How Do Plant Mitochondria Avoid Importing Chloroplast Proteins? Components of the Import Apparatus Tom20 and Tom22 from Arabidopsis Differ from Their Fungal Counterparts[1]

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Mitochondria and plastids import proteins using mechanisms that have many features in common and yet each mechanism is sufficiently selective that only the appropriate set of proteins is imported. Protein selectivity is determined by discrete targeting sequences in mitochondrial and plastid precursor proteins and by large protein complexes that reside in the outer membrane of each organelle. In the mitochondrion, the translocase in the outer mitochondrial membrane controls protein import. One of the key subunits of this complex, the integral membrane protein Tom22, is well conserved in organisms that lack plastids such as yeast and filamentous fungi, nematodes, insects, birds, and mammals. The sequence of plant Toms 22 are different and reveal features that probably arose after the arrival of the chloroplast approximately 800 million years ago.

The translocase in the outer mitochondrial membrane, or TOM complex, on the surface of mitochondria is a fascinating example of a molecular machine that has evolved to overcome a fundamental problem. In eukaryotic cells, the reaction steps of metabolic pathways are compartmentalized by internal membranes so that distinct enzyme activities have to be sent to each cellular compartment. The targeting of these enzymes occurs before they are assembled, using specific sequences on the nascent polypeptides (Schatz and Dobberstein, 1996). Most mitochondrial proteins are made as precursors with an amino-terminal targeting sequence: the TOM complex recognizes the mitochondrial-targeting sequence, binds the mitochondrial precursor protein productively, and initiates its translocation into the mitochondrion (Fig. 1). This first stage of the import pathway does not require energy from ATP hydrolysis or a transmembrane potential (for review, see Neupert, 1997; Pfanner et al., 1997; Schatz, 1997; Verschoor and Lithgow, 1999).

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CHOOSING BETWEEN PROTEINS FOR THE MITOCHONDRIA OR THE PLASTID

In plants, mitochondrial protein targeting is made even more complicated by the presence of a chloroplast. Like proteins targeted to the mitochondrion, chloroplast-targeted proteins are made as precursors with an N-terminal transit peptide that is cleaved during import. Chloroplast transit peptides have some of the features of mitochondrial target peptides. In general, mitochondrial-targeting peptides contain a segment of 12 to 15 amino acids that can form a basic, amphipathic helix (von Heijne, 1986). Transit peptides also have a central region rich in basic amino acids and a carboxy-terminal region that is predicted to be amphipathic (von Heijne et al., 1989). Indeed, several plastid transit peptides look enough like mitochondrial-targeting peptides that they can direct import of chloroplast proteins into fungal mitochondria; the TOM complex of fungi is unable to distinguish plastid-transit sequences from mitochondrial-targeting sequences (Hurt et al., 1986; Brink et al., 1994). However, the plant TOM complex can clearly distinguish a plastid-transit sequence from a mitochondrial-targeting sequence (Glaser et al., 1998). How does the plant TOM complex deal with the problem of selectively importing proteins into mitochondria? Although we still do not have a complete answer to this question, a combination of biochemical studies and DNA sequence comparisons is starting to provide clues. However, to understand the significance of these studies, we must first review protein import by the TOM complex in cells that lack a plastid.

ORGANIZATION OF THE TOM COMPLEX AND THE CENTRAL ROLE OF TOM22

In the yeast *Saccharomyces cerevisiae*, there are up to eight proteins in the TOM complex and these are named according to their apparent M_rs on SDS-polyacrylamide gels (Fig. 1). In the fungi *Neurospora crassa*, there are up to six proteins in the TOM complex, but otherwise the size, primary structure, and

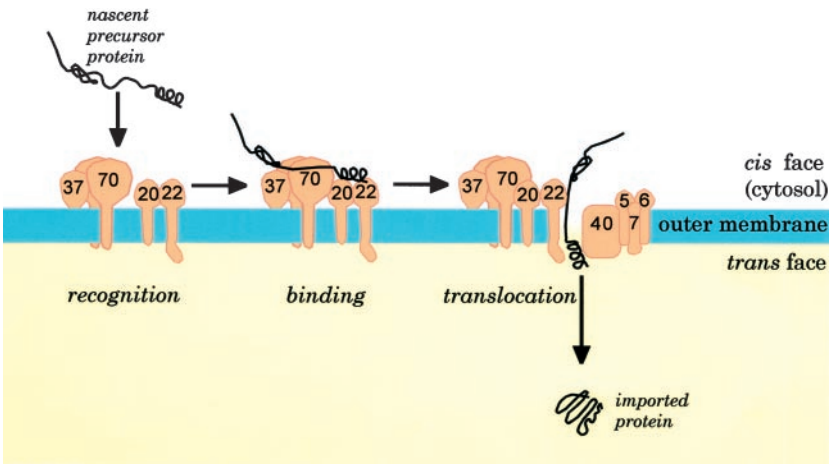

Figure 1. The TOM complex of *S. cerevisiae*. The multiple subunits of the TOM complex are named according to their apparent M_rs (Pfanner et al., 1996). After recognition of a mitochondria-specific presequence, mitochondrial precursor proteins bind to the receptor subunits Tom70/71, Tom37, Tom20, and Tom22 on the cis side of the membrane. Subsequently, precursor proteins are transferred from Tom22 into the channel subunit Tom40 and then bound by domains of Tom22 and Tom40 on the trans side of the membrane (Bolliger et al., 1995; Rapaport et al., 1998; Kanamori et al., 1999). Components of the protein import apparatus in the intermembrane space and inner membrane complete the translocation process (Neupert, 1997; Pfanner et al., 1997; Koehler et al., 1999).

domain organization of the various subunits is conserved (Neupert, 1997; Pfanner et al., 1997). The TOM complex from *N. crassa* has been purified and examined by electron microscopy, revealing the existence of a core complex that comprises Tom22, Tom40, and the small Tom subunits (Tom5, Tom6, and Tom7). Together, these proteins provide a translocation channel through the outer mitochondrial membrane. The other subunits act as receptors for incoming precursor proteins and are loosely associated with this core complex (Ahting et al., 1999). The Tom22 and Tom40 subunits are literally essential to life in yeast as deleting the gene encoding either protein is lethal (Baker et al., 1990; Lithgow et al., 1994).

The Tom22 subunit acts as both an acidic receptor for the basic targeting peptide of precursor proteins

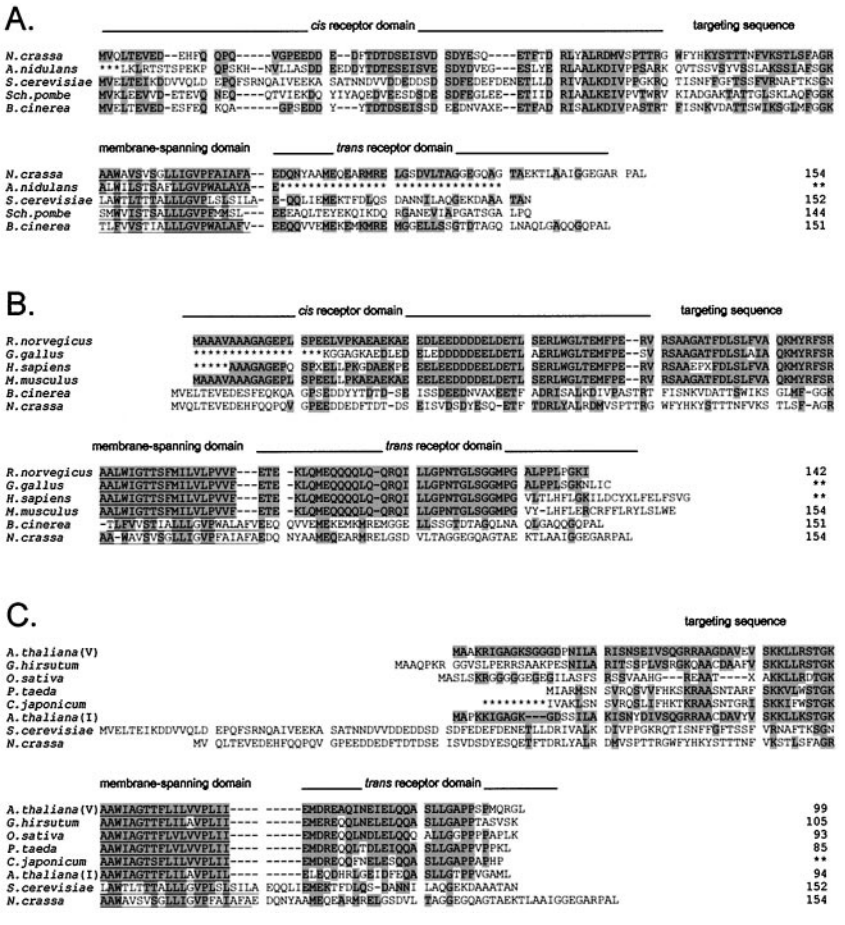

Figure 2. ClustalW sequence analysis of Tom22 from various organisms. A, Tom22 homologs from four other fungi (*Aspergillus nidulans, S. cerevisiae, Schizosaccharomyces pombe*, and *Botyritis cinerea*) are compared with *N. crassa* with conserved residues highlighted. The four domains of Tom22 have been experimentally defined for *Sc*Tom22. Asterisks denote that the *A. nidulans* sequence is derived from a partial cDNA (accession no. AA784604). B, Tom22 homologs from three other vertebrates (*Gallus gallus,* humans [*Homo sapiens*], and *Mus musculus*) are compared with *Rattus norvegicus* with conserved residues highlighted. The sequences were identified by BLAST analysis with short segments of *Nc*Tom22. The sequence cluster was aligned to the sequences from *N. crassa* and *B. cinerea,* and the functional domains of the fungal proteins are labeled. Asterisks denote that the *G. gallus* and human sequences are derived from partial cDNAs (accession nos. AI982019 and T35592). C, Tom22 homologs from four other plants (cotton [*Gossypium hirsutum*], rice [*Oryza sativa*], loblolly pine [*Pinus taeda*], and Japanese cedar [*Cryptomeria japonica*]) are compared to Arabidopsis sequences encoded on chromosome 1 (Arabidopsis I) and chromosome 5 (Arabidopsis V) with conserved residues highlighted. The sequences were identified by BLAST analysis with short segments of *Nc*Tom22, *Sc*Tom22, and *Os*Tom22. The sequence cluster was aligned to the sequences from *N. crassa* and yeast, and the functional domains of the fungal proteins are labeled. Asterisks denote that the Japanese cedar sequence is derived from a partial cDNA (accession no. AU036888).

and as a core component of the translocation channel itself. Figure 2A shows an alignment of the Tom22 sequence from yeast, *N. crassa*, and three other fungi (*S. pombe*, *A. nidulans*, and *B. cinerea*). In yeast, where the complete genome sequence is now known, there is a single Tom22 gene; this appears also to be true for other organisms as well. The aligned sequences reveal considerable conservation of primary structure in the Tom22 protein from these five evolutionary divergent fungi.

The fungal Tom22 is made up of four domains: the cis receptor domain exposed in the cytosol, a short mitochondrial-targeting segment, a single membrane-spanning domain, and a small trans receptor domain located in the mitochondrial intermembrane space. The trans receptor domain is the least conserved region in the various fungal sequences shown in Figure 2. However, all fungi and other organisms (see below) seem to have a domain in this position with the same size and amino acid composition as the yeast sequence. Yeast mutants lacking this trans domain of Tom22 show protein import defects consistent with the proposed role of the C-terminal region of Tom22 as a trans receptor (Bolliger et al., 1995; Moczko et al., 1997; Kanamori et al., 1999). It is thought that the trans domains of both Tom22 and Tom40 help to draw translocating precursor proteins through the outer membrane by anchoring them in the intermembrane space (Lithgow et al., 1994; Bolliger et al., 1995; Mayer et al., 1995; Schatz, 1997; Rapaport et al., 1998; Kanamori et al., 1999).

The membrane-spanning domain of Tom22 interacts with Tom40, Tom5, Tom6, and Tom7 to form the translocation channel in the mitochondrial outer membrane, stabilizing the structure of the core complex (Ahting et al., 1999; van Wilpe et al., 1999).

The N-terminal cis domain of Tom22 has at least three roles in the TOM complex. The main role is as a receptor that binds mitochondrial presequences. In addition, regions within the cis domain also promote assembly of Tom22 into the TOM complex (Rodriguez-Cousiño et al., 1998) and contain the sequence that targets Tom22 to mitochondria (Egan et al., 1999).

In yeast, the cis receptor domain is crucial: A truncated version of Tom22 lacking the first 65 amino acids localizes to the mitochondrial outer membrane but cannot rescue the lethal phenotype of Δ*tom22* cells (Egan et al., 1999). Antibodies that recognize the cis receptor domain of Tom22 block precursor binding to isolated mitochondria (Hönlinger et al., 1995; Mayer et al., 1995). Point mutations in critical acidic residues confirm that this domain takes part in precursor binding on the mitochondrial surface (Bolliger et al., 1995). Recombinant cis domain can bind precursor proteins in vitro through interactions that are likely to be largely electrostatic between the acidic receptor domain and the basic part of the mitochondrial presequence (Schatz, 1997; Brix et al., 1999; Komiya et al., 1998).

Sequencing projects have recently found sequences encoding Tom22 from rats, chickens, mice, and humans, as well as a range of invertebrate animals. Figure 2B is an alignment of the vertebrate sequences showing that the basic plan of metazoan and fungal Toms 22 is conserved. Although not yet verified experimentally, the predicted sequences for the Toms 22 of the various organisms shown in Figure 2B are consistent with the domain structure outlined for the fungal protein.

THE PLANT TOM COMPLEX: CONSERVATION AND SURPRISES

Studies on mitochondrial protein import in plants have turned up some remarkable findings recently. Using blue native PAGE, a technique that allows the resolution of intact protein complexes from detergent-solubilized membranes, Jänsch et al. (1998) showed that the sizes of some individual subunits from the potato TOM complex differed from the known sizes of subunits in the yeast complex. In particular, no homologs of Tom37 or Tom22 were apparent and there was an additional protein of around 9 kD. The absence of Tom37 from the plant complex was not so surprising, since this subunit is also missing from the *N. crassa* complex, and the Tom37 subunit might have a function unique to the mitochondria of yeast (Gratzer et al., 1995) and mammals (Armstrong et al., 1997, 1999). The apparent absence of Tom22 in plants was a total shock: How could the TOM complex of plant mitochondria function without this pivotal subunit?

Perhaps more surprising is that various sequencing projects have revealed homologs of Tom22 in a variety of plants. Using iterative BLAST analysis of short sequence segments from the various fungal sequences, we identified homologs sequenced from two dicot species, Arabidopsis and cotton; a grass, rice; and two gymnosperms, loblolly pine and Japanese cedar. However, in contrast to the extensive conservation seen in fungal and metazoan Toms 22, the predicted plant proteins range in size from 11.1 (in cotton) to 9.2 kD (in loblolly pine). By both size and amino-terminal sequence, the available cDNAs correspond to the partially sequenced, additional 9-kD subunit found in the TOM complex purified from potato mitochondria (Jänsch et al., 1998).

What causes the difference in size between plant Tom22 homologs and the rest? Whereas the Tom22 homologs from fungi (Fig. 2A), mammals, and birds (Fig. 2B), the nematodes *Caenorhabditis elegans* and *Ancylostoma caninum*, the fly *Drosophila melanogaster*, and the blood fluke *Schistostoma masoni* (data not shown) all have an acidic cis receptor domain at their N-terminal end, this domain is missing from the available plant sequences. Hence, the plant proteins

are smaller. But while this finding suggests that the subunit composition of the TOM complex in plants and other organisms is conserved, it also suggests the function of the Tom22 subunit may have been modified during the evolution of plants.

Tom22 from angiosperms (Arabidopsis, rice, and their allies) and gymnosperms have the same structure. This suggests that the acidic cis receptor domain was lost before these lineages diverged roughly 130 million years ago in the early Cretaceous period. Our hypothesis is that this change occurred even earlier as a response to the arrival of the chloroplast. The subsequent evolution of this endosymbiotic relationship has involved transfer of genes from the chloroplast to the nucleus and the development of mechanisms to target gene products back to the chloroplast. Loss of the cis receptor domain of Tom22 may therefore have been an adaptive change associated with an increased requirement for selectivity in protein targeting. If this hypothesis is correct, then we would predict that the cis receptor domain is also missing in organisms, such as the green algae, that represent the most basal green plant lineages. Unfortunately, Tom22 sequences from representatives of these lineages are not currently available in the DNA databases. We can, however, speculate on how changes to Tom22 appear to allow the TOM complex to distinguish mitochondrial-targeting peptides from chloroplast transit peptides.

A MORE DISCRIMINATING TOM COMPLEX IN PLANTS?

The function of the acidic cis receptor domain of Tom22, although normally essential for viability in other organisms, might be unnecessary or even a liability, in plants. In fungi, the acidic cis receptor domain provides a binding site for the basic presequence of mitochondrial precursors (Bolliger et al., 1995; Mayer et al., 1995; Brix et al., 1999; Komiya et al., 1998). In plants, this simple electrostatic interaction might be counterproductive for selective binding of mitochondrial precursor proteins; the targeting sequences for precursor proteins destined for the plastids are also positively charged. It may be that to solve the problem of discriminating between basic, amphipathic, helical sequences on mitochondrial proteins and the basic, less-structured targeting sequences on plastid proteins, the most acidic receptor domain has been deleted from the plant TOM complex. The acid-chain hypothesis for precursor recognition and binding suggests sequential interaction with the Tom20, Tom22, and Tom5 cis receptor domains before a precursor protein can be inserted into the translocation channel (Schatz, 1997). We anticipate the overall mechanism, whereby a precursor protein effectively surfs from one domain to another, is likely to be conserved in plant mitochondria. However, the absence of the very acidic Tom22 receptor domain from the acid chain in plants might enable the TOM complex to prevent plastid proteins from ever binding productively.

This modification to the plant TOM complex is likely to have further consequences. First, the receptor domain of Tom20 is distinct in plants, and may have been modified to compensate for the absence of the Tom22 domain. Certainly, the Tom20 homologs from potato (*Solanum tuberosum*), cultivated soybean (*Glycine max*), Arabidopsis, cotton, and loblolly pine differ considerably primary structure from the fungal and animal Toms 20 (Fig. 3). In particular, the plant proteins have a slightly higher proportion of acidic residues (15.3% for *At*Tom20, compared with 13.11% for *Sc*Tom20), and have their transmembrane anchor at the carboxy-terminal end of the protein. Since antibodies bound to Tom20 inhibit protein import into potato mitochondria (Heins and Schmitz, 1996), the receptor must be tail-anchored with a N_{out}-C_{in} topology.

Second, the changes in receptor structure are likely to have evolved in parallel with modifications of the mitochondrial-targeting sequences in plant proteins. Tom20 and Tom22 act together as the primary binding site for the targeting sequence of most mitochondrial proteins (Bolliger et al., 1995; Mayer et al., 1995; Komiya et al., 1998; Brix et al., 1999), and the differences in the plant Tom20 and Tom22 receptors would seem to demand some differences in the ligands to which they bind. This notion is supported by observations that plant mitochondrial presequences are longer than those found in fungi and animals (Sjoling and Glaser, 1998), they can be post-translationally modified (von Stedingk et al., 1999), and that positive residues outside the amphipathic region were as crucial for import as were positive residues within this region (Tanudji et al., 1999). In which period of evolutionary time these modifications occurred is not clear, but we suggest that both the mitochondrial-targeting signals and receptor structure are subtly modified in higher plants to prevent productive binding of non-mitochondrial (especially plastid) proteins by the TOM complex.

ARE THERE FURTHER SURPRISES IN STORE?

The other subunits of the plant TOM complex seem conserved, at least with respect to size (Jänsch et al., 1998), and all indications to date show the translocation stage of protein import into mitochondria is conserved between plants, fungi, and animals (for review, see Braun and Schmitz, 1999). In terms of the TOM complex, the only other subunit that is essential for viability in yeast is Tom40, the major component of the translocation channel (for review, see Bains and Lithgow, 1999). Full-length or near full-length sequences encoding obvious plant homologs of Tom40 from Arabidopsis, cultivated tomato (*Lycopersicon esculentum*), corn (*Zea mays*), cotton, and culti-

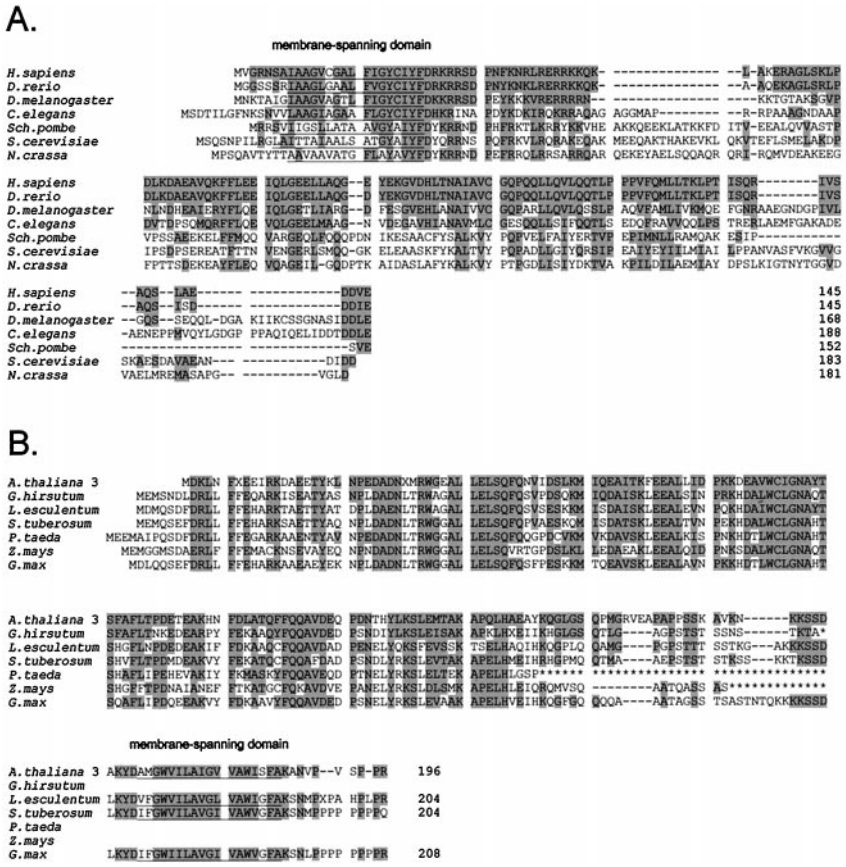

Figure 3. ClustalW sequence analysis of Tom20 from various organisms. A, Tom20 homologs from zebrafish (*Danio rario*), fruitfly, the nematode, and three fungi (*S. cerevisiae, S. pombe*, and *N. crassa*) are compared to human Tom20 with conserved residues highlighted. B, Tom20 homologs from cotton and tomato. Potato, loblolly pine, corn, and soybean are compared to Tom20 inferred for Arabidopsis from the genomic sequence of chromosome 3, with conserved residues highlighted. Asterisks denote that the cotton, loblolly pine, and corn sequences are derived from partial cDNAs (accession nos. AI728132, AI812948, and AI820306, respectively). In all cases, transmembrane domains were predicted using dense alignment surface (DAS) method (http://www.biokemi.su.se/~server/DAS/tmdas.cgi) and are shown underlined.

vated soybean are deposited at GenBank, revealing conservation of both size and primary structure to the known fungal sequences (T. Lithgow, unpublished data).

AT LEAST TWO GENES ENCODE EACH TOM SUBUNIT IN ARABIDOPSIS

An additional finding from analysis of the plant sequences is that Tom20, Tom22, and the small subunit Tom7 are encoded by at least two genes in Arabidopsis (T. Lithgow, unpublished data). Genes encoding Tom20 are present on chromosomes 1, 3, and 5 (*AtTOM20*-I, *AtTOM20*-III, and *AtTOM20*-V), and genes encoding Tom7 and Tom22 are each present on chromosomes 1 and 5 (*AtTOM7*-I, *AtTOM7*-V and *AtTOM22*-I, *AtTOM22*-V). Our preliminary reverse transcriptase-PCR analysis shows the *AtTOM22*-I gene is widely expressed, being present in mRNA preparations made from leaves, roots, and flowers, and from cells cultured in the dark with Suc as a carbon source (D. Macasev and T. Lithgow, unpublished data). To date, we have been unable to detect the transcript from *AtTOM22*-V in any RNA preparation; however, both genes are expressed under some conditions since each gene has a corresponding cDNA in the Ohio State transcriptome collection (GenBank accession nos. AI993339 and AI999522) and expression of the *AtTOM22*-V gene might be developmentally regulated.

The *At*Tom20 isoforms are largely similar, whereas the two *At*Tom22 isoforms differ only in the primary structure of their trans domains (50% similar, Fig. 2C). It will be of interest to see whether the genes are expressed at the same time in the same tissue yielding subtly different TOM complexes in the outer membrane, or whether the gene pairs are differentially transcribed to provide for developmental or tissue-specific responses. Other plant mitochondrial proteins are encoded by small gene families, such as proteins of the electron transport chain, the ATP synthase complex, the adenine nucleotide translocator family, and alternative oxidase (for review, see McCabe et al., 2000), as well as other components of the protein-import machinery including the processing peptidase, mtHSP70, mtGrpE, and mtHSP60 (for review, see Braun and Schmitz, 1999).

CONCLUDING REMARKS

Major genome and transcriptome sequencing projects in several species of plants are generating a wealth of information, and analysis of the data can provide models on which to base future experiments to tease apart the function of the machinery mediating various cellular processes. Comparative sequence

analyses of several components of the TOM machinery suggest that it is highly conserved in plants and have provided insight into features such as the ubiquitous need for the trans receptor domain that were not obvious from previous analyses of the protein from *N. crassa* and yeast alone. Perhaps most important, the absence of the crucial cis receptor domain specifically from plant Tom22 and changes in the partner receptor Tom20 hint at a rearrangement of the TOM complex's receptor function. A fascinating possibility is that the truncation of Tom22 was required for mitochondria and plastids to cohabit within the same cell. While a detailed phylogenetic analysis awaits sequencing of Tom22 from more primitive plants, experiments are under way in several laboratories to understand how precursor proteins are selected for import into mitochondria, chloroplasts, and other organelles of the plant cell.

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