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Pleiotropic mutants hypersensitive to heavy metals and to oxidative stress in *Chlamydomonas reinhardtii*

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Abstract

Insertional mutagenesis was used in *Chlamydomonas reinhardtii* to isolate original mutants hypersensitive to multiple drugs and physical agents. Out of 5200 transformants analyzed, 13 mutants belonging to seven phenotypic classes were isolated. Five were exclusively sensitive to cadmium and represented two loci. The other mutants were pleiotropic and presented a cross sensitivity to several (2-6) of the following agents : cadmium, copper, lead, paraquat, hydrogen peroxide, UVC and light. In all mutants analyzed, the hypersensitive phenotype was most probably due to a single mutational event. The sensitivity of several pleiotropic mutants to a broad range of physical and chemical agents suggests that the disrupted genes are involved in multiple stress responses.

Keywords: *Chlamydomonas reinhardtii;* Heavy metal; Oxidative stress; Insertional mutagenesis; Pleiotropic mutant

1. Introduction

In response to heavy metals and to various oxidative stress agents, living organisms have evolved multiple scavenging systems, including mechanisms that allow them to escape the deleterious effects of reactive oxygen species (ROS). In the yeast *Saccharomyces cerevisiae,* the *YAP1* gene that encodes a b-ZIP transcription factor belonging to the AP-1 family is involved in multiple stress responses [1,2]. The YAP1 protein controls the expression of a number of genes, including those coding for glutathione reductase, γ-glutamyl-cysteine synthetase, thioredoxin, an ATP-binding cassette protein involved in cadmium (Cd) tolerance and other antioxidant proteins [2,3]. Genes homologous to *YAP1* have been isolated from *Schizosaccharomyces pombe, Kluyveromyces lactis* and *Candida albicans* [2]. The disruption of the genes encoding these b-ZIP transcription factors in yeasts confers a pleiotropic drug hypersensitivity phenotype [1,2]. In humans, the AP-1 protein, which plays a crucial role in cell proliferation, differentiation and morphogenesis, is also induced by oxidative stress agents like H_2O_2, UVC, UVA and ionizing radiation [4].

While the responses to heavy metals [5-7] and to oxidative stress [8] are well documented in plants, pleiotropic mutants are missing and the transcriptional regulation of genes involved in these stress responses is poorly understood. Here, we use insertional mutagenesis to produce original pleiotropic mutants of the unicellular green alga *Chlamydomonas reinhardtii.* These mutants which are hypersensitive to heavy metals and to oxidative stress should allow further characterization of genes involved in multiple stress responses.

2. Materials and methods

2.1. C. reinhardtii strains, culture, transformation and genetic analysis

Mutants deficient in cell-wall biosynthesis and auxotrophic for arginine, strains 325 *(cwl5 arg7-8 mt$^+$)* and 365 *(cwl5 arg7 mt$^-$),* were used in this study. The *arg7* and *arg7-8* mutations affect the *ARG7* gene encoding argininosuccinate lyase. These strains and their arg$^+$ transformants were grown on Tris-acetate-phosphate (TAP) agar medium [9], supplemented with 100 mg l^{-1} arginine, when required. Cell suspensions were prepared in minimal medium [9] deprived of nitrogen source (M-N). Except otherwise stated, cells were grown at 25°C under continuous light (70 μE m^{-2} s^{-1}).

Transformation was performed using 0.6 μg *Bam*HI-linearized pASL (pBCKS$^+$+*Chlamydomonas ARG7* gene [10]) as previously described [11]. Crosses, maturation of zygotes and random analysis of meiotic products were carried out according to published procedures [9].

2.2. Phenotypic analysis

To select sensitive mutants, cells from transformed clones were transferred onto fresh TAP agar medium (60 colonies per plate), then replica-plated onto TAP containing 400 µM $CdCl_2$ or 0.4 µM paraquat. The sensitive clones were further investigated as follows: TAP agar plates containing each one of the toxic agents ($CdCl_2$, $CuSO_4$, $Pb(NO_3)_2$, $HgCl_2$, H_2O_2 or paraquat) were spotted with 15 µl of cell suspensions (10^6 and 10^7 cells ml^{-1}). For the UVC treatments, the TAP plates spotted with the different cell suspensions were exposed for 20, 30 or 40 s to a 254 nm UV light source, incubated for 24 h in the dark (to avoid photoreactivation), then transferred to the light. The results of replica-plating and spot analysis were observed after 2-3 days.

The survival to a short exposure to high Cd concentrations was determined as follows: 10^7 cells suspended in 100 µl M-N liquid medium containing 4, 8 and 16 mM Cd were incubated in the light (70 µE m^{-2} s^{-1}) for 1 h. Dilutions of the suspensions were then spread onto TAP agar medium together with $2x10^7$ cells of strain 325. The presence of an auxotrophic cell carpet enhanced the plating efficiency of wall-less prototrophic cells spread at low density [12]. The survival rates were calculated after 7 days.

2.3. DNA preparation and hybridization procedures

Total *Chlamydomonas* DNA was extracted and purified according to G. Gloeckner (personal communication). About $2x10^8$ cells were resuspended in 700 µl of CTAB buffer (2% (w/v) cetyltrimethylammonium bromide, 100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% (v/v) β-mercaptoethanol) and incubated at 65°C for 1 h. After two phenol-chloroform extractions, DNA was precipitated with 0.7 volume isopropanol, resuspended in TE and treated with ribonuclease A (20 µg ml^{-1}). Southern blot analysis was carried out as previously described [10] using the 130-bp ***Stu*I**-***Sph*I** fragment from exon 11 of the *Chlamydomonas ARG7* gene as a probe.

Table 1: Growth of the Cd mutants in the presence of Cd

Clones	Cd (µM)				
	0	100	200	300	400
C1	++++	+++	++	+	+
Cd48	++++	+++	++	++	-
Cd47	++++	+++	++	+	-
Cd32	++++	+++	++	+/-	-
Cd51	++++	++	+	-	-
Cd29	++++	+	+/-	-	-
Cd46	++++	++	-	-	-
Cd30	++++	+	-	-	-
Cd34	++++	+	-	-	-
Cd41	++++	+	-	-	-
Cd43	++++	+	-	-	-
Cd50	++++	+/-	-	-	-

Growth, recorded after 3 days of incubation in the light, varied from very good (++++) to no growth (—). C1: control (strain 325 transformed with pASL and showing a wild phenotype).

3. Results

3.1. Isolation and phenotypic characterization of pleiotropic hypersensitive mutants

Cells from the wall-less arginine-requiring strain 325 were transformed with plasmid pASL carrying the *ARG7* gene [10] and arg^+ transformants were selected on TAP agar medium. With a view to isolating mutants sensitive to Cd and to the redox cycling drug paraquat, 5200 arg^+ transformants were replica-plated onto plates containing 400 μM $CdCl_2$ or 0.4 μM paraquat. At these concentrations of the toxic agents, the non-transformed strain, as well as most arg^+ transformants, were still able to grow. Eleven mutants sensitive to Cd (Cd mutants) and two mutants sensitive to paraquat (PQ mutants) were isolated following this procedure.

To define more accurately the phenotype of each sensitive strain, the mutant cells were suspended in liquid medium and 15 μl of two different cell suspensions (10^6 and 10^7 cells ml^{-1}) were spotted onto plates containing various concentrations of Cd (100-400 μM) or paraquat (0.3-0.7 μM). As shown in Table 1, Cd mutants sharply differed in their sensitivity to the metal, the minimal inhibitory Cd concentrations ranging from 200 to 400 μM. The two PQ mutants were also differently sensitive to the drug: PQ42 was still able to grow on TAP+0.6 μM paraquat but not on TAP+0.7 μM, while PQ44 cells stopped growing on TAP+0.6 μM paraquat or higher. None of the Cd-sensitive mutants were sensitive to paraquat and vice-versa. Moreover, we noted that all mutants, except PQ42, grew normally on medium deprived of inhibitor.

Four Cd-sensitive mutants were also examined for their response to a short exposure to high Cd concentrations. The results of a typical experiment are shown in Fig. 1. At all Cd concentrations, the survival rates were lower in the mutants than in a non-sensitive arg^+ transformant (strain C1) taken as a control. The results obtained with Cd32, Cd34 and Cd47 confirmed those presented in Table 1. In contrast, Cd46, which was very sensitive to a continuous exposure to the metal, was only slightly affected by a short treatment. In this experiment, the survival rate of the control was unaffected by 4 and 8 mM Cd but dropped to 76% after exposure to 16 mM Cd. This could explain why in all strains the survival rate (expressed as a percentage of control) was greater at 16 mM than at 8 mM.

The mutants were then analyzed for their possible cross sensitivity to copper, lead, mercury, hydrogen peroxide (H_2O) and UVC light (Table 2). Their sensitivity to a direct transfer from low light (5 μE m^{-2} s^{-1}) to a higher light intensity (70 μE m^{-2} s^{-1}) was also investigated. The analyses were achieved using a range of inhibitor concentrations. As a result of these phenotypic analyses, the 13 clones were distributed between seven classes (Table 2). Five mutants (Cd30, Cd34, Cd41, Cd43, Cd47) were sensitive only to Cd (class 1), the Cd47 being clearly less sensitive than the other four (see Table 1). The eight remaining mutants presented multiple sensitivity phenotypes allowing the definition of classes 2 to 7 (Table 2). The mutants of classes 2 and 3 were sensitive to two or three agents, while the other Cd mutants (classes 4 and 5) were hypersensitive to multiple drugs and physical agents. The two PQ mutants, which were differently sensitive to paraquat (see above), were also differently sensitive to other toxic agents, but not to metals.

Fig. 1. Survival rate (as a percentage of C1 control) of Cd32, Cd34, Cd46 and Cd47 submitted to a short exposure (1 h) to 4, 8 and 16 mM Cd. ND: not determined.

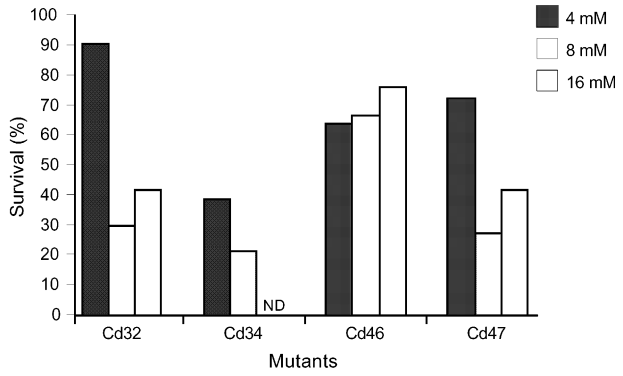


Table 2: Phenotypes of the hypersensitive mutants

Classes	Clones	Stress factors							
		Cd	Cu	Pb	Hg	H_2O_2	paraquat	UVC	light
WT	C1	-	-	-	-	-	-	-	-
1	Cd30	+	-	-	-	-	-	-	-
	Cd34	+	-	-	-	-	-	-	-
	Cd41	+	-	-	-	-	-	-	-
	Cd43	+	-	-	-	-	-	-	-
	Cd47	+	-	-	-	-	-	-	-
2	Cd29	+	-	-	-	-	-	-	+
	Cd50	+	-	-	-	-	-	-	+
3	Cd48	+	+	-	-	-	-	-	+
	Cd51	+	+	-	-	-	-	-	+
4	Cd32	+	+	-	-	+	-	+	+
5	Cd46	+	+	+	-	+	-	+	+
6	PQ42	-	-	-	-	+	+	-	-
7	PQ44	-	-	-	-	-	+	+	+

The phenotypes were determined by spotting 15 µl of cell suspensions (10^6 and 10^7 cells ml^{-1}) on TAP added with Cd (100-400 µM), Cu (200-500 µM), Pb (0.6-1.2 mM), Hg (4-10 µM), H_2O_2 (0.5-1.5 mM) or PQ (0.3-0.7 µM). The UVC and light sensitivities were determined as described in Section 2 and in text. +: sensitive phenotype (no growth); - : insensitive (wild) phenotype. C1: control (see Table 1).

3.2. Genetic analysis

The mutants exclusively sensitive to Cd (class 1) were crossed with each other and the meiotic progeny were analyzed for the presence of wild-type Cd-resistant recombinants. In the crosses involving Cd30, Cd34, Cd41 and Cd43, no recombinant was obtained, which indicates that the four mutations are allelic or very closely linked. In contrast, recombinants were produced in the crosses involving Cd47, indicating that the Cd47 mutation represents a second locus. It has to be pointed out that the four allelic mutants display the same sensitivity to Cd (Table 1).

In order to determine whether the mutations conferring the drug sensitivity are due to the insertion of a single copy of the *ARG7* gene, excluding any other insertional event, crosses between the arg+-sensitive mutants (all mt^+) and an arg^--resistant strain (strain 365 mt^-) were performed. The Cd29, Cd48, PQ42 and PQ44 mutant strains were not able to mate and were not submitted to this genetic analysis. In the nine crosses, the progeny were analyzed for their sensitivity or resistance to Cd and for their arg+/arg- character (Table 3).

An absolute cosegregation of arg+ and Cd^s phenotypes was observed only in the cross Cd34x365 (Table 3). The presence of a single insertion in mutant Cd34 was confirmed by a molecular analysis of this strain and of some meiotic products issued from the cross. Total DNA (3 µg) was restricted with *Pvu*II (a *Pvu*II site is located near 3' end of the *ARG7* gene), blotted then hybridized to the ^{32}P-labelled *ARG7* probe (Fig. 2). Besides the 3.5-kb fragment corresponding to the endogenous *arg7* mutant allele, an additional 2.9-kb fragment, visible in the original Cd34 mutant, was detected in arg+ Cd^s segregants, but not in arg^- Cd^R products, indicating a cosegregation of Cd^s phenotype and plasmid insertion.

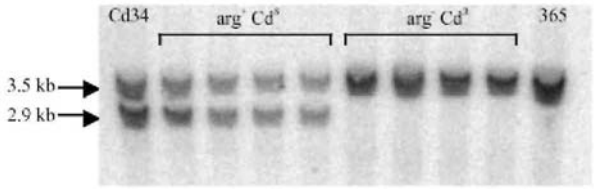
In cross Cd30x365, arg^+ Cd^R recombinants were produced in addition to the two parental classes (Table 3), which indicates that the gene disruption by a functional *ARG7* gene is accompanied by the integration of at least one additional functional *ARG7* copy. Four phenotypic classes were obtained in the other seven crosses. This suggests that in each of these Cd^s strains, the mutation results from the insertion of an inactive *ARG7* gene and that the arg+ phenotype originates from the integration of at least another pASL copy elsewhere in the genome. Southern blot analysis revealed that several Cd mutants contain more than one intact or truncated copy of the pASL plasmid in their nuclear genome (data not shown).

Two observations suggested however that sensitivity phenotypes were due to mutations at single loci in each clone. First, Cd^s and Cd^R characters segregated approximately in a 1:1 ratio in most crosses (Table 3). Second, we found that, in the pleiotropic mutants, the multiple sensitivity phenotype cosegregated with the Cd^s character (data not shown).

Table 3: Segregation of arg+/arg^- and Cd^s/Cd^R phenotypes in crosses of nine arg^+ Cd^s mutants with an arg^- Cd^R strain (365)

Crosses	No. of analyzed products	No. of parental products		No. of recombinant products	
		arg+ Cd^s	arg^- Cd^R	arg+ Cd^R	arg^- Cd^s
Cd30 x 365	57	34	10	13	0
Cd32 x 365	117	5	54	8	50
Cd34 x 365	120	73	47	0	0
Cd41 x 365	116	40	24	29	23
Cd43 x 365	166	57	36	31	42
Cd46 x 365	210	63	101	20	26
Cd47 x 365	117	51	32	17	17
Cd50 x 365	58	22	3	16	17
Cd51 x 365	73	17	13	19	24

Fig. 2. Southern blot analysis of the *Pvu*II DNA restriction fragments from Cd34, strain 365 and eight meiotic products (four arg^+ Cd^s and four arg^- Cd^R) obtained in the 365xCd34 cross (a fragment of the *ARG7* gene was used as a probe).



4. Discussion

Insertional mutagenesis was used to produce *Chlamydomonas reinhardtii* mutants hypersensitive to multiple agents. Out of 5200 transformants analyzed, 13 mutants were selected: five were exclusively sensitive to Cd while eight were pleiotropic and distributed into six phenotypic classes, depending on their response to the different agents (Table 2). Most of the pleiotropic mutants harbored more than one copy of the transforming plasmid (a frequent feature in *Chlamydomonas* transformants, see [10,13] for example). However, their multiple sensitivity phenotype is unlikely to result from several independent insertional events since the sensitivity to Cd

cosegregated in crosses with the sensitivity to other toxic agents.

The class 1 mutants are exclusively sensitive to Cd. In the yeast *S. pombe* and in *Arabidopsis thaliana,* several Cd-sensitive mutants affected in the metabolism of glutathione and phytochelatins have been isolated [5,7]. Cd-sensitive strains have also been isolated in *Chlamydomonas* [14] but to our knowledge, they have never been further characterized. The five class 1 mutants are distributed into two groups: four mutants are allelic and display the same sensitivity while the fifth one (Cd47) is less sensitive and represents a second locus. It is rather surprising that four of the five Cd-sensitive mutants are allelic since the integration of plasmids into the *Chlamydomonas* genome appears to be random [10]. It could be proposed that this apparent specificity is related to the procedure used for the selection.

Mutants belonging to the other classes (Table 2) are all pleiotropic. To our knowledge, such mutants have never been described in photosynthetic organisms. The Cd32 and Cd46 mutants, which are sensitive to several heavy metals, H_2O_2, UVC and light, are of particular interest. Their sensitivity to a broad range of chemical and physical agents suggests that the disrupted genes are involved in multiple stress responses. Like the *yapl* disruptant of *S. cerevisiae,* which is deficient in a b-ZIP transcription factor belonging to the AP-1 family [2], both mutants display hypersensitivity to Cd and H_2O_2. However, Cd32 and Cd46, unlike the yeast mutant [1], are insensitive to paraquat (Table 2), *o*-phenanthroline and cycloheximide (data not shown). On the other hand, the human AP-1 transcription factor is involved in the response to UV light [4] and in the transcriptional activation of metallothio-nein-encoding genes [15]. Metallothioneins are generally considered to be involved in Cu tolerance in plants [5,6] as well as other organisms. A mutation affecting transcription factor related to AP-1 in *Chlamydomonas* might lead to the observed pleiotropic phenotype.

The two PQ mutants also represent phenotypic classes that have not been isolated before in plants. PQ42 is sensitive to paraquat and H_2O_2, while PQ44 is sensitive to paraquat, UVC and light. These mutants could be affected in mechanisms involved in ROS detoxification [16]. In this respect, a *Chlamydomonas* mutant with reduced catalase activity and displaying increased sensitivity to light in the presence of methionine has been described [17].

A further development of this work will be the molecular characterization of the hypersensitive mutants. Since in the mutant Cd34 the sensitive phenotype results from a single insertional event, the inactivated gene could be cloned by plasmid rescue followed by the screening of a *Chlamydomonas* genomic library and complementation experiments. In most of the other mutants submitted to the genetic analysis, the untagging of the mutations (occurrence of both recombinant classes) would make plasmid rescue and direct cloning of the corresponding genes impossible. However, these genes could be isolated by complementation using a genomic cosmid library.

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