

RESEARCH PAPER

The polygalacturonase gene *BcMF2* from *Brassica campestris* is associated with intine development

Li Huang, Jiashu Cao*, Aihong Zhang, Yiqun Ye, Yuchao Zhang and Tingting Liu

Laboratory of Cell & Molecular Biology, Institute of Vegetable Science, Zhejiang University, Hangzhou 310029, China

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Abstract

Brassica campestris Male Fertility 2 (*BcMF2*) is a putative polygalacturonase (PG) gene previously isolated from the flower bud of Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* Makino, syn. *B. rapa* ssp. *chinensis*). This gene was found to be expressed specifically in tapetum and pollen after the tetrad stage of anther development. Antisense RNA technology was used to study the function of *BcMF2* in Chinese cabbage. Scanning and transmission electron microscopy revealed that there were deformities in the transgenic mature pollen grains such as abnormal location of germinal furrows. In addition, the homogeneous pectic exintine layer facing the exterior seemed to be overdeveloped and predominantly occupied the intine, thus reversing the normal proportional distribution of the internal endintine layer and the external exintine layer. Since it is a continuation of the intine layer, the pollen tube wall could not grow normally. This resulted in the formation of a balloon-like swelling structure in the pollen tube tip in nearly 80% of the transgenic pollen grains. Premature degradation of tapetum was also found in these transgenic plants, which displayed decreased expression of the *BcMF2* gene. *BcMF2* might therefore encode a new PG with an important role in pollen wall development, possibly via regulation of pectin's dynamic metabolism.

Key words: *Brassica campestris*, *Brassica rapa*, Chinese cabbage, intine, PG, polygalacturonase, pollen wall.

Introduction

An important feature of the *Arabidopsis* pollen transcriptome is the existence of a large number of genes related to cell wall synthesis and regulation (Becker *et al.*, 2003; Pina *et al.*, 2005). Analysis of Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* Makino, syn. *B. rapa* ssp. *chinensis*) pollen gene expression also reveals that genes related to cell wall synthesis and regulation occupy a large proportion of the pollen transcriptome (Huang *et al.*, 2008). A unique characteristic of the microspore is the pollen wall. In trinucleate pollen species, although there is species-specific variation, the pollen wall is generally composed of the outer exine and inner intine. The exine, facing the exterior, comprises two layers: the innermost and featureless nexine; and the outer sculpted sexine, which presents multiple species-specific pores and furrows (Shukla *et al.*, 1998). The intine is the innermost layer and

is located adjacent to the pollen plasma membrane, which is a highly ordered complex of polysaccharides and structural proteins similar to other plant cell walls (Owen and Makaroff, 1995). The intine also consists of two layers: a granular exintine with pectin and protein inclusions facing the exterior, and a microfibrillar cellulosic endintine facing the interior. The exine is formed from the material released by tapetum (sporophytic origin), while the intine is secreted by the microspore (gametophytic origin) during the ring-vacuolated microspore stage (Owen and Makaroff, 1995). Both layers of the pollen wall are important for pollen hydration, activation, germination, and pollen tube growth (Blackmore and Barnes, 1990; Owen and Makaroff, 1995). Thus, abnormalities of the pollen wall often render the pollen grain unable to fulfil its biological function.

* To whom correspondence should be addressed. E-mail: jshcao@zju.edu.cn

Abbreviations: *BcMF2*, *Brassica campestris* Male Fertility 2; *bcms*, *Brassica campestris* male sterile; cDNA-AFLP, cDNA-amplified fragment length polymorphism; DAPI, 4', 6-diamidino-2-phenylindole; PG, polygalacturonase; RT-PCR, reverse transcription-PCR; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

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As an indispensable part of pollen development, pollen wall formation is a complicated process involving the activity of numerous genes. Several mutants aberrant in exine formation have been characterized (Aarts *et al.*, 1997; Paxson-Sowers *et al.*, 1997, 2001; Ariizumi *et al.*, 2003), but relatively few mutants defective in intine formation have been found. Aside from genetic screening, isolating genes or proteins specific to cell wall metabolism has provided a feasible way to elucidate the molecular mechanism underlying pollen wall formation. Such experiments were further facilitated by the release of the *Arabidopsis* genome. A large number of genes, representing many gene families, are considered to be involved in cell wall synthesis and regulation, such as a number of cDNA clones encoding pectin-degrading enzymes including polygalacturonase (PG), pectic lyases, pectin methylesterase (PME), and polymethylgalacturonases. The PG family of enzymes dominates plant cell function, functioning as hydrolases and as loosening enzymes in the degradation of pectin and the disintegration of the cell wall. A high level of exo-PG activity has been detected in the pollen of many species (Hadfield *et al.*, 1998). Many pollen-specific PG genes have also been cloned, and it appears that a number of PG family members might be present in pollen (Allen *et al.*, 1993; Honys and Twell, 2003). It is speculated that PG in a pollen tube disintegrates the style cell wall, allowing pollen tubes to pass through. Alternatively, PG could act on a pollen tube's own cell wall, thus accelerating its growth (Brown *et al.*, 1990). However, most of the current research on pollen PG genes remains at the level of cloning and expression pattern identification. There is negligible direct evidence regarding the role of PG genes in the development of pollen or pollen tubes.

A previous study identified a male-sterile mutant which lacks mature pollen, *Brassica campestris male sterile (bcms)*, among *B. campestris ssp. chinensis* (Huang *et al.*, 2008). Genome-wide transcriptional profiling was performed on the flower buds of *bcms* and the wild type using cDNA amplified fragment length polymorphism (cDNA-AFLP) technology together with genome array analysis. This methodology detected five putative PG genes expressed exclusively in wild-type flower buds (Wang *et al.*, 2005; Huang *et al.*, 2008; genome array data not shown). All of these genes [*BcMF2* (*Brassica campestris Male Fertility 2*), *BcMF6*, *BcMF9*, *BcMF16*, and *BcMF17*] display typical exo-PG structure in the deduced amino acid sequences, but possess distinct nucleotide sequences. These putative PG genes were previously considered important in pollen development, but the exact roles were not fully delineated. Here, a novel investigation of one such gene, *BcMF2*, is presented. Sequence analysis revealed that *BcMF2* shares 83% and 82% similarity with the nucleotide and amino acid sequences, respectively, of the *Arabidopsis* exo-PG gene *PGA4* (At1g02790). The *PGA4* gene was detected early in the *Arabidopsis* pollen transcriptome and was considered pollen specific; however, its exact role in pollen development is not yet clear (Honys and Twell, 2003). In this study, the function of *BcMF2* in pollen development was investigated

with antisense RNA technology. Possible effects of *BcMF2* on pollen and the pollen wall are discussed; the results obtained in this study may provide valuable insight regarding PG genes and pollen.

Materials and methods

Plant materials

The *bcms* mutant of *B. campestris ssp. chinensis* Makino was found in the field in the 1980s. It was maintained by crossing it with its wild-type ancestor. The plants were then cultivated in the experimental farm of Zhejiang University.

RNA extraction, cDNA synthesis, and reverse transcription-PCR analysis

Total RNA was extracted from different tissues using Trizol reagent (Gibco, Berlin, Germany), while the poly(A)⁺ RNA was isolated from the total RNA with an Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). The first-strand cDNA and double-stranded cDNA were then synthesized using a SMART[™] cDNA Library Construction Kit (Clontech, Mountain View, USA) according to the manufacturer's instructions.

Two rounds of reverse transcription-PCR (RT-PCR) were conducted with two independently isolated total RNA samples as templates. The RT-PCR was performed for 18, 23, 28, 33, and 38 cycles to determine the linearity of the PCR, and ultimately 28 cycles was determined as the optimal number. A 350 bp *Actin-1* fragment was amplified under the same conditions to serve as a positive control. The gene specificity of the RT-PCR products was then confirmed by sequencing.

In situ hybridization

Flower buds at different developmental stages were fixed in 4% formaldehyde phosphate-buffered saline (PBS) solution with 0.1% Triton X-100 and 0.1% Tween-20, serially dehydrated, cleared with dimethylbenzene, and embedded in paraffin. Sections (8 μm) of flower buds were hybridized to specific digoxigenin-labelled RNA probes (Roche). Templates for the *BcMF2*-specific probes were obtained from amplification with specific primers. The sense and antisense probes were synthesized using an SP6/ T7 transcription kit (Roche).

Antisense RNA construction and plant transformation

Partial cDNA sequences (nucleotides 232–680) of *BcMF2* were amplified by PCR and cloned in the antisense orientation into the binary vector pBI121. Restriction digestion, PCR, and DNA sequencing confirmed the correct orientation of the fragment. The antisense *BcMF2* RNA construct was transferred into the Chinese cabbage mediated by *Agrobacterium tumefaciens* as described by Yu *et al.* (2004). As a control, an empty vector pBI121 was also transferred into the Chinese cabbage.

PCR analysis, Southern hybridization, and northern blotting

For PCR and Southern hybridization, genomic DNA was extracted from ~0.5–1 g of fresh young leaves of putative transformed and non-transgenic plants according to the procedures described by Cao *et al.* (1995). PCR was performed to detect a 680 bp fragment of the NPTII gene in the pBI121 vector. Genomic DNA was digested with *EcoRI* for Southern hybridization to confirm the integration of the antisense RNA fragment of *BcMF2* into the plant genome. The digested genomic DNAs were fractionated on 0.8% agarose gels, transferred onto a nylon membrane, and hybridized to α -³²P labelling probes. The DNA probes were prepared by labelling the NPTII gene using a Random Primer DNA Labeling Kit Ver.2 (Dalian, China) according to the manufacturer's instructions.

Northern blotting of total RNA from flower buds and flowers was performed to detect the expression of *BcMF2* in the transgenic or control plants. Total RNA (20 μ g) was separated by electrophoresis on a 1.2% formaldehyde agarose gel, followed by blotting onto a nylon membrane (Hybond-N+, Amersham Pharmacia), and probed with α -³²P labelling, as for the procedure in Southern hybridization. Hybridization was performed as previously described (Cho *et al.*, 1997). The Southern and Northern hybridized membranes were scanned with a Typhoon 9210 scanner (Amersham Bioscience, Uppsala, Sweden).

Pollen analysis, microscopy, and pollen germination observation

For pollen staining, anthers were dissected to release pollen grains followed by incubation in different histochemical stains. To stain nuclei and cellulose, 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes) was used. Solutions of DAPI were prepared and used as previously described (Regan and Moffatt, 1990). Aniline blue (0.1%) was used to stain the callose in pollen and observed with epifluorescence optics in a Leica microscope.

For *in vitro* pollen germination, pollen grains were collected and cultured in the culture medium [15% sucrose (w/v); 0.4 mmol l⁻¹ HBO₃; 0.4 mmol l⁻¹ Ca(NO₃)₂; 0.1% agar (w/v)]. The pollen grains spread on the agar plates were cultured immediately at 20–25 °C, 100% relative humidity. The germinating pollen grains were counted under a microscope. Three independent *BcMF2* transgenic lines and the corresponding control lines were examined and more than five flowers from each plant were tested. From each culture, at least 300 pollen grains were examined to calculate an average germination rate, and 20 pollen tubes were measured for the average pollen tube length.

For environmental scanning electron microscopy (SEM), individual pollen grains from either control or *BcMF2* transgenic plants were mounted on SEM (Philips XL-40) stubs and coated with palladium–gold using standard techniques and vacuum desiccation. Digital images were then taken.

For transmission electron microscopy (TEM), anthers were fixed with 2.5% glutaraldehyde (containing 0.01% Tween-20) overnight, and rinsed in 0.1 M phosphate buffer, then transferred to 1% osmic acid for 1 h. Specimens were washed again in phosphate buffer as above, and dehydrated through an ethanol series to 80% ethanol. Samples were then embedded in Spurr's resin, with the ultrathin sections stained with uranyl acetate and lead citrate before being viewed in a JEM-1230 electron microscope operating at 80 kV.

Results

BcMF2 is a PG gene expressed in pollen

To investigate the *BcMF2* gene further, its deduced amino acid sequence was compared with those of other published plant PGs. In addition to the sequences formerly used to classify the plant PGs by Markovič and Janeček (2001), other known PGs were also downloaded from the database (Supplementary Table 1 available at *JXB* online) and then the PG phylogenetic tree was reconstructed using the maximum parsimony (MP) method. This resulted in a tree with four main clades, similar to the one previously reconstructed (Torki *et al.*, 2000; Athanasiou *et al.*, 2003) (Fig. 1). *BcMF2* belongs to clade C, in which it was expected to observe pollen expression of the corresponding PG genes, encoding exo-PG (Torki *et al.*, 2000). If the hypothesis that clade C is comprised entirely of genes expressed in pollen (Torki *et al.*, 2000) is true, the separation of *BcMF2* to clade C may suggest a pollen-expressed pattern.

To test this hypothesis, the expression pattern of *BcMF2* was analysed. Its expression was first investigated in different tissues using RT-PCR and Northern blotting. Flower buds of five different sizes, open flowers, germinal siliques, scapes, and leaves were selected as materials. The developmental stage of the various sized flower buds was determined by cytological observation. In wild-type Chinese cabbage, flower buds with a diameter \leq 1.0 mm (stage I flower buds) corresponded to the onset of microspore mother cell formation or the period leading up to it. Flower buds with a diameter of ~1.6 mm (stage II flower buds) represented the meiosis period. Flower buds with a diameter of ~2.2 mm (stage III flower buds) corresponded to the tetrad period. Flower buds with a diameter of 2.8 mm (stage IV flower buds) represented the uninucleate pollen period. Finally, flower buds with the largest diameter were the opening flower buds (stage V flower buds). At this point, opened anthers and a pollen-spreading appearance could be observed (Huang *et al.*, 2008). Materials from the same tissue obtained from the *bcms* mutant were used as negative controls.

Both RT-PCR and Northern blotting results showed that transcription of *BcMF2* first occurred in the stage III flower buds. The expression of *BcMF2* reached its maximum in the stage IV flower buds and decreased gradually thereafter as development proceeded, though it was comparatively high in the stage V flower buds and the open flowers. Expression continued until the formation of siliques. Notably, no *BcMF2* expression was detected in the leaves or scapes of

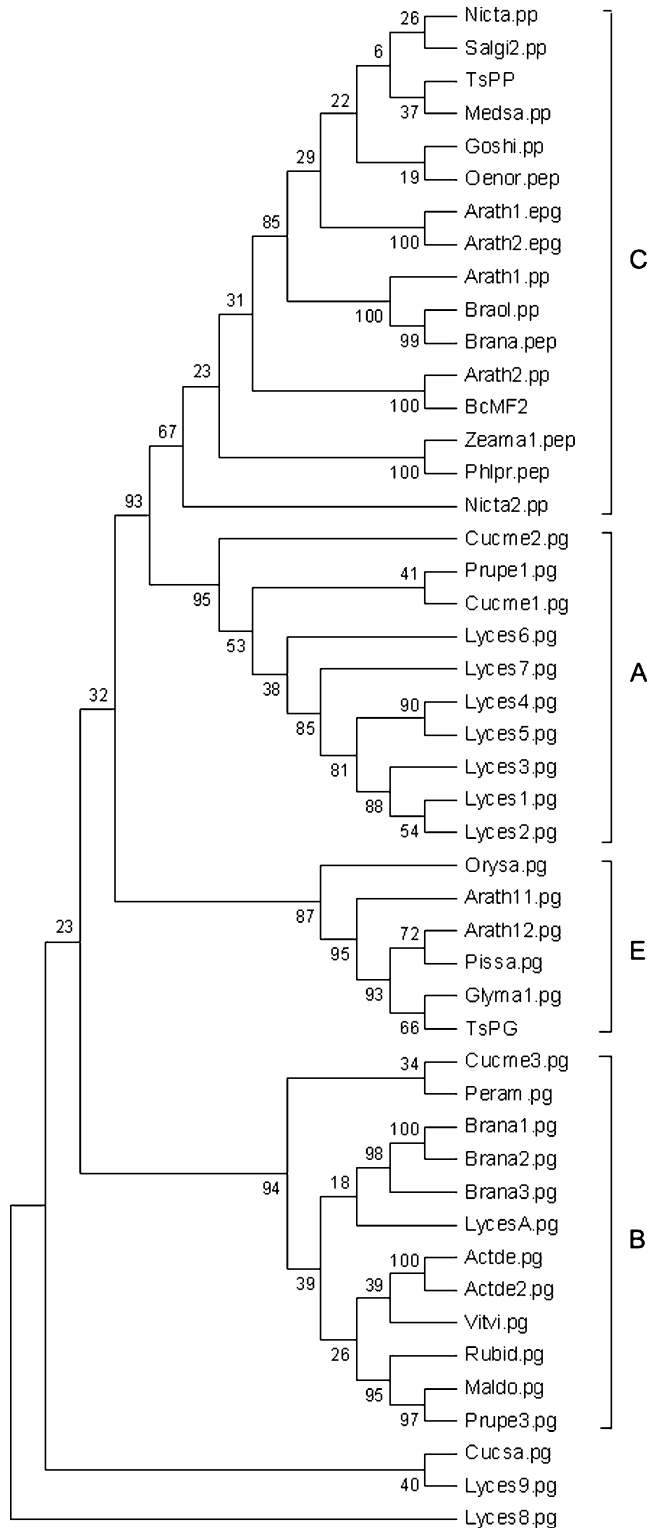


Fig. 1. BcMF2 protein in the molecular phylogenetic trees based on the amino acid sequence of plant PGs using the maximum parsimony (MP) method. Numbers on the tree represented confidence values from bootstrap test (1000 replicates).

stage II or stage I flower buds prior to meiosis (Fig. 2). Meanwhile, *BcMF2* expression was not detected in any tissue of the *bcms* mutant (data not shown).

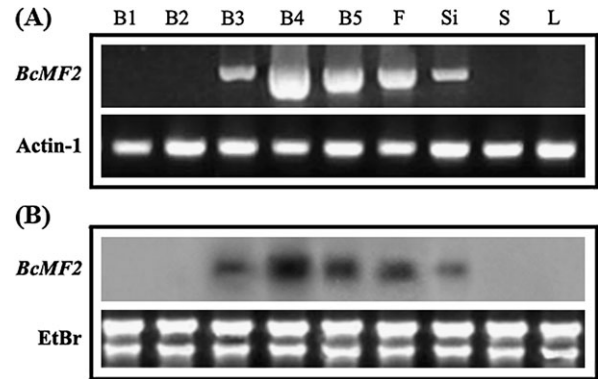


Fig. 2. Spatial and temporal expression pattern of *BcMF2* from Chinese cabbage. (A) RT-PCR analysis of the expression pattern of *BcMF2*. (B) Northern blotting analysis of the expression pattern of *BcMF2*. (B1–B5) The flower buds of Chinese cabbage at stages I–V, respectively. F, Si, S, and L indicate the flowers, germinal siliques, scape, and leaves, respectively. Transcripts for the ubiquitously expressed *Actin-1* gene were used as controls.

To investigate further the cellular location of *BcMF2* mRNA, *in situ* hybridization analysis was performed on transverse sections of flower buds at different developmental stages. Hybridization signals were first detected in the tapetal cells and in the microspore tetrads of the anther (Fig. 3B), though the signals were comparatively weak. Stronger signals were found in anthers at the uninucleate microspore stage (Fig. 3C). Strong signals were also shown in the tapetum at early degradation stage and in the binucleate microspore; at this time, the signal in microspores was stronger than that in previous stages (Fig. 3D). In anthers at the mature pollen stage, the tapetum was completely degraded; during this period, the *BcMF2* signal in the mature pollen grains reached its peak (Fig. 3). There was also strong expression in the ovules of old flower buds at the anther dehiscence stage (Fig. 3L). No expression signal was observed either in the anthers prior to meiosis (Fig. 3A) or in the sense control (Fig. 3F–J, M).

Inhibition of BcMF2 expression results in abnormal pollen tube growth and consequent reduction in male fertility

To investigate the exact role of *BcMF2* in the development of pollen or anther, antisense RNA was used specifically to inhibit *BcMF2* expression in Chinese cabbage. Antisense RNA expression vectors under the constitutive cauliflower mosaic virus (CaMV) 35S promoter were introduced to a normal fertile plant by the *Agrobacterium*-mediated method. After kanamycin screening, PCR, and Southern blotting analysis, a total of seven transformed lines (named *bcmf2*) were obtained. At the same time, control transformed plants were created with a pBI121 empty vector.

Northern blotting of five randomly selected transgenic plants from each transgenic line revealed that *BcMF2* expression was fairly inhibited in the flower buds of transgenic plants. Three of the five transgenic lines had no

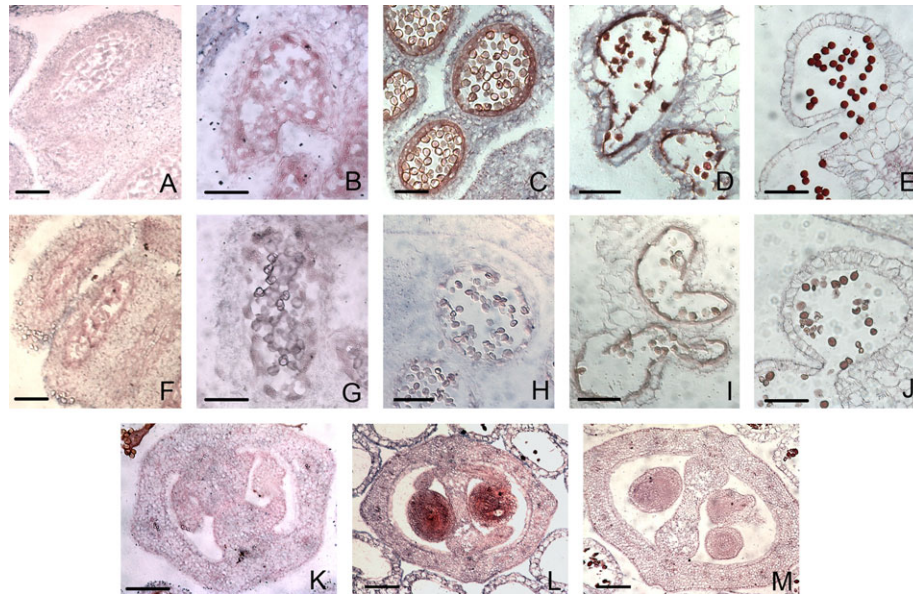


Fig. 3. Analysis of the expression pattern of *BcMF2* in Chinese cabbage using *in situ* hybridization. (A–E) Sections of anthers at pollen mother, tetrads, uninucleate, binucleate, and mature pollen stages, respectively, hybridized with a *BcMF2* antisense probe. (F–J) Section of anthers at pollen mother, tetrad, uninucleate, binucleate, and mature pollen stages, respectively, hybridized with a *BcMF2* sense probe as a negative control. (K) Section of anthers at the uninucleate pollen stage hybridized with a *BcMF2* antisense probe showing no expression in ovules. (L) Section of anthers at the mature pollen stage hybridized with a *BcMF2* antisense probe showing strong expression in ovules. (M) Section of anthers at the mature pollen stage hybridized with a *BcMF2* sense probe as a control. Scale bars = 20 μm .

expression signals in the flower buds; in the other two, a weak hybridization signal was detected, but it was significantly lower than expression levels in normal plants (Fig. 4). In contrast, a high expression level of *BcMF2* was detected in the flower buds of the wild-type Chinese cabbage from which the gene was originally isolated. High levels were also observed in the empty vector-transformed plant, selected as control plants in this experiment. As expected, no hybridization signal was detected in the flower buds of the *bcms* mutant. These results verified that the antisense gene effectively inhibited gene expression.

It was found that all the transgenic plants had normal vegetative growth; they flowered normally and had normal sepal, petal, gynoecium, nectary, and other floral organs. Seven *bcmf2* plants from three transgenic lines were also compared with empty vector-transformed plants. The plants were shown by Northern blotting analysis to lack *BcMF2* expression in flower buds and had shorter filaments and shrunken anthers, with a relatively small amount of pollen (Fig. 5A). Three *bcmf2* plants from three different lines, all displaying this phenotype, were chosen for further investigation. To test the fertility of the transgenic plants, self-pollination tests were performed during the budding period. The transgenic plants were able to bear fruit pods when pollinated with enough pollen grains. However, comparing the transgenic self-pollinated fruit pods with those of the empty vector-transformed plants revealed size reductions of ~50% in the transgenic plants. Each empty vector-transformed plant could produce a fruit pod with ~15 seeds, whereas the fruit pods of transgenic plants could only produce between one and five seeds (Fig. 5B). A test-cross

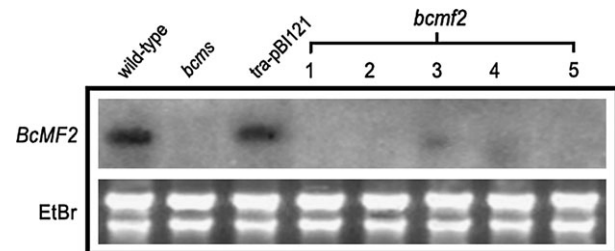


Fig. 4. Detection of the expression of *BcMF2* in flower buds of antisense transgenic Chinese cabbage by northern blotting. *bcms*, *bcms* mutant of Chinese cabbage; tra-pBI121, transformed plants with an empty pBI121 vector; 1–5 refers to five different transgenic lines of *bcmf2*.

was then performed, pollinating transgenic plants with the normal pollen of an empty vector-transformed plant. The resulting fruit pod showed no difference in fruit size or seed number in comparison with the self-pollinated fruit pods of empty vector-transformed plants. These facts demonstrate that the female fertility and ovule development of these transgenic plants are normal, such that the reduction of fruit set rate should be attributed to the abnormality in male organs. Aniline blue staining showed that the callose in transgenic pollen was normal (Fig. 5C, D), while DAPI staining showed that transgenic pollen underwent normal karyokinesis (Fig. 5E, F). Furthermore, when enough transgenic pollen grains were collected and germinated *in vitro*, it was found that the germination rate reached 84%. This was similar to the 83% germination rate of the empty vector-transformed plant pollen (calculated after a

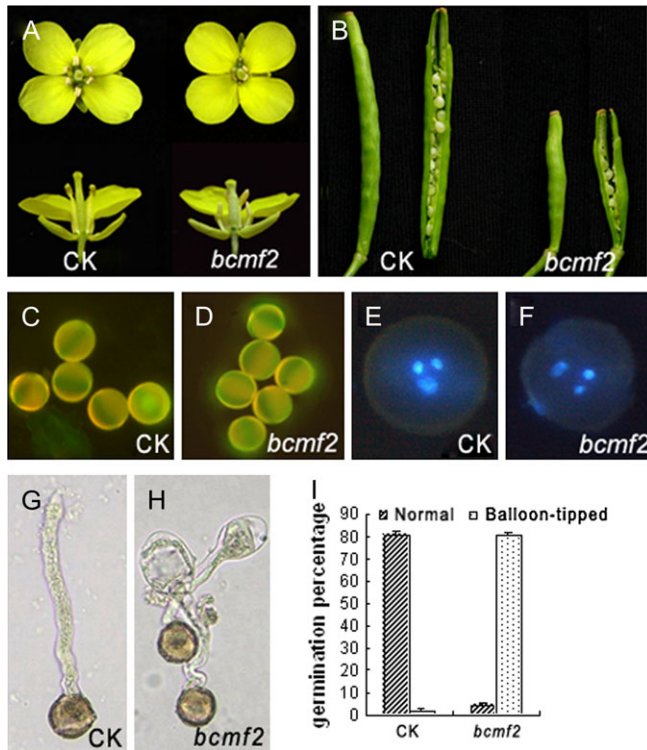


Fig. 5. Characterization of *bcmf2* transgenic Chinese cabbage. (A) Top and side view of the flowers from empty vector-transformed plants (CK) and *bcmf2* transgenic plants. (B) Siliques with seeds of empty vector-transformed plants (CK) and *bcmf2* transgenic plants. (C and D) Aniline blue staining of the callose of pollen from empty vector-transformed plants (CK) and *bcmf2* transgenic plants. (E and F) Staining of the nuclei of the pollen from empty vector-transformed plants (CK) and *bcmf2* transgenic plants. (G and H) *In vitro* germination showed normal pollen tube growth of the empty vector-transformed plant (CK) and the balloon-tipped pollen tube of the *bcmf2* transgenic plants. (I) Comparison of the percentage growth of the pollen tube between the empty vector-transformed plant (CK) and *bcmf2* transgenic plants.

4 h incubation). It seemed that the transgenic pollen lacked any abnormality. At 6 h after incubation, the germination rate of transgenic pollen grains was still maintained at 84%. However, ~80% of the pollen tubes formed abnormally; these pollen tubes exhibited a balloon-swelling structure in the tip after the tube had grown to a certain length. Only ~4% of the transgenic pollen grains grew normal pollen tubes. Even for this segment of the population, the pollen tubes eventually stopped growing and exhibited inclusions of disorderly distribution (Fig. 5H, I). For the empty vector-transformed plants, the proportion of pollen germinated with normal tube growth reached 81%; balloon-tipped structures appeared at a rate of only ~2% (Fig. 5G, I). To test further whether the pollen tube growth was normal *in vivo*, an attempt was made to observe pollen tube growth in the pistil after pollination. However, no high-quality images are available because the bulk of vascular bundles in the large, thick pistils of Chinese cabbages also stain with aniline blue, and this interfered

with discrimination of pollen tubes. Nonetheless, the much lower fruit set rate for transgenic plants as compared with empty vector-transformed plants after self-fertilization may provide indirect but persuasive evidence for abnormality in the *in vivo* pollen tube elongation of these transgenic plants. Furthermore, the seeds harvested from transgenic plants were pollinated by pollen grains with normal pollen tube growth. In contrast, pollen tubes with a swelling at the tip could not reach the ovules.

BcMF2 inhibition leads to pollen deformities with abnormal intine development

In order to determine the mechanism underlying abnormal pollen tube growth in *bcmf2* transgenic plants, pollen morphology was investigated further with SEM. The pollen development process was examined using TEM. SEM showed that nearly 100% of the pollen from *bcmf2* plants had deformities (Fig. 6A, B). Compared with the normal ovular shape of pollen grains, with three evenly distributed germinal furrows (Fig. 6C, D), the pollen grains of transgenic plants were irregular in shape with a disordered distribution of germinal furrows.

Pollen development initially seemed to be normal in the transgenic plants. The cell nuclei exhibited a normal division and differentiation process; the cell organelles formed normally; all of the distinct developmental stages including uninucleate, binucleate, and trinucleate could be observed (Fig. 7B, G, H). These observations supported previous results obtained by aniline blue staining and DAPI staining, showing normal callose metabolism and normal karyokinesis. However, close scrutiny revealed that the pollen shape was somewhat different from that of control pollen. This difference, probably due to abnormal germinal furrow formation, could be detected at the early uninucleate microspore stage. In normal control pollen, germinal furrow formation followed pollen wall formation. At the free microspore stage, the exine began to take shape, exhibiting clear tectum and baculum layers (Fig. 7D). At the uninucleate stage, the exine layers underwent further thickening; however, in the regions of the three prospective germinal furrows, no exine deposition occurred. Moreover, these regions were depressed towards the interior, leading to a cloverleaf pattern on the microspore (Fig. 7E). In the case of transgenic pollen, germinal furrows were indicated by a regional lack of exine, but the cloverleaf pattern was replaced by an irregular shape caused by the disordered distribution of germinal furrows (Fig. 7B). In late-stage uninucleate pollen, the outer and inner layers of intine began to form in control pollen, where the intine within the germinal furrows was much thicker than that in the other regions (Fig. 7F). At this stage, the intine of the transgenic pollen also began to form, but the germinal furrow's intine layer was thickened to a greater extent than in the control plants. Moreover, coverage was significantly increased in most pollen grains, creating the appearance of only one or two germination furrows. The germinal furrow frequently accounted for almost half of the pollen circumference in



Fig. 6. Scanning electron microscope of pollen from *bcmf2* transgenic Chinese cabbage. (A and B) Deformities in pollen grains of the *bcmf2* transgenic plants. (C and D) Normal pollen grains of empty vector-transformed plants (CK).

transgenic pollen (Fig. 7C). At the early binucleate pollen stage, intine within the germinal furrow of both the control (Fig. 7J) and transgenic pollen underwent further thickening. However, thickening was clearly becoming more extreme in the transgenic pollen (Fig. 7G). When pollen developed to the trinucleate and maturation stages, the intine grew further in both the control (Fig. 7K, L) and transgenic pollen (Fig. 7H, I). However, the relative proportion of exintine and endintine in the mature intine differed between the transgenic and control pollen, potentially misinterpreted as varying intine composition. Outside the germinal furrow region, the transgenic pollen had no obvious demarcation between the exintine and endintine layers. Notably, the homonymous granular exintine layer facing the exterior predominantly occupied the intine (Fig. 8A). In the same region of the control pollen, although the demarcation of exintine and endintine layers also was not clearly delineated, the intine appeared more microfibrillar, mainly occupied by the endintine layer (Fig. 8B). The demarcation was clear, however, in the region inside of the germinal furrow. A multilamellar intine within the germinal furrow was visible in both transgenic and control pollen: a granular exintine towards the exterior and a microfibrillar endintine towards the interior, beneath the nexine. However, in the transgenic pollen, the granular exintine overdeveloped to a thicker layer and occupied predominantly the intine layer. The internal microfibrillar layer occupied only a relatively small proportion of the total (Fig. 8C). In contrast, the internal microfibrillar endintine occupied a large proportion in control pollen, where the external granular exintine occupied a small proportion (Fig. 8D). Therefore, the abnormal thickening of the intine in transgenic plants was due to overaccumulation of the granular

exintine. Exine of the transgenic pollen had relatively normal development, including the formation of tryphine at late pollen maturity stages. From these results, it is concluded that the deformity of the pollen grains detected by SEM might result from abnormal pollen wall development. The abnormal thickening of intine results in an imbalanced force upon the pollen wall, causing the twisted shape observed in transgenic pollen grains.

Inhibition of BcMF2 expression results in early degradation of the anther tapetum

The anther tapetum development process in *bcmf2* transgenic plants was also observed with TEM. No obvious defect in morphology or organellar distribution was detected, but the degradation process of tapetum seemed to be accelerated. Prior to the late uninucleate pollen stage, the tapetum development of the transgenic plants was not significantly different from that of the empty vector-transformed plant. However, for late-stage uninucleate pollen, it was observed that a large number of elaioplasts emerged in the anther tapetum of the transgenic plants, which forecast late degradation of tapetum (Fig. 9A). At the early binucleate pollen stage, the elaioplasts increased in number and were further enlarged (Fig. 9B). In late-stage binucleate and trinucleate pollen, the tapetum underwent serious degradation and fell off. The anther locule was full of material being shed at the last stage (Fig. 9C). However, in empty vector-transformed plants, a large amount of elaioplasts began to appear in the late-stage binucleate pollen. The tapetum degradation process was noticeably slower than in the transgenic plants (Fig. 9D, E). At the trinucleate pollen stage, the tapetum was still relatively complete (Fig. 9F).

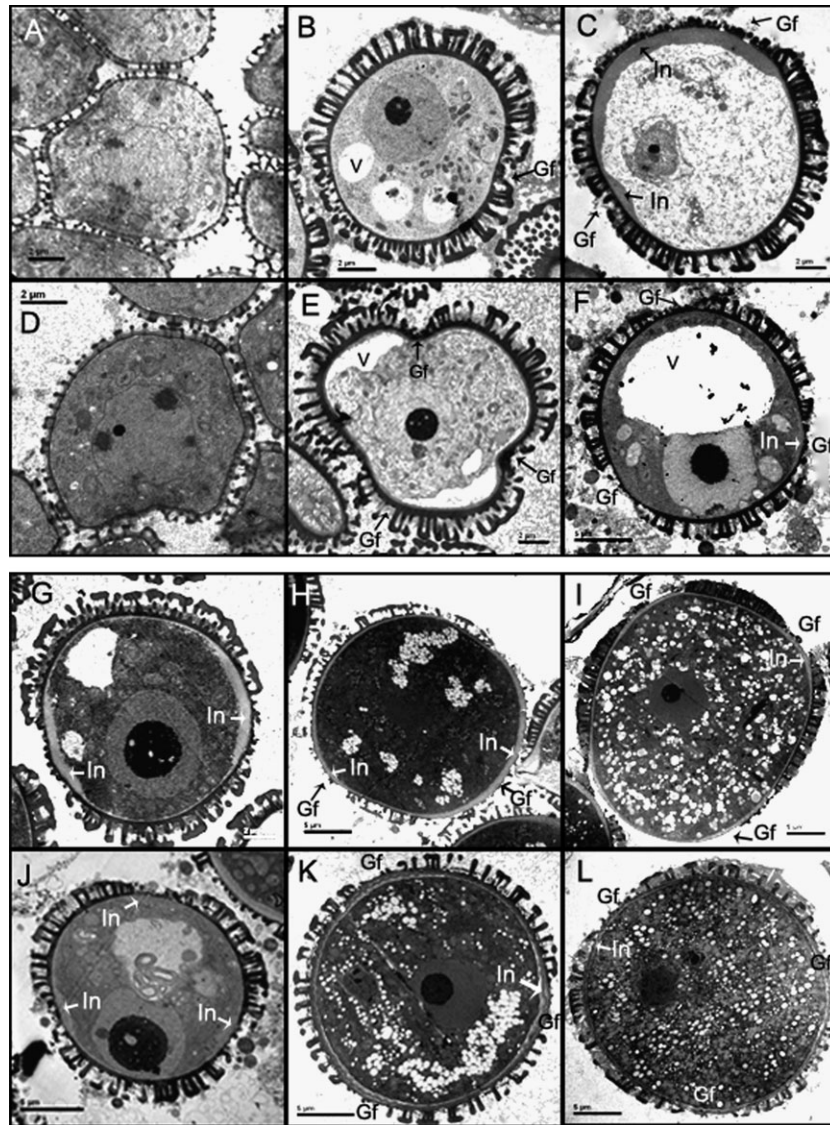


Fig. 7. Transmission electron micrographs of pollen development of *bcmf2* transgenic Chinese cabbage. (A) The free microspore of *bcmf2* transgenic plants was normal. (B) Loss of the cloverleaf pattern of the microspore at the early uniuucleate pollen stage in *bcmf2* transgenic plants. (C) The great extent of the thickening in intine within the germinal furrow of *bcmf2* transgenic plants at the late uniuucleate pollen stage. (D–F) The free microspore, early-stage uniuucleate microspores, and late-stage uniuucleate pollen of empty vector-transformed plants. (G–I) The uneven distribution of the germinal furrow and an abnormal thickening of the intine observed in *bcmf2* transgenic plants from binucleate to mature pollen stage. (J–L) Development of the microspore of empty vector-transformed plants from the binucleate to mature pollen stage showing three evenly distributed germinal furrows. Gf, germinal furrow; In, intine; V, vacuole.

Consistent with the morphological observations, as well as the self-pollination and test-cross tests which showed normal ovule function in the *bcmf2* transgenic plants, there was no apparent phenotype at the ultrastructural level associated with inhibited expression of *BcMF2* in the ovule.

Discussion

BcMF2 is a new PG gene that modulates intine development

The *BcMF2* gene, previously isolated from Chinese cabbage, includes the typical PG sequence in its predicted protein sequence (Wang *et al.*, 2005). The gene also shares high

similarity with *Arabidopsis* PGA4 mRNA (At1g02790). PG is a hydrolase and a loosening enzyme that disintegrates the cell wall structure through the degradation of pectin. Most of the PGs are expressed in the late stages of pollen development (Robert *et al.*, 1993; John *et al.*, 1994; Tebbutt *et al.*, 1994) and are considered to be necessary for pollen maturation and pollen tube growth (Allen *et al.*, 1993; Honys and Twell, 2003; Pina *et al.*, 2005). During pollen germination, hydrated pollen grains release many proteins anchored in the pollen wall, including PG. Thus, most previous studies concluded that PG in the pollen tube disintegrated the style cell wall, allowing the pollen tube to pass through the style. Alternatively, some studies concluded that PG acted on the pollen tube's own cell wall, and

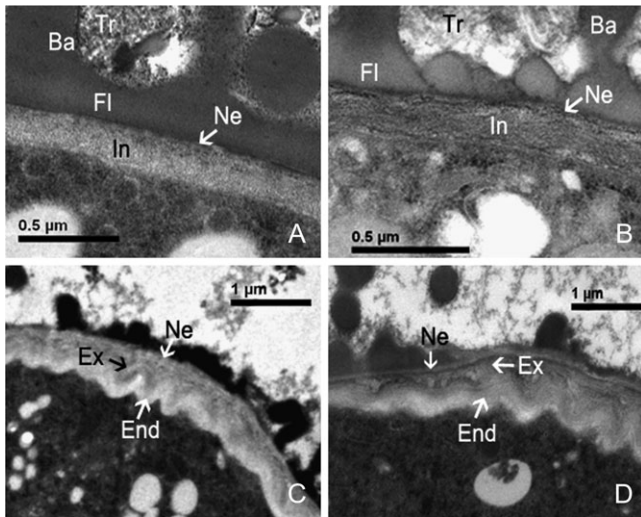


Fig. 8. Transmission electron micrographs of late intine formation in *bcmf2* transgenic Chinese cabbage. (A) Mature intine outside of the germinal furrow region of a *bcmf2* transgenic plant showing a homonymous granular exintine layer facing the exterior predominantly occupying the intine. (B) Mature intine outside of the germinal furrow region of empty vector-transformed plants showing more microfibrillar endintine facing the interior predominantly occupying the intine. (C) Mature intine inside of the germinal furrow region of a *bcmf2* transgenic plant had a larger granular exintine layer and a small microfibrillar endintine layer. (D) Mature intine inside of the germinal furrow region of empty vector-transformed plants showed an apparent large proportion of the internal microfibrillar endintine and a small proportion of the external granular exintine. Ba, bacula; End, endintine; Ex, exintine; Fl, foot layer; In, intine; Ne, nexine; Tr, tryphine.

accelerated its growth amidst style tissue (Brown *et al.*, 1990). However, to date, there is little direct evidence regarding the role of PG genes in the development of pollen or of pollen tubes, though many pollen-specific PG genes have been identified (Allen *et al.*, 1993; Honys and Twell, 2003). One of the pollen-associated PG genes that has been well described is *QRT3* in *Arabidopsis*. *QRT3* is specifically and transiently expressed in the tapetum during the phase when microspores separate from their meiotic siblings. Mutation of *QRT3* results in failed degradation of the mother pollen cell wall and subsequent tetrad separation. It is presumed that the *QRT3* protein plays a direct and specific role in degradation of the pectic polysaccharides comprising the mother pollen cell wall (Rhee *et al.*, 2003). Notably, molecular evolution analysis attributed *BcMF2* protein to clade C, a clade comprised entirely of PG genes expressed in pollen, flower buds, or flowers. Expression pattern analysis showed that *BcMF2* was expressed preferentially in the developing tapetum and microspores from the tetrad stage onward. These results suggest that *BcMF2* may encode a new PG with a modulatory role in pollen development.

In order to study *BcMF2*'s function, antisense RNA expression vectors under the constitutive CaMV 35S promoter were introduced to a normal fertile plant. The activity of the CaMV 35S promoter in pollen and anthers has always been controversial. Great variability in CaMV 35S promoter activity is observed in comparisons between the pollen and anthers of tobacco and *Arabidopsis* (Wilkinson *et al.*, 1997). However, this promoter is highly active in floral organs and in the pollen of transgenic strawberry plants (de Mesa *et al.*, 2004). Furthermore, the CaMV 35S promoter has already been used as a positive control when analysing the activity of a tapetum-expressed promoter in *B. napus* (Robert and Hong, 1999). In the present study, placing the antisense

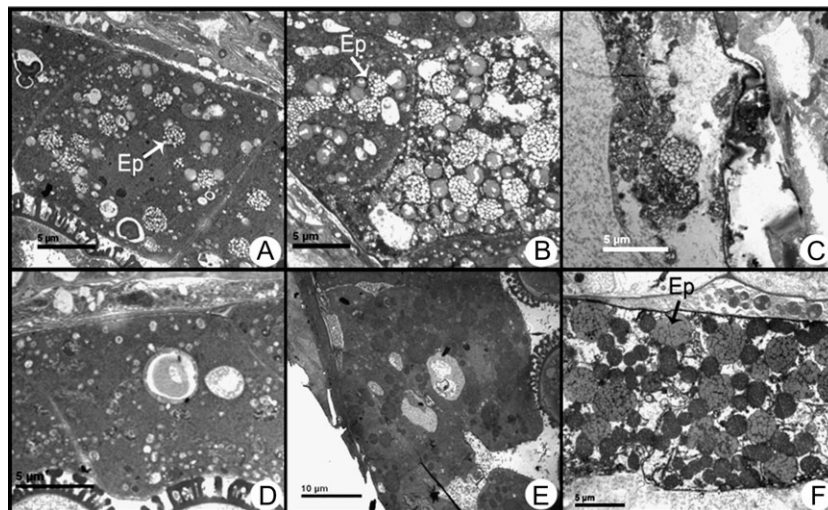


Fig. 9. Transmission electron microscopy of tapetum development of *bcmf2* transgenic Chinese cabbage. (A) A large number of elaioplasts emerged in the *bcmf2* tapetum at the late uninucleate stage. (B) At the early binucleate stage, the elaioplasts increased and enlarged further in the *bcmf2* tapetum. (C) Tapetum in the *bcmf2* transgenic plant begins to degenerate during the late binucleate stage. (D and E) A few elaioplast appeared in tapetum at the late uninucleate stage and the early binucleate stage of the empty vector-transformed plants. (F) At the trinucleate pollen stage, tapetum of the empty vector-transformed plants was still relatively complete. Ep, elaioplast.

RNA vector under the CaMV 35S promoter resulted in substantial gene inhibition in Chinese cabbage (*B. campestris*) flower buds. The construct did, however, show small differences in the level of target gene expression among various transgenic plants. This result was consistent with that of previous studies showing normal CaMV 35S promoter activity in the pollen and anthers of Chinese cabbage (Cao *et al.*, 2006; Li *et al.*, 2007; Zhang *et al.*, 2008). Therefore, it is feasible to use the CaMV 35S promoter as a substitute when the gene's own promoter is not available in investigations of Chinese cabbage pollen or anther genes.

Self-fertilization and test-cross revealed that the transgenic *bcmf2* plant with inhibited *BcMF2* expression displayed substantially reduced male fertility, but normal female fertility. The most dramatic aberrations in *bcmf2* transgenic plants (detected by SEM) were pollen deformities characterized by the disordered location of germinal furrows. TEM revealed that pollen grains underwent an abnormal thickening in the intine region due to the overdevelopment of the granular exintine. The external exintine layer occupied most of the intine, thus reversing the typical proportions of internal endintine and external exintine layers. Disordered intine distribution is also found in *Arabidopsis ms33* mutants, although the pattern is reversed. In *ms33* pollen, the endintine is much thicker than the exintine at the bicellular stage. Intine formation occurs prematurely, affecting pollen grain desiccation and therefore pollen viability (Fei and Sawhney, 2001). These differences in the phenotypes observed for the *ms33* mutant and the *bcmf2* plant show that *BcMF2* and *MS33* have different activity patterns in the context of intine formation. In general, the granular exintine in intine is composed of pectin and various proteins, while the microfibrillar endintine consists of cellulose. The *BcMF2*-encoded PG, a hydrolase enzyme active in the degradation of pectin, may be necessary for pectin's dynamic metabolism. The abnormal thickening of the exintine, filled with pectin, that follows inhibition of *BcMF2* expression may result from a decline in PG activity and subsequent abnormalities in pectin's dynamic metabolism in the intine.

It has been demonstrated that germinal furrow formation is accompanied by development of intine. Within the germinal furrow, there is a layer of tissue connecting the aperture foramen and intine, called the *zwischenkorpe* layer (El-Ghazaly and Jensen, 1987; Marquez *et al.*, 1997). This term actually denotes the outer intine. Thus, the disorder in *bcmf2* germinal furrows may be a consequence of abnormal intine formation. Abnormal thickening of intine might not adequately balance the force in the pollen wall and consequently result in pollen grains with a twisted shape.

Pollen tube arrest in antisense BcMF2 transgenic plants may result from confused pectin metabolism

During *in vitro* pollen germination, most of the pollen tubes in *bcmf2* transgenic plants formed a balloon-shaped swelling structure at the tip after growing to a certain length, then halted growth. Experiments have been conducted to

try to determine if there was any connection between intine formation and pollen tube elongation. Significantly, the pollen tube cell wall is a continuation of the intine layer. Not long after pollen grains contact water, the exine (outer layer of the pollen wall) abruptly breaks after pressure accumulation due to expansion of the intine. The intine swells after being released from the exine; in optimal conditions, the germinating pollen cell will emerge from one end of the pollen grain and the extending pollen tube then travels through the appropriate hole (the germinal furrow) of the intine. As the pollen tube emerges, the intine layer becomes continuous with the cell wall of the tube (Stone *et al.*, 2004). It is widely accepted that pollen tubes grow by tip extension, with highly active cytoplasmic streaming transporting vesicles and organelles towards the tip; these vesicles and organelles then deposit cell wall components. Recent research has also revealed the highly anisotropic growth behaviour of the cell wall of angiosperm pollen tubes (Taylor and Hepler, 1997; Geitmann, 1999). At least two regions exist in the axial direction, the approximately hemispherical growing tip and the cylindrical shank. The tip region is generally characterized by a single wall layer (Lancelle and Hepler, 1992) and is known to be composed mainly of newly synthesized methyl esterified pectins (Geitmann *et al.*, 1995). This pectin layer can continue along the entire length of the pollen tube and forms the outer layer of the cell wall in the pollen tube shank, where a secondary wall layer is generally formed adjacent to the plasma membrane. Turgor is believed to be the primary motive force behind tip growth, and the pectin layer has also been demonstrated to play an important role in the process (Geitmann and Steer, 2006).

Pollen tube growth is a dynamic complex system, and a balloon-swelling structure in the tip can exert various influences, for example the transition from anisotropic to isotropic growth. In *bcmf2* plants, pollen tube elongation was interrupted after the tube grew to a certain length. Therefore, it seems that the initial segment of the pollen tube remains relatively normal. However, elongation did not proceed normally and ultimately resulted in halted growth. The balloon-shaped swelling tip in *bcmf2* did not continue to expand indefinitely at the apex after it was shaped. This highlights the significant departure of this transgenic phenotype from the typical trajectory of pollen tube growth. Here, a possible explanation for this phenotype is proposed. The primary inner turgor behind tip growth may encounter the suddenly immobilized growth wall, forcing formation of a balloon-shaped swelling structure in the tip, similar to the blowing of a bubble. The bubble does not burst, possibly due to the thickened intine in the *bcmf2* pollen tube tip. In this hypothetical model, *BcMF2* encodes a PG necessary for pectin's dynamic metabolism in the pollen tube. Inhibiting protein expression may cause disordered pectin metabolism, beginning at an early stage of intine development and extending to pollen tube wall formation. As the initial pollen tube wall is continuous with the pollen grain intine, the enzymatic activity localized within the intine is incorporated into the

lateral tube walls (Edlund *et al.*, 2004). Alternatively, it has been widely shown that genes encoding proteins related to cell wall biosynthesis and regulation are highly expressed in pollen (Becker *et al.*, 2003; Honys and Twell, 2003). It is therefore not surprising that the *BcMF2* gene was expressed early in pollen development. However, at present, the mechanism underlying *BcMF2* gene involvement in pectin metabolism cannot be explained. To resolve this puzzle, more experimental evidence is needed.

The bcmf2 phenotype may suggest a link between tapetum and intine development

Another impressive phenotype resulting from the inhibition of *BcMF2* expression was premature tapetum degradation. The exine formed from material released by the tapetum demonstrates a direct correlation of tapetum development with exine formation. Currently, there are a number of tapetum-expressed genes that are known to affect exine development. For example, mutating *MS2*, which codes for fatty acid reductase, prevents exine formation (Aarts *et al.*, 1997). There are relatively few reports on an association between tapetum and intine development. In contrast to exine, intine is secreted from the microspore at the vacuolation stage and is produced by gamete differentiation. However, two mutants defective in intine formation, *ms33* and *ms1*, were also found to be defective in tapetum degeneration. As mentioned above, *ms33* mutants displayed early intine formation as well as early tapetum degeneration (Fei and Sawhney, 2001). In the *ms1* mutant, reduced intine was accompanied by abnormal tapetum development (Vizcay-Barrena and Wilson, 2006). It should be noted that, in these mutants, both intine formation and exine development were affected, leading the authors of both studies to speculate that abnormal exine development may lead to disordered intine. When the pollen grain undergoes dehydration, intine must adapt to the particular stage of exine development, with consequent pollen grain volume changes, despite independent exine and intine development (Shukla *et al.*, 1998). Moreover, exine development depends on the accumulation of materials secreted by the tapetum. Therefore, tapetum development affected the development of intine by influencing the formation of the outer wall (Fei *et al.*, 2001). However, exine abnormalities have not been observed in *bcmf2* pollen. Thus, the relationship between tapetum development and intine cannot be entirely explained by abnormal exine development that leads to disordered intine. A distinct link between the development of tapetum and intine is therefore proposed. However, further experiments need to be conducted to elucidate the underlying mechanism.

Pollen-associated genes such as BcMF2 may also be expressed in the embryo

In situ hybridization analysis showed expression of *BcMF2* in the ovules of flower buds in later stages of development. However, inhibited expression of *BcMF2* did not affect

ovule development or ovule function. Interestingly, it was reported that *QRT3*, the PG gene in *Arabidopsis* influencing degradation of pectic polysaccharides in the pollen mother cell wall, also showed strong expression in the ovules of open flowers. However, there is no apparent phenotype associated with the *qrt3* mutation in the ovules either at the histological level or in terms of ovule function (Rhee *et al.*, 2003). In addition, both RT-PCR and Northern blotting results showed that the expression of *BcMF2* continued in siliques after fertilization. Interestingly, no expression signal was detected in siliques from the *bcms* mutant. It is noteworthy, however, that this expression pattern was not unique for *BcMF2*. When the spatial and temporal expression patterns of 20 specifically or predominantly accumulated transcripts in wild-type flower buds compared with *bcms* mutants were investigated, it was found that 12 of them showed expression in wild-type siliques but not in those of *bcms* mutants (Huang *et al.*, 2008). As a matter of fact, siliques harvested from wild-type and *bcms* mutant plants were not identical. Following pollen abortion, no fertilization of ovules in *bcms* siliques occurred. However, siliques from wild-type plants were filled with fertilized ovules after self-pollination. Otherwise, the ovules in the *bcms* mutants were normal, demonstrated by crossing the *bcms* mutant with its wild-type ancestor. Based on all of these results, it is presumed that *BcMF2* may not be expressed in the ovule but rather in the embryo.

The old Chinese cabbage flower buds which were collected in this research corresponded to the anther dehiscence and pollen dispersal stage. Hence, it is likely that the gynoeceum had been pollinated. If this were true, it would be expected that *BcMF2* inhibition would not affect ovule function in the transgenic plants. Similarly, it is proposed that *QRT3* is expressed in the embryo but not in the ovule. The characteristic self-pollination in *Arabidopsis* may lead to fertilization in the open flower. Furthermore, this pattern of expression exists not only in the PG gene family but also in many other genes associated with pollen development. Two possible explanations for why these male reproductive organ-expressed genes appeared again in the embryo were proposed. First, these genes may be essential for development after the zygotic stage. Secondly, the phenomenon may be due to paternal genomic imprinting in the plant. Further investigation is required to address the question conclusively.

Supplementary data

Supplementary data are available at *JXB* online.

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