Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 10716–10720, December 1991
Genetics

Functional analysis in yeast of cDNA coding for the mitochondrial Rieske iron-sulfur protein of higher plants

(polymerase chain reaction/complementation/*Zea mays*/*Nicotiana tabacum*/complex III)

JINTAI HUANG, FRIEDHELM STRUCK*, DALE F. MATZINGER, AND CHARLES S. LEVINGS III†

Department of Genetics, Box 7614, North Carolina State University, Raleigh, NC 27695-7614

Contributed by Charles S. Levings III, August 26, 1991

ABSTRACT **cDNA clones coding for the nuclear-encoded mitochondrial Rieske iron-sulfur protein (RISP) have been isolated from maize and tobacco. Complementation analysis of hybrid proteins consisting of different domains of plant and yeast RISPs showed that the carboxyl two-thirds of the plant protein is functionally equivalent to that of the yeast protein. The amino terminus of the RISP, however, seems to be species specific because this region is not interchangeable between plant and yeast proteins. Complementation analysis of hybrid proteins also identified a structurally conserved domain probably essential for the function of bc_1 complex RISPs. A specific domain from the plant RISP was found to cause temperature-sensitive respiratory growth in yeast. We have demonstrated that yeast can serve as a model system to study the structural and functional relationships of plant gene products that are enzymatic components of the mitochondrial respiratory chain.**

The ubiquinol:cytochrome *c* oxidoreductase (EC 1.10.2.2), commonly referred to as complex III or cytochrome bc_1 complex, catalyzes electron transfer in the inner mitochondrial (mt) membrane respiratory chain (1, 2). A similar complex, the cytochrome b_6f complex, catalyzes electron transfer in the thylakoid membranes of algae, plants, and some photosynthetic bacteria (3). The bc_1 complex has been isolated from several prokaryotes and from mitochondria of lower and higher eukaryotes (for review, see refs. 4–6). It is an oligomeric structure that consists of subunits encoded by nuclear and mt genes. The subunit composition of the bc_1 complex varies among species, but all bc_1 complexes contain the Rieske iron-sulfur protein (RISP) (7), which includes a high potential 2Fe-2S cluster coordinately liganded to two cysteines and two histidine residues (5). The similar oligomeric b_6f complex also contains the RISP (8). The RISP, which participates in the proposed Q cycle (for review, see ref. 4), is required for mt respiration (9, 10). Current data suggest that the RISP is also required for electron transfer from plastoquinol to cytochrome *f* in the b_6f complex (3, 4).

In all eukaryotes the RISP is encoded by a nuclear gene, translated as a precursor protein in the cytoplasm, posttranslationally imported into mitochondria or chloroplasts, processed, and assembled into the bc_1/b_6f complex, respectively. The gene sequence coding for the RISP has been reported in *Neurospora crassa* (11), *Saccharomyces cerevisiae* (12), rat (13), the photosynthetic bacterium *Rhodobacter capsulatus* (14), the cyanobacterium *Nostoc*, and other bacteria (ref. 15 and references therein). In higher plants the gene sequence of the chloroplast homolog has been determined for *Spinacea oleracea* (16). Here we report the isolation of cDNAs coding for the mt RISP in maize and tobacco.‡ We show that these clones encode functional proteins by complementing a RISP-deficient yeast mutant using a domain-swapping assay. These studies have identified universal and species-specific components of the RISP and demonstrate the feasibility of using yeast as a model system to study the structural and functional relationships of a plant gene product that is a component of an enzyme complex in the mt respiratory chain.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Synthesizing a Partial cDNA with Polymerase Chain Reaction (PCR). Two degenerate oligonucleotide primers (17), based on two highly conserved amino acid sequences found in all RISPs of the bc_1 complex (see Fig. 1*a*), were used in a PCR (18, 19) to amplify a partial cDNA from a tobacco leaf cDNA library. PCR products were cloned into M13mp19 vector. Clones were screened with PCR (20) and identified by nucleotide sequencing (21). A 23-mer oligonucleotide (5′-CCAAATGCTGGTGATTTTGGTGG-3′) derived from the PCR clones was used as a gene-specific probe to screen a tobacco leaf cDNA library.

Screening cDNA Libraries. A cDNA library in pUC12, made from *Nicotiana tabacum* var. Xanthi leaf cDNA was provided by M. A. Conkling (Department of Genetics, North Carolina State University). A cDNA library in λZAP, made from embryo cDNA of *Zea mays*, inbred line Va26, was obtained from A. L. Kriz (Department of Agronomy, University of Illinois). Screening and isolation of positive clones were performed according to standard procedures (22, 23). The tobacco leaf cDNA library was screened with the 23-mer (see above) probe (24). The *Z. mays* cDNA library was screened using cloned tobacco cDNA as a probe. The *in vivo* excision of recombinant phagemid from the positive λZAP clones was carried out as described (Stratagene).

DNA Sequencing. The cloned tobacco and maize cDNA inserts were subcloned in both orientations in M13mp18/M13mp19 and pBluescript II KS$^+$ (Stratagene), respectively, for sequencing. Unidirectional, overlapping deletions were generated with exonuclease III/S1 nuclease (25). Nucleotide sequencing (21) was performed with single-stranded DNA (26).

Isolation of Proteins and Immunoblot Analysis. Chloroplasts and mitochondria were isolated from tobacco leaves or dark-grown maize seedlings, respectively, as described (27). Yeast mitochondria were isolated from cells grown in HAT medium (see below) according to Daum *et al.* (28). Total protein extracts from whole yeast cells were prepared by trichloroacetic acid precipitation (29). Immunoblot analysis (30) was performed using an antiserum raised against the RISP of *Neurospora* (kindly provided by U. Harnisch, In-

Abbreviations: RISP, Rieske iron-sulfur protein; mt, mitochondrial; ORF, open reading frame.

*Present address: Institut für Genbiologische Forschung, Mascher-oder Weg 1, D-3300 Braunschweig, F.R.G.
†To whom reprint requests should be addressed.

‡The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M77224 (MR2.4) and M77225 (TR12)].

stitut für Biochemie, Universität Düsseldorf, Düsseldorf, F.R.G.) and with goat/anti-rabbit alkaline phosphatase conjugate (Sigma) as recommended by the manufacturer. *In vitro* transcription and translation used commercially available kits (Stratagene). Immunoprecipitation of the *in vitro* translation product was basically as described (31).

Construction of Yeast Expression Plasmids. The multicopy, yeast/*Escherichia coli* shuttle plasmid YEp352 (32) vector was used for construction of expression plasmids. Restriction sites were introduced into the cDNA clones of maize (pMR2.4) and tobacco (pTR12) coding for the RISP and into the *RIP1* gene of yeast (12) by site-directed mutagenesis (33, 34) to facilitate construction. Construction details are not described here but are available upon request.

Yeast Strains, Transformation, and Growth Media. The yeast strain JPJ1 (*MATa his3 ura3 leu2 trp1 can1 rip1Δ2::LEU2*), deficient in the RISP (10), was provided by B. L. Trumpower (Department of Biochemistry, Dartmouth Medical School). Yeast transformation was performed by a modified LiOAc method (35). Transformants were grown in HAT medium [0.6% yeast nitrogen base minus amino acids (Difco), 2% glucose, supplemented with histidine, adenine, and tryptophan at 20 μg/ml each] or EG medium (1% yeast extract, 2% peptone, 2% glycerol) where appropriate. Solid media contained 2% agar (Difco). To test the temperature-sensitive growth of yeast transformants, yeast cells were patched on HAT master plates and replicated onto EG plates. Replica were incubated at designated temperatures for 4 days.

RESULTS

Synthesis of a Partial cDNA Specific for the bc_1-Type RISP. Fig. 1*a* shows an alignment of several partial RISP sequences; all are deduced from nucleotide sequences except the beef protein sequence (36). Each contains two identical peptide sequences that encompass the putative ligands of the 2Fe-2S cluster. The sequence internal to the two identical regions, however, can be grouped into two types: the bc_1 complex type and the b_6f complex type. This internal region differs between the two types but is highly conserved within each type (Fig. 1*a*). These features were exploited to clone the mt (bc_1-type) homolog of the *RISP* gene from tobacco and maize by PCR (18, 19). Two degenerate oligonucleotides (17) were designed with their 3′ ends perfectly matched with the bc_1 but not with the b_6f sequences (Fig. 1*b*) and used as opposing primers for PCR with a mixture of supercoiled plasmids from a tobacco leaf cDNA library. An expected 102-base-pair (bp) fragment was obtained when PCR products were fractionated by agarose gel electrophoresis (data not shown). The 102-bp fragment was cloned, and the nucleotide sequences of seven isolates were determined. Four of these isolates contained an open reading frame (ORF) whose deduced amino acid sequence is similar to that of the bc_1 type but not to the b_6f type (Fig. 1*a*), suggesting they represent a fragment of the mt RISP.

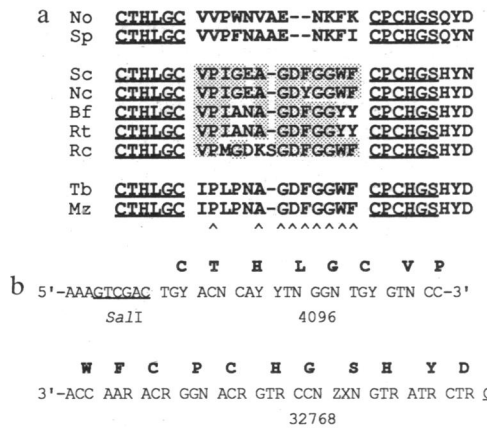

FIG. 1. (*a*) Alignment of a highly conserved amino acid sequence (single-letter code) found in the RISPs from different species: *Nostoc* (No, ref. 15), spinach (Sp, ref. 16), *S. cerevisiae* (Sc, ref. 12), *N. crassa* (Nc, ref. 11), beef (Bf, ref. 36), rat (Rt, ref. 13), *R. capsulatus* (Rc, ref. 14), tobacco (Tb, this work), and maize (Mz, this work). Two identical regions encompassing the putative ligands of the 2Fe-2S cluster are underlined. Amino acids conserved in three of the five published bc_1 complex-type RISPs are shaded. Carets below the Mz/Tb sequence designate conserved residues in the bc_1-type RISPs. (*b*) Nucleotide sequence of the degenerate primers used for PCR. Appendant restriction sites are underlined. Bold letters above the primer sequence indicate the amino acid sequence from which the primer was derived. Numbers underneath the primer are the fold of degeneracy. The code for the nucleotide is N = A, G, C, or T; R = A or G; Y = C or T; X = C or G; Z = A or T.

Isolation of cDNA Clones Coding for the mt RISP from Maize and Tobacco. Based on sequence of the partial cDNA obtained by PCR amplification, a 23-mer oligonucleotide (see *Materials and Methods*) specific for the mt homolog was prepared and used to screen the tobacco cDNA library for clones coding for the mt RISP. From the positive clones, a clone designated TR12 was selected, which contained the largest insert [≈1.2 kilobases (kb)]. The predicted amino acid sequence of TR12 is presented in Fig. 2. The TR12 clone is a partial cDNA because it lacks the coding sequence for the amino terminus of the putative transit peptide (see below).

The nucleotide sequence of MR2.4, one of the positive maize cDNA clones obtained, was determined for both strands except for ≈100 bp in the 3′ nontranslated region. The cDNA contains 1197 bp [excluding the poly(A) tail] and an ORF of 819 bp, which begins with the first ATG translational initiation codon at 108 and could code a protein of 29.8 kDa (Fig. 2). The only other ATG in the frame occurs at 522 and is conserved among all bc_1 complex RISPs. Starting at this methionine, the amino acid sequence encoded by the maize cDNA ORF is 70% and 63% identical to *Neurospora* (11) and yeast (12), respectively. This result agrees with previous observations that the carboxyl terminus of the RISP is highly conserved among phylogenetically divergent species. Sequence similarity between the maize and tobacco proteins extends farther into the amino-terminal region. Starting at proline 68, the amino acid sequence of the maize protein is 90% identical to that of tobacco (Fig. 2).

Protein Products of the mt *RISP* Genes in Plants. mt proteins, isolated from tobacco leaves, etiolated maize seedlings (27), and yeast cells (28), were analyzed using an antiserum raised against *Neurospora* RISP. As shown in Fig.

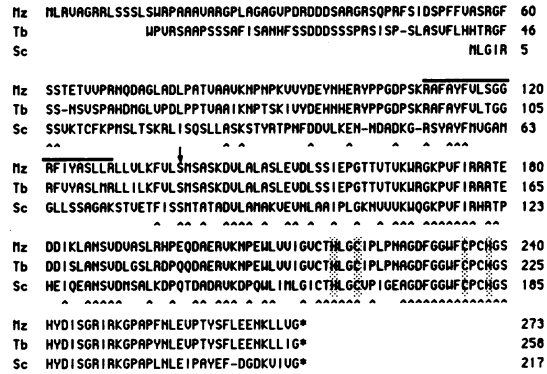

FIG. 2. Alignment of deduced amino acid sequence of maize (Mz), tobacco (Tb), and yeast (Sc) RISPs. Carets designate identical amino acid residues in all three sequences. The putative ligands for the Fe-S cluster (10) are shaded. The amino acid sequence thought to interact with cytochrome *b* (37) is overlined. A vertical arrow indicates the amino terminus of the functionally equivalent domain in the plant and yeast proteins.

3, the antiserum specifically cross-reacted with a polypeptide of ≈22 kDa from yeast cells expressing the *RIP1* gene coding for the yeast RISP (12). This 22-kDa polypeptide was not detected in the yeast strain JPJ1 (10) in which the *RIP1* gene is deleted (cf. lanes 1 and 2, Fig. 3), suggesting the antiserum specifically recognizes the yeast RISP. Two polypeptides of 22.8 kDa and 24 kDa from maize seedling mitochondria cross-reacted with the antiserum (Fig. 3, lane 3). The tobacco leaf mitochondria, however, apparently contain only one polypeptide of about 23 kDa that cross-reacted with the antiserum (Fig. 3, lane 4). The antiserum specifically recognizes the plant mt RISPs because it detects the protein product when the tobacco coding sequence is expressed in yeast (see below). The antiserum did not detect polypeptides in this size range from chloroplasts (Fig. 3, lane 5). The *in vitro* translational product of MR2.4 detected by immunoprecipitation was a single, ≈30-kDa polypeptide, suggesting that the mt RISP of maize may contain a transit peptide of ≈7.5 kDa (Fig. 3, lane 6). The result also confirms the specificity of the heterologous antiserum to the plant RISP.

Complementation of a Yeast Mutant Deficient in the RISP. To characterize how the structure of the mt RISP is related to its function(s) and how this protein interacts with other components of the bc_1 complex, we used the mt *RISP* gene from plants to complement a yeast mutant, which is deficient for this protein. A diagram of protein domains based upon functional and/or secondary structural properties is presented in Fig. 4*a*. All yeast expression plasmids were expressed in the multicopy, *E. coli*/yeast shuttle vector YEp352 (32) under the control of the yeast *RIP1* gene promoter (12). We first expressed plasmid pMZR10 (Fig. 4*b*) in yeast strain JPJ1, which lacks a functional RISP and, therefore, is incapable of respiratory growth (10). Plasmid pMZR10 is a transcriptional fusion between the *RIP1* promoter (nucleotides −321 to −1; ref. 12) and the entire coding region of the maize cDNA clone MR2.4 (nucleotides 57–934). The transit peptide of the plant RISP might function to direct import and processing of the protein in yeast because transit peptides from plant mt proteins are known to function in yeast (39, 40) and vice versa (27, 41). Nevertheless, yeast cells transformed by pMZR10 did not restore respiratory competency (Fig. 4*b*). We were unable to detect the protein product, although the fusion gene was transcribed (data not shown). Similarly, the expression plasmid pYTR, which is a translational fusion between the transit peptide of the yeast RISP (amino acids 1–39, including 9 amino acids of the mature protein) and the mature tobacco RISP (amino acids 62–258; Fig. 2 and Fig. 4*a*), failed to restore respiratory growth to JPJ1 (Fig. 4*b*). Western blot analysis indicated that the fusion protein was translated and imported into mitochondria because it was recovered from mitochondria after exogenous proteinase K treatment (data not shown). Because mt RISPs from divergent species are highly conserved in the carboxyl terminus,

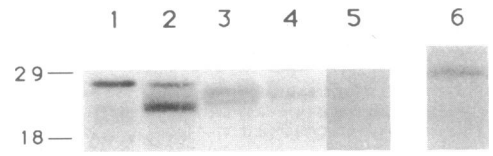

FIG. 3. Analysis of plant and yeast mt RISPs. For Western blot analysis, 30 μg of total mt or chloroplast proteins was separated on SDS/PAGE, transferred to nitrocellulose, and probed with an antiserum against the *Neurospora* RISP. Lane 1, yeast cells transformed with YEp352; lane 2, yeast cells expressing *RIP1*; lane 3, purified mitochondria from maize line Va35; lane 4, purified mitochondria from tobacco line Sc58; lane 5, purified chloroplasts from maize line Va35; lane 6, an autoradiogram of immunoprecipitated *in vitro* translational product of MR2.4. Molecular mass standards (in kDa) are shown on the left. The upper band in lanes 1 and 2 is nonspecific.

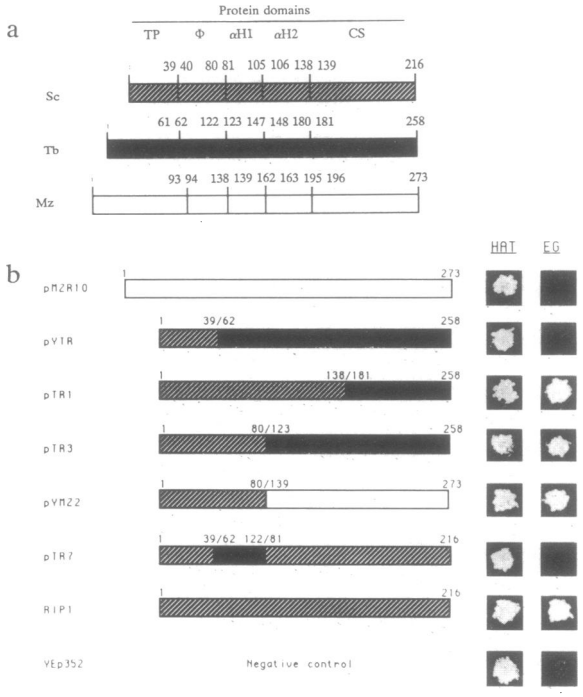

FIG. 4. Complementation analysis of plant/yeast hybrid proteins in yeast strain JPJ1. (*a*) Diagram of arbitrary domains of yeast (Sc), tobacco (Tb), and maize (Mz) RISPs. Secondary structures were derived by the Chou–Fasman method (38). Domain TP contains the transit peptide; domain φ, the region thought to interact with cytochrome *b* (37); domains αH1 and αH2, the two largest α-helices (10); and domain CS, the catalytic sites (10). Numbers designate the amino acid position at the junction between domains. (*b*) Complementation analysis. Constructs used for expression in JPJ1 are shown on the left; domains and numbers correspond to those in *a*. Growth of yeast transformants on fermentable glucose (HAT) and nonfermentable glycerol (EG) is shown on the right. Negative control, yeast cells transformed only with the YEp352 vector.

we substituted the carboxyl terminus of the yeast protein with that of the plant homolog. The expression plasmid pTR1, which codes for a fusion protein consisting of the amino terminus of the yeast protein (amino acids 1–138) and the catalytic site of the tobacco protein (amino acids 181–258, Fig. 2 and Fig. 4*a*), completely restored respiratory growth to JPJ1 (Fig. 4*b*). Two other expression plasmids, pTR3 and pYMZ2, also complemented JPJ1 but with lower efficiency than pTR1 (Fig. 4*b*). These results suggested that the amino terminus of the mt RISP functions differently in yeast and higher plants. Moreover, the expression plasmid pTR7, which codes for a fusion protein in which the amino-terminal, putative membrane-bound domain of the yeast protein (amino acids 40–80, ref. 10) is replaced with that of the tobacco protein (amino acids 62–122; Fig. 4*a*), also fails to complement JPJ1 (Fig. 4*b*). Western blot analysis revealed that the fusion protein encoded by pTR7 was not processed to its mature form (J.H., F.S., C.S.L., unpublished data).

A Specific Region from the Maize/Tobacco RISP Caused Temperature-Dependent Respiratory Growth in Yeast. Changing amino acid Thr-85 to Ile-85 or Pro-102 to Leu-102, respectively, in the yeast RISP confers temperature-sensitive respiratory growth to JPJ1 (10). In maize and tobacco RISPs, a serine and a glutamic acid occur at the positions corresponding to Thr-85 and Pro-102, respectively, in the yeast protein (Fig. 2). To investigate whether this domain could cause temperature-sensitive growth in yeast, a series of expression plasmids were constructed (Fig. 5). Expression plasmid pYT6 codes for a hybrid protein in which amino acid residues 81–105 of the yeast RISP (10) are replaced by residues 123–147 from the tobacco homolog (Fig. 5). pYT6

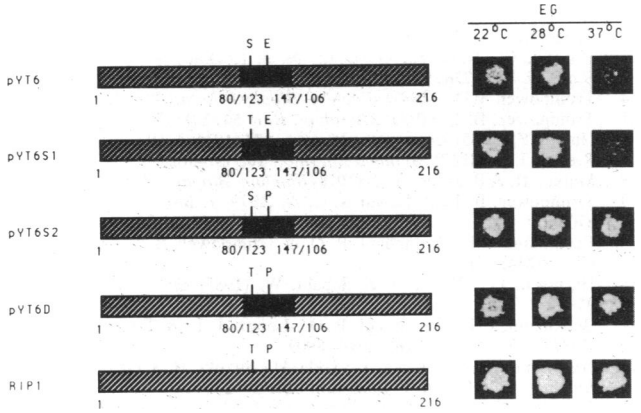

FIG. 5. A specific domain from the plant mt RISP causes temperature-sensitive respiratory growth in yeast. Yeast transformants expressing the constructs (*Left*) are grown on nonfermentable glycerol (EG) at designated temperatures (*Right*). Amino acid residues (single-letter code) are shown above each construct. Numbers below each construct indicate the position of the junction (see Fig. 4*a*).

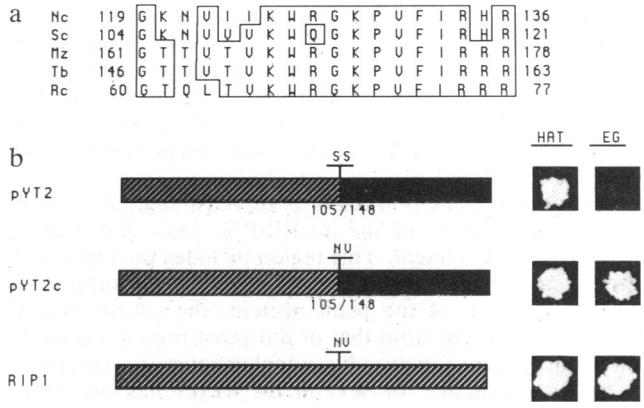

FIG. 6. Structurally conserved domain essential for the function of bc_1-type RISPs. (*a*) Alignment of the structurally conserved sequences from *Neurospora* (Nc, ref. 11), yeast (Sc, ref. 12), maize (Mz, this work), tobacco (Tb, this work), and *R. capsulatus* (Rc, ref. 14). Amino acids identical in three of the five sequences are boxed. Numbers indicate the position of the first (left) and last (right) amino acid residues, respectively, of the protein sequences. (*b*) Yeast transformants expressing the constructs growing on fermentable glucose (HAT) and nonfermentable glycerol (EG). Amino acid residues (single-letter code) are shown above each construct. Numbers below each construct designate the junction (see Fig. 4*a*).

restored respiratory growth to JPJ1 only at ≤30°C but not at 37°C, a temperature-sensitive growth response typical in yeast (Fig. 5, pYT6). When we changed Ser to Thr and Glu to Pro as in the yeast protein, we found that either changing both residues (pYT6D) or changing only Glu to Pro (pYT6S2) completely eliminated temperature sensitivity; however, changing only Ser to Thr (pYT6S1) did not eliminate temperature sensitivity (Fig. 5).

Identification of a Structurally Conserved Domain Essential for RISP Function. During the domain-swapping analysis we found that expression plasmid pYT2 failed to complement JPJ1 to respiratory competency (Fig. 6*b*). This result was unexpected because pYT2 contains a carboxyl-terminal region of the plant protein shorter than that in pTR3 (Fig. 4*b*), which restored respiratory competency to JPJ1. When pYT2 was constructed, two amino acids were changed at the junction between the yeast and plant proteins: Gln-106 to Ser and Val-107 to Ser, respectively (numbering according to the yeast RISP sequence, ref. 10). A computer sequence alignment analysis revealed that the junction is located in a region that is conserved among RISPs of bc_1 complexes from species as divergent as higher plants, single-cell fungi, and photosynthetic bacteria (Fig. 6*a*). Furthermore, analysis of secondary structure of this domain by the Chou–Fasman (38) method revealed that all sequences shown in Fig. 6*a* have a nearly identical β-sheet–turn–β-sheet profile (data not shown). The two amino acid changes, however, were predicted to eliminate the first β-sheet structure (data not shown). When the two serine residues were changed to the wild-type yeast sequence (pYT2c, Fig. 6*b*), the construct complemented JPJ1 to respiratory competency (Fig. 6*b*).

DISCUSSION

cDNA clones coding for the mt RISP have been isolated from maize and tobacco by screening cDNA libraries with a gene-specific probe. These cDNA clones are identified as the mt RISP gene based on the following criteria. (*i*) They contain the highly conserved region that is characteristic of bc_1 complex RISPs (Fig. 1*a*). (*ii*) The deduced amino acid sequences are similar to mt RISPs from *N. crassa* and *S. cerevisiae* but different from the chloroplast RISP from spinach. (*iii*) The hydropathy profile of the maize and tobacco RISPs is similar to the *Neurospora* and yeast proteins but different from the chloroplast RISP of spinach [window size = 7, scale = Kyte–Doolittle (42); data not shown]. (*iv*) The carboxyl two-thirds of the plant proteins is functionally equivalent to the yeast mt RISP.

The maize cDNA clone MR2.4 predicts an ORF of 819 bp that could code for a protein of 29.8 kDa. The *in vitro* translation product of pMR2.4 is a single polypeptide of ≈30 kDa (Fig. 3), in agreement with the predicted size. Western blot analysis detected two immunoreactive polypeptides with apparent molecular masses of 24 kDa and 22.8 kDa from isolated maize mitochondria (Fig. 3), suggesting the maize mt RISP is, like other mt RISPs, translated as a precursor protein, and its transit peptide is removed during translocation. In *Neurospora* (43) and yeast (44), the processing of mt RISPs occurs in two steps. A highly conserved three-amino acid motif has been identified to be common to this class of transit peptides (45). This motif includes an arginine at position −10, a hydrophobic residue at −8, and serine, threonine, or glycine at −5 relative to the mature amino terminus. The deduced protein sequence of MR2.4 contains an arginine at position 58, a phenylalanine at 60, and a threonine at 63 (Fig. 2). This analysis predicts that the amino terminus of the mature maize RISP is proline 68. The predicted molecular mass of this mature protein is 22.7 kDa, close to the 22.8-kDa polypeptide detected by Western blotting (Fig. 3). This suggests that the 22.8-kDa band corresponds to the mature maize RISP; the identity of the 24-kDa polypeptide detected from the isolated maize mitochondria is unclear (Fig. 3). Possible explanations are (*i*) nonspecific cross-reaction with the heterologous antiserum, (*ii*) an intermediate form of processing, (*iii*) a different form of the mature RISP resulting from posttranscriptional/posttranslational modification, and (*iv*) a different RISP encoded by an independent gene. Our Southern and Northern analyses suggest that there are several copies of the gene coding for the mt RISP in higher plant genomes and that their expression may be differentially regulated (J.H., F.S., and C.S.L., unpublished data). The 5′ end of the reading frame predicted from the tobacco cDNA clone TR12 is still open, an indication that TR12 is an incomplete cDNA (Fig. 2). Nevertheless, a similar three-amino acid motif, common to transit peptides cleaved in two steps, is also found in the amino-terminal region of the tobacco protein (arginine at 44, phenylalanine at 46, and asparagine at 49, Fig. 2), suggesting that the tobacco mature protein starts with the alanine at 54 and has a molecular mass of 22.6 kDa. The molecular mass of the immunoreactive polypeptide detected by Western blotting is

23 kDa (Fig. 3). Taken together, these results suggest that the mt RISP in higher plants is also translated as a precursor and imported into mitochondria where the cleavage of the transit peptide probably occurs in two steps as in *Neurospora* and yeast. The predicted amino terminus for the mature maize or tobacco protein and the proposed two-step processing event must be confirmed.

The complementation analysis in yeast suggests that the carboxyl two-thirds of the mt RISP in yeast and plants is functionally equivalent. This region includes the highly conserved catalytic site common to all bc_1 types. In contrast, the amino one-third of the plant protein, the species-specific region, is different from that of the yeast protein because it does not restore function by complementation. Analysis of secondary structure of several mt RISPs has revealed a hydrophobic domain in the amino-terminal region of the mature protein, which was proposed to transverse or bind to the mt inner membrane and fix the RISP in the bc_1 complex (10, 11). More recent studies with the RISP from beef heart mitochondria show that the hydrophobic domain contains a nontransmembrane core region of 18 amino acids, which could form an amphipathic structure and interact with other subunits in the bc_1 complex to determine its relative position in the complex (37). The reason that expression plasmids pMZR10, pYTR, and pTR7 cannot complement the deficient yeast strain JPJ1 may be due to a faulty interaction between the amino terminus of the plant protein and other subunits in the bc_1 complex of yeast. In higher plants, combining a nuclear genome with a distantly related cytoplasm (e.g., interspecific cross) often leads to cytoplasmic male sterility, which might result from a nonfunctional interaction/assembly of a mt enzyme complex consisting of nuclear and cytoplasmically encoded subunits. Our complementation analysis in yeast may, in fact, mimic this process. Other factors also appear to be involved because the fusion protein encoded by pTR7 was not processed to its mature form, even though the native cleavage site for the yeast protein is retained (data not shown). Together these results suggest that the amino terminus of the mt RISP is crucial for correct conformation, processing, and interacting with other components of the bc_1 complex.

Another interesting finding from our complementation analysis is that a specific region from the plant RISP can cause temperature-sensitive respiratory growth in yeast (Fig. 5). A glutamic acid residue at position 144 (numbering according to the tobacco protein sequence, Fig. 2) is responsible for the temperature-sensitive growth; an amino acid change at the corresponding position in the yeast RISP also causes temperature-sensitive growth (10). Interestingly, Glu-144 is very close to the structurally conserved domain found to be essential for the function of the bc_1-type RISPs (Fig. 6*a*). A large number of genes, including those for chloroplast functions and possibly those for heat-shocked proteins, are involved in heat tolerance in plants (46). Genes coding for mt functions are also associated with high temperature tolerance of plants (47, 48), and mt respiration differs in heat tolerance between species (47). Our results suggest that yeast is useful for studying the relationship between structure and thermal stability of plant gene products.

We thank M. A. Conkling and A. L. Kriz for cDNA libraries, U. Harnisch for antiserum, B. Trumpower for yeast strain JPJ1 and the *RIP1* clone, and A. Myers for plasmid YEp352. We also thank Jane Suddith and Mary Clark for excellent technical assistance, Suzanne Quick for editorial assistance, and members of our laboratory for critical reading of the manuscript. This work was supported, in part, by grants from the National Science Foundation (C.S.L.) and the R. J. Reynolds Tobacco Company (J.H. and D.F.M.).

1. Rieske, J. S. (1976) *Biochim. Biophys. Acta* **456,** 195–247.
2. Tzagoloff, A. (1982) *Mitochondria* (Plenum, New York).
3. Hauska, G., Hurt, E., Gabellini, N. & Lockau, W. (1983) *Biochim. Biophys. Acta* **726,** 97–133.
4. Trumpower, B. L. (1981) *Biochim. Biophys. Acta* **639,** 129–155.
5. Trumpower, B. L. (1990) *Microbiol. Rev.* **54,** 101–129.
6. Hatefi, Y. (1985) *Annu. Rev. Biochem.* **54,** 1015–1069.
7. Rieske, J. S. (1967) *Methods Enzymol.* **10,** 357–362.
8. Malkin, R. & Bearden, A. J. (1978) *Biochim. Biophys. Acta* **505,** 147–181.
9. Trumpower, B. L. & Edwards, C. A. (1979) *J. Biol. Chem.* **254,** 8697–8706.
10. Beckmann, J. D., Ljungdahl, P. O. & Trumpower, B. L. (1989) *J. Biol. Chem.* **264,** 3713–3722.
11. Harnisch, U., Weiss, H. & Sebald, W. (1985) *Eur. J. Biochem.* **149,** 95–99.
12. Beckmann, J. D., Ljungdahl, P. O., Lopez, J. L. & Trumpower, B. L. (1987) *J. Biol. Chem.* **262,** 8901–8909.
13. Nishikimi, M., Hosokawa, Y., Toda, H., Suzuki, H. & Ozawa, T. (1989) *Biochem. Biophys. Res. Commun.* **159,** 19–25.
14. Gabellini, N. & Sebald, W. (1986) *Eur. J. Biochem.* **154,** 569–579.
15. Kallas, T., Spiller, S. & Malkin, R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5794–5798.
16. Steppuhn, J., Rother, C., Hermans, J., Jansen, T., Salnikow, J., Hauska, G. & Herrmann, R. G. (1987) *Mol. Gen. Genet.* **210,** 171–177.
17. Mack, D. H. & Sninsky, J. J. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 6977–6981.
18. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) *Science* **230,** 1350–1354.
19. Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. & Erlich, H. (1986) *Cold Spring Harbor Symp. Quant. Biol.* **51,** 263–273.
20. Güssow, D. & Clackson, T. (1989) *Nucleic Acids Res.* **17,** 4000.
21. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
22. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987) *Current Protocols in Molecular Biology* (Wiley, New York).
23. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.
24. Wallace, R. B. & Miyada, C. G. (1987) in *Guide to Molecular Cloning Techniques*, eds. Berger, S. L. & Kimmel, A. R. (Academic, San Diego), pp. 432–442.
25. Heinrich, P. (1987) in *Current Protocols in Molecular Biology*, eds. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (Wiley, New York), pp. 7.3.1–7.3.8.
26. Vieira, J. & Messing, J. (1987) *Methods Enzymol.* **153,** 3–11.
27. Huang, J., Hack, E., Thornburg, R. W. & Myers, A. M. (1990) *Plant Cell* **2,** 1249–1260.
28. Daum, G., Böhni, P. C. & Schatz, G. (1982) *J. Biol. Chem.* **257,** 13028–13033.
29. Hurd, H. K., Roberts, C. W. & Roberts, J. W. (1987) *Mol. Cell. Biol.* **7,** 3673–3677.
30. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
31. Dixon, L. K. & Leaver, C. J. (1982) *Plant Mol. Biol.* **1,** 89–102.
32. Hill, J. E., Myers, A. M., Koerner, T. J. & Tzagoloff, A. (1986) *Yeast* **2,** 163–167.
33. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
34. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382.
35. Schiestl, R. H. & Gietz, R. D. (1989) *Curr. Genet.* **16,** 339–346.
36. Schägger, H., Borchart, U., Machleidt, W., Link, T. A. & Von Jagow, G. (1987) *FEBS Lett.* **219,** 161–168.
37. González-Halphen, D., Vázquez-Acevedo, M. & García-Ponce, B. (1991) *J. Biol. Chem.* **266,** 3870–3876.
38. Chou, P. Y. & Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47,** 251–276.
39. Bowler, C., Alliotte, T., Van Den Bulcke, M., Bauw, G., Vandekerckhove, J., Van Montagu, M. & Inzé, D. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 3237–3241.
40. Chaumont, F., O'Riordan, V. & Boutry, M. (1990) *J. Biol. Chem.* **265,** 16856–16862.
41. Schmitz, U. K. & Lonsdale, D. M. (1989) *Plant Cell* **1,** 783–791.
42. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132.
43. Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H. & Neupert, W. (1986) *Cell* **47,** 939–951.
44. Fu, W., Japa, S. & Beattie, D. S. (1990) *J. Biol. Chem.* **265,** 16541–16547.
45. Hendrick, J. P., Hodges, P. E. & Rosenberg, L. E. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4056–4060.
46. Mascarenhas, J. P. (1984) in *Applications of Genetic Engineering to Crop Improvement*, eds. Collins, G. B. & Petolino, J. G. (Nijhoff, Dordrecht, The Netherlands), pp. 391–425.
47. Lin, T.-Y. & Markhart, A. H., III (1990) *Plant Physiol.* **94,** 54–58.
48. Diezengremel, P. & Chauveau, M. (1978) in *Plant Mitochondria, Proceedings of the International Symposium on Plant Mitochondria Held in Marseille, France*, eds. Doucet, G. & Lance, C. (Elsevier, Amsterdam), pp. 267–274.