Maize Tapetum Xylanase Is Synthesized as a Precursor, Processed and Activated by a Serine Protease, and Deposited on the Pollen*

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Pollen coat contains ingredients that interact with the stigma surface during sexual reproduction. In maize (Zea mays L.) pollen coat, the predominant protein is a 35-kDa endoxylanase, whose mRNA is located in the tapetum cells enclosing the maturing pollen in the anthers. This 2.0-kb mRNA was found to have an open reading frame of 1,635 nucleotides encoding a 60-kDa pre-xylanase. In developing anthers, the pre-xylanase protein appeared prior to the 35-kDa xylanase protein and enzyme activity and then peaked and declined, whereas the 35-kDa xylanase protein and activity continued to increase until anther maturation. An acid protease in the anther extract converted the inactive pre-xylanase to the active 35-kDa xylanase in vitro. The protease activity was inhibited by inhibitors of serine proteases but unaffected by inhibitors of cysteine, aspartic, or metallic proteases. Sequence analysis revealed that the 60-kDa pre-xylanase was converted to the 35-kDa xylanase with the removal of 198 and 48 residues from the N and C termini, respectively. During in vitro and in vivo conversions, no intermediates of 60–35 kDa were observed, and the 35-kDa xylanase was highly stable. The pre-xylanase was localized in the tapetum-containing anther wall, whereas the 35-kDa xylanase was found in the pollen coat. The significance of having a large non-active pre-xylanase and the mode of transfer of the xylanase to the pollen are discussed. A gene encoding the barley (Hordeum vulgare L.) tapetum xylanase was cloned; this gene and the gene encoding the seed aleurone-layer xylanase had strict tissue-specific expressions.

A major step in sexual reproduction in plants is the interaction between the male gamete-containing pollen and the pollen-receiving stigma in flowers (1–4). This interaction manifests at the contact between the pollen coat and the surface structures of the stigma. The coat of pollen contains special constituents that are essential to the initial sexual contact and thus the success of fertilization. Its chemical compositions vary, depending on the species. In insect or self-pollinating species, the coat is thin (13, 14), and major biochemical studies have been carried out only with maize (15). The coat of maize pollen contains undefined neutral lipids and a predominant protein. This protein is a 35-kDa xylanase (EC 3.2.1.8). Upon contact with the stigma surface, the pollen would release the xylanase to hydrolyze the xylan on the stigma surface. The hydrolysis would generate a gap on the stigma for the pollen tube to penetrate into the pollen tube track to reach the ovules.

The 35-kDa xylanase on the maize pollen coat has been extracted and purified to homogeneity (15). The enzyme is an active endoxylanase, as it catalyzes the in vitro hydrolysis of internal xylose-xylose linkages in oat-spelt xylan. Its gene has been cloned with use of reverse transcriptase (RT)1-PCR on the basis of its N-terminal amino acid sequence. The transcript is present in the tapetum cells enclosing the pollen locule in the anthers and is absent in the microspore or pollen interior. At the late stage of anther development, the tapetum cells lyse and deposit the xylanase onto the pollen, where it becomes the predominant protein. The xylanase transcript has 2.0 kb, which is very long for encoding the xylanase of 35 kDa. Initial sequencing of the transcript showed an ORF of 933 nt, which could encode the 35-kDa xylanase completely and was separated from another ORF of 525 nt at the 5′ upstream region. We subsequently re-sequenced the transcript and the gDNA (to be shown in this report) and found that the two ORFs are actually linked in-frame to be a longer ORF of 1,635 nt. The protein encoded by this ORF and its relationship to the active xylanase were unknown and have been studied by us.

A recent report (16) showed that during germination of barley seed, the aleurone layer contains a long xylanase-mRNA similar to that in the maize tapetum reported earlier (15). This barley mRNA produces a large precursor, which is processed to become an active xylanase of about 30–34 kDa. The barley aleurone xylanase is released at a late stage of germination to the adjacent storage endosperm, where it hydrolyzes the remaining cell walls to release sugars for germination. The maize and barley xylanases are in two different tissues and organs and carry out very dissimilar physiological functions. Their
relationship at the gene and protein levels was unknown and has been investigated by us.

In this report, we present our findings on the maize tapetum-pollen xylanase. The xylanase is synthesized initially as a large, inactive 60-kDa pre-xylanase, which is processed to become the active 35-kDa xylanase. This is the first tapetum-exported enzyme shown to have undergone an extensive processing and activation. The processing occurred by an acidic serine protease, which is highly specific at defined sites at both the N and C termini of the pre-xylanase. We also describe the cloning of a barley gene encoding the tapetum xylanase. This gene and the gene encoding the aleurone-layer xylanases have strict tissue-specific expressions.

**EXPERIMENTAL PROCEDURES**

**Plant Materials—Maize (Zea mays L.; B73) plants were grown in a greenhouse at 28 °C with a 14/10-h day/night cycle (15). Pollen was collected on sunny mornings when the anthers had just opened. Anthers of five developmental stages were obtained on the basis of the following criteria. At stage I, the anthers was still embedded in the shoot apex. The anthers filled up about one-third of each floret. Each microspore mother cell had undergone meiosis to produce a tetrad of microspores, which were still encased within a callous wall. At stage II, part of the tassel was visible from the shoot. The anther had reached about one-half of the floret. Young microspores had been released from the dissolved callous wall, and the outer pollen wall (exine) had been synthesized. At stage III, the anther had protruded completely out of the top of the plant. The anthers filled up about two-thirds of the floret. The microspores had enlarged and contained multiple small vacuoles. The first mitosis had occurred, and the microspores were binucleate. At stage IV, the anthers filled up the floret completely. Second mitosis in the microspores had occurred, and the microspores were trinucleate. At stage V, the anthers became yellow. Some of the florets on the tassel were open, and the pollen was ready to be released.**

**Barley (Hordeum vulgare L.; cv. Himalaya) seed was soaked in water for several hours and allowed to germinate and grow on several layers of cheesecloth in a dark chamber at 16–18 °C. The aleurone layers were dissected from seed that had germinated for 1 to 10 days and combined as one sample for RT-PCR. Five-day-old whole seedlings were used for gDNA extraction. The seedlings were allowed to grow in potted soil in a greenhouse at 28 °C for various durations. Each reaction mixture was used to analyze for proteins (30 μl) by immunoblotting and xylanase activity (800 μl) by an enzyme assay (to be described). To test the effect of protease inhibitors on the protease hydrolysis of the 60-kDa pre-xylanase, the inhibitor was added to the protease reaction mixture described in the preceding paragraph and incubated for 4 h. The inhibitors and their final concentrations in the reaction mixture were as follows: aprotinin, 1.5 μM; phenylmethylsulfonyl fluoride, 1 mM; cystatin, 42 μM; E-64, 10 μM; pepstatin, 1 μM; trypsin inhibitor, 100 μM; and EDTA, 10 mM. To test the effect of pH on the protease hydrolysis of the 60-kDa pre-xylanase, the crude anther protease fraction was mixed with the partially purified 60-kDa pre-xylanase in a 0.1 M buffer at 30 °C for 2 h. The buffer included sodium acetate, pH 4, 4.5, 5, and 5.5; potassium phosphate, pH 6; and Tris-HCl, pH 7, 8, and 9. To test the protease activities in anthers of different developmental stages, an extract of 0.5 μl of total anther protein of each developmental stage was incubated with the partially purified 60-kDa pre-xylanase in 0.1 M sodium acetate, pH 5.0, at 30 °C for 2 h. The total anther extract was prepared by homogenizing 50 anthers in 1 ml of 0.05 M Hepes-NaOH, pH 7.5.**

**Determination of the Cleavage Sites of the 60-kDa Pre-xylanase in Its Conversion to the 35-kDa Xylanase—The proteins in the partially purified 60-kDa pre-xylanase, 55-kDa protein, and the pollen-coat (containing the 35-kDa xylanase) fractions were separated with the use of 12.5% (w/v) SDS-PAGE and small gels (8 × 8 cm). The gel slices containing the 60-, 55- and 35-kDa proteins were obtained. Each gel slice was cut vertically into ~1-mm pieces. The pieces were washed three times with 400 μl of 50% acetonitrile, air dried, and soaked in 50 mM ammonium bicarbonate for 15 min and then soaked in 100% acetonitrile for 5 min. The acetonitrile was removed, and the gel pieces were dried in a Speed Vac for 30 min. The gel pieces were rehydrated in 50 μl of a trypsin solution (0.01 μg/μl) and incubated at 37 °C for 16 h. A 50-μl solution of 50% acetonitrile and 5% trifluoroacetic acid was added, and the mixture was incubated at 37 °C for 1 h. The supernatant containing the tryptic-digested fragments was collected after centrifugation at 5,000 × g for 15 min. The gel pieces in the pellet were re-extracted once with acetonitrile and trifluoroacetic acid, and the combined supernatants were dried with the use of a Speed Vac. The extracted protein fragments were subjected to mass spectrometry (MALDI-TOF) analysis with the use of Voyager DE-STR (PerSeptive Biosystems, Framingham, MA) according to the manufacturer’s instructions. All the major fragments detected between 900 and 2,400 m/z (920.53, 1150.61, 1914.81, 1975.32, 1992.01, 2034.38, and 2388.13) in the 35-kDa xylanase sample were also found in the 35-kDa pre-xylanase sample but not in the 60-kDa pre-xylanase sample. A fragment of 565.42 m/z was present in the 35-kDa xylanase sample but not in the 60-kDa pre-xylanase sample; this peak corresponded to the RASATTD at the C terminus of the 35-kDa xylanase. The N-terminal sequence of the 35-kDa xylanase was determined by Edman degradation to be RLGFPPGNAVTEKIELLPA (15).**

**SDS-PAGE and Immunoblotting—All procedures followed those described earlier (15). Proteins were separated with the use of 12.5% (w/v) SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Blue and then destained. For immunoblot analyses, proteins fractionated on the gel were transferred to a nitrocellulose filter membrane and then subjected to immunodetection. Rabbit antibodies were raised against the purified recombinant protein of the mature 35-kDa xylanase (15).
Xylanase Activity Assay—RBB-xylan (4-O-methyl-D-glucurono-D-xylan linked covalently to Remazol Brilliant Blue R, obtained from Sigma) was used as the substrate. The xylanase reaction mixture of 900 μl contained an enzyme sample (either the anther or pollen extracts or the protease reaction mixture; see “Analyses of the Anther Protease Activity on the 60-kDa Pre-xylanase and the 55-kDa Protein”), 2.57 mg of RBB-xylan, and sodium acetate, pH 5.0, to make a final buffer concentration of 0.1 M. The reaction was allowed to proceed at 30 °C. At time intervals, an aliquot of 200 μl of the reaction mixture was mixed with 400 μl of 95% ethanol to terminate the reaction. The mixture was shaken gently and left at 24 °C for 30 min. After centrifugation at 16,000 × g for 10 min, the supernatant was collected, and its absorbance at 595 nm was determined with the use of a spectrophotometer. Each enzyme assay was monitored at four time intervals within a 12-h period to ensure linearity of the reaction. During the long incubation period, 0.5 μl of a protease inhibitor mixture (Sigma) was added to the xylanase reaction mixture every 2 h to prevent proteolytic activities.

DNA Sequencing—The inserted maize DNA in a xylanase genomic clone λ-pXYN (15) and a full-length cDNA clone pXYN-60 (to be described) were sequenced with internal primers by the DNA Sequencing Core Laboratory, University of Maine. A comparison of their sequences allowed us to determine the site of the intron.

RNA Extraction and cDNA Synthesis—Total RNA was extracted with the use of guanidine hydrochloride (18) from maize anthers of different developmental stages and from barley anthers and aleurone layers of combined developmental stages. For a nonspecific gene probe, a 653-bp fragment corresponding to a small 5′-terminal portion of the ORF and the 3′-UTR was amplified with the use of a 5′ primer, 5′-GCTCTAAGGTCTACCA-CAGGG-3′, and a 3′ primer, 5′-CCAGAACCGATGCTTCCCTC-3′. For a gene-specific probe, a 455-bp fragment corresponding to a small 5′-terminal portion of the ORF and the 3′-UTR was amplified with the use of a 5′ primer, 5′-AATTGGCGCCAGAGGCGC-3′, and a 3′ primer, 5′-TGCA-TGACTAAGGATTTTTC-3′. The fragments were purified with the use of Millipore Microcon YM-30 (Millipore, Bedford, MA) and then ^32P labeled with the use of a Multiprimer DNA labeling kit (Amersham Biosciences).

RT-PCR Analysis of HvXYN1 and HvXYN2 Transcripts in Barley Anthers and Aleurone Layers—Total RNA from barley anthers or aleurone layers of combined developmental stages was used to synthesize first-strand cDNA. The cDNAs were used as a template to do PCR with the use of one gene-specific primer described in the preceding paragraph. The PCR fragments were analyzed with a 1% agarose gel. For a barley actin gene, a 5′ primer (5′-GGTATCTCCCTACCAACGGC-3′) and a 3′ primer (5′-CAGACGTGTAGCCTCTCTCTAG-3′) were designed from two conserved nucleotide sequence regions of a maize actin gene (GenBankTM, M65681) and a barley partial actin EST (GenBankTM, AF214440), respectively.

Genomic DNA Blot Hybridization of HvXYN1 and HvXYN2—gDNA was extracted from barley seedlings by cetyltrimethylammonium bromide (20). Each hybridization reaction mixture contained 30 μg of gDNA and one restriction enzyme (to be listed in the legend of Fig. 8). After digestion, the DNA fragments were separated with the use of a 1% agarose gel in 0.2× TBE buffer containing 0.5% bovine serum albumin and washed with water twice, shaken in 1.5 M NaCl and 0.5 M NaOH for 1 h, and equilibrated in 1 M Tris-HCl, pH 8.0, and 1.5 M NaCl for 1 h. After equilibration, the DNA fragments were blotted onto a Hybond-N membrane (Amersham Biosciences). The membrane was pre-hybridized at 65 °C in potassium phosphate, pH 7.2, 7% SDS, 1% bovine serum albumin, and 0.1 M EDTA for 4 h, hybridized with ^32P-labeled gene-specific or gene-nonspecific probes overnight, and then washed with 2× SSC, 0.1% SDS for 20 min, 1× SSC, 0.1% SDS for 20 min, and 0.1× SSC, 0.1% SDS for 20 to 30 min, all at 65 °C.

RESULTS

The Maize 35-kDa Xylanase Transcript Has 2 kb and an ORF of 1,635 nt—We obtained the sequences of the gDNA and cDNA of the maize xylanase gene (ZmXYN1) after re-sequencing and revising a few misplaced bp of the sequences reported earlier (15). Fig. 1 shows a 2.1-kb gDNA sequence that encodes the complete transcript and has a short intron of 106 nt. This sequence and its 1.7-kb 5′ upstream promoter region can be found in GenBank™ (AF149016). The transcript has 2.0 kb, as deduced from the cDNA sequence and by RNase protection assay, and an ORF of 1,635 nt. If the entire ORF is used to encode a protein, the transcript would have 23 nt of 5′-UTR and about 300 nt of 3′-UTR. Within the ORF are 30 possible ATG codons, 21 of which are in-frame with the sequence corresponding to the known 35-kDa xylanase sequence (15). These 21 ATG codons are distributed throughout the ORF (Fig. 1). Seven of them are at the 5′ region preceding the sequence encoding the final 35-kDa xylanase (its N-terminal sequence is FPFG—referring to the amino acid sequence starting at FFPF shown in Fig. 1). Any of these seven ATG codons could be the initiation codon. If the first ATG codon is used, the protein would have 60.59 kDa, and if the sixth and seventh ATG codons are used, the protein would have 41.37 and 40.66 kDa, respectively. We used in vitro transcription to test whether the transcript produces a large precursor of about 60 kDa or a precursor of a molecular mass fairly similar to that of the 35-kDa xylanase.
FIG. 1. Nucleotide and deduced amino acid sequences of a maize xylanase gene, ZmXYN1. A 2.1-kb sequence corresponding to the mRNA plus an intron is indicated. This sequence and its upstream 1.7-kb sequence are available in GenBank™ (AF149016). The transcript contains 30 possible ATG codons, 21 of which are in-frame with the known 35-kDa xylanase sequence. The positions of these ATG codons are indicated with vertical bars on the line symbolizing the DNA, and those vertical bars with a circle at the top represent the in-frame ATG codons. The two vertical bars labeled with ATG are the first and the sixth in-frame ATG codons described under “Results.” The corresponding 60-kDa pre-xylanase (544 residues) and the 35-kDa active xylanase (298 residues) are illustrated as longitudinal boxes. The DNA sequences cloned in pXYN-60 and pXYN-45 are shown. Two vertical arrows along the amino acid sequence indicate the locations of the proteolytic cuts of the pre-xylanase to form the active xylanase.
pXYN-60 Transcript Containing the Full-length ZmXYN1 ORF Produced a Major Protein of 60 kDa during in Vitro Translation—We constructed pXYN-60 containing the entire ORF, which would encode a protein of 60.59 kDa. The plasmid was subjected to in vitro transcription, and the transcript was used to do in vitro translation with the use of the wheat-germ system. The transcript produced one major protein of about 60 kDa and several minor and smaller proteins (Fig. 2). The molecular mass of this major protein estimated by its mobility in the current SDS-PAGE system was about 61 kDa; for convenience, we termed the protein a 60-kDa protein. Thus, the first ATG codon (calculated to produce a protein of 60.59 kDa), or less likely the second ATG codon (calculated to produce a protein of 58.52 kDa), was used as the initiation codon. A few other ATG codons downstream were also used, albeit less frequently, to initiate translations to generate smaller proteins. For comparison, we also constructed pXYN-45 containing the 3'1.1 kb of the ORF, which includes the two ATG codons preceding the sequence encoding the final 35-kDa xylanase (Fig. 1). The pXYN-45 transcript produced two major proteins, of about 45 and 40 kDa, and a few minor and smaller proteins (Fig. 2). In this transcript, apparently both the first ATG codon (calculated to produce a protein of 41.37 kDa) and the second ATG codon (calculated to produce a protein of 40.66 kDa) were used as the initiation codon. The major and minor proteins produced by the pXYN-45 transcript are identical in size to the minor proteins produced by the pXYN-60 transcript, and this similarity indicates that initiation of in vitro translation occurred at the same preferred ATG codons along the ORF. Overall, the findings show that the first ATG codon in the ZmXYN1 ORF is used to initiate translation in vivo to produce a 60-kDa precursor. The existence and properties of this 60-kDa precursor were then explored.

In Developing Anthers and Germinating Pollen, the Appearance of the 35-kDa Xylanase Protein, Its Putative 60-kDa Precursor, and Xylanase Activity Were Correlated—An anther consists of several layers of sporophytic cells. The innermost layer is the tapetum, which encloses the developing gametophytic microspores in the locule. Most of the constituents in the coat of the microspores are derived from the sporophytic tapetum. The predominant protein in the coat of mature maize pollen is an active xylanase of 35 kDa (15). We examined the developmental profiles of the 35-kDa xylanase protein, its putative 60-kDa

![Fig. 2. In Vitro translation products from pXYN-60 and pXYN-45 transcripts.](image-url)
precursor, and xylanase activity in the anthers. The amounts and the molecular varieties of the proteins in the total anther extract increased and reached the highest levels at stage 5, as revealed with the use of SDS-PAGE (Fig. 3A). The major proteins in mature pollen were fairly similar in molecular masses to those in stage-5 anthers. As the pollen germinated, the molecular varieties of the proteins remained very similar, whereas the amounts decreased (Fig. 3A).

Antibodies raised against the pollen-coat 35-kDa xylanase (15) were used to determine the amount of the xylanase protein in the total anther extracts (Fig. 3B). The 35-kDa xylanase protein was absent at stages 1 and 2 and appeared and increased from stages 3 to 5. It was present in mature pollen and decreased in germinated pollen.

In addition to the 35-kDa xylanase protein, two proteins of about 60 and 55 kDa in the anther extract were recognized by the antibodies (Fig. 3B). The 35-, 60-, and 55-kDa proteins were not recognized by the IgG from pre-immunized rabbits (data not shown). The 60-kDa protein was absent at stage 1 and appeared at stage 2; its level peaked at stage 3 and declined at stages 4 and 5. It was absent in mature or germinated pollen. The rise and fall of this 60-kDa protein in the anthers in comparison with the delayed appearance and the continuous rise of the 35-kDa xylanase protein could signify a precursor-product relationship.

The antibody-recognized 55-kDa protein was already present in the anther extract at stage 1, and its amount in the anthers remained fairly constant from stages 1 to 4 and was reduced at stage 5. It was also present in mature pollen in an amount similar to that in stage-5 anthers and disappeared in germinated pollen. This 55-kDa protein was present in the microspore/pollen interior and contained no xylanase activity (to be described later). Its identity is unknown, and it had no apparent relationship with the 35-kDa xylanase in the pollen coat other than its being recognized by the antibodies.

Xylanase activity in the total anther extract was absent at stages 1 and 2 and rose continuously from stage 3 to stage 5, when the anther matured (Fig. 3C). Xylanase activity of a magnitude similar to that in stage-5 anthers was found in mature pollen (Fig. 3C). Essentially all the xylanase activity in mature pollen was present in the coat fraction, and no activity was found in the interior fraction (to be described later). The activity in the total extract of mature pollen decreased by about 50% in pollen (plus tube) that had germinated for 15 and 60 min when the length of the tube was about 0.5 and 10 times the diameter of the pollen, respectively. The developmental profile of xylanase activity followed that of the 35-kDa xylanase protein but not of the putative 60-kDa xylanase precursor, which would have no xylanase activity.

**Mass Spectrometry (MALDI-TOF) Analyses Revealed That the 60-kDa Protein and the 35-kDa Xylanase Shared Many Identical Trypsin Fragments**—If the 60-kDa protein is a precursor of the 35-kDa xylanase, they should share an identical sequence segment. This possibility was examined. The 60- and 55-kDa proteins in partially purified fractions and the 35-kDa xylanase extracted from the mature pollen surface were resolved with the use of SDS-PAGE, and the proteins in the gel were subjected to trypsin digestion and then mass spectrometry (MALDI-TOF) analyses. Seven identical, major fragments present in the 35-kDa xylanase sample were also found in the 60-kDa protein sample. They represented trypsin fragments of the 35-kDa xylanase on the basis of analyses of its amino acid sequence (Fig. 1) with the use of the website, prospector.ucsf.edu/ucsfhtlm4.0/msfit.htm. These fragments were not found in the 55-kDa protein sample. On the basis of the N- and C-terminal sequences of the 35-kDa xylanase (see “Experimental Procedures”), we conclude that the 35-kDa xylanase (298 residues) was produced from the 60-kDa pre-xylanase after the removal of 198 and 48 residues from the N and C termini, respectively (Fig. 1).

The 60-kDa Pre-xylanase Was Converted to the Active 35-kDa Xylanase In Vitro—The total extract of stage-2 anthers contained the antibody-recognized 55- and 60-kDa protein but minimal 35-kDa xylanase or xylanase activity (Fig. 3, B and C). The two proteins in stage-2 anther extract were partially separated from each other, and the possible conversion of these two proteins to the active 35-kDa xylanase in vitro was examined. They were incubated in a medium of pH 5 with a minimal amount of the 16,000 × g supernatant of the total extract of stage-3 anthers. This anther extract supernatant was a source of the protease that converted the 60-kDa pre-xylanase to the 35-kDa xylanase (Fig. 3). A minimal amount of this supernatant was used, and so its contents of the 55- and 60-kDa proteins, as well as the 35-kDa xylanase, were negligible in comparison with the amount of the added 55- or 60-kDa protein. During the incubation, the 60-kDa protein was converted to the 35-kDa xylanase protein concomitant with an increase in xylanase enzyme activity (Fig. 4). The conversion was complete after 2 h, and the 35-kDa xylanase protein persisted without further degradation after 4 and 17 h (Fig. 4). In contrast, the 55-kDa protein remained intact after 2 h of incubation, and no 35-kDa protein or xylanase activity appeared during the incubation. The findings provide corroboratory evidence that the 60-kDa protein is the xylanase precursor possessing no enzymic activity.

During the in vitro conversion of the 60-kDa pre-xylanase to the 35-kDa xylanase, no intermediates of 60–35 kDa were detected (see Fig. 4 and Fig. 5). The lack of intermediates in
in vivo was also recognized (Fig. 3). The 35-kDa xylanase after its formation in vitro and in vivo is quite resistant to the native protease. This statement is made, because (a) the 35-kDa xylanase in the pollen coat was resolved as a sharp protein band on a SDS-PAGE gel (to be shown in Fig. 6); (b) N-terminal sequencing of this protein revealed only one N-terminal sequence (data not shown); and (c) once formed in vivo or in vitro, the 35-kDa xylanase was resistant to further degradation (see Figs. 3–5). The lack of intermediates and the high stability of the 35-kDa xylanase suggest that the proteolysis of the 60-kDa pre-xylanase to the 35-kDa xylanase was very specific.

The Processing Protease Was an Acidic Serine Protease and Appeared Concomitantly with the 35-kDa Xylanase during Anther Development—The activities of the protease that converted the 60-kDa pre-xylanase to the active 35-kDa xylanase during anther development (Fig. 3B). At stage 2, the 60-kDa pre-xylanase appeared, and the 35-kDa xylanase and the protease were absent. Then at stage 3, the protease appeared and converted the accumulating 60-kDa pre-xylanase to the active 35-kDa xylanase, concomitantly with the appearance of xylanase activity.

The 60-kDa Pre-xylanase Was Localized in the Anther Wall, the 35-kDa Xylanase Was Localized in the Microspore/Pollen Coat, and the Antibody-recognized 55-kDa Protein Was Localized in the Microspore/Pollen Interior—Stage-4 anthers contained the 60-kDa pre-xylanase, the 35-kDa xylanase, and the antibody-recognized 55-kDa protein (Fig. 3). They were dissected into an anther wall fraction (which included the tapetum) and a microspore fraction. The microspores were separated further into a coat fraction (whose constituents were derived largely from the tapetum) and an interior fraction. The proteins in the various fractions were analyzed by immunoblotting after SDS-PAGE (Fig. 6). The 60-kDa pre-xylanase was located exclusively in the anther wall fraction, whereas the 35-kDa xylanase and the 55-kDa protein were present only in the microspores. Of the microspores, the 35-kDa xylanase and the 55-kDa protein were restricted to the coat fraction and the interior fraction, respectively. Similarly, in mature pollen, the 35-kDa xylanase and the 55-kDa protein were present in the coat fraction and the interior fraction, respectively. The mature pollen coat fraction contained 90–98% (varied from experiment to experiment) of the xylanase activity, and the remaining activities were recovered in the pollen interior fraction, presumably representing the leftover 35-kDa xylanase (data not shown).

The presence of the 60-kDa pre-xylanase in the anther wall is consistent with the earlier finding that the mRNA is present in the tapetum cells (15). This pre-xylanase is synthesized and apparently processed by a serine protease in the tapetum cells immediately before or after cell lysis, such that the 35-kDa xylanase can be recovered only in the coat of the microspores or
pollen. The 55-kDa protein was already present in stage-1 anthers and persisted throughout development (Fig. 3). Apparently, it was synthesized early on inside the microspores and remained there when the microspores became mature pollen. Its identity is unknown.

There is No Apparent Sequence Signal in the 60-kDa Pre-xylanase to Have Targeted the Protein to Specific Organelles—This was revealed with the use of the website, psort.nibb.ac.jp/form.html. This website program indicates a possibility that the protein is targeted to the peroxisomes because of the presence the tripeptide AKL in the 60-kDa pre-xylanase and the 35-kDa xylanase. We discount this possibility, because the tripeptide AKL, unlike those in peroxisomal proteins, is not present at or very near the C terminus, and because we (data not shown), as well as others (13, 14), did not observe apparent peroxisomes in the tapetum cells. We subfractionated the extract of stage-3 anthers by differential centrifugation after the microspores (and the attached 35-kDa xylanase) in the extract had been removed by a short and low speed centrifugation. The differential centrifugation partitioned the plastids, the mitochondria, the endoplasmic reticulum, and the peroxisomes into pellets (22), and the 60-kDa protein was recovered in the 100,000 g supernatant (data not shown). Presumably, the 60-kDa pre-xylanase is synthesized and located in the cytosol.

The Tapetum Xylanase and the Aleurone-layer Xylanase in Barley Were Encoded by Different Genes—A recent report indicates that in the aleurone layer of germinated barley seed, a xylanase is synthesized initially as a larger precursor (16), a process similar to that of the xylanase in the maize floral tapetum. We explored whether the two tissues of very diverse origins in cereals used the same or different genes to synthesize the xylanase. On the basis of the maize tapetum-expressed gene (ZmXYN1) and the barley aleurone-layer-expressed gene (HvXYN1) (16), we designed primers to obtain a RT-PCR fragment of a putative barley tapetum-expressed xylanase gene (termed HvXYN2) (GenBank™, AF539980). The fragment would encode 319 residues of the C-terminal portion of the protein. Its amino acid sequence is more similar to ZmXYN1 than to HvXYN1 (Fig. 7, A and C). We tested the expressions of HvXYN1 and HvXYN2 in the anthers and the aleurone layers of barley. Fig. 7B shows that HvXYN2 transcript was restricted to the anthers, presumably the tapetum, whereas HvXYN1 transcript was present only in the aleurone layer. Thus, the xylanases of the tapetum and aleurone layers in barley are products of different genes, whose expressions are highly tissue-specific.

Southern blot hybridization of barley DNA was carried out with the use of seven to nine restriction enzymes and two sets of probes, one set being the gene-specific 3′-UTR sequences and another set being the ORF sequences of HvXYN1 and HvXYN2 (Fig. 8). The two gene-specific probes detected one restriction fragment from each of the restriction enzyme reactions. For HvXYN2, the ORF probe detected one to two restriction frag-

\[ \text{RT-PCR of the barley anther and aleurone-layer RNA with the use of gene-specific primers of HvXYN1 and HvXYN2. A barley actin gene, which is known to be expressed ubiquitously, was used to monitor that about the same amount of its transcript was present in the two samples.}
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\[ C, \text{ a phylogenetic tree of eight cereal xylanases constructed on the basis of their amino acid sequences by the GrowTree function in SeqWeb, Version 2, of the GCG Wisconsin Package (Accelrys, Burlington, MA). TaXYN1 (AAD41893) is derived from a wheat gDNA sequence. OsXYN1.1 (BAB64626) and OsXYL1.2 (BAB64624) are derived from gDNA sequences; the 3′ region of OsXYN1.1 is also present as ESTs from seedling roots, ZmXYN2 (AY104702 and TC126452) and TaXYN2 (TC34113) are derived from ESTs from maize (ears, anthers plus pollen, and seed) and wheat spike, respectively. The scale represents the number of substitutions per 100 residues.} \]
HvXYN gene, show that in barley, the tapetum xylanase is encoded by one unknown. Because of a restriction map of the ORF probe (map not shown).

ments in each of the enzyme reactions (Fig. 8), as expected because of a restriction map of the ORF probe (map not shown). For HvXYN1, the ORF probe identified in most of the enzyme reactions one to two extra restriction fragments other than those expected from digestion of HvXYN1 alone. The findings show that in barley, the tapetum xylanase is encoded by one gene, HvXYN2, whereas the aleurone-layer xylanase is encoded by HvXYN1 and likely one or more related genes.

A phylogenetic tree of the amino acid sequences of eight cereal xylanases from the present report and a BLAST search reveals at least three groups of xylanases (Fig. 7C). The tapetum xylanases from maize (ZmXYN1) and barley (HvXYN2) form a group relatively distinct from the other two groups. The aleurone-layer xylanases from barley (HvXYN1) and a xylanase from wheat (TaXYN1) form another group. Thus, the barley tapetum xylanase is more related to the barley aleurone-layer xylanase (HvXYN2) than to the maize tapetum xylanase. Two rice xylanases (OsXYN1.1 and OsXYN1.2) form a third group. Genes encoding the two rice enzymes are in tandem (~3 kb apart) on chromosome 1, and one of them is also available as short ESTs from a seedling-root cDNA library (see legend of Fig. 7). The expression of the two rice genes and the properties of their encoded xylanases have not been reported. Two xylanases derived from ESTs from maize (ZmXYN2, from anther plus pollen, ears, and seed) and wheat (TaXYN2, from spike) are distantly related to the two tapetum xylanases; their identities and relationship to the tapetum and other xylanases are unknown.

**DISCUSSION**

In flowers, the tapetum controls the maturation of the microspores to pollen. Several proteins known to be exported from the tapetum contain an obvious cleavable N-terminal signal peptide for the endoplasmic reticulum and presumably are secreted via the established protein secretory pathway. They include β-1,3-glucanase (23), lipid transfer protein (24), a glycine-rich protein (24), protease/amylase inhibitors (25), and A3 of unknown function (26). Their secretion occurs at an earlier stage of microspore development before lysis of the tapetum cells. In maize, neither the 60-kDa pre-xylanase nor the active 35-kDa xylanase has an apparent targeting signal sequence for the endoplasmic reticulum, and thus no pre-packaging of the to-be-transferred xylanase in vesicles is apparent. We envision that the pre-xylanase or xylanase is present in the cytosol and is transferred to the pollen surface after lysis of the tapetum cells.

The transfer and processing of the tapetum xylanase to the pollen surface in maize bears limited similarities to that of the tapetum oleosins in the insect or self-pollinating species of *Brassica* and *Arabidopsis* (5–10). Oleosins also do not have an apparent subcellular targeting signal sequence, but they are associated with neutral lipids in the tapetosomes (5). Oleosins in *Arabidopsis* are not processed (6). Those in *Brassica* are subjected to one proteolytic fragmentation (7, 10), and both fragments are transferred to the pollen coat. Oleosins have no enzymic activity, and their role on the pollen is supposed to be for water uptake to aid germination. Thus, whether they are fragmented, as well as their high variations in sizes and sequences (6), may have no functional relevance as long as their amphipathicity for water uptake is maintained. The xylanase in maize tapetum is synthesized initially as a precursor and then processed extensively and specifically to become the active xylanase in the pollen coat. The physiological relevance for this highly regulated processing is intriguing, and the following considerations are noted. (a) The mature xylanase is an active enzyme, and therefore its size and sequence should be fairly rigid. (b) The pre-xylanase or xylanase on its way from the lysed tapetum cells to the pollen surface will initially encounter its substrate (i.e. the inner tapetum cell wall). If the active enzyme were to tangle up with the substrate, its enzymic activity, its further movement to the pollen coat, and its release from the pollen coat to the stigma could be jeopardized. These conditions could be prevented if the pre-xylanase were the initial transfer form and had its to-be-removed N- and C-terminal regions covering up the active site. (c) The processing protease has optimal activities in an acidic medium. This protease would be active only after the tapetum cell vacuoles had lysed or in the acidic anther locule fluid. (d) The proteolytic processing of the pre-xylanase is precise, and the produced active xylanase is highly resistant to further proteolysis by the processing protease or other proteases in the anther extract. On the basis of these considerations, we speculate that the pre-xylanase is proteolyzed in the anther locule after cell lysis, such that the active enzyme is deposited immediately onto the pollen.

The tapetum-pollen xylanase and the aleurone-layer xylanase of maize and barley and possibly other cereals have obvious similarities but important differences. They share a similar pattern of precursor-product processing, they catalyze the same chemical reaction, and they are released extracellularly during programmed cell death. Nevertheless, the differences are more significant. First, they have very different physiological functions. The pollen xylanase is discharged early to hydrolyze the stigma wall for the entry of the pollen tube, whereas the aleurone-layer xylanase is released late in germination to scavenge the remaining xylan wall of the aleurone-layer and storage-endosperm cells to support seed germination. Second, the pre-xylanase processing mechanism in the tapetum is more sophisticated, as is revealed in the precise proteolysis of the precursor without any detectable intermediates and the high stability of the final active xylanase. In the aleu-
rone layer, the processing produced many intermediates (16), and active enzymes of molecular masses of 55 and 30 kDa in wheat (27), and 29 (28), 34 (29), and 41 kDa (30) in barley have been reported. In addition, the aleurone-layer xylanase is likely to be encoded by more than one gene (this report). A more sophisticated regulation of the pre-xylanase processing in the tapetum-pollen is advantageous, because the enzyme will need to maintain its activity and other properties for functioning at a different developmental stage, at a future period, in a distant organ, or even in a different organism. This sophistication may not be needed in the aleurone layer, because the enzyme will perform its function of scavenging the leftover xylan cell wall at the late stage of germination.

REFERENCES
1. Bewley, J. D., Hempel, F. D., McCormick, S., and Zambryski, P. (2000) in Biochemistry and Molecular Biology of Plants (Buchanan, B. B., Grissem, W., and Jones, R. L., eds) pp. 988–1043, American Society for Plant Physiology, Rockville, MD
2. Heslop-Harrison, Y. (2000) Ann. Bot. 85, 5–13
3. Mascarenhas J. P. (1993) Plant Cell 5, 1303–1314
4. McCormick, S. (1998) Curr. Opin. Plant Biol. 1, 18–25
5. Wu, S. S. H., Platt, K. A., Ratnayake, C., Wang, T. W., Ting, J. T. L., and Huang, A. H. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12711–12716
6. Kim, H. U., Haieh, K., Ratnayake, C., and Huang, A. H. C. (2002) J. Biol. Chem. 277, 22677–22684
7. Ting, J. T. L., Wu, S. S. H., Ratnayake, C., and Huang, A. H. C. (1998) Plant J. 16, 541–551
8. Ruitter, R. K., van Eldik, G. J., van Herpen, R. M. A., Schrauwen, J. A. M., and Wallens, G. J. (1997) Plant Cell 9, 1621–1631
9. Mayfield, J. A., Feigl, A., Johnstone, S. E., and Preuss, D. (2001) Science 292, 2482–2485
10. Piffanelli, P., Ross, J. H. E., and Murphy, D. J. (1997) Plant J. 11, 549–562
11. Doughty, J., Hedderison, F., McCubbin, A., and Dickinson, H. (1993) Proc. Nat. Acad. Sci. U. S. A. 90, 467–471
12. Kachroo, A., Nasrallah, M. E., and Nasrallah, J. B. (2002) Plant Cell 14, S227–S238
13. Horner, H. T., Hall, V. L., and Vargo, A. M. (1993) Protoplasma 173, 48–57
14. Loukides, C. A., Broadwater, A. H., and Bedinger, P. A. (1995) Am. J. Bot. 82, 1017–1023
15. Bih, F. Y., Wu, S. S. H., Ratnayake, C., Walling, L. L., Nothnagel, E. A., and Huang, A. H. C. (1999) J. Biol. Chem. 274, 22884–22894
16. Caspers, M. P. M., Lok, F., Sinjorgo, M. K. M., van Zeijl, M. J., Nielsen, K. A., and Cameron-Mills, V. (2001) Plant J. 26, 191–204
17. Sains, J. P., and Dube, S. D. (1986) Mycol. Res. 3, 227–232
18. Verwoerd, T. C., Dekker, B. M. M., and Hooftman, A. (1989) Nucleic Acids Res. 17, 2362
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
20. Suen, D. F., Wang, C. K., Lin, R. F., Kao, Y. Y., Lee, F. M., and Chen, C. C. (1997) Theor. Appl. Genet. 94, 331–337
21. Beynon, R. J., and Bond, J. S. (1989) in Proteolytic Enzymes: A Practical Approach, IRL Press, Oxford
22. Wang, S. M., and Huang, A. H. C. (1987) J. Biol. Chem. 262, 2270–2274
23. Hird D. L., Worrall, D., Hodge, R., Smartt, S., Paul, W., and Scott, R. (1993) Plant J. 4, 1023–1033
24. Koltunow, A. M., Truettner, J., Cox, K. H., Wallroth, M., and Goldberg, R. B. (1996) Plant Mol. Biol. 19, 611–622
25. Staiger, D., Kappeler, S., Muller, M., and Apel, K. (1994) Planta 192, 221–231
26. Cleemput, G., van Laere, K., Hessing, M., van Leuven, F., Torrekens, S., and Delcourt, J. A. (1997) Plant Physiol. 115, 619–627
27. Dashek, W. V., and Chrispeels, M. J. (1977) Planta 134, 251–256
28. Benjavongkulchai, E., and Spencer, M. S. (1986) Planta 169, 415–419
29. Banik, M., Garrett, T. P., and Fischer, G. B. (1996) Plant Mol. Biol. 31, 1163–1172