Biochemical and Biophysical Research Communications xxx (2018) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The zinc-finger transcription factor *BcMF20* and its orthologs in Cruciferae which are required for pollen development

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ARTICLE INFO

Article history:
Received 17 June 2018
Accepted 19 June 2018
Available online xxx

Keywords:
Brassica campestris ssp. *chinensis*
Pollen wall development
TFs
Tapetum
Vacuole

ABSTRACT

Brassica campestris Male Fertility 20 (*BcMF20*) is a typical zinc-finger transcription factor that was previously isolated from flower buds of Chinese cabbage (*Brassica campestris* ssp. *chinensis*). By applying expression pattern analysis, it can be known that *BcMF20* was specifically and strongly expressed in tapetum and pollen, beginning from the uninucleate stage, and was maintained during the mature-pollen stage. As *BcMF20* was highly conserved in Cruciferae, it can be indicated that this zinc-finger transcription factor is important during the growth of Cruciferae. In this study, 12 C2H2-type zinc-finger TFs which shared high homology with *BcMF20* were found from NCBI via BLAST. A new molecular phylogenetic tree was constructed by the comparison between *BcMF20* and these 12 C2H2-type zinc-finger TFs with NJ method. By analyzing this phylogenetic tree, the evolution of *BcMF20* was discussed. Then, antisense RNA technology was applied in the transgenesis of *Arabidopsis thaliana* to get the deletion mutants of *BcMF20*, so that its function during the pollen development can be identified. The results showed: *BcMF20* are in the same clade with three genes from *Arabidopsis*. The inhibition of *BcMF20* expression led to smaller amounts of and lower rate in germination of pollen and lower rate in fruit setting in certain transgenetic plants. This also led to the complete collapse of pollen grains. By SEM and TEM, pollen morphology and anther development processes were observed. In the middle uninucleate microspore stage, a relatively thin or even no primexine was formed in microspores. This may result in the malformation of the pollen wall and finally cause the deformity of pollens. Above all, it can be indicated that *BcMF20* may act as a part of regulation mechanisms *of TAZ1* and *MS1*. Together they play a role in a genetic pathway in the tapetum to act on proliferation of tapetal cells and keep the normal development of pollens.

1. Introduction

Cruciferae crops are important crops in agricultural production. During their production, the male sterile line is widely utilized to produce F1 hybrid seeds, which means the Cruciferae species have taken great advantages from the heterosis phenomenon in hybrid cultivars [7]. Usually, male sterile means a plant has its anther, pollen or male gametes lacking or non-functional. The reason for this phenomenon is complicated. According to the genetic characteristics, male sterile can be listed as: genic male sterility (GMS), genic-cytoplasmic male sterility (G-CMS) and cytoplasmic male sterility (CMS). Meanwhile, in the perspective of molecular biology in plant development, pollen abortion is the phenotype of male sterile [18]. Thus, having a full understanding of mechanism in pollen development is the key to study male sterile.

Zinc-finger transcription factor (TF) is a kind of protein which has finger-like domains. It stabilizes its self-folded finger-like polypeptide dimensional conformation by binding Zn^{2+}, in which way the expression of certain genes can be adjusted. This zinc-binding domain was found first in Xenopus oocytes by Miller in 1985 [16], now it can be found in many animals, plants and

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https://doi.org/10.1016/j.bbrc.2018.06.108
0006-291X/

microorganism. Among all the eukaryotic TFs, Cys2/His2 (C2H2) zinc-finger protein is one of the kinds that were studied relatively the most clearly. Some of these proteins are known to play central roles in plant development, whereas others are involved in general transcription. To date, several studies have showed that some C2H2 zinc-finger proteins get involved in reproductive growth. In petunia, C2H2-type zinc-finger proteins, which are called the EPF family, have been implicated in floral-organ specific transcriptional regulation [22–24]. Silencing the genes which encodes them would result in bad consequences like re-mature degradation of the tapetum, aberrant meiosis or pollen abortion [12,13]. In Ref. [14], C2H2 zinc-finger genes expressed accordingly during anther development. In *Arabidopsis*, *SUPERMAN* (*SUP*) and *KNUCLES* (*KNU*), two of the C2H2-type zinc-finger proteins, are necessary for the proper development of reproductive organs [2,19]. On the other hand, studies showed that the overexpression of a petunia zinc-finger gene, *Lateral Shoot-Inducing Factor* (*LIF*), alters cytokinin metabolism and plant forms in transgenic petunia. In Ref. [17]; there was a dramatic increase in lateral shoots and reduced plant height. Also, *LIF* overexpression caused a decrease in cell numbers in stem, leaves and flowers with cell enlargements.

In our previous studies, the *BcMF20* gene, a homolog of the Atlg26620 gene which encodes a putative C2H2 zinc-finger protein, was successfully isolated from Chinese cabbage and its homologous genes from 18 varieties of Brassica or *Raphanus* in Cruciferae were cloned. By analysing these genes and constructing a molecular evolutionary tree via Neighbor-joining (NJ) method, it can be known that *BcMF20* was highly conservative in Cruciferae and exhibited high homology (approximately 82%) with C2H2 TF At1g26610 from *Arabidopsi*. This gene expressed specifically in the developing pollen grains and the tapetum from the uninucleate pollen stage to mature pollen stage and had a very high-level expression in the stage 4 and 5 flower buds. Expression of *BcMF20* also was detected inside anthers and in the tapetal cell layer and the pollen. This indicated that this gene may play an important role in pollen development among Cruciferae species [3,4]. To expand the acknowledgement of the C2H2 gene family in pollen or tapetum development, *BcMF20* was still selected for further characterization in this study.

In this study, 12 C2H2-type zinc-finger TFs which shared high homology with *BcMF20* were searched and selected from NCBI via BLAST. NJ method was applied to construct a molecular phylogenetic tree by comparing *BcMF20* and the selected 12 C2H2-type zinc-finger TFs. The transgenesis of *Arabidopsis thaliana* which resulted in the deletion mutants of *BcMF20* was transformed by the method Floral Dip so that the functions of *BcMF20* during the pollen development can be identified.

2. Materials and methods

2.1. Plant materials and growth conditions

BcMF20 gene was isolated from Chinese cabbage. Transgenesis of *Arabidopsis thaliana* and its normal plants were grown in chambers with controlled environment at 20–22 °C under a long photoperiod (16 h light and 8 h dark). The soil was a mixture of vermiculite and peat moss.

2.2. Construction and analysis of phylogenetic tree

To search the database, Basic Local Alignment Search Tool (BLAST) network service from NCBI World Wide Web server was applied according to [1]. Homolog alignment was determined by MegAlign in the DNAStar software and phylogenetic tree was established by the NJ method in the ClustalX software.

2.3. Construction of antisense expression plasmids and plant transformation

Antisense RNA technology was used specifically to inhibit the *BcMF20* ortholog in *Arabidopsis thaliana* with the constitutive cauliflower mosaic virus (CaMV) 35 S promoter. The whole cDNA sequence from amplified fragments of *BcMF20* was obtained through PCR and were used as antisense gene fragments. Antisense fragments were cloned into the binary vector pBI121(named *BcMF2*0 RNA construct). The correction of these fragments was confirmed via kanamycin screening, PCR, and DNA southern sequencing. Using the agrobacterium-mediated method and the Floral Dip method, the antisense *BcMF2*0 RNA construct was transferred into *Arabidopsis thaliana* (named *35s-bcmf20*) while the empty vector pBI121 was transferred into *Arabidopsis thaliana* as a control (named CK).

2.4. Observations on morphology and germination process of transplant's pollen with microscopy

For *in vitro* pollen germination, pollen grains were collected and cultured in the culture medium [15% sucrose (w/v); 0.4 mmol L^{-1} HBO_3; 0.4 mmol L^{-1} $Ca(NO_3)$; 0.1% agar (w/v)]. The pollen grains were cultivated on agar plates that were placed at 20–25 °C for 4–6 h in humidity of relatively 100%. 5 and 3 independent transgenic lines were selected from F1 and F2 generations respectively. Microscopes were used to observe the germinating pollen grains. The pollen grains with lengths exceeding 1/2 of pollen grain diameter were selected and recorded as the germinating pollens. At the same time, the corresponding control lines were examined. More than 5 flowers were tested from each plant. For each culture, at least 300 pollen grains were examined to calculate the average germination rate.

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

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

3. Results

3.1. Similarity and evolution of 12 selected C2H2-type zinc-finger TFs with BcMF20

For comparison of genes from the closely related species in Cruciferae family, a phylogenetic tree (Fig. 1) were constructed. The results showed that *BcMF20* and three genes from *Arabidopsis*, including At1g26610, belonged to the same clade. They were then showed in a same clade with two other four-fingered proteins from petunia.

3.2. Inhibition of BcMF20 ortholog expression results in smaller amount and lower germination rate of pollen and lower rate of fruit setring

During the cultivation of *35s-bcmf20*, when comparing with CK, only 1/8 of them showed short, round leaf blades, while others had

Please cite this article in press as: Y.-Y. Han, et al., The zinc-finger transcription factor *BcMF20* and its orthologs in Cruciferae which are required for pollen development, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.06.108