BcMF8, a putative arabinogalactan protein-encoding gene, contributes to pollen wall development, aperture formation and pollen tube growth in *Brassica campestris*

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- *Background and Aims* The arabinogalactan protein (AGP) gene family is involved in plant reproduction. However, little is known about the function of individual AGP genes in pollen development and pollen tube growth. In this study, *Brassica campestris male fertility 8* (*BcMF8*), a putative AGP-encoding gene previously found to be pollen specific in Chinese cabbage (*B. campestris* ssp. *chinensis*), was investigated.
- *Methods* Real-time reverse transcription–PCR and *in situ* hybridization were used to analyse the expression pattern of *BcMF8* in pistils. Prokaryotic expression and western blots were used to ensure that *BcMF8* could encode a protein. Antisense RNA technology was applied to silence gene expression, and morphological and cytological approaches (e.g. scanning electron microscopy and transmission electron microscopy) were used to reveal abnormal phenotypes caused by gene silencing.
- *Key Results* The *BcMF8* gene encoded a putative AGP protein that was located in the cell wall, and was expressed in pollen grains and pollen tubes. The functional interruption of *BcMF8* by antisense RNA technology resulted in slipper-shaped and bilaterally sunken pollen with abnormal intine development and aperture formation. The inhibition of *BcMF8* led to a decrease in the percentage of *in vitro* pollen germination. In pollen that did germinate, the pollen tubes were unstable, abnormally shaped and burst more frequently relative to controls, which corresponded to an *in vivo* arrest of pollen germination at the stigma surface and retarded pollen tube growth in the stylar transmitting tissues.
- *Conclusions* The phenotypic defects of antisense *BcMF8* RNA lines (*bcmf8*) suggest a crucial function of *BcMF8* in modulating the physical nature of the pollen wall and in helping in maintaining the integrity of the pollen tube wall matrix.

Key words: AGP, arabinogalactan proteins, *Brassica campestris*, Chinese cabbage, aperture, intine, pollen tube, pollen wall development, aperture formation.

INTRODUCTION

As a biological protector of male sperm, pollen is covered by the pollen wall. Despite the morphological diversity of the pollen wall, its fundamental structure shows a significant similarity among different species. The outer layer of the pollen wall, which is called the exine, is composed largely of sporopollenin, which usually comprises the outer sexine and inner nexine layers. The intine is the inner layer of the pollen wall underlying the exine, consisting of hydrolytic enzymes, hydrophobic proteins, cellulose, hemicellulose and pectic polymers (Ariizumi and Toriyama, 2011). Both layers of the pollen wall are important for pollen actions (Owen and Makaroff, 1995). Although the general pattern of pollen wall development and the components are clear (Ariizumi and Toriyama, 2011), the entire dynamic regulatory network of pollen wall synthesis is largely undefined, which may involve a considerable amount of different kinds of molecules (Seifert and Blaukopf, 2010).

Arabinogalactan proteins (AGPs), extensively glycosylated hydroxyproline-rich glycol-proteins, are abundant throughout the plant kingdom, mainly at cell surfaces, where they are thought to have important functions in plant growth and development, especially in plant reproduction (Showalter, 2001). By investigating the distribution of AGP epitopes and mRNA transcripts, results show that AGPs are highly expressed in male organs (pollen, pollen tube and sperm), female organs (stigma, style and ovary) and the embryo. In many species, such as *Nicotiana alata*, *Lilium longiflorum*, maize and tomato, AGPs have significant functions in anther development, discrimination and adhesion between the pollen and stigma, pollen tube germination and growth, fertilization and embryo development (Du *et al.*, 1996; Geitmann and Steer, 2006; Lee *et al.*, 2008, 2009). Although these studies showed that the reproduction process is associated with changes in the distribution of AGP epitopes or some AGP mRNA transcripts, they did not identify the precise function of a specific AGP protein or corresponding gene acting during reproduction. In recent years, several AGP mutants have been generated to understand the mechanisms of AGP function (van Hengel and Roberts, 2003; Gaspar *et al.*, 2004; Sun *et al.*, 2004; van Hengel *et al.*, 2004; Seifert and

Blaukopf, 2010). However, few studies directly support the functions of AGP-encoding genes in plant reproduction, except for the recent specific function found for *AtAGP6*, *AtAGP11* and *FLA3* in pollen development, and *AtAGP18* in the initiation of female gametogenesis in arabidopsis (Garcia and Calzada, 2004; Pereira *et al.*, 2006; Levitin *et al.*, 2008; Coimbra *et al.*, 2009, 2010; Li *et al.*, 2010).

In a previous study, a genic male sterility (GMS) system named '*Bcajh97-01A/B*' was constructed in *B. campestris* ssp. *chinensis* (Huang *et al.*, 2008*a*). '*Bcajh97-01A/B*' is a sibling line segregated in a 1:1 ratio with homozygous male-sterile plants ('*Bcajh97-01A*') and heterozygous male-fertile plants ('*Bcajh97-01B*'). Moreover, the male-sterile plants do not produce any functional pollen, and exhibit dramatic changes in the transcriptional profile of downstream genes in flower buds, among which one gene encoding a putative classical AGP, namely *BcMF8*, was isolated (Huang *et al.*, 2008*a*). *BcMF8* was found to be pollen specific and started to be expressed at the uninucleate stage, which suggested its important function in pollen development (Huang *et al.*, 2008*b*).

In the present study, the function of *BcMF8* was determined by specifically degrading the endogenous *BcMF8* transcript by antisense RNA technology. The pollen grains of the *bcmf8* lines exhibited slipper-shaped and bilaterally sunken pollen. Most of the pollen tubes could not grow normally, whereas pollen germination at the stigma surface and pollen tube elongation in the style tissue and transmitting tract were retarded before 8 h after pollination (HAP) *in vivo*, and fewer pollen tubes could reach the base of the transmitting tract at 24 HAP. Furthermore, the downregulation of *BcMF8* resulted in abnormal pollen intine development and pollen aperture formation, which possibly contributed to pollen tube growth. These results may provide valuable insight regarding AGP genes in pollen development and pollen tube growth.

MATERIALS AND METHODS

Plant material

The *Brassica campestris* ssp. *chinensis* (Chinese cabbage) GMS line system '*Bcajh97-01A/B*' was cultivated in the experimental farm of Zhejiang University (Huang *et al.*, 2008*a*).

Real-time RT–PCR

Total RNA was extracted from the closed flower buds from the whole inflorescence and pistils at 1, 2, 4, 8, 12 and 24 HAP using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. cDNA was then synthesized using a Reverse Transcription System A3500 (Promega, Madison, WI, USA) according to the manufacturer's instructions. Three technical repeats were performed. Real-time reverse transcription–PCR (RT–PCR) was achieved with the gene-specific primer pair P1 (5′-CCACCACGCAGACCAAGGCA-3') and P2 (5'-GTCCATCAACAACCTCTG-3') using GoTaq® qPCR Master Mix (Promega) on a BioRad CFX96 Real-time PCR Detection System according to the manufacturer's instructions. *UBC-10* was used as the normalization control.

In situ *hybridization*

Flower buds and pistils of sterile plants at 1, 3, 10 and 24 HAP were fixed in 4 % paraformaldehyde PBS (phosphate-buffered saline) solution and embedded in Tissue-Tek® O.C.T. Compound (SAKURA, USA). A 14 μm section of tissue was hybridized to specific digoxigenin (DIG)-labelled RNA probes (Roche, Branchburg, Germany). Templates for the gene-specific probes were obtained through amplification with the primer pair P3 (5'-ATGGCACGTCAATTTGTCGTA-3') and P4 (5'-GAAG ATGGTCCATCAACAACC-3'). The sense and antisense probes were synthesized using an Sp6/T7 transcription Kit (Roche).

Western blots

The coding sequence of *BcMF8* was cloned into the pET32a(+) vector at *Sac*I and *Hin*dIII sites with the gene-specific primer pair P5 (5'-CGAGCTCATGGCACGTCAA TTTGTCGTATTG-3') and P6 (5'-CCCAAGCTTTTGGAGA GAGAAGAAGAAGAATCC-3'). A Trx-Tag, small His-Tag and S-Tag sequence were included in the fusion protein (LaVallie *et al.*, 1993). The recombinant vector was then introduced into the *Escherichia coli* strain DE3. The expression of the BcMF8 fusion protein in *E. coli* cells was induced at 30 °C by adding isopropyl-β-D-thiogalactoside to a final concentration of 0·5 mmol L^{-1}. Cultures were grown for an additional 4 h. The BcMF8 fusion protein was purified using a His-Bind Kit following the manufacturer's protocols (NovaGen, Madison, WI, USA), and detected by western blot using an anti-His antibody (Huaan, Hangzhou, China). The empty pET32a(+) vector was also induced under the same conditions.

Sub-cellular localization of BcMF8−enhanced yellow fluorescent protein (eYFP) fusion protein in onion epidermal cells

The *BcMF8*-coding open reading frame (ORF) fragment with a modified stop codon was amplified with the gene-specific primer pair P7 (5'-CACCATGGCACGTCAATTTGTCGT ATTG-3') and P8 (5'-TTGGAGAGAGAAGAAGAAGAAT CC-3'). The resulting cDNA fragment was cloned into the Gateway entry vector pENTR (Invitrogen), and the entry vector was recombined with the destination binary vector pB7YWG2,0 (Karimi *et al.*, 2002). The fusion vector of BcMF8−eYFP was transiently transformed into onion epidermal cells by particle bombardment (Gan, 1989). eYFP-dependent fluorescence was analysed 24 h after introduction under a fluorescence microscope (ECLIPSE 90i; Nikon). To visualize the eYFP distribution, onion epidermal cells were plasmolysed in 0·3 g mL^{-1} sucrose for 3 min.

Generation of antisense BcMF8 *RNA constructs and plant transformation*

A 566 bp partial cDNA sequence of *BcMF8* was amplified using the oligonucleotide primer pair P9 (5'-AATGGCAC GTCAATTTGTCGT-3', forward, with a *Bam*HI restriction endonuclease site) and P10 (5'-CGCATCCAAAAATGTT ACAA-3', reverse, with an *Xba*I restriction endonuclease site). The resulting *BcMF8* fragment was then inserted in the antisense orientation into the binary vector pBI121 with the constitutive

Cauliflower mosaic virus (CaMV) 35S promoter and the NPTII reporter gene. The fusion vector pBI*35S::BcMF8A*, as well as a control empty vector, pBI121, were transformed into the fertile Chinese cabbage mediated by *Agrobacterium tumefaciens* as described by Yu *et al.* (2004).

PCR analysis and Southern hybridization

For PCR and Southern hybridization, genomic DNA was extracted from fresh young leaves using the cetyl trimethyl ammonium bromide extraction method (McDonald and Martinez, 1990). PCR was performed to identify the transformed lines by detecting the reporter gene, NPTII, in the pBI121 vector, with the primer pair P11 (5'-CAGGATCTCCTGTCATCTCACC-3') and P12 (5'-GGCGATACCGTAAAGCACG-3'). Genomic DNA was then digested with *Eco*RI for Southern hybridization to confirm the integration of the antisense *BcMF8* RNA fragment into the plant genome. Southern hybridization was performed according to Sambrook *et al.* (1989). The digested genomic DNA was electrophoresed in 1 % agarose gels, transferred by capillary action onto a nylon membrane (Amersham Biosciences, Buckinghamshire, UK) and hybridized to DIG-labelled DNA probes. The DNA probes were prepared by labelling the NPTII gene using a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche) according to the manufacturer's instructions.

Phenotypic and cytological analysis of pollen

Anthers were dissected from unopened floral buds and subsequently dyed with aniline blue (0·1 % aniline blue in 0·1 M K_2HPO_4-KOH, pH 11) to stain callose in pollen. Alexander stain (Alexander, 1969) was used to detect the vitality of pollen, whereas 4',6-diamidino-2-phenylindole (DAPI) solution (Regan and Moffatt, 1990) was used to investigate the nuclei of pollen.

Electron microscopy

For scanning electron microscopy (SEM), individual pollen grains were spread on SEM carriers, coated with gold–palladium in an Eiko Model IB5 ion coater for 4–5 min, and observed in a Hitachi Model TM-1000 scanning electron microscope. Digital images were then taken. For transmission electron microscopy (TEM), anthers of different development stages were fixed with 2·5 % glutaraldehyde in phosphate buffer (pH 7·0) overnight, and post-fixed with 1 % OsO_4 in phosphate buffer for 1 h. The specimens were then dehydrated through a graded series of ethanol, and embedded in Spurr resin. Ultrathin sections (70 nm) were obtained and stained with uranyl acetate, followed by alkaline lead citrate, and observed in a Hitachi Model H-7650 transmission electron microscope. Digital images were then recorded.

Pollen germination

Pollen grains for the pollen germination *in vitro* assay were collected and cultured in a medium containing 15 % sucrose, 0·4 mM HBO_3, 0·4 mM $Ca(NO_3)_2$ and 0·1 % agar. The pH was adjusted to 5·8. The pollen grains were grown at 20 °C with 100 % relative humidity in the dark for 4 h. The germinating

pollen grains were examined to calculate an average percentage germination and to what extent they were misshapen. To investigate pollen germination *in vivo*, the pre-emasculated mature flowers of the antisense transgenic and control plants were self-pollinated. Given that *B. campestris* is a typical sporophytic self-incompatibility species, selfing is induced using immature material in which the S phenotype is not yet expressed (bud pollination) to break down self-incompatibility (Mable, 2008). Flowers of the maternal parents were emasculated and pollinated at 2 d before anthesis. The pollinated pistils were collected at 0, 2, 8, 16 and 24 HAP, and fixed with a mixture of acetic acid and ethanol (3:1). The fixed pistils were rinsed three times with distilled water, softened with 8 M NaOH overnight, washed in distilled water and stained in decolorized aniline blue for 3 h in the dark. The stained pistils were observed and photographed with a Leica DMLB fluorescence microscope (Wetzlar, Germany) under UV light.

Analysis of seeds

Self-pollination tests were performed during the budding period to investigate the fertility of the transgenic plants. Flowers of the maternal parents were emasculated at 2 d before anthesis and pollinated with their own mature pollen. Ten siliques of each line were analysed to count the number of seeds.

RESULTS

BcMF8 *is expressed in pollen tubes and pollinated stigma at the early stage of pollen tube growth*

Results of previous work have shown that *BcMF8* is pollen specific, and expressed at the onset of the uninucleate stage to the mature pollen stage (Huang *et al.*, 2008*b*). In the present study, the expression pattern of *BcMF8* in the pollinated pistils was supplemented. Real-time RT–PCR results showed that the *BcMF8* expression level was significantly higher in the pollinated pistils than in those that were unpollinated, especially in the pollinated pistils at 1 and 2 HAP (Fig. 1).

In situ hybridization was performed to visualize the precise location of *BcMF8* mRNA in the pistils. A specific hybridization
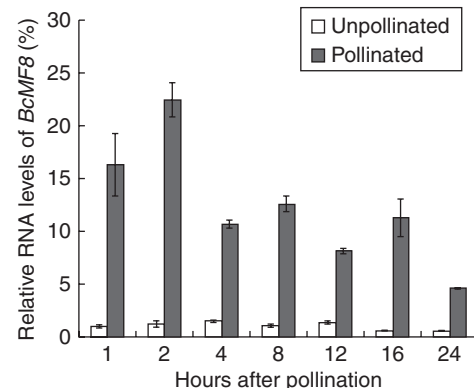

FIG. 1. Analysis of the expression of *BcMF8* in pistils of the *Brassica campestris* ssp. *chinensis* genic male-sterile line system ('*Bcajh97-01A/B*') using real-time RT–PCR. Increased expression levels in the pollinated pistils at 1, 2, 4, 8, 12 and 24 HAP were detected. Ubiquitously expressed *UBC-10* was used as an internal control. Standard errors for three independent experiments are also shown.
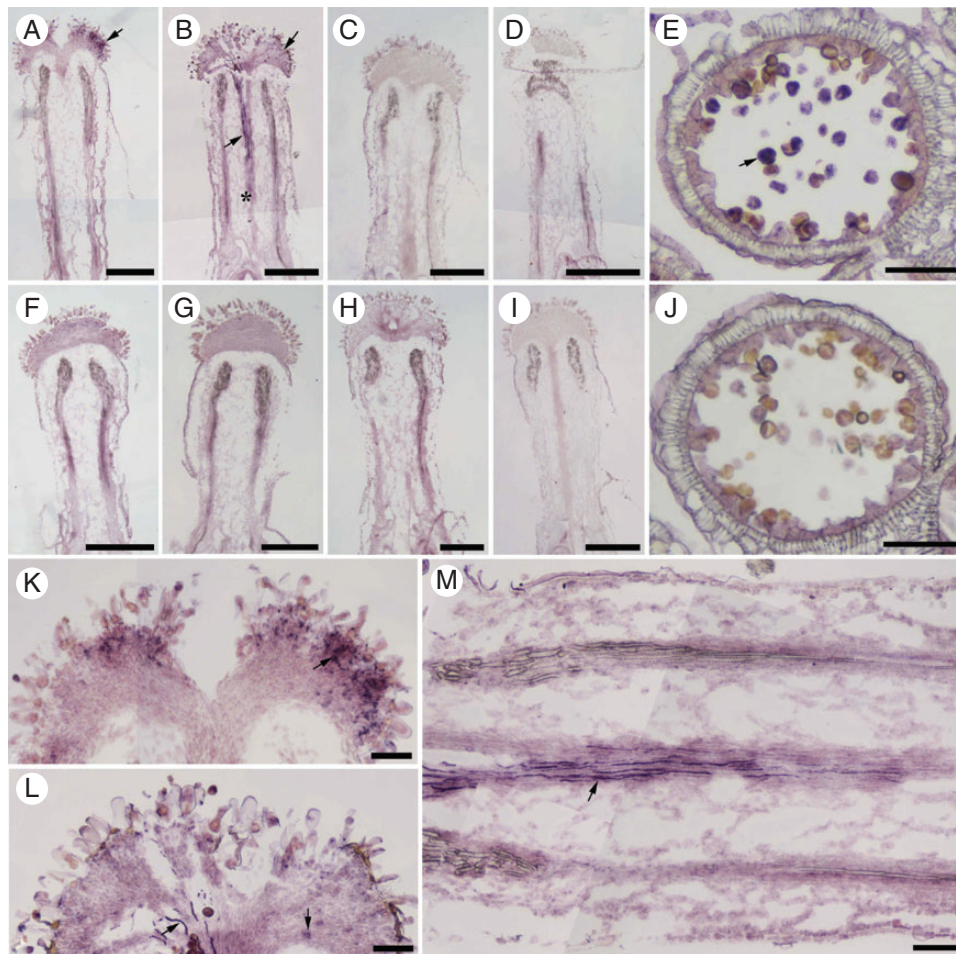
FIG. 2. The expression of *BcMF8* in pollen tubes of the *Brassica campestris* ssp. *chinensis* genic male-sterile line system ('Bcajh97-01A/B') based on *in situ* hybridization. (A–D) Longitudinal sections of the pollinated pistils at 1, 3, 10 and 24 HAP hybridized with a *BcMF8* antisense probe. A specific hybridization signal (arrowhead) was detected at the stigma at 1 HAP (A). A stronger hybridization signal (arrowhead) was detected at the stigma at 3 HAP and in the growing pollen tubes, which penetrated the style tissue and almost reached the upper end of the transmitting tract of the pistil (asterisk) (B). No hybridization signal was detected in the pollinated pistils at 10 HAP (C) and 24 HAP (D). (E) A section of anther at the binucleate stage hybridized with a *BcMF8* antisense probe. Specific hybridization signals were observed in the pollen (arrowhead). (F–I) Longitudinal sections of the unpollinated pistils at 1, 3, 10 and 24 HAP hybridized with a *BcMF8* antisense probe. No hybridization signal was detected in the unpollinated pistils. (J) A section of anther at the binucleate stage hybridized with a *BcMF8* sense probe as a negative control. (K and L) Magnified images of the corresponding stigma in A and B showing the hybridization signal (arrowhead). (M) Magnified image of the corresponding style in B showing the hybridization signal (arrowhead) in the pollen tubes. Scale bars = 500 μm (A–D, F–I); 100 μm (E, J–M).

signal was detected in the pollen and stigma of the pollinated pistil at 1 HAP (Fig. 2A, K). A stronger hybridization signal was detected in the stigma at 3 HAP, and in the growing pollen tubes when the pollen tubes penetrated the style tissue and almost reached the upper end of the transmitting tract of the pistil (Fig. 2B, L, M). No signal could be detected in the pollinated pistils at 10 and 24 HAP (Fig. 2C, D), or in the unpollinated pistils (Fig. 2F–I) and sense control (Supplementary Data Fig. S1).

BcMF8 protein exhibits extracellular localization

To establish that *BcMF8* can encode a protein, the *BcMF8* ORF was cloned and expressed in an *E. coli* strain DE3 T7 RNA polymerase expression system. Total protein was extracted from cultures and purified. Western blot analysis showed that the protein induced in the empty pET32a(+) vector, which contained a Trx-Tag, small His-Tag and S-Tag, was 20·5 kDa. The

purified BcMF8 fusion protein, which also contained a Trx-Tag, small His-Tag and S-Tag, was approx. 40 kDa, which indicates that the molecular mass of the BcMF8 protein characterized by SDS–PAGE was approx. 20 kDa (Fig. 3). This mass was slightly higher than that predicted by the deduced amino acid sequence in ExPASy (http://web.expasy.org/compute_pi/), which is 13·2 kDa.

Sequence analysis of BcMF8 using the SignalP 4·0 Server revealed a secretory signal sequence at the N-terminus, with a most probable cleavage site between amino acid residues 20 and 21. WoLF PSORT Protein Subcellular Localization Prediction showed that the BcMF8 protein was localized both intracellularly and extracellularly. An eYFP gene was fused to BcMF8 under the control of the constitutive CaMV 35S promoter to investigate the sub-cellular location of BcMF8. The fusion vector was then transformed into onion epidermal cells. The results showed that the BcMF8–eYFP transgenic cells,

including the nuclei, displayed a widespread distribution of fluorescence (Fig. 4A, B). In the plasmolysed BcMF8–eYFP cells, fluorescence was observed in the plasma membrane and cell wall (Fig. 4C, D). These results indicated that the *BcMF8* product was a secreted protein, and exhibited an extracellular localization.

Inhibition of BcMF8 *expression results in a pollen morphological defect with ectopic intine development and abnormal aperture formation*

To characterize the biological function of *BcMF8* in pollen development and pollen tube growth, antisense RNA technology was used to reduce specifically *BcMF8* expression in Chinese cabbage. Fusion vector (Supplementary Data Fig. S2) was obtained and transformed into fertile Chinese cabbage mediated by *A. tumefaciens*. PCR detection (Supplementary Data Fig. S3) and Southern hybridization analysis (Supplementary Data Fig. S4) identified eight independent *bcmf8* lines (named *bcmf8-1* to *bcmf8-8*). Control transformed plants were simultaneously obtained with empty vector pBI121 (named CK). Real-time RT–PCR analysis validated the significantly

FIG. 3. Western blot of the purified BcMF8 fusion protein. Lane 1, purified BcMF8 protein fused with a Trx-Tag, small His-Tag and S-Tag; lane 2, 20·5 kDa protein induced in the empty pET32a(+) vector, containing a Trx-Tag, small His-Tag and S-Tag.

reduced expression of *BcMF8* in antisense RNA lines (>60 % reduction compared with CK; Fig. 5).

Further investigation revealed that these lines had consistent phenotypes. They displayed normal vegetative growth and flowered normally, with no defects in the sepal, petal, gynoecium, nectar or other floral organs (data not shown). However, obvious defects in pollen morphology in these lines were assessed by SEM (Fig. 6). Three lines, namely *bcmf8-2*, *bcmf8-4* and *bcmf8-5*, displayed stronger and more obvious defects than the others. Compared with the normal-shaped pollen grains of CK (Fig. 6A), pollen grains from *bcmf8* were slipper shaped and bilaterally sunken, and also lost the even aperture distribution pattern (Fig. 6B, C). Statistically, 64 and 69 % of pollen from *bcmf8-2* and *bcmf8-5*, respectively, had morphological defects (Fig. 6D), and almost 100 % of the pollen from *bcmf8-4* line was abnormal (Fig. 6D). However, DAPI staining showed that the *bcmf8* pollen underwent normal karyokinesis. Aniline blue staining indicated that callose was synthesized normally at the pollen meiosis stage and was degraded at the onset of the uninucleate microspore stage in the *bcmf8* pollen. Alexander staining also showed that the vitality of the *bcmf8* pollen was not significantly different from that of the control (data not shown).

Further examinations of the anther and pollen development process were performed by TEM. All the pollen development stages, including the early uninucleate, late uninucleate, binucleate and trinucleate stages, could be observed (Fig. 7). The TEM results demonstrate that the cell nuclei exhibited normal division and differentiation, and the cell organelles, anther tapetum and other layers showed normal formation throughout the pollen development process (data not shown). These findings reinforced the previous results obtained by DAPI staining and aniline blue staining, which indicate that the *bcmf8* pollen was capable of normal callose metabolism and karyokinesis. The only ectopic structure observed was the pollen wall. In normal control pollen, the exine was formed with visible tectum and baculum layers at the early uninucleate microscope stage (Fig. 7A). Three apertures started to form with the development of the pollen wall. At the late uninucleate microscope stage, the intine began to take shape inside the prospective aperture region, whereas no exine occurred (Fig. 7B). This even formation of intine led to a binucleate microscope stage with three evenly distributed apertures (Fig. 7C). Compared with the control pollen, the pollen from *bcmf8-2*, *bcmf8-4* and *bcmf8-5* displayed unusual but similar development processes. Prior to the late uninucleate microscope stage, no obvious distinction was found between the *bcmf8* pollen (Fig. 7E) and CK pollen (Fig. 7A).
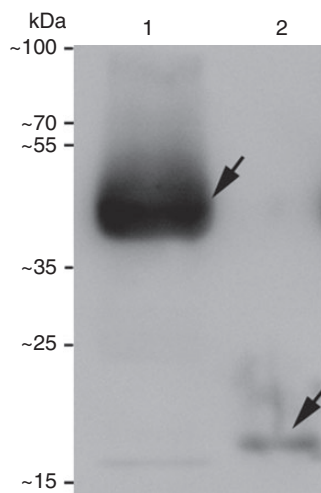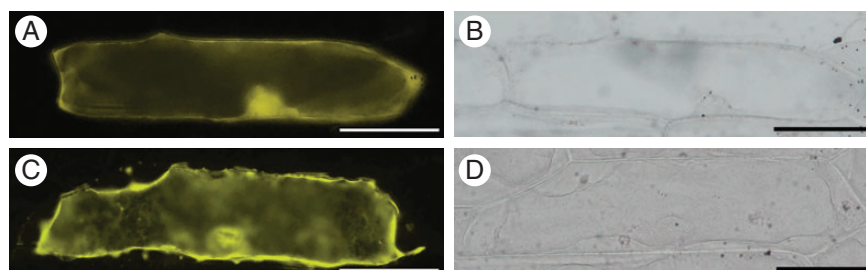

FIG. 4. Subcellular localization of BcMF8–eYFP fusion protein in onion epidermal cells. (A) Fluorescence image of unplasmolysed BcMF8–eYFP transgenic cells. (C) Fluorescence image of plasmolysed BcMF8–eYFP transgenic cells. (B) and (D) Bright-field image. Scale bars = 100 μm.

However, at the late uninucleate microscope stage, a remarkable thickening in intine both inside and outside the aperture region was detected in the *bcmf8* pollen (Fig. 7F, J, L). Moreover, at the binucleate microscope stage, rather than three evenly distributed apertures, four apertures were found in almost 80 % of pollen grains from the *bcmf8* lines (Fig. 7G). When pollen developed to the trinucleate and maturation stages, the aperture distribution was still uneven (Fig. 7H). Previous results obtained by SEM revealed that the shape of the *bcmf8* pollen was quite different from that of CK, and this difference could be associated with intine development and formation of four apertures.
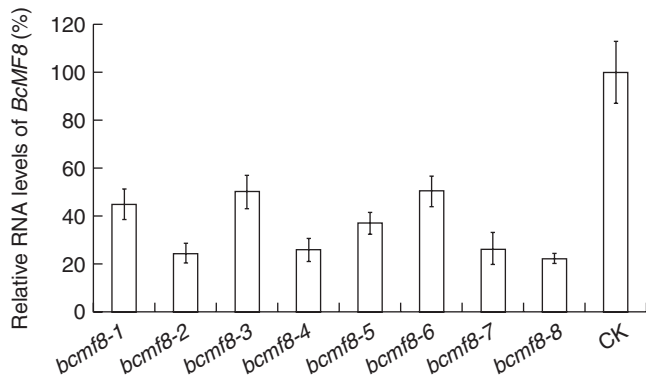
BcMF8 *inhibition retards pollen tube growth, resulting in significantly fewer seeds*

In view of the defect of the *bcmf8* pollen and upregulated *BcMF8* transcript in the pollinated pistils, *in vitro* and *in vivo* pollen tube growth were investigated. The *bcmf8* pollen tubes were unstable when cultured *in vitro*. Although an average pollen germination of 63 % could be obtained in optimized conditions, only a small portion exhibited normal pollen tube elongation (Fig. 8E). In the germinated *bcmf8-2* pollen, 30·2 % of pollen tubes were bead like (Fig. 8C, E), whereas 17·3 % had burst (Fig. 8D, E). In the germinated *bcmf8-4* pollen, 17·7 % of tubes were bead like (Fig. 8E), whereas 47·4 % had burst (Fig. 8E). In the germinated *bcmf8-5* pollen, 3·3 % of tubes were balloon tipped (Fig. 8B, E), 6·4 % were bead like (Fig. 8E) and 18·8 % had burst (Fig. 8E). In contrast, >90 % of the control pollen tubes could elongate normally, and none burst or displayed abnormal shapes (Fig. 8A, E).

Self-pollination tests were performed during the budding period to investigate the fertility of the *bcmf8* lines. The *bcmf8* lines could bear fruit pods when pollinated with enough pollen grains. However, each silique of CK could produce about 18 seeds, whereas only around five seeds could be produced in the *bcmf8* silique (Fig. 8F). To investigate the cause of the reduction in seeds, the *in vivo* germination of the *bcmf8* pollen on the stigma was examined. The results showed an initial growth of *bcmf8* tubes on the surface of stigmatic cells, and the style tissue was significantly affected (Fig. 9). At 8 HAP, the CK pollen tubes penetrated through the style tissue, exited the placenta, travelled along the funicular surface and finally entered as far as half of the length of the transmitting tract (Fig. 9A). The *bcmf8* pollen tubes could not even penetrate through the style tissue completely

FIG. 5. Analysis of the *BcMF8* mRNA levels in the inflorescence of the *bcmf8* lines by real-time RT–PCR. The expression levels of *BcMF8* in the inflorescence in *bcmf8* lines were significantly less than those in the empty vector pBI121 (CK), which was defined as 100 %. Ubiquitously expressed *UBC-10* was used as an internal control. Standard errors for three independent experiments are also shown.
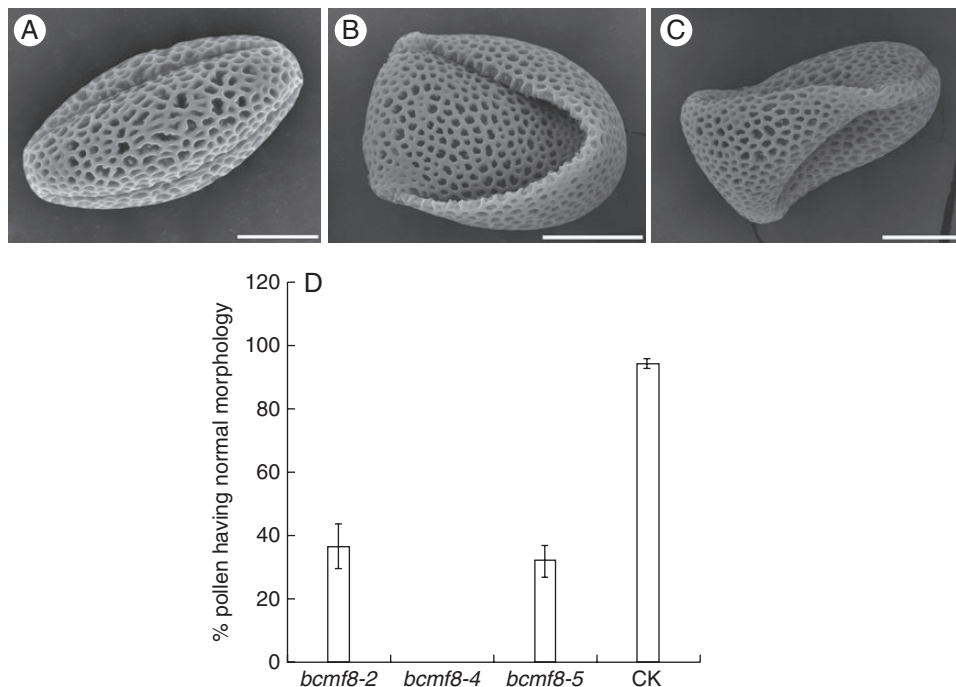

FIG. 6. Morphological defects of pollen grains from the *bcmf8* lines. (A) Scanning electron micrographs of normal pollen of empty vector pBI121-transformed plants (CK). (B and C) Scanning electron micrographs showing the slipper-shaped and bilaterally sunken pollen of the *bcmf8* lines. (D) Histogram showing the normal percentage of pollen in *bcmf8-2*, *bcmf8-4* and *bcmf8-5*. Standard errors are also shown. Scale bars = 100 μm.
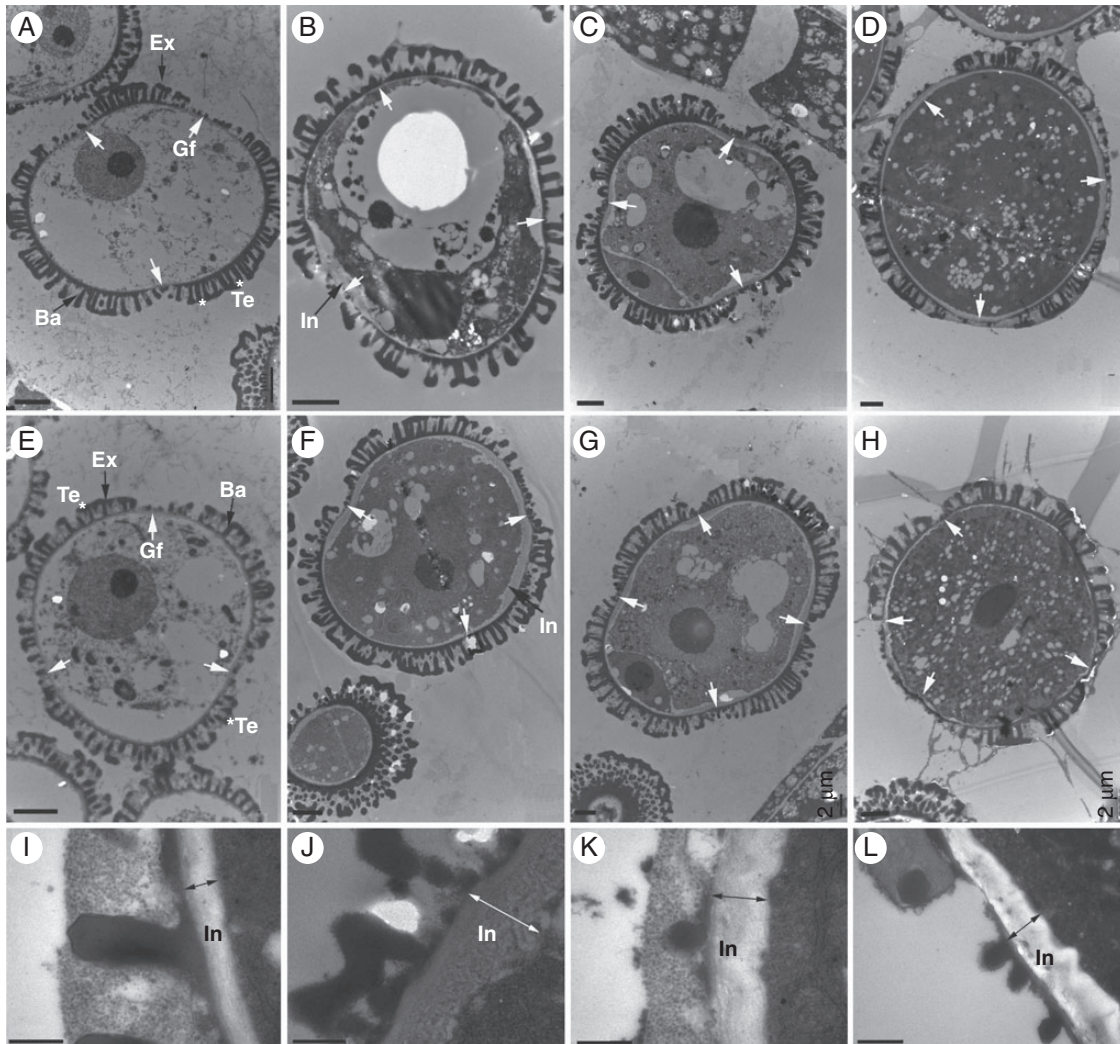
FIG. 7. Transmission electron micrographs of pollen development in the *bcmf8* lines. (A–D) Micrographs of pollen from empty vector pBI121-transformed plants (CK) showing normal pollen morphology with three apertures and a normal development process from the early uninucleate microscope stage to the mature pollen stage. The pollen at the early uninucleate (A), late uninucleate (B), binucleate (C) and trinucleate (D) microscope stages was observed. (E) Micrograph of pollen from the *bcmf8* lines showed normal morphology with three apertures at the early uninucleate microscope stage. (F) Micrograph of pollen from the *bcmf8* lines at the late uninucleate microscope stage showing a remarkable thickening in intine (black arrow) both inside and outside the aperture region. (G) Micrograph of pollen from the *bcmf8* lines at the binucleate microscope stage showing the number of potential apertures to increase to four instead of three as normal. (H) The uneven distribution of the apertures in the *bcmf8* pollen at the mature pollen stage. (I) Mature intine outside the aperture region of the control pollen. (J) Mature intine outside the aperture region of the *bcmf8* pollen. There was a remarkable thickening outside the aperture region, compared with the control pollen. (K) Mature intine inside the aperture region of the control pollen. (L) Mature intine inside the aperture region of the *bcmf8* pollen. Gf, aperture; In, intine; Ex, exine; Te, tectum; Ba, baculum. Scale bars = 2 μm (A–H); 0·5 μm (I–L).

(Fig. 9B). Despite this retardation, the *bcmf8* tubes still managed to elongate after 8 HAP, and finally entered the ovule at the base of the transmitting tract (Fig. 9D), similar to the CK tubes (Fig. 9C). However, markedly fewer pollen tubes could be observed within the *bcmf8* pistils (Fig. 9D) compared with those in CK (Fig. 9C) under the condition where all the pistils were pollinated with enough pollen grains (Fig. 9E–H). Numerous *bcmf8* pollen grains could not germinate (Fig. 9F, H), and remained on the stigma surface. In contrast, most of the CK pollen grains could germinate, pass through the style and enter the ovules (Fig. 9E, G). This result confirms that *BcMF8* influenced pollen germination at the stigma, and contributed to tube growth in the stylar transmitting tissues of pistils. Although the *bcmf8* lines could produce seeds, the significant

decrease in the number of fruit pods was caused by the retardation of pollen tube growth.

DISCUSSION

BcMF8 *is expressed in the pollen tube and activated in the stigma by pollination*

Pollen, pollen wall function and pollen tube growth have been the focus of substantial research because of their biological importance and uniqueness (Becker and Feijo, 2007). In an increasing number of cases, genes with essential gametophytic functions in pollen, including AGP, PG and PME genes, have been discovered by characterizing loss-of-function phenotypes caused by
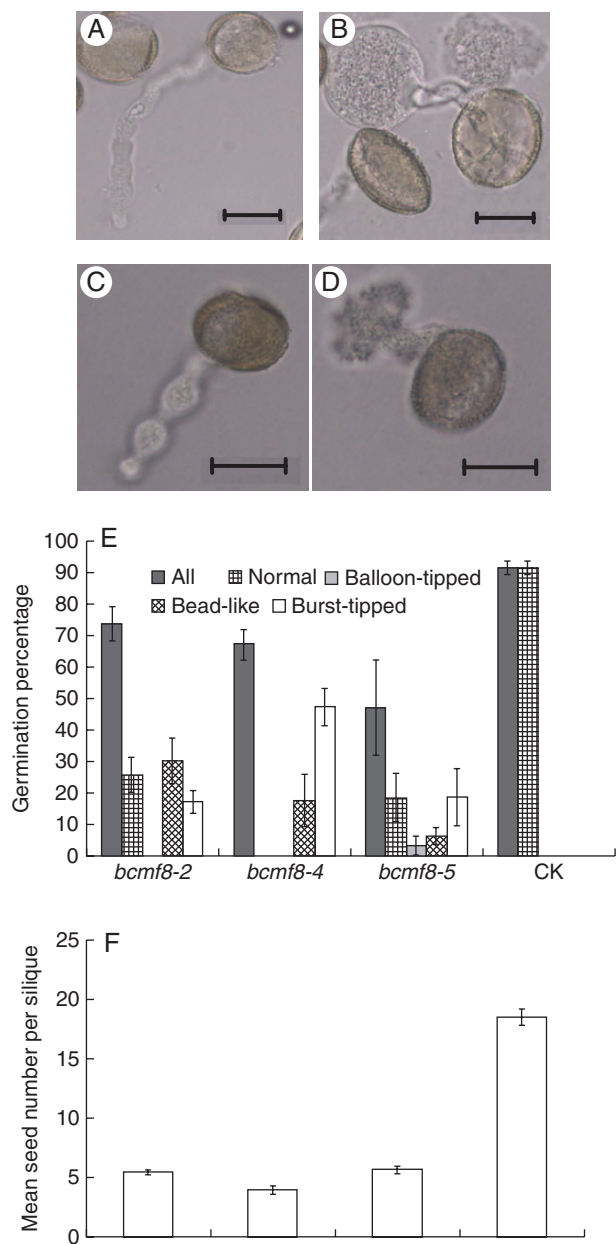
FIG. 8. *BcMF8* inhibition specifically disrupted the *in vitro* pollen tube growth and affected seed set. (A) Pollen from empty vector pBI121-transformed plants (CK) was normal. (B–D) Pollen from the *bcmf8* lines was abnormal. The pollen tubes of the *bcmf8* lines were balloon-tipped (B), bead-like (C) or burst-tipped (D). (E) The germination percentage ($\pm$ s.e.) of the *bcmf8* pollen. (F) The *bcmf8* siliques produced significantly fewer seeds than those of CK. Mean seed numbers ($\pm$ s.e.) per silique of the *bcmf8* lines are shown. Scale bars = 20 μm (A–D).

knockout or knockdown mutations (Twell, 2010). AGPs are widely distributed within the plant kingdom. In the arabidopsis genome, 47 putative AGPs have been found (Gaspar *et al.*, 2001; Borner *et al.*, 2002, 2003). Much biochemical and immunohistochemical evidence indicates that AGPs contribute to different aspects of plant growth and development, specifically sexual reproduction (Garcia and Calzada, 2004; Pereira *et al.*, 2006; Coimbra *et al.*, 2007; Qin *et al.*, 2007; Chen *et al.*, 2008; Lee *et al.*, 2009).

Several AGPs are expressed in a high tissue-specific manner, such as *AtAGP31*, which has high expression in vascular tissues (Liu and Mehdy, 2007); AtAGP30 is exclusively expressed in roots (van Hengel and Roberts, 2003); and *AtAGP6* and *AtAGP11* are expressed specifically in pollen (Pereira *et al.*, 2006; Levitin *et al.*, 2008).

Our previous work showed that *BcMF8* is a pollen-specific gene, whose transcript is expressed at the uninucleate stage and maintained throughout the pollen at the pollination stage (Huang *et al.*, 2008*b*). In the present study, real-time RT–PCR and *in situ* hybridization results reveal that *BcMF8* was also expressed in pollen tubes in the pollinated pistils. Thus, *BcMF8* is a late pollen gene according to the classification by Mascarenhas (1990), and may function in pollen development and pollen tube growth. Notably, *BcMF8* was also activated by pollination and expressed in the stigma during 0 HAP to 3 HAP. During the process of pollen germination, many genes show significant changes (Wang *et al.*, 2008; Qin *et al.*, 2009), and the stigmas produce proteins secreted to the exudate for pollen–pistil interactions (Quiapim *et al.*, 2009). *AGPNa3*, a *Nicotiana* stigma-specific gene, is the only AGP gene that functions in pollen–pistil interactions after pollination (Du *et al.*, 1996; Quiapim *et al.*, 2009). The prevalence of AGPs in reproductive tissues has led to the speculation that they have significant functions therein, ranging from serving as nutrient resources to involvement in cell–cell recognition in plant reproduction (Cheung and Wu, 1999). Taking into account the expression of *BcMF8* in the stigma during the early stage of pollen germination, *BcMF8* possibly participates in pollen–pistil interactions.

BcMF8 is a cell wall and secreted protein

A common feature among the diverse AGPs is that they are highly glycosylated cell wall or secreted proteins, in which >90 % (w/w) carbohydrates are covalently attached to a core protein backbone (Showalter, 2001; Liu and Mehdy, 2007). SDS–PAGE analysis showed that the molecular mass of the BcMF8 protein was approx. 20 kDa, which was 6·8 kDa larger than the molecular weight of 13·2 kDa calculated by ExPASy. Meanwhile, an isoelectric point (pI) of 4·31 was predicted by ExPASy, which may explain why the BcMF8 protein ran more slowly than predicted, as a charged protein can show a very different migration in the electric field even if the sequence is completely correct without any unexpected modifications (Takano *et al.*, 1988). It is worth noting that the BcMF8 protein induced in the *E. coli* expression system was immature, which means that it was not decorated by polysaccharide units.

Arabinogalactan proteins are expressed throughout the plant kingdom, mainly at cell surfaces (Showalter, 2001). Sub-cellular localization of BcMF8 illustrated that BcMF8 was a secreted protein, and exhibited a plasma membrane and extracellular localization. Despite the expected GPI (glycophosphatidylinositol) anchor based on the deduced amino acid sequence (Huang *et al.*, 2008*b*), the BcMF8 protein was still extracellularly secreted. Although GPI could anchor the AGP to the luminal face of the endoplasmic reticulum membrane and the Golgi membrane (Showalter, 2001), the BcMF8 protein could still be released from the plasma membrane for cell wall and extracellular destinations by the action of an endogenous phospholipase,
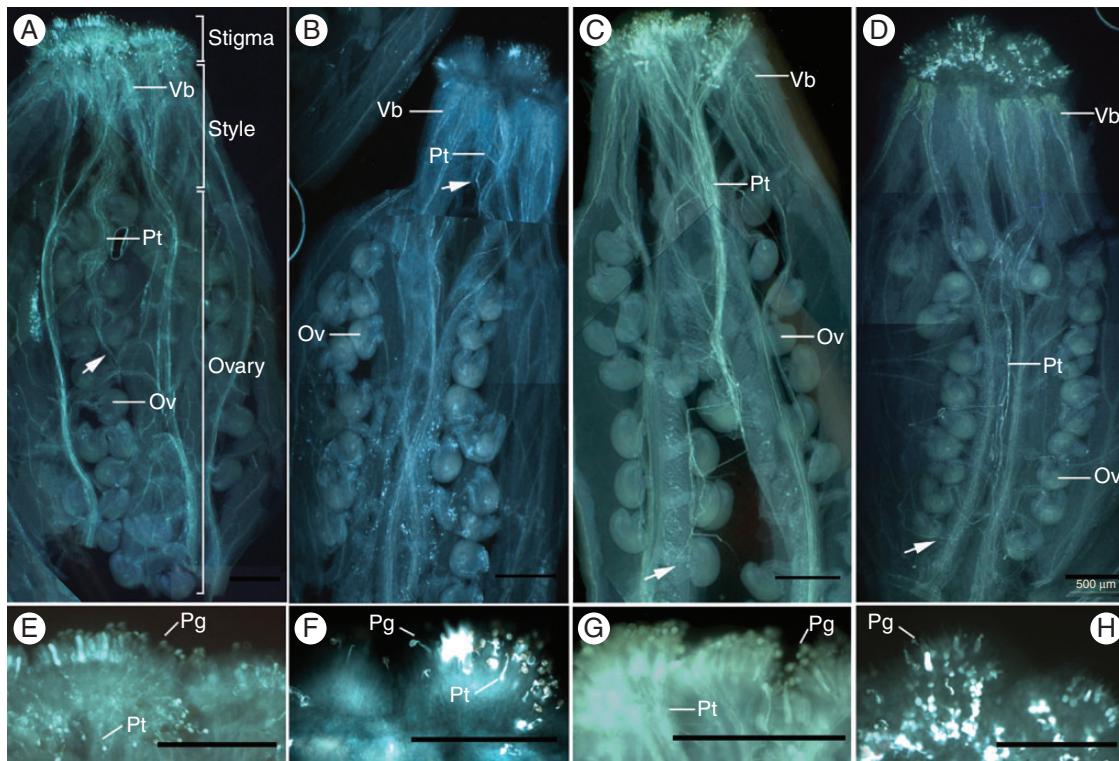
FIG. 9. *In vivo* pollen germination and pollen tube growth in the *bcmf8* lines. (A) Pollen tube growth in the CK female organs at 8 HAP. Pollen tubes had passed through the style tissue and reached approximately half the length of the transmitting tract. (B) Pollen tubes growth in the *bcmf8* female organs at 8 HAP. Part of the pollen germinated at the stigma but could not penetrate through the style tissues. (C) Pollen tube growth in the CK female organs at 24 HAP. The pollen tubes had almost reached the base of the ovary. (D) Pollen tube growth in the *bcmf8* female organs at 24 HAP. Markedly fewer pollen tubes could be observed within the *bcmf8* pistils. (E–H) Magnified images of the corresponding stigma in A–D showing the pollen grains on the stigma. Numerous *bcmf8* pollen grains could not germinate (F, H). Arrows show the position at which the pollen tubes arrived. Vb, vascular bundle; Pt, pollen tube; Ov, ovule; Pg, pollen grain. Scale bars = 500 μm.

similar to its homologous gene *AGP11* in arabidopsis (Borner *et al.*, 2003).

BcMF8 *is important for pollen morphogenesis and aperture formation*

Although some AGP genes are associated with plant vegetative growth or signalling, such as *LeAGP1*, *AGP17*, and *AGP30* (van Hengel and Roberts, 2003; Gaspar *et al.*, 2004; van Hengel *et al.*, 2004; Sun *et al.*, 2004), few molecular results demonstrate the involvement of specific AGPs in pollen function.

At the time of writing, one fasciclin-like AGP gene, *FLA3*, and two homologous members, *AGP6* and *AGP11*, have been proven to contribute to pollen development. *FLA3*-RNA interference (RNAi) transgenic plants displayed pollen abortion during the transition from uninucleate microspores to bicellular pollen, with abnormal cellulose distribution and an abnormal intine layer, suggesting that *FLA3* was involved in microspore development, and may affect pollen intine formation possibly by participating in cellulose deposition (Li *et al.*, 2010). *AGP6* and *AGP11* are specifically expressed in stamens, pollen grains and pollen tubes (Levitin *et al.*, 2008); their products have 68 % amino acid sequence similarity (Gaspar *et al.*, 2001) and share functional redundancy because neither the *agp6* nor the *agp11* mutant shows obvious phenotypic alterations (Levitin *et al.*, 2008;

Coimbra *et al.*, 2009). However, the pollen of plants harbouring mutated *AGP6* and *AGP11* and loss of function in *AGP6* and *AGP11* inside each locule were devoid of content and had a collapsed appearance, showing a condensed cytoplasm, membrane blebbing and the presence of small lytic vacuoles. In addition, the aborted pollen underwent incomplete mitotic division (Coimbra *et al.*, 2009). Further study which revealed reduced germination and elongation of mutant *agp6 agp11* pollen, as well as precocious germination inside the anthers, indicated that AGPs possibly interfere with the timing of pollen germination (Coimbra *et al.*, 2010). However, these investigations could explain neither the mechanism by which AGPs influence the pollen function and sequential pollen tube growth, nor the exact process in which AGPs participate.

Despite the 80·29 % sequence similarity to *AGP11*, the single *BcMF8* knockdown transgenic plants had obvious phenotypic alterations in *B. campestris*. The antisense *BcMF8* transgenic lines produced slipper-shaped and bilaterally sunken pollen, which displayed abnormal intine programming. These results suggest that the *BcMF8* product was one of the most important components of the pollen intine layer. Plasma membrane AGPs may help in maintaining the integrity of the plasma membrane and cell wall matrix (Johnson *et al.*, 2003; MacMillan *et al.*, 2010). *BcMF8* functioned to maintain normal intine formation, providing a stable interior environment for microspore development.

Arabinogalactan proteins are complex compounds of hydroxyproline-rich core proteins, which are decorated by arabinose- and galactose-rich polysaccharide units (Showalter, 2001). Reversible glycosylation is a complicated process, which is implicated in polysaccharide biosynthesis through the conversion of sugar nucleotides into polysaccharides or other carbohydrates (Drakakaki *et al.*, 2006). The core protein backbone and carbohydrate epitopes are essential in AGP function (Showalter, 2001). Therefore, we hypothesized that the defective pollen without a well-defined intine layer in *bcmf8* may result from a lack of a specific protein backbone for the attachment of specific polysaccharides.

A remarkable thickening of *bcmf8* intine was observed not only inside but also outside the prospective aperture region, where exine should occur, which resulted in the number of potential apertures increasing to four instead of three as normal. Although aperture number, positions and morphology vary greatly across different species, apertures usually have precise shapes, sizes, decorations and locations within a given species. The discovery of INAPERTURATE POLLEN1 (INP1) provided the first step in understanding at the molecular level of how such functional apertures took shape on the areas where no exine was deposited (Dobritsa and Coerper, 2012). INP1 was specifically involved in the formation of the pollen surface apertures, which resulted from the restriction of exine deposition at specific sites. The *bcmf8* mutant described in this study underwent unregulated intine programming, as uneven intine thickening occurred at four areas both inside and outside the prospective aperture region. Following the uneven exine deposition at those corresponding domains, four unevenly distributed apertures were observed instead of three. The increase in aperture size and uneven location were indirectly caused by the inaccurate intine formation.

The pollen wall is more complex than other cell walls in plants. Although several genes are associated with pollen wall development, including the pollen mother cell primary wall (Rhee *et al.*, 2003; Francis *et al.*, 2006), callose (Enns *et al.*, 2005) and exine (Sowders *et al.*, 2001; Ariizumi and Toriyama, 2011), few studies have investigated the mechanism underlying intine development. Our previous work indicated the involvement of two other genes, *BcMF2* and *BcMF9*, which belong to the PG gene family, in intine development and pollen tube growth through the regulation of dynamic pectin metabolism (Huang *et al.*, 2009*a*, *b*). *BcMF8* might have a redundant function with *BcMF2* and *BcMF9* in intine development but has special characteristics in aperture formation.

Real-time RT–PCR analysis and *in situ* hybridization illustrated that *BcMF8* was expressed in the pollen tubes. The *in vitro* germination study showed that the *BcMF8* pollen tube was highly unstable when grown in culture medium. Although it was able to germinate *in vitro*, the resulting pollen tube failed to elongate. This finding indicates that the antisense transgenic lines altered the mechanical characteristics of the pollen tube wall and created the imbalance of the apical wall dynamics.

In accordance with this conjecture, retarded pollen tube growth *in vivo* was also observed. Knockdown of *BcMF8* not only led to the reduced germination rate of the pollen tubes on the surface of stigmatic cells, but also retarded the pollen tube penetrating the style tissue and transmitting tract. Despite the *bcmf8* pollen tube entering the base of the transmitting tract, significantly fewer seeds of *bcmf8* were produced, which resulted in a reduction in male fertility. Thus, this putative AGP, BcMF8, had a crucial function in modulating the physical nature of the growing pollen tube wall, and helped in maintaining the integrity of the pollen tube wall matrix. Previous studies indicated that the style and transmitting tract are composed of cells that secrete a specialized nutrient-rich extracellular matrix (ECM) (Sassen, 1974). Most AGPs are implicated in pollen tube nutrition and guidance (Cheung and Wu, 1999; Wu *et al.*, 2000). Tissue-specific proteins display a gradient of increasing glycosylation from the stigma to the ovary, thereby inducing the pollen tube to elongate along the style and transmitting tracts. We hypothesized that the *BcMF8* product has a special distribution in the pollen tubes. The inhibition of *BcMF8* expression in *bcmf8* may disturb the balance of polysaccharide distribution in the pollen tubes, as well as the interaction between the pollen tubes and ECM, resulting in retarded pollen tube growth and fewer seeds.

Given the efficiency of antisense technology, partial pollen tubes were suggested to reach the base of the ovary, enter the ovules and produce the seeds, because *BcMF8* was not completely inhibited. The possibility that complete disruption of the *BcMF8* gene would result in a stronger phenotype or null mutation, determined using using RNAi or artificial microRNA technology, which has higher efficiency of gene silencing, could not be eliminated. The AGP family in *B. campestris* had quite a substantial number of members, similar to the arabidopsis genome in which 47 putative AGPs have been found (Gaspar *et al.*, 2001; Borner *et al.*, 2002, 2003). Other AGP family members and genes that are critical for pollen germination and tube growth might share functional redundancy with *BcMF8*.

BcMF8 *product is essential for pollen tube growth in female floral tissue*

As the continuation of the intine layer, quite a lot is known about pollen tube growth (Stone *et al.*, 2004). During this process, gene products involved in cell rescue (stress/abiotic/heat-related function), proteins with binding functions and cellular transport are over-represented, except for the pre-stored transcripts and proteins in pollen (Wang *et al.*, 2008). Many genes are critical for pollen germination and tube growth (Shi and Yang, 2010). The AGP gene family makes up an important part of these genes (Pereira *et al.*, 2006).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: pistils of *Brassica campestris* ssp. *chinensis* genic male-sterile line system ('*Bcajh97-01A/B*') hybridized with a *BcMF8* sense probe. Figure S2: construction of the expression vector pBI*35S::BcMF8A*. Figure S3: detection of the marker gene *NPTII* in the *bcmf8* lines by PCR amplification. Figure S4: detection of the marker gene *NPTII* in the *bcmf8* lines by Southern blot.

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