The Maize Tapetum Employs Diverse Mechanisms to Synthesize and Store Proteins and Flavonoids and Transfer Them to the Pollen Surface

Yubing Li, Der Fen Suen, Chien-Yu Huang, Shung-Yee Kung, and Anthony H.C. Huang*

Center for Plant Cell Biology, Department of Botany and Plant Sciences, University of California, Riverside, California 92521 (Y.L., D.F.S., C.-Y.H., S.-Y.K., A.H.C.H.); and Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan 11529 (S.-Y.K., A.H.C.H.)

In anthers, the tapetum synthesizes and stores proteins and flavonoids, which will be transferred to the surface of adjacent microspores. The mechanism of synthesis, storage, and transfer of these pollen-coat materials in maize (Zea mays) differs completely from that reported in Arabidopsis (Arabidopsis thaliana), which stores major pollen-coat materials in tapetosomes and elaioplasts. On maize pollen, three proteins, glucanase, xylanase, and a novel protease, ZmPCP, are predominant. During anther development, glucanase and xylanase transcripts appeared at a mid developmental stage, whereas protease transcript emerged at a late developmental stage. Protease and xylanase transcripts were present only in the anther tapetum of the plant, whereas glucanase transcript was distributed ubiquitously. ZmPCP belongs to the cysteine protease family but has no closely related paralogs. Its nascent polypeptide has a putative amino-terminal endoplasmic reticulum (ER)-targeting peptide and a propeptide. All three proteins were synthesized in the tapetum and were present on mature pollen and Huang, 2005, 2007). Early in development, the tapetum cells are involved in active secretion of molecules into the locule for maturation of the microspores from tetrads to solitary entities, on which the outer exine wall gradually appears. Late in development, when the microspores become binucleate with a single large and then multiple smaller vacuoles, the tapetum cells become warehouses for temporary storage. In Brassicaceae species, they are packed with two predominant storage organelles, the elaioplasts and tapetosomes. Elaioplasts are plastids containing abundant steryl ester droplets and minimal thylakoids, and tapetosomes are storage organelles, each having numerous oleosin-coated alkane droplets linked ionically with flavonoid-containing vesicles. At the conclusion of development, the tapetum cells undergo programmed cell death (PCD; Wu and Cheung, 2000; Huang et al., 2011) and release the storage materials, which become the coat of mature pollen. These materials include steryl esters from the elaioplasts as well as alkanes, oleosins, and flavonoids from the tapetosomes. Steryl esters and alkanes are lipids waterproofing the pollen. Oleosins are amphipathic proteins aiding the storage of coat materials in the tapetum cells and the emulsification of these materials on the pollen surface and, subsequently, assisting water uptake from the stigma to the pollen for germination. Flavonoids are UV-absorbing molecules for protecting the nucleic acids in...
haploidic pollen (Winkel-Shirley, 2001; Hsieh and Huang, 2007).

Tapetum cells in other plant species have been less investigated (Hesse et al., 1993). Among these other species, maize (Zea mays) has been the most studied. Electron microscopy (EM) studies have shown that tapetum cells at a late developmental stage in maize do not possess elaioplasts and tapetosomes (Skvarla and Larson, 1966; Horner et al., 1993; this report), so the pollen-coat materials have to be synthesized, stored, and delivered via other mechanisms.

Maize pollen surface components external to the plasma membrane can be separated into two biochemically distinct morphological parts: the wall in the interior and the noncovalently linked coat external to it (Suen et al., 2003). The pollen wall consists largely of sporopollenin and includes transiently associated proteins (Chay et al., 1992; Allen and Lonsdale, 1993; Rubinstein et al., 1995; Wu et al., 2001; Li et al., 2003; Suen et al., 2003; Bosch et al., 2005), which are synthesized in the microspore or pollen interior and secreted to the wall and exterior before, during, or immediately after pollen germination. These proteins include polygalacturonase and several wall-modulating proteins, which consist of expansin, pectin methylesterase, profilin, cation-binding protein, pollen allergen (trypsin inhibitor), extensin, and others. The transcripts of these proteins in the microspores and mature pollen appear late during anther development, and their levels persist or increase after germination (Suen et al., 2003). 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 wall of the cells along the pollen tube track in the carpel for advancement of the pollen tube toward the ovary.

The maize pollen coat, which is on the surface of the pollen wall, contains proteins, flavonoids, and lipids. These coat molecules are largely if not exclusively synthesized in the adjacent tapetum (Hesse et al., 1993). The coat consists of only three major proteins: a 70-kD 1,3,1,4-β-glucanase (Suen et al., 2003; termed glucanase hereafter), a 35-kD endoxylanase (Bih et al., 1999; termed xylanase), and a previously mentioned but unstudied 25-kD protein (Suen et al., 2003). In developing anthers, the transcripts of the glucanase and xylanase appear earlier than those of the microspore-synthesized wall proteins (see above). The glucanase transcript is present in the tapetum and many other maize organs (via genome database searches [Suen et al., 2003] and experimentation [this report]), and the function of the glucanase in the pollen coat is unknown. This glucanase differs from the well-known tapetum-synthesized and -secreted glucanase that hydrolyzes the tetrad microspore wall; the latter enzyme appears at a very early stage of anther development (Suen et al., 2003). The xylanase transcript is restricted to the tapetum in anthers and nowhere else in a whole plant. The nascent xylanase is an inactive precursor of 60 kD, which is processed at both the N and C termini to the active 35-kD enzyme (Wu et al., 2002). Maize and other cereals have a type II cell wall, which contains predominantly cellulose and hemicellulose, and xylan is a major component of hemicellulose (Carpita and McCann, 2000). Use of an antisense approach has revealed that the pollen-coat xylanase acts on the stigma cell wall, thereby creating an opening for the entry of the emerging pollen tube (Suen and Huang, 2007). The maize pollen coat contains flavonoids, which are mostly quer cetin and isorhamnetin glycosides (Ceska and Styles, 1984). In diverse plant species, pollen coat flavonoids protect the haploid genome against UV irradiation (Hsieh and Huang, 2007), and in some species such as maize, they are also involved in pollen germination and tube growth (Mo et al., 1992). Pollen coat flavonoids are derived from the tapetum (Stanley and Linskens, 1974). The flavonoids in Brassicaceae tapeta are synthesized in the endoplasmic reticulum (ER) and then temporarily stored in the tapetosomes (Hsieh and Huang, 2007). Their synthesis and storage in tapeta of other species, including maize, is unknown. Minimal lipids are present in the pollen coat of maize, a wind-pollinating species, and no or few lipid droplets are present in the tapetum cells immediately before tapetum PCD.

The mechanisms whereby maize tapetum cells synthesize the pollen-coat proteins and flavonoids, store them in the cells, and transfer them to the pollen surface are unknown. In this report, we present experimental findings to delineate these mechanisms, which differ completely from those in the studied Brassicaceae tapetum cells. We also report the identification and characterization of the previously unstudied maize pollen-coat 25-kD Cys protease.

RESULTS

The Maize 25-kD Pollen-Coat Protein Is a Cys Protease, Whose Nascent Polypeptide Has an N-Terminal ER-Targeting Peptide and a Propeptide

Mature maize pollen was washed with diethyl ether to yield a coat fraction. Proteins in the fraction were resolved by SDS-PAGE (Fig. 1A). Three proteins predominated and could represent less than 1% of the total pollen proteins via our visual estimation of the protein stains on the gel. They appeared as sharp bands on a SDS-PAGE gel and thus did not seem to represent degraded proteins from dead tapetum cells. The 70-kD protein was a glucanase (Suen et al., 2003), and the 35-kD protein was a xylanase (Bih et al., 1999; Wu et al., 2002). The 25-kD protein has been observed but not studied (Suen et al., 2003), and we explored its identity and properties. A 14-residue sequence near the N terminus of the 25-kD protein was obtained by microsequencing (Supplemental Fig. S1). This sequence matches an assembled but incomplete EST sequence of a maize gene in The Institute for Genomic Research (TC342635), and
we further obtained the complete sequence, termed *Zea mays* pollen coat protease (ZmPCP) in this report (GenBank accession no. NP_001146834). The deduced polypeptide has 35.3 kD and 352 residues, including, beginning from the N terminus, a 28-residue ER-targeting sequence, a 113-residue N-terminal propeptide (NTPP; which would cover the active site of a hydrolase for more refined control of the in vivo activity), and a 211-residue mature protein. The NTPP has a peptidase C1A motif, ERFNIN (Supplemental Fig. S1), that is conserved among studied plant, mammalian, and microbial Cys proteases (Groves et al., 1998). The mature protein has the characteristics of a Cys protease, possessing the catalytic residues Gln, Cys, His, and Asn of C1 family peptidases (Supplemental Fig. S1). The ER-targeting signal was predicted with SignalP 4.0, and the other domains were analyzed with the National Center for Biotechnology Information Conserved Domain Database. A search of proteins with sequences similar to that of ZmPCP in GenBank retrieved two closely related homologs. One is from sorghum (*Sorghum bicolor*; GenBank accession no. EER94674; 85% identity with ZmPCP) and the other is from rice (*Oryza sativa*; GenBank accession no. EECC8502; 58% identity). The sorghum and rice proteins were retrieved from results of large-scale DNA/RNA sequencing (Tanaka et al., 2008; Paterson et al., 2009), and their properties have not been studied and reported. A BLAST for maize proteins related to ZmPCP retrieved 17 distantly related paralogs (sequence identity ranging from 40% to 50%), of which 12 are predicted proteins from large-scale DNA/RNA sequencing without functional analysis (Alexandrov et al., 2009). The other five paralogs have been studied, including AAB70820 (Mir1), AAB88262 (Mir2), and AAB88263 (Mir3) in calli and leaves (Pechan et al., 1999; Lopez et al., 2007) and BAA08244 (CCP1) and BAA08245 (CCP2) in developing and germinated seeds (Domoto et al., 1995) and senescing leaves (Griffiths et al., 1997). An unrooted phylogenetic tree of these 17 maize Cys proteases and ZmPCP is shown in Figure 1B, and an alignment of their sequences is shown in Supplemental Figure S1.
shown in Supplemental Figure S1. ZmPCP falls into the same clade (SEN102) with three other predicted maize Cys proteases (GenBank accession nos. ACG25394, ACG30386, and ACF82315 in Fig. 1B); these three other maize Cys proteases have not been characterized and reported. A member of the SEN102 clade was first identified in daylily (*Hemerocallis*) tapel, in which the protease gene transcription is up-regulated during flower senescence, and the protease may be involved in protein hydrolysis at a late stage of senescence (Valpuesta et al., 1995; Guerrero et al., 1998).

A rice protease (*OsCP1*; GenBank accession no. BAF16127) has been reported to be tapetum specific, and its transcript peaks during early anther development (Lee et al., 2004). Its amino acid sequence is less similar to those of the 18 maize Cys proteases (complete sequences are shown in Supplemental Fig. S1). We studied this rice protease gene (*OsCP1*) in rice anthers by reverse transcription (RT)-PCR. Our results confirmed the early appearance of the *OsCP1* transcript during anther development (data not shown). The early appearance (developmental stages of maize are defined in the following section; developmental stages of rice were defined earlier [Huang et al., 2011]) of this rice tapetum protease transcript (*OsCP1*) suggests that the protease is not directly involved in tapetum PCD at a late stage of development.

We synthesized a short peptide of the sequence QARRYACSRSRAAQ, which is unique to ZmPCP (Supplemental Fig. S1). Polyclonal antibodies were raised against this peptide for further studies of the protease.

**Transcripts of the Three Pollen-Coat Hydrolases Were Present in the Tapetum of Anthers but Had Different Temporal Profiles**

The three hydrolases were the predominant proteins in the pollen-coat fraction (Fig. 1A). Glucanase transcript in anthers was present in tapetum cells and not in microspores or mature pollen (Suen et al., 2003). Nevertheless, it also existed in many other maize organs (Fig. 1D). Xylanase transcript in anthers was also present in tapetum cells and absent in microspores and mature pollen (Wu et al., 2002) as well as other maize organs (Fig. 1D). Protease transcript was similar to xylanase transcript in being restricted to tapetum cells (Fig. 1C) and absent in pollen and other organs (Fig. 1D).

Developing anthers were divided into four stages (see “Materials and Methods”) for the study of the appearance of the transcripts and proteins of the three pollen-coat hydrolases. Briefly, at stage 1, the microspores were in a tetrad structure. At stage 2, the microspores were solitary and had the exine wall. At stages 3 and 4, the microspores were binucleate and trinucleate, respectively. During anther development, glucanase and xylanase transcripts appeared at stages 2 and 3 (Fig. 1E). Protease transcript was absent in stages 2 and 3 and emerged only at stage 4. The developmental profiles of these three transcripts reflected those of their encoded proteins (Fig. 2). Both glucanase and xylanase appeared in stage-3 anthers and peaked in stage-4 anthers; they were also present in pollen (Fig. 2) and specifically restricted to the pollen coat (Wu et al., 2002; Suen et al., 2003). Xylanase appeared initially as a 60-kD precursor, which was...
then converted to the final active 35-kD xylanase (Wu et al., 2002). Protease appeared only in stage-4 anthers and then in the pollen coat. The antibody preparations against these three hydrolases were highly specific in immunoblotting, recognizing only antigens of the expected molecular masses in the tapetum cells; the only exception was that the antibodies against the xylanase recognized an unknown protein of 55 kD of the microspore/pollen interior (Fig. 2; Wu et al., 2002). These antibodies were suitable for immunofluorescence microscopy.

**Tapetum Cells Had Small Vacuoles Distributed throughout the Cytoplasm and Secretory Vesicles Concentrated near the Locular Side**

A maize anther has four layers of cells enclosing the locule, in which microspores mature. Cells of the outer three anther layers are highly vacuolated, usually each with a large central vacuole. Cells of the innermost layer, the tapetum, had dense cytoplasm throughout anther development (Fig. 3).

Each tapetum cell at stages 1 to 2 had one or two nuclei, which began to disintegrate at stage 3. During development, the cell had abundant rough ER and some Golgi, mitochondria, and proplastids. Numerous vacuoles of 0.5 to 2 μm in diameter were distributed throughout the cytoplasm. Many secretory vesicles of 0.2 to 0.5 μm in diameter were present; they, in comparison with the vacuoles, were smaller, with a more electron-dense, granular matrix, and concentrated near the locular side of the cytoplasm (Fig. 3B). At stage 4, the cell became elongated, concomitant with elongation of the anther; its wall facing the locule had dissolved, and the cell gradually detached from the three outer anther cell layers.
Immunofluorescence Confocal Laser Scanning Microscopy of Tapetum Cells Localized Glucanase in ER Vesicles near the Locule, Xylanase in the Cytosol, and Protease in Vacuoles

The subcellular locations of the three hydrolases in anthers of different developmental stages were examined with immunofluorescence confocal laser scanning microscopy (CLSM). Figure 4 shows the results at the cellular level in one anther lobe. Glucanase was barely detected in a stage-2 anther but localized in the tapetum in stage-3 and -4 anthers. In these anthers, most glucanase was present in the tapetum near the locule and between adjacent tapetum cells connecting to the locule. Some glucanase was present on the microspore surface in stage 3 and less so in stage 4. Xylanase exhibited a similar developmental pattern but was distributed evenly, rather than near the locule, in tapetum cells. In addition, the microspores in stage-2 to -4 anthers showed antigenic signal (Fig. 4). This signal represented an unknown 55-kD protein present inside the microspores throughout development; this protein was recognized by the antibody preparation via immunoblotting (Fig. 2; Wu et al., 2002). Protease was present only in tapetum cells in stage-4 anthers and was absent on the microspores.

Immunofluorescence CLSM at the subcellular level in tapetum cells was performed with antibodies against the hydrolases and known subcellular organelle markers (Fig. 5). Glucanase was located near the locule in a tapetum cell in a stage-3 anther, whereas calreticulin, a marker of the ER, was distributed in the ER network throughout the cell (Fig. 5A). At the outermost region of the cell nearest the locule, glucanase (shown in red) existed in the absence of calreticulin (green), whereas slightly interior to this region, glucanase and calreticulin overlapped (appearing as yellow). The former region could represent the wall outside the plasma membrane, and the latter region could correspond to the nearby proplastid where abundant secretory vesicles existed (Fig. 3). Glucanase was not detected in other regions of the ER, presumably because the secretory vesicles were only transiently associated with specific regions of the ER facing the locule. Thus, glucanase was associated with ER-derived secretory vesicles and was secreted.

Xylanase in a tapetum cell in a stage-3 anther was distributed throughout the cytoplasm and neither overlapped with glucanase or calreticulin nor exhibited a specific pattern (Fig. 5A). Thus, xylanase was in the cytosol.

Protease was scarce in stage-3 anthers (Fig. 2), so we examined its subcellular location in tapetum cells in stage-4 anthers. At this developmental stage, tapetum cells had lost most of their cell wall and were partly detached from the outer anther cell layers. Unlike glucanase and xylanase, protease was associated with small subcellular particles of 1 to 2 \(\mu\)m in diameter throughout the cytoplasm (Fig. 5B). The protease-associated particles were also the locations of \(\gamma\)-TIP (for tonoplast intrinsic protein; Pedrazzini et al., 1997)

Figure 4. Bright-field differential interference contrast and immunofluorescence (IF) microscopy images of maize anther lobes. One lobe of an anther of each progressive developmental stage is shown. Immunofluorescence images were obtained after treating samples with antibodies against the indicated proteins. Cy5-conjugated secondary antibodies were used. An excitation wavelength of 648 nm and an emission wavelength of 645 to 700 nm were instituted, under which autofluorescence of the pollen wall was not detected. Identical CLSM settings (laser power and detection gain) were used for all images of each hydrolase. In the anther lobe, several layers of outer anther wall cells (A) and the tapetum (T) together enclosed the locule (L) in which microspores (M) matured.
and V-PPase (for vacuolar pyrophosphatase; Sarafian et al., 1992), both markers of cell vacuoles. They were not ER-derived secretory vesicles, because V-PPase (Fig. 5B) and protease (data not shown) did not overlap with glucanase-associated secretory particles near the locule. Antibody preparations against tobacco \((\text{Nicotiana tabacum})\) \(\gamma\)-TIP and pea \((\text{Pisum sativum})\) V-PPase were specific for the respective vacuolar enzymes in maize anther extracts, as revealed in immunoblot analyses (Fig. 5C). The overall results indicate that protease was present in vacuoles of tapetum cells at a late stage of anther development.

The above subcellular localization results on the three hydrolases with CLSM are consistent with computer software predictions on the basis of amino acid sequences. Two software programs, PSORT (Nakai and Kanehisa, 1991) and TargetP (Nielsen et al., 1997; Emanuelsson et al., 2000), suggest that the three hydrolases are not in chloroplasts or mitochondria. Glucanase is predicted to be in the secretory pathway; its nascent polypeptide has a putative 23-residue N-terminal ER-targeting signal but no ER retention signal; therefore, glucanase may be associated with the ER secretory machinery. Xylanase is predicted not to associate with subcellular structures. Protease is predicted to be in the ER secretory pathway; its sequence of having a putative 28-residue N-terminal ER-targeting signal and an immediate downstream 113-residue NTPP (identified via a comparison with other proteases; Hatzugai et al., 2006) leads to the prediction of its location in vacuoles.

**Subcellular Fractionation of Tapetum Cells by Density Gradient Centrifugation Separated the Three Hydrolases into Different Fractions**

The subcellular locations of the three hydrolases were further explored by biochemical subcellular fractionation. An extract of stage-3 and -4 anthers, obtained after mild grinding to yield mostly tapetum subcellular materials and after removing the glucanase-containing tapetum wall fragments by filtration (see “Materials and Methods”), was subjected to Suc density gradient centrifugation (Fig. 6). Xylanase, prote-
ase, and glucanase peaked at different gradient fractions, although their peaks overlapped.

Xylanase was present only in fractions at the top of the gradient. These fractions contained cellular materials (cytosol) that were not associated with subcellular structures.

Protease migrated into the gradient, peaking at fraction 7 (density of 1.07 g cm$^{-3}$). It was also present in the top fractions. The distribution of protease along the gradient followed that of V-PPase, a vacuole marker. These findings are consistent with protease being in vacuoles in tapetum cells. The small vacuoles in tapetum cells were heterogeneous in size, as shown by EM (Fig. 3) and CLSM (Fig. 5B), and perhaps also in contents. Their content is largely water and other small molecules; therefore, vacuoles had a low buoyant density. This low density, plus the small and heterogeneous sizes of the vacuoles, resulted in a wide distribution of the organelles along the gradient.

Glucanase moved into the gradient and peaked at fraction 9 (density of 1.09 g cm$^{-3}$), and no soluble glucanase was at the top of the gradient. The observed buoyant density of the glucanase is that expected of small secretory vesicles containing glucanase after 4 h of centrifugation (Huang et al., 1983). Calreticulin, a marker of the ER, had a wider distribution along the gradient but encompassed the glucanase peak fractions. This wide distribution is expected, because calreticulin was associated with all ER present throughout the cell and secretory vesicles containing glucanase located near the locule side of the cell (Fig. 5A).

Fraction 9 possessing peak glucanase in the gradient contained largely vesicles with morphology and size similar to those of secretory vesicles in situ observed by EM (Fig. 6C). These secretory vesicles had an electron-opaque matrix and differed from the vacuoles that possessed a distinctly less opaque matrix. Our attempts to observe isolated vacuoles by EM in fractions 7 and 8 possessing peak protease were unsuccessful, probably because the fragility of the organelles and the fractions being highly contaminated with cytosolic materials prevented proper processing of the fractions for EM.

Flavonoids in Tapetum Cells Were Present in ER Subdomains and Not in Vacuoles

Pollen-coat flavonoids are derived from the tapetum (Stanley and Linskens, 1974). In this study of developing maize anthers, flavonoids were almost absent in stages 1 and 2 but abundant in stages 3 and 4 and then in the coat of mature pollen, as revealed in a thin-layer chromatography (TLC) analysis (Fig. 7A). Maize anther and pollen-coat flavonoids were present as flavonoid glycosides (Ceska and Styles, 1984), whose flavonoid moieties were mostly quercetin and isorhamnetin (Fig. 7A); these findings are consistent with those of maize pollen flavonoids reported earlier (Ceska and Styles, 1984).

We examined the subcellular locations of the flavonoids in stage-3 and -4 tapetum cells. Fixed anthers were sectioned into small segments, stained with di-
phenylboric acid 2-aminoethyl ester (DPBA), and then subjected to immunofluorescence analyses (see “Materials and Methods”). Stage-3 and -4 tapetum cells did not have an extensive ER network of a secretory nature facing the locular side; rather, the ER network, localized with the ER marker calreticulin, was distributed throughout the cytoplasm (Figs. 3B and 7B). DPBA flavonoids were colocalized with portions of the calreticulin, which presumably represented ER subdomains (Fig. 7B); this colocalization is similar to that in the Brassica tapetum, in which the flavonoids are initially associated with the ER (Hsieh and Huang, 2007). In contrast, DPBA flavonoids were not colocalized with V-PPase, a marker of vacuoles, and thus flavonoids were absent in the vacuoles.

The Three Hydrolases and Flavonoids Were Deposited on the Pollen Surface at a Late Stage of Anther Development

Microspores removed from stage-3 and -4 anthers and mature pollen were subjected to immunoblot treatment for observation of the three hydrolases on their surface (Fig. 8). Microspores of stages 3 and 4 had little hydrolases on the surface. A trace amount of glucanase, but not the other two hydrolases, could be detected on the microspore surface at stage 3 (Fig. 8); this observation is consistent with the secretory nature of glucanase (Figs. 4A and 5). Slightly more glucanase and a trace amount of protease appeared on the microspore surface at stage 4 (Fig. 8), when the tapetum began its PCD; these observed hydrolase signals in relatively small amounts might be authentic or represent technical background noise. Finally, all three hydrolases became abundant on mature pollen when the tapetum PCD was complete. These findings are consistent with the tapetum cells maintaining their integrity and possessing the hydrolases (glucanase retained in the wall or present transiently on its way to the microspore surface and xylanase and protease in the protoplasts) until stage 4, when PCD commenced.

Similarly, in stage-3 anthers, DPBA flavonoids were present mainly in tapetum cells and almost absent on the microspore surface (Fig. 7C). After tapetum PCD in late stage 4, most of the flavonoids were present on the microspore surface (Fig. 7C).

DISCUSSION

In anthers, tapetum cells synthesize, store, and transfer proteins and nonprotein molecules, especially flavonoids, to the microspore surface, where the molecules carry out specific functions. The mechanisms of these syntheses, storage, and transfers, as well as their protein constituents, are largely unknown. Recent studies have shown that in Brassicaceae species, tapetum cells synthesize proteins (oleosins), alkanes and steryl esters, and flavonoids and store them in tapetosomes and elaioplasts for bulk and simultaneous dis-
charge to the microspore surface upon PCD (Hsieh and Huang, 2005, 2007). Maize and rice tapetum cells do not have storage tapetosomes and elaioplasts. Instead, maize tapetum cells use three different mechanisms for the synthesis, storage, and transfer of the three pollen-coat proteins and yet another mechanism for flavonoids. Glucanase, with its N-terminal ER-targeting signal peptide in the nascent protein, is synthesized in the ER and secreted via the subcellular secretory pathway; it likely digests the wall of the tapetum cell and perhaps subsequently coordinates with the pollen-coat xylanase in hydrolyzing the stigma wall for pollen tube penetration. Xylanase is synthesized and stored in the cytosol as a large inactive protein and upon PCD is activated via proteolysis and released to the microspore surface; it functions after release from the pollen surface onto the stigma to hydrolyze the stigma wall for pollen tube penetration (Suen and Huang, 2007). Protease, with its N-terminal ER-targeting signal peptide in the nascent protein, is synthesized on the ER as a large inactive protein and reaches via the subcellular secretory pathway numerous small vacuoles; it is likely released from the vacuoles upon PCD to proteolyze the cellular contents and then is retained on the pollen surface, where it possibly exerts an additional function on the stigma similar to those for the xylanase and glucanase. Flavonoids are synthesized on the cytosolic side of the ER (Winkel-Shirley, 2001) and transferred to the ER lumen and then the secretory vesicles; they protect the haploid genome from UV irradiation (Hsieh and Huang, 2007) and play an additional role as regulators in pollen germination and tube growth (Mo et al., 1992).

The functions of pollen-coat proteins that originated from tapetum cells have been examined only recently, and minimal information is available. Self-incompatibility signal proteins in the pollen coat of several plant species, including those of *Brassica*, come from sporophytic cells, presumably the tapetum (Kachroo et al., 2002; Kao and Tsukamoto, 2004). In *Brassica* and *Arabidopsis* (*Arabidopsis thaliana*), the amphipathic oleosins are the predominant proteins in the pollen coat and originated from the storage tapetosomes in tapetum cells (Wu et al., 1997; Ting et al., 1998). A mutational loss of the major pollen-coat oleosin led to the pollen grain being inefficient in taking up water from the stigma for germination and pollen tube growth (Mayfield and Preuss, 2000). This observation may reflect the amphipathic oleosins (1) serving as an essential ingredient for water uptake from the stigma to the pollen, (2) acting as an emulsifying agent for the proper coating of the pollen coat materials that include very hydrophobic lipids (alkanes), fairly hydrophobic steryl esters, and relatively hydrophilic flavonoids (Hsieh and Huang, 2004), (3) carrying the highly hydrophobic alkanes from the tapetum tapetosomes, in which small alkane droplets are stabilized with surface oleosins, to the pollen surface, and/or (4) being a nonfunctional leftover after their stabilization of alkane droplets in storage tapetosomes in tapetum cells. Regardless, oleosins are absent in the pollen coat of maize and rice and likely many other species.

The function of xylanase, one of the three pollen-coat proteins in maize, has been documented. Pollen-coat xylanase acts on the stigma surface, where it hydrolyzes the stigma xylan wall to generate a hole for entry of the pollen tube. Before xylanase is released

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Figure 8. Bright-field, autofluorescence, and immunofluorescence (IF) microscopy images of stage-3 and -4 maize microspores and mature pollen. Microspores removed from anthers and mature pollen were subjected to immunoblot treatment. Cy5-conjugated secondary antibodies were used. An excitation wavelength of 648 nm was used, and an emission wavelength of 645 to 700 nm was instituted. For visualization of the autofluorescence of microspores/pollen, an excitation wavelength of 488 nm and an emission wavelength of 530 to 580 nm were used. Identical CLSM settings (laser power and detection gain) were used for all images.

targeting signal peptide in the nascent protein, is synthesized in the ER and secreted via the subcellular secretory pathway; it likely digests the wall of the tapetum cell and perhaps subsequently coordinates with the pollen-coat xylanase in hydrolyzing the stigma wall for pollen tube penetration. Xylanase is synthesized and stored in the cytosol as a large inactive protein and upon PCD is activated via proteolysis and released to the microspore surface; it functions after release from the pollen surface onto the stigma to hydrolyze the stigma wall for pollen tube penetration (Suen and Huang, 2007). Protease, with its N-terminal ER-targeting signal peptide in the nascent protein, is synthesized on the ER as a large inactive protein and reaches via the subcellular secretory pathway numerous small vacuoles; it is likely released from the vacuoles upon PCD to proteolyze the cellular contents and then is retained on the pollen surface, where it possibly exerts an additional function on the stigma similar to those for the xylanase and glucanase. Flavonoids are synthesized on the cytosolic side of the ER (Winkel-Shirley, 2001) and transferred to the ER lumen and then the secretory vesicles; they protect the haploid genome from UV irradiation (Hsieh and Huang, 2007) and play an additional role as regulators in pollen germination and tube growth (Mo et al., 1992).
from tapetum cells, it is present as an inactive 60-kD prexylanase (Wu et al., 2002) residing in the cytosol (this report). The protein in the pollen coat is an active 35-kD xylanase, with 198 and 48 residues of the N and C termini, respectively, of the prexylanase having been removed. The proteolytic processing of the prexylanase in vivo and in vitro is precise, and the final active xylanase is highly resistant to further proteolysis. If xylanase were released from tapetum cells as an active form early before PCD, it would encounter its substrate in the tapetum wall facing the locule before it could reach the microspore surface. Such an undesirable condition is prevented via storage of the prexylanase in the cytosol, where no xylan is available, before its activation and release upon PCD.

The functions of the other two maize pollen-coat proteins, glucanase and protease, remain to be elucidated. We can speculate on their functions from available information, especially the findings here of the mechanisms of storage and transfer of the two proteins. The gene encoding the glucanase is expressed not just in the anther tapetum but also in diverse organs and tissues. Its transcript in the anther appears first at stage 2 and peaks at stage 3; the developmental profile of its encoded protein follows slightly after that of the transcript. During stages 2 and 3, the microspores have already become solitary; thus, this glucanase is not the glucanase for the hydrolysis of the callose wall of the microspore tetrad (Suen and Huang, 2007). This glucanase is synthesized and secreted in stage-2 and -3 tapetum cells, which still maintain the wall facing the locule (Fig. 5A), and the majority of the enzyme is not detected on the microspore surface until PCD (Fig. 8). It is initially concentrated in vesicles near the locular side of the cell and then secreted. Its detection in the tapetum wall and not in the locule fluid (Figs. 4 and 5) could be due to its high concentration during its action in the tapetum wall and/or its low concentration in the locule fluid on its way to the microspore surface. This glucanase could function in hydrolyzing the tapetum cell wall facing the locule and remains with the wall remnant until PCD, after which it is absorbed onto the microspore surface. Alternatively, or in addition, the glucanase in the pollen coat may work coordinately with xylanase (Suen et al., 2003) in generating a hole in the stigma wall for entry of the pollen tube. The gene encoding this glucanase is expressed in diverse tissues and organs (Fig. 1D) and thus may play a role of modulating the wall linkage and thus properties for general cell growth and development.

The pollen-coat protease, ZmPCP, is synthesized at a very late stage, stage 4, of anther development. This is the only known tapetum protease that appears at such a late stage of anther development in any species. The protease may be directly related to PCD of the tapetum, either alone or together with other proteases. The protease is synthesized, presumably in the rough ER, and processed during or after its transfer to the vacuole. According to the current concept of plant PCD (Hatsugai et al., 2006; Trobacher et al., 2006), PCD-related proteases, whether trigger or downstream proteases, are present in vacuoles and are self-activated upon lysis of the tonoplast and plasma membrane. PCD proteases would be stable after self-activation, resisting further proteolysis by other identical protease molecules or different proteases. The maize tapetum protease could be the tapetum-specific trigger protease, because its gene is expressed only in the tapetum at a very late stage of development in the whole plant. If so, the enzyme could be present in the pollen coat due to a fortuitous absorption of the very stable protease left over after PCD and serves no future function. However, we cannot rule out that the protease plays an additional role on the stigma surface.

Pollen-coat flavonoids are derived from the tapetum (Stanley and Linskens, 1974). The locations of flavonoids at the subcellular level or even the tissue level in different plant cells are largely unknown due to a major technical difficulty. Most plant flavonoids, with a few exceptions such as the colorful flavonoids in large central cell vacuoles, are colorless glycosides, which could not be detected in situ with known flavonoid dyes for CLSM. Recently, our laboratory developed an in situ glycoside hydrolysis procedure that could remove the sugar moieties from flavonoid glycosides mildly, such that subcellular structures and protein antigenicity can be maintained for immunofluorescence colocalization studies. With this method, we demonstrated that in Brassicaceae tapeta, flavonoids are present first in the ER and then packaged in tapetosomes (Hsieh and Huang, 2007). In this study with maize tapetum cells, the flavonoids after synthesis in the ER remain largely in ER subdomains rather than move to the vacuoles, as could have been speculated. It is possible that this flavonoid location in ER subdomains is common in tapeta of plant species with no tapetosomes and may even be so in many non-tapetum cells in plants.

MATERIALS AND METHODS

Plant Materials

Maize (Zea mays; inbred B73) plants were grown in a greenhouse. Anthers were classified into four developmental stages (Bih et al., 1999). At stage 1, the anthers filled about one-third of the floret. Each microspore mother cell had become a tetrad of microspores. At stage 2, the anthers filled up about one-half of the floret. Microspores had been released from the tetrad and were covered with the exine. At stage 3, the anthers filled up about two-thirds of the floret. Microspores had become larger and binucleate, and the single large vacuole developed into multiple small vacuoles. At stage 4, the anthers filled up the floret completely. Microspores were trinucleate. Starch accumulates in the microspore, which was dark as observed by bright-field microscopy. Fresh pollens were collected as described (Wu et al., 2002).

Total Anther Extraction

Anthers in different stages were homogenized in 1× SDS-PAGE loading buffer (Suen and Huang, 2007) with a mortar and pestle. The homogenate was boiled and then centrifuged at 16,000g for 10 min. The supernatant was used as the total anther extract.
Preparation of Pollen Total and Coat Extracts

Fresh pollen was homogenized in chlorormormethanol (2:1, v/v) with a mortar and pestle, and the extract was dried under a stream of nitrogen (Suen et al., 2003). This was the pollen total extract. Fresh pollen was washed with diethyl ether (1 g of pollen per 10 mL of ether) in a capped tube, and the tube was subjected to repeated inversions. The tube was centrifuged at 800 g for 10 min. The supernatant was retained and dried under a stream of nitrogen. This was the pollen coat fraction.

Subcellular Fractionation

All solutions contained 0.05 M HEPES-NaOH, pH 7.5. Stage-3 and -4 anthers in a 0.6 M Suc solution at 4°C were chopped with a razor blade and then homogenized gently with a mortar and pestle. The homogenate was filtered through a Nitex cloth (20- x 20-μm pore size). The filtrate retained most microsomes (approximately 70 μm in diameter) and unlysed outer anther cells that were more difficult to break than wall-depleted tapetum cells. The filtrate was placed on a linear gradient (0.8-2.3 M Suc solution) in a 5-mL tube. The gradient was centrifuged at 28,000 rpm for 3 h at 4°C in a Beckman SW55 rotor. Fractions of 0.2 mL each were collected from the bottom of the gradient. Densities of fractions were checked with a refractometer (Milton Roy) and analyzed for proteins by SDS-PAGE and immunoblotting and for organelles by EM.

SDS-PAGE and Immunoblot Analyses

SDS-PAGE of 12.5% (w/v) polyacrylamide and immunoblotting were performed as described (Wu et al., 1997). Preparations of anti-35-KD xylanase (Bih et al., 1999), anti-glucanase (Suen et al., 2003), anti-protease (our preparation), anti-calreticulin (Coughlan et al., 1997), anti-V-PPase (Sarafian et al., 1992), anti-glucanase (Suen et al., 2003), anti-protease (our preparation), anti-β-TIP (Pedrazzini et al., 1997) as well as horseradish peroxidase-conjugated goat anti-rabbit-IgG were used. A synthetic polypeptide of the sequence QARRYACSRSRAAQ, unique to the maize pollen-coat protease (Supplemental Fig. S1), was used to prepare rabbit antibodies via a commercial source (Kim et al., 2002).

Flavonoid Extraction and Analyses

Preliminary tests showed that most flavonoids in maize anther extracts were in glycosylated form. Total extracts of anthers of different developmental stages were acidified in 2 v/v HCl at 80°C for 1 h. Deglycosylated flavonoids in samples were extracted twice with an equal volume of isooamyl alcohol at 80°C. The samples were dried under a stream of nitrogen gas, and the residues were dissolved in methanol for TLC. TLC plates were developed in toluene:ethyl acetate:formic acid/water (50:40:10, v/v/v/v) and sprayed with 0.5% (w/v) DPBA (Sigma D9754-1G) in methanol for flavonoid staining (van der Meer et al., 1989). For TLC plates, observed and photographed on top of a UV light source. For double flavonoid labeling with DPBA (Sigma-Aldrich) and immunolabeling, Cy3-conjuated Fab fragment goat anti-rabbit IgG (H+L) and Cy5-conjugated goat anti-rabbit IgG antibodies (1:200; Jackson ImmunoResearch Laboratories product no. 211-005-109) for 1 h at 20°C were used. After washing with PBST three times each for 5 min, sections were mounted with antifade solution (Molecular Probes; S-2828), and observed by confocal microscopy. For double immunolabeling, sections were incubated with primary antibodies at 4°C for 2 h and then washed with PBST three times each for 5 min. Fresh sections were then mounted with antifade solution and observed with a Leica SP2 confocal microscope. DPBA and cyanine 5 were excited with 488- and 633-nm lines, respectively; the emissions were detected at 490 to 530 nm and 650 to 750 nm, respectively.

EM

Anthers were fixed with paraformaldehyde and glutaraldehyde, postfixed with OsO4, dehydrated, embedded in Spurr’s resin, sectioned, poststained with uranyl acetate and lead citrate, and observed with a Philips EM400 electron microscope (FEI), all as described (Platt et al., 1998).

Fraction 9 (in approximately 0.75 M Suc) representing the glucanase peak in the Suc gradient (Fig. 6) was diluted with an equal volume of 2.5% glutaraldehyde and 0.75 M Suc for 5 h at 4°C. The mixture was diluted to 0.6 M Suc with 0.4× Suc solution in 0.05 M HEPES-NaOH, pH 7.5. The diluted mixture was placed on top of a membrane (Amicon cellulose membrane YM-10) in a 0.8-mL centrifuge tube (Beckman-Coulter) and centrifuged at 28,000 rpm for 3 h at 4°C. Materials retained on the membrane were covered with a drop of 5% agar and, after agar solidification, washed twice with 0.1 M potassium phosphate, pH 7.2. The sample was fixed with 1% OsO4 in 0.1 M potassium phosphate, pH 7.2, for 4 h at 4°C and subjected to dehydration, embedding, sectioning and poststaining as described for anther sections.

DNA Extraction and RT-PCR

Total RNA was extracted (Kim et al., 2002) from anthers of different developmental stages, mature pollen, and pollen germinated for 20 min in a liquid medium (Suen and Huang, 2007). The cDNA template for PCR was synthesized from total RNA with an oligo(dT)15 primer (Sambrook et al., 1989). For the gene encoding the pollen-coat 70-kD β-glucanase, the 5′′-primer (5′-CAGATCGAGGGCCAAACG-3′′) and 3′′-primer (5′-CTTGGAGGC-CCGGCGTTC-3′′) were designed from the coding region of GLA (ZmGLA5; GenBank accession no. AY344622). For the gene encoding β-glucanase, the 5′′-primer (5′-GGGAGGCATGACCGCTTACT-3′′) and 3′′-primer (5′-CTTGGTGACCGCTTGCGCC-3′′) were designed from the coding region of XYN (ZmXYN1; GenBank accession no. AF149016). For the gene encoding the pollen-coat protease, the 5′′-primer (5′-ATGGCCTCTCTCGTGCGG-3′′) and 3′′-primer (5′-TTATTGTATGATTCACATC-3′′) were designed from the coding region of the maize est (GenBank accession no. TC4Z2605) in The Institute for Genomic Research database (http://www.jcvi.org); the complete cDNA sequence was obtained via DNA sequencing and registered as GenBank accession number EU117211. For the gene encoding the pollen-wall 10-kD extensin protein, a 5′′-primer (5′-CAACAAATGCGTGGCTACG-3′′) and 3′′-primer (5′-GCTGAGTGTGGCTATCAG-3′′) were designed from the coding region of a maize est (GenBank accession no. AY111779). For a maize actin gene, the 5′′-primer (5′-GGTATCCCTCCACACAGC-3′′) and 3′′-primer (5′-CAGACACTGACTCCCTC-3′′) were designed from the coding region of MaZ56 (GenBank accession no. U60514). The purified PCR product was 32P labeled with the Prime-A-Gene labeling kit (Promega) and employed as a probe for RNA-blot hybridization. For RNA-blot hybridization, each sample of 30 μg of total RNA was fractionated by electrophoresis with a 1.2% formaldehyde gel and then blotted onto a Hybond-N membrane (Amersham Bioscience). The radiolabeled membrane was prehybridized at 65°C in potassium phosphate, pH 7.2, 7.5% SDS, 1% bovine serum albumin, and 0.1× SS/EDTA, pH 8.0, for 4 h, hybridized with 32P-labeled probes (preceding paragraph) for 12 h, then washed with 2× SSC, 0.1% SDS for 20 min, 1× SSC, 0.1% SDS for 20 min, and 0.1× SSC, 0.1% SDS for 20 min, all at 65°C.

Fluorescence Confocal Microscopy

The procedures followed those described (Li et al., 2002). Anthers were fixed in 4% paraformaldehyde and 0.5% potassium phosphate buffer, pH 7.4, for 12 h at 4°C. Fixed anthers were dehydrated in serial ethanol solutions and then embedded in paraffin. Paraffin sections 6 μm thick were placed on a coverslip and sealed with nail polish. Primary antibodies were anti-calreticulin, anti-xylanase, anti-glucanase, anti-protease-1, and anti-VPPase. Confocal images were collected with a Leica TCS SP2 confocal microscopy system with an HCX PL APO 63×/1.2 water immersion objective. Confocal images of sequential scans and three-dimensional image reconstruction of sequential confocal images were taken with Leica confocal software (LCS Lite). Images were processed with Adobe Photoshop software.

For double flavonoid labeling with DPBA (Sigma-Aldrich) and immunolabeling (Hsieh and Huang, 2007), anther sections were fixed in 4% paraformaldehyde, 1× PBS (10 mM potassium phosphate, pH 7.4, 138 mM NaCl, and 2.7 mM KCl), and 0.15 M Suc at 4°C for 16 h. After fixation, sections were washed with PBST (1× PBS and 0.1% Tween 20) for 10 min twice and treated with 0.4 M HCl at 56°C for 30 min, then washed with PBST for 10 min twice. Sections after acid treatment were blocked with a blocking solution (3% milk, 1× PBS) for 25°C in 1 h and then treated with a 1:50 dilution of primary antibodies in 1% milk and 1× PBS at 25°C for 1 h. After washing with PBST for 10 min, sections were treated with PBDA and secondary antibody (0.5% PBDA, 0.01% Triton X-100, 10% glycerol, 1× PBS, and 1:100 cyanine 5-conjugated goat antibodies against rabbit IgG [Jackson ImmunoResearch Laboratories]) for 2 h at 25°C and then washed twice. Finally, sections were mounted with antifade solution and observed with a Leica SP2 confocal microscope. DPBA and cyanine 5 were excited with 488- and 633-nm lines, respectively; the emissions were detected at 490 to 530 nm and 650 to 750 nm, respectively.
In Situ Hybridization

Riboprobe was synthesized with the DIG Nonradioactive RNA Labeling Kit (Boehringer). A PCR fragment of ZmPCP (preceding section) was cloned into pGEM-T vector (Promega). The plasmid was digested with NotI or SpeI for the synthesis of sense or antisense riboprobes. The product was alkali hydrolyzed with sodium carbonate buffer (120 mM Na2CO3, 80 mM NaHCO3, pH 10.3) at 65°C for 70 min. The riboprobe product was precipitated and then dissolved in water pretreated with diethyl pyrocarbonate.

For procedures in situ hybridization followed those described (Qu et al., 2003). Anthers of stage 2.5 were fixed with 4% paraformaldehyde for 4 h and then treated with protease K in prehybridization buffer (100 mM Tris-HCl and 50 mM EDTA, pH 8.0) at 37°C for 30 min. After being rinsed with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl) twice, the sections were incubated in TBS containing 2 mg ml⁻¹ Gly at 20°C for 2 min and then postfixed with 3.7% formaldehyde in PBS at 20°C for 20 min. Sections were washed with TBS for 5 min twice, acetylated with acetic anhydride (0.25% acetic acid in 0.1M triethanolamine-HCl, pH 8.0) at 20°C for 10 min, and dehydrated with serial ethanol solutions. A hybridization solution (10 mM Tris-HCl, pH 6.8, 10 mM sodium phosphate buffer, pH 6.8, 5 mM EDTA, pH 8.0, 40% [v/v] deionized formamide, 10% [v/v] dextran sulfate, 300 mM NaCl, 1 mg ml⁻¹ yeast tRNA, RNase inhibitor [Promega], and 400 ng ml⁻¹ digoxigenin-labeled riboprobe) was added onto the sections. After being treated with 50% formamide at 50°C for 12 h, sections were washed twice with 2× SSC at 37°C and incubated with 20 μg ml⁻¹ RNase A (Promega) at 37°C for 30 min, followed by washes each for 1 h with 2× SSC, 1× SSC, and 0.1× SSC at 65°C. Immunological detection was performed with the Nucleic Acid Detection Kit (Boehringer Mannheim) according to the supplier’s protocol. Samples were observed under bright field with a Leica DMLB microscope.

Phylogenetic Analysis

Full-length amino acid sequences of 17 maize Cys proteases closest to that of ZmPCP were obtained from BLAST of GenBank using the ZmPCP sequence as the query. An unrooted phylogenetic tree was constructed with the use of the neighbor-joining method based on the matrix of multiple sequence alignments of the 18 Cys proteases created through ClustalW. CLC Sequence Viewer (6.5.2; www.ccbio.com) was used for phylogenetic tree construction, and bootstrapping analysis was performed (100 replications).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers ACG3680, ACG39911, ACF87422, ACF79746, ACG42266, BAA88244, BAA88245, ACG23594, ACG31086, ACF82135, ACG38442, AAB88263, AAB88262, ACF87906, AAB70820, ACG38044, and ACF84854.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Sequence alignments of 17 maize Cys proteases and one rice Cys protease most similar to ZmPCP.

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