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Article

Insertional Mutagenesis as a Strategy to Open New Paths in Microalgal Molybdenum and Nitrate Homeostasis

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Abstract: Molybdenum (Mo) is a vital micronutrient for nearly all living organisms, serving as a cofactor for molybdoenzymes that catalyze essential redox reactions in nitrogen metabolism. Among these enzymes nitrate reductase plays a crucial role in nitrate assimilation. Maintaining Mo homeostasis—including uptake, storage, and utilization—is critical to avoid both deficiency and toxicity. Our research focuses on uncovering novel molecular components involved in Mo homeostasis, particularly in connection with nitrate assimilation, using *Chlamydomonas reinhardtii*, a model green microalga. To achieve this, we generated over 5,000 *Chlamydomonas* transformants through insertional mutagenesis using a paromomycin resistance cassette (*AphVIII*) and screened them for altered growth on nitrate and under different Mo concentrations. We identified four strains showing altered growth pattern when using nitrate as a nitrogen source or exhibiting increased sensitivity or resistance to Mo. The genomic alterations in these strains were identified. Notably, both a Mo-resistant and a Mo-sensitive transformant had disruptions in genes encoding ABC-type transport proteins, indicating a potential role for these proteins in Mo transport. Additionally, two strains were unable to grow on nitrate. One of them had a mutation in the *CNX7*, a gene involved in Mo cofactor biosynthesis, while the other had a mutation in *BAT1*, an amino acid transporter. The *BAT1* mutant represents an interesting case study, as this gene has not previously been associated with nitrate metabolism. These findings enhance our understanding of Mo and nitrate homeostasis mechanisms and open new paths for engineering microalgae with improved nitrogen assimilation.

Keywords: *Chlamydomonas*; nitrogen; transformation; ABC superfamily; BAT1

1. Introduction

Microalgae have emerged as a significant source of innovation in various fields, including bioremediation, biofuel production, biomass generation, biofertilizer development, and the creation of high-value-added products [1]. Among microalgae, the unicellular green alga *Chlamydomonas reinhardtii* (hereafter referred to as *Chlamydomonas*) has become a key focus of study, with numerous important advancements being made across these areas [2]. *Chlamydomonas* is used as a model organism for studying various processes, including chloroplast dynamics, photosynthesis, mineral nutrition, cell cycles, and flagellar biology, among others [3]. Some advantages of using *Chlamydomonas* are that it is easy to cultivate under various conditions, including in synthetic mineral media, and it reproduces rapidly, with a generation time of about 8 hours, allowing for quick biomass production [4]. Moreover, a wide variety of laboratory techniques are available for working with *Chlamydomonas*, including methods in molecular biology, physiology, and biochemistry. Additionally, this organism can undergo both asexual and sexual reproduction, facilitating genetic studies. *Chlamydomonas* also has a fully sequenced haploid genome, and stable mutants can be generated and isolated [5].

All organisms require mechanisms to maintain an adequate concentration of essential metals within their cells [6]. Situations may arise where certain metals are either scarce, leading to deficiency, or present in excess concentrations, causing potentially lethal toxicity. Therefore, it is crucial to ensure the cellular availability of the metals while preventing the potential toxic effects of their accumulation [7]. This set of mechanisms is known as metal homeostasis. Metal homeostasis is a highly regulated process ensuring proper metal uptake, storage, transport, and distribution [8]. It involves specialized mechanisms for absorption from the environment, intracellular compartmentalization, and long-distance transport. Additionally, all these processes must be regulated and coordinated to match the biological demand for the nutrient and its availability [9]. For this reason, organisms have developed metals storage systems through compartmentalization or by binding to small molecules, such as proteins or organic acids, which help create metal reserves while preventing their toxic effects [10].

In the case of Molybdenum (Mo), it is a biologically active transition metal that plays a crucial role in the active sites of various enzymes (molybdoenzymes) found in bacteria, fungi, plants, and animals [11]. The importance of Mo in living organisms was first studied by Arnon & Stout using tomato plants [12]. They observed that plants grown in purified nutrient solutions—designed to remove metal contaminants—developed deficiency symptoms leading to impaired growth. However, when these plants were treated with a diluted solution of molybdic acid, the deficiency symptoms disappeared, allowing for recovery and the resumption of normal growth. Subsequent research has established the role of Mo in promoting plant growth and its involvement in redox processes related to nitrogen and sulfur metabolism, phytohormone biosynthesis, and xenobiotic detoxification [13].

All cells take up Mo in the form of the oxyanion molybdate (MoO_4^{2-}), which is the most abundant form of Mo in aqueous solution at pH above 4.2 [14]. In bacteria, Mo uptake is mediated by different protein systems from the ABC superfamily [15]. In eukaryotes, two molybdate transport systems are known: MOT-1 and MOT-2, both first identified in *Chlamydomonas*. Neither of these proteins shows similarity to members of the ABC superfamily [16,17]. In general, Mo enzymes catalyze redox reactions that involve the transfer of two electrons to or from a substrate, accompanied by the transfer of an oxygen atom, either derived from or incorporated into water. This process changes the oxidation state of Mo from IV to VI [18]. In prokaryotes, Mo in the form of iron-molybdenum cofactor (FeMo-co) is essential for nitrogenase, the key enzyme involved in biological nitrogen fixation [19]. In eukaryotes, five molybdoenzymes have been found: nitrate reductase (NR), sulfite oxidase (SO), aldehyde oxidase (AO), xanthine dehydrogenase (XDH), and the mitochondrial amidoxime reducing component (mARC) [20]. In these enzymes, Mo must be coordinated with molybdopterin (MPT), which is a complex heterocycle featuring a pyran fused to a pterin ring, to become biologically active, forming the Mo cofactor (Moco) [21]. In plants, the proteins involved in Moco synthesis are designated as CNX.

NR is involved in the assimilation of nitrate as a nitrogen source. It catalyzes the reduction of nitrate to nitrite, which is then further reduced to ammonium and incorporated into carbon skeletons to form amino acids. It functions as a homodimer, and each monomer, contains three prosthetic groups: Moco in the N-terminal domain, citb5/heme in a central domain, and flavin adenine dinucleotide (FAD) in the C-terminal domain [22]. The mARC protein is involved in the reduction of N-hydroxylated compounds and has been reported to participate in the synthesis of nitric oxide from nitrite [23]. To carry out its catalytic activity, the mARC protein requires other associated proteins as electron donors [24]. SO detoxifies sulfite to sulfate in sulfur metabolism. Its structure varies across organisms: animal SO, found in mitochondria, has both heme and Moco domains; plant SO, located in peroxisomes, only has the Moco domain; *Chlamydomonas* possesses a SO with both domains [25]. In humans, Mo deficiency is primarily linked to the loss of SO activity, resulting in sulfite accumulation and disruptions in the catabolism of sulfur-containing amino acids. This leads to severe neurological degeneration, which typically causes premature death within the first few weeks of life [26]. XDH catalyzes purine degradation by sequentially oxidizing hypoxanthine to xanthine and xanthine to uric acid. This enzyme functions as a dimer, each monomer containing three domains: an

N-terminal Fe₂-S₂ cluster, and FAD domain, and a C-terminal Moco domain [27]. AO derives from a duplication of the *XDH* gene, with both showing some sequence similarity and prosthetic groups [28]. However, AO exhibits greater substrate specificity than XDH, with its substrates being purines, pteridines, aldehydes, and aromatic and aliphatic heterocycles [29].

In plants, deficiency in Mo, and consequently in its cofactor, leads to the loss of activity in these enzymes and the inability to grow on nitrate as a nitrogen source [30]. In summary, without Mo, plants cannot efficiently process nitrogen, which severely affects their growth and development. Engineering plants for efficient nitrogen assimilation is a major challenge, but it has the potential to significantly reduce the need for intensive nitrogen fertilization in agricultural soils [31]. To achieve this goal, it is essential to understand the various elements involved in Mo homeostasis, as it has a critical role for nitrate assimilation. However, most of the Mo homeostasis components are still unknown in plants. Therefore, our primary objective is to identify new potential molecular components involved in Mo and nitrate homeostasis using *Chlamydomonas* as a model organism by obtaining and characterizing insertional mutants affected in these processes. Mutants affected in genes involved in Mo homeostasis are likely less efficient in dealing with Mo toxicity or deficiency conditions, thus showing abnormal growth under these conditions. To obtain Mo- and nitrate-related mutants, a DNA cassette conferring resistance to the antibiotic paromomycin (*AphVIII*) was randomly inserted into the *Chlamydomonas* genome. The growth ability of the resulting paromomycin-resistant transformants was evaluated under different Mo and nitrogen availability conditions, compared to the parental strain. Four transformants were selected for further analysis: two exhibited impaired growth on nitrate and two displayed abnormal responses to Mo, showing either enhanced resistance or increased sensitivity. The identification of the affected genes in these mutants revealed two ABC-type proteins potentially involved in molybdate transport. Additionally, two genes related to nitrate metabolism were identified: *CNX7*, which is involved in Mo cofactor biosynthesis, and *BAT-1*, a putative amino acid transporter whose role in nitrate metabolism remains to be elucidated.

2. Materials and Methods

2.1. *Chlamydomonas* Strains and Growth Conditions

The *Chlamydomonas reinhardtii* strains used in this study included the cell wall-deficient strain 704 [32] and the transformants generated using the *AphVIII* marker. Cells were cultured for 3-6 days in a temperature-controlled chamber at 25 °C under continuous light (15-30 W/m²) provided by white LED panels. Cultures were grown in Tris-Acetate-Phosphate (TAP) medium following previously described methods [4]. Depending on the required volume, 25 mL, 50 mL, or 100 mL Erlenmeyer flasks were used as culture vessels. Strains were maintained on solid TAP medium in sterile Petri dishes and refresh every 2-3 months to ensure viability. For molybdate-free conditions, a trace element solution prepared without adding molybdate was used.

2.2. Growth Test

Growth on solid agar medium. Following transformation, agar plates were incubated in a growth chamber for 7 days. Colonies growing on plates containing both ammonium and paromomycin were selected with sterile toothpicks and resuspended in 20 µL of liquid culture medium. Cells concentration was determined, and 5 µL containing approximately 500 cells were spotted onto agar plates with the corresponding media. After 7 days of incubation under standard growth conditions, plates were photographed to assess colony development. To analyze growth differences in liquid media, a sequential process was carried out, including a pre-inoculum step. For the pre-inoculum, the different *Chlamydomonas* strains under study were taken from solid agar cultures and transferred to liquid medium. These liquid cultures were kept in the growth chamber for 2-3 days until they reached the exponential growth phase. Afterwards, the cells were centrifuged at 3,000 × g for 1 min, washed, and resuspended in fresh medium. Cell concentration was adjusted to

50,000 cells/mL for each condition. After 7 days of growth, chlorophyll concentration was analyzed as an indicator of biomass accumulation.

2.3. Quantification of *Chlamydomonas* Cell Growth

The quantification of *Chlamydomonas* growth was quantified using two complementary approaches: direct cell counting and chlorophyll content measurement. To determine the cell number, an automatic cell counter (Microcellcounter F-500, Sysmex) was used. Cells were diluted in isotonic saline solution at a ratio of 5 μ L of culture per 10 mL of diluent. To quantify chlorophyll concentration, a 1 mL sample of cells was centrifuged, and the cell pellet resuspended in 1 mL of 100 % (v/v) acetone. The suspension was incubated at 4 °C in the dark for 8 hours to allow complete pigments extraction. The sample was then centrifuged again, and the absorbance of the supernatant was measured at 652 nm to estimate total chlorophyll content [33].

2.4. Preparation of the Selectable Marker for Insertional Mutagenesis

For the generation of insertional mutants, the *Chlamydomonas* strain 704 was transformed using the *AphVIII* gene as a selectable marker. This gene confers resistance to the antibiotic paromomycin and has been used in other functional genomics studies in *Chlamydomonas* [34]. The *AphVIII* gene, derived from *Streptomyces rimosus*, encodes an aminoglycoside 3'-phosphotransferase type VIII enzyme that inactivates aminoglycoside antibiotics such as paromomycin. To allow this gene to be expressed in *Chlamydomonas*, its coding sequence was placed under the control of a chimeric *Chlamydomonas* promoter, consisting of the small subunit of rubisco (*RbcS2*) promoter and the *Hsp70A* terminator, which together provide robust transcriptional activity. The resulting plasmid was named pSI104 [35]. For the PCR amplification of the *AphVIII* gene from the pSI104 plasmid, the following primers and conditions were used: Primer pair Parcomp1-F (5'-GCGGGGAGCTCGCTGAGG-3') and Parcomp1-R (5'-CGCTTCAAATACGCCAG-3'). Amplification was performed using PhusionTM High-Fidelity DNA Polymerase (Thermo Scientific). The PCR program consisted of initial denaturation at 98 °C for 30 s, followed by 30 cycles of denaturation at 98 °C for 10 s and combined annealing and extension at 72 °C for 1 min, concluding with a final extension at 72 °C for 5 min (Figure 1A). The resulting PCR product was analyzed on an agarose gel, verified to correspond to the expected size (~2 kb), sequenced to confirm its identity, and quantified. This DNA, hereafter referred to as the *AphVIII* marker, will be used in the subsequent transformation experiment for insertional mutagenesis.

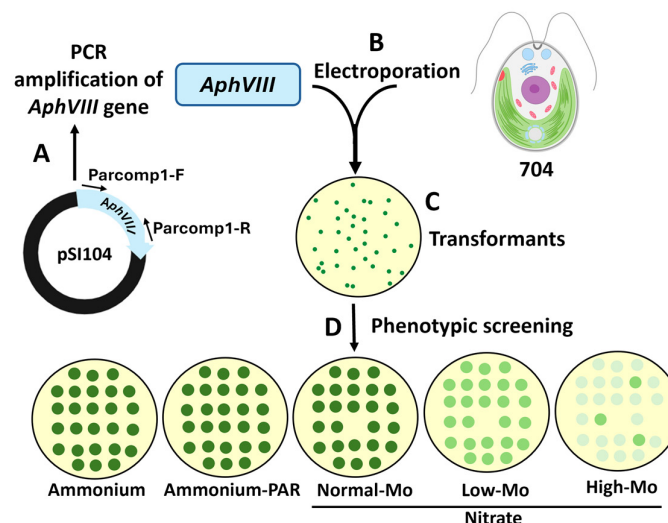


Figure 1. Schematic representation of the transformation process via insertional mutagenesis and selection of the transformants. A) The *AphVIII* marker gene was amplified by PCR from the vector pSI104. Parcomp1-F

and Parcomp1-R are the primers used for the PCR amplification of *AphVIII*. B) The *Chlamydomonas* strain 704 was transformed with *AphVIII* by electroporation. C) illustrates a culture plate with paromomycin 25 µg/mL (PAR) in which transformants resistant to paromomycin can be observed. D) Each of the transformants was grown individually, afterward the growth was analyzed using a drop test on culture plates with the indicated concentrations of ammonium or nitrate and molybdate. Ammonium (8 mM ammonium and 1 µM molybdate), Ammonium-PAR (8 mM ammonium, 1 µM molybdate, and 25 µg/mL paromomycin), Normal-Mo (4 mM nitrate and 1 µM Mo), Low-Mo (4 mM nitrate and no added Mo), High-Mo (4 mM nitrate and 10 mM Mo).

2.5. *Chlamydomonas* Transformation

For the generation of *Chlamydomonas* transformants, a electroporation method was used with some modifications (Figure 1B) [36]. *Chlamydomonas* cells were cultured in liquid medium until they reached the exponential growth phase. They were then harvested by centrifugation at $3,000 \times g$ for 2 minutes and resuspended in TAP medium to a final density of 3×10^6 cells/mL. The transformation was carried out by exposing 250 µL of the cell suspension to an electrical pulse of 600 mV for 20 ms in a 4 mm gap electroporation cuvette. The amount of DNA used in the process was 10 ng. The electric pulse was applied using a Gene Pulser Xcell system (Bio-Rad). After the electroporation procedure, the cells were kept in the cuvette for 10 minutes to facilitate initial recovery. Subsequently, the cells were transferred to a tube containing 10 mL of TAP medium supplemented with 40 mM sucrose and incubated at 25°C on an orbital shaker at 50 rpm under continuous light for 12-16 hours. After the recovery period, the cells were centrifuged at $3,000 \times g$ for 2 minutes. The resulting pellet was resuspended in 500 µL of TAP medium with 40 mM sucrose and plated onto selective medium containing 25 µg/mL paromomycin.

2.6. Genomic DNA Isolation from *Chlamydomonas*

Genomic DNA was isolated using a modified phenol-chloroform method [37]. *Chlamydomonas* cells corresponding to 15 mL of an exponential growing culture were harvested by centrifugation at $3,000 \times g$ for 2 min. The cell pellet was resuspended in 700 µL of lysis buffer (50 mM Tris-HCl, pH 8.0; 5 mM EDTA; 300 mM NaCl). SDS (sodium dodecyl sulfate) was then added to a final concentration of 2 %. The suspension was gently vortexed and incubated for 10 min at 4 °C to facilitate cell lysis. Subsequently, nucleic acid extraction was performed by adding an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1v/v/v) pre-saturated with 50 mM Tris-HCl, pH 8.0. The mixture was vortexed vigorously for 1 min and then centrifuged at $13,000 \times g$ for 5 min at 4 °C (the initial centrifugation was done for 10 minutes at 4°C). The aqueous (upper) phase was carefully collected, and additional extraction was repeated as necessary until a clean interface was obtained. To remove residual phenol, an equal volume of chloroform saturated with water was added to the aqueous phase. The mixture was then vortexed vigorously for 1 min and centrifuged at $13,000 \times g$ for 5 min at 4 °C. Genomic DNA was then precipitated by adding two volumes of cold 100 % ethanol and centrifuged at $13,000 \times g$ for 20 min at 4 °C. The nucleic acid pellet obtained then was washed with 500 µL of cold 70 % ethanol and allowed to dry at room temperature for 10 min. Finally, the genomic DNA pellet was resuspended in 25 µL of nuclease-free water. To remove RNA contamination, 1 µL of RNase (100 µg/mL) was added to each sample.

2.7. DNA Quantification

DNA was quantified using a NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific). Additionally, DNA content was semi-quantitatively estimated through agarose gel electrophoresis. Samples were run alongside λ phage DNA standards of known concentration, and relative quantities were estimated by comparing band intensities under UV illumination.

2.8. Purification of DNA Fragments from Agarose Gels

DNA fragments of interest were excised using a sterile scalpel and transferred to a 1.5 ml tube. Gel-purified DNA was obtained using the NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel), following the manufacturer's instructions.

2.9. Identification of the Genomic Region Adjacent to the *AphVIII* Marker Insertion

The strategy used to identify the *Chlamydomonas* genomic regions adjacent to the insertion of the *AphVIII* marker was an adaptation of the Inverse Polymerase Chain Reaction method [38], with the following modifications. Isolated *Chlamydomonas* genomic DNA was digested with the restriction enzyme *AvaI* (New England Biolabs). This enzyme cuts very frequently in the *Chlamydomonas* genome but only once in the *AphVIII* marker. Genomic digestion was carried out using 1-5 µg of DNA and 10-20 units of *AvaI* enzyme in a final volume of 20 µl, with incubation for 8-16 h at 37 °C. This digestion generated two DNA fragments per insertion sites: one containing the 5' region of the *AphVIII* construct along with a genomic DNA fragment of variable size (left border), and another fragment containing the 3' region of the *AphVIII* construct with the corresponding genomic segment (right border). Following the digestion, the ligation of the DNA fragments was performed using T4 DNA ligase enzyme (Roche) at 16 °C for 16 h. The ligated DNA served as a template for a PCR amplification using specific primers for the *AphVIII* cassette. To minimize non-specific amplifications, a nested PCR approach was used. The primers used for the first round were FL1 (CGCCGGGCTTGCTCGTC) and FL2 (CAGCGTGCTTGACAGATTGACTTG) for the left border and FR1 (CCAGAGCTGCCACCTTGACA) and FR2 (AGCTGGCCACGAGGAGGAC) for the right border. The primers used for the second round were SL1 (TCGGGGTTCGCGGGCTTTTAT) and SL2 (CCCTCCCCGGTGCTGAAGAA) for the left border and SR1 (CCACCACCCCGAAGCCGATAA) and SR2 (TACCGGCTGTTGGACGAGTTCTTCTG) for the right border. In the first round, 1 µl of the ligation mixture was used as the template DNA, and in the second round, 1 µl of the product resulting from the first round was used as the template. In both rounds, a master mix reaction was prepared using a thermostable Taq polymerase (Biotools), following the manufacturer's protocol. The program used in each of the amplification rounds was as follows. For the first round: initial denaturation at 96 °C for 3 min, followed by 25 cycles of: denaturation at 96 °C for 1 min, primer annealing at 60 °C for 1 min, and extension at 72 °C for 2 min. Finally, an extension at 72 °C for 10 min. The conditions for the second round were the same as the first, except for increasing the number of PCR cycles to 40.

2.10. Bioinformatics Tools

The following databases and software tools were used in this study: Phytozome (<https://phytozome-next.jgi.doe.gov/>) for retrieving and analyzing genome sequences of *Chlamydomonas*; and NCBI BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for nucleotide and protein sequence analysis. ClustalW (<https://www.genome.jp/tools-bin/clustalw>) and BioEdit software were used for nucleotide and protein sequence alignments, respectively. The DNASTar (Lasergene) software suite (<https://www.dnastar.com/>) included: SeqMan for managing and analyzing DNA sequencing data and PrimerSelect for primer design. MEME (Multiple Expectation Maximization for Motif Elicitation) (<http://meme-suite.org/>) was employed to identify conserved protein domains. Cello2go (<http://cello.life.nctu.edu.tw/cello2go/>) facilitated the prediction of protein subcellular localization, ProtParam (<https://web.expasy.org/protparam/>) was used to determine the physicochemical properties of proteins based on their sequences, and BioRender (<https://biorender.com/>) was utilized for creating scientific illustrations.

3. Results

3.1. Generation of an Insertional Mutant Collection

The *Chlamydomonas* parental strain selected was the strain 704. The key distinguishing feature of this strain, compared to wild-type *Chlamydomonas*, is that it has a mutation in the genes involved in cell wall formation. This mutation results in a thinner cell wall, enabling highly efficient

transformation and the generation of a large number of transformants [32]. Aside from the cell wall mutation, strain 704 does not possess any additional mutations affecting Mo or nitrate homeostasis, making it an ideal choice for the objectives of this study. The DNA selected for the transformation of strain 704 was the *AphVIII* gene, which confers resistance to the antibiotic paromomycin and serves as a selectable marker. The *AphVIII* gene was amplified by PCR from the plasmid pSI104 (Figure 1A). This marker has been successfully employed in other functional genomics strategies in *Chlamydomonas* [34]. *Chlamydomonas* lacks the ability for homologous recombination, so during transformation, the genetic marker is inserted randomly into its genome [39]. This random insertion can have significant consequences. If the marker is inserted within a gene sequence, it can disrupt the gene's structure and function, potentially causing a mutation.

A total of 150 transformations were performed using *AphVIII*, including several negative controls lacking DNA. One example of the transformation results is shown in Figure 2. On average, 20-50 transformants were obtained per selection plate, yielding approximately 5,200 paromomycin-resistant transformants. The transformation efficiency achieved was approximately 3,500 transformants per μg of DNA, which is highly comparable to the efficiencies reported in previous studies [40]. The transformants were sufficiently separated to allow for individual isolation and long-term preservation of each of them (Figure 2).

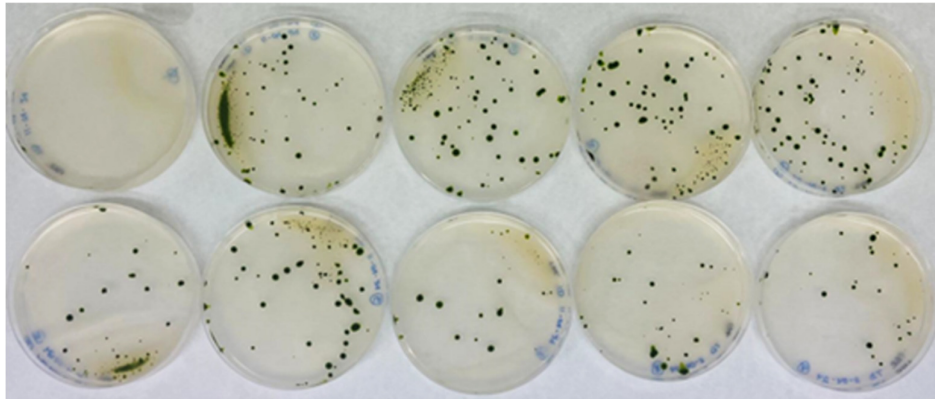


Figure 2. Representative example of transformation plates obtained in the same batch. The figure shows the results of 9 transformations plus a control from the 150 transformations that were performed. The top-left plate shows the control plate, in which the transformation was done without the DNA *AphVIII* marker. The other plates included DNA from the *AphVIII* marker gene in the transformation. The culture medium of the plates was Ammonium-PAR (8 mM ammonium, 1 μM molybdate, and 25 $\mu\text{g}/\text{mL}$ paromomycin). Each of these plates is an actual image corresponding to what is schematically illustrated in Figure 1C.

3.2. Phenotypic Analysis of Transformants in Relation to Mo and Nitrate Homeostasis

Each paromomycin-resistant colony was individually isolated and preserved; from this point onward, they will be referred to as the transformants. The transformants were named based on their position within the selection plate. To analyze potential phenotypes related to Mo homeostasis, the entire collection of 5,200 transformants were spot-cultured on agar plates containing different Mo concentrations and nitrogen sources (Figure 1D). The conditions chosen for the phenotypic screening of the transformants were as follows. The *Chlamydomonas* culture medium with ammonium as nitrogen source and containing the standard concentration of molybdate 1 μM (referred to as Ammonium). A medium identical to the previous one, supplemented with paromomycin to verify the presence of the *AphVIII* marker in the transformants (referred to as Ammonium-Par). *Chlamydomonas* can grow in culture media using either ammonium or nitrate as a nitrogen source. However, Mo is essential only in nitrate-containing media, as it is necessary for the nitrate reductase enzyme, which is crucial for assimilating nitrate as a nitrogen source that as mentioned requires Mo as a cofactor. For this reason, nitrate was chosen as the nitrogen source in the culture media to analyze

the effect of Mo on the growth. Therefore, the transformants were screened in a medium where ammonium was replaced with nitrate, containing the same amount of Mo as the standard *Chlamydomonas* medium (referred to as Nitrate normal-Mo). This condition will also help us detect strains incapable of growing on nitrate. Additionally, two other media were used to further investigate Mo-related phenotypes. A medium with nitrate but prepared without adding Mo (referred to as Nitrate low-Mo). This condition was used to study the efficiency of molybdate assimilation under low-Mo stress. And a medium with nitrate supplemented with 10 mM Mo (referred to as Nitrate high-Mo). This condition was used to examine Mo toxicity and tolerance. The parental strain 704, along with the 5,200 transformants, were then cultivated on these plates for comparative analysis.

The growth of the parental strain 704 and all the transformants was observed and compared in these media. After analyzing the 5,200 transformants, all except the parental strain 704 (Figure 3) were able to grow in ammonium-paromomycin medium, confirming that all the transformants carry the *AphVIII* marker inserted into their genome. Four of these transformants exhibited a phenotypic behavior different from the parental strain 704. Figure 3 shows actual images of the spot test for the parental strain and these four transformants that displayed differential phenotypic behavior in these media compared to strain 704. As previously reported, the parental strain 704 was able to grow in nitrate medium with normal Mo concentrations [32]. The screening revealed that two transformants (81.90 and 8.2) were unable to grow in nitrate-containing media, regardless of the presence or absence of Mo. This suggests that the insertion of the *AphVIII* marker affected genes involved in the nitrate metabolism in these transformants.

Strain	Ammonium	Ammonium	Nitrate	Nitrate	Nitrate
		-Par	normal-Mo	low-Mo	high-Mo
704					
2.2					
9.34					
81.90					
8.2					

Figure 3. Comparison of the growth of the parental *Chlamydomonas* strain 704 and four selected transformants on agar plates. Approximately 500 cells from each of the indicated strains were spotted on the media specified at the top of the figure. The media compositions were as follows: Ammonium (8 mM ammonium and 1 μ M molybdate), Ammonium-PAR (8 mM ammonium, 1 μ M molybdate, and 25 μ g/mL paromomycin), Normal-Mo (4 mM nitrate and 1 μ M Mo), Low-Mo (4 mM nitrate and no added Mo), High-Mo (4 mM nitrate and 10 mM Mo). All culture media contained 1.6% agar. The plates were incubated in the growth chamber for 7 days. Afterward, the plates were photographed, and the results are shown. Shown data are representative of three biologically independent replicates.

Initially, we thought that strain 704 would not be able to grow in nitrate media without Mo added (Nitrate low-Mo), as it is required for nitrate reductase. However, strain 704 exhibited reduced growth in low-Mo media compared to its growth in normal-Mo media, as indicated the less intense green spot color. Nevertheless, it still managed to grow in the low-Mo media (Figure 3). This suggests that even in media prepared without intentionally added Mo, trace amounts of Mo must be present as an impurity in some of the reagents used to prepare the standard culture medium. That’s why we have termed this medium without added Mo as “Low-Mo”. Despite no intentional addition of Mo, it must contain trace amounts that allow for some growth. These trace amounts of Mo are sufficient for

strain 704 to grow in nitrate-containing media; however, although the 704 strain can grow using trace amounts of Mo, its growth is significantly reduced compared to media containing the normal-Mo concentration (1 μ M) (Figure 3). This observation suggests that while 704 can survive on minimal Mo levels, optimal growth requires Mo at higher concentrations. However, among the transformants that grew in low-Mo nitrate media, none showed altered growth compared to the parental strain. Therefore, our initial idea of finding transformants with altered low Mo assimilation was not successful. In high-Mo medium (10 mM), the growth of the parental strain 704 was noticeably diminished, and two transformants (2.2 and 9.34) showed appreciable differences compared to the parental strain. Transformant 2.2 was more resistant to high-Mo concentrations, and the transformant 9.34 was more sensitive to high-Mo than the parental 704 strain.

This initial screening on agar plates enabled us to perform a large-scale selection of transformants, identifying four strains as promising candidates. However, this visual assessment provides only qualitative results. Therefore, to confirm the phenotypes observed in the initial screening and to quantify growth more precisely, we cultivated the selected transformants in liquid medium, which allows for accurate chlorophyll quantification. The amount of chlorophyll is directly related to the growth of microalgae, allowing for precise quantification of their growth. As shown in Figure 4, the growth of strain 704 and transformants 2.2 and 9.34 was approximately 40% lower in Low-Mo nitrate medium compared to Normal-Mo nitrate medium. Transformants 81.90 and 8.2 exhibited a complete inability to grow in nitrate-containing media confirming the result obtained in the agar plate. Transformant 2.2 exhibited approximately 50% greater growth than the parental strain in nitrate high-Mo medium, whereas mutant 9.34 showed notably lower growth under de same condition. Overall, the results from the liquid cultures confirmed the phenotypes observed in the initial screening.

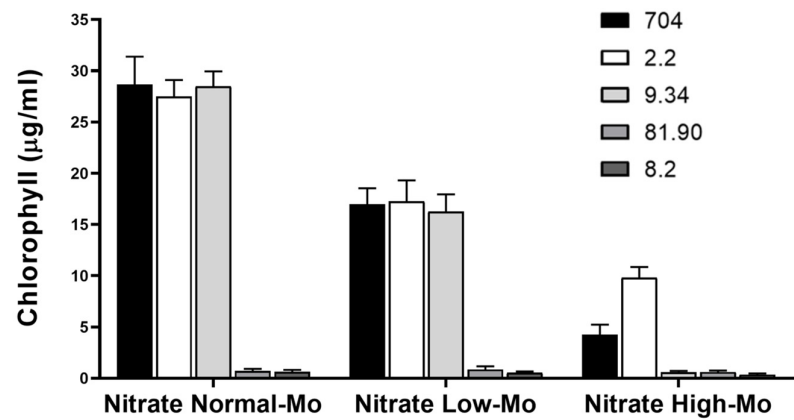


Figure 4. Comparison of the growth of the parental *Chlamydomonas* strain 704 and four selected transformants in liquid media. The indicated *Chlamydomonas* strains were inoculated in the specified liquid media at an initial concentration of 50,000 cells/mL. The media compositions were as follows: Nitrate Normal-Mo (4 mM nitrate and 1 μ M Mo), Nitrate Low-Mo (4 mM nitrate and no added Mo), Nitrate High-Mo (4 mM nitrate and 10 mM Mo). Chlorophyll content was quantified after 7 days of growth as a measure of biomass accumulation. Results represent the mean of three independent experiments $\pm$ standard deviation.

3.3. Localization of the *AphVIII* Marker Insertion in the Transformants

After selecting the four transformants, the next step was to determine the genomic region disrupted by the insertion of the *AphVIII* marker. This is important because the gene in which the insertion occurred could be responsible for the observed phenotype of resistance or sensitivity to high Mo or inability to grow in nitrate. To localize the *AphVIII* marker insertion, a ligation-mediated PCR strategy was employed, based on the Inverse Polymerase Chain Reaction method [38]. This process involved the next steps (Figure 5). Isolation of total genomic DNA from each mutant. Digestion of

the genomic DNA with the restriction endonuclease *AvaI*. This enzyme was chosen because it cuts only once within the *AphVIII* marker and frequently throughout the *Chlamydomonas* genome. Ligation of the resulting DNA fragments to produce a large number of circular DNA molecules. Some of these circular DNA fragments should contain part of the *AphVIII* marker along with adjacent *Chlamydomonas* genomic DNA. The ligation products obtained were used as templates for a first round of PCR, followed by a second, nested PCR round using the product from the initial PCR as a template. The nested PCR approach enhances both the specificity and yield of the desired amplicons. For this purpose, specific *AphVIII* marker primers were employed to enable the amplification of both the right and left sides of the insertion site. Further details about the procedure are provided in the Materials and Methods section. This strategy is particularly effective for amplifying unknown genomic sequences adjacent to the known *AphVIII* marker sequence.

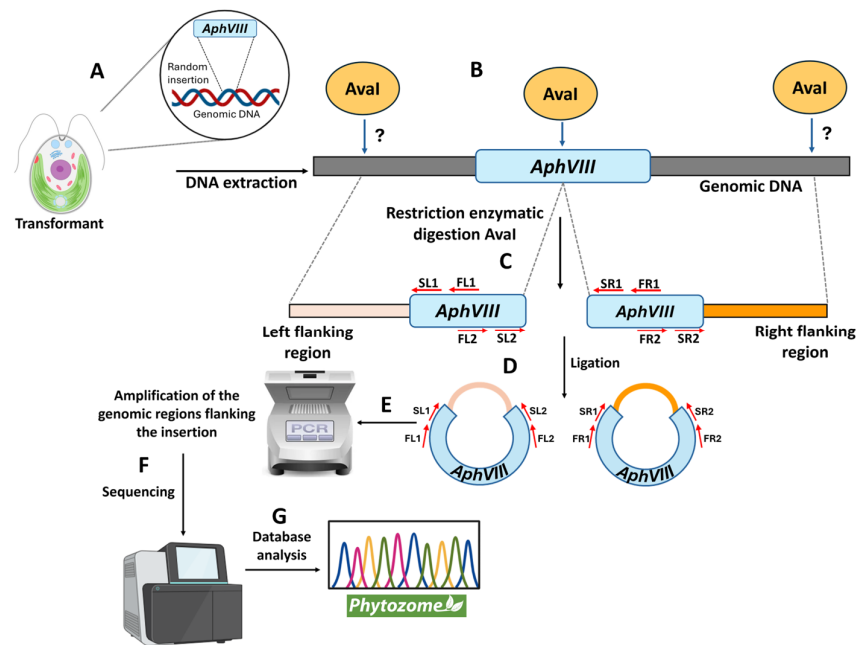


Figure 5. Schematic representation of the procedure to identify the genomic region flanking the *AphVIII* marker insertion. The figure illustrates the different stages of the process for identifying the genomic insertion site of the *AphVIII* marker. A) *Chlamydomonas* is transformed with *AphVIII* marker. The *AphVIII* marker randomly inserts into an unknown location in the *Chlamydomonas* genome. Since the insertion site varies, the position of the genomic *AvaI* sites is unpredictable (indicated by a question mark) B) The genomic DNA is isolated and digested with the restriction enzyme *AvaI*. C) The left side of the insertion was considered the genomic region adjacent to the 5' end of *AphVIII*, while the right side corresponded to the genomic region adjacent to the 3' end of *AphVIII*. D) The fragments resulting from the digestion are ligated. E) The ligation DNA is used as a template for PCR amplification using the indicated primers sets (FL1, FL2, FR1, FR2, SL1, SL2, SR1 and SR2). F) After the purification from the agarose gel, the PCR products are sequenced. G) The sequences obtained were analyzed using the *Chlamydomonas* Phytozome. For more specific details, see Materials and Methods.

The bands obtained after the PCR of the four transformants are shown in Figure 6. As can be seen, amplification failed for some primer pairs, while others were successful. However, in all transformants, the amplification of at least one border was achieved, allowing us to identify the genomic insertion site. All bands larger than 300 bp were excised from the agarose gel and sent for sequencing. By sequencing the PCR products, we were able to identify the genomic regions flanking the *AphVIII* insertion site, thus revealing the precise location of the marker in the *Chlamydomonas* genome and identifying potentially disrupted genes.

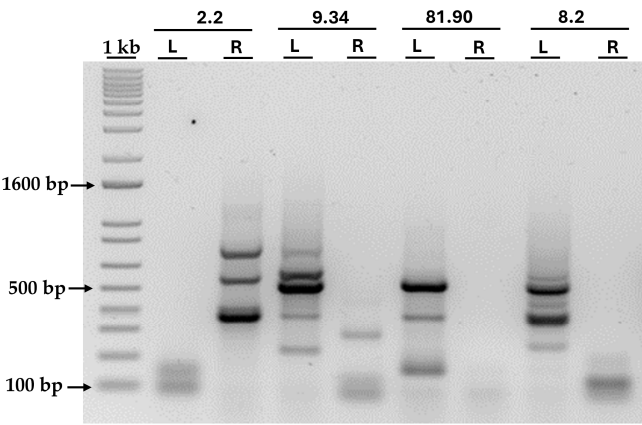


Figure 6. PCR amplification of the genomic DNA adjacent to the *AphVIII* insertion. The figure shows the agarose gel after loading the products from the second round of PCR amplification of the genomic DNA adjacent to the *AphVIII* insertion in the four selected transformants. Above the lanes, the names of the transformants and the primer pairs used are indicated: L (primers pair for the left border) and R (primers pair for the right border). More details about the procedure can be found in the Materials and Methods section. “1Kb” represents the molecular weight marker, next to it, the size in base pairs (bp) of three representative bands is indicated.

The sequencing results obtained were compared with the *Chlamydomonas* genome database (phytozome), considering as valid those sequences that presented a clear junction between the *AphVIII* and a known chromosomal region. Table 1 summarizes the results, indicating the chromosome, the gene locus, and the predicted function of the affected gene.

Table 1. Characterization of *Chlamydomonas* transformants identified in this study.

Transformants	Chromosome	Locus affected	Predicted funtion (protein type)
2.2	5	cre05.g234400	Solute transport (ABC transport)
9.34	3	cre03.g191350	Solute transport (ABC transport)
81.90	8	cre08.g382545	Moco synthesis (CNX7)
8.2	7	cre07.g348040	Amino acid transporter (BAT1)

The table provides details on the transformant number, the chromosome where the insertion is located, the affected locus (gene ID), and the predicted function of the disrupted gene, including protein type.

Transformants 2.2 and 9.34 had the marker insertions in genes with homology to ABC-type transporters: *Cre05.g234400* in transformant 2.2 and *Cre03.g191350* in transformant 9.34. ABC-type transporters are a family of membrane proteins that have been implicated in the transport of various types of substrates across membranes. These substrates include a wide range of molecules. Notably, in bacteria Mo is among the substrates known to be transported by certain ABC-type transporters [41]. Their possible transmembrane topology and subcellular localization of these possible transporters were studied using the TMHMM 2.0 and WoLF PSORT tools respectively. The results indicated that both proteins have a high probability of being located in the plasma membrane. The protein encoded by the gene *Cre05.g234400* is predicted to have 4 possible transmembrane elements, while the protein encoded by the gene *Cre03.g191350* is predicted to have 9 transmembrane elements.

Regarding the transformants unable to grow on nitrate: In 81.90, the marker insertion occurred in the *Cre08.g382545* gene, which shares 40.1% homology with the *CNX7* gene found in the higher plant *Arabidopsis thaliana*, suggesting a similar function. *CNX7* is crucial for the biosynthesis of Moco, and this mutation accounts for the mutant’s inability to grow on nitrate as the sole nitrogen source. Without a functional *CNX7* gene, the strain cannot produce the Moco necessary for activating nitrate

reductase, the key enzyme in nitrate assimilation. As a result, mutant 81.90 should lack active nitrate reductase, making it incapable of utilizing nitrate for growth. In transformant 8.2, the marker was inserted in the gene *Cre07.g348040*. Notably, the protein encoded by this gene shares 61% homology with the Amino-acid permease BAT1 found in *Arabidopsis* [42], suggesting a potentially similar function. The possible transmembrane topology and subcellular localization of BAT1 were analyzed using the TMHMM 2.0 and WoLF PSORT tools, respectively. The results predict that BAT1 contains 11 transmembrane domains and has a high probability of being localized to the plasma membrane. The transformant 8.2 presents an interesting case in our study. Its inability to grow on nitrate is intriguing, given that, as far as is known, BAT1 is not directly involved in nitrate assimilation, unlike CNX7. In the discussion section, we will propose possible explanations for this unexpected phenomenon.

4. Discussion

Mo homeostasis includes aspects that remain unknown, such as Mo efflux transport (critical for long-distance transport), mechanisms of Mo storage (necessary to provide a rapid Mo source under conditions of high demand), and cellular strategies for Mo sensing and regulation (essential for coordinating Mo availability with cellular demand) [43]. Identifying the molecular players involved in these processes is crucial for improving nitrogen assimilation, as NR, a key enzyme in nitrogen metabolism, depends on Mo [23]. Therefore, a comprehensive understanding of Mo homeostasis is critical not only to meet the increased metal demand associated with nitrogen assimilation but also to prevent potential Mo deficiencies that could impair metabolic function.

Our working hypothesis is that *Chlamydomonas* has homeostatic mechanisms to regulate intracellular Mo concentration, and that these mechanisms are shared by higher plants. Supporting this hypothesis is the fact that much of the current knowledge on plant Mo homeostasis has been derived, directly or indirectly, from studies on *Chlamydomonas* [44]. The success of this model has been previously demonstrated, as the two known families of molybdate-specific transporters in eukaryotes (MOT1 and MOT2) were first identified in *Chlamydomonas* [16,17]. Both MOT1 and MOT2 belong to the Major Facilitator Superfamily (MFS), a large group of membrane transport proteins that facilitate the movement of small solutes across cell membranes. MOT1 is present not only in algae but also in higher plants and fungi, where it mediates high-affinity molybdate uptake [16]. In *Arabidopsis thaliana*, MOT1 transporters have been reported to play a role in efficient Mo uptake from the soil [45], with their absence leading to impaired plant growth [46]. Members of the MOT1 family have also been identified as specific Mo transporters in other eukaryotic organisms, such as *Medicago truncatula* and rice [47–49]. MOT2 was also first identified in *Chlamydomonas*, and subsequent research using heterologous expression have suggested its involvement in Mo transport in humans [17].

We conducted a screening in nitrate-containing medium under three different Mo concentrations: normal concentration (1 μ M), no added Mo (low-Mo), and with High-Mo (10 mM). Initially, we hypothesized that 704 would not grow in the absence of added Mo. However, it did show growth, albeit reduced (Figures 3 and 4). This suggests that even when Mo is not added to the culture medium, trace amount may be present as contaminants from other reagents used to prepare the culture medium, possibly in sulfate or phosphate, which are structural analogs to molybdate [50,51]. One of the aims of this screening was to identify genes involved in Mo homeostasis under limiting conditions. From this perspective, the screening with low Mo was not successful.

In contrast, the high-Mo conditions yielded more promising results. We used 10 mM Mo, a concentration previously known to be toxic in *Chlamydomonas* [52], with the aim of identifying transformants that were either more resistant or more sensitive than the parental strain 704 to these high concentrations. The objective was to identify genes responsible for regulating and detoxifying excess Mo. There are several potential reasons for the toxicity of high Mo concentrations. In general, elevated metal concentrations can lead to the generation of reactive oxygen species (ROS) through Fenton-type reactions, resulting in damage to cellular component [53]. Moreover, Mo acts as a copper antagonist [54], and excessive Mo levels can disrupt copper homeostasis. Such disruption has been

linked to disorders like Menkes and Wilson diseases, characterized by copper deficiency and overload, respectively [55]. In plants, since copper is essential for photosynthesis [56], imbalances caused by high Mo levels could impair this process. The antagonistic relationship between these two metals has been proposed as a key factor in their metabolism. It has been suggested that copper interferes with Moco biosynthesis by inhibiting the magnesium-dependent Mo insertion reaction catalyzed by CNX1G [57]. Another possible explanation for the toxic effect of high Mo concentrations is its chemical similarity to other oxyanions, particularly sulfate and phosphate. Molybdate can be non-specifically taken up via sulfate transport systems, as reported for SHST1, a high-affinity sulfate transporter from *Stylosanthes hamata* which facilitates Mo uptake [58], illustrating the overlap in transport mechanisms between these two ions. Another possible explanation for the toxic effect of high concentrations of Mo could be related to phosphate. The phosphate transport system may also play a role in the non-specific absorption of molybdate. Molybdate and phosphate are highly similar in size, charge, and tetrahedral structure, leading to potential overlap in their transport mechanisms [59]. This is evidenced by observations in tomato plants, where a deficiency in phosphate leads to an increased uptake of molybdate, suggesting cross-talk between these transporter systems [60].

Apart from MOT1 and MOT2, no other Mo transport systems have been identified in eukaryotic organisms. However, more transport systems could exist, particularly those involved in exporting Mo out of the cell to prevent toxic accumulation. Two of the genes identified in this study, *Cre05.g234400* and *Cre03.g191350*, encode ABC-type transport proteins, which may represent novel mechanisms for regulating Mo transport. ABC transporters are essential components of cellular homeostasis and survival across all life forms due to their ability to handle a wide range of substrates with high specificity and efficiency [61]. These substrates include nutrients, metabolites, peptides, metals, ions, and amino acids, which are moved across cellular membranes into or out of the cytoplasm. Their main characteristic is a common architecture consisting of transmembrane domains (TMDs) and nucleotide-binding domains (NBDs). TMDs are embedded in the membrane and are responsible for substrate recognition and translocation. ABC transporters operate via an active transport, requiring ATP hydrolysis to move substrates against their concentration gradient [62]. Interestingly, two families of ABC-type transporters have been identified in bacteria involved in the transport of molybdate: the MolBC-A and the ModABC system. The MolBC-A functions as a low-affinity system and is active during periods of high extracellular molybdate concentration [63]. It works by transferring molybdate from the periplasmic binding protein MolA to the transmembrane domain MolB. In contrast, the ModABC system acts as a high-affinity Mo transporter and includes ModA, a periplasmic binding protein; ModB, the membrane-spanning domain; and ModC, the ATPase that powers the transport process [41].

The ABC-type transporters identified in this study could be involved in either importing Mo into the cell or exporting it to the extracellular environment. Among the transformant analyzed, mutant 2.2, which carries a mutation in the gene *cre05.g234400*, exhibited higher resistance to elevated Mo concentrations compared to the parental strain. This phenotype suggests that the ABC transporter encoded by *cre05.g234400* may be involved in Mo uptake or storage. Its disruption could reduce Mo entry into the cell or promote enhanced sequestration of excess Mo, thereby conferring increased tolerance to high Mo concentrations. In contrast, the transformant 9.34, which carries a mutation in the gene *Cre03.g191350*, displayed lower tolerance to elevated Mo concentrations compared to the parental strain. This observation suggests a potential defect in genes involved in Mo efflux mechanisms. If the ABC transporter encoded by *Cre03.g191350* is involved in Mo export, its mutation could impair the cell's ability to eliminate excess Mo, leading to toxic accumulation and increased sensitivity. Consequently, we hypothesize that *Cre03.g191350* encodes a transporter potentially involved in Mo the efflux. Future research focused on characterizing Mo transport activity in these mutants versus the wild type will elucidate the specific roles of these ABC transporters in Mo homeostasis.

Once Mo enters the cell, it can be either immediately utilized for Moco synthesis or stored to ensure a reserve for future metabolic needs. In bacteria, two distinct Mo storage systems have been

identified: one involving molybdate binding to Molbindin-type proteins, and another utilizing MoSto proteins [64,65]. However, no homologs of these bacterial storage proteins have been found in eukaryotes. Our study did not identify any proteins with homology to known Mo storage components, suggesting that eukaryotes may rely on alternative strategies for Mo storage or may have a reduced requirement for long-term Mo reserves.

Before being inserted into molybdoenzymes, Mo must be coordinated to MPT to become biologically active in eukaryotes, and form Moco [21]. Moco is synthesized through a highly conserved pathway, from bacteria to mammals [66]. The first step of Moco biosynthesis involves the conversion of GTP into cyclic pyranopterin monophosphate (cPMP), a reaction catalyzed by the proteins CNX2 and CNX3 [67]. The second step involves the transfer of two sulfur atoms to cPMP, forming MPT through catalysis by the MPT synthase complex, which consists of two small CNX7 subunits and two large CNX6 subunits [11]. Subsequently, the MPT synthase must be re-sulfurated by the MPT synthase sulfurase CNX5 [68]. In the third step, MPT is activated by adenylation catalyzed by CNX1G, resulting in the MPT-AMP intermediate [69,70]. In the final step, MPT-AMP is deadenylated by CNX1E, a process that enables the insertion of Mo into MPT, resulting in the formation of active Moco [71]. After its synthesis, Moco binds to proteins that store, protect and transport it, such as the MCP (Moco carrier protein) that was first identified in *Chlamydomonas* [72,73]. In our screening process, we were unable to identify any transformants in the MCP. Furthermore, we did not find any mutants in other proteins that perform a similar putative function. Based on these data, we cannot exclude the possibility that other proteins may perform a function analogous to MCP in *Chlamydomonas*.

Although the main goal of this work was to identify genes involved in Mo homeostasis, the screening strategy also led to additional findings. Transformants resistant to paromomycin were initially selected in ammonium-containing medium, and their Mo homeostasis-related phenotypes were later evaluated in nitrate-containing medium. This two-step process enabled the identification of mutants with defects not only in Mo homeostasis but also in nitrate utilization. Specifically, we identified two mutants incapable of growing on nitrate regardless of Mo availability. One of them harbored a mutation in the gene *Cre08.g382545*, which encodes CNX7 protein, the small subunit of the MPT synthase. This phenotype is consistent, as without CNX7, the synthesis of an active Moco cannot occur, nitrate reductase cannot function properly, and growth on nitrate is not possible. We are uncertain why, out of the 5,200 mutants analyzed, only mutant 81.90 harbors a mutation in a CNX gene—specifically CNX7—and why no transformants were identified in other CNX genes. This result could be due to random chance, or it may reflect the essential roles that Moco might play in pathways beyond nitrate metabolism. While Moco is known to be critical for nitrate assimilation via enzymes such as nitrate reductase and mARC, it may also be involved in ammonium metabolism, where as mentioned other molybdoenzymes such as sulfite oxidase, aldehyde oxidase, and xanthine dehydrogenase play key roles [18]. The broader metabolic relevance of Moco, including in ammonium metabolism, could cause cofactor-deficient mutants exhibiting slower growth rates and delayed colonies formation. Consequently, such slow-growing mutants may have been overlooked during our initial screening, which was performed in ammonium-containing media supplemented with paromomycin (Figure 2). This remarkably low recovery of mutants with alteration in CNX genes suggests that disrupting these genes is particularly challenging. In this sense, a previous mutant library screen aimed at identifying genes involved in nitrate assimilation recovered only a single mutant in the CNX2 gene among 22,000 transformants isolated [74].

The mutant 8.2 presents an intriguing case in our study, as the marker is inserted in the gene *Cre07.g348040*, which encodes a protein that sharing similarities with the bidirectional amino acid permease BAT1. This mutant exhibits an inability to grow on nitrate, which is particularly interesting since the affected gene is not directly part of the nitrate assimilation pathway. In *A. thaliana*, BAT1 is notable for its dual transport activity -both export and import of amino acids- hence its name: Bidirectional Amino acid Transporter 1 [42]. BAT1 belongs to a family of integral membrane proteins that mediate amino acids across cell membranes and contains conserved domains common to amino

acid transporters found in both prokaryotes and eukaryotes, suggesting that it is part of an evolutionary ancient family of transporters preceding the divergence of these two domains of life [75]. BAT1 is essential for the uptake of amino acids, which serve as organic nitrogen sources. However, its connection to the nitrate-dependent phenotype of mutant 8.2 is not immediately clear, given that nitrate is an inorganic nitrogen source. This unexpected phenotype suggests a possible link between amino acid transport and nitrate metabolism, or alternatively, an unknown function of BAT that is essential for nitrate utilization. In plants, nitrate is absorbed from the soil and reduced to nitrite and subsequently to ammonium, which is then incorporated into amino acids, primarily glutamine [76]. Glutamine is not only the most abundant amino acid in many plant species, but also the first organic nitrogen compound formed in this pathway. It acts both as a crucial metabolic intermediate and as a signaling molecule involved in the regulation of nitrogen metabolism [77]. It has been suggested that the internal amino acid pools, particularly glutamine, can act as nitrogen status signal, modulating nitrate uptake and transporters expression. In support of this idea, exogenously applied amino acids, particularly glutamine, have been shown to decrease nitrate influx and downregulate transporter transcription in root tissue [78]. This regulatory mechanism may occur both at the transcriptional level and through post-translational modification. BAT1 has been reported to transport several amino acids, including alanine, arginine, glutamate, and lysine, with a particularly high affinity for glutamine, and its expression is strongly induced in the presence of glutamine [79]. Moreover, recent studies indicate that glutamine availability directly influences the expression of key nitrate transporters, such as NRT2.4, which are vital for nitrate-dependent growth [80]. Specifically, glutamine depletion has been observed to inhibit the expression of these transporters, suggesting that glutamine acts as an internal signal that helps plants fine-tune their nitrogen uptake mechanisms based on their current nitrogen status. Based on this evidence, we hypothesize that mutant 8.2 may have an altered transport mechanism for an essential amino acid, most likely glutamine, which could impair its ability to properly regulate nitrate uptake and metabolism. This would explain its inability to grow efficiently on nitrate as the sole nitrogen source. Further experimental validation is required to verify this hypothesis and to determine the precise role of BAT1 in nitrate-related pathways.

5. Conclusions

Through an extensive insertional mutagenesis screen, this study has identified novel candidate genes involved in Mo and nitrate homeostasis in *Chlamydomonas*. Among the most relevant findings, we uncovered two ABC transporter family genes potentially involved in molybdate transport, one gene essential for the Moco biosynthesis, and the *BAT1* gene, a putative bidirectional amino acid transporter with a possible regulatory role in nitrate metabolism. These findings expand our understanding of the genetic network involved in Mo uptake, cofactor biosynthesis, and nitrogen assimilation. The identification of these genes provides a solid foundation for future investigations aimed at elucidating their specific biological functions and interactions in Mo and nitrate metabolisms. Moreover, our findings contribute to a better understanding of nutrient uptake and utilization, the regulation of Mo-dependent processes, and the connection between amino acid transport and nitrate assimilation. The insights gained have potential implications for both agricultural and environmental applications, advancing our knowledge in the broader field of nutrient metabolism.

Author Contributions: M T-J and A.L designed research. E.L-M performed most of the experimental work. E.L-M; M T-J and A.L analyzed data. M T-J and A.L wrote the paper. All authors discussed the results and commented on the manuscript. All authors have read and agreed to the published version of the manuscript.

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