



## MOLECULAR CLONING AND EXPRESSION ANALYSIS OF *CML27* IN *BRASSICA OLERACEA* POLLINATION PROCESS

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To keep genetic diversity, flowering plants have developed a self-incompatibility system, which can prevent self-pollination. It has been reported that calcium concentration in pistil papilla cells was increased after self-pollination in transformed self-incompatible *Arabidopsis thaliana*. In this study, we found that *CML27* changed its expression level for both mRNA and protein when compared to transcriptome and proteome. At the same time, *CML27* was expressed in the anther and pistil at a high level and reached up to 5-fold up-regulated expression in the pistil at 1 h post-pollination when compared to 0 min. In order to find out potential proteins that may interact with BoCML27, BoCML27 was expressed in and isolated from *E. coli*. After its co-incubation with *Brassica oleracea* pistil proteins, the products were separated on SDS-PAGE gels. We found a specific band at the position between 130–180 kDa. Through LC-MS-MS (Q-TOF) analysis, eight proteins were identified from the band. The proteins include 26S proteasome non-ATPase regulatory (26S), Phospholipase D, alpha 2 (PLD $\alpha$ 2) involved in Ca<sup>2+</sup> binding and Coatomer subunit alpha-2-like (Coatomer) involved in vesicle mediated transport. All of these identified proteins provide new insights for the self-incompatibility response in *B. oleracea*, specific for increasing Ca<sup>2+</sup> concentration in pistil papilla cells.

**Keywords:** *Brassica oleracea*, pollination, *CML27*, LC-MS-MS (Q-TOF) assays

### INTRODUCTION

A self-incompatibility system has developed in angiosperm plants and it functions in rejecting self-pollen, promoting outcrossing and keeping genetic diversity. In Brassicaceae, the self-incompatibility system is controlled by a single polymorphic *S*-locus gene, acting as a model system to study intercellular signal transduction (Takayama et al., 2001). At present, the self-incompatibility response in Brassicaceae is thought to be a signal transmission pathway, flowing from SCR to SRK

and ARC1 stepwise. Consequently, ARC1 degrades pollen compatible factor Exo70A1, resulting in pollen rejection (Joshua et al., 1996; Stephenson et al., 1997; Stone et al., 2003; Samuel et al., 2009). In addition to the above-mentioned factors, Thioredoxin-*h*-like proteins 1 and 2 (THL1/THL2), *M*-locus protein kinase (MLPK) and *S*-locus glycoprotein (SLG) are also involved in the self-incompatibility response (Nasrallah et al., 1985; Mazzurco et al., 2001; Kakita et al., 2007).

Calcium ion (Ca<sup>2+</sup>) is an important second messenger in plants, and it functions in signal

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transduction. It was reported that  $\text{Ca}^{2+}$  assembled in pollen tube tips and led to polarized growth (Iwano et al., 2004). In transformed self-incompatible *A. thaliana*, SCR-SRK complex induced  $\text{Ca}^{2+}$  concentration increasing in pistil papilla cells, leading to pollen rejection (Iwano et al., 2015). With respect to mRNAs of key regulatory components in the self-incompatibility response, like SRK and ARC1, their mRNA levels increased during the first 30 min, but decreased at 1 h post-pollination (Wang et al., 2014).

In this study, calcium binding protein 27 (CML27) changed expression levels at both mRNA and protein. RT-PCR assay revealed that it was highly expressed in pollen grains, pistils and 5-fold up-regulated expressed in the pistil at 1 h post-pollination when compared to 0 min. In order to detect CML27 potential interacting proteins in the pistil during the pollination process, GST pull down assay was performed. One band specifically located between 130-180 kDa was separated. LC-MS-MS (Q-TOF) assays identified eight proteins, which can be categorized into several gene groups through gene ontology and KEGG analysis. These gene groups include gene silencing, protein metabolism, regulation of translation,  $\text{Ca}^{2+}$  binding and vesicle mediated transport. For these identified proteins, their potential relationships with CML27 are predicted and their roles in the early self-incompatibility response in *B. oleracea* are postulated.

## MATERIALS AND METHODS

### PLANT GROWTH CONDITIONS AND POLLINATION

The self-incompatible cultivars of *Brassica oleracea* L. var. *capitata* L. were grown under natural conditions (25°C, 16 h light/8 h dark). For pollination, floral buds were covered with plastic bags and the bags were removed during pollination with self-pollen. For the gene expression analysis in different tissues, leaves, floral buds, petals, sepals, pollen grains and pistils were collected at the anthesis. For transcript expression analysis and protein extraction, *B. oleracea* plants were self-pollinated and redundant pollen was removed; they were harvested at 0, 15, 30, and 45 min, 1 h and 2 h. Samples were stored at -80°C until used for RNA and protein extraction.

### POLLEN TUBE GROWTH AFTER POLLINATION AT ANTHESIS

Using pollen grains collected at the anthesis stage, pollen germination and pollen tube growth assays were performed. *B. oleracea* pistils were pollinated with

pollen, collected at 1, 2, 24 and 48 h, fixed in 3:1 (v/v) ethanol: acetic acid for 6 h, washed with ultrapure water three times, and macerated in 1N NaOH at 60°C for 20 min sequentially. Having been washed with ultrapure water three times, the pistils were stained with 0.1% aniline blue for 6 h. The stained pistils were examined by fluorescence microscopy as described previously (Shiba et al., 2001; Haffani et al., 2004). Ten pistils of each group (1, 2, 24 and 48 h) were analyzed for manifestation.

### RNA EXTRACTION AND RT-PCR ASSAY

The leaves, flower buds, sepals, petals, pollen, non-pollinated pistils and pollinated pistils at 0 min, 15 min, 30 min, 45 min, 1 h and 2 h of post-pollination were used for RNA extraction. Total RNA was extracted using the RNeasy Plant Kit (Qiagen, Beijing, China) and 1 µg of total RNA was used for cDNA synthesis. CML27 and reference *Actin3* transcripts were amplified by using perspective primer sets. The primers are shown as follows:

CML27-F: 5'-ATGGCAAGTGGTAACCCTG-3',

CML27-R: 5'-CTAGGCCGTCGAACCACCGTTC-3'

*Actin3*-F: 5'-ATGGAAACATCGTCCTCAGTGGTG-3',

*Actin3*-R: 5'-AGTGCTGAGGGATGCCAAGATG-3'.

For RT-PCR, the Phanta Max Super-Fidelity DNA Polymerase was recruited to amplify CML27 and *Actin 3* in flower organs and the pistil during the pollination process. The total volume for the reaction mixture was 25 µL and the PCR was performed using the following protocol: 25 cycles for *Actin3* and 28 cycles for CML27; 95°C 30 s; 95°C 15 s; 56°C 15 s; 72°C 30 s; 72°C 5 min. Quantity one software was used for detecting CML27 relative expression levels in flower organs and the pollination process.

### PROTEIN EXTRACTION

Pistil protein was extracted using a plant active protein extraction kit (Sangon, Shanghai, China). Briefly, pistils (approximately 300 mg) were frozen and ground in liquid nitrogen, followed by the suspension in a 5 mL centrifuge tube with 2 mL buffer A, and then in a vortex tube for 2 min at room temperature, following 5 min in a centrifuge at 8000 rpm/min under 4°C. The aqueous part was crude soluble proteins.

### GST-CML27 PULL DOWN ASSAY

For expression recombinant proteins in *E. coli* cells, the full-length of CML27 cDNA was cloned into prokaryotic expression vector pGEX-4T-1, and then, the plasmid was transformed into *E. coli* DE3 cells to express recombinant protein GST-CML27. The primers are shown as follows:

**CML27-F:** 5'-GATCTGGTTCCGCGTGGATCCATGGTGCTGGTTCTGGCATTGGGGGATC-3',  
**CML27-R:** 5'-GATGCGGCCGCTCGAGTCGACCTAAGGAGCAGAGCTGGAAGTGG-3'.

The expressed GST, GST-CML27 and pistil proteins + GST-CML27 co-incubation proteins were purified with Beaver Beads™ GSH. For GST pull down assay, GST-CML27 proteins were bound with Glutathione Sepharose 4B beads, and then GST-CML27 proteins were co-incubated with 2 mL extracted pistil crude soluble proteins at room temperature for 2 h, during which the co-incubated mixture was softly vortexed every 10 min. After co-incubation, the beads were washed three times with washing buffer, and the bead-bound protein complex was eluted and subjected to SDS-PAGE electrophoresis separation followed by Coomassie Blue 250 staining and western-blot assay. In western-blot assay, 10 µL of each sample was separated by 12% (w/v) SDS-PAGE, and GST-CML27 fusion proteins were detected by anti-GST antibodies as described by the manufacturer (Sigma, St Louis, USA).

#### PROTEINS IDENTIFIED BY LC-MS-MS (Q-TOF)

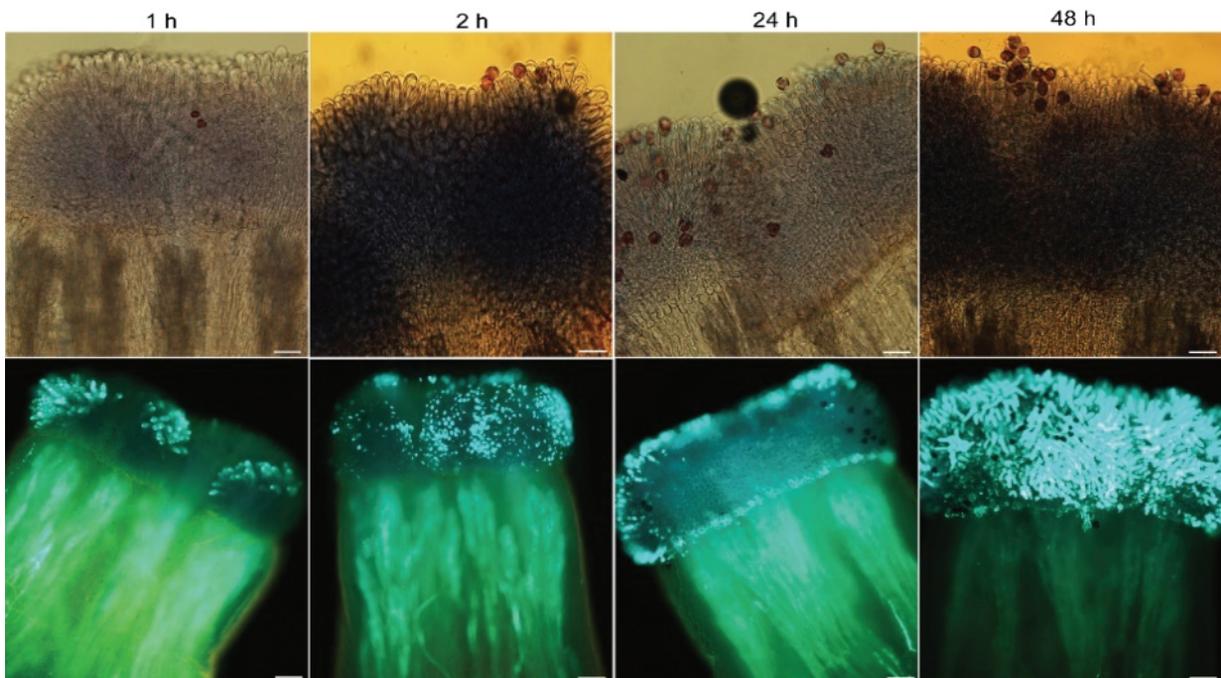
The specific band located between 130–180 kDa was excised from SDS-PAGE gels. The excised gel was further trypsin-digested and analyzed using

liquid chromatography electrospray ionisation tandem mass spectrometry (LC-MS-MS) according to the manufacturer's protocol. The resulting peaks were used to identify the corresponding proteins in *B. oleracea* database in uniprot (<http://www.uniprot.org/>), using Mascot 2.3.01 ([www.matrixscience.com](http://www.matrixscience.com)) search engine. Searches were performed with the following parameters: trypsin as proteolytic enzyme, allowing for one missed cleavage; carbamidomethylation of cysteine as a fixed modification; oxidation of methionine and Gln→Pyro-Glu (N-term Q) as a variable modification. Only the proteins having a Mowse score above 85 (significant  $p < 0.05$ ) are thought to be identified proteins.

## RESULTS

### POLLEN GERMINATION ON PISTIL

The number of pollen grains and pollen tubes on the pistils of *B. oleracea* increased gradually after pollination. During the first hour, there was a limited number of pollen grains adhered and germinated on pistil epidermal cells, but this number increased to about 50 at 48 h. After 48 h, these adhered pollen grains displayed poor germination while pollen tubes did not penetrate into pistils anymore (Fig. 1).



**Fig. 1.** Morphology of pollen grains and pollen tubes on *Brassica oleracea* pistils after pollination. Bars = 100 µm

## CML27 DIFFERENTIALLY EXPRESSED AT BOTH MRNA AND PROTEIN LEVELS IN PISTIL

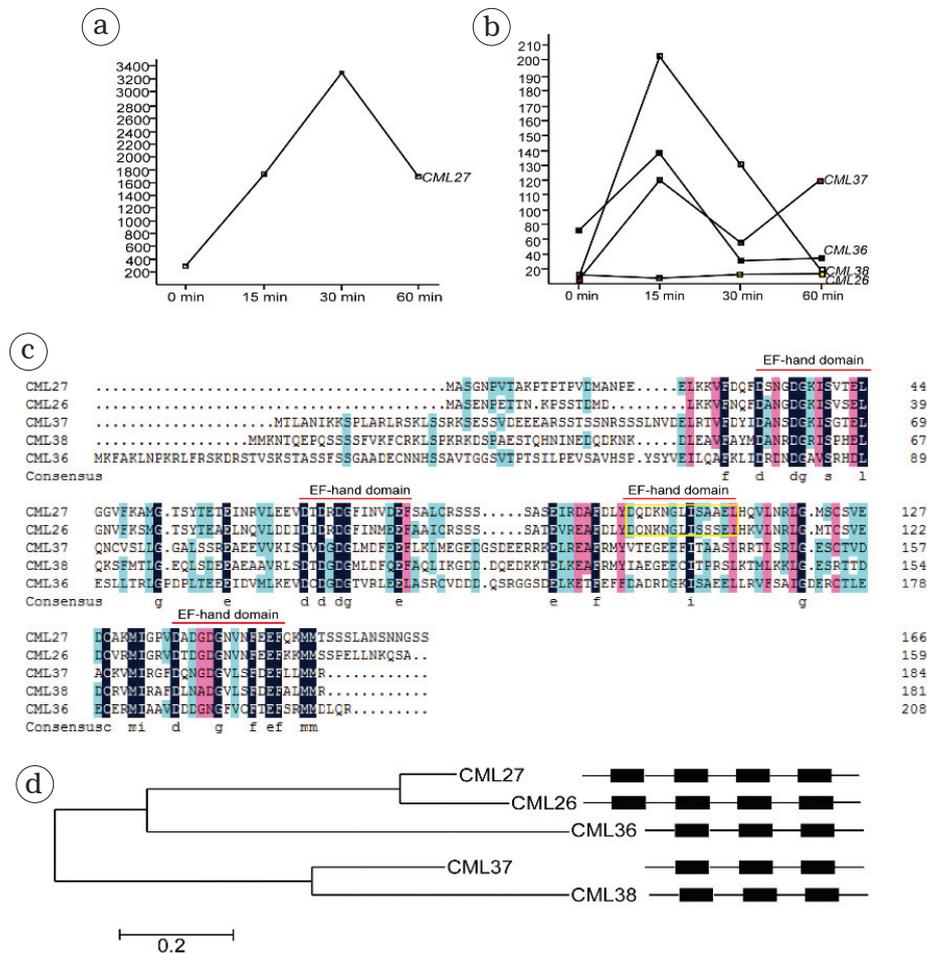
To investigate *CML* genes with different expression levels in the pollination process, we surveyed RNA-sequence and proteomics data of self-incompatible *B. oleracea* plants. In RNA-sequence data, five *CML* genes were expressed at different levels, namely *CML26*, *CML27*, *CML36*, *CML37* and *CML38*. Among them, *CML27* was up-regulated after pollination and reached its peak at 30 min, followed by an immediate down-regulation until 60 min after pollination (Fig. 2a). Similarly, the level of *CML36*, *CML37* and *CML38* increased during the initial 15 min, followed by an immediate decrease at 30 min. While the level of *CML36* and *CML37* increased, *CML38* kept decreasing until 60 min (Fig. 2b). Through alignment analysis on protein sequences, we found that *CML26* and *CML27* contained four EF-hand domains, while *CML36*, *CML37* and *CML38* contained three EF-hand

domains (Figs. 2c, 2d). Phylogenetic analysis showed that *CML27* and *CML26* can be categorized into one clade (Fig. 2d).

Through analysis on SDS-PAGE gels, the spot corresponding to *CML27* showed a decrease at 1 h and an increase at 2 h in pistils after pollination, indicating the slight down-regulated expression at 1 h, and up-regulated expression at 2 h (Fig. 3a). Matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF-MS) coupled with peptide mass fingerprinting (PMF) identified its peptide fragments 69.41% matched to At*CML27* (Figs. 3b, 3c).

## CML27 CDNA CLONING AND ITS EXPRESSION LEVEL IN FLOWER ORGANS AND POLLINATION PROCESS

According to the proteomics data, using un-pollinated pistil cDNA as template, we cloned the full-length of *CML27* cDNA sequence. To analyze

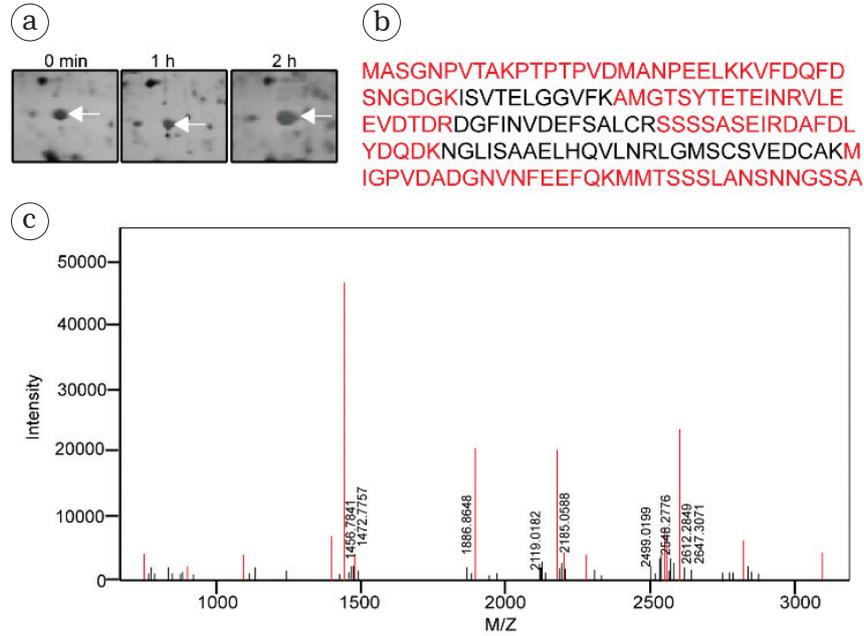


**Fig. 2.** Differentially expressed *CML*s in the pollination process. **(a)** the pattern of *CML27* expression after pollination, **(b)** *CML26*, *CML36*, *CML37* and *CML38* differentially expressed in the pollination process at mRNA levels, **(c)** alignment analysis of protein sequence on *CML26*, *CML27*, *CML36*, *CML37* and *CML38*, **(d)** phylogenetic analysis of *CML26*, *CML27*, *CML36*, *CML37* and *CML38*.

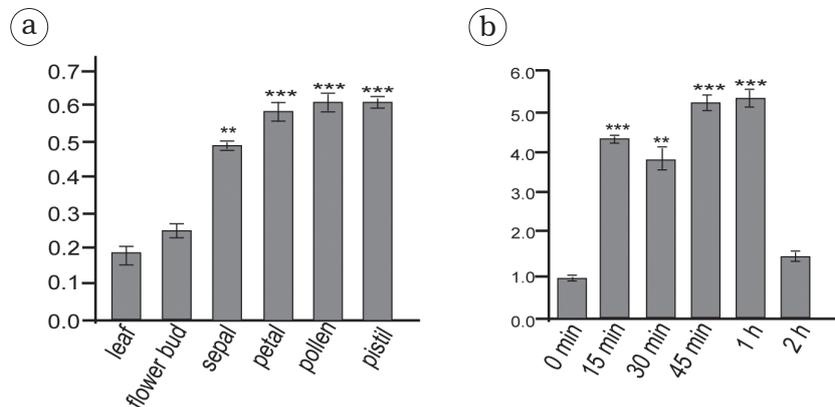
the expression of *CML27* in *B. oleracea* floral organs, semi-quantitative RT-PCR analysis was performed. *CML27* was expressed in petals, pollen and pistils at a higher level, but at a lower level in leaves and flower buds (Fig. 4a). We also analyzed *CML27* levels in the pistils at the early stages of pollination (Fig. 4b). *CML27* expression was immediately induced at 15 min post-pollination, then reduced 20% of its level at 30 min. After 45 min, the level of *CML27* reached its peak, with a 5-fold increase comparing to 0 min. After 2 h, its level significantly decreased (Fig. 4b).

ONE SPECIFIC BAND LOCATED IN CML27-GST + PISTIL PROTEINS CO-INCUBATION LANE

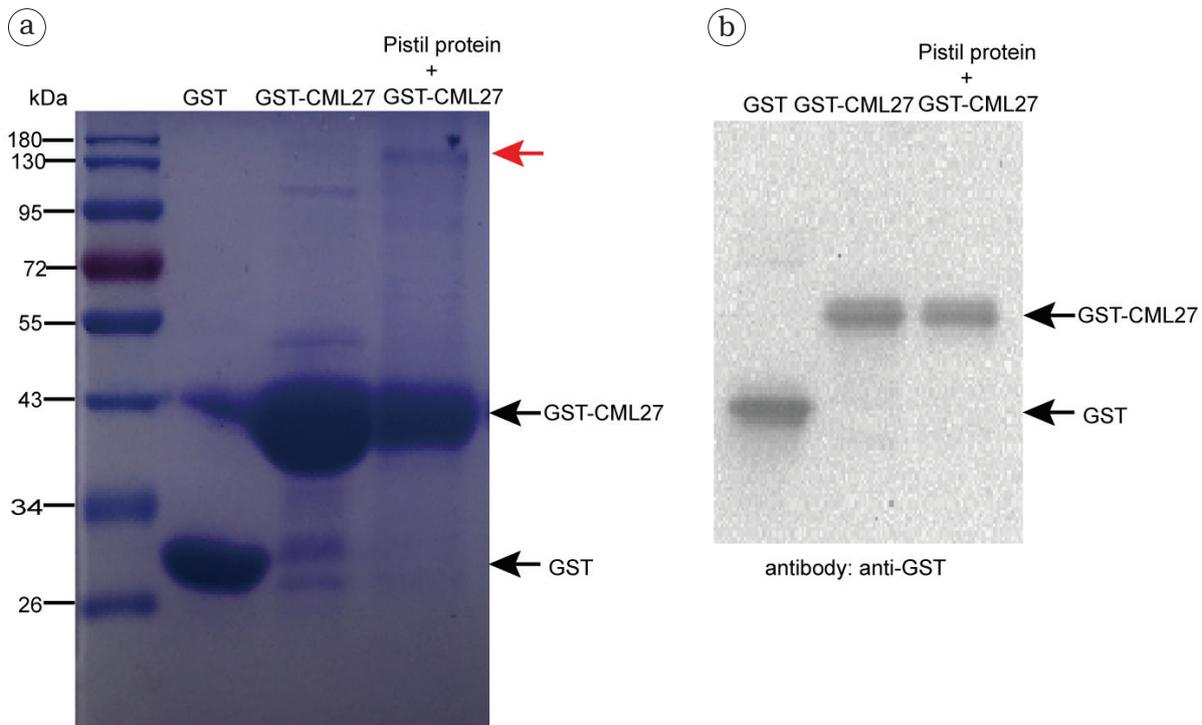
To investigate potential *CML27* interacting proteins in the pistil, GST pull down assay was performed. On SDS-PAGE gels, GST-*CML27* and pistil proteins + GST-*CML27* lanes showed highly expressed GST-*CML27* protein located in 43 kDa size (Fig. 5a). Western-blot assay also confirmed bands specific for GST and GST-*CML27* proteins in each lane (Fig. 5b). It is worth noting that one band was located between 130–180 kDa size in the pistil proteins + GST-*CML27* lane (Fig. 5a).



**Fig. 3.** The protein level of *CML27* expressed differently after pollination. (a) protein spots of *CML27* on SDS-PAGE gel after pollination, (b) the peptide components of target spots on gel matched to *AtCML27*, (c) the first protein mass spectrometry result of *BoCML27*.



**Fig. 4.** Transcription levels of *CML27* in different flower organs and RT-PCR analysis of *CML27* during the early pollination processes. (a) Transcription levels of *CML27* in flower organs, (b) Transcription levels of *CML27* during the early pollination processes.



**Fig. 5.** *In vitro* analysis of the interactions of CML27 with pistil proteins. CML27 was cloned into PGEX-4T-1 vector. The GST, GST-CML27 and pistil proteins + GST-CML27 co-incubation protein were purified, GST protein was used as control. (a) Bound proteins were eluted and stained with Coomassie Brilliant Blue 250 and separated by 12% SDS-PAGE gel. (b) *In vitro* pull-down assays. 10  $\mu$ L purified GST, 40  $\mu$ L purified GST-CML27 and 40  $\mu$ L purified pistil proteins + GST-CML27 co-incubation elution fragments were used in pull-down assays. Immunoblots were performed using anti GST antibody.

The SDS-PAGE specific band, which was located between 130–180 kDa size was cut down and subjected to LC-MS-MS assay. The band was digested with trypsin and then analyzed using liquid chromatography electrospray ionisation tandem mass spectrometry LC-MS-MS (Q-TOF). The identified peptide fragments were subjected to search in *B. oleracea* L. database in uniprot, the protein scores greater than 85 are significant ( $p < 0.05$ ). Finally, we identified eight proteins and the proteins were listed in Table 1.

## DISCUSSION

Angiosperms have evolved self-incompatibility through a pistil-specific recognition system. Interestingly, *Brassica* plants have a dry pistil, which prevents incompatible pollen grains at the early stage of pollination (Dickinson, 1995). A number of molecular components in the self-incompatibility signaling pathway have been identified in both pollen and the pistil organs (Stone et al., 2003; Takasaki et al., 2000; Shiba et

al., 2001). For further investigated potential candidate genes involved in self-incompatibility, RNA-sequence and proteomics were explored to study pistil molecules in the pollination process (Samuel et al., 2011; Wang et al., 2014; Zhang et al., 2015; Zeng et al., 2017). The mRNA levels of key regulatory components in the self-incompatibility signaling pathway, including the levels in *SRK* and *ARC1* have been shown to increase during the initial 30 min, followed by a reduction at 1 h after pollination (Wang et al., 2014). In our RNA-sequence and proteomics analysis, five CML genes were differently expressed in the pollination process at mRNA levels, among which only CML27 was expressed differently at both mRNA and protein levels (Figs. 2, 3).

In transformed self-incompatible *A. thaliana*, once pollen lands on the pistil, SRK recognizes SCR and induces  $Ca^{2+}$  rapidly gets into the sites on pistil papilla cells, which pollen attached (Iwano et al., 2015). In the pollination process, CML27 was up-regulated 5-fold at 1 h (Fig. 4b). In *B. oleracea*, SRK7 kinase domain interacted with CaM12 *in vitro* and *in vivo*, and a further analysis showed

that the interaction between SRK7 kinase domain and CaM12 depended on its four EF-hand domains (Xu et al., 2013). Proteomics analysis revealed that the protein profile in the pistil changed after either compatible or incompatible pollination at 1 h (Chen et al., 2013; Zeng et al., 2017). In GST pull down assay, we separated one specific band located between 130-180 kDa size in the pistil proteins + GST-CML27 lane. LC-MS-MS assay identified eight protein candidates. These proteins are involved in gene silencing, protein metabolism, regulation of translation, Ca<sup>2+</sup> binding and vesicle mediated transport (Fig. 5, Table 1).

The SP11 methylation inducer, which is located in the flanking region of a dominant SP11 allele, can interfere with pollen self-incompatibility phenotype through transcriptional gene silencing of the recessive SP11 allele (Tarutani et al., 2010). Argonaute (AGO1) is an RNA binding protein, involved in RNA silencing. Its conserved PAZ domain binds to short interfering RNAs (siRNAs) and microRNAs (miRNAs), and thus regulates gene silencing (Baumberger et al., 2005). We postulated that AGO1 may function in regulating SP11 or other gene silencing related genes in the pollen rejection response by binding to their mRNA.

26S proteasome non-ATPase regulatory subunit 1 (26S), Ubiquitin carboxyl-terminal

hydrolase (UCTH) and Cystine lyase CORI3 (CORI3) are involved in protein metabolism. In self-incompatible *B. napus*, multiple ubiquitinated bands and their up-regulated ubiquitination levels suggested that more than one substrate was the target for ubiquitination in the self-pollination process (Stone et al., 2003). The ubiquitin-proteasome system is a major protein degradation pathway in eukaryotic cells. It contains at least 32 different subunits which form a barrel-shaped 20S proteolytic core and two regulatory particles capped on either end. 26S is a non-ATPase subunit of 26S proteasome (Wolf and Hilt, 2004). UCTH is one of deubiquitinating enzymes; it binds to small adducts and ubiquitins to generate free monomeric ubiquitin (Jin et al., 2009). CORI3 is the first identified plant cystine lyase and it belongs to a group of eight *A. thaliana* genes, annotated as putative tyrosine aminotransferases (Lopukhina et al., 2001). All of those identified protein metabolism related genes may function in the degradation of pollen compatible factors in the pistil.

Among the eight proteins, two proteins are involved in regulation of translation. Among them, elongation factor 2 (EF-2) catalyzes the translocation of peptidyl-tRNA from A-site to P-site on the ribosome, while EF-2 kinase phosphorylated

TABLE 1. List of identified proteins in pistil proteins + GST-CML27 co-incubation lane.

Accession No.	Protein name [ Taxonomy]	Mowse score	MW (kDa)	Total matches	Significant matches	Total sequences	Significant sequences	confidence
Gene silencing								
tr A0A0D3DJR1	Argonaute 1	907	117.7	41	26	24	15	7.20E-05
Protein metabolism								
tr A0A0D3B4K1	26S proteasome non-ATPase regulatory	201	108.7	10	5	5	4	4.20E-05
tr A0A0D3AHZ4	Ubiquitin carboxyl-terminal hydrolase 12	613	131.4	34	12	24	9	1.3E-05
tr A0A0D3A5M6	Cystine lyase CORI3-like	627	47.6	38	23	13	10	9.80E-06
Regulation of translation								
tr A0A0D3AXH2	Elongation factor 2	937	94.7	56	31	23	15	9.10E-06
tr A0A0D3C8E5	Elongation factor 1-alpha 1-like	414	52.2	38	28	10	9	5.90E-06
Ca <sup>2+</sup> binding								
tr A0A0D3ADA8	Phospholipase D alpha 2	507	92.4	29	12	17	8	6.90E-06
Vesicle mediated transport								
tr A0A0D3A0Z0	Coatomer subunit alpha-2-like	230	137.8	15	2	11	2	0.00014

EF-2 by a highly conserved and specific Ca<sup>2+</sup>/calmodulin dependent kinase (Nairn et al., 2001). In vitro, elongation factor 1- $\alpha$  (EF-1 $\alpha$ ) depends on Ca<sup>2+</sup>/CaM to modulate the dynamic microtubule assembling (Moore et al., 1998). It is postulated that EF-2 may function in pollen rejection depending on CML27 regulated Ca<sup>2+</sup> concentration. Meanwhile, EF-1 $\alpha$  may function in pistil microtubule assembling which also depends on CML27 regulated Ca<sup>2+</sup> concentration.

One protein is involved in Ca<sup>2+</sup> binding activity. *Arabidopsis* has multiple *phospholipase D* genes which display different requirements for Ca<sup>2+</sup>. In Phospholipase D alpha (PLD $\alpha$ ) deficient mutants, they improved tolerance to freezing (Walti et al., 2002). In pollinated pistil, CML27 functions in Ca<sup>2+</sup> transportation and PLD $\alpha$  binding to Ca<sup>2+</sup>.

One protein is involved in vesicle mediating transport. Coatomer functions in retrograding Golgi-to-ER transport and retrieval of dilysine-tagged proteins back to the ER. Yeast coatomer mutant displayed a modest defect in secretion at the non-permissive temperature (Letourneur et al., 1994). It coincides with the concept that retrograded Golgi-to-ER transportation is related to the self-incompatibility response (Samuel et al., 2011).

## CONCLUSION

In our study, *CML27* was differentially expressed at both mRNA and protein levels in the pollination process. In the pollination process, *CML27* was up-regulated expressed at 1 h post-pollination and significantly down-regulated expressed at 2 h. RT-PCR assay revealed that *CML27* was expressed in both the pollen and pistil at a high level. GST pull down assay showed one band specifically located between 130-180 kDa size in the pistil proteins + GST-CML27 lane. LC-MS-MS (Q-TOF) identified eight different proteins in this band, which are involved in five biological pathways: AGO1 is involved in gene silencing; 26S, UCHL1 and COR13 are involved in protein metabolism; EF-2 and EF-1 $\alpha$  are involved in regulation of translation; PLD $\alpha$ 2 is involved in Ca<sup>2+</sup> binding; Coatomer is involved in vesicle mediating transport. Taking together these protein functions, *CML27* may function in increasing Ca<sup>2+</sup> concentration in pistil papilla cells and lead to pollen rejection.

## AUTHORS' CONTRIBUTIONS

Xiao ping Lian responsible for gene cloning, expression analysis and writing manuscript, Jing Zeng responsible for GST pull down assays and

revising manuscript, He cui Zhang and Xiao hong Yang responsible for data analysis, Liang Zhao and Li Quan Zhu responsible for revising manuscript.

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