Sex as a response to oxidative stress: a twofold increase in cellular reactive oxygen species activates sex genes

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Organisms are constantly subjected to factors that can alter the cellular redox balance and result in the formation of a series of highly reactive molecules known as reactive oxygen species (ROS). As ROS can be damaging to biological structures, cells evolved a series of mechanisms (e.g. cell-cycle arrest, programmed cell death) to respond to high levels of ROS (i.e. oxidative stress). Recently, we presented evidence that in a facultatively sexual lineage—the multicellular green alga *Volvox carteri*—sex is an additional response to increased levels of stress, and probably ROS and DNA damage. Here we show that, in *V. carteri*, (i) sex is triggered by an approximately twofold increase in the level of cellular ROS (induced either by the natural sex-inducing stress, namely heat, or by blocking the mitochondrial electron transport chain with antimycin A), and (ii) ROS are responsible for the activation of sex genes. As most types of stress result in the overproduction of ROS, we believe that our findings will prove to extend to other facultatively sexual lineages, which could be indicative of the ancestral role of sex as an adaptive response to stress and ROS-induced DNA damage.

Keywords: oxidative stress; reactive oxygen species; facultatively sexual species; sexual induction; *Volvox carteri*

1. INTRODUCTION

Life is based on a complex and finely tuned network of reduction–oxidation (redox) reactions that is under homeostatic control. Cells are constantly subjected to factors that can alter this redox balance, often resulting in an increase in the level of a series of partly reduced and highly reactive forms of oxygen collectively known as reactive oxygen species, or ROS (e.g. the superoxide anion, O_2^- ; the hydroxyl radical, $OH\cdot$; and hydrogen peroxide, H_2O_2). Although ROS are by-products of normal metabolism and can act as secondary messengers in various signal transduction pathways (see Van Breusegem *et al.* 2001 and Mittler 2002 for reviews), increased intracellular levels of ROS (i.e. oxidative stress) can alter cellular functions and damage many biological structures, most importantly DNA (e.g. Marnett & Plataras 2001). Consequently, cells evolved a large spectrum of responses to cope with oxidative stress and its consequences, such as tolerance, acclimatization, cell-cycle arrest, senescence and programmed cell death. Recently, we have suggested that in facultatively sexual lineages sex is an additional response to oxidative stress, which could be indicative of the ancestral role of sex as an adaptive response to stress-induced ROS and their DNA-damaging effects (Nedelcu & Michod 2003).

To explore this hypothesis, we are using a facultatively sexual lineage, the multicellular green alga *Volvox carteri*. In this haploid lineage, sex is triggered by environmental stress (i.e. increased temperatures of the vernal waters

these algae live in) via a 30 kDa glycoprotein sexual inducer (SI) produced by the somatic cells of both females and males. The SI acts on the asexual reproductive cells (gonidia) of both sexes and alters their developmental pathway such that in the next generation sexual females (bearing eggs) and males (bearing sperm packets) will be produced. Following fertilization of the eggs, desiccation-resistant and over-wintering diploid zygospores are formed; their germination takes place only under favourable environmental conditions and involves meiosis, which restores the haploid asexual state. Sex is easily induced in the laboratory either by subjecting asexual cultures to a 2 h heat shock (42.5 °C) or via the addition of the SI to asexual cultures (Starr 1970; Kirk & Kirk 1986).

On the basis of our findings that the efficiency of both heat stress and SI to induce sex in *V. carteri* are drastically reduced in the presence of two antioxidants, we suggested that sex is a response to increased levels of ROS, and that ROS activate genes involved in sexual induction and development (Nedelcu & Michod 2003). Here we show that (i) sexual induction is triggered by an approximately twofold increase in ROS levels (induced either by the natural sex-inducing stress, i.e. heat, or by blocking the mitochondrial electron transport chain), and (ii) ROS are responsible for the activation of two sex genes, the SI gene and the so-called ‘clone B’, a gene induced in response to the SI (Amon *et al.* 1998).

Although in many lineages sex is an obligatory part of the life cycle and is associated with reproduction, in prokaryotes and lower eukaryotes sex is facultative and occurs in response to stress. The proximate and ultimate causes of the connection between stress and sex in facultatively sexual lineages have been unclear. The data presented

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Figure 1. Relative changes ($p < 0.0001$; t -test) in ROS levels (normalized with the control as 1); means ($\pm$ s.d.) of 5–10 independent experiments are reported. (a) An approximately twofold increase (1.9 ± 0.27) in ROS levels in heat-stressed cultures (Hs), relative to unstressed cultures (C) during the 2 h sex-inducing heat stress (42.5°C). (b) High levels of ROS (2.20 ± 0.44) reached in the first 10 min of the heat stress. (c) Drastic decrease (0.69 ± 0.10) in ROS levels by the end of the first 30 min of heat stress. (d) A ca. 1.4-fold (1.39 ± 0.17) and twofold (2.1 ± 0.35) increase in ROS levels (relative to unstressed cultures) during a mild heat stress (2 h at 40°C) carried out in the absence (hs) and presence (hs+AA) of antimycin A (AA; $1\ \mu\text{M}$ final concentration), respectively; (e) decrease in ROS levels (0.48 ± 0.07) in cultures heat stressed in the presence (+Cat) of catalase ($5\ \mu\text{g ml}^{-1}$) relative to cultures heat stressed in the absence of catalase (–Cat).

here corroborate our previous data on the inhibitory effect of antioxidants on sexual induction and development in *V. carteri* (Nedelcu & Michod 2003), and collectively point to an unequivocal causal relationship between oxidative stress and sex in this species. Furthermore, because most forms of stress result in the overproduction of ROS (e.g. Mittler 2002), we expect that this relationship will extend to other facultatively sexual lineages, which will provide a novel insight into both the mechanistic basis of facultative sexual reproduction and the adaptive significance of sex. Specifically, in these lineages, gametogenesis is triggered by increased levels of stress-induced ROS in the anticipation of further stress and DNA damage that can be repaired during the meiotic event associated with the germination of the zygospore and the start of a new generation (Nedelcu & Michod 2003).

2. MATERIAL AND METHODS

(a) Strain and culture conditions

A *V. carteri* female strain, Eve, was kindly provided by Dr David L. Kirk (Washington University). Synchronous cultures of asexual females were grown in standard *Volvox* medium, at 28°C on a 16 L : 8 D cycle (Kirk & Kirk 1986).

(b) Sex induction

Cultures of asexual females (five per millilitre) bearing young asexual embryos were subjected to a 42.5°C heat stress for 2 h (Kirk & Kirk 1986). For induction via the SI, the medium in which the heat-stressed females released the SI (i.e. the conditioned medium) was collected 18–20 h after the heat shock and was used to induce sex in unstressed females (bearing young embryos) (Kirk & Kirk 1986). Catalase (Sigma; $12\ 800\ \text{units mg}^{-1}$) was added to cultures (30 min to 1 h before the stress) at $5\ \mu\text{g ml}^{-1}$ final concentration. Antimycin A (AA; $1\ \text{mM}$ stock in ethanol) was added immediately before the mild heat stress (40°C for 2 h) at a final concentration of $1\ \mu\text{M}$ (controls were also treated with the same concentration of ethanol as in the AA experiments).

(c) Detection of ROS

Cellular production of ROS was measured by using 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCF-DA}$; Molecular Probes; $5\ \text{mM}$ stock in ethanol, stored in the dark at -20°C) and a fluorospectrophotometer (PicoFluor, Turner Design) with excitation and emission wavelengths at 475 ± 15 and $515 \pm 10\ \text{nm}$, respectively. $\text{H}_2\text{DCF-DA}$ (at $5\ \mu\text{M}$ final concentration) diffuses passively into cells, where its acetate groups are cleaved by intracellular esterases; the resulting H_2DCF is non-fluorescent but becomes fluorogenic upon oxidation to DCF by intracellular H_2O_2 and other ROS (e.g. Mihre *et al.* 2003). Fluorescence was measured in the supernatant (Maxwell *et al.* 1999) of both the control and the stressed cultures, and the values were normalized with the fluorescence of the respective medium (i.e. unheated or heated), to account for the increase in fluorescence owing to the cell-independent (i.e. heat) auto-oxidation of the dye.

For fluorescence microscopy, we used a carboxymethyl derivative of $\text{H}_2\text{DCF-DA}$, 5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein ($\text{CM-H}_2\text{DCFDA}$; Molecular Probes; $15\ \text{mM}$ stock in methanol; stored at -20°C), whose subsequent oxidation yields a fluorescent adduct that is trapped inside the cell, thus providing a much better retention in live cells. The dye was added (at $15\ \mu\text{M}$ final concentration) before the heat stress; after the stress, cells were washed in PBS buffer, fixed in 4% formaldehyde and examined with a Nikon Optiphot-2 microscope with an epifluorescence attachment fitted with an Endow GFP filter set (HQ470/40 excitation, Q495LP dichroic, HQ525/50 bandpass emission; Chroma Technology Corp.). Digital photographs were taken with a Spot-2 CCD camera (Diagnostic Instruments, Inc.).

(d) RNA extraction and RT-PCR

RNA was extracted from 20 females (as described in Hallmann & Sumper 1994), immediately after the heat stress (for SI), or after 2 h of exposure to the SI (for clone B). RT-PCR was performed using the SuperScript One-Step RT-PCR System (Life Technologies) and a Stratagene Robocycler; RNA

Figure 2. Fluorescence microscopy visualization of ROS during the sex-inducing heat stress in *Volvox carteri*. The same concentration of CM-H₂DCFDA (15 μ M final) and exposure time were used for all images (both control and heat stressed), and intensity and background were normalized against the control (i.e. unstressed cultures). (a) Control: unstressed female with embryos (magnification, $\times 75$). (b,c) Heat-stressed female with embryos (magnification, $\times 75$). (d,e) Embryos within the mother colony (magnification, $\times 150$). (f) Juvenile (magnification, $\times 150$).

levels were normalized using actin mRNA as a control (Hallmann *et al.* 2001).

(e) Sequence analyses

BLAST (Altschul *et al.* 1990) searches were performed using WU-BLASTN (at www.biology.duke.edu/chlamy-genome/) and BLASTX, TBLASTX and TBLASTN at www.ncbi.nlm.nih.gov/BLAST/. CLUSTAL W (Thompson *et al.* 1994) implemented in BioEDIT (www.bioexchange.com/tools/software.cfm) was used for alignments.

(f) Statistical analyses

Statistical tests (means, standard deviations, *t*-tests and regressions) were performed using JMP IN (v. 4.0.4); sample sizes and numbers of experiments are indicated in figure legends.

3. RESULTS

To address our previous suggestion that the initiation of the sexual pathway in *V. carteri* is a response to increased levels of ROS (Nedelcu & Michod 2003), we have assessed the relative change in ROS levels during the sex-inducing heat stress, using the fluorogenic compound H₂DCF-DA (e.g. Ikeda *et al.* 1999; Maxwell *et al.* 1999). Experiments were carried out in both dark (to avoid the light-induced auto-oxidation of the dye over the duration of the heat stress) and light (in case the dark constitutes an additional stress on these photosynthetic organisms, or the sexual induction requires photosynthetic activity). Using a linear additive multiple regression model we found that the increase in fluorescence in heat-stressed relative to control cultures was significant ($p < 0.0001$) in

both dark and light experiments, and there was no significant difference ($p = 0.1526$) in the relative increase in fluorescence between dark and light treatments.

Overall, we found that: (i) the 2 h sex-inducing heat stress results in an approximately twofold increase in fluorescence levels relative to unstressed cultures (figure 1*a*); (ii) most ROS were produced in the first 10 min of stress (figure 1*b*); and (iii) by the end of the first 30 min, ROS levels dropped below those in the unstressed cultures (figure 1*c*). In the first case, the dye was added before the stress (to allow the heat-induced ROS molecules to oxidize the dye as they are produced, thus providing a cumulative measure of the total ROS produced during the 2 h stress), whereas in the two latter cases, the dye was added at the end of the 10 and 30 min stress, respectively (ensuring that the measurements are indicative of the level of ROS at that point in time; Ikeda *et al.* (1999)). The overproduction of ROS in heat-stressed cultures was also confirmed by fluorescence microscopy (figure 2).

To provide support for a causal relationship between ROS and sexual induction in *V. carteri*, we investigated the expression of two sex genes, the SI gene and clone B, in the presence and absence of the ROS scavenger catalase. If these genes are ROS-activated, catalase should reduce their transcript levels. First, we showed that exogenous catalase could decrease the heat stress-induced ROS levels (figure 1*e*). Then, we amplified a region spanning most of the SI transcript (i.e. 586 bp) from females heat stressed in either the absence or presence of catalase. Consistent with a ROS activation of the SI gene, we found that the SI transcript levels in cultures stressed in the presence of catalase are decreased relative to those in cultures stressed in the absence of catalase (figure 3*a*).

We have shown previously that in addition to diminishing the effect of the heat stress in inducing sex, antioxidants reduced the efficiency of the SI itself in triggering sexual development, thus suggesting that the inducer's mode of action also involves ROS (Nedelcu & Michod 2003). To further address this suggestion, we investigated clone B's transcript levels in response to the SI, in the presence and absence of catalase. Consistent with a pathway that involves ROS, we found that clone B transcript levels were lower in cultures induced in the presence of catalase, when compared with those in cultures induced in the absence of catalase (figure 3*c*).

We have also amplified clone B directly from heat-stressed cultures (rather than from cultures induced indirectly, via the addition of the SI as above) and noted that in the presence of catalase, clone B transcripts were almost undetectable (figure 3*d*). This is probably due to the fact that in this case (because the SI is already expressed and fully functional by the end of the heat stress; Kirk & Kirk 1986) catalase has the opportunity to interfere with the activation of both the SI gene and clone B. These results are consistent with our previous observation that the efficiency of antioxidants in reducing the sex response is highest when sex is induced via the heat stress (relative to when sex is induced via the SI; Nedelcu & Michod (2003)).

Because we observed that a milder heat stress (i.e. 2 h heat stress at 40 °C) that alone does not trigger sex will do so if carried out in the presence of 1 µM of AA—a compound that triggers the overproduction of O₂⁻ by

Figure 3. ROS activation of the SI gene (*a,b*) and clone B (*c,d*); actin was used to normalize the RNA levels (RNA was extracted at the end of the 2 h of heat stress—for the SI, and after 2 h of exposure to the SI—for clone B). The numbers indicate the size (in bp) of the amplified transcripts. (*a*) SI transcript levels from cultures heat stressed (2 h at 42.5 °C) in the absence (-Cat) and presence (+Cat) of catalase; correspondingly increasing amounts of RNA (lanes 1, 2 and 3) have been used for RT-PCR. (*b*) SI transcript levels from cultures heat stressed (2 h at 40 °C) in the absence (-AA) and presence (+AA) of antimycin A. (*c*) Clone B transcript levels in cultures induced via the addition of conditioned medium in the absence (-Cat) and presence (+Cat) of catalase. (*d*) Clone B transcript levels in cultures heat stressed in the absence (-Cat) and presence (+Cat) of catalase.

specifically blocking the mitochondrial electron transport chain (e.g. Maxwell *et al.* 1999), we investigated the increase in ROS levels and the expression of the SI gene in cultures subjected to this composite stress. We found that (i) sexual induction in this case is also associated with an approximately twofold increase in ROS levels relative to unstressed cultures (figure 1*d*), (ii) AA elevates the relative increase in ROS levels from *ca.* 1.4-fold (in cultures subjected to the mild heat stress only) to an approximately twofold value (figure 1*d*), and (iii) the SI gene is expressed only in cultures heat stressed in the presence of AA (figure 3*b*).

4. DISCUSSION

Heat stress is known to increase the level of cellular ROS (e.g. Ikeda *et al.* 1999), and such an oxidative burst can trigger the expression of a number of genes coding for proteins involved in various stress responses (e.g. Mittler 2002). Here we have shown that the sex-inducing heat stress in *V. carteri* also results in an increase in the level of cellular ROS. In addition, as reported in other systems (e.g. Ikeda *et al.* 1999), most ROS were produced in the

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Cr  FPKQCVTTPGATPFAIAPAMSSMAGRTKDTT-AYCFSITLVTPNSATSLCG-RSTSLLKAEFYADDKKR---RAITG
Vc  FPSCRCER---ELTPFAIKSAATQLTSRNPGVVNLYCFEIGIVNSGSGACYTEPASQNLSKSVYAQAAQRDRLSAFGV
      **.*:*      *****.* :.:.*. . . .  ***.* :*...*.:.  *.* *...*: :*  *:
      : . . . . . :*: . *.: : *:.  **:* ***** :***** : *.: :**  *...**  :*: * :** *

VYVQPKTGTGKWLSATWGAVDEQTVKATPLNWSKDQANGGKICLELKNDTPLSDFCLP---ANDGVCWGNVFDTKNCCP
LLAGAPVSNMTYLTPRWDSLNMTISN--LNFSKTQANGTRICLELFKPTTINEFCEREGASGSFCWALFNDNNCVP
      : . . . . . :*: . *.: : *:.  **:* ***** :***** : *.: :**  *...**  :*: * :** *

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Figure 4. Amino acid sequence alignment of the translated sequence of a *Chlamydomonas reinhardtii* (Cr) EST expressed in response to nutrient stress exhibits 48% similarity (with regions showing as much as 78% similarity) to the *Volvox carteri* (Vc) SI sequence; identical (stars) and similar (semicolons/full stops) amino acids are in bold.

first 10 min of stress—indicating a fast and intense cellular response to the heat stress, and by the end of the first 30 min ROS levels drop below those in the unstressed cultures—suggesting a very rapid and extensive activation of cellular antioxidant systems.

A causal relationship between increased levels of ROS and sexual induction is supported by the effect of the ROS scavenger catalase on the sex response (Nedelcu & Michod 2003) and on the expression of two sex genes (this work). The relatively large size of catalase complexes probably impedes their entering cells; nevertheless, exogenous catalase is known to effectively scavenge stress-induced H_2O_2 produced intracellularly and diffusing outside cells, or produced at the cell membrane level (e.g. Sestili *et al.* 1996; Torii *et al.* 1997). Indeed, we have shown here that catalase decreases the heat-induced cellular ROS (figure 1e) and the SI transcript levels (figure 3a), which together argue for the SI gene being activated by elevated cellular ROS levels. In addition, we found that the activation of the SI gene requires reaching a threshold ROS value (i.e. a twofold increase in ROS levels relative to unstressed cultures; figure 1d). On the other hand, the precise role of ROS in clone B's activation awaits further investigation, as two scenarios can be envisioned. It is possible that the observed inhibitory effect of catalase on the SI-induced expression of clone B (figure 3c) is a result of catalase's direct effect on the SI (i.e. by affecting the redox status of the cysteine residues in the SI). Alternatively, catalase can be scavenging ROS that are responsible for the activation of clone B. The latter scenario assumes that the SI triggers a biotic-like stress response via the production of H_2O_2 by membrane-bound NADPH oxidases (Nedelcu & Michod 2003). This scenario is consistent with the fact that many of the genes activated by the SI (including clone B) are also induced by wounding (Amon *et al.* 1998), and code for proteins that in plants are implicated in defence mechanisms against pathogen infections (and whose genes are ROS activated) (Desikan *et al.* 1998; Hallmann *et al.* 2001).

Exactly how ROS are involved in activating the sexual pathway in *V. carteri* remains to be further investigated. Several observations argue that the sexual pathway in *V. carteri* is under a very fine and precise control, within a complex network of signalling pathways and cellular stress responses among which cross-talk is possible. For instance, the sex response is extremely sensitive to the overall redox state of cells, as cultures in the stationary phase are less susceptible to sexual induction (A. M. Nedelcu and R. E. Michod, unpublished data). Furthermore, sex is part of a series of cellular responses to increasing levels of stress, as the sex-inducing heat stress can trigger

cell-cycle arrest or apoptosis if the density of the culture is higher or the duration or temperature of the heat stress is slightly higher (Nedelcu & Michod 2003).

Because various agents that induce the synthesis of heat-shock proteins in other species did not elicit sexuality in *V. carteri*, Kirk & Kirk (1986) concluded that 'the activation of the inducer production appears to be a heat-shock-specific response not a generalized stress response'. Nevertheless, our findings collectively show that in *V. carteri* sex (and the expression of the SI gene) is triggered by an approximately twofold increase in ROS levels, regardless of the type of stress and ROS source (figure 1a,d). Such a general internal signal for sex can explain the fact that in *V. carteri* sex can be experimentally induced via various factors with no obvious common features but which are all likely to trigger ROS production (e.g. heat, various aldehydes including glutaraldehyde and formaldehyde, or UV irradiation; Starr & Jaenicke (1988)).

As most types of environmental stress promote an increase in the cellular ROS levels (e.g. Mittler 2002), we believe that the involvement of ROS extends to other stress-induced sexual processes in other facultatively sexual lineages, which can be indicative of the ancestral role of sex as an additional response to oxidative stress. Interestingly, in *Chlamydomonas reinhardtii* (which is the closest unicellular relative of *V. carteri*), sex is induced by nitrogen depletion, and our preliminary data suggest that nitrogen stress is associated with an increase in ROS levels in this lineage (A. M. Nedelcu, unpublished data). In this context, it is noteworthy that we identified in the *Chlamydomonas* database (www.biology.duke.edu/chlamy_genome/) a *C. reinhardtii* expressed sequence tag (EST) (963048C01.y1) that showed 54% nucleotide sequence identity (and 48% amino acid similarity) to the *V. carteri* SI sequence (figure 4). In addition, six out of the seven cysteine residues present in the *V. carteri* sequence are also conserved in the *C. reinhardtii* EST; cysteine residues are often sites for post-translational ROS-induced regulation of proteins, including some involved in stress responses (e.g. Zheng *et al.* 1998). Interestingly, this EST was obtained from a library that combined cDNA from cells grown under nutrient stress (including nitrogen stress). The similarity between the *V. carteri* SI and this *C. reinhardtii* EST sequence may not be coincidental. It is conceivable that the two sequences have evolved from a sequence associated with the sex-inducing stress response in their last common ancestor, which, as the lineages leading to *C. reinhardtii* and *V. carteri* occupied new habitats (soil and small temporary ponds), became activated by the main stress associated with the new environment, namely

nitrogen depletion and heat stress. We think that a general signal for sex (such as an increase in ROS levels) would have allowed lineages to adopt the same adaptive strategy regardless of the type of stress encountered, and thus to rapidly adapt to new environments.

The evolution of sex remains one of the great unsolved problems in biology. Three major hypotheses have been postulated to account for the origin of sex: the 'variation', the 'selfish DNA', and the 'DNA repair and mutation' hypotheses (e.g. Maynard Smith 1978; Michod & Levin 1988; Michod 1995). If the association between stress-induced ROS and sex in *V. carteri* extends to other facultatively sexual lineages, this could be indicative of the ancestral role of sex in coping with ROS-induced DNA damage (Bernstein *et al.* 1985; Michod 1995). Furthermore, it will provide evidence for the suggested role of fluctuating levels of DNA damage (Szathmáry *et al.* 1990) and recombinational repair of double-strand DNA breaks (Cavalier-Smith 2002) in selecting haploid-diploid life cycles during the early evolution of eukaryotes. Finally, the ability of mitochondrially produced ROS to induce sex provides support for the proposed role of ROS in the evolution of sex during the acquisition of mitochondria and the origin of the eukaryotic cell (Blackstone 1995).

The authors thank D. L. Kirk for the *Volvox carteri* strain and C. A. Solari for help with the statistic analyses. Support from the UNB and NSERC to A.M.N. and from NSF and the UA College of Science to R.E.M. is also acknowledged.

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