BcMF27, a Pectin Methylesterase Gene, Regulates Pollen Development And Pollen Tube Growth in *Brassica Campestris*

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Research Article

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Abstract

Functional pollen grains are an essential ingredient of successful reproduction in flowering plants and are protected by outer walls. Pectin methylesterases (PMEs) modify pectin, a structural component of pollen intine. However, there are few studies on PMEs. Artificial microRNA (amiRNA) and overexpression technology was performed to investigate the function of pollen-specific PME gene, *BcMF27*, in pollen development. Knockdown of *BcMF27* led to pollen wall collapse, 20% of which unknown material adhered to. Wall-collapsed pollen had abnormally thick intine outside of the germinal furrows. A portion of the cytoplasm was degraded in the remaining pollen with unknown material on the wall, in addition to a thick intine. Overexpression of *BcMF27* resulted in 66.67% pollen wall disruption, causing an abnormally thick intine. In addition, functional interruption of *BcMF27* gave rise to pollen tubes twisted in vitro. Taken together, *BcMF27* contributes to the intine morphogenesis during pollen development and stabilizes pollen tube elongation. This research can promote knowledge of PMEs function and the molecular mechanism in pollen wall construction.

Key Message

Unbalanced expression of *BcMF27* induced pollen morphology defects and pollen tubes twist. Therefore, *BcMF27* contributes to pollen construction and maintains pollen tube stability.

Introduction

Pollen wall is the outer structure of pollen grain, protecting the male gametophyte from physical and biological stress. The pollen wall plays an important role in pollen development, the key procedure of the angiosperm life cycle (Zhang et al. 2016). The elaborately organized pollen wall consists of the exine and intine. The major component of the exine is sporopollenin. The intine comprises pectin, structural proteins, and microfibrillar cellulose (Huang et al. 2009). Pectin is a structurally covalently linked hetero-polysaccharide (Mohnen 2008). PMEs (EC 3.1.1.11) catalyze demethylation of HG at the C-6 to release the free negatively charged carboxyl group and reduce the degree of methylesterification (DM) (Pelloux et al. 2007). De-esterified pectin molecules link to each other by calcium ions to form an “egg-box” super-molecular gel (Yoneda et al. 2010). The pectic gel is then integrated into the wall to strengthen wall rigidity (Roger et al. 2001). Therefore, the demethylation of pectin by PMEs affects pollen development by participating in wall construction.

PMEs exist ubiquitously in the whole plant life cycle (Pelloux et al. 2007) and have important biological significance in plant growth and development, including pollen development, cell elongation, stem morphogenesis, cell adhesion and separation, seed coat mucilage extrusion, abiotic stress, and biological stress (Micheli 2001; Hongo et al. 2012; Sénéchal et al. 2014; Levesque-Tremblay et al. 2015; Turbant et al. 2016; Yue et al. 2018a). PMEs belong to a large gene family (Markovič and Janeček 2004), and some PME genes are expressed specifically in pollen grains, for example, *NtPPME1* from *Nicotiana tabacum* (Bosch and Hepler 2006; Wang et al. 2013) and *AtPPME1* and *VGD1* from *Arabidopsis thaliana* (Jiang

2005; Tian et al. 2006; Chen and Ye 2007), which have been confirmed to affect pollen tube growth. Moreover, Francis et al. (2006) observed that *QRT1* was involved in the separation of tetrad. *AtPME48* regulated pollen germination (Leroux et al. 2015). *BcMF23a* contributed to pollen development and pollen tube growth (Yue et al. 2018a). *BcPME37c* influenced pollen intine formation (Xiong et al. 2019). However, studies on the biological function of PMEs, particularly in pollen development, still remain limited.

Previously, *BcMF27* from *B. campestris* was identified as a putative pectin methylesterase gene. *BcMF27* was expressed significantly in mature pollen and pollinated pistils (Yue et al. 2018b). In this study, artificial microRNA (amiRNA) and overexpression technology was used to further determine the biological function of *BcMF27*. Knockdown and overexpression of *BcMF27* led to intine formation outside of the germinal furrows, pollen morphology abortion, and pollen tube twisting. According to these results, it is suggested that *BcMF27* is necessary for pollen development and pollen tube growth by participating in pollen intine and pollen tube wall, respectively.

Materials And Methods

Construction of vectors and plant transformation

The amiRNA sequence was designed according to the procedure in the Web MicroRNA Designer (WMD3–Web MicroRNA Designer; 5'-TAAGCAACATACACTGCGCGA-3'), and miR164a was used as the gene backbone. The incorporated sequence was integrated into the binary vector pCAMBIA1301 between *Xba*I and *Hind*III with a constitutive CaMV35S promoter. The cDNA sequence of *BcMF27* (Yue et al. 2018b) was cloned and inserted into the same vector between the restriction sites. Then, amiRNA, overexpression, and empty vectors were induced in *B. campestris* ssp. *chinensis* var. *parachinensis* by *Agrobacterium tumefaciens* to obtain transgenic lines according to Yu et al. (2004). Transgenic and control plants were cultivated in an illumination incubator at 22°C with a photoperiod of 16 h light/8 h dark.

Detection of positive transgenic plants

To identify the transformed lines, PCR was performed with genomic DNA extracted from fresh young leaves using the primers 5'-CCAGGCTTTACACTTTATGC-3' and 5'-GCGATTAAGTTGGGTAACGC-3'. Total RNA of the whole inflorescence from positive plants was extracted by the Trizol® reagent (Invitrogen, Carlsbad, CA, USA) and then used to synthesize the first-strand cDNA with the PrimerScript RT reagent kit (TaKaRa, Japan). Real-time RT-PCR was performed with the SYBR Premix Ex Taq Kit (TaKaRa, Japan) using a Bio-Rad CFX96 Real-time PCR Detection System (Bio-Rad, USA). UBC10 was used as a control. The primer sets were as follows: BcMF27F 5'-ATGGCGTTTCAGGATTTCGAC AA-3' and BcMF27R 5'-TCACGCATCATAAAGACCAAGC-3'; and UBC10F 5'-GGGTCCT ACAGACAGTCCTTAC-3' and UBC10R 5'-ATGGAACACCTTCGTCCTAAA-3'. Three biological repeats were performed. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression levels of *BcMF27* (Livak and Schmittgen 2001).

Pollen microscopy observation

Scanning electron microscopy (SEM) of mature pollen and transmission electron microscopy (TEM) of pollen during pollen development were performed according to Lin et al. (2014). Semi-thin anther sections (2 μm) from anthers collected during pollen development were stained by dimethyl blue and photographed with a fluorescent microscope (Leica, Germany). The pollen abortion percentage was analyzed.

Pollen germination in vitro

Pollen germination in vitro was performed according to Lin et al. (2014). Pollen tubes were observed and photographed with a fluorescent microscope. The rate of pollen germination and percentage of abnormal pollen tubes were calculated. Three biological repeats were performed.

Results

Up- and downregulation of BcMF27 resulted in pollen abnormality

To determine the function in pollen development, amiRNA and overexpression vectors of *BcMF27* were constructed (Fig. 1a, b) and induced in *B. campestris* ssp. *chinensis* var. *parachinensis*. Positive transgenic lines were confirmed by PCR analysis and named BcMF27-amiR and *BcMF27*^{OE} (Fig. 1c). The *BcMF27* transcripts from the inflorescence of BcMF27-amiR and *BcMF27*^{OE} plants were detected by real-time RT-PCR (Fig. 1d). The results showed that the expression of *BcMF27* decreased in the inflorescence of BcMF27-amiR1-3 plants and increased in the inflorescence of *BcMF27*^{OE}-1-3 plants although variation of the *BcMF27* transcript was not remarkable.

No difference was observed in vegetative growth and flower organs development among BcMF27-amiR and *BcMF27*^{OE} and control transgenic plants (Suppl. Fig. S1). However, SEM indicated that the pollen of BcMF27-amiR and *BcMF27*^{OE} plants was defective (Fig. 2). Of pollen from BcMF27-amiR plants, 93.56% possessed collapsed walls (Fig. 2b, e, f, h). The surface of 29% of abnormal pollen walls locally accumulated unknown material (Fig. 2e). Of *BcMF27*^{OE} pollen, 66.67% had collapsed walls (Fig. 2c, g, i). Pollen from control plants had an ellipsoid shape and normal reticular structure, and the deformity rate was only 13.75% (Fig. 2a, d, h, i).

To clarify anther and pollen development in BcMF27-amiR and *BcMF27*^{OE} transgenic plants, semi-thin sections of BcMF27-amiR and *BcMF27*^{OE} pollen were collected (Fig. 3). Tapetum development in BcMF27-amiR (Fig. 3f–j) and *BcMF27*^{OE} (Fig. 3k–o) plants was identical to that of control plants (Fig. 3a–e). BcMF27-amiR (Fig. 3f–i) and *BcMF27*^{OE} (Fig. 3k–n) pollen developed normally from the pollen mother cell stage to the binucleate stage. However, BcMF27-amiR pollen (Fig. 3j) exhibited two kinds of malformed shapes and *BcMF27*^{OE} pollen (Fig. 3o) presented an irregular shape compared with control pollen at the trinucleate stage (Fig. 3e).

To further detail BcMF27-amiR and *BcMF27*^{OE} pollen development, TEM was performed (Fig. 4). BcMF27-amiR pollen development normally proceeded to the uninucleate stage (Fig. 4f–h, r) in accordance with the control (Fig. 4a–c, q). At the binuclear stage, the intine thickened normally at germinal furrow regions in control pollen (Fig. 4d, t), but 95.45% of BcMF27-amiR pollen intines formed abnormally outside germinal furrows (Fig. 4i, u). Furthermore, 36.36% of BcMF27-amiR pollen with an additional germinal aperture displayed partial cytoplasm degradation and unknown material on the surface of pollen (Fig. 4j, x) and the remaining defective pollen still included four germinal furrows (Fig. 4p, z), while the control pollen contained a normal organized wall with three germinal furrows and a dense cytoplasm (Fig. 4e, w) at the trinucleate stage. *BcMF27*^{OE} pollen showed normal development from the pollen mother cell stage to the binuclear pollen stage (Fig. 4k–n, s, v), whereas 52% of pollen exhibited aberrant intine deposition outside the germinal furrow regions at the mature pollen stage (Fig. 4o, y).

Functional disruption of BcMF27 caused morphologically unstable pollen tubes

Alexander staining was performed for pollen viability analyses (Suppl. Fig. S2). It was observed that, similar to control pollen (Suppl. Fig. S2a), BcMF27-amiR (Suppl. Fig. S2b) and BcMF27^{OE} (Suppl. Fig. S2c) pollen grains were purplish red, which indicated normal BcMF27-amiR and BcMF27^{OE} pollen viability.

In BcMF27-amiR and *BcMF27*^{OE} transgenic plants, pollen germination in vitro was checked to determine the effect of *BcMF27* expression imbalance on pollen germination and pollen tube growth (Fig. 5). The average germination rates of BcMF27-amiR and *BcMF27*^{OE} pollen were 74.95% and 71.86%, respectively. The pollen germination of BcMF27-amiR and *BcMF27*^{OE} was hardly affected compared with control pollen (80.68%; Fig. 5d). However, BcMF27-amiR (Fig. 5b) and *BcMF27*^{OE} (Fig. 5c) had twisted tubes compared with the positive control (Fig. 5a). The percentage of twisted pollen tubes in BcMF27-amiR1, BcMF27-amiR2, and BcMF27-amiR3 (91.67%, 85.22%, and 94.58%, respectively) were significantly increased (Fig. 5d). Similarly, the percentage of twisted pollen tubes was significantly increased in *BcMF27*^{OE}*-1*, *BcMF27*^{OE}*-2*, and *BcMF27*^{OE}*-3* (50%, 78.33%, and 41.11%, respectively; Fig. 5d). However, pollen from BcMF27-amiR (Fig. 6b) and *BcMF27*^{OE} (Fig. 6c) on control pistils germinated normally in vivo compared to that from control (Fig. 6a) at 4 h after pollination. The average seed number per silique from BcMF27-amiR, *BcMF27*^{OE}, and control plants was not significantly different (data not shown).

Discussion

The elaborately decorated pollen wall protects pollen from stress and consists of the exine and intine. Pectin is a structural component of the intine and is involved in the modification of wall characteristics. PMEs hydrolyze pectin and demethylate pectin molecules linked to each other by calcium ion bonds; they are then integrated into the intine to strengthen wall rigidity. Therefore, PMEs have an essential function in pollen wall construction and pollen development.

Yue et al. (2018a) observed that knockdown of *BcMF23a* caused shape deformities and defective intine construction in pollen. Therefore, it was inferred that *BcMF23a* influenced pollen development *via* intine formation. *BcMF27*, the characteristic pollen-specific pectin methylesterase, was remarkably expressed in mature pollen (Yue et al. 2018b). BcMF27-amiR and $BcMF27^{OE}$ mature pollen walls collapsed and the intine thickened abnormally outside of the germinal apertures induced by variation of the *BcMF27* transcript, which was similar to the phenotype of *bcmf23a* pollen. In addition, in 36.36% of BcMF27-amiR pollen, the cytoplasm partially disappeared, which also occurred in *bcmf23a* pollen. Thus, it was estimated that the imbalance of *BcMF27* expression led to abnormal intine formation, which further affected pollen morphology construction.

The pollen tube is an extension of the pollen intine and grows rapidly in the pistil (Geitmann and Steer 2006). Pollen tube walls are mainly composed of pectin (Chen and Ye 2007), which control the inflexibility of pollen tubes. The remodeling of the pollen tube wall involves many enzymes, such as glycoside hydrolases (GHs), xyloglucan endo-transglucosylase hydrolase (XTHs), and PMEs (Yokoyama and Nishitani 2001; Tung et al. 2005; Mollet et al. 2013). PMEs regulate the DM of pectin to adjust the mechanical properties of the pollen tube, such as stiffness and elasticity (Staunton et al. 2011; Chebli et al. 2012; Zhang et al. 2018).

Many pollen-specific PME genes have demonstrated an important function in pollen germination and pollen tube growth (Kim et al. 2020). *AtPME48* belongs to the pollen-specific PME gene (Leroux et al. 2015). Functional interruption of *AtPME48* resulted in the appearance of two pollen tubes in vitro, delayed growth of pollen tubes in the pistil, and increased the DM of *pme48* pollen grains. It was concluded that *AtPME48* affected pollen germination by influencing the formation of the pollen intine. *VGD1* was also expressed in pollen and the pollen tube (Jiang 2005). *VGD1* knockout caused the pollen tube to burst in vitro, and pollen tube growth in vivo was postponed, causing the reduction of PME activity in the pollen tube, although the pollen grain displayed normal morphology. ZmGa1P and ZmPME10-1, two pollen-specific PMEs, assembled to modulate pectin esterification in pollen tube wall, which affected pollen tube growth (Zhang et al. 2018). Similarly, *BcMF27* was specifically expressed in pollen and pollen tubes, and down- and upregulation of *BcMF27* led the tube to be significantly twisted. In addition, pollen fertility and germination in vitro/vivo were not affected by pollen defects incuced by the regulation of *BcMF27*. Therefore, according to these results, disruption of *BcMF27* function directly reduced PME activity in the pollen tube, causing depressed rigidity in the tube wall, which destabilized pollen tube growth. The functional interruption of *BcMF27* and *BcMF23a* led to intine construction abnormality, pollen morphology deformity, and pollen tube growth instability (Yue et al. 2018a). However, the transcript of *BcMF27* appeared remarkably in pollen tubes, as well as mature pollen, while *BcMF23a* was expressed in pollen but not in the pollen tube (Lin et al. 2017; Yue et al. 2018a). Consequently, *BcMF27* not only was involved in intine formation to influence pollen development but also directly contributed to pollen tube growth by modifying pollen tube walls.

Declarations

Author contribution statement JC and YY conceived and designed the research. YY conducted the experiments, analyzed the data, and wrote the manuscript. JC revised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest Not applicable.

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Figures

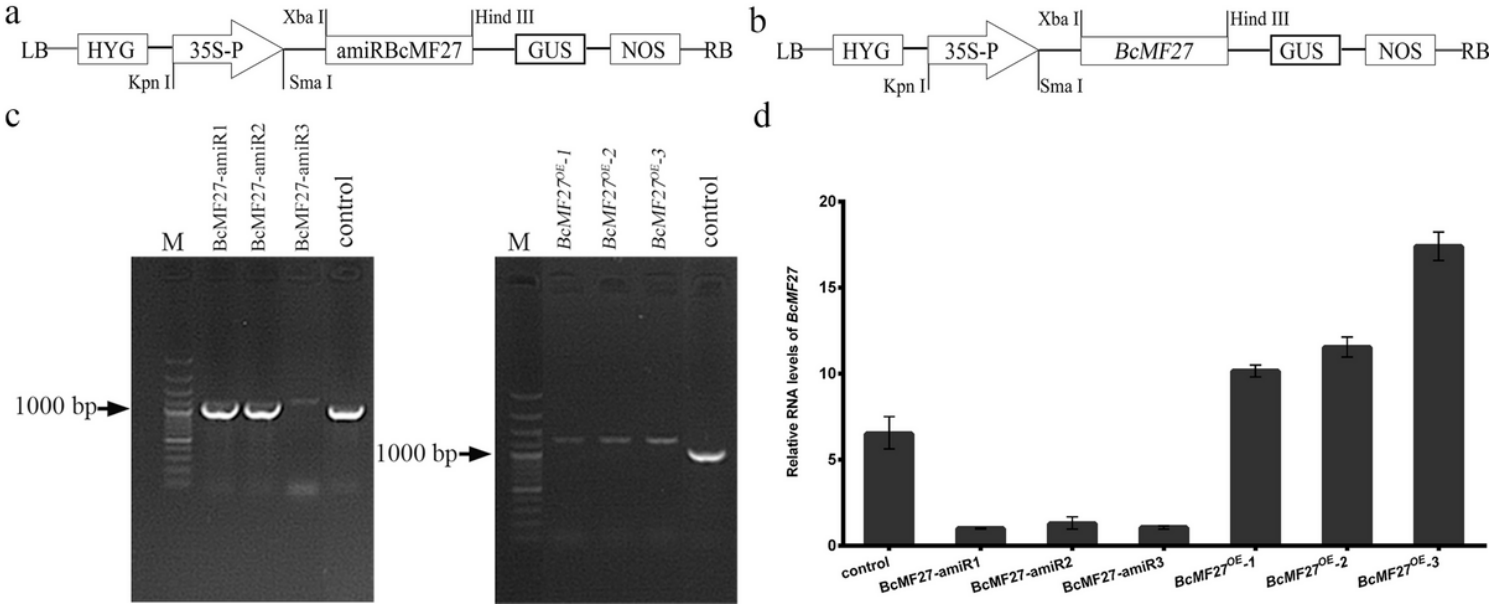

Figure 1

Construction of amiRNA (a) and overexpression (b) vectors of BcMF27 from Brassica campestris ssp. chinensis. (c) PCR detection of positive plants transformed with amiRNA, overexpression and empty vectors. (d) Real-time RT-PCR analysis of BcMF27 in the inflorescences of BcMF27-amiR and BcMF27OE transgenic plants. ±Standard errors for three independent experiments are shown; UBC10 was used as the internal control

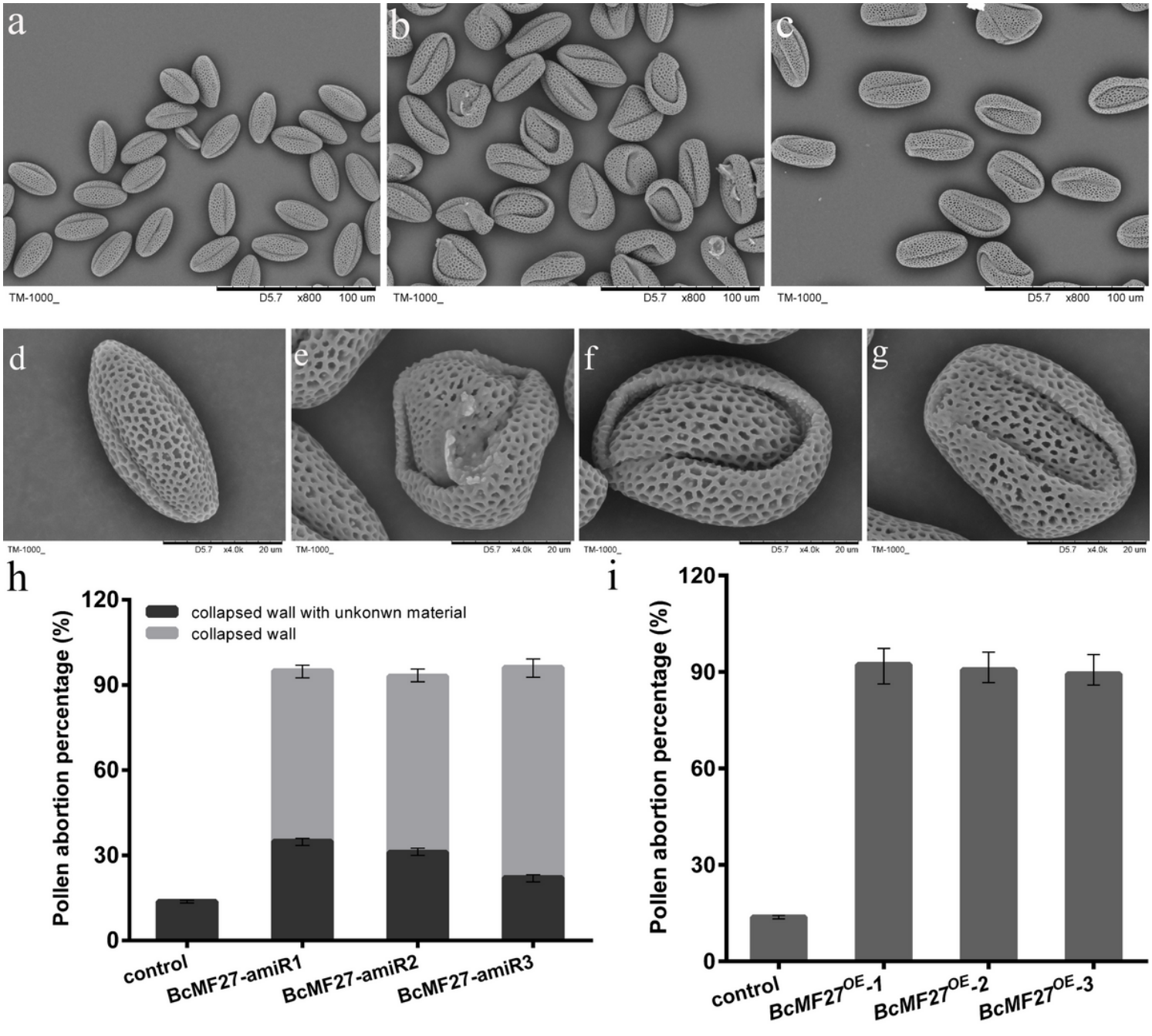

Figure 2

Scanning electron microscopy observation of pollen from BcMF27-amiR and BcMF27OE plants. (a–c) Pollen of the control, BcMF27-amiR, and BcMF27OE plants, respectively. (d) Magnified images of (a). (e and f) Magnified images of (b). (g) Magnified images of (c). (h and i) Pollen abortion percentage of

BcMF27-amiR and BcMF27OE plants. The values are the mean±standard error. Scale bars 100 μm in a–c, Scale bars 20 μm in d–j

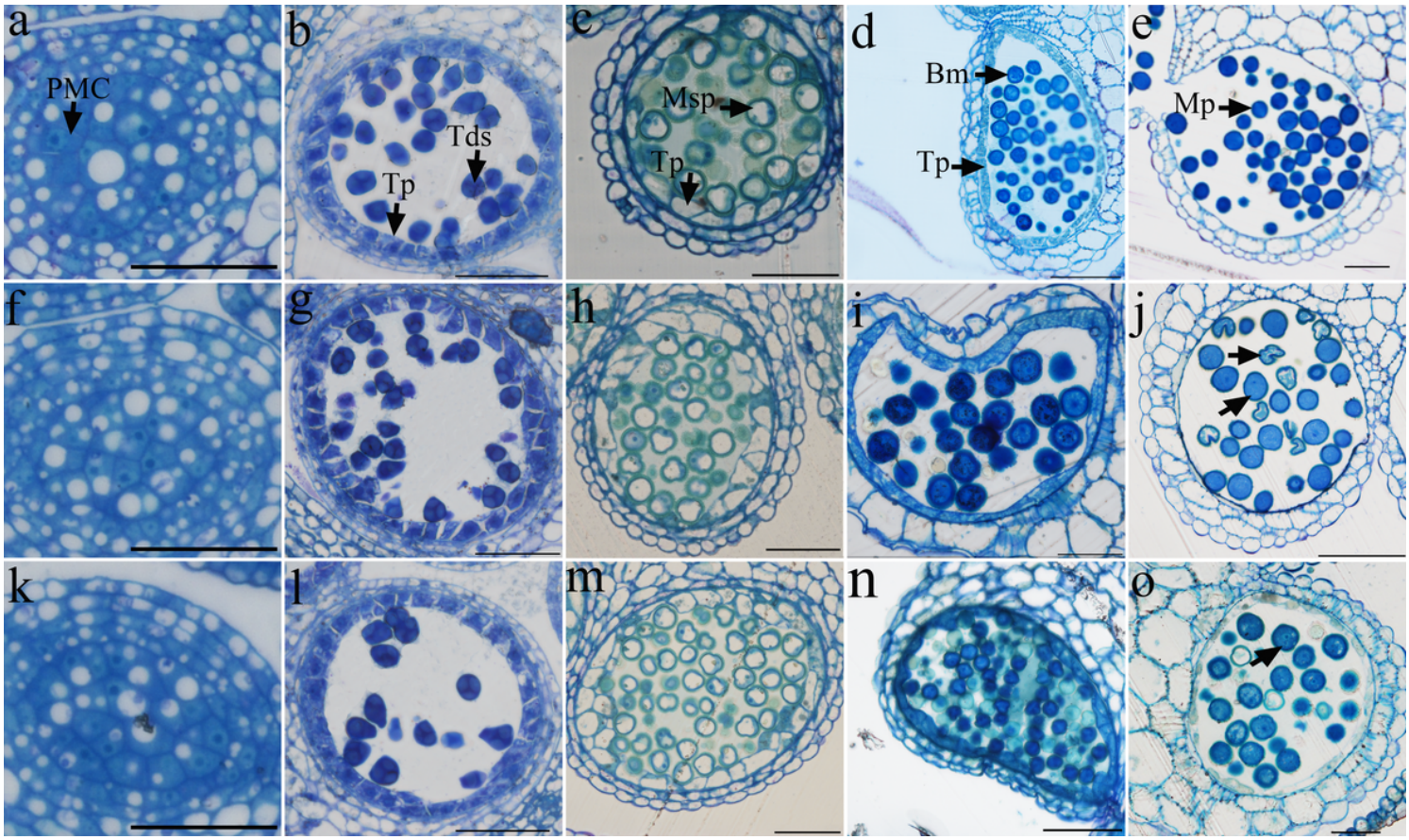

Figure 3

Semi-thin section observation of pollen from BcMF27-amiR and BcMF27OE plants. (a–o) Semi-thin sections of anthers in the control (a–e), BcMF27-amiR (f–j), and BcMF27OE (k–o) plants at the pollen mother cell stage (a, f, k), tetrad stage (b, g, l), uninucleate stage (c, h, m), binucleate stage (d, i, n), and trinucleate stage (e, j, o). PMC, pollen mother cell; Tp, tapetum; Tds, tetreds; Msp, microspore; Bm, binucleate microscope; MP, mature pollen. Scale bars 50 μm in a–o

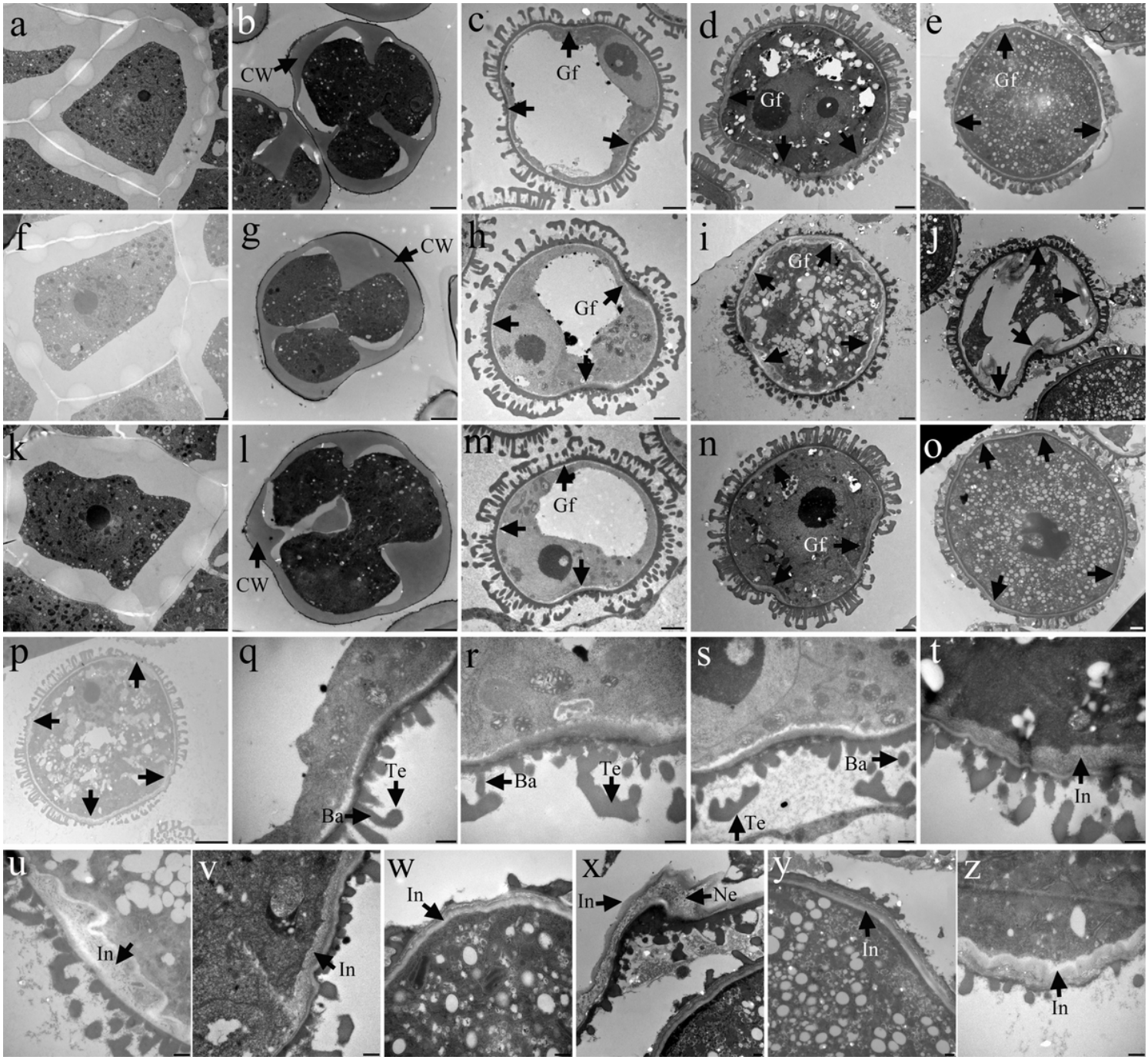

Figure 4

Transmission electron microscopy observation of microspore development in BcMF27-amiR and BcMF27OE plants. (a–p) pollen of control (a–e), BcMF27-amiR (f–j and p), and BcMF27OE (k–o) plants at pollen mother cell stage (a, f, and k), tetrad stage (b, g, and l), uninucleate stage (c, h, and m), binucleate stage (d, i, and n), and trinucleate stage (e, j, o, and p). (q–s) Magnified images of the corresponding aperture region in c, h, and m. (t–v) Magnified images of pollen wall in d, i, and n. (w–z) Magnified images of the pollen wall in e, j, o, and p. CW, callose wall; Gf, aperture; Te, tectum; Ba, baculum; Ne, nexine; In, intine. Scale bars 5 μm in a–p; 2 μm in q–z

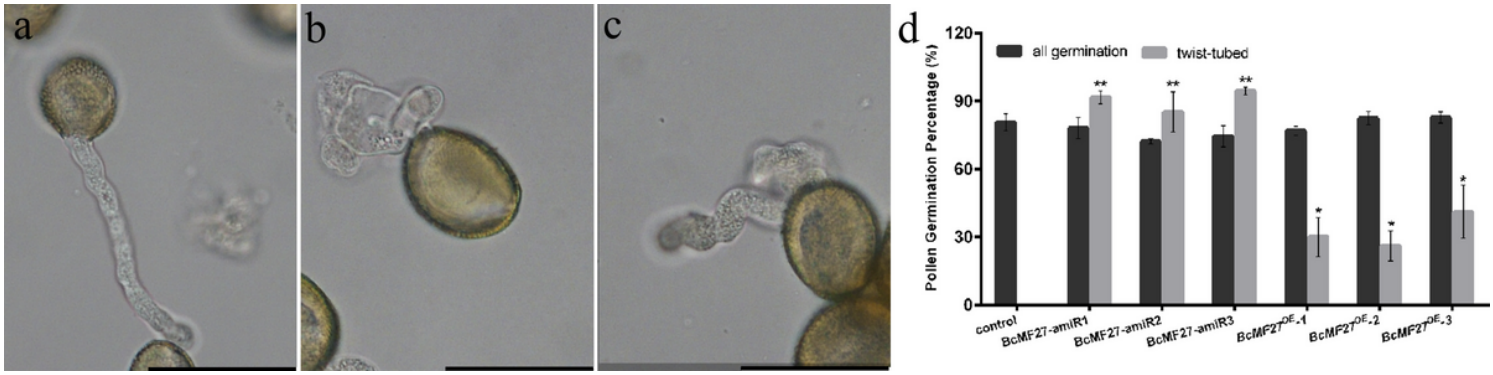

Figure 5

Pollen germination and pollen tube growth in vitro in BcMF27-amiR and BcMF27OE plant. (a–c) Pollen germination of control (a), BcMF27-amiR (b), and BcMF27OE (c) plant in vitro. (d) Pollen germination frequencies of control, BcMF27-amiR, and BcMF27OE plants and abnormal pollen tube percentage in all pollen germinated in vitro. *p < 0.05 and **p < 0.01 vs. the control. The values are the mean ± standard error. Scale bars 50 μm

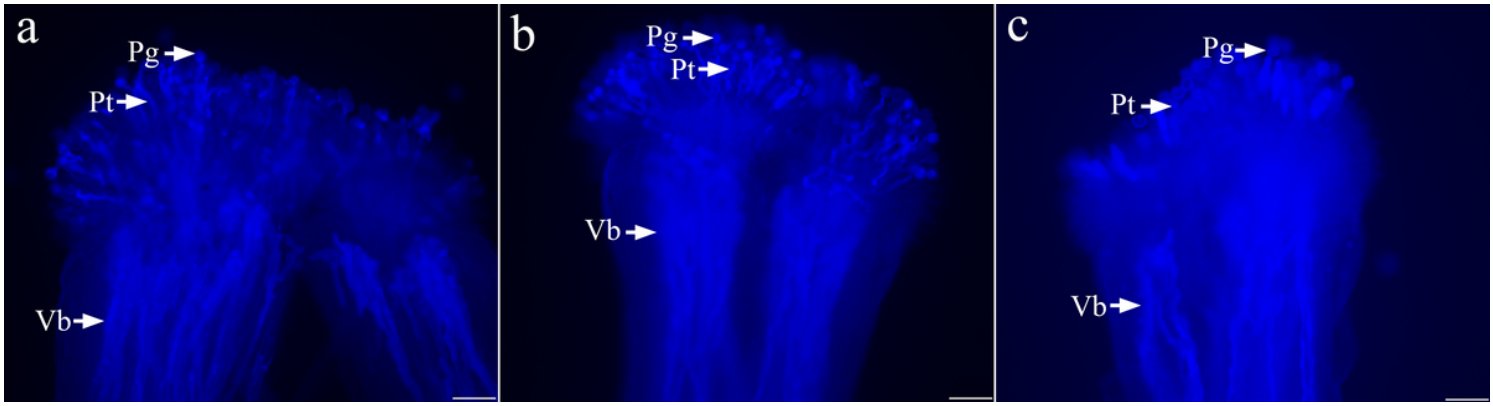

Figure 6

Pollen germination and pollen tube growth of BcMF27-amiR and BcMF27OE on control pistils in vivo. (a) Control pollen tube growth at 4 h after self-pollination. (b) BcMF27-amiR pollen tube growth at 4 h after pollination. (c) BcMF27OE pollen tube growth at 4 h after pollination. PG, pollen grains; Vb, vascular; Pt, pollen tube. Scale bars 50 μm

Supplementary Files

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