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1 # Epigenetics of floral homeotic genes in relation to sexual dimorphism in the
2 dioecious plant *Mercurialis annua*

3

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13

14 **Highlights**

15 Sex determination in *Mercurialis annua* is not related to epigenetics of floral homeotic genes
16 but appears to be modulated by an unknown gender-specific regulator(s) that affects hormonal
17 homeostasis.
18

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19 **Abstract**

20 In plants, dioecy characterizes species carrying male and female flowers on separate plants
21 and occurs in about 6% of angiosperms. To date, the molecular mechanism(s) underlying
22 sexual dimorphism is essentially unknown. The ability of gender-reversal by hormone
23 application suggests that epigenetics might play an important role in sexual dimorphism.
24 Proteome analysis of nuclei derived from flower buds of females, males and feminized males
25 of the dioecious plant *Mercurialis annua* revealed differentially expressed proteins related to
26 nucleic acid binding proteins, hydrolases and transcription factors, including floral homeotic
27 genes. Further analysis showed that class B genes are mainly expressed in male flowers, while
28 class D genes, as well as *SUPERMAN*-like genes, were mainly expressed in female flowers.
29 Cytokinin-induced feminization of male plants was associated with down-regulation of male-
30 specific genes concomitantly with up-regulation of female-specific genes. No correlation
31 could be found between the expression of class B and D genes and their DNA methylation or
32 chromatin conformation. Thus, our results ruled out epigenetic control over floral identity
33 genes as the major determinants regulating sexual dimorphisms. Rather, determination of sex
34 in *M. annua* might be controlled upstream of floral identity genes by a gender-specific factor
35 that affects hormonal homeostasis.

36

37 **Key words**

38 *Mercurialis annua*, Dioecy, Floral homeotic gene, MADS-box gene, ABCDE model,
39 SUPERMAN-like gene, Sex determination

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41 **Introduction**

42 The majority of angiosperms are hermaphrodites and monoecious (sexually
43 monomorphic), whereby both male and female organs are found on the same individual plant.
44 In contrast, only about 6% of the angiosperms are dioecious (sexually dimorphic) where male
45 and female flowers are carried on separate individual plants (Renner and Ricklefs, 1995;
46 Charlesworth, 2002). It has been hypothesized that dioecy has evolved independently from
47 hermaphrodites through mutations in various families of plants (Charlesworth and
48 Charlesworth, 1978; Charlesworth, 2002). Based on developmental aspects, dioecious plants
49 are categorized into two types: type-I, unisexual flowers developed *via* abortion of
50 reproductive organs that exhibit rudiments of the aborted organs, and type-II, to which
51 *Mercurialis annua* belongs, where unisexual flowers do not have rudiments of the opposite
52 sex (Mitchell and Diggle, 2005).

53 Most studies related to the regulation of flower development were performed in model
54 plants such as *Arabidopsis thaliana* that has hermaphroditic flowers with four concentric
55 whorls: sepals, petals, stamens and carpels. The homeotic genes that regulate the development
56 of such flowers were described by the ABCDE model (Coen and Meyerowitz, 1991; Krizek
57 and Fletcher, 2005). These gene classes were termed according to the whorl in which they
58 function: class A genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*), class B genes *APETALA3*
59 (*AP3*), *TOMATO MADS-BOX GENE6* (*TM6*) and *PISTILLATA* (*PI*), class C gene *AGAMOUS*
60 (*AG*), class D genes *SHATTERPROOF1* (*SHP1*), *SHATTERPROOF2* (*SHP2*) and
61 *SEEDSTICK* (*STK*) also known as *AGAMOUS-like1* (*AGL1*), *AGAMOUS-like5* (*AGL5*) and
62 *AGAMOUS-like11* (*AGL11*), respectively, and class E genes *SEPALATA1* (*SEP1*) to
63 *SEPALATA* (*SEP4*). Most of these genes encode MIKC-type MADS-box domain containing
64 transcription factors. The MADS-box proteins form homo- and/or heterodimers that bind
65 specific DNA sequences, CC(A/T)$_6$GG, called CArG box and the pairs of CArG-boxes are
66 brought into close proximity by DNA looping (Nurrish and Treisman, 1995; Davies *et al.*,
67 1996; Riechmann *et al.*, 1996; Mendes *et al.*, 2013). The protein dimers further form
68 functionally active tetrameric protein complex termed, 'floral quartet', that specifically
69 controls differentiation of flower whorls (Theissen and Saedler, 2001; Smaczniak *et al.*,
70 2012). Accordingly, combination of A+E genes specify sepals, A+B+E genes specify petals,

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71 B+C+E genes specify stamens, C+E genes specify carpels and D+E genes specify ovule
72 identity (Theissen and Saedler, 2001; Soltis *et al*., 2007).

73 As floral homeotic MADS-box genes control the expression of other regulatory
74 genes, these genes are considered as key factors for development of the floral organs, both
75 perianth parts and sex organs (Wuest *et al*., 2012; O'Maoileidigh *et al*., 2013; Stewart *et al*.,
76 2016). However, the regulation of these genes is not fully understood. In *Arabidopsis*,
77 *SUPERMAN* (*SUP*) is required for proper development of the reproductive organs inasmuch
78 as mutation of the *SUP* gene has led to extra stamens formation at the expense of carpel
79 development (Jacobsen and Meyerowitz, 1997; Sakai *et al*., 1995). The *SUP* transcription
80 factor is proposed to act as a negative regulator of class B genes (i.e., *AP3* and *PI*) to maintain
81 boundaries between the stamen and the carpel whorls (Bowman *et al*., 1992; Yun *et al*., 2002;
82 Prunet *et al*., 2017) and its role appears to be conserved among dicot and monocot plants
83 (Nandi *et al*., 2000). Additionally, *SUP* gene is required for development of the outer
84 integument of the ovule (Gaiser *et al*., 1995). In dioecious *Silene latifolia*, *SUPERMAN*
85 orthologous gene, *SlSUP* was associated with female flower development (Kazama *et al*.,
86 2009). Ectopic expression of *SUP* in tobacco plants was shown to induce increased
87 feminization via enhancing cytokinin related processes (Nibau *et al*., 2011). A recent report
88 addressing gender-specific methylation in the sex determining region of *Populus balsamifera*
89 identified the PbRR9 gene showing a clear pattern of gender-specific methylation. PbRR9
90 encode for a protein member of the two-component response regulator (type-A) gene family
91 involved in cytokinin signaling (Brautigam *et al*., 2017).

92 Only limited research has been made to elucidate the role of floral homeotic genes in
93 sexually dimorphic dioecious plants. This is surprising in view of the apparent advantage of
94 separation of the reproductive organs between female and male plants, which makes it
95 experimentally more amenable to investigation of the developmental regulation of each
96 gender in plants. We have shown recently dimorphic responses of *M. annua* plant genders to
97 stress that may be attributed to female plants' capacity to survive stress and complete the
98 reproductive life cycle (Orlofsky *et al*., 2016). A few of the more studied dioecious species
99 include *Silene latifolia* and *Rumex acetosa* of the type-I flowers and *Thalictrum dioicum* and
100 *Spinacia oleracea* of the type-II flowers. It was shown that in the case of *S. latifolia* and *R.*
101 *acetosa*, class B and C floral organ identity genes are expressed early in development of male
102 and female flowers (Hardenack *et al*., 1994; Ainsworth *et al*., 1995), while in *T. dioicum* and

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103 *S. oleracea*, class B and C genes are differentially expressed at floral initiation (Di stilio *et al*.,
104 2005; Pfent *et al*., 2005). Sather *et. al*. (2010) showed that silencing of class B genes in *S.*
105 *oleracea* is able to alter the floral gender of males into hermaphrodites or females due to
106 transformation of stamens into carpels.

107 The annual dioecious (type-II) *M. annua* L. (Euphorbiaceae) is a unique model plant
108 for the study of dioecism, since it has a short life cycle, which enables molecular-genetic
109 studies, in contrast to most dioecious plants, which are woody perennials. It is a common
110 roadside herb native to the drought and high-sunlight prone Mediterranean basin, which has
111 spread into Europe, North America and Australia (Durand and Durand, 1991; Pannell *et al*.,
112 2008). The diploid species (2n=16) is a strictly dioecious, while polyploid species are not
113 (Thomas, 1958; Durand and Durand, 1991; Pannell *et al*., 2008). The dioecious *M. annua* has
114 an interesting genetic system of sex determination lacking heteromorphic sex chromosome.
115 Identification of male specific molecular markers and recent genetic analyses have revealed
116 that males possess homomorphic XY chromosomes, but the molecular mechanism of sex
117 determination is not clear yet (Khadka *et al*., 2002, 2005; Russell and Pannell, 2015; Veltsos
118 *et al*., 2018). Furthermore, sex expression in *M. annua* can be reversed by exogenously
119 applied plant growth hormones. Accordingly, auxins have a masculinizing effect while
120 cytokinins have a feminizing effect (Delaigue *et al*., 1984). The ability of gender-reversal by
121 hormone application suggests that the gene(s) required for the development of both type of
122 flowers are genetically functional but might be restrained by epigenetic means in the floral
123 primordia, even when lacking vestiges of the opposite sex, thus being still sexually bi-potent.

124 Here we attempted to study the epigenetic regulation of floral identity genes in *M.*
125 *annua* and the relationship with sex determination. We report that differential expression of
126 floral homeotic genes was associated with sexual dimorphism in *M. annua* and cytokinin was
127 involved in their transcriptional control. The possible involvement of epigenetic regulation of
128 the examined floral genes was ruled out.

129

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130 **Materials and methods**

131 **Plant growth condition**

132 Dioecious *Mercurialis annua* (Euphorbiaceae), Belgian origin, was used in this study. Seeds
133 were sown in trays containing standard gardening soil and the seedlings were transplanted
134 into pots (2.5 L) and grown in a controlled climate growth chamber at 27 °C with photoperiod
135 regime of 14 h light/10 h dark and light intensity of approximately 400 µmol m^{-2} sec^{-1}.

136 **Feminization of male plants by 6-benzylaminopurine treatment**

137 At the onset of flowering (about 25-day-old plants), male plants were separated from female
138 plants. Feminization of the isolated male plants was done by spraying 1 mg L^{-1} 6-
139 benzylaminopurine (BAP) three times daily as described (Durand and Durand 1991; Khadka
140 *et al*., 2005). Inflorescence bud samples were collected and either used immediately for nuclei
141 isolation or stored at -80˚C until analyzed.

142 **Proteomic analysis**

143 Nuclei isolated from flower buds were subjected to proteome analysis by the proteomic
144 services of The Smoler Protein Research Center at the Technion, Israel. The samples
145 were digested by trypsin, analyzed by LC-MS/MS on Q-Exactive (Thermo) and identified by
146 Discoverer1.4 software against *Ricinus communis, Jatropha curcas* and *Arabidopsis* protein
147 databases. All the identified peptides were filtered with high confidence, top rank and mass
148 accuracy. High confidence peptides were passed the 1% FDR threshold (FDR =false
149 discovery rate, is the estimated fraction of false positives in a list of peptides). The peak area
150 on the chromatogram of the protein was calculated from the average of the peptides from each
151 protein. PANTHER classification tool was used for categorization of differentially expressed
152 proteins (Mi *et al*., 2013).

153 **Nucleic acid extraction and cDNA synthesis**

154 Genomic DNA was extracted using the PureLink Genomic DNA Mini Kit according to the
155 manufacturer's protocol (ThermoFisher Scientific). Total RNA was extracted using the

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156 RNeasy Mini Kit (Qiagen). The first strand of cDNA was synthesized from 1 μg DNase
157 (Epicentre)-treated total RNA using Verso cDNA Synthesis Kit (ThermoFisher Scientific).

158 **Isolation of genes and partial promoter sequences**

159 Floral homeotic cDNA clones were prepared by PCR using *M. annua* flower cDNA as
160 template and appropriate degenerate primers (based on conserved regions of *A. thaliana*,
161 *Ricinus communis* and *Jatropha curcas*; for primer sequences see Supplementary file 1) for
162 the recovery of class B (*AP3*, *PI*, *TM6*), class C/D (*AG*, *AGL5* and *AGL11*), as well as two
163 *SUPERMAN*-like (*SUP*-like) gene products. PCR conditions were: 95 °C, 2 min; 40 cycles of
164 95 °C, 30 s; 65-45 °C, 30 s; 72 °C, 60 s; followed by 72 °C, 5 min. The PCR products were
165 purified using QIAquick gel extraction kit, then cloned into pJET1.2 plasmid vector
166 (ThermoFisher scientific) and sequenced at the Biotechnology Center, Ben-Gurion University
167 of the Negev, Beer-Sheva, Israel.

168 To obtain full cDNA sequence, 3'- RACE was performed as described by Yadav *et al*. (2012)
169 and 5′-RACE was performed using a 5'-Full RACE Core Set kit (TaKaRa). The purified PCR
170 products were directly sequenced as above.

171 Based on phylogenetic analyses (see supplementary text for details), the class B genes were
172 designated as *MaPI* for *PI* ortholog, *MaAP3* for *AP3* ortholog, *MaTM6* for *TOMATO MADS*
173 *BOX GENE6* ortholog. The AGAMOUS-like genes were designated as *MaAG1* for
174 *AGAMOUS* ortholog (class C), *MaAGL1* for *STK*/*AGL11* ortholog (class D) and *MaAGL3* for
175 *SHP2*/*AGL5* ortholog (class D). NCBI GenBank accession numbers: KR781112-6.

176 The upstream promoters of *MaAP3*, *MaAGL1*, *MaPI*, *MaSL1* and *MaSL2* were isolated by
177 semi-random sequence walking strategy modified from Aquino and Figueiredo (2004).
178 Briefly, a gene specific primer was used for linear amplification of specific DNA segment for
179 20 high stringency cycles (95 ˚C, 30 s; 60 ˚C, 30 s; 72 ˚C, 2 min). Then random walking
180 primer was added and a low stringency cycle (95 ˚C 30 s, 35 ˚C 30s, 72 ˚C 2 min) was used
181 for unspecific binding and amplification. Then, 30 high stringency cycles were used for
182 exponential amplification. The desired fragments were screened by semi-nested PCR using
183 asymmetrical ratio (1:5) of walking primer and nested gene specific primer. The products of
184 interest were purified, cloned and sequenced as above.

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185 For reference, a 135 bp of Actin gene was amplified using primers designed from conserved
186 region of mRNA of *J. curcas*, *R. communis* and *Populus trichocarpa*. The amplified product
187 of *M. annua ACTIN* (*Act*) gene was confirmed by direct sequencing from both ends.

188 **Gene expression analysis**

189 Quantification of the gene expression level was done by quantitative or semi-quantitative RT-
190 PCR analysis using gene specific primers. qPCR was carried out using Perfecta SYBR green
191 supermix (Quanta Biosciences). Amplification was conducted on an Applied Biosystems®
192 7500 Real-Time PCR Systems. All reactions were performed from three biological samples
193 and each with three technical replicates. The PCR conditions were: 94 °C for 15 s, 40 cycles
194 of 94 °C for 5 s, 60 °C for 30 s. Each reaction was normalized against the expression of *Actin*
195 gene. The relative changes in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak
196 and Schmittgen, 2001).

197 **Micrococcal nuclease accessibility assay**

198 Micrococcal nuclease (MNase) accessibility assay was performed as described (Zhao *et al*.,
199 2001). Nuclei prepared from male and feminized *M. annua* flower buds were incubated with
200 MNase for various durations, and the DNA was resolved on agarose gel. MNase treatment
201 resulted in a nucleosomal ladder. The recovery of DNA after MNase treatment was checked
202 by PCR.

203 **DNA methylation analysis**

204 For cytosine methylation analysis, chop-PCR (methylation-sensitive enzyme digestion
205 followed by PCR) and bisulfite sequencing were performed as described (Yadav *et al*., 2018).
206 In chop-PCR, genomic DNA was treated with methylation sensitive restriction enzymes
207 *Hpa*II or *Msp*I and subjected to PCR to amplify various gene fragments containing the
208 restriction site 'CCGG'.

209 Bisulfite conversion was done by adding a mixture of sodium bisulfite, hydroquinone and
210 urea, and incubated at 55 °C for 16 hrs. The samples were desalted using PCR purification kit
211 and desulfonated by adding NaOH to a final concentration of 0.3 M. Then, DNA was purified

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212 by QIAquick PCR purification kit (Qiagen). The bisulfite converted DNA was used for PCR
213 amplification of promoter and gene-body of MaAP3 and MaSL1 genes. The PCR products
214 were cloned into pJET1.2 vector. At least 10 individual clones from each region were
215 sequenced by Macrogen, Netherlands. The sequences were analyzed and scored using
216 Kismeth online service (Gruntman *et al*. 2008).

217 **Statistical analysis**

218 The data representing the average values of three biological replicates each with three
219 technical chemical replicates and error bars representing the standard deviations were
220 graphed. Student's t-test was used to determine the statistical significance of differences at the
221 p-level of 0.01. Error bars indicate SE of the mean (n=3).

222

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223 **Results**

224 **Feminization of male *Mercurialis annua*: setting up the experimental system**

225 The female and male *M. annua* plants have distinct inflorescence morphology (Fig. 1A and
226 1B). In female plants, flowers developed directly at leaf axils with short pedicels, while in
227 male plants, clusters of flowers developed on long pedunculated inflorescences. Feminization
228 of male flowers by the cytokinin, 6-Benzylaminopurine (BAP), caused development of female
229 flowers that yielded fertile seeds (Fig. 1C) on male inflorescences (Khadka *et al*., 2005).

230 **Proteome analysis of flower bud nuclei**

231 To identify regulatory genes involved in sexual dimorphism and BAP induced sex alteration
232 of *M. annua*, we performed proteome analysis of nuclear proteins derived from flower buds of
233 female, male and males treated with BAP for 4, 8, 12 and 16 days. The proteome data showed
234 a total of 1443 proteins. Nuclear proteins including core histone proteins H2A, H2B, H3 and
235 H4 displayed the highest intensities among the proteins identified in this analysis
236 (Supplementary file 2, S1). The major difference between the genders was that 52 proteins
237 that were present in female flowers were absent in male flowers, while 244 proteins that were
238 present in males were absent in female flowers. Among the 52 proteins expressed only in
239 female flowers, 49 proteins were up-regulated in feminized males (Supplementary file 2, S2).
240 Out of the 244 male-specific proteins, 84 proteins were disappeared in the course of
241 feminization (Supplementary file 2, S3). The change in protein expression was as follows: 39
242 proteins disappeared after 4 days of BAP treatment, 15 after 8 days, 12 after 12 days and 18
243 proteins disappeared after 16 days of BAP treatment.

244 Multiple classes of proteins were identified by categorization analysis of the differentially
245 expressed proteins in feminized male. The major up-regulated protein classes were nucleic
246 acid binding proteins, transcription factors and cytoskeleton proteins (Fig. 2A), and the major
247 down-regulated protein classes were hydrolases, nucleic acid binding proteins, ligases and
248 transferases (Fig. 2B). Among differentially expressed proteins, four floral organ identity
249 MADS-box transcription factors were identified. The class E proteins, *SEPALLATA1* (*SEP1*)
250 and *SEPALLATA3* (*SEP3*), and the class D protein *SHP2*/*AGL5* were up-regulated during
251 feminization, reaching a maximum at day 16 (Fig. 2C, D and E). In contrast, the class B

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252 protein *PISTILLATA* (*PI*) was down-regulated within 4 days and disappeared completely
253 afterwards (Fig. 2F).

254 **Differential expression of floral homeotic genes**

255 The proteome data prompted cloning and analysis of *M. annua* orthologs of floral homeotic
256 MADS-box genes. The RNA expression pattern of the isolated genes in female and male
257 flowers, at bud and opened-flower stages was investigated (Fig.3). The class B genes *MaPI*
258 and *MaAP3* were highly expressed in male flowers and poorly expressed in female flowers
259 (Fig. 3A and 3B). The class C gene, *MaAG1* was strongly expressed with similar level in
260 female and male flowers (Fig. 3C). In contrast, the class D genes *MaAGL1* and *MaAGL3* were
261 strongly expressed in female flowers and poorly expressed in male flowers (Fig. 3D and 3E).
262 Moreover, the expression level of most floral genes was significantly different ($p<0.01$)
263 between the floral bud stage and the open flower stage; the expression of *MaAP3* and *MaAG1*
264 was higher at flower bud developmental stage, while the expression of *MaPI* and *MaAGL3*
265 was higher at open flower developmental stage (Fig. 3A, B, C and E).

266 The flower organ specificity of gene expression (Fig. 4) showed that *MaPI* and *MaAP3* were
267 almost exclusively expressed in male flowers, noteworthy that *MaPI* was also strongly
268 expressed in peduncles. *MaTM6* gene expression was evident in flowers of female and male
269 plants. The *MaTM6* expression was relatively higher in flowers and low in vegetative organs
270 of female plants. In the male, *MaTM6* expression was highest in flowers, moderate in leaves
271 and peduncles, and very low in stem and roots. *MaAGL1* and *MaSL1* were exclusively
272 expressed in flowers of female plants. *MaAG1* was expressed at moderate level in flowers of
273 both sexes, and at lower level in peduncle of male plants. *MaAGL3* was highly expressed in
274 flowers of female and slightly lower expression in flowers and peduncles of male plants.

275 BAP-induced feminization of male plants resulted in changes in expression patterns of
276 floral genes (Fig. 5). The expression of the class B identity gene *MaTM6* was not significantly
277 affected by feminization, while *MaPI* and *MaAP3* were down-regulated. In contrast, the
278 expression of class C/D floral genes, namely, *MaAG1, MaAGL1* and *MaAGL3* as well as
279 *MaSL1* was up-regulated by feminization. A significant up-regulation of class C and D genes
280 was observed at 8-11 days of BAP treatment.

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281 **Epigenetic regulation of floral genes**

282 Epigenetics has often been implicated in sex determination in dioecious plants (Janousek *et*
283 *al*., 1996; Brautigam *et al*., 2017). We thus wanted to address the involvement of epigenetic
284 mechanisms in the regulation of floral gene expression. To this end, we first examined the
285 chromatin configuration of promoters of several floral genes by micrococcal nuclease
286 (MNase) assay. The MNase-treated nuclei from male and feminized male flowers (after 14
287 days of BAP treatment) showed similar progressive digestion of genomic DNA with notable
288 nucleosomal ladders (Fig. 6A). MNase-digested DNAs was used as templates for PCR
289 analysis of promoter regions of several genes. The results showed (Fig. 6B) two major
290 digestion pattern reflecting open and relatively close chromatin configuration. Yet no notable
291 differences in digestion pattern could be observed between male and feminized male flowers.
292 Accordingly, group I consists the promoter regions of class B genes *MaPI* and *MaAP3*
293 showing higher sensitivity to MNase digestion similarly to actin, a constitutively expressed
294 gene. Group II, which composed of the class D gene *MaAGL1* as well as *MaSL1* and *MaSL2*
295 were more resistant to MNase digestion (Fig. 6B). Thus, it appears that class B genes that
296 assume an open chromatin conformation in male flowers remained open upon feminization,
297 while class D assumes a relatively close configuration in male and feminized male flowers.

298 To examine the role of DNA methylation in the control of chromatin configuration
299 and expression of floral genes, the status of cytosine methylation at the promoter regions of
300 several differentially expressed genes, namely, *MaSL1, MaSL2* and *MaAGL1* was assayed by
301 chop-PCR using the methylation sensitive enzymes *Hpa*II and *Msp*I. Notably, both enzymes
302 recognize the CCGG site but differ in their sensitivity to cytosine methylation. While *Hpa*II is
303 sensitive when either of cytosine is methylated, *Msp*I is sensitive only when the external
304 cytosine is methylated, allowing distinguishing between CG and CHG methylation. Chop-
305 PCR revealed no differences in CpG methylation status of the examined genes in female and
306 male flowers. However, CHG methylation appeared to be absent from the promoter regions of
307 *MaAGL1* and *MaSL2* genes in male flowers inasmuch as no recovery of PCR fragment could
308 be detected from *Msp*I digest (Fig. 7A). We also perform bisulfite sequencing of *MaAP3* and
309 *MaSL1* promoter and gene body regions showing no differences in DNA methylation status
310 between male and female flowers. The promoter regions of both genes were highly
311 methylated at all cytosine contexts (CG, CHG and CHH) while their gene bodies were
312 essentially unmethylated (Fig. 7B).

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313 # Discussion

314 The annual dioecious *Mercurialis annua* is a unique experimental system to study
315 mechanisms underlying plant sexual dimorphism. A major advantage of this species is the
316 possibility to feminize male plants that produce viable seeds. The change of the fate of the
317 male flower development by cytokinin treatment of the plants (Louis *et al*., 1990; Duran and
318 Durand, 1991), enabled investigation of gene regulation at various levels: proteome, mRNA
319 and epigenetics. The data obtained in this study regarding the expression of floral identity
320 genes are consistent with their known function in determining sexual identity of floral organs
321 in various plant species. It has been shown previously that class B genes were highly
322 expressed in male flowers, of the type-II dioecious plants *Thalictrum dioicum* and *Spinacia*
323 *oleracea* (Di Stilio *et al.,* 2005; Pfent *et al.,* 2005). In agreement, our results showed that male
324 flowers are characterized by a strong expression of class B genes, *MaPI* and *MaAP3*,
325 concomitantly with suppression of female identity genes such as *MaAGL1* (class D) and
326 *MaSL1*. The involvement of cytokinin in sex determination has been reported in a variety of
327 plant species including the oilseed crops *Plukenetia volubilis* and *Jatropha curcas* (Pan and
328 Xu, 2011; Fu *et al*., 2014).

329 **Expression pattern of floral genes**

330 Proteome analysis of BAP feminized males showed differential expression of several protein
331 families including nucleic acid binding proteins, hydrolases, ligases, transferases and
332 transcription factors. Interestingly, floral homeotic MADS-box gene product homologs of
333 *Arabidopsis* class E genes, SEP1, SEP3 and class D gene SHP2/AGL5 were up-regulated and
334 homolog of class B gene, PI*,* involved in specification of petals and stamens, was down-
335 regulated in feminized males. The proteins, SEP1 and SEP3 were implicated in regulation of
336 all four flower whorls of *Arabidopsis* (Zahn *et al*., 2005); while in other plants species, *SEP*-
337 like genes play diverse roles in growth and development including plant architecture, ovule
338 development, fruit ripening, inflorescence architecture and reproductive meristem fate
339 (Uimari *et al*. 2004; Biewers, 2014). In *Gerbera hybrida* two duplicated orthologs of SEP-like
340 gene *GRCD1* and *GRCD2* were sub-functionalized for stamen and carpel identity,
341 respectively. The *Mercurialis* orthologs of SEP1 and SEP3 proteins presented here might
342 have a role in female flower specification. The up-regulation of SHP2/AGL5 in feminized
343 males is consistent with SHP role in carpel development in *Arabidopsis*. Accordingly,

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344 constitutive expression of SHP genes in *Arabidopsis* resulted in a partial conversion of the
345 first whorl sepals into carpel-like structures demonstrated by extensive proliferation of
346 stigmatic papillae (Favaro *et al*., 2003; Pinyopich *et al*., 2003). The PI protein, which was
347 down-regulated in feminized males was involved in controlling the development of whorls 2
348 and 3 in *Arabidopsis*, *Antirhinum* and tomato (Trobner *et al*., 1992; Goto and Mayerowitz,
349 1994; Guo *et al*., 2016). Thus, these results suggest that the cytokinin switched-off the male
350 control genes (e.g., *PISTILLATA*) concomitantly with up-regulation of female identity genes
351 consequently leading to the replacement of stamen by carpels, as in the development of
352 normal dioecious female flower.

353 An earlier study, using a cell-free translation system with RNAs derived from *M. annua* male
354 and female flowers demonstrated peptide variation between the two sexes and that cytokinin-
355 induced feminization of male flowers has led to the expression of female-specific peptides
356 (Deligue *et al*., 1984). Similarly, we found that cytokinin-induced feminization of *M. annua*
357 male flowers was associated with upregulation of female-specific floral genes concomitantly
358 with downregulation of male-specific genes. The effect of cytokinin on floral gene expression
359 was reported previously (Estruch *et al*., 1993). Accordingly, the expression of the cytokinin-
360 synthesizing gene *IPT* in transgenic tobacco plants resulted in abnormal flower development
361 concomitantly with a notable decrease in accumulation of class B genes (*DEFA*, *GLO*) and
362 class C gene (*PLENA*) (Estruch *et al*., 1993). In *Arabidopsis,* exogenous application of BAP
363 was reported to promote differentiation of carpeloid tissue and suppress stamen development.
364 This is similar to the effect obtained by overexpressing *SUP* in tobacco plants leading to the
365 proposition that *SUP* may regulate sex determination pathways by promoting female organ
366 differentiation *via* its effect on cytokinin signaling (Nibau *et al*., 2011). Alternatively,
367 cytokinin may affect male and female flower development *via* controlling *SUP* expression.
368 Indeed, in *M. annua* as well as in the dioecious *Popolus tomentosa* and *Silene latifolia* the
369 *SUP*-like genes exhibited female flower-specific expression (Kazama *et al*., 2009; Song *et al*.,
370 2013). In *Arabidopsis*, *sup* mutant was associated with the ectopic expression of *AP3* gene in
371 the fourth whorl (Bowman *et al*., 1992), therefore *SUP* was proposed to function as a negative
372 regulator of *AP3*. The concomitant expression of class B and SUP-like genes in male flower
373 buds suggests that *SUP*-like gene(s) might not be a transcriptional regulator of class B genes
374 in *M. annua*. An alternative possibility is that the *SUP* gene expression in male flower buds is
375 negatively regulated post-transcriptionally.

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376 The expression of class B gene, *MaAP3*, was restricted to male flowers, while *MaTM6*
377 *(AP3*-related*)* and *MaPI*, were expressed in flowers as well as in peduncles (Fig. 4). It is
378 noteworthy that *TM6*, which is absent in *A. thaliana,* was also expressed in leaves of *M.*
379 *annua* male plants and weakly in other vegetative organs. The broader expression pattern of
380 *TM6* orthologs was reported in *Carica papaya* (*CpTM6-2*) and *Vitis vinifera* (*VvTM6*);
381 *CpTM6-2* was expressed at a low level in sepals and at a high level in leaves (Ackerman *et*
382 *al*., 2008), while *VvTM6* was expressed throughout the plant, though displaying high levels in
383 flowers and berries (Poupin *et al*., 2007). It has been proposed that a gene duplication event of
384 the *paleoAP3* genes resulted in two types, *euAP3* and *TM6* lineages that are distinguished by
385 their C-terminal regions (Kramer *et al*., 1998). These duplicated genes probably adopted, to
386 some extent, different functions (sub-functionalization) demonstrated by their tissue-specific
387 patterns of expression and the effect of their loss-of-function on flower development (Eckardt,
388 2006).

389 The expression of class C gene, *MaAG1* was similar in male and female flowers of *M.*
390 *annua* suggesting it may not involve in gender determination. This is consistent with previous
391 reports showing that the C class *AG* genes are involved in the floral quartet specifying both
392 stamens and carpels (reviewed in Theissen *et al*., 2016). The class D genes, *MaAGL1* and
393 *MaAGL3* were highly expressed in female flowers; *MaAGL3* was also expressed in male
394 flowers as well as in peduncles (Fig. 4). Our results suggest that class B genes *MaAP3*, *MaPI*
395 together with class C *MaAG1* have a role in determining the identity of male floral organs.
396 These gene products may participate in the floral quartet that controls gene expression and
397 male reproductive organ identity (Theissen *et al*., 2016). On the other hand, class D genes
398 *MaAGL1* and *MaAGL3* together with class C and class E genes may form a floral quartet that
399 specifies female floral organs, carpels and ovules. Notably, in seed plants the class B genes
400 have been suggested to have a primary role in sex-determination (Winter *et al*., 1999).
401 Accordingly, expression of both, class B and class C genes specify male reproductive organs
402 while the expression of only class C genes specify female reproductive organs. Thus,
403 switching from male to female and *vice versa* can be solely driven by changes in the spatio-
404 temporal expression of class B genes (Winter *et al*., 1999; Theissen and Melzer, 2007). Our
405 data however showed that induction of feminization was associated not only with turning off
406 expression of class B genes but also with up-regulation of class C/D genes, which might be
407 crucial for the development of female flower in otherwise male plants of *M. annua*.

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408 **Epigenetics and sex determination**

409 Our data showed that there is no clear relationship between floral homeotic genes and
410 their epigenetic makeup (Table 1). Gene expression is primarily regulated at the chromatin
411 level where gene transcription requires open chromatin to allow for the transcription
412 machinery to approach the gene locus. The analysis of chromatin accessibility by MNase
413 assay revealed that in male flowers class B genes *MaPI* and *MaAP3* assume an open
414 chromatin conformation similar to the constitutively expressed gene *Actin*. On the other hand,
415 class D genes *MaAGL1* and SUP-like genes *MaSL1* and *MaSL2* appeared to acquire a
416 relatively close conformation, which is consistent with the lack of expression in male flowers.
417 Surprisingly, however, upon feminization and up-regulation of *MaAGL1* and *MaSL1* no
418 apparent change in accessibility of chromatin to MNase was evident. This suggests that
419 chromatin can assume different levels of open chromatin conformation that provide another
420 regulatory layer for control of gene expression (Ishihara *et al*., 2010; Kotomura *et al*., 2015).
421 Similarly, no change in chromatin accessibility was observed for the down-regulated class B
422 genes, *MaPI* and *MaAP3*, whose transcription was possibly halted in an open chromatin
423 environment by other means (e.g., suppressor proteins).

424 The nature of gene regulation by DNA methylation is not fully understood, but
425 generally DNA methylation has been implicated in regulating chromatin structure and
426 function (Niederhuth and Schmitz, 2017). DNA methylation was detected at promoters but
427 not at gene-bodies of the examined floral genes. Interestingly, methylation status of all tested
428 genes was similar in both sexes despite of their differential expression. In *Arabidopsis,* gene
429 methylation was reported to correlate with gene expression level; gene-body methylation was
430 correlated with constitutively and highly expressed genes, while promoter methylation was
431 correlated with weakly expressed genes, which are usually tissue-specific (Zhang *et al*., 2006;
432 Zilberman *et al*., 2007). However, in this work, a consistent correlation between DNA
433 methylation and expression of the floral genes in *M. annua* was not found (Table 1 and Fig.
434 7). The floral genes *MaAP3*, *MaAGL1*, *MaSL1* and *MaSL2* were normally transcribed in spite
435 of being heavily methylated at their promoters. Thus, it appears that DNA methylation at
436 promoter regions of *M. annua* floral genes had a positive effect on floral gene expression, in
437 contrast to the commonly known effect of suppression of expression by methylation,
438 particularly when transposable elements are concerned (Lisch, 2009). This finding can
439 plausibly be explained by lowering the affinity of repressors to their binding sites by DNA

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440 methylation. There are indeed reports that showed likewise that DNA methylation at
441 promoters contributes to transcriptional activation of certain tissue-specific genes (Neissen *et*
442 *al*., 2005; Weber *et al*., 2007; Rishi *et al*., 2010; Bahar Halpren *et al*., 2014).

443 We concluded that determination of sex organ identity in *M. annua* does not primarily
444 involve epigenetic regulation of floral homeotic genes. Rather, the gender identity of a
445 dioecious flower seems to be controlled up-stream in the regulatory pathway by a gender-
446 specific regulator(s) that affects hormonal homeostasis. This is further supported by a recent
447 report that identified only a handful number of epigenetically-regulated genes within the sex-
448 determining region of *Populus balsamifera* (Brautigam *et al*., 2017). In that work it was
449 shown that both the promoter and gene body of PbRR9 were methylated. Since this gene is a
450 member of the two-component response regulator (type-A) gene family, which is involved in
451 cytokinin signaling, it would be important to explore further the role of genes involved in
452 hormonal homeostasis in sex determination in *M. annua*.

453

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454 **Supplementary data**

455 Supplementary file 1

456 Text: Sequence and phylogenetic analysis of floral MADS-box and *SUPERMAN-like* genes

457 Fig. S1. Phylogenetic analysis of class B genes from *M. annua*, *A. thaliana* and various
458 taxonomic groups.

459 Fig S2: Phylogenetic analysis of *AG*-like genes from *M. annua*, *A. thaliana* and various
460 taxonomic groups.

461 Fig S3: Phylogenetic analysis of *SUPERMAN-like* genes from *M. annua*, *A. thaliana* and
462 various taxonomic groups.

463 Table S1: List of primers used in this study

464 Supplementary file 2 (Excel Sheets)

465 S1: List of proteins identified in proteomic analysis

466 S2: Proteins exclusively present in female flower buds that appeared following BAP treatment

467 S3: Proteins exclusively present in male flower buds that disappeared following BAP
468 treatment

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477

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Figure legends

Figure 1. **Morphological observation of dioecious *M. annua***. (**A**) Female plant. (**B**) Male inflorescence. (**C**) Feminized male inflorescence, BAP-hormone was sprayed 3 times daily for 4 weeks. Note that feminized male produced bi-carpellet flowers (some are indicated by arrows).

Figure 2. Proteome analysis of nuclei isolated from female, male and BAP treated male flower buds. Categorization analysis of down-regulated (**A**) and up-regulated (**B**) proteins following male feminization. The LFQ intensity of SEPALLATA1 (**C**), SEPALLATA3 (**D**), SHP2/AGAMOUS-like5 (**E**) and PISTILLATA (**F**) proteins in female, male and BAP treated males for 4, 8, 12 and 16 days, is shown. F, Female; M, Male; DABAP, Days after initiation of BAP treatment. Note that LFQ (label-free quantification) intensity reflects the relative amounts of the proteins, which was calculated using peptide intensities normalized between the samples.

Figure 3. Expression of MADS-box genes in flower buds and open flowers of female and male plants of *M. annua*. Relative expression of (**A**) *MaPI*, (**B**) *MaAP3*, (**C**) *MaAG1*, (**D**) *MaAGL1* and (**E**) *MaAGL3* genes determined using RT-qPCR. Y-axis shows relative transcript level of genes normalization to *Actin* gene. The values are average of three biological replicates. Values denoted by different numbers of asterisks are significantly different (Students *t*-test, $P < 0.01$). Error bars indicate the standard error of the mean (n=3).

Figure 4. Expression pattern of floral homeotic genes in different organs of female and male plants of *M. annua*. Expression of class B, C, D and SUPERMAN-like genes was determined using semi-quantitative PCR using cDNAs derived from RNA prepared from the indicated organs. Actin was used as ubiquitously expressed reference gene. M, molecular size markers in base pairs.

Figure 5. A time course of the expression of floral genes during BAP-induced feminization. 25-day-old *M. annua* plants were sprayed 3 times daily with water (control male) or with cytokinin (BAP-treated male). Newly emerging inflorescences were collected on the indicated days, RNA was prepared and subjected to cDNA synthesis. The expression of the indicated floral genes was determined by semi-quantitative PCR using cDNAs as templates. The class to which floral homeotic genes belong is indicated on the left. Actin was used as reference gene.

Figure 6. Analysis of chromatin configuration of selected floral genes by micrococcal nuclease assay. (**A**) Nuclei prepared from male and feminized male flower buds (BAP-treated for 14 days, before female flowers are visible) were treated with MNase for the indicated time periods. DNA was extracted from MNase-treated nuclei and resolved on 1.5% agarose gel. M, molecular size marker in

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base pairs. (**B**) Assessment of chromatin configuration of promoters of the indicated genes was performed by PCR using DNA recovered from MNase-treated nuclei (shown in A). Group I refers to male-related identity genes and Group II to Female-related identity genes. Actin was used as reference for open chromatin configuration. M, molecular size markers in base pairs.

Figure 7. Transcriptionally active floral genes are methylated in both genders of *M. annua.* (**A**) Analysis of DNA methylation at promoters of MaAGL1, MaSL1 and MaSL2 genes by Chop-PCR. A fragmet of the *MaSL1* gene lacking CCGG site was used as control. Left panel is a control of undigested DNA (Ud). H, *HpaII*; M, *MspI*; L, molecular size markers in base pairs. (**B**) Analysis of methylation at promoter and gene-body of *MaAP3* and *MaSL1* genes by bisulfite sequencing. The percentage of cytosine methylation for each fragment was determined from at least 10 different clones.

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Table 1: Summary of the expression level of floral genes in relation to their epigenetic constraints.

Gene name	expression			DNA methylation			Sensitivity to Mnase			Score		
	Female	Male	F-male	Female	Male	F-male	Female	Male	F-male	Female	Male	F-male
MaPI	-	++	-					high	high	S	O E	O S
MaAP3	-	+++	-	mALL	mALL			high	high	m S	O m E	O S
MaActin	+++	+++	+++					high	high	E	O E	O E
MaAGL3	+++	+	+++							E	e	E
MaAGL1	+++	-	++	mALL	mCG			low	low	m E	PO m S	PO E
MaSL1	++	-	++	mALL	mALL			low	low	m E	PO m S	PO E
MaSL2	++	-	-	mALL	mCG			low	low	m E	PO S	PO S
MaAG1	+++	++	+++							E	E	E

-, no expression; +, low expression; ++/+++, high expression; mAll, methylated at all C context; mCG, methylated at the CG context only; S, silent; E, expressed; e, low expression; O, Open chromatin; PO, Partial open chromatin.

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Figure 1

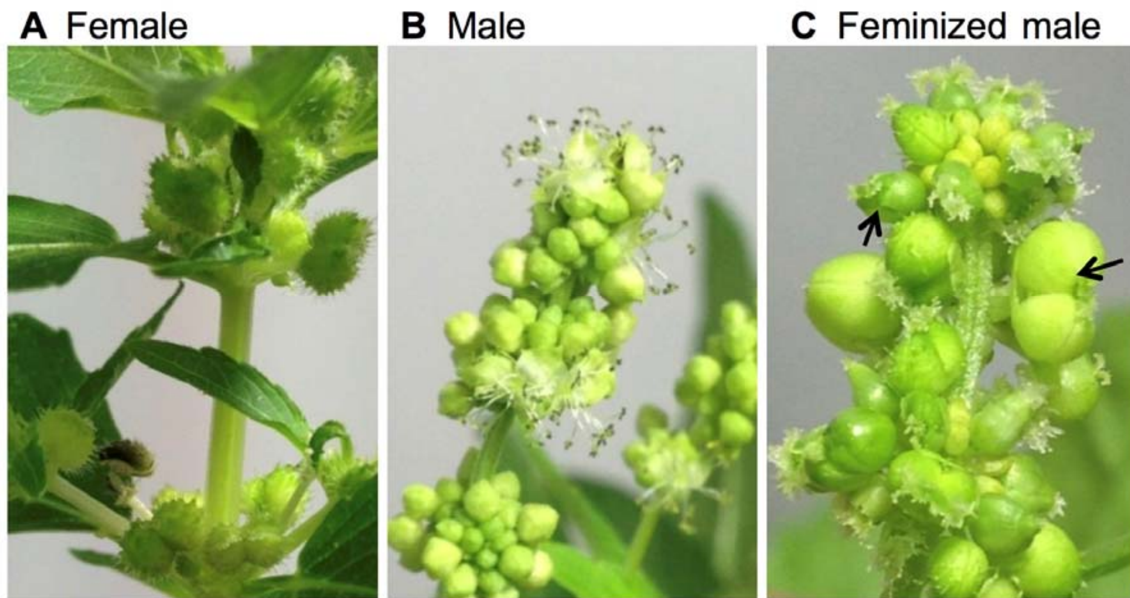

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Fig. 2

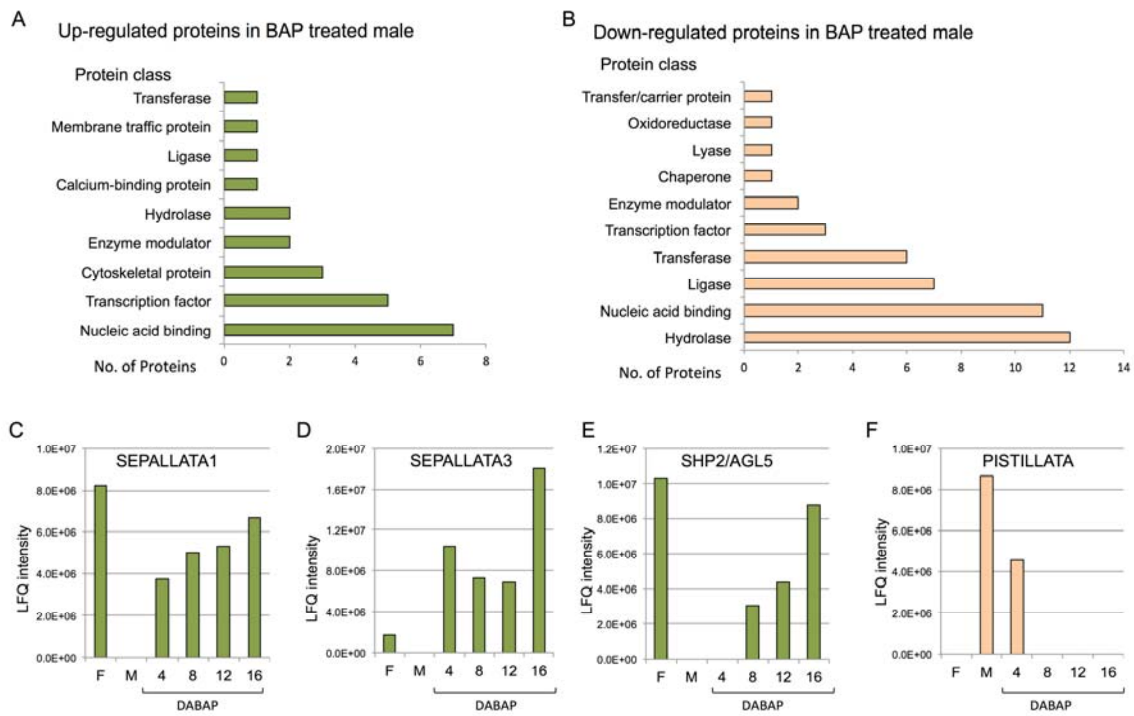

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Fig. 3

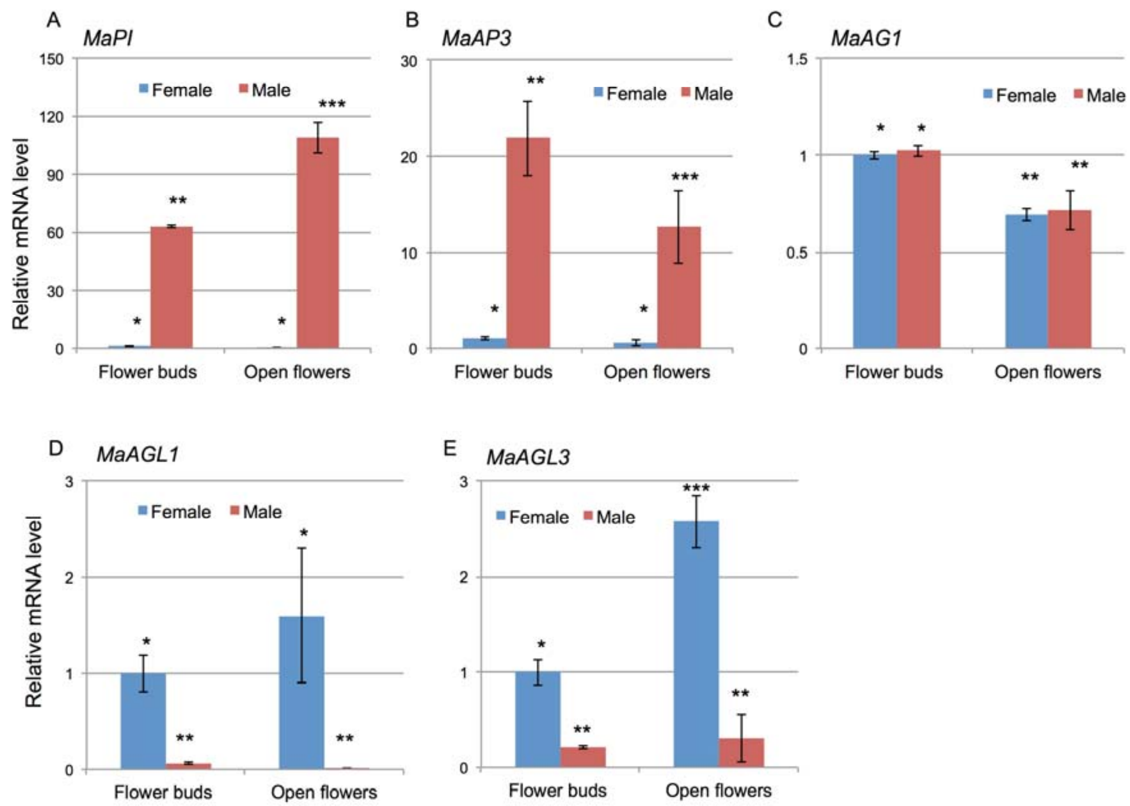

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Fig. 4

Female
Male
Flower Stem Leaf Root
Flower Stem Leaf Root Peduncle
M bp
Class
B
MaPI
MaAP3
MaTM6
C
MaAG1
D
MaAGL1
MaAGL3
SUP
MaSL1
MaSL2
Actin
-300
-400
-500
-500
-400
-200
-300
-300
-200

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Fig. 5

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Fig. 6

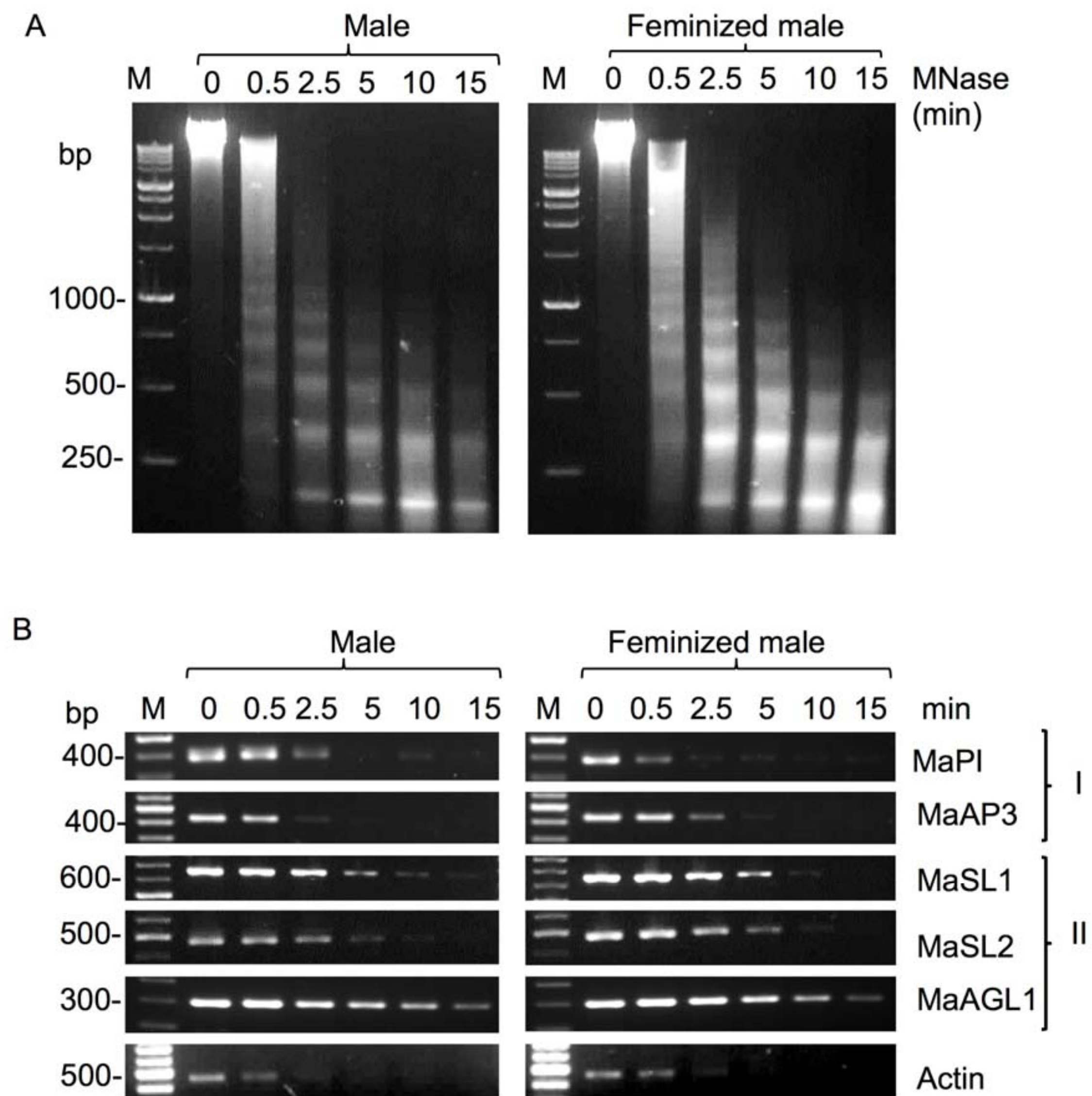

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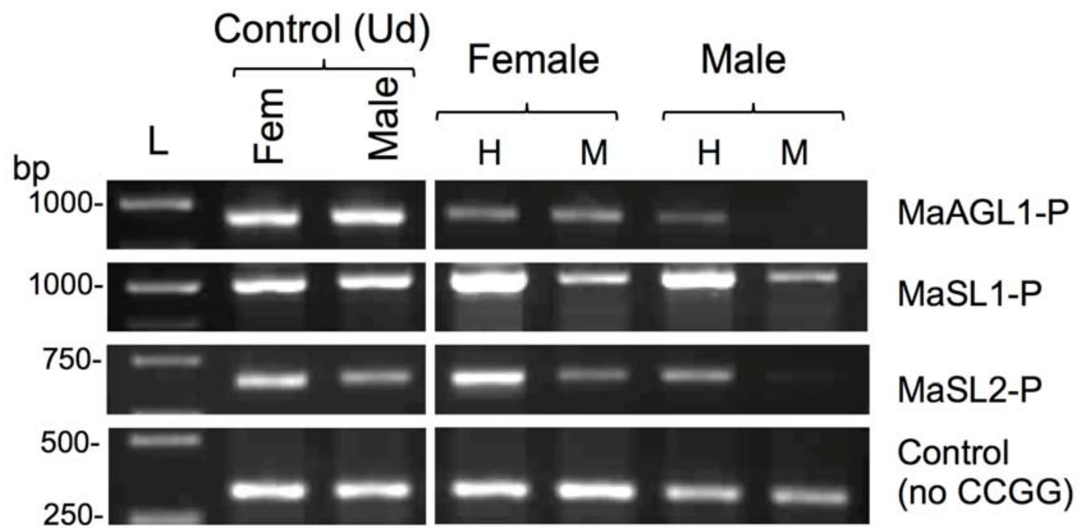
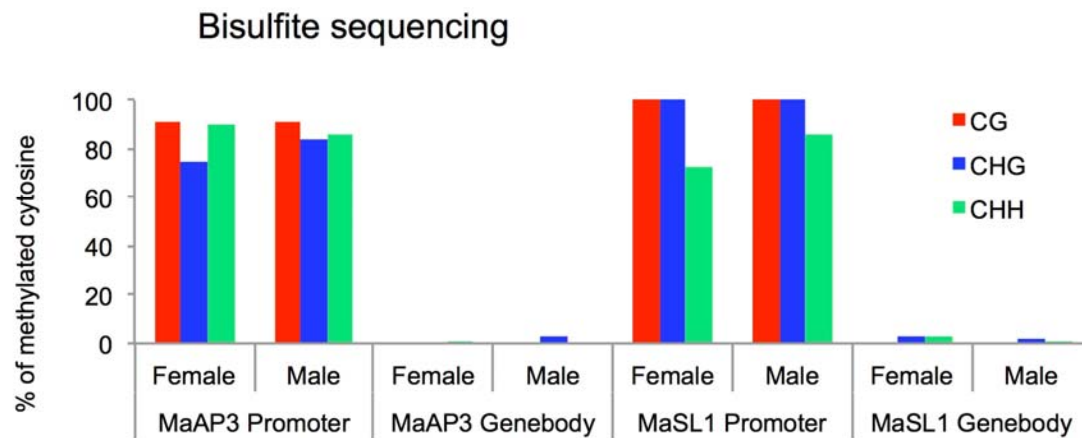
Fig. 7





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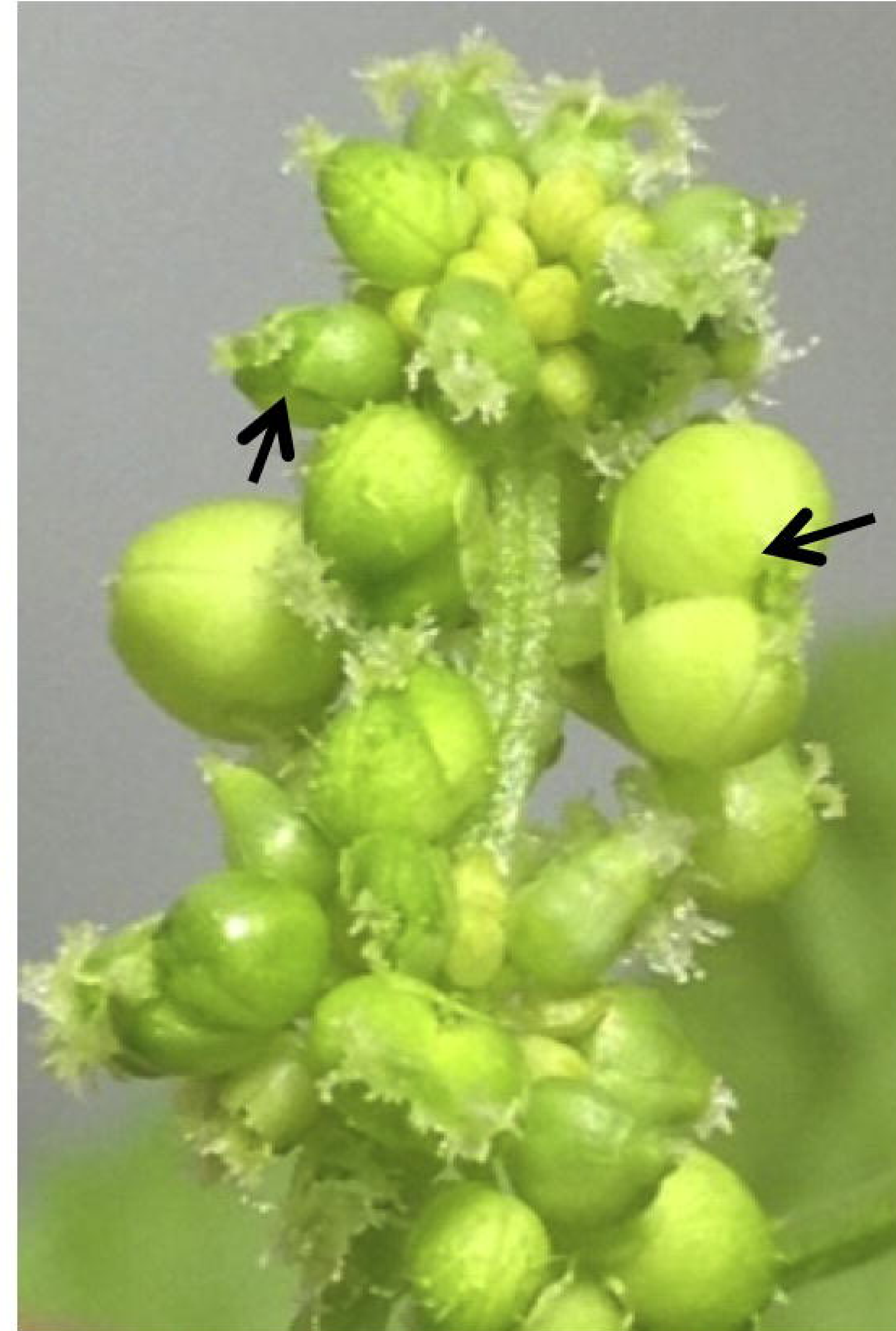

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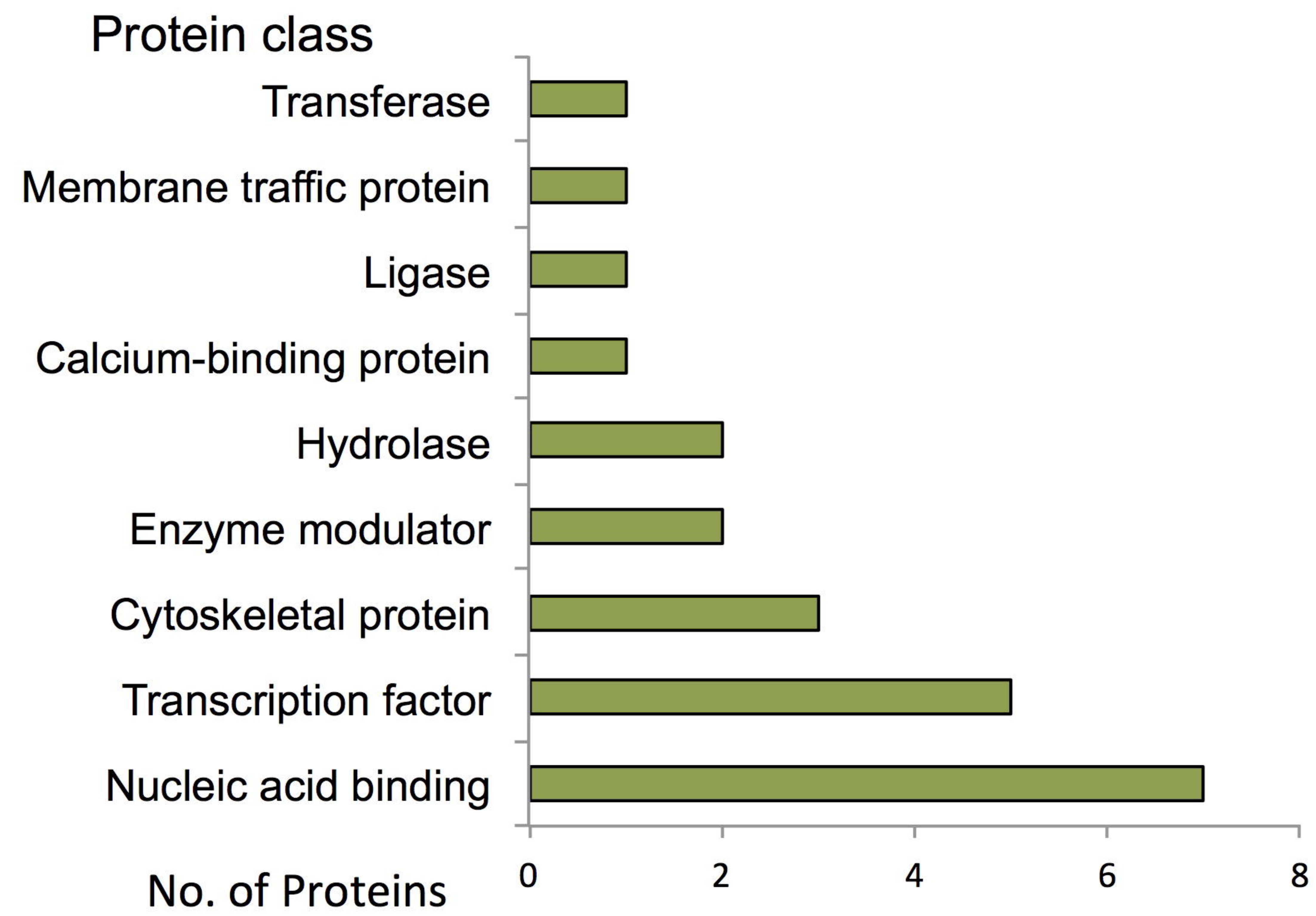

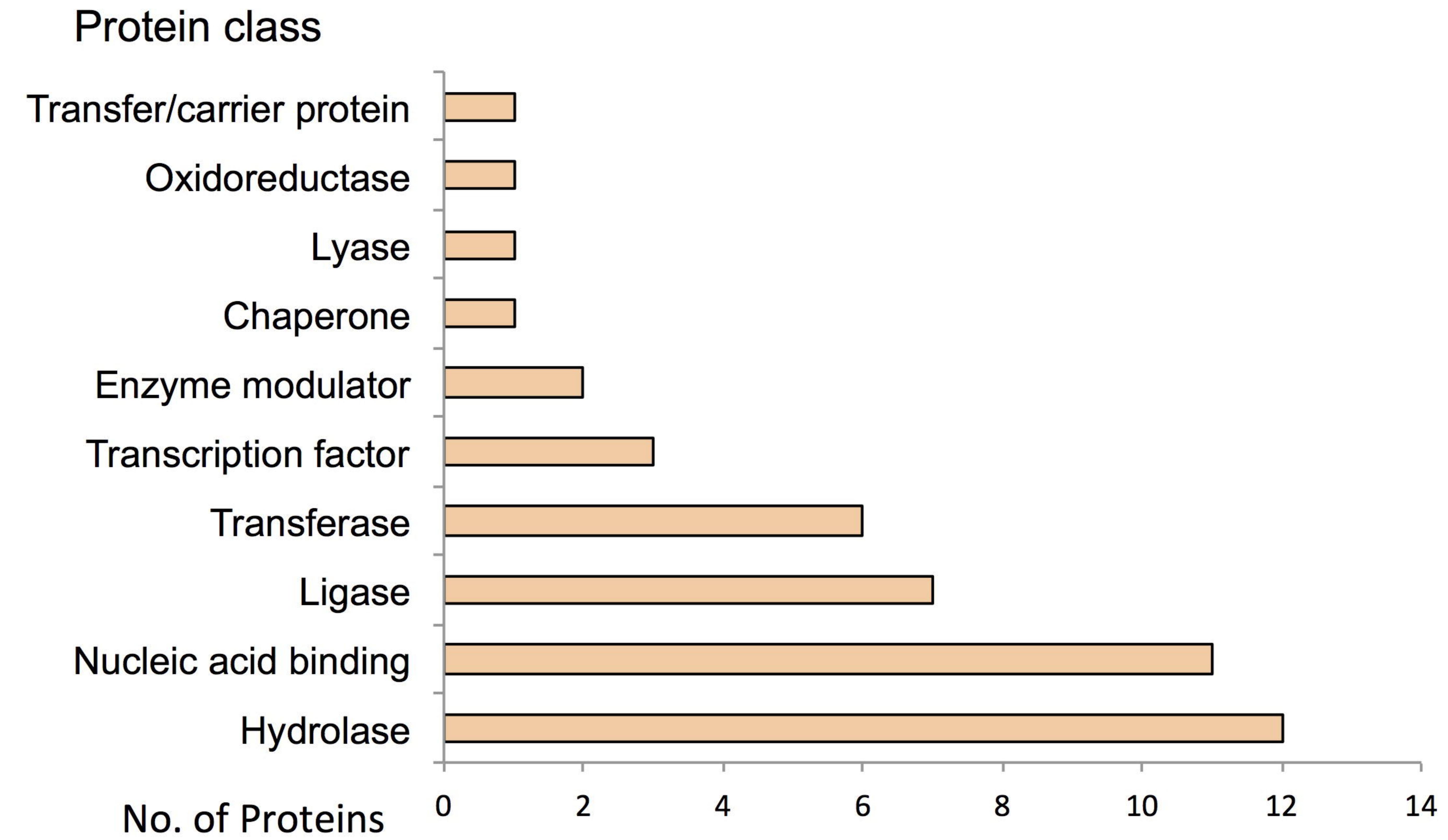

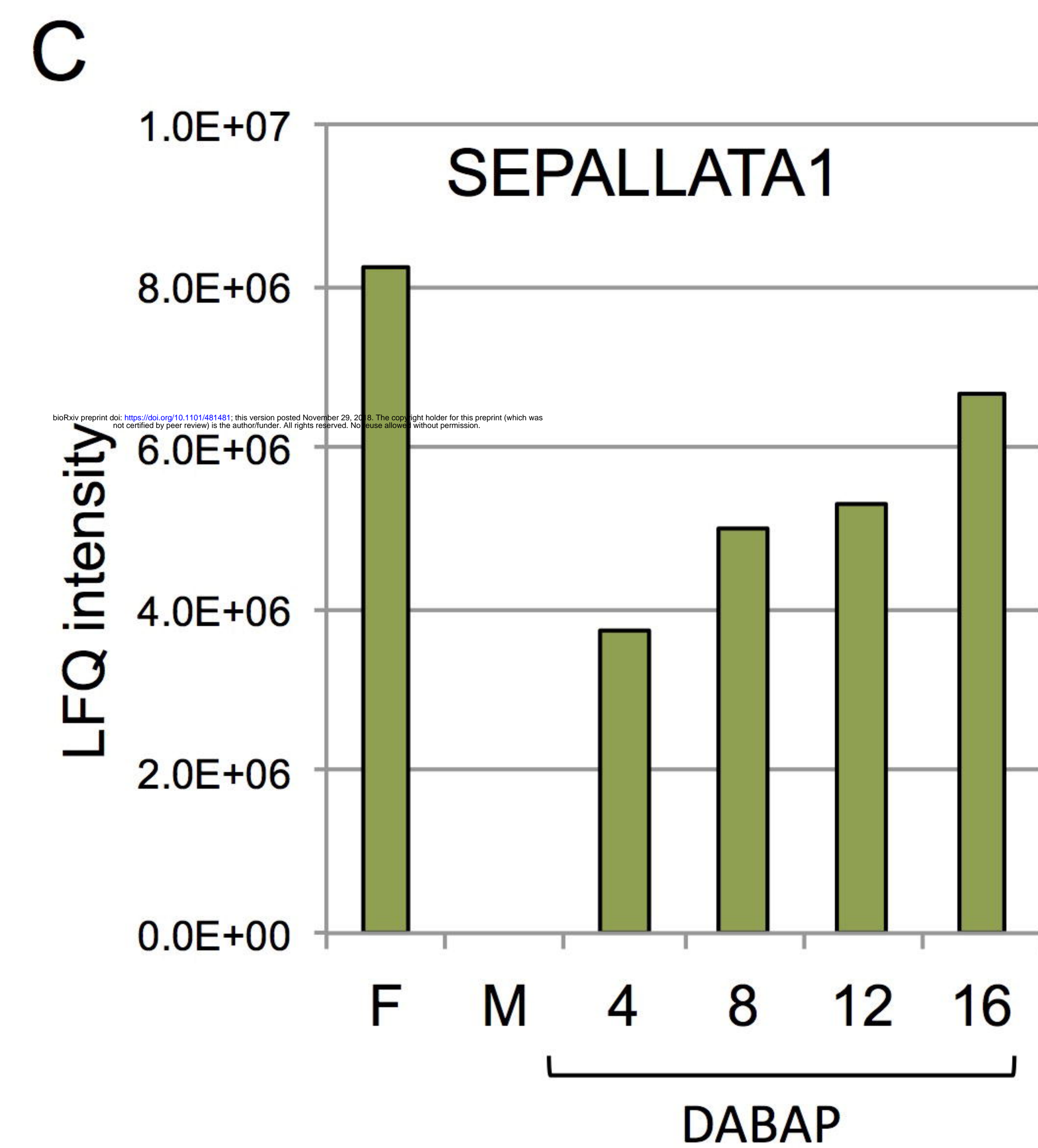

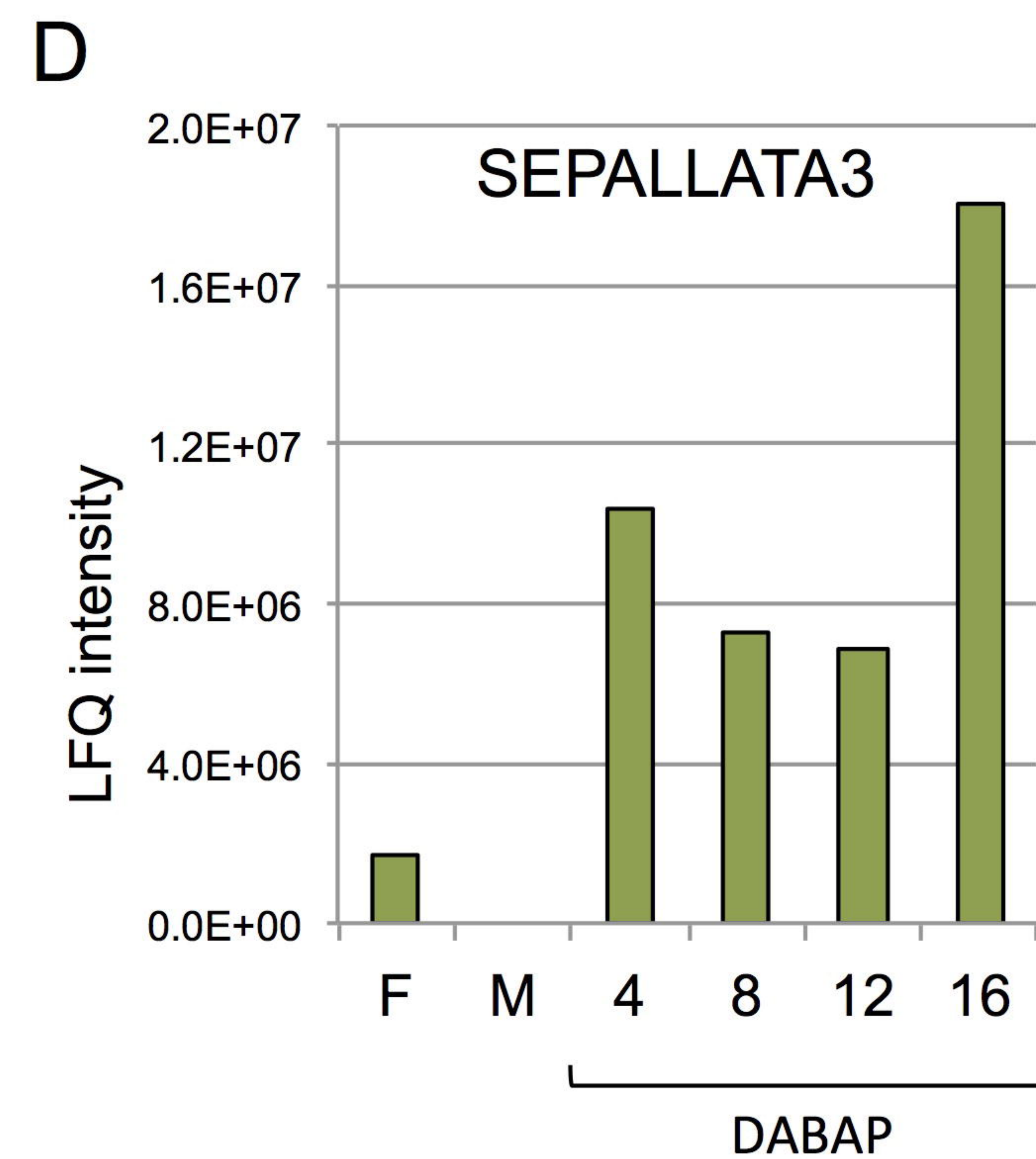

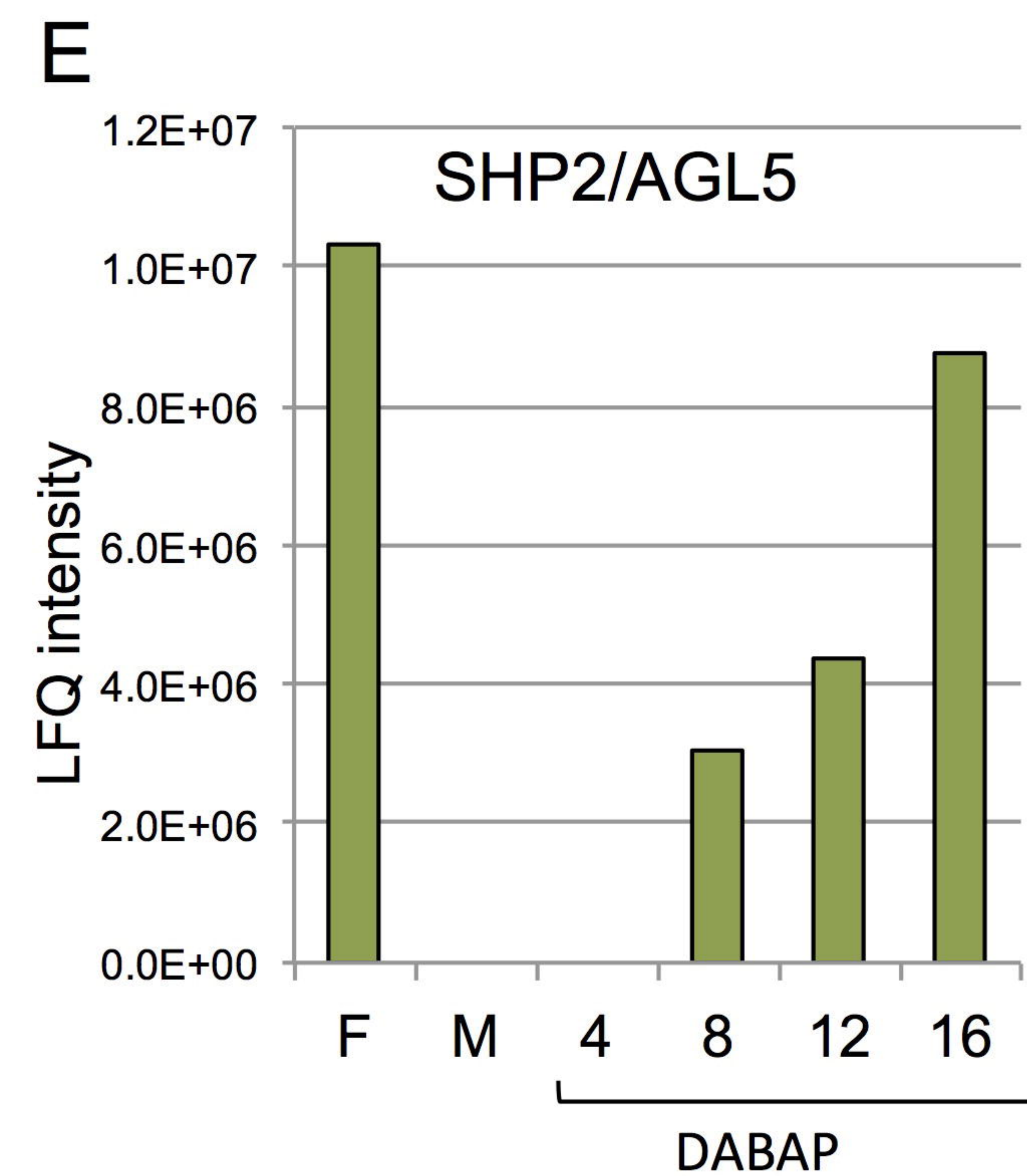

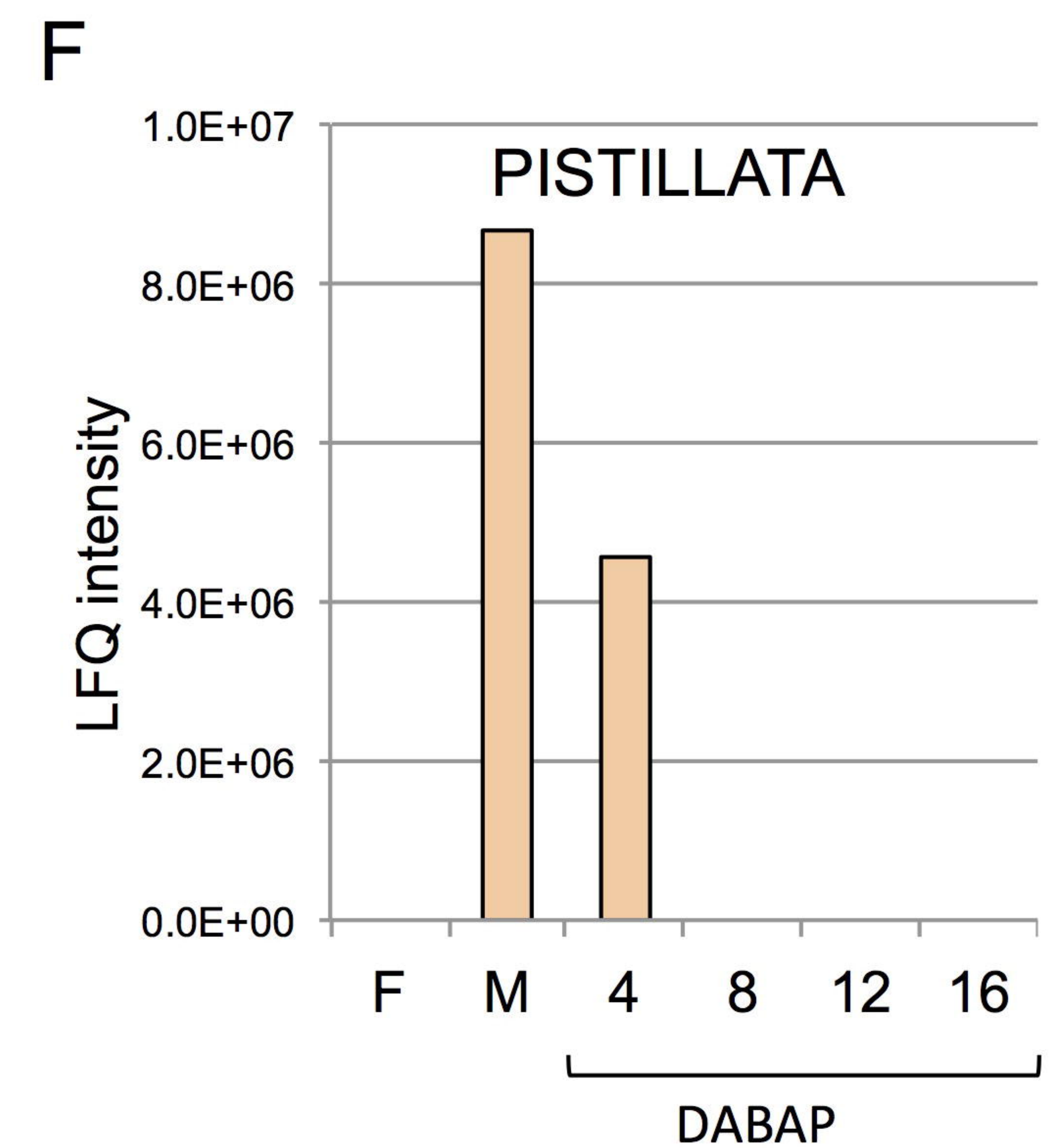

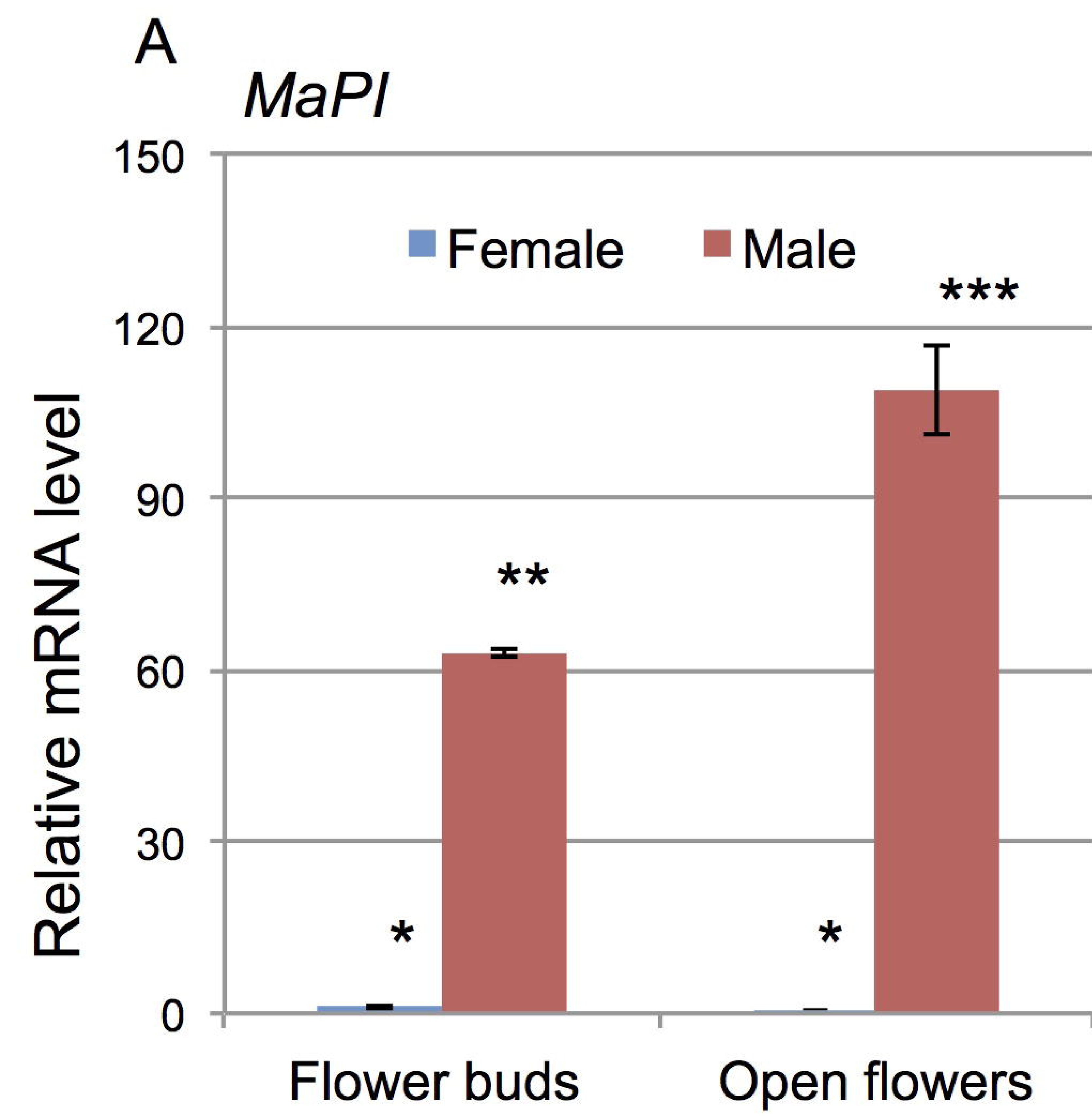

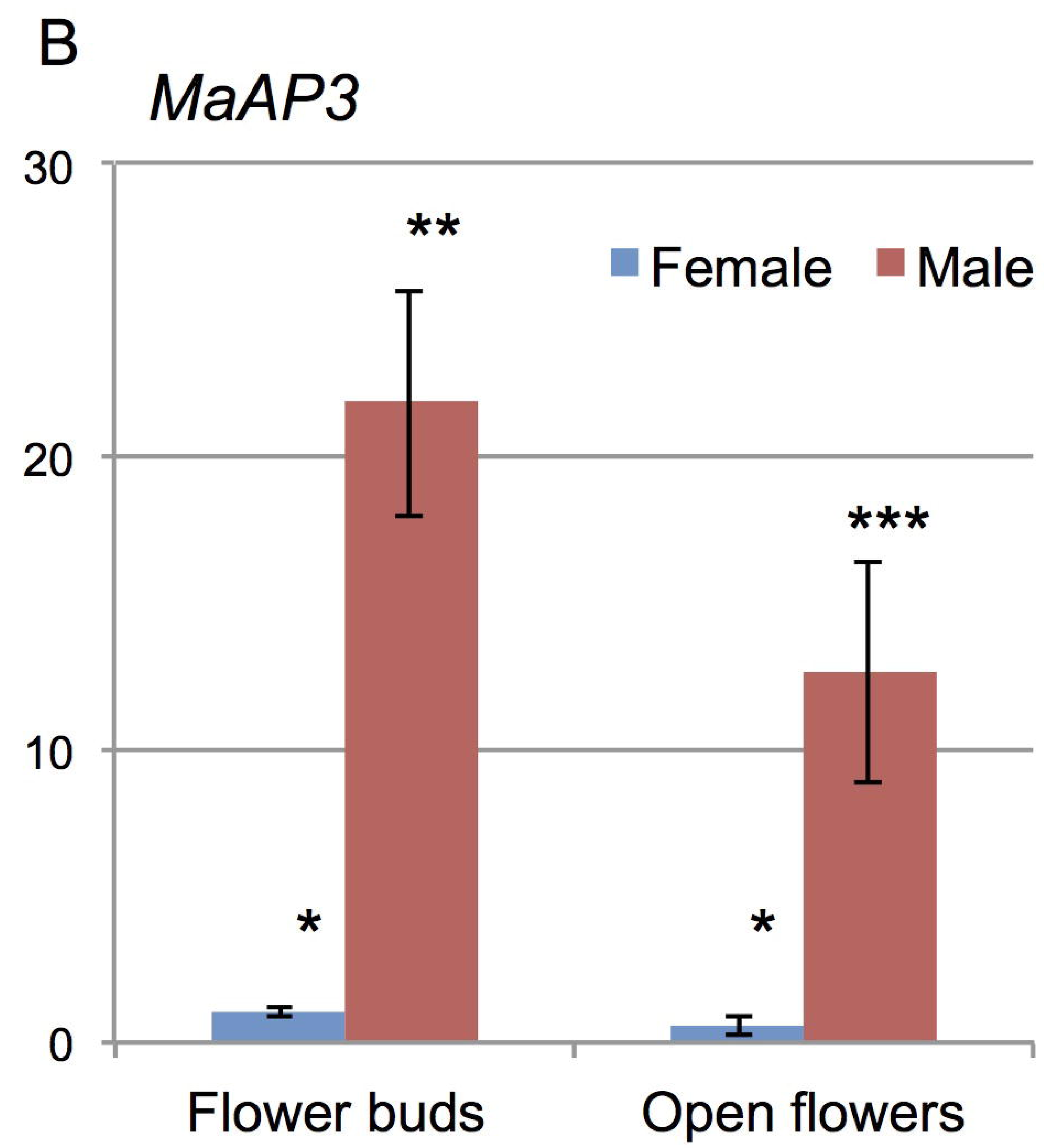

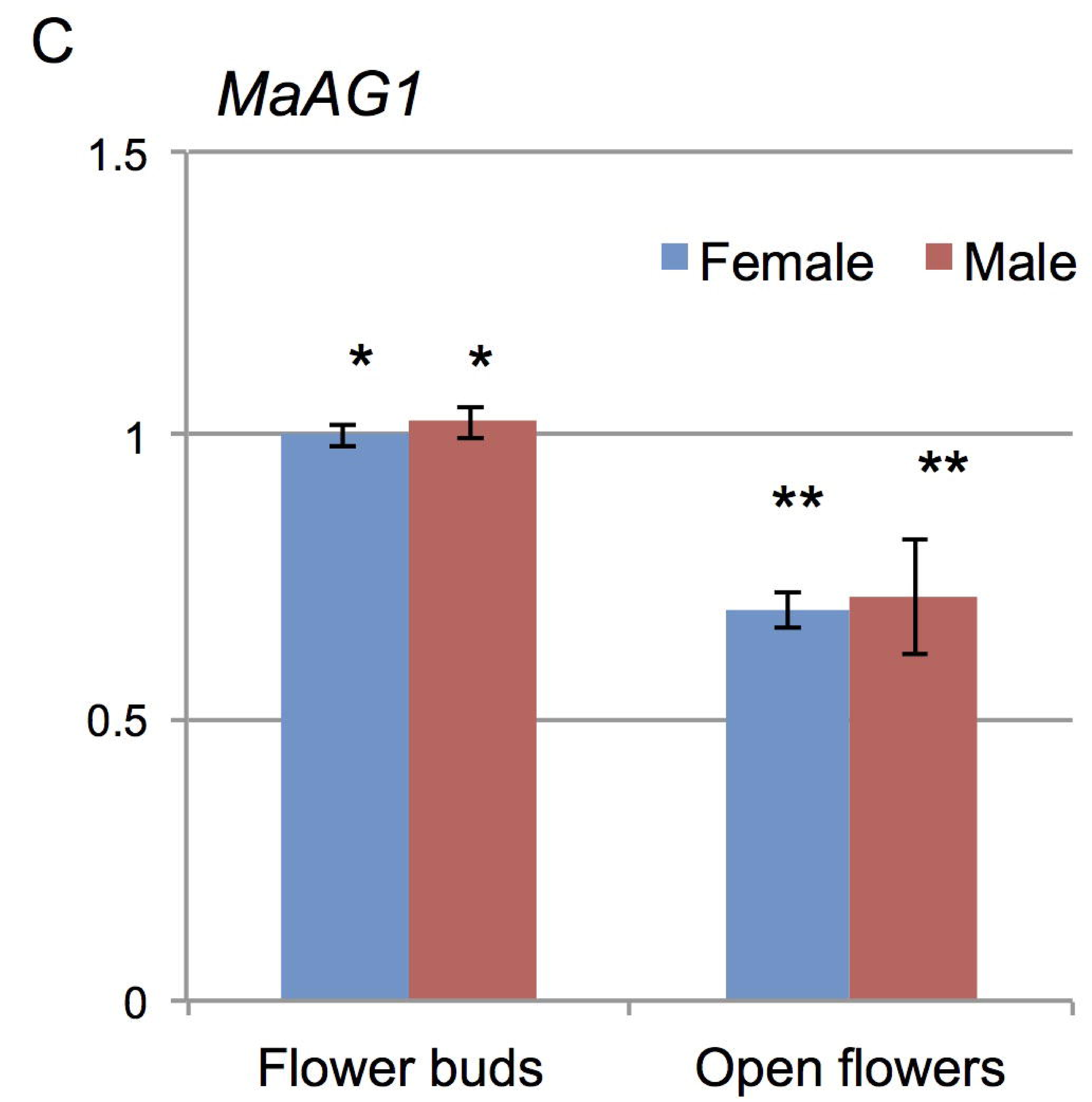

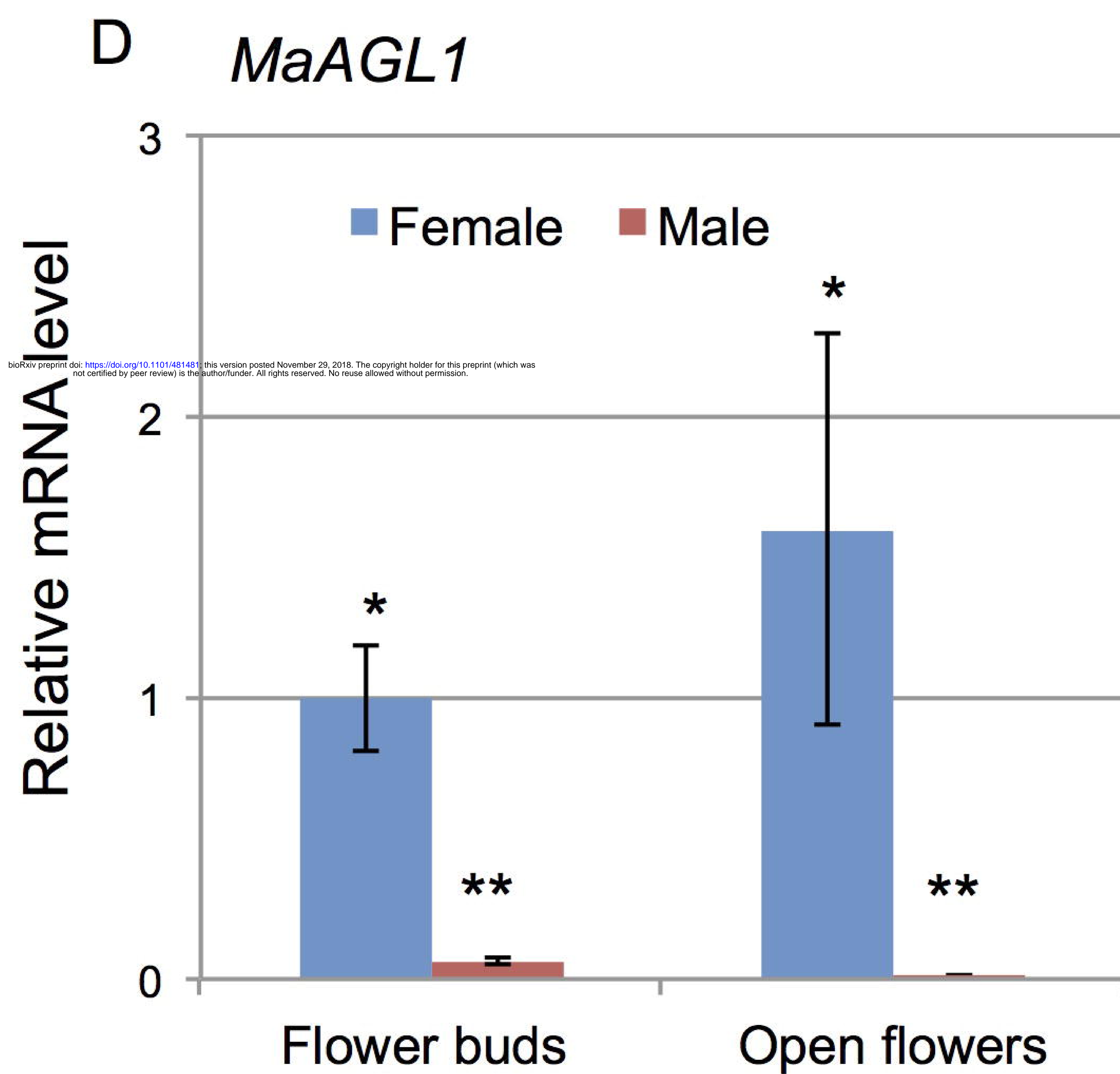

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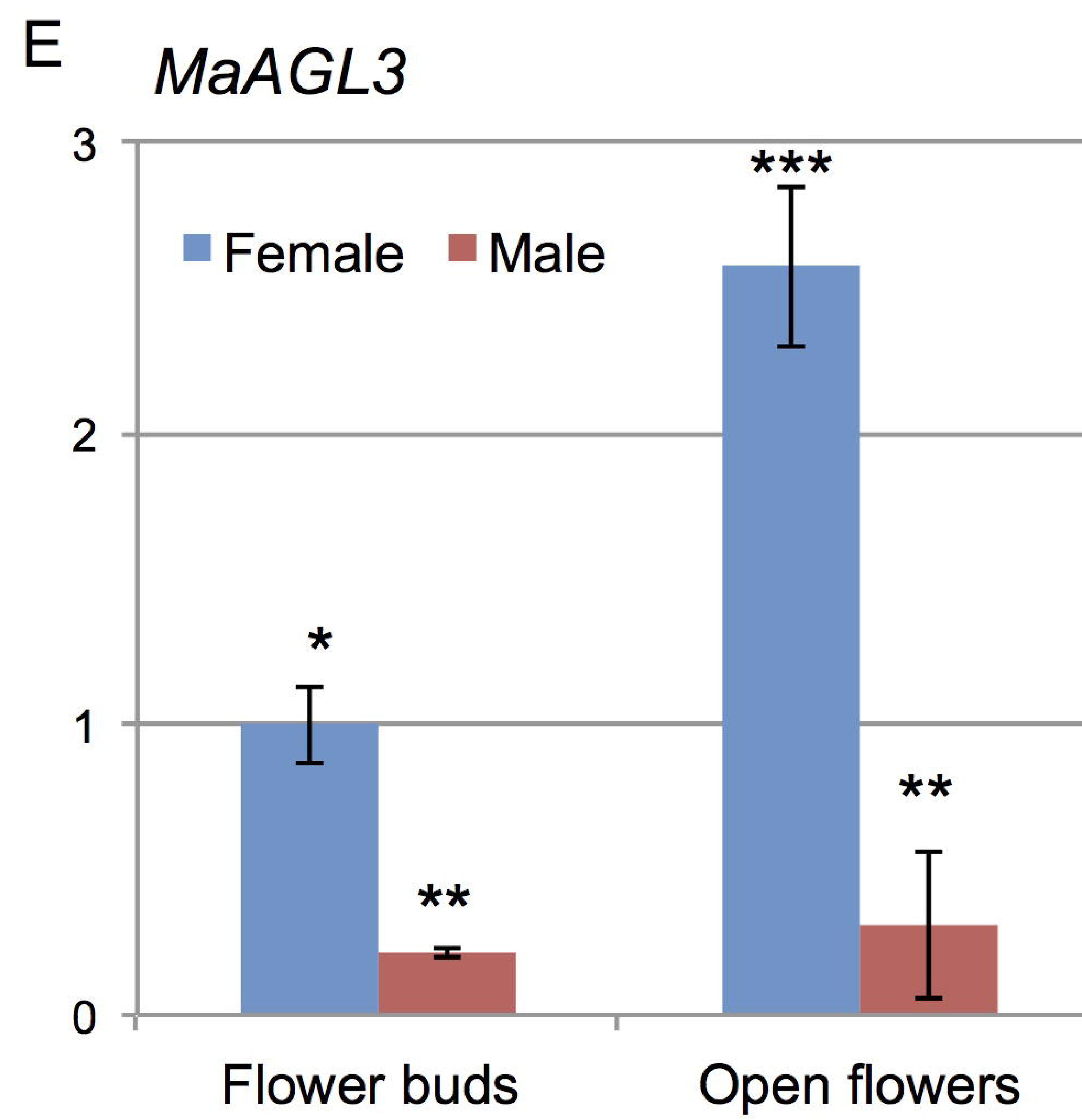

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A

Control (Ud)

Fem Male

Female

H M

Male

H M

L

bp

1000-

1000-

750-

500-

250-

MaAGL1-P

MaSL1-P

MaSL2-P

Control (no CCGG)

B

Bisulfite sequencing

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