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A mitochondrial transcript with homology to the 3′ end of the chloroplast psbA gene is present only in the atrazine-resistant biotype of *Chenopodium album*

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Summary. The 3′ end of the chloroplast (ct) psbA gene is present in the mitochondrial genome of *Chenopodium album* and is expressed in mitochondria from atrazine-resistant plants. Short specific chloroplast psbA gene probes reveal a 270 bp homolog with the 3′ end of the chloroplast psbA gene. The homology starts close to the codon 264 of this gene; this codon is known to be involved in atrazine resistance when mutated in the chloroplast DNA. Comparison of susceptible and resistant plants has not given evidence of modification in the mitochondrial (mt) genome; however, the homologous 3′ end of the ct psbA gene present in the mitochondria is expressed as part of a 0.8 kb transcript only in atrazine-resistant plants and not in susceptible ones.

Key words: ct psbA gene – mtDNA – Gene expression – Atrazine resistance – Promiscuous DNA

Introduction

The high turnover protein, Mr 32,000 daltons, is one of the two major products of in vitro protein synthesis of chloroplasts from light-grown higher plants (Edelman et al. 1985; Ellis 1981; Crouse et al. 1985). This protein is involved in photosynthetic electron transport in photosystem II (Mattoo et al. 1981). The gene for this protein is present as a single copy in the higher plants chloroplast genomes (Bedbrook et al. 1978; Driesel et al. 1980) and is called psbA. It codes for an abundant chloroplast RNA whose translation is light-regulated (Mattoo et al. 1984). The 32kd protein appears to be the target of urea derived herbicides such as diuron and atrazine (Arntzen et al. 1982). A single point mutation at codon 264 (ser → gly) of the psbA gene is present in atrazine-resistant plants (*Solanum nigrum* (Goloubinoff et al. 1984), *Amaranthus hybridus* (Hirschberg and Mac Intosh 1983), *Chenopodium album* (Bettini et al. 1986)). In higher plants, this mutation is accompanied by a slowing down of electron transfer between the primary (Qa) and the secondary (Qb) quinones of the photosystem II reaction centers (Arntzen et al. 1979). Mutations at other positions in the psbA gene leading to DCMU (3-3-, 4-dichlorophenyl,-1,1-dimethylurea) and atrazine (2-chloro 4 ethylamine 6-(isopropylamino) s-triazine) resistance and not accompanied by a slowing down of electron transfer have been found in mutants of *Chlamydomonas reinhardii* (Erickson et al. 1985) but never in higher plants.

Chloroplast DNA has been found to be present in the mitochondrial genome of many different higher plants (Stern and Lonsdale 1982; Stern and Palmer 1984; Timmis and Scott 1983; Dron et al. 1985). Because no function has been ascribed to these sequences, they have been considered as selfish DNA in plant mitochondrial genomes. Recently, in maize male-sterile T cytoplasms only it has been found that such sequences could be expressed after site-specific recombination: a $tRNA^{arg}$ gene of chloroplast origin is involved in the creation of a new open reading frame coding for a potential 12,961 Mr polypeptide (Dewey

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et al. 1986). In *Sorghum* (Bayley-Serres et al. 1986) a similar recombination within mtDNA sequences is involved in the modification of a cytochrome c oxidase subunit 1 gene.

Chenopodium album as spinach, is a member of the family of Chenopodiaceae. It possesses a chloroplast DNA map highly related to spinach and identical to *Atriplex triangularis* (Palmer 1982). As usual in higher plants, the ct psbA gene is unique, located in the large single copy region close to the left junction of the inverted repeat (Driesel et al. 1980). The DNA primary sequence of this gene is highly related to that of spinach (Zurawski et al. 1982), and as in other weeds a single base pair mutation (A to G in the first position of codon 264) is observed in atrazine-resistant *Chenopodium album* plants (Bettini et al. 1986).

In this paper we show that the 3′ end of the chloroplast psbA gene is present in the mitochondrial genome of *Chenopodium album;* and that the mitochondrial 3′ end of the psbA gene is transcribed only in atrazine-resistant plants as a 0.8 kb mitochondrial transcript. No transcript for this region has been detected in susceptible plants.

Materials and methods

Plant material

The susceptible (S) and resistant (R) *Chenopodium album* plants were isolated and selected by J. Gasquez and H. Darmency (Dijon, France) (Gasquez and Compoint 1981). They were grown in a greenhouse under controlled temperature and light conditions (16 h light at 23 °C, 8 h dark at 18 °C).

DNA isolation

For nuclear DNA preparations, 5 g of liquid nitrogen frozen leaves were used. They were ground in a mortar and resuspended in 0.4 *M* sucrose, 50 m*M* Tris-HCl pH 8.0, 2 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, 2 m*M* mercaptoethanol. The mixture was filtered through Blutex nylon (50 μ), and centrifuged 10 mn at 1,000 RPM at 4 °C (IEC centrifuge). The pellet was resuspended in 0.25 *M* sucrose, 50 m*M* Tris-HCl pH 8.0, 2 m*M* $CaCl_2$ (buffer 2) and centrifuged again 10mn, 1,000RPM at 4 °C. The pellet was resuspended in 10 ml of the same buffer and loaded on a 25 ml sucrose cushion (2 *M* sucrose, 50 m*M* Tris-HCl pH8.0, 2 m*M* $CaCl_2$). The sample was centrifuged 45 mn at 4 °C and 18,000 RPM (JA 20 Beckman centrifuge) and the resulting pellet resuspended in buffer 2. This pellet contained the nuclei, they were washed by centrifugation 10 mn, 2,500 RPM and lysed overnight at 4 °C in 50 m*M* Tris-HCl pH 8.0, 20 m*M* Na_4EDTA, 1% Sarkosyl and 1 mg/ml pronase. Ethidium Bromide (1 μg/ml) and CsCl were added to the lysate and isopycnic centrifugation was performed as described (Maniatis et al. 1982).

Chloroplast and mitochondrial DNA

Chloroplast and mitochondrial DNAs were extracted from young green leaves of the different biotypes of *Chenopodium album* as described (Dron et al. 1985). Chloroplasts and mitochondria were separated using differential centrifugation steps. Mitochondria were further purified on discontinuous sucrose gradients. The resulting organelles were finally lysed in the presence of sodium lauroyl sarcosinate and pronase (1 mg/ml). CsCl isopycnic gradients were as described (Maniatis et al. 1982). Preparation of the DNA probes, electroelution, agarose gel electrophoresis, nitrocellulose transfer, and DNA/DNA hybridizations were performed according to Maniatis et al. (1982).

The spinach ct psbA gene was a gift of Hans Bohnert (Univ. of Arizona, Tucson). The tobacco ct rbcL gene was a gift of Masahiro Sugiura (Nagoya, Japan), and the wheat mt cytochrome oxidase gene was a gift of Bernard Lejeune (Orsay, France).

RNA isolation

Total and chloroplast RNAs. RNA was prepared from young green seedlings of *Chenopodium album* S and R biotypes. Chloroplasts were prepared at 4 °C as previously described but using sterile glassware and media. The isolated chloroplats were resuspended in 4 *M* Guanidinium thiocyanate, 0.2 *M* Tris-HCl pH 8.0, 2% lauroyl sarcosinate and 0.1% mercaptoethanol. RNA was purified through a cushion of 5.2 *M* CsCl, 0.1 *M* EDTA for 12 h at room temperature at 40,000 RPM in a Ti50 rotor (L65 Beckman centrifuge). The RNA pellet was resuspended in 10 m*M* Tris-HCl pH 7.4, 5 m*M* EDTA, 1% SDS and precipitated with 2 volumes of ethanol. The RNA was finally resuspended in a sterile solution of distilled water.

Mitochondrial RNA. Mitochondria were purified as previously described (Dron et al. 1985) in sterile buffers. Pellets of purified mitochondria were resuspended in 50 m*M* Tris-HCl pH 8.0, 0.3 *M* KCl, 10 m*M* MgAc, 5 m*M* EDTA, 2% Triton X100, 2 m*M* mercaptoethanol and frozen in liquid nitrogen. After thawing at 4 °C, the mixture was centrifuged 10 mn at 10,000 RPM (SS34 rotor, Sorvall RC2b centrifuge). The supernatant contains polysomes and the other species of RNA. For extraction of total mitochondrial RNA, 2 volumes of a solution containing 2% triisopropylnaphthalene sulfonate, 12% sodium amino salicylate and 0.1 *M* NaCl in 20 m*M* Tris-HCl pH 7.4 were added. The solution was chilled 5 mn on ice, extracted twice with 10 m*M* Tris-HCl pH 8.0, 1 m*M* EDTA-saturated phenol and precipitated with 2 volumes of ethanol.

For Northern experiments 3 μg of RNA for each sample were electrophoresed in denaturing agarose formaldehyde gels as described by Maniatis et al. (1982) and transferred to Genescreen (NEN). Hybridization of the resulting blot with electroeluted DNA probes (spinach psbA Hind III 900 bp (Zurawski et al. 1982), tobacco rbcL BamHI 1.1 kbp (Shinozaki and Sugiura 1982), and wheat cytochrome oxidase II Sal I 2.4 kbp DNA fragments (Quetier et al. 1985) were as proposed by the manufacturer (NEN). Filters were then exposed to Kodak X-Ray films.

Results

Characterization of a mtDNA sequence presenting high homology to the 3′ end of the ct psbA gene

We used different parts of the spinach ct DNA fragment Bam5 (Paillard et al. 1985) (Fig. 1) as probes to discriminate between the 5′ and the 3′ ends of the

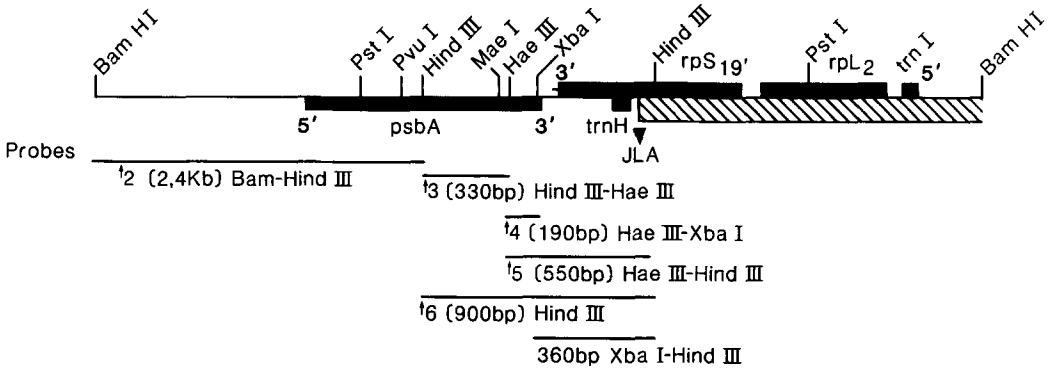

Fig. 1. Restriction map of the spinach chloroplast Bam5 restriction DNA fragment (Zurawski et al. 1982; Zurawski et al. 1984). The *bars under the map* represent the psbA short specific restriction DNA probes which have been used in the different experiments. *Probes 3* and *6* have been cloned in pUC19 and pBR325 respectively (Bettini et al. 1986), the other ones have been electroeluted from Bam5. The *black boxes* represent the genes lying on the two strands (psbA, trnH, rps19', rpL2, trnI) and the *hatched box* represent the left junction of the ct inverted repeat (JLA)

chloroplast psbA gene. The spinach Bam5 fragment was digested by the described restriction enzymes (Fig. 1) and electrophoresed on 5% polyacrylamide gels; the resulting fragments (probes 2, 3, 4, 5) were electroeluted and nicktranslated. They were hybridised to southern blots of Hpa II and EcoRI-restricted ctDNA from susceptible plants (ctS), or mtDNA from susceptible (mtS) and resistant (mtR) plants. The single ctEcoRI 4.1 kbp and the single Hpa II 1.2 kbp hybridization pattern correspond to the chloroplast DNA fragments containing the chloroplast psbA gene (Fig. 2). Probes 4 and 5 (3' end of the ct psbA gene) hybridize to the same ct DNA fragments (EcoRI 4.1 kbp and Hpa II 1.2 kbp) but also to specific mitochondrial Hpa II 0.8 kbp and EcoRI 8.2 kbp DNA fragments which are not present in the chloroplast DNA (Fig. 3) while probe 2 (BamHI-HindIII 2.4 kbp) does not hybridize to any of the mitochondrial DNA fragments (not shown). This result shows that sequences corresponding to the 3' end of the psbA gene are present in the mitochondrial genome of *Chenopodium album*. The 190 bp HaeIII-XbaI (probe 4) fragment which is internal to the psbA gene in the 3' end region hybridizes strongly relative to probe 3. The HindIII-BamHI 1.6 kbp fragment (right end of the map) that includes part of rpS19' and all of rpL2 does not hybridize to mtDNA (not shown). This data shows that at least a region of the chloroplast psbA gene is present in the mt genome, and that the 3' end of the gene is present in a mt 360 bp XbaI-HindIII fragment. To determine the approximate 5' end of the homology between ct and mt DNAs at the psbA gene level, we have used probe 3 (HindIII-HaeIII 330 bp fragment, Fig. 2A, B). This fragment contains the A to G base pair change related to atrazine resistance in higher plants (MaeI restriction site in Fig. 1). The results show that this probe hybridizes only weakly to the same HpaII fragments as probe 4 and 5, suggesting that of the mt/ct DNA homology extends only a short distance upstream from the HaeIII restriction site and probably diverges very close to the psbA gene codon 264 which is involved in atrazine resistance.

By using HpaII, EcoRI (Figs. 2 and 3), MaeI (Fig. 4) and also HindIII, BamHI, SalI, HaeIII (not shown) we have not been able to observe any evidence of recombination in the region of homology between the mtDNA and the chloroplast psbA gene in atrazine susceptible or resistant plants. In each case (Figs. 2 and 3) the different probes used, spanning the overall mt/ct psbA gene homology, hybridize exactly to the same mt restriction fragments either in susceptible or in resistant plants. This demonstrates that there is no major DNA recombination in this mtDNA region after setting up of atrazine resistance. Furthermore, it appears that there is no recombination in the total mitochondrial DNA insofar as there is no difference in the DNA restriction patterns (Fig. 2, staining gel). In the chloroplast psbA gene of resistant plants there is a mutation at codon 264 (Bettini et al. 1986). A MaeI restriction site which overlaps the mutation can be used to discriminate between susceptible and resistant plants. We have used this restriction enzyme (Fig. 4) to search for this mutation in the mitochondrial psbA gene copy by using the ct HindIII 900 bp DNA fragment (probe 6, Fig. 1) as a probe. The results show that although there is a difference at the chloroplast DNA level as already shown (Bettini et al. 1986), there is no difference at the mtDNA level, the upper band visualized in mtS is due to partial digestion.

No homology to the ct psbA gene was found in the nuclear DNA of either susceptible or atrazine-resistant plants (not shown).

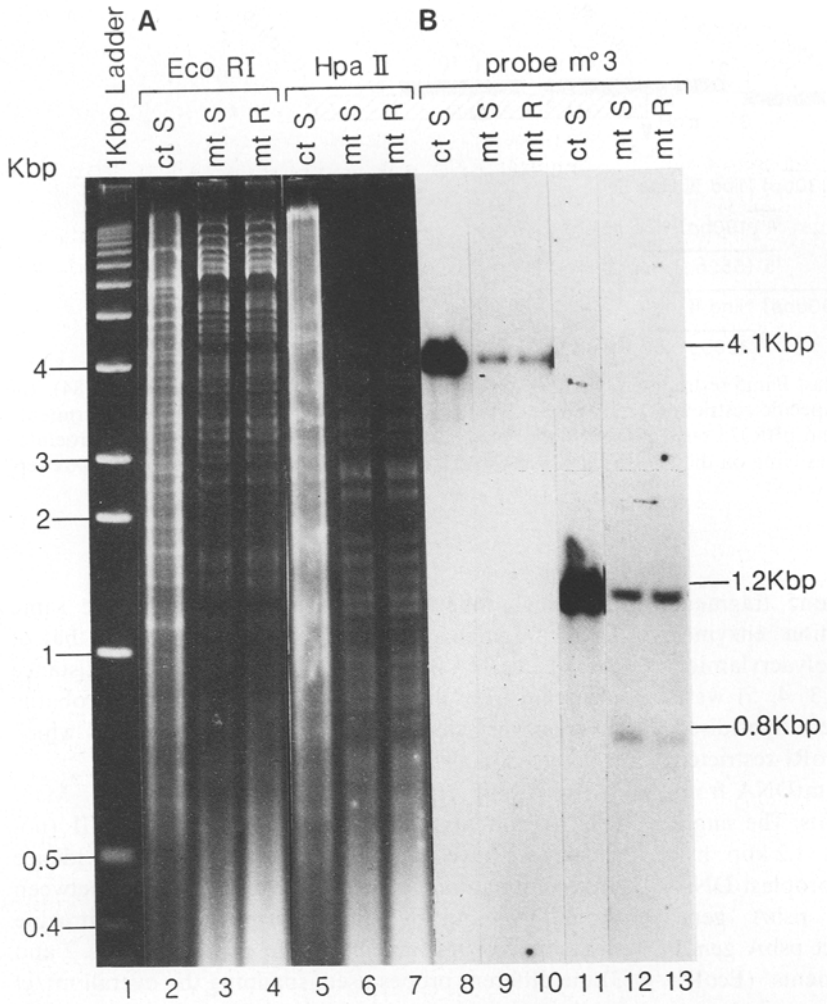

Fig. 2 A, B. Localization of the homology between the ct psbA gene and the mitochondrial genome of *Chenopodium album.* **A** Ethidium Bromide staining of an agarose gel (0.8%) in which were electrophoresed EcoRI and HpaII restriction digests of chloroplast and mitochondrial DNA. *Lane 1:* 1 kb ladder marker, *lane 2, 3, 4:* EcoRI digests of ctDNA from S and of mtDNA from S and R plants; *lanes 5 to 7:* HpaII digests of the same DNAs except that in *lane 5* ctDNA from R plants has been used. **B** Southern blot analysis of electroeluted probe 3 HindIII–HaeIII 330bp in the mtDNA and ctDNA from S and R plants. *Lanes 8 to 13* correspond to *lanes 2 to 7* in **A**

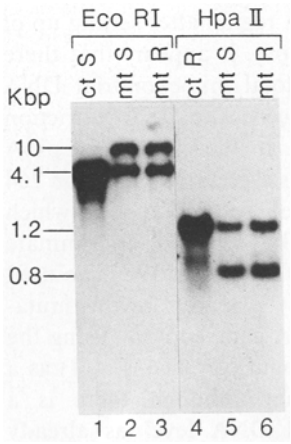

Fig. 3. EcoRI and HpaII-restricted DNA from chloroplast or mitochondrial RNAs hybridized against probe 5. Hybridization with probe 4 produces an identical result (not shown)

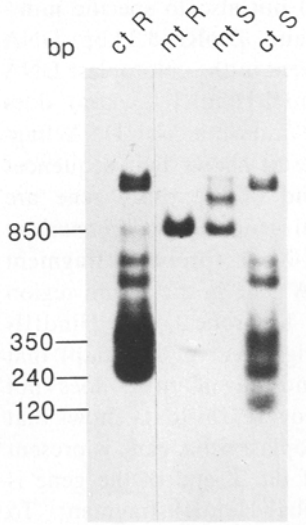

Fig. 4. Southern blot analysis of mt- and ctDNAs isolated from atrazine S and R plants probed with the ct 900bp HindIII probe 6. The DNAs were restricted with enzyme MaeI (C TAG), this enzyme discriminates between atrazine S and R ctDNAs (5, this figure). The figure corresponds to the autoradiogram of the hybridization between the blot issued from the agarose gel and a nicktranslated probe 6 (HindIII 900 bp). The marker refers to a nicktranslated pBR322 HinfI DNA digest

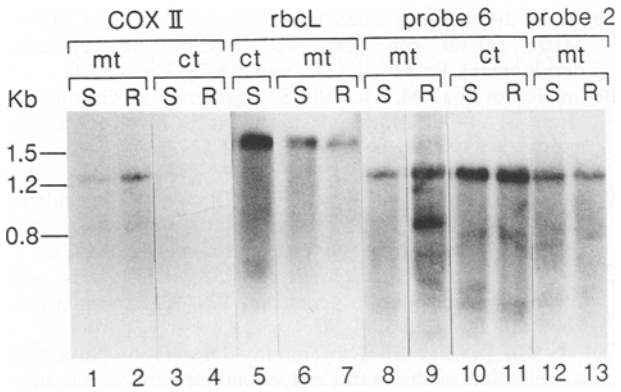

Fig. 5. Northern blot analysis of the psbA messenger RNA in chloroplast and mitochondria from atrazine S and R plants. The RNA was isolated as described in "Materials and methods", electrophoresed in formaldehyde denaturing gels, transferred to Genescreen and analysed with the probes indicated: *lanes 1–4,* probe coxII from wheat; *lanes 5–7,* probe rbcL from tobacco; *lanes 8–11,* using psbA 900 bp probe 6; *lanes 12, 13* probe 2. The sizes of the different transcripts are indicated. mt: RNA from mitochondria; ct: RNA from chloroplasts. S is for susceptible plants and R is for resistant. V.

Analysis of the psbA gene transcripts in the chloroplasts and mitochondria of susceptible and resistant plants of Chenopodium album

The psbA gene of *Chenopodium album* is transcribed as a unique abundant transcript of 1.2 kb in the chloroplasts of light grown plants (Edelman et al. 1985, Fig. 3). Different spinach probes (Fig. 1) have been used to study the steady state level of this transcript in susceptible and resistant plants. The chloroplasts and mitochondrial RNAs were purified separately, electrophoresed on denaturing formaldehyde gels and transferred to Genescreen filters. They were then probed with different DNA inserts corresponding to different parts (probes 2 and 4) of the spinach chloroplast psbA gene (Fig. 1). The rbcL and coxII probes have been used to check cross-contamination between ct and mt RNAs. The results (Fig. 5) show that a 1.2 kb transcript (probe 6) is present in each lane (8 to 11), corresponding to the normal single chloroplast gene. In susceptible plants, only this 1.2 kb transcript is visible as far as total chloroplast and mitochondrial compartments are concerned. The presence of this transcript in the mitochondrial RNA is due to chloroplast contamination as shown also for the large subunit of Ribulose biphosphate carboxylase (lanes 5–7, Fig. 5). In plants resistant to atrazine, a single 1.2 kb transcript in the chloroplasts hybridizes to probe 6 and two transcripts 1.2 and 0.8 kb in the mitochondria. The 0.8 kb transcript appears to be specifically present in total and mitochondrial RNAs extracted from plants resistant to atrazine. It is related only to the mitochondrial 3′ end of the psbA gene as demonstrated by the lack of hybridization to probe 2 (lanes 12, 13). We cannot estimate the exact amount of this transcript because the coxII probe used as a control for mitochondrial RNA was heterologous (wheat, lanes 1–4), but we have loaded five times more RNA in the mt lanes than in the chloroplast ones in order to obtain the same amount of ct psbA 1.2 kb transcript in all lanes (mt and ct). The 0.8 kb transcript of mitochondria from resistant plants is approximately five times less abundant than the ct psbA gene transcript, and is absent in susceptible plants.

Discussion

The 3′ end of the ct psbA gene is present in the mt genome of susceptible and resistant Chenopodium album plants

We have shown that the 3′ end of the ct psbA gene is present in the mitochondrial DNA genome of *Chenopodium album.* The probes we have chosen suggest that the homology between the chloroplast psbA gene and mitochondrial DNA must start very close to codon 264 (where mutation leading to atrazine resistance has been demonstrated in at least three different higher plants (Goloubinoff et al. 1984; Hirschberg and Mac Intosh 1983; Bettini et al. 1986)).

The mt DNA of *Chenopodium album* hybridizes strongly to the HaeIII-HindIII 550 bp probe 5 while it hybridizes weakly to the HindIII-HaeIII 330 bp probe 3 fragment. In maize (Paillard et al. 1985) S1 Cms cytoplasm, a part of the 3′ end of the ct psbA gene, has also been found in the mitochondria. Two main regions of homology have been reported (Sederoff et al. 1986), the shorter one of about 80 bp between amino acids 215 and 240 of the 32 kd protein and the longer one from amino acid 264 to the end of the coding psbA gene sequence. It has been proposed (Sederoff et al. 1986) that the region between these two conserved blocks was involved in recombination events after being introduced from chloroplasts to mitochondria. From our mapping data the beginning of the homology should also start in this region in *Chenopodium album* mt DNA. We cannot say if the transfer happens once in evolution and is then distributed through the plant kingdom, or if we are dealing with separate transfers from chloroplasts to mitochondria.

A mitochondrial psbA gene transcript in Chenopodium album plants presenting resistance to atrazine

As shown before, there is a partial 3′ end copy of the cp psbA gene in the mitochondrial DNA from *Chenopo-*

dium album. A mutation in the chloroplast psbA gene product at position 264 is correlated with the appearance of atrazine resistance in *Chenopodium album* (Bettini et al. 1986) as in other different crops (Goloubinoff et al. 1984; Hirschberg and Mac Intosh 1983). We have shown in this paper that the partial mt DNA copy of this ct DNA gene does not appear to be mutated at the same position 264. However, although mitochondrial RNA is contaminated by chloroplast RNA, a 0.8 kb transcript homologous only to the 3′ end of the psbA gene is expressed in mitochondria from plants resistant to atrazine and not in susceptible plants. The controls show clearly that there is no trace of this RNA transcript in the chloroplasts. It is not known whether any protein product is translated from this 0.8 kb mRNA containing homology to psbA. Recently, it has been shown using in vitro transcription that ctDNA inserted in the mt genome has not lost its transcriptional potentiality (Carlson et al. 1986). Our results suggest that this is also true in vivo. From the mapping experiments it seems that the 0.8 kb mtDNA transcript should overlap ctpsbA coding, non-coding sequences, and probably mtDNA sequences. We do not know if the 0.8 kb mRNA putative product would be involved in atrazine resistance. In maize S male sterile cytoplasms a DNA recombination event is involved in the appearance of a new transcript and corresponding polypeptide product (Dewey et al. 1986). There is no obvious analogous DNA recombination event in *Chenopodium,* but a transcript appears only in resistant plants. The mechanism leading to the expression of this gene is still unknown, but its elucidation may provide some insight into the biology of ct/mt interactions.

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