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Functional expression and characterisation of a gut facilitative glucose transporter, NlHT1, from the phloem-feeding insect *Nilaparvata lugens* (rice brown planthopper)

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Abstract

Phloem-sap feeding Hemipteran insects have access to a sucrose-rich diet but are dependent on sucrose hydrolysis and hexose transport for carbon nutrition. A cDNA library from *Nilaparvata lugens* (rice brown planthopper) was screened for clones encoding potential transmembrane transporters. A selected cDNA, *NlHT1*, encodes a 53 kDa polypeptide with sequence similarity to facilitative hexose transporters of eukaryotes and prokaryotes, including GLUT1, the human erythrocyte hexose transporter. NlHT1 was expressed as a recombinant protein in the methylotropic yeast *Pichia pastoris*, and was identified in a membrane fraction isolated from transformed yeast cells. Transport experiments using membrane vesicles containing NlHT1 showed that the protein is a saturable, sodium independent transporter, with a relatively low affinity for glucose (K_m 3.0 mM), which can be inhibited by cytochalasin B. Competition experiments with fructose demonstrate NlHT1 is glucose specific. *In situ* localisation studies revealed that *NlHT1* mRNA is expressed in *N. lugens* gut tissue, mainly in midgut regions, and that expression is absent in hindgut and Malpighian tubules. NlHT1 is therefore likely to play an important role in glucose transport from the gut, and in carbon nutrition *in vivo*. This is the first report of a facilitative glucose transporter from a phloem-feeding insect pest.

Keywords: Hexose transport; *Pichia pastoris*; Major facilitator superfamily; *In situ* hybridisation

1. Introduction

Phloem-sap feeding insects from the order Hemiptera cause persistent yield reductions in many globally important crop species. The brown planthopper, *Nilaparvata lugens*, is a major rice pest in many parts of Asia (Holt et al., 1996); single season losses, as a consequence of *N. lugens* infestation, were predicted at US$30 million in Thailand and Vietnam during the 1990/1991 growing season (Gallagher et al., 1994). Rice crop damage is caused by removal of photoassimilates, blocking of phloem elements (Watanabe and Kitagawa, 2000) and transmission of plant viruses (Hibino, 1996). Control of *N. lugens* infestation is reliant on broad-spectrum insecticides, the overuse of which has encouraged resistant strains to appear. Resistance to commonly used organophosphorous, carbamate and pyrethroid insecticides is now widespread (Karunaratne et al., 1999; Small and Hemingway, 2000a, b; Hemingway et al., 1999; Hasui and Ozaki, 1984; Vontas et al., 2001). Due to the global importance of rice, estimated to supply 20% of the world's calorific intake (statistics derived from IRRI, world rice statistics), new control measures must be addressed.

We have focused on hexose transport as a potential target for the development of novel insecticides for phloem-feeding insects, like *N. lugens*. These insects have access to a carbon rich diet, the main constituent of which, in many plant species, including rice, is sucrose (Fisher, 2000; Fukumorita and Chino, 1982). Sucrose is present in plant phloem sap at high concentrations, often in the range of 0.5–1 M and is responsible for a high osmotic pressure,

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often up to five times that of the feeding Hemipterans body fluids (Douglas et al., 2006). Phloem-feeding insects circumvent this osmotic problem by removing sucrose, rendering the gut contents isoosmotic with the body fluids surrounding the gut, and preventing the loss of water to gut contents. Two processes are involved in maintaining osmotic balance: sucrose hydrolysis and transport, and sucrose conversion. Sucrose conversion into longer chain oligosaccharides decreases the osmotic potential of gut contents, but causes a loss of carbon as a nutritional source. In many species, the majority of carbon ingested as sucrose is converted into longer chain oligosaccharides and voided as honeydew (Fisher et al., 1984; Walters and Mullin, 1988; Rhodes et al., 1997; Wilkinson et al., 1997; Ashford et al., 2000). However, some ingested carbon must be available for nutrition, and because sucrose is not transported across Hemipteran gut wall, carbon nutrition is wholly reliant on sucrose hydrolysis into its constituent parts, glucose and fructose, and transport of these monosaccharides across the gut (Rhodes et al., 1996; Ashford et al., 2000). The processes of osmotic balance and nutrition effectively compete for ingested carbon, and a dynamic balance must exist between sucrose hydrolysis and hexose transport across the gut wall, and hexose conversion to oligosaccharides. The regulation of these processes in phloem feeders is a fundamental part of their adaptation to utilising a physiologically 'extreme' food source, with a high sucrose content. Phloem sap is only utilised as a sole carbon source by Hemipteran insects, which include agronomically important pests such as aphids, whitefly and planthoppers (Douglas et al., 2006). Therefore, characterisation of hexose transport has the potential to generate novel targets for Hemipteran-specific insect control.

The importance of sucrose hydrolysis and partitioning of carbon between excretion and hexose transport and utilisation in Hemipteran metabolism has been demonstrated by the drastic impact of α-glucosidase inhibitors on normal physiology of the pea aphid *Acyrthosiphon pisum* (Karley et al., 2005) and the silverleaf whitefly *Bemisia argentifolii* (Salvucci, 2000). Oral uptake of the broad-spectrum α-glucosidase inhibitor bromoconduritol reduced α-glucosidase and trehalose synthase activity in protein extracts from whiteflies, and caused unusual carbon partitioning *in vivo*. Oral delivery of acarbose, another broad-spectrum α-glucosidase inhibitor, completely eliminated extractable sucrase activity in *A. pisum*. When acarbose treated aphids were maintained on artificial diets with 750 mM sucrose, a high level of mortality was observed (mean survival 2.8 days), and aphids died of osmotic dysfunction. However, when acarbose treated aphids were maintained on diets with 200 mM sucrose, mean survival was similar to that of starved aphids (4.2 days). Death in these aphids appeared to be a result of carbon starvation rather than osmotic dysfunction. Thus, when sucrose hydrolysis is inhibited, aphids have access to a sufficient carbon supply, but are unable to utilise it.

As a first step in the development of novel insecticides targeting sucrose metabolism in phloem-feeding insects, the present paper reports the identification and functional characterisation of a glucose-specific transporter, located in gut tissue, from rice brown planthopper (*N. lugens*; NlHT1). The data presented also show that the yeast *Pichia pastoris* is a viable expression system that can be used to produce functional insect transport proteins.

2. Materials and methods

2.1. N. lugens insect cultivation

The rice brown planthopper *N. lugens* was maintained on susceptible 2–3 month old rice plants (*Oryza sativa* var. TN1), under controlled environmental conditions (27 °C, 80% relative humidity and a 16 h light:8 h dark regimen).

2.2. Isolation of insect RNA and first strand cDNA synthesis

Total RNA was isolated from whole insects, and from dissected guts, using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions. Poly-A^+ RNA was purified using polyATtract system (Promega). First-strand cDNA was synthesised at 42 °C from either total RNA or poly-A^+ RNA using M-MLV reverse transcriptase (Promega) and a poly-dT primer, according to standard procedures.

2.3. DNA sequencing and sequence analysis

DNA sequencing reactions were carried out using BigDye Terminator with AmpliTaq DNA polymerase (ABI Biosciences). Reaction products were analysed on automated sequencer (ABI Prism 3730; DBS Genomics, Department of Biological and Biomedical Sciences, University of Durham). Sequence data was used in BLAST similarity searches (Altschul et al., 1990) against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/), and identification of sequence features in encoded polypeptides was performed using the CBS prediction servers (http://www.cbs.dtu.dk/services/). Protein domains and functional sites were identified by both InterProScan (Zdobnov and Apweiler, 2001) and superfamily domain search (Gough et al., 2001).

2.4. Cloning putative transporters from N. lugens

cDNA libraries were constructed from poly-A^+ RNA isolated from dissected guts and whole adult *N. lugens*, using Lambda Zap II XR cDNA library construction kit (Stratagene). The cDNA was size fractionated to remove fragments <500 bp, directionally cloned into ZAP II vector and packaged into phagemids using Gigapack III Gold packaging extract (Stratagene). Mass excision of the cDNA library generated subclones in pBluescript SK(-) vector, from which individual clones were randomly

selected and sequenced over 5′ and 3′ ends. Sequences were annotated by BLAST similarity searches against GenBank database. Genes with similarity to sugar transporters were identified and the entire coding sequence was reamplified by PCR from *N. lugens* gut-specific first-strand cDNA using a proofreading polymerase. Truncated sequences were completed by RACE (rapid amplification of cDNA ends) and sequence data from several independent clones was obtained and checked for PCR errors.

2.5. Localisation of NlHT1 RNA by in situ hybridisation and RT–PCR

N. lugens guts were dissected in 0.9% (w/v) NaCl, fixed in MEMFA (0.1 M MOPS, 2 mM EDTA, 1 mM $MgSO_4$, 3.7% formaldehyde) for 1 h at room temperature and stored in 100% ethanol at −20 °C until further processing. Guts were rehydrated through a graded series of ethanol, rinsed in PBS with 0.1% (v/v) Tween, and incubated with 10 μg ml^{-1} proteinase K for 1 min at room temperature. Guts were hybridised overnight at 60 °C with either sense or antisense DIG-dUTP labelled RNA probes (corresponding to 1–1056 bp of *NlHT1* open reading frame) in 50% formamide, 5 × SSC, 5 mg ml^{-1} *Troula* yeast RNA Type VI (Sigma), 100 mg ml^{-1} heparin, 1 × Denhardt's, 0.1% (v/v) Tween, 0.1% (w/v) CHAPS and 10 mM EDTA. Extensive washes in 2 × SSC and 0.2 × SSC at 60 °C were followed by washes at room temperature with maleic acid buffer (MAB; 0.1 M maleic acid, 0.15 M NaCl, 0.1% Tween pH 7.5), and blocked in 5% (w/v) BSA, 2% (w/v) Boehringer Mannheim Blocking Reagent in MAB buffer for 2 h at room temperature. Guts were then incubated with anti-DIG antibody linked to alkaline phosphatase (Roche) at a dilution of 1:2000 in blocking solution at 4 °C overnight. The antibody was detected after extensive washes at room temperature in MAB by a colour reaction with BCIP/NBT (Roche). Endogenous phosphatase activity was blocked by adding levamisole (5 mM) to the colour substrate.

NlHT1 transcript distribution was determined by RT–PCR on *N. lugens* tissues, which included whole insect, head, gut and whole insect without gut. Tissues were dissected from adult insects and homogenised in 10 μl RDD buffer (Qiagen) and lysates were treated with RNase-free DNase I (Qiagen), according to manufacturer's instructions. After DNA digestion total RNA was purified using Tri-reagent (Sigma) and RNA pellets were resuspended in 14 μl RNase-free water. Total RNA was used for first-strand cDNA synthesis using M-MLV reverse transcriptase (Promega), according to standard procedures. First-strand cDNA was amplified by PCR using primers for full-length *NlHT1* and amplification products were analysed on a 1.1% agarose gel.

2.6. SDS–PAGE and western blotting

SDS–PAGE was carried out in 12% polyacrylamide gels containing 0.1% SDS, on a discontinuous pH system (Laemmli, 1970), using ATTO AE6450 equipment. Samples were mixed with sample buffer (4:1) containing 225 mM Tris, pH 6.8, 5% SDS, 50% glycerol, 250 mM DTT, 0.05% bromophenol blue and heated to 95 °C for 5 min prior to loading. Gels were either stained with Coomassie Blue R-250 or transferred to nitrocellulose by electroblotting. For experiments on the recombinant protein, transferred proteins were probed with anti-myc antibody (Cell Signaling Technology) at 1:1000 dilution. Specifically bound antibodies were visualised using peroxidase-coupled secondary antibodies in conjunction with ECL substrate (Amersham), and then exposed to film.

2.7. Construction of expression vector pPICZB-NlHT1

The full-length *NlHT1* coding sequence was amplified by polymerase chain reaction from gut-specific cDNA using sense primer, 5′-ATGAATTCAATAATGTCTACCAAGGCAACG-3′, which incorporated an optimised start consensus for protein expression in yeast (Romanos et al., 1992) and a *Eco*RI site (underlined); and antisense primer, 5′-TCTCTAGACCAAATTTTGCAGGTTTC-3′, which contained an *Xba*I site (underlined). The PCR product was cloned into *Eco*RI and *Xba*I sites of the pPICZB vector (Invitrogen) to obtain *NlHT1* in frame with myc-epitope and 6 × His-tag at the C-terminus. *NlHT1* sequence was verified by DNA sequencing on both strands. Vectors pPICZB-*NlHT1* and pPICZB were linerarised by *Sac*I digestion at 37 °C for 3 h and purified by phenol chloroform extraction, and concentrated by ethanol precipitation. 10 μg of linearised DNA was resuspended in 10 μl sterile water and used to transform chemically competent *P. pastoris* (strain X-33) using EasyComp transformation kit (Invitrogen). *P. pastoris* transformants were selected on YPG-zeocin plates (1% yeast extract, 2% peptone, 1% glycerol, 2% agar, 100 μg μl^{-1} zeocin) and individual colonies were screened for mut^{+} phenotype and expression levels (evaluated in small-scale cultures).

2.8. Protein expression in P. pastoris

A single colony identified as a 'high-level expresser' was used to inoculate 50 ml BMG minimal medium (100 mM potassium phosphate, pH 6.0, 1.34% (w/v) yeast nitrogen base, 4×10^{-5}% (w/v) biotin and 1% (v/v) glycerol) in a 250 ml baffled shake flask. Cells were grown at 30 °C with shaking (280 rpm) until mid-log phase (OD λ_{600} nm = 2–6, approximately 18 h). Cells were collected by centrifugation (3000 × *g*, 5 min at room temperature) and diluted to λ_{600} nm = 1.0 with BMM (BMG medium in which glycerol was replaced with 1% methanol) which induced expression of recombinant NlHT1. Cells were maintained in standard culture conditions, and 100% methanol was added every 12 h to a final concentration of 1% (v/v). Cells were cultured for 72 h post-methanol induction and then

harvested by centrifugation ($3000 \times g$, 5 min at room temperature).

2.9. Membrane transport in whole cells

Methanol induced cells transformed with pPICZB-*NlHT1* (NlHT1$^+$ cells) and pPICZB (NlHT1$^-$ cells) were harvested by centrifugation ($3000 \times g$, 5 min), washed twice in ice-cold water and resuspended in 50 mM potassium phosphate buffer (pH 7.0) to give 10 OD_{600} ml^{-1}. Uptake experiments were initiated by addition of 1.1 µl of labelled glucose solution (100 mM glucose, 0.1 µCi D-[1-^{3}H]-glucose, [20 Ci mmol^{-1}; ICN]) to 100 µl *Pichia* cells. At different time intervals transport was stopped by rapid-filtration of a 100 µl aliquot through a pre-wetted 0.45 µm nitrocellulose membrane (Sartorius) under vacuum. Filters were washed with 3 ml of water and counted in 5 ml scintillation fluid (Ecoscint A, National Diagnostics).

2.10. Membrane preparation for transport studies

Membrane vesicles were prepared from *Pichia* transformed with pPICZB-*NlHT1* (NlHT1$^+$ vesicles) and *Pichia* transformed with pPICZB vector (NlHT1$^-$ vesicles). BMM induced cells were washed in ice-cold breaking buffer (50 mM sodium phosphate, pH 7.4, 5% glycerol) and resuspended in 0.1 volumes of breaking buffer supplemented with 1 mM EDTA and 1 mM PMSF. An equal volume of acid washed glass beads (0.5 mm diameter) was added to the suspension and cells were disrupted by vortexing five times for 1 min with cooling on ice. Unbroken cells were removed by centrifugation ($8000 \times g$ at 4 °C for 10 min) and membranes were collected from the clarified supernatant by high-speed centrifugation ($100{,}000 \times g$ at 4 °C for 30 min). Membranes were resuspended at 20 µg µl^{-1} membrane protein in 20 mM HEPES (pH 7.4), 100 mM D-mannitol, and membrane vesicles were prepared by passing the suspension 20 times through a 25-gauge needle. Aliquots of both membrane vesicles were flash frozen, stored at −80 °C, and used within 1 week of preparation.

2.11. Transport assay of NlHT1 in membrane vesicles

Uptake experiments were initiated by addition of 2 µl membrane vesicles (40 µg membrane protein) to 18 µl transport solution (20 mM HEPES, pH 7.4, 99 mM D-mannitol, 1 mM D-glucose, 0.2 µCi D-[1-^{3}H]-glucose, [20 Ci mmol^{-1}; ICN]). At different time intervals transport was stopped by rapid-filtration of a 20 µl aliquot through a pre-wet 0.45 µm nitrocellulose membrane (Sartorius) under vacuum. Filters were washed with 3 ml of ice-cold stop solution (20 mM HEPES, pH7.4, 100 mM mannitol, 10 µM cytochalasin B) air dried and counted in 5 ml scintillation fluid (Ecoscint A, National Diagnostics). Transport activity in the presence of an inwardly directed NaCl gradient was performed by including NaCl to a final concentration of 18 mM in the transport assay. For inhibition studies, cytochalasin B was included at a final concentration of 9 µM in the transport assay. Sugar preference was determined by adding 5 mM unlabelled sugars to a standard transport solution, and D-mannitol was decreased to 94 mM to maintain an osmotic balance on either side of the vesicle membrane. Kinetic parameters K_m and V_{max} were estimated by using a Michaelis–Menten model (PRISM version X (GraphPad, San Diego). Glucose uptake into membrane vesicles was measured after 60 s at glucose concentrations 1–5 mM, transport of D-[1-^{3}H]-glucose was corrected for dilution of specific activity by unlabelled glucose. All experiments were performed in triplicate and uptakes are displayed as mean values (+/−) standard error. Uptake experiments were performed at room temperature and background uptake into NlHT1$^-$ membrane vesicles was subtracted from transport into NlHT1$^+$ vesicles.

3. Results

3.1. Sequence analysis of NlHT1

A sequence encoding part of a facilitative sugar transporter cDNA (*N. lugens* hexose transporter 1; NlHT1) was identified in an *N. lugens* gut cDNA library by a random screening approach. The sequence was verified and completed by RT–PCR and RACE, with multiple PCR products being sequenced to eliminate PCR-based errors. The complete cDNA sequence has been deposited in GenBank (Accession number: EF028238). *NlHT1* is 1786 bp long and contains a 123 bp 5′ untranslated region (UTR), a 1461 bp open reading frame, and a 202 bp 3′ UTR which contains a putative polyadenylation consensus sequence (AATAAA), 154 and 17 bp from the beginning of the poly(A) tail. The protein encoded by the longest open reading frame is 486 amino acids with a predicted M_r of 53,066 and has an isoelectric point of 7.5. When compared by BlastP software to the global protein database, excluding predicted polypeptides, the NlHT1 polypeptide is most similar to the CG10960-PB gene product from *Drosophila melanogaster* (42% identity, 63% similarity). A phylogenetic comparison using ClustalX software shows that NlHT1 also shares significant similarity (18–34%) to representative facilitative hexose transporters from both prokaryotes and eukaryotes, including the human erythrocyte facilitative transporter, GLUT1 (21.6% similarity) (Fig. 1C). NlHT1 shares a number of features characteristic of members of facilitative sugar transporter superfamily (Interpro, IPR003663), including 12 predicted transmembrane α-helicies and characteristic sequence motifs. In particular, loop 2 and loop 8 contain a GRR/K motif in positions characteristic of this superfamily of transporters. Regions TM5 and TM7 contain highly conserved glutamine residues that participate in exofacial ligand binding (Mueckler et al., 1994). Region

A

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                                                                                ACGCGGGGAGTACAGTGTTCTGAAGCTGAAGGA
AAGAAGGGATTGAAATCTATTAATTAAATAATTCATCTAGATTCGTTTTTGTGTATGCTAAATTGTTGCTGAGGCAAGTTGCAAGCAATA

ATGAGCACCAAGGCAACGACTGTCAGTGCTCAGACCCTGGTGTCGACTGCCGTCCCGGCGGCCAAACTACCGCAGTATGTGGCTGCTCTC +90
 M  S  T  K  A  T  T  V  S  A  Q  T  L  V  S  T  A  V  P  A  A  K  L  P  Q  Y  V  A  A  L   30
                                                                                TM1

ATTGCCACGATCGGAGGCTTCTGCCTTGGCACAGTGCTGGGATGGACGTCTCCGGTGCTGACGTCACTGTCCGACTACTACGGCTTCGAA +180
 I  A  T  I  G  G  F  C  L  G  T  V  L  G  W  T  S  P  V  L  T  S  L  S  D  Y  Y  G  F  E   60

GTGAACGTGGACTCGCAGGCGTGGATCGGATCCATCATGGCGATTGGCGCCATGGTCGGCGGTCTGCCTATGAGTTGGATGCTGGATACG +270
 V  N  V  D  S  Q  A  W  I  G  S  I  M  A  I  G  A  M  V  G  G  L  P  M  S  W  M  L  D  T   90
                TM2                                                             (R1)

TTCGGCCGCAAGTCCACCATCATCATCCTCACCGTGCCCACCGTCGCCGCTTGGATGATGATCATTTTTGCACCCAGTGTGACAGTAATT +360
 F  G  R  K  S  T  I  I  I  L  T  V  P  T  V  A  A  W  M  M  I  I  F  A  P  S  V  T  V  I  120
             TM3

TGCATAGCCAGGTTCATACTTGGTTTCACCACCGGAGCATATGCAGTTGCCGTGCCATTATACACCAGCGAAATAAGTGAGAACGAGATC +450
 C  I  A  R  F  I  L  G  F  T  T  G  A  Y  A  V  A  V  P  L  Y  T  S  E  I  S  E  N  E  I  150
 TM4

CGAGGAACGTTAGGAACATATTTTCAACTACAACTTACAATTGGAATAACATCAGCTTACATTCTGGGAAGTCTGCTACCCATCTTCTGG +540
 R  G  T  L  G  T  Y  F  Q  L  Q  L  T  I  G  I  T  S  A  Y  I  L  G  S  L  L  P  I  F  W  180
             TM5                (R2)                                             TM6

ATGACAATGGTGTGTGGATGCATACCGGTTGTGTTGGCCCTGGCTATGTTGATCATCCCCGAGACTCCGACCTACTACTTAAAAAAATTC +630
 M  T  M  V  C  G  C  I  P  V  V  L  A  L  A  M  L  I  I  P  E  T  P  T  Y  Y  L  K  K  F  210

AGGGTTGATGAGGCAAGGAAAGCTTTGCAGTGGTTCCGTGGCAGCCATTATGATGTTGAACCCGAGCTTATGCTTTTGAAAGCAAATTTG +720
 R  V  D  E  A  R  K  A  L  Q  W  F  R  G  S  H  Y  D  V  E  P  E  L  M  L  L  K  A  N  L  240

GATCAGATGGAAGCAGAGCGTGTACCTTTCACCCAAGCGTTTGTGACAACACCAGCTAAACGCGGTCTAGTGGTTGGACTCGGTGTCATG +810
 D  Q  M  E  A  E  R  V  P  F  T  Q  A  F  V  T  T  P  A  K  R  G  L  V  V  G  L  G  V  M  270
                                                                   TM7

TTCTTCCAACAAGTTCAAGTGGAGTCAATGCTGTCATATTCTACGCCCGAAAGTATTTTTAAGGCTGCTGGCAGTTCGATGAGTCCAAGT +900
 F  F  Q  Q  V  Q  V  E  S  M  L  S  Y  S  T  P  E  S  I  F  K  A  A  G  S  S  M  S  P  S  300
 (R3)

CTGCAAACAATTATAGTTGGTCTGATTATGGTTGTGATGACATGGGTTGCTACGCTGGCGATCGACCGTGCCGGCAGAAGACCACTGCTT +990
 L  Q  T  I  I  V  G  L  I  M  V  V  M  T  W  V  A  T  L  A  I  D  R  A  G  R  R  P  L  L  330
 TM8                                                                (R4)          TM9

CTAATCTCCGCATCAATCATGGCTATTTGCACTGCGATTCTCGGTGTTTACTTTTTATTACTTGAGAAAACTCCAGATTTCGCAAAAACG +1080
 L  I  S  A  S  I  M  A  I  C  T  A  I  L  G  V  Y  F  L  L  L  E  K  T  P  D  F  A  K  T  360

ATCGGGTCGGTTCCGATTGTCAGTTTGTCAATTTTCATTATTGTATTCTCGTTGGGATTTGGACCAATTCCGTGGATGTTCATGTCAGAG +1170
 I  G  S  V  P  I  V  S  L  S  I  F  I  I  V  F  S  L  G  F  G  P  I  P  W  M  F  M  S  E  390
             TM10                                                  (R5)

ATATTCCCTCCACAGATCAAGGGACCAGCATGCTCGATAGCCTGCTTTTTCAACTGGTTCTCTGTATTCATGGTTACAAAATTCTTCGGT +1260
 I  F  P  P  Q  I  K  G  P  A  C  S  I  A  C  F  F  N  W  F  S  V  F  M  V  T  K  F  F  G  420
                            TM11                    (R6)

GATTTGCAATCGAAGTTTGGATCGTATGGAACTTTCTGGATTTTCTCTGGAATTTCCATAGCTGGAACATTCTTTGTTCTGAATCTGGTA +1350
 D  L  Q  S  K  F  G  S  Y  G  T  F  W  I  F  S  G  I  S  I  A  G  T  F  F  V  L  N  L  V  450
                         TM12

CCTGAGACCAAGGGAAAGTCTATGGAGGAAATCCAAAAGGAGCTAGGAGCAACCCCTCAAATGACACCTGAGGATAGAATGGAGAATGGA +1440
 P  E  T  K  G  K  S  M  E  E  I  Q  K  E  L  G  A  T  P  Q  M  T  P  E  D  R  M  E  N  G  480

CAGAAACCTGCAAAATTTTGA                                                           +1461
 Q  K  P  A  K  F  *                                                             486

GCCAGAACTTTAAAATAAATGTTCCTGTGTGATACTGTGTGATTCTATCAATACTGTATTGATAGCCTAGTGATAGATGAATAATTTGTA +1551
CCATATTTTTAAATTATCACATAAGATGAAGAGAATAGTGTATAGACTTCCCTGTGTACAAATAAAGTATTAATATCGTAAGTAAAAAAA +1641
AAAAAAAAAAAAAAAAAAAAA                                                           +1663
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B

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          R1      R2         R3         R4      R5      R6
NlHT1   DTFGRK  LQLTIG  MFFQQVQVESM  RAGRR  FGPIPWM  NWF
DmHT1   NMIGRK  FQLMIT  MFFQQVCGINA  KLGRR  YGPVPWL  NWL
AaHT1   KMIGRK  FQLLVT  MFFQQLSGINA  KAGRR  FGPIPWL  NWL
PfHT1     GRR   HQLFIT  SGLQQFTGINV  KLGRK  YGPVLWI  NWV
araE     RLGRK  YQLMVT  QAMQQFTGMNI  KAGRK  AAPVVWI  NWV
GLUT1    RFGRR  HQLGIV  QLSQQLSGINA  RAGRR  PGPIPWF  NWT
```

C

	NlHT1	DmHT1	AaHT1	PfHT1	araE
GLUT1	21.6	24.0	24.2	22.6	21.2
araE	22.0	25.7	25.6	20.1	
PfTH1	18.9	19.3	16.5		
AaHT1	38.1	56.3			
DmHT1	40.3				

Fig. 1. NlHT1 nucleotide and encoded protein sequence and analysis of related hexose transporters. (A) *NlHT1* nucleotide and encoded protein sequence (GenBank accession number EF028238; encoded protein ABM01870). Transmembrane regions, as predicted by TMHMM are underlined (TM 1–12). Residues conserved amongst members of the facilitative sugar transporter superfamily are shaded grey and numbered (R1–R6). (B) Alignment of conserved residues R1–R6 from *NlHT1* and sequences from the facilitative sugar transporter superfamily from phylogenetically distant organisms: DmHT1, *Drosophila melanogaster* (NP648605; product of gene CG10960); AaHT1, *Aedes aegypti* sugar transporter (EAT44903); PfHT1, *Plasmodium falciparum* hexose transporter 1 (CAA10374); AraE, *Escherichia coli* arabinose transporter (P0AE24); GLUT1, human glucose transporter 1 (AAA52571). (C) Overall sequence similarity by ClustalX analysis of hexose transporters shown in the alignment in panel (B).

TM10 contains a proline residue that is required for conformational flexibility (Tamori et al., 1994), and a tryptophan residue that is involved in binding to cytochalasin B (Garcia et al., 1992). Region TM11 contains a tryptophan residue essential for transport activity (Garcia et al., 1992).

3.2. Localisation of NlHT1 by in situ hybridisation and RT–PCR

The distribution of *NlHT1* transcript in dissected gut tissues from *N. lugens* reared on plants was visualised by *in situ* hybridisation of a DIG-labelled synthetic *NlHT1* RNA probe. The anti-sense probe hybridised strongly to midgut regions distal to the anterior end of the gut (Fig. 2A), but staining was absent in hindgut and Malpighian tubules. Hybridisation of a sense *NlHT1*-probe to similar tissues was undetectable along the full length of the gut (Fig. 2B).

RT–PCR on RNA extracted from whole insects, and organs from insects, was carried out to confirm the localisation determined by *in situ* hybridisation. RNA encoding *NlHT1* was readily detectable in gut tissue and whole insects, and was undetectable in head tissues and whole insects with gut tissue removed (Fig. 2C).

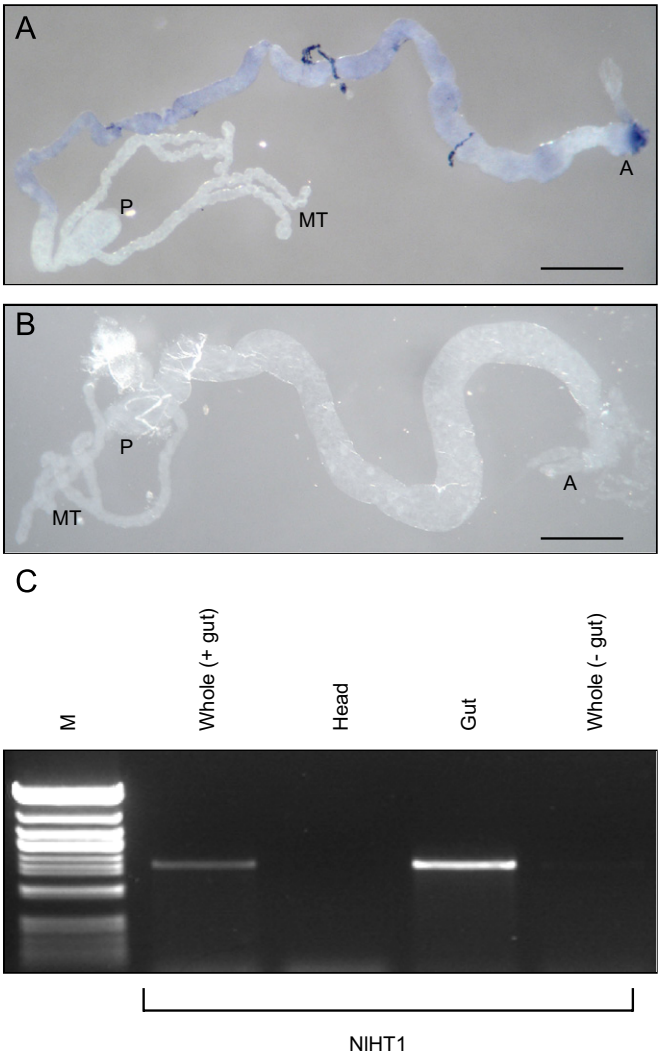

Fig. 2. Localisation of *NlHT1* mRNA in *N. lugens* gut tissue. Detection of *NlHT1* transcript by *in situ* hybridisation in dissected *N. lugens* gut using (A) antisense and (B) sense DIG-labelled *NlHT1* RNA probe. Each panel shows a complete gut, including foregut and hindgut regions. Colorimetric detection was used to detect hybridised probe. A, anterior end of gut; P, posterior end of gut; MT, Malpighian tubules (bar 100 μm). (C) Detection of *NlHT1* transcript by RT–PCR, using RNA extracted from whole insect and tissues as indicated.

3.3. Overexpression of NlHT1 in P. pastoris

To establish and characterise transport properties of NlHT1, we introduced an optimised yeast initiation sequence at the start of the *NlHT1* cDNA and cloned the sequence into a methanol-inducible yeast expression vector. The construct was transformed into *P. pastoris* (strain X-33). Transformants were grown in shake flasks in a glycerol minimal medium to generate cell biomass and repress recombinant NlHT1 expression (Fig. 3, lane 1). Replacing glycerol in the growth medium with methanol induced expression of recombinant NlHT1 (rNlHT1). Under these conditions, rNlHT1 accumulated and was detectable with anti-myc antibody in western blot as a 40 kDa protein on SDS–PAGE gels (Fig. 3, lane 2). Membrane fractions generated by mechanical disruption of yeast cells followed by high-speed centrifugation contained rNlHT1 (Fig. 3, lane 3).

3.4. Functional characterisation of NlHT1

Initial uptake experiments with intact *Pichia* cells demonstrated time dependent transport of glucose into both wild type-*Pichia* (NlHT1$^-$ cells) and *Pichia* transformed

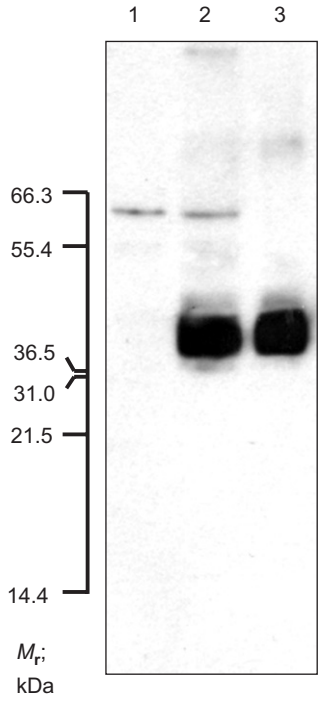

Fig. 3. Western blot of recombinant NlHT1 in *Pichia pastoris*, using anti-myc antibody. Lane 1, uninduced *Pichia* total cell protein; lane 2, induced *Pichia* total cell protein, 48 h post-induction; lane 3, induced *Pichia* membrane protein, 48 h post induction. All loadings contain equivalent amounts of protein.

with NlHT1 (NlHT1$^+$ cells) (Fig. 4A). However, the kinetics of glucose uptake into NlHT1$^-$ cells was greater than NlHT1$^+$ cells, and after 5 min of transport NlHT1$^-$ cells had transported approximately 1.7 times more glucose than NlHT1$^+$ cells. Due to the large background uptake in NlHT1$^-$ cells subsequent experiments were performed in membrane vesicles prepared from both NlHT1$^+$ and NlHT1$^-$ cells. Membrane vesicles prepared from recombinant *P. pastoris* overexpressing rNlHT1 (NlHT1$^+$ vesicles) demonstrated time dependent transport of glucose (Fig. 4B) and background activity in control vesicles prepared from *P. pastoris* transformed with empty vector (NlHT1$^-$ vesicles) was low (Fig. 4B).

Competition experiments carried out under conditions with osmotic balance inside and outside vesicles showed that radiolabelled glucose uptake was inhibited by unlabelled glucose (i.e., glucose was not accumulated above equilibrium values; data not shown). In contrast, glucose uptake was not inhibited in the presence of excess fructose, showing that the NlHT1 transporter is specific for glucose (Fig. 5B). Glucose transport was not dependent on, or stimulated by, the presence of sodium ions (Fig. 5C) and transport was inhibited by the presence of transport inhibitor cytochalasin B (Fig. 5D). For kinetic analysis, transport was stopped after 60 s, where uptake was still linear and values were above background levels. Transport assays were carried out at varying concentrations of glucose, and the resulting assay data were used to estimate the K_m for ligand binding. A Lineweaver–Burk plot gave a value 3.0 mM for K_m (Fig 6).

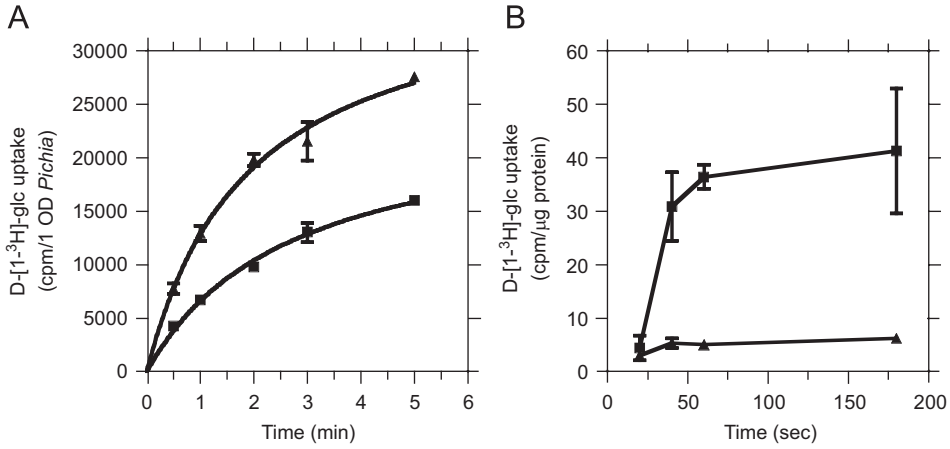

Fig. 4. Uptake of 0.9 mM D-glucose labelled with trace amounts of D-[1-^{3}H]-glucose (0.2 µCi per assay) into: (A) 1 OD unit *Pichia pastoris* cells transformed with empty vector (NlHT1$^-$ cells, triangles) and 1 OD unit *Pichia pastoris* cells transformed with NlHT1 (NlHT1$^+$ cells; squares); (B) membrane vesicles prepared from NlHT1 cells (40 µg membrane protein; triangles) and membrane vesicles prepared from NlHT1$^+$ cells (40 µg membrane protein; squares). Uptake in membrane vesicles shows low background in the control, whereas uptake in whole cells shows a high background in the control, with lower uptake in NlHT1$^+$ cells.

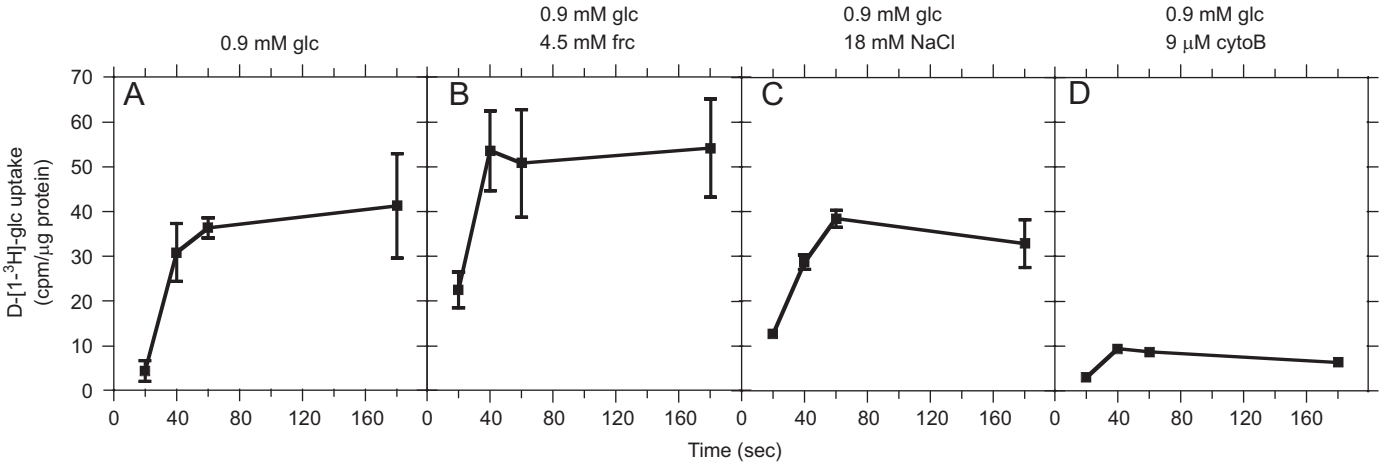

Fig. 5. Uptake of 0.9 mM D-glucose labelled with trace amounts of D-[1-^{3}H]-glucose (0.2 µCi per assay) into membrane vesicles prepared from NlHT1$^+$ cells (see Fig. 4) in the presence of (A) 0.9 mM D-glucose; (B) 0.9 mM D-glucose, 4.5 mM β-D-fructose; (C) 0.9 mM D-glucose, 18 mM NaCl; (D) 0.9 mM D-glucose, 9 µM cytochalasin B.

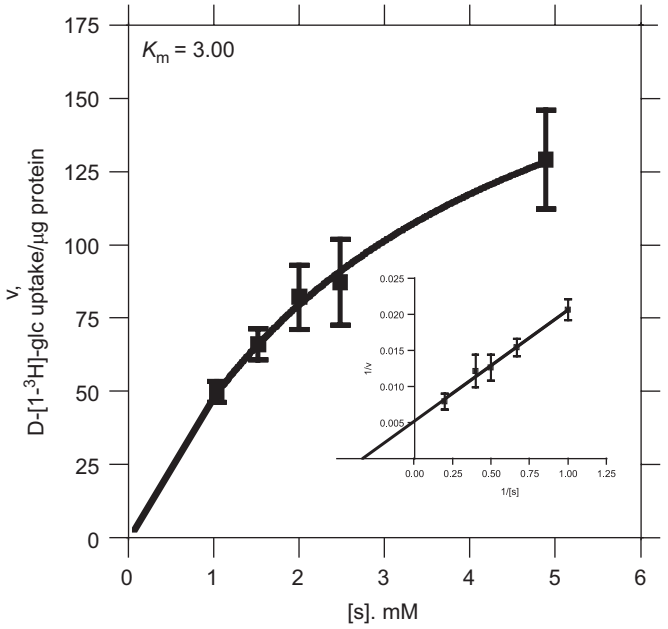

Fig. 6. Transport kinetics of D-glucose uptake into membrane vesicles prepared from NlHT1$^+$ cells (see Fig. 4). Michaelis–Menten and Lineweaver–Burk plots showing transport of D-[1-^{3}H]-glucose (0.2 µCi per assay) in the presence of increasing concentrations of unlabelled D-glucose. Counts min^{-1} D-[1-^{3}H]-glucose transported were corrected for dilution of the specific activity caused by the addition of unlabelled glucose.

4. Discussion

We have identified and functionally expressed a glucose-specific transporter, NlHT1, which is present in gut tissues of the rice brown planthopper, *N. lugens*. NlHT1 was overexpressed using the methylotropic yeast *P. pastoris*, which has been previously used for characterisation of other membrane transporters, including, ATP binding cassette (ABC) transporter (Cai and Gros, 2003), mammalian intestinal peptide transporter (Doring et al., 1997) and human sodium–glucose cotransporter (Tyagi et al., 2005). In addition, a facilitative glucose transporter from rat, GLUT1, has been expressed in yeast *Saccharomyces cerevisiae*, and membrane preparations showed glucose transport (Kasahara and Kasahara, 1996).

Glucose uptake assays in *Pichia* cells transformed with NlHT1 suggested that the transporter was present in a functional form in the cell membrane, as these cells showed a reduction (nearly twofold) in glucose uptake compared to untransformed cells. Yeast (*S. cerevisiae*) contains both facilitative and proton gradient-driven glucose transporters, the former being used at high glucose concentrations in the medium, the latter at low concentrations of extracellular glucose (Boles and Hollenberg, 1997). Similar genes are present in *Pichia* spp. (Weierstall et al., 1999). Since the *Pichia* cells had been grown in minimal medium, with low glucose concentration, the active transport system for glucose would be present to accumulate the sugar against its concentration gradient. The presence of a facilitative glucose transporter in the induced NlHT1$^+$ cells would thus have caused leakage of glucose from the cells, leading to reduced uptake in transport assays, as observed. The NlHT1$^+$ cells also grew less well than untransformed cells after induction, consistent with glucose leakage from cells into the medium.

In order to be able to use *P. pastoris* as a system for functional characterisation of NlHT1, membrane vesicles prepared from the cells had to be used as an assay system. The active glucose uptake system was not functional in these vesicles, and endogenous facilitative transporters were not present in significant amount, as transport of glucose into *Pichia* membrane vesicles prepared from cells transformed with empty vector (NlHT1$^-$ vesicles) was low. The low levels of background uptake from endogenous facilitative transporters is likely to be a consequence of culturing *Pichia* cells in glucose-free media, and supplying either glycerol or methanol as a sole carbon source. The small size of *N. lugens* would make generation of enough insect material for transport characterisation in gut membranes very difficult (and would only measure net transport as a result of multiple transporters being present), but the use of *Pichia* as a recombinant expression system allowed the study of the properties of a single transporter species to be readily undertaken. To our knowledge, this is the first example of expression and functional characterisation of an insect facilitative glucose transporter. Although the data presented show that *Pichia* membrane vesicles can be used as a model system for the characterisation of insect facilitative transporters, this system could be improved by using mutant yeast strains deficient in endogenous transporters. The use of mutants would allow assays to be carried out with intact cells rather than membrane vesicles, which would be technically more straightforward, and more sensitive.

Expression of NlHT1 in *P. pastoris* was under the control of alcohol oxidase promoter *AOX1*, and upon induction with methanol recombinant protein is detected with anti-myc antibody. rNlHT1 migrates as a 40 kDa band on SDS–PAGE, which compares to 55.6 kDa of the predicted recombinant protein. Anomalous migration of membrane transport proteins by SDS–PAGE is common phenomenon, and is due to large hydrophobic membrane spanning regions (Banker and Cotman, 1972). The recombinant protein is present in a membrane fraction prepared from transformed yeast cells, consistent with correct insertion into cell membranes, and preparations of membrane vesicles from *P. pastoris* transformed with NlHT1 (NlHT1$^+$ vesicles) were functionally active in glucose transport. The primary sequence of NlHT1 predicts it to be a facilitative glucose transporter (on the basis of sequence similarity to other facilitative transporters) and the transport data obtained were consistent with this role.

RT–PCR showed that expression of *NlHT1* is gut-specific, and the *in situ* hybridisation showed that *NlHT1* transcript was detectable throughout the midgut, where hexose transport would be expected to occur, but was absent from the posterior part of the gut, including the rectum and Malpighian tubules. The posterior region of the gut is thought to be relatively impermeable, and the gut contents in this region will contain hexose oligomers to be voided as honeydew, which are therefore unsuitable for transport. The Malpighian tubules are primarily an excretory rather than an absorptive organ, and thus sugar transport from gut to haemolymph might not be expected in this region. Although the RT–PCR results suggested that NlHT1 is an abundant transcript in gut tissue, it is unlikely to be the sole hexose transporter. Subsequent mass sequencing of a cDNA library from whole adult insects generated partial sequences for other facilitative hexose transporters, although the NlHT1 sequence was not represented in this screen of 7680 clones. The putative hexose transporters showed significant sequence differences when compared with NlHT1. The sequence data obtained suggested that these other putative transporters may show specificities towards other substrates, since the residues present in TM7, thought to be responsible for hexose transport selection, differ from those in NlHT1.

Hexose transporters similar to NlHT1 have been identified in other insect species at the level of nucleotide sequence, and annotated as facilitative hexose transporters by similarity to functionally characterised mammalian transporters. Similar transporters have been identified through genome sequencing projects in *D. melanogaster, Bombyx mori, Anopheles gambiae*, and *A. pisum* but these transporters remain uncharacterised. Analysis of the *Drosophilsa* genome sequence reveals 25 putative facilitative sugar transporters. Comparison of these sequences with each other and NlHT1 using ClustalX shows that NlHT1 is phylogenetically closest to the product of CG1208 (this sequence comparison is based on a different algorithm to the Blast software, which compares sequence similarity in a pairwise fashion, and suggests that the product of CG10960 is most similar to NlHT1). In *Drosophila*, CG1208 is expressed in gut tissues, and expression of this gene is up-regulated in response to carbon starvation, and down-regulated in response an excess of carbon (Zinke et al., 2002), as would be expected for a sugar transporter, although CG1208 has not yet been functionally characterised. The human erythrocyte facilitative glucose transporter (GLUT1) is the closest relation to NlHT1 with functional characterisation. Indeed, GLUT1 is a glucose-specific transporter and has an estimated K_m of 1.2 mM for glucose (Wheeler and Hinkle, 1981), similar to that of the K_m estimation of NlHT1. The rat GLUT1 transporter expressed in *S. cerevisiae* had an estimated K_m of 3.4 mM (Kasahara and Kasahara, 1996), which again is similar to the value for NlHT1.

The estimation of K_m for NlHT1 in the present paper is subject to error in that the concentration of glucose in the medium could not be increased to a level sufficient to nearly saturate the transporter. A high value for K_m (glucose) in NlHT1 is not in conflict with its proposed role as a major hexose transporter. Although rice phloem sap does not contain glucose, it contains high concentrations of sucrose (0.1–0.7 M; Fukumorita and Chino, 1982), and the gut in rice brown planthopper contains high levels of α-glucosidase activity (unpublished data), probably due to an enzyme similar to the membrane-bound α-glucosidase identified in the pea aphid *A. pisum* (Price et al., 2007). As a result of sucrose hydrolysis in the gut of *N. lugens*, glucose is likely to be present at concentrations well above that of the predicted K_m of NlHT1.

The specificity of NlHT1 for glucose, and its failure to transport fructose at comparable rates, was unexpected, although mammalian GLUT1 is also glucose-specific and does not transport fructose. In the pea aphid, fructose is preferentially transported over glucose, and glucose residues are polymerised into oligosaccharides and voided as honeydew (Fisher et al., 1984; Walters and Mullin, 1988; Rhodes et al., 1997; Wilkinson et al., 1997; Ashford et al., 2000). However, although high osmotic pressure of ingested diet is a common problem amongst phloem-sap feeding Hemipterans, multiple strategies for rendering the ingest isoosmotic have evolved. The main haemolymph sugar in insects from most orders is trehalose, a disaccharide formed from two glucose units linked with a 1-1α-glycosidic bond (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside). Phloem feeders like aphids and whitefly accumulate trehalose at high concentrations in the haemolymph, as an adaptive mechanism to osmotic problems created by ingestion of an osmotically challenging diet (Moriwaki et al., 2003a). In contrast, trehalose is found in *N. lugens* haemolymph at comparable concentrations to other insect species, and at relatively low concentration compared to aphids and whitefly. The predominant haemolymph sugar in *N. lugens* is myo-inositol (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol), which is produced from glucose and is present at concentrations three to five times that of trehalose (Moriwaki et al., 2003b). Adaptation to water stress by accumulation of sugars, in particular sugar alcohols (polyols) such as glycerol, sorbitol, myo-inositol and mannitol is an adaptive mechanism to osmotic shock used by many organisms (Yancey et al., 1982). It can be speculated that glucose transport across the gut wall in *N. lugens* is important for both carbon nutrition and osmoregulation by the conversion of transported glucose into myo-inositol and accumulation of this polyol.

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