

Identification of an RNA–protein complex involved in chloroplast group II intron *trans*-splicing in *Chlamydomonas reinhardtii*

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In *Chlamydomonas reinhardtii*, the *psaA* mRNA is assembled by a process involving *trans*-splicing of separate transcripts, encoded at three separate loci of the chloroplast genome. At least 14 nuclear loci and one chloroplast gene, *tscA*, are needed for this process. We have cloned *Raa3*, the first nuclear gene implicated in the splicing of intron 1. The predicted sequence of *Raa3* consists of 1783 amino acids and shares a small region of homology with pyridoxamine 5'-phosphate oxidases. *Raa3* is present in the soluble fraction of the chloroplast and is part of a large 1700 kDa complex, which also contains *tscA* RNA and the first *psaA* exon transcript. These partners, in association with other factors, form a chloroplast RNP particle that is required for the splicing of the first intron of *psaA* and which may be the counterpart of eukaryotic snRNPs involved in nuclear splicing.

Keywords: *Chlamydomonas reinhardtii*/chloroplast/group II intron/RNA–protein complex/*trans*-splicing

Introduction

Group II introns have been found mainly in organellar genes from fungi, algae and higher plants, and also in eubacterial genes. Although many of these group II introns are able to catalyse their excision and exon splicing *in vitro*, proteins are required *in vivo* for splicing (Waldherr *et al.*, 1993; Guo *et al.*, 1997; Jenkins *et al.*, 1997; Vogel *et al.*, 1997; Hollander and Kück, 1999). These introns can be folded in a characteristic secondary structure with a central core and six protruding stem–loop domains, which act *in cis* as catalytic sites for splicing. The splicing mechanism of group II introns involves two consecutive *trans*-esterification reactions that result in the formation of an intermediate intron lariat–exon and, finally, in the splicing of the two exons (Michel and Ferat, 1995). The same reactions also occur during splicing of nuclear introns, except that they are catalysed by *trans*-acting snRNPs and splicing factors. Structural similarities exist between some of the snRNPs and the *cis*-acting catalytic domains of group II introns. It has therefore been proposed that group II introns represent evolutionary precursors of nuclear introns (Cech, 1986; Sharp, 1991; Weiner, 1993).

In the green alga *Chlamydomonas reinhardtii*, the chloroplast *psaA* gene, encoding one of the two major polypeptides of the photosystem I (PSI) reaction centre,

consists of three exons that are separated on the circular genome (Kück *et al.*, 1987). Each exon is flanked by group II intron sequences. Maturation of *psaA* mRNA depends on two separate *trans*-splicing reactions (Choquet *et al.*, 1988). Splicing of the exon 1 and exon 2 transcripts also requires *tscA* RNA encoded by the chloroplast genome. This RNA is part of the structure of the group II intron, which is, in this case at least, tripartite (Goldschmidt-Clermont *et al.*, 1991).

At least 14 nuclear mutants affecting *trans*-splicing of *psaA* were isolated (Girard *et al.*, 1980; Goldschmidt-Clermont *et al.*, 1990). These can be grouped in three classes depending on which intron fails to be spliced. Mutants of class A are defective in the splicing of exons 2 and 3, mutants of class C are defective in the splicing of exons 1 and 2, and mutants of class B are affected in the splicing of all three exons. Furthermore, one class B mutant was reported to be deficient in both *tscA* RNA maturation and in the splicing of the second intron (Hahn *et al.*, 1998). Recently, the gene affected in one of the class A mutants was cloned and shown to encode a protein related to pseudouridine synthases (Perron *et al.*, 1999). To gain further insights into the first *psaA* *trans*-splicing reaction, we have rescued a class C mutant by genomic complementation. The gene, called *Raa3*, for RNA maturation of *psaA*, encodes a predicted polypeptide of 176 kDa that does not share significant sequence identity with any other known protein, except for a small domain present in pyridoxamine 5'-phosphate oxidase (PDX). The protein is enriched in a soluble fraction of the chloroplast and is part of a 1700 kDa complex that also includes the chloroplast *tscA* RNA, the *psaA* exon 1 transcript and other unknown proteins.

Results

Cloning of the *Raa3* gene

M18 is a nuclear mutant lacking PSI activity that has been shown to be deficient in the *trans*-splicing of the chloroplast *psaA* precursor RNAs (Girard *et al.*, 1980). The mutant strain is unable to grow photoautotrophically and is highly photosensitive, in particular it is unable to grow on acetate-containing medium at 60 $\mu\text{E}/\text{m}^2\text{s}$. Based on its *psaA* RNA phenotype, M18 is a class C mutant affected in the *trans*-splicing of exons 1 and 2, and lacks detectable levels of PsaA protein.

The nuclear gene *Raa3* affected in M18 was isolated by genomic rescue, using an indexed cosmid library for transformation (see Materials and methods for details). A single cosmid was isolated that complements the mutant phenotype of M18 with high efficiency (200 colonies per 3×10^7 cells). The transformed cells have wild-type fluorescence transients, grow on minimal medium under all light conditions and accumulate mature *psaA* mRNA

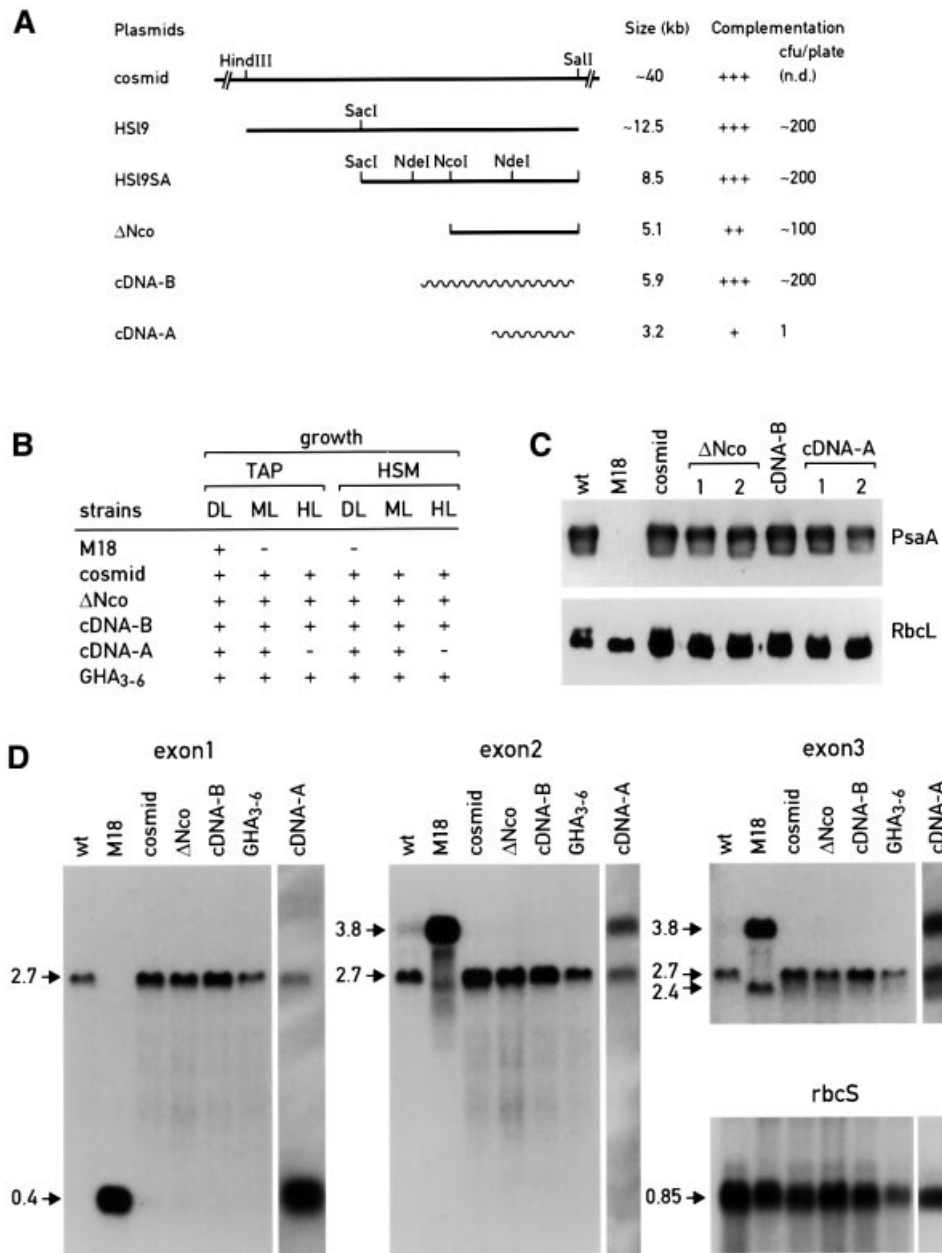


Fig. 1. Genomic complementation of M18. (A) Mapping of *Raa3*. Cosmid represents the entire cosmid of ~40 kb isolated from the indexed cosmid library that is able to rescue the M18 mutant. HSI9 is a *HindIII*–*SalI* subfragment of the cosmid. HSI9SA is the longer *SacI* subfragment of HSI9. Δ Nco is an *NcoI* subclone of HSI9SA. cDNAs-B and -A are two independently isolated cDNAs (see Materials and methods). (B) Photoautotrophic growth and photosensitivity of M18 and the rescued strains obtained by transformation of M18 with the constructs described in (A). GHA3-6 is the M18 strain transformed with a HSI9SA fragment containing the entire sequence of *Raa3* and an HA tag at the 3'-terminal end of the coding sequence. + and – indicate normal and absent growth, respectively. DL, dim light (6 μ E/m²s); ML, medium light (60 μ E/m²s); HL, high light (600 μ E/m²s); TAP, Tris–acetate–phosphate medium; HSM, high salt minimal medium. (C) Immunoblot analysis. Total cell proteins (10 μ g) from the indicated strains were separated by SDS–PAGE, blotted and decorated with PsaA and RbcL antibodies. (D) RNA blot analysis. The blots were hybridized with the three different *psaA* exon probes and *RbcS* as a loading control. The sizes of the major transcripts are indicated: the 2.7, 3.8, 2.4 and 0.4 kb bands correspond to the mature *psaA* mRNA, the exon 2–exon 3 precursor (containing the 3' end of intron 1), the exon 3 precursor and the exon 1 precursor, respectively.

and PsaA protein (Figure 1). The cosmid containing nearly 40 kb of genomic DNA was digested with *HindIII*, *SacI*, *NcoI* or *SalI*. The fragments produced were still able to rescue M18, indicating that these enzymes cut outside of the *Raa3* gene. A subcloned 12.5 kb *SalI*–*HindIII* fragment, HSI9, was able to complement M18. This fragment was trimmed further by digestion with *SacI* or *NcoI*. The resulting plasmids, HSI9SA and Δ Nco, were still able to rescue M18. The latter fragment was the

smallest genomic fragment identified able to restore the wild-type phenotype (Figure 1A).

A cDNA of 3.3 kb was isolated by screening a cDNA library with a subfragment of Δ Nco. When cloned into Bluescript SK, this cDNA (cDNA-A) was able to complement the M18 mutant. However, this occurred only at a low frequency (<1 transformant per 3×10^7 cells) and the transformants were sensitive to high light (600 μ E/m²s; Figure 1B). Since the sequence of cDNA-A revealed an

open reading frame (ORF) that extended beyond its 5' end, a second screen was performed with a probe corresponding to the first 300 bp of cDNA-A to obtain a full-length cDNA. A new cDNA of 5.9 kb was isolated (cDNA-B) that fully restores the wild-type phenotype upon transformation of M18. Immunoblot analysis revealed that in all cases the rescued strains accumulate near wild-type levels of PsaA protein, even in the case of the transformants obtained with the incomplete cDNA-A (Figure 1C). Similarly, RNA blot analysis showed that all the transformants obtained accumulate the mature *psaA* RNA (Figure 1D). However, the transformant obtained with cDNA-A also accumulates the 0.4 kb exon 1 and the 3.8 kb exon 2–exon 3 precursor RNAs, indicating that the first *psaA trans*-splicing reaction is inefficient.

Southern blot analysis using >10 restriction enzymes revealed no difference between the wild-type and the mutant DNA restriction patterns when the cDNA was used as hybridization probe (data not shown). To test whether the mutant phenotype was linked to the region encoding the cDNA, M18 was crossed with the polymorphic strain S1D2, and the DNA of the progeny was examined. Analysis of four tetrads revealed co-segregation between the mutant or wild-type phenotype and the restriction fragment polymorphism of the parental strains (data not shown). This suggests that the cDNA corresponds to the gene affected in M18 or that the two are closely linked. Finally, in order to establish this correspondence without ambiguity, the *Raa3* gene from the M18 allele was amplified by PCR using a proof-reading *Pfu* polymerase and sequenced. A small rearrangement was observed at position 1152 of the mature protein that causes a frameshift and results in a truncation of the C-terminal end of the protein. These results indicate that the isolated cosmid and cDNA correspond to the gene that is altered by the mutation of M18. This gene was named *Raa3*.

***Raa3* sequence analysis**

Both cDNAs and the genomic fragment Δ Nco were sequenced. The cDNA-B contains an ORF of 1783 amino acids encoding a polypeptide of 180.5 kDa (Figure 2A). The presence of an upstream stop codon in-frame with the ATG initiation site, and the match between the size of the predicted protein and that observed by immunoblot analysis (see below), indicate that the cDNA encodes the entire protein. The 5'-untranslated region (5'-UTR) of the cDNA is very short and neither a polyadenylation signal nor a poly(A) tract is present in the 3'-UTR, suggesting that parts of the 5'- and 3'-UTRs are missing in this cDNA. A putative transit peptide for import into the chloroplast is present at the N-terminal end, with an abundance of arginine, serine and alanine residues. A potential cleavage site, Val–Ser–Gly, is present at position 41, which would give rise to a predicted mature protein of 1742 amino acids with a molecular mass of 176 kDa. Comparison of the genomic and cDNA sequences of *Raa3* reveals the presence of four introns of 135, 120, 178 and 153 bp, which are confined within only 450 bp of the genomic *Raa3* sequence (see Figure 2A).

Both the cDNA-A and the genomic fragment Δ Nco lack part of the 5' end of the gene, thus truncating the N-terminal 801 and 453 amino acids of the mature protein, respectively. Since these two fragments are capable of

complementing M18, although only partially in the case of cDNA-A, it appears that the N-terminal portion of *Raa3* is dispensable for its function.

Database searches detected significant sequence identity between a small portion of *Raa3* and one domain of PDXs (Figure 2B). These enzymes are implicated in vitamin B6 biosynthesis and are known to bind flavin mononucleotide (FMN) as immediate electron acceptor cofactor. The function of the shared domain and its significance for *Raa3* function are still unknown. An unusual feature of *Raa3* is the presence of several stretches of alanine, serine, proline and arginine residues, which are scattered throughout the sequence.

***Raa3* is a chloroplast soluble protein**

Immunoblot analysis with an antiserum raised against recombinant *Raa3* protein revealed a band close to 200 kDa that is absent from M18 and present in the wild type and in the M18 strain rescued with the cosmid (Figure 3A). Since the genetic evidence indicates that *Raa3* is involved in *psaA trans*-splicing and its sequence reveals a putative transit peptide, the protein is expected to be localized in the chloroplast. To test this hypothesis, intact chloroplasts were prepared from GHA3-6, a strain in which the *Raa3* protein has been tagged with a triple haemagglutinin (HA) epitope at its C-terminal end. The tagged protein is fully functional since it rescues M18 at the same rate as the untagged version (data not shown). The protein can be identified readily with an HA antibody, which produces significantly less background than the antibody raised against *Raa3*. The amount of *Raa3* protein was determined in total cell extracts and the chloroplast fraction, which was subdivided further into a soluble and insoluble chloroplast fraction. Figure 3B shows that the tagged *Raa3* is absent from wild-type and M18 cells, present in GHA3-6, and that it is enriched in the chloroplast fraction in comparison with the total cell extract. This indicates that *Raa3* is indeed a chloroplast protein. The protein is found mostly in the chloroplast soluble fraction, but small amounts can also be detected in the insoluble fraction. The absence of the cytoplasmic protein eIF4A in the chloroplast extract demonstrates that this fraction is not significantly contaminated with cytosolic proteins. The immunoblot with the chloroplast membrane protein PsaA of PSI shows that the soluble chloroplast fraction is not contaminated with membrane proteins. We conclude that *Raa3* is present mostly in the chloroplast stromal phase.

A surprising result is that the genomic clone Δ NcoI and the cDNA-A construct, which lack the 5' end of the *Raa3* gene, are still able to rescue the M18 mutant. Since most chloroplast transit sequences are encoded by the 5' end of the gene, the truncated *Raa3* protein would not be expected to be imported into the chloroplast. However, earlier studies have shown that signal sequences specific for a membrane transport system lack a strict consensus and that they can be replaced by a surprisingly large number of randomly generated peptides (Schatz and Dobberstein, 1996). It is therefore possible that upon integration of the transforming DNA into the nuclear genome, transit-like sequences were fused in-frame with the truncated *Raa3* sequence. Such a mechanism would predict that in each transformant, the size of the *Raa3* protein is different. Immunoblots with cell extracts from the transformants

A

1	MKADLATAKG	SSPAFSAPRT	YRARLLSRCL	NKCFNTVLVS	GGAAESRFRG
51	GATVGKAGAH	DARGVGDLD	FLQQEATPYE	VNVPPYLILP	PLKSNEATTH
101	RRAGDSGGQG	PAAAGGRGQA	RHGRRQAAAS	AATTVSAAPO	TGATKPAATA
151	KTTTPQRPRGS	DADAGSRAQS	QYGFDPGPGG	GALKGAVDAA	SDAAPDVAAA
201	SPAPAGISDQ	LSTPACPPER	EPQAGKPRAS	GRAPAAPGVG	PQDVGGSSGA
251	CAPAPDESHM	GLTHRDQGH	ERISQTAGEA	WKAGAVAAPP	AAPTFSPFGL
301	AAAPTRLASS	ALGTHSSDGD	MRRVAVPGRDT	PSLSAVAGPV	TLGSSSSSSS
351	GRNSSNSNT	STSSTSNVGT	ITSNVGVNGA	SPQERLMAAR	RAVVTMQWNT
401	HLGRRGRSFA	PLPTGGMSIA	TSAASSSTSS	ASSSSSMNDG	SNAKKTSDA
451	VSLPVGQQA	AEQPHVPTAP	GGPSQTGASA	VAAQAPSSAM	PTAAMAATMG
501	SATIGSAATL	PTAAVSSAA	AEGTQPSGLL	LAGGRPALLG	RTIQGRIARL
551	QAAREALRAA	RHARVGAAMQ	PPPVQARPVQ	GQSGQVPQVG	QGPVQSQPGR
601	RQEPAAATK	LHVADGLPAR	PVQPAVSATD	LQTDATAAS	APFPVSDASL
651	GSTEPLAASA	PTTSLASASG	PAIGTSSSNA	HSSAAAEEA	AGTVRAPTDT
701	AAPAASPAAT	APALASTPFA	TPAAAAPLEP	PEVVAARDLL	GRLSRYQPAG
751	RDDGPAVLAP	HARYSPAAYQ	AAAAAAAQ	QLLLAPLSLP	QLAAVVAGYA
801	AAGHRHEPLL	EALAGVALAK	AGGAGGIGGG	AAGAVPRGQA	ASELKRSLT
851	FRAACVFLTA	LARLGYRGA	VTRLAAALAV	WLARQLNTGA	VTPRAKWGT
901	WLAALWAYA	TLEQLPAAAP	TPPPPPASAA	AASTAPRAAE	SRPAAAPAPA
951	EATATRTPLL	TAPQLAPQRA	SASPDLELAR	GGVLLFTEAA	EAVRVAPGWL
1001	HLMDGREALW	SLWAFRKA	AYGRGEGTAS	GAVYVAAAAG	YAMLQSGGAG
1051	GGGMGQAGGA	GRGGAGAGAA	ALPLVVAAPR	YEANPLVELK	LADRATSLFP
1101	QLDPGQLAEA	HVLLLECGLA	EDELQPALTA	LRRCVIARAD	SIRPQPLAVM
1151	VATLAVMQVR	DVVWLSALAM	ACRNKMINMS	PDQIVAVLHA	FGSVLRFHHL
1201	LLFHAADVVC	SCPGMWRLGG	MGAGEVLRV	GAFATRHYE	GRLRAAAER
1251	MLQLGSTGST	AGQRAGLLQS	LCSLHYRHNG	LLRIVVADTF	GLADLSPGSA
1301	GQPRNMGSSA	KLKAGGTA	AGAAAPGLPP	SLLVAVAEAC	GQLQHRPPGL
1351	LQALHASRAA	VWPRVGLAQR	ATLCWALLVL	TGGLPAQYLP	ADRPRDAKRH
1401	VRQSA	AAAAQDQRQ	EQHQHQEQ	LLAKALVEYL	QALGAGVQRW
1451	PPPAPSSYHL	QLLVACTVLA	SCTPPPAPAH	MQQQPQSSGT	GCQKSRRRM
1501	RYRRSRGAVL	QQQLKEELDK	LPASAMQRAL	EVQRARSAA	LGWAHEVAS
1551	VVREVLQEAS	VDARADSEGR	QPLPHWLQ	PPATLLSAAV	STGVEVCDGA
1601	MLVDVAVELE	VEAGLPRPQV	KGRGRGRRTA	ARATTDVQGA	EQAVVTEEAA
1651	YEHSHPLRQR	LQLALDLCPL	PPPPRTAAP	VLSAAYAPGA	GGAGTSATAE
1701	VTANRTTGAP	RSLAVGAAAG	GAVIRNSRWL	LSGAGALRRR	LLTHAGWLVV
1751	PVRERQWKDL	RSAEQQRVV	REWLKAVLLH	AQQ	

B

Raa3	1534	RARSAALGGWAHEVASV--REVLQEASVDARADSEGRQ--PLPPHWLG	1579
ZYMMO	107	RARKSQLGAWASEQSRPLPARDVFEKRIADIEARYEGKD-VPRPPYWTG	154
YEAST	139	RPRGSKIGAWASRQSDVIKREELDELTAQKNTERFKDAEDIPCPDYWGG	188
MYCLE	136	RPRGSQLGAWASHQSRPIASRTALLDQLLEVTVRFADSELIPLPNWGG	185
ECOLI	131	RPRDSQIGAWVSKQSSRISARGILESKFLELKQKFQOGE-VPLPSFWGG	179
MYXA	184	RARGSQVGAWASLQSQPLATREELEARVAEVEQKYAGQP-VPRPPHWSG	232
SYNY	144	RPRGSQLGAWASPSQSRIVGDRQLELDNLRWEKQYENQS-IPRPPHWGG	192
O74250	140	RARGSKLGAWASRQSDVIKREELDELTAQKNTERFKDAEDIPRPPYWGG	189

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Fig. 2. *Raa3* sequence. (A) Deduced amino acid sequence of *Raa3*. The putative transit peptide is framed and the domain related to pyridoxamine 5' phosphate oxidases is shaded. The asterisk indicates the site of the mutation of M18. The black arrow and the black inverted triangle correspond to the 5' ends of the Δ NcoI fragment and of cDNA-A, respectively. The sites corresponding to the four introns are indicated by open inverted triangles. (B) Amino acid comparison of the homologous domains of pyridoxamine 5' phosphate oxidases and *Raa3*. Sequences were aligned using ClustalW (gap open 10; gap ext 0.2; Blosum matrix). * = conserved residues (shaded), : = conservative substitutions, · = semi-conservative substitution (Thompson *et al.*, 1994). Accession Nos for PDXs are *Zymomonas mobilis*: AF179611; yeast, P38075; *Mycobacterium leprae*, O33065; *E. coli*, P28225; *Myxococcus xanthus*, P21159; *Synechocystis* PCC 6803, P74211; O74250.

obtained with Δ NcoI revealed that this was indeed the case (Figure 3A). The amount of *Raa3* protein in the cDNA-A transformants was too low to be detected. These results do not exclude the alternative possibility that the chloroplast

targeting sequence of *Raa3* is not localized within its N-terminal part. The transformant obtained with the full-length cDNA-B produces a protein that is significantly shorter than the wild-type *Raa3* protein (Figure 3A). This

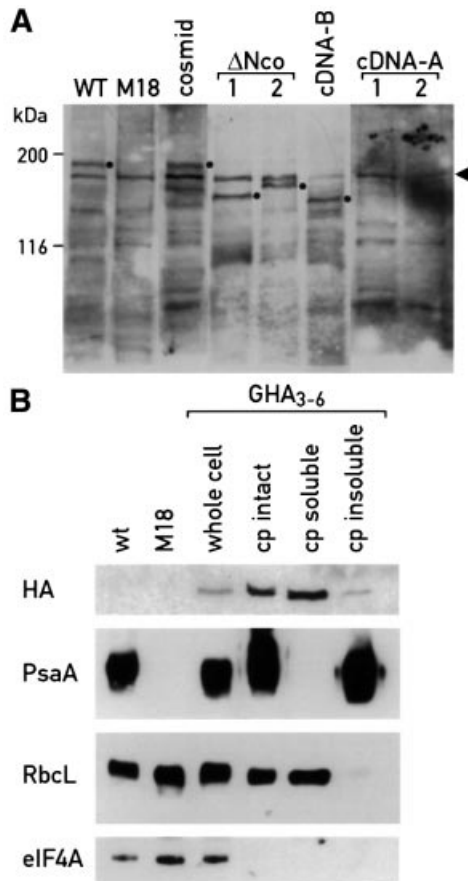


Fig. 3. Accumulation and subcellular localization of Raa3. **(A)** Immunoblot analysis of Raa3 in wild-type and M18 rescued with the genomic (cosmid and Δ Nco) and cDNA (cDNA-A and -B) clones. The antibody used was directed against recombinant Raa3. Dots indicate Raa3 and its truncated versions. The arrowhead indicates a non-specific band that is also present in M18. **(B)** Immunoblot analysis of Raa3 in whole-cell extracts from wild-type and M18 and from cell fractions of M18 transformed with the Raa3-HA construct (total cell, intact chloroplasts, soluble and insoluble chloroplast fractions) prepared as described in Materials and methods. To detect Raa3, 100 μ g of proteins were loaded on a 6% polyacrylamide gel and decorated with α HA. For PsaA, RbcL and eIF4A detection, 10 μ g of proteins were loaded on a 10% polyacrylamide gel and decorated with the corresponding antibodies.

is most likely due to the fact that the plasmid used for the transformation lacks a nuclear promoter. Thus, the cDNA needs to be fused to a promoter and surrogate transit peptide-coding sequence in order to be expressed upon integration into the nuclear genome. Because the N-terminal region of Raa3 is dispensable, a deletion of the 5' end of the Raa3 coding sequence can be tolerated in the transformants.

Raa3 is part of a large complex

At least seven nuclear loci have been shown previously to be involved in the *trans*-splicing of the transcripts of exons 1 and 2 of *psaA* (Goldschmidt-Clermont *et al.*, 1990). The factors encoded by these loci could potentially interact and form a catalytically active complex. To test for the presence of such a complex, soluble extracts from the Raa3-HA-tagged strain were fractionated by size exclusion chromatography and the fractions were analysed by immunoblotting. In extracts containing heparin to preserve RNA from degradation, Raa3 elutes in fractions 6–13,

with its maximum in fraction 8 (Figure 4). Molecular weight markers indicate that this fraction corresponds to a size of 1700 kDa. As a protein of 176 kDa, free Raa3 would be expected to elute between fractions 19 and 20. Its larger apparent size indicates that it is part of a complex.

To determine whether RNA is associated with this complex, the same experiment was conducted in the absence of heparin, but in the presence of RNase. Under these conditions, Raa3 fractionates as a free protein, eluting in fractions 17–20 (Figure 4A). This shows that one or more RNA molecules are part of the complex. RB60, another protein known to associate with RNA (Danon and Mayfield, 1994), behaves similarly: its complex shifts to a smaller size upon treatment with RNase, as found previously (Figure 4B; Boudreau *et al.*, 2000). A general effect of RNase on protein complex stability can be ruled out by the fact that this treatment does not alter the elution profile of Rubisco (Figure 4B).

The M18 mutation belongs to one of the seven identified nuclear loci required for the first *psaA* *trans*-splicing reaction. It is thus possible that some of the remaining six loci encode factors that are part of the Raa3 complex. Loss of any of these subunits could compromise the stability of the complex and thus of Raa3 itself. To test this possibility, protein extracts from six PSI mutants representing the different complementation groups were tested by immunoblotting. Raa3 was exclusively missing in mutants M18 and L138A, which belong to the same complementation group, but accumulated to wild-type levels in the other mutants (Figure 4C). Thus, there is no evidence for a role for these factors in the stability of the Raa3 complex. However, since the nature of the mutations has not been determined, an association of these factors with the complex remains possible.

***tscA* RNA and the *psaA* exon 1 precursor transcript are associated with the Raa3 complex**

To identify the RNA associated with the Raa3 complex, an RNA blot analysis was performed on the different fractions obtained after size exclusion chromatography. One possible candidate is *tscA* RNA, since this small RNA, encoded by the chloroplast genome, has been postulated to represent the middle portion of the discontinuous group II intron 1 of *psaA* (Goldschmidt-Clermont *et al.*, 1991). The analysis revealed that *tscA* RNA co-fractionates with Raa3 (Figures 4A and 5A), suggesting that both are part of the same 1700 kDa complex. In M18 extracts, *tscA* RNA elutes as a complex of 1500 kDa, consistent with the idea that the complex is missing a subunit (Figure 5A). The *psaA* exon 1 precursor transcript also co-fractionates with the Raa3 complex, but does not co-fractionate with *tscA* RNA in M18 (Figure 5A), suggesting that the Raa3 protein plays an important role in maintaining the stability of the large RNA–protein complex. To confirm the presence of *tscA* RNA in the complex, the *tscA* gene was deleted from GHA3-6 (see Materials and methods). The characterization of this strain shows that both Raa3 and the *psaA* exon 1 transcript are present in a smaller complex of 900 kDa (Figure 5A and B). The effect of the deletion appears to be specific, as the Rubisco complex preserves its normal size in the absence of *tscA* (Figure 5C). To test whether the co-fractionation of the RNA with the protein complex during size exclusion

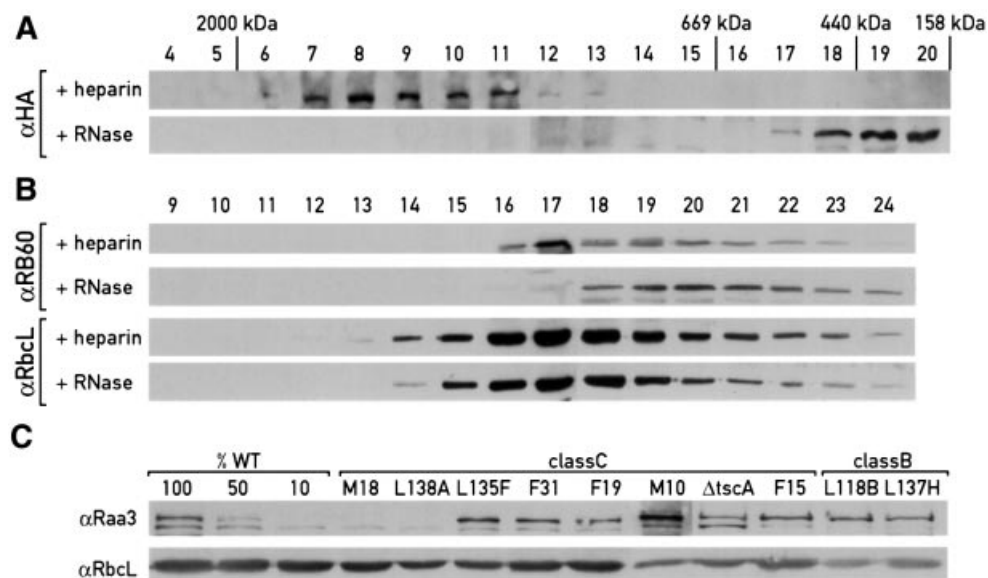


Fig. 4. Raa3 is part of a multimolecular complex. (A) Immunoblot analysis of cell extracts of GHA3-6 fractionated by size exclusion chromatography. A soluble fraction (see Materials and methods) was prepared in the presence of 0.5 mg/ml heparin (+heparin), or incubated on ice without heparin and with 100 μ g/ml RNase (+ RNase), and subjected to size exclusion chromatography. Part of each fraction (90%) was electrophoresed on a 6% polyacrylamide gel, blotted and reacted with HA antiserum. Molecular weight size standards are included. (B) The remaining part of the fractions (10%) was electrophoresed on a 10% polyacrylamide gel, blotted and decorated with antibodies directed against RB60 and RbcL. (C) Immunoblot analysis of total cell extracts from wild type (with dilution series) and representatives from the different class C and class B *psaA* complementation groups probed with Raa3 and RbcL antibodies.

chromatography is specific, the elution profile of *atpH* mRNA, an unrelated chloroplast RNA with a size similar to that of *tscA* RNA, was examined. This RNA was present in fractions different from those containing *tscA* RNA, and the elution profile was the same in the wild-type and M18 mutant strains (Figure 5A). Taken together, these data indicate that Raa3, *tscA* RNA and the *psaA* exon 1 transcript are part of the same large RNP complex.

Discussion

Genetic analysis of *psaA* *trans*-splicing in the chloroplast of *C.reinhardtii* has revealed the existence of several nucleus-encoded splicing factors involved in this process, which fall into three classes. Some are specific for the splicing of exons 1 and 2, others are specific for the splicing of exons 2 and 3, and additional factors are required for both *trans*-splicing reactions. By genomic complementation of some of these *psaA* *trans*-splicing mutants, we have recently identified several of these factors, such as Raa1 and Raa2, which are both involved in the splicing of exons 2 and 3 (Perron *et al.*, 1999; M.Goldschmidt-Clermont, unpublished results). Here we have presented the first characterization of another nucleus-encoded factor, Raa3, specifically involved in the *trans*-splicing of exons 1 and 2. This factor has been isolated by rescuing a nuclear mutant, M18, deficient in the first *psaA* *trans*-splicing reaction.

Unusual features of Raa3

We have localized Raa3 in the stromal phase of the chloroplast using cell fractionation and specific antibodies. Surprisingly, this factor has a different location from that of Raa2, which is associated through ionic interactions with a low density chloroplast membrane fraction that also contains RNA-binding proteins involved in translation

(Zerges and Rochaix, 1998; Perron *et al.*, 1999). The predicted precursor polypeptide encoded by the *Raa3* gene consists of 1783 amino acids. Raa3 contains several long stretches of alanine, serine, proline and arginine residues and is unrelated to known proteins, with the exception of a small region that is homologous to a domain of PDX, an enzyme implicated in the biosynthesis of vitamin B6, which contains FMN as a cofactor. However, this domain does not correspond to the catalytic site of the enzyme and its role has not yet been elucidated. We reported previously that Raa2 contains two domains found in pseudouridine synthases. However, the activity of this enzyme does not appear to be required for *trans*-splicing (Perron *et al.*, 1999).

Surprisingly, the N-terminal 453 residues of Raa3 are dispensable for its function, as shown by the fact that the corresponding transformant is able to grow photoautotrophically and accumulates wild-type levels of PsaA. Raa3 is still functional, at least partially, even after the removal of 801 N-terminal residues. Interestingly, in this case, the cells still accumulate near wild-type levels of PsaA, but they are sensitive to high light. One possibility, which will need to be explored further, is that PSI may be mis-assembled and that Raa3 may also have a role in this process. Similar findings have been reported for the chloroplast nucleus-encoded Nac2 factor involved in *psbD* mRNA stability in *C.reinhardtii* in which removal of the N-terminal half of the protein does not compromise its function (Boudreau *et al.*, 2000). Since homologous recombination occurs at a very low rate in the nuclear genome of *C.reinhardtii* (Kindle, 1998), it is likely that upon integration into the genome, the truncated genes are fused accidentally to sequences that may act as surrogate transit sequences, a phenomenon that is well documented in other systems (Schatz and Dobberstein, 1996). The observation that the size of Raa3 varies in

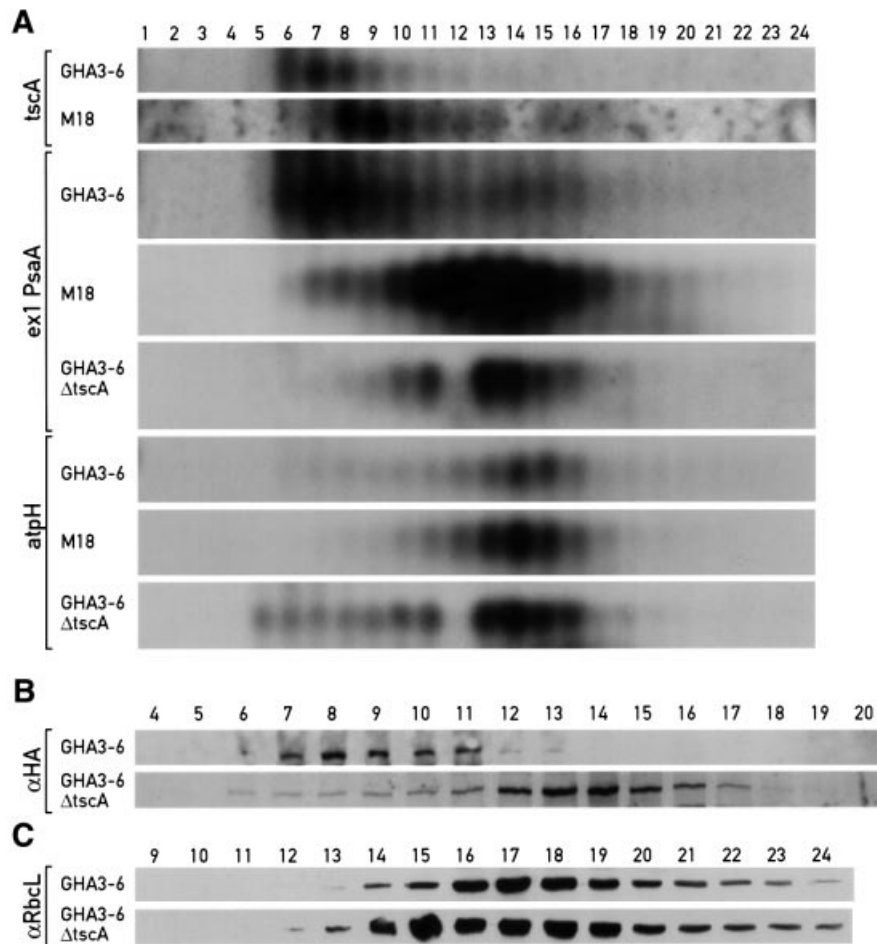


Fig. 5. The Raa3 complex includes *tscA* RNA and the *psaA* exon 1 transcript. (A) RNA blot analysis of cell extracts from GHA3-6 and M18 fractionated by size exclusion chromatography. Soluble extracts were prepared in the presence of heparin (0.5 mg/ml). The RNA was separated on a 1.25% formamide-agarose gel, blotted and hybridized with labelled probes of *tscA*, *psaA* exon1 and *atpH*. The absence of an RNA signal in fraction 12 of GHA3-6 $\Delta tscA$ is due to the accidental loss or degradation of the RNA of this strain during the lengthy preparation procedure. (B and C) Immunoblot analysis of cell extracts of the GHA3-6 strain and its derivative lacking *tscA* (GHA3-6 $\Delta tscA$). Samples were handled as described in Figure 4. Blots were decorated with antibodies against HA and RbcL.

each transformant obtained with truncated versions of the *Raa3* gene (see Figure 3A) is compatible with this proposal.

***Raa3* is part of a high molecular weight complex containing *tscA* RNA and *psaA* exon 1 transcript**

Raa3 is part of a high molecular weight complex of 1700 kDa. *Raa1* and *Raa2* are also part of a complex that is clearly distinct from that of *Raa3* (K.Perron, M.Goldschmidt-Clermont and J.-D.Rochaix, unpublished results). The first intron of *psaA* is particularly interesting because it is tripartite, with its middle section, *tscA* RNA, encoded by a chloroplast locus distinct from those of exons 1 and 2. The *tscA* RNA is associated with the *Raa3* complex based on the following observations: (i) co-fractionation of *tscA* RNA with the *Raa3* complex during size exclusion chromatography (Figure 5); (ii) shift of the *Raa3* complex to a smaller size in mutant strains lacking *tscA* (Figure 5); (iii) shift of the *tscA* RNA-containing complex to a smaller size in the *Raa3* M18 mutant (Figure 5); and (iv) shift to a reduced size when the *Raa3* complex is treated with RNase (Figure 4). Similar arguments can be made for the association of the *psaA* exon 1 transcript with the *Raa3* complex.

Several soluble chloroplast proteins from higher plants have been identified that are associated with RNA (Schuster and Gruissem, 1991; Nakamura *et al.*, 1999). Some of these proteins interact with chloroplast mRNAs as well as with intron-containing precursor tRNAs. However, the function of these protein-RNA complexes has not yet been elucidated. The existence of the *Raa3* complex raises the question of how it relates to the RNA-protein particles involved in the splicing of nuclear introns, especially since group II introns are considered as the evolutionary precursors of nuclear introns (Cech, 1986). It has been proposed that the tripartite structure of the *psaA* intron 1 could reflect an intermediate in the evolution of nuclear introns from group II introns (Goldschmidt-Clermont *et al.*, 1991; Sharp, 1991). According to this hypothesis, the catalytic *cis*-acting group II intron RNA domains would have been gradually replaced by *trans*-acting RNA-protein particles or snRNPs. In this respect, it will be interesting to characterize the other components of the *Raa3* complex fully and to test whether additional parallels can be drawn between this complex and eukaryotic snRNPs.

Materials and methods

Strains and media

Chlamydomonas reinhardtii wild-type and mutant strains were grown as described (Harris, 1989). TAP (Tris–acetate–phosphate) and HSM (high-salt minimal) media were solidified with 2% Bacto agar (Difco) for plate cultures. The mutant M18 has been described previously (Girard *et al.*, 1980; Goldschmidt-Clermont *et al.*, 1990).

Nucleic acid techniques

Procedures for standard molecular techniques were performed as described (Sambrook *et al.*, 1989; Ausubel *et al.*, 1998). The bacterial host was DH5 α . Total *C.reinhardtii* DNA was isolated according to Rochaix *et al.* (1988). Total *C.reinhardtii* RNA was prepared with Tri reagent[®] according to the manufacturer's instructions (Sigma, Fluka Chemie, Switzerland). For RNA blot experiments, 5 μ g of total RNA were electrophoresed through a 1.25% agarose gel containing formaldehyde and MOPS buffer, transferred to Hybond-N⁺ membrane (Amersham) and probed with double-stranded DNA labelled by random priming with [α -³²P]dATP. For *psaA* exon 1, the probe was the 400 bp *HindIII* fragment of R21; for exon 2, the 140 bp *AvaI*–*KpnI* fragment; and for exon 3, the 750 bp *BstXI* fragment in the middle of this exon.

To delete *tscA*, an *EcoRV*–*BglII* fragment from R12 was cloned in pUC 18. Two new restriction sites were introduced at each extremity of the *tscA* gene: *MluI* at the 3' end and *ClaI* at the 5' end using the one-tube PCR protocol (Picard *et al.*, 1994). The recyclable *aadA* cassette was inserted into an *NsiI* site upstream of *tscA* (Fischer *et al.*, 1996). This construct was reintroduced in the R12 fragment. The gene was deleted using the newly introduced sites.

Genomic complementation by transformation

The genomic indexed cosmid library used was described by Zhang *et al.* (1994). Cells were transformed with DNA purified from 96-well microtitre plates and selection was on TAP plates under light (60 μ E/m²). After testing 80 microtitre plates, one transformant was obtained. Cosmid DNA was prepared from the 12 columns and the eight rows of the positive plate, and tested for its ability to rescue M18 under photoautotrophic growth conditions. One rescuing cosmid (61F15) of nearly 40 kbp was isolated. This cosmid was digested with *HindIII* and *SalI*, yielding nine fragments that were tested for complementation of M18. A fragment (HS19) of 12.5 kb able to complement M18 was digested with *SacI*, yielding fragments of 8.5 and 4 kb. The 8.5 kb fragment (HS19SA) that complemented M18 was cloned in pSK (*EcoRV*). This plasmid was trimmed further by deleting a *HindIII* (polylinker)–*NcoI* subfragment, producing Δ Nco. A *NcoI*–*NdeI* fragment of ~2.3 kbp from HS19SA was used to screen a cDNA library constructed by Dr Hans Sommer (Max-Planck Institute, Köln, Germany). One positive cDNA of 3.2 kb was obtained by screening 400 000 phages. When cloned into pSK, this cDNA (cDNA-A) was able to complement M18 at a low frequency. A second screening was performed using a 300 bp PCR fragment complementary to the 5' end of Δ Nco, and two cDNAs of identical size (5.8 kb) were obtained by screening 600 000 phages. When cloned into pSK, these cDNAs (cDNA-B) were able to complement M18 with a high frequency. cDNAs-A and -B as well as the genomic fragment Δ Nco and part of the fragment HS19SA were sequenced using a DNA sequencer (ABI prism, 377 DNA sequencer; Perkin Elmer). To determine the mutation affecting *Raa3* in M18, gene fragments were amplified by PCR from total mutant DNA, gel purified and sequenced. The *Raa3* cDNA and genomic sequences have been submitted to the DDBJ/EMBL/GenBank databases under the accession Nos AF310674 and AF310675, respectively.

HA tagging

To clone the triple HA tag, a *SpeI* site was introduced in the Δ Nco fragment, just upstream of the stop codon, using three primers and the one-tube mutagenic PCR method (Picard *et al.*, 1994). The HA tag was amplified using two primers each containing a *SpeI* site, gel purified, digested with *SpeI* and cloned in Δ Nco. The 5' end of *Raa3* was cloned in Δ Nco-HA by introducing an *XhoI* (polylinker)–*NheI* fragment from HS19SA.

Nuclear and chloroplast transformation

The nuclear transformation was performed as described (Kindle, 1998; Stevens *et al.*, 1996) with the following modifications. Cell wall-deficient cells (*cw15*) were collected by centrifugation and resuspended in TAP at a concentration of 10⁸ cells/ml. Aliquots (0.3 ml) of the cell suspension were mixed with 300 mg of glass beads (0.4 mm; Thomas scientific) and

1.5 μ g of transforming DNA, and vortexed at a maximal speed for 15 s. After addition of 0.3 ml of TAP, the contents of the tube were spread on a TAP plate, incubated in the light (60 μ E/m²) and selected for light resistance.

Deletion of *tscA* was achieved by chloroplast transformation (Finer *et al.*, 1992). Transformants were restreaked four times and the homoplasmy was verified by Southern blot analysis.

Fluorescence transients

Fluorescence transients were measured as described (Bennoun and Delepeleire, 1982; Fenton and Crofts, 1990). Cells were grown on TAP plates in the dark for 7 days.

Antiserum production and purification

The 1689 bp *BamHI*–*BstYI* fragment from Δ Nco (coding for amino acids 1104–1667) was cloned into the *BamHI* site of pET 15b and the plasmid was introduced into *Escherichia coli* BL21(DE3)pLys S. For expression, transformed cells were grown at 37°C to an A₆₀₀ of 0.3 in Luria–Bertani medium supplemented with ampicillin (100 mg/ml). Isopropyl- β -D-thiogalactopyranoside (IPTG; 1 mM) was added and incubation was continued for 2 h. Under these conditions, the majority of the protein was found in the inclusion bodies. Cells were collected and lysed by sonication, and the inclusion bodies were purified by four rounds of centrifugation at 12 000 g and sonication to resuspend the pellet. The final pellet was resuspended in 1 \times loading buffer (25 mM Tris pH 7.0, 10% glycerol, 0.2% β -mercaptoethanol, 0.01% bromophenol blue) and the proteins were loaded on an SDS–polyacrylamide gel. The gel was stained with a solution containing 0.1% Coomassie Blue in 20% ethanol and destained with a 10% ethanol solution. The band containing the protein was cut from the gel. The fragment of gel was squashed and incubated for 16 h at 4°C in a solution containing 15 mM NH₄HCO₃, 0.025% SDS, 1 mM dithiothreitol (DTT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The solution was centrifuged briefly and the supernatant conserved. This elution was repeated three times and all the supernatants were pooled and lyophilized. The protein concentration was estimated by SDS–PAGE and comparison with standard protein markers.

Polyclonal antiserum was raised in mouse by peritoneal injection of 20 μ g of purified protein and Freund's adjuvant every 20 days, with six injections in total. The antiserum was purified by affinity chromatography on Raa3–Sepharose prepared by binding recombinant protein to cyanogen bromide-activated Sepharose.

Protein extracts

A pellet of 4 \times 10⁶ cells was resuspended in lysis buffer containing protease inhibitors (50 mM Tris pH 6.8, 2% SDS, 10 mM EDTA, 5 mM ϵ -aminocaproic acid, 1 mM benzamidine–HCl, 25 μ g/ml pepstatin A, 10 μ g/ml leupeptin), incubated at 37°C for 30 min and centrifuged for 5 min in an microfuge. The resulting supernatant was used as total protein extract.

To prepare soluble and insoluble fractions, the pellet was resuspended in lysis buffer without SDS, passed through a French press (small cell, 300 p.s.i.) and centrifuged at 100 000 g for 45 min at 4°C. The resulting supernatant was the soluble fraction. The pellet was washed with 1 ml of lysis buffer without SDS and centrifuged again at 100 000 g for 15 min at 4°C to remove contaminating soluble proteins. The pellet was resuspended in lysis buffer and proteins from the insoluble fraction were extracted as described for the total protein extracts.

Chloroplast isolation was performed as described (Zerges and Rochaix, 1998) with addition of protease inhibitors (5 mM ϵ -aminocaproic acid, 1 mM benzamidine–HCl, 25 μ g/ml pepstatin A, 10 μ g/ml leupeptin, Sigma inhibitor cocktail P8849) in all solutions. Briefly, *cw15* cells were broken in a Bionebulizer (Gascol Appartus Co., Terre Haute, IN) at 22 p.s.i. Broken cell extracts were fractionated on a continuous Percoll gradient (10–80%). The lower of the two bands corresponding to intact chloroplasts was collected, washed once and lysed osmotically. The soluble and insoluble fractions were prepared as described for total soluble and insoluble proteins. Protein concentration was determined with the bicinchonic acid assay (Sigma).

Immunoblot analysis

Proteins were separated by SDS–PAGE using acrylamide concentrations of 6% (w/v) (for Raa3) or 10% (w/v) (for PsaA, RbcL and eIF4A) and electroblotted to nitrocellulose membranes (Protran, 0.45 μ m; Schleicher & Schuell, Inc., Keen, NH). Filters were incubated with specific antibodies, and visualized by the enhanced chemiluminescence method (SuperSignal; Pierce).

Size exclusion chromatography

The soluble protein extracts of the M18 strain complemented with the Raa3-HA protein were prepared as follows. A 100 ml culture at 2×10^6 cells/ml was centrifuged and resuspended in 0.7 ml of breaking buffer [20 mM HEPES pH 7.8, 10 mM MgCl₂, 50 mM KCl, 10 mg/ml heparin, protease inhibitors (5 mM ϵ -aminocaproic acid, 1 mM benzamide-HCl, 25 μ g/ml pepstatin A, 10 μ g/ml leupeptin, Sigma inhibitor cocktail P8849)] and lysed through the French press (small cell, 300 p.s.i.). A first centrifugation at 40 000 g for 45 min at 4°C to remove cellular debris and insoluble components was followed by a second centrifugation at 100 000 g for 45 min at 4°C. The clear supernatant was loaded on a Superose 6 PC3.2/30 column (Pharmacia Biotech, Uppsala, Sweden) using the SMART System (Pharmacia Biotech). A 50 μ l aliquot (700 μ g) of the proteins was injected into the column and elution was performed at 4°C with a buffer containing 20 mM HEPES-KOH pH 7.8, 50 mM KCl and 10 mM MgCl₂ at a rate of 40 μ l/min. Twenty-four 50 μ l fractions, eluted between volumes of 600 and 1800 μ l after injection, were collected. Three consecutive fractionations were performed and each pooled 150 μ l fraction was precipitated by addition of 8 vols of ethanol. Proteins were pelleted by centrifugation (15 min at 15 000 g), washed with 70% ethanol and resuspended in 10 μ l of loading buffer. The fractions were loaded on a 6% SDS-polyacrylamide gel. The molecular weight range was estimated by comparison with size standards included in the HMW Gel Filtration Calibration Kit (Pharmacia Biotech, Sweden).

Total RNA was prepared from pooled 150 μ l fractions obtained by three consecutive runs as above. After phenol-chloroform extraction and ethanol precipitation, the pellets were resuspended in 4.5 μ l of sterile water, stained with ethidium bromide and electrophoresed through a 1.5% agarose-formaldehyde denaturing gel.

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