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A partially sex-reversed giant kelp sheds light into the mechanisms of sexual differentiation in a UV sexual system

Dieter G. Müller[1], Enora Gaschet[2], Olivier Godfroy[2], Josselin Gueno[2], Guillaume Cossard[2], Maritta Kunert[3], Akira F. Peters[4], Renato Westermeier[5], Wilhelm Boland[3], J. Mark Cock[2], Agnieszka P. Lipinska[2,6] and Susana M. Coelho[2,6]

[1]Fachbereich Biologie der Universität Konstanz, Konstanz 78457, Germany; [2]UPMC Univ Paris 06, CNRS, Integrative Biology of Marine Models, Station Biologique de Roscoff, Sorbonne Université, Roscoff CS 90074, F-29688, France; [3]Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Jena 07745, Germany; [4]Bezhin Rosko, Santec 29250, France; [5]Instituto de Acuicultura, Universidad Austral de Chile, Casilla 1327, Puerto Montt, Chile; [6]Max Plank Institute for Developmental Biology, Tübingen, Germany

Author for correspondence:
Susana M. Coelho
Email: susana.coelho@tuebingen.mpg.de

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Summary

- In UV sexual systems, sex is determined during the haploid phase of the life cycle and males have a V chromosome whereas females have a U chromosome. Previous work in the brown alga *Ectocarpus* revealed that the V chromosome has a dominant role in male sex determination and suggested that the female developmental programme may occur by 'default'.
- Here, we describe the identification of a genetically male giant kelp strain presenting phenotypic features typical of a female, despite lacking the U-specific region. The conversion to the female developmental programme is however incomplete, because gametes of this feminized male are unable to produce the sperm-attracting pheromone lamoxirene. We identify the transcriptomic patterns underlying the male and female specific developmental programmes, and show that the phenotypic feminization is associated with both feminization and de-masculinization of gene expression patterns. Importantly, the feminization phenotype was associated with dramatic downregulation of two V-specific genes including a candidate male-determining gene.
- Our results reveal the transcriptional changes associated with sexual differentiation in a UV system, and contribute to disentangling the role of sex-linked and autosomal gene expression in the initiation of sex-specific developmental programmes.
- Overall, the data presented here imply that the U-specific region is not required to initiate the female developmental programme, but is critical to produce fully functional eggs, arguing against the idea that female is the 'default' sex in this species.

Introduction

Females and males often differ dramatically in appearance and behaviour. These differences, which are referred to as sexual dimorphism, are principally the result of natural and/or sexual selection for traits that influence the fitness of each sex. Genetically, however, females and males are nearly identical differing by only a few genes located on sex-specific chromosomes (such as Y chromosomes in mammals, W chromosomes in birds, or U and V chromosomes in mosses and many algae; Coelho *et al.*, 2018, 2020; Umen & Coelho, 2019). Consequently, sexually dimorphic traits are to a large extent a result of differential expression of (autosomal) genes that are present in both sexes (Grath & Parsch, 2016).

Autosomal sex biased gene expression patterns in diploid XY and ZW systems have been intensively investigated in recent years (reviewed in Grath & Parsch, 2016). Such studies have revealed that sex-biased gene expression is abundant in many animal and plant species, although its extent may vary greatly among tissues or developmental stages. In species with genetic sex determination, sex chromosome-specific processes, such as dosage compensation, also may influence sex-biased gene expression. The molecular mechanisms that lead to sex-biased gene expression, however, are yet to be elucidated (Grath & Parsch, 2016).

In contrast to the well-studied XY and ZW systems, knowledge about sex-biased gene expression, sexual dimorphism and the link between sex-chromosomes and control of sex-biased gene expression in UV haploid sexual systems remains relatively scarce. UV systems are abundant among Eukaryotes, but are largely understudied (Coelho *et al.*, 2018; Umen & Coelho, 2019). In UV systems, sex is determined after meiosis by a male or a female sex-determining region (SDR). After meiosis, if a daughter cell inherits the U-chromosome (containing a U-specific region), it will develop into a female individual (female gametophyte) that at maturity will produce female structures (oogonia) and female

gametes (eggs). If the daughter cell inherits a V-chromosome, it will develop into a male individual (male gametophyte), producing male reproductive structures (antheridia), where male gamete cells are produced by mitosis at maturity. Sexual dimorphism can be minor, as in the brown alga *Ectocarpus*, where male and females differ very little, but sexual dimorphism may be more marked in algae such as the giant kelp *Macrocystis pyrifera* (Luthringer *et al.*, 2015; Mignerot & Coelho, 2016). In the latter, male and female gametophytes can be differentiated by size, colour and shape. Males are pale or bluish, the cells are small, and the overall shape is often filamentous. Females are yellow-green, gold, or brown with round or ovoid shapes. Antheridia on males liberate biflagellated sperm. Females form the strongly pigmented eggs (Westermeier *et al.*, 2007). The presence of shared orthologues in the SDRs of the Ectocarpales and the giant kelp indicates that the U and V chromosomes is these organisms are derived from the same ancestral autosome (Lipinska *et al.*, 2017).

The SDRs of UV systems contain sex-determining gene(s) that initiate the sexual determination, by regulating sex-specific patterns of expression of downstream effector autosomal genes. In *Ectocarpus*, genetic analysis has shown that the male V-specific region (VSR) is dominant over the female U-specific region, and that the female developmental programme is triggered in the absence of the VSR (and presence of the U-specific region) (Müller, 1975; Ahmed *et al.*, 2014). These results have led to the idea that a master male sex-determining gene(s) is located on the VSR (Ahmed *et al.*, 2014; Lipinska *et al.*, 2017) and that female sex may be initiated 'by default', in the absence of the VSR (Ahmed *et al.*, 2014). The U-specific genomic region may therefore not be strictly necessary for initiation of the female developmental programme, with female development exclusively relying on autosomal sex-biased gene expression. This idea is further supported by the fact that female-specific genes on the U-specific region present signs of degeneration and relatively low levels of expression (Ahmed *et al.*, 2014; Avia *et al.*, 2018). Hence, an individual with a V chromosome with an impaired male master sex-determining gene(s) would be anticipated to develop into a phenotypic female. A variant or mutant line where the genotypic sex is uncoupled from phenotypic sex would be the ideal system to test this hypothesis and to understand the role of sex-specific and (autosomal) sex-biased genes in the events leading to male and female developmental programmes.

We describe here the identification of a variant strain of an organism with UV sex chromosomes, the giant kelp *Macrocystis pyrifera*, that exhibits a range of phenotypic features typical of a female, despite being genetically male. The availability of this feminized line, together with closely related male and female lines, provided access to the molecular events underlying the initiation of the male vs female developmental programme in this ecologically important organism and to disentangle the role of sex-linked genes and autosomal gene expression in the initiation of sex-specific development.

We show that a considerable proportion of the transcriptome of the giant kelp is sex-biased, and that the morphological feminization of the variant line is associated with extensive feminization and de-masculinization of autosomal gene expression patterns. We show that two genes within the VSR exhibit a significantly reduction in transcript abundance in the feminized line, suggesting their involvement in sexual differentiation. Our observations indicate that the female programme in this UV system is not initiated entirely by default, and that the U-specific region may be required to fully express the female developmental programme. Taken together, our results provide the first illustration of how male- and female-specific developmental programmes in a haploid sex determination system may, at least partially, be uncoupled from sex chromosome identity.

Materials and Methods

Biological material

A *Macrocystis pyrifera* sporophyte was collected at Curanue, Chiloé, Chile. Meiospores were isolated and clonal cultures of male and female gametophytes were established and propagated as described by Westermeier *et al.* (2007). Male and female gametophyte clones (Westermeier *et al.*, 2010) were selected for the present study (Supporting Information Fig. S1). Axenic subclones were initiated by antibiotic treatment as described by Müller *et al.* (2008) and maintained on 1% agar in seawater with transfers every three-months. For clone isolation, culture medium was prepared with a commercial salt mixture (hw-Professional, Wiegandt, Krefeld, Germany) in demineralized water and adjusted to 3% salinity with an optical refractometer. Nutrients were added with 20 ml l^{-1} PES-enrichment (Starr & Zeikus, 2004; Coelho *et al.*, 2012). Colchicine treatment was performed using a disk of filter paper of 6 mm diameter loaded with 1 mg of colchicine (Fluka, Honeywell Research Chemicals, Illkirch, France), which was placed in the centre of an agar plate filled with gametophyte material, with good contact between agar and paper in order to allow diffusion of the colchicine into the agar. The agar plates were sealed with parafilm and subjected to culture conditions (12 ± 2°C and 2–3 µE m^{-2} s^{-1} from daylight type fluorescent lamps for 14 h : 10 h, light : dark cycles) for 12 to 16 wk until selected regenerates were isolated. Male algal individuals presenting a feminization phenotype were subsequently cultivated for 4 wk, cut in small pieces and re-isolated several times, to ensure the absence of mosaicism. Genetic sex of the feminized individuals was verified using PCR (Ahmed *et al.*, 2014; Lipinska *et al.*, 2015a).

Pheromone measurements

Female eggs of *M. pyrifera* produce a pheromone (lamoxirenee) that attracts male gametes. To induce gametogenesis in wild-type female and Mpyr-13-4 lines, light intensity was increased to 30 µE m^{-2} s^{-1} and culture medium was refreshed every 4 d. Oogonia and eggs were produced between 9–16 d after culture in these conditions.

For each of the tested strains, 25 ml of fertile gametophyte cultures containing 6.5×10^4 eggs (wild-type female) or 3.1×10^5 eggs-like (Mpyr-13-4) were introduced in 50 ml Greiner Cellstar tissue culture tubes placed horizontally to increase surface area.

Most algal pheromones known are hydrophobic, cycloaliphatic unsaturated hydrocarbons, comprising eight to eleven carbon atoms [1]. The sperm-releasing pheromone in the Laminariales carries an additional epoxy moiety, but is still a volatile and hydrophobic compound (Maier *et al.*, 2001). To efficiently trap these pheromones, which are released in only minute amounts from fertile gametes, Solid-phase micro-extraction (SPME) was used (Maier *et al.*, 1996). SPME fibres and the holder were obtained from Supelco (Bellefonte, PA, USA). For volatile extraction, a poly-dimethyl-siloxane (100 µm PDMS) fibre was used (red fibre). Prior to use, the SPME fibres were conditioned according to the manufacturer's instructions. After 14 h exposure time of the fibre in the culture medium, the fibre was thermally desorbed in gas chromatography-mass spectrometry (GC-MS) injection port followed by separation of the volatiles under programmed conditions using an ISQ LT and Trace 1310 (ThermoFisher Scientific GmbH, Dreieich, Germany) device equipped with a ZB5 column (30 m, 0.25 mm inner diameter (i.d.), 0.25 µm film thickness) linked to a guard column (10 m, Phenomenex, Aschaffenburg, Germany). Helium (1.5 ml min^{-1}) served as the carrier gas. Separation of compounds was achieved under programmed conditions from 50°C (2 min isotherm), followed by heating at 10°C min^{-1} to 200°C and at 50°C min^{-1} to 280°C. The GC injector (splitless, splitless time 2 min), transfer line and ion source were set at 230, 280 and 250°C, respectively. Mass spectra were recorded in electron impact (EI) mode at 70 eV, 35–350 *m/z*.

Generation of transcriptomic sequence data

The algal strains used, sequencing statistics, and accession numbers are listed in Supporting Information Table S1. Gametophytes were grown at 14°C and 2–3 µE m^{-2} s^{-1} from daylight type fluorescent lamps for 14 : 10 (light : dark) cycles for several weeks. Vegetative gametophytes were then transferred to conditions to induce fertility for 1 wk (20 µE m^{-2} s^{-1}), and were collected before sexual structures were visible.

RNA-sequencing (RNA-Seq) analysis was carried out to compare the relative abundances of gene transcripts in the different samples. For each sample, total RNA was extracted from two independent bulks of approximately 1000 male individuals and two bulks of 1000 female individuals (two biological replicates for each sex) using the Qiagen Mini kit (http://www.qiagen.com) as previously described (Lipinska *et al.*, 2015b; Arun *et al.*, 2019). RNA quality and quantity were assessed using an Agilent 2100 bioanalyser, associated with an RNA 6000 Nano kit.

For each replicate, the RNA was quantified and complementary DNA (cDNA) was synthesized using an oligo-dT primer. The cDNA was fragmented, cloned, and sequenced by Fasteris (Plan-les-Ouates, CH-1228, Switzerland) using an Illumina Hiseq 2000 set to generate 150-bp single-end reads. Data quality was assessed using FastQC (Wingett & Andrews, 2018). Reads were trimmed and filtered using Trimmomatic (Bolger *et al.*, 2014) with average quality > 28, a quality threshold of 24 (base calling) and a minimal size of 60 bp.

Filtered reads were mapped to the *M. pyrifera* haploid genome (Lipinska *et al.*, 2019) using TopHat2 (Kim *et al.*, 2013) with the Bowtie2 aligner (Langmead & Salzberg, 2012).

More than 70% of the sequencing reads for each library could be mapped to the genome (Table S1). The mapped sequencing data were then processed with FeatureCounts (Liao *et al.*, 2014) to obtain counts for sequencing reads mapped to exons and counts by gene. Transcript abundances, measured as transcript per million (TPM) were strongly correlated between biological replicates of each sample (Fig. S5).

Expression values were represented as TPM; any genes with TPM value above the fifth percentile is considered as expressed. This resulted in a total of 19 208 genes with expression in at least one of the three strains.

Identification of sex-biased genes

The filtering steps described earlier yielded a set of expressed genes in the transcriptome that were then classified based on their sex-expression patterns. Differential expression analysis was performed with the DESeq2 package (Love *et al.*, 2014) (Bioconductor). Genes were considered to be male-biased or female-biased if they exhibited at least a two-fold difference in expression between generations with a false discovery rate (FDR) of < 0.05. Sex-biased genes were defined as sex-specific when the TPM was below the fifth percentile for one of the sexes. Full lists of sex-biased genes can be found in Table S3. Note that sex-linked genes (Lipinska *et al.*, 2017) were removed from the analysis.

Flow cytometry

Flow cytometry was performed as described earlier for brown algal tissues (Bothwell *et al.*, 2010). Gametophyte tissue was finely cut with a razor blade and nuclei were isolated by suspension in nuclei buffer (30 mM MgCl, 120 mM trisodium citrate, 120 mM sorbitol, 55 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), pH 8, 5 mM EDTA supplemented with 0.1% (v/v) Triton X-100 and 5 mM sodium bisulphite; pH 8.0), and their DNA content was measured immediately by flow cytometry. Between 600 and 13 200 nuclei were analysed in each sample. Male wild-type gametophytes were considered to be haploid and were used as an internal reference for the determination of ploidy. The nucleic acid-specific stain SYBR Green I (http://www.invitrogen.com) was used at a final dilution of 1 : 10 000. Samples were analysed using a FACSort flow cytometer (http://www.bsbiosciences.com).

Results

Identification of a genetically male line exhibiting female-like morphology

During a screen of colchicine treated *M. pyrifera* gametophytes, we identified a genetically male line (Mpyr-13-4) that exhibited morphological features resembling those of female gametophytes (Figs 1, S1). To determine whether the observed phenotypes were

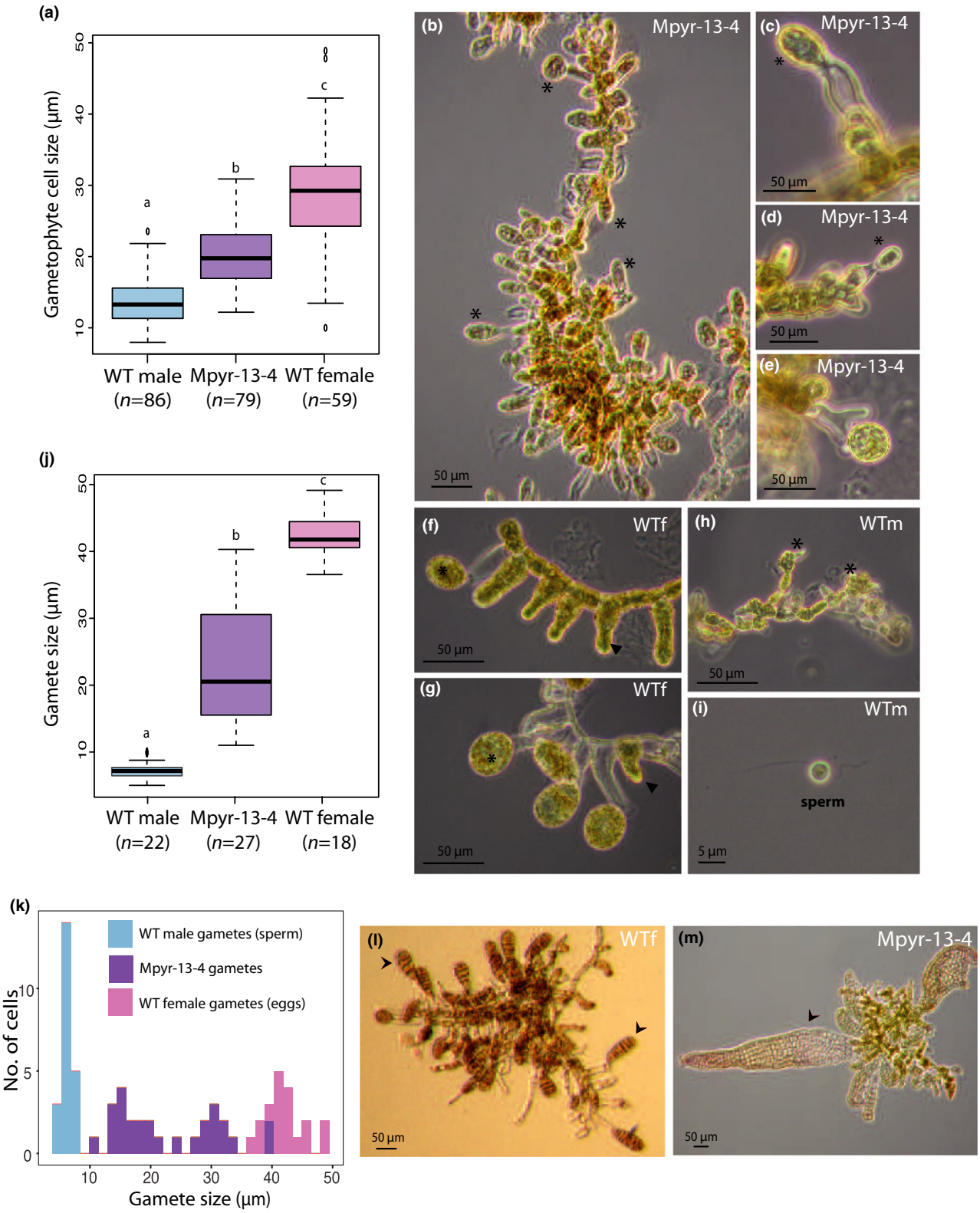


Fig. 1 Phenotypic characterization of wild-type male and female gametophytes and the variant Mpyr-13-4 line. (a) Sizes of gametophyte cells in wild-type male, wild-type female and Mpyr-13-4 individuals. (b–e) Fertile Mpyr-13-4 gametophytes showing several egg-like structures of different sizes (asterisks). (f,g) Fertile wild-type female gametophyte with extruded eggs (asterisks) and oogonia (arrow head). (h) Fertile wild-type male gametophyte with antheridia (asterisks) and sperm (i,j) Gamete sizes in wild-type males, wild-type females and Mpyr-13-4. Different letters above the plots indicate significant differences (Wilcoxon rank sum test, *P*-value < 0.001). (k) Distribution of the gamete sizes for wild-type male, wild-type female and Mpyr-13-4 variant lines. (l) Parthenogenetic sporophytes in wild-type females (arrow heads). (m) Parthenogenetic sporophytes in Mpyr-13-4 (arrow heads).

due to polyploidy or aneuploidy induced by the colchicine treatment, we measured the ploidy level of Mpyr-13-4 using flow cytometry. This analysis showed no evidence for chromosome doubling (Fig. S2), indicating that the colchicine treatment had not caused large scale chromosomic modifications.

Phenotypic characterization of the feminized line compared with wild-type males and females

A detailed morphometric study of gametophytes of the Mpyr-13-4 strain showed the cells of this strain were significantly larger than those of the wild-type male strain (Wilcoxon test $W = 31$, P-value = 6.27e-10) (Fig. 1a), exhibiting intermediate size between those of cells of male and female wild-type strains.

Following induction of gametogenesis, Mpyr-13-4 gametophytes formed reproductive structures (Fig. 1b–e) that strongly resembled wild-type female oogonia (Fig. 1f,g) and not male antheridia (Fig. 1h–i). At maturity, Mpyr-13-4 gametophytes produced egg-like cells, lacking the flagella typically present in male gametes, with diameters ranging from 10 to 40 µm. Wild-type eggs are typically around 40 µm in diameter (Fig. 1k).

In absence of fertilization by gametes of the opposite sex, wild-type female gametes of *M. pyrifera* initiate parthenogenetic development within 48 h (Fig. 1l). Wild-type male gametes do not undergo parthenogenesis. Mpyr-13-4 gametes, regardless of their size, initiated parthenogenetic development within 48 h (Fig. 1m).

Note that the female-like phenotypes of Mpyr-13-4 were stably maintained through (asexual) vegetative reproduction, and were not affected by culture conditions (10°C, 15°C, high/low white light, red light).

Taken together, these analyses indicated that despite being genetically male, Mpyr-13-4 individuals exhibit a feminized phenotype, with several phenotypic features more typical of females, including gametophyte cell size, presence of oogonia-like structures, gamete cell size and parthenogenetic capacity.

Mpyr-13-4 gametes do not produce the male-attracting pheromone lamoxirene

In order to test if gametes produced by the feminized strain were fully functional female gametes, we performed test crosses (Westermeier *et al.*, 2007). When Mpyr-13-4 gametes were confronted with active sperm from an unrelated, wild-type *M. pyrifera* male line, no gamete interaction was observed, and no zygotes were produced.

Wild-type female gametes attract male gametes by producing a pheromone (lamoxirene; (Maier *et al.*, 2001). This pheromone is responsible both for the release of sperm from male antheridea and for the attraction of sperm towards the eggs. We therefore investigated whether Mpyr-13-4 gametes were capable of producing lamoxirene. GC-MS analysis of *M. pyrifera* detected lamoxirene in control wild-type female gametophytes, but failed to detect lamoxirene or other C11 hydrocarbons in the feminized Mpyr-13-4 fertile gametophytes (Fig. 2). This observation suggests that despite presenting phenotypic features typical of a female, the Mpyr-13-4 strain is not fully recapitulating the female developmental programme.

Gene expression in Mpyr-13-4 compared with wild-type male and female gametophytes

To determine whether the morphological feminization of the variant male strain was associated with changes in terms of transcriptional landscape compared with wild-type male and female gametophytes, transcript abundances were measured by RNA-Seq analysis of wild-type male, wild-type female and Mpyr-13-4 gametophytes (Table S1 and Methods section for details).

This analysis identified 17 922, 18 436 and 17 744 expressed genes (defined as TPM > fifth percentile) in males, females and the Mpyr-13-4 strain, respectively, of the total of 22 242 genes that have been annotated in the *M. pyrifera* genome (Lipinska *et al.*, 2019; Table S2; Figs S3, S4). Hence, 81%, 82% and 80% of the total number of annotated genes were detected as being expressed in wild-type male, wild-type female and Mpyr-13-3 gametophytes, respectively.

The majority (approximately 94%) of the transcriptome was expressed in all three samples but a slightly greater number of genes were uniquely expressed in wild-type females than in wild-type males and Mpyr-13-4 (3%, 1.8% and 0.69% of their transcriptomes, respectively; Fig. S4). Interestingly, we noticed that the variant Mpyr13-4 strain shared more expressed genes with wild-type females than with wild-type males (610 and 327 genes respectively; Fig. S4). Moreover, a sample distance matrix showed a closer relationship between wild-type female and Mpyr-13-4 samples compared with wild-type male (Fig. S5).

Sex-biased gene expression in wild-type and Mpyr-13-4 lines

In order to examine if the line Mpyr-13-4 was feminized and/or demasculinized at the level of gene expression, we next analysed the patterns of expression of sex-biased genes in each of the samples.

DESeq2 analyses identified 5442 genes that were differentially expressed between males and females, indicating that a considerable proportion (24.5%) of the transcriptome of *M. pyrifera* exhibits sex-biased expression (Tables S2, S3). Approximately the same numbers of genes were found to be male-biased (2785) and female-biased (2657) (Tables S2, S3; Fig. 3a). Consistent with the female-like phenotype of Mpyr13-4, the transcriptomic profile of this strain was more similar to the wild-type female profile (only 5.8% differentially expressed genes) than the wild-type males gene expression landscape (20.8% differentially expressed genes) (Tables S2–S4; Fig. 3a).

Hierarchical clustering of expression levels was used to visualize gene transcription patterns for wild-type male, female and variant Mpyr-13-4 samples. Wild-type female and Mpyr-13-4 samples consistently clustered together, indicating that the transcriptome of the Mpyr-13-4 strain was more similar to that of a wild-type female than to that of a male (Fig. 3b). Principal

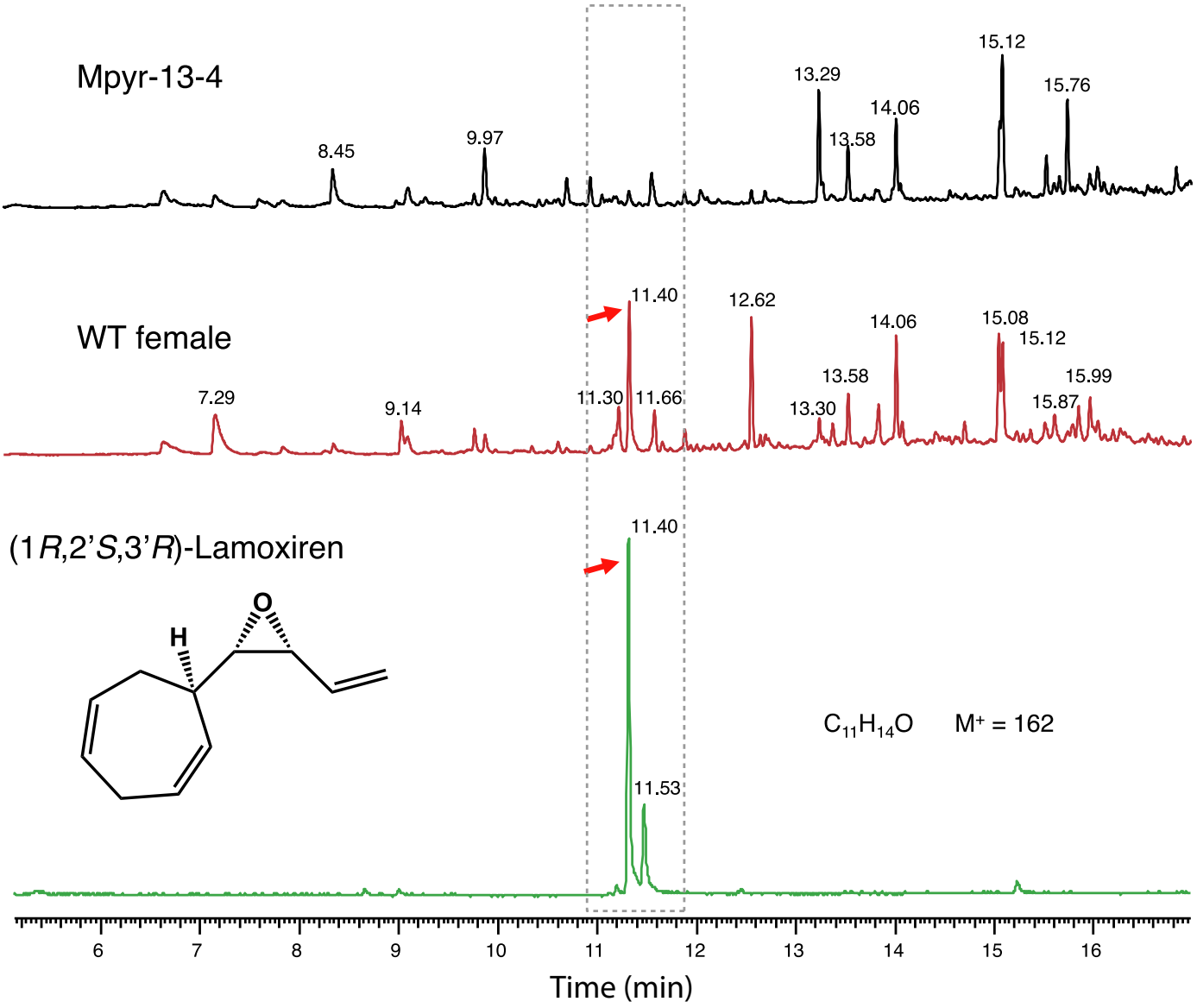


Fig. 1 Gaschromatography-massspectrometry (GS-MS) assay for lamoxirene detection in wild-type females and Mpyr-13-4 lines. Red arrows indicate the peak of lamoxirene.

component analysis (PCA) further supported this conclusion (Fig. S3).

In order to examine how sex-biased gene expression is affected by the phenotypic feminization of the variant Mpyr-13-4 strain, we next focused on the sets of genes that had been defined as sex-biased in the wild-type strains. The median level of expression (measured as $\log_2$(TPM + 1)) of the male-biased gene set in Mpyr-13-4 gametophytes was 79.4% lower than that observed in wild-type males; (Wilcoxon test, $P < 2.2E\text{-}16$, Fig. 4a), suggesting these genes are transcriptionally 'de-masculinized' in Mpyr-13-4. In contrast, female-biased genes were expressed at a higher level in Myr-13-4 (31.6% more) than in wild-type males (Wilcoxon test, $P < 2.2E\text{-}16$, Fig. 4a), suggesting that Mpyr-13-4 is transcriptionally 'feminized'. Finally, genes with unbiased expression ($n = 13\,766$) showed no average change in expression between wild-type male and females and variant strain (Fig. S6).

Transcript abundance fold changes (FCs) in pairwise comparisons between wild-type males, wild-type females and in Mpyr-13-4 also revealed a pattern of simultaneous feminization and de-masculinization of the transcriptome of Mpyr-13-4 (Fig. 4b).

However, we noticed that a subset of female-biased genes that was completely silenced in wild-type males was also not expressed in Mpyr-13-4 (Figs 3b, 4b). Conversely, male biased genes that were silenced or lowly expressed in wild-type females were expressed in Mpyr-13-4 (Figs 3b, 4b).

Taken together, these observations indicate that although the transcriptome of Mpyr-13-4 was clearly feminized and de-masculinized, there was not a complete shift to the female transcriptional programme.

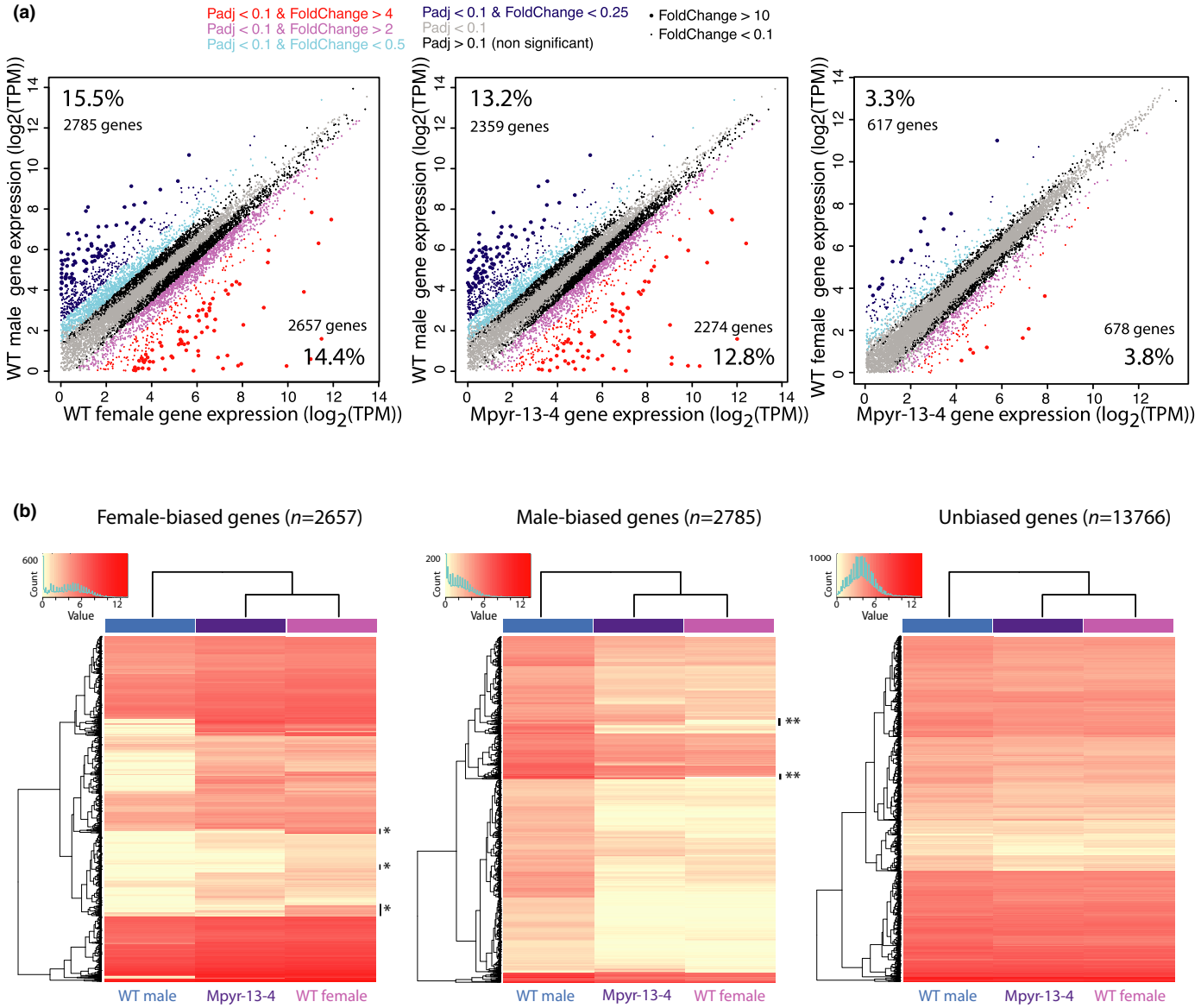
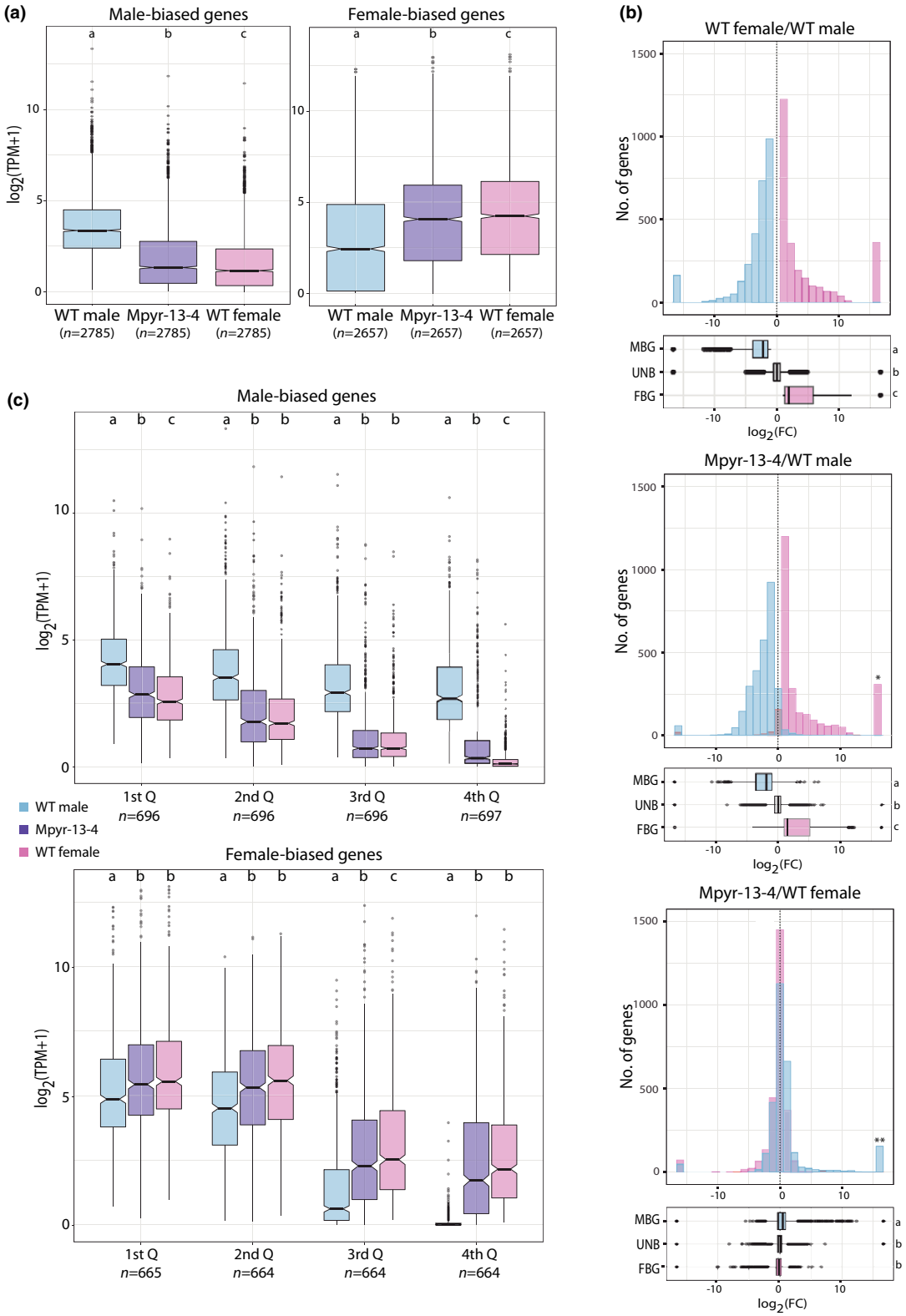


Fig. 3 Sex-biased gene expression (a) Comparisons of gene transcript abundances (log_2(TPMs)) in wild-type male, wild-type female and the Mpyr-13-4 line. Genes whose expression was zero in one of the samples were removed from the plot. The percentages of biased genes in each sample are indicated in the upper left-hand and lower right-hand corners. (b) Heat maps and hierarchical clustering of gene expression for wild-type male, wild-type female and Mpyr-13-4 variant line. Shown is the relative expression for autosomal male-biased, female-biased and unbiased genes. Hierarchical gene clustering is based on Euclidean distance for average log_2 expression of each gene for the three samples.

To examine in more detail the relationship between degree of sex-biased gene expression (FC in transcript abundance between the wild-type female and male) and transcript abundance, the sex-biased genes were grouped according to FC differences between the male and female samples and median transcript abundances plotted for wild-type males, wild-type females and Mpyr-13-4. The global pattern of de-masculinization and feminization of Mpyr-13-4 transcription appeared to be strongly

Fig. 4 (a) Abundances of transcripts per million (TPMs) of male- and female-biased genes in wild-type males, wild-type females and Mpyr-13-4. (b) Transcript abundance fold changes in pairwise comparisons between wild-type males, wild-type females and in Mpyr-13-4. Upper panel: wild-type female vs wild-type male. Positive values in the *x*-axis correspond to higher expression in females, negative values to higher expression in males. Middle panel: gene expression in Mp-13-4 vs wild-type male gametophytes. Positive values on the *x*-axis correspond to higher expression in the variant Mpyr-13-4, negative values to higher expression in wild-type males. Lower panel: positive values on the *x*-axis correspond to higher expression in Mpyr-13-4, negative values to higher expression in wild-type females. Colours indicate male-biased (blue), female-biased (pink) and unbiased (grey) genes. For clarity, unbiased genes are omitted from the histogram. Different letters indicate significant differences (Wilcoxon rank sum test, *P*-value < 0.01). (c) Male-biased (upper panel) and female-biased (lower panel) genes ranked by level of bias (log_2(TPM + 1)). Numbers of genes are indicated below the plots. Note that only genes with TPM > 0 are included in the plots. Significant differences detected following Wilcoxon tests (*P*-values < 0.01) are indicated as different letters above the plots.

correlated with the degree of sex-bias (Fig. 4c). Downregulation of the male-biased gene set in Mpyr-13-4 was more pronounced for the genes that showed the highest levels of male-bias, suggesting that the male-biased genes that exhibit the greatest FCs make the greatest contribution to male-specific traits in the variant strain. Similarly, stronger upregulation was observed in Mpyr-13-4 for the genes that exhibited the highest levels of female-bias (Fig. 4c).



Taken together, these results indicate the transcriptome of Mpyr-13-4 is both de-masculinized and feminized, with the strongest effect on genes that exhibit strong sex-biased expression patterns (i.e. highest FC between wild-type male and female).

Predicted functions of genes involved in feminization

An analysis of gene ontology (GO) terms associated with the sex-biased genes and with the genes differentially expressed in Mpyr-13-4 was carried out using BLAST2GO (Conesa & Götz, 2008). The aim of this analysis was to search for enrichment in particular functional groups and to relate gene function to phenotypic feminization. Significant enrichment of specific GO categories related to microtubule- and flagella-related processes was detected for genes that were downregulated in Mpyr-13-4 compared with wild-type males (Table S5). These genes may be involved in the production of flagellated gametes, which are not produced by Mpyr-13-4. Note that these two GO categories were also enriched in the set of male-biased genes expressed in male gametes identified by Lipinska *et al.* (2013) and in fertile male gametophytes (Lipinska *et al.*, 2015b, 2019). The female-biased genes that were upregulated in Mpyr-13-4 compared to wild-type males were enriched in GO terms related to metabolism and photosynthesis (Table S5).

We identified a set of 312 genes likely involved in the female developmental programme whose regulation depends on the presence of the U-specific region (genes that were over-expressed in wild-type females compared with wild-type males and Mpyr-13-4). GO term analysis revealed an enrichment in metabolic pathways-related and oxidation-reduction functions (Table S5).

Expression of male sex-determining region genes in the Mpyr-13-4 line

The feminized phenotype of the Mpyr-13-4 strain could be consistent with modification of the expression of a sex-determining gene or genes carried by the VSR. We thus specifically focused on the expression patterns of previously identified male-linked *M. pyrifera* genes (Lipinska *et al.*, 2017). For the majority (five out of seven) of these genes, similar transcript abundances were detected in wild-type males and the Mpyr-13-4 strain (Fig. 5). However, two genes, gHMG.13001750 and gSDR.13001840, were markedly downregulated in the Mpyr-13-4 line compared with wild-type males. The orthologues of these two genes in *Ectocarpus* are Ec-13-001750 and Ec-13-001840, respectively. Interestingly, Ec-13-001750 is the candidate master-sex-determining gene in *Ectocarpus* sp. (Ahmed *et al.*, 2014).

Discussion

Transcriptome de-masculinization and feminization underlie the phenotype of Mpyr-13-4

Gametophytes of the giant kelp variant line Mpyr-13-4 exhibited female phenotypic characteristics despite being genetically male. We used wild-type male and female lines to examine whether the degree of sex-biased gene expression is associated with phenotypic sexual dimorphism, and to understand the role of gene expression in encoding the variant morphology. Our results reveal a simultaneous de-masculinization and feminization of the transcriptome of the Mpyr-13-4 line and underline the association between sex-specific phenotypes and sex-specific transcriptomic patterns. The overall pattern of de-masculinization and feminization of Mpyr-13-4 transcription was strongly correlated with the degree of sex-bias (measured as FC in expression between wild-type male and wild-type female). De-masculinization of gene expression in the variant line was more pronounced for genes that exhibited the strongest male-bias (highest FC), possibly suggesting that the most extreme male-biased genes make the greatest contribution to male-specific traits in the giant kelp. Similarly, feminization of the variant transcriptome manifested itself as increased expression of female-biased genes, and was more marked for the genes with highest FCs between wild-type male and wild-type female. A similar tendency was observed in animals with ZW systems, where masculinization of gene expression was associated with increased male sexual dimorphism, and the genes with most extreme sex-bias make the greatest contribution to sex-specific traits (Pointer *et al.*, 2013). Our results therefore extend the link between sexually dimorphic transcription and sexually dimorphic phenotypes to haploid sex determining systems.

Despite a clear de-masculinization and feminization of the transcriptome of the variant line, there was not a full shift to the female transcriptomic programme and, accordingly, the phenotype of the variant line did not fully recapitulate the female developmental programme.

The female sex-determining region may be required to fully express the female developmental programme

Despite the phenotypic resemblance to a female, the Mpyr-13-4 gametophyte produced nonfunctional eggs, which failed to attract male gametes. GC-MS analyses revealed absence of pheromone production in the eggs-like cells of the variant line, so it appears that the factor(s) required to initiate the pheromone pathway is absent or not functional in this line. Pheromone production may be regulated directly (or indirectly) by a gene(s) located in the SDR, similar to the case of yeast where the mating type linked Ste-20 regulates mating through pheromone response pathways (Leberer *et al.*, 1992). One gene within the *M. pyrifera* sex-linked region is a Ste-20-like serine-threonine kinase (Lipinska *et al.*, 2017), but currently there is no evidence for a role of this gene in pheromone production in brown algae.

Interestingly, examination of the predicted functions of the list of genes that were over-expressed in wild-type females but not in wild-type males nor Mpyr-13-4, i.e. presumably the genes that may be regulated by the U-specific region, revealed an enrichment in metabolism-related and oxidation-reduction functions. In pheromone producing stages of yeast, significant increases in the transcript levels of a multitude of metabolic enzymes are also observed (Williams *et al.*, 2016). It is therefore possible that among these wild-type female-limited genes there are

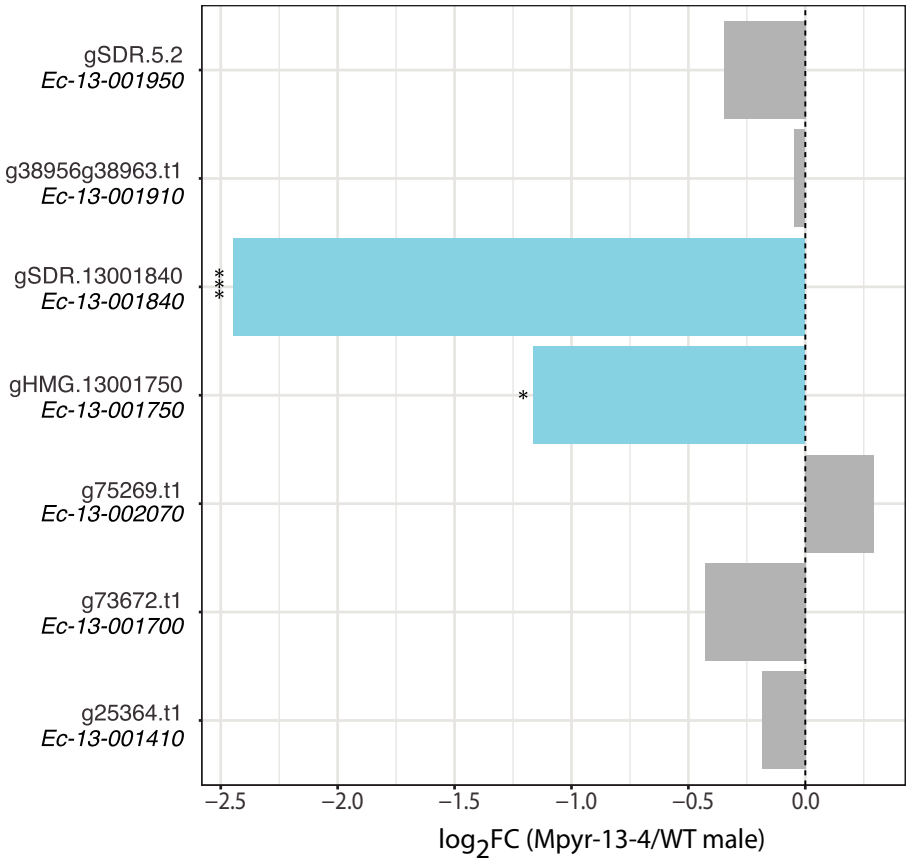


Fig. 5 Differential expression (DESeq2) of *Macrocystis pyrifera* orthologues of Ectocarpus male sex-determining region (SDR) genes in Mpyr-13-4 vs wild-type males. Negative values represent downregulation (log_2(FC)) in the variant Mpyr-13-4 strain relative to the wild-type male. Asterisks indicate significant differences in expression level: *, $P < 0.01$; ***, $P < 0.0001$.

components of the giant kelp pheromone biosynthetic cascade or its regulators. Another possibility is that autosomal genes potentially involved in the pheromone biosynthesis pathway may have been affected in the Mpyr13-4 line.

Taken together, our observations are coherent with incomplete sex-reversal of Mpyr-13-4, and suggest that the female SDR is required to fully express the female programme of development in this UV system.

Role of the sex-determining region and autosomal gene expression in the initiation of sex-specific developmental programmes

Although Mpyr-13-4 is not a fully male-to-female sex reversed line, analysis of the Mpyr-13-4 line contributed to understanding the role of sex-linked genes and autosomal gene expression in the initiation of the male and female developmental programmes. Our observations indicate that many of the female developmental features depend exclusively on autosomal gene expression and do not require the presence of the female SDR. One particularly interesting feature was the capacity of the variant line to undergo parthenogenesis, a trait that is typical of females of oogamous brown algal species (Luthringer *et al.*, 2015). In the model brown alga *Ectocarpus*, parthenogenesis is a complex genetic trait under the control of the SDR, together with at least two additional autosomal loci (Mignerot *et al.*, 2019). It has been suggested that either the male SDR produces a repressor of parthenogenesis, or, alternatively, the female SDR produces an activator of parthenogenesis (in either case the activator or repressor could be directly encoded by the SDR or produced indirectly as part of the male or female sex-differentiation programmes). Taking into consideration the parthenogenetic development of the egg-like cells of the Mpyr-13-4 line (which lack the female SDR), observed in this study, it appears clear that the female SDR is not required for parthenogenesis to be initiated in kelps. Therefore, parthenogenesis may involve a repressor being produced by the male SDR, as part of the male-differentiation programme or, alternatively, parthenogenesis could be induced as part of the female sex-differentiation programme but independently of the female SDR.

The transcriptional landscape underlying sexual differentiation in the giant kelp

Sex-biased gene expression has been characterized for the brown alga *Ectocarpus*, a near-isogamous species with a low level of sexual dimorphism (Lipinska *et al.*, 2015b; Luthringer *et al.*, 2015). Here we show that substantially more genes are sex-biased in the giant kelp (about 24% of the transcriptome, compared with less than 10% in *Ectocarpus* (Lipinska *et al.*, 2015b)). The higher proportion of sex-biased genes in the giant kelp is consistent with the higher level of phenotypic sexual dimorphism in this organism, where male and female gametophytes have clearly distinct morphologies (Muller *et al.*, 1979).

V-linked genes potentially required for male developmental patterns

Two genes located within the giant kelp male SDR exhibited a significant reduction in transcript abundance in the Mpyr-13-4 line, suggesting their involvement in the variant phenotype. One of these genes is an HMG-domain protein coding gene, with orthologues present with the VSR across a range of brown algae and thought to be the master male sex determining gene for this group of organisms (Ahmed *et al.*, 2014; Lipinska *et al.*, 2017). The other gene is a membrane-located protein-coding gene with a putative sugar binding and cell adhesion domains. Both genes belong to a small set of genes that have been conservatively sex linked across all the brown algae studied so far (Lipinska *et al.*, 2019). Our observations suggest that these ancestrally male-linked genes are involved in the development of male functional characteristics, although mechanistic studies will be necessary to fully validate their role in male sex determination and differentiation.

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Author contributions

DGM and SMC planned and designed the research; DGM, WB, MK, AFP, JG, RW and SMC performed experiments; EG, APL, JMC, GC, OG and SMC analysed data; SMC wrote the manuscript with input from all authors.

ORCID

J. Mark Cock https://orcid.org/0000-0002-2650-0383
Susana M. Coelho https://orcid.org/0000-0002-9171-2550

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Pedigree of the *Macrocystis pyrifera* strains used in this study.

Fig. S2 Flow cytometry analysis of a wild-type male line (top) and the Mpyr-13-4 line (bottom) of *Macrocystis pyrifera*.

Fig. S3 PCA was used to compare transcript abundance patterns across samples. The two dimensions represent 79% and 20% of the variance.

Fig. S4 Venn diagram showing the sets of expressed genes (TPM > fifth percentile) in wild-type male, wild-type female and variant Mpyr-13-4 lines and the overlap between the three sets.

Fig. S5 Expression heatmap of sample-to-sample distances on the matrix of variance-stabilized data for overall gene expression.

Fig. S6 Box-plots representing the levels of expression ($\log_2$(TPM + 1)) of sex-biased genes in wild-type males, Mpyr-13-4 and wild-type females of *Macrocystis pyrifera*.

Table S1 List of strains used and genome and transcriptome assembly statistics.

Table S2 Number of biased genes from DESeq2 analysis and categories of sex-biased genes with different levels of fold change (FC) between the three samples. Only genes with transcript per million (TPM) > fifth percentile in at least one of the samples were considered for the analysis.

Table S3 Differential expression levels (DESeq2, FC > 2, P_{adj} < 0.05) between wild-type male, wild-type female and Mpyr-13-4 samples.

Table S4 Gene expression (measured as transcript per million (TPM)) in wild-type males, wild-type females and Mpyr-13-4 variant strain.

Table S5 Gene ontology terms significantly enriched among biased genes (Fisher exact test, false discovery rate (FDR) < 5%).

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