The ARG9 Gene Encodes the Plastid-Resident N-Acetyl Ornithine Aminotransferase in the Green Alga Chlamydomonas reinhardtii

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Here we report the characterization of the Chlamydomonas reinhardtii gene ARG9, encoding the plastid resident N-acetyl ornithine aminotransferase, which is involved in arginine synthesis. Integration of an engineered ARG9 cassette in the plastid chromosome of the nuclear arg9 mutant restores arginine prototrophy. This suggests that ARG9 could be used as a new selectable marker for plastid transformation.

In the green alga Chlamydomonas reinhardtii, the arg9-1 and arg9-2 mutations result in arginine auxotrophy because of a deficiency in N-acetyl ornithine aminotransferase activity (NAOAT) (6). Of the two arg9 mutants originally isolated (6), only the arg9-2 strain was found to be an arginine auxotroph, while the arg9-1 mutant had reverted to wild type. We reasoned that the arg9-2 mutation mapped to the structural gene for NAOAT and identified a candidate ARG9 gene (XP_001698091), based on the similarity of the predicted gene product to the Saccharomyces cerevisiae NAOAT, Arg8p. Three full-length cDNAs were sequenced and identified. Both the ARG9 genomic DNA and full-length cDNAs restored arginine prototrophy when introduced into the nucleus of the arg9-2 mutant (data not shown). Sequencing the ARG9 genomic locus in the arg9-2 strain identified a G-to-A transition at codon 317, resulting in a glycine-to-arginine mutation at a strictly conserved residue in NAOATs.

Next, we tested if Chlamydomonas ARG9 could functionally replace the Arg8 protein in yeast by expressing the ARG9 cDNA in an arg8-null mutant that is deficient in NAOAT. Figure 1 shows that expression of the ARG9 cDNA from the plasmid-borne PGK1 promoter is able to partially restore arginine prototrophy. Since the yeast arg8 mutant can be complemented by the Chlamydomonas ARG9 protein, it is likely that the algal protein expressed in yeast is targeted to the mitochondria, where NAOAT typically functions in fungi (10). Indeed, the N-terminal extension of the candidate ARG9 protein exhibits features typical of a plastid- or mitochondrial-targeting sequence, such as the propensity to form an amphiphilic α-helix (7).

The sublocalization of the ARG9 protein was examined by immunoblot analysis using an antibody raised against Arg8p, the S. cerevisiae NAOAT that is resident in the mitochondrial matrix. The anti-Arg8p antibody cross-reacted with species in mitochondrial and plastid fractions of Chlamydomonas cells (Fig. 2). We identified the 48-kDa species in the plastid fraction as the ARG9 protein, based on the predicted size of the mature protein. This species was also present in the arg9-2 strain, suggesting that the arg9-2 mutant accumulates a non-functional ARG9 protein. The cross-reacting species detected in the mitochondrial fraction have higher electrophoretic mobilities and could correspond to nonspecific bands, splicing variants of the ARG9 transcript that specify a mitochondrial protein or dually targeted ARG9 protein. Complementation experiments described below indicate that the primary site of action of ARG9 is the plastid. Based on our analyses, we concluded that NAOAT is located in the plastid in Chlamydomonas, but we cannot exclude the possibility that it also operates in the mitochondrion. The operation of plastid-localized enzymes involved in arginine biosynthesis in Chlamydomonas is also supported by studies suggesting that argininosuccinate lyase could also be resident in the chloroplast (1).

We reasoned that the ARG9 gene relocated to the plastid chromosome of an arg9-2 strain should be able to restore arginine prototrophy if the ARG9 protein is successfully expressed and active in the organelle. For this experiment, we chose the ARG9 cDNA from Arabidopsis thaliana, whose nuclear genome has a codon bias (44% GC) closer to that of the plastid genome than the nuclear genome of Chlamydomonas.

FIG. 1. Heterologous functional complementation of the S. cerevisiae arg8 mutant by the C. reinhardtii ARG9 cDNA encoding NAOAT. The yeast arg8 mutant (NB880) was transformed by the yeast expression vector pFL61, by pFL61/ARG8 (expressing the yeast ARG8 gene), and by pFL61/ARG9 (expressing the Chlamydomonas ARG9 cDNA). Dilution series (10×) of each transformant were plated on synthetic complete medium with or without arginine and incubated at 28°C for 7 or 14 days, respectively.

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Moreover, the frequency of codons in the *Chlamydomonas* plastid genes made it likely that the *Arabidopsis* cDNA would be translated by the set of plastid tRNAs. Using an existing spectinomycin resistance cassette (*aadA*) for plastid transformation, we designed an *arg9* (c for chloroplast) cassette for expression of the *Arabidopsis* NAOAT in the chloroplast of *Chlamydomonas* (3). As a proof of concept, we first targeted the *aadA* cassette to an integration site on the plastid chromosome that is neutral with respect to chloroplast function (4). A spectinomycin-resistant (Spec) transformant was generated by using an antibody against a known protein resident in the mitochondria (Aox) or in the chloroplast (cyt). Lanes 1 to 3, *arg9*-2 transformants complemented by genomic *ARG9*; lane 4, *arg9*-2 mutant.

![FIG. 2. ARG9 localizes to the plastid in Chlamydomonas.](image)

The purity of each fraction was verified by using an antibody against a known protein resident in the mitochondria (Aox) or in the chloroplast (cyt). Lanes 1 to 3, *arg9*-2 transformants complemented by genomic *ARG9*; lane 4, *arg9*-2 mutant.

![FIG. 3. Plastid transformation of the *arg9*-2 strain with the *arg9* cassette restores arginine prototrophy.](image)

(A) Molecular analysis of the plastid transformants was performed by PCR using diagnostic primers (F and R) lying outside the region of homology in the transforming DNA (see below). PCR amplification products were separated by electrophoresis in agarose gel and ethidium bromide stained. The gel was imaged using an imaging system (Kodak Image Station 2000R). PstI-digested λ phage DNA was used as a size marker. DNAs extracted from strain CC125 (wild type [WT]), an *arg9*-2 Spec cDNA would be translated by the set of plastid tRNAs. Using an existing spectinomycin resistance cassette (*aadA*) for plastid transformation, we designed an *arg9* (c for chloroplast) cassette for expression of the *Arabidopsis* NAOAT in the chloroplast of *Chlamydomonas* (3). As a proof of concept, we first targeted the *aadA* cassette to an integration site on the plastid chromosome that is neutral with respect to chloroplast function (4). A spectinomycin-resistant (Spec) transformant was generated by using an antibody against a known protein resident in the mitochondria (Aox) or in the chloroplast (cyt). Lanes 1 to 3, *arg9*-2 transformants complemented by genomic *ARG9*; lane 4, *arg9*-2 mutant.

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using diagnostic primers lying outside the regions of homology between the aadA and arg9c cassettes and were found to contain the arg9c cassette (Fig. 3A and B). As expected, the clones were still arginine prototrophs but had lost the spectinomycin resistance (data not shown), indicating that they had become homoplasmic for the arg9c cassette. This suggests that the arg9c cassette was able to replace the aadA cassette in the plastid chromosome and could be successfully expressed to complement the arg9-2 mutation. Interestingly, we found that the level of growth on medium without arginine of the plastid transformants expressing arg9c was comparable to that of nuclear transformants complemented with ARG9 genomic DNA (Fig. 3C).

To provide additional proof of the successful relocation of the arg9 gene to the plastid chromosome, we tested for non-Mendelian segregation of the arginine prototrophy phenotype in the plastid transformants. In genetic crosses of C. reinhardtii, nuclear markers segregate according to Mendelian genetics, but chloroplast genes are inherited uniparentally from the mt+ parent (5). Hence, it is expected that the Arg+ trait can be transmitted to the progeny only if the plastid transformant is of the mt+ sexual type. We crossed an Arg+ plastid transformant (mt+ arg9-2 arg9c) to an mt− arg9-2 mutant and performed bulk segregation of the progeny. As expected, all 40 spores examined in the progeny were arginine prototrophs because they inherited the arg9c cassette from the mt+ parent. An Arg+ (mt− arg9-2 arg9c) spore from this progeny was then used to do the reciprocal cross with the mt+ arg9-2 mutant. We examined 42 spores, and all of them were arginine auxotrophs, as expected, because the plastid trait cannot be transmitted by the mt− parent.

In order to show functional complementation of the arg9-2 mutation, we prepared cellular extracts and measured NAOAT activity in both plastid transformants and nuclear transformants complemented with Chlamydomonas ARG9 genomic DNA. The arg9-2 mutation results in very little detectable NAOAT activity, while the enzymatic activity was significantly restored in both nuclear and plastid transformants (Fig. 4). We concluded that integration of the A. thalliana ARG9 cDNA into the plastid chromosome of C. reinhardtii resulted in the successful expression of an enzymatically active NAOAT. Our results indicate that the arg9c cassette could be developed as a marker for chloroplast transformation in Chlamydomonas. The manipulation of the chloroplast genome of microalgae for the commercial production of recombinant molecules is a recent and promising advance in biotechnology (8). For obvious reasons, the use of arginine as selection for plastid transformation will be of significant value, considering that all markers employed so far are derived from bacterial antibiotic resistance genes (8).

The arg9-2 mutation is a nonreverting mutation (≤10−10). We took advantage of this property and tested the effectiveness of the ARG9 gene as a marker for insertional mutagenesis in the nucleus. In Chlamydomonas, integration of transforming DNA into the nuclear genome occurs via nonhomologous recombination events that are presumed to occur at random loci (2). Thus, nuclear markers such as the ARG7 gene that encodes argininosuccinate lyase are routinely used as tool to generate insertional mutants (2). About 3,000 arginine prototrophic transformants were generated by using the arg9-2 mutant as a recipient strain and the ARG9 gene as a transforming DNA. Since we are interested in a separate project, isolating mutants deficient for mitochondrial function, we screened for candidate mutants on the basis of their slow-growth phenotype in the dark, a phenotypic trait of complex I-deficient mutants (9). Out of 3,000 insertional transformants, two arginine prototrophic mutants displaying a slow-growth phenotype in the dark. Co-segregation of the slow-growth phenotype with the Arg+ trait was observed, suggesting that insertion of the ARG9 gene interrupts a gene controlling respiration. However, neither mutant was deficient for complex I activity, as determined by enzymatic measurement or in-gel staining (data not shown).

We concluded that the slow-growth phenotype is probably due to a defect in other respiratory enzymes, but this was not investigated further. In conclusion, our results show that ARG9 could also be used efficiently as an insertional marker to generate nuclear mutants of interest.
Nucleotide sequence accession number. The cDNAs sequenced in this study have been deposited in the GenBank database under accession number EU711276.

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