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A conserved RWP-RK transcription factor VSR1 controls gametic differentiation in volvocine algae

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Abstract

Volvocine green algae are a model for understanding the evolution of mating types and sexes. They are facultatively sexual, with gametic differentiation occurring in response to nitrogen starvation (-N) in most genera, and to sex inducer hormone (SI) in *Volvox*. The conserved RWP-RK family transcription factor (TF) MID is encoded by the *minus* mating type (*MT*) locus or male sex-determining region (SDR) of heterothallic volvocine species and dominantly determines *minus* or male gametic differentiation. However, the factor(s) responsible for establishing the default *plus* or female differentiation programs have remained elusive. We performed a phylo-transcriptomic screen for autosomal RWP-RK TFs induced during gametogenesis in unicellular isogamous *Chlamydomonas reinhardtii* (Chlamydomonas) and in multicellular oogamous *Volvox carteri* (Volvox), and identified a single conserved ortho-group we named Volvocine Sex Regulator (VSR1). Chlamydomonas *vsr1* mutants of either mating type failed to mate and could not induce expression of key mating-type-specific genes. Similarly, Volvox *vsr1* mutants in either sex could initiate sexual embryogenesis, but the presumptive eggs or androgonidia (sperm packet precursors) were infertile and unable to express key sex-specific genes. Yeast two-hybrid assays identified a conserved domain in VSR1 that interacts with itself or with the conserved N terminal domain of MID. *In vivo* co-immunoprecipitation experiments demonstrated association of VSR1 and MID in both Chlamydomonas and Volvox. These data support a new model for volvocine sexual differentiation where VSR1 homodimers activate expression of *plus*/female gamete-specific-genes, but when MID is present MID-VSR1 heterodimers are preferentially formed and activate *minus*/male gamete-specific-genes.

Significance Statement

Sex and recombination are conserved features of eukaryotic life cycles, but sex determination mechanisms are diverse, and are poorly understood in most major taxa. Our study identified a long-sought regulator of sexual differentiation in volvocine green algae—the RWP-RK family transcription factor (TF) VSR1— leading to the first complete paradigm for mating type or sex determination in this lineage. VSR1 is expressed in gametes where it homodimerizes and activates *plus*/female specific genes. When the dominant sex-linked *minus*/male RWP-RK family TF MID is present it preferentially heterodimerizes with VSR1 to activate *minus*/male genes. The widespread association of RWP-RK TFs with gamete differentiation and sex determining regions in the green lineage suggests that a similar paradigm may operate throughout the plant kingdom.

Main Text

Introduction

Sexual reproduction is a ubiquitous part of eukaryotic life cycles. A key feature of eukaryotic sex is the specification of gamete types which can help ensure that two non-related individual gametes fuse during fertilization and enable the transition to zygotic differentiation (1). In isogamous species, which include many single celled protistan lineages, gametes are defined by mating types whose numbers can range from two in most taxa to many thousands in some fungi (2). Many multicellular taxa are anisogamous or oogamous with two sexes that are defined as male and female based on size and motility of their gametes, with males producing many small motile gametes and females producing fewer large and sometimes immotile gametes. An astonishing diversity of mechanisms have evolved for specifying mating types and sexually dimorphic gametes, including genetic mechanisms governed by mating-type loci or sex chromosomes, and epigenetic mechanisms where a single individual can produce gametes of either mating type or of either sex. Genetic programs that specify gamete differentiation have been well studied in animals and fungi, but much less is known about the logic of gamete specification in other taxonomic groups. Green algae and land plants share common ancestry, but knowledge of mechanisms for gamete differentiation and sex determination in this group is incomplete, especially in green algae where molecular information is only known for a few species (3).

Green algae in the volvocine lineage have mating type and sex determination systems that are some of the best understood among algae and other protists. Volvocine algae include *Chlamydomonas reinhardtii* (Chlamydomonas), a bi-ciliate unicellular species with two mating types, and *Volvox carteri* (Volvox), a multicellular species with dimorphic sexes and UV sex chromosomes (4–6). Like most green algae, volvocines are haplontic with mating type or sex determined in the haploid phase by mating type or sex determining loci (Figure 1). Sex in volvocine algae is facultative and sexual reproduction that is triggered by absence of nitrogen (-N) in Chlamydomonas and most other volvocine genera, or by a sex inducer hormone (SI) in Volvox. Upon fertilization a diploid zygote is formed which immediately differentiates into a dormant spore. When germinated the spore undergoes meiosis to produce new haploid progeny.

First discovered in the mating type *minus* (*MT-*) locus of Chlamydomonas, the *MID* (*MINUS DOMINANCE*) gene encodes a small RWP-RK family transcription factor (TF) and is responsible for *minus* gamete differentiation: introduction of a *MID* gene into a *MT+* strain, or its presence in a heterozygous diploid dominantly cause *minus* differentiation (7–9). Conversely, in *mid* mutants a default program of *plus* gamete differentiation occurs. *MID* is conserved across volvocine species and is found in the *MT-* locus or male SDR of all heterothallic species (6). In Volvox *MID* was found to be a dominant determinant of spermatogenesis, with oogenesis occurring in its absence, though other aspects of dimorphic sexual development were not controlled by MID (10). Although direct targets of MID activation or repression are not established, they likely include gamete type specific genes including minus genes *GSM1* and *SAD1* which encode a homeodomain TF required for zygote specification and the *minus* sexual agglutinin respectively, and *plus* genes *GSP1* and *SAG1* which encode a homeodomain TF partner for GSM1 and the *plus* sexual agglutinin binding partner for SAD1 (11, 12).

Although presence/absence of *MID* is the key determinant of *minus* or male gamete differentiation in volvocine algae, it has remained unclear how the default or ground state *plus* or female differentiation program is specified in its absence. Although extensive mutagenesis screens have been done in Chlamydomonas, no mutants that specifically interfere with plus differentiation have been identified (13, 14). Moreover, mutants that impact gametogenesis of both mating types are difficult to investigate because they are sterile (15), so to date no factors

that impact plus or female gamete differentiation have been positively identified in volvocine algae and this important aspect of their sexual life cycle has remained missing.

Here we used a phylogenomically informed search to identify a key missing gamete differentiation factor in volvocine algae, a conserved RWP-RK TF we designated as VSR1 (volvocine sex regulator). VSR1 was induced in both mating types of Chlamydomonas in response to -N, while in Volvox its expression was induced by SI in both sexes. *vsr1* mutants were validated or created for Chlamydomonas and Volvox where they caused sterile phenotypes in both mating types and sexes and failed to express key gamete differentiation genes. Yeast two-hybrid experiments identified a region of VSR1 that could mediate both self-interaction and interaction with the N-terminal domain of cognate MID proteins. Interactions between MID and VSR1 in Chlamydomonas and in Volvox were further validated *in vivo* using co-immunoprecipitation. Our data support a model where VSR1, when expressed alone self-dimerizes and activates *plus* or female differentiation genes, and that when co-expressed with MID preferentially forms VSR1-MID heterodimers which can activate *minus* or male gamete differentiation genes. These data provide a new paradigm for mating type or sex determination by RWP-RK TFs and have implications for the evolution of gamete differentiation that is likely controlled by RWP-RK TFs in other green lineage species.

Results

Phylogenomics search for conserved volvocine gametogenesis regulators. We reasoned that the default gametic differentiation state of volvocine algae that is adopted when gametogenesis cues are present (-N for Chlamydomonas and +SI for Volvox) but when Mid is absent but could be controlled by an autosomal transcription factor (TF) that is expressed in both mating types or sexes. The activity of this hypothetical TF would need to be modified or inhibited by Mid in *minus* or male gametes since Mid is dominant (8, 10). We further guessed that this TF might be in the RWP-RK family. In Chlamydomonas extensive transcriptome responses to -N treatment and gametogenesis have been documented, including the induction of several RWP-RK TFs, but most of the transcriptome changes in -N are related to starvation and growth arrest and not to gametogenesis or sex (11, 12, 16, 17). Having an orthogonal set of data for Volvox where gametogenesis occurs in nutrient replete conditions provided a potential phylogenetic filter to identify conserved gametogenesis genes. Using time series data from V. carteri (Hamaji et al, in preparation) and previously published data on *plus* and *minus* gamete gene expression from *C. reinhardtii* (11, 12) we searched for related RWP-RK TFs that were upregulated in both species by their respective cues for gametogenesis. A single candidate sub-family of autosomal RWP-RK TFs fit this pattern (Figure 1). This subfamily had a single member in Volvox, *VcRWP2* (Vocar.0014s0106), and two closely related paralogs in Chlamydomonas, *CrRWP4* (Cre03.g149350) and *CrRWP11* (Cre03.g149400) that are adjacent to each other on Chromosome 3. All three of these genes showed increased expression in response to the respective gametogenesis cues in each species. We subsequently identified single *RWP2* candidate orthologs in other multicellular volvocine genera (a lineage comprising Tetrabaenaceae, Goniaceae, and Volvocaceae or namely TGV clade), and the same tandem duplication in two other *Chlamydomonas* species that are closely related to *C. reinhardtii*, *C. incerta* and *C. schloesseri*, but only a single ortholog in *Edaphochlamys debaryana* (18). These findings indicate a likely origin of the duplication within Metaclade-C of the volvocine algae but not in the TGV clade that contains all the multicellular volvocine genera (Figure S 1). The paralogous duplicates in the Metaclade-C *Chlamydomonas* species are highly similar in their predicted C-terminal RWP-RK domains, and share different degrees of similarity in their long N terminal domains (Figure S 1).

***Chlamydomonas rwp11/vsr1* mutants are mating defective.** *Chlamydomonas rwp4* and *rwp11* mutants were obtained from the CLiP collection of insertional strains which are in a *MT-* strain background (19) (Figure 2A, Figure S 2A). The *rwp4 MT*-strain was confirmed by genotyping and

found to mate and produce viable offspring. Both *MT+ rwp4* and *MT- rwp4* progeny mated successfully with strains of opposite mating type, and this mutant was not pursued further (Table S 1). The *MT- rwp11* mutant strain could not mate, and was blocked prior to agglutination with wild-type *MT+* gametes. It was also unable to agglutinate or mate with wild-type *MT-* gametes meaning that it had not adopted a pseudo-*plus* mating program as is observed in *mid* mutants (8). In order to pursue the *rwp11* mutant further a rescuing construct containing a N-terminal hemagglutinin tagged version of the *RWP11* gene driven by a strong promoter/terminator from the *RPL23* gene (20) (pHA-CrVSR1; Figure S 3A was introduced into the *rwp11* mutant strain by transformation. Multiple independent transformants were recovered that expressed the tagged transgene constitutively (Figure S 3B) and these strains had restored ability to mate with an isogenic *MT+* parental strain CC-5125 (Figure 2C). Segregation behavior of the *rwp11* mutation and rescuing construct were as expected, enabling the characterization of the *rwp11* phenotype in both mating types (Table S 2). *rwp11* mutant progeny of both mating types were sterile, but those which also inherited the rescuing transgene were mating proficient (Table S 3, Figure S 3 and Figure S 4). During the process of outcrossing and genotyping *rwp11*, a second insertion cassette and paromomycin resistance marker was discovered in the original *rwp11* CLiP strain in an unlinked gene, *SYP1* (Cre13.g588550), encoding a predicted vesicle trafficking protein. The presence/absence of the *syp1* mutation did not affect the mating phenotypes of any of the progeny strains that were tested (Table S 3) meaning that this second mutation is unrelated to the mating defect of *rwp11*. Progeny in which the *syp1* mutation was segregated away from the *rwp11* mutation were chosen for further study. We renamed the *RWP11* gene *VSR1* (volvocine sex regulator 1).

Volvox *vsr1* mutants are defective for gamete differentiation. In order to test whether the *Volvox carteri* VSR1 homolog encoded by VcVSR1 (originally *RWP2*, Vocar.0014s0106) was involved in sexual differentiation or mating we used a gene editing method based on CRISPR-Cas9 to make edited alleles (21). We made multiple independent edits in either the fourth or seventh exons of *VSR1* in a male strain (Figure 3, Figure S 5). All VcVSR1 frame-shift male strains tested had normal vegetative phenotypes that were indistinguishable from wild type (Figure 3C,G). In response to SI, *vsr1* mutants underwent a typical male early embryogenesis program to produce spheroids with 128 small somatic cells and 128 large cells that resembled uncleaved androgonidia (Figure 3D,H). However, unlike wild-type males, the androgonidial-like cells in *vsr1* mutants did not cleave into sperm packets, but instead underwent a vegetative embryogenesis program and reentered the vegetative life cycle. This novel phenotype is different from that seen in sexually induced males with *mid* knockdowns where the post-embryogenesis large cells trans-differentiated into eggs (10).

Using a representative allele, *vsr1*$^{5.1}$, a rescue experiment was performed by transforming the mutant with construct pRSF-VcVSR1 containing the *VSR1* gene and its native promoter with a terminator sequence from the *NitA* gene. Tandem StrepII and FLAG epitope tags were inserted in the N-terminus of *VSR1* in pRSF-VcVSR1 to facilitate detection of the VSR1 protein (Figure 3K). Several of the transformants produced with this construct showed a rescued phenotype where sperm packet development occurred normally after sexual induction. These strains also produced detectable tagged VSR1 protein of the expected size on immunoblots in sexually induced spheroids (Figure 3). These results indicated that defects in *VcVSR1* were causative for the male gametogenesis defect that was observed in the *vsr1* mutant strain. Immunofluorescent staining of the sperm cells from the rescued Vcvsr1 male strain with anti-StrepII antibody showed that tagged transgenic VcVSR1 localized to nuclei as expected for a predicted transcription factor (Figure S 6).

The rescued Vcvsr1 male strain was then used in a cross to a wild-type female strain (Figure 3, Figure S 7). Normal segregation patterns were observed in progeny, some of which were Vcvsr1 mutant females (Table S 4, Figure S 8). Like the case in males, all female Vcvsr1 segregants without a segregating rescuing construct had normal vegetative phase phenotypes, but were sterile. Upon sexual induction they underwent a normal sexual early embryogenesis program to make spheroids that had around 2000 somatic cells and 32-48 large egg-like cells. However, the

egg-like cells in the female *vsr1* mutants could not be fertilized when incubated with mature sperm from a wild-type male, despite the sperm being able to enter the females through a fertilization pore and adhere to the egg-like cells (Figure 3H). Eventually the egg-like cells underwent vegetative embryogenesis and reentered the vegetative life cycle, though with different timing than unfertilized wild-type eggs which experience a delay before the-differentiating (Figure 3F,G). We also noted that the vegetative embryos produced by the egg-like cells of Vcvsr1 mutants frequently failed to invert, though this phenotype disappeared in all subsequent vegetative generations (Figure 3G). This one-time inversion phenotype may be related to the size or expansion capacity of the vesicle surrounding the egg-like cells which is known to influence inversion (22) As expected, male Vcvsr1 mutants behaved as they did in the parental mutant strain, and all female spheroids that either received a rescuing construct or that inherited the wild-type VSR1 allele underwent normal female sexual development (Figure S 8).

***VSR1* is required to express mating type/sex specific genes.** Chlamydomonas gametes express several mating-related autosomal genes that are specific to either *plus* (e.g. *SAG1*, *GSP1*) or *minus* (e.g. *SAD1*, *GSM1*) gametes (11, 12, 23). Quantitative reverse transcription and PCR (qRT-PCR) was used to determine whether these marker genes were expressed normally in *vsr1* gametes. These experiments revealed that *vsr1* mutants, but not parental or rescued strains, were defective for expressing mating-type specific genes (Figure 4A, B). We also noted that the pHA-VSR1 rescued strains, which express *VSR1* constitutively, did not show ectopic expression of mating-related genes in either mating type, a result consistent with rescued strains of either mating type not being able to mate in the presence of nitrogen (data not shown). Together our data show that VSR1 in Chlamydomonas is necessary for gametic differentiation and mating of both *plus* and *minus* mating types, and the sterile phenotype of *vsr1* mutants is wholly or partly due to defects in activating mating-type specific gene expression. *VSR1* is the first gene identified in Chlamydomonas that is necessary for differentiation of both gamete types.
Using life cycle stage specific transcriptome data from wild type *V. carteri* (manuscript in preparation) we identified candidate sexual phase genes with different expression patterns. Volvox has single orthologs of the *GSP1* and *GSM1* homeodomain protein coding genes that were designated *VcGSP1* and *VcGSM1* (Figure 4C, D). Using qRT-PCR we determined that *VcGSP1*was expressed only in female sexual spheroids and *VcGSM1* only in male sexual spheroids, indicating that members of this heterodimeric pair retained the same expression polarity as in Chlamydomonas with respect to *MT* or SDR haplotype (Figure 4C, D). In summary Vsr1 is a conserved volvocine putative RWP-RK family transcription factor that is essential for gametic differentiation and controls both mating-type-specific or sex-specific autosomal gene expression. Moreover, in Volvox VcVsr1 also appears required for expression of a subset of genes that are normally expressed in both sexes.

VSR1 proteins interact with themselves and with MID proteins via a novel protein interaction domain. A simple model that could explain bi-stable and mutually exclusive gamete differentiation programs involves Vsr1 forming homodimers (or other homomeric forms) in the absence of Mid to activate expression of *plus* or female gamete differentiation genes. When Mid is coexpressed with Vsr1, Mid-Vsr1 heterodimers (or higher order heteromeric forms) with *minus*/male promoter specificity would outcompete Vsr1 homodimers to activate *minus* or male gamete differentiation genes. This model predicts that Mid and Vsr1 associate with each other, and that Vsr1 can self-associate.
We tested Vsr1 and Mid associations with themselves and each other using a yeast two-hybrid (Y2H) assay. Full length Mid produced a negative result when co-expressed as bait and prey, so lacks the ability to homodimerize in this assay (Figures 5A-D, Figure S 9). In the case of CrVsr1 or VcVsr1, full length bait constructs showed auto-activation, so could not be used to test self-association. Using CrMID as the bait construct, a positive interaction was detected with CrVSR1 as prey. Truncations of CrVSR1 prey constructs showed that a 261 aa region in its N-terminus (CrVSR1$^{431-691}$) was sufficient to interact with CrMID. We termed this region of VSR1 as its dimerization domain (DD). The DD sequences of Vsr1 are conserved across volvocine algae but

are not a previously defined domain detectable using automated NCBI BLAST searching. Using Volvox constructs we found Bait-VcMID and Prey-VcVSR1 or Prey-VcVSR1$^{485-694}$ (DD-equivalent, 210 AA) exhibited interaction, confirming that this domain and its interaction with a cognate Mid protein is functionally conserved (Figure 5B). MID proteins are small, with a C terminal RWP-RK domain and a conserved N terminal region. Using Chlamydomonas constructs with just the MID N domain or MID C domain as bait, VSR1 was found to interact only with the N domain and this interaction may explain why this part of MID is conserved (Figure 5D).

Because VSR1 autoactivates when used as bait in Y2H assays, the full-length protein could not be used as a bait. We therefore tested the VSR1-DD region (CrVSR1$^{431-691}$) as bait since this fragment did not autoactivate. When co-expressed with full length VCrVSR1 as prey, a positive interaction was detected indicating that VSR1 can self-interact through its DD. When just the CrVSR1 DD was expressed in both bait and prey constructs the interaction was also detected, and a similar result was found for the DD of VcVSR1. These data support the idea that VSR1 can homo-dimerize through its DD, and this same domain can interact with the MID N-terminal domain.

To determine if the MID-VSR1 interaction occurs *in vivo* we used a co-immunoprecipitation (Co-IP) assay from algal lysates. We constructed strains of Chlamydomonas or Volvox expressing epitope tagged *MID* and *VSR1* transgenes, (Figure 5E, F; Figure S 10). For Chlamydomonas we prepared whole cell lysates from gametes of a double transgenic strain *vsr1-1::HA-CrVSR1 MID-FLAG* or a strain with only the *MID-FLAG* transgene as a control. IPs were performed using anti-HA antibodies to capture VSR1 and immunoblots were probed with anti-FLAG antibodies to detect MID. FLAG signal was detected only in IPs from the strain co-expressing the HA-VSR1 transgene and MID-FLAG, but not the control strain indicating that these two proteins likely interact *in vivo*. Similar results were obtained for Volvox male strains co-expressing a FLAG and StrepII tagged VcVSR1 protein and an OLLAS-tagged MID protein, or expressing only OLLAS-tagged MID (Figure 5F). In summary, our results indicate that VSR1 contains a conserved dimerization domain (DD) in its N terminal region and this domain facilitates self-interaction, most likely to form homodimers, as well as interaction with the N terminal domain of MID, most likely to form heterodimers.

Discussion

VSR1 is a key missing regulator for volvocine algal gametic differentiation and sex determination. Several decades ago mutant screens were performed to identify mating and gametogenesis genes in Chlamydomonas (13, 14, 24). These screens identified mating defective or *imp* (impotent) mutants of various types, including *imp-11* which was later found to have a mutation in the *MID* gene and caused a *plus* differentiation phenotype (8, 14). However, further work on several *imp* mutants was not feasible because they were unable to mate or mated very poorly. This conundrum precluded further analysis on genes that might be required for plus gamete differentiation. Here we made use of phylogenomics and phylotranscriptomics to identify a key missing factor for *plus*/female gamete differentiation, the conserved RWP-RK transcription factor VSR1. The success of this approach rested on the different induction conditions for gametogenesis in Chlamydomonas versus Volvox which served as a powerful filter against the myriad stress and starvation genes induced in Chlamydomonas by -N. While our search for VSR1 was narrowly focused on one TF family, it could easily be generalized to find a larger set of genes that could have conserved functions in volvocine gametogenesis. Other factors besides VSR1 may be required for volvocine gametic differentiation, but VSR1 fulfills a key role of helping to establish the gametic default or ground state of *plus* or female in two distant volvocine cousins—*C. reinhardtii* and *V. carteri*. By extension, it seems likely that this role for VSR1 is conserved across the entire volvocine clade where we have identified candidate VSR1 orthologs in species with sequenced genomes (Figure S 1).

The tandem duplication of VSR1 (RWP11) and RWP4 that seemed to have occurred only in *C. reinhardtii* and two close relatives is somewhat puzzling. The RWP-RK domains of these two

predicted TFs are highly similar, though their N terminal regions are more diverged. However, their mutant phenotypes are completely different, ruling out the possibility of redundant function: *rwp4* mutants have no detectable phenotype in the sexual cycle, while *rwp11* (*vsr1*) mutants are completely sterile. Importantly, some DD residues which are conserved in volvocine VSR1 proteins are absent from RWP4 which may render RWP4 incapable of interacting with itself or with MID. Nonetheless, the retention of this duplication over several speciation events and the induction of RWP4 expression by -N suggests some role in the sexual cycle or nitrogen starvation response. On the other hand, most volvocine species have only a single VSR1 ortholog, so whatever role RWP4 acquired seems specific to Chlamydomonas and its closest relatives and not a core feature of volvocine mating type or sex determination.

The identification of VSR1 as being required for gametogenesis in both mating types or sexes indicates a dual role in promoting expression of mating-type or sex-specific genes whose expression patterns are mutually exclusive. One way to reconcile these mutually exclusive roles would involve modification of VSR1 by association with MID, converting it from either a homodimer in plus/female (or heterodimer with another factor) to MID-VSR1 heterodimers in minus/males with presumably different DNA binding specificity. Data from Y2H and Co-IP data are consistent with this simple model since the VSR1 DD we identified has the capacity for homodimerization with the N terminus of MID and with itself, a finding consistent with mutual exclusivity VSR1 self interaction versus interaction with MID (Figure 5). This model further predicts that the MID-VSR1 interaction is stronger than the VSR1-VSR1 interaction, and that VSR1 homodimers and MID-VSR1 heterodimers have different DNA sequence binding specificity, with VSR1 homodimers binding and activating plus/female genes and MID-VSR1 heterodimers binding and activating minus/male genes. While these additional predictions remain to be directly tested, our finding that VSR1 is required for expression of key mating type or sex specific genes for both mating types or sexes of Chlamydomonas and of Volvox support its role as a dual function transcription factor for gametogenesis. Although the VSR1 and MID DDs are not detectably conserved in other species, studies with plant RWP-RK transcription factors identified PB1 domains in NLP type RWP-RKs that mediate interactions between NLPs and between NLPs and TCP type TFs (25). Thus it seems that dimerization and combinatorial DNA binding is a feature of at least some RWP-RK proteins.

A new paradigm for control of sexual differentiation in volvocine algae. Molecular mechanisms for sex or mating type determination have been studied extensively in fungi and metazoans where a great diversity of epigenetic and genetic sex determination systems have evolved (1). The model we propose here for MID and VSR1 (Figure 5G) is different from previously described systems as the VSR1 protein is required in both mating types or sexes, and interacts directly with a *minus*/male transcription factor MID to modify its function. In many fungi each haploid mating-type locus encodes a transcription factor idiomorph that is required for mating type specific gene expression, and for activating the diploid program after fertilization (26). In the ascomycete budding yeast *S. cerevisiae*, a variant system has arisen that is superficially similar to the case in volvocine algae. **a** mating-type differentiation is the default when *MATα* genes *α1* and *α2* are absent, just as *plus* is the default in Chlamydomonas when MID is absent. However, unlike the case in Chlamydomonas where the presence of MID dominantly converts a *plus* gamete to a *minus* gamete, the *MATα* locus is co-dominant with *MATa* and the coexpression of a1 from the MATa locus with *α1* and *α2* represses haploid cell type gene expression programs (27). In eutherian mammals the Y-chromosome gene SRY is somewhat analogous to MID in being a dominant specifier of male differentiation with the ground state or default being female. However, the regulatory logic is different since the male differentiation regulator on the Y chromosome, SRY, and its coregulator SF1 are at the top of a hierarchy in which male sexual differentiation is maintained by pushing gonadal precursor cells into a state of stable SOX9 expression, but neither of these three male-expressed TFs play a role in maintaining female gonadal differentiation which occurs when SOX9 expression is turned off (28). Although no evidence is available, it seems likely that MID plays a direct role in activating minus or male genes as a heterodimer with VSR1, which is unlike the case for SRY whose only target may be

SOX9 (28). Interestingly, the dominance relationship between MID and VSR1 would make this system compatible with a transition to a diploid XY sex determination system with MID as the Y linked male determining factor, though no natural diploid species have been found among volvocine algae.

Deep Conservation of RWP-RK TFs in green lineage gamete specification. Conserved RWP-RK transcription factors in the RKD family are key factors for egg cell differentiation in embryophytes. In Arabidopsis RKD family members are required for egg cell differentiation (29–31), and the single RKD homolog in the liverwort *Marchantia polymorpha* (MpRKD) is required for egg cell differentiation and also plays a role in spermatogenesis. While no partner proteins for these land plant RKDs have been identified, the dual role of MpRKD in egg cell differentiation and spermatogenesis hints that it may also be modified by association with male or female factors interactions to support its dual role in male and female gametogenesis, analogous to MID-VSR1 and VSR1-VSR1. Sex and mating type determination in most green algae outside of the volvocine lineage are less well understood, but intriguingly, RWP-RK family TFs are implicated in mating or sexual differentiation in two other diverged Chlorophyte algal lineages and in the charophyte alga *Closterium*. In the prasinophyte grade of algae within the Mamiellophyceae clade, a putative heteromorphic mating-type locus has been identified in *Ostreococcus*, with one of the two haplotypes encoding a RWP-RK family member (32). A candidate RWP-RK TF was also found in what could be a mating locus region of another member of this group, *Micromonas* (33). In the green seaweed Ulva partita, a RWP-RK family TF called RWP1 was present in the male mating locus and expressed in gametes (34). Like MID, RWP1 is a small protein (120 aa), but is not in the same sub-family as MID, though it does share some motif similarity outside of the RWP domain. Intriguingly, in *Ulva mutabilis*, a RWP-RK family TF (UMSL057_0048) was found to be induced during gametogenesis of a mating type *plus* strain, and the closest match to this family member in volvocine algae is the VSR1 subfamily, though the similarity is only found in the RWP-RK domain (35). No information on the expression pattern of UMSL057_0048 in male strains was reported, but if it is expressed in both sexes as an autosomal gene, then it may associate with RWP1 using the same regulatory logic for mating type specification as in volvocine algae. *Closterium peracerosum–strigosum–littorale* (Closterium) are unicellular charophyte algae in the Zygnematophyceae clade that undergo sex via conjugation. Recently a small mating type *minus* encoded RWP-RK protein, CpMinus1 was shown to function analogously to MID in volvocine algae (36). Its RWP-RK domain is its only conserved domain and was grouped into the RKD(A) subfamily while VSR1 and MID proteins are in the RKD(B) and RKD(C) subfamilies, respectively. Taken together, the available information across the green lineage suggests an ancestral role for RWP-RK TFs in governing sexual differentiation. Although molecular phylogenies place MID, RWP1, mamiellophycean *MT* locus RWPs, and streptophyte RKDs in possibly different clades, it should be noted that the RWP-RK domain is short, and building reliable phylogenies across large phylogenetic distances using the RWP-RK domain often gives conflicting results. For this reason, it is reasonable to speculate that a single clade of RWP-RK TFs mediated gamete differentiation and mating-type specification at the base of the Viridiplantae or even earlier, and that rapid evolution of these genes, as has been demonstrated directly for MID (37), might obscure a common origin among modern descendant lineages.

Mating type specification and the emergence of sexes. In species with mating types, gametes are usually isogamous with little or no morphological differentiation between types. In many fungi where mating type specific gene expression is required for differentiation of mating types, each Mat locus has its own TF that directly regulates mating type specific genes (38). In multicellular taxa with separate sexes, two additional features often evolve. One is more complex gamete differentiation to produce male and female gamete types, and the second is integration of gametogenesis into a body plan where males and females produce distinct types of support tissues and organs. In these sexually dimorphic systems sex determination mechanisms can control both somatic and gametic differentiation through hierarchical or parallel developmental mechanisms that may govern thousands of genes either directly or indirectly (39). This emergent

property of sexes in multicellular organisms is evident even in Volvox where at least two levels of control over sexual differentiation have been observed. Male and female sexual spheroids each have their own unique embryogenesis programs that produce a characteristic pattern of sexual somatic cells and germ cell precursors. Previously it was shown that the MID pathway was largely responsible for differentiation of gametic precursor cells, but that it did not control sexual embryogenesis which instead must be controlled by the Volvox U and V sex chromosomes (10, 40). Our data for VSR1 further supports this emergent role for sex chromosomes in dimorphic embryogenesis in Volvox. Mutation of Volvox vsr1 caused both male and female germ cell precursors to revert to a vegetative reproductive program, but did not impact the early sexual embryogenesis patterning. In addition, our molecular data point to a more complex and hierarchical program of sexual gene expression in Volvox where some sexual genes are under the control of just sex inducer but not VSR1/MID or sex chromosomes, some are under the control of both sex inducer and VSR1 in both sexes, and others are sex specific and controlled by the VSR1-MID pathway. The genes which are expressed in both sexes and are also dependent on VSR1 present an interesting case as their expression does not appear to be sensitive to the presence of MID (Figure S 9). There are several possibilities for how these VSR1-dependent, MID-independent genes might be controlled. One possibility is that they respond to VSR1 homodimers that could be formed in both sexes prior to when MID is expressed. However, since MID protein is present even in vegetative stage male Volvox spheroids (10) this temporal regulation mechanism seems unlikely. A second possibility is that promoters for these genes have response elements for both VSR1 homodimers and for VSR1-MID heterodimers, thereby allowing activation in both males and females. Finally, it is possible that in Volvox, VSR1 has another partner which is expressed in both sexes and which creates a novel heterodimer that controls different genes than either VSR1-MID heterodimers or VSR1 homodimers. The added complexity of Volvox sex-related gene expression provides an opportunity to understand how the core gametogenesis and mating type programs coevolved with multicellularity and integrated with genetic programs for sexually dimorphic development.

Molecular analyses of target genes and sequence binding specificity of VSR1 homodimers and MID-VSR1 heterodimers in Chlamydomonas and Volvox will also be useful to understand whether the core recognition sequence changed during the transition to oogamy in Volvox and whether the MID-VSR1 protein interaction coevolved as well. It is notable that Volvox MID did not function in Chlamydomonas. Reciprocally, Chlamydomonas MID or chimeras between the N terminal and C terminal domains of Chlamydomonas and Volvox MID did not function in Volvox (10). We speculate that part of this incompatibility may be due to inability of MID and VSR1 to undergo cross species interactions, though there may also be changes in DNA binding specificity. Notably, Gonium and Pleodorina MID proteins showed partial function in Volvox, so MID in these species is predicted to retain some interaction with Volvox VSR1 (40). A second aspect of VSR1 regulation that must have changed during the evolution of the Volvox lineage is the cue for VSR1 expression going from nitrogen starvation to sex inducer. Little is known about the perception and signaling mechanism of sex inducer, but it likely involves activation of one or more transcription factors that control expression of VSR1 and other sex-inducer-dependent genes.

Materials and Methods

***V. carteri and C. reinhardtii* strains and culture conditions.** *Eve* (*Volvox carteri.* f. *nagariensis* UTEX 1885) and *AichiM* (*Volvox carteri.* f. *nagariensis* NIES 398) were obtained from stock centers http://web.biosci.utexas.edu/utex/ and http://mcc.nies.go.jp/, respectively. All *Volvox* strains were cultured in Standard Volvox Medium (SVM) at 32°C (unless otherwise specified) and synchronized on a 48 hour developmental cycle with a 16hr:8hr light:dark regime(41) and with a combination of 100 µE blue (465 nm) and 100 µE red (465 nm) LED lights with bubbling aeration. Female *VcMID-GO* transgenic lines were grown at 28°C for vegetative cultures and 32°C to obtain sexual cultures. Sexual development was induced by adding pre-titered sex inducer to juveniles 36 hours prior to embryonic cleavage. Life-cycle cultures including vegetative phase and

sexual phase for deep sequencing were collected according to the marked time points in Figure S 11. Sexual induction and mating tests were done as described previously (10, 40).
The *Chlamydomonas reinhardtii* strains used in this study were obtained through the Chlamydomonas Resource Center (http://www.chlamycollection.org/): CC3712 (*mid* deletion mutant), CC1690 (21gr wild-type MT+), CC1691 (6145C wild-type MT-), and CLiP strains from the Chlamydomonas Library Project (19): CC5325 (MT-, parental line of CLiP mutant), CC5155 (MT+, isogenic to CC5325), *rwp4* mutant (LMJ.RY0402.060132), and *rwp11* (*vsr1*) mutant (LMJ.RY0402.189640). Strains were grown in liquid Tris-acetate-phosphate medium at ~25°C or on TAP plates with 1.5% agar (42).

The sgRNA design and sgRNA vector construction. VcVSR1 sgRNA vectors that target VcVSR1 (Phytozome Vocar.0014s0106) (43) was generated by incorporating the VcVSR1 sgRNA into the plasmid pVsgRNA stuffer (21): digesting pVsgRNA stuffer with BtgZI and ligating annealed 24-base oligonucleotides that create 20-base protospacer sequences just upstream of the sgRNA scaffold. The Cas-Designer tool at CRISPR RGEN Tools (http://www.rgenome.net/cas-designer/; (44) was used to select gene target sequences; targets were picked from coding exons in the 5' half of the gene, generally the ones with ≥ 55% GC content, out-of-frame score ≥ 66, no mismatches to sgRNA, and no potential off-targets with zero, one or two mismatches were chosen. Complementary oligonucleotides were designed to generate the protospacer sequence with 4-base extensions at their 5′ ends to create BtgZI overhangs complementary to those created by BtgZI digestion of pVsgRNA-stuffer plasmid (Table S 3). 10 µl of each oligonucleotide at a concentration of 100 pmol was mixed with 20 µl annealing Buffer (10 mM Tris pH 7.5, 1 mM EDTA, 50 mM NaCl) and annealed by heating in a thermal cycler for 5 min at 95°C, then cooling to 25°C at a rate of 1°C per second, repeated three cycles. Annealed oligonucleotides were diluted 1:5 with 1xTE, and 1 µl of this dilution was ligated with 25 ng BtgZI-digested pVsgRNA-stuffer to generate the targeting vectors. To confirm the correct annealing, ligation products were sequenced with primer M13R.

***V. carteri vsr1* mutant generation and rescue.** CRISPR-Cas9 editing to make *vsr1* mutants was done as described previously using an *in vivo* expression system that co-expresses Cas9 enzyme along with a guide RNA (gRNA) in a plasmid vector (21). Oligos used to generate gRNA sequences and their insertion into the expression vector are described in Table S 5. All transformations of Volvox were done as described previously (10) with 10µg/ml Hygromycin B used for selection of edited transformants. The AichiM *vsr1* mutant candidates from the Hygromycin B selection medium were tested for sexual differentiation, and multiple independent candidates were found that did not develop sperm packets and were characterized further by amplifying the edited region using PCR (Table S 6) and sequencing. Edited male mutant line vsr1#5_4 (*vsr1-1*) was used for rescue experiments. Construct pRSF-VcVSR1 was co-transformed into *vsr1-1* along with pPmr3 which encodes a paromomycin resistance marker (45) with selection of rescued strains in medium containing 10µg/ml of paromomycin. Eve *vsr1-1* mutants were generated by crossing Eve and a selected rescued male strain *vsr1*::RSF-VcVSR1. Around 70 meiotic progeny were scored by genotyping (sex determining region, *VSR1* locus, and presence/absence of pRSF-VcVSR1 construct) and phenotypic analysis of sexual development. Genotyping *vsr1-1* was facilitated by use of a *Hpy1*88I restriction site polymorphism created by the edited mutation (Figure S 8, Table S 6 and Table S 7). Strains for Co-IP experiments with MID and VSR1 were generated by transforming either wild-type Eve or rescued female strain *vsr1*::RSF-VSR1#1 with plasmid VcMID-GO and selecting for paromomycin resistance carried by the plasmid. Pseudo-male transformants were identified by sexual induction and scoring for sperm packet formation as described previously [2], and MID expression was confirmed by immunoblotting. Selected female VcMID-GO expressing and female *vsr1*::RSF-VSR1 VcMID-GO expressing transformants were used for Co-IP experiments.

Chlamydomonas RNA extraction and cDNA synthesis. Vegetative and gamete RNA isolation were done as previously described (9) with the following modifications: *C. reinhardtii* cultures

were grown to confluence on TAP plates (9, 42) for four days under continuous light at room temperature. Cells were washed off of the plates with either TAP medium and placed immediately into 200ml TAP media (for vegetative samples) with density of ~$5.0x10^6$ cells/mL at 25 ℃ for 4 hours with air bubbling Erlenmeyer, or with nitrogen-free (N-free) HSM media and placed immediately into 200ml nitrogen-free (N-free) HSM media with density of ~$5.0x10^6$ cells/mL at 25℃ for 4 hours in large unshaken Erlenmeyer flasks. For each sample, 200 mL of cells were collected in 250mL Centrifuge Bottles and Tween-20 was added to a final concentration of 0.005%. The samples were centrifuged at 3,000g for 3 minutes, the supernatant decanted and pellet was resuspended in 1ml TAP or N-free HSM medium, and the suspension was moved to 1.5ml Eppendorf tubes with $5x10^7$cells/tube. Suspensions were centrifuged at 3,000g for 3 minutes, supernatants removed, and cell pellets were snap frozen in liquid nitrogen and stored at -80C. RNA was extracted from frozen pellets with Trizol (Invitrogen, Carlsbad CA) according to the manufacturer's protocol. RNA was further purified using RNEasy columns with DNAse digestion (Qiagen) according to the manufacturer's protocol. cDNA preparation was performed as described previously (40).

Volvox PCR genotyping, RNA preparation and cDNA synthesis. PCR genotyping, PCR amplification conditions, RNA and cDNA preparation and RT-PCR on *V. carteri* strains were performed as described previously (10, 20) using primers listed in Table S 6. RNA was purified using RNEasy columns (Qiagen) according to the manufacturer's protocol.

Quantitative RT-PCR (qRT-PCR). cDNAs were diluted 1:20 in H_2O. Each 10 µL RT-PCR contained 2.5 µL of diluted cDNA, 1 µL 10 X Choice Taq buffer (Denville Scientific, Cat# C775Y44), 1X SYBR Green I (Invitrogen), 0.6 µL $MgCl_2$(50mM $MgCl_2$, Invitrogen), 1 µL dNTP mix (2mM each dNTP, OMEGA BIO-TEK, Cat#101414-958), 1U Choice Taq (Denville Scientific, Cat# C775Y44), 0.8 µL primers (mix with 5µM each primer). Real-time RT-PCR analysis was carried out using a LightCycler-® 480 Instrument II (Roche Diagnostics, USA) with the following cycling conditions: 95C 3", 57C 20', 72C 20' for 50 cycles. Melt curves and gel electrophoresis were used to confirm the presence of a single amplification product of the correct size in each reaction. For all primer sets, a standard dilution curve was prepared using cDNAs pooled from all samples (Table S 6). Relative cDNA levels were calculated using the best-fit curve from the standard dilution of each primer set using supplied software from Roche and then normalized against the internal reference gene cDNA signal. Each reaction was run with three biological and two technical replicates.

Cloning *VcVSR1* genomic DNA, *VcVSR1* cDNA and *CrVSR1* genomic DNA. For Volvox VSR1, PCR was used to amplify genomic fragments and cDNAs of *VSR1* from wild type female strain EVE. Genomic DNA was isolated as described previously (46), and cDNAs were prepared as described above. The high GC content and low complexity of *VSR1* necessitated using a modified 10X dNTP mix for PCR amplifications containing 0.5 µM dGTP, 1.5 µM 7-deaza-2'deoxy dGTP, and 2 µM each dATP, dCTP, dTTP. Phusion enzyme (NEB) was used for amplifications according to the manufacturer's instructions. PCR products were amplified using 32 cycles of 98°C for 20 s, 60-68°C for 20s, and 72°C for 30s-120s.

Two separate fragments that together covered the whole *VcVSR1* gene were amplified from female genomic DNA (isolated as described above) using primer sets VcVSR1_F1-2(907)/VcVSR1-R23(904) and VcVSR1-F23(903)/ VcVSR1_R6-2(908). The two PCR fragments were assembled into Chlamydomonas vector AR:Luciferase:RBCS2 (10, 47) that was digested with XhoI/BamHI to remove the luciferase fragment and using Gibson Assembly® Master Mix (NEB, E2611) to create vector pAR-VcVSR1 containing a full length genomic clone of VcVSR1 from predicted ATG to stop codon. pAR-VcVSR1 was further modified by inserting FLAG(F) and StrepII(S) epitope tags at the 5'end and replacing the Chlamydomonas AR promoter with the Volvox NitA promoter. To do so, the vector pVcCas9-1 (21) was used as a template with amplification primer set NitA3'UTR_F4(sg44)/ NitA 5'UTR_R4(sg45) to generate a NitA promoter fragment (21). Epitope tags FLAG(F) and StrepII(S) block fragment were amplified with primer

set gBlock_TAP_F1(sg46)/ gBlock_TAP_R2 (sg49) from the following synthetic fragment 5'-GGCTCCTGGAGCCACCCCCAGTTCGAGAAGGGCGGCGGCAGCGGCGGTGGTTCGGGCGGCGGCAGCTGGTCGCATCCCCAGTTCGAGAAGGGCAGCTACCCCTACGACGTGCCCGATTACGCGCACCACCACCACCACCATGACATCCCCACCACTGCGAGCGAGAACCTGTACTTCCAGGGCGAGCTGGACTACAAGGACCACGACGGCGACTACAAGGACCATGACATTGACTACAAGGACGACGACGACAAGGACATCCCCACCACCGCTTCG-3'. Two VcVSR1 gene fragments were amplified from the vector pAR-VcVSR1 with the primer sets VcVSR1-F41(sg48/))/VcVSR1-R23(904) and VcVSR1-F23(903)/VcVSR1-R35(sg43). Then the two VcVSR1 genomic fragments, tandem epitope tag fragment and NitA promoter fragment were assembled with the backbone of pVcCas9-1 digested by SpeI/BamHI following the recipe of Gibson assembly (NEB) to get vector pNitA-SF-VcVSR1. To generate the construct pRSFVcVSR1, the pNitA-SF-VcVSR1 vector backbone was amplified with the primer set HRcas9-vector_R(sg125)/NitA3'UTR_F4(44). The VcVSR1 native 5'UTR and promoter were amplified from genomic DNA by primer set VcVSR1_5'UTR_F(122)/ VcVSR1_5'UTR_R(123). The tandem epitope tag FLAG(F) and Strep II(S) was amplified with primer set gBlock_TAP_F4 (sg124)/ gBlock_TAP_R2 (sg49) and the vector pNitA-SF-VcVSR1 was cut with MluI and PacI to recover a 9.2kb VcVSR1 genomic fragment. Then the four fragments were assembled following the recipe of Gibson assembly (NEB) to get the vector pRSF-VcVSR1. All the clones were verified by Sanger sequencing.

For Chlamydomonas VSR1, a 9.5 kb fragment containing the full-length genomic region of CrVSR1 was obtained by digesting BAC clone 9498 using enzymes SspI/Mfe (http://www.chlamy.org/bac_details.html), ligated to vector pBlueScript II KS (48) amplified with primers pBlueCrVSR1SSP1R(782)/ pBlueCrVSR1MfeIF(783) (http://www.chlamy.org/bac_details.html) and digested with SspI/Mfe to get pKSCrVSR1 (Primers described in Table S 5). Two fragments that cover the whole length of CrVSR1 genomic DNA were amplified from pKSCrVSR1 with primer set CrVSR1_XhoI_F(780)/CrVSR1-mid-R(795) and CrVSR1-mid-F(794)/CrVSR1_BamHI_R(781), and assembled using Gibson assembly (NEB) with the vector from pRPL23:Luc:RPL23 (20) digested with enzymes XhoI/BamHI to excise the luciferase gene and create pRPL23CrVSR1. To generate AphVII-RPL23CrVSR1, the AphVII expression cassette was amplified from pKS-aph7-lox (49) with primer set XbaI-Hyg-F and XbaI-Hyg-R. The amplicon was digested with XbaI and ligated to XbaI digested pRPL23CrVSR1 to generate the vector pAphVII-RPL23CrVSR1. To generate the vector pRPL23-3HA-CrVSR1, four fragments were assembled. First, vector pAphVII-RPL23CrVSR1 was digested with SpeI/NsiI to recover a ~11kb fragment with a small portion of VSR1 removed from the 5' region. Second, the 3xHA_F (sg234)/ 3xHA_R3 (sg238) primers were used to amplify the triple HA epitope tag from 3xHA-MAT3 (50). Third, Primers RPL23_SpeI-F(sg264) and RPL23_R (sg265) were used to amplify a fragment from AphVII-RPL23CrVSR1 between the SpeI site and ATG at the start site. Fourth, the primer set CrVSR1_atg_f (sg232)/ CrVSR1_NsiI_R (sg267) were used to amplify the fragment from AphVII-RPL23CrVSR1 between the ATG and the internal NsiI site as the fourth fragment. Then the four fragments were assembled following the recipe of Gibson assembly (NEB) to generate the construct pHA-CrVSR1. All the clones were confirmed by Sanger sequencing.

The plasmid pVcMID-GFP-Ollas (pVcMID-GO) was created as follows: the two oligos Ollas_VcMid_Ve, gcgaatgaattgggacctaggttgatgggcaagctttctagatagcggccgcGGATCCATTTGCGATTGCTTGCTCTTCGTA and Ollas-GFP_R, atcaacctaggtcccaattcattcgcaaagccactagtcatggtagatctggctgcCTTGTACAGCTCGTCCATGCCGT were annealed and used to create a double stranded fragment by running PCR program, 20 cycles of 98°C for 20 s, 68°C for 20s, and 72°C for 10s with Phusion enzyme (NEB) and HF buffer, and the product was used as a reverse primer to pair with primer VcMid-GFP_F to amplify GFP from pMF124cGFP (51) to create a GFP-OLLAS fusion fragment. The plasmid pVcMID-BH (10) was digested with NheI and BamHI, and the 3.5kb backbone was assembled with GFP-OLLAS fragments using Gibson assembly (NEB) to generate pVcMID-GFP-Ollas (pVcMID-GO).

Transformation, gametogenesis, whole cell extract preparation and immunoblotting of Chlamydomonas extracts. Transformation of Chlamydomonas and preparation of gametes was done as described previously (40). Whole cell extract preparation and immunoblotting of Chlamydomonas cultures were performed as described previously (40, 52) with the following modifications: Antibody dilutions were: rat-anti-HA (1:5000) (3F10, Roche, Switzerland), Anti-FLAG rabbit polyclonal (Rockland 600-401- 383) was used at 1:5,000 to detect the FLAG epitope tag. Signal was detected by chemiluminescence (Luminata Forte Western HRP Substrate, Millipore) using an Azure c300 Gel Imaging System (Azure Biosystems chemiluminescence).
Chlamydomonas immunoprecipitation (IP)
An outcrossed *MT- vsr1* CLiP mutant (LMJ.RY0402.189640) expressing pHA-CrVSR1 was crossed with *MT+* wild type strain 21gr to generate a *MT+* progeny expressing HA-CrVSR1. This progeny strain was then crossed with CC3712 *MT- mid-2::CrMID-6XFLAG* (Geng,2018) to generate a double tagged strain *MT- mid-2:: HA-CrVSR1::CrMID-6XFLAG* for Immunoprecipitation (IP) along with the rescued *mid-2 CrMID-6XFLAG* parental strain used as a negative control. Gametes of Chlamydomonas double tagged strain *vsr1*::HA-CrVSR1 MID-FLAG and control strain CC3712::MID-FLAG were collected and used to prepare whole cell lysates that were subjected to IP with anti-HA magnetic beads (Pierce™ 88837) as described previously (40, 50, 52). Bound proteins were eluted with 2xSDS sample buffer by boiling at 95℃ for 5 min, separated by SDS-PAGE, and detected by immunoblotting as described above.

Volvox extract preparation and immunoblotting. Preparation of whole-cell lysates and immunoblotting of *V. carteri* cultures were performed as described previously (10) with the following modifications: culture pellets were resuspended in PBS with protease and phosphatase inhibitors (P9599, Sigma-Aldrich; 10 mM PMSF, 10 mM benzamidine, 5 mM EDTA, 5 mM EGTA, 50 mM MG-132, 10 mM ALLN, 1 mM NaF, and 1 mM Na2VO4). Anti-FLAG rabbit polyclonal (Rockland 600-401- 383) was used at 1:5,000 to detect the FLAG epitope tag. OLLAS epitope tag antibody (L2) (Novus Biologicals LLC, NBP1-06713) was used at 1:3,000 to detect the OLLAS epitope tag. Antigens were detected by chemiluminescence (Luminata Forte Western HRP Substrate, Millipore) using an Azure c300 Gel Imaging System (Azure Biosystems chemiluminescence) with a Chem Mode.

Volvox Immunoprecipitation (IP). Two pseudo-male strains of Volvox (females expressing MID-GO) were generated–one coexpressing a pSF-VcVSR1 tagged transgene in a *vsr1* mutant background, and a control strain expressing only MID-GO in a *VSR1* wild-type background. Upon sexual induction both strains produced sperm packets. Whole cell lysates were prepared from mature sexually induced spheroids with sperm packets and were subjected to IP with anti-FLAG magnetic agarose beads (Pierce,A36797) using a similar procedure as the Co-IP done in Chlamydomonas. Bound proteins were eluted, separated by SDS-PAGE, and detected by immunoblotting as described above.

Immunofluorescent Staining and Microscopy. Immunofluorescent staining and microscopy on sperm packets were performed as described previously (10) with the following modifications: male *vsr1::SF-VcVSR1* or wild type male control samples were processed in parallel and imaged under identical conditions. After fixation, blocking and washing, cover slips with adhered spheroids were incubated overnight in anti-StrepII 1:500 (Abcam, ab76949) in 20% blocking buffer at room temperature or at 4°C and washed 6x10 min with 20% blocking buffer in PBS 0.05% Tween 20. After washing out unbound primary antibody cover slips were incubated with AlexaFluor 488 conjugated goat anti-mouse secondary antibodies (Invitrogen) 1:500 in 20% blocking buffer and incubated for 1 h at room temperature (for 4 h at 4°C for somatic cells staining) in the dark. Following six washes with 20% blocking buffer diluted in PBS and 0.05% Tween 20, the coverslips were incubated for 15 min in 2 mg/ml DAPI and then washed for 5 min in PBS. Excess liquid was removed, and the coverslips were mounted with VectaShield (Vector Labs). Microscopy was performed with a Leica DMI6000 B using DIC optics or using the following filter cube sets and illumination with a Prior Lumen 200 light source: A4: excitation BP 360/40;

dichroic 400; emission BP470/40, L5: excitation BP 480/40; dichroic 505; emission BP 527/30. Images are representative of results from at least three independent experiments.

Phylogenomic analysis. Alignments of RWP-RK domains from all predicted family members in *C. reinhardtii* and *V. carteri* plus Mid proteins from additional volvocine species in Figure 1 were done using the Muscle algorithm in MEGAX (53, 54). MEGAX was used to identify the best fit model, LG+G with gamma shape 1.34. An unrooted maximum likelihood phylogeny was estimated using PhyML with aLRT values calculated for branch support. The resulting tree was converted to a cladogram and a subtree containing RWP-RK members that were sex or mating related was used to create Figure 1. Estimates of sex induced gene expression were derived from previously published data from Chlamydomonas (11, 12) and from unpublished Volvox transcriptome data shown in Figure 1. Amino acid similarity between *VSR1* paralogs of core *Reinhardtinia* were done with the PLOTCON software contained in the EMBOSS package (55).

Yeast two hybrid assay. Plasmids for yeast two-hybrid experiments were constructed using NEBuilder DNA assembly (NEB), Ligation High kit (TOYOBO), or T4 DNA ligase (NEB) to assemble PCR and restriction fragments as summarized in Table S 6 and Table S 7. The pGBKT7 or pGADT7 backbone vectors were linearized with EcoRI/SalI or EcoRI/XhoI, respectively. Required fragments were amplified with the KOD FX Neo polymerase (TOYOBO) following the manufacturer's instruction. Isolated colonies were inoculated and mini-prepped with the plasmid mini kit (QIAGEN). Inserted fragments were sequenced and confirmed (GENEWIZ). Yeast two-hybrid experiments were conducted using Matchmaker Yeast Two-Hybrid Kit (Clontech) following the product instruction. *Saccharomyces cerevisiae* strain AH109 was co-transformed with bait and prey plasmids and selected on synthetic complete (SC) medium agar plates without leucine or tryptophan (-LW). Resultant colonies were assayed in serial dilution for production of adenine and histidine to grow on SC plates without adenine/histidine/leucine/tryptophan (-AHLW).

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Figures and Tables

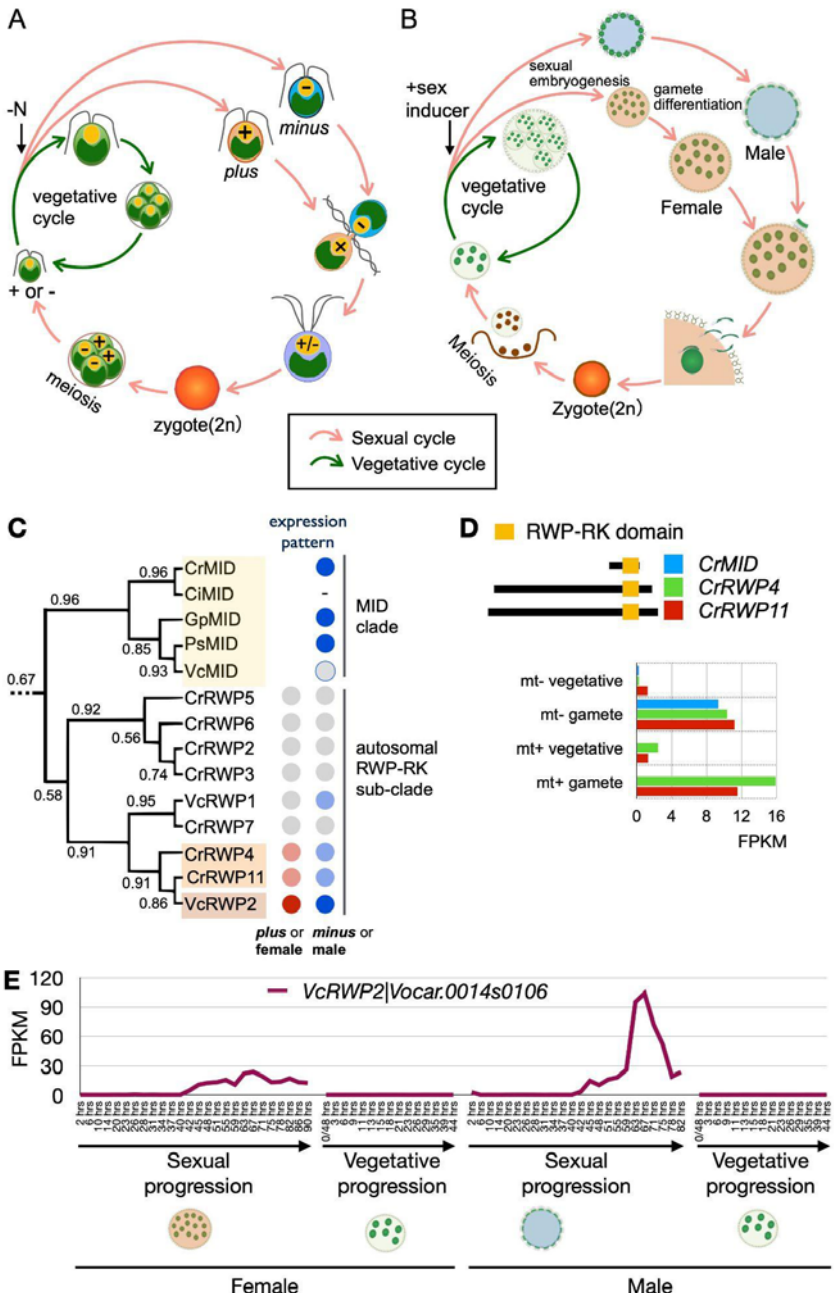

Figure 1. Life cycles of *Chlamydomonas reinhardtii* (Chlamydomonas) and *Volvox carteri* (Volvox) (A, B) and phylogenomic screening of volvocine RWP-RK family genes expressed during sexual reproduction (C-E). A. Chlamydomonas life cycle. During vegetative reproduction (green arrows) cells grow and divide by multiple fission. The sexual cycle (pink arrows) is triggered when nitrogen is absent (-N). Gametic differentiation occurs under the control of the *MT+* and *MT-* mating-type loci that specify *plus* or *minus* gametes. Ciliary agglutination is followed by fertilization and differentiation into a diploid zygospore. Upon germination meiosis occurs yielding four recombinant haploid progeny. B. Volvox life cycle. During vegetative reproduction of both sexes (green arrows) large stem cells in each spheroid called gonidia undergo embryogenesis and produce a new generation of spheroids. Exposure to sex inducer triggers the sexual cycle (pink arrows) leading to dimorphic embryogenesis in males and females

under the control of the male (*MTM*) or female (*MTF*) sex determining regions (SDRs). Post embryonic maturation of sexual germ cell precursors leads to their differentiation into sperm packets or eggs. Sperm packets produced by males swim to females, enter the female extracellular matrix (ECM) and fertilize the large immotile egg cells that then develop into dormant diploid zygospores. Upon germination a single meiotic product survives and develops into a new haploid recombinant progeny. (C) Phylogenetic subtree of selected RWP-RK family genes in volvocine algae. MID genes (restricted to *minus*/male) are indicated at the top and autosomal RWP-RK genes at the bottom. For the mRNA expression patterns, gray circles indicate weak or no induction (<2-fold) in sexual versus vegetative phases; pink indicates 5-10 fold induction and red >10-fold induction in *plus*/female sexual phase; pale blue indicates 5-10 fold induction and dark blue indicate >10 fold induction in minus/male sexual phase. Colored rectangles highlight gene families of special interest. (D) Expression patterns of Chlamydomonas sexually expressed RWP-RK genes. FPKM values are extracted from Joo et al. 2017. Protein domain schematics with RWP-RK domain in pale orange are shown next to the color key for mRNA expression of each gene. (E) Volvox *RWP2* expression pattern across the vegetative and sexual development cycles. Arrows indicate time progression in the four sample types starting from pre-cleavage spheroids for vegetative samples and from addition of sex inducer for sexual samples: female sexual, female vegetative, male sexual, and male vegetative.

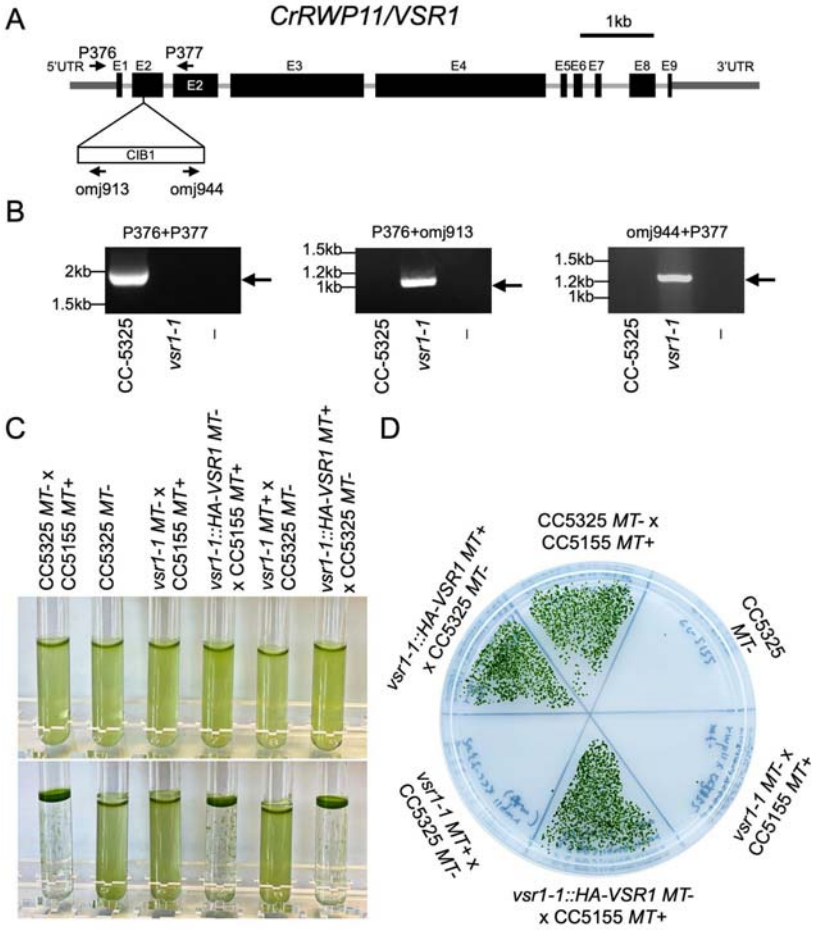

Figure 2. Sterility phenotype of Chlamydomonas *vsr1* mutants. A. Diagram of *RWP11* (*VSR1*) locus and location of CIB cassette in exon 2 (E2) of mutant strain LMJ.RY0402.189640 whose mutant allele is designated as *vsr1-1*. Locations and names of PCR primers used for genotyping are shown above. Exons are indicated by thick black boxes and labeled E1-E9. 5' and 3' untranslated regions (UTRs) are indicated by thinner dark gray lines. B. PCR genotyping of *VSR1* and *vsr1-1* alleles. PCR products were run on agarose gels and imaged after staining with RedSafe dye. Strain CC5325 *MT-* is the parent strain of *vsr1-1* and serves as a wild-type control. C. Mating reactions between pairs of indicated strains (or CC5325 as a negative control). Top row of tubes shows gamete suspensions just after mixing. Bottom row shows tubes after an overnight incubation. Successful mating results in buoyant mats of zygote pellicle on the surface and sides of the tube and a clear appearance. D. Matings from panel C were spread in sectors on an agar plate prior to pellicle formation, allowed to mature, and chloroform treated to kill unmated gametes prior to germination. Colonies are formed by germinated zygospores.

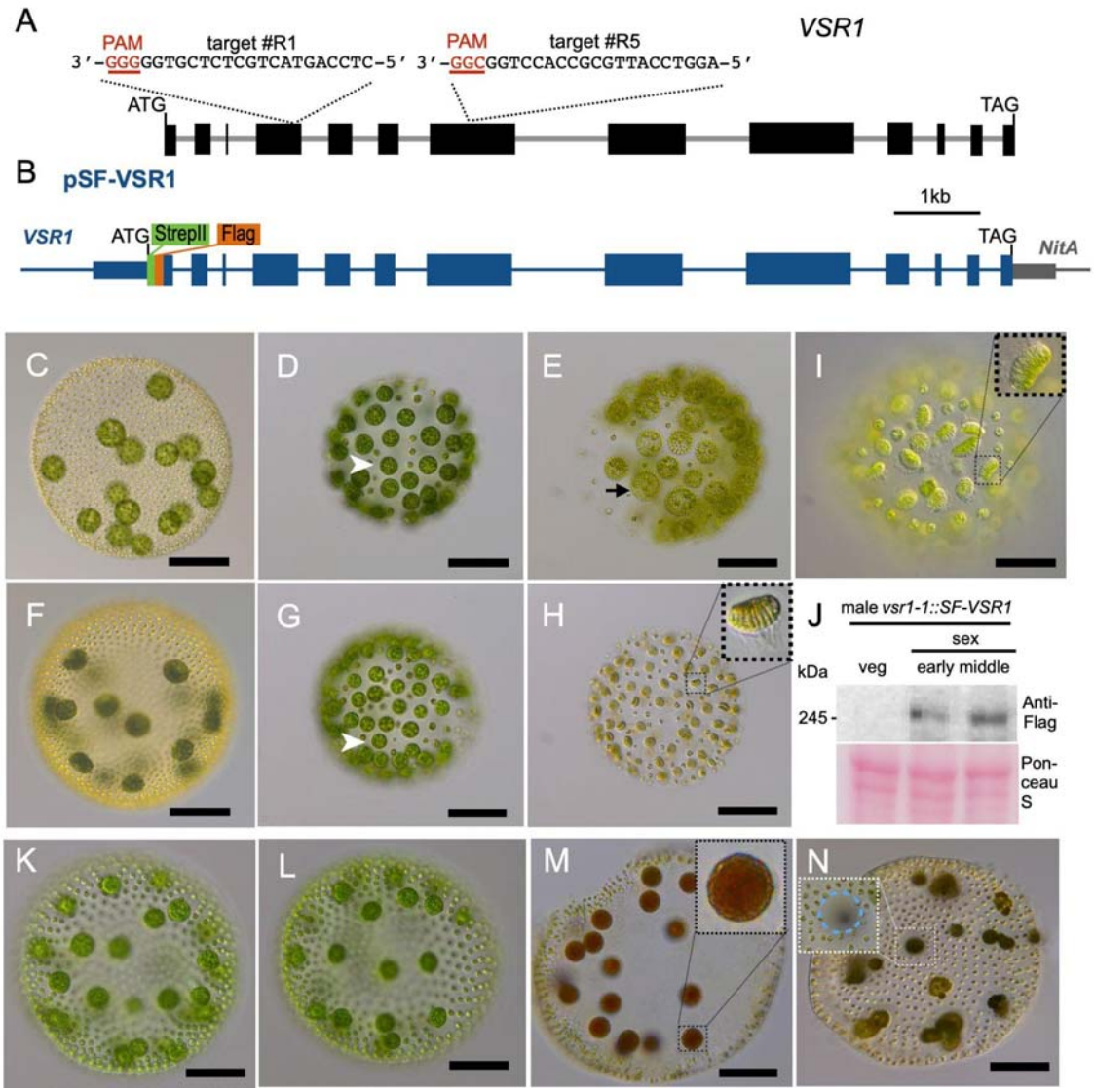

Figure 3. Isolation and characterization of Volvox *VSR1* edited mutants and rescued strains. (A) Diagram of Volvox *VSR1* locus showing two different editing sites in detail including the PAM site that specifies cleavage by targeted Cas9 guide RNA RNP complexes. Dark boxes represent exons. Start and termination codons are also shown. (B) Diagram of pSF-VSR1 construct used to rescue the *vsr1-1* mutation. Coding sequences are wide bars, medium width bars are UTRs, and thin lines are intronic or non-coding regions. Native *VSR1* sequences are in blue. StrepII and FLAG epitope tag sequences at the N terminus are in green and orange, respectively. The *NitA* 3'UTR and terminator replace the native sequences past the stop codon and are shown in gray. (C-E). Micrographs of male *vsr1-1* mutant taken from vegetative cultures (C), from post-embryonic sexual males with uncleaved presumptive androgonidia with an example shown by a white arrowhead (D), and from a stage when the presumptive androgonidia had undergone vegetative embryogenesis to produce vegetative juveniles (black arrowhead) instead of sperm packets (E). (F-H) Micrographs of a wild-type male strain taken at equivalent stages as the *vsr1* mutant in panels C-E, respectively. A mature male with sperm packets is shown in panel H, with a magnified sperm packet in the inset. I. Rescued *vsr1-1::SF-VSR1* male strain at sexual maturity with restoration of sperm packets. A magnified sperm packet is shown in the inset. All scale bars

are 50 µm. J. Immunoblot detection of FLAG epitope tagged VSR1 in a rescued *vsr1-1* strain. Samples were taken from vegetative (veg) and from early (48 hrs), and middle (59 hrs) stages of sexual (sex) development as depicted in Fig. S2. (K) Wild-type sexual female with eggs. (L) Sexually induced *vsr1-1* female mutant spheroids with egg-like cells. (M) Fertilized wild-type female with maturing zygotes. Inset in (M) shows a magnified view of a zygote. (N) Post-mating *vsr1-1* sexual females with fertilization pore at sperm entry point (inset) but no zygotes. The egg-like cells behave as unfertilized *vsr1-1* mutants and undergo vegetative embryogenesis.

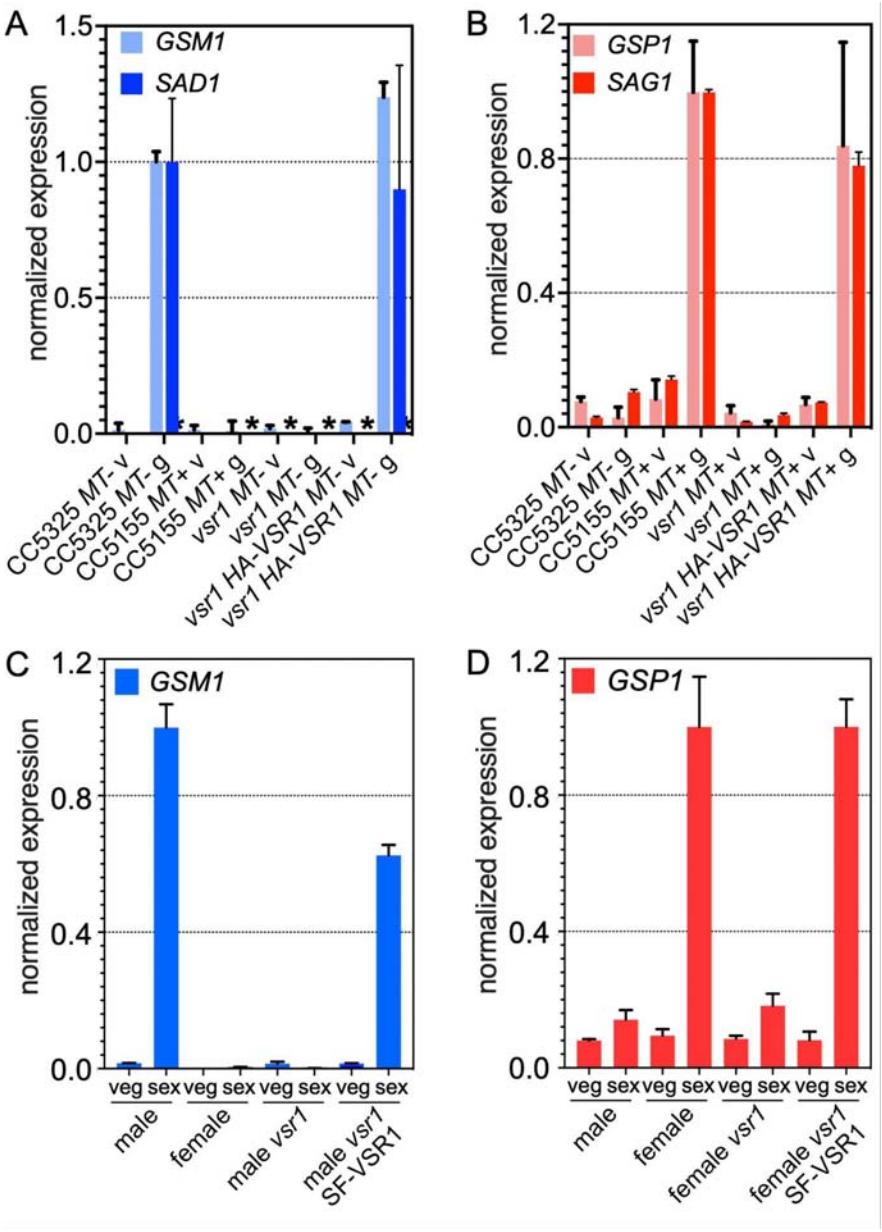

Figure 4. Impact of *vsr1* mutants on cell-type-specific gamete transcript accumulation. (A, B) Expression patterns of *minus* and *plus* gamete specific genes in Chlamydomonas wild type (CC5325 or CC5155), *vsr1-1* mutants, and rescued *vsr1-1* mutant strains expressing HA-VSR1. Transcript levels of vegetative cells (v) or gametic cells (g) were measured using quantitative RT-PCR (qRT-PCR) with values normalized to internal control ribosomal protein gene *RPL36a* and then re-scaled as relative expression to wild type *minus* or *plus* gamete values, respectively. A, *minus* genes *GSM1* and *SAD1*; B, *plus* genes *GSP1* and *SAG1*. * No expression detected. (C, D) Expression patterns of Volvox *GSP1* (female-specific) and *GSM1* (male-specific) orthologs from qRT-PCR normalized to 18S rRNA, and then re-scaled to wild type male or female values, respectively. RNA samples were derived from vegetative cultures (veg) or mature sexual cultures (sex) from indicated genotypes.

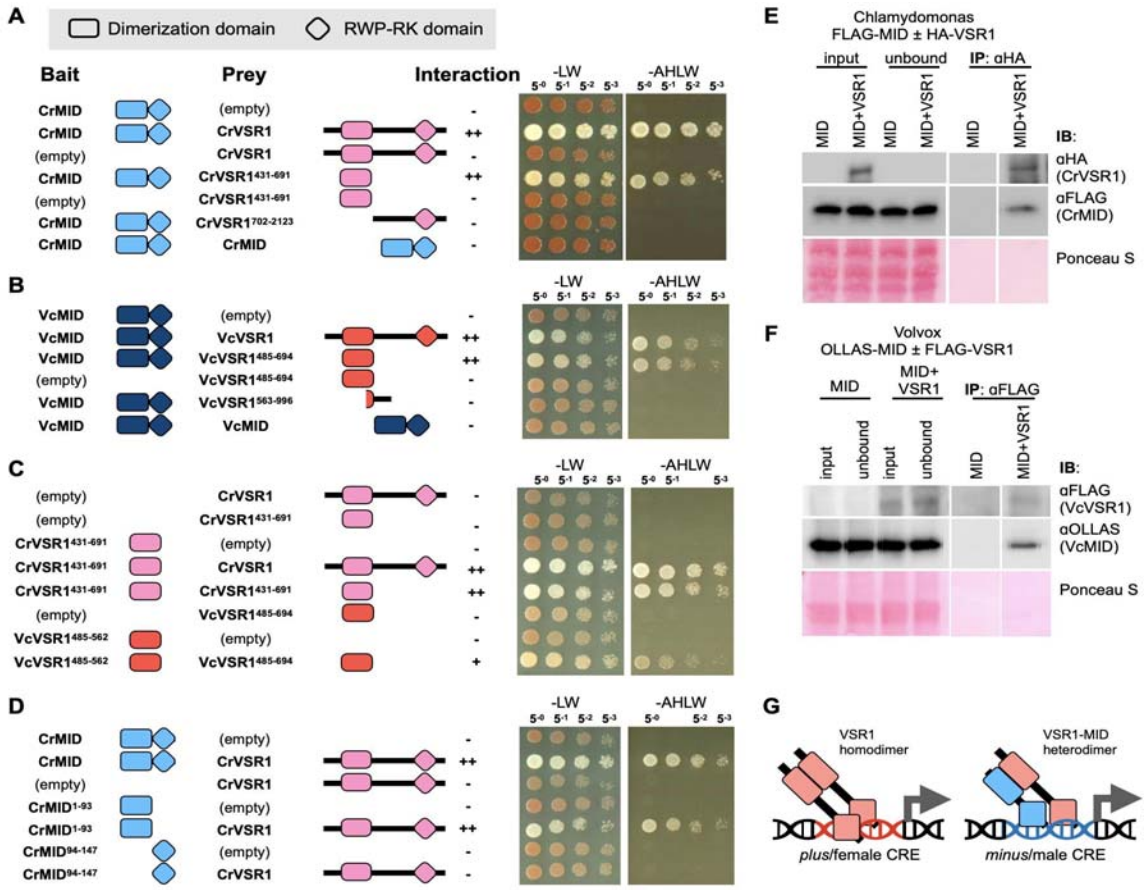

Figure 5. VSR1 interacts with itself or with MID via an N-terminal domain. (A-D) Yeast two hybrid experiments with schematics of bait and prey constructs shown on the left, and dilutions of yeast expressing indicated construct pairs spotted on the right. Results are summarized as - (no interaction), + (weak interaction) or ++ (strong interaction). The RWP-RK domains are depicted as diamonds and the protein dimerization domains (DDs) are depicted as rectangles. VSR1 is shown in pink (Chlamydomonas) or red (Volvox), and MID is shown in light blue (Chlamydomonas) or dark blue (Volvox). Empty indicates empty vector controls. Growth of two independent co-transformormants spotted in 1:5 serial dilutions on permissive media (-LW) or interaction-dependent media (-AHLW) are shown. White colonies on -LW and growth on -AHLW indicate interaction between introduced bait and prey proteins. (E,F) Co-immunoprecipitation (IP) of VSR1 and MID in Chlamydomonas (E) and Volvox (F). (E) Co-IPs for Chlamydomonas were done in two strains, both expressing a FLAG-epitope tagged transgenic MID protein rescuing a *mid-2* deletion allele (7). One strain also expressed a transgene encoding a HA-epitope tagged VSR1 protein as described in Figure S 3A. Immunoblots (IBs) with indicated antibodies are shown in each row with the Ponceau S stained membrane below. Input and unbound lanes were loaded with 1/60 of the total sample. Pellet lanes were loaded with 1/10 of the total sample. (F) Co-IP with αFLAG from sexually induced Volvox pseudo-males expressing OLLAS-tagged MID with and without FLAG tagged VSR1. Loading amounts were the same as in (E) for input, unbound and pellet fractions. (G) Model for bi-potential gamete differentiation based on VSR1-VSR1 homodimers binding to and activating promoters of *plus*/female genes and VSR1-MID heterodimers binding to and activating *minus*/male genes. See main text for additional details.