An N-acetylglucosamine transporter required for arbuscular mycorrhizal symbiosis in rice and maize.

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Abstract

Most terrestrial plants, including crops, engage in beneficial interactions with arbuscular mycorrhizal fungi. Vital to the association is mutual recognition involving the release of diffusible signals into the rhizosphere. Previously, we identified the maize *no perception 1* (*nope1*) mutant defective in early signaling. Here, we report cloning of *ZmNOPE1* on the basis of synteny with rice. *NOPE1* encodes a functional homolog of the *Candida albicans* N-acetylglucosamine (GlcNAc) transporter *NGT1*, and represents the first plasma membrane GlcNAc transporter identified from plants. In *C. albicans*, exposure to GlcNAc activates cell signaling and virulence. Similarly, in *Rhizophagus irregularis* treatment with rice wild type but not *nope1* root exudates induced transcriptome changes associated with signaling function, suggesting a requirement of NOPE1 function for presymbiotic fungal reprogramming. (122 words)

Introduction

Arbuscular Mycorrhizal (AM) symbiosis entails a mutually beneficial relationship between plants and fungi in which plant roots exchange photoassimilated compounds for fungus-delivered soil mineral nutrients. The resulting interaction profoundly influences plant, including crop performance. For the symbiosis to begin, plant roots and AM fungi (AMF) signal each other in the soil via secretion of diffusible compounds [1]. These include fungal chitin-based molecules (reviewed in [2,3]), which are detected by the Lysin Motif (LysM) containing receptor-like kinases (RLKs) at the plasma membrane of legumes and rice, respectively [4,5]. Central for the perception of AMF however are the α/β hydrolase DWARF 14 LIKE (D14L) and the F-box protein DWARF 3 (D3) [6]. Mutations in either *D14L* or *D3* renders rice incapable of sensing the AMF and leads to the complete loss of susceptibility to the fungus [6]. The recognition substrate is at present unknown but could either be an AMF-released or a plant-derived molecule.

Several plant-derived factors are known to stimulate morphological changes in AMF that are thought to promote fungal-host encounters [1]. This includes flavonoids that promote AMF hyphal tip elongation [7], 2-hydroxid fatty acids (2-OH-FA) triggering hyphal branching [8], and root-secreted strigolactones (SL) that induce profuse hyphal ramification [9]. Moreover, the SL induced morphological switch is preceded by a sharp change in fungal metabolism characterized by increased cellular respiration, mitochondria biogenesis, and mitotic activity [10,11]. Despite their pre-symbiotic effect on AMF growth behavior, plant SL or flavonoid biosynthetic mutants are still partially or fully colonized, respectively [12,13], and the relevance of 2-OH-FAs for symbiosis establishment is unknown. Once the fungus has reached the plant's surface, cutin monomers induce hyphopodium differentiation, the anchoring structure that aids AMF entering the root epidermal cell layer [14]. However, as the fungal genome lacks genes for the *de novo* biosynthesis of certain fatty acids [15] cutin may have in addition a nutritional role.

To better understand the mechanisms that lead to symbiosis establishment, we analyzed the maize *no perception1* (*nope1*) mutant which lacks proper hyphopodium differentiation on the root surface, suggesting an impairment in signaling between the fungus and the plant during pre-symbiotic signal exchange [16]. Interestingly, we found that *NOPE1* encodes an N-acetylglucosamine (GlcNAc) transporter, so far not described in plants but homologous to the Ngt1 transporter described in fungi [17]. This is significant as GlcNAc has been shown to stimulate the fungal pathogen *C. albicans* to undergo morphological changes and increase expression of virulence genes that promote pathogenic interactions with the host [18]. Our analyses provide the first evidence that a previously unknown plant GlcNAc transporter plays a role in the initiation of root colonization by AMF.

Results

Cloning of maize *NOPE1* using synteny with rice

The maize *nope1* mutant is unable to establish AM symbioses [16]. Limited physical interaction of *nope1* with AMF suggested a failure in pre-symbiotic plant-fungal recognition that preceded fungal colonization of the root. Genetic mapping linked the *NOPE1* locus to the marker UMC1336, located at 86.4 Mb on chromosome 10. On the basis of our previous molecular characterization of AM symbiosis in rice, we searched for rice candidate genes showing a transcriptional response to AMF located in a region syntenic to maize *nope1* [19]. We identified the AM symbiosis-induced rice gene *LOC_Os04g01520*, which showed a >6 fold induction in colonized relative to control roots (*OsAM156* [19]), and was located at 0.4 Mb on rice chromosome 4 (Figure 1A, underlined). According to rice expression atlas data, *LOC_Os04g01520* transcripts accumulate to low levels across different plant organs, including leaves, stem and embryo tissue, with the highest accumulation in anthers (http://ricexpro.dna.affrc.go.jp/).

LOC_Os04g01520 encodes a single copy gene in the rice genome. Based on full cDNA analysis (http://getentry.ddbj.nig.ac.jp/getentry/ddbj/AK101964?filetype=html) and supporting ESTs, the gene model predicted two exons and one intron (Figure 1B), producing an ORF of 1404 bp. The 5' and 3' RACE PCR analysis indicated a transcriptional start point at -70 bp and a 184 bp 3'UTR sequence. The gene product of *LOC_Os04g01520* consists of 476 residues and has a predicted molecular weight of 50.34 kD.

To investigate the role *LOC_Os04g01520* plays during AM symbiosis, we identified two lines *4A-01057* and *3A-02512* from the POSTECH mutant collection [20] with T-DNA insertions 158 bp downstream of ATG within the first exon, and 22 bp upstream of the 3' intron splice-junction, respectively (Figure 1B). RT-PCR based analysis of *LOC_Os04g01520* mRNA levels in line *4A-01057* using primers either spanning the insertion site or a more 3' region failed to detect transcript (Figure 1C). In line *3A-02512* however, transcripts of *LOC_Os04g01520* accumulated to wild type size and levels, suggesting that the T-DNA insertion had been removed together with the intron during splicing, and therefore this allele was no longer considered for our study. Genomic PCR amplification and sequencing of the amplicon from line *4A-01057* confirmed mutation at the predicted site and revealed the additional presence of ~800 bp of the backbone vector (pGA2517) on the T-DNA right-border side (Figure 1B).

Co-cultivation of plants segregating for the T-DNA insertion *4A-01057* with *R. irregularis* identified a significant reduction ($p \leq 0.05$ for all structures tested) of fungal root colonization in homozygous mutant plants (Figure 2A). Comparing fungal colonization structures on wild type and mutant roots showed aberrant hyphopodia on the root surface of *4A-01057* that were absent from wild type roots (Figure 2B-C). Closer inspection of hyphopodia morphology on *4A-01057* revealed multiple unsuccessful penetration attempts (Figure 2D, arrowheads) and extensive hyphal septation, a sign of fungal stress (Figure 2C, arrows). Infrequently, the fungus succeeded in invading the root cortex and produced arbuscules that were of wild type morphology (Figure 2E-

F). Germination, overall vegetative and reproductive development, and seed production of *4A-01057* plants were equivalent to the wild type. To corroborate that the mutant phenotype of line *4A-01057* was indeed linked to the insertion at *LOC_Os04g01520*, we reintroduced a wild type copy of the gene under the native promoter into line *4A-01057* and found that wild-type levels of AM fungal colonization were restored in eleven independent transformants (Figure 2A, G; Supplemental Figure S1), confirming that the mutation in gene *LOC_Os04g01520* caused the AM phenotype of line *4A-01057*. The quantitative and qualitative phenotype of the rice insertion mutant was thus equivalent to the reported maize *nope1* mutant [16] and the *4A-01057* allele was accordingly designated *OsNOPE1*.

To investigate whether NOPE1 was required for susceptibility to diverse fungal species, known to effectively invade rice roots [21,22], wild type and *Osnope1* mutant roots were inoculated with *Piriformospora indica* and *Magnaporthe oryzae*. Both fungi invaded mutant and wild type root tissue equivalently well (Supplemental Figure S2) suggesting that NOPE1 might be specifically required for interaction with AMF.

Mutation of maize *NOPE1* recapitulates the AM phenotype of *Osnope1*.

The maize *nope1* mutant arose in a *Mutator* mutagenized population [16]. Poor seed viability and suppression of the phenotype of the original allele prevented further characterization of the maize *nope1* mutation; instead, we undertook a reverse genetics approach to verify the role of the maize homologue of *OsNOPE1* in AM symbiosis. The maize genome contains a single gene, *GRMZM2G176737*, showing high similarity to *OsNOPE1* (BLASTP; score=488, ID=83%, e-value=8.2xe^{-61}). *GRMZM2G176737* is located on Chromosme 10 near to the original mapped position of the *nope1* mutation (Figure 1A, underlined). To determine the role of *GRMZM2G176737* during AM symbiosis, a *Dissociation* (*Ds)*- tagged maize population was screened for transposon insertions at this locus [23,24]. A *Ds* element was identified that had inserted

147 within the first exon at 600 bp downstream of ATG, resulting in disruption of gene function as
148 reflected by the lack of corresponding transcript acummulation (Figure 3B). Microscopic
149 inspection of inoculated maize roots confirmed that despite fungal proliferation on the mutant root
150 surface the hyphae were septated. Hyphopodia were malformed and failed to penetrate while wild
151 type roots supported extensive development of intraradical fungal structures (Figure 3C and D).
152 Quantification of intraradical fungal structures revealed significantly lower fungal colonization of
153 homozygous individuals ($p < 0.05$) as compared to the wild-type (Figure 3E). Also transcript
154 levels of the maize homologues of the rice AM marker genes *OsAM3* [21] and *OsPT11* [25],
155 *GRMZM2G135244* (*ZmAM3*) and *GRMZM5G881088* (*ZmPT6*) [26], were lower in inoculated roots
156 homozygous for the transposon insertion as compared to hemizygous and wild-type siblings
157 (Figure 3F).

158 Reproduction of compromised early AMF-interaction of the original *nope1* mutant [16] in both
159 loss of function alleles of rice *LOC_Os04g01520* (*OsNOPE1*) and maize *GRMZM2G176737*
160 (*ZmNOPE1*) strongly indicates that *GRMZM2G176737* and *LOC_Os04g01520* correspond to the
161 *NOPE1* gene.

162

163 **NOPE1 belongs to the Major Facilitator Superfamily and occurs in all land plant species.**
164 Computational analysis of protein topology (http://phobius.binf.ku.dk/) and domains
165 (http://smart.embl-heidelberg.de/) predicted that NOPE1 has 12 transmembrane domains, no
166 signal peptide and contains a domain of unknown function, DUF895, between amino acids 49 and
167 181 (Supplemental Figure S3A). The NOPE1 protein is predicted to belong to the Major
168 Facilitator Superfamily (MFS, Pfam e-value 4.9e-13) of membrane transport proteins, thereby
169 suggesting NOPE1 to be involved in the transport of small molecules across membranes.

170 Blast Search based on available sequence data (http://blast.ncbi.nlm.nih.gov/Blast.cgi),
171 indicated that genes encoding NOPE1 are present in the genomes of all land plants investigated,

including non-mycorrhizal plant species (Supplemental Figure S3B). The genome of the model legume *Medicago truncatula* contains two copies of *NOPE1* orthologs; *MtNOPE1a* (*Medtr3g093270*) and *MtNOPE1b* (*Medtr3g093290*) and the corresponding gene products share 63% identity (79% positive) and 64% identity (81% positive), respectively with OsNOPE1, providing a plausible explanation for the lack of recovery of *NOPE1*-associated AM phenotypes from forward genetic screens in legumes. The model plant *Arabidopsis thaliana* is unable to form AM symbiosis, but yet two *NOPE1* orthologs *At1g18000* and *At1g18010* are present. At the genomic level, the two genes are identical in DNA, and protein sequence and share 57% identity (71% positive) at the protein level with rice NOPE1, indicating that the protein serves functions beyond AM symbiosis. The Arabidopsis electronic Fluorescent Pictograph indicated that both Arabidopsis genes are expressed constitutively throughout the plant with highest transcript accumulation in cauline leaves and flowers https://bar.utoronto.ca/eplant/ [27]. To determine a possible role of the Arabidopsis *NOPE1* genes for overall plant development, RNAi-based silencing lines were generated that targeted both genes simultaneously. We selected line *AtMNC42* and *AtMNC58* which displayed the strongest down-regulation for both genes (Supplemental Figure S4A) but were unable to find evidence for any altered developmental phenotype when inspecting germination, root and shoot architecture, flowering and seed setting.

Rice NOPE1 mediates N-acetylglucosamine transport in *Candida albicans*.

In order to investigate the mechanism by which NOPE1 influences AM symbioses, we sought to identify putative homologs with functional annotation. As no land plant orthologs had been previously functionally characterised, we adopted a reciprocal best hit (RBH) strategy between *Oryza sativa* and the human pathogenic fungus *Candida albicans.* RBH identified the functionally characterised protein Ngt1 as a putative orthologue of NOPE1 (41% identity and 59% positive). Ngt1 mediates N-acetylglucosamine (GlcNAc) transport across the plasma membrane of *C.*

albicans, enabling growth on GlcNAc as the only source of carbohydrates [28]. A key feature of the *C. albicans* virulence is the cells' ability to reversibly shift from isotropic budding to polarized filamentous growth in response to environmental signals [29]. Amongst the stimuli triggering this morphological switch is GlcNAc, which induced filamentous hyphal growth [30]. Deletion of *NGT1* impairs *C. albicans* GlcNAc uptake, preventing cells from switching morphology and from proliferating on GlcNAc-containing medium [17,31]. To address whether OsNOPE1 is a functional GlcNAc transporter, a *C. albicans* codon-optimized version of the rice *OsNOPE1*, driven by the native fungal *NGT1* promoter was transformed into the *C. albicans* mutant *ngt1*Δ. Remarkably, three different assays demonstrated that rice *OsNOPE1* restored the function ofcan complement *C. albicans ngt1Δ* cells (Figure 4A-C): the expression of *OsNOPE1* restored the ability of the *ngt1Δ* mutant *C. albicans* to grow on GlcNAc medium, to undergo filamentous hyphal differentiation upon GlcNAc treatment, and to take up radioactive GlcNAc from the medium. Rice *OsNOPE1* therefore mediated GlcNAc uptake in *C. albicans*, indicating functional conservation of the protein across plant and fungal kingdoms.

In order to determine substrate specifity of OsNOPE1 with regard to transport of other related hexose sugars, competition assays were performed in which an excess of cold hexoses was provided together with [^{3}H]GlcNAc. Control studies showed that the addition of a 2-fold excess of cold GlcNAc led to a partial decline in the uptake of radioactive GlcNAc and there was nearly complete inhibition at 20-fold excess cold GlcNAc (Supplemental Figure S5). OsNOPE1 showed strong specificity for transporting GlcNAc, since a 200-fold excess of glucosamine, dextrose, fructose or galactose did not significantly impact on the amount of [^{3}H]GlcNAc transported into the cells. A 200-fold excess of N-acetylmannosamine partially competed with [^{3}H]GlcNAc ($p <$ 0.01 by non-parametric one way ANOVA), suggesting that the N-acetyl moiety may be important for transport substrate specificity. Overall, these results demonstrated that OsNOPE1 exhibits a high specificity for transporting GlcNAc, similar to *C. albicans* Ngt1 (Supplemental Figure S5).

NOPE1 mediates N-acetylglucosamine influx in plants.

The efficient rice NOPE1 transport activity in *C. albicans* predicted that OsNOPE1 may mediate GlcNAc transport across the plasma membrane also in plants. We verified subcellular localization of At1g18000 in stably transformed *A. thaliana* plants by using the constitutively active Arabidopsis ubiquitin promoter to express the in-frame fused *YFP-AtNOPE1a*. Three independent lines were analyzed to determine reproducible localization patterns. Propidium iodide was applied to counterstain the plant cell wall. Transgenic lines showed a reproducible and clear signal consistent with plasma membrane localization, here shown for line *At4731y-4*, (Figure 5A). It is therefore conceivable that absence of the NOPE1 transport activity at the plasma membrane leads to the early AM phenotype of the rice and maize *nope1* mutants.

To confirm that OsNOPE1 can transport GlcNAc in its native plant environment, we measured [^{3}H]GlcNAc root uptake in rice seedlings[32] over time using wild type, *OsNOPE1* and the genetically complemented mutant line C4. While the uptake rates observed for the wild type and the complemented C4 line were 2.37 (R^2 = 0.992) and 2.00 pmol (R^2 = 0.991) [^{3}H]GlcNAc/60 min and plant, respectively, that of the *Osnope1* mutant was 0.624 pmol (R^2 = 0.960) [^{3}H]GlcNAc /60 min and plant, thus significantly slower ($p < 0.05$; Figure 5B). To test if NOPE1 supported transport of GlcNAc across plant cell membranes in another system, we measured the [^{3}H]GlcNAc uptake in protoplasts derived from the *A. thaliana At1g18000* overexpression line, *At4731y-4* and compared this uptake to that of the RNAi-silencing line *AtMNC58* (Supplemental Figure S6). Uptake of [^{3}H]GlcNAc displayed a linear increase over time and reached significantly higher uptake rates in the overexpression line *At4731y-4* with 2 ± 0.05 pmol [^{3}H]GlcNAc/g chlorophyll at 25 min ($p < 0.01$) as compared to 1.25 ± 0.1 pmol [^{3}H]GlcNAc/g chlorophyll acquired by protoplasts of the RNAi-silenced line *AtMNC58* (Supplemental Figure S6). Please note that the 0.5 min value does not correspond to specific uptake but to unspecific adsorption of

medium to the protoplasts. Taken together, NOPE1 mediated GlcNAc uptake in whole roots and leaf protoplasts of mycorrhizal and non-mycorrhizal plant species, respectively.

In addition, we quantified GlcNAc efflux in the rice seedling system. Roots of wild type rice seedlings were first loaded by incubation with 100 µM [^{3}H]GlcNAc and then transferred to a solution either containing unlabeled GlcNAc at 50X concentration or no GlcNAc. Monitoring the levels of [^{3}H]GlcNAc retained in the roots revealed that in both cases GlcNAc was released from the roots, but that at shorter times, significantly less ($p < 0.05$) remained in the roots when the external medium contained no GlcNAc (2.21 ± 0.10 [^{3}H]GlcNAc/plant) relative to roots incubated in high GlcNAc medium (3.30 ± 0.36 [^{3}H]GlcNAc/plant, Figure 5C), indicating that substrate availability at the external side partially inhibited efflux.

In summary, NOPE1 mediated GlcNAc uptake into plant cells, which conceivably also facilitated GlcNAc efflux across the plasma membrane, however export might be catalyzed by additional or alternative transporters.

Distinct transcriptional responses of *R. irregularis* to rice wild type and *nope1* root exudates.

The NOPE1-mediated GlcNAc transport across the plasma membrane may be required for symbiosis establishment by contributing to plant perception of AMF. AM specific marker genes from rice offer sensitive diagnostics and are routinely used to molecularly phenotype distinct stages of the interaction in rice [21,33-37]. Examining the mRNA levels of four AM marker genes (*AM1*, *AM3*, *AM14* and *PT11*) in control and inoculated wild type and *Osnope1* roots demonstrated that in the mutant these genes exhibited about two order of magnitude lower induction than in the wild type (Figure 5D). The low marker gene expression levels correlated with the low colonization level (Figure 2A). However, the mutant may alternatively be affected in the uptake of compounds whose signaling functions are not monitored by these marker genes.

An equally plausible scenario could be that NOPE1 function is required for the release of compounds that activate AMF towards symbiosis. As diffusible compounds released by plant roots such as SLs alter hyphal branching patterns [8,9], we used *Gigaspora rosea* to assess hyphal ramification upon treatment with plant exudates. No difference was found in the number of hyphal apices and overall hyphal growth behaviour in response to exudates from wild type and *Osnope1* (Supplemental Figure S7). This is consistent with GlcNAc belonging to another chemical compound class than SLs, 2-OH fatty acids or flavonoids which trigger specific hyphal growth patterns (for review see [1]).

To more sensitively and comprehensively examine possible fungal responses to root-released compounds, RNAseq analysis was performed on pre-germinated *R. irregularis* spores exposed to either rice wild type or *Osnope1* root exudates at 1h, 24h and 7 days post treatment. Signifcantly differentially expressed genes (d.e.) between treatment and mock condition were defined by False Discovery Rate (FDR) ≤ 0.05. Remarkably, wild-type vs mutant exudate treatment resulted in a much larger number of d.e. genes at 1h post treatment, corresponding to 1249 vs 388 genes, respectively, whereas the volume of the transcriptional response was more comparable at later time points (Table 1A and B). Next, we compared the d.e. gene lists of the two treatments at each time point and applied a 2-fold cut-off [Fold Change (FC) ≥ 2] (Table 2A). Treatment with wild-type exudates led to the induction of 92 genes at 1h, which were not induced when the fungus was exposed to *Osnope1* root exudates (Figure 6A top and bottom, Table 2A). At 24h and 7d treatment with wild-type exudates resulted in the induction of 283 and 901 genes, respectively (Figure 6A top, Table 2A and B). In contrast, although *Osnope1* exudate treatment induced 343 genes at 24h, only 256 genes had increased transcript levels at 7d (Figure 6A top and bottom, Table 2A and B). Therefore, exposure to wild-type and *Osnope1* exudates led to distinctly different transcriptional response patterns in the fungus. Time resolved gene ontology (GO) analysis on those genes which met the filtering citeria of FDR < 0.05 and FC > 2 (Table 2A and B), revealed an

overrepresentation of genes associated with the GO terms protein kinase and ATPase activity amongst the 92 d.e. genes (Figure 6B, Table 3A) suggesting an early induction of fungal signaling activities. At 24h either treatment had led to a significant change in the fungal transcriptome (Table 2A and B), however GO analysis suggested that while the fungus switched to an elevated oxidative status in response wild-type exudates, it induced stress responses upon mutant exudate treatment (Table 3B). While the 'stress response signature' remained for the fungal transcriptome at 7d post treatment with mutant root exudate, exposure to wild-type exudates induced fungal genes, associated with GO terms corresponding to a higher energetic and metabolic status (Figure 6B, Table 3C). Together these data are consistent with an early and transient activation of fungal signaling, followed by the activation of genes involved in primary metabolism in wild type but not *Osnope1* root exudate treated fungus.

RNAseq results were validated by qRT-PCR analysis on a subset of representative d.e. genes using two selection criteria: (1) predicted to encode proteins with a potential role in processes involved in the initial recognition and interaction with the host plant (cell wall modification, transport, signaling) and (2) showing pronounced differences in the level of transcript accumulation at both 1h and 24h (Supplemental Figure S8A-C), only at 24h (Supplemental Figure S8D and E), and at 7 days (Supplemental Figure S8F and G). Interestingly, the *R. irregularis NGT1* homologue MIX9501_16_76 [38] displayed a basal expression level throughout the experiment (Gene Expression Omnibus, accession n° GSE65595), and was not induced in response to treatment with either exudates or by GlcNAc treatments (Supplemental Figure S9). The specific fungal transcriptional response to rice wild type root exudates is consistent with the hypothesis that the NOPE1 function leads to adequate reprogramming of *R. irregularis* for host colonization. However, application of GlcNAc to *R. irregularis*-inoculated *Osnope1* mutant plants for seven weeks at 1 mM, 10 mM or 100 mM GlcNAc did not complement the mutant phenotype. This may be due to application of GlcNAc outside the biologically active concentration, or that

either the development of a GlcNAc gradient or efflux of a GlcNAc-conjugate might be necessary for stimulating the fungus.

On the assumption that wild type roots release the critical GlcNAc compound, we examined whether the presence of wild type exudates would restore AM colonization of the mutant when cocultivated within the same container. Indeed, roots of *Osnope1* were fully colonized when grown together with wild type but not with mutant 'donor' plants (Supplemental Figure S10), lending further support for NOPE1 being directly or indirectly required for the root exudation of an AMF activating compound.

Discussion

We report here the discovery of NOPE1 as a plasma membrane GlcNAc transporter required for the initiation of AM symbiosis in rice and maize. Current knowledge of the molecular mechanisms that plants employ to attract and reprogram mutualistic AMF in the rhizosphere is largely limited to the stimulatory effects that SLs exert on fungal metabolism and development. It has been anticipated that additionally secreted bioactive molecules are necessary to stimulate the fungus for symbiosis ([39], for review see [1]). While there was no difference in the ability to induce pre-symbiotic hyphal branching, wild-type and *Osnope1* root exudates triggered distinct transcriptional responses in the fungus. After 1h exposure to wild type exudates, transcripts assigned to GO terms associated with signaling functions were induced, consistent with the conditioning of the fungus by the plant prior to contact formation. This hypothesis was further supported by the restoration of normal levels of colonization of *Osnope1* by co-cultivation with wild-type plants, indicative for bioactive molecules in the wild-type exudates. We therefore hypothesize that NOPE1 fulfills distinct roles, complementary to SL, in stimulating the fungus for the interaction.

NOPE1 represents a functional GlcNAc transporter and mediates efficient GlcNAc import in *C. albicans*, rice and Arabidopsis. GlcNAc occurs prevalently in microbial environments as a building unit of either fungal and bacterial cell walls or of microbial signaling molecules such as rhizobial nod-factors. Whether GlcNAc uptake by the plant contributes to microbe perception or influences root exudate composition, e.g. via signaling remains at present unclear. Analysis of the expression of a small number of marker genes revealed quantitative differences between wild type and mutant that were proportional to the level of root colonization.

Wild-type roots were shown to be able to acquire and release GlcNAc; as uptake depended on functional NOPE1, efflux might also require NOPE1. However, the chemical identity of the bioactive molecule is at present unknown as addition of GlcNAc to inoculated mutant plants did not recover wild-type colonization levels. At this stage, the biological role of NOPE1 in the release of bioactive compounds into the rhizosphere remains to be determined.

Plants produce detectable quantities of cellular GlcNAc; mass spectrometry-based analysis detected abundant levels of GlcNAc monomers in leaves of wild type *Arabidopsis thaliana* [40]. Even though corresponding data are not available from roots a similar scenario can be expected due to the important intracellular roles GlcNAc plays in e.g. the posttranslational modification of lipids and proteins or also in influencing diverse cellular processes as glycans. For example interference with biosynthesis of the activated substrate for GlcNAc transfer, UDP-GlcNAc, in rice roots impaired cell expansion in the root elongation zone, leading to a short root phenotype, thereby demonstrating the importance of the GlcNAc metabolism for root functioning [41].

Host-secreted GlcNAc is known to act as a potent signaling molecule for a number of microbial organisms including the facultative human pathogenic fungus *C. albicans* [18]. Exposure to GlcNAc leads to the induction of invasive hyphal growth and the expression of virulence genes such as the adhesins that facilitate attachment to host cells (for review see [28]). Also the thermally dimorphic pathogenic fungi *Histoplasma capsulatum* and *Blastomyces dermatitidis* respond to

treatment with GlcNAc by a similar yeast-to-filament switch [42]. In these facultative human pathogens GlcNAc additionally functions as a source of sugar. The utilization of plant-derived GlcNAc as a substrate was recently reported for the plant-pathogenic bacteria *Xanthomonas campestris* pv. *campestris* while infecting leaves of *Brassica oleracea* [43]. In these bacteria, availability of GlcNAc-containing compounds induces numerous bacterial glycosyl hydrolases, which enzymatically release *N*-glycans from extracellular plant *N*-glycosylated proteins. The closely related vector-borne phytopathogenic bacterium *Xylella fastidiosa* enzymatically digests GlcNAc polymers (chitin) available in the foregut of the insect vector and also uses the acquired GlcNAc as a nutrient source [44]. Remarkably, the effects of host-provided GlcNAc on microbial physiology extend towards the mutualistic bioluminescent squid-vibrio model where host-derived GlcNAc mono- and dimers act as a regulators of shifting the bacterial metabolism to provide optimal symbiont services to the host [45].

Our study identifies the first GlcNAc transporter in plants and introduces the importance of NOPE1 in interspecies communication between rice and *R. irregularis*, thereby suggesting NOPE1 to represent an evolutionarily ancient and ecologically prevalent protein for the interaction with Glomeromycotan fungi.

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Author contribution: M.N., R.S., N.G., E.M., J.B.K., T.P.B. and U.P. designed the experiments. M.N., R.S., S.N., B.B., C.K., A.S., G.A., K.A., A.R., C.G., and C.R., performed the experiments. C.R. performed bioinformatics and statistical analyses of the RNAseq data. M.N., R.S., C.R., E.M., J.B.K. and U.P. wrote the manuscript.

R. irregularis RNAseq reads have been released at Gene Expression Omnibus (accession n° GSE65595). Correspondence and requests for materials should be addressed to U.P. (up220@cam.ac.uk). The authors declare no conflict of interest.

FIGURE 1 (1 column)

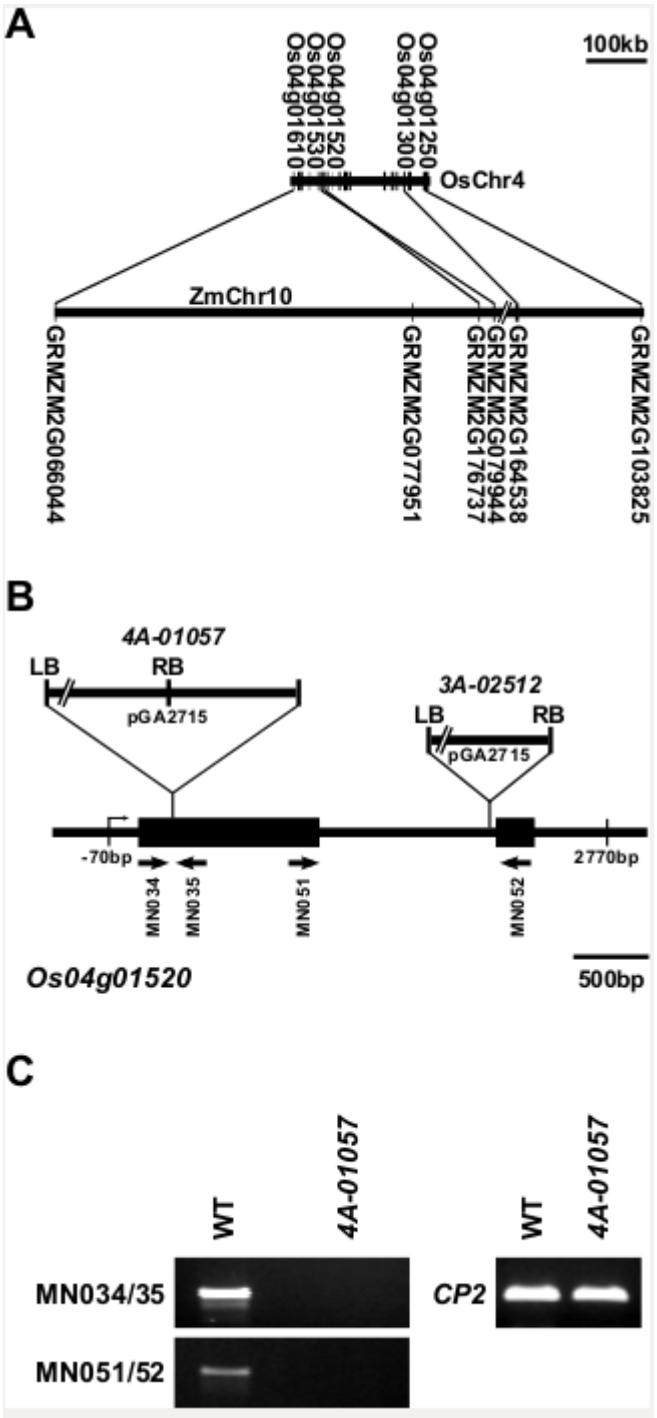


Figure 1. **Identification of syntenic rice and maize *NOPE1* candidate genes. A**, Physical map of the *nope1* interval in syntenic regions of rice chromosome 4 (OsChr4) and maize chromosome 10 (ZmChr10). Maize and rice shown on a common scale with arrows indicating orientation. Double bar indicates a break in the representation of the maize chromosome. Annotated gene models are shown by bars and orthologous pairs connected with a dotted line. Only those rice genes assigned orthologs in the maize region are labeled by name. Maize and rice *NOPE1* homologs are indicated (underline). **B**, Structure of the rice gene *LOC_Os04g01520* and position of T-DNA insertions present in the lines in lines *4A-01057* and *3A-02512*. Sites of transcriptional initiation and termination are indicated relative to the start of translation, at -70bp and 2770bp,

respectively. Arrows represent primers used in **C,** RT-PCR-based analysis of *LOC_Os04g01520* transcript levels in wild type (WT)and *4A-01057*. LB, left border; RB right border; *CP2, CYCLOPHILLIN (LOC_Os02g02890).*

FIGURE 2 (2 columns)

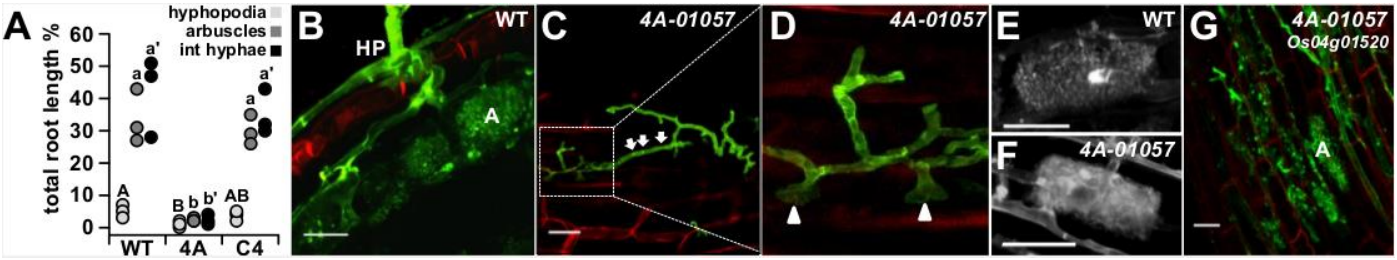


Figure 2.Coloniztion by *R. irregularis* is disrupted in the rice insertion line *4A-01057*. A, Percentage root length colonization in individuals segregating for the T-DNA insertion *4A-01057* at 6 wpi. Points represent individual plants. Means groups assigned for each fungal structure indicated by letters (adj. $p < 0.05$); **B-G,** WGA-staining of fungal structures and propidium-iodide counterstained plant cell walls of rice roots inoculated with *R. irregularis* at 6wpi as examined by laser scanning confocal microscopy. **B**, Hyphopodium (HP) and arbuscules differentiation in a wild type (WT) root **C,** Misshapen and highly septate (arrows) hyphopodial hypha on the surface of the root of a plant homozygous for the *4A-01057* insertion. **D,** Detail of hyphopodia on the surface of the roots of a *4A-01057* homozygous plant, showing several aborted penetration attempts (arrowhead). Morphologically equivalent arbuscules formed in the roots of wild type (**E**) and *4A-01057* homozygous (**F**) plants. **G**, Arbuscules formed in root cortical cells of the complemented line C4. HP, hyphopodium; A, arbuscule.scale bar = 50µm.

FIGURE 3 (1 column)

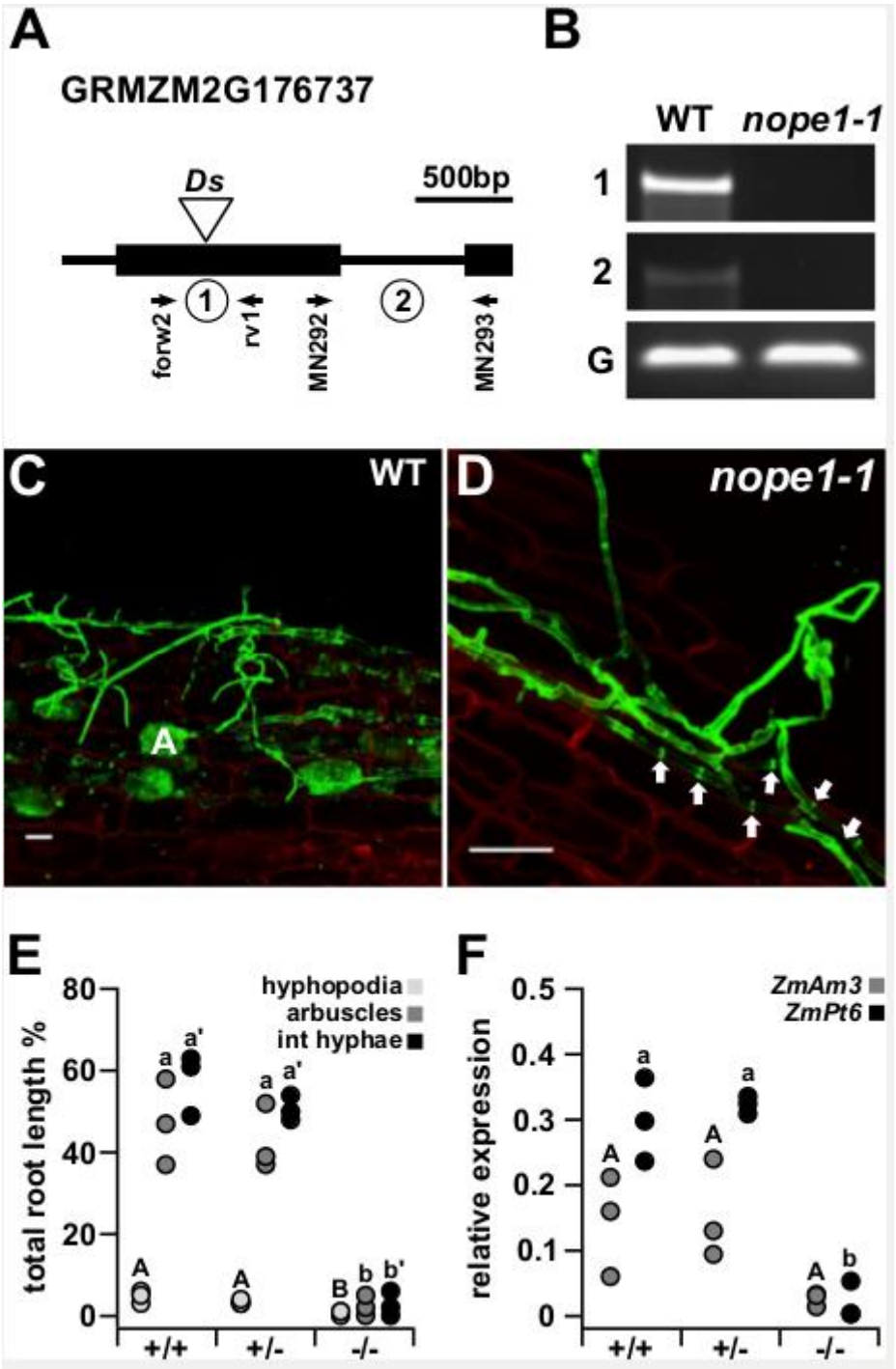
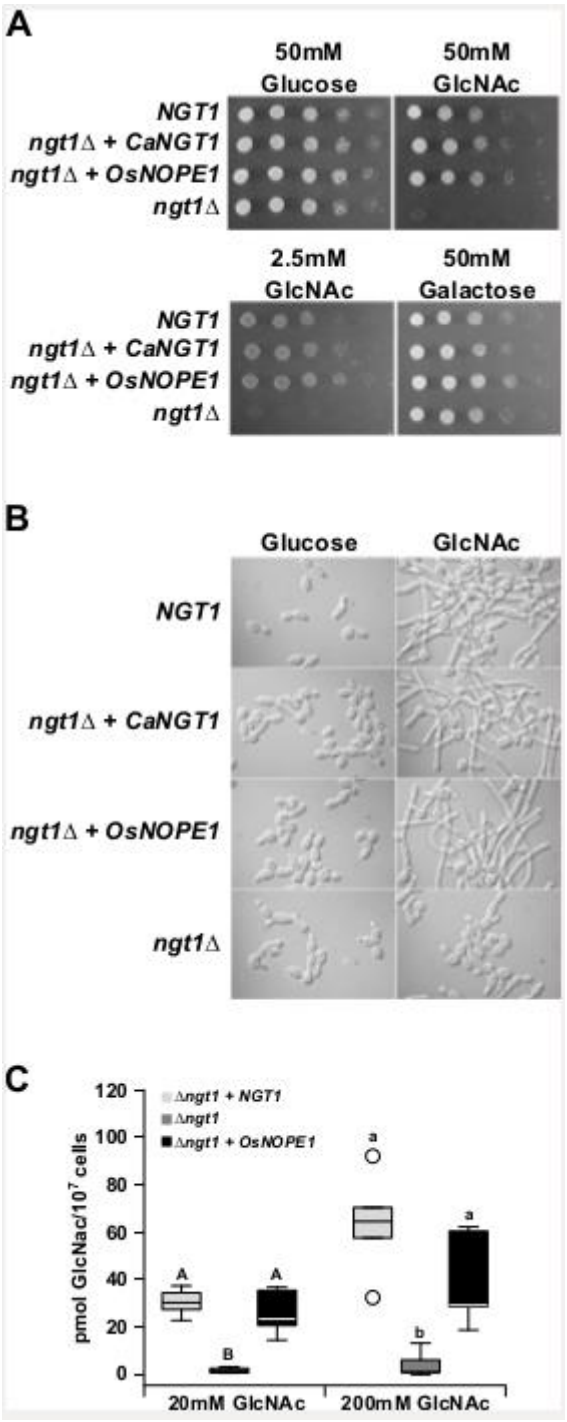


Figure 3. Mutation of the maize gene *GRMZM2G176737* reproduces the *Osnope1* phenotype. **A**, *GRMZM2G176737* gene structure and position of *Ds* transposon insertion (triangle). Arrows indicate position of primers used in B; forw2, ZmLph2-forw2; rv1, ZmLph2-rv1 **B**, RT-PCR analysis of *GRMZM2G176737* transcript accumulation in the roots of plants homozygous for the *Ds* insertion *GRMZM2G176737::Ds* using primers flanking (1) and downstream (2) of the insertion site (reaction 1); *GAPDH, GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE* (*GRMZM2G046804*). **C** and **D,** WGA-staining of fungal structures and propidium-iodide counterstained plant cell walls of maize roots inoculated with *R. irregularis* at 6 wpi, as examined by laser scanning confocal microscopy. **C**, On wild type roots the fungus

develops normal hyphopodia and extensively colonizes the root forming frequent arbuscules. **D**, A hyphopodium on roots of *GRMZM2G176737::Ds* appears misshaped with mutiple septa (arrows) and fails to penetrate the root tissue. A, arbuscule. Scale bar = 50μm. **E**, Percentage *R. irregularis* root length colonization of individuals segregating *GRMZM2G176737::Ds* at 6 wpi (+/+, wild type, +/-, heterozygote, –/- homozygous mutant). Points represent individual plants. Means groups were calculated *post hoc* indepently for each structure and are indicated by letters ($p<0.05$). **F,** qRT-PCR-based analysis of transcript accumualtion of *ZmAM3* (*GRMZM2G135244*) and *ZmPT6* (*GRMZM5G881088*) in maize plants segregating *GRMZM2G176737::Ds* at 6wpi with *R. irregularis.* Expression values were normalized against *GAPDH.* Points indicate individual plants. Means groups were calculated *post hoc* indepently for the two transcripts and are indicated by letters ($p < 0.05$).

577 **FIGURE4 (1 Column)**



578

579 **Figure 4**. **Rice NOPE1 mediates GlcNAc transport in *C. albicans*. A**, Ten-fold cell dilution
580 series of *C. albicans* strains spotted onto plates with indicated sugar. **B**, *C. albicans* strains grown
581 overnight in glucose containing medium and resuspended in fresh medium containing either 50
582 mM glucose or 50 mM GlcNAc. **C,** [H^3]GlcNAc uptake in *C. albicans* strains at 20mM and
583 200mM GlcNAc. GlcNAc, N-acetylglucosamine. Boxes show 1st quartile, median and 3rd
584 quartile. Whiskers extend to the most extreme points within 1.5x box length; outlying values

beyond this range are shown as unfilled circles. Means groups were calculated *post hoc* indepently for the two GlcNAc treatments and are indicated by letters ($p < 0.05$). For description of strains see Supplemental Information Table 5.

FIGURE 5 (1.5 columns)

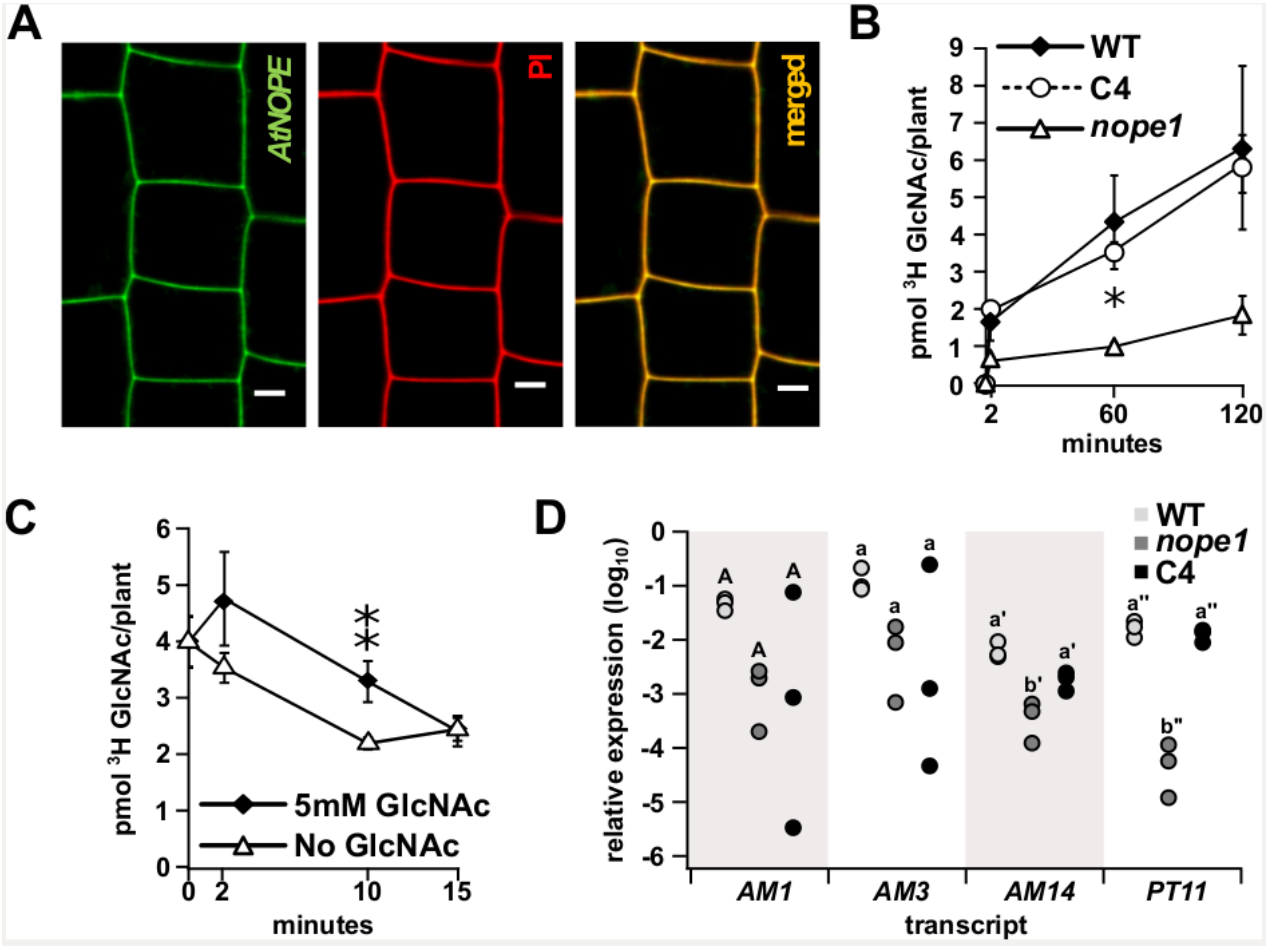


Figure 5. NOPE1 mediates GlcNAc transport in rice and Arabidopsis. A, Laser scanning confocal microscopy of *A. thaliana* roots expressing Ubq_{prom}::*YPF*::*AtNOPE1* (line *At4731y-4*). YFP-AtNOPE1 signal shown in green (left). Corresponding cells stained with Propidium Iodide (PI) shown in red (centre). Overlay (yellow, right). Scale bar: 5µm. **B,** Time course of [^{3}H]GlcNAc uptake in roots of O*snope1*, wild type and genetically complemented mutant line C4. Means and SEs of three biologically independent experiments are shown (*$P \leq 0.05$) **C,** Time course of [^{3}H]GlcNAc export activity of wild type rice roots at 0 and 5mM (50X) GlcNAc external concentration. Means and SEs of three biological replicates are shown. (**$p \leq 0.01$). D, qRT-PCR-based analysis of *AM1*, *AM3*, *AM14*, and *PT11* marker transcript accumulation in the roots of wild type, O*snope1*, and the complemented line C4, at 6 wpi with *R. irregularis*. Expression values were normalized against *CYCLOPHILLIN* (*LOC_Os02g02890*). Points represent individual plants. Means groups were calculated *post hoc* indepently for each transcript and are indicated by letters ($p < 0.05$).

FIGURE 6 (2 columns)

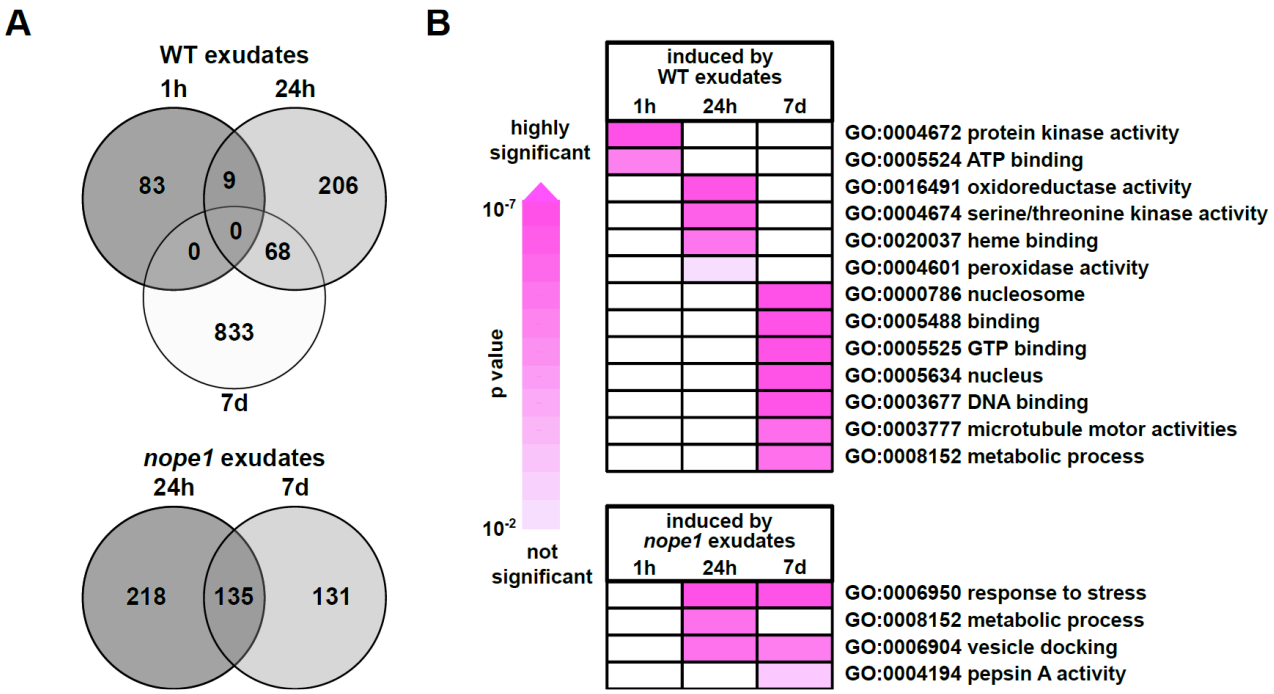


Figure 6. *R. irregularis* transcriptional response to root exudates from rice wild type and *Osnope1* mutant plants. A, Venn diagrams indicating number of significantly induced fungal genes ($P \leq 0.05$, one way ANOVA) in response to treatment with exudates from wild type relative to *Osnope* (top) and *Osnope* relative to wild type (bottom). **B,** Time-resolved Gene Ontology analysis for Biological Process terms ($p \leq 0.01$) for fungal genes induced when treated with root exudates from wild type (top) or from *Osnope1* (bottom). The colour code indicates the significance of gene enrichment (p-value).