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A chimeric and truncated mitochondrial *atpA* gene is transcribed in alloplasmic cytoplasmic male-sterile tobacco with *Nicotiana bigelovii* mitochondria

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Abstract Protoplast fusions were performed between two sexually produced alloplasmic male-sterile tobacco cultivars, with cytoplasms from *Nicotiana bigelovii* [Nta (big)S] and *N. undulata*[Nta(und)S], both of which exhibit homeotic-like phenotypes affecting the petal and stamen whorls. Among the fusion products obtained, both novel male-sterile and pollen-producing cybrid plants were identified. Of the pollen-producing cybrid plants, all of which were indehiscent, some had flowers with stamens that appeared normal when compared to male-fertile tobacco plants. Other hybrid plants were incompletely restored as they exhibited petaloid structures on the anther-bearing pollen-producing stamens. In this study, gel-blot analyses with mitochondrial gene-probes were conducted comparing the mitochondrial DNA of cybrids and male-sterile parents. It was found that the flower morphology typical of the Nta(big)S parental plants, as well as of the novel male-sterile cybrids, coincided with the presence of a chimeric *atpA* gene copy where an open reading frame of unknown origin was found to be linked in-frame to the 3′-end of a truncated *atpA* gene. RNA gel-blot hybridizations revealed the presence of *atpA* transcripts in the male-sterile parent Nta(big)S and novel male-sterile cybrids, but which were absent in cybrids capable of pollen production.

Key words CMS · Cybrids · Chimeric *atpA* · Gene · Mitochondria · *Nicotiana*

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Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait that has been associated with modifications of the mitochondrial genome in higher plants (Lonsdale 1987; Levings and Dewey 1988; Hanson 1991). Besides occurring as spontaneous mutants in a population of normal plants, CMS lines can be obtained by intra-or inter-specific sexual crosses, resulting in the combination of the nucleus of one species with the cytoplasm of another (Kaul 1988).

In tobacco, CMS correlates with homeotic-like mutant phenotypes involving petal and stamen development (Bonnett et al. 1991, Kofer et al. 1991a). Stamen development is arrested and frequently the stamens are modified to petalodes, and might include stigmatoids or ovules. Petals may be shortened or may fail to fuse. This variation in flower development is accounted for by the hypothesis of Rosenberg and Bonnet (1983), suggesting that a series of steps along the pathway of androgenesis require the cooperative action of the cytoplasmic and nuclear genomes. The different floral morphologies of alloplasmic CMS tobacco cultivars can thus be predicted to be due to a variation in the activity of several mitochondrial genes. The results obtained from fusion experiments performed between different male-sterile tobacco cybrids (Aviv and Galun 1986), and several different sexually produced alloplasmic and male-sterile tobacco cultivars (Kofer et al. 1990), largely confirm this model. Cybrid plants were obtained with recombined biparental male-sterile and novel male-sterile stamen morphologies (Kofer et al. 1990, 1991a), as well as cybrids with restored pollen producion (Aviv and Galun 1986; Kofer et al. 1990, 1991b).

The cybrid plant differed in their flower morphologies and their degree of male-fertility (Kofer et al. 1990). The offspring of cybrids derived from callus 28, obtained from fusions between Nta(big)S and Nta(und)S, displayed a split corolla as found in the Nta(big)S parent, and distinctive male-sterile stamen morphologies com-

bining features of both fusion parents in novel arrangements (Kofer et al. 1991a). By contrast offspring of cybrid 29-1 (derived from callus 29) produced flowers with pollen-bearing anthers on petaloid stamen structures, while the offspring from cybrid 29-3 (also derived from callus 29) showed a variety of floral phenotypes (Kofer et al. 1991b). Some of the offspring from 29-3 developed flowers with petaloid stamens; some, however, had flowers with normal-appearing stamens when compared to fertile tobacco, although the anthers were indehiscent. A third group of 29-3 offspring had flowers with petaloid stamens as well as flowers with the normal-appearing stamens. These flowers were usually formed on separate branches. Both the flowers with petaloid stamens as well as the flowers with normal-appearing stamens produced fertile pollen. As the anthers did not dehisce, self-pollination and pollination of the CMS parents was possible by removing pollen from the anthers and applying it to the stigma.

In this study we have used DNA and RNA gel-blot hybridizations to further analyze this cybrid material. A novel *atpA* sequence was found in the male-sterile cybrids of the Nta(big)S phenotype. Sequence analysis and molecular analysis revealed a transcribed chimeric and truncated copy of the *atpA* gene.

Materials and methods

Plant material

Novel male-sterile and male-fertile cybrids were obtained from fusions between cytoplasmic male-sterile tobacco cultivars with organelles from *Nicotina bigelovii* and *N. undulata*, as described by Kofer et al. (1990, 1991b). These two male-sterile cultivars [referred to as Nta(big)S and Nta(und)S] resulted from the introgression of the nucleus from *N. tabacum* to the cytoplasms of the respective donor species and have been backcrossed for 12–16 generations. Thus, the parental materials represent highly inbred sexually produced lines.

Cybrids from callus 28 exhibited flowers with novel male-sterile morphology. All cybrid plants from callus 28 exhibited a split corolla as found in the Nta(big)S parent; however stamen morphologies combined subsets of both parental stamen features in novel combinations (Kofer et al. 1991a). The cybrids that were restored to male-fertility (Kofer et al. 1991b) were categorized into two groups. Both types were found among the progeny of cybrid plants raised from the same cybrid callus (29) and pollinated with *N. tabacum*. One of those cybrids gave rise to R_1 progeny plants having either petaloid or abnormal stamens. This plant was designated 29-1. The other cybrid, 29-3, yielded R_1 plants having both normal-appearing and petaloid-stamen types. Some of these plants had flowers with only normal-appearing stamens, other plants had only petaloid stames, and still others had both types on separate floral branches. Progeny of both cybrids (29-1 and 29-3) produced stamen structures with anthers containing normal pollen, although the anthers did not dehisce. After pollination with *N. tabacum*, mtDNA was isolated from offspring of cybrid 29-3 (about 70 plants) with normal-appearing stamens.

DNA isolation and gel-blot hybridizations

For the isolation and electrophoresis of mtDNA, the procedure of Bland et al. (1985) as modified by Håkansson et al. (1988) was used. Total DNA was isolated from plant leaves according to Bernatzky and Tanksley (1986) as modified by Landgren and Glimelius (1990), except for the extraction-buffer which was supplemented with β-mercapto-ethanol (50 mM). After endonuclease digestion and subsequent electrophoresis in 0.4 or 0.6% agarose, the gels were transferred to nylon filters (Pall Biodyne membrane, Pall BioSupport Corporation, Glen Cove, N.Y.) according to standard procedures (Sambrook et al. 1989). Filters were hybridized, washed and re-hybridized as described by Landgren and Glimelius (1990).

RNA isolatiion and gel-blot hybridizations

The mtRNA isolation and the gel-blot hybridization analyses were performed as described earlier (Håkansson and Glimelius 1991). Total cell RNA was isolated from young leaves as well as from 1–3 mm undeveloped flower buds (measured from base to top of sepals). At this stage the first flower bud is still covered by the top leaves, which have to be folded aside in order to see the developing bud. Before RNA isolation the flower rudimient was cut clean from sepals and stem tissues and RNA was isolated according to Verwoerd et al. (1989).

Isolation and sequence analysis of a truncated *atpA* gene copy

*Hin*dIII-digested mtDNA isolated from Nta(big)S was ligated to *Hin*dIII-digested pUC-18 after which *E. coli*-XL1 bacteria were transformed. The bacterial library was screened by colony hybridization by first using the 716-bp 5′-end of the *Zea mays atpA* gene as a probe (upstream of the conserved *Bam*HI site; Braun and Levings 1985), then filters were screened with the 1:85b *atpA* gene 3′-end clone from *N. debneyi* (downstream from the conserved *Bam*HI site; Håkansson 1992). Clone 2:38 (5 kb long), hybridizing to the *N. debneyi* 3′-probe but not to the *Z. mays* 5′-probe, was subsequently subcloned in pUC-18 as a 1.7-kb *Pst*I-*Hin*dIII fragment. This insert was re-cloned as a *Bam*HI - *Hin*dIII fragment in pGem-7Zf(-) (Promega, Madison, USA) to yield plasmid P2. A series of unidirectional deletions of plasmid P2 where constructed by the use of *Exo*III (Henicoff 1984) as described in "Erase a Base" by Promega (Promega, Madison, USA). Plasmids representing different deletions on both strands were re-ligated and transformed into *E. coli*-XL1. The size of the deletion in the transformants was estimated by agarose-gel electrophoresis. DNA sequencing was carried out on double-stranded plasmid DNA by the dideoxy chain-termination method (Sanger et al. 1977) according to the instructions supplied with the sequencing kit (*Taq* DyeDeoxy Terminator Cycle Sequencing Kit, Applied Biosystems, Inc, USA). A Perkin Elmer Cetus DNA Thermal Cycler 480 (Perkin Elmer Corp., Emeryville, Calif., USA) and an Applied Biosystems Model 370A was used. The primers used for "cycle sequencing" were the pUC/M13 forward and reverse sequencing primers (Kabi-Pharmacia, Uppsala, Sweden).

Mitochondrial gene probes

The initial screening for differences in the mitochondrial DNA organization were done using the probes listed in Table 1. Other probes used in hybridization experiments were 1:85b, which is an *atpA* 3-end clone from *N. debneyi* (Håkansson 1992), and BLSC1, covering the 3′-flanking region and part of the coding region of the *atpA* gene in *Z. mays* (Small et al. 1987). Probes were radiolabelled with 1.85×10^6 Bq (^{32}P) dCTP (Oligolabelling Kit, Kabi-Pharmacia, Uppsala, Sweden).

Results

DNA gel-blot hybridizations identify a region correlating with the CMS-phenotype of Nta(big)S

Blots of mtDNA from offspring of the pollen-producing cybrids 29-1 and 29-3, as well as the male-sterile

Table 1 The heterologous mitochondrial gene probes, and their species origin, used in this investigation to screen for differences in DNA organisation among fusion parents and cybrid offspring

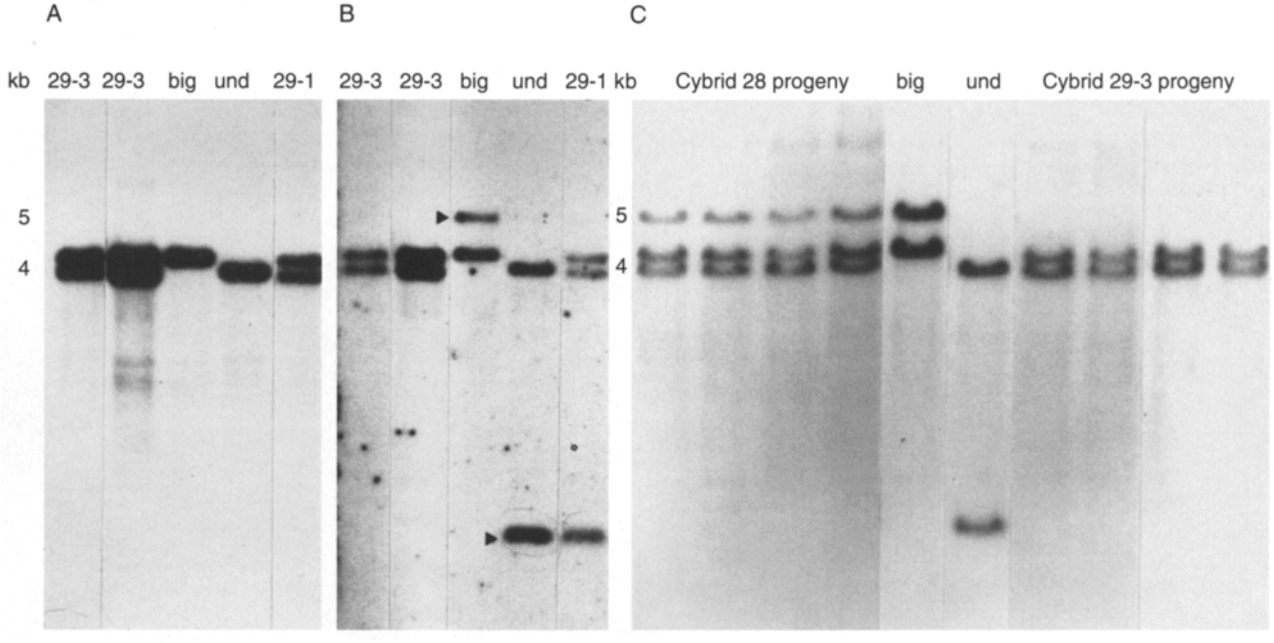
Probe[a]	Species	Reference
atp6	*Zea mays*	Dewey et al. 1985
atp9	*Nicotiana tabacum*	Bland et al. 1986
atpA	*Zea mays*	Braun and Levings 1985
coxI	*Triticum aestivum*	Bonen et al. 1987
cytb	*Zea mays*	Dawson et al. 1984
orf25[b]	*Petunia hybrida*	Stamper et al. 1987
rrn18/rrn5	*Zea diploperennis*	Gwynn et al. 1987
rrn26	*Zea mays*	Dale et al. 1984

[a] Probes were kindly provided by Dr. M. R. Hanson, Cornell University and Dr. C. S. Levings III, North Carolina University
[b] The petunia *orf25* gene is 100% homologous to tobacco *orf25* (M. R. Hanson, personal communication)

parental cultivars, were analysed for differences in mitochondrial organization by the use of different restriction enzymes and heterologous probes of mitochondrial genes (Table 1). From the 29-3 offspring, mtDNA was isolated from plants with flowers having normal-appearing stamens. Of the probes tested, only that corresponding to the region of *atpA* from *Z. mays* (Braun and Levings 1985) displayed differences in hybridization patterns between sterile and pollen-producing material. Figure 1a shows the hybridization of mtDNA from pollen-producing cybrids and male-sterile parental cultivars to the *Z. mays atpA* 5′-end probe (shown in Fig. 3 B). The mtDNA from cybrid 29-1 and progeny of cybrid 29-3 hybridized with two fragments (about 4 kb) to this probe, identical in size to each of the fragments from Nts(und)S Nta(big)S.

To further investigate the mtDNA in the vicinity of the *atpA* gene, probes derived from the *atpA* 3′-regions of *Z. mays* and *N. debneyi* (shown in Fig. 3 B) were hybridized to DNA gel-blots from offspring of novel male-sterile cybrids from callus 28, offspring from the pollen-producing cybrids 29-1 and 29-3 as well as from male-sterile parental cultivars. With the *Z. mays* BLSC1 probe, the mtDNA from progeny of 29-3 hybridized with two fragments around 4 kb (Fig. 1B) which were the same as was found with the *Z. mays* probe covering the 5′-part of the *atpA* gene, as shown in Fig. 1A. In contrast, mtDNA of both male-sterile parents hybridized with an additional 3′-specific fragment, thus possessing mtDNA regions not present in the progeny of cybrid 29-3 (Fig. 1B; arrows). As further shown in Fig. 1B, the 29-1 cybrid retained the fragment specific for the 3′-part of the gene of Nta(und)S, but not that of Nta(big)S. As shown in Fig. 1C, probe 1:85b gave the same hybridization pattern as probe BLSC1 when hybridized to progeny of cybrid 29-3 and male-sterile parents. In all progeny from cybrids from callus 28 the 1:85b probe hybridized to the two mtDNA fragments of about 4 kb specific for Nta(und)S and Nta(big)S. In CMS parent Nta(big)S and in all novel male-sterile offspring of cybrid 28 the 1:85b probe further hybridized to an additional fragment of identical size (about 5

Fig. 1 A–C DNA gel-blot hybridization to *Hin*dIII digests of DNA from progeny of male-sterile cybrids from callus 28, progeny of pollen-producing cybrids 29-1 and 29-3, and male-sterile parents. **A** mtDNA gel-blot hybridization of male-sterile parents Nta(big)S, marked as *big*, and Nta(und)S, marked as *und*, two progeny of 29-3 and one progeny of cybrid 29-1 using a probe covering the 5′-region of the *atpA* gene. **B** mtDNA gel-blot hybridization to mtDNA of Nta(big)S and Nta(und)S (marked as *big* and *und* respectively), two progeny of 29-3 and progeny of cybrid 29-1 using a *Z. mays* probe (BLSC1) covering the 3′-flanking region and part of the coding region of the *atpA* gene. **C** Gel-blot hybridization to total-DNA of progeny of cybrids from callus 28, progeny from cybrid 29-3 as well as sterile parentals Nta(big)S and Nta(und)S marked as *big* and *und* respectively, using the *Nicotiana debneyi* probe 1:85b, homologous to BLSC1



kb). This fragment was not found in the progeny from the pollen-producing 29-3 cybrid. Thus, all male-sterile cybrids tested had an additional *atpA* fragment not present in the pollen-producing cybrids.

Transcription analysis of the *atpA* gene region

RNA-blot hybridizations were performed to investigate if the presence of the additional *atpA* fragment in plants of the Nta(big)S phenotype was associated with differences in the transcription pattern of this region.

The 5′-and 3′-end *atpA* probes were hybridized to mtRNA gel-blots. Figure 2 demonstrates that in both cases differences were detected in transcription patterns between the two parental CMS cultivars Nta(big)S and Nta(und)S on one hand and also between parental materials and the 29-3 cybrid offspring on the other hand. The probe covering the 5′-part of the *atpA* gene located two major transcripts in the CMS cultivar Nta(und)S and three major transcripts in Nta(big)S (Fig. 2A). In agreement with data from the DNA blot-hybridization with this probe (Fig. 1A), the transcription pattern of the 29-3 cybrid progeny was mainly the sum of the parental patterns. The same results were obtained when RNA from young flower buds was analyzed (data not shown).

The 3′-end *atpA* probes identified the same transcripts as the 5′-probe, both in leaves (Fig. 2B) and in young flower buds (data not shown); one additional transcript was detected in each of the male-sterile parental cultivars (approximately 1500 and 4000 nt; Fig. 2B). The additional transcript of Nta(big)S was found in all offspring from cybrid 28, whereas neither of the transcripts were found in the pollen-producing cybrid offspring.

Fig. 3 Nucleotide sequence and structure of a truncated *atpA* gene from Nta(big)S. **A** Nucleotide sequence of the region within clone P2 containing the truncated *atpA* copy isolated from the male-sterile parent Nta(big)S. In regions of similarity, the Nta(big)S sequence is aligned with the *trans*-intron distal to exon c of the *A. thaliana nad5* gene marked as *Ath nad5i* (Brandt et al. 1992), and the *Z. mays* mitochondrial *atpA* gene marked as maize (Braun and Levings 1985). Amino-acid sequences predicted from the *atpA* gene sequences of Nta(big)S and *Z. mays* are given above and below the nucleotide sequences, respectively. *Dashes* designate nucleotide gaps, *colons* indicate identical nucleotides. *Stars* indicate *Z. mays* amino-acid positions identical to those predicted in the Nta(big)S protein. The Nta(big)S sequence is deposited under Accession number X76110 in the EMBL Data Library. The aligned sequences of Brandt et al. (1992) and Braun and Levings (1985) are numbered according to Accession numbers X67105 and M16222, respectively. **B** A comparison of the sequenced *atpA*-gene region in Nta(big)S and *atpA* gene from *Z. mays* (Braun and Levings 1985). A clone representing the *N. debneyi atpA* gene 3′-end and part of the 3′-untranslated region is also shown (Håkansson 1992). *Open boxes* represents *atpA* gene open reading frames. The *hatched vertical line* indicates the recombination point in Nta(big)S *atpA*. The *grey box* marks an unidentified open reading frame fused in-frame to the truncated *atpA* sequence of Nta(big)S. The *hatched area* indicates a sequence with homology to the intron distal to the *trans*-spliced *nad5* exon c in *A. thaliana* (Brandt et al. 1992). The positions of *Z. mays* probe 5′ (Braun and Levings 1985), BLSC1 (Small et al. 1987) and *N. debneyi* 1:85b (Håkansson 1992) are shown by *horizontal black bars.*

Cloning of a truncated copy of *atpA* from Nta(big)S

The 5-kb *Hin*dIII DNA fragment specific to parent Nta(big)S and progeny of cybrids from callus 28 was cloned from mtDNA of Nta(big)S. After sub-cloning of this *atpA* regioin, a 1.7-kb *Pst*I-*Hin*dIII fragment (clone P2) was sequenced. This sequence, shown in Fig. 3A, contains an open reading frame of 774 bp potentially encoding 258 amino acids whereby the first 38 amino acids, of unknown origin, are coupled in-frame to a truncated *atpA* gene. This *atpA* sequence is about half the length of the *Z. mays atpA* gene with near sequence

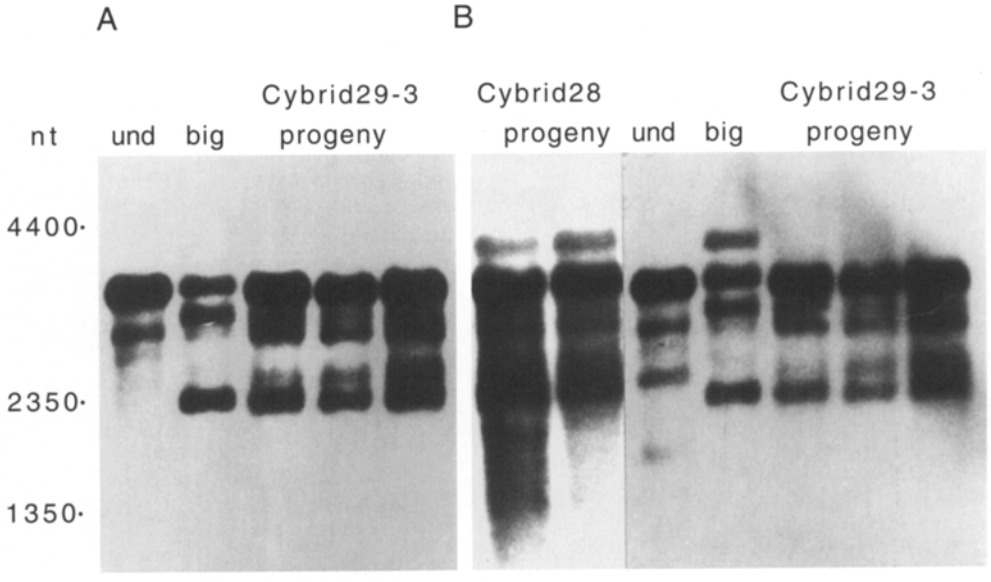


Fig. 2 A, B mtRNA gel-blot hybridizations to progeny of cybrids from callus 28, progeny of cybrid 29-3 and male-sterile parents. Nta(und)S is marked as *und*, and Nta(big)S is marked as *big*. **A** Hybridization with a *Z. mays* probe covering the 5′-region of the *atpA* gene. **B** Hybridization with probe BLSC1 coveriing the 3′-flanking part of the coding region of *atpA*

A

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Nta(big)S  CTGCAGCCTCCCGGACTTCATATTCATCATTTTAGAAAGCGAGGGGAAGGGATCTCTTTTCTGCAACG--GAAAAAAAACGGAGCAGATTTGACTCGGCACAACCTAACGATACATCCAA  118
Ath nad5i  CTGCAGCCCCACGGACTTCATATTCCTCATTTTAGAAAGCGGAGGGAAGGGATCTCTTTTCTGCAACGAAAAAAAAAAAACGGAGCAGATTTGACTCGGCACAACCTAACGATACATCCAA 1728

Nta(big)S  TACCAATGATCTGTGCCTAGAATGCGTTGCTAGATCTCTGCTCTAGAAAGCTATACAGGCAACAACGAACGCGCTTTGACCCCGGGCTTCCCTTTCATTACTTCTTTTAGAAAAGATCAT  238
Ath nad5i  TACCAATGATCTGTGCCTAGAATGCGTTGCTAGATCTCTGTTCTAAAAAGCTATACAGGCAACAATAAAGTTGACCAGGGGCGGGTCTTCCCTTTCATTACTTATTTCACGA         1840

           M S V A L C P T M P R C D T  14
Nta(big)S  CAGAGAGGAGACAACCCGCTCAAAAGACCCCAATTTTCCGACCAAGACAATCAAAGATAGCCGGCGGGGGCGCGACTTATGTCCGTGGCCCTATGCCCGACCATGCCGAGGTGTGATACC  358

           K R A I A T T D L P I G L E E C A P L S L V H H R P P G R E A F P G D V F Y L H  54
Nta(big)S  AAGAGAGCCATCGCAACCACTGATCTACCCATTGGGCTAGAAGAGTGCGCTCCTCTATCTCTCGTTCACCATCGACCACCAGGTCGTGAGGCTTTCCCAGGGGATGTTTTCTATTTACAT  478
Maize      CATGCATTAATTATATATGATGATCTAAGTAAACAGGCGGTGGCATATCGACAAATGTCATTATTGTTACGCCGACCACCAGGCCGTGAGGCTTTCCCCGGGGATGTTTTCTATTTACAT  978
           H A L * I Y D * * S K Q A V A Y R Q M * * L L R * * * * * * * * * * * * * * * *  304

           S R L L E R A A K R S D Q T G A G S L T A L P V I E T Q A G D V S A Y I P T N V  94
Nta(big)S  TCCCGTCTCTTAGAAAGAGCGGCTAAACGATCGGACCAGACAGGCGCAGGTAGCTTGACCGCCTTACCCGTCATTGAAACACAAGCTGGAGACGTATCGGCCTATATTCCCACCAATGTG  598
Maize      TCCCGTCTCTTAGAAAGAGCCGCTAAACGATCGGACCAGACAGGTGCAGGTAGCTTGACTGCGTTACCCGTGATTGAAACACAAGCTGGAGACGTATCGGCCTATATCCCCACCAATGTG 1098
           * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *  344

           I P I T D G Q I C L E T E L F Y R G I R P A I N V G L S V S R V G S A A Q L K T  134
Nta(big)S  ATCCCCATTACTGATGGACAAATCTGTTTGGAAACAGAGCTCTTTTATCGCGGAATTAGACCTGCGATTAACGTCGGCTTATCTGTCAGTCGCGTCGGGTCTGCCGCTCAGTTGAAAACT  718
Maize      ATCTCCATTACAGATGGACAAATCTGTTTGGAAACAGAGCTCTTTTATCGCGGAATTAGACCAGCTATTAACGTTGGCTTATCCGTCAGTCGCGTCGGGTCCGCCGCTCAGTTGAAAGCT 1218
           * S * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * A  384

           M K Q V C G S S K L E L A Q Y R E V A A L A Q F G S D L D A A T Q A L L N R G A  174
Nta(big)S  ATGAAACAAGTCTGCGGTAGTTCAAAACTGGAATTGGCACAATATCGCGAAGTGGCCGCCCTTGCTCAATTTGGCTCAGACCTTGATGCTGCGACTCAGGCATTACTCAATAGAGGTGCA  838
Maize      ATGAAACAAGTCTGCGGTAGTTCAAAACTGGAATTGGCACAATATCGCGAAGTGGCCGCCTTCGCTCAATTTGGGTCAGACCTTGATGCTGCGACTCAGGCATTACTCAATAGAGGTGCA 1338
           * * * * * * * * * * * * * * * * * * * * F * * * * * * * * * * * * * * * * * * *  424

           R L T E V P K Q P Q Y A P L P I E K Q I L V I Y A A V N G F C D R M P L D R I S  214
Nta(big)S  AGGCTGACAGAAGTACCGAAACAACCACAATATGCACCACTGCCAATTGAAAAACAAATTCTAGTCATTTATGCAGCTGTCAATGGATTCTGTGATCGAATGCCACTAGACAGAATTTCT  958
Maize      AGGCTTACAGAAGTGCCCAAACAACCACAATATGAGCCACTTCCAATTGAAAAACAAATTGTTGTTATTTATGCTGCTGTCAACGGCTTCTGTGATCGAATGCCACTAGACAGAATTTCT 1458
           * * * * * * * * * * * E * * * * * * * * V * * * * * * * * * * * * * * * * * * *  464

           Q Y E R A I P N S V K P E L L Q S F L E K G G L T N E R K M E P D T F L K E S A  254
Nta(big)S  CAATATGAGAGAGCCATTCCAAATAGTGTCAAACCAGAATTACTACAATCCTTTTTAGAAAAAGGTGGCTTAACTAACGAAAGAAAGATGGAACCAGATACATTCTTAAAAGAAAGTGCT 1078
Maize      CAATATGAAAAAAACATTCTAAGTACTATTAATCCTGAATTACTAAAATCCTTCTTAGAAAAAGGTGGCTTAACTAACGAAAGAAAGATGGAACCTGATGCTTCTTTAAAAGAAAGCGCT 1578
           * * * K N * L S T I N * * * * K * * * * * * * * * * * * * * * * A S * * * * * *  504

           L A F I stop  258
Nta(big)S  TTAGCTTTTATTTAATACAATACAAGGAAAGGAAAAAGGTCTCTTAGGGGTACCACAAATAATGGACTTACTAGTTCTTTTTCTATCTATGAAGTAGGACGAGTGTCCTACCTAATTCAA 1198
Maize      TTAAATTTATGAGAAGCAAAACTAAACTAATGAGAATGAGTACCACTATTTCTTGGGATAAGAATGCTTCTTCACCAGCAACGGCGAAACTACACTACCAATAACGATGGGAAGAGATAA 1698
           * N L stop  507

Nta(big)S  TGCTAGGTAACCAAGTTAGTATCCTAGCGGAGCTCTTGAAGGGGGGAACATCCTAATGGATGAGTATGAGCACGTTATACATCTCATATTAGACCCCCAGAAAAAAAAAAGAAAAGAAAA 1318

Nta(big)S  AGCAAAAATAGCTAAAAACAGCCTGCTTCCTTTATCATATATATGAGATGGGCGGGCCTTTTTAAGCAAAAGGTGATCCACTATCGGTTGTAGTCTTCTTGACATATCAAGTCAAGAGGG 1438

Nta(big)S  CGAGGCTCCTAGCAATGGGGGGAAAGGGGAGTGGGTAAGCGGGCTCCTTTCACTTGACTGGTTAGTTAGGAGTGATCTTTTTTCGCACATCTCCACTCTTTTTCTCTTTCAAACTAGGAG 1558

Nta(big)S  GATTGGCATTTGGCCAAGATGTGATCTATCTCTTTCCTTGGTTGTAGTGGAACTTTGGTTTTATTTAGCGAGGAACCGTTTCACTCGGCGTCGAATGATCAAA                 1661
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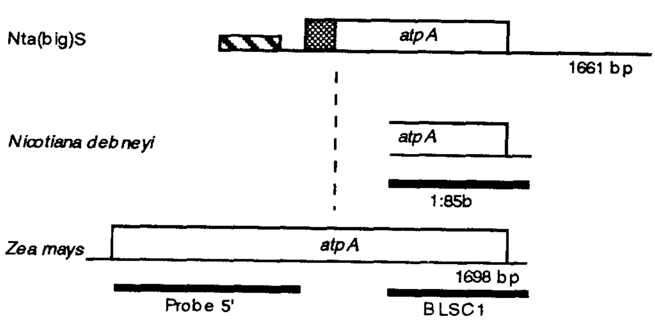
B



identity after nucleotide 431 (*Z. mays* nucleotide 930, Fig. 3 A). From this position to the end of the Nta(big)S reading frame the sequence is 95% and 92% homologous to the *Z. mays atpA* gene at the nucleotide and predicted amino-acid levels, respectively. The 3'-noncoding regions do not show any significant homology. After searching the Genebank and NIH data bases no significant similarity was found between previously published sequences and the P2 sequence from nt 226 to 430. However, from the 5'-end to nt 225 of clone P2, an 89.0% sequence homology to the downstream *trans*-intron of *Arabidopsis thaliana* NADH dehydrogenase (*nad5*) exon c (Brandt et al. 1992) was found. A similar homology (90.0%) from nt 138 to the 5'-end of clone P2 was found in the corresponding region of the *nad5* exon c intron from wheat (Pereira de Souza et al. 1991).

Discussion

DNA gel-blot experiments detected differences between all analysed novel male-sterile progeny from cybrid 28 and pollen-producing progeny from callus 29. However, only probes located in or near the *atpA* gene identified differences between these sterile and pollen-producing cybrid materials. This region was investigated further by the use of 5'- and 3'-end probes of the *atpA* coding sequences from *Z. mays* and *N. debneyi*. Apart from detecting presumably functional copies of the *atpA* gene, the *atpA* 3'-end hybridized to an additional 5-kb *Hind* III fragment as well as to a 4000-nt transcript in both the male-sterile Nta(big)S parental plants and novel male-sterile progeny of cybrids from callus 28. Neither the additional 5-kb *Hin*dIII fragment nor the 4000-nt transcript were detected in offspring of cybrids 29-3 and 29-1 with pollen-producing, petiolated or normal-appearing stamens. It was further found that the fertile cytoplasmic donor, *N. bigelovii*, contained neither the additional *Hin*dIII fragment nor the 4000-nt *atpA* transcript (data not shown). The 5-kb *Hin*dIII fragment, found in the male-sterile Nta(big)S and novel male-sterile cybrid 28 progeny, thus correlates with the flower morphology, split corolla and fringed anthers typical for these materials.

The *atpA* 3'-end-specific mtDNA *Hin*dIII fragment that was detected in Nta(und)S was present in offspring from cybrid 29-1, but absent in offspring of cybrid 29-3. Since the 29-1 offspring produced pollen, the presence of this *atpA* 3'end-specific fragment is not correlated with sterility. Thus, it is possible that the male-sterile phenotype in Nta(und)S is associated with mitochondrial sequences different from those of Nta(big)S. An indication for the involvement of other mtDNA regions in flower development, in addition to the 3'-end of the *atpA* gene, can be inferred from the observation that the offspring of cybrids 29-1 and 29-3, still express other CMS-related traits, such as shortened corolla, indehiscent anthers which produce functional pollen and, in some cybrids, petaloid tissues attached to pollen-bearing anthers. These findings are consistent with the previously stated hypothesis wherein the different floral morphologies of cytoplasmic male-sterile tobacco cultivars are indicative of variation in the activity of several not yet identified mitochondiral genes (Rosenberg and Bonnett 1983; Kofer et al. 1991a).

Changes associated with the *atpA* gene region, although not involving the *atpA* gene itself, have been correlated to the appearance of CMS in several plant species. The frequency of structural changes leading in many cases to the formation of chimeric genes in the *atpA* gene region could be explained by the fact that the gene has been found to be a part of the repeats involved in the recombination of mtDNA in several genera, including *Oenothera* (Schuster and Brennicke 1986), *Z. mays* (Small et al. 1987), *Pisum sativum* (Morikami and Nakamura 1987), *Glycine max* (Chanut et al. 1993) and *Phaseolus vulgaris* (Janska and Mackenzie 1993). In *Helianthus annus* and *P. vulgaris*, co-transcription of *atpA* and CMS-specific ORFs downstream from the *atpA* gene was suggested to be the cause of male-sterility. A rearrangemment in the *atpA* 3'-flanking region, involving an inversion and an insertion, created *orfH522* in a CMS line of *H. annus* (Köhler et al. 1991, Laver et al. 1991). The nuclear restoration of CMS in *H. annuus* has been shown to be concomitant with a tissue-specific post-transcriptional down-regulation of the *orf*H522 15-kDa polypeptide in male florets (Monéger et al. 1994). Three ORFs have been found downstream from the *atpA* gene in male-sterile *P. vulgaris* (Chase and Ortega 1992), and reversion to fertility is accompanied by the deletion of two of these reading frames (Johns et al. 1992). Further indications for the association of *atpA* to CMS in several tobacco CMS systems were obtained by investigations of sexually produced alloplasmic male-sterile *N. tabacum* cultivars with *N. debneyi*, *N. repanda* or *N. suaveolens* cytoplasms, as changes in *atpA* transcription patterns were detected in two of the cultivars (Håkansson and Glimelius 1991). In both cases the introgression of the nuclear restorer genes from the cytoplasmic donor species restored the transcription patterns along with male-fertility.

A sequence analysis was conducted on a subclone (P2) of the 5-kb *Hin*dIII fragment unique to the Nta(big)S phenotype. It was found that P2 contained an unidentified ORF of 38 amino-acid codons, fused in-frame to a truncated copy of the *atpA* gene of 220 amino-acid codons. Based on the mtDNA the predicted amino-acid sequence of the truncated Nta(big)S *atpA* copy is nearly identical to the corresponding region of the *Z. mays* protein, and identical to that of *N. plumbaginifolia* (Chaumont et al. 1988). A further increase in the homology to other *atpA* proteins was obtained after sequence determination of edited cDNA molecules (Bergman et al. 1994). The truncated *atpA* gene copy does not encompass the conserved adenine nucleotide-binding folds A and B proposed by Walker et al. (1982). We can thus speculate that if the chimeric ORF containing the truncated Nta(big)S *atpA* copy is expressed as a protein product which could fold into an *atpA*-like

protein, then potentially it would be capable of competing with the complete atpA protein for assembly into subunit F_1 of the mitochondrial ATPase complex. This could result in ATPase complexes which would not be capable of optimal ADP binding during ATP synthesis.

The presence of sequences, most likely originating from the *trans*-spliced intron of *nad5* exon c, in the near proximity of the truncated *atpA*-like gene copy of Nta(big)S raises some questions. Has the *atpA* gene been recombined into the intron of *nad5* exon c, or has a reverse-transcribed copy of the intron inserted upstream of the recombined *atpA* gene? An indication of transcriptional activity initiated upstream of the clone P2 was found as nearly all of the transcripts detected by the *atpA* 3′-end probe BLSC1 also were detected by a probe derived from the first 316 bp of clone P2 (data not shown). We do not yet know from what promoter these transcripts initiate. It is, however, of interest to note that the approximately 230 bp homology of P2 to the region of *nad5* exon c corresponds to a sequence downstream from the last intron domain in the proposed secondary RNA structure (Knoop and Brennicke 1993), and that the P2 homology ends downstream from the point of sequence divergence in this region between *A. thaliana* and *Oenothera* (Knoop et al. 1991; Brandt et al. 1992). Thus, if the truncated *atpA* gene is co-transcribed with the *nad5* exon c it would not necessarily disturb the maturation of *nad5* RNA. Investigations have been initiated to determine if the region upstream of the Nta(big)S *atpA* copy continues further into the nad5 intron and includes a nad5 exon.

During evolution cytoplasmic and nuclear genomes in a given species or genus have adapted their genetic information to cooperatively direct essential processes, including photosynthesis, respiration, and some aspects of plant morphogenesis. The combination of a nucleus with a foreign cytoplasm may result in a disturbance of the cooperation between nuclear and cytoplasmic factors which becomes manifiested as functional or developmental disorders, one of them CMS. Future investigations are aimed to clarify the possible involvement of this *atpA* region in CMS of tobacco. Gene transfer with edited cDNA from the chimeric *atpA* copy is in progress to determine whether or not expression of this gene is sufficient to confer one or several of the phenotypic changes typical of the male-sterile Nta(big)S cultivar.

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