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Substrate Specificity and Molecular Cloning of the Lily Endo-β-Mannosidase Acting on *N*-Glycan

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Endo-β-mannosidase, which hydrolyzes the Manβ1-4GlcNAc linkage in the trimannosyl core structure of *N*-glycans, was recently purified to homogeneity from lily (*Lilium longiflorum*) flowers as a heterotrimer [Ishimizu, T., Sasaki, A., Okutani, S., Maeda, M., Yamagishi, M., and Hase, S. (2004) *J. Biol. Chem.* 279, 38555–38562]. Here, we describe the substrate specificity of the enzyme and cloning of its cDNA. The purified enzyme hydrolyzed pyridylaminated (PA-) Man_nManα1-6Manβ1-4GlcNAcβ1-4GlcNAc (n = 0–2) to Man_nManα1-6Man and GlcNAcβ1-4GlcNAc-PA. It did not hydrolyze PA-sugar chains containing Manα1-3Manβ and/or Xylβ1-2Manβ. The best substrate among the PA-sugar chains tested was Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA with a K_m value of 1.2 mM. However, the enzyme displayed a marked preference for the corresponding glycopeptide, Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc-peptide (K_m value 75 μM). These results indicate that the substrate recognition by the enzyme involves the peptide portion attached to the *N*-glycan. Sequence information on the purified enzyme was used to clone the corresponding cDNA. The monocotyledonous lily enzyme (952 amino acids) displays 68% identity to its dicotyledonous (*Arabidopsis thaliana*) homologue. Our results show that the heterotrimeric enzyme is encoded by a single gene that gives rise to three polypeptides following posttranslational proteolysis. The enzyme is ubiquitously expressed, suggesting that it has a general function such as processing or degrading *N*-glycans.

Key words: endo-β-mannosidase, mannosidase, *N*-glycan, plant, processing.

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; PA-, pyridylamino-; *p*NP, *p*-nitrophenyl. The structures and abbreviations for the sugar chains are listed in Fig. 1.

Several endo-type hydrolases that act in the vicinity of the reducing-end of *N*-glycans attached to proteins have been described in the literature. These include peptide *N*-glycanase (*1*), endo-β-*N*-acetylglucosaminidase (*2*) and endo-β-mannosidase (*3*, *4*). We previously reported the partial purification of endo-β-mannosidase from flowers of the monocotyledonous lily (*Lilium longiflorum*) plant and determined its substrate specificity (*3*). The enzyme hydrolyzed Man_nManα1-6Manβ1-4GlcNAcβ1-4GlcNAc-PA (-PA; pyridylaminated) (n = 0–2) to Man_nManα1-6Man and GlcNAcβ1-4GlcNAc-PA. However, the endo-β-mannosidase did not hydrolyze β1,4-mannohexaose or *p*-nitrophenyl β-mannoside (*p*NP β-Man), in contrast to β-mannanases and β-mannosidases, respectively. The lily enzyme also failed to hydrolyze *N*-glycans containing Manα1-3Manβ and/or Xylβ1-2Manβ structures. The complementary substrate specificities of the lily endo-β-mannosidase and an α-mannosidase such as jack bean α-mannosidase, which prefers a Manα1-3Manβ linkage for hydrolysis, suggest that these two enzymes cooperatively process or degrade the trimannosyl core *N*-glycan to generate the chitobiose structure as the *N*-glycan (*4*, *5*).

Recently, endo-β-mannosidase was purified to homogeneity from lily flower, and a cDNA encoding endo-β-mannosidase was cloned from the dicotyledonous plant, *Arabidopsis thaliana* (*4*). Sequence analysis showed that endo-β-mannosidase is plant-specific and should be classified into a new enzyme family distinct from other glycosidases, including β-mannosidase. We have now determined the substrate specificity of the purified lily endo-β-mannosidase. The enzyme recognizes several features of the substrate, including the peptide portion attached to the *N*-glycan. In addition, the molecular cloning of a cDNA encoding the endo-β-mannosidase from lily will be described.

MATERIALS AND METHODS

Materials—The lily endo-β-mannosidase was purified as described earlier (*4*). Jack bean α-mannosidase and almond peptide *N*-glycanase were purchased from Seikagaku Kogyo (Tokyo, Japan). The Shodex Asahipak NH2-P column (0.46 × 5 cm) was from Showa Denko (Tokyo, Japan). An Inertsil ODS-3 column (0.46 × 25 cm) was purchased from GL Sciences (Tokyo, Japan). PA-sugar chains listed in Fig. 1 were prepared as reported previously (*6*, *7*). The glycopeptides (M3B-peptides) were obtained from the pronase digests of ovomucoid from Japanese quail egg white and then purified by Sephadex G-25 gel chromatography. Analysis of this sample, using matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF MS) and pyridylamina-

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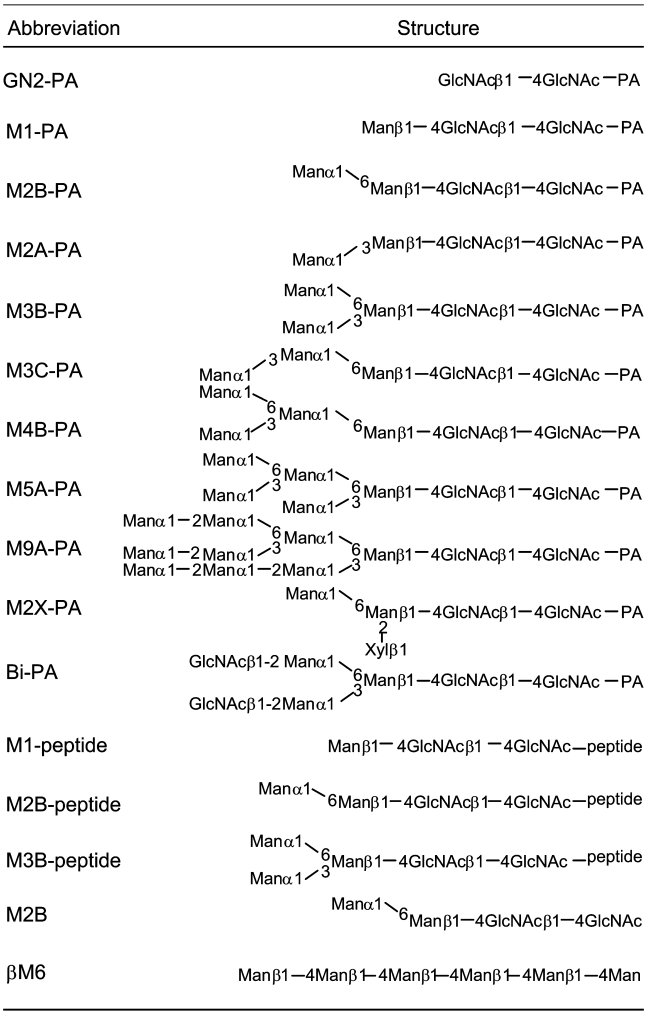

Fig. 1. **Sugar chain structures and abbreviations used in the present study.**

tion of the carbohydrate portion (*8*), showed it to consist of a mixture of glycopeptides of 4 to 6 amino acid residues with an M3B structure (data not shown). M2B- and M1-peptides were prepared by partial and complete digestion, respectively, of M3B-peptides with jack bean α-mannosidase. The amount of glycopeptide was quantified by measuring the fluorescence of PA-derivatives of sugar chains obtained by *N*-acetylation following hydrazinolyzation of the glycopeptide using Taka-amylase A as an internal standard. M2B was prepared by the hydrolysis of M2B-peptides with almond peptide *N*-glycanase. *p*NP α-Man and *p*NP β-Man were purchased from Nacalai Tesque (Kyoto, Japan), and Manα1-6Man was from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest grade commercially available.

Assay of Endo-β-Mannosidase Activity—Endo-β-mannosidase activity toward PA-sugar chains as substrate was measured as described previously (*3*, *4*). Briefly, the enzyme solution containing 12.5 μM PA-sugar chains in 16 μl of 0.16 M ammonium acetate buffer, pH 5.0, was incubated at 37°C for 30 min. The product, GN2-PA, was quantified by its fluorescence using size-fractionation HPLC.

When glycopeptides were used as substrate, the freeze-dried reaction mixture was hydrazinolyzed and then *N*-acetylated, and the products were pyridylaminated by the procedure reported previously (*8*). Excess reagents were removed with a Shodex Asahipak NH2-P column according to the method of Nakakita *et al*. (*9*). The resultant PA-sugar chains were quantified by reversed-phase HPLC using a known amount of PA-GlcNAc as a standard.

When M2B with a free reducing-end was used as a substrate, the hydrolysates were lyophilized and then pyridylaminated. The PA-sugar chains thus obtained were quantified by size-fractionation HPLC as described below. When *p*-nitrophenyl derivatives were used as substrates, the reaction products were quantified by measuring their absorbance at 400 nm.

The optimum pH for enzyme activity and stability was investigated using M2B-PA as a substrate in 0.2 M sodium citrate phosphate buffer, pH 2.5 to 8.0. One unit of enzyme activity was defined as the amount of enzyme that released 1 nmol of GN2-PA from M2B-PA per minute under the conditions used.

HPLC—Size-fractionation HPLC was performed on a Shodex Asahipak NH2-P column at a flow rate of 0.6 ml/min by isocratic elution. The eluent used was 3% (v/v) acetic acid in acetonitrile:water (400:85, v/v) adjusted to pH 7.3 with triethylamine. PA-derivatives were detected by their fluorescence using an excitation and an emission wavelength of 310 nm and 380 nm, respectively. HPLC was carried out at 25°C using a Beckman model 332 chromatograph equipped with a Hitachi model 650-10M fluorescence spectrophotometer.

Reversed-phase HPLC was performed on an Inertsil ODS-3 column at a flow rate of 1.5 ml/min. The eluents used were 0.1 M ammonium acetate buffer, pH 6.0 (Eluent A) and 0.1 M ammonium acetate buffer, pH 6.0, containing 1.0% 1-butanol (Eluent B). The column was equilibrated with 3% Eluent B. After injecting a sample, the proportion of Eluent B was increased linearly to 50% in 45 min. PA-sugar chains were detected by their fluorescence as described above.

Preparation of a Crude Enzyme Solution from Each Organ of Lily—Flowers, pistils, stamens, stems, leaves and bulbs of lily (5 g of each) were ground separately in a mortar under liquid nitrogen. Proteins were extracted by stirring with 20 ml of 0.1 M sodium phosphate buffer pH 6.0 at 4°C for 1 h. The homogenate was centrifuged at 46,000 × *g* at 4°C for 30 min, and the supernatant was used as a crude enzyme solution.

PCR-Based Cloning of a cDNA Encoding Lily Endo-β-Mannosidase—Total RNA was prepared from lily leaves as described previously (*10*). Poly (A)$^+$RNA was isolated from the total RNA using PolyATtract mRNA isolation system (Promega, Madison, WI). Double-stranded cDNA was synthesized from poly (A)$^+$RNA with a cDNA synthesis kit (Takara Bio, Otsu, Japan). Lily endo-β-mannosidase cDNA cloning strategy is shown in Fig. 2. Degenerate primers (F1, 5′-CNCARTAYGTNGARGGNTGGG-3′; F2, 5′-TAYGTNGARGGNTGGGAYTGG-3′; R1, 5′-CCYTGDATYTANACNCKNGT-3′; and R2, 5′-ATYTANACNCKNGTNCCRTC-3′) were designed based on internal amino acid sequences of the purified lily endo-β-mannosidase: TQYVEGWDW for F1 and F2; DGTRVYIQG for R1 and R2 (*4*). The primers were used for nested PCR using

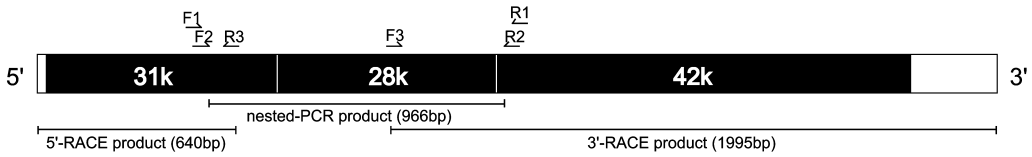

Fig. 2. **Cloning strategy.** The lily endo-β-mannosidase cDNA is depicted. The black rectangle represents the cDNA ORF, and the order of three polypeptides (28K, 31K, and 42K) in lily endo-β-mannosidase is shown. Open rectangles represent the cDNA 5′- and 3′-UTRs. The arrows indicate primers for PCR and RACE. PCR conditions are described in text. Bars represent PCR and RACE products.

Ex Taq polymerase (Takara Bio) according to the manufacturer's instructions. The first PCR employing primers F1 and R1 was performed using a program of 30 cycles at 98°C for 10 s, 52°C for 30 s, 70°C for 1 min with a final extension of 70°C for 10 min. The amplified products were used as template DNA for a second PCR. The same program was utilized for this second PCR using primers F2 and R2. The amplified PCR fragment was subcloned into pGEM-T Easy vector (Promega). Nucleotide sequences of the insert of several clones were determined by the dideoxynucleotide chain-terminating method using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and Applied Biosystems 377 DNA sequencer.

The primer F3 (5′-GGGGAGGAGGATTGGCTGAG-3′) was synthesized from the nucleotide sequence of the amplified PCR fragment. F3 was then used in 3′ rapid amplification of cDNA ends (3′ RACE) with a Generacer kit (Invitrogen, Carlsbad, CA). Five micrograms of total RNA was used for first-strand synthesis. PCR cycling consisted of 30 cycles at 98°C for 10 s, 62°C for 30 s, and 70°C for 2 min. Nucleotide sequence of the amplified fragment was determined as described above.

Primer R3 (5′-AGCTATCTTCACAGGCCCACTTGTG-3′) was synthesized from the nucleotide sequence of the amplified PCR fragment and used in 5′ rapid amplification of cDNA ends (5′ RACE) with a Generacer kit. Five micrograms of total RNA was used for first strand synthesis. PCR cycling consisted of 30 cycles at 98°C for 10 s, 58°C for 30 s, and 70°C for 1 min. Nucleotide sequence of the amplified fragment was determined as described above.

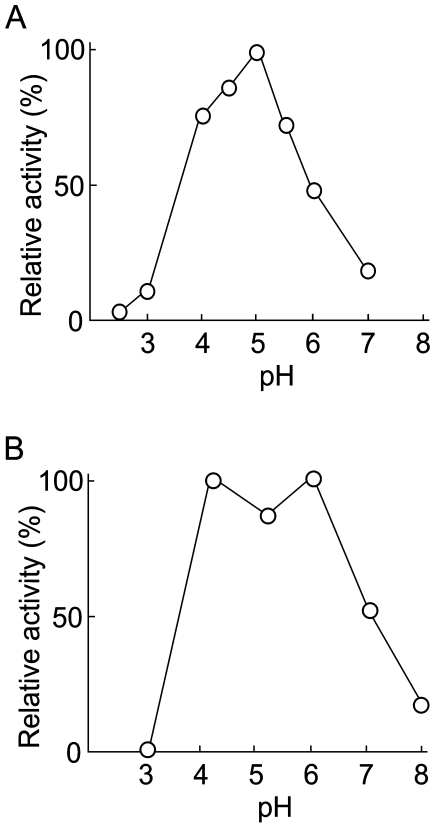

Fig. 3. **pH dependence and pH stability of the lily endo-β-mannosidase.** Enzyme activity toward M2B-PA was measured in 0.2 M sodium citrate phosphate buffer (pH 2.5 to 8.0). A, pH dependence of the enzyme. The value obtained at pH 5.0 was taken as 100%. B, Stability of the enzyme after 2 h of pre-incubation with each buffer. The value obtained at pH 6.0 was taken as 100%.

RESULTS AND DISCUSSION

Characterization of the Purified Lily Endo-β-Mannosidase—The purified lily enzyme displayed optimal activity at pH 5.0 when M2B-PA was used as a substrate (Fig. 3A). The enzyme exhibited no activity below pH 3 or above pH 7. The enzyme activity was relatively stable after incubation for 2 h between pH 4 and pH 6 (Fig. 3B), but unstable below pH 3 and above pH 8. The addition of either 10 mM EDTA or 2 mM Ca^{2+} had no affect on the activity of the purified enzyme. These characteristics were also observed for the partially purified lily enzyme (*3*) and the arabidopsis enzyme expressed in *Escherichia coli* (*4*).

Substrate Specificity of the Purified lily Endo-β-Mannosidase—The substrate specificity of the purified enzyme was studied using PA-sugar chains, free sugar chains, glycopeptides and *p*-nitrophenyl derivatives. The relative hydrolysis rates are summarized in Table 1. M2B-PA was the best substrate among the PA-sugar chains studied. M3C-PA and M4B-PA underwent considerable hydrolysis, and M1-PA was partially hydrolyzed. However, the lily enzyme did not hydrolyze PA-sugar chains containing the Manα1-3Manβ structure, such as M2A-PA, M3B-PA, M5A-PA, M9A-PA and Bi-PA. Furthermore, M2X-PA, containing Xylβ1-2Manβ, was not hydrolyzed. These results show that the purified enzyme recognizes not only the Manβ1-4GlcNAc linkage but also the adjacent Manα1-6Manβ linkage and/or hydroxyl groups at the C-2 and C-3 positions of the Manβ residue. The purified enzyme did not hydrolyze β1,4-mannohexaose, indicating that the activity was different from β-mannanase. In addition, neither *p*NP α-Man nor *p*NP β-Man was modified by the lily enzyme. The results are similar to those obtained with the partially purified lily enzyme (*3*) and the recombinant arabidopsis enzyme (*4*).

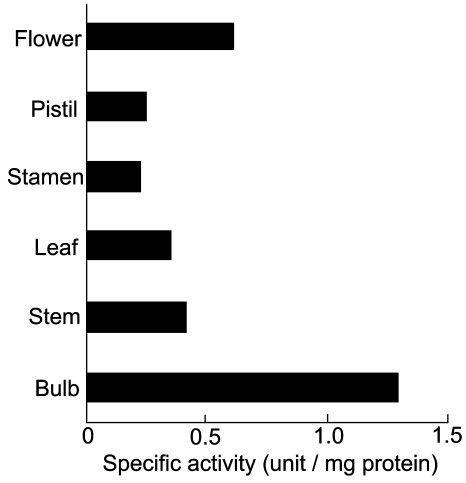

Fig. 4. **Endo-β-mannosidase activities in each lily organ. Specific activities are shown as the mean of three independent experiments.** The amount of protein was determined using the BCA protein assay reagent with bovine serum albumin as a standard.

Table 1. **Substrate specificity of the purified endo-b-mannosidase. Relative hydrolysis rates to that of M2B-PA are shown.**

Substrate	Hydrolysis rate (%)
M2B-PA	100
M3C-PA	48
M4B-PA	42
M1-PA	4.0
M2A-PA	<0.01
M2X-PA	<0.01
M3B-PA	<0.01
M5A-PA	<0.01
M9A-PA	<0.01
Bi-PA	<0.01
M2B	86
M2B-peptide	300
M1-peptide	9.8
M3B-peptide	<0.01
*p*NP α-Man	<0.01
*p*NP β-Man	<0.01
βM6	<0.01

The hydrolysis rates of M4B-PA by the lily and arabidopsis enzymes differed, and this may be due to the amino acid sequence difference between the two enzymes. In this study we further analyzed aglycon specificities. M2B, with a free reducing-end, was hydrolyzed at a slightly slower rate than M2B-PA. Furthermore, M2B-peptide was hydrolyzed 3 times faster than M2B-PA. The apparent K_m values for M2B-PA and M2B-peptide were 1.2 mM and 75 μM, respectively. The purified enzyme hydrolyzed M1-peptide 2.5 times faster than the corresponding PA-sugar chain. These results indicate that the enzyme recognizes a region of the substrate molecule around the reducing end of the *N*-glycan, including the peptide portion. Molecular recognition across a large region of the substrate was also observed for other carbohydrate-related enzymes such as cytosolic α-mannosidase (*11*) and brain-type β-galactosyltransferase (*12*). The enzyme may act directly on the *N*-glycans of glycoproteins, such as S-RNases, to produce a chitobiose structure (*5*).

Distribution of Endo-β-Mannosidase in the Lily Organs—The distribution of endo-β-mannosidase among lily organs was investigated. The specific activity of the crude enzyme prepared from each organ is shown in Fig. 4. Endo-β-mannosidase activity was detected in all the organs investigated. The specific activity was highest in the bulb, but it was only two times that in the flower. A similar expression pattern was observed for the arabidopsis endo-β-mannosidase gene (At1g09010) (*13*). Although it showed stronger expression in the silique, it was expressed in all organs investigated (young leaf, leaf, stem, bud, flower, silique, and root). The higher expression of endo-β-mannosidase in storage tissues (bulb or silique) may relate to the accumulation of storage proteins, because the *Arabidopsis* mutant harboring T-DNA in a gene encoding glucosidase I, which is associated with *N*-glycan trimming, accumulated a low level of storage proteins in its seeds (*14*). The ubiquitous distribution of endo-β-mannosidase suggests that, rather than an organ-specific role, it may have a common function in all organs, such as *N*-glycan degradation and/or processing.

Molecular Cloning of cDNA Encoding the Lily Endo-β-Mannosidase—The purified lily enzyme was composed of three polypeptides with molecular masses of 28 kDa, 31 kDa and 42 kDa. *N*-terminal and internal amino acid sequences were determined (*4*). All sequences were homologous to the arabidopsis endo-β-mannosidase expressed in *E. coli* as a single polypeptide (*4*). These results suggest that the lily endo-β-mannosidase is encoded by a single cDNA and that the translation product is subject to proteolytic processing. A segment of the *N*-terminal amino acid sequence of the 42-kDa polypeptide (ETEDPSQYLDGTRVYIQGSMWEGFA) and internal amino acid sequence of the 31-kDa polypeptide (DVATQYVEGWDW) were used to design a degenerate pair of nested PCR primers (based on the underlined sequences) (Fig. 2). Lily leaf cDNA was used as a template for the PCR. A specific 966-bp fragment was amplified that encoded 5 peptide fragments previously obtained from a lysylendopeptidase digest of the purified endo-β-mannosidase (*4*). The complete cDNA was subsequently obtained by a combination of 3′- and 5′-RACE strategies using primers designed from the nucleotide sequence of the 966-bp PCR product (Fig. 2). The resulting 640-bp fragment from 5′-RACE contained a putative start codon. The 3′-RACE product (1995-bp) contained a stop codon and a 53-bp poly $(A)^+$ tail. Nucleotide sequencing of the cDNA encoding endo-β-mannosidase revealed a 2,859-bp open reading frame encoding a polypeptide of 952 amino acid residues with a predicted molecular mass of 108 kDa (Fig. 5). This molecular mass roughly corresponded to the sum of the three polypeptides of the purified lily endo-β-mannosidase. All the amino acid sequences determined from the purified enzyme (*4*) were present in the deduced amino acid sequence of the lily endo-β-mannosidase. We therefore conclude that the lily endo-β-mannosidase is encoded by a single gene and that posttranslational proteolytic digestion gives the mature enzyme. At least three proteolytic sites exist in the lily enzyme, as shown in Fig. 5.

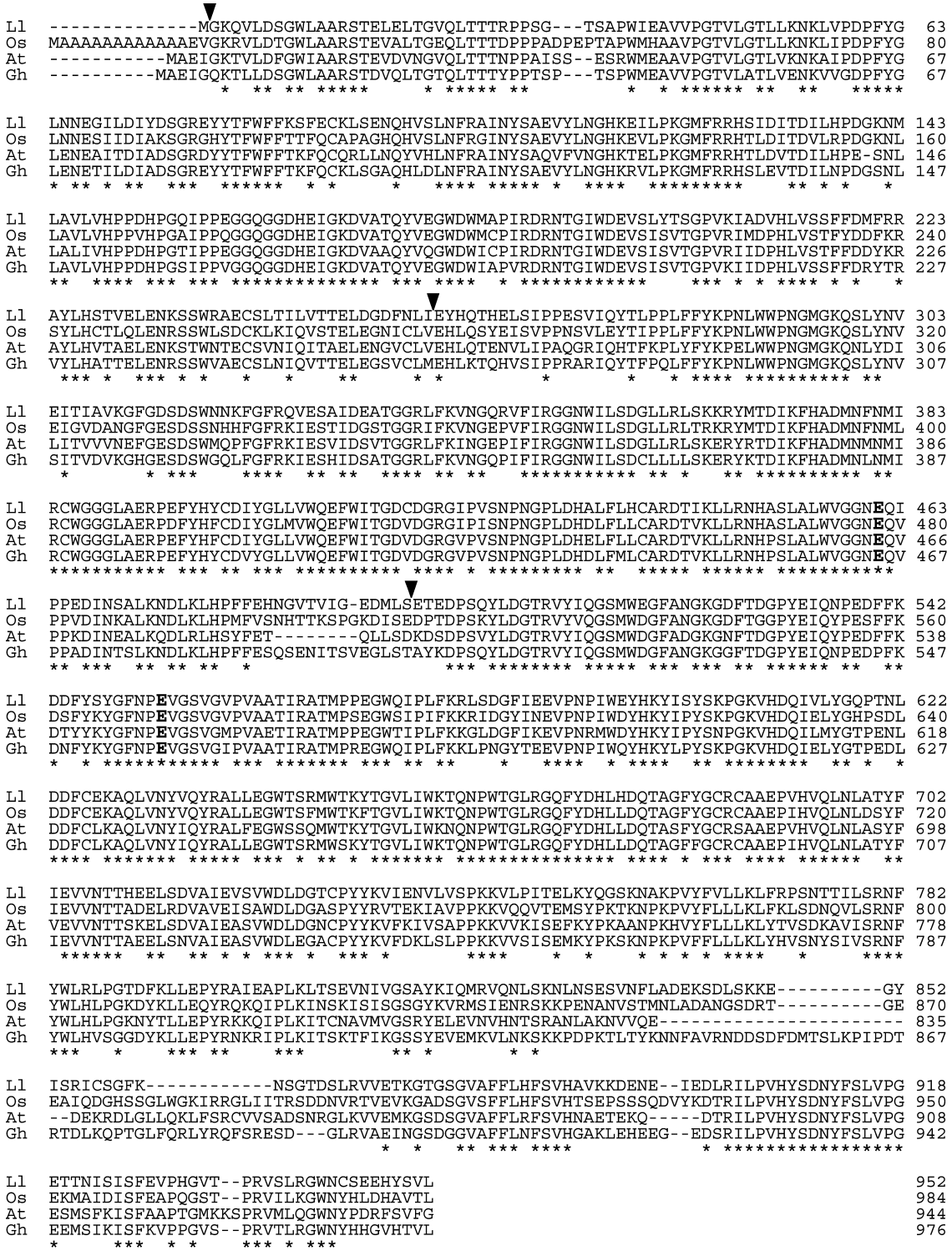

Fig. 5. **Amino acid sequence alignment of the lily endo-β-mannosidase and plant homologues.** Sequences were aligned using the ClustalW program (*15*). The conserved amino acid residues are marked by asterisks. Gaps are marked by dashes. Putative proteolytic sites are shown by an inverted triangle. The proposed catalytic nucleophile (Glu-553) and proton donor (Glu-461) are showed in bold. Ll, *Lilium longiflorum* endo-β-mannosidase (AB185918); Os, *Oryza sativa* putative glycosyl hydrolase (AK119412/AC107085) (*16*); At, *Arabidopsis thaliana* endo-β-mannosidase (AB122060) (*4*); Gh, *Gossipium hirsutum* putative endo-β-mannosidase (AY187062).

Comparison of the lily (monocotyledonous) enzyme with the arabidopsis (dicotyledonous) enzyme showed them to possess a high level of sequence identity (68%). An amino acid sequence alignment of the lily and arabidopsis endo-β-mannosidases and their homologues (putative glycosyl hydrolases) from *Oryza sativa* and *Gossipium hirsutum* is shown in Fig. 5. Conserved amino acid residues are found throughout the length of the polypep-

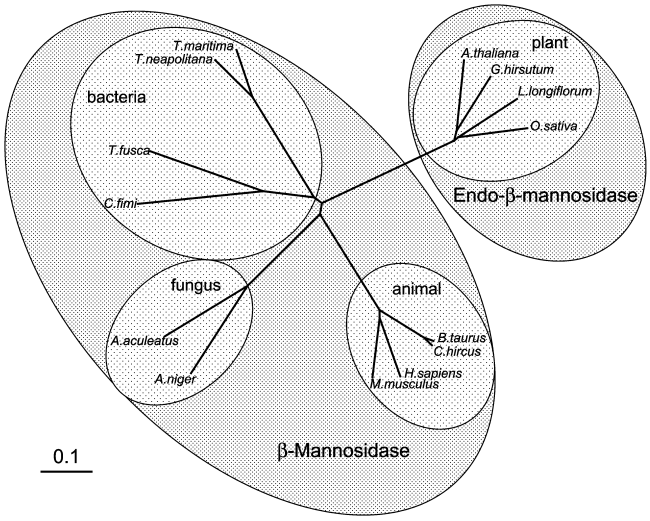

Fig. 6. **Neighbor-joining phylogenetic tree of amino acid sequences of endo-β-mannosidases and β-mannosidases.** The phylogenetic tree, based on the full-length amino acid sequence, was constructed using a neighbor-joining method (*17*). Sequences of the β-mannosidases are from *Bos taurus* (U17432) (*18*), *Capra hircus* (U46067) (*19*), *Homo sapiens* (U60337) (*20*), *Mus musculus* (AF306557) (*21*), *Aspergillus aculeatus* (AB015509) (*22*), *Aspergillus niger* (AJ251874) (*23*), *Thermotoga maritima* (AE001806) (*24*), *Thermotoga neapolitana* (AY033395) (*24*), *Thermobifida fusca* (AJ489440) (*25*) and *Cellulomonas fimi* (AF126472) (*26*). The *bar* represents the number of nucleotide substitutions.

tide chain. The proposed proton donor and nucleophile of the arabidopsis enzyme (Glu-461 and Glu-553, respectively), which are critical for enzyme activity (*4*), were also conserved in the four sequences. The sequence identity between the four proteins ranges from 66 to 73%, indicating that the two putative glycosyl hydrolases have endo-β-mannosidase activity. Phylogenetic analysis of these four plant proteins showed they cluster together and are clearly distinguished from β-mannosidases of animal, fungal and bacterial origin (Fig. 6). In conclusion, our sequence analysis reveals that, as suggested previously (*4*), endo-β-mannosidase is a plant-specific enzyme. The phylogenetic tree also supports the idea that the *O. sativa* and *G. hirsutum* proteins are endo-β-mannosidases.

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