Nitrogen affects cluster root formation and expression of putative peptide transporters

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Abstract

Non-mycorrhizal Hakea actites (Proteaceae) grows in heathland where organic nitrogen (ON) dominates the soil nitrogen (N) pool. Hakea actites uses ON for growth, but the role of cluster roots in ON acquisition is unknown. The aim of the present study was to ascertain how N form and concentration affect cluster root formation and expression of peptide transporters. Hydroponically grown plants produced most biomass with low molecular weight ON > inorganic N > high molecular weight ON, while cluster roots were formed in the order no-N > ON > inorganic N. Intact dipeptide was transported into roots and metabolized, suggesting a role for the peptide transporter (PTR) for uptake and transport of peptides. HaPTR4, a member of subgroup II of the NRT1/PTR transporter family, which contains most characterized di- and tripeptide transporters in plants, facilitated transport of di- and tripeptides when expressed in yeast. No transport activity was demonstrated for HaPTR5 and HaPTR12, most similar to less well characterized transporters in subgroup III. The results provide further evidence that subgroup II of the NRT1/PTR family contains functional di- and tripeptide transporters. Green fluorescent protein fusion proteins of HaPTR4 and HaPTR12 localized to tonoplast, and plasma- and endomembranes, respectively, while HaPTR5 localized to vesicles of unknown identity. Grown in heathland or hydroponic culture with limiting N supply or starved of nutrients, HaPTR genes had the highest expression in cluster roots and non-cluster roots, and leaf expression increased upon re-supply of ON. It is concluded that formation of cluster roots and expression of PTR are regulated in response to N supply.

Key words: Cluster root, heathland, organic nitrogen, peptide transporters, Proteaceae, roots.

Introduction

Organic nitrogen (ON) is emerging as a potentially important soil-derived N source for plants, although the contribution of ON to plant N supply is not well understood (reviewed by Näsholm et al., 2009). Better knowledge of the strategies of plants for acquiring ON is important for understanding nutrient cycles, conservation of biodiversity, and design of sustainable bioproduction systems. The heathland plant Hakea actites grows in soil dominated by ON (Schmidt and Stewart, 1997) and uses amino acids, peptides, and proteins as N sources (Schmidt and Stewart, 1999; Schmidt et al., 2003; Paungfoo-Lonhienne et al., 2008). Hakea actites was chosen as a model to examine how ON is acquired and metabolized, because in contrast to most heathland plants, which rely on fungal and/or bacterial symbioses to access N (Read, 1991), H. actites does not form symbioses but produces cluster roots. Cluster roots are formed by a number of species in selected plant families, but occur in most species of the Southern hemisphere Proteaceae family.

Cluster roots are a strategy for nutrient acquisition in extremely oligotrophic habitats (Lambers et al., 2008) and
are formed in response to low nutrient supply (reviewed by Dinkelaker et al., 1995; Neumann and Martinoia, 2002; Shane and Lambers, 2005). Cluster roots enhance the plant’s access to phosphorus and other soil nutrients (Dinkelaker et al., 1995; Neumann and Martinoia, 2002) and may have a role for accessing N (Pate and Jeschke, 1993; Schmidt et al., 2003). It has been shown that cluster roots exude a range of inorganic and organic compounds, as well as enzymes, including acid phosphatases (Dinkelaker et al., 1995) and proteases (Schmidt et al., 2003; Paungfoo-Lonhienne et al., 2008).

There is evidence that plants use di- and tripeptides as an N source (Schmidt et al., 2003; Komarova et al., 2008). Transport of peptides across membranes is mediated by transporters of three gene families: members of the NRT1/PTR (nitrate transporter 1/peptide transporter) family which facilitate transport of di- and tripeptides; members of the OPT (oligopeptide transporter) family transporting tetra- and pentapeptides; and members of the ABC (ATP-binding cassette) family which mediate transport of larger peptides, although the latter has not been confirmed in plants (reviewed by Rentsch et al., 2007). While a large number of putative PTR genes exist in plants, functionality has been confirmed for comparatively few transporters (Rentsch et al., 2007; reviewed by Tsay et al., 2007). In Arabidopsis, peptide transporters AtPTR1 and AtPTR5 facilitate uptake of di- and tripeptides into roots and germinating pollen, respectively, and overexpression of AtPTR5 increased growth of Arabidopsis with peptides as the sole N source (Komarova et al., 2008). Thus, the presence and expression of PTRs in roots may determine the plant’s ability to use peptides for growth. It is currently not known whether the main function of PTRs is uptake of peptides from the growth medium into the root and/or inter- and intracellular transport of peptides.

A partial cDNA fragment of a putative PTR was previously cloned from H. actites (HaPepT1); this cDNA had increased levels of expression during cluster root development (Schmidt et al., 2003). The partial HaPepT1 cDNA is identical to HaPTR4, one of the transporters characterized here. The aim of this study was to examine formation of cluster roots as well as functionality and expression of peptide transporters from H. actites in the natural habitat and in controlled conditions with different concentrations and forms of N.

**Materials and methods**

**Hydroponic plant culture**

In the first experiment, the aim was to determine how cluster roots are formed and peptide transporters are expressed. *Hakea actites* (W.R. Barker) seedlings were initially cultivated without addition of an N source, and then transferred to nutrient solution with limiting or adequate N supply (inorganic N, ON). Plants were grown from seed in water–vermiculite for 3 months (April to June 2005) in a naturally lit glasshouse (60% full sunlight, maximum temperature 28 °C). Subsequently, plants were cultivated in hydroponic culture containing 4.0 l of nutrient solution (200 μM CaCl₂, 100 μM MgSO₄, 50 μM KH₂PO₄, 50 μM K₂SO₄, 2.5 μM H₂BO₃, 0.25 μM MnSO₄, 0.025 μM ZnSO₄, 0.025 μM CuSO₄, 0.025 μM NH₄MO₄O₂₉, 5 μM Fe-EDTA, pH adjusted to 6.0) and grown for a further 7 months. Nutrient solutions were supplemented with 15 μM or 150 μM N as ammonium nitrate, glycine, or bovine serum albumin (BSA). The control received no N. Each pot contained three seedlings, and each treatment consisted of three pots. The nutrient solution was changed weekly. Tissues of 7-month-old plants were sampled, and immediately submerged in liquid N₂ and stored at −80 °C. Additional plants were divided into shoots, non-cluster roots, and cluster roots for biomass analysis (dried for 48 h at 60 °C).

In the second experiment, the aim was to investigate the effect of the plant’s original nutrient status on cluster root formation. Four-month-old H. actites from a commercial nursery (Wallum Nurseries Pty, Cleveland, Australia) were grown in University of California (UC) potting mix (Matkin and Chandler, 1957) and watered daily for 4 months with tap water. Roots were washed free of soil, and seedlings of uniform size were selected and transplanted to 4.0 l plastic pots. Nutrient solution and growth conditions were the same as for the plants initially cultivated without N described above. Plants were grown for an additional 6 months (June 2005 to January 2006).

The third experiment was to determine expression of HaPTR genes under conditions of protein re-supply after starvation. *Hakea actites* seeds were germinated in April 2004, grown in UC potting mix for 2 months, and grown hydroponically for 10 months with low nutrient conditions supplied only with tap water. Seedlings were grown for an additional 7 months under ‘nutrient starvation’ conditions with distilled water amended with 10 μM CaSO₄ and replaced weekly. Three pots were re-supplied with 150 μM N in the form of protein (BSA) for 3 d.

Hydroponic pots were covered with aluminium foil to prevent algal growth, and pots were rotated frequently. Young and mature cluster roots, non-cluster roots, and leaves were taken for RNA isolation from the first and third experiments; plants in the second experiment (grown initially at high N) did not produce cluster roots.

**Field-grown plants**

*Hakea actites* seedlings were collected in October 2006 from wallum heathland [Beerwah Scientific Area No. 1 (26.9°S, 152.9°E)] where mature *H. actites* shrubs grow to ~2 m height. During regular fires, imposed every 7 years, mature plants die and release seeds, which germinate in the ash bed. Small plants of up to 50 cm in size were collected from an area which had been burnt 2 years previously. *Hakea actites* produces cluster roots at 10–20 cm intervals along lateral roots in the upper 10 cm of soil (Schmidt and Stewart, 1999). Roots were carefully excavated, and adhering soil was...
removed by gently shaking the roots and washing them in deionized water. Ten young cluster roots were placed in microcentrifuge tubes and stored immediately in liquid N₂ for RNA extraction. Roots and leaves were collected from the same plants. Tissues were sampled from five individual plants.

Uptake of the dipeptide Gly-Gly

Axenic _Hakea_ plants (six seeds per plate) were grown for 4 weeks on N-free nutrient medium (see hydroponic culture) on agar plates (0.3% phytagel, Phytotechnology Laboratories, Lenexa, KS, USA) and individual roots of similar size were supplied with 25 μmol of Gly-Gly (99%, Sigma). Three-quarters of each cotyledon was removed when Gly-Gly was added to reduce the seedlings' reliance on stored N. Roots were extracted after 5 min, 4 h, or 15 h, and rinsed three times in 0.5 mM CaCl₂. Roots were immediately placed in liquid N₂. Frozen roots were homogenized and resuspended in 250 μl of methanol (20%). Samples were shaken on a vortex and stored at 4 °C overnight, centrifuged at 23 000 g for 10 min at 4 °C to condense the tissue into a pellet, and 40 μl of supernatant was mixed with 140 μl of borate buffer and 20 μl of AccQTag™ reagent (AccQTag™ derivitization kit), and analysed for peptide and amino acids (UPLC, Waters, Milford, MA, USA, equipped with a BEH C₁₈ 1.7 μm 2.1×100 mm column and a tunable UV detector at 254 nm). A solvent gradient from 0.1% trifluoroacetic acid (TFA) + 5% acetonitrile to 80% acetonitrile was used and amino acids were separated using agarose gel electrophoresis to monitor RNA degradation. Only RNA preparations with A₂₆₀/A₂₈₀ > 1.8 were used. _HaPTR_ genes were isolated using 3' and 5' rapid amplification of cDNA ends (RACE) PCR (FirstChoice RLM-RACE; Ambion) following the manufacturer's instructions. A control reaction (absence of reverse transcriptase) was used to check for residual genomic DNA. Primers used for _HaPTR4_, 5', and 12, were respectively: 5'-CATCGACCAGAGGCGTGTTCAC-3', 5'-GCACTGACTGAATGGAGATGATCAGTT-3', 5'-GATCTTTAG-3'. 18S small subunit nuclear rRNA, primers 5'-CGGCGGCCCTTGAAA-3' and 5'-TTGAGTGAGG-3' were used. 18S rRNA was the internal control of transport expression after demonstrating that its expression was similar in all tissues (data not shown). Quantitative real-time PCR was performed using the 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR® Green PCR Master Mix (Applied Biosystems). Ten minutes of enzyme activation was used followed by 45 cycles of 15 s at 95 °C, and 15 s at 60 °C. Elongation was not required because PCR products were very short (~100 bp) and amplification was completed before the denaturation step. After amplification, melting curve analysis was performed to verify the formed product. The measured Ct values were converted to relative copy numbers using the ACt method. The values were normalized by comparing them with 18S rRNA.

RNA expression analysis

For quantitative real-time-PCR, total RNA from non-cluster roots, cluster roots, and leaves was converted to cDNA using SuperScript™ III Reverse Transcriptase (Invitrogen, La Jolla, CA, USA) following the manufacturer's instructions. A control reaction (absence of reverse transcriptase) was used to check for residual genomic DNA. Amplified full-length cDNAs were cloned into pGEM-T Easy vector. Sequencing data of three independent PCR products were analyzed and organized by the ConTigExpress program from Vector NTI Suite 6.0 (Informax Inc., North Bethesda, MD, USA).

Isolation of _HaPTR_ constructs

Total RNA from _Hakea_ roots was isolated as previously described (Mason and Schmidt, 2002). DNA was removed with 2 U of DNase (Turbo DNA-free™; Ambion, Austin, TX, USA, 30 min at 37 °C). Total RNA was quantified using a NanoDrop ND-1000 spectrophotometer and separated using agarose gel electrophoresis to monitor RNA degradation. Only RNA preparations with A₂₆₀/A₂₈₀ ≥ 1.8 were used. _HaPTR_ genes were isolated using 3' and 5' rapid amplification of cDNA ends (RACE) PCR (FirstChoice RLM-RACE; Ambion) following the manufacturer’s instructions. 3' RACE PCR was accomplished using a degenerate primer 5'-GTGTNWSNWSNTTTGGNGCNGATCAGTT-3', respectively: 5'-AACGAAAACCTGGCA-3', 5'-AACACCTTGCCCAATCTTCTCAGT-3', 5'-TTGGACTTGTCAACTCTGGATGC-3', 5'-AAACACCTTGCCCAACTCTTCGTG-3', 5'-AAGAGCTCTTACATTTATTTATCTCTGGATG-3'.

Full-length cDNAs were amplified by using specific primers designed against regions before the ATG and after the stop codon of each gene: _HaPTR4_, 5'-CGCAAAATCCACCCA-GATCTTTAG-3' and 5'-AGTGGTAAACATCCTA-3', 5'-AGTTTTAATATA-ATTTCAGGAGTGTATC-3' and 5'-CCGGGTAA-ATGCCCATATAAAGGC-3', 5'-CTTCAGGCG-3', 5'-TTCTGTGC-3', and 5'-TTCTGTGC-3'.

PCR fragments corresponding to the open reading frame (ORF) of _HaPTR4_, _HaPTR5_, and _HaPTR12_ cDNA were amplified using primers with restriction sites for all vectors used, i.e. for complementation of _Saccharomyces cerevisiae_ mutants, transient protoplast expression, and cRNA synthesis (Xenopus oocyte expression). The following primers were used: _HaPTR4_, 5'-GGCCCCG-GCTAGCATGGGTCTCTGGAGAGATTGCT-3',
containing peptides to support growth. Growth assays were carried out as described previously with slight modifications (Hauer et al., 2000): yeast transformants were selected on proline medium containing 1.7 g l^{-1} yeast N base without amino acid and ammonium sulphate, 20 g l^{-1} glucose, 1 g l^{-1} proline (as a nitrogen source), 191 μM methionine, supplemented with 100–200 μM leucine-containing peptides (LLL, KLLHG, or SFLLRN).

GenBank accession numbers

Sequences have been submitted to GenBank under the accession numbers EF608213 (HaPTR4), EF608214 (HaPTR5), and EF608215 (HaPTR12).

Statistical analysis

Data were analysed using STATISTICA (Statsoft, Tulsa, OK, USA). Significant differences between treatments were determined by analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc test.

Results

N form and concentration affect biomass allocation and cluster root formation

To assess effects of N concentration and form on cluster root formation, plants were grown hydroponically in nutrient solution with 15 μM (low) or 150 μM (adequate) N supplied as NH_{4}NO_{3}, glycine, or protein (BSA). BSA was chosen as the treatment to represent ON of higher molecular mass than glycine, and microbial conversion of BSA in the non-axenic growth conditions was intentional. Plants supplied with 150 μM N produced significantly (P < 0.05) more biomass than plants in no-N or 15 μM N treatments. Biomass production with 15 μM or 150 μM was in the order glycine>NH_{4}NO_{3}>BSA (Table 1). Plants grown with 150 μM glycine had the lowest allocation of biomass to roots (Table 1).

Plants grown with adequate N (150 μM N) did not produce cluster roots (Fig. 1, Table 1). At 13 weeks, no-N plants produced on average 1.3 cluster roots per pot (three plants per pot) (Table 1). At 15 weeks, cluster roots were produced by every plant in the no-N treatment (data not shown). At 22 weeks, three cluster roots were produced on average in each pot in the no-N treatment, while in low N treatments (BSA, glycine, and NH_{4}NO), cluster roots were only produced in one pot per treatment (Table 1). To increase cluster root production, phosphorus was omitted in the nutrient solutions from week 22 in all treatments. In week 24, all plants in the low BSA treatment had cluster roots, and the numbers of cluster roots had increased in no-N and low BSA treatments. Most cluster roots were produced by plants grown in low BSA and no-N treatment 4 weeks after transfer to phosphorus-free medium (Table 1). Cluster roots were smaller in the BSA treatment than in the other treatments.
Intact dipeptide is taken up into roots

Gly-Gly was offered as the sole N source to axenic *H. actites* seedlings to determine if intact dipeptides are taken up into the root. Soluble Gly-Gly in roots increased 43- and 63-fold after 4 h and 15 h, respectively (Fig. 2). Glycine increased 46- and 25-fold after 4 h and 15 h, respectively. Serine increased 1520- and 4150-fold after 4 h and 15 h incubation with Gly-Gly, respectively (Fig. 2). Concentrations of 19 amino acids remained constant in the soluble pool of root tissue over the incubation period (data not shown). Low levels of ammonium were detected in the tissue after 15 h incubation (8±3 pmol g⁻¹ dry weight, data not shown).

**Table 1. Dry weight and number of cluster roots of hydroponically grown *Hakea actites***

Plants were grown without N for 3 months and grown for an additional 7 months with different N treatments. Low-N-treated plants commenced producing cluster roots 13 weeks after commencement of hydroponic culture with different N treatments, while plants grown in adequate N did not produce cluster roots. Phosphorus was omitted from the growth media after week 22. The average of three pooled seedlings in three pots ± SD is shown. Means marked with different letters are significantly different (P <0.05, ANOVA LSD post hoc test). The dry weight of plants was determined in week 26.

<table>
<thead>
<tr>
<th>N source (µM)</th>
<th>Concentration</th>
<th>Dry weight</th>
<th>No. of cluster roots per pot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shoot (g)</td>
<td>Roots (g)</td>
<td>Cluster roots</td>
</tr>
<tr>
<td>No N</td>
<td>0</td>
<td>1.8±0.14a</td>
<td>0.75±0.08a</td>
</tr>
<tr>
<td>NH₄NO₃ 15</td>
<td>6.2±0.27b,c</td>
<td>2.1±0.4b</td>
<td>37a</td>
</tr>
<tr>
<td>NH₄NO₃ 150</td>
<td>47.0±4.0d</td>
<td>14.9±1.3d</td>
<td>0</td>
</tr>
<tr>
<td>Glycine 15</td>
<td>9.3±1.5c</td>
<td>3.2±0.39b</td>
<td>35±28a</td>
</tr>
<tr>
<td>Glycine 150</td>
<td>60.9±6.5e</td>
<td>15.1±1.7d</td>
<td>0</td>
</tr>
<tr>
<td>BSA 15</td>
<td>3.9±0.2a,b</td>
<td>1.7±0.2a,b</td>
<td>49±27a,b</td>
</tr>
<tr>
<td>BSA 150</td>
<td>33.9±3.0f</td>
<td>9.6±0.2c</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cluster roots were produced by plants in one pot only.

**Fig. 1.** Roots of 9-month-old hydroponically grown *Hakea actites* initially cultivated without N and then grown for 26 weeks with different N forms. No-nitrogen (A) and a low N concentration (15 µM N) of NH₄NO₃, glycine, or BSA (B, C, D). Plants grown with a higher N concentration (150 µM N) did not produce cluster roots. Cluster roots are highlighted by white circles. Scale bar = 20 mm.

| N affects cluster roots and peptide transporter |

To investigate the role of peptide transporters in cluster roots, HaPTRs were isolated. Full-length cDNAs were named *HaPTR4*, *HaPTR5*, and *HaPTR12*. Hydropathy...
ized nitrate, peptide, and carboxylate transporters of NRT1/PTR family, which contains functionally characterized nitrate, peptide, and carboxylate transporters of Arabidopsis, faba bean (Vicia faba), barley (Hordeum vulgare), and alder (Alnus glutinosa). HaPTRs of subgroup II, whereas HaPTR5 and HaPTR12 belong to subgroup III (Fig. 3). Phylogenetic analysis revealed that HaPTR4 was identical to the partial cDNA of HaPepT1 (Schmidt et al., 2003) and, with −80% identity, highly homologous to (putative) PTRs of almond (Prunus dulcis: PdPTR2, AF213936), tomato (Solanum lycopersicum: LeNTR1, AF016713), faba bean (V. faba: VfPTR1) (Miranda et al., 2003), and Arabidopsis (AtPTR2) (Frommer et al., 1994; Rentsch et al., 1995). HaPTR5 shared the highest similarity with HaPTR12 (72% identity), Arabidopsis At1g22540 (60% identity), and At1g72140 and At1g72130 (51% identity, each). The two latter genes are expressed to a higher level in root than in shoot of Arabidopsis (Tsai et al., 2007). Protoplasts transformed with the HaPTR12-GFP construct showed localization at the plasma membrane (Fig. 4F) while the GFP–HaPTR12 construct showed fluorescence at the plasma membrane as well as some endomembranes (Fig. 4E), possibly due to incomplete targeting.

HaPTR4 mediates transport of di- and tripeptides

To determine gene function, cDNAs were expressed in the peptide transport-deficient yeast strain LR2 (Rentsch et al., 1995). This yeast strain is unable to grow on media containing peptides as the sole N source or on media containing histidine in the form of di- or tripeptides. In contrast to HaPTR5 and HaPTR12, which did not support growth, HaPTR4 mediated growth on 1 mM His-Ala as the sole source of histidine, similar to cells expressing the Arabidopsis di- and tripeptide transporter AtPTR2 (Fig. 5). HaPTR4 also mediated growth on the di- and tripeptides Arg-Glu, Ala-Asp, His-Lys, Leu-Leu, Phe-Ala, Val-Ala, Ala-Ala-Lys, and Leu-Leu-Leu as the sole N source (data not shown). Thus, HaPTR4 appears to have a low selectivity towards the amino acid side chain of the tested di- and tripeptides.

To determine if HaPTRs transport larger peptides, yeast strain BY4730 was used which is auxotrophic for leucine and methionine and can use peptides as a source for these amino acids provided that the mutation in the yeast oligopeptide transporter gene is complemented by a homologue. None of the HaPTRs mediated growth of the yeast mutant BY4730 when supplied with leucine-containing tetra-, penta-, or hexapeptides in the growth medium. When expressed in Xenopus oocytes, no transport functions of HaPTR5 and 12 were demonstrated, despite testing diverse compounds (data not shown).

Expression in prooplasts indicates localization of HaPTRs in different membranes

To demonstrate intracellular localization, GFP fusion proteins were transiently expressed in tobacco prooplasts. Free GFP showed characteristic fluorescence in the cytosol (Fig. 4A). AtProT2–GFP fusion protein was the positive control for proteins localized at the plasma membrane (Fig. 4B). Protoplasts expressing HaPTR4–GFP or GFP–HaPTR4 showed localization at the tonoplast (Fig. 4C). Protoplasts expressing HaPTR5–GFP or GFP–HaPTR5 showed fluorescence in small vesicular structures (Fig. 4D). To investigate the possibility that HaPTR5 localizes to the mitochondrial membrane, colocalization with a mitochondrial-specific dye, Mitotracker (Molecular Probes™, Eugene, OR, USA), was performed; however, no clear co-localization was observed (data not shown). Protoplasts transformed with the HaPTR12–GFP construct showed localization at the plasma membrane (Fig. 4F) while the GFP–HaPTR12 construct showed fluorescence at the plasma membrane as well as some endomembranes (Fig. 4E), possibly due to incomplete targeting.
HaPTR5 was significantly higher at 10 h than at all other times (Fig. 6).

A longer term response of HaPTR expression to protein resupply was determined in plants grown under nutrient starvation condition for 7 months and resupplied for 3 d with protein as the N source (experiment 3). Expression of HaPTR genes in cluster roots under conditions of nutrient starvation was up to 2- to 4-fold higher than after re-supply with protein (Fig. 7A–C). In contrast, expression of HaPTR genes was lower in leaves of nutrient-starved than in N-resupplied plants (Fig. 7A–C). The lower expression of HaPTR genes was lower in leaves of nutrient-starved than in N-resupplied plants (Fig. 7A–C). The lower expression of HaPTR genes in plants with higher N supply was in agreement with expression of hydroponically grown plants (experiment 1). Overall, expression of HaPTR genes was higher in plants grown without N or with 15 µM N than in plants grown with 150 µM N, except in cluster roots in the 15 µM glycine treatment (Table 2). Expression of HaPTRs was comparatively low because, following standard practice, expression of target genes is compared with the expression of highly abundant 18S rRNA.

Expression levels of HaPTR genes in field-grown plants in all tissues were considerably higher than in plants from the controlled condition (Fig. 7). HaPTR genes were expressed more strongly in root tissues than in leaves (Fig. 7D). Expression of HaPTR4 was significantly higher in cluster roots than in other tissues, while expression of HaPTR5 and HaPTR12 was highest in roots (Fig. 7D).

**Discussion**

N form had a strong effect on biomass production and cluster root formation of *H. actites*. The biomass of plants grown with limiting N supply was highest with glycine as the N source, followed by inorganic N and protein. To enhance cluster root formation, phosphorus was omitted from the nutrient solution in the last 4 weeks of growth. No cluster roots were produced by plants supplied with adequate N irrespective of the N source, while numerous large cluster roots were formed by plants grown without N,
accounting for 11% of total root dry weight. Growth-limiting supply with inorganic N or amino acid N resulted in few and small cluster roots (1–2% of root dry weight), while plants supplied with protein as the N source produced many small cluster roots (3% of root dry weight). The results indicate that under N-limiting growth conditions, N form affects cluster root formation. Similar observations were made in other species: Myrica gale produced cluster roots more readily when supplied with urea than when supplied with nitrate (Crocker and Schwintzer, 1993), and Gymnostoma papuanum formed cluster roots in the order nitrate>no added N >N_2 fixation>ammonium (Racette et al., 1990), while N form did not affect cluster root formation in Myrica cerifera (Louis et al., 1990). This highlights that taxa differ in their responses to cluster root formation, although the plants’ nutritional status and supply of other nutrients, especially phosphorus and iron, also affect cluster root formation (Dinkelaker et al., 1995; Neumann and Martinoia, 2002; Shane and Lambers, 2005).

N supply determines cluster root initiation in several taxa. Formation of cluster roots in Hakea species increased with low N and low phosphorus supply and decreased with high N and low phosphorus supply (Lamont, 2003). Here, omission of phosphorus increased cluster root formation only under low N supply. In contrast, cluster root formation was greater in M. gale and Grevillea robusta supplied with adequate N than in those grown without N (Moore and Keraitis, 1966; Crocker and Schwintzer, 1993). However, these seemingly conflicting findings have to be viewed in an ecological context. Cluster-rooted Proteaceae have the highest abundance on highly weathered soils poor in phosphorus and N, while other cluster-rooted species such as N_2-fixing species occur in habitats with higher phosphorus or N status (Lambers et al., 2008). In its heathland habitat, H. actites produces abundant cluster roots in the organic matter-rich upper soil layer, which may indicate that nutrients are accessed from organic compounds, but does not exclude the possibility that nutrients are derived from mineral sources.

Proteolytic enzymes are exuded from cluster roots and non-cluster roots (Paungfoo-Lonhienne et al., 2008). To assess the possible role of cluster roots for accessing organic N forms, membrane transporters were examined because transport of peptides across biomembranes is a likely prerequisite for uptake and metabolism of peptide-based N. While HaPTR4 was most similar to the functionally characterized di- and tripeptide transporters AtPTR1, AtPTR2, and AtPTR5 (60, 73, and 59% identity, respectively), HaPTR5 and HaPTR12 were less similar (41% and 43% identity, respectively) to HaPTR4. HaPTR4 mediated transport of di- and tripeptides, but no transport function could be assigned to HaPTR5 and HaPTR12. Similarly to AtPTR1, AtPTR2, and AtPTR5, HaPTR4 had a low selectivity for di- and tripeptides when expressed in yeast, agreeing with other reports and indicating that di- and

![Fig. 4. Localization of fusion proteins of HaPTR with GFP in tobacco (Nicotiana tabacum) protoplasts. Confocal laser scanning microscope pictures (left) and corresponding bright field images (right) of tobacco protoplasts transiently expressing fusion proteins of HaPTRs with GFP or free GFP. Free GFP (A), ProT2–GFP (B), HaPTR4–GFP (C), HaPTR5–GFP (D), GFP–HaPTR12 (E), and HaPTR12–GFP (F). Merged images show GFP fluorescence (green) and chlorophyll fluorescence (red). Protoplast diameter ~40 μm.](image-url)
tripeptide transporters have no strict side chain specificity (Becker and Naider, 1980; Payne and Smith, 1994). Similarly to AtPTR1 and AtPTR2 (Chiang et al., 2004; Dietrich et al., 2004), HaPTR4 did not facilitate transport of oligopeptides including tetra-, penta-, and hexapeptides. Therefore, the results provide further evidence that genes in the PTR subgroup II encode functional di- and tripeptide transporters in herbaceous and woody taxa.

Examining membrane localization, GFP fusion in tobacco protoplasts showed that HaPTR4 was localized at the tonoplast, similar to AtPTR2, which was identified as a tonoplast protein in proteome analysis (Shimaoka et al., 2004). Vacuoles perform multiple functions, including storage and degradation of proteins (Bassham and Raikhel, 2000; De, 2000), and localization of HaPTR4 at the tonoplast may point to a role for HaPTR4 for transport of oligopeptides including tetra-, penta-, and hexapeptides. Therefore, the results provide further evidence that genes in the PTR subgroup II encode functional di- and tripeptide transporters in herbaceous and woody taxa.

PTRs localized at the plasma membrane include AtPTR1, AtPTR5, and HvPTR1 (Waterworth et al., 2000; Dietrich et al., 2004; Komarova et al., 2008). Using atptr1 knockout and AtPTR5-overexpressing Arabidopsis, Komarova et al. (2008) showed, for the first time, that AtPTRs facilitate transport of di- and tripeptides into roots, confirming that peptides are an N source for non-mycorrhizal plants. It is demonstrated here that intact Gly-Gly was taken up by roots. Although the experiment does not allow differentiation between glycine originating from Gly-Gly cleaved prior to uptake into the root and glycine generated from Gly-Gly in planta, H. actites rapidly metabolizes glycine to serine when supplied with glycine as the sole N source (Schmidt and Stewart, 1999). There is now good evidence that dipeptides are taken up into roots intact, and the ecological relevance of peptides as N sources has now to be established.

To investigate a possible function of the HaPTRs, tissue-specific expression was studied in H. actites seedlings grown in heathland and controlled conditions. Expression of HaPTR genes was generally higher in cluster roots and non-cluster roots than in leaves, indicating a role for HaPTRs for nutrient acquisition and/or root function. In cluster roots and non-cluster roots of field-grown plants, expression of HaPTR4 was 5- and 3-fold higher than in leaves, respectively, while expression of the two uncharacterized transporters HaPTR5 and HaPTR12 was 25- and 4-fold higher in non-cluster roots than in leaves. Starved of nutrients, cluster roots had mostly higher expression of
Expression of HaPTR4 and HaPTR12 decreased after 6 h incubation with protein as the N source, similar to the expression pattern of the di- and tripeptide transporter HcPTR2A of Hebeloma cylindrosporum (Benjdia et al., 2006). In contrast to HcPTR2A which had higher expression with inorganic N sources (Benjdia et al., 2006), HaPTR genes were expressed more strongly in the presence of ON sources. Considering the expression of HaPTR genes at different stages of cluster root development, there is

Table 2. Expression ratio of peptide transporter genes HaPTR4, HaPTR5, and HaPTR12 in hydroponically grown Hakea actites

Plants were cultivated without N for 3 months and then grown for 7 months with different N treatments. Data represent averages ±SDs of three independent replicates. Expression values were normalized by comparing them with 18S rRNA, and comparative values are the expression results of plants grown on different N compared with expression of plants grown without N supply. See Table 1 for biomass and number of cluster roots.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Transporter</th>
<th>Values</th>
<th>No N</th>
<th>15 μM N concentration</th>
<th>150 μM N concentration</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BSA</td>
<td>Glycine</td>
<td>NH₄NO₃</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cluster root</td>
<td>HaPTR4</td>
<td>Expression (×10⁻⁶)</td>
<td>6.67±4.28</td>
<td>4.77±4.05</td>
<td>11.75±7.49</td>
</tr>
<tr>
<td></td>
<td>Comparative</td>
<td>1</td>
<td>1.4</td>
<td>0.6</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>HaPTR5</td>
<td>Expression (×10⁻⁶)</td>
<td>6.36±3.82</td>
<td>5.45±4.05</td>
<td>12.17±8.13</td>
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<tr>
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<td>1.2</td>
<td>0.5</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>HaPTR12</td>
<td>Expression (×10⁻⁶)</td>
<td>4.65±3.64</td>
<td>2.16±0.50</td>
<td>14.57±1.02</td>
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<tr>
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<td>2.2</td>
<td>0.3</td>
<td>n/a</td>
</tr>
<tr>
<td>Non-cluster root</td>
<td>HaPTR4</td>
<td>Expression (×10⁻⁶)</td>
<td>15.78±15.43</td>
<td>23.10±24.96</td>
<td>8.82±9.63</td>
</tr>
<tr>
<td></td>
<td>Comparative</td>
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<td>0.7</td>
<td>1.8</td>
<td>2.6</td>
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<tr>
<td></td>
<td>HaPTR5</td>
<td>Expression (×10⁻⁶)</td>
<td>10.03±9.29</td>
<td>14.33±15.33</td>
<td>4.00±4.02</td>
</tr>
<tr>
<td></td>
<td>Comparative</td>
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<td>0.7</td>
<td>2.5</td>
<td>2.3</td>
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<tr>
<td></td>
<td>HaPTR12</td>
<td>Expression (×10⁻⁶)</td>
<td>7.14±7.28</td>
<td>7.26±7.47</td>
<td>4.15±5.21</td>
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<tr>
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<td>1.7</td>
<td>3.2</td>
</tr>
<tr>
<td>Leaves</td>
<td>HaPTR4</td>
<td>Expression (×10⁻⁶)</td>
<td>28.32±21.62</td>
<td>20.12±15.18</td>
<td>24.75±5.43</td>
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<tr>
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<td>2.5</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
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<td>Expression (×10⁻⁶)</td>
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<td>7.40±5.54</td>
<td>11.79±4.53</td>
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<tr>
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<td>1.5</td>
<td>0.9</td>
<td>2.2</td>
</tr>
</tbody>
</table>

n/a, plants produced no or insufficient (15 μM NH₄NO₃) clusters.
evidence that expression of HaPTR4 increases throughout cluster root development (Schmidt et al., 2003). This observation was supported by initial results of quantitative real-time PCR, and transcript levels and tissue-specific localization of HaPTRs have to be ascertained, for example with mRNA in situ localization in cluster roots throughout development.

Overall, the studied HaPTR genes were expressed to a greater extent in cluster roots than in other tissues under conditions of nutrient or N starvation. Similarly, expression of the di- and tripeptide transporter HcPTR2A of H. cylindrosporum (Benjdia et al., 2006), and ammonium transporters AtAMT1;1, AtAMT1;3, and LeAMT1.1 (Lauter et al., 1996; Gazzarrini et al., 1999; von Wirén et al., 2000) increased in response to N deficiency. It was argued that up-regulation of N transporters increases N uptake from the soil under conditions of low N availability. In contrast, the PTR transporter VjPTR1 had lower transcript levels in roots when Leu-Leu or glutamine was present (Miranda et al., 2003), while amino acids down-regulated HvPTR1 protein activity at the post-translational level (Waterworth et al., 2005).

In summary, it is proposed that H. actites is a useful model for studying ON acquisition. While only HaPTR4 could be fully characterized, the higher expression of the studied HaPTRs in cluster roots and non-cluster roots compared with leaves, and the strong effect of N levels, form, and concentration on expression levels indicate that the studied HaPTRs are involved in root processes.

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**References**


