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Analysis of Sialyltransferase-Like Proteins from *Oryza sativa*

Shou Takashima[1,*,¶], Tomoko Abe[2,†], Shigeo Yoshida[2,‡], Hiroyuki Kawahigashi[3], Tamio Saito[4,§], Shuichi Tsuji[5] and Masafumi Tsujimoto[1]

[1]Cellular Biochemistry Laboratory and [2]Plant Functions Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198; [3]Plant Biotechnology Department, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602; [4]Plant Molecular Biology Laboratory, RIKEN Tsukuba Institute, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074; and [5]Future Science and Technology Joint Research Center, Institute of Glycotechnology, Tokai University, 1117 Kitakaname, Hiratsuka, Kanagawa 259-1292

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Sialic acids are widely distributed among living creatures, from bacteria to mammals, but it has been commonly accepted that they do not exist in plants. However, with the progress of genome analyses, putative gene homologs of animal sialyltransferases have been detected in the genome of some plants. In this study, we cloned three genes from *Oryza sativa* (Japanese rice) that encode sialyltransferase-like proteins, designated OsSTLP1, 2, and 3, and analyzed the enzymatic activity of the proteins. OsSTLP1, 2, and 3 consist of 393, 396, and 384 amino acids, respectively, and each contains sequences similar to the sialyl motifs that are highly conserved among animal sialyltransferases. The recombinant soluble forms of OsSTLPs produced by COS-7 cells were analyzed for sialyltransferase-like activity. OsSTLP1 exhibited such activity toward the oligosaccharide Galβ1,4GlcNAc and such glycoproteins as asialofetuin, α1-acid glycoprotein, and asialo-α1-acid glycoprotein; OsSTLP3 exhibited similar activity toward asialofetuin; and OsSTLP2 exhibited no sialyltransferase-like activity. The sialic acid transferred by OsSTLP1 or 3 was linked to galactose of Galβ1,4GlcNAc through α2,6-linkage. This is the first report of plant proteins having sialyltransferase-like activity.

Key words: glycoprotein, oligosaccharide, *Oryza sativa*, sialyl motif, sialyltransferase.

Abbreviations: The ganglioside designations are according to the nomenclature of Svennerholm (*1*). The animal sialyltransferase designations are according to the nomenclature of Tsuji *et al*. (*2*). BLAST, the basic local alignment search tool; BSM, bovine submaxillary mucin; EST, expressed sequence tag; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HPTLC, high performance thin layer chromatography; RT, reverse transcription; Sia, sialic acids; STLP, sialyltransferase-like protein.

Sialic acids (Sia) such as *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) are derivatives of a negatively charged acidic sugar, neuraminic acid, and usually occur at the terminal ends of carbohydrate groups of glycoconjugates. Because of their negative charge and wide occurrence in exposed positions of cell-surface molecules, sialic acids function as key determinants of oligosaccharide structures that may mediate a variety of biological phenomena such as cell–cell communication, cell–substrate interaction, adhesion, and protein targeting. For the synthesis of sialylglycoconjugates, a family of glycosyltransferases called sialyltransferases catalyzes the transfer of a sialic acid from CMP-Sia to an acceptor carbohydrate. All sialyltransferases from vertebrates characterized to date have type II transmembrane topology and contain highly conserved motifs called sialyl motifs L (Long), S (Short), and VS (Very Short) (*3*–*5*). Sialyl motif L is characterized by a 45–60–amino acid region in the center of the protein and it has been shown to be involved in the binding of a donor substrate, CMP-Sia (*6*). Sialyl motif S is located in the COOH-terminal region and consists of a 20–30–amino acid stretch. It has been shown to be involved in the binding of both the donor and acceptor substrates (*7*). Sialyl motif VS is also located in the COOH-terminal region, within which one glutamic acid residue is always found separated by four amino acid residues from a highly conserved histidine residue. This motif is thought to be involved in the catalytic activity (*5*, *8*). Recently, the existence of a new motif (motif III), which is located between sialyl motifs S and VS and has the consensus sequence of (H/y)Y(Y/F/W/h)(E/D/q/g), has been reported (*9*). This motif is also involved in the catalytic activity (*9*). Although bacterial sialyltransferases reported so far do not have sialyl motifs, sialyltransferases from a virus and insects also have sialyl motifs (*10*, *11*). Based on the high sequence conservation of sialyl motifs L and S, PCR-based cloning of sialyltransferase cDNAs has been extensively performed (reviewed in Refs. *12* and *13*). So far, cDNA cloning of 20 members of the mammalian sialyltransferase family has been performed, and these

[*]Present address: Glyco-chain Functions Laboratory, RIKEN-FRS, 2-1 Hirosawa, Wako, Saitama 351-0198.

[†]Present address: Accelerator Research Program, Application and Development Group, RIKEN-FRS, 2-1 Hirosawa, Wako, Saitama 351-0198.

[‡]Present address: Plant Science Center, RIKEN, 1-7-22 Suehiro, Tsurumi, Yokohama 230-0045.

[§]Present address: Antibiotics Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198.

[¶]To whom correspondence should be addressed. Tel: +81-48-467-9616, Fax: +81-48-467-9617, E-mail: staka@riken.jp

enzymes have been grouped into four families according to the carbohydrate linkages they synthesize: β-galactoside α2,3-sialyltransferase (ST3Gal I-VI), β-galactoside α2,6-sialyltransferase (ST6Gal I and II), GalNAc α2,6-sialyltransferase (ST6GalNAc I-VI), and α2,8-sialyltransferase (ST8Sia I-VI) (*14*).

Sialic acids are widely distributed among living creatures, from bacteria to mammals, but it has been commonly accepted that they do not exist in plants (*15*), although the possibility remains that sialylated glycoconjugates may exist in plant cells (*16*, *17*). Interestingly, however, putative gene homologs for mammalian sialyltransferases and CMP-sialic acid transporters have been detected in the genome and/or expressed sequence tag (EST) databases of some plants, such as *Arabidopsis thaliana* (thale-cress) and *Oryza sativa* (Japanese rice) (*15*, *17*, *18*). This suggests that plants have potential ability of sialylation despite the absence of sialic acids in them. To elucidate this issue, we have cloned three genes from *O. sativa*, each encoding a protein having sialyl motif–like sequences, and analyzed the enzymatic activity of the proteins. Here, we report for the first time that plant proteins having sialyl motif–like sequences actually have sialyltransferase-like activity.

EXPERIMENTAL PROCEDURES

Materials—Fetuin, asialofetuin, bovine submaxillary mucin (BSM, type I-S), α1-acid glycoprotein, ovomucoid, lactosyl ceramide (LacCer), GA1, CMP-NeuAc, Galβ1,3GalNAc, Galβ1,3GlcNAc, Galβ1,4GlcNAc, and Triton CF-54 were purchased from Sigma. Paragloboside and lactose were from Wako (Tokyo, Japan). CMP-[^{14}C]NeuAc (12.0 GBq/mmol, 925 kBq/ml) was from Amersham Biosciences. Sialidases (NANase I and II) were from Glyko Inc. Asialo-BSM, asialo-α1-acid glycoprotein, and asialoovomucoid were prepared as described previously (*19*, *20*).

Isolation of OsSTLP Genes—*O. sativa* DNA sequences encoding proteins having sialyl motif–like sequences were obtained using the Basic Local Alignment Search Tool (BLAST) algorithm to search the nucleotide databases at the National Center for Biotechnology Information with sialyl motif sequences of mammalian sialyltransferases as queries. Three *O. sativa* putative proteins having sialyl motif–like sequences were deduced (GenBank accession Nos. AK107493, AK058389, and AK104762 for cDNA sequences (*21*) and GenBank accession Nos. AP003289, AP003794, AP008207, AP008218, AL929350, DP000011, AL662969, AL731626, and AP008210 for genomic sequences), which we tentatively designated *O. sativa* sialyltransferase-like proteins (OsSTLP) 1, 2, and 3, respectively. Comparison of cDNA and genomic sequences of the OsSTLP1, 2, and 3 genes revealed that each of them consists of a single exon, so we used genomic DNA as a template for PCR amplification to avoid difficulties in amplifying cDNA of low expression genes. The OsSTLP1 gene was amplified by PCR (LA Taq with GC buffer, Takara) using two primers, 5′-GCGATGAAGCGGCCG-CTGCGGCGCCCGTTC-3′ (CB265+, nucleotides 134–163 of GenBank accession No. AK107493) and 5′-GTCGAC-CACCTTGCGCGCACGCGCTCTAAT-3′ (CB267-, complementary to nucleotides 1314–1343 of GenBank accession No. AK107493; the synthetic *Sal*I site is underlined) with *O. sativa* genomic DNA as a template. The OsSTLP2 gene was similarly amplified by PCR using 5′-GGGATGAAGCGCCGCCACCTCCCGCCCGTC-3′ (CB361+, nucleotides 35–64 of GenBank accession No. AK058389) and 5′-CACCTCGAGGTCACCGTCAC-CAGTGCAACC-3′ (CB363–, complementary to nucleotides 1215–1244 of GenBank accession No. AK058389; the synthetic *Xho*I site is underlined) as primers and *O. sativa* genomic DNA as a template. These PCRs were performed as follows: 94°C for 60 s, 40 cycles of (94°C for 60 s, 50°C for 60 s, and 72°C for 120 s), and 72°C for 7 min. The amplified DNA fragments were cloned into pBluescript II SK(+) vector. The OsSTLP3 gene was amplified by PCRs using two primer sets, 5′-GTGCTCTCTCTCC-TCTGATACTGCAGAGG-3′ (CB379+, nucleotides 1–30 of GenBank accession No. AK104762) and 5′-ACC-ACCATCCGGCGCGGCGGCCGCGGCGCC-3′ (CB380-, complementary to nucleotides 171–200 of GenBank accession No. AK104762), and 5′-GAATTCCGCTGCTCC-CAGCTGCGCCATTCC-3′ (CB365+, nucleotides 135–164 of GenBank accession No. AK104762; the synthetic *Eco*RI site is underlined) and 5′-CGTCGACTGTCAGC-GGTAGAACGACACGGG-3′ (CB366-, complementary to nucleotides 1197–1226 of GenBank accession No. AK104762; the synthetic *Sal*I site is underlined), with *O. sativa* genomic DNA as a template. The PCRs were performed as described above, and the amplified fragments were cloned into pBluescript II SK(+) vector, and then combined using a common *Not*I site at nucleotide 177. The nucleotide sequences were confirmed by the dideoxy termination method using an ABI PRISM 377 DNA sequencer (Applied Biosystems).

Construction of Expression Vectors—The expression vector of the soluble OsSTLP1 was constructed as follows. The DNA fragment encoding a truncated form of OsSTLP1 lacking the first 26 amino acids was amplified by PCR using primers 5′-TCCGAATTCCGCAGGTCG-GTGGGCCCGGCT-3′ (CB266+, nucleotides 209–238 of GenBank accession No. AK107493; the synthetic *Eco*RI site is underlined) and CB267- with the cloned OsSTLP1 gene as a template, and it was cloned into pBluescript II SK(+) vector. Then the 1.1-kb *Eco*RI–*Sal*I fragment was excised and subcloned into the *Eco*RI–*Xho*I sites of the expression vector pcDSA. The resulting plasmid, designated pcDSA-OsSTLP1, encodes a soluble fusion protein consisting of the IgM signal peptide, the *Staphylococcus aureus* protein A IgG-binding domain, and the truncated form of OsSTLP1. The expression vector of the soluble OsSTLP2 was constructed as follows. The DNA fragment encoding a truncated form of OsSTLP2 lacking the first 21 amino acids was amplified by PCR using primers 5′-GAATTCCGCCGCCGCCTCCTCGTGCTGCAG-3′ (CB362+, nucleotides 98–127 of GenBank accession No. AK058389; the synthetic *Eco*RI site is underlined) and CB363- with the cloned OsSTLP2 gene as a template, and it was cloned into pBluescript II SK(+) vector. Then the 1.1 kb *Eco*RI–*Xho*I fragment was excised and subcloned into the *Eco*RI–*Xho*I sites of the expression vector pcDSA, which was designated pcDSA-OsSTLP2. The expression vector of the soluble OsSTLP3 was constructed as follows. The DNA fragment encoding a truncated form of OsSTLP3 lacking the first 25 amino acids was amplified

by PCR using primers CB365+ and CB366– and cloned into pBluescript II SK(+) vector as described above. Then the 1.1-kb *Eco*RI–*Sal*I fragment was excised and subcloned into the *Eco*RI–*Xho*I sites of the expression vector pcDSA, which was designated pcDSA-OsSTLP3.

Preparation of Soluble OsSTLPs—For production of soluble forms of OsSTLPs, COS-7 cells were transfected with above pcDSA-vectors using Lipofectamine™ Reagent (Invitrogen) and cultured as described previously (*19*). The protein A-fused OsSTLPs expressed in the medium were adsorbed onto IgG-Sepharose gel (Amersham Biosciences) and used as the enzyme source.

Enzymatic Assays and Product Characterization—We examined whether OsSTLPs have similar activity to animal sialyltransferases by employing the animal sialyltransferase assay (*14*). In brief, enzymatic activity was measured in 50 mM MES (4-morpholineethanesulfonic acid) buffer (pH 6.0), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% Triton CF-54, 100 μM CMP-[^{14}C]NeuAc, an acceptor substrate, and an enzyme preparation, in a total volume of 10 μl. As acceptor substrates, 10 μg of glycoproteins, 5 μg of glycolipids, or 10 μg of oligosaccharides were used. The enzyme reaction was performed at 37°C for 3–20 h. For glycoproteins, the reaction was terminated by the addition of SDS-PAGE loading buffer, and the reaction mixtures were directly subjected to SDS-PAGE. For glycolipids, the reaction mixtures were applied to a Waters Sep-Pak Vac C_{18} column (100 mg), and purified glycolipids were subjected to high-performance thin-layer chromatography (HPTLC, Silica-Gel 60; Merck) with a solvent system of chloroform, methanol, and 0.02% $CaCl_2$ (55:45:10). For oligosaccharides, the reaction mixtures were directly subjected to HPTLC with a solvent system of 1-propanol, aqueous ammonia, and water (6:1:2.5). The radioactive materials were visualized and quantified with a Fuji BAS2000 radioimage analyzer. The intensity of the radioactivity was converted into moles using the radioactivities of various amounts of CMP-[^{14}C]NeuAc (12.0 GBq/mmol, 925 kBq/ml) as standards. Quantification was performed within the linear range of the standard radioactivity.

For kinetic analysis, the reaction was performed as described above except for the use of various concentrations of acceptor substrate Galβ1,4GlcNAc or donor substrate CMP-[^{14}C]NeuAc. Kinetic parameters were determined from Lineweaver-Burk plots.

For linkage analysis of sialic acids, [^{14}C]NeuAc-incorporated Galβ1,4GlcNAc with protein A–fused OsSTLP1 and [^{14}C]NeuAc-incorporated asialofetuin with protein A-fused OsSTLP3 were digested with linkage-specific exosialidase: NANase I (specific for α2,3-linked sialic acids; Glyko, Inc.) or NANase II (specific for α2,3- and α2,6-linked sialic acids; Glyko, Inc.). After treatment, reaction mixtures were subjected to HPTLC for oligosaccharides with a solvent systems of 1-propanol/aqueous ammonia/water (6:1:2.5) or SDS-PAGE for glycoproteins. The radioactive materials were visualized with the BAS2000 radioimage analyzer.

For *Sambucus nigra* lectin blotting, asialofetuin (10 μg) was incubated with 1 mM CMP-NeuAc and protein A–fused OsSTLP1 or OsSTLP3 at 37°C for 20 h, and the reaction products were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The blotted membrane was subjected to *Sambucus nigra* lectin blotting analysis (*22*) and glycochains containing the Siaα2,6Galβ1,4GlcNAc structure were detected. Fetuin and asialofetuin were also analyzed as positive and negative controls, respectively.

For preparation of de-*N*-glycosylated asialofetuin, the [^{14}C]-sialylated asialofetuin was treated with recombinant *N*-glycanase (glycopeptidase F, Takara), which is free of protease activity, according to the manufacturer's instructions. The [^{14}C]-sialylated asialofetuin and de-*N*-glycosylated asialofetuin were then subjected to SDS-PAGE, and the radioactive materials were visualized with the BAS2000 radioimage analyzer.

Expression Analysis of the OsSTLP Genes—Total RNA was extracted from 3-week-old *O. sativa* seedlings using ISOGEN (Wako), and first-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitogen). Relative expression levels of OsSTLP mRNAs were estimated by semiquantitative RT-PCR using the above first-strand cDNA as a template. For the analysis of OsSTLP1 gene expression, OsSTLP1 specific primers CB266+ and CB267– were used. For the analysis of OsSTLP2 gene expression, OsSTLP2 specific primers CB362+ and CB363– were used. For the analysis of OsSTLP3 gene expression, OsSTLP3 specific primers CB365+ and CB366– were used. As a control, *O. sativa* glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene expression was also measured using G3PDH-specific primers 5′-AAAGGAGGCTTCTCTTACAGTGGCCAGAAG-3′ (CB400+, nucleotides 871–900 of GenBank accession No. AF357884) and 5′-TCAGCCCATGGTGTAGGATGGAGATGGTAG-3′ (CB401–, complementary to nucleotides 1495–1524 of GenBank accession No. AF357884). PCRs were performed as follows: 94°C for 60 s; 40 cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 120 s for OsSTLP genes and 30 cycles for G3PDH gene; and 72°C for 7 min. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and then visualized under UV light.

RESULTS

Cloning of OsSTLP Genes—Using the BLAST algorithm to search the *O. sativa* nucleotide sequence databases with sialyl motif sequences of mammalian sialyltransferases as queries, we found three genes which encode proteins having sialyl motif–like sequences. We cloned these genes by PCR and tentatively designated them as the OsSTLP1, 2 and 3 genes. The OsSTLP1, 2, and 3 genes consist of a single exon located on chromosome 1, 12, and 4 and encode a protein of 393, 396, and 384 amino acids, respectively. Their GC contents are 71.2%, 72.1%, and 71.1%, respectively. Hydropathy analysis (*23*) of each of deduced proteins OsSTLP1, 2, and 3 indicated one prominent hydrophobic sequence in the NH_2-terminal region of each protein, predicting that these proteins have type II transmembrane topology characteristic of many glycosyltransferases cloned to date. The overall amino acid sequence of OsSTLP1 showed 43.9% and 61.3% sequence identity with OsSTLP2 and 3, respectively, and OsSTLP2 showed 48.2% sequence identity with OsSTLP3 (Fig. 1). Although OsSTLP1, 2, and 3 have sialyl motif-like sequences, their overall amino acid sequences did not otherwise show any significant similarity with mammalian

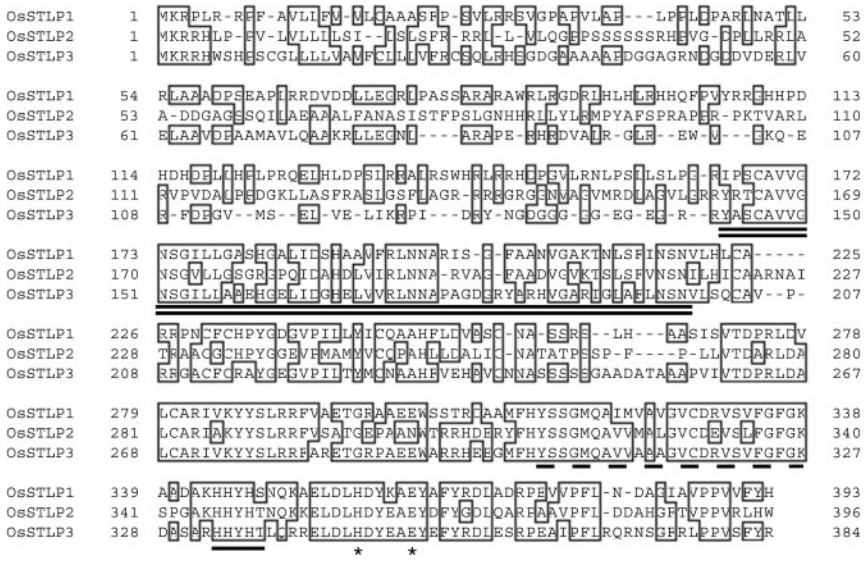

Fig. 1. **Sequence comparison of OsSTLP1, 2, and 3.** The conserved amino acid residues are boxed. Sequences corresponding to sialyl motifs L, S, and III are doubly underlined, dashed underlined, and singly underlined, respectively. The corresponding His and Glu residues that are conserved in sialyl motif VS are marked with asterisks.

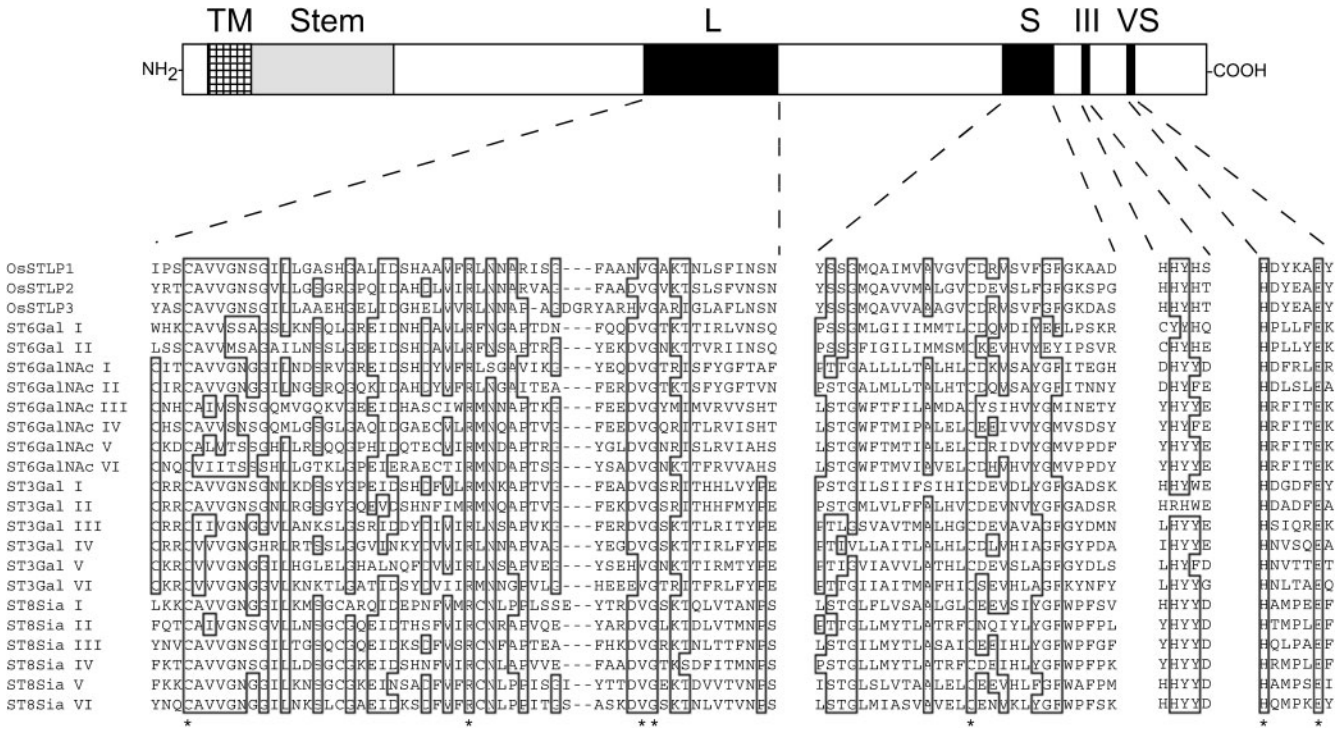

Fig. 2. **Comparison of the sialyl motif–like sequences of OsSTLPs and the sialyl motif sequences of 20 mouse sialyltransferases.** The sialyl motif–like sequences of OsSTLPs and the sialyl motif sequences of 20 mouse sialyltransferases are aligned. The conserved amino acid residues are boxed. The highly conserved amino acid residues are marked with asterisks. TM, transmembrane domain; L, sialyl motif L; S, sialyl motif S; III, sialyl motif III; VS, sialyl motif VS.

sialyltransferases. There are highly conserved amino acid residues in the sialyl motifs of mammalian sialyltransferases (*14*), and some of these amino acid residues are also conserved among the sialyl motif–like sequences of OsSTLP1-3 (Fig. 2), suggesting that the plant sialyl motif-like sequences may function as sialyl motifs.

Enzymatic Activity of OsSTLPs—To facilitate determination of the enzymatic activity of OsSTLPs, we constructed the expression plasmids pcDSA-OsSTLP1, 2, and 3, which allow expression of OsSTLPs lacking the transmembrane domain as secretable proteins fused with the IgG-binding domain of *Staphylococcus aureus* protein A. These expression plasmids were then transfected into COS-7 cells, and the protein A–fused OsSTLPs secreted into the medium were adsorbed onto IgG-Sepharose resin for use as the enzyme sources. Enzymatic activity was measured with CMP-[^{14}C]NeuAc as a donor substrate of sialic acid and various oligosaccharides, glycoproteins from animals, and glycolipids as acceptor substrates according to the animal sialyltransferase assay

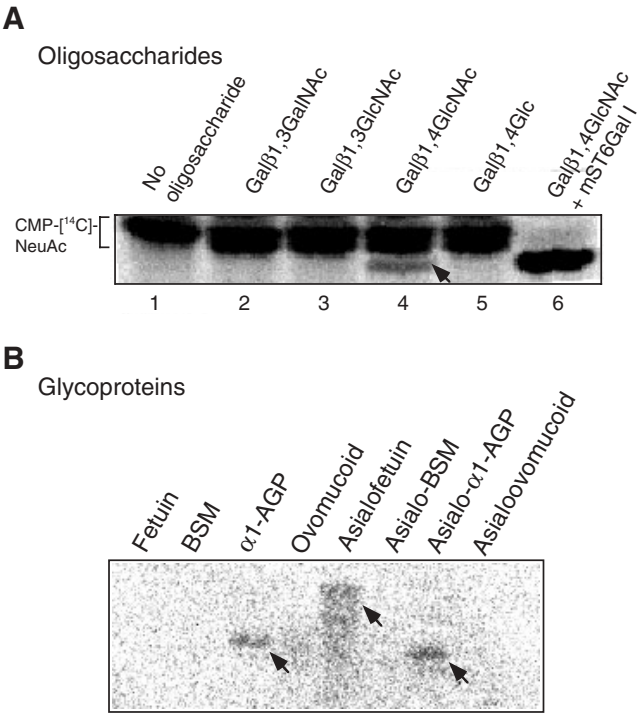

Fig. 3. **Analysis of sialyltransferase-like activity of OsSTLP1.** The various oligosaccharides (10 μg/lane, A) or glycoproteins (10 μg/lane, B) indicated were incubated with CMP-[^{14}C]NeuAc and protein A–fused OsSTLP1 at 37°C for 20 h, and the reaction mixtures were analyzed by high performance thin-layer chromatography (HPTLC) for oligosaccharides with a solvent system of 1-propanol, aqueous ammonia, and water (6:1:2.5) or by SDS-PAGE for glycoproteins. The [^{14}C]NeuAc incorporated products (indicated by arrowheads) were visualized and quantified with a Fuji BAS2000 radioimage analyzer. (A) The standard [^{14}C]-NeuAcα2,6Galβ1,4GlcNAc produced by mouse α2,6-sialyltransferase ST6Gal I (mST6Gal I) (*14*) was applied in lane 6. (B) BSM, bovine submaxillary mucin; α1-AGP, α1-acid glycoprotein.

(*14*). Note that the assay conditions are quite artificial and unphysiological for plant OsSTLPs.

As shown in Fig. 3 and Table 1, OsSTLP1 exhibited sialyltransferase-like activity toward an oligosaccharide Galβ1,4GlcNAc and such mammalian glycoproteins as asialofetuin, α1-acid glycoprotein, and asialo-α1-acid glycoprotein. These glycoproteins are considered to have the Galβ1,4GlcNAc structure at the nonreducing end of some of their carbohydrate groups. However, OsSTLP1 did not exhibit sialyltransferase-like activity toward glycolipids examined in this study, including paragloboside, which has a Galβ1,4GlcNAc structure at the nonreducing end of its carbohydrate group.

OsSTLP3 exhibited sialyltransferase-like activity toward asialofetuin (Table 1), but not toward other glycoproteins, glycolipids, and oligosaccharides examined.

OsSTLP2 exhibited no sialyltransferase-like activity toward any of the acceptor substrates examined (Table 1).

Linkage Specificity of OsSTLP1 and 3—The linkages of the incorporated sialic acids were also examined. Galβ1,4GlcNAc was [^{14}C]-sialylated with OsSTLP1, then treated with linkage-specific exosialidases (Fig. 4A). The incorporated sialic acid residue, [^{14}C]NeuAc, was resistant to treatment with α2,3-linkage–specific exosialidase (NANase I), but it was digested by α2,3- and α2,6-linkage–specific exosialidase (NANase II). Note that Galβ1,4GlcNAc [^{14}C]-sialylated by OsSTLP1 co-migrated with [^{14}C]-NeuAcα2,6Galβ1,4GlcNAc produced by mouse α2,6-sialyltransferase ST6Gal I (Fig. 3A). Asialofetuin was similarly [^{14}C]-sialylated with OsSTLP3 and treated with linkage-specific exosialidases (Fig. 4B). The incorporated sialic acid residue was resistant to treatment with α2,3-linkage–specific exosialidase, but it was digested by α2,3- and α2,6-linkage–specific exosialidase. In addition, when asialofetuin was sialylated with OsSTLP1 or OsSTLP3, the reaction products were detected by the lectin blot with *Sambucus nigra* agglutinin, which binds the Siaα2,6Galβ1,4GlcNAc structure (Fig. 4C). The Siaα2,6Galβ1,4GlcNAc structures produced by OsSTLP1 or OsSTLP3 were involved in *N*-glycans of asialofetuin (Fig. 4D). These results indicate that although assay conditions are unphysiological, OsSTLP1 and 3 can transfer sialic acid onto galactose of the Galβ1,4GlcNAc structure on oligosaccharides or some glycoproteins through α2,6-linkage. The apparent K_m value of OsSTLP1 for Galβ1,4GlcNAc was estimated to be 2.5 mM, which was comparable to that of mammalian α2,6-sialyltransferase ST6Gal I (2–10 mM) (*24*). However, the apparent K_m value of OsSTLP1 for CMP-NeuAc was estimated to be 33.3 mM, which was much higher than that of ST6Gal I (30–50 μM) (*24*).

Expression of the OsSTLP Genes—The expression levels of the OsSTLP genes in 3-week-old *O. sativa* seedlings were examined by RT-PCR (Fig. 5). Significant levels of the OsSTLP1 gene expression were detected in leaf and stalk, and very low expression was also detected in root. The expression of the OsSTLP2 gene was detected in stalk only at this stage. It should be noted that the EST clones of the OsSTLP2 gene (GenBank accession Nos. AU078330, AU078331, AU163987, CK053361, and C20201) have been cloned from panicle at ripening stage. The expression of the OsSTLP3 gene was not detected in any tissues at this stage under the conditions employed. However, an EST clone of the OsSTLP3 gene (GenBank accession No. CA755189) has been cloned from roots of 3,4-week-old plants, which had undergone the 200 mM NaCl treatment for 19 h, suggesting that the expression of the OsSTLP3 gene may be induced by stress.

DISCUSSION

We have shown here for the first time that plant proteins having sialyltransferase-like activity exist in *O. sativa*. OsSTLP1 and 3 transferred NeuAc to the galactose of the terminal Galβ1,4GlcNAc structure on *N*-glycans of some glycoproteins through α2,6-linkage. But we could not detect any sialyltransferase-like activity of OsSTLP2. We also examined whether an *A. thaliana* protein homologous to OsSTLP (GenBank accession No. NM_100701; tentatively designated AtSTLP1) has sialyltransferase-like activity. However, like OsSTLP2, AtSTLP1 had no detectable sialyltransferase-like activity (data not shown). In a sense, it is natural that OsSTLP2 and AtSTLP1 did not exhibit sialyltransferase-like activity, because there should be no CMP-NeuAc in plants (*15*) and it is not considered to be a physiological substrate of plant STLPs.

Table 1. **Analysis of sialyltransferase-like activity of OsSTLP1, 2, and 3.** Various acceptor substrates were incubated in the standard assay mixture using soluble OsSTLP fused with protein A as an enzyme source. Each substrate was used at the concentration of 0.5 mg/ml for glycolipids and 1 mg/ml for glycoproteins and oligosaccharides. Relative rates are calculated as a percentage of the incorporation obtained with asialofetuin. *R* represents the remainder of the *N*-linked oligosaccharide chain. *0.0374 pmol/h/ml medium; **0.0234 pmol/h/ml medium.

Acceptors	Representative structures of carbohydrates	Relative rate (%)		
		OsSTLP1	OsSTLP2	OsSTLP3
Oligosaccharides				
Type II	Galβ1,4GlcNAc	371.7	0	0
Type I	Galβ1,3GlcNAc	0	0	0
Type III	Galβ1,3GalNAc	0	0	0
Lactose	Galβ1,4Glc	0	0	0
Glycoproteins				
Fetuin	NeuAcα2,3Galβ1,3GalNAc-*O*-Ser/Thr NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAc-*O*-Ser/Thr NeuAcα2,6(3)Galβ1,4GlcNAc-R	0	0	0
Asialofetuin		100*	0	100**
BSM	NeuAcα2,6GalNAc-*O*-Ser/Thr GlcNAcβ1,3(NeuAcα2,6)GalNAc-*O*-Ser/Thr	0	0	0
Asialo-BSM		0	0	0
Ovomucoid	NeuAcα2,3Galβ1,4GlcNAc-R	0	0	0
Asialoovomucoid		0	0	0
α1-Acid glycoprotein	(NeuAcα2,6(3))Galβ1,4GlcNAc-R	31.3	0	0
Asialo-α1-acid glycoprotein		37.4	0	0
Glycolipids				
Lactosylceramide	Galβ1,4Glcβ1-Cer	0	0	0
GA1	Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1-Cer	0	0	0
Paragloboside	Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1-Cer	0	0	0

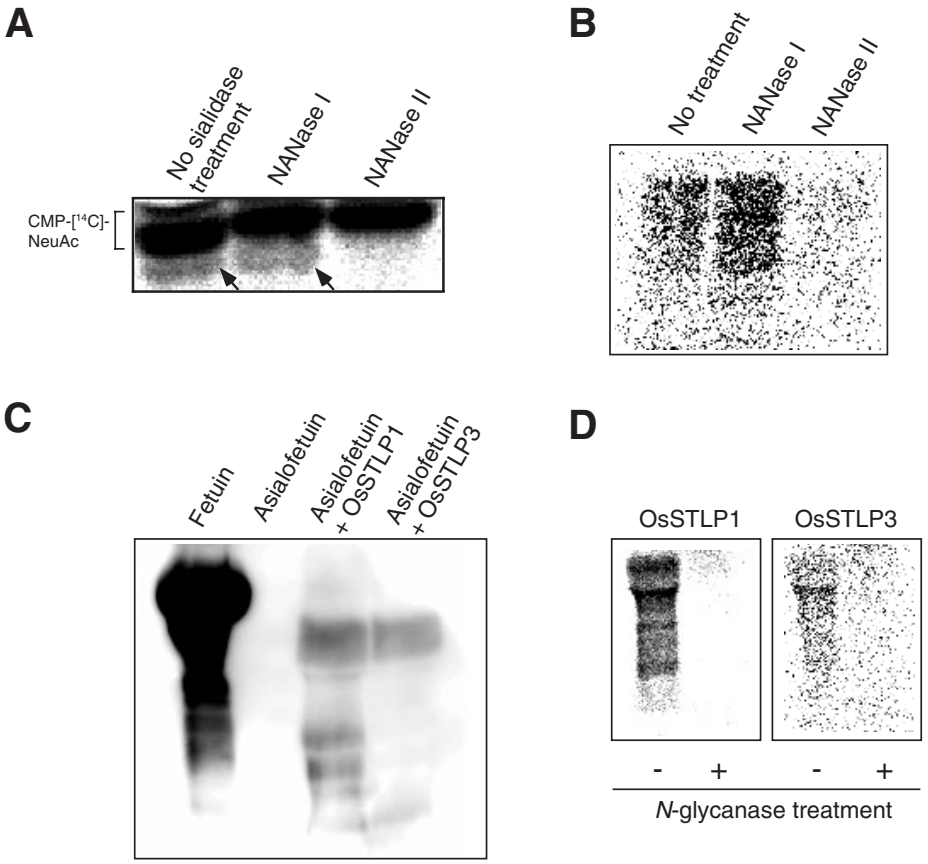

Fig. 4. **Linkage analysis of incorporated sialic acids by OsSTLP1 and 3.** (A) [^{14}C]NeuAc-incorporated Galβ1,4GlcNAc sialylated with OsSTLP1 (left, indicated by an arrowhead) was treated with α2,3-linkage–specific exosialidase (NANase I, middle) or α2,3- and α2,6-linkage–specific exosialidase (NANase II, right), and the reaction mixtures were analyzed by HPTLC. (B) [^{14}C]NeuAc-incorporated asialofetuin sialylated with OsSTLP3 (left) was treated with NANase I (middle) or NANase II (right), and the reaction mixtures were analyzed by SDS-PAGE. (C) Asialofetuin was incubated with CMP-NeuAc and OsSTLP1 or 3 at 37°C for 20 h, and the reaction products were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The blotted membrane was subjected to *Sambucus nigra* lectin blotting analysis and glycochains containing Siaα2,6Galβ1,4GlcNAc structures were detected. Fetuin and asialofetuin were also analyzed as positive and negative controls, respectively. (D) Asialofetuin (10 μg/reaction) was [^{14}C]-sialylated with OsSTLP1 or 3 and then treated with *N*-glycanase. The resulting glycoproteins were subjected to SDS-PAGE, and the radioactive materials were visualized with a BAS2000 radioimage analyzer.

The acceptor glycoproteins used in this study were from animals and were not physiological substrates of plant STLPs at all. However, although the assay system is quite artificial, it is still interesting that some plant proteins having sialyl motif–like sequences have the ability to use CMP-NeuAc as a donor substrate and the Galβ1,4GlcNAc structure as an acceptor substrate and exhibit sialyltransferase-like activity.

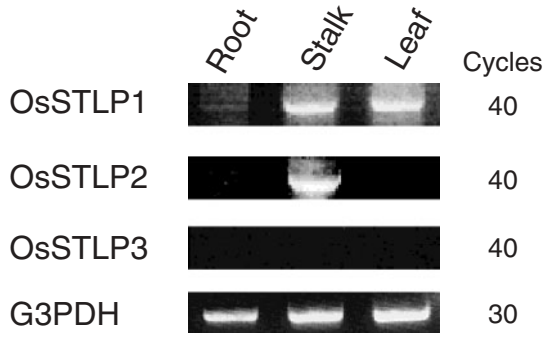

Fig. 5. **Expression analysis of the OsSTLP1, 3, and 3 genes.** Relative expression levels of the OsSTLP1, 2, and 3 genes in 3-week-old *O. sativa* seedlings were measured by semiquantitative RT-PCR.

The apparent K_m value of OsSTLP1 for CMP-NeuAc was much higher than that of mammalian α2,6-sialyltransferase, also suggesting that CMP-NeuAc is not a physiological substrate of STLPs. STLPs may utilize a compound that has a similar structure to CMP-NeuAc as a physiological substrate. It is likely that OsSTLP1 and 3 have broad substrate specificity and can utilize CMP-NeuAc as a donor substrate, whereas OsSTLP2 and AtSTLP1 have narrow substrate specificity and cannot utilize CMP-NeuAc as a donor substrate. Although sialic acids, which have a nine-carbon framework structure, have not been considered to exist in plants, the eight-carbon acidic sugar 3-deoxy-D-manno-2-octulosonate (KDO) is involved in a plant pectin component, rhamnogalacturonan II (*25*). There are some structural similarities between CMP-Sia and the sugar nucleotide form of KDO, CMP-KDO. Thus, CMP-KDO may be a physiological donor substrate of STLPs, although

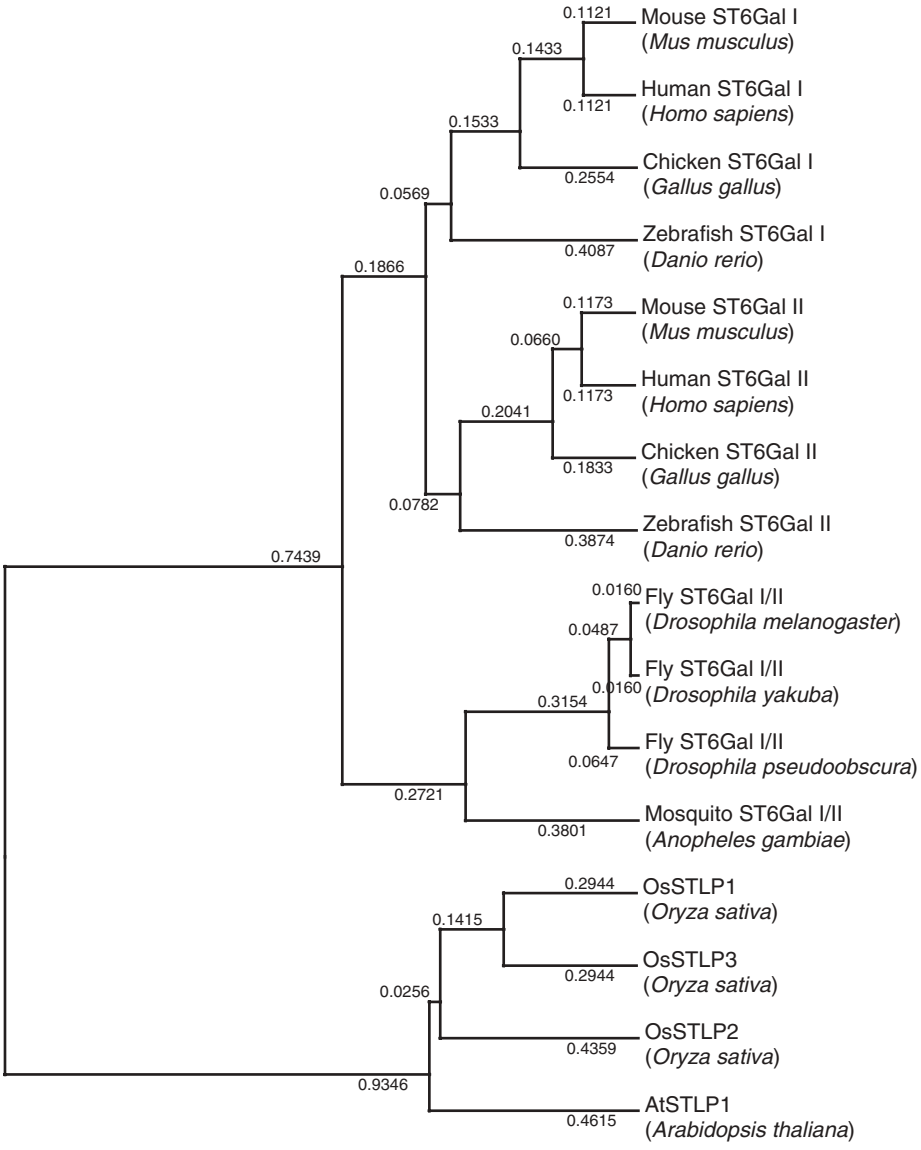

Fig. 6. **The phylogenetic tree of the ST6Gal family sialyltransferases and plant STLPs.** The phylogenetic tree was constructed by the unweighted pair group method with arithmetic mean (UPGMA).

KDO in rhamnogalacturonan II is linked to galacturonic acid through α2,3-linkage (*26*) and is unlikely to be transferred by STLPs which have α2,6-sialyltransferase–like activity. It is also possible that a new type of sialic acid that has not been detected by ordinary methods because of its very small amount and/or structural features exists in plants and is transferred by STLPs onto their physiological acceptor substrates.

It should be also noted that the terminal Galβ1,4GlcNAc structure that OsSTLP1 and 3 utilize as an acceptor substrate has not been detected in plants (*27*). Therefore, more preferable acceptor substrates of OsSTLP1 and 3 may exist in plants. It is also possible that STLPs are involved in the defense system of plants and recognize Galβ1, 4GlcNAc or a similar structure of pathogens. STLPs have type II transmembrane topology characteristic of many glycosyltransferases cloned to date, and are most likely to be members of a glycosyltransferase family. But if STLPs do not act as a glycosyltransferase in plants and have other physiological functions, elucidation of such functions may help predict the unknown functions which mammalian sialyltransferases may have. Identification of physiological substrates and elucidation of physiological functions of STLPs are problems to be solved.

From an evolutional point of view, there are two types of sialyltransferases. Those from eukaryotes have sialyl motifs, and those from prokaryotes do not. It seems that they have evolved independently. Among eukaryotic sialyltransferases, the β-galactoside α2,6-sialyltransferase family (ST6Gal family) is considered to have appeared first, followed by the GalNAc α2,6-sialyltransferase family (ST6GalNAc family), the β-galactoside α2,3-sialyltransferase family (ST3Gal family), and finally the α2,8-sialyltransferase family (ST8Sia family) (*18*). At present, neither sialyltransferase nor sialic acid has been detected in nematodes such as *Caenorhabditis elegans*, but sialyltransferases of the ST6Gal family have been detected in insects, suggesting that the first precursor gene of the animal sialyltransferase series might be a member of the ST6Gal family from insects (*11*, *18*). The insect sialyltransferases constitute a new group ortholog to the common ancestor (ST6Gal I/II) that was present before the split of animal ST6Gal I and II subfamilies (*18*). Both ST6Gal I and II utilize Galβ1,4GlcNAc structure as the acceptor substrate, but their enzymatic properties are different (*14*, *28*, *29*). The plant STLPs have sialyl motif–like sequences and some of them exhibit α2,6-sialyltransferase–like activity toward the Galβ1,4GlcNAc structure. We therefore investigated the evolutional relation between plant STLPs and animal α2,6-sialyltransferases. The phylogenic analysis reveals that plant STLPs branch out from the tree before the split of insect and vertebrate sialyltransferases, and constitute a new group (Fig. 6). The divergence of plant and metazoan is estimated to have taken place 1.2 billion years ago, whereas the appearance of sialyltransferase activity in animals is estimated to be a more recent event (*18*, *30*). In addition, it should be noted that OsSTLP genes and the AtSTLP1 gene consist of a single exon, whereas all animal sialyltransferase genes so far analyzed consist of multiple exons (*18*). These suggest that plant STLP genes and animal sialyltransferase genes evolved independently and in different manners. It is not clear whether plant STLPs and animal sialyltransferases actually share a common ancestor, but if so, it seems that they split at very early stage from the ancestor.

At present, one promising host for the production of therapeutically valuable mammalian proteins is plant. Unlike in bacterial systems, mammalian proteins are glycosylated on the proper glycosylation sites in a plant system. However, the fact that the structure of plant *N*-glycans differs from those of mammals may affect the biological activity of plant-made mammalian glycoproteins and/or cause undesirable immune responses in mammals. The most critical difference is the absence of sialic acid on plant glycoconjugates. To obtain sialylated plant-made mammalian glycoproteins, the input and expression of several mammalian genes, such as genes related to CMP-sialic acid biosynthesis pathway, will be required. In part, the sialylation machinery present in plants may be available. In that case, sialyltransferase-like activity of plant STLPs would be a promising candidate for the sialyltransferase activity.

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