On the Complexity of Chloroplast RNA Metabolism: psaA Trans-splicing Can be Bypassed in Chlamydomonas

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Abstract

In the chloroplast, the posttranscriptional steps of gene expression are remarkably complex. RNA maturation and translation rely on a large cohort of nucleus-encoded proteins that act specifically on a single target transcript or a small set of targets. For example in the chloroplast of Chlamydomonas, trans-splicing of the two split introns of psaA requires at least 14 nucleus-encoded proteins. To investigate the functional significance of this complex trans-splicing pathway, we have introduced an intron-less copy of psaA in the chloroplast genomes of three mutants deficient in trans-splicing and of the wild type. We find that the intron-less psaA gene rescues the mutant phenotypes. The growth of strains with the intron-less psaA is indistinguishable from the wild type under the set of different experimental conditions that were investigated. Thus, the trans-splicing factors do not appear to have any other essential function and trans-splicing of psaA can be bypassed. We discuss how these observations support the hypothesis that complex RNA metabolism in the chloroplast may in part be the result of a nonadaptive evolutionary ratchet. Genetic drift may lead to the accumulation of chloroplast mutations and the recruitment of compensatory nuclear suppressors from large preexisting pools of genes encoding RNA-binding proteins.

Key words: chloroplast, RNA processing, splicing, constructive neutral evolution, Chlamydomonas, synthetic biology.

Introduction

The plastid is an essential organelle of photosynthetic eukaryotes, where vital processes such as photosynthesis and biosynthesis of important metabolites take place. The plastid is governed by two separate genomes, in the nucleus and in the organelle. During the course of evolution, many genes from the ancestral cyanobacterial endosymbiont of the plastid have been transferred to the nucleus or have been lost. Consequently, only about a hundred proteins are encoded in the plastid genome, and most of the plastid proteome is nucleus-encoded and imported.

The genomes of mitochondria, which are endosymbiotic descendants of proteobacteria, have followed a similar evolutionary path. Mitochondrial genomes typically contain even fewer genes, and mitochondria also import the majority of their proteome (Millar et al. 2005). During evolution, new genes have been recruited to participate in organelle biogenesis. Most prominent amongst them are the members of helical-repeat protein families such as the PPR proteins (pentatrico peptide repeat) in vascular plants or the OPR (octatrico peptide repeat) proteins in Chlamydomonas (Schmitz-Linneweber and Small 2008; Eberhard et al. 2011; Rahire et al. 2012; Barkan and Small 2014; Hammani et al. 2014). Some of these families have vastly expanded in different lineages of the Viridiplantae during the coevolution of the organelles and their hosts, so that, for example, the PPR family counts hundreds of members in land plants but less than a dozen in Chlamydomonas (Johnson et al. 2010; Tourasse et al. 2013). Conversely, the OPR protein family contains more than 40 members in Chlamydomonas but only one in higher plants (Rahire et al. 2012; Kleinknecht et al. 2014).

Chloroplast gene expression includes a remarkably complex set of posttranscriptional steps. Polycistronic transcripts are cleaved by endonucleases and trimmed by exonucleases (Barkan and Goldschmidt-Clermont 2000; Eberhard et al. 2008; Stern et al. 2010). The resulting transcripts are protected by 3′-hairpin-loops or by specific proteins that can bend at the 5′- or 3′-end and inhibit the progression of exonucleases (Drager et al. 1998; Nickelsen et al. 1999; Vaistij, Goldschmidt-Clermont, et al. 2000; Pfalz et al. 2009; Barkan 2011; Ghulam et al. 2013; Loizeau et al. 2014). Introns belonging to group I or group II are removed and the exons are spliced, usually “in cis” (from a single precursor) but sometimes “in trans” (from independent transcripts) (Glanz and Kuck 2009; de Longevialle et al. 2010; Stern et al. 2010; Barkan 2011). Furthermore, specific C residues are edited to U in the plastid mRNAs of many plants, although not in liverworts (Marchantiidae) or chlorophyte algae such as Chlamydomonas (Stern et al. 2010). Editing can create a start codon for translation or change the primary sequence of the encoded protein. At each of these
steps, large numbers of nucleus-encoded proteins intervene, most of which are strikingly specific for single transcripts or small subsets of plastid transcripts. Translation of the plastid mRNAs also requires the action of specific proteins that bind the 5′-untranslated region (5′-UTR) (Barkan 2011; Lyska et al. 2013). Specific assembly factors finally intervene in the assembly of the protein subunits, together with their cofactors and pigments, into large multimolecular complexes (Lyska et al. 2013).

Some aspects of chloroplast gene expression reflect the endosymbiotic bacterial origin of the organelle, such as the structure of its 70 S ribosomes or the nature of the plastid-encoded polymerase which is of eubacterial origin. Other traits of plastid gene expression contrast with features of the ancestral prokaryote. For example plastid genes with similar functions, such as those encoding the subunits of the photosystems, are often scattered in different transcription units, in contrast to the organization of the prokaryotic genome based on operons of coregulated genes clustered in the same transcription unit. Operons are apparently not replaced by posttranscriptional regulons in the chloroplast, on the contrary expression of different subunits of the same complex often depends on different nucleus-encoded proteins. This can be illustrated with the example of the photosystem II subunits encoded in the Chlamydomonas chloroplast. Mutations of TBC1 or TBC2 affect the translation of psbC with no apparent effect on translation of the other subunits (Rochaix et al. 1989; Auchincloss et al. 2002).

Likewise, the stability of psbD mRNA is specifically lost in mutants of Nac2, a TPR protein that protects the 5′-end of the transcript, with no apparent effect on the levels of psbA, psbB, or psbC mRNAs (Kuchka et al. 1989). Conversely for MBB1, which is strictly required for mRNA stability of the polycistronic transcription unit comprising both psbB/T and psbH, the mbb1 null mutation has no effect on the mRNAs for other photosystem II (PSII) subunits which are transcribed separately (Monod et al. 1992).

Trans-splicing of psaA in the Chlamydomonas chloroplast is a paradigmatic example of the large number and strict substrate specificities of the nucleus-encoded proteins involved in posttranscriptional steps of gene expression in the organelle. The psaA gene is split in three separate exons scattered in distant loci of the plastid genome (Kück et al. 1987). The exons are transcribed separately and are flanked by sequences that can assemble to form the conserved structures found in other group II introns. A fourth locus, tscA, encodes a short noncoding RNA that is required to complete the structure of intron 1 (Goldschmidt-Clermont et al. 1991). Two steps of splicing "in trans" are required to form the mature psaA mRNA (Choquet et al. 1988). This type of trans-splicing occurs in the plastids and mitochondria of many organisms and in a variety of different genes (Goldschmidt-Clermont and Kuck 2009). For example, psaA is trans-spliced in Chlamydomonas but is an intron-less gene in higher plant plastids, and conversely rps12 is trans-spliced in higher plants but not in Chlamydomonas. Genetic analysis of PSI-deficient Chlamydomonas mutants has revealed that at least 14 nuclear loci are required for trans-splicing of psaA (Goldschmidt-Clermont et al. 1990). Most of these genes are required specifically for splicing of only one of the two split introns. At least seven genes are essential for trans-splicing of the first intron, some of which are necessary for processing of tscA from a polycistronic precursor (Hahn et al. 1998; Rivier 2000; Balczun et al. 2005; Glanz et al. 2012). Five loci are required for trans-splicing of the second intron (Perron et al. 1999), and two are involved in splicing of both the introns (Merendino et al. 2006).

There may be multiple reasons for the remarkable complexity of chloroplast RNA metabolism. One is the need for regulation in response to developmental or environmental changes, in coordination with nuclear gene expression. An example is provided in Chlamydomonas by two proteins required for petA expression, Mca1 and Tca1, whose accumulation is reduced under conditions of nitrogen deprivation when the cytochrome b6f complex is down-regulated (Raynaud et al. 2007; Wei et al. 2014). Another source of complexity may stem from the need to precisely regulate the intricate assembly of the photosynthetic complexes which are composed of many subunits and numerous cofactors and pigments. In a mechanism that is thought to ensure the stoichiometric production of the different chloroplast subunits, negative feedback regulation is exerted by unassembled subunits on the translation of their cognate mRNAs (Choquet and Wollman 2002, 2009). These negative feedback loops, dubbed control by epistasy of synthesis, regulate the assembly of all the photosynthetic complexes in Chlamydomonas and also seem to govern the assembly of Rubisco in tobacco plastids (Wostrack and Sint 2007).

Another source of complexity in RNA metabolism may be “debugging the chloroplast genetic program” (Maier et al. 2008), a possibility that we like to call the “spoiled kid hypothesis”. In this view, mutations accumulate over time in the chloroplast genome, compensated by suppressor mutations in the nucleus, driving an evolutionary ratchet. Such suppressors may be essential for optimal chloroplast function to ensure photosynthesis and other important metabolic pathways of the plastid. Their evolutionary fixation may be facilitated because the plastids are sexually isolated due to their uniparental inheritance. When a plastid mutation severely affects RNA metabolism, the theory of constructive neutral evolution (CNE) proposes that suppression may involve a preexisting nucleus-encoded factor which restores adequate gene expression, and allows a step towards “irremediable complexity” (Gray et al. 2010; Lukes et al. 2011). The whims of the plastid—the “spoiled kid”—are thus compensated by a liberal nuclear genome. In the long term, this might allow the occurrence and persistence of apparently futile steps of gene expression, such as RNA editing or trans-splicing.

To address the complexity of plastid RNA metabolism, Chlamydomonas is an ideal system because it offers a large set of nuclear mutants affected in chloroplast gene expression, together with the possibility to manipulate the chloroplast genome by transformation. We sought to determine whether trans-splicing of psaA in the Chlamydomonas chloroplast, with the numerous nucleus-encoded proteins required in this process, plays an essential function. To address this question, we turned to a simple implementation
of the concepts of synthetic biology. We constructed an intron-less version of the psaA gene, and introduced it by chloroplast transformation in different nuclear mutants deficient in psaA trans-splicing or in the wild type (WT) (fig. 1). We report that the intron-less gene rescued photoautotrophic growth of the mutants, and that the transformed lines had no significant growth phenotype under a variety of laboratory conditions that were tested. These observations support the hypothesis that the complexity of chloroplast RNA metabolism may in part be due to nonadaptive evolutionary processes.

**Results**

**Chlamydomonas Chloroplast Transformants with Intron-less psaA**

We assembled a vector for chloroplast transformation (psaA-Δi) that contains an intron-less version of the psaA gene (supplementary fig. S1A, Supplementary Material online). In this vector exons 1, 2, and 3 of psaA were fused and placed under the control of the psaA-ex1 promoter/5’-UTR. The flanking sequences in the vector were chosen such that after chloroplast transformation and homologous recombination, the intron-less gene would replace the resident psaA exon3. A selectable marker (aadA), that confers resistance to spectinomycin, was placed downstream of the psaA-ex3 sequence (Goldschmidt-Clermont 1991). This vector was used to transform Chlamydomonas WT cells, as well as the raa1, raa2, and raa3 mutants, representing the three classes of trans-splicing mutants (Choquet et al. 1988; Goldschmidt-Clermont et al. 1990). The raa1 mutant is deficient in processing of tscA from a polycistronic precursor and in trans-splicing of both the split introns (Merendino et al. 2006). The raa2 mutant is defective in trans-splicing of exons 2 and 3, whereas raa3 is affected in trans-splicing of exons 1 and 2 (Perron et al. 1999; Rivier 2000). The transformants were selected on spectinomycin-containing medium and subcultured until homoplasmy was achieved. This was assessed by polymerase chain reaction (PCR) on genomic DNA (supplementary fig. S1B, Supplementary Material online), which showed the presence of the intron-less psaA gene and the absence of any detectable parental genome.

**Chlamydomonas** is haploid in the vegetative state, and mutants deficient in photosynthesis may accumulate additional secondary mutations upon prolonged cultivation (Spreitzer and Ogren 1983; Girard-Bascou et al. 1992). The psaA trans-splicing mutants are PSI-deficient and hence nonphotosynthetic, so that any secondary mutations that would affect photosynthesis might have gone unnoticed because they would not have any further growth phenotype.

**FIG. 1.** Chloroplast bypass or nuclear rescue of trans-splicing mutants. The chloroplast is shown on the right (green) with a schematic representation of the psaA trans-splicing pathway. The three exons of psaA (e1, e2, and e3) and the noncoding tscA RNA map to four separate loci on the chloroplast genome (not to scale). They are transcribed separately and the four RNAs assemble to form the conserved structures of two group II introns, which are spliced “in trans” to produce the mature psaA mRNA. In the nucleus (blue) at least 14 loci (only RAA1 is shown here as an example) encode proteins that are imported in the chloroplast where they are required for trans-splicing of psaA. A mutation in any of these genes (red crosses and red dotted arrow) prevents the maturation of psaA mRNA. To try to bypass the nuclear mutation and the trans-splicing pathway (bypass, shown in brown on the right), an intron-less copy of psaA (psaA-Δi) is introduced at the exon3 locus by chloroplast transformation. Nearly-isogenic lines are obtained by rescuing the nuclear mutation with a genomic fragment containing a WT copy of the mutant gene (rescue, shown in pink on the left).
(Girard-Bascou et al. 1992). PSI-deficient mutants are also sensitive to photodamage, and may acquire secondary mutations that relieve photosensitivity (Spreitzer and Ogren 1983). Such mutations would change the properties of the mutant host strains that were transformed with the intron-less psaA gene. To try to circumvent this problem and allow a valid comparison of the growth properties of the transgenic lines, we also derived nearly isogenic lines by transforming the three splicing mutants with the respective WT genes (RAA1, RAA2, or RAA3) inserted in the nucleus (fig. 1). The nuclear transformants were selected for photoautotrophic growth on minimal medium. Several lines were isolated for each mutant to monitor possible variations due to position effects or copy number of the transformed nuclear gene.

**Trans-splicing Mutants Rescued with Intron-less psaA Have Normal Growth Phenotypes**

For each mutant, we compared the growth properties of at least three lines rescued with the WT copy of the nuclear gene with those of two lines rescued with the intron-less psaA-Δi gene in the chloroplast (fig. 2A, supplementary figs. S2 and S3, Supplementary Material online). All the transformants were capable of photoautotrophic growth on minimal medium and were not sensitive to higher light intensities, in contrast to the parental splicing mutants which do not grow on minimal medium and are sensitive to light when grown on acetate-containing medium. This suggests that expression of PsA and assembly of PSI are rescued in the three trans-splicing mutants carrying the intron-less copy of psaA. This was confirmed by immunoblotting, which showed that they all accumulated PsA protein in amounts similar to the WT (fig. 3A and supplementary fig. S4, Supplementary Material online). RNA blot hybridization further showed that the intron-less psaA mRNA accumulates in these strains to levels similar to the trans-spliced mRNA in the corresponding strains rescued with the respective WT gene (fig. 3B). The accumulation of psaA mRNA was similar to the WT, but did vary in the different genetic backgrounds. This confirms the importance of comparing nearly isogenic lines, the mutant strains bypassed with the intron-less psaA versus the controls rescued with the WT gene (fig. 1).

As might be expected from these results which showed that PsA was present at normal levels, no changes were detectable in the growth rates of liquid cultures of the three trans-splicing mutants bypassed with the intron-less psaA, as compared with the WT or the controls rescued with the respective nuclear genes (fig. 2B and supplementary fig. S3, Supplementary Material online). These results show that trans-splicing can be bypassed and does not have an essential function. They also suggest that the trans-splicing factors do not have essential roles other than in psaA maturation under normal growth conditions.

**Acclimation to Iron Limitation**

In conditions of mixotrophic growth (in acetate-containing medium in the light), iron deprivation causes dramatic changes in the photosynthetic electron transfer chain (Moseley et al. 2002; Terauchi et al. 2010; Hohner et al. 2013). The relative amount of PSI strongly decreases and the light-harvesting antennae undergo a reorganization that involves proteolytic processing of Lhca3 (Naumann et al. 2005). The effect of iron limitation on PSI was readily confirmed when *Chlamydomonas* cells were transferred to medium lacking iron and the PsA subunit was monitored by immunoblotting (fig. 4). Within 48 h the amount of PsA dropped to low levels, and when iron was restored, PsA rapidly reaccumulated to normal amounts. We did not observe any significant difference in the response to iron deprivation or in the recovery after iron restoration, for the mutant lines with the intron-less psaA-Δi compared with the WT, or compared with the mutant lines rescued with the respective nuclear genes (fig. 4 and supplementary fig. S5, Supplementary Material online). Thus, trans-splicing does not appear to play an important role in the acclimation of the *Chlamydomonas* cells to iron limitation or in the recovery upon iron supplementation.

**Growth under Competition**

The mutant lines rescued with the intron-less psaA gene seemed to grow at normal rates. However, we considered the possibility that trans-splicing might offer a growth advantage—or disadvantage—that would only be apparent in conditions of competition. To investigate this possibility a transgenic line carrying the intron-less psaA-Δi gene in a WT nuclear background was compared with a WT control that expresses psaA by trans-splicing. A WT genetic background was chosen for this sensitive assay to avoid any unwanted effect of cryptic mutations that may have appeared in the genetic background of the splicing mutants. The cultures were grown in minimal medium with diurnal light/dark cycles, conditions where photosynthesis and light acclimation are expected to play an important role. The cultures were inoculated with an equal mixture of the two genotypes, and they were serially subcultured every week. During the course of the experiments, the proportions of the two genotypes were determined by plating on solid media in the presence or absence of spectinomycin. Although both genotypes grow on normal medium, only the strain with the intron-less psaA gene and the linked *aadA* selection marker forms colonies on spectinomycin. We did not observe any apparent selective advantage for the WT line, where trans-splicing is operating, compared with the intron-less genotype (fig. 2C). As a control for any possible effect of the antibiotic resistance marker inserted downstream of psaA ex3, we also obtained transformants with the *aadA* cassette inserted at the same site but lacking the intron-less psaA gene (*aadA*-control). When grown in competition with the WT, this control line did not show any significant difference. These data indicate that under the conditions tested, trans-splicing does not confer a significant growth advantage or disadvantage to the *Chlamydomonas* cells.
Acclimation to Higher Temperature or to Oxygen Limitation

At high temperatures, trans-splicing of psaA is retarded (Choquet et al. 1988). To investigate whether psaA trans-splicing could have an effect on acclimation to elevated temperatures, three Chlamydomonas lines were compared, the

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WT, a line carrying the intron-less psaA-Δi in the WT background and a line with only the aadA marker as a control. Serial dilutions of cultures grown at 25 °C were spotted on plates containing acetate-containing medium or minimal medium, and transferred to 32 °C. No differences in the plating efficiency or growth of the two lines compared with the WT could be observed (supplementary fig. S6, Supplementary Material online), indicating that trans-splicing does not significantly influence the acclimation to the higher temperature or growth at the elevated temperature.

Acclimation to growth under low oxygen conditions was also tested in a similar set of experiments. The two lines and the WT were spotted on acetate medium or on minimal medium, and then sealed in anaerobiosis bags (supplementary fig. S6, Supplementary Material online). No differences in their plating efficiency or growth compared with the WT were observed.

**Discussion**

The *Trans*-Splicing Pathway of *psaA* Maturation Can be Bypassed

In the chloroplast genome of *Chlamydomonas* cells, the *psaA* gene contains two fragmented introns that are spliced “in trans.” By replacing *trans*-spliced *psaA* with an intron-less version in the chloroplast genome, we were able to rescue the photosynthetic deficiency of three mutants defective in *psaA* trans-splicing. The three mutants are representative of the three classes of phenotypes, blocked in splicing of either intron 1 (*raa*3, class C), intron 2 (*raa*2, class A), or both the introns (*raa*1, class B). Unlike the parental strains which are PSI-deficient and cannot grow on minimal medium, the three mutants rescued with the intron-less *psaA* gene showed normal photoautotrophic growth. These results indicate that these *trans*-splicing factors do not have any other roles that would be essential for the viability of the *Chlamydomonas* cells under the conditions that were tested. In preliminary experiments, we previously observed that several other nuclear mutants defective in *trans*-splicing could also be rescued with the intron-less *psaA* gene (Rivier 2000). Why the *psaA* introns have not been lost during evolution as could be engineered in the intron-less *psaA* strain is a matter of speculation, it is possible that their loss could be hindered by the split organization of the *trans*-spliced introns.

Immunoblot analysis showed that the rescued strains accumulate WT levels of PsaA protein. To account for any effect of additional mutations that might have appeared in the background of the *trans*-splicing mutants, we created a set of nearly isogenic control lines where *psaA* trans-splicing was restored by transformation with the corresponding WT nuclear gene. The importance of using these controls was apparent when the amounts of *psaA* mRNA were compared by RNA blot hybridization. The levels of the mature mRNA were similar in the nearly isogenic strains rescued with the intron-less *psaA* gene compared with the strains rescued with the respective WT nuclear gene; however, the amount seemed to vary in the different genetic backgrounds of the three original mutants. It thus appears that the regulation of *psaA* accumulation at the translational or posttranslational levels must compensate for these small differences in mRNA abundance, as was previously observed for many chloroplast mRNAs in *Chlamydomonas* (Eberhard et al. 2002). As *psaA* mRNA is apparently not limiting for PsaA accumulation, it should probably not be expected that the bypass of *trans*-splicing confers a growth advantage. Our results also indicate that the *psaA* trans-splicing pathway does not play an essential role because it can be bypassed by providing an intron-less version of the gene. This is particularly striking because *psaA* trans-splicing involves four different chloroplast transcripts and a least 14 nucleus-encoded proteins in *Chlamydomonas* (Choquet et al. 1988; Goldschmidt-Clermont et al. 1990, 1991). For splicing “in cis,” a similar observation was made for the introns in the *psbA* and *rnl* genes of *Chlamydomonas*, and for the two introns of *ycf3* in the tobacco chloroplast, which could be deleted and replaced with an intron-less version of the respective genes without any apparent effect on the phenotype (Johanningmeier and Heiss 1993; Minagawa and Crofts 1994; Holloway and Herrin 1998; Petersen et al. 2011). This is also reminiscent of the introns in the mitochondria of the yeast *Saccharomyces cerevisiae*, which can be removed without apparent consequences on cell viability (Seraphin et al. 1987). These observations fit with the hypothesis that group I and group II introns originated as selfish transposable retroelements, capable of inserting in the genome with little phenotypic consequence as they splice out precisely from the RNA product (Lambowitz and Zimmerly 2004). That splicing of these introns in the chloroplast requires nucleus-encoded factors implies that the ancestral self-splicing ribozymes have degenerated and become dependent on gene products of their hosts (Barkan 2011). In the case of *psaA* trans-splicing, these nucleus-encoded factors are particularly numerous as at least 14 have been identified originally and it is not known whether those that were identified more recently with biochemical or genetic approaches are included in this set or represent additional components (Goldschmidt-Clermont et al. 1990; Glanz and Kuck 2009; Glanz et al. 2012; Jacobs et al. 2013).
The question thus arises, whether trans-splicing has evolved to serve functions that might be beneficial to the chloroplast or the host cell, in particular for the regulation of gene expression and the assembly of PSI. We investigated this possibility by testing the potential role of the trans-splicing pathway in the acclimation of Chlamydomonas to various environmental conditions. As psaA encodes a major subunit of PSI, light intensity is an important external factor, in particular because PSI mutants are highly sensitive to light even though they are grown auxotrophically on medium containing acetate. No difference was observed in the growth of the lines with the intron-less psaA compared with the controls under different light intensities, in the presence or absence of acetate. To test this with more sensitivity, cells with the intron-less psaA were grown in competition with WT cells for 4 weeks, but no significant competitive advantage or disadvantage was observed. The lines with intron-less psaA also grew like the controls upon a shift to elevated temperatures or to anoxic conditions. Under iron limitation in mixotrophic cultures, the abundance of PSI centers is strongly down-regulated but quickly restored when iron is resupplied (Moseley et al. 2002; Hohner et al. 2013). Similar to the controls, the lines with intron-less psaA down-regulated the amount of PSI in response to iron limitation and quickly restored PSI levels when it was again available. The environmental conditions that we examined constitute a diverse spectrum and we did not identify any conditions where bypassing of psaA trans-splicing had detectable consequences for growth. It cannot be ruled that under another stress condition, or under a combination of different stresses, a phenotype might still become apparent. We nevertheless conclude that the psaA trans-splicing pathway is largely dispensable.

Evolution of Complex RNA Metabolism in the Chloroplast

Posttranscriptional RNA editing provides another striking example of complex but apparently futile RNA metabolism. Numerous C residues in plant chloroplast and mitochondrial transcripts are converted to U residues, a process which requires a large cohort of site-specific editing factors (Shikanai and Fujii 2013). In principle, the need for editing could be circumvented by mutating the C residues to U at the DNA level. There is only very limited evidence that editing plays any regulatory role (Peeters and Hanson 2002; Stern et al. 2010; Shikanai and Fujii 2013). Cytbrids with the nuclear genome of Atropa belladonna (deadly nightshade) and the plastome of Nicotiana tabacum (tobacco) have an albino phenotype. This was ascribed to defective editing of a site in the plastid atpA mRNA, which is specific to the tobacco chloroplast and for which an editing factor is missing in the nightshade nuclear genome (Schmitz-Linneweber et al. 2005). A mutation of C-to-U in the tobacco chloroplast DNA that bypassed editing at this site had no apparent phenotypic consequence in tobacco. Furthermore, the mutation rescued the albino phenotype in the Atropa cybrids. These observations can be interpreted in the context of the coevolution of the plastid and the nucleus to suggest that when the plastid is placed in a different nuclear background, chloroplast mutations may be revealed due to the lack of the corresponding nuclear suppressors. The role of nucleus-chloroplast genome incompatibility in speciation is well recognized (Greiner et al. 2011).

If the complexity of chloroplast RNA maturation does not reflect essential functions, the possibility has to be considered that it has evolved as consequence of an evolutionary ratchet whereby mutations in the chloroplast are suppressed by mutations in the nuclear genome. In this view, the nucleus-encoded factors are required for debugging the chloroplast genetic program (Maier et al. 2008). Such an evolutionary drift could be favored by the sexual isolation of the plastids, due to their uniparental mode of inheritance in many plant species. This evolutionary pathway requires that the mutations in the organelle have low fitness cost to the organism when they arise, otherwise the mutants would be lost from the population. One possibility is that the individual chloroplast mutations could have rather minor consequences, but this is contradicted by the strong phenotypes of some mutations that cause the loss of nucleus-encoded trans-splicing or editing factors. The theory of CNE provides a more plausible alternative, namely that the nuclear suppressors may actually preexist the mutation that they eventually suppress and are thus easy to recruit (Covello and Gray 1993; Lynch 2007; Gray et al. 2010; Lukes et al. 2011; Lynch et al. 2011). The CNE theory explains how this type of transgenomic suppression mechanism could be part of a “drive toward irremediable complexity”. A pool of potential preexisting suppressors for chloroplast mutations could be constituted by proteins that have functions in RNA metabolism and can be co-opted for the new task. An early example for the CNE theory was provided by the mitochondrial group I introns of Neurospora that require a tyrosyl tRNA synthetase as splicing factor (Akins and Lambowitz 1987; Gray et al. 2010). In Chlamydomonas, a typical example is the Raa2 protein which is a member of the family of pseudouridine synthases but does not require this enzymatic activity to play its role in psaA trans-splicing (Perron et al. 1999). Likewise, the maize chloroplast splicing factor CRS2 shows strong sequence similarity to tRNA hydrolases, and RNC1 belongs to the family of RNase III but has lost this enzymatic activity (Jenkins and Barkan 2001; Watkins et al. 2007).

Another large pool of preexisting genetic suppressors of chloroplast mutations could be constituted by the numerous members of the helical repeat families of RNA-binding proteins, such as the PPR proteins (Tourasse et al. 2013; Barkan and Small 2014), TPR/HAT proteins (tetratricopeptide repeat/half a TPR) (Boudreau et al. 2000; Vaistij, Boudreau, et al. 2000; Hammami et al. 2014) or OPR proteins (Eberhard et al. 2011; Rahire et al. 2012). The paradigmatic PPR family in higher plants contains 450–600 members with characteristic degenerate repeats of a 35 amino-acid sequence. Each repeat module binds one base of the RNA target through a few amino-acid residues which play a critical role in sequence-specific recognition (Barkan et al. 2012; Ke et al. 2013; Yagi et al. 2013; Yin et al. 2013). Members of the PPR protein family are known to act in the chloroplast as editing factors, as facilitators of intron splicing, as blockers of exonuclease
progression or as translation factors (Barkan 2011; Shikanai and Fujii 2013; Barkan and Small 2014). Such a wide pool could facilitate the evolutionary recruitment of RNA-binding proteins capable of suppressing new mutations that arise in chloroplast RNA. Their specificity could further evolve through changes in the few critical residues that govern sequence recognition. Similarly, the psaA trans-splicing factor Raa1 belongs to the family of OPR proteins that are predicted to form a structural scaffold similar to the PPR repeats and to bind RNA specifically (Merendino et al. 2006; Rahire et al. 2012).

In conclusion, our results show that the complex psaA trans-splicing pathway, with its numerous nucleus-encoded factors, can be bypassed in the chloroplast of Chlamydomonas under a variety of growth conditions that were tested. These results bring new experimental support for the theory of CNE to explain certain aspects of the complexity of chloroplast RNA metabolism.

Materials and Methods

Chlamydomonas Cultures

The Chlamydomonas reinhardtii WT and the mutant strains L137H (raa1) (Merendino et al. 2006), L136F (raa2) (Perron et al. 1999), and M18 (raa3) (Rivier et al. 2001) were grown in Tris-acetate-phosphate medium (TAP) or in high salt minimal medium (HSM) (Rochaix et al. 1988) to densities of 1–2 × 10^6 cells/ml in the dark or under fluorescent lights (60 μE m⁻² s⁻¹) at 25 °C.

For growth tests, 10 μl of cell culture at 2 × 10^6 cells/ml were spotted with 1:10 serial dilutions on agar plates and grown under 60 μE m⁻² s⁻¹ light as indicated. To test the effect of a shift to anaerobiosis, immediately after spotting the plates were inserted in Anaerocult P plastic bags containing Anaerocult C mini reagent (Merck).

For growth curves, cultures in stationary phase were diluted 50-fold in fresh medium and cell density was monitored by counting using a Neubauer ultraplane hemacytometer or by measuring optical density at 750 nm (with OD 750 0.075 corresponding to 0.5 × 10^6 cells ml⁻¹ in our conditions).

For competition experiments, the cells were grown in HSM with 12-h-light/12-h-dark cycles (60 μE m⁻² s⁻¹). Two independent cultures were grown in parallel in a volume of 20 ml, in one culture 0.5 × 10^7 WT cells were mixed to 0.5 × 10^7 WT (psaA-Δi) cells, in the other 0.5 × 10^7 WT cells were mixed to 0.5 × 10^7 WT (psaA-Δi) cells. At weekly intervals 200 μl aliquots of a 250-fold dilution of these mixed cultures were spotted on TAP plates and on TAP plates supplemented with 100 μg/ml spectinomycin. At the same times the cultures were diluted into fresh HSM medium at a density of 0.5 × 10^6 cells ml⁻¹.

Iron Deprivation

For growth under iron limitation, glassware was treated with 50 mM ethylenediaminetetraacetic acid (EDTA) pH 8 and washed with milliQ water. TAP-Fe was prepared with trace elements lacking Fe. For supplementation, Fe was added at 18 μM from a stock solution of 50 mM FeSO₄ chelated with 134 mM EDTA.

Chlamydomonas cells from cultures at 2 × 10^6 cells/ml were harvested by centrifugation and resuspended in TAP-Fe at 0.5 × 10^6 cells/ml. After 24 h and 48 h, cells were diluted in TAP-Fe to 0.5 × 10^6 cells/ml. After the dilution at 48 h, Fe was supplemented to 18 μM. Samples for immunoblot analyses were taken from cultures at 2 × 10^6 cells/ml at different timepoints.

Transformation Vectors

The plasmid psaA-Δi (pOS200, Supplementary fig. 1, Supplementary Material online) was assembled by first cloning the HindIII fragment containing psaA exon1 (483 bp) in Bluescript SK(+) to yield pEx1. The HincII fragment from pKR150 containing part of exon3 (Redding et al. 1998) was cloned in pEx1 digested with HindIII and Nael, yielding pEx1Ex3. A cDNA of psaA obtained by RT-PCR was digested with Scal (in ex1) and Ncol (in ex3) and inserted in pEx1Ex3 digested with the same enzymes to yield pEx123. The EcoRV-BstXI fragment from pEx123 (promoter of ex1-ex1:ex2:ex3 fragment) was inserted in pKR150 (Redding et al. 1998) digested with Affil (blunted) and BstXI.

The adaA-control plasmid (pOS200Δ) is derived from psaA-Δi (pOS200) by digestion with Stul and Alel and religation, removing the promoter of exon 1 as well as exons 1 and 2.

The cosmid used to complement the mutants are from an ordered cosmid library (Zhang et al. 1994; Perron et al. 1999; Rivier et al. 2001; Merendino et al. 2006).

Transformation

Chlamydomonas cells were transformed by Helium-gun bombardment with cosmids containing nuclear genomic DNA or chloroplast transformation vectors psaA-Δi (pOS200) or pOS200Δ (see above). Nuclear transformants were selected on HSM plates for photoautotrophic growth; chloroplast transformants were selected on TAP plates supplemented with 100 μg/ml spectinomycin (pOS200 and pOS200Δ). They were grown under illumination (60 μE m⁻² s⁻¹), subcultured several times to obtain homoplasmic strains and genotyped by PCR as follows.

Genotyping

Homoplasmicity of the chloroplast insertions was determined by PCR (Supplementary figs. S1 and S2, Supplementary Material online) on total DNA extracts (Cao et al. 2009) using the following protocol: 5 min at 95 °C/40 cycles (1 min at 95 °C, 1 min at 54–60 °C [depending on the Tm of the oligos], 1 min at 72 °C)/40 cycles.

The absence of the parental genome (where psaA exon3 is flanked by the 3′-part of split intron 2 rather than exon2) is revealed with the primers "psaA ex3 5′-UTR for" (ggcggaggctggaaattaag) and “psaA ex3 rev” (ggcggaggctggaaattaag) (Tm 60 °C) that give a PCR1 product of 290 bp with WT DNA and 850 bp with transformed DNA. For psaA-Δi (pOS200) transformants, the presence of the intron-less psaA
gene is revealed with the primers “psaAfor1” (atgacattag-tactccagagcctg) and “psaArev1” (ccacagtcgctacttcacctc) (Tm 54 °C) that give a PCR product of 1,000 bp (PCR2) consisting of exon 1, exon 2, and a part of exon 3.

For aadA-control pOS200 Δ transformants, the primers “Prom cyt rev” (gcatcagttttatcgtacactc) and “exon3 for” (Tm54 °C) (catctggtaagtttcaaaaggtc) give a PCR product of 476 bp in the presence of the WT genome. The primers “pOS200 for2” (gcataacgtaaatcctgcaaag) and “aadA Prom seq” (ggagacgcaagtttcaaaaggt) give a PCR product (Tm 58 °C) of 1,127 bp in the presence of transformed genome.

RNA Analysis
Chlamydomonas cells from 50-ml cultures (2 × 10^6 cells/ ml) were harvested by centrifugation and washed once with 10 ml of 20 mM Tris pH 7.9, and the pellets were stored at −70 °C. For RNA extraction, 5 ml of Tri Reagent (Sigma-Aldrich, Chemie, Buchs, Switzerland) and 500 µl of glass beads (G8772; Sigma-Aldrich) were added to the frozen pellets, and the suspensions were vigorously agitated for 2 min. The homogenates were split into 5 Phase Lock Gel tubes (5 PRIME, Hamburg, Germany). 400 µl of chloroform was added followed by thorough mixing. After 10-min centrifugation at 14,000 × g at 4 °C, 200 µl of chloroform was added followed by thorough mixing. After 10 min centrifugation at 14,000 × g at 4 °C, the RNA was precipitated from the aqueous phase with 1 ml isopropanol, collected by centrifugation and washed with 70% ethanol. Total RNA (5 µg) was analysed by formaldehyde agarose gel electrophoresis, transfer to Nylon membranes and hybridization using probes labeled with 32P-dATP by random priming (Ausubel et al. 1998).

Protein Analysis
Chlamydomonas cells (5 ml, 2 × 10^6 cells/ ml) were collected by centrifugation, washed with water, and resuspended in lysis buffer (100 mM Tris–HCl pH 6.8, 4% SDS, 20 mM EDTA, protease inhibitor cocktail [Sigma-Aldrich]). After 30 min at room temperature, cell debris were removed by centrifugation, and total proteins were analysed by SDS–PAGE (12% acrylamide) and immunoblotting. Labeling of the membranes with antisera against PsaA (subunit PSI), CF1 (subcomplex of chloroplast ATP synthase), D1 (PsBA subunit of PSI), or DnaJ was carried out at room temperature in 1 × Tris-Buffered Saline (50 mM Tris–HCl pH 7.6, 150 mM NaCl), 0.1% Tween 20 and 5% nonfat powder milk. After washing the membranes, the antibodies were revealed with a peroxidase-linked secondary antibody (Promega, Amriswil, Switzerland) and visualized by enhanced chemiluminescence.

Supplementary Material
Supplementary figures S1–S6 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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