Current Biology 18, 895–899, June 24, 2008 ©2008 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2008.05.037

Report

A Diatom Gene Regulating Nitric-Oxide Signaling and Susceptibility to Diatom-Derived Aldehydes

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Summary

Diatoms are unicellular phytoplankton accounting for ~40% of global marine primary productivity [1], yet the molecular mechanisms underlying their ecological success are largely unexplored. We use a functional-genomics approach in the marine diatom *Phaeodactylum tricornutum* to characterize a novel protein belonging to the widely conserved YqeH subfamily [2] of GTP-binding proteins thought to play a role in ribosome biogenesis [3], sporulation [4], and nitric oxide (NO) generation [5]. Transgenic diatoms overexpressing this gene, designated *PtNOA*, displayed higher NO production, reduced growth, impaired photosynthetic efficiency, and a reduced ability to adhere to surfaces. A fused YFP-PtNOA protein was plastid localized, distinguishing it from a mitochondria-localized plant ortholog. *PtNOA* was upregulated in response to the diatom-derived unsaturated aldehyde 2E,4E/Z-decadienal (DD), a molecule previously shown to regulate intercellular signaling, stress surveillance [6], and defense against grazers [7]. Overexpressing cell lines were hypersensitive to sublethal levels of this aldehyde, manifested by altered expression of superoxide dismutase and metacaspases, key components of stress and death pathways [8, 9]. NOA-like sequences were found in diverse oceanic regions, suggesting that a novel NO-based system operates in diatoms and may be widespread in phytoplankton, providing a biological context for NO in the upper ocean [10].

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Results and Discussion

Cloning and Expression of *PtNOA*

The recent availability of genomic resources and genetic tools for the centric diatom *Thalassiosira pseudonana* and the pennate diatom *Phaeodactylum tricornutum* [11, 12] provide unprecedented opportunities to explore the function of genes encoding components of signaling and stress pathways. Examination of the *P. tricornutum* genome (http://genome.jgi-psf.org/Phatr2/Phatr2.home.html) revealed two gene models (Phatr2_56150 and Phatr2_37004) encoding proteins containing a conserved YqeH domain with 50% sequence similarity to AtNOA1 (nitric oxide-associated protein 1) [13] from *Arabidopsis thaliana*, which is required for nitric oxide (NO) production in response to bacterial elicitors, phytohormones, and senescence [5, 14, 15]. Multiple alignment of several YqeH-contaning protein sequences from diatoms, *A. thaliana*, and bacteria exhibited high conservation of sequence motifs characteristic of the YqeH subfamily of P loop-containing GTPases [16] (Figure S1 available online). We focused on one of the putative *P. tricornutum* orthologs (Phatr2_56150; designated PtNOA) because of better gene-prediction models and higher expressed-sequence tag (EST) coverage. *PtNOA* contained a conserved YqeH, as well as a predicted GTPase domain (COG1161). Analysis of the *T. pseudonana* genome revealed a single predicted protein (Thaps3_21823) with similar conserved domains (denoted TpNOA).

We used a reverse-genetics approach to elucidate the physiological and cellular roles of PtNOA in *P. tricornutum* in response to stress conditions. Overexpressing transgenic clones containing a Hemagglutinin (HA) epitope tag fused to the carboxy terminus of PtNOA were detected by western blot, and two highly expressing lines, denoted OE1 and OE2, were selected (Figure 1A). A comparison of transgene and endogene *PtNOA* expression levels by qRT-PCR revealed 10- and 30-fold-higher transcript abundance in OE1 and OE2, respectively (Figure S2), corroborating the relative protein levels observed by western blotting (Figure 1A). Given that two eukaryotic orthologs of YqeH-containing proteins were recently suggested to modulate NO production in response to various stress conditions [5, 17], we compared NO production in wild-type cells and OE2 transformant cells. OE2 cells had an average 2.4-fold increase in the basal rate of NO production over wild-type cells when the NO-specific fluorescent probe DAF-FM diacetate was used (Figure 1B). Electron paramagnetic resonance (EPR) analysis of spin-trapped NO directly confirmed higher (1.85-fold) basal NO production in the OE2 line compared to wild-type cells (Figure 1C).

Enhanced NO production compromised basal cell physiology. *PtNOA*-overexpressing clones (OE1, OE2) both displayed impaired growth during exponential phase with respect to wild-type cells; maximum specific growth rates (μ_{max}) were 1.01, 0.88, and 1.46 d^{-1}, respectively (Figure 1D), consistent with the previous observations that application of an NO donor to wild-type *P. tricornutum* cells resulted in a dose-dependent suppression of growth and induction of cell death [6]. Furthermore, application of 20 μM cPTIO, a NO scavenger, rescued the growth of OE1 and OE2 by 4- and 2.6-fold, respectively.

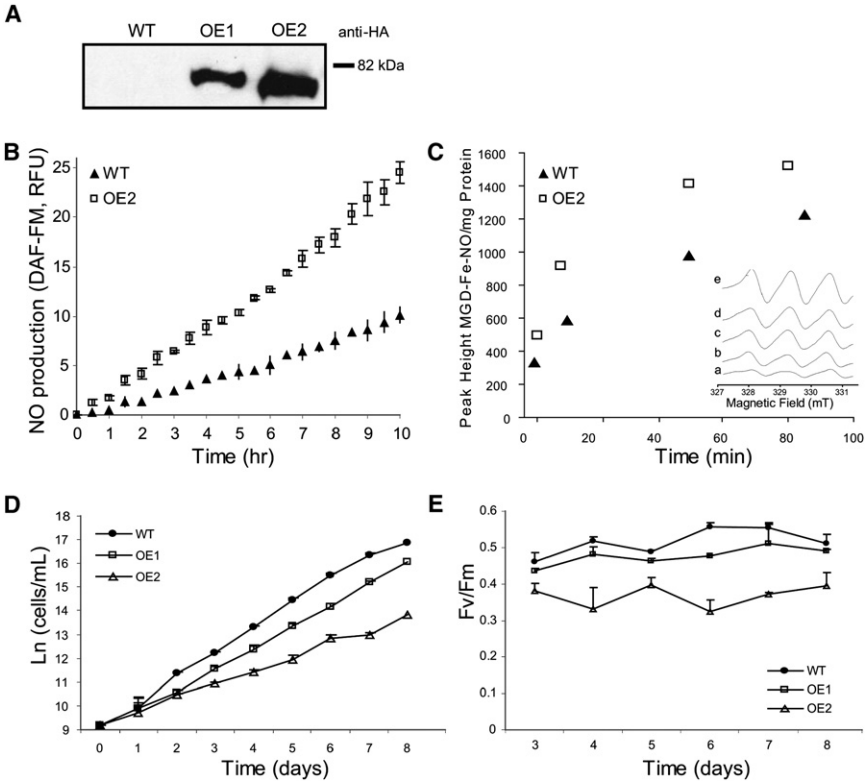

Figure 1. Characterization of *PtNOA*-Overexpressing Transgenic *P. tricornutum* Lines

(A) Western-blot analysis with anti-HA primary antibody to detect HA-tagged PtNOA in cell extracts from transgenic diatoms (OE1, OE2) as compared with wild-type cells, which showed no cross hybridization.

(B and C) Relative rates of basal NO production in wild-type (WT, triangles) and in *PtNOA*-overexpresing line OE2 (squares) as determined by (B) DAF-FM diacetate-derived fluorescence and by (C) EPR analysis of spin-trapped NO, $(MGD)_2$-Fe(II)-NO. (C, inset) The EPR spectra of the spin-trapped nitroxide radical in cell lysates of OE2 at (C_a) 4, (C_b) 11, (C_c) 50, and (C_d) 80 min are compared to that produced chemically by (C_e) 100 μM NOC-5 and normalized to equal protein concentration.

(D and E) Time course of cell growth (D) and photosynthetic efficiency (E) in wild-type (WT, •) and *PtNOA*-overexpressing lines (OE1, □; OE2, △). Representative data from six experiments are shown in (B) and three experiments in (C), and data in (D) and (E) are means ± standard deviation (SD) from three experiments.

Assessment of photosynthetic health by fluorescence induction and relaxation (FiRE) indicated a significant reduction in photochemical efficiency of PSII (Fv/Fm) in OE1 and OE2 cells relative to wild-type cells. Average Fv/Fm values were 0.47, 0.36, and 0.51 for OE1, OE2, and wild-type cells, respectively (Figure 1E). Hence, enhanced NO production, suppression of growth, and reduction of photosynthetic activity in *P. tricornutum* were proportional to the relative expression of *PtNOA* in the transgenic lines. Similarly, higher production of NO reduces photosynthetic and photorespiration efficiencies in plants [18].

PtNOA Is Localized in the Chloroplast

In silico analyses of PtNOA revealed significant probability of targeting to the chloroplast (Supplemental Results), which we tested with a YFP reporter fused to the carboxy terminus of PtNOA. Western-blot analysis verified that the YFP signal was indeed derived from a fusion of YFP and PtNOA (Figure 2A). Cells expressing PtNOA-YFP displayed strong punctate YFP signals localized within the plastid; wild-type cells showed no YFP signal (Figure 2B). Cells transformed with YFP alone or with YFP fused to the amino terminus of PtNOA displayed fluorescent signals in the cytosol and not the plastid ([19] and data not shown). Fluorescence topography and three-dimensional deconvolution images provided better resolution of the punctate YFP signal in PtNOA-YFP transformants (Movie S1). Merged chlorophyll autofluorescence and YFP showed a localized signal in a ring-like structure within the plastid. Plastid localization was further confirmed by immunogold labeling of ultrathin sections with an anti-GFP antibody (Figure 2C). Gold particles were dispersed close to or within the pyrenoid and were not observed in sections from wild-type cells. A similar cluster formation in *P. tricornutum* chloroplasts was recently observed for a β-carbonic anhydrase [20].

AtNOA1 was reported to be localized to the mitochondria in plants [14]. However, the *A. thaliana* genome contains another sequence that encodes a YqeH-domain from the same subfamily of GTP-binding proteins (At3G57180) and shows a higher similarity to PtNOA and is predicted to be targeted to the chloroplast. Furthermore, Bayesian phylogenetic analysis of the YqeH-containing proteins grouped At3G57180 with a variety of unicellular photoautotrophs within a "green" plastid lineage, including sequences derived from planktonic samples from diverse oceanic regions. PtNOA and TpNOA formed a unique branch (Figure S3).

PtNOA Modulates Threshold Sensitivity to Stress

Diatoms accurately sense diatom-derived aldehydes via a calcium-dependent, NO-based system at a distinct threshold, above which programmed cell death (PCD) is triggered [6, 21]. These aldehydes are derived both from plasma-membrane-localized phospholipid and chloroplast-membrane-localized glycolipid precursors and are rapidly induced by wounding and under nutrient deprivation [22, 23]. We examined the response of PtNOA to the reactive aldehyde 2E,4E/Z-decadienal (DD). *PtNOA* transcript abundance increased up to 10-fold after 6 hr in response to subthreshold DD concentrations (3.3 μM) of this model oxylipin (Figure S4), corroborating its presence in an EST library derived from *P. tricornutum* cells exposed to DD (http://www.biologie.ens.fr/diatomics/EST3). Expression of *TpNOA* was also responsive to low levels of DD and was strongly upregulated (25-fold) after 24 hr exposure (data not shown).

Overexpression of *PtNOA* dramatically altered the cellular threshold response to sublethal DD concentrations (Figure 3). OE1 and OE2 cells exhibited hypersensitivity to concentrations of DD that were sublethal to wild-type cells. A DD concentration of only 3.3 μM was sufficient to suppress growth

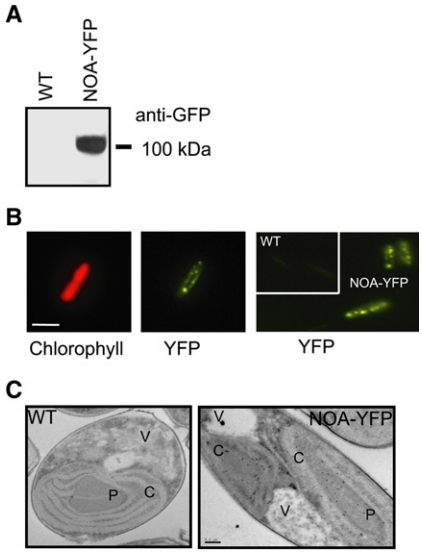

Figure 2. Subcellular Localization of NOA-YFP

(A) Western-blot analysis with anti-GFP primary antibody to detect YFP-tagged PtNOA in cell extracts from transgenic diatoms (*NOA-YFP*) and wild-type cells, which showed no crosshybridization.

(B) Subcellular localization of PtNOA-YFP to the plastid, as depicted by the punctate YFP signals inside the chloroplast (the red signal is chlorophyll autofluorescence). Wild-type cells showed no YFP signal under the same microscope settings compared to a bright yellow signal in PtNOA-YFP cells.

(C) Transmission electron microscopy (TEM) images of immunogold labeling of ultrathin sections from PtNOA-YFP transformants and wild-type cells with the use of an anti-GFP antibody. Gold particles are localized within the pyrenoid (P) inside the chloroplast (C) and excluded from the vacuole (V). Scale bars in (B) and (C) represent 10 and 0.2 μm, respectively.

of both *PtNOA* transformants, whereas wild-type cells reached near-maximal growth rates (Figure 3A). OE2 cells, with their higher levels of *PtNOA* expression and NO production (Figures 1A and 1B), exhibited the highest susceptibility to subthreshold DD concentrations (3.3 μM). The observed reduction in cell viability was also accompanied by a dramatic decrease in the photochemical efficiency of PSII. Partial recovery was observed after 3 days in OE1 but not in OE2 cells (Figure 3B). Similar trends were also observed for 6.6 μM DD treatment with a sharp decline in both growth and photosynthetic activity, but with no apparent recovery in either transformed line (Figures 3C and 3D). In comparison, wild-type cells reached cell densities more than two orders of magnitude higher after 5 days of exposure. Our results demonstrate that modulation of *PtNOA* expression and NO production determines the threshold response of diatoms to DD and their susceptibility to cytotoxic effects.

Effect of Modulated NO Production on Downstream Stress Pathways

To elucidate the predisposition of *PtNOA*-overexpressing lines to stress, we examined downstream effects on stress-related proteins. Recently, a novel plastid-targeted, superoxide dismutase (MnSOD) was identified in a diatom and was proposed to be an important component in response to oxidative stress [8]. Western-blot analysis of crude extracts from wild-type, OE1, and OE2 cells revealed a distinct band of approximately 24 kDa (Figure 4A) when probed with a polyclonal antibody raised against a purified, recombinant *T. pseudonana* MnSOD [8]. This protein closely corresponded with the predicted molecular weight of the *P. tricornutum* MnSOD protein (24.7 kDa). Notably, OE1 and OE2 cell extracts showed a substantial decrease in the level of MnSOD relative to wild-type cells, indicative of a compromised ability to cope with oxidative stress. Similarly, *Atnoa* mutant seedlings exhibited higher levels of H_2O_2 compared to wild-type seedlings in response to stress [14]. Furthermore, downregulation of SOD1 in PC12 neuronal cells leads to cell death via the NO pathway [24]. Colocalization of PtNOA and MnSOD in the chloroplast may be of relevance for managing reactive oxygen species (ROS) metabolism and susceptibility to reactive aldehydes to regulate diatom stress surveillance.

Once ROS production exceeds the cellular antioxidant capacity, a biochemical cascade leading to PCD is induced [25]. We specifically examined the expression of metacaspases, which are involved in stress response and PCD activation in yeast [26] and phytoplankton [9, 27]. Protein extracts from wild-type and OE1 and OE2 cells crossreacted with a polyclonal antibody raised against a purified, recombinant *Emiliania huxleyi* metacaspase (EhMC) [27] (Figure 4B). Immunoreactive proteins in *P. tricornutum* ranged in size from ~25 kDa to ~110 kDa and corresponded with the predicted molecular weights of several putatively annotated metacaspase proteins (32.8, 34.7, 40.5, 45.5, 73.4 KDa). Several putative metacaspases were strongly upregulated in the OE1 and OE2 cells (e.g., 22, 32.8, 40.5, 45.5, 73.4, and 110 kDa) and were either absent or showed reduced expression in wild-type cells (Figure 4B). No immunoreactive bands were observed when the same extracts were incubated with preimmune rabbit serum, supporting their identity as metacaspases. The same cell extracts also showed higher basal caspase-8 activities, with a 2.5-fold increase in OE2 cells with respect to wild-type cells. Application of the broad caspase inhibitor z-VAD-FMK to cells 1 hr prior to addition of 3.3 μM DD delayed but did not abolish the detrimental effect on OE2 cell growth; treated cells reached on average 25% higher cell abundances over the first 4 days following treatment (data not shown).

The findings that overexpression of *PtNOA* led to overproduction of NO, decreased MnSOD protein levels, enhanced expression of metacaspases, and elevated caspase activity (Figures 1 and 4) suggest that it plays a key role in the activation of PCD. Indeed, NO can act as either a pro- or an antiapoptotic signal in both animals and plants [28]. In *A. thaliana*, NO regulated both metacaspases and caspase-like activity, and a caspase-1 inhibitor blocked NO-induced cell death [29, 30]. Overexpression of the PtNOA ortholog *mAtNOS1* in human neuroblastoma cells induced NO production in the mitochondria and led to cytochrome C release and apoptosis [31]. PCD may represent a biochemical mechanism by which compromised cells are eliminated from a population through an intraspecies competition based around susceptibility to environmental stress and threshold responses to dose-dependent chemical signals [32].

PtNOA Is Implicated in the Control of *P. tricornutum* Adhesion to Substrata and Biofilm Formation

Microbial mats and biofilms represent ecological niches that allow for the accumulation of high cell densities of benthic diatoms, like the oval morphotype of *P. tricornutum* [33], and their derived infochemicals. Adhesion of diatoms to surfaces, and therefore biofilm formation, is mediated by cell-surface properties and secretion of extracellular polymeric substances. It has been hypothesized that increased intracellular NO production on a hydrophilic surface could be regarded as

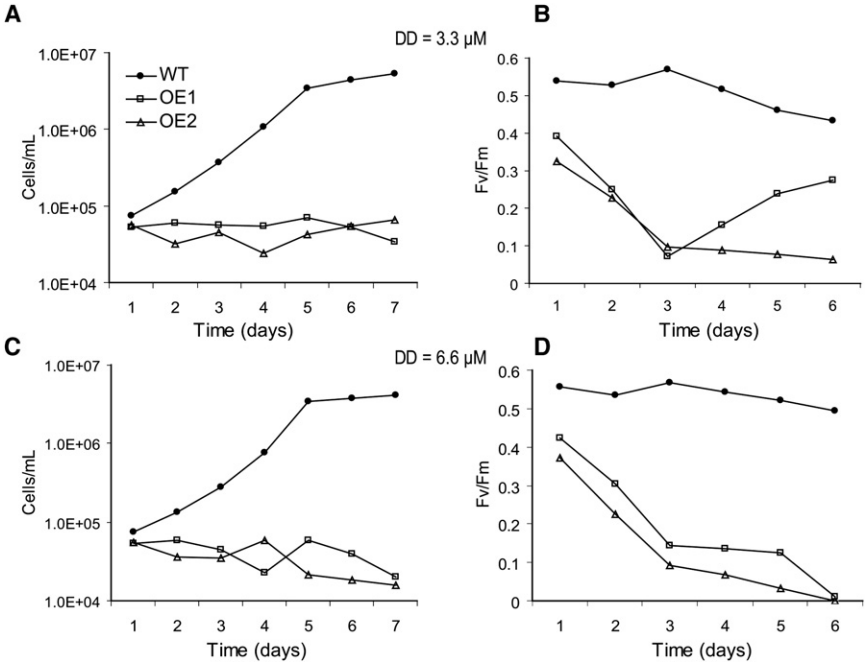

Figure 3. *PtNOA*-Overexpressing Lines Are Hypersensitive to Diatom-Derived Unsaturated Aldehyde

Time course of cell growth (A and C) and photosynthetic efficiency (B and D) of wild-type (WT, •) and *PtNOA*-overexpressing lines (OE1, □; OE2, △) in response to (2*E*,4*E*/*Z*)-decadienal concentrations.

a form of stress response, the significance of which in nature is that low adhesion would facilitate motility and the search for surfaces more conducive to adhesion and biofilm formation [34]. Indeed, we observed that OE2 cells displayed an 80% reduction in biofilm formation compared to wild-type and OE1 cells (Figure 4C). To further explore this observation, we subjected wild-type and OE1 cells to a range of hydrodynamic shear stresses and quantified their attachment strength to substrata with different surface properties. OE1 cells attached less strongly to glass substratum than the wild-type cells (Figure S5). Only 2 Pa was required to remove 50% of OE1 cells from the hydrophilic glass surface compared to a 4-fold-higher shear stress required to remove 50% of wild-type cells (Figure S5). The adhesion strength of OE1 cells was also reduced on the hydrophobic silicone (polydimethylsiloxane elastomer [PDMSE]) surface compared with wild-type cells (Figure S5). These findings support the hypothesis that NO-mediated stress signaling plays an important role in diatom perception of substratum properties.

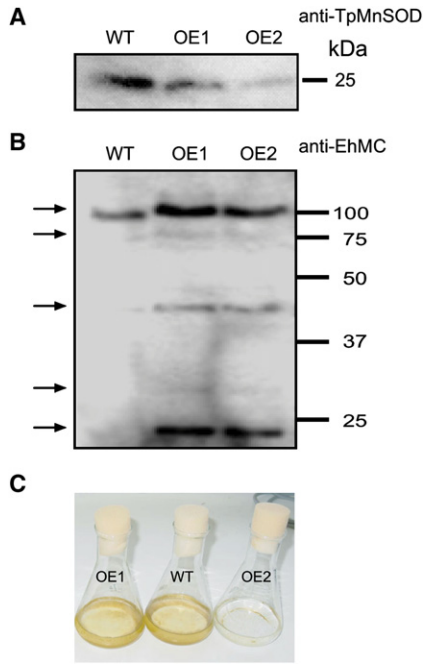

Figure 4. *PtNOA* Overexpression Leads to Impaired Stress Responses

(A and B) Immunoblotting with a polyclonal antibody generated against a purified recombinant *T. pseudonana* MnSOD (TpMnSOD [A]), or a purified recombinant *E. huxleyi* metacaspase protein (EhMC [B]) on cell extracts from exponentially growing wild-type and *PtNOA* transformants. Molecular weights are indicated. Arrows highlight differentially expressed metacaspases between wild-type and OE1 and OE2 cells.

(C) Biofilm formation of oval, wild-type (WT), and *PtNOA*-overexpressing lines seen as cells attaching to the bottom of the flasks, after removal of suspended cells. OE2 showed markedly reduced attachment strength of oval cells, although initial inocula were identical in all three cultures.

Conclusions

The conservation of proteins containing YqeH domains across kingdoms and their prevalence in oceanic samples may indicate a novel conserved signaling pathway. In at least three eukaryotic experimental systems (including diatoms in this study), these proteins have been shown to be involved in NO signaling, whereas in bacteria they play important roles in ribosome biogenesis and in sporulation. Consistently, other eukaryotic orthologs (e.g., in yeast and in mouse) have been implicated in mitochondrial-ribosome biogenesis, protein translation, and ROS metabolism [14, 35, 36]. We now provide information about a chloroplast-localized member of the YqeH family and propose that it is involved in controlling fundamental cellular processes such as photosynthetic efficiency, oxidative stress, and PCD, via management of NO signaling. Our results also provide an intriguing mechanistic backdrop for measurable NO concentrations in surface waters of the equatorial Pacific, where NO production was suggested to originate solely from abiotic nitrite photolysis [10] or from cycles of bacterial denitrification and nitrification [37]. Future studies using transgenic approaches for manipulating genes in key signaling and stress-related pathways in diatoms will provide the opportunity to gain further insights into their role in controlling algal population dynamics and other trophic-level interactions in the aquatic environment.

Supplemental Data

Additional results, experimental procedures, five figures, and one movie are available at http://www.current-biology.com/cgi/content/full/18/12/895/DC1/.

Acknowledgments

We thank Valentin Starovoytov for transmission electron microscopy (TEM) analysis, Faye Rosin for her assistance with the Deltavision microscope, Felisa Wolfe-Simon, Christiane Lichtlé, Agnès Meichenin, Marc Heijde, Liti Haramaty, and Hui Lui for technical assistance. We thank Nigel Crawford for providing valuable advice during the course of this work and Kim Thamatrakoln for valuable feedback. This work was supported by a Marie Curie Intra-European fellowship to A.V. (EIF-515066), the EU-funded DIATOMICS project to J.A.C. and C.B. (LSHG-CT-2004-512035), the Agence Nationale de la Recherche (ANR) to C.B., and grants from the National Science Foundation IOB-0414536 to K.D.B. and P.G.F. and IOS-0717494 to K.D.B. and A.V.

Received: February 29, 2008
Revised: May 5, 2008
Accepted: May 15, 2008
Published online: June 5, 2008

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