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1 **Evolution of sex-biased gene expression in a dioecious plant**

2

3

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14

15 **Abstract**

16 Separate sexes and sex-biased gene expression have repeatedly
17 evolved in animals and plants, but the underlying changes in gene
18 expression remain unknown. Here we studied a pair of plant species, one
19 in which separate sexes and sex chromosomes evolved recently and one
20 which maintained hermaphrodite flowers resembling the ancestral state,
21 to reconstruct expression changes associated with the evolution of dioecy.
22 We find that sex-biased gene expression has evolved in autosomal and
23 sex-linked genes in the dioecious species. Most expression changes
24 relative to hermaphrodite flowers occurred in females rather than males,
25 with higher and lower expression in females leading to female-biased and
26 male-biased expression, respectively. Expression changes were
27 commoner in genes located on the sex chromosomes than the
28 autosomes and led to feminisation of the X and masculinisation of the Y
29 chromosome. Our results support a scenario in which sex-biased gene
30 expression evolved during the evolution of dioecy to resolve intralocus
31 sexual conflicts over the allocation of resources.

32

33 Females and males of many plant and animal species differ in
34 morphological, physiological and ecological characteristics, despite their
35 overall genetic similarity ^{1,2}. Such sexual dimorphism can evolve through
36 sex-limited or sex-biased expression of genes that are present in both
37 sexes, or through complete sex-linkage, when a gene or allele is
38 restricted to the genome of just one sex ¹⁻⁵. Sex-biased and sex-limited
39 gene expression, and enrichment of such genes in fully sex-linked
40 regions, are well documented in animals ^{1,6-8}, including humans ⁹, and
41 have recently been studied also in plants and algae ¹⁰⁻¹³, but the
42 evolutionary changes that actually led to expression differences between
43 the sexes remain unknown.

44 Sex-biased gene expression can evolve through changes in
45 expression in either one or both sexes. For example, female-biased
46 expression may evolve by increased expression in females, decreased
47 expression in males, or a combination of both (Fig. 1a, scenarios I-III). In
48 many dioecious organisms, these evolutionary changes cannot be
49 studied because separate sexes evolved too long ago. Species in which
50 they evolved more recently, such as some plants, are therefore of great
51 interest, because gene expression changes can be inferred from
52 comparisons with related species without separate sexes, which should
53 often represent the ancestral state (Fig. 1b). Such changes provide new
54 information about the role of sex-linked and sexually antagonistic genes
55 in the evolution of separate sexes ¹⁴.

56 Here, we study the evolution of sex-biased gene expression in
57 dioecious *Silene latifolia* Poiret (White Campion), a plant model for sex

58 chromosome evolution ¹⁵⁻²⁰. In the genus *Silene*, gynodioecy, the co-
59 existence of hermaphrodites and females in the same population,
60 represents the ancestral state, and dioecy (separate sexes) has evolved
61 at least twice independently ²¹. *Silene vulgaris* (Moench) Garcke is
62 gynodioecious and closely related to *S. latifolia* ¹⁵, in which female and
63 male flowers and inflorescences are sexually dimorphic ²² (Fig. 2a) and
64 both fully and partially sex-linked quantitative trait loci affecting sexually
65 dimorphic traits have been inferred ²³. *Silene latifolia* has an XY sex-
66 determination system with heteromorphic sex chromosomes that have
67 evolved within the past 5-10 MY ^{24,25}. Its Y chromosome is much less
68 degenerated than in ancient animal sex chromosome systems ^{17,18,26}.

69 We used comparative mRNA-seq transcriptome sequencing to
70 assess gene expression differences between *S. latifolia* females and
71 males and investigate evolutionary changes in gene expression in each
72 sex from the likely ancestral state represented by flowers of *S. vulgaris*
73 hermaphrodites. We further test for differential representation of sex-
74 biased genes on the sex chromosomes and autosomes, and assess
75 allele-specific changes in the expression of sex-linked genes to test for
76 feminisation and masculinisation of the X and Y chromosome,
77 respectively. We find that the evolution of sex-biased expression in *S.*
78 *latifolia* primarily involved changes in females, and that the different
79 selective forces acting on the sex chromosomes have not yet led to
80 accumulation of genes with female-biased and male-biased expression
81 on the X and Y chromosome, respectively, but to subtler expression

82 changes causing feminisation of the X and masculinisation of the Y
83 chromosome.

84

85 **Results**

86 **The extent of sex-biased gene expression.**

87 We analysed mRNA-seq data from Illumina 100 bp paired-end reads
88 obtained from developing flower buds and rosette leaves of female and
89 male *S. latifolia*, and flower buds of hermaphrodite *S. vulgaris*. We
90 obtained 145 Gb of RNA-seq from flower buds of seven female and
91 seven male *S. latifolia* individuals, and 41 Gb from rosette leaves from
92 four individuals of each sex. From the flower buds of five *S. vulgaris*
93 hermaphrodites we obtained 33 Gb of transcriptome data. 58% and 57%
94 of the *S. latifolia* reads from flower buds and rosette leaves, respectively,
95 and 44% of the *S. vulgaris* reads, matched sequences in the *S. latifolia*
96 flower bud reference transcriptome (for details see Supplementary Table
97 1). The lower percentage for *S. vulgaris* probably reflects sequence
98 divergence between the two species ²⁴.

99 We used flower buds in our expression analyses because sexual
100 dimorphism in *S. latifolia* is stronger for flower and inflorescence traits
101 than for other characters ²². However, some sex differences in gene
102 expression in buds must be due simply to the presence or absence of the
103 sex organs (referred to as “primary differences” in Fig. 1b). Therefore,
104 before quantifying sex-bias in gene expression, or counting numbers of
105 genes with evolved expression differences between the sexes, we

106 excluded all 903 contigs exhibiting sex-limited expression in this data set
107 (i.e. expressed in buds of only one sex in *S. latifolia*). Among the
108 remaining 11,366 *S. latifolia* contigs with at least some expression in
109 buds of both sexes, many showed significant sex differences in
110 expression (Supplementary Fig. 1, Supplementary Table 2). The results
111 are robust to different normalisation procedures and estimators of gene
112 expression differences (Supplementary Fig. 2), and, for 16 genes tested,
113 agree well with qRT-PCR results (Supplementary Fig. 3; Spearman
114 correlation; $\rho=0.92$; $p<0.0001$). GO analysis revealed several biological
115 processes that are significantly over-represented among female-biased
116 genes but under-represented among male-biased genes, or vice versa,
117 suggesting that sex-biased expression has evolved to support contrasting
118 biological functions in *S. latifolia* females and males. Specifically, female-
119 biased contigs are enriched for transcription factors involved in cell-cycle
120 and developmental functions, but depleted in genes involved in
121 catabolism (Supplementary Table 3), while male-biased contigs are
122 enriched in genes involved in carbohydrate, lipid, and secondary
123 metabolite metabolism, transport, and responses to various stimuli, and
124 depleted in genes involved in nucleic acid metabolism and protein
125 synthesis and modification.

126 We divided the contigs expressed in buds into autosomal, sex-linked
127 (defined as contigs having both X- and Y-linked alleles) and X
128 hemizygous contigs (sex-linked, but with an expressed copy on the X
129 only). These categories were inferred from SNPs segregating in a full-sib
130 family, using a probabilistic model ²⁷. 2,142 *S. latifolia* bud-expressed

131 autosomal contigs (16.8% of such contigs) had significantly sex-biased
132 expression (Supplementary Table 2), 7.2% with female and 9.6% with
133 male-biased expression (Fig. 2b). Sex-biased expression is commoner
134 among the 936 fully sex-linked contigs (28.8% overall, with respectively
135 13.8% and 15.0% having female and male-biased expression; Fig. 2b
136 and Supplementary Table 2). Female-biased expression of sex-linked
137 genes may reflect either higher expression in females or lower expression
138 in males caused by reduced expression of the Y-linked allele if dosage
139 compensation is absent or incomplete. These alternatives are notoriously
140 difficult to distinguish ²⁸. Incomplete dosage compensation is widely
141 observed in animals ¹. In *S. latifolia*, evidence for dosage compensation
142 has been reported ^{20,29}, but not all genes are fully compensated ^{16,17,26}.
143 Apparent female-biased expression due to incomplete dosage
144 compensation should be displayed in both flower buds and vegetative
145 tissues ⁸. In order to exclude such genes, we therefore examined sex-
146 biased expression also in rosette leaves (which show overall less sex-
147 biased expression, see below). Of the 86 sex-linked contigs with female-
148 biased expression in flower buds that were sufficiently expressed in
149 rosette leaves, only 16 (18.6%) had female-biased expression
150 (Supplementary Fig. 4). Excluding these from our analysis, the sex
151 chromosomes still have a highly significant overrepresentation of contigs
152 with sex-biased expression (Fisher's exact test, $p < 0.0001$), but no
153 longer of contigs with female-biased expression (Fisher's exact test,
154 $p=0.7303$). The apparent over-representation of female-biased genes on

155 the sex chromosomes therefore probably reflects incomplete dosage
156 compensation in *S. latifolia*.

157 Many fewer genes showed sex-biased expression in rosette leaves than
158 in flower buds (Fisher's exact test, $p < 0.0001$), consistent with sexual
159 dimorphism in *S. latifolia* being stronger for flower and inflorescence traits
160 ²². This difference was observed for both autosomal and sex-linked
161 contigs (18.7-fold and 3.84-fold lower, respectively, see Fig. 2b and
162 Supplementary Table 2). Similar findings in *Rumex hastatulus* ³⁰ suggest
163 that sex bias may generally be low in plant vegetative tissues. As in buds,
164 genes with sex-biased expression in rosette leaves were over-
165 represented on the sex chromosomes (Fig. 2b). Among contigs
166 expressed in rosette leaves, female-bias was commonest, with 0.6% of
167 autosomal and 4.1% of sex-linked contigs showing female bias, and male
168 bias being significantly rarer (0.3% of autosomal and 3.4% of sex-linked
169 contigs, Supplementary Table 2, Fisher's exact test, $p < 0.0001$). The
170 higher frequency of female-biased contigs in leaves contrasts with our
171 finding of a higher proportion of contigs with male-biased expression in
172 buds. In *Asparagus officinalis* flower buds, genes with higher male than
173 female expression also predominated ¹¹, potentially reflecting sexual
174 selection acting on floral and inflorescence traits ^{31,32}.

175 **Evolution of sex-biased gene expression**

176 To investigate the evolutionary changes that have led to the observed
177 sex-biased gene expression in *S. latifolia*, we also estimated gene
178 expression in hermaphrodite flowers of gynodioecious *S. vulgaris*.

179 Because few genes show sex-biased expression in vegetative tissues of
180 *S. latifolia* we focus on expression changes in buds. Expression levels of
181 genes with no sex bias in expression in *S. latifolia* (white bars in Fig. 3)
182 are largely unchanged in females and males, relative to *S. vulgaris*
183 hermaphrodite flower buds, indicating that much of the gene expression
184 changes between the two species relates to the evolution of separate
185 sexes. Our results reveal fundamental changes in the transcriptome
186 associated with the evolution of dioecy, resulting in both male- and
187 female biases in expression (Fig. 1b), and revealing the changes that led
188 to them. For both autosomal and sex-linked contigs in *S. latifolia* (Fig. 3),
189 we found that the evolution of sex-biased expression mainly involves
190 changes in females: female-biased expression (red bars in Fig. 3) is due
191 primarily to higher expression in *S. latifolia* female buds, and the many
192 genes with male-biased expression (blue bars in Fig. 3) primarily result
193 from lower expression in females than in *S. vulgaris* hermaphrodites,
194 implying many changes in autosomal and X-linked genes. For both
195 autosomal and sex-linked contigs, gene expression changes in males are
196 much smaller than those in females, although the variances are high for
197 the more limited number of sex-linked contigs (Fig. 3). Similar patterns
198 were also found for both X-hemizygous contigs and contigs whose
199 genomic locations are unknown (called “undefined contigs” in
200 Supplementary Fig. 5).

201 For most contigs whose expression was studied, we inferred whether
202 they are autosomal or sex-linked, which required expression in both
203 sexes, and therefore genes with primary expression changes due simply

204 to loss of sex organs and functions (see above) are not included.
205 Secondary gene expression changes, including up- or down-regulation of
206 genes following establishment of a unisexual type in a population (Fig.
207 1b), are of greater interest, and may often benefit the sex in which
208 expression is changed ³³. Assuming that expression changes affect
209 fitness and are under selection, rather than reflecting neutral divergence
210 (evidence for selection is described below), three evolutionary scenarios
211 are possible (Fig. 1a). When an expression change is advantageous only
212 for one sex, increased expression of a gene may occur specifically in that
213 sex, with the other sex retaining the ancestral expression level. For
214 example, if the ancestral expression state is optimal for males but
215 suboptimal for females, a female-specific increase will be advantageous,
216 whereas selection favours retaining the ancestral expression state in
217 males, as it is already optimal (Scenario I). Similarly, in scenario II, the
218 ancestral expression state exceeds the optimum for males, favouring a
219 male-specific reduction in expression. In scenario III, the expression level
220 of a gene in the ancestral hermaphrodite (before dioecy evolved) was
221 non-optimal for both sexes, potentially because of trade-offs ³⁴, and this
222 was adjusted by evolutionary changes in both sexes after dioecy evolved.
223 Large expression changes in opposite directions in both sexes suggest
224 the evolution of changes in response to sexual antagonism at such genes,
225 increasing their expression in the sex where high expression is
226 advantageous, and reducing it in the other sex (Fig. 1a; scenario III).
227 Patterns corresponding to scenarios I and II are also compatible with the
228 hypothesis that sexual antagonisms underlie evolutionary changes in

229 gene expression between the sexes. If the optimal expression for one sex
230 is the same as the ancestral state, sex-biased gene expression can
231 evolve during the evolution of unisexuality, when expression is optimised in
232 the other sex to resolve ancestral antagonistic effects.

233 We inferred the directions of changes in expression by comparing the
234 expression of female- and male-biased genes in dioecious *S. latifolia* with
235 expression levels in hermaphroditic flowers of *S. vulgaris* (Supplementary
236 Fig. 6). Approximately half of the genes with sex-biased expression could
237 be assigned to scenarios I to III (Fig. 1a). Other changes leading to sex-
238 biased expression are possible, such as increased (or decreased)
239 expression in both sexes, relative to hermaphrodite flowers, but to
240 different extents; these, however, cannot be distinguished from species-
241 specific changes in expression between *S. latifolia* and *S. vulgaris* and
242 are therefore not discussed. Of the autosomal contigs with male-biased
243 expression, only a small proportion (14.9%) evolved through increased
244 expression in males (Fig. 4a, I, blue bar), whereas 39.4% have
245 undergone reduced expression in females (Fig. 4a, II, blue bar). In
246 marked contrast, a large percentage (42.1%) of autosomal genes with
247 female-biased expression in *S. latifolia* are more strongly expressed in *S.*
248 *latifolia* females than in hermaphroditic *S. vulgaris* flowers (Fig. 4a, I, red
249 bar), while only 11.2% evolved lower expression in males (Fig. 4a, II, red
250 bar). The results are similar for the smaller number of sex-linked (Fig. 4a)
251 and X-hemizygous contigs and contigs with undefined locations
252 (Supplementary Fig. 7). Thus sex-biased expression in the dioecious *S.*
253 *latifolia* evolved primarily through expression changes in females, rather

254 than males: higher expression in females led to female-biased expression,
255 of many genes, whereas male biases evolved mainly through reduced
256 expression in females.

257 **The role of selection in the evolution of sex-biased gene expression**

258 We estimated the proportions of genes with evidence for directional
259 selection underlying gene expression changes under scenarios I and II
260 above by computing ΔX values^{35,36}. Our results indicate that the great
261 majority of expression changes in females, but not in males, have been
262 driven by selection. Fig. 4 a-b shows the fractions of genes in the top
263 25% of ΔX values whose directions of change correspond to scenarios I
264 and II. We estimate that only about 50% of these autosomal expression
265 changes in males can be attributed to directional selection, whereas our
266 estimates are much higher for expression changes in females (73% for
267 up-regulations creating female-biased contigs, and 96% for down-
268 regulations creating male-biased contigs). Expression changes in one sex,
269 without change in the other, were much commoner than significant
270 expression changes in opposite directions in the two sexes (scenario III in
271 Fig. 1a); almost all of these genes are male-biased in *S. latifolia* (Fig. 4c).
272 In animals, male-biased genes are also often commoner than female-
273 biased genes, and tend to evolve more rapidly, possibly because sexually
274 antagonistic selection is stronger in males¹. Consistently, male-biased
275 expression in *Drosophila* was inferred to result primarily from adaptive
276 changes in the male transcriptome³⁷. In *S. latifolia*, although there are
277 many male-biased genes, these do not indicate stronger sexually

278 antagonistic selection in males, because they evolved through reduced
279 expression in females.

280 **Sex-biased expression on sex chromosomes**

281 In dioecious species, tertiary changes in gene expression may follow the
282 evolution of sex chromosomes with non-recombining regions (Fig. 1b,
283 Step iii) and include expression changes that are specific to the X and Y
284 chromosome. Overall, the expression changes inferred for sex-linked
285 contigs are consistent with those for autosomal contigs, but the proportion
286 of genes with changes in males was slightly higher than for autosomal
287 contigs (Fig. 4). Evidence for a selective advantage of expression
288 changes on the sex chromosomes was again strongest for changes in
289 females (82% and 100% of contigs with higher and lower expression in
290 females, respectively, are in the top 25% of ΔX values; Fig. 4b), and
291 higher proportions of contigs were inferred to have decreased expression
292 as a consequence of selection than to have undergone increases (Fig.
293 4b), suggesting that selection may be strong to reduce fitness costs at
294 loci on the sex chromosomes that have fixed sexually antagonistic alleles.

295 In animals such as *Drosophila*⁷ and mice⁶ with evolutionarily much older
296 sex chromosomes, most functional Y-linked genes have been lost as a
297 consequence of Y chromosome degeneration, rendering the majority of
298 X-linked genes hemizygous in males. Dosage compensation systems
299 have evolved in these species, compensating for low expression from
300 degenerated Y-linked genes and X chromosomes have evolved an
301 overrepresentation of genes with female-biased expression³⁸. Such a

302 feminisation of the X is predicted by theory for hemizygous loci (reviewed
303 in ¹) whereas the Y chromosomes are enriched for genes with male-
304 biased expression among their few remaining genes (masculinization) ³⁹⁻
305 ⁴². In *S. latifolia*, up to 45% of Y-linked genes are not expressed ²⁰.
306 Nevertheless, Y chromosome degeneration is much less extensive than
307 in old animal sex chromosomes, and X-hemizygous genes studied are
308 apparently not dosage compensated ^{16,17,26}. Together, these differences
309 prevent direct comparison with the much older animal sex chromosomes.

310 To assess whether subtler gene expression changes have evolved on the
311 *S. latifolia* X and Y chromosome, we examined sex-linked genes with
312 copies expressed from both the X and Y chromosome. Expression ratios
313 of these genes (per X-linked allele, see Supplementary Methods)
314 revealed that genes with equal expression in both sexes express their X-
315 linked alleles with equal intensity in females and males (Fig. 5a).
316 However, the expression per X-linked allele is significantly higher for
317 contigs with female-biased expression, and lower when the gene is male-
318 biased (Fig. 5a; Wilcoxon-test, $p < 0.0001$ for both comparisons),
319 suggesting subtle feminisation and de-masculinisation of the *S. latifolia* X
320 chromosome.

321 To assess whether the Y chromosome has similarly evolved a degree of
322 masculinisation, a simple comparison of expression ratios of Y-linked
323 versus X-linked alleles may be inappropriate if dosage compensation in *S.*
324 *latifolia* ^{20,29} is achieved through increased X/Y expression in males.
325 Instead, therefore, we compared the expression of Y-linked alleles in *S.*
326 *latifolia* with that of the homologous genes in hermaphrodite *S. vulgaris*

327 (not sex-linked). Compared with contigs without sex bias in expression,
328 contigs with male-biased expression in *S. latifolia* indeed had higher Y/S.
329 *vulgaris* allele expression ratios ($Y/0.5*AA$ in Fig. 5b; Wilcoxon-test, $p <$
330 0.01), and Y-linked alleles of contigs with female-biased expression had
331 lower ratios (Wilcoxon-test, $p < 0.001$). Consistent patterns were seen for
332 Y/X expression ratios in males (Supplementary Fig. 8; the ratios are
333 correlated; Spearman correlation, $\rho = 0.598$, $p < 0.001$; Supplementary
334 Fig. 9). The weakly negative median expression ratio of contigs with
335 unbiased expression is in agreement with other studies evidencing Y
336 chromosome degeneration in *S. latifolia* ^{17,18,20,26,29,43,44} and the higher
337 ratio of contigs with male-biased than female-biased expression supports
338 a scenario in which degeneration of Y-linked genes is retarded by haploid
339 selection acting on pollen-expressed genes ¹⁷. We conclude that the *S.*
340 *latifolia* Y chromosome has undergone some masculinisation and thus
341 contributes to male-biased expression of sex-linked genes.

342 **Discussion**

343 Our finding that sex-biased gene expression in a dioecious plant has
344 most often evolved through decreased transcription, predominantly in
345 females, is consistent with the occurrence of sexual antagonism in the
346 hermaphrodite ancestor, specifically with intralocus sexual conflicts in
347 which high expression levels of many genes benefitting male functions in
348 the hermaphrodite, are detrimental in females ³³. Similarly, the smaller,
349 but still substantial, number of genes that underwent expression changes
350 only in males suggests that males benefit from changed expression of

351 some genes that experienced trade-offs in the hermaphrodite, but were
352 fixed for female beneficial/male detrimental alleles ³⁴. Together, our
353 results suggest that conflict over gene expression in hermaphrodite
354 flowers of *S. vulgaris* led to an outcome closer to the optimum for male
355 than female functions, and that sex-biased gene expression may have
356 been involved in re-allocating resources during the evolution of dioecy in
357 *S. latifolia*, resolving such conflicts. Such resource reallocation was
358 apparently more important for females than for males, compatible with
359 female plants often being resource limited ^{14,45}, and with Darwin's ⁴⁶ idea
360 that resource compensation is a major factor in the evolution of dioecy.

361 While our results support the hypothesis that sex-biased expression has
362 evolved to reduce intralocus sexual conflict, it remains unknown what
363 fraction of genes with sex-biased expression evolved through conflict
364 resolution, as sex-biased expression alone does not necessarily imply the
365 past existence of sexual antagonism ⁴⁷. All three scenarios in Fig. 1a are
366 compatible with intralocus sexual conflict, and these patterns apply to
367 approximately 50% of contigs with female-biased expression (53.7% for
368 autosomal contigs and 51.0% for sex-linked ones), and approximately
369 60% of contigs with male-biased expression (60.4% for autosomal and
370 64.2% for sex-linked contigs) (Supplementary Fig. 6). The remaining
371 genes with male or female-biased expression may have evolved sex-
372 biased expression under other selective forces acting during the evolution
373 of separate sexes, for example to compensate for negative pleiotropic
374 fitness effects of the sterility mutations involved, and of primary
375 expression changes, or because upstream regulatory elements causing

376 sex-biased expression may affect other genes where the change is
377 maladaptive.

378 We conclude that the sex chromosomes, as in animals, contribute to
379 sexual dimorphism in *S. latifolia*, as genes with sex-biased expression are
380 over-represented on these chromosomes. However, in contrast to
381 animals with evolutionarily much older sex chromosomes^{6,7,40,48}, our
382 analysis of the contribution of X and Y-linked alleles to sex-biased gene
383 expression in this plant detected no accumulation of genes with female-
384 biased expression on the X chromosome, or male-biased expression on
385 the Y. Instead, we find evidence for opposing selective forces acting on
386 the *S. latifolia* X and Y chromosome, leading to tertiary expression
387 differences between X- and Y-linked alleles, a subtle form of feminisation
388 of the X chromosome, and masculinisation of the Y. These expression
389 changes may represent a hitherto unknown early stage of sex
390 chromosome evolution that precedes the accumulation of genes with
391 expression biased to one sex or the other.

392 Our study demonstrates considerable expression changes in this recently
393 evolved dioecious species and illustrates the value of studying closely
394 related species, only one of which has evolved separate sexes and sex
395 chromosomes. Including a close relative with hermaphrodite flowers
396 allowed us to infer that sex-biased gene expression in dioecious *S.*
397 *latifolia* has evolved primarily through secondary expression changes of
398 many genes in females. Our results support the long-standing hypothesis
399 that the evolution of sex-biased gene expression reduces the detrimental
400 effects of sexually antagonistic alleles present in the ancestral

401 hermaphrodite that became fixed at autosomal, and particularly at sex-
402 linked, loci in the dioecious descendant. This resolution often reduces
403 expression in the disadvantaged sex, but sometimes increases
404 expression in the sex in which a derived allele was favoured in the
405 dioecious descendant. Our comparative approach can be used to
406 compare expression changes in genes that became fully sex linked at
407 different times during the evolution of a sex chromosome system, and
408 can be applied to studying other plant and animal lineages in which
409 separate sexes have evolved recently.

410

411

412 **Methods**

413 **Transcriptome sequencing, assembly and identification of sex-** 414 **linked genes**

415 Multiple females and males of *S. latifolia* and hermaphrodite individuals of
416 *S. vulgaris* were used in this study (Supplementary Table 1). High quality
417 RNA was extracted from small flower buds at developmental stages B1-
418 B2 ⁴⁹ and from fully developed rosette leaves. Individually tagged RNA-
419 seq libraries were sequenced on Illumina HiSeq2000 machines using
420 100bp paired-end reads.

421 Illumina short reads and Roche 454 Genome Sequencer FLX reads
422 derived from multiple tissues of *S. latifolia* were combined to assemble *de*
423 *novo* a reference transcriptome encompassing 46,178 contigs (for details
424 see Supplementary Methods). We used SEX-DETECTOR ²⁷ to classify

425 contigs as autosomal, sex-linked, X-hemizygous or undefined based on
426 SNPs segregating in one *S. latifolia* population.

427 **Analyses of sex-biased expression**

428 For gene expression analysis, RNA-seq reads derived from flower buds
429 and rosette leaves of *S. latifolia* and *S. vulgaris* were individually mapped
430 to the reference transcriptome. Numbers of mapped reads were extracted
431 per contig and sample and significantly differentially expressed contigs
432 between female and male *S. latifolia* individuals were identified in both
433 flower buds and rosette leaves.

434 Apparent sex-biased gene expression in flower buds of female and male
435 plants may arise trivially, when genes with sex-limited expression (i.e.
436 genes that are expressed in one sex only) are not expressed in the sex
437 that does not form the corresponding tissue (for example, apparent male-
438 biased gene expression may occur in *S. latifolia* for anther-specific genes,
439 simply because no anthers are formed in female flowers and the
440 corresponding genes are not expressed), or when genes are expressed
441 at similar levels in both female and male organs, and thus have reduced
442 expression when the organs are not developed in one sex. These contigs
443 representing primary expression changes (Fig. 1b) were excluded from
444 further analyses. They encompassed 839 contigs that were expressed
445 exclusively in males, and 64 contigs expressed exclusively in females.

446 To assess differences in the prevalence of contigs with sex-biased versus
447 unbiased expression, and female-biased versus male-biased expression
448 between autosomal and sex-linked contigs, we used Fisher's exact tests.

449 **Expression divergence between *S. vulgaris* and *S. latifolia*.**

450 To test whether directional selection has affected expression levels, we
451 used the ΔX approach^{35,36}. ΔX was calculated as $\Delta X = \text{mean}(X_{\text{SL females or}}$
452 $\text{males}) - \text{mean}(X_{\text{SV}}) / \text{sd}(X_{\text{SL females or males}})$.

453 Expression differences between *S. latifolia* females or males and *S.*
454 *vulgaris* hermaphrodites were divided by the standard deviation for all
455 contigs, estimated separately for the two sexes in *S. latifolia*. For
456 categories I and II of Fig. 1a, we computed the percentages of contigs
457 displaying outlier expression divergence values between the two species
458 (defined as $\Delta X \geq 75$ percentile across all contigs).

459 **Allelic expression estimates of sex-linked contigs**

460 Allelic contribution of the X and Y chromosomes to gene expression of
461 sex-linked genes were calculated using sex linked SNPs and were
462 compared to the autosomal ancestral stage in *S. vulgaris* (Supplementary
463 Methods).

464 **Data availability**

465 RNA-seq data and the reference transcriptome have been deposited in
466 the European Nucleotide Archive (ENA) under accession number
467 PRJEB14171. Further data that support the findings of this study are
468 available from the corresponding author upon reasonable request.

469

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607

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619 **Author contributions**

620 Designed the experiments and formulated predictions: N.Z., A.W.,
621 G.A.B.M., D.C.; Performed the experiments: N.Z.; Analysed the data N.Z.,
622 R.T., A.M.; Wrote the manuscript: N.Z., A.W., D.C., G.A.B.M., R.T., A.M.

623 **Competing interests**

624 The authors declare no competing financial interests.

625

626

627

628 **Figures**

629 **Fig. 1**

630 **Hypothetical scenarios for the evolution of sex-biased gene**

631 **expression. a**, Evolution of female-biased expression from a
632 hermaphroditic ancestral state. I: expression increased in females, II:
633 expression decreased in males, III: expression increased in females and
634 decreased in males. **b**, Evolutionary changes in gene expression
635 associated with the transition from hermaphroditism to dioecy and the
636 evolution of non-recombining sex chromosomes. Primary mutations lead
637 to gynodioecy (or androdioecy) and subsequently to dioecy ⁵⁰. Secondary
638 expression changes lead to sex-biased gene expression. Tertiary
639 expression changes on sex chromosomes cause feminisation or
640 masculinisation of X and Y (or Z and W) chromosomes.

641

642 **Fig. 2**

643 **Sexual dimorphism and sex-biased gene expression in *S. latifolia*. a,**

644 Sexual dimorphism in female and male flowers. **b,** Proportions of contigs

645 with female-biased (red), male-biased (blue) and unbiased (white)

646 expression for 12,708 contigs with inferred autosomal inheritance and

647 936 fully sex-linked contigs in flower buds and rosette leaves.

648

649 **Fig. 3**

650 **Expression changes in genes with sex-biased expression in *S.***

651 ***latifolia*. a-b**, Expression differences (median with 95% confidence

652 intervals) in **(a)** autosomal and **(b)**, sex-linked contigs between *S. latifolia*

653 females and males relative to *S. vulgaris* hermaphrodites for contigs with

654 female-biased (red), male-biased (blue), and unbiased (white) expression

655 in flower buds. Positive values correspond to genes with higher

656 expression than in *S. vulgaris* hermaphrodites.

657

658 **Fig. 4**

659 **Evolutionary changes leading to sex-biased gene expression in *S.***

660 ***latifolia*. a-b**, Autosomal (**a**) and sex-linked (**b**) contigs with elevated

661 expression in the sex with higher expression (scenario I in Fig. 1a) and

662 reduced expression in the sex with lower expression (scenario II of Fig.

663 1a) relative to *S. vulgaris* hermaphrodites for female- (red) and male-

664 biased (blue) contigs. ΔX values indicate percentages of contigs that are

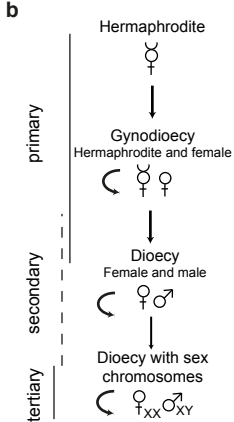
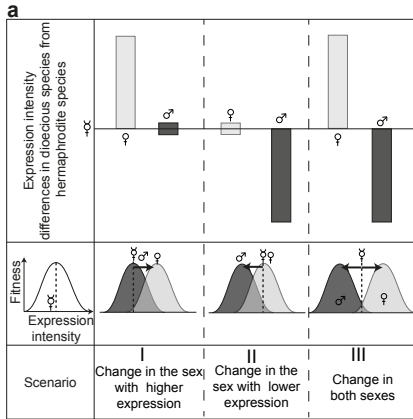
665 outliers for expression divergence. **c**, Contigs with significant expression

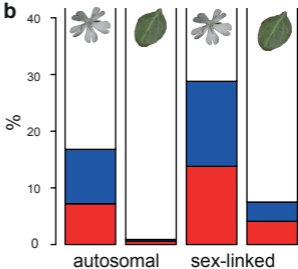
666 changes in opposite directions in the two sexes (scenario III in Fig. 1a).

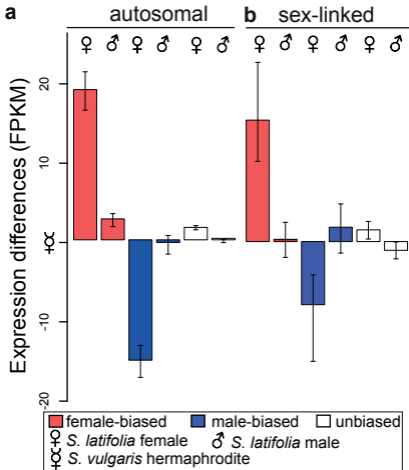
667 Numbers in parentheses correspond to numbers of contigs in each

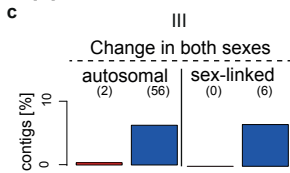
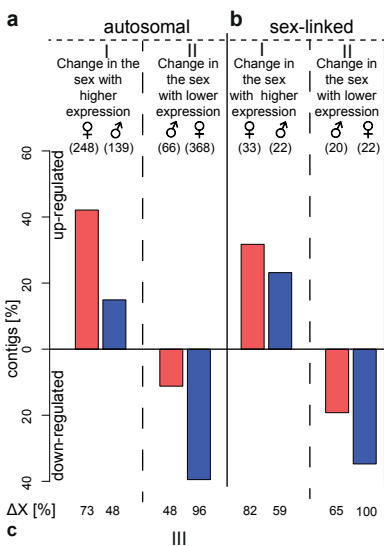
668 category.

669 **Fig. 5: Tertiary expression changes on *S. latifolia* sex chromosomes.**
670 **a**, X female/male expression ratios in flower buds for contigs with female-
671 biased (red), male-biased (blue), and unbiased expression (white). This
672 ratio is significantly larger for contigs with female- than male-biased or
673 unbiased expression (Wilcoxon-test, $p \leq 0.0001$). **b**, Expression in males
674 as the ratios of Y expression to that in the cosexual ancestor for the same
675 three expression bias categories (colours as in a). Compared with contigs
676 with unbiased expression, ratios are significantly larger for male-biased
677 and smaller for female-biased contigs (Wilcoxon-test, $p \leq 0.01$ and $p \leq$
678 0.0001 , respectively).



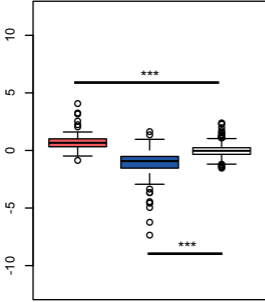






a

$\log_2(0.5 \cdot XX_{\text{females}} / X_{\text{males}})$



b

$\log_2(Y/0.5 \cdot AA_{SV})$

