

Molecular Cloning and Characterization of *BcMF22*, a Novel Gene Related to Pollen Development and Fertilization in *Brassica campestris* ssp. *chinensis*

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Published online: 4 January 2012
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Abstract The *BcMF22* (*Brassica campestris* Male Fertility 22) gene was isolated from Chinese cabbage-pak-choi (*B. campestris* L. ssp. *chinensis* Makino, syn. *B. rapa* ssp. *chinensis*). The cDNA clone of *BcMF22* was 1,870 bp in length and contained an open reading frame (ORF) of 1,260 bp, while the genomic sequence contained no introns. Sequence prediction indicated that *BcMF22* might encode a methyltransferase. Spatial and temporal expression analyses indicated *BcMF22* was preferentially expressed in pollen. Transcripts of *BcMF22* were first detected in pollen mother cells and continued to be present in mature pollen. In addition, transcripts were detected in pollinated pistils, approximately 4 h after pollination (4HAP). These results indicated that *BcMF22* might be a pollen-preferential gene and was closely involved in pollination.

Keywords *Brassica campestris* ssp. *chinensis* · *BcMF22* · Pollen · Pistil · HAP

Abbreviations

BcMF22 *Brassica campestris* Male Fertility 22
ORF Open reading frame
RACE Rapid amplification of cDNA ends
HAP Time after pollination
qRT-PCR Quantitative real-time RT-PCR

Introduction

Both pollination and fertilization are important processes in plant reproduction. Pollination involves the transfer of

pollen from the anthers to stigmatic surfaces, and it is critical for subsequent fertilization and for seed set.

Pollen development is a process regulated by a wide variety of genes (Kim et al. 2010; Li et al. 2010, 2011; Singh and Grover 2010; Tsuwamoto et al. 2010). Transcriptional profiles of pollen tissues have revealed that several specific genes are involved during the development of mother cell meiosis into mature pollen (Becker et al. 2003; Honys and Twell 2003; Whittle et al. 2010). In tobacco (*Nicotiana tabacum*), there were about 11,000 anther-specific mRNAs (Kamalay and Goldberg 1980), in maize (*Zea mays*), approximately 2,000 to 7,000 pollen-specific genes among 20,000 pollen expressed mRNAs (Wing et al. 1990), and in *Arabidopsis*, there were 992 pollen expressed mRNAs, nearly 40% of which were pollen-specific (Honys and Twell 2003). Moreover, there were almost 9.7% of the 13,997 male gametophyte-expressed mRNAs that were gametophyte-specific in *Arabidopsis* (Honys and Twell 2004). So, a large number of pollen-specific transcripts are being recognized in plants. In recent years, many pollen-related or pollen-specific genes were isolated, characterized and analyzed. These genes include “early genes” (Zhang et al. 2011a, b), “late genes” and genes expressed in both periods of male organ development (Chen et al. 2011; Fourgoux-Nicol et al. 1999; Honys and Twell 2004; Kato et al. 2010; Robert et al. 1993; Zhang et al. 2011). However, these studies are mainly associated with genes which were involved in pollen development, and the current knowledge on the constant process of pollen development and fertilization is scarce, which is greatly appealing to us.

Recently, many works focused on the microarray analysis in plants providing important information on the genome-wide regulatory networks of pollination and fertilization (Boavida et al. 2011; Lan et al. 2004; Wang et al. 2008). However, such transcriptional profile cannot provide a complete outline of the molecular events during this

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process. So, isolation and identification pollen development and fertilization-related genes and illustration their functions are demanding.

In our previous work, we found that one homologous gene of *At1g58120* was upregulated in flower buds and the pollinated pistils (unpublished data) by ATH1 microarray of ‘Aijiaohuang’ genic male sterile AB line *Bajh97-01A/B* in Chinese cabbage-pak-choi (*B. campestris* L. ssp. *chinensis*, Makino, syn. *B. rapa* ssp. *chinensis*). Thus, we speculate that this gene might be one that participated both in pollen development and fertilization, which is valuable for understanding this entire process in plants. In this paper, we analyzed the expression pattern of the homologous gene of *At1g58120*, *BcMF22* in Chinese cabbage-pak-choi using quantitative real-time RT-PCR (qRT-PCR) and in situ hybridization and discussed its association with pollen development and fertilization.

Materials and Methods

Plant Material

A Chinese cabbage-pak-choi ‘Aijiaohuang’ genic male sterile system named *Bajh97-01A/B* was selected in our laboratory (Cao et al. 2006). It comprised a homozygous male sterile line *Bajh97-01A* and a heterozygous male fertile line *Bajh97-01B*. The segregation ratio of male fertile plant *Bajh97-01B* and the sterile plant *Bajh97-01A* is 1:1. When comparing it to the male fertile plant *Bajh97-01B*, the anther of a sterile plant could not produce pollen grains; however, the other floral tissues such as the sepal, petal and pistil remain normal and the female functions are not affected (Huang et al. 2010). All the plants were cultivated in the experimental farm of Zhejiang University.

At the flowering stage, the tissue samples were marked and frozen in liquid nitrogen immediately, and then stored at -80°C . Developmental stages of flower buds were selected as described in our previous work (Huang et al. 2008): four different organs (open flowers, germinal siliques, scapes and leaves) in fertile plants and five floral parts (petals, sepals, stamens, pistils and nectarys) of flower buds in sterile and fertile plants at Stage V. In addition, we pollinated the pistil of the *Bajh97-01A* lines (sterile plants) with the pollen grains from the *Bajh97-01B* lines (fertile plants), and then selected pistils at 1, 2, 4, 8, 12 and 24 h after pollination (HAP) and the corresponding controls in sterile plants.

Bioinformatics Analysis

Database searches were performed at the NCBI World Wide Web server using the Basic Local Alignment Search Tool (BLAST) network service (<http://blast.ncbi.nlm.nih.gov/>

[Blast.cgi](http://blast.ncbi.nlm.nih.gov/)). Gene sequence was analyzed by using the DNASTar software (DNASTAR, Madison, WI, USA). The nucleotide sequence and the deduced amino acid sequence of gene were analyzed by GENETYX. The instability index of the gene was computed by the ProtParam (<http://ca.expasy.org/tools/protparam.html>), and its transmembrane regions were predicted by TMHMM (<http://www.cbs.dtu.dk/services>).

DNA, RNA Extraction and cDNA Synthesis

DNA was extracted from the fresh leaves of the fertile line *Bajh97-01B* using cetyl-trimethylammonium bromide (CTAB) plant DNA extraction method. Total RNA was extracted from the flower buds at five developmental stages and five floral parts of the flower bud in both the fertile and sterile plants at Stage V, different organs in fertile plants, and pistils at 1, 2, 4, 8, 12, and 24 HAP and the corresponding controls using Trizol reagents (Invitrogen, USA) according to the manufacturer's instruction. The first-stranded cDNA was then synthesized using a SMARTTM PCR cDNA synthesis kit (Clontech, USA) according to the manufacturer's instruction.

Isolation of cDNA and DNA Sequences

The full length cDNA was amplified by homologous cloning; P1 and P2 primers (Table 1) were designed according to *At1g58120*. The genomic DNA sequence was amplified using the same primer. A 3'-rapid amplification of its cDNA ends was performed to obtain its 3' end sequence. The gene-specific primer, P3 (Table 1), was designed according to its full length cDNA. The 3' end anchor primers, P4 (Table 1), was designed according to the cDNA Synthesis Kit. Because we failed to obtain the 5' end sequence by 5'RACE, we then performed BLAST (http://brassica.bbsrc.ac.uk/BrassicaDB/blast_form.html) to

Table 1 Primers used in the experiments

Primer name	Primer sequences
P1	5'-ATGGTGTTTGTACTGTCCAAAAC-3'
P2	5'-TCAACCCACCATTGATGC-3'
P3	5'-GAGTGGTAGGGCTTATTGGGAG-3'
P4	5'-AGGCCGAGGCGGACATGTTTTT-3'
P5	5'-GGATTCATTGATAACCAGACAT-3'
P6	5'-GACTCAAGACTTCAGCGCG-3'
RT1	5'-ATCCTCACCGTCCAAC-3'
RT2	5'-CATCACCGTGTAGTCAGAGC-3'
Actin-1-F	5'-CGCTTAACCCGAAAGCTAAC-3'
Actin-1-R	5'-TACGCCCACTAG CGTAAAGA-3'
SP1	5'-ACGCGTCGACATCCTCACCGTCCAACTCAA-3'
SP2	5'-GTTGAGTTGGACGGTGAGGATCCCGGGGA-3'

find the homology of the gene and found that GR4545551 fragment in *Brassica napus* had 83% similarities with it. The primers of P5 and P6 (Table 1) were designed according to the GR455551 fragment. Then, a 5' end sequence of the gene was performed by the homologous fragment of GR4545551.

PCR was carried out in a total volume of 25 μ l containing 0.5 μ l template (0.5 μ g), 2.5 μ l 10 \times PCR buffer, 0.5 μ l 10 mmol/l dNTPs, 0.5 Taq polymerase (5 U/ μ l), 1 μ l gene-specific primer (10 mM) and 19 μ l ddH₂O. The PCR program was as follows: 94°C for 3 min, followed by 34 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min, and the final extension step at 72°C for 7 min.

The amplified PCR products were purified using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), cloned into a pGEM-T easy vector (Promega, USA), and then transformed into *Escherichia coli* competent cells. The recombinant clone was sequenced by Invitrogen Co. (Shanghai, CN).

Gene Expression Analysis by qRT-PCR

For gene expression analysis, first-stranded cDNAs from the flower buds at the five developmental stages and five floral parts of both the fertile and the sterile plants, four different organs of fertile plants, and pistils at different times after pollination of sterile plants were used as templates.

The qRT-PCR primers were selected from a specific region in the gene. Gene-specific primers (RT1 and RT2) and internal control gene *Actin-1* (GenBank accession number: EU012495) primers (actin-1-F and actin-1-R) were shown in Table 1. Analysis was performed using an Mx3005P multiplex quantitative PCR system (Stratagene, La Jolla, CA, USA). Amplification was carried out with the following cycling parameters: heating for 3 min at 95°C, 40 cycles of denaturation at 95°C, annealing for 15 s at 55°C, and extension for 30 s at 72°C. Triplicate quantitative PCR experiments were performed for each sample; the expression values obtained were normalized against *Actin-1*. Analysis of

Fig. 1 ORF with 1,260 bp and the deduced amino acid sequence of the *BcMF22* gene (GenBank Accession No: JN398670). There is no intron in the *BcMF22* sequence. The ORF of *BcMF22* gene is composed of 419 amino acids. The deduced protein with a transmembrane (underlined). Initiation and stop code are shown in **bold**. Asterisk indicates the termination codon

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1  ATGGTGTTTTGTACTGTCCAAAACATTCACTCAGTGTGTCAAAGCATTCTTCACTACCTC
   M V F V T V Q N I H S V C Q K H F H Y L
61  TTGAGAAAGAAAGACGGTTCGAGCTATTCTCTTGGCTCTGAGGATTTCTCCCCCAAGGCT
   L R K K D G S S Y S L G S E D S S P K A
121 TCTCGCGCTGAAGTCTTGTAGTCTCTTTATGAGATCAACCCCTTTTGGCTTCTTGTCTGTG
   S R A E V L S L F M R S T L L A F L F L
181 TCATTTACTTGGTTGAGTCTGCTTAAGCATGAACTGATGCAACCGCTGCATCTAAATCG
   S F T W L S L L K H E T D A T A A S K S
241 GTTGAACCCGACCACACAGGCTATTGCCTTCGCTTCTTAATGACTTAGAGAAGGAAGGT
   V E P D H H R L L P S L L N D L E K E G
301 CTCTTTAACTTGGAGATAAAGCACTCCTCCTTAGCGAAGGAGATGGTGATCAGGTCCT
   L F K L G D K A L L L S E G D G D Q V T
361 GGTAATTCATACTCTCAAACAATCATCGAAACCGAGGTGCTTGTGTATCTGCAAGCGAT
   G N S Y S Q T I I E T E V S T A S S D
421 GAGGAGATGAAGAGAATGGTCCCAAGCGAGACCTTTGATTTTGCCTTTGCACATTCTCGT
   E E M K R M V P S E T F D F A F A H S R
481 CACATTGACTCTGTTGAGTTTCTAGACAGGACTCTCAAAGTTGGCGGTATCCTCACCGTC
   H I D S V E F L D R T L K V G I L T V
541 CAACTCAACCATCATGATCTTCTTACACATTTCTTAAACATTTAAACTACGAAATAGTC
   Q L N H H D L P T H F L K H L N Y E I V
601 TACATGAGGAGCTCTGACTACACGGTGATGGCGATGAGAAAAACAGAGCAAAAAACAGAGC
   Y M R S S D Y T V M A M R K T E Q K Q S
661 ATAGGCGCCACTGGGAGAAAGCTCCTCACTATCACAGATGAGGAGGTCAAGAAAAGAGCT
   I G A T G R K L L T I T D E E V K K R A
721 TTGAGTAAGCTAGAAGATGTTCTCCTTGAACCACCAAGAGCAGCTTCAAGGAAATCAAGA
   L S K L E D V L L E P P R A A S R K S R
781 ACCTACTTGAACCGACAAGGTACCTCCCGGACCTTATGGGAGATAGTTTGGACCTCGAG
   T Y L K R T R Y L P D L M G D S L D L E
841 AGCTACTCGAGGCGGGTATTTATCGATGTGGGTGATGGAAAAGGAAGCAGCGGAACAGAA
   S Y S R R V F I D V G D G K G S S G T E
901 TGGTTTGTAAAAATACCCACAAGGAAGCTGAGGTTTGTAGATGTACAAGATTGAGACA
   W F V K N Y P T R K L R F E M Y K I Q T
961 GTAAACGATGAGATGAGCATAGAGTCTGAAAATATGGGGATAACGGGAGTGGCTGAAAGAG
   V N D E M S I E S E N M G I T E W K E
1021 AATGTGAAGGATGAGGAATACGTGGTAATGAAGGCAGAGGCCGAAGTGGTGGAGGAGATG
   N V K D E E Y V V M K A E A E V V E E M
1081 ATGAGGAACAAGTCGATAAAAAATGGTGGATGAGCTTTTCTTGGAGTGCAAGCCAAAGGGT
   M R N K S I K M V D E L F I T E W K E G
1141 TTAGCGCTAAGGGGAAGGAGGAAGATGCAGAGTAAGAGTGGTAGGGCTTATTGGGAGTGT
   L A L R G R R K M Q S K S G R A Y W E C
1201 TTGGCTCTATATGGCAAACCTAGAGATGAAGGTGTTGCTGTGCATCAATGGTGGGGTTGA
   L A L Y G K L R D E G V A V H Q W W G *

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the relative gene expression data was done using the $2^{-\Delta - \Delta C_T}$ method (Livak and Schmittgen 2001).

In Situ Hybridization

The inflorescences of *Bajh97-01B* and pollinated pistils of *Bajh97-01A* were used for in situ hybridization. Samples were fixed in 4% paraformaldehyde PBS solution with 0.1% Triton X-100 and 0.1% Tween-20, serially dehydrated, cleared with dimethylbenzene, and embedded in paraffin. Then, 7- μ m thick sections of flower buds and pistil were hybridized to specific digoxigenin (DIG)-labeled RNA probes (Roche, Branchburg, NJ, USA). Templates for the gene-specific probes were obtained from amplification with a primer pair SP1 and SP2 (Table 1). The sense and antisense probes were synthesized and DIG-labeled using a SP6/T7 transcription kit (Roche). Images were taken by a Leica DMLE camera (Leica, Wetzlar, Germany).

Results

The Structure Characterization Defined *BcMF22* as a New Gene

The gene was isolated in Chinese cabbage-pak-choi by homology cloning according to *Arabidopsis At1g58120*. The full-length cDNA was 1,870 bp, with a 418-bp 5'-untranslated region and a 192-bp of 3'-untranslated region

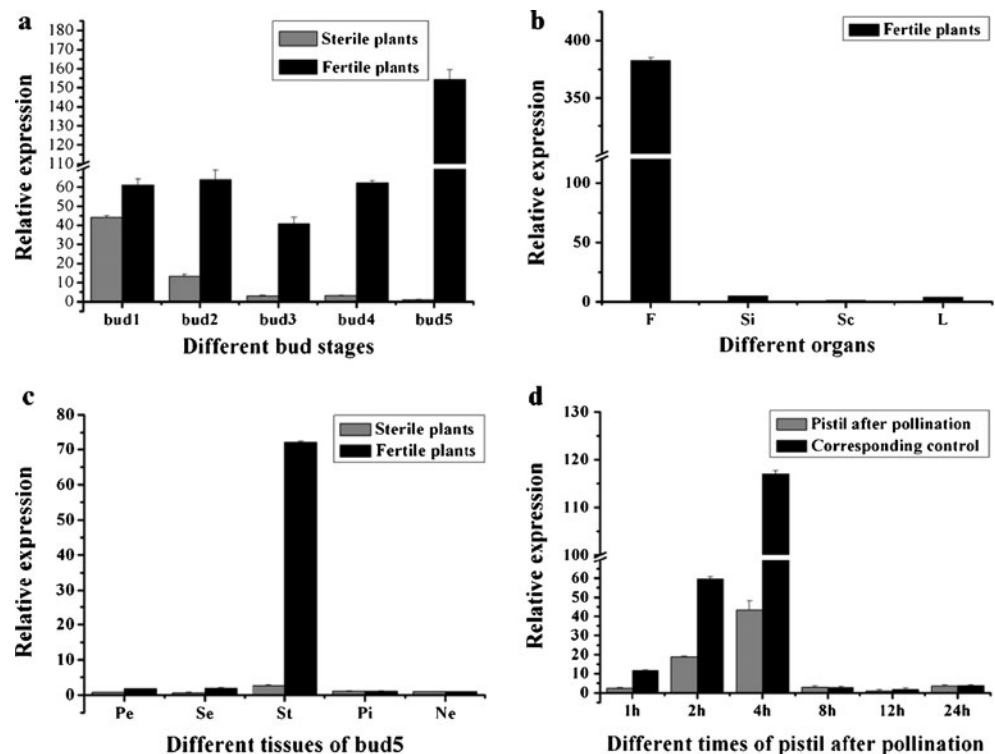
(Fig. 1). The 1,260-bp DNA sequence, amplified with the gene-specific primers, contained no introns. The NCBI BLAST showed that its nucleotide sequence had 86% similarities with *At1g58120*.

The ORF consists of 1,260 bp, encoding a 419 amino acid polypeptide with molecular weight of 48.062 kDa and an isoelectric point of 6.69 (GenBank Accession No: JN398670) (Fig. 1). Among the 419 amino acids, 60 were strongly basic (+) (K and R), 62 were strongly acidic (–) (D and E), 136 were hydrophobic (A, I, L, F, W and V), and 68 were polar (N, C, Q, S, T and Y). Its instability index was computed as 45.68 by the ProtParam (<http://ca.expasy.org/tools/protparam.html>), which was classified as an unstable protein. Furthermore, the aliphatic index was 84.42, which defined it as aliphatic. The deduced amino acid sequence was predicted by TMHMM (<http://www.cbs.dtu.dk/services>) database, in which it contains one transmembrane region located in the first 60 amino acids. The gene only shared a significant sequence similarity to *At1g58120* of *Arabidopsis*, which encodes a methyltransferases. Secondary structure composition prediction by PredictProtein server showed that there were 29.6% helix, 18.6% sheet, and 51.8% loop in the deduced protein.

BcMF22 is Differently Expressed in Reproductive Organs

The relative expression patterns of the gene in different stages of flower buds, different organs, floral parts, and pollinated pistils were investigated by qRT-PCR.

Fig. 2 Relative expression analysis of *BcMF22* in various tissues by qRT-PCR. **a** Flower buds (bud1–bud5) of *Bajh97-01A/B* at Stages I–V, respectively. **b** Open flowers, siliques, scapes and leaves (F, Si, Sc and L) of the *Bajh97-01B*. **c** Petal, sepal, stamen, pistil and nectar (Pe, Se, St, Pi and Ne) of the flower buds at Stage V, respectively. **d** *Black column* indicates pistils at different hours after pollination (1, 2, 4, 8, 12 and 24 h), *gray column* indicates the corresponding control



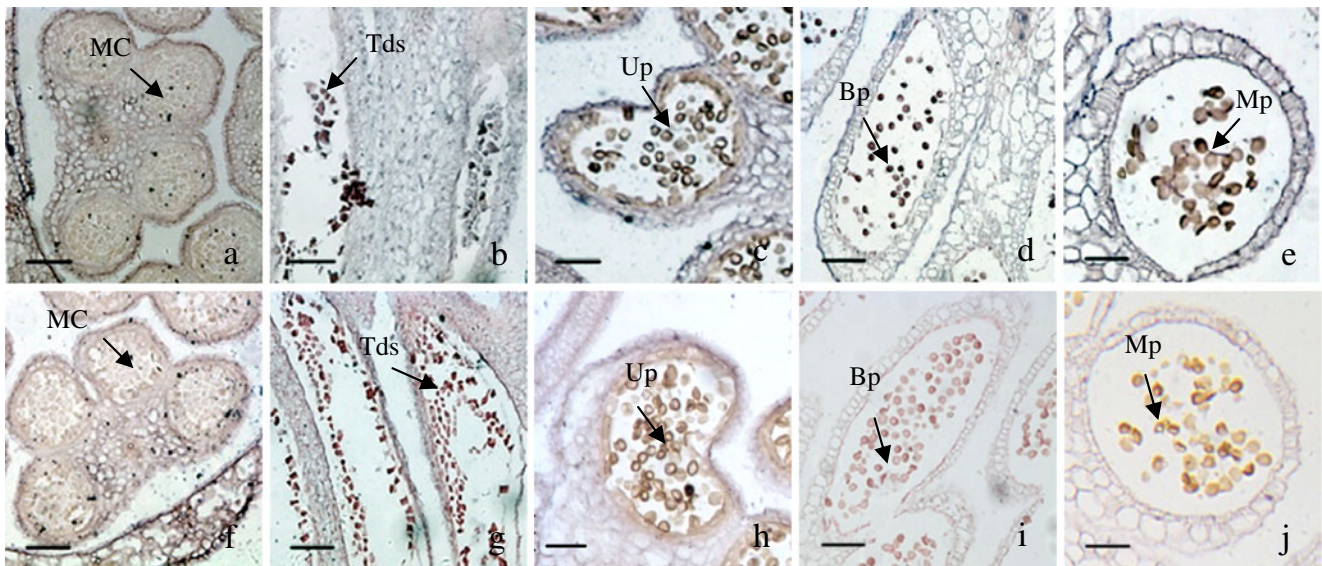


Fig. 3 Detection of *BcMF22* mRNA in different anther developmental stages in *Brassica campestris* ssp. *chinensis* by in situ hybridization. **a–e** Sections of anthers at pollen mother (Stage I), tetrads (Stage II), unilcate (Stage III), binucleate (Stage IV), and mature pollen (Stage V), respectively, hybridized with a *BcMF22* antisense RNA probe. **f–j** Sections of

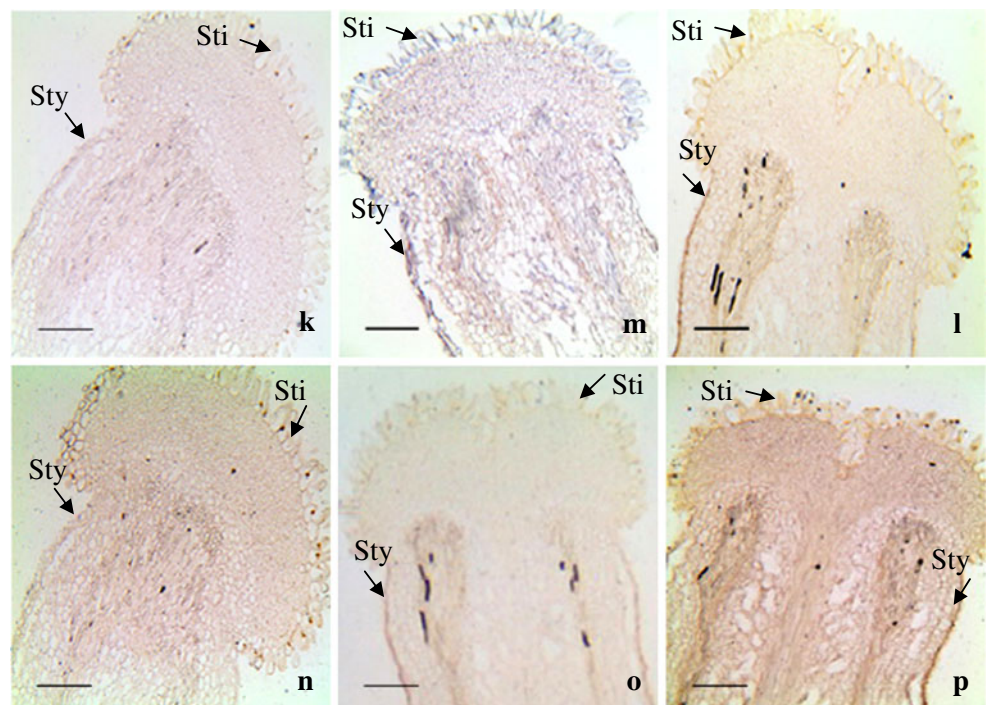
the corresponding developmental stages hybridized with a *BcMF22* sense RNA probe as a control. Probes were labeled with digoxigenin-UTP. The transcript-specific hybridization signal is visualized as blue black color. *MC* pollen mother cell, *Tds* tapetum, *Up* unilcate, *Up* binucleate, *Bp* binucleate, *Mp* mature pollen. Scale bars = 20 μ m

Our data showed that the gene was constantly expressed in flower buds at five developmental stages in fertile plants (Fig. 2a). However, the expression levels of the gene in the flower buds of fertile plants show an evident increase compared to those of the sterile plants, except Stage I (pollen mother stages), and the dramatic difference appeared at

Stage V (mature pollen stage) (Fig. 2a). Therefore, the gene was named as *B. campestris* Male Fertility 22 (*BcMF22*).

The expression pattern of *BcMF22* in the different organs was checked in fertile plants, and the results demonstrated that *BcMF22* was expressed at higher level in open flowers than in other organs (siliques, scapes and leaves) (Fig. 2b).

Fig. 4 Analysis of the expression pattern of *BcMF22* in pistil at different times after pollination in *Brassica campestris* ssp. *chinensis* by in situ hybridization. **k–l** Sections of pistil at 1, 4 and 24 HAP, respectively, hybridized with a *BcMF22* anti-sense RNA probe. **n–p** Sections of the corresponding stages hybridized with a *BcMF22* sense RNA probe as a control. Probes were labeled with digoxigenin-UTP. The transcript-specific hybridization signal is visualized as blue black color. *Sti* stigma, *Sty* style. Scale bars = 50 μ m



However, *BcMF22* was expressed at a low expression level in germinal siliques, roots and leaves.

Furthermore, the expression pattern of *BcMF22* in the floral parts of fertile and sterile open flowers were also detected, and the results showed that *BcMF22* was expressed at higher transcript levels in stamens of fertile plants as compared to other floral parts (petals, sepals, pistils and nectarys) (Fig. 2c). However, transcript levels of *BcMF22* were very low in each floral part of sterile plants (Fig. 2c).

When we address the expression pattern in the pollinated pistils of *BcMF22* in sterile plants, qRT-PCR results illustrated that *BcMF22* was expressed at higher levels in pistils at 1, 2 and 4 HAP than their corresponding controls (Fig. 2d). Moreover, *BcMF22* mRNA was rather highly expressed in pistils at 4 HAP compared to pistils after other times of pollination (Fig. 2d). However, *BcMF22* showed a detectable relative expression in nonpollinated pistils and pistils at 8, 12 and 24 HAP (Fig. 2d) but at a very low level.

BcMF22 is Highly Expressed in the Pollen

To further address the expression pattern and the cellular location of *BcMF22* mRNA during pollen development and pollinated pistils, in situ hybridization was performed on transverse sections of flower buds at different developmental stages and pistil at 1, 4 and 24 HAP. Specific hybridization signals were first detected in the pollen mother cells and continued to the mature pollen grains (Fig. 3b) but not in other tissues, and the *BcMF22* signal in the mature pollen grains reached its peak (Fig. 3e). There was also strong expression in the stigma and style of pistils at 4 HAP (Fig. 4m). However, no signal was observed at pollen mother stages (Fig. 3a), 1 and 24 HAP (Fig. 4k, l), as well as the sense control (Fig. 3f–j, 4n–p).

Discussion

Analysis based on bioinformatics tools indicated that *BcMF22* encoded a methyltransferase. The results of qRT-PCR showed that *BcMF22* was significantly upregulated in flower buds of different stages compared to the sterile ones and reached its maximum expression level at Stage V (mature pollen stage). Moreover, *BcMF22* was also specially expressed in the stamens of fertile plants. Since sterile flower buds differ from the fertile ones only in the failure of pollen formation and the pistils in sterile plants is normal (Huang et al. 2010), we, therefore, could speculate that *BcMF22* is a pollen-preferential gene. Cellular location of *BcMF22* mRNA demonstrated that it was specially expressed in pollen, and its maximal mRNA level appeared at the mature pollen stage.

This result agrees well with the results of the qRT-PCR analysis. Additionally, researchers (Mascarenhas 1975) reported that the RNAs required for germination and early tube growths were already present in the mature pollen grain. Biochemical analysis showed that the mRNAs, required for germination, are synthesized during pollen maturation and persisted in the pollen grain until they are utilized for translation during the germination process (Mascarenhas 1988). So, *BcMF22* showed the highest expression in the fertile flower buds at mature pollen stages that might be because of accumulated mRNA of *BcMF22* was prepared for the next step of pollen germination and pollen tube growth.

In this paper, we pollinated the pistils of the *Bajh97-01A* (sterile plants) with pollen grains from the *Bajh97-01B* (fertile plants) for fixing the expression mode of *BcMF22* in pollinated pistils. The qRT-PCR results showed that the expression levels of *BcMF22* gradually increased during the three points of time after pollination (1, 2 and 4 HAP), and then declined from 4 HAP. Cellular localization of *BcMF22* showed that the signal of hybridization was detected in the stigma and style of pistils at 4 HAP. There were studies which have already demonstrated that the pollen tubes passed through the stigma surface at 1HAP, and corresponded to the phase in which the pollen tubes had just penetrated the style at 4 HAP (Jiang et al. 2005; Tansengco et al. 2004; Wu et al. 2008). In *Arabidopsis*, the researchers found that during the first 0.5 HAP, most pollen grains have hydrated, germinated and invaded the stigmatic papilla cells; at 3.5 HAP, pollen tubes are growing through the style transmitting tissue cells (Boavida et al. 2011). As is known to all, after the pollen tube grows through the carpel's style, the sex cell nuclei from the pollen grains migrate into the ovule to fertilize the egg cell and endosperm nuclei within the female gametophyte in a process termed double fertilization. Thus, the expression features of *BcMF22* and the cellular localization in pistil are strong evidence for *BcMF22* that is closely related to fertilization in Chinese cabbage-pak-choi.

So, our work indicates that *BcMF22* is a pollen-preferential gene and closely related to fertilization. However, more experiments should be performed to elucidate the function of *BcMF22* in pollen formation and fertilization.

Acknowledgement This work was supported by the Natural Science Foundation of China (No. 30871715), the Natural Science Foundation of Zhejiang Province (2010 C12004) and the Key Sci-Technology Project of Zhejiang Province (No. Y3100300).

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