The surface of a pollen grain consists of an outermost coat and an underlying wall. In maize (\textit{Zea mays} L.), the pollen coat contains two major proteins derived from the adjacent tapetum cells in the anthers. One of the proteins is a 35-kDa endoxylanase (Wu, S. S. H., Suen, D. F., Chang, H. C., and Huang, A. H. C. (2002) \textit{J. Biol. Chem.} 277, 49055–49064). The other protein of 70 kDa was purified to homogeneity and shown to be a \(\beta\)-glucanase. Its gene sequence and the developmental pattern of its mRNA differ from those of the known \(\beta\)-glucanases that hydrolyze the callose wall of the microspore tetrad. Mature pollen placed in a liquid medium released about nine major proteins. These proteins were partially sequenced and identified via GenBank\textsuperscript{TM} data bases, and some had not been previously reported to be in pollen. They appear to have wall-loosening, structural, and enzymatic functions. A novel pollen wall-bound protein of 17 kDa has a unique pattern of cysteine distribution in its sequence (six tandem repeats of \(\text{CX}_3\text{CX}_{10–15}\)) that could chelate cations and form signal-receiving finger motifs. These pollen-released proteins were synthesized in the pollen interior, and their mRNA increased during pollen maturation and germination. They were localized mainly in the pollen tube wall. The pollen shell was isolated and found to contain no detectable proteins. We suggest that the pollen-coat \(\beta\)-glucanase and xylanase hydrolyze the stigma wall for pollen tube entrance and the pollen secrete proteins to loosen or become new wall constituents of the tube and to break the wall of the transmitting track for tube advance.

In plants, sexual reproduction is initiated when the pollen grain (male component) lands on the stigma of the style (female component) in the flowers (for reviews, see Refs. 1–3). The pollen produces a tube that penetrates the stigma and grows through the wall of adjacent cells in the transmitting track in the style to reach the ovary, where the tube delivers the male gametes to fertilize the eggs. Pollen tube penetration of the stigma and advancement in the style are critical steps in sexual reproduction; yet biochemical information on this process is limited.

The surface of a pollen grain makes the initial contact with the stigma. It includes an outermost coat and an underlying wall (1–3). The coat consists of lipids and proteins, which are initially synthesized and accumulated in the tapetum cells that enclose the pollen locule in the anthers. Upon lysis of the tapetum cells, the accumulated lipids and proteins are discharged onto the microspores (maturing pollen), forming the bulk of the pollen coat. In some species, the coat also contains minor proteins involved in self-incompatibility, which are synthesized in the pollen interior (4). The underlying wall consists of sporopollenin and other polymers embedded with proteins; it is derived from the tapetum and the pollen interior (3).

The pollen coat consists of lipids and proteins, and its composition varies, depending on the species. The coat in insect- or self-pollinating species, of which \textit{Brassica} and \textit{Arabidopsis} are the best studied (5–7), is thick; steryl esters and very non-polar lipids are the major lipids, and oleosins are the predominant proteins. The lipids are for waterproofing, whereas the amphipathic oleosins may act as a wick for water uptake to initiate germination.

The pollen coat in wind-pollinating species is thin (3, 8), and major biochemical studies have been carried out only with maize (8, 9). Maize pollen coat contains undefined neutral lipids and a few proteins of 25, 35, and 70 kDa. Only the 35-kDa protein, which is the most abundant, has been characterized and shown to be an active endoxylanase. It is initially synthesized as a large, inactive precursor in the tapetum and then activated after cleavage by a serine protease at both the N and C termini. The precursor or the mature 35-kDa xylanase does not have an apparent signal sequence that could target the protein to specific organelles; the protein is presumed to be present in the cytosol and is released to the anther locule and then the pollen surface after lysis of the tapetum cells. After landing on the stigma, the pollen would release the xylanase to hydrolyze the stigma wall for the entry of the pollen tube into the transmitting track. The characteristics of the other coat proteins of 70 and 25 kDa are presented in the current report.

Imbibed pollen quickly releases proteins that were present in the coat or the wall, or are secreted from the pollen interior. These proteins include expansin, extensin, polygalacturonase, trypsin inhibitors, and a few others (2, 10–12). Most of them are also categorized as pollen allergens because of their allergenic properties (13). Some of these proteins were shown to have their mRNA present in mature pollen. The relative amounts of these individual proteins in the whole pollen are unknown. It is also unclear whether these proteins are present in the coat or the wall of the mature or germinated pollen and thus whether they will exert their functions on the stigma or inside the style. Another uncertainty is whether these proteins and their mRNA are present continuously in the pollen or

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Maize Pollen Coat and Wall Proteins

EXPERIMENTAL PROCEDURES

Plant Materials—Maize (Zea mays L., B73) plants were grown in a greenhouse, and fresh pollen and anthers were collected as described (9). Anthers of five developmental stages were selected on the basis of the following criteria (8): At stage 1, the tassel 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 callosum. At stage 2, the upper portion of the tassel had protruded from the shoot apex. The anthers filled up about one-half of the floret. Young microspores had been released from the dissolved callosum wall, and the outer pollen wall (exine) had been synthesized. At stage 3, 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 become larger and contained multiple small vacuoles. The first mitosis had occurred, and the microspores were binucleate. At stage 4, the anthers filled up the floret completely. Second mitosis in the microspores had occurred, and the microspores were trinucleate. At stage 5, the tassel was yellow. Some of the florets on the tassel were open, and the pollen was ready to be released.

Pollen Germination—A sample of 5 mg of freshly collected pollen was placed in 1.5 ml of liquid germination medium containing 100 ppm Ca(NO₃)₂, 10 ppm H₂BO₃, 37.5 ppm lysine, 5 ppm cysteine, 0.05 ppm glutamic acid, and 15% (w/v) sucrose (14). More than 80% of the pollen germinated, and the length of the pollen tube was about 0.5 and 4 times the diameter of the pollen after 10 and 30 min, respectively.

Preparation of an Anther Wall Fraction and a Micropore Fraction—Each anther of developmental stage 3 was placed in a drop of solution containing 0.05 M sodium acetate, pH 5.2, and 0.4 M sucrose on a dish under a dissecting microscope. The microspores were gently scraped from the anther wall. The microspores and the anther wall, which still retained about 10% of the original microspores, were collected separately. Each of the two fractions from 10 anthers were combined and subjected to RNA extraction.

Preparation of a Total Pollen Fraction, a Pollen-Released Protein Fraction, an Interior Fraction, and a Pollen Shell Fraction—Fresh pollen was homogenized rigorously in 0.1 M sodium acetate, pH 5.2, with a mortar and pestle to yield a total protein fraction. A sample of 20 mg of fresh pollen was placed in a 1.5-ml microcentrifuge tube containing 1 ml of 0.01 M sodium acetate, pH 5.2 or the germination medium described in the preceding paragraph. The tube was sonicated for 10, 20, or 60 min and centrifuged at 8,000 × g for 3 min. The supernatant was collected as the pollen-released protein fraction. The pellet was homogenized gently in 0.1 ml of 0.01 M sodium acetate, pH 5.2 with a small mortar and pestle, and the homogenate was referred to as the pollen interior fraction. The homogenate was put onto a sucrose gradient in a 1.5-ml microtube for 10, 20, or 60 min and centrifuged at 8,000 × g for 30 min. The interface materials between the 1 and 1.5 M sucrose solution were collected. The sample was mixed with three volumes of 0.05 M HEPE-NaOH, pH 7.5, and centrifuged at 8,000 × g for 30 min. The pellet was resuspended with a small volume of 0.05 M HEPE-NaOH, pH 7.5, and centrifuged in a cuffed tube referred to as the pollen shell fraction.

Extraction of a Coat Fraction by Diethyl Ether from Pollen and Partition of Its Proteins into an Aqueous Medium for Enzymatic Studies—Freshly collected pollen was mixed with diethyl ether (1 g of pollen/10 ml of ether) for 1 min in a capped tube by repeated inversions. The ether layer was collected after centrifugation for 10 min at 8,000 × g. The ether preparation was reduced to 1 ml under a stream of nitrogen and used as the coat fraction for SDS-PAGE. The other proteins in this coat fraction were partitioned into an equal volume of 0.05 M sodium acetate, pH 5.2 for fast protein liquid chromatography (FPLC). ¹

Cation Exchange FPLC—The procedure was as described earlier (8). All solutions contained 0.05 M sodium acetate, pH 5.2. The aqueous pollen coat sample (~30-µg proteins in 3 ml) described in the preceding paragraph was filtered through a 0.2-µm syringe filter and applied to a pre-equilibrated Mono S HR 5/5 FPLC column (Amersham Biosciences). Solutions of 5 ml of 0 M NaCl, 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 ions were collected for protein purification by SDS-PAGE and β-glucanase and xylanase activities. The fractions containing the purified 70- and 35-kDa proteins had the peak activities of β-glucanase and xylanase, respectively and were retained for additional analyses of enzyme activities.

Enzyme Activity Assay—β-glucanase activity was measured by monitoring the appearance of reducing ends from substrates at time intervals. Laminarin from Laminarin digitate (Sigma) was used as the substrate. The β-glucanase reaction mixture of 0.3 ml contained the purified 70-kDa protein fraction and 0.3 mg laminarin in a 0.05 M buffer, which included sodium acetate, pH 4.0, 5.0, and 5.5; succinate-NaOH, pH 5.5, and 6; phosphate-NaOH, pH 6, 7, 8, and 8.5; CHES-NaOH, pH 8.5, 9, and 10. The reaction was allowed to proceed at 30 °C and terminated by the addition of 0.9 ml of β-hydroxybenzoic acid hydrizide reagent and then heating (15). After the mixture was cooled, the absorbance of the reaction mixture was read at 410 nm with a spectrophotometer. The enzyme activity was monitored at four time intervals within a 6-h period to ensure linearity of the reaction. The activities of the purified 70-kDa β-glucanase fraction, the purified coat xylanase fraction, and Aspergillus niger β-glucanase (EC 3.2.1.4, from Sigma) on various substrates (xylan, lichenan, carbomethylcellulose, and polygalacturonic acid; Sigma) in 0.05 M sodium acetate, pH 5.2, were assayed as described for laminarin. Activities of the various enzyme preparations on 0.15 mg of β-glucopyranoside (Sigma) were assayed as described for laminarin. The activities of the various enzyme preparations on 0.15 mg of β-glucopyranoside (Sigma) were assayed as described for laminarin.

SDS-PAGE of Proteins for Separation, Antibody Preparation, and Peptide Microsequencing—Acrylamide (12.5%, w/v) SDS-PAGE was performed as described (5). The proteins in the gels were stained with Coomassie Blue. For antibody preparation, gel slices containing the 10- and 17-kDa proteins from the pollen-released and interior fractions, respectively, were cut from the gel and used to produce antibodies in chicken (5). For microsequencing, fragmented proteins in a gel were transferred to a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA) and sequenced at the Genome Institute at the University of California, Riverside ( Riverside, CA). Alternatively, the proteins in the gel were subjected to trypsin digestion (for the 14-, 25-, and 70-kDa proteins) and their fragments sequenced at the Iowa State University Protein Facility (Ames, IA). The obtained sequences of the 10-, 14-, 17-, 20-, 25-, 35-, 38-, 47-, and 70-kDa proteins were TTPLTLPQVKGSKPG, DFDEPGHLAP, KKKRAAESGEAAAK, ATLEACRTAGTPDFDI, FVVTGIRICDNCRAG, LPALQSP-LK, GPPKVPKGKNIATY, RFFIGVGDDY, EKEESKGIDAKA, and YFYGVSLSGG, respectively.

Analysis of Nucleotide and Amino Acid Sequences—We used the National Center for Biotechnology Information (NCBI's) TIGR (www.tigr.org) and GenBank (www.ncbi.nlm.gov) databases to search for EST nucleotide sequences or protein sequences. The GCG Program (gsc.unc.edu) was used to compare nucleotide sequences or amino acid sequences, to construct phylogenetic trees and to translate nucleotide sequences into amino acid sequences. Subcellular locations of proteins were analyzed by use of the PSORT Program in Expasy (us.expasy.org).

RNA Extraction and RT-PCR—Total RNA was extracted (17) from maize anthers of different developmental stages, mature pollen and germinated (after 15–30 min) pollen. A sample of 1 µg of total RNA was

¹ The abbreviations used are: FPLC, fast protein liquid chromatography; ER, endoplasmic reticulum; EST, expressed sequence tag; β-glucanase and xylanase activities.
treated with RNAase-free DNase and then used to synthesize first-strand cDNA with an oligo(dT) primer (18). The cDNA was used as a template for RT-PCR with a pair of primers. For the gene encoding the pollen-coat 70-kDa β-glucanase, the 5′ primer (5'-CGACTGACGGGGCCACCGC-3′) and 3′ primer (5'-CTTGTGACCAGGGCCGCTGCC-3′) were designed from the coding region of the new maize gene ZmGLA3 (AY344692), which is different from those of the two known maize β-glucanases (to be described in Fig. 3). For the gene encoding the pollen-coat xylanase, the 5′ primer (5'-GGAGGCGTACCCAGCTTACT-3′) and 3′ primer (5'-CTTGGTGGACCGTGTCCG-3′) were designed from the coding region of the maize gene ZmXYV1 (AF149016). For the gene encoding the pollen-released 10-kDa protein, the 5′ primer (5'-CAGCAATGGGTCCCTACG-3′) and 3′ primer (5'-GCTGAGTTGT- GACAACAGGC-3′) were designed from the 3′-UTR sequences of the EST clone, AY111779. For the gene encoding the pollen-released 14-kDa protein, the 5′ primer (5'-TATGGTCTGTCCTAAGGTT-3′) and 3′ primer (5'-TGGTATTGAAATGAGTCG-3′) were designed from the 3′-UTR of a maize prolin gene, ZmPRO1, X73279. For the gene encoding the pollen-wall 17-kDa protein, the 5′ primer (5'-GCCCTTGAATACTAAAGAC-3′) and 3′ primer (5'-ACATTTCCTGCTGACATG-3′) were designed from the 3′-UTR of a maize EST clone, TC148758. For the gene encoding the pollen-released 20-kDa protein, the 5′ primer (5'-GATCTCTTCTCCACGGACG-3′) and 3′ primer (5′-ATTCACCTCATTCCAACCTC-3′) were designed from the 3′-UTR of a maize EST clone, TC123256. For the gene encoding the pollen-released 23-kDa protein, the 5′ primer (5′-ACACCCCGGTCGCACTG-3′) and 3′ primer (5′-AGATGTCGAAGATGGATCTC-3′) were designed from the 3′-UTR of a maize EST clone, TC130551. For the gene encoding the pollen-released 35-kDa protein, the 5′ primer (5′-GTGCTGTGATACTTTTAAAGCGC-3′) and 3′ primer (5′-ACACCAACATCATGATCAGGC-3′) were designed from the 3′-UTR of a maize EST clone, TC148755. For the gene encoding the pollen-released 47-kDa protein, the 5′ primer (5′-CGACTGACCACCTCCTCCTA-3′) and 3′ primer (5′-GAGAGAGAGAGACAAGAGGT-3′) were designed from the 3′-UTR of a maize EST clone, TC130551. For the gene encoding the pollen-released 10-kDa protein, the 5′ primer (5′-CGACTGTGCAATCATCCAGG-3′) and 3′ primer (5′-ACACCAACATCATGATCAGGC-3′) were designed from the 3′-UTR of a maize EST clone, ZmXYV1. For the gene encoding the pollen-released 14-kDa protein, the 5′ primer (5′-GAGCTGGGTTGTAATTAGG-3′) and 3′ primer (5′-GGATGTCGAAGATGGATCTC-3′) were designed from the 3′-UTR of a maize EST clone, AY111779.

RESULTS AND DISCUSSION

### Mature Pollen Wall Was Separated into Fractions of Distinct Structures or Origins

Mature pollen was separated into four distinct fractions, the coat, a pollen-released protein fraction, the interior, and the shell. These fractions were analyzed for their protein constituents by SDS-PAGE.

A coat fraction was obtained by washing the mature pollen with diethyl ether. SDS-PAGE revealed three visible protein bands, which represented only a very small proportion of the total pollen proteins (Fig. 1A). N-terminal sequencing and FPLC revealed that the 70- and 35-kDa protein bands each represented one protein (to be described) and that the 25-kDa protein band contained at least two fragments of different proteins via a search of GenBank™ protein data bases. These fragments were not studied further.

Fractions of pollen-released proteins and pollen interior proteins were obtained by the following procedure. Mature pollen was shaken gently in a liquid medium of 10 mM sodium acetate, pH 7.2, 7% SDS, 1% bovine serum albumin, and 0.1 M EDTA, pH 8.0 for 4 h; hybridized with 32P-labeled probes (preceding paragraph) 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 all 65°C.

RT-PCR Analysis of ZmGLA1, ZmGLA2, and ZmGLA3 Expression in Maize Anthers, Mature Pollen, and Germinated Pollen—Total RNA from maize anthers of different developmental stages, mature pollen or germinated pollen was extracted and used to synthesize first-strand cDNA, as described in a preceding section. The cDNA was used as templates for PCR with a pair of gene-specific primers. For ZmGLA1, the 5′ primer (5′-GGTTGAGGGCCCTCTGATG-3′) and 3′ primer (5′-ACACCGCTAATAGGTTAGG-3′) were designed from the 3′-UTR of a maize β-glucanase Exo1 EST clone, AY103742 (gDNA, AF225411). For ZmGLA2, the 5′ primer (5′-AGATGGTACCGAACTAAGAAA-3′) and 3′ primer (5′-AGAAGTAGAGATGCTCTATATG-3′) were designed from the 3′-UTR of a maize β-glucanase (Exo1) EST clone, AF061707. For ZmGLA3, the 5′ primer (5′-GCCCGAGGATCAGGATAGC-3′) and 3′ primer (5′-CAATCTTCTTTTACCTACCTC-3′) were designed from the 3′-UTR of our newly registered maize gene (GenBank™ AT344632). For a maize actin gene, the 5′ primer (5′-GGTATGATGTCATCTCC-3′) and 3′ primer (5′-GAGGCCTGAGATGCTCTCC-3′) were designed from the 3′-UTR of a maize actin gene (GenBank™, Mza56, U60514). The PCR fragments were analyzed with 1% agarose gels.

### Rapid Amplification of cDNA Ends (5′-RACE)—It was performed with the use of a 5′-RACE system (Invitrogen) according to the provided instructions. The first-strand cDNA was synthesized with a primer, 5′-CTTGGGAGGGCCAGATG-3′. Primers were designed from the 3′ and 5′ ends of the 5′-RACE products by methods similar to those used to synthesize first-strand cDNA with an oligo(dT) primer (18). The cDNA was used as a template for RT-PCR with a pair of primers. For the gene encoding the pollen-coat 70-kDa β-glucanase, the 5′ primer (5′-CACTGACGGGGCCACCGC-3′) and 3′ primer (5′-CTTGGTGGACCGTGTCCG-3′) were designed from the coding region of the maize gene ZmXYV1 (AF149016). For the gene encoding the pollen-released 10-kDa protein, the 5′ primer (5′-CAGCAATGGGTCCCTACG-3′) and 3′ primer (5′-GCTGAGTTGTGACAACAGGC-3′) were designed from the 3′-UTR sequences of the EST clone, AY111779. For the gene encoding the pollen-released 14-kDa protein, the 5′ primer (5′-TATGGTCTGTCCTAAGGTT-3′) and 3′ primer (5′-TGGTATTGAAATGAGTCG-3′) were designed from the 3′-UTR of a maize prolin gene, ZmPRO1, X73279. For the gene encoding the pollen-wall 17-kDa protein, the 5′ primer (5′-GCCCTTGAATACTAAAGAC-3′) and 3′ primer (5′-ACATTTCCTGCTGACATG-3′) were designed from the 3′-UTR of a maize EST clone, TC148758. For the gene encoding the pollen-released 20-kDa protein, the 5′ primer (5′-GATCTCTTCTCCACGGACG-3′) and 3′ primer (5′-ATTCACCTCATTCCAACCTC-3′) were designed from the 3′-UTR of a maize EST clone, TC123256. For the gene encoding the pollen-released 23-kDa protein, the 5′ primer (5′-ACACCCCGGTCGCACTG-3′) and 3′ primer (5′-AGATGTCGAAGATGGATCTC-3′) were designed from the 3′-UTR of a maize EST clone, AY111779. For the gene encoding the pollen-released 35-kDa protein, the 5′ primer (5′-GTGCTGTGATACTTTTAAAGCGC-3′) and 3′ primer (5′-ACACCAACATCATGATCAGGC-3′) were designed from the 3′-UTR of a maize EST clone, ZmXYV1.

Microscopy—Samples of pollen before and after protein release were observed under a Zeiss Axioskop microscope and then photographed. For immunofluorescence microscopy, germinated pollen grains having different turgescence, which is not visible from the stained SDS-PAGE gel because of their relatively minute amounts (Fig. 1A) but were revealed by immunoblotting with antibodies against the 35-kDa proteins (data not shown).

A pollen shell fraction, representing the pollen after the pollen-released and interior proteins had been removed, was obtained by gently grinding the interior fraction and subjecting the ground materials to sucrose gradient centrifugation. A clean fraction of individual pollen shells was obtained (Fig. 1D). The aperture on each pollen shell was visible by high magnification microscopy (inset in Fig. 1D). No proteins were detected.
in this shell fraction (Fig. 1A). Thus, no abundant proteins were tightly associated with the pollen wall.

The Coat 70-kDa Protein Is an Active β-Glucanase Whose Gene/Protein Has Not Been Previously Studied—Of the two coat proteins, the 35-kDa protein has been shown to be an endoxylanase (9). We purified the 70-kDa protein to homogeneity from the coat fraction by FPLC (Fig. 2A) and subjected it to trypsin digestion. One trypsin fragment was sequenced. The sequence of this fragment, YFVGSVLSGG, closely matches those of proteins encoded by two maize genes (9 of 10 residues, β-glucanase ExoI, termed *ZmGLA1* for convenience in the current report; and 7 of 10 residues, β-glucanase ExoII, termed *ZmGLA2* in the current report) (Fig. 3A); the former protein was studied in relation to its β-glucanase activity in young shoots (19). It also closely matches those of proteins encoded by two barley genes (9 of 10 residues, β-glucanase ExoI, *HvGLA1*; and 7 of 10 residues, β-glucanase ExoII, *HvGLA2*); both barley

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**Fig. 1.** Separation of maize pollen into fractions of distinct origins. Panel A, SDS-PAGE of proteins in mature pollen (total) and separated fractions of coat, pollen-released fraction, interior fraction, and shell. Different proportions of individual samples were applied to the lanes, and these proportions relative to an equal amount of pollen are shown. Asterisks indicate proteins described in text: the 35- and 70-kDa proteins in the coat; the 10-, 14-, 20-, 23-, 25-, 35-, 38-, and 47-kDa proteins in the pollen-released fraction; and the 17-kDa protein in the interior fraction. Molecular mass markers in kDa are indicated on the right. Panels B–D, light microscopic photographs of fresh pollen, pollen after the released proteins had been removed, and shells, respectively. Inset in panel D is an enlarged photo of a shell, showing an aperture on its surface. The scale bar represents 100 μm.

**Fig. 2.** Separation of the pollen coat proteins and analysis of the 70-kDa protein for β-glucanase activity. Panel A, SDS-PAGE analysis of proteins in the coat fraction and the FPLC fractions containing the purified 25-, 35-, (xylanase), and 70-kDa proteins. Molecular mass markers were on the far left lane, and their values in kDa are indicated on the left margin. Panel B, activity of the 70-kDa protein (β-glucanase) on laminarin in buffers of different pH values. The buffers were sodium acetate, pH 4.0, 5.0, and 5.5; succinate-NaOH, pH 5.5 and 6.0; phosphate-NaOH, 6.0, 7.0, 8.0, and 8.5; CHES-NaOH, 8.5, 9.0, and 10. Panel C, activities of maize coat 35-kDa xylanase and 70-kDa β-glucanase, and a commercial preparation of *Aspergillus* β-glucanase on different substrates, including *p*-nitrophenyl-β-glucopyranoside (4-NPG). The values of 100 relative activities represent 17 μmol/h/mg protein of maize xylanase, 68 μmol/h/mg protein of maize β-glucanase, and 1,857 μmol/h/mg protein of the *Aspergillus* β-glucanase preparation.
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Fig. 3. Characterization of the maize β-glucanases. Panel A, a pileup of the N-terminal amino acid sequences of cereal β-glucanases. The enzymes are from maize (ZmGLA1 from β-glucanase ExoI derived from the DNA sequence of AF225411, ZmGLA2, from β-glucanase ExoII, AF064707; ZmGLA3, our registered gene AY344825), barley (HvGLA1 from β-glucanase ExoI, AF102968; HvGLA2 from β-glucanase ExoII, U46003; HvGLA3 (a partial EST), assembled from BU995244, BU995245, and BU468423) and rice (OsGLA1, TC124353; OsGLA2, TC113312; OsGLA3, TC81322). ZmGLA1, HvGLA1, and ZmGLA2 have been shown to be exo-β-glucanases; for convenience, all the cereal enzymes are termed β-glucanase (GLA) in this figure. The shaded sequences were those used in the current study to identify the coat protein β-glucanase to ZmGLA3; specifically, a valine (in bold) in lieu of isoleucine is present in OsGLA3 and ZmGLA3. The underlined sequences represent putative ER-targeting signal peptide. Panel B, RT-PCR results with RNA from anther walls and microspores separated from stage-3 anthers and primers specific for ZmGLA3, the maize xylanase gene (ZmXYN1), and the gene encoding the pollen 10-kDa protein (AY111779). The anther wall sample had about one-third of the amount of the transcript of a maize actin gene in the microspore sample. Panel C, RT-PCR results with RNA from anthers of developmental stages 1–5 and mature (M) and 15–30-min germinated (G) pollen, and primers specific for maize ZmGLA1, 2 or 3. Approximately equal amounts of transcript of a maize actin gene were present in the various samples. Panel D, a phylogenetic tree of cereal β-glucanases constructed on the basis of their amino acid sequences. The scale bar represents 10 substitutions per 100 residues.

proteins were studied in relation to their β-glucanase activities in seeds (20). It completely matches a protein encoded by a full-length rice EST sequence (10 of 10 residues, OsGLA3) derived from maturing flowers. We used the rice EST sequence to search for and obtain two related, short maize gene sequences (BZ402366 and TC163747). On the basis of these rice and maize gene sequences, we designed primers for RT-PCR and obtained the 5′-sequence of a maize cDNA; this gene, termed ZmGLA3, encodes the coat 70-kDa protein (see next section).

Whether the coat 70-kDa protein was an active β-glucanase was tested by assaying its enzymic activities on various substrates (Fig. 2C). It was active on laminarin (mostly 1,3-β-glucan), β-nitrophenyl-β-glucopyranoside (4-NPG), and li-chenan (mostly 1,3:1,4-β-glucan), and fairly inactive on carboxymethylcellulose (1,4-β-glucan), oat-spelled xylan (1,3-β-xylan), and 1,4-β-polygalacturonan. This pattern of activity contrasts with that of the coat xylanase (9), which was active mostly on oat-spelled xylan (Fig. 2C). A commercial β-glucanase (the preparation could contain contaminants of related enzymes) from Aspergillus was active on most of the tested substrates (Fig. 2C). The coat 70-kDa protein was most active at pH 5.5 on laminaran (Fig. 2B). Thus, the coat protein is an acidic β-glucanase that can act on 1,3-β-glucan and 1,3:1,4-β-glucan and is termed β-glucanase in the remaining discussion.

We explored whether the coat β-glucanase was synthesized in the tapetum or the pollen interior. Individual anthers were dissected into a pure microspore fraction and an anther wall fraction, which contained all of the outer anther cells, the inner tapetum, and about 10% of the original microspores. RT-PCR analyses revealed that the transcripts of ZmGLA3, encoding the coat β-glucanase and the gene encoding the pollen-released 10-kDa protein (to be described) were present exclusively in the anther wall fraction and the microspore fraction, respectively (Fig. 3B). Apparently, the coat β-glucanase is similar to the coat xylanase (Ref. 9 and Fig. 3B) in that they are synthesized in the tapetum cells and released into the locule and then onto the microspore surface.

The sequence of the nascent coat β-glucanase, as deduced from the gene sequence, contains a putative N-terminal signal peptide (Fig. 3A). The enzyme is apparently secreted from the tapetum cells via the endoplasmic reticulum (ER)-vesicle-exte-rior secretory pathway. This release mechanism is different from that for the xylanase, which is apparently a cytosolic protein that is released from the tapetum cells after cell death and activated by extensive proteolysis (9).

The Pollen Coat β-Glucanase Is Different from the Well Known β-Glucanase That Hydrolyzes the Callose Wall of the Microspore Tetrad—During early microsporogenesis, the tapetum secretes β-glucanases that hydrolyze the callose wall of the microspore tetrad to produce solitary microspores (21). These biochemical findings were initially obtained from large lily flowers. The peaking of the enzyme activities during anther development coincided with the formation of solitary microspores, and thereafter the enzyme activities disappeared. In subsequent studies, genes encoding β-glucanase in anthers or tapetum in several species were cloned (22) and assumed to be those encoding the β-glucanase that hydrolyzed the callose wall of the microspore tetrad.

We examined whether the coat β-glucanase is the same enzyme that hydrolyzes the callose wall of the microspore tetrad—

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rad. Developing anthers were divided into five stages (see “Experimental Procedures”). Microspores were in the tetrad phase in stage 1 and the solitary phase in stage 2. The transcript of the gene (ZmGLA3) encoding the coat β-glucanase was absent in stage 1, appeared in stage 2, peaked at stage 3, and declined in stage 4 (Fig. 3C). Clearly, the coat β-glucanase is not the enzyme that hydrolyzes the callose wall of the microspore tetrad. In contrast, transcripts of the other two known maize β-glucanase genes (ZmGLA1 and ZmGLA2) were highest in stage 1 and disappeared gradually thereafter. It is likely that ZmGLA1 and ZmGLA2 encode the recognized β-glucanases that hydrolyze the callose wall of the microspore tetrad. Nevertheless, the possibility of other genes encoding unknown β-glucanases for callose hydrolysis cannot be excluded. We conclude that the coat β-glucanase is different from those hydrolyzing the callose wall of microspore tetrad in the anthers. The coat β-glucanase could, along with the coat xylanase (9), hydrolyze the stigma wall during pollen germination and tube growth.

A phylogenetic tree of related β-glucanases in cereals available in GenBank™ was constructed on the basis of amino acid sequences (Fig. 3D). These β-glucanases presumably can hydrolyze 1,3;1,4-β-glucan. They can be divided into three groups, each consisting of a maize β-glucanase. Members of the group that includes the maize coat β-glucanase have not been previously characterized, whereas those in the other two groups have been studied (the maize ExoI (ZmGLA1) in seedling shoots, Ref. 19 and the barley ExoI and -II (HvGLA1 and 2) in germinated seed, Ref. 20, shoots, roots, and leaves, Ref. 22). The current results show that the β-glucanases in the latter two groups are present not only in vegetative organs but also in anthers. The finding that the three maize β-glucanases scatter in the tree indicates that their genes are not products of recent gene divergence but encode specific β-glucanases for different physiological functions. Several studied β-glucanases in dicots are relatively dissimilar to the cereal β-glucanases and are not included in the phylogenetic tree.

Proteins Were Released from the Pollen and Tube During Germination—We studied the liberation of the proteins of the pollen-released protein fraction (Fig. 1A). We allowed mature pollen to germinate in a liquid germination medium (of a pH of ~5.0) and then analyzed the medium by SDS-PAGE for proteins that had been released from the pollen. Many specific proteins were present in the medium after a 10-min incubation (Fig. 4). At the end of this incubation, the length of the tube reached about half the diameter of the pollen grain. Attempts to analyze the released proteins after a longer time of incubation were unsuccessful because the longer tubes broke during removal of the grains to recover the medium. We switched to a medium of 10 mM sodium acetate, pH 5.2, in which the pollen did not germinate. The durations of incubation are indicated. Different proportions of individual samples were applied to the lanes; these proportions relative to an equal amount of pollen are indicated. Total pollen extract and the coat fraction were used as references. Molecular mass markers in kDa are indicated on the right.

The Pollen-released Proteins Could Loosen or Form New Tube Walls or Loosen or Hydrolyze the Transmitting Track Wall—After separation by SDS-PAGE (Fig. 4), the major pollen-released proteins, or their fragments after trypsin digestion, were subjected to N-terminal sequencing. The proteins were identified via the GenBank™ or TIGR data bases according to amino acid sequences. Their identities suggest that they could loosen and form new tube walls for tube elongation or loosen and hydrolyze the transmitting track wall for tube advance. We briefly describe the proteins.

The 10- and 35-kDa proteins are identified as expansin-like allergen and β-expansin, respectively (Ref. 10 and Table 1). These two specific β-expansins have not been previously reported, even though several pollen expansins of maize and other cereals are known (24, 25). The 35-kDa β-expansin has the binding and catalytic domains of an expansin, which catalyzes the break-and-reunion of non-covalent bonds in the cell wall (10). It may loosen the wall of the tube or the transmitting track for tube advance. The 10-kDa expansin-like allergen has only the binding domain (in the allergen group II/III, Ref. 13) and may retard cellulose crystallization at the tube tip or the transmitting track for tube advance (26).

The 14- and 23-kDa proteins are identified as pollen allergens described in maize (27) and Lolium (28), respectively (Table 1). The 14-kDa allergen is also known as a profilin (27). Profilins are known to be allergens released from pollen of several cereals (13). The possible function of profilin, which is abundant in maize pollen (Fig. 1), is puzzling. Profilin is supposed to be involved in cytoskeleton organization inside the cell. Unlike all other pollen-released proteins we studied (Table 1), the nascent 14-kDa profilin does not have a putative N-terminal ER-targeting signal peptide. Thus, the protein is supposed to be cytosolic and not released to the exterior. It is likely that profilins can somehow be easily released from pollen and perform a yet-to-be-determined function on the tube wall or in the transmitting track. The 23-kDa allergen is also known to be related to trypsin inhibitors in the amino acid sequence, except...
that the inhibitor active site in the allergen is absent (28). Its function in the pollen as a released protein is unknown.

A 20-kDa protein is identified as a wall-modulating protein previously studied as an inhibitor of pectin methylesterase (29) or inhibitor of invertase (30) in non-pollen tissues (Table I). The common features of these two enzymes include the presence of a sugar-binding motif and the reversibility of the enzymatic reactions. The 20-kDa maize pollen protein released from germinated pollen may modulate the activities of these two enzymes or other related enzymes in the tube wall or the transmitting track wall and facilitate tube elongation. Alternatively, its sugar-binding motif may retard cellulose crystallization at the tube tip and enhance tube growth (26).

The 25- and 38-kDa proteins are tentatively identified as wall structural proteins called extensins (Table I), which have motifs for binding among themselves and to sugar residues of the wall (31–33). The 25-kDa protein could be a hydroxyproline-rich chimeric extensin (33) because the protein was absent in the pollen shell fraction that remained associated with the pollen after our washing/germination procedure (Figs. 1 and 4). This association was not tight, as the 25-kDa protein could be a hydroxyproline-rich chimeric extensin (7 of 9 sequenced residues identical (NP_200917)), which was so identified via a studied Chlamydomonas protein (34). The 38-kDa protein could be a glycene-rich extensin because of its similarity to a bacteria Ca$^{2+}$-binding protein (8 of 10 sequenced residues identical (NP_437273, Ref. 35)), which in turn is similar to an Arabidopsis extensin (AAK07681, Ref. 36). Maize pollen-synthesized extensins of higher molecular weights have been reported (37).

Finally, a 47-kDa protein is identified as a polygalacturonase (Table I). The gene encoding this exact maize polygalacturonase has been described (11). Polygalacturonate is a major component of pectin in the cell wall, even though monocot cell walls do not have abundant pectin. The polygalacturonase could hydrolyze the pectin between adjacent cells in the transmitting track and facilitate tube advance.

A Novel, Abundant, and Potentially Cation-chelating Protein with Three Consecutive Finger Motifs Is Associated with the Wall of the Pollen and Pollen Tube—An abundant 17-kDa protein, representing 1–2% of the total pollen proteins, was not readily released from the mature pollen in a mildly acidic medium (Figs. 1 and 4). It is identified (14 of 14-sequenced residues) as a protein encoded by a maize EST (TC84818) derived from mixed anthers and pollen (Fig. 5A). The maize protein is closely related to a sorghum protein encoded by an EST (Fig. 5A). The maize EST sequence has 1,153 nt and an open reading frame that encodes a protein of 192 residues. The N-terminal 25 residues of this protein are predicted (via PSORT in ExPASy) to represent a removable ER-targeted sequence. In our study, 30 residues had been removed from the N terminus of the nascent protein to produce the mature protein (Fig. 5A), which has 162 residues and 17,814 daltons.

Other than having a removable N-terminal, ER-targeting signal peptide, the 17-kDa protein has no other recognized organelle-targeting sequences, transmembrane segment, or glycosylation site. The mature protein is hydrophilic and has a predicted pI of 8.8. Although it is a secretory protein and present as the processed form in mature pollen (Fig. 1), it remained associated with the pollen after our washing/germination procedure in a pH 5.2 medium (the presumed pH in cell wall) that removed many other pollen-released proteins in their entirety (Figs. 1 and 4). This association was not tight, because the protein was absent in the pollen shell fraction that was isolated after gentle grinding and gradient centrifugation (Fig. 1A). In germinated pollen, the protein was associated with the wall of the protruded tube (Fig. 5C).

A striking feature of the sequence of the 17-kDa protein is the presence of 14 cysteine residues in a unique pattern not found in any other protein. The N terminus of the mature protein has a 21-residue sequence that possesses no cysteine but many hydrophilic residues (Fig. 5A). This sequence is followed by six tandem repeats of CX$_2$CX$_{10-15}$ (C for cysteine and X for any residue other than cysteine) and then by a seventh slightly modified CX$_2$CX$_{10-15}$ repeat at the C terminus. The six tandem repeats are also present in a protein derived from a sorghum EST sequence, which does not have the seventh repeat (Fig. 5A). Two such tandem repeats could form a finger loop motif that brings the four cysteine residues to proximity via binding of a divalent cation (Fig. 5B). Thus, the 17-kDa protein could form three consecutive finger motifs. Finger motifs binding to zinc are present in steroid hormone receptors (a single finger, with four cysteines) and transcription factors.

### Table I

| Protein (kDa) | Identified as | Sequenced residues matching those in identified proteins | N-terminal, ER-targeting sequence | Number of residues from ORF (including N-signal peptide) | Whether the proteins have been studied earlier (i.e., not just derived from EST sequences) |
|--------------|--------------|----------------------------------------------------------|---------------------------------|----------------------------------------------------------|----------------------------------------------------------------------------------|
| 35 | Xylanase | AF149016 | (19/19) | No | 544 | Studied by us (9) |
| 70 | β-Glucanase |AY544632 | 10/10 | Yes | 644 | Novel |
| 10 | Expansin-like allergen | AY111779 | 15/15 | Yes | 120 | Similar proteins studied in pollen of other species |
| 14 | Allergen profilin | CAA51719 | 10/10 | Yes | 130 | Exact protein studied in pollen of maize |
| 17 | Wall modulating allergen (trypsin inhibitor) | TC148758 | 15/15 | Yes | 192 | Novel |
| 20 | Allergen | TC132555 | 15/15 | Yes | 172 | Similar protein studied in meristems of tobacco |
| 23 | Extensin | TC130551 | 15/15 | Yes | 164 | Similar proteins studied in pollen of maize and other species |
| 25 | Extensin | NP_200917 | 7/9 | Yes | 344 | Similar proteins (larger size) studied in non-pollen organs of Arabidopsis and other species |
| 35 | β-Expansin | TC148755 | 14/15 | Yes | 270 | Similar protein studied in shoots of rice |
| 38 | Extensin | AAR07681 | 8/10 | Yes | 744 | Similar protein studied in bacteria |
| 47 | Polygalacturonase | X57827 | 12/12 | Yes | 410 | Exact protein studied in pollen of maize |

The proteins are listed according to their size, and their resolution by SDS-PAGE can be found in Fig. 1. Proteins having molecular weights higher than those derived from the open reading frame (ORF) might have been glycosylated.
Preliminary experiments were performed to test whether the 17-kDa protein could bind to $\text{Ca}^{2+}/\text{H}^{+}$, an abundant cation in cell wall, so that its movement during SDS-PAGE would be retarded (39). No such retardation was observed (data not shown). Whether the binding would occur and be observed

**Fig. 5. Characterization of the maize pollen 17-kDa protein.** Panel A, a pileup of the amino acid sequences of the maize protein (derived from the DNA sequence of TC148758) and a closely related sorghum protein (from TC54813). The maize sequence obtained by N-terminal sequencing of the mature protein is in blue. The cysteine residues are in red. The putative N-terminal ER targeting sequences are underlined. Panel B, a model of a finger motif in the presence of a chelated divalent cation ($\text{Ca}^{2+}/\text{H}^{+}$) by $\text{C} \cdot \text{X} \cdot \text{C}$ tandem repeats of $\text{C} \cdot \text{X} \cdot \text{C} \cdot \text{X}$, in the maize 17-kDa protein. Panel C, immunofluorescence microscopy with use of chicken antibodies against the protein and cyanine 3-linked donkey secondary antibodies against chicken IgY. Germinated pollen grains having different tube lengths were observed under a microscope for fluorescence (circled 1, 3, and 5) or in a bright field (circled 2, 4, and 6). Germinated pollen grain in circled 5 and 6 was subjected to similar treatments but without chicken antibodies. Scale bar represents 40 $\mu$m.

**Fig. 6.** RNA blot hybridization of RNA extracted from anthers of developmental stages 1–5 and mature (M) and 15–30-min germinated (G) pollen. High stringent washing was applied to the radioactive blot so that only RNAs from the designated genes were observed (see “Experimental Procedures”). The genes encode tapetum-synthesized coat 35- and 70-kDa proteins and microspore-synthesized released proteins (listed and named in Table I). Ethidium bromide-stained 25 S and 16 S rRNA in the gel reveal that equal amounts of RNA in each sample were used.

**Fig. 7.** Localization of the maize 10-kDa expansin-like allergen in the pollen tube wall by immunofluorescence microscopy. Chicken antibodies against the protein and cyanine 3-linked donkey secondary antibodies against chicken IgY were used. Germinated pollen grains having different tube lengths were observed under a fluorescence microscope for fluorescence (circled 1, 3, and 5) or in a bright field (circled 2, 4, and 6). Germinated pollen grain in circled 5 and 6 was subjected to similar treatments but without chicken antibodies. Scale bar represents 40 $\mu$m.

17-kDa protein could bind to $\text{Ca}^{2+}$, an abundant cation in cell wall, so that its movement during SDS-PAGE would be retarded (39). No such retardation was observed (data not shown). Whether the binding would occur and be observed
under specific incubation and analysis conditions remains to be elucidated.

We speculate the functions of the abundant 17-kDa protein. The sequence of six tandem repeats of CX_{10–15} is flanked by 21 residues at the N terminus and 33 residues at the C terminus. The N-terminal 21-residue sequence does not conform to any known functional motif in other proteins. The C-terminal 33-residue sequence contains a slightly modified CX_{10–15} repeat that is absent in the songburn analog (Fig. 5A). It is likely that the functioning of the 17-kDa protein rests largely on its six tandem repeats of CX_{10–15}. These repeats could form three consecutive finger motifs that could chelate cations and act as a signal receptor. Divalent cations such as calcium are present in the cell wall; they chelate polysaccharides and maintain the cell wall structure. The 17-kDa protein could chelate divalent cations in the cell wall and thus increase wall fluidity of the pollen tube wall for tube extension, or of the transmitting track to guide the tube advancement. Or, the three consecutive finger motifs, which are known to be versatile in specific binding to proteins (38), could interact with signal proteins in the transmitting track to guide the tube to the ovules. It is also possible that the cysteine residues form numerous sulfide linkages to generate a unique protein structure for a yet-to-be-determined function.

The Appearance of the Transcripts of the Coat Proteins in the Tapetum Preceded That of the Pollen-released Proteins in the Microspores During Anther Development—The transcripts of the coat xylanase (9) and β-glucanase (Fig. 3B) were present in the tapetum and not in the microspores of the anthers. The levels of the transcripts of both enzymes in anthers peaked at stages 2 to 3 of development (Figs. 3C and 6). In contrast, the transcripts of the pollen-released proteins were present in the microspore and not the tapetum, as exemplified in the transcript of the 10-kDa expansin being localized in the microspores (Fig. 3B) and shown in their presence in mature and germinated pollen (Fig. 6). The levels of some of these pollen-released protein transcripts, especially the 10-kDa expansin transcript, remained fairly similar throughout development from stage 4 to germinated pollen (Fig. 6); it is unknown whether these transcripts in the mature and germinated pollen were those synthesized in the maturing microspores or were newly synthesized after pollen maturation. The levels of some other transcripts, especially the 23-kDa trypsin inhibitor and the 35-kDa allergen still in the wall and that stored or newly synthesized from the pollen after 10 to 30 min germination (Figs. 1 and 4).

Therefore, what we observed (Fig. 7) was the expansin-like allergen still in the wall and that stored or newly synthesized in vivo, they should be slowly released to the exterior. Most appear to interact with the wall of the pollen tube or the transmitting track of the style for advancement of the tube. The novel 17-kDa protein could act as a receptor to interact with signals in the style, perhaps to guide the tube to the ovules. We studied only the abundant pollen-released proteins. Minor proteins for possible signaling and other functions may exist and need to be studied.

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