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CHARACTERISATION OF NEW RADIO RESISTANT STRAINS OF *Chlamydomonas reinhardtii*

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Abstract. We have constructed radioresistant strains of *Chlamydomonas reinhardtii* by mating a highly radioresistant strain (AK-9-9) with the cell-wall-less mutant (CW15) strain. The characteristics of 5 hybrid strains (H-3, H-4, H-7, H-12, H-18) and both parental strains CW 15(+) and AK-9-9(-) were investigated. Only strain H-3 was found to have a higher radioresistance than that of the highly radioresistant parental strain AK-9-9. With respect to the plastid pigments, the parental and hybrid strains can be divided into 2 groups: those having a higher content of chlorophyll '*a*' and '*b*' and carotenoids, namely CW-15, AK-9-9, H-3 and H-4; and those that have a lower content – strains H-7, H-12 and H-18. Strain H-3 was found to possess a lower proline content and higher SOD activity compared to the other strains. Data from transmission electron microscopy study show that the strain H-3 manifests all features characteristic of its parental CW15 (+) strain, with regard to protoplast organisation. The cell wall structure is similar to the parental AK-9-9. Thus, the H-3 is a useful radioresistant hybrid strain of *Chlamydomonas reinhardtii* in which to perform radiation and biochemical studies.

Keywords: *Chlamydomonas reinhardtii*, radioresistance, proline, pigments, ultrastructure.

AIMS AND BACKGROUND

Radioresistant mutants are scarce; up to now most studies have focused on radiosensitive strains. However, the molecular mechanisms of radioresistance exhibited by microorganisms may give us a further understanding of the response of mammalian cells and especially the response of radioresistant tumors. In addition, it may provide further insight into the response of plant cells to radiation,

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particularly from the point of view of the adaptive response to radiation and other environmental genotoxins.

Previously several radioresistant strains of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* were characterised[1]. The main aim of this work was to derive and characterise new radioresistant strains of *Chlamydomonas reinhardtii* by mating one of the previously derived highly radioresistant strains (AK 9-9) with the cell-wall-less mutant (CW15) strain. Unfortunately we were not able to derive cell-wall-less radioresistant hybrid strains on our experiments. Nevertheless, these characteristics make strain H-3 potentially useful in future radiobiological investigations

EXPERIMENTAL

Cell strains used. Chlamydomonas reinhardtii CW 15 (+) was characterised as cell-wall-less (*Chlamys*) strain, but its protoplast structure is typical for the genus *Chlamydomonas*[2]. The radioresistant strain AK 9-9 was derived from the wild-type strain 137C (+) by chemical mutagenesis with 2-amino-6-hydroxyaminopurine and is canavanine (*can*) resistant (500 mg/l) (Ref. 3).

Cell culture. Cultures of cells were routinely grown in *Euglena gracilis* (EG) liquid and solid media. Solid and liquid (L2) minimal media described previously[4] were used in survival experiments. L_2 supplemented with *can* (500 mg/l) was used to assess can-resistance of hybrid clones.

Mating protocol. Both mating and zygote clone analyses were performed according to the standard methods described by Harris[2]. Briefly, 5-7-day old cultures grown on L2 nutrient agar plates were transferred to low nitrogen medium 2 days before the gametogenesis. Appropriate cell numbers of both strains were suspended separately in nitrogen-free liquid medium and left overnight to facilitate gametogenesis. Gametes of both strains AK-9-9 *mt* (–) and CW 15 mt (+) were mixed and left for several hours in the light to allow mating to occur. 500 µl of mating suspension was then pipetted on to the maturation plates (complete medium containing nitrogen, with 4% agar).

Maturation plates were exposed to light for 24 h and then transferred to the dark (incubator, $t = 24°C$). After 7 days of maturation time zygotes were exposed to chloroform vapour for 30 s to kill any residual unmated cells.

More than 90 zygotic clones were isolated and analysed for their radio- and canavanine-resistance.

Irradiation and survival assays. A gamma-ray ^{137}Cs source, giving a dose-rate of approximately 4 Gy per minute was used. For survival measurement 3-5-day old cell suspension (stationary phase) with a density about10^6 cells/ml was irradiated with a range of doses up to 300 Gy. For survival measurements, cells at

appropriate dilutions were plated on solid L_2 medium and incubated under illumination. Cell clones were counted 10 days after plating.

Transmission electron microscopy. For transmission electron microscopy (TEM) the material was fixed in phosphate-buffered 3% glutaraldehyde (pH 7.4) for 12 h at 4°C and post-fixed in 2% $KMnO_4$ for 2 h at room temperature. After dehydration the material was embedded in the Spurr's epoxy-resin. Ultrathin sections were cut using ultramicrotome (Reichert, Austria). Sections were examined with a JEOL 1200 EX (Japan) electron microscope.

Plastid pigments. The content of plastid pigments (chlorophyll and carotenoids) of algal cells was determined spectrophotometrically according to Arnon[5]. Cells were harvested by centrifugation, sonicated and extracted with 80% (v/v) acetone at 4°C.

Proline estimation. Proline (Pro) was extracted and its concentration determined spectrophotometrically following the method of Bates et al.[6] Briefly, samples from algal cells were homogenised in 3% (w/v) sulphosalicylic acid and centrifuged. The supernatant fluid was treated with acetic acid and acid-ninhydrin and boiled for 1 h. Pro content was expressed in $\mu m/g^{-1}$ FW.

Superoxide dismutase assay. Superoxide dismutase (SOD, EC 1.15.1.1) activity assay was based on the method of Beauchamp and Fridovich[7] which measures inhibition of the photochemical reduction of nitro-blue tetrazolium (NBT) at 560 nm. The reaction mixture contained 50 mM phosphate buffer, pH 7.8, 0.1 mM EDTA, 15 mM methionine, 75 mM NBT, 16.7 mM riboflavin. One unit of SOD was defined as the amount of enzyme causing half the maximum inhibition of NBT to blue formazan, and SOD activity of the extracts was expressed as SOD units/mg protein^{-1}. Protein content was estimated by the method of Bradfor[8] using bovine serum albumin as a standard.

Statistics. All experiments were repeated at least for three times. For all tests divergence from the control was significant at $p \leq 0.01$ and $p \leq 0.05$.

RESULTS AND DISCUSSION

It was shown previously that the parental cell-wall-less strain *C. reinhardtii* CW15 expresses the wild type survival and adaptive response[9]. The other parental strain AK-9-9 was shown to be highly radioresistant[3]. Screening for radioresistance was carried out on more than 90 zygotic clones (data not shown). Only 5 cloned strains (H-3, H-4, H-7, H-12 and H-18) were classified as radioresistant and chosen for further analysis. It was found that the radioresistance of 4 of these strains (H-4, H-7, H-12 and H-18) varied between those of the two paren-

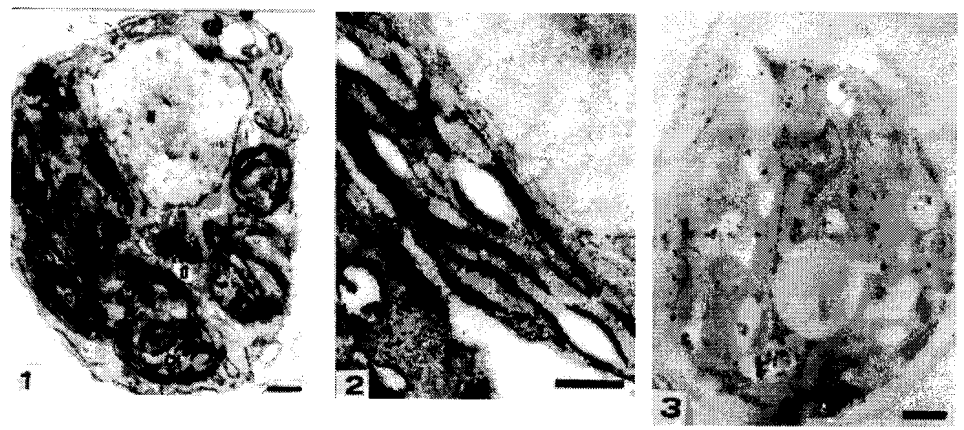
tal strains. Strain H-3 showed the highest radioresistance, higher than that of the parental AK9-9 strain[3].

These 5 clones were compared with parental strains, using TEM, focussing on the protoplast organisation and the cell wall structures. The parental CW 15 strain is placed in class C (cell wall absent or produced in very reduced quantity compared to wild type cells). The protoplast structure of CW 15 (mating type +) is typical for the genus *Chlamydomonas* (Figs 1 and 2). The single chloroplast is situated parentally. The inner membrane system is constructed from normally structured stroma thilakoids and low granas (2-6 thilakoids). There are pyrenoid and stigma in the chloroplast. In the cytoplasm there are a lot of small mitochondria and the nucleus is situated in the center of the protoplast. Dictiosomes are big, single, situated close to the nucleus. They are in very active condition.

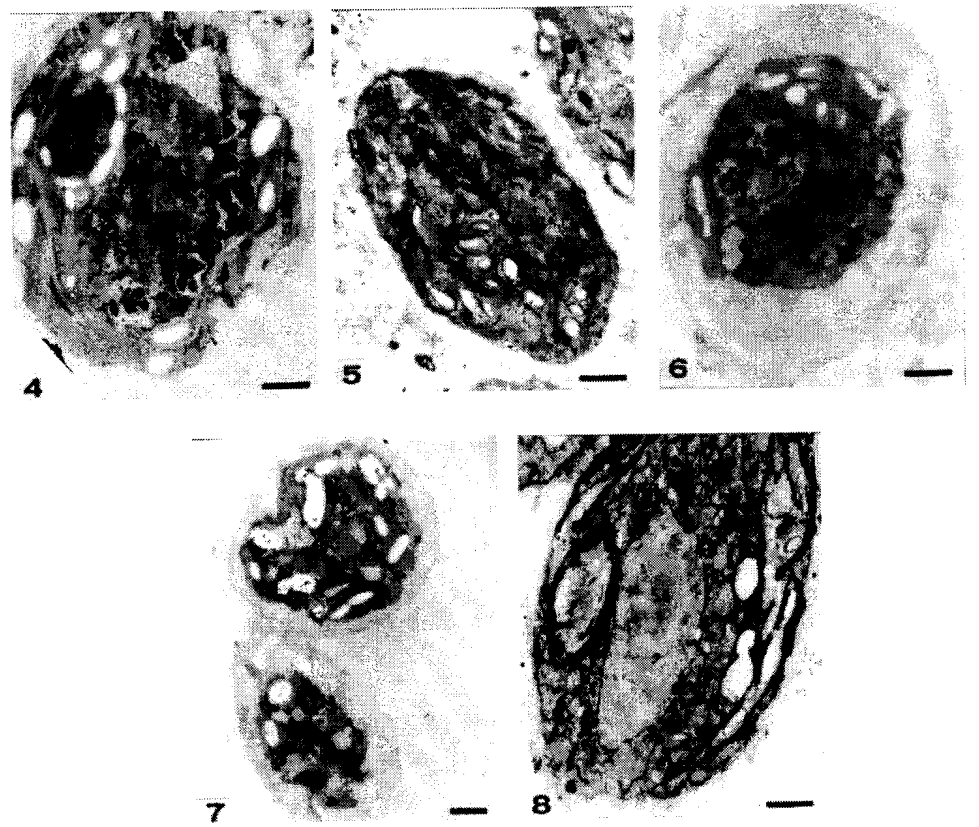
The parental strain AK 9-9 (mating type) and hybrid strains (H-3, H-4, H-7, H-12, H-18) possess a glycoprotein cell wall with clearly shaped papillae (Figs 3-8). The protoplast organisation of AK 9-9, H-4, H-7, H-12 and H-18 is different from that of CW-15 and H-3. The protoplast of H-4, H-7 and H-18 is more electron-dense (Figs 5-7). The density of the chloroplast is higher, too, with hardly distinguishable thilacoids. Only hybrid H-3 has a protoplast structure similar to that of the strain CW-15 (Fig. 8).

However, the strains showed a shape typical for Chlamydomonas. There was no evidence of 'amoeboid'-shaped and/or flat appearance of the colonies, typical for wall-deficient mutants[2] belonging to group B (i.e. mutants with altered cell walls).

The typical cell wall of genus Chlamydomonas consists of glycoproteine, three layers structured, with distinctly shaped two layers. In the cell wall of



Figs 1-3. Cells of the parental strains CW 15 (+) (1, 2) and AK-9-9 (3). Ch – chloroplast, N – nucleus, D – dictyosome; scale bar = 0.5 μM



Figs 4-8. Cells of hybrid strains: H-12 (4), H-4 (5), H-7 (6), H-18 (7) and H-3 (8). Scale bar=1 μM

experimental material strains AK 9-9, H-3, H-4 and H-12 the inner layer (the middle of the typical cell wall) is homogeneous with bigger thickness which is about 200-250 nm, visibly lighter in electron-microscopical observation (Figs 3,4,5 and 8). The outer layer (peripheral of the typical cell wall) is structured from loose situated fibriles and is considerably thinner, up to 80 nm. The third layer (the most internal of the tree) is probably very slightly structured and the thickness is not more than 20-30 nm. Two of the examined strains (H-7 and H-18) are different from this model of structural wall organisation (Figs 6 and 7). They possess bigger thickness of the cell wall.

In strain H-7 both layers are clearly distinguishable. The outer is strongly developed and irregularly wide (better represented). Its thickness is about 500 nm. It could be supposed that the greater envelope is uniformly structured. The outer layer is with less thickness than the inner. We suppose that greater total thickness of the wall of H-7 and H-18 in comparisson with strains AK 9-9, H-3, H-4 and H-12 is due to the better developed inner layer.

In this investigation we have obtained new data about the ultrastructure of protoplast and cell wall of 5 new strains from genus *Chlamydomonas*. CW-15 is a strain without a cell wall and possesses structural organisation of the protoplast typical for genus *Chlamydomonas*[2]. Strain AK 9-9 has normally structured cell wall. Electronmicroscope data obtained for all hybrid strains indicate that only H-3 possesses almost the same protoplast as CW-15 and the cell wall is very similar to AK 9-9. This is a reason to consider that H-3, as a strain with a typical for the genus structural organisation of the cell, will present the biggest interest for future investigations.

Plants possess a number of antioxidant molecules (e.g. ascorbic acid, α-tocoferol, carotenoids) as well as enzymes that protect them against oxidative damage[10]. It is now well documented that carotenoids are involved in the protection of the photosynthetic apparatus against photo-inhibitory damage by singlet oxygen, which is produced by the excited triplet-state of chlorophyll. Carotenoids can directly deactivate ($^1O_2$) and can also quench the excited triplet-state of chlorophyll, thus indirectly reducing the formation of $^1O_2$ species[11,12]. The carotenoid content of the cells varies slightly, depending on the culture conditions. For example, in *Plectonema bryanum* it is increased both at low and high temperature[13], which is obviously connected with the role of carotenoids in protecting the chlorophyll[14].

In this study, chlorophyll and carotenoids were estimated in the wild strain 137C, both parental strains and in the 5 new strains mentioned above. It was found that the hybrid strains could be divided into two groups according to their chl. *a* and chl. *b* content. Both the parental strains and both new strains H-3 and H-4 belong to the first group, with a high content of chl. *a* and chl. *b*. H-7, H-12 and H-18 belong to the second group, with a lower chlorophyll content. We found that both parental strains and H-3 have higher carotenoid content when compared to those of the strains H-4, H-7, H-12 and H-18 (Table 1).

Table 1. Pigment content (mg g^{-1} fresh weight) of *Chlamydomonas reinhardtii* hybrid strains

Strains	Chlorophyll '*a*'	Chlorophyll '*b*'	Carotenoids
CW 15(+)	1.5293±0.02	0.815625±0.008	0.3431±0.002
AK 9-9	1.4713±0.02	0.75302±0.004	0.3566±0.005
H-3	1.6737±0.012**	0.8561±0.005*	0.4137±0.012*
H-4	1.5258±0.025**	0.8485±0.02*	0.2066±0.002**
H-7	1.0831±0.055**	0.5657±0.001**	0.2760±0.003**
H-12	1.1132±0.001**	0.68291±0.09**	0.2411±0.013**
H-18	1.0559±0.06**	0.6274±0.017**	0.2833±0.0046**

Values, significantly different from the control (parental strains) are indicated by: * ($p \leq 0.05$); ** ($p \leq 0.01$).

Accumulation of Pro is a widespread plant response to environmental stresses of biotic and abiotic origin[15]. However, the precise role of Pro accumulation in plants is still a matter of debate. Because of high concentrations observed at stress impact, Pro has a clear role as an osmotic agent, but other functions, including radical detoxification and regulation of cellular redox status, are also suggested[16]. The metabolic source of Pro accumulation is contributed to increased Pro synthesis, decreased Pro catabolism, or protein degradation[15,17]. It was reported that in the presence of abiotic stresses, Pro accumulation in plant tissues provides an adaptive advantage counteracting the effect of the stress as a physiological mechanism and enhances the stability of proteins and membranes[18]. It may also serve to minimise the effect of a particular form of cell damage by simply adjusting the intracellular osmotic potential[17]. We found, that the Pro content is higher in the wild strain 137C (+) than for the strains AK-9-9, CW 15 (+) and H-3. The most radioresistant strain (H-3) possessed the lowest Pro content (Table 2). This result confirms our earlier report of lower Pro content in radioresistant mutants of *Chlamydomonas reinhardtii* (AK-9-9) and *Chlorella vulgaris* (GK-1,GK-3, 260 and 23/R) than their parental wild-type and radiosensitive strains[1].

Table 2. SOD activity, proline in percentage to the control strain [137C (+)]

Strains	SOD activity	Proline
137C (+)	100.00	100.00
AK-9-9 (-)	159.23	30.64
CW-15 (+)	147.18	42.13
H-3	380.28	19.18
LSD $p \leq 0.05$	43.7861	13.4791

Initial values for control cells were: SOD–14.2731±0.6650 units/mg protein (LSD $p \leq 0.05$-43.7861); proline – 20.4215±0.2521 μmol g^{-1} FW; (LSD $p \leq 0.05$-13.4791)

Plants have the capacity to eliminate active oxygen species with an efficient scavenging system[19]. SOD is considered to have a key role in the antioxidant defence system, since it regulates the cellular concentration of O_2^- and H_2O_2. The importance of the antioxidant defence system is demonstrated by the fact that overproduction of several scavengers of active oxygen species in different transgenic plants leads to a significant protection against oxidative stress and is the foremost enzyme in the detoxifying process[20]. An increase in SOD activity is observed in many plants more tolerant to oxidative stress, induced by SO_2, NO_3^-, by intensive light, or by high and low temperatures. In the investigations performed on transgenic plants over-expressing SOD, an increase in tolerance to oxidative stress is often, but not always observed[21]. Our results (Table 2) show that the wild

type *Chlamydomonas reinhardtii* (strain 137C+) has a lower SOD activity than those of the other strains. The SOD activity of H-3 is higher than that of parental strains CW-15 (+) and AK-9-9 possibly accounting for its increased radioresistance.

CONCLUSIONS

New radioresistant strains are derived by mating CW15 X AK-9-9 which contributes to enlarge *Chlamydomonas reinhardtii* collection of radioresistant strains. The only strain displayed the highest level of radioresistance, higher than its parental radioresistant strain AK-9-9 is H-3. The most appropriate in future radiobiological investigations is H-3, because this strain possesses typical for the genus structural organisation of the cell, lower proline content, higher pigment content and SOD activity – several typical characteristics for radioresistant strains.

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