Slow development restores the fertility of photoperiod-sensitive male-sterile plant lines

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One-sentence summary: Slow development is a general mechanism for Photoperiod-sensitive Genic Male Sterility.

Short title: Slow development restores fertility of PGMS lines

Author Contributions
Z.-N.Y. conceived and designed the experiment with C.Z. and T.X.. C.Z., T.X. and M.-Y.R. performed the observation of fertility conversion of the lines under short-photoperiod and low light intensity. C.Z., M.-Y.R., J.Z. and Q.-S.S measured the plant growth rate under short-day photoperiod. T.X., Y.W.Q and Y.F.Z. performed the cytological observations of cal5s-2 under different conditions. T.X., M.J.H. and L.S. performed the fertility analysis and plant growth rate measurement of cal5s-2 under high temperature. Z.N.Y., P.X. and C.Z. wrote the paper.

Abstract
Photoperiod- and thermo-sensitive genic male sterility (P/TGMS) lines are widely used in crop breeding. The fertility conversion of Arabidopsis thaliana TGMS lines including cal5s-2, which is defective in callose wall formation, relies on slow development under low temperatures. In this study, we discovered that cal5s-2 also exhibits PGMS. Fertility of cal5s-2 was restored when pollen development was slowed under short-day photoperiods or low light intensity, suggesting that slow
development restores the fertility of *cals5-2* under these conditions. We found that several other TGMS lines with defects in pollen wall formation also exhibited PGMS characteristics. This similarity indicates that slow development is a general mechanism of PGMS fertility restoration. Notably, slow development also underlies the fertility recovery of TGMS lines. Further analysis revealed the pollen wall features during the formation of functional pollens of these P/TGMS lines under permissive conditions. We conclude that slow development is a general mechanism for fertility restoration of P/TGMS lines and allows these plants to take different strategies to overcome pollen formation defects.

**Introduction**

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

Much progress about TGMS mechanism has been made in recent years. A number of TGMS loci have been identified in rice. In AnngongS-1, a pre-mature stop codon is introduced in the *RNase Zsi* gene (Zhou et al., 2014). The genome of HengnongS-1 contains a mutation in the homolog of Arabidopsis *MALE STERILITY1 (MS1)* (Qi et al., 2014). An artificial TGMS rice line was created by silencing a UGPase (Chen et al., 2007). In addition, TGMS in *indica* rice Peai64S is determined by the mutation of the genetic locus for a long noncoding RNA (*PMS1T and PMS3*) (Zhou et al., 2012). Similar to rice, several TGMS loci have been identified in Arabidopsis, including the *PLANT U-BOX 4 (PUB4)*, *MYB33*, and *MYB65* (Millar and Gubler, 2005). Recently, a new TGMS locus,
**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.
Further analysis revealed that slow development was responsible for fertility restoration in these PGMS lines, revealing a shared mechanism for PGMS and TGMS.
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,
and their sizes were 17.0 μm to 19.3 μm after two and four days’ growth, respectively (Figure 1E). These results demonstrated that SD photoperiod significantly slowed microspore development. A similar delay in microspore development was observed under the low temperature conditions which can restore the fertility of cals5-2 and other TGMS lines (Zhu et al., 2020). Therefore, both low temperatures and SD photoperiod restore the fertility of cals5-2 plants through slowing microspore development.

Low light intensity restores the fertility of cals5-2.

In addition to photoperiod, light intensity is another important factor that affects light signaling and general energy production. Thus, we further investigated whether the fertility of cals5-2 can be restored by low light intensity. We placed cals5-2 in the growth chamber with low light intensity (45 μmol m⁻² s⁻¹) for ten days. The control cals5-2 plants were grown under the normal conditions with light intensity of 103 μmol m⁻² s⁻¹. Under low light intensity, seeds were produced normally (Figure 2A and Figure S1) and fertile pollen grains were observed in cals5-2 (Figure 2B). We further analyzed the pollen development process under different light intensity. Pollen maturation under normal conditions and under low light intensity took about three and four days, respectively (Figure 2C). By measuring microspore diameters at two and four days after release from tetrad, we found the microspores were significantly smaller at both time points under the low light intensity condition (Figure 2D). Therefore, low intensity of light also slowed
microspore development. Taken together, low light intensity restores *cals5-2* fertility through slowing its microspore growth, which is the same as the low temperature and SD photoperiod.

**Slow development overcomes sexine defects and restores the formation of functional pollen in *cals5-2*.**

After meiosis, all four microspores are enclosed in callose wall. Primexine, formed between the callose wall and microspore plasma membrane, is responsible for pollen wall pattern establishment (Xu et al., 2016). *CalS5* encodes a callose synthase involved in callose wall formation. Peripheral callose wall is absent in *cals5-2* which leads to defective exine pattern formation and male sterility under normal conditions (Dong et al., 2005). We used the callose stain aniline blue to reveal the wall features of tetrad in fertility restored plants. The peripheral callose
of the fertile *cals5-2* tetrads was found to be absent, similar to what was observed in normal (sterile) conditions (Figure 3A–C). We further observed microspore development of fertile and sterile *cals5-2* plants using a Scanning Electronic Microscope (SEM). The pollen surface of the microspore was rough and abnormal in the fertility restored plants of *cals5-2*, again similar to *cals5-2* male sterile plants under normal conditions (Figure 3D). Thus, even though the pollen grains became fertile in the restored plants under SD photoperiod and low temperatures, their callose wall and pollen wall remained defective. Furthermore, we stained the pollen wall with Tinopal and DiOC2. The intine can be stained purple by Tinopal, and the lipid contents and sexine of the mature pollen grain can...
be stained red by DIOC$_2$ (Gu et al., 2014). The intine layer and lipid contents were not visible in pollen grains of cals5-2 plants under normal (sterile) conditions (Figure 3E). In contrast, in the fertile cals5-2 plants, the intine layer and lipid contents were clearly visible in the fertile cals5-2 pollen grains under SD photoperiod or low temperature (Figure 3E). This indicates that cals5-2 microspore could overcome pollen wall pattern defect to further develop into mature functional pollen under these permissive conditions.

During anther development, the pollen wall materials from the tapetum deposit outside of the microspore to form a wall with a reticular pattern. Both callose wall and primexine are important for pollen wall establishment (Xu et al., 2016). TEM observation revealed that the primexine remained absent in cals5-2 tetrads under low temperature (Figure 3N), but some electron dense (pe-like) materials were visible between the plasma membrane and the thin wall (TW; Figure 3N and O). Globular sporopollenin was randomly deposited on the plasma membrane at the late tetrad stage under both low temperature and normal conditions (Figure 3J-M, P and Q). Thus, callose wall is essential for primexine formation. The defective callose wall and primexine are in agreement with defective pollen wall pattern in the restored cals5-2 plant under low temperature. Under normal conditions, the cals5-2 nexine layer was discontinuous (Figure 3L). However, under low temperature, the nexine layer was continuous and intact at the microspore stage (Figure 3P) and the intine layer also exhibited good integrity at the mature pollen stage (Figure 3Q). These results show that the formation of functional mature pollen in the fertility restored cals5-2 is associated with restoration of nexine and intine layers, while the outer sexine wall remained defective. Slowed development may therefore overcome certain pollen wall pattern defects to support its further development into functional pollen.

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

Our results suggest that slowed development led to the fertility restoration of P/TGMS lines under permissive conditions (Figure 1 and 2). To further test this hypothesis, we investigated whether faster pollen development could affect the fertility of P/TGMS lines. cals5 has several alleles, and cals5-5 is a weak allele with a T-DNA insertion in the third intron (Nishikawa et al., 2005; Figure S2A).
The expression level of CalS5 in cals5-5 was found to be about 54.9% of that of wild-type (Figure S2B). cals5-5 was fully fertile under normal conditions (Figure 4A). In cals5-5, pollen production and callose wall formation were similar to those of wild type (Figure 4B; Figure S2D). To find out whether high-temperature could cause male sterility in the mutant, cals5-5 was placed under high temperature (28°C). The wild type control was fully fertile at both 23°C and 28°C (Figure 4A). However, the fertility of cals5-5 was severely compromised at 28°C (Figure 4A). The average seed set of the cals5-5 was significantly decreased (Figure 4C), and few pollen grains were produced in cals5-5 anthers at 28°C (Figure 4B). It was shown that high temperature may impact the mRNA splicing in the rice ugp1-RNAi plants (Chen et al., 2007). The intron of CalS5 contains a T-DNA insertion in cals5-5 plants. Thus, we further investigated whether the transcription and splicing of CalS5 in cals5-5 had the similar effect to the ugp1-RNAi line. We analyzed the
expression level of the full-length mature CalS5 transcript in wild type and cals5-5 under normal and high temperature by RT-PCR. Results showed that the expression of CalS5 was reduced in cals5-5 compared with in wild type (Figure S2C). However, the CalS5 transcripts under 28°C was slightly higher than the ones under 23°C either in wild type or in cals5-5, respectively (Figure S2C). This result indicates that the full-length CalS5 transcripts in cals5-5 under high temperature was not reduced compared with that under low temperature. Thus, the sterility in cals5-5 under high temperature was not due to the temperature sensitive mRNA splicing. As there were no pollen grains in cals5-5 at 28°C, we analyzed the growth rate of the buds from the tetrad stage to mature pollen grain. It took two days for the buds to grow from 0.2 cm (tetrad stage) to 1.58 cm (mature pollen stage) at 28°C, while it took three days to complete the same process at 23°C (Figure 4D). These results indicate that high temperature accelerates the growth rate of plants which is associated with male sterility in cals5-5.

SD photoperiod restores fertility of several other TGMS lines

The pollen wall is composed of outer exine (the outer sexine and inner nexine) and inner intine. The development of the exine requires the synthesis of wall materials for sexine and nexine and the assembly of these materials to form a particular pollen wall pattern (sexine pattern) (Xu et al., 2016). ACoS5 and CYP703A2 are responsible for the synthesis of sexine material. The acos5-2 and cyp703a2 single mutants are male sterile and have defective pollen wall formation (Morant et al., 2007; de Azevedo Souza et al., 2009). NPU is responsible for the primexine formation. npu-2 is also male sterile. In npu-2, primexine cannot be formed, and pollen wall pattern is defective (Chang et al., 2012). RVMS is responsible for the synthesis of membrane material of microspores. The rvms line also showed male sterility and defective pollen development (Zhu et al., 2020). Previous investigation shows that acos5-2, cyp703a2 and rvms are all TGMS lines (Zhu et al., 2020). We found npu-2 is also a TGMS line as its fertility was restored under low temperature (Figure S3). Here, we further investigated their fertility under SD photoperiod. As shown in Figure 5, they exhibited full siliques of seeds under the tested conditions. Results from Alexander staining assay further indicated the production of fertile pollen (Figure 5A-D). Thus these TGMS lines also showed PGMS features that
their fertility is restored under SD photoperiod. This indicates that slow development is a general mechanism for fertility restoration of PGMS lines, and both PGMS and TGMS may commonly function through slowed growth to rescue the fertility. To further understand the pollen wall development of these lines under SD conditions, SEM observations were then performed. We found that the pollen grains of these lines mostly turned round and plump indicating morphological restoration (Figure 5). The pollen surface of the functional npu-2 pollen grains exhibited regular reticulated pattern under the permissive conditions (Figure 5C). The formation of the primexine was restored in npu-2 under the permissive condition (Figure S4), indicating the full recovery of the pollen wall pattern. However, the cell wall surface of the pollen grains of acos5-2 and cyp703a2
remained rough and irregular, similar to what was observed for *cals5-5* (Figure 5A and B). Thus, despite the pollen wall defects in these lines under low temperature, SD photoperiod, and low light intensity, the pollen fertility can be restored through slow development (Figure 1 and 2).
Discussion

The discovery of a PGMS line in the japonica rice variety NongKen 58S in 1973 initiated the research and breeding of two-line hybrid rice in China and led to the discovery and development of TGMS germplasm (Fan and Zhang, 2018). The mechanisms underlying the EGMS fertility recovery remain of great interests to plant scientists and agronomists. Our previous study shows that Arabidopsis *cals5-2* is a TGMS line and that slowed development is a general mechanism for fertility restoration of all investigated TGMS lines (Zhu et al., 2020). In the present study, we found that *cals5-2* is also a PGMS line and its fertility restoration under SD photoperiod is associated with slowed development (Figure 1). We show that other TGMS lines, *cyp703a2*, *npu-2*, and *rvms*, also feature PGMS characteristics (Figure 5). Furthermore, low light intensity, another environmental factor that results in slowed pollen development, also restored pollen fertility of the P/TGMS line (Figure 2). Thus, we propose that slowed development is the mechanism for the fertility restoration of PGMS, and both TGMS and PGMS lines share the common mechanism for fertility restoration. For most plants, suboptimal conditions including low temperature, low light intensity or SD photoperiod are known to result in slowed growth and development. For instance, we previously established that pollen development was significantly slowed in rice and Brassica under low temperature conditions (Zhu et al., 2020). Reproductive processes like anther development and pollen formation are quite conserved in plants (Wilson and Zhang, 2009; Gomez et al., 2015). It is likely that slowed development is also involved in fertility restoration of rice P/TGMS lines. In rice, PGMS and TGMS are two types of male sterile lines widely used for hybrid breeding. However, results from agriculture practice already indicate the possible interactions between PGMS and TGMS (Fan and Zhang, 2018). For example, photoperiod and temperature were found to act synergistically in PGMS and TGMS lines (He et al., 1987). In addition, the same genetic locus for a noncoding RNA that determines PGMS in *japonica* rice Nongken 58S but TGMS in *indica* rice Peiai64S (Ding et al., 2012; Zhou et al., 2012). All these information support a common mechanism for the fertility restoration shared by both PGMS and TGMS lines.

The restoration of fertility in TGMS and PGMS plants depends on the successful formation of functional pollen. The genes determining P/TGMS such as *CalS5*,
NPUS, CYP703A2, ACoS5 and RVMS play different roles in pollen formation. This is reflected by our findings which reveal multiple mechanisms are involved in the recovery of functional pollen (Figure 6). CalS5 is responsible for callose wall formation and ACoS5 and CYP703A2 for synthesis of sexine materials. Under normal conditions, loss of any of these genes results in a defective sexine layer, pollen rupture and male sterility (Dong et al., 2005; Morant et al., 2007; de Azevedo Souza et al., 2009). However, our results suggest that in these P/TGMS lines, permissive conditions relieve the requirement for an intact sexine, thereby restoring fertility (Figure 3 and 5). It indicates that slow development overcomes sexine defect during the formation of fertile pollen, which leads to the fertility restoration. Every plant species has unique and reticular pollen wall pattern. Callose wall and primexine are critical for pollen wall pattern establishment (Xu et al., 2016). In npu-2 line, primexine is absent and pollen wall pattern is defective (Chang et al., 2012). However, in fertility-restored npu-2 plant, primexine was observed and its pollen wall pattern was established (Figure S4). Several other genes were reported to be involved in primexine formation (Paxson-Sowders et al., 2001; Ariizumi et al., 2004; Guan et al., 2008; Hu et al., 2014; Li et al., 2017; Suzuki et al., 2017). The slower pace of development may overcome the loss of NPU’s function in coordination of these genes to allow primexine formation. Nevertheless, in fertility-restored cals5-2 plants, primexine remained absent and the

**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.
pollen wall pattern was still defective (Figure 3). Therefore, primexine is critical for pollen wall pattern establishment. RVMS encodes a member of the GDSL lipase family that may provide materials for plasma-membrane formation during microspore enlargement. In Arabidopsis, this gene family contains about 108 members (Zhu et al., 2020). Under permissive conditions, the remaining RVMS lipase activity in the *rvms* mutant or other members in this family may provide sufficient plasma membrane for slow microspore development. In conclusion, slow development, as a general mechanism, may allow different P/TGMS lines to adopt different strategies to overcome pollen formation defects and to restore fertility under permissive condition.

Normal plant growth and reproduction rely on suitable environmental conditions such as certain temperature, photoperiod and light intensity. Based on the responses to environmental factors, EGMS has been divided into four types: PGMS, TGMS, reverse PGMS (rPGMS) and rTGMS (Chen and Liu, 2014; Fan and Zhang, 2018). rPGMS lines show male sterility under SD conditions and normal fertility under LD conditions. rTGMS lines show male sterility under normal temperature and fertility under high temperature. In this work, we showed that light intensity also affected fertility of male sterile lines (Figure 2). Together with previous work (Zhu et al., 2020), we show slow development is the common mechanism for the fertility restoration of TGMS and PGMS lines. The responses of rP/TGMS lines to environmental change are opposite to that of P/TGMS lines. rP/TGMS may have different mechanism to restore their fertility.

**Materials and Methods**

**Plant growth conditions**

Arabidopsis (*Arabidopsis thaliana*) plants, *cals5-2* (Columbia, SALK_026354) and *cals5-5* (Columbia, SALK_072226) were obtained from the Arabidopsis Biological Resource Center (ABRC). Plants were grown on soil under normal conditions (16h light/ 8h dark, 103 μmol m⁻² s⁻¹, 23°C) in a greenhouse. For low temperature treatments, the plants were grown for five weeks in the same conditions as described above, and then transferred into a low temperature greenhouse (16h light/ 8h dark, 103 μmol m⁻² s⁻¹, 18°C). Similarly, for the short-day photoperiod treatment, the plants were first grown for five weeks under normal conditions and
then were transferred to a short-day photoperiod growth chamber (8h light/ 16h
dark, 103 μmol m⁻² s⁻¹, 23°C) for two weeks. For the low light intensity treatment,
after five weeks growth under normal conditions, the plants were transferred into
low light intensity conditions (16h light/ 8h dark, 43 μmol m⁻² s⁻¹, 23°C) for two
weeks. For the high temperature treatment, the plants were grown for five weeks
under normal temperature and then transferred to a growth chamber with 16h light/
8h dark, 103 μmol m⁻² s⁻¹, at 28°C for seven days.

**Genetic analysis and expression analysis**

To obtain the *cals5-2* and *cals5-5* homozygous mutants, the primers of genetic
analysis for locus identification were designed using SIGnALiSe ct Tools
(http://signal.salk.edu/isects.html). PCR primers used to amplify the specific
genomic fragments for each allele were: *cals5-2* (CalS5-2-F 5’-TGCTTCTGTGGTGCTCACAGG-3’, CalS5-2-R 5’-GCATACAAATTTGAGTGTCCAT-3’) and *cals5-5* (CalS5-5-F 5’-CCAGATTTCCGGTTTTCTTTC-3’, CalS5-5-R 5’-TGTGGATTTCTCCATCGGTAG-3’). Total RNA was isolated from Arabidopsis
Col-0 inflorescences using Trizol reagent (Invitrogen). cDNA was reverse-
transcribed from RNA using the SuperScript First-Strand Synthesis System
(Invitrogen). The relative expression levels of CalS5 in the two mutant alleles were
assayed by RT-PCR/RT-qPCR using gene-specific primers: CalS5-RT-F, 5’-TTCAGGGAAGGGGATCTGAAGG-3’; CalS5-RT-R, 5’-TCAGAGAAAGTTAGTCTGAGACTT-3’; CalS5qRT3F, 5’-GGGGATGCTATTCCACTTCTTGA-3’; CalS5qRT3R, 5’-CAACTCCCATGATGTACTCATACCC-3’. The RT-PCR program for CalS5 used
was as follows: 94°C for 5 min, (94°C for 15 s, 55°C for 15 s, 72°C for 3 min) ×36
cycles, 72°C for 5 min. The qPCR was performed as described (Peng et al., 2019).
All reactions were repeated three times with β-TUBULIN as the normalizing gene.

**Pollen viability assay and callose wall and intine staining**
Pollen grains were collected on a slide. They were stained according to the Alexander staining method (Alexander, 1969), and the photos of the stained pollen grains were taken using an Olympus optical microscope with an Olympus digital camera. Aniline blue staining of callose was performed as described in a previous report (Zhang et al., 2007). The anthers were fixed in Carnoy’s fixative for 2h and then the tetrads were separated to a glass slide. The tetrads were stained with 0.1% (m/v) aniline blue. The pictures of callose staining were taken with an Olympus BX-51 microscope (Olympus). T&D staining assay was performed as described in a previous report (Lou et al., 2014). The inflorescence of WT and mutant were embedded into spur resin, and the sections of pollen were put on the surface of the 50°C dryer. Then the desiccation of sections was performed, and the sections were stained with toluidine blue for 5 min (10 mg/ml), Tinopal for 15 min (10 mg/ml) (Sigma, USA) and DiOC2 for 5 min (5 mg/ml) (Sigma).

**SEM and TEM sample preparation and observation**

For Scanning Electron Microscopy (SEM) observation, pollen grains were applied by flicking the anthers of mature flowers over mounting tape on a stub. The pollen grains were allowed to air dry for about 30 min, sputter coated with gold, and observed with a JSM-840 microscope (JEOL, Japan). For Transmission Electron Microscopy (TEM) observation, the same-stage inflorescences of WT and mutant were fixed in 0.1 M phosphate buffer (pH 7.2) with 2.5% glutaraldehyde (v/v). After 7 days of fixation, the material was washed thoroughly with phosphate buffer (pH 7.2). After that, the material was gradient dehydrated to 100% ethyl alcohol and the inflorescences were then embedded and polymerization was performed (65°C, 24h). Finally, Ultra-thin sections (70–100 nm thick) were made and observed using a TEM microscope (JEOL, Japan).

**The measure of pollen growth rate.**

The tetrad-stage buds of WT were marked and retained at 8:00 am under the different conditions. The anthers were carefully separated and dissected. The anthers of different stages were respectively fixed in Carnoy’s fixative for two hours, and the pollen grains were separated on a glass slide using a needle. The pollen grains were stained in toluidine blue solution, and then observed and
measured using an Olympus optical microscope. To determine the microspore or pollen diameters, images were captured using a BX51 Olympus microscope and measured using ImageJ software (NIH). The F-test was performed to compare the diameters between normal condition and low light intensity conditions.

**Accession Numbers**
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers CalS5, AT2G13680; NPU, AT3G51610; CYP703A2, AT1G01280; AcoS5, AT1G62940; RVMS1, AT4G10950.

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

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

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

Supplemental Figure S4. Primexine formation and pollen wall pattern are restored in *npu*-2 at low temperature.

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**Figure Legends**
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.

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.

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 ones 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.

**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 bud 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.

**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.

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.
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