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Genetics

A cytoplasmic male sterility-associated mitochondrial protein causes pollen disruption in transgenic tobacco

(callose deposition/common bean)

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ABSTRACT **In higher plants, dominant mitochondrial mutations are associated with pollen sterility. This phenomenon is known as cytoplasmic male sterility (CMS). It is thought that the disruption in pollen development is a consequence of mitochondrial dysfunction. To provide definitive evidence that expression of an abnormal mitochondrial gene can interrupt pollen development, a CMS-associated mitochondrial DNA sequence from common bean, *orf239*, was introduced into the tobacco nuclear genome. Several transformants containing the *orf239* gene constructs, with or without a mitochondrial targeting sequence, exhibited a semisterile or male-sterile phenotype. Expression of the gene fusions in transformed anthers was confirmed using RNA gel blotting, ELISA, and light and electron microscopic immunocytochemistry. Immunocytological analysis showed that the ORF239 protein could associate with the cell wall of aberrant developing microspores. This pattern of extracellular localization was earlier observed in the CMS common bean line containing *orf239* in the mitochondrial genome. Results presented here demonstrate that ORF239 causes pollen disruption in transgenic tobacco plants and may do so without targeting of the protein to the mitochondrion.**

The mitochondrion, as a cellular center for energy metabolism, serves essential functions in the development of eukaryotic organisms. In plants, one of the developmental transitions that appears to be particularly influenced by mitochondrial function is male reproductive (pollen) development. Mutations in the mitochondrial genome most commonly result in the inability of the plant to shed viable pollen. This phenomenon, known as cytoplasmic male sterility (CMS) and observed in more than 150 plant species, has presented the opportunity to investigate the special functions provided by mitochondria at this crucial developmental stage.

The association of CMS with abnormal mitochondrial gene expression has been established in many plant species including maize (1, 2), petunia (3, 4), sunflower (5–8), and common bean (9, 10). In all cases, pollen disruption in CMS lines is accompanied by the expression of novel mitochondrial DNA sequences that contain protein-coding open reading frames. To date, there are two important issues regarding CMS yet to be resolved. (*i*) Definitive evidence that these aberrant mitochondrial genes actually cause pollen disruption is not yet available in any plant system, and (*ii*) the molecular mechanisms effecting the disruption of pollen development are still not understood.

In CMS common bean, male-sterile plants contain a novel mitochondrial DNA sequence (designated *pvs*) of unknown origin that is associated with the pollen sterility phenotype by various genetic criteria (10, 11). The 3.7-kb *pvs* sequence contains at least two open reading frames, *orf239* and *orf98*. Immunohistochemical studies have shown that the protein product of *orf239*, 27 kDa in size, is present only in the reproductive tissues (12), implying that it is the gene responsible for the pollen disruption event. Immunogold labeling studies using polyclonal antibodies against the ORF239 protein have shown that the protein localizes to the callose layer and primary cell wall of developing pollen (12). Because pollen disruption in CMS common bean can be accompanied by the failure of normal callose deposition, it is possible that the specific localization of the ORF239 peptide at the periphery of the cell may interfere with normal cell wall deposition, leading to incomplete cytokinesis, whereas the mitochondria serve only as the sites of ORF239 protein synthesis. To provide decisive evidence of ORF239 as the causative agent in the disruption of pollen development and to investigate the biological implications of the observed extracellular localization of ORF239 to the cell wall, we have conducted transformation experiments in *Nicotiana tabacum*, an unrelated plant species.

MATERIALS AND METHODS

Transgene Constructions. For the development of transgene constructions, we used two different plant gene promoters: a pollen-specific promoter, P_{Lat52} (13, 14), and a strong constitutive promoter, P_{SP} (15). The mitochondrial transit sequence used was derived from the *Nicotiana plumbaginifolia* β subunit of mitochondrial F1-ATPase (*atp2-1*; ref. 16). The pollen-specific promoter P_{Lat52}, the mitochondrial transit sequence, and the *orf239* sequences used in the constructions were amplified by PCR to facilitate cloning. Promoter P_{SP} is composed of three tandem copies of the *Agrobacterium tumefaciens* octopine synthase activator, one copy of the mannopine synthase 2′ activator, and the mannopine synthase 2′ promoter (15). It was cloned into a modified pBI101 transferred DNA binary vector, with *Xba*I–*Not*I–*Sac*I cloning sites at its 3′ end, followed by a poly(A) tail signal. This vector is designated pATC940. P_{Lat52}, composed of 666 bp immediately upstream to the ATG codon, was PCR-amplified from tomato total genomic DNA using two primers that were designed to introduce a *Hin*dIII site at the 5′ end and an *Xba*I site at the 3′ end. The primer sequences are 5′-CGCAAGCTTGGATAAGGG TAGCTCTATCTA-3′, and 5′-CCTCTAGATTGGAATT TTTTTTTTTGGTGT-3′, respectively. The mitochondrial transit sequence was the N-terminal 60-aa sequence of *atp2-1*. This peptide targets foreign proteins to the mitochondria and is cleaved between the 54th and 55th amino acids after translocation in transgenic tobacco (16). The DNA sequence was amplified from *N. plumbaginifolia* total genomic DNA using two primers introducing an *Xba*I site at the 5′ end and a *Kpn*I site at the 3′ end. The primer sequences are 5′-CCTCTAGACCATGGCTTCTCGGAGGCTTCT-3′ and 5′-CCGGTACCGCTGCGGAGGTAGCGTACTG-3′, respectively. The mitochondrial *pvs-orf239* DNA sequence was am-

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Abbreviations: CMS, cytoplasmic male sterility; W38, Wisconsin 38.

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plified from a CMS bean mitochondrial cDNA clone containing the *pvs* sequence. With use of specially designed primers, convenient cloning sites were introduced to the *orf239* fragment. The 5′ primer 5′-CCGGTACCCATGTTC CTCCCATTCAACCCA-3′ contains a *Kpn*I site that, upon ligation, forms an in-frame chimeric gene with the 183-bp transit sequence of *atp2-1* (Fig. 1). The 61st amino acid of ATP2-1 is changed from Ala to Val followed immediately by the first AUG condon of ORF239.

The procedures for cloning into manipulating vectors are as follows. A PCR-amplified *orf239* fragment was digested with *Kpn*I and *Sac*I, and then ligated to the *Kpn*I–*Sac*I site of the Phagemid vector pBS(+) (Stratagene), resulting in the construction con1. The PCR-amplified *atp2-1* fragment was digested with *Xba*I and *Kpn*I, and ligated to *Xba*I- and *Kpn*I-digested con1. The resulting construction was designated con2. To clone the *Lat52* promoter, the PCR-amplified fragment was digested with *Hin*dIII and *Xba*I and ligated to *Hin*dIII–*Xba*I-digested pBS(+), forming the construction con5. The *Hin*dIII–*Xba*I *Lat52* fragment from con5 was then ligated to the *Hin*dIII- and *Xba*I-digested con1 and con2, respectively, resulting in constructions con6 and con7. The DNA sequences of these constructions were then confirmed. To place the chimeric genes under the control of P_{SP} in pATC940, the insert fragments from con1 and con2 were released using *Xba*I and *Sac*I, and ligated into the *Xba*I–*Sac*I cloning sites of the modified binary vector pATC940. The resulting constructions were designated con8 and con3, respectively (Fig. 1). To insert the chimeric constructions con6 and con7, which contain the pollen-specific promoter P_{Lat52}, into a transferred DNA binary vector, the chimeric expression cassettes were excised from con6 and con7 using *Hin*dIII and *Sac*I, and used to replace the *Hin*dIII–*Sac*I *uidA* gene fragment of pBI101. The resulting constructions were designated con10 and con9, respectively (Fig. 1). Constructions con3, con8, con9, and con10 were used for plant transformation experiments.

Plant Materials and Transformation Procedures. The four transgene constructions were mobilized into *A. tumefaciens* LBA4404 by a triparental mating procedure (17) using *Escherichia coli* MM294 harboring the mobilizing plasmid pRK2013 (18). *A. tumefaciens* transconjugants were selected on AB minimal medium plates containing 0.5% sucrose, 10 μg/ml rifampicin, and 50 μg/ml kanamycin. Leaf disks from 6-week-old sterile shoot tip cultures of tobacco [*N. tabacum* cv. Wisconsin 38 (W38)] were transformed by a leaf disk transformation method (19). Shoots were rooted and propagated as described by Ni *et al.* (15).

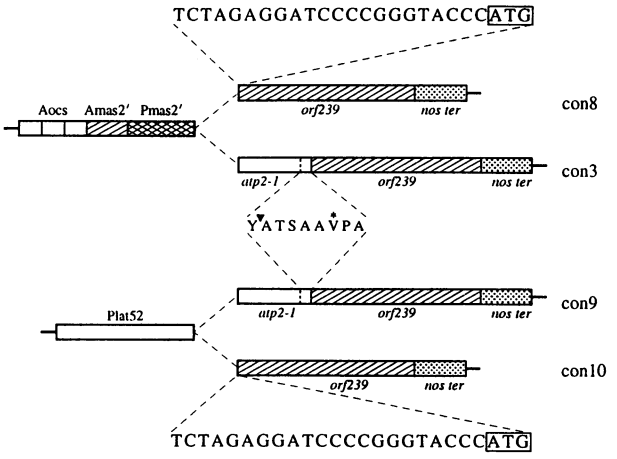

FIG. 1. Construction of *orf239* chimeric genes. The diagram shows the strategy for cloning each gene construct into the transferred DNA binary vector pBI101. *Aocs*, activator of the octopine synthase gene (trimer); *Amas2′*, activator of mannopine synthase 2′ gene; Pmas2′, promoter of the mannopine synthase 2′ gene; PLat52, promoter of the tomato pollen-specific gene *Lat52*. The boxed ATG is the first codon of *orf239*. ▼, Site of peptide cleavage; *, the amino acid altered as a consequence of cloning.

Transgenic Phenotype Evaluation. Putative transformants were characterized using several criteria. Regenerated seedling leaf tissues were processed for PCR, DNA gel blot, and RNA gel blot analyses to test for presence of the transgene and its transcripts. Total genomic DNA was prepared following the protocol described by Vallejos *et al.* (20), and the RNA preparation procedure was taken from Powlowski *et al.* (21). DNA and RNA gel blot analyses were according to standard procedures (22).

Regenerated tobacco plants were grown to flowering. At flowering, sterile plants were classified based on plant morphology, flower morphology, and seed set. Male-sterile plants were classified as those that demonstrated a compacted floral head (also evident in shorter plant stature); collapsed, gray anthers recessed below the level of the stigma (Fig. 2); and little or no seed set within capsules. Semisterile plants were those that demonstrated recessed anthers but intermediate phenotypes for all other characters. Pollinations using wild-type pollen (W38) allowed us to confirm female fertility.

ELISA Assays. We experienced certain technical difficulties in extracting enough ORF239 protein for ELISA. In anthers, ORF239 was detectable only in developing microspores when controlled by promoter P_{Lat52}, and in developing microspores and stomia when controlled by promoter P_{SP}. This greatly diluted the ORF239-containing cells when whole anther tissues were used for protein extraction. Furthermore, ORF239 has a very strong affinity for cell wall material (12), and we experienced difficulty in eluting this protein from walls using buffers that would not interfere with ELISA. To overcome these intrinsic problems, we used a modified ELISA procedure. Nitrocellulose disks (7 mm in diameter) were incubated with 250 μl of a solution of total protein from bud samples at 100 μg/250 μl solution concentration in 10 mM Tris·HCl buffer (pH 7.2). After incubation for 12 hr at 4°C, the disks were washed 3 times for 10 min in Tris·HCl buffer, and the remaining sites were blocked with Tris·HCl buffer containing

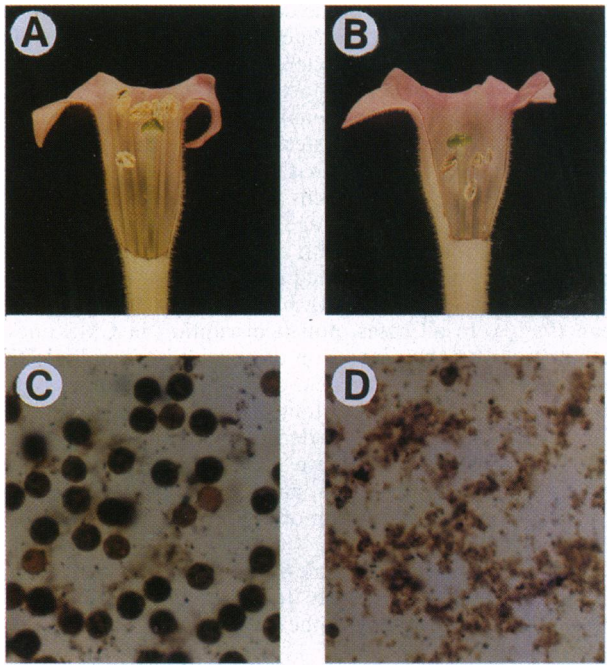

FIG. 2. Flower morphology and pollen shed of a male-sterile transformant. (*A*) A nontransformed wild-type (W38) floral structure. (*B*) A male-sterile (T_0) flower containing the *orf239* gene fusion. (*C*) Anther locule contents of W38 stained with potassium iodide. (*D*) Anther locule contents of the male-sterile transformant stained with potassium iodide.

5% nonfat dry milk for 12 hr at room temperature. The disks were processed using the ELISA protocol described previously (12). A final sample volume of 100 μl was used for readings at A_{405}.

Light and Electron Microscopic Immunocytology. Procedures for bud fixation, embedding, sectioning, and immunocytological analysis were exactly as those described previously (12).

RESULTS

Chimeric gene constructions (Fig. 1) were developed using the mitochondrial gene *orf239* with or without a mitochondrial transit sequence. We assume that the ORF239 product alone, without a mitochondrial transit sequence, is not capable of targeting to the mitochondrion. The NH_2 terminus of ORF239 does not contain the common features of a mitochondrial transit sequence; instead of a basic sequence, the putative helix-forming regions contain many acidic residues that do not appear amphiphilic (10). The two promoters, P_{SP} and P_{Lat52}, were used to direct the expression of *orf239* gene fusions in developing microspores. P_{SP}, a recently described chimeric promoter (15), when linked to the *uidA* gene, conferred somewhat uneven β-glucuronidase activity (not all microspores stained and intensity varied) during early microsporogenesis, including premeiosis (data not shown). P_{Lat52}, isolated from tomato, is reported to direct pollen-specific expression in transgenic tobacco plants postmeiotically (13, 14). Pvs-ORF239 in common bean is expressed premeiotically in the pollen mother cell (12). Although neither promoter is optimal for simulating *pvs-orf239* expression premeiotically, they represented the most promising promoters that had been reported at the onset of these experiments. To date, a promoter conferring high-level expression at the pollen mother cell stage has not, to our knowledge, been reported.

The chimeric genes were ligated to a derivative of the transferred DNA binary vector pBI101, resulting in four constructions designated con3, con8, con9, and con10 (Fig. 1). The constructions were then introduced into *A. tumefaciens* and used to infect tobacco leaf disks. A total of 108 transgenic plants were obtained and grown to maturity. The vegetative growth (i.e., growth rate, plant morphology) of transformants was uniform and similar to that of nontransformed regenerated control plants. At flowering, transgenic plants containing con3, con8, or con9 exhibited a male-fertile, semisterile, or sterile phenotype; transgenic plants containing con10 exhibited either a male-fertile or a semisterile phenotype (Table 1).

Table 1. Fertility evaluation based on seed production in T_0 plants confirmed to contain the *orf239* transgene

Plant type	No. of plants	Classification			Seeds per capsule, mg	ORF239 expression/no. of plants tested*
		Fertile	Semisterile	Sterile		
W38	5	5	0	0	155 ± 36†	0/1
con3	37	28	—	—	136 ± 49	0/3
		—	6	—	37 ± 28	
		—	—	3	0	3/3
con8	25	22	—	—	139 ± 41	
		—	2	—	41 ± 28	
		—	—	1	0	1/1
con9	26	20	—	—	120 ± 48	0/2
		—	2	—	10 ± 8	
		—	—	4	0	3/3
con10	20	17	—	—	140 ± 49	
		—	3	—	13 ± 7	

*ORF239 expression in developing pollen was assayed immunocytologically; here it is presented as the number of plants expressing ORF239 per the number of plants tested. In all cases, multiple buds per plant were assayed.

†The standard deviation was derived from variation among capsules of tested plants.

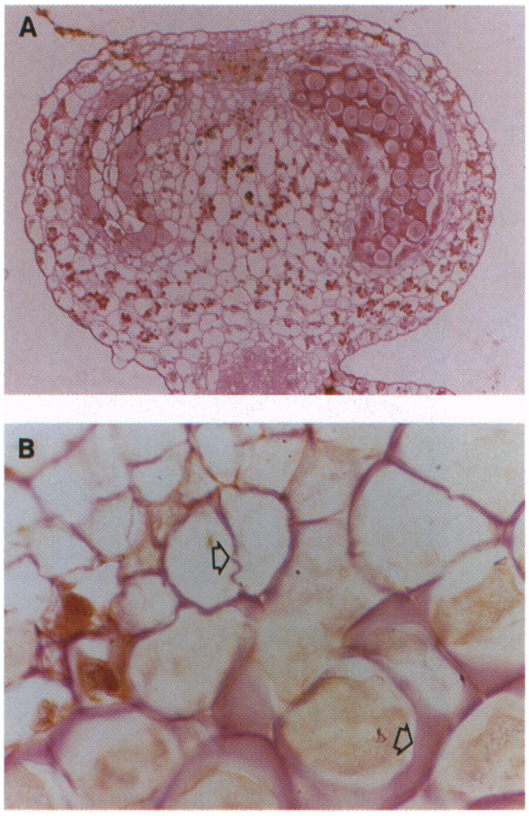

FIG. 3. Developmental aberrations of male-sterile transformants at the cellular level. (*A*) A cross-section of a male-sterile (T_0) anther showing unsynchronized development of microspores among locules of the same anther. (×330.) (*B*) A section of a male-sterile anther locule premeiosis showing variable callose deposition (arrows) among developing pollen mother cells. (×2000.)

This classification was based on flower morphology and seed-set. All the sterile plants exhibited similar developmental changes that were not observed in regenerants that did not contain the transgene, including increased number of flowers, delayed apical senescence, and slightly retarded vegetative growth during flowering. In most cases, the filaments failed to extend and remained below the level of the stigma (Fig. 2*B*). Anthers on male-sterile plants were shriveled and shed no or greatly reduced amounts of pollen (Fig. 2*D*). In the most severe case, the anther locules collapsed and anthers failed to dehisce. Abnormal patterns of microsporogenesis were observed, including unsynchronized pollen development within the same anther (Fig. 3*A*) and variable callose deposition of developing microspores (Fig. 3*B*), as well as nonfunctional but morphologically normal pollen. This observation was based on results of standard pollen germination tests conducted to determine the viability of the morphologically normal pollen from sterile

Table 2. Segregation of the *orf239* transgene and incomplete penetrance of the male sterility phenotype in T_1 testcross populations

Plant type	No. of plants	Plus transgene*		Minus transgene‡
		Fertile†‡	Sterile‡	
con3	36	12 (0/2)	3 (2/2)	21 (0/1)
con8	37	16 (0/2)	5 (2/2)	16

*The presence of transgene was determined using PCR assay.

†Semisterile plants were included in the fertile class to prevent overestimation of the transgene effect.

‡ORF239 expression in developing pollen was assayed immunocytologically; here it is presented in parentheses as the number of individuals expressing ORF239 per the number of individuals tested. In all cases, multiple buds per plant were assayed.

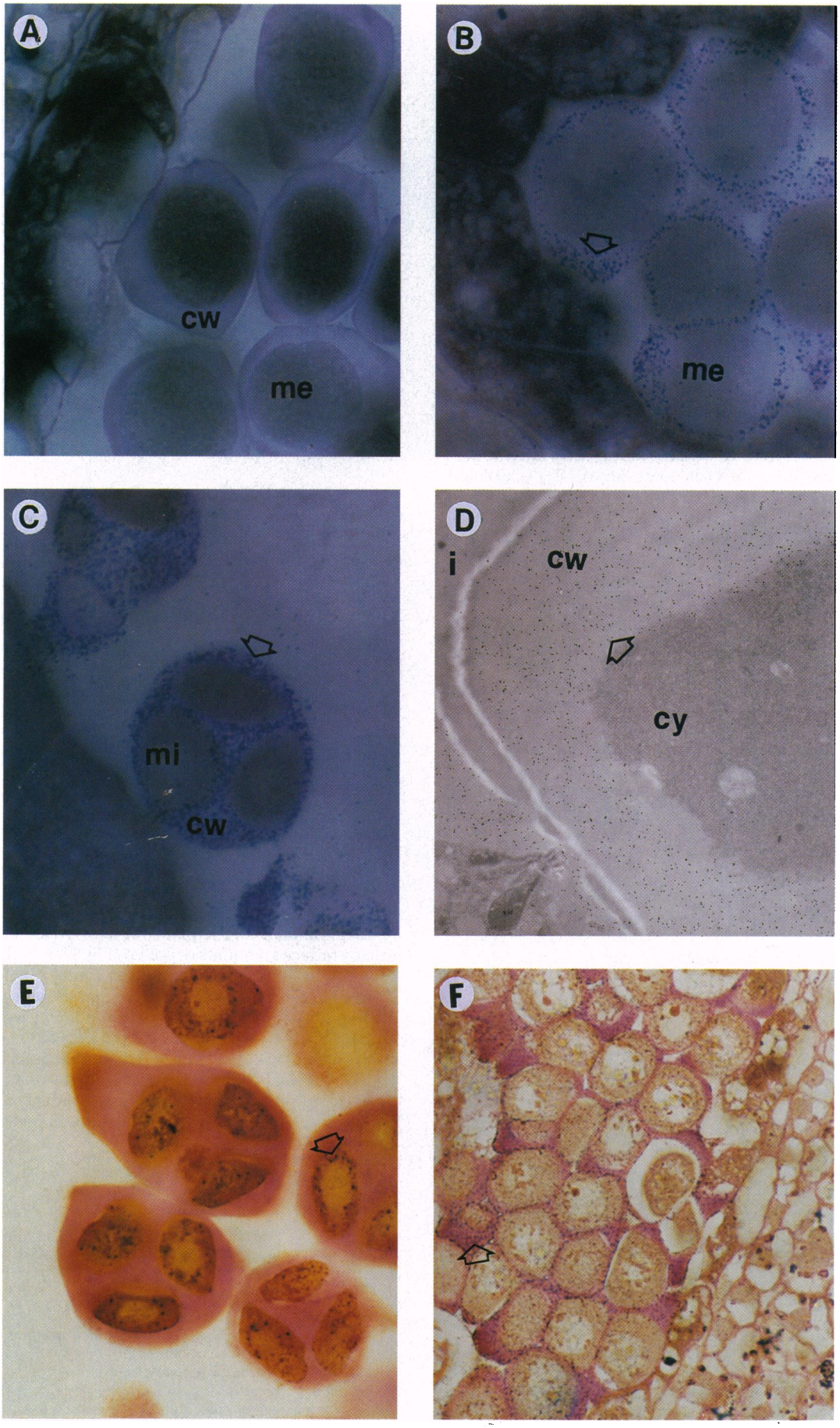

FIG. 4. *(Legend appears on opposite page.)*

and semisterile transgenic plants. The germination frequency of pollen from nontransformed plants was about 50–60%; however, in both sterile and semisterile plants tested (two for each construct), pollen germination frequency was about 1% (data not shown).

When the male-sterile plants were cross-pollinated using pollen from nontransformed wild-type plants, fruit capsules developed and normal seed set was obtained, indicating that female fertility was unaffected. Examination of the cross-pollinated progeny showed that male sterility was transmitted to the T_1 generation. Successful sexual transmission (T_1 segregation) of male sterility was observed in progeny from three crosses derived from independent transformants containing either con3 or con8 under the control of promoter P_{SP}; we failed to obtain sterile progeny from the three independent crosses to transformants containing con9 controlled by promoter P_{Lat52}.

Integration of the gene fusions into the tobacco genome was confirmed in all the sterile and semisterile T_0 plants using both PCR assays and DNA gel blot analyses (data not shown). Segregation for the transgenes approximating the expected 1:1 (+transgene/−transgene) ratio in the T_1 generation was observed using PCR assays (Table 2).

Expression of the transgenes in developing microspores of sterile and semisterile T_0 transformants was demonstrated at both the transcription and translation levels. RNA gel blot analyses of total anther RNA using the *orf239* DNA sequence as a probe showed that the expected transgene mRNAs (i.e., 0.92 kb for con3 and con9, and 0.72 kb for con8 and con10) were present in the anther tissues (data not shown). *orf239* transcripts were also detected in vegetative (leaf) tissues of both fertile and sterile transgenic T_0 plants containing the P_{SP} promoter (data not shown). Enzyme-linked immunosorbent assays (ELISA), modified for optimal sensitivity, were carried out to test for the presence of ORF239 protein using rabbit polyclonal antibodies raised against a truncated form of the ORF239 protein (12). The transgene protein product was detectable in the anther tissues of male-sterile and semisterile transformants but not in anther or leaf tissues of fertile transformants (five plants were tested). Total anther protein (100 μg per assay) extracted from fully male-sterile and semisterile T_0 plants (six plants were sampled) produced absorbance (A_{405}) readings ranging from 2.5 to 3.5 times higher than background readings taken with BSA (100 μg) and 1.9 to 2.5 times higher than samples taken from untransformed regenerants. Total anther proteins purified from male-sterile and semisterile T_1 segregants (five plants were sampled) again produced absorbance readings 2.0 to 2.5 times the level obtained from untransformed and transformed fertile regenerants. BSA samples spiked with purified ORF239 expression product at 5, 10, 25, and 50 ng produced a linear curve for the interval (10–20 ng) corresponding to that of the sample readings. Consequently, although transcripts were detected in both male-sterile and male-fertile transformants, the ORF239 protein was only detected in the male-sterile T_0 and T_1 plants. These results provide strong evidence to support the assumption that ORF239 expression is sufficient to interrupt pollen development. The most important aspect of these findings is the observation that partially or fully male-sterile plants were obtained using all four types of *orf239* gene fusions, with and without a mitochondrial targeting sequence. These observations imply that ORF239 causes pollen disruption even without localizing to mitochondria.

An interesting and unexpected observation was made when total genomic DNAs from both wild-type and transgenic tobacco plants were hybridized with the *orf239* fragment using DNA gel blot analysis. A *pvs*-homologous sequence was detected in both wild-type and transgenic plants. We have determined, by DNA sequence, RNA gel blot, ELISA, and immunocytological analyses that the *orf239*-homologous sequence is not expressed in tobacco anthers and that, were it expressed, sufficient sequence divergence exists to render any putative protein product highly unrelated to ORF239 (unpublished data).

Because of the possibility that a male-sterile phenotype might be observed in transgenic tobacco as a consequence of the plant tissue culture and regeneration process (23), we evaluated the association of ORF239 expression with sterility in more detail. To examine the expression and intracellular localization pattern of ORF239 protein in transgenic tobacco plants, immunocytochemical examinations of aborting microspores at stages before and after callose deposition were conducted using both light and electron microscopy. Sectioned bud tissues from T_0 transgenic plants containing three of the four *orf239* constructs (con3, con8, or con9) and wild-type tobacco W38 were incubated with anti-ORF239 antibodies. A strong signal, similar to that demonstrated in Fig. 4 *B* and *C*, was observed within developing microspores from all seven sterile or semisterile T_0 transgenic plants examined regardless of transgene construction. No signal was detectable in the male-fertile T_0 transgenic plants (five plants were tested). Similar immunocytological evaluations were made of four male-sterile and four fertile T_1 segregants (con3 and con8, Table 2). These studies also revealed intense labeling in all four male-sterile plants with no expression detected in the fertile T_1 transformants. The level and timing of *orf239* expression varied among floral buds, with some buds showing highest expression as early as the pollen mother cell stage (Fig. 4*B*) and others at the tetrad stage (Fig. 4*C*). ORF239, when attached to the mitochondrial transit sequence, was localized primarily to the periphery of the cells in the transformants (Fig. 4 *B* and *C*). Electron microscopic analyses performed using floral buds from these sterile plants clearly showed that ORF239 was localized within the cell wall layer (Fig. 4*D*). However, in male-sterile T_0 and T_1 plants that contained *orf239* without a mitochondrial transit sequence (con8), ORF239 localized within the microspores, with no clear association to the microspore wall (Fig. 4*E*). This observation implies that transport to the mitochondrion, although perhaps not essential for pollen abortion, may be important for the peripheral localization of ORF239. These results also indicate that the association of ORF239 with the pollen wall may be an effect secondary to its mode of action in causing pollen abortion. In all cases, ORF239 expression was uniform throughout the anther and limited to gametophytic tissues (Fig. 4*F*).

FIG. 4. Immunogold localization of the ORF239 product during microsporogenesis in transgenic tobacco. (*A*) An anther section of nontransformed wild-type (W38) control showing no immunogold labeling of meiocytes when incubated with antibodies against ORF239. (×2450.) (*B*) An anther section of a male-sterile T_0 transformant (con9) showing the presence of ORF239 within the callose layer of meiotic cells after incubation with ORF239 polyclonal antibodies. (×2450.) (*C*) An anther section of a T_0 male-sterile plant (con9, includes the mitochondrial transit sequence) at tetrad stage after incubation with the ORF239 antibodies. (×4050.) (*D*) An electron micrograph of an aborted microspore section after incubation with ORF239 antibodies. Sample was not post-stained and osmium was omitted to exclude the possibility of masking the antigen. (×13,800.) (*E*) An anther section of a male-sterile T_1 transformant (con8, without the mitochondrial transit sequence) showing immunogold labeling of the cytoplasm with little or no labeling of the wall of developing microspores. (×3500.) (*F*) An anther section of a male-sterile T_1 transformant (con3) demonstrating uniform labeling with gold particles of callose throughout the anther locule, and no labeling of surrounding sporophytic tissues. (×1600.) The arrows in *B–F* indicate the immunogold labeling. me, Meiocytes; cw, callose wall layer; mi, microspores; i, intercellular space (resin); cy, cytoplasm. The procedures for light and electron microscopy were as described (12).

DISCUSSION

The results of this study indicate that introduction of each of the *orf239* gene constructions, with and without a mitochondrial transit sequence, resulted in aberrant pollen function. In all male-sterile or semisterile transgenic T_0 or T_1 plants that were tested, the ORF239 protein was detected in developing microspores but not in vegetative tissues. In none of the fertile transgenic T_0 or T_1 plants did we observe the ORF239 protein in vegetative or anther tissues tested. Consequently, although incomplete penetrance of the male-sterility phenotype precluded complete T_1 cosegregation of male sterility with the presence of the transgene, perfect correlation was observed between the male-sterility phenotype and expression of the ORF239 protein.

The findings from this study are important not only in their implication that ORF239 expression is sufficient to cause male sterility but also because they suggest that ORF239 may act without impairing mitochondrial function. The means by which ORF239 acts to interfere with normal pollen development is not yet clear.

In neither the T_0 nor the T_1 generation did we observe a fully male-sterile phenotype in 100% of the transgenic plants. This incomplete penetrance may be the consequence of transgene inactivation, although we suggest a more likely explanation is insufficient expression at the critical premeiotic stage at which ORF239 acts. In CMS bean, expression of ORF239 occurs within a rather narrow developmental window (12). Duplicating this pattern of expression transgenically would require a specialized promoter. Consequently, we observed several fertile transgenic con3 and con8 T_0 plants that demonstrated high levels of *orf239* transcript in vegetative tissues but no detectable ORF239 protein in the developing pollen mother cells or microspores (data not shown). Furthermore, because P_{Lat52} is considered to be a promoter functioning primarily late in microsporogenesis (13, 14), we speculate that under normal physiological conditions, this promoter would not direct the expression of *orf239* at the right developmental stage, i.e., before meiosis. In the T_0 generation, we clearly observed, using RNA gel blot, immunocytochemical, and ELISA analyses, P_{Lat52}-directed *orf239* expression premeiotically in male-sterile transformants containing con9, but this male-sterile phenotype was not transmitted to the T_1 progeny. This result may be the consequence of some degree of relaxation in developmental regulation of expression in the transgenic T_0 regenerants.

In transgenic experiments testing CMS-associated sequences from both CMS petunia (24) and *cms-T* maize (23), similar conclusions were reached regarding the requirement in these systems for proper developmental expression and localization of the CMS-inducing protein to observe the male-sterile phenotype. In petunia, where the CMS-associated sequence *pcf* expresses premeiotically, no male-sterile phenotype was observed transgenically using the CaMV35S promoter. Similarly in *cms-T* maize, transgenic expression of the *T-urf13* sequence directed by the CaMV35S promoter resulted in methomyl sensitivity but no associated male-sterile phenotype.

ORF239 represents the only currently known plant mitochondrially encoded protein that is located outside of mitochondria and is apparently biologically active in interactions with cellular components other than the mitochondrion. We have yet to determine whether ORF239 exits the mitochondrion by active transport or by mitochondrial lysis in either CMS common bean or transgenic tobacco. Those studies are currently underway. Immunocytological data presented here, however, do suggest that association with the mitochondrion is essential for the subsequent localization of ORF239 to the pollen wall.

We observed male sterility as a consequence of transgene constructions that did not contain a mitochondrial transit sequence (con8). These results imply that aside from any effects ORF239 may have on mitochondria, it need not be targeted to the mitochondrion to cause microspore abortion. Whether or not these results will have implications for other CMS mutations remains to be seen. Comparison of results from CMS petunia (24), *cms-T* maize (23), and those presented here suggest that quite distinct mechanisms of action by these three sterility-associated products give rise to the pollen abortion phenotype and, in the case of bean, that microsporogenesis is vulnerable to other factors in addition to energy demands. The radical and complex changes in cell development that occur during meiosis and pollen development are perhaps also subject to interference by particular aberrant proteins in the cell. The plant mitochondrial genome is clearly susceptible to dominant mutations that give rise to a distinct phenotype, and mitochondria apparently possess mechanisms for exporting deleterious protein products (25, 26). It is possible in CMS bean that such protein translocation, although perhaps beneficial to mitochondria, would result in the impairment of other cellular functions during microsporogenesis.

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