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Genes Regulating the *ABORTED MICROSPORES* (*AMS*)-Mediated Male Sterility Networks in Melon (*Cucumis melo* L.)

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Author Contributions

YS designed and supervised this experiment. LW carried out all tests of yeast system. DD verified the yeast system and analyzed part of bioinformatics, and wrote a manuscript. XW and PJ conducted data analysis and prepared the tables and figures. DL and FZ analyzed and screened the transcriptome data. DW gives specific modification opinions and constructive suggestion for submission. YS contributed substantially to revisions. All coauthors reviewed and approved the manuscript before submission. LW and DD contributed equally to this work.

Data Availability

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Abstract

The male sterile plants have higher heterosis in the production of hybrid seeds. The *ABORTED MICROSPORES* (*AMS*) gene has been demonstrated to be a candidate gene for ms-5. However, the genetic mechanism underlying *AMS*-mediated male sterility (MS) regulatory networks in melon (*Cucumis melo* L.) is still not clearly understood. In the present study, we used transcriptome sequencing analysis, yeast hybridization technology, quantitative real-time polymerase chain reaction (qRT-PCR), and bioinformatics analyzed to systematically investigate the *AMS*-mediated MS regulatory networks in melon. A set of 15 proteins interacting with *AMS*, including the *C. melo* L. *Zinc Ribbon protein 1* (*CmZR1*) gene, was identified using the yeast one-hybrid (Y1H) system and further confirmed using the yeast two-hybrid (Y2H) assay. The interaction of the *CmZR1* protein with the *C. melo* L. *Pectin Methylesterase Inhibitor 1* (*CmPMEI1*) protein was identified and further verified by the glutathione S-transferase (GST) pull-down technique. Bioinformatics analyzed the physical and chemical properties, gene structure, and kinship of the melon *PMEI* family. We proposed a partial regulatory network for melon MS in which the interaction of *CmPMEI1* protein with *CmZR1* protein regulates the expression of the *AMS* gene for pollen abortion. These findings provide important information for increasing the understanding of the molecular mechanism of the MS regulatory network in melon.

Additional key words: bioinformatics analysis, gene-protein interaction, *pectin methylesterase inhibitor*, yeast system, *zinc ribbon protein*

Introduction

Melon (*Cucumis melo.* L) is an important crop of the family Cucurbitaceae and is planted worldwide. Melon has high heterosis for fruit and seed production. Male sterility (MS) is one of the significant traits and has been reported in a wide range of higher plants. This plays a crucial role in

low-cost hybrid seed production by eliminating the need for emasculation and cross-pollination. MS enhances heterosis in the plant breeding industry and forms an important area of study in developmental biology (Kaul, 1988; Sorensen et al., 2003). The occurrence of MS is closely related to pollen development. The formation of tapetum is the key to anther development. A number of principal tapetum transcription factors in anthers have been identified, including *Dysfunctional Tapetum 1* (*DYT1*) (Zhang et al., 2006; Feng et al., 2012), *ABORTED MICROSPORES* (*AMS*) (Sorensen et al., 2003; Xu et al., 2010; Xu et al., 2014), basic Helix-Loop-Helix (*bHLH*) transcription factors, Male Sterility 1 (*MS1*) (Wilson et al., 2001; Yang et al., 2007) in *Arabidopsis*, the ATP synthase subunit alpha (*ATPA*) gene in ramie (*Boehmeria nivea*) (Duan et al., 2009), the *ATPA* gene in non-heading Chinese cabbage (Jiang et al., 2019), the *Zmms1* gene in maize (Lu et al., 2018), and the *MS2* gene in wolfberry (Cheng et al., 2018). Mutation of these genes at different stages of development results in pollen abortion (Ma, 2005). Moreover, the abnormal expression of other genes, including *RAFTIN1* and *Aps* (Zhai et al., 2018) in the anthers causes MS by affecting the early cell and tapetum formation, meiosis, or pollen maturation (Sanders et al., 1999; Wilson et al., 2001; Boavida et al., 2005; Hord et al., 2006; Zhang et al., 2007; Zhu et al., 2008; Wilson and Zhang, 2009).

AMS has been demonstrated to be a major regulator of pollen wall development. The pollen wall not only provides mechanical protection for male gametophytes but also protects them from microbial attacks and environmental stress; thus, playing a vital role in specific pollen stigma recognition (Zinkl et al., 1999; Scott et al., 2004). It has been reported that the *AMS* mutants of *Arabidopsis thaliana* failed to accumulate lipid spore powder protein precursors (Xu et al., 2014) and altered the expression of anther genes (Xu et al., 2010). *AMS* is highly expressed in the tapetum, pollen mitosis I, and two-cell pollen periods to regulate the secretion of the dull layer, the formation of pollen wall materials, and transport of the tapetum to the ventricle in microspores and immature pollen grains. Its absence results in the vacuolation of tapetum cells and degradation of microspores (Sorensen et al., 2003; Xu et al., 2010). Ma et al. (2012) compared the *AMS* anther transcriptome with those of *Sporocyteless* (*SPL*)/*Nozzle* (*NZZ*) and *Excess Microsporocytes* 1 (*EMS1*)/*Extra Sporogenous Cells* (*EXS*) anthers, revealing both overlapping and different regulatory gene sets, including transcription factors and other proteins. Using yeast two-hybrid screening, Sorensen et al. (2003) studied the interaction between *ASH1*-related 3 *(ASHR3)* with the putative *Myc bHLH* transcription factor *AMS*, which is a key regulator of anther development and stamen length.

In addition to *AMS*, zinc ribbon (ZR) protein (zinc finger protein transcription regulation family) and pectin methylesterase inhibitor (PMEI) protein play significant roles in pollen wall development (Borg et al., 2014; Jin, 2017). The C2H2-type zinc finger proteins involved in pollen development act on the mechanism regulating tapetum degradation (Vizcay-Barrena and Wilson, 2006). The gene encoding the C2H2 zinc finger protein, *DZA1*/*DZA2* participates in the second meiosis process of spermatogenesis (Borg et al., 2014). Pectin is one of the main components of the plant cell wall (Carpita and Gibeaut, 1993). The wall of the pollen tube tip is composed of a single layer of pectin and does not contain cellulose or callose like other plant cell walls (Li et al., 1994; Ferguson et al., 1998). Pollen tube growth inhibition is associated with pectin esterification (Li et al., 1996). This leads to the subsequent degradation of pectin under the combined action of polygalacturonase and pectin lyase (Louvet et al., 2006; Pelloux et al., 2007). The early activity of the *pectin methylesterase (PME)* promoter in *Brassica napus* during the tetrad isolation indicated that the *PME* isoform might play a role in tetrad division (Albani et al., 1991). Mutations in *Arabidopsis thaliana pollen-specific pectin methylesterase 1 (AtPPME1)*, a type I PME, resulted in the loss of a regular pollen tube tip shape and slower growth (Tian et al., 2006). The diversity of

PME expressed in *Arabidopsis* pollen suggests that other members of this protein family are associated with the growth of pollen tubes and may influence different aspects of this process (Pina et al., 2005). Similarly, mutation of the type II *PME* gene, *VANGUARD 1* (*vgd1*), which has the highest expression level in the *Arabidopsis* pollen tube, leads to a slow growth of the style and conduction pathway, leading to a decrease in male fertility (Jiang et al., 2005).

In melon, five single recessive genes, designated *MS-1* to *MS-5*, were originally identified as responsible for Genic Male Sterility (GMS) (Bohn and Whitaker, 1949; McCreight, 1983; Lecouviour et al., 1990; Pitrat, 2002; Park et al., 2004). Sheng et al. (2017) used Specific Length Amplified Fragment Sequencing (SLAF-seq) to map *AMS* as the candidate gene for MS-5. These results suggest that *AMS* may be a key candidate gene for MS in melon. However, the molecular mechanisms involved in *AMS* regulation of MS in melon are still unclear. The objective of the present study, therefore, was to understand the molecular mechanisms of the *AMS* regulatory network. We systematically elucidated the *AMS*-mediated pollen abortion mechanisms using yeast hybridization to identify proteins with potential key roles in the regulation of microsporogenesis. The expression and interactions of these candidate genes were further investigated at different developmental stages of MS and MF (Male Fertility) flower buds using transcriptomics, qRT-PCR, and bioinformatics. The findings on the MS network regulatory system will provide crucial information for the study of MS study at the transcription level in melon.

Materials and Methods

Plant Materials

The MS and MF melon lines were provided by Dr. James D. McCreight, Department of Agriculture Crop Improvement and Protection Research Center in Salinas, CA, USA. These lines were planted in a plastic greenhouse at Bayi Agricultural University Experimental station (125.03° latitude and 46.58° longitude), Daqing, Heilongjiang Province, China. Use conventional water and fertilizer management for cultivation. Samples (10 g) of flower buds (the tetrad stage and mononuclear pollen stage) with diameters of 1 and 2 mm were collected from the plants for construction of the yeast hybridization cDNA library. The samples were frozen in liquid nitrogen at −80°C before transcriptome sequencing, qRT-PCR and PME enzyme activity determination. To reduce potential differences between samples, three samples were used for transcriptome sequencing analysis and qRT- PCR.

Screening of Proteins Interacting with the *AMS* Gene

The cDNA library was constructed by Matchmaker library and a screening kit (Clontech, Mountain View, CA, USA) was used according to the manufacturer's instructions. Yeast one-hybrid (Y1H) experiments were performed as previously described (Dai et al., 2020). Briefly, the cDNA was cloned into the vector, pGADT7 to construct an AD-fusion library for the yeast strain Y1HGold, a strain that does not grow in the absence of uracil. The sequence of the *AMS* promoter was then cloned upstream of the reporter gene promoter. Subsequently, the bait vector, pAbAi containing the *AMS* core sequence was transformed into the Y1HGold strain. The cDNA pool with flanking end sequences homologous to the prey vector, pGADT7-Rec was constructed using SMART™ MMLV RT (Takara Bio, Japan). The cDNA and the linear pGADT7-Rec vector were then co-transformed into the Y1HGold strain. Finally, after co-transformation, the cells were plated on the

SD/-Leu/AbA to select colonies based on the prey proteins which activated the AbA^r reporter. Positive colonies were analyzed by colony PCR and sequencing.

Screening of Proteins Interacting with *CmZR1*

Yeast two-hybrid (Y2H) interactions were conducted using the mating yeast strain, Y187 carrying the full-length pGBKT7-*CmZR1* bait construct with the AD fusion library at 30°C. The culture was plated on selective media Quadruple Dropout Supplements (QDO) containing X-a-Gal (20 $mg \cdot L^{-1}$) for the nutritional reporter genes, *HIS3* and *MEL1* to identify positive two-hybrid interactions. The putative positive clones were transformed with empty vectors, pGADT7 and pGADT7-Rec as control to verify the interactions. Y187 cells transformed with pGBKT7-*CmZR1* full-length and deletion constructs were tested for autoactivation of the *HIS3* and *MEL1* reporter genes. The yeast double hybrid test refers to the method of Thorstensen et al. (2008).

Glutathione S-transferase (GST) Pull-Down Verification of the Y2H Assay

The PGEX-6P-1 plasmid was transformed into the *E. coli* BL21 strain. Isopropyl β-d-1-thiogalactopyranoside (0.6 mM) was added to the cells which were then incubated at 20°C for 4 h. The bacterial proteins were sonicated and purified by GST agarose beads and separated using SDS-PAGE to determine the expression of GST and the GST-*CmPMEI1* fusion protein. The GST and *CmPMEI1*-GST proteins were detected by pull-down assay using 20 μL of samples from the control group or experimental group, 50 ng of GST protein or *CmPMEI1*-GST protein, 10 μL of *CmZR1* solution, 1:50,000 of GST antibody, and exposure for 10 s. For the detection of the HIS-*CmZR1* protein, 20 μL of sample from the experimental group, 50 ng of GST protein or *CmPMEI1*-GST protein, 10 μL of *CmZR1* solution, 1:1,000 of *HIS* antibody, and hypersensitive exposure for 2 min. The proteins were transferred to a PVDF membrane for western blot analysis. The conditions for the western blot were as follows: 50 ng of *CmPME*-GST and GST protein, 1:5,000 dilution of GST antibody, exposure for 10 s, 50 ng of HIS-*CmZR1* and HIS control protein, 1:1,000 dilution of *HIS* antibody, and hypersensitive exposure for 15 min.

RNA Extraction and Transcriptome Sequencing

Total RNA from the melon flower buds was extracted using an RNA Mini Kit (Tiangen, Beijing, China), according to the manufacturer's protocol. The flower buds from MS and MF plants were mixed as a single biological replication, respectively. The experiments were performed in triplicate independently for the MS and MF lines.

mRNA was isolated from the total RNA using oligo-dT magnetic beads and fragmented with fragmentation buffer for the synthesis of cDNA. The short fragments were purified for the end repair, and then connected with sequencing adapters. Finally, the cDNA (−250 bp)in length were used for the PCR amplification. The sequences were quantified and determined for quality using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA)and ABI Step One Plus RT-PCR System (Thermo Fisher, Waltham, MA, USA)after which the cDNA libraries were sequenced using the Illumina sequencing platform HiSeq 4,000 (Beijing Biomarker, Beijing, China).

Table 1. Primers of selected genes used in this study

No.	Gene (CuGenDB id)	Forward sequence	Reverse sequence
1	*AMS* gene (MELO3C021653T1)	CGCTGGGACTGAGAACAATA	TAGCCAGTTGGGTTCATTTG
2	*CmZR1* protein (MELO3C020021P1)	TCGAAATCACGGTAGGAA	GGACGAGTGCGGTAAGAC
3	*CmPMEI1* protein (MELO3C006821P1)	AGTCGGAGACACCGTAGA	TAGCAGCACTCACCCAAG
4	*β-actin* (*AB033599*)	GGTGATGAAGCTCAGTCCAA	TGTAGAAGGTGTGATGCCAAA

qRT-PCR

The qRT-PCR assay was performed to verify the gene screened from the yeast system. The total RNA for the transcriptome sequencing was used for the qRT-PCR also. Reverse transcription and PCR assays were performed using a Thunderbird SYBR qPCR Mix kit (TOYOBO, Osaka, Japan), according to the manufacturer's instructions. The qRT-PCR reaction program is: pre-denaturation 95°C 60 s; 40 cycles (95°C, 15 s, 58°C, 30 s, 72°C, 60 s); dissolution curve (65°C, 5 s, 95°C, 5 s). The primer used in the qRT-PCR was shown in Table 1 and the assay was performed with three biological samples.

Bioinformatics Analysis of Melon *CmPMEI* Gene Family

Sequence Retrieval and Analysis of the *PMEI* Gene Family

The *CmPMEI* gene was obtained from yeast two-hybrid results. Screening of the*PMEI* gene family was performed using the *Cucurbitaceae* database (http://cucurbitgenomics.org/organism/18) for Melon. Next, the reported amino acid sequences of *PMEI* proteins from *Cucumis sativus* L. and *Arabidopsis thaliana (L.)* Heynh. were retrieved using the information available on the National Center for Biotechnology Information (NCBI) protein database (https://www.ncbi.nlm.nih.gov/protein) and the UniProt protein database (https://www.uniprot.org/).

Sequence Alignment and Phylogenetic Tree Construction

Multiple sequence alignments of the amino acid sequences were performed using MEGA 7.0 software. The phylogenetic trees were constructed separately for the *PMEI* using the neighbor-joining method with the bootstrap values set at 1,000 replicates.

Prediction of Conserved Sequences and Gene Structure Analysis

The conserved domains in the sequences were predicted using the SMART database and their distribution was analyzed by IBS software version 2.0. The conserved motifs of *PMEI* were analyzed through the MEME (http://meme-suite.org/).

Characteristics of Gene Structure Analysis

The accession numbers, length of the coding sequence, and the amino acid numbers of *PMEI* were obtained from the SMART database (http://smart.embl－heidelberg.de/). The physical and chemical properties, such as molecular formula, molecular weight, and isoelectric point (pI) were obtained from ExPASy (http://web.expasy.org/cgi-bin/protparam/protparam).

Determination of PME Enzyme Activity

The PME enzyme activity was determined as previously described (Yang et al., 2012). Briefly, pre-chilled 8.8% NaCl (5mL) was added to the samples (3 g) which were then homogenized in an ice bath. The sample was then centrifuged at 10,000 rpm, at 4°C for 2 min. The pH of the crude enzyme extract was adjusted to 7.5 with 0.1 M NaOH and the sample was stored store at 4°C for later use. To determine the activity, 0.5% (w/v) citrus pectin solution (4 mL) and 0.01% bromothymol blue (0.3 mL) were added to 300 μL of the crude enzyme extract. The color of the indicator was changed due to the action of the enzyme and the pectin. The absorbance at 620 nm was measured after 2 min of reaction, and the change in absorbance ($\triangle A_{620}$/min·g) was used to indicate the enzyme activity. The experiments were performed in three biological replicates.

Results

Protein Interaction with the *AMS* Gene

The positive PCR products of the co-transformation separated on agarose gel 1.5% showed bands of 500 − 2,000 bp for the genes in the positive clones of the prey plasmid. In total, 15 positive clones interacting with the *AMS* core elements were identified by sequencing analysis (Dai et al., 2020). The sequences were investigated using BLAST (www.ncbi.nlm.nih.gove/BLAST) to identify potential homologs and protein functions. Among these 15 proteins, three were found to be unknown proteins with no functional annotations. However, sequence comparison showed that there was high homology of the *LOC103496635* (MELO3C020021P1) gene identified by the Y1H system to zinc ribbon protein motif genes. Therefore, we hypothesized that *LOC103496635* is the gene responsible for early pollen development and was named as *C. melo* L. Zinc Ribbon protein 1 (*CmZR1*).

Screening of Proteins Interacting with *CmZR1* and GST Pull-Down Verification

The *CmZR1* gene was amplified by PCR and electrophoresed to recover the whole sequence of the bait gene (Fig. 1). The recombinant plasmid-positive strain was cultured in combination with the DNA library, and the microscopic examination showed normal growth of the yeast cells (Fig. 1E). The conjugated growth and various sizes of the insertion fragments of the library were found by using the pressure screening and the general primer PCR system. The gene size carried by the aggregates in the conjugated growths was different, with a range of 500 − 2,000 bp (Fig. 1B − D).

A total of 19 proteins interacting with *CmZR1* were identified by the Y2H system (some proteins were duplicated) (Table 2). The domains of these proteins were analyzed, showing that the 22 proteins had a high probability of having transcription factor activity. The aligned *PMEI* protein sequence closely resembled the *LOC103483991* (MELO3C006821P1) gene sequence, named as *CmPMEI1*.

The point-to-point verification method was used to confirm further the reliability of the *CmPMEI1* genes screened from the Y2H system. The bait plasmid pGBKT7-*CmZR1* and the prey plasmid pGADT7-*CmPMEI1* were co-transformed to Y2HGold yeast cells for the co-transformation and dot-plate verification. The recombinant bait plasmid showed no toxic effects on yeast cells and no self-activation effects on the Y2HGold yeast reporter gene suggesting an interaction between the *CmPMEI1* and *CmZR1* protein (Fig. 2).

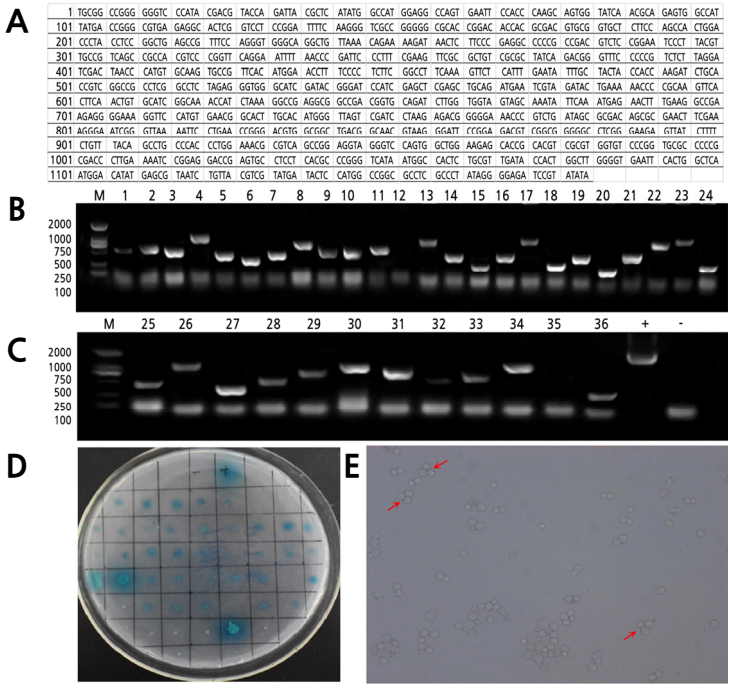

Fig. 1. *CmZR1* gene sequencing data and Y2H binding growth positive screening: *CmZR1* gene sequencing results (A); Agarose gel electrophoresis maps of PCR products of positive colonies interacting with *CmZR1* gene (B-C), M (Marker 2000/1000/750/500/250/100 bp), + (positive control), - (negative control); The combined growth yeast cells were transferred to QDO/X/A (SD/-Leu/-Trp/-His/-Ade/Aba/X) medium (D); Combined with growth yeast cell inverted microscope photographs (E).

Table 2. Functional annotations of positive clones for the Y2H system

NO.	NCBI ID	Functional annotations	Uniprot ID
1	XP_008446602.1	Ribulose diphosphate carboxylase small chain, chloroplastic	A0A1S3BG44
2	XP_008445444.1	protein SPIRAL1-like	A0A1S3BCR3
3	XP_008460580.1	NADH dehydrogenase [ubiquinone] 1 beta sub complex subunit 3-B	A0A1S3CD83
4	XP_016902843.1	GDSL esterase/lipase APG isoform X4	A0A1S4E3N9
5	XP_008439417.1	glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	A0A1S3AYP5
6	XP_008456727.1	peptidyl-prolyl cis-trans isomerase CYP28, chloroplastic	A0A1S3C403
7	XP_008450314.1	succinate dehydrogenase subunit 6, mitochondrial	A0A1S3BP04
8	XP_008437646.1	ubiquitin-60S ribosomal protein L40	A0A1S3AV41
9	XP_008455733.1	10 kDa chaperonin-like	A0A1S3C2U3
10	XP_008439427.1	peptidyl-prolyl cis-trans isomerase FKBP13, chloroplastic isoform X1	A0A1S3AYS0
11	XP_008457647.1	triosephosphate isomerase, chloroplastic	A0A1S3C5Y3
12	XP_008448849.1	putative glucuronosyltransferase PGSIP8	A0A1S3BLA3
13	XP_008437098.1	autophagy-related protein 8f	A0A1S3ASU6
14	XP_008439100.1	Convertase/Pectin Methyl Esterase Inhibitor Domain Superfamily	A0A1S3AXX1
15	ASY96376.1	cytochrome b6/f complex subunit VIII (chloroplast)	A0A249RY55
16	XP_008451480.1	cytochrome c oxidase subunit 5C-like	A0A1S3BRM0
17	XP_008452588.1	ethylene-responsive transcription factor ERF011-like	A0A1S3BU56
18	XP_008448618.1	glutathione S-transferase U8-like	A0A1S3BJH8
19	ASY96481.1	Ycf1 (chloroplast)	A0A249RXX6

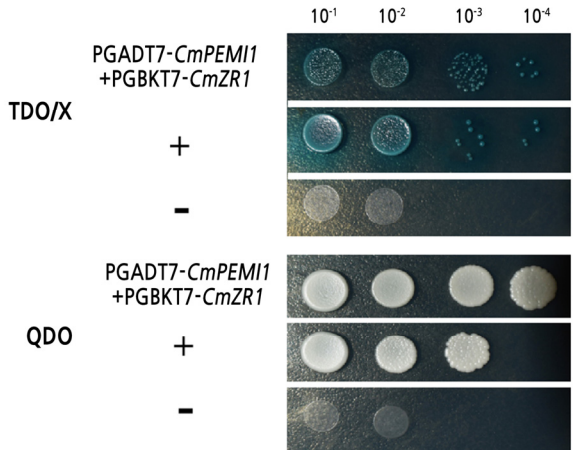

Fig. 2. Point-to-point verification of *CmPMEI1* protein and *CmZR1* protein: TDO/X, SD/-Leu/-Trp/-His/X; QDO, SD/-Leu/-Trp/-His/-Ade.

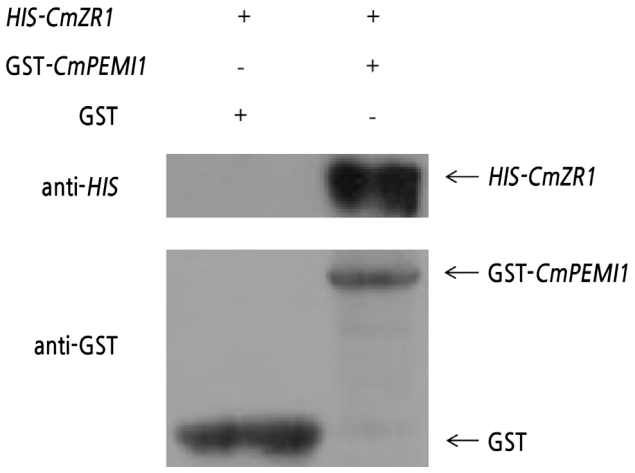

Fig. 3. GST pull-down results.

The GST pull-down test result is shown in Fig. 3. It can be concluded that GST-*CmPMEI1* protein can interact with HIS-*CmZR1* protein.

Transcriptome Sequencing of Differentially Expressed Genes (DEGs)

Twelve DEG libraries were constructed from the tetrad and monocyte stages. We detected a total of 820 differentially expressed genes (Dai et al., 2019). The genes, *CmZR1* and *CmPMEI1,* were screened by the yeast system and subjected to qRT-PCR analysis. The qRT-PCR results showed that the expression of these genes was consistent with the sequencing results (Fig. 4). Compared with the MS plants, *AMS* was expressed significantly higher in the MF lines, whereas the *CmZR1* gene was more highly expressed at the early stage of anther development in the MF lines. The expression trend of the *CmPMEI1* gene was similar to that of the *CmZR1* gene, with no expression difference between the MS and MF plants at the mononuclear pollen stage (Fig. 4).

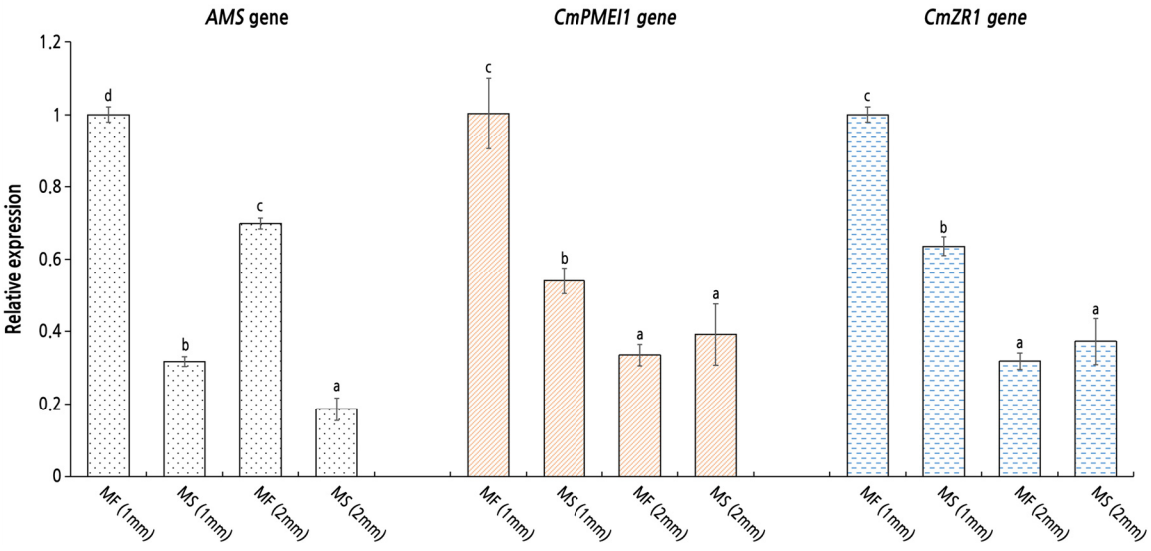

Fig. 4. qRT-PCR verification of genes related to melon MS regulation network: MF, Male Fertility; MS, Male sterility.

Although transcriptome sequencing revealed similar expression trends of the *CmZR1* gene and the *CmPMEI1* gene in MS and MF plants (Fig. 4), compared with the MF plants, the *CmZR1* gene and the *CmPMEI1* gene were expressed to a lesser extent in the MS plants at the early stage of mononuclear pollen development (length of flower bud = 2 mm), suggesting inhibition of *CmZR1* and *CmPMEI1* gene expression. The *CmZR*1 gene identified by the Y2H was located upstream of the *AMS* gene and interacted with the *CmPMEI1* gene.

Phylogenetic Analysis of Melon, *CmPMEI*

CmPMEIs were divided into two sub-groups according to the phylogenetic tree (Suppl. Table 1s) (Fig. 6). The sub-group I contained 31 genes, including the *CmPMEI1* gene screened by Y2H. The sub-group II consisted of 39 genes, mainly composed of *PMEI* genes of melon and cucumber. Within each sub-group, most melon *PMEI* and their cucumber homologs formed a phylogenetic branch at higher values.

Prediction of Conserved Sequences and Gene Structure Analysis

The conservative domain of melon *PMEI* genes analyzed by SMART software showed that the conserved domains of the melon *PMEI* genes were relatively simple, and had only one conserved domain *PMEI* (Suppl. Fig. 1s).

Analysis of Pectin Methylesterase Inhibitor Genes

The lengths of the *PMEI* genes ranged from 160 to 252 residues, with an average molecular weight of about 20,887 Da, and isoelectric points of 4.40 − 9.73. Subcellular localization analysis showed that most of the *PMEI* genes were expressed in chloroplasts and vacuoles (Suppl. Table 2s).

PME Enzyme Activity

For MF plants, the PME activity of tetrad stage (0.136 units) and mononuclear pollen stage (0.236 units) flower buds was significantly different ($p < 0.05$). For MS plants there was significant different of PME activity between tetrad stage (0.151 units) and mononuclear pollen stage (0.202 units) flower buds ($p < 0.05$). The PME enzyme activity of MS plants was higher than that of MF plants at the tetrad stage (length of flower bud = 1 mm). The PME enzyme activity of MF plants was higher than that of MS plants at the mononuclear pollen stage (length of flower bud = 2 mm). At the mononuclear pollen stage, the PME activity in MS plants was 14.7% lower than that of MF plants (Supp. Fig. 2s).

Discussion

Melon has a high intraspecific genetic variation and a small genome (450 Mb). Therefore, it has become a representative crop for genetic studies on important traits in the *Cucurbitaceae* family. Understanding the regulatory mechanisms of the MS network would be extremely beneficial for the utilization of heterosis. Several MS regulatory networks have been developed (Sorensen et al., 2003; Xu et al., 2010; Xu et al., 2014), in which the transcription factor *AMS* has been identified as a key candidate gene for MS (Ma et al., 2012; Xu et al., 2014; Xiong et al., 2016). *AMS* regulates different genes in the tapetum by forming complexes with other proteins such as *DYT1-bHLH* homodimers (Ma et al., 2012; Xu et al., 2014), thus affecting the normal expression of the transcription factor *MS188. MS188* is a petal cell-specific transcription factor for pollen wall formation and interacts with *AMS* to form a complex for activation of the expression of *CYP703A2* and other genes, leading to MS (Xiong et al., 2016). To the best of our knowledge, this is the first report, demonstrating a partial pathway related to the MS network in melon. Using a yeast expression system, we identified a number of proteins such as *CmZR1* with potential key roles in the regulation of anther development and microsporogenesis. The expression and interactions of these candidate genes were further investigated at different developmental stages of MS and MF flower buds using transcriptome sequencing and qRT-PCR. The *CmZR1* gene upstream of the *AMS* gene interacted with the *CmPMEI1* gene leading to the male sterility in melons (Fig. 5). These findings in melon provide the information necessary to study the MS network at the transcription level in melon.

The *CmZR1* gene belongs to the zinc finger protein family and participates in zinc ribbon protein synthesis regulation. Zinc finger proteins self-fold to form a “finger”-like structure that binds to RNA/DNA molecules, thus playing a vital role in the plant development and response to environmental stresses (Chen et al., 2019; Li et al., 2020). Previous studies have revealed the involvement of zinc finger proteins such as C2H2-zfp and BcMF20 in the development of the tapetum, sperm cell differentiation, and meiosis (Kapoor et al., 2002; Kapoor and Takatsuji, 2006; Borg et al., 2014; Han et al., 2018).

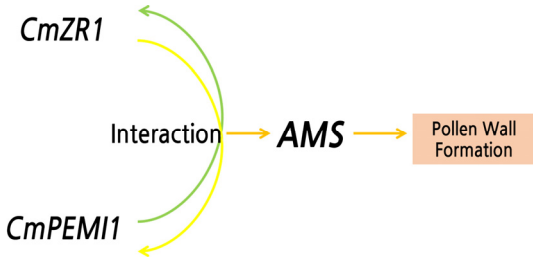

Fig. 5. *AMS*-related MS regulatory network.

Besides, Denes et al. (2000) demonstrated a preferential expression pattern of pollen development-related factors *C2H2-zfp* (*Maz1* gene) in the early stage of *Arabidopsis* anther development. The mutation in the *Maz1* leads to the abnormal deposition of the cytoplasm in the tetrad stage has established an essential role in the defective formation of the cytoplasm and microspores, leading to the reduction of pollen wall formation and pollen fertility (Lyu et al., 2019). The specific zinc finger transcription factor, the inhibition of *BCMF20* expression in *Arabidopsis* mutants with *BCMF20* gene deletion resulted in impaired pollen germination as well as low germination and seed setting rates (Han et al., 2018). Here, we studied the expression of the zinc finger protein gene *CmZR1* upstream of *AMS* in the early stage of melon flower bud development. The *CmZR1* gene was located upstream of *AMS* and affected the regular expression pattern, ultimately leading to MS in melon.Transcription factors bind not only to protein promoter regions but also to other proteins, to form a complex. In the present study, the results suggest that the interaction between the *CmPMEI1* and *CmZR1* genes upstream of the *AMS* gene regulates abnormal expression of the *AMS* gene, resulting in MS in melon (Fig. 5).

Observation of different flower buds development stages of melon indicated that the male flower differentiation was in the tetrad stage when the diameter of melon bud was less than 1.5 mm, in the mononuclear pollen stage when the diameter of melon bud was less than 2 mm, and pollen mature stage when the flower bud was ≈ 4mm (Wang et al., 2009). Previous research indicated significant different were detected in tetrads periods in pollen development for male sterility and fertility lines, because of pollen sac were empty in male sterile plants and pollen grain were not export may reduce male sterility in melon ms5 (Sheng et al., 2016). PME is a key enzyme in pectin metabolism (Louvet et al., 2006; Pelloux et al., 2007) and regulates the plant cell wall's mechanical strength and chemical properties through demethylation of pectin, thus promoting cell development. The cell wall consists of a polymeric network of crystalline cellulose microfibers embedded in the hydrophilic matrix of hemicellulose and pectin (Denes et al., 2000). Previous studies have demonstrated that PME has a key role in the modification of the cell wall and regulates various physiological processes (Kagan-Zur et al., 1995; Guglielmino, 1997; Wakeley et al., 1998; Futamura et al., 2000; Micheli et al., 2000; Ren and Kermode, 2000; Li et al., 2002). Furthermore, studies by Paynel et al. (2014) showed that Kiwi *PMEI* inhibits PME activity in *Arabidopsis* and regulates the root elongation and simultaneously induces the pollen tube burst (Paynel et al., 2014). The change in mechanical strength and stiffness of the pollen tube wall are also considered to impact the elongation of pollen tube and its interaction with female flower tissues (Franklin-Tong, 1999). The PME enzyme activity of MS plants was higher than that of MF plants at the tetrad stage, but for mononuclear pollen stage, the PME enzyme activity of MS plants was lower than that of MF plants .Although the significant increasing of PME activity were all appeared in both MS and MF plant when comparing tetrad and mononuclear pollen stages, respectively, but there were no significant different between 2mm stage in MS vs MF plants. For investigated if PME activity were related to male sterility in pollen development stages, PME activity was also detected in pollen mature stage (flower bud was ≈ 4mm) and significant difference between MS and MF plants in this stage were identified (Suppl. Fig. 2s). based on the results, the PME enzyme may related to pollen development which induce male sterility of melon. And the similar results were also identified in Chinese Cabbage-pak-choi (Liu, 2006) .

By analyzing the bioinformatics of the melon *PMEI* gene, it was found that the length of the melon *PMEI* gene in between 160 − 252 aa, which was similar to the length of the cassava *MePMEI1* protein reported so far (Zhou et al., 2019). The resemblance between the melon and *Arabidopsis PMEI* gene families was not high while the melon genes closely resembled those of the cucumber *PMEI* family was highly similar (Fig. 6). These differences may be due to differences

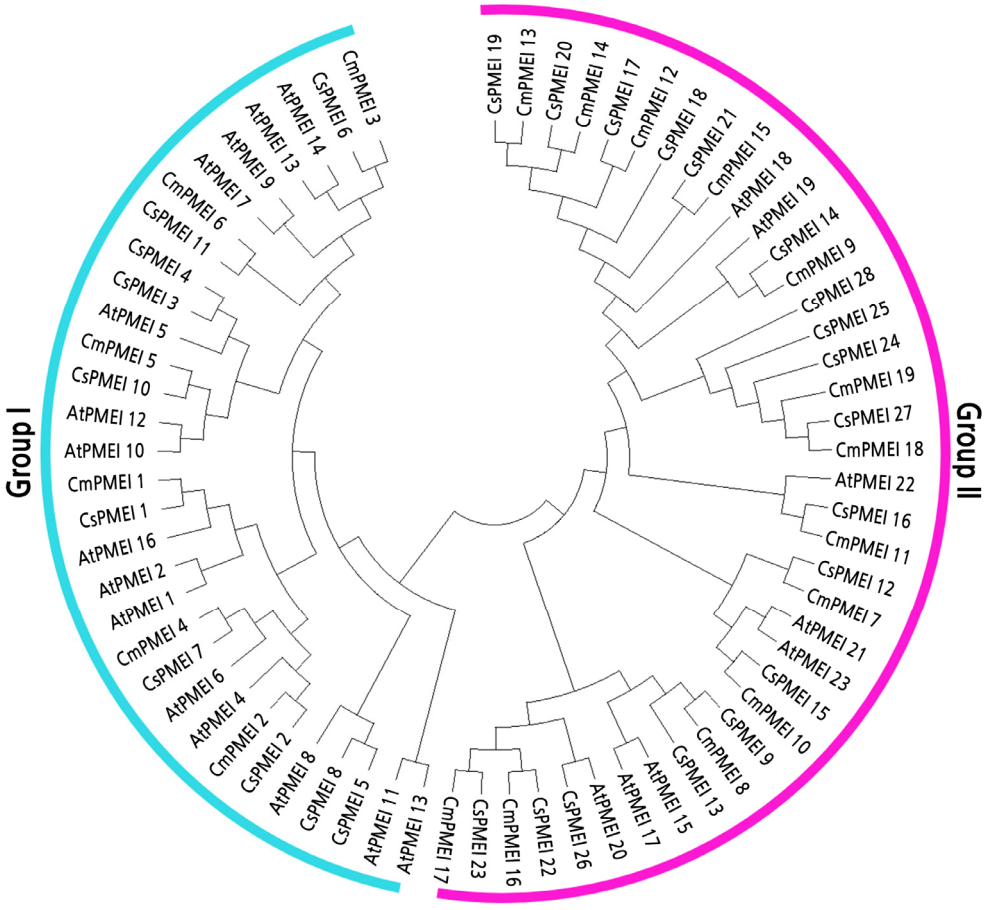

Fig. 6. Phylogenetic analysis of Pectin methylesterase inhibitor gene family.

between species. The subcellular localization of the multiple genes of the melon *PMEI* gene family was found to be in the cytoplasm and vacuoles. This is consistent with findings in other plants (Zhou et al., 2019). Therefore, we speculated that a low *CmPMEI1* expression in the early stage of flower bud development caused demethylation of pectin, thus changing the mechanical strength and chemical properties of the cell wall, affecting fertility in the melon.

To understand the regulatory mechanisms of the MS network in melon, the *AMS* was used as a target gene to screen the downstream interacting partners through Chromatin Immunoprecipitation (ChIP) experiments. However, because of its low success rate in melons, no DNA fragment binding to *AMS* was detected in living cells. Therefore, further studies are needed to investigate the regulatory network downstream of *AMS*.

Conclusion

Using transcriptome sequencing and qRT-PCR, we observed the differential expression between *CmZR1* and *CmPMEI1* in fertile and sterile male melon plants. Yeast two-hybrid analysis showed that *CmZR1* and *CmPMEI1* interacted with and regulated the transcription factor *AMS*. We have, thus, identified a primary pathway related to male sterility in melon.

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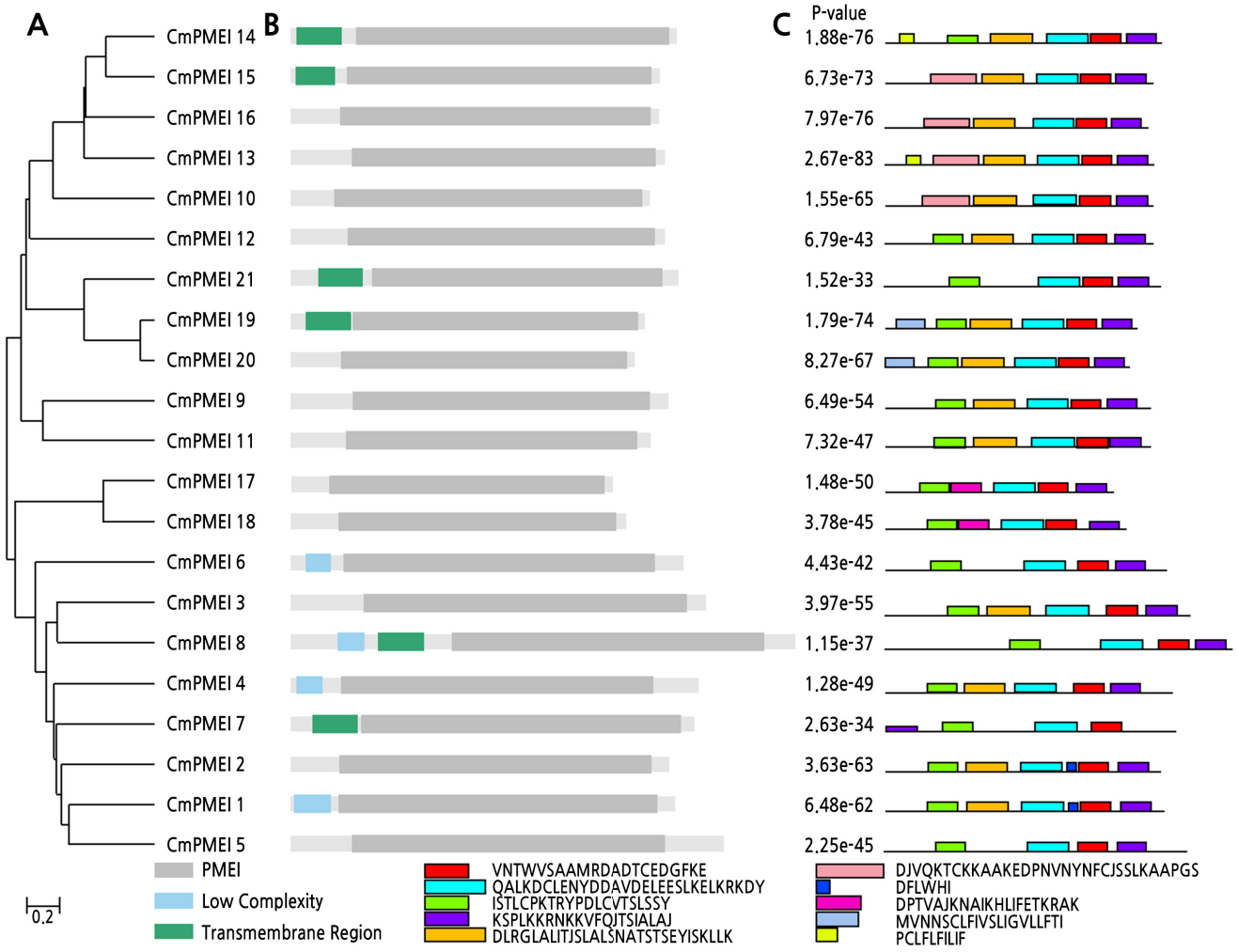
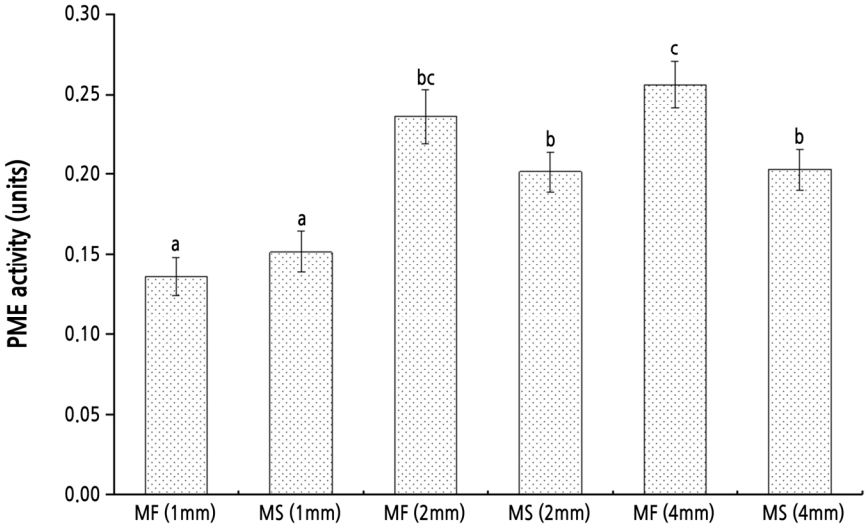


Supplementary Fig. 1s. Analysis of Conserved Domains of Pectinase Inhibitors and Prediction of Conserved Motifs: Phylogenetic tree analysis of melon *PMEI* gene family (A); Conserved Domains analysis of melon *PMEI* gene family (B); Prediction of conserved motif in melon *PMEI* gene family (C).



Supplementary Fig. 2s. Determination of PME activity: 1 unit equals 1 OD600/min·g.

Supplementary Table 1s. Phylogenetic analysis of Pectin methylesterase inhibitor gene family gene list

No	Name	Uniprot ID	Amino Acid Sequence
1	CmPMEI1	A0A1S3AXX1	MAKSLSLLLLLLSILTISASAFNGGASNFIKSKCSAATYPDLCVQSLSSFASTIQRNPRQLVQTALAVSLSRAQST RSFVWKLTKFSGLKPRERAALKDCMEEVGDTVDRLNKSVEELKRVSGSKKKDFLWHISNVETWVSAAMTDE NTCSDGFAGSALNGRIKSSVRGRIVDVTRVISNALSLINKYAETQS
2	CmPMEI2	A0A5A7T9V3	MANLKISLPLLITLIALHNAATTGSPSTSFIESSCKVTRYPALCVQSLSTYANTIRQSGRQLARTALSVSLSKALLA AAFVAKLGKGGGMKGLEYQAVKDCIENMGDSVDRLSQSVKELGDLRRTAGRDFLWHMNNVQTWVSAALT DETTCLDGFAGRRLDGQTKAGIRRRITLVAQITSNALALVNRFADENH
3	CmPMEI3	A0A5A7TTZ6	MKPQRPLILSLLFAATLFHLRPVFANEDTRNSSNATANCIEFIRTSCGITLYPDVCYTSLSRYANDIQQDPASLTRI AITISLANSRRMAAYVSNLSHAGDYGADRRAASALHDCFTNFDDAVDEIRGSLKQMRQINDVDAPSFRFQMSN VQTWMSAALTDQETCTDGFEDVADGPMKEDVCAKAEKVKKHTSNALALVNSFVEKRIP
4	CmPMEI4	A0A5A7UKZ9	MTVLSLLLLLLLSLSLSAAHGGGGAVSQDLIRSSCLQARYPTLCIRTLSSYAGSVKTPRDLAQVTISVSLSLAQN LSEYLSDSLRKASRQQRAAVDDCVDQIGDSVEELSNTLGVLRHLPCGDDRRKFRLEMGNAKTWVSAALTNEE TCLDGFKEVDGEVKLDVKRRIVKVAKVTSNALFMINRLDSGNSTGKEDVGRGGDNDK
5	CmPMEI5	A0A5D3D1R1	MMRPPSTRASIAALLALISILPWLTHSAKTSYVQEACRVTLHQDLCIQSLSPFSSTAKRSPTKWARAGVSVTITE AKKVAALLGRLKNNKRMKGRNRAAVLDCVEVFDAAIDELHRSLGVLRRLSRRNFDSQMGDLTTWVSAALTD EDTCVEGFEGERGKVVSLLRNRVVKVGYITSNALALVNKLATSGFETALNM
6	CmPMEI6	A0A5D3DJE3	MESQILKSSLTLFFFFIILTTFTPSAVASSSSTVRPVKPHIRKACKPTPYPRLCETALSLYASQTKRNQQELCRAAM VSSLKAAQNATSIISKLSRRKMSAYETEVIGDCIDDLKDSVDELRRASTAIKSLSRSKDVDFQLNSIKTWTSAAL TDIITCTDGLSGGSGWKVNKKVKKEVKNCSINVVRQISNALSLINNFNYK
7	CmPMEI7	A0A5A7T3U1	MDQINALKGYGKLTHHNLDLEHQIPPPPSKPNSKFPNNHNYSLPLRFAAAISALLLTALIISLIVGVYTHNSTPDN KSSSNNAAHTISIICNVTRYPNSCFTSISSLNSSPQPDPELILNLSLQVSLNELSNISRWVKTLGAEGDGGAAAALK DCQSQIEDAISQVNESVAEMRGGSGEMTLTESKIGNIQTWMSSAMTNEESCLDGVEEMDSTSFEEVKRRMKKS IEYVSNSLAIVANIHVILDKFNMPLH
8	CmPMEI8	A0A5A7UBK3	MKSIAIAVAFLVISLCQFQILAQTPQTPTTSGGNDLITKTCSSTPHVEMCKTILQSSPNSKGADLYGLAQIVMNIA ASNVSNIYESINQLQNGTTVDSFLDSCLTDCLESYQDAIDQIEDSVTALEFKAYNDVNTWVSAAMSDAATCDM GFKEKKQGYQSPIAQMTTVFDQICKIILAINKLLSQGNSN
9	CmPMEI9	A0A5A7TAE5	MENPIAKSILFLLCVPTLIQLANGLDIVQHYCKLAAKTDPYVDYKLCVQTLKANPNSEDADFKDLVMISINQAK ANATEIRSEISELMKRTSEKWGKYSLNCLKSCLELYSEAVSDLKKALRGLKMEDYETAKTAVSAAMDAPVSCE DGYKEKDGEVSPLSETNDGFFQLVAISLAFINMC
10	CmPMEI10	A0A5D3E196	MKFFSAILLVLCLCMAAQRLDAASQANKEGIEKIEKMCAQTNYKDLCVTSLTSDPNSFPADKMGLALVALRLA SSNASDISESIKVMLNETSQNEPAVQQGLFDCLDEYLDASQQLDDSIAAIIAKAYGDVEKWVHAAVADVRTCE NSFPTKPSVLTPRNEEFIKLCDIALSISRIAEEN
11	CmPMEI11	A0A5D3DQ82	MGMKNFSISLIFFAIPLIFFHKNGVSLASADQTLIQKTCTNTLYYKLCMSSLKSDPSSLTADTKGLAIIMASIGAA NATATSTYLSSQLPTSSSAATNANNNKTKLLRQCSEKYAFAAEALRESLKDLADETYDYAYMHVSAAADYAN VCRDAFKGFPAVSYPAKLGRREEGLKRICRVVLGILDLLGW
12	CmPMEI12	A0A5A7TAH1	MKPKFSLSPFMTTPLPCLLLFIMIFNISTTIVVADLVQKTCKKCETDDPNVNYNFCISSFRAHSGSDSTDLRKLGA ISLSLIQKNLSSSLEYVEKLLQNKEIDSYRRVRLNDCLDVYSDAIVNVEEGKKAYKEKHYDDANIKVSSVMDA ARVCEDGFREKEGVSSPLTKWNKDLFQLAAIALSIVNMYP
13	CmPMEI13	A0A5A7TEK0	MSSLSHSILIPCLFLFMLIFSNFPITQSSNNNNNTSSLIYKTCKASSEQDPNISFDFCITSLKPAATKHRHGDTSLRR LGLMTIYLIRHNMSSTRHHIKKLQRKKGPGDPFVKLCLTDCLELYSDAIPMVKQARKDYKAGRYADANSKISS VMDDCSTCEEGFEEKEGVVSPLTNRNHNAFELSAIALSIINML
14	CmPMEI14	A0A5A7TCY0	MAIPHYFSVFILSIFFFSNFLIIQSSKTIKTADLIYKTCKKISREDPNISFNFCLTSLKLATNHSRCTDVRHLGLLSIG LLYRNVTSTCHHITKLVKNKKLDPFVKSCLDDCLELYSDAIPTVKQAMKDYKSKRYDDVNVAIGSVMDAATT CEDGFKERKGVASPLKKRDENAFELGAIVLSIMSLVR
15	CmPMEI15	A0A5A7U1K7	MSFTYTHRYIALAFSFVVLLNFEPISANDIVSRTCETSAARDPNVRLDFCLRSLAAAPGSDTADLYELGAISIRLI GRNATSTQRYIERLLKNEKKKSSSDSYIRPRLSDCEELYSDAVETVGEAAAEYGRKRYDEVNVKLSSVMDAVT TCEDGFKEMESRVSPLTKRNGDVFELAAIALCILDLRP
16	CmPMEI16	A0A5A7SYM0	MGENHLVSFSMVFLFAAVFIGQAHGSSLCDKAAFPALCRSTLKGASDPTSALKNAIKHLIFETRRAKVSSLRIGS LKSLVVCKQNFDDAIDDLETSLAYMQKKDIASLKINLSAAMTDYSTCDDAVIESGEQKKASRVLNTDNLLEQL AANCLYLASLLK
17	CmPMEI17	A0A5D3CVX8	MAKKQSVSFSPVFLVVATAVVVLFAGQAQGVDICAQAEYVPLCRSVVKGASDPTVAIKTAIQHLSFETKRAKT ASSILGNKQAIDACTQNYDSALDNLQKSLEYLQIKDLPSLRVMLSGALSFYVSCTDAVAEVSTFGVVKMAKNV EKTDTALQHLAGNCLHIASLLK
18	CmPMEI18	A0A5A7V7V7	MGKNKEIKMVYNSCLFIVSLIGVLLFTINVASSTDVVSTICPKTSNPQFCSSVLKSAGTTDLKGLAVYTLNLAHT NARKSLTLANSLAKTTTNPQLKQRYSSCVESYDEAVGDIENVQKDLALGDFNGVNIVTSGAMTEIDDCQDKFA QPPKDTSLLLKNGKTLNDICSIILVISNLL
19	CmPMEI19	A0A5A7VHV0	MANNSCLIIVSLVGVLLFTIISNVASSNDVVSTICPKTSNPSFCSSVLKSAGTTDLKGLAVYTLNLAHTNAHKSLT LAKLLATTTTNPQLKQRYSSCAESYDETVGDIENAQKDLALGDFNAVNIVTSGAMTEIDDCQDKFAQPPKDTS LLLKNGKTLNDIYNIILVISNLL
20	AtPMEI 1	Q9LVA4	MAKQYLFVLLSISYLLSLELTAATAASQTGASKKAINFIQSSCKTTTYPALCVHSLSVYANDIQTSPKRLAETAI AVTLSRAQSTKLFVSRLTRMKGLKKREVEAIKDCVEEMNDTVDRLTKSVQELKLCGSAKDQDQFAYHMSNA QTWTSAALTDENTCSDGFSGRVMDGRIKNSVRARIMNVGHETSNALSLINAFAKTY
21	AtPMEI 2	Q9STY5	MAKQIFYTLFLFLLSTAILTASSSAPRAAITSKRAINFIQASCKATTYPTVCVNSLTGYANSIQTSPRRLAETALNV TVTQAQSTKVFVWRLGRFTSLKKREIQAVKDCIEEIHDAVDRLTMSIHEVKMCGSAKGRDQFWFHMSNAQTW TSAALTNANTCSDGFAGRVMDGRVKNSVRARILNLGRGTSNALALINAFAKKY
22	AtPMEI 3	Q9SB37	MARNFELSLILFVLYLSTAAIVMARNLEEESSGDTEFIKASCETTSYPDRCFQSLSSYASEIKKQPRKLAETALAV SIARAKSAKTYVSEMTDYKGITKRQHEAVADCLEEMGDTVDRLSNSLKELKHLEEGDSGEDFWFCLSNVRTW TSAALTDETACMDGFGGKAMAGELKSLIRTHIVSVAEETSNALALINDFASKH
23	AtPMEI 4	Q9STH2	MEPKLTHLCYCLLLFLPLLCQSTIAKPSSSPNPSSSINFIVSSCRVTRYQTLCVKCLAAFADKIRRNENQLAQTAL AVTLVRVQSTTIYVGKLTKARRIKRREYLAVKDCVENLGDGLEMLAQSMRELKQVGRSGRDRDEFLWRLSNV ETWVSAALTDETTCLDGFDGKVMDGVVKSAIRRRVVHVARVTSNALALVNRFAARHKS

Supplementary Table 1s. Continued

No	Name	Uniprot ID	Amino Acid Sequence
24	AtPMEI 5	Q9LVA3	MGESFRLFNHHHFLTTFLIIIAMLKLVHTTTTTTTTTTTTNTEFVKSSCTFTTYPRLCFSSLSTHASLIQTSPKLMAH AALNITLASAKVTSAMMVRLSNSRLKPKEVSAMRDCVEELGDTLEELRKSIGEMCQLSGSNYEVYISDIQTWV SAALTDVNTCTDGFEGEDMDGKVKVLVRGRILVIAHLTSNALALINHFASIHG
25	AtPMEI 6	Q84WE4	MAPTQNLFLVAIAFAVIFTASTVHGRHNGAEDIVHSSCEHASYPSLCVRTLSSYSGPTITNRRDLAQAAIKISLSH AQSAAKKLAVVRDSVGKKKQEKAALVDCVEMIGDSVDELSRTLGVLKHLRVSGGSAKEFRWQMSNAQTWA SAALTDDDTCLDGFQGMDDGEIKTEVKQWMTKVARVTSNALYMVNQLDETRGKPHDVHL
26	AtPMEI 7	F4HXW0	MLTRNKEEINRVKNLKLKMGRQLYTTTVLYLVTLLFICRTISAVRFPPEQPTTDDLDFIRTSCNTTLYPDVCYTS LAGYASAVQDNPARLAKLAIGVSLSRAKYTAAYLSKLSRRAASAAVHDCVSNVGDAVDQMRGSLRQLREMN HRRPGDPAFRFQMSNVQTWMSAALTDEETCTDGVTEEMEDGETKTAICDRVADVKRFTSNALALVNTYANN GA
27	AtPMEI 8	Q9SI74	MNILSQTQILHLSIAILLFITTSSSSLSPSSSSPSLSPSPPSSSPSSAPPSSLSPSSPPPLSLSPSSPPPPPPSSSPLSSLSPSL SPSPPSSSPSSAPPSSLSPSSPPPLSLSPSSPPPPPPSSSPLSSLSPSSSSSTYSNQTNLDYIKTSCNITLYKTICYNSLSP YASTIRSNPQKLAVIALNLTLSSAKSASKFVKNISHGGGLTRLEVVAVADCVEEIGDSVTSLQDSIRELDSINYK DSAKFEMVMSDVETWVSAALTNDDTCMDGFSLVKTAVKDLVRRHVVEVARLTSNALALINMYASTQENFS
28	AtPMEI 9	Q9ZNU5	MVTVSQSHTTTFLFFTTFLLIFGSISAVRLLPRPNTTTTNDLDFIRTSCNATLYPDVCFTSLSGYASAVQDSPARL AKLAIGVSLSQAKSTAAFLSKLSRSAAKYSGDGHQTASAVIRDCVSNVEDAVDEMRGSLRQLRDMNGRGGGT AARRSVETFRFQMSNVQTWMSAALTDEDTCTDGFEDMDEGGLIKTTVCDRLEEVKRLTSNALALVNTYANN GAP
29	AtPMEI 10	Q9SB38	MLRFVVLSLTLMVFINSSNFPKTAATPPGTYQNHTTYVKTACNSTTYPTMCYNCLSSYSSTIKSDPIKLCTTSLN LNVKSAKNATLVVSNLLQKAKAAKSHEVSILKDCVDEMKDTIDELKQAVAEMKYVRGGGKTTEEHLKNVKT WVSSALTDEGTCTDGFEEGRVNVETKKKVKKAISELSKTTSNTLALLTHYLSY
30	AtPMEI 11	O81309	MAANNKLFFVLLSLFPLIIFSATATSSKDYDTKAYVHSWCRTTLYPKLCVRSMSRYVRSRAVQNPRDLARFAL KASLYRAKYTKAFLLKEVKNLETTLRPQYYASVHDCLDQIRDSVNQLSLAIAELDRVSRRQGKSQGDLHWHIN NLQTWTSTALTDAETCVSQFPGRRMSKLKATIKGKVKNVEETTSNALAFIEHYAAARYRARRP
31	AtPMEI 12	Q9FHN2	MSQVLYSLTIVFVFFASTNIQKTSGSASSYSQNHKTFVKTACNSTTYPDKCYKSLSSYSSNIKSDPIKLCTTALNL NVKSAKEATSVVSKLLKMSQKSTAGRKGKMLPEALILKDCLEEMKDTIIELKQAITEMKNLQDGGSMAEHITN VRTWVSSALTDEGTCTDGFEEVKVNKETKKKVNKVVEELATTTSNTLALITNLRY
32	AtPMEI 13	Q1PFE5	MVTMMRPTLLILLFSTFLPQILTVDPPLLPSNGSDFIRLACNTTLYPDLCFSTLSSFANSIQNDSNRLARVAISLTL HNTLHLLSYLQNAYNRDHPTPVLRDCFENLKDAVDGMRGSMKQMKELVSASGSIESFRFQMSNVKTWLSAA LTDEYTCTDGFKDVHEDDSIKDDVCSRVDDVKKLTSNALALVNRYADESIIN
33	AtPMEI 14	O49297	MKLSLHQPLLFFFLASVLPLILTVHSQSDDSDFIRTSCNTTLYPDLCFSSLSSFSSSVHNDPALLARAAISVTLTKT LDLASYLANITTLQPESNEDGAHHPTAAAVFHDCFDNLKDAVEEMKGSMKQMRELVSTGSLESFRFQMSNVQ TWLSAALTDEETCTDGFKDIHDEPRKDDICARVDDVKKMTSNALALVNRCVDKAIH
34	AtPMEI 15	Q9LZI3	MKTPMSSSITFALVFFLLSLNPTSSLPSKRESYVQNACSVTRYQDLCAKTLLPFASVAKNSPSKWARAGVSVAIT DNKDVLRHLLKTRLSTIGKRDRIALSDCRELLQDSLDSLHKSLAVLRTLRASEFQQQMSDLATWLSSSLTDKDT CLDGFEKTSTRSSSTVRMIRKRVTTSMYLSSNSLALLNKLAANGL
35	AtPMEI 16	Q9LVA5	MAKQYQALFLLFSVFYLFSSVLTTATVNPAGTTTKALNFIQSSCKSTTYQSLCVETLSVYANTIKTSPRHLLDAA ITVSLNQALSTKLFISHLRKSQFQILQDCAPSTDTFSTDCECSVQALQEVVNCNSWTDCLFHVKNAEVCAISGES HSVENTCSSPFADPGKISARGRISDAVRKSLHTRFSKLRQEINNAKMLFEAFPNKH
36	AtPMEI 17	O22244	MTSSSSSPITFTLLLLLSLLVALNPNPSLASTGSNINTNDIVTQYSTYVRNACNVTRYNRLCVRTLWPFAIVARN NTSKWARASVAVTITDTKRVLRLLLKTQRSAVGESERIALSDCRELFVDSLDNLYKSLAVLRTLNADEFQRQIS DLATWLSAALTDDDTCLDGFEETSSRTRTVRMVRRKATKCMRLCSNALALLKKLAFDGL
37	AtPMEI 18	Q9FFW0	MKLSQVFYIIFLFFLVSQVKSTSDMIDQTCKSCAAKSTIFDYNFCVSSLNNSPIALPSPTNLSSLALVPMLQALDN ATATASTIQQLLISDDDGSFRSACLRDCLELYEDATDRLEEAVRVFITRKELGTVNVMVSAAMESAVTCENGFR ERDDGDGGGGGVTTWTSPIGDENHKLFEFGQIALCIFNMLSSSVTSLSF
38	AtPMEI 19	Q9FJR7	MKFLLYLVTFFVLSNGLANGQTLIRNSCKKATATSPKFKYNLCVTSLETNPQAKTAKDLAGLVMASTKNAVT KATTLKGTVDKIIKGKKVNKMTAMPLRDCLQLYTDAIGSLNEALAGVKSRNYPTVKTVLSAAMDTPSTCETG FKERKAPSPVTKENDNLYQMILIPLAFTNMLK
39	AtPMEI 20	Q9SAC5	MAGRDTFQLRFAAVAASAAVLIFLMAGQVAESRMINICSHTAYPSLCRPLVKRVTSPRKATHRTIQALEAKTK LALAETARFKNGNQAVSTCYETLGDAVYNLASARKSIRKRDVPAMNTYLTAAVSDYGACVDGFIETQQVNAI QNAVVDLRKISSNCLALSTLIR
40	AtPMEI 21	F4JHA1	MKMAGRIFLLFLSVYVTVAIADKAFCVASLTSRPEAATATAPKLGVIALSIASSNASDTSFYIKAKLKQKNLEPA LEDTLDDCSKNYLDAVAQLDDSLAALMQNSFIDVDIWLNTAISDGEACENALNDRAGNDAELARRNTNLLKL CKDALLINTILTP
41	AtPMEI 22	Q9LNF2	MAANLRNNAFLSSLMFLLLIGSSYAITSSEMSTICDKTLNPSFCLKFLNTKFASPNLQALAKTTLDSTQARATQT LKKLQSIIDGGVDPRSKLAYRSCVDEYESAIGNLEEAFEHLASGDGMGMNMKVSAALDGADTCLDDVKRLRS VDSSVVNNSKTIKNLCGIALVISNMLPRN
42	AtPMEI 23	F4I1W9	MKQSAGVLFLCIVLISFVTGNANSGMISDLCKHSDDPNLCLSSITSRPESGEFAGTSNQIEIIAISAASANASATSS YIKQKLSNEDLEPAIEDTLEDCQKDYQDAVEQLDDSISAMLADAHTDVDVWLSAAISAIESCGSALGSRAGND AELSQRNEVFLKLCKNALMINKMLT
43	CsPMEI 1	A0A0A0L8D7	MAKSLSLLLLLLSILTISASAFNGGASSFIKSKCSAATYPDLCVQSLSSFSSTIQRNPRQLVQTALAVSLSHAQSTR SFVWKLTKFSGLKPRERAALKDCMEEVGDTVDRLNKSVEELKRVSGSKKKDFQWHISNVETWVSAAMTDEN TCSDGFAGSALNGRIKSSVRGRIVDVTRVISNALSLINKYAENQS
44	CsPMEI 2	A0A0A0KR60	LHNAATTGSAATSFIESSCKVTRYPALCVQSLSTYANVIRQSGRQLARTALSVSLSKARLASAFVAKLGKGGG MKGLEYQAVKDCIENMGDTVDRLSQSVKELGDLRQTAGRDFLWHMNNVQTWVSAALTDETTCLDGFAGRR LDGQIKAEIRRRITLVAQITSNALALVNRFADENH
45	CsPMEI 3	A0A0A0LB27	TEFIRSSCSSTTYPRLCFSSLSVHANAIQTSPRLLATAALSVSLSSVKSTATQILKLSHSHGLPSRDVSALNDCLEE LSDSVDSLAASISEMPKLRGTNFDLAMSNVQTWVSAALTDETTCSEGFQGKTVNGGVKAEVRTKIVNIAQLTS NALSLINRIADLH
46	CsPMEI 4	A0A0A0L8D1	MGISNSKKSLILFQLILQLTLIHSAITPQSSTEFIKSSCSSTTYPRLCFSSLSVHANAIQTSPRLLATAALSVSLSSVK STATQILKLSHSHGLPSRDVSALDDCLEELSDSVDSLAASISEMPKLRGTNFDLAMSNVQTWVSAALTDETTCS EGFQGKTVNGGVKGVVRTKIVNIAQLTSNALSLINQIGDLH

Supplementary Table 1s. Continued

No	Name	Uniprot ID	Amino Acid Sequence
47	CsPMEI 5	A0A0A0KP63	MENPLPTLLPLLLLLLIISDQTQILSVAASTLPRKSSAGIRTNTEYVRTSCSTTSYPRLCYNSLSVYAGKIKTNPKT LALAALHVNLAAARSSAASMRRLAKTRGLRRRDASAISDCVEEVGDSVFELQRAIRELGRPRGYDFMGLISDIE TWVSSALTDEETCMEGFGGRRVNGVSVKAKVRRHIVRVAHLTSNSLALINSYASSAAVEEGVLP
48	CsPMEI 6	A0A0A0KPD9	MKPQRPLILSLLFAATLFYLRPVSADEDTPNSPNATANCMEFIRTSCGITLYPDVCYTSLSRYANDIQQDPASLT RIAITISLANSRRMAAYVSNLSHVGDNGADRRAASALHDCFTNFDDAVDEIRGSLKQMRQINDVDAPSFRFQM SNVQTWMSAALTDQETCTDGFEDVADGPMKEDVCAKAEKVKKHTSNALALVNSFVEKKIP
49	CsPMEI 7	A0A0A0LWR8	AISQDLIHSSCLQASYPTLCIRTLSSYAGAVKTPRDLAQATISVSLSLAQNLSEYLSDSLRQASRQQRAAVDDCV DQIGDSVEELSNTLGVLRHLPCGDDRRKFRLEMGNAKTWVSAALTNEETCLDGFKEVDGEVKLDVKRRILKV AKVTSNALFMINRLDVTNGGGFTAVE
50	CsPMEI 8	A0A0A0L1P3	MKSSYFPLPVKAILLILLINQSNIIANSQPINDTQFIKTTCQSTPYPDLCLSSLSDSAATIHSSCHLMTVAALTVALT HTRSTSSAIESLAKSSNALTPRDSYVIRDCIEEFGDSVEELKMAVEELKDNNKSRSETEDIRTWVSAALTDDDTC MDGLVGDAMNGNVKESIKEMVVNVAQLTSIALSLVSLLK
51	CsPMEI 9	A0A0A0KAX0	MLRAPSRRASIIALLALISILPWLTHSAKTSYVQEACRVTRHQDLCIQSLSPFSSAAKRSPTKWARAGVSVTITEA KKVAGLLGRLKNNKRMKGRNRAAVLDCVEVFEAAIDELHRSLGVLRRLSRRNFDAQMGDLTTWVSAALTDE DTCVEGFEGEEGKVVTLLRNRVVKVGYITSNALALVNKLAASSFETTINM
52	CsPMEI 10	A0A0A0L671	MESQILKSSLTLLIFFFIILTTFTPSAVSSSSSTVRPVQPHIRKACKPTPYPRLCETALSLYASQTKRNQQELCRAA MVSSLKAAQNATSIISKLSRRKMSAYEAEVIGDCIDNLKDSVDELRRASTAIKSLSRSKDVDFQLNSIKTWTSAA QTDVITCTDGLSGGSGWKVSKMLKKEVKNCSINVVRQISNALFLINNFNYK
53	CsPMEI 11	A0A0A0LVF4	LIVGVYIHNSTPDNKSSSNNAAHTISIVCNVTRYPNSCFTSIFSLNSSPQPDPELILNLSLQVSLNELSNMSRWLKS VGGEGDGGAAAALKDCQSQIEDAISQVNDSVAEMRGGSGEKTLTESKIGNIQTWMSSAMTNEESCLEGVEEM DATSFEEVKRRMKKSIEYVSNSLAIVANIHVILDKFNMPLH
54	CsPMEI 12	A0A0A0M1H8	MKSIAIAVAFLVVSLCQFQILAQTPQTPTTSGGNDLISKTCSSTSYSEMCKTILQSSPNSKGADLYGLAQIVMNV AADNVSSIYENINQLQNGTSVDSFLDSCLTDCLESFQDAIDQIEDSVTALEFKAYNDVKTWISAAMSDVATCDS GFKEKQGYQSPIAQMTSVFDQICSIILSINQLLSQGNTN
55	CsPMEI 13	A0A0A0KAX0	DSWGIDEQQADEKEKQGSPSCVKVFEATIDELHRSYGVLRRLSRRNFDAQMGDLTTWVNTALTNEDTCIEGFE GERGKVVNLLPLVLKLR
56	CsPMEI 14	A0A0A0LHE9	YGLDIVQHSCKLAAKTDPYVDYKLCVQTLKASPNSKDAEFKDLVVISINQSKANATEIGSEISELMKRRSEKWG QYSLNCLKSCLELYSEAVSDLEKALKGLKMEDYETAKTGVSAAMDAPVSCEDGYKEKDGEVSPLSEINDGFF QLVAISLAFINMC
57	CsPMEI 15	A0A0A0KY96	ILLVLCLCMAPQRLDAAGAAQEEGLGMIQKMCAQTNYKDLCITSLTSDPNSFPADKMGLALVALRLASSNASD ISESIKVMLNETSQNNEPTVQQALFDCLDEYLEASQQLDDSIAAIIAKAYGDVQEWVRVAVTNVRTCESSFPTK PSVLTPRNEEFIKLCDIALSITKIAETN
58	CsPMEI 16	A0A0A0KJ03	MGMKNFSISLIFFAIPLIFFHKNNGVSLASADQTLIQKTCTNTLYYKLCMSSLKSDPASLTADTKGLAVIMASIG AANATATASYLSSQLPTSSSGAGANNNKTKLLRQCSEKYAFAAEALRESLKDLGDETFDYAYMHVSAAADYA NVCRDAFKGFPAVSYPTKLGRREEGLKRICRVVLGILDLLGW
59	CsPMEI 17	A0A0A0K9X7	MKPKFSPFMTTPLPSLLLFIIIFNISTTFVVADLVQKACKKCEIDDPNINYNFCTSSFRAHSGSDSTDLRKLGAISLS LIQRNLSSSFEYVEKLLQNKEIDSYKRVRLNDCLDVYSDAIVTVEEGKKAYKEKHYDDANIKVSAVMDSARVC EDGFREKEGVSSPLTKWNKDMFQLAAIALSIINMHP
60	CsPMEI 18	A0A0A0LUJ3	MMIMISTSFLFSFTIVFILIFSSFSQTYSNPHNIIQETCKKSAASTPNLTYKFCVTSLESDTRSRYANLHKLGLISMD LLRHNVTSTRREIKKLLRNKKMEEFIKGCLKDCVELYSDAVPTLKEAKREYKNRNYKDANIKVSSIMEAPTTCE NGFKEKEGIISPLTKNNSDVFQLAALTLSIINMNLHDIQ
61	CsPMEI 19	A0A0A0KBR8	MFFSNFPITQSSNNNNTSSLIYKTCKASSEQDPNISFNFCVTSLKPAATKHRHGDTSLRRLGLITIYLIRHNMSNT RHHIKKYLHKNKGPRDPFVKLCLTDCLELYSDAIPTVKQARKDYKAGRYADANLKISSVMDDCSTCEEGFKEK DGVISPLTNRNHNAFELSAIALSIINMLC
62	CsPMEI 20	A0A0A0K951	DLIYKTCKKISREDPNVSFNFCLASLKLATNHSRCTDVRHLGLFSIGFLCRNVTSTYHHITKLVRNKKLDPFVKL CLDDCLELYTDAIPTVKQAMKDYKSKRYDDANVAISSVMDAATTCEDGFKERKGVASPLKKRDGDAFELGAI ALSIMSLLG
63	CsPMEI 21	A0A0A0L7T4	MSITYMHRFIALALSFFVLFNLRSISANDTLSRACELSAASDPNVRLDFCLQSLAAAPGSDTADLYELGALSIKLI AWNATSTRRYIERLLKNEKKSPDPYVRPRLSDCEELYIDAIKAVGDAAFEYGRNRYEEVNVKLSSVMDAVTTC EDGFKEMEGRVSPLTKRNGDVFELTAIALSILNLRP
64	CsPMEI 22	A0A0A0KQ52	MGEKHLVSFSMVFLLVAVFISQAQGSALCDEAAFPALCRSTVKGASDPTSALKITIEHLIFETKRAKDSSLKIGSL KSLGVCKQNFDDAVDDLQSSLAYMQKKDIPSLKINLSAALTFYSTCDDAVVESGDQKKASTVLSNDLLLQHLA ANCLHLSTLLK
65	CsPMEI 23	A0A0A0KQ68	MAKKQLVSFSPVFLVVAAAAAVVVLFAGQAQGVDICVHSEYIPLCRSVVKGASDPTAAIKTAIGHLLFETKRA KTSSVVLGNEQAISACNQNYDLALDNLQKSLEYLQSKDLASLRVMLSGALSSYVSCTDAVAEVSSFGVVKMA KNVEQTDTTLQHLAGNCLHIASLLK
66	CsPMEI 24	A0A0A0KCV8	MANNSCLIIISLVGVLSFTIISNVASSNDVVSTICPKTSNPQFCSSVLKSAGTTNLKGLAVYTLNLARTNAEKSLT LANSLAKTATNPQLKQRYSSCAESYDEAIGDIENAQKDLALGDFTGVNIVTSGAMTNIGDCQD
67	CsPMEI 25	A0A0A0KEW6	VVPSRATTTPSNDVASSICPKTRNPPFCVDVLKSAGSTDLKVLATYTLNLANENALKSTNLAKSLAAMTTNPPL KNQYLSCYESYEEATSDIENAKSNLASGDFNGVNIATSGVMTSVSDCLDSFKQLRIDPSLLKDGKTLNDVCSIIL VISNLLP
68	CsPMEI 26	A0A0A0KHY0	TGSPNVLTTTICPKTRNSTFCKTVLKPVGKSDAALLKVANYTLTFAHTTTVEGLHHAQRLATEATDPLLKQRYS ECSRRFDFVTKGLEEAIEALAKGGYIPLSHATGAAVVEADRCVNMFKKPPPEPSKLPEKAKNIGDICDIAVSVSN ILTEDY
69	CsPMEI 27	A0A0A0KCV8	VTSSNNVVSTICLKTSNSPFCLSLLKSACTTNLKGLIVYTLNLAHTNARNTFTLANSLAKKTTIIPQLKQLYSSCV ESYDEDFARGDFNGVNIVTSGDMTNIDEYQDKFAQPSKDTSLLLKNDKTLKNICTIILVISTPL
70	CsPMEI 28	A0A0A0LK13	MNCCLNLTSFIPALLLAAFSLTVFPSHGIPHENLVTICSKTSNPSLCEKILNNDSRTVSANLPKLSLICSNLAKKQA DQNLDTFYKLSKNESDPEEKKSFEHCVKYYHEIQSNIQKAYQFSQQKIFRENVMNKLMILCCDISISVNQCAAA NGHHSVNV

Supplementary Table 2s. Physicochemical properties and subcellular localization of Pectin methylesterase inhibitor

ID	Amino acid number/aa	Molecular weight /Da	Isoelectric point	Subcellular localization
CmPMEI1	193	21013.12	9.73	Chlo/ Vacu/ Extr
CmPMEI2	194	20928.04	9.65	Chlo/ Vacu/ E.R
CmPMEI3	208	23067.02	5.8	Chlo/ Extr
CmPMEI4	204	22029.08	7.61	Chlo/ Vacu/ Extr
CmPMEI5	197	21589.05	9.96	Chlo/ Extr/ Vacu
CmPMEI6	202	22338.81	9.72	Chlo/ Extr
CmPMEI7	252	27424.98	5.45	Cyto/ E.R./ Nucl
CmPMEI8	189	20400.15	4.4	Extr/ Vacu/ Mito
CmPMEI9	181	20051.11	4.91	Extr/ Mito/ E.R._plas
CmPMEI10	180	19642.43	4.54	Extr/ Vacu/ chlo
CmPMEI11	189	20524.61	8.93	Extr/ Vacu/ Mito
CmPMEI12	189	21321.59	6.72	Extr/ Vacu/ Chlo
CmPMEI13	194	21755.04	8.83	Extr/ Chlo/ Cyto
CmPMEI14	188	21213.81	9.13	Extr/ Chlo/ Nucl
CmPMEI15	187	20854.62	5.2	Chlo/ Extr/ Cyto
CmPMEI16	160	17338.02	7.62	Chlo/ E.R._plas/ Plas
CmPMEI17	168	17895.78	8.59	Extr/ Chlo/ Cyto
CmPMEI18	178	19142.04	6.55	Chlo/ Extr/ Mito
CmPMEI19	172	18328.95	5.22	Chlo/ Extr/ Vacu

Note: Chlo: Chloroplast. Vacu：Vacuole. Extr：Extracellular. E.R: Endoplasmic reticulum. Mito: Mitochondria. Nucl: Nucleus. Cyto: Cytoplasm. Plas: Plasmodesma.