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Sex as a response to oxidative stress: stress genes co-opted for sex

Aurora M. Nedelcu*

Department of Biology, University of New Brunswick, Fredericton, New Brunswick, Canada E3B 6E1

Despite a great deal of interest, the evolutionary origins and roles of sex remain unclear. Recently, we showed that in the multicellular green alga, *Volvox carteri*, sex is a response to increased levels of reactive oxygen species (ROS), which could be indicative of the ancestral role of sex as an adaptive response to stress-induced ROS. To provide additional support for the suggestion that sex evolved as a response to oxidative stress, this study addresses the hypothesis that genes involved in sexual induction are evolutionarily related to genes associated with various stress responses. In particular, this study investigates the evolutionary history of genes specific to the sexual induction process in *V. carteri*—including those encoding the sexual inducer (SI) and several SI-induced extracellular matrix (ECM) proteins. Surprisingly, (i) a highly diversified multigene family with similarity to the *V. carteri* SI and SI-induced pherophorin family is present in its unicellular relative, *Chlamydomonas reinhardtii* (which lacks both a SI and an ECM) and (ii) at least half of the 12 identified gene members are induced (as inferred from reported expressed sequence tags) under various stress conditions. These findings suggest an evolutionary connection between sex and stress at the gene level, via duplication and/or co-option.

Keywords: sex; oxidative stress; co-option; gene duplication; pherophorins; *Volvox carteri*; *Chlamydomonas reinhardtii*

1. INTRODUCTION

Despite a great deal of interest, the evolutionary origins and roles of sex remain unclear. Because (i) in prokaryotes and many lower eukaryotes, sex is facultative and occurs in response to stress and (ii) most forms of stress result in the overproduction of potentially damaging reactive oxygen species (ROS), we suggested that sex evolved as an adaptive response to stress-induced ROS (Nedelcu & Michod 2003; Nedelcu *et al.* 2004). A general cellular signal for sex (i.e. an increase in ROS levels regardless of the inducing factor) would allow an evolutionary lineage to respond with the same adaptive strategy to a new stress, and thus to adapt more rapidly to new environments. This hypothesis makes two testable predictions: (i) sex in facultatively sexual lineages is triggered by elevated ROS levels and (ii) genes involved in sexual induction are evolutionarily related to genes associated with various stress responses.

The green algal group, Volvocales, is an excellent model-system with which to address these predictions. In the two most studied members of the group, the multicellular *Volvox carteri* and its unicellular relative, *Chlamydomonas reinhardtii*, sex is triggered by two very distinct environmental factors: heat-stress and nitrogen-deprivation, respectively. Nevertheless, in both *V. carteri* (Nedelcu & Michod 2003; Nedelcu *et al.* 2004) and *C. reinhardtii* (A. M. Nedelcu & M. English, unpublished data) sexual induction is associated with ROS over-production. To address the second prediction, namely, that genes involved in sexual induction are evolutionarily related to stress genes, the present study investigates the evolutionary history of genes that are specific to the sexual induction process in *V. carteri*.

The sexual process in *V. carteri* involves the release of a soluble 30 kDa glycoprotein (the sexual inducer (SI)—or pheromone) that acts at concentrations as low as 10^{-16} M through yet to be deciphered mechanisms involving changes in the extracellular matrix (ECM) of this multicellular organism (Sumper *et al.* 1993). Surprisingly, SI's only known similarity is to the C-terminus of a family of glycoproteins found in *V. carteri*'s ECM, the pherophorins (*aka* perphorins), some of which are induced by the SI itself (Sumper *et al.* 1993). In contrast to the SI, pherophorins consist of two globular domains separated by a rod-shaped proline-rich region (P-link) of variable size (Hallmann 2003; figure 1*a*). At least 13 different pherophorins (some being encoded by as many as 10 gene copies; Godl *et al.* 1995) are thought to be present in *V. carteri* (Hallmann 2003). Interestingly, while pherophorins I and III are expressed constitutively, pherophorins II, S and DZ are induced in response to the SI as well as wounding (see Hallmann 2003 for a review).

In addition to pherophorins, several other genes expressed in response to the SI have been identified in *V. carteri*, including clone A (coding for a chitinase/lysozyme) and clone B (coding for a protein with a cysteine-protease and three chitin-binding domains) (Amon *et al.* 1998). Remarkably, all these sequences are also induced by mechanical stress and code for ECM proteins that in plants are implicated in defence mechanisms (Hallmann *et al.* 2001).

To investigate the evolutionary history of genes that are specific to the sexual induction process in *V. carteri*, this study (i) searched the available genome sequence of *C. reinhardtii* for sequences with similarity to *V. carteri*'s

* (anedelcu@unb.ca).

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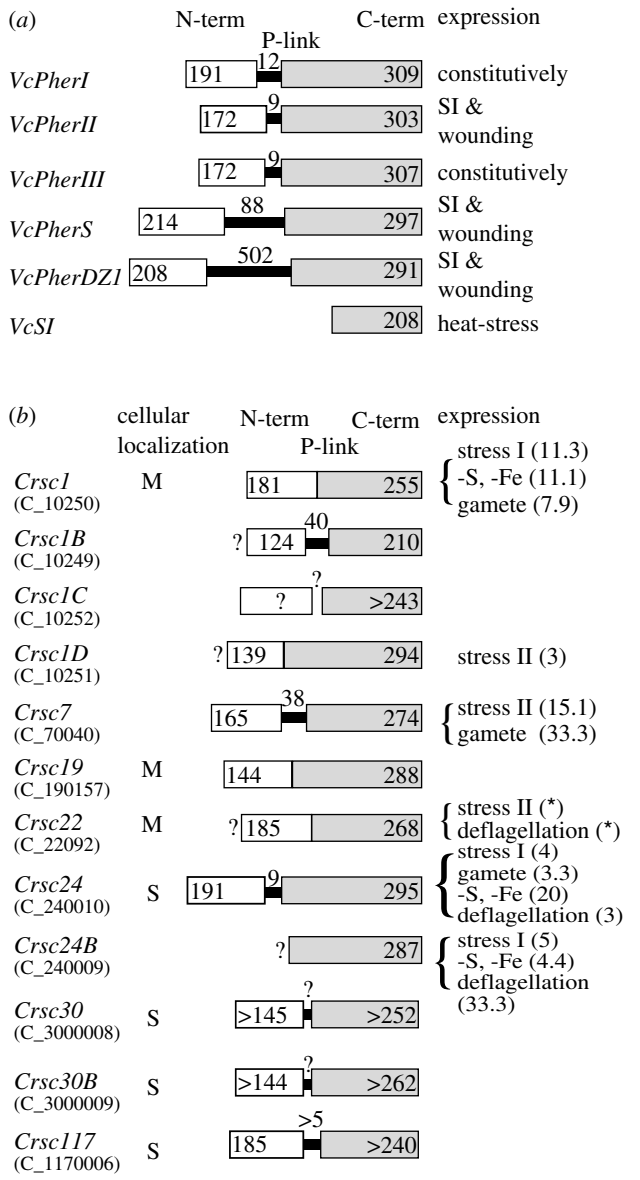

Figure 1. A comparative analysis (organization and expression) of *V. carteri* and *C. reinhardtii* Pher-like sequences (the length in amino acids of each domain—where known, is indicated). (*a*) Organization and expression patterns for *V. carteri* (*Vc*) pherophorin I, II, III S, DZ1 (*PherI, PherII, PherIII, PherDZ1*) and sexual inducer (SI). (*b*) Organization (? indicates missing or incomplete data), cellular localization (M, mitochondria-targeted; SP, secretory pathway) and expression patterns for the 12 *C. reinhardtii* (*Cr*) Pher-like sequences reported in this study (B, C, D denote sequences found on the same scaffold; gene models in the JGI *Chlamydomonas* genome database v2.0 are indicated in brackets). Expression patterns are based on an *in silico* analysis (see §2 and table S1) of reported ESTs in various cDNA libraries (numbers indicate EST corrected ratios between stress and standard libraries, as a proxy for the difference in expression levels in stress versus standard libraries; only sequences and stress conditions with ratios higher than three are indicated; asterisks indicate sequences with ESTs found only in stress libraries).

pherophorins, clone A and clone B and (ii) performed an *in silico* analysis of reported expressed sequence tags (ESTs) corresponding to the sequences identified. Surprisingly, a highly diversified multigene family with similarity to the *V. carteri* pherophorin family was found, and at least half of the 12 gene members are induced (as inferred from ESTs) under various stress conditions. These findings suggest an evolutionary connection between sex and stress at the gene level, via duplication and/or co-option.

2. MATERIAL AND METHODS

V. carteri SI (X12581), pherophorin I (X69801), II (X69802), III (X82446), S (Y07752), DZ1 (AJ429230), clone A (AF058716) and clone B (AF058717) amino acid sequences were retrieved from GenBank and Blasted (tblastn) against the *C. reinhardtii* v2.0 database at the Joint Genome Institute (JGI; http://genome.jgi-psf.org/chlre2/chlre2.home.html). Sequences were aligned using ClustalW (Thompson *et al.* 1994). Phylogenetic analyses (gaps and unalignable regions excluded) were performed using MrBayes v3.0B4 (Huelsenbeck & Ronquist 2001). SignalP 3.0 and TargetP v1.01 were used for signal peptide and cellular localization predictions (Emanuelson *et al.* 2000; Nielsen *et al.* 1997).

The absolute number of ESTs per library corresponding to each of the *C. reinhardtii* sequences reported in this study are from the JGI *Chlamydomonas* genome v2.0 gene model analyses; expected numbers (i.e. the number of ESTs expected if the sequences in stress libraries were expressed at the same level as in 'standard' libraries) were calculated relative to the number of ESTs sequenced from standard libraries (i.e. grown under 'standard' conditions), and were corrected for differences in total numbers of ESTs (deposited in the NCBI EST database at http://www.ncbi.nlm.nih.gov/dbEST/) between libraries (table S1, Electronic Appendix). For full description of *C. reinhardtii* stress libraries and their use see (Shrager *et al.* 2003) or go to http://www.chlamy.org/libraries.html.

3. RESULTS

(a) *A pherophorin-like family in the unicellular Chlamydomonas reinhardtii*

Volvox carteri pherophorin amino acid sequences were Blasted against the JGI *C. reinhardtii* v2.0 genome database. Unexpectedly (as *C. reinhardtii* lacks both an SI and an ECM), a rather large number of sequences with similarity to the pherophorin family was found. Figure 1*b* shows the organization of 12 such sequences, as inferred from comparisons with reported *V. carteri* pherophorins. *C. reinhardtii* pherophorin-like (Pher-like) sequences were found on seven genome scaffolds (*sc1, sc7, sc19, sc22, sc24, sc30, sc117*), some of which contain more than one such region (in opposite orientation—on *sc1*, or in tandem—on *sc24* and *sc30*), with less (i.e. *sc30*) or more (i.e. *sc1*) dramatic differences among same-scaffold 'copies' (figure 1*b*). Noteworthy, in *V. carteri*, at least three out of the 10 copies of PherII-related genes (Godl *et al.* 1995), and the six copies (differing only in intronic sequences) of the SI-encoding region (Schmitt *et al.* 1992) are also organized as tandem repeats.

The Pher-like sequences differ both in size (although complete sequence information is not available for all sequences) and the presence/absence of the P-link that separates the N- and C-domains in all *V. carteri* pherophorins (figure 1*a*,*b* and supporting figure S1). The number of introns is also variable, from as few as two in *Crsc7* to as many as 11 in *Crsc19*; notably, the location of the most 3′-end intron in *V. carteri* PherI and SI is also shared by half of the *C. reinhardtii* Pher-like sequences

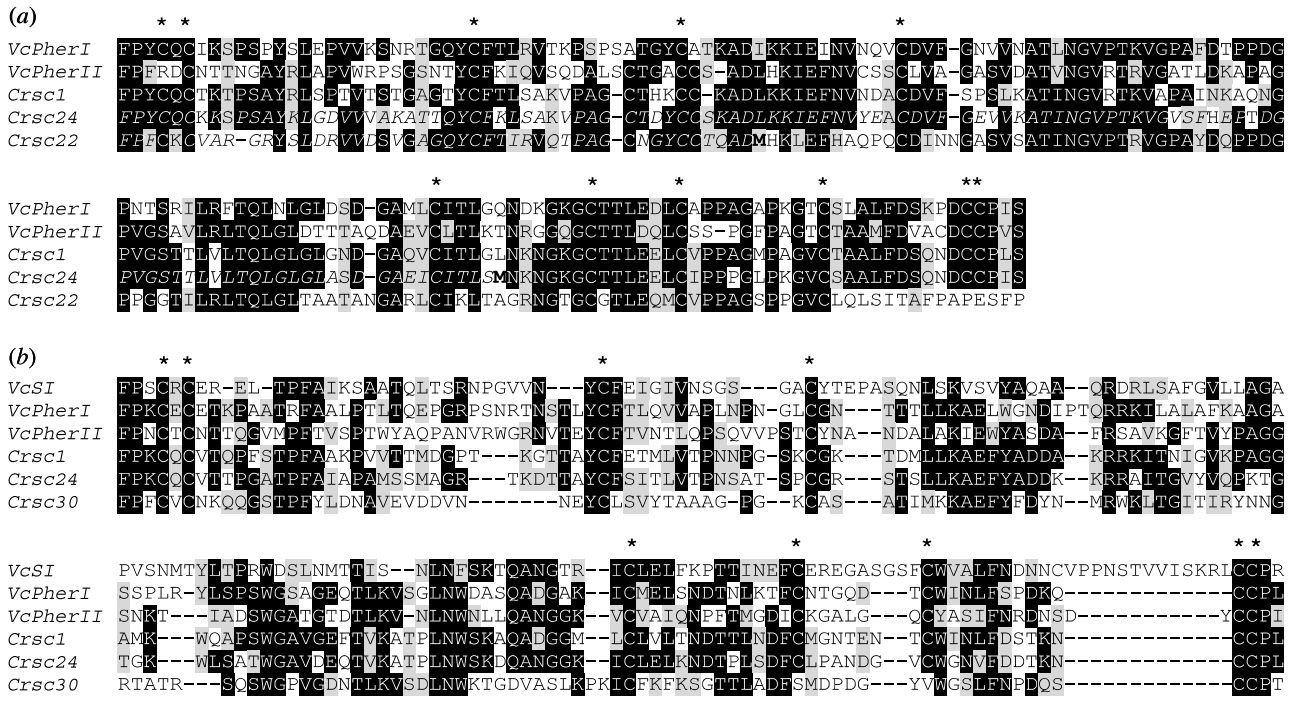

Figure 2. Partial alignment of (*a*) the N-domain (i.e. position 28–185 in *V. carteri* PherI; italics denote sequences identified in this study, which extend the coding regions upstream of the position annotated as start codon in the *C. renhardtii* v2.0 assembly gene models) and (*b*) the C-domain (i.e. position 349–505 in PherI) of selected *V. carteri* and *C. reinhardtii* Pher-like sequences (abbreviations are as in figure 1). Shading for identities and similarities is set at 50%; asterisks indicate conserved cysteine residues. A complete alignment is provided as Electronic Appendix (figure S1).

(i.e, *Crsc1, Crsc1B, Crsc1C, Crsc19, Crsc22* and *Crsc117*). Nevertheless, despite differences in gene structure and organization, all Pher-like genes are expressed (see below), and their deduced amino acid sequences are similar to each other and to the *V. carteri* pherophorin family in both their N- and C-domains (with N-domain being more conserved; figure 2 and figure S1 in Electronic Appendix). Remarkable is the strong conservation of cysteine residues and the cysteine–cysteine–proline (CCP) motif at the end of both N- and C-domains (discussed later).

(b) *Gene duplication and Pherophorin evolution*

Interestingly, both Bayesian (figure 3) as well as parsimony, minimum evolution and maximum-likelihood (data not shown) analyses using the alignable portion of the C-domain (figure 3*a*) or of both N- and C-domains—where applicable and available (figure 3*b*), suggest that the *V. carteri* pherophorins evolved from at least two *C. reinhardtii* Pher-like ancestors. In other words, at least two Pher-like sequences were already present in the last common ancestor of *C. reinhardtii* and *V. carteri*, which implies that the diversification of this gene family likely started before the divergence of the two lineages. Another aspect that adds complexity to the evolution of pherophorins is the fact that the N- and the C-domain exhibit similarity to each other, suggesting that they are also the result of a duplication event. However, when compared with other N- and C- domains, the N-domains are more closely related among themselves than they are to their corresponding C-domains (and the same is true of C-domains) (figure 3*c*), arguing that the duplication event that gave rise to the two domains took place before the radiation of the Pher-like sequences.

In this context, it should be mentioned that two additional sequences, GAS28 in *C. reinhardtii* and SSG 185 in *V. carteri,* are also related to the pherophorin family. The *V. carteri* SSG185 ('*S*ulphated *S*urface *G*lycoprotein') is one of the main components of the ECM cellular zone (along with PherI) (Ertl *et al.* 1989), whereas the *C. reinhardtii* GAS28 is expressed during the late phase of gametogenesis (Rodriguez *et al.* 1999). Nevertheless, in these cases, the N- and the C- domains are more related to each other than either is to the N- or C-domains of other members of the pherophorin family (figure 3*d*), suggesting that they are the result of independent duplication events relative to that (those) responsible for the diversification of the pherophorin family. A diagram depicting putative events responsible for the evolution and diversification of this gene family is presented in figure 4. The assessment of the relative involvement of the potential mechanisms responsible for sequence duplication in this family (i.e. unequal crossing over, retroposition, or chromosomal duplication) awaits the completion of the *C. reinhardtii* genome sequence.

(c) *Pherophorin-like sequences are induced by various types of stress*

All Pher-like sequences reported in figure 1*b* are expressed (table S1, Electronic Appendix). To address the potential roles these Pher-like sequences have in *C. reinhardtii*, an *in silico* analysis (Shrager *et al.* 2003) was carried out (see §2 and table S1). Remarkably, at least half of the 12 *C. reinhardtii* Pher-like sequences are induced (as inferred from reported ESTs) under one or more stress conditions (including nitrogen/phosphate/S/Fe deprivation, pH-induced deflagellation, osmotic and oxidative stress) and/or gametogenesis, with *Crsc24* being expressed under most stress conditions (table S1 and figure 1*b*). Many Pher-like sequences are also expressed at low levels under standard conditions, which is consistent

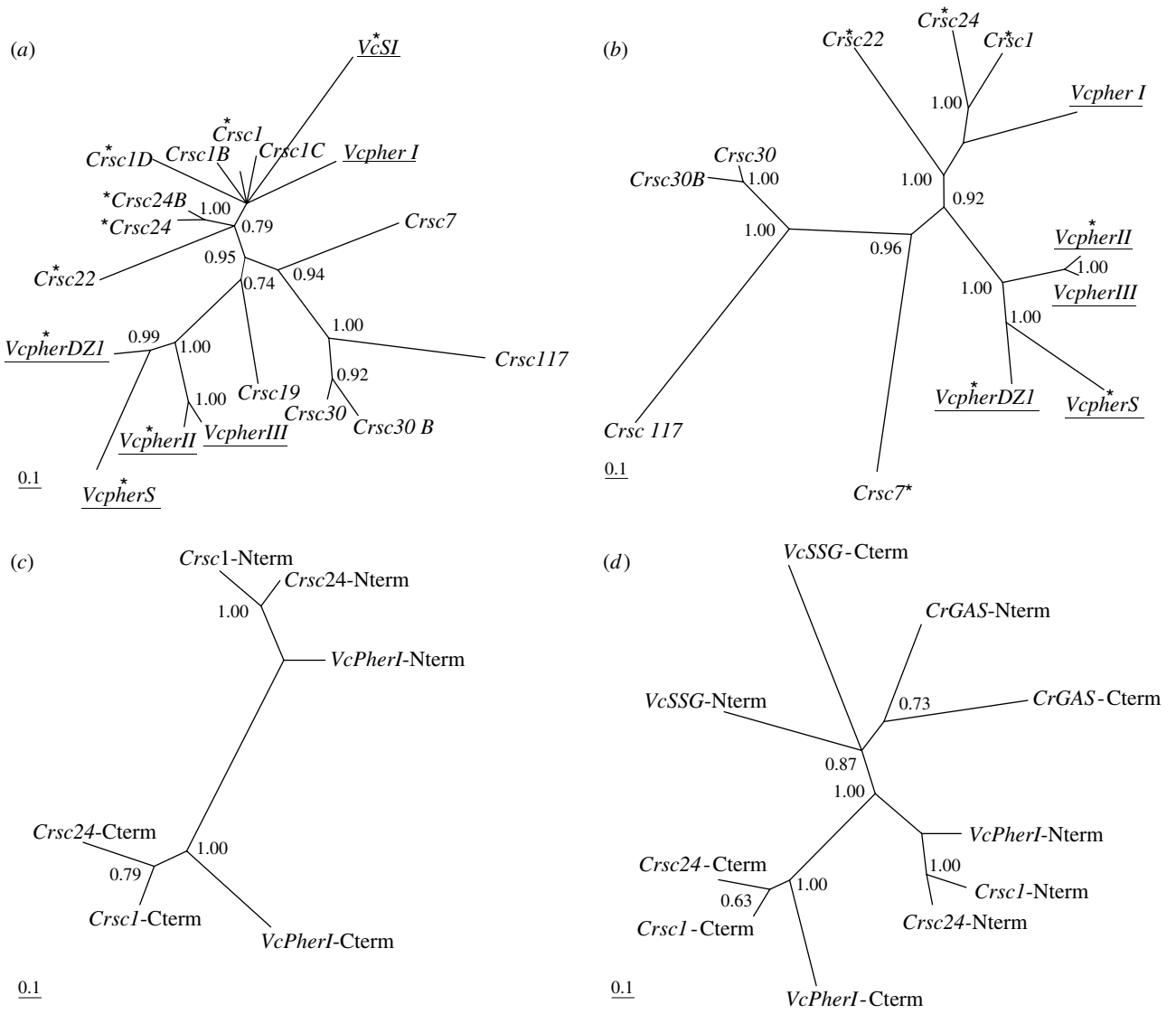

Figure 3. Bayesian analyses (unrooted trees; mixed amino acid model; 3 500 000 generations; 100 sample frequency; 5000 burn in) of *V. carteri* pherophorin and *C. reinhardtii* Pher-like sequences. (*a*) and (*b*) N-domains only (133 sites) and both N- and C-domains (239 sites), respectively; *V. carteri* sequences are underlined and italicized, and asterisks mark sex- and/or stress-induced sequences. (*c*) N- versus C-domains (142 sites). (*d*) N- versus C-domains, including GAS28 and SSG185 (131 sites). Numbers represent posterior probability distributions of trees (Huelsenbeck & Ronquist 2001).

with the expression patterns of *V. carteri* PherII and of several genes induced during gametogenesis in *C. reinhardtii* (e.g. GAS28; Rodriguez *et al.* 1999).

A search for signal peptides—where the N-terminus was available, revealed interesting differences among Pher-like sequences in terms of the cellular pathway they enter and their final destination. For instance, while *Crsc24, Crsc30, Crsc30B* and *Crsc117* are likely entering the secretory pathway (as all pherophorins do), *Crsc1, Crsc19* and *Crsc22* appear to be targeted at mitochondria (figure 1*b*). To support this split is also the distribution of the P-link that separates the N- and C-domains in pherophorins: a proline-rich region is present in the secreted *Crsc24, Crsc30, Crsc30B* and *Crsc117* but absent in the potentially mitochondria-targeted *Crsc1*, *Crsc19* and *Crsc22* (figure 1*b*).

Although to be further confirmed, a mitochondrial localization of some of the Pher-like sequences would support their involvement in stress-responses as mitochondria are thought to be a site for ROS production during abiotic stresses (Mittler 2002), and our previous work showed that the expression of the SI gene in *V. carteri* can be induced by blocking the mitochondrial electron transport chain (Nedelcu *et al.* 2004). An association with oxidative stress is further suggested by the number and strong conservation of cysteine residues in both pherophorin and Pher-like sequences (figure 2 and figure S1 in Electronic Appendix). As the participation of intermolecular disulphide bridges in cross-linking during ECM self-assembly was proved unlikely (Ender *et al.* 2002; Sumper *et al.* 2000), the cysteine residues might be involved in redox signaling (e.g. Kuge *et al.* 2001; Toledano *et al.* 2004).

(**d**) *Other sex-gene homologues induced by stress*

The available *C. reinhardtii* genome sequence was also searched for potential clone A and clone B homologues. Clone A and B code for a chitinase/lysozyme and a cysteine protease/chitin-binding protein, respectively, and are known to be induced during sexual induction and

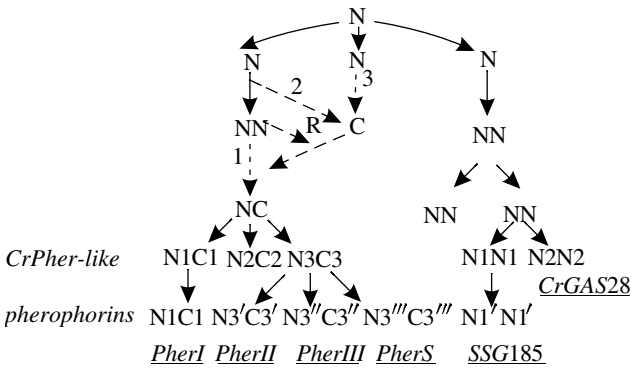

Figure 4. Putative evolution of the multigene pherophorin family. The ancestral sequence containing both an N- and C-domain (NC) could have evolved from either a duplicated sequence (NN) and subsequent divergence (NN→NC; pathway 1), or through a recombination event (R) involving an N or NN sequence (pathway 2) and an already diverged C-like sequence (pathway 3); other pathways are also possible.

wounding in *V. carteri* (Amon *et al.* 1998). A sequence with 44% identity (60% similarity) to clone B (over the first 438 aa; i.e. the cysteine protease domain) was found in the JGI database (gene model C_80056). Remarkably, the only EST reported for this sequence is from a stress library that includes direct oxidative stress conditions (i.e. exogenous addition of H_2O_2; table S1). This finding is consistent with our previous report that clone B activation in *V. carteri* is mediated by ROS (Nedelcu *et al.* 2004). Likewise, the *C. reinhardtii* genome contains a sequence with similarity to clone A (gene model C_140024), which appears to be also induced under stress conditions (table Sl). These two sequences provide additional examples of stress-induced genes possibly co-opted into the *V. carteri*'s sexual pathway; as no other algal sequences with similarity to these two genes are known, phylogenetic analyses could not be performed.

4. DISCUSSION

The SI and the inducible pherophorins are thought to be 'evolutionarily derived from an ancient member of the pherophorin family that originally served a structural function within the ECM' (e.g. Hallmann *et al.* 1998). This view implies that pherophorins evolved in multicellular Volvocales (specifically, in those that have an ECM), and predicts that a subsequent diversification took place during the evolution of the sexual induction system in *V. carteri.* Thus, the presence of such a large number of Pher-like sequences in a unicellular lineage in which neither a SI nor an ECM are produced is puzzling at first.

However, if viewed in the context of stress-responses, the diversification of the *C. reinhardtii* Pher-like sequences before the evolution of an ECM is less surprising. High rates of gene birth and death are known for genes involved in physiological processes that vary greatly among species, such as immunity, reproduction and sensory systems (Zhang 2003), and the stress response likely fits into this category. The average rate of origin of new duplicate genes in eukaryotes is thought to be on the order of 0.01 per gene per million years (Lynch & Conery 2000). The rather high number of copies among both pherophorin and Pher-like sequences in *V. carteri* and *C. reinhardtii*, which are thought to have diverged from a common ancestor 50 Myr ago (Rausch *et al.* 1989), suggests that there is strong positive selection acting on this gene family.

Gene duplication can lead to species-specific gene functions, which can facilitate species specific adaptations (Zhang 2003), and this might have been the case during the evolution of the heat-induced SI and SI-inducible pherophorins in *V. carteri*. Differential gene duplication in geographically isolated populations can also cause reproductive isolation and speciation (Lynch 2002). In this context, it is noteworthy that two genetically incompatible *V. carteri* strains from distinct geographical regions differ in the number of SI gene copies (i.e. one versus six) (Kirk 1998).

Remarkably, many of the *C. reinhardtii* Pher-like sequences are expressed under more than one type of stress. This multifunctionality offers an evolutionary explanation as to why some of the *V. carteri* counterparts are also induced by stresses other than those associated with sexual induction. As most *C. reinhardtii* Pher-like sequences show various degrees of divergence (figure S1 in Electronic Appendix), it appears that selection has acted on diversifying function rather than increasing dosage; the latter would have resulted in preserving identical copies, which is likely the case for the five or six tandem repeats encoding identical SI polypeptides in *V. carteri*. However, additional data are needed to asses the relative role of direct co-option and/or co-option of a duplicated element (via sub-functionalization) as evolutionary mechanisms associated with the diversification of the Pher-like sequences and the functional shift(s) in this multigene family (Ganfornina & Sanchez 1999). On the other hand, if no other sequences with similarity to clone A and B are found in the genome of *C. reinhardtii*, direct co-option events have to be invoked for the functional shifts in these cases.

Overall, this study suggests an evolutionary connection between sex and other stress-responses at the gene level, which provides additional support for the hypothesis that sex evolved as an additional response to oxidative stress. But why would important constitutively expressed ECM structural components (such as Pherophorin I, III and SSG185) be related to stress-induced proteins? The answer may be found in a behaviour believed to be a precondition for the evolution of multicellularity in this group: under less than optimal conditions, *C. reinhardtii* secretes an extracellular mucilage that holds cell together. Nutrient-stress is known to also induce a multicelluar stage in myxobacteria and slime moulds (e.g. Kaiser 2001), and the findings reported here suggest an analogous process in an evolutionary rather than developmental context, and a mechanistic basis for a potential connection between stress and multicellularity.

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