Biogenesis of photosynthetic complexes in the chloroplast of *Chlamydomonas reinhardtii* requires ARSA1, a homolog of prokaryotic arsenite transporter and eukaryotic TRC40 for guided entry of tail-anchored proteins

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Received 28 March 2012; revised 13 November 2012; accepted 15 November 2012; published online 28 December 2012.

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SUMMARY

*as1*, for antenna size mutant 1, was obtained by insertion mutagenesis of the unicellular green alga *Chlamydomonas reinhardtii*. This strain has a low chlorophyll content, 8% with respect to the wild type, and displays a general reduction in thylakoid polypeptides. The mutant was found to carry an insertion into a homologous gene, prokaryotic arsenite transporter (*ARSA*), whose yeast and mammal counterparts were found to be involved in the targeting of tail-anchored (TA) proteins to cytosol-exposed membranes, essential for several cellular functions. Here we present the characterization in a photosynthetic organism of an insertion mutant in an *ARSA*-homolog gene. The ARSA1 protein was found to be localized in the cytosol, and yet its absence in *as1* leads to a small chloroplast and a strongly decreased chlorophyll content per cell. ARSA1 appears to be required for optimal biogenesis of photosynthetic complexes because of its involvement in the accumulation of TOC34, an essential component of the outer chloroplast membrane translocon (TOC) complex, which, in turn, catalyzes the import of nucleus-encoded precursor polypeptides into the chloroplast. Remarkably, the effect of the mutation appears to be restricted to biogenesis of chlorophyll-binding polypeptides and is not compensated by the other *ARSA* homolog encoded by the *C. reinhardtii* genome, implying a non-redundant function.

**Keywords:** ARSA, tail-anchored proteins, TOC34, *Chlamydomonas*, protein targeting, chloroplast.

INTRODUCTION

The unicellular green alga *Chlamydomonas reinhardtii* is suitable for random nuclear genetic transformation, and the availability of nuclear genome sequence information (Merchant *et al.*, 2007) makes it an organism of choice for both forward and reverse genetic studies. In addition, the ability of *C. reinhardtii* to grow heterotrophically in the dark makes photosynthetic function dispensable and amenable to mutational analysis. Although the electron transport reactions associated with photosynthesis occur in the chloroplast, the multimeric thylakoid complexes contain only a few chloroplast-encoded polypeptides, the large majority being nuclear-encoded and needing to be imported into the plastid upon translation by cytosolic ribosomes. The enzymes catalyzing the synthesis of required cofactors are also nuclear-encoded; their expression and biogenesis must be strictly coordinated and controlled in order to optimally respond to developmental and external stimuli (Pogson *et al.*, 2008). Nuclear mutagenesis could therefore affect genes involved in or controlling the targeting of nucleus-encoded photosynthesis-related proteins to the chloroplast and the biogenesis of photosystems.

Random insertion mutagenesis of *C. reinhardtii* and phenotype screening identified a mutant severely affected in chlorophyll content, about 8% of the wild-type level, named *as1*, for antenna size mutant 1 (Bonente *et al.*, 2011). The light-harvesting antenna size of both photosystems is significantly reduced; this clear phenotype
of antenna versus reaction center reduction is confirmed by a higher \( \beta \)-carotene content and chlorophyll \( a \)/chlorophyll \( b \) ratio (Bonente et al., 2011). The present work reports on further characterization of \( \textit{ast} \); in particular the ‘pale green’ phenotype is associated with an insertion mutation in an \textit{ARSA}-homolog gene on chromosome 5.

\textit{ARSA} proteins are found in bacteria where they have two ATP\textsubscript{ase} domains within a single polypeptide chain and, together with the transmembrane partner ARSB, seem to be involved in active arsenite extrusion and resistance (Kuroda et al., 1997; Zhou et al., 2000; Borgese and Righi, 2010; Borgese and Fasana, 2011). \textit{ARSA} homologs are conserved between archea and eukaryotes, where they have acquired a novel function in the targeting of tail-anchored (TA) proteins (Rabu et al., 2009; Borgese and Righi, 2010; Borgese and Fasana, 2011). The TA proteins constitute a distinct class of integral membrane proteins, whose targeting information resides on the C-terminus rather than the N-terminus of the polypeptide. Since the only membrane-targeting sequence emerges from the ribosome upon completion of translation, TA proteins insert into their target membranes by post-translational mechanisms. The defining feature of a TA protein is the presence of a single transmembrane (TM) segment, typically of 20 amino acids, very close to the C-terminus, at no more than 30 residues. This TM segment provides both the targeting signal for the delivery of the protein to the correct subcellular compartment and the anchor that retains the polypeptide in the lipid bilayer once integration has taken place (Kutay et al., 1993; Borgese et al., 2007), although targeting information may be additionally be located outside the tail anchor (Dhana et al., 2010). Irrespective of the compartment in which they reside, TA proteins are oriented in the membrane such that the N-terminal region faces the cytosol where it can perform its biological function. As a consequence TA proteins are not embedded into the internal membranes of organelles (Kriechbaumer et al., 2009; Rabu et al., 2009). This protein group, present in all domains of life and in all cytosol-exposed membranes, performs essential cellular functions such as vesicle trafficking (e.g. the vesicle-associated membrane protein synaptobrevin-2), ubiquitination, apoptosis, protein translocation (e.g. Sec61\( \beta \) and Sec61\( \gamma \); subunits of the Sec61 translocon complex of the endoplasmic reticulum (ER)), signal transduction, transcription, enzymatic reactions and electron carrying (e.g. different isoforms of cytochrome \( b_5 \) targeted to the ER, mitochondria or plastids). Over 10 and 50 TA proteins are predicted to be expressed in prokaryotes (Borgese and Righi, 2010) and yeast (Beilharz et al., 2003), respectively, while over 400 are predicted in humans (Kalbfleisch et al., 2007) and plants (Kriechbaumer et al., 2009). Studies performed in mammals (Mukhopadhyay et al., 2006; Stefanovic and Hegde, 2007) and yeasts (Schuldiner et al., 2008) have shown that \textit{ARSA} homologs are involved in the delivery of TA proteins to target membranes. The mammalian \textit{ARSA} homolog TRC40 interacts with newly synthesized Sec61\( \beta \) in cross-linking experiments and is peripherally associated with membranes (Stefanovic and Hegde, 2007). A homozygous knockout of the mouse \textit{ASNA1} gene caused embryonic lethality (Mukhopadhyay et al., 2006), suggesting that the \textit{ASNA1} pathway could be essential for the biogenesis of some strictly \textit{ASNA1}-dependent TA proteins. The structure of the yeast homolog GET3 (Guided Entry of Tail-anchored proteins-3; Schuldiner et al., 2008) was resolved, showing it to be a homodimer whose monomers, each carrying an ATP\textsubscript{ase} domain and a methionine \( \alpha \)-helical domain, are linked by two cysteine residues coordinating a zinc ion. In the active state, the dimer interface exposes a large hydrophobic groove implicated in TA binding (Hu et al., 2009) and interacts with GET1 and GET2 membrane proteins to form a receptor (Schuldiner et al., 2008) and with GET4 and GET5 in the cytosol to form the trans-membrane recognition complex (Jonikas et al., 2009). The yeast \textit{GET3} knockout, although presenting a reduced fitness, is viable, suggesting the existence of alternative routes for TA protein insertion in the absence of \textit{GET3} (Maggio et al., 2007; Rabu et al., 2009). Although there are bioinformatic predictions of over 400 TA proteins in Arabidopsis, including 138 TA proteins putatively localized in plastids (Kriechbaumer et al., 2009), only a few plastidial TA proteins have been documented, including an isoform of cytochrome \( b_5 \) (Maggio et al., 2007), TOC33 and TOC34 of the chloroplast outer membrane translocon (May and Soll, 1998; Qbadou et al., 2003; Dhanoa et al., 2010) and the novel outer envelope TA protein OEP9 of unknown function (Dhana et al., 2010). While in yeast and human only one \textit{ARSA}-homolog protein is encoded, two and three \textit{ARSA} genes are present in \textit{Chlamydomonas} and Arabidopsis, respectively. Different isoforms of \textit{ARSA} could be required in photosynthetic eukaryotes where the presence of plastids, in addition to the other cellular compartments, could increase the level of complexity in TA protein targeting and biogenesis. In the present work we show that an insertional mutation in the \textit{ARSA}-homolog gene on chromosome 5 of \textit{C. reinhardtii} profoundly affects the biogenesis of photosynthetic complexes in the chloroplast and is not compensated by a different \textit{ARSA}-homolog protein, encoded by a gene on chromosome 3, suggesting a non-redundant function. Despite the mutation strongly affecting chloroplast structure and function, the encoded protein was found to be located in the cytosol and to control the insertion of TOC34 which is a TA protein. These results suggest that \textit{ARSA1} affects the biogenesis and activity of the macromolecular complex importing nuclear encoded chloroplast proteins.
RESULTS

Identification of an asra1 mutant of Chlamydomonas reinhardtii

Random insertion mutagenesis of the nuclear genome of a cw15 strain of C. reinhardtii (also called wild type in the present paper) was performed with a linearized pSL18 plasmid containing the paromomycin resistance cassette, as described in Bonente et al. (2011). A mutant with a residual chlorophyll content of 8% of the wild-type level was isolated for a reduced yield of in vivo chlorophyll fluorescence and an altered pigment content (mutant formerly called as1) (Bonente et al., 2011). To identify the insertion site, we proceeded to resequence the entire nuclear genome through an ILLUMINA® platform. The software SOAP v. 2.20 was used to align the 100 bp reads against either the sequence of the pSL18 plasmid or the C. reinhardtii genome sequence available online on the NCBI website (Li et al., 2009). The software BLAT was used to distinguish putative genomic regions flanking a plasmid sequence into a read and then aligned against all the reads to confirm the result. The insertion occurred in a predicted ARSA-homolog gene on chromosome 5 (Cre05.g230350 Chlamydomonas reinhardtii v4.3, g5570 v5.3). A second ARSA-homolog gene, ARSA2, not mutated in as1, is annotated on chromosome 3 (Cre03.g204800 (http://www.phytozome.net)). Complementary DNA clones for ARSA1 are available (Kazusa Institute, Tokyo, Japan), indicating that the gene is indeed expressed (the encoded amino acid sequence is reported in Figure S1 in the Supporting Information), aligned with those of homologous Chlamydomonas gene sequences.

Figure 1. Mapping and characterization of the mutation in the ARSA1 gene. (a) Schematic representation of the insertion of the pSL18 cassette into the genomic sequence encoding for the second ARSA1 ATPase domain. The genome deletion caused by the insertion is marked by the dashed line. Arrows indicate primers used in (b) and (c). Also the pSL18 cassette itself underwent some rearrangements, including head-to-tail concatamerization and deletion of 899 bp close to the flanking border (dashed line). (b) Polymerase chain reaction on genomic DNA from cw15, as1 and two rescued clones (r2, r3). Primers were either designed to amplify a fragment at the 5’ end of the ARSA1 gene, the 3’ end of the ARSA1 gene or the ARSA1_CDS3. The ARSA1_CDS3 primer set was not able to amplify a 500 bp product on the wild-type genome due to the presence of introns. (c) Reverse transcriptase-PCR, showing ARSA1 expression at the transcript level only in the cw15 and r2 rescued clone, using the ARSA1_CDS3 primer set as in (b). Expression of CBL is shown as a positive control. (d) Random progeny analysis of the cross between cell wall-less as1 mt- and the cell wall-containing wild type S34 mt+. Progeny colonies were tested on TRIS-acetate-phosphate (TAP) for resistance to paromomycin (15 μg ml⁻¹) and on minimal medium for photoautotrophy at 50 μE photons m⁻² sec⁻¹. Of 52 progeny colonies, 21 colonies (left panel small stars) grew on TAP-paromomycin, while 31 died on minimal medium (right panel).
resistance carried by the insertion cassette, indicating a mutation tagged by the transforming DNA. An example of such genetic analysis is shown in Figure 1d. The mutant was then transformed with the ARSA1 cDNA. Since photoautotrophy is compromised in as1 at 50 μm photons m⁻² sec⁻¹, a limiting light intensity considering the strongly reduced absorption cross-section of the mutant (Figures 1d and 6), selection of transformants was performed by directly selecting for a rescued phenotype in minimal medium at the aforementioned irradiance. No colonies were observed in the untransformed control, indicating absence of spontaneous revertants. Clones having the ARSA1 cDNA cassette integrated in the genome and expressing the corresponding transcript (Figures 1b,c) showed rescue of the mutated phenotype (Figures 3, 4, 6 and 7), indicating that the observed pale green phenotype is indeed due to the insertion in the ARSA1 gene.

The arsa1 mutation affects the accumulation of photosystem polypeptides

as1 mutant has higher chlorophyll a/b ratio (6.4 versus 2.7 in cw15; Table 1) and a lower chlorophyll/carotenoid ratio (1.5 versus 3.2 in cw15; Table 1), suggesting a phenotype of antenna versus core complex reduction. Compared to the 8% chlorophyll content of as1, transformants for the ARSA1 cDNA (Figure 1b, c) display different levels of chlorophyll content (from 46% in r2 to 65 and 70% in r1 and r2, respectively, as compared with the wild type). The expression of ARSA1 in these clones is sufficient to recover chlorophyll a/b and chlorophyll/carotenoid ratios close to the wild-type level (Figure 3, Table 1).

Since chlorophyll biosynthesis is coordinated to the expression of chlorophyll-binding proteins, the mutant is expected to have a reduced accumulation of such polypeptides in thylakoid membranes. Thylakoid proteins were thus separated by electrophoresis in denaturing conditions (SDS-PAGE), followed by Coomassie staining of the gel to give an overview of the polypeptide profile in thylakoids (Figure 4a). Annotation of protein bands is based on fractionation and immunoblotting as reported in Bassi and Wollman (1991). Moreover, a detailed investigation of several photosynthetic polypeptides was performed by immunoblotting with specific antibodies on total protein extract (on a per cell basis in Figure 4b and on a per chlorophyll basis in Figure S2). The mutant shows a general reduction in the level of thylakoid polypeptides per cell as compared with cw15 (Figure 4a); in particular, the accumulation of photosystem II core subunits appears to be more affected than the accumulation of photosystem I core polypeptides, ATPase and cytochrome f of the cytochrome b₆f complex (Figures 4b and S2). In addition, light-harvesting chlorophyll a/b-binding (LHC) subunits of both photosystems are strongly reduced (Figure 4a, b). Among the LHCI polypeptides of the photosystem I antenna system, LHCA3 and LHCA9 were the most reduced, followed by LHCA4 and LHCA8 (Figure S2). However, the content of soluble chloroplast proteins such as ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) was significantly higher in the mutant versus cw15 on a per cell basis. Markers for mitochondria and the ER (CPLX1 and BIP2, respectively) were present at the same level in both genotypes. We then proceeded to analyze cell organization by electron microscopy (Figure 5). We observed that in the wild type the chloroplast occupies most of the cell volume and is packed with thylakoid membranes. In contrast, as1 cells exhibit...
much smaller chloroplasts with a lower density of thylakoid membranes, suggesting impaired biogenesis of chloroplast membrane structures.

The **arsa1** mutation compromises photoautotrophic but not heterotrophic growth

In order to verify the effect of the **arsa1** mutation on photosynthesis and photoautotrophy we compared growth on acetate-supplemented medium (TRIS-acetate-phosphate, TAP), allowing for heterotrophic growth, with growth on minimal medium (high salt, HS), selective for photoautotrophy. As shown in Figure 6, photoautotrophic growth on HS medium was severely impaired at 50 μm photons m⁻² sec⁻¹, probably due to the very low chlorophyll level insufficient to sustain light harvesting and photosynthesis in limiting light. This phenotype was partially rescued at higher light (400 μm photons m⁻² sec⁻¹ in Figure 6), suggesting that the mutant is not light-sensitive, and almost completely rescued on TAP. Equal respiration rates were measured, expressed as oxygen uptake in the dark (18 × 10⁶ μm O₂ 10⁻⁶ cells⁻¹ h⁻¹ versus 18.9 in cw15; Table 2), suggesting normal respiratory activity and thus unaffected mitochondrial biogenesis.

The **arsa1** mutation affects accumulation of the TOC34 subunit of the translocon of the outer chloroplast membrane (TOC) complex

Eukaryotic ARSA homologs have been proposed to target TA proteins to membranes, based on studies performed so far in yeast and mammals (Stefanovic and Hegde, 2007; Schuldiner et al., 2008). The TA proteins are defined as integral membrane proteins exposed to the cytosol rather than embedded into the internal membranes of organelles (Kriechbaumer et al., 2009; Rabu et al., 2009). Therefore, thylakoid membranes do not comprise TA proteins. However, the outer chloroplast membrane is predicted to contain TA proteins (May and Soll, 1998; Qbadou et al., 2003; Maggio et al., 2007; Kriechbaumer et al., 2009; Dhanoa et al., 2010) that may also utilize different sorting pathways (Maggio et al., 2007; Dhanoa et al., 2010). In the present work we investigated the accumulation of TOC34, which is a TA protein (May and Soll, 1998; Qbadou et al., 2003; Dhanoa et al., 2010). By immunoblotting with a specific antibody raised against Arabidopsis TOC34, no reactivity was detected in the mutant in contrast to cw15 (Figure 7).

We verified that the anti-TOC34 antibody did not react with purified thylakoid membranes, while it did recognize a 44-kDa SDS-PAGE band in the intact chloroplast preparation, consistent with location of TOC34 in the chloroplast envelope membrane (Figure 7b). This result suggests a role for ARSA1 in the biogenesis of TOC34, which is a core component of the TOC complex. Consequently, accumulation of TOC34 is compromised in the **arsa1** mutant. The TOC complex has evolved to perform the physical task of transporting the nucleus-encoded precursor proteins across the chloroplast envelope (Li and Chiu, 2010) and represents the first step in the chloroplast protein targeting machinery. Impaired biogenesis of TOC34 could explain the reduced accumulation of photosynthetic complexes, due to reduced protein import activity, and the pale green/yellow phenotype of **arsa1** is consistent with the reported phenotype of an Arabidopsis thaliana mutant for the homolog TOC component (Jarvis et al., 1998; Kubis et al., 2003). Remarkably, TOC34 accumulation was restored to different extents in transformants for the ARSA1 cDNA (r2 and r3 in Figure 7a). In particular, the higher level of TOC34
polypeptide in r2 as compared with r3 correlates to the extent of rescue of the wild-type phenotype, i.e. the chlorophyll content (Table 1). This result implies that absence of TOC34 in as1 was indeed due to the arsa1 mutation. We then proceeded to verify whether the inhibitory effect of the mutation was selective for the accumulation of TOC34 or if other TOC subunits were affected. To this aim we used antiserum raised against plant TOC159 which showed a good reaction in Chlamydomonas at the corresponding apparent molecular mass, implying TOC159 accumulated to the same level in cw15 and as1 on a cell basis (Figure 7c). Instead, the antiserum against plant TOC 75 showed no reaction on the extract from algae.

The ARSA1 protein is localized in the cytosol

TOC34 is localized in the outer chloroplast envelope membrane (Ferro et al., 2002); thus, the machinery for its insertion is expected to be cytosolic. In order to locate ARSA1 in the Chlamydomonas cell we have produced an antibody by using as antigen the recombinant ARSA1 protein expressed in E. coli. The antiserum recognized a single band around the molecular weight of 80 kDa in SDS-PAGE which was absent in as1 and was present in cw15 (Figure 8a). We then proceeded to fractionation of C. reinhardtii cells into intact chloroplasts and mitochondria, highly purified thylakoid membranes, microsomal and cytosol preparations as described in Experimental Procedures. Probing with antibodies directed against marker proteins (Figure 8b) confirmed the purity of the fractions: in fact, nitrite reductase was only found in intact chloroplasts, not in thylakoids, where the chlorophyll a/b binding protein LHCB4 was, instead, detected. CPLX1 (49-kDa subunit of mitochondrial complex I) was only found in the mitochondrial fraction while UGPase (UDP-glucose pyrophosphorylase) was detected in the cytosolic fraction. Finally, BIP2 (binding protein 2) was found in both the microsomal fraction and the cytosol, consistent with the partial loss of this soluble ER marker during the formation of right-side-out microsomal vesicles. All these antigens could be detected in the whole cell extract. When these fractions, loaded in the gel on a protein basis, were probed with anti-ARSA1 antibody, a signal was found in the cytosol fraction with a faint trace in microsomes (Figure 8b). In order to verify the possibility of an interaction between ARSA1 and microsomes, we proceeded to step centrifugation of the total cell extract at 20 000, 40 000 and 100 000 g and probed the pellet obtained after each centrifugation with respect to the whole cell extract and the 100 000 g supernatant loaded on a protein basis. Although the immunoblot signal was stronger in the soluble fraction and
fainter in the pellets, a band was still detected in the 100 000 g pellet containing microsomes (figure S3). Thus, although ARSA1 is to a large extent soluble in the cytosol, we cannot exclude a low level of interaction with microsomes.

**DISCUSSION**

This study reports the identification and characterization of an arsa1 mutant of *C. reinhardtii*, generated by random insertion mutagenesis of the nuclear genome and screened for an altered pigment content/composition and a lower chlorophyll fluorescent yield (mutant formerly called as1) (Bonente et al., 2011). In bacteria, ARSA proteins are involved in active arsenite extrusion and resistance while ARSA-homolog genes have acquired a novel function in targeting of TA proteins in eukaryotes (Rabu et al., 2009; Borgese and Fasana, 2011). So far ARSA-homologs have been studied in yeast and mammals (Mukhopadhyay et al., 2006; Stefanovic and Hegde, 2007; Schuldiner et al., 2008) while nothing is known about their function in plants. A homozygous knockout of the mouse ASNA1 gene caused embryonic lethality (Mukhopadhyay et al., 2006), suggesting the existence of some strictly ASNA1-dependent TA proteins fulfilling essential cellular functions. The homologous mutation in yeast didn’t lead to lethality, indicating some redundancy in TA proteins targeting pathways, but all the same pleiotropic effects were observed (Schuldiner et al., 2008). In eukaryotic photosynthetic organisms, in addition to TA proteins targeted to other cytosol-exposed membranes a specific plastidial set of TA proteins could increase the level of complexity in the biogenesis of TA proteins. Consistently, while a single ARSA-homolog is present in yeast and mammals, plants have more than one gene in the nuclear genome, i.e. two and three in *Chlamydomonas* and Arabidopsis, respectively. The present mutant for the ARSA-homolog gene on chromosome 5 of *C. reinhardtii* displays profound effects on the accumulation of chlorophyll-binding photosynthetic proteins in chloroplast thylakoids (Figure 4). ARSA1 seems to be specifically involved in chloroplast function and its mutation only mildly altered the function of other cellular compartments, if at all. As a matter of fact, the arsa1 mutation had a much stronger effect on phototrophic growth than on heterotrophy (Figure 6). Moreover, respiration rate was unaltered, suggesting maintenance of mitochondrial function. This could reflect a major role of ARSA1 in the biogenesis and targeting of plastidial TA proteins. The
Oxygen evolution saturates; also known as the compensation point, Chl, chlorophyll. Uptake by respiration equals oxygen evolution by photosystem II.

The cyanobacterium engulfed by a mitochondriate eukaryote chloroplast arose from a photosynthetic prokaryote (a cyanobacterium) that was not compensated by the distinct chloroplast surface by two homologous GTPases, TOC159 and TOC34. Together with the channel TOC75, both TOC34 and TOC159 constitute the core components of the TOC complex, thus excluding thylakoids. As a consequence, the non-TA protein TOC159 was unaffected (Figure 7c). In all cases, the absence of TOC34 would impair the import of precursor proteins across the double membrane envelope of the chloroplast (Liu and Chiu, 2010). The transit peptide, exposed by a precursor protein, is recognized at the chloroplast surface by two homologous GTPases, TOC159 and TOC34. Together with the channel TOC75, both TOC34 and TOC159 constitute the core components of the TOC complex, firmly interacting with each other (Waegemann and Sol, 1991). From the absence of any signal when assaying either whole cell extracts or membrane fractions using the antibody raised against Arabidopsis TOC34 in as1, while a band at the expected mobility was observed in cw15 (Figure 7), we can hypothesize a role for ARSA1 in the targeting and biogenesis of TOC34. Critical in this respect is the cellular location of ARSA1 that was carefully assessed by raising a polyclonal antibody in rabbit using highly purified recombinant ARSA1 expressed in E. coli that we used for detection in cell fractions (Figure 8). Recovery of ARSA1 in the cytosolic fraction rather than in the chloroplast fraction or thylakoid membranes rules out the possibility that ARSA1 might reside in the chloroplast stroma to promote insertion of proteins into the thylakoid membrane. These findings are highly significant and consistent with ARSA1 being needed for the insertion of the TA protein TOC34 in the outer chloroplast membranes. Instead, accumulation of the non-TA protein TOC159 was unaffected (Figure 7c). In turn, the absence of TOC34 would impair the import of nucleus-encoded photosynthetic proteins. While photosystem core complexes (PSAA, D1, CP43, CP47; Figures 4 and S2) are encoded by the plastid genome, subunits of the light-harvesting antenna complexes (LHC) are encoded by the nucleus (LHCII, LHCB4-S LHCA1-9; Figures 4 and S2) and then imported into the chloroplast where they are folded together with chlorophylls and carotenoid molecules.

### Table 1 Pigment content and composition of Chlamydomonas reinhardtii cw15, as1 mutant and complemented r1, r2, r3 clones. Data are expressed as mean ± SD (n = 4)

<table>
<thead>
<tr>
<th></th>
<th>cw15</th>
<th>as1</th>
<th>r1</th>
<th>r2</th>
<th>r3</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg chl/10^6 cells</td>
<td>3.72 ± 0.33</td>
<td>0.30 ± 0.04</td>
<td>2.44 ± 0.23</td>
<td>2.63 ± 0.23</td>
<td>1.72 ± 0.18</td>
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<tr>
<td>Chl a/chl/b</td>
<td>2.70 ± 0.22</td>
<td>6.40 ± 0.61</td>
<td>2.95 ± 0.28</td>
<td>3.20 ± 0.32</td>
<td>2.88 ± 0.29</td>
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<tr>
<td>Chl/Car</td>
<td>3.20 ± 0.34</td>
<td>1.50 ± 0.18</td>
<td>2.23 ± 0.22</td>
<td>2.52 ± 0.22</td>
<td>2.14 ± 0.21</td>
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</table>

Chl, chlorophyll; Car, carotenoid.

### Table 2 Photosynthetic parameters extrapolated from light saturation curves, measured with a Clark-type electrode. Experimental data for oxygen exchange rates were curve-fitted with a hyperbolic tangent function (Falkowski and Raven, 1997) using the Microsoft Excel least-squares solver algorithm (Huesemann et al., 2008). Data are expressed as mean ± SD (n = 4)

<table>
<thead>
<tr>
<th></th>
<th>cw15</th>
<th>as1</th>
<th>r1</th>
<th>r2</th>
<th>r3</th>
</tr>
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<tbody>
<tr>
<td>R_{dark} (10^{-2} µM O_2 cell^{-1} h^{-1})</td>
<td>(--) 18.90 ± 1.51</td>
<td>(--) 18.00 ± 1.43</td>
<td></td>
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<tr>
<td>R_{max} (µM O_2 mg cell^{-1} h^{-1})</td>
<td>106.18 ± 8.94</td>
<td>85.80 ± 6.83</td>
<td></td>
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<tr>
<td>a (µM photons m^{-2} sec^{-1})</td>
<td>0.51 ± 0.01</td>
<td>0.31 ± 0.02</td>
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<tr>
<td>l_b (µM photons m^{-2} sec^{-1})</td>
<td>213.20 ± 17.01</td>
<td>276.90 ± 22.15</td>
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<tr>
<td>l_b (µM photons m^{-2} sec^{-1})</td>
<td>37.60 ± 2.99</td>
<td>58.90 ± 4.71</td>
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R_{dark}, oxygen uptake in the dark by respiration; R_{max}, light-saturated maximum oxygen evolution rate; a, the slope of the initial linear increase of the light saturation curve that is a measure of the photon use efficiency; l_b, light intensity at which photosynthetic oxygen evolution saturates; l_b, light intensity at which oxygen uptake by respiration equals oxygen evolution by photosystem II, also known as the compensation point, Chl, chlorophyll.

Mutation in ARSA1 was not compensated by the distinct ARSA-homolog protein, encoded by a gene on chromosome 3, suggesting a non-redundant function. ARSA1 protein has two ATPase domains within a single polypeptide, in contrast to ARSA2 and ARSA-homologs found in other eukaryotic systems (Figures 2 and S1). In this respect, ARSA1 is more similar to prokaryotic ARSA proteins and could suggest an endosymbiotic origin.

Tail-anchored proteins are embedded in cytosol-exposed membranes, thus excluding thylakoids. As a consequence, polypeptides of photosynthetic complexes, that are reduced in the present mutant, cannot be direct substrates for ARSA1. However, the outer chloroplast membrane is predicted to contain TA proteins, including the TOC34 subunit of the translocon of the TOC (May and Soll, 1998; Qbadou et al., 2003). It is generally accepted that the chloroplast arose from a photosynthetic prokaryote (a cyanobacterium) engulfed by a mitochondriate eukaryote (Raven and Allen, 2003). Since its endosymbiotic beginning, the chloroplast has become fully integrated into the biology of the host eukaryotic cell. The transfer of genetic information from the chloroplast genome to the host nuclear genome resulted in the loss of most plastid genes, whose function was assumed by nucleus-encoded proteins (Martin and Herrmann, 1998). New proteins were added to facilitate its biogenesis and coordinate its function. This process required evolution of a protein translocation system to facilitate the post-translational return of endosymbiont proteins back to the organelle. The translocon of the chloroplast performs the physical task of translocating the precursor proteins across the double membrane envelope of the chloroplast (Li and Chiu, 2010). The transit peptide, exposed by a precursor protein, is recognized at the chloroplast surface by two homologous GTPases, TOC159 and TOC34. Together with the channel TOC75, both TOC34 and TOC159 constitute the core components of the TOC complex, firmly interacting with each other (Waegemann and Sol, 1991). From the absence of any signal when assaying either whole cell extracts or membrane fractions using the antibody raised against Arabidopsis TOC34 in as1, while a band at the expected mobility was observed in cw15 (Figure 15), we can hypothesize a role for ARSA1 in the targeting and biogenesis of TOC34.
The LHC polypeptides of both photosystems II and I are first recognized by translocases of the outer and inner chloroplast envelopes (TOC and TIC, respectively) (Li and Chiu, 2010) and once in the stroma, targeted to thylakoids thanks to a chloroplast signal recognition particle (SRP)-dependent pathway (Asakura et al., 2004; Schunemann, 2004). Impaired biogenesis of TOC in the present 

arsa1

mutant could explain the observed depletion of LHC polypeptides (Figure 4). Interestingly, plastid-encoded subunits of photosystem core complexes are also reduced in the mutant, especially those of photosystem II (Figure 4). Since plastid-encoded photosystem core subunits have no need for import machinery, the 

arsa1

mutation could have an indirect effect by causing the reduced import of nucleus-encoded proteins of the transcription and translation machinery of the chloroplast (Gutensohn et al., 2004). Alternatively, this could be due to the lack of nuclear factors controlling specifically the assembly and stability of photosystem core complexes (Rochaix, 2011). In addition, import of enzymes involved in chlorophyll biosynthesis could be impaired (Reinbothe et al., 2005), limiting the availability of chlorophyll for chlorophyll-binding protein folding.

It should be noted that TOC34 is present in a single copy in 

C. reinhardtii

(Kalanon and McFadden, 2008) in contrast to Arabidopsis that has two paralogs (AtTOC33 and AtTOC34). Different paralogs exhibit distinct expression profiles and form functionally different TOC complexes, allowing the chloroplast to maintain import of non-abundant, housekeeping proteins while simultaneously import- ing highly abundant photosynthetic subunits (Bauer et al., 2000). In Arabidopsis, TOC33 was proposed to be involved preferentially in the import of photosynthetic proteins, that are deficient in the 

atTOC33

mutant, leading to a pale green phenotype especially in the early stages of leaf development when the capacity of plastids to import proteins has to be maximal to ensure the assembly of the photosynthetic apparatus (Jarvis et al., 1998; Kubis et al., 2003). TOC33 can substitute for TOC34 in the 

atTOC34

mutant; however the double homozygous 

TOC34

/ 

TOC33

mutation was found to be embryo lethal in Arabidopsis, suggesting an essential function provided by TOC34/TOC33 (Constan et al., 2004). Differently, because of the presence of a single copy of TOC34 in wild-type 

C. reinhardtii

only one TOC complex is reasonably assembled.

TOC34 is a core component of the TOC complex, firmly interacting with the other subunits (Waegemann and Soll, 1991). Impaired biogenesis of TOC34 in the 

ars1

mutant could thus compromise overall translocon function. However, the present 

ars1

mutant which has a pale green/yellow phenotype that resembles the reported phenotype of the

Figure 8. Localization of ARSA1 protein. (a) Reaction of the  

α-ARSA1

antiserum (1:4000) versus 

cw15

and 

as1

total cell extract (10 μg protein per lane). (b) Localization of ARSA1 in 

Chlamydomonas reinhardtii

cell fractions. Samples from the cell fractionation procedure as described in Experimental Procedures (10 μg protein per lane) were run in a 12% Laemmii SDS-PAGE gel, blotted on a polyvinylidene difluoride membrane and revealed using specific antibodies directed to the following marker proteins: LHCB4 (thylakoid membranes); NR, nitrite reductase (chloroplast stroma); CPLX1 (mitochondria); BIP2 (endoplasmic reticulum); UDP-pyruvoglucosidase (cytosol).

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atTOC33 mutant rather than the double mutant atTOC34/TOC33 (Gutensohn et al., 2004), still accumulates 8% of the wild-type chlorophyll level and is photosynthetically active, particularly in high-light conditions. Interestingly, accumulation of phosphoribulose kinase (PRK), a nucleus-encoded chloroplast protein involved in the Calvin–Benson cycle, is largely unaffected in the mutant (Figure 4B). Similarly, the levels of some photosynthetic components, namely Rubisco as well as the cytochrome b6f complex and ATP synthase, that comprise subunits encoded by both nuclear (Rubisco large chain) and chloroplast genes (ATP, cytochrome f), are unaffected or reduced to a lesser extent than the observed depletion in chlorophyll-binding polypeptides of the photosystems (Figures 4 and S2), suggesting import of the required nucleus-encoded assembling partners, despite the arsa1 mutation. Similar to the case of photosystems I and II, accumulation of these protein complexes is dependent on the presence of all assembling partners and the expression of chloroplast-encoded subunits is controlled by nucleus-encoded factors (Albus et al., 2010; Rahire et al., 2012). These results suggest residual chloroplast protein import to be the primary factor in determining the as1 phenotype, that could derive from somehow lasting TOC activity catalyzed by TOC159, which is accumulated at similar level in cw15 and as1 (Figure 7c). Alternatively, chloroplast protein import might be less dependent on TOC34 activity in algae than it is in plants (Constan et al., 2004). Although the TOC/TIC translocon is the generally acknowledged import pathway into chloroplasts, an alternative import mechanism was reported for glycosylated proteins that are imported into plastids by first going through the endomembrane system in particular conditions, such as in the presence of an impaired TOC function in as1 mutant. This hypothesis would require further investigations but would also make the present mutant a valuable model for studying chloroplast protein import, that could shed light on unexpected complexity.

EXPERIMENTAL PROCEDURES

Chlamydomonas strains and culture conditions

The wild-type strain utilized was cw15 mt~ (mating type minus) (Harris, 1989). Insertion mutagenesis was performed using cw15 mt~ as the genetic background, as described in Bonente et al. (2011). Wild-type S34 mt+, kindly provided by F. A. Wollman (UMR CNRS/UMPC Institut de Biologie Physico-Chimique, Paris, France) was employed for backcross analysis (Harris, 1989). The as1 mutant was transformed by electroporation (Shimogawara et al., 1998) with ARSA1 cDNA (accession AV644541, clone HCL090c04; Kaszu Institute, Tokyo, Japan) (Asamizu et al., 2004), excised from pBluescript II SK+ by digestion with KpnI and BamHI. All C. reihardtii strains were grown at a controlled temperature of 25°C, with 45 r.p.m. agitation in a 16-h light/8-h dark photoperiod in TAP or minimal HS medium (Harris, 1989). For the growth tests in Figure 6,2 ml of 1 x 10^4 or 1 x 10^5 cells ml^-1 were inoculated on TAP or HS medium and subjected to the light conditions indicated.

DNA extraction, sequencing and PCR analysis

Isolation of genomic DNA was performed according to the manufacturer's manual (Purification of total DNA from plant tissue, mini protocol, Qiagen, http://www.qiagen.com/). Primers ARSA1_Fw Gen5′ and ARSA1_Rv Gen5′ amplify a 500 pb fragment at the 5′ end of the gene, ARSA1_Fw Gen3′ and ARSA1_Rv Gen3′ amplify a 500 pb fragment at the 3′ end of the gene, which is deleted in the mutant, and were designed to include introns. Primers ARSA1_Fw CDS3′ and ARSA1_Rv CDS3′ were designed according to the coding sequence of the cDNA clone (Kaszu Institute, Tokyo, Japan) and used to confirm the transformants generated by the complementation experiment. For the sequence of the aforementioned primers see Table S1. Genomic DNAs from Chlamydomonas algae were extracted from nuclei using a modified protocol developed from Zhang et al. (1995) for plant nuclei. Library preparation was performed using Illumina DNA-seq Sample Preparation protocol (Illumina, Inc., http://www.illumina.com/) starting from 4 μg of nucleus-extracted genomic DNA. Libraries were quality tested and quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, http://www.agilent.com/), then processed with the Illumina Cluster Generation Station following the manufacturer’s recommendations. Sequencing of the genomic DNA of as1 was carried on an illumina® platform as described. The illumina® Genome Analyzer Ilx was programmed to run for 76 sequencing cycles in the pair-end set-up. Raw data were processed using Illumina Pipeline v. 1.5. The software SOAP v. 2.20 was used to align the 100 bp reads against the C. reinhardtii genome sequence available online on the NCBI website (http://www.ncbi.nlm.nih.gov/) (Li et al., 2009). The software BLAT was used to distinguish putative genomic regions flanking a pSL16 plasmid sequence into a read in order to identify insertion sites used to construct the map in Figure 1a.

Pigment analysis

Chlorophyll and carotenoids were extracted in 80% acetone. The absorption spectrum was recorded using an Aminco DW-2000 spectrophotometer (Oils, http://www.oilsweb.com/) and fitted with spectra of individual pigments (Croce et al., 2002).

Protein analysis

The unstacked thykalois used for the SDS-PAGE in Figure 4a were obtained from cultures grown in TAP at 50 μM photons m^-2 sec^-1. Cells were collected during the exponential phase, frozen in liquid nitrogen and resuspended in 0.1 x Tricine KOH pH 7.8, 0.5% milk powder before sonication (two cycles of 5 sec). The sample was centrifuged for 10 min at 10 000 g and the pellet was resuspended in 25 μM HEPES KOH pH 7.5, 10 mM EDTA. Debris was removed by centrifugation for 1 min at 500 g and finally thykalois were collected at 10 000 g and resuspended in 25 μM HEPES KOH pH 7.5, 10 mM EDTA, 50% glycerol. Thykalois
polypeptides were diluted in loading buffer (1% running buffer, 2% SDS, 5% β-mercaptoethanol, 10% glycerol), analysed by TRIS-sulfate SDS-PAGE 10-20% (Bassi and Wollman, 1991) and visualized by Coomassie staining. For immunoblots, total protein extracts were obtained from cells grown in TAP under 50 μm photons m⁻² sec⁻¹ illumination, collected and resuspended in loading buffer. Protein concentration was determined by colorimetric measurement with biocinchonic acid (Pierce, http://www.piercenet.com/). The antibodies against Arabidopsis TOC34 and BiP2 were purchased from Agrisera (http://www.agrisera.com/).

Sample preparation for transmission electron microscopy

Chlamydomonas reinhardtii cells grown in TAP medium under 50 μm photons m⁻² sec⁻¹ illumination were harvested during the exponential growth phase and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer for 2 days at 4°C and rinsed three times for 30 min each with 0.1 M cacodylate buffer. Cells were then post-fixed for 2 h at 4°C using 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in increasing concentrations of ethanol (up to 100% ethanol) and embedded in araldite. Ultrathin sections of 40 nm were examined with a FEI Tecnai T12 electron microscope operating at 100 kV accelerating voltage at the Department of Biology, University of Padova.

Recombinant protein expression, purification and antibody production

The ARSA1 sequence encoding the mature protein was amplified by RT-PCR with the ARSA1_FW and ARSA1_Rv HindIII primers (Table S1) and cloned into the BamHI and HindIII restriction sites of the pRSFduet-1 vector (Millipore, http://www.millipore.com/) for expression in BL21 E. coli. The protein eluted with 250 mM imidazole. The ARSA1 recombinant protein was further purified from low-level contaminants by SDS-PAGE followed by electroelution of the excised Coomassie-stained band. In total, 100 μg of the purified protein was combined with Freund’s adjuvant to form a stable emulsion that was injected subcutaneously into a rabbit. This procedure was repeated after 3 weeks, and 1 week after injection blood was collected from the central ear artery and the antibody’s titer was checked by immunoblot.

Cell fractionation and immunoassay analysis

Cw15 cells were mildly broken by passing through a 27-gauge needle at a low flow rate (Mason et al., 2006). Unbroken cells and chloroplasts were pelleted at 3000 g for 5 min while the supernatant was submitted to serial rounds of centrifugations at, respectively 20,000 g, 40,000 g and 100,000 g in order to pellet different microsomal fractions. Intact chloroplasts and mitochondrial preparations were obtained according to the procedure described in Jans et al. (2008). Highly purified thylakoids were obtained as previously described (Bassi and Wollman, 1991).

The protein content of the cell fractions was determined by biocinchonic acid protein assay (Pierce) and the same amounts of proteins were loaded on 12% acrylamide gels for SDS-PAGE (Laemmli, 1970). Proteins were then transferred to polyvinylidene difluoride membranes and immunostained with different antibodies directed versus marker antigens for distinct cell compartments: LHCBl4 (thylakoid membranes), nitrite reductase (chloroplast stroma), CPMX1 (mitochondria), BiP2 (endoplasmic reticulum), UDP-pyrogulosidase (cytosol).

ACKNOWLEDGMENTS

The authors thank the Centro di Genomica Funzionale of the University of Verona for the help in analyzing ILLUMINA® genome sequence data obtained at the Institute of Applied Genomics, University of Udine, by Professor Michele Morgante. Professor Claire Remacle (Université de Liège) is thanked for help with isolation of intact chloroplasts and mitochondria and for the kind gift of Cpmx1 antibody. Professor Juergen Soll (Ludwig Maximilian Universität München) is thanked for the kind gift of anti-TOC75 and TOC159 antibodies and Professor Rudolf Tishner (University of Göttingen) for the anti-nitrite reductase antibody. The FP7 EEC project ‘SUNBIOPATH’ and the CARIVERONA foundation for grant “BIO-MASSE DI OGGI E DI DOMANI” are acknowledged for financial support.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Sequence alignment of ARSA-homolog proteins. The cDNA of Chlamydomonas reinhardtii ARSA1.

Figure S2. Immunoblot analysis of photosynthetic polypeptides on thylakoid preparations of cw15 and as1 mutant, loaded on a per chlorophyll basis.

Figure S3. Reactivity of anti-ARSA1 antisera against fractions from differential centrifugation of Chlamydomonas cw15 cells.

Figure S4. Reaction of the as1-ARSA1 antisera versus cw15 and as1 total cell extract and versus ARSA1 recombinant protein purified from Escherichia coli by Ni⁺⁺ column.

Table S1. Primers used to characterize the arsa1 mutation and for cloning and expression of recombinant ARSA1 in Escherichia coli.

REFERENCES


