Plant Physiology Preview. Published on December 5, 2016, as DOI:10.1104/pp.16.01024

1 **Running title: SUF4 regulates the *EC1* genes**

2 Corresponding authors:

3 Simona Masiero tel.0039-02-50315039 email simona.masiero@unimi.it

4 Stefanie Sprunck tel.0049-941-943-3005 email stefanie.Sprunck@ur.de

5

6 **SUPRESSOR OF FRIGIDA (SUF4) supports gamete fusion via regulating Arabidopsis EC1**

7 **gene expression**

8

9

10 Francesca Resentini[1], Philipp Cyprys[2], Joshua G. Steffen[3], Svenja Alter[2,#], Piero Morandini[1], Chiara

11 Mizzotti[1], Alan Lloyd[3], Gary N. Drews[3], Thomas Dresselhaus[2], Lucia Colombo[1,4], Stefanie

12 Sprunck[2*], Simona Masiero[1*]

13

14 [1,] Dipartimento di Bioscienze, Università degli Studi di Milano, 20133 Milano, Italy

15 [2,] Lehrstuhl Zellbiologie und Pflanzenbiochemie, Biochemie-Zentrum Regensburg, Universität

16 Regensburg, Universitätsstrasse 31, D-93053 Regensburg, Germany

17 [3,] Department of Biology, University of Utah, Salt Lake City, Utah 84112, USA

18 [4,] Istituto di Biofisica, CNR, Università di Milano, Italy

19 # present address: Institut für Humangenetik, Molekulargenetische Diagnostik, Breisacher Str. 33,

20 D-79106 Freiburg i. Br., Germany

21 * Corresponding authors

22

23

24

25

26

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.
Copyright 2016 by the American Society of Plant Biologists

27 **One sentence summary**

28 In Arabidopsis gamete fusion requires the C_2H_2 transcription factor SUF4, which regulates the
29 expression of the *EC1* (*EGG CELL 1*) gene family

30

31 **Key words: yeast one-hybrid, gamete fusion, *EC1* promoter, SUF4, transcription factor,**
32 **histone modification, *MOM1***

33

34 This work was funded by the German Research Council (DFG) (grants SP 686/1–2 and SFB924 to
35 SS) and by PPP Vigoni 2009 by the DAAD (German Academic Exchange Service) to TD and LC.
36 FR was funded by a PhD fellowship from Università degli Studi di Milano. SM was supported by
37 the CARIPLO Foundation (2011-2257).

38

39 **Author contributions**

40 SM, SS, GND, LC and TD conceived/designed experiments. FR, PC, JGS, SA, AL, PM, CM, SS
41 and SM performed experiments. FR, PM, SA, GND, SS and SM analysed data. FR, SS and SM
42 wrote the paper.

43

44

45

46

47

48

49

50

51

52

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

53 **ABSTRACT**

54 The *EC1* gene family of *Arabidopsis thaliana* comprises five members that are specifically
55 expressed in the egg cell and redundantly control gamete fusion during double fertilization. We
56 investigated the activity of all five *EC1* promoters in promoter-deletion studies and identified SUF4
57 (SUPPRESSOR OF FRIGIDA 4), a C_2H_2 transcription factor, as a direct regulator of the *EC1* gene
58 expression. In particular, we demonstrated that SUF4 binds to all five *Arabidopsis EC1* promoters,
59 thus regulating their expression. The down regulation of *SUF4* in homozygous *suf4-1* ovules results
60 in reduced *EC1* expression and delayed sperm fusion, which can be rescued by expressing SUF4-
61 GUS under control of the *SUF4* promoter. To identify more gene products able to regulate *EC1*
62 expression together with SUF4, we performed co-expression studies that led to the identification of
63 *MOM1 (Morpheus' Molecule 1)*, a component of a silencing mechanism that is independent of
64 DNA methylation marks. In *mom1-3* ovules both *SUF4* and *EC1* genes are down regulated and *EC1*
65 genes show higher levels of Histone 3 lysine 9 acetylation, suggesting that MOM1 contributes to
66 the regulation of *SUF4* and *EC1* gene expression.

67
68
69
70
71
72
73
74
75
76
77

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

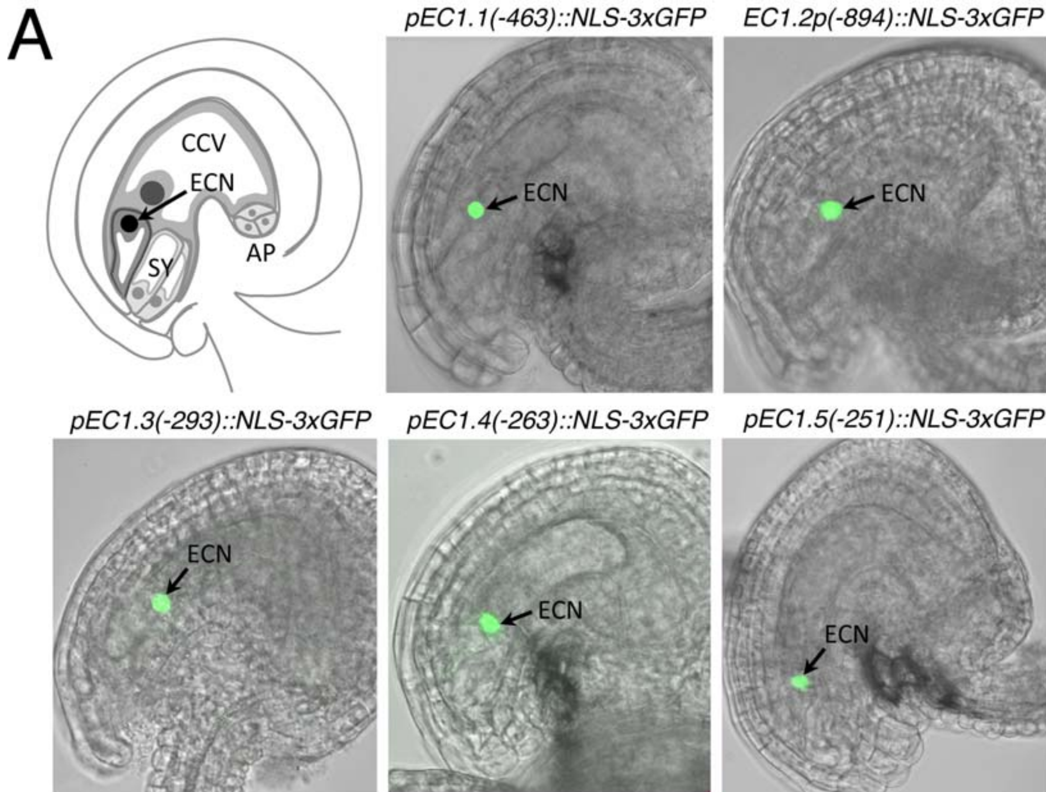
78 **INTRODUCTION**

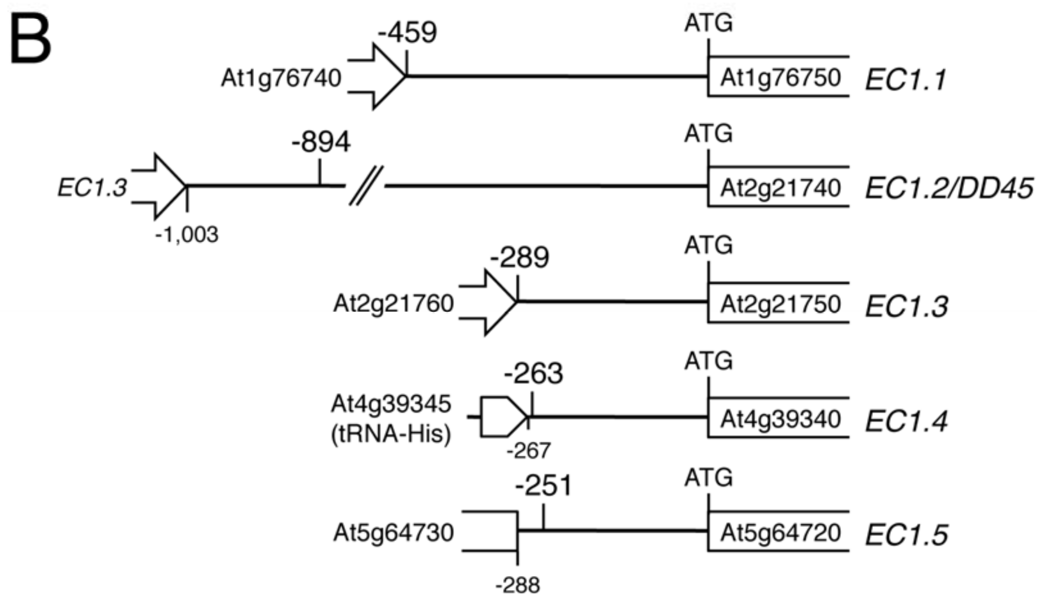
79 The female gametophyte (FG) of flowering plants, also called the embryo sac, is the haploid
80 generation that produces the two female gametes, the egg cell and central cell. The development of
81 the FG of *Arabidopsis thaliana* is a morphologically well-described multistep process (from FG1 to
82 FG7, Drews and Koltunow, 2011). The mature embryo sac of *Arabidopsis thaliana* consists of four
83 different cell types which possess distinctive morphologies and hold defined positions within the
84 FG: three antipodal cells are located at the chalazal pole of the FG (the proximal end of the ovule),
85 a homo-diploid central cell with a large vacuole occupies the center of the FG, while the egg cell
86 and two adjacent synergid cells are located at the micropylar (distal) end of the FG (Schneitz et al.,
87 1995) (Scheme in Fig. 1A). The entire FG is enclosed by the maternal tissues of the ovule.

88 The molecular mechanisms regulating the establishment of cell identities within the FG are largely
89 unknown, although several embryo sac defective mutants have been isolated (Christensen et al.,
90 1997; Pagnussat et al., 2005; Gross-Hardt et al., 2007; Pagnussat et al., 2007; Matias-Hernandez et
91 al., 2010; Masiero et al., 2011) and the impact of the phytohormones auxin and cytokinin on cell
92 specification in the developing FG became evident (Pagnussat et al., 2009; Yuan et al., 2016).

93 Besides genetic screens, a number of molecular approaches have been employed to clarify the
94 mechanisms controlling embryo sac cell differentiation, such as differential gene expression
95 analyses between wild-type and female gametophyte defective mutants (Yu et al., 2005; Johnston et
96 al., 2007; Jones-Rhoades et al., 2007; Steffen et al., 2007), microarray expression analysis of laser-
97 dissected female gametophytic cells (Wuest et al., 2010) or exhaustive sequencing of expressed
98 sequence tags (ESTs) from the cDNAs of manually isolated cells (Kumlehn et al., 2001; Le et al.,
99 2005; Marton et al., 2005; Yang et al., 2006; Koszegi et al., 2011). Isolation of egg cells and two-
100 celled embryos from wheat, by micromanipulation and subsequent EST analyses, resulted in the
101 identification of the large, egg cell-specific transcript EST cluster termed *TaEC-1* (*Triticum*
102 *aestivum EC1*; Sprunck et al., 2005). *TaEC-1* messengers encode small proteins having six
103 conserved cysteine residues and a predicted secretion signal sequence. Five *EC-1-related* genes are

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.





104 present in the Arabidopsis genome, namely *EC1.1*, *EC1.2*, *EC1.3*, *EC1.4* and *EC1.5*, all exclusively

105 expressed in egg cells (Sprunck et al., 2012). Simultaneous silencing of all five *EC1* genes prevents

106 the fusion of the two male gametes with the egg cell and central cell during double fertilisation. The

107 observed sperm-activating effects of EC1 peptides suggest that EC1 proteins are secreted by the egg

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

108 cell to promote sperm activation and thereby achieve rapid fusion with the female gametes
109 (Sprunck et al., 2012; Rademacher and Sprunck, 2013).
110 To shed light on *EC1* gene regulation, we investigated the promoter activities of all five *EC1* genes
111 in deletion studies and used the yeast one-hybrid approach to identify putative Arabidopsis *EC1.1*
112 transcriptional regulators. Among them, we identified the C_2H_2 zinc finger transcription factor
113 SUPPRESSOR OF FRIGIDA4 (SUF4; Kim and Michaels, 2006). *In vivo* and *in vitro* evidence
114 indicate that SUF4 is able to regulate all five *EC1* genes; furthermore, *suf4-1* mutants show a mild
115 *ec1* phenotype of delayed sperm fusion which can be rescued by the expression of *pSUF4::SUF4-*
116 *GUS*. Bioinformatics approaches demonstrated that *SUF4* is co-expressed with *MOM1* (*Morpheus'*
117 *Molecule 1*, Amedeo et al., 2000) and expression studies showed that *SUF4* is down regulated in
118 *mom1-3*. Real time RT-PCR analyses and genetic evidence indicate that MOM1 also controls *EC1*
119 expression by modulating the Histone 3 lysine 9 acetylation of the *EC1* loci.
120
121

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

122 **RESULTS**

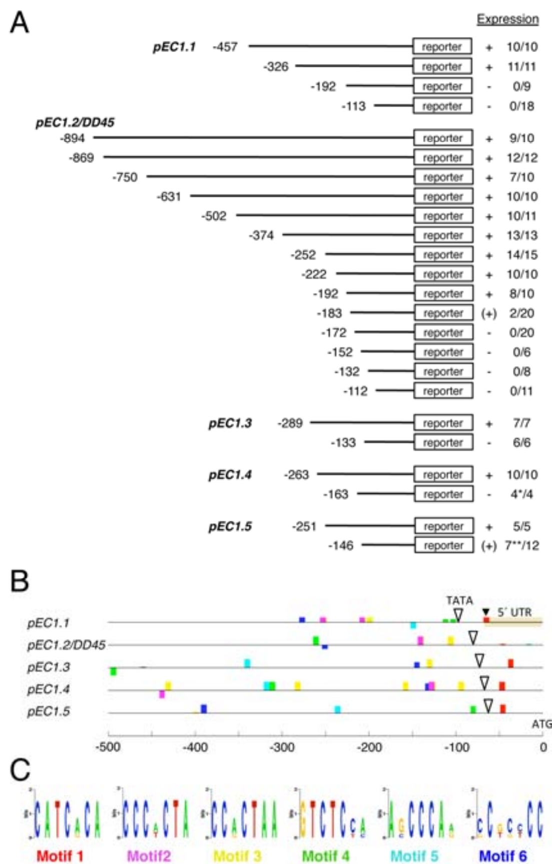
123

124 ***EC1* promoters drive egg cell-specific expression of a nuclear-localized GFP reporter**

125 The promoter activities of *EC1.1* and *EC1.2/DD45* in the Arabidopsis egg cell have been reported
126 previously (Ingouff et al., 2009; Steffen et al., 2007; Sprunck et al., 2012), while the upstream
127 regulatory sequences of *EC1.3*, *EC1.4* and *EC1.5* have not been investigated to date. To compare
128 the activity of all five *EC1* promoters we performed promoter-reporter studies using the nuclear-
129 localized 3xGFP (NLS-3xGFP) as a reporter (Fig. 1A). Notably, all the *EC1* promoters are able to
130 drive a strong egg cell-specific expression of the reporter. Compared to the genomic regions 5'
131 upstream of the start codons of *EC1.1* and *EC1.2/DD45* (-459 and -1,003 bp, respectively) the 5'
132 upstream genomic regions of *EC1.3*, *EC1.4* and *EC1.5* are only 289 bp (*EC1.3*), 267 bp (*EC1.4*)
133 and 287 bp (*EC1.5*) in length (Fig. 1B).

134 With the aim to narrow down the *EC1* promoter regions sufficient to drive egg cell-specific gene
135 expression, we generated a series of 5′-deletion constructs and investigated the ability of the deleted
136 promoter fragments to drive reporter gene expression *in vivo* (Fig. 2A). Transgenic plants for the
137 generated *EC1.1* and *EC1.2* promoter deletion constructs revealed that important cis-regulatory
138 elements for egg cell-specific expression are located between -326 and -192 bp upstream of the
139 translation start site of *EC1.1* and -192 to -172 bp upstream of the translation start site of *EC1.2*.
140 Further upstream promoter deletions of *EC1.1* and *EC1.2* did not affect the reporter activity. The
141 *EC1.3(-133)* and the *EC1.4(-163)* promoter deletions lost their ability to drive expression of NLS-
142 3xGFP in the egg cell. However, one of four independent *pEC1.4(-163)::NLS-3xGFP* lines
143 exhibited ectopic fluorescence in the nuclei of sporophytic cells of the ovule. Seven of the 12
144 independent lines transgenic for *pEC1.5(-146)::NLS-3xGFP* showed expression of the reporter in
145 the egg cell, but five of these lines revealed a very weak reporter activity, whilst two of the five
146 lines showed ectopic expression of the NLS-3xGFP reporter in sporophytic cells of the ovule (Fig.
147 2A).

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.



148

149 **Conserved sequence motifs in the *EC1* promoter regions**

150 To identify transcription factor binding sites for TATA binding proteins (TBPs) we used AthMap
151 (http://www.athamap.de/index.php) and detected a putative TATA box in every *EC1* promoter (Fig.
152 2B; Supplemental Table S1). The TATA box consensus sequence is TATAAA (*EC1.1*, *EC1.2*,
153 *EC1.3*, *EC1.4*) and TATATAT (*EC1.5*), respectively. The position of the predicted TATA box
154 relative to the start codon (ATG) is -99 nt for *EC1.1*, -77 nt for *EC1.2*, -73 nt for *EC1.2*, -64 nt for
155 *EC1.4* and -62 nt for *EC1.5* (Fig. 2B). The distance of the annotated transcription start site for
156 *EC1.1* (black triangle in Fig. 2B) to the predicted TATA box is 31 nt, matching with the reported
157 average distance of a TATA box to the transcription start site of 31.7 nt (Molina and Grotewold,
158 2005).

159 To map conserved DNA motifs in the -500 bp upstream regions, relative to the start codons of the
160 *EC1* genes, we used the online tool Cistome (Austin et al., 2016; Bio-Analytic Resource at
161 http://BAR.utoronto.ca). The comparison of all five *EC1* promoters revealed that their allover

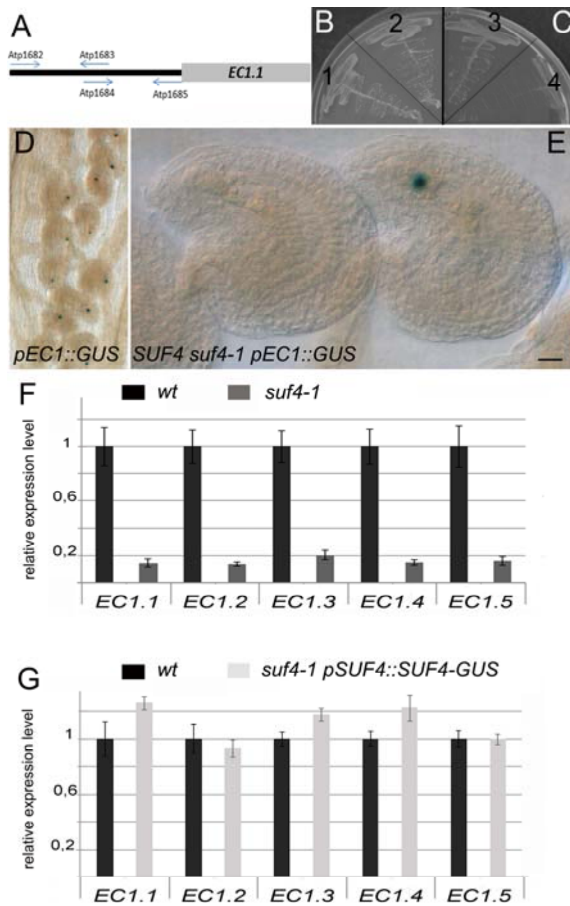
Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

162 sequence similarity is not very high. However, Cistome mapped a number of conserved DNA
163 motifs in at least four out of five promoters (Fig. 2B, C). Motif 1 [CATC(A/G)CA] (Fig. 2C) is
164 present in all five *EC1* promoters and locates to the core promoter region, downstream of the
165 predicted TATA boxes (Fig. 2B). The spatial proximity of motif 1 to the predicted TATA boxes (12
166 to 33 nt downstream of TATA) and the match of motif 1 with the annotated transcription start site
167 for *EC1.1* (Fig. 2B) suggest that this motif is close to, or part of, the initiator element, which is
168 described as a loosely conserved element containing an adenosine at the transcription start site and a
169 C as the nucleotide preceding it, surrounded by a few pyrimidines (Smale and Kadonaga, 2003).
170 Motif 2 [(CCC(A/T)TA] and motif 3 [CC(A/G)CTAA] (Fig. 2C) share overlapping sequence
171 identity and appear repeatedly in the -500 bp upstream regions of *EC1.1*, *EC1.2* and *EC1.4*.
172 However, the -500 bp upstream region of *EC1.5* lacks both motifs and just one motif 3 is detected
173 in the *EC1.3* promoter. Motif 5 [A(G/C)CCCA(A/G)] appears in the -500 bp upstream regions of all
174 *EC1* genes except *EC1.2*. Only motif 4 [GTCTC(C/T)(A/C)] and motif 6
175 [(C/G)C(G/T)(C/G)(C/T)CC] are detected in all five *EC1* promoters. Nevertheless, our promoter
176 deletion studies (Fig. 2A) indicate that a major role for these motifs in mediating egg cell-
177 specificity is not very likely.
178

179 **SUF4 positively regulates the transcription of *EC1* genes**

180 To dissect the molecular network controlling egg cell differentiation, we employed the *EC1.1*
181 promoter as bait in two yeast one-hybrid screens. The 463 bp *EC1.1* upstream regulatory region was
182 divided in two bait fragments (Fig. 3A) that were integrated into the MATα yeast strain Y187, and
183 subsequently mated with yeast strain AH109 previously transformed with a normalized total plant
184 Arabidopsis cDNA library (Costa et al., 2013; H. Sommer and S. Masiero, unpublished data). More
185 than seven million diploid clones were analysed in each single screening, 31 positive clones
186 matched a total of nine different proteins (Supplemental Table S2). All these clones were able to
187 grow on media lacking histidine and leucine and supplemented with 20 mM 3-AT (3-Amino-1,2,4-

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.



188 triazole a HIS3 competitive inhibitor). One of the transcription factors identified was the C_2H_2 zinc

189 finger protein SUF4.

190 SUF4 binds the proximal fragment of the *EC1.1* promoter (from -245 bp to -1 bp before the ATG;

191 Fig. 3A). The full length *SUF4* cDNA was cloned into pGADT7 and re-introduced in the yeast

192 strain containing the proximal region of the *EC1.1* promoter. *HIS3* reporter gene activation

193 confirmed the ability of SUF4 to bind the *EC1.1* promoter fragment (Fig. 3B,C).

194 To confirm that SUF4 controls *EC1.1* expression, transgenic plants homozygous for *pEC1.1(-*

195 *457)::GUS* (Ingouff et al., 2009) and with 97.36% GUS-positive egg cells (n = 455 ovules, Fig. 1D)

196 were crossed with homozygous *suf4-1* plants. The F_1 progeny plants were used to perform GUS

197 assays on mature pistils collected 24 hours after emasculation. The ratio expected for marker gene

198 expression in the female gametes of heterozygous plants is 50% (Yadegari and Drews, 2004). If

199 SUF4 positively regulates *EC1.1* we would expect a reduction of GUS activity in egg cells from

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

200 50% to 25%. We analysed 1,392 ovules and detected enzyme activity in only 356 egg cells (25.6%;
201 Fig. 1E, Supplemental Table S3). We also analysed the F_2 segregating population and we examined
202 circa 300 ovules produced by *suf4-1* mutants homozygous for the *pEC1.1(-457)::GUS* T-DNA
203 insertion (as suggested by the fact that all the progeny seedlings survived to BASTA application)
204 and none showed GUS activity, although these plants were GUS positive in PCR analyses.
205 In addition, we also crossed homozygous *pEC1.2(-893)::GUS* plants (Sprunck et al., 2012) with
206 *suf4-1*. In the F_1 developing carpels, 301 female gametophytes (24.6%) were GUS positive out of
207 the 1,225 analysed (Supplemental Table S3), suggesting that SUF4 also controls *EC1.2* expressions.
208 Real time RT-PCR analyses using cDNAs from *suf4-1* pistils confirmed *EC1.1* and *EC1.2* down
209 regulation and provides evidence that SUF4 also regulates the other *EC1* gene family members
210 *EC1.3, EC1.4* and *EC1.5* (Fig. 3F). To confirm that SUF4 is a true regulator of the Arabidopsis
211 *EC1* genes we analyzed *EC1* expression in *pSUF4::SUF4-GUS* plants complementing *suf4-1* (Kim
212 and Michaels, 2006). Kim and Michaels (2006) introduced *pSUF4::SUF4-GUS* into the *suf4-1*
213 mutant background demonstrating that the chimeric SUF4-GUS is biological active, as these plants
214 displayed a late flowering phenotype. Real time RT-PCR analyses using cDNAs from *suf4-1 suf4-1*
215 *pSUF4::SUF4-GUS pSUF4::SUF4-GUS* pistils showed that also the expression of the five
216 Arabidopsis *EC1* genes is fully restored (Fig. 3G).
217

218 **SUF4 is expressed in the developing female gametophyte**

219 We also used the *suf4-1 pSUF4::SUF4-GUS* line (Kim and Michaels, 2006) to study SUF4 protein
220 expression during embryo sac development. SUF4-GUS activity, driven by the genomic
221 *pSUF4::SUF4* locus, is neither detected in ovule primordia, in the diploid megaspore mother cell
222 (mmc), nor during meiosis (Fig. 4A,B). SUF4-GUS becomes visible immediately after meiosis
223 (Fig. 2C) when it localizes in the nucleus of the functional megaspore and persists during
224 megagametogenesis (Fig. 2D-G). In the seven-celled embryo sac (female gametophyte stage 6;
225 FG6) of stage 3-V ovules according to Schneitz et al. (1995), SUF4-GUS is detectable in all eight

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

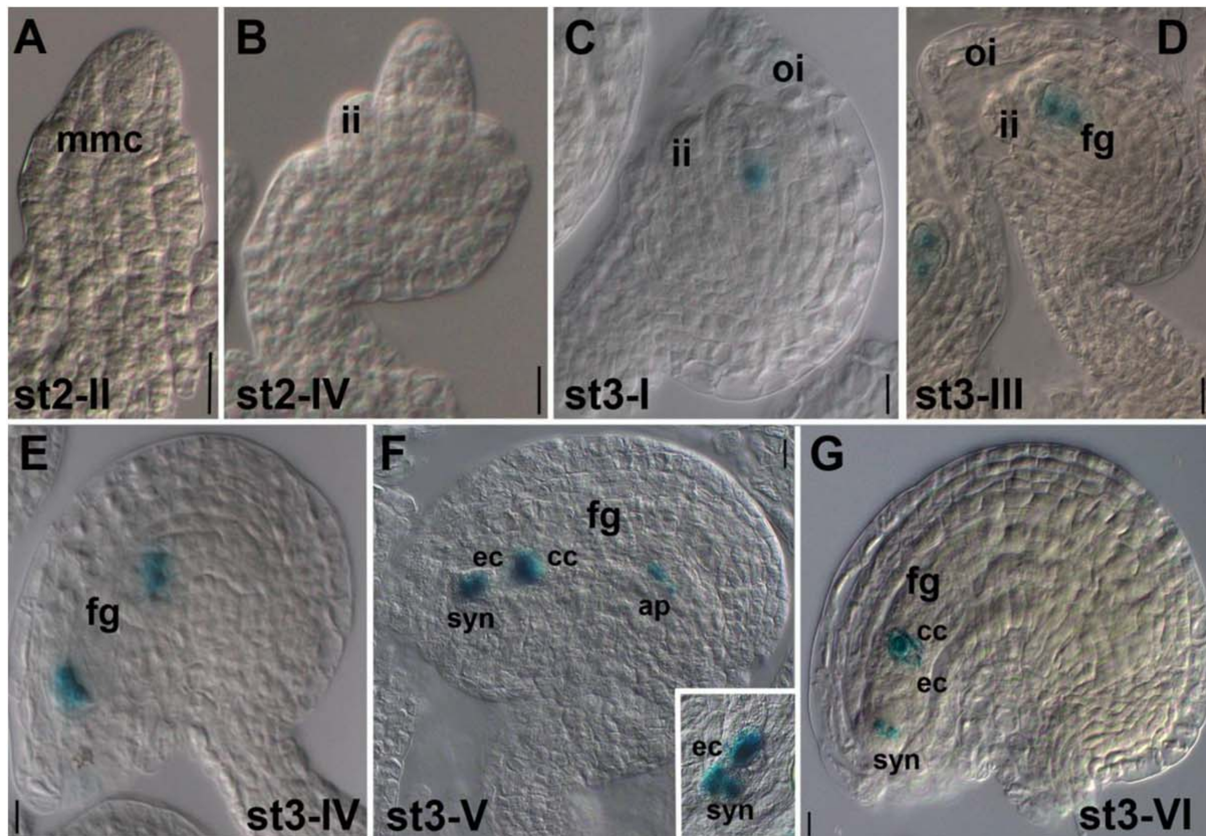


Figure 4. SUF4 is expressed in developing female gametophyte.
(**A,B**) *pSUF4::SUF4-GUS* activity is neither detected in the mmc (A) nor in the tetrad of megaspores (B).
(**C**) SUF4-GUS is detected in developing ovules from stage 3-I on, initially in the nucleus of the functional megaspore forming the haploid female gametophyte.
(**D,E**) SUF4-GUS expression persists in the developing embryo sac.
(**F**) In the seven-celled embryo sac (stage 3-V), SUF4-GUS is detected in all the seven nuclei.
(**G**) At stage 3-VI, SUF4-GUS is no longer expressed in the egg cell but only in the nuclei of central cell and synergid cells.
Ovule stages are according to Schneitz et al. (1995).
ap, antipodal cells; cc, central cell; ec, egg cell; fg, female gametophyte; ii, inner integument; mmc, megaspore mother cell; oi, outer integument; syn, synergid cells
Scale bars: 20 μm

226 nuclei, including the two polar nuclei of the central cell and the egg cell nucleus (Fig. 2F).
227 However, in the mature stage 3-VI ovule (FG7), SUF4-GUS is no longer detected in the egg cell
228 nucleus (Fig. 2G). Such peculiar expression pattern indicates that SUF4, detected during egg cell
229 differentiation, is removed during egg cell maturation. This suggests a possible role for SUF4 in the
230 developing egg cell and makes SUF4 a suitable marker to discriminate between immature egg cells,
231 not yet competent for fertilisation, and mature egg cells.

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

232

233 **SUF4 binds to *EC1* promoters**

234 Recombinant SUF4, expressed either as 6xHIS-SUF4-STREPII or as a 6xHIS-MBP-SUF4 fusion in
235 *E. coli*, was purified and used for *in vitro* DNA binding assays. Electrophoretic mobility shift assays
236 (EMSAs) were performed to confirm the interaction between SUF4 and the *EC1.1* promoter, as
237 well as with all other Arabidopsis *EC1* genes (Fig. 5). A 108 bp *EC1.1* promoter fragment, covering
238 part of the proximal fragment that has been used in the yeast one hybrid screening (Fig. 3A) and is
239 known to be necessary for egg cell expression (Fig. 2A), was radioactively labelled with [α-32P].
240 This fragment showed significant binding to increasing amounts of purified 6xHIS-SUF4-STREPII
241 (Fig. 5A). Competition experiments confirmed that SUF4-binding to the *EC1.1* promoter fragment
242 is displaced by the cold probe (Fig. 5B).

243 We used 6xHIS-MBP-SUF4 and the fusion protein 6xHIS-MBP as a control to show that MBP-
244 tagged SUF4 is able to specifically bind the radioactively labelled fragments of all five *EC1*
245 promoters (Fig. 5C).

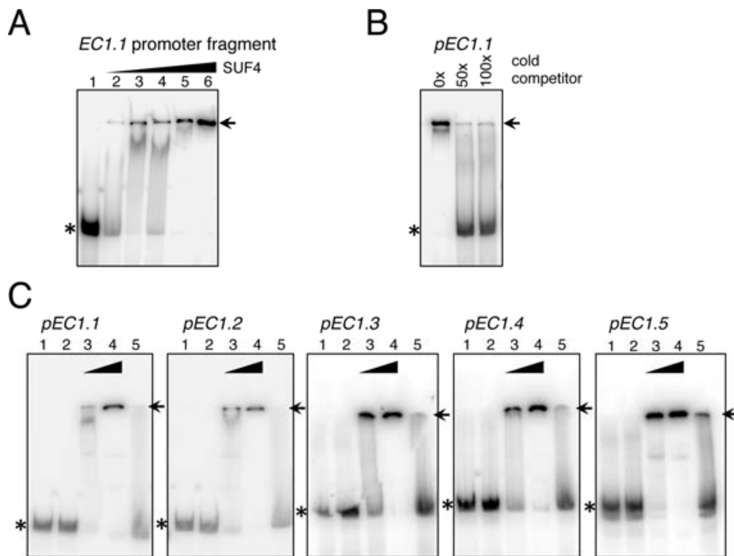
246 In summary, the DNA binding assays, together with the yeast data and the loss of GUS reporter
247 activity of p*EC1.1(-457)::GUS* and *pEC1.2(-893)::*GUS in the *suf4-1* mutant (Supplemental Table
248 S3) clearly prove that SUF4 binds to and activates *EC1* promoters. This is furthermore supported by
249 real time RT-PCR analyses of *EC1* gene expression in *suf4-1* and in the complemented *suf4-1* line
250 (Fig. 3F,G), suggesting that SUF4 binding to *EC1* promoter sequences is necessary to promote *EC1*
251 gene activation.

252

253 ***suf4-1* shows a moderate *ec1* phenotype**

254 The simultaneous down-regulation of *EC1.2* and *EC1.3* by RNA interference in the homozygous
255 triple mutant *ec1.1/ec1.4/ec1.5* (termed *ec1-RNAi*) severely affects double fertilisation (Sprunck et
256 al., 2012). The sperm cells, delivered into *ec1-RNAi* mutant ovules, do not fuse with the two female
257 gametes causing polytubey, multiple sperm delivery, and reduced seed set (Sprunck et al., 2012).

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.



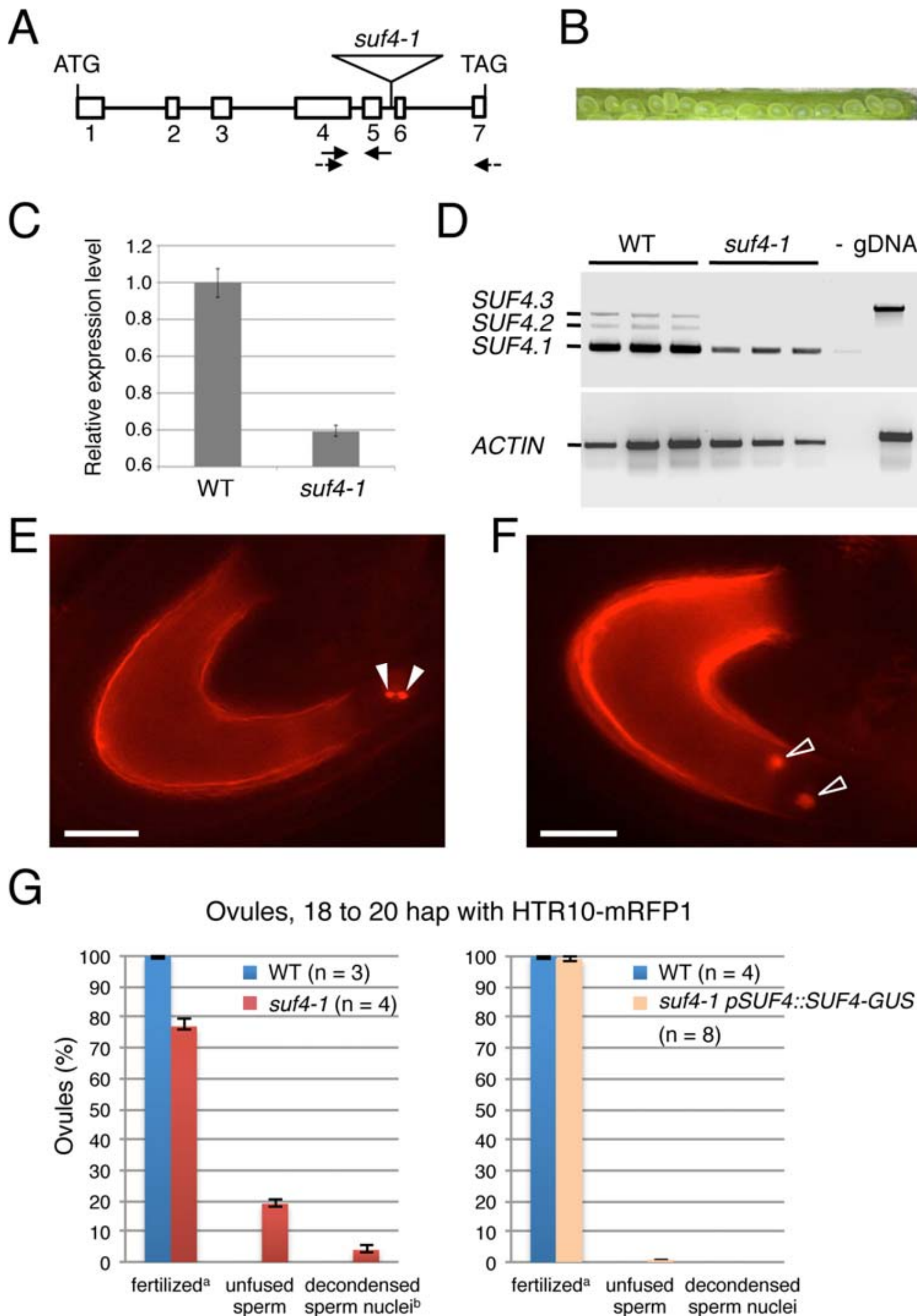
258 We therefore analyzed the siliques of homozygous *suf4-1* plants but no seed set defects have been
259 observed (Fig. 6B). However, the presence of functional *SUF4.1* transcripts revealed that *suf4-1* is
260 not a null mutant (Fig. 6C, D). This is likely the reason why *suf4-1* is still able to accumulate lower
261 *EC1* transcript levels (Fig. 3F).

262 To investigate sperm cell behaviour during double fertilization, we emasculated the pistils of the
263 wild type and of homozygous *suf4-1* plants and pollinated them with the sperm cell marker line
264 HTR10-mRFP1 (Ingouff et al., 2007). With this marker line successful plasmogamy and on-going
265 karyogamy of male and female gametes are recognizable by the spatial separation of the two sperm
266 nuclei and the decondensation of sperm chromatin, respectively.

267 When we prepared *suf4-1* pistils 18 to 20 HAP (hours after pollination) we detected a significant
268 portion of *suf4-1* ovules (23%; 53 of 232 ovules) exhibiting either non-fused sperm cells or sperm
269 cells delayed in fusion (Fig. 6E-G). These phenotypes were not observed in wild type ovules (Fig.
270 6G) where gamete fusion is accomplished 6 to 9 HAP (Sprunck et al., 2012). Seed set is not
271 affected in *suf4-1* siliques, suggesting that unfused sperm cells do fuse later. Late-fusing sperm cells
272 have also been described in individual *ec1*-RNAi lines (Rademacher and Sprunck, 2013) and are
273 likely a result of variable *EC1.2* and *EC1.3* knockdown efficiencies in the triple *ec1.1/ec1.4/ec1.5*
274 mutant by the *EC1.2*/*EC1.3* RNAi construct.

275 Importantly, the delay in sperm fusion was reversed when pistils of the double homozygous line

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.



276 *suf4-1 pSUF4::SUF4-GUS* were pollinated with the sperm marker line HTR10-mRFP (Fig. 6G,
277 right chart; 400 ovules analyzed), indicating that the complementation with *pSUF4::SUF4-GUS* is
278 able to rescue the moderate *ec1* phenotype in *suf4-1*.
279 Altogether, the observed delayed gamete fusion phenotype in *suf4-1* ovules and the lack of
280 undeveloped seeds in *suf4-1* siliques suggests that the down-regulation of *SUF4*, and in turn the
281 down-regulation of *EC1* gene expression, impairs rapid sperm fusion without abolishing it.

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

282

283 **MOM1 participates with SUF4 in regulating the *EC1* genes**

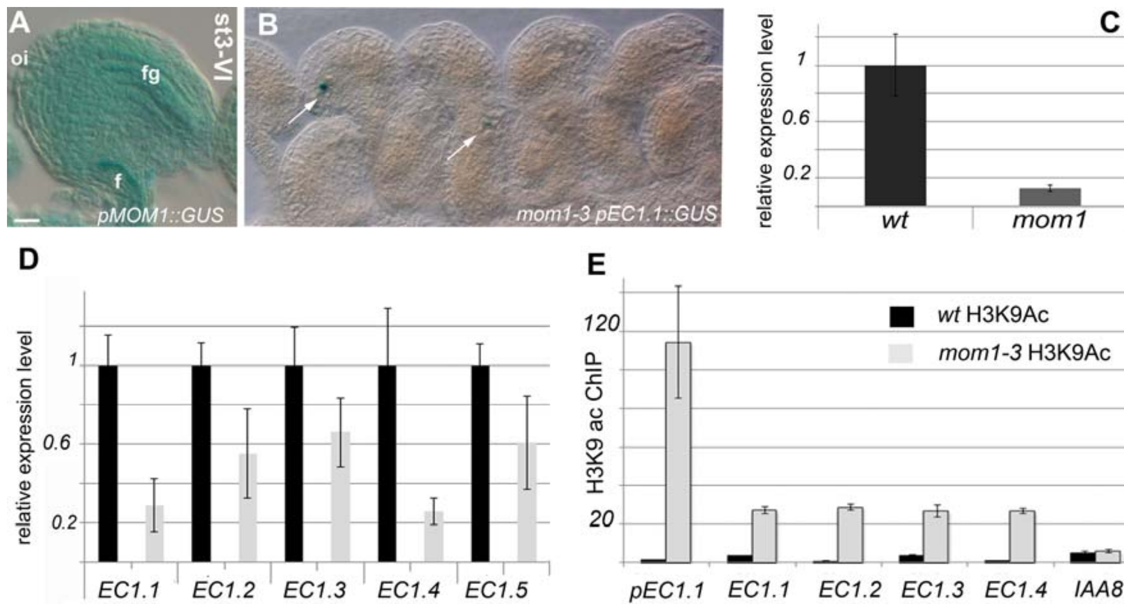
284 To better understand how SUF4 can regulate *EC1* gene expression, we performed correlation
285 analyses on around 1,700 microarray-based transcriptomic measurements (Menges et al., 2008).
286 Gene co-expression often highlights a functional linkage between genes and we observed that
287 *MOM1* shows a significant correlation value with *SUF4* (Supplemental Table S4). We focused on
288 MOM1, since it modulates "epigenetic stress memory" (Iwasakia and Paszkowski, 2014). MOM1 is
289 a CHD3 chromatin-remodelling factor, which has nucleosome remodelling and histone
290 deacetylation activities (Tong et al., 1998).

291 *MOM1* messenger is detected in siliques (3-6 DAP), leaves and inflorescences (Supplemental
292 Figure S1A). In transgenic *pMOM1::GUS* plants, GUS activity was found in the placenta tissue
293 when ovule primordia arise (Supplemental Figure S1B). In developing ovules, *MOM1* is expressed
294 from stage 2-III on (Supplemental Figure S1D). In mature ovules (stage 3-VI), *MOM1* promoter
295 activity is detected in the sporophytic tissues of the ovule and in the mature female gametophytes
296 although the reporter gene activity is weak (Fig. 7A). Furthermore, *MOM1* expression in the
297 embryo sac is corroborated by transcriptome analyses (Yu et al., 2005; Johnston et al., 2007; Zhang
298 et al., 2015).

299

300 To investigate the impact of MOM1 on *SUF4* and *EC1* gene expression, we performed real time
301 RT-PCR analyses and crossed homozygous *pEC1.1(-457)::GUS* plants with *mom1-3*. In the F_2
302 segregating population we looked for homozygous *mom1-3* plants also homozygous for the
303 *pEC1.1(-457)::GUS* insertion. In these plants GUS enzymatic activity was detected in 68 to 73% of
304 egg cells analysed (four plants and three carpels per plant were analysed, n = 589). Coherently, in
305 *mom1-3* mutants hemizygous for *pEC1.1(-457)::GUS* the enzymatic activity was detected in a
306 range from 25% to 37% of analysed egg cells (Fig. 7B). Quantitative RT-PCR analyses with *mom1-*
307 *3* inflorescences showed that *SUF4* expression is downregulated (Fig. 7C). Although the members

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.



308 of the *EC1* gene family are also downregulated in *mom1-3* (Fig. 7D), the reduction in *EC1*
309 expression is not as strong as observed in *suf4-1* (Fig. 3E).
310
311 In an attempt to clarify *EC1* family gene regulation by MOM1, we explored their epigenetic
312 landscape focusing on Histone 3 lysine 9 acetylation (H3K9ac). ChIP experiments revealed that in
313 *mom1-3*, especially the *EC1.1* promoter region shows a higher level of H3K9ac (Fig. 7E) but also
314 the tested *EC1* gene loci displayed higher H3K9ac levels compared to the wild type, while the *IAA8*
315 gene locus was not affected in *mom1-3* (Fig. 7E). Altogether our data indicate that histone
316 modifications also participate in *EC1* regulation, as we show that in *mom1-3* flowers the epigenetic
317 landscape of these loci changes towards a state that favours the transcription, thus counteracting
318 *SUF4* reduction recorded in *mom1-3* mutant plants.
319
320
321

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

322 **DISCUSSION**

323 The few-celled female gametophyte (FG) of flowering plants has become an attractive model
324 system to study the mechanisms involved in pattern formation and differentiation of distinct cell
325 types (Sprunck and Gross-Hardt, 2011). Considerable progress has been made in the past decade
326 towards the identification of genes involved in the differentiation of FG cells (e.g., Evans, 2007;
327 Pagnussat et al., 2007; Gross-Hardt et al., 2007; Moll et al., 2008; Pagnussat et al., 2009; Krohn et
328 al., 2014; Yuan et al., 2016). Nevertheless, not much is known about the transcriptional regulatory
329 network involved in egg-cell specification. One exception is the RKD subfamily of plant-specific
330 RWP-RK transcription factors provoking an egg cell-like transcriptional profile when ectopically
331 expressed in Arabidopsis seedlings (Koszegi et al., 2011) and acting in egg and sperm cell
332 differentiation in the liverwort *Marchantia polymorpha* (Koi et al., 2016; Roevekamp et al., 2016).
333 In this work we used the egg cell-specific *EC1.1* promoter as a tool to identify transcription factors
334 participating in egg cell differentiation. We show that all five Arabidopsis *EC1* promoters drive egg
335 cell-specific reporter gene expression and share some common DNA sequence motifs. In 5´
336 deletion studies we observed that relatively short proximal promoter regions are sufficient to drive
337 egg cell-specific expression, indicating that important cis-regulatory elements for egg cell
338 specificity are present in these regions. Using the yeast one-hybrid technique, we aimed to identify
339 transcription factors binding to the *EC1.1* promoter, which has been used as developmental marker
340 for the egg cell (e.g., Ingouff et al., 2009; Voelz et al., 2012; Denninger et al., 2014; Kong et al.,
341 2015; Mendes et al., 2016). The yeast one hybrid system detects protein-DNA interactions *in vivo,*
342 as prey proteins can acquire their native configuration (Lopato et al., 2006). The yeast one hybrid
343 technique is a simple, rapid and sensitive tool (Reece-Hoyes et al., 2011) that nevertheless suffers
344 certain limitations such as its inability to identify transcription factors that bind the target DNA only
345 if post-translationally modified, or those which are members of higher order complexes (Deplancke
346 et al. 2006).
347 Unequivocal evidence for cis-regulatory motifs involved in egg cell specific promoter activity is not

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

348 yet given. We therefore split the 463 bp 5′ upstream region of the *EC1.1* promoter into two bait
349 fragments. This facilitates the interaction of transcription factors with the *EC1.1* regulatory
350 sequences even without a detailed knowledge of the key cis-regulatory elements. Quite large
351 promoter fragments have already been used successfully as bait in yeast one-hybrid screenings
352 (Roccaro et al., 2005, Brady et al., 2011), although it is common to perform yeast one-hybrid
353 screenings using multiple copies of small bait elements, such as cis-regulatory motifs (Tran et al.,
354 2004; Lopato et al., 2006). One potential difficulty using larger promoter fragments is the presence
355 of several cis-regulatory elements, which might be bound by yeast DNA-binding proteins activating
356 the transcription of the reporter gene even without any prey GAL4AD chimeric protein.
357 Nevertheless, we did not experience self-activation for either of the two *EC1.1* bait fragments.
358

359 Our *in vitro* and *in vivo* data indicate that SUF4 exerts a direct positive regulation on the *EC1* gene
360 family. SUF4 is a C_2H_2 protein, already identified in secondary genetic screenings performed to
361 isolate loci able to suppress the *Col-FRIGIDA* late flowering phenotype. SUF4 binds the *Flowering*
362 *Locus C* (*FLC*) promoter and subsequently recruits FRIGIDA (FRI) and FR-LIKE1 (FRL1) (Choi
363 et al., 2011). FRI acts as a scaffold protein, forming a transcription activator complex that recruits,
364 amongst others, chromatin modifiers to regulate *FLC*. Repression of *FLC* causes early flowering,
365 and it is accompanied by covalent histone modification, like H3K9 and H3K14 deacetylation and
366 H3K9 and H3K27 methylation (Sung and Amasino, 2004).

367 SUF4 binds the *FLC* promoter through the A/T rich consensus sequence 5’-
368 CCAAATTTTAAGTTT-3’ (Choi et al., 2011). Although we have not been able to recognise this
369 consensus sequence in the *EC1* promoters, it is well accepted that interacting proteins may
370 modulate a transcription factors binding specificity. Indeed, SUF4 interacts with several proteins,
371 like MEDIATOR 18 (MED18, Lai et al., 2014), members of the Spindle Assembly Checkpoint
372 complex (SAC; Bao et al., 2014) and with LUMINIDEPENDS (Kim et al., 2006). SUF4 also
373 contains a BED-finger domain with DNA-binding ability, named after the *Drosophila* proteins

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

374 BEAF and DREF (Aravind, 2000). Interestingly, the human ZBED1-6 proteins (Zinc BED,
375 Mokhonov et al., 2012) act as transcriptional regulators by modifying the local chromatin structure
376 upon binding to GC-rich sequences.

377 In eukaryotic organisms, transcription factors regulate gene expression through binding to *cis*-
378 regulatory specific sequences in the promoters of their target genes. Nevertheless, also the
379 chromatin structure actively participates in gene regulation favouring or not the access of the DNA
380 binding proteins to their regulatory sites. Indeed, the chromatin structure is modulated in a highly
381 cell-specific manner, as extensively reported for flowering time regulation (He, 2009) and flower
382 development (Gan et al., 2013).

383 Our data on the SUF4-dependent *EC1* expression in egg cells and on the strong down regulation of
384 *SUF4* in *mom1-3* mutant ovules (accompanied by an enrichment of H3 lysine 9 acetylation in *EC1*
385 loci) suggest a complex regulation of *EC1* gene expression involving chromatin remodelling. We
386 provide evidence that SUF4 is involved in regulating *EC1* gene expression in the developing egg
387 cell, while in the mature egg cell SUF4 is not detectable anymore. It is therefore possible that SUF4
388 participates in the recruitment of chromatin modifiers in the developing egg cell to promote *EC1*
389 gene expression.

390 We were able to show that histone modifications participate in *EC1* gene regulation, at least in
391 *mom1-3* flowers. *MOM1*, which is co-expressed with *SUF4*, was identified during a genetic screen
392 set up to monitor the release of Transcriptional Gene Silencing (TGS) of a cluster of transgenes
393 (Amedeo et al., 2000). Remnants of the *gypsy*-like retrotransposon *Athila* are also transcriptionally
394 activated in *mom1-3* mutants (Habu et al., 2006). The C-terminal region of MOM1 is similar to the
395 C terminus of eukaryotic enhancer of polycomb proteins, which have roles in heterochromatin
396 formation. However, the mechanism by which MOM1 contributes to chromatin changes is still
397 quite elusive as *mom1-3* mutants display none or poor alterations of the epigenetic landscape of the
398 released loci (Vaillant et al., 2006). Nevertheless, Numa and collaborators (2010) demonstrated that

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

399 MOM1 targets also map in euchromatic regions. By ChIP experiments they have shown that the
400 promoter of *SDC* (*SUPPRESSOR OF drm1 drm2 cmt39*), a MOM1 target, is enriched in H3K9me2.
401 *SDC* is activated in *mom1-3* and ChIP experiments revealed that the level of di-methylated histone
402 H3 lysine 9 (H3K9me2) in tandem repeats of the *SDC* promoter is reduced (Numa et al., 2010).
403 The *EC1* loci in *mom1-3* flowers are enriched in H3K9ac and both *SUF4* and *EC1* genes are
404 differentially expressed in *mom1-3* ovules, suggesting that MOM1 also participates in remodelling
405 the chromatin organization of *SUF4* and thus regulates its transcriptional activity. However,
406 whether or not the chromatin status of *SUF4* is changed in *mom1-3*, or whether SUF4 and MOM1
407 are directly interacting to regulate *EC1* gene expression in the developing egg cell remains to be
408 investigated.
409 The observed enrichment of H3K9ac in *EC1* loci of *mom1-3* flowers indicates that MOM1 affects
410 the modification of histones in *EC1* genomic loci. Histone tail acetylation results in chromatin
411 decondensation and thus in remodelling the chromatin organization into transcriptionally active
412 chromatin, as lysine acetylation removes the positive charge of this amino acid, favouring
413 chromatin relaxation and access to transcription factors and other transcriptional co-activators. In
414 *mom1-3* the epigenetic landscape of *EC1* loci therefore changes towards a state that favours the
415 transcription. Our studies revealed, however, that SUF4-binding to the *EC1* promoter sequences is
416 necessary to promote *EC1* gene activation but *SUF4* is strongly down regulated in *mom1-3*.
417 Although *EC1* expression is lower in *mom1-3* compared to the wild type it is not as reduced as in
418 *suf4-1,* suggesting that the *SUF4* reduction and the resulting down-regulation of *EC1* genes is
419 partially counteracted in *mom1-3* female gametophytes. However, in addition to H3K9 acetylation,
420 other altered epigenetic events such as histone methylation, histone phosphorylation or DNA
421 methylation may also be involved in the regulation of *EC1* gene expression.
422 The identification of egg cell-specific genes, the analyses of their promoter activities and the
423 characterization of transcriptional regulatory networks acting during egg cell differentiation are
424 essential to improve our understanding of how this important cell becomes specified and how it

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

425 acquires its unique features and functions in sexual reproduction. The discovery of SUF4 and
426 MOM1 as regulators of the egg cell-specific *EC1* gene family of Arabidopsis is a first important
427 step towards the identification of the egg cell transcriptional regulatory network. Nevertheless, we
428 are only just beginning to understand how the complex expressional control of the *EC1* genes is
429 achieved.
430
431
432
433 **Material and Methods**
434 **Plant material**
435 *suf4-1* mutants and *suf4-1 pSUF4:SUF4::GUS* seeds were donated by S.D. Michaels, *mom1-3*
436 mutants by J. Paszkowski. Plants were grown under long-day conditions (14 h light/10 h dark) at
437 22°C. Gentotyping has been done using gene specific primers, specific T-DNA primers and primers
438 able to anneal to the GUS gene. All primers are listed in Supplemental Table S5.
439 **Constructs for promoter-reporter studies**
440 All five *EC1* upstream regulatory sequences were cloned as PCR fragments extending in 5´
441 directions from the -1 position (referring to the respective start codon) towards the previous gene
442 (Fig. 1A). *EC1* promoters were amplified from genomic DNA of *Arabidopsis thaliana* (accession
443 Columbia-0) using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) and the
444 primer pairs EC1.1p(-463bp)_fw/EC1.1p_rev, EC1.2p(-894)_fw/EC1.2p_rev, EC1.3p(-
445 289)_fw/EC1.3p_rev, EC1.4p(-263)_fw/EC1.4p_rev and EC1.5p(-251)_fw/EC1.5p_rev (primer
446 sequences are available in Supplemental Table 5) The PCR products were cloned into the
447 Gateway® Entry vector pENTR™/D-TOPO® (ThermoFischer Scientific). Subsequently, the
448 promoter fragments were transferred into a Gateway-compatible version of the pGreenII-based
449 vector NLS:3GFP:NOSt (Takada and Jürgens, 2007) termed pGII_GW:NLS:3GFP:NOSt (Zheng et
450 al., 2011) by LR reaction using Gateway® LR Clonase II Enzyme Mix (ThermoFischer Scientific).

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

451 For deletion studies with NLS-3xGFP as a reporter, 5´ truncated promoter fragments were
452 amplified using genomic DNA of *Arabidopsis* (Col-0) as a template and the primer combinations
453 EC1.3p(-133)_fw/EC1.3p_rev, EC1.4p(-163)_fw/EC1.4p_rev and EC1.5p(-156)_fw/EC1.5p_rev
454 (Supplemental Table 5). *EC1.3*, *EC1.4* and *EC1.5* promoter deletions were cloned into
455 pENTR™/D-TOPO® and recombined into pGII_GW:NLS:3GFP:NOSt. For studies with GFP as a
456 reporter *EC1.2* promoter, deletion fragments were generated by PCR using primers introducing
457 unique restriction enzyme sites (PstI, BamHI; Supplemental Table 5). The PCR fragments were
458 digested and ligated with pBI101.GFP (Yadegari et al., 20000). The binary vectors *pEC1.1(-*
459 *457)::GUS* and *pEC1.2(-893)::GUS* have been described previously (Ingouff et al., 2009; Sprunck
460 et al., 2012). *pEC1.1(-457)::GUS* served as a template to generate the deletion constructs *pEC1.1(-*
461 *326)::GUS* and *pEC1.1(-192)::GUS*, applying the forward primers EC1.1p(-326)_fw and EC1.1p(-
462 192)_fw (Supplemental Table 5). The deletion construct *pEC1.1(-113)::GUS* was generated by
463 digesting *pEC1.1(-457)::GUS* with PmeI and HpaI, followed by religation. All constructs were
464 sequence verified.
465 T-DNA constructs with p*EC1.2* in pBI101.GFP were introduced into *Agrobacterium tumefaciens*
466 strain LBA4404 by electroporation. *Arabidopsis* plants (Col-0) were transformed using a modified
467 floral dip procedure (Clough and Bent, 1998) . Transformed progeny were selected by germinating
468 surface-sterilized T1 seeds on growth medium containing antibiotics (30 µg/mL kanamycin sulfate)
469 supplemented with 15 µg/mL cefeotaxime. Resistant seedlings were transplanted to soil 10 days
470 after germination. The *pEC1::NLS3xGFP* expression vectors were delivered into *Agrobacterium*
471 *tumefaciens* strain GV3101 pSOUP, *pEC1::GUS* expression vectors were delivered into strain
472 GV3101 pMP90RK. *Arabidopsis* plants (Col-0) were transformed by floral dip. T1 seeds were
473 collected, sown on soil and vernalized for 3 days at 4°C in the dark. Starting three days after
474 germination, BASTA® resistant seedlings were selected by spraying three times with 200 mg/l
475 BASTA® (Bayer Crop Science) supplemented with 0.1 % Tween. Transgene identity was verified
476 by PCR.

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

477 **Cloning of *pMOM1:GUS***

478 For the *pMOM1:GUS* construct, a 1.1 kb genomic region upstream of the *MOM1* ATG start codon
479 was amplified by Phusion® High-Fidelity DNA Polymerase (Finnzymes; see Supplemental Table
480 S5). The product was cloned in the pBGWFS7 vector (Karimi et al., 2002) using the Gateway®
481 system (ThermoFischer Scientific). The construct was verified by sequencing and used to transform
482 Arabidopsis *Col-0* plants (Clough and Bent, 1998). GUS assays have been done according to
483 Colombo et al. (2008).

484 **Yeast Experiments and cloning**

485 The *EC1.1* upstream regulatory region of 463 bp was amplified as two distinct fragments using
486 primer pairs pAtEC1.11 plus EcoRI_fw/pAtEC1.11 plus XbaI_rev and pAtEC1.12 plus
487 EcoRI_fw/pAtEC1.12 plus XbaI_rev (Supplemental Table S5), digested and ligated into the
488 EcoRI/XbaI-digested pHISi vector (Clontech). The two bait plasmids were linearised with XhoI and
489 used to transform yeast strain Y187. A whole normalised total plant cDNA library (H. Sommer and
490 S. Masiero, unpublished) was cloned in pGADT7-rec and introduced into yeast strain AH109. The
491 yeast containing the expression library was mated with modified Y187 strains (containing the
492 *EC1.1* regulatory regions) as described in Clontech user manual PT4085-1. Diploids were selected
493 on medium lacking Leu and His and supplemented with 20 mM 3-AT (Sigma-Aldrich). Plasmids
494 were extracted from positive colonies and retransformed into Y187 to discard the false positives.

495 **Purification of recombinant SUF4 and Electrophoretic Mobility Shift Assays (EMSAs)**

496 Expression vectors for recombinant protein expression in *E. coli* were cloned using the
497 GATEWAY® system (Invitrogen). The coding sequence of *SUF4* was amplified by PCR from
498 inflorescence cDNA (Supplemental Table S5) and cloned into pENTR/D-TOPO. LR-Clonase
499 reactions were performed using the SUF4 entry vector and the destination vector pET-53-DEST®
500 (Novagen) and pDEST-HisMBP (Nallamsetty et al., 2005), respectively. The resulting expression
501 vectors were used to express a 6xHis-SUF4-StrepII fusion protein and a 6xHIS-MBP-SUF4 fusion
502 protein. After expressing 6xHis-SUF4-StrepII in *E. coli* RosettaTM(DE3) (Novagen) the soluble

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

503 fraction of the crude cell extract was purified by Immobilized Metal Ion Affinity Chromatography
504 (IMAC) under native conditions using Ni-NTA-Agarose (Qiagen) and gravity flow columns,
505 following the manufacturer´s instructions. The 6xHis-MBP and 6xHis-MBP-SUF4 recombinant
506 proteins were expressed in *E. coli* BL21-Codon Plus(DE3)-RIPL cells (Stratagene) and purified
507 under native conditions using TALON® Metal Affinity Resin (Clontech).
508 The *EC1* promoter fragments were amplified with terminal *Xba*I restriction sites via PCR using Taq
509 Polymerase (Fermentas), resulting in fragments for *EC1.1* (108 bp), *EC1.2* (115 bp), *EC1.3* (167
510 bp), *EC1.4 (*199 bp) and *EC1.5* (189 bp) (primer sequences are available in Supplemental Table
511 S5). The purified promoter fragments were digested with *Xba*I and radioactively labelled using
512 Klenow enzyme (Fermentas) and [α-32P]dATP. Unincorporated [α-32P]dATP was removed by
513 spin-column chromatography (Illustra ProbeQuant G-50 Micro columns; GE Healthcare).
514 For the EMSAs the radioactively labelled promoter fragments (10 or 18 ng) were incubated with
515 different amounts of SUF4 (10 to 400 ng) in 1x EMSA-buffer (10 mM Tris-HCl, pH 7.5; 100 mM
516 KCl; 1 mM EDTA; 0.1 mg/ml BSA; 100 µM ZnCl2; 6% glycerol; 1 mM DTT) in 20 µl reaction
517 volumes for 1h at 4°C. Afterwards, the reactions were separated on a 5% polyacrylamide gel in
518 TAE buffer (40 mM Tris and 2.5 mM EDTA, pH 7.8) at 10 V/cm gel length for 1h. For the
519 competitor assays, the respective unlabelled probe was added in excess (50x and 100x) to the
520 binding mixture. Gel images were obtained using autoradiography (Cyclone Phosphoimager
521 A431201, Packard Inc.).
522 **Comparative promoter studies**
523 For motif discovery we used the online tool Cistome (https://bar.utoronto.ca/cistome/cgi-
524 bin/BAR_Cistome.cgi) to map conserved sequence motifs in the -500 bp upstream regions of *EC1*
525 genes, relative to their translation start sites. Cistome predicts cis-elements in the promoters of sets
526 of co-expressed genes. The cis-element prediction program MEME (Multiple Em for Motif
527 Elicitation) (Bailey et al., 2009) was selected, with the following parameters: Width, 7; Number of

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

528 Motifs, 6; Mode: oops. Transcription factor binding sites for TATA binding proteins (TBPs) were
529 mapped using AthMap (http://www.athamap.de/index.php).

530 **Correlation analysis**

531 Calculation of the Pearson correlation coefficient and the microarray dataset employed were as
532 described previously (Menges et al., 2008; Berri et al., 2009).

533 **ChIP and quantitative PCR analyses**

534 For ChIP experiments, chromatin was extracted from Arabidopsis *Col-0* and *mom1-3* mutant
535 flowers (before fertilization occurs). ChIP experiments were done as previously described (Mizzotti
536 et al., 2014). Real time PCR analyses were performed on input and immunoprecipitated samples
537 and % of input was calculated. *IAA8* (At2g22670) was used as a reference as it carries the H3K9ac
538 mark (Mizzotti et al., 2014). Quantitative expression analyses were performed using the iQ5 Multi
539 Color real-time PCR detection system (Bio-Rad). Primers used for ChIP experiments are listed in
540 Supplemental Table S5.

541

542

543 **Acknowledgments**

544 We thank Scott D. Micheals for *suf4-1* and *suf4-1 pSUF4::SUF4-GUS* seeds, J. Paszkowski for
545 *mom1-3* seeds and M. Ron for making the Gateway-compatible pGreenII-based vector
546 GW::NLS:3GFP:NOSt available to us. M. Kammerer is acknowledged for cloning and plant care and
547 M. Grasser for her support in the gel-shift assays.

548

549

550 **Figure legends**

551

552 **Figure 1. *EC1* promoter regions drive egg cell-specific expression.**

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

553 **(A)** Egg cell-specific reporter activity in mature ovules. Green fluorescent egg cell nuclei (arrows)
554 indicate that all five promoters of the Arabidopsis *EC1* gene family are functional and specifically
555 active in the egg cell.
556 **(B)** Schemes illustrating the genomic regions 5' upstream of the sense strands of *EC1* coding
557 sequences. The position in a DNA sequence is designated relative to the predicted start codon
558 (ATG) of the EC1 open reading frame. AGI codes for *EC1* genes and adjacent gene loci are given.
559 Note that the promoter regions of *EC1.3*, *EC1.4* and *EC1.5* are short (-289 to -267 bp) but sufficient
560 to drive egg cell-specific expression.
561 AP, antipodal cells; CCV, central cell vacuole; ECN, egg cell nucleus; SY, synergid cells.
562
563 **Figure 2. *EC1* promoter deletion studies and mapping of putative cis-regulatory motifs.**
564 **(A)** Scheme summarizing the results from *EC1* promoter deletion studies. A series of 5' deletion
565 constructs was tested for reporter activity in transgenic plants. "Expression" describes the observed
566 reporter activity as present (+), weakly present ((+)), or absent (-) in the egg cell. Numbers indicate
567 individual transgenic lines for a given deletion construct showing reporter activity, compared to the
568 total number of lines transgenic for this construct. *, 1 out of 4 lines showed misexpression of the
569 reporter in sporophytic cells; **, 5 of 7 lines showed only very weak reporter activity and 2 of these
570 5 lines showed misexpression in sporophytic cells.
571 **(B)** Conserved sequence motifs (colored boxes) mapped in the -500 upstream regions of the five
572 *EC1* genes by Cistome (https://bar.utoronto.ca/cistome/cgi-bin/BAR_Cistome.cgi) using the
573 prediction program MEME. Open triangles mark the position of TATA box motifs identified by
574 AthMap (http://www.athamap.de/index.php). The transcription start site of *EC1.1* is labeled with a
575 black triangle.
576 **(C)** Sequence logos of mapped sequence motifs shown in (B). Motif 2 and motif 3 show high
577 sequence similarity.
578

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

579 **Figure 3.** ***SUF4*** **regulates** ***EC1.1*** **in yeast and** ***in planta***

580 (**A** to **C**) Yeast one-hybrid analysis of interactions between SUF4 and *pEC1.1*. The *EC1.1* promoter

581 was divided into two bait fragments, arrows indicate primers used for bait construction (A).

582 Transformed yeast strains with the proximal fragment of the *EC1.1* promoter were grown on either

583 permissive (**B**) –His –Leu medium or selective (**C**) –His-Leu with 5 mM 3-AT medium. 1 and 4,

584 pGADT7 without any insert (negative control); 2 and 3, pGAD-SUF4.

585 (**D**) GUS staining of homozygous *pEC1.1(-457)::GUS* plants. All egg cells show reporter activity.

586 (**E**) SUF4 is important for *EC1.1* promoter activity *in planta*. *suf4-1* mutants were crossed with

587 homozygous *pEC1.1(-457)::GUS* plants. In the F1 carpels only 25%, instead of the expected 50%,

588 of egg cells were GUS positive, therefore *pEC1.1(-457)::GUS* activation relies on SUF4.

589 (**F**) All five *EC1* genes are down regulated in *suf4-1* mutant pistils, as indicated by real time RT-

590 PCR analyses. To normalise the expression level, we used *UBIQUITIN10* or *ACTIN8* (data not

591 shown). The expression of each *EC1* gene has been calibrated to 1 in wild-type pistils.

592 (**G**) The normal *EC1* gene expression is restored *in suf4-1 suf4-1 pSUF4::SUF4-GUS*

593 *pSUF4::SUF4-GUS* pistils. The expression of each *EC1* gene has been calibrated to 1 in wild-type

594 pistils.

595 Scale 20 µm

596

597 **Figure 4. SUF4 is expressed in developing female gametophyte.**

598 (**A,B**) *pSUF4::SUF4-GUS* activity is neither detected in the mmc (A) nor in the tetrad of

599 megaspores (B).

600 (**C**) SUF4-GUS is detected in developing ovules from stage 3-I on, initially in the nucleus of the

601 functional megaspore forming the haploid female gametophyte.

602 (**D,E**) SUF4-GUS expression persists in the developing embryo sac.

603 (**F**) In the seven-celled embryo sac (stage 3-V), SUF4-GUS is detected in all the seven nuclei.

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

604 (**G**) At stage 3-VI, SUF4-GUS is no longer expressed in the egg cell but only in the nuclei of
605 central cell and synergid cells.
606 Ovule stages are according to Schneitz et al. (1995).
607 ap, antipodal cells; cc, central cell; ec, egg cell; fg, female gametophyte; ii, inner integument; mmc,
608 megaspore mother cell; oi, outer integument; syn, synergid cells
609 Scale bars: 20 μm
610
611 **Figure 5. SUF4 binds to all five *EC1* promoters.**
612 **(A)** Gel shift assay without (1) and with 10 (2), 50 (3), 100 (4), 200 (5), and 400 ng (6) of
613 recombinant 6xHIS-SUF4-STREPII, added to a radioactively labelled 108 bp *EC1.1* promoter
614 fragment covering the DNA region used as bait in the yeast one-hybrid screening.
615 (**B**) Gel shift assay with the 50-fold (50x) and 100-fold (100x) excess of unlabelled *EC1.1* promoter
616 fragment as a cold competitor, added to the reaction mix with 200 ng of 6xHIS-SUF4-STREPII.
617 Control reaction is without cold competitor (0x).
618 (**C**) 50 and 150 ng of recombinant 6xHIS-MBP-SUF4 and 150 ng of 6xHIS-MBP as control, mixed
619 with 10 ng of radioactively labelled *EC1* promoter fragments. Lane (1), radioactively labelled
620 promoter fragment only; (2) radioactively labelled promoter fragment with 150 ng 6xHIS-MBP tag
621 only; (3) radioactively labelled promoter fragment with 50 ng 6xHIS-MBP-SUF4; (4) radioactively
622 labelled promoter fragment with 150 ng MBP-SUF4; (5) radioactively labelled promoter fragment
623 with 150 ng MBP-SUF4 and 100-fold excess of cold competitor (unlabelled promoter fragment).
624
625 **Figure 6. *suf4-1* ovules show a moderate *ec1* phenotype.**
626 (**A**) Genomic organization of *SUF4*, composed of 7 exons and 6 introns. The T-DNA in *suf4-1* is
627 inserted in intron 5, 2,325 bp downstream of the predicted translation start site.
628 (**B**) Siliques of homozygous *suf4-1* show normal seed set.
629 (**C**) Quantitative RT-PCR analyses revealed that residual *SUF4* transcript is detectable in *suf4-1*.

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

630 (**D**) Three alternative splicing variants of *SUF4* (*SUF4.1*, *SUF4.2*, *SUF4.3*) are expressed in pistils
631 of the wild type. The functional splicing variant *SUF4.1* (Kim and Michaels, 2006) is also
632 detectable in pistils of homozygous *suf4-1* plants.
633 (**E**,**F**) Phenotype of *suf4-1* pistils, pollinated with the sperm cell marker line *HTR10-mRFP1*.
634 Fluorescence microscopy 18-20 hours after pollination revealed ovules with unfused sperm cells
635 (arrowheads in E) or sperm cell nuclei with decondensed chromatin (dashed arrowheads in F). At
636 that time, gamete fusion in wild type ovules has been accomplished (not shown).
637 (**G**) Quantification of *suf4-1* ovule phenotypes shown in (E, F). n = number of pistils (Col-0: 167
638 ovules, *suf4-1*: 232 ovules). In the complemented line *suf4-1 pSUF4::SUF4-GUS* (graphic to the
639 right) the *suf4-1* phenotype of unfused or delayed-fusing sperm cells is not detectable. n = number
640 of pistils (Col-0: 178 ovules, *suf4-1*: 400 ovules). Error bars = SEM; scale bars = 20 µm. [a,] fertilized
641 ovules, no HTR10-mRFP1 fluorescence visible; [b,] includes two ovules with decondensed sperm
642 chromatin and two additional unfused sperm cells.
643
644 **Figure 7. *MOM1* is expressed in developing ovules and participates in *SUF4* and *EC1***
645 **expression.**
646 (**A**) GUS activity driven by *pMOM1::GUS* is detected in the female gametophyte and in the
647 sporophytic tissues of mature ovules.
648 (**B**) In *mom1-3* mutants hemizygous for *pEC1.1(-457)::GUS*, enzymatic GUS activity is detected in
649 25-36% of the analysed egg cells (arrows). 589 ovules have been analysed.
650 (**C**) Quantitative RT-PCR analyses to monitor *SUF4* expression in *mom1-3* flowers. *SUF4*
651 expression is reduced compared with wild type (wt) flowers.
652 (**D**) In *mom1-3* mutant pistils, all five *EC1* genes are down regulated as shown by quantitative RT-
653 PCR analysis.
654 (**E**) Chromatin immunoprecipitation (ChIP) using an antibody antiH3K9ac. ChIP enrichment was
655 evaluated by quantitative PCR analyses. *EC1* genes are enriched in H3K9ac in *mom1-3*

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

656 inflorescence in comparison to wild type ones. Immunoprecipitation (IP) efficiency was tested by
657 quantifying H3K9ac marks in the *IAA8* locus (Zou et al., 2010). Ct values were used to calculate the
658 IP/Input signal. ChIP enrichments are presented as the percentage (%) of bound/input signal.
659 fg, female gametophyte; f, funiculus; ii, inner integument; oi, outer integument
660 Scale bars: 20 μm
661
662 **Supplemental data legends**
663 **Supplemental Table S1. Predicted transcription factor binding sites for TATA binding**
664 **protein (TBP).**
665 **Supplemental Table S2. List of proteins able to bind the *EC1.1* promoter in yeast**.
666 **Supplemental Table S3. SUF4 affects activity of *EC1.1* and *EC1.2* promoters.**
667 **Supplemental Table S4. List of genes co-expressed with *SUF4***
668 **Supplemental Table S5. Primers used in this work**.
669
670 **Supplemental Figure 1. *MOM1* and *IAA8* expression pattern**
671 (**A**) Quantitative RT-PCR analyses to monitor *MOM1* expression in leaves, inflorescences, and
672 developing siliques (4-8 DAF). Error bars stand for s.e.m..
673 (**B-D**) GUS activity driven by *pMOM1::GUS* is detected in developing ovules, ovule stages are
674 indicated (ovule stage according Schneitz et al., 1995)
675 (**E**) *IAA8* is similarly expressed in wild type and *mom1-3* inflorescences. Error bars stand for s.e.m..
676 fm, functional megaspore; female gametophyte; ff, funiculus; ii, inner integument; oi, outer
677 integument op, ovule primordium; pl, placenta;
678 Scale bars: 20 μm
679
680
681

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

682

683

684

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

685

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

Parsed Citations

Amedeo P, Habu Y, Afsar K, Mittelsten Scheid O, Paszkowski J (2000) Disruption of the plant gene MOM releases transcriptional silencing of methylated genes. Nature 405: 203-206
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Aravind L (2000) The BED finger, a novel DNA-binding domain in chromatin-boundary-element-binding proteins and transposases. Trends Biochem Sci 25:421-423
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS (2009) MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37: W202-W208
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Bao Z, Zhang, N, Hua J (2014) Endopolyploidization and flowering time are antagonistically regulated by checkpoint component MAD1 and immunity modulator MOS1. Nature Commun 5: 5628.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Berri S, Abbruscato P, Faivre-Rampant O, Brasileiro ACM, Fumasoni I, Satoh K, Kikuchi S, Mizzi L, Morandini P, Pè ME, Piffanelli P (2009) Characterization of WRKY co-regulatory networks in rice and Arabidopsis. BMC Plant Biol 9: 120
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Brady SM, Zhang L, Megraw M, Martinez NJ, Jiang E, Yi CS, Liu W, Zeng A, Taylor-Teeples M, Kim D, Ahnert S, Ohler U, Ware D, Walhout AJ, Benfey PN (2011) A stele-enriched gene regulatory network in the Arabidopsis root. Mol Syst Biol 7: 459-10.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Choi K, Kim, J, Hwang, HJ, Kim, S, Park, C, Kim, SY, Lee I (2011) The FRIGIDA complex activates transcription of FLC, a strong flowering repressor in Arabidopsis, by recruiting chromatin modification factors. Plant Cell 23: 289-303
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Christensen CA, King EJ, Jordan JR, Drews GN (1997) Megagametogenesis in Arabidopsis wild type and the Gf mutant. Sex Plant Reprod 10: 49-64.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735-743.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Colombo M, Masiero S, Vanzulli S, Lardelli P, Kater MM, Colombo L (2008) AGL23, a type I MADS-box gene that controls female gametophyte and embryo development in Arabidopsis. Plant J 54: 1037-1048
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Costa M, Nobre MS, Becker JD, Masiero S, Amorim MI, Pereira LG, Coimbra S (2013) Expression-based and co-localization detection of arabinogalactan protein 6 and arabinogalactan protein 11 interactors in Arabidopsis pollen and pollen tubes BMC Plant Biol 13: 7

Denninger P, Bleckmann A, Lausser A, Vogler F, Ott T, Ehrhardt DW, Frommer WB, Sprunck S, Dresselhaus T, Grossmann G (2014) Male-female communication triggers calcium signatures during fertilization in Arabidopsis. Nat Comm 5: 4645
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Drews GN, Koltunow AMG (2011) The female gametophyte Arabidopsis book. 9: e0155
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Evans MMS (2007) The indeterminate gametophyte1 Gene of Maize Encodes a LOB Domain Protein Required for Embryo Sac and Leaf Development. Plant Cell 19: 46-62
Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Gan ES, Huang J, Ito T (2013) Functional roles of histone modification, chromatin remodeling and microRNAs in Arabidopsis flower development In International Review of Cell and Molecular Biology (Kwang, WJ ed) Waltham, MA: Academic Press: 115-161

Gross-Hardt R, Kagi C, Baumann N, Moore JM, Baskar R, Gagliano WB, Jurgens G, Grossniklaus U (2007) LACHESIS restricts gametic cell fate in the female gametophyte of Arabidopsis. PLOS Biol 5: e47
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Habu Y, Mathieu O, Tariq M, Probst AV, Smathajitt C, Zhu T, Paszkowski J (2006) Epigenetic regulation of transcription in intermediate heterochromatin. EMBO Rep 7: 1279-1284
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

He Y (2009) Control of the transition to flowering by chromatin modifications. Mol Plant 2: 554-564
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Ingouff M, Hamamura Y, Gourgues M, Higashiyama T, Berger F (2007) Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. Curr Biol 17: 1032-1037
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Ingouff M, Sakata T, Li J, Sprunck S, Dresselhaus T, Berger F (2009) The two male gametes share equal ability to fertilize the egg cell in Arabidopsis thaliana. Curr Biol 19: R19-R20
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Iwasaki M, Paszkowski J (2014) Identification of genes preventing transgenerational transmission of stress-induced epigenetic states Proc Natl Acad Sci USA 23: 8547-8552
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Johnston AJ, Meier P, Gheyselinck J, Wuest SEJ, Federer M, Schlagenhauf E, Becker JD, Grossniklaus U (2007) Genetic subtraction profiling identifies genes essential for Arabidopsis reproduction and reveals interaction between the female gametophyte and the maternal sporophyte. Genome Biology 8, R204
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Jones-Rhoades MW, Borevtz JO, Preuss D (2007) Genome-wide expression profiling of the arabidopsis female gametophyte identifies families of small, secreted proteins. PLoS Genet 3, e171
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Karimi M, Inze D, Depicker A (2002) GATEWAYTM vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7: 193-195
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Kim S, Choi K, Park C, Hwang HJ, Lee I (2006) SUPPRESSOR OF FRIGIDA4, encoding a C2H2-Type zinc finger protein, represses flowering by transcriptional activation of Arabidopsis FLOWERING LOCUS C. Plant Cell 18: 2985-2998
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Kim SY, Michaels SD (2006) SUPPRESSOR OF FRI 4 encodes a nuclear-localized protein that is required for delayed flowering in winter-annual Arabidopsis. Development 133: 4699-4707
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Koi S, Hisanaga T, Sato K, Shimamura M, Yamato K, Ishizaki K, Kohchi T, Nakajima, K. (2016) An evolutionarily conserved plant RKD factor controls germ cell differentiation. Curr Biol 26:1775-1781
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Kong J, Lau S, Jurgens G (2015) Twin plants from supernumerary egg cells in Arabidopsis. Curr Biol 25: 225-230-291
Pubmed: Author and Title
Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Koszegi D, Johnston AJ, Rutten T, Czihal A, Altschmied L, Kumlehn J, Wust SEJ, Kirioukhova O, Gheyselinck J, Grossniklaus U, Baumlein H (2011) Members of the RKD transcription factor family induce an egg cell-like gene expression program. Plant J 67: 280
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Krohn NG, Lausser A, Juranic M, Dresselhaus T (2012) Egg cell signaling by the secreted peptide ZmEAL1 controls antipodal cell fate. Dev. Cell 23: 219-225.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Kumlehn J, Kirik V, Czihal A, Altschmied L, Matzk F, Lorz, H, Baumlein H (2001) Parthenogenetic egg cells of wheat: cellular and molecular studies Sex Plant Reprod 14: 239-243
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Lai Z, Schluttenhofer CM, Bhide K, Shreve J, Thimmapuram J, Lee SY, Yun DJ, Mengiste T (2014) MED18 interaction with distinct transcription factors regulates multiple plant functions. Nat Commun 5: 3064
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Le Q, Gutiérrez-Marcos JF, Costa LM, Meyer S, Dickinson HG, Lörz H, Kranz E, Scholten S (2005) Construction and screening of subtracted cDNA libraries from limited populations of plant cells: a comparative analysis of gene expression between maize egg cells and central cells. Plant J 44: 167-178
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Lopato S, Bazanova N, Morran S, Milligan AS, Shirley N, Langridge P (2006) Isolation of plant transcription factors using a modified yeast one-hybrid system. Plant Methods 2: 3-17.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Márton ML, Cordts S, Broadhvest J, Dresselhaus T (2005) Micropylar pollen tube guidance by Egg Apparatus 1 of maize. Science 307: 573-576.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Masiero S, Colombo L, Grini PE, Schnittger A, Kater MM (2011) The emerging importance of type I MADS box transcription factors for plant reproduction. Plant Cell 23: 865-872
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Matias-Hernandez L, Battaglia R, Galbiati F, Rubes M, Eichenberger C, Grossniklaus U, Kater, MM, Colombo L (2010) VERDANDI is a direct target of the MADS domain ovule identity complex and affects embryo sac differentiation in Arabidopsis. Plant Cell 22: 1702-1715
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Mendes AM, Guerra RF, Castelnovo B, Silva-Velazquez Y, Morandini P, Manrique S, Baumann N, Groß-Hardt R, Dickinson H, Colombo L (2016) Live and let die: a REM complex promotes fertilization through synergid cell death in Arabidopsis. Development 143: 2780-2790
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Menges M, Doczi R, Okresz L, Morandini P, Mizzi L, Soloviev M, Murray JAH, Bogre L (2008) Comprehensive gene expression atlas for the Arabidopsis MAP kinase signalling pathways. New Phytol 179: 643-662
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Mizzotti C, Ezquer I, Paolo D, Paloma-Romero P, Guerra RF, Battaglia R, Rogachev I, Aharoni A, Kater MM, Caporali E, Colombo L (2014) SEEDSTICK is a master regulator of development and metabolism in the Arabidopsis seed coat. PLOS Genetics 10: e1004856
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

Mokhonov VV, Theendakara VP, Gribanova YE, Ahmedli NB, Farber DB (2012) Sequence-specific binding of recombinant Zbed4 to DNA: insights into Zbed4 participation in gene transcription and its association with other proteins. PLoS ONE 7: e35317
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Molina C, Grotewold E (2005). Genome wide analysis of Arabidopsis core promoters. BMC Genomics 6, 25
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Moll C, von Lyncker L, Zimmermann S, Kagi C, Baumann N, Twell D, Grossniklaus U, Gross-Hardt R (2008) CLO/GFA1 and ATO are novel regulators of gametic cell fate in plants. Plant J 56: 913-921
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Nallamsetty S, Austin BP, Penrose KJ, Waugh DS (2005) Gateway vectors for the production of combinatorially-tagged His6-MBP fusion proteins in the cytoplasm and periplasm of Escherichia coli- Protein Sci 14: 2964-2971
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Numa H, Kim J, Matsui A, Kurihara Y, Morosawa T, Ishida J, Mochizuki Y, Kimura H, Shinozaki K, Toyoda T, Seki M, Yoshikawa M, Habu Y (2010) Transduction of RNA-directed DNA methylation signals to repressive histone marks in Arabidopsis thaliana. EMBO J 29: 352-362
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pagnussat GC, Yu HJ, Ngo QA, Rajani S, Mayalagu S, Johnson CS, Capron A, Xie LF, Ye D, Sundaresan V (2005) Genetic and molecular identi?cation of genes required for female gametophyte development and function in Arabidopsis. Development 132: 603-614
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pagnussat GC, Yu HJ, Sundaresan V (2007) Cell-fate switch of synergid to egg cell in Arabidopsis eostre mutant embryo sacs arises from misexpression of the BEL1-like homeodomain gene BLH1- Plant Cell 19: 3578-3592
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Pagnussat GC, Alandete-Saez M, Bowman JL, Sundaresan V (2009) Auxin-dependent patterning and gamete specification in the Arabidopsis female gametophyte. Science 324: 1684-1689.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Rademacher S, Sprunck, S (2013) Downregulation of egg cell-secreted EC1 is accompanied with delayed gamete fusion and polytubey. Plant Signal & Behavior 8, e27377
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Reece-Hoyes JS, Barutcu AR, McCord RP, Jeong JS, Jiang L, MacWilliams A, Yang X, Salehi-Ashtiani K, Hill DE, Blackshaw S, Zhu H, Dekker J, Walhout AJ (2011) Yeast one-hybrid assays for gene-centered human gene regulatory network mapping. Nat Methods 8: 1050-1052
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Roccaro M, Li Y, Masiero S, Saedler H, Sommer H (2005) ROSINA (RSI), a novel protein with DNA-binding capacity, acts during floral organ development in Antirrhinum majus. Plant J 43: 238-250.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Rövekamp M, Bowman JL, Grossniklaus U (2016) Marchantia MpRKD regulates the gametophyte-sporophyte transition by keeping egg cells quiescent in the absence of fertilization. Curr Biol 26: 1782-1789
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Schneitz K, Hulskamp M, Pruitt, RE (1995) Wild-type ovule development in Arabidopsis thaliana: A light microscope study of cleared whole-mount tissue. Plant J 7: 731-749
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

Smale ST, Kadonaga JT (2003) The RNA polymerase II core promoter. Annu Rev Biochem 72: 449-479
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Sprunck S, Baumann U, Edwards K, Langridge, P,Dresselhaus T (2005) The transcript composition of egg cells changes significantly following fertilization in wheat (Triticum aestivum L). Plant J 41: 660-672
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Sprunck S, Groß-Hardt R (2011) Nuclear behavior, cell polarity, and cell specification in the female gametophyte. Sex Plant Reprod 24:123-136
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Sprunck S, Rademacher S, Vogler F, Gheyselinck J, Grossniklaus U, Dresselhaus T (2012) Egg-cell secreted EC1 triggers sperm cell activation during double fertilization. Science 338: 1093-1097
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Steffen JG, Kang I, Macfarlane J, Drews GN (2007) Identification of genes expressed in the Arabidopsis female gametophyte. Plant J 51: 281-292
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Sung S, Amasino RM (2004) Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature 427: 159-164
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Tong JK, Hassig CA, Schnitzler GR, Kingston RE, Schreiber SL (1998) Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. Nature 395: 917-921
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Tran L-SP, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell 16:2481-2498
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Vaillant I, Schubert I, Tourmente S, Mathieu O (2006) MOM1 mediates DNA-methylation-independent silencing of repetitive sequences in Arabidopsis. EMBO Rep 7: 1273-12
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Voelz R, von Lyncker L, Baumann N, Dresselhaus T, Sprunck S, Groß-Hardt, R (2012) LACHESIS-dependent egg-cell signaling regulates the development of female gametophytic cells. Development 139: 498-502
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Wuest SE, Vijverberg K, Schmidt A, Weiss M, Gheyselinck J, Lohr M, Wellmer F, Rahnenfuhrer J, von Mering C, Grossniklaus U (2010) Arabidopsis female gametophyte gene expression map reveals similarities between plant and animal gametes. Curr Biol 20: 506-512
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Yadegari R, Drews GN (2004) Female gametophyte development. Plant Cell 16: S133-S141
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Yadegari R, Kinoshita T, Lotan O, Cohen G, Katz A, Choi Y, Nakashima K, Harada JJ, Goldberg RB, Fischer RL, Ohad N (2000) Mutations in the FIE and MEA genes that encode interacting Polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms. Plant Cell 12: 2367-2381Yang H, Kaur N, Kiriakopolos S, McCormick S (2006) EST generation and analyses towards identifying female gametophyte-specific genes in Zea mays L. Planta 224: 1004-1014
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

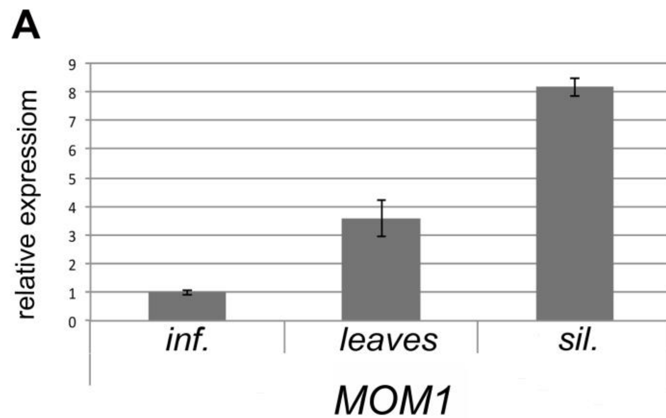
Yu HJ, Hogan P, Sundaresan V (2005) Analysis of the female gametophyte transcriptome of Arabidopsis by comparative expression
Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.

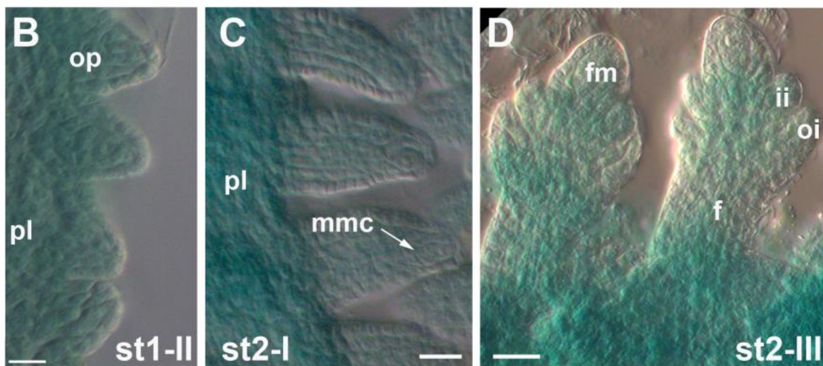
profiling. Plant Physiol 139: 1853-1869
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

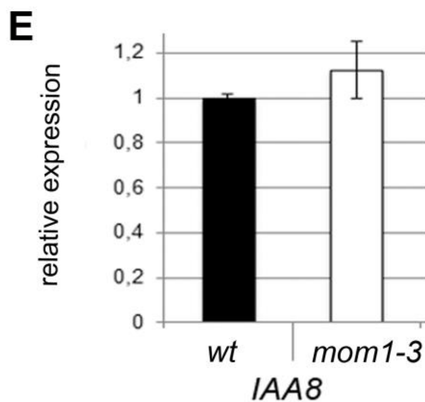
Yuan L, Liu Z, Song X, Johnson C, Yu X, Sundaresan V (2016) The CKI1 Histidine Kinase Specifies the Female Gametic Precursor of the Endosperm. Dev Cell. 37: 34-46
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Zhang L, Wang L, Yang Y, Cui J, Chang F, Wang Y, Ma H (2015) Analysis of Arabidopsis floral transcriptome: detection of new florally expressed genes and expansion of Brassicaceae-specific gene families. Front Plant Sci 5: 802
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only Title Only Author and Title

Downloaded from www.plantphysiol.org on December 21, 2016 - Published by www.plantphysiol.org
Copyright © 2016 American Society of Plant Biologists. All rights reserved.







Supplemental Figure 1. ***MOM1 expression pattern***
(**A**) Quantitative RT-PCR analyses to monitor *MOM1* expression in leaves, inflorescences, and developing siliques (4-8 DAF). Error bars stand for s.e.m..
(**B-D**) GUS activity driven by *pMOM1::GUS* is detected in developing ovules, ovule stages are indicated (ovule stage according Schneitz et al., 1995)
(**E**) *IAA8* is similarly expressed in wild type and *mom1-3* inflorescences. Error bars stand for s.e.m..
fm, functional megaspore emale gametophyte; f, funiculus; ii, inner integument; oi, outer integument op, ovule primordium; pl, placenta;
Scale bars: 20 µm

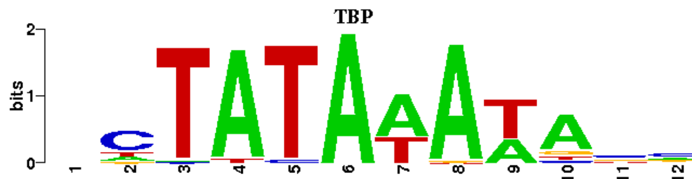
Supplemental Table S1. Predicted transcription factor binding sites for TATA binding protein (TBP).

TBP sites, mapped in the -500 upstream region of *EC1* genes. Predictions are according to AthMap (http://www.athamap.de/index.php). The sequence logo of TBP DNA binding motif (Franco-Zorilla et al., 2014) is shown below.

Gene	Factor	Family	Position	Relative orientation	Relative distance	Max. Score	Threshold Score	Score
At1g76750.1	TBP	- other	28815898	+	-36	10.98	5.16	7.6
At2g21740.1	TBP	- other	9289511	+	-68	10.98	5.16	7.11
At2g21740.1	TBP	- other	9289664	+	-221	10.98	5.16	5.16
At2g21750.1	TBP	- other	9290888	+	-64	10.98	5.16	8.09
At4g39340.1	TBP	- other	18293561	+	-55	10.98	5.16	10.44
At5g64720.1	TBP	- other	25890092	+	-53	10.98	5.16	6.88

Name: **TBP**

Logo:



Matrix:

```
A |  48  27   5 162   0 171 106 166  65 125  22  51
C |  46 108   2   0   7   0   0   0   2  14  72  72
G |  29   9   0   0   0   0   0   3   0  17  48  27
T |  48  27 164   9 164   0  65   2 104  15  31  19
```

Max. score: 10.98

Threshold: 5.16

Supplemental Table S2. List of proteins able to bind the *EC1.1* promoter in yeast.

The proteins listed have been found more than once as *pEC1.1* interactors.

Accession	Description
At1g0782/ At1g07820/At3g53730	Histone 4
At2g46980	ASY3, a coiled-coil domain protein
At1g07820	Histone superfamily protein
At1g69690	AtTCP15
At1g01960	Embryo sac Development Arrest 10 (EDA10)
At1g30970	SUF4
At5g62470	Myb 96
At3g27670	RESURRECTION1, RST1
At5g22880	Histone H2B

Supplemental Table S3. SUF4 affects activity of *EC1.1* and *EC1.2* promoters.

GUS analyses performed with mature ovules of F1, obtained by crossing homozygous *suf4-1* mutants with homozygous *pEC1.1(-457)::GUS* or *pEC1.2(-893)::GUS* lines (*suf4-1 suf4-1xpEC1.1(-457)::GUS pEC1.1(-457)::GUS* and *suf4-1 suf4-1xpEC1.2(-893)::GUS)*. Note that the χ^2 statistics indicate that SUF4 regulates expression of *pEC1.1(-457)::GUS*, as well as *pEC1.2(-893)::GUS*.

Genotypes	Ovules						
	observed GUS+	observed GUS-	total	expected GUS+	expected GUS-	χ^2 value*	p-value
SUF suf4-1 EC1.1::GUS +/-	356 25,6%	1,036 74,4%	1,392	348 25%	1,044 75%	0.24	0.5<P<0.75
SUF suf4-1 EC1.2::GUS +/-	301 24,6%	924 75,4%	1,225	306.3 25%	918.7 75%	0.12	0.5<P<0.75

GUS+, GUS positive ovules; GUS-, GUS negative ovules

Supplemental Table S4. List of genes co-expressed with *SUF4*.

Accession	Description	Pearson coefficient (Log)
At1g30970	Zinc finger (C2H2 type) family protein, contains Pfam domain	1
At1g78930	Mitochondrial transcription termination factor-related /	0,6780
At5g63200	Tetratricopeptide repeat (TPR)- protein	0,6676
At1g50910	Unknown protein	0,6624
At5g47400	Unknown protein	0,6601
At1g08060	**MOM1**	**0,6555**
At3g44530	Transducin family protein	0,6551
At4g18600	Unknown protein	0,6484
At5g06100	Myb transcription factor (MYB33)	0,6471
At2g35540	DNAJ heat shock	0,6455
At2g47820	Expressed protein	0,6441
At5g20200	Nucleoporin-related protein	0,6407
At5g18770	F-box protein	0,6381
At1g77410	Putative beta-galactosidase	0,6378
At2g43980	Inositol 1,3,4-trisphosphate 5/6-kinase	0,6353
At4g35930	F-box family protein	0,6345
At5g05350	Unknown protein	0,6295
At5g05130	SNF2 domain-containing protein /	0,6268
At4g25540	DNA mismatch repair protein (MSH3)	0,6254
At1g52620	Pentatricopeptide (PPR) repeat-containing protein	0,6248
At5g13470	Unknown protein	0,6222
At2g40950	bZIP transcription factor	0,6206
At2g28330	Unknown protein	0,6200

Supplemental Table S5. Primers used in this work.

Name	Sequence
Promoter analysis	
EC1.2p(-869)_fw(PstI)	TGATTACGCCCTGCAGCGTTTATACAAGGACAGAG
EC1.2p (-750)_fw(PstI)	TGATTACGCCCTGCAGAGGTAACTTGTCCAAGAAG
EC1.2p(-631)_fw(PstI)	TGATTACGCCCTGCAGTATATTTGTATTCGAGATTGTG
EC1.2p(-502)fw(PstI)	TGATTACGCCCTGCAGCACATCGACAACACTATAGC
EC1.2p(-374)_fw(PstI)	TGATTACGCCCTGCAGCATAAAGTTCAATGAGTAGT
EC1.2p (-252)_fw(PstI)	TGATTACGCCCTGCAGCGCTACTGATTCAACATG
EC1.2p(-222)_fw(PstI)	TGATTACGCCCTGCAGCTAAATACGTTTCTACAGTCAAA
EC1.2p(-212)_fw(PstI)	TGATTACGCCCTGCAGTTCTACAGTCAAATGCTTTAAC
EC1.2p(-192)_fw(PstI)	TGATTACGCCCTGCAGACGTTTCATGATTAAGTGACTAT
EC1.2p(-183)_fw(PstI)	TGATTACGCCCTGCAGATTAAGTGACTATTTACCGTC
EC1.2p(-172)_fw(PstI)	TGATTACGCCCTGCAGTATTTACCGTCAATCCTTTCC
EC1.2p(-152)_fw(PstI)	TGATTACGCCCTGCAGCCATTCCTCCCACTAATCCA
EC1.2p(-132)_fw(PstI)	TGATTACGCCCTGCAGACTTTTTAATTACTCTTAAATCAC
EC1.2p(-112)_fw(PstI)	TGATTACGCCCTGCAGTCACCACTAAGCTTCGAATC
EC1.2p_rev(BamHI)	TGCTCACCATGGATCCACTTGTGTTAGAAGCCATTA
EC1.1p(-463)_fw	caccTGCCTTATGATTTCTTCGGTTT
EC1.1p(-326)_fw	TTCTATTGAACTAAATCAAACGAGTTT
EC1.1p(-192)_fw	CTAATTATCTCACAATCTAATGAGCTTC
EC1.1p_rev	TTCTCAACAGATTGATAAGGTCGA
EC1.2p(-894)_fw	caccAAGCATTTGCGTTTGGTTTATCATTGCG
EC1.2p_rev	TATTCTTTCTTTTTGGGGTTTTTGTTT
EC1.3p(-293)_fw	caCCACTCTTGTTATACAATCTTCAAAGTTTCTATA
EC1.3p(-133)_fw	caccACTAAAAACCATCTTTAATCATAATTAAA
EC1.3p_rev	TGTGTTTTCTTTTGAAGATTTTGTG
EC1.4p(-263)_fw	caccGTTGCTCTTGCTGCATCAAATACATA
EC1.4p(-163)_fw	caccACTAATCATTTACTTTCACTTATCCCT
EC1.4p_rev	TGGTGTGGTTTTTGTTTGGAATTTTA
EC1.5p(-251)_fw	caccGGGTTTCCATAAAGCCCAATTTAGTT
EC1.5p(-146)_fw	caacTAATTAAATTAACCACGATCACTGTGATAATTAC
EC1.5p_rev	TGCTTCTTTTTGTGTTATGGATTTTTTGT

Housekeeping and control genes	
ACT3 (AT3G53750)_fw	GATTTGGCATCACACTTTCTACAATG
ACT3 (AT3G53750)_rev	GTTCCACCACTGAGCACAATG
ACT8 (AT1G49240)_fw	CTCAGGTATTGCAGACCGTATGAG
ACT8 (AT1G49240)_rev	CTGGACCTGCTTCATCATACTCTG
UBQ10 (AT4G05320)_fw	GGAAAAAGGTCTGACCGACA
UBQ10 (AT4G05320)_rev	CTGTTCACGGAACCCAATTC
IAA8 for ChIP experiment_fw	GAACGTAACTACTTGGGTCT
IAA8 for ChIP experiment_rev	GTCTTTAGAAGGTAGCAAC
IAA8 RTqPCR_fw	CAATGGCTTCTTCTACTTCG
IAA8 RTqPCR_rev	CCAATCACCGTCTTTATCT
Primers for quantitative PCR	
EC1.1_rev	AGTCATTGCCATCACAGTAACC
EC1.1_fw	CTTGTGTACAGGCTCAAGCTTG
EC1.2_fw	GGCTTCTAACACAAGTTTCCTC
EC1.2_rev	ATGACCTCGACGGCTTGACA
EC1.2_rev	CCGAGTTTGGTCTCACCGTT
EC1.2_fw	CTCTTCTCCTCGTTCTCAACG
EC1.4_fw	ATGGCTTCGAACACTACTTTCC
EC1.4_rev	CCATCAGTCCTCCACTTTGG
EC1.5_rev	CGGTTCACTCGTACCGGTTTGA
EC1.5_fw	GACCTTTCATGTCATCACTGTCG
Yeast constructs	
pAtEC1.11 plus EcoRI_fw	CGAATTCTGCCTTATGATTTCTTCGG
pAtEC1.11 plus XbaI_rev	CGTCTAGATAATTAGTGGGTCTGTTTAGG
pAtEC1.12 plus EcoRI_fw	CGAATTCCAGACCCACTAATTACG
pAtEC1.12 plus XbaI_rev	CGTCTAGACTCAACAGATTGATAAGG
Other primers	
GUS_rev	TCATTGTTTGCCTCCCTGCTG
SUF4-1_fw	GTATGGCAAATGCCACCTC
SUF4-1_rev	CTGAGATTCGTCTGTCTATCGC
TDNA pSKI015 derivative (Dinneny et al. 2004)	CATTTTATAATAACGCTGCGGACATCTAC
SUF4 alternative splicing_fw	GGGGCTCAGCAACCATCTCAT

SUF4 alternative splicing_rev	ATCCGCCCAGCAAGCCTACT
SUF4 CDS pENTR-D-TOPO_fw	CACCATGGGTAAGAAGAAGAAGAG
SUF4 CDS_rev	CTAAAACGCCATCCGCCC
MOM1.3_fw	AAGCAGTTGTCTTCTACCAG
MOM1.3_rev	TTGCCGCTTATTTTGCCTAG
T-DNA SALK LBb1 primer	GCGTGGACCGCTTGCTGCAACT
MOM1 promoter_fw gateway	GGGGACAAGTTTGTACAAAAAAGCAGGCTGATCGACTCTAAACATTATGGG
MOM1 promoter_rev gateway	GGGGACCACTTTGTACAAGAAAGCTGGGTCATATTCACTGAGAGCACGCAA

Supplemental table cited literature

Franco-Zorrilla JM, López-Vidriero I, Carrasco JL, Godoy M, Vera P, Solano R (2014) DNA-binding specificities of plant transcription factors and their potential to define target genes. Proc Natl Acad Sci USA 111: 2367-72