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Specific mitochondrial proteins in pollen: Presence of an additional ATP synthase β subunit

(male gametogenesis/pollen mitochondria isolation/two-dimensional protein electrophoresis/*in organello* protein synthesis/protein isoforms)

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ABSTRACT **A protocol was designed to obtain a pure fraction of pollen mitochondria from the diploid species *Nicotiana sylvestris*, the female parent of the allotetraploid *Nicotiana tabacum*. Most organelles were morphologically intact and able to perform *in organello* mitochondrial (mt) protein synthesis. As revealed by two-dimensional protein electrophoresis, numerous quantitative differences exist between leaf and pollen mt proteins. Moreover, additional mt polypeptides, named R (for reproductive), encoded by either nuclear or mitochondrial genes, are found in pollen. The most abundant R polypeptide, R1 (M_r 53,000, pI 5.6), is nuclearly encoded, is membrane bound, and cross-reacts with an antibody directed against the β subunit of the mt ATP synthase (ATPase). N-terminal microsequence analysis showed that the two ATPase β subunits present in leaves (β1 and β2) and the R1 pollen-specific subunit are encoded by distinct genes. A similar additional ATPase β subunit was observed in pollen mitochondria from *Petunia*, suggesting that this polypeptide is of general importance for male gametophytic development in Solanaceaes.**

In higher plants, the mature male gametophyte consists of two or three cells, depending on the species, derived from the haploid tetrad cells issued from meiosis. Tetrad cells differentiate into free microspores that undergo an asymmetric division called the first pollen mitosis. The resulting binucleate pollen grain is composed of two highly differentiated cells, the large vegetative cell, which assumes most of the metabolism, and the generative cell, which forms the two sperm cells by the so-called second pollen mitosis, either before or after pollen tube germination.

During this short but complex process, mitochondria play an essential role, as indicated by cytological, physiological, and genetic evidence. First, mitochondria are much more abundant in anther cells and pollen than in other types of plant cells (1). This increase may be related to the high energy requirement of microgametogenesis. In early stages, the inner anther tissue (tapetum) serves as the source of nutrients for meiotic cells and young microspores (2). The tapetum degenerates from the stage of microspore vacuolization and exine (the outer pollen wall) synthesis. As pollen is nonphotosynthetic, containing only undifferentiated plastids and amyloplasts, its ATP has to be supplied solely by mitochondria. In such a demanding process, a small amount of mitochondrial (mt) dysfunction could be limiting, and this may explain why cytoplasmic male sterility (cms) is the most common phenotype due to mt DNA mutation (3). In most cms, vegetative growth is normal, and sterility is caused by abnormalities occurring at different stages of anther and pollen development. For example, in maize cms T (4) and in *Petunia* (5), the tapetum degenerates precociously, and expression of cms-specific mt polypeptides is higher in reproductive somatic organs than in vegetative ones (6–8). In other cases, such as in maize cms S, anther development seems unaffected; microspores are formed but are unable to develop into functional pollen (9). In such cases, it would be of interest to analyze the molecular features of sterility in the pollen itself. However, to our knowledge, no characterization of plant mt proteins from pollen has been reported. Mitochondria are semiautonomous organelles, the genome of which encodes only a small number of the several hundred mt polypeptides. The nuclearly encoded polypeptides are imported from the cytosol (10). In plants, the mt genome is larger than in other organisms (11). In addition to some subunits of enzymes of the oxidative phosphorylation pathway, mt DNA encodes ribosomal proteins, maturases, and numerous still unidentified polypeptides.

Here we describe the isolation of pollen mitochondria from *Nicotiana sylvestris*, the diploid female parent of the cultivated *Nicotiana tabacum*; both species have a similar mt genome (12). In *N. sylvestris*, microsporogenesis is closely related to floral ontogenesis and is highly synchronous in all five anthers of the same flower, so that microspores and pollen at a precise stage of development can be collected (13). Several changes in both nuclearly or mitochondrially encoded mt proteins occur during microspore differentiation and are maintained up to the mature pollen stage. One of the most striking differences consists of the presence of an additional ATP synthase (ATPase) β subunit. N-terminal microsequencing showed that the pollen-specific mt ATPase β subunit is encoded by a different gene. A similar subunit was observed in *Petunia* pollen, suggesting it could have an essential role in microsporogenesis of Solanaceaes.

MATERIALS AND METHODS

Isolation of Pollen Mitochondria. Anthers from 100 to 300 flowers, according to the stage, were collected at 4°C in 0.4 M sucrose, 0.01 M KH_2PO_4 (pH 7.6), 0.001 M EGTA, 2% bovine serum albumin, 2-mercaptoethanol at 2 ml/liter, and phenylmethylsulfonyl fluoride at 0.2 g/liter. Depending on the anther stage, two different protocols were used for subsequent isolation of mitochondria:

(*i*) After the exine has formed, microspores need to be completely separated from somatic cells in order to be broken and to obtain reasonable amounts of mitochondria. Preparations of microspores and pollen devoid of anther cells were obtained as described (13), with a few modifications. Anthers were disrupted in a Potter grinder, and the solution was filtered under low vacuum through a 100-μm nylon net, which

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Abbreviations: mt, mitochondrial; cms, cytoplasmic male sterility; 2D, two-dimensional.

retained the anthers. Pollen grains were recovered on a 20-μm nylon net by a second filtration and crushed rapidly with mortar and pestle without buffer addition. After sufficient crushing, as judged by microscopic examination (about 90% broken pollen), 50 ml of isolation buffer was added, and debris, nuclei, and proplasts were pelleted at 1500 × *g* for 5 min. Mitochondria were then pelleted at 12,000 × *g* for 10 min and further purified on a three-layer Percoll gradient [13%, 22%, and 45% (wt/vol) Percoll] (14).

(*ii*) Young microspores issued from meiosis are too small to be efficiently sorted out from somatic cells; however, as they are not yet surrounded by a resistant wall, they can easily be broken within anthers. Whole anthers were crushed in 50 ml of isolation buffer, and mitochondria were isolated as described above.

Electron Microscopy. Gametophytic cells and mitochondrial pellets were fixed for 2–6 hr (depending on the stage) in 2% glutaraldehyde/0.05 M sucrose/0.05 M phosphate buffer, pH 7, postfixed in 1.5% osmium tetroxide in the same buffer for 90 min at 0°C, preembedded in fibrinogen, dehydrated through a graded series of alcohol and propylene oxide, and finally embedded in epon. Organelle volumes were estimated by using the formula for the volume of a prolate ellipsoid, $V = 4/3\ \pi ab^2$, where a is the major semiaxis and b is the minor semiaxis.

Purification of Membrane and Soluble mt Protein Fractions. The matrix and membrane fractions were obtained by osmotic shock followed by three freeze–thaw cycles in 50 mM Tris·HCl, pH 7.5/1 mM octyl glucopyranoside and centrifugation at 80,000 × *g* for 30 min. The supernatant was referred to as the soluble matrix fraction, and the pellet was referred to as the membrane fraction (15).

Two-Dimensional (2D) Gel Electrophoresis. First (isoelectric focusing)- and second (SDS/PAGE)-dimension electrophoresis were performed as described by O'Farrell (16), with a few modifications concerning essentially the solubilization procedure (15, 17). mt proteins were delipidated in 80% (vol/vol) ice-cold acetone, dried, and solubilized in 30 μl of a solution containing 0.05 g of SDS, 0.5 ml of 2-mercaptoethanol, and 2.85 g of urea in a final volume of 5 ml at pH 7.4. Ten microliters of a solution containing 1 ml of 10% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) plus 0.1 ml of carrier ampholytes (LKB; pH 5–8)) were added, followed by 40 μl of a solution made of 10% CHAPS, 0.1 ml of carrier ampholytes, and 2.85 g of urea in a 5-ml final volume. Twenty to 50 μl of the sample was loaded on isoelectric focusing rod gels, 10 cm long and 3 mm in diameter, and run at 500 V for 15 hr. For a final volume of 10 ml, the IEF mixture was made of 5.5 g of urea, 2.2 ml of distilled water, 2 ml of CHAPS, 1.6 ml of a 26% acrylamide solution (25 g of acrylamide plus 1 g of *N,N'*-methylenebisacrylamide), 0.4 ml of ampholytes (pH 5–8), 20 μl of ammonium persulfate, and 15 μl of *N,N,N',N'*-tetramethylethylenediamine. Rod gels were submitted to second-dimension analysis on 12% acrylamide slab gels, 100 × 100 × 1 mm, in Tris/glycine buffer (25 mM Tris/192 mM glycine) at 130 V for 5 hr. Gels were stained with 0.1% Coomassie brilliant blue R-250 or by using a silver nitrate procedure (18).

***In Organello* Protein Synthesis.** Mitochondria were isolated aseptically from the pollen of 250 flowers or 2 g of young green leaves. Plant material was sterilized with 2% calcium hypochlorite for 5 min. *In organello* protein synthesis was performed according to ref. 14, in an incubation medium containing 50 μCi (1 Ci = 37 GBq) of [^{35}S]methionine and 40 μg of erythromycin, a potent inhibitor of bacterial and plastid translation (7). mt proteins were subjected to 2D electrophoresis as described above. After Coomassie blue staining, the gels were dried and exposed to Amersham Hyperfilm-β_{max} x-ray film.

Western Blotting. After 2D electrophoresis, proteins were transferred onto nitrocellulose membranes in Tris/glycine buffer and 20% (vol/vol) methanol at 150 V for 1 hr in a mini-V 8·10 BRL electrotransfer apparatus. Immunoblotting was performed according to Towbin *et al.* (19), with an antiserum directed to the β subunit of *Vicia faba* ATP synthase, kindly provided by M. Boutry (University of Louvain, Louvain la Neuve, Belgium).

Protein Microsequence Determination. Spots corresponding to the analyzed polypeptide were cut out from dried Coomassie blue gels (6–10 gels), pooled, rehydrated in distilled water, and submitted to either N-terminal or internal sequence determination after digestion by endoprotease Lys-C.

N-Terminal Sequences. Pooled gel pieces were put in the well of a 10% acrylamide gel, overlaid with Laemmli buffer (20) for 1 hr, with several changes of the buffer until pH 8 was reached, and then submitted to one-dimensional electrophoresis. Proteins were transferred to Trans-Blot nylon membranes in borate buffer (50 mM borate/50 mM Tris) at 35 V overnight. Membranes were briefly (2–5 sec) immersed in 0.1% amido black/45% methanol/7% acetic acid and extensively rinsed in distilled water. Colored spots were excised, and sequence determination carried out in an Applied Biosystems 470 gas-phase peptide sequencer. Phenylthiohydantoin amino acids were detected with an on-line 120A analyzer.

Internal Sequences. Polypeptides were digested according to the method of Kawasaki *et al.* (21), with the following modifications. Gel pieces were incubated with endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer Mannheim) (2 μg/ml in 0.1 M Tris·HCl, pH 9/0.06% SDS) at 37°C for 20 hr. Digested peptides were separated by reverse-phase HPLC on a double column line [DEAE Aquapore AX300 (Applied Biosystems) plus C_{18} (Vydac)]. Microsequencing of HPLC fractions of interest was carried out as described for N-terminal sequences.

RESULTS

Highly Pure Preparations of Mitochondria Were Obtained from *N. sylvestris* Pollen. In *N. sylvestris*, the first pollen mitosis takes place about 2 days after meiosis, and gametophytic maturation, up to the release of mature pollen at anthesis, covers an additional period of about 8 days (13). During the whole process, microsporogenesis is highly synchronous in all five anthers of a single flower (each anther contains ≈70,000 pollen grains) and correlates with precise configurations of other floral parts. This allows the collection of gametophytes at well-defined developmental stages. Tetrad cells, microspores, and binucleated pollen were examined by electron microscopy. Fig. 1 shows the characteristic aspect of microspore cytoplasm, just before the first pollen mitosis (Fig. 1*a*), and of a pollen grain 1 day before anthesis (Fig. 1*b*). As already observed in maize (1), mitochondria are much more abundant in gametophytic cells than in other cell types, and their volume is unusually reduced, around 0.01 μm^3, due to their rapid division in pollen mother cells (1, 22). mt volume increases markedly after first pollen mitosis, reaching about 0.06 μm^3 in mature pollen. The scarce plastids are significantly larger, ≈1.5 μm^3, and are undifferentiated.

mt fractions, recovered at the 22%/45% interface of Percoll gradients, were examined by electron microscopy (Fig. 1*c*). They did not contain significant amounts of contaminating plastids. Most of the mitochondria appeared morphologically intact, with outer double limiting membrane and internal cristae.

Numerous Differences Distinguish Pollen from Leaf mt Proteins. Peptide compositions of mitochondria isolated from leaf, anthers, and male gametophytes at different stages were

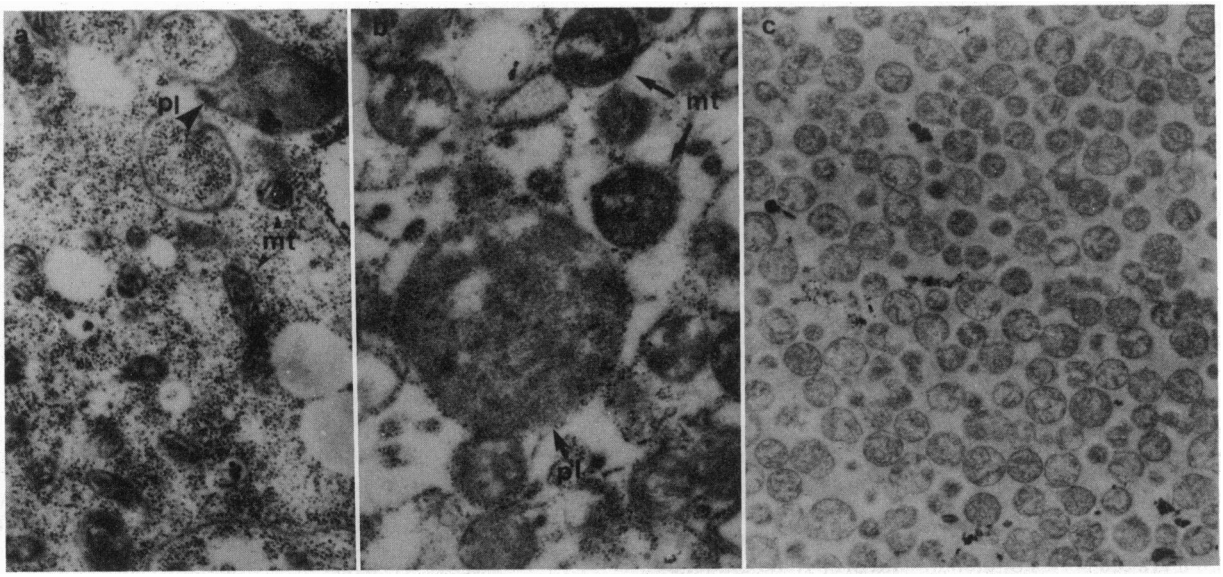

FIG. 1. Electron micrographs of *N. sylvestris* mitochondria *in situ* and after purification. (*a*) Microspore before first pollen mitosis. (×27,000.) (*b*) Binucleate pollen 1 day before anthesis. (×27,000.) (*c*) Mitochondria isolated from pollen taken 1 or 2 days before anthesis (mature pollen). (×13,500.) mt, Mitochondria; pl, plastids.

compared by 2D electrophoresis. Gels were stained either by Coomassie blue or by the more sensitive silver nitrate procedure when necessary (i.e., for tetrads and microspores).

On leaf gels, about 80 spots, called L, were routinely detected by Coomassie blue staining (Fig. 2*a*), and more than 200 were detected by silver staining (data not shown). The molecular weight and isoelectric point of the two major spots designated A (M_r 58,000, pI 6.2) and B (M_r 53,000, pI 5.3) were similar to those described in maize (23), *V. faba* (24), and potato (15) for, respectively, the α (mitochondrially encoded) and β (nuclearly encoded) subunits of the mt ATP synthase (ATPase) complex. As already observed in the other species, the β spot was composed of two overlapping polypeptide spots, which have been called β1 (left) and β2 (right).

On binucleate pollen gels (Fig. 2*b*), the relative intensity of several L spots, such as L1 and L2, decreased, while additional polypeptides, not detectable on leaf profiles and called R for reproductive, could be observed. The major new spots were R1 (M_r 53,000, pI 5.6), R2 (M_r 58,000, pI 6.4), and R3 (M_r 38,000, pI 6.8). Several other R polypeptides of lower abundance were clearly visible on silver nitrate-stained gels. On anther gels, only traces of R1, R2, and R3 could sometimes be detected. Microscopic examination revealed that anther preparations contain broken pollen grains; these traces may be due to contamination by pollen mitochondria.

To demonstrate the mt origin of R polypeptides, it is necessary to rule out the possibility of contamination of pollen mt preparations by other organelles. Although in pollen the most common organelles are mitochondria, and electron micrographs of pollen mt fractions did not reveal the presence of intact plastids or peroxysomes (Fig. 1), a low level of contamination could not be discounted, and several experiments were carried out to test this possibility. Pollen fractions corresponding to amyloplasts and peroxysomes were purified as described in ref. 25 and analyzed by 2D electrophoresis. None of the spots obtained from these fractions corresponded to any of the pollen mt-specific spots (data not shown). The same test was done for purified chloroplastic membranes from *N. sylvestris* leaves and peroxysomes isolated from potato tubers. Taken together, the control experiments strongly suggest that the presence of R polypeptides is not the result of contamination of pollen mitochondria by other organelles or debris.

The time course of the appearance of R polypeptides during male gametogenesis was determined. As could be expected from the smaller size of their mitochondria, mt protein yields from tetrads or microspores were much lower than from binucleate pollen. Just after meiosis, because tetrad cells could not be sorted out from somatic cells, mitochondria were purified from whole anthers. At this stage, R2 and R3 could only be detected as traces. The R1 spot was first detected later in gametophytic development, in vacuolated microspores just before first pollen mitosis.

R Polypeptides Are Found in Either Membrane or Soluble mt Fractions and Most Are Nuclearly Encoded. Soluble and insoluble fractions of mt proteins from pollen were prepared as described in *Materials and Methods*. Most polypeptides were recovered in the soluble fraction—namely, R2 and R3 (Fig. 2*d*). In contrast, R1 was recovered mainly in the membrane fraction, together with the α and β subunits of the mt ATPase (Fig. 2*c*).

Leaf and pollen mt proteins synthesized *in organello* were compared by 2D electrophoresis followed by autoradiography (Fig. 2 *e* and *f*). Both leaf and pollen autoradiographs showed a major spot corresponding to the α subunit of mt ATPase, known to be mitochondrially encoded in higher plants (23, 24). In addition, about 40 labeled spots were present; some of them varied quantitatively between leaves and pollen (arrows). On leaf patterns, none of these labeled spots could be superimposed on Coomassie blue-stained spots. In contrast, several of the pollen autoradiographic spots corresponded to Coomassie blue-stained polypeptides, suggesting that they are encoded by the mt genome. However, R1, R2, and R3 did not correspond to any autoradiographic spot and are probably encoded by the nuclear genome.

The R1 Polypeptide Is a mt ATPase β Subunit. R1 was identified as an additional ATPase β subunit by using an antiserum directed against the *V. faba* mt ATPase β subunit (Fig. 3 *a* and *b*). We previously checked that this antiserum recognized specifically the β subunit of the *N. sylvestris* mt ATPase and not its chloroplastic counterpart. To characterize the nature of R1—either modification of one of the mt ATPase β subunits, β1 or β2, present in leaf mitochondria or additional form encoded by a different nuclear DNA sequence—protein microsequence analyses were performed. Table 1 gives all three N-terminal sequences and compares

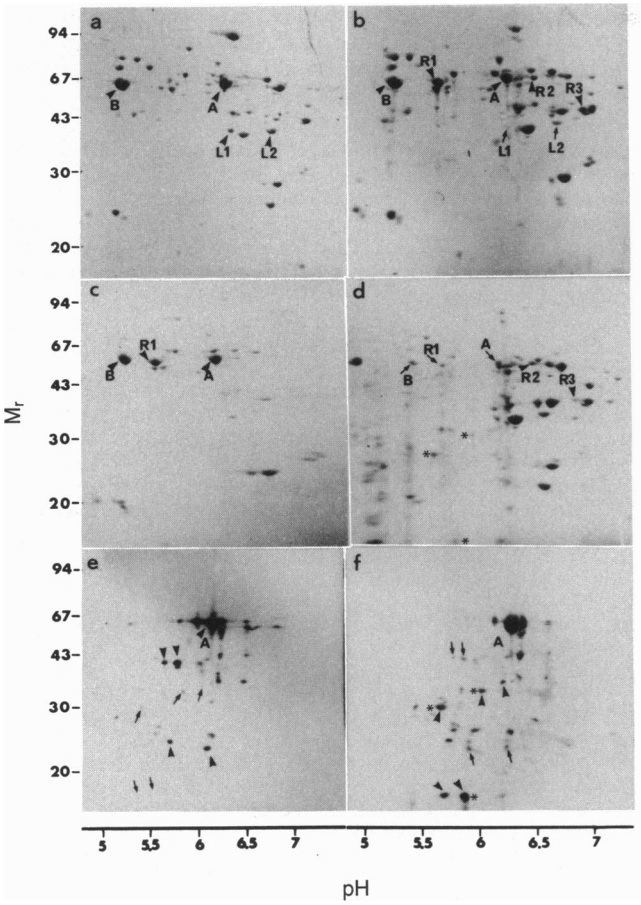

FIG. 2. Two-dimensional protein patterns of mitochondria isolated from leaf and mature pollen. (*a–d*) Coomassie blue-stained gels from leaf (*a*), mature pollen (*b*), pollen membrane fraction (*c*), and pollen soluble fraction (*d*). (*e* and *f*) Autoradiographs of *in organello* mt protein synthesis from leaf (*e*) and mature pollen (*f*). A and B, α and β subunits, respectively, of the mt ATP synthase. The β spot is composed of two close polypeptides, called β1 (left) and β2 (right) in the text. L1 and L2, leaf spots decreased in pollen; R (reproductive), spots only detectable in pollen; arrowheads, appearance or increase; arrows, decrease of the considered polypeptide on autoradiographs; asterisks, autoradiographic spots corresponding to Coomassie-blue stained polypeptides. $M_r \times 10^{-3}$ are given at left.

them with other published plant sequences (26–30). The β1 sequence is nearly identical to that of the related species *N. plumbaginifolia*. The β2 sequence shows several differences, grouped either after the conserved N terminus or just before the consensus sequence (GKITD– – –). After the six conserved N-terminal residues, the R1 sequence diverges from the other β-subunit sequences. The most striking differences

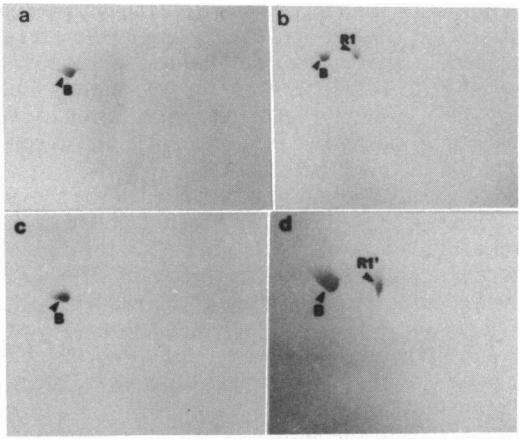

FIG. 3. Western blots of 2D gels of mt proteins from leaf and pollen probed with an antiserum directed against the β ATPase of *V. faba*. (*a*) *N. sylvestris* leaf. (*b*) *N. sylvestris* pollen. (*c*) *P. hybrida* leaf. (*d*) *P. hybrida* pollen. B, β subunit of the mt ATPase; R1 and R1′, additional β subunits in pollen.

are the loss of proline residues and the addition of lysine residues in this region. Analysis of an internal sequence, obtained by endoprotease Lys-C digestion, also reveals the presence of an additional lysine in R1, as compared to the corresponding region of the *N. plumbaginifolia* mt ATPase β subunit (Table 1). Lysine is a positively charged amino acid, and a higher frequency of such a residue would explain why R1 is more basic than β1 and β2. Several additional differences in the core portion of the protein must exist between R1 and the other ATPase β subunits, since their HPLC profiles after Lys-C cleavage are very different (data not shown).

A Similar R1 Polypeptide Is Present in *Petunia hybrida* Pollen. To determine if the presence of an additional form of mt β ATPase in pollen is limited to *N. sylvestris* or can also be observed in other plant species, pollen mitochondria from different *Petunia* varieties were isolated with the same protocol as for *N. sylvestris* and compared to *Petunia* leaf mitochondria. In all cases, an additional spot recognized by the β ATPase antiserum, R1′, was found in *Petunia* pollen (Fig. 3 *c* and *d*).

DISCUSSION

In *N. sylvestris*, it is possible to obtain highly purified fractions of mitochondria from microspores up to the mature binucleate pollen stage. A previous report of mitochondria isolation from pollen concerned only mature pollen at anther dehiscence (31). mt protein yield increases markedly with gametophytic development, in relation to larger mt size (Fig. 1*a*) or to higher metabolic activity. Indeed, pollen *in organello* mt protein syntheses were much more efficient just

Table 1. β-ATPase microsequence comparisons between *N. sylvestris* and other plant species

Plant	Sequence		Ref(s).
	N-terminal	R1 internal	
Maize	ASSAAA–QAAPA–TPPPATGK–TGGG	KVVDL–APYQRG	28, 29
Rice	ATAAAA—KER—PPAPATGKATGGG	KVVDLVAPYQRG	30
Sweet potato	XXXAAA–PAEKP—AAKPAXN–E		27
N. plumbaginifolia	ATSAAA–PASQPSTPPK–SGS–EPSG	KVVDL–APYQRG	26
N. sylvestris β1	ATSAAA–PASQP–TPPKPSGS–EPSG		
N. sylvestris β2	ATSAAA–EKPKA–TPPKPSGN–EITG		
N. sylvestris R1	ATSAAA–KATPA–PQKKTPGS–XIG	KVVDL–APYK	

X, not determined. Dashes indicate gaps in sequence for the best alignment. After the last glycine residue of the N-terminal sequences shown, a consensus sequence (GKITD– – –) found in all plant mt ATPase β subunits determined previously begins. The internal sequence of R1 was obtained after endoprotease Lys-C cleavage.

prior to anthesis. High metabolic activity in mature pollen has been described in maize and *Tradescantia*, where most of the pollen-specific cytoplasmic mRNAs are synthesized after the first pollen mitosis (32). However, other authors found that in maize the maximum level of cytoplasmic protein synthesis (33), as well as the highest expression of some mt genes (34), was reached in young microspores.

In *N. sylvestris*, qualitative differences can be shown by 2D electrophoresis between leaf, anther, and pollen mt proteins. Several additional polypeptides, named R for reproductive, are present in anther and pollen. Traces of R2 and R3 could be observed after tetrad formation, whereas R1 appears later in development, just before first pollen mitosis. According to the classification of Mascarenhas (35), the corresponding R1 gene can be classified as a late gametophytic gene, involved in pollen maturation.

In contrast with the situation in leaf, where only the mt ATPase α subunit can be identified on 2D autoradiographs, in pollen a correspondence between several autoradiographic spots and Coomassie blue-stained spots was established. This interesting result will allow the characterization of mt-encoded proteins preferentially expressed during the reproductive pathway. To our knowledge, differential peptide mt synthesis has only been reported in maize for floral organs, before and after pollinization, as compared to seedling shoots (7). In *N. sylvestris* mitochondria, some of the most striking differences between leaf and pollen concern nuclear-encoded polypeptides, and one of them has been characterized in this paper. Indeed, immunoscreening revealed that, in addition to the two mt ATPase β subunits found in leaves (β1 and β2), the R1 spot is also a mt ATPase β subunit. In maize (23) and potato (15) mitochondria isolated from somatic tissues, two or three close ATPase β spots have been observed, but their exact nature was not determined. Hack and Leaver (23) suggested that the presence of two spots for maize could be artifactual or that they might originate from a modification of the same polypeptide. Here we show, by N-terminal microsequencing, that the three *N. sylvestris* polypeptides are encoded by three distinct nuclear genes. The N terminus of R1 (ATSAAA) is identical to those of mt ATPase β subunits in *N. plumbaginifolia* (26) and in *N. sylvestris* (this paper) and shows no homology with the N terminus of the tobacco chloroplastic ATPase β subunit (MRINPTTS) (36). This demonstrates that the presence of R1 is not due to contamination of pollen mitochondria by other components. In *N. plumbaginifolia*, two different β ATPase genes were cloned (26). Southern hybridizations of a *N. plumbaginifolia* cDNA probe (gift from M. Boutry) to blots of *N. sylvestris* nuclear DNA digested by various restriction enzymes gave a number of signals (three to nine), depending on the enzyme used, which is compatible with the presence of three β ATPase nuclear genes (data not shown). Whether the R1 polypeptide is not synthesized in leaf cells or is not imported into leaf mitochondria is still an open question. Northern hybridizations of pollen and leaf cytoplasmic RNAs using the *N. plumbaginifolia* cDNA as the probe did not give information about this point, because a single signal at about 2.2 kb was detected, suggesting that all three gene transcripts have approximately the same length.

The presence of R polypeptides is not restricted to *N. sylvestris* pollen. Indeed, an additional mt ATPase β subunit was detected in several *Petunia* varieties and should be looked for in other species. Isoforms of respiratory proteins may play an essential role in gametophytic development. In mammals, an increasing amount of data indicates some function of mt isoforms in cellular respiration and more generally suggests that isoforms modulate the activity of holoenzymes (37). Also it would be of interest to compare pollen mt proteins of fertile and male sterile plants. This should be done for either nuclear or cytoplasmic mutants, as we have recently shown that *N. sylvestris* nuclear male sterility genes are implicated in the control of mt gene expression (38). Preliminary results indicate mt protein differences between fertile and sterile pollen (39).

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