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Different fates of the chloroplast tufA gene following its transfer to the nucleus in green algae

(elongation factor Tu/gene transfer/organelle genomes/gene duplication/multigene family)

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ABSTRACT Previous work suggested that the tufA gene, encoding protein synthesis elongation factor Tu, was transferred from the chloroplast to the nucleus within the green algal lineage giving rise to land plants. In this report we investigate the timing and mode of transfer by examining chloroplast and nuclear DNA from the three major classes of green algae, with emphasis on the class Charophyceae, the proposed sister group to land plants. Filter hybridizations reveal a chloroplast tufA gene in all Ulvophyceae and Chlorophyceae and in some but not all Charophyceae. One chlorophycean alga, Coleochaete orbicularis, is shown to contain an intact but highly divergent chloroplast tufA gene, whose product is predicted to be nonfunctional in protein synthesis. We propose that a copy of the tufA gene was functionally transferred from the chloroplast to the nucleus early in the evolution of the Charophyceae, with chloroplast copies of varying function being retained in some but not all of the subsequently diverging lineages. This proposal is supported by the demonstration of multiple tufA-like sequences in Coleochaete nuclear DNA and in nuclear DNA from all other Charophyceae examined.

Chloroplasts and mitochondria encode only a small subset of the proteins necessary for their function, the rest being encoded in the nucleus and posttranslationally imported into the organelles. Characterization of organelle proteins encoded by nuclear genes shows that many are eubacterial in nature (1, 2). According to endosymbiotic theory these genes arose by direct transfer from the organelles, which once were free-living eubacteria. The conservation of gene content among organelles of distantly related taxa suggests that most gene transfer occurred early in organelle evolution (3, 4). However, evidence of modern gene transfer has been accumulating (5, 6), suggesting that the process continues, albeit at a greatly reduced rate.

The plant tufA gene encodes the chloroplast protein synthesis elongation factor Tu (EF-Tu). A chloroplast-localized tufA has been sequenced from Euglena gracilis (7) and from chlorophycean (Chlamydomonas reinhardtii, ref. 5) and ulvophycean (Codium fragile, M. Kuhsel and J. D. P., unpublished data) green algae. However, tufA is missing from the chloroplast DNA (cpDNA) of all examined land plants, including a bryophyte (Marchantia polymorpha, ref. 8), and has been found in the nuclear DNA (ncDNA) of the land plant Arabidopsis thaliana (5). Phylogenetic analysis suggests that tufA was transferred from the chloroplast to the nucleus within the green algal lineage giving rise to land plants (5).

Five classes of green algae are recognized, with most taxa being assigned to the classes Charophyceae, Chlorophyceae, and Ulvophyceae (9). To further characterize the transfer of the tufA gene, we have investigated its structure and subcellular location in members of these three classes of green algae by a combination of filter hybridization and gene sequencing. Among the Charophyceae, an unusual chloroplast tufA was found in the genus Coleochaete, the proposed sister group to land plants (10, 11).

MATERIALS AND METHODS

Filaments of Spirogyra maxima and Sirogonium melanosporum were obtained from unialgal cultures grown in soil/water medium (12) on a 16:8 hr light/dark cycle at 20°C ± 2°C under fluorescent light at an illumination of 50 µmol m−2 s−1. N. translucens and Ch. connivens were grown in aquaria in a soil/water solution in a greenhouse. Coleochaete orbicularis was grown at 20°C in D11 solution (13) under 24-hr fluorescent light. Plants of Cladophora sp. were collected from a stream at the Matthaei Botanical Gardens of the University of Michigan.

cpDNAs and ncDNAs of the above algae were extracted from total DNA preparations (14) by centrifugation in cesium chloride and bisbenzimide H33258 (15). Other algal DNA were generously provided by J. E. Boynton (Duke University), A. W. Coleman (Brown University), M. Li-Weber (Max-Planck-Institute, F.R.G.), C. Lemieux (Universtes Laval, Quebec), and R. Meints (Oregon State University). Nonflowering land plant cpDNAs were prepared as described (16). Crucifer ncDNAs were extracted from Percoll-gradient isolated nuclei (17). Brassica campestris mitochondrial DNA was purified using DNase I (18) and cpDNA by density gradient centrifugation (19).

The single Coleochaete orbicularis chloroplast tufA was mapped to two adjacent cpDNA PstI fragments of 2.1 and 8.6 kilobases (kb), and a 5.8-kb HindIII fragment was found to overlap the junction between the two PstI fragments. Complete sequencing of the 2.1-kb PstI fragment showed that it contained the bulk of the tufA gene. The remaining 5′ end of the gene was then determined from the 5.8-kb HindIII fragment using synthetic primers. Sequences were determined for both strands by dyeoxy chain-termination, and all restriction sites were sequenced across.

Restriction enzyme digestion, agarose gel electrophoresis, Southern transfer, preparation of 32P-labeled probes, and hybridization were performed as described (20) with minor modifications (5). Hybridizations were at 60°C, and filters were washed after hybridization, once at room temperature and three times at 60°C in 0.3 M NaCl/30 mM sodium citrate/0.5% SDS.

Deduced protein sequences were aligned by eye, maximizing the alignment of identical and conserved amino acids (21), while introducing a minimum of gaps. Amino acids were scored for all informative sites as equally weighted, unordered, multistate characters. Gaps were scored as missing data. Phylogenetic trees were calculated by parsimony criteria.

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Abbreviations: cpDNA, chloroplast DNA; ncDNA, nuclear DNA; EF-Tu, elongation factor Tu.

The sequence reported in this paper has been deposited in the GenBank data base (accession no. M34286).
RESULTS

Distribution of tufA in cpDNAs of Green Algae and Land Plants. Within the three classes of green algae analyzed, a tufA-specific probe shows strong hybridization to cpDNAs from all examined members of the Ulvophyceae and Chlorophyceae and some but not all members of the Charophyceae (Fig. 1). Of the three orders of Charophyceae examined, members of the Charales (Chara and Nitella) show strong tufA signals, a member of the Coleochaetales (Coleochaete) gives only weak signals, and no signal at all can be detected in the Zygnematales (Spirogyra and Sirogonium). No tufA hybridization is seen to cpDNA from several basally derived branches of tracheophytes (Fig. 1), further supporting the previous designation of the Charophyceae as the ancestral origin of the land plants. Within the three classes of green algae analyzed, a tufA-specific probe shows strong hybridization to all cpDNAs tested (Fig. 1) except for the chloroplast rbcL gene (6). This is also consistent with the notion that tufA is absent from all land plant cpDNAs (5, 8, 22). Note that all hybridizations were conducted under conditions (Fig. 1) in which a probe for the chloroplast rbcL gene hybridized strongly to all cpDNAs tested. The apparent absence of tufA in cpDNAs from three classes of green algae suggests that the gene was ancestrally present in green algal cpDNA (5). This is also consistent with the proposed endosymbiotic origin of chloroplasts. The apparent lack of tufA in some charophycean and all land plant cpDNAs supports the previous designation of the Charophyceae as the algal lineage giving rise to land plants (11) and suggests that transfer of the gene occurred in the charophycean lineage.

An Unusually Divergent Chloroplast tufA from Coleochaete. Of the weakly hybridizing tufA signals of Coleochaete (Fig. 1) only the 2.1-kb Pst I fragment was found to correspond to a cpDNA fragment. This and an overlapping HindIII fragment were further examined by sequencing, and a single open reading frame of 1245 bp encoding an EF-Tu-like sequence was found. This deduced EF-Tu sequence of 415 amino acids aligns throughout its length with all other chloroplast and cyanobacterial EF-Tus except for extensions of three amino acids each at the amino and carboxyl termini. None of the internal insertions/deletions characteristic of other eubacterial, eukaryotic, or mitochondrial EF-Tus are found.

The Coleochaete sequence is, however, unusually divergent. Sequence similarity is <55% overall with all other known EF-Tus (Table 1). In contrast, the lowest level of similarity found among all other chloroplast EF-Tus is 71% (Codium fragile versus Cryptomonas ß). The EF-Tus of Arabidopsis thaliana, a flowering plant, and Thermotoga maritima, a member of probably the earliest diverging group of eubacteria (27), are still 64% identical (Table 1).

The Coleochaete sequence also differs considerably at what are otherwise conserved amino acid positions. Of the 297 positions that are identical in sequence in nearly all cyanobacterial and chloroplast EF-Tus, 105 are altered in Coleochaete (Fig. 2). Of these changes, ~25% involve nonconservative amino acid substitutions (21). The Coleochaete EF-Tu also differs at 22 positions that are nearly universally conserved in all eubacteria (27), archeabacteria, and eukaryotes (Fig. 2).

Mutations at amino acid positions 24, 236, and 394 (Fig. 2), corresponding to positions 20, 222, and 375 of Escherichia coli EF-Tu (24), have been characterized in E. coli. Position 24 lies in a phosphate-binding loop (28), and a glycine substitution at this position in E. coli results in a 10-fold reduction in GDP-binding and a 3-fold reduction in the overall rate of protein synthesis (29). Coleochaete EF-Tu contains the potentially much more disruptive substitution of a phenylalanine at this position (21, 30). Substitutions of either an aspartate at position 236 or a valine or threonine at position 394 in E. coli all result in the production of frameshifting errors in vivo and a decrease in cell growth rate (31). As shown in Fig. 2, both positions are altered in Coleochaete EF-Tu.

The Coleochaete EF-Tu is also unusual in having a net charge of +21, whereas all other EF-Tus are close to neutrality, ranging from +4 (Chlamydomonas, Codium) to -7 (Micrococcus luteus, ref. 32). The predicted extension of the Coleochaete sequence at the amino and carboxyl termini are also unique among EF-Tus. Thus, in a number of respects, the Coleochaete cpDNA sequence encodes by far the most divergent EF-Tu known.
The Coleochaete tufA Is Derived From a Chloroplast tufA. The exceptional divergence of the Coleochaete tufA sequence raises the possibility that the gene was not originally present in the chloroplast but was acquired by it from a foreign source via lateral gene transfer. Although the lack of internal insertions/deletions characteristic of other EF-Tus suggests that the Coleochaete sequence is of chloroplast/cyanobacterial origin, cladistic analysis was used to further investigate the evolutionary origin of the gene.

A single shortest tree constructed from parsimony analysis of 15 EF-Tu sequences places the Coleochaete EF-Tu well within a clade of chloroplast-encoded proteins (Fig. 3). The exceptionally long terminal branch leading to Coleochaete further emphasizes the extensive divergence unique to this lineage. Thus, it seems that the Coleochaete sequence was derived from a green algal chloroplast tufA gene, which has evolved at an accelerated rate in the lineage leading to Coleochaete.

Distribution of tufA in Green Algal ncdNA. The sporadic distribution of tufA in the cpDNA of charophyan algae suggests that the gene may have been established in the nucleus early in the evolution of the lineage. Examination of ncdNAs from three of the six recognized orders of Charophyceae (9) shows strong tufA hybridization in all ncdNAs tested (Fig. 4). The weaker signals seen in the Zygnematales are roughly in proportion to the smaller amounts of DNA loaded in these lanes (Fig. 4). In contrast, no signal was found in the ncdNA of the chlorophyan alga Chlamydomonas (ref. 35; S.L.B., unpublished data).

The strength of the signals and their similarity in intensity among all of the chlorophyan ncdNAs (when normalized relative to the amount of DNA loaded in each lane) suggest that these signals are not due to bacterial DNA contamination, although this possibility cannot be ruled out. However, since the algae used were obtained from separate sources, and grown at different times in various media and under different conditions, the chances of their being contaminated to similar extents seems unlikely.

The presence of multiple tufA-hybridizing bands in the Charophyceae ncdNAs is in contrast to the single tufA found

Table 1. Percent amino acid identity* among EF-Tu sequences

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<th>Sequence</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>8</th>
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<td>48.4</td>
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<td>51.2</td>
<td>51.9</td>
<td>53.2</td>
<td>53.2</td>
<td>50.4</td>
<td>54.4</td>
<td>53.4</td>
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</table>

Sources of additional tufA sequences: Anacystis nidulans (23), Arabidopsis thaliana and Chlamydomonas reinhardtii (5), Codium fragile (M. Kuhsel and J.D.P., unpublished data), Cyanophora paradoxa (M. Kraus and W. Loeffelhardt, personal communication), Cryptomonas (S. Douglas, personal communication), Escherichia coli (24), Euglena gracilis (7), Saccharomyces cerevisiae (mitochondrion, ref. 25), Spirulina platensis (26), and Thermotoga maritima (27).

*Adjusted for missing data.

Fig. 2. Comparison of Coleochaete EF-Tu with EF-Tu consensus sequences. The Coleochaete deduced amino acid sequence is presented in its entirety together with consensus sites for cyanobacteria and chloroplasts (cp-consensus); eubacteria, cyanobacteria, and chloroplasts (eub-consensus); and eubacteria, euarcheota, and archaea bacteria (all-consensus). The cp-consensus includes sites identical for 6 or more of 8 chloroplast and cyanobacterial sequences, the eub-consensus includes sites identical for 11 or more of 13 eubacterial and organellar sequences, and the all-consensus includes sites identical for 23 or more of 27 total sequences. Changes in the Coleochaete sequence relative to the consensus sequences are indicated below as very conservative (+), conservative (−), nonconservative (+), and very nonconservative (++) changes, as defined by Dayhoff et al. (21). Amino acid positions at which Coleochaete differs from all consensus sequences are enclosed in boxes. Sites specifically discussed in the text are denoted with arrowheads.
Fig. 3. The Coleochaete tufA is derived from a green algal chloroplast gene. The tree shown is the single shortest tree found by cladistic analysis of 15 eu bacterial EF-Tu amino acid sequences. The tree has a total length of 1108 steps and a consistency index of 0.66, excluding autapomorphies. Thermotoga maritima, representing the earliest known branch of eu bacteria (27), is used to root the tree. Horizontal branches are drawn to scale with lengths indicated numerically above the branches. tufA sequences in addition to those reported in Table 1 are Arabidopsis thaliana (33), Micrococcus luteus (32), and Thermus thermophilus (34).

in Arabidopsis thaliana (5). However, multiple tufA signals are also found in the ncDNAs of all crucifers examined other than Arabidopsis (Fig. 5), suggesting that tufA exists as a multigene family in these nuclear genomes. Reduction in copy number of multigene families in Arabidopsis, whose nuclear genome is unusually small in size, has been noted previously (36).

DISCUSSION

The tufA gene appears to be present in the cpDNA of all ulvophycean and chlorophycean green algae based on filter hybridization (Fig. 1) and the sequencing of an apparently "normal" chloroplast tufA from a member of each class (Fig. 3: Codium, Ulvophyceae; Chlamydomonas, Chlorophyceae). However, tufA is missing from the cpDNAs of all land plants based on its absence from a bryophyte (8), probably the earliest diverging group of land plants (11), and from all other lineages of vascular plants examined (Fig. 1; refs. 5 and 22). This suggests that tufA was probably transferred to the nucleus after separation of the major green algal classes, since all appear to have at least a vestige of the gene in their cpDNA, but before the emergence of land plants. If this is the case, then the transfer must have occurred in the lineage leading to land plants, the Charophyceae.

The actual distribution of tufA within cpDNA of the Charophyceae—i.e., its apparent presence in Charales but absence from Zygmenlates and land-plants—is not congruent with the phylogeny of green plants as proposed by Bremer et al. (11). Their analysis, based on a broad range of phenotypic characters, places the Coleochaetales and Charales as closer to land plants than the Zygmenlates and the Coleochaetales as the sister group to land plants. This branching pattern is also supported by the apparent shared gain of a cpDNA tRNA intein by Coleochaetales, Charales, and land plants (37) and by the results of 5S ribosomal RNA sequence analysis (38). Possible explanations of the distribution of tufA are (i) transfer of the gene to the nucleus early in the charophycean lineage, with subsequent loss of the cpDNA copy in at least two descendant lineages (land plants and Zygmenlates); (ii) two independent transfers of the gene, one in the Zygmenlates and one in the Coleochaetales/land plant lineage; or (iii) a single transfer in the common ancestor of Zygmenlates and land plants, with these two being sister groups. We favor the first of these explanations for the following reasons.

The hybridization of tufA to ncDNA of Zygmenlates, Charales, and Coleochaetales (Fig. 5) supports transfer of tufA early within the Charophyceae, in a common ancestor to all three orders. This common ancestor would then have retained copies of the gene in the chloroplast and the nucleus through the divergence of each lineage, after which the cpDNA copy followed distinct evolutionary paths in each. In the Zygmenlates, the cpDNA copy would have been lost entirely—in parallel with its loss from the common ancestor of land plants—whereas in the Charales it appears to have been retained. In Coleochaete, the cpDNA copy also seems to have been retained but may no longer encode a functional elongation factor (see below).

One possibility that cannot be ruled out is that the tufA gene transfer occurred even earlier in a common ancestor of the Charophyceae and other classes of green algae. This explanation is most consistent with the phylogenetic analysis (Fig. 3), in which Coleochaete and Arabidopsis do not come out as sister taxa. However, the extreme divergence of the
*Coeleochaete* EF-Tu suggests that it may not be an accurate representative of the charophyte lineage. Parallel, the duplication transfers of the gene in the Zygome-

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