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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 identified and sequenced. 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 mitochondrion-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 nonfunctional 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*.

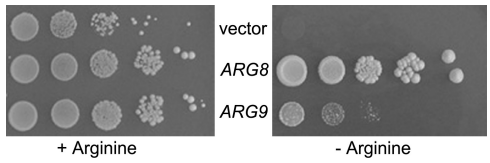

FIG. 1. Heterologous functional complementation of the *S. cerevisiae arg8* mutant by the *C. reinhardtii ARG9* cDNA encoding NAOAT. The yeast *arg8* mutant (NB880) was transformed by the yeast expression vector pFL61, by pFL61/*ARG8* (expressing the yeast *ARG8* gene), and by pFL61/*ARG9* (expressing 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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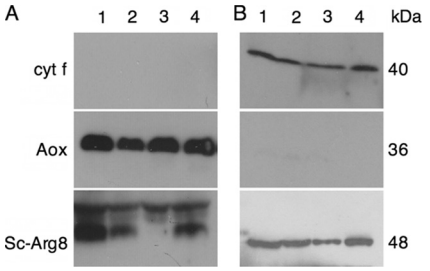

FIG. 2. ARG9 localizes to the plastid in *Chlamydomonas*. Proteins from mitochondrion-enriched (A) and chloroplast-enriched (B) fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immobilized on a nylon membrane. The membrane was immunodecorated using antibodies against Arg8p, Aox, or cytochrome *f*. 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 *f*). Lanes 1 to 3, *arg9-2* transformants complemented by genomic *ARG9*; lane 4, *arg9-2* mutant.

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* (*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^r) transformant was generated by biolistic transformation of the *mt*$^+$ *arg9-2* mutant (4). The presence of the *aadA* gene at the expected location in the plastid chromosome was detected by diagnostic PCR (Fig. 3A and B). Next, we replaced the *aadA* cassette with the *arg9^c* cassette, which expresses the *A. thaliana* ARG9 protein from the same promoter and terminator as the *aadA* cassette (plasmid KS/*arg9^c*) (Fig. 3B). Based on our alignments of NAOATs, we reasoned that the first 48 residues of ARG9 might include the plastid-targeting sequence and were therefore not necessary if ARG9 is synthesized on plastid ribosomes. Replacement of the *aadA* marker by the *arg9^c* cassette is expected to occur by homologous recombination between the *atpA* promoter and *rbcL* terminator sequences, which are common to the *aadA* and *arg9^c* cassettes (Fig. 3B). The *mt*$^+$ *arg9-2* Spec^r strain was bombarded with the pKS/*arg9^c* plasmid and transformants were selected on medium lacking arginine. A few transformants were able to grow on the selective medium (Arg$^+$ transformants), suggesting that ARG9 was successfully expressed from the plastid genome. Molecular analysis of two independent Arg$^+$ transformants showed that they were heteroplasmic for the presence of the *aadA* and *arg9^c* cassettes and were also Spec^r, as expected (data not shown). In a previous study, we showed that the segregation of plastid markers is facilitated if heteroplasmic transformants are converted to gametes, presumably because gametes undergo a reduction of the copy number of their chloroplast genomes (4). To achieve homoplasmy for the *arg9^c* marker, we induced gametogenesis of two *arg9-2* Spec^r Arg$^+$ plastid transformants and retrieved vegetative clones by plating the gametes on medium without arginine. Two independent clones were further analyzed by

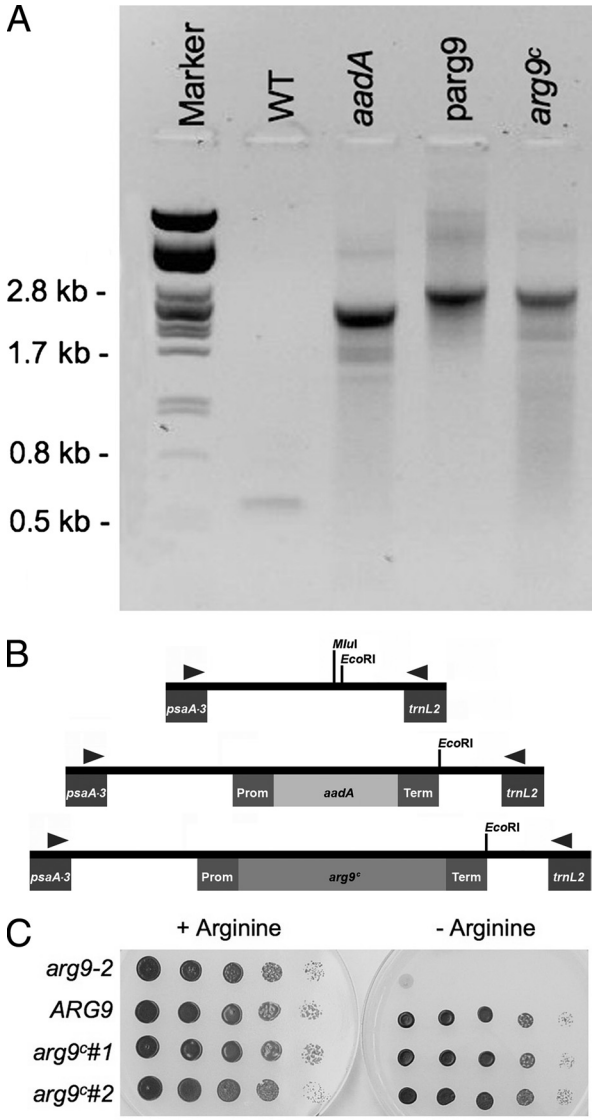

FIG. 3. Plastid transformation of the *arg9-2* strain with the *arg9^c* cassette restores arginine prototrophy. (A) Molecular analysis of the plastid transformants was performed by PCR using diagnostic primers (F and R) lying outside the region of homology in the transforming DNA (see below). PCR amplification 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^r transformant (*aadA*), an *arg9-2* Arg$^+$ transformant (*arg9^c*), and plasmid pmp-*arg9^c* (parg9) were used as templates in the PCR. The plasmid pmp-*arg9^c* contains the *arg9^c* cassette and 5 kb of chloroplast DNA flanking the region of homology between the *aadA* and *arg9^c* cassettes. (B) Illustration of expected PCR products using the diagnostic primers F (left arrowhead) and R (right arrowhead) and plastid DNAs from the wild-type strain (WT), Spec^r transformant (*aadA*), and Arg$^+$ transformant (*arg9^c*) as templates. The neutral site lies between the *psaA-3* and *trnL2* genes on the chloroplast genome. The MluI site is lost in the cloning of the *aadA* cassette. The expected sizes for the PCR products are 600 bp (WT), 2,533 bp (*aadA*), and 2,971 bp (*arg9^c*). (C) Growth of the untransformed *arg9* mutant (*arg9-2*), *arg9-2* transformed by pARG9g (containing a 7-kb genomic DNA fragment with the *ARG9* gene), and two independent *arg9-2* plastid transformants expressing the *arg9^c* cassette (*arg9^c*#1 and *arg9^c*#2) on unsupplemented and arginine-supplemented Tris-acetate-phosphate medium. Cells (4×10^6 for each strain) were serially diluted (10 times for each dilution), plated, and incubated for 4 days.

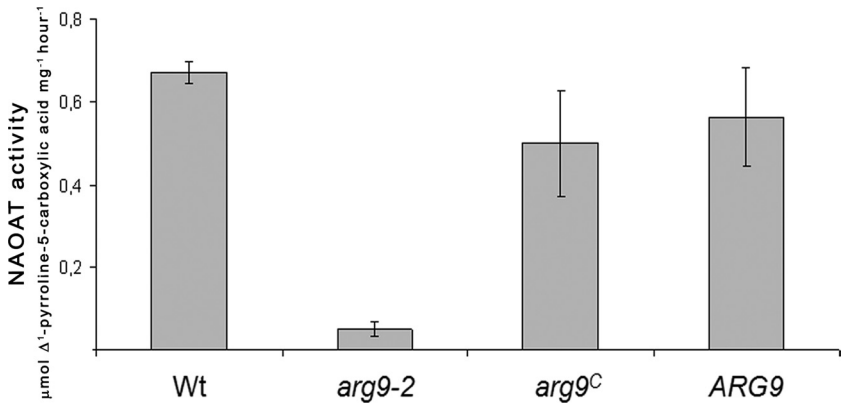

FIG. 4. Restoration of NAOAT activity in nuclear and plastid transformants. Soluble extracts of the *cw15 mt*$^-$ strain (wild type [Wt]), the *cw*$^-$ *arg9-2* strain, the *arg9-2* strain transformed with pARG9g (*ARG9*), and the *arg9-2* strain transformed with the *arg9*^{c} cassette (*arg9*^{c}) were assayed. Results for only one representative of each plastid or nuclear transformant are shown. For each strain, at least three biological replicates were included in the experiments. Error bars indicate standard deviations.

using diagnostic primers lying outside the regions of homology between the *aadA* and *arg9*^{c} cassettes and were found to contain the *arg9*^{c} 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 *arg9*^{c} cassette. This suggests that the *arg9*^{c} 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 *arg9*^{c} 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*^{c} 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 arg9*^{c}) 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 *arg9*^{c} cassette from the *mt*$^+$ parent. An Arg$^+$ (*mt*$^-$ *arg9-2 arg9*^{c}) spore from this progeny was selected, and diagnostic PCR confirmed the presence of the *arg9*^{c} cassette in the chloroplast genome (data not shown). This spore 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. thaliana ARG9* cDNA into the plastid chromosome of *C. reinhardtii* resulted in the successful expression of an enzymatically active NAOAT. Our results indicate that the *arg9*^{c} 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 prototrophs displayed a slow-growth phenotype in the dark. Cosegregation 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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