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The proteins encoded by two tapetum-specific transcripts, *Sa*tap35 and *Sa*tap44, from *Sinapis alba* L. are localized in the exine cell wall layer of developing microspores

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Abstract. By differential screening of a copy DNA (cDNA) library from flowering *Sinapis alba* L. apices against cDNAs from vegetative apices, two cDNA clones were isolated representing transcripts that are expressed transiently at an early stage of tapetum development. The *Sa*tap35 cDNA encodes a polypeptide with a predicted molecular weight of 12.7 kDa and an isoelectric point of 10.4. The *Sa*tap44 cDNA codes for a putative 12.4-kDa polypeptide with an isoelectric point of 7.5. The deduced amino-acid sequences display 76% sequence identity and contain an N-terminal stretch of hydrophobic amino acids which has characteristics of secretory signal sequences. In-vitro transcription of the cDNAs and translation of the resulting RNAs in the presence of canine pancreatic microsomes demonstrates that the two proteins are translocated into the microsomes and that the putative preproteins are proteolytically processed to the mature forms. By immunoelectron microscopy the *Sa*TAP35 and *Sa*TAP44 proteins were detected at the developing peritapetal membrane between the tapetal cytoplasm and the adjacent middle layer of the anther wall. Furthermore, labelling was observed within the locule in association with globules resembling pro-Ubisch bodies which appeared at the tetrad stage. During the early vacuolate stage of microspore development the young exine was strongly labelled. The exine and the peritapetal membrane both are composed of sporopollenin, and the pro-Ubisch bodies are thought to contain sporopollenin precursors. Thus, *Sa*TAP35 and *Sa*TAP44 might be involved in sporopollenin formation and/or deposition.

Key words: Exine – Flower-specific cDNAs – Maltose-binding protein fusions – Peritapetal membrane – Pro-Ubisch bodies – Signal sequence – *Sinapis* – Tapetum

Introduction

In higher plants, pollen formation occurs in a specialized floral organ, the stamen. It requires the interaction of several cell types within the anther. Sporogenous cells divide mitotically, forming diploid meiocytes. The meiocytes undergo meiosis to give rise to tetrads of haploid microspores. These are initially encaged in a callose wall which is degraded once pollen wall formation has started on the surface of the young microspores. During the subsequent microspore development the pollen wall is further elaborated and reserves are deposited. The tapetum, the innermost wall layer of the anther surrounding the locular space, is of importance for sporogenesis. Its lifetime is confined to a short period during anther development. The tapetum represents a secretory tissue providing nourishment to the developing microspores (Nave and Sawhney 1986; Sawhney and Nave 1986). Furthermore, the tapetum synthesizes and secretes callase, a β-1,3-glucanase activity which degrades the callose wall encaging the tetrads of microspores and thus releases the microspores into the locule (Frankel et al. 1969). Additionally, the tapetum synthesizes sporopollenin precursors that become incorporated into the exine, the outer cell wall layer of pollen grains (Echlin 1971; Vithanage and Knox 1980). The exine outer layer also seems to play an important role in recognition of pollen to prevent self-fertilization. Sporophytic control of the pollen phenotype has been hypothesized to result from expression of the *S*-locus gene in the tapetum with transfer of the *S*-locus gene product to the pollen surface upon dissolution of the tapetum (Heslop-Harrison 1975). Indeed, in anthers of transgenic *Arabidopsis thaliana* plants, reporter-gene expression driven by the *S*-locus glycoprotein promoter of *Brassica* was spatially restricted to the tapetal cell layer and temporally restricted to the period from the beginning of exine deposition until tapetal cell degeneration (Toriyama et al. 1991).

The important role of the tapetum in pollen formation is demonstrated by natural male-sterile mutants exhibi-

Abbreviations: cDNA = copy DNA; GST = glutathione S-transferase; IPTG = isopropyl thiogalactopyranoside; MBP = maltose-binding protein; SDS = sodium dodecyl sulfate

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ting various degrees of tapetal dysfunction (Bino 1985; Kaul 1988). Recently, male-sterile plants have been obtained by expressing cytotoxic genes under the control of tapetum-specific promoters, thereby selectively destroying the tapetal cell layer and interfering with microspore development (Koltunow et al. 1990; Mariani et al. 1990). These genetic ablation methods in conjunction with restorer-gene systems have practical applications in the control of plant male fertility in hybrid seed production (Mariani et al. 1992).

Despite the detailed knowledge about tapetum function, to date little is known about individual gene products participating in cellular processes unique to the tapetum. We have exploited the synchronous flower formation of mustard plants (*Sinapis alba* L.) exposed to an inductive long day (Bernier 1988) to construct stage-specific cDNA libraries. Differential screening of a library representing transcripts expressed at day 10 after induction against cDNAs from vegetative apices has led to the identification of de-novo-induced cDNAs (Majewski 1990; Melzer et al. 1990). The corresponding transcripts *Sa*tap35 and *Sa*tap44, which are encoded by a small multigene family in mustard, are expressed specifically in the tapetal cell layer in flower buds of 1 to 1.5 mm size (Staiger and Apel 1993). Here we present a detailed characterization of the encoded proteins *Sa*TAP35 and *Sa*TAP44 and provide evidence that the proteins are associated with the exine outer layer of developing microspores.

Materials and methods

Plant material. Mustard plants (*Sinapis alba* L.; Asgrow, Ebnet, Freiburg, FRG) were grown in a phytotron with a daily 8-h light period at 20° C and a 16-h dark period at 16° C. Plants were induced to flower after 56 d by extending the daily illumination period to 16 h.

To define the developmental stages of flower buds we used bud length as an easily scorable character. This is based on the fact that the inflorescence of mustard plants bears flower buds in concentrically arranged whorls such that the age and the size of a flower bud increases with its distance from the inflorescence axis. To establish the correlation between bud length and anther elongation, which in turn is closely correlated with anther development (Erickson 1948; Koltunow et al. 1990; Scott et al. 1991), we measured the length of anthers dissected from *S. alba* buds. A linear correlation was found between anther length and bud length up to 4-mm buds, i.e. during the entire life span of the tapetum (data not shown).

In-vitro transcription, translation and protease-protection assay. The cDNA clones *Sa*tap35 and *Sa*tap44 representing transcripts expressed specifically in the tapetal cell layer of the anther were isolated from a flower-specific cDNA library as described previously (Staiger and Apel 1993). Templates for in-vitro synthesis of RNA were amplified from the Bluescript SK subclones of these cDNAs using polylinker primers flanking the T3 and T7 promoters by polymerase chain reaction (PCR). In-vitro transcription of the gel-purified fragments was performed in the presence of 0.5 mM ATP, CTP, GTP and UTP. After 1 h at 37° C the DNA template was digested with RNase-free DNase and the RNA was recovered by phenol extraction and ethanol precipitation. Translation assays containing 1 µg RNA, 7.5 µl of wheat-germ extract (Boehringer, Mannheim, FRG), 740 MBq [^{35}S]methionine (Dupont, Regensdorf, Switzerland) in a final volume of 25 µl were incubated at 30° C for 1 h. For microsomal uptake experiments, 1.5 µl of canine pancreatic microsomes (Boehringer) were included in the translation reaction. In-vitro translations were terminated by adding cold methionine to a final concentration of 8 mM and cycloheximide to 0.2 mM. One third of each sample was kept as untreated control. One third was supplemented with 10 mM $CaCl_2$ and incubated with 1 mg · ml^{-1} proteinase K for 1 h at room temperature. To the third aliquot, 10 mM $CaCl_2$ and 1% Triton X-100 were added before protease treatment (Walter and Blobel 1983; Bernhard et al. 1991). Protease digestion was subsequently inhibited by 1 mM phenylmethylsulfonyl fluoride prior to the electrophoretic separation of the polypeptides on 13% SDS polyacrylamide gels.

Cloning and expression of bacterial fusion proteins. The coding regions of the proteins *Sa*TAP35 and *Sa*TAP44 lacking the signal peptide were PCR-amplified from phage DNA of the respective lambda gt10 cDNA clones *Sa*tap35 and *Sa*tap44 (Staiger and Apel 1993). For the construction of TAP35 fusions the 5′ primer 5′ GCAATCAGAACCAACATCACC 3′ (nucleotide positions 114–132) and the 3′ primer 5′ CTTAAATCTTCAAGCGTTTCCT 3′ (nucleotide positions 378–357) were used to amplify the region of the cDNA *Sa*tap35 corresponding to amino acids 34–117 of *Sa*TAP35 (see Fig. 1). Likewise, PCR amplification of the cDNA *Sa*tap44 with the 5′ primer 5′ CCTAAGACCGCAGTCAGAAC 3′ (nucleotide positions 109–128) and the 3′ primer 5′ TTATCATGCGTTTCCTCCATC 3′ (nucleotide positions 367–347) generated a fragment corresponding to amino acids 31–114 of *Sa*TAP44 (see Fig. 1). The gel-purified fragments were inserted into the blunt-ended EcoRI site of the vector $pMBP_{cRI}$ (Biolabs, Allschwill, Switzerland) and the SmaI site of the vector pGEX-2T (Pharmacia, Dübendorf, Switzerland), respectively. Recombinant plasmids were verified by sequencing and transferred into a protease-deficient *E. coli* strain (lon$^-$ htpR$^-$). Transformed cells were grown in Luria broth, 1% glucose, 100 µg ampicillin and 15 µg tetracycline per litre at 30° C and the expression of the fusion proteins was induced by addition of 0.3 mM isopropyl thiogalactopyranoside (IPTG).

For purification of maltose-binding-protein (MBP) fusions, bacteria from 1 l of induced culture were pelleted, resuspended in 50 ml lysis buffer (10 mM sodium phosphate, pH 7.0; 0.5 M NaCl; 0.25% Tween 20, (Fluka, Buchs, Switzerland); 10 mM EDTA; 10 mM EGTA; 1 mM dithiothreitol (DTT) and frozen in liquid nitrogen. After thawing the bacterial pellet on ice, 1 µM each of pepstatin, leupeptin, chymostatin and antipain was added and the suspension was subjected to three 10-s cycles of sonication. The lysate was clarified at 14000 · *g* for 20 min. The supernatant containing most of the fusion protein in a soluble form was applied to an amylose matrix (Biolabs) by batch absorption at 4° C. After washing the resin with four changes of column buffer (10 mM sodium phosphate, pH 7.0; 0.5 M NaCl; 1 mM DTT; 1 mM EGTA) which was supplemented with 0.25% Tween 20 and four changes of column buffer without Tween 20, bound protein was recovered by elution with 15 mM maltose in column buffer. The solution was dialyzed five times against 100 volumes of 100 mM Tris-HCl (pH 8), 100 mM NaCl and concentrated with a Centricon 30 (Amicon, Wallisellen, Switzerland). Cleavage of the purified fusion protein with Factor Xa (Biolabs), a protease in the blood clotting cascade, was performed in 20 mM Tris-HCl (pH 8), 100 mM NaCl and 2 mM $CaCl_2$ at room temperature.

Affinity-purification of the glutathione S-transferase fusions on glutathione agarose (Sigma, Buchs, Switzerland) was performed according to Ausubel et al. (1987).

Immunization. About 300 µg of purified fusion protein MBP-TAP35 or 600 µg MBP-TAP44 was emulsified with an equal volume of Freund's adjuvant (Difco, Detroit, Mich., USA) and injected subcutaneously into rabbits. Booster injections were administered at four week intervals.

The IgG fraction of preimmune sera and antisera were purified by Protein A-Sepharose (Pharmacia) chromatography according to established protocols (Harlow and Lane 1988).

Affinity purification of the antisera. The fusion proteins GST-TAP35 and GST-TAP44 (GST-glutathiones-transferase), which had been purified on a glutathione-agarose matrix, were coupled to Affigel-10 following the instructions of the supplier (BioRad, Glattbrugg, Switzerland). The serum directed against *Sa*TAP35 and the corresponding preimmune serum were purified over Affigel containing GST-TAP35 and the serum directed against *Sa*TAP44 and the corresponding preimmune serum were purified over Affigel containing GST-TAP44. Bound antibodies were recovered by sequential elution with 100 mM glycine-HCl (pH 2.8) and 100 mM triethylamine-HCl (pH 11.5) (Harlow and Lane 1988).

Immunoprecipitation. In-vitro translations of poly(A)-containing RNA from flower buds or in-vitro transcripts of the cDNAs were precipitated using 8 µl of Protein-A-Sepharose-purified serum or preimmune serum according to Anderson and Blobel (1983). Immunoprecipitated proteins were resolved on 13% SDS polyacrylamide gels and the gels were subjected to fluorography (Bonner and Laskey 1974).

Western blot. Anthers were dissected from size-selected flower buds and frozen in liquid nitrogen. Protein extracts were prepared using the following solvents: *Native extract:* 20 mM Tris-HCl (pH 7.8), 50 mM NaCl, 20% (v/v) glycerol; 1 h on ice. *Sodium dodecyl sulfate extract:* Laemmli sample buffer adjusted to 1% SDS (Laemmli 1970); 10 min at 90° C. *Acidic extract:* 84 mM sodium citrate, 32 mM Na_2HPO_4, 6 mM sodium ascorbate (pH 2.8) (Antoniew et al. 1980); 1 h on ice. The extract was subsequently neutralized with 1 M NaOH on ice. *Alkaline extract:* 1 M NaOH; 1 h on ice. The extract was subsequently neutralized with 10% acetic acid on ice. *Calcium-chloride extract:* 0.5 M $CaCl_2$ (Nagahashi and Seibles 1986); 1 h on ice. *Phenol-formic acid:* 50% (w/v) phenol, 25% (v/v) formic acid (Apel and Schweiger 1972); 1 h at room temperature. For the experiment described in Fig. 5B, C, equal amounts of pulverized anthers were subjected to the extraction with different solvents. All buffers were supplemented with a cocktail of pepstatin, chymostatin, antipain and leupeptin and 5 mM β-mercaptoethanol. Insoluble material was pelleted and the supernatants were dialyzed against 62.5 mM Tris-HCl (pH 6.8), 0.1% SDS, 5 mM β-mercaptoethanol and 20% (v/v) glycerol. Protein extracts were resolved on 13% SDS polyacrylamide gels and electroblotted onto a nitrocellulose membrane. The antisera against MBP-TAP35 and MBP-TAP44 were purified on a Protein A-Sepharose column and used at a 1:1000 dilution. The western blots were developed using alkaline phosphatase coupled to goat anti-rabbit IgG (Sigma; 1:9000 dilution) or [^{35}S]-labelled goat anti-rabbit IgG (Amersham, Buckinghamshire, UK; 1:3000 dilution) as secondary antibody, following established protocols (Harlow and Lane 1988).

Immunogold labelling and electron microscopy. Anthers were dissected from flower buds 1–4 mm in length and subjected to cryofixation using a commercial high-pressure freezer (Balzers Union, Balzers, Liechtenstein) according to Studer et al. (1989). Freeze-substitution was performed in anhydrous acetone containing 2% uranyl acetate, 1% glutaraldehyde and 8% methanol and the specimens were embedded in LR Gold (London resin company, EMS, Fort Washington, Pa., USA).

Ultrathin sections of silver interference colour were mounted on Collodium-coated nickel grids. Sections were blocked in PBS (6 mM Na_2HPO_4; 1 mM KH_2PO_4; pH 7.4; 137 mM NaCl; 3 mM KCl)/50 mM glycine and PBG (PBS supplemented with 0.5% bovine serum albumin and 0.2% cold-water fish gelatine). Incubations with the affinity-purified primary antibodies (diluted 1:30 in PBG) were performed for 2 h at room temperature. After washing in PBG the grids were treated with 1:75 diluted Protein A-gold (10 nm; Biocell, Lausanne, Switzerland) for 1 h at room temperature. Post-staining was done with 2% uranyl acetate and lead citrate (Reynolds 1963) for 7 min each. Sections were examined with a Hitachi 600 electron microscope at 100 kV.

Results

SaTAP35 and SaTAP44 are synthesized as precursor proteins. The deduced amino-acid sequences of the tapetum-specific cDNAs *Sa*tap35 and *Sa*tap44 share 76% sequence identity (Staiger and Apel 1993; Fig. 1). Database searches did not reveal any homology with known proteins. *Sa*tap35 encodes a putative 117-amino-acid polypeptide of 12.7 kDa with an isoelectric point of 10.6. *Sa*tap44 contains an open reading frame, predicting a 115-amino-acid polypeptide of 12.4 kDa molecular mass and an isoelectric point of 7.5. Conspicuously, both proteins contain strongly hydrophobic regions meeting the criteria for signal sequences (von Heijne 1983, 1986) at their respective amino termini. Potential signal-peptidase cleavage sites conforming to the −3, −1 rule (von Heijne 1983, 1986) are present either between Ser and Gln at positions 22/23 in *Sa*TAP35 and 23/24 in *Sa*TAP44 or between Ser and Leu at positions 27/28 in *Sa*TAP35 and 28/29 in *Sa*TAP44, marked by the arrowheads in Fig. 1.

To analyze the role of the amino-terminal hydrophobic sequences, the cDNAs were transcribed in vitro and the resulting RNAs translated in a wheat-germ cell-free system under conditions leading to processing of signal sequences by the secretory pathway. In the absence of microsomal membranes a prominent translation product was produced from the *Sa*tap44 cDNA (Fig. 2, lane 1). The polypeptide was readily digested by proteinase K in the absence (lane 2) or presence (lane 3) of Triton X-100. When dog pancreas microsomal membranes were present during the translation an additional lower-molecular-weight translation product accumulated (lane 4). This lower-molecular-weight polypeptide was resistant to proteolysis while the higher-molecular-weight species was not (lane 5). Addition of Triton X-100 concomitantly with proteinase K rendered the lower-molecular-weight product protease-sensitive (lane 6). This indicates that the amino terminus functions as a signal peptide and that the mature polypeptide is sequestered in the endoplasmic reticulum (ER).

The same experiment was performed with RNA from flower buds employing a specific antiserum directed

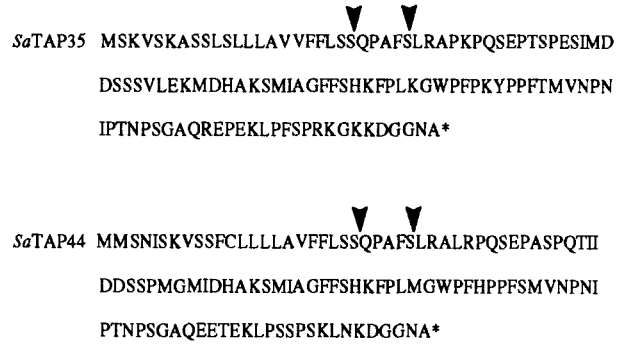


Fig. 1. Amino-acid sequences of the *Sa*TAP35 and *Sa*TAP44 proteins deduced from the nucleotide sequences of the *Sa*tap35 and *Sa*tap44 cDNAs from *Sinapis alba* (Staiger and Apel 1993). Two potential signal-peptidase cleavage sites predicted according to von Heijne (1983, 1986) are indicated by *arrowheads*

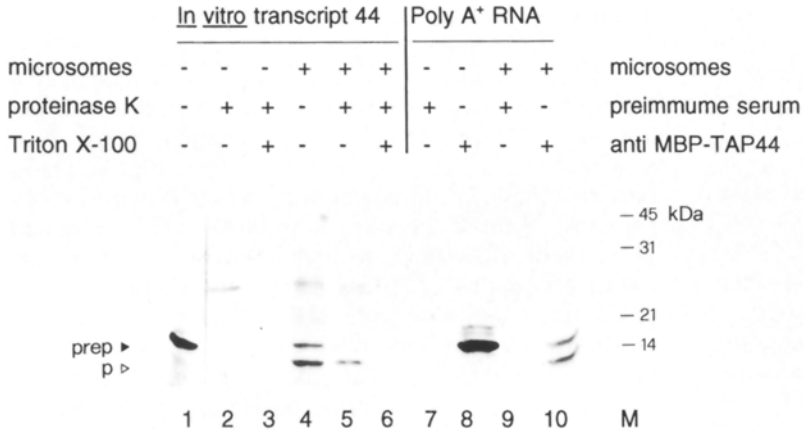


Fig. 2. Processing of in-vitro translation products *Sa*TAP44 by canine microsomal membranes, and protease sensitivities. In-vitro transcript from the cDNA *Sa*tap44 was translated in the wheat-germ system without microsomes *(lanes 1–3)* or in the presence of microsomes *(lanes 4–6)*. The reaction products were subsequently incubated with proteinase K alone *(lanes 2, 5)* or with proteinase K in the presence of Triton X-100 *(lanes 3, 6)*. Polyadenylated RNA (1 μg) from 1- to 2-mm flower buds was translated without the addition of microsomes *(lanes 7, 8)* or in the presence of microsomes *(lanes 9, 10)*. Protein *Sa*TAP44 was immunoprecipitated from total translation products by a specific serum directed against MBP-TAP44 (see below; *lanes 8, 10*). Control precipitation was performed with preimmune serum *(lanes 7, 9)*. The two substoichiometric bands slightly larger than the precursor protein of *Sa*TAP44 *(lane 8)* are likely to represent translation products of related transcripts (see below). The position and size of the molecular weight markers (*M*) are indicated. *prep*, precursor protein; *p*, processed protein

against a bacterially expressed *Sa*TAP44 fusion protein (described below) to detect *Sa*TAP44 proteins by immunoprecipitation. When the wheat-germ system was programmed with poly(A)-containing RNA from 1- to 2-mm buds a prominent protein with the size of the precursor protein could be immunoprecipitated from total translation products by the antiserum (Fig. 2, lane 8). When dog pancreas microsomes were included in the translation assay, two proteins corresponding to the precursor protein and the processed form were immunoprecipitated (lane 10). The preimmune serum did not precipitate either of the two polypeptides (lane 7, 9).

The in-vitro translation product of in-vitro-transcribed *Sa*tap35 cDNA was found to be processed by dog pancreas microsomes in the same manner (data not shown).

Production of antisera against MBP fusions. Fusion constructs were designed to express the proteins *Sa*TAP35 and *Sa*TAP44 in *E. coli*. Chimeric proteins were generated by inserting the coding region of cDNAs *Sa*tap35 and *Sa*tap44 lacking the N-terminal signal sequence into the cytoplasmic MBP vector, which expresses the fusion protein in the *E. coli* cytoplasm due to a deletion of the *malE* signal sequence (di Guan et al. 1988) and into the glutathione S-transferase (GST) vector (Smith and Johnson 1988). The following parallel constructs were made: amino acids 34–117 of *Sa*TAP35 fused to MBP (pMBP-TAP35) and to GST (pGST-TAP35); amino acids 31–114 of *Sa*TAP44 fused to MBP (pMBP-TAP44) and to GST (pGST-TAP44). The MBP fusion proteins were used for immunization after large-scale purification, as shown for MBP-TAP35 in Fig. 3. In *E. coli* cells harbouring the pMBP vector alone IPTG induced the synthesis of a 51-kDa MBP-β-galactosidase fusion

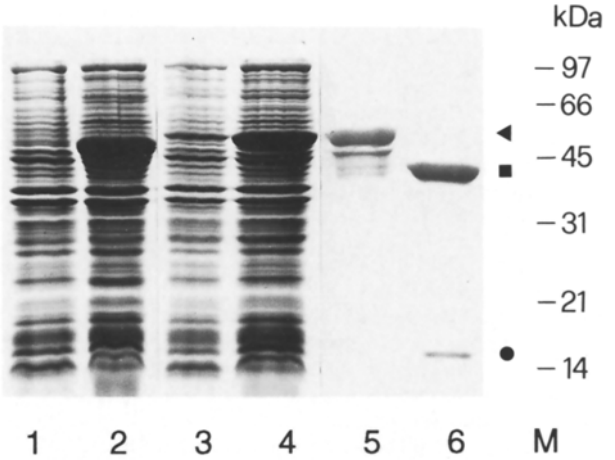


Fig. 3. Purification of MBP fusions by amylose affinity chromatography. Purification was monitored by analyzing aliquots of each step on a 13% SDS polyacrylamide gel. Crude protein extract from *E. coli* harbouring the vector $pMaI_{cRI}$ before *(lane 1)* and 2 h after IPTG induction *(lane 2)*. Crude protein extract from *E. coli* harbouring the chimeric construct pMBP-TAP35 before *(lane 3)* and after IPTG induction *(lane 4)*. Fusion protein MBP-TAP35 purified by one cycle of affinity chromatography *(lane 5)*. Factor Xa protease cleavage of the purified fusion protein *(lane 6)*. The position and size of the molecular-weight markers (*M*) are indicated. *Triangle*, fusion protein MBP-TAP35; *square*, MBP carrier protein; *circle*, *Sa*TAP35

(Fig. 3, lanes 1, 2) whereas upon IPTG induction of cells transformed with pMBP-TAP35 a prominent 55-kDa band appeared corresponding to the MBP-TAP35 fusion (Fig. 3, lane 3, 4). The chimeric protein was purified in a single step on an amylose matrix. The affinity-purified fraction contained, in addition to the intact 55-kDa fusion protein, substoichiometric bands ranging from

55 kDa to 42 kDa, the size of the carrier protein (Fig. 3, lane 5). These bands most likely represent truncated fusion proteins in which the TAP35 extension has been degraded to various extents. The *Sinapis* portion of the protein could be released by cleavage of the fusion protein with the protease Factor Xa (Fig. 3, lane 6).

The antisera raised against the affinity-purified fusion proteins MBP-TAP35 and MBP-TAP44 were tested for the ability to immunoprecipitate polypeptides *Sa*TAP35 and *Sa*TAP44 from total in-vitro translation products of flower bud RNA. As shown in Fig. 4, the serum directed against MBP-TAP35 precipitated two polypeptides (lane 3) and the serum directed against MBP-TAP44 precipitated one polypeptide (lane 5). The preimmune sera were inactive (lanes 2, 4). As observed for the *Sinapis*-derived portion of the MBP fusion protein (Fig. 3, lane 6) the polypeptides migrated at slightly higher apparent molecular weights than expected from the deduced amino-acid sequences.

Since the coding regions of the proteins *Sa*TAP35 and *Sa*TAP44 are 76% identical at the amino acid level the antisera were tested for cross-reactivity with the antigens. Immunoprecipitation was performed with in-vitro translation products of in-vitro-transcribed *Sa*tap35 and *Sa*tap44 cDNAs. In-vitro translation of *Sa*tap35-derived RNA yielded, in addition to the full-length protein, a lower-molecular-weight band (Fig. 4, lane 6). We believe this to be due to an internal translation start at Met 44 because the RNA derived from a truncated cDNA of *Sa*tap35 lacking the ATG start codon and most of the signal sequence up to position 20 is translated into a polypeptide of the same apparent size as this lower-molecular-weight band (data not shown). Both polypeptides are precipitated by the serum directed against MBP-TAP35 (Fig. 4, lane 7) but not by the antiserum for MBP-TAP44 (lane 8). The in-vitro translation product of *Sa*tap44-derived RNA (Fig. 4, lane 9) is recognized by both antisera (Fig. 4, lanes 10, 11). These data suggest that (i) the serum against MBP-TAP35 reacts with both proteins *Sa*TAP35 and *Sa*TAP44 whereas the serum against MBP-TAP44 recognizes specifically protein *Sa*TAP44 under the conditions employed, and (ii) the larger polypeptide immunoprecipitated from bud RNA translations corresponds to protein *Sa*TAP44 whereas the smaller polypeptide corresponds to protein *Sa*TAP35. The GST-TAP35 and GST-TAP44 fusion proteins were employed for construction of an affinity matrix for the enrichment of antibodies directed against epitopes of the *Sinapis alba* proteins TAP35 and TAP44 from the antisera directed against the MBP-TAP35 and MBP-TAP44 fusion proteins (see *Materials and methods*).

Identification of Satap35- and Satap44-encoded proteins. Anthers of size-selected flower buds were analyzed for the presence of proteins *Sa*TAP35 and *Sa*TAP44. The western blot shown in Fig. 5A illustrates that the serum directed against MBP-TAP44 recognizes a doublet band of an apparent molecular weight of approx. 14 kDa extracted from anthers of 1.5-mm buds (lane 4). The preimmune serum shows no signal (lane 2). Since the serum directed against MBP-TAP44 does not recognize *Sa*TAP35

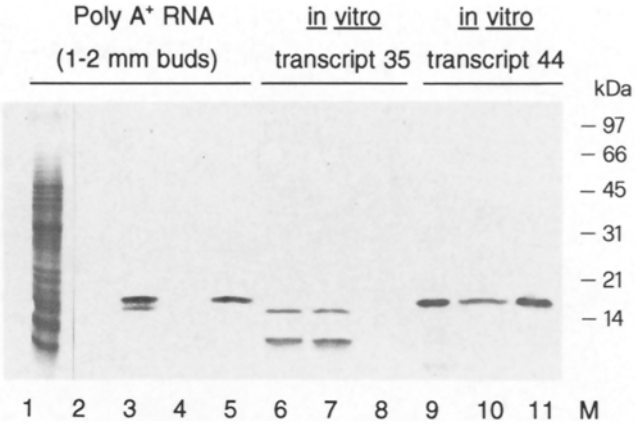


Fig. 4. Immunoprecipitation of proteins *Sa*TAP35 and *Sa*TAP44. Total in-vitro translation products of poly(A)-containing RNA from 1- to 2-mm flower buds *(lane 1)* were immunoprecipitated with serum directed against MBP-TAP35 *(lane 3)* and the corresponding preimmune serum *(lane 2)* or with serum directed against MBP-TAP44 *(lane 5)* and the corresponding preimmune serum *(lane 4)*. In-vitro translation products from in-vitro-transcribed cDNA *Sa*tap35 *(lanes 6–8)* or *Sa*tap44 *(lanes 9–11)* were immunoprecipitated with antiserum against MBP-TAP35 *(lanes 7, 10)* or antiserum against MBP-TAP44 *(lanes 8, 11)*. The positions and sizes of the molecular-mass markers (*M*) are indicated

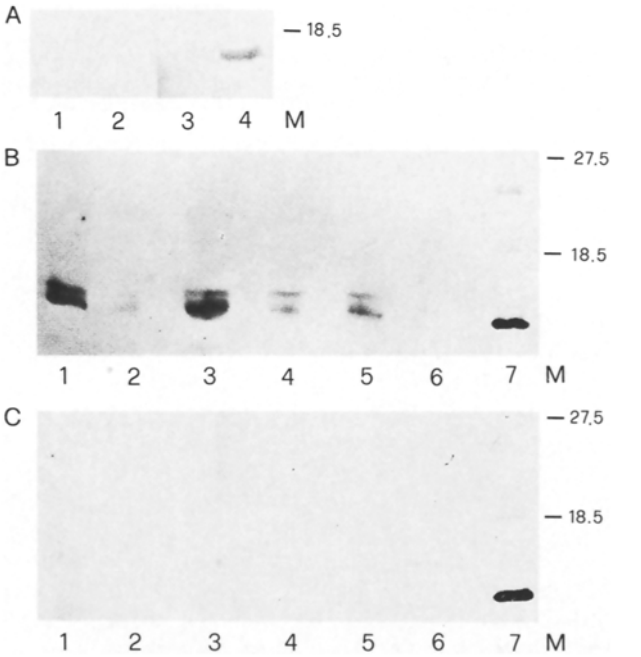
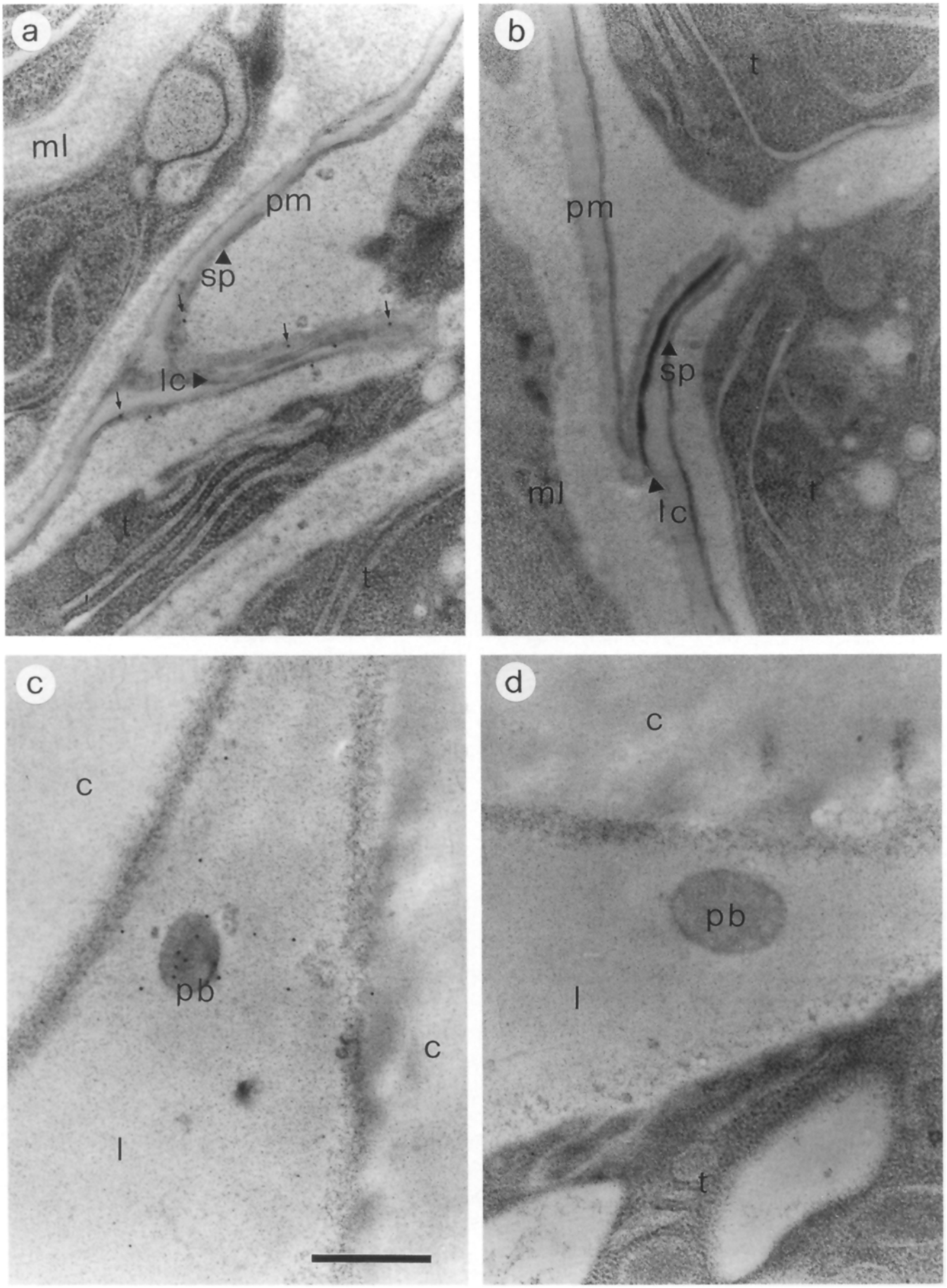


Fig. 5A–C. Immunoblot analysis of protein *Sa*TAP44. **A** The blot of a 13% SDS-polyacrylamide gel was probed with antiserum raised against MBP-TAP44 or the corresponding preimmune serum and developed with alkaline-phosphatase-conjugated secondary antibody. *Lanes 1* and *3* contain protein extracts from 2-mm anthers; *lanes 2* and *4* contain protein extracts from 1.5 mm anthers. **B, C** Protein gel blots of anther extracts from 1.5-mm buds (**B**) or from 2- to 3-mm buds (**C**) were probed with the antiserum against MBP-TAP44. *Lane 1*, phenol/formic acid extract; *lane 2*, native extract; *lane 3*, SDS extract; *lane 4*, $CaCl_2$ extract; *lane 5*, acidic extract; *lane 6*: alkaline extract. *Lane 7* contains purified bacterially expressed MBP-TAP44 cleaved with protease Factor Xa. ^{35}S-labelled secondary antibody was used. The positions and sizes of the molecular-weight markers (*M*) are given in kDa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in the immunoprecipitation (Fig. 4, lane 8) these different bands might correspond to the products of the family of *Sa*tap44 genes present in the *S. alba* genome. The fact that the serum against MBP-TAP44 immunoprecipitates *Sa*TAP44 as well as two additional proteins of low abundance (seen only on longer exposures as in Fig. 4) from flower bud RNA translation products supports this hypothesis. Alternatively, the protein bands could represent modified forms of *Sa*TAP44. No immunoreactive band was detected in anther extracts from 2-mm buds (Fig. 5A, lane 3). Under the assumption that the proteins might have become modified in a way that they cannot be released by boiling homogenized anthers in SDS-containing buffer, we tested the efficiency of various buffers to extract immunodetectable protein. Figure 5B shows that under native conditions (lane 2) almost no *Sa*TAP44 protein was released from anthers of 1.5-mm buds. A small amount of solubilized *Sa*TAP44 protein was recovered by extraction under acidic conditions (lane 5) or with high salt (lane 4). Incubation of anthers with a mixture of phenol and formic acid (lane 1) was almost as efficient as boiling in SDS-containing buffer (lane 3). None of these buffer systems, however, was effective in recovering significant amounts of *Sa*TAP35 or *Sa*TAP44 from anthers of 2- to 3-mm buds (shown for *Sa*TAP44 in Fig. 5C). Bacterially expressed *Sa*TAP44 released from the purified MBP-TAP44 fusion protein by Factor Xa cleavage was loaded in lane 7.

Immunoelectron-microscopical localization of SaTAP35 and SaTAP44 within the anther. To determine the subcellular localization of *Sa*TAP35 and *Sa*TAP44, anthers were subjected to cryofixation and freeze-substitution in acetone and subsequently embedded in LR Gold resin. Ultrathin sections were incubated with the affinity-purified antibodies against MBP-TAP35 and MBP-TAP44 and the corresponding affinity-purified preimmune sera. Gold deposition was detected in a developmentally regulated manner. During the tetrad stage when microspores are still encaged in the callose wall, specific labelling of the precursor of the peritapetal membrane was observed with both the serum against MBP-TAP35 (Fig. 6a) and MBP-TAP44 (data not shown). No background label was seen on control sections treated with the preimmune serum (Fig. 6b). The peritapetal membrane marks the boundary between the tapetum and the middle layers of the anther wall and consists of a lipoid layer covered with sporopollenin precursors (Heslop-Harrison 1969; Dickinson 1970; Dickinson and Bell 1972). Additionally, gold deposition was found within the locule, particularly associated with pro-Ubisch-body-like globules (Fig. 6c). Again, the preimmune serum was inactive (Fig. 6d). Pro-Ubisch bodies are thought to arise in the tapetum. After their extrusion into the anther cavity they become covered with sporopollenin precursors that are secreted from the tapetum. No staining by the antibodies was detectable within the tetrad or the callose wall.

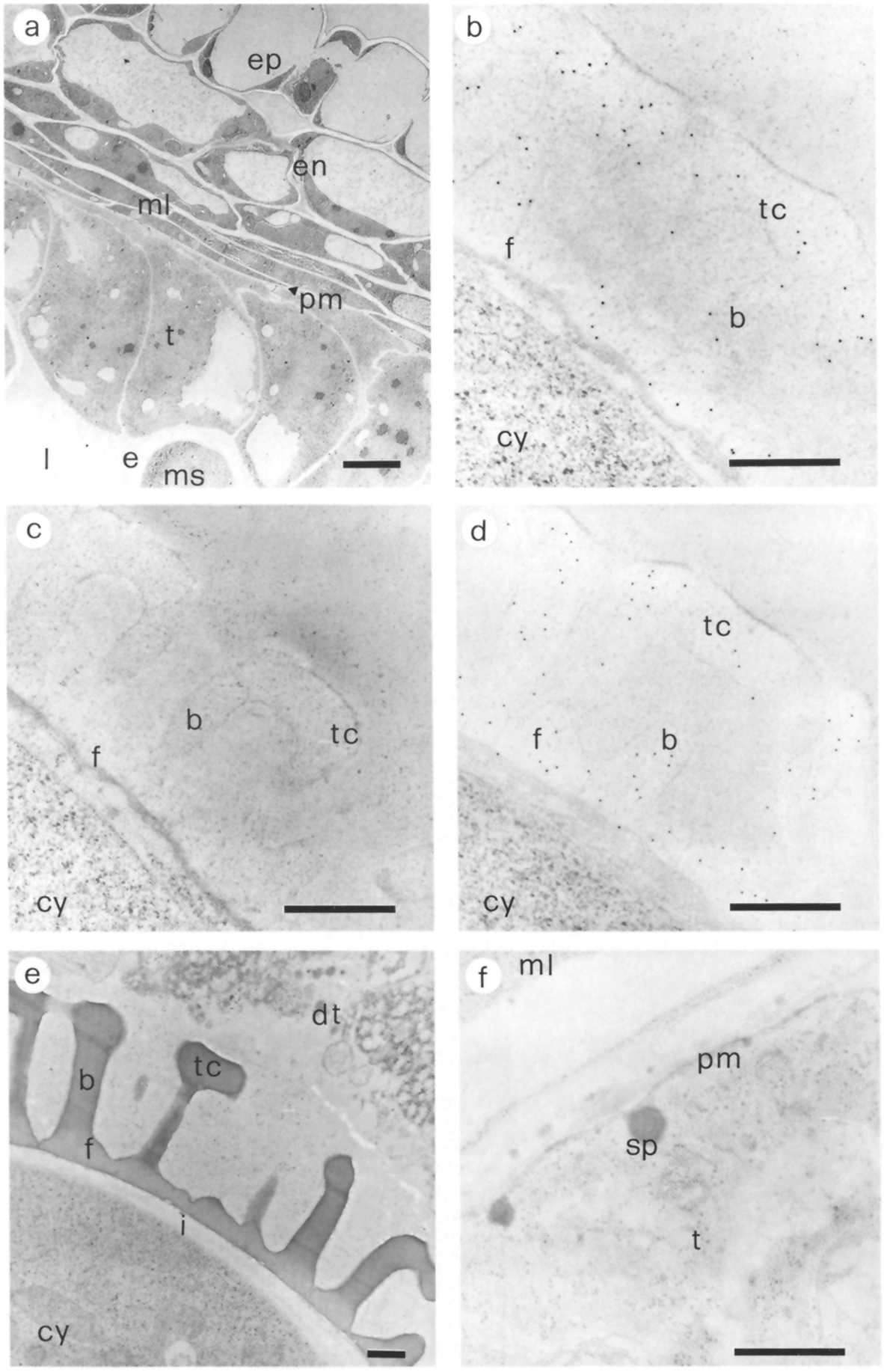
Figure 7a shows a cross-section of an anther at the early vacuolate stage of microspore development illustrating the wall layers and the microspores after release from the callose wall has been completed. At this stage the precursor of the peritapetal membrane was further elaborated and labelling was greatly diminished (data not shown). Details of a microspore surface are shown in Fig. 7b. The serum against MBP-TAP35 specifically labels the exine. Preincubation of the serum with purified fusion protein MBP-TAP35 greatly reduced the labelling of the exine, indicating that the protein *Sa*TAP35 is largely responsible for the signal (data not shown). No gold deposition was observed on sections treated with the preimmune serum (Fig. 7c). Specific labelling of the exine was also detected with the antibody against MBP-TAP44 (Fig. 7d). Figure 7e shows a section of a microspore from 3-mm buds incubated with the serum against MBP-TAP35. At this stage the baculae of the exine are greatly enlarged (see the different scale bars in Fig. 7b and 7e, respectively) and exine polymerization is more advanced, as can be seen by the different staining properties compared to the young exine (see Fig. 7b). The antibodies displayed no reaction in this cross-section. Figure 7f shows the mature peritapetal membrane in anthers from 4-mm buds with large depositions which now exhibit the same staining properties as the adult exine and therefore presumably represent sporopollenin. At no time was specific labelling of the microspore cytoplasm by the antibodies observed.

Fig. 6a–d. Immunocytochemical localization of *Sa*TAP35 and *Sa*TAP44 in anthers of *Sinapis alba* at the tetrad stage of microspore development. **a, b** The precursor of the peritapetal membrane (*pm*) surrounds the tapetum (*t*). *ml*, middle layer; *sp*, sporopollenin; *lc*, lipoid core. **c, d**, The locule (*l*) containing globules resembling pro-Ubisch bodies (*pb*) can be seen in the vicinity of the callose wall (*c*) of the microspores. Sections were treated with affinity-purified serum against MPB-TAP35 (**a, c**) or the corresponding preimmune serum (**b, d**). *Arrows* in **a** mark the gold-labelled antibodies. ×45000; bar = 0.5 μm

Discussion

In this manuscript we describe two proteins encoded by tapetum-specific transcripts. These proteins are localized in the exine during early microspore development. The cDNAs *Sa*tap35 and *Sa*tap44 were originally identified by differentially screening cDNAs of flowering and vegetative mustard apices (Majewski 1990; Melzer et al. 1990) and correspond to transcripts expressed transiently in the tapetal cell layer of the anther (Staiger and Apel 1993).

Based on histological and ultrastructural investigations of the tapetum, which is enriched for rough endoplasmic reticulum and dictyosome-derived vesicles (Murgia et al. 1991) it has been suggested that the tapetum primarily fulfills secretory functions. The finding that a large proportion of tapetum-specific proteins identified to date carry hydrophobic N-terminal signal sequences has advanced the hypothesis that these proteins are released into the locule either directly or following storage in the tapetal vacuoles (Koltunow et al. 1990; Nacken et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al. 1991; Paul et al. 1992). Data on the subcellular localization are not yet available in these cases.

The amino termini of the deduced amino acid sequences for *Sa*TAP35 and *Sa*TAP44 contain some characteristics of signal sequences as well. Indeed, we could demonstrate processing of in-vitro translation products by canine pancreatic microsomes, suggesting a cotranslational insertion of the precursor proteins into the endoplasmic reticulum.

Using affinity-purified antibodies directed against MBP-TAP35 and MBP-TAP44, immunogold labelling was detected in the anther cavity in association with structures made of sporopollenin and/or sporopollenin precursor substances. Thus, during the tetrad stage of microspores, labelling was localized on the forming peritapetal membrane and on globules resembling pro-Ubisch bodies. After the release of the microspores labelling was found on the exine. These results show that the *Sa*TAP35 and *Sa*TAP44 proteins are indeed secreted from the tapetum into the locule. To our knowledge, this is the first demonstration that proteins synthesized in the tapetum are transferred to the microspore surface in an apparently temporally regulated manner prior to dissolution of the tapetum.

The typical exine consists of three distinct layers. The outermost layer is designated the tectum. Between the interior layer, the foot layer, and the tectum, rod-like connections are found which are known as the baculae (Heslop-Harrison 1971). The exine is composed largely of sporopollenin, a polymer noted for its resistance to chemical solvents or hydrolases (Southworth 1990). At present it is assumed that sporopollenin is a polymer built of long-chain aliphatic substances and varying degrees of phenolic compounds (Schulze-Osthoff and Wiermann 1987; Guilford et al. 1988; Wiermann and Gubatz 1992). It is assumed that the tapetum secretes into the locule sporopollenin precursors which subsequently polymerize on the surface of the forming exine. During microspore maturation, further chemical changes occur whereby the sporopollenin initially susceptible to acetolysis becomes acetolysis-resistant (Southworth 1990). On the electronmicrographs it is obvious that the staining properties of the exine change during this "hardening" process (see Fig. 7B, E). It is not known how proteins are associated with the exine. They could either be attracted to the surface of sporopollenin by charge or hydrophobic interactions or they could be embedded within or even be covalently attached to the sporopollenin. Our finding that denaturing solvents, such as SDS or phenol/formic acid, are required to release the *Sa*TAP35 and *Sa*TAP44 proteins from anthers suggests their tight association with the exine.

A clue as to the function of *Sa*TAP35 and *Sa*TAP44 may derive from their developmental expression: after dissolution of the callose wall encaging the microspore tetrads, further sporopollenin accretion onto the surface of the microspores takes place. Proteins associated with the exine at this early stage may serve a function related to sporopollenin deposition. The specific labelling of the forming peritapetal membrane and of the presumed pro-Ubisch bodies points in this same direction. The peritapetal membrane surrounds the tapetum and encloses the locule with the developing microspores like a sac (Heslop-Harrison 1969). It consists of an outer lipoid layer upon which by-products of the tapetal sporopollenin synthesis condense (Dickinson 1970; Dickinson and Bell 1972). The pro-Ubisch bodies are constructed according to the same principle: sporopollenin is deposited upon a lipoidal core (Echlin and Godwin 1968a; Dickinson and Bell 1972). Their function is as yet not understood. It has been hypothesized that they might represent by-products of tapetal activity necessary for synthesis of the peritapetal membrane (Dickinson and Bell 1972). An earlier view that Ubisch bodies might serve as a transport mechanism for sporopollenin precursors from the tapetum to the microspore surface (Echlin and Godwin 1968a, b) has been discarded (Heslop-Harrison and Dickinson 1969) because this would require that sporopollenin polymerized onto the globules becomes remobilized for the incorporation into the exine. Notably, both the exine and the peritapetal membrane are not labelled further by the antibodies against MBP-TAP35 and MBP-TAP44 after sporopollenin has undergone additional polymerization, a process not yet fully understood. Furthermore, it is assumed that the first sporopollenin depositions on the microspore surface derive from precursors formed within the microspore cytoplasm as long as the microspores are still encaged in the callose wall (Echlin and Godwin 1968b). Since we did not detect specific staining of the microspore cytoplasm, *Sa*TAP35 and *Sa*TAP44 might only be involved in sporopollenin deposition from tapetal precursors.

A prerequisite for the immunoelectron-microscopical localization of the proteins within the locule was the development of an improved method for freeze-substitution and low-temperature embedding in LR Gold resin after high-pressure freezing of whole anthers (data not shown). Conservation of the ultrastructure was found unsatisfactory after embedding in Lowicryl, a resin commonly used for immunocytochemistry (Humbel et al. 1983). High-pressure freezing and freeze-substitution followed by embedding in epoxy resin, in which immunological reactions with our antibodies were not possible (data not shown), has previously been

Fig. 7a–f. Immunocytochemical localization of *Sa*TAP35 and *Sa*TAP44 in the exine. **a** Cross-section of an anther from 2-mm buds (early vacuolate microspore stage) showing the epidermis (*ep*), endothecium (*en*), middle layers (*ml*), tapetum (*t*) and the locule (*l*) containing microspores (*ms*) with the exine outer layer (*e*). *pm*, precursor of the peritapetal membrane. × 2250; bar = 5 μm. **b–d** Details of microspore wall showing parts of the microspore cytoplasm (*cy*) and the forming exine. *tc*, tectum; *b*, bacula; *f*, footlayer. Sections were treated with affinity-purified antiserum against MBP-TAP35 (**b**), the corresponding affinity-purified preimmune serum (**c**) and the affinity-purified serum against MBP-TAP44 (**d**). × 45000; bar = 0.5 μm. **e** Cross-section of an anther from 3-mm buds incubated with affinity-purified antiserum against MBP-TAP35. *dt*, tapetum beginning to degenerate; *i*, intine. × 15000; bar = 0.5 μm. **f** Cross-section of an anther from 4-mm buds showing the mature peritapetal membrane (*pm*) with sporopollenin depositions (*sp*). *ml*, middle layer. × 45000; bar = 0.5 μm

shown to be suitable for ultrastructural investigation on intine development, the inner cell wall layer of pollen in *Ledebouria socialis* (Hess 1993).

The expression of the *Sa*tap35 and *Sa*tap44 cDNAs begins as soon as the tapetum has been established, is maximal in 1.5-mm buds (tetrad stage to release of microspores) and ceases in 2- to 2.5-mm buds (free microspore stage to early vacuolate microspore stage) before dissolution of the tapetal cells (Staiger and Apel 1993). With this expression pattern, *Sa*tap35 and *Sa*tap44 belong to the earliest tapetum-specific transcripts known to date (Koltunow et al. 1990; Smith et al. 1990; Scott et al. 1991).

The *Sa*TAP35 and *Sa*TAP44 proteins were detected by protein gel blots in anther extracts from 1.5-mm buds but not from 2- to 3-mm buds. Similarly, the immunogold label in the exine decreased in sections from 3-mm buds in which exine formation has been largely completed, suggesting that the proteins are short-lived. Alternatively, the proteins might become incorporated into the exine in such a way that they cannot be released from 2- to 3-mm buds by conventional solvents and/or that they escape detection by the antibodies.

Previously, allergenic proteins in rye grass pollen have been detected in the exine (Staff et al. 1990). These allergens behave differently in a number of aspects as compared to *Sa*TAP35 and *Sa*TAP44: the allergens are not only detected during early microsporogenesis but are also still present in mature pollen grains. The soluble pollen allergens are extracted in water within minutes. In contrast, denaturing agents are required to liberate *Sa*TAP35 and *Sa*TAP44. The allergen mRNA is expressed within the pollen, i.e. it represents a product of haploid gene expression. Consequently, allergens are immunologically detectable in the pollen cytoplasm and are obviously secreted from the cytoplasm during pollen development. Exine-associated proteins have also been isolated by SDS-extraction from highly purified maize pollen exines and found to constitute about 0.2% of total mature pollen protein (Chay et al. 1992). A subset of these proteins could be recovered from exines of developing microspores, indicating that the profile of exine-associated proteins changes during microspore development.

Conclusion. As the expression of *Sa*tap35 and *Sa*tap44 cDNAs is spatially restricted to the tapetal cell layer and temporally limited to early tapetum development, the *Sa*TAP35 and *Sa*TAP proteins are unlikely to perform functions that are essential for any vegetative tissue or common to different reproductive organs. On the basis of their localization within the anther it is tempting to speculate that the proteins are involved in a unique aspect of tapetum cell function, namely the elaboration of the pollen wall, a male-specific structure. An antisense-RNA approach to block the synthesis in the tapetum of either or both of the two proteins *Sa*TAP35 and *Sa*TAP44 may provide information about the function of the two gene products.

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