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Sugar transporter genes of the brown planthopper, *Nilaparvata lugens*: A facilitated glucose/fructose transporter

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ABSTRACT

The brown planthopper (BPH), *Nilaparvata lugens*, attacks rice plants and feeds on their phloem sap, which contains large amounts of sugars. The main sugar component of phloem sap is sucrose, a disaccharide composed of glucose and fructose. Sugars appear to be incorporated into the planthopper body by sugar transporters in the midgut. A total of 93 expressed sequence tags (ESTs) for putative sugar transporters were obtained from a BPH EST database, and 18 putative sugar transporter genes (*Nlst1–18*) were identified. The most abundantly expressed of these genes was *Nlst1*. This gene has previously been identified in the BPH as the glucose transporter gene *NlHT1*, which belongs to the major facilitator superfamily. *Nlst1*, *4*, *6*, *9*, *12*, *16*, and *18* were highly expressed in the midgut, and *Nlst2*, *7*, *8*, *10*, *15*, *17*, and *18* were highly expressed during the embryonic stages. Functional analyses were performed using *Xenopus* oocytes expressing NlST1 or 6. This showed that NlST6 is a facilitative glucose/fructose transporter that mediates sugar uptake from rice phloem sap in the BPH midgut in a manner similar to NlST1.

1. Introduction

The brown planthopper (BPH) is the most serious insect pest of rice plants. It is a vascular feeder and transmits rice plant viruses, as well as causes severe damage when feeding (Hibino, 1996; Sogawa, 1982). The phloem sap in the vascular bundles of rice contains a considerable concentration of sugars, which are synthesized in the leaves and transferred to other tissues. These phloem sap sugars are a major energy source for the BPH (Sogawa, 1982). Elucidation of the mechanism of sugar uptake into the gut and hemolymph is important in order to understand energy acquisition in plant sap-feeding insects and to identify new targets for the control of these pests.

Sugar transporters play an essential role in controlling carbohydrate transport in a diverse array of organisms, from bacteria to mammals, and are responsible for mediating the movement of sugars into cells (Mueckler, 1994). Two categories of sugar transporters are currently known; the facilitated sugar transporter and the Na^+/sugar symporter or H^+/sugar symporter (Wood and Trayhurn, 2003). The proteins of the facilitated sugar transporter family move sugars along gradients from regions of high concentration to those of lower concentration. The Na^+/sugar symporter or H^+/sugar symporter acts as a secondary active membrane transporter that moves sugars via electrochemical membrane gradients of Na^+ or H^+ ions, respectively. In the mammalian intestine, sugars are transported not only by facilitated transports but also by sodium-dependent glucose transporter in a process driven by an electrochemical membrane potential (Wright, 1993). Epithelial cells in the human small intestine take up sugars through the intestinal brush-border membrane via the sodium-dependent glucose transporter (SGLT1) and passively export the sugars through the basal membrane via facilitated sugar transporter 2 (GLUT2) (Drozdowski and Thomson, 2006; Wright et al., 2007). If these sugar transporters are deficient, the result is a disease involving glucose-galactose malabsorption.

Sugar transporters may offer a new potential target for the control of insect pests. However, relatively little information is currently available on insect sugar transporters (Burchmore et al., 2003; Chen et al., 2006; Caccia et al., 2007; Kikawada et al., 2007; Price et al., 2007b, 2010; Kanamori et al., 2010). The sugar transporter *NlHT1*, which was previously identified in the BPH, is a facilitative glucose transporter and is specifically expressed in the midgut (Price et al., 2007b). Other sugar transporters are also expected to be present and to function in the gut of the BPH for the

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uptake of sugars from the phloem sap of rice plants. The principal carbohydrate in the phloem sap of rice plants is sucrose, a disaccharide composed of glucose and fructose (Kawabe et al., 1980; Fukumorita and Chino, 1982; Hayashi and Chino, 1990). In general, disaccharides are difficult to transport across the cellular membranes of the hemipteran gut wall (Rhodes et al., 1997). To avoid this difficulty, sucrose is hydrolyzed by sucrase in the hemipteran midgut into its two component monosaccharides (Price et al., 2007a). The glucose transporter NlHT1 mediates the uptake of glucose, but not fructose, in the midgut (Price et al., 2007b). Because fructose is routinely used as an energy source, another transporter for fructose uptake is believed to be present in the midgut of the BPH. Recently, the facilitated sugar transporter Ap_ST3 was identified in *Acyrthosiphon pisum*; Ap_ST3 has been found to transport both glucose and fructose (Price et al., 2010).

In the present study, we sought to identify candidate sugar transporter genes using sequence data to search the cDNA expressed sequence tag (EST) database described by Noda et al. (2008). Expression of BPH sugar transporter (*Nlst*) genes was examined in various tissues and developmental stages from embryo to adult. Functional analyses were performed on 2 *Nlst* genes, using the *Xenopus laevis* oocyte expression system.

2. Materials and methods

2.1. Insect

The rice brown planthopper (BPH), *Nilaparvata lugens* (strain: Izumo), was reared and maintained on rice seedlings at 26 °C with cycles of 16 h light/8 h dark. All experiments were performed at the same temperature and under the same light-dark conditions.

2.2. RNA extraction

Total RNA was isolated from female adults using an RNeasy Mini kit (Qiagen) and used for sequence analyses of BPH putative sugar transporter genes. Gene expression levels were examined in various tissues: head, thorax, abdomen, midgut, ovary, salivary glands, Malpighian tubules, and fat body. The tissues were dissected from newly emerged (0 day) females, and RNAs were extracted. Testes were dissected from newly emerged (0 day) males. Gene expression levels were analyzed at different developmental stages: 1st (N1), 2nd (N2), 3rd (N3), 4th (N4), and 5th (N5) instar nymphs, and young (0 day) adult females. Three embryonic stages (stages I, II, and III) were used for RNA extraction. The embryos were obtained from eggs oviposited into the rice plants at the 3- or 4-leaf stage. Embryonic developmental stages (Fig. S1) were determined using the criteria described by Nasu and Suenaga (1958). Briefly, the embryo stage I corresponds to the developmental period within 24 h of oviposition (blastoderm stage), the stage II corresponds to the developmental stage at about 96 h after oviposition (blastkinesis stage), and the stage III corresponds to the developmental stage at about 168 h after oviposition (anaphase of eye pigmentation stage).

2.3. cDNA cloning of sugar transporter genes

Preliminary nucleotide sequences of candidate BPH sugar transporter genes were obtained from ESTs. Full-length cDNA sequences of candidate sugar transporter genes were determined by rapid amplification of cDNA ends (RACE) using the SMART RACE kit for 3′-RACE (Clontech) and the 5′-RACE kit (Invitrogen). The primers used for the 3′- and 5′-RACE are listed in Supplemental Tables S1 and S2. The PCR products were cloned into the pGEM-T vector (Promega), and the sequencing templates were prepared by colony PCR. The sequence analysis was performed with an ABI Prism 3730 using BigDye Terminator (ABI Biosciences). Several clones were sequenced for each PCR product, and the sequence data were analyzed with GENETYX-MAC, ver.13 (GENETYX). Transmembrane regions were predicted from the cDNA sequences using the TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/) programs (Hirokawa et al., 1998; Sonnhammer et al., 1998). The putative BPH sugar transporter genes were named *Nlst1* to *Nlst18*, with the ranking based on the number of EST clones.

2.4. Quantitative RT-PCR

First-strand cDNA was synthesized using 100 ng of total RNA and the PrimeScript RT reagent kit (Takara) in accordance with the manufacturer's protocols. Real-time RT-PCR was performed using SYBR Green PCR Master Mix (Roche Applied Science) with a LightCycler 480 (Roche Applied Science). Ribosomal protein L4 gene (*RP-L4*) was used for normalization. The specific primers are listed in Supplemental Table S3. Total RNA samples for RT-PCR were independently prepared 3 times.

2.5. Phylogenetic tree

The phylogenetic relationships of the candidate *Nlst* genes were examined using the putative amino acid sequences. The amino acid sequences of insect sugar transporter family members from *Drosophila melanogaster*, *Bombyx mori*, *Anopheles gambiae*, *Apis mellifera* and *Acyrthosiphon pisum* were obtained from the National Center for Biotechnology Information (NCBI). Putative insect sugar transporter genes were predicted using a protein BLAST search in the NCBI database with a cutoff E value of 10^{-7} against insect genome sequences using the NlST1 sequence as the query. Genes that were predicted to possess fewer than 10 or more than 12 transmembrane helices in the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/) were discarded. Amino acid alignment was performed using ClustalW. An unrooted tree was constructed with the neighbor-joining method using MEGA, ver. 4 (Tamura et al., 2007) and 284 amino acid residues; the tree was evaluated with a bootstrap analysis of 1000 replications.

2.6. cRNA preparation

Expression vectors carrying BPH sugar transporter genes were constructed from PCR products amplified with specific primers containing restriction sites (Table S4). The PCR products of *Nlst1* and *Nlst6* were digested with the restriction enzymes *Eco*RV and *Eco*RI. They were cloned into pT7XbG2-AcGFP1 vectors (DDBJ accession number AB255038) digested with *Eco*RV and *Eco*RI. The template DNA for capped RNA (cRNA) preparation was amplified using high-fidelity DNA polymerase (KOD plus, Toyobo) and primers containing the T7 and T3 promoters. The *Nlst1* and *Nlst6* cRNAs were obtained using the mMESSAGE mMACHINE T7 kit (Ambion) according to the manufacturer's standard protocols.

2.7. Expression of NlST transporters in Xenopus oocyte membranes and evaluation of transport activity using HPLC and radiolabeled isotopes

The ability of selected *Nlst* genes to mediate sugar transport was screened using the *Xenopus* oocyte method as described previously (Kikawada et al., 2007). Briefly, cRNA was injected into *Xenopus laevis* oocytes, and the oocytes were incubated in Modified Barth's saline (MBS) buffer for 72 h at 20 °C. Oocytes displaying GFP-fluorescence in the cellular membrane were used for the sugar uptake

assays. The oocytes were incubated in 105 mM sugar solutions for 2 h at 20 °C, washed three times with sterilized MBS, and homogenized in 80% ethanol containing 1% sorbitol. For the HPLC analyses, the homogenate was centrifuged at 15,000 g for 20 min to remove debris, and the supernatant was dried thoroughly with centrifugation. The pellets were diluted with distilled water. Sugar incorporated into the oocytes was measured by HPLC (HPX-87C, BioRad). Oocytes injected with AcGFP1 cRNA were used as a negative control to evaluate endogenous sugar uptake. Experiments were repeated 3 times.

Sugar uptake assays were also performed using ^{14}C-labeled glucose and ^{14}C-labeled fructose (GE Healthcare). Oocytes expressing NlST were incubated with radiolabeled sugars and washed 3 times with MBS. The oocytes were homogenized in an 80% ethanol solution, and the fluids were centrifuged at 15,000 g for 20 min. The supernatant was added to a solid scintillation material (Ready Cap, Beckman Coulter) and, after drying thoroughly at 75 °C, radioactivity was measured using a scintillation counter (Quanta Smart, Perkin Elmer). Endogenous sugar transport activities in *Xenopus* oocytes were examined using cells expressing AcGFP1. For kinetic analysis of Michaelis-Menten components, sugar uptake assays were performed at a range of concentrations from 0 mM to 12 mM for 20 min at room temperature. K_m and V_{max} values were analyzed using PRISM 5 (GraphPad). To analyze the characteristics of the sugar transporters with regard to Na^+ and H^+ ions, a Na^+-free MBS buffer was prepared with choline chloride instead of NaCl, and MBS buffers of various pHs were prepared. A pH of 7.8 was normally used.

Statistical analyses were conducted, including a Student's t-test and one-way ANOVA followed by Tukey's multiple comparison tests (Prism ver. 5, GraphPad).

3. Results

3.1. Database search

The cDNA EST library described by Noda et al. (2008), which contains more than 37,000 ESTs, was searched for putative sugar transporter genes. A BLASTP analysis was performed using 18 *Drosophila* sugar transporter sequences as a query. This search yielded 89 clones with putative BPH sugar transporter gene (*Nlst*) sequences, using a cutoff *E* value of 10^{-3}.

3.2. Identification of BPH sugar transporter genes

In order to determine how many *Nlst* genes were present among the 89 EST clones, the cDNA sequences of the clones were extended by 3′- and 5′-RACE. Analysis of the full-length cDNAs indicated that the 89 EST sequences were derived from 18 sequences (excluding some EST clones that did not code sugar transporter genes). The 18 gene sequences (accession numbers AB549994–AB550011) were identified as belonging to the major facilitator superfamily on the basis of amino acid sequence similarities. The BPH EST library was again searched using the 18 full-length cDNA sequences. This search yielded 93 EST clones, all of which were derived from sugar transporter genes (Table 1).

The most highly expressed of the sugar transporter genes was *Nlst1*, to which 47 of the 93 EST clones belonged. *Nlst1* corresponds to the *NlHT1* gene of the BPH that was previously reported by Price et al. (2007b). However, the amino acid sequences were slightly different: *Nlst1* has 485 residues, whereas *NlHT1* has 486 residues. Overall, *Nlst1* showed 97.5% identity with *NlHT1*. In general, facilitated sugar transporters have 12 transmembrane (TM) regions (Joost and Thorens, 2001). We therefore used the TMHMM and SOSUI programs to predict possible transmembrane regions in the *Nlst* genes. Twelve transmembrane regions were predicted for *Nlst2*, *8*, *9*, and *13* by both programs; for *Nlst1*, *3*, *4*, *6*, *7*, *11*, and *12* by TMHMM; and for *Nlst15*, *16*, and *17* by SOSUI. Neither program identified 12 transmembrane structures in *Nlst5*, *10*, *14,* or *18*.

Table 1
BPH sugar transporter genes.

Gene	EST[a]	Clone name[b]	ORF[c]	TMHMM	SOSUI
Nlst1[d]	47	MB0516	486	12	11
Nlst2	9	HT3692	486	12	12
Nlst3	8	MB1507	466	12	10
Nlst4	5	MB3064	478	12	11
Nlst5	4	OC1141	487	9	11
Nlst6	3	MB1040	495	12	11
Nlst7	3	MB0106	507	12	11
Nlst8	2	SG4295	499	12	12
Nlst9	2	NA6316	566	12	12
Nlst10	2	EA7235	504	10	10
Nlst11	1	AD0895	475	12	11
Nlst12	1	AD3130	527	12	11
Nlst13	1	EA7530	544	12	12
Nlst14	1	MA0017	450	10	11
Nlst15	1	MA5086	530	11	12
Nlst16	1	MB3026	549	11	12
Nlst17	1	NB0289	494	11	12
Nlst18	1	OC0495	478	10	11

Transmembrane regions identified by TMHMM and SOSUI programs.
[a] The number of clones found in the BPH cDNA EST database.
[b] Representative clone names are shown.
[c] The number of amino acid residues in the open reading frame.
[d] *Nlst1* corresponds to *NlHT1* reported by Price et al. (2007b).

3.3. Expression profiles of BPH sugar transporter genes

The expression levels of the *Nlst* putative sugar transporter genes were examined in the whole body, head, thorax, abdomen, midgut, ovary, testis, salivary glands, Malpighian tubules and fat body of young adults using real-time RT-PCR (Fig. 1). The different *Nlsts* showed variable levels of expression in the tested tissues. Clear evidence of expression in the midgut was obtained for *Nlst1*, *4*, *6*, *9*, *12*, *16*, and *18*. Expression of *NlHT1* (*Nlst1*) in the midgut was also reported by Price et al. (2007b). *Nlst7*, *8*, *9*, *12*, *13*, *15*, *16*, and *18* were highly expressed in the Malpighian tubules. However, *Nlst9*, *12*, *16*, and *18* were expressed in the midgut as well as the Malpighian tubules. *Nlst5*, *8*, *10*, *11*, and *13* were expressed in the fat body. Clear expression of *Nlst3* was found in the testis, and clear expression of *Nlst17* was found in the salivary glands. *Nlst14* was expressed in various tissues, and *Nlst10* showed a low level of expression in all tissues (Fig. 1).

Expression of the *Nlst* genes at different stages of development, from embryo to adult, was also examined (Fig. 2). Three embryo stages were studied (Fig. S1). We found that *Nlst2*, *7*, *8*, *10*, *15*, *17*, and *18* were expressed in embryo stages I, II, and III. *Nlst7*, *15*, *17*, and *18* had a high level of expression only in embryo stage III. Other *Nlst* genes were constitutively expressed from the nymph to the adult stages (N1, N2, N3, N4, N5, and female adults). However, we were unable to determine unambiguously the pattern of expression of *Nlst14*.

3.4. Phylogenetic analysis

The phylogenetic relationships of insect sugar transporter genes were analyzed using the neighbor-joining method for amino-acid multiple-sequence alignment (Fig. 3). NlST transporters were interspersed in the tree, suggesting that NlST transporters had diversified before the insects evolved into each group.

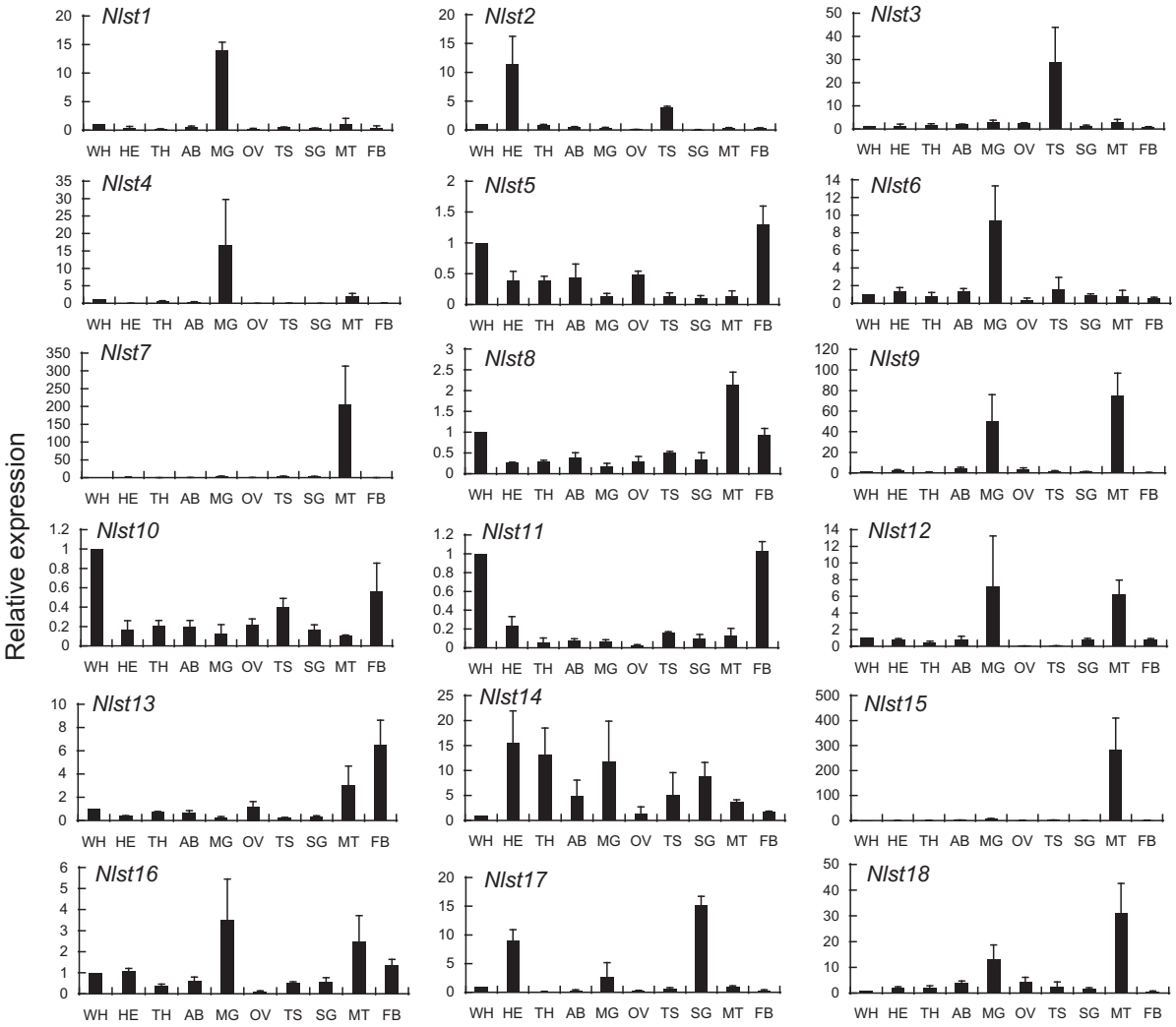

Fig. 1. Real-time RT-PCR analysis of the expression of BPH sugar transporter genes in various tissues. Tissue name abbreviations: WH, whole-body; HE, head; TH, thorax; AB, abdomen; MG, midgut; OV, ovary; TS, testis; SG, salivary glands; MT, Malpighian tubules and FB, fat body. Error bars represent standard deviation. Tissue samples were independently prepared 3 times. *RP-L4* was used for normalization.

3.5. Characterization of NlST transporters

NlST6 was selected for the sugar transport activity experiment because it was specifically expressed in the midgut. To evaluate sugar transport, *Nlst6* cRNA was injected into *Xenopus* oocytes. GFP fluorescence of NlST6::AcGFP1 fusion proteins was detected on the cellular membrane of the *Xenopus* oocytes (Fig. S2A, E). No fluorescence was detected on the cellular membrane of oocytes injected with AcGFP1 cRNA (Fig. S2F); similarly, no GFP fluorescence was observed in water-injected control oocytes (Fig. S2C, D, G, H).

Nlst1 (*NlHT1*), which was previously reported to be a glucose transporter (Price et al., 2007b), was also examined. NlST1 was expressed in *Xenopus* oocyte membranes and tested for sugar transport activity. The HPLC analysis detected glucose clearly in the NlST1-expressing oocytes, but fructose was below the detection limits (Fig. 4A). Incorporation of sugars into oocytes was also examined using radiolabeled ^{14}C-glucose and ^{14}C-fructose (Fig. 4B). Glucose uptake was confirmed by the tracer experiments. However, fructose incorporation in oocytes expressing the NlST1::AcGFP1 fusion protein was not different from the controls in which AcGFP1 was expressed in the oocytes. These results are in good agreement with those reported by Price et al. (2007b).

Glucose and fructose were examined in the *Xenopus* oocytes expressing NlST6::AcGFP1. As a negative control, the endogenous transport activity was examined in the oocytes injected with AcGFP1 cRNA. The HPLC analysis indicated that sugar uptake by the endogenous transporters was negligible. In contrast, NlST6 was found to transport glucose and fructose but not 2-deoxy-D-glucose (2-DOG), which is often used as a substitute sugar for glucose (Fig. 4C). Considerable fructose uptake was noted. NlST6 transport activity was then measured using radiolabeled ^{14}C-glucose and ^{14}C-fructose (Fig. 4D). The radioisotope assays clearly indicated that glucose and fructose were incorporated into oocytes expressing NlST6, which is in agreement with the results of the HPLC analysis.

3.6. NlST6 is a facilitated glucose/fructose transporter

The sequence homology analysis suggested that *NlST6* belongs to the major facilitator superfamily of transporters. To determine which sugar transporter group (i.e., facilitative, Na^+/sugar, or H^+/sugar transporters) *NlST6* belongs to, sugar uptake was examined under conditions of Na^+ deficiency and across a range of pH values. The Na^+ dependency of sugar uptake was examined in a Na^+-free buffer (Fig. 5A, B). NlST6-mediated transport of ^{14}C-fructose was

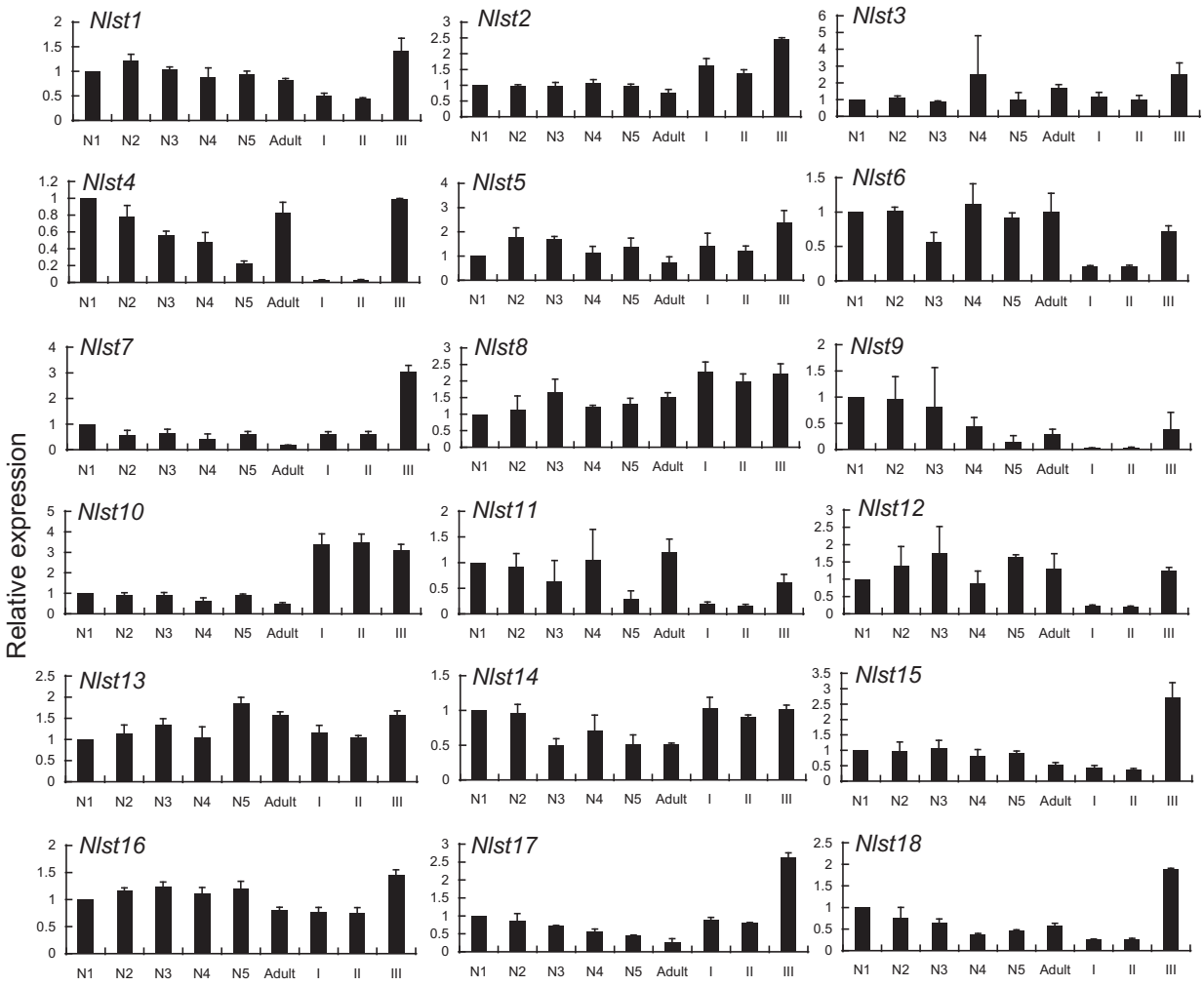

Fig. 2. Real-time RT-PCR analysis of the expression of BPH sugar transporter genes at various developmental stages. Stage name abbreviations: N1–N5, 1st to 5th instar nymphs; I, II, and III indicate BPH embryonic stages. (See detailed information in Materials and methods, and Fig. S1). Error bars represent standard deviation. Tissue samples were independently prepared 3 times. *RP-L4* was used for normalization.

not affected by Na^+ deficiency (Fig. 5B). However, ^{14}C-glucose uptake by NlST6 was not suppressed but, rather, slightly stimulated under Na^+-deficient condition (Fig. 5A). The influence of $[H^+]$ concentration on ^{14}C-glucose and ^{14}C-fructose uptake by NlST6 was examined using MBS buffers with pH values ranging from 4.0 to 9.0 (Fig. 5C, D). Although the ^{14}C-glucose uptake was not influenced by pH, ^{14}C-fructose uptake was significantly influenced by pH in these experiments. Therefore, the fructose uptake assay was reexamined using HPLC analysis and by replacing the Tris in the MBS buffer with 2-(*N*-morpholino)ethanesulfonic acid for the lower pH values (4 and 6). This examination clearly showed no significant difference in fructose uptake by NlST6 between the different pH values (Fig. S5). These results indicate that NlST6 is not an electrochemical membrane potential symporter but is a facilitative glucose/fructose transporter operated by sugar gradients. NlST1 was also found to be a facilitated glucose transporter (Fig. S3A, B), in agreement with the results of Price et al. (2007b).

Because NlST6 shows transport activity for both glucose and fructose, competitive assays were performed with radioisotope-labeled sugars and unlabeled sugars. Uptake of 1 mM ^{14}C-glucose mediated by NlST6 was inhibited by 5 mM unlabeled fructose (Fig. 6A). Similarly, 1 mM ^{14}C-fructose uptake was inhibited by 5 mM unlabeled glucose (Fig. 6B). Inhibition assays with cytochalasin B, a glucose-uptake inhibitor, were also performed with NlST6. ^{14}C-glucose uptake by NlST6 was markedly inhibited by cytochalasin B (Fig. 6C). ^{14}C-fructose uptake was also inhibited, although the degree of inhibition was low (Fig. 6D). ^{14}C-glucose uptake was markedly inhibited by cytochalasin B in NlST1 (Fig. S3C).

3.7. Kinetics analyses

The kinetics values of NlST6 for glucose were calculated; the $K_m = 2.3 \pm 0.6$ mM and $V_{max} = 7.1 \pm 0.8$ (Fig. 7A). The values for fructose were also calculated: $K_m = 11.8 \pm 4.3$ mM and $V_{max} = 165.6 \pm 34.7$ (Fig. 7B). For NlST1 and glucose, $K_m = 2.0 \pm 0.4$ mM and $V_{max} = 37.9 \pm 2.8$ (Fig. S3D).

4. Discussion

Planthoppers are phloem feeders and cause damage by their severe sucking of phloem sap in rice plants. The phloem sap is rich in sugars and amino acids; feeding transports these nutrients into the gut of the planthopper, usually in excess amounts (Sogawa, 1982). Some of the nutrients are taken into the planthopper body through the midgut cells, and the rest are excreted as honeydew. The sugars are thought to be absorbed with the aid of sugar transporters on the midgut cells, as occurs in the mammalian stomach or intestine (Baker and Thummel, 2007).

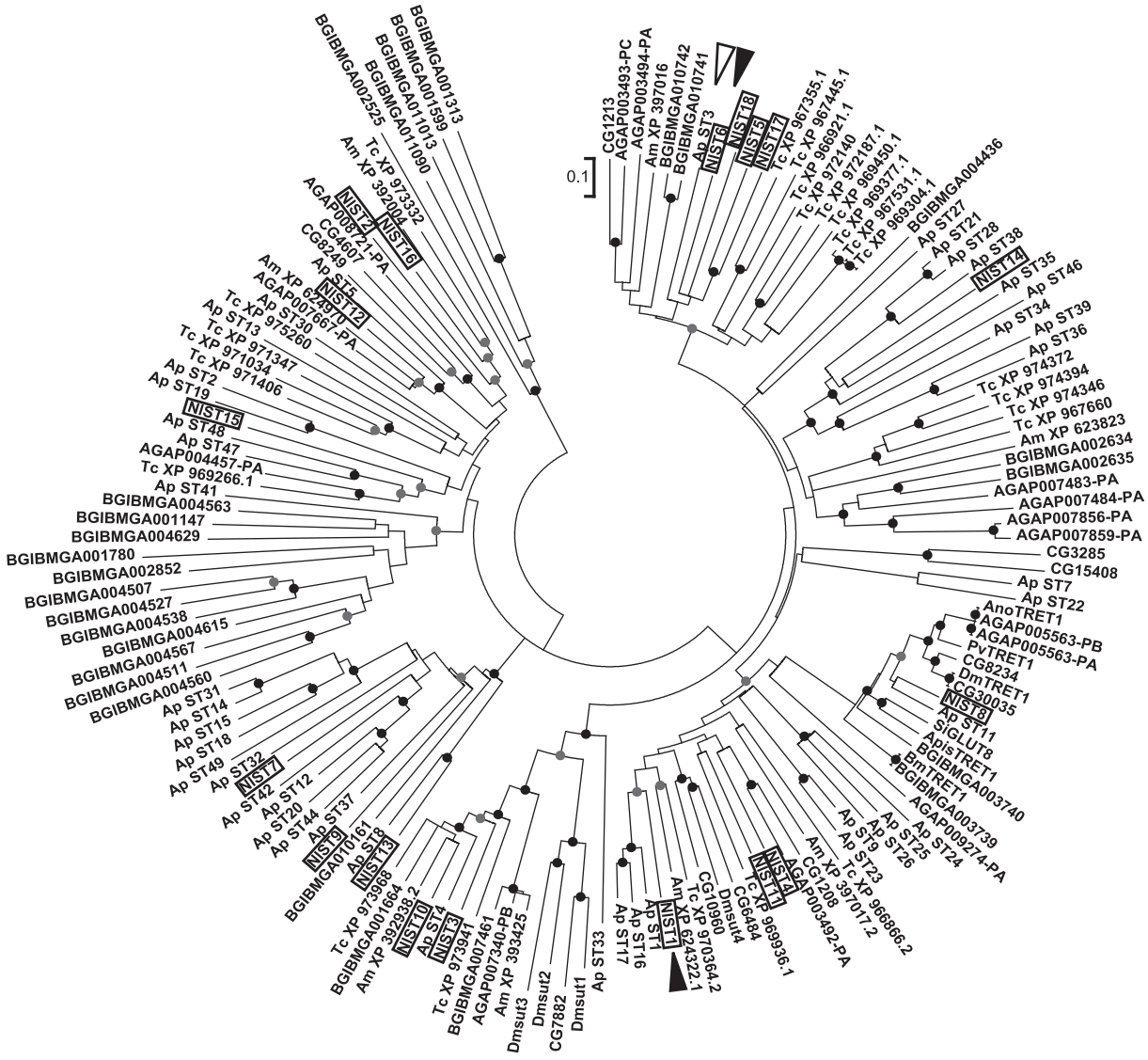

Fig. 3. Phylogenetic tree of insect putative sugar transporter genes. Putative sugar transporter genes in insects were identified from insect genome sequences using the predicted amino acid sequence of *Nlst1* as a query in a BLASTP search of the NCBI database with a cutoff E value of 10^{-7}. Genes that were predicted to have 10–12 transmembrane domains by TMHMM were included in the analysis. Amino acid alignment was performed using ClustalW. The unrooted tree was constructed with the neighbor-joining method using MEGA, ver. 4. The reliability of the trees was evaluated with 1000 bootstrap replications. Circles show the nodes whose bootstrap values were above 90% (black) and 70% (gray). BPH sugar transporter genes are boxed. Scale bar represents 0.1 amino acid substitutions per site. Initial letters show the insect species: CG or Dm, *Drosophila melanogaster*; BGIBMGA, *Bombyx mori*; AGAP, *Anopheles gambiae*; Tc, *Tribolium castaneum*; Am, *Apis mellifera*; and Ap, *Acyrthosiphon pisum*. pvTRET1 is derived from *Polypedilum vanderplanki*, DmTRET1 from *D. melanogaster*, BmTRET1 from *B. mori*, AnoTRET1 from *A. gambiae*, ApisTRET1 from *A. mellifera*, and SiGLUT8 from *Solenopsis invicta*. Closed arrowheads represent NlST1 and NlST6, and an open arrowhead represents Ap_ST3.

Therefore, the transporters on the membranes are key molecules for energy acquisition in planthoppers. Price et al. (2007b) reported that the BPH glucose transporter gene *NlHT1*, corresponding to *Nlst1* in the present study, is expressed in the midgut. We found that the BPH had at least 18 putative *Nlst* transporter genes, 8 of which are expressed in the midgut. Of these, *Nlst1* showed the highest level of gene expression in the midgut on the basis of the number of EST clones. NlST1 is responsible for glucose uptake in the midgut. Rice plants contain sucrose, a disaccharide composed of glucose and fructose, as the primary sugar component of phloem sap (Hayashi and Chino, 1990), and sucrose, glucose, and fructose are all excreted into honeydew by the BPH (Sogawa, 1982). NlST1 does not mediate cellular uptake of fructose, which is produced along with glucose by sucrose degradation (Price et al., 2007b) (Fig. 4A, B). This study revealed that fructose is taken up by NlST6 in the midgut.

BPH sugar transporter genes are predicted to show conserved features, such as distinctive patterns of hydrophobic and loop regions, across the evolutionary spectrum (Wallin and von Heijne, 1998; Stevens and Arkin, 2000). Some of the *Nlst* genes were predicted by the TMHMM and SOSUI programs to possess 12 transmembrane regions, although *Nlst5*, *10*, *14,* and *18* were not predicted to possess the expected 12 transmembrane regions. All of the *Nlst* genes in the transporter superfamily contain a GRR/K motif in loop 2 as a set of conserved charged residues (Fig. S4). However, another GRR/K motif, in loop 8, is replaced by GXR/K (X indicates residues other than R) in *Nlst2*, *5*, *6*, *17*, and *18*. Although some transporter genes have lost the conserved transporter family motifs, these transporters may nevertheless still have a transport function. Indeed, we found here that NlST6 could transport sugars despite loss of the GRK motif in loop 8 (Fig. S4). The BPH transporter genes are scattered in the phylogenetic tree of insect putative sugar

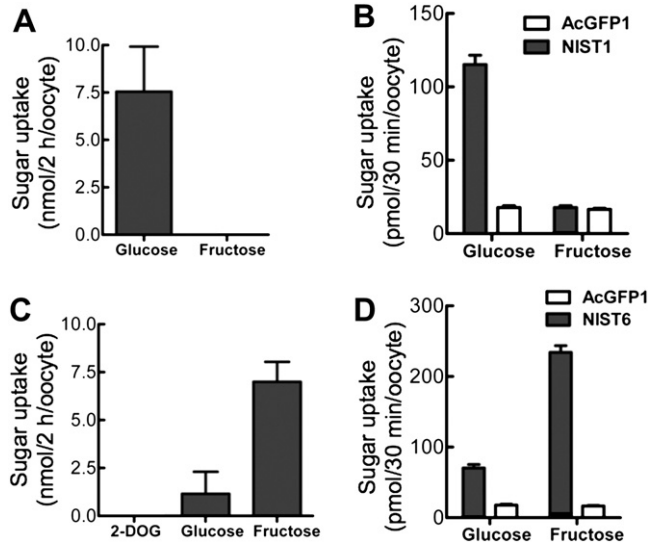

Fig. 4. Sugar uptake analyses using HPLC and radioisotope-labeled sugars. Transporters were expressed on the cellular membrane of *Xenopus* oocytes by injecting cRNA of *Nlst1* (A, B) or *Nlst6* (C, D). Error bars represent standard error ($n = 3$). Five oocytes were analyzed in each assay. A and C, HPLC analyses; B and D, radioisotope tracer analyses. Sugar uptake assays using radioisotope were performed in 1 mM ^{14}C-glucose or ^{14}C-fructose solutions for 30 min at 20 °C. The white bar represents the endogenous transporter activity in oocytes expressing AcGFP1 cRNA. 2-DOG, 2-deoxy-D-glucose.

transporter genes (Fig. 3), as was already shown by Price et al. (2010) in *Acyrthosiphon pisum*. This shows that the sugar transporter genes differentiated in ancestral species in the insect lineage.

Xenopus oocytes are useful for functional assays of sugar transporters, as seen in the study of the trehalose transporter

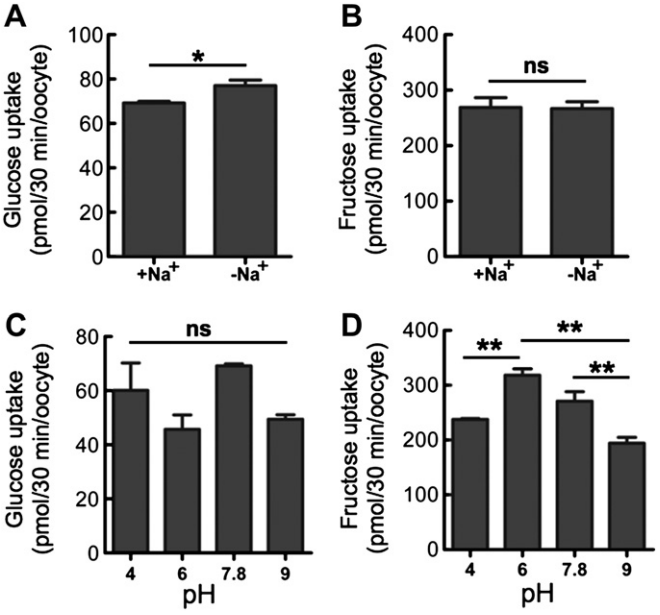

Fig. 5. NlST6 is a facilitated glucose/fructose transporter. Glucose and fructose uptake via NlST6 was examined under Na^+-free conditions (A, B) and different pH conditions (C, D). Sugar uptake assays were performed for 30 min at 20 °C at a concentration of 1 mM ^{14}C-glucose or ^{14}C-fructose. Five oocytes were used in each assay. The net transport content was calculated by subtracting the endogenous transport activities of *Xenopus* oocytes. Error bars represent standard error ($n = 3$). A and B: Statistical analyses by Student's *t*-test. C and D: Statistical analyses by one-way ANOVA before Tukey's multiple comparison tests. "ns" indicates no significant difference; an asterisk indicates a significant difference (* $P < 0.05$, ** $P < 0.001$). A: $P = 0.0467$, B: $P = 0.9689$, C: $P = 0.0753$, D: $P = 0.0005$.

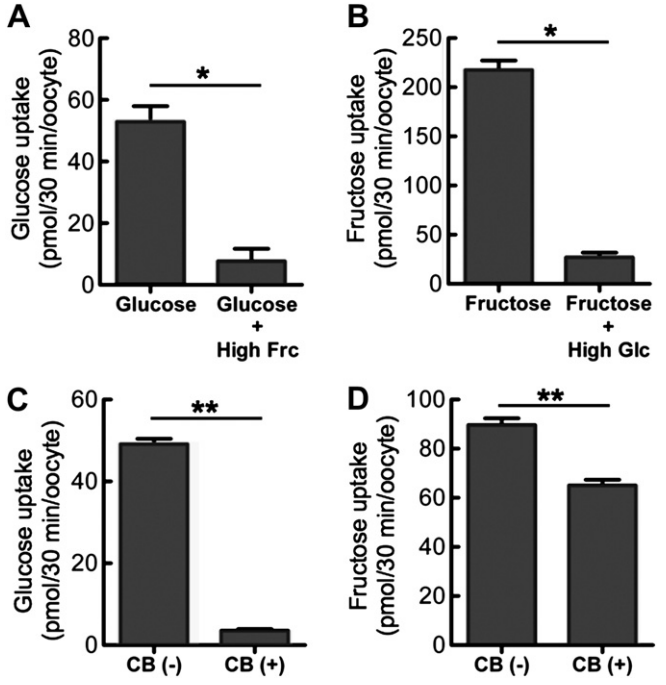

Fig. 6. Analyses of competition for NlST6 between glucose and fructose and of inhibition by cytochalasin B. Experimental conditions were similar to those in Fig. 5. Error bars represent standard error ($n = 3$). A: ^{14}C-glucose uptake was measured in a solution containing 1 mM ^{14}C-glucose plus 5 mM unlabelled radioisotope fructose (High Frc). B: ^{14}C-fructose uptake was measured in a solution containing 1 mM ^{14}C-fructose plus 5 mM unlabelled radioisotope glucose (High Glc). C and D: Inhibitory assays with cytochalasin B (CB). *Xenopus* oocytes expressing NlST6 were incubated for 30 min in a solution of 0.8 mM ^{14}C-glucose and ^{14}C-fructose with 10 μM CB. Statistical analyses were evaluated by Student's *t*-test. "ns" indicates no significant difference, an asterisk indicates a significant difference (* $P < 0.05$, ** $P < 0.001$). A: $P = 0.0006$, B: $P = 0.0007$, C: $P < 0.0001$, D: $P = 0.0023$.

(Kikawada et al., 2007). *Xenopus* oocyte expression systems have a high degree of expression for the exogeneous receptors or membrane transporters compared with other expression systems such as yeast, *Escherichia coli* or eukaryotic cells (Sobczak et al., 2010). Both HPLC analysis and isotope radioactivity detection of sugars were used for the functional assay in the *Xenopus* oocytes.

The effect of Na^+ and H^+ on the sugar uptake activity of the transporters was tested under Na^+-deficiency condition and varying pH conditions, respectively. Although the sugar uptake of NlST6 was shown to be influenced by Na^+ and H^+ in 2 experiments

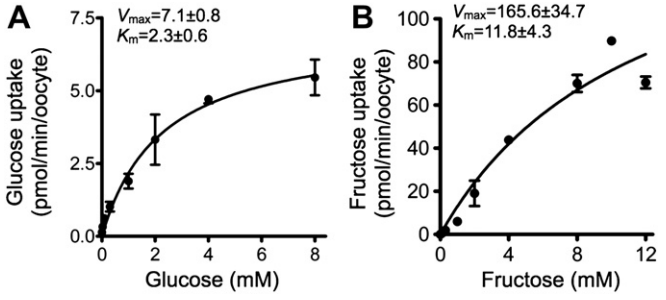

Fig. 7. Analysis of the kinetics of NlST6 for glucose (A) and fructose (B). Oocytes expressing NlST6 were incubated with various concentrations of radioisotope-labeled sugars for 20 min. Sugar uptake is shown as a curve fitted using the Michaelis–Menten equation. Five oocytes were used for each assay. Endogenous transport activities in *Xenopus* oocytes were subtracted from NlST6 activities. Error bars represent standard error ($n = 3$).

in statistical evaluation, these observations are considered to be a result of experimental variability. Sugar uptake activity of a Na^+ symporter should be decreased under Na^+-deficient conditions, suggesting that uptake promotion appears to be within the experimental variability. The pH conditions showed a significant effect on fructose uptake by NlST6 (Fig. 5D). However, the present experimental data do not show that NlST6 is a H^+ symporter. First, the H^+ effect is usually much more apparent than that of NlST6, as shown in human (Uldry et al., 2001) and *Leishmania* (Drew et al., 1995) H^+ myo-inositol symporters. Second, we reexamined the effect of H^+ on fructose uptake using HPLC, which indicated no significant effect of pH on the fructose uptake into *Xenopus* oocytes (Fig. S5). Therefore, we concluded that NlST6 is a facilitated sugar transporter that mediates glucose and fructose uptake.

In the analysis of NlST6 kinetics, we found a K_m value for glucose and fructose uptake of 2.3 mM and 11.8 mM, respectively. That is, NlST6 was more effective for fructose transport than for glucose transport. The transport ability of NlST6 is similar to those of Ap_ST3 in *A. pisum* and GLUT7 in humans, which transport both glucose and fructose (Li et al., 2004; Price et al., 2010). NlST6 shows 43.9% identity and 88.9% similarity to AP_ST3 and 26% identity and 48% similarity to GLUT7. Phylogenetic analysis also indicated that the *Nlst6* and *Ap_ST3* genes are closely related (Fig. 3). The NlST6 and Ap_ST3 transporters seem to play similar roles in the midguts of the two hemipterous insects. The rice plant is nearly the sole host plant for the BPH. Therefore, the sucrose in the phloem sap of rice plants is the main sugar source for the BPH. Digested monosaccharides from sucrose, glucose, and fructose are the major sugars taken up in the BPH. The transporters other than NlST1 and NlST6 in the midgut might also function in the uptake of glucose and fructose; if so, functional redundancy for sugar uptake among transporter genes should be clarified by exploring the functions of the other genes expressed in the midgut. If the other transporters mediate uptake of other sugars, the identities of these sugars and the significance of their expression in the midgut deserve investigation.

Facilitative sugar transporters use gradients to move sugars across cell membranes from regions of high sugar content to those of low sugar content (Wood and Trayhurn, 2003). It has not yet been determined whether NlST1 and NlST6 are located on the intestinal brush-border membranes or basal membranes. However, sugar uptake by sucking insects such as planthoppers and aphids can be achieved through such transporters because of the high sugar content of plant phloem sap compared with insect hemolymph (Downing, 1978; Douglas, 2006; Price et al., 2007b, 2010). The sucrose concentration of the rice phloem sap is about 500 mM (15–20%) (Hayashi and Chino, 1990), which stimulates significant sucking in the BPH (Sakai and Sogawa, 1976). The BPH possesses trehalose and myo-inositol in the hemolymph at high concentrations, whereas glucose and fructose are at low concentrations (Moriwaki et al., 2003; unpublished data). The BPH has a hemolymph sugar concentration of about 1% based on its blood sugar concentration of 5.8–8.3 mM trehalose and 31.6–34.2 mM myo-inositol (Moriwaki et al., 2003). The considerable difference in sugar concentration between the midgut lumen and the hemolymph appears to promote sugar diffusion from the midgut lumen to the hemolymph.

Nlst gene expression levels varied between different tissues. Eight *Nlst* genes showed high levels of expression in the midgut, as mentioned above. The fat body metabolizes disaccharides such as trehalose and stores sugars as a form of glycogen. As *Nlst5*, *8*, *10*, *11*, and *13* are expressed in the fat body, they are candidate genes to work in the fat body for sugar mobilization. NlST transporters expressed in the Malpighian tubules might function in the export of excess sugars to maintain a stable sugar concentration in the hemolymph. Also, *Nlst* genes were actively expressed in the embryonic stages. The expression of *Nlst2*, *7*, *10*, *15*, *17* and *18* during the embryonic stages suggests that they have an important role in sugar mobilization during embryo development.

Sugar transporters appear to play a pivotal role in sugar metabolism and energy acquisition. We still do not have any evidence that defects in the sugar transporters suppress normal developmental growth in insects, an effect known as malabsorption in mammalians (Wood and Trayhurn, 2003). Studying the functions of the other sugar transporters in the BPH is required to clarify the whole picture of the transporters in connection with sugar metabolism in this species. In particular, functional redundancy or specificity of transporters should be clarified. The transporters showing important specific function appear to be good candidate targets for control of the growth and development of this rice pest.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ibmb.2010.07.008.

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