

Article

Regulation of Pollen Viability, Pollen Tube Growth and Seed Development in Maize by Application of Cysteine Protease ZmPCP

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Abstract

In the process of maize production, extreme meteorological conditions such as drought and high temperature are often the main environmental stress factors affecting pollination efficiency. Previous studies have shown that, under adversity, the germination rate of pollen grains on the filaments of female spikes directly affects the success rate of reproduction and ultimately determines the grain yield. This study focuses on a cysteine protease named ZmPCP. The expression of this protease in maize pollen is significantly higher than in other tissues, and its specific function has not been clearly defined. Its localization in the cell membrane or apoplast was further confirmed by transient transfection experiments and plasmolysis. The interaction between ZmPCP and ZmSNAP33 was verified by yeast two-hybrid technology and a GST pull-down experiment, indicating that ZmPCP may affect pollen germination and stress resistance by regulating vesicle transport. Secondly, by analyzing the pollen germination rate of maize inbred lines B104, *ZmPCP*-KO and *ZmPCP*-OE transgenic maize plants, we found that *ZmPCP* overexpression could significantly enhance pollen viability and pollen tube growth under drought stress. After 1 h of short-term drying treatment, the pollen germination rate of the *ZmPCP*-OE line was maintained at 44%, which was significantly higher than that of the other lines. In addition, the observation of pollen tube growth showed that *ZmPCP* overexpression could promote the extension of pollen tubes in the filament. Moreover, a transcriptome sequencing analysis revealed the regulatory effects of *ZmPCP* on pollen in multiple biological processes, including stress response, carbohydrate metabolism, growth and development, cell wall material metabolism, signal transduction, etc. The involved pathways of these differential genes indicate that *ZmPCP* enhances pollen drought tolerance and promotes pollen tube growth through a “metabolism signal structure”. In the germination experiment on the seventh day, the germination rate of *ZmPCP*-OE maize seeds was the lowest, indicating that its overexpression inhibited seed germination. At the same time, *ZmPCP*-overexpressing *Arabidopsis* showed a significant advantage in taproot growth under high-concentration ABA stress. ZmPCP provides an important theoretical basis for regulating the pollination process and improving the pollination efficiency of maize varieties through interaction with ZmSNAP33.

Keywords: pollen; viability; pollen tube growth; subcellular localization; yeast two-hybrid; seed development; maize



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1. Introduction

Maize (*Zea mays* L.), as one of the world's three major staple crops, occupies a central position in China's agricultural economy. In 2024, its production reached approximately 295 million tons, with a year-on-year increase of 2.1% [1]. It serves not only as a source of human food and industrial raw materials but also as a critical source of livestock and poultry feed, playing an irreplaceable role in ensuring food security and promoting agricultural modernization [2]. However, influenced by global warming, the frequency of extreme weather events such as high temperatures and droughts has increased, significantly reducing maize cross-pollination efficiency. During the critical period of 10 to 15 days before flowering, the viability of pollen and silks is essential for kernel number, and this stage is highly vulnerable to abiotic stress interference [3]. Drought stress suppresses pollen viability because the loss of pollen moisture results in increased pollen mortality, associated with poor sucrose synthase activity [3]. In maize, two sucrose synthase (SUS) genes are downregulated in pollen under drought conditions, limiting the conversion of sucrose into D-fructose and thereby compromising pollen performance [4]. Successful fertilization in maize further relies on the rapid growth of pollen tubes, which grow at rates of up to 1 cm per hour through elongated styles (silks) before releasing their sperm cells within ovules [5]. Pollen tubes secrete numerous small proteins to facilitate communication with diverse maternal tissues during their transit from the stigma through the style's transmitting tract toward ovules. For example, rapid alkalization factors (RALFs) regulate hydration as well as cell wall integrity during pollen germination, tube growth and reception in the model plant *Arabidopsis* [6]; *ZmRALF2/3* mutations disrupt pectin distribution, altering cell wall organization and thickness, which triggers pollen tube rupture [5].

Cysteine proteases (CPs) represent an important class of enzymes that are widely involved in pollen development [7], seed germination [8] and resistance to abiotic stresses [9]. Pollen coat proteins, including several CPs, are key components regulating pollen hydration, germination, and pollen tube elongation, with their molecular mechanisms being a research focus in plant reproductive biology [10]. In maize, for example, β -extensin EXPB1 binds to glucuronyl arabinoxylans, and its downregulation reduces pollen competitiveness [11]. In addition, the maize pollen coat also accumulates β -1,3-glucanase, endoxylanase, and exopolysaccharidase, which specifically hydrolyze cell wall polysaccharides [12,13]. *ZmPCP*, a pollen-specific CP cysteine protease belonging to the papain-like cysteine protease (C1-papain subfamily), is highly expressed in mature pollen. Extensive studies in multiple plant species have demonstrated crucial roles for CPs in pollen development. In *Arabidopsis*, *AtCEP1* is an important executor in the process of tapetal programmed cell death (PCD), and its overexpression leads to early tapetal PCD and pollen abortion [7]. Reduced expression of *AtCP51* results in early tapetum degeneration and defective pollen exine formation [14]. In tobacco, knockout of *NtCP56* causes delayed tapetal breakdown and the production of abortive pollen [15]. In rice, *OsCP1*, which is strongly expressed in anthers, is mutated by T-DNA insertion, leading to pollen degeneration [16]. Previous studies indicate that *ZmPCP* overexpression leads to abnormal morphology of pollen germination pores, reduced germination rates, and decreased fertility while simultaneously enhancing plant drought resistance [17]. Conversely, knockout experiments demonstrate the opposite effect, suggesting that *ZmPCP* plays a dual role in pollen development and stress response. In addition, the *ZmPCP*-OE and *ZmPCP*-KO maize lines used in this study were generated and characterized in our previous work [17], providing validated genetic materials for the functional analyses presented here.

Beyond reproductive tissues, CPs also play essential roles in seed development. In cereals, CPs mediate the degradation of seed storage proteins to release amino acids and nutrients that are necessary for seedling growth [8]. For instance, the *OsCP6* gene positively

regulates rice seed germination. The metabolism of endosperm storage and hydrolysis of storage proteins in the *oscp6-1* mutant decreased, and cysteine protease activity decreased under low-nitrogen conditions during early seedling development [18].

Moreover, CPs are important components of plant responses to abiotic stress, particularly drought and waterlogging [9]. In sweet potato, the overexpression of *SPCP3* in *Arabidopsis* increases drought sensitivity, whereas ectopic expression of *SPCP2* enhances drought tolerance [19].

Integrating these observations, we hypothesize that ZmPCP (*Zea mays* pollen-specific cysteine protease) modulates maize yield stability by differentially regulating reproductive development and stress-response pathways. The objectives of this study are to: (1) resolve molecular evolutionary relationships and sequence conservation of ZmPCP in *Zea mays*, *Zea mays* ssp. *mexicana* (Mexican teosinte), and *Arabidopsis thaliana*; (2) characterize the ZmPCP protein interaction network through subcellular localization, yeast two-hybrid (Y2H) assays, and GST pull-down experiments; (3) evaluate transgenic plants under 28 °C drought stress to assess pollen viability, pollen tube elongation, and transcriptomic profiles (RNA-seq), thereby clarifying its regulatory role in pollen tube growth; and (4) investigate ZmPCP functions in seed germination and root development using transgenic maize and *Arabidopsis* models. This work aims to elucidate the core mechanism of ZmPCP in pollen germination, providing novel genetic targets for maize improvement to counteract climate-impaired pollination efficiency.

2. Results

2.1. Phylogenetic and Structural Analysis Reveals Evolutionary Proximity of ZmPCP Between Maize and Mexican Teosinte

To elucidate the phylogenetic relationships of ZmPCP, this study constructed a phylogenetic tree of cysteine proteases (CPs) among maize (*Zea mays*), its wild progenitor Mexican teosinte (*Zea mays* ssp. *mexicana*), and the model eudicot *Arabidopsis thaliana*. Based on functional and structural characteristics, the CP family was classified into nine subfamilies (Figure 1A). A phylogenetic analysis revealed that ZmPCP possesses conserved domains of the CP family, belonging to the THI group within the papain-like cysteine protease subfamily C1A. Notably, maize and Mexican teosinte CPs exhibit closer evolutionary proximity than those of *Arabidopsis thaliana* (Figure 1A). Further multiple sequence alignment of homologous proteins in the THI subgroup branch demonstrated 100% sequence identity between ZmPCP and Zmex03t011636_P01 from Mexican teosinte, while identity with Zmex01t001605_P01 reached 99.14% (Figure 1B). A conservation analysis confirmed that all these proteins contain the characteristic catalytic triad (Cys–His–Asn) of the C1A subfamily.

Protein tertiary structures predicted by AlphaFold2 indicated highly conserved spatial configurations among ZmPCP, Zmex03t011636_P01, and Zmex01t001605_P01 (Figure 2). The core domains (Inhibitor_29 and Peptidase_C1) of these proteins form a highly conserved core catalytic fold composed of tightly packed β -sheets and surrounding α -helices (colored in varying shades of blue). In contrast, the structural model of AtTHI1 exhibited noticeable deviations from ZmPCP, including differences in the overall fold architecture (Figure 2). This structural divergence corresponds with the low sequence identity between AtTHI1 and ZmPCP (40.17%) (Figure 1B), likely attributable to their distant phylogenetic relationship.

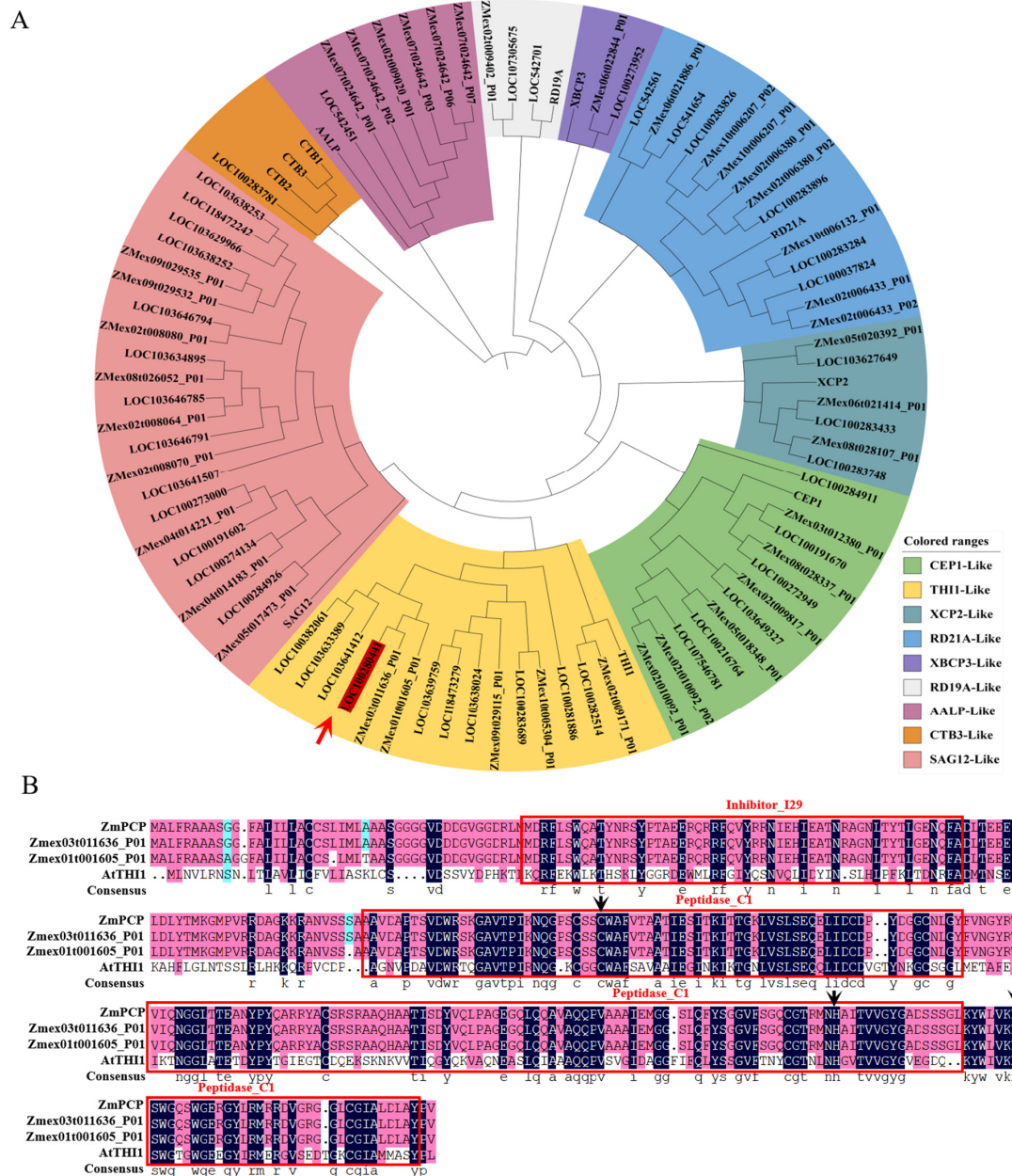


Figure 1. ZmPCP bioinformatics analysis. (A) Phylogenetic tree of ZmPCP in the cysteine protease family of maize, Mexican teosinte and *Arabidopsis thaliana*. The red arrow indicates ZmPCP. (B) Sequence alignment of ZmPCP with similar THI1 proteins. The black arrow indicates Cys, His and Asn.

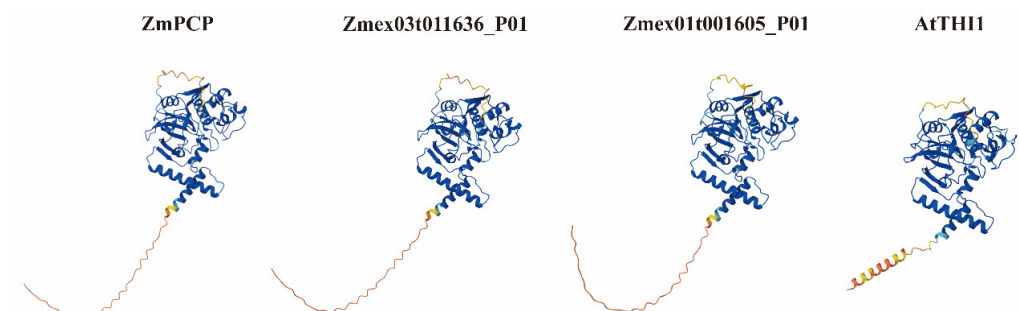


Figure 2. Three-dimensional structure of ZmPCP and its closely related THI1 protein. AlphaFold produces a per-residue confidence score (pLDDT) between 0 and 100. Some regions with low pLDDT may be unstructured in isolation. Model confidence: dark blue: very high (pLDDT > 90); light blue: confident (90 > pLDDT > 70); yellow: low (70 > pLDDT > 50); orange: very low (pLDDT < 50).

2.2. Subcellular Localization of ZmPCP

To determine the subcellular localization of ZmPCP, the gene was cloned from maize cDNA and fused with GFP in a pCAMBIA1302 vector under the CaMV35S promoter. The recombinant plasmid was transformed into *Agrobacterium tumefaciens* GV3101 and transiently expressed in *Nicotiana benthamiana* leaves. Confocal microscopy revealed ZmPCP-GFP fluorescence specifically enriched at the cell periphery, distinct from the cytoplasmic distribution in GFP controls. Subsequent plasmolysis induced by the 10% NaCl treatment confirmed that the fluorescence remained stably associated with the cell wall (Figure 3), indicating that ZmPCP is synthesized intracellularly and targeted to the apoplastic space via the secretory pathway, consistent with bioinformatic predictions.

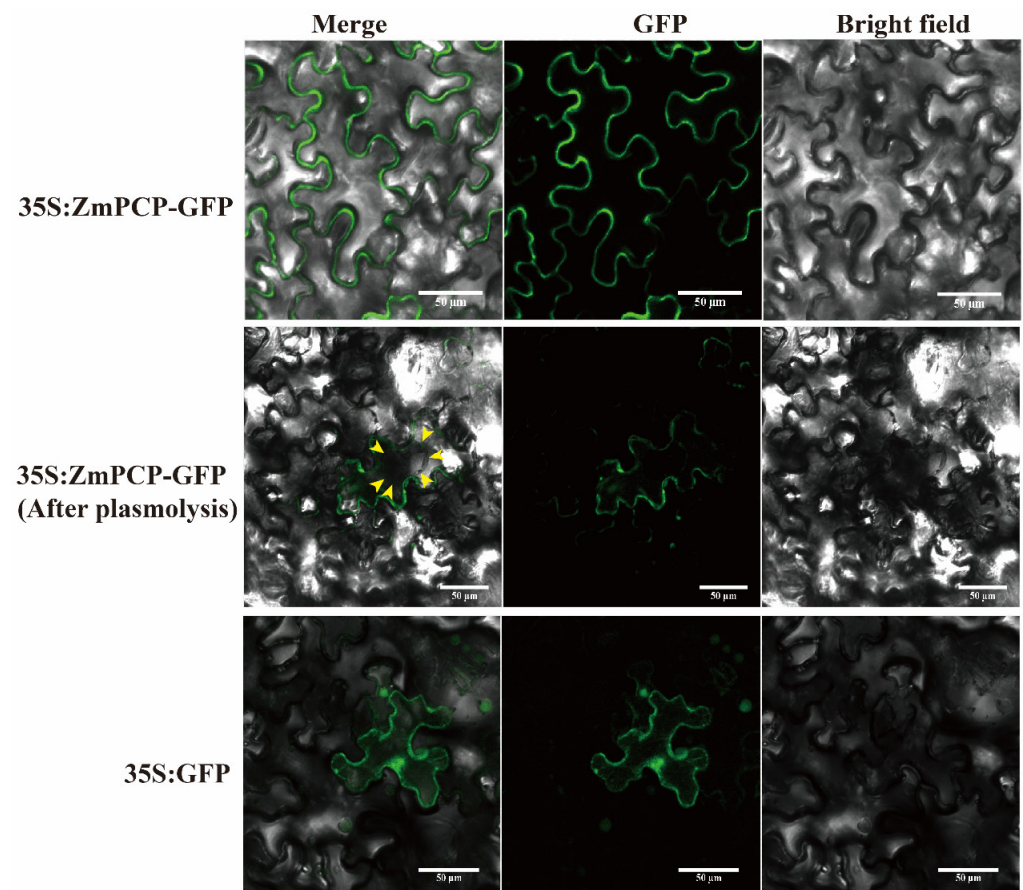


Figure 3. Subcellular localization of ZmPCP in tobacco cells. Scale bar = 20 µm. Note: yellow arrows indicate the position of the plasma membrane after plasmolysis.

2.3. Interaction of ZmPCP with ZmSNAP33

To validate the predicted interaction between ZmPCP and ZmSNAP33 via bioinformatics analysis, this study employed a yeast two-hybrid (Y2H) assay. The preliminary control experiments demonstrated that: (1) the positive control (pGADT7-T + pGBKT7-53) exhibited growth on both SD/-Trp-Leu (double dropout; DDO) and SD/-Trp-Leu-His-Ade (quadruple dropout; QDO) media; (2) the negative control (pGADT7-T + pGBKT7-Lam) showed no growth on QDO medium (Figure 4A), confirming system validity. Subsequent autoactivation and toxicity tests revealed that the pGADT7 + pGBKT7-ZmPCP co-transformants grew on DDO medium (indicating no toxicity) but failed to grow on QDO medium (excluding autoactivation) (Figure 4A). Ultimately, interaction assays confirmed that pGADT7-ZmPCP + pGBKT7-ZmSNAP33 co-transformants grew normally on DDO medium (confirming no toxicity of both proteins) and formed distinct colonies on QDO medium (Figure 4B), demonstrating a specific interaction between ZmPCP and ZmSNAP33.

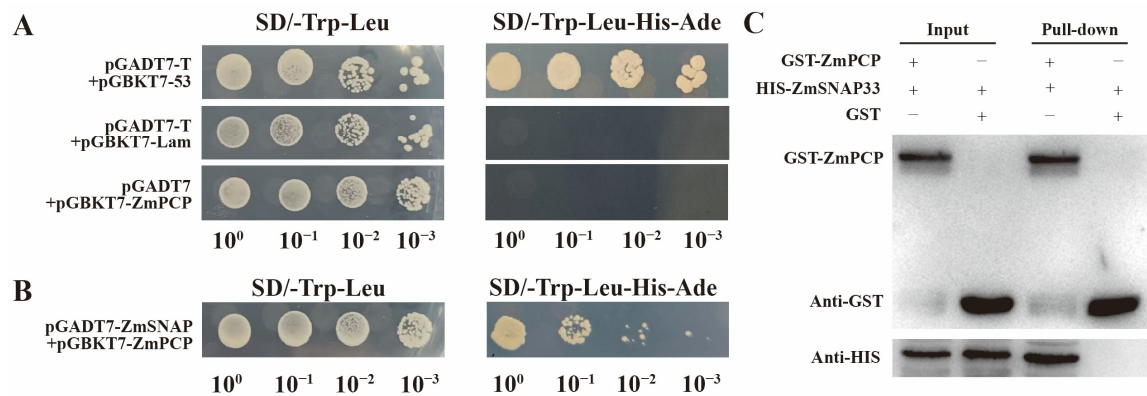


Figure 4. The interactions of ZmPCP with ZmSNAP33. **(A)** Yeast two-hybrid interaction analysis of ZmPCP and ZmSNAP33 with pGADT7-T and pGBKT7-53 as the positive control and pGADT7-T and pGBKT7-lam as the negative control. Validation of ZmPCP toxicity and autoactivation. **(B)** Interaction between ZmPCP and ZmSNAP33. **(C)** GST pull-down assay demonstrating the interaction between ZmPCP and ZmSNAP33. Please refer to Supplementary Figures S1 and S2 for the original images.

Furthermore, GST pull-down assays were employed to substantiate this interaction *in vitro*. GST (empty vector control) or GST-ZmPCP was incubated with His-ZmSNAP33 and bound to glutathione-agarose beads. After washing, centrifugation, SDS-PAGE, and Western blot analysis, no His-ZmSNAP33 signal was detected in the GST control group, whereas GST-ZmPCP significantly captured His-ZmSNAP33 (Figure 4C), confirming the specific binding. This interaction occurred independently of cofactors, indicating that ZmPCP may directly recognize ZmSNAP33 through specific domains, thereby unambiguously demonstrating their direct interaction *in vitro*.

2.4. Analysis of the Effects of ZmPCP on Pollen Viability and Drought Resistance

To investigate the effect of ZmPCP on pollen viability, the I₂-KI staining method was employed for preliminary detection of untreated B104 (wild type), ZmPCP-KO (knockout line), and ZmPCP-OE (overexpression line) pollen (Figure 5A). The results show that the pollen viability of B104 and ZmPCP-KO ranged from 87% to 93%, whereas that of ZmPCP-OE was $83 \pm 2.7\%$, with a significant difference observed (Figure 5B).

In exploring the drought-resistance function of ZmPCP, a 28 °C drought treatment revealed that, under untreated conditions, the germination rate of ZmPCP-OE was significantly lower than that of the wild-type and knockout lines ($p < 0.05$), indicating that ZmPCP overexpression inhibited normal germination. After the 0.5 h treatment, the germination rates of B104 and ZmPCP-KO decreased by 22% to 23%, while ZmPCP-OE maintained a higher germination rate of $44 \pm 5.8\%$. After the 1 h treatment, this trend continued, with the germination rate of ZmPCP-OE ($43.5 \pm 2.9\%$) being significantly higher than that of the other lines ($p < 0.01$), demonstrating an early advantage in drought resistance. After the 1.5 h treatment, the germination rate of ZmPCP-OE remained significantly higher. By the 2 h treatment, the germination rates of all the lines declined to below 7%, but ZmPCP-OE still maintained a relative advantage (Figure 5C,D). In summary, although ZmPCP overexpression suppressed the basal germination ability of pollen, it significantly enhanced its tolerance to drought stress.

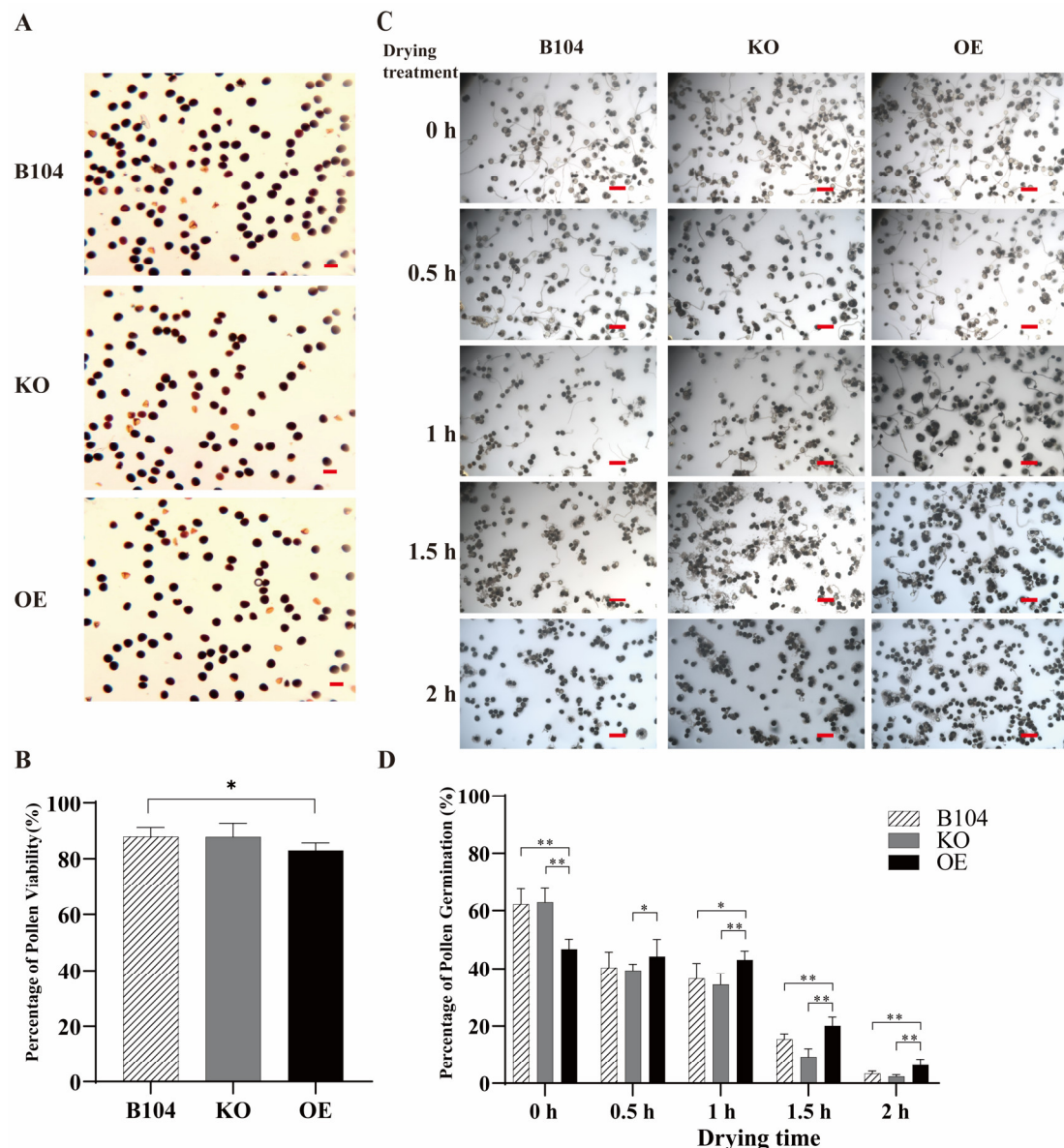


Figure 5. Effect of *ZmPCP* on pollen viability and germination after desiccation treatment (28 °C) for different durations. (A) Untreated pollen stained with I₂-KI. Bar = 100 µm. (B) Viability statistics of untreated pollen (I₂-KI staining). Bar = 300 µm. The experimental data were obtained from three biological replicates and analyzed for significant differences using Student's *t*-test (* *p* < 0.05). (C) Pollen germination images under desiccation treatment (28 °C) at different time points. Please refer to Supplementary Figure S5 for the original images. (D) Pollen germination rates under desiccation treatment (28 °C) at different time points. The experimental data were obtained from three biological replicates and analyzed for significant differences using Student's *t*-test (* *p* < 0.05, ** *p* < 0.01).

2.5. *ZmPCP* Promotes Pollen Tube Growth

To investigate the effects of the maize *ZmPCP* gene on pollen tube growth, silk samples from B104 (wild type), *ZmPCP*-KO, and *ZmPCP*-OE were collected at 0.5 h intervals after self-pollination. The maximum pollen tube elongation distance within the field of view was measured from the stigma attachment point (Figure 6A).

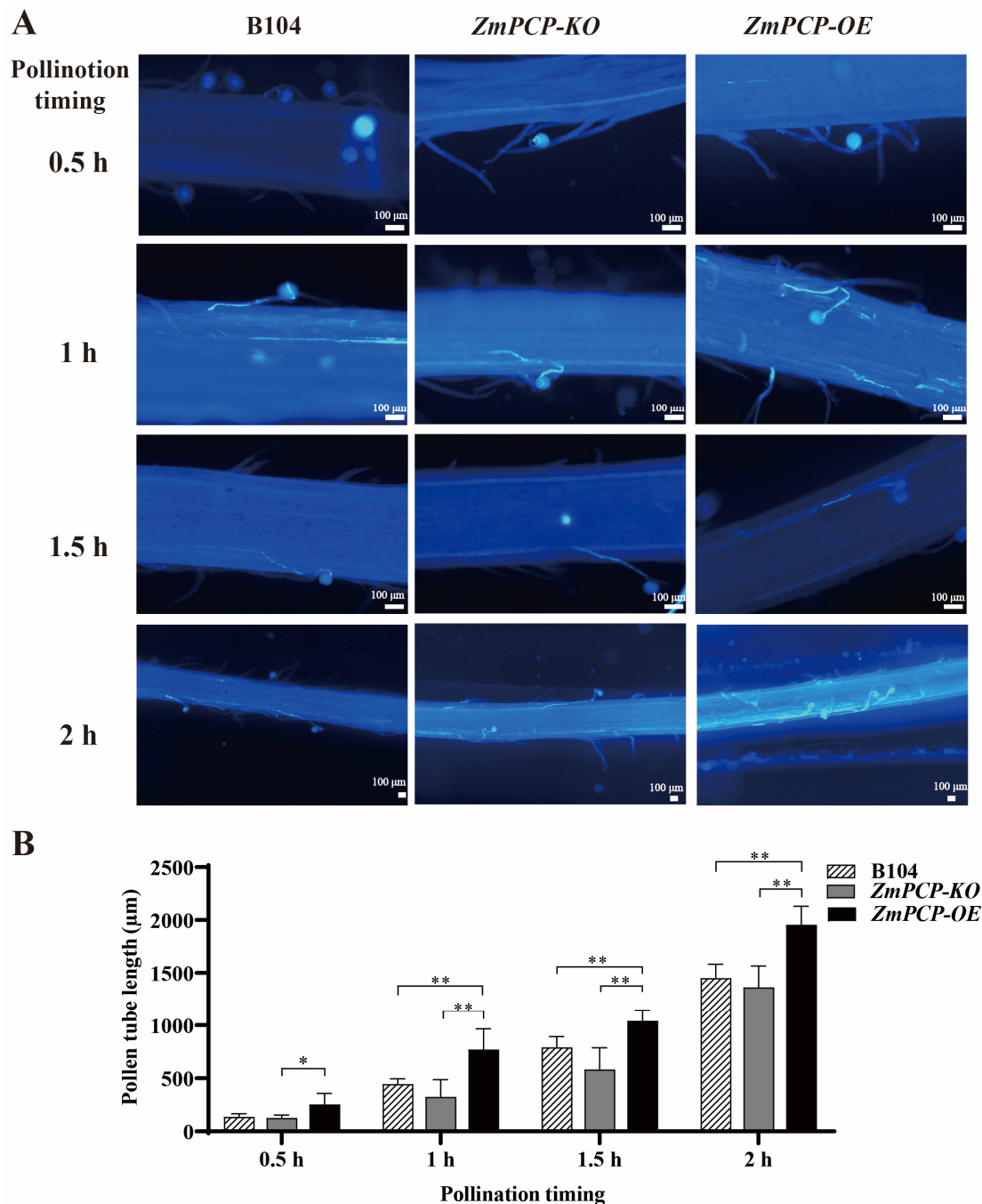


Figure 6. Effect of *ZmPCP* on pollen tube elongation. **(A)** Fluorescence microscopy images showing pollen tube elongation in the styles of B104, *ZmPCP-KO*, and *ZmPCP-OE* lines under self-pollination. Scale bar = 100 μm . **(B)** Quantification of pollen tube length in the styles of B104, *ZmPCP-KO*, and *ZmPCP-OE* lines. The experimental data were obtained from three biological replicates and analyzed for significant differences using Student's *t*-test (* $p < 0.05$, ** $p < 0.01$).

The results demonstrated a significant positive correlation ($p < 0.01$) between the *ZmPCP* expression levels and pollen tube growth kinetics. The B104 pollen tubes elongated progressively from $136 \pm 5.3 \mu\text{m}$ at 0.5 h to $1452 \pm 28 \mu\text{m}$ at 2 h; *ZmPCP-KO* exhibited significantly reduced lengths versus B104 at all time points (e.g., $1363 \pm 22 \mu\text{m}$ at 2 h; $p < 0.05$); *ZmPCP-OE* displayed accelerated growth throughout, reaching $253 \pm 9.1 \mu\text{m}$ (1.86-fold of B104) at 0.5 h and achieving $1956 \pm 31 \mu\text{m}$ at 2 h (1.44-fold longer than *ZmPCP-KO*) (Figure 6B). This dynamic change is consistent with the typical pattern of pollen tube elongation in maize, in which pollen tubes begin to elongate early after pollination and

show an accelerating trend with time [20]. These findings indicate that *ZmPCP* deficiency significantly suppresses pollen tube elongation ($p < 0.01$), while its overexpression enhances this process, demonstrating that *ZmPCP* acts as a positive regulator of pollen tube growth and directly determines elongation velocity.

2.6. RNA-Seq Analysis of Pollen

To investigate the regulatory role of *ZmPCP* in maize pollen development, we performed an RNA-seq analysis on mature pollen from *ZmPCP* overexpression (OE) and knockout (KO) lines under normal growth conditions, with three biological replicates. Applying a threshold of $|\log_2(\text{Fold Change})| > 1$ and adjusted $p < 0.05$, we identified 1664 differentially expressed genes (DEGs), including 673 upregulated and 991 downregulated genes (Figure 7A,B).

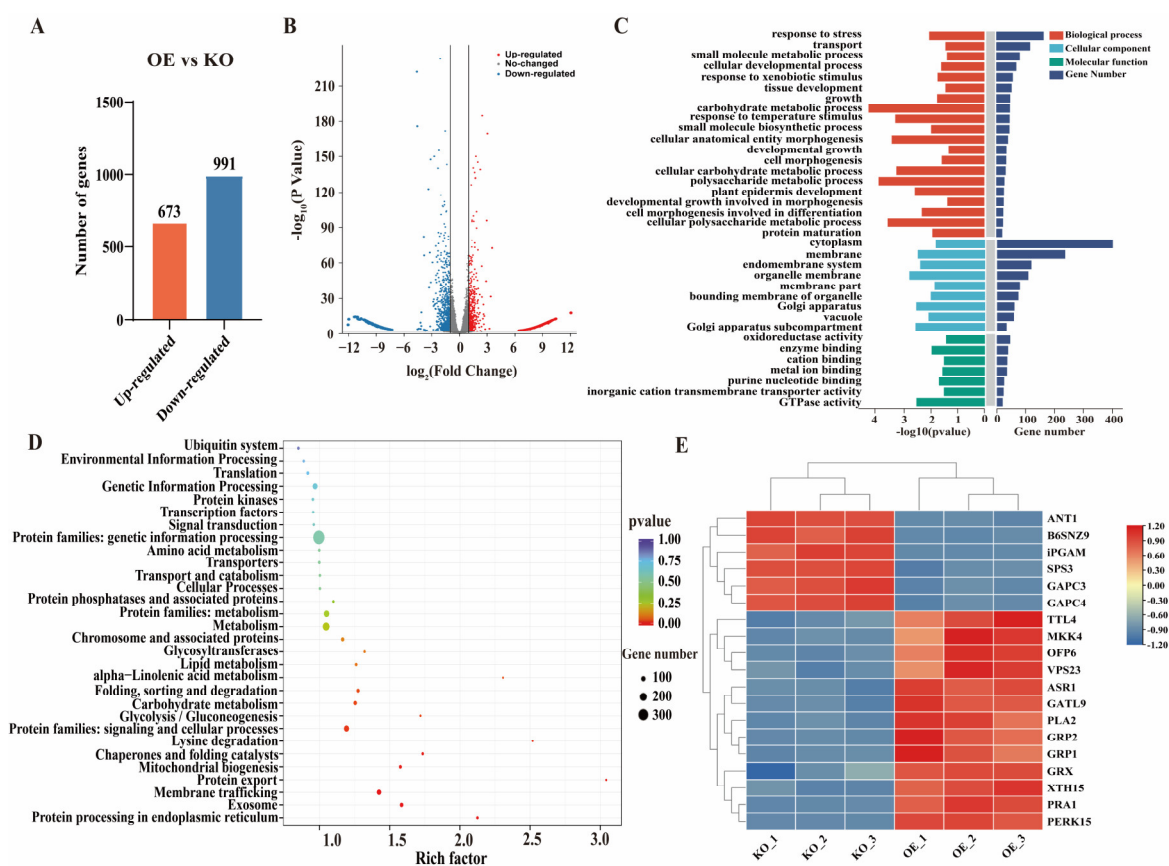


Figure 7. RNA-Seq analysis of *ZmPCP*-OE and *ZmPCP*-KO mature pollen. (A) Number of differentially expressed genes. Upregulated and downregulated genes are derived from *ZmPCP*-OE and compared with *ZmPCP*-KO. (B) Volcano plot analysis of differentially expressed genes. Blue indicates that the differentially expressed genes are significantly downregulated, while red indicates that they are significantly upregulated. (C) GO enrichment analysis of differentially expressed genes. (D) KEGG enrichment analysis of differentially expressed genes. (E) Heatmap of differentially expressed genes. Blue indicates lower expression levels; red indicates higher expression levels. Dendrogram shows clustering results.

A Gene Ontology (GO) enrichment analysis revealed significant enrichment of pollen DEGs in biological processes related to stress response, transmembrane transport, and cellular development. The subcellular localization predictions (plasma membrane/endomembrane system) suggest that *ZmPCP* may function through the apoplastic pathway. Molecular functions were primarily associated with oxidoreductase activity and ion transport ($p < 1 \times 10^{-5}$), indicating that *ZmPCP* modulates pollen energy supply by

regulating redox homeostasis and nucleotide metabolism (Figure 7C). A KEGG pathway analysis further identified enrichment in 30 key pathways, including carbohydrate, lipid, and amino acid metabolism, as well as genetic information processing, signal transduction, and cellular structure maintenance. These findings suggest that *ZmPCP* coordinates a “metabolism-signaling-structure” mechanism during pollen development.

These results indicate that *ZmPCP* acts as a cross-pathway regulatory hub, coordinating a “metabolism-signaling-structure” trinity mechanism during pollen development. To decipher the molecular mechanisms underlying drought resistance and pollen tube growth dynamics, we systematically analyzed pollen transcriptomes and prioritized functionally characterized DEGs (Table S4). The literature evidence implicates drought-responsive genes *Zm00001eb275850* (*GRP2*), *Zm00001eb388380* (*GRP1*), *Zm00001eb255210* (*TTL4*), *Zm00001eb110700* (*PLA2*), *Zm00001eb351170* (*GRX*), and *Zm00001eb407710* (*ASR1*) in stress tolerance [21–26]. It also indicates energy metabolism genes *Zm00001eb252870* (*ANT1*), *Zm00001eb184000* (*GAPC3*), *Zm00001eb246370* (*GAPC4*), *Zm00001eb368990* (*iPGAM*), and *Zm00001eb275240* (*SPS3*) in ATP synthesis [27–31]. Further, it outlines vesicle trafficking/cell wall dynamics genes *Zm00001eb290310* (*PRA1*), *Zm00001eb047590* (*OFP6*), *Zm00001eb117750* (*PERK15*), *Zm00001eb259630* (*VPS23*), *Zm00001eb194800* (*GATL9*), and *Zm00001eb312000* (*XTH15*) in polar growth [32–37]. Finally, signaling regulators are indicated, including *Zm00001eb257340* (*MKK4*), *Zm00001eb149520* (*B6SNZ9*), and *Zm00001eb286870* (*CML15*), in transduction cascades [38–40].

In the *ZmPCP*-OE line, the expression levels of those genes associated with stress response, pollen tube elongation, cell wall metabolism, and vesicle trafficking were significantly elevated compared to the *ZmPCP*-KO line ($p < 0.05$). This transcriptional profile corresponds to enhanced drought tolerance and accelerated pollen tube growth. Paradoxically, the expression of specific energy metabolism-related genes was downregulated in *ZmPCP*-OE, which may contribute to the observed reduction in the pollen germination rate.

2.7. Overexpression of *ZmPCP* Inhibits Maize Seed Germination

To investigate the impact of *ZmPCP* on maize seed germination, the germination rates of the B104 (wild type), *ZmPCP*-KO, and *ZmPCP*-OE lines were compared on day 7 of soil-based cultivation. The germination rates were 94% (B104), 93% (*ZmPCP*-KO), and 69% (*ZmPCP*-OE), respectively (Figure S4), with *ZmPCP*-OE exhibiting significantly reduced germination compared to the wild-type and knockout lines ($p < 0.05$). These results indicate that *ZmPCP* overexpression suppresses maize seed germination and functions as a negative regulator in this process.

2.8. Promotion of Germination and Seedling Growth in *Arabidopsis* Under Stress Through *ZmPCP* Overexpression

To investigate the functional role of *ZmPCP* in stress responses, the recombinant vector pCambia1302-*ZmPCP* was transformed into *Arabidopsis*. Transgenic lines were selected via antibiotic resistance screening and systematically validated using molecular biology approaches. A genomic PCR analysis (Figure 8A) confirmed the successful integration of *ZmPCP* into the *Arabidopsis* genome, with the wild type (WT) serving as the negative control; RT-qPCR assays (Figure 8B) demonstrated significantly elevated *ZmPCP* transcript levels in the OE3 and OE6 lines, meeting the overexpression criteria [41], with OE3 and OE6 exhibiting relatively smaller variance. Homozygous T3 lines were selected for functional assays.

Under simulated abiotic stress conditions, *ZmPCP* overexpression markedly altered the seed germination dynamics. In the standard MS medium, both overexpression lines (OE3 and OE6) and WT exhibited >90% germination at 24 h, reaching 100% by 48 h. Under 300 mM mannitol stress, the germination rates at 24 h were significantly reduced (OE3:

29% $\pm$ 3.1; OE6: 21% $\pm$ 2.8 vs. WT: 35% $\pm$ 3.5; $p < 0.05$), with completion delayed to 72 h (Figure 8C). The ABA treatment similarly suppressed germination, with the overexpression lines consistently lagging behind the WT during 24–48 h. Notably, under the 2 μ M ABA treatment, OE6 exhibited a more obvious reduction in germination rate than OE3, which may be due to small changes in transgenic insertion sites and genetic backgrounds leading to phenotypic differences that are not strictly proportional to expression level. These results indicate that *ZmPCP* modulates germination timing in response to osmotic stress and ABA, thereby participating in seed germination regulation.

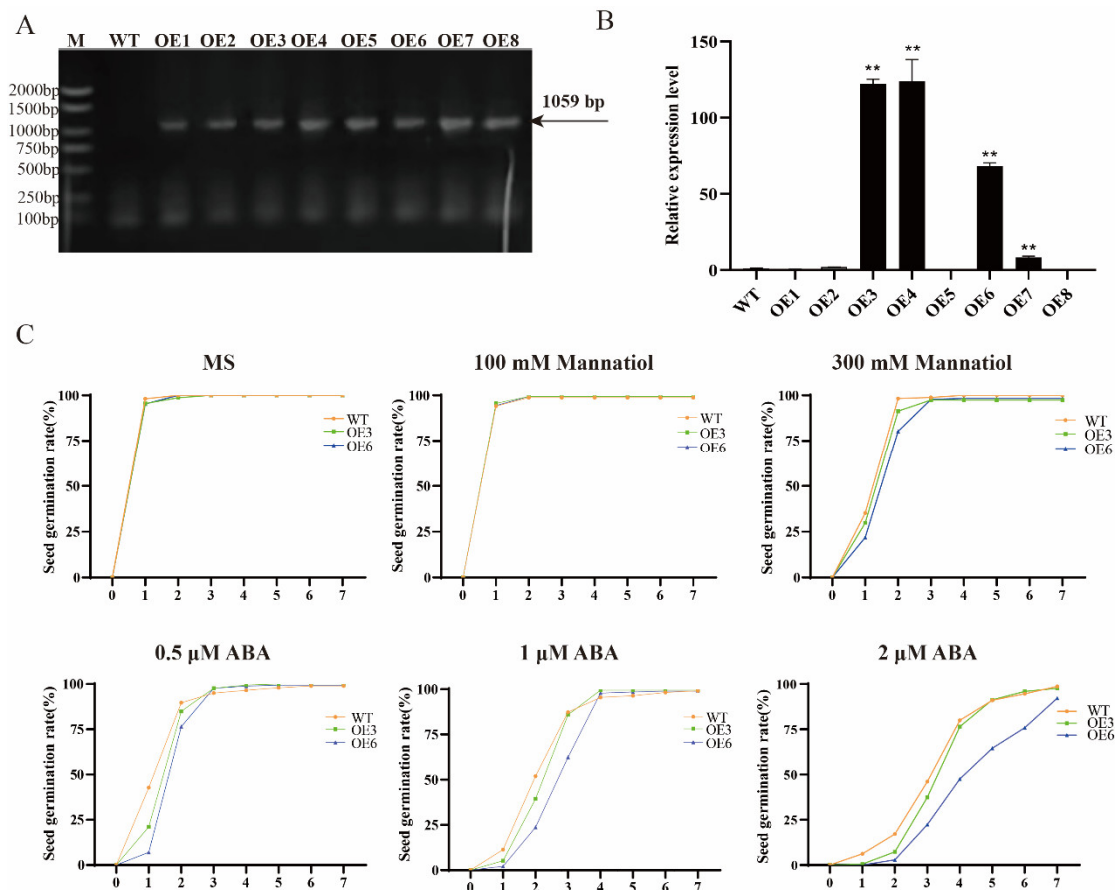


Figure 8. The effects of *ZmPCP* overexpression on seed germination. (A) PCR verification of *ZmPCP* in transgenic *Arabidopsis* lines. Please refer to Supplementary Figure S3 for the original image. (B) Validation of *ZmPCP* expression levels in transgenic *Arabidopsis*. The experimental data were obtained from three biological replicates and analyzed for significant differences using Student's *t*-test (** $p < 0.01$). (C) Germination rate of *Arabidopsis thaliana* cultured for 7 days on MS solid medium containing different concentrations of mannitol and ABA.

Further phenotypic analysis of the seedlings revealed no significant differences in root length among WT, OE3, and OE6 under control conditions (Figure 9A). At low ABA concentrations (0.5–1 μ M), the root lengths of the overexpression lines were marginally greater than WT (not statistically significant). At 2 μ M ABA, the OE3 and OE6 roots (14 $\pm$ 2.1 mm and 12.9 $\pm$ 1.4 mm, respectively) exceeded WT (10.6 $\pm$ 1.5 mm) by 21.7–32.1% ($p < 0.01$) (Figure 9B). This demonstrates that *ZmPCP* overexpression enhances root adaptation to high-concentration ABA stress, potentially through modulation of stress signaling pathways.

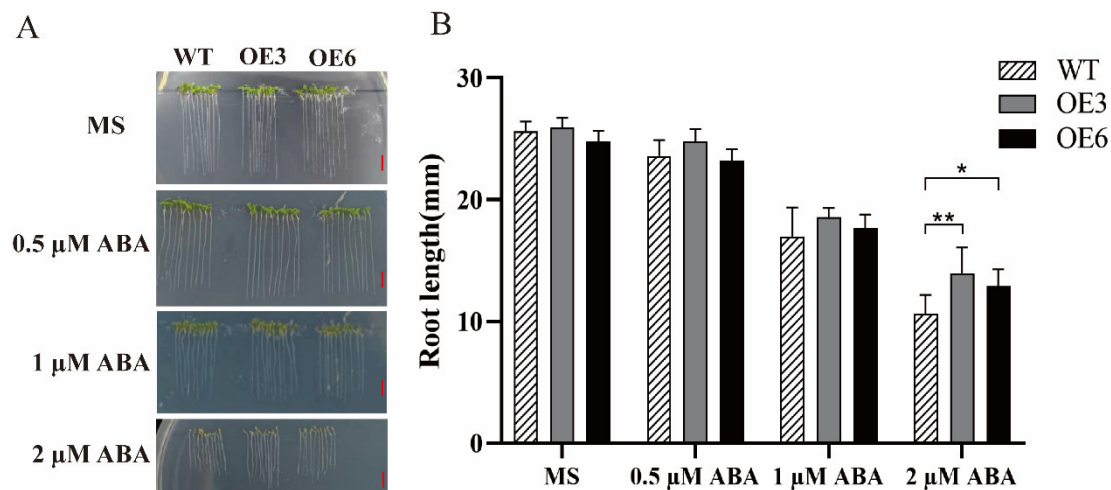


Figure 9. Roots of transgenic Arabidopsis under ABA treatment. (A) Roots grown for 7 days under different concentrations of ABA, with a scale bar of 5 mm. Please refer to Supplementary Figure S6 for the original images. (B) Data of root lengths of plants grown for 7 days under different concentrations of ABA. The experimental data were obtained from three biological replicates and analyzed for significant differences using Student's *t*-test (* $p < 0.05$, ** $p < 0.01$).

3. Discussion

Papain-like cysteine proteases (PLCPs) serve as crucial regulators in plants, extensively participating in developmental processes and stress responses. Functional studies on identified PLCP members in maize (such as Mir1 and CP1B) remain limited [42–44]. This study reveals that ZmPCP (belonging to the TH11 clade of the C1A subfamily) significantly enhances maize drought resistance by improving cellular osmotic adjustment and membrane stability and additionally regulates pollen germination [17,45]. The protein encoded by this gene is an apoplastic secretory protein possessing a conserved Peptidase_C1 domain, exhibiting high homology with its ortholog in *Zea mays* ssp. *parviglumis* (*Mexican teosinte*), suggesting evolutionary conservation (Figure 1). Subcellular localization confirmed ZmPCP as an apoplastic secretory protein (Figure 3), consistent with the results from immunogold electron microscopy in pollen [46]. Notably, ZmPCP overexpression alters pollen pore morphology, indicating its potential involvement in cell wall remodeling (e.g., pectin degradation) through extracellular proteolysis. This mechanism is analogous to that of Arabidopsis CP1, which cleaves extensins to promote polar growth [47].

Furthermore, this study identified an interaction between ZmPCP and the membrane-localized SNARE protein ZmSNAP33 (Figure 5). SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) family proteins were initially discovered in yeast. Based on cluster analyses of their SNARE domain amino acid sequences, SNAREs are classified into four subgroups: Qa-SNAREs, Qb-SNAREs, Qc-SNAREs, and R-SNAREs. They are also categorized as vesicle-associated SNAREs (v-SNAREs) or target membrane SNAREs (t-SNAREs) [48]. SNARE proteins play essential roles in vesicle trafficking, immune secretion, exocytosis during pollen tube growth, and the fusion of secretory vesicles with the plasma membrane at the pollen tube apex [20]. SNAP25-type SNAREs, which are located on the plasma membrane, function as key components of the SNARE complex by assembling with a syntaxin (Qa-SNARE) and a vesicle-anchored R-SNARE to mediate membrane fusion [49]. ZmSNAP33 encodes a SNAP25-type SNARE protein, and its Arabidopsis homolog AtSNAP33 has been reported to cause delayed pollen hydration on mutant stigmas [50]. One of the cellular responses in the stigma associated with pollen hydration involves vesicle trafficking in the stigmatic papilla, which is presumed to deliver cargo that facilitates water release to the pollen grain [51]. Moreover, OCP, featuring cys-

teine protease activity and whose loss of function leads to abnormal pollen development, interacts with OsRACK1A or OsSNAP32 physically, in vitro and in vivo, and can suppress the expression of OsSNAP32 [52]. Therefore, through its interaction with ZmSNAP33, ZmPCP may regulate the vesicle trafficking in the stigmatic papilla during the process of pollen hydration (Figure 10). In addition, several W-boxes (WRKY transcription factor binding sites, TTGAC), MYB binding sites (WAACCA), and ABREs (abscisic acid responsive elements, AAACGTGA) have been identified in the promoter region of *OsSNAP32*. These cis elements suggest that *OsSNAP32* participates in various signal transduction pathways related to plant responses to biotic and abiotic stresses [49]. Accordingly, its maize homolog ZmSNAP33 may have a pleiotropic role in responding to environmental stressors, potentially mediated through its interaction with ZmPCP (Figure 10).

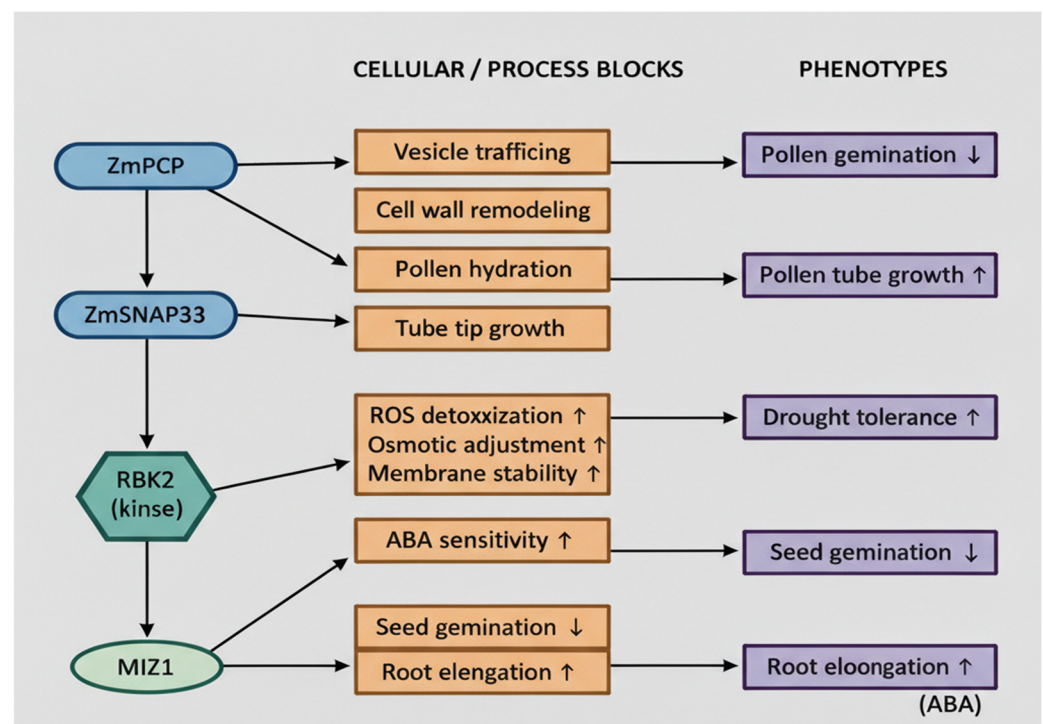


Figure 10. The possible functional scheme of ZmPCP. ZmSNAP33: a SNAP25-type SNARE protein; SNARE: soluble N-ethylmaleimide-sensitive-factor attachment protein receptor; RBK2: a pathogen-induced receptor-like cytosolic kinase 2; MIZ1: MIZU-KUSSEI 1. Upward arrows (↑) denote an increase or enhancement of the corresponding cellular process or phenotype, whereas downward arrows (↓) indicate a decrease or reduction in the related process or phenotype.

Pollen fertility is a critical factor for successful plant sexual reproduction and is regulated by diverse genetic, environmental, and physiological–biochemical factors. Our results demonstrate that *ZmPCP* plays a crucial regulatory role within this process. Overexpression of *ZmPCP* reduced the germination rate of non-desiccated pollen, potentially due to disrupted energy metabolism or aberrant cell wall remodeling. Several genes associated with energy metabolism were significantly upregulated in *ZmPCP*-KO pollen compared with *ZmPCP*-OE (e.g., *Zm00001eb184000* (*GPC3*), *Zm00001eb246370* (*GPC4*), *Zm00001eb149730* (*PGAM*), and *Zm00001eb368990* (*PGAM*) in Table S2). This pattern indicates that *ZmPCP* overexpression may restrict energy metabolism during early pollen development, potentially contributing to reduced pollen activity. The mutations of *NtiPGAM* (2, 3-bisphosphoglycerate-independent phosphoglycerate mutase) impaired glycolysis, limited the energy supply, and ultimately led to defective pollen development and pollen tube growth in the mutant plants [53]. These parallels suggest that *ZmPCP* influences pollen

performance in part through modulation of carbohydrate metabolic pathways. Despite its negative effect on germination, *ZmPCP* overexpression markedly enhanced drought tolerance after a 1 h desiccation treatment, and drought resistance was significantly enhanced, a mechanism involving the accumulation of osmolytes (e.g., sugars) and upregulation of antioxidant enzyme activity [54]. Additional transcriptomic evidence supports this mechanism: several stress-response genes were upregulated in *ZmPCP*-OE pollen, including a pathogen-induced receptor-like cytosolic kinase *RBK2* (*Zm00001eb165930*), a member of the Arabidopsis RLCK-VIb family known to enhance ROS detoxification and improve drought and salt tolerance [55,56]. Meanwhile, *MIZU-KUSSEI 1* (*MIZ1*) (*Zm00001eb069080*) was also upregulated, which may play a role in drought stress [57]. *AtMIZ1* was also shown to improve drought avoidance and increase root cell viability under hydro-stimulated conditions in Arabidopsis [58]. Notably, although *ZmPCP* overexpression inhibited germination, it promoted pollen tube elongation, with its expression level positively correlated with pollen tube growth rate. Transcriptome profiling further supports a role for *ZmPCP* in regulating cell wall synthesis: the genes involved in cell wall synthesis were downregulated in *ZmPCP*-KO compared with *ZmPCP*-OE pollen (e.g., *Zm00001eb275850* (*GRP2*), *Zm00001eb388380* (*GRP1*), *Zm00001eb303890* (*RALF*), and *Zm00001eb117750* (*PERK15*) in Table S3). This is consistent with observations in Arabidopsis, where the *perk5-1 perk12-1* double mutant exhibits shorter pollen tubes, reduced pollen transmission, and decreased seed set due to altered cell wall polysaccharide composition and disrupted ROS homeostasis [34]. Together, these findings suggest that *ZmPCP* promotes pollen tube elongation by coordinating cell wall synthesis, modification, and redox signaling at the growing tip (Figure 10).

A pollen transcriptome analysis further revealed that differentially expressed genes were predominantly enriched in maize stress responses, growth and development, carbohydrate metabolic processes, signal transduction, and protein secretion capacity. *ZmPCP* likely functions as a cross-pathway regulatory node, exerting significant control over pollen germination and pollen tube growth by modulating the expression of genes associated with stress responses, energy metabolism, vesicle trafficking, cell wall structure, and signal transduction (Figure 10).

During seed germination, *ZmPCP* overexpression significantly reduced maize seed germination rates, contrasting with the promotive role of *HvPap-1* [59]. ABA inhibits seed germination, while ABA-deficient mutants germinate more rapidly than the wild type under the same conditions [60]. The reduced germination of *ZmPCP*-overexpressing seeds suggests the possible involvement of ABA-associated regulatory pathways. Consistent with this, under stress treatments with mannitol and ABA, germination of *ZmPCP*-overexpressing Arabidopsis seeds exhibited a certain delay but did not affect overall germination (Figure 8). Previous reports have shown that ABA can regulate the expression of *MIZ1* [57]. Therefore, it is possible that *ZmPCP* influences ABA-associated processes. Under high-concentration ABA treatment, the root length of *ZmPCP*-overexpressing Arabidopsis lines was significantly longer than that of the wild type, indicating that *ZmPCP* coordinately regulates germination and root growth through the ABA signaling pathway [9,61]. The enhanced ABA responsiveness may be related to the *ZmPCP*-induced upregulation of *RBK2* (*Zm00001eb165930*), a kinase associated with cell wall biosynthesis and ROS-related signaling processes [55]. Notably, PLCP family members (e.g., *RD21*) have been demonstrated to enhance plant resistance by degrading viral pathogenicity factors [62], suggesting that *ZmPCP* may possess analogous immune-regulatory potential.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

To analyze the effects of *ZmPCP* (gene ID: LOC100280441) on seed germination of maize, the seeds of maize inbred lines B104, *ZmPCP*-OE and *ZmPCP*-KO (generated in our previous work) [17] were germinated in soil mixture composed of peat soil and vermiculite thoroughly mixed at a 1:1 ratio and watered every 3 d. The seedlings were grown in plastic pots (7 cm top diameter, 5 cm bottom diameter, and 7.5 cm height), with five plants per pot, under controlled conditions of 27/22 °C (day/night) and 60% relative humidity with a 14/10 h photoperiod and a light intensity of 2000 Lux. Count the germination rate of maize plants when they grow to the seventh day. All *Arabidopsis thaliana* plants and *Nicotiana benthamiana*, grown in the same soil mixture and in pots of the same specifications, were maintained under a 16 h light/8 h dark cycle with approximately 60% relative humidity at 22 °C in a plant growth chamber. All experiments were conducted with at least three independent biological replicates.

4.2. Bioinformatics Analysis of *ZmPCP*

Genomic data and annotation files for maize (B73) and Mexican teosinte (*Zea mays* ssp. *mexicana*) were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/> (accessed on 30 January 2024)) and ZEAMAP (<https://db.cngb.org/zeamap/> (accessed on 30 January 2024)), respectively. *Arabidopsis thaliana* cysteine protease gene sequences were acquired from the TAIR database (<https://www.arabidopsis.org/> (accessed on 30 January 2024)). Genomic data extraction and conversion to protein sequences were performed using TBtools (version 2.323) [63]. Candidate family members were identified via bidirectional Blast alignment ($E\text{-value} \leq 1 \times 10^{-5}$) against published *Arabidopsis* cysteine protease protein sequences as Query Seqs. Redundant sequences were curated through motif analysis (MEME Suite: <https://meme-suite.org/meme/> (accessed on 30 January 2024)), domain validation (NCBI CDD: <https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi> (accessed on 30 January 2024)), and InterPro structural analysis (<https://www.ebi.ac.uk/interpro/> (accessed on 30 January 2024)). Multiple sequence alignment was conducted using MUSCLE in MEGA 11 software (default parameters), followed by neighbor-joining (NJ) phylogenetic tree construction (Bootstrap 1000–1500 replicates; Jones–Taylor–Thornton model). The evolutionary tree was visually refined using iTOL (version 7) (<https://itol.embl.de/> (accessed on 30 January 2024)). Protein tertiary structures of *ZmPCP* homologs (Mexican teosinte and *Arabidopsis*) were predicted via AlphaFold2 to analyze evolutionary relationships and structural divergence.

4.3. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Referring to the method of Li [17], the main steps are as follows: Total RNA in maize tissues was extracted using RNA-Solv[®] Reagent (Omega Bio-Tek, Norcross, GA, USA), and a 2 µg RNA sample was used to synthesize the first-strand cDNA via the 5 × All-In-One MasterMix with AccuRT Genomic DNA Removal Kit (Applied Biological Materials Inc., Richmond, BC, Canada). The qRT-PCR was carried out using a Hieff qPCR SYBR Green Master Mix kit (YEASEN Biotechnology, Shanghai, China), and the gene-specific primers of PCP can be referred to in the previous research of our research group [17]. The quantification method $2^{-\Delta\Delta CT}$ was used to assess the relative expression level of PCP after normalization based on *ZmUBI* expression [64]. Data were represented as relative expression (mean ± SD) from three biological replicates.

4.4. Subcellular Localization

Nicotiana benthamiana was selected due to its high efficiency in *Agrobacterium*-mediated transient expression, enabling rapid assessment of protein subcellular localization [65]. To determine the subcellular localization of ZmPCP, the CDS region of ZmPCP was cloned downstream of CaMV 35S promoter and into the pCambia1302-GFP vector to construct the 35S: ZmPCP-GFP expression vector. The recombinant vector was transformed into *Agrobacterium* GV3101 and infiltrated into tobacco leaf epidermal cells using the *Agrobacterium*-mediated method, incubating at 28 °C for 2 d. Fluorescence signals were observed using a confocal laser microscope (Nikon, Tokyo, Japan).

4.5. Identification of ZmSNAP33 as a Candidate Interacting Partner of ZmPCP and Yeast Two-Hybrid Assay

To identify potential interacting partners of ZmPCP, we first referred to previous studies reporting that OsCP (an oryzain alpha-chain precursor featuring cysteine protease activity) interacts with OsSNAP32 (synaptosome-associated protein of 32 kD) physically in vitro and in vivo in rice [52]. Using the OsSNAP32 protein sequence as a query, we performed BLASTP searches against the maize proteome and identified several SNAP25 homologs, including protein SNAP33 (GeneID:100217287). These ZmSNAP33 candidates were subsequently cloned and subjected to yeast two-hybrid screening with ZmPCP. Through this stepwise validation, the ZmSNAP33 protein reported in this study was identified as the interacting partner of ZmPCP.

The interactions of ZmPCP and ZmSNAP33 were studied using the Gal4-based yeast two-hybrid (Y2H) system, following the method of Liang [66] and the manufacturer's instructions (Zoman Bio., Beijing, China). The CDS of ZmPCP was cloned into the decoy vector (pGBKT7) to generate the bait vector pGBKT7-ZmPCP, and the CDSs of ZmSNAP33 were cloned into pGADT7 as prey. pGBKT7-53/pGADT7-T and pGBKT7-Lam/pGADT7-T were used as positive and negative controls, respectively. After co-transformation into Y2HGold competent cells (Zoman Bio., Beijing, China), transformants were first selected on SD/-Leu/-Trp medium, and interaction was tested on SD/-Leu/-Trp/-His/-Ade medium. One ZmSNAP33 candidate showing consistent growth on selective medium was identified as the interacting partner of ZmPCP. Primers used for vector construction are listed in Table S1.

4.6. Prokaryotic Protein-Induced Expression and Protein Purification

The coding sequences (CDSs) of ZmPCP and ZmSNAP33 were cloned into the pGEX4T-1 and pET-30a (+) vectors, respectively, using NcoI and SpeI for digestion and subsequent ligation. The constructed vectors were then transformed into *Escherichia coli* BL21(DE3) cells. Clones were selected using kanamycin (50 µg/mL) as a selection marker. *E. coli* cells harboring the genes of interest were grown overnight at 37 °C with shaking at 200 rpm in 5 mL of LB liquid medium supplemented with 50 µg/mL kanamycin. Subsequently, 1 mL of this saturated culture was inoculated into 50 mL of fresh LB medium containing 50 µg/mL kanamycin and cultured at 37 °C until the optical density at 600 nm (OD₆₀₀) reached 0.6. The incubation temperature was then lowered to 16 °C, and protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 14 h. Purification of ZmPCP-GST was performed using the GST-tag Protein Purification Kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's instructions, while ZmSNAP33-His was purified using a His-tagged protein purification kit (CWBI, Taizhou, China) as per the manufacturer's protocol. Finally, 12% SDS-PAGE electrophoresis was used to analyze the expression product and the purified protein.

4.7. GST –Pull-Down and Western Blot

To confirm the direct interaction of ZmPCP with ZmSNAP33, we performed a GST pull-down assay. ZmPCP-GST or GST proteins were incubated with ZmSNAP33-His protein in 100 µL of binding buffer for 2 h at 4 °C with continuous rotation [67]. After incubation, 30 µL of pre-equilibrated GST agarose gel was added, followed by incubation at 4 °C with gentle agitation (40 rpm) for 16 h (overnight). A 20 µL sample was taken as input, while the remaining samples were washed thoroughly and subjected to SDS-PAGE. For the Western blot analysis, proteins separated by SDS-PAGE were transferred to PVDF membranes. After blocking the membrane, it was incubated with the primary antibody (diluted 1:2000), followed by the secondary antibody (diluted 1:5000). After TBST washing, protein bands were visualized using ECL substrate and imaged with a chemiluminescence detection system. Band intensities were quantified using ImageJ software (v1.8.0) with normalization to input controls. The primers used in this experiment are listed in Supplemental Table S1.

4.8. Pollen Viability and Germination *in Vitro*

Pollen viability was examined with I₂-KI staining methods [68]. Pollen grains that were stained blue after 8 min of staining in 0.5% I₂-KI solution were also counted as viable. The proportion of about 50 pollens with different staining depth was counted under the microscope (Eclipse Ni-U, Nikon, Tokyo, Japan) to evaluate the maturity or vitality of the population, and photos were taken for preservation [17]. *In vitro* germination experiments were carried out. The collected pollens were placed in disposable Petri dishes. After drying at 28 °C for 0, 0.5, 1, 1.5 and 2 h, an appropriate amount of pollen was spread on 1 × 1 cm pollen germination solid medium (the components included 100 mg/L Ca (NO₃)₂, 10 mg/L H₃BO₃, 10 mg/L MgCl₂, 15% *w/v* sucrose and 1.0% *w/v* agar). After incubation at 28 °C for 4 h, the germination of no less than 200 pollen particles in each field was counted and photographed with a microscope (Eclipse Ni-U, Nikon, Tokyo, Japan). A pollen grain was considered germinated when its tube length reached pollen diameter [69]. All viability and germination tests were conducted in three biological replications.

4.9. Aniline Blue Staining of Pollen Tubes

Pollen collected from maize plants was evenly distributed to self-pollinated silks, and silk samples were collected at 0.5 h, 1 h, 1.5 h and 2 h after pollination. The samples were fixed in Carnoy stationary solution (absolute ethanol: chloroform: glacial acetic acid mixed at 6:3:1 volume ratio) for 24 h. After fixation, the silks were rinsed in deionized water and then softened in 4 M KOH solution for 4 h, followed by another rinse with deionized water. The softened silks were then incubated in aniline blue staining solution (0.1 M phosphate buffer containing 0.1% aniline blue, pH = 7.0) for 12 h. After staining, the silks were washed with deionized water, cut into 1.5 cm segments, mounted in 50% glycerol, and examined under the ultraviolet filter of fluorescence microscope (Eclipse Ni-U, Nikon, Tokyo, Japan) with near-UV excitation [70].

4.10. Transcriptome Analysis of Pollen

In this study, mature pollen from ZmPCP-OE and ZmPCP-KO maize plants grown under normal conditions was used for transcriptome sequencing. Three biological replicates were prepared for each genotype. Total RNA per sample was used for cDNA library construction and Illumina sequencing (BGI Genomics Co. Ltd., Beijing, China), and RNA-seq data were processed, assembled, and annotated. Bioinformatic analysis was performed using TBtools software. First, clean data were aligned to the reference genome using the Hisat2 plugin. Then, read counts for each gene were quantified with StringTie. The differentially expressed genes (DEGs) were conducted using the DESeq2 plugin (version 2.323) to com-

pute the fold change and p -value for each gene. Genes satisfying $|\log_2(\text{fold change})| \geq 1$ and $p\text{-value} < 0.05$ were identified as differentially expressed. Finally, TBtools plugins were utilized to generate a volcano plot of differentially expressed genes and to perform both GO and KEGG enrichment analyses.

4.11. Heterologous Expression of *ZmPCP* in *Arabidopsis thaliana*

Arabidopsis is a preferred model for physiological, biochemical, genetic, and molecular studies due to its compact and well-annotated genome, ease of cultivation and manipulation, short life cycle, and high seed yield [71].

The CDS of *ZmPCP* was inserted into the pCAMBIA1302 overexpression vector (Takara Bio, Inc. Shiga, Japan). *Agrobacterium tumefaciens* GV3101 (Zoman, Beijing, China) harboring the vector p pCAMBIA1302-*ZmPCP* under 35S promoter was transformed into *Arabidopsis* using the floral dip method. The positive lines were selected using 50 mg/L hygromycin for further analysis. The T2 plants that showed 100% resistance to hygromycin were considered homozygous transformants in the T3 generation, and then the T3 homozygous transgenic lines OE-1 and OE-2 with high expression levels of *ZmPCP* were used for further analysis as the experimental materials.

4.12. Germination Assessment

A total of 63 wild-type (WT) and transgenic (*ZmPCP*-overexpressing lines OE-3 and OE-6) *Arabidopsis thaliana* seeds were surface-sterilized and planted on the Murashige and Skoog (MS) medium supplemented with mannitol (0, 100, or 300 mM) or ABA (0, 0.5, 1, or 2 μM) in triplicate. All the seeds were planted in a tissue culture incubator under a 16/8 h day/night cycle at 22 °C for germination and vertically cultivated after 4 days. The germination (emergence of radicles) was monitored daily. Subsequently, the root lengths of seedlings treated with 0.5, 1, or 2 μM ABA were measured and photographed on days 7 of vertical cultivation.

4.13. Statistical Analysis

All statistical data were calculated from three replications using DPS 8.0 software package and are reported as means $\pm$ standard error (SE). Differences between different plant lines or treatments were considered significant if $p < 0.05$.

5. Conclusions

In summary, *ZmPCP*, functioning as an extracellular protease, regulates cell wall remodeling and drought resistance via *ZmSNAP33*-mediated vesicle trafficking. *ZmPCP*-overexpressing maize exhibited significantly lower pollen viability and seed germination rates compared to wild-type and knockout lines. Nevertheless, *ZmPCP* overexpression conferred significantly enhanced tolerance to drought stress (desiccation treatment) in pollen, mitigating the reduction in pollen viability. Accelerated pollen tube growth was observed in *ZmPCP*-overexpressing maize plants under self-pollination compared to wild-type and knockout lines. However, the upstream regulators of *ZmPCP*, its downstream target proteins, and the underlying pollination recognition mechanism require further elucidation. This study provides a theoretical foundation and genetic resources for breeding maize with improved drought tolerance and high pollen viability while also offering new insights into the mechanisms of cysteine proteases.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants15050677/s1>, Figure S1: The original image of anti GST-*ZmPCP*; Figure S2: The original image of anti-HIS; Figure S3: The original image of PCR verification of *ZmPCP* in transgenic *Arabidopsis* lines; Figure S4: Germination rates of B104, *ZmPCP*-KO and

ZmPCP-OE maize seed lines; Figure S5: The original image of viability statistics of untreated pollen (I₂-KI staining); Figure S6: The original image of roots grown for 7 days under different concentrations of ABA; Table S1: Primer sequences; Table S2: RNA-seq analysis showed the expression levels of up-regulated genes in differential genes; Table S3: RNA-seq analysis showed down-regulated gene expression levels in differential genes; Table S4: Reports related to the genes with significantly differential expression in RNA-seq.

Author Contributions: H.L. and W.W. (Wei Wang) designed and supervised the project. W.W. (Wenkang Wang) performed the experiments and bioinformatics analysis. Y.L. wrote the manuscript and prepared the figures and tables and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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