In plants, the pollen coat covers the exine wall of the pollen and is the outermost layer that makes the initial contact with the stigma surface during sexual reproduction. Little is known about the constituents of the pollen coat, especially in wind-pollinated species. The pollen coat was extracted with diethyl ether from the pollen of maize (Zea mays L.), and a predominant protein of 35 kDa was identified. On the basis of the N-terminal sequence of this protein, a cDNA clone of the Xyl gene was obtained by reverse transcriptase-polymerase chain reaction. The deduced amino acid sequence of the 35-kDa protein shared similarities with the sequences of many microbial xylanases and a barley aleurone-layer xylanase. The 35-kDa protein in the pollen-coat extract was purified to homogeneity by fast protein liquid chromatography and determined to be an acidic endoxylanase that was most active on oat spelt xylan. Northern and in situ hybridization showed that Xyl was specifically expressed in the tapetum of the anther after the tetrad microspores had become individual microspores. Southern hybridization and gene-copy reconstruction studies showed only one copy of the Xyl gene per haploid genome. Analyses of the genomic DNA sequence of Xyl and RNase protection studies with the transcript revealed many regulatory motifs at the promoter region and a leader region of the transcript. The Xyl transcript had a 562-nucleotide (nt) 5'-leader, a 54-nt sequence encoding a putative signal peptide, a 933-nt coding sequence, and a 420-nt 3'-untranslated sequence. The unusually long 5'-leader had an open reading frame encoding a putative 175-residue protein whose sequence was most similar to that of a microbial arabinoxidase. The maize xylanase is the first enzyme documented to be present in the pollen coat. Its possible role in the hydrolysis of the maize type II primary cell wall (having xylose, glucose, and arabinose as the major moieties) of the tapetum cells and the stigma surface is discussed.

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studies, those in the pollen coat that are deposited on the surface of these pollen walls are unknown.

Recently, the surface proteins and lipids of the pollen coat of *Brassica* have been studied by several laboratories (14–17). The pollen coat can be extracted with an organic solvent such as cyclohexane or diethyl ether, and the extraction apparently does not damage the pollen protoplast, which can be stained with the vital dye fluorescein diacetate. The pollen coat contains neutral esters as the major lipid constituents, which initially accumulate in the plastids of the tapetum cells at the final stage of anther development (16). The predominant proteins in the pollen coat are related to seed oleosins, which have been well studied as abundant structural proteins on the storage oil bodies of seeds (18). In the anther, oleosins accumulate initially in novel triacylglycerol-containing organelles of the tapetum cells (16). Subsequently, the proteins are specifically cleaved, and their fragments are released from the organelles and the disintegrating tapetum cells to the pollen surface. The functions of the abundant neutral esters and oleosin fragments in the pollen coat are unknown. Findings similar to those on *Brassica* have been obtained from *Arabidopsis*, although to a limited extent (19). A mutant of *Arabidopsis* defective in long acyl chain synthesis produces pollen without pollen coat; these pollen do not germinate on the stigma (20). *Brassica* and *Arabidopsis* are insect- or self-pollinated species, and their pollen has a thick coat, which is sticky and contains abundant lipids. In contrast, pollen of wind-pollinated species such as the cereals has lesser amounts of pollen coat, which are non-sticky and contain a lesser amount of lipids. The pollen coat in the wind-pollinated species should be quite different from that in insect- or self-pollinated species in constituents and function.

We have studied the pollen coat from maize, a wind-pollinated species. Maize pollen coat contains a minimal amount of lipids and one dominant protein constituent. This protein is an endoxylanase encoded by a single copy gene expressed in the limited extent (19). A mutant of *Arabidopsis* defective in long acyl chain synthesis produces pollen without pollen coat; these pollen do not germinate on the stigma (20). *Brassica* and *Arabidopsis* are insect- or self-pollinated species, and their pollen has a thick coat, which is sticky and contains abundant lipids. In contrast, pollen of wind-pollinated species such as the cereals has lesser amounts of pollen coat, which are non-sticky and contain a lesser amount of lipids. The pollen coat in the wind-pollinated species should be quite different from that in insect- or self-pollinated species in constituents and function.

**Experimental Procedures**

**Plant Materials**—Maize (Zea mays L., Mo17) organs were obtained from plants grown as described below. The embryos were dissected from mature kernels that had been soaked in water for several hours. Kernels were germinated and grown on several layers of cheesecloth in a dark moist chamber at 28–30 °C. Four-day-old etiolated seedlings (4-day-old hypocotyl) were harvested. The other seedlings were transferred to a greenhouse at 25 °C with 14/10 h day/night cycle. Roots, shoot apex, stem, and leaves were collected from plants having five leaves (about 1 month old). Anthers of different developmental stages, silks, and whole kernels (about half mature) were obtained from older plants. Pollen was collected on sunny mornings when the anthers had just opened.

The anthers were classified into four stages based on their developmental status during microsporogenesis (1, 22). At stage I, the tassel was still embedded in the shoot apex. The anthers filled about one-third of the floret. Each microspore mother cell had undergone meiosis to produce a tetrad of microspores, which were still encased within a calllose wall. At stage II, part of the tassel had protruded from the shoot apex. The anthers filled up about half of the floret. Young microspores had been released from the dissolved calllose wall, and the outer pollen wall (exine) had been synthesized. At stage III, the tassel 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 many small vacuoles. The first mitosis had occurred, and the microspores were binucleate. At stage IV, the tassel became yellow. The anthers filled up the floret completely. Second mitosis had occurred, and the microspores were trinucleate.

**Extraction of Pollen Coat from Pollen by Diethyl Ether and Its Partition into an Aqueous Medium for Enzymatic Studies**—Pollen coat (approximately 300 mg of pollen/10 ml of ether) was extracted with diethyl ether (20) as described in the preceding paragraph. The ether extract was allowed to evaporate to 1 ml under a stream of nitrogen. The proteins in the ether mixture were partitioned into an equal volume of 0.05 M sodium acetate, pH 5.0. The aqueous sample was used to assay for xylanase activity directly or subjected to cation exchange FPLC for the isolation of the xylanase.

**Preparation of Coat and Interior Fractions from Pollen**—Pollen was washed with diethyl ether to remove the coat as described above, and the leftover pollen was homogenized in chloroform/methanol (2:1, v/v) to yield an interior fraction.

**Cation Exchange FPLC**—All solutions described in this paragraph contained 0.05 M sodium acetate, pH 5.0. The aqueous pollen coat sample (approximately 30 µg of proteins in 4 ml) was filtered through a 0.2-µm syringe filter and then applied to a pre-equilibrated Mono S HR 5/5 FPLC column (Pharmacia, Uppsala, Sweden). Solutions of 6 ml of 0.25 M NaCl, 15 ml of a linear gradient of 0.25–0.75 M NaCl, 5 ml of 1 M NaCl, and 5 ml of 2 M NaCl were applied successively to the column. Chromatographic fractions of 0.5 ml each were collected and analyzed for xylanase activity and protein constituents by SDS-PAGE.

**Xylanase Activity Assay**—Xylanase activity was measured by monitoring the appearance of reducing ends at time intervals. Xylan from oat spelt (Sigma) was used as the substrate. The xylanase reaction mixture of 300 µl contained enzyme and 0.3 mg of oat spelt in 0.15 M sodium acetate, pH 5.0 (or another buffer, see Fig. 6). The reaction was allowed to proceed at 30 °C and terminated by the addition of 900 µl of 0.2 M sodium hydroxide and acetic acid reagent (23) followed by heating. After heating, the absorption of the reaction mixture was determined at 240 nm with a spectrophotometer. The enzyme activity was monitored at four time intervals within a 6-h period to ensure linearity of the reaction. Oat spelt xylan and other potential substrates including birchwood xylan, 4-O-methylglucuronoxylan, pectin, carboxymethyl cellulose, and polygalacturonic acid were obtained from Sigma, and arabinan was purchased from Megazyme Inc. (Warriewood, Australia).

**Viscosity Test**—The effect of the xylanase on the viscosity of a solution of oat spelt xylan was measured semiquantitatively (24). The reaction mixture was prepared as follows. A mixture of oat spelt xylan and water (50 mg of xylan/ml) was centrifuged at 2,000 × g for 5 min to pellet undissolved xylan (about 60%). The supernatant was mixed with 0.2 volumes of the pollen coat xylanase and concentrated buffer to make the final mixture 0.15 M sodium acetate, pH 5.0. A volume of 250 µl of the mixture was allowed to flow gravitationally in a 0.2-mI viscosity pipette (Fisher Inc., Pittsburgh, PA). The flow times for 0.2 ml of buffer alone was 6.7 s, whereas that of the xylan solution prior to the addition of enzyme was 12.2 s. Duplicated samples were used for colorimetric determination of the xylanase activity as described above.

**HPLC Separation of Monosaccharides and Oligosaccharides**—Maize pollen coat fraction and oat spelt xylan were mixed and incubated in 0.5 ml of 10 µM sodium acetate, pH 5.0, for 0, 3, or 6 h. An aliquot of 50 µl was used for the colorimetric determination of xylanase activity. Another aliquot of 50 µl was used for HPLC analysis of monosaccharides and oligosaccharides, as follows. Three volumes of 95% ethanol were added, and the mixture was centrifuged to remove precipitated polymers. The resulting supernatant was filtered (0.2 µm pore size), evaporated, and analyzed by reversed phase high performance liquid chromatography. The monosaccharides present in the reaction mixture were monitored at their respective absorbance maxima (25).
orated, and redissolved in 0.3 ml of water. This solution was injected into CarboPac PA1 guard and separation columns in a high performance anion-exchange chromatograph attached to a pulsed amperometric detection system ( Dionex Corp., Sunnyvale, CA). The columns were equilibrated in 7.5 mM NaOH prior to sample injection, and elution programs had gradient programs beginning with water and concluding with 150 mM sodium acetate in 100 mM NaOH. Peaks observed in the chromatogram were compared with standards for monosaccharides found in the pollen coat fraction (mostly glucose and fructose) or resulting from total acid hydrolysis of oat spelt xylan (xylose, glucose, arabinose, galactose, and 4-O-methylglucuronic acid). Peaks observed in the 0-h lane were considered to represent baseline amounts of sugars and other chemicals present in the pollen coat fraction, and subsequent changes in the 3- and 6-h mixtures were referenced to this baseline. Peaks absent in the 0-h mixture but appearing in the 3- and 6-h mixtures at retention times longer than those of the monosaccharide standards were considered to represent enzyme-generated oligosaccharides, although structural analyses were not performed on these compounds. While the gradient elution used in this analysis separates oligosaccharides up to at least tetrasaccharide size, the sum of all chromatographic peaks observed accounted for only a small proportion of the reducing ends generated during the 3- and 6-h incubations. The additional reducing ends were likely on larger oligosaccharides or polymers removed by the ethanol precipitation or retained by the guard column.

SDS-PAGE and Blotting for Immunoreactivity and Microsequencing—All procedures followed those described earlier (25). Proteins were separated by 10 or 12.5% (w/v) SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Blue and then destained. For protein blot analyses, gel-fractionated proteins were transferred to a nitrocellulose filter membrane as described. The filter was subjected to immunodetection. Rabbit antibodies were raised against purified recombinant XYL (to be described). For sequence analyses, gel-fractionated XYL from pollen coat was transferred to a membrane for N-terminal sequencing.

RNA Extraction and RT-PCR—Total RNAs were extracted with guanidine-HCl (26) from anthers of different developmental stages and various organs.

RT-PCR was performed using the Life Technologies, Inc. 3′-rapid amplification of cDNA ends system (Life Technologies, Gaithersburg, MD) with total RNAs from stage II anthers. oligo primers used for PCR amplification were based on the N-terminal sequence of XYL, RLGF-PFGNAVTEKIGLPA. One degenerate primer number 501 (5′-TYGC- CNTYGGNAAYGC-3′) was based on RLGFPP. The other degenerate primer number 50′ (5′-ACCAARGATHIYNTAGC-3′) is a genomic degenerate oligonucleotide, NAVTKE. The 3′-end primer was (dT12-adapter (5′-GACTCGATGC- GACATCGA(dT)12)-3′). An Xyl 1.3-kb fragment was obtained and subcloned into the vector pGEM-T, and the product was called p-Xyl.

DNA Sequencing—The 1.3-kb Xyl CDNA insert was digested by restriction enzymes into 0.3–0.6-kb fragments containing overlapping regions. The fragments were purified and converted to single-stranded DNA templates by asymmetric PCR. Sequencing of these fragments was used to determine the vector pGEM-T and the product was called p-Xyl.

Ribobrope Synthesis—Ribobrope synthesis followed the protocol provided by Promega (Madison, WI). A fragment covering about 80% of the coding region of Xyl, starting at the 5′ terminus to a Spal site (nt 722 to 1492, Fig. 3), was obtained from p-Xyl by restriction enzyme digestion as a template for making 5′-UTP-labeled Ribobrope for RNA and DNA blot hybridization, and for gDNA library screening. Similarly, a fragment covering about 90% of the coding region of Xyl, starting at the 5′ terminus to a Sall site (nt 722 to 1566, Fig. 3) was obtained as a template for making digoxigenin-11-UTP-labeled ribobrope for in situ hybridization.

RNA Blot Hybridization—RNAs were electrophoresed in a glyoxal-dimethyl sulfoxide denaturing gel and blotted onto a nitrocellulose filter (27). The filter was hybridized with Xyl antisense ribobrope as at 42°C for 16 h. The blot was washed in 2 × SSC (SSC had 0.15 M NaCl, 0.015 M Na3 citrate, pH 7.0) and 0.1% (w/v) SDS for 5 min and twice at 65°C in 0.1 × SSC and 0.1% (w/v) SDS for 15 min. The blots were exposed to Hyper-film MP (Amersham Pharmacia Biotech) at −80°C with a Du Pont phosphorimager. The stringent programs beginning with water and concluding with 150 mM sodium acetate in 100 mM NaOH.

In Situ Hybridization—Antibodies were fixed in 2.5% (v/v) glutaraldehyde and 0.1 M NaPO4, pH 7.0, embedded in paraffin, and sectioned into 10-μm pieces. The sections were prepared for in situ hybridization according to Jackson (28) with use of digoxigenin-11-UTP-labeled sense and antisense ribobrope of Xyl. Controls were performed using sense and antisense ribobrope of a maize polygalacturonase gene (EMBL AccX62384) expressed in the microspores during late microsporogenesis (11). A conserved region of this gene from nt 531 to 698 was amplified from maize anther mRNA by RT-PCR using the end sequences as primers, and the fragment was subcloned into pBS-SK for ribobrope synthesis.

The RNA probes were subjected to mild alkali hydrolysis to generate fragments of 75–100 nt, which were added (200–500 ng/ml) to the hybridization buffer (28). The sections were allowed to hybridize at 50°C for 16 h. The slides were washed in several changes in 2 × SSC and 50% (v/v) formamide at 50°C, and then twice in 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. The sections were treated with RNase A, NA, and slides were washed in 2 × SSC and 50% formamide at 50°C for 1 h and then several times in 130 mM NaCl, 10 mM sodium phosphate, pH 7.0, and stored in this buffer at 4°C overnight.

Ribobrope were detected using the DIG/Genius nonradioactive nucleic acid detection kit (Roche Molecular Biochemicals, Indianapolis, IN). The slides were examined after 12–24 h for proper color development. Color development was terminated by washing the slides in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA and passing them through a dehydration series of ethanol. Slides were washed in xylene and mounted in permount for microscopic examination.

Genomic DNA Blot Hybridization—Maize B73 genomic DNA provided by Dr. Todd Young (University of California, Riverside, CA) was digested with BamHI, EcoRIV, HindIII, and PstI, and the fragments were separated by electrophoresis in a 0.6% (w/v) agarose gel. Blotting, pre-hybridization (10 × SCP at 65°C for 6 h), hybridization (10 × SCP at 65°C for 12 h with 10% (v/v) of 32P-labeled riboprobes, and washes (2 × SCP at 65°C) were as described (27). Ten times SCP had 1 M NaCl, 0.3 μM Na2PO4, and 0.01 μM Na2EDTA. In gene copy studies, 1, 2, 5, and 10 copy equivalents were run in parallel lanes in the gel for the above genomic blot hybridization. One genome equivalent of p-Xyl was calculated to be 7.7 ng.

Isolation and Sequencing of a Xyl Genomic Clone—A maize (B73) genomic library constructed in λ-GEM11 provided by Dr. J. C. Walker (University of Missouri) was screened using an Xyl ribobrope. Three λ-g-Xyl clones were isolated. Restriction mapping of these clones with Apal, EcoRI, and SacI suggested that the three clones were identical. A 5.5-kb EcoRV fragment from λ-g-Xyl was digested with EcoRI. The resulting two fragments of 2.5 and 3 kb were subcloned into a pBlue- script-SK (Stratagene, La Jolla, CA) for sequencing. The genomic sequences spanning the EcoRI site were determined by sequencing with internal primers.

RNAse Protection Assays—RNAseq protection assays were performed as described (27). Seven antisense riboprobes were synthesized using the same template (λ-g-Xyl). The protected DNA fragments were analyzed in a 5% (w/v) urea sequencing gel, and M13 DNA templates sequenced with the −40 primer were used as markers for sequence length.

To determine the exact location of the intron, RT-PCR amplified fragments spanning the splice junctions were obtained from another RNAs. The 5′-Xyl primer was 5′-CACGGTTACCCAGAGAATC-3′. DNA fragments were converted to single-stranded DNA templates by asymmetric PCR. The protected DNA fragments were subcloned into pBS-SK for sequencing.

Construction of Plasmid Containing Xyl for Synthesis of XYL in Escherichia coli—The coding region of Xyl corresponding to XYL precursor protein (with a 18-residue signal peptide) was amplified by PCR and cloned into the vector pGEM-T (promoterless). The resulting DNA fragments were digested with XbaI and XhoI, ligated into the vector pGEM-T, and transformed into *E. coli* MG1655. Transformed colonies were selected on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (1 mM), and the selected colonies were.XPath A. The plasmid DNA was isolated and sequenced to confirm the correct sequence.

Production and Purification of Recombinant XYL in E. coli—The expression plasmids harboring pQE30 (Qiagen, Valencia, CA) were transformed into *E. coli* strain M15 (pREP4).

The recombinant XYL in E. coli harboring pQE30-His-xyl-pre-Xyl to produce recombinant XYL in the cell pellet supplied by Qiagen (Valencia, CA) using isopropyl-β-D-thiogalactopyranoside. The optimal concentration of isopropyl-β-D-thiogalactopyranoside (0.05 mM), temperature (37°C), and time (3 h) to produce large amounts of the recombinant protein were determined empirically. After induction, the cells were harvested by centrifugation at 4000 × g for 15 min at 4°C. The cells were resuspended in 10 ml of sonication buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl). Lysozyme at a final concentration of 1 mg/ml was added, and the mixture was incubated at room temperature for 30 min. Phenylmethylsulfonyl fluoride at a final concentration of 0.1% was added, and the mixture was incubated at 37°C for 30 min. The cell-free supernatant was applied to a nickel-NTA agarose column (Qiagen, Valencia, CA), and the column was washed extensively to remove the non-specific adsorbed proteins. The recombinant XYL was eluted from the column with an elution buffer containing 250 mM imidazole, and dialyzed against a standard buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM DTT. The protein concentrations were estimated by the Bradford assay (29), and the purified protein was analyzed by SDS-PAGE.
concentration of 0.4 M was added to the mixture. The cell suspension, in a tube in an ice bucket, was sonicated by a series of 20-s bursts in a sonicator (Braun-Sonic, Allentown, PA) until $A_{600\ nm}$ dropped to 0.1. The mixture was centrifuged at 12,000 \times g for 10 min at 4 °C to produce a supernatant and a pellet. The supernatant (10 ml) was subjected to TALON metal-affinity resin (CLONTECH, Palo Alto, CA) purification of the recombinant XYL using the procedure provided by the company. The purified recombinant XYL was used to raise antibodies against XYL.

RESULTS

Maize Pollen Coat Contained a Predominant Protein of 35 kDa—Eight different organic solvents were used to extract the pollen coat from freshly harvested, mature maize pollen. These solvents included, in descending order of hydrophobicity, hexane (dielectric constant, 1.8), heptane (1.8), cyclohexane (2.0), carbon tetrachloride (2.2), benzene (2.2), diethyl ether (4.3), chloroform (4.8), and methanol (32). Proteins in the extracted fractions were resolved by SDS-PAGE. Fig. 1 shows that solvents of moderate hydrophobicity extracted a major protein of 35 kDa and several minor proteins. A visible protein band at the 25-kDa region of the gel was the second most intensive band on the gel; it was made up of at least two proteins of similar molecular mass that could not be distinguished visually after photography. The 35-kDa protein (termed XYL, representing xylanase, to be documented) and the 25-kDa proteins were not extracted by very hydrophobic (heptane and hexane) or very hydrophilic (methanol) solvents. When water or an aqueous medium was used, the protoplast emerged from the enclosing pollen wall and was osmolyzed. When 0.8 M sucrose was used, only the 25-kDa proteins were present in the extract (data not shown). After extraction with diethyl ether, the pollen could be stained with the vital dye fluorescein diacetate, suggesting that the pollen protoplast was still viable. Whether the protoplasts also showed positive signals in other viability tests is unknown. The above pattern of extraction of the pollen coat from freshly harvested, mature maize pollen.

To investigate the location of XYL in the pollen, we separated the pollen into a coat fraction and an interior fraction and analyzed them by SDS-PAGE and immunoblotting. Fig. 2 shows that the pollen coat proteins represented substantially less than 1% of the total pollen proteins. XYL was detected in the coat fraction and not in the interior fraction by immunoblotting using antibodies against XYL (synthesized by bacteria). The antibodies also recognized a protein of about 68 kDa present in the pollen interior (Fig. 2); the identity of this protein was unknown.

The Amino Acid Sequence of XYL, as Deduced from a Xyl cDNA Clone, Shared Similarities with Xylanases—The N-terminal sequence of XYL of the pollen coat fractionated by SDS-PAGE was determined to be RLGPFGNAVTKEILGLPA. Nested 5’ primers based on the underlined sequences were synthesized and used in conjunction with the 3’ (dT)$_{17}$-adapter and anther poly(A) RNA for 3’-rapid amplification of cDNA ends to obtain a 1.3-kb Xyl cDNA. Fig. 3 shows the sequence of this 1.3-kb Xyl cDNA and the corresponding genomic DNA region (to be described). The Xyl cDNA sequence had a single ORF encoding a protein of 311 amino acids. The protein had a predicted pI of 9.45 and a molecular weight of 35,229. The N-terminal 19-residue sequence matches the N-terminal sequence of XYL determined by microsequencing. Fig. 4 is a hydropathy plot of the XYL sequence and shows that the protein was hydrophilic, with the hydrophilic residues dispersed...
throughout the polypeptide. The hydrophilicity of XYL reflected the outcome of the organic solvent extraction of the pollen coat (Fig. 1), in which only moderately hydrophobic solvents removed the protein from the pollen surface. Presumably, the protein is located at a semihydrophobic region on the pollen surface, such that very hydrophilic or hydrophobic solvents could not extract the protein.

A search for amino acid sequences of genes similar to that of XYL has revealed 30 microbial genes known to encode xylanases (EC 3.2.1.8). All of these xylanases belong to glycosyl hydrolase Group F, which comprises high-molecular weight endoxylanases and a few cellulases (30, 31). The best matches also included a recently reported (32) barley aleurone xylanase of 44 kDa. The maize XYL shared 42 and 30% identity in the amino acid sequence with the barley and \textit{Penicillium} enzymes, respectively. Fig. 5 is an alignment of the sequences of five xylanases from diverse organisms. The XYL and other xylanases share high similarities over regions surrounding the enzymatic active site, including two nucleophilic residues of glutamic acid and asparagine and one proton-donor residue of arginine (30, 31).

XYL Was an Active Endoxylanase—The similarity between the amino acid sequence of XYL and other microbial endoxylanases suggests that the maize protein is an endoxylanase. Therefore, the pollen coat proteins were assayed for xylanase activity. The activity, as determined by monitoring the generation of reducing ends in oat spelt xylan, was highest at pH 5.0 within the range of pH 4–10 (Fig. 6A). On different cell wall extracts, the activity was high on oat spelt xylan, barely detectable on birchwood xylan, 4-O-methyl glucuronoxylan, and pectin, and absent on carboxymethyl cellulose, polygalacturonic acid, and arabinan (Fig. 6B). The activity versus pH profile on birchwood xylan (data not shown) was also similar to that on oat spelt xylan (Fig. 6A), although the activity on...
birchwood xylan was much lower. The findings that the maize xylanase was most active on xylan extracted from the spelt of oat, another cereal closely related to maize, may be of physiological relevance. It is possible that the active site of the maize enzyme recognizes not just the $\beta$-1,4-xylan backbone, but also the side chain moieties.

A comparison among the amino acid sequences of XYL and microbial xylanases places the maize enzyme and some microbial enzymes into Group F xylanases, which are mostly endoxylanases (30). The maize xylanase was tested to see if indeed it was an endohydrolase. During the course of hydrolysis of oat spelt xylan by the maize xylanase (Fig. 7A), the generation of reducing ends far exceeded the generation of xylose, other monosaccharides, and oligosaccharides (containing at least 2–4 sugar residues as detected in the HPLC analysis). Presumably, larger oligosaccharides with exposed reducing ends were also produced but had been removed by the initial alcohol precipitation and the guard column before the HPLC analysis. The findings support the notion that the maize xylanase was an endohydrolase. In addition, during the hydrolysis of oat spelt xylan by the maize enzyme, the viscosity of the reaction mixture dropped considerably after cleavage of only a small portion of the oligosaccharide linkages in the xylan (Fig. 7B). This observation is again consistent (33) with the maize xylanase being an endo- rather than an exohydrolase.

The xylanase was purified to homogeneity from the pollen coat proteins by cation exchange FPLC (Fig. 8). The pollen coat proteins were resolved into individual proteins as peaks in the chromatography, and one of the peaks coincided with the peak of xylanase activity. This peak fraction contained solely XYL, as revealed by SDS-PAGE. Approximately 1 mg of XYL was obtained from 50 mg of pollen. The purified xylanase generated 17.4 mmol of reducing ends/h/mg of protein. This activity was less that those in commercial microbial xylanases (10,000 mmol of reducing ends/h/mg of protein), shown in the Sigma catalog. A direct comparison of the specific activities of enzymes that act on polymer substrates may be inappropriate. In addition, the commercial microbial enzymes have been selected for their high activities, versatility on diverse substrates, and durability for industrial uses, and they are relatively heat and SDS resistant. On the contrary, the maize xylanase was easily denatured by heat (enzyme treated at 50 °C for 30 min retained less than 10% activity; data not shown) and SDS (enzyme treated with 0.005% (w/w) SDS retained less than 10% activity; data not shown).

**FIG. 5.** Alignment of the amino acid sequences of five representative xylanases. The enzymes included the xylanases of maize (AF063865), barley (U59312), a fungus (Penicillium chrysogenum, P29417), a Eubacterium (Streptomyces halstedii, U41627), and an unclassified organism (Thermoanaerobacter saccharolyticum, P36917). Amino acid residues identical to those in XYL are shadowed. Arbitrary numbering is on the top. Dots denote potential gaps. Indicates potential residues R, N, E, at the active site determined for the microbial xylanases (30).
Fig. 7. **Time course of hydrolysis of oat spelt xylan by the xylanase in the maize pollen coat.** The production of reducing ends was compared with the production of monosaccharides and small oligosaccharides (with 0.6 μg of pollen coat proteins, upper panel) or the change in the viscosity of the reaction media (with 0.5 μg of pollen coat proteins and 4 × xylan concentration, lower panel). Shown in the upper panel, the majority of the reducing ends produced apparently belonged to large oligosaccharides that had been removed by alcohol precipitation and a guard column before the samples were analyzed by HPLC. Note that the scale on the y axis for the reducing ends is 20 times that for the saccharides. Shown in the lower panel, the viscosity dropped considerably after cleavage of only a very small proportion (0.2%, equivalent to 38 nmol reducing ends, after 7 h) of the glycoside linkages in the xylan. In the viscosity test, the flow rates of 100 and 0% represent those to larger oligosaccharides that had been removed by alcohol precipitation and a guard column before the samples were analyzed by HPLC.

**Anther**—Proteins present on the surface of pollen are likely to be synthesized in the tapetum cells, although one small polypeptide on the surface of *Brassica* pollen has been shown to be synthesized in the pollen interior (34). Fig. 9 shows an RNA blot hybridized to a Xyl coding sequence probe. An mRNA of 2.0 kb was detected in developing anthers but not in various other maize organs. Anthers were classified into four developmental stages according to the morphological features of the tassels and the microspores (see “Experimental Procedures”). The *Xyl* mRNA was undetectable in stage I anthers, abundant in stage II anthers, and present in substantially lesser amounts in stage III and stage IV anthers (Fig. 9). In stage II anthers, the microspores and pollen (11). Polygalacturonase mRNA was detected with the antisense probe, but not the sense probe, in the microspores in stage IV anthers (Fig. 10). The developmental changes of the amount of *Xyl* mRNA in the tapetum as detected by *in situ* hybridization (Fig. 10) were consistent with those in the anthers detected by RNA blot hybridization (Fig. 9).

**Genomic DNA Blot Hybridizations Revealed One or a Small Number of Xyl Copies per Maize Haploid Genome**—Maize genomic DNA was subjected to DNA blot hybridization using the Xyl coding region probe. Fig. 11 illustrates that a single Xyl fragment of 9, 4.5, and 3 kb was produced from *Bam*HI, *Eco*RV, and *Hind*III, respectively. *Pst*I digestion did not produce a detectable fragment; it generated a 0.2-kb fragment, which was not resolved in the gel. Subsequent sequence data (to be described) confirmed the presence of the expected restriction sites of the above enzymes within or near the coding region of Xyl. A comparison of the intensity of the hybridization signal detected in maize genomic DNA with copy number reconstruction lanes revealed that the maize haploid genome contained one or a small number of Xyl gene copies.

**RNase Protection Studies Delineated the Intron and Tran-**
scription Initiation Sites on Xyl-g-DNA—A segment of 3685 nucleotides of a genomic clone (l-g-Xyl), including the 5′-flanking sequence, the complete Xyl coding region, and the 3′-untranslated region was sequenced (Fig. 3). A comparison between the sequences of g-Xyl and the 1.3-kb Xyl cDNA revealed no introns in the coding region and the 3′-untranslated region.

Two translational start codons were in-frame and upstream of the sequence encoding the N terminus of the mature XYL (RLGF. . . . ) that was determined by microsequencing. The putative presequence had either 18 or 11 residues. The first AUG codon being the initial one on the mRNA, and its 2′-nnt being G rather than C as for the second AUG codon, are more in line with the consensus of those of translation start codons (35). It is likely that there is a cleavable signal peptide of 18 rather than 11 residues associated with the nascent protein.

RNase protection assays were performed to map the location of potential introns and the Xyl transcriptional initiation site. Seven antisense RNA probes were synthesized from the Xyl genomic subclones. Each probe was hybridized to total RNA extracted from stage II anthers. After RNase digestion, the protected RNA fragments were analyzed by gel electrophoresis. Fig. 12 summarizes these findings. The results indicated that an intron was present in the 5′ leader region between nt 259 and 354 and was bordered by the nucleotides GT and AG (Fig. 3) that match the splicing consensus sequence well. They also revealed that the transcriptional initiation site was staggered.

In diverse genes, the sequences adjacent to the transcription initiation site do not share obvious similarities, but there is a tendency for the first base of the transcript to be adenine flanked on both sides by pyrimidine (36). The termini of the two

**FIG. 9.** RNA blot hybridizations of Xyl mRNAs in maize organs. The upper panels show the RNA blots hybridized to a Xyl riboprobe. Total RNAs (10 μg) prepared from different organs were electrophoresed in agarose gels. Lanes 1–10 represent mature kernel embryos, 1-month-old roots, 1-month-old shoots, 4-day-old hypocotyl, 1-month-old internode, leaves, anthers (a mixture of different stages), mature pollen, maturing kernel, and silk, respectively. The right panel includes anthers of developmental stages I to IV and mature pollen (Po). The lower panels show gels identical to those used in the upper panels, except that the gels were stained with ethidium bromide. The sizes of RNA markers are shown on the right.

**FIG. 10.** In situ detection of Xyl transcripts in maize anthers. Sections of anthers of stages I to IV were hybridized with a digoxigenin-11-rUTP labeled antisense riboprobe of Xyl and then treated with antibodies against digoxigenin (top four panels). A photograph of stage II anther treated with sense riboprobe and an enlarged photograph of the stage II anther treated with antisense riboprobe are in the lower panel. The lower panel also has photographs of stage IV anthers treated with a polygalacturonase gene (PG) antisense and sense riboprobes. PG mRNA is known to accumulate within the mature maize microspores.
most abundant Xyl mRNAs correspond to the A and C nt in the multiple initiation site region TAATCGGCCAG. The A but not the C nt is flanked by pyrimidines; for this reason, the A nt was designated nucleotide 1 of the Xyl transcript. This designation reflected the observed fragments of 94 nt with probe 6, 258 nt with probes 1, 2, and 5, and 174 nt with probes 3 and 4 (Fig. 12). On the basis of this assigned transcription initiation site, the length of the 5′ leader of the Xyl transcript is 562 nt (Fig. 3).

The 5′-Flanking Region of Xyl Has Many Potential Regulatory Sequence Motifs—More than 30 genes that are specifically expressed in the tapetum have been reported. Most of these genes were characterized as cDNAs. The 5′-flanking regions of several of them (TA29 of tobacco (37), A6 of Arabidopsis (38), olnB4 of Brassica (39), and OsG3 of rice (40)) have been sequenced, and the length of the sequences required for transcription to occur have been determined. The 1.6-kb sequence of the 5′-flanking region of Xyl and the 5′-flanking regions of the above four genes were compared, as were those of genes that are not specifically expressed in the tapetum. Many motifs found in the 5′-flanking region of Xyl (Fig. 3) were also present in these other genes. For example, they include the PB-core (at nt −38), TATA box (−43), G box (−57), 52/56 box (−883), and 56/59 box (−384, −154, and −18) of many genes, as well as, of tapetum specifically expressed genes, five repeats of the R5 (−1295, −1228, −542, −379, and −150) and six other motifs (−72, −99, −279, −687, −882, and −1108) of tobacco TA29 (37), one motif (−163) of Brassica A6 (38), one motif (−201) of Brassica olnB4 (39), and two motifs (−499 and −299) of rice OsG3 (40). They presumably are involved in the control of the temporal, hormonal, tissue-specific, and quantitative expression of the gene in the maize plant.

The Xyl Transcript Had a Long 5′ Leader Sequence with an ORF—The Xyl transcript has a 562-nt 5′ leader, a 54-nt sequence encoding a putative signal peptide, a 933-nt coding sequence, and a 420-nt 3′-untranslated region (Fig. 3). The 5′ leader is unusually long for an eukaryotic mRNA. None of the known genes that are specifically expressed in the tapetum have an mRNA with a 5′ leader longer than 200 nt, and only the transcripts of homeobox gene ATH1 (41) and plastid psbD gene in Arabidopsis (42) have long 5′ leaders of 830 and 950 nt, respectively.

The Xyl 5′ leader could potentially form many hairpin secondary structures (predicted by the Greedy Program; data not shown), which together possess free energy of −150 kcal/mol. Whether the long 5′ leader sequence or its secondary structures prohibit or enhance interaction with the 40 S ribosomes, interact with specific binding proteins, or form regulatory secondary structures with the relatively long 3′-untranslated region (420 nt) remains to be determined (35).

The 562-nt 5′ leader has a long ORF encoding a putative protein of 175-amino acid residues. Unlike the ORF for XYL, the 5′ leader ORF does not have an apparent sequence encoding a signal peptide. Searches for proteins with sequences similar to the sequence of this putative protein indicated that the deduced peptide sequence was most similar to a Bacteroides ovatus arabinosidase (GenBank, Q59128). There was 25.7% identity and an additional 28.1% similarity between the two overlapping sequences of 167 residues in the maize putative protein and the Bacteroides arabinosidase. The work on the Bacteroides arabinosidase has not been published, and unlike xylanases, arabinosidases have not been studied extensively, and the residues lining the active sites are not known.

Whether the similarity between the putative protein encoded by the 5′ leader of Xyl transcript and the Bacteroides arabinosidase is fortuitous remains to be examined. Nevertheless, it is intriguing that arabinosidase (releasing arabinose) and xylanase (releasing xylose) could be encoded by the same mRNA in maize, whose Type II primary cell wall contains xylose, arabinose, and glucose as the most abundant sugar residues.

DISCUSSION

The maize pollen coat xylanase is synthesized in the tapetum. Apparently it has a cleavable signal peptide and is secreted to the locule via the intracellular secretory pathway. Unlike the tapetum β-glucanase, which is secreted to the locule for the hydrolysis of the callose cell wall of the microspore tetrad (6), the xylanase is secreted after the microspore tetrad has become solitary microspores. It accumulates on the pollen surface, and unlike the Brassica oleosin fragments, remains intact and active. This is the only enzyme well documented to be present in the pollen coat, even though several other enzymes have been reported to be present in the intine of pollen (see Introduction). These latter enzymes, whenever studied by cloning, are products of genes in the pollen gametophyte, whereas the maize xylanase is the product of genes in the sporophytic tapetum. In addition, the maize xylanase is present mostly, and probably exclusively, in the pollen coat, as revealed by immunoblotting studies.

The physiological function of the maize pollen xylanase can be speculated. As described above, the enzyme is not involved in the hydrolysis of the callose wall of the microspore tetrad. The cell walls of the tapetum cells are hydrolyzed (5) sometime before or during the peak abundance of the xylanase mRNA. If the enzyme is involved in the hydrolysis of the tapetum cell wall, or the stomium cell wall for anther dehiscence, it is intriguing that the enzyme is retained intact and active, and abundantly in the pollen coat. Alternatively, the xylanase may be deposited in the pollen coat to exert a physiological role after the pollen has landed on the stigma. On the stigma surface, the enzyme may hydrolyze the cell wall and thus aid the initial penetration of the pollen tube into the stigma interior. The penetration does not require the action of a cutinase, because the stigma trichomes have a thin and discontinuous cuticle (43). It is also possible that the xylanase may release regulatory oligosaccharides from the stigma cells for a yet to be discovered regulatory process. To carry out one or more of the above possible functions, the xylanase has the appropriate enzymatic activity because the maize cell wall has a Type II organization, containing xylose as a major sugar moiety (21), and it is much more active on the xylan of oat (a related cereal) than birch (a dicot). Alternatively, the xylanase may have a non-enzymatic role. For example, similar to the Brassica oleo-
The occurrence of a very long 5’ leader sequence containing an extensive ORF in Xyl mRNA is very unusual among eukaryotic mRNAs. It is intriguing that this ORF could encode an arabinosidase that is closely related enzymatically to the xylanase encoded by the primary ORF. If the 5’ leader ORF does produce an arabinosidase, it will be interesting to examine how the two enzymes act concertedly in hydrolyzing the cell wall in the sexual reproductive machinery and exert their physiological function during microsporogenesis. If so, it will also be intriguing to investigate how the arabinosidase, which is devoid of a recognizable signal peptide, is secreted from the tapetum cells to the locule. The ORF for arabinosidase is located at the 5’ upstream of the ORF for xylanase, and the two ORFs are not in-frame (separated by 11 nt). Thus, the two proteins would not have been produced as a poly-protein for secretion. Whether the arabinosidase is inserted into the intracellular secretory pathway via a piggy-backing mechanism similar to that for some peroxisomal enzymes remains to be studied.

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