Up-regulation of genes involved in *N*-acetylglucosamine uptake and metabolism suggests a recycling mode of chitin in intraradical mycelium of arbuscular mycorrhizal fungi

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Abstract

Arbuscular mycorrhizal (AM) fungi colonize roots and form two kinds of mycelium, intraradical mycelium (IRM) and extraradical mycelium (ERM). Arbuscules are characteristic IRM structures that highly branch within host cells in order to mediate resource exchange between the symbionts. They are ephemeral structures and at the end of their life span, arbuscular branches collapse from the tip, fungal cytoplasm withdraws and the whole arbuscule shrinks into fungal clumps. The exoskeleton of an arbuscule contains structured chitin, which is a polymer of *N*-acetylglucosamine (GlcNAc), whereas a collapsed arbuscule does not. The molecular mechanisms underlying the turnover of chitin in AM fungi remain unknown. Here, a GlcNAc transporter, RiNGT, was identified from the AM fungus *Rhizophagus irregularis*. Yeast mutants defective in endogenous GlcNAc uptake and expressing *RiNGT* took up ^{14}C-GlcNAc, and the optimum uptake was at acidic pH values (pH4.0–4.5). The transcript levels of *RiNGT* in IRM in mycorrhizal *Lotus japonicus* roots were over 1000 times higher than those in ERM. GlcNAc-6-phosphate deacetylase (*DAC1*) and glucosamine-6-phosphate isomerase (*NAG1*) genes, which are related to the GlcNAc catabolism pathway, were also induced in IRM. Altogether, data suggest the existence of an enhanced recycling mode of GlcNAc in IRM of AM fungi.

Key words

arbuscular mycorrhizal fungi; chitin recycling; *N*-acetylglucosamine (GlcNAc) transporter; *Rhizophagus irregularis*

Introduction

The roots of many plant species are colonized with arbuscular mycorrhizal (AM) fungi. The extraradical mycelium (ERM) of AM fungi takes up soil minerals, such as phosphorus and nitrogen, and transfers them to the host roots by their syncytial mycelium (Smith et al. 2001). AM fungi extensively colonize root tissues to form intraradical mycelium (IRM), which consists of longitudinally developed intercellular/intracellular hyphae and arbuscules. An arbuscule, which is a characteristic structure of AM fungi, is formed in root cortical cells and highly branches in order to mediate resource exchange with the host plant (Gutjahr and Parniske 2013). The life span of an arbuscule is completed within 7–12 days from the beginning of trunk formation (Toth and Miller 1984; Alexander et al. 1989). Early morphological studies have demonstrated that at the end of the life span of an arbuscule, arbuscular branches collapse from the tip, fungal cytoplasm withdraws and the whole arbuscule shrinks into fungal clumps (Bonfante-Fasolo 1984). Recent live imaging studies of rice (*Oryza sativa*) roots that were colonized with *Rhizophagus irregularis* have suggested that most arbuscules collapse within a few days (Kobae and Hata 2010; Kobae and Fujiwara 2014; Kobae et al. 2014a). Even though well-developed arbuscules appear physically tough structures (Saito 1995; Solaiman et al. 2000), the arbuscular branches can collapse quite rapidly (Alexander et al. 1989; Kobae and Hata 2010), suggesting that the exoskeletal elements of arbuscules are degraded during this process.

The characteristic component of fungal cell walls is chitin (Rich et al. 2014), which is a long-chain polymer of *N*-acetylglucosamine (GlcNAc). As wheat germ agglutinin (WGA) has a strong affinity for GlcNAc oligomers and polymers, several studies have applied fluorescein-labelled WGA (e.g. Alexa Fluor dyes) to determine the spatial pattern of AM fungal colonization in host roots and the morphology of

arbuscules within colonized roots (Bonfante-Fasolo et al. 1990; Vierheilig et al. 2005); however, fluorescein-labelled WGA does not enable the visualization of the collapsed arbuscules (Bonfante-Fasolo et al. 1990). Electron microscope localization of gold-labelled WGA has also demonstrated that crystalline chitin is present in both intercellular and extraradical hyphal walls, and in an amorphous form on young, thin arbuscular branches, but not on completely collapsed arbuscules (Bonfante-Fasolo et al. 1990). It is thus conceivable that the chitin that accumulates on arbuscular branches is degraded during arbuscule turnover. In support of this, plant genes encoding chitinase, which break down glycosidic bonds in chitin to produce GlcNAc, are selectively expressed in arbuscule-containing cells (Bonanomi et al. 2001; Elfstrand et al. 2005; Hogekamp et al. 2011). In mycorrhizal *Medicago truncatula* the predicted protein sequence of Mtchit3-3 possesses a secretion signal peptide (Elfstrand et al. 2005), suggesting that GlcNAc is produced at the symbiotic interfaces.

The molecular mechanisms of GlcNAc recycling have been well established in *Candida albicans* (Konopka 2012) but, to the best of our knowledge, neither the fate of GlcNAc produced during arbuscule turnover nor the molecular mechanisms related to recycling of GlcNAc in AM fungi have been described. Here, a GlcNAc transporter, RiNGT, was identified in the *R. irregularis* genome database that is homologous to the GlcNAc-specific transporter of *C. albicans*, NGT1 (Alvarez and Konopka 2007; Gilmore et al. 2013). Transcript levels of *RiNGT* in IRM of mycorrhizal *Lotus japonicus* roots were over 1000 times higher than those in ERM. Moreover, *R. irregularis* genes encoding GlcNAc-6-phosphate deacetylase (DAC1) and glucosamine-6-phosphate isomerase (NAG1), which are related to the GlcNAc catabolism pathway, were also induced in IRM, suggesting an enhanced recycling mode of GlcNAc in the IRM of the

AM fungus.

Materials and Methods

Database searches

Protein sequences responsible for GlcNAc transport and metabolism of *C. albicans* were retrieved from the National Center for Biological Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). The sequences were employed to search for protein sequences derived from the whole genome sequence of *R. irregularis* DAOM 197198 (Tisserant et al. 2013) with BLASTP (*R. irregularis* database v1.0, http://genome.jgi.doe.gov/Gloin1/Gloin1.home.html). RiNGT protein sequence was employed to search homologous sequences in 16 fungal species with BLASTP on the integrated database of Department of Energy Joint Genome Institute (http://genome.jgi.doe.gov/) and in the genome database of *C. albicans* (SC5314 Assembly 19, http://www.candidagenome.org/). Phylogenetic trees were constructed using Molecular Evolutionary Genetic Analysis (MEGA) v6.06 (Tamura et al. 2013). The distances between branches were calculated by the neighbor-joining method based on the p-distance model with 1,000 bootstrap replicates.

RNA isolation

L. japonicus L. cv Miyakojima MG-20 was grown in a mesh bag culture system (Kikuchi et al. 2014), in which a mycorrhizal compartment (MC) and a hyphal compartment (HC) were separated by a cone-shaped

37 μm nylon mesh bag (Nippon Rikagaku Kikai, Tokyo). Four plants were inoculated with 1,000 spores of *R. irregularis* (Premier Tech, Riviere-du-Loup, Canada) in the mesh bag. The nylon mesh did not allow the penetration of roots but allowed that of hyphae. Plants were cultivated in a growth chamber at 25°C with 16 h day/8 h night cycle and thinned to two plants per pot after 1 week. Plants received deionized water every other day for the first week and nutrient solution (4 mM NH_4NO_3, 1 mM K_2SO_4, 0.75 mM $MgSO_4$, 2 mM $CaCl_2$, 50 μM Fe-Na-EDTA, 50 μM KH_2PO_4, pH 5.4) for the second to fourth week. At the beginning of fifth week, ERM in HC were harvested by wet sieving and roots were isolated from the ERM using a stereomicroscope. Roots and ERM from two pots were pooled for each sample. Roots (IRM) and ERM were frozen in liquid nitrogen and stored at −80°C until RNA extractions.

Quantitative RT-PCR analysis

Total RNA was extracted from ERM (20–40 mg) or roots (20–30 mg) with RNeasy Plant Mini kit (Qiagen). Genomic DNA was digested with RNase-free DNase I (Qiagen). However, DNA contamination derived from *R. irregularis* was detected in a no RT control reaction in which no reverse transcriptase was added. To eliminate the DNA contamination from the RNA solution completely, RNA was treated with Turbo DNA free (Life Technologies) once or twice and purified using RNAiso Plus (Takara). Total RNA was reverse transcribed with oligo-dT and random primers and a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative PCR was performed using Power SYBR Green Master Mix (Life Technologies) with a StepOne™ Real-Time PCR system (Life Technologies) according to the manufacturer's instructions, and a thermal cycle program of 95°C for 10 s and 40 cycles of 95°C for 15 s and 60°C for 60 s. Target genes

and corresponding primers are listed in Supplementary Table 1. The levels of transcripts were evaluated by the comparative Ct method using the *RiEF1β* gene as internal standard. All reactions were performed with three biological replicates. Significant differences in gene expression between samples were detected using Student's t test.

Heterologous expression of RiNGT in S. cerevisiae

To generate DNA encoding the *RiNGT* open reading frame, cDNA from maize roots colonized with *R. irregularis* (Kobae et al. 2014b) was used to amplify the DNA fragments by PCR with the specific primers 5' - GCTCGAGATGTTACGGTATTCAAGTCCATTAGTTCA -3' and 5' - AGTCGACTTAAATAGCATCCGAGTTAGTCTCACCAC -3'. The amplified gene was ligated into pCR-Blunt II-TOPO vector (Life Technologies) and sequenced. An expression vector was constructed in yeast by the homologous recombination method (Ma et al. 1987). The pKT10, URA3-marked yeast expression vector (Tanaka et al. 1990), was linearized with *KpnI*, and *RiNGT* DNA was amplified by PCR using primers with appropriate homology to the cut end of pKT10 (5'-GTTTCGAATAAACACACATAAACAGAATCGCCCTTGCTCGAG -3' and 5'-TTCAACCAAGTCGACTCAATCAATCAATTCGCCCTTAGTCGAC -3'). The vector and the insert DNA were purified with GENECLEAN II Kit (MP Biomedicals) and were introduced into *S. cerevisiae* EBY.VW4000 strain, which lacks endogenous monosaccharide transporters (Wieczorke et al. 1999), using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Gietz and Woods 2002). PCR-positive colonies that grew on maltose medium without uracil were selected (Wieczorke et al. 1999).

GlcNAc transport assay

Three distinct colonies of *S. cerevisiae* EBY.VW4000 containing the pKT10 control vector or *RiNGT*/pKT10 were each grown in 4 ml AHCW/maltose medium (0.17% yeast nitrogen base without amino acid, 0.5% ammonium sulfate, 1% casein hydrolysate, 0.002% adenine sulfate dehydrate, 0.002% tryptophan, 50 mM potassium phosphate, pH 5.5, 1% maltose). The cells were harvested by centrifugation, washed twice with 4 ml ultrapure water and then adjusted to a suspension in ultrapure water at $O.D._{600}$=10. Cell pellets were prepared from 1 mL suspensions in 1.5 ml microcentrifuge tubes and resuspended in 1 ml GlcNAc uptake solution (1 mM GlcNAc, 50 mM potassium-phosphate buffer, pH 5.5) with 1 µl ^{14}C-GlcNAc (PerkinElmer, 7.4 kBq μl^{-1}, 1.85–2.29 GBq $mmol^{-1}$) to start the GlcNAc transport assay at 30°C. Samples (200 µl) were withdrawn at given intervals and yeast cells were collected by vacuum filtration through a mixed cellulose ester membrane filter (pore size 0.45 µm, diameter 13 mm, Advantec). Yeast cells on the membrane filter were washed immediately with 1 ml of cold GlcNAc uptake solution without ^{14}C-GlcNAc. Incorporation of radioactivity was determined in a liquid scintillation counter. For the optimum pH determination, 10 mM citrate-phosphate buffer was used instead of 50 mM potassium-phosphate buffer to prepare universal pH GlcNAc uptake solutions (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0). The uptake solutions contained 10 mM GlcNAc.

Results

Identification of a GlcNAc transporter from *R. irregularis*

In the model for GlcNAc metabolism in *C. albicans*, exogenous GlcNAc is taken up by NGT1 (Alvarez and Konopka 2007) (Fig. 1). A search in the *R. irregularis* database (http://genome.jgi.doe.gov/Gloin1/Gloin1.home.html) for sequences with similarities to NGT1 gave only one protein sequence in the genome, which we named RiNGT (JGI protein ID: 327720). It was predicted to be an MFS (Major Facilitator Superfamily) transporter (Interpro Accession: IPR011701) with 12 transmembrane-spanning domains. A phylogenetic tree was generated with 195 aligned BLASTP homologues to RiNGT, including 160 sequences from 16 fungal species (top 10 homologous sequences in each species) (Supplementary Table 2), 31 NGT protein sequences from 21 fungal species described by Gilmore et al. (2013), and previously known monosaccharide transporters of Glomeromycota fungi (*Geosiphon pyriformis* MST1, Schüβler et al. 2006; *R. irregularis* MST2, Helber et al. 2011), a monosaccharide transporter of grass endophyte (*Neotyphodium lolii* MSTN, Rasmussen et al. 2012) and a sucrose transporter from a plant pathogen (*Ustilago maydis* SRT1, Wahl et al. 2010). This analysis showed RiNGT to be a close homologue of GlcNAc transporters (Supplementary Fig. 1). A simplified phylogenetic model rebuilt to include only putative NGT sequences suggested that some fungal species belonging to Ascomycota had both the NGT1 and NGT2 sub-families (Gilmore et al. 2013), whereas the fungi belonging to Zygomycota and Glomeromycota that were analyzed had only one NGT family (Supplementary Fig. 2).

To investigate whether RiNGT was capable of transporting GlcNAc, the gene was expressed in *S. cerevisiae* EBY.VW4000 strain, which is genetically deprived of all hexose transporters that are required for GlcNAc uptake (Scarcelli et al. 2012). *RiNGT* was overexpressed in EBY.VW4000 cells under the control of the yeast GAPDH promoter and their ability to take up ^{14}C-GlcNAc was evaluated, compared with vector

only control strains. The results demonstrated that EBY.VW4000 cells carrying *RiNGT* rapidly took up ^{14}C-GlcNAc within a few minutes, whereas the cells carrying the empty vector did not show significant GlcNAc uptake (Fig. 2A). The optimum ^{14}C-GlcNAc uptake into the transformed yeast cells was at acidic pH values (pH 4.0–4.5) (Fig. 2B).

Transcript levels of genes related to GlcNAc metabolism in IRM and ERM of *R. irregularis*

To obtain insights into the GlcNAc recycling pathway of AM fungi, the *R. irregularis* database was searched for sequences of genes with similarities to those encoding *C. albicans* protein related to GlcNAc recycling (Konopka 2012). As shown in the schematic model of GlcNAc recycling (Fig. 1), exogenous GlcNAc taken up by NGT can enter the anabolic pathway to form UDP-GlcNAc through reactions with GlcNAc kinase (HXK1), GlcNAc phosphomutase (AGM1), UDP-GlcNAc pyrophosphorylase (UAP1) and chitin synthase (CHS), or it can be catabolized and used for energy through the reactions with HXK1, GlcNAc-6-phosphate deacetylase (DAC1) and glucosamine-6-phosphate isomerase (NAG1) to form fructose-6-phosphate. A BLASTP analysis identified two orthologous sequences for HXK1 (JGI protein IDs: 147891 and 346548) and a single orthologous sequence for NGT (JGI protein ID: 327720), AGM1 (JGI protein ID: 73628), UAP1 (JGI protein ID: 335273), DAC1 (JGI protein ID: 335796) and NAG1 (JGI protein ID: 190217) in *R. irregularis*. In *C. albicans*, chitin synthesis is achieved by CHS1, CHS2, CHS3 and CHS8 (Munro et al. 2003; Lenardon et al. 2007), whereas at least 10 CHS sequences are found in *R. irregularis* (JGI protein IDs: 67365, 121158, 318355, 35412, 339241, 328520, 68035, 344952, 349919, and 348546).

To investigate the spatial regulation of the GlcNAc recycling pathway, the transcript levels were

analyzed for some of the above candidate genes for GlcNAc recycling in IRM and ERM, prepared from *L. japonicus* mycorrhiza grown in the mesh bag culture system, from which high-quality RNA of IRM and ERM can be isolated (Kikuchi et al. 2014). Quantitative RT-PCR demonstrated that *RiNGT* was selectively expressed in the IRM, that the transcripts of *DAC1* and *NAG1* were more abundant in IRM rather than in ERM, whereas the transcripts of *HXK1a*, *AGM1* and *UAP1* were more abundant in ERM rather than in IRM (Fig. 2C). These results suggested that the GlcNAc catabolic pathway was active in IRM, but that the GlcNAc anabolic pathway or *de novo* chitin synthesis was active in ERM. A fungal *CHS* gene (JGI protein ID: 121158) has been reported to be highly up-regulated in *R. irregularis–Medicago truncatula* symbiotic roots (Tisserant et al. 2013) but its expression was not significantly up-regulated either in IRM nor ERM of *L. japonicus* mycorrhiza (Fig. 2C). Our RNA-seq analyses using IRM and ERM samples of *R. irregularis* (Katsuharu Saito, unpublished data) did not find any CHS gene that is significantly expressed in ERM compared to IRM.

Discussion

In this study, we identified a GlcNAc transporter (RiNGT) in the AM fungus *R. irregularis*, and showed that its gene expression was selectively up-regulated in IRM. The yeast strain expressing *RiNGT* significantly took up ^{14}C-GlcNAc compared with the empty vector control, indicating that RiNGT has GlcNAc transporter activity. The optimum uptake was at acidic pH values, which was consistent with the proposed pH value of apoplastic spaces around arbuscules (Guttenberger 2000; Smith et al. 2001; Krajinski et al. 2014). In addition, gene expression related to GlcNAc catabolism (*DAC1* and *NAG1*) was also up-regulated in the IRM. These

results indicate that a recycling mode of GlcNAc should exist in IRM. Morphological studies have suggested that arbuscules have a short lifespan and collapse within a few days (Toth and Miller 1984; Alexander et al. 1989; Kobae and Hata 2010; Kobae and Fujiwara 2014). In *M. truncatula*, a class-III chitinase gene was strongly induced in a mycorrhiza-specific manner (Salzer et al. 2000), and transcripts were detected in host cells containing developing and mature arbuscules but not in cells touching intraradical hyphae (Bonanomi et al. 2001). In addition, transcripts of AM fungal chitinase have also been detected in IRM (Tisserant et al. 2012), although the location of gene expression has not been determined. It is thus conceivable that a particular proportion of chitin in IRM could be degraded by plant/fungal chitinases and that AM fungi could withdraw the dissociated GlcNAc to recycle it during intraradical development. Our results raise the possibility that GlcNAc may be taken up by RiNGT.

The exact biological role of chitin recycling in AM fungi remains to be understood. The cell wall of filamentous fungi is a highly dynamic structure subject to constant change during cell expansion, hyphal branching and septum formation (Adams 2004). The morphological changes of the cell wall may depend on the activities of hydrolytic enzymes, which may be implicated in the maintenance of wall plasticity and produce GlcNAc (Duo-Chuan 2006). Young arbuscules located in the apical portion of the infection unit represent the amorphous state of chitin, which reflects the plasticity of cell wall to allow the repeated branching (Bonfante-Fasolo et al. 1990). Therefore, the enhanced chitin recycling may be functionally associated with the development of the IRM of AM fungi. Functional analysis of RiNGT using gene silencing technique (Helber et al. 2011) is needed to understand the role of this protein.

The transcript levels of the *RiNGT* and GlcNAc catabolic pathway genes were selectively

up-regulated in IRM, suggesting a low recycling mode of chitin in ERM hyphae. Supporting this, the ERM is likely to persist for several months, contrary to the short life span of the IRM (Tisdall and Oades 1980; Treseder et al. 2010). Expression of genes related to chitin biosynthesis is likely to be higher in the ERM compared to the IRM, as indicated by the transcript levels of *AGM* and *UAP*, which are required for chitin synthesis in *C. albicans* (Mio et al. 1998; 2000). This suggests that chitin metabolism in the ERM may be directed towards its synthesis rather than its degeneration. Consistent with this, the biomass of the ERM of AM fungi is about 10 times higher than that of the IRM (Olsson et al. 1999). Although the expression level of a *CHS* gene (JGI protein ID: 121158) in ERM was not higher than that in IRM, the *R. irregularis* genome has at least nine additional CHS genes; therefore, other CHSs may contribute to produce chitin in ERM. It is possible that the low cell wall recycling activity in ERM is responsible for the prolonged life span of ERM. Importantly, the remnants of fine ERM attach to the particles of organic matter in soil (Mosse and Hepper 1975), eventually leading to the assumption that ERM may maintain soil structure with organic matter and aggregate formation (Bago and Cano 2005). Future studies exploring the molecular mechanisms of chitin remodeling and its recycling, which are responsible for the asymmetric carbon deposition in IRM and ERM, may provide insights into the ecological role of AM roots.

In the human pathogens *Histoplasma capsulatum* and *Blastomyces dermatitidis*, extracellular GlcNAc has a role as an inducer of the yeast-to-filamentous phase transition (Gilmore et al. 2013) and, notably, NGT1 and NGT2 are required for efficient yeast-to-filament conversion. Moreover, because O-linked GlcNAc is found in all multicellular eukaryotes and has been implicated in a variety of fundamental cellular processes such as signal transduction (Wells et al. 2001), GlcNAc uptake by RiNGT

may also play a role in the signal processes during AM fungal colonization of roots. Furthermore, given that short-chain chitin oligomers (COs) can elicit defense responses in plants (Shibuya and Minami 2001), the levels of chitin derivatives in the symbiotic interfaces may be strictly controlled. Earlier studies have suggested that symbiotic chitinases that cleave short-chain chitin oligomers could, in turn, attenuate defense responses in arbuscule-containing cells (Salzer et al. 2000). In addition, some chitin-derived molecules (lipochitoologosaccharides and COs) secreted by AM fungi are proposed to be molecular signals involved in the initial stages of the root colonization process (Maillet et al. 2011; Genre et al. 2013). Therefore, the GlcNAc recycling may have a role in establishing symbiotic AM associations and in intraradical colonization. The finding that a putative GlcNAc recycling system is selectively up-regulated in IRM of AM fungi opens new avenues in the study of GlcNAc metabolism of these mycosymbionts, which might be related to resource utilization and signal transduction.

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Figures and Tables

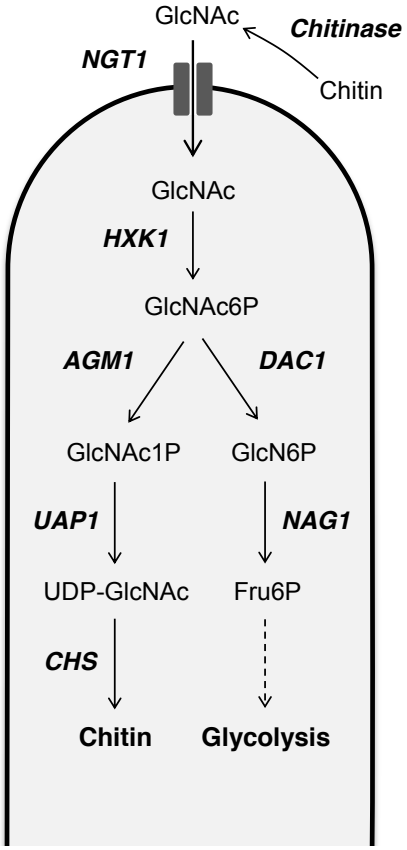

Fig. 1
Proposed model of GlcNAc recycling in fungal mycelium
Exogenous *N*-acetylglucosamine (GlcNAc) taken up by NGT1 (GlcNAc transporter) can enter the anabolic pathway to form UDP-GlcNAc via the reactions with HXK1 (GlcNAc kinase), AGM1 (GlcNAc phosphomutase), UAP1 (UDP-GlcNAc pyrophosphorylase) and CHS (chitin synthase), or it can be catabolized and used for energy via the reactions with HXK1, DAC1 (GlcNAc-6-phosphate deacetylase) and NAG1 (glucosamine-6-phosphate isomerase) to form fructose-6-phosphate.

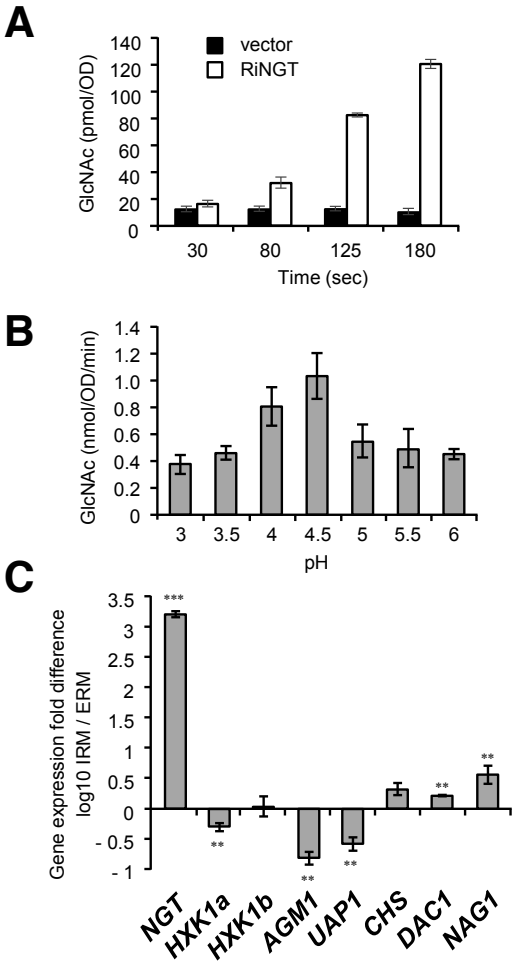

Fig. 2

The existence of GlcNAc recycling mode in intraradical mycelium of AM fungi

(A) Uptake of ^{14}C-GlcNAc by RiNGT-expressing (white bars) and control yeast EBY.VW4000 strain (black bars). (B) The pH optimum for 14C-GlcNAc uptake by RiNGT in yeast is in the acidic pH range. (C) Expression profile (quantitative RT-PCR) of GlcNAc transport and metabolism genes of *Rhizophagus irregularis* in intraradical mycelium (IRM) and extraradical mycelium (ERM). Gene expression was normalized to the expression of the constitutively expressed gene *RiEF1β*. The fold differences in gene expression levels in IRM relative to ERM are depicted. Mean and SE values of three biological replicates are shown. *** $P < 0.005$, ** $P<0.01$, Student's t test (IRM versus ERM).

Supplementary materials

Article Title
Up-regulation of genes involved in N-acetylglucosamine uptake and metabolism suggests a recycling mode of chitin in intraradical mycelium of arbuscular mycorrhizal fungi

Journal
Mycorrhiza

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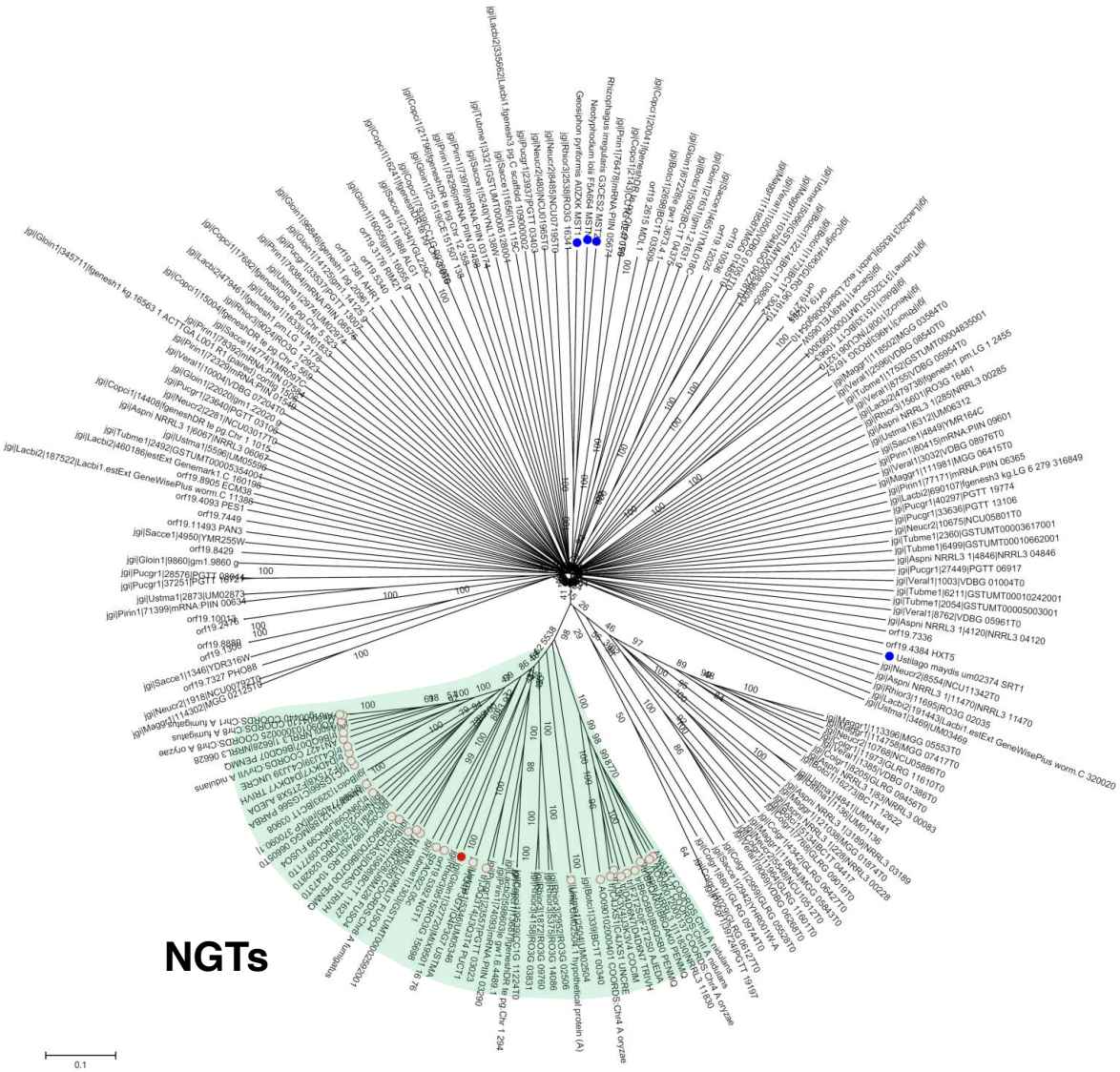
Gene name	Forward (5' - 3')	Reverse (5' - 3')
NGT	TGGCGCAGCACTTTTGTG	CGTTCGGTAGGGTAAGATAACATGA
HXK1a	CGATTGCCAACTGGTATGGA	GCGCAAATTAGTCCCACCTAAG
HXK1b	GGAATCCCAACTGGCAAAGA	ACATTCGTAAATTTGTACCTCCAAGA
AGM1	AAAACAATTCGATCTGCTGAAGGT	ATGCTCGTAATTTTTCGATTGCT
UAP1	TGAACGCGTCAACCGAATC	CGGTACCGGGAGCAATTTC
CHS1	CGGCACAATTTAGGGATATAGTGA	GGTTCCCCATGAATCAAACTAGTAA
DAC1	TTTGGAAGAGTTGGTTAATTTTGGT	AATACGGTCGCGGACGAA
NAG1	GGCGTTAGCTCTTGCCAAGT	CGCCGAAACGGTAAACATG
EF1β	CCCATGCAGCTCGATGGTA	TGCCAGGAAGTGAAGAAAATGA

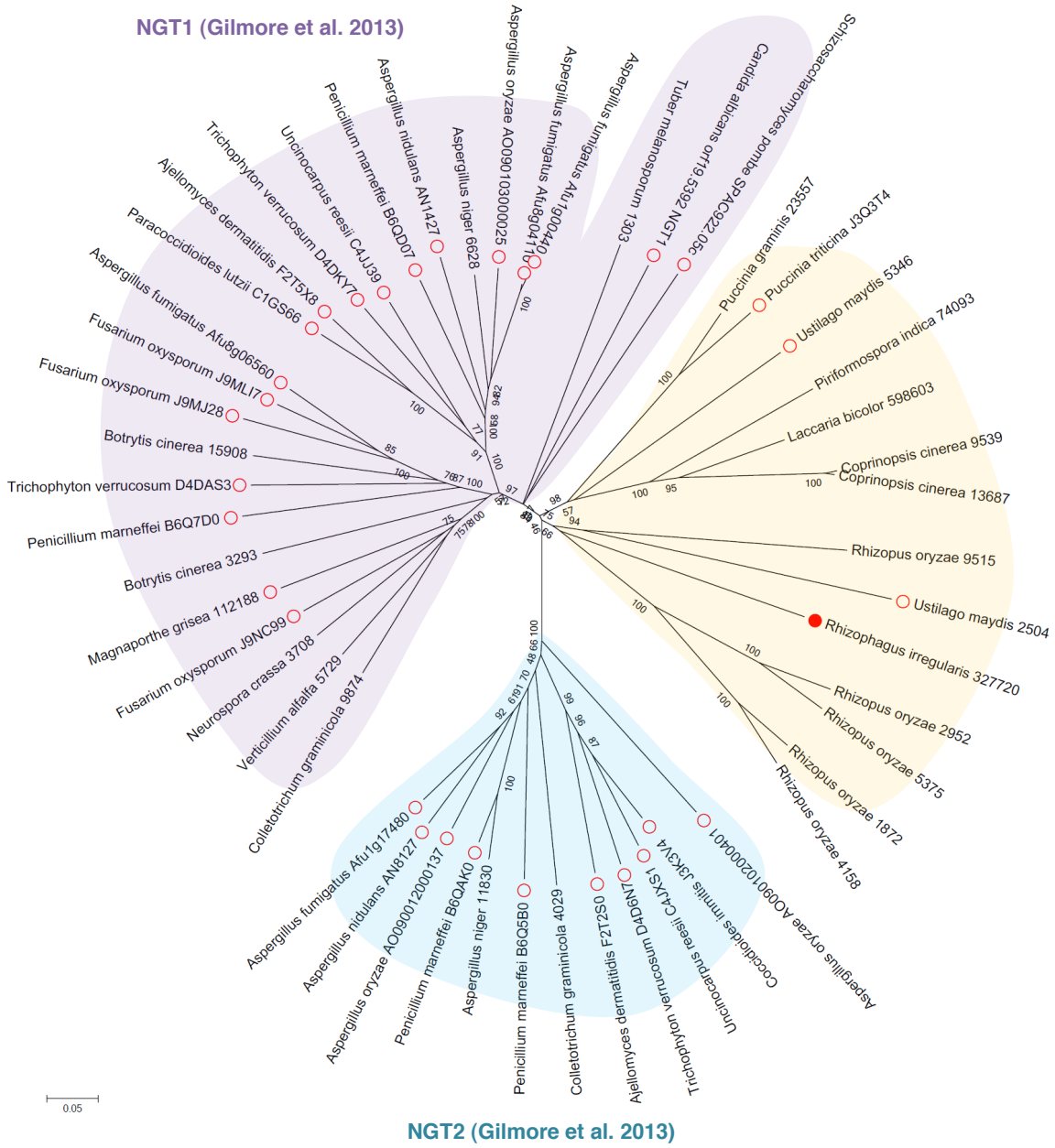
Supplementary Table 1
Primer sequences used in Quantitative RT-PCR analysis

species	link
Colletotrichum graminicola	http://genome.jgi.doe.gov/Colgr1/Colgr1.home.html
Rhizophagus irregularis	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Gloin1
Laccaria bicolor	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Lacbi2
Magnaporthe grisea	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Maggr1
Piriformospora indica	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Pirin1
Verticillium alfalfa	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Veral1
Puccinia graminis	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Pucgr1
Ustilago maydis	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Ustma1
Coprinopsis cinerea	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Copci1
Saccharomyces cerevisiae	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Sacce1
Tuber melanosporum	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Tubme1
Aspergillus niger	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Aspni_NRRL3_1
Botrytis cinerea	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Botci1
Neurospora crassa	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Neucr2
Rhizopus oryzae	http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Rhior3
Candida albicans	http://www.candidagenome.org/SearchContents.shtml

Supplementary Table 2
Fungal species used in BlastP analyses and the web links



Supplementary Fig. 1
Unrooted phylogenetic trees of fungal proteins homologous to RiNGT
The dendrogram was generated with Mega 6.0 software (Tamura et al. 2013) using ClustalW and the neighbor-joining method. Bootstrap tests were performed using 1,000 replicates. Red open circles indicate NGT proteins that were reported by Gilmore et al. (2013). Red closed circle indicates RiNGT. Blue closed circles indicate previously known monosaccharide transporter of glomeromycotan fungi (*Geosiphon pyriformis* MST1, Schüßler et al. 2006; *R. irregularis* MST2, Helber et al. 2011), monosaccharide transporter of grass-endophyte (*Neotyphodium lolii* MSTN, Rasmussen et al. 2012), and sucrose transporter of plant pathogen (*Ustilago maydis* SRT1, Wahl et al. 2010).



Supplementary Fig. 2
Unrooted phylogenetic trees of fungal NGT proteins
The dendrogram was generated with Mega 6.0 software (Tamura *et al.* 2013, *Mol. Biol. Evol.*, **30**: 2725–2729) using ClustalW and the neighbor-joining method. Bootstrap tests were performed using 1,000 replicates. The accession numbers are given after the name of species. Red open circles indicate NGT proteins that were reported by Gilmore et al. (2013) PLoS Genet 9: e1003799. Red closed circle indicates RiNGT.