Maize Pollen Coat Xylanase Facilitates Pollen Tube Penetration into Silk during Sexual Reproduction*

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Der Fen Suen and Anthony H. C. Huang1

From the Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, California 92521

Cell wall hydrolases are well documented to be present on pollen, but their roles on the stigma during sexual reproduction have not been previously demonstrated. We explored the function of the tapetum-synthesized xylanase, ZmXYN1, on maize (Zea mays L.) pollen. Transgenic lines (xyl-less) containing little or no xylanase in the pollen coat were generated with use of an antisense construct of the xylanase gene-coding region driven by the XYN1 gene promoter. Xyl-less and wild-type plants had similar vegetative growth. Electron microscopy revealed no appreciable morphological difference in anther cells and pollen between xyl-less lines and the wild type, whereas immunofluorescence microscopy and biochemical analyses indicated an absence of xylanase on xyl-less pollen. Xyl-less pollen germinated as efficiently as wild-type pollen in vitro in a liquid medium but less so on gel media of increasing solidity or on silk, which is indicative of partial impaired water uptake. Once germinated in vitro or on silk, the xyl-less and wild-type pollen tubes elongated at comparable rates. Tubes of germinated xyl-less pollen on silk did not penetrate into the silk as efficiently as wild-type pollen. Xyl-less pollen germinated in vitro and on silk, the xyl-less and wild-type pollen tubes elongated at comparable rates. Tubes of germinated xyl-less pollen on silk did not penetrate into the silk as efficiently as tubes of wild-type pollen, and this lower efficiency could be overcome by the addition of xylanase to the silk. For wild-type pollen, coat xylanase activity on oat spilled xylan in vitro and tube penetration into silk were inhibited by xylose but not glucose. The overall findings indicate that maize pollen coat xylanase facilitates pollen tube penetration into silk via enzymatic xylan hydrolysis.

Sexual reproduction in plants is the union of male gametes in pollen and eggs in the female carpel of flowers. A pollen grain lands and germinates on the carpel surface, termed the stigma, and produces a tube that penetrates the stigma into the carpel. The tube carrying male gametes elongates toward the ovary and produces a tube that penetrates the stigma into the carpel. The mechanism of this step is unknown, in part, because of the difficulty in studying the in vivo process. The stigma wall is overlaid with an incomplete layer of cutin, and thus the crucial physical barrier is the wall alone (7). Wall hydrolases and wall-modulating proteins are known to be present on or rapidly secreted from pollen. Their presence was observed by in situ staining (10) and by chemical analyses of extracts of pollen coat, subfractions of the pollen wall, or rapidly secreted materials from pollen (11–18). These proteins could be remnants of lysed tapetum cells stuck onto the pollen having no further function. Alternatively, they could be components specifically synthesized for hydrolysis of the stigma wall for tube entry or hydrolysis and modulation of the tube track wall inside the carpel for advancement of the pollen tube.

Pollen wall hydrolases and wall-modulating proteins have been best studied with maize. Maize is monoecious and self-compatible, and its flowers are large and produce abundant pollen. Maize pollen surface crest (19) can be separated into two biochemically distinct, morphological parts: the wall and the coat external to it (17).

Maize pollen wall and its transiently associated proteins include wall hydrolases and modulating proteins (11–18). These proteins are synthesized in the pollen interior and secreted to the wall and exterior before, during, or immediately after germination. They include polygalacturonase and several wall-modulating proteins, which consist of expansin, profilin, cation-binding protein, pollen allergen (trypsin inhibitor), extensin, and other potential wall modulators. The mRNAs of these proteins in the microspores (immature pollen) and mature pollen appear late during anther development, and their levels persist or increase after germination (17). Thus,
these wall hydrolases and modulating proteins likely exert a role after the pollen tube has penetrated the stigma and may hydrolyze and modulate the cell wall of the cells forming the pollen tube track in the carpel for advancement of the pollen tube toward the ovary.

Maize pollen coat, which is located on top of the pollen wall, contains proteins and lipids. These coat molecules are largely if not exclusively synthesized in the adjacent tapetum, which is a one-celled layer enclosing the anther locule where pollen mature (20). The coat proteins consist of only a few molecular species, the predominant one being a xylanase (EC 3.2.1.8) (14). Their mRNAs are present exclusively in the tapetum and appear earlier than those of the pollen-synthesized wall proteins (preceding paragraph) during anther development. The xylanase is synthesized as a larger precursor of 60 kDa in the tapetum and is processed at both the N and C termini to the active 35-kDa enzyme (21). These coat proteins may be present fortuitously as remnants after death of the tapetum or, especially the predominant xylanase, play a vital and yet unknown physiological role in the initiation of sexual reproduction.

Maize and other cereals have a Type II cell wall, which contains predominantly hemicellulose and cellulose (22). The hemicellulose backbone consists of a polymer of xylose (xylan) with short branches of arabinose and glucuronic acid. The xylanase in the maize pollen coat has been purified to homogeneity, with short branches of arabinose and glucuronic acid. The xylanase in maize pollen coat has been purified to homogeneity, with short branches of arabinose and glucuronic acid. The xylanase in maize pollen coat has been purified to homogeneity, with short branches of arabinose and glucuronic acid. The xylanase in maize pollen coat has been purified to homogeneity, with short branches of arabinose and glucuronic acid.

EXPERIMENTAL PROCEDURES

Plant Materials—Anthers and pollen were collected from maize (Zea mays L., B73) plants grown in a greenhouse at 28 °C with 14-h/10-h day/night cycle. Anthers of five developmental stages were selected on the basis of the following criteria (14). 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 callose wall. 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 callose 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. 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.

Cloning and Sequencing of Xyl Antisense Construct—A 2.0-kb ZmXYN1 fragment was amplified from a genomic clone (14) by PCR with 5′-GCGGCGATGAAGATAAATTTAACATGC-3′ and 3′-GGATCCCTTGATAGGCAGCCTGGTGCAC-3′ primers; the underlined sequences represent those recognized by the applied restriction enzymes. This fragment contains a 1.2-kb ZmXYN1 5′ promoter sequence plus the 5′-untranslated region and a 744-bp sequence (preXYN1) encoding the N-terminal 248 residues of the 60-kDa prexylanase; the 248-residue polypeptide encoded by the latter sequence would be removed in in vivo processing of the prexylanase to the mature 35-kDa xylanase. A 931-bp ORF2 encoding the mature 35-kDa xylanase was amplified from the same genomic clone by PCR with 5′-GGGTACCTCGGCCTCCCGCGATACGC-3′ and 3′-GGGTACCTCTACGTTAGAACCTAAAGTCGC-3′ primers. The 2.0- and 931-bp fragments were cloned into pBluescript II SK(+) (pBSK) vector and pGEM-T, respectively. A 0.3-kb nopaline synthase terminator (NOS-ter) was cut from pBI101.1 with use of SacI and EcoRI and then cloned into pUC19. The NOS-ter fragment was cut from this pU19 with BamHI and EcoRI and inserted into pBS containing (ZmXYN1 promoter + N-preXYN1). The ORF of ZmXYN1 encoding the 35-kDa xylanase was cut from pGEM-T containing the insert with use of BamHI and inserted into pBSK containing ZmXYN1 promoter + N-preXYN1 + NOS-ter at the position between the N-preXYN1 and NOS-ter sequences. The antisense orientation and the sequence of the ORF of ZmXYN1 were confirmed with results from restriction enzyme digestions and sequencing, respectively.

Establishment of Xyl Antisense Transgenic Lines—Immature embryos of maize (Z. mays L., B73) were bombarded with the plasmid containing the above-mentioned antisense XYN1 construct and a plasmid containing a bar gene (23) with use of a gene gun. After bombardment, calli were initiated from the embryos in a selection medium for bialaphos resistance. A portion of each selected callus was used to detect the inserted antisense XYN1 in the genome by PCR. Calli containing the proper antisense XYN1 construct were regenerated into plantlets on MSS media (MS medium plus vitamins, 3% sucrose, and 0.3% gelrite but minus hormones) in Petri dishes (25 × 100 mm) at 25 °C in 80–100 μE/m²/s light (16/8-h photoperiod). The bombardment and regeneration of plantlets were performed at the Plant Transformation Facility at Iowa State University. When the leaves and roots of a plantlet reached about 4–6 cm long, the plantlet was transferred to soil and grown in a greenhouse at 28 °C with a 14/10-h photoperiod. Regenerated transgenic plants (T₀) were pollinated with wild-type (inbred B73) pollen, and the seeds so produced were termed T₁. T₀ plants grown from T₁ seeds produced flowers, and the pollen was subjected to immunoblot analyses for XYN1 (a following section). Individual T₁ plants that produced pollen with little or no XYN1

2 The abbreviations used are: ORF, open reading frame; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
were retained and selfed, and the seeds produced were termed T2. Plants grown from T2 seeds were used for experiments.

PCR Analysis of Transgenic Lines—Genomic DNA was extracted from transformed calli by cetyltrimethylammonium bromide (24). PCR analysis involved use of 5′ (5′-GGGAGGTACTGACCGCTTACT-3′) and 3′ (5′-CGAGAGGTACTGAGTGTCC-3′) primers and yielded a 3.0-kb fragment, representing the promoter and the coding regions of XYN1 (shown in Fig. 1). The PCR products were analyzed by electrophoresis in a 1% agarose gel with ethidium bromide staining and photographed under UV. Calli whose DNA gave the 3.0-kb fragment represented transformed lines that had the correct inserted antisense XYN1 construct in the genome. These calli were regenerated into mature plants (T0).

Genomic DNA Blot Hybridization of XYN1—Genomic DNA was extracted was obtained as

Preparation of Total Pollen Protein and Pollen Coat Fraction—A total pollen-protein fraction was obtained as follows. A sample of 5.5 mg of mature pollen was ground in 100 µl of 2× SDS-PAGE sample buffer (1×: 0.0625 M Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 0.002% bromophenol blue, and 10% glycerol) in a microcentrifuge tube with a mini-pestle, and the homogenate was boiled for 20 min. A pollen coat fraction was obtained as follows. One gram of pollen was mixed with 10 ml of diethyl ether for 3 min in a capped tube by repeated inversions. The tube was centrifuged for 10 min at 8,000 × g, and the ether layer was collected. The volume of the ether preparation was reduced to 500 µl under a stream of nitrogen. The ether preparation was used directly for lipid analyses by TLC or partitioned with 1 volume of 50 mM sodium acetate, pH 5.0. The aqueous sodium acetate fraction (lower layer) was collected and used as the pollen coat protein fraction.

Detection of Pollen Coat Xylanase and β-Glucanase by SDS-PAGE and Immunoblot Analyses and of Lipids by TLC—All procedures followed those described earlier (14). The total proteins of pollen from wild-type and transgenic plants were subjected to 12.5% (w/v) SDS-PAGE. After electrophoresis, the gel was stained with Coomasie Blue and then destained or subjected to immunoblot analysis as follows. The proteins on the gel were transferred to a nitrocellulose membrane. Rabbit antibodies were raised against the purified recombinant mature 35-kDa XYN1 (14). The antibody preparation recognized the pollen coat mature 35-kDa XYN1 and also a 55-kDa pollen interior protein, whose identity is unknown (21). The latter immunodetected protein was used as a control of equal loading of total pollen proteins from different transgenic maize lines into a gel for comparison. Serial-diluted total pollen-protein fraction of the wild type (1×, 2×, 4×, and 8×) and serial-increased total pollen-protein fractions individual transgenic lines (1× and 2×) were used to estimate the loss of xylanase in the transgenic lines. Transgenic lines producing pollen xylanase with less than 20% of that in wild-type were used; they are called the xyl-less line or pollen.

The above total pollen protein sample after SDS-PAGE was also used for immunoblot analyses of the coat β-glucanase. Rabbit antibodies were raised against a synthetic polypeptide of DKDDLAKSAEGYE, which was present in the maize pollen coat β-glucanase but absent in all other known maize β-glucanases (17). Coat lipids were applied to a TLC plate (silica gel 60 A, Whatman Inc., Clifton, NJ). The plate was developed in hexane-diethyl ether-acetic acid (95:5:2, v/v/v) and stained in an iodine chamber for 24 h.

Reverse Transcriptase-PCR Analysis of Antisense Gene Expression—Total RNA was extracted from maize anthers, leaves, immature kernels, and roots as described earlier (25). A sample of 1 µg of total RNA was treated with RNase-free DNase and then used to synthesize first-strand cDNA with an oligo(dT)12–18 primer (17). The cDNA was used as a template for reverse transcriptase-PCR with a pair of primers. For the sense transcript, the 5′ primer (5′-GGGAGGTACTGACCGCTTACT-3′) and 3′ primer (5′-CGAGAGGTACTGAGTGTCC-3′) were used for a 0.13-kb fragment (shown in Fig. 1). For the antisense transcript, 5′ primer (5′-CGCGGCCACAACGTCTTCTC-3′) and 3′ primer (5′-CGAGAGGTACTGAGTGTCC-3′ were used for a 3-kb fragment. The PCR product was electrophoresed on a 1.2% agarose gel, which was stained with ethidium bromide and then observed under UV.

Pollen Germination in Vitro—Old pollen and anthers on tassels of plants were removed in the afternoon a day before pollen collection. Fresh pollen was collected from each plant in the morning by shaking the tassel in a plastic bag. For in vitro germination, a sample of 5 mg of pollen was placed in 2 ml of liquid germination medium (100 ppm Ca(NO3)2, 10 ppm H3BO3, 37.5 ppm lysine, 5 ppm cysteine, 0.05 ppm glutamic acid, and 15% (w/v) sucrose (8)) or on solid germination media. Solid media had the same composition as that of the liquid medium but with the addition of 0.7, 1.4, or 2.1% (w/v) of phytagel (Sigma catalog number P-8169). Each gel medium was placed in a 100 × 15-mm Petri dish, which was then air-dried under a laminar hood for 2 h to remove water on the medium surface. The dish
was sealed with a parafilm and stored at 4 °C. It was warmed to room temperature immediately before use. Pollen was spread on the gel, and the dish was covered to keep moisture. The pollen was allowed to germinate at 25 °C for periods of time described under “Results.” Germinated and non-germinated pollen grains were observed with a Leica DMLB microscope and counted.

**Pollen Germination on and Tube Penetration into Silk**—
Fresh silks on each wild-type plant were covered with a paper bag after the silks started to emerge. All silks used in one experiment were obtained from the same plant. When the silks that had emerged from the cob were 8 cm long, their tips turned slightly pink. These 8-cm silks were cut, and their lower ~1-cm portions were placed in a cylindrical vial (5 cm tall and 1.3 cm internal diameter) containing 2 ml of water. Pollen grains were dusted onto the top internal diameter) containing 2 ml of water. Pollen grains were treated instead. The top 6- to 7-cm portions of the 8-cm segments (preceding paragraph) before being dusted with pollen were first immersed in a solution of 0.4 μg/μl of BSA, 0.4 μg/μl of a Thermomyces lanuginosus xylanase (Sigma catalog number X-0627), 0.1M xylose or glucose, or a blank solution for 5 min. Therefore, the silks were treated with any tested solution for 5 min. Therefore, the silks were treated instead. The top 6- to 7-cm portions of the 8-cm segments (preceding paragraph) before being dusted with pollen were first immersed in a solution of 0.4 μg/μl of BSA, 0.4 μg/μl of a Thermomyces lanuginosus xylanase (Sigma catalog number X-0627), 0.1M xylose or glucose, or a blank solution for 5 min and then dried in air for 10 min. The silk segments were then treated as described in the preceding paragraph.

**Measurement of Pollen Tube Length**—
Pollen was germinated in a liquid or 2.1% phytagel medium as described in the preceding paragraph. When the pollen germination ratio was about 10% (after 5 min in liquid and 20 min in gel medium), 10 or more germinated pollen grains were randomly chosen, and the lengths of tubes were measured at time intervals. For convenience of estimation by microscopy, the unit of tube length was the diameter of a pollen grain (~70 μm).

**Statistical Analyses**—S.E. values shown in all figures that illustrate percents of germination or tube penetration were calculated from the following equation,

\[
\sqrt{\frac{p(1-p)}{n}}
\]

(Eq. 1)
in which \( p \) (percent germination) = \( x/n \), \( n \) = the number of pollen grains used, and \( x \) = the number of pollen grains germinated. The \( n \) was about 200 for pollen germination in vitro and about 200–400 for pollen germination on or penetration into silk. The S.E. values of tube elongation (Fig. 4) were calculated from the following equation,

\[
\sqrt{n-1 \sum_{i=1}^{n}(x_i - \bar{x})^2}
\]

(Eq. 2)
in which \( x \) = fold of pollen diameter, \( \bar{x} \) = average of 10 randomly picked tubes, and \( x_i \) = fold of each sample. Z test was used to test whether the difference between two data points was significant.

**Measurement of Xylanase Activity by Viscosity Assay**—All procedures followed those described earlier (14). An amount of 200 mg of oat spelled xylan (Sigma catalog number X-0627) was mixed with 2 ml of water in a capped tube with continuous, slow inversion. The mixture was centrifuged at 2,000 × g for 10 min. The supernatant was used as the substrate for xylanase. Each enzyme assay contained 1 ml of oat spelled xylan, 20 μl of pollen coat fraction (about 6.5 μg proteins), or 0.01 unit of \( T. \) lanuginosus xylanase, sodium acetate (0.15 M final concentration), and water to a final volume of 1.2 ml. When desired, the assay mixture also contained 0.1 M xylose or glucose. The assay mixture was placed at 30 °C. At time intervals, a volume of 0.15 ml of the assay mixture was used for viscosity testing. The volume was taken into a 0.2-ml viscosity pipette and then allowed to flow down vertically. The flow time for a 0.15-ml reaction mixture without enzyme was 6.3 s.

**Electron Microscopy**—For transmission EM, all steps were as described previously (26). Anthers at different stages from the wild-type and a xyl-less plant were cut and fixed in a fixative solution (2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.0) for 2 h. The samples were washed with 0.1 M sodium phosphate buffer, pH 7.0, post-fixed with 1% of OsO\(_4\) in 0.1 M sodium phosphate buffer, pH 7.0, for 2 h, washed with the buffer without osmium, then dehydrated, embedded, sectioned, and post-stained with uranyl acetate and lead citrate. For scanning EM, pollen was fixed with 2.5% glutaraldehyde solution overnight at 4 °C, washed with 0.1 M sodium phosphate buffer, pH 7.0, and post-fixed in 1% of OsO\(_4\) for 2 h at 4 °C. The samples were then washed with the buffer and dehydrated in an ethanol series. The dehydrated samples were critical point-dried (CPD 020, Balzers Union, Furstentum, Liechtenstein) and mounted onto aluminum.
Xylanase and Pollen Tube Entry into Silk

Transgenic Lines Containing No Xylanase on Pollen Were Generated by an Antisense Technique—No T-DNA insertion mutant of XYN1 of maize or rice was available for study of the phenotype of xylanase-less pollen. Thus, we used an antisense technique to knock-out or knockdown the maize pollen coat xylanase. An antisense construct containing a 1.2-kb XYN1 promoter region, a 744-bp ORF encoding the removable, N-terminal 248 residues of the 60-kDa pre-xylanase, and a 35-kDa N-terminal xylanase. An antisense construct containing a 1.2-kb XYN1 promoter region, a 744-bp ORF encoding the removable, N-terminal 248 residues of the 60-kDa pre-xylanase, and a 35-kDa N-terminal xylanase.

RESULTS

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for the antisense XYN1 fragment and immunoblot detection of pollen XYN1 from the wild-type (wt) and various T1 transgenic lines produced from selfing of the same T1 line. The gDNA was digested with EcoRI and allowed to hybridize to a 0.5-kb probe (shown in A), which could hybridize the native XYN1 (indicated with an arrow on the right side) and the antisense fragment (all other visible fragments). Pollen from the same wild-type and T1 transgenic lines were subjected to SDS-PAGE and immunoblot analyses with antibodies against XYN1 (detected to be described in D). Only some of the T1 transgenic lines lost the 35-kDa XYN1 completely or partially. D. SDS-PAGE of wild-type (wt) pollen coat and total extract of the wild type (left panel), SDS-PAGE, and immunoblot analyses of pollen total extract of the wild-type and a xyl-less line (middle panel), and TLC of lipids of the pollen coat of the wild-type and a xyl-less line (right panel). Left panel, SDS-PAGE showed the wild-type pollen coat (pc) possessing 70-kDa glucanase, 35-kDa XYN1, and a minor protein of 27 kDa; these proteins represented a very small fraction of the proteins in the total pollen extract (loading amounts were 20× and 1× an equal amount of pollen). Positions of molecular markers are shown on the left. Middle panel, SDS-PAGE and immunoblot analyses of total extract of wild-type and xyl-less pollen with anti-glucanase (GLA1) or anti-XYN1 revealed the selective absence of the 35-kDa XYN1 in xyl-less pollen. A pollen interior 55-kDa protein of unknown identity that reacted with the XYN1 antibody preparation (21) was observed and served as an indicator for equal loading of the wild-type and xyl-less pollen samples (also in C). Right panel, TLC of lipids extracted from wild-type and xyl-less pollen coat illustrated no major difference between the 2 samples. Lipid standards were C-29 alkane, cholesteryl palmitate, and triolein (from top to bottom).
Xylanase and Pollen Tube Entry into Silk

the whole 60-kDa pre-xylanase to make the antisense construct because of the availability of the former DNA fragment, which we employed earlier to prepare the mature xylanase in bacteria for antibody preparation (14). We included the ORF encoding the sense N-terminal, removable 25-kDa fragment, whose function is unknown, to mimic the native 5’ terminus of the mRNA. Several calli initiated from transformed embryos after selection culturing contained the antisense construct in the proper size and orientation (Fig. 1B); they were regenerated into adult plants (T0). T0 plants were pollinated with wild-type pollen to generate T1 seeds. T1 plants were checked for the contents of pollen coat xylanase, and those that had no detectable xylanase were retained. These T1 plants were selfed to produce T2 seeds. T2 plants were found to have many copies of the antisense XYN1 per native XYN1. Although T2 plants generated from one individual T1 line possessed many copies of the antisense XYN1 in an identical pattern of restriction fragments, they produced pollen having highly variable XYN1 quantities. Exemplified results of T2 plants generated from selfing of one T1 plant are shown in Fig. 1C. Different T2 plants possessed 10–30 copies of the antisense XYN1 per native XYN1 in an identical pattern of restriction fragments (digestion with EcoRI) but highly variable amounts of pollen XYN1. These latter amounts ranged from 100% of that in the wild type to a non-detectable quantity.

The non-matching between the identical pattern of antisense XYN1 restriction fragments and the variable pollen XYN1 in the T2 plants generated from selfing of one T1 plant could be explained as follows. These T2 plants might have obtained via Mendelian inheritance different pattern of the antisense fragments that was not detectable by the DNA hybridization procedure with EcoRI digestion; however, digestion with HindIII instead of EcoRI also revealed an identical pattern of the antisense fragments in them (data not shown). Alternatively, the T2 plants might have undergone different epigenetic events (DNA and/or histone methylation), such that their antisense fragments were subjected to different degrees of silencing. This silencing could have occurred because the multiple copies of the antisense fragment generated from transcription formed double-stranded RNAs automatically or through RNA-dependent RNA polymerase action, and the double-stranded RNAs were processed by Dicer into small interfering RNAs that could direct DNA methylation (27, 28).

Because of the uncertainty of which of the T2 lines generated by selfing of the same T1 plant produced pollen with no pollen-coat xylanase, we adopted the following strategy. We grew different T2 transgenic lines to maturity, collected organ and pollen samples and preliminarily processed them for future experiments, then estimated the amounts of pollen coat xylanase in these lines. T2 transgenic lines whose pollen contained no detectable xylanase (conservatively termed 0–20% of the wild-type pollen xylanase) were used for further study. They are termed xyl-less lines, and their pollen, xyl-less pollen. We detected the amount of xylanase by immunoblotting rather than analyzing its enzyme activity because the amount of pollen coat xylanase from one T2 plant was insufficient for accurate enzyme activity determination, germination test, and other examinations. Vegetative growth of xyl-less plants in greenhouses was indistinguishable from that of wild-type plants.

In three xyl-less lines studied, reverse transcriptase-PCR revealed the antisense transcript of the proper size and orientation in anthers. However, it was also present, albeit in smaller quantities, in leaves, immature kernels, and roots (data not shown). One possibility is that our 1.2-kb XYL1 promoter sequence, which contained a putative tapetum-specificity motif sequence (14), was not sufficient to confer high anther/tapetum specificity. In wild-type plants, the XYL1 transcript was present only in the tapetum among diverse tissues and organs (14). Thus, in xyl-less plants, the presence of the antisense construct in other organs did not affect their vegetative growth or our experimental purposes.

Xyl-less Lines and the Wild Type Exhibited No Appreciable Difference in Anther Structures by Electron Microscopy, but Xyl-less Pollen Exhibited No Coat Xylanase by Immunofluorescence Microscopy—Transmission electron microscopy of anthers of various developmental stages, and mature pollen of xyl-less lines and the wild type revealed no difference in cell and subcellular morphology. As anthers matured, the tapetum cells gradually lost their cell wall, especially on the side facing the locule (Fig. 2A). They acquired the characteristics of secretory cells by having abundant rough endoplasmic reticulum and secretory vesicles located toward the locule side. Microspores in anthers of xyl-less lines and the wild type underwent similar developmental changes (Fig. 2A) as described in the first paragraph under “Experimental Procedures.” Scanning EM also revealed no appreciable difference in shape and surface morphology, including the aperture, between xyl-less and wild-type pollen (Fig. 2B). The pollen aperture had a central exine that did not cover an annular area.

Nevertheless, immunofluorescence microscopy showed that xyl-less pollen had little or no surface xylanase, which was present on wild-type pollen (Fig. 2C), and the result reiterates our biochemical findings (Fig. 1D). Both xyl-less and wild-type pollen had the same amount of coat β-glucanase and the same amount and type of lipids (Fig. 1D).

Xyl-less Pollen Germinated in Vitro as Efficiently as Wild-type Pollen Did in a Liquid Medium but Less So on Gel Media of Increasing Solidity—Germination in the current study is defined as observable protrusion of the tube and not tube elongation. A maize pollen grain does not change its shape upon water uptake to initiate germination, unlike the pollen grains of Brassicaceae species. Thus, we could not use swelling as a simple indicator of water uptake and germination but had to observe every pollen grain carefully for tube protrusion. Xyl-less pollen germinated as well as wild-type pollen did in liquid medium; ~80% of the pollen germinated within 20 min (Fig. 3, A and B). These percents of germination of wild-type pollen in liquid medium are similar to those reported earlier (8, 29). Both types of pollen germinated less efficiently on gel media of increasing solidity, xyl-less pollen more so than wild-type pollen. On a 0.7% (w/v) gel, 17% of xyl-less and 33% of wild-type pollen germinated in 20 min. The disparity in percent germination of the two types of pollen became more pronounced, with 48% to 56% to 67%
germination, when the solidity of the gel increased from 0.7% to 1.4% to 2.1%, respectively (Fig. 3, A and B). The percents of germination of both types of pollens on gel media increased with time, and the disparity gradually disappeared. A time course of pollen germination on 2.1% gel is exemplified in Fig. 3C.

Results shown in Fig. 3 and to be described that deal with pollen germination and tube penetration into silk were from experiments performed at least three times. Results of a similar pattern were observed, and one set of the results is shown in this report.

After Germination in Liquid or on Gel Media, Xyl-less Pollen Tubes Elongated as Rapidly as Wild-type Pollen Tubes Did—Xyl-less pollen germinated in vitro as efficiently as wild-type pollen did in a liquid medium but less so on gel media of increasing solidity (Fig. 3B). However, after germination, the tubes of both types of pollen elongated at a similar rate. Fig. 4 shows the elongation rates of the 2 types of pollen placed on 2.1% gel. Even though xyl-less pollen germinated slower than wild-type pollen did (Figs. 3C and 4), the tubes from both types of germinated pollen elongated at a similar rate. Thus, the disparity of the two types of pollen on gel media (Fig. 3B) applies only to initiation of germination (i.e. protrusion of the tube) rather than subsequent tube elongation.

Xyl-less Pollen Germinated on Silk Less Efficiently than Wild-type Pollen Did, and This Defect Could Not be Overcome by Application of Xylanase to the Silk—Xyl-less pollen germinated on silk less efficiently than wild-type pollen did (Fig. 3B). We tested whether the addition of external xylanase would alleviate the low germination rate of xyl-less pollen. We could not add external xylanase directly to the surface of xyl-less pollen because the viability of maize pollen is severely affected by many factors (29). In our preliminary test, treatment of wild-type pollen with a blank solution briefly (e.g., 5 min) reduced its germination rate by more than 50%. Therefore, we soaked the silk for 5 min in a solution containing commercial *Thermomyces* xylanase, BSAs, or no extra ingredient (blank) and allowed the two types of pollen to germinate on treated and then briefly dried silk. Silk treated with a blank solution was a slightly less favorable medium for germination for both types of pollen (Fig. 5B). The addition of xylanase or BSA to the silk did not alleviate the low germination rate of xyl-less pollen (Fig. 5, A and B). This lack of alleviation suggests that for the coat xylanase to aid water uptake for germination, it has to be intimately associated with the pollen surface.

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**FIGURE 2.** Microscopy images of anthers and pollen of the wild-type (wt) and a xyl-less line. A, transmission electron microscopy images showing (upper panels) a portion of a tapetum cell (between stages 1 and 2) containing a conspicuous nucleus and packed cytoplasm adjacent to the locule (light region on the right) and (lower panels) a portion of a stage 5 microspore containing both intine (I) and exine (E). B, scanning EM images showing a mature pollen grain with an aperture and coarse surface (upper panels) and a magnified pollen surface with a granular structure (lower panels). C, fluorescence microscopy images showing pollen treated with anti-XYN1 IgG and then anti-IgG conjugated to cyanine 5 observed with a bright-field (upper panels) or fluorescence (lower panels) setting. Bright-field and fluorescence imaging of the wild-type and xyl-less pollen used the same confocal laser scanning microscopy settings (laser power and detection gain). In the fluorescence images, the background of the wild-type sample was faintly lighter than the xyl-less sample because of the fluorescence emitted from the pollen.
FIGURE 3. Germination of wild-type (wt) and xyl-less pollen in various media. A, light microscopy images of pollen germinated in liquid and on 2.1% (w/v) gel media for 20 min. Inset shows one germinated xyl-less pollen grain. B, percentage of pollen germination in a liquid medium or on gel media of different solidity (percent, w/v) or on silk for 20 min. The S.E. values were calculated as described under “Experimental Procedures.” The difference between the percent germination of wild-type and xyl-less pollen in each medium is indicated on top of the paired bars. The asterisk indicates that the difference in germination between wild-type and xyl-less pollen was significant (p < 0.05). C, percentage of pollen germination on 2.1% gel at time intervals.

FIGURE 4. Length of tubes of germinated wild-type (wt) and xyl-less pollen in liquid and on gel media. Pollen was allowed to germinate in liquid or on 2.1% (w/v) gel media. Lengths of the tubes from 10 randomly picked, germinated pollen were recorded by use of a light microscope at time intervals. The length of each tube was expressed as fold of tube length to the diameter of pollen grain. The S.E. values were calculated as described under “Experimental Procedures.”

FIGURE 5. Germination of wild-type (wt) and xyl-less pollen on silk. A, light microscopy of pollen germinated on silk for 20 min. Pollen grains that had germinated are circled, and their just-protruded tube is indicated with an arrow. B, percentage of germination of pollen on silk untreated or treated with a commercial xylanase, BSA, or a blank solution at time intervals. The S.E. values were calculated as described under “Experimental Procedures.”
Xyl-less Pollen Tubes Penetrated Silk Less Efficiently than Wild-type Pollen Tubes Did, and This Defect Could Be Overcome by Application of Xylanase—When xyl-less and wild-type pollen were placed on silk, they germinated and their tubes elongated. After 20 min, the tubes began to penetrate the silk into the carpel interior. Xyl-less pollen tubes did not penetrate the silk as efficiently as wild-type pollen tubes did (Fig. 6, A and B). Xyl-less pollen tubes that failed to penetrate the silk elongated along (Fig. 6 A) or curved at the silk surface. After 60 min, ~40% of the xyl-less pollen tubes, compared with ~70% of the wild-type pollen tubes, penetrated the silk. These percents reached ~50 and ~90%, respectively, after 90 min. Prior application of xylanase to the silk restored the ability of xyl-less pollen tubes to penetrate to almost the same level as the wild-type pollen tubes. The restoration was ineffective when BSA or a blank solution instead of xylanase was pre-applied to the silk. Thus, loss of xylanase on xyl-less pollen was the cause of the reduced ability of the tubes to penetrate the silk.

The Efficiency of Germination on and Tube Penetration into Silk of Pollen from Different Transgenic Lines and the Wild Type Were Dependent on the Amount of Coat Xylanase—In the current study, with the exception of this section, we examined germination on and tube penetration into silk of pollen of transgenic lines with 0–20% xylanase (termed xyl-less pollen). We repeated our examinations by comparing pollen of the wild type (100%) and transgenic lines containing 40–60% (termed 50% for convenience) and 0–20% (termed 10%, only in this section) coat xylanase. Fig. 7 shows that pollen from the two transgenic lines exhibited reduced efficiency in germination on and tube penetration into silk after 90 min, depending on the residual amounts of xylanase.

For Wild-type Pollen, Xylose Inhibited the Coat Xylanase Activity in Vitro but Did Not Affect Germination in a Liquid Medium, and Application of Xylose to Silk Hinder Pollen Tube Penetration—Ideally, we would test coat xylanase enzymatic activity of wild-type pollen on silk. Technical difficulties complicated such a direct detection. The xylanase activity is an endohydrolase, and free xylose would not be released and detected. Observing pollen action on silk at a physiological condition would necessitate application of a limited number of pollen grains per silk area, and the expected minimal number of xylose ends generated from xylan breakage in the silk wall would hinder biochemical detection. Pollen coat also contains a
glucanase, which could hydrolyze glucan of cellulose and complicate the detection of breakage of xylan in the hemicellulose. To circumvent these difficulties, we took advantage of our observation that the pollen coat xylanase enzymatic activity was inhibited by xylose but not glucose and tested the effect of xylose on pollen germination and tube penetration into silk.

Xylose inhibited pollen coat xylanase enzymatic activity on xylan in vitro (Fig. 8A). In this enzyme activity assay, we wanted to add xylose to the assay medium and therefore could not use the common assay procedure of detecting the released xylose or the exposed reducing ends on xylan by colorimetry. Instead, we used a viscosity assay procedure, which detected the reduction in viscosity of a xylan resuspension after it had been broken down into smaller chains by endoxylanase activity (14, 30). In our assay, xylanase activity on oat spelled xylan was detected in the presence of coat xylanase, or Thermomyces xylanase, but not in the absence of enzyme. The coat xylanase activity was inhibited by 0.1 M xylose but not glucose.

Although xylose inhibited coat xylanase enzymatic activity, it had no effect on germination of wild-type pollen in a liquid medium. Fig. 8B shows that wild-type pollen germinated similarly in a liquid medium in the absence or presence of 0.1 M xylose or glucose.

Also, addition of 0.1 M xylose or glucose to silk did not affect pollen germination on the treated silk (Fig. 8C). As noted earlier (Fig. 5B), pretreatment of silk with a blank solution slightly reduced the percentage of pollen germination on the silk after 90 min. Regardless, pretreatment of silk with xylose, glucose, and a blank solution all slightly reduced the percentage of pollen germination to a similar extent.

However, pretreatment of silk with 0.1 M xylose but not glucose inhibited tube penetration into the silk (Fig. 8C). The findings show that xylanase on maize pollen aids penetration of the tube into silk because of its xylanase enzyme activity on the silk wall.

**DISCUSSION**

**Three Different Molecular Actions of Pollen Coat Ingredients on the Stigma Have Been Recognized**—Recent studies have recognized three molecular actions of pollen on the stigma. First, initiation of signal transduction for water uptake in self-incompatibility systems has been delineated (5–6). Second, the physical requirements of pollen-surface materials for water uptake/germination have been documented. A mutational loss of a pollen coat lipid (a common phenotype in many Arabidopsis wax mutants described by the Arabidopsis Biological Resource Center) or protein including oleosin (31) and xylanase (this report) leads to a reduced ability of the mutant pollen to take up...
water and thus undergo fertilization. Third, as documented in the current report, the enzymatic action of xylanase on the stigma xylan creates an opening for tube penetration. Numerous cell wall digestive enzymes and modulating proteins are known to be on the pollen surface or quickly released from hydrated pollen, but their action on the stigma wall has not been previously reported. Some of these proteins appear to exert their action not on the stigma but in the pollen tube track in the style (18).

In the current experiments, tubes of xyl-less pollen were still able to penetrate silk into the carpel interior, even though the penetration efficiency had been substantially reduced (by ~50% in xyl-less versus wild-type pollen; Fig. 6). Two possible explanations are offered. First, the xyl-less pollen we used contained 0–20% of the wild-type pollen xylanase, even though we observed no xylanase on the immunoblot. A trace amount of leftover xylanase would still be able to perform wall hydrolysis, albeit with less efficiency. Second, the tapetum-synthesized pollen coat materials in maize include two other, even though less abundant, hydrolyases (a β-glucanase and a protease) (Fig. 1D; Ref. 17). Whether these other hydrolyses are leftover tapetum proteins with no further function or are required to act collaborative or synergistically with the xylanase for optimal hydrolysis of the silk wall remains to be elucidated.

Xylanase and Pollen Tube Entry into Silk

Loss of Pollen Coat Xylanase Impairs Water Uptake on Silk or Solid Media—Xyl-less pollen germinated as efficiently as wild-type pollen did in a liquid medium but less so on gel media of increasing solidity (Fig. 3). This finding suggests that the presence of xylanase on the pollen surface provides efficient water uptake into pollen for germination. After germination in a liquid or on a gel medium (Fig. 4), xyl-less pollen tubes elongated as rapidly as wild-type pollen tubes did. Presumably, germination involves uptake of water through the aperture of a pollen grain; xylanase and other hydrophilic components on the pollen surface help with contact of the grain to the medium and movement of water along the pollen surface to the aperture. After germination, water is taken up directly and easily into the pollen tube and not through the aperture, and thus the presence of xylanase on the pollen becomes irrelevant.

The xylanase molecule is overall very hydrophilic, as revealed in a hydrophatic plot (14). Our calculation shows that a pollen grain of 70-μm diameter has 5.8 pg of the 35-kDa xylanase. If we assume that a pollen grain is completely spherical and has xylanase evenly distributed on its surface and that the xylanase molecule has a globular structure, the xylanase molecules would occupy about 10% of the pollen surface. Therefore, the coat xylanase aiding direct water uptake should be minimal. The pollen surface also contains flavonoids (~400 pg per pollen; Ref. 32) and perhaps sugars and amino acids in greater abundance. These other hydrophilic or amphipathic molecules on pollen should be the major components mediating direct water uptake.

Xyl-less pollen germinated less efficiently than wild-type pollen did on silk and gel media of increasing solidity. This loss of efficiency should have more to do with the contact of the pollen grain surface with the solid media than to a slight decrease in the osmotic potential of the gel of increasing solidity (the osmotic potentials of the media containing 0, 0.7, 1.4, and 2.1% gel were −1.34, −1.56, −1.67, and −1.78 MPa, respectively, determined with a Wescor Dew Point Microvoltmeter), because pollen has a very low water potential (~20 MPa in maize pollen; Ref. 33). The pollen grain has a granular, coarse surface (Fig. 2B), and thus water uptake depends greatly on its proper contact with the solid medium. It is unlikely that the loss of xylanase from the exine surface affected the membrane or water potential of the pollen. Both wild-type and xyl-less pollen grains rapidly burst at similar rates when placed in water, with their protoplasts disintegrating after emergence from the aperture (data not shown). The reduced water uptake efficiency of xyl-less pollen is similar to that of mutant pollen of other species with a lost coat ingredient, such as lipid (many wax mutants, described by the Arabidopsis Biological Resource Center) or protein (31). We suggest that the loss of xylanase, or another protein or lipid, on the pollen surface would impair the semifluid and amphipathic status of the coat materials, optimized by evolution or breeding, to act as a waterproofing agent and also a wick for water uptake. The main function of xylanase is related to its enzymatic activity on tube penetration into silk.

Wall Hydrolyses and Modulating Proteins on the Pollen Surface—Crest Can Be Divided into Two Functional Groups with Distinct Origins—Maize pollen is the best studied system in terms of wall hydrolyses and modulating proteins associated with the pollen surface crest. Findings presented in the current and earlier reports have allowed us to divide these proteins into two groups. Members of one group, including mostly the endoxylanase, are synthesized in the tapetum cells and transferred to the microspore surface as coat materials (14, 17). Members of the other group, including polygalacturonase and wall-modulating proteins such as extensin and calcium-chelating proteins, are synthesized in and secreted from the pollen interior during late maturation of pollen (microspore) or after the mature pollen have germinated (17). We suggest that the tapetum-synthesized endoxylanase on pollen is responsible for the initial hydrolysis of the carpel wall xylan after pollen landing, thus creating an opening for entry of the pollen tube, and that the pollen-synthesized hydrolyses and wall-modulating proteins are for the subsequent hydrolysis and modulation of the carpel interior wall, thereby generating a path for tube advancement to the ovary. The pollen-synthesized hydrolyses and wall-modulating proteins could also function to loosen the pollen tube wall for growth, generate sugars as wall precursors for tube growth, and produce oligosaccharides as signal molecules. These potential functions of the pollen-synthesized polygalacturonase (34) and pectin methylesterase (35) in the pollen tube track have been suggested or described.

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