainpine>; Canales et al. 2014) by BLAST search of known
253 sequences from *Arabidopsis*. The homology of these membrane proteins was studied using
254 Consensus Constrained TOPology prediction (CCTOP; <http://cctop.enzim.ttk.mta.hu>) server
255 that integrates 10 different topology prediction methods incorporating parameters such as
256 charge bias, helix length and hydrophobicity (Dobson et al. 2015). According to CCTOP, all
257 the PpAAP proteins contained 11 transmembrane domains (Table 1), with N- and C-terminal
258 regions and inter transmembrane domains of variable length (Fig. S1). The amino acid
259 transporter AtAAP5 from *Arabidopsis* was also included in the analysis as a reference. As
260 depicted in Fig. S1, the topology predicted for AtAAP5 was quite similar to that of PpAAP1.
261 The genes identified in *P. pinaster* encode proteins with a similar number of amino acid
262 residues, from 466 to 500, and with a molecular mass ranging from 51 to 58.5 kDa (Table 1).
263 These results are quite similar to those described for members of the AAP family characterized
264 in other plant species such as *A. thaliana*, *O. sativa* and *P. trichocarpa* (Yuan et al. 2005;
265 Tuskan et al. 2006; Rentsch et al. 2007; Ouyang et al. 2007; Taylor et al. 2015).

266 A phylogenetic analysis of 51 AAP protein sequences from *P. pinaster* (10), *O. sativa*
267 (19), *P. trichocarpa* (14) and *A. thaliana* (8) revealed that AAPs are divided into four main
268 groups (Fig. 1), as described previously (Couturier et al. 2010; Taylor et al. 2015). The cluster
269 1 included three maritime pine proteins (PpAAP5, PpAAP6 and PpAAP7) together with only
270 sequences from *O. sativa* in good agreement to Taylor et al. (2015). As previously described
271 by Okumoto et al. (2002), AtAAP7 was the most distal member of the *Arabidopsis* AAP family
272 and grouped together with three PpAAP proteins included in cluster 2 exhibiting 50-52 %
273 identity (PpAAP8, PpAAP9 and PpAAP10). On the other hand, three AAPs from *Arabidopsis*
274 (AtAAP1, AtAAP6 and AtAAP8) were included in cluster 3 in which no AAP proteins from
275 *P. pinaster* were present. Four putative AAPs from *P. pinaster*, PpAAP1, PpAAP2, PpAAP3,
276 and PpAAP4 were included in the cluster 4 together with AtAAP2, AtAAP3, AtAAP4 and
277 AtAAP5 from *Arabidopsis*. These proteins present a high degree of identity at the protein level
278 (60 to 69 %; Table S2). All *Arabidopsis* AAPs in cluster 4 appears to be involved in loading
279 of amino acids into the phloem (Tegeeder and Ward, 2012), and AtAAP5 has been previously
280 shown to be involved in the root uptake of basic amino acids (Svennerstan et al. 2008). The

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3 281 closest *P. pinaster* homologs of AtAAP5, involved in root uptake of basic amino acids, were
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5 282 PpAAP1, PpAAP2, PpAAP3 and PpAAP4.

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7 283 To explore the potential function of *PpAAPs* genes phylogenetically related to *AtAAP5*
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9 284 the relative expression levels of *PpAAP1*, *PpAAP2*, *PpAAP3*, and *PpAAP4* genes were
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11 285 investigated at the ConGenIE database (Cañas et al. 2017). Among these genes only *PpAAP1*
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13 286 and *PpAAP2* presented expression levels in developing roots (Fig. 2A). *PpAAP1* transcripts
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15 287 were highly abundant in developing root vascular tissue (DRV) whereas *PpAAP2* was mainly
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17 288 expressed in the root meristem (RM). The relative abundance of *PpAAP1* and *PpAAP2*
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19 289 transcripts in the roots was confirmed by qPCR analysis (Fig. 2B). In comparison to *PpAAP1*
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21 290 and *PpAAP2*, the expression of *PpAAP3* and *PpAAP4* was barely detectable (Fig. S2). *PpAAP1*
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23 291 and *PpAAP2* were therefore selected as potentially involved in the primary uptake of arginine
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25 292 from the soil.

26 293 27 294 *Effect of arginine availability on the expression of PpAAP1 and PpAAP2*

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29 295 To study whether *PpAAP1* and *PpAAP2* expression are affected by the exogenous supply of
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31 296 arginine, 2 week-old pine plantlets were cultured for two days in hydroponic media in the
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33 297 absence (control) or the presence of arginine at a concentration of 2.25 mM (Fig. 3A). Time-
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35 298 course analysis of the expression of these genes was performed by qPCR in three sections of
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37 299 the root, upper part (1), medium part (2) and apical part including the root tip (3).

38
39 300 Fig. 3B shows that the expression of *PpAAP1* was upregulated in response to arginine
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41 301 availability with maximal values observed after 48 hours of culture and localized in the apical
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43 302 and in the medium part of the root. In contrast, no major differences were found in the transcript
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45 303 levels of *PpAAP2* between control and arginine supplied plants at the end of the treatment (Fig.
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47 304 3C). It should be noted that the transcript levels of *PpAAP2* were much lower than those
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49 305 *PpAAP1*. PpAAP1 protein was immunolocalized in maritime pine roots using antibodies raised
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51 306 against a 20 amino acid sequence specific for PpAAP1 (Fig. 4). The protein localization in the
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53 307 apex of the roots was consistent with the observed expression profile of *PpAAP1* in the same
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55 308 tissues (Fig.2). These results suggest that PpAAP1 may play an important role in the root
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57 309 arginine uptake and was selected for functional analysis.

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60 311 *Functional analysis of PpAAP1 in yeast*

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62 312 To determine whether *PpAAP1* encodes a functional amino acid permease, a yeast
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64 313 complementation test was performed by transforming the mutant strain 22Δ8AA with the
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66 314 maritime pine *PpAAP1* cDNA driven by the *PMAl* promoter (plasma membrane *ATPase*) in

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3 315 the yeast expression vector pDR196 (Rentsch et al. 1995). The mutant yeast strain 22Δ8AA is
4 316 unable to use efficiently arginine, aspartate, glutamate and proline as sole N source (Fischer et
5 317 al. 2002). A positive control with ammonium as the nitrogen source was included. As
6 318 additional controls, the strain 22Δ8AA was transformed with AtAAP5 and the empty vector
7 319 pDR196. Yeast growth was evaluated after three days in a media supplemented with aspartic
8 320 acid, proline, lysine and arginine as the sole N source. Fig. 5A shows that yeast transformation
9 321 with pDR196 harbouring the *PpAAP1* coding sequence conferred to strain 22Δ8AA the ability
10 322 to grow with proline and aspartate and particularly low growth was observed with arginine and
11 323 lysine.

12 324 To determine the transport properties of *PpAAP1*, the uptake of L-[¹⁴C]-arginine by
13 325 yeast cells that expressed *PpAAP1* was quantified. Expression of *PpAAP1* in the yeast mutant
14 326 22Δ8AA gave an increase of L-[¹⁴C]-arginine over time compared to cells transformed with
15 327 the empty vector (Fig. 5B). *PpAAP1*-mediated L-[¹⁴C]-arginine uptake was concentration
16 328 dependent and showed saturated kinetics with an apparent K_m value of $101.13 \pm 35.45 \mu\text{M}$ and
17 329 a V_{max} of $7.26 \pm 1.3 \text{ nmol min}^{-1} 10^8 \text{ cells}^{-1}$ (Fig. 5B).

30 330 31 331 *Growth of Arabidopsis aap5 mutants under high concentration of arginine*

32 332 *Arabidopsis* wild-type and a T-DNA insertion line defective in AtAAP5 (*aap5-1*; Svennerstam
33 333 et al. 2008) were grown with or without arginine for 21 days and phenotypic differences were
34 334 examined (Fig. S4). The mutant line had a T-DNA insertion located in the *AtAAP5* fifth exon
35 335 (Fig. S4A). *Arabidopsis* wild-type and *aap5-1* plants displayed similar root development after
36 336 growing with 3 mM nitrate as the sole N source (Fig. S4B). However, phenotypic differences
37 337 between wild-type and mutant plants were noticeable when arginine was also present in the
38 338 media at 1 mM concentration (Fig. S4C). The presence of arginine at high concentration
39 339 severely affected the growth of wild-type plants, which exhibited shorter roots and enhanced
40 340 secondary root development. In contrast, the growth of mutant plants was not affected by
41 341 arginine displaying the same phenotype of plants grown on nitrate as the sole nitrogen source.
42 342 Primary roots of *aap5-1* seedlings were significantly longer reaching an average length of 11.9
43 343 ± 2.1 cm, whereas roots of wild-type seedlings reached an average length of only 3.7 ± 1.1 cm.
44 344 These differences suppose an increase of approximately 65-74 % in the root growth of mutants
45 345 compared to wild-type plants (Fig. S4D). These phenotypic differences are attributable to the
46 346 role of AtAAP5 in arginine acquisition (Svennerstam et al. 2008). As shown in Fig. S4E, the

347 relative expression levels of *AtAAP5* observed in wild-type plants were not present in the
348 mutant line.

349

350 *Growth of aap5-1 mutants overexpressing PpAAP1 under low and high arginine*
351 *concentrations*

352 To investigate whether *PpAAP1* could be a functional ortholog of *AtAAP5*, the full-length
353 cDNA of *PpAAP1* was cloned into a gateway destination vector pGWB2 for overexpression in
354 *Arabidopsis* (Fig. 6). The resulting construct was used to transform the *aap5-1* mutant (Fig.
355 6A). The growth of mutant plants overexpressing *PpAAP1* (*35S::PpAAP1*) was compared at
356 nitrate 3 mM and different arginine concentrations from the micromolar range (10 μ M, 50 μ M,
357 and 100 μ M) to 1 mM. After three weeks of growth at 100 μ M of arginine no clear phenotypic
358 differences were observed in wild-type plants whereas *PpAAP1* transgenic lines exhibited a
359 considerable reduction of root length. However, this nutritional effect was undetectable at low
360 arginine concentration (10 μ M) (Fig. S5). Under high arginine levels (1 mM), the development
361 of both wild-type and transgenic plants was dramatically affected. Fig. 6B shows that
362 transgenic lines complementing *aap5-1* mutants (*35S::PpAAP1-1* and *35S::PpAAP1-5*)
363 exhibited a reduction of root length comparable to that of observed in wild-type plants. In
364 contrast, *aap5-1* plants were substantially larger and accumulated more biomass than wild-
365 type plants growing under the same conditions of culture, a phenotype previously described by
366 Svennerstam et al. (2008). In fact, our results also show that the biomass reduction observed in
367 *35S::PpAAP1-1* and *35S::PpAAP1-5* plants (L1: 89% \pm 8 % and L5: 85% \pm 7 %) was even
368 higher than in wild-type plants (36% \pm 9 %) (Fig. 6C).

369

370 *Molecular and functional analysis of PpAAP1-overexpressing plants*

371 To further characterize *PpAAP1*-overexpressing plants the relative expression levels of
372 *PpAAP1* and *AtAAP5* were compared in wild-type, *aap5-1*, and *PpAAP1*-overexpressing lines.
373 Fig. 7A shows that high levels of *PpAAP1* transcripts were detected in the lines *35S::PpAAP1-*
374 *1* and *35S::PpAAP1-5*. Furthermore, their relative expression levels were much higher than
375 those of the endogenous *AtAAP5* gene in wild-type plants. These results are consistent with the
376 highest reduction of root development observed in these plants growing at high arginine
377 concentration.

378 To assess that *PpAAP1* is effectively functioning as an arginine transporter in
379 *Arabidopsis*, the arginine root uptake capability of *PpAAP1*-overexpressing plants was
380 measured. Transgenic lines *35S::PpAAP1-1* and *35S::PpAAP1-5*, *aap5-1*, and wild-type plants

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3 381 exhibited identical development after 21 days of growth only with nitrate in sterile conditions.
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5 382 After growing, roots of intact plants were placed in a 30 μ M dual-labelled arginine solution
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7 383 ($^{13}\text{C}_6^{15}\text{N}_4$ -L-arginine-HCL) at pH 5.8 for 2h. Plants were collected and roots were dried to
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9 384 quantify root ^{15}N content. Arginine acquisition by roots was much higher in both *PpAAP1*-
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11 385 overexpressing lines (L1 and L5) than in wild-type plants and negligible in the *aap5-1* mutant
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13 386 (Fig. 7B). The observed uptake rates of ^{15}N in transgenic lines *35S::PpAAP1-1* and
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15 387 *35S::PpAAP1-5* were 4 and 3-fold higher than those observed in wild-type plants. Fig. 7B also
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17 388 shows that uptake capability of *aap5-1* was reduced by 90 % compared with wild-type plants.
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19 389 To further study the molecular basis of the contrasting phenotypes observed in the presence of
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21 390 arginine the relative content of ammonium was determined in wild-type, *aap5-1*, and *PpAAP1*-
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23 391 overexpressing lines. Figure 7C shows that the internal content of ammonium significantly
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25 392 increased with arginine nutrition in wild-type and *PpAAP1* transgenic lines but no differences
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27 393 were observed in the *aap5-1* mutant. In summary, these results indicate that the overexpression
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29 394 of *PpAAP1* was able to rescue the arginine transport capability lost by T-DNA insertional
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31 395 mutagenesis in *Arabidopsis*, and concomitantly, restored an accumulation of ammonium *in*
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33 396 *planta*.

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399 Discussion

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401 *The family of amino acid permeases in maritime pine*

402 The present work aims to unravel how pines detect and incorporate amino acids by
403 identification of a transporter involved in arginine uptake by roots. Taking advantage of the
404 current available genomic resources in *P. pinaster*, the members of the amino acid permease
405 AAP family have been investigated in this gymnosperm species.

406 The AAP family in *P. pinaster* is composed with at least ten members and can be
407 phylogenetically grouped into three clusters of plant AAPs (Fig. 1). Three genes were included
408 into cluster 1, other three in cluster 2, and four genes in cluster 4. Pine transporters of the cluster
409 4 were selected to further study because they were phylogenetically close to AtAAP5, an amino
410 acid permease involved in arginine root uptake in *Arabidopsis* and exhibiting a high degree of
411 sequence identity (Table S2). PpAAPs of cluster 4 were expressed in almost all pine tissues.
412 These wide expression patterns are consistent with the hypothesis that many transporters are
413 expressed in different tissues at different developmental stages, and therefore have multiple
414 functions in plant N metabolism (Liu and Bush, 2006). Nevertheless, only *PpAAP1* and
415 *PpAAP2* exhibited high relative expression levels in root developing tissues (Fig. 2), suggesting
416 that they could be involved in the root uptake of amino acids from soil. This assumption is
417 further supported by immunodetection of PpAAP1 transporter in the membranes of maritime
418 pine roots (Fig. 4). Consistently, *AtAAP5* was also found to be expressed through the plant but
419 preferentially in the *A. thaliana* root cortex and endodermis (Brady et al. 2007; Gifford et al.
420 2008).

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422 *PpAAP1 is a high-affinity arginine transporter which expression is induced in maritime pine* 423 *roots in response to arginine*

424 Interestingly, the expression of *PpAAP1* was upregulated by externally supplied arginine
425 whereas *PpAAP2* expression was unaffected (Fig. 3). Moreover, the highest abundance of
426 *PpAAP1* transcripts in response to arginine nutrition was found to be located in the apical and
427 intermediate regions of the root suggesting a role of PpAAP1 in the uptake of arginine by
428 maritime pine roots.

429 Previous functional studies on AAP transporters have been performed by
430 complementation of yeast strains deficient in amino acid uptake at different concentrations of
431 externally supplied amino acids (Hirner et al. 2006; Forsum et al. 2008) and revealed that they

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3 432 are able to recognize and transport a wide spectrum of amino acids. The results obtained in this
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5 433 work suggest that PpAAP1 actively mediates uptake of arginine and lysine (Fig.5) with strong
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7 434 inhibition of yeast growth at relatively high concentrations of these basic amino acids. The
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9 435 observed capability to grow with proline is also consistent with previous studies in which the
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11 436 expression of several AAP family members can restore yeast growth on a medium containing
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13 437 proline as the sole nitrogen source (Tegeeder et al. 2000; Okumoto et al. 2002).

14 438 Determination of the kinetic behaviour for L-[¹⁴C]-arginine uptake revealed that
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16 439 PpAAP1 works as a functional arginine transporter at micromolar ranges from 10 to 100 μ M
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18 440 (Fig. 5). Typically, amino acid concentrations in the soils of agricultural systems, and
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20 441 temperate and boreal forests are in the micromolar range (Jämtgård et al. 2010). PpAAP1 was
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22 442 able to transport arginine with high affinity showing an apparent K_m value of approximately
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24 443 100 μ M (Fig. 5), lower than the value reported for *Arabidopsis* AtAAP5 140 μ M (Fischer et
25
26 444 al. 2002) and rice OsAAP3 1.02 mM (Taylor et al. 2015).

27 446 *PpAAP1 is responsible for in planta arginine uptake*

28 447 According to the results reported by Bonner et al. (1996) all amino acids except glutamine may
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30 448 cause growth inhibition, a phenomenon named general amino acid inhibition. However,
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32 449 Forsum et al. (2008) identified some amino acids that promote growth in *Arabidopsis* when
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34 450 supplied as the sole N source. Arginine had a positive effect on growth when supplied as the
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36 451 sole N source at low concentrations but when supplied at high concentration in combination
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38 452 with nitrate, it significantly hampers biomass production. Thereafter, screening of a series of
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40 453 T-DNA mutants lacking amino acid uptake mechanisms in *Arabidopsis* revealed that AtAAP5
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42 454 mutants displayed enhanced growth relative to wild-type plants when grown on 1 mM arginine
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44 455 (Svennerstam et al. 2008).

45 456 To elucidate whether PpAAP1 plays a role in the root uptake of arginine at field-
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47 457 relevant concentrations, *Arabidopsis* transgenic plants overexpressing PpAAP1 were obtained
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49 458 for the AtAAP5 mutants, and its growth was assessed under several arginine concentrations
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51 459 ranging from 10 μ M to 1 mM. Strong phenotypic alterations were identified on mutant plants,
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53 460 which exhibited much longer roots than wild-type plants when growing under high arginine
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55 461 supply (Fig. S4). The molecular basis of these phenotypical differences was explained by the
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57 462 mutants' inability to take up arginine that was reduced more than an 85 % respect to wild-type
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59 463 plants (Svennerstam et al. 2008). However, this nutritional effect was not observed in the
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3 464 growth of untransformed plants at low arginine concentrations (micromolar range) (Fig. S5),
4 465 likely reflecting that arginine is metabolically assimilated under these nutritional conditions.

5 466
6 In contrast, the exposure to high levels of arginine had a strong negative influence on
7 467 plant development for both wild-type and transgenic plants (Fig. 6). A dramatic inhibition of
8 468 plant biomass was evident in *35S::PpAAP1* plants at 1 mM arginine, even greater than
9 469 observed in wild-type plants (Fig. 6). Although the overexpression of *PpAAP1* causes delayed
10 470 growth at high arginine concentrations (1 mM), it should be noted that growth inhibition was
11 471 also apparent at lower arginine concentrations (Fig. S4). Therefore, it can be concluded that
12 472 the phenotypic appearance of *35S::PpAAP1* plants at low and high arginine concentration was
13 473 consistent with an efficient transport of this amino acid.

14 474
15 475 Moreover, the [¹⁵N]-arginine uptake assays showed that PpAAP1 was able to rescue
16 476 the inability of *aap5-1* mutants for arginine recognition and transport. Whereas arginine uptake
17 477 of *aap5-1* mutants was reduced by 90 %, the complementation with the maritime pine *PpAAP1*
18 478 conferred to transgenic *35S::PpAAP1* plants the ability to acquire arginine, more efficiently
19 479 than wild-type plants (Fig. 7). The acquisition of arginine resulted in ammonium accumulation
20 480 suggesting its metabolic utilization *in planta* (Fig. 7). Furthermore, these data suggest that the
21 481 ability of arginine incorporation and metabolism is limited in *Arabidopsis*, and nitrogen excess
22 482 accumulates as free ammonium in plant tissues. Consistently, the accumulation of ammonium
23 483 could also be responsible of the observed inhibition of yeast growth in the presence of basic
24 484 amino acids. Ammonium toxicity triggers stress in roots and shoots and explain the reduction
25 485 in biomass and inhibition of root length of wild-type and PpAAP1 overexpressing plants in the
26 486 presence of arginine (Li et al. 2014).

27 487
28 488 Taken together, the above findings fully support a role for PpAAP1 as an efficient
29 489 arginine transporter in maritime pine. However, additional studies are necessary to figure out
30 490 the specific function of other members of the *P. pinaster* AAP family in amino acid uptake and
31 491 long-distance transport of nitrogen.

32 492
33 493 Identification of amino acid transporters in conifers and the clarification of their specific
34 494 roles and potential redundancy in function is currently a challenging objective. New advances
35 495 in this research area will help to clarify how pines are able to incorporate amino acids from soil
36 496 and allocate them through the whole plant body.

37 495 **Supplementary data**

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3 497 **Supplementary tables**

4 498 **Table S1.** List of the specific primer sequences used in this research.

5 499 **Table S2.** Identity percentages among members of the *P. pinaster* and *A. thaliana* amino acid
6 permease (AAP) family. Highest identities are shaded in grey colour.

7 500
8 501 **Table S3.** Accession numbers of AAPs from *P. pinaster*, *A. thaliana*, *P. trichocarpa* and *O.*
9 *sativa*.

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11 503

12 504 **Supplementary figures**

13 505 **Fig. S1.** Predicted transmembrane domains of *P. pinaster* AAP proteins.

14 506 **Fig. S2.** Expression profiles of *PpAAP* genes

15 507 **Fig. S3.** Specificity of PpAPP1 antibodies.

16 508 **Fig. S4.** Characterization of the *Arabidopsis AtAAP5* mutants.

17 509 **Fig. S5.** Plant phenotypes growing at low arginine concentrations

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19 511

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21 513

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24 515
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34 525 **Conflict of interest**

35 526

36 527 None declared

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3 **653 Figure legends**

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7 **655 Figure 1. Phylogenetic analysis of maritime pine amino acid permeases.** An unrooted,
8 neighbour-joining (NJ)-based tree of the amino acid permease (AAP) family. The analysis was
9 performed as described in the 'Materials and methods' section and the tree was generated using
10 MEGA version 7.0. Branch lengths (drawn in the horizontal dimension only) are proportional
11 to phylogenetic distances. Bootstrap values are indicated (1000 replicates). Names of the
12 species are abbreviated with a two-letter code *P. pinaster* (Pp), *A. thaliana* (At), *P. trichocarpa*
13 (Pt) and *O. sativa* (Os). Accession numbers are provided as supplementary data in Table S3.
14 Clusters are depicted in different colours. Sequences from *P. pinaster* are indicated in bold.
15 AtAAP5, PpAAP1, PpAAP2, PpAAP3 and PpAAP4 are marked by asterisks.
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665 Figure 2. Relative expression levels of *PpAAP1* and *PpAAP2* in maritime pine seedlings.

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26 (A) Images captured using the exImage tool at ConGenIE.org
27 (<http://v22.popgenie.org/microdissection/>) indicating the expression and tissue localization for
28 *PpAAP1* and *PpAAP2*. Gene expression is shown in relative units. Red colour indicates high
29 level of expression and yellow colour low expression level. ExImage uses variance-stabilizing
30 transformation values for absolute expression generated by aligning RNA-Seq reads to the
31 reference genome and gene annotation with aligned read numbers. (B) Quantitative PCR
32 (qPCR) analysis of *PpAAP1* and *PpAAP2*. Total RNA was isolated from different organs of
33 one-month-old *P. pinaster* seedlings. The expression data were normalized using *EF1α* as
34 reference gene. Each value is the mean ± SE of three biological replicates. T, root tip; R, root;
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677 Figure 3 Differential expression of *PpAAP1* and *PpAAP2* in maritime pine seedlings

678 **supplied with arginine as a nitrogen source.** (A) Two-week-old *P. pinaster* seedlings were
679 grown in a hydroponic solution without arginine (control) or supplemented with arginine 2.25
680 mM. Root samples were collected after 1 (1H), 4 (4H), 8 (8H), 24 (24H) and 48 (48H) hours.
681 RNA was prepared from three sections of maritime pine roots, each of 3 cm in length, taken
682 from the upper part to the tip (1, 2, 3), and the relative expression levels of *PpAAP1* (B) and
683 *PpAAP2* (C) were determined by qPCR. Data were normalized to *EF1α* as a reference gene.
684 Specific primers used are listed in Table S1. Bars represent mean values of three assays with

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3 685 three biological replicates each \pm standard deviation. Different letters above bars indicate
4 686 significant differences between samples and were calculated using Student's t-test ($P < 0.01$).

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8 688 **Figure 4. Immunolocalization of PpAAP1 in the roots of maritime pine.** Confocal laser
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10 689 scanning micrographs of horizontal (A) and longitudinal (B) sections of root tips. The
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12 690 localization of PpAAP1 signals was detected using CF 488A conjugates on the green channel
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14 691 of the microscope. In the upper panels, green channel allows to localize AAP1-fluorescence
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16 692 signals associated to cell membranes meanwhile red channel reveals the cellular distribution in
17
18 693 root tips using bright field. In the lower panels, control micrographs of root sections.
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20 694
21 695 **Figure 5. Functional and kinetic analysis of PpAAP1 in yeast.** (A) Yeast strain 22 Δ 8AA
22 696 was transformed with the yeast expression vector pDR196 harbouring the coding sequence of
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24 697 *PpAAP1*, *AtAAP5* and empty pDR196 as a control. Growth was assayed on N-free medium
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26 698 containing 0.5 % ammonium sulphate (NH_4^+) as positive control, 10 μM and 0.5 mM arginine,
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28 699 or 0.5 mM lysine, proline and aspartic acid as sole N source. Two independent yeast colonies
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30 700 of each construction were grown in liquid URA-free SD minimal medium to reach stationary
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32 701 phase. Cells were pelleted, washed with sterile water and serially diluted (1, 1:10, 1:100 and
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34 702 1:1000). A volume of 10 μl corresponding to each dilution was spotted onto plates. The plates
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36 703 were incubated at 30 $^\circ\text{C}$ and photographed after 3 days. Yeast growth assays were repeated at
37
38 704 least three times with similar results. (B) Concentration-dependent kinetics of L-[^{14}C]-arginine
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40 705 uptake by the yeast strain 22 Δ 8AA transformed with either empty pDR196 or with pDR196
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42 706 harbouring the *PpAAP1* construct. The values are expressed as the means \pm standard deviation
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44 707 (SD) of three replicates; the following values were calculated for kinetic parameters: K_m :
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46 708 $101.13 \pm 35.45 \mu\text{M}$; V_{max} : $7.26 \pm 1.3 \text{ nmol min}^{-1} 10^6 \text{ cells}^{-1}$.

47 709
48 710 **Figure 6. Growth of *A. thaliana aap5-1* mutant overexpressing maritime pine *PpAAP1*.**
49 711 (A) *PpAAP1* construct in PGWB2 Gateway destination vector with CaMV35S::HPT-NosT
50
51 712 marker used to transform *Arabidopsis* mutants; (B). Wild-type (*wt*), *aap5-1*, and transgenic
52
53 713 lines 35S::*PpAAP1-1* (*L1*) and 35S::*PpAAP1-5* (*L5*) were grown on N-free half-strength MS
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55 714 medium supplemented with 3 mM nitrate and 1 mM arginine for 21 days (Bar 1 cm); (C) Plant
56
57 715 biomass of *Arabidopsis* wild-type (*wt*), *aap5-1*, 35S::*PpAAP1-1* and 35S::*PpAAP1-5* grown
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59 716 on N-free half-strength MS medium supplemented with 3 mM nitrate and 1 mM arginine for
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61 717 21 days; (D) Root length of plants grown on N-free half-strength MS medium supplemented

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3 718 with 3 mM nitrate and 1 mM arginine for 21 days. Asterisks indicate significant differences
4 719 and were calculated using Student's t-test ($P < 0.01$). No significant differences were detected
5 720 in the root length of plants grown without arginine. Bars represent mean values \pm standard
6 721 deviation.

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11 **Figure 7. Functional complementation of arginine uptake in *Arabidopsis aap5-1* mutants**

12 **by overexpression of maritime pine *PpAAP1*.** (A) Relative expression levels of *AtAAP5* and

13 724 *PpAAP1* genes in plants grown on N-free half-strength MS medium supplemented with 3 mM

14 725 *PpAAP1* genes in plants grown on N-free half-strength MS medium supplemented with 3 mM

15 726 nitrate and 1 mM arginine for 21 days. ND, not detected. EF1 α was used as reference gene.

16 727 Specific primers used are listed in Table S1. (B) Root ^{15}N uptake after 2 h culture in a 30 μM

17 728 dual-labelled arginine solution of plants grown on N-free half-strength MS medium

18 729 supplemented only with 3 mM nitrate for 21 days. Three replicate samples, each comprising

19 730 five plants, were incubated for line. Roots were dried at 60 $^{\circ}\text{C}$ and analysed for ^{15}N content at

20 731 the University of Málaga Research Facility (Unit of Atomic Spectrometry) using a flash EA

21 732 1112 elemental analyser coupled to a Delta V Advantage isotope ratio mass spectrometer

22 733 (IRMS; Thermo Electronic Corporation). Results were expressed as ^{15}N content by milligram

23 734 of dry root (DW, dry weight). Asterisks indicate significant differences between samples

24 735 referred to *aap5-1* mutant and were calculated using Student's t-test ($P < 0.01$). (C) Ammonium

25 736 content in wild-type (*wt*), *aap5-1*, and transgenic lines *35S::PpAAP1-1 (L1)* and *35S::PpAAP1-*

26 737 *5 (L5)* plants grown on N-free half-strength MS medium supplemented only with 3 mM nitrate

27 738 (white bar) and together with 1 mM arginine (black bar) for 21 days. Asterisks indicate

28 739 significant differences and were calculated using Student's t-test ($P < 0.005$). Bars represent

29 740 mean values of three biological replicates \pm standard deviation.

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3 Forest trees have access to diverse nitrogenous compounds in the soil such as ammonium, nitrate and amino acids. Recent progress has been made in the identification and characterization of ammonium and nitrate transporters. However, much more limited is our current knowledge of amino acid transport systems despite their relevance to fully understanding nitrogen nutrition in trees.

8 In the present study, we have identified 10 genes encoding putative amino acid permeases of the AAP family in maritime pine (*Pinus pinaster* Ait.). Four members of this family, PpAAP1, PpAAP2, PpAAP3, and PpAAP4 were phylogenetically related to AtAAP5, involved in arginine transport in *Arabidopsis thaliana*. One of these genes, *PpAAP1*, exhibited enhanced expression levels in maritime pine roots when arginine was externally supplied. PpAAP1 was functionally characterized by complementation of a yeast mutant strain defective in the transport of arginine, allowing yeast to take up [¹⁴C]-arginine with high affinity. Furthermore, PpAAP1 was able to restore the severely affected root uptake of arginine displayed by AtAAP5 T-DNA mutants. Uptake rates of ¹⁵N-labelled arginine were significantly higher in *PpAAP1*-overexpressing plants when compared to wild-type and AtAAP5 mutant plants. Taken together, our results indicate that PpAAP1 is a high-affinity arginine transporter in maritime pine.

21 **Keywords:** AAP family, amino acid transport, arginine uptake, organic nitrogen nutrition, root uptake, *Pinus pinaster*

27 Introduction

28
29 Nitrogen uptake is a key process for plant growth and productivity. Nitrogen (N) is available
30 to plants as an inorganic form in soil, such as ammonium and nitrate, and also as organic
31 compounds such as amino acids and peptides (Rentsch et al. 2007). Due to its paramount
32 importance as constituent of essential biomolecules, plants have evolved different ways and
33 mechanisms to incorporate, transport and store N in different organs and tissues (Xu et al.
34 2012).

35 Plant species differ in their preferred N source, absorbing this nutrient by the roots
36 primarily in inorganic and organic forms (Tegeger and Masclaux-Daubresse, 2018). Conifers
37 from boreal forests have a marked preference for ammonium over nitrate due to their natural
38 distribution in ammonium-rich soils (Öhlund and Näsholm, 2004; Miller and Hawkins, 2007;
39 Cañas et al. 2016). In some forest ecosystems, organic N in the form of amino acids and
40 peptides is also quantitatively important and may represent a major source of N for tree
41 nutrition (Lipson and Näsholm, 2001; Näsholm et al. 2009). However, the rates of amino acid
42 uptake are highly dependent on substrate abundance (Wright, 1962) and their natural
43 concentrations in soils are usually found in the lower micromolar range (Öhlund and Näsholm,
44 2004; Jones et al. 2005). Among them, arginine has the highest nitrogen to carbon ratio, which
45 makes it especially suitable as a source of organic N.

46 The first evidence that amino acids are taken up by plants started to appear at the
47 beginning of the 20th century (Hutchinson and Miller, 1912). Since then, molecular cloning
48 and functional complementation in yeast have revealed that plants possess a large number of
49 different amino acid transporters. In *Arabidopsis thaliana*, *Populus trichocarpa* and *Oryza*
50 *sativa*, at least 100, 134 and 96 genes respectively were annotated as putative amino acid
51 transporters (Yuan et al. 2005; Tuskan et al. 2006; Ouyang et al. 2007; Pratelli and Pilot, 2014).

52 Plant genes encoding putative amino acid transporters can be classified into two major
53 groups: the amino acid transporter family (ATF) and the amino acid polyamine choline (APC)
54 superfamily (Wipf et al. 2002). Most research has been performed on *Arabidopsis* and much
55 less is known about amino acid transport systems in other plant species (Pratelli and Pilot,
56 2014).

57 In *Arabidopsis*, the ATF family, also called amino acid/auxin permease family (AAP),
58 is the largest family consisting of 46 members (Rentsch et al. 2007). The ATFs can be divided
59 into six subfamilies, being the amino acid permease family (AAP) the best characterized with
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3 60 8 members. When examined in heterologous expression systems, *Arabidopsis* AAPs generally
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5 61 display an affinity for neutral and acidic amino acids, with the exceptions of AtAAP3 and
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7 62 AtAAP5, which efficiently mediate transport of basic amino acids (Fischer et al. 2002;
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9 63 Okumoto et al. 2004). Especially important for long-distance transport of arginine is AtAAP5,
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11 64 which transports arginine and lysine with high affinity (Svennerstam et al. 2008) having a role
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13 65 in the uptake of basic amino acids by roots (Svennerstam et al. 2011). Screening of *Arabidopsis*
14
15 66 T-DNA insertional mutants showed that *aap5* mutants displayed a clear phenotypic divergence
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17 67 when high levels of arginine were present in the growth media and the root uptake of basic
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19 68 amino acids was severely affected (Svennerstam et al. 2008).

20
21 69 In conifers, arginine is a key amino acid for N storage and mobilization that constitutes
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23 70 a large proportion of the amino acid content in the seed storage proteins (Cañas et al. 2016;
24
25 71 Llebrés et al. 2018). In the soil of boreal forests, amino acids such as arginine represent an
26
27 72 important fraction of the total nitrogen that is available for tree nutrition (Inselsbacher and
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29 73 Näsholm, 2012). However, in spite of the relevance of arginine for nitrogen nutrition and
30
31 74 metabolism no specific transporter for this amino acid has been yet identified in conifers.

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33 75 In this study, 10 full-length cDNA sequences of putative AAPs have been identified in
34
35 76 maritime pine using the available information of *P. pinaster* transcriptome (Canales et al. 2014).
36
37 77 **The expression level of one of them, PpAAP1, specifically increased in the roots when**
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39 78 **maritime pine plantlets were supplied with arginine.** We have functionally characterized
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41 79 *PpAAP1* in yeast to determine its kinetics and ability for arginine transport. In addition,
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43 80 transgenic *Arabidopsis* overexpressing *PpAAP1* were generated to further study its function
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45 81 during plant development with arginine. Altogether, our results suggest that PpAAP1 is an
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47 82 efficient arginine transporter in maritime pine.
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85 **Materials and methods**

86

87 *Plant material, transformation and growth conditions*

88 Seeds of *Pinus pinaster* Aiton were imbibed in distilled water for 24 h and grown in vermiculite
89 as a substrate. Seedlings were cultivated in a plant growth chamber at 24 °C and 16 h
90 photoperiod, light intensity of 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and watered twice a week with distilled water.
91 For expression analysis, cotyledons, hypocotyls and roots from one-month-old pine seedlings
92 were collected separately, frozen in liquid nitrogen and stored at -80 °C. For hydroponic
93 cultivation, two-week-old seedlings grown in vermiculite were harvested and individually
94 transferred to a 2 L pot with a nutritive solution (Canales et al. 2010) containing (mg/L):
95 Na_2HPO_4 (20), KCl (40), CaCl_2 (40), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (20), chelated Fe (5.6), $\text{NaMoO}_4 \cdot 7\text{H}_2\text{O}$
96 (0.001), $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ (0.006), H_3BO_3 (0.09), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.7). Seedlings
97 were grown either without a nitrogen source or supplemented with 2.25 mM of arginine. To
98 avoid contamination the nutrient solutions adjusted to pH 5.8 were changed every day. Root
99 samples were harvested after 1, 4, 8, 24 and 48 hours of treatment with the nutrient solution,
100 frozen in liquid nitrogen and stored at -80 °C until use for expression analysis. Three biological
101 replicates for each time point were harvested.

102 The full-length *PpAAP1* sequence was retrieved from the SustainPine Database v.3.0
103 (Canales et al. 2014). PCR amplification was performed from 100 ng of *P. pinaster* cDNA
104 using *PpAAP1* specific primers listed in Table S1. The PCR product was recombined into
105 pDONR207 (Invitrogen, Germany) and cloned into the destination vector pGWB2, which
106 contains a hygromycin selectable marker gene and the CaMV 35S promoter to drive the
107 expression of the transgene. The construct was verified by sequencing.

108 Seeds of *Arabidopsis thaliana* AtAAP5 T-DNA insertion line (*aap5-1*, SALK-041999,
109 were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK). Seeds were
110 grown in soil at 24 °C and 16 h light photoperiod for 8 weeks and the presence of the T-DNA
111 insert was verified by PCR using specific primers previously described by Svennerstam et al.
112 (2008). Homozygous mutants were transformed by the floral dipping method (Clough and Bent,
113 1998) with *Agrobacterium tumefaciens* strain C58C1 containing *PpAAP1* in pGWB2.
114 Transgenic seedlings were selected on half-strength Murashige and Skoog (MS; Murashige
115 and Skoog, 1962) medium containing 20 $\mu\text{g mL}^{-1}$ hygromycin B (Duchefa Biochemie, Haarlem,
116 the Netherlands).

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3 117 For *in vitro* studies, *Arabidopsis* sterile seeds were germinated on plates containing N
4 118 free half-strength MS media with 0.8 % plant agar (Duchefa Biochemie, Haarlem, the
5 119 Netherlands), 0.5 % sucrose, 3 mM potassium nitrate and several concentrations of arginine
6 120 (10 μ M, 50 μ M, 100 μ M or 1 mM). The media was buffered to pH 5.8 with MES (Sigma).
7 121 Plants were grown on vertical plates (to avoid roots penetrating the agar surface) in a growth
8 122 chamber under long day (16 h light/ 8 h dark) at 22 °C and photosynthetic photon flux density
9 123 of 150 μ mol m⁻² s⁻¹. *A. thaliana* wild-type (ecotype Columbia-0) plants were used as controls
10 124 in all experiments described. Root length and biomass accumulation were measured.
11 125

126 *RNA isolation and qPCR analysis*

127 Total RNA was extracted as described by Canales et al. (2012). Samples were ground in liquid
128 nitrogen and 100 mg of each were extracted with 650 μ L extraction buffer containing 3 %
129 cetyltrimethylammonium bromide (CTAB), 100 mM Tris pH 8.0, 2 M NaCl, 2 % PVP-40 and
130 30 mM ethylenediaminetetraacetic acid (EDTA). Residual DNA was removed by a treatment
131 with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) and preparation purified using
132 Nucleospin® Gel and PCR Clean-Up (Macherey-Nagel, Düren, Germany). Total RNA was
133 quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham,
134 USA). cDNA synthesis was performed from 500 ng of total RNA in 10 μ L reaction volume
135 using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) under the following
136 conditions, 25 °C for 5 min, 46 °C for 20 min and 95 °C for 1 min. Real-time PCR was carried
137 out on a CFX384 Real-Time System C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA)
138 using RT-qPCR SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA) under the
139 following conditions, 95 °C for 2 min (1 cycle), 95 °C for 1 s and 60 °C for 5 s (40 cycles). A
140 reaction mixture was performed in a total reaction volume of 10 μ L containing primers (0.5 μ M
141 each), cDNA (10 ng), and 5 μ l of SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA,
142 USA). Three biological replicates with three technical replicates were performed per sample
143 type. *Elongation factor alpha 1* (EF1 α) was used as a reference gene. Gene-specific primers
144 used are listed in Table S1.
145

146 *Immunolocalization of PpAAP1 under nitrogen starvation*

147 One-month-old seedlings were harvested and transferred to a hydroponic nutritive
148 solution containing macronutrients and micronutrients with no source of nitrogen (Canales et
149 al. 2010). After 2 days, root tips were transferred to 4% (v/v) paraformaldehyde and 0.01%
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3 150 (v/v) Triton X-100 in 0.1 M PBS pH 7.4 and vacuum infiltrated. Fixation was then performed
4
5 151 overnight at 4 °C. Root tips were sectioned on a vibratome into 80 µm transverse and 0.5 mm
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7 152 longitudinal slices. The samples were permeabilized with 0.05% (v/v) Tween-20 in 0.1 M PBS
8
9 153 pH 7.4 for 1 h and blocked with 5% (w/v) bovine serum albumin (BSA) in 0.1 M PBS pH 7.4
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11 154 for 1 h at room temperature (RT). Then, the sections were incubated with primary antibodies
12
13 155 specific for PpAAP1 (1:200) [PpAAP1-5'-H2N-GSIEGIIQDLKSYKPFHTAY-CONH2-3']
14
15 156 (Biomedal SL, Seville, Spain) in 0.1 m PBS pH 7.4 containing 1% (w/v) BSA at 4 °C
16
17 157 overnight. **A negative control incubated with non-immune serum was also prepared.** The
18
19 158 samples were rinsed with 0.1 M PBS containing 0.01% (v/v) Triton X-100 and then incubated
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21 159 with 2 µg mL⁻¹ **of fluorescent secondary antibodies (CF 488A conjugates, Sigma-Aldrich)**
22
23 160 (1:100) for 3 h at room temperature in the dark. Finally, the sections were washed twice with
24
25 161 0.1 M PBS pH 7.4 and stored in the dark at 2-8 °C. Antibody specificity is shown in Figure S3
26
27 162 using recombinantly produced PpAAP1 according to Cantón et al. (1996).

28
29 163 Confocal microscopy was performed using a Leica SP5 HyD confocal microscope
30
31 164 equipped with argon, krypton and HeNe lasers and an acousto-optical beam splitter (AOBS)
32
33 165 head system (Leica Microsystems, Bannockburn, IL, USA) capable of spectrally separating the
34
35 166 emissions from green fluorescent protein (GFP) and yellow fluorescent protein (YFP). The
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37 167 488 nm excitation line of an argon ion laser was used to excite GFP and YFP. The confocal
38
39 168 detectors included 2 × HyD and 3 × PMT + 1 for reflection. Line averaging was performed over
40
41 169 16 frames for single images. The images were processed using confocal software and
42
43 170 Fiji ImageJ software (version 4.1.1).

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45 172 *Yeast growth and transformation*

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47 173 The *Saccharomyces cerevisiae* strain 22Δ8AA is a mutant lacking multiple amino acid uptake
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49 174 systems (Mata gap1-1, put4-1, uga4-1, Δcan1, Δapl1, Δlyp1, Δhip1, Δdip5, ura3-1; Fischer et
50
51 175 al. 2002). The coding sequences corresponding to *PpAAP1* and *AtAAP5* were subcloned in the
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53 176 yeast expression vector *pDR196* and introduced into yeast strain 22Δ8AA by heat shock
54
55 177 according to Dohmen et al. (1991). Colonies harbouring PpAAP1, AtAAP5 and the empty
56
57 178 pDR196 vector were selected on a synthetic minimal defined medium without uracil, and
58
59 179 containing 0.67 % yeast nitrogen base without amino acids, 2 % glucose, 0.01 % (adenine,
60
180 cysteine, leucine, lysine, threonine, tryptophan), 0.005 % (aspartic acid, histidine,
181 isoleucine, methionine, phenylalanine, serine, tyrosine, valine) and 2 % agar. **The**
182 **transformants were grown in liquid URA-free SD minimal medium to reach stationary phase.**

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3 183 Cells were pelleted, washed with sterile distilled water and serially diluted (1, 1:10, 1:100 and
4 184 1:1000). A volume of 10 μ l corresponding to each dilution was spotted onto plates. Growth
5 185 complementation assays were performed in N-free minimal media (0.17 % yeast nitrogen base
6 186 without amino acids and ammonium sulphate, 2 % glucose and 2 % agar) containing arginine
7 187 (10 μ M and 0.5 mM), or 0.5 mM lysine, proline or aspartic acid as the sole nitrogen source.
8 188 AtAAP5 was used as control in this study and empty pDR196 vector as a negative control. For
9 189 positive control N-free media was supplemented with 0.5 % ammonium sulphate. The growth
10 190 of two independent yeast colonies was examined after 3 days of culture at 30°C.

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19 192 *Transport measurement of [¹⁴C]-arginine in yeast*

20 193 PpAAP1 transport activity was determined by measuring the initial uptake rate of [¹⁴C]-
21 194 arginine. Yeast cells were grown to reach logarithmic phase, harvested at OD₆₀₀ of 0.6, washed,
22 195 and resuspended in ice-cold buffer (50 mM KH₂PO₄, pH 5.0), to a final OD₆₀₀ of 5. Before the
23 196 uptake measurements, an aliquot of yeast cells was supplemented with 20 mM glucose and
24 197 incubated at 30 °C for 5 min. Uptake was initiated by the addition of 100 μ L of radioactive
25 198 substrate mixture, containing L-[¹⁴C]-arginine (specific activity 274.3 mCi mmol⁻¹, Perkin
26 199 Elmer, Boston, MA, USA) at several concentrations ranged between 2.5 μ M to 150 μ M.
27 200 Accumulated counts were measured 30 s, 1 min, 2 min and 4 min after the addition of the
28 201 radioactive substrate. Radioactivity (count per min) was measured in a scintillation counter
29 202 (Beckman LS6000IC). Transport measurements were performed by triplicate and the values
30 203 were well fitted by a Michaelis-Menten model. For determination of kinetic parameters uptake
31 204 assays were performed with PpAAP1-pDR196 and an empty vector under the same conditions
32 205 according to Gietz and Woods (2002). Transport activity was analyzed using Origin (Pro),
33 206 Version 2018b (Origin Lab Corporation, Northampton, MA, USA) and fitting with non-linear
34 207 curve fit, growth/sigmoidal category.

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49 209 *Uptake measurement of [¹⁵N]-arginine in Arabidopsis*

50 210 *Arabidopsis* wild-type, *aap5-1* and two independent homozygous lines (T3 generation plants)
51 211 overexpressing *PpAAP1* [*35S::PpAAP-1* (L1), *35S::PpAAP-5* (L5); see below] were grown on
52 212 plates containing 0.8 % agar, 0.5 % sucrose, N-free half-strength MS medium and 3 mM nitrate
53 213 under long day (16 h light/ 8 h dark) conditions, 22 °C and photosynthetic photon flux density
54 214 of 150 μ mol m⁻² s⁻¹. After 21 days plants were harvested and washed thoroughly in 0.5 mM
55 215 CaCl₂. Roots of the intact plants were then submerged for 2 h in 1 mL of the uptake solution

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3 216 per plant on a shaking table. Uptake solution of 0.5 mM CaCl₂ pH 5.8 included 30 μM dual-
4 217 labelled arginine ([¹³C₆¹⁵N₄]-L-arginine-HCL, ThermoFisher, USA). Three replicate samples,
5 218 each comprising five plants, were incubated for each line. Plants were collected and washed
6 219 three times in 0.5 mM CaCl₂. Roots were excised, dried at 60 °C, weighed and homogenized.
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8 220 Finally, samples were analysed for ¹⁵N content at the University of Málaga Research Facility
9 221 (Unit of Atomic Spectrometry) using a flash EA 1112 elemental analyser coupled to a Delta V
10 222 Advantage isotope ratio mass spectrometer (IRMS; Thermo Electronic Corporation).
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224 *Determination of ammonium content*

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17 225 *Arabidopsis* wild-type, *aap5-1*, *35S::PpAAP-1* (L1) and *35S::PpAAP-5* (L5) plants were
18 226 grown on plates under the same conditions described above. After 21 days, three replicate
19 227 samples comprising five plants each, were harvested and ground in liquid nitrogen. Ammonium
20 228 was extracted with 2% 5-sulfosalicylic acid (SSA) (100 mg FW mL⁻¹). The extracts were
21 229 centrifuged at 13.000 g for 15 min and supernatants were recovered (Ferrario-Méry et al. 1998).
22 230 Ammonium content was determined following the phenol hypochlorite method (Solórzano et
23 231 al. 1969).
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233 *Sequence analysis of P. pinaster AAP family*

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32 234 Full-length amino acid sequences of plants AAPs were retrieved using PLAZA 3.0 database
33 235 (<http://bioinformatics.psb.ugent.be/plaza/>). Protein alignment was performed with the
34 236 ClustalW2 package. For phylogenetic analyses, the alignments were imported into the
35 237 Molecular Evolutionary Genetics Analyses software (MEGA) version 7.0 (Kumar et al. 2016).
36 238 Phylogenetic analyses were conducted using the neighbour-joining (NJ) method with the
37 239 Poisson correction model for distance computation, the pairwise deletion option and bootstrap
38 240 test with 1000 replicates (Couturier et al. 2010). Names of the species are abbreviated using a
39 241 two-letter code *P. pinaster* (Pp), *A. thaliana* (At), *P. trichocarpa* (Pt) and *O. sativa* (Os).
40 242 Accession numbers are provided as supplementary data in Table S3. Transmembrane domains
41 243 were predicted by CCTOP algorithm (Constrained Consensus TOPology,
42 244 <http://cctop.enzim.ttk.mta.hu>; Dobson et al. 2015). The expression localization data of *PpAAP*
43 245 genes is available at ConGenIE.org (<http://v22.popgenie.org/microdissection/>).
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248 Results

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250 *Identification and phylogenetic analysis of AAP family members in maritime pine*

251 A total of 10 members of the AAP family were identified in the *P. pinaster* database
252 (<http://www.scbi.uma.es/sustainpine>; Canales et al. 2014) by BLAST search of known
253 sequences from *Arabidopsis*. The homology of these membrane proteins was studied using
254 Consensus Constrained TOPology prediction (CCTOP; <http://cctop.enzim.ttk.mta.hu>) server
255 that integrates 10 different topology prediction methods incorporating parameters such as
256 charge bias, helix length and hydrophobicity (Dobson et al. 2015). According to CCTOP, all
257 the PpAAP proteins contained 11 transmembrane domains (Table 1), with N- and C-terminal
258 regions and inter transmembrane domains of variable length (Fig. S1). The amino acid
259 transporter AtAAP5 from *Arabidopsis* was also included in the analysis as a reference. As
260 depicted in Fig. S1, the topology predicted for AtAAP5 was quite similar to that of PpAAP1.
261 The genes identified in *P. pinaster* encode proteins with a similar number of amino acid
262 residues, from 466 to 500, and with a molecular mass ranging from 51 to 58.5 kDa (Table 1).
263 These results are quite similar to those described for members of the AAP family characterized
264 in other plant species such as *A. thaliana*, *O. sativa* and *P. trichocarpa* (Yuan et al. 2005;
265 Tuskan et al. 2006; Rentsch et al. 2007; Ouyang et al. 2007; Taylor et al. 2015).

266 A phylogenetic analysis of 51 AAP protein sequences from *P. pinaster* (10), *O. sativa*
267 (19), *P. trichocarpa* (14) and *A. thaliana* (8) revealed that AAPs are divided into four main
268 groups (Fig. 1), as described previously (Couturier et al. 2010; Taylor et al. 2015). The cluster
269 1 included three maritime pine proteins (PpAAP5, PpAAP6 and PpAAP7) together with only
270 sequences from *O. sativa* in good agreement to Taylor et al. (2015). As previously described
271 by Okumoto et al. (2002), AtAAP7 was the most distal member of the *Arabidopsis* AAP family
272 and grouped together with three PpAAP proteins included in cluster 2 exhibiting 50-52 %
273 identity (PpAAP8, PpAAP9 and PpAAP10). On the other hand, three AAPs from *Arabidopsis*
274 (AtAAP1, AtAAP6 and AtAAP8) were included in cluster 3 in which no AAP proteins from
275 *P. pinaster* were present. Four putative AAPs from *P. pinaster*, PpAAP1, PpAAP2, PpAAP3,
276 and PpAAP4 were included in the cluster 4 together with AtAAP2, AtAAP3, AtAAP4 and
277 AtAAP5 from *Arabidopsis*. These proteins present a high degree of identity at the protein level
278 (60 to 69 %; Table S2). All *Arabidopsis* AAPs in cluster 4 appears to be involved in loading
279 of amino acids into the phloem (Tegeeder and Ward, 2012), and AtAAP5 has been previously
280 shown to be involved in the root uptake of basic amino acids (Svennerstan et al. 2008). The

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3 281 closest *P. pinaster* homologs of AtAAP5, involved in root uptake of basic amino acids, were
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5 282 PpAAP1, PpAAP2, PpAAP3 and PpAAP4.

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7 283 To explore the potential function of *PpAAPs* genes phylogenetically related to *AtAAP5*
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9 284 the relative expression levels of *PpAAP1*, *PpAAP2*, *PpAAP3*, and *PpAAP4* genes were
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11 285 investigated at the ConGenIE database (Cañas et al. 2017). Among these genes only *PpAAP1*
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13 286 and *PpAAP2* presented expression levels in developing roots (Fig. 2A). *PpAAP1* transcripts
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15 287 were highly abundant in developing root vascular tissue (DRV) whereas *PpAAP2* was mainly
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17 288 expressed in the root meristem (RM). The relative abundance of *PpAAP1* and *PpAAP2*
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19 289 transcripts in the roots was confirmed by qPCR analysis (Fig. 2B). In comparison to *PpAAP1*
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21 290 and *PpAAP2*, the expression of *PpAAP3* and *PpAAP4* was barely detectable (Fig. S2). *PpAAP1*
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23 291 and *PpAAP2* were therefore selected as potentially involved in the primary uptake of arginine
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25 292 from the soil.

26 294 *Effect of arginine availability on the expression of PpAAP1 and PpAAP2*

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28 295 To study whether *PpAAP1* and *PpAAP2* expression are affected by the exogenous supply of
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30 296 arginine, 2 week-old pine plantlets were cultured for two days in hydroponic media in the
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32 297 absence (control) or the presence of arginine at a concentration of 2.25 mM (Fig. 3A). Time-
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34 298 course analysis of the expression of these genes was performed by qPCR in three sections of
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36 299 **the root, upper part (1), medium part (2) and apical part including the root tip (3).**

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38 300 Fig. 3B shows that the expression of *PpAAP1* was upregulated in response to arginine
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40 301 availability with maximal values observed after 48 hours of culture and localized in the root
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42 302 tip and in the medium part of the root. In contrast, no major differences were found in the
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44 303 transcript levels of *PpAAP2* between control and arginine supplied plants at the end of the
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46 304 treatment (Fig. 3C). It should be noted that the transcript levels of *PpAAP2* were much lower
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48 305 than those *PpAAP1*. PpAAP1 protein was immunolocalized in maritime pine roots using
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50 306 antibodies raised against a 20 amino acid sequence specific for PpAAP1 (Fig. 4). The protein
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52 307 **localization in the apex of the roots was consistent** with the observed expression profile of
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54 308 *PpAAP1* in the same tissues (Fig.2). These results suggest that PpAAP1 may play an important
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56 309 role in the root arginine uptake and was selected for functional analysis.

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58 311 *Functional analysis of PpAAP1 in yeast*

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60 312 To determine whether *PpAAP1* encodes a functional amino acid permease, a yeast
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62 313 complementation test was performed by transforming the mutant strain 22Δ8AA with the
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64 314 maritime pine *PpAAP1* cDNA driven by the *PMAl* promoter (plasma membrane *ATPase*) in

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3 315 the yeast expression vector pDR196 (Rentsch et al. 1995). The mutant yeast strain 22Δ8AA is
4 316 unable to use efficiently arginine, aspartate, glutamate and proline as sole N source (Fischer et
5 317 al. 2002). A positive control with ammonium as the nitrogen source was included. As
6 318 additional controls, the strain 22Δ8AA was transformed with AtAAP5 and the empty vector
7 319 pDR196. Yeast growth was evaluated after three days in a media supplemented with aspartic
8 320 acid, proline, lysine and arginine as the sole N source. Fig. 5A shows that yeast transformation
9 321 with pDR196 harbouring the *PpAAP1* coding sequence conferred to strain 22Δ8AA the ability
10 322 to grow with proline and aspartate and particularly low growth was observed with arginine and
11 323 lysine.

12 324 To determine the transport properties of *PpAAP1*, the uptake of L-[¹⁴C]-arginine by
13 325 yeast cells that expressed *PpAAP1* was quantified. Expression of *PpAAP1* in the yeast mutant
14 326 22Δ8AA gave an increase of L-[¹⁴C]-arginine over time compared to cells transformed with
15 327 the empty vector (Fig. 5B). *PpAAP1*-mediated L-[¹⁴C]-arginine uptake was concentration
16 328 dependent and showed saturated kinetics with an apparent K_m value of $101.13 \pm 35.45 \mu\text{M}$ and
17 329 a V_{max} of $7.26 \pm 1.3 \text{ nmol min}^{-1} 10^8 \text{ cells}^{-1}$ (Fig. 5B).

30 330 31 331 *Growth of Arabidopsis aap5 mutants under high concentration of arginine*

32 332 *Arabidopsis* wild-type and a T-DNA insertion line defective in AtAAP5 (*aap5-1*; Svennerstam
33 333 et al. 2008) were grown with or without arginine for 21 days and phenotypic differences were
34 334 examined (Fig. S4). The mutant line had a T-DNA insertion located in the *AtAAP5* fifth exon
35 335 (Fig. S4A). *Arabidopsis* wild-type and *aap5-1* plants displayed similar root development after
36 336 growing with 3 mM nitrate as the sole N source (Fig. S4B). However, phenotypic differences
37 337 between wild-type and mutant plants were noticeable when arginine was also present in the
38 338 media at 1 mM concentration (Fig. S4C). The presence of arginine at high concentration
39 339 severely affected the growth of wild-type plants, which exhibited shorter roots and enhanced
40 340 secondary root development. In contrast, the growth of mutant plants was not affected by
41 341 arginine displaying the same phenotype of plants grown on nitrate as the sole nitrogen source.
42 342 Primary roots of *aap5-1* seedlings were significantly longer reaching an average length of 11.9
43 343 ± 2.1 cm, whereas roots of wild-type seedlings reached an average length of only 3.7 ± 1.1 cm.
44 344 These differences suppose an increase of approximately 65-74 % in the root growth of mutants
45 345 compared to wild-type plants (Fig. S4D). These phenotypic differences are attributable to the
46 346 role of AtAAP5 in arginine acquisition (Svennerstam et al. 2008). As shown in Fig. S4E, the

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3 347 relative expression levels of *AtAAP5* observed in wild-type plants were not present in the
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5 348 mutant line.

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9 350 *Growth of aap5-1 mutants overexpressing PpAAP1 under low and high arginine*
10 351 *concentrations*

11 352 To investigate whether *PpAAP1* could be a functional ortholog of *AtAAP5*, the full-length
12 353 cDNA of *PpAAP1* was cloned into a gateway destination vector pGWB2 for overexpression in
13 354 *Arabidopsis* (Fig. 6). The resulting construct was used to transform the *aap5-1* mutant (Fig.
14 355 6A). The growth of mutant plants overexpressing *PpAAP1* (*35S::PpAAP1*) was compared at
15 356 nitrate 3 mM and different arginine concentrations from the micromolar range (10 μ M, 50 μ M,
16 357 and 100 μ M) to 1 mM. After three weeks of growth at 100 μ M of arginine no clear phenotypic
17 358 differences were observed in wild-type plants whereas *PpAAP1* transgenic lines exhibited a
18 359 considerable reduction of root length. However, this nutritional effect was undetectable at low
19 360 arginine concentration (10 μ M) (Fig. S5). Under high arginine levels (1 mM), the development
20 361 of both wild-type and transgenic plants was dramatically affected. Fig. 6B shows that
21 362 transgenic lines complementing *aap5-1* mutants (*35S::PpAAP1-1* and *35S::PpAAP1-5*)
22 363 exhibited a reduction of root length comparable to that of observed in wild-type plants. In
23 364 contrast, *aap5-1* plants were substantially larger and accumulated more biomass than wild-
24 365 type plants growing under the same conditions of culture, a phenotype previously described by
25 366 Svennerstam et al. (2008). In fact, our results also show that the biomass reduction observed in
26 367 *35S::PpAAP1-1* and *35S::PpAAP1-5* plants (L1: 89% \pm 8 % and L5: 85% \pm 7 %) was even
27 368 higher than in wild-type plants (36% \pm 9 %) (Fig. 6C).

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43 370 *Molecular and functional analysis of PpAAP1-overexpressing plants*

44 371 To further characterize *PpAAP1*-overexpressing plants the relative expression levels of
45 372 *PpAAP1* and *AtAAP5* were compared in wild-type, *aap5-1*, and *PpAAP1*-overexpressing lines.
46 373 Fig. 7A shows that high levels of *PpAAP1* transcripts were detected in the lines *35S::PpAAP1-*
47 374 *1* and *35S::PpAAP1-5*. Furthermore, their relative expression levels were much higher than
48 375 those of the endogenous *AtAAP5* gene in wild-type plants. These results are consistent with the
49 376 highest reduction of root development observed in these plants growing at high arginine
50 377 concentration.

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56 378 To assess that *PpAAP1* is effectively functioning as an arginine transporter in
57 379 *Arabidopsis*, the arginine root uptake capability of *PpAAP1*-overexpressing plants was
58 380 measured. Transgenic lines *35S::PpAAP1-1* and *35S::PpAAP1-5*, *aap5-1*, and wild-type plants

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3 381 exhibited identical development after 21 days of growth only with nitrate in sterile conditions.
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5 382 After growing, roots of intact plants were placed in a 30 μ M dual-labelled arginine solution
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7 383 ($^{13}\text{C}_6^{15}\text{N}_4$ -L-arginine-HCL) at pH 5.8 for 2h. Plants were collected and roots were dried to
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9 384 quantify root ^{15}N content. Arginine acquisition by roots was much higher in both *PpAAP1*-
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11 385 overexpressing lines (L1 and L5) than in wild-type plants and negligible in the *aap5-1* mutant
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13 386 (Fig. 7B). The observed uptake rates of ^{15}N in transgenic lines *35S::PpAAP1-1* and
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15 387 *35S::PpAAP1-5* were 4 and 3-fold higher than those observed in wild-type plants. Fig. 7B also
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17 388 shows that uptake capability of *aap5-1* was reduced by 90 % compared with wild-type plants.
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19 389 To further study the molecular basis of the contrasting phenotypes observed in the presence of
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21 390 arginine the relative content of ammonium was determined in wild-type, *aap5-1*, and *PpAAP1*-
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23 391 overexpressing lines. Figure 7C shows that the internal content of ammonium significantly
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25 392 increased with arginine nutrition in wild-type and *PpAAP1* transgenic lines but no differences
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27 393 were observed in the *aap5-1* mutant. In summary, these results indicate that the overexpression
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29 394 of *PpAAP1* was able to rescue the arginine transport capability lost by T-DNA insertional
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31 395 mutagenesis in *Arabidopsis*, and concomitantly, restored an accumulation of ammonium *in*
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33 396 *planta*.

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399 Discussion

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401 *The family of amino acid permeases in maritime pine*

402 The present work aims to unravel how pines detect and incorporate amino acids by
403 identification of a transporter involved in arginine uptake by roots. Taking advantage of the
404 current available genomic resources in *P. pinaster*, the members of the amino acid permease
405 AAP family have been investigated in this gymnosperm species.

406 The AAP family in *P. pinaster* is composed with at least ten members and can be
407 phylogenetically grouped into three clusters of plant AAPs (Fig. 1). Three genes were included
408 into cluster 1, other three in cluster 2, and four genes in cluster 4. Pine transporters of the cluster
409 4 were selected to further study because they were phylogenetically close to AtAAP5, an amino
410 acid permease involved in arginine root uptake in *Arabidopsis* and exhibiting a high degree of
411 sequence identity (Table S2). PpAAPs of cluster 4 were expressed in almost all pine tissues.
412 These wide expression patterns are consistent with the hypothesis that many transporters are
413 expressed in different tissues at different developmental stages, and therefore have multiple
414 functions in plant N metabolism (Liu and Bush, 2006). Nevertheless, only *PpAAP1* and
415 *PpAAP2* exhibited high relative expression levels in root developing tissues (Fig. 2), suggesting
416 that they could be involved in the root uptake of amino acids from soil. This assumption is
417 further supported by immunodetection of PpAAP1 transporter in the membranes of maritime
418 pine roots (Fig. 4). Consistently, *AtAAP5* was also found to be expressed through the plant but
419 preferentially in the *A. thaliana* root cortex and endodermis (Brady et al. 2007; Gifford et al.
420 2008).

421

422 *PpAAP1 is a high-affinity arginine transporter which expression is induced in maritime pine* 423 *roots in response to arginine*

424 Interestingly, the expression of *PpAAP1* was upregulated by externally supplied arginine
425 whereas *PpAAP2* expression was unaffected (Fig. 3). Moreover, the highest abundance of
426 *PpAAP1* transcripts in response to arginine nutrition was found to be located in the apical and
427 intermediate regions of the root suggesting a role of PpAAP1 in the uptake of arginine by
428 maritime pine roots.

429 Previous functional studies on AAP transporters have been performed by
430 complementation of yeast strains deficient in amino acid uptake at different concentrations of
431 externally supplied amino acids (Hirner et al. 2006; Forsum et al. 2008) and revealed that they

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3 432 are able to recognize and transport a wide spectrum of amino acids. The results obtained in this
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5 433 work suggest that PpAAP1 actively mediates uptake of arginine and lysine (Fig.5) with strong
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7 434 inhibition of yeast growth at relatively high concentrations of these basic amino acids. The
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9 435 observed capability to grow with proline is also consistent with previous studies in which the
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11 436 expression of several AAP family members can restore yeast growth on a medium containing
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13 437 proline as the sole nitrogen source (Tegeeder et al. 2000; Okumoto et al. 2002).

14 438 Determination of the kinetic behaviour for L-[¹⁴C]-arginine uptake revealed that
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16 439 PpAAP1 works as a functional arginine transporter at micromolar ranges from 10 to 100 μ M
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18 440 (Fig. 5). Typically, amino acid concentrations in the soils of agricultural systems, and
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20 441 temperate and boreal forests are in the micromolar range (Jämtgård et al. 2010). PpAAP1 was
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22 442 able to transport arginine with high affinity showing an apparent K_m value of approximately
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24 443 100 μ M (Fig. 5), lower than the value reported for *Arabidopsis* AtAAP5 140 μ M (Fischer et
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26 444 al. 2002) and rice OsAAP3 1.02 mM (Taylor et al. 2015).

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28 446 *PpAAP1 is responsible for in planta arginine uptake*

29 447 According to the results reported by Bonner et al. (1996) all amino acids except glutamine may
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31 448 cause growth inhibition, a phenomenon named general amino acid inhibition. However,
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33 449 Forsum et al. (2008) identified some amino acids that promote growth in *Arabidopsis* when
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35 450 supplied as the sole N source. Arginine had a positive effect on growth when supplied as the
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37 451 sole N source at low concentrations but when supplied at high concentration in combination
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39 452 with nitrate, it significantly hampers biomass production. Thereafter, screening of a series of
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41 453 T-DNA mutants lacking amino acid uptake mechanisms in *Arabidopsis* revealed that AtAAP5
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43 454 mutants displayed enhanced growth relative to wild-type plants when grown on 1 mM arginine
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45 455 (Svennerstam et al. 2008).

46 456 To elucidate whether PpAAP1 plays a role in the root uptake of arginine at field-
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48 457 relevant concentrations, *Arabidopsis* transgenic plants overexpressing PpAAP1 were obtained
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50 458 for the AtAAP5 mutants, and its growth was assessed under several arginine concentrations
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52 459 ranging from 10 μ M to 1 mM. Strong phenotypic alterations were identified on mutant plants,
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54 460 which exhibited much longer roots than wild-type plants when growing under high arginine
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56 461 supply (Fig. S4). The molecular basis of these phenotypical differences was explained by the
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58 462 mutants' inability to take up arginine that was reduced more than an 85 % respect to wild-type
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60 463 plants (Svennerstam et al. 2008). However, this nutritional effect was not observed in the

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3 464 growth of untransformed plants at low arginine concentrations (micromolar range) (Fig. S5),
4 465 likely reflecting that arginine is metabolically assimilated under these nutritional conditions.

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6 466 In contrast, the exposure to high levels of arginine had a strong negative influence on
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8 467 plant development for both wild-type and transgenic plants (Fig. 6). A dramatic inhibition of
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10 468 plant biomass was evident in *35S::PpAAP1* plants at 1 mM arginine, even greater than
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12 469 observed in wild-type plants (Fig. 6). Although the overexpression of *PpAAP1* causes delayed
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14 470 growth at high arginine concentrations (1 mM), it should be noted that growth inhibition was
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16 471 also apparent at lower arginine concentrations (Fig. S4). Therefore, it can be concluded that
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18 472 the phenotypic appearance of *35S::PpAAP1* plants at low and high arginine concentration was
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20 473 consistent with an efficient transport of this amino acid.

21 474 Moreover, the [¹⁵N]-arginine uptake assays showed that PpAAP1 was able to rescue
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23 475 the inability of *aap5-1* mutants for arginine recognition and transport. Whereas arginine uptake
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25 476 of *aap5-1* mutants was reduced by 90 %, the complementation with the maritime pine *PpAAP1*
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27 477 conferred to transgenic *35S::PpAAP1* plants the ability to acquire arginine, more efficiently
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29 478 than wild-type plants (Fig. 7). **The acquisition of arginine resulted in ammonium accumulation**
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31 479 **suggesting its metabolic utilization in planta** (Fig. 7). Furthermore, these data suggest that the
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33 480 ability of arginine incorporation and metabolism is limited in *Arabidopsis*, and nitrogen excess
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35 481 accumulates as free ammonium in plant tissues. Consistently, the accumulation of ammonium
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37 482 could also be responsible of the observed inhibition of yeast growth in the presence of basic
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39 483 amino acids. Ammonium toxicity triggers stress in roots and shoots and explain the reduction
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41 484 in biomass and inhibition of root length of wild-type and PpAAP1 overexpressing plants in the
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43 485 presence of arginine (Li et al. 2014).

44
45 486 Taken together, the above findings fully support a role for PpAAP1 as an efficient
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47 487 arginine transporter in maritime pine. However, additional studies are necessary to figure out
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49 488 the specific function of other members of the *P. pinaster* AAP family in amino acid uptake and
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51 489 long-distance transport of nitrogen.

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53 490 Identification of amino acid transporters in conifers and the clarification of their specific
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55 491 roles and potential redundancy in function is currently a challenging objective. New advances
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57 492 in this research area will help to clarify how pines are able to incorporate amino acids from soil
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59 493 and allocate them through the whole plant body.

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495 **Supplementary data**

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3 497 **Supplementary tables**

4 498 **Table S1.** List of the specific primer sequences used in this research.

5 499 **Table S2.** Identity percentages among members of the *P. pinaster* and *A. thaliana* amino acid
6 500 permease (AAP) family. Highest identities are shaded in grey colour.

7 501 **Table S3.** Accession numbers of AAPs from *P. pinaster*, *A. thaliana*, *P. trichocarpa* and *O.*
8 502 *sativa*.

9 503

10 504 **Supplementary figures**

11 505 **Fig. S1.** Predicted transmembrane domains of *P. pinaster* AAP proteins.

12 506 **Fig. S2.** Expression profiles of *PpAAP* genes

13 507 **Fig. S3.** Specificity of PpAPP1 antibodies.

14 508 **Fig. S4.** Characterization of the *Arabidopsis AtAAP5* mutants.

15 509 **Fig. S5.** Plant phenotypes growing at low arginine concentrations

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18 512 **Acknowledgements**

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31 525 **Conflict of interest**

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33 527 None declared

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3 **653 Figure legends**
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6 **654**

7 **655 Figure 1. Phylogenetic analysis of maritime pine amino acid permeases.** An unrooted,
8 neighbour-joining (NJ)-based tree of the amino acid permease (AAP) family. The analysis was
9 performed as described in the 'Materials and methods' section and the tree was generated using
10 MEGA version 7.0. Branch lengths (drawn in the horizontal dimension only) are proportional
11 to phylogenetic distances. Bootstrap values are indicated (1000 replicates). Names of the
12 species are abbreviated with a two-letter code *P. pinaster* (Pp), *A. thaliana* (At), *P. trichocarpa*
13 (Pt) and *O. sativa* (Os). Accession numbers are provided as supplementary data in Table S3.
14 Clusters are depicted in different colours. Sequences from *P. pinaster* are indicated in bold.
15 AtAAP5, PpAAP1, PpAAP2, PpAAP3 and PpAAP4 are marked by asterisks.
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24 **665**

25 **666 Figure 2. Relative expression levels of *PpAAP1* and *PpAAP2* in maritime pine seedlings.**

26 (A) Images captured using the exImage tool at ConGenIE.org
27 (<http://v22.popgenie.org/microdissection/>) indicating the expression and tissue localization for
28 *PpAAP1* and *PpAAP2*. Gene expression is shown in relative units. Red colour indicates high
29 level of expression and yellow colour low expression level. ExImage uses variance-stabilizing
30 transformation values for absolute expression generated by aligning RNA-Seq reads to the
31 reference genome and gene annotation with aligned read numbers. (B) Quantitative PCR
32 (qPCR) analysis of *PpAAP1* and *PpAAP2*. Total RNA was isolated from different organs of
33 one-month-old *P. pinaster* seedlings. The expression data were normalized using *EF1 α* as
34 reference gene. Each value is the mean \pm SE of three biological replicates. T, root tip; R, root;
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677 **Figure 3 Differential expression of *PpAAP1* and *PpAAP2* in maritime pine seedlings**

678 **supplied with arginine as a nitrogen source.** (A) Two-week-old *P. pinaster* seedlings were
679 grown in a hydroponic solution without arginine (control) or supplemented with arginine 2.25
680 mM. Root samples were collected after 1 (1H), 4 (4H), 8 (8H), 24 (24H) and 48 (48H) hours.
681 RNA was prepared from three sections of maritime pine roots, each of 3 cm in length, taken
682 from the upper part to the tip (1, 2, 3), and the relative expression levels of *PpAAP1* (B) and
683 *PpAAP2* (C) were determined by qPCR. Data were normalized to *EF1 α* as a reference gene.
684 Specific primers used are listed in Table S1. Bars represent mean values of three assays with

685 three biological replicates each \pm standard deviation. Different letters above bars indicate
 686 significant differences between samples and were calculated using Student's t-test ($P < 0.01$).

687

688 **Figure 4. Immunolocalization of PpAAP1 in the roots of maritime pine.** Confocal laser
 689 scanning micrographs of horizontal (A) and longitudinal (B) sections of root tips. The
 690 localization of PpAAP1 signals was detected using CF 488A conjugates on the green channel
 691 of the microscope. In the upper panels, green channel allows to localize AAP1-fluorescence
 692 signals associated to cell membranes meanwhile red channel reveals the cellular distribution in
 693 root tips using bright field. In the lower panels, control micrographs of root sections.

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695 **Figure 5. Functional and kinetic analysis of PpAAP1 in yeast.** (A) Yeast strain 22 Δ 8AA
 696 was transformed with the yeast expression vector pDR196 harbouring the coding sequence of
 697 *PpAAP1*, *AtAAP5* and empty pDR196 as a control. Growth was assayed on N-free medium
 698 containing 0.5 % ammonium sulphate (NH_4^+) as positive control, 10 μM and 0.5 mM arginine,
 699 or 0.5 mM lysine, proline and aspartic acid as sole N source. Two independent yeast colonies
 700 **of each construction were grown in liquid URA-free SD minimal medium to reach stationary**
 701 phase. Cells were pelleted, washed with sterile water and serially diluted (1, 1:10, 1:100 and
 702 1:1000). A volume of 10 μl corresponding to each dilution was spotted onto plates. The plates
 703 were incubated at 30 $^\circ\text{C}$ and photographed after 3 days. Yeast growth assays were repeated at
 704 least three times with similar results. (B) Concentration-dependent kinetics of L-[^{14}C]-arginine
 705 uptake by the yeast strain 22 Δ 8AA transformed with either empty pDR196 or with pDR196
 706 harbouring the *PpAAP1* construct. The values are expressed as the means \pm standard deviation
 707 (SD) of three replicates; the following values were calculated for kinetic parameters: K_m :
 708 $101.13 \pm 35.45 \mu\text{M}$; V_{max} : $7.26 \pm 1.3 \text{ nmol min}^{-1} 10^6 \text{ cells}^{-1}$.

709

710 **Figure 6. Growth of *A. thaliana aap5-1* mutant overexpressing maritime pine *PpAAP1*.**
 711 (A) *PpAAP1* construct in PGWB2 Gateway destination vector with CaMV35S::HPT-NosT
 712 marker used to transform *Arabidopsis* mutants; (B). Wild-type (*wt*), *aap5-1*, and transgenic
 713 lines 35S::*PpAAP1-1* (*L1*) and 35S::*PpAAP1-5* (*L5*) were grown on N-free half-strength MS
 714 medium supplemented with 3 mM nitrate and 1 mM arginine for 21 days (Bar 1 cm); (C) Plant
 715 biomass of *Arabidopsis* wild-type (*wt*), *aap5-1*, 35S::*PpAAP1-1* and 35S::*PpAAP1-5* grown
 716 on N-free half-strength MS medium supplemented with 3 mM nitrate and 1 mM arginine for
 717 21 days; (D) Root length of plants grown on N-free half-strength MS medium supplemented

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3 718 with 3 mM nitrate and 1 mM arginine for 21 days. Asterisks indicate significant differences
4 719 and were calculated using Student's t-test ($P < 0.01$). No significant differences were detected
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6 720 in the root length of plants grown without arginine. Bars represent mean values \pm standard
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11 **Figure 7. Functional complementation of arginine uptake in *Arabidopsis aap5-1* mutants**

12 **by overexpression of maritime pine *PpAAP1*.** (A) Relative expression levels of *AtAAP5* and

13 724 *PpAAP1* genes in plants grown on N-free half-strength MS medium supplemented with 3 mM

14 725 *PpAAP1* genes in plants grown on N-free half-strength MS medium supplemented with 3 mM

15 726 nitrate and 1 mM arginine for 21 days. ND, not detected. EF1 α was used as reference gene.

16 727 Specific primers used are listed in Table S1. (B) Root ^{15}N uptake after 2 h culture in a 30 μM

17 728 dual-labelled arginine solution of plants grown on N-free half-strength MS medium

18 729 supplemented only with 3 mM nitrate for 21 days. Three replicate samples, each comprising

19 730 five plants, were incubated for line. Roots were dried at 60 $^{\circ}\text{C}$ and analysed for ^{15}N content at

20 731 the University of Málaga Research Facility (Unit of Atomic Spectrometry) using a flash EA

21 732 1112 elemental analyser coupled to a Delta V Advantage isotope ratio mass spectrometer

22 733 (IRMS; Thermo Electronic Corporation). Results were expressed as ^{15}N content by milligram

23 734 of dry root (DW, dry weight). Asterisks indicate significant differences between samples

24 735 referred to *aap5-1* mutant and were calculated using Student's t-test ($P < 0.01$). (C) Ammonium

25 736 content in wild-type (*wt*), *aap5-1*, and transgenic lines *35S::PpAAP1-1 (L1)* and *35S::PpAAP1-*

26 737 *5 (L5)* plants grown on N-free half-strength MS medium supplemented only with 3 mM nitrate

27 738 (white bar) and together with 1 mM arginine (black bar) for 21 days. Asterisks indicate

28 739 significant differences and were calculated using Student's t-test ($P < 0.005$). Bars represent

29 740 mean values of three biological replicates \pm standard deviation.
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Table 1. The AAP family of amino acid transporters in maritime pine (*P. pinaster*). The molecular characteristics of the individual members of the AAP family are shown. *AtAAP5* of *Arabidopsis thaliana* is also included as a reference.

Name	Gene ID	FLcDNA (bp)	Protein (aa)	MW (Da)	Transmembrane domains (CCTOP)
<i>PpAAP1</i>	isotig28092	2267	489	54.01	11
<i>PpAAP2</i>	isotig32920	1791	492	54.69	11
<i>PpAAP3</i>	isotig44810	2040	496	54.35	11
<i>PpAAP4</i>	isotig44984	2009	499	54.64	11
<i>PpAAP5</i>	unigene165	1995	499	54.54	11
<i>PpAAP6</i>	unigene2046	2195	533	58.55	11
<i>PpAAP7</i>	unigene41903	2083	482	52.84	11
<i>PpAAP8</i>	isotig44509	2103	482	53.53	11
<i>PpAAP9</i>	isotig22404	1797	451	51.33	11
<i>PpAAP10</i>	isotig43366	2412	465	51.53	11
<i>AtAAP5</i>	At1g44100	2151	480	52.54	11

AtAAP5 of *Arabidopsis thaliana* is also included as a reference.

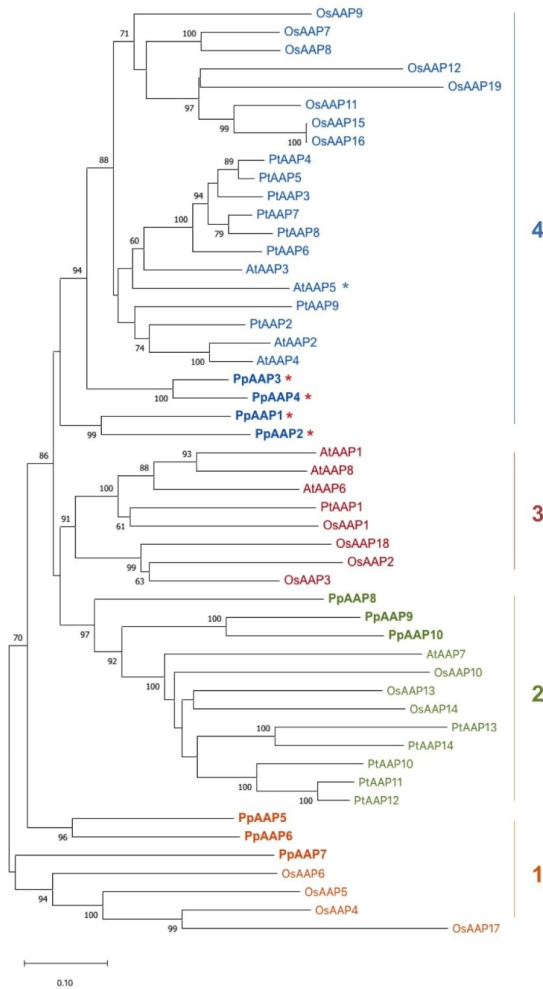


Figure 1. Phylogenetic analysis of maritime pine amino acid permeases. An unrooted, neighbour-joining (NJ)-based tree of the amino acid permease (AAP) family. The analysis was performed as described in the 'Materials and methods' section and the tree was generated using MEGA version 7.0. Branch lengths (drawn in the horizontal dimension only) are proportional to phylogenetic distances. Bootstrap values are indicated (1000 replicates). Names of the species are abbreviated with a two-letter code *P. pinaster* (Pp), *A. thaliana* (At), *P. trichocarpa* (Pt) and *O. sativa* (Os). Accession numbers are provided as supplementary data in Table S3. Clusters are depicted in different colours. Sequences from *P. pinaster* are indicated in bold. AtAAP5, PpAAP1, PpAAP2, PpAAP3 and PpAAP4 are marked by asterisks.

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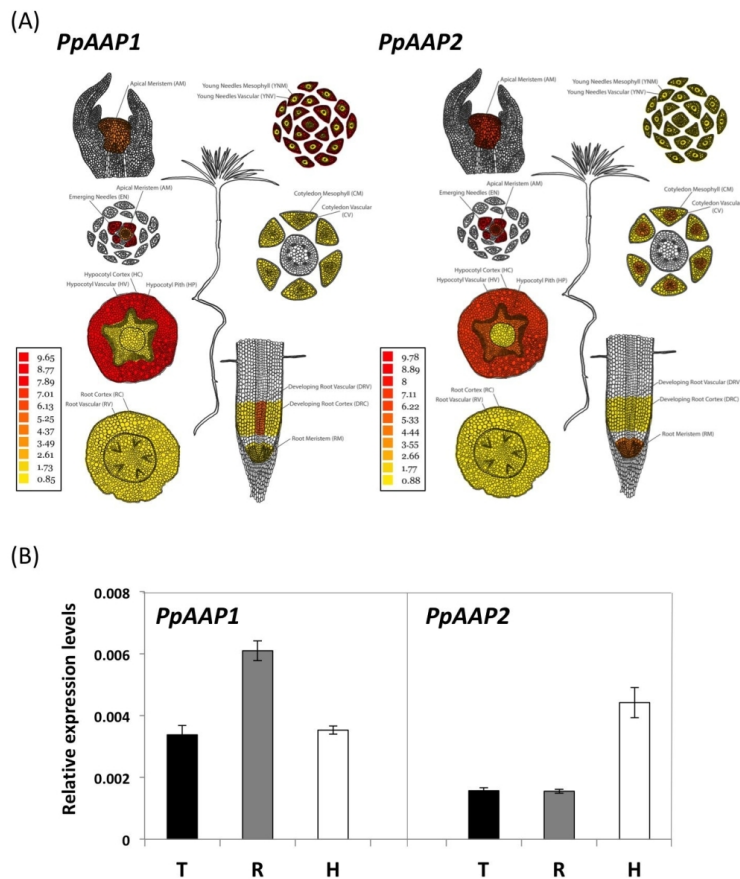


Figure 2. Relative expression levels of PpAAP1 and PpAAP2 in maritime pine seedlings. (A) Images captured using the exImage tool at ConGenIE.org (<http://v22.popgenie.org/microdissection/>) indicating the expression and tissue localization for PpAAP1 and PpAAP2. Gene expression is shown in relative units. Red colour indicates high level of expression and yellow colour low expression level. ExImage uses variance-stabilizing transformation values for absolute expression generated by aligning RNA-Seq reads to the reference genome and gene annotation with aligned read numbers. (B) Quantitative PCR (qPCR) analysis of PpAAP1 and PpAAP2. Total RNA was isolated from different organs of one-month-old *P. pinaster* seedlings. The expression data were normalized using EF1α as reference gene. Each value is the mean ± SE of three biological replicates. T, root tip; R, root; H, hypocotyl.

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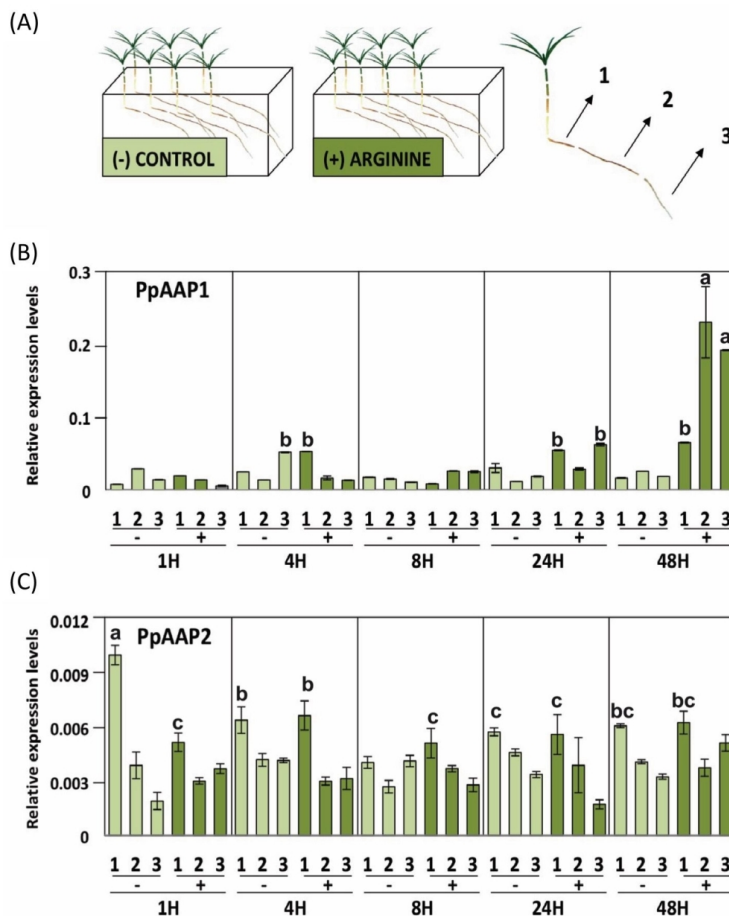


Figure 3 Differential expression of PpAAP1 and PpAAP2 in maritime pine seedlings supplied with arginine as a nitrogen source. (A) Two-week-old *P. pinaster* seedlings were grown in a hydroponic solution without arginine (control) or supplemented with arginine 2.25 mM. Root samples were collected after 1 (1H), 4 (4H), 8 (8H), 24 (24H) and 48 (48H) hours. RNA was prepared from three sections of maritime pine roots, each of 3 cm in length, taken from the upper part to the tip (1, 2, 3), and the relative expression levels of PpAAP1 (B) and PpAAP2 (C) were determined by qPCR. Data were normalized to EF1 α as a reference gene. Specific primers used are listed in Table S1. Bars represent mean values of three assays with three biological replicates each \pm standard deviation. Different letters above bars indicate significant differences between samples and were calculated using Student's t-test ($P < 0.01$).

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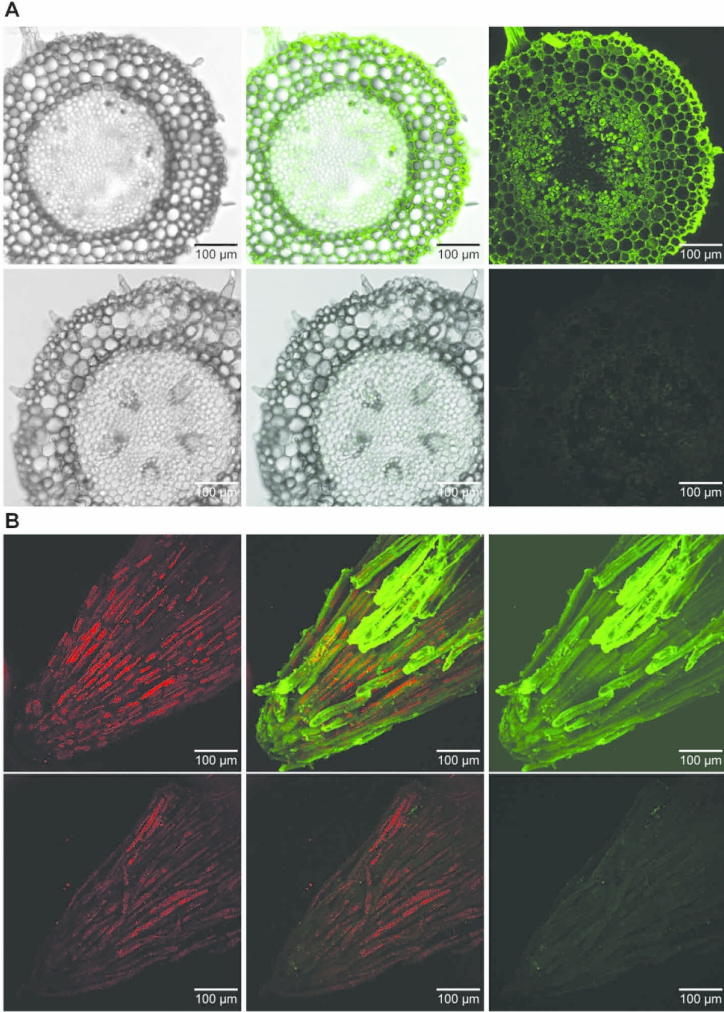


Figure 4. Immunolocalization of PpAAP1 in the roots of maritime pine. Confocal laser scanning micrographs of horizontal (A) and longitudinal (B) sections of root tips. The localization of PpAAP1 signals was detected using CF 488A conjugates on the green channel of the microscope. In the upper panels, green channel allows to localize AAP1-fluorescence signals associated to cell membranes meanwhile red channel reveals the cellular distribution in root tips using bright field. In the lower panels, control micrographs of root sections probed with non-immune antibodies.

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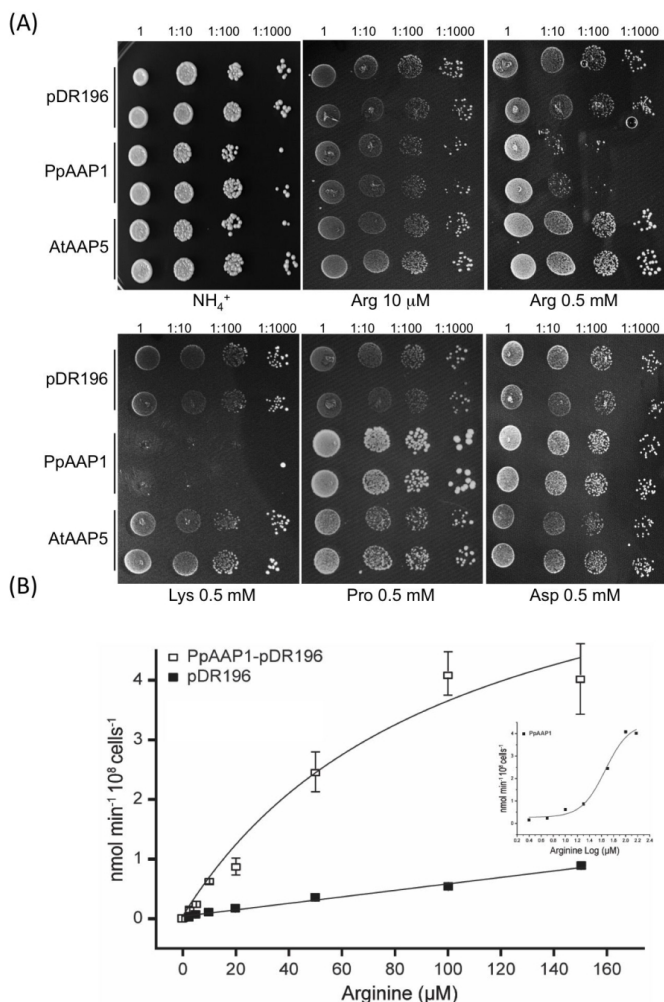


Figure 5. Functional and kinetic analysis of PpAAP1 in yeast. (A) Yeast strain 22 Δ 8AA was transformed with the yeast expression vector pDR196 harbouring the coding sequence of PpAAP1, AtAAP5 and empty pDR196 as a control. Growth was assayed on N-free medium containing 0.5 % ammonium sulphate (NH_4^+) as positive control, 10 μM and 0.5 mM arginine, or 0.5 mM lysine, proline and aspartic acid as sole N source. Two independent yeast colonies of each construction were grown in liquid N-free minimal medium media to reach stationary phase. Cells were pelleted, washed with sterile water and serially diluted (1, 1:10, 1:100 and 1:1000). A volume of 10 μl corresponding to each dilution was spotted onto plates. The plates were incubated at 30 $^\circ\text{C}$ and photographed after 3 days. Yeast growth assays were repeated at least three times with similar results. (B) Concentration-dependent kinetics of L-[14C]-arginine uptake by the yeast strain 22 Δ 8AA transformed with either empty pDR196 or with pDR196 harbouring the PpAAP1 construct. The values are expressed as the means \pm standard deviation (SD) of three replicates; the following values were calculated for kinetic parameters: K_m : $101.13 \pm 35.45 \mu\text{M}$; V_{max} : $7.26 \pm 1.3 \text{ nmol min}^{-1} 10^6 \text{ cells}^{-1}$.

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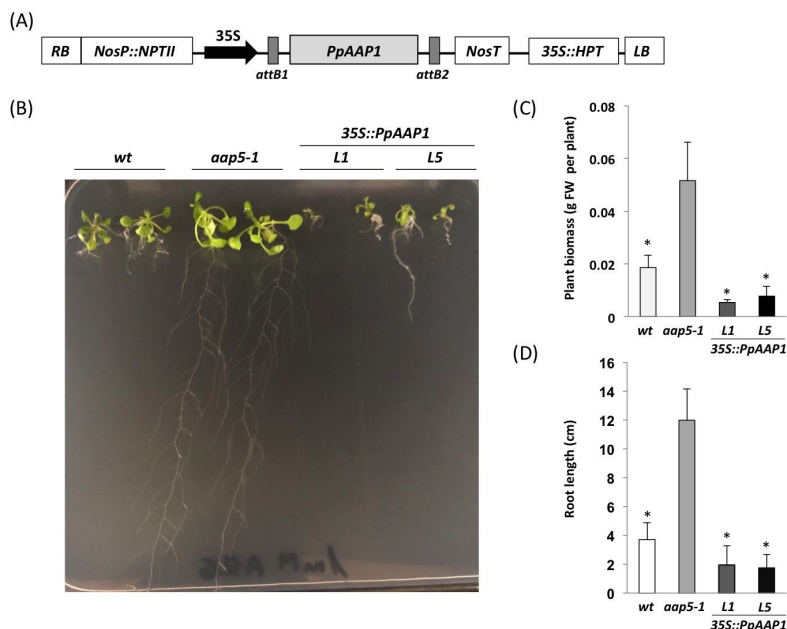


Figure 6. Growth of *A. thaliana* *aap5-1* mutant overexpressing maritime pine PpAAP1. (A) PpAAP1 construct in PGWB2 Gateway destination vector with CaMV35S::HPT-NosT marker used to transform *Arabidopsis* mutants; (B). Wild-type (wt), *aap5-1*, and transgenic lines 35S::PpAAP1-1 (L1) and 35S::PpAAP1-5 (L5) were grown on N-free half-strength MS medium supplemented with 3 mM nitrate and 1 mM arginine for 21 days (Bar 1 cm); (C) Plant biomass of *Arabidopsis* wild-type (wt), *aap5-1*, 35S::PpAAP1-1 and 35S::PpAAP1-5 grown on N-free half-strength MS medium supplemented with 3 mM nitrate and 1 mM arginine for 21 days; (D) Root length of plants grown on N-free half-strength MS medium supplemented with 3 mM nitrate and 1 mM arginine for 21 days. Asterisks indicate significant differences and were calculated using Student's t-test ($P < 0.01$). No significant differences were detected in the root length of plants grown without arginine. Bars represent mean values \pm standard deviation.

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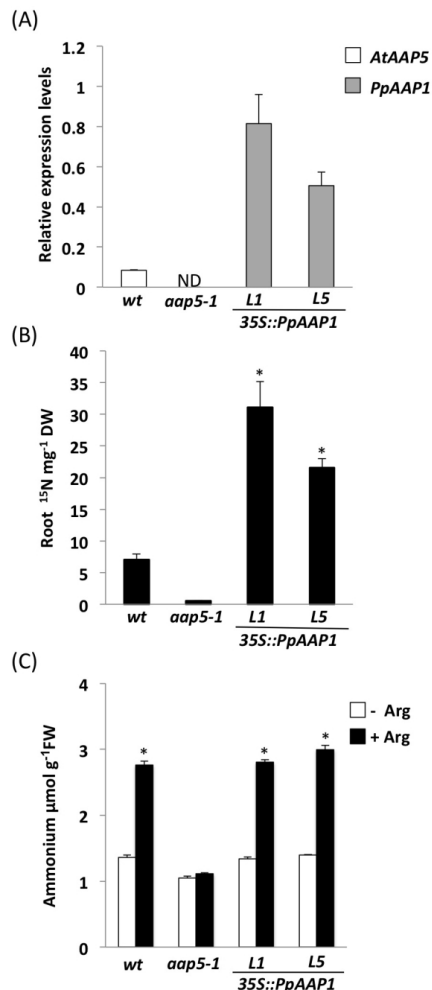


Figure 7. Functional complementation of arginine uptake in Arabidopsis *aap5-1* mutants by overexpression of maritime pine *PpAAP1*. (A) Relative expression levels of *AtAAP5* and *PpAAP1* genes in plants grown on N-free half-strength MS medium supplemented with 3 mM nitrate and 1 mM arginine for 21 days. ND, not detected. EF1 α was used as reference gene. Specific primers used are listed in Table S1. (B) Root ^{15}N uptake after 2 h culture in a 30 μM dual-labelled arginine solution of plants grown on N-free half-strength MS medium supplemented only with 3 mM nitrate for 21 days. Three replicate samples, each comprising five plants, were incubated for line. Roots were dried at 60 $^{\circ}\text{C}$ and analysed for ^{15}N content at the University of Málaga Research Facility (Unit of Atomic Spectrometry) using a flash EA 1112 elemental analyser coupled to a Delta V Advantage isotope ratio mass spectrometer (IRMS; Thermo Electronic Corporation). Results were expressed as ^{15}N content by milligram of dry root (DW, dry weight). Asterisks indicate significant differences between samples referred to *aap5-1* mutant and were calculated using Student's t-test ($P < 0.01$). (C) Ammonium content in wild-type (*wt*), *aap5-1*, and transgenic lines *35S::PpAAP1*-1 (L1) and *35S::PpAAP1*-5 (L5) plants grown on N-free half-strength MS medium supplemented only with 3 mM nitrate (white bar) and together with 1 mM arginine (black bar) for 21 days. Asterisks indicate significant

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3 differences and were calculated using Student's t-test ($P < 0.005$). Bars represent mean values of three
4 biological replicates \pm standard deviation.

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