Sexual Pheromone Induces Diffusion of the Pheromone-Homologous Polypeptide in the Extracellular Matrix of *Volvox carteri*\(^\text{\textsuperscript{7}}\)

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**NOTES**

*Volvox carteri* is one of the simplest multicellular green algae, comprising 2,000 to 4,000 biflagellate somatic cells arranged in a monolayer surface around a hollow sphere and 16 reproductive cells called gonidia residing below the somatic cells (13). In the asexual life cycle, the matured gonidium undergoes 11 to 12 rapid cell divisions and gives rise to the somatic cells and gonidia; however, when exposed to a sexual pheromone, gonidia of the male and female spheroids undergo modified cell divisions, and sperm packets and eggs are produced instead of the asexual gonidia, respectively (12). The sexual pheromone (20) (the sugar residues of the glycoprotein of *V. carteri* is one of the simplest multicellular green algae, comprising 2,000 to 4,000 biflagellate somatic cells arranged in a monolayer surface around a hollow sphere and 16 reproductive cells called gonidia residing below the somatic cells (13). In the asexual life cycle, the matured gonidium undergoes 11 to 12 rapid cell divisions and gives rise to the somatic cells and gonidia; however, when exposed to a sexual pheromone, gonidia of the male and female spheroids undergo modified cell divisions, and sperm packets and eggs are produced instead of the asexual gonidia, respectively (12). The sexual pheromone of *V. carteri* is a 32-kDa glycoprotein that works at a very low concentration of less than 10\(^{-16}\) M (20, 21), which implies that a special mechanism amplifies the sexual-pheromone signaling within the spheroid during the sexual-induction process. Interestingly, this sexual-induction process necessitates a continuous 6- to 8-h exposure of the asexual spheroid to the sexual pheromone before the initiation of gonidial cleavage, and during this period, the sexual-induction signal is assumed to be transmitted to the putative surface receptor of the gonidium (6).

The nature of the sexual-induction mechanism of *V. carteri* is largely unknown. However, several molecular clues have been accumulated to date; the earliest biochemical responses of the spheroid to the sexual pheromone occurs in the somatic cells, which is evidenced by the synthesis of an extracellular matrix (ECM) protein, pherophorin II, as well as of many other proteins (1, 2, 3, 4, 8, 11, 20, 22). Pherophorin II is also a glycoprotein that consists of three domains: the N-terminal domain, whose sequence is related to a motif of another ECM protein, SSG185 (this motif is shared among other types of pherophorins as well); the polyproline spacer; and the C-terminal domain, whose amino acid sequence is homologous to that of the sexual pheromone (8, 20). An impressive fact is that this C-terminal domain of pherophorin II is liberated from the other domains by proteolytic cleavage slowly after the sexual induction, with the same kinetics as those of the sexual-induction process (20). This phenomenon invokes the liberated pheromone-like C-terminal domain to mediate the signaling process between the sexual pheromone and the gonidium. Therefore, in this work, green fluorescent protein (GFP) was fused to the C terminus and/or the N terminus of pherophorin II (5) and *V. carteri* was transformed with these fusion gene constructs (18) (hereinafter referred to as the PIIC-GFP and PIIN-GFP transformants, respectively) to study the behavior of the each domain of pherophorin II in the sexually induced spheroid.

Figure 1A shows the scheme of the PIIC- and PIIN-GFP fusion proteins. Figure 1B shows a Western blot of the soluble fraction of the 153-48 strain (a recipient strain for transformation), in which the PIIC- and PIIN-GFP transformants in 1% sodium dodecyl sulfate (SDS)–0.5 M NaCl were boiled for 5 min and detected by anti-GFP antibody, suggesting that GFP-tagged pherophorin II was synthesized successfully in both transformants. However, the Western blot of the ECM protein fraction, which was insoluble in boiled 1% SDS–0.5 M NaCl solution (called the “ECM ghost”) but thereafter solubilized in 1% Triton X-100–0.1 M EDTA at 65°C for 20 min as detected with anti-GFP antibody, showed that only the PIIC-GFP fusion protein was proteolytically cleaved appropriately (Fig. 1C). This result suggests that the insertion of GFP into the N-terminal domain of pherophorin II, which might cause a steric obstruction, inhibited further processing of this fusion protein. Thus, this transformant can serve as a control in distinguishing the localization of the cleaved C-terminal domain of the PIIC-GFP fusion protein from that of intact pherophorin II, although it has to be noted that it was still not a strict control because it is not known whether the PIIN-GFP fusion protein migrates through the ECM in the same way as the intact PIIC-GFP fusion protein. Next, I performed a pulse-chase labeling experiment with \(^{35}\)SO\(_4\)\(^{2-}\) using the soluble fraction of the ECM ghosts of the PIIC-GFP transformant after the Triton X-100–EDTA treatment, prepared as described in the work of Wenzl and Sumper (20) (the sugar residues of the glycoprotein of *V. carteri* ECM is sulfated frequently [19]). As shown in Fig. 1D, the deduced molecular mass suggests that the band (band (b)) represents the cleaved C-terminal domain.
The result of Western blotting using anti-GFP antibody (Fig. 1C) supports the notion that the band of the corresponding molecular mass is the cleaved C-terminal domain fused to GFP. These results suggest that the PIIC-GFP fusion protein was also cleaved proteolytically, as is the native protein. Next, the GFP signal in the ECM structure (14) (shown in Fig. 2A) of the PIIC-GFP transformant was detected at each time point after the application of the sexual pheromone with a fluorescence image system (Till Photonics, Gräfelfing, Germany) as described by Fuhrmann et al. (5). The result in Fig. 2B shows that the GFP signal that was initially seen in the somatic cells (1 h later; bright spots in the surface view) spreads into amorphous cellular zone 2 (CZ2) and the fibrous CZ3 layer of the somatic cells (2 h later, surface view; signals in the compartment surrounding each somatic cell and the boundary of the compartment, respectively). Thereafter, the intense signal was detected at CZ3 of the gonidia (3 h later; signal at the compartment boundary surrounding each gonidium in the inner view). However, in the PIIN-GFP transformant, the GFP signal was detected only weakly in CZ2 and -3 of the somatic cells (surface view) and in the inner space of the spheroid (inner view) even at 14 h after pheromone exposure, whereas rather intense signals were found in the somatic cells (Fig. 2C, surface view). These signals in the PIIC- and PIIN-GFP transformants were absent in the 153-48 strain (Fig. 2D). To obtain more-detailed images, the GFP signals of the PIIC- and PIIN-GFP transformants and the 153-48 strain at 8 h after the application of the sexual pheromone were captured by a confocal laser scanning microscope (model LSM 5 Pascal; Zeiss, Jena, Germany). The surface view of the PIIC-GFP transformant showed the clear GFP signal in the honeycomb structure of CZ3 of the somatic cells (Fig. 3A), but the view of the inner section of the spheroid exhibited the GFP signal at CZ3 of the gonidia (Fig. 3B). Furthermore, a significantly intense signal was found in CZ4 and the deep zone of the PIIC-GFP transformant. In the PIIN-GFP transformant, however, only a weak signal was detected in CZ2, -3, and -4 of the somatic cells (Figs. 3C and 3D), in addition to the point-like signal in the somatic cells. The signal detected in strain 153-48 shows the background signal mainly from the endogenous chloroplast (Fig. 3E and F). A more important result was obtained from a magnified image of the gonidium of the PIIC-GFP transformant, in which the GFP signal was found at CZ1 (Fig. 3A). In this picture,cleaving gonidium was already detached from CZ1, but CZ1 is directly juxtaposed to the plasma membrane of the precleavage gonidium (14), which is supposed to receive the sexual-induction signal. Neither the PIIN-GFP transformant nor strain 153-48 showed such signals at CZ1s of their gonidia, although weak signals were seen in CZ2, -3, and -4 of the somatic cells of the PIIN-GFP transformant (Fig. 4B and C).

The sexual-induction signal received first by the somatic cells must be transmitted to its final target, namely, the gonidium. Therefore, inevitably, the induction signal has to pass through CZ1, -2, and -3 of both the somatic cell and the gonidium. The results in this study showed that PIIC-GFP liberated from its parental molecule diffused gradually to CZ1 of the gonidium, which implies the role of pherophorin II as a transmitter/amplifier of the sexual-induction signal. In addition, the results of the confocal images of the PIIN-GFP transformant, in which the PIIN-GFP fusion protein cannot be cleaved proteolytically...
and thus is expected to exhibit the same localization pattern as the intact PIIC-GFP fusion protein, suggests that intact pherophorin II can diffuse into CZ2, -3, and -4 of the somatic cells as well as the deep zone of the spheroid; however, proteolytic cleavage is necessary for further diffusion of the C-terminal domain to CZ1 of the gonidium. This result correlates well with the fact that the inhibition of pherophorin II cleavage suppresses sexual induction (7).

Images of the sexually induced spheroid expressing the pherophorin-deep zone 1 (DZ1)-GFP fusion protein showing the GFP signal at CZ1 of the gonidium, similar to Fig. 3B, were observed although not mentioned previously (4). The sexual induction of \textit{V. carteri} triggers a series of genes in addition to pherophorin II and pherophorin-DZ1, as mentioned earlier. Therefore, it is probable that the concerted actions of these multiple gene products is necessary to amplify and transmit the sexual-induction signal within the spheroid. This hypothesis explains that the applications of the artificially synthesized C-terminal domain of pherophorin II alone cannot induce the sexuality of \textit{V. carteri} (7, 20). However, the fact that there are
In the sexual induction of V. carteri, which implies an important role of pherophorin II, two types of mutant phenotype, one refractory to the sexual induction and another constitutively sexually induced, correspond well to the expression status of the pherophorin II transcript, which suggests the existence of such a signal driven by ECM remodeling has not yet been clarified (9).

Another hypothesis is related to the wound-healing process of the spheroid, which is inferred from two considerations of the evolution of the sexual-induction system of V. carteri, described below. First, in the volvocine-lineage algae, the unicellular Chlamydomonas reinhardtii and other colonial species, such as Pleodorina starrii, are induced sexually by the lack of a nitrogen source in the environment but not by a factor such as sexual pheromone (15, 16). However, V. carteri developed a more intricate ECM structure than those species in the course of multicellular evolution (14, 19), and the sexual-induction system of V. carteri seems to have evolved in relation to the establishment of such a complex ECM structure, considering that the sexual pheromone triggers the expression of several of the ECM proteins, including several types of the pherophorin family proteins (9, 10). In reference to this, many genes encoding pherophorin-related proteins have been isolated from the volvocine-lineage algae ranging from C. reinhardtii to V. carteri (10, 17), which implies that the sexually induced pherophorins of V. carteri were evolutionarily derived from the ancestral genes of these pherophorin-related ECM proteins (notably, one of them, GAS28 of C. reinhardtii, is expressed specifically in the gamete [17]), which originally would be the fundamental components of the ECM architecture and unrelated to the pheromone-based sexual-induction process.

Second, the expression of all of the sexually induced genes of V. carteri tested to date are also induced by the wounding of the spheroid (1, 3, 4, 9, 10, 11). Therefore, one possible scenario is that the sexual-induction system of V. carteri has evolved from the fundamental ECM components which were originally used in the wound-healing process of the spheroid to facilitate their survival; that is, the same set of genes as those expressed in the wound-healing process have become utilized in the sexual-induction process in the course of the evolution of the elaborate ECM architecture required for more-sophisticated multicellularity. Several unknown extra factors must be involved in the sexual-induction process because the wounding of the spheroid per se does not induce the sexuality of the spheroid (1), but at least both of these processes appear to share a common signal transduction pathway, which makes sense given that both wound healing and durable zygote formation, the ultimate consequence of sexual induction, occur as the results of a response to an adverse environment (1). In fact, it is realistically presumable that both of these processes occur at the same time in the severely desiccating pond in nature in order to carry out sexual induction in the unwounded spheroid and to bring up the gametes within the normal sexual spheroid.
of the next generation, which will raise the adaptive value of this species.

Taking the two considerations mentioned above into account, GFP signals detected in CZ3, CZ4, and the deep zone of the PIIC-GFP transformant (Fig. 3A and B and 4A) can be interpreted as follows. These ECM regions are the fundamental extracellular framework and the interstitial tissues that support the multicellular architecture; therefore, the signals in these regions might represent the function of this molecule in the wound-healing process rather than in the sexual-induction process. Such a versatile function of this molecule would be of great use for the survival of the spheroid in nature, which requires both of these processes at the same time, as mentioned above. Nevertheless, the wound-healing mechanism would be of great advantage also for other colonial species of volvocine-lineage algae even if they do not have the pheromone-based sexual-induction system. Although the existence of such a wound-healing mechanism in those colonial species has not yet been examined, at least it is apparent that there is a molecular link between the wound-healing and sexual-induction processes of _V. carteri_, and therefore, the evolutionary study which connects these two processes might shed light on the riddle of the sexual-induction mechanism of _V. carteri_.

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REFERENCES

1. Amon, P., E. Haas, and M. Sumper. 1998. The sex-inducing pheromone and wound trigger the same set of genes in the multicellular green alga _Volvox_. Plant Cell 10:781–789.
2. Aono, N., T. Inoue, and H. Shiraishi. 2005. Genes specifically expressed in sexually differentiated female spheroids of _Volvox carteri_. J. Biochem. 138:375–382.
3. Ender, F., A. Hallmann, P. Amon, and M. Sumper. 1999. Response to the sexual pheromone and wounding in the green alga _Volvox_: induction of an extracellular glycoprotein consisting almost exclusively of hydroxyproline. J. Biol. Chem. 274:35023–35028.
4. Ender, F., K. Godl, S. Wenzl, and M. Sumper. 2002. Evidence for autocatalytic cross-linking of hydroxyproline-rich glycoproteins during extracellular matrix assembly in _Volvox_. Plant Cell 14:1147–1160.
5. Fuhrmann, M., W. Oertel, and P. Hegemann. 1999. A synthetic gene coding...
for the green fluorescent protein (GFP) is a versatile reporter in *Chlamydomonas reinhardtii*. Plant J. 19:353–361.

6. Gilles, R., C. Gilles, and L. Jaenicke. 1984. Pheromone-binding and matrix-mediated events in sexual induction of *Volvox carteri*. Z. Naturforsch. 39c: 584–592.

7. Godl, K., A. Hallmann, A. Rappel, and M. Sumper. 1995. Pherophorins: a family of extracellular matrix glycoproteins from *Volvox* structurally related to the sex-inducing pheromone. Plant J. 19:781–787.

8. Godl, K., A. Hallmann, S. Wenzl, and M. Sumper. 1997. Differential targeting of closely related ECM glycoproteins: the pherophorin family from *Volvox*. EMBO J. 16:25–34.

9. Hallmann, A. 2003. Extracellular matrix and sex-inducing pheromone in *Volvox*. Int. Rev. Cytol. 227:131–182.

10. Hallmann, A. 2006. The pherophorins: common, versatile building blocks in the evolution of extracellular matrix architecture in Volvocales. Plant J. 45:292–307.

11. Hallmann, A., P. Amon, K. Godl, M. Heitzer, and M. Sumper. 2001. Transcriptional activation by the sexual pheromone and wounding: a new gene family from *Volvox* encoding modular proteins with (hydroxy)proline-rich and metalloproteinase homology domains. Plant J. 26:583–593.

12. Hallmann, A., K. Godl, S. Wenzl, and M. Sumper. 1998. The highly efficient sex-inducing pheromone system of *Volvox*. Trends Microbiol. 6:185–189.

13. Kirk, D. L. 1998. *Volvox*: molecular-genetic origins of multicellularity and cellular differentiation. Cambridge University Press, Cambridge, United Kingdom.

14. Kirk, D. L., R. Birchem, and N. King. 1986. The extracellular matrix of *Volvox*: a comparative study and proposed system of nomenclature. J. Cell Sci. 80:207–231.

15. Matsuda, Y., T. Shimada, and Y. Sakamoto. 1992. Ammonium ions control gametic differentiation and dedifferentiation in *Chlamydomonas reinhardtii*. Plant Cell Physiol. 33:909–914.

16. Nozaki, H., T. Mori, O. Misumi, S. Matsunaga, and T. Kuroiwa. 2006. Males evolved from the dominant isogametic mating type. Curr. Biol. 16: R1018–R1020.

17. Rodriguez, H., M. A. Haring, and C. F. Beck. 1999. Molecular characterization of two light-induced, gamete-specific genes from Chlamydomonas reinhardtii that encode hydroxyproline-rich proteins. Mol. Gen. Genet. 261:267–274.

18. Schiedlmeier, R., R. Schmitt, W. Müller, M. M. Kirk, H. Gruber, W. Mages, and D. L. Kirk. 1994. Nuclear transformation of *Volvox carteri*. Proc. Natl. Acad. Sci. USA 91:5080–5084.

19. Sumper, M., and A. Hallmann. 1998. Biochemistry of the extracellular matrix of *Volvox*. Int. Rev. Cytol. 180:51–85.

20. Sumper, M., E. Berg, S. Wenzl, and K. Godl. 1993. How a sex pheromone might act at a concentration below 10^{-16} M. EMBO J. 12:831–836.

21. Tschochner, H., F. Lottspeich, and M. Sumper. 1987. The sexual inducer of *Volvox carteri*: purification, chemical characterization and identification of its gene. EMBO J. 6:2203–2207.

22. Wenzl, S., and M. Sumper. 1986. Early event of sexual induction in *Volvox*: chemical modification of the extracellular matrix. Dev. Biol. 115:119–128.