Supporting Information

Zhang et al. 10.1073/pnas.1016144108

SI Materials and Methods

Plant Material and Growth Conditions. The *brittle culm14* (*bc14*) mutant was isolated from a *japonica* cultivar, *NE17*. An F_2 population used for mapping was generated by crossing *bc14* with a polymorphic *indica* cultivar, *Kasalath*. All of the rice plants were cultivated in the experimental fields at the Institute of Genetics and Developmental Biology in Beijing or Sanya (Hainan Province, China) during the natural growing seasons.

Measurement of the Breaking Force. Age-matched second internodes and leaves of wild-type and *bc14* plants were cut into segments of equal length and width and immediately used for measurement. The stretching force of the rice samples before being broken was measured with a digital force/length tester (5848 microtester, Istron). The newton is used as the unit of breaking force.

Microscopy. The second internodes from age-matched wild-type and *bc14* plants were used for fresh hand-cut section preparations. The sections (~20 μm thick) were stained with Toluidine blue (0.01% wt/vol) and analyzed under a light microscope (Leica). For transmission electron microscopy observations, the second internodes of the culms from adult plants were successively fixed in 2.5% glutaraldehyde and 2% OsO_4 and dehydrated by an ethanol gradient (30, 50, 70, 90, and twice in 100%, each for 30 min). The samples were infiltrated and embedded with a Spurr resin (Sigma). The ultrathin sections (80 nm) were prepared on an Ultracut E ultramicrotome (Leica), stained with uranyl acetate and lead citrate, and viewed on a Hitachi H7500 microscope.

Complementation Tests. The genomic fragment containing the entire ORF was inserted into the binary vector pCAMBIA1300 to generate the pBC14F construct. The *GUS* gene was inserted in frame into pBC14F just before the stop codon to generate the pBC14GUS vector. The construct (pBC14cDT) containing the CDS of *BC14* driven by the maize ubiquitin promoter was fused with a 3× Flag-tag and 6× histidine (His) tag and cloned into the pCAMBIA 1300 vector. All of the constructs were transformed into *bc14* plants via *Agrobacterium tumefaciens* (*EHA105* strain) infection, as described previously (1). T_0/T_1 transgenic plants harboring these constructs were used for phenotypic and GUS activity assays.

Bioinformatics Analysis of OsNST1. The full-length cDNA of *Oryza sativa Nucleotide Sugar Transporter1* (*OsNST1*) was obtained from the Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp/). Domain prediction for OsNST1 was performed using the Pfam database (http://pfam.sanger.ac.uk/) and National Center for Biotechnology Information (NCBI) Conserved Domains database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). A search for OsNST1 homologs in plants and animals was performed using the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree of OsNST1 homologs was generated using MEGA4 (2) with 1,000 bootstrap replicates. TargetP (v1.1), SignalP (v3.0), and SecretomeP (v2.0) servers (http://www.cbs.dtu.dk/services/) were used to identify putative signal peptides in OsNST1 for prediction of its subcellular localization.

Yeast Strains and Growth Conditions. The *Saccharomyces cerevisiae* strains used in this study included the following mutant strains: UDP-GlcNAc transporter mutant *Δyea4* (Y10244, MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YEL004w::kanMX4*) (3), GDP-mannose transporter mutant *vrg4-2* (NDY5, MATα *ura3–52 leu2–211*) (4), and UDP-galactose transporter mutant *Δhut1* (Y11048, MATα *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YPL244c::kanMX4*) (5), and the wild-type strain Y4741 (MATα *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). *Δyea*, *Δhut1*, and Y4741 were purchased from EUROSCARF. The *vrg4-2* strain was a kind gift from Neta Dean (Stony Brook University, New York). For cultivation and maintenance, the strains were grown on YPD medium containing 1% yeast extract, 2% Bacto peptone, and 2% glucose at 30 °C.

Subcellular Localization. For the transformation of rice protoplasts, EGFP was in frame fused to the N or C terminus of *OsNST1* and inserted into the pUC19 vector between the CaMV 35S promoter and the nopaline synthase terminator. Cotransfections were performed in rice protoplast cells. Briefly, the above-mentioned constructs were cotransfected with the RFP-tagged Golgi marker sialyltransferase (6) or the mCherry-tagged Golgi marker Man49 (7), as previously described (8). The transfected cells were observed with a confocal laser scanning microscope (Leica TCS SP5).

Complementation Assay of Yeast Nucleotide Sugar Transporter Mutants. The full-length cDNA of *OsNST1* was cloned in frame with the 6× His tag into the high-level expression vector pDR196 (9). This construct and the empty vector were introduced into the *S. cerevisiae* strains described above. Yeast transformations were performed using the lithium acetate/polyethylene glycol method, as described previously (10). The transformed mutant and wild-type strains were grown on synthetic complete medium without uracil (SC-ura) at 30 °C (11). Drug sensitivities were tested by spotting 5 to 10 μL of gradually diluted yeast cultures on YPD/SC-ura plates with or without supplementation with 5 mM neomycin (for *Δyea4*), 50 μg/mL Congo red (for *vrg4-2*), or 50 μg/mL leflunomide (for *Δhut1*).

Transport Assay. OsNST1 was subcloned into the pYES2 vector with a 6× His or a 3× FLAG tag at its N terminus. OsNST1 and the empty vector were introduced into the *S. cerevisiae* strain, 23344c (MATα *ura3-52*) (12), by the lithium acetate/polyethylene glycol method. Once the transformed yeast clones had grown to an OD_{600} of 3.0 in SC-ura liquid medium, they were induced in a larger volume (1:10) of SC-ura replaced glucose with galactose (2% wt/vol) at 28 °C for 24 h. The culture was collected and converted to spheroplasts using eight units of lyticase per OD_{600} unit (1.4 M sorbitol, 50 mM potassium phosphate, pH 7.5, 10 mM NaN3, 0.33% β-mercaptoethanol). Cells were pelleted and ground in ice-cold membrane buffer (0.8 M sorbitol, 10 mM triethanolamine, pH 7.2, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) with a homogenizer. The suspension was successively centrifuged at 1,000 × *g* and 10,000 × *g* for 10 min, and finally at 100,000 × *g* for 60 min to obtain a pellet fraction enriched with Golgi vesicles. GDPase assays, which reveal the latency of the extracted Golgi-rich fractions, were analyzed as described previously (13). Briefly, 10 μg of the Golgi-rich fractions were incubated in reaction buffer (10 mM $CaCl_2$, 0.1% Triton X-100, 2 mM GDP or ADP, and 200 mM imidazole buffer, pH 7.6) in a final volume of 0.1 mL. The reactions were incubated for 20 min at 30 °C and stopped by the addition of 10 μL of 10% SDS. The inorganic phosphate concentration was determined by the Ames method

(14). GDPase activity was calculated as the difference between GDP and ADP hydrolysis.

The nucleotide sugar uptake assay was conducted by incubating 50 μg of Golgi-rich vesicles in a reaction mixture containing 0.5 μCi of radioactive nucleotide sugars (0.3–30 Ci/mmol), 10 mM Tris–HCl (pH 7.0), 0.8 M sorbitol, and 2 mM $MgCl_2$ in a final volume of 50 μL at 30 °C for 8 min. The radioactive substrates used in this study were purchased from American Radiolabeled Chemicals [UDP-[^{3}H]Glc (30 Ci/mmol), UDP-[^{3}H]Gal (15 Ci/mmol), UDP-[^{3}H]GlcA (10 Ci/mmol), UDP-[^{3}H] GalNAc (5 Ci/mmol), and UDP-[^{14}C]Xyl (0.3 Ci/mmol)] and PerkinElmer [UDP-[^{3}H]GlcNAc (20 Ci/mmol)]. The reaction was stopped by adding 0.5 mL of ice-cold stop solution [10 mM Tris–HCl (pH 7.0), 0.8 M sorbitol, and 2 mM $MgCl_2$]. After filtration through a 0.45-μm nitrocellulose filter (Millipore), the radioactivity associated with the filters was detected by a liquid scintillation spectrometer (1450 MicroBeta Wallac TaiLux; Perkin-Elmer). For the uptake inhibition assay, increasing amounts of unlabeled nucleotide sugars were added to the reaction mixture that contained 0.5 μCi of UDP-[^{3}H] Glc and 10 μM unlabeled UDP-Glc. The transport reaction and radioactivity detection were performed as described above.

Cell Wall Analysis. The monosaccharide composition was determined by GC-MS (Agilent), as described previously (8). In brief, 2 mg of destarched alcohol insoluble residue (AIR) was hydrolyzed in 2 M trifluoroacetic acid (TFA) at 121 °C for 90 min. The supernatants were air dried and reduced with sodium borohydride (10 mg/mL in 1 M ammonium hydroxide). The generated alditol acetates were extracted in ethyl acetate and analyzed by an Agilent 7890 GC system equipped with a 5975C MSD (Agilent). For the measurement of uronic acids, 2 mg of AIR was methanolyzed in 1 M methanolic HCl at 80 °C overnight. After trimethylsilane derivatization, the silylated sugars were then extracted in hexane and analyzed by GC-MS on a DB-1 column (Agilent).

Approximately 50 mg of destarched AIRs of wild-type and mutant plants was sequentially fractionated with 0.5% hot ammonium oxalate and 1 N and 4 N potassium hydroxide (KOH). The soluble fractions were adjusted to pH 7.0 using acetic acid on ice. All fractions were extensively dialyzed against water and freeze-dried. The fractionated cell wall material was used to prepare alditol acetate derivatives, which were submitted to GC-MS analysis, as described above.

For the crystalline cellulose analysis, the material that remained after TFA treatment was hydrolyzed in Updegraff reagent (acetic acid:nitric acid:water, 8:1:2 vol/vol) at 100 °C for 30 min. The cooled pellets were washed and hydrolyzed with 72% sulfuric acid. The cellulose content was quantified by the anthrone assay (15).

Gene Expression. For RNA in situ hybridization, RNA probes (289–594 nt) for *OsNST1* were generated by in vitro transcription (Invitrogen). Various tissues of 4-wk-old seedlings were fixed in 4% paraformaldehyde prepared in PBS buffer (4 mM sodium phosphate, pH 7.2) and embedded in wax (Sigma). Hybridization was performed on 10-μm thick wax sections using probes labeled with digoxigenin (Roche) (16). For the GUS activity assay, 3-mo-old T_0 and 10-d-old T_1 pBC14GUS transgenic plants were used. The tissues were stained in 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) in staining buffer (50 mM sodium phosphate, pH 7.2, 0.1% Triton X-100, and 20% methanol) at 37 °C for 1 to 12 h. After being cleared in 70% ethanol, the tissues were mounted in water and observed under a light microscope (Leica).

1. Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6:271–282.
2. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599.
3. Roy SK, Chiba Y, Takeuchi M, Jigami Y (2000) Characterization of yeast Yea4p, a uridine diphosphate-N-acetylglucosamine transporter localized in the endoplasmic reticulum and required for chitin synthesis. *J Biol Chem* 275:13580–13587.
4. Dean N, Zhang YB, Poster JB (1997) The *VRG4* gene is required for GDP-mannose transport into the lumen of the Golgi in the yeast, *Saccharomyces cerevisiae*. *J Biol Chem* 272:31908–31914.
5. Fujimura H (2001) The *Saccharomyces cerevisiae MLF6/YPL244C* gene, which encodes a possible yeast homologue of mammalian UDP-galactose transporter, confers resistance to the immunosuppressive drug, leflunomide. *Curr Microbiol* 43:93–95.
6. Lee MH, et al. (2002) ADP-ribosylation factor 1 of *Arabidopsis* plays a critical role in intracellular trafficking and maintenance of endoplasmic reticulum morphology in *Arabidopsis*. *Plant Physiol* 129:1507–1520.
7. Nelson BK, Cai X, Nebenführ A (2007) A multicolored set of in vivo organelle markers for co-localization studies in *Arabidopsis* and other plants. *Plant J* 51:1126–1136.
8. Li M, et al. (2009) Rice cellulose synthase-like D4 is essential for normal cell-wall biosynthesis and plant growth. *Plant J* 60:1055–1069.
9. Rentsch D, et al. (1995) *NTR1* encodes a high affinity oligopeptide transporter in *Arabidopsis*. *FEBS Lett* 370:264–268.
10. Agatep R, Kirkpatrick R.D., Parchaliuk D.L., Woods R.A., Gietz R.D. (1998) Transformation of *Saccharomyces cerevisiae* by the lithium acetate/single-stranded carrier DNA /polyethylene glycol protocol. *Technical Tips Online* 3(1):133–137.
11. Sherman F (1991) Getting started with yeast. *Methods Enzymol* 194:3–21.
12. Marini AM, Soussi-Boudekou S, Vissers S, Andre B (1997) A family of ammonium transporters in *Saccharomyces cerevisiae*. *Mol Cell Biol* 17:4282–4293.
13. Abeijon C, Mandon EC, Robbins PW, Hirschberg CB (1996) A mutant yeast deficient in Golgi transport of uridine diphosphate *N*-acetylglucosamine. *J Biol Chem* 271: 8851–8854.
14. Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* 8:115–118.
15. Updegraff DM (1969) Semimicro determination of cellulose in biological materials. *Anal Biochem* 32:420–424.
16. Li Y, et al. (2003) *BRITTLE CULM1*, which encodes a COBRA-like protein, affects the mechanical properties of rice plants. *Plant Cell* 15:2020–2031.

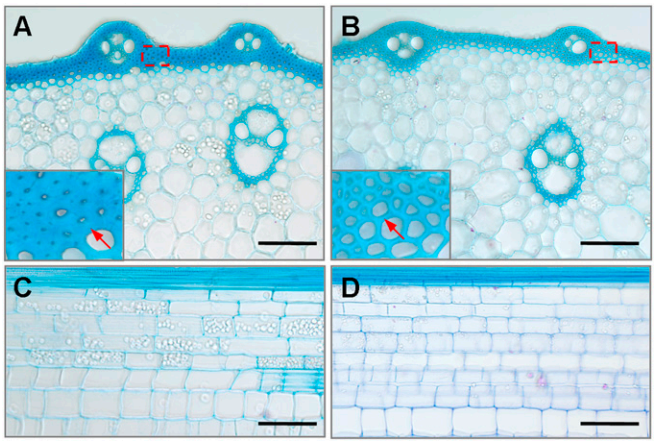

Fig. S1. Anatomical analysis of the *bc14* and wild-type culms. (*A* and *B*) Fresh hand-cut cross-sections of wild-type (*A*) and *bc14* (*B*) culms. The red-dotted parts are enlarged and embedded as insets in *A* and *B*. The reduced wall thickness in *bc14* is indicated by arrows. (*C* and *D*) Fresh hand-cut longitudinal sections of wild-type (*C*) and *bc14* (*D*) culms showing indistinguishable cell sizes and lengths in wild type and *bc14*. (Scale bars, 100 μm.)

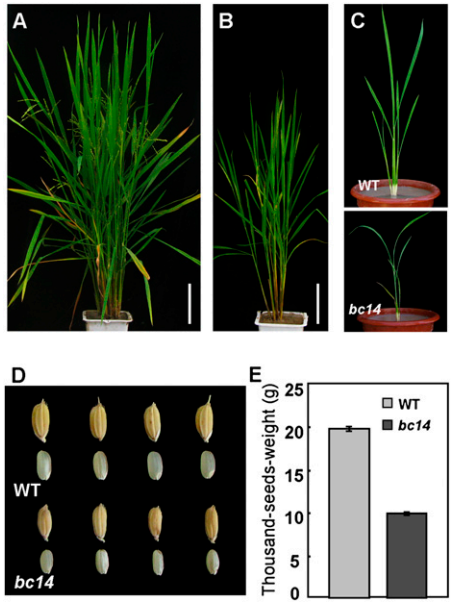

Fig. S2. Phenotypic characterization of *bc14* plants. (*A* and *B*) Mature wild-type (*A*) and *bc14* (*B*) plants. (Scale bars, 15 cm.) (*C*) One-mo-old wild-type and *bc14* seedlings. (*D*) Seeds of the wild-type and *bc14* plants. (*E*) Quantification of thousand-seeds-weight of wild-type and *bc14* plants (mean of three replicates ± SEM).

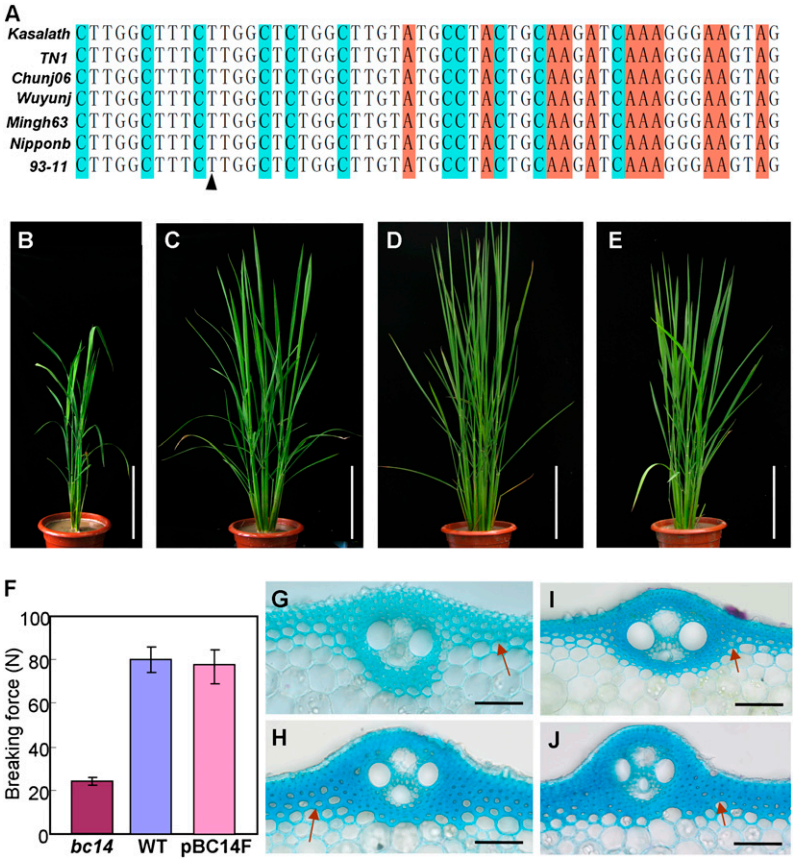

Fig. S3. Complementation assay. (*A*) DNA sequences of *OsNST1* in seven rice varieties, covering the mutation point (indicated by an arrowhead) in *bc14*. (*B–E*) Two-mo-old *bc14* plants (*B*) and pBC14F- (*C*), pBC14cDT- (*D*), and pBC14GUS-transformed plants (*E*). (Scale bars, 20 cm.) (*F*) Breaking force of *bc14*, wild-type, and transgenic plants (mean of three replicates ± SEM). (*G–J*) Fresh hand-cut cross-sections of culms of *bc14* (*G*) and those transformed with pBC14F (*H*), pBC14cDT (*I*), and pBC14GUS (*J*). The arrows indicate the wall thickness of sclerenchyma cells in *bc14* and transgenic plants. (Scale bars, 60 μm.)

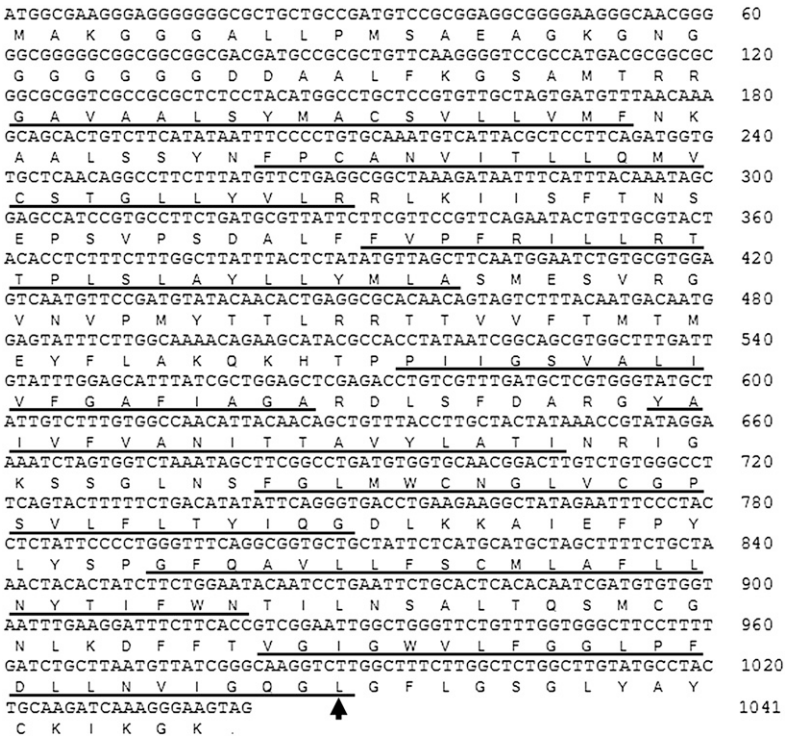

Fig. S4. The coding sequence and deduced amino acid sequence of *OsNST1*. The transmembrane domains are underlined. The arrow indicates the mutated site.

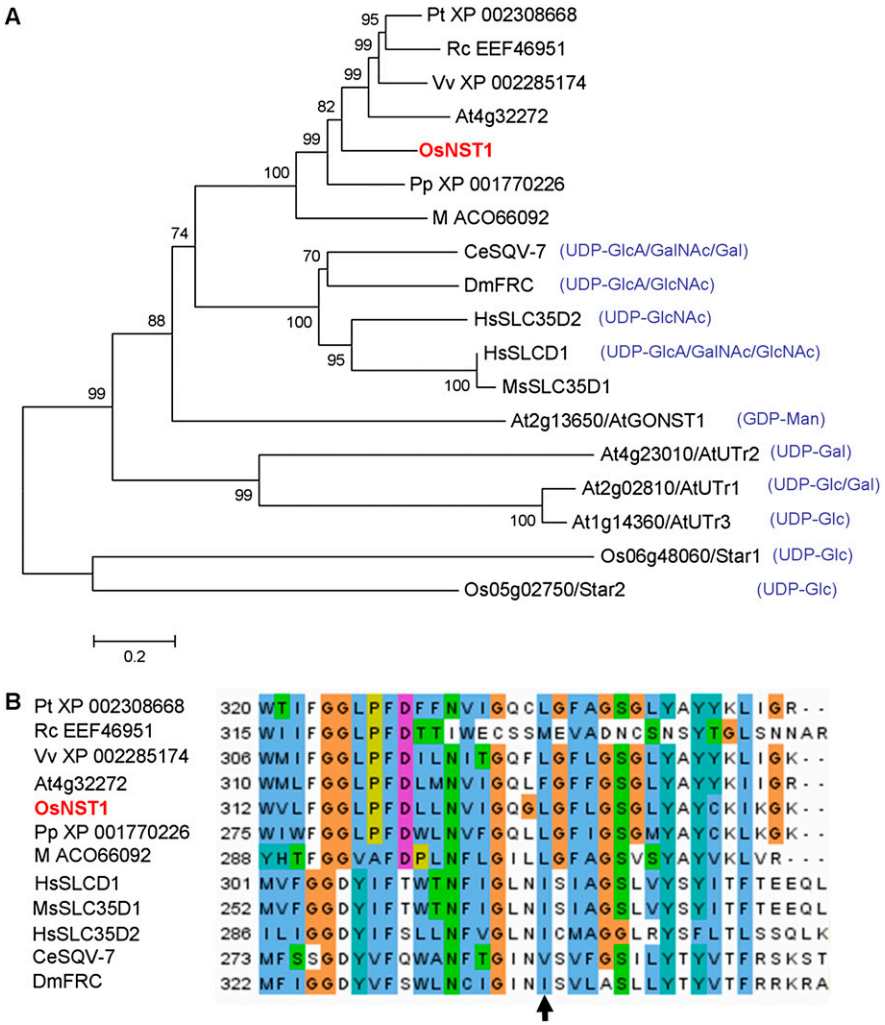

Fig. S5. Bioinformatic analysis of OsNST1. (*A*) Phylogenetic tree of OsNST1 and its homologs in plants and animals. The numbers at each node represent the bootstrap support (percentage) and the scale bar is an indicator of genetic distance based on branch length. The identified transport activities of nucleotide sugar transporters (NST) are shown in parentheses. (*B*) Sequence alignment of OsNST1 and its homologs from different plants and animals, to reveal conservation of the residue mutated in OsNST1. Pt, *Populus trichocarpa*; Rc, *Ricinus communis*; Vv, *Vitis vinifera*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Pp, *Physcomitrella patens*; Mp, *Micromonas pusilla*; Hs, *Homo sapiens*; Ms, *Mus musculus*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*.

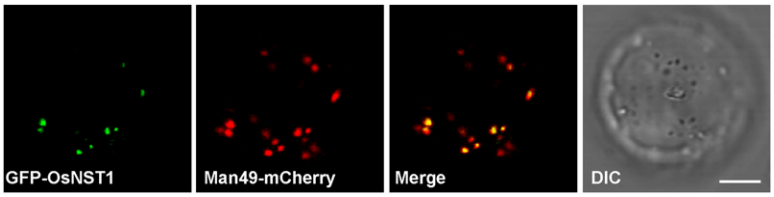

Fig. S6. OsNST1 is resident in the Golgi apparatus. Rice protoplast cells coexpressing GFP-OsNST1 (N-terminal tagging) with Man49-mCherry. (Scale bar, 5 μm.)

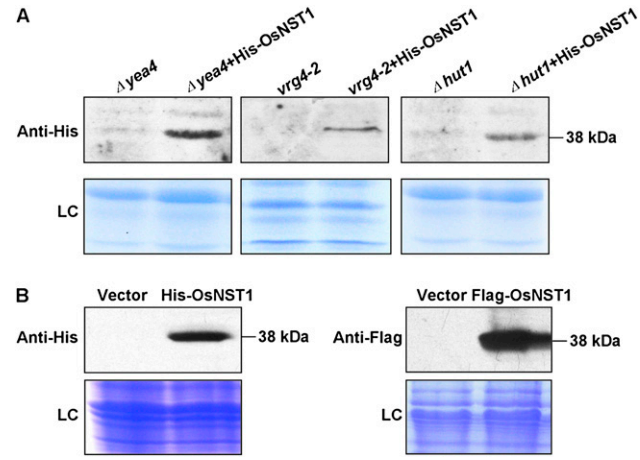

Fig. S7. Protein gel blotting the expressed OsNST1 in transformed yeasts. (*A*) Analysis of the total proteins isolated from untransformed and transformed yeast NST mutants. (*B*) Analysis of the yeast Golgi-rich fraction before the uptake assay. Total proteins and the Golgi fraction prepared from His/Flag-OsNST1 and empty vector transfectants were submitted to SDS/PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with anti-His and anti-FLAG antibodies, respectively. LC, loading control stained with Coomassie Brilliant blue.

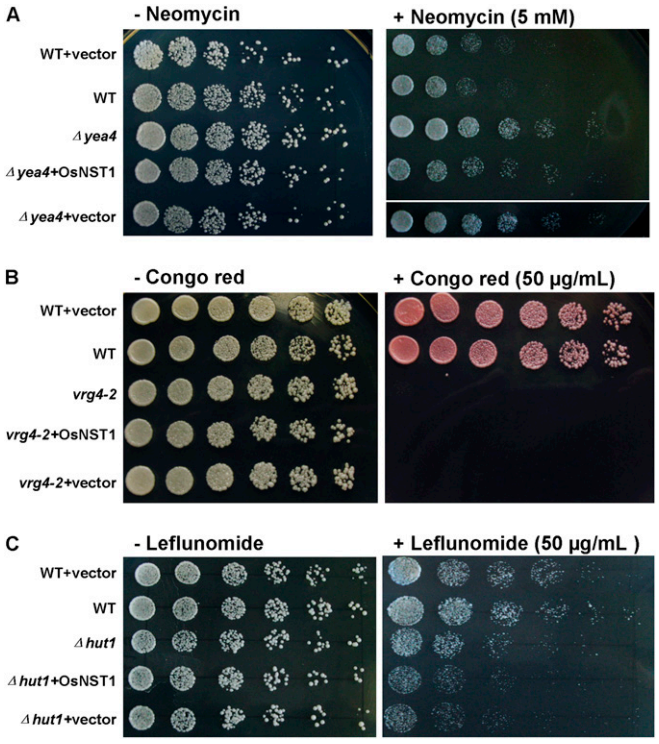

Fig. S8. Complementation assay of yeast NST mutants. (*A*) *Δyea4* is the UDP-GlcNAc transporter mutant. (*B*) *vrg4* is the GDP-mannose transporter mutant. (*C*) *Δhut1* is the UDP-galactose transporter mutant. The mutant and wild-type strains expressing the empty vector (+vector) or His-OsNST1 (+OsNST1) were gradually diluted and spotted on medium supplemented without (–) and with (+) corresponding screening chemicals, as described in *SI Material and Methods*.

Table S1. Molecular markers used for the *BC14* gene cloning

Markers	Type	Primer sequences	Position on Chr. 2
RM262	SSR	F: CATTCCGTCTCGGCTCAACT R: CAGAGCAAGGTGGCTTGC	20.8 Mb
STS-2–89.9	SSR	F: ATCCTGATCTCTCCTGTGCC R: AGATGTAAACCCAATAGGACC	22.7 Mb
RM3515	SSR	F: GGAAAGAAGATATGCCATGC R: AGAGAGAATCAGAAACACCAAC	24.0 Mb
RM13617	SSR	F: ACCATATAGCGTGTATGCAACC R: TGCACACATGTACACAGTACACC	24.8 Mb
RM525	SSR	F: GGCCCGTCCAAGAAATATTG R: CGGTGAGACAGAATCCTTACG	28.3 Mb
STS1	SSR	F: CTCGGGAAGCGGTGGGAT R: TAGCTCTGCCAATAGCGGC	24,183 Kb
STS2	SSR	F: ATGGGATTCGCCTAGACACC R: CAGAGAAGAATACGAGTCCGAC	24,197 Kb
STS3	SSR	F: GGAACTCGGAAGCAGTGGA R: GAAACGCTCGCTGCCTCA	24,255 Kb
STS4	SSR	F: TGTCTACGAAGACACGAAACAC R: GAGAAGGCGGCAATAAGGTT	24,333 Kb
STS5	SSR	F: TAAAAGTGGAGTCATCCGTCC R: GAGTCCAGATGTTCGCAAGG	24,484 Kb

SSR, simple sequence repeat.

Table S2. Integrity of yeast Golgi-rich vesicles for the uptake assay

Yeast stains	GDPase (–)*	GDPase (+)*	Latency (%)
Empty vector	0.19 ± 0.01	7.99 ± 0.02	98
His-OsNST1	0.45 ± 0.01	9.12 ± 0.02	95
Flag-OsNST1	0.32 ± 0.01	8.35 ± 0.01	96

Results are the average of two independent assays.
*One unit of GDPase activity is defined as 1 μmol of inorganic phosphate released by 1 mg protein under standard assay conditions, as described in *SI Materials and Methods*. The "–" and "+" indicate without and with Triton X-100 treatment, respectively.