Promoted ABA Hydroxylation by Capsicum annuum CYP707As Overexpression Suppresses Pollen Maturation in Nicotiana tabacum

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Abscisic acid (ABA) is a key signaling molecule that mediates plant response to stress. Increasing evidence indicates that ABA also regulates many aspects of plant development, such as seed germination, leaf development, and ripening. ABA metabolism, including ABA biosynthesis and degradation, is an essential aspect of ABA response in plants. In this study, we identified four cytochrome P450 genes (CaCYP707A1, 2, 3, and 4) that mediate ABA hydroxylation, which is required for ABA degradation in Capsicum annuum. We observed that CaCYP707A-mediated ABA hydroxylation promotes ABA degradation, leading to low levels of ABA and a dehydration phenotype in 35S:CaCYP707A plants. Importantly, seed formation was strongly inhibited in 35S:CaCYP707A plants, and a cross-pollination test suggested that the defect in seed formation is caused by improper pollen development. Phenotypic analysis showed that pollen maturation is suppressed in 35S:CaCYP707A1 plants. Consequently, most 35S:CaCYP707A1 pollen grains degenerated, unlike non-transgenic (NT) pollen, which developed into mature pollen grains. Together our results indicate that CaCYP707A mediates ABA hydroxylation and thereby influences pollen development, helping to elucidate the mechanism underlying ABA-regulated pollen development.

Keywords: ABA hydroxylation, CaCYP707A, Capsicum annuum, cytochrome P450, pollen maturation, pollen viability, seed formation

INTRODUCTION

Plant hormones regulate plant physiology and growth by governing endogenous developmental programs and plant responses to environmental signals. Abscisic acid (ABA) is an essential hormone that mediates plant defense against environmental stresses (Sah et al., 2016). For example, plants with reduced ABA response show reduced tolerance to abiotic stresses (drought, salinity, and oxidative stresses), whereas plants with enhanced ABA response exhibit improved tolerance (Verslues and Bray, 2006; Dong et al., 2011; Vishwakarma et al., 2017). In addition, increasing evidence shows that ABA is deeply involved in aspects of plant development such as germination,
ABA hydroxylation is involved in pollen maturation and seed formation. These findings suggest that CaCYP707As-mediated ABA hydroxylation is involved in pollen maturation and seed formation.

### MATERIALS AND METHODS

#### Plant Materials and Growth Conditions

*Nicotiana tabacum* cv. Xanthi-nc was used to generate 35S:CaCYP707A and NT control plants. *Capsicum annuum* L. cv. Bukang was used for isolation of CaCYP707As and for characterization of CaCYP707A expression in response to drought. All plants were grown at 22 ± 1°C with a controlled light (120 µmol m⁻² s⁻¹) cycle (16 h light/8 h dark).

#### Isolation of CYP707As and Multiple Alignment

To isolate CYP707As, total RNA extraction and cDNA biosynthesis from *C. annuum* leaves and flowers were carried out as described previously (Kim et al., 2019). The full-length CaCYP707A cDNAs were amplified by
PCR from the *C. annuum* cDNA and cloned into pGEM-T Easy Vectors (Promega, United States). The inserted sites were sequenced and aligned with their homologs from Arabidopsis and tomato using the PRALINE toolbox (Simossis and Heringa, 2005). CaCYP707A protein sequences were deduced from the nucleic acid sequences and uploaded to GenBank with the following accession numbers: CaCYP707A1 (MT198680), CaCYP707A2 (JQ828939), CaCYP707A3 (MT198681), and CaCYP707A4 (MT198682). Primer information for isolation of CaCYP707A genes is listed in Supplementary Table 1.

Subcellular Localization of CaCYP707As

**Subcellular localization analysis of CaCYP707As**

Subcellular localization analysis was carried out as previously described (Voinnet et al., 2003; Kim et al., 2016), with a slight modification. 35S:CaCYP707A-GFP plasmids were constructed by inserting full-length of CaCYP707A cDNAs into a pBI1121-GFP plasmid carrying the 35S promoter and the GFP gene; these were inserted into tobacco leaves (*Nicotiana benthamiana*) by agro-infiltration. To verify the subcellular localization of CaCYP707A-GFP, endoplasmic reticulum (ER)-targeting mCherry protein (ER-mCherry) and plasma membrane (PM)-targeting mCherry protein (PM-mCherry) plasmids were used (Nelson et al., 2007); these were co-introduced into tobacco leaves together with the 35S:CaCYP707A-GFP plasmids. After 5 days, GFP and mCherry fluorescence signals were observed with a laser-scanning confocal microscope (Leica TCS SP5, Germany). Primer information for construction of 35S:CaCYP707A-GFP is listed in Supplementary Table 1.

**Analysis of ABA Hydroxylation Activity of CaCYP707As**

Full-length CaCYP707A cDNAs were ligated to pCW vectors and introduced into the *Escherichia coli* strain Rosetta (DE3) pLysS. The expression and purification of CaCYP707As were conducted as previously described (Kim et al., 2008). The concentrations of isolated CaCYP707A proteins were determined using the co-difference spectra method (Omura and Sato, 1964). The isolated CaCYP707A proteins were incubated with substrate (0.1 mM ABA), reductase, and a NADPH-generating system (NGS) in 0.1 M KPi buffer (pH 7.4). As the reductase, CaCPR1, which was confirmed to have reductase activity in a previous study (Lee et al., 2014), was used. The NGS contained 10 mM glucose-6-phosphate, 0.5 mM NADP+, and 1 IU/ml glucose 6-phosphate dehydrogenase (Spaans et al., 2015). The negative controls were conducted without NGS solution. After a 30-min incubation at 27°C, the reaction was terminated with 25 µl of 1 N HCl. The reaction products were collected by addition of ethyl acetate (three times) and dried using a stream of nitrogen gas. Analysis of product formation was conducted using an HPLC system (Shimadzu, Japan) with a UV detector and a Gemini C18 column (4.6 mm × 150 mm, 5 µm, Phenomenex). The mobile phase solution was 0.05% acetic acid and 45% methanol in water (v/v). The flow rate was 1 ml/min, and detection was conducted at 254 nm. Primer information for insertion into pCW vectors is listed in Supplementary Table 1.

**Generation of 35S:CaCYP707A Transgenic Plants**

To construct 35S:CaCYP707A genes, full-length CaCYP707A cDNAs were inserted into the pBI1121 binary vector with the 35S promoter for overexpression of CaCYP707As. The recombinant plasmids were introduced into tobacco using *Agrobacterium tumefaciens* strain LBA4404–mediated transformation as described previously (Oh et al., 2005), with a slight modification. For transgenic plant generation, tobacco leaves (*N. tabacum* cv. Xanthi-nc) were cut into small disks and incubated with *A. tumefaciens* for 10 min, after which the explants were placed on co-culture medium. After 2 days, explants were transferred to shoot induction medium [Murashige and Skoog medium containing 3% (w/v) sucrose, 1 µg/ml zeatin, 0.01 µg/ml 1-naphthaleneacetic acid, 0.1 µg/ml gibberellic acid, 50 µg/ml kanamycin, and 250 µg/ml cefotaxime, pH 5.8] and then transferred to new medium every 2 weeks. After 6–8 weeks, the developing shoots were excised from the callus and transferred to rooting medium [Murashige and Skoog medium containing 1.5% (w/v) sucrose, 2 µg/ml 1-naphthaleneacetic acid, 50 µg/ml kanamycin, and 250 µg/ml cefotaxime, pH 5.8]. After the root induced, these regenerated plants were transferred to soil. Primer information for construction of 35S:CaCYP707A genes is listed in Supplementary Table 1.

**Phenotypic Analysis of Pollen Development**

For the analysis of pollen ultrastructure, anthers were collected from tobacco flowers at early and middle developmental stages and then were fixed and observed as described previously (Lee et al., 2017), with minor modifications. In brief, the anthers were fixed in 0.05 M sodium cacodylate buffer (pH 7.2) containing 2% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde and postfixed with 1% (w/v) osmium tetroxide (Sigma-Aldrich, United States) in the same buffer. After a series of ethanol dehydrations, each sample was embedded in LR White resin (Sigma-Aldrich, United States). For light-microscopic observation, semi-thin section samples were cut with a diamond knife at 2-µm increments in an ultramicrotome (RMC MT-X, Sigma-Aldrich, United States) and stained with 0.5% (w/v) toluidine blue O (Sigma-Aldrich, United States). These samples were visualized using an Axio Lab A1 light microscope (Zeiss, Germany). For transmission electron microscopy (TEM), ultra-thin (80- to 100-nm) section samples were sliced using the instruments named above and collected on a nickel grid (carbon-film coated, 150 mesh). These samples were stained with 4% (w/v) uranyl acetate and 0.4% (w/v) lead citrate. Images of the ultra-thin section samples were obtained using a JEM-2100F microscope/camera (Jeol Ltd., Japan). For phenotypical analysis of pollen by scanning electron microscopy (SEM), pollen at the anthesis stage of flowering were collected with carbon sticky tape and coated with platinum. The images of pollen were captured with a JSM-IT300 microscope/camera (Jeol Ltd., Japan).
**Pollen Viability Assay**

To test pollen viability, the pollen collected from flowers at the anthesis stage were incubated in staining solutions containing 12.5 μg/ml fluorescein diacetate (FDA) and 5 μg/ml propidium iodide (PI) for 5 min. Fluorescent signals (green signals for FDA; red for PI) were observed by fluorescence microscopy (Leica DM LB2, Germany).

**Quantitative PCR Analysis**

*Capsicum annuum* leaves were detached from 5-week-old plants and then air-dried (drought-stressed) for 6 h in the dark condition. Total RNA was extracted from these samples, and cDNA was biosynthesized as described previously (Kim et al., 2019). To analyze changes of CaCYP707A expression in response to drought, quantitative PCR (qPCR) analysis was conducted with using a Rotor-Gene 6000 real-time amplification system (Corbett Research, Australia). The reaction mix consisted of cDNA, gene-specific primers, and QuantiTect SYBR Green Master Mix (Qiagen, Germany). All qPCR experiments were run three times in three biological replicates. The relative expression of the target gene for each repeated experiment was calculated by the 2^{-ΔΔCt} method as described previously (Livak and Schmittgen, 2001). Primer information for qPCR is listed in Supplementary Table 1.

**Quantification of ABA Concentration**

Abscisic acid concentrations were measured as previously described (Han et al., 2012), with slight modifications. To analyze ABA concentrations in leaves and anthers, 100 mg samples of leaves were collected from 6-week-old tobacco plants and 100 mg of anthers were collected from the flowers at the anthesis stage, respectively. The collected samples were suspended in 1.5 ml of ABA extraction solution [0.45 mM butylated hydroxytoluene and 2.5 mM citric acid monohydrate in 80% (v/v) methanol] and incubated overnight at 4°C. Supernatants were collected and dried under vacuum conditions. Extracted ABA amounts were measured using an ABA ELISA quantitation kit (Agrisera, Sweden) according to the manufacturer’s instructions.

**RESULTS**

**Identification of CYP707As in *C. annuum***

Abscisic acid hydroxylation, which is involved in ABA degradation, regulates the ABA response in plants (Nambara et al., 2010; Ma et al., 2018). CYP707As mediate ABA hydroxylation (Krochko et al., 1998). Through bioinformatics approaches, we identified four CYP707A genes in the *C. annuum* genome, similar to the numbers in Arabidopsis and tomato (Kushiro et al., 2004; Nitsch et al., 2009). The four predicted *C. annuum* CaCYP707A proteins (CaCYP707A1, 2, 3, and 4) are highly conserved, as are those of Arabidopsis and tomato (Supplementary Figure 1). These findings suggest that the CaCYP707A genes encode CYP proteins that contribute to ABA hydroxylation. Because ABA is hydroxylated in ER by the activity of ER-localized CYP707As (Seco, 2014), we investigated subcellular localization of CaCYP707A1, 2, 3, and 4 by visualizing localization of GFP-fused CaCYP707A1, 2, 3, and 4 proteins. CaCYP707A1-GFP, CaCYP707A2-GFP, and CaCYP707A3-GFP signals were detected in the ER, where they co-localized with an ER marker. These findings suggest that the CaCYP707A1, 2, and 3 genes encode CYPs that are responsible for ABA hydroxylation in the ER. By contrast, CaCYP707A4 localized to the plasma membrane (Figure 1).

**ABA Hydroxylation Activity of CaCYP707As**

We next investigated whether the CaCYP707As had ABA hydroxylation activity (Figure 2). Previous studies demonstrated that CYP707As hydroxylate ABA to 8′-hydroxy ABA only in the presence of NADPH. The 8′-hydroxy ABA is spontaneously isomerized into PA, so that ABA hydroxylation by CYP707As results in production of 8′-hydroxy ABA or PA (Saito et al., 2004). When the CaCYP707A1 protein was incubated with ABA in the presence of NAPDH, 8′-hydroxy ABA and PA peaks were detected. By contrast, the peaks were not observed in NAPDH-free conditions in which CaCYP707As cannot act, indicating that CaCYP707A1 have the activity of ABA hydroxylation (Figure 2A). Similar to CaCYP707A1 proteins, CaCYP707A2, CaCYP707A3, and CaCYP707A4 proteins gave rise to 8′-hydroxy ABA and PA peaks only in the presence of NADPH, indicating that they also have ABA hydroxylation activity (Figures 2B–D). These findings indicate that the CaCYP707A genes encode CYPs responsible for ABA hydroxylation in *C. annuum*, and suggest that CaCYP707A is involved in ABA metabolism. This idea is partially supported by the result that transcriptional expression of CaCYP707A genes is regulated by abiotic stress such as dehydration conditions (Supplementary Figure 2).

**CaCYP707A Negatively Regulate Seed Formation**

To understand the function of CaCYP707A genes in plant development, we generated transgenic tobacco plants overexpressing CaCYP707A1 (35S:CaCYP707A1), CaCYP707A2 (35S:CaCYP707A2), CaCYP707A3 (35S:CaCYP707A3), and CaCYP707A4 (35S:CaCYP707A4). In the 35S:CaCYP707A transgenic plants, the ABA concentration was approximately 2–6 fold lower than that of NT grown in the same conditions for 6 weeks (Supplementary Figure 3). In addition, the transgenic plants exhibited a dehydration-like phenotype under normal growth conditions, unlike NT plants (Supplementary Figure 4). Because overexpression of CaCYP707As reduces ABA levels by promoting hydroxylation of ABA, a key hormone of stomatal closure, it was suggested that the dehydration-like phenotype of CaCYP707As-overexpressing plants might be caused by preventing ABA-induced stomatal closure.

Importantly, we observed that 35S:CaCYP707A plants showed defects in seed formation, although there was no obvious difference in floral structure between NT and 35S:CaCYP707A plants. The total number of seeds harvested...
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FIGURE 1 | Subcellular localization of CaCYP707As. Subcellular localization of CaCYP707A1, 2, 3, and 4 proteins fused to GFP was investigated by visualizing fluorescent signals. ER-specific localization of CaCYP707A1, 2, and 3 proteins and PM-specific localization of CaCYP707A4 was verified through co-localization of ER-targeting mCherry (ER-mCherry) and PM-targeting mCherry (PM-mCherry), respectively. CYP707A1, 2, 3, and 4 indicate tobacco transiently expressing 35S:CaCYP707A1-GFP, 2-GFP, 3-GFP, and 4-GFP. Green and red indicate fluorescence signals of GFP and mCherry, respectively. Scale bars = 10 \( \mu \text{m} \). More than three individuals of the indicated plants were analyzed, and the experiments were performed three times with similar results.

from NT plants was approximately 25,000. However, total numbers of seeds collected from transgenic plants grown in the same conditions were much lower; 35S:CaCYP707A1 plants produced ~800–1,400, 35S:CaCYP707A2 plants produced 2,000–4,200, 35S:CaCYP707A3 plants produced 1,000–3,000, and 35S:CaCYP707A4 plants produced 1,000–8,000 (Figure 3). The transgenic plants had produced similar or slightly lower numbers of flowers compared to wild-type plants. Consequently, the ratios of seed number and flower number in the transgenic plants were low compared to that of NT plants. This result also implies that the lower number of seeds in the transgenic plants is caused by defects in fertilization but not by defects in flower development. To explore this, we performed cross-pollination test. Seed formation in CaCYP707As-overexpressing plants was increased by cross-pollination using NT pollens. By contrast, seed formation in NT plants was decreased by cross-pollination using the pollens of CaCYP707As-overexpressing plants (Figure 4). This finding indicated that the suppressed seed formation in 35S:CaCYP707A plants is caused by pollen defects in fertilization, suggesting that CaCYP707A are possibly involved in pollen development.

CaCYP707A Genes Regulate Pollen Maturation

To understand the role of CaCYP707A genes in pollen development, NT and 35S:CaCYP707A1 anthers were collected from flowers at two different stages, and the morphology of the pollen was visualized by semi-thin sectioning and toluidine blue staining (Figure 5). In the early stage NT flowers, all pollen grains displayed a shrunken shape, and the phenotype was almost identical to that of 35S:CaCYP707A1 pollen grains. However, the morphologies of NT and 35S:CaCYP707A1 pollen in middle-stage flowers differed. In the middle-stage NT flowers, most pollen grains were mature pollen grains (MPGs) with a spherical shape and only a few pollen grains were degenerated pollen grains (DPGs) with a shrunken shape (Xu et al., 2017; Zhu et al., 2017). By contrast, in middle-stage 35S:CaCYP707A1 flowers,
FIGURE 2 | ABA hydroxylation activity of CaCYP707As. Characterization of CaCYP707A1 (A), CaCYP707A2 (B), CaCYP707A3 (C), and CaCYP707A4 (D) function in ABA hydroxylation. CYP707A1, 2, 3, and 4 proteins expressed in E. coli were incubated with ABA in the presence (left; +NGS) or absence (right; –NGS) of a NADPH-generating system, and the catabolites were analyzed by HPLC. The peaks marked by red and blue arrows are 8’-hydroxy ABA (8’-OH) and phaseic acid (PA), respectively, whereas the peaks marked by black arrows are abscisic acid (ABA). The experiments were performed three times with similar results.
most pollen grains were DPGs, and a few pollen grains showed an intermediate phenotype between those of DPGs and MPGs. These findings indicate that 35S:CaCYP707A1 transgenic plants have defects in pollen maturation, suggesting that overexpression of CaCYP707A1 negatively regulates maturation of pollen.

To further explore their maturation, we examined the ultrastructure of 35S:CaCYP707A1 pollen by TEM (Figure 6). Similar to the results from light microscopy, we observed morphological differences between NT and 35S:CaCYP707A1 pollen only in the middle-stage flowers with mature pollen grains, but not in early stage flowers with immature pollen grains. Notably, TEM analysis showed that ER development, which is tightly linked to pollen maturation (Kim et al., 2011), was different between NT and 35S:CaCYP707A1 pollen in middle-stage flowers. In middle-stage flowers, we observed well-developed ER only in NT pollen, but not in 35S:CaCYP707A1 pollen. However, this difference in ER formation was not observed between NT and 35S:CaCYP707A1 pollen in early stage flowers (Figure 6). These findings support the conclusion that overexpression of CaCYP707A1 suppresses pollen maturation.

CaCYP707As Negatively Regulate Pollen Viability

To further test the hypothesis that CaCYP707A1 negatively regulates maturation of pollen, we analyzed the morphologies of the NT and CaCYP707A1-overexpressing plant pollen at the anthesis stage using SEM. In NT plants, most pollen grains had similar size and shape. The 35S:CaCYP707A1 flowers formed a few pollen grains whose phenotype (size and shape) was similar to that of the NT pollen; however, most pollen in the transgenic flowers was smaller and more irregular compared to the pollen of NT flowers (Figure 7). This SEM result supports the TEM result showing that maturation of pollen is defective in 35S:CaCYP707A1 plants. In addition, the result showing that ABA concentration in the anthers of 35S:CaCYP707A1 plants is much lower than that of NT plants (Supplementary Figure 5) suggests that CaCYP707A-promoted ABA hydroxylation inhibits pollen maturation. Similar to CaCYP707A1, overexpression of CaCYP707A2, CaCYP707A3 or CaCYP707A4 also suppressed pollen maturation (Supplementary Figure 6), further supporting the function of CaCYP707As in pollen development.

To address how the defect in pollen maturation leads to the inhibited seed formation in 35S:CaCYP707As plants, we tested pollen viability in NT and 35S:CaCYP707A1 plants. To do this, we stained pollens at the anthesis stage using FDA and PI to visualize viability (Figure 8). In this assay, viable pollen exhibit green fluorescence signals upon FDA staining, whereas non-viable pollen display red fluorescence signals upon PI staining. In NT plants, most pollen displayed green fluorescence signals upon FDA staining. In contrast to the NT
plants, most pollen from 35S:CaCYP707A1 plants showed red fluorescence signals upon PI staining, whereas only a small portion of the pollen exhibited green fluorescence signals (Figures 8A,B). We obtained nearly identical results for the pollen viability of plants expressing CaCYP707A2, 3, and 4. Most pollen in 35S:CaCYP707A2, 3, and 4 plants displayed red signals corresponding to PI, and only a few pollen exhibited green signals corresponding to FDA (Figures 8C–E). When we determined the ratio of the number of pollen with FDA signals to the number of pollen with PI signals, there was an obvious difference in pollen viability (Figure 8F). The ratios of the transgenic plants were much lower than those of the NT controls. This finding indicates that CaCYP707A-promoted ABA hydroxylation negatively regulate pollen viability, explaining why seed formation was inhibited in 35S:CaCYP707A plants.

**DISCUSSION**

The plant CYPs are the third largest gene family in higher plants (Nelson and Werck-Reichhart, 2011). Unlike bacterial CYPs, most plant CYPs with transmembrane domains are mainly located at the ER, and some are predicted to be located at the membranes of other organelles such as PM and plastids (Schuler et al., 2006; Bak et al., 2011; Yin-Ping et al., 2019). CYPs act as monooxygenases, and their activity is regulated by NADPH and NADPH-cytochrome P450 reductase (CPR), which provide electrons to CYPs (Jensen and Moller, 2010; Bak et al., 2011). In plants, CYPs are involved in plant growth and physiology, mediating a variety of metabolic processes, including metabolism of hormones such as auxin, cytokinin, and ABA (Kim and Tsukaya, 2002; Mizutani, 2012). In this study, we identified four CYP707As (CaCYP707A1, 2, 3, and 4) in C. annuum and
characterized their function in ABA degradation. Production of 8′-hydroxy ABA is a key step in ABA degradation (Saito et al., 2004), and our enzymatic assay showed that the CaCYP707As mediate production of 8′-hydroxy ABA from ABA only in the presence of NADPH. In addition, overexpression of CaCYP707A genes reduced ABA levels and induced a dehydration phenotype, indicating that the CaCYP707As mediate ABA degradation by controlling hydroxylation of ABA.

In this study, we observed that seed formation is suppressed by overexpression of CaCYP707A genes. The total numbers of seeds formed in 35S:CaCYP707A1 plants were ~20-fold lower than those of NT plants, and the defective phenotype was also observed in other transgenic plants overexpressing CaCYP707A2, 3, and 4. This indicates that CaCYP707A1-mediated ABA degradation regulates seed formation. Previous studies using ABA biosynthesis mutants support this finding. ABA-deficient mutant aba2-1 shows suppressed ABA response and forms fewer seeds per siliquae in Arabidopsis (Sharma and Verslues, 2010; Cheng et al., 2014), and a knockdown mutant of NCED, which is responsible for ABA biosynthesis, also exhibits lower levels of ABA than NT control plants, leading to fewer seeds per fruit (Sun et al., 2012). Together, these findings suggest that CaCYP707A-mediated degradation of ABA induces defects in seed formation, and the cross-pollination result showing that NT pollen rescued the defective seed phenotype in 35S:CaCYP707A plants further indicates that the defective seed phenotype is due to defects in pollen development in CaCYP707A-overexpressing plants.

A recent study by Dai et al. (2018) using NCED suggested that ABA is involved in pollen development in tomato. In that study, the authors showed that RNAi gene silencing of NCED reduced the ABA levels in the RNAi transgenic tomato and suppressed pollen development. Interestingly, overexpression of NCED also
FIGURE 7 | Overexpression of CaCYP707A1 promotes formation of immature pollen. Pollen maturation was investigated by SEM. Pollen of NT and 3SS:CaCYP707A1 (line # 1) were collected from the anthesis stage of flowers (left), and morphological patterns of the pollen were captured by SEM (right). Flw and CYP707A1 indicate flowers and 3SS:CaCYP707A1 plants, respectively. Scale bars = 1 cm in flowers and 20 µm in SEM images. Two individuals of the indicated plants were analyzed, and the experiments were performed two times with similar results.

FIGURE 8 | Suppressed pollen viability in 3SS:CaCYP707A plants. Pollen viability was visualized by FDA and PI staining. Pollen collected from the flowers (anthesis stage) of NT and 3SS:CaCYP707A transgenic plants were stained with a mixture of FDA and PI for 5 min, and then fluorescent signals were observed by fluorescence microscopy [A], NT; [B], CaCYP707A1 #1; [C], CaCYP707A2 #1; [D], CaCYP707A3 #1; and [E], CaCYP707A4 #1. More than three individuals of the indicated plants were analyzed with similar results. [F] Quantification of the ratio of FDA (green) to PI (red) signals in the indicated plants (n > 3). The values are the means of three biological replicates, and error bars are S.D. #1 and #2 indicate individual lines of the indicated transgenic plants. Asterisks indicate significant differences between the transgenic plants and NT controls (Student t-test p < 0.01). Scale bars = 100 µm. The experiments were performed at least three times with similar results.
suppressed ABA levels, suggesting that pollen development is sensitive to ABA homeostasis. A study by Saini and Aspinall (1982) showing that exogenous ABA affects pollen development partially supports this hypothesis. In our study, we traced pollen development with age, and found that maturation of immature pollen grains is suppressed in 35S:CaCYP707A transgenic plants. This indicated that overexpression of CaCYP707A genes suppresses pollen maturation, and ultra-thin sectioning and TEM results visualizing ER formation further supported this finding. Establishment of the intracellular membrane network during pollen maturation is characterized by ER development (Rodriguez-Garcia et al., 1995; Piffanelli et al., 1998). Therefore, the TEM results that middle-stage 35S:CaCYP707A1 pollen had a poorly developed ER unlike the same developmental stage of NT pollens with a well-developed ER support the hypothesis that CaCYP707A negatively regulates pollen maturation. In addition, our SEM and pollen viability results show that the defect in pollen maturation affects pollen development and pollen viability. NT pollen grains at the anthesis stage had similar size and shape, and also were viable, whereas pollen grains of 35S:CaCYP707A1 plants were irregular in shape and non-viable. Additionally, in response to exogenous ABA treatment, pollen maturation in NT and CaCYP707A1-overexpressing plants tended to be reduced and enhanced, respectively (Supplementary Figure 7). This indicates that overexpression of CaCYP707A1 suppressed by promoting ABA hydroxylation, and also suggests that ABA homeostasis is involved in pollen maturation. This finding is partially supported by a previous study by Dai et al. (2018), which shows that ABA homeostasis affects pollen development (Dai et al., 2018). Together with the results showing that CaCYP707A1 overexpression reduced ABA levels in anthers, these findings suggest that overexpression of CaCYP707As suppresses pollen maturation and seed formation by promoting ABA hydroxylation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

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AUTHOR CONTRIBUTIONS

HMK and YHJ planned and designed all aspects of the research. HMK and CHY analyzed the data and provided technical guidance in enzyme assay experiments. HMK and SHM, and SYP participated phenotypical analysis of plant. HMK, GJ, and YHJ performed the data interpretation and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.583767/full#supplementary-material

Supplementary Figure 1 | Multiple alignment of CaCYP707As amino acid sequence.

Supplementary Figure 2 | CaCYP707As expression in response to drought.

Supplementary Figure 3 | ABA concentration of CaCYP707As transgenic plant leaves.

Supplementary Figure 4 | Dehydration phenotype of CaCYP707As transgenic plants.

Supplementary Figure 5 | ABA concentration of CaCYP707A1 transgenic plant anther.

Supplementary Figure 6 | Morphological patterns of CaCYP707A2, 3, and 4 transgenic plant pollen.

Supplementary Figure 7 | Pollen maturation of CaCYP707A1 transgenic plant under ABA treatment.

Supplementary Table 1 | A list of primer sequences used in this study.
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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