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Isolation and characterization of pigment deficient insertional mutants in the chlorophyte *Chlamydomonas reinhardtii*

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Abstract

In the present work, a large collection of mutants was obtained by insertional mutagenesis from 704 (cw15 Arg7+ Nia1 : Ars mt+) strain of *Chlamydomonas reinhardtii*, using a linearized fragment of the pSI03 plasmid. About 2100 insertional mutants resistant to the antibiotic paromomycin were isolated and screened to identify *Chlamydomonas reinhardtii* mutants: i) sensitive to high light, ii) with an altered pigment composition and iii) with an irregular response to high light stress. The insertion site in 15 of the selected mutants was amplified by Restriction enzyme site-directed amplification (RESDA) PCR or inverse PCR. Cloning, sequencing and alignment of the amplified DNA with the *Chlamydomonas* database allowed the identification of the region adjacent to the insertion in most of the mutants studied. We observed that in 77 % of the transformants analysed, insertion took place in intragenic regions. Almost half of the mutants (46 %) had insertions in genome loci with unknown functions. Among disrupted genes with known functions, we found genes involved in a great diversity of functions, from flagella motion to regulatory or signal transduction processes, suggesting that the sensitivity to high light and the synthesis of pigments are complex and very regulated processes.

Keywords: Carotenoids, *Chlamydomonas reinhardtii*, microalgae, mutagenesis, pigments, zeaxanthin

Introduction

The unicellular green alga *Chlamydomonas reinhardtii* has become, during the last decades, a popular model system to understand gene functions due to its molecular, genetic and physiological features together with the availability of easy methods for its stable genetic transformation and the existence of complete genomic and cDNA (Complementary Deoxyribonucleic Acid) databases [1,2]. Its role as a model system is particularly important to study photosynthesis and responses to light [3-5] due to the possibility of *Chlamydomonas* to grow either photoautotrophically in mineral media and light or heterotrophically in acetate and dark. The study of *Chlamydomonas* pigment-less mutants generated by traditional mutagenesis has offered interesting information about photoprotective mechanisms in microalgae and plants [4], but the identification of the gene affected in these kinds of mutants is not easy.

In the recent years, new approaches to generate mutants and understand gene function in microalgae and higher plants, in which homologous recombination does not occur at a high frecuency, have been developed [6].

Among reverse genetic approaches, developed to generate point mutations in specific genes: the use of zinc-finger nucleases, which allows the generation of small delections and/or insertions within the gene of interest through custom-designed chimeric endonucleases [7] and TILLING (Targeting Induced Local Lesions In Genomes), which combines traditional chemical mutagenesis with rapid mutational screening to discover induced lesions [8,9], are commonly used in plants but their high cost and laboriousness have limited their use in microalgas [10].

RNA silencing technique has been successfully used to suppress endogenous transcript levels in *Chlamydomonas* [11-13] but it is difficult to obtain a complete loss-of-function RNA-silenced strain and there may be off-target effects induced by the introduced construct [6,10].

Exploring indexed insertional mutant libraries by PCR has been a popular approach to isolate strains with lesions in specific target genes in higher plants. A similar collection of mutants has been stablished for *Chlamydomonas reinhardtii* but maintenance of a collection of microalgal mutants is not easy [6].

On the contrary, forward genetic approaches allow the isolation of insertional mutants with specific phenotypes, which can be analysed by several easy methods to identify the gene affected, and have demonstrated to be a powerful tool to study the relationship existing between a gene sequence and its function in plant and microalgal cells. The technique is based on nuclear transformation of the microalga with an exogenous DNA marker, which is randomly integrated into the genome. After screening for a particular phenotype and analysis of the disrupted genes, it is possible to establish a functional relation between the affected gene and the phenotype selected. This approach has allowed isolation of mutants affected in

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different metabolic and physiological aspects [14] and has become a popular method for forward genetics studies because identification of the affected genomic region is generally easier than location of genomic lesions caused by traditional mutagenesis procedures based on chemical or physical agents. In *C. reinhardtii,* this technique has allowed the isolation of mutants affected in flagellar function [15], phototaxis [16], microtubules [17], carbon assimilation [18], H_2 production [19], photosynthesis [20], nitrogen [21] and sulphur metabolism [22].

The most complete functional genomic analysis of photosynthesis in *Chlamydomonas* by insertional mutagenesis was carried out by Dent and Coworkers [20]. They introduced a linearized plasmid carrying the *ble* gene from *Streptoalloteicus hindustanus* that confers resistance to bleomicin [23] and generated 2760 insertional mutants that were screened for light sensitive and nonphotosynthetic phenotypes. They characterized the insertion site in 50 of the isolated mutants, finding mutants affected in very diverse metabolic, physiological and regulatory genes.

In the present work, using a linerized fragment of the pSI03 plasmid [24], 2100 insertional mutants resistant to the antibiotic paromomycin have been isolated and screened to identify *Chlamydomonas reinhardtii* light-sensitive and pigment-deficient mutants. Mutants which have higher violaxanthin content (B20), higher pigments concentration (A7), lower chlorophyll content (C13) and total lack of chlorophylls and carotenoids (C2), together with three mutants that synthesize small quantities of zeaxanthin in response to high light stress (Z7, Z34 and Z198), have been isolated and physiologically characterized. The insertion site in 15 of the selected mutants has been characterized by RESDA PCR (Polymerase Chain Reaction) [25] or inverse-nested PCR [26], contributing to increase the information generated by previous photosynthetic functional genomic analysis in *Chlamydomonas* [20].

Methods

Microorganisms and culture conditions

Chlamydomonas reinhardtii cell-wall deficient strain 704 (Cw15, Arg7, mt+) was kindly provided by Dr. Roland Loppes [27] and cultured photomixotrophically in liquid or agar solidified TAP (Tris-Acetate-Phosphate) medium [2] at 25 ˚C under continuous white light irradiation of 100 µE m^{-2} s^{-1}, unless other intensity is indicated. Light sensitive mutants obtained were kept in the dark or very low light intensity (15 µE m^{-2} s^{-1}). The *Escherichia coli* strain used for *in vivo* amplification of DNA was DH5α, cultured in LB (Luria Broth) medium as previously described [28].

High irradiance treatment

For high irradiance experiments *Chlamydomonas reinhardtii* cultured in TAP plates were illuminated with an adjustable halogen lamp (Philips 500 W). The plates were located under a glass thermostatic bath, connected with a refrigerated circulator (LAUDA E300), with water at a constant temperature of 25 ˚C to avoid overheating of the plates. Light intensity was measured by a Delta OHM quantum photo radiometer equipped with a PAR sensor.

Generation of insertional mutants in *Chlamydomonas reinhardtii*

Insertional mutants were generated by transformation of the *C. reinhardtii* strain 704 (Cw15, Arg7, mt+) with a fragment of the plasmid pSI103 [24] which harbours the *AphVIII* gene from *Streptomyces rimosus*, coding for an aminoglycoside 3'phosphotransferase that confers resistance to the antibiotic paromomycin, under the control of the strong constitutive promoters *RbcS2* (Ribulose Bisphosphate Carboxylase Small Subunit 2) and Hsp70A (Heat Shock Protein 70A) and terminated by the 3' untranslated region of *RbcS2*. The fragment was exscinded from plasmid pSI103 by digestion with the endonucleases enzymes *Not*I and *Kpn*I (**Figure 1.1**).

Nuclear transformation of *Chlamydomonas reinhardtii*

Transformation was carried out using the glass-bead method of Kindle [29] with minor modifications. *C. reinhardtii* cells were grown to a cell density of about 10^7 cells per ml, harvested by centrifugation and resuspended in fresh TAP medium to obtain a 100 fold concentrated cell suspension. The concentrated cell suspension (0.6 ml) was added to a conical tube containing 0.3 g of sterile glass beads (0.4-0.6 mm diameter), 0.2 ml of 20 % polyethylene glycol (MW8000) and about 100 ng of the desired plasmid. Cells were vortexed for 8 s, resuspended in 50 ml of fresh sterile TAP medium and incubated at dim light overnight. After this incubation in the absence of antibiotic, the cells were pelleted and spread onto TAP solid medium plates with paramomycin (30 µg ml^{-1}). Transformed colonies will be visible after 4 or 5 days.

Screening of light sensitive and pigment deficient mutants

Transformed cells showing resistance to paromomycin were inoculated in 5 ml of TAP liquid medium with paromomycin (15 µg ml^{-1}) and grown under dim light as described in Microorganisms and culture conditions for 5 days. The absorbance at 660 nm was estimated and adjusted to the same optical density at 660 (OD_{660}) in all the cultures by adding fresh sterile TAP medium. 5 ml of each culture were collected by centrifugation (1200 x g, 5 min) and resuspended in 200 µl of TAP medium. Two TAP-paromomycin plates were symmetrically spotted in an order array with 20 µl-drops of each culture and subjected to a two-step screening strategy. In the first screening step one replicate was kept at dim light (100 µE m^{-2} s^{-1}) and the other was exposed to high light intensity (800 µE m^{-2} s^{-1}). After two days, the growth of the transformants in each plate was evaluated (**Figure 2**). The mutants which died, grew more slowly than the control or

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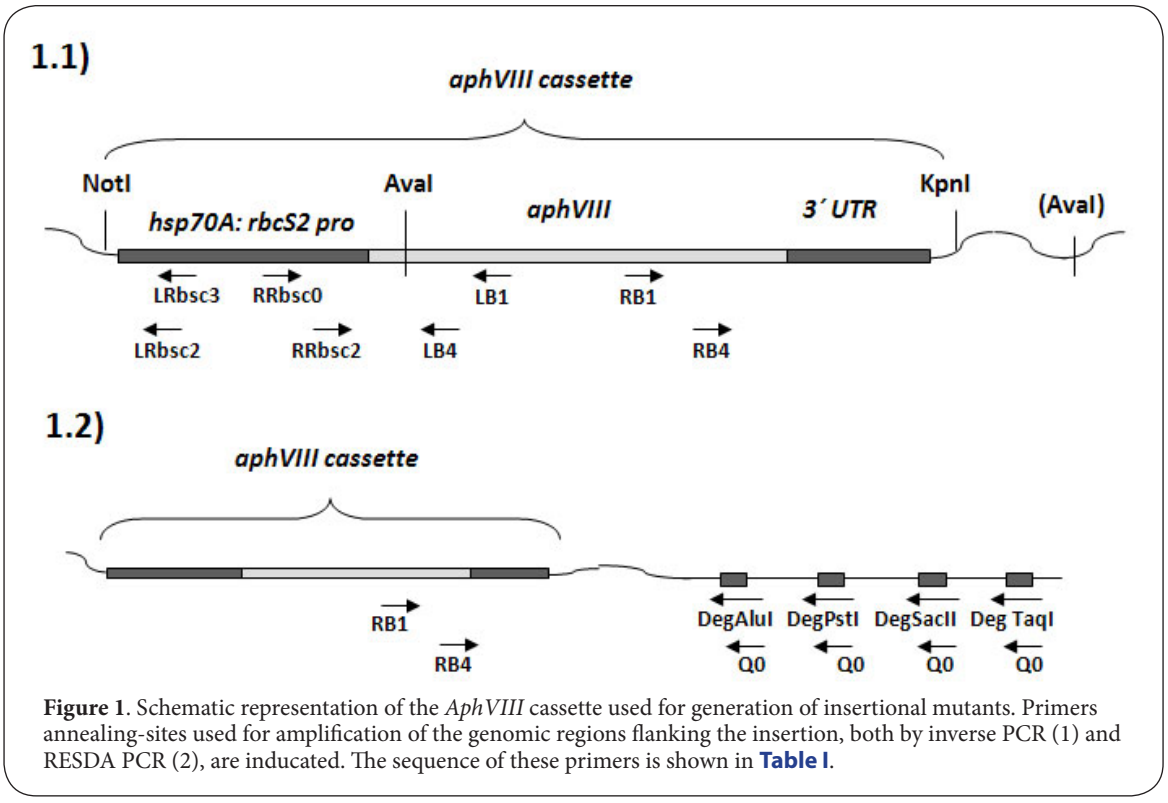

Figure 1. Schematic representation of the *AphVIII* cassette used for generation of insertional mutants. Primers annealing-sites used for amplification of the genomic regions flanking the insertion, both by inverse PCR (1) and RESDA PCR (2), are inducated. The sequence of these primers is shown in **Table I**.

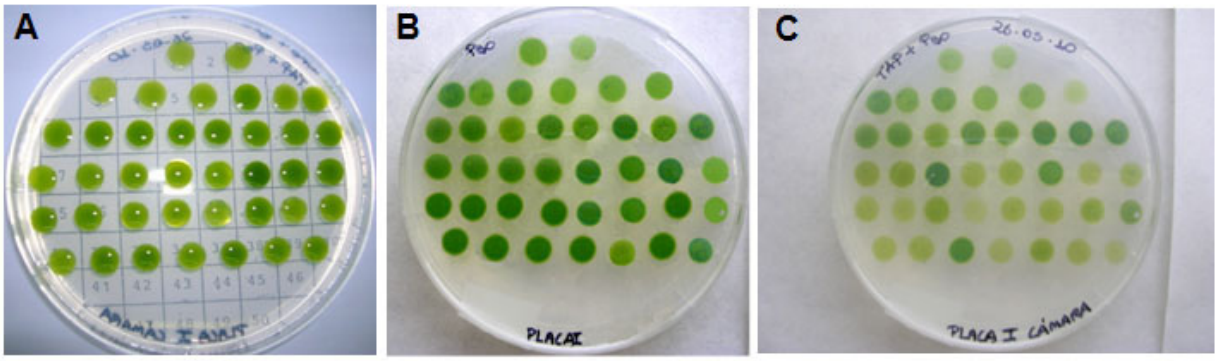

Figure 2. Screening of high light sensitive transformants. Drops of the well grown paromomycin resistant transformant cultures were symmetrically spotted on two plates in an order array (**A**). One replicate was kept at dim light and the other was exposed to high light intensity (800 μE m^{-2} s^{-1}). After two days, the growth of each transformant at dim light (**B**) and at high light intensity (**C**) was evaluated.

showed deficient pigmentation were selected as high light-sensitive mutants. In the second step, light sensitive selected lines were cultured in TAP liquid medium with paromomycin (15 μg ml^{-1}) at low light intensity (15 μE m^{-2} s^{-1}) and pigment composition of each selected mutant was chromatographically determined as described in Analysis of pigments.

Screening of mutants deficient in zeaxanthin synthesis in response to high light

Transform-cells showing resistance to paromomycin were inoculated in 5 ml of TAP liquid medium (15 μg ml^{-1}) and grown at standard conditions for 5 days. The volume in each tube was adjusted with fresh sterile TAP medium to obtain the same absorbance at 660 nm in all the tubes. Then, the cultures were subjected to a 24 h dark pre-treatment and immediately transferred to high light (800 μE m^{-2} s^{-1}) for 2 h. The concentration of violaxanthin, anteraxanthin and zeaxanthin in each transformant was chromatographically determined as described in Analysis of pigments and the de-epoxidation state calculated as the ratio [(ant + zea) /

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Table I. Primers used.

Primer	Sequence (5`--- 3`)	Uses
NRfor	GCGCTGCCCTCCGTCACCTTCC	Estimation of the number of *NR* genes by qPCR
NRre	CAGCCGCACGCCCGTCCAGTAG	
Parafor	GAGGATCTGGACGAGGAGCGGAA	Estimation of the number of *AphVIII* genes by qPCR genes
Pararev	CCCTCAGAAGAACTCGTCCAACAGC	
RB1	AGCTGGCCCACGAGGAGGAC	Identification of the region adjacent to the marker gene by iPCR (3' end)
LB1	CCAGAGCTGCCACCTTGACA	
RB4	TGGTTCGGGCCGGAGTGTTC	
LB3	CCACCACCCCGAAGCCGATAA	
Rbsc0	CCCTCCCCGGTGCTGAAGAAT	Identification of the region adjacent to the marker gene by iPCR (5' end)
LRbsc3	GTCGCGTATGTATAAGTGCTCGTTTG	
Rbsc2	CCGGTTGTGAGTGGGTTGTTGT	
LRbsc2	CGTTCGGGGTCGCGGGCTTTTA	
RB1	AGCTGGCCCACGAGGAGGAC	Identification of the region adjacent to the marker gene by RESDA
Deg *Alu*	CCAGTGAGCAGAGTGACGIIIIIINNSWCAGCTT	
Deg *PstI*	CCAGTGAGCAGAGTGACGIIIIIINNSWCAGCTT	
Deg *SacII*	CCAGTGAGCAGAGTGACGIIIIIINNSCTGCAGW	
Deg *TaqI*	CCAGTGAGCAGAGTGACGIIIIIINNSCCGCGGW	
RB4	TGGTTCGGGCCGGAGTGTTC	
Q0	CCAGTGAGCAGAGTGACG	

(ant + zea + viol)] Those that showed a de-epoxidation state significantly lower than the control cells submitted to the same treatment were selected and rescreened in the same way two additional times.

Small scale genomic DNA extraction

The pellet obtained after centrifugation of 2 ml of *C. reinhardtii* culture was resuspended in 300 µl of lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA), 3 % dodecilsulfato sódico (SDS) vortexed during 5 min and incubated at 4 ˚C for 5 min. DNA was extracted with phenol/chloroform and precipitated with ethanol absolute at -20 ˚C for 3-4 h. The pellet was washed with ethanol 70 %, dried and resuspended in 5 mM Tris-HCl, pH 8. The genomic DNA suspension (1 µl) was used to carry out the PCR reaction.

Standard polymerase chain reaction

The PCR amplification was performed from 1 µl of genomic DNA in a total volume of 25 µl containing 10 pmol of each primer, 0.2 mM desoxirribonucleotids (dNTPs), 0.5 U *Taq* DNA polymerase from Biotools (B&M Labs, Madrid, Spain), 2.5 µl of specific 10X buffer (containing 2.5 mM $MgCl_2$), and 1 % dimethylsulfoxide (DMSO). The PCR program was: 0.5 min at 96 °C, 0.5 min at annealing temperature, and the required time at 72 °C for 30 cycles.

Inverse PCR

Inverse PCR was preformed by digesting 500 ng of genomic DNA with the restriction endonuclease *Ava*I, which has a unique restriction site inside the cassette used in the insertional mutagenesis experiments and has a high number of target sites in the genome of *Chlamydomonas*. The resulted digestions were precipitated by adding ethanol 95 % and 3 M sodium acetate and incubation for 1-2 h at -20 ˚C, washed with ethanol 70 % and ligated with T4 ligase. The ligation was used as a template for nested PCR using different pairs of inverted primers (**Table I**). Each PCR reaction was carried out at the standard conditions described in Standard polymerase chain reaction, excepting that cycling conditions were 1x (96 ˚C, 5 min) 35x (95 ˚C, 1 min; 60 ˚C, 1 min; 72 ˚C, 3 min) 1x (72 ˚C, 10 min).

RESDA PCR

Restriction enzyme site-directed amplification PCR [**25**], RESDA-PCR, is modified from Thermal asymmetric interlaced TAIL-PCR [**31**] and basically involves using specific primers for the marker gene together with degenerated primers. The design of these degenerated primers is based on the presence of frequent restriction sites randomly distributed in the microalgal genome. The degenerated primer consists of the chosen restriction site with several highly degenerated nucleotides in the 3´end linked by a polyinosine bridge to a specific Qo sequence in the 5´end (see **Figure 1.2**). Two amplification rounds at the cycling conditions indicated below, allowed the amplification of fragments with lengths between 0.3 and 2 Kb that were sequenced for identification of the genomic marker adjacent region. For the first round: Primers RB1 and Deg*Alu*I, Deg*Pst*I, Deg*Sac*II and Deg*Taq*I (sequence

detailed in **Table I** and in [25]) and cycling conditions 1x (95 ˚C, 5 min), 20x (95 ˚C, 1 min; 60 ˚C, 1 min; 72 ˚C, 3 min), 10x (95 ˚C, 1 min; 40 ˚C, 1 min; 72 ˚C, 3 min); 1x (72 ˚C, 10 min) were used. For the second round the primers were RB4 and Qo (sequence detailed in **Table I** and in [17]) and the cycling conditions: 1x (96 ˚C, 5 min) 35x (95 ˚C, 1 min; 60 ˚C, 1 min; 72 ˚C, 3 min) 1x (72 ˚C, 10 min).

Analysis of the genomic regions flanking the insertion

The products amplified by inverse PCR or RESDA-PCR were separated by agarose gel electrophoresis. The fragments obtained were isolated with a gel extraction kit (Quiagen, Germany) and sequenced (Stabvida, Portugal). The resulted sequences were analyzed by comparison with *Chlamydomonas* genome database [31].

Analysis of pigments

Carotenoids and chlorophylls were extracted with 80 % acetone. The separation and chromatographic analysis of pigments were preformed in a Merck Hitachi High-performance liquid chromatography (HPLC) equipped with a Diode-array detector as described by Young and coworkers [32], using a RP-18 column and a flow rate of 1 ml min^{-1}. The mobile phase consisted of: solvent A, ethyl acetate; solvent B acetonitrile/ water (9:1, v/v) and the gradient programme applied was: 0-16 min 0-60 % A; 16-30 min 60 % A; 30-35 min 100 % B. Injection volume was 100 µl. Pigments detection was carried out at 450 nm. Pigments standards were supplied by SIGMA or DHI (Hoersholm, Denmark).

Quantitative Real-Time PCR (QPCR)

Real time PCR was performed on a Mx3000P Multiplex Quantitative PCR System from Stratagene using the Brilliant SYBR Green QPCR Master Mix (Stratagene). Each determination was carried out in triplicate using genomic DNA as template and 10 pmoles of the indicated primers in a final volume of 25 µl. Cycling conditions were: 10 min at 95 ˚C for activation of the hot start Taq polymerase and 40 cycles for the melting (30 s at 95 ˚C), annealing (30 s at 60 ˚C) and extension (30 s at 72 ˚C). The fluorescence measurement was made at the end of the annealing step. A dissociation curve (30 s at 95 ˚C, 30 s at 55 ˚C and 30 s at 95 ˚C) was applied at the end of the amplification reaction to check possible formation of dimmers.

Results

Generation of insertional mutants

Insertional mutants were obtained by nuclear transformation of *Chlamydomonas reinhardtii*, strain 704, with a 1800 bp lineal DNA fragment, which contains the paromomycin resistant *AphVIII* gene from *Streptomyces rimosus* under the control of the Hsp70A:*RbcS2* promoters and the *RbcS2* terminator region [24]. This cassette, obtained by the digestion of pSI103 with *Not*I and *Kpn*I restriction enzymes (**Figure 1.1**), was randomly integrated by non-homologous recombination into the nuclear genome of *C. reinhardtii*, causing disruption of nuclear genes and generating random mutants. About 2100 mutants were isolated at dim light in the presence of the antibiotic paromomycin and screened for light sensitivity, pigment deficiency and lack of zeaxanthin in response to high light stress phenotypes as described below. The mean transformation efficiency was about 270 transformants per µg of DNA. Mutants were maintained in TAP-agar plates in the presence of paromomycin to minimize gene silencing of the inserted DNA. The *AphVIII* cassette was chosen as selectable marker because its high level of expression has already been shown [23] and the selective antibiotic paromomycin lacks the mutagenic effect reported for bleomycin [14]. No more than 100 ng of DNA per transformation were used in mutagenesis experiments since this small quantity of DNA is enough to ensure a good number of transformants minimizing the integration of multiple copies of the marker DNA, as has been set up by González-Ballester and coworkers [21]. Single integration is desirable to establish an unequivocal relation between the observed phenotype and the disrupted gene.

Screening of insertional mutants sensitive to high light and with altered pigment composition

Our objective was to identify tagged mutants with an altered composition of pigments, to gain more knowledge about the synthesis of chlorophylls and carotenoids in *Chlamydomonas*. Some of these mutants can be easily identified by direct observation, because if they have a great deficiency in pigments they will appear as white or pale yellow colonies. But those mutants which have a small pigment deficiency or lack any minor carotenoid will not show an obvious colour difference. To avoid loosing these partial pigment deficient transformants, we have designed a two steps screening strategy: In the first step we isolated a collection of light-sensitive mutants, whose pigment composition was systematically analysed by HPLC in the second step.

The first screening step was carried out exposing the insertional mutants to high light (800 µE m^{-2} s^{-1}), as described in Screening of light sensitive and pigment deficient mutants. In this way we were able to select 14 light sensitive mutants among the 1200 paromomycin resistant mutants obtained. Two of these mutants were obviously deficient in pigments since they were practically white (mutant M2) or pale orange (mutant C13) in dim light and unable to live at standard light intensity. Mutant M2 practically lacks carotenoids and chlorophylls and mutant C13 has very low content of chlorophylls and slightly lower content of carotenoids than the control non-transformed cells. The chromatographic pigment spectra and the coloration of the colonies of these two mutants are shown in **Figure 3** in comparison with the pigment profile of control cells.

The other 12 light-sensitive mutants were cultured in TAP medium at low light intensity, their carotenoids and chlorophyll content was determined by HPLC analysis,

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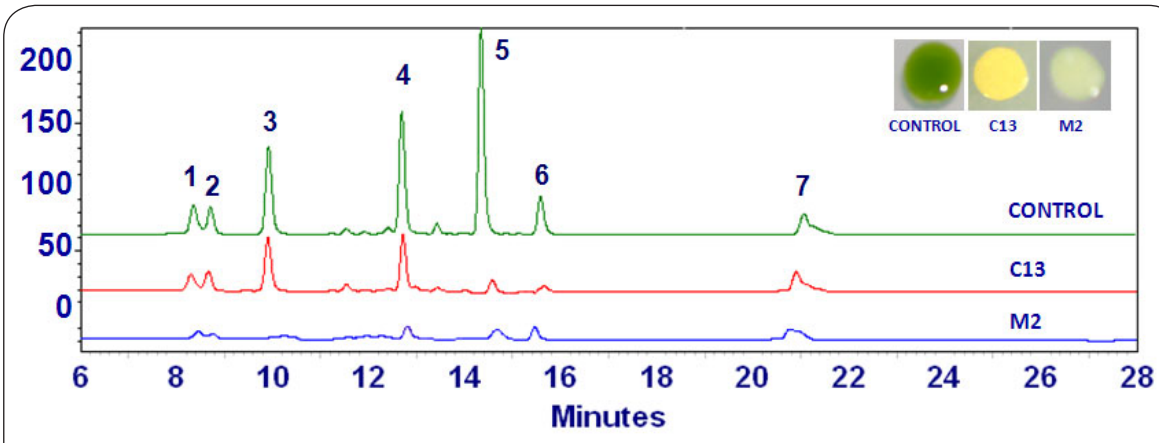

Figure 3. Analysis of pigment-deficient mutants. Pigment phenotype and HPLC-chromatograms registered at 450 nm for C13 and M2 mutants cultured at dim light and comparison with control *Chlamydomonas* cells cultured in the same conditions are shown. Peaks identification: Loroxanthin (1), Neoxanthin (2), Violaxanthin (3), Lutein (4), Chlorophyll b (5), Chlorophyll a (6), β-Carotene (7).

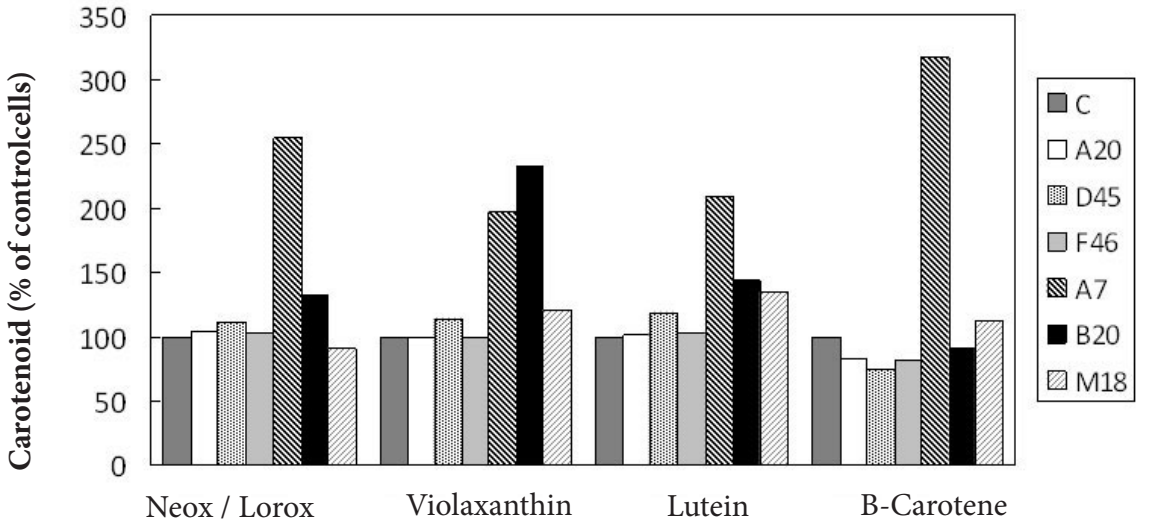

Figure 4. Carotenoid content in some of the light sensitive mutants. Carotenoid content is expressed as a percentage of the carotenoids content in control cells. The values represent the average of three measures.

referred to the dry weight and expressed as the percentage over the corresponding carotenoid concentration in control cells. The carotenoids content of some of these mutants is shown in **Figure 4**. This rescreening allowed us to select two additional mutants that, beside the sensitivity to high light, had an altered pigment profile. Mutant B20 had very high content of the epoxidated carotenoid violaxanthin, which reached an intracellular level of 0.9 mg g^{-1} DW, 2.5 fold the level observed in control *Chlamydomonas* cells cultured in the same conditions. While mutant A7 had higher content of all the carotenoids, mainly of b-carotene, which was three fold the content of control culture, and violaxanthin, which content was more than two-fold the value in control cells. The other light sensitive mutants (A4, A20, D45, F46, M3, M4, M5, M12, M17 and M18) had normal pigment composition, but the marker- gene insertion site was also studied in these transformants (**Table II**).

Screening of insertional mutants with an altered response to high light stress

The main short-time response in many higher plants and microalgae when transferred from low to high light intensity is the synthesis of zeaxanthin from violaxanthin through the xanthopyll cycle [**33**,**34**]. In *Chlamydomonas* this response to high light shifts is well documented [**4**]. The synthesis of zeaxanthin in *Chlamydomonas* cells after exposure to a high light stress [**35**] and other kind of stress conditions [**36**] has been previously reported. It has been demonstrated that this

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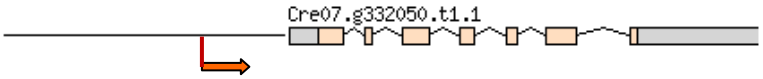
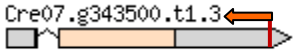
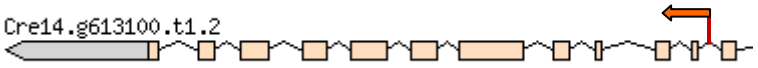
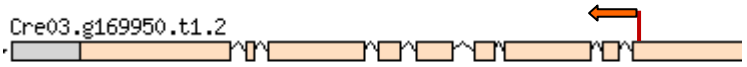
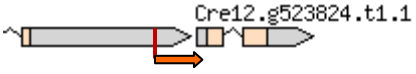
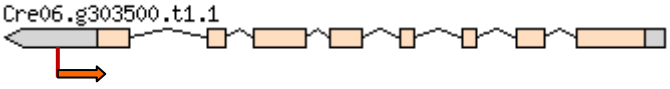
Table II. Analysis of the genomic regions flanking the insertion of the marker gene for some of the obtained mutants.

Mutant	Insertion site	Protein	Scheme of the insertion site
Phenotype HLSENS: sensitivity to high light			
A4	12:9186350	No functional annotations for this locus (Cre12. g13733)	
A20	16:7167887	FERROUS IRON TRANSPORTER (Cre01. g16784)	
D45	ND	(possible insertion of several tandem copies of the marker)	-
F46	7:2832774	No functional annotations for this locus (Cre07. g332050)	
M3	7: 4504328	ZINC FINGER MYND domain containing protein (cre07.g343500)	
M4	14: 1405326	FLAGELLAR OUTER DYNEIN ARM-DOCKING COMPLEX 3 (ODA-DC·) (Cre014. g617550)	
M5	14: 807625	No functional annotations for this locus (Cre014. g613100)	
M12	5:3180164	No functional annotations for this locus (Cre05. g239600)	
M17	3:3764505	JUMONJI domain containing protein (Cre03. g169950)	
M18	12:4609072	No functional annotations for this locus (Cre03. g523824)	
Phenotype PIGMENTS: Altered pigment composition			
A7	6:7947320	G-PATCH NUCLEIC ACID BINDING PROTEIN (Cre06. g303500)	
B20	ND	-	
C13	5:1831326	MAGNESIUM CHELATASE, SUBUNIT D (Cre05. g422000)	
M2	ND	-	
Phenotype ZEALESS: Deficient synthesis of zeaxanthin in response to high light stress			
Z7	23: 9766550	PROTEIN TYROSINE KINASE (Cre23: g9766550)	
Z34	ND	Insertion in a repetitive DNA region of the genome	-
Z198	3: 4120575	No functional annotations for this locus (Cre03. g181900)	

Localization of the marker insertion site and description of the protein encoded by the interrupted gene, when available, are shown. Dark grey: untranslated regions. Light grey: exons. Lines: introns. The Arrows indicate the point and the sense of the insertions. In the insertion site column the chromosome: nucleotide position for the insertion is indicated.

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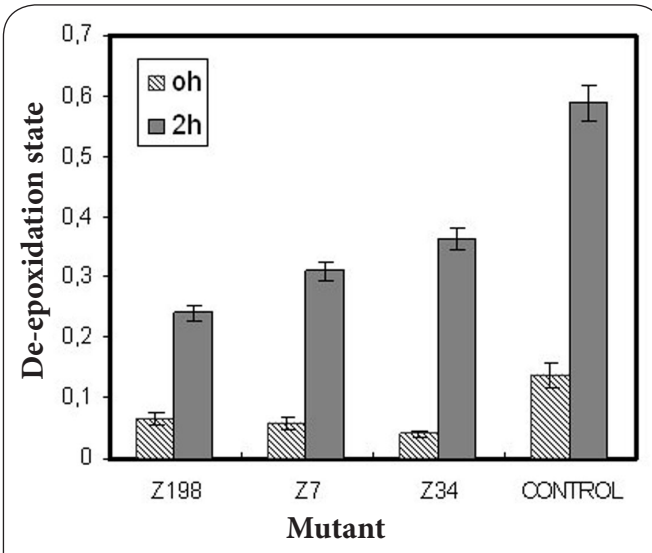

Figure 5. De-epoxidation state of the xanthophyll cycle in dark pretreated Control and ZEALESS mutants of *Chlamydomonas reinhardtii*. Dark pretreated cells were transferred to high light (800 µE m^{-2} s^{-1}) and the de-epoxidation state, expressed as the ratio [(ant + zea) / (ant + zea + viol)], was determined after 2 h.

zeaxanthin is produced from violaxanthin via the xanthophyll cycle [33], but neither the enzyme nor the gene encoding this putative violaxanthin de-epoxidase (*VDE*) has been identified yet in *Chlamydomonas*. In the sequenced genome of this chlorophyte no orthologues to plant or other algal *VDE* genes have been identified [37].

About 900 insertional mutants were obtained and screened to check their response when submitted to high light stress. The mutants were picked up and cultured in 5 ml TAP liquid media with paromomycin 15 µg ml^{-1} until the exponential phase of growth, then they were transferred to dark, where they stayed for 24 h. After the dark pretreatment the cultures were submitted to high light intensity (800 µE $m^{-2}s^{-1}$). The ability of the stressed mutants to epoxidise violaxanthin producing the quenching xanthophylls antheraxanthin and zeaxanthin, expressed as the de-epoxidised state, was determined after 2 h of treatment and compared with that of control *C. reinhardtii* cells submitted to the same conditions. This allowed us to isolate 3 mutants (Z7, Z34 and Z198) in which the de-epoxidation state after the high light shock was significantly lower than the de-epoxidation state of control cells in the same stress conditions (**Figure 5**).

The standard intracellular level of the quenching xanthophylls, zeaxanthin and antheraxanthin, is very low in the dark. Their concentration was about 14 % of the total cycle xanthophylls pool in control cells and between 4 and 6 % in the studied mutants. Under high light stress, the de-epoxidation state increases until 0.58 in control cells, while the de-epoxidation state in the selected mutants reaches values between 0.24 and 0.32. The lowest de-epoxidation state under high light stress was observed for mutant Z198, with a value of 0.24, which is less than half the de-epoxidation state of control cells under the same high light stress conditions.

In **Figure 6**, the time course evolution of the xanthophyll cycle components for control *Chlamydomonas* cells and for the three selected zeaxanthin deficient mutants (ZEALESS mutants) submitted to light stress is plotted. After 24 h of dark pretreatment the cultures were exposed to high light intensity (800 µE m^{-2} s^{-1}) for 6 hours. In these conditions, the zeaxanthin intracellular concentration in the mutants Z34 and Z7, was about half the concentration in control untransformed cells, while in mutant Z198 the zeaxanthin content did not even reach one fourth the zeaxanthin level of control cells. The decrease in violaxanthin content was much slower and minor in the mutants than in the control. On the contrary, the production of the intermediate antheraxanthin is similar in control and mutant cells, being only slightly lower in the mutant Z198. This could suggest that both sequential steps involved in violaxanthin de-epoxidation to antheraxanthin and zeaxanthin are differentially regulated. Although in the three mutants production of zeaxanthin and violaxanthin de-epoxidation was partially inhibited, no mutant with total lack of de-epoxidation activity was obtained.

Identification of the genomic regions flanking the insertion

To establish an unequivocal relationship between the observed phenotype and the affected gene it is necessary to ensure a unique insertion event. The DNA was kept to the minimum in the transformation experiments and the number of copies of the tag DNA was checked by Real Time PCR using the single-copy gene *Nitrate Reductase 1* (*Nia1*) as reference. This method has been previously validated by comparison with southern blot analysis [21]. The number of integrations was calculated using the delta Ct method [38] and specific primers of very similar efficiencies for *AphVIII* and *Nia1* (**Table I**). Efficiencies of the primers were previously determined [21]. We found that practically all of the mutants analyzed had a single copy of the tag DNA in their genomes. The only exception was mutant D45 that seems to have several copies of the marker gene. Two different methods were used to determine the genomic DNA flanking the inserted marker gene: RESDA-PCR [25] or inverse PCR over ligation [26].

RESDA-PCR basically involves using specific primers for the marker gene together with degenerated primers, designed on the basis of the presence of frequent restriction sites randomly distributed in the microalgal genome [25]. A schematic representation of the RESDA strategy with indication of the annealing sites for the primers is shown in **Figure 1.2**. This method allowed the amplification of the flanking region in 3 of the 17 mutants analysed. Inverse PCR over ligation is based on the fact that the chosen restriction enzyme, which cuts once inside the *AphVIII* gene, will also cut outside the insert, in the flanking genomic regions. On the basis of this

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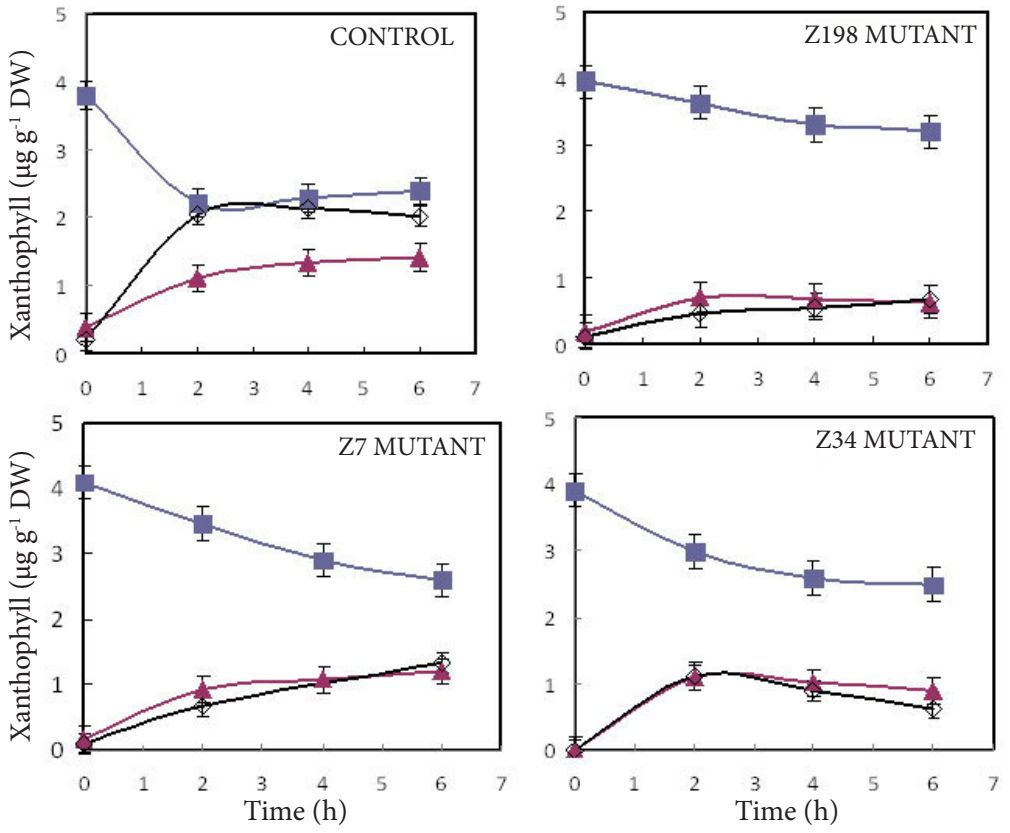

Figure 6. Time-course evolution of the xanthophyll cycle components in *C. reinhardtii* upon transition from dark to high light. Dark pretreated control and ZEALESS mutant cells were transferred to high light (800 µE m^{-2} s^{-1}) and the concentration of zeaxanthin (●), antheraxanthin (♦) and violaxanthin (■) was quantified by HPLC after 2, 4 and 6 hours of growth in these conditions.

hypothesis the genomic DNA of the mutant is digested with the chosen restrictase and ligated. The ligation resulted is used as a template for the inverse PCR. The approximate situation of the primers designed to amplify the genomic region flanking the insertion is shown in **Figure 1.1**. This method allowed the identification of the flanking region in other 12 of the 17 mutants analysed. In two mutants (B20 and M2) no amplification was possible by either of the approaches used and the flanking region could not be identified. The sequences of the region adjacent to the insertion were compared with the genome sequence of *Chlamydomonas* [**31**].

Identification of the genes responsible for the observed phenotype

The sequences of the genomic regions flanking the insert allowed the identification of the gene disrupted by integration of the marker *AphVIII* gene. All the sequences obtained by amplification from the 13 mutants successfully analyzed, included a short fragment homologous to the 3`UTR region of *RbcS2* located in chromosome 2, which confirmed specificity of the amplification, and a fragment that showed very high homology with the genome of *Chlamydomonas*. These fragments were compared with the latest version of the *Chlamydomonas* genome database at the joint genome center [**31**] by the BLAST tool. The insertion site and the protein encoded by the interrupted gene, when available, are shown in **Table II**. In four of the mutants studied, identification of the gene affected by the integration was not possible. In mutants B20 and M2 several attempts did not yield any amplification. This could mean that the insertion has taken place in a region of low accessibility or that silencing epigenetic phenomena that modify the structure of the marker gene are interfering with the alignment of primers during PCR amplifications as proposed by González-Ballester and coworkers [**21**]. In mutant D45, predicted region coincides with the region of the chromosome 8, where is located the promoter of the *Hsp70A* gene and within the region of chromosome 2 corresponding to the *RbcS2* promoter, which are part of the same insertional cassette, indicating the possible insertion of several tandem copies of the marker gene, as was already predicted when determining the number of integrations. In mutant Z34 the amplified DNA fragment showed 100 % homology with several regions of the genome located in different chromosomes, so the region affected by the integration could not be identified in

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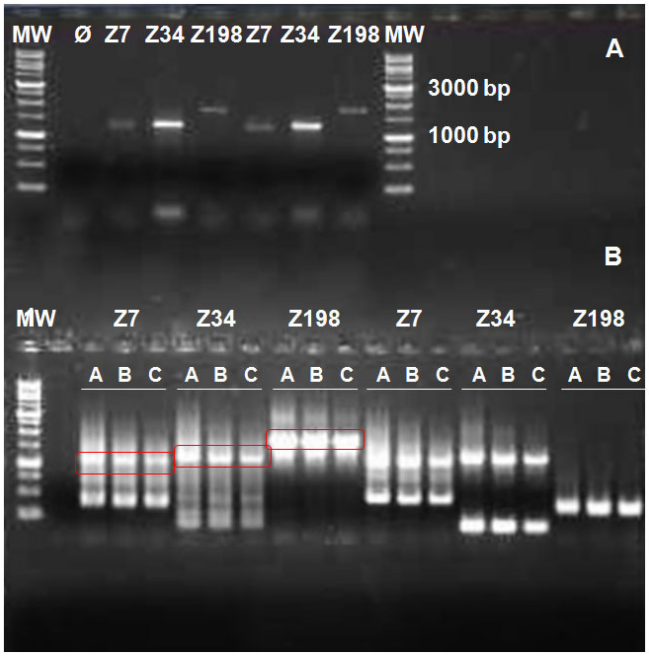

Figure 7. Agarose gel analysis of the inverse PCR products obtained for 3 insertional mutants. Genomic DNA of transformants Z34, Z198 and Z7 was isolated, digested and ligated as described in the materials and methods section. The resulting ligations were used as template for nested PCR. In the first amplification round (**A**) RB1 and LB1 primers were used with the resulting ligation as template. In the second round (**B**) primers RB4 and LB4 were used for reamplification of different dilutions of the PCR products obtained in the first round. Lines 1 and 2: 1Kb ladder DNA marker; a, b and c correspond to dilutions 1:1, 1:10, 1:100, respectively.

this case because the marker gene has probably been inserted in a repetitive region of the genome. In *Chlamydomonas* more than 20 % of the genome is repetitive DNA [1].

Six of the disrupted genes in the analysed mutants encoded predicted proteins of unkown funtions, while the other 7 genes encoded known proteins with a great variety of functions. **Table II** shows the resume of the genes affected in the 13 mutants successfully analysed. Among the light-sensitive mutants we found mutants affected in the function and assembly of the flagella, such as the mutant M4, in which the tag DNA is inserted at the beginning of the gene encoding the subunit 3 of the Flagellar Outer Dynein arm-docking complex subunit 3 (oda-dc 3). We also found mutants affected in nucleic acid binding proteins with known conserved domains, such as the Jumonji (M17) or the Mynd Zinc Finger (M3), involved in regulation of the transcription. A mutant affected in the D-Tyrosil-tRNA (Tyr) Deacylase (A4), an editing enzyme that removes D- amino acids from mischarged tRNAs. And mutants affected in transduction pathways, such as the Putative Mitocondrial GTPase protein mutant (A20).

Among the pigment deficient mutants, C13 has an insertion in the gene encoding the D Subunit of Mg-Chelatase, which catalyzes the first committed step of chlorophyll synthesis, the insertion of Mg^{2+} into the protoporphyrin ring to yield Mg-protoporphyrin IX [39], and is a key regulatory point in the chlorophyll biosynthetic pathway [40]. In A7 mutant the tag is inserted in a putative regulatory protein, the nucleic acid binding protein, which contains the D111/G-patch dominium, a motif found in many Ribose Nucleic Acid (RNA) Binding proteins.

For mutant C13 a detailed study of the insertion site was carried out. An additional set of primers were designed to identify the genomic region at the 5`end of the inserted cassette (**Figure 1**) and it was revealed that in this mutant the insertion was clean without deletions, and that the tag DNA was placed in the third intron of the interrupted gene, causing the synthesis of a truncated and non-functional version of the Mg-chelatase subunit D. A phenotype similar to that of mutant C13 has been found in other *C. reinhardtii* mutants affected in the Mg-chelatase subunits H [41] and M [42].

The last group of mutants, deficient in their response to high light stress, are unable to synthetize normal quantities of zeaxanthin when shifted to high light conditions. The gene affected by the insertion in mutant Z198 corresponds to a predicted protein of unknown function, while in mutant Z7, the tag is inserted in a gene encoding a protein Tyrosine Kinase, involved in signal transduction pathway. In Z34 it was not possible to identify the insertion site, the DNA sequence preceding the marker resulted to hit with multiple zones of the genome in all cases with homologies near 100 %, indicating that it was a repetitive region of the genome. The predicted protein in mutant Z198 is a hypothetical protein with 218 residues which has also been found in other related algae such as *Volvox carteri* and *Micromonas*, it has certain homology with bacterial metal responsive transcripcional regulator factors of the MerR family. Detailed analysis of the genomic region flanking the marker gene in these three mutants is presented in **Figure 7**.

Discussion

Reverse genetic approaches, such as RNA interference [43] or PCR, exploring of tagged mutant collections [14] allow the knock out of specific known genes and have helped to the functional characterization of a good number of genes in *Chlamydomonas*. However, insertional mutagenesis is the best approach to identify uncharacterized genes related to a certain phenotype. The latest version of the nuclear genome of *Chlamydomonas*, ver 5.3, was released in January 2012. It comprises 111 Mb arranged in 17 linkage groups (chromosomes) and 37 additional unmapped scaffolds, with about 17728 protein encoding loci. Some of these loci encode alternate transcript making a total of 19529 protein coding transcripts, however there is a large number of predicted proteins of unknown function, confirming that an effort is necessary to understand the function of genes which cannot be assigned by sequence comparison [1].

In our analysis most of the transformants (94 %) had a single *AphVIII* insertion. Cloning, sequencing and aligment of the DNA fragments, amplified by RESDA or inverse PCR in each transformant, with the *Chlamydomonas* database has allowed the identification of the region adjacent to the insertion in most of the mutants studied. We observed that in 77 % of the transformants analysed, the insertion took place in intragenic regions. This rate is similar to that observed by Dent and coworkers [**20**] and González-Ballester and coworkers [**21**] in functional genomic studies of photosynthesis and the regulation of nitrate assimilation, respectively. In all cases the marker insertion has occurred within a predicted gene or very near one, so identification of the gene responsible for the observed phenotype, has been done considering that the gene interrupted is the most probable responsible of the observed phenotype. If integration of the tag gene takes place with big genome deletions of upto 20 Kb, as has been reported in some insertional mutagenesis experiments, other genes placed near the insertion site could also be affected and be responsible for the observed phenotype, but since we do not have the evidence of the deleted genomic area in most of the mutants, this possibility has not been considered here. Only in mutant C13 the flanking region at both sides of the insertion have been amplified and identified, revealing that the tag insertion took place without a deleted nucleotide.

To identify the region flanking the marker inserted, both the RESDA-PCR [**25**] and inverse PCR after digestion with an enzyme having a unique restriction site in the marker gene, [**26**] were successfully used. Most of the mutants were solved by inverse PCR and those mutants recalcitrant to inverse PCR were successfully solved by RESDA. Only 4 mutants remained unresolved, probably due to the insertion of several concatamerized copies of the marker gene, as happens in mutant D45; due to insertion in a genome repetitive element, as observed in mutant Z34; or because of insertion in a difficult region of the genome, or modifications of the marker gene after the insertion that make difficult the aligment of primers during PCR, as proposed by González-Ballester and coworkers [**25**].

Almost half of the mutants (46 %) had insertions in genome loci with unknown functions. Among disrupted genes with a known function we found genes involved in a great diversity of functions, from flagella motion to regulatory or signal transduction processes, suggesting that the sensitivity to high light and the synthesis of pigments are complex and very regulated processes and that a large number of mutants should be generated to saturate the genes encoding proteins involved in these processes. Here we describe the generation and characterization of 13 of the obtained mutants, as a first step to the generation of a large collection of mutants which will be mainly focussed on mutants unable to synthetize zeaxanthin in response to high light stress. This strategy has already allowed the isolation of Z7, Z34 and Z198 mutants, in which the xanthophyll cycle is partially inhibited. The de-epoxidation state in these mutants is less than half the de-epoxidation state in control cells cultured in the same conditions, due to a big decrease in the zeaxanthin synthesis and in the de-epoxidation of violaxanthin. Although neither mutant Z7, which is affected in a protein with homology to protein tyrosine kinase, nor Z198, which is affected in a gene which encodes a predicted protein of unknown function, are *VDE* mutants, it is clear that they must be affected in regulatory or signalling processes very related to violaxanthin de-epoxidase.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
M. Vila has acquired some experimental data, contributed to data analysis and to writing of the manuscript.
E. Díaz-Santos and M. de la Vega have acquired experimental data and performed statistical analysis.
I. Couso has made critical revision of the maunsucript.
R. León has conceived and designed the research, has analyzed obtained data and written the manuscript.

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