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# A Yeast Mitochondrial Presequence Functions as a Signal for Targeting to Plant Mitochondria in Vivo

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**To date, the presequence of the mitochondrial β-subunit of ATPase from tobacco is the only signal sequence that has been shown to target a foreign protein into plant mitochondria in vivo. Here we report that the presequence of a yeast mitochondrial protein directs bacterial β-glucuronidase (GUS) specifically into the mitochondrial compartment of transgenic tobacco plants. Fusions between the presequence of the mitochondrial tryptophanyl-tRNA-synthetase gene from yeast and the GUS gene have been introduced into tobacco plants and yeast cells. In both systems, proteins containing the complete yeast mitochondrial presequence are efficiently imported in the mitochondria. Measurements of GUS activity in different subcellular fractions indicate that there is no substantial misrouting of the chimeric proteins in plant cells. In vitro synthesized GUS fusion proteins have a higher molecular weight than those found inside yeast and tobacco mitochondria, suggesting a processing of the precursors during import. Interestingly, fusion proteins translocated across the mitochondrial membranes of tobacco have the same size as those that are imported into yeast mitochondria. We conclude that the processing enzyme in plant mitochondria may recognize a proximate or even the same cleavage site within the mitochondrial tryptophanyl-tRNA-synthetase presequence as the matrix protease from yeast.**

## INTRODUCTION

A great number of proteins synthesized on cytoplasmic ribosomes of a plant cell are translocated across or integrated into distinct cellular compartments or membranes. In most cases, these polypeptides are made as a precursor molecule with an amino-terminal extension not found in the mature protein. This signal sequence mediates the targeting into the right cytoplasmic compartment (reviewed by Ellis and Robinson, 1987; Verner and Schatz, 1988). In some cases, however, carboxy-terminal or internal sequences of a protein may be involved in targeting (Opperdoes, 1988). For several chloroplast proteins, it has been shown that their $NH_2$-terminal transit peptides have sufficient information to direct a foreign protein into the chloroplast stroma of transgenic tobacco (Schreier et al., 1985; van den Broeck et al., 1985; Smeekens et al., 1987). In similar experiments, it has been demonstrated that plastids of developmentally different tissues of transformed tomato plants have the same ability to import fusion proteins (de Boer et al., 1988). In contrast, very little is known about the import of nuclearly encoded proteins into the mitochondrial compartment of plant cells. Until now, only the presequence of the β-subunit of the plant mitochondrial ATPase complex has been shown to target a passenger protein into tobacco mitochondria in vivo (Boutry et al., 1987). This presequence seems to be much longer (more than 80 amino acids) than yeast mitochondrial presequences, although its exact length has not yet been determined. It has been speculated that targeting to the mitochondrial compartment may require more specific information within the signal sequence in plants than in yeast, as plant cells contain chloroplasts in addition to the normal complement of eukaryotic cytoplasmic organelles. Targeting signals for yeast mitochondria seem to be highly degenerate, as demonstrated by import experiments with random fragments of bacterial DNA fused to the $NH_2$ terminus of a passenger protein (Baker and Schatz, 1987). Even a chloroplast transit peptide may direct an attached polypeptide with low efficiency into yeast mitochondria (Hurt et al., 1986a, 1986b), although this does not seem to apply for other transit peptides (Smeekens et al., 1987).

We have started to investigate the import of proteins into plant mitochondria and to analyze signals involved in mitochondrial targeting. A useful way of studying these signals is the construction of gene fusions between putative targeting sequences and a reporter gene. We have applied this approach to test the ability of a yeast mitochondrial presequence to target a foreign protein into plant mitochondria in vivo. Tobacco plants and yeast cells have

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been transformed with fusion genes, and the subcellular distribution of the reporter enzyme has been determined. This opens the possibility to compare in vivo import efficiencies of the same proteins in different systems and to learn more about plant mitochondrial import using the well-characterized yeast system as a model.

## RESULTS

### Vector Construction and Expression of the Fusion Genes in Transgenic Tobacco and Yeast

Different lengths of the presequence of the yeast gene for mitochondrial tryptophanyl-tRNA-synthetase (MSW) (Myers and Tzagoloff, 1985) have been fused to the $NH_2$ terminus of the $\beta$-glucuronidase (GUS) reporter gene. The strategy for producing these gene fusions is outlined in Figure 1. As described in Methods, DNA fragments encoding parts of the 5′ region of the MSW gene were enzymatically modified and cloned into one of a series of plasmids, pBI201.1-3, described by Kavanagh, Jefferson, and Bevan (1988). These plasmids allow in-frame fusions between $NH_2$-terminal additions and the GUS gene. The fusion gene referred to as *E-GUS* comprises 82 nucleotides of the MSW gene and 27 nucleotides of the 5′ nontranslated region. The *A-GUS* and *X-GUS* fusions have the same 5′ nontranslated region together with 308 and 328 nucleotides of the coding region of the MSW gene, respectively. The E-GUS protein has an incomplete mitochondrial presequence, whereas the A-GUS and X-GUS proteins comprise the complete presequence and different parts of the mature MSW protein in front of GUS (Figure 1b). To express the chimeric genes in plants, they were placed under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The GUS gene without any presequence driven by the same promoter served as a negative control. All the constructs were cloned into a derivative of the binary vector pBIN19 (Bevan, 1984) and introduced into tobacco. The same fusion genes were cloned into the multi-copy yeast vector, pAAH5 (Ammerer, 1983), where they are expressed under the control of the constitutive alcohol dehydrogenase 1 gene. Yeast cells transformed with these pAAH5 derivatives have been used in the experiments described below.

Transgenic tobacco plants and transformed yeast cells were analyzed for the presence of the GUS fusion proteins. As shown in Figure 2, GUS protein from the control transformants has the same size as purified bacterial GUS. The fusion proteins have a higher molecular weight corresponding to their $NH_2$-terminal fusions in all three plants. Additionally, a second band of higher electrophoretic mobility occurs in those plants expressing a chimeric protein with a complete mitochondrial presequence. These bands

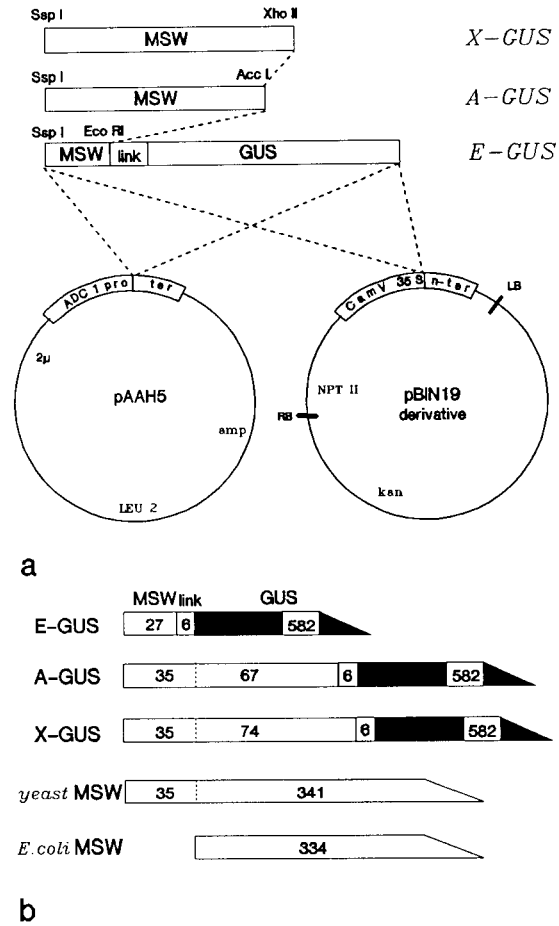


**Figure 1.** Gene Constructions and Fusion Proteins.

**(a)** Vector constructions used for plant and yeast transformation. A pBIN19 derivative (Bevan, 1984) was used for the expression of fusion genes in tobacco. The same constructs were expressed in yeast using plasmid pAAH5 (Ammerer, 1983). In the gene fusions referred to as *E-GUS* and *X-GUS*, a linker (link) of 17 nucleotides connects part of the 5′-coding region of the MSW gene with the GUS gene, whereas for the *A-GUS* fusion, 16 nucleotides permit the continuity of reading frame. The DNA constructs were end-modified and cloned into appropriate restriction sites (see Methods) between the promoter (pro) and terminator (ter) regions of the yeast alcohol dehydrogenase gene (ADC 1) as well as the CaMV 35S promoter and the nopaline synthetase terminator (n-ter). Leu 2, yeast leucin 2 gene; NPT II, chimeric nopaline synthase::neomycin phosphotransferase gene; amp, bacterial $\beta$-lactamase gene; kan, bacterial neomycin phosphotransferase gene.

**(b)** Fusion proteins with various lengths of the yeast tryptophanyl-tRNA-synthase (MSW) in front of GUS. The number of amino acids of each part of the fusion proteins is indicated. A broken line separates the mature part of the yeast MSW protein from its putative presequence. The exact length of the presequence has not been determined yet but may be deduced from the start of homology between the yeast and the *Escherichia coli* MSW protein.

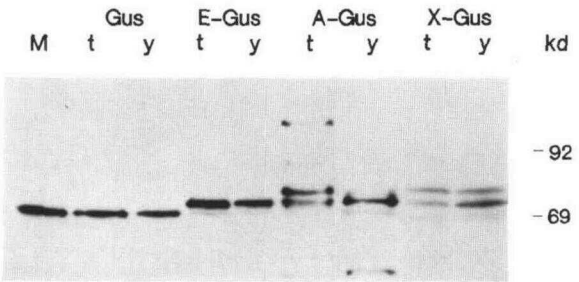


**Figure 2.** Protein Gel Blot of Protein Isolated from Transgenic Tobacco (t) and Yeast (y).

Each lane contains either 10 $\mu$g to 25 $\mu$g of total plant protein or 4 $\mu$g to 10 $\mu$g of total yeast protein. The blot was probed with antibodies raised against bacterial $\beta$-glucuronidase. The first lane from the left contains 8 ng of purified GUS as a marker (M). kd, kilodaltons.

have the same size as the fusion proteins extracted from yeast and are localized within mitochondria.

To determine whether the fusion proteins are enzymatically active and to compare GUS activity in different transformants, leaf tissue and yeast cells were homogenized and incubated under standard assay conditions (see Methods) with the fluorogenic substrate 4-methylumbelliferone glucuronide (MUG). All three fusion proteins exhibit GUS activity in plant and yeast cells, but the one with the longest amino-terminal addition generally has a reduced activity level.

To find out whether a reduced mRNA level is responsible for the decreased activity in *X-GUS* transformants, the transcription of the fusion genes has been monitored in transgenic tobacco. Total RNA was isolated from equal amounts of leaf tissue of plants exhibiting maximum levels of GUS activity. The RNA gel blot in Figure 3 shows that some differences in the steady-state mRNA levels of different constructs occur. *X-GUS* transformants have the lowest transcript levels, suggesting that differences in mRNA abundance or stability contribute to the reduced reporter enzyme activity in these plants. Furthermore, the hybrid proteins with the longest $NH_2$-terminal additions (A-GUS, X-GUS) may be degraded more rapidly in the cytoplasm. Their steady-state levels seem to be generally lower than for the E-GUS fusion or the control as determined by protein gel blot analysis of plants exhibiting similar transcription levels of the fusion genes. A possible explanation is an aberrant folding of these proteins.

## Reporter Enzyme Activity in Different Subcellular Fractions of Tobacco and Yeast

To determine the ability of the MSW presequence to direct GUS into the mitochondrial compartment in a homologous (yeast) and a heterologous (tobacco) system, we have analyzed the subcellular distribution of reporter enzyme activity. Yeast cells expressing the chimeric genes were fractionated into mitochondria and supernatant as described. Cross-contamination between both fractions was monitored with antibodies against hexokinase, a marker enzyme of the cytoplasm, and citrate synthase, a marker enzyme of the mitochondrial matrix. As illustrated in Figure 4, control yeast cells expressing the unmodified GUS gene alone contain little GUS activity in the mitochondria but high levels in the supernatant. In contrast, all yeasts expressing fusion proteins exhibit an elevated level of GUS activity in the mitochondrial fraction. Although the targeting efficiency of the construct with an incomplete presequence (E-GUS) seems to be rather low, both fusion proteins with a complete signal sequence lead to high levels of specific activity in the mitochondria. This activity is protected from proteinase K, suggesting that the fusion proteins are imported into yeast mitochondria in vivo. Although the import efficiency seems to be very high for the A-GUS fusion, the X-GUS protein shows a reduced ability to enter the mitochondria, as indicated by its presence in the supernatant. Possibly, its conformation prevents efficient translocation or makes the $NH_2$-terminal addition highly prone to protease attack.

Transgenic tobacco plants have similar characteristics in the subcellular distribution of the fusion proteins. As illustrated in Figure 5, proteinase K-treated plastids and mitochondria of the control plant and the *E-GUS* transform-

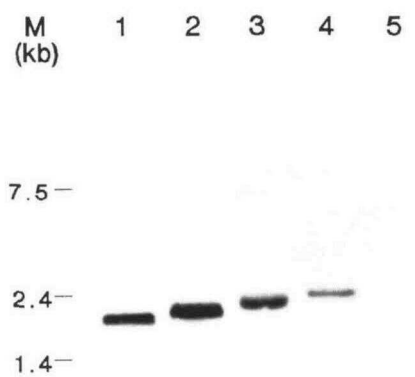


**Figure 3.** RNA Gel Blot of Total RNA of Transgenic Tobacco.

The same transformants were used as in Figure 2. Lane 1, *GUS*; lane 2, *E-GUS*; lane 3, *A-GUS*; lane 4, *X-GUS*; lane 5, untransformed tobacco. The filter was probed with an isolated DNA fragment comprising the coding region of the GUS gene. Transcript sizes vary according to the length of the introduced DNA constructs. kb, kilobase pairs.

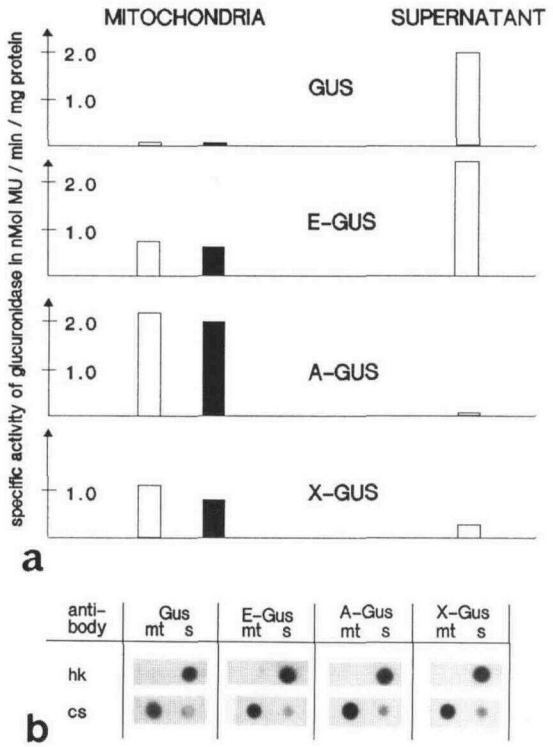


**Figure 4.** Reporter Enzyme Activity and Presence of Marker Enzymes in Subcellular Fractions of Transformed Yeast Cells.

**(a)** GUS activity in the mitochondrial fraction and the supernatant of transformed yeast cells. The specific activity of GUS has been determined fluorometrically and is shown. The solid bars indicate the specific activity after proteinase K treatment.
**(b)** Dot blots of aliquots from the fractions used in **(a)**. The nitrocellulose blots (about 0.5 $\mu$g of protein per dot) were reacted with antibodies against hexokinase (hk), a marker enzyme of the cytoplasm, and citrate synthase (cs), a marker enzyme of the mitochondrial matrix.

ants display very little GUS activity. In contrast, A-GUS and X-GUS fusion proteins exhibit the highest specific activity in the mitochondrial fraction, resembling the activity profiles found in yeast. An obvious difference to yeast expressing the A-GUS gene is the relative amount of specific activity present in the supernatant. As discussed later, this activity may reflect the presence of the precursor protein in the cytoplasm of transgenic tobacco. However, a high amount of the specific activity of the fusion proteins with a complete yeast mitochondrial presequence is protected against externally added protease and co-purifies with the proteinase K-protected activity of the mitochondrial matrix enzyme malate dehydrogenase. We conclude that a certain amount of the A-GUS and X-GUS fusion proteins may have been imported into plant mitochondria in vivo.

### Submitochondrial Location of the Chimeric Proteins

To elucidate the submitochondrial location of the chimeric proteins in yeast and plant mitochondria, GUS activity was measured in different submitochondrial fractions. As illustrated in Figure 6, all three constructs tested exhibit the highest level of specific activity in the soluble fraction of yeast and plant mitochondria. The activity found in the membrane fraction may occur because of cross-contami-

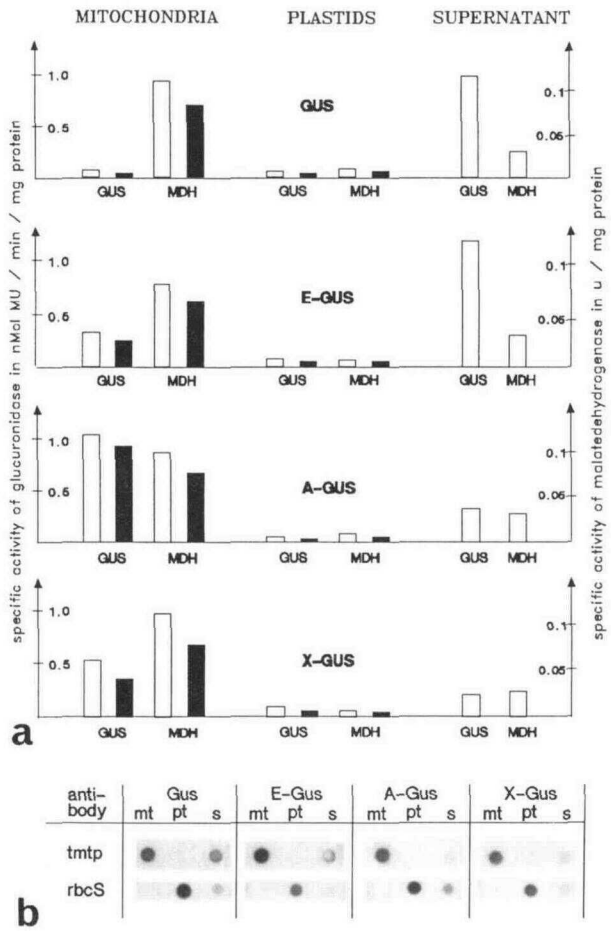


**Figure 5.** Reporter Enzyme Activity and Presence of Marker Enzymes in Subcellular Fractions of Transformed Tobacco Plants.

**(a)** GUS and MDH activity in organellar fractions and the supernatant. The activity of MDH has been determined spectrophotometrically and is shown. The solid bars indicate the specific activity after proteinase K treatment.
**(b)** Dot blots of aliquots from the subcellular fractions (mt, mitochondria; pt, plastids; s, supernatant) used in **(a)**. Each dot contains an equal amount of protein (2 $\mu$g). The nitrocellulose blots were reacted with antibodies raised against total mitochondrial proteins (tmtp) and the small subunit of ribulose bisphosphate carboxylase (rbcS).

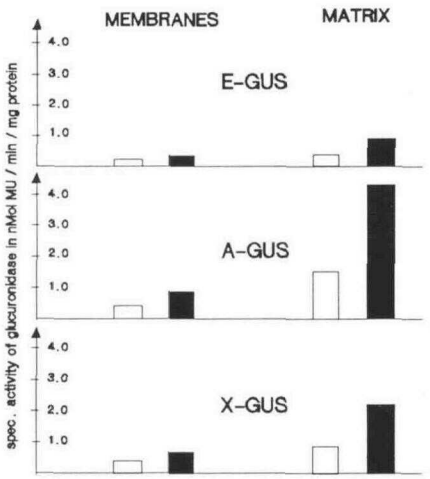


**Figure 6.** GUS Activity in Different Submitochondrial Fractions.

The activity has been determined in transformed plants (open bars) and yeast (solid bars).

nation with matrix material, as some background activity is encountered in membrane fractions of yeast and plants. A certain amount of the activity of the soluble mitochondrial fraction may come from the intermembrane space, but, as yeast mitoplasts exhibit only slightly reduced GUS activity levels in comparison to yeast mitochondria (data not shown), we assume that most of the fusion proteins entering mitochondria reside in the matrix. Thus, the targeting information of the MSW presequence seems to be sufficient for correct intramitochondrial sorting.

### Processing of the Fusion Proteins during Import into Plant and Yeast Mitochondria

Our finding that GUS activity is highly enriched in the mitochondrial fraction of tobacco and yeast transformants raised the question of whether the *GUS fusion proteins* are processed during import into mitochondria. Thus, we tried to determine: (1) whether processing occurs for all the fusion proteins upon translocation across yeast mitochondrial membranes, and (2) whether plant mitochondria are able to process a yeast mitochondrial presequence. Chimeric proteins from yeast and tobacco were isolated from proteinase K-treated mitochondria and analyzed by SDS-PAGE. These gels were either incubated with MUG subsequently to visualize GUS bands under UV light or they were reacted with antibodies raised against GUS. As shown in Figure 7, the A-GUS fusion proteins have the same size within yeast homogenate and yeast mitochondria. In contrast, tobacco transformants expressing the *A-GUS* construct exhibit an additional band of higher molecular weight in the homogenate that is not present in protease-treated mitochondria. The mitochondrial fraction from these plants only contains a single band of lower molecular weight that co-migrates with the corresponding protein isolated from yeast mitochondria. This suggests that all the A-GUS fusion protein synthesized in yeast is imported into mitochondria and processed, whereas only part of the total amount synthesized in plants is imported and processed.

To determine whether the additional protein band in the tobacco homogenate really represents a precursor form of the imported fusion protein, full-length fusion polypeptides were synthesized in an in vitro transcription/translation system (see Methods). The protein gel blot shown in Figure 8 compares these polypeptides with GUS proteins from yeast and plant homogenates. As the E-GUS fusion protein synthesized in vitro has the same size as the proteins from yeast and plant homogenates, we conclude that this protein is not processed upon import into the mitochondria. In contrast, the A-GUS fusion protein made in vitro has a higher molecular weight than the protein from yeast homogenate and co-migrates with the additional higher molecular weight band found in plant homogenate, suggesting that this band represents a precursor form of the smaller protein found in mitochondria.

## DISCUSSION

Fusion proteins consisting of GUS and $NH_2$-terminal pieces of the MSW protein from yeast can be synthesized stably

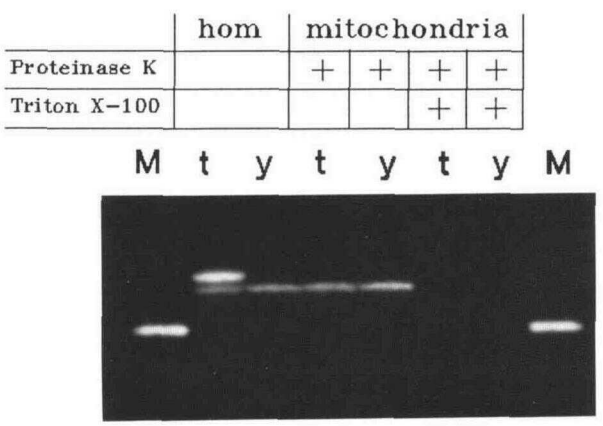


**Figure 7.** Activity Gel of Fusion Proteins from *A-GUS* Transformants.

The proteins were isolated from total homogenate (hom) and purified mitochondria of tobacco (t) and yeast (y) and were separated on a 5% SDS-polyacrylamide gel. GUS bands were visualized by UV illumination after incubating the gel in 1 mM MUG for 30 min. Purified bacterial GUS was used as a marker (M).

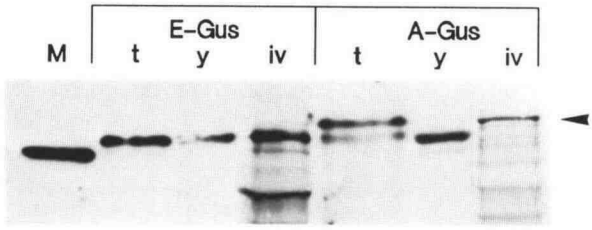


**Figure 8.** Protein Gel Blot of E-GUS and A-GUS Fusion Proteins.

The proteins were synthesized in vitro (iv) or isolated from tobacco (t) and yeast (y) homogenate. The arrow points to the putative precursor form of the A-GUS protein. Antibody and marker (M) are as in Figure 2.

in the cytoplasm of yeast and plant cells if appropriate control regions are used for the expression of the chimeric genes. Our finding that the GUS protein is only imported into the mitochondrial compartment if a presequence is attached to its amino terminus is in line with other studies on mitochondrial import (Glaser et al., 1988; reviewed by van Loon et al., 1988). Our data suggest that the general mechanism by which nuclearly encoded proteins are imported into yeast and plant mitochondria may be very similar, as a yeast mitochondrial presequence directs a passenger protein efficiently into tobacco mitochondria in vivo.

Even the incomplete presequence of the MSW gene that is not processed seems to increase slightly the amount of reporter enzyme activity in the mitochondrial fraction of transgenic plants. Although it is difficult to deduce from our data that the fusion protein with a truncated presequence is really imported into plant mitochondria, it seems likely that this protein is imported with low efficiency in yeast. This result is in accordance with those reported by Hurt et al. (1985) and Vassarotti et al. (1987), who showed that as few as 10 to 12 amino acids of a yeast mitochondrial presequence are able to direct a protein into the mitochondrial matrix. However, the efficiency of import depends on the size of the $NH_2$-terminal addition, as a chimeric protein having the presequence and part of the mature MSW protein in front of GUS is quantitatively imported into yeast mitochondria. We cannot exclude that part of the mature MSW polypeptide is involved in mitochondrial import, as reported for $F_0$-ATPase subunit 9 from *Neurospora* (Pfanner et al., 1987), although most mitochondrial presequences described so far contain sufficient targeting information themselves (Roise and Schatz, 1988).

The targeting efficiency of the signal sequence of the MSW protein is generally lower in tobacco than in yeast. In plant homogenates of transformants with the complete presequence in front of GUS, an additional band of higher molecular weight is present that does not occur in yeast cells expressing the same construct. This band has the same size as the corresponding protein synthesized in vitro and is not detectable in the mitochondrial fraction after protease treatment. We conclude that only part of the total amount of fusion protein produced in transgenic tobacco is imported into the mitochondria.

As shown for several mitochondrial presequences, the information for intramitochondrial sorting is normally contained within the amino-terminal part of the signal peptide (Douglas, McCammon, and Vassarotti, 1986; Hurt and van Loon, 1986). According to the location of the tryptophanyl-tRNA-synthetase in the mitochondrial matrix, its presequence seems to target the GUS protein into the right intramitochondrial compartment. Most of the specific GUS activity is found in the soluble fraction of yeast and plant mitochondria, suggesting that the fusion proteins reach the matrix space. It may be concluded that the matrix targeting domains within yeast mitochondrial presequences also function for targeting to the matrix of plant mitochondria.

Interestingly, the fusion proteins are selectively imported into the mitochondrial compartment of tobacco, suggesting that the yeast signal sequence contains sufficient information for the specific targeting of a passenger protein in living plant cells. At least there is no substantial misrouting into the plastid compartment, as very little GUS activity is found in the chloroplast fraction of transformed plants. This result is unexpected since the presequences of mitochondrial and chloroplast proteins are similar because they have a bias for basic and hydroxylated amino acids and are generally hydrophilic (Ellis and Robinson, 1987; Verner and Schatz, 1988). Additionally, Hurt et al. (1986a, 1986b) reported that the amino-terminal two-thirds of the ribulose bisphosphate carboxylase can direct an attached passenger protein into yeast mitochondria, although with very low efficiency. On the other hand, Boutry et al. (1987) showed that, in living plant cells, no misrouting of chimeric proteins having a chloroplast or a mitochondrial signal sequence in front of the same reporter gene is detectable. Our data suggest that the signal that specifies the mitochondrial compartment as a target is similar in yeast and plants. The nature of this signal is still unknown, although it is believed that the organelle-specific information is encrypted as a secondary or tertiary structure of the signal sequence (Verner and Schatz, 1988).

The translocation of precursor proteins into mitochondria is normally accompanied by proteolytic removal of the presequence (Hurt and van Loon, 1986). The GUS fusion proteins with a complete yeast mitochondrial presequence also seem to be processed upon import into yeast and plant mitochondria. GUS protein in protease-treated mitochondria has a lower molecular weight than the complete precursor protein synthesized in vitro. As the protease-protected fusion proteins have the same size in yeast and tobacco, as determined by SDS-PAGE, we conclude that a piece of similar or identical size is removed from the

precursor proteins during translocation across the mitochondrial membranes. This suggests that the processing enzyme in plant mitochondria may recognize a proximate or even the same cleavage site within the yeast mitochondrial presequence. It is tempting to speculate that there is a conserved mechanism for the import of proteins into yeast and plant mitochondria, with an import machinery having striking similarities in both systems.

## METHODS

### Plasmids and Construction of Gene Fusions

Standard procedures were used for recombinant DNA work essentially as described by Maniatis, Fritsch, and Sambrook (1982). To fuse different lengths of the $NH_2$ terminus of the MSW gene with the GUS gene restriction fragments generated by the appropriate enzymes cutting at a SspI site upstream of the ATG and at an EcoRI, an AccI and a XhoII site within the coding sequence of the MSW gene were isolated from plasmid pG181/ST2 described and kindly supplied by Myers and Tzagoloff (1985). The 5′ overhangs of these fragments were filled in with Klenow enzyme to generate blunt ends. This allows the subsequent in-frame fusion of the DNA fragments with the GUS gene by cloning into the SmaI site of an appropriate pBI201.1-3 vector (Kavanagh, Jefferson, and Bevan, 1988) having a linker of 16 to 18 nucleotides between the SmaI site and the ATG codon of the GUS gene. The junctions of the fragments inserted into one of these vectors were sequenced to ensure the continuity of reading frame through the fusion.

### Plant Transformation

The gene fusions were introduced into the vector pBI121 (Jefferson, Kavanagh, and Bevan, 1987) using the XbaI and the SstI sites to replace the GUS gene contained therein. The constructs were mobilized into *Agrobacterium tumefaciens*, strain 4404 (Hoekema et al., 1983), in a triparental mating event using the helper plasmid pRK2013. Shoots emerging from transformed calli were rooted on selective medium containing 0.1 mg of kanamycin per milliliter and maintained in sterile culture.

### Yeast Strains and Transformation

Host strains to serve as recipients for the expression of GUS were DC 04, described by Carle and Olson (1985), and AH 216 (Yaffe and Schatz, 1984). The strains were transformed according to the method of Ito et al. (1983) with derivatives of vector pAAH5 (Ammerer, 1983) harboring inserts of the GUS fusion genes in the unique HindIII site (U. Schmitz, D. Lonsdale, and R. Jefferson, submitted). Transformants were selected for a $LEU2^+$ phenotype on minimal plates (0.67% yeast nitrogen base, DIFCO, 2% glucose, 2% agar) supplemented with uracil, tryptophan, methionine, or histidine. Individual transformants were grown in lactate medium (Daum, Böhni, and Schatz, 1982) after preselection in minimal medium.

### RNA Isolation and RNA Gel Blot

RNA was isolated from tobacco leaves after grinding the tissue in liquid nitrogen as described by Jones, Dunsmuir, and Bedbrook (1985). The RNA was electrophoretically separated on formaldehyde agarose (1%) gel systems according to Maniatis, Fritsch, and Sambrook (1982) and subsequently transferred to GeneScreen-*Plus* filters as recommended by the suppliers (Du Pont-New England Nuclear). The DNA used as a labeled probe was an EcoRI/SalI fragment isolated from plasmid pRAJ275 (Jefferson, Burgess, and Hirsch, 1986), which comprises the complete coding region of the GUS gene. The DNA probe was radioactively labeled using random primers (Feinberg and Vogelstein, 1983). Hybridization was performed at 65°C for 20 hr in 3 × SSC, 1% SDS. The filter was washed at 65°C in 1 × SSC, 0.1% SDS, and 0.3 × SSC several times and exposed to x-ray film.

### Protein Isolation and Protein Gel Blot

Proteins from tobacco were isolated by grinding leaf tissue in a buffer containing 50 mM Tris/HCl, pH 7.5, 0.5 M 2-mercaptoethanol, 2% SDS, and 2 mM phenylmethanesulfonyl fluoride (PMSF). The amount of protein was determined with the method of Bradford (1976) using a kit supplied by Bio-Rad. If necessary, protein samples were concentrated with a Centricon device purchased from Amicon. The samples used for GUS activity gels were subsequently electrophoresed on 5% SDS-polyacrylamide gels (Laemmli, 1970). GUS protein bands were visualized according to Kavanagh, Jefferson, and Bevan (1988) except that the bicarbonate wash was omitted. Proteins used for protein gel blot analysis were boiled for 5 min and separated on 7.5% SDS-polyacrylamide gels. Electroblotting onto nitrocellulose membranes was performed overnight, and GUS protein was specifically immunostained using antibodies raised against purified bacterial GUS. The blots were incubated with $^{125}I$-labeled protein A as described by the supplier (Amersham), and radioactive bands were visualized by autoradiography.

Proteins from yeast were isolated from spheroplasts that were prepared and washed repeatedly as described below. For protein extraction and electrophoresis, the methods outlined above were applied. Dot blots of aliquots from different subcellular fractions of transformed yeast were reacted with antibodies against citrate synthase and hexokinase from yeast. Dot blots of aliquots from the mitochondrial and the plastid fractions of transgenic tobacco were incubated with antibodies raised against purified proteinase K-treated mitochondria and with antibodies against the small subunit of ribulose bisphosphate carboxylase of wheat, kindly supplied by Stewart Payne. Immunoreactive dots were identified as described above.

### In Vitro Transcription/Translation of *GUS* Constructs

To synthesize RNA of the three fusion genes, the DNA constructs with terminal HindIII linkers (see above) were cloned into the polylinker of a Bluescript vector (Stratagene). The recombinant plasmids were linearized using the unique SalI site, and the genes were expressed under the control of the T7 promoter according to the supplier's instructions. The mRNA was extracted with phenol, precipitated with ethanol, and stored at −70°C in sterile

water. The transcripts were translated in a reticulocyte lysate purchased from Amersham, United Kingdom, according to the supplier's instructions.

### Purification of Chloroplasts and Plant Mitochondria

To obtain highly intact mitochondria, leaf tissue submerged in ice-cold ST buffer (0.4 M sorbitol, 50 mM Tris/HCl, pH 8.0, 1 mM DTT, 0.5% BSA) was sliced into very small pieces using new razor blades. All subsequent steps were carried out at 4°C, and, in the ST buffer used for washing organellar fractions, DTT and BSA were omitted. The homogenate was filtered through four layers of muslin and centrifuged at 1200*g* for 5 min. The chloroplasts of the pellet were further purified and fractionated on Percoll step gradients as described by Bartlett, Grossman, and Chua (1982). The mitochondria in the supernatant were pelleted at 12,500*g* and purified on Percoll step gradients according to Boutry et al. (1984). Proteinase K treatment of organellar fractions was performed at 20°C for 30 min (0.2 mg of proteinase K/mL). The enzyme was inactivated by addition of PMSF (2 mM), and the organelles were subsequently centrifuged through a sucrose cushion (0.3 M sucrose, 50 mM Tris/HCl, pH 8.0, 1 mM PMSF) at 15,000*g* for 10 min.

Washed mitochondria (2 mg of protein/mL) from Percoll gradients were lysed in the presence of 0.5% octylpolyoxyethylene (a kind gift of Dr. J. Rosenbusch, Biocenter, Basel, Switzerland), which is an excellent detergent for the separation of the membrane and matrix fraction (Vestweber and Schatz, 1988). The lysed mitochondria were subfractionated by centrifugation at 144,000*g* for 30 min, and the pellet containing mitochondrial membranes was vigorously vortexed in GUS extraction buffer (see below). The supernatant representing matrix and intermembrane space was adjusted to the same buffer conditions for measurements of GUS activity.

### Isolation of Yeast Mitochondria

Yeast transformants were grown overnight in lactate medium (see above) and converted to spheroplasts in the presence of Zymolyase 20,000 (Seikagaku Kogyo) following the method of Daum, Böhni, and Schatz (1982). For the preparation of yeast mitochondria, mitoplasts, and submitochondrial fractions, the protocol of the same authors was used.

### Measurement of GUS and Malate Dehydrogenase (MDH) Activity

GUS activity was measured with the fluorometric assay using MUG (Sigma) as a substrate. Plant tissue and yeast spheroplasts were homogenized in Eppendorf tubes in the presence of GUS extraction buffer (Jefferson, 1987). For the determination of reporter enzyme activity, the method described by Jefferson was adapted. Aliquots of homogenates and subcellular fractions were incubated in microtiter plates at 37°C in the presence of 1 mM MUG. The production of 4-methylumbelliferone (MU) in each well was measured at three different timepoints with a Fluoroscan II (Flow Laboratories), and the kinetics of the reaction were calculated using the program "Plates" (D. Wolfe and R. A. Jefferson, in preparation). The protein concentration in each sample was determined with the method of Bradford (1976) using a Bio-Rad kit. All data were normalized to yield values expressed in nanomoles of product (MU) produced per milligram of protein per minute. The activity of MDH was measured in the presence of 1.5 mM reduced $\beta$-NAD (Sigma) by addition of 7.6 mM oxalacetate to the reaction as described by Ochoa (1955). The decrease in optical density ($OD_{340}$) was monitored at intervals of 15 sec, and enzyme kinetics were calculated. Standard curves of MDH activity were produced in the same way with purified MDH purchased from Sigma to determine the relative specific activity in subcellular and submitochondrial fractions. Specific activity is expressed as units per milligram of protein. (One unit of enzyme is defined as the amount that causes a decrease in $OD_{340}$ of 0.01 per minute under the above conditions.)

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