THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Evolution of sex-biased gene expression in a dioecious plant

OPEN ACCESS

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

2

3

4 Niklaus Zemp[1], Raquel Tavares[2], Aline Muyle[2], Deborah Charlesworth[3], Gabriel A. B. Marais[2] & Alex Widmer*[1]

6

7 *[1] ETH Zurich, Institute of Integrative Biology, Universitätstrasse 16, 8092 Zürich, Switzerland*

9 *[2] Université Lyon 1, CNRS, UMR 5558, Laboratoire de Biométrie et Biologie Evolutive, F-69622 Villeurbanne, France*

11 *[3] University of Edinburgh, Institute of Evolutionary Biology, Edinburgh EH9 3JT, Midlothian, Scotland*

13 ** corresponding author*

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; ϱ=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 (<u>before</u> 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 optimisd 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 males [1]. Consistently, male-biased

275 expression in *Drosophila* was inferred to result primarily from adaptive

276 changes in the male transcriptome [37]. In *S. latifolia*, although there are

277 many male-biased genes, these do not indicate stronger sexually

12

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 mice [6] 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 expression [38]. 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, ρ =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.

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

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

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

Allelic expression estimates of sex-linked contigs

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

Data availability

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

References

1   Ellegren, H. & Parsch, J. The evolution of sex-biased genes and sex-biased gene expression. *Nat Rev Genet* **8**, 689-698 (2007).

2   Mank, J. E. Sex chromosomes and the evolution of sexual dimorphism: lessons from the genome. *Am Nat* **173**, 141-150 (2009).

3   Stewart, A. D., Pischedda, A. & Rice, W. R. Resolving intralocus sexual conflict: genetic mechanisms and time frame. *J Hered* **101 Suppl 1**, S94-99 (2010).

4   van Doorn, G. S. Intralocus sexual conflict. *Ann N Y Acad Sci* **1168**, 52-71 (2009).

5   Bonduriansky, R. & Chenoweth, S. F. Intralocus sexual conflict. *Trends Ecol Evol* **24**, 280-288 (2009).

6   Reinius, B. *et al.* Abundance of female-biased and paucity of male-biased somatically expressed genes on the mouse X-chromosome. *BMC Genomics* **13**, 607 (2012).

7   Meisel, R. P., Malone, J. H. & Clark, A. G. Disentangling the relationship between sex-biased gene expression and X-linkage. *Genome Res* **22**, 1255-1265 (2012).

8   Ellegren, H. Emergence of male-biased genes on the chicken Z-chromosome: Sex-chromosome contrasts between male and female heterogametic systems. *Genome Res* **21**, 2082-2086 (2011).

9   Kang, H. J. *et al.* Spatio-temporal transcriptome of the human brain. *Nature* **478**, 483-489 (2011).

10   Lipinska, A. *et al.* Sexual dimorphism and the evolution of sex-biased gene expression in the brown alga *Ectocarpus*. *Mol Biol Evol* **32**, 1581-1597 (2015).

11   Harkess, A. *et al.* Sex-biased gene expression in dioecious garden asparagus (*Asparagus officinalis*). *New Phytol* **207**, 883-892 (2015).

12   Robinson, K. M. *et al. Populus tremula* (European aspen) shows no evidence of sexual dimorphism. *BMC Plant Biol* **14**, 276 (2014).

13   Zemp, N., Tavares, R. & Widmer, A. Fungal infection induces sex-specific transcriptional changes and alters sexual dimorphism in the dioecious plant *Silene latifolia*. *PLoS Genet* **11**, e1005536 (2015).

14   Barrett, S. C. H. & Hough, J. Sexual dimorphism in flowering plants. *J Exp Bot* **64**, 67-82 (2013).

15   Bernasconi, G. *et al. Silene* as a model system in ecology and evolution. *Heredity* **103**, 5-14 (2009).

16   Bergero, R., Qiu, S. & Charlesworth, D. Gene loss from a plant sex chromosome system. *Curr Biol* **25**, 1234-1240 (2015).

17   Chibalina, M. V. & Filatov, D. A. Plant Y chromosome degeneration is retarded by haploid purifying selection. *Curr Biol* **21**, 1475-1479 (2011).

516 18 Marais, G. A. B. *et al.* Evidence for degeneration of the Y
517   chromosome in the dioecious plant *Silene latifolia*. *Curr Biol* **18**,
518   545-549 (2008).
519 19 Nicolas, M. *et al.* A gradual process of recombination restriction in
520   the evolutionary history of the sex chromosomes in dioecious
521   plants. *PLoS Biol* **3**, 47-56 (2005).
522 20 Papadopulos, A. S. T., Chester, M., Ridout, K. & Filatov, D. A.
523   Rapid Y degeneration and dosage compensation in plant sex
524   chromosomes. *Proceedings of the National Academy of Sciences*
525   **112**, 13021-13026 (2015).
526 21 Mrackova, M. *et al.* Independent origin of sex chromosomes in two
527   species of the genus *Silene*. *Genetics* **179**, 1129-1133 (2008).
528 22 Delph, L. F., Knapczyk, F. N. & Taylor, D. R. Among-population
529   variation and correlations in sexually dimorphic traits of *Silene*
530   *latifolia*. *J Evol Biol* **15**, 1011-1020 (2002).
531 23 Delph, L. F., Arntz, A. M., Scotti-Saintagne, C. & Scotti, I. The
532   genomic architecture of sexual dimorphism in the dioecious plant
533   *Silene latifolia*. *Evolution* **64**, 2873-2886 (2010).
534 24 Marais, G. A. B. *et al.* Multiple nuclear gene phylogenetic analysis
535   of the evolution of dioecy and sex chromosomes in the genus
536   *Silene*. *PloS One* **6**, e21915 (2011).
537 25 Rautenberg, A., Sloan, D. B., Alden, V. & Oxelman, B.
538   Phylogenetic relationships of *Silene multinervia* and *Silene* section
539   Conoimorpha (Caryophyllaceae). *Syst Bot* **37**, 226-237 (2012).
540 26 Bergero, R. & Charlesworth, D. Preservation of the Y
541   transcriptome in a 10-million-year-old plant sex chromosome
542   system. *Curr Biol* **21**, 1470-1474 (2011).
543 27 Muyle, A. *et al.* SEX-DETector: a probabilistic approach to study
544   sex chromosomes in non-model organisms. *Genome Biol Evol*,
545   (2016).
546 28 Mank, J. E. Sex chromosome dosage compensation: definitely not
547   for everyone. *Trends Genet* **29**, 677-683 (2013).
548 29 Muyle, A. *et al.* Rapid de novo evolution of X chromosome dosage
549   compensation in *Silene latifolia*, a plant with young sex
550   chromosomes. *PLoS Biol* **10**, e1001308 (2012).
551 30 Hough, J., Hollister, J. D., Wang, W., Barrett, S. C. H. & Wright, S.
552   I. Genetic degeneration of old and young Y chromosomes in the
553   flowering plant *Rumex hastatulus*. *Proc Natl Acad Sci USA* **111**,
554   7713-7718 (2014).
555 31 Moore, J. C. & Pannell, J. R. Sexual selection in plants. *Curr Biol*
556   **21**, R176-182 (2011).
557 32 Delph, L. F. & Herlihy, C. R. Sexual, fecundity, and viability
558   selection on flower size and number in a sexually dimorphic plant.
559   *Evolution* **66**, 1154-1166 (2012).
560 33 Vicoso, B., Kaiser, V. B. & Bachtrog, D. Sex-biased gene
561   expression at homomorphic sex chromosomes in emus and its
562   implication for sex chromosome evolution. *Proc Natl Acad Sci USA*
563   **110**, 6453-6458 (2013).
564 34 Jordan, C. Y. & Connallon, T. Sexually antagonistic polymorphism
565   in simultaneous hermaphrodites. *Evolution* **68**, 3555-3569 (2014).

566 35 Moghadam, H. K., Pointer, M. A., Wright, A. E., Berlin, S. & Mank,
567   J. E. W chromosome expression responds to female-specific
568   selection. *Proc Natl Acad Sci USA* **109**, 8207-8211 (2012).
569 36 Ometto, L., Shoemaker, D., Ross, K. G. & Keller, L. Evolution of
570   gene expression in fire ants: the effects of developmental stage,
571   caste, and species. *Mol Biol Evol* **28**, 1381-1392 (2011).
572 37 Connalon, T., & Knowles, L.L. Intergenomic conflict revealed by
573   patterns of sex-biased gene expression. *Trends Genet* **21**, 495-
574   499 (2005).
575 38 Disteche, C. M. Dosage compensation of the sex chromosomes.
576   *Annu Rev Genet* **46**, 537-560 (2012).
577 39 Zhou, Q. & Bachtrog, D. Sex-specific adaptation drives early sex
578   chromosome evolution in *Drosophila*. *Science* **337**, 341-345 (2012).
579 40 Skaletsky, H. *et al.* The male-specific region of the human Y
580   chromosome is a mosaic of discrete sequence classes. *Nature*
581   **423**, 825-837 (2003).
582 41 Bellott, D. W. *et al.* Mammalian Y chromosomes retain widely
583   expressed dosage-sensitive regulators. *Nature* **508**, 494-499
584   (2014).
585 42 Cortez, D. *et al.* Origins and functional evolution of Y
586   chromosomes across mammals. *Nature* **508**, 488-493 (2014).
587 43 Charlesworth, B. & Charlesworth, D. The degeneration of Y
588   chromosomes. *J Roy Stat Soc Ser B (Stat Method)* **355**, 1563-
589   1572 (2000).
590 44 Bachtrog, D. Y-chromosome evolution: emerging insights into
591   processes of Y-chromosome degeneration. *Nat Rev Genet* **14**,
592   113-124 (2013).
593 45 Asikainen, E. & Mutikainen, P. Pollen and resource limitation in a
594   gynodioecious species. *Am J Bot* **92**, 487-494 (2005).
595 46 Darwin, C The different forms of flowers on plants of the same
596   species. John Murray, London (1877).
597 47 Innocenti, P. & Morrow, E. H. The sexually antagonistic genes of
598   *Drosophila melanogaster*. *PLoS Biol* **8**, e1000335 (2010).
599 48 Zhou, Q. & Bachtrog, D. Chromosome-wide gene silencing
600   initiates Y degeneration in *Drosophila*. *Curr Biol* **22**, 522-525
601   (2012).
602 49 Matsunaga, S. *et al.* Isolation and developmental expression of
603   male reproductive organ-specific genes in a dioecious campion,
604   *Melandrium album* (S*ilene latifolia*). *Plant J* **10**, 679-690 (1996).
605 50 Charlesworth, B. & Charlesworth, D. A model for the evolution of
606   dioecy and gynodioecy. *Am Nat* **112**, 975-997 (1978).

607

608 Correspondence and requests for materials should be addressed to A.W.

Acknowledgements

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.

Competing interests

624 The authors declare no competing financial interests.

625

626

627

24

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. Δ X values indicate percentages of contigs that are
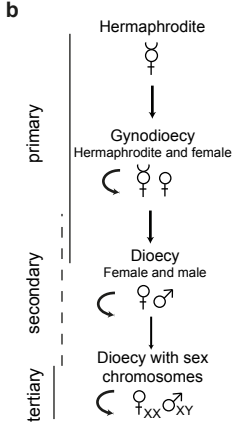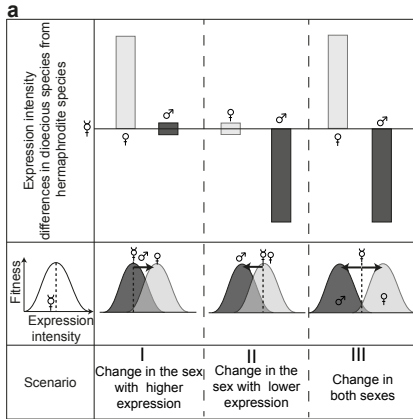
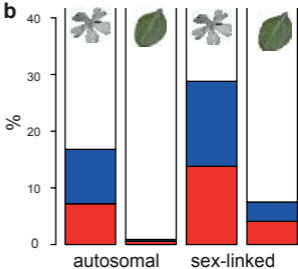665 outliers for expression divergence. **c**, Contigs with significant expression

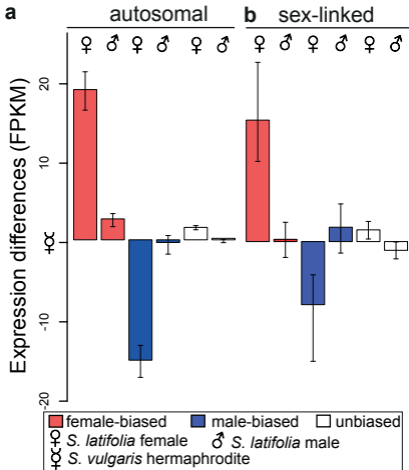666 changes in opposite directions in the two sexes (scenario III in Fig. 1a).

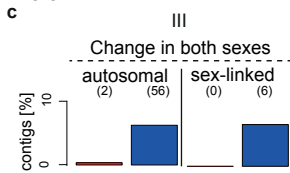667 Numbers in parentheses correspond to numbers of contigs in each

668 category.

Fig. 5: Tertiary expression changes on *S. latifolia* sex chromosomes.

a, X female/male expression ratios in flower buds for contigs with female-biased (red), male-biased (blue), and unbiased expression (white). This ratio is significantly larger for contigs with female- than male-biased or unbiased expression (Wilcoxon-test, p ≤ 0.0001). **b**, Expression in males as the ratios of Y expression to that in the cosexual ancestor for the same three expression bias categories (colours as in a). Compared with contigs with unbiased expression, ratios are significantly larger for male-biased and smaller for female-biased contigs (Wilcoxon-test, p ≤ 0.01 and p ≤ 0.0001, respectively).

a

Expression intensity differences in dioecious species from hermaphrodite species

Fitness / Expression intensity

Scenario I: Change in the sex with higher expression

Scenario II: Change in the sex with lower expression

Scenario III: Change in both sexes

b

Hermaphrodite ☿

primary

Gynodioecy
Hermaphrodite and female ☿ ♀

secondary

Dioecy
Female and male ♀ ♂

tertiary

Dioecy with sex chromosomes ♀$_{XX}$ ♂$_{XY}$

a ♀ ♂ 10 mm

b autosomal sex-linked

a autosomal   **b** sex-linked

Expression differences (FPKM)

female-biased   male-biased   unbiased
⚲ *S. latifolia* female   ♂ *S. latifolia* male
⚲ *S. vulgaris* hermaphrodite

a autosomal   **b** sex-linked

a

Change in the sex with higher expression	Change in the sex with lower expression
♀ ♂	♂ ♀
(248) (139)	(66) (368)

ΔX [%]   73 48   48 96

b

Change in the sex with higher expression	Change in the sex with lower expression
♀ ♂	♂ ♀
(33) (22)	(20) (22)

ΔX [%]   82 59   65 100

III

c

Change in both sexes

autosomal	sex-linked
(2) (56)	(0) (6)

contigs [%]
up-regulated — down-regulated

a

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

b

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