Influence of different host associations on glutamine synthetase activity and ammonium transporter in *Santalum album* L.

P. Deepa¹ · A. Yusuf¹

Received: 11 February 2016 / Revised: 24 June 2016 / Accepted: 31 July 2016 / Published online: 9 August 2016
© Prof. H.S. Srivastava Foundation for Science and Society 2016

Abstract The present study was aimed at understanding the role of different hosts in ammonium transporter1;2 expressions and glutamine synthetase(GS) activity and their effects on the growth parameters in the sandal. Sandal plant associated with leguminous host expressed better growth parameters. GS activity of leguminous hosts alone and in host associated sandal was analyzed using GS transferase assay. Highest GS activity was expressed in *Mimosa pudica*—sandal association compared to other leguminous and non-leguminous host associations. The association of N₂ fixing host with sandal enhanced C and N levels in order to maintain the C/N value. The role of ammonium transporters in N nutrition of sandal-host association was elucidated by cloning AMT1;2 from the leaves, haustoria and roots of host associated sandal and quantifying the relative expression by the 2^{−ΔΔC_T} method. *SaAMT1;2* was strongly up-regulated in leaves, roots and haustoria of leguminous host associated sandal compared to non-leguminous host associations. The relative increase in *SaAMT1;2* expressions and up-regulated GS activity positively affected the growth parameters in sandal when associated with leguminous hosts.

Keywords Host- parasite interaction · Glutamine synthetase activity · Transferase assay · Haustoria · C/N ratio · Ammonium transporter · qRT-PCR

Abbreviations

AMT Ammonium transporter

BLAST	Basic local alignment search tool
CTAB	Cetyltrimethyl ammonium bromide
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
GS	Glutamine synthetase
HATS	High-affinity ammonium transporter system
IUCN	International Union for Conservation of Nature
LATS	Low-affinity ammonium transporter system
PMSF	Phenylmethylsulfonyl fluoride
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone

Introduction

Indian sandal wood (*Santalum album* L.) is an economically precious perennial tree, facing extinction and listed as vulnerable in the red data book of IUCN (Kumar et al. 2012). During its life cycle sandal plant's association with leguminous host roots contribute to better growth characteristics. Association with *Sesbania formosa*, *Acacia trachycarpa* and *Acacia ampliceps* improved total N, C, P, K, Na, fructose, malic acid and proline contents in sandal xylem sap (Radomiljac et al. 1998). *Pongamia pinnata* and *Vigna unguiculata* used as annual primary host under nursery conditions increased total chlorophyll content, performance index, sturdiness quotient, shoot–root ratio and Dicksons quality index of associated sandal (Ramya 2010). Meristematic glandular haustorium, a unique character of the family *Santalaceae* establishes within 30 days after seed germination if provided with suitable host roots (Nagaveni and Srimathi 1985). The number, size, shape and colour of sandal haustoria are dependent on the type of attached host plants. Leguminous host association induces numerous large-sized haustoria, whereas non-leguminous

✉ A. Yusuf
akkara69@yahoo.co.in

¹ Interuniversity Centre for Plant Biotechnology, Department of Botany, University of Calicut, Kerala 673635, India

hosts produced less number of small sized non-specific haustoria. During the establishment of sandal-*Tithonia diversifolia* association, a bell-shaped haustorium constitutes a meristematic peripheral hyaline body and central penetration peg with an ellipsoidal disc at the end. Sandal-host plant interface constitutes layers of parenchymatous tissue with scattered vascular elements supports selective cross-membrane transport of nutrients between xylem tissues of the host and parasite than massive flow of nutrients through vascular elements (Deepa and Yusuf 2015; Tennakoon and Cameron 2006).

Preferential assimilation of NH_3 over nitrate is mediated by glutamine synthetase (GS; EC.6.3.1.2) which exists as two isozymes (GS1 and GS2) having different organellar localizations and metabolic functions. GS1 is located in leaf cytosol and a non-photosynthetic portion of the cells while GS2 is found in the chloroplast of photosynthetic cells (Ishiyama et al. 2004; Bernard and Habash 2009; Wang et al. 2015). Up-regulation of GS1 and GS2 emancipates NH_4^+ toxicity by catalyzing the condensation of ammonium and δ carboxyl group of glutamate to form glutamine (Lea and Ireland 1999). In non-leguminous roots, GS expression is regulated by NH_4^+ or NO_3^- affecting total N metabolism while in root nodules multiple gene controlled GS1 has enhanced expressions thus assimilating microsymbiont fixed ammonium (Bowles et al. 2015; Masalkar and Roberts 2015).

Nitrogen uptake and transport in plants are accomplished by differential expression of nitrate and ammonium transporters coded by multigene families (Brouquisse et al. 2001). Ammonium transporter proteins (AMTs) are ubiquitous integral membrane transport proteins, mediate ammonium transport across the plasma membrane in all domains of life. High-affinity ammonium transport system (HATS) and low-affinity ammonium transport system (LATS) are located in plant roots assisting controlled NH_4^+ uptake, of which HATS is expressed at low external NH_4^+ concentrations for efficient substrate capture and LATS is induced at higher external NH_4^+ concentrations (Glass et al. 2002; Yang et al. 2008). AMTs regulate scavenging and recapturing of ammonium and balance N nutrition in bacteria, fungi, animals and plants (von Wiren and Merrick 2004). Root AMTs play an important role in ammonium homeostasis which prevents cytoplasmic ammonium toxicity and progressive cell death (Zhou et al. 2015).

AMT1 gene family was first identified from *Saccharomyces* and *Arabidopsis* (Gazzarrini et al. 1999), subsequently cloned from *Oryza sativa* (Suenaga et al. 2003), *Medicago truncatula* (Straub et al. 2014), *Lotus japonicus* (Salvemini et al. 2001), *Brassica napus* (Pearson et al. 2002), *Lycopersicon esculentum* (von Wiren et al. 2000), *Populus trichocarpa* (Couturier et al. 2007), *Sorghum bicolor* (Koegel et al. 2013) etc. Among woody plants

constitutive organ-specific AMT1 transcription was detected only in *Populus* (Couturier et al. 2007), *Citrus* (Camanes et al. 2007) and *Pyrus* (Li et al. 2015). Plant AMT transcript levels are controlled by light duration, circadian rhythm and availability of nutrients including ammonium, nitrates, glutamine and sucrose. Members of AMT1 sub-family preferentially expressed in roots, while *LeAMT1;3*, *LjAMT1;1* and *LjAMT1;2* showed a higher level of expression in shoots (von Wiren et al. 2000; Apuzzo et al. 2004).

Previous reports suggest that leguminous hosts are essential for normal growth and development of sandal of which N_2 fixing woody hosts including *Sesbania formosa*, *Acacia trachycarpa* and *Acacia ampliceps* enhanced the growth of associated sandal compared to sandal grown with *Eucalyptus camaldulensis* or without the host (Radomiljac et al. 1998). But the molecular evidence to support such findings is lacking.

The present study was focused on the GS specific activity, maintenance of C/N ratio and *SaAMT1;2* transcript levels in sandal-host association.

Materials and methods

Plant material

Sandal seeds were procured from Institute of Wood Science and Technology, Bangalore, India. The seeds were scarified using 98 % (v/v) H_2SO_4 and sown in root trainers containing potting mixture (sand, cow dung and soil in 1:1:1 ratio). After one month, the seedlings were transferred to polythene bags containing the same soil mixture. These seedlings were divided into eight treatment groups. Each group included ten plants. One group maintained as no host control plants. The other seven groups were co-cultivated with seven different hosts of which five were leguminous (*Mimosa pudica*, *Centrosema pubescens*, *Clitoria ternatea*, *Arachis glabrata* and *Vigna radiata*) and two were non-leguminous (*Digitaria ciliaris* and *Vetiveria zizanioides*) plants. The experiments were repeated at least thrice. Host seedlings with two true leaves were planted about 0.5 cm apart from sandal seedling. The hosts were pruned at regular intervals to control overgrowth. The plants were maintained under full sunlight and watered regularly without any fertilizer application. After 6 months, sandal leaves, roots and haustoria were collected for biochemical and molecular studies.

Extraction of RNA

Total RNA from fresh sandal leaves (150 mg) was isolated using modified CTAB method (Rubio-Pina and Zapata-

Perez 2011). Extraction buffer contained 2 % (w/v) CTAB, 150 mM Tris–HCl (pH 8), 25 mM EDTA (pH 8) and 2 M NaCl. The samples were homogenized in liquid N₂ and the powder was transferred to 900 µl of pre-heated (65 °C) CTAB buffer. Fresh PVPP [2 % (w/v)] was added at the time of homogenization. Proteinase K (2 µl, 10 mg/ml) and 2 % (v/v) β-mercaptoethanol were added to the extract and mixed well. Samples were incubated at 60 °C for 5 min with frequent mixing. The homogenized extract was centrifuged at 4 °C for 10 min at 10,000 rpm and the supernatant was transferred to a new Eppendorf tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the supernatant and centrifuged at 4 °C for 10 min at 10,000 rpm. The supernatant was extracted twice with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), followed by extraction with chloroform: isoamyl alcohol (24:1). 0.25 vol. of 10 M LiCl was added to the supernatant and incubated overnight at 4 °C. The sample was centrifuged at 4 °C for 20 min at 10,000 rpm and the pellet was dissolved in 50 µl sterile double distilled water. RNA was precipitated with 0.1 vol. of 3 M sodium acetate and 2.5 vol. of 96 % (v/v) ethanol by incubating at –80 °C for 30 min. The samples were centrifuged at 10,000 rpm for 20 min at 4 °C to pellet the RNA. RNA pellet was washed with 70 % (v/v) chilled ethanol and air dried at room temperature and re-dissolved in 20 µl sterile double distilled water. Co-precipitated DNA from the samples was eliminated by treating with 1U of DNaseI and the purified RNA was stored at –80 °C.

Total RNA from sandal roots and haustoria were isolated using modified SDS-acid phenol method (Deepa et al. 2014). 100 mg of fresh samples was grind along with 2 % (w/v) PVP to a fine powder in mortar and pestle using liquid nitrogen. The samples were transferred to a sterile Eppendorf tube containing 1 ml of pre-warmed (65 °C) extraction buffer [2 % (w/v) SDS, 100 mM Tris–HCl—pH 8 and 25 mM EDTA—pH 8]. β-mercaptoethanol, 1 % (v/v) and equal volume of acid phenol:chloroform were added to the sample and mixed the contents by inverting the tubes. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was transferred to fresh Eppendorf tube and added 0.3 vol. of 5.0 M sodium acetate (pH 5.2) and 0.7 vol. of acid phenol:chloroform. The contents were mixed by inverting the tubes and incubated for 10 min on ice. The sample was centrifuged at 12,000 rpm for 10 min at 4 °C and supernatant was collected. 0.1 vol. of 3 M sodium acetate (pH 5.2) and equal volume of isopropanol were added to the sample and mixed well. The sample incubated at –20 °C for 1 h and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with 75 % (v/v) ethanol and centrifuged at 8000 rpm for 5 min at 4 °C. The pellet was air dried and

dissolved in 20 µl sterile double distilled water. The dissolved RNA was stored in –80 °C until use. Co-precipitated DNA was eliminated using 1U DNaseI (Takara, Japan) treatment.

Quantitative and qualitative assessment of RNA samples

Quality and quantity of RNA samples were assessed using a nanodrop spectrophotometer (Thermo, USA). The samples were run on 1.2 % formaldehyde agarose gel and visualized to verify the RNA quality using gel documentation system.

Primer designing

Eleven AMT1 gene sequences of different plants [*A. thaliana* (3), *O. sativa* (3), *B. napus* (1), *S. lycopersicum* (1), *M. hupehensis* (1), *C. trifoliata* (1) and *P. trichocarpa* (1)] were retrieved from NCBI database (<http://www.ncbi.nlm.nih.gov>). These multiple sequences were aligned to obtain a consensus sequence using Multalin with hierarchical clustering (<http://www.multalin.toulouse.inra.fr/multalin/>). Degenerate primers were designed from conserved regions of the aligned AMT1 sequences (Fig. 1).

Expression of AMT cDNA in different tissues

Purified RNA was converted into cDNA and amplified using One-Step Reverse Transcription-PCR Kit (Takara, Japan). PCR mixture contained 6.0 µl 5X One Step RT-PCR buffer, 1.0 µl 10 mM dNTP mix, 1.0 µl (10 pmol/µl) degenerate primers (F: 5' ATGCAGCTYGGBTYYGC 3' and R: 5' ANCCRWACCANCC RAACC 3'), 2.0 µl One Step RT-PCR enzyme mix and 0.5 µl template RNA (100 ng). The reaction conditions were 50 °C for 30 min to activate One-Step Reverse Transcriptase enzyme followed by initial denaturation at 95 °C for 15 min and then subjected to 35 cycles comprising: 95 °C for 20 s, 51 °C for 40 s, 72 °C for 1 min and 72 °C for 5 min. Amplified cDNA was separated on a 1 % TAE-agarose/EtBr gel by loading 5.0 µl of the sample. The bands were visualized under UV light using gel documentation system (Cell Biosciences, USA). 1.5 kb DNA ladder (Takara, Japan) was used as molecular size marker. The PCR product was purified and sequenced. The obtained sequence was analyzed using BLAST (<http://www.ncbi.nlm.nih.gov>) program to find out the homology of the sequence.

qRT-PCR analysis

First strand cDNA was synthesized using 100 ng of RNA in the presence of oligo (dT)₁₈ primer and Revert Aid

Fig. 1 Multiple sequence alignment of 11 AMT1 sequences from different plant species used for developing consensus sequence

Reverse transcriptase enzyme (Thermo Scientific, USA) after digesting with 1U of DNase I as per the supplier's instruction. 2.0 μ l of cDNA (~50 ng) was used for qRT-PCR assay and the reaction was performed in triplicates with forward (5' GGCTCTCTTCCACCAACAG 3') and reverse primers (5' AAGGAGGAGTAGATGAGGTA

GG 3') targeting 120 bp of *SaAMT1;2* because the whole cDNA in a Step One Real-time PCR system (Applied Biosystems, USA). Actin was used as an internal control with forward (5' GGTACTCTTTTACCACCAACAG 3') and reverse primers (5' AGTCTCTAGCTTCTGCTC ATAG 3'). qRT-PCR was carried out using SYBR green

Prime Script RT Reagent Kit (Perfect Real-time, Takara, Japan). The reaction mixture contained 10 µl SYBR Premix Ex TaqII (2×), 0.4 µl ROX reference dye (50×), 1.0 µl forward primer (10 µM), 1.0 µl reverse primer (10 µM) and 2.0 µl cDNA (~50 ng). The assay mixture was subjected to denaturation at 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 60 °C for 30 s followed by 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. Relative quantification of *SaAMT1;2* in the samples were carried out using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

GS extraction

Fresh leaf samples (0.1 g) from 6 months-old host associated sandal and their host plants were collected separately for GS transferase assay. The samples were homogenized completely with 2.0 ml of 100 mM Tris–HCl (pH 7.0–7.2) using a pre-cooled mortar and pestle. 10 mM DTT, 1 % (w/v) PVP and 1.0 mM PMSF were added fresh at the time of homogenization. The extract was collected in a 2.0 ml Eppendorf tube and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was used for GS transferase assay and total protein estimation.

GS transferase assay was carried out according to the protocol of Shapiro and Stadtmann (1970). The assay mixture contained 0.25 ml reaction buffer [1.0 M Imidazole–HCl (pH 7), 0.2 M Glutamine (pH 7), 1.0 M Potassium arsenate (pH 7), 0.1 M MnCl₂, 0.0047 g ADP–Na and 0.1042 g Hydroxylamine–HCl], 0.15 ml double distilled water and 0.1 ml supernatant. The mixture was incubated at 30 °C for 20 min in a shaking water bath, and 1.0 ml of stop solution [10 % (w/v) FeCl₃ in 0.2 N HCl, 24 % (w/v) TCA and 54 % (v/v) HCl mixed in the ratio of 1:1:1] was added to the reaction mixture to stop the reaction, mixed well and kept for 15 min at ambient temperature for colour development. A blank to which stop solution was added prior to the addition of sample served as control. The absorbance due to the γ -glutamyl hydroxamate formed was measured at 540 nm using a UV–VIS spectrophotometer. The gradient concentration of γ -glutamyl hydroxamate was used as the standard.

Total protein was determined according to the protocol of Lowry et al. (1951) using BSA as standard and the absorbance was measured at 650 nm using UV–VIS spectrophotometer.

CNS analysis

Leaves from 6 months-old sandal plants associated with different hosts were dried at 60 °C and blend to a fine powder using mortar and pestle. Total carbon, nitrogen and sulfur were measured using Euro Vector CHNS Analyzer

(Model No. EA3000). The analysis was carried out with 1.5–2.5 mg of powdered sample at 980 °C and the quantity of total C, N and S were estimated using Sulphanilamide as the standard reference. C/N ratio was calculated from the estimated relative C and N.

Results

AMT1 gene isolation from leaf, root and haustorium of sandal

The coding sequence of sandal AMT1 from the leaf, root and haustorium of the associations yielded cDNA of size 564, 574 and 579 bp respectively using One-Step RT PCR (Fig. 2). BLAST analysis of these sequences showed the best identity with AMT1;2 of other plants, confirming the obtained sequence as AMT1;2 and named as *SaAMT1;2* and submitted to NCBI.

Expression of *SaAMT1;2* in host associated sandal plants

Transcript levels of *SaAMT1;2* in the leaf, root and haustorium of different sandal-host associations were quantified using qRT-PCR and $2^{-\Delta\Delta C_T}$ method (Table 1). *SaAMT1;2* was organ specifically expressed in all host-sandal associations. *SaAMT1;2* was strongly up-regulated in leguminous host associated sandal leaves, haustoria and roots compared to non-leguminous host associations. Highest *SaAMT1;2* transcript level was observed in the leaves of sandal-*Mimosa* association while a decrease in transcript levels was observed in the order of *Centrosema*, *Clitoria*, *Vetiveria*, *Vigna* and *Arachis* associations. Haustorial *SaAMT1;2* expression was higher in the sandal-*Mimosa* association and were decreased in the order *Centrosema*, *Clitoria*, *Vetiveria*, *Vigna* and *Arachis* association. The roots of sandal-*Mimosa* association showed highest *SaAMT1;2* expressions followed by *Clitoria*, *Centrosema*, *Vigna*, *Vetiveria* and *Arachis* association. However, lesser *SaAMT1;2* expressions was noticed in the leaf, root and haustorium of sandal associated with *Digitaria* compared to control plants. The sandal-*Vetiveria* association showed slight up-regulation of *SaAMT1;2* transcript levels compared to other non-leguminous hosts, can be correlated with rhizospheric N₂ fixing microbial association of *Vetiveria*.

GS specific activity in sandal associated with different hosts

Sandal grown along with leguminous hosts showed highest GS specific activity compared to non-leguminous host

Fig. 2 Partial cDNA sequences of *SaAMT1;2* from the **a** leaf, **b** root and **c** haustorium of sandal-host associations

(a)

TACGCGGCAGATGCAGTGACATCATGCTCACCAACGTGCTCGACGCCGCCCCGGGG
GCCTCTTCTACTACCTCTTTGGGTTTCGCCTTCGCCTTCGGGTCCCCCTCGGGTTCCTTC
ATCTTCCGCCACTTCTACTTCTCAAACATATCCCCCTCTCGCTCTCCGACTACAGATT
CTTCCTCTATCATCGGGCCTTCGGCATCCGCGACGCCGGGATCACCAATGGATCCAT
CGTTGAGCGCACACAGGACGTGCGCTACCTCATCTACTCTCTCTCTTTTCGGATTTC
GTCTACCCCGTACCCTCCCACGGGTTCTGGGCCGCCGACCGCTGGGGCGTGCCTCAC
CCCCCACGGATCTGCTCTTCATTTCCAGCGTCATCGACTTCTTGGGTCCGGCGCGGC
TCCACCTCTTCGGCGGGGTCGGCCGCCTCTGGGGGGCCCTCTCGATGGAACGCCCA
TCGGTTCGGGTCGACCGATCCGGTCGGGAAGTGGGTTTCCATGCGCGGCCACAACCC
CACTCTCGTGGTGTCTCGGGACCTTCTTGCTGTGGTTCGGCTGGTATAG

(b)

GAGGTAATGTCGCGCAGATGCATGTACATCATGCTCACCAACGTGGTTCGACGCCGC
CCTGGGGGCTCTTCTACTACCTCTTTGGCTTCGCCTTCGCCTTCGGGTCTGGCTCCC
ATTCTTCTCTTCCGCCGCTTCTTCTTCTCAAGTCCATCCCCCTCCTCGCTCTCCGAG
TATGATTCTTCTTCATCCTCGTGCCTTCGGCATTCCCGACGCCGGGATCCCGAATGG
ATCCCTCCTCGAGCGCACACGGACGTGCGCTACCTCATCTACTCTCTCTCTTTT
CCTGACCGCTATCCCTTACCCTCCCATGGGTTCTGGGCTGCGGACCGCCGGGGCGTG
CCTACCCCCCCCACGGATCTGCTCGTCAGTTCCAGCGTCCGCGATCTCTTCGGGCC
GGCTGCCTCTGTGTCTTCGGCGGCGTCTGTGCGCTCTGGAGGGCCCTCCGTCATCGC
CCGCGGATCGGCCGGTTCGACCGATCCGGCCGGGAAGTGGGGCTCCATGGCCACAG
CGCTCCCTCTCTCTCCTCGCTCCTTCATGCTCTGCTTGGGTGGGCTGGGATAGGCTA

(c)

AAAAGGTTTCTGATCGCGCAGATGCATGAACATCATGCTCACCAACGTGGTTCGACGC
CGCCCTGGGGAGCCTCTTCTACTACCTCTTTGGCTTCGCCTTCGCCTTCGGCGCTGGC
TGGATTCTTCTCTTCTTCCGCTTCTACTTCTTCGCGCTGCATGACATTTTCAGGGG
GACTAATGATTCTTCTTTCATCCTCGTCCCTGGGCTTCGCGATCGCGTCTCCCGAA
TCGATCCCGCGAGCGCACACGGACGTGCGCTACCTCATCTACTCTCTCTCTTTT
CCTGACCGCTACTATCCCTTACCCTCCCATGGGTTCTGGGCCGCGGACCTCTGGGGC
ATGCCTCACCCACCACCGATCTGCTCGTCAGTTCCAGCGGCCCTCGACTTACTTCGC
GCCGGCGGCCGCTGTGTCTTCGGCGGGGTCGGTTCGCTGTGGAGGGCCCTCATCGAT
CGAACGCGCATCGGCCGGTTCGATCGATCCGGCCGGGAAGCGGGGTTCCATGGCCA
CAGCGCCTCCCTCTCCCTCGTCCGTCCCTTCATGCTCTGCTGTGGGCTGGTTGGGATA
GGCTA

associations. The GS specific activity of sandal in association with different host species was ranked as *Mimosa* > *Centrosema* > *Clitoria* > *Vigna* > *Arachis* > *Vetiveria* > *Digitaria* (Fig. 3). The selected annual leguminous hosts showed species-specific variations in GS activity as evident from higher GS activity of *Centrosema*, *Clitoria* and *Mimosa* and lesser activity of *Arachis* and *Vigna*. Of the non-leguminous hosts, *Vetiveria* exhibited more GS activity than *Digitaria* (Fig. 4). Augmentation in GS activity of sandal-legume association positively affected sandal growth parameters like stem height, collar diameter, leaf length and breadth, the number of leaves, lateral branches etc., while *Digitaria*-sandal associations and control plants showed low GS activity and fewer growth parameters (Figs. 5, 6). However, sandal-*Vetiveria* association exhibited better growth and higher GS activity even though the host is a monocotyledon.

CNS analysis in host associated sandal plants

Highest C and N were observed in the sandal-leguminous host associations compared to sandal-*Digitaria* association and control plants (Fig. 7). Among the leguminous host associations, *Mimosa* associated sandal showed the highest percentage of C and N. The presence of S was detected only in *Mimosa* associated sandals. The leguminous host-sandal associations showed lowest C/N ratio whereas sandal associated with non-leguminous *Digitaria* and no host control plants showed highest C/N ratios. The low C/N ratio in the leguminous host associated sandal is due to the inherent mechanism present in the N₂ fixing hosts to maintain a stable C–N metabolism by coordinating the rate of photosynthesis in relation to N₂ fixation, which is absent in the monocotyledonous hosts suggestive of their higher C/N ratio.

Table 1 Relative expression level of *SaAMT1;2* in leaves, roots and haustoria of sandals associated with different leguminous and non-leguminous hosts

Host associated with sandal plant	Sandal plant-tissue type	C_T (Mean $\pm$ SE)	ΔC_T (Mean $\pm$ SE)	$\Delta\Delta C_T$ (Mean $\pm$ SE)	$2^{-\Delta\Delta C_T}$ (Mean $\pm$ SE)
Control	Leaf	33.89 $\pm$ 0.11	6.78 $\pm$ 0.34	–	–
Control	Root	29.18 $\pm$ 0.36	–4.26 $\pm$ 0.46	–	–
Control	Hauistoria	33.10 $\pm$ 0.21	1.94 $\pm$ 0.03	–	–
<i>M. pudica</i>	Leaf	22.07 $\pm$ 0.12	–5.05 $\pm$ 0.11	–11.82 $\pm$ 0.23	3710.7 $\pm$ 204
<i>M. pudica</i>	Root	21.23 $\pm$ 0.15	–9.37 $\pm$ 0.19	–7.95 $\pm$ 0.43	269.00 $\pm$ 29
<i>M. pudica</i>	Hauistoria	23.03 $\pm$ 0.15	–8.22 $\pm$ 0.19	–10.16 $\pm$ 0.16	1162 $\pm$ 52
<i>C. pubescens</i>	Leaf	24.02 $\pm$ 0.09	–3.09 $\pm$ 0.32	–9.87 $\pm$ 0.07	935.3 $\pm$ 17
<i>C. pubescens</i>	Root	22.17 $\pm$ 0.12	–8.43 $\pm$ 0.26	–7.01 $\pm$ 0.26	133.3 $\pm$ 09
<i>C. pubescens</i>	Hauistoria	22.93 $\pm$ 0.12	–8.22 $\pm$ 0.15	–10.17 $\pm$ 0.12	1157.3 $\pm$ 55
<i>C. ternatea</i>	Leaf	25.10 $\pm$ 0.10	–2.01 $\pm$ 0.13	–8.79 $\pm$ 0.21	451.0 $\pm$ 23
<i>C. ternatea</i>	Root	22.03 $\pm$ 0.15	–8.57 $\pm$ 0.35	–7.15 $\pm$ 0.22	145.0 $\pm$ 09
<i>C. ternatea</i>	Hauistoria	24.03 $\pm$ 0.15	–7.12 $\pm$ 0.09	–9.07 $\pm$ 0.07	537.2 $\pm$ 10
<i>A. glabrata</i>	Leaf	26.16 $\pm$ 0.12	–0.95 $\pm$ 0.11	–7.76 $\pm$ 0.23	219.3 $\pm$ 14
<i>A. glabrata</i>	Root	24.04 $\pm$ 0.21	–6.56 $\pm$ 0.36	–5.14 $\pm$ 0.15	36.3 $\pm$ 03
<i>A. glabrata</i>	Hauistoria	26.67 $\pm$ 0.23	–4.49 $\pm$ 0.35	–6.43 $\pm$ 0.35	67.3 $\pm$ 04
<i>V. radiata</i>	Leaf	26.06 $\pm$ 0.17	–1.05 $\pm$ 0.08	–7.83 $\pm$ 0.28	232.3 $\pm$ 13
<i>V. radiata</i>	Root	23.10 $\pm$ 0.10	–7.50 $\pm$ 0.32	–6.08 $\pm$ 0.26	68.7 $\pm$ 03
<i>V. radiata</i>	Hauistoria	26.4 $\pm$ 0.49	–4.76 $\pm$ 0.35	–6.7 $\pm$ 0.34	134.0 $\pm$ 03
<i>V. zizanioides</i>	Leaf	25.06 $\pm$ 0.13	–2.06 $\pm$ 0.11	–8.83 $\pm$ 0.23	467.7 $\pm$ 26
<i>V. zizanioides</i>	Root	22.98 $\pm$ 0.05	–7.62 $\pm$ 0.33	–6.2 $\pm$ 0.32	77.3 $\pm$ 04
<i>V. zizanioides</i>	Hauistoria	26.13 $\pm$ 0.29	–5.02 $\pm$ 0.08	–6.97 $\pm$ 0.09	125.2 $\pm$ 07
<i>D. ciliaris</i>	Leaf	32.01 $\pm$ 0.19	4.89 $\pm$ 0.07	–1.88 $\pm$ 0.29	4.0 $\pm$ 0.20
<i>D. ciliaris</i>	Root	28.02 $\pm$ 0.09	–2.58 $\pm$ 0.29	–1.16 $\pm$ 0.44	2.3 $\pm$ 0.30
<i>D. ciliaris</i>	Hauistoria	28.17 $\pm$ 0.21	–2.99 $\pm$ 0.01	–4.93 $\pm$ 0.03	30.7 $\pm$ 3.40

The values are mean $\pm$ SE, N = 3 and significant at 5 % level using single factor ANOVA

Fig. 3 GS activity of sandal associated with leguminous and non-leguminous hosts. Different host-sandal associations are represented on the X-axis and GS activity on the Y-axis. The values are mean $\pm$ SE, N = 3 and significant at 5 % level using single factor ANOVA **Fig. 4** Differential GS activity of the hosts used for sandal-host association studies. The values are mean $\pm$ SE, N = 3 and significant at 5 % level using single factor ANOVA

Fig. 5 Morphological parameters of sandal plants associated with different leguminous and non-leguminous hosts. The values are mean $\pm$ SE, N = 10 and significant at 5 % level using single factor ANOVA

Fig. 6 Morphological parameters of sandal plants associated with different leguminous and non-leguminous hosts. The values are mean $\pm$ SE, N = 10 and significant at 5 % level using single factor ANOVA

Fig. 7 Relative CNS contents and C/N ratio in different sandal-host associations and control plants. Sulfur content was present only in *Mimosa*—sandal associations. The values are mean $\pm$ SE, N = 3 and significant at 5 % level using single factor ANOVA

Discussion

Nutrient status of the associated hosts is the key factor influencing the overall morphological growth of sandal. The present study was focused on the role of leguminous (*M. pudica*, *C. pubescens*, *C. ternatea*, *A. glabrata* and *V. radiata*) and non-leguminous (*D. ciliaris* and *V. zizanioides*) hosts on sandal growth supported by molecular and biochemical cues. Within 3 months of planting *Mimosa* developed a large number of haustorial connections with adjacent sandal roots contribute to improved nutrient transport and better morphological growth (Deepa and Yusuf 2015). The increase in GS specific activity observed of sandal-leguminous host associations justifies the haustorial acquisition of host ammonium. The prolonged time period for establishing root modifications/associations in other leguminous hosts (*C. pubescens*, *C. ternatea*, *A. glabrata* and *V. radiata*) account for less haustorial nutrient transport and hence the low GS specific activity. Our results are in agreement with the induction of numerous haustoria on sandal roots associated with *Acacia ampliceps* and *Sesbania formosa* (Radomiljac et al. 1998).

The higher GS specific activity shown by the non-leguminous *Vetiveria* compared to *Digitaria*, *Vigna* and *Arachis* is due to the colonization of abundant free-living N_2 fixing microbes in the *Vetiveria* rhizosphere contribute to nitrification of the soil, thus facilitating N_2 uptake by the *Vetiveria* fibrous root system (Leaungvutiviroj et al. 2010). However, when it is translated to sandal-*Vetiveria* association the GS activity was lesser due to the lack of a well-defined host-parasite interaction.

C, N and S analysis in sandal-host associations also supported the positive influence of leguminous hosts on sandal growth. Legume-sandal associations exhibited better C and N contents, and *Mimosa* exhibited better quantity than other sandal-host associations. Interestingly, *Mimosa* showed higher S content which has pronounced role in Periodic Leaf Movement Factor—PLMF1 (Gallic acid 4-o-(β -D-glucopyranosyl-6-sulfate), responsible for nyctinastic movement (Kallas et al. 1990). The higher C/N ratio exhibited by control plants and *Digitaria* associated sandal is due to the lesser N contribution to sandal by these hosts. High GS activity and balanced C/N values in legume sandal association confirmed N nutrition transport through the sandal-host interface.

The host specific differential up-regulation of *SaAMT1;2* in the leguminous/non-leguminous host-sandal associations throw light into the mechanisms of ammonium transport across the host-parasite membrane barriers. This host-parasite interaction is suggestive that the low N concentration in the plant account for the up/down regulation

of AMTs. *Mimosa*-sandal associations persistently up-regulated haustorial *SaAMT1;2* transcript level within 3 months to uptake NH_4^+ from host roots, corroborate with the earlier results that during the seedling phases the N levels will be reduced in seedlings and to compensate the loss, plants develop specific AMTs (Straub et al. 2014). The low *SaAMT1;2* transcript levels exhibited in other leguminous host-sandal association is due to nonspecific haustorial development suggestive that the *SaAMT1;2* is a host induced transporter system. These results are further justified in case of non-nitrogen-fixing *Digitaria* associated sandals with slight haustorial *SaAMT1;2* up-regulation compared to control plants due to N starvation. Further clues to the haustoria mediated N uptake through *SaAMT1;2* was clear from the slight increase in haustorial *SaAMT1;2* transcriptional level in the *Vetiveria*-sandal association which develop haustoria and has N_2 assimilation ability due to the presence of N_2 fixers in *Vetiver* rhizosphere compared to *Digitaria* and *Vigna* associated sandals. Our results are in agreement with *PtAMT1;2* of Poplar roots—mycorrhizal association in which *PtAMT1;2* was over expressed during fungal NH_3 supply, in turn, resulted in higher plant total metabolism (Couturier et al. 2007).

Leaf *SaAMT1;2* transcript levels in *Mimosa*, *Arachis*, *Vigna* and *Vetiveria* associated sandal were higher compared to haustorial and root *SaAMT1;2*. The increased levels of NH_4^+ in the roots and haustoria create an imbalance in the leaf N status resulting in enhanced leaf *SaAMT1;2* transcript levels affecting the transport of root assimilated NH_4^+ to the leaves. The lesser leaf *SaAMT1;2* expression level in N_2 deficient *Digitaria*-sandal association and N_2 fixing *Clitoria*, *Arachis* and *Vigna* associated sandals, and increase in *Vetiveria*-sandal association suggesting the role of N acquisition in up-regulating the transporter level. These suggest that a concentration gradient driven influx of NH_4^+ during higher N availability, corroborating the *AMT1;2* up-regulations reported in *Brassica napus* leaves, under N^+ supply (Pearson et al. 2002).

The root *SaAMT1;2* transcript levels are dependent on host association resulting in lesser transcript levels in non-legume associated sandal roots. Roots of N_2 rich *Vetiveria*-sandal association exhibited higher *SaAMT1;2* transcript levels than *Vigna* and *Arachis* association, suggesting our result's concurrence with *LeAMT1;2* and *OsAMT1;2* expressions in the roots of tomato and rice under N resupply (Gazzarrini et al. 1999; von Wiren et al. 2000; Kumar et al. 2003).

Our study confirmed the correlation between GS activity and *SaAMT1;2* transcript levels in the host associated sandal which enhanced total N and plant growth as reported in *Boehmeria nivea* and *Brassica napus* (Bowles et al. 2015; Zheng et al. 2015). Highest leaf and haustorial

SaAMT1;2 expressions in leguminous host associated sandal, in turn, up-regulated GS activity to assimilate acquired host NH_4^+ , whereas the reduced leaf and haustorial *SaAMT1;2* levels in non-leguminous host associated sandal with less GS induction suggests that the sandal utilizes the minimal NH_4^+ supplied by non-leguminous hosts. This improved sandal GS activity and *SaAMT1;2* expressions in legume-sandal associations bettered total plant growth affecting the levels of total proteins, chlorophylls and sugar contents (Deepa and Yusuf 2015). The *SaAMT1;2* sequences obtained can be used for further genetic transformation of sandal so that the intermediary host can be avoided in the nurseries.

Acknowledgments The authors are thankful to the Director, Interuniversity Centre for Plant Biotechnology, Department of Botany, University of Calicut, Kerala for providing the facilities.

References

- Apuzzo E, Rogato A, Simon-Rosin U, El Alaoui H, Barbulova A, Betti M, Dimou M, Katinakis P, Marquez A, Marini AM, Udvardi MK (2004) Characterization of three functional high-affinity ammonium transporters in *Lotus japonicus* with differential transcriptional regulation and spatial expression. *Plant Physiol* 134(4):1763–1774
- Bernard SM, Habash DZ (2009) The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. *New Phytol* 182:608–620
- Bowles TM, Philipp AR, Lowie EJ (2015) Root expression of nitrogen metabolism genes reflects soil nitrogen cycling in an organic agroecosystem. *Plant Soil* 392(1–2):175–189
- Brouquisse R, Masclaux C, Feller U, Raymond P (2001) Protein hydrolysis and nitrogen remobilisation in plant life and senescence. In: Lea PJ, Morot-Gaudry J-F (eds) *Plant nitrogen*. Springer, Berlin, pp 275–293
- Camanes G, Cerezo M, Primo-Millo E, Gojon A, Garcia-Agustin P (2007) Ammonium transport and *CitAMT1* expression are regulated by light and sucrose in *Citrus* plants. *J Exp Bot* 58(11):2811–2825
- Couturier J, Montanini B, Martin F, Brun A, Blaudez D, Chalot M (2007) The expanded family of ammonium transporters in the perennial poplar plant. *New Phytol* 174(1):137–150
- Deepa P, Yusuf A (2015) Histological and biochemical evaluation of *Santalum album* L. seedlings cocultivated with different hosts. *Ann Plant Sci* 4(03):1016–1021
- Deepa K, Sheeja TE, Santhi R, Sasikumar B, Cyriac A, Deepesh PV, Prasath D (2014) A simple and efficient protocol for isolation of high quality functional RNA from different tissues of turmeric (*Curcuma longa* L.). *Physiol Mol Biol Plants* 20(2):263–271
- Gazzarrini S, Lejay L, Gojon A, Ninnemann O, Frommer WB, von Wiren N (1999) Three functional transporters for constitutive, diurnally regulated, and starvation-induced uptake of ammonium into *Arabidopsis* roots. *Plant Cell* 11(5):937–947
- Glass AD, Britto DT, Kaiser BN, Kinghorn JR, Kronzucker HJ, Kumar A, Okamoto M, Rawat S, Siddiqi MY, Unkles SE, Vidmar JJ (2002) The regulation of nitrate and ammonium transport systems in plants. *J Exp Bot* 53(370):855–864
- Ishiyama K, Inoue E, Watanabe-Takahashi A, Obara M, Yamaya T, Takahashi H (2004) Kinetic properties and ammonium-

- dependent regulation of cytosolic isoenzymes of glutamine synthetase in *Arabidopsis*. *J Biol Chem* 279(16):16598–16605
- Kallas P, Meier-Augenstein W, Schildknecht H (1990) The structure-activity relationship of the Turgorin PLMF 1 in the sensitive plant *Mimosa pudica* L. in vitro binding of [14 C-Carboxyl]-PLMF 1 to plasma membrane fractions from *Mimosa* leaves and bioassays with PLMF I-isomeric compounds. *J Plant Physiol* 136(2):225–230
- Koegel S, Ait Lahmidi N, Arnould C, Chatagnier O, Walder F, Ineichen K, Boller T, Wipf D, Wiemken A, Courty PE (2013) The family of ammonium transporters (AMT) in *Sorghum bicolor*: two AMT members are induced locally, but not systemically in roots colonized by arbuscular mycorrhizal fungi. *New Phytol* 198(3):853–865
- Kumar A, Silim SN, Okamoto M, Siddiqi MY, Glass ADM (2003) Differential expression of three members of the AMT1 gene family encoding putative high-affinity NH_4^+ transporters in roots of *Oryza sativa* subspecies indica. *Plant, Cell Environ* 26(6):907–914
- Kumar AA, Joshi G, Ram HM (2012) Sandalwood: history, uses, present status and the future. *Curr Sci* 103(12):1408–1416
- Lea PJ, Ireland RJ (1999) Nitrogen metabolism in higher plants. *Plant Amino Acids Biochem Biotechnol* 1–47
- Leaungvutiviroj C, Piriyaipin S, Limtong P, Sa saki L (2010) Relationships between soil microorganisms and nutrient contents of *Vetiveria zizanioides* (L.) Nash and *Vetiveria nemoralis* (A.) Camus in some problem soils from Thailand. *Appl Soil Ecol* 46:95–102
- Li H, Cong Y, Chang YH, Lin J (2015) Two AMT2—type ammonium transporters from *Pyrus betulaefolia* demonstrate distinct expression characteristics. *Plant Mol Biol Report* 1–13
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta\text{CT}}$ method. *Methods* 25(4):402–408
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. *J Biol Chem* 193(1):265–275
- Masalkar PD, Roberts DM (2015) Glutamine synthetase isoforms in nitrogen-fixing soybean nodules: distinct oligomeric structures and thiol-based regulation. *FEBS Lett* 589(2):215–221
- Nagaveni HC, Srimathi RA (1985) Germinative capacity of floating and sinking sandal seeds. *Indian For* 111(8):615–618
- Pearson JN, Finnemann J, Schjoerring JK (2002) Regulation of the high-affinity ammonium transporter (*BnAMT1*; 2) in the leaves of *Brassica napus* by nitrogen status. *Plant Mol Biol* 49(5):483–490
- Radomiljac AM, McComb JA, Pate JS, Tennakoon KU (1998) Xylem transfer of organic solutes in *Santalum album* L. (Indian sandalwood) in association with legume and non-legume hosts. *Ann Bot* 82(5):675–682
- Ramya R (2010) Physiological and genetic diversity studies on regeneration of *Santalum album* L. PhD thesis. Cochin University of Science and Technology
- Rubio-Pina JA, Zapata-Perez O (2011) Isolation of total RNA from tissues rich in polyphenols and polysaccharides of mangrove plants. *Electron J Biotechnol* 14(5):11
- Salvemini F, Marini AM, Riccio A, Patriarca EJ, Chiurazzi M (2001) Functional characterization of an ammonium transporter gene from *Lotus japonicus*. *Gene* 270(1):237–243
- Shapiro BM, Stadtmann ER (1970) Glutamine synthetase (*E. coli*). *Methods Enzymol* 17A:910–922.25
- Straub D, Ludwig U, Neuhauser B (2014) A nitrogen-dependent switch in the high affinity ammonium transport in *Medicago truncatula*. *Plant Mol Biol* 86(4–5):485–494
- Suenaga A, Moriya K, Sonoda Y, Ikeda A, von Wiren N, Hayakawa T, Yamaguchi J, Yamaya T (2003) Constitutive expression of a novel-type ammonium transporter *OsAMT2* in rice plants. *Plant Cell Physiol* 44(2):206–211
- Tennakoon KU, Cameron DD (2006) The anatomy of *Santalum album* (Sandalwood) haustoria. *Botany* 84(10):1608–1616
- von Wiren N, Merrick M (2004) Regulation and function of ammonium carriers in bacteria, fungi, and plants. In: Boles E, Krämer R (eds) *Molecular mechanisms controlling transmembrane transport*. Springer, Heidelberg, pp 95–120
- von Wiren N, Lauter FR, Ninnemann O, Gillissen B, Walch-Liu P, Engels C, Jost W, Frommer WB (2000) Differential regulation of three functional ammonium transporter genes by nitrogen in root hairs and by light in leaves of tomato. *Plant J* 21(2):167–175
- Wang X, Wei Y, Shi L, Ma X, Theg SM (2015) New isoforms and assembly of glutamine synthetase in the leaf of wheat (*Triticum aestivum* L.). *J Exp Botany* p erv388
- Yang X, Kalluri UC, Jawdy S, Gunter LE, Yin T, Tschaplinski TJ, Weston DJ, Ranjan P, Tuskan GA (2008) The F-box gene family is expanded in herbaceous annual plants relative to woody perennial plants. *Plant Physiol* 148(3):1189–1200
- Zheng JS, Yu CM, Chen P, Wang YZ, Tan LT, Chen JK, Xiong HP (2015) Characterization of a glutamine synthetase gene *BnGS1-2* from ramie (*Boehmeria nivea* L. Gaud) and biochemical assays of BnGS1-2-over-expressing transgenic tobacco. *Acta Physiologiae Plantarum* 37(1):1–10
- Zhou Y, Bai L, Song CP (2015) Ammonium homeostasis and signaling in plant cells. *Sci Bull* 60(8):741–747