Pheromones

DOI: 10.1002/anie.201208175

Metabolomics Enables the Structure Elucidation of a Diatom Sex Pheromone**

Jeroen Gillard, Johannes Frenkel, Valerie Devos, Koen Sabbe, Carsten Paul, Martin Rempt, Dirk Inzé, Georg Pohnert, Marnik Vuylsteke,* and Wim Vyverman**

Diatoms are unicellular photosynthetic organisms that often dominate primary production in pelagic and benthic aquatic ecosystems.[1,2] Despite their central role in the biosphere, little is known about their pheromone chemistry and their lifecycle, which is characterized by asexual population growth alternating with short bursts of sexual reproduction.[3,4] Diatoms are unique among microalgae in that sexual reproduction is only possible below a species-specific sexual size threshold (SST).[5] This SST is intimately linked to the characteristic cell division of diatoms. Due to their rigid biomineralized cell wall, mitotic division results in a reduction in size (Figure 1).[6] Size is restored typically by sexual reproduction. Upon germination, a zygote generates a large initial cell, which begins a new round of vegetative proliferation.[6,7] In the ancestral group of predominantly planktonic centric diatoms, environmental cues induce meiosis in cells below the SST, resulting in the formation of eggs and flagellated sperm.[8] However, in the youngest and most species-rich pennate raphid diatoms,[9] which have adopted a primarily benthic lifestyle, it is the pairing of diploid cells that triggers the production of isogametes.[6] Although the SST is known to control the mating capacity of pennate diatoms and indirect evidence suggests the involvement of pheromones, the regulatory principles underlying the differentiation of mating cells and the identity of the pheromones remain unknown.[6,10]

We studied the physiological and metabolic changes associated with sexual reproduction in *Seminavis robusta*, a model for studies of pennate diatom lifecycles, with the goal to elucidate its pheromone chemistry.[7,11] Mating is strictly size-dependent with a narrowly defined SST of (51.6 ± 0.5) μm (Figure 2a). Like most pennate diatoms, *S. robusta* is a heterothallic species, that is, a species with morphologically identical but physiologically distinct cell types between which fertilization can take place. Mixing G1 phase-synchronized cells[12] below the SST revealed the physiological differences between cells of different mating types (Figure 2b). Depending on the density of the partners, pairs or clusters of a migrating mating type (designated MT^+) around an attracting cell (designated MT^-) were observed. The increased motility of MT^+ in the presence of medium from a mating culture suggested the involvement of regulatory pheromones. We developed a bioassay to unambiguously prove and quantify the effect of such pheromones. Therefore hydrophilic/lipophilic-balanced solid-phase extraction cartridges (HLB-SPE) were loaded with media of *S. robusta* cultures. The SPE absorbent beads were then removed from the cartridges and served as pheromone sources. Behavioral responses towards such beads were monitored using light microscopy. Beads loaded with medium from a mating culture attracted MT^+ cells below the SST proving that the pheromone can be extracted (Figure 2b, movie in the Supporting Information). Attraction by MT^- cells was dependent on cell size and on prior perception of sexually mature (i.e. below the SST) mating partners. Extracts of MT^- cultures below the SST were only active when the MT^- cells were previously conditioned with medium from MT^+ below the SST (Figure 2c). Extracts of MT^- above the SST were not active, even after conditioning with medium from MT^+ below the SST (Figure S1 in the Supporting Information). Likewise, the MT^+ motility response is primed by MT^- signals. Attraction to pheromone-loaded beads only occurred if MT^+ cells were

[*] Dr. J. Gillard,[$] [+] V. Devos,[+] Prof. Dr. K. Sabbe, Prof. Dr. W. Vyverman
Laboratory of Protistology and Aquatic Ecology
Department of Biology, Ghent University, 9000 Gent (Belgium)
E-mail: wim.vyverman@ugent.be
Homepage: http://www.pae.ugent.be/academic.html
J. Frenkel,[+] C. Paul, Dr. M. Rempt, Prof. Dr. G. Pohnert
Institute for Inorganic and Analytical Chemistry
Bioorganic Analytics, Friedrich Schiller University
07743 Jena (Germany)
E-mail: georg.pohnert@uni-jena.de
Homepage: http://www.uni-jena.de/prof_pohnert.html
Prof. Dr. D. Inzé, Prof. Dr. M. Vuylsteke
Department of Plant Systems Biology, VIB
9052 Gent (Belgium)
and
Department of Plant Biotechnology and Bioinformatics
Ghent University, 9052 Gent (Belgium)
E-mail: mavuy@psb.vib-ugent.be
Homepage: http://www.psb.ugent.be/marnik-vuylsteke
[$] Present address: J. Craig Venter Institute
San Diego, CA 92121 (USA)
[+] These authors contributed equally to this work.

[**] Financial support (G.P., J.F., C.P.) from the Volkswagen Foundation, the IMPRS ("Exploration of Ecological Interactions with Molecular and Chemical Techniques"), and the Jena School for Microbial Communication is acknowledged. J.G. and V.D. were funded by the Institute for the Promotion of Innovation by Science and Technology (IWT)—Flanders", J.G. by an EMBO short-term fellowship (ASTF 410.00-2008). W.V., K.S., D.I., and M.V. acknowledge Belspo for funding the Belgian-coordinated "Culture Collections of Microorganisms (BCCM)" and Ghent University Research Funding (BOFGOA 01G01911).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201208175.

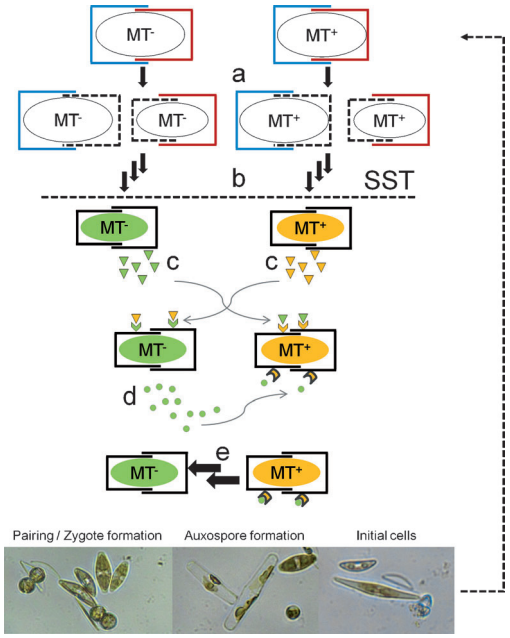

Figure 1. Size reduction–restitution cycle involving mating differentiation and cell pairing in the diatom *Seminavis robusta*. The biomineralized diatom shell is made up of two unequal thecae depicted as blue and red open boxes. a) During mitosis new thecae (dashed lines) are synthesized inside the shell, resulting in b) a decrease in the mean cell size of the population. c) Below the sexual size threshold (SST) differentiation into sexual mating types (MT^+ or MT^-) occurs. Both mating types produce chemical cues (triangles) that activate mating behavior in the respective partner. d) MT^- releases the attraction pheromone L-diproline (green dots). e) MT^+ becomes motile and is attracted to the diproline source. Cell pairing occurs, followed by meiosis, gametogenesis, zygote formation, and cell size restoration.

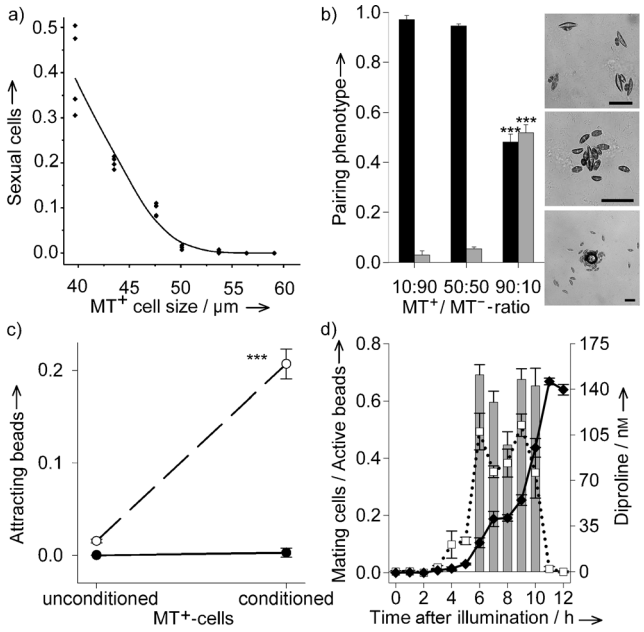

Figure 2. Regulation of pheromone production. a) Assessment of sexual proficiency as a function of cell size in *S. robusta*. Shown is the proportion of sexual cells in cultures of small MT^- cells (< SST) that were mixed in equal proportion with MT^+ cells of different cell size. b) Proportions of pairs (black bars, upper microscopic picture) and clusters (gray bars, middle picture) in mixed cultures of different mating type ratios (mean ± standard error, $n = 3$, ***: $p < 0.001$). Larger MT^- and smaller MT^+ cells, both below the SST, were used for distinction. Bottom picture shows an active bioassay bead surrounded by attracted MT^+ cells. Bars = 50 µm. c) Attraction ability of beads loaded with extracts from conditioned (dashed line) and unconditioned (solid line) MT^- cells on unconditioned and conditioned MT^+ cells (mean ± standard error, $n = 5$). d) Proportion of mating cells in a culture containing both mating types (—◆—), proportion of MT^+-attracting beads loaded with medium extracts from the same mating culture (•••••□•••••), and concentration of diproline in the medium (gray bars) (mean ± standard error, $n = 3$).

previously treated with medium from MT^- cells below the SST (Figure 2c). Diatoms thus use multiple chemical signals to ensure the presence of a sexually mature mating partner before investing in sexual response. The combined action of these reciprocal mating signals is concentration dependent and enhances mate-finding success (Figure S2 in the Supporting Information). This corroborates field observations on pennate diatom populations where sexual reproduction was only observed in sufficiently dense populations of sexually mature mating partners.[13,14]

Since we were able to control the attraction chemistry of *S. robusta*, we could identify pheromones by comparing exometabolomes. Comprehensive profiling of released metabolites from MT^- cultures under different physiological conditions in combination with a multivariate data analysis enabled the identification of the attraction pheromone and helped to efficiently reduce the effort usually required for bioassay-guided fractionation.[15] The media of conditioned and unconditioned MT^- cultures were extracted using HLB-SPE and submitted to ultra-performance liquid chromatography/mass spectrometry (UPLC/MS) to comprehensively record the exo-metabolome (Figure 3a).[16] Subsequently, metabolites up-regulated in conditioned cultures were identified using principal component analysis. One metabolite (pseudomolecular ion $[M+H]^+$, m/z 195) stood out as most significantly up-regulated and was isolated by targeted fractionation using preparative HPLC (Figure 3). This metabolite was active in the attraction bioassay and structure elucidation was further pursued. Fragmentation data from tandem and electron-impact MS suggested a 2,5-diketopiperazine (di-L-prolyl diketopiperazine, in the following diproline) as candidate pheromone (Figure 3d and Figure S3). This molecule was produced by the algae only during the mating process and the attraction assay was only positive when diproline was detectable; these findings support a specific role for diproline as a pheromone (Figure 2d). Enantioselective synthesis starting from L- or D-proline and co-injection in UPLC/MS confirmed the structure (Figure 4).[17] Circular dichroism indicated that the pheromone is derived from L-proline. Bacterial origin of this metabolite was excluded since mating activity was not affected in axenic cultures (Figure S4).

For activity testing, synthetic diproline was loaded on SPE beads and supplied to conditioned MT^+ cultures. L-Diproline elicited a significant attraction of MT^+ cells at concentrations corresponding to roughly 2 pmol mg^{-1} beads. We observed

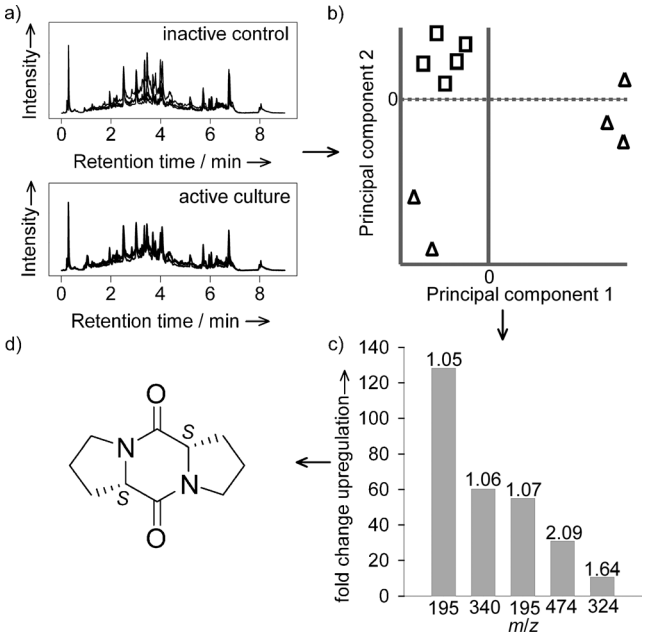

Figure 3. Workflow for the comparative metabolomic approach for pheromone identification. a) Extracts from unconditioned (top) and conditioned (bottom) MT$^-$ medium were profiled, resulting in complex UPLC/MS data. b) Principal component analysis of mass/retention time pairs revealed pronounced grouping of the MT$^-$ extracts (triangles: unconditioned, squares: conditioned). c) Five most up-regulated mass/retention time pairs (numbers above bars in min) pairs in conditioned MT$^-$ cultures. d) Structure of the pheromone.

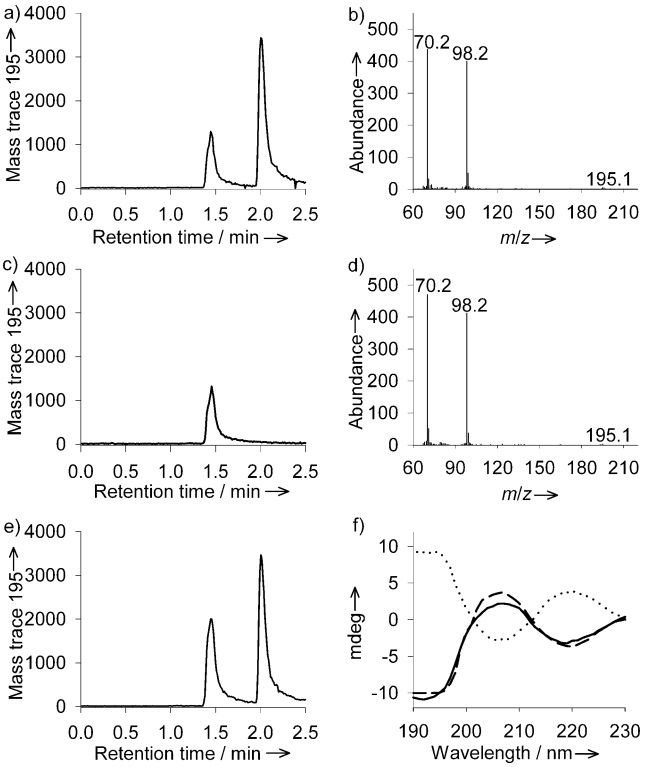

Figure 4. Characterization of the pheromone L-diproline. a) LC–MS chromatographic profile of the pheromone extract (mass trace *m/z* 195, signal of the internal standard caffeine at 2.1 min) and b) corresponding MS/MS spectrum. c, d) Synthetic L-diproline and e) co-injection of extract and synthetic standard. f) Circular dichroism spectra of synthetic L- (solid line) and D-diproline (dotted line) and the natural pheromone (dashed line).

concentration-dependent attraction with a potential saturation of the receptors at 200 nmol mg^{-1} beads (ca. 715 nM, Figure S5). Despite uncertainties regarding absolute quantification of local pheromone concentrations, the activities of synthetic L-diproline and L-diproline from mating cultures (1.5 nM to 165 nM, Figure 2d) are comparable. Similar concentrations will most likely be reached in natural biofilms where cell densities are even higher than in our assays. In synchronized cultures of conditioned MT$^-$ cells and mixtures of both mating types, pheromone production and mating can only be detected 5 h after illumination (Figure 2d, Figure S6). Pheromone production is thus strictly light dependent,which is in line with an earlier report on light as a trigger for mating in the pennate diatom *Haslea ostrearia*.[18] Light-induced activation of pheromone production further enhances the encounter probabilities of sexually mature cells. L-diproline is detected up to 10 h after illumination, coinciding with a loss of attraction capacity of the medium extracts (Figure 2d). Since L-diproline is stable under assay conditions, active degradation or resorption of the pheromone must occur. A rapid decline of L-diproline concentration was also observed in conditioned MT$^-$ cultures lacking MT$^+$ (Figure S6), suggesting that the pheromone producer can actively prevent cells being misled by outdated signals or that associated bacteria metabolize the pheromone. Surprisingly, D-diproline was active in a concentration range comparable to that of its isomer, which might indicate a "swipe card" recognition mechanism that is determined by electronic rather than geometric properties of a signal.[19] An alternative explanation for the activity of both enantiomers might be found in the observed degradation process. This could involve racemization or other transformations that generate an active product from D-diproline. Diproline is unprecedented as a pheromone but the compound is found in several organisms including higher plants and fungi where it occurs in substantially higher concentrations and serves as an antibacterial agent.[20] Interestingly, the pheromone chemistry of *S. robusta* is fundamentally different from that of related brown algae which also belong to the heterokonts and rely on very apolar fatty acid derived hydrocarbons to attract conspecific gametes.[21]

Taken together, the pennate diatom *S. robusta* employs a sophisticated multistage control of cell pairing mediated by several pheromones, of which diproline represents the first elucidated diatom pheromone. This strategy allows cells to time sexual investment through sensing the presence of mature partners (Figure 1). Our findings provide a mechanistic explanation of earlier indirect observations of pheromone-mediated sexualization and attraction.[10] Given the pivotal role of pheromone signaling in *S. robusta*, we expect that the present and further studies on pheromone diversity and reception in diatoms will contribute to our understanding of their remarkable evolutionary and ecological success.

Received: October 11, 2012
Published online: December 12, 2012

Keywords: algae · cell cycle · mass spectrometry · metabolomics · pheromones

[1] D. G. Mann, *Phycologia* **1999**, *38*, 437 – 495.
[2] C. B. Field, M. J. Behrenfeld, J. T. Randerson, P. Falkowski, *Science* **1998**, *281*, 237 – 240.
[3] P. von Dassow, M. Montresor, *J. Plankton Res.* **2011**, *33*, 3 – 12.
[4] C. Bowler, A. De Martino, A. Falciatore, *Curr. Opin. Plant Biol.* **2010**, *13*, 623 – 630.
[5] L. Geitler, *Bot. Rev.* **1935**, *1*, 149 – 161.
[6] V. A. Chepurnov, D. G. Mann, K. Sabbe, W. Vyverman in *International Review of Cytology, Vol. 237* (Ed.: K. W. Jeon), Elsevier Academic Press, San Diego, **2004**, pp. 91 – 154.
[7] V. A. Chepurnov, D. G. Mann, P. von Dassow, P. Vanormelingen, J. Gillard, D. Inze, K. Sabbe, W. Vyverman, *BioEssays* **2008**, *30*, 692 – 702.
[8] E. V. Armbrust, *Nature* **2009**, *459*, 185 – 192.
[9] C. Bowler, et al., *Nature* **2008**, *456*, 239 – 244.
[10] S. Sato, G. Beakes, M. Idei, T. Nagumo, D. G. Mann, *PLoS ONE* **2011**, *6*, e26923.
[11] V. A. Chepurnov, D. G. Mann, W. Vyverman, K. Sabbe, D. B. Danielidis, *J. Phycol.* **2002**, *38*, 1004 – 1019.
[12] J. Gillard, et al., *Plant Phys.* **2008**, *148*, 1394 – 1411.
[13] D. d'Alelio, M. R. d'Alcala, L. Dubroca, D. Sarno, A. Zingone, M. Montresor, *Limnol. Oceanogr.* **2010**, *55*, 106 – 114.
[14] K. E. Holtermann, S. S. Bates, V. L. Trainer, A. Odell, E. Virginia Armbrust, *J. Phycol.* **2010**, *46*, 41 – 52.
[15] E. K. Prince, G. Pohnert, *Anal. Bioanal. Chem.* **2010**, *396*, 193 – 197.
[16] A. Barofsky, C. Vidoudez, G. Pohnert, *Limnol. Oceanogr. Methods* **2009**, *7*, 382 – 390.
[17] D. Nakamura, K. Kakiuchi, K. Koga, R. Shirai, *Org. Lett.* **2006**, *8*, 6139 – 6142.
[18] J.-L. Mouget, R. Gastineau, O. Davidovich, P. Gaudin, N. A. Davidovich, *FEMS Microbiol. Ecol.* **2009**, *69*, 194 – 201.
[19] J. C. Brookes, A. P. Horsfield, A. M. Stoneham, *J. R. Soc. Interface* **2009**, *6*, 75 – 86.
[20] L. Huberman, N. Gollop, K. Y. Mumcuoglu, E. Breuter, S. R. Bhusare, Y. Shai, R. Galun, *Med. Vet. Entomol.* **2007**, *21*, 127 – 131.
[21] G. Pohnert, W. Boland, *Nat. Prod. Rep.* **2002**, *19*, 108 – 122.

