1 **Slow development restores the fertility of photoperiod-sensitive male-sterile**

2 **plant lines**

3

4 **Author names and affiliations:**

5 Cheng Zhang[#,1], Te Xu[#,1], Meng-Yi Ren[#,1], Jun Zhu[1], Qiang-Sheng Shi[1], Ya-Fei

6 Zhang[1], Yi-Wen Qi[1], Min-Jia Huang[1], Lei Song[1], Ping Xu[*,1], Zhong-Nan Yang[*,1]

7 [1]Shanghai Key Laboratory of Plant Molecular Sciences, College of Life Sciences,

8 Shanghai Normal University, Shanghai, 200234, China

9 #These authors contributed equally to this work.

10 * Correspondence: Zhong-Nan Yang (znyang@shnu.edu.cn) and Ping Xu

11 (PingXu_ARDNOR@shnu.edu.cn)

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13 **One-sentence summary:** Slow development is a general mechanism for

14 Photoperiod-sensitive Genic Male Sterility.

15

16 **Short title:** Slow development restores fertility of PGMS lines

17

18 **Author Contributions**

19 Z.-N.Y. conceived and designed the experiment with C.Z. and T.X.. C.Z., T.X. and

20 M.-Y.R. performed the observation of fertility conversion of the lines under short-

21 photoperiod and low light intensity. C.Z., M.-Y.R., J.Z. and Q.-S.S measured the

22 plant growth rate under short-day photoperiod. T.X., Y.W.Q and Y.F.Z. performed

23 the cytological observations of *cals5-2* under different conditions. T.X., M.J.H. and

24 L.S. performed the fertility analysis and plant growth rate measurement of *cals5-2*

25 under high temperature. Z.N.Y., P.X. and C.Z. wrote the paper.

26

27 **Abstract**

28 Photoperiod- and thermo-sensitive genic male sterility (P/TGMS) lines are widely

29 used in crop breeding. The fertility conversion of *Arabidopsis thaliana* TGMS lines

30 including *cals5-2*, which is defective in callose wall formation, relies on slow

31 development under low temperatures. In this study, we discovered that *cals5-2* also

32 exhibits PGMS. Fertility of *cals5-2* was restored when pollen development was

33 slowed under short-day photoperiods or low light intensity, suggesting that slow

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34 development restores the fertility of *cals5-2* under these conditions. We found that
35 several other TGMS lines with defects in pollen wall formation also exhibited
36 PGMS characteristics. This similarity indicates that slow development is a general
37 mechanism of PGMS fertility restoration. Notably, slow development also
38 underlies the fertility recovery of TGMS lines. Further analysis revealed the pollen
39 wall features during the formation of functional pollens of these P/TGMS lines
40 under permissive conditions. We conclude that slow development is a general
41 mechanism for fertility restoration of P/TGMS lines and allows these plants to take
42 different strategies to overcome pollen formation defects.

43

44 **Introduction**

45 Hybrid breeding, using three-line and two-line systems, is applied in increasing
46 crop yields to meet the rising needs for food worldwide (Li et al., 2007). The three-
47 line system involves a cytoplasmic male-sterile line, a restorer line, and a
48 maintainer line. The two-line system only requires a male sterile line and a restorer
49 line, which simplifies the breeding procedures and has been widely used in
50 numerous crops, including rice, maize, cotton, and rape (Virmani and Ilyas-
51 Ahmed;, 2001). The most extensively used male sterile lines in the two-line system
52 exhibit T̲hermo-sensitive G̲enic M̲ale S̲terility (TGMS) and/or P̲hotoperiod-
53 sensitive G̲enic M̲ale S̲terility (PGMS). TGMS lines are sterile under normal
54 temperature range, but turn fertile under low temperatures while PGMS lines are
55 sterile under long-day (LD) photoperiod, but are fertile under short-day (SD)
56 photoperiod (Chen and Liu, 2014).

57 Much progress about TGMS mechanism has been made in recent years. A number
58 of TGMS loci have been identified in rice. In AnnongS-1, a pre-mature stop codon
59 is introduced in the *RNase Z^{S1}* gene (Zhou et al., 2014). The genome of
60 HengnongS-1 contains a mutation in the homolog of Arabidopsis *MALE*
61 *STERILITY1* (*MS1*) (Qi et al., 2014). An artificial TGMS rice line was created by
62 silencing a UGPase (Chen et al., 2007). In addition, TGMS in *indica* rice Peiai64S
63 is determined by the mutation of the genetic locus for a long noncoding RNA
64 (*PMS1T and PMS3*) (Zhou et al., 2012). Similar to rice, several TGMS loci have
65 been identified in Arabidopsis, including the *PLANT U-BOX 4* (*PUB4*), *MYB33,*
66 and *MYB65* (Millar and Gubler, 2005). Recently, a new TGMS locus,

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REVERSIBLE MALE STERILE1 (*RVMS*), was identified. RVMS encodes a GDSL lipase that hydrolyzes triglycerides into glycerol and hexadecanoic acid which are the components of plasma membrane (Zhu et al., 2020). The male sterile mutants *acyl-coa synthetase5-2* (*acos5-2*), *cyp703a2*, *callose synthase 5-2* (*cals5-2*), and *ruptured pollen grain* (*rpg1*) have defective pollen walls (Dong et al., 2005; Morant et al., 2007; Guan et al., 2008; de Azevedo Souza et al., 2009). These lines also show the TGMS phenotype (Zhu et al., 2020). Analysis of the underlying mechanism revealed that slow development induced by low temperature is involved in the fertility conversion in these TGMS lines (Zhu et al., 2020).

The first PGMS line was discovered in 1973 in the japonica rice variety NongKen 58S (Shi, 1985). Then, a number of PGMS lines derived from NongKen 58S with significant hybrid vigor, were created for application in agriculture (Fan et al., 2016). Photoperiod was considered to be the only environmental regulator of fertility conversion of the sterile lines until the discovery of TGMS in the late 1980s (Sun et al., 1989; Chen 2001). In the summer of 1989 in China, low temperatures unusually led to unexpected fertility restoration in several rice sterile lines including W6541S, AnnongS-1, and HengnongS-1 (Chen, 2001). In 1991, the unusual high autumn temperatures caused unintentional sterility in the 7001S rice line (Chen, 2001). Later on, it was found that both photoperiod and temperature may contribute to the sterility or fertility recovery (He et al., 1987). In addition, researchers found that in *O. sativa,* japonica varieties are mainly affected by photoperiod, while indica varieties are mainly affected by temperature (Sun et al., 1991). Scientists also discovered that mutation of a noncoding RNA shows different Environmental Genic Male Sterility (EGMS) traits: PGMS in japonica rice Nongken 58S and TGMS in indica rice Peiai64S (Ding et al., 2012). Nevertheless, the mechanism of the fertility conversion in PGMS lines and how it is coordinated by photoperiod and temperature are still unclear.

In Arabidopsis, the callose synthase CalS5 is required for the callose wall formation during early pollen wall development (Dong et al., 2005). The *cals5-2* knockout line exhibits the TGMS phenotype and its fertility can be restored by low temperature (Zhu et al., 2020). In the present study, we found that *cals5-2* is also a PGMS line. Slow development under SD photoperiod or low light intensity restores its fertility. We found several other TGMS lines also showed the PGMS phenotype.

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100 Further analysis revealed that slow development was responsible for fertility
101 restoration in these PGMS lines, revealing a shared mechanism for PGMS and
102 TGMS.
103
104

Results

***cals5-2* exhibits PGMS.**

It was shown that the fertility and pollen production of *cals5-2* line can be restored under low temperature (Zhu et al., 2020). In the present study, we investigated whether its fertility was also photoperiod-sensitive. A typical photoperiod for growing Arabidopsis is 16 hours (16h) of light and 8 hours (8h) of darkness at 23°C, the LD condition. To test its sensitivity to photoperiod, we grew the *cals5-2* line under SD photoperiod with 8h of light and 16h of darkness at 23°C. Under LD, *cals5-2* plants were severely sterile with few pollen grains (Figure 1A). However, under the SD photoperiod, the seed set of *cals5-2* was significantly restored (Figure 1C), similar to that observed under low temperature (Figure 1B). In addition, results from Alexander staining showed the formation of mature pollen grains (purple) in *cals5-2* under SD condition (Figure 1C). Thus, SD photoperiod restores pollen formation and fertility of *cals5-2*.

The pollen development process, microgametogenesis, depends on timely coordination of meiosis, mitosis, cell growth and expansion (Sanders et al., 1999). Our previous work showed that the pollen development is slowed under low temperature (Zhu et al., 2020). To analyze whether the development is also slowed under SD conditions, we compared the pollen growth under LD and SD conditions using two different approaches. First, we analyzed the growth rate of *cals5-2* from tetrad to mature pollen grain under LD and SD conditions. One tetrad encloses four microspores in callose wall, which can be easily observed using light microscopy. In Arabidopsis, mature pollen grains contain three nuclei which are easy to identify. Under the LD condition, a few fertile pollen grains can be occasionally observed in *cals5-2* anthers (Figure 1A). It took about three days for tetrads to develop into mature pollen grains under LD photoperiod (n=10; Figure 1D), compared to about four days under the SD photoperiod (n=12; Figure 1D). The second approach was to measure the size of developing microspores during microgametogenesis, as previously described (Zhu et al., 2020). Under the LD condition, the microspores released from the tetrad were about 13.0 μm in diameter. The microspore size was 19.4 μm and 25.3 μm after two and four days' growth, respectively (Figure 1E). Under the SD condition, the released microspores were about 12.7 μm in diameter,
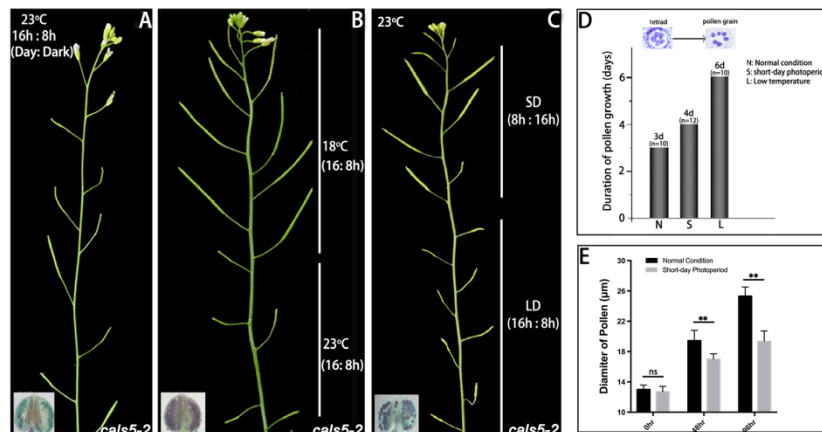
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Figure 1. *cals5-2* is a PGMS line. (**A**) *cals5-2* exhibited severely reduced male fertility under normal conditions. Few pollen grains were produced in *cals5-2* anther. (**B**) The fertility of *cals5-2* was restored under low temperature. (**C**) The fertility of *cals5-2* was restored under SD condition and pollen maturation was recovered. The images of plants shown in A, B and C were digitally extracted for comparison. Bars in A, B and C=20μm. (**D**) Pollen maturation was delayed by 1 day under SD conditions compared with LD conditions. (**E**) Diameters of microspores at different developmental stages. 20 microspores or pollen grains were used to measure the diameter at each developmental stage, respectively. One-way ANOVA was performed to compare the diameter of microspores. * $P<0.05$; ** $P<0.001$; ns, not significant ($P>0.05$). Error bars represent the standard deviation (SD) of pollen diameters. The diameter of microspores at 48h and 96h under a SD photoperiod was significantly reduced compared to a LD photoperiod.

137   and their sizes were 17.0 μm to 19.3 μm after two and four days' growth,

138   respectively (Figure 1E). These results demonstrated that SD photoperiod

139   significantly slowed microspore development. A similar delay in microspore

140   development was observed under the low temperature conditions which can restore

141   the fertility of *cals5-2* and other TGMS lines (Zhu et al., 2020). Therefore, both low

142   temperatures and SD photoperiod restore the fertility of *cals5-2* plants through

143   slowing microspore development.

144

145   **Low light intensity restores the fertility of *cals5-2*.**

146   In addition to photoperiod, light intensity is another important factor that affects

147   light signaling and general energy production. Thus, we further investigated

148   whether the fertility of *cals5-2* can be restored by low light intensity. We placed

149   *cals5-2* in the growth chamber with low light intensity (45 μmol m^{-2} s^{-1}) for ten

150   days. The control *cals5-2* plants were grown under the normal conditions with light

151   intensity of 103 μmol m^{-2} s^{-1}. Under low light intensity, seeds were produced

152   normally (Figure 2A and Figure S1) and fertile pollen grains were observed in

153   *cals5-2* (Figure 2B). We further analyzed the pollen development process under

154   different light intensity. Pollen maturation under normal conditions and under low

155   light intensity took about three and four days, respectively (Figure 2C). By

156   measuring microspore diameters at two and four days after release from tetrad, we

157   found the microspores were significantly smaller at both time points under the low

158   light intensity condition (Figure 2D). Therefore, low intensity of light also slowed
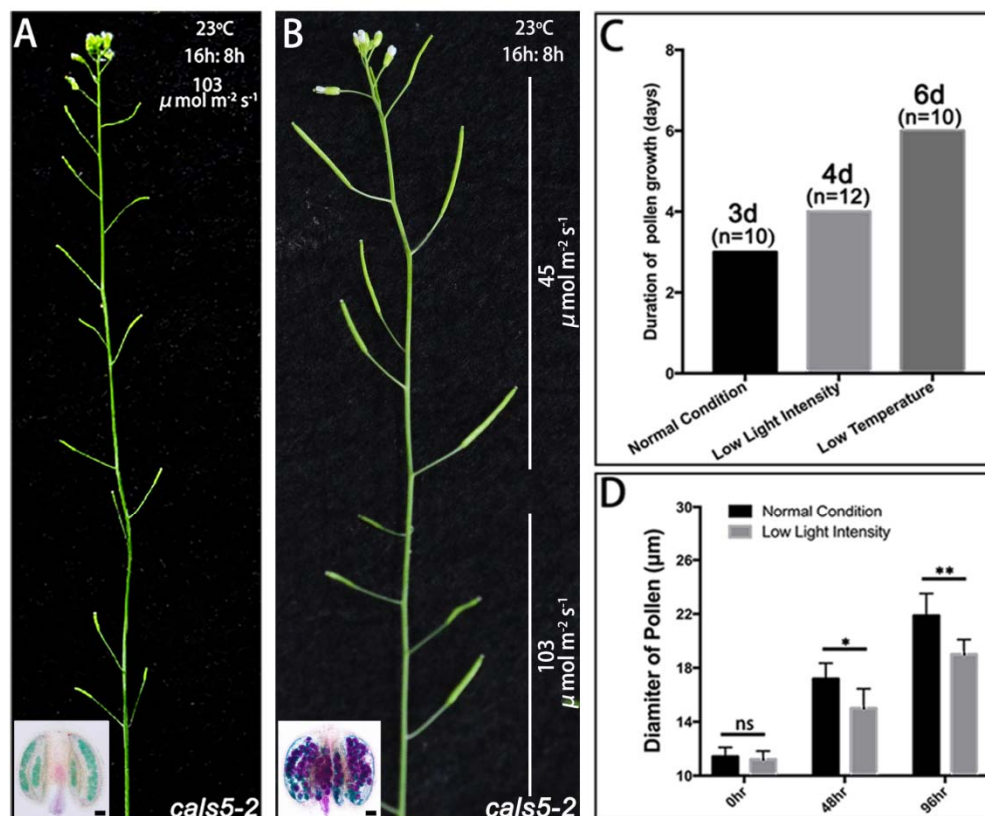
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Figure 2. Fertility of *cals5-2* is restored under low light intensity. (A) *cals5-2* showed severe male sterility under normal conditions. Pollen production was abnormal in *cals5-2* anther. **(B)** The fertility of *cals5-2* was restored under low light intensity. Pollen grains in *cals5-2* were produced under low light intensity. Bars in A and B=20μm. **(C)** Pollen growth, from tetrad to mature pollen, was delayed by one day delayed under low light intensity (n=12). **(D)** Diameter of microspores at different developmental stages. 20 microspores or pollen grains were used to measure the diameter at each developmental stage, respectively. One-way ANOVA was performed to compare the diameter of microspores. * $P<0.05$; ** $P<0.001$; ns, not significant ($P>0.05$). Error bars represent the SD of pollen diameters. The diameter of microspores at 48h and 96h was significantly smaller under low light intensity compared to normal conditions.

159   microspore development. Taken together, low light intensity restores *cals5-2*

160   fertility through slowing its microspore growth, which is the same as the low

161   temperature and SD photoperiod.

162

163   **Slow development overcomes sexine defects and restores the formation of**

164   **functional pollen in *cals5-2*.**

165   After meiosis, all four microspores are enclosed in callose wall. Primexine, formed

166   between the callose wall and microspore plasma membrane, is responsible for

167   pollen wall pattern establishment (Xu et al., 2016). *CalS5* encodes a callose

168   synthase involved in callose wall formation. Peripheral callose wall is absent in

169   *cals5-2* which leads to defective exine pattern formation and male sterility under

170   normal conditions (Dong et al., 2005). We used the callose stain aniline blue to

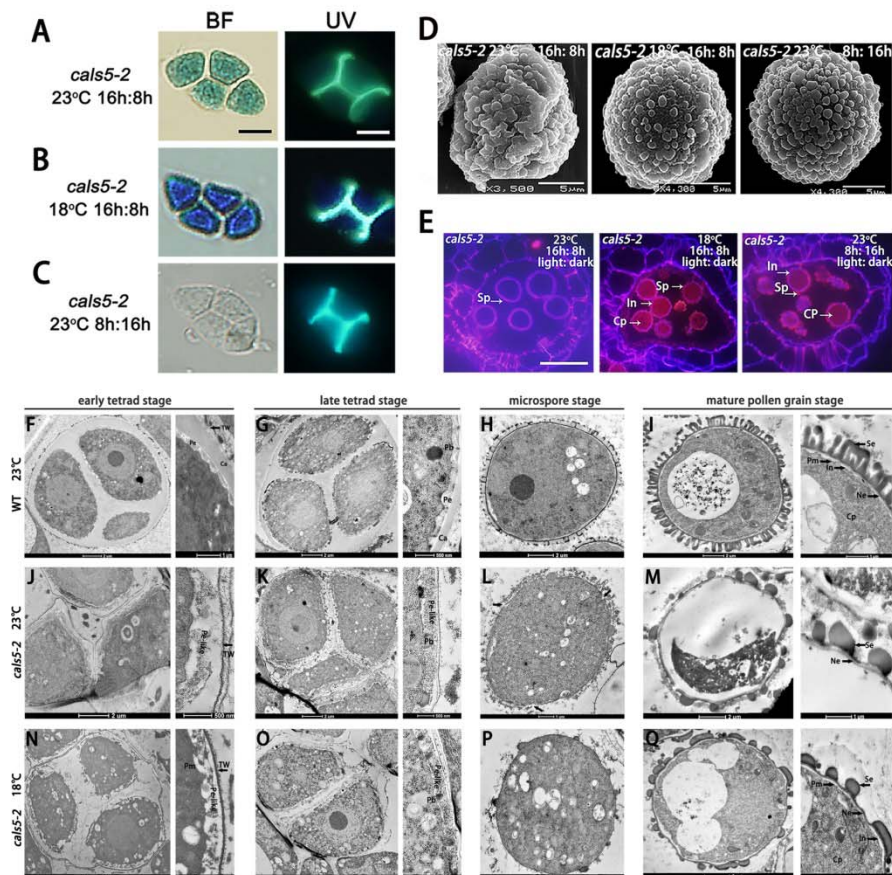171   reveal the wall features of tetrads in fertility restored plants. The peripheral callose

Figure 3. Fertility-restored *cals5-2* line exhibits pollen wall defects but recovered pollen wall integrity. (**A–C**) Results from aniline blue staining of the callose wall of tetrads. Callose deposition was not recovered in *cals5-2* under low temperature or SD conditions. Bars=10μm. The scale bar in A also represents the one in B and C. (**D**) SEM observation of the mature pollen of *cals5-2* plants. The pollen surface remained rough and irregular under low temperature or SD conditions. (**E**) Tinopal and DIOC2 staining of *cals5-2* anther and pollen grains. The intine of *cals5-2* pollen was intact under low temperature or SD conditions. Cp, Cytoplasm; Sp, Sporopollenin; In, Intine. Bars=20μm. (**F–I**) TEM images of wild type pollen from the early tetrad stage to mature pollen stage at 23°C. (**J–M**) TEM images of *cals5-2* pollen from the early tetrad stage to mature pollen stage under 23°C. The callose wall and primexine were not observed around microspores in tetrads. The nexine was discontinuous, and intine formation was disrupted at 23°C. (**N–Q**) TEM images of *cals5-2* pollen from the early tetrad stage to mature pollen stage at 18°C. The callose wall and primexine were not restored, but the integrity of nexine and intine was much better maintained than those under normal temperature (23°C). Ca, Callose wall; TW, Tetrad Wall; Pb, Probacular; Pe, primexine; Pm, Plasma membrane; Pe-like, Primexine-like materials; In, Intine; Se, Sexine; Ne, Nexine; Cp, Cytoplasm.

172 wall of the fertile *cals5-2* tetrad was found to be absent, similar to what was

173 observed in normal (sterile) conditions (Figure 3A–C). We further observed

174 microspore development of fertile and sterile *cals5-2* plants using a Scanning

175 Electronic Microscope (SEM). The pollen surface of the microspore was rough and

176 abnormal in the fertility restored plants of *cals5-2*, again similar to *cals5-2* male

177 sterile plants under normal conditions (Figure 3D). Thus, even though the pollen

178 grains became fertile in the restored plants under SD photoperiod and low

179 temperatures, their callose wall and pollen wall remained defective. Furthermore,

180 we stained the pollen wall with Tinopal and DiOC$_2$. The intine can be stained

181 purple by Tinopal, and the lipid contents and sexine of the mature pollen grain can

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182   be stained red by $DIOC_2$ (Gu et al., 2014). The intine layer and lipid contents were

183   not visible in pollen grains of *cals5-2* plants under normal (sterile) conditions

184   (Figure 3E). In contrast, in the fertile *cals5-2* plants, the intine layer and lipid

185   contents were clearly visible in the fertile *cals5-2* pollen grains under SD

186   photoperiod or low temperature (Figure 3E). This indicates that *cals5-2* microspore

187   could overcome pollen wall pattern defect to further develop into mature functional

188   pollen under these permissive conditions.

189   During anther development, the pollen wall materials from the tapetum deposit

190   outside of the microspore to form a wall with a reticular pattern. Both callose wall

191   and primexine are important for pollen wall establishment (Xu et al., 2016). TEM

192   observation revealed that the primexine remained absent in *cals5-2* tetrads under

193   low temperature (Figure 3N), but some electron dense (pe-like) materials were

194   visible between the plasma membrane and the thin wall (TW; Figure 3N and O).

195   Globular sporopollenin was randomly deposited on the plasma membrane at the

196   late tetrad stage under both low temperature and normal conditions (Figure 3J-M, P

197   and Q). Thus, callose wall is essential for primexine formation. The defective

198   callose wall and primexine are in agreement with defective pollen wall pattern in

199   the restored *cals5-2* plant under low temperature. Under normal conditions, the

200   *cals5-2* nexine layer was discontinuous (Figure 3L). However, under low

201   temperature, the nexine layer was continuous and intact at the microspore stage

202   (Figure 3P) and the intine layer also exhibited good integrity at the mature pollen

203   stage (Figure 3Q). These results show that the formation of functional mature

204   pollen in the fertility restored *cals5-2* is associated with restoration of nexine and

205   intine layers, while the outer sexine wall remained defective. Slowed development

206   may therefore overcome certain pollen wall pattern defects to support its further

207   development into functional pollen.

208

209   **Fertility of *cals5-5* is reduced under high temperature**

210   Our results suggest that slowed development led to the fertility restoration of

211   P/TGMS lines under permissive conditions (Figure 1 and 2). To further test this

212   hypothesis, we investigated whether faster pollen development could affect the

213   fertility of P/TGMS lines. *cals5* has several alleles, and *cals5-5* is a weak allele

214   with a T-DNA insertion in the third intron (Nishikawa et al., 2005; Figure S2A).
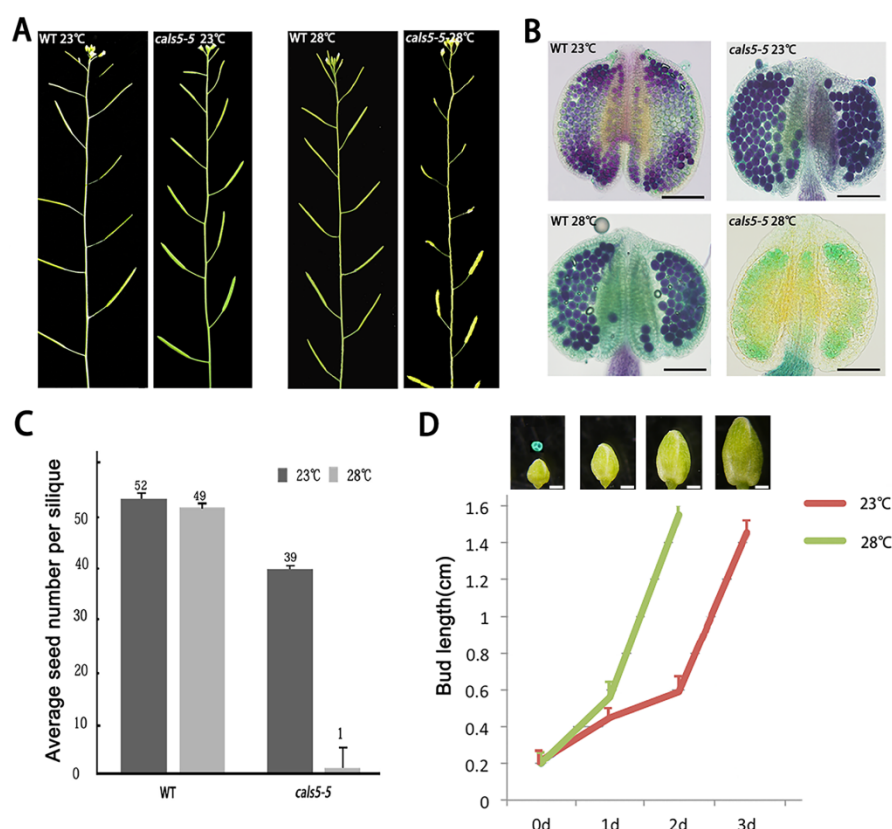
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Figure 4. Fertility of *cals5-5* is significantly reduced under high temperature. (A) Fertility of *cals5-5* was reduced at 28°C, while the fertility of the wild type plants was normal. The images of plants shown in A were digitally extracted for comparison. **(B)** Results from Alexander staining of the pollen grains. A number of pollen grains were observed in the wild type anther but not in the *cals5-5* anther under high temperature. Bars=50μm. **(C)** The seed number per pod in the wild type and *cals5-5* plants growing at 23°C and 28°C (n=12). Error bars represent the SD of seed setting per silique. There was almost no seed in the *cals5-5* siliques under high temperature. **(D)** Growth rate of the pollen from the tetrad to mature stage in wild type plants. The tetrad over the first flower represents that it was at the tetrad stage. Error bars represent the SD of bud length (n=10). The buds developed much quicker at 28°C than at 23°C. Bars=100μm.

215   The expression level of *CalS5* in *cals5-5* was found to be about 54.9% of that of

216   wild-type (Figure S2B). *cals5-5* was fully fertile under normal conditions (Figure

217   4A). In *cals5-5*, pollen production and callose wall formation were similar to those

218   of wild type (Figure 4B; Figure S2D). To find out whether high-temperature could

219   cause male sterility in the mutant, *cals5-5* was placed under high temperature

220   (28°C). The wild type control was fully fertile at both 23°C and 28°C (Figure 4A).

221   However, the fertility of *cals5-5* was severely compromised at 28°C (Figure 4A).

222   The average seed set of the *cals5-5* was significantly decreased (Figure 4C), and

223   few pollen grains were produced in *cals5-5* anthers at 28°C (Figure 4B). It was

224   shown that high temperature may impact the mRNA splicing in the rice *ugp1*-RNAi

225   plants (Chen et al., 2007). The intron of *CalS5* contains a T-DNA insertion in

226   *cals5-5* plants. Thus, we further investigated whether the transcription and splicing

227   of *CalS5* in *cals5-5* had the similar effect to the *ugp1*-RNAi line. We analyzed the

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228   expression level of the full-length mature *CalS5* transcript in wild type and *cals5-5*
229   under normal and high temperature by RT-PCR. Results showed that the expression
230   of *CalS5* was reduced in *cals5-5* compared with in wild type (Figure S2C).
231   However, the *CalS5* transcripts under 28°C was slightly higher than the ones under
232   23°C either in wild type or in *cals5-5*, respectively (Figure S2C). This result
233   indicates that the full-length *CalS5* transcripts in *cals5-5* under high temperature
234   was not reduced compared with that under low temperature. Thus, the sterility in
235   *cals5-5* under high temperature was not due to the temperature sensitive mRNA splicing.
236   As there were no pollen grains in *cals5-5* at 28°C, we analyzed the growth rate of
237   the buds from the tetrad stage to mature pollen grain. It took two days for the buds
238   to grow from 0.2 cm (tetrad stage) to 1.58 cm (mature pollen stage) at 28°C, while
239   it took three days to complete the same process at 23°C (Figure 4D). These results
240   indicate that high temperature accelerates the growth rate of plants which is
241   associated with male sterility in *cals5-5*.

242

243   **SD photoperiod restores fertility of several other TGMS lines**
244   The pollen wall is composed of outer exine (the outer sexine and inner nexine) and
245   inner intine. The development of the exine requires the synthesis of wall materials
246   for sexine and nexine and the assembly of these materials to form a particular
247   pollen wall pattern (sexine pattern) (Xu et al., 2016). ACoS5 and CYP703A2 are
248   responsible for the synthesis of sexine material. The *acos5-2* and *cyp703a2* single
249   mutants are male sterile and have defective pollen wall formation (Morant et al.,
250   2007; de Azevedo Souza et al., 2009). NPU is responsible for the primexine
251   formation. *npu-2* is also male sterile. In *npu-2*, primexine cannot be formed, and
252   pollen wall pattern is defective (Chang et al., 2012). RVMS is responsible for the
253   synthesis of membrane material of microspores. The *rvms* line also showed male
254   sterility and defective pollen development (Zhu et al., 2020). Previous investigation
255   shows that *acos5-2*, *cyp703a2* and *rvms* are all TGMS lines (Zhu et al., 2020). We
256   found *npu-2* is also a TGMS line as its fertility was restored under low temperature
257   (Figure S3). Here, we further investigated their fertility under SD photoperiod. As
258   shown in Figure 5, they exhibited full siliques of seeds under the tested conditions.
259   Results from Alexander staining assay further indicated the production of fertile
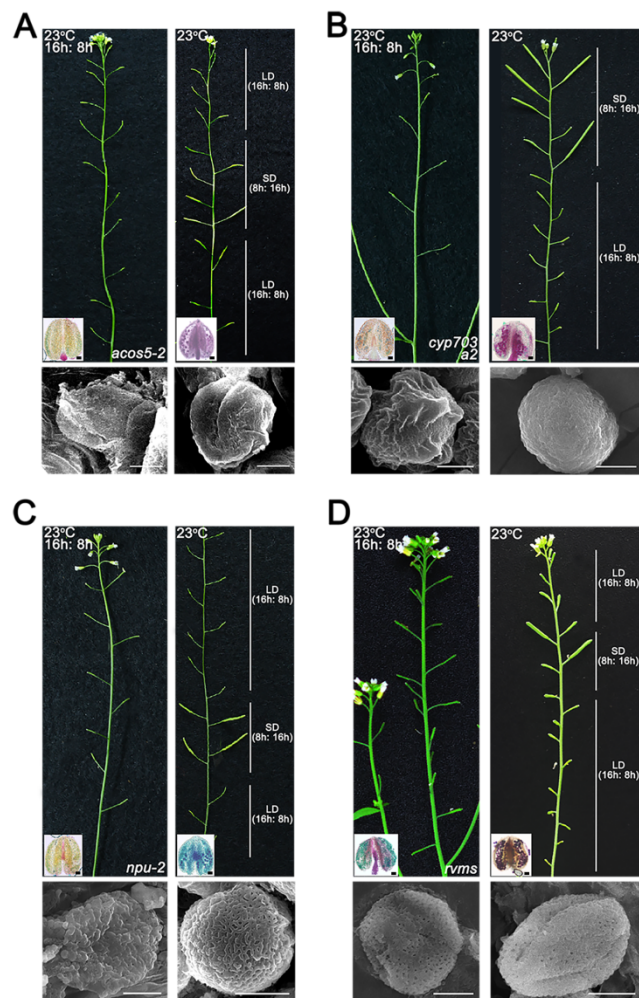260   pollen (Figure 5A-D). Thus these TGMS lines also showed PGMS features that

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Figure 5. Fertility restoration of TGMS lines under SD photoperiod. (**A**) *acos5-2* is male sterile under normal conditions, but its fertility was restored under short photoperiod or low light intensity. SEM observations showed that the defective pollen surface of *acos5-2* remained defective under SD photoperiod or low light intensity by SEM observations. (**B**) *cyp703a2* is male sterile under normal conditions, but its fertility was largely restored under SD photoperiod or low light intensity. (**C**) *npu-2* showed male sterility under normal conditions, but its fertility was significantly restored under SD photoperiod or low light intensity. SEM observations revealed that the pollen surface was restored to a normal reticulated pattern under SD photoperiod or low light intensity. (**D**) *rvms* exhibited male sterility under normal conditions, but was fertile under SD photoperiod or low light intensity. The pollen wall of *rvms* remained rough and irregular under SD photoperiod or low light intensity. Bars for pollen staining=20μm, and bars for SEM=5μm.

261   their fertility is restored under SD photoperiod. This indicates that slow

262   development is a general mechanism for fertility restoration of PGMS lines, and

263   both PGMS and TGMS may commonly function through slowed growth to rescue

264   the fertility. To further understand the pollen wall development of these lines under

265   SD conditions, SEM observations were then performed. We found that the pollen

266   grains of these lines mostly turned round and plump indicating morphological

267   restoration (Figure 5). The pollen surface of the functional *npu-2* pollen grains

268   exhibited regular reticulated pattern under the permissive conditions (Figure 5C).

269   The formation of the primexine was restored in *npu-2* under the permissive

270   condition (Figure S4), indicating the full recovery of the pollen wall pattern.

271   However, the cell wall surface of the pollen grains of *acos5-2* and *cyp703a2*

12

272   remained rough and irregular, similar to what was observed for *cals5-5* (Figure 5A

273   and B). Thus, despite the pollen wall defects in these lines under low temperature,

274   SD photoperiod, and low light intensity, the pollen fertility can be restored through

275   slow development (Figure 1 and 2).

276

277

Discussion

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279   The discovery of a PGMS line in the japonica rice variety NongKen 58S in 1973

280   initiated the research and breeding of two-line hybrid rice in China and led to the

281   discovery and development of TGMS germplasm (Fan and Zhang, 2018). The

282   mechanisms underlying the EGMS fertility recovery remain of great interests to

283   plant scientists and agronomists. Our previous study shows that Arabidopsis *cals5-*

284   *2* is a TGMS line and that slowed development is a general mechanism for fertility

285   restoration of all investigated TGMS lines (Zhu et al., 2020). In the present study,

286   we found that *cals5-2* is also a PGMS line and its fertility restoration under SD

287   photoperiod is associated with slowed development (Figure 1). We show that other

288   TGMS lines, *cyp703a2*, *npu-2,* and *rvms*, also feature PGMS characteristics (Figure

289   5). Furthermore, low light intensity, another environmental factor that results in

290   slowed pollen development, also restored pollen fertility of the P/TGMS line

291   (Figure 2). Thus, we propose that slowed development is the mechanism for the

292   fertility restoration of PGMS, and both TGMS and PGMS lines share the common

293   mechanism for fertility restoration. For most plants, suboptimal conditions

294   including low temperature, low light intensity or SD photoperiod are known to

295   result in slowed growth and development. For instance, we previously established

296   that pollen development was significantly slowed in rice and Brassica under low

297   temperature conditions (Zhu et al., 2020). Reproductive processes like anther

298   development and pollen formation are quite conserved in plants (Wilson and

299   Zhang, 2009; Gomez et al., 2015). It is likely that slowed development is also

300   involved in fertility restoration of rice P/TGMS lines. In rice, PGMS and TGMS are

301   two types of male sterile lines widely used for hybrid breeding. However, results

302   from agriculture practice already indicate the possible interactions between PGMS

303   and TGMS (Fan and Zhang, 2018). For example, photoperiod and temperature were

304   found to act synergistically in PGMS and TGMS lines (He et al., 1987). In addition,

305   the same genetic locus for a noncoding RNA that determines PGMS in *japonica*

306   rice Nongken 58S but TGMS in *indica* rice Peiai64S (Ding et al., 2012; Zhou et al.,

307   2012). All these information support a common mechanism for the fertility

308   restoration shared by both PGMS and TGMS lines.

309   The restoration of fertility in TGMS and PGMS plants depends on the successful

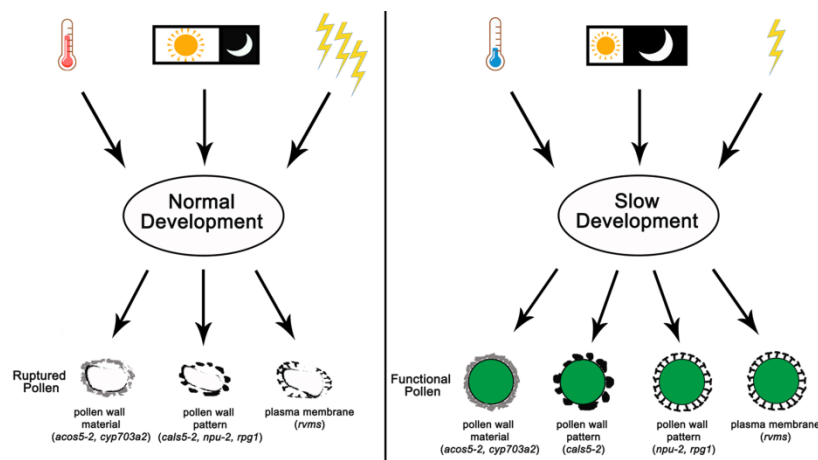310   formation of functional pollen. The genes determining P/TGMS such as *CalS5*,

14

Figure 6. A schematic model for slow development functions in the fertility restoration of different T/PGMS lines. Temperature, photoperiod and light intensity are all crucial for the fertility restoration of T/PGMS lines. Under restrictive conditions, pollen development is rapid, leading to the pollen rupture due to various pollen defects in different T/PGMS lines (left). However, under permissive conditions, pollen development is slowed down, which promotes the functional pollen formation (right). The restored pollen remains morphological defects in some lines or show restored cell wall features in others under all tested conditions, suggesting that multiple molecular mechanisms are involved.

311   *NPU*, *CYP703A2*, *ACoS5* and *RVMS* play different roles in pollen formation. This

312   is reflected by our findings which reveal multiple mechanisms are involved in the

313   recovery of functional pollen (Figure 6). CalS5 is responsible for callose wall

314   formation and ACoS5 and CYP703A2 for synthesis of sexine materials. Under

315   normal conditions, loss of any of these genes results in a defective sexine layer,

316   pollen rupture and male sterility (Dong et al., 2005; Morant et al., 2007; de

317   Azevedo Souza et al., 2009). However, our results suggest that in these P/TGMS

318   lines, permissive conditions relieve the requirement for an intact sexine, thereby

319   restoring fertility (Figure 3 and 5). It indicates that slow development overcomes

320   sexine defect during the formation of fertile pollen, which leads to the fertility

321   restoration. Every plant species has unique and reticular pollen wall pattern. Callose

322   wall and primexine are critical for pollen wall pattern establishment (Xu et al.,

323   2016). In *npu-2* line, primexine is absent and pollen wall pattern is defective

324   (Chang et al., 2012). However, in fertility-restored *npu-2* plant, primexine was

325   observed and its pollen wall pattern was established (Figure S4). Several other

326   genes were reported to be involved in primexine formation (Paxson-Sowders et al.,

327   2001; Ariizumi et al., 2004; Guan et al., 2008; Hu et al., 2014; Li et al., 2017;

328   Suzuki et al., 2017). The slower pace of development may overcome the loss of

329   NPU's function in coordination of these genes to allow primexine formation.

330   Nevertheless, in fertility-restored *cals5-2* plants, primexine remained absent and the

15

331	pollen wall pattern was still defective (Figure 3). Therefore, primexine is critical for
332	pollen wall pattern establishment. *RVMS* encodes a member of the GDSL lipase
333	family that may provide materials for plasma-membrane formation during
334	microspore enlargement. In Arabidopsis, this gene family contains about 108
335	members (Zhu et al., 2020). Under permissive conditions, the remaining RVMS
336	lipase activity in the *rvms* mutant or other members in this family may provide
337	sufficient plasma membrane for slow microspore development. In conclusion, slow
338	development, as a general mechanism, may allow different P/TGMS lines to adopt
339	different strategies to overcome pollen formation defects and to restore fertility
340	under permissive condition.
341	Normal plant growth and reproduction rely on suitable environmental conditions
342	such as certain temperature, photoperiod and light intensity. Based on the responses
343	to environmental factors, EGMS has been divided into four types: PGMS, TGMS,
344	reverse PGMS (rPGMS) and rTGMS (Chen and Liu, 2014; Fan and Zhang, 2018).
345	rPGMS lines show male sterility under SD conditions and normal fertility under LD
346	conditions. rTGMS lines show male sterility under normal temperature and fertility
347	under high temperature. In this work, we showed that light intensity also affected
348	fertility of male sterile lines (Figure 2). Together with previous work (Zhu et al.,
349	2020), we show slow development is the common mechanism for the fertility
350	restoration of TGMS and PGMS lines. The responses of rP/TGMS lines to
351	environmental change are opposite to that of P/TGMS lines. rP/TGMS may have
352	different mechanism to restore their fertility.
353	

354	**Materials and Methods**
355	**Plant growth conditions**
356	Arabidopsis (*Arabidopsis thaliana*) plants, *cals5-2* (Columbia, SALK_026354) and
357	*cals5-5* (Columbia, SALK_072226) were obtained from the Arabidopsis Biological
358	Resource Center (ABRC). Plants were grown on soil under normal conditions (16h
359	light/ 8h dark, 103 μmol m^{-2} s^{-1}, 23°C) in a greenhouse. For low temperature
360	treatments, the plants were grown for five weeks in the same conditions as
361	described above, and then transferred into a low temperature greenhouse (16h light/
362	8h dark, 103 μmol m^{-2} s^{-1}, 18°C). Similarly, for the short-day photoperiod
363	treatment, the plants were first grown for five weeks under normal conditions and

16

364   then were transferred to a short-day photoperiod growth chamber (8h light/ 16h

365   dark, 103 μmol m^{-2} s^{-1}, 23°C) for two weeks. For the low light intensity treatment,

366   after five weeks growth under normal conditions, the plants were transferred into

367   low light intensity conditions (16h light/ 8h dark, 43 μmol m^{-2} s^{-1}, 23°C) for two

368   weeks. For the high temperature treatment, the plants were grown for five weeks

369   under normal temperature and then transferred to a growth chamber with 16h light/

370   8h dark, 103 μmol m^{-2} s^{-1}, at 28°C for seven days.

371

372   **Genetic analysis and expression analysis**

373   To obtain the *cals5-2* and *cals5-5* homozygous mutants, the primers of genetic

374   analysis for locus identification were designed using SIGnALiSect Tools

375   (http://signal.salk.edu/isects.html). PCR primers used to amplify the specific

376   genomic fragments for each allele were: *cals5-2* (CalS5-2-F 5'-

377   TGCTTCTGTGGTGGTCACAGG-3',   CalS5-2-R   5'-

378   GCATACCAAATTTGAGTGTCCAT-3')   and   *cals5-5*   (CalS5-5-F   5'-

379   CCAGATTTCCGGTTTTCTTTC-3',   CalS5-5-R   5'-

380   TGTGGATTTCTCCATCGGTAG-3'). Total RNA was isolated from Arabidopsis

381   Col-0 inflorescences using Trizol reagent (Invitrogen). cDNA was reverse-

382   transcribed from RNA using the SuperScript First-Strand Synthesis System

383   (Invitrogen). The relative expression levels of *CalS5* in the two mutant alleles were

384   assayed by RT-PCR/RT-qPCR using gene-specific primers: CalS5-RT-F, 5'-

385   TTCAGGGAAGGGGATCTGAAAG-3';   CalS5-RT-R,   5'-

386   TCAGAGAAAGTTAGTCTGAGACTT-3';   CalS5qRT3F,   5'-

387   GGGGATGCTATTCCACTTCTTGA-3';   CalS5qRT3R,   5'-

388   CAACTCCCATGATGTACTCATACCC-3'. The RT-PCR program for *CalS5* used

389   was as follows: 94°C for 5 min, (94°C for 15 s, 55°C for 15 s, 72°C for 3 min) ×36

390   cycles, 72°C for 5 min. The qPCR was performed as described (Peng et al., 2019).

391   All reactions were repeated three times with *β-TUBULIN* as the normalizing gene.

392

393   **Pollen viability assay and callose wall and intine staining**

17

394   Pollen grains were collected on a slide. They were stained according to the

395   Alexander staining method (Alexander, 1969), and the photos of the stained pollen

396   grains were taken using an Olympus optical microscope with an Olympus digital

397   camera. Aniline blue staining of callose was performed as described in a previous

398   report (Zhang et al., 2007). The anthers were fixed in Carnoy's fixative for 2h and

399   then the tetrads were separated to a glass slide. The tetrads were stained with 0.1%

400   (m/v) aniline blue. The pictures of callose staining were taken with an Olympus

401   BX-51 microscope (Olympus). T&D staining assay was performed as described in

402   a previous report (Lou et al., 2014). The inflorescence of WT and mutant were

403   embedded into spur resin, and the sections of pollen were put on the surface of the

404   50°C dryer. Then the desiccation of sections was performed, and the sections were

405   stained with toluidine blue for 5 min (10 mg/ml), Tinopal for 15 min (10 mg/ml)

406   (Sigma, USA) and $DiOC_2$ for 5 min (5 mg/ml) (Sigma).

407

408   **SEM and TEM sample preparation and observation**

409   For Scanning Electron Microscopy (SEM) observation, pollen grains were applied

410   by flicking the anthers of mature flowers over mounting tape on a stub. The pollen

411   grains were allowed to air dry for about 30 min, sputter coated with gold, and

412   observed with a JSM-840 microscope (JEOL, Japan). For Transmission Electron

413   Microscopy (TEM) observation, the same-stage inflorescences of WT and mutant

414   were fixed in 0.1 M phosphate buffer (pH 7.2) with 2.5% glutaraldehyde (v/v).

415   After 7 days of fixation, the material was washed thoroughly with phosphate buffer

416   (pH 7.2). After that, the material was gradient dehydrated to 100% ethyl alcohol

417   and the inflorescences were then embedded and polymerization was performed

418   (65°C, 24h). Finally, Ultra-thin sections (70–100 nm thick) were made and

419   observed using a TEM microscope (JEOL, Japan).

420

421   **The measure of pollen growth rate.**

422   The tetrad-stage buds of WT were marked and retained at 8:00 am under the

423   different conditions. The anthers were carefully separated and dissected. The

424   anthers of different stages were respectively fixed in Carnoy's fixative for two

425   hours, and the pollen grains were separated on a glass slide using a needle. The

426   pollen grains were stained in toluidine blue solution, and then observed and

18

427   measured using an Olympus optical microscope. To determine the microspore or

428   pollen diameters, images were captured using a BX51 Olympus microscope and

429   measured using ImageJ software (NIH). The F-test was performed to compare the

430   diameters between normal condition and low light intensity conditions.

431

432   **Accession Numbers**

433   Sequence data from this article can be found in the GenBank/EMBL data libraries

434   under accession numbers CalS5, AT2G13680; NPU, AT3G51610; CYP703A2,

435   AT1G01280; AcoS5, AT1G62940; RVMS1, AT4G10950.

436

437   **Supplemental data**

438   Supplemental Figure S1. Seed production analysis of *cals5-2* under different

439   conditions.

440

441   Supplemental Figure S2. Characterization of two alleles of *cals5*.

442

443   Supplemental Figure S3. Fertility of *npu-2* is restored under low temperature.

444

445   Supplemental Figure S4. Primexine formation and pollen wall pattern are restored

446   in *npu-2* at low temperature.

447

456

457   **Figure Legends**

458   **Figure 1. *cals5-2* is a PGMS line.**

19

459   (**A**) *cals5-2* exhibited severely reduced male fertility under normal conditions. Few

460   pollen grains were produced in *cals5-2* anther. (**B**) The fertility of *cals5-2* was

461   restored under low temperature. (**C**) The fertility of *cals5-2* was restored under SD

462   condition and pollen maturation was recovered. The images of plants shown in A,

463   B and C were digitally extracted for comparison. Bars in A, B and C=20μm. (**D**)

464   Pollen maturation was delayed by 1 day under SD conditions compared with LD

465   conditions. (**E**) Diameters of microspores at different developmental stages. 20

466   microspores or pollen grains were used to measure the diameter at each

467   developmental stage, respectively. One-way ANOVA was performed to compare

468   the diameter of microspores. * $P<0.05$; ** $P<0.001$; ns, not significant ($P>0.05$).

469   Error bars represent the standard deviation (SD) of pollen diameters. The diameter

470   of microspores at 48h and 96h under a SD photoperiod was significantly reduced

471   compared to a LD photoperiod.

472

473   **Figure 2. Fertility of *cals5-2* is restored under low light intensity.**

474   (**A**) *cals5-2* showed severe male sterility under normal conditions. Pollen

475   production was abnormal in *cals5-2* anther. (**B**) The fertility of *cals5-2* was restored

476   under low light intensity. Pollen grains in *cals5-2* were produced under low light

477   intensity. Bars in A and B=20μm. (**C**) Pollen growth, from tetrad to mature pollen,

478   was delayed by one day delayed under low light intensity (n=12). (**D**) Diameter of

479   microspores at different developmental stages. 20 microspores or pollen grains

480   were used to measure the diameter at each developmental stage, respectively. One-

481   way ANOVA was performed to compare the diameter of microspores. * $P<0.05$; **

482    $P<0.001$; ns, not significant ($P>0.05$). Error bars represent the SD of pollen

483   diameters. The diameter of microspores at 48h and 96h was significantly smaller

484   under low light intensity compared to normal conditions.

485

486   **Figure 3. Fertility-restored *cals5-2* line exhibits pollen wall defects but**

487   **recovered pollen wall integrity.**

488   (**A–C**) Results from aniline blue staining of the callose wall of tetrads. Callose

489   deposition was not recovered in *cals5-2* under low temperature or SD conditions.

490   Bars=10μm. The scale bar in A also represents the ones in B and C. (**D**) SEM

491   observation of the mature pollen of *cals5-2* plants. The pollen surface remained

20

492   rough and irregular under low temperature or SD conditions. (**E**) Tinopal and
493   DIOC2 staining of *cals5-2* anther and pollen grains. The intine of *cals5-2* pollen
494   was intact under low temperature or SD conditions. Cp, Cytoplasm; Sp,
495   Sporopollenin; In, Intine. Bars=20μm. (**F–I**) TEM images of wild type pollen from
496   the early tetrad stage to mature pollen stage at 23°C. (**J–M**) TEM images of *cals5-2*
497   pollen from the early tetrad stage to mature pollen stage under 23°C. The callose
498   wall and primexine were not observed around microspores in tetrads. The nexine
499   was discontinuous, and intine formation was disrupted at 23°C. (**N–Q**) TEM
500   images of *cals5-2* pollen from the early tetrad stage to mature pollen stage at 18°C.
501   The callose wall and primexine were not restored, but the integrity of nexine and
502   intine was much better maintained than those under normal temperature (23°C). Ca,
503   Callose wall; TW, Tetrad Wall; Pb, Probacular; Pe, primexine; Pm, Plasma
504   membrane; Pe-like, Primexine-like materials; In, Intine; Se, Sexine; Ne, Nexine;
505   Cp, Cytoplasm.

506

507   **Figure 4. Fertility of *cals5-5* is significantly reduced under high temperature.**
508   (**A**) Fertility of *cals5-5* was reduced at 28°C, while the fertility of the wild type
509   plants was normal. The images of plants shown in A were digitally extracted for
510   comparison. (**B**) Results from Alexander staining of the pollen grains. A number of
511   pollen grains were observed in the wild type anther but not in the *cals5-5* anther
512   under high temperature. Bars=50μm. (**C**) The seed number per pod in the wild type
513   and *cals5-5* plants growing at 23°C and 28°C (n=12). Error bars represent the SD of
514   seed setting per silique. There was almost no seed in the *cals5-5* siliques under high
515   temperature. (**D**) Growth rate of the pollen from the tetrad to mature stage in wild
516   type plants. The tetrad over the first flower bud represents that it was at the tetrad
517   stage. Error bars represent the SD of bud length (n=10). The buds developed much
518   quicker at 28°C than at 23°C. Bars=100μm.

519

520   **Figure 5. Fertility restoration of TGMS lines under SD photoperiod.**
521   (**A**) *acos5-2* is male sterile under normal conditions, but its fertility was restored
522   under short photoperiod or low light intensity. SEM observations showed that the
523   defective pollen surface of *acos5-2* remained defective under SD photoperiod or
524   low light intensity by SEM observations. (**B**) *cyp703a2* is male sterile under normal

conditions, but its fertility was largely restored under SD photoperiod or low light intensity. (**C**) *npu-2* showed male sterility under normal conditions, but its fertility was significantly restored under SD photoperiod or low light intensity. SEM observations revealed that the pollen surface was restored to a normal reticulated pattern under SD photoperiod or low light intensity. (**D**) *rvms* exhibited male sterility under normal conditions, but was fertile under SD photoperiod or low light intensity. The pollen wall of *rvms* remained rough and irregular under SD photoperiod or low light intensity. Bars for pollen staining=20μm, and bars for SEM=5μm.

Figure 6. A schematic model for slow development functions in the fertility restoration of different T/PGMS lines.

Temperature, photoperiod and light intensity are all crucial for the fertility restoration of T/PGMS lines. Under restrictive conditions, pollen development is rapid, leading to the pollen rupture due to various pollen defects in different T/PGMS lines (left). However, under permissive conditions, pollen development is slowed down, which promotes the functional pollen formation (right). The restored pollen remains morphological defects in some lines or show restored cell wall features in others under all tested conditions, suggesting that multiple molecular mechanisms are involved.

22

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