Genome-wide identification, phylogeny and expression analysis of the PME and PMEI gene families in maize

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Pectins, the major components of cell walls in plants, are synthesized and secreted to cell walls as highly methyl-esterified polymers and then demethyl-esterified by pectin methylesterases (PMEs). The PMEs are spatially regulated by pectin methylesterase inhibitors (PMEIs). In this study, 43 and 49 putative PME and PMEI genes were identified in maize, respectively. Gene structure and motif analysis revealed that members in the same paralogous pairs or in the same subgroup generally had common motif compositions and gene structure patterns, which indicates functional similarity between the closely related ZmPME/PMEI genes. Gene ontology annotation analysis showed that most of the ZmPME/PMEI genes are involved in cell wall modification and pectin catabolic process with molecular functions of pectinesterase or pectinesterase inhibitor activities. There are 35 ZmPME/PMEI genes expressed higher in anthers than in other tissues from the NimbleGen maize microarray data, and the semiq-RT-PCR assay revealed most of these ZmPME/PMEIs specially expressed in anthers and pollens, indicating they possibly had role in anther and pollen development. In addition, these ZmPME/PMEI genes were highly expressed in the fertile anthers, while lowly or no expressed in sterile anthers. This further indicated these genes might be involved in the development of anther and pollen.
For example, OsPMEI28 overexpression in rice had an effect on the growth process, which resulted in a dwarfed phenotype\textsuperscript{31}, and overexpression of the PMEI5 resulted in a higher demethylesterification of seeds and reduced the PME activity, which was accompanied by an earlier and faster germination process compared to wildtype in Arabidopsis\textsuperscript{32}.

In recent years, many reports have shown that some PME/PMEIs regulate plant stress resistance and pollen development. AtPMEI10, AtPMEI11 and AtPMEI12 were identified as upregulated in response to B. cinerea infection\textsuperscript{33}. Expression profile of the genes TaPME1-2, TaPME21-1/24, TaPME38, TaPME63 and TaPME67\textsuperscript{44} was induced in the susceptible cv. Bobwhite and repressed in the resistant cv. Sumai 334\textsuperscript{34}. The transgenic rice overexpressing OsPMEI4 showed higher PME activity and AI content in root tip cell wall, and became more sensitive to AI stress\textsuperscript{35}. In flax, 48 (77.4\%) PME genes and 53 (80.3\%) PMEI genes had higher expression level in the flowers\textsuperscript{36}. In Arabidopsis, 15 PMEs were highly expressed in pollen and 10 of these contained PRO regions\textsuperscript{37}. These suggest that the PME/PMEIs might play important roles in pollen development. Mutations of VANGUARD1 (VGD1), the type I PME gene with the highest expression levels in Arabidopsis pollen tubes, resulted in retarded growth in the style and transmitting tract and subsequent reduction in male fertility\textsuperscript{38}. In maize, the ZmCS of PMEs has a role in pollen tube elongation\textsuperscript{39} and ZmGa1P, a pollen–expressed PME gene, can confer the male function in the maize unilateral cross-incompatibility (UCI) system\textsuperscript{40}.

In this study, genome-wide identification of ZmPME/PMEI genes was firstly conducted in maize, and the phylogenetic tree, gene structure, conservative motif, expression, gene ontology annotations were also examined. In addition, semi-qRT-PCR assay was conducted to verify the gene expression pattern of some ZmPME/PMEI genes highly expressed in anthers, since more than half the genes highly expressed in anthers according to the NimbleGen maize microarray data. To further evaluate their possible roles on pollen development, gene expression of some ZmPME/PMEI\textsuperscript{21} genes in fertile and sterile anthers was also investigated. Results in this study would provide useful information for further investigate the function of maize PME/PMEIs, especially on the development of anther and pollen.

Results

Identification of ZmPME/PMEI genes in maize. To identify putative ZmPME/PMEI genes in maize genome, we searched the maize genome annotation data with known plant PME/PMEI domains (pfam01095/ pfam04043) as a query using HMMER 3.0 package\textsuperscript{41}. In total, we obtained 43 putative PME genes and 49 putative PMEI genes in maize. These genes were designated as ZmPME1-43 and ZmPMEI1-49 (Fig. 1 and Supplementary Table 1), of them, 20 genes (PMEI-20) had PRO region (which showed similarities with the PMEI domain) and the PME domain. Each ZmPME/PMEI gene model was selected by analyzing the similarity between the ZmPME/PMEI genes and homologous genes, as most of the ZmPME/PMEI genes had more than one transcript in the MaizeGDB database (https://www.maizegdb.org/). Then we randomly selected 15 ZmPME/PMEIs for reverse transcription polymerase chain reaction (RT-PCR) to assess the veracity of the ZmPME/PMEI genes models. The results indicated that the 15 ZmPME/PMEI genes were expressed in maize pollen and only a single amplicon was found (Supplementary Fig. S1). The most identified ZmPME/PMEI genes encode proteins with 150-250 amino acids (aa). Their amino acid sequence identity and isoelectric points (pI) are 4.28 to 10.23. These ZmPME/PMEIs are distributed on all the 10 maize chromosomes, and chromosomes 1, 2, 3, 7 and 8 have more ZmPME/PMEIs than others (Supplementary Fig. S2).

Phylogenetic analysis. Phylogenetic trees were constructed by using MEGA 7.0 with the neighbor-joining model. In order to analyze the evolutionary relationships among the predicted ZmPMEs and ZmPMEIs, we aligned maize acid sequences with 101 and 106 predicted PMEs and PMEIs from rice and Arabidopsis. On the basis of phylogeny, the PMEs and PMEIs families in plants were subdivided into 5 and 12 groups, respectively (Supplementary Fig. S3). PMEs in each group and PMEIs in groups I to III, V, VI and VIII are all from the three species (Supplementary Fig. S3), indicating that these ZmPME/PMEIs might have the conserved function in evolution.

Meanwhile, according to cluster analysis, the ZmPME/PMEI families could be divided into 5 and 8 subfamilies, respectively (Fig. 1). The PMEI domains may be derived from duplication and divergence of the PRO domain and have rapidly evolved\textsuperscript{42}. So, we constructed the PMEI phylogenetic tree used the protein sequences of all the ZmPMEs and the 20 ZmPMEIs containing PRO region. The ZmPMEI subfamily I includes the same genes in the ZmPME subfamily I (except ZmPME21 and ZmPME22), both of the subfamilies are the largest subfamily, and the genes had signal prediction or transmembrane region domain. For ZmPMEs, genes in the subfamilies II and III do not have signal prediction or transmembrane region domain (except ZmPME27); while genes in the subfamily IV have signal prediction domain (except ZmPME32 and -35); and the pI of subfamily V are higher than 8. For ZmPMEIs, genes in the subfamilies II and VII have signal prediction domain, and their pI are higher than 7 (except ZmPMEI26, -37 and -41); most genes in the subfamilies III, IV and VIII expressed higher in anthers than in other tissues (Fig. 2). Homologous ZmPME/PMEI genes were identified 24 paralogous pairs in maize (Supplementary Table 2). The value of the nonsynonymous substitution rate (Ka) to the synonymous substitution rate (Ks) substitutions (Ka/Ks) can be used as an indicator which could reflect selection pressure of a gene or a gene region during evolution. To infer the influence of selection on the evolution of the maize, we estimated Ka/Ks values for all of them (Supplementary Table 2). The Ka/Ks values of all the homologous genes are between 0.0033 and 0.3889, suggesting that most of the ZmPME/PMEI genes undergone negative selection and evolved slowly. The Ka/Ks values of maize PMEs paralogs are significantly lower than that of the PMEIs homologs (P < 0.005).
Gene structure and motif analysis of the ZmPME/PMEI families. Gene structures of the ZmPME/PMEI genes were constructed by aligning the extracted genomic sequences to predicted cDNA sequences of the maize PME/PMEI genes. As can be seen from Supplementary Fig. S5, most of the ZmPME genes in subfamily I have 2 exons, and the ZmPME members in the subfamily III have 5 introns (except ZmPME28). In addition, most of the ZmPME genes contain 1–10 introns, and most of the ZmPMEI genes do not have any intron.

Analysis of the ZmPME/PMEI protein sequences with MEME (http://meme-suite.org/tools/meme) revealed 6 conserved motifs of the ZmPME genes, and 9 conserved motifs of the ZmPMEI genes (Supplementary Table 3). Of the ZmPMEs, 36 proteins contain motifs 1, 2, 4, 5 and 6 (except ZmPME21, -23, -24, -25, -27, -29, -32, -34 and -43, Supplementary Fig. S6). For the ZmPMEIs, the proteins in the subfamily I (containing both PME and PMEI domains) have motifs 1 to 9 (except ZmPME1 to ZmPME16), and the rest of ZmPMEIs contain motifs 8 and 9 (except a few ZmPMEI genes, Supplementary Fig. S7).

As expected, most closely related members have a common motif composition and gene structure pattern, which indicates functional similarity between the ZmPME/PMEI proteins in paralogous pairs or in the same subfamily (Fig. 3). For the ZmPME genes, proteins in the subfamilies IV and V contain motifs 1–5 (except ZmPME32, -34 and -43) and the intron phase 2, 0, 0 and 2 separating the PME domain (except ZmPME32, Fig. 3a,b). Proteins in the subfamily VII of the ZmPMEIs have motifs 8 and 9 (except ZmPMEI37 and -41), while that in the subfamilies II, III and IV have motifs 7, 8 and 9 (except ZmPMEI35 and -40, Fig. 3c,d), and most of ZmPME genes in the subfamily IV have 5 exons.
The PME and PMEI domains of the ZmPME/PMEIs, the PME domains in *E. chrysanthemi* (GenBank: Y00549), carrot (SwissProt Accession No. P83218) and *A. aculeatus* (Swiss-Prot code Q12535); and the PMEI domains in kiwi (SwissProt Accession number No. P83326) and *Arabidopsis* (*AthPMEI-1*, Accession Number NP_175256; *AthPMEI-2*, Accession Number NP_188348) were analyzed by T-Coffee (http://tcoffee.org/) and displayed by ESPript 3.0 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi, Supplementary Figs. S9 and S10). The ZmPMEs contain five characteristic sequence fragments (44_GxYxE, 113_QAVAL, 135_QDTL, 157_DFIFG, 223_LGRPW; carrot numbering), and several highly conserved aromatic residues (Supplementary Fig. S9). The ZmPMEIs contain four conservative Cys residues, which were connected by two disulfide bridges (first to second and third to fourth) and do not have the fifth conservative Cys residue which has a free thiol group comparing to kiwi and *Arabidopsis*.

To further understand the structure of the ZmPME/PMEI proteins, three-dimensional (3D) structure of the PME/PMEI domains in ZmPME3 and ZmPMEI2 were analyzed by I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/), and exhibited by Chimera1.8.1 (http://www.cgl.ucsf.edu/chimera/, Fig. 4). ZmPME3 has high similarity with the PME (PDB 1GQ8) from Carrot40 (C-score = 1.71, TM-score = 0.95 ± 0.05, RMSD = 2.8 ± 2.0, Fig. 4b). Furthermore, ZmPMEI2 has high similarity with the PMEI (PDB 1xg2B) from kiwi30 (C-score = 1.7, TM-score = 0.86 ± 0.07, RMSD = 2.7 ± 2.0, Fig. 4d). Superposition of the known PME structures of carrot and maize (ZmPME3, Fig. 4b), and PMEI structures of kiwi and maize (ZmPMEI2, Fig. 4d) confirm the similarity of the folding topologies.

Gene ontology (GO) annotation and subcellular localization of the ZmPME/PMEI proteins. The 92 ZmPME/PMEI genes (except ZmPME28) were assigned a total of 37 GO terms (Fig. 5 and Table 1). Among them, 175, 78 and 167 proteins were assigned terms under molecular function, cellular component and biological process, respectively. Under biological process, 41 ZmPME genes were related to pectin catabolic process and 73 genes (all of ZmPMEI genes and ZmPME21, -22, -23 and -24) were involved in negative regulation of catalytic activity. Under cellular component, 41 ZmPME genes were assigned to cell part. Under molecular function, most of the ZmPME genes and a few ZmPMEI genes had pectinesterase activity and aspartyl esterase activity; and most ZmPMEI genes and a few ZmPME genes had pectinesterase inhibitor activity or enzyme inhibitor activity. In addition, we analyzed the GO annotations for each subfamily. The same annotations exist in different genes of different subfamilies (e.g., the PMEI genes in the subfamilies II and III), and there are also different annotations for genes in the same subfamily (e.g., the PME genes in the subfamily II, Supplementary Table 4). These results suggested that different genes in the same subfamily may have different roles in the evolution process.

Subcellular localization of the 92 ZmPME/PMEIs were predicted using TargetP (http://www.cbs.dtu.dk/services/TargetP/) and WoLF PSORT (https://wolfpsort.hgc.jp/). Majority of the proteins (77, 83.7%) were revealed as signal peptides by TargetP; five (5.4%) are located in mitochondria; and ten are not assigned (Supplementary Table 1).
Moreover, the WoLF PSORT predicted a number of ZmPME/PMEIs (93.5%) locating to chloroplast or extracellular (Supplementary Table 1). In addition, a ZmPMEI gene (ZmPMEI16) was found to be targeted to chloroplast by an in vivo transient expression assay (Supplementary Fig. S8). This consistent with the prediction of WoLF PSORT.

**Expression assay of the ZmPME/PMEI genes.** The NimbleGen maize microarray data (ZM37) including 60 tissues representing 11 major organ systems and various developmental stages of the B73 maize inbred line was employed to analyze the expression pattern of the ZmPME/PMEI genes. All of the ZmPME/PMEI genes (except...
one ZmPME gene and 13 ZmPMEI genes) expression data was used to draw Heatmap. Of them, 35 ZmPME/PMEI genes had a much higher expression level in anthers than in other tissues (Fig. 2), these ZmPME/PMEI genes may be related to the development of anther or pollen. In general, expression pattern was similar for genes within the same paralogous gene pairs (e.g., ZmPMEI1/-17, Supplementary Table 1 and Fig. 2), indicating they might be formed by segmental duplication and retained their function. However, the expression profiles of the four paralogous gene pairs (ZmPME12/-13, ZmPME14/-15, ZmPME16/-17 and ZmPME29/-38) were fundamentally different in different tissues, suggesting that these genes may have differentiated with different roles.

To confirm the organ-specific expression of ZmPME/PMEI genes shown by the microarray data, 14 ZmPME/PMEI genes specifically expressed in anthers, ZmPME24 (not including in the maize microarray data) and ZmPME30 (expressed low in all tissues) were selected for conducting semi-q-RT-PCR. Semi-q-RT-PCR was
performed with total RNA isolated from the roots, leaves, ears, immature tassels, pollens, anthers, whole seed (after pollinated), endosperm and embryo of the B73 inbred line. Fourteen of them, ZmPME3, -5, -7, -11, -23, -31, -42, -43 and ZmPMEI2, -16, -25, -31, -32, -44 matched well with the microarray data, all of these ZmPME/PMEI genes expressed significantly higher in the anthers or pollens than in other organs. It is interesting to note that ZmPME23 was specifically expressed in the anthers. The gene not included in the microarray data, ZmPME24, was also specifically expressed in the anthers and pollens. However, expression of only one gene, ZmPME30, did not match with the microarray data, it had higher expression level in the anthers and pollens but showed little or no expression in roots, leaves, ears and seed according to the semiq-RT-PCR assay (Fig. 6a).

To further analyze the possible role of the ZmPME/PMEI genes on anther or pollen development, expression of the 13 genes (which had identified specifically expression in the anthers or pollens of B73 inbred line) was investigated in the anthers of three fertile and three sterile individuals of a maize backcrossing population derived from a cytoplasmic male sterility (CMS) line. The results showed that all of the selected ZmPME/PMEI genes were differentially expressed in the fertile and sterile anthers, although some ZmPMEI genes showed lower expression in the sterile anthers (Fig. 6b).

**Discussion**

Genome-wide analysis has identified the PMEs and PMEIs in nearly all vascular plants and in multiple gene members. Up to now, the function of a number of the PME genes have been studied in *Arabidopsis*42, rice9, pea43, wheat44, and cotton45; and the PMEI genes in *Arabidopsis*46,47, rice13, broccoli48, and Chinese cabbage49. Most of them involved in plant growth and various stress responses (reviewed by Wormit and Usadel50). In maize, however, only three ZmPMEs (ZmC5, ZmPme3 and ZmGa1P) and one ZmPMEI (ZmPMEI1) were characterized.

| Classification          | GOs ID       | Num | Annotation                                                                 |
|-------------------------|--------------|-----|---------------------------------------------------------------------------|
| **Biological Process (167)** | GO:0000079   | 1   | regulation of cyclin-dependent protein serine/threonine kinase activity   |
|                         | GO:00000723  | 1   | telomere maintenance                                                      |
|                         | GO:00006281  | 1   | DNA repair                                                                |
|                         | GO:0006310   | 1   | DNA recombination                                                         |
|                         | GO:0006412   | 1   | translation                                                               |
|                         | GO:0006508   | 1   | proteolysis                                                               |
|                         | GO:0007088   | 1   | regulation of mitotic nuclear division                                     |
|                         | GO:0008152   | 1   | metabolic process                                                          |
|                         | GO:0008284   | 1   | positive regulation of cell proliferation                                 |
|                         | GO:0032508   | 1   | DNA duplex unwinding                                                      |
|                         | GO:0042545   | 41  | cell wall modification                                                    |
|                         | GO:0043086   | 73  | negative regulation of catalytic activity                                 |
|                         | GO:0045490   | 42  | pectin catabolic process                                                  |
|                         | GO:0045787   | 1   | positive regulation of cell cycle                                         |
| **Cellular Component (78)** | GO:0000307   | 1   | cyclin-dependent protein kinase holoenzyme complex                        |
|                         | GO:00005576  | 13  | extracellular region                                                       |
|                         | GO:00005618  | 41  | cell wall                                                                 |
|                         | GO:00005634  | 3   | Nucleus                                                                   |
|                         | GO:00005737  | 1   | cytoplasm                                                                 |
|                         | GO:00005840  | 1   | ribosome                                                                  |
|                         | GO:00005886  | 1   | plasma membrane                                                           |
|                         | GO:0016020   | 4   | membrane                                                                  |
|                         | GO:0016021   | 12  | integral component of membrane                                            |
|                         | GO:0048046   | 1   | Apoplast                                                                  |
| **Molecular Function (175)** | GO:00003678  | 1   | DNA helicase activity                                                     |
|                         | GO:00003735  | 1   | structural constituent of ribosome                                         |
|                         | GO:00004564  | 1   | beta-fructofuranosidase activity                                           |
|                         | GO:00004672  | 1   | protein kinase activity                                                   |
|                         | GO:00004857  | 34  | enzyme inhibitor activity                                                 |
|                         | GO:0005524   | 1   | ATP binding                                                               |
|                         | GO:0008234   | 1   | cysteine-type peptidase activity                                           |
|                         | GO:0016538   | 1   | cyclin-dependent protein serine/threonine kinase regulator activity        |
|                         | GO:0016787   | 1   | hydrolase activity                                                        |
|                         | GO:0019901   | 1   | protein kinase binding                                                    |
|                         | GO:0030599   | 52  | pectinesterase activity                                                   |
|                         | GO:0045330   | 41  | aspartyl esterase activity                                                |
|                         | GO:0046910   | 39  | pectinesterase inhibitor activity                                         |

Table 1. Gene ontology (GO) annotations of the ZmPME/PMEI genes.
and found to be involved in pollen tube elongation 37,38,51,52. Thus genome-wide identification, evolution, and expression analysis of the PME/PMEI families in maize will facilitate to understanding of the function of the gene families.

In this study, 43 and 49 ZmPMEs and ZmPMEIs were identified in the maize genome, which were divided into 5 and 8 subfamilies (Supplementary Table 1; Fig. 1), respectively. The number of ZmPMEs is less than that identified in Arabidopsis 7, and Gossypium raimondii 11, while that of ZmPMEIs is much more than that identified in Sorghum bicolor 12, and Brachypodium distachyon 53. We identified 24 paralogous pairs in maize, but all Ka/Ks values of paralogous are less than 1 (Supplementary Table 2), implying that ZmPME/PMEI genes evolved mainly under the influence of stabilizing selection. The result of Ka/Ks analysis of PME, PRO and PMEI domains reveals that the homologous gene pairs of Arabidopsis, rice and sorghum experienced purifying selection 12 and the PME homologous gene pairs of G. arboreum, G. raimondii and G. hirsutum also experienced stabilizing selection 45. Thus ZmPME/PMEI genes might play important role in growth and development of plants.

Intragroup ZmPME/PMEIs have conserved gene structure and motif composition, indicating that ZmPME/PMEIs in the same group could have the same function and they might come from a common ancestor. For instance, the ZmPME subfamilies IV and V contain motifs 1–5 (except ZmPME32, -34 and -43) and most intron phases 2, 0, 0 and 2 separating the PME domain (Fig. 3a,b). Of the ZmPMEs, most members in the subfamilies III, IV and VIII expressed higher in anthers than in other tissues (Fig. 2), and most members in the subfamily VII have motifs 8 and 9 (Fig. 3c,d). The PME and PMEI domains alignment implying that the ZmPME domains have five characteristic sequence fragments (44_GxYxE, 113_QAVAL, 135_QDTL, 157_DFIFG, 223_LGRPW; carrot numbering, Supplementary Fig. S9), which have all been shown to be functionally important in carrot 54; the ZmPMEI domains have four conservative Cys residues (Supplementary Fig. S10), which are connected by two disulfide bridges, but do not have the fifth conservative Cys residue in comparison with that in kiwi 29 and

Figure 6. Semiq-RT-PCR analysis of some ZmPME/PMEI genes in the nine different tissues of B73 inbred line and in the anthers of three fertile and three sterile individuals of a maize backcross population. (a) The total RNA of nine tissues including seedling roots, seedling leaves, 2-cm immature ears, non-emerged immature tassels, anthers, pollen, whole seeds after pollination for 18 days, endosperm and embryo after pollination for 20 days of B73 inbred line was isolated and used to perform the semiq-RT-PCR of the ZmPME/PMEI genes. (b) The total RNA of anthers of three sterile and three fertile individuals was isolated and used to perform the semiq-RT-PCR of the ZmPME/PMEI genes. Beta actin was used for internal controls to normalize the RNA contents in each sample. Primers used are shown in Supplementary Table 5. In the figure, the PCR products were separated with the same experiment condition and that gels were processed in parallel. The original gels were presented in Supplementary Fig. S11.
Arabidopsis\textsuperscript{55}. The structure of the carrot PME is almost completely superimposable to the structure of tomato\textsuperscript{50}. In this study, the 3D structure of ZmPME3 and ZmPME12 are highly similar to that from Carrot\textsuperscript{48} and kiwi\textsuperscript{29}, respectively. This indicated that the PME and PMEI domains were highly conserved in different plant species.

In recent years, there are many reports on the function of PME/PMEI genes. Overexpression of PMEIs in Arabidopsis thaliana caused aberrant growth morphology of the stems\textsuperscript{56}; ZmPMEI1 expression negatively correlates with the PME activity during the early stage of grape berry development of Grapevine\textsuperscript{57}. The expression pattern of the ZmPME/PMEI genes from the NimbleGen maize microarray data showed 35 ZmPME/PMEI genes had a much higher expression level in the anthers than in other tissues (Fig. 2), and semiq-RT-PCR analysis of different tissues from B73 inbred line verified that they had much higher expression in the anthers or pollens (Fig. 6a). In addition, semiq-RT-PCR analysis of some ZmPMEI/PMEI genes showed that 13 ZmPMEI/PMEI genes were differentially expressed in the anthers of fertile and sterile individuals derived from a maize S-type CMS line (Fig. 6b). Similar results also have been reported in other plant species. For example, antisense expression of a pollen-specific PMEI from broccoli (Brassica oleracea) in Arabidopsis triggered silencing of the orthologous Arabidopsis gene At1g10770 and resulted in male sterility\textsuperscript{46}; the expression of the pectin methyl esterase gene (At3g06830) was significantly lower in male-sterile line than in male-fertile line at the <1 mm anther length stage of Brassica napus\textsuperscript{46}; in cotton, transcriptome analysis showed that many pectin methyl esterase genes highly expressed in flowering buds of fertile plants compared to those of the CMS-D8 line\textsuperscript{48}. This implied that a number of ZmPMEI/PMEI genes might play an important role in anther and pollen development, however, their detailed roles in male function of maize need to be further studied in future.

Materials and Methods

Identification of the PME/PMEI genes in maize. Maize genome sequences were downloaded from the Maize Genome Database (Maize GDB; https://www.maizegdb.org/). Local HMMER3.0\textsuperscript{49} (E-value-10) searches were performed using the Hidden Markov Model (HMM) profile in the Pfam database (http://pfam.janelia.org/search/sequence) to screen all candidate ZmPME/PMEI gene sequences. Candidate genes were retained that contained known conserved domains and passed checks against the Pfam (http://pfam.janelia.org/) and SMART (http://smart.embl-heidelberg.de/) databases for presence of the PME/PMEI domains (PF01095/PF04043). Bioinformatics analyses were performed on the ZmPME/PMEI protein sequences, and physical and chemical parameters (e.g., MW, pI) were calculated using ExPASy (http://www.expasy.ch/tools/pi_tool.html). TargetP (http://www.cbs.dtu.dk/services/TargetP/) and WoLF PSORT (https://wolfpsort.hgc.jp/) were used to predict the subcellular of ZmPME/PMEIs.

Analysis of gene structures and conserved motif of the ZmPME/PMEI genes. Several ZmPME/PMEI genes had more than one gene model annotated in MaizeGDB (https://www.maizegdb.org/). To confirm the putative alternative splicing transcripts, transcript-specific primers (Supplementary Table 5) were designed to amplify corresponding DNA isolated from B73 seedlings and cDNA derived from B73 pollen RNA. Conserved PME/PMEI domains and gene structures producing validated transcripts were drawn and displayed using the Gene Structure Display Server\textsuperscript{48} (GSDS2.0; http://gds.cbi.pku.edu.cn/index.php).

Sequence alignment of ZmPME/PMEIs domain was conducted by T-Coffee (http://tcoffee.org/503/index.html), 3D structures were predicted through I-TASSER\textsuperscript{61} (http://zhanglab.ccmb.med.umich.edu/ITASSER/) and visualized by Chimera1.13.1 (http://www.cgl.ucsf.edu/chimera/).

Phylogenetic and multiple alignment analyses. The PME/PMEI protein sequences were aligned using ClustalX 2.0\textsuperscript{62} (http://www.clustal.org/clustal2/) with the default parameters. Phylogenetic tree was drawn with the neighbor-joining method using software MEGA7.0\textsuperscript{63} (molecular evolutionary genetics analysis, https://www.megasoftware.net/) using pairwise deletion; 1,000 replicates were used for bootstrap analysis and the cut-off value was 90\%.

Calculation of synonymous (Ks) and non-synonymous (Ka) substitutions. To identify homologous pairs of genes, the transcript sequences of the ZmPME/PMEIs were investigated by BLASTN searches\textsuperscript{45}. Paralogous pairs within the genome of maize were defined as follows: the aligned sequences were longer than 6 and 50 residues\textsuperscript{66}. If the amino acid shorter than 300 bp, the aligned region had an identity ≥ 40\% and Wang et al\textsuperscript{12}, and the protein sequences were downloaded from the Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/) and the Rice Genome Annotation Project websites (http://rice.plantbiology.msu.edu/analyses_search_locus.shtml).

We used the MEME system (http://meme.sdsc.edu/meme/meme.html) to identify conserved motifs with parameters set as: number of repetitions, arbitrary; maximum number of patterns, 20; optimal width of the motif, between 6 and 50 residues\textsuperscript{64}.

Gene ontology (GO) annotation. The translated ZmPME/PMEIs protein sequences were annotated using the Blast2GO5.2.4 program to assign GO terms\textsuperscript{67} (http://amigo.geneontology.org/amigo/term/). GO analysis e-value is 1.0E-6. GO terms are provided under three main categories, biological process, cellular component, and molecular function.

Localization of fluorescent protein-tagged ZmPMEI16. Full-length ORF of ZmPMEI16 was isolated by PCR using primers ZmPMEI16-pM999-F (5′-AGCAGATCTATCGATGAATTCGAGCTACACCA-3′) and ZmPMEI16-pM999-R (5′-TCTCTGGCCATGGCGCTTGATCATCTATGTTGGAAGCG-3′). The resulting fragment was digested with EcoRI and XbaI and inserted between the corresponding sites of pM999-EGFP (provided by professor Liwen Jiang), which expressed an engineered version of the green-fluorescent
protein (GFP) under the control of the cauliflower mosaic virus 35S promoter. The plasmid pZmPMEI16-GFP and pMV999-EGFP were used for transient expression experiments in maize protoplasts\(^6\). Samples were analyzed by confocal laser scanning microscopy using a Leica TCS-SP8 operating system as described by Ravan et al.\(^6\).

**Expression analysis of the ZmPME/PMEI genes in different tissues.** To investigate the spatiotemporal expression patterns of the ZmPME/PMEI genes, RMA-normalized data for ZmPME/PMEI genes were downloaded from PLEXdb (http://www.plexdb.org/). A heat map was produced by Hemi 1.0.3.7- Heatmap illustrator.

**Semi-quantitative reverse transcription PCR (semi-q-RT-PCR).** Total RNA of the B73 inbred line was extracted using the Trizol reagent (Invitrogen, USA) according to the manufacturer’s recommendations. In addition, anthers on tassels (about to exert from the upmost leaves) were collected from sterile and fertile plants of a backcrossing population derived from a maize S-type CMS, and RNA was also extracted using the same method. First-strand cDNA was synthesized from 0.05–5 μg of total RNA (20μL reaction volume) using TransScript First-Strand cDNA Synthesis Super Mix (TransGen Biotech). All gene-specific primers were designed by primer 3 (http://primer3.ut.ee/) as shown in Supplementary Table 5. The maize gene Actin1 (GenBank ID: NM_001155179) was used as an internal control. The semi-q-RT-PCR assays were repeated for two or three times (biological replications).

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**References**

1. Cosgrove, D. J. Wall structure and wall loosening. A look backwards and forwards. Plant Physiol. **125**, 131–134 (2001).
2. Mohnen, D. Pectin structure and biosynthesis. *Curr Opin Plant Biol.* **11**, 266–27 (2008).
3. Domozycz, D. S., Serfis, A., Kiemle, S. N. & Gretz, M. R. The structure and biochemistry of charophyte cell walls: I. Pectins of *Porphyridium cruentum*. *Protoplasma* **230**, 99–115 (2007).
4. Willats, W. G. T., Mccartney, L., Mackie, W. & Knox, J. P. Pectin: cell biology and prospects for functional analysis. *Plant Mol Biol.** **47**, 9–27 (2001).
5. Balestrieri, C., Castaldo, D., Giovane, A., Quaglialu, L. & Servillo, L. *A glycoprotein inhibitor of pectin methylesterase in kiwifruit (Actinidia chinensis)*. *Eur J Biochem.** **193**, 183–187 (1990).
6. Micheli, F. Pectin methylesterases: cell wall enzymes with important roles in plant physiology. *Trends Plant Sci.* **6**, 414–419 (2001).
7. Louvet, R. *et al.* Comprehensive expression profiling of the pectin methylesterase family during silique development in *Arabidopsis thaliana*. *Planta** **224**, 782–791 (2006).
8. Horowitz, B. B., Osipa-Giraldo, M. D. & Vijai, G. The Pectin Methylesterase Gene Complement of *Phytophthora sojae*: Structural and Functional Analyses, and the Evolutionary Relationships with Its Oomycete Homologos. *Plos One.** **10**, e0142096 (2015).
9. Yang, X. *et al.* Association of specific pectin methylesterases with Al-induced root elongation inhibition in rice. *Physiol Plant.** **148**, 502–511 (2013).
10. Pinzón-Latorre, D. & Deyholos, M. K. Characterization and transcript profiling of the pectin methylesterase (PME) and pectin methylesterase inhibitor (PMEI) gene families in *Brassica campestris ssp. chinensis*. *Plant Mol Biol.** **92**, 379–396 (2016).
11. Liu, Q. X., Talbot, M. & Lewellyn, D. J. Pectin Methylesterase and Pectin Remodelling Differ in the Fibre Walls of Two Gossypium Species with Very Different Fibre Properties. *Plos One.** **8**, e65131 (2013).
12. Wang, M. *et al.* A comparative genome analysis of PME and PMEI families reveals the evolution of pectin metabolism in plant cell walls. *Plos One.** **8**, e720822 (2013).
13. Nguyen, H. P., Jeong, H. Y., Kim, H., Kim, Y. C. & Lee, C. Molecular and biochemical characterization of rice pectin methylesterase inhibitors (OsPMEI). *Plant Physiol Biochem.** **101**, 105–112 (2016).
14. Liu, T. *et al.* Genome-Wide Identification, Molecular Evolution, and Expression Profiling Analysis of Pectin Methylesterase Genes in *Brassica campestris ssp. chinensis*. *Int J Mol Sci.** **19**, 1338 (2018).
15. Coutinho, P. M., Stam, M., Blanc, E. & Henrissat, B. Why are there so many carbohydrate-active enzyme-related genes in plants? *Trends Plant Sci.** **8**, 0–565 (2003).
16. Giovane, A. *et al.* Pectin methylesterase inhibitor. *Biochim Biophys Acta.* **849**, 245–252 (2004).
17. Bosch, M., Cheung, A. Y. & Hepler, P. K. Pectin Methylesterase, a Regulator of Pollen Tube Growth. *Plant Physiol.** **138**, 1334–1346 (2005).
18. Pelloux, J., Rusterucci, C. & Mellerowicz, E. J. New insights into pectin methylesterase structure and function. *Trends Plant Sci.* **12**, 9–271 (2007).
19. Catoire, L., Pierron, M., Morvan, C., Penhoat, C. H. & Goldberg, R. Investigation of the Action Patterns of Pectinmethylesterase Isoforms through Kinetic Analyses and NMR Spectroscopy Implications In Cell Wall Expansion. *J Biol Chem.* **273**, 33150–33156 (1998).
20. Denès, J. M., Baron, A., Renard, C. M., Pèan, C. & Drilleau, J. F. Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. *Eur J Biochem.** **327**, 385–393 (2000).
21. Goldberg, R., Morvan, C., Jaunneau, A. & Jarvis, M. C. Methyl-esterification, de-esterification and gelation of pectins in the primary cell wall. *Prog Biotechnol.* **14**, 151–172 (1996).
22. Wed, F., Zhu, Y. & Hawes, M. C. Effect of Pectin Methylesterase Gene Expression on Pea Root Development. *Plant Cell.* **11**, 1129–1140 (1999).
23. Gaff, J., Tiznado, M. E. & Handa, A. K. Characterization and functional expression of a ubiquitously expressed tomato pectin methylesterase. *Plant Physiol.** **114**, 1547–1556 (1997).
24. Frenkel, C., Peters, J. S., Tieman, D. M., Tiznado, M. E. & Handa, A. K. Pectin Methylesterase Regulates Methanol and Ethanol Accumulation in Ripening Tomato (*Lycopersicon esculentum*). *Fruit J Biol Chem.* **273**, 4293–4295 (1998).
25. Brummell, D. A. & Harpster, M. H. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. *Plant Mol Biol.** **47**, 311–340 (2001).
26. Bordenave, M. *et al.* Pectinmethylesterase isoforms from Vigna radiata hypocotyl cell walls: kinetic properties and molecular cloning of a cDNA encoding the most alkaline isoform. *Plant Mol Biol.** **31**, 1039–1049 (1996).
27. Pilling, I., Willnitter, L. & Fisahn, J. Expression of a *Petunia inflata* pectin methyl esterase in *Solanium tuberosum* L. enhances stem elongation and modifies cation distribution. *Planta.** **210**, 391–399 (2000).
28. Pina, C. Gene Family Analysis of the *Arabidopsis* Pollen Transcriptome Reveals Biological Implications for Cell Growth, Division Control, and Gene Expression Regulation. *Plant Physiol.** **138**, 744–756 (2005).
29. Camardella, L. *et al.* Kiwifruit pectin methylesterase. *Eur J Biochem.** **267**, 4561–4565 (2000).
30. Di Matteo, A. *et al.* Structural Basis for the Interaction between Pectin Methylesterase and a Specific Inhibitor Protein. *Plant Cell.** **17**, 849–858 (2005).
31. Nguyen, H. P., Jeong, H. Y., Jeon, S. H., Kim, D. & Lee, C. Rice pectin methylesterase inhibitor28 (OsPMEI28) encodes a functional PMEI and its overexpression results in a dwarf phenotype through increased pectin methylesterification levels. J Plant Physiol. 208, 17–25 (2017).
32. Müller, K. et al. Demethylesterification of Cell Wall Pectins in Arabidopsis Plays a Role in Seed Germination. Plant Physiol. 161, 305–316 (2013).
33. Lionetti, V. et al. Three Pectin Methylesterase Inhibitors Protect Cell Wall Integrity for Arabidopsis Immunity to Botrytis. Plant Physiol. 173, 1844–1863 (2017).
34. Zega, A. & D’Ovidio, R. Genome-wide characterization of pectin methyl esterase genes reveals members differentially expressed in tolerant and susceptible wheat in response to Fusarium graminearum. Plant Physiol Biochem. 108, 1–11 (2016).
35. Chen, L. Q. & Ye, D. Roles of Pectin Methyltransferases in Pollen-Tube Growth. J Integ. Plant Biol. 49, 94–98 (2007).
36. Jiang, L. et al. VANGUARD1 Encodes a Pectin Methylesterase That Enhances Pollen Tube Growth in the Arabidopsis Style and Transmitting Tract. Plant Cell Plant. 17, 584–596 (2005).
37. Wakeley, P. R., Rogers, H. L., Rozyczka, M., Greenland, A. J. & Hussey, P. J. A maize pectin methyl-esterase-like gene, ZmC5, specifically expressed in pollen. Plant Mol Biol. 37, 187–192 (1998).
38. Zhang, Z. et al. A PECTIN METHYLTRANSFERASE gene at the maize Ga1 locus confers male function in unilateral cross-incompatibility. Nat Commun. 9, 3678 (2018).
39. Eddy, S. R. & Pearson, W. R. Accelerated Profile HMM Searches. Nucleic Acids Res. 34, 1063–1070 (2006).
40. Johansson, K. et al. Crystal structure of plant pectin methyl esterase. FEBS Lett. 514, 243–249 (2002).
41. Sekhon, R. S. et al. Genome-wide atlas of transcription during maize development. Plant J. 66, 553–563 (2011).
42. Tian, G. W., Chen, M. H., Zaltsman, A. & Citovsky, V. Pollen-specific pectin methyl esterase involved in pollen tube growth. Dev Biol. 294, 0–91 (2006).
43. Gómez, M. D., Renau-Morata, B., Beltrán, J. P. & Cañas, L. A. PsPMEP, a pollen-specific pectin methylesterase of pea (Pisum sativum). Plant Reprod. 26, 245–254 (2013).
44. El-Moneim, D. A. et al. Pectin methyl esterase gene and aluminum tolerance in Secale cereale. Environ Exp Bot. 107, 125–133 (2014).
45. Li, W. et al. Genome-wide identification, phylogeny, and expression analysis of pectin methyltransferases reveal their major role in cotton fiber development. BMC Genomics. 17, 1000 (2016).
46. Wolf, S., Grsic-Rausch, S., Rausch, T. & Greiner, S. Identification of pollen-expressed pectin methyl-esterase inhibitors in Arabidopsis. FEBS Lett. 555, 0–555 (2003).
47. Chen, J. et al. A cold-induced pectin methyl-esterase inhibitor gene contributes negatively to freezing tolerance but positively to salt tolerance in Arabidopsis. J Plant Physiol. 222, 67–78 (2018).
48. Zhang, G. Y., Feng, J., Wu, J. & Wang, X. W. BoPMEI1, a pollen-specific pectin methyl esterase inhibitor, has an essential role in pollen tube growth. Plant. 231, 1323–1334 (2010).
49. Tan, C. et al. Pectin methyl esterase inhibitor (PMEI) family can be related to male sterility in Chinese cabbage (Brassica napus ssp. pekinensis). Mol Genet Genomics. 293, 343–357 (2017).
50. Worum, A. & Usadel, B. The Multifaceted Role of Pectin Methyltransferases (PMEs). Int J Mol Sci. 19, 2878 (2018).
51. Worrild, M. et al. External application of gametophyte-specific ZmPMEI1 induces pollen tube burst in maize. Plant Reprod. 26, 255–266 (2013).
52. Moran, L. A., N. Muszynski, M. G., Huffman, R. D. & Scott, M. P. A Pectin Methyltransferase ZmPme3 Is Expressed in Gametophyte factor 1-s (Ga1-s) Siliks and Maps to that Locus in Maize (Zea mays L.). Front Plant. 8, 1926 (2017).
53. Wolf, S., Mouille, G. & Pelloux, J. Homogalacturonan Methyl-Esterification and Plant Development. Mol Plant. 2, 851–860 (2009).
54. Markovic, O. & Janecek, S. Pectin methyltransferases: sequence-structural features and phylogenetic relationships. Carbohydr Res. 339, 2281–2295 (2004).
55. Iothon, M., Wolf, S., Aloy, P., Greiner, S. & Scheffzek, K. Structural Insights into the Target Specificity of Plant Invertebrate and Pectin Methyltransferase Inhibitory Proteins. Plant Cell. 16, 3437–3447 (2004).
56. Müller, K. et al. Overexpression of a pectin methyl esterase inhibitor in Arabidopsis thaliana leads to altered growth morphology of the stem and defective organ separation. Plant Signal Behav. 8, e26464 (2013a).
57. Lionetti, V., Riaiola, A., Mattei, B. & Bellincampi, D. The Grapevine VvPMEI1 Gene Encodes a Novel Functional Pectin Methyltransferase Inhibitor Associated to Grape Berry Development. PlosOne. 10, e0133810 (2015).
58. Zyu, Y. et al. A separation defect of tapetum cells and microspore mother cells results in male sterility in Brassica napus: the role of abscisic acid in early anther development. Plant Mol Biol. 72, 111–123 (2010).
59. Suzuki, H., Rodriguez-Uribe, L., Xu, J. & Zhang, J. Transcriptome analysis of cytoplasmic male sterility and restoration in CMS-D8 cotton. Plant Cell Rep. 32, 1531–1542 (2013).
60. Hu, B. et al. GSDS 2.0: an upgraded gene feature visualization server. Bioinformatics. 31, 1296 (2014).
61. Roy, A., Kucukural, A. & Zhang, Y. I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc. 5, 725–738 (2010).
62. Larkin, M. A. et al. Clustal W and Clustal X version 2.0. Bioinformatics. 23, 2947–2948 (2007).
63. Kumar, S., Stecher, G. & Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 33, 1870–4 (2016).
64. Bailey, T. L., Williams, N., Misek, C. & Li, W. W. MEME: discovering and analyzing DNA and protein sequence motifs. Nucleic Acids Res. 34, 369–373 (2006).
65. Altschul, S. F. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389 (1997).
66. Blanc, G. & Wolfe, K. H. Widespread paleopolyploidy in model plant species inferred from age distributions of duplicate genes. Plant Cell. 16, 1667–78 (2004).
67. Conesa, A. & Götz, S. Blast2GO: A comprehensive suite for functional analysis in plant genomics. Int J Plant Genomics. 2008, 619832 (2008).
68. Lei, H., Bui, F., Feng, Y., Guo, Y. & Qiao, N. Preparation of Maize Leaf Protoplasts and Establishment of Transient Transformation System. Journal of Changzhi University (2018).
69. Ravanelli, S. et al. Tetrahydrofurfuryl biosynthesis in plants: molecular and functional characterization of dihydrofurfuryl synthetase and three isofoms of folypholyglutamate synthetase in Arabidopsis thaliana. Proc Natl Acad Sci USA. 98, 15360–15365 (2001).

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Author contributions

B.Y., J.Z. and Y.Z. designed the study. P.Z., H.W., X.Q. and K.C. performed the experiments. P.Z. and B.Y. wrote the manuscript.
Competing interests
The authors declare no competing interests.

Additional information
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