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Expression in yeast of the T-URF13 protein from Texas male-sterile maize mitochondria confers sensitivity to methomyl and to Texas-cytoplasm-specific fungal toxins

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The mitochondrial gene T-*urf13* from maize (*Zea mays* L.) with Texas male-sterile (T) cytoplasm codes for a unique 13 kd polypeptide, T-URF13, which is implicated in cytoplasmic male sterility and sensitivity to the insecticide methomyl and to host-specific fungal toxins produced by *Helminthosporium maydis* race T (HmT toxin) and *Phyllosticta maydis* (Pm toxin). A chimeric gene coding for T-URF13 fused to the mitochondrial targeting peptide from the *Neurospora crassa* ATP synthase subunit 9 precursor was constructed. Expression of this gene in the yeast *Saccharomyces cerevisiae* yielded a polypeptide that was translocated into the membrane fraction of mitochondria and processed to give a protein the same size as maize T-URF13. Methomyl, HmT toxin and Pm toxin inhibited growth of yeast cells expressing the gene fusion on medium containing glycerol as sole carbon source and stimulated respiration with NADH as substrate by isolated mitochondria from these cells. These effects were not observed in yeast cells expressing T-URF13 without a targeting peptide. The results show that T-URF13 is sufficient to confer sensitivity to methomyl and the fungal toxins in a heterologous eukaryotic system, and suggest that mitochondrial localization of T-URF13 is critical for these functions.

Key words: cytoplasmic male sterility/HmT and Pm toxins/methomyl/mitochondrial protein import/T-URF13

Introduction

Maize with the Texas (T) cytoplasm is male-sterile and specifically susceptible to two fungal diseases, southern corn leaf blight caused by *Helminthosporium (Bipolaris) maydis* race T (teleomorph *Cochliobolus heterostrophus*) and yellow leaf blight caused by *Phyllosticta maydis* (teleomorph *Mycosphaerella zeae-maydis*) (for reviews see Ullstrup, 1972; Laughnan and Gabay-Laughnan, 1983). These fungi produce toxins with similar structures, called HmT toxin and Pm toxin respectively, that specifically affect the membranes of T mitochondria (Miller and Koeppe, 1971; Comstock *et al.*, 1973; Payne *et al.*, 1980; Danko *et al.*, 1984); their effects include mitochondrial swelling, uncoupling of respiration with NADH or succinate as substrate, inhibition of respiration with malate as substrate, dissipation of the membrane potential, and leakage of NAD^+ and calcium ions (Payne *et al.*, 1980; Bervillé *et al.*, 1984; Holden and Sze, 1984, 1987). The insecticide methomyl affects T mitochondria similarly and thus is a functional analog of these toxins (Koeppe *et al.*, 1978; Klein and Koeppe, 1985). The molecular mechanism underlying the action of the toxins and methomyl is unknown.

Several lines of evidence implicate a protein of approximate molecular mass 13 kd, T-URF13, in causing cytoplasmic male sterility and susceptibility to HmT and Pm toxins and to methomyl. T-URF13 is found specifically in mitochondria of T cytoplasm; it is not present in the normal (N) cytoplasm (Forde *et al.*, 1978). Male fertility can be restored to plants with T cytoplasm by the nuclear genes *Rf1* and *Rf2* in combination (Laughnan and Gabay-Laughnan, 1983), and the level of T-URF13 in mitochondria of these plants is significantly reduced relative to near-isogenic male-sterile lines (Forde and Leaver, 1980; Dewey *et al.*, 1987). Fertile plants derived from T cytoplasm have been obtained by regeneration from tissue culture (Gengenbach *et al.*, 1977; Brettell *et al.*, 1980). Almost invariably, these plants are resistant to HmT toxin and no longer synthesize T-URF13 (Dixon *et al.*, 1982; Wise *et al.*, 1987a). Despite these observations, the interrelations between T-URF13 and the phenotypes of cytoplasmic male sterility and toxin sensitivity are unclear. Plants restored to fertility by the action of *Rf1* and *Rf2* are still affected by HmT toxin (Barratt and Flavell, 1975), and the *Rf1* gene without *Rf2* suppresses synthesis of T-URF13 without making plants fertile (Dewey *et al.*, 1987).

The gene coding for T-URF13, T-*urf13*, has been isolated (Dewey *et al.*, 1986, 1987; Wise *et al.*, 1987a). The T-*urf13* gene is unique to T mitochondrial DNA, and the open reading frame is deleted or truncated in fertile, toxin-resistant plants derived from T tissue cultures (Fauron *et al.*, 1987; Rottmann *et al.*, 1987; Wise *et al.*, 1987b). With the identification of T-*urf13* it has become feasible to test directly whether T-URF13 causes either male sterility or susceptibility to HmT and Pm toxins and methomyl by expressing the gene in cells that do not normally contain this protein. In this way, Dewey *et al.* (1988) were able to show that respiration in *Escherichia coli* expressing T-*urf13* is susceptible to HmT toxin and methomyl, providing the first direct evidence that T-URF13 is specifically responsible for the toxin sensitivity and disease susceptibility characteristic of maize with Texas cytoplasm.

One means of investigating the effect of T-URF13 on plant cells, particularly with regard to cytoplasmic male sterility, is to introduce the protein into normal plant mitochondria. In plants, a method of transforming mitochondria directly

has not been reported. The alternative is to transform cells with a chimeric nuclear gene coding for a mitochondrial targeting peptide fused to T-URF13, with the expectation that T-URF13 will be expressed from this gene and translocated into mitochondria in a functional form. Little is known about targeting of polypeptides to mitochondria in plants, and few nuclear genes coding for mitochondrial proteins are available to supply the leader peptide coding sequences. In contrast, mitochondrial targeting in yeasts has been the subject of intensive study (for reviews see Attardi and Schatz, 1988; Grivell, 1988; Hartl *et al.*, 1989), and genetic alteration of mitochondria through nuclear transformation has been accomplished (Banroques *et al.*, 1986; Nagley *et al.*, 1988). We therefore sought to introduce T-URF13 into mitochondria of the yeast *Saccharomyces cerevisiae*.

We report here that T-URF13 can indeed be synthesized in yeast and targeted to mitochondria, provided a suitable leader peptide is chosen. Growth of cells containing T-URF13 in their mitochondria is inhibited by methomyl, HmT toxin and Pm toxin, and all three substances affect the electron transport process in these cells. Thus, the effects of T-URF13 in yeast resemble its effects in *E.coli* and its postulated effects in maize. Since there seems to be considerable conservation of mitochondrial targeting functions among eukaryotes (Hartl *et al.*, 1989; Schmitz and Lonsdale, 1989), it may now be possible to use the same leader peptide to target T-URF13 to plant mitochondria, so as to study directly its effect on plants and in particular on male fertility.

Results

Synthesis and mitochondrial targeting of T-URF13 in yeast

In order to determine whether the T maize mitochondrial polypeptide T-URF13 could be targeted to yeast mitochondria, recombinant genes coding for T-URF13 with or without a mitochondrial targeting peptide were expressed in yeast from the plasmids pJTH1 and pJTH2 respectively (Figure 1; see Materials and methods for a description of all the T-URF13 fusion genes used in this study). In both cases a site-specific mutation was introduced into the T-*urf13* coding sequence to compensate for a presumed difference between the maize mitochondrial and universal genetic codes (Fox and Leaver, 1981). Both forms of T-*urf13* were transcribed from the promoter of the yeast nuclear gene *COX5a*, coding for the precursor to subunit V_a of cytochrome oxidase (Koerner *et al.*, 1985; Cumsky *et al.*, 1987). In pJTH1 the *COX5a* promoter is fused to part of the *Neurospora* nuclear gene *oli*$^+$ coding for the precursor to subunit 9 of ATP synthase (prN9). The first ATG codon downstream of the *COX5a* transcription initiation site is the amino-terminal codon of prN9. Sixty-nine amino-terminal codons of *oli*$^+$ are included in pJTH1, comprising the entire mitochondrial targeting peptide and three amino acids of mature subunit 9. This sequence is fused to T-*urf13* in its first initiation codon so that expression of the gene gives the fusion protein prN9/T-URF13; this fusion replaces the initiator methionine codon of T-*urf13* with a glutamic acid codon. In the control plasmid pJTH2, the entire T-*urf13* coding region was fused to the 5′ end of the *COX5a* gene immediately downstream of the subunit V_a targeting

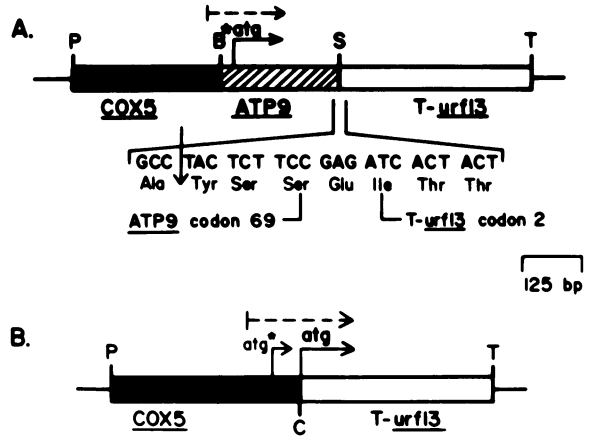

Fig. 1. Gene fusions for expression of T-*urf13* in yeast. (**A**) pJTH1; (**B**) pJTH2. Both gene fusions were cloned in the yeast–*E.coli* shuttle vector YEp352; details of their construction are given in Materials and methods. Genes used are *COX5a*, the yeast gene coding for subunit V_a of cytochrome oxidase (Koerner *et al.*, 1985; Cumsky *et al.*, 1987); *ATP9*, the *Neurospora oli*$^+$ gene coding for subunit 9 of ATP synthase (Viebrock *et al.*, 1982; Gearing and Nagley, 1986); T-*urf13*, the mitochondrial gene from maize T cytoplasm coding for T-URF13 (Dewey *et al.*, 1986). Restriction endonuclease recognition sites are indicated for *Pst*I (P), *Sau*3AI (S), *Sst*I (T), *Bcl*I (C) and a *Bam*HI–*Bgl*II junction (B*). Additional symbols indicate the presumed positions of transcriptional initiation (|- - -→) and translational initiation (┌——→), the matrix protease processing site of ATP synthase subunit 9 (↓) and the disabled translational initiation site of *COX5a* (*——→).

peptide. A site-specific mutation was then introduced to eliminate the *COX5a* initiation codon, so that the first ATG codon sequence in the transcript is the T-*urf13* initiation codon.

pJTH1 and pJTH2 were introduced into the *ura3*$^-$ strains W303 and αW303-11B respectively, and transformants were selected for the wild-type *URA3* gene present on both plasmids. Expression of T-*urf13* in these transformants was detected using polyclonal anti-T-URF13 antiserum. The specificity of this antiserum is shown by its reaction with a 15 kd mitochondrial protein found only in T maize mitochondria (Figure 2A; note that in this gel system T-URF13 migrates at an apparent molecular mass of 15 kd, even though the T-*urf13* nucleotide sequence predicts a protein of 12 961 daltons). Whole cell extracts from three pJTH1 transformants, pJTH1-1, pJTH1-2 and pJTH1-3, were examined for the presence of anti-T-URF13 immunoreactive proteins. In contrast to the parent strains, all three transformants contained two immunoreactive bands of apparent molecular masses 21 and 15 kd (Figure 2B). The 21 kd band corresponds to the size predicted for unprocessed prN9/T-URF13 fusion protein, suggesting that the larger protein has not been imported into mitochondria, while the 15 kd band may represent imported T-URF13 from which the prN9 leader has been cleaved by the matrix processing protease. Mitochondria isolated from these transformants contained predominantly the 15 kd band, although some 21 kd immunoreactive material was also present (Figure 2C). The pJTH2 transformant tested, pJTH2-1, also contained a 15 kd immunoreactive band, which was present in the mitochondrial fraction (Figure 2B and C).

Protease protection assays showed that the prN9 targeting peptide was able to deliver T-URF13 into mitochondria (Figure 2C). The 15 kd protein in the mitochondrial fraction

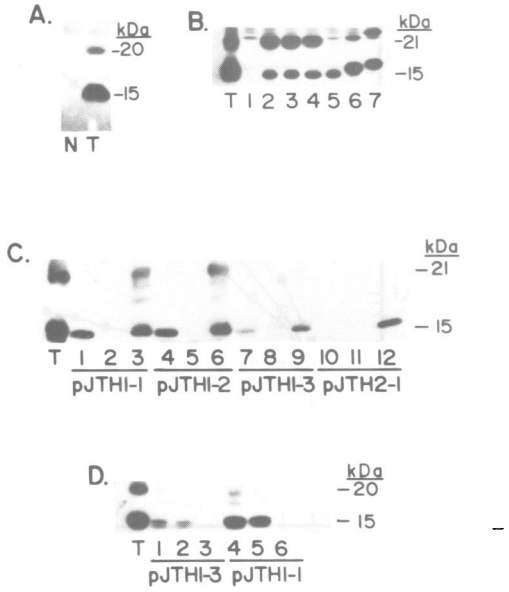

Fig. 2. Immunodetection of T-URF13. Total cellular proteins or mitochondrial proteins were separated by SDS–PAGE (12% acrylamide), transferred to nitrocellulose and challenged with anti-T-URF13; antibody binding was detected with [^{125}I]protein A. Apparent molecular masses were estimated from standards of known molecular mass run in a parallel gel in the same apparatus and stained with Brilliant Blue R (not shown). Maize mitochondria in (**A**) and (**B**) were isolated by centrifugation through a sucrose cushion, and those in (**C**) and (**D**) by density gradient centrifugation. (**A**) Polyclonal anti-T-URF13 antiserum reacts with a protein of ~15 kd found only in mitochondria from T cytoplasm. **Lane N**, mitochondrial proteins from maize inbred W64AN (N cytoplasm); **lane T**, mitochondrial proteins from maize inbred W64AT (T cytoplasm). The immunoreactive material migrating at ~20 kd appears consistently and specifically in T maize mitochondria, and is probably an anomalously migrating aggregate of T-URF13 (C.Lin and E.Hack, unpublished results). (**B**) Expression of T-URF13 in yeast. **Lane T**, 30 μg mitochondrial proteins from W64AT maize; **lane 1**, W303 (parent strain); **lane 2**, pJTH1-1; **lane 3**, pJTH1-2; **lane 4**, pJTH1-3; **lane 5**, pJTH2-1; **lane 6**, pCMS1-1; **lane 7**, pCMS2-1. Lanes 1–7 contain 30 μg total yeast protein. Three bands migrating above the 21 kd protein are due to reaction of anti-T-URF13 with endogenous yeast proteins. (**C**) Proteinase K treatment of yeast mitochondria (30 μg protein) isolated from pJTH1 (pJTH1-1, pJTH1-2 and pJTH1-3) and pJTH2 (pJTH2-1) transformants. **Lanes 3, 6, 9, 12** contain proteins from untreated mitochondria; **lanes 1, 4, 7, 10** contain proteins from mitochondria treated with proteinase K as described in Materials and methods; **lanes 2, 5, 8, 11** contain proteins from mitochondria treated with proteinase K in the presence of Triton X-100. **Lane T** contains 30 μg B73T maize mitochondrial proteins. (**D**) T-URF13 is associated with mitochondrial membranes of yeast transformants containing pJTH1 (pJTH1-3 and pJTH1-1). Isolated mitochondria were fractionated into membrane and soluble portions as described by Fujiki *et al.* (1982). Total mitochondrial proteins (30 μg) were loaded onto the gel along with the membrane or soluble portions extracted from an equivalent amount of mitochondria. **Lanes 1** and **4** contain total mitochondrial protein, **lanes 2** and **5** contain the membrane fraction, and **lanes 3** and **6** contain the soluble fraction. **Lane T** contains 30 μg B73T maize mitochondrial protein.

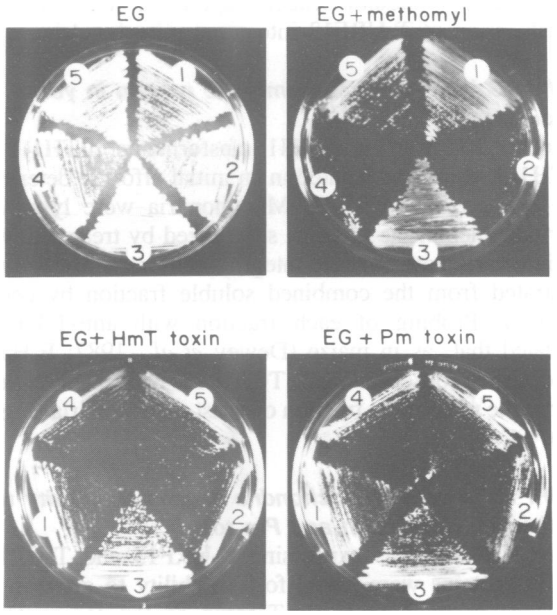

Fig. 3. Methomyl, HmT toxin and Pm toxin inhibit growth of yeast strains containing pJTH1. Yeast transformants and an isogenic parent strain were streaked on EG plates minus or plus methomyl (5 mM), HmT toxin (0.5 μg/ml) or Pm toxin (0.5 μg/ml). Plates were incubated for 72 h (control, methomyl) or 48 h (toxins) at 30°C and then photographed. **Section 1**, pJTH2-1; **section 2**, pJTH1-1; **section 3**, wild-type parent (strain W303); **section 4**, pJTH1-2; **section 5**, pJTH1-3.

of the pJTH1 transformants was protected from digestion by proteinase K, whereas the 21 kd protein was completely digested by this treatment. Disruption of mitochondrial membranes with detergent rendered the 15 kd protein susceptible to proteolysis, indicating that this form of T-URF13 is located within the organelle. The leaderless T-URF13 found in the mitochondrial fraction of the pJTH2 transformant was not protected from digestion by proteinase K (Figure 2C). As a control in these experiments, digestion of the mitochondrial matrix protein hsp60 (McMullin and Hallberg, 1988; Reading *et al.*, 1989) by proteinase K was also monitored (not shown). In all cases, including the pJTH2-1 mitochondria where leaderless T-URF13 was sensitive to the proteinase K, hsp60 was protected from proteolysis by intact mitochondrial membranes.

Two additional T-URF13 fusion proteins were expressed in yeast strain W303, from the plasmids pCMS1 and pCMS2 (see Materials and methods). pCMS1 codes for fusion protein prC5/T-URF13, containing the 20 amino acid amino-terminal leader peptide of cytochrome oxidase subunit V_a (Koerner *et al.*, 1985; Cumsky *et al.*, 1987) fused directly to the amino terminus of T-URF13; expression of the fusion gene is driven by the same *COX5a* promoter used in pJTH1 and pJTH2. pCMS2 codes for fusion protein prA2/T-URF13, in which T-URF13 is fused to the 19 amino acid amino-terminal leader peptide of F_1-ATPase subunit β and 14 amino acids of mature subunit β; expression of this gene is driven by the promoter of the β subunit gene, *ATP2* (Bedwell *et al.*, 1987). Yeast transformants containing these plasmids, pCMS1-1 and pCMS2-1, express appreciable amounts of protein that cross-reacts with anti-T-URF13 antiserum. The apparent sizes of the principal bands in the two transformants, 16.5 kd for pCMS1-1 and 17.5 kd for pCMS2-1 (Figure 2B), correspond to the sizes of the predicted fusion proteins. Processing of the two precursors could not be detected in whole cell extracts (Figure 2B). In both cases, some of the fusion protein co-fractionated with mitochondria, but in neither case were anti-T-URF13 immunoreactive proteins protected by mitochondrial membranes from digestion with proteinase K (not shown). Thus, neither the cytochrome oxidase subunit V_a nor the

F_1-ATPase β subunit leader peptide is able to effect translocation of T-URF13 into yeast mitochondria.

T-URF13 is an integral membrane protein in yeast mitochondria

Mitochondria from two pJTH1 transformants, pJTH1-1 and pJTH1-3, were fractionated in an initial effort to determine the location of T-URF13. Mitochondria were lysed and extrinsic membrane proteins solubilized by treatment with sodium carbonate, and the integral membrane fraction was separated from the combined soluble fraction by centrifugation. Probing of each fraction with anti-T-URF13 revealed that, as in maize (Dewey *et al.*, 1987; E.Hack, unpublished observations), T-URF13 is located entirely within the membrane fraction of yeast mitochondria (Figure 2D).

T-URF13 in yeast mitochondria causes sensitivity to methomyl, HmT toxin and Pm toxin

Yeast transformants expressing T-URF13 and T-URF13 fusion proteins were tested for the ability to grow in the presence of methomyl, HmT toxin or Pm toxin. Three independent transformants containing prN9/T-URF13 (pJTH1-1, pJTH1-2 and pJTH1-3), a transformant containing leaderless T-URF13 (pJTH2-1) and the wild-type parent strain were spread on rich ethanol/glycerol medium (EG) or on EG supplemented with methomyl, HmT toxin or Pm toxin. The pJTH1 transformants showed significantly reduced growth in the presence of methomyl or either toxin, compared to the wild-type strain or pJTH2-1 (Figure 3). In similar experiments, growth of transformants containing prC5/T-URF13 (pCMS1-1) or prA2/T-URF13 (pCMS2-1) on methomyl or either toxin was indistinguishable from growth of wild-type cells (not shown). These results suggested that mitochondrial localization of T-URF13 causes sensitivity to methomyl and the fungal toxins.

Two quantitative experiments were performed to confirm these results. First, the growth rates of pJTH1-1 and pJTH2-1 in liquid EG medium were determined in the presence and absence of methomyl. pJTH2-1, which expresses leaderless T-URF13, grew at approximately the same rate whether or not methomyl was present. In contrast, pJTH1-1, expressing prN9/T-URF13, grew normally in the absence of methomyl but extremely slowly in its presence (Figure 4).

In the second experiment, the various transformants were grown to early stationary phase in minimal glucose medium lacking uracil (WO-U), which should minimize plasmid loss, and ~200 cells were plated on solid media in the presence or absence of methomyl or Pm toxin. After 4 days of growth, the number of colonies formed on each plate was determined (Figure 5; data for pJTH1-1 and pJTH1-2 not shown). For all three pJTH1 transformants tested, the colony-forming efficiency on EG medium in the presence of either methomyl or Pm toxin was ~25% of that observed on EG alone. In contrast, the colony-forming efficiency of pJTH2-1, pCMS1-1 and pCMS2-1 was approximately the same in the presence of either compound as in its absence. For all strains, the colony-forming efficiency in the presence of methomyl on rich glucose medium (YPD; Figure 5) or rich galactose medium (YPGal; not shown) was 80–110% of that on the same medium in the absence of methomyl. Thus, methomyl

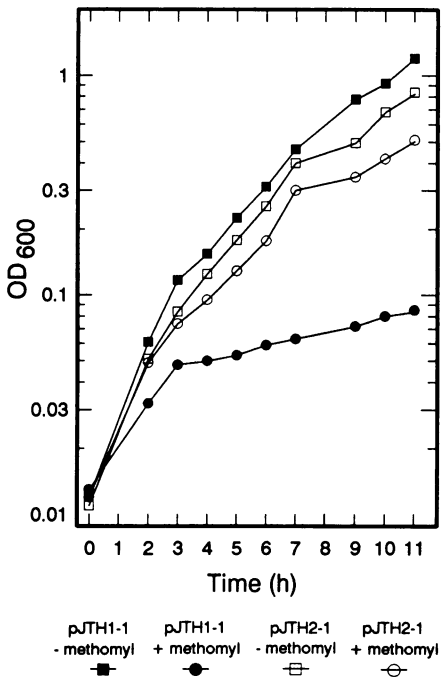

Fig. 4. Growth rate of yeast transformants in liquid EG medium plus or minus methomyl (5 mM), measured by optical density at 600 nm. (■—■), pJTH1-1 minus methomyl; (●—●), pJTH1-1 plus methomyl; (□—□), pJTH2-1 minus methomyl; (○—○), pJTH2-1 plus methomyl. In each case, the initial OD_{600} was 0.01.

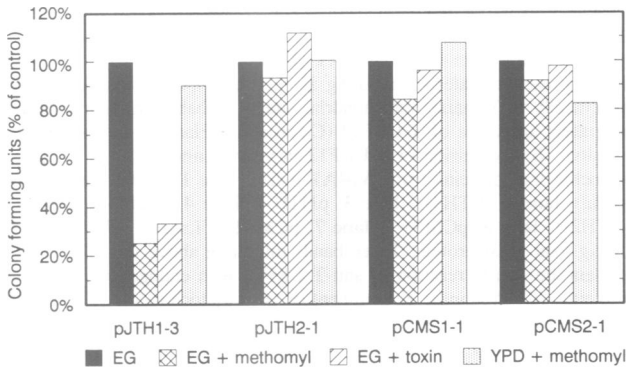

Fig. 5. Effect of methomyl and Pm toxin on growth of colonies from single yeast cells. Yeast transformants pJTH1-3, pJTH2-1, pCMS1-1 and pCMS2-1 were grown in liquid WO-U medium to early stationary phase. The cell density was determined with a hemocytometer and adjusted with sterile water to 5000 cells/ml. Forty microliters of the suspension, containing ~200 cells, was plated on EG, EG plus 5 mM methomyl, EG plus 5 μM Pm toxin or YPD plus 5 mM methomyl. Plates were incubated at 30°C for 4 days and the number of colonies on each plate was counted. For each strain, the number is expressed as a percentage of the number of colonies that grew on the control EG plate (which ranged from 123 to 231).

only prevented growth of pJTH1 transformants on a non-fermentable substrate, glycerol.

If the presence of T-URF13 in yeast is the cause of methomyl and toxin sensitivity, then loss of pJTH1 by mitotic segregation should allow cells to grow on EG in the presence of these compounds. This prediction was tested by replicating colonies formed on EG in the presence of methomyl or Pm toxin to WO-U medium. All such colonies derived from pJTH1 transformants were found to require uracil, and thus

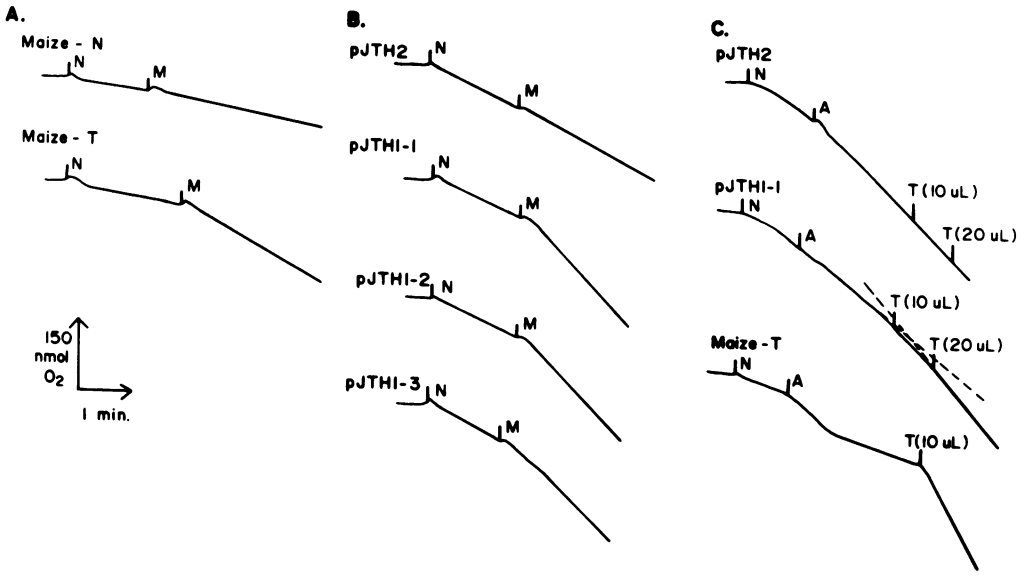

Fig. 6. Effect of methomyl and HmT toxin on rates of respiration by isolated mitochondria. Oxygen uptake rates were measured with a Clark oxygen electrode as described in Materials and methods. (**A**) Mitochondria from W64AN and W64AT maize. Times of additions are indicated for NADH (N) and methomyl (M; final concentration 5 mM). (**B**) Mitochondria from the indicated yeast transformants (pJTH2-1 is labeled pJTH2) with additions as in (A). (**C**) W64AT maize mitochondria and yeast mitochondria assayed in the presence of HmT toxin (indicated volume of a 170-fold concentration of crude *Helminthosporium maydis* race T culture filtrate, prepared according to Yoder *et al.*, 1977 and dissolved in water). Times of addition are indicated for NADH (N), ADP (A) and HmT toxin (T).

had lost the plasmid. In contrast, ~90% of the pJTH2-1, pCMS1-1 or pCMS2-1 colonies growing on EG plus methomyl or Pm toxin had retained their plasmids. Thus, both methomyl and Pm toxin select against pJTH1 but not against pJTH2, pCMS1 or pCMS2. Plasmid loss explains the observation that 25% of the cells from pJTH1 cultures were able to form colonies on EG plus methomyl or Pm toxin.

Taken together, these data indicate that the presence of prN9/T-URF13 in yeast cells causes them to become sensitive to methomyl and the toxins when grown under respiratory conditions. Sensitivity is not induced by leaderless T-URF13, prC5/T-URF13 or prA2/T-URF13.

Methomyl, HmT toxin and Pm toxin affect electron transport in yeast mitochondria containing T-URF13

The effects of T-URF13 on electron transport were assayed by measuring the rates of oxygen consumption by purified mitochondria in the presence of methomyl. In agreement with previous results (Koeppe *et al.*, 1978), we found that with NADH as substrate mitochondria isolated from N maize were not affected by methomyl, whereas T mitochondria showed a significant increase in the rate of oxygen reduction upon addition of this compound (Figure 6A). Mitochondria from wild-type yeast cells (not shown) or from pJTH2-1 (Figure 6B) showed no change in the rate of oxygen consumption upon addition of methomyl to the reaction mixture. In contrast, mitochondria from three independent methomyl-sensitive pJTH1 transformants (pJTH1-1, pJTH1-2 and pJTH1-3) did show a significant increase in the rate of oxygen consumption when methomyl was added (Figure 6B).

The same assay was used to measure the effect of HmT and Pm toxins on yeast mitochondria containing T-URF13. Addition of HmT toxin gave a slight increase in oxygen consumption by mitochondria from pJTH1-1 cells, but had no effect on mitochondria from pJTH2-1 (Figure 6C), pCMS1-1 or pCMS2-1 (not shown). The identical concentration of HmT toxin applied to T maize mitochondria caused a much more substantial increase in the rate of oxygen reduction. The results shown in Figure 6(C) were obtained with a partially purified toxin preparation. Highly purified HmT toxin and Pm toxin also stimulated oxygen consumption by mitochondria from the sensitive transformants pJTH1-1 and pJTH1-2, but had no significant effect on oxygen consumption by mitochondria from wild-type cells or the pJTH2 transformant (Table I).

The effects of methomyl, HmT toxin and Pm toxin on oxygen uptake by whole cells and spheroplasts were also tested (Table I). Methomyl or toxin increased the rate of

Table I. Effects of methomyl, HmT toxin and Pm toxin on oxygen consumption by yeast cells, spheroplasts and mitochondria containing the T-URF13 protein

Strain	Stimulation of oxygen consumption (%)[a]								
	Methomyl (5 mM)			HmT toxin (1 μg/ml)[b]			Pm toxin (1 μg/ml)[b]		
	C[c]	S[d]	M[e]	C	S	M	C	S	M
W303	−6	ND[f]	ND	3	ND	9	4	2	7
pJTH2-1	−6	14	14	8	5	6	9	10	6
pJTH1-1	27	74	124	32	26	16	66	59	20
pJTH1-2	30	76	131	23	29	23	54	67	23

[a] % stimulation was calculated as: 100 × {[O_2 consumption rate after toxin or methomyl addition)/(rate before addition)]−1}.
[b] Purified HmT or Pm toxin, from 200 μg/ml stock in DMSO. With 0.5% (v/v) DMSO alone, there was generally a 5−10% increase in O_2 consumption.
[c] C, whole cells.
[d] S, spheroplasts.
[e] M, mitochondria (NADH as substrate).
[f] Not determined.

uptake by transformants containing pJTH1 but had no significant effect on those containing pJTH2. The effects of these compounds on whole cells and spheroplasts thus corresponded qualitatively to their effects on mitochondria, but there were differences in their relative potency in the three assays.

Discussion

We have directly tested the effects of the T-URF13 protein in a eukaryotic organism by using an amino-terminal leader peptide to introduce it into yeast mitochondria. In many cases, a single leader peptide can effectively target a variety of different proteins into mitochondria (Attardi and Schatz, 1988; Grivell, 1988; Hartl *et al.*, 1989), but the nature of the passenger protein can also influence the transport process. The leader peptide of *Neurospora* ATP synthase subunit 9 (prN9) appears to be particularly effective in targeting integral membrane proteins to mitochondria; this leader directed the import of functional yeast ATP synthase subunit 8, normally the product of the mitochondrial *AAP1* gene, whereas the leader peptide from yeast cytochrome oxidase subunit 6 was not able to do so (Gearing and Nagley, 1986; Nagley *et al.*, 1988). In the current study, prN9 was fused to T-URF13, which is an integral membrane protein in maize (Dewey *et al.*, 1987; E.Hack, unpublished observations), and the hybrid protein prN9/T-URF13 was translated in the cytosol of yeast.

A fraction of the T-URF13 synthesized with the prN9 leader peptide becomes internalized in mitochondria, as shown by its co-fractionation with the mitochondrial pellet and resistance to digestion by proteinase K; in contrast, the amino-terminal targeting peptides of cytochrome oxidase subunit V_a and F_1-ATPase subunit β are unable to deliver T-URF13 to the mitochondrial interior. The internalized protein is converted from the 21 kd prN9/T-URF13 to a 15 kd form. The size of this form is consistent with removal of the amino-terminal leader peptide by cleavage, associated with import, at the natural processing site for production of mature ATP synthase subunit 9 in *Neurospora*. If this processing site is used in yeast, the predicted product, compared to authentic maize T-URF13, would carry four extra amino acids at the amino terminus with loss of the T-URF13 amino-terminal methionine (Figure 1). In a previous study using the prN9 mitochondrial targeting peptide, the natural processing site was indeed used by the yeast mitochondrial import system (Gearing and Nagley, 1986), but at present we do not know the exact processing site in the prN9/T-URF13 fusion protein.

Import of T-URF13 into yeast mitochondria is relatively inefficient; the majority of T-URF13 accumulates in the precursor form and is not processed into the 15 kd protein. This inefficient import of prN9/T-URF13 is not surprising, considering that both the amino-terminal leader peptide and the passenger protein are heterologous in *S.cerevisiae*. The majority of the prN9/T-URF13 does not co-fractionate with mitochondria; the subcellular location of this protein could not be determined, as it was not detectable in a post-mitochondrial supernatant fraction. Therefore, prN9/T-URF13 appears to be unstable upon cell lysis if it is not internalized in mitochondria, presumably because it is susceptible to proteolytic digestion.

Leaderless T-URF13 produced from pJTH2 accumulates in cells to much lower levels than does the prN9/T-URF13 fusion protein produced from pJTH1 (Figure 2B), even though both genes are expressed from the *COX5a* promoter. The basis for this difference in expression levels is not known; possible explanations are differential rates of translation initiation or different rates of protein turnover by proteolysis. Surprisingly, T-URF13 produced from pJTH2 is found associated with mitochondria. This association must, however, occur on the outer face of mitochondria, since the protein is highly susceptible to digestion with proteinase K. Association of leaderless T-URF13 with mitochondria is likely to reflect a non-specific affinity for membranes, since the protein is also detected in a high-speed, microsomal pellet fraction (not shown).

Cells that contain T-URF13 internalized in their mitochondria are sensitive to methomyl, as assayed by the effects of this compound on cell growth and on oxygen consumption by isolated mitochondria or whole cells. Such cells are unable to grow in the presence of methomyl unless they lose the pJTH1 plasmid. In contrast, cells or mitochondria containing leaderless T-URF13 are insensitive to methomyl, and methomyl does not select for loss of the pJTH2 plasmid. These data indicate that T-URF13 must be located inside mitochondria to confer sensitivity. Alternatively, it is possible that the total level of T-URF13 in the cell, with or without an amino-terminal leader, might determine sensitivity. If this were the case, the lack of response to methomyl in pJTH2-1 would simply be due to the low steady-state level of T-URF13 produced from pJTH2 compared to the level of precursor (prN9/T-URF13) plus processed T-URF13 produced from pJTH1 (Figure 2B). Two lines of evidence favor the former explanation. Most importantly, NADH oxidation by isolated mitochondria is affected by methomyl and toxins only in pJTH1 strains, yet mitochondria from both pJTH1 and pJTH2 strains contain similar amounts of T-URF13 (compare lane 12 to lanes 3, 6 and 9 in Figure 2C). Therefore, T-URF13 located on the external surface of mitochondria fails to confer sensitivity to methomyl as measured by oxygen uptake rates, while an equivalent amount of the protein located within mitochondria does cause sensitivity in this assay. Indirect evidence for the importance of mitochondrial localization comes from the pCMS1-1 and pCMS2-1 strains. These accumulate T-URF13 fusion proteins (prC5/T-URF13 and prA2/T-URF13 respectively) to levels comparable to those of prN9/T-URF13, but neither fusion protein is internalized within mitochondria to appreciable levels, neither strain is sensitive to methomyl, and methomyl does not select for loss of pCMS1 or pCMS2. Thus, we suggest that T-URF13 located within mitochondria, rather than excess T-URF13 in the form of prN9/T-URF13, is the causative agent of susceptibility to methomyl.

Like methomyl, HmT toxin and Pm toxin inhibit respiratory growth of yeast cells containing mitochondrial T-URF13 and also stimulate oxygen consumption by whole cells, spheroplasts and isolated mitochondria. In T maize, methomyl and toxins at the concentrations used here have similar effects on mitochondria, whereas the yeast mitochondria are substantially more sensitive to methomyl than to the toxins. There are several differences between the yeast system and T maize that could account for this difference in the effects of T-URF13. The abundance of T-URF13 in yeast mitochondria is much less than in T maize mitochondria

(Figure 2C), and the amino-terminal sequence is probably different. Moreover, the effect of T-URF13 may be influenced by intrinsic differences in the structure and/or function of yeast and maize mitochondria. Expression of T-URF13 in *E.coli*, however, confers similar degrees of susceptibility to methomyl and HmT toxin (Dewey *et al.*, 1988), even though some of the components of the electron transport system in this prokaryote are distinctly different from those in mitochondria (Poole and Ingledew, 1987).

The observation that methomyl, HmT toxin and Pm toxin only prevent growth of strains containing mitochondrial T-URF13 on a non-fermentable substrate raises the possibility that T-URF13 exerts its effects on yeast cells by interfering with respiration or gluconeogenic growth. Since pJTH1 transformants grow in the presence of methomyl on both the repressing sugar glucose and the non-repressing sugar galatose, repression of transcription from the *COX5a* promoter by glucose (Cumsky *et al.*, 1987; Myers *et al.*, 1987) is unlikely to explain this result.

Now that it has been shown that T-URF13 can be targeted to yeast mitochondria and make them susceptible to methomyl and to the fungal toxins, it will be possible to determine whether the prN9/T-URF13 gene fusion behaves the same way in plants. Targeting of proteins to mitochondria is known to have features that are highly conserved between animals and fungi. Conservation of function is indicated by the recent finding that a fungal targeting peptide is able to target a foreign protein to tobacco mitochondria (Schmitz and Lonsdale, 1989). Analyses of transgenic tobacco plants expressing prN9/T-URF13 are in progress. These experiments will test whether the prN9 targeting peptide can deliver T-URF13 to plant mitochondria, and, if so, will examine the effects of T-URF13 in a heterologous higher plant system.

Materials and methods

Strains and growth media

Saccharomyces cerevisiae strains αW303-11B (*MATα leu2 ura3 trp1 his3 ade2*) or W303 (*MATa/MATα leu2/leu2 his3/his3 ade2/ade2 trp1/trp1 ura3/ura3*) were grown in the following media: WO-U [0.67% yeast nitrogen base minus amino acids (Difco), 2% glucose, supplemented with adenine, tryptophan, histidine and leucine at 20 μg/ml each]; YPD (1% yeast extract, 2% glucose, 2% peptone); YPGal (1% yeast extract, 2% galactose, 2% peptone); EG (1% yeast extract, 2% peptone, 2% glycerol). Solid media for yeast contained 2% agar (Difco). For tests of the effect of methomyl and toxins on growth, 100 μl of filter-sterilized Lannate L (Du Pont; concentration 1.48 M) or 150 μl of a 100 μg/ml solution of purified toxin in DMSO (from H.W.Knoche, University of Nebraska, Lincoln, NE) was spread per 30 ml plate.

Yeast transformation (Dieckmann and Tzagoloff, 1983) used WO-U supplemented with 1.2 M sorbitol. *Escherichia coli* strain TG-1 (K12, Δ(*lac-pro*), *supE, thi*$^-$, *hsdD5*/F' *traD36, proA*$^+$*B*$^+$, *lacI*^{q}, *lacZ*ΔM15), used for amplification of plasmids and production of single-stranded DNA, was grown by standard methods (Maniatis *et al.*, 1982). The *dam*$^-$ *E.coli* strain GM33 was used for amplification of plasmids to be digested with *Bcl*I.

DNA manipulations

DNA manipulations were performed using standard procedures (Maniatis *et al.*, 1982; Ausubel *et al.*, 1987). Oligonucleotides were synthesized by the Iowa State University Nucleic Acid Facility using a Biosearch 8750EX automated DNA synthesizer. Single-stranded DNA was prepared from pUC118 and pUC119 based phagemids as described by Vieira and Messing (1987). Oligonucleotide-directed site-specific mutagenesis was by the 'Eckstein' method (Taylor *et al.*, 1985), using a commercial kit (Amersham Corp., Arlington Hts, IL). Nucleotide sequence analysis was by the chain termination method (Sanger *et al.*, 1977).

Table II. Plasmids coding for T-URF13 fusion proteins

Plasmid	Promoter	Leader peptide source	Expressed protein
pJTH1	*COX5a*	ATP synthase subunit 9[a]	prN9/T-URF13
pJTH2	*COX5a*	–	T-URF13
pCMS1	*COX5a*	Cytochrome oxidase subunit V_a	prC5/T-URF13
pCMS2	*ATP2*	ATP synthase subunit β	prA2/T-URF13

[a]From *N.crassa* (all other genes and leader peptides from *S.cerevisiae*).

Plasmid constructions

The plasmids used in this study and the T-URF13 fusion proteins for which they code are shown in Table II. The construction of these plasmids is described below.

T-urf13 coding region. A 1.7 kb *Hin*dIII−*Bgl*II fragment of maize mitochondrial DNA containing the T-*urf13* coding region (nt 1 to nt 1722 of the sequence described by Dewey *et al.*, 1986) was subcloned into pUC119. Three changes were made in the T-*urf13* sequence by oligonucleotide-directed site-specific mutagenesis. First, the T residue at nt 1247 was changed to a G. This mutation eliminated a *Bcl*I site within the T-*urf13* coding region without changing the codon identity, thereby facilitating use of a *Bcl*I site within the initiation codon (nt 1216) for construction of gene fusions. Second, the CGG codon at nt 1473, which is believed to code for tryptophan in maize mitochondria but arginine in the universal genetic code (Fox and Leaver, 1981), was changed to the tryptophan codon TGG. Third, the G at nt 1606 was changed to a C, introducing an *Sst*I site at nt 1601, 41 nt downstream of the T-*urf13* termination codon. The 1.6 kb *Hin*dIII−*Sst*I fragment (nt 1−1601) containing T-*urf13* with all three changes was subcloned into pUC119 to form plasmid pTI4. The nucleotide sequence of the entire T-*urf13* coding region was determined in pTI4, and no mutations were found other than the three changes described above (not shown).

COX5a promoter and mitochondrial targeting peptide. The yeast nuclear gene *COX5a*, coding for subunit V_a of cytochrome oxidase, is located within a *Pst*I fragment containing nt −273 to nt 299 (Koerner *et al.*, 1985; Cumsky *et al.*, 1987; nt 1 is the A of the *COX5a* initiation codon). This fragment was subcloned in pUC119, and two restriction enzyme recognition sites were introduced by site-specific mutagenesis. First, the sequence ATGATCA was inserted following nt 60, creating a *Bcl*I site immediately following the C-terminal codon of the subunit V_a mitochondrial targeting peptide (Koerner *et al.*, 1985). The plasmid containing this mutation, pVL, was further modified by replacement of the A at nt −19 with a T. This mutation creates a *Bgl*II site at nt −24, 2 or 7 nt respectively downstream of the two major transcription initiation sites of *COX5a* (Cumsky *et al.*, 1987). This plasmid was denoted pVP.

Neurospora ATP synthase subunit 9 mitochondrial targeting peptide. Plasmid pUC19N9L (P.Nagley and R.J.Devenish, Department of Biochemistry, Monash University, Clayton, Australia) contains the *Neurospora crassa oli*$^+$ gene coding for the precursor to ATP synthase subunit 9 (prN9; Viebrock *et al.*, 1982), engineered to contain *Bam*HI sites 28 bp upstream of the initiation codon and 5 bp downstream of the termination codon (Gearing and Nagley, 1986).

Yeast expression plasmid pJTH1. The *Bam*HI−*Sau*3AI fragment from pUC19N9L coding for the mitochondrial targeting peptide of prN9 (nt 30−257, Viebrock *et al.*, 1982; Gearing and Nagley, 1986) was ligated to the *Bcl*I−*Sst*I fragment from pTI4 containing mutagenized T-*urf13* (nt 1216−1601, Dewey *et al.*, 1986), using pUC119 as the cloning vector. Restriction mapping and nucleotide sequence analysis of the resulting plasmid determined the relative orientation of the two coding regions and verified that the segment of *oli*$^+$ and T-*urf13* were fused in-frame (see Figure 1). The fused coding sequence was then ligated to the *COX5a* promoter, in a yeast−*E.coli* shuttle vector. The *Bam*HI−*Sst*I fragment containing the fused *oli*$^+$ and T-*urf13* coding regions, and the *Pst*I−*Bgl*II fragment from pVP containing the *COX5a* promoter and transcription initiation sites (nt −273 to −24, Cumsky *et al.*, 1987), were ligated in a three-fragment reaction into YEp352 (Hill *et al.*, 1986) digested with *Pst*I and *Sst*I. The product of this ligation, pJTH1, was mapped using restriction enzymes to confirm that the promoter, mitochondrial targeting sequence and T-*urf13* coding sequence were present in the necessary sequence and orientation (not shown).

Yeast expression plasmid pJTH2. The *Pst*I–*Bcl*I fragment of pVL, containing the *COX5a* promoter and mitochondrial targeting sequence (nt −273 to 60, Cumsky *et al.*, 1987), and the *Bcl*I–*Sst*I fragment of pTI4, containing the T-*urf13* coding region (see above), were ligated in a three-fragment reaction into pUC118 digested with *Pst*I and *Sst*I. Ligation of these two *Bcl*I sites results in an in-frame fusion between T-URF13 and the subunit V_a mitochondrial targeting sequence, with the initiator methionine codon of T-URF13 immediately following the C-terminal codon of the targeting peptide. The G residue at nt 3 of *COX5a* was then changed to a T, eliminating the *COX5a* initiation codon and leaving the initiation codon of T-*urf13* as the first ATG sequence present in the transcript. The mutated *Pst*I–*Sst*I fragment was then cloned in YEp352, resulting in the yeast expression plasmid pJTH2.

Yeast expression plasmid pCMS1. pCMS1 is identical to pJTH2, except that the *COX5a* initiation site has been left intact. Thus, the amino-terminal 20 amino acids of subunit V_a are fused to T-URF13, and expression of the fusion gene construct is driven by the *COX5a* promoter.

Yeast expression plasmid pCMS2. An *Eco*RI–*Pvu*II fragment containing the promoter and mitochondrial targeting sequence of the yeast nuclear gene *ATP2*, coding for the F_1-ATPase β subunit (Bedwell *et al.*, 1987), was subcloned into pUC118. A deletion series starting at the downstream *Pvu*II site was constructed with nuclease *Bal*31, and *Bam*HI linkers were attached to the ends of the deleted plasmids. Nucleotide sequence analysis revealed a clone containing a *Bam*HI site at codon 34 of *ATP2*. The *Eco*RI site upstream of the *ATP2* promoter was then changed to a *Pst*I site using an oligonucleotide adaptor (5′-AATTCTGCAG-3′). Finally, pCMS2 was constructed by ligating the *Pst*I–*Bam*HI fragment of *ATP2* and the *Bcl*I–*Sst*I fragment of T-*urf13* from pTI4 (see above) into YEp352 digested with *Pst*I and *Sst*I, in a three-fragment ligation. Ligation of the compatible *Bam*HI and *Bcl*I sites results in an in-frame fusion between T-URF13 and the β subunit mitochondrial targeting sequence plus 14 amino acids of the mature polypeptide; the initiator methionine codon of *ATP2* is the expected translational start site of the fusion protein.

Antibodies to T-URF13

For purification of T-URF13 protein, mitochondria were isolated from T maize etiolated shoots and immature ears (inbreds B37T, B73T and W64AT) by differential and sucrose density gradient centrifugation (Leaver *et al.*, 1983). Total proteins from T mitochondria were separated by preparative SDS–PAGE and the 15 kd protein band seen only in T mitochondria was collected from these gels (Lin, 1987). Purified T-URF13 was injected into rabbits to raise polyclonal antibodies (Lin, 1987). The antiserum used for the experiments reported here, denoted anti-T-URF13, was from a single bleeding of one rabbit.

Cell fractionation and immunodetection of T-URF13

Maize mitochondria were isolated by differential and sucrose density gradient centrifugation (Leaver *et al.*, 1983) from etiolated shoots grown in vermiculite for 4–5 days at 30°C; in some experiments (see figure legends), centrifugation through a 0.6 M sucrose cushion (Day and Hanson, 1977) was substituted for the density gradient.

Yeast mitochondria were prepared from cells grown to early stationary phase in EG medium, as described by Daum *et al.* (1982). Soluble and membrane fractions were prepared from purified mitochondria by treatment with sodium carbonate followed by centrifugation at 100 000 g_{max} for 30 min (Fujiki *et al.*, 1982). Total protein fractions from whole yeast cells were prepared by trichloroacetic acid precipitation, as described by Hurd *et al.* (1987). Protein concentrations were determined by the Bio-Rad (Bio-Rad Laboratories, Richmond, CA) protein assay (Bradford, 1976).

Protein fractions were separated by SDS–PAGE and transferred to nitrocellulose membranes (Towbin *et al.*, 1979). The membranes were incubated with anti-T-URF13 and membrane-bound IgG was detected by incubation with ^{125}I-labeled protein A (Du Pont–NEN Research Products, Wilmington, DE) as described by Schmidt *et al.* (1984). For protease protection assays, isolated mitochondria were incubated with proteinase K (Boehringer-Mannheim Biochemicals, Indianapolis, IN) at 0.22 mg/ml for 30 min at 0°C, in the presence or absence of 1% Triton X-100. The incubation was terminated by the addition of 1 mM phenylmethylsulfonyl fluoride (Pfanner and Neupert, 1987).

Measurement of oxygen uptake rates

Oxygen consumption rates were measured in isolated mitochondria with a Clark oxygen electrode at 25°C as described (Ohnishi *et al.*, 1966). The reaction medium for both yeast and plant mitochondria contained 0.6 M mannitol, 10 mM potassium phosphate (pH 6.5), 20 mM Tris–HCl (pH 6.5), 10 mM KCl, 1 mM EDTA and 0.3% BSA. Each assay was carried out in 3 ml of reaction medium with a mitochondrial protein concentration of 0.2 mg/ml. NADH (0.5 mM) was used as substrate. Oxygen consumption by whole yeast cells or spheroplasts was measured as described by Dewey *et al.* (1988) for whole *E.coli* cells, except that the reaction medium for spheroplasts contained 1.2 M sorbitol as an osmotic stabilizer.

Methomyl or toxin was added to the reaction after a steady rate of oxygen consumption was established. Methomyl was diluted from Lannate L. HmT toxin was either a 170-fold concentration of crude *Helminthosporium maydis* race T culture filtrate, prepared according to Yoder *et al.* (1977) and dissolved in water, or purified toxin dissolved in DMSO at 200 μg/ml (see figure legends). Pm toxin was purified toxin dissolved in DMSO at 200 μg/ml.

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