Targeted migration of pherophorin-S indicates extensive extracellular matrix dynamics in *Volvox carteri*

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SUMMARY

Hydroxyproline-rich glycoproteins (HRGPs) constitute a major group of proteins of the extracellular matrix (ECM). The multicellular green alga *Volvox carteri* is a suitable model organism in which to study the evolutionary transition to multicellularity, including the basic principles and characteristics of an ECM. In *Volvox*, the ECM is dominated by a single HRGP family: the pherophorins. Our inventory amounts to 117 pherophorin-related genes in *V. carteri*. We focused on a pherophorin with an unexpected characteristic: pherophorin-S is a soluble, non-cross-linked ECM protein. Using transformants expressing a YFP-tagged pherophorin-S we observed the synthesis and secretion of pherophorin-S by somatic cells *in vivo*, and we then traced the protein during its conspicuous migration to the ECM around prehatching juveniles and its localized concentration there. Our results provide insights into how an ECM zone surrounding the progeny is remotely affected by distantly located parental somatic cells. In view of the properties and migration of pherophorin-S, we conclude that pherophorin-S is likely to act as an ECM plasticizer to allow for dynamic ECM remodeling.

Keywords: extracellular matrix (ECM), *Volvox carteri*, pherophorins, sex-inducer protein, green algae, confocal laser scanning microscopy, multicellularity, *Chlamydomonas reinhardtii*.

INTRODUCTION

One of the most important innovations in the history of life has been the evolutionary transition from unicellular to multicellular species, with the division of labor between germline and somatic cells. To investigate this evolutionary transition, the spheroidal green alga *Volvox carteri* and its close relatives have long been seen as suitable model organisms. These volvocine algae diverged relatively recently from unicellular relatives, and extant species display a range of organizational complexity, from unicellular and colonial genera to multicellular genera with full germ-soma division (Kirk, 1998, 2001, 2003, 2005; Hallmann, 2003; Nozaki, 2003; Schmitt, 2003; Kirk and Kirk, 2004; Michod et al., 2006). During the evolution of multicellularity, a key step was the development of a complex, multifunctional extracellular matrix (ECM) out of the simple cell wall of a unicellular ancestor. In volvocine algae, ECM development is supposed to be one of only five essential steps on the path to differentiated multicellularity (Kirk, 2005). The surprisingly elaborated ECM of *V. carteri* consists of many region-specific and anatomically distinct structures arranged in a defined spatial pattern (Kirk et al., 1986; Hallmann, 2003) (Figure 1). These ECM structures are supposed to be modified under physiological, metabolic or developmental control. The architecture, chemical properties and the building blocks of the *Volvox* ECM, and its potential influence on developmental events, therefore, have been the subject of a number of studies (Sumper and Hallmann, 1998; Hallmann, 2003). The cell walls or ECMs of both unicellular volvocine algae, like *Chlamydomonas*, and multicellular volvocine algae, like *Volvox*, are known to be assembled mainly from hydroxyproline-rich glycoproteins (HRGPs), which not only dominate the ECM composition of green algae but also represent a main constituent of the ECMs of embryophytic land plants (Miller et al., 1972; Sommer-Knudsen et al., 1998; Sumper and Hallmann, 1998; Hallmann, 2003; Showalter and Basu, 2016).

The first ECM glycoprotein of *Volvox* to be identified and characterized was SSG185 (Ertl et al., 1989). This sulfated HRGP was suggested to be one of the main components of CZ3 (Figure 1). A few years later, pherophorins I and II were characterized and roughly assigned to the CZ (Sumper et al., 1993); a special feature of pherophorin II was its synthesis in response to the sex-inducer protein. The identification of further pherophorins, pherophorin III (Godl et al., 1995), pherophorin-DZ1 (Ender et al., 2002),...
Among the numerous pherophorins, fewer than 10 have been characterized in more detail (Sumper and Hallmann, 1998; Hallmann, 2003; Hallmann, 2006). All pherophorins investigated have been glycoproteins, i.e. they contained covalently attached oligosaccharide chains (glycans). The building blocks of these glycans are predominantly the sugars arabinose and galactose, which indicates the O-linked glycosylation of serine, threonine and hydroxyproline. Notably, the amino acid hydroxyproline, produced by the post-translational hydroxylation of proline, is the dominant amino acid of the rod-shaped hydroxyproline-rich domain of pherophorins (Hallmann, 2003; Hallmann, 2006).

Therefore, we pursued to identify pherophorins by 35S-sulfate in vivo pulse-labeling studies (Wenzl and Sumper, 1981; Wenzl and Sumper, 1982). It was also demonstrated that some of the pherophorins contain a quite uncommon phosphodiester between two arabinose residues (Holst et al., 1989; Godl et al., 1997; Ender et al., 2002). For two purified pherophorins, pherophorin-DZ1 and pherophorin-DZ2, polymerization has even been shown in vitro, producing an insoluble fibrous network (Ender et al., 2002). The chemistry behind the polymerization remained unclear, however.

Only one of the characterized pherophorins, pherophorin-S, showed a quite unexpected characteristic for an ECM protein: it seemed to stay soluble without cross-linking with itself or other ECM components (Godl et al., 1997). The ‘S’ of pherophorin-S thus stands for ‘soluble’. What could be the role of a soluble ECM glycoprotein? Actually, all extracellular proteins must be bound to the ECM somehow in order to prevent diffusion into the surrounding pond water. The biochemical analysis of ECM fractions suggested a pherophorin-S localization in the deep zone (DZ), the innermost part of the Volvox spheroid (Figure 1), where it could be trapped by the surrounding ECM components (Godl et al., 1997). Notably, pherophorin-S also contains phosphodiester bridges between arabinose residues (Godl et al., 1997), even if the protein is soluble and does not show any cross-linking.

In this work, first we carried out a sequence database analysis to provide a current inventory of the existing pherophorin-related genes in V. carteri. The large family of pherophorin-related genes identified stresses the requirement of numerous different ECM glycoproteins to build, maintain and modify a complex ECM during the course of ontogenesis.

Following the pherophorin inventory, we focused on a particular pherophorin with a very special characteristic when it concerns an ECM protein: the soluble pherophorin-S. We show that expression of the pherophorin-S gene is enhanced by the sex-inducer protein, and we trace the synthesis, migration and concentration of fluorescence-tagged pherophorin-S in vivo. Moreover, we demonstrate that not only the sex-inducer protein but also
heat, salt and oxidative stress are able to increase pherophorin-S expression, and thus its local accumulation.

Finally, we provide a scenario that shows the action of the soluble ECM glycoprotein pherophorin-S together with lytic ECM enzymes to soften and then to dissolve the ECM. Pherophorin-S seems to be an ECM plasticizer involved in the hatching process and in the course of the intracorporal fertilization of eggs inside sexual females.

RESULTS

Database analysis and inventory of pherophorin-related genes in V. carteri

After sequencing the V. carteri genome, it was believed that there are 49 pherophorin-related genes in V. carteri (Prochnik et al., 2010). A recent search for genes that are computationally annotated as pherophorins in the V. carteri genome (v2.1) of Phytozome 12 (Goodstein et al., 2012), however, resulted in 97 pherophorin genes. Our detailed BLAST searches (Altschul et al., 1990; Johnson et al., 2008; Boratyn et al., 2013) of the V. carteri genome (v2.1) in Phytozome 12 using pherophorins as query sequences then revealed a total of 117 pherophorin genes. We thus found 20 pherophorin-related genes that are not annotated. Of the 117 pherophorin genes in the V. carteri genome, 68 genes are without a gene name and have not yet been described in a publication. Using an earlier nomenclature for pherophorins (Prochnik et al., 2010), we therefore continue the gene designation and call these pherophorin-related genes phV43-pherophorin110. An overview of all pherophorin-related genes of V. carteri is given in Table S1.

Gene and mRNA sequences of pherophorin-S

In 1997, peptides, mRNA fragments and genomic fragments of pherophorin-S from V. carteri were investigated for the first time to obtain the complete amino acid sequence (Godl et al., 1997). The coding sequence and the deduced amino acid sequence of pherophorin-S, but not the genomic sequence, have previously been deposited in the GenBank data library (accession no. Y07752). The gene of pherophorin-S (phS) has not been annotated in the current version 2.1 of the V. carteri genome, for unknown reasons. Nevertheless, we identified the phS gene on scaffold 4: the start codon is at nucleotide position 943321-943323 on the forward strand. Using the RNA-seq data available (Klein et al., 2017) we identified a very short 5’-UTR of 1803 bp (Figure S1). The genomic size is approximately 7 kb, including the UTRs and the promoter region. We also closed a previous sequence gap in exon 3 (Figure S1). The total pre-mRNA of phS is 6649 bp in length and contains seven introns. Once spliced, the mRNA is 3613 bp in length, but only 1800 bp of it represents a coding sequence. The fact that the 3’-UTR of phS covers half of the mRNA could indicate that the 3’-UTR contains regulatory regions that post transcriptionally influence gene expression. The completed gene structure of phS is shown in Figure S2.

Generation of V. carteri transformants with stable genomic integration of a chimeric phS::yfp gene

To visualize and track the expression and target location of pherophorin-S protein in vivo, a chimeric gene was constructed that allows for the expression of a fusion protein in which the C terminus of pherophorin-S is fused via a pentaglycine interpeptide bridge (Gly5) to a yellow fluorescent protein (YFP, mVenus) (Figure 2a). The chimeric phS::yfp gene is driven by the endogenous V. carteri phS promoter region and terminated by the endogenous V. carteri phS terminator region (Figure 2a) to allow for unaltered regulation of phS expression. The yfp coding sequence used has previously been codon-adapted for C. reinhardtii (Lauersen et al., 2015) but also works well in V. carteri (Tian et al., 2018). The nita’ transformants obtained were investigated for stable genomic integration of the co-transformed, chimeric phS::yfp gene by PCR amplification using their genomic DNA as a template (Figure 2b). The 309-bp fragments obtained indicate the presence of the chimeric phS::yfp gene in the corresponding transformants. The correct identity of the amplified DNA fragments was further confirmed by sequencing (Figure 2c).

The sex-inducer protein increases the phS transcript abundance

The glycoprotein pherophorin-S has been identified as a radioactive band of approximately 100 kDa (on an 8% SDS polyacrylamide gel) in a pulse-chase experiment with [35S]-sulfate after treating vegetatively grown Volvox spheroids with the sex-inducer protein for 40 min (Godl et al., 1997). It remained unclear, however, whether the sex-inducer enhances: (i) the transcription of the phS gene, with subsequent translation, protein glycosylation and sulfation; (ii) the translation of stored phS mRNA, with subsequent protein glycosylation and sulfation; (iii) the post-translational glycosylation of existing pherophorin-S protein, with subsequent sulfation; or (iv) the sulfation of hydroxyl groups in the sugar moiety of pre-existing glycosylated pherophorin-S.

To clarify this matter, we first analyzed whether the addition of the sex inducer triggers changes in the phS transcript abundance using a Volvox strain with an unmodified phS gene in its natural genomic environment (recipient strain Tnit-1013). The quantitative real-time reverse transcriptase (RT)-PCR results in Figure 3(a) show that phS transcript abundance quickly increases about 12-fold in less than 1 h, and then the phS transcript abundance decreases very slowly; 14 h after sex induction it is still

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about 7.5-fold higher than under the uninduced condition. The kinetics of the phS transcript abundance after sex induction match well with the kinetics of the [$^{35}$S]sulfate-labelled pherophorin-S band in pulse-chase experiments after sex induction, indicating that the sex-inducer protein effectively enhances the transcription of phS, followed immediately by translation, protein glycosylation and sulfation.

The chimeric phS:yfp gene in our corresponding Volvox transformants also comprises the endogenous phS promoter. Thus, we checked by real-time RT-PCR whether the sex inducer also triggers changes in abundance of the chimeric phS:yfp transcript of transformants. In this experiment, transcript abundance was investigated 14 h after sexual induction and was compared with transcript abundance in cultures grown vegetatively. In fact, in all transformants the phS:yfp gene is sex inducible (Figure 3b). Compared with the uninduced condition, the induction factor varies between 3.5 and 7.5, however. Yet the phS:yfp transcript abundance in the transformant strain PhS-YFP-6-1 showed exactly the same induction behavior as the unmodified phS gene of the recipient strain TNit-1013 (Figure 3b). The results demonstrate that the required regulatory sequences for induction have not been destroyed or cut off inadvertently during construction or genomic integration of the chimeric phS:yfp gene.

Transformants produce fluorescence-tagged pherophorin-S protein
Stable transformants expressing the phS:yfp gene were examined with an LSM780 confocal laser scanning microscope (CLSM) for the presence of fluorescence. Our aim was to distinguish YFP fluorescence unequivocally from autofluorescence. A major pigment contributing to autofluorescence is the chlorophyll in the chloroplast, which occupies a large portion of the cell’s volume. Carotenoid pigments of the eyespot apparatus also show weak autofluorescence, however. Therefore, lambda scans were performed that allow for the separation of spatially overlapping emission signals. More precisely, fluorescence spectra of selected cellular and extracellular regions of sexually induced transformants were measured and compared, both with each other and with the known YFP spectrum (Kremers et al., 2006). The same cellular and extracellular regions were also scanned in the untransformed, sexually induced recipient strain as a control. After excitation at 488 nm, only the fluorescence emitted from regions in the ECM of transformants (see below) peaked at

![Figure 2. Schematic diagram of the transformation vector used and proof of integration of the chimeric gene construct into the recipient genome. (a) Vector pPhS-YFP carries a fragment of Volvox carteri genomic DNA containing the pherophorin-S gene (phS), including its seven introns, a short linker sequence, which codes for five glycines (Gly5), and the coding sequence of yfp (mVenus) coding sequence has been codon-adapted for Chlamydomonas reinhardtii Lauersen et al., 2015), but also works well in V. carteri (Tian et al., 2018b). (b) The stable integration of the transformed pPhS-YFP vector into the genome of transformants was verified by PCR amplification using genomic DNA of putative transformants as a template. The amplified fragment, which is indicated in (a) (PCR), is specific for the pPhS-YFP vector. Amplification of a fragment of an unaltered phS gene is excluded because one primer is located on the yfp coding sequence. The agarose gel electrophoresis shows the result with the untransformed recipient strain and four transformants obtained. The expected size of the amplified fragment was 309 bp. Lane M refers to the molecular weight marker. (c) Obtained sequence of the amplified fragment. The 309-bp PCR fragment contains 72 bp of exon 8 of phS (white background), a 15-bp fragment coding for a flexible pentaglycine interpeptide bridge (Gly5, blue background) and 222 bp of the yfp coding sequence (yellow background). The PCR primers used are indicated. © 2020 The Authors. The Plant Journal published by Society for Experimental Biology and John Wiley & Sons Ltd, The Plant Journal, (2020), 103, 2301–2317]
approximately 530 nm (Figure 4a). Both the position of the peak and the shape of the spectrum are typical for the YFP variant used (Kremers et al., 2006).

PhS:YFP protein synthesis increases up to 14-fold after sex induction

The phS:yfp transformants, which express the chimeric phS:yfp gene under control of the endogenous phS promoter, were analyzed under vegetative (uninduced) and sex-induced conditions for YFP fluorescence by CLSM, and the YFP fluorescence detected was quantified. To this end, fluorescent regions of the ECM (see below) were excited at 514 nm and the fluorescence signal was detected between 520 and 550 nm and quantified using ZEN BLACK (Figure 4b). Even vegetatively grown (uninduced) transformants revealed significant levels of YFP fluorescence. The PhS:YFP protein synthesis strongly increased after sex induction, however, and the induction factors ranged between 4 and 14 (Figure 4b). Fluorescence spectra (lambda scans) were conducted to ensure that the measured fluorescence originated only from YFP (Figure 4a). The increase of PhS:YFP protein synthesis after sexual induction of phS:yfp transformants roughly corresponds to the sex-induced increase of phS transcript abundance in these strains.

In vivo localization of PhS:YFP protein during embryogenesis of sexual development

The pherophorin-S protein was previously identified in an ECM fraction of sexually induced Volvox spheroids (Godl et al., 1997). The ECM fraction has been assigned to the DZ (Figure 1); however, the identification of pherophorin-S in such an extract can only provide a very rough spatial resolution of its distribution in the organism. Moreover, earlier experiments dealt only with a single stage in development (Godl et al., 1997). Therefore, it was our aim to characterize precisely both the spatial and temporal distributions of PhS:YFP in the corresponding transformants by CLSM. We focused on sexually induced algae because protein synthesis is strongly induced under these conditions, suggesting a role of pherophorin-S in sexual development. Moreover, the induction of phS:yfp transcript abundance by the addition of the sex inducer gives us a starting point for immediately following the strong increase in PhS:YFP protein synthesis, which can then be traced by CLSM. In order to distinguish unequivocally YFP fluorescence from autofluorescence in the CLSM, we verified the YFP fluorescence spectrum in the ECM of transformants (Figure 4a) and also investigated the untransformed V. carteri parent strain TNit-1013 for autofluorescence, as a control. As expected, strain TNit-1013 showed only weak chlorophyll autofluorescence in the chloroplast and very weak carotenoid autofluorescence in the eyespot, but no fluorescence in the ECM of any stage of vegetative or sexual development (Figure S3).

We added the sex-inducer protein to vegetatively grown phS:yfp transformants at the developmental stage shortly before the hatching of their juveniles to then follow the PhS:YFP synthesis and its distribution in the developing

Figure 3. Inducibility of the phS promoter by the addition of the sex-inducer protein. (a) The induction kinetics of phS transcript abundance after the addition of the sex-inducer protein was investigated in a Volvox strain with an unmodified phS gene (including its endogenous promoter) in its natural genomic environment (recipient strain TNit-1013). Induction was started at 0 min by the addition of the sex-inducer protein. The data represent three biological replicates (n = 3). Error bars indicate one standard deviation from the mean. (b) The sex inducibility of the phS transcript abundance of strain TNit-1013 was compared with the sex inducibility of the phS:yfp transcript abundance of three independent transformant strains, which integrated the chimeric phS:yfp construct into their genomes. The phS:yfp gene is driven by the endogenous phS promoter. To determine induction factors, transcript abundance was investigated 14 h after sexual induction (sex-induced) and set in relation to the transcript abundance in uninduced algae (vegetative). The sex-inducer protein was added at a concentration of 10⁻¹² M. Transcript abundance was analyzed by quantitative real-time RT-PCR. Oligonucleotide primer pairs were specific either for the unmodified phS gene (recipient strain TNit-1013) or for the chimeric phS:yfp gene (transformant strains). The relative transcript abundance was calculated using the $2^{-\Delta\Delta C_t}$ method (Bustin, 2000; Pfaffl, 2001). The data represent three biological replicates with three technical replicates each. Error bars indicate one standard deviation from the mean. Asterisks indicate the level of statistical significance (Student’s t-test, *P < 0.05, **P < 0.01, ***P < 0.005).
juveniles (Figure 5). Freshly hatched Volvox juveniles already produce fluorescent PhS:YFP protein (Figure 5a). The synthesized PhS:YFP protein appears at the surface of the spheroid in the ECM structures CZ1, CZ2 and CZ3, around the somatic cells, and begins to spread, probably by diffusion, into deeper ECM zones, i.e. into CZ4 (Figure 1). PhS probably cannot pass through the boundary zone (BZ), which seems to prevent PhS from diffusing into the surrounding medium (Figure 5a). Remarkably, the ECM structures CZ1, CZ2 and CZ3 around the immature gonidia (Figure 1) show hardly any fluorescence (Figure 5a). This indicates that the PhS:YFP protein is produced by somatic cells. Later in development, when the gonidia have matured inside the young adults and the first cleavage of embryogenesis lies ahead, the highest concentration of PhS:YFP is still detectable in the ECM structures CZ1, CZ2 and CZ3 of somatic cells, but the spread of PhS:YFP into deeper ECM zones increases continuously (Figure 5b). At this stage, small quantities of PhS:YFP protein also entered the ECM structures CZ2 and CZ3 around the mature gonidia, but not their CZ1. With advancing embryogenesis inside the adults, the PhS:YFP concentration in the cellular zones CZ2 and CZ3 around the dividing embryo increases, and in the 16-cell stage these zones show the strongest PhS:YFP fluorescence overall (Figure 5c). There is still no PhS:YFP fluorescence in the CZ1 around the embryo, however. This indicates that neither PhS:YFP nor pherophorin-S are able to penetrate the CZ1 around embryos, i.e. the glycoprotein vesicle. In this context, it should be noted that dyes (e.g. 4',6-diamidino-2-phenylindole, DAPI) added to Volvox cultures from outside get into the interior of the spheroid, and also into the interior of the somatic cells, but they are not able to penetrate the CZ1 around gonidia or embryos and, thus, do not get into the interior of gonidia or embryos. These observations could imply that the CZ1 structure around gonidia or embryos constitutes a considerable barrier between the gonidium or embryo and its surrounding parent. The fact that the cells of the dividing embryo detach somewhat from the surrounding CZ1 makes it easier to see that the extracellular space between the plasma membranes of the embryonic cells and their surrounding CZ1 does not contain any notable levels of PhS:YFP fluorescence, even though there is a considerable presence of PhS:YFP fluorescence on the outside of the CZ1 (Figure 5c). To illustrate this, we quantified the PhS: YFP fluorescence in this extracellular space under standard conditions and also produced CLSM images with overamplified YFP fluorescence (Figure S4). Moreover, there is also no notable PhS:YFP fluorescence inside the gonidia or embryos (Figures 5c4 and S4). Furthermore, this is in accordance with the RNA-seq data available (Klein et al., 2017) that reveal an approximately 15-fold overexpression of phS mRNA in somatic cells compared with gonidia. Taken together, this shows that gonidia and dividing...
embryos produce no notable quantities of PhS:YFP and that the observed PhS:YFP protein comes from the parent’s somatic cells. During embryogenesis, PhS:YFP fluorescence also spread into the innermost ECM zone, the DZ (Figure 5c). In return, the PhS:YFP fluorescence intensity in the cellular zones CZ2 and CZ3 around the somatic cells decreased. This also indicates that the production of PhS:YFP protein by the parent’s somatic cells has declined at this stage, which corresponds to the induction kinetics of the phS transcript abundance (Figure 3a). Further on in their development the embryos undergo inversion inside their parent, and at the same time more and more PhS:YFP protein enters the ECM zones around the embryos and a shift of PhS:YFP protein from the CZ2 and CZ3 zones to the CZ1 zone emerges. Then, when egg cells within the juveniles, which are still inside their parent, become visible, the PhS:YFP fluorescence intensity at CZ1 gradually increases and CZ1 becomes the place with the highest concentration of PhS:YFP (Figure 5d). In return, the PhS:YFP fluorescence intensity in all other ECM zones gradually decreases, which indicates that PhS:YFP binds with high affinity to an unknown target at CZ1. The PhS:YFP protein eventually seems to stick firmly to the CZ1. There is (still) no detectable PhS:YFP fluorescence in the space between the egg-bearing juveniles and their surrounding CZ1 (Figure 5d), and also not inside the egg-bearing juveniles. All the other ECM zones now contain only very low levels of PhS:YFP.

In vivo localization of PhS:YFP protein during the hatching of egg-bearing juveniles

The hatching of egg-bearing juveniles from their parent is a highly selective enzymatic process. In this process, birth canals for the juveniles must be formed (Kirk, 1998). To this end, lytic enzymes are secreted by the juveniles to specifically degrade the parental ECM (Fukada et al., 2006), and these enzymes start with the disintegration of the CZ1 (Nishimura et al., 2017). In addition, lytic enzymes are also secreted by the parental somatic cells. These enzymes accumulate in the parental ECM around the somatic cells and start to disintegrate it (Nishimura et al., 2017). The birth canals are cooperatively formed by lytic enzymes secreted by the juveniles and those secreted by parental somatic cells (Nishimura et al., 2017).

When the CZ1 is disintegrated by the lytic enzymes of the juveniles, the PhS:YFP protein seems to lose its footing in the ECM and once the juveniles hatch through the birth canals, a haze and streaks of fluorescent PhS:YFP protein leak from the holes. A significant level of PhS:YFP protein also sticks to the edges of the holes created (Figure 6), indicating that PhS:YFP protein might inhibit the autocatalytic repair mechanisms of the ECM (Ender et al., 2002). At the edges of the created holes, PhS:YFP protein seems to mainly stick to the BZ and to the bottom of the somatic cell sheet, which corresponds to CZ3. Thus, two fluorescent rings become visible at each birth canal, one at the outer rim and one at the inner rim of the holes in the somatic cell sheet (Figure 6).

In vivo localization of PhS:YFP protein in matured egg-bearing adults

After hatching, the (female) juveniles develop into adults with matured eggs and the somatic cells of the adults start to produce PhS:YFP protein, which then spreads into deeper ECM zones. The PhS:YFP protein accumulates continuously and becomes more or less evenly distributed across all ECM zones (Figure 7). There might be slightly more PhS:YFP protein in CZ3 than in the other ECM zones. On the whole, all eggs are surrounded by PhS:YFP-containing ECM. At this stage, the egg-bearing adult females are ready for fertilization by sperm. Contrary to the earlier situation around egg-bearing juveniles, in which CZ1 became the place with a strong concentration of PhS:YFP, there is not significantly more PhS:YFP accumulation in the immediate vicinity of the eggs than in other ECM zones.

Overall model of the synthesis, migration and concentration of pherophorin-S

Based on our numerous CLSM images of the different developmental stages of transformants expressing the phS:YFP gene, we developed an overall model of the synthesis, migration and concentration of pherophorin-S (Figure 8). After sexual induction, pherophorin-S is synthesized and excreted by somatic cells (Figure 8a). It then spreads into deeper ECM zones and enters the ECM structures CZ2 and CZ3 around the mature gonidia (Figure 8b). During embryogenesis, pherophorin-S concentration in the ECM zones CZ2 and CZ3 around the dividing embryo increases and pherophorin-S also spreads into the innermost ECM zones of the spheroid (Figure 8c,d). Then a shift of pherophorin-S protein from the CZ2 and CZ3 zones to the CZ1 around the embryos occurs, and CZ1 becomes the place with the highest concentration of pherophorin-S (Figure 8e). When the birth canals for the juveniles are formed, pherophorin-S spreads from CZ1 into the adjacent, pre-digested ECM around the parent’s somatic cells (Figure 8f). Once the juveniles hatch through the birth canals created, a haze and streaks of pherophorin-S protein leak from the holes (Figure 8g). Pherophorin-S also sticks to the edges of the created holes (Figure 8g). After hatching, the (female) juveniles develop into adults with matured eggs and the adults’ somatic cells start to produce pherophorin-S, which then spreads into deeper ECM zones (Figure 8h). Somewhat later, pherophorin-S is more or less evenly distributed across all ECM zones and all eggs are surrounded by pherophorin-S-containing ECM (Figure 8i). In the natural course, matured sperm packets released from sexual adult males

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will then dock onto the egg-bearing female adults, the sperm packets then break up into individual sperm and these sperm penetrate the ECM of the female to fertilize the eggs inside the female to produce diploid zygotes.

**Stress-induced expression of PhS:YFP protein**

In *Volvox*, the switch to the sexual mode of reproduction is not only triggered by the sex inducer but also by increased temperatures, i.e. heat shock (Kirk and Kirk, 1986), which typically is a sign of a pond drying up in late summer. Only because the sexual cycle produces heavily walled, dormant zygotes (zygospores), the species can resist tough conditions like drought, heat and cold for a long period of time. In addition to increased temperatures, oxidative stress seems to trigger sexual development (Nedelcu and Michod, 2003; Nedelcu et al., 2004). These findings suggest

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**Figure 5.** Localization of PhS:YFP in sexually induced *Volvox carteri* females before, during and after embryogenesis. The sex-inducer protein was added to transformants expressing the *phS:yfp* chimeric gene under the control of the wild-type *phS* promoter (strain PhS-YFP-6-1), and then they were analyzed *in vivo* for the localization of the PhS:YFP fusion protein. (a-d1, column 1) Transmission-photomultiplier tube detection (trans-PMT): overview of whole organisms. (a3–d3, column 3) Trans-PMT: detailed view of one gonidium, embryo or juvenile, respectively, with its surrounding structures. (a2–d2, a4–d4, columns 2 and 4) Fluorescence images of the specimen shown in (a1–d1, a3–d3, columns 1 and 3). Overlay of YFP fluorescence of PhS:YFP protein (green) and chlorophyll fluorescence (magenta). (a1–a4) Freshly hatched juvenile with immature gonidia. (b1–b4) Young adult with mature gonidia. (c1–c4) Adult with embryos at the 16-cell stage. (d1–d4) Adult with juveniles after embryonic inversion. The egg cells within the juveniles are already visible. Scale bars: 50 µm.
a connection between a stress response and the induction of sexual development.

In view of the above, we examined whether the phS promoter is not only inducible by the sex inducer but also by stress stimuli. To this end, we exposed the transformants expressing the phS:yfp chimeric gene (strain PhS-YFP-6-1) and 48 h later, adult female algae were analyzed in vivo for the localization of the PhS::YFP fusion protein. (a1) Transmission-photomultiplier tube detection (trans-PMT): birth canal of an adult with a just-hatched juvenile above. The sexually induced juvenile contains egg cells. The hatched juvenile is approximately 520 µm in diameter, whereas the birth canal has a diameter of only approximately 470 µm (90% of the hatched juvenile). (b1) Trans-PMT: lateral view of a birth canal of an adult. (a2, b2) fluorescence images of the specimen shown in (a1, b1). Overlay of YFP fluorescence of PhS::YFP protein (green) and chlorophyll fluorescence (magenta). Scale bars: 100 µm.

**Figure 6.** Localization of PhS::YFP in a sexually induced Volvox carteri female after the hatching of juveniles. The sex-inducer protein was added to transformants expressing the phS:yfp chimeric gene (strain PhS-YFP-6-1) and 48 h later, adult female algae were analyzed in vivo for the localization of the PhS::YFP fusion protein. (a1) Transmission-photomultiplier tube detection (trans-PMT): birth canal of an adult with a just-hatched juvenile above. The sexually induced juvenile contains egg cells. The hatched juvenile is approximately 520 µm in diameter, whereas the birth canal has a diameter of only approximately 470 µm (90% of the hatched juvenile). (b1) Trans-PMT: lateral view of a birth canal of an adult. (a2, b2) fluorescence images of the specimen shown in (a1, b1). Overlay of YFP fluorescence of PhS::YFP protein (green) and chlorophyll fluorescence (magenta). Scale bars: 100 µm.

**DISCUSSION**

In the life of a *V. carteri* alga, ECM deposition begins after the completion of all cell divisions and after inversion, which is the process by which the embryo turns itself right-side-out at the end of embryogenesis. The ECM to be synthesized is dominated by a single HRGP family: the pherophorins (Godl *et al.*, 1995; Godl *et al.*, 1997; Sumper and Hallmann, 1998; Hallmann, 2003; Hallmann, 2006). When ECM deposition starts, the spheroid has a diameter of approximately 60 µm (Kirk, 1998). Then, the juvenile alga not only needs to synthesize and secrete huge amounts of various HRGPs, which enable its massive organismal expansion, but the HRGPs also have to build the known region-specific, anatomically distinct ECM structures, and these structures must be able to expand together with the whole spheroid (Sumper and Hallmann, 1998; Hallmann, 2003; Hallmann, 2006). In an adult *V. carteri* alga at the stage shortly before the release of its matured juveniles, the ECM constitutes about 99% of the volume of the spheroid and its diameter is up to 2 mm, which corresponds to an increase in volume by a factor of more than 30 000 within roughly 24 h. This clearly
demonstrates that the ECM must be a highly dynamic structure that allows for permanent expansion and remodeling. During the course of ontogenesis, ECM components also need to be locally removed to make room for new components. The most conspicuous ECM decomposition happens when large holes need to be formed to allow for the hatching of the matured juveniles. But there is a big challenge: the ECM of the mother spheroid directly above the juvenile needs to be fully removed, whereas the ECM of the hatching juveniles must remain intact and completely unharmed. Therefore, it is not enough to simply produce large quantities of lytic enzymes to achieve this.

Figure 8. Model of the synthesis, migration and concentration of pherophorin-S during the development of sexual Volvox carteri females. Schematic cross section of a sexually developing V. carteri female, emphasizing the localization of pherophorin-S (green) in the given extracellular matrix (ECM) zones and the presumed flows of pherophorin-S (green arrows) during development. (a) Freshly hatched juvenile with immature gonidium. (b) Young adult with mature gonidium. (c) Adult with embryo at the four-cell stage. (d) Adult with embryo at the 16-cell stage. (e) Adult with juvenile after embryonic inversion; the juvenile contains egg cells. (f) Adult with egg-bearing juvenile; lytic enzymes start to digest birth canals into the parent’s ECM. (g) Adult and its freshly hatched egg-bearing juvenile. (h) Adult with matured eggs. (i) Adult with matured eggs; a sperm packet released from a sexual adult male has docked onto the female and some sperm has begun to penetrate the ECM of the female. CZ, cellular zone; DZ, deep zone. The cellular zone has the subzones CZ1, CZ2, CZ3, and CZ4. The deep zone has the subzones DZ1 and DZ2.
similar situation exists when sperm need to penetrate the ECM of the egg-bearing female adults in order to reach the eggs inside the female for fertilization (Hallmann, 2011). The female’s ECM needs to be softened to allow the sperm to swim to the eggs, but the sperm must not be harmed.

Not only do multicellular algae like Volvox face the need for digesting older ECM structures without impairing younger ECM structures, but even unicellular algae are confronted with such a situation. For example, soon after cell division, daughter cells of C. reinhardtii have to degrade their parental ECM (cell wall) to allow for hatching (Harris et al., 2009). The corresponding lytic enzyme in C. reinhardtii is the vegetative lytic enzyme VLE (Matsuda et al., 1995). As mentioned above, there are also 27 pherophorins in C. reinhardtii (Merchant et al., 2007; Prochnik et al., 2010). Among those, pherophorin PhC6 (Hallmann, 2006) exhibits the highest amino acid similarity with Volvox pherophorin-S (Figure S5). Remarkably, RNA-Seq data (Strenkert et al., 2019) show that expression of the \textit{phC6} mRNA peaks shortly before the hatching of the C. reinhardtii daughter cells (Figure S5). This could be a hint that the molecular repertoire for selectively removing older ECM structures (e.g. during hatching) evolved already in unicellular ancestors of \textit{V. carteri} and that already an ancestral lytic process required not only lytic enzymes but also ECM components related to pherophorin-S.

**A scenario for the synergistic action of pherophorin-S and lytic ECM enzymes**

Aside from the HRGP pherophorin-S, two lytic enzymes seem to be of importance for the ECM degradation of the hatching process in \textit{V. carteri}: VheA (Fukada et al., 2006) and Lsg2 (Nishimura et al., 2017) (Figure 10). The protease VheA is secreted by the juveniles (Figure 10a) and is able to disintegrate the CZ1 around the juveniles (Fukada et al., 2006; Nishimura et al., 2017). It should be noted that this ECM zone is misleadingly also called ‘gonidial vesicle’, even though it contains no membrane and, moreover, is not only present at the gonidial stage but also at the subsequent stages, until its disintegration shortly before the hatching of the formed juveniles. As we show in this paper, the HRGP pherophorin-S accumulates at the CZ1 around the juveniles, the ‘gonidial vesicle’, and seems to adhere to this ECM zone (Figures 5d and 10a). The strong concentration of pherophorin-S at the CZ1 (Figure 5d) indicates a very high affinity of pherophorin-S for a component of the CZ1. The second lytic ECM enzyme, Lsg2, is secreted only by the parental somatic cells (Figure 10b) (Nishimura et al., 2017). Once VheA disintegrates the CZ1 around the juveniles (Figure 10b), pherophorin-S becomes liberated (Figure 10c). It is then located in the liquefied space around the juveniles together with VheA and disintegrated ECM components (Figure 10c). While this is happening, Lsg2 diffuses into the ECM that surrounds the somatic cells and pre-digests this parental ECM.

Why seems the action of the two lytic enzymes to be not enough for making holes into the parental ECM under natural conditions? Earlier considerations about the generation of holes for hatching included only the lytic enzymes VheA (Fukada et al., 2006) and Lsg2 (Nishimura et al., 2017), but the corresponding assays for confirmation worked only under non-physiological conditions that included pre-heating of the substrate algae at 55°C, which kills the cells and denatures the proteins, and the addition of very high lytic enzyme concentrations that resulted in an atypical, destroyed-looking phenotype (Nishimura et al., 2017). Under natural \textit{in vivo} conditions, large numbers of new HRGPs are synthesized and secreted by somatic cells at any developmental stage after embryonic inversion (Sumper and Hallmann, 1998; Hallmann, 2003). Thus, the ECM production does not stop before hatching. In addition, autocatalytic cross-linking of at least two HRGPs, the pherophorins D21 and D22, allows for ECM self-assembly and even healing of ECM damage (Ender et al., 2002), a process that counteracts the formation of birth canals for the hatching of the juveniles. A reasonable function of the soluble pherophorin-S could thus be that it hampers ECM

**Figure 9.** Induction of PhS:YFP protein expression after stress stimulation or the addition of the sex inducer. Transformants expressing the \textit{phS}YFP chimeric gene under the control of the wild-type \textit{phS} promoter (strain \textit{PhS-YFP-6-1}) were subject to stress stimulation (oxidative, salt and heat stress) or sexual induction with the sex inducer. Then the expression of the PhS:YFP protein was analyzed \textit{in vivo} by quantification of the YFP fluorescence and the results were compared with the YFP fluorescence obtained from untreated, vegetatively grown algae. Oxidative stress was generated by the addition of 100 \textmu M H2O2, salt stress was produced by adding 75 mM NaCl and heat stress was caused by increasing the temperature to 42.5°C for 100 min and then to 45°C for 20 min. Sex induction was performed as described above. In the confocal laser scanning microscope (CLSM) analysis, regions of interest were excited at 514 nm and the fluorescence signal was detected between 520 nm and 550 nm. Quantification was carried out using \textsc{Zen Black} 2.1. The data represent three biological replicates, with three or four technical replicates each. Error bars indicate one standard deviation from the mean. Asterisks indicate the level of statistical significance (Student’s \textit{t}-test, * \textit{P} < 0.05, ** \textit{P} < 0.01, *** \textit{P} < 0.005).

polymerization as an inhibitor. Parts of pherophorin-S could resemble the corresponding parts of cross-linked pherophorins, but pherophorin-S cannot then be integrated into the HRGP meshwork. In this way, pherophorin-S could act like an ECM plasticizer.

At the parental ECM position, right above the juvenile, the two lytic ECM enzymes and pherophorin-S coincidently take effect on the ECM meshwork. The simultaneous presence of these three ECM components seems to create the weakest area in the parental ECM (Figure 10d). There, the ECM first softens then begins to dissolve, and, after a short time, holes develop (Figure 10e). These birth canals allow for the hatching of the juveniles (Figure 10f).

When sperm need to pass through the ECM of female adults to reach the eggs for fertilization, the female’s ECM also needs to be softened to allow the sperm to penetrate the spheroid. This agrees well with our finding that pherophorin-S is distributed more or less evenly across all ECM zones of egg-bearing females (Figure 7). Thus, at this stage in development, pherophorin-S again seems to act as an ECM plasticizer. At this developmental stage, the simultaneous presence of lytic enzymes has not been investigated but can be expected.

Increased pherophorin-S levels after stress stimuli and sex induction might allow for faster ECM remodeling

As we showed, pherophorin-S protein levels increase by a factor of 3–4 after the application of oxidative stress or salt stress (Figure 9). Moreover, pherophorin-S expression was even found to be approximately 10 times higher in heat-stressed algae compared with untreated algae, which was similar to the induction factor after addition of the sex-inducer protein, which resulted in a 14-fold induction (Figure 9). Our heat-stress conditions were as described earlier (Kirk and Kirk, 1986), and these conditions mean that the treated algae switch from vegetative to sexual development. As expected, the heat-treated PhS:YFP-expressing transformants also produced egg-bearing sexual progeny at a rate of approximately 98%. The heat-induced switch to sexual development requires a remodeling of the ECM (Sumper and Hallmann, 1998), and for this to happen ECM components need to be modified or removed to make room for new ECM components. A similar ECM remodeling seems to be triggered by the addition of the sex-inducer protein (Sumper and Hallmann, 1998; Hallmann, 2003), and it is quite obvious that other external stimuli, like oxidative stress or salt stress, also effect changes of the ECM structures. External (stress) stimuli indicate environmental changes that can require immediate adaptation to reduce a disadvantage, to obtain a competitive advantage or even to protect the life of the alga. Two of the stresses that we tested, i.e. heat stress (Kirk and Kirk, 1986) and oxidative stress (Nedelcu and Michod, 2003; Nedelcu et al., 2004), are also able to induce the switch to sexual development, which is otherwise triggered by the sex inducer. Therefore, the induction of pherophorin-S synthesis by these stresses could also be a secondary effect caused by the induction of the sexual pathway, which also leads to ECM remodeling. The reaction speed of any ECM remodeling could be increased by increasing the synthesis rate of the components required for ECM reconstruction, which include ECM plasticizers (e.g. pherophorin-S), ECM-degrading enzymes (e.g. VheA and Lsg2) and structural building blocks of the ECM (i.e. different HRGPs).

Remarkably, highly dynamic ECM remodeling is not only found in Volvox or other multicellular (green) algae, but also in animals (Lu et al., 2011; Matusiewicz, 2011; Sonbol, 2018). As in Volvox, animal ECMs are modified and remodeled throughout development, and most conditions of stress induce intense ECM remodeling (Jean et al., 2011). When the ECM dynamics of animal tissues become deregulated or disturbed, it leads to various defects and diseases, including cancer (Lu et al., 2011; Matusiewicz, 2011; Chakravarthy et al., 2018; Sonbol, 2018). The further investigation and comparison of algae and animal ECM characteristics could help to shed more light on the fundamental biochemical requirements for dynamic ECM remodeling and its evolutionary development.

CONCLUSION

Our results provide insights into how an ECM zone that surrounds the developing progeny is remotely affected by the distantly located parental somatic cells. Based on the characteristics of the pherophorin-S glycoprotein, its synthesis by somatic cells, its conspicuous migration and its localized concentration in the ECM around prehatching juveniles, we conclude that pherophorin-S is likely to act as an ECM plasticizer. Pherophorin-S probably acts together with known lytic ECM enzymes to soften and then to dissolve the ECM to allow for the hatching of juveniles. Softening of the ECM by pherophorin-S could also help sperm in the course of the intracorporal fertilization of eggs inside sexual females. The observed increase of pherophorin-S levels after sex induction and stress stimuli might allow for an accelerated ECM remodeling, which includes the quick removal of existing ECM components to make room for newly synthesized ECM components. The more dynamic the ECM remodeling is, the more adaptable the organism is when environmental conditions change.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

The wild-type Volvox carteri f. nagariensis strain Eve10 (female), which originates from Japan, has been described previously (Starr, 1969; Starr, 1970; Kianianmomeni et al., 2008). A non-revertible nitrate reductase-deficient (nitA-) descendant of Eve10,
strain TNit-1013 (Tian et al., 2018), was used as a recipient strain for transformation experiments. The recipient strain was grown in standard Volvox medium (Provasoli and Pintner, 1959), supplemented with 1 mM ammonium chloride (NH₄Cl) as a nitrogen source. Transformants with a complemented nitrate reductase gene were grown in standard Volvox medium without ammonium chloride. Cultures were grown at 28°C in a cycle of 8 h dark/16 h cool fluorescent white light (Starr and Jaenicke, 1974) at an average of approximately 100 μmol photons m⁻² s⁻¹ photosynthetically active radiation (PAR) in glass tubes or Fernbach flasks. The glass tubes had caps that allow for gas exchange. Fernbach flasks were aerated with approximately 50 cm³ sterile air per min.

Vector construction

Standard cloning methods (Sambrook and Russell, 2001) and overlap extension PCR (Higuchi et al., 1988) were used for the construction of the expression vector, plasmid pHs-YFP (Figure 2), carrying the chimeric phs:yfp gene. Plasmid pHs-YFP contains a 5.7-kb fragment of V. carteri genomic DNA harboring the phs gene under the control of its own promoter (Figure S6). The fragment begins 0.9 kb upstream of the phs start codon and ends right in front of the phs stop codon. It is flanked by an artificial XbaI site and an artificial XhoI site (Figures 2 and S6). Our sequencing results of the genomic phs sequence (Figure S1) coincide with the corresponding phs sequence of the published V. carteri genome (v2.1, scaffold 4, nucleotides 942447–950704) (Prochnik et al., 2010) in Phytozome 12.1.6 (Goodstein et al., 2012); however, the phs gene in genome version 2.1 contained a 141-bp sequence gap, which we closed (Figure S1). For unknown reasons, in genome version 2.1 no gene model has been created for the phs gene on scaffold 4 (nucleotides 942447–950704).

In order to facilitate the fusion of the genomic phs fragment with yfp (mVenus), artificial Xhol and HindIII sites were added on both sides of the intronless yfp reporter gene (Kremers et al., 2006; Lauersen et al., 2015) and, simultaneously, a short linker sequence that codes for a flexible pentaglycine interpeptide bridge was inserted in front of the yfp gene (Figures 2 and S6). The 0.7-kb yfp sequence was previously codon-adapted to C. reinhardtii (Fukada et al., 2006; Nishimura et al., 2017).

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complete phS 3′-UTR (1.8 kb) and 0.8 kb of downstream sequence (Figures 2 and S6).

The chimeric phSyfp gene was assembled within the pBlue-script II SK(+) (Stratagene, now Sigma-Aldrich, https://www.sigma-aldrich.com) vector backbone.

Stable nuclear transformation of *V. carteri* by particle bombardment

Stable nuclear transformation of *V. carteri* strain TNit-1013 was performed as described previously (Scheidielmeier et al., 1994), but with some modifications and using a Biologic PDS-1000/He i (Bio-Rad, https://www.bio-rad.com) particle gun (Hallmann and Wodniok, 2006). The target algae were co-bombarded with the selectable plasmid pVcNR15 (Gruber et al., 1996), carrying the *V. carteri* nitrate reductase gene, and the non-selectable plasmid pHs-YFP. Plasmid pVcNR15 is able to complement the nitrate reductase deficiency of the recipient strain. For the selection of transformants, the nitrogen source of the Volvox medium was switched from ammonium to nitrate and the algae were then incubated under standard conditions in Petri dishes (9 cm in diameter) filled with a volume of approximately 35 ml of liquid medium. After incubation for at least 6 days, the Petri dishes were inspected for green and living transformants.

Isolation of genomic DNA

Genomic DNA was isolated from Volvox algae as previously described (Edwards et al., 1991), with minor modifications. The purity of DNA was determined by measuring the 260/280 and 260/230 ratios using a Nanodrop 1000 (ThermoFisher Scientific, https://www.thermofisher.com) UV-Vis spectrophotometer for the 260/280 ratio a value of approximately 1.8 indicated pure DNA and for the 260/230 ratio it was a value of 2.0–2.2. The DNA was quantified by agarose gel electrophoresis at 260 nm. The quality of DNA was further verified by agarose gel electrophoresis.

Genomic PCR

In order to verify the stable integration of the chimeric phSyfp gene into the genome of *V. carteri* transformants, PCR reactions with genomic DNA as a template were carried out as previously described (Lauersen et al., 2015). For reverse transcription and amplification of a fragment of the wild-type phS transcript, total RNA of the untransformed recipient strain was used as a template and the oligonucleotide primers used were 5′-TCGACTTGTTGCTGGGCTTC-3′ and 5′-CTTTAGAACTTACAGCAAGGC-3′. The amplified fragment was expected to be 147 bp in length containing 31 bp of phS exon 7, 67 bp of phS exon 8, 6 bp of an artificial HindIII restriction site, a 15-bp fragment coding for a flexible pentaglycine interpeptide bridge (Gly5) and 28 bp of the yfp (mVenus) coding sequence, which was codon adapted for C. reinhardtii previously (Lauersen et al., 2015). For reverse transcription and amplification of a fragment of the wild-type phS transcript, total RNA of the untransformed recipient strain was used as a template and the oligonucleotide primers used were 5′-TCGACTTGTTGCTGGGCTTC-3′ and 5′-ATCTTAAGCTTACAGCAAGGC-3′. The amplified fragment was expected to be 110 bp in length containing 31 bp of phS exon 7 and 79 bp of phS exon 8. For reverse transcription and the amplification of a fragment of the reference transcript (eef1, eukaryotic translation elongation factor 1α2 gene), the total RNA of transformants or the untransformed recipient strain was used, respectively. The oligonucleotide primers used were 5′-GAGCTTAGCATGCACAGGACACTAAGC-3′ and 5′-ATGCAAGGACACSCACAG-3′ and the amplified fragment was expected to be 132 bp in length. Previously, the eef1 gene has been established as a suitable reference gene for quantitative real-time RT-PCRs in *V. carteri*, particularly when vegetative and sex-induced algae are compared with each other (Kianianmomeni and Hallmann, 2013). The cycling conditions in the CFX96 Touch™ Real-Time PCR Detection System were as follows: reverse transcription was at 55°C for 2 min, followed by polymerase activation at 95°C for 20 min, and 40 cycles of DNA amplification with 95°C for 2 sec, 55°C for 2 sec and 72°C for 2 sec. Melting curves were recorded to check for the amplification of a single specific product. The relative transcript abundance was calculated using the 2^{-ΔΔCt} method (Bustin, 2000; Pfaffl, 2001).

Confocal laser scanning microscopy

For life cell imaging, algae were grown under standard conditions and examined using an inverted LSM780 confocal laser scanning microscope (Carl Zeiss GmbH) equipped with 63× LCI Plan-Neofluar and 10× Plan-Apochromat objectives (Carl Zeiss GmbH, https://www.zeiss.com). The confocal pinhole diameter of the microscope was set to 1 Airy unit, which corresponds to an optical section of 0.8 μm. The fluorescence of the YFP within the PhS:YFP fusion protein was excited by an argon-ion (Ar+) laser at 514 nm, we used an MBS 458/514 main beam splitter and the fluorescence was detected between 520 and 550 nm. The fluorescence of
chlorophyll was detected at 650–700 nm. Fluorescence intensity was recorded in bidirectional scan mode for YFP and chlorophyll in two channels simultaneously. Transmission images were obtained in a third channel using a transmission-photomultiplier tube detector (trans-PMT). Images were captured with a bit depth of 12 bits per pixel (4096 gray levels) and analyzed using ZEN BLACK 2.1 digital imaging software (ZEN 2011; Carl Zeiss GmbH). Image processing and analysis was performed with FIJI (IMAGEMJ).

For the quantification of the sex-induced expression of the PhS::YFP fusion, vegetatively grown cultures of transformants were treated with 100 µM sex-inducer protein at a concentration of 10–2 M. For quantification of the PhS::YFP expression after stress stimulation, transformants were instead treated with 100 µM H2O2 (oxidative stress), 75 mM NaCl (salt stress) or they were incubated at 42.5 °C for 100 min followed by 45°C for 20 min (heat stress). After 14 h of further incubation under standard conditions, the algae were concentrated on a low spheroids per ml, and at the stage shortly after the hatch of juveniles, were treated by the addition of the sex-inducer protein at a concentration of 10–12 M. For quantification of the PhS::YFP expression after stress stimulation, transformants were instead treated with 100 µM H2O2 (oxidative stress), 75 mM NaCl (salt stress) or they were incubated at 42.5 °C for 100 min followed by 45°C for 20 min (heat stress). After 14 h of further incubation under standard conditions, the algae were concentrated on a 100-µm mesh nylon screen and analyzed by confocal laser scanning microscopy, as described above. All images were taken with the same set-up and with the same settings. To facilitate the comparability of the images, the focal plane was always adjusted to the center plane of the spherical algae, which was achieved by searching in z-direction for somatic cells at the furthest distance from the center of the spheroid. Regions of interest with 4000 pixels were manually selected, while taking care that the fluorescence signal was uniform and evenly distributed within the given area. Quantification of PhS::YFP protein expression was carried out using ZEN BLACK 2.1. Three biological replicates with three or four technical replicates each were performed for every experimental condition.

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AUTHOR CONTRIBUTIONS
BH conducted the experimental and analytical work and wrote the first draft of the article. AH (corresponding author) conceived and coordinated the study, critically evaluated the data, and finalized the article. Both authors read and approved the final version for publication.

CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this article and its supplementary information files. Plasmids are available upon request.

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SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article.

Figure S1. Genomic sequence of the Volvox carteri phs gene.
Figure S2. Schematic structure of the phs gene, phs mRNA and pherophorin-S protein.
Figure S3. Absence of autofluorescence from the ECM of the untransformed Volvox carteri strain Tn1013.
Figure S4. Absence of Phs::YFP fluorescence from the extracellular space between the plasma membranes of the embryonic cells and their surrounding CZ1.
Figure S5. Pherophorin-S of Volvox carteri is similar to the hatch-specific pherophorin PhC6 expressed in Chlamydomonas reinhardtii.
Figure S6. Sequence of the chimeric phsYfp gene in vector pHs-YFP.
Table S1. Overview of pherophorin-related genes in Volvox carteri.

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