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BrEXL6, a GDSL lipase gene of *Brassica rapa*, functions in pollen development

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Abstract

Multiple allele-inherited male sterility has been widely used by breeders of *Brassica rapa* L. ssp. *pekinensis,* but the molecular mechanisms of male sterility are not yet clear. In this study, we isolated the full-length cDNA of a new gene (not included in the *Brassica* database). This gene, comprising 1 054 bp, encodes a 39.99 kDa protein with a Gly-Asp-Ser-Leu (GDSL)-lipase domain that is a member of the lipolytic protein GDSL family. The sequence of candidate gene is the most similar to extracellular lipase 6 (*EXL6*) of *Arabidopsis* and was therefore designated *BrEXL6* and submitted to *NCBI* (accession No. JX131630.1). Reverse transcription semi-quantitative PCR and Western blot analysis showed that *BrEXL6* and its encoded protein were significantly more expressed in fertile buds than in sterile buds. Quantitative PCR and *in situ* hybridization showed that *BrEXL6* was highly expressed in the anthers of fertile buds, especially anthers at the pollen-development stages, but only weakly expressed in other tissues and floral organs of fertile plants and whole sterile plants. These results suggest that *BrEXL6* is a pollen development-related gene. The results of this study provide clues for understanding the mechanisms underlying multiple allele-inherited male sterility.

Additional key words: Chinese cabbage, gene expression, *in situ* hybridization, male sterility, Western blot.

Introduction

The utilization of heterosis is one of the major genetic techniques for increasing the yield of *Brassica* plants. Several pollination control systems have been developed for hybrid production in *Brassica* plants, including self-incompatibility, cytoplasmic male sterility (CMS), genic male sterility (GMS), and the use of chemical hybridizing agents. The GMS system is regarded as especially promising alternative, primarily due to the production of plants with complete and stable male sterility, with no negative cytoplasmic effects on yield, as it was found for some CMS lines. In a previous test-crossing study, we discovered the multiple allele inherited male sterile AB line in Chinese cabbage line AB01 (Feng *et al*. 1995, 1996), which is inherited stably, with 100 % male sterility and no negative cytoplasmic effects. Therefore, this line has become the focus of many breeders.

To date, the multiple allele-inherited male sterility gene has been transferred to various ecotypes of Chinese cabbage by conventional hybridization breeding, and some high-quality male sterile lines have been successfully bred (Feng *et al*. 1996, Li *et al*. 2009). Recently, several molecular markers of male sterility have been identified in Chinese cabbage (Feng *et al*. 2009, Wang *et al*. 2010), and some fertility-related genes have been isolated (Ji *et al*. 2011a,b). However, the functions of these genes are not clear yet. Identifying the functions of most fertility-related genes in Chinese cabbage would help us to understand the mechanisms underlying male sterility.

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Abbreviations: CMS - cytoplasmic male sterility; ESTs - expressed sequence tags; EXL - extracellular lipase 6; GDSL - Gly-Asp-Ser-Leu; GMS - genic male sterility; ORF - open reading frame; RT-sqPCR - reverse-transcription semi-quantitative PCR; SSH - suppression subtractive hybridization.
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In this study, we analyzed one of the multiple allele inherited fertility-related genes that was isolated by suppression subtractive hybridization (SSH) (Ji *et al*. 2011b). Bioinformatic analysis revealed that this is a new gene not included in the *Brassica* database, but it was predicted to be a member of the Gly-Asp-Ser-Leu (GDSL) lipase family based on the characteristic architecture of the GDSL-motif. The GDSL family was first proposed by Upton and Buckley in 1995, who found that most lipases have the sequence GxSxG, which accounts for the turn in these proteins and is considered to be one of the identifying features of lipases. Owing to this and other structural similarities, these enzymes are often treated as a single family (Upton and Buckley 1995). Even individual oleosins exhibit extensive divergence between ecotypes, yet the entire cluster remains intact (Mayfield *et al*. 2001). GDSL esterases/lipases are a newly discovered subclass of lipolytic enzymes that are in focus of research due to their multifunctional properties (Chepyshko *et al*. 2012). These enzymes were found to be involved in flower development and peroxisome activity, as revealed by genome sequence analyses in *Arabidopsis thaliana* (Hu *et al*. 2003, Reumann *et al*. 2004). However, the relationship of GDSL lipase genes to pollen development in Chinese cabbage remains unclear.

In this study, with the purpose of elucidating the role of a pollen development related gene, *BrEXL6* was isolated and its expression profile in different tissues of Chinese cabbage was compared.

Materials and methods

Plants: The Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) line AB01, a previously bred GMS AB line, was used in this study. This line has a 1:1 proportion of sterile (AB01-1, MsMs) to fertile (AB01-2, $Ms^{f}Ms$) progeny and is therefore considered to be a stable system. The AB01 line used in the current study was reproduced continuously by performing sister-line crosses (A×B and A×B) for more than 10 years (Ji *et al*. 2011b). After a period of vernalization at 4 °C for approximately two weeks, the seeds of AB01 were sown in a plot, and seedlings with four to five leaves were transplanted to a field at the Shenyang Agricultural University, China. Fertility of plants was identified during the flowering period by visual examination.

Samples (three biological replicates) of both sterile and fertile plants were obtained separately, immediately frozen in liquid nitrogen, and stored at -85 °C. The samples included flower buds, stems, leaves, and roots, as well as distinct floral parts including petals, sepals, anthers, and stigmas. The anthers were separated from the buds at six stages of development: stage I (> 0 and ≤ 1.5 mm), stage II (> 1.5 and ≤ 2 mm), stage III (> 2 and ≤ 2.5 mm), stage IV (> 2.5 and ≤ 3 mm), stage V (> 3 and ≤ 3.5 mm), and stage VI (> 3.5 mm). The developmental stages of flower buds were selected as described by Ji *et al.* (2011c).

Acquiring the candidate expressed sequence tags (ESTs) and their cloning: Previously, a subtractive cDNA library was constructed and a series of differentially expressed ESTs were identified (Ji *et al*. 2011b). In the current study, one of these differentially expressed ESTs was selected randomly and sequenced. Total RNA was extracted from the flower buds using *Trizol* reagent (*Invitrogen*, Carlsbad, USA), and poly (A^{+})-mRNA was isolated using *PolyAtract* mRNA isolation system II (*Promega*, Madisdon, WI, USA). A full-length cDNA library was constructed using a *Zap* cDNA synthesis kit and a *Gigapack II Gold* cloning kit (*Stratagene*, La Jolla, CA, USA) using the buds of *B. rapa* L. ssp. *pekinensis* and inbred line Chiifu. A full-length clone for the candidate EST was selected based on sequencing data analysis. The homologous sequence of the differentially expressed EST was searched in the full-length cDNA library. Gene-specific primers for the candidate EST were designed based on the homologous sequences in the cDNA library. The thermocycling conditions were as follows: an initial denaturation (94 °C, 3 min), followed by 30 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 30 s), extension (72 °C, 1 min), and a final extension step (72 °C, 5 min). The PCR products were purified using an *AxyPrep* DNA gel extraction kit (*Axygen Biosciences*, Union City, CA, USA), cloned into the pGEM-T Easy vector (*Tiangen*, Beijing, China), and transformed into *Escherichia coli* competent cells. After identification of recombinant clones by colony PCR, the clones were sequenced by *Huada Gene Inc.*, Shenzhen, China.

Sequence analysis of the candidate gene: The open reading frame (ORF) of the candidate gene was identified using *DNAstar* software. A *BLASTN* search was performed against the *Brassica* database (http://brassicadb.org/) to find the introns, then these introns were verified by full-length DNA PCR and sequencing. The primers for obtaining the full-length DNA sequence of the candidate gene were: F1 (210 - 675), 5′-AGATACCGACGGGCAGAT-3′; R1 (210 - 675), 5′-GGAATCACTCCCATCACC-3′; F2 (651 - 953), 5′-AATTCGCGGTGATGGGAGTG-3′; and R2 (651 - 953), 5′-CCGGGTTTCGGCATGGTA-3′. Then, a *BLASTX* search of the GenBank database (http://blast.ncbi.nlm.nih.gov/) was performed. Signal peptide prediction was carried out by submitting the amino acid sequence to *Signal P 4.0* (http://www.cbs.dtu.dk/services/SignalP/). *SOPMA*, and *Swiss-Model*

analysis were also performed (http://swissmodel.expasy.org/).

Bioinformatic analysis of the full-length deduced protein of the candidate gene: The intra-domain features of the deduced amino acid sequence were predicted by examining the amino acid sequence of the candidate gene and all of its homologous amino acid sequences using *DNAMAN* software and analyzed using the multiple sequence alignment programs. The molecular mass (Mr) values were calculated based on the predicted amino acid sequence of the candidate gene (http://web.expasy.org/ cgi-bin/compute_pi/pi_tool), and homolog alignment was performed with *ClustalX1.83*. The phylogenetic tree was constructed with *MEGA4.1*.

Differentially expressed gene analysis by reverse transcription semi-quantitative PCR: The differences in gene expression between fertile buds and sterile buds were detected by RT-sqPCR. The gene-specific primers were designed based on the sequence of this EST. Total RNA was extracted using an *RNAprep* pure plant kit (*Tiangen*) from the fertile and sterile buds, and the first-strand cDNAs were synthesized with oligo (dT) 15 primers using *M-MLV* reverse transcriptase (*Promega*) and recombinant RNA ribonuclease inhibitor (*Takara*, Tokyo, Japan). *Brlact7* (GenBank accession No. JN120480) was used as a reference (Ji *et al*. 2011c). The gene-specific primers were EP1, 5′-TTGTTTCGC ATCAGGTGG-3′ and EP2, 5′-CGTAGACGA GCGGGAGT-3′. The primers for *Brlact7* were 5′-ATC TACGAGGGTTATGCT-3′ and 5′-CCACTGAGGACG ATGTTT-3′. The PCR conditions were as follows: 94 °C for 5 min, followed by 30 cycles of amplification (94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s), and an extension at 72 °C for 5 min. The RT-PCR products were electrophoresed on a 1 % agarose gel.

Western blot analysis: Protein extracts were separated by sodium dodecylsulphate (SDS)-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes, and probed with antibodies against *BrEXL6* (131220001, 1:4 000) or *Brlact7* (131220002, 1:4 000) (*Abgent Biotechnology*, *Suzhou Co.*, China). Proteins of interest were detected with peroxidase conjugated *AffiniPure Goat Anti-Rabbit lgG* (H+L) antibody (1:5 000, *ZB-2301*, *ZSGB-Bio*, Beijing, China) and visualized with a *Pro-light HRP* chemiluminescent kit (*PA112*, *Tiangen*, Beijing, China), according to the manufacturer's protocol.

Gene expression analysis by real time quantitative PCR: The primers were designed based on the gene sequence of *Brlact7* from *B. campestris* (GenBank accession JN120480) (Table 1 Suppl.). The analysis was performed with the *Bio-Rad* (Hercules, USA) *IQ5* real-time PCR System following the instruction manual for the *Dan SYBR Green Premix Ex Taq* kit (*Tiangen*, Beijing, China). The thermocycling conditions were as follows: initial polymerase activation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 20 s, 57 °C for 20 s, and 72 °C for 30 s, and a final extension at 72 °C for 10 min. To confirm that only one specific PCR product was amplified, a melting cycle, in which the PCR product was denatured from 65 to 95 °C, was added at the end of the thermocycler profile to produce the melting curves. Each reaction was performed in triplicate. PCR efficiencies were calculated using standard curves from six serial five-fold dilutions of pooled cDNAs for each gene and were > 95 % (Table 1). The relative gene expression was calculated using the Pfaffl method using *Brlact7* as a reference. Dunnett's test (α = 0.05) was conducted for the evaluation of significance of mean values. The data were analyzed using *DPS 7.05* software with *OriginPro7.5* drawing software.

***In situ* hybridization:** Flower buds at different developmental stages (I - VI) were fixed in 4 % (m/v) formaldehyde-PBS solution, serially dehydrated, cleared with dimethylbenzene, and embedded in paraffin. Then, 10 μm thick sections of flower buds were hybridized to specific digoxigenin (DIG)-labeled RNA probes (DIG RNA labeling kit *SP6/T7*, *Roche*, Basel, Switzerland). Templates for the *BrEXL6*-specific probes were obtained by amplification with the specific forward primer 5′-GGC TGCAGTTGTTTCGCATCAGGTGG-3′ and reverse primer 5′-GGGGTACCCGTAGACGAG CGGGAGT-3′. The sense and antisense probes were synthesized and DIG labeled using a *SP6/T7* transcription kit (*Roche*). The sections were observed under a *Nikon Eclipse 80i* microscope (*Nikon*, Tokyo, Japan). Images of the anthers at different stages were captured with a *Nikon DS-Ri1* camera.

Results

Previously, we constructed cDNA libraries from sterile and fertile Chinese cabbage using SSH (Ji *et al.* 2011b). A fragment of 558 bp (Fig. 1*A*) was obtained from a fertile-tester cDNA library. A full-length cDNA library was constructed using the buds of *B. rapa* ssp. *pekinensis* and the inbred line Chiifu. The homologous sequences of the differently expressed EST were found in the full-length cDNA library. A 1 054 bp full-length cDNA of the candidate EST was amplified (Fig. 1*B*) from line AB01 according to its homologous sequence in the full-length cDNA library of Chiifu.

Sequence alignment showed that the differentially expressed fragment was located in region 278 - 835 bp (Fig. 1 Suppl.) of the full-length cDNA sequence. Open

reading frame analysis of the cDNA by *DNAman* showed that the largest ORF of this candidate EST was composed of 1 032 bp starting from the start codon (ATG) at nucleotide number 11 and ending at the stop code (TGA) at nucleotide number 1 042. Computational analysis of the cDNA sequence showed that this gene encodes a putative polypeptide of 343 amino acids with an estimated molecular mass of 39.99 kDa. A signal peptide sequence at the amino terminus was also identified by *SignalP4.0* analysis. The first 20 amino acids at the N-terminus constituted the signal peptide. The most likely signal peptide cleavage site was predicted between amino acid residues 22 and 23 (Fig. 1 Suppl.). The predicted protein sequence contain one GDSL-lipase domain (LFAFGDSILDTG) from amino acid residue 30 to 41, as predicted with the *Prosite* program (Fig. 1 Suppl.). In addition, 3D structural modelling of the candidate protein was performed online with *Swiss-Model* software. Analysis of the intricate spatial architecture showed that one model of the candidate protein was similar to that of the lipolytic protein of the GDSL family.

A *BLASTN* search of the *Brassica* database (http://brassicadb.org/) revealed that the candidate gene is

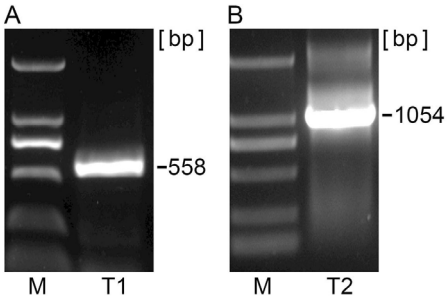

Fig. 1. The candidate expressed sequence tag (EST) and full-length cDNA of the candidate gene. *A* - Differentially expressed EST acquired from a subtractive cDNA library. *B* - Full-length cDNA of the candidate gene. M - markers, T1 - EST, T2 - full-length cDNA.

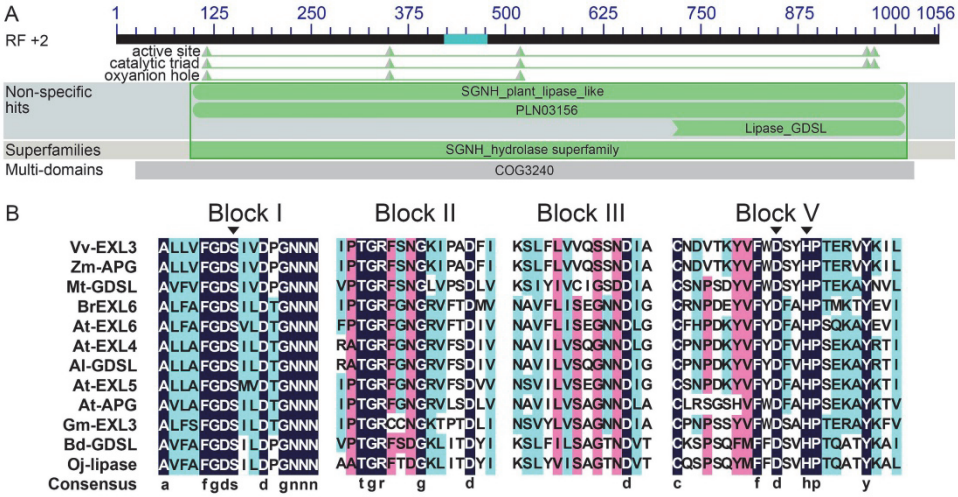

Fig. 2. Functional prediction of the candidate gene. *A* - Putative conserved domains of the candidate gene, as determined by *BLASTX* analysis. *B* - Multiple alignments of amino-acid sequences of four highly conserved residues in the candidate gene with those of other plant lipases obtained from the *NCBI* databases (http://www.ncbi.nlm.nih.gov/). The aligned proteins included Vv-EXL3 (*Vitis vinifera,* XP_002279353.2), Zm-APG (*Zea mays*, NP_001150904.1), Mt-GDSL (*Medicago truncatula*, XP_003603778.1), AtEXL3 - 6 (NP177718, NP177719, NP565122, NP177721), At-GDSL (*Arabidopsis. thaliana*, AT5G63170), At-APG (*Arabidopsis thaliana*, AC022472.2), Gm-EXL3 (Glycine *max*, XP_003527440.1), Bd-GDSL (*Brachypodium distata*, XP_003566161.1), and Oj-lipase (*Oryza sativa* var. *japonica,* NP_001055799.1). The downward *arrowheads* show the conserved Ser (S), Asp (D), and His (H) residues that form a putative lipase catalytic triad. The conserved blocks are marked above the alignment. Consensus amino acid residues are indicated with *lowercase letters* at the bottom of the figure.

located in region No. 12970852–12972746 of the A02 chromosome (chromosome V1.5). The results of *BLASTN* suite-2 sequence analysis between candidate gene and A02: 12970852–12972746 showed that there were four introns located in regions 241, 375, 607, and 868, respectively, which was verified by full-length DNA PCR and sequencing, but no homologous cDNA was found in *B. rapa* (CDS). A *BLASTX* search of the *NCBI* database revealed that this gene is most similar to *EXL6* of *A. thaliana* (74 % maximum identity; accession No. NP_177721.1, AT1G75930) (Table 2 Suppl.). Furthermore, the *BLASTX* search also revealed that the full-length cDNA of the candidate gene encoded an SGNH hydrolase-type esterase domain (Fig. 2*A*). Enzymes containing the SGNH hydrolase-type esterase domain function as esterases and lipases, and SGNH hydrolase-type esterase domains contain a unique hydrogen bond network that stabilizes their catalytic centers; such domains usually contain the catalytic triad Ser/Acid/His (http://www.ebi.ac.uk/interpro/IEntry?ac

=IPR013830).

We investigated the evolutionary relationships among the selected plant proteins, finding high sequence similarity among the candidate protein and other plant lipase proteins. The results show that the lipolytic enzyme sites in all of the selected sequences were highly similar to that of the candidate gene (Fig. 2*B*). GDSL-lipases exhibit the GDSL motif GxSxxxxG, in which the active site serine is located near the N-terminus, as well as a Gly-Asp-Ser-(Leu) [GDS (L)] motif in conserved block I. The deduced amino acid sequence of the protein shared the following features: a putative Ser-containing GDSL-like motif close to the N-terminus (Leu is replaced by Ile37) and five consensus sequences, including FGDSIXDTGNN, TGRFXNGRVXXD, GXNDI, LYXGARXFXVXGXXPXGCXP, and CXNPXXYV FXDXXHPTEKA in Blocks I, II, III, and V, respectively. The residues Ser36, Asp174 (or Asp318), and His321 may represent the catalytic triad SDH. The residues Gly68 and Gly73 in Block II and Asn170 in Block III may act as catalytic residues. These results suggest that the candidate gene belongs to the GDSL lipase family. Phylogenetic analysis showed that the candidate gene had maximum homology with the GDSL lipases gene *EXL6* of *A. thaliana* (Fig. 2 Suppl.). Therefore, we named the candidate gene *BrEXL6* and submitted it to the GenBank databases under accession No. JX131630.1.

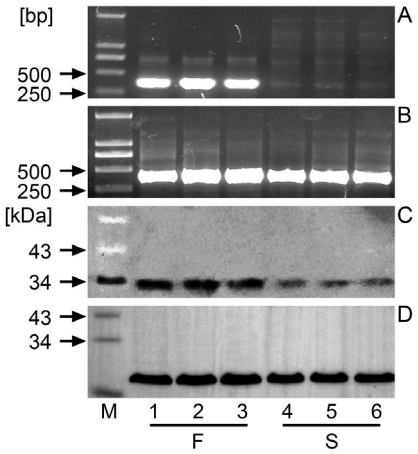

Fig. 3. Differences in cDNA and protein expression of *BrEXL6* between fertile and sterile buds. *A, B* - Differential expression of candidate genes (*A*) and the housekeeping gene (*B*) as determined by reverse transcription semi-quantitative PCR. *C, D* - the differential expression of the proteins translated from the candidate gene (*C*) and the housekeeping gene (*D*), as determined by Western blot analysis. M - markers, F - fertile buds, S - sterile buds.

Reverse transcription semi-quantitative PCR indicates that the expression of the housekeeping gene *Brlact7* (Ji *et al*. 2011a) was similar between fertile and sterile buds, but *BrEXL6* exhibited significantly different expression in fertile and sterile buds; this gene was highly expressed in fertile buds, but its expression was almost undetectable in sterile buds (Fig. 3*A*). The results of Western blot analysis verified these results, as the protein translated from *BrEXL6* was strongly expressed in fertile buds but only a weak signal was detected in sterile buds (Fig. 3*C*), indicating that the difference in transcriptional level significantly influenced the translation of this gene.

Further, we investigated the expression patterns of *BrEXL6* in different organs and tissues, and in different

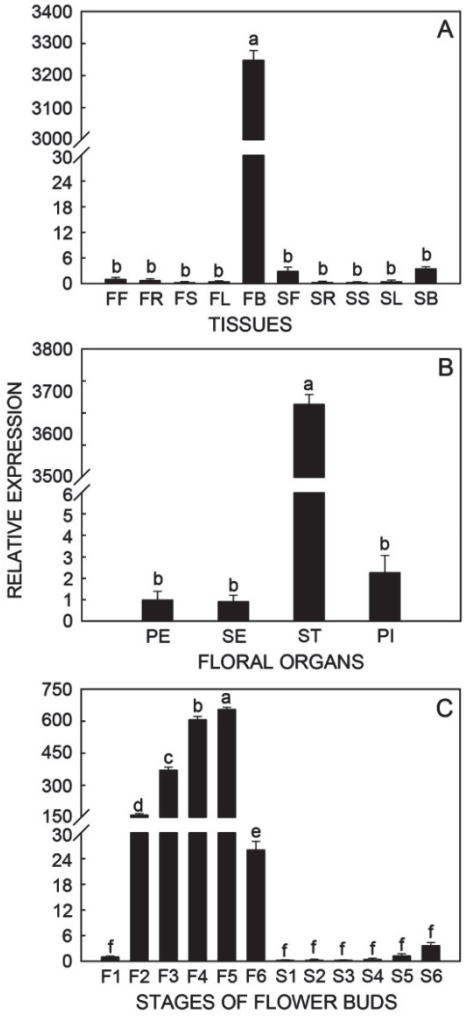

Fig. 4. Spatial and temporal expression analysis of *BrEXL6* by real-time quantitative PCR. Relative expressions were compared with the expression of *Brlact7* using the Pfaffl method. *A* - Expression analysis in different tissues of fertile plant: FF - fiber, FR - root, FS - stem, FL - leaf, FB - bud; and of sterile plant: SF - fiber, SR - root, SS - stem, SL - leaf, and SB - bud. *B* - Expression analysis in different floral organs: PE - petal, SE - sepal, ST - stamens, and PI - pistils, *C* - Expression analysis in anthers of different stages: F1 - F6 - stage I - VI in anthers of fertile buds; S1 - S6 - stages I -VI in anthers of sterile buds. Means ± SDs, $n = 3$; different lettters indicate significant differences at $P \leq 0.05$. as calculated by Duncan's multiple range test.

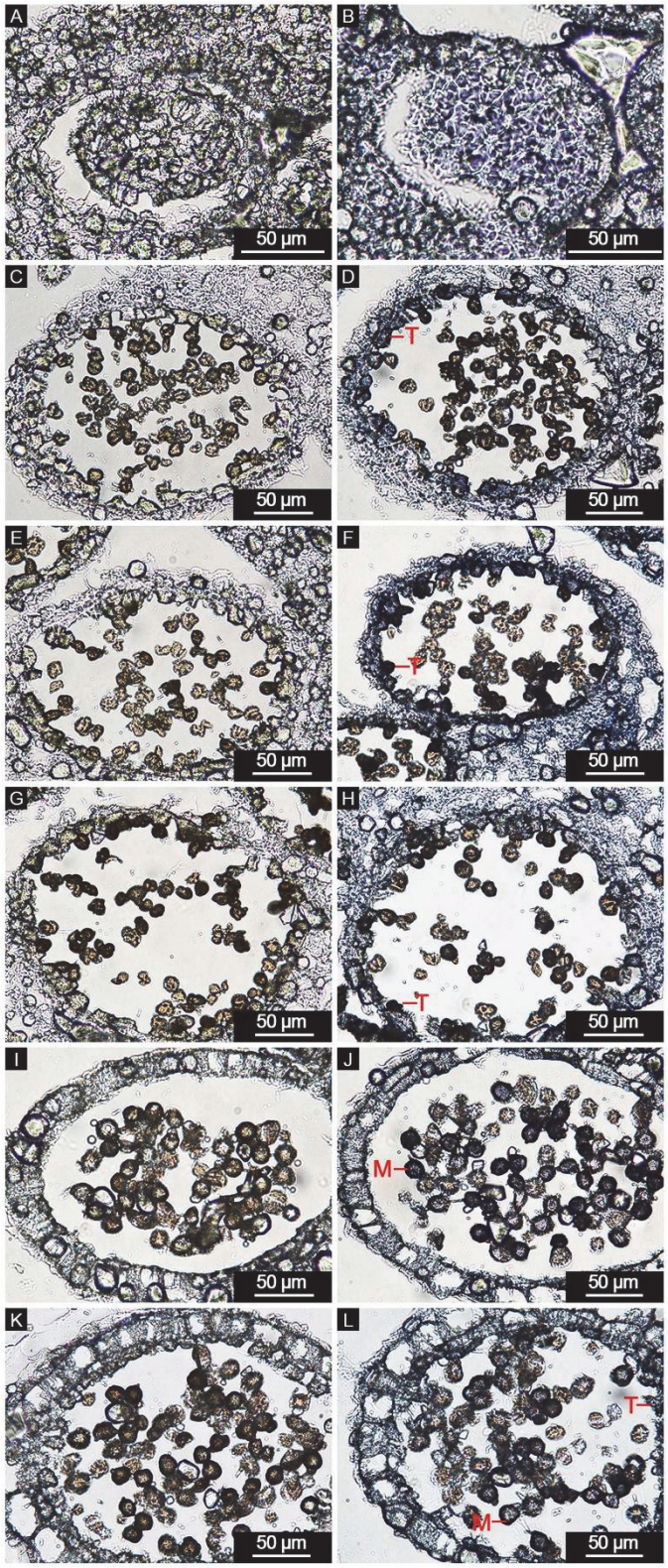

Fig. 5. Analysis of *BrEXL6* expression during anther development in male fertile Chinese cabbage line AB01 using *in situ* hybridization. *A* to *F* - cross-sections of anthers in different developmental stages (I - VI) hybridized with a *BrEXL6* sense probe as a negative control. *G* to *L* - cross-sections of anthers at the corresponding developmental stages hybridized with a *BrEXL6* antisense probe. T - tapetum, M - mature pollen grain.

developmental stages. In fertile plants, the *BrEXL6* was strongly expressed in buds, but its expression was very low in roots, stems, or leaves (Fig. 4*A*). To examine the site of *BrEXL6* expression in fertile buds, we analyzed its expression in sepals, petals, stamens, and pistils. The results show that *BrEXL6* was almost not expressed in sepals, petals, or pistils, but it was highly expressed in stamens (Fig. 4*B*). We also performed real-time qPCR analysis of anthers from buds in different developmental stages. We found a significantly higher expression ($P < 0.05$) in stages II - VI than in other stages. This gene was initially expressed in stage II, the expression reached a peak in stages IV and V, followed by a decrease in stage VI (Fig. 4*C*).

To further dissect the cellular location of *BrEXL6* mRNA during pollen and anther development, *in situ* hybridization analysis was performed on cross-sections of the flower buds from fertile plants (AB01) in different developmental stages (I - VI) (Fig. 5). A high expression was initially detected in tapetal cells in stages II and III. In stages V and VI, the hybridization signals were also detected in mature pollen grains (Fig. 5), but no signals were detected in negative control anthers.

Discussion

With the completion of genome sequencing and functional genomics studies of many plant species, the number of identified fertility-related genes has recently increased. There are 140 flower genes listed in the *Brassica* database (http://brassicadb.org/brad/flowerGene.php). However, identifying all of these genes has not uncovered the molecular mechanism of multiple allele-inherited male sterility in *B. rapa*. We previously isolated several fertility-related ESTs by SSH and cDNA-amplified fragment length polymorphism (AFLP) analysis (Ji *et al*. 2011a,b), but the full-length sequences and function of these genes have not been clear yet. In the present study, one new fertility-related gene (not included in the *Brassica* database) was identified, and the full-length cDNA of this gene was cloned (Fig. 1*B*). This gene is predicted to encode a 39.99 kDa protein, which was verified by Western blot analysis (Fig. 3*C*). GDSL lipases represent a subfamily of lipolytic enzymes, which possess a conserved catalytic triad (Ser, Asp, and His), like other members of the lipase and esterase families. GDS (L)-motif proteins are generally referred to as GDSL esterases and lipases (Akoh *et al*. 2004). Sequence analysis of *BrEXL6* at both the amino acid and nucleotide levels revealed that it is highly similar (74 % maximum identity) to *EXL6* (At1g75930), a GDSL lipase gene of *A. thaliana* (Table 2 Suppl.), and it was therefore designated *BrEXL6*. *BrEXL6* possesses a consensus sequence (also known as a block) in its deduced amino acid sequence and contained major sequence features of GDSL lipases (Fig. 2*B*), such as a flexible active-site serine located near the N-terminus, five conservative blocks, and a Ser-Asp-His triad in its deduced amino acid sequence (Akoh *et al*. 2004, Ling *et al*. 2006).

The expression pattern of *BrEXL6* is associated with morphogenesis and development. For example, some genes are differentially expressed in different tissues (Ten *et al*. 2001), and the expression of some genes is altered after stress treatment (Wang *et al*. 2015). Hu *et al*. (2003) found that many floral genes are expressed at low levels, and 97 genes are expressed at higher levels in flowers than in leaves. Some GDSL lipase genes are highly expressed in flowers, such as two GDSL-motif lipases (At5g33370 and At3g04290), which are predominantly expressed in buds and are candidates for anther-specific proteins with overlapping functions (Hu *et al*. 2003). In the current study, *BrEXL6* gene and BrEXL6 protein, like *EXL6* of *A. thaliana* (Dong *et al*. 2013), were found to be significantly upregulated in fertile plants as compared with sterile plants, as revealed by real-time PCR and Western blot analyses (Fig. 3). This suggests that *BrEXL6* is a fertility-related gene. Quantitative PCR showed that among roots, stems, leaves, and buds of fertile and sterile plants, *BrEXL6* was only highly expressed in fertile buds, but it was expressed at very low levels in other tissues (Fig. 4*A*), which suggests that *BrEXL6* is a bud-specific gene. To determine which part of the bud exhibits high *BrEXL6* expression, we compared its expression patterns in different flower organs. The results show that *BrEXL6* was strongly expressed in anthers but not in other floral organs, such as sepals, petals, and stigmas, suggesting that this gene is related to anther development. Further, we found that this gene was initially expressed at stage II, its expression reached a peak at stages IV and V, followed by a decrease at stage VI (Fig. 4*C*), which was a similar expression pattern to extracellular lipases (EXL4, 5, and 6) of *A. thaliana*. The results of Xu *et al*. (2014) show that extracellular lipases are highly expressed in the middle and late stages of anther development in *A. thaliana*. To investigate whether *BrEXL6* expression is correlated with pollen development, *in situ* hybridization was performed in fertile flower buds at different developmental stages. The results show that *BrEXL6* was exclusively expressed in the tapetum and mature pollen grain (Fig. 5). The results of Xu *et al*. (2014) show that *EXL6* of *A. thaliana* is expressed in the tapetum and that the protein is present in the tapetum and pollen coat. From these findings, we can conclude that *BrEXL6* of *B. rapa* ssp. *pekinensis* has a similar function to *EXL6* of *A. thaliana* and it is involved in pollen development.

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