Targeting of fusion proteins to organelles in yeast and tobacco: investigation of the molecular basis of toxin susceptibility in mitochondria of maize (Zea mays L.) with the Texas male-sterile cytoplasm

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Targeting of fusion proteins to organelles in yeast and tobacco: Investigation of the molecular basis of toxin susceptibility in mitochondria of maize (*Zea mays* L.) with the Texas male-sterile cytoplasm

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Iowa State University, 1990
Targeting of fusion proteins to organelles in yeast and tobacco: Investigation of the molecular basis of toxin susceptibility in mitochondria of maize (Zea mays L.) with the Texas male-sterile cytoplasm

by

Jintai Huang

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GENERAL INTRODUCTION

Cytoplasmic Male Sterility

Overview
The inability of plants to produce functional pollen when female reproductive function is completely or nearly normal has been termed male sterility. Male sterility is of more widespread occurrence in higher plants than female sterility, possibly because the male sporophyte and gametophyte are more vulnerable to environmental and physiological stress than are the protected ovule and embryo sac. In addition, the identification of male sterility is technically easier than that of female sterility (Kaul, 1988).

Male sterility can arise spontaneously or can be induced by physical and chemical mutagens through mutations in nuclear genes, cytoplasmic genes, or both. This type of male sterility is heritable. The male-sterile phenotype can also be induced by physiological or ecological stresses such as those resulting from temperature, photoperiod, or climate (for reviews see Edwardson, 1970; Kaul, 1988). In this case, the phenotype cannot be transmitted to the next generation. Heritable male sterility, or genetic male sterility, includes three types (Kaul, 1988). The first type is genic male sterility, where the male-sterile phenotype is controlled by
one or more nuclear genes and is inherited in a Mendelian pattern. The second type, gene-cytoplasmic male sterility, conventionally called cytoplasmic male sterility (CMS), is controlled by the interaction between nuclear genes and cytoplasmic genes. This type of male sterility is inherited through the maternal parent in a non-Mendelian fashion. There may be a third type where male sterility is controlled solely by cytoplasmic genes, although the existence of this type remains to be determined (Kaul, 1988).

CMS occurs mainly through interspecific, intraspecific, or intergeneric crosses, probably because simultaneous or successive mutations in both nuclear and cytoplasmic genes in the same cell, a rare event, would otherwise be needed. Cross hybridization, on the other hand, provides an efficient way to combine independent nuclear and cytoplasmic mutations. An example of this is the first report of CMS in plants by Bateson and Gairdner (1921), who found that male-sterile progeny could be produced by crossing two strains of flax in one direction but not in the other. CMS has been documented in 16 species and 271 species crosses as of 1988 (Kaul, 1988). Among these are many economically important crops, such as sorghum (Stephens, 1937), rice (Hsu, 1945), and maize (Rhoades, 1931; 1933). CMS has been studied extensively, primarily because of its breeding value and its potential application in hybrid seed production (Stephens and Holland,
1954; Duvick, 1959). Besides, CMS has for a long time been the only well-documented non-lethal mitochondrial dysfunction in higher plants.

**Mitochondrial and chloroplast genomes**

Plant cells have, besides the nucleus, two other types of DNA-containing organelles, the chloroplast (Sager and Ishida, 1963; Chun et al., 1963) and the mitochondrion (Luck and Reich, 1964), that are capable of synthesizing some of their own proteins. The cytoplasmic factors that interact with nuclear genes to confer male sterility would therefore be carried by either the chloroplast (cp) or the mitochondrial (mt) genome.

Chloroplasts are transmitted predominantly through the maternal parent (Sears, 1980), although in some species they are transmitted biparentally (Metzlaff et al., 1981) or even strictly through the paternal parent (Szmidi et al., 1987; Neale and Sederoff, 1989). Chloroplast genomes of angiosperms are relatively constant in size and conformation. In the great majority of angiosperms examined, chloroplast DNA (cpDNA) varies in size over the relatively narrow range of 135-160 kilobase pairs (kbp) (Palmer, 1985). In most plants, the chloroplast genome consists of a unicircular molecule with two large segments of unique sequence separated by a large inverted repeat sequence of 20-28 kbp, part of which encodes
ribosomal RNAs (Whitfeld and Bottomley, 1983). The chloroplast genome of higher plants, therefore, has a relatively constant structure and molecular homogeneity.

Mitochondria are transmitted strictly through maternal parent in most eukaryotes examined (Boynton et al., 1987; Neale and Sederoff, 1989). In contrast to chloroplast genomes, mitochondrial genomes of higher plants are characterized by a more complicated physical structure, apparent molecular heterogeneity and widely varying sizes (for reviews see Leaver and Gray, 1982; Lonsdale, 1984; Pring and Lonsdale, 1985; Newton, 1988). They vary in size from 208 kbp in a Brassica species (Palmer and Herbon, 1987) to about 2500 kbp in muskmelon (Ward et al., 1981). Plant mitochondrial genomes usually contain one or more repeated sequences (Pring and Lonsdale, 1985), but repetitive DNA does not contribute significantly to their large size (Ward et al., 1981; Borck and Walbot, 1982). Although mitochondrial genomes of higher plants are substantially larger than typical mammalian (15-18 kbp) or fungal (18-80 kbp) mitochondrial genomes (Tzagoloff and Myers, 1986; Cantatore and Saccone, 1987), all eukaryotic mitochondrial genomes appear to code for a similar group of products, including rRNAs, tRNAs, and proteins of inner membrane enzyme complexes (for reviews see Attardi, 1985; Tzagoloff and Myers, 1986; Newton, 1988; Levings and Brown, 1989). One conceivable reason for the apparently
unnecessarily large size of plant mitochondrial genomes is
that they may contain more genetic information than
mitochondrial genomes of other eukaryotes. The α-subunit of
ATP synthase is encoded by mitochondrial genes in plants
(Boutry et al., 1983; Hack and Leaver, 1983; Braun and
Levings, 1985) but by nuclear genes in animals (Attardi, 1985)
and yeast (Tzagoloff and Myers, 1986). Subunit 9 of ATP
synthase is encoded by a mitochondrial gene in plants, and
also in the yeast Saccharomyces cerevisiae, but by a nuclear
gene in mammals and Neurospora (van den Boogaart et al., 1982;
Hack and Leaver, 1984; Dewey et al., 1985). The plant
mitochondrial genome codes for a 5S rRNA that is not encoded
by mitochondrial genomes of other organisms (Leaver and Gray,
1982). Also, there is evidence that plant mitochondrial
genomes can code for ribosomal proteins (Leaver and Gray,
1982; Bland et al., 1986; Li et al., 1988); none of the
approximately 85 mitochondrial ribosomal proteins appears to
be encoded by mammalian mtDNA (Schieber and O'Brien, 1985).
Another unique feature of plant mitochondrial genomes that may
be related to their unusual size and organization is the
relatively high frequency of rearrangements that involve both
intra- and inter-molecular recombination (Palmer, 1985;
Levings and Brown, 1989). Plant mitochondrial genomes are
often organized as multiple circular molecules that are
derived from a master chromosome as the result of
recombination between repeated sequences in the master chromosome (Lonsdale et al., 1984; Palmer and Shields 1984; Quetier et al., 1985).

**Chloroplast genomes and cytoplasmic male sterility**

Some early studies indicated a correlation between chloroplasts and CMS. Biochemical and genetic analysis of chloroplast-encoded proteins (Chen et al., 1976; 1977) and restriction endonuclease fragment analysis of chloroplast DNAs (Frankel et al., 1979) suggested involvement of the chloroplast genome in CMS in tobacco. Correlations between ctDNA and CMS have also been described in other plant species. That chloroplasts might carry CMS genes in barley was suggested by Ahokas (1978). Differences in restriction fragment patterns of ctDNA from CMS and male-fertile sorghum (Pring et al., 1982) and *Brassica* (Vedel et al., 1982) may implicate chloroplasts as the bearers of cytoplasmic genes responsible for CMS in these plants. By using two-dimensional polyacrylamide gel electrophoresis (PAGE), several differences in number and relative positions of the separated restriction fragments of ctDNA between CMS lines and their respective maintainers were found in maize, wheat and rape (Li and Liu, 1983). Changes in ctDNA might be involved in CMS in these plant species too.
Mitochondrial genomes and cytoplasmic male sterility

Despite the various suggestions of chloroplast DNA involvement, more detailed analysis indicates that it is the mitochondrial genome, not the chloroplast genome, that is the carrier of the cytoplasmic genes involved in CMS. By studying male-sterile cytoplasmic hybrids obtained by fusing protoplasts of different tobacco lines, it was found that there was no relationship between ctDNA and CMS in somatic hybrids of *N. tabacum* and *N. sylvestris*, since chloroplast segregation and transmission were independent of CMS and fully random (Belliard et al., 1979; Aviv and Galun, 1980; Aviv et al., 1980). In contrast, differences were found in mtDNA between male fertile and CMS lines. Similar analysis supported the hypothesis that mtDNA is involved in CMS in tobacco (Bonnett and Glimelius, 1983). Analysis of somatic hybrids in petunia also indicated involvement of mtDNA in cytoplasmic male sterility (Boeshore et al., 1983; Izhar et al., 1983; Clark et al., 1985).

Evidence is now overwhelming that the mitochondrial genome contains the cytoplasmic genes that interact with nuclear genes to confer CMS (Leaver and Gray, 1982; Hanson and Conde, 1985; Pring and Lonsdale, 1985). Restriction endonuclease fragment analysis of mtDNA from male-fertile and male-sterile lines of a single plant species (usually with the same nuclear background) reveals a close correlation between
CMS and altered mtDNAs. In many plant species examined, distinct restriction pattern differences are found between mtDNAs of the male-fertile and CMS lines. These include maize (Levings and Pring, 1976; Pring and Levings, 1978; Levings et al., 1979; Thompson et al., 1980; Borck and Walbot, 1982), sugar beet (Powling, 1982; Powling and Ellis, 1983; Boutin et al., 1987), *Brassica* (Vedel et al., 1982; Vedel et al., 1987), petunia (Izhar et al., 1983; Kool et al., 1983), sorghum (Pring et al., 1979; Conde et al., 1982), faba beans (Boutry and Briquet, 1982), wheat (Quetier and Vedel, 1977), and rice (Yamaguchi and Kakiuchi, 1983; Mignouna et al., 1987). In nearly all cases where ctDNA is examined, however, no obvious differences are detected between ctDNA of male-fertile and CMS lines (Pring and Levings, 1978).

An independent line of evidence indicating involvement of mtDNA in CMS came from analysis of proteins synthesized by isolated mitochondria. There are three types of male-sterile cytoplasm in maize, each characterized by the presence or absence of specific polypeptides (Forde et al., 1978; Forde and Leaver, 1980), and there is a close correlation between the polypeptides synthesized and the type of cytoplasm (see below). Mitochondria from three male-sterile cytoplasms of sorghum synthesize high molecular mass proteins that are not synthesized by mitochondria from the male-fertile Kafir cytoplasm (Dixon and Leaver, 1982; Bailey-Serres et al.,
1986). Correlation of specific polypeptides with either fertile or sterile cytoplasm was also found in tobacco (Boutry et al., 1984; Li et al., 1988), faba beans, sugar beet and wheat (Boutry et al., 1984).

Some of the most convincing evidence for the involvement of mitochondrial gene products in CMS has been obtained from studies in petunia. A mtDNA region, named the S-pcf locus, was identified in petunia somatic hybrids because it always co-segregated with cytoplasmic male sterility in somatic hybrids (Boeshore et al., 1983; Izhar et al., 1983; Boeshore et al., 1985). Sequencing of this region revealed a chimeric gene called pcf (Young and Hanson, 1987), which is composed of the 5'-flanking and amino-terminal segment of the ATP synthase subunit 9 gene (atp9), parts of the cytochrome oxidase subunit II (coxII) coding region, and the carboxyl terminus and the 3'-flanking region of an unidentified reading frame (urfS). The pcf gene was transcribed in leaves, ovaries, and anthers of CMS plants, but the transcripts were 4 to 5 times more abundant in anthers than in leaves, whereas the abundance of atp9 gene transcripts was the same in all tissues tested (Young and Hanson, 1987). The pcf gene could code for a protein of 38 kDa. Using antibodies raised against oligopeptides prepared from the deduced amino acid sequence, Nivison and Hanson (1989) identified a protein of 25 kDa that was specifically synthesized by mitochondria from CMS petunia.
plants. The amount of the 25 kDa protein was greatly reduced in a petunia line with male sterile cytoplasm but carrying the dominant fertility restorer (Rf) gene. That the 25 kDa protein was specifically synthesized by mitochondria of CMS petunia plants and its expression was suppressed by the Rf gene that confers fertility to the CMS line suggests a direct association of this protein with cytoplasmic male sterility in petunia.

**Cytoplasmic male sterility in maize**

Cytoplasmic male sterility in maize was first reported by Rhoades (1931, 1933). The majority of the cytoplasms from various sources can be classified into one of four types (Laughnan and Gabay-Laughnan, 1983). These are the fertile or normal (N) cytoplasm, the Texas (T) male-sterile cytoplasm (cms-T), the USDA (S) male-sterile cytoplasm (cms-S), and the Charrua (C) male-sterile cytoplasm (cms-C). The three types of CMS in maize were originally identified on the basis of their differing response to nuclear genes, called restorer of fertility (Rf), that confer fertility to maize plants carrying a male-sterile cytoplasm (Duvick, 1965; Beckett, 1971).

Two dominant restorer genes, Rf1 and Rf2, are required for complete restoration of fertility to cms-T plants. Restoration of fertility in cms-T is sporophytic; a cms-T plant that is heterozygous at both restorer gene loci will
produce all normal pollen even though only one-fourth of the pollen grains carry both restoring alleles. $Rf_1$ and $Rf_2$ have been assigned to chromosome 3 (Duvick et al., 1961) and chromosome 9 (Snyder and Duvick, 1969), respectively. A single dominant gene, $Rf_3$, is required for fertility restoration to cms-S plants. The mode of fertility restoration in cms-S is gametophytic; a cms-S plant heterozygous for the restorer gene will produce only one-half normal pollen since only one-half of the pollen grains carry the dominant $Rf_3$ allele. $Rf_3$ is located on chromosome 2 (Laughnan and Gabay, 1975). Exactly how many loci are involved in fertility restoration to cms-C plants is uncertain. Kheyr-Pour and co-workers concluded that a single dominant gene, $Rf_4$, is involved in cms-C fertility restoration (Kheyr-Pour et al., 1981), but other studies indicated that two other restorer genes, $Rf_5$ and $Rf_6$, are also required (Josephson et al., 1978; Laughnan and Gabay-Laughnan, 1983). Fertility restoration in cms-C is sporophytic, but complete restoration of fertility is found only in certain nuclear backgrounds (Beckett, 1971).

**Genome organization of maize mitochondria**

The maize mitochondrial genome is organized typically as reported in other higher plants (Palmer and Shields, 1984; Quetier et al., 1985). The N mitochondrial genome is 570 kbp
in size as determined by restriction mapping and can be represented on a single circular master chromosome. The master chromosome contains six repeated sequences, five direct and one inverted, that are active in recombination (Lonsdale et al., 1984). The circular master chromosome of the cms-T genome is 540 kbp in length and its organization is very different from that of the N mitochondrial genome (Fauron and Havlik, 1989; Fauron et al., 1989). The N and cms-T genomes share approximately 500 kbp of common sequences, but there is considerable variation in sequence organization as the result of structural alterations from large and small permutations. The N mitochondrial genome contains a total of 70 kbp of sequences not present in the cms-T genome; the cms-T genome has 40 kbp of sequences not found in the N genome. These differences can be accounted for by the presence of different repeated sequences, the absence in cms-T of a 12 kbp sequence representing an integrated form of plasmids S1 and S2 (see below), the presence or absence of 9 kbp of chloroplast sequences, and a number of sequences of unknown origin unique to either N or cms-T DNA (Fauron and Havlik, 1989).

**Plasmids in maize mitochondria**

In addition to the main chromosome, plant mitochondria contain many minilinear or minicircular DNA or RNA plasmids (Pring and Lonsdale, 1985; Finnegang and Brown, 1986).
Mitochondria of the S cytoplasm contain two episomal linear DNA plasmids, the 6.4 kbp S1 and the 5.4 kbp S2 (Pring et al., 1977), that are not present in other cytoplasms. Sequences homologous to S1 and S2 have been identified in the main mitochondrial genome of N cytoplasm, but are apparently absent from T and C male-sterile cytoplasms (Lonsdale et al., 1981; Thompson et al., 1980). Recombination between S1 and S2 and their homologous sequences in the main cms-S mitochondrial genome causes linearization of the main genome accompanied by integration of the free S1 and S2 plasmids (Schardl et al., 1984). Spontaneous reversion to fertility occurs at unusually high rates in field-grown cms-S plants because of either nuclear gene mutations or cytoplasmic changes (Laughnan et al., 1981). Reversion to fertility was not associated with a change in status of the S plasmids in all nuclear backgrounds (Levings et al., 1980; Escote et al., 1985; Schardl et al., 1985). It seems that cms-S pollen sterility is not strictly dependent on the presence of the S plasmids and that nuclear genes influence maintenance of and changes in the elements. It has been speculated that cms-S sterility may be related to the disruptive effect of rearrangements associated with the S plasmids rather than to information encoded by the elements (Levings and Brown, 1989).

Mitochondria of N, cms-T, and cms-C maize also contain linear or circular DNA plasmids (for reviews see Pring and
Lonsdale, 1985; Sederoff and Levings, 1985). The size of DNA plasmids of mitochondria was proposed as diagnostic for each type of cytoplasm (Kemble and Bedbrook, 1980; Kemble et al., 1980; Weissinger et al., 1982). For instance, in a survey of 31 North American cytoplasms, a 2.1 kbp linear DNA plasmid was found to be present in all cms-T plants, while a larger 2.35 kbp linear plasmid was found in all other cytoplasms (Kemble et al., 1980). When, however, Mexican races of maize were surveyed, the 2.1 kbp plasmid was found in some lines carrying cytoplasms with an N-type restriction enzyme digestion pattern (Kemble et al., 1983). This 2.1 kbp plasmid was also found in the universal restorer line Ky21, which was shown, by restriction endonuclease fragment analysis, to have a male-fertile cytoplasm (Newton and Walbot, 1985). The DNA plasmids in the mitochondrial genome appear to be optional in that they may occur in variable arrays within a species and their absence does not affect normal mitochondrial functions. One exception to this is the forementioned 2.35 kbp and 2.1 kbp plasmids in maize, which were found to carry an essential gene coding for tRNA_{Trp} (Marechal et al., 1987). It has been proposed that the universal maintenance of either the 2.35 kbp (in N, C, and S maize) or the 2.1 kbp (mainly in T maize) plasmid may result from the fact that they contain an essential gene.
Maize mitochondria and cytoplasmic male sterility

As for many other higher plant species, mitochondria are believed to be the carrier of cytoplasmic factors responsible for cytoplasmic male sterility in maize. Restriction endonuclease fragment analysis of maize mtDNA first led Levings and Pring to propose that it is the mtDNA that is involved in male sterility (and disease susceptibility, see below) in maize (Levings and Pring, 1976; Pring and Levings, 1978; Levings and Pring, 1979). Substantial differences in restriction fragment patterns of mtDNA from N, cms-T, cms-C and cms-S were reproducibly found, irrespective of the endonuclease used (Pring and Levings, 1978; Koncz et al., 1980; Borck and Walbot, 1982). No such differences were observed with ctDNAs, except that the HindIII digestion pattern of cms-S was slightly different from that of N cytoplasm (Pring and Levings, 1978). Each type of cytoplasm is characterized by distinctive mtDNA bands, although the majority of the bands are common to all four cytoplasms. The types of maize cytoplasm identified on the basis of fertility restoration genetics correlate well with studies at the molecular level. A limited extent of polymorphism was observed in mtDNAs isolated from a number of inbred lines carrying N cytoplasm (Levings and Pring, 1977), especially in the regions of mtDNA from N cytoplasm that are homologous to the S plasmids (McNay et al., 1983). Similarly, a limited
amount of diversity was detected in restriction analysis of members of the cms-C group (Pring et al., 1980; Pring et al., 1987). These observed changes did not result in a fragment pattern which resembled that of mtDNA from the N, T, or S cytoplasm.

Comparative light and electron microscopy studies also implicate maize mitochondria in causing cytoplasmic male sterility. Following meiosis, the mitochondria of the tapetal cells of cms-T anthers underwent a change in their ultrastructure and then mitochondria of microspores degenerated (Warmke and Lee, 1977, 1978; Lee and Warmke, 1979). These changes were not observed in anthers of plants of the same inbred line but with normal cytoplasm. An independent study found, however, that mitochondria in cms-T plants did not always follow this pattern of degeneration (Colhoun and Steer, 1981). When cms-C anthers were studied by the same techniques, abnormalities were observed in the tapetal cells at the tetrad stage of pollen development, but mitochondria were not affected (Lee et al., 1979). Abnormalities were observed in pollen development in cms-S plants and mitochondria showed structural changes when pollen aborted (Lee et al., 1980). Again, these abnormalities were not seen in the corresponding tissues of plants with normal cytoplasm.

Analysis of proteins synthesized by isolated mitochondria
also indicates the involvement of mtDNA in CMS of maize. Mitochondria of the three male-sterile cytoplasms of maize synthesize specific polypeptides that are not synthesized by those of normal cytoplasm (Forde et al., 1978; Forde and Leaver, 1980). Cms-T mitochondria specifically synthesize a 13 kDa polypeptide that is not found in mitochondria from N, C, or S cytoplasm. In addition, cms-T mitochondria lack a 21 kDa polypeptide that is synthesized by all other cytoplasms. Interestingly, synthesis of the 13 kDa polypeptide is suppressed in the fertility-restored cms-T line (Forde and Leaver, 1980). Similarly, cms-C mitochondria synthesize an additional polypeptide of 17 kDa that replaces a 15 kDa polypeptide in N mitochondria. Synthesis of the 17 kDa polypeptide is not, however, suppressed by fertility restoration (Forde and Leaver, 1980). Mitochondria from cms-S maize synthesize a number of high molecular mass polypeptides that are not synthesized by any other mitochondria. Several of these polypeptides are encoded by the S1 and S2 plasmids (Manson et al., 1986; Zabala et al., 1987; Zabala and Walbot, 1988). These polypeptides appear not to be related to the CMS phenotype since they are synthesized in both sterile and fertile plants of cms-S maize.
The Texas male-sterile cytoplasm

The Texas male-sterile cytoplasm (cms-T) of maize is perhaps the most extensively studied cytoplasm among higher plants. It is unique not only because of its practical breeding value and potential application in hybrid seed production (Duvick, 1959), but also because of its close association with another well documented higher plant mitochondrial dysfunction (see below). Maize plants with T cytoplasm are completely male sterile but female fertile. Two nuclear genes, Rf1 and Rf2, can together restore fertility completely in a sporophytic fashion (see above). These features make cms-T cytoplasm ideal for commercial production of hybrid seeds. Indeed, the use of cms-T cytoplasm in hybrid maize seed production was so successful that by 1970 more than 85% of maize grown in the United States was cms-T (Laughnan and Gabay-Laughnan, 1983). However, utilization of cms-T in hybrid seed production had to be abandoned because it turned out that cms-T maize was highly susceptible to two fungal diseases, southern corn leaf blight (Tatum, 1971) caused by Helminthosporium maydis (Cochliobolus heterostrophus), race T, and yellow corn leaf blight (Scheifele et al., 1969) caused by Phyllosticta maydis (Mycosphaerella zeae-maydis) (for reviews see Ullstrup, 1972; Gregory et al., 1977; Laughnan and Gabay-Laughnan, 1983). Both pathogens produce toxins, called HmT toxin and Pm toxin, respectively, that consist of a mixture of
C₃₅ to C₄₅ linear polyketals (Karr et al., 1974; Kono and Daly, 1979; Danko et al., 1984). HmT and Pm toxins specifically affect the membranes of cms-T but not N, C, or S mitochondria (Miller and Koeppe, 1971; Comstock et al., 1973; Barratt and Flavell, 1975; Payne et al., 1980; Danko et al., 1984); their effects include mitochondrial swelling, uncoupling of respiration with NADH or succinate 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; Holden and Sze, 1987). Cms-T maize lines carrying Rf1 and Rf2 genes are male fertile and mitochondria from these lines were found to be significantly less susceptible to toxins (Barratt and Flavell, 1975). The insecticide methomyl is specifically toxic to cms-T plants (Humaydan and Scott, 1977), producing physiological effects similar to those of HmT and Pm toxin on T mitochondria (Koeppe et al., 1978; Klein and Koeppe, 1985).

As discussed earlier, cms-T mitochondria synthesize a unique 13 kDa protein (Forde et al., 1978). This 13 kDa protein, named T-URF13, is not synthesized by N, C, or S mitochondria, and its level is significantly reduced in the presence of fertility restorer genes (Forde and Leaver, 1980). Using end-labeled mtRNA from T or N cytoplasm to hybridize to a cms-T mtDNA library, a 9.0 kbp BamHI fragment, designated TURF2B, was identified that hybridized much more intensely to
mtRNA from T than to mtRNA from normal cytoplasm (Dewey et al., 1986). Significant hybridization of T mtRNA was confined to three consecutive HindIII fragments, totaling 3547 bp, internal to the 9.0 kbp region. Sequencing of this 3547 bp region, designated TURF2H3, showed that it originated by multiple recombination events involving portions of the flanking and/or coding region of the maize mitochondrial 26S rRNA gene, the ATP synthase subunit 6 gene, and the chloroplast tRNAArg gene. Two open reading frames (ORFs) were identified within TURF2H3. The larger one, designated ORF25, can code for a polypeptide of ca. 25 kDa. ORF25 is believed to be an essential mitochondrial gene, since a homologous transcript was found in all four maize cytoplasms and in mitochondria from bean, wheat, pea, rice and tobacco (Dewey et al., 1986; Stamper et al., 1987). The smaller open reading frame, T-urfl3, can code for a polypeptide of ca. 13 kDa. T-urfl3 is unique to cms-T mtDNA; its transcript was found only in T mitochondria (Dewey et al., 1986). T-urfl3 codes for the same 13 kDa protein (T-URF13) that was first reported by Forde and co-workers (Forde et al., 1978; Dewey et al., 1987; Wise et al., 1987a). The Rf1 gene, which causes the reduction in the amount of T-URF13 protein in mitochondria, seems to alter the pattern of transcripts of T-urfl3. The other fertility restorer gene, Rf2, has no effect on the T-urfl3 transcript pattern, nor is it involved in
suppressing T-URF13 translation. The function of Rf2 in fertility restoration is unknown.

Several lines of evidence suggest that T-URF13 is responsible for cytoplasmic male sterility and susceptibility to HmT and Pm toxins and to methomyl. First, T-URF13 is synthesized specifically by cms-T mitochondria and the gene coding for it is found only in cms-T mtDNA (Forde et al., 1978; Dewey et al., 1986; Dewey et al., 1987; Wise et al., 1987a). Second, the transcript pattern of T-urfl3 is altered in fertile maize plants with T cytoplasm but carrying the fertility restorer genes (Dewey et al., 1987), the synthesis of T-URF13 is suppressed in these plants, and mitochondria isolated from them have reduced susceptibility to HmT toxin (Barratt and Flavell, 1975). The most convincing evidence come from analysis of fertile revertants obtained from tissue cultures of cms-T maize (Gengenbach et al., 1977; Brettell et al., 1980). These fertile revertants are no longer susceptible to HmT toxin. The T-urfl3 coding sequence either is deleted or has undergone a frame-shift mutation, so that T-URF13 is not synthesized or only a truncated version is synthesized (Dixon et al., 1982; Umbeck and Gengenbach, 1983; Fauron et al., 1987; Rottmann et al., 1987; Wise et al., 1987b). More recently, it has been shown that when T-URF13 is expressed in E. coli, respiration of E. coli transformants is susceptible to HmT and Pm toxins and to methomyl (Dewey et
al., 1988; Braun et al., 1989a). Using site-directed mutagenesis, Braun and co-workers have shown that the amino terminal 83 residues of T-URF13 are enough to confer toxin or methomyl sensitivity in *E. coli* (Braun et al., 1989b). The authors also have identified two dicyclohexylcarbodiimide (DCCD) binding sites in the T-URF13 protein and shown that one of these sites, the aspartate at position 39, is involved in conferring the previously observed DCCD protection against toxins or methomyl (Bouthyette et al., 1985; Holden and Sze, 1987). Substitution mutations at position 39 also eliminate toxin sensitivity. It is clear, therefore, that the T-URF13 protein causes susceptibility to toxins and methomyl. Whether or not the same protein is responsible for cytoplasmic male sterility is still unknown.

**Protein Import into Mitochondria and Chloroplasts**

**Overview**

Eukaryotic cells are divided into distinct compartments or organelles by intracellular membranes. Each organelle performs specialized functions mediated by a characteristic group of proteins. A typical eukaryotic cell normally contains at least six different types of organelles; they are
the nucleus, endoplasmic reticulum (ER), Golgi apparatus, peroxisomes, lysosomes (or vacuole), and mitochondria. Plant cells and algae are peculiar in that they contain in addition the photosynthetic organelle, the chloroplast. Mitochondria and chloroplasts have their own genomes and independent transcription and translation machinery, so that they can synthesize their own proteins. Still, most mitochondrial and chloroplast proteins, and probably all proteins of other organelles, are encoded by nuclear genes, synthesized in the cytosol, and subsequently translocated to the organelle. All cytosolically-synthesized organellar proteins are sorted by two main pathways. In the first, the secretory pathway, synthesis and translocation are usually tightly coupled processes (for review see Schatz, 1986); this is called cotranslational translocation. Proteins destined for the ER, Golgi apparatus, lysosomes (or vacuoles), and plasma membranes are translocated by this pathway; proteins are translocated first into ER, from there to the Golgi apparatus, and then to other destinations via vesicles. In the second pathway, proteins that are destined for the nucleus, peroxisomes, chloroplasts and mitochondria are translocated posttranslationally; the translation and translocation processes are clearly separated (for reviews see Hay et al., 1984; Douglas et al., 1986; Grivell, 1988). Present knowledge about protein translocation has led researchers to focus on several
aspects of this process. Questions can be raised as to what signal directs a protein to its correct organelle, how a protein maintains its translocation-competent state when it is synthesized, how a protein interacts with target membranes, what the energy requirement is for protein translocation, and what cytosolic or target membrane factors are involved in the translocation process.

**Targeting signals for imported mitochondrial and chloroplast proteins**

The discovery that imported mitochondrial (Maccecchini et al., 1979) and chloroplast (Dobberstein et al., 1977; Chua and Schmidt, 1978) proteins are synthesized in the cytosol as larger precursors with an amino terminal extension that is not found in the organellar form of the protein suggests that the targeting signal is carried within the amino terminus of the precursor protein itself. It is now believed that most mitochondrial and chloroplast proteins are synthesized with such a defined amino terminal extension, termed the leader peptide, targeting peptide, or transit peptide, that directs their organelle-specific translocation and that is proteolytically removed upon importation (for recent reviews see Attardi and Schatz, 1988; Grivell, 1988; Hartl et al., 1989; Keegstra, 1989; Keegstra et al., 1989). Some mitochondrial proteins, however, are synthesized with a non-
cleavable targeting peptide at their amino terminus. For example, the 70 kDa outer membrane protein in yeast has an amino terminal portion that carries information for targeting (and for outer-membrane sorting) but is not cleaved off during import (Hase et al., 1983; Riezman et al., 1983a; Hase et al., 1984). Two other outer-membrane proteins, the 29 kDa porin in yeast (Mihara et al., 1982; Mihara and Sato, 1985) and the 31 kDa porin in Neurospora (Pfaller et al., 1985; Kleene et al., 1987), also do not have a cleavable leader peptide. Sometimes a protein may contain targeting information in discrete internal regions. An example of this is the ADP/ATP carrier, a mitochondrial inner membrane protein, which carries a targeting signal in residues 72 through 97 (Smagula and Douglas, 1988). This targeting signal is probably duplicated in the ADP/ATP carrier since two other homologous domains were found in the protein (Arends and Sebald, 1984; Adrian et al., 1986). There are other proteins whose carboxyl terminus is important for efficient translocation (Ness and Weiss, 1987; Stuart et al., 1987).

Targeting signals are necessary and usually sufficient for directing protein transport into organelles. The necessity of leader peptides for protein translocation has been demonstrated in experiments showing that precursor proteins lacking leader peptides cannot be imported into mitochondria (Riezman et al., 1983b; van Loon and Young, 1986).
or chloroplasts (Mishkind et al., 1985; Anderson and Smith, 1986). On the other hand, foreign proteins fused to leader peptides can be imported into mitochondria (Hurt et al., 1984; Horwich et al., 1985b) and chloroplasts (van den Broeck et al., 1985). Sequences that are not specific for mitochondrial protein import can also target passenger proteins to mitochondria. A so called "cryptic mitochondrial targeting signal" has been identified within the cytosolic protein dihydrofolate reductase (DHFR) of mouse (Hurt and Schatz, 1987). A high percentage of random fragments of total E. coli DNA codes for polypeptides that can restore mitochondrial import to a truncated cytochrome oxidase subunit IV (COXIV) precursor lacking most of its own leader peptide (Baker and Schatz, 1987). These findings suggest that any stretch of amino acids, irrespective of its source, could be a potential targeting signal as long as it meets certain biochemical and physical requirements.

**Structural properties of targeting peptides**

It would be reasonable to expect that leader peptides from precursor proteins destined for the same organelle, for example, the mitochondrion, would share related amino acid sequences. When the amino acid sequences of the leader peptides of a number of mitochondrial precursors are analyzed, however, no significant primary sequence similarities are
detected (von Heijne, 1986; Hartl et al., 1989).

Nevertheless, the mitochondrial leader peptides from various eukaryotic organisms do share several common features: a) they are rich in positively charged residues (mainly arginines); b) they contain few, if any, acidic amino acid residues; c) they generally have a high content of hydroxylated and small hydrophobic residues; and d) in most cases they show a tendency to form an amphiphilic α-helix (Hartl et al., 1989).

The positively charged residues in a leader peptide have been demonstrated to be critical for its function. When three arginine residues were substituted with glycines in the leader peptide of human ornithine transcarbamoylase (OTC), the modified precursor protein was no longer imported into mitochondria (Horwich et al., 1985a). Using a yeast complementation assay system, Vassarotti and co-workers showed that spontaneous mutations could restore import to F1 ATPase β-subunit whose leader peptide had been deleted (Vassarotti et al., 1987). Analysis of the surrogate leader peptides found in the amino-terminus of the truncated F1 precursor indicated that they were generated through mutations in which acidic residues had been replaced by basic or neutral residues. Similarly, Bibus et al. (1988) identified six different mutations in a truncated yeast mitochondrial protein that restored its import. All six mutations generated new amino-
terminal extensions that were rich in basic, hydroxylated, and hydrophobic amino acids and thus resembled authentic mitochondrial leader peptides (Bibus et al., 1988). Bedwell and co-workers addressed the question of sequence and structural requirements for a mitochondrial protein import signal by saturation cassette mutagenesis (Bedwell et al., 1989). They found that at least two basic residues were necessary for the ATPase F1 leader peptide to function. On the other hand, acidic amino acids are found to interfere actively with import competence (Bedwell et al., 1989).

Hydrophobic residues are also important for the function of a leader peptide. Among the six mutations identified by Bibus et al. (1988), one mutation changed a hydrophilic threonine into a hydrophobic isoleucine and restored import to the truncated precursor protein. Skerjanc and co-workers used predictive secondary structure computations as a guide to localize the putative membrane binding region in the OCT leader peptide and found that replacement of leucine residues at positions 5, 8, and 9 with a less hydrophobic residue, alanine, reduced the rate of precursor import four to five fold compared to the wild type (Skerjanc et al., 1988).

Theoretical structure analysis of a number of mitochondrial leader peptides suggests that they have the ability to form amphiphilic helices, i.e., an $\alpha$-helix with a hydrophobic face and a highly positively-charged face (von
Experimental data have shown that amphiphilicity is an essential feature for the function of the leader peptide (Roise et al., 1988). In the mutational analysis of several mitochondrial leader peptides, it was found that any mutation that created or stabilized an amphiphilic structure would improve import (Horwich et al., 1985a; Vasarotti et al., 1987; Bedwell et al., 1989). All the peptides encoded by random segments of *E. coli* DNA that directed the import of COXIV exhibited the potential to form amphiphilic helices (Baker and Schatz, 1987). The necessity of amphiphilicity for mitochondrial leader peptide function has been tested by constructing completely artificial leader peptides consisting of only the three amino acids leucine, serine, and arginine, apart from the initiating methionine (Allison and Schatz, 1986). Six such artificial leader peptides were chemically synthesized and three of them could direct the import of a passenger protein into mitochondria *in vivo*. Circular dichroism measurements in the presence of detergent micelles showed that all three functional artificial leader peptides could form an amphiphilic secondary structure; two were largely α-helical while the third existed almost exclusively as β-sheet (Allison and Schatz, 1986; Roise et al., 1988). No mitochondrial targeting function was detected with the other three artificial peptides. These contained the same number of
positive charges as the functional peptides, but were essentially non-amphiphilic because most of the hydrophobic leucines had been replaced by glutamines. These results strongly support the view that a positively charged amphiphilic structure, either an α-helix or a β-sheet, is an essential common feature of mitochondrial leader peptides.

When a number of chloroplast leader peptides were analyzed, no primary sequence similarities were found (Keegstra et al., 1989). Chloroplast and mitochondrial leader peptides share many common features (Verner and Schatz, 1988; von Heijne, 1988; Keegstra et al., 1989). Both types of leader peptides are rich in basic and hydroxylated residues and contain few, if any, acidic residues, and therefore contain net positive charges. Some preliminary analysis has indicated that chloroplast leader peptides may not form amphiphilic helices as most mitochondrial leader peptides do, but may form amphiphilic β-sheet structures (Keegstra et al., 1989). It is remarkable that mitochondrial and chloroplast leader peptides are structurally so alike, yet apparently maintain strict organellar specificity of in vivo protein targeting (Boutry et al., 1987).
Precursor proteins must assume a competent conformation to be imported

Fusion proteins that consist of a mitochondrial leader peptide and a passenger protein have been shown to provide a useful approach to study protein import. Several studies with fusion proteins indicate that, in addition to the amino-terminal leader peptide, other properties of a precursor protein are also very important for protein translocation. For example, the leader peptide of mitochondrial manganese superoxide dismutase was able to direct DHFR but not cytosolic invertase as a passenger protein into mitochondria (van Steeg et al., 1986). Yeast mitochondrial ATP synthase subunit 8 could be translocated into mitochondria when it was fused to the leader peptide of ATP synthase subunit 9 of Neurospora, but not when fused to the leader peptide of COXIV of yeast (Gearing and Nagley, 1986; Nagley et al., 1988). It is speculated that cytosolically synthesized precursor proteins must be soluble in the cytosol and maintain a loose, unfolded conformation in order to be translocated.

Being soluble in the cytosol seems not to be a problem for hydrophilic precursors. For integral membrane proteins, which are very hydrophobic, the question that arises is what features confer solubility to their precursors. The positively charged leader peptides that are very important for the targeting of proteins certainly play a role also in this
regard. An excellent example is the *Neurospora* ATP synthase subunit 9. The mature part of this protein consists of 81 amino acids and is very hydrophobic. The precursor, however, carries a highly hydrophilic 66 amino-acid leader peptide (Viebrock et al., 1982) that reduces the overall hydrophobicity of the precursor protein. ATP synthase subunit 9 is transported through the cytoplasm as high molecular mass aggregates (Schmidt et al., 1983). In these aggregates, the leader peptide probably faces the surrounding aqueous medium, thus conferring solubility to the whole complex. Indeed, aggregate formation is probably common for most precursor proteins (Neupert and Schatz, 1981).

Recent evidence suggests that lack of tertiary structure is a prerequisite for import competence (Zimmermann and Meyer, 1986). Precursor proteins can be trapped in translocation contact sites so that the amino-terminus protrudes into the matrix space, while the carboxy-terminal portions are still outside the outer membrane (Schleyer and Neupert, 1985; Hartl et al., 1986). A portion of the precursor protein thus spans the two mitochondrial membranes. Since the trans-membrane distance is larger than the diameter of the assembled polypeptide chain, the precursor polypeptide must be at least partially unfolded. The importance of a loose conformation for translocation competence was directly shown with a mitochondrial precursor fusion protein containing the COXIV
leader peptide fused to DHFR; import of this protein into isolated yeast mitochondria was completely blocked by stabilizing its folded structure with folate analogs such as methotrexate (Eilers and Schatz, 1986). Similarly, addition of copper to import reactions blocked translocation of a fusion protein containing 61 residues of yeast copper metallothionein because of the tertiary conformation of the metallothionein portion induced by binding of the metal ligand (Chen and Douglas, 1987b). Precursor proteins destined for chloroplasts also have to be in an unfolded state in order to be imported; import of the precursor to 5-enolpyruvylshikimate-3-phosphate synthase (pEPSPS) into chloroplasts was inhibited by the herbicide glyphosate, a specific ligand that binds to pEPSPS (della-Cioppa and Kishore, 1988). These results demonstrate that precursor proteins must be at least partly unfolded in order to be translocated across membranes. In support of this view, import of the COXIV precursor protein is dramatically accelerated by presenting it to mitochondria as nascent peptidyl-tRNA chains (Verner and Schatz, 1987). When a COXIV/DHFR fusion protein (Eilers and Schatz, 1986) was denatured by urea (Eilers et al., 1988) or when its tertiary structure was destabilized by point mutations (Vestweber and Schatz, 1988), its import was much more efficient at 25°C than that of the native protein, could occur at a much lower
temperature, and was not blocked by 150 nM methotrexate, which effectively blocks the import of the native protein.

The observations that precursors form aggregates in the cytosol and that an unfolded conformation is necessary for import appear to be contradictory. This implies that some cytosolic factors must be involved in unfolding. It has been proposed that ATP-dependent unfolding enzymes, termed unfoldases, are required to keep precursor proteins destined for different organelles in a translocation-competent conformation (Rothman and Kornberg, 1986). Recent evidence suggests that a group of proteins called chaperonins, usually similar or related to heat shock proteins (hsp), could be the putative unfoldases (Pelham, 1986). Transient association of newly synthesized unfolded proteins with the GroEL heat-shock protein, a chaperonin, was reported in *E. coli* (Bochkareva et al., 1988). Members of the 70 kDa heat-shock protein (hsp70) family are involved in ATP-dependent unfolding of precursor proteins destined for import into different organelles (Pelham, 1986). Purified yeast hsp70-like proteins stimulated translocation of precursor proteins into different organelles both *in vivo* and *in vitro* (Chirico et al., 1988; Deshaies et al., 1988; Zimmermann et al., 1988). Heat shock proteins or hsp-related proteins are also involved in ATP-dependent unfolding of chloroplast precursor proteins (Roy, 1989).

Normal protein translocation into mitochondria and
chloroplasts requires ATP (for reviews see Eilers and Schatz, 1988; Hartl et al., 1989; Keegstra et al., 1989). Although nothing firm is known about how ATP supports protein translocation, the accumulated body of evidence suggests that, for mitochondrial protein import, it participates in the unfolding of precursors, probably by providing energy required for unfoldase activities via hydrolysis outside the mitochondrial inner membrane (Chen and Douglas 1987a; Eilers et al., 1987; Pfanner et al., 1987b). For example, it was reported that yeast ATPase β-subunit precursor lacking an internal tetramer-forming domain could be imported into mitochondria in the absence of ATP (Chen and Douglas, 1988). Similarly, a water-soluble form of porin obtained by subjecting membrane-derived porin to an acid-base treatment did not require ATP for import (Pfanner et al., 1988b). ATP utilization in chloroplast protein import may be different from that in mitochondria in that ATP is hydrolyzed inside chloroplasts (Pain and Blobel, 1987; Theg et al., 1989; for review see Keegstra et al., 1989). It is difficult, though, to image how internal ATP can be utilized by unfolding activities outside chloroplasts. Mitochondrial protein import also requires a membrane potential (Schleyer et al., 1982; Eilers et al., 1987), which is not required for chloroplast protein import (Keegstra et al., 1989). In at least one case, precursor unfolding was stimulated by the potential across the
mitochondrial inner membrane (Eilers et al., 1988).

Involvement of membrane receptors in import

An early step in the import pathway of mitochondrial or chloroplast precursor proteins is the specific binding of precursors to the organelar membranes (Riezman et al., 1983b; Cline et al., 1985). The binding step is expected to be specific to account for the organelle specificity observed in plant cells (Boutry et al., 1987). It is likely that this specific binding is in essence the mutual recognition of precursors and membrane receptors, with possible involvement of other factors in the cytosol.

Several lines of evidence suggest that proteinaceous receptors on the mitochondrial or chloroplast surface perform important functions for specific binding (for reviews see Hartl et al., 1989; Keegstra et al., 1989). In a number of studies, isolated mitochondria or chloroplasts were pretreated with low concentrations of proteinases such as trypsin, proteinase K, or thermolysin. This treatment significantly inhibited the specific binding and subsequent import of precursor proteins (Zwizinski et al., 1984; Cline et al., 1985; Pfanner et al., 1987a). Specific binding of precursors to mitochondria could occur in the absence of a membrane potential, but it required ATP (Pfanner et al., 1987b). Although previous reports indicated that binding of
chloroplast precursors did not require ATP (Cline et al., 1985), recent studies showed that ATP was required for chloroplast precursor binding, but at a much lower concentration than required for driving import (Olsen et al., 1989). This observation provides compelling evidence that binding of precursors to chloroplasts is a protein-mediated event. In addition, binding sites on the mitochondrial or chloroplast surface could be saturated when precursor proteins were presented in a high concentration (Pfisterer et al., 1982; Pfaller et al., 1985). This resembles the kinetics of enzymatic reactions.

At least four different types of binding/import receptors have been proposed to exist for mitochondrial precursor proteins: for porin, for the ADP/ATP carrier, for the ATP synthase β-subunit, and for cytochrome C (Hartl et al., 1989). For example, pre-treatment of mitochondria with elastase inhibited import of porin, Fe/S protein, the ADP/ATP carrier and ATP synthase subunit 9, but not of ATP synthase subunit β, whereas pre-treatment with trypsin inhibited the import of all these proteins (Zwizinski et al., 1984; Pfaller et al., 1988). Thus, the receptor for ATP synthase subunit β seems to be different from those for the other four precursors. It is unlikely, however, that each precursor protein has a specific receptor. A synthetic peptide consisting of the 27 N-terminal amino acid residues of the rat OTC precursor was able
to block the import of authentic pre-OTC and of two other mitochondrial precursors, the matrix protein malate dehydrogenase and the inner membrane protein thermogenin (Gillespie et al., 1985). Similarly, the chemically synthesized leader peptide of ornithine aminotransferase completely inhibited the import of several mitochondrial precursors plus authentic ornithine aminotransferase (Ono and Tuboi, 1988). A "general insertion protein" (GIP) in the mitochondrial outer membrane has been proposed to be common to the import pathways of several precursor proteins (Pfaller et al., 1988; Pfaller et al., 1989). GIP appears to be different from the specific membrane receptors; it is not exposed to external proteases and the number of GIP sites seems to be about tenfold higher than the number of specific receptor sites (Pfaller et al., 1988). The inefficient import of non-mitochondrial precursors into mitochondria appears to occur through GIP (Pfaller et al., 1989), without the involvement of a specific receptor.

Attempts have been made to identify membrane receptors involved in protein import into mitochondria and chloroplasts. A specific outer mitochondrial membrane protein has been implicated as a possible receptor for mitochondrial import (Gillespie, 1987). At least two different proteins have been identified as potential receptors for chloroplast import. Using a heterobifunctional, photoactivatable cross-linking
reagent, Cornwell and Keegstra (1987) have identified a 66 kDa envelope surface protein that is associated with specific binding to the precursor to the small subunit of ribulose-1,5-bisphosphate carboxylase (prSS). Pain and co-workers (1988) raised antibodies against antibodies that were directed against a synthetic peptide analog of the C-terminal 30 residues of the leader peptide of pea prSS. These anti-idiotypic antibodies were able to block the import of prSS into chloroplasts, suggesting that they were mimicking the leader peptide, interacting with the same binding site on the putative import receptor. In addition, they cross-reacted with a major 30-kDa protein of the envelope membrane. The authors concluded that this 30-kDa protein was the receptor for prSS (Pain et al., 1988). Using an approach similar to that of Cornwell and Keegstra (1987), Kaderbhai et al. (1988) also identified a 30-kDa protein which they showed to be the phosphate translocator. The phosphate translocator is unlikely to be a receptor. Although various approaches have come to different conclusions about the identity of the prSS receptor, they may all be correct. The 66-kDa and 30-kDa proteins may be different subunits of a single receptor, or they may be separate proteins involved in precursor import.
**Processing of precursor proteins**

Since the majority of cytosolically synthesized mitochondrial and chloroplast precursor proteins carry an extra N-terminus (the leader peptide) that is not found in the mature form of the protein, there must be a mechanism by which the leader peptide is cleaved off. Both mitochondria and chloroplasts contain processing peptidases that remove the leader peptide during or after translocation. Processing peptidase activity was first detected in hypotonic extracts of yeast mitochondria (Bohni et al., 1980). It has also been characterized for mitochondria from mammals (Ou et al., 1989) and Neurospora (Hawlitschek et al., 1988) and for pea chloroplasts (Robinson and Ellis, 1984).

The processing peptidases of mitochondria and chloroplasts show similar properties: a) solubility in the absence of detergent; b) neutral pH-optimum; c) inhibition by divalent cation chelators such as EDTA, ortho-phenanthroline, and batho-phenanthroline; d) insensitivity to a number of known proteinase inhibitors, such as phenylmethylsulfonyl fluoride (PMSF); e) stimulation by divalent cations such as Co$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$; and f) no requirement for ATP (for reviews see Cashmore et al., 1985; Hartl et al., 1989).

The processing peptidase has been most extensively studied in Neurospora (Hawlitschek et al., 1988) and yeast (Yaffe et al., 1985; Pollock et al., 1988; Witte et al.,.
1988). Two polypeptides involved in processing have been identified for *Neurospora* mitochondria. One polypeptide, the 57 kDa "matrix processing peptidase" (MPP), is located in the mitochondrial matrix and carries the catalytic center for proteolytic cleavage. The other one, the 52 kDa "processing enhancing protein" (PEP), is attached to the inner (matrix) surface of the inner membrane and enhances MPP function (Hawlitschek et al., 1988). MPP alone has a low processing activity, whereas PEP alone has no apparent activity. Upon recombining both, full processing activity is restored. It is suggested the PEP might also contribute to the specificity of the cleavage reaction (Hartl et al., 1989).

Temperature-sensitive mutants in yeast have contributed considerably to our understanding of the processing peptidase. Schatz and colleagues have isolated yeast mutants characterized by strongly reduced protein import at non-permissive temperature (Yaffe and Schatz, 1984; Yaffe et al., 1985). Two complementation groups, namely mas1 and mas2 (mas for mitochondrial assembly), have been identified. The wild type mas1 gene encodes a 48 kDa protein and the mas1 mutant was shown to be defective in the matrix-localized processing activity in vitro (Yaffe et al., 1985; Witte et al., 1988). An antiserum raised against the 48 kDa polypeptide cross-reacted with the 52 kDa PEP of *Neurospora* (Witte et al., 1988). These two polypeptides share about 70% sequence
homology (Hawlitschek et al., 1988). Therefore, the 48 kDa protein is a counterpart of PEP in yeast. Pollock and coworkers have recently identified two yeast temperature sensitive mutants, mif1 and mif2 (mif for mitochondrial import function), that are characterized by the accumulation of mitochondrial precursors at non-permissive temperatures (Pollock et al., 1988). mif1 and mif2 were found to be identical with the mas1 and mas2 mutations described by Schatz’s group. mif2 encodes a polypeptide of 53 kDa. Biochemical analysis of the phenotype of the mif2 mutation revealed that it was defective in the catalytic component of the processing enzyme (Pollock et al., 1988). This 53 kDa polypeptide might be the counterpart of MPP in yeast. Interestingly, a processing peptidase can be bifunctional; subunit 1 of the cytochrome reductase complex of Neurospora crassa, a protein necessary for electron transport in the respiratory chain, has been found to be identical to PEP (Schulte et al., 1989).

A partially-purified soluble processing proteinase from pea chloroplasts has been characterized (Robinson and Ellis, 1984). This protease processed the precursor of wheat and barley plastocyanin as well as prSS from pea, suggesting that the processing enzyme is neither precursor-specific nor species-specific. Similar results have been obtained in studying mitochondrial precursor processing (for review see
Hartl et al., 1989). For example, the native processing site of *Neurospora* ATP synthase subunit 9 precursor can be correctly recognized by yeast matrix processing peptidase (Gearing and Nagley, 1986), and a yeast mitochondrial leader peptide can apparently be processed by plant mitochondrial processing peptidase (Schmitz and Lonsdale, 1989). It seems reasonable, then, to speculate that mitochondrial or chloroplast precursors would have consensus sequences that can be recognized by mitochondrial or chloroplast processing protease. Analysis of 38 mitochondrial precursors from various sources showed that no such primary sequence conservation could be found at or around the cleavage site (Hartl et al., 1989), although in 22 cases out of 38 a positively charged residue was found at position -2 (second residue upstream of the cleavage site). Chloroplast precursor proteins also do not have a conserved primary sequence at the processing site (Keegstra et al., 1989). It is likely that processing peptidases recognize a conformational signal in the precursor (Roise and Schatz, 1988). For example, the matrix processing peptidase does not cleave denatured precursor proteins (Bohni et al., 1983; Ohta and Schatz, 1984).

**Specific protein targeting in plants**

The picture of protein targeting in plants is more complicated than in other eukaryotes because plant cells
contain a family of organelles, the plastids, that other eukaryotic organisms (except for photosynthetic algae) do not have. Mitochondrial and chloroplast leader peptides share common features, and there are many similarities between protein import into mitochondria and chloroplasts (Keegstra, 1989). These similarities raise the question of how organelle specificity is determined in plant cells. Hartl et al. (1986) showed that a part of the leader peptide from prSS of chloroplasts directed the transport of two different passenger proteins into yeast mitochondria *in vitro* and/or *in vivo*. The efficiency of import was very low compared to that directed by an authentic yeast mitochondrial leader peptide. More recently, it has been reported that native chloroplast prSS can be imported into yeast mitochondria *in vitro* with very low efficiency (Pfaller et al., 1989). The authors propose that non-specific protein transport can occur inefficiently through a pathway mediated by the non-specific import receptor, the general insertion protein (GIP) located in the mitochondrial outer membrane.

*In vivo* protein targeting in higher plants is probably strictly organelle specific. Boutry et al. (1987) fused a passenger protein, chloramphenicol acetyltransferase (CAT), to either a mitochondrial or a chloroplast leader peptide and studied the targeting specificity of the fusion proteins in transgenic tobacco plants. They found that whether CAT was
translocated to mitochondria or to chloroplasts depended on
the leader peptide in front of it. Moreover, a yeast
mitochondrial leader peptide directed the import of a
passenger protein specifically into mitochondria in transgenic
tobacco (Schmitz and Lonsdale, 1989). Import of a protein
into chloroplasts directed by a mitochondrial leader peptide
has not been reported. The mechanism underlying organellar
specificity of protein targeting is unknown.

Objectives and Rationale

The objectives of my dissertation research were to test
directly the role of the T-URF13 protein (see Part A, General
Introduction) in the causation of toxin susceptibility and
cytoplasmic male sterility. The overall rationale of the
experiments is that if T-URF13 is responsible for toxin
susceptibility and/or cytoplasmic male sterility, a
heterologous organism that does not normally contain this
protein may exhibit these phenotypes when it expresses T-
URF13. T-URF13 is encoded by a mitochondrial gene in maize.
Since techniques for direct transformation of mitochondria
have not yet been developed for practical use, T-urfl3 was
genetically engineered so that the protein would be
synthesized in the cytosol of the heterologous organism, then translocated into mitochondria. Therefore, fusion genes consisting of a mitochondrial leader peptide and the T-urf13 coding region were constructed and used to transform the nuclear genome, with the expectation that a functional form of T-URF13 would be translocated into mitochondria by the leader peptide.

My first project was to study the role of T-URF13 in the causation of toxin/methomyl susceptibility. Yeast was chosen as the heterologous recipient because it has been genetically and biochemically well studied and would be an ideal model system to study the biochemical and physiological effects of T-URF13. In addition, protein translocation in yeast is well studied and there are many yeast mitochondrial leader peptides to chose from.

To investigate the role of T-URF13 in causing cytoplasmic male sterility, the second project of my dissertation research, a plant recipient must be used so that any effect of T-URF13 on pollen fertility can be examined. Tobacco was chosen because genetic transformation and plant regeneration systems have been well established in tobacco.

In order to transform the nuclear genome of tobacco and to deliver the T-URF13 protein into tobacco mitochondria, a fusion gene must be constructed as for yeast. Unfortunately, few plant mitochondrial leader peptides are available to be
chosen for this purpose, in contrast to the many yeast mitochondrial leader peptides that are available. Thus, it seemed that it would be desirable to try to target T-URF13 to plant mitochondria with a yeast mitochondrial leader peptide. Although mitochondrial leader peptides are functionally conserved among eukaryotes (see part B, General Introduction), functioning of a yeast mitochondrial leader peptide as a targeting signal in higher plants had not been reported when my project started. For this reason, I investigated the possibility of using a yeast mitochondrial leader peptide to direct the transport of a model passenger protein, chloramphenicol acetyltransferase, into plant mitochondria. Besides providing a means for designing experiments to target T-URF13 to plant mitochondria, this study also provides valuable information about the general problem of mitochondrial protein targeting in plants, which has not been extensively studied.

Explanation of Dissertation Format

This dissertation includes an abstract of the dissertation, a general introduction, three independent sections, and a summary of the dissertation. In the General
Introduction, I have given a brief literature review on two separate topics, cytoplasmic male sterility in higher plants and protein targeting into mitochondria and chloroplasts, both of which are the subjects of my research.

Section I represents a manuscript that has been accepted for publication by the EMBO Journal. The results reported in this section were obtained by the persons named in the author list. Mrs. S.-H. Lee, a graduate student in the Department of Biochemistry and Biophysics, Iowa State University, did all the DNA manipulations at the 3'-ends of the COXVα and ATP2 leader peptides, which I used to construct the expression plasmids pCMS1 and pCMS2. Mr. C. Lin, a former graduate student in Dr. E. Hack's lab, prepared the antibodies against T-URF13. Dr. R. Medici, a visiting scholar from Italy, did the site-specific mutagenesis that eliminated the translation initiation codon of the COXVα leader peptide. The rest of the results represent my own work under the supervision of Dr. A. Myers and Dr. E. Hack.

Section II deals with the subject of protein targeting in higher plants. This section communicates results that have not yet been published. All the results in this section represent my own work under the supervision of Drs. R. W. Thornburg, E. Hack, and A. Myers.

Section III represents some preliminary results obtained in an effort to reveal the molecular basis of cytoplasmic male
sterility in maize with the Texas male-sterile cytoplasm. These results did not allow a firm conclusion to be drawn. Additional work is in progress.

Finally, the main results and conclusions of my research work are given in the Summary section.
SECTION I. EXPRESSION IN YEAST OF THE T-URF13 PROTEIN FROM TEXAS MALE-STERILE MAIZE MITOCHONDRIA CONFFERS SENSITIVITY TO METHOMYL AND TO TEXAS-CYTOPLASM-SPECIFIC FUNGAL TOXINS
The mitochondrial gene T-urfl3 from maize (Zea mays L.) with Texas male-sterile (T) cytoplasm codes for a unique 13-kilodalton polypeptide, T-URF13, which is implicated in the causation of 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.
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 Koepppe, 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; Holden and Sze, 1987). The insecticide methomyl affects T mitochondria similarly and thus is a functional analog of these toxins (Koepppe et al., 1978; Klein and Koepppe, 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 kDa, 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 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; Dewey et al., 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-urfl3 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-urfl3 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.
MATERIALS AND METHODS

Strains and growth media

Saccharomyces cerevisiae strains W303-11B (MATα leu2 ura3 trpl his3 ade2) or W303 (MATα/MATα leu2/leu2 his3/his3 ade2/ade2 trpl/trpl 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 (DuPont; 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. E. coli strain TG-1 (K12, Δ(lac-pro), supE, thy−, hsdR5/F' traD36, proA+R', lacIq, lacZ4M15), 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 BclI.

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).

Plasmid constructions

The plasmids used in this study and the T-URF13 fusion proteins for which they code are shown in Table 1-1. The construction of these plasmids is described below.

T-urfl3 coding region: A 1.7-kb HindIII-BglII fragment of maize mitochondrial DNA containing the T-urfl3 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-urfl3 sequence by oligonucleotide-directed site-specific mutagenesis. First, the T residue at nt 1247 was
Table 1-1. Plasmids coding for T-URF13 fusion proteins

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter</th>
<th>Leader peptide source</th>
<th>Expressed protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJTH1</td>
<td>COX5a</td>
<td>ATP synthase subunit 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>prN9/T-URF13</td>
</tr>
<tr>
<td>pJTH2</td>
<td>COX5a</td>
<td>--------</td>
<td>T-URF13</td>
</tr>
<tr>
<td>pCMS1</td>
<td>COX5a</td>
<td>Cytochrome oxidase subunit Va</td>
<td>prC5/T-URF13</td>
</tr>
<tr>
<td>pCMS2</td>
<td>ATP2</td>
<td>ATP synthase subunit beta</td>
<td>prA2/T-URF13</td>
</tr>
</tbody>
</table>

<sup>a</sup>From *N. Crassa* (All other genes and leader peptides from *S. cerevisiae*).
changed to a G. This mutation eliminated a BclI site within the T-urf13 coding region without changing the codon identity, thereby facilitating use of a BclI 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 SstI site at nt 1601, 41 nt downstream of the T-urf13 termination codon. The 1.6 kb HindIII-SstI fragment (nt 1 - nt 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 V₅ of cytochrome oxidase, is located within a PstI 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 BclI site immediately following the C-terminal codon of the subunit V₅ mitochondrial targeting peptide (Koerner et al.,
The plasmid containing this mutation, pVL, was further modified by replacement of the A at nt -19 with a T. This mutation creates a BglII site at nt -24, two or seven nucleotides, 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 BamHI 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 BamHI-Sau3AI fragment from pUC19N9L coding for the mitochondrial targeting peptide of prN9 (nt 30 - nt 257, Viebrock et al., 1982; Gearing and Nagley, 1986) was ligated to the BclI-SstI fragment from pTI4 containing mutagenized T-urfl3 (nt 1216 to nt 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 segments of **oli**⁺ and T-urfl3 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 BamHI-SstI fragment containing the fused oli* and T-urfl3 coding regions, and the PstI-BglII fragment from pVP containing the COX5a promoter and transcription initiation sites (nt -273 to nt -24, Cumsky et al., 1987), were ligated in a three-fragment reaction into YEp352 (Hill et al., 1986) digested with PstI and SstI. The product of this ligation, pJTH1, was mapped using restriction enzymes to confirm that the promoter, mitochondrial targeting sequence, and T-urfl3 coding sequence were present in the necessary sequence and orientation (not shown).

Yeast expression plasmid pJTH2: The PstI-BclI fragment of pVL, containing the COX5a promoter and mitochondrial targeting sequence (nt -273 to nt 60, Cumsky et al., 1987), and the BclI-SstI fragment of pTI4, containing the T-urfl3 coding region (see above), were ligated in a three-fragment reaction into pUC118 digested with PstI and SstI. Ligation of these two BclI sites results in an in-frame fusion between T-URF13 and the subunit Vα 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-urfl3 as the first ATG sequence present in the transcript. The mutated PstI-SstI fragment was then cloned in YEp352, resulting in the yeast expression plasmid
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 Va are fused to T-URF13, and expression of the fusion gene construct is driven by the COX5a promoter.

Yeast expression plasmid pCMS2: An EcoRI-PvuII fragment containing the promoter and mitochondrial targeting sequence of the yeast nuclear gene ATP2, coding for the F1-ATPase β subunit (Bedwell et al., 1987), was subcloned into pUC118. A deletion series starting at the downstream PvuII site was constructed with nuclease Bal31, and BamHI linkers were attached to the ends of the deleted plasmids. Nucleotide sequence analysis revealed a clone containing a BamHI site at codon 34 of ATP2. The EcoRI site upstream of the ATP2 promoter was then changed to a PstI site using an oligonucleotide adaptor (5'-AATTCTGCAG-3'). Finally, pCMS2 was constructed by ligating the PstI-BamHI fragment of ATP2 and the BglI-SstI fragment of T-URF13 from pTI4 (see above) into YEp352 digested with PstI and SstI, in a three-fragment ligation. Ligation of the compatible BamHI and BglI 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-polyacrylamide gel electrophoresis and the 15 kDa 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 d 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 x 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 125I-labeled protein A (DuPont-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.
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-urfl3 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 Va 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 oli1+ 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 oli1+ are included in pJTH1, comprising the entire mitochondrial targeting peptide and three amino acids of mature subunit 9. This sequence is fused to T-urfl3 in its first initiation codon so that expression of the gene gives
Fig. 1. Gene fusions for expression of T-urfl3 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 of cytochrome oxidase (Koerner et al., 1985; Cumsky et al., 1987); ATP2, the Neurospora olig gene coding for subunit 9 of ATP synthase (Viebrock et al., 1982; Gearing and Nagley, 1986); T-urfl3, the mitochondrial gene from maize T cytoplasm coding for T-URF13 (Dewey et al., 1986). Restriction endonuclease recognition sites are indicated for PstI (P), Sau3AI (S), SstI (T), BglII (C), and a BamHI-BglII 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 (↑——).
A.

\[ \text{COX5} \quad \text{ATP9} \quad \text{T-urfl3} \]

GCC TAC TCT TCC GAG ATC ACT ACT
Ala Tyr Ser Ser Glu Ile Thr Thr

ATP9 codon 69

125 bp

B.

\[ \text{COX5} \quad \text{T-urfl3} \]

COX5

T-urfl3

125 bp
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 Va targeting 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 aw303-11B, 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 kDa
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 kDa, even though the T-urf13
nucleotide sequence predicts a protein of 12,961 Da). 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 kDa and 15 kDa (Figure 2B). The
21 kDa band corresponds to the size predicted for unprocessed
prN9/T-URF13 fusion protein, suggesting that the larger
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 \(^{125}\text{I}\)-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 approximately 15 kDa 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 an approximately 20 kDa 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 kDa 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.
protein has not been imported into mitochondria, while the 15 kDa 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 kDa band, although some 21 kDa immunoreactive material was also present (Figure 2C). The pJTH2 transformant tested, pJTH2-1, also contained a 15 kDa immunoreactive band, which was present in the mitochondrial fraction (Figure 2B,C).

Protease protection assays showed that the prN9 targeting peptide was able to deliver T-URF13 into mitochondria (Figure 2C). The 15 kDa protein in the mitochondrial fraction of the pJTH1 transformants was protected from digestion by proteinase K, whereas the 21 kDa protein was completely digested by this treatment. Disruption of mitochondrial membranes with detergent rendered the 15 kDa 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
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 Va (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 F1-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-l and pCMS2-l, 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 kDa for pCMS1-l and 17.5 kDa for pCMS2-l (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 \( \beta \) 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 approximately 200 cells were plated on solid media in the presence or absence of methomyl or Pm toxin. After four 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
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.
EG + methomyl

EG + HmT toxin

EG + Pm toxin
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.
I:

Time (h)

- methomyl + methomyl - methomyl + methomyl

pJTH1-1 pJTH1-1 pJTH2-1 pJTH2-1

OD600
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. 40 μl of the suspension, containing approximately 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).
efficiency on EG medium in the presence of either methomyl or Pm toxin was approximately 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 only prevents 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 had lost the plasmid. In contrast, approximately 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
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-l 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).
Table 1-2. Effects of methomyl, HmT toxin and Pm toxin on oxygen consumption by yeast cells, spheroplasts and mitochondria containing the T-URF13 protein

<table>
<thead>
<tr>
<th>Strain</th>
<th>Stimulation of oxygen consumption&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methomyl (5 mM)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>W303</td>
<td>-6</td>
</tr>
<tr>
<td>pJTH2-1</td>
<td>-6</td>
</tr>
<tr>
<td>pJTH1-1</td>
<td>27</td>
</tr>
<tr>
<td>pJTH1-2</td>
<td>30</td>
</tr>
</tbody>
</table>

<sup>a</sup>% stimulation was calculated as: \( \frac{[O_2 \text{ consumption rate after toxin or methomyl addition]} - 1}{[rate \text{ before addition}] \times 100. \)

<sup>b</sup>Purified HmT toxin or Pm toxin, from 200 ug/ml stock in DMSO. With 0.5% (v/v) DMSO alone there was generally a 5-10% increase in \( O_2 \) consumption.

<sup>c</sup>C, whole cells.

<sup>d</sup>S, spheroplasts.

<sup>e</sup>M, mitochondria.

<sup>f</sup>Not determined.
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 6C 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 1-2).

The effects of methomyl, HmT toxin, and Pm toxin on oxygen uptake by whole cells and spheroplasts were also tested (Table 1-2). Methomyl or toxin increased the rate of 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 Va and F1-ATPase subunit β are unable to deliver T-URF13 to the mitochondrial interior. The internalized protein is converted from the 21 kDa prN9/T-URF13 to a 15 kDa 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 kDa 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-l 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-l and pCMS2-l 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 galactose, 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.
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SECTION II. A YEAST MITOCHONDRIAL LEADER PEPTIDE TARGETS BACTERIAL CHLORAMPHENICOL ACETYLTRANSFERASE INTO MITOCHONDRIA AND CHLOROPLASTS IN TOBACCO
ABSTRACT

Targeting of endogenous chloroplast or mitochondrial proteins in a plant cell seems to be organelle specific. Nevertheless, a part of a chloroplast leader peptide can direct the transport of a passenger protein into yeast mitochondria both in vitro and in vivo. A yeast mitochondrial leader peptide can target bacterial β-glucuronidase specifically to tobacco mitochondria in vivo. Targeting of a protein to chloroplasts directed by a mitochondrial leader peptide has not, to our knowledge, been previously reported. Here we present evidence that a mitochondrial leader peptide from the yeast cytochrome oxidase subunit Vα (COXVα) directs transport of chloramphenicol acetyltransferase (CAT) into both chloroplasts and mitochondria of transgenic tobacco plants, as well as into mitochondria of yeast. The specific activity of CAT in mitochondria and chloroplasts is comparable. Varying growth conditions may alter the distribution of CAT activity between the chloroplasts and mitochondria in a single plant. The proteins that are found inside chloroplasts and mitochondria of transgenic tobacco plants have a smaller molecular mass than that of the in vitro synthesized precursor protein, and have a similar molecular mass in chloroplasts and in mitochondria of tobacco and yeast. Our data suggest that
either the native processing site or a nearby site in the yeast COXV \textsubscript{a} leader peptide can be recognized by the processing proteases of chloroplasts and mitochondria of tobacco plants. That a single leader peptide possesses dual targeting specificity may provide a potential experimental system for studying the mechanism of specific protein targeting in plants.
INTRODUCTION

Mitochondria and plastids contain DNA and are capable of synthesizing some of their own proteins. The majority of their proteins, however, are coded for by nuclear genes, synthesized on cytosolic ribosomes, and post-translationally imported into the organelle (Ellis, 1981; Ellis and Robinson, 1987). Most of these imported proteins are synthesized as precursors with an amino terminal segment, termed the leader peptide, that is proteolytically removed upon importation (Ellis and Robinson, 1987; Verner and Schatz, 1988). Numerous studies of the import of fusion proteins consisting of organellar leader peptides attached to heterologous passenger proteins show that the leader peptide usually contains all the information needed to target a protein to the proper subcellular location (for recent reviews see Grivell, 1988; von Heijne, 1988; Hartl et al., 1989), although in some cases the passenger protein can influence import (Gearing and Nagley, 1986; van Steeg et al., 1986; Ness and Weiss, 1987; Section I) or sorting within the organelle (Smeekens et al., 1986; Smeekens et al., 1987). Moreover, the features of targeting peptides that are recognized by the import machinery of the organelle are highly conserved. For example, leader peptides from Neurospora and humans target proteins into yeast
mitochondria (Banrogues et al., 1986; Cheng et al., 1987; Nagley et al., 1988; Section I), and a yeast mitochondrial leader peptide can target the *E. coli* β-glucuronidase protein to tobacco mitochondria (Schmitz and Lonsdale, 1989).

In plants, protein import into plastids has been extensively studied (Keegstra 1989; Keegstra et al., 1989) but there have been few studies on protein import into mitochondria (Boutry et al., 1987; White and Scandalios, 1987; White and Scandalios, 1989). Targeting of proteins to plastids and mitochondria is probably strictly organelle-specific (Boutry et al., 1987), and the import machinery is likely to be different for the two organelles (Verner and Schatz, 1988; Keegstra et al., 1989). Nevertheless, plastid and mitochondrial leader peptides share common features. They are rich in hydroxylated and basic residues and contain few, if any, acidic residues, and therefore have net positive charges (Verner and Schatz, 1988; von Heijne, 1988). They are also predicted to have an amphiphilic conformation (von Heijne, 1986; Keegstra et al., 1989). The existence of these common features raises the question of how targeting specificity is achieved, and suggests that under some circumstances leader peptides could be functionally interchangeable between chloroplast and mitochondrial proteins. Indeed, a part of the leader peptide from a *Chlamydomonas* chloroplast protein, the small subunit of
ribulose-1,5-bisphosphate carboxylase, can deliver two different passenger proteins into yeast mitochondria, although with less efficiency than an authentic yeast mitochondrial leader peptide (Hurt et al., 1986). Two other plastid leader peptides failed, however, to target a passenger protein into yeast mitochondria (Smeekens et al., 1987). The apparent difference between the targeting specificity in the heterologous system and in plant cells suggests that the mitochondrial import apparatus in plant cells may have somewhat different requirements from the apparatus in yeast cells for protein import.

In order to investigate further the relation between targeting specificity in yeast and plant cells, we have examined the function of the leader peptide of yeast cytochrome oxidase subunit V, a mitochondrial protein, in transgenic tobacco plants. We present evidence that this leader peptide functions in plants as a protein targeting signal with dual specificity, targeting bacterial chloramphenicol acetyltransferase (CAT) into mitochondria and also into chloroplasts. The subunit V leader-CAT fusion protein is processed to a smaller mature protein in both organelles. Our findings provide a potential experimental system to study the mechanism by which proteins are targeted specifically to mitochondria or plastids in plants.
DNA manipulations

DNA manipulations were performed using standard procedures (Maniatis et al., 1982; Ausubel et al., 1987). Oligonucleotide adaptomers were synthesized by the Iowa State University Nucleic Acid Facility using a Biosearch 8750EX automated DNA synthesizer. Single-stranded DNA was prepared as described by Vieira and Messing (1987), and nucleotide sequence analysis was by the chain termination method (Sanger et al., 1977).

Construction of expression plasmids

pJTH5: A 464 base pair (bp) BglII-SstI fragment containing the COX5a sequence which codes for the prC5 mitochondrial targeting peptide was obtained from the plasmid pTCB, a derivative of the plasmid pVP (Section I), and subcloned into pUC119 digested with BamHI and SstI. The insert was isolated from the resulting plasmid as a HindIII-SstI fragment and, in a three-fragment ligation into pUC119, attached to a 1 kilobase pair (kbp) EcoRI-HindIII fragment containing the 35S promoter (Odell et al., 1985). The resulting plasmid, pCCT, contains a unique BglI site located immediately downstream of the last triplet of the prC5 leader.
coding region (Section I). Thus, digestion of pCCT with SstI and BclI liberates a fragment containing the 35S promoter fused to the prC5 leader coding sequence, plus the entire sequence of pUC119. This fragment was ligated into the binary vector pGA492 (An, 1986), digested with KpnI and SstI, to produce pJTH5. The KpnI site of pGA492, located in the multiple cloning site (MCS) upstream of the CAT coding region, was ligated to the BclI site of pCCT using a BclI-KpnI adaptomer (5'-GATCCTAG-3'). The nucleotide sequence of pJTH5 from the 35S transcription start site into the CAT coding region was determined; Figure 1 presents this sequence.

pJTH6: pJTH6 was constructed by a series of cloning steps similar to those described for pJTH5. There are two differences between these plasmids, both derived from changes in the HindIII-BclI region of pCCT. The first change is a mutation in the initiation codon of prC5, changing it from an ATG to an ATT codon (Section I). The second change, located immediately downstream of the HindIII site, is such that the 5' end of the 35S promoter of pJTH6, compared to that of pJTH5, is six nt shorter and slightly altered in sequence (Figure 1).

pJTH7: The KpnI-EcoRI fragment of pGA492 (An, 1986), containing the 5'-half of the CAT coding region, and the EcoRI-BamHI fragment of pGA425 (An, 1986), containing the 3'-half of the CAT coding region, were cloned in a three fragment
ligation into pBluescript (Stratagene) digested with Kpnl and BamHI. The reconstituted CAT coding region was collected from the resulting plasmid as a Kpnl-SstI fragment, and subcloned into pUC119 to produce the intermediate plasmid pCAT12. The PstI-BclI fragment of plasmid pVL (Section I), which contains the COX5a promoter and leader peptide coding sequence, was then purified and cloned into pCAT12 digested with PstI and Kpnl. The BclI-Kpnl adaptomer described above was used to facilitate this ligation, which produced intermediate plasmid pCAT13. The yeast expression plasmid pJTH7 was then formed by subcloning the PstI-SstI fragment of pCAT13, which contains the COX5a promoter, the COX5a leader sequence and the CAT coding sequence, into the yeast-E. coli shuttle vector YEp352 (Hill et al., 1986). The nucleotide sequence of pJTH6 from the COX5a transcription initiation site into the CAT coding region is shown in Figure 1.

pJTH8: pJTH8 was formed by a series of ligation steps similar to those described for plasmid pJTH7. pJTH8 is identical to pJTH7 except for the replacement of the prC5 ATG initiation codon by an ATT codon and the insertion of 24 nt in the 5' flanking region of the COX5a transcript, as compared to the latter plasmid. The nucleotide sequence of pJTH8 is compared to that of pJTH7 in Figure 1.
The nucleotide sequence from the expected transcription initiation site through the third codon of CAT is shown for pJTH5, pJTH6, pJTH7, and pJTH8. Nucleotide 1 is the transcription start site of the 35S promoter (pJTH5 and pJTH6) or the COX5α promoter (pJTH7 and pJTH8). Amino acid sequences of open reading frames beginning with an ATG initiation codon are indicated above the nucleotide sequences, using the single letter code. Triple asterisks (**) indicate termination codons. Single asterisks (*) indicate the position of a G residue that has been mutated to a T, to eliminate the COX5α initiation codon.

pJTH5) nt 1-14: 35S promoter.
    nt 15-44: MCS linker.
    nt 45-122: COX5α.
    nt 123-182: MCS linker.
    nt 183+: CAT.

pJTH6) nt 1-14: 35S promoter.
    nt 15-38: MCS linker.
    nt 39-116: COX5α.
    nt 117-176: MCS linker.
    nt 177+: CAT.

pJTH7) nt 1-91: COX5α.
    nt 92-151: MCS linker.
    nt 152+: CAT.

pJTH8) nt 1-12: COX5α.
    nt 13-37: MCS linker.
    nt 38-115: COX5α.
    nt 116-175: MCS linker.
    nt 176+: CAT.
Strains and growth media

The parent strain for S. cerevisiae transformation was αW303-11B (MATα leu2 ura3 trpl his3 ade3). To maintain selection for the URA3 marker allele of the CAT expression plasmids pJTH7 and pJTH8, transformants were grown in WO-U medium (0.67% yeast nitrogen base minus amino acids [Difco], 2% glucose, supplemented with adenine, tryptophan, histidine, and leucine at 20 μg/ml each. Yeast transformants also were propagated in EG medium (1% yeast extract [Difco], 2% peptone [Difco], 2% glycerol). Solid media for yeast contained 2% agar (Difco).

E. coli strain TG-1 (K12, *lac-pro*, supE, thi\(^{-}\), hsdD5/F' traD36, proA\(^{+}\)B\(^{+}\), lacI\(^{q}\), lacZ\(^{M15}\), 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 propagation of plasmids to be digested with BclI.

Agrobacterium tumefaciens strain LBA4404 (Clontech), used for transformation of tobacco, was grown in YEP medium ((1% peptone, 1% yeast extract, 0.1% NaCl, 1.5% Phytagar [Difco]). A. tumefaciens transformants were selected on YEP+TET+KAN (YEP supplemented with 3 μg/ml tetracycline and 10 μg/ml kanamycin).
Plant transformation

Plant expression plasmids pJTH5 and pJTH6 were introduced into *A. tumefaciens* by direct transformation, essentially as described (An et al., 1988), and transformants were selected on YEP+TET+KAN. Plasmids were reisolated from transformed *A. tumefaciens* cells by a modification of the plasmid quick-screen procedure originally described by An et al. (1988). Briefly, each plasmid preparation used 3 ml of cells cultured overnight in liquid YEP+TET+KAN. All incubation steps were done on ice. The addition of 30 μl phenol to the cell lysate was omitted. Instead, the supernatant was extracted once with water-saturated phenol and washed twice with diethyl ether. The DNA pellet was resuspended in 20 μl of TE (10 mM Tris-HCL, pH8.0, 1 mM EDTA) buffer. Two to five μl was used for digestion with appropriate restriction enzymes to ensure that the *A. tumefaciens* transformants contained structurally unaltered plasmid.

For plant transformation, leaf slices from sterile *N. tabacum* cv. Xanthi) were co-cultured with *A. tumefaciens* cells for 2 days (An et al., 1988). The bacterial cells were washed away and transformed tobacco calli were selected on a Murashige-Skoog (MS) agar medium (Murashige and Skoog, 1962) containing 3% sucrose, kanamycin (200 mg/l), cefotaxime (250 mg/l), and benzyladenine (0.5 mg/l). Shoots emerging from transformed calli were transferred to rooting
medium (MS agar medium without hormones and containing 50 kanamycin at mg/l). Plants with regenerated roots were transferred to peat pots and maintained in a high-humidity chamber before being transferred to a greenhouse or fluorescent light culture room (8 h light/16 h dark; 30 to 35 microeinstens m\(^{-2}\) s\(^{-1}\)).

**Fractionation of tobacco cells**

Plants were kept in the dark for 48 hours, then exposed to light for 2 hours before leaves were harvested. Ten to fifteen grams of leaf tissue devoid of mid-vein was sliced with a new razor blade into 30-45 ml of ice-cold Grinding Medium (GM; 0.4 M sorbitol, 50 mM Tris-HCl, pH 8.0, 2 mM EGTA, 1 mM DTT, 0.1% ascorbic acid, 0.5% BSA) and homogenized with a Waring blender at full speed for 2 seconds. All subsequent steps were performed at 4\(^{\circ}\)C. The homogenate was filtered through 4 layers of Miracloth. One ml of crude cell homogenate was saved, while a second one ml aliquot was centrifuged at full speed in a Beckman Microfuge for 10 min to obtain crude organelle and post-organelar (S10) fractions. The remaining homogenate was centrifuged at 1,700 x g\(_{\text{max}}\) for 5 min. The pellet was resuspended in 1.5 ml of GM and fractionated on a two-step Percoll gradient as described (Boutry et al., 1987). Intact chloroplasts were recovered by collecting the fraction at the 80%-40% interface and washed 3X.
with Wash Medium (WM; 0.4 M sorbitol, 50 mM Tris-HCl, pH 7.5, 2 mM EGTA). Mitochondria in the supernatant from the 1,700 \( g_{\text{max}} \) first spin were pelleted at 21,000 \( g_{\text{max}} \) for 10 min and further purified on a three-step Percoll gradient especially designed for purification of mitochondria from green tissues (Jackson et al., 1979). Purified mitochondria were obtained by collecting the fraction at the 21%-45% interface, and washed 3X with WM.

Soluble and membrane fractions of chloroplasts and mitochondria were prepared either by disrupting the membranes with an Artec Sonic Dismembrator, model 150 (two 5 s bursts) in the presence of 1 M NaCl, followed by centrifugation at 100,000 \( g_{\text{max}} \) for 30 min. The pelleted membrane fraction was suspended in 10 mM Tris-HCl, pH 7.5.

**Transformation and fractionation of yeast cells**

Yeast transformation was as described (Dieckmann and Tzagoloff, 1983); transformants were selected on WO-U plates supplemented with 1.2 M sorbitol. Cells were grown to early stationary phase in liquid EG medium, and mitochondrial and S10 fractions were prepared as described (Daum et al., 1982). Soluble and membrane fractions of yeast mitochondria were prepared as described above for tobacco mitochondria and chloroplasts.
Protease protection assay

Purified chloroplasts or mitochondria (1 mg protein/ml) were incubated with proteinase K (Boehringer-Mannheim Biochemicals, Indianapolis, IN) at a final concentration of 0.2 mg/ml for 30 min at 0°C, in the presence or absence of 1% Triton X-100. The incubation was terminated by addition of phenylmethylsulfonyl fluoride to 2 mM. The treated samples were then assayed for the presence of CAT by enzymatic assay or immunoblotting, as described below.

Detection of CAT, hsp60, and rbcL by immunoblotting

Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Protein fractions were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Towbin et al., 1979). The membranes were incubated with antiserum against purified CAT, hsp60, (McMullin and Hallberg, 1988), or rbcL (Berry et al., 1985), and membrane-bound IgG was detected by incubation with 125I-protein A (DuPont-NEN Research Products, Wilmington, DE) as described (Schmidt et al., 1984).

Measurement of CAT activity

Cellular and organellar fractions of tobacco or yeast were prepared as described above. An aliquot from each fraction containing 1.2 µg protein (tobacco) or 0.05 µg
protein (yeast) was assayed for CAT activity as described (Gorman et al., 1982), using $^{14}$C-chloramphenicol as substrate.
RESULTS

Gene constructs for expression of fusion proteins

Figure 2 represents two gene fusions constructed to test whether a mitochondrial targeting peptide from the yeast Saccharomyces cerevisiae causes transport of a reporter protein into tobacco mitochondria. The targeting peptide used is that of the cytochrome oxidase subunit Va precursor (prC5), coded for by the yeast gene COX5a (Koerner et al., 1985; Cumsky et al., 1987). The reporter protein is chloramphenicol acetyl transferase (CAT), coded for by the CAT gene of bacterial transposon Tn9 (Alton and Vapnek, 1979).

Table 2-1 describes the plasmids used and the fusion proteins for which they code. Plasmid pJTH5 codes for fusion protein prC5/CAT, comprising the amino terminal 20 residues of prC5, 20 amino acids coded for by a multiple cloning site (MCS) linker region, and all 219 amino acids of mature CAT. This region of prC5 contains the entire mitochondrial targeting sequence, since the native protein is cleaved on the C-terminal side of residue 20 upon transport into mitochondria (Koerner et al., 1985). Transcription of the fusion gene in pJTH5 is controlled by the cauliflower mosaic virus 35S RNA promoter (Odell et al., 1985), and the dule terminator of the transcripts 6a and 6b of the octopine T-DNA (An, 1986) located
adjacent to the 3' end of the CAT coding region.

Figure 2B shows the composition of prC5/CAT and pCAT. For expression of prC5/CAT, the initiation codon of COX5a is located 63 nucleotides (nt) downstream of the transcription initiation site predicted for the 35S RNA promoter, and is the first ATG sequence of a continuous open reading frame that includes the CAT coding sequence (see Methods). Another methionine codon is located 17 nt downstream of the predicted transcription start site, 5' to the COX5a initiation codon. This open reading frame extends for only six codons, terminating at a TAG sequence 26 nt upstream of the prC5 initiation codon, and does not preclude expression of the downstream open reading frame coding for prC5/CAT (see below). The next ATG sequence present in the transcript is codon 21 of prC5/CAT, the first codon of the MCS located between COX5a and CAT. Control plasmid pJTH6 is similar to pJTH5, except that the initiation codon of COX5a has been eliminated. Thus, the prC5 targeting peptide is not included in the fusion protein. In pJTH6, the first ATG codon of the open reading frame that contains CAT is located within the MCS. Translation initiation at this methionine codon would produce a protein, pCAT, that is 20 residues longer at the amino terminus than native CAT and lacks a mitochondrial targeting peptide (Figure 2B).

The gene fusions coding for prC5/CAT and the leaderless
Figure 2. Gene constructions and fusion proteins

A) Diagramatic representation of the gene constructions used for expression in plants of a protein fusion between the yeast mitochondrial targeting peptide from cytochrome oxidase subunit Va and chloramphenicol acetyl transferase (prC5/CAT), or a form of chloramphenicol acetyl transferase lacking a mitochondrial targeting peptide (pCAT). The promoter is the 35S RNA promoter (P-35S) of cauliflower mosaic virus, and the fusion genes are adjacent at their 3' ends to the dule terminator (T-6a'5b!) of the transcripts 6a and 6b of the octopine T-DNA. Regions of the yeast gene COX5a and the E. coli gene CAT are indicated, as is a coding region (MCS) derived from a multiple cloning site linker upstream of the chloramphenicol acetyl transferase coding region. Dotted arrows indicate the transcribed regions of each fusion gene, pointing in the direction of transcription. Solid arrows indicate the location of the ATG initiation codon in the open reading frame that codes for chloramphenicol acetyl transferase. The asterisk (*) indicates the former position of an ATG codon altered by site-directed mutagenesis to an ATT codon, to eliminate translation of the mitochondrial targeting peptide. Construction of these gene fusions, and the nucleotide sequence of the region critical to this study, are described in detail in the Methods section. The diagram is not drawn to scale.

B) Diagramatic representation of the fusion proteins prC5/CAT and pCAT. The number of amino acids coded for by each region of the proteins is indicated. The sequence of the amino terminal regions of these proteins is presented in the Methods section.
A) 

prC5/CAT 

\[ \text{ATG} \] 

\[ \text{P-35S} \quad \text{COXVa} \quad \text{MCS} \quad \text{CAT} \quad \text{T-t6a'6b'} \]

pCAT 

\[ \text{ATG} \] 

\[ \text{P-35S} \quad \text{COXVa} \quad \text{MCS} \quad \text{CAT} \quad \text{T-t6a'6b'} \]

B) 

20 20 219 

\[ \text{prC5/CAT} \] 

\[ \text{pCAT} \] 

\[ \text{Native CAT} \] 

\[ \text{COXVa} \quad \text{MCS} \quad \text{CAT} \]
Table 2-1. CAT expression plasmids

<table>
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</tr>
</thead>
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<td>pGA492&lt;sup&gt;a&lt;/sup&gt;</td>
<td>prC5/CAT</td>
</tr>
<tr>
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<td>pCAT</td>
</tr>
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<td>Tobacco</td>
<td>YEp352&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>pJTH8</td>
<td>Tobacco</td>
<td>YEp352</td>
<td>pCAT</td>
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<sup>a</sup>An (1986).
<sup>b</sup>Hill et al. (1986).
control protein pCAT were expressed in yeast from the episomal plasmids pJTH7 and pJTH8, respectively. Both fusion proteins are expressed in yeast from the COX5a promoter. The coding sequences of the pCAT and prC5/CAT fusion genes in pJTH6 and pJTH7 are identical to those in the plant transformation plasmids pJTH5 and pJTH6 (see Methods).

**Expression and sub-cellular targeting of CAT in yeast and tobacco**

Figure 3 shows that CAT is expressed to approximately the same specific activity in yeast cells transformed either with the prC5/CAT expression vector pJTH7 or with the pCAT expression vector pJTH8. However, fractionation of the transformed cells into mitochondrial and post-mitochondrial supernatant (S10) fractions revealed a significant difference in the sub-cellular locations of the two CAT proteins. CAT activity from prC5/CAT co-fractionates with both the mitochondrial and S10 fractions, while the leaderless protein pCAT appears to reside entirely in the S10 fraction. Immunoblot analysis using antibodies against CAT confirmed this result (data not shown). Thus, the mitochondrial targeting peptide of prC5 functions to transport CAT into mitochondria in this homologous system.

Figure 4A indicates that similar levels of CAT activity are found in transgenic tobacco plants transformed with either
Figure 3. Expression and sub-cellular localization of CAT activity in yeast


Lanes 1,4: Total cell homogenate.
Lanes 2,5: S10 (post-mitochondrial) supernatant.
Lanes 3,6: Mitochondria.
Lane 7: Negative control with no protein added to the reaction.
Lane 8: Positive control with purified CAT added to the reaction.
Figure 4. Expression and subcellular localization of CAT activity in tobacco

A) CAT activity is measured as in Figure 1 (1,3-diAC: 1,3-diacetyl [14C]-chloramphenicol). Lanes denoted "pCAT" show CAT activity in protein fractions from a pJTH6 transformant, and those marked "prC5/CAT" show activity in a pJTH5 transformant.

Lanes 1,6: Total cell homogenate.
Lanes 2,7: S10 (post-organellar) supernatant.
Lanes 3,8: Crude organellar pellet.
Lanes 4,9: Chloroplasts.
Lanes 5,10: Mitochondria.
Lanes 11,12: Negative and positive controls, respectively, as in Figure 1.

B) Immunoblotting analysis of sub-cellular fractions. Aliquots of the protein fractions analyzed for CAT activity were subjected to SDS-PAGE, and probed by Western blotting for the presence of the chloroplast stromal protein rbcL and the mitochondrial matrix protein hsp60. Lane designations are as in part A) above.
pJTH5 or pJTH6. However, the specific activity of CAT in total cell homogenates was approximately two orders of magnitude less in tobacco than in yeast. To determine the location of CAT in plant cells, leaf tissue from the transformants was fractionated into a crude organellar pellet, post-organellar S10 supernatant, mitochondria, and chloroplasts. CAT assays revealed that pCAT is located exclusively in the S10 fraction, while prC5/CAT produces CAT activity in both the S10 and crude organellar fractions. Upon further fractionation of the crude organellar pellet into mitochondria and chloroplasts, CAT activity from prC5/CAT was detected in both organelles. In the example shown in Figure 4A, CAT specific activity is approximately equal in the two organelles. These results indicate that a yeast mitochondrial targeting peptide is able to direct a passenger protein to plant mitochondria, and to chloroplasts as well. Identical results were obtained in analysis of two independent tobacco transformants expressing prC5/CAT (see below).

Figure 4B shows the result of an immunoblot analysis performed to detect cross-contamination between the chloroplast and mitochondrial fractions. Probing the fractions with antiserum against a chloroplast polypeptide, the large subunit of ribulose bisphosphate carboxylase/oxygenase (rbcL), revealed that the mitochondrial fraction was slightly contaminated with chloroplasts.
However, probing with antiserum against the mitochondrial protein hsp60 (McMullin and Hallberg, 1988) showed that the chloroplast fraction is not contaminated with mitochondria. Thus, the presence of CAT activity in chloroplasts is due to targeting of prC5/CAT to this organelle. This targeting is dependent on the presence of the prC5 targeting peptide, since the leaderless protein pCAT does not co-fractionate with chloroplasts (Figure 4A, Lane 4).

**Import of CAT to the interior of mitochondria and chloroplasts**

Several experiments determined that the mitochondrial targeting peptide of prC5 is able to cause transport of CAT to the interior of plant mitochondria and chloroplasts. First, as shown in Figure 5A, washing of the intact organelles with 1 M NaCl did not release a significant amount of CAT activity, an indication that the co-fractionation of CAT with mitochondria and chloroplasts is not due to non-specific protein-protein interactions. After sonication of the organelles and separation of sub-organelar soluble and membrane fractions, the great majority of CAT activity was found to be released into the soluble fraction. Figure 5B shows that the fractionation procedure was effective, as judged from immunoblot analyses that detect the soluble mitochondrial and chloroplast marker proteins hsp60 and rbcL, respectively. These results suggest that CAT is located
Figure 5. Sub-organellar localization of CAT

A) Sub-organellar fractionation. CAT activity was assayed as in Figure 1. Lanes denoted "Chloro." show CAT activity in protein fractions derived from purified chloroplasts of a pJTH5 transformant, and those denoted "Mito." show activity in fractions derived from mitochondria of the same plant.

Lanes 1,5: Supernatant from a 1 M NaCl wash of the organelles.

Lanes 2,6: Organellar pellets from the 1 M NaCl wash.

Lanes 3,7: Supernatant from the sonicated organelles, containing the soluble protein fraction.

Lanes 4,8: Pellet from the sonicated organelles, containing the membrane bound proteins.

B) Immunoblot analysis of sub-organellar fractions. Fractions were analyzed by Western blotting for the presence of the stromal marker rbcL and the matrix marker hsp60, as in Figure 4.

C) Protease protection assay. CAT activity was analyzed as in Figure 2. Chloroplasts and mitochondria from a pJTH5 transformant were treated with proteinase K in the presence or absence of 1% Triton X-100, as indicated.
A)

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<thead>
<tr>
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<tr>
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1 2 3 4 5 6 7 8

B)

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1 2 3 4 5 6 7 8

C)

<table>
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<td></td>
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<tr>
<td>1-AC</td>
<td></td>
<td></td>
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<tr>
<td>C</td>
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Proteinase K
Triton X-100
within mitochondria and chloroplasts, in the soluble fraction of the organelles. The results were confirmed by protease protection experiments in which CAT activity in purified mitochondria or chloroplasts was assayed following treatment with proteinase K. Figure 5C shows that in both organelles the CAT activity was protected from digestion with externally added proteinase K. However, addition of detergent to disrupt organelle membranes rendered most of the CAT activity susceptible to protease. Treatment with detergent alone had no effect on CAT activity (data not shown). In all the analyses described above, similar results were obtained with the homologous transport system in which prC5/CAT was targeted to yeast mitochondria (data not shown).

**Processing of prC5/CAT during import into organelles**

In order to determine whether prC5/CAT was processed when imported into chloroplasts or mitochondria, aliquots of chloroplast or mitochondrial proteins were separated by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) and analysed by immunoblotting using antibodies raised against purified *E. coli* CAT. Processing of the precursor was characterized in the homologous transport system, where prC5 is expected to be cleaved by the mitochondrial processing protease (Bohni et al., 1980).
Figure 6. Detection of CAT in plant mitochondria and chloroplasts by immunoblotting

Mitochondrial and chloroplast proteins from yeast and tobacco transformants expressing prC5/CAT were separated by SDS-PAGE and probed for the presence of CAT by Western blotting. In addition, the yeast mitochondria were treated with proteinase K in the presence and absence of Triton X-100, as in Figure 5C.

Lane 1: Tobacco chloroplasts.
Lane 2: Tobacco mitochondria.
Lane 3: Yeast mitochondria.
Lane 4: Yeast mitochondria treated with proteinase K.
Lane 5: Yeast mitochondria treated with proteinase K plus 1% Triton X-100.
Lane 6: Native CAT purified from E. coli.

Lanes 1 and 2 were exposed to X-ray film for 4 days, and Lanes 3-6 for one day.
Figure 6 shows that purified yeast mitochondria from a transformant expressing prC5/CAT contain two immunoreactive bands of apparent molecular masses 29 kDa and 27 kDa, respectively. The 29 kDa band corresponds to the predicted size of the prC5/CAT precursor protein and it co-migrates with the major in vitro translation product produced from the COX5a-CAT fusion gene (data not shown). Furthermore, addition of proteinase K to these mitochondria completely eliminates the 29 kDa band (Figure 6), suggesting that this protein is prC5/CAT that has attached to the outer surface of mitochondria without being imported.

In contrast to the 29 kDa protein, the 27 kDa form of CAT seen in untreated mitochondria is protected from digestion with proteinase K, unless mitochondrial membranes are lysed by the addition of detergent (Figure 6). Therefore, the 27 kDa band is an internal mitochondrial protein; its size is the same as that expected if prC5/CAT were cleaved at the native processing site of prC5. This protein appears to be approximately 2 kDa larger than native CAT (Figure 6), as expected from the presence of the MCS linker region upstream of CAT in the fusion gene constructions (see Figure 2B).

Digestion of the 27 kDa and/or 29 kDa immunoreactive bands with proteinase K results in the appearance of a new immunoreactive band not seen in untreated mitochondria, with an apparent molecular mass of approximately 25 kDa (Figure 6).
Since the 25 kDa band is resistant to proteinase K even when membranes are disrupted by detergent, we presume that this is a protease resistant form of CAT. Although this protein appears to be just slightly smaller than native CAT, it retains only residual enzymatic activity. This conclusion is based on the observations that 1) treatment of yeast mitochondria with proteinase K and detergent leaves only the protease resistant CAT protein, yet eliminates the majority of CAT activity (data not shown; see Figure 5C), and 2) treating purified CAT with an excess of proteinase K strongly reduces, but does not eliminate, enzyme activity (data not shown).

The chloroplast and mitochondrial CAT proteins produced in the heterologous transport systems were compared to those found in yeast (Figure 6). In both organelles, one prominent immunoreactive band was found which co-migrates with the 27 kDa CAT protein shown to be internal to yeast mitochondria. Since CAT activity was shown to be internal to tobacco chloroplasts and mitochondria, prC5/CAT produced in the plant is proteolytically processed upon transport into the organelles. The size of the processed products is consistent with cleavage at the native processing site of prC5, but at present we have no data bearing on the specific nature of the proteolytic processing of prC5/CAT in plant mitochondria and chloroplasts.
Growth conditions influence the transport of CAT into chloroplasts

The sub-cellular location of CAT was determined in independent tobacco transformants expressing prC5/CAT, propagated under different growth conditions. Transformants JT51 and JT58 were grown for sixteen weeks in the greenhouse or under fluorescent lights in a culture room (8 h light/16 h dark), respectively, before sub-cellular fractionation of leaf tissue. Table 2-2 indicates that the total level of CAT expressed was similar in the two plants. Figure 7 shows, however, that the distribution of CAT in chloroplasts and mitochondria was different: the CAT specific activity in chloroplasts and mitochondria was approximately equal in the greenhouse grown plant, while mitochondrial CAT activity in the plant grown in the culture room was approximately three times greater than chloroplast CAT activity. These results suggest that the efficiency of CAT transport into chloroplasts relative to its transport into mitochondria is affected by growth conditions.

To rule out the possibility that the different efficiencies of CAT transport are due to inherent differences in independent transformants JT51 and JT58, the two plants were switched to the opposite growth condition for a period of three weeks, then the sub-cellular fractionation experiment was repeated. Figure 7 shows that moving transformant JT51
from the greenhouse to the culture room caused a reversal of the pattern of CAT localization, so that mitochondria exhibited significantly more CAT specific activity than did chloroplasts. Likewise, the CAT localization pattern in transformant JT58 also was reversed when this plant was moved from the culture room to the greenhouse, so that mitochondria and chloroplasts now contained approximately equal amounts of CAT per unit protein. Quantitation of these results is presented in Table 2-2. Leaves used in these assays are the pre-existing ones that are apparently not expanded.
Figure 7. CAT activities in chloroplasts and mitochondria under different growth conditions

CAT activity was determined as in Figure 2. In all panels the upper band is 3-acetyl $[^{14}\text{C}]$-chloramphenicol, the middle band is 1-acetyl $[^{14}\text{C}]$-chloramphenicol, and the lower band is $[^{14}\text{C}]$-chloramphenicol. Activity was assayed in chloroplasts (C) and mitochondria (M) from two independent pJTH5 transformants, plants JT51 and JT58. Plants were grown either in the greenhouse (GH) or in a culture room (CR). JT51 was placed in the greenhouse immediately after the plant was transferred from regeneration medium to soil, and grown for 16 wk before the GH cell fractions were collected. The plant was then transferred to the culture room and grown for an additional three weeks before the CR cell fractions were collected. JT58 was kept in the culture room for 16 wk after transfer from regeneration medium to soil, then assayed for CAT activity in mitochondria and chloroplasts. The plant was then transferred to the greenhouse for 3 wk before assay of the GH fractions.
<table>
<thead>
<tr>
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Table 2-2. Targeting of CAT in different growth conditions

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<th>Time&lt;sup&gt;b&lt;/sup&gt;</th>
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<th>Ratio&lt;sup&gt;d&lt;/sup&gt;</th>
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<sup>a</sup>GH: Greenhouse. CR: Fluorescent light culture room.

<sup>b</sup>Plant JT51 was grown continuously in the greenhouse, assayed for CAT activity, switched to the culture room for 3 wk, then assayed again. Plant JT58 was grown continuously in the culture room, assayed, switched to the greenhouse, then assayed again.

<sup>c</sup>CAT activity is expressed as the percent acetylated chloramphenical, relative to the total chloramphenicol.

<sup>d</sup>Ratio of CAT specific activity in chloroplasts to CAT specific activity in mitochondria.
Biogenesis of both plastids and mitochondria depends on post-translational import of precursor proteins synthesized on cytosolic ribosomes. Since plastids and mitochondria contain largely distinct sets of proteins, protein targeting and import are generally assumed to be specific for each compartment. The most direct evidence for specific targeting comes from experiments (Boutry et al., 1987) in which either a mitochondrial leader peptide, from the $\beta$-subunit of ATP synthase (Boutry and Chua, 1985) or a chloroplast leader peptide, from the small subunit of Rubisco, from *Nicotiana plumbaginifolia* (Poulsen et al., 1986) was fused to CAT as a reporter protein. Whether the CAT protein was targeted to chloroplasts or mitochondria in transgenic tobacco plants depended on the leader peptide; no misrouting was found. Nevertheless, the transport mechanisms of plastids and mitochondria exhibit significant similarities, raising questions about how specific targeting is achieved. The present study shows that a single targeting leader peptide can be recognized by the transport systems of both mitochondria and chloroplasts. These results raise the possibility that not all natural targeting is specific, so that certain endogenous proteins might be transported independently into
two distinct subcellular organelles.

Our observations contrast with those of Schmitz and Lonsdale (1989), who found that the leader peptide of yeast mitochondrial tryptophanyl-tRNA synthetase directed a reporter protein, β-glucuronidase (GUS), specifically to mitochondria in transgenic tobacco plants. This difference in results is unlikely to be due to the use of a different reporter protein, since CAT showed no affinity for either mitochondria or plastids in the absence of the prC5 leader peptide (Figure 3A) and Boutry et al. (1987) observed specific targeting of CAT. It is more likely that the difference in targeting specificities of the prC5 and tryptophanyl-tRNA synthetase leader peptides is due to inherent differences in their structure.

Mitochondrial leader peptides from different eukaryotes share significant structural similarities (von Heijne, 1986), although there is little conservation of amino acid sequence among them. They are rich in basic and hydroxylated residues and contain few, if any, acidic residues, so that they have net positive charges. They are predicted to form amphiphilic structures, most commonly α-helices (von Heijne, 1986; Verner and Schatz, 1988), and it has been proposed that amphiphilicity of a leader peptide is a fundamental requirement for mitochondrial protein import (Roise et al., 1988). By the criteria of von Heijne (1986), the
tryptophanyl-tRNA synthetase leader peptide has properties typical of a mitochondrial targeting peptide; moreover, it is predicted to be able to form an amphiphilic α-helix by the criteria of Kyte and Doolittle (1982) (not shown). In contrast, the prC5 leader peptide is atypical. As an α-helix, it would be the least amphiphilic of 25 leader peptides examined by von Heijne (1986), while Cumsky et al. (1987) propose that it is likely to form not the usual α-helix but an antiparallel β-pleated sheet.

It has been more difficult to discern common elements in the structure of plastid leader peptides than in the structure of mitochondrial leader peptides. Plastid leader peptides share several common features with those of mitochondria, such as having net positive charges and being rich in hydroxylated residues (for reviews see Verner and Schatz, 1988; von Heijne, 1988; Keegstra et al., 1989). As with mitochondria, amphiphilicity is probably important, but many chloroplast leader peptides seem unlikely to form α-helices (Verner and Schatz, 1988; Keegstra et al., 1989). Some of them instead contain regions that may form amphiphilic β-structures (Steppuhn et al., 1987; Tyagi et al., 1987), like the unusual mitochondrial leader peptide of prC5. Thus, the unusual properties of the prC5 leader peptide may be responsible for its ability to target proteins to plastids.

Pfaller et al. (1988, 1989) have suggested that there are
two pathways of import of proteins into mitochondria, although the two pathways overlap at a certain point. One pathway is mediated by specific receptors of the mitochondrial membrane, and import through this pathway is highly efficient. Import through the other pathway is mediated by a group of non-specific receptors, the "General Insertion Proteins" (GIP), and is relatively inefficient. Both pathways are able to operate in vivo and in vitro. Importation directed by some artificial leader peptides and by at least one chloroplast leader peptide takes place by the inefficient pathway (Pfaller et al., 1989). It is possible that there is a similar inefficient import pathway in plastids; import of prC5/CAT into plastids could occur by such a pathway. Our data indicate, however, that the specific CAT activity is approximately the same in chloroplasts and mitochondria, at least when assayed under favorable growth conditions. In mitochondria, the two pathways can most easily be distinguished by in vitro import experiments, so that it will be desirable to examine the import into chloroplasts of the prC5/CAT fusion protein in vitro.

Mitochondria isolated from yeast transformants expressing the gene coding for prC5/CAT contained two polypeptides that cross-reacted with anti-CAT antibodies (Figure 5, lane 3), a 29 kDa polypeptide that was susceptible to proteinase K digestion, and a 27-kDa polypeptide that was protected by
mitochondrial membranes from proteolysis (Figure 5, lane 4). Since the 29-kDa polypeptide is the same size as the protein synthesized from RNA transcribed in vitro from the prC5/CAT gene (not shown), we conclude that it represents precursor that is associated with the mitochondria but has not been imported. It is unusual for the precursor to a mitochondrial protein to be detectable in cell extracts, but very large amounts of CAT were produced in the yeast cells: we estimate that CAT made up as much as 0.5 to 1% of mitochondrial protein. This large amount of protein may overload the protein import machinery of the mitochondria.

In transgenic plants expressing the prC5/CAT fusion protein, only one immunoreactive band co-fractionating with chloroplasts and mitochondria was detected; its molecular mass was 27 kDa in both organelles (Figure 5, Lanes 1 and 2), approximately the same size as the form of the protein inside yeast mitochondria. No precursor was detected in homogenates or in the post-organellar supernatant (not shown). Since the specific activity of CAT in the transgenic tobacco plastids and mitochondria was 50- to 100-fold less than in the yeast mitochondria, it is likely that the prC5/CAT precursor protein was transported rapidly into organelles, so that the precursor did not accumulate. The 27 kDa polypeptide was protected from proteinase K digestion (Figure 4 and not shown), and therefore was located inside chloroplasts and mitochondria. This 27 kDa
polypeptide may be a processed product generated by the stromal processing protease (Robinson and Ellis, 1984) or the matrix processing protease. The similarity in molecular mass of the protein in chloroplasts and mitochondria of yeast and tobacco suggests that the processing enzymes of tobacco chloroplasts and mitochondria recognize nearby cleavage sites or even the same cleavage site in prC5 as the yeast matrix processing protease. It is also possible, however, that the 27 kDa protein was generated through non-specific proteolysis.

The specific activity of CAT was comparable in plastids and mitochondria of the prC5/CAT transformants analyzed, with chloroplasts showing slightly higher CAT activity. The relative specific activity of CAT in chloroplasts and mitochondria was, however, influenced by the conditions under which a plant was grown: when plants were grown under low light intensity in a culture room, they had a lower specific activity of CAT in their plastids than when grown under high light intensity in a greenhouse. The total amount of CAT in the plant under the different growth conditions showed little apparent difference, and immunoblot analysis indicated that the content of rbcL was similar in chloroplasts (not shown). Plants grown under these different conditions may contain plastids that are developmentally, biochemically, or physiologically different. At present, we do not know precisely what caused the differential distribution of CAT
activity between chloroplasts and mitochondria, and whether only the import of the prC5/CAT fusion protein into chloroplasts was affected or the import of endogenous chloroplast proteins was affected as well.
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SECTION III. AN INITIAL EFFORT IN STUDYING THE
MOLECULAR BASIS OF CYTOPLASMIC MALE STERILITY IN MAIZE
(Zea mays L.) WITH THE TEXAS MALE-STERILE CYTOPLASM
INTRODUCTION

Mitochondria of maize (Zea mays L.) with the Texas (T) male-sterile cytoplasm synthesize a unique 13 kDa protein, T-URF13 (Forde et al., 1978; Dewey et al., 1987; Wise et al., 1987a and 1987b). T-URF13 has been implicated in causing both cytoplasmic male sterility (CMS) and susceptibility to two fungal pathotoxins, HmT toxin produced by Helminthosporium maydis, race T, and Pm toxin produced by Phyllosticta maydis, and to the insecticide methomyl (for reviews see Ullstrup, 1972; Gregory et al., 1977). T-URF13 is constitutively synthesized in mitochondria of all tissues of cms-T plants examined (Newton and Walbot, 1985), but mitochondrial degeneration has been observed only in anthers (Warmke and Lee, 1977, 1978). Two dominant nuclear genes, Rf1 and Rf2, can restore fertility to maize plants with the T male-sterile cytoplasm (Laughnan and Gabay-Laughnan, 1983). In a fertility-restored plant, synthesis of T-URF13 is greatly reduced (Forde and Leaver, 1980). Rf1 alone has been shown to suppress T-URF13 expression (Dewey et al., 1986; Dewey et al., 1987). Male fertile revertants can be obtained from tissue cultures of T maize (Gengenbach et al., 1977; Brettell et al., 1980). These fertile revertants no longer synthesize T-URF13 (Dixon et al., 1982); the T mitochondrial gene (T-urfl3) that
codes for T-URF13 (Dewey et al., 1986) is either deleted or mutated (Umbeck and Gengenbach, 1983; Fauron et al., 1987; Rothman et al., 1987; Wise et al., 1987b). Recent studies have directly proven the role of T-URF13 in the causation of susceptibility to toxins and methomyl. Expression of T-URF13 in E. coli caused respiration of E. coli to be susceptible to HmT and Pm toxins and to methomyl (Dewey et al., 1988). Yeast cells expressing a mitochondrial form of T-URF13 are also susceptible to toxins and methomyl; their growth was inhibited, and mitochondria were uncoupled by these compounds (Section I). Whether T-URF13 is also responsible for the CMS phenotype is, however, still unknown. We attempted to express T-URF13 in a heterologous plant, tobacco, which normally does not contain T-URF13 protein. We expected that if T-URF13 is responsible for CMS in maize, expression of a mitochondrial form of T-URF13 would be likely to cause mitochondrial dysfunction in tobacco anthers, and hence male sterility. This report communicates some preliminary results. Even though no firm conclusion can be drawn from these results, we believe that this approach, with some modification, would still be useful to investigate the molecular basis of cytoplasmic male sterility in cms-T maize.
MATERIALS AND METHODS

Construction of expression plasmids

PJTH9: Expressing plasmid PJTH9 was constructed by directly fusing pCCT and pRT99 linearized by the enzyme SstI. Construction of pCCT has been described (see Section II, Methods). The binary vector pRT99 was provided by Dr. R. W. Thornburg, Department of Biochemistry and Biophysics, Iowa State University.

PJTH10: PJTH10 was constructed by direct fusion of SstI-linearized pCCT10 and pRT99. Plasmid pCCT10 is a derivative of pCCT which included two changes in the HindIII-BclI region of pCCT (see pJTH6 in Section II, Methods).

PJTH11: The BamHI-Sau3AI fragment from pUC19N9L coding for the mitochondrial leader peptide from Neurospora ATP synthase subunit 9 (prN9) (Viebrock et al., 1982; Gearing and Nagley, 1986) was ligated to the BclI-SstI fragment from pTI4 (Section I) containing T-urf13 (Dewey et al., 1986), using pUC119 as the cloning vector. The resulting plasmid was designated pN9LT13. The expression plasmid PJTH11 was constructed by replacing the BamHI-SstI fragment of the binary vector pBI121 (Clontech) with the BamHI-SstI fragment from pN9LT13.
Plant transformation and identification of transformants

Plant transformation and regeneration and maintenance of transgenic tobacco plants have been described (see Section II, Methods). Transgenic plants were identified by DNA hybridization (Southern, 1975) using $^{32}$P-labeled T-urf13 coding region as probe. Probes were labeled by the random primer methods (Feinberg and Vogelstein, 1983), using a labeling kit (Amersham) as described by the manufacturer. DNA from leaf tissues of transgenic tobacco was isolated as described (Davis et al., 1980). Prehybridization, hybridization, and washing were all carried out at 65°C.

Characterization of transgenic plants

Leaf-disc assay: Leaves were harvested from greenhouse-grown plants and sterilized by soaking in 70% (v/v) ethanol for 1 min, then in 15% bleach (5%, w/v, sodium hydrochlorite) for 20 min, and rinsed 3x with sterile water. Sterilized leaves were cut into small discs with a paper punch and discs were put on top of solid MS medium (see Section II, Methods) containing various concentrations of methomyl. MS medium was supplemented with 2 µg/l NAA and 0.5 µg/l BAP. Methomyl (Lannate L, Dupont) was sterilized by filtration through a 0.22 µm filter (Co-Star) and spread on MS plates. Plates containing leaf discs were kept in a culture room (18 h, 26°C day/8 h, 16°C night) for 3 to 5 weeks before being scored.
Leaf-injection assay: Methomyl and HmT and Pm toxins were injected into tobacco leaves through the lower epidermis as described (Turner and Martinson, 1972). Either H$_2$O (for methomyl) or DMSO (for toxin) was used as control. Leaves were checked for reaction towards toxins and methomyl (Turner and Martinson, 1972) 16 to 18 h after injection.

Immunodetection of T-URF13: Fractionation of tobacco cells, purification of mitochondria and chloroplasts, quantitation of proteins, SDS-PAGE, and western blotting have been described (see Section II, Methods).

Pollen fertility: Young buds (2 to 3 cm) were collected from greenhouse-grown plants at ca. 10 a.m., soaked in 70% (v/v) ethanol, and either used immediately or stored at 4°C. Pollen grains were stained with I$_2$/KI solution and examined under a microscope.

Miscellaneous

DNA manipulations, E. coli strains, the Agrobacterium tumefaciens strain, the tobacco variety (Section II, Methods), antibodies against T-URF13, HmT toxin, Pm toxin, methomyl, and measurement of O$_2$ consumption rate (Section I, Materials and Methods) have been described previously.
RESULTS AND DISCUSSION

Gene constructions for expression of fusion proteins

We (Section II) and others (Schmitz and Lonsdale, 1989) have demonstrated the feasibility of using heterologous leader peptides to target two proteins, chloramphenicol acetyltransferase (CAT) and β-glucuronidase, to mitochondria in transgenic tobacco plants. The objectives of the study reported here were to investigate whether the T-URF13 protein could also be targeted to mitochondria in tobacco and to examine its effects on tobacco plants. Three fusion genes (Figure 1) containing the T-urf13 open reading frame were used. The construction of expression plasmids containing these fusion genes is described in Materials and Methods. The first expression plasmid, pJTH9, contains a gene that codes for a fusion protein consisting of the leader peptide from the yeast cytochrome oxidase subunit Va precursor fused to T-URF13 (prC5/T-URF13); the prC5 leader peptide targets CAT to mitochondria, and chloroplasts, in tobacco (Section II), but fails to target T-URF13 to the interior of mitochondria in yeast (Section I). The second expression plasmid, pJTH10, codes for leaderless T-URF13, as a control. The third expression plasmid, pJTH11, contains a gene that codes for a fusion protein consisting of the leader peptide from the
Fig. 1. Gene fusions for expression of T-urfl3 in tobacco

A. Fusion genes cloned into the binary vector pGA492

NPTII: neomycin phosphotransferase gene from Tn5
P-35S: cauliflower mosaic virus 35S RNA promoter
COX5: leader sequence of cytochrome oxidase
     subunit V of yeast
T-urfl3: the T maize mitochondrial gene coding
         for T-URF13
T-PIN1: terminator of the proteinase inhibitor I
         gene of potato
LB: T-DNA left border
RB: T-DNA right border

Locations of the translation initiation codon
(ATG) for pJTH9 and pJTH10 are as indicated.
Symbols for restriction enzymes are: B, BamHI; C, BclI; E, EcoRI.

B. Fusion genes cloned into the binary vector pBI121

oli+: leader sequence of Neurospora ATP synthase
      subunit 9 gene
T-NOS: terminator of the noponine synthase gene
Symbols for restriction enzymes: H, HindIII; S, SstI. Other symbols are as designated in A.
A) pJTH9 and pJTH10

B) pJTH11
Neurospora ATP synthase subunit 9 precursor fused to T-URF13 (prN9/T-URF13). This leader peptide targets T-URF13 to the interior of mitochondria in yeast, but its function in plants has not previously been examined. All three fusion proteins are expressed under the control of the cauliflower mosaic virus 35S promoter and the PIN-1 terminator. The proteins encoded by pJTH9, pJTH10, and pJTH11 are identical to those encoded by the yeast expression plasmids pCMS1, pJTH2, and pJTH1, respectively (see Section I).

Screening for transgenic plants

Transformants were identified by DNA hybridization (Southern, 1975). Genomic DNAs were isolated from 10 plants transformed with pJTH9 (JT30 through JT39) and 9 plants transformed with pJTH10 (JT41 through JT49), digested with EcoRI, and separated on a 0.8% agarose gel. The resolved DNA fragments were transferred to a nylon membrane (Micron Separations Inc.) and the membrane was probed with the 32p-labeled EcoRI-SstI fragment from pJTH9 (see Figure 1), which contains the cauliflower mosaic virus (CaMV) 35S RNA promoter, the COX5a presequence, and the T-urfl3 coding region. In both expression plasmids, pJTH9 and pJTH10, there are two EcoRI sites between the right and left borders that define the integrated DNA region (Figure 1). EcoRI digestion of genomic DNAs isolated from successful transformants will therefore
Fig. 2. DNA hybridization analysis of transgenic tobacco

A. DNA hybridization analysis of tobacco plants transformed with pJTH9 and pJTH10

lanes 1 to 10: transformants JT30 through JT39
lanes 12 to 20: transformants JT41 through JT49
lane 11: pJTH9 digested with EcoRI

B. DNA hybridization analysis of tobacco plants transformed with pJTH11

lane 1: transformant NT-1A
lane 2: transformant NT-1B
lane 3: transformant NT-1C
lane 4: transformant NT-1D
lane 5: transformant NT-1E
lane 6: transformant NT-11
lane 7: transformant NT-12
lane 8: transformant NT-13
lane 9: transformant NT-14A
lane 10: transformant NT-14B
lane 11: transformant NT-14D
lane 12: transformant NT-14E
lane 13: transformant NT-16
lane 14: transformant CT-1H
lane 15: transformant CT-2
lane 16: pJTH11 digested with HindIII and SstI
generate a 4.2 kbp internal fragment homologous to the probe. Figure 2A shows that the expected 4.2 kbp EcoRI fragment was detected in at least 5 of the 10 pJTH9 transformants (JT31, JT32, JT37, JT38 and JT39) and in 4 of the 9 pJTH10 transformants (JT43, JT45, JT46 and JT49). This 4.2 kbp fragment was not seen in plants transformed by the vector pRT99 (not shown). One pJTH10 transformant, JT47, contained a 5.8 kbp fragment showing homology to the probe (Figure 2A); it is likely that some rearrangement had occurred in JT47. When genomic DNAs were digested with the restriction enzyme BamHI and probed with the same EcoRI-SstI fragment, only one band was detected in those plants having the 4.2 kbp EcoRI fragment (not shown). The sizes of the BamHI fragments varied in different transgenic plants, except for JT45 and JT46. This was expected because the integrated DNA region has only one BamHI site and the second site has to be in the genomic DNA. The BamHI fragment detected in JT45 and JT46 was the same size, indicating that these two plants were probably derived from a single transformation event.

Similarly, genomic DNAs were isolated from regenerated tobacco plants transformed with pJTH11 and from control plants transformed by the vector pBI121, digested with HindIII and SstI, and probed with $^{32}$P-labeled HindIII-SstI fragment of pJTH11 (see Figure 1). A HindIII and SstI double digestion generates a 1.45 kbp fragment internal to the integrated DNA
region. As shown in Figure 2B, a band of ca. 1.45 kbp was detected in all pJTH11 transformants but one and was missing from the control. One additional band, ca. 1.25 kbp, was present in all plants, suggesting it is due to non-specific hybridization (Figure 2B). The results of the DNA hybridization analysis show that at least some of the regenerated tobacco plants had been successfully transformed.

**Analysis of transgenic plants**

Positive transgenic plants, as revealed by the DNA hybridization analysis, were tested for their possible susceptibility to methomyl by leaf-disc assay (see Materials and Methods). Concentrations of methomyl used were 1 mM, 2 mM, 3 mM, 4 mM, 6 mM, and 8 mM. A concentration of 8 mM completely inhibited the growth of leaf-discs of all samples, including pJTH9 and pJTH11 transformants, the leaderless pJTH10 transformants, and the non-transformed control. Concentrations lower than 3 mM had no inhibitory effect, while 3 mM, 4 mM and 6 mM methomyl showed a moderate inhibitory effect on leaf-disc growth (not shown). There was, however, no tight correlation between growth inhibition and the presence of the fusion genes; the inhibition seemed nearly random and was perhaps due to the non-specific toxic effect of methomyl. This assay needs to be repeated under more defined conditions.
Inoculation of leaves of T. maize plants with toxin causes chlorotic streaks (Turner and Martinson, 1972). Tobacco plants transformed with pJTH9, pJTH11, and the control plasmid pJTH10 were tested for their response to toxins and to methomyl by a similar inoculation assay (see Materials and Methods). HmT and Pm toxins (1 \( \mu \)g/ml, 2 \( \mu \)g/ml, 4 \( \mu \)g/ml, 16 \( \mu \)g/ml) and methomyl (1 mM, 2 mM, 4 mM, 6 mM, 8 mM) were injected into tobacco leaves through the lower epidermis. Injection of methomyl at all concentrations tested had no visible effect, but injection of both toxins caused chlorosis-like streaks (not shown). As was the case in the leaf-disc assay, however, the formation of chlorotic streaks was random and was likely resulted from non-specific effects of the solvent, DMSO.

Pollen fertility of transgenic plants was checked as described in the Materials and Methods. Normal, fertile pollen grains stain dark brown and have a regular shape, whereas abnormal or sterile pollen grains are stained only slightly and are irregular in shape. More than 90% dark-brown pollen grains with regular shape were observed in both transformants and untransformed controls. Therefore, transgenic plants with the plasmids pJTH9 and pJTH11 are male fertile. This absence of sterility could be because not enough T-URF13 is produced in the transgenic tobacco plants. It appeared that a critical level of about 33% maximum
synthesis of T-URF13 was necessary to confer male sterility in maize (Dixon et al., 1982). Flavell (1974) proposed that an anther-specific substance produced during normal pollen development may trigger mitochondrial degeneration. This substance may not be produced by tobacco anthers.

When T-URF13 is expressed in *E. coli* or in yeast, it causes *E. coli* or isolated yeast mitochondria to be susceptible to toxins and methomyl (Dewey et al., 1988; Section I). Mitochondria were isolated from transformants and control plants; the rate of $O_2$ consumption by the mitochondria in the presence of toxins and methomyl was measured. $O_2$ consumption by mitochondria isolated from pJTH11 transformants was not significantly affected (not shown). However, this experiment needs to be repeated since the amount of mitochondria used in these assays was small.

Immunodetection of T-URF13 in transgenic plants

Mitochondria and chloroplasts were isolated from all pJTH9 and pJTH10 transformants that were positive in the DNA hybridization analysis and from 6 pJTH11 transformants that showed possible sensitivity to 3 to 5 mM methomyl in the leaf-disc assay. Isolated mitochondria and chloroplasts were analyzed by immunoblotting with antibodies against T-URF13 (see Section I). No anti-T-URF13 cross-reacting polypeptide was detected in mitochondria of pJTH9 and pJTH10.
transformants, or in chloroplasts and mitochondria of pJTH11 transformants. Among many attempts, only once was a polypeptide that cross-reacted with the anti-T-URF13 antibodies detected in chloroplasts from pJTH9 and pJTH10 transformants (not shown). All plants containing this immunoreactive band were positive in DNA hybridization analysis, while no band was present in chloroplasts from the control plant JT28 and a pJTH9 transformant, JT36, that does not contain the 4.2 kbp EcoRI fragment. The size of the immunoreactive band (ca. 17 kDa) was, however, greater than that of the mature T-URF13 protein. Its identity remains to be determined; it cannot be the precursor, prC5/T-URF13, since the size of the band is the same in transgenic plants transformed by pJTH9 (with leader) and pJTH10 (leaderless).

There are several possible explanations for the difficulty in detecting T-URF13 protein in the transgenic plants. The amount of T-URF13 produced may be beyond the detection limit, the T-URF13 precursors may be unstable, presumably because of proteolysis, the coding regions of the fusion gene may not be appropriately transcribed, or the transcripts may be turned over rapidly. Northern hybridization analysis needs to be done to ensure the fusion genes are correctly transcribed. Certain translation enhancing sequences from tobacco mosaic virus have been shown to increase the translation level of a foreign gene 16- to 18-
fold in tobacco cells (Gallie et al., 1989). It would be desirable to construct fusion genes containing the translation enhancing sequence in order to increase the amount of T-URF13 in transgenic plants.
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SUMMARY AND GENERAL DISCUSSION

Cytoplamic male sterility (CMS) is very useful in large-scale hybrid seed production because it eliminates the necessity of hand emasculation of female parents. One particular type of male-sterile cytoplasm in maize, the Texas (T) male-sterile cytoplasm (cms-T), has been successfully and extensively utilized in commercial production of hybrid corn seed. Unfortunately, plants with T cytoplasm is highly susceptible to two fungal diseases, caused by Helminthosporium maydis race T and Phyllosticta maydis. The susceptibility of cms-T plants to H. maydis led to the abandonment of its use in hybrid corn seed production. H. maydis and P. maydis produce toxins, called respectively HmT toxin and Pm toxin, that specifically affect cms-T mitochondria. Evidence is now overwhelming that the mitochondrial genome of higher plants contains the cytoplasmic genes that interact with nuclear genes to confer CMS. Especially in maize, the T-URF13 protein encoded specifically by T mitochondria has been implicated in CMS and susceptibility to the toxins and methomyl.

One aspect of this research was to test directly the hypothesis that T-URF13 is responsible for both CMS and toxin/methomyl sensitivity. The simplest strategy for testing a hypothesis like this is to introduce the gene of interest,
if available, into a heterologous organism that normally does not contain this gene and to see if expression of this gene causes a corresponding phenotype(s) in the recipient organism. Yeast was used as a recipient to test the role of T-URF13 in causing toxin/methomyl sensitivity. Expression of T-URF13 in yeast cells made them susceptible to HmT and Pm toxins and to methomyl (Section I). The growth of yeast transformants was inhibited, and the respiration rate of whole yeast cells, spheroplasts, and isolated mitochondria was stimulated by these substances. Thus, a single protein, T-URF13, is sufficient to confer toxin/methomyl susceptibility to a heterologous eukaryotic organism. These results provide direct evidence that T-URF13 is the causative agent of susceptibility to HmT toxin, Pm toxin and methomyl in maize with the T cytoplasm.

How T-URF13 causes toxin/methomyl sensitivity is still unknown. Experimental results in Section I suggested that T-URF13 must be localized in the mitochondrion and must assume a correct orientation or conformation in the mitochondrial membrane, most likely in the inner membrane, in order to be functional. Yeast cells expressing pJTH2 (leaderless) were not susceptible because T-URF13 was not translocated into mitochondria. In yeast cells expressing pCMS1 (COXVp leader) or pCMS2 (ATP2 leader), T-URF13 was perhaps partially imported into mitochondria, because some putative processed T-URF13
products were detected in mitochondria isolated from these cells which were still susceptible to externally-added proteinase K (not shown). These yeast cells were not sensitive to toxins or methomyl, probably because either the orientation (C-terminus outside) or the conformation (loose, unfolded) of T-URF13 was not appropriate.

One possibility is that T-URF13 functions by interfering with electron transport in the respiratory chain. Yeast transformants expressing pJTH1 were sensitive only when they were grown on a non-fermentable carbon source where, respiratory function of mitochondria is necessary for growth (Section I). It has been suggested that T-URF13 is associated with enzyme complexes involved in oxidative phosphorylation (Wise et al., 1987b; R. Dewey, cited in Levings and Brown, 1989). T-URF13 might disturb normal functions of these enzymes by forming complexes with them. From this point of view, yeast serves as an excellent model system to study the mechanism of T-URF13 function.

Another interesting observation made in Section I is that yeast transformants were not as sensitive to toxins as to methomyl. Some possible explanations have been discussed (Section I). Still another possibility is that the observed difference was due to a codon change in T-URF13. Codon CGG was proposed to code for tryptophan in mitochondria instead of arginine as in the "universal" code (Fox and Leaver, 1981).
Recent studies on RNA editing in plant mitochondria (Covello and Gray, 1989; Gualberto et al., 1989) imply that this issue may need to be reconsidered. In plasmid pJTH1, CGG was changed to TGG; this change may have altered the primary amino acid sequence of T-URF13, and hence susceptibility to the toxins. It would be desirable to express a native T-URF13 in yeast to see if there is any difference in the susceptibility towards methomyl or toxins. Also, the actual amino acid sequence of the mature T-URF13 protein in pJTH1 transformants should be determined.

The possibility that T-URF13 is also responsible for cytoplasmic male sterility was tested (Section III). In this case, T-URF13 was expressed in tobacco plants so that any effect of T-URF13 on pollen fertility could be examined. The results presented in Section III are inconclusive at present. As has been discussed, however, more definitive results should be obtained if the expression of T-URF13 in transgenic plants could be increased.

In order to test the function of T-URF13 in tobacco, a fusion gene as in yeast must be constructed so that T-URF13 can be imported into mitochondria by a mitochondrial leader peptide. The possibility of using a heterologous mitochondrial leader peptide as protein targeting signal in plants was explored (Section II). Very interestingly, the yeast COXVα leader peptide was found to possess dual targeting
specificity; it directed the import of the same reporter protein (CAT) into both mitochondria and chloroplasts in transgenic tobacco plants.

Mechanisms underlying protein import are largely unknown at present. Even less is known about the specificity of protein targeting. The novel finding that a single leader peptide can direct a protein to different organelles provides a potential experimental system to understand organelle-specific protein targeting. It also raises the question of whether endogeneous protein targeting is always as specific as has been expected or believed.
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APPENDIX
pTI4
(4.8 kb)
pVL
(-3.77 Kb)
pATP2L
(-3.6 Kb)

II. ATP2 leader sequence
(MCS): Sites between KpnI and BamHI in the MCS of pBluescript

B/B*: BamHI/BgII junction
pCCT10
(4.52 Kb)
pUCC
(～3.7 Kb)

ATG* : inactivated ATG
pTCB
(-3.9 Kb)
pNQLT13
(4.08 kb)

pNQLORF13 – BamHI, SstI

pTCB – BglII, SstI
pJTH1

(6.06 kb)

pN9L T13 - PstI, SstI → pJTH1

γEP352 - PstI, SstI
pCMSI
(- 5.9 Kb)
B/C: BamHI/BclI junction

ILL: ATP2 leader
CAT(\*

\text{(ATG*)}: initiation codon for pJTH6 (Leaderless)

\text{(SmaI)}: replaces SalI and XbaI in pJTH6
pJTH7
(- 6.25 kb)

B/K : Bcl I/ KpnI adaptomer junction
B/K : BclI/KpnI junction
pIVT2
(-3.8 kb)
pJTH9
(-17.6 kb)

pCCT - SacI → pJTH9
pRT99 - SacI → pJTH9

(ATG*): initiation codon for pJTH10 (Leaderless)

(SmaI): replaces SalI and XbaI in pJTH10