The Plant Cell, Vol. 2, 1249–1260, December 1990

A Yeast Mitochondrial Leader Peptide Functions in Vivo as a Dual Targeting Signal for Both Chloroplasts and Mitochondria

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A fusion protein was expressed in transgenic tobacco and yeast cells to examine the functional conservation of mechanisms for importing precursor proteins from the cytosol into mitochondria and chloroplasts. The test protein consisted of the mitochondrial leader peptide from the yeast precursor to cytochrome oxidase subunit Va (prC5) fused to the reporter protein chloramphenicol acetyltransferase. This protein, denoted prC5/CAT, was transported into the mitochondrial interior in yeast and tobacco cells. In both organisms, the mitochondrial form of prC5/CAT was smaller than the primary translation product, suggesting that proteolytic processing occurred during the transport process. prC5/CAT also was translocated into chloroplasts in vivo, accumulating to approximately the same levels as in plant mitochondria. However, accumulation of prC5/CAT in chloroplasts relative to mitochondria varied with the conditions under which plants were grown. The chloroplast form of prC5/CAT also appeared to have been proteolytically processed, yielding a mature protein of the same apparent size as that seen in mitochondria of either tobacco or yeast. Chloramphenicol acetyltransferase lacking a mitochondrial targeting peptide did not associate with either chloroplasts or mitochondria. The results demonstrated that in plant cells a single leader peptide can interact functionally with the protein translocation systems of both chloroplasts and mitochondria, and raised the possibility that certain native proteins might be shared between these two organelles.

INTRODUCTION

Mitochondria and plastids contain DNA and are capable of synthesizing some of their constituent proteins. The majority of organelle proteins, however, are coded for by nuclear genes, synthesized in the cytosol, and post-translationally imported into the organelle. Most imported proteins are synthesized as precursors with an amino-terminal extension, termed the leader peptide, that is removed proteolytically upon translocation across the organelle membranes (Ellis and Robinson, 1987; Verner and Schatz, 1988). Numerous studies using fusion proteins consisting of an organelle leader peptide fused to a heterologous passenger protein have shown that in most cases the leader peptide contains all the information necessary to target a protein to its proper subcellular location (for recent reviews, see Grivell, 1988; Von Heijne, 1988; Hartl et al., 1989), although in some cases information within the mature protein itself can influence import or sorting to a suborganellar compartment (Gearing and Nagley, 1986; van Steeg et al., 1986; Ness and Weiss, 1987; Smeekens et al., 1986, 1987).

Translocation of a cytosolic precursor protein into chloroplasts or mitochondria is thought to require a specific interaction between a receptor protein on the organelle surface and the leader peptide (Hartl et al., 1989; Keegstra et al., 1989; Sollner et al., 1989). The general features of leader peptides important for this interaction appear to be highly conserved. For example, leader peptides from *Neurospora* and humans efficiently target proteins into mitochondria of the yeast *Saccharomyces cerevisiae* (Banroques et al., 1986; Cheng et al., 1987; Nagley et al., 1988), a yeast mitochondrial leader peptide directs the *Escherichia coli* protein β-glucuronidase to tobacco mitochondria (Schmitz and Lonsdale, 1989), and a plant mitochondrial targeting peptide directs transport into yeast mitochondria (Bowler et al., 1989). Leader peptides that direct precursor proteins to chloroplasts are generally similar in amino acid composition to their mitochondrial counterparts, exhibiting a high percentage of basic residues,

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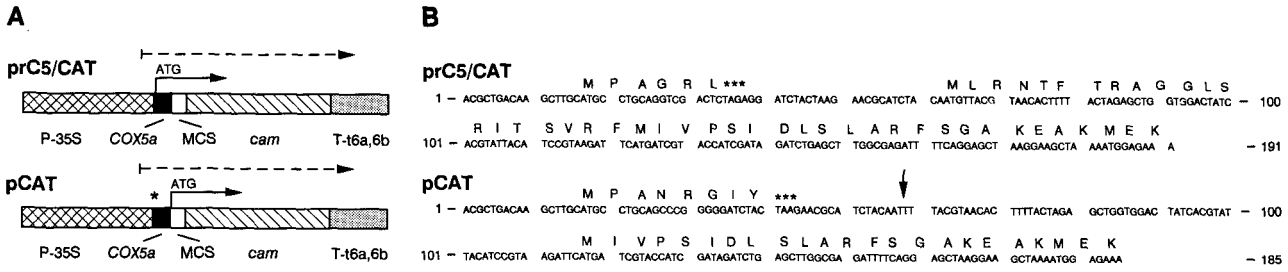

Figure 1. Gene Constructions and Fusion Proteins.

(A) Fusion genes producing prC5/CAT and pCAT in tobacco. Transcribed regions are marked by dashed arrows (– – →), and the initiation codons of the CAT open reading frames are marked by solid arrows (→). A nucleotide substitution in the pCAT gene that eliminates the prC5 initiation codon is marked by an asterisk (*). The diagram is not drawn to scale. P-35S, cauliflower mosaic virus 35S promoter; *COX5a*, yeast cytochrome oxidase subunit Va 5′-flanking and coding sequence; MCS, multiple cloning site; *cam*, chloramphenicol acetyltransferase coding sequence; T-t6a,6b, terminator region of octopine synthase Ti plasmid transcripts 6a and 6b. See Methods for a detailed description of each fusion gene.

(B) Partial nucleotide sequence of prC5/CAT and pCAT fusion genes. In each case, nucleotide 1 is the transcription initiation site of the P-35S promoter, and the last nucleotide shown is the ninth of the native CAT coding sequence. Open reading frames in the transcripts beginning with ATG codons are indicated by the single-letter amino acid code above the nucleotide sequence. Asterisks (***) mark termination codons. The arrow (↓) indicates a T residue that has been altered from a G in the *COX5a* sequence, to eliminate the prC5 initiation codon. The composition of each sequence is as follows. prC5/CAT: nucleotides 1 to 14, P-35S; 15 to 39, MCS; 40 to 122, *COX5a*; 123 to 182, MCS; 183–, *cam*. pCAT: nucleotides 1 to 14, P-35S; 15 to 33, MCS; 34 to 116, *COX5a*; 117 to 176, MCS; 177–, *cam*.

frequent hydroxylated residues, few acidic residues, and a predicted amphiphilic structure (Verner and Schatz, 1988; Von Heijne, 1988; Keegstra, 1989; Von Heijne et al., 1989). The similar characteristics of chloroplast and mitochondrial leader peptides raise the question of how specific targeting is achieved so that mitochondria and chloroplasts contain distinct sets of proteins, and suggest that in some cases leader peptides could be functionally interchangeable between chloroplast and mitochondrial proteins.

Dual targeting to chloroplasts and mitochondria is suggested by the finding that a portion of the leader peptide from the small subunit of *Chlamydomonas reinhardtii* ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcS), a chloroplast protein, directs transport in vitro and in vivo into yeast mitochondria (Hurt et al., 1986). However, the efficiency of import was very low compared with that directed by an authentic yeast mitochondrial leader peptide. The full-length targeting peptide of rbcS from pea directs transport into *Neurospora crassa* mitochondria in vitro, again with very low efficiency (Pfaller et al., 1989). In an in vivo study of the chrysophyte alga *Ochromonas dancia*, a homolog of rbcS was detected by immunological methods in mitochondria, an observation that could be explained by dual targeting of the rbcS precursor to both chloroplasts and mitochondria (Lacoste-Royal and Gibbs, 1985). Such dual targeting cannot be a general phenomenon. Boutry et al. (1987) found that in tobacco cells transport of a reporter protein to chloroplasts and mitochondria was strictly specific, depending on the origin of the leader peptide to which it was attached. Similar results have also been obtained in in vitro import experiments (Whelan et al., 1990). Finally, in tobacco cells, a yeast mitochondrial targeting peptide directed transport specifically to mitochondria; no targeting to chloroplasts could be detected (Schmitz and Lonsdale, 1989).

To investigate further the evolutionary conservation of organelle targeting mechanisms, the leader peptide from the precursor to the yeast mitochondrial inner membrane protein cytochrome oxidase subunit Va was fused to the reporter protein chloramphenicol acetyltransferase (CAT), and the resultant fusion protein, prC5/CAT, was expressed in transgenic tobacco plants. Both mitochondria and chloroplasts in the transgenic plant cells were found to contain the reporter protein in their interior in a form that apparently was processed from the primary translation product by proteolytic cleavage. The results demonstrated that a single targeting peptide can be recognized by the translocation systems of both chloroplasts and mitochondria, and suggested that the endogenous proteolytic maturation enzymes of these two organelles can act upon the same precursor protein.

RESULTS

Gene Constructs for Expression of Fusion Proteins

Figure 1 represents two gene fusions constructed to test whether a mitochondrial targeting peptide from the yeast

Table 1. CAT Expression Plasmids

Plasmid	Organism	Backbone	CAT Protein
pJTH5	Tobacco	pGA425[a]	prC5/CAT
pJTH6	Tobacco	pGA425	pCAT
pJTH7	Yeast	YEp352[b]	prC5/CAT
pJTH8	Yeast	YEp352	pCAT

[a] An (1986).
[b] Hill et al. (1986).

S. 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 CAT, coded for by the *cam* gene of bacterial transposon Tn9 (Alton and Vapnek, 1979).

Table 1 lists the plasmids used in this study 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 native CAT. The entire mitochondrial targeting sequence of prC5 is present because the native protein is cleaved on the C-terminal side of residue 20 upon transport into mitochondria (Cumsky et al., 1987). Transcription of the fusion gene in pJTH5 is controlled by the cauliflower mosaic virus 35S RNA promoter (Odell et al., 1985), and the dual terminator of transcripts 6a and 6b of the octopine synthase Ti plasmid (Barker et al., 1983) is located adjacent to the 3′ end of the CAT coding region. 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 1B). The gene fusions coding for prC5/CAT and the leaderless 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 pJTH7 and pJTH8 are identical to those in the plant transformation plasmids pJTH5 and pJTH6.

Figure 1B shows the primary amino-terminal sequence of prC5/CAT and pCAT. In the case of prC5/CAT, the initiation codon of *COX5a* is located 63 nucleotides (nt) downstream of the 35S RNA transcription initiation site and is the first ATG sequence of a continuous open reading frame that includes the CAT coding sequence (see Methods). A second methionine codon is located upstream of the *COX5a* initiation codon, 17 nt from the transcription start site. This open reading frame extends for six codons, terminating at a TAG sequence 26 nt upstream of the prC5/CAT initiation codon. A similar upstream initiation codon and short open reading frame is located in the pCAT transcript (Figure 1B). As shown below, these upstream initiation codons and open reading frames do not prevent expression of prC5/CAT or pCAT.

Expression and Subcellular Targeting of CAT in Yeast and Tobacco

Figure 2A shows that CAT was 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 (lanes 1 and 4). However, fractionation of the transformed cells into mitochondrial and postmitochondrial supernatant (S10) fractions revealed a significant difference in the subcellular locations of the two CAT proteins. CAT activity from prC5/CAT cofractionated with both the mitochondrial and S10 fractions (Figure 2A, lanes 2 and 3), whereas the leaderless protein pCAT appeared to reside almost entirely in the S10 fraction (Figure 2A, lanes 5 and 6). Immunoblot analysis using antibodies against CAT confirmed this result (data not shown). Therefore, in this homologous system, the mitochondrial targeting peptide of prC5 functions to transport CAT into mitochondria.

Figure 2B indicates that similar levels of CAT activity were found in transgenic tobacco plants transformed with either pJTH5 or pJTH6 (lanes 1 and 6). However, the specific activity of CAT in total cell homogenates was approximately 2 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, postorganellar S10 supernatant, mitochondria, and chloroplasts. CAT assays revealed that pCAT was located predominantly in the S10 fraction (Figure 2B, lanes 2 and 3); scintillation counting showed that the CAT specific activity in the organelle pellet (lane 3) was less than 5% of that in the S10 supernatant (lane 2). In contrast, expression of prC5/CAT yields significant CAT activity in both the S10 and crude organellar fractions (Figure 2B, lanes 7 and 8). Upon further fractionation of the crude organellar pellet into mitochondria and chloroplasts, CAT activity from prC5/CAT was detected in both organelles (Figure 2B, lanes 9 and 10). Similar results were obtained in analyses of five independent tobacco transformants expressing prC5/CAT (data not shown). In the example shown in Figure 2B, 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 both mitochondria and chloroplasts.

Figure 2C shows the result of an immunoblot analysis performed to determine the degree of cross-contamination

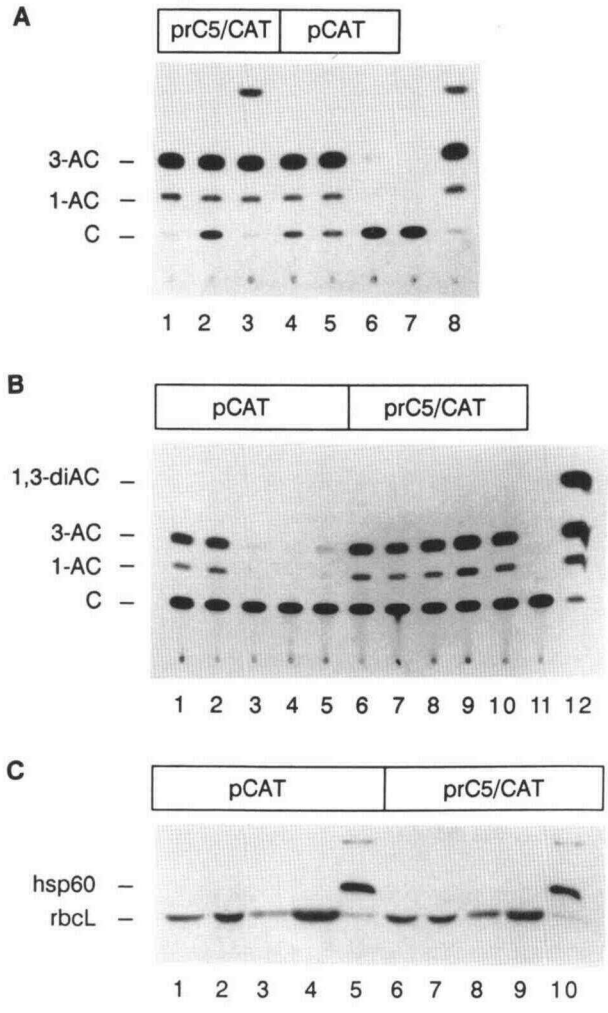

Figure 2. CAT Expression and Subcellular Localization in Transgenic Yeast and Tobacco.

CAT activity was measured by appearance of acetylated forms of ^{14}C-chloramphenicol in thin-layer chromatographs. C, ^{14}C-chloramphenicol; 1-AC, 1-acetyl-^{14}C-chloramphenicol; 3-AC, 3-acetyl-^{14}C-chloramphenicol; 1,3-diAC, 1,3-diacetyl-^{14}C-chloramphenicol. The expressed fusion protein, pCAT or prC5/CAT, is indicated for each sample.

(A) CAT expression in yeast. Lanes 1 and 4, total cell homogenate; lanes 2 and 5, postmitochondrial supernatant; lanes 3 and 6, mitochondria; lane 7, negative control with no protein added to the reaction; lane 8, positive control with purified CAT added to the reaction.

(B) CAT expression in tobacco. Lanes 1 and 6, total cell homogenate; lanes 2 and 7, postorganellar supernatant; lanes 3 and 8, crude organellar pellet; lanes 4 and 9, purified chloroplasts; lanes 5 and 10, purified mitochondria; lanes 11 and 12, negative and positive controls, respectively, as in **(A)**.

(C) Immunoblotting analysis of subcellular fractions. Aliquots of the protein fractions analyzed for CAT activity were subjected to SDS-PAGE and probed by protein gel blotting for the presence of the chloroplast stromal protein rbcL and the mitochondrial matrix protein hsp60. Lane designations are as in **(B)**. The absence of hsp60 from the chloroplast fraction was confirmed by a radiographic exposure 10 times longer than that shown here (data not shown). Lanes 1 to 3 and 6 to 8 contained 100 μg of protein; lanes 4, 5, 9, and 10 contained 30 μg of protein.

between chloroplasts and mitochondria in the organelle fractions. Probing protein samples from each fraction with antiserum against a chloroplast polypeptide, the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL), revealed that the mitochondrial fractions were slightly contaminated with chloroplasts (Figure 2C, lanes 5 and 10). Antiserum against the mitochondrial protein hsp60 (McMullin and Hallberg, 1988) was used to detect cross-contamination of the chloroplast fraction with mitochondria. Based on the intensity of the hsp60 signal in the mitochondrial fractions (Figure 2C, lanes 5 and 10), mitochondrial contamination in the chloroplast fraction at the level of 5% of the total protein or greater would be detected clearly. However, hsp60 was not detectable in the chloroplast fractions (Figure 2C, lanes 4 and 9), either in the autoradiograph shown or in an exposure 10 times longer (data not shown). Therefore, CAT activity in the chloroplast fraction was not due to cross-contamination with mitochondrial proteins. The immunoblot analysis also showed that the postorganellar supernatant contained high levels of rbcL and, thus, was contaminated with stromal proteins derived from broken chloroplasts. Therefore, CAT activity in the S10 fraction could be due to prC5/CAT that remained in the cytosol and/or to CAT proteins that had been translocated into chloroplasts.

Import of CAT to the Interior of Mitochondria and Chloroplasts

Suborganellar fractionation and protease protection experiments determined that the mitochondrial targeting peptide of prC5 directed transport of CAT to the interior of plant mitochondria and chloroplasts. Figure 3A shows that washing the intact organelles with 1 M NaCl released only a small proportion of the total CAT activity (quantified by scintillation counting as less than 5%) into the supernatant (lanes 1 and 5), whereas the majority of the activity remained associated with the organelle pellet (lanes 2 and 6). Thus, cofractionation of CAT with mitochondria and chloroplasts was not due to nonspecific protein-protein interactions on the organelle exterior. After sonication of the organelles and separation of suborganellar soluble (Figure 3A, lanes 3 and 7) and membrane (Figure 3A, lanes 4 and 8) fractions, the great majority of CAT activity was found in the soluble fractions. Figure 3B shows that the fractionation procedure was effective, as judged from immunoblot analyses that detected the soluble mitochondrial

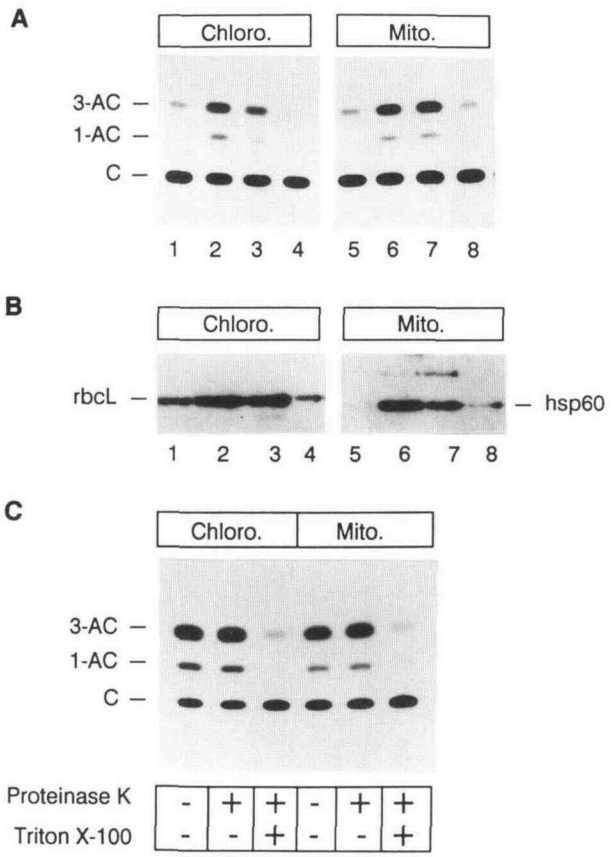

Figure 3. Suborganellar Location of CAT Activity.

(A) Suborganellar fractionation. CAT activity was assayed as in Figure 2. "Chloro." and "Mito." indicate chloroplast and mitochondrial fractions, respectively, from a single tobacco transformant expressing prC5/CAT. Lanes 1 and 5, supernatant from a 1 M NaCl wash of the organelles; lanes 2 and 6, organellar pellets from the 1 M NaCl wash; lanes 3 and 7, supernatant from the sonicated organelles, containing the soluble protein fraction; lanes 4 and 8, pellet from the sonicated organelles, containing the membrane-bound proteins.

(B) Immunoblot analysis of suborganellar fractions. Fractions were analyzed by protein gel blotting for the presence of rbcL and hsp60, as in Figure 2C. Liberation of some rbcL from chloroplasts by the 1 M NaCl wash is most likely explained by organelle breakage.

(C) Protease protection assay. CAT activity was analyzed as in *Figure 2. Before the assays,* organelles were treated with proteinase K in the presence or absence of 1% Triton X-100, as indicated.

and chloroplast marker proteins hsp60 and rbcL, respectively. These results suggest that CAT is located 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 after treatment with proteinase K. Figure 3C shows that in both organelles the CAT activity was protected from digestion with externally added proteinase K (second and fifth lanes from the left). However, addition of detergent to disrupt organelle membranes rendered most of the CAT activity susceptible to protease (Figure 3C, third and sixth lanes from the left). Treating the organelles with detergent alone had no appreciable 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

To determine whether prC5/CAT was processed during translocation across chloroplast or mitochondrial membranes, aliquots of chloroplast or mitochondrial proteins were separated by SDS-PAGE and analyzed 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. Figure 4 shows that purified yeast mitochondria from a trans-

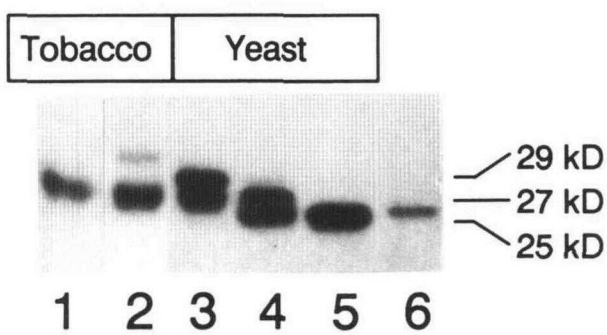

Figure 4. Detection of CAT in Plant Mitochondria and Chloroplasts by Immunoblotting.

Organelle proteins were probed for the presence of CAT by protein gel blotting as in Figure 2C, using polyclonal antiserum reactive with CAT (R.W. Thornburg, unpublished data). Yeast mitochondria were treated with proteinase K in the presence or absence of Triton X-100, as indicated. 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 have been exposed to x-ray film for 4 days, and lanes 3 to 6 are a 1-day exposure. Lanes 1 and 2 contained 30 μg of chloroplast and mitochondrial proteins, respectively. Lanes 3 to 5 contained 15 μg of yeast mitochondrial protein.

formant expressing prC5/CAT contained two immunoreactive bands with apparent molecular masses of 29 kD and 27 kD, respectively (lane 3). The 29-kD band corresponds to the predicted size of the prC5/CAT precursor protein and, in fact, comigrates with the major in vitro translation product produced from the *COX5a-cam* fusion gene (data not shown). Furthermore, addition of proteinase K to these mitochondria completely eliminated the 29-kD band (Figure 4, lane 4), suggesting that this protein is prC5/CAT that has attached to the outer surface of mitochondria without being imported.

In contrast to the 29-kD protein, the 27-kD 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 4, lanes 4 and 5). Therefore, the 27-kD 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 kD larger than native CAT (Figure 4, lane 6), as expected from the presence of the MCS linker region upstream of *cam* in the fusion gene constructions (see Figure 1B).

Treatment of the 27-kD and/or 29-kD immunoreactive bands with proteinase K resulted in the appearance of a new immunoreactive band of an apparent molecular mass of 25 kD, which is not seen in untreated mitochondria (Figure 4, lanes 4 and 5). The 25-kD protein presumably is a protease-resistant form of CAT because it is resistant to proteinase K even when membranes are disrupted by detergent. As in the case of tobacco mitochondria and chloroplasts, proteinase K treatment of yeast mitochondria in the presence of Triton X-100 severely reduced the CAT activity present (data not shown). Therefore, although the 25-kD protein appears to be just slightly smaller than native CAT, it retains only residual enzymatic activity.

The chloroplast and mitochondrial CAT proteins produced in the heterologous transport systems were compared with those found in yeast (Figure 4, lanes 1 and 2). In both organelles, one prominent immunoreactive band was found that comigrates with the 27-kD CAT protein internal to yeast mitochondria. This protein is presumed to be internal to tobacco mitochondria and chloroplasts because CAT activity cofractionated with the soluble, interior portion of the organelles (Figure 3A). Therefore, prC5/CAT produced in the plant most likely is proteolytically processed upon transport into both mitochondria and chloroplasts. The size of the processed products was consistent with cleavage at the native processing site of prC5; however, at present we have no data bearing on the specific nature of the proteolytic processing of prC5/CAT in plant mitochondria and chloroplasts. The identity of the higher molecular weight protein in tobacco mitochondria that reacts with antibodies against CAT (Figure 4, lane 2) is not known. However, this protein does not appear to be a precursor form of CAT because (1) its apparent molecular weight is greater than that of prC5/CAT and (2) it is not susceptible to external proteinase K (data not shown).

Growth Conditions Influence the Transport of CAT into Chloroplasts

The subcellular location of CAT was compared in four independent tobacco transformants expressing prC5/CAT propagated under varying growth conditions. Transformants JT51 and JT58 were grown for 16 weeks in the greenhouse (approximately 360 μE m^{-2} sec^{-1}) or under fluorescent lights (25 to 30 μE m^{-2} sec^{-1}) in a culture room (8 hr light/16 hr dark), respectively, before subcellular fractionation of leaf tissue. Table 2 presents quantitative results of CAT assays, indicating that in the two plants the total CAT activity in organelles was within a factor of two in all cases. However, the data of Table 2 showed the distribution of CAT in chloroplasts and mitochondria to be significantly different in the two plants. Specifically, in the greenhouse-grown plant JT51, CAT specific activity was 1.5-fold higher in chloroplasts than in mitochondria, whereas chloroplast CAT activity in the fluorescent light-grown plant JT58 was approximately 8 times less than the specific activity in mitochondria. These results suggest that the accumulation of CAT in chloroplasts relative to its level of accumulation in mitochondria is affected by growth conditions.

To rule out the possibility that the observed differences in CAT activity were due to inherent differences in the independent transformants JT51 and JT58, the two plants were switched to the opposite growth condition for a

Table 2. Targeting of CAT in Different Growth Conditions

Plant	Organelle	Condition[a]	Time[b]	CAT[c]	Ratio[d]
JT51	Chloroplast	GH	16 wk	37	1.61
JT51	Mitochondria	GH	16 wk	23	
JT51	Chloroplast	CR	3 wk	17	0.57
JT51	Mitochondria	CR	3 wk	29	
JT58	Chloroplast	GH	3 wk	40	1.05
JT58	Mitochondria	GH	3 wk	38	
JT58	Chloroplast	CR	16 wk	4	0.13
JT58	Mitochondria	CR	16 wk	31	

[a] GH, greenhouse; CR, fluorescent light culture room.
[b] Plant JT51 was grown continuously in the greenhouse, assayed for CAT activity, switched to the culture room for 3 weeks, and then assayed again. Plant JT58 was grown continuously in the culture room, assayed, switched to the greenhouse, and then assayed again.
[c] CAT activity is expressed as the percent acetylated chloramphenicol, relative to total chloramphenicol.
[d] Ratio of CAT specific activity in chloroplasts to CAT specific activity in mitochondria, within a given plant at each time point.

period of 3 weeks before repeating the subcellular fractionation experiment. Pre-existing leaves that apparently were not expanded during the 3-week growth period were used in this second set of assays. Table 2 shows that after moving transformant JT51 from the greenhouse to the culture room, a reversal in the pattern of CAT localization was observed so that mitochondria then exhibited approximately 1.7 times higher specific activity than did chloroplasts. Likewise, transformant JT58 also exhibited a reversed CAT localization pattern upon being moved from the culture room to the greenhouse so that mitochondria and chloroplasts contained approximately equal amounts of CAT activity per unit of protein. Two additional independent transformants were assayed by a similar scheme (data not shown). In both cases, the plants were originally grown in the greenhouse, and CAT specific activity was greater in the chloroplast fraction than in the mitochondrial fraction. Each plant was then moved to the culture room for 3 weeks, after which time the CAT localization pattern had reversed to an extent similar to that shown quantitatively in Table 2 for plant JT51. Thus, the accumulation of CAT protein in chloroplasts of plants expressing prC5/CAT seems to be related to the growth conditions, not to a particular transgenic plant.

DISCUSSION

Biogenesis of both chloroplasts and mitochondria depends upon post-translational import of precursor proteins synthesized on cytoplasmic ribosomes. Because these two organelles appear to contain entirely distinct sets of proteins, the targeting and import processes required for biogenesis generally are assumed to be specific for each compartment. However, the targeting mechanisms of chloroplast and mitochondrial precursor proteins exhibit many similarities (Verner and Schatz, 1988; Keegstra et al., 1989; Von Heijne et al., 1989; Franzen et al., 1990), raising the question of how specific targeting is achieved. This question is emphasized further by the current study, which shows that a single targeting peptide can be recognized functionally by the transport systems of both mitochondria and chloroplasts. These results also raise the possibility that not all targeting to chloroplasts and mitochondria is specific and that some proteins might be transported independently into two distinct subcellular organelles.

Translocation of prC5/CAT into tobacco mitochondria confirms the results of Schmitz and Lonsdale (1989), who demonstrated conservation of mitochondrial targeting function between yeast and tobacco by showing that in transgenic plants the leader peptide of yeast mitochondrial tryptophanyl tRNA synthetase directed a reporter protein, β-glucuronidase (GUS), specifically to mitochondria. However, the results presented here differ significantly from the former study in the fact that the prC5 targeting peptide causes translocation of the passenger protein into chloroplasts as well as the expected transport into mitochondria. Depending on growth conditions, CAT accumulation in chloroplasts can be approximately the same as, or significantly greater than, accumulation in mitochondria. Therefore, assuming that there are no large differences in the stability of CAT in these organelles, the rates of transport across chloroplast and mitochondrial membranes are expected to be similar. The data raise the question of whether the mitochondrial leader peptide of prC5 interacts specifically with a receptor molecule on the surface of tobacco mitochondria and chloroplasts or, alternatively, transport occurs by way of a nonspecific pathway such as that proposed by Pfaller et al. (1989), which appears to bypass proteinaceous receptors. Assuming that the transport rates are similar in chloroplasts and mitochondria, the mechanism of transport, i.e., specific or nonspecific, is likely to be the same for both organelles. Analyzing the uptake of prC5/CAT by mitochondria and chloroplasts in vitro will characterize the actual efficiency of transport across the organelle membranes as well as any requirements for specific interactions between the prC5 targeting peptide and receptor molecules on the surface of either organelle.

The prC5 targeting peptide has several unusual properties compared with other mitochondrial targeting peptides that may be responsible for its ability to target a foreign protein to chloroplasts. Thus, the different targeting specificities of prC5 and tryptophanyl tRNA synthetase fusion proteins in tobacco cells may be caused by differences in the secondary and/or tertiary structures of the two targeting peptides, such that the prC5 structure resembles a typical chloroplast targeting peptide. Most mitochondrial targeting peptides are predicted by computer algorithms to assume an amphiphilic α-helical structure, although a minority may fold into an amphiphilic β-sheet (Von Heijne, 1988; Von Heijne et al., 1989). Chloroplast targeting peptides, despite their resemblance in primary structure to those of mitochondrial proteins, may not form α-helices but instead may contain regions of β-sheet (Steppuhn et al., 1987; Tyagi et al., 1987; Verner and Schatz, 1988; Keegstra et al., 1989; Von Heijne et al., 1989). By using computer-aided analysis, Cumsky et al. (1987) predicted that the secondary structure of the prC5 leader peptide is an amphiphilic, antiparallel β-sheet. Other indications of the unusual nature of prC5 with respect to mitochondrial import are that it is imported in vitro at the same rate and efficiency at 0°C as at 30°C, and is imported into protease-treated mitochondria with the same efficiency as into untreated mitochondria (M. Cumsky, personal communication). Either of these conditions abolishes in vitro translocation of most mitochondrial precursor proteins. Further analysis of the intracellular targeting of prC5/CAT and mutant derivatives provides an opportunity to

test the involvement of β-sheet and/or other essential structural elements in targeting to chloroplasts.

A trivial explanation for the observed differences in targeting function between the leader peptides of prC5 and tryptophanyl tRNA synthetase (Schmitz and Lonsdale, 1989) may result from the different passenger proteins used: CAT and GUS, respectively. This is unlikely because in these studies CAT showed no affinity for either mitochondria or chloroplasts in the absence of the prC5 leader peptide. Furthermore, Boutry et al. (1987) found that in transgenic plant cells the targeting of CAT to chloroplasts or mitochondria depended on the specific targeting peptide to which it was fused; no dual targeting was detected. Another consideration is the possibility that the 20-amino-acid residues coded for by the MCS (MCS peptide) located between the prC5 and CAT coding regions may contribute a structural element that alters the specificity of the prC5 leader peptide, causing it to function as a dual targeting signal. This is not likely to be the case because the MCS peptide by itself does not target CAT to chloroplasts or mitochondria and because analysis of the MCS peptide sequence by the Chou-Fasman secondary structure prediction method did not reveal any similarity to either a typical mitochondrial leader peptide or to the proposed secondary structure of chloroplast leader peptides (Von Heijne et al., 1989; data not shown). Furthermore, interaction of these 20 amino acids with the prC5 leader peptide would require alteration of the secondary and/or tertiary structure of the targeting sequence, most likely preventing its function in organelle targeting. Thus, dual targeting of prC5/CAT to chloroplasts and mitochondria is likely to be a specific property of the prC5 targeting peptide.

Cytochrome oxidase subunit Va is an integral protein of the mitochondrial inner membrane. In tobacco cells, however, CAT protein targeted to mitochondria or chloroplasts by the prC5 targeting peptide was located in the soluble fraction of either organelle, not the membrane fraction as might be expected if intraorganelle targeting were determined entirely by the targeting peptide. The same result was obtained with yeast mitochondria expressing prC5/CAT (data not shown). These are the expected results because the structural information required to direct subunit Va to its correct location is located within the mature protein, not in the targeting peptide (Glaser et al., 1990).

Mitochondria from yeast cells expressing prC5/CAT contain two polypeptides that react with anti-CAT antibodies, one of approximately 29 kD and one of approximately 27 kD (Figure 4). The smaller protein is located within mitochondria because it is protected from protease digestion, whereas the larger protein is external to the mitochondrial membranes. Presumably, the 27-kD protein is produced by proteolytic cleavage of the 29-kD protein during import into mitochondria. A similar accumulation of unprocessed precursor protein on the external surface of yeast mitochondria was seen previously using a different fusion protein containing the prC5 leader peptide (Huang et al., 1990). In contrast, the unprocessed form of prC5/CAT did not accumulate on the external surface of tobacco mitochondria or chloroplasts. Instead, these organelles contained a single CAT protein located in the organelle interior, which comigrated in SDS-PAGE with the processed form of prC5/CAT from yeast (Figure 4). The fact that the total level of CAT activity produced in yeast was approximately 100 times more than that in tobacco may explain the buildup of unprocessed prC5/CAT on the external face of yeast mitochondria that is not seen in plants. Thus, the transport system of yeast mitochondria might be unable to translocate prC5/CAT as rapidly as the protein is synthesized and binds to the mitochondrial exterior. In tobacco, the lower rate of prC5/CAT production may prevent such a saturation of the mitochondrial and chloroplast transport systems.

The CAT proteins found inside tobacco mitochondria and chloroplasts appear to be proteolytic cleavage products derived from prC5/CAT. The molecular weight of mature CAT in plant organelles appears to be identical to that of mature CAT produced during translocation into yeast mitochondria. Therefore, the processing proteases of plant organelle biogenesis systems, including those of the chloroplast, may recognize the same structural signals as the processing protease of the yeast mitochondrial matrix. Suggestive evidence that this is the case was found previously for the yeast mitochondrial tryptophanyl tRNA synthetase leader peptide imported into tobacco mitochondria (Schmitz and Lonsdale, 1989) and the tobacco manganese superoxide dismutase imported into yeast mitochondria (Bowler et al., 1989). However, because no precursor form of CAT was detected on the external surface of chloroplasts or mitochondria, or in the cytosol (data not shown), the data presented here do not rule out the possibility that the apparent processing of prC5/CAT was due to nonspecific proteolytic cleavage. In vitro import experiments will definitively address the question of whether the prC5 leader peptide is processed by the chloroplast protein maturation system.

An interesting observation is that specific activity of CAT in plastids relative to that in mitochondria was influenced by conditions under which the plant was grown. Although many environmental factors may be involved in this response, one obvious difference in the two growth conditions was the light intensity. Growth under relatively low light conditions in the culture room resulted in less accumulation of CAT in chloroplasts than when plants were grown under relatively high light conditions in the greenhouse. The total amount of CAT activity in leaf tissue showed little apparent difference in either growth condition, and immunoblot analysis indicated that the plastid content of rbcL was independent of the growth conditions (data not shown). Thus, plants grown under these alternative conditions may contain plastids that differ biochemically and physiologically in such a way as to affect targeting and transport of the foreign protein, and perhaps

endogenous proteins as well. For example, the density of receptors on the chloroplast surface might increase in higher light conditions, or these conditions might increase the expression of cytosolic factors that affect translocation into plastids (Waegemann et al., 1990).

METHODS

DNA Manipulations

DNA manipulations were performed using standard procedures (Ausubel et al., 1987; Sambrook et al., 1989). 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 (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-bp BglII-SstI fragment containing the *COX5a* sequence, which codes for the prC5 mitochondrial targeting peptide, was obtained from the plasmid pVP (Huang et al., 1990) and subcloned into pUC119 (Vieira and Messing, 1987) digested with BamHI and SstI. The insert was isolated from the resultant plasmid as a HindIII-SstI fragment and, in a three-fragment ligation into pUC119, attached to an EcoRI-HindIII fragment containing the 35S promoter (Odell et al., 1985). The resultant plasmid, pCCT, contains a unique BclI site located immediately downstream of the last triplet of the prC5 leader coding region (Huang et al., 1990). 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) and digested with KpnI and SstI to produce pJTH5. The KpnI site of pGA492, located in the MCS upstream of the CAT coding region, was ligated to the BclI site of pCCT using a BclI-KpnI adaptomer (5′-GATCGTAC-3′). The nucleotide sequence of pJTH5 from the 35S transcription start site into the CAT coding region was determined (Figure 1B).

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 (Huang et al., 1990). The second change is located immediately downstream of the HindIII site, such that the 5′ end of the 35S promoter transcript of pJTH6, compared with that of pJTH5, is 6 nt shorter and slightly altered in sequence (Figure 1B).

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, containing the 3′ half of the CAT coding region, were cloned in a three-fragment ligation into pBluescript (Stratagene Cloning Systems) digested with KpnI and BamHI. The reconstituted CAT coding region was collected from the resultant plasmid as a KpnI-SstI fragment and subcloned into pUC119 to produce the intermediate plasmid pCAT12. The PstI-BclI fragment of plasmid pVL (Huang et al., 1990), which contains the *COX5a* promoter and leader peptide coding sequence, was then purified and cloned into pCAT12 digested with PstI and KpnI. The BclI-KpnI 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 consists of the *COX5a* promoter, the prC5 leader peptide coding sequence, and the CAT coding sequence, into the yeast-*Escherichia coli* shuttle vector YEp352 (Hill et al., 1986).

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 with the latter plasmid.

Strains and Growth Media

The parent strain for *Saccharomyces cerevisiae* transformation was aW303-11B (*MATa leu2 ura3 trp1 his3 ade2*). 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, Δ(*lac-pro*), *supE*, *thi*$^-$, *hsdD5*/F′ *traD36*, *proA*$^+$*B*$^+$, *lacI*^{q}, *lacZΔM15*), used for amplification of plasmids and production of single-stranded DNA, was grown by standard methods (Ausubel et al., 1987). The dam$^-$ *E. coli* strain GM33 was used for propagation of plasmids to be digested with BclI. *Agrobacterium tumefaciens* strain LBA4404 (Clonetech Laboratories, Inc., Palo Alto, CA), 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 previously (An et al., 1988), and transformants were

selected on YEP+TET+KAN. Plasmids were isolated from transformed *A. tumefaciens* cells by a modification of the plasmid quick-screen procedure originally described by An et al. (1988). 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 of 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 suspended in 20 μL of 10 mM Tris HCl, pH 8.0, 0.1 mM EDTA buffer. A 2-μL to 5-μL sample 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 tobacco plants (*Nicotiana tabacum* cv Xanthi) were cocultured 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 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 mg/L kanamycin). Plants with regenerated roots were transferred to peat pots and maintained in a high-humidity chamber before transferring to a greenhouse or fluorescent light culture room.

Fractionation of Tobacco Cells

Plants were kept in the dark for 48 hr and then exposed to light for 2 hr before leaves were harvested. Ten grams to 15 g of leaf tissue devoid of midvein was sliced with a new razor blade into 30 mL to 45 mL of ice-cold grinding medium (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 sec. All subsequent steps were performed at 4°C. The homogenate was filtered through four layers of Miracloth. One milliliter of crude cell homogenate was saved, whereas a second 1-mL aliquot was centrifuged at full speed in a Beckman Microfuge for 10 min to obtain crude organelle and S10 fractions. The remaining homogenate was centrifuged at 1700*g* for 5 min. The pellet was suspended in 1.5 mL of grinding medium 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% to 40% interface and washed three times with wash medium (0.4 M sorbitol, 50 mM Tris-HCl, pH 7.5, 2 mM EGTA). Mitochondria in the supernatant from the 1700*g* first spin were pelleted at 21,000*g* for 10 min and further purified on a three-step Percoll gradient designed for isolation of mitochondria from green leaves (Jackson et al., 1979). Purified mitochondria were obtained by collecting the fraction at the 45% to 21% interface and were washed three times with wash medium.

Soluble and membrane fractions of chloroplasts and mitochondria were prepared by disrupting the organelles with an Artec Sonic Dismembrator, model 150 (two bursts, 5 sec each) in the presence of 1 M NaCl followed by centrifugation at 100,000*g* 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/mL protein) were incubated with proteinase K (Boehringer-Mannheim) 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). 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 (R.W. Thornburg, unpublished results), hsp60 (McMullin and Hallberg, 1988), or rbcL (Dr. B. Nikolau, Department of Biochemistry, Iowa State University, personal communication), and membrane-bound IgG was detected by incubation with ^{125}I-protein A (DuPont-New England Nuclear Research Products) 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 of protein (tobacco) or 0.05 μg of protein (yeast) was assayed for CAT activity as described (Gorman et al., 1982), using ^{14}C-chloramphenicol as substrate. CAT activity was quantified by excising radioactive spots from TLC plates and counting ^{14}C radioactivity with a scintillation counter.

ACKNOWLEDGMENTS

This research was supported by an Agricultural Biotechnology Research Award from Iowa State University.

Received August 22, 1990; accepted October 8, 1990.

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