RESEARCH ARTICLE

Caffeoyl Shikimate Esterase has a Role in Endocarp Lignification in Peach (*Prunus persica* L.) Fruit

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Abstract

Caffeoyl shikimate esterase (CSE) is a key enzyme in lignin synthesis in *Arabidopsis thaliana*. To determine the role of CSE in lignification of the endocarp in peach (*Prunus persica* L.) fruit, we cloned and characterized the *P. persica* CSE homolog, which we designated PpCSE. The 954 - bp PpCSE gene encoded a 317 - amino acid polypeptide. PpCSE expression patterns in the mesocarp and endocarp changed during peach fruit development. There was no significant difference between the expression levels of PpCSE in the mesocarp and endocarp at 39 and 44 days after full bloom (DAFB), but the expression level of PpCSE in the endocarp at 50 and 55 DAFB was 80.73 and 72.75 times higher, respectively, than that in the mesocarp. During peach fruit development, PpCSE expression in the endocarp increased rapidly; the relative PpCSE expression level at 50 DAFB was 122.70 times higher than that at 39 DAFB. At the protein level, CSE was detected in the peach fruit endocarp at 50 and 55 DAFB. Our study suggests that PpCSE expression during peach fruit development is closely related to the degree of endocarp lignification.

Additional key words: immunoblotting, fruit development, gene cloning, lignin deposition, protein expression analysis

Introduction

Peach (*Prunus persica* L.), a member of the Rosaceae family, produces drupes (indehiscent fruit) consisting of three parts: exocarp (skin), mesocarp (fleshy fruit), and endocarp (hard outer covering encasing the seed). The hardened endocarp provides a physical barrier around the seed, protecting it from disease and herbivory (Doster and Michailides, 1999). Currently, pit - splitting is a serious concern for peach fruit cultivators because it renders the fruit vulnerable to pests and diseases, reduces the quality and taste of the fruit, and makes storage, transportation, and processing more difficult. Pit - splitting is a phenomenon with endocarp splitting. During the middle stage of fruit development, the

crack appeared in endocarp of pit - splitting fruit, white callus could be found in the endocarp, although no change can be found in appearance. In a previous study, we demonstrated that the endocarp of splitting fruit was not completely lignified (Shi *et al.*, 2013). However, in the endocarp of normal peach fruit, lignin is synthesized at a high level and accumulates to a greater degree than in other lignified tissues (Koukios *et al.*, 2005; Mendu *et al.*, 2011, Dardick *et al.*, 2010).

Lignin is a complex polymer formed by the coupling of oxidized monolignols that mainly serves to keep glycan polymers in place in cell walls, producing thickened and lignified walls that confer mechanical strength and enable plants to stand upright (Amthor, 2003; Baucher *et al.*, 1998; Chen *et al.*, 2007; Simmons *et al.*, 2010; Sticklen, 2008). The complete process of lignin synthesis is not fully understood. Vanholme *et al.* (2013) assessed gene expression in *Arabidopsis thaliana* and demonstrated the key role of caffeoyl shikimate esterase (CSE) in lignin biosynthesis. CSE can hydrolyze caffeoyl shikimate to generate caffeate and shikimate, thus performing an important role in lignin biosynthesis. *Arabidopsis* mutants lacking CSE retain their ability to grow; however, their lignin content is decreased to 36% of that of normal plants. *Arabidopsis* mutants lacking CSE show no sagging or lodging, although nutrient transport is impaired and vascular contraction occurs. When specimens of *Arabidopsis* mutants lacking CSE were dried, chopped, and processed, they were found to contain 300% more cellulose than normal plants.

In this study, we investigated the molecular basis of lignification in the endocarp of the peach cultivar 'Okubao' to. 'Okubao' originated in Japan and is popular in China. 'Okubao' is a late maturing variety, pit - splitting phenomena of fruit is not common in cultivation. Because significant lignin accumulation occurs in the endocarp and CSE is the key enzyme in lignin biosynthesis, we cloned the CSE homolog in the drupe of peach and determined its expression at the mRNA and protein levels by quantitative PCR (qPCR) and immunoblotting. The results presented in this study illuminate the function of CSE in lignification of the peach endocarp and provide a theoretical foundation for future studies aimed at elucidating the molecular mechanism of endocarp lignification and resolving pit - splitting in peach fruit.

Materials and Methods

Plant Materials

Three neighboring peach trees (*P. persica* cv. Okubao) located in the experimental orchard of Beijing University of Agriculture (Changping District, Beijing, China) were the source of the fruit used in this study. Drupes were harvested from each tree at 24, 29, 34, 39, 44, 50, 55, and 60 days after full bloom (DAFB). Intact drupes were randomly divided into two groups consisting of ten drupes each. The endocarps and mesocarps of one group of drupes were separated, cut into small pieces, pooled, packaged, frozen in liquid nitrogen, and stored at –80°C for further RNA extractions and protein analyses. The other group of drupes was used for to measure lignin content and observe lignin deposition.

Observation of Lignin Deposition and Determination of Lignin Content

Lignin deposition was observed using a phloroglucinol - HCl reagent (Abeles and Biles, 1991). Each drupe was cut along the ventral suture and covered completely with phloroglucinol solution [5% phloroglucinol, 85% ethanol (v / v)] for approximately 30 min, after which the section was exposed to 1 M HCl. Tissue containing lignin was identified by its pink color after exposure to 1 M HCl. The tissue sections were observed and photographed. Lignin content was measured according to the method of Kirk and

Obst (1988).

RNA Extraction and cDNA Synthesis

Total RNA was extracted using the EASYspin Plant RNA Rapid Extraction Kit according to the manufacturer's instructions (Beijing Bomaide Co., Beijing, China). First - strand cDNA was synthesized from 4 μg total RNA using the SuperScript III First - Strand Synthesis System (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. Reverse transcription was performed at 50°C for 50 min using oligo (dT) primers in a volume of 20 μL.

Cloning of *CSE*

Arabidopsis CSE amino acid sequences (http://www.ncbi.nlm.nih.gov/protein/Q9C942.1) were used as querying probes to perform BLAST searches of the *P. persica* genomic database with Phytozome v. 10.2 (http://phytozome.jgi.doe.gov/pz/portal. html). The CSE amino acid sequence with the highest similarity and lowest e - value was selected. The predicted coding sequence (CDS) of CSE in *P. persica* (ppa008845m) was highly homologous with the Arabidopsis CSE. Based on the predicted CDS of the CSE in *P. persica*, specific primers (5′ - CGGAAAAGCGATCAAGGAC - 3′ and 5′ - CTGTATGTACTGGCACACCCT - 3′) were designed and synthesized by Sangon Biotech (Beijing, China).

PCR was carried out in a 20 μL reaction volume using a Bio - Rad Thermocycler. The PCR products were separated by 1.0% agarose gel electrophoresis. The target DNA fragment was purified with the Axyprep DNA Gel Extraction Kit (Aygen Bioscience, Union City, CA, USA), ligated into the pMD - 19T vector, and transformed into DH5α competent cells. Positive clones were identified by PCR and sequenced by Sangon Biotech (Beijing, China).

Phylogenetic Analysis

For phylogenetic analysis, we searched for homologous *CSE* genes in other species using BLAST online (https://blast.ncbi.nlm. nih.gov/Blast.cgi) Multiple protein sequences were aligned by DANMAN using default parameters (Thompson *et al.*, 1994). A phylogenetic tree was generated using MEGA5.0 (Tamura, 2011). The bootstrap value shown next to the branches were tree - inferred from 1,000 replicates. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

PpCSE Gene Expression Assays

qPCR was performed using a C1000TM Thermal Cycler (Bio - Rad, Hercules, CA, USA) with SYBR Green detection chemistry. Gene - specific primers (5′ - CGGAAAAGCGATCAAGGAC - 3′ and 5′ - CTGTATGTACTGGCACACCCT - 3′) were designed for qPCR. Translation elongation factor EF - 1 alpha (TEF2, gi: 852415) was used as a reference gene (specific primers: 5′ - GTTGCCTT - GGTCGGTCTTGA - 3′ and 5′ - ATTGAACAGCAACACGCACAA - 3′).

Preparation of Protein Extracts

Crude protein extracts were produced from peach drupe endocarps and mesocarps by the method of Deng *et al.* (1997). Protein concentrations were determined using a Non - Interfering Protein Assay Kit (Calbiochem, Millipore Co., Billerica, MA, USA) with bovine serum albumin (BSA) as a standard.

PpCSE Protein Expression Assays

A 19 - amino acid peptide (13 - MPEEDYYTSQGVRNTKSFC - 32) and a 17 - amino acid peptide (301 - CDMREWIDERVERY - GPK - 317) were commercially synthesized, purified by HLPC, verified by mass spectrometry. The purified peptide was coupled with KLH (keyhole limpet hemocyanin) and used by BGI labs (Beijing, China) to inoculate rabbits, from which affinity - purified (protein G column) antibodies were obtained. The purified antibodies were designated as anti - PpCSE antibodies.

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS - PAGE) and Immunoblotting

The samples were prepared for SDS - PAGE by standard methods. Briefly, samples were electrophoresed in 12% Precise Protein Gels, transferred onto nitrocellulose membranes in a Mini Trans - Blot Cell (Bio - Rad), and reacted with the anti - PpCSE antibodies at a dilution of 1:1,000 (BGI Labs, Inc., Beijing, China). The appropriate level of dilution was determined by a titration experiment. Pre - immune serum was used as a control. Anti - mouse alkaline phosphatase - labeled secondary antiserum was used for PpCSE detection at a 1 : 5,000 dilution (Sigma - Aldrich, St. Louis, MO, USA) using the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Beijing). Beta - actin was used as a reference protein.

Results

Lignification in the Peach Drupe Endocarp

To detect lignin deposition, dissected peach drupes were stained with phloroglucinol - HCl, which causes tissue containing lignin to turn pink. The lignin deposition process is shown in Fig. 1A - E. No obvious color reaction was detected at 29 DAFB (Fig. 1A). Punctate staining began to increased from 34 to 39 DAFB. The extent of lignification was significant at 44 DAFB; the tissue around the vascular bundle showed lignin deposition, while a rapid expansion of color was detected from the seed cavity to the peripheral region of the endocarp (Fig. 1D). At 50 DAFB, parenchyma cells were filled with lignin, indicating that a large amount of lignin was deposited in the endocarp. At 50 DAFB, the lignification process was complete and the endocarp was difficult to cut using normal tools. During the detection period, the phloem of the vascular bundle (arrows in Fig. 1E), which transports nutrients, was not stained,

We determined the lignin content of the endocarp throughout fruit development (Fig. 1F). Lignin content increased gradually from 24 to 60 DAFB, consistent with the results of the staining experiment described above.ai

Cloning and Analysis of PpCSE

A CSE homolog was cloned from the fruit of *P. persica* and designated PpCSE The nucleotide sequence of PpCSE was 954 bp in length and encoded a protein of 317 amino acids with a theoretical isoelectric point (pI) of 6.5 and a theoretical molecular weight of 35.8 kDa. The characteristics of PpCSE were similar to those of the *Arabidopsis* CSE homolog.

The predicted amino acid sequence of PpCSE was aligned with the CSE homologs from other species (Fig. 2). The *A. thaliana* CSE sequence reported in functional studies and that of *Malus domestica* were used for comparison. According to BLASTP analysis of sequence identity (Fig. 2), the analysis of the PpCSE amino acid sequence showed that the protein had no transmembrane domains and no signal peptide in the coding region.

A phylogenetic tree was constructed with PpCSE and CSE homologs from *A. thaliana*, *P. mume* (plum), and other
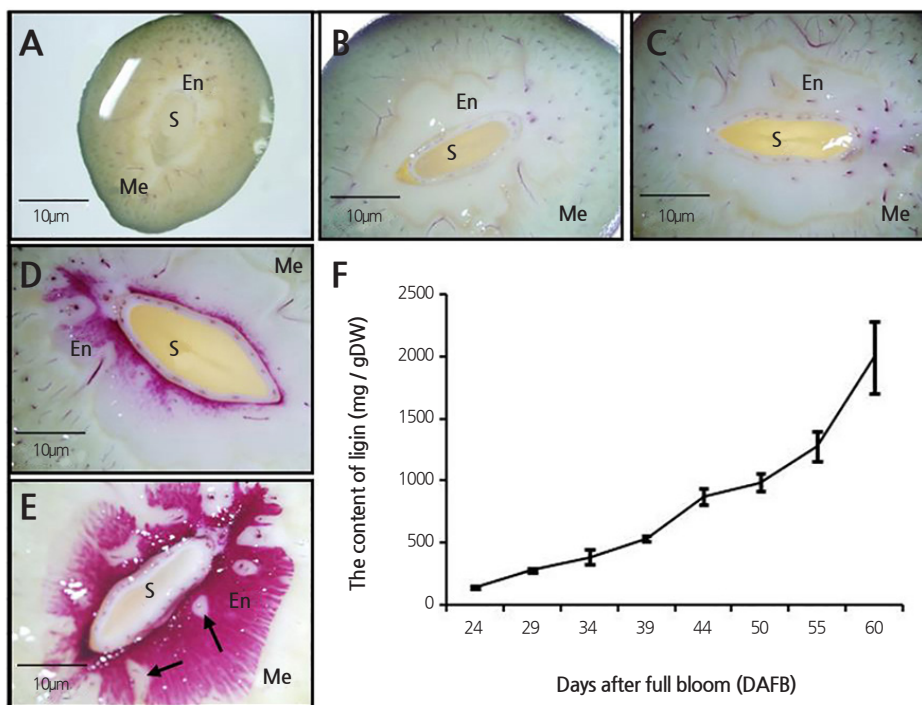
Fig. 1. Lignin deposition in developing peach fruit and quantitative determination of lignin content in the peach endocarp. (A) At 29 DAFB, the dissected peach fruit showed scattered punctate staining with phloroglucinol; (B) at 34 DAFB, the number of pink dots increased; (C) at 39 DAFB, pink dots were observed inside the endocarp; (D) at 44 DAFB, pink staining in the tissue of the endocarp around the seed increased; (E) at 50 DAFB, the endocarp tissue was highly stained. In the vascular bundle, the xylem was lignified but the phloem was not. (F) Quantitative determination of lignin content. Data are the means ± SE (n = 3). S: seed; En: endocarp; Me: mesocarp. Arrows indicate the phloem of vascular bundle.

species. As shown in Fig. 3, the CSE homologs of *P. persica* and *P. mume* were in the same branch of the phylogenetic tree and were the most similar of the homologs, with 99% sequence similarity. The CSE homologs from *Pyrus × bretschneideri* (pear) and *M. domestica* were located in another branch and were 91% and 90% similar with PpCSE, respectively.

Comparison of PpCSE Gene Expression in the Mesocarp and Endocarp of Peach Fruit

The relative expression level of PpCSE in peach fruit was determined by qPCR. PpCSE was expressed in the mesocarp and endocarp of peach fruit (Fig. 4). PpCSE expression was not detected before 39 DAFB. No significant differences between the expression levels of PpCSE in the mesocarp and endocarp appeared before 44 DAFB. At 50 and 55 DAFB, PpCSE expression was significantly increased in the endocarp. The expression level of PpCSE in the endocarp at 50 and 55 DAFB was 80.73 and 72.75 times higher than that of the mesocarp, respectively. At 50 and 55 DAFB, the relative expression level of PpCSE in the endocarp was 122.70 and 115.76 times higher than that at 39 DAFB, respectively. In the mesocarp, the relative expression level of PpCSE at 44 DAFB was 14.16 times higher than that measured at 39 DAFB, but was far lower than that of the endocarp at the same time point. There was a strong positive correlation between PpCSE expression and lignin content (Fig. 1), which was greatest at 50 and 55 DAFB. At 60 DAFB, the endocarp was too hard to cut for gene expression assays.
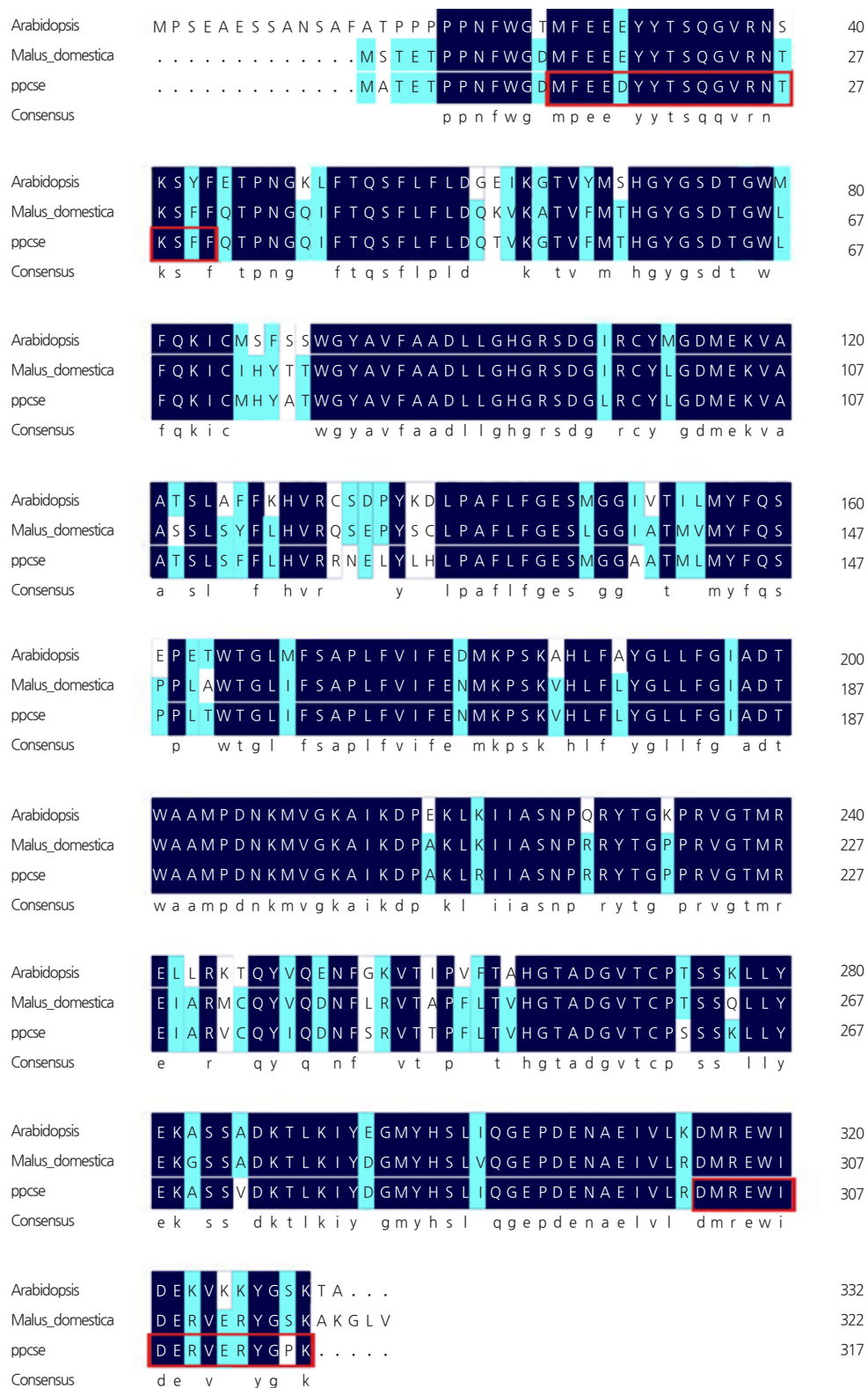
Fig. 2. Multiple sequence alignment analysis of the CSE homologs from *Arabidopsis thaliana*, *Malus domestica*, and *Prunus persica*. Red boxes represent the sequences used in antibody production.
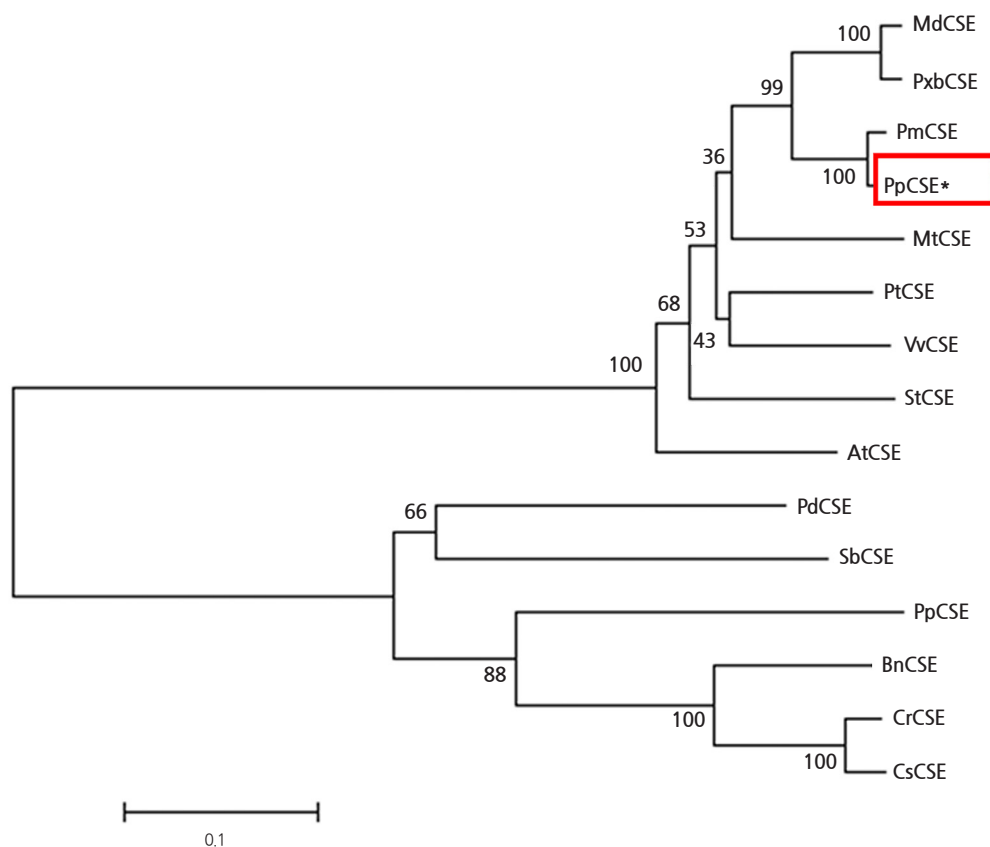
Fig. 3. Phylogenetic analysis of PpCSE and CSE homologs from different species. Accession numbers used to build the tree are as follows: *Malus domestica*: *MdCSE*, XP_0083776061; *Pyrus × bretschneideri*: *PxbCSE*, XP_009348432; *Prunus mume*: *PmCSE*, XP_008239016; *Prunus persica*: PpCSE, XP_007210524; *Medicago truncatula*: *MtCSE*, XP_003610038; *Populus trichocarpa*: *PtCSE*, XP_002298118; *Vitis vinifera*: *VvCSE*, XP_002285258; *Solanum tuberosum*: *StCSE*, XP_006341666; *Arabidopsis thaliana*: *AtCSE*, XP_175685; *Phoenix dactylifera*: *PdCSE*, XP_008792151; *Sorghum bicolor*: *SbCSE*, XP_002463034; *Prunus persica*: PpCSE, XP_007211531; *Brassica napus*: *BnCSE*, XP_013746612; *Capsella rubella*: *CrCSE*, XP_006303706; *Camelina sativa*: *CsCSE*, XP_010500894. Red boxes represent the name of CSE gene cloned in peach. Numbers at tree nodes indicate the genetic distances in substitutions per site. The scale bar indicates the average number of amino acid substitutions per site.

Comparison of PpCSE Protein Expression in the Mesocarp and Endocarp of Peach Fruit

Fig. 5 shows the results of an immunoblot analysis using an antibody raised against a 19 - amino acid sequence of the predicted N - terminal region of PpCSE (Fig. 2). The target band containing the PpCSE protein was found to have a size of 31.7 kDa. PpCSE protein was not detected in the mesocarp at any developmental stage. In the endocarp, the expression level of PpCSE increased during development. The PpCSE expression level in the endocarp was highest at 50 and 55 DAFB, indicating that the pattern of PpCSE expression was closely related to lignin deposition in the endocarp, which coincided well with the results of gene expression.
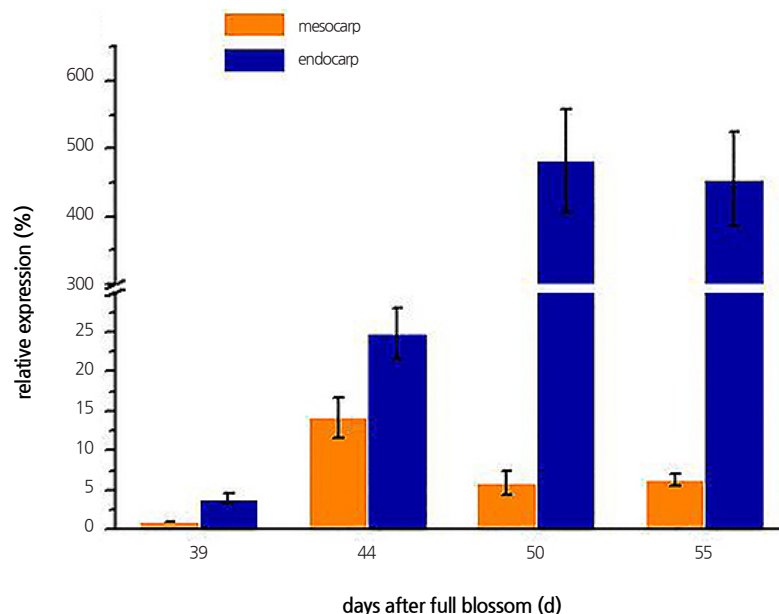
Fig. 4. PpCSE expression during peach fruit development. No significant differences were observed between the expression levels of PpCSE in the mesocarp and endocarp before 44 DAFB. At 50 and 55 DAFB, PpCSE expression was significantly increased in the endocarp. PpCSE expression in the endocarp increased rapidly with fruit development; the relative PpCSE expression level at 50 DAFB was 122.7 times higher than that at 39 DAFB.
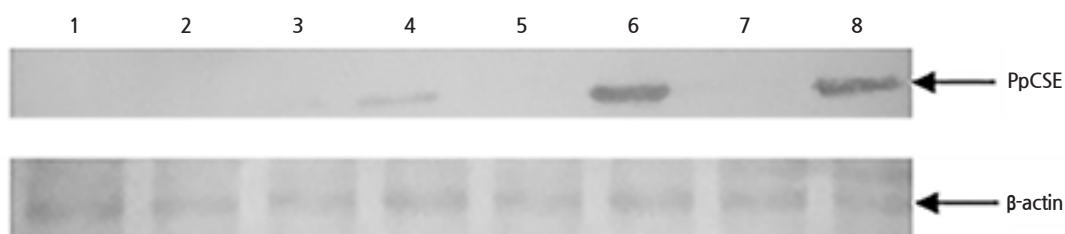

Fig. 5. Immunoblot analysis of PpCSE protein in different tissues during peach fruit development. Bands 1, 3, 5, and 7 show the expression level of PpCSE in the mesocarp at 39, 44, 50, and 55 DAFB, respectively. Bands 2, 4, 6, and 8 show the expression level of PpCSE in the endocarp at 39, 44, 50, and 55 DAFB, respectively.

Discussion

Lignin is a complex phenylpropanoid polymer that includes three types of monomers: syringyl lignin (S), guaiacyl lignin (G), and hydroxyl - phenyl lignin (H). At present, it is generally believed that synthesis of monolignol monomers is divided into three processes: shikimate synthesis, phenylpyruvic acid synthesis, and lignin monomer formation. The phenylpyruvic acid pathway includes the reaction from phenylalanine to hydroxy - cinnamic acid, which then forms a compound with coenzyme A. During this pathway, ρ - coumarate is the substrate for the formation of caffeoyl - CoA, which is converted to ρ - coumaroyl - CoA by 4 - coumarate - CoA ligase (4 - CL). After this step, caffeoyl - CoA is generated from ρ - coumaroyl shikimate and caffeoyl shikimate by hydroxycinnamoyltransferase (HCT). Caffeoyl - CoA is the starting material for the

synthesis of the G and S monolignol monomers (Douglas, 1996; Marie *et al.*, 1998). In *Arabidopsis*, recent studies have demonstrated the important function of CSE in the process of lignin formation (Vanholme *et al.*, 2013). The activities of CSE and 4 - CL enable the reversion reaction catalyzed by HCT to be bypassed, leading to the formation of caffeoyl - CoA. *Arabidopsis* mutants lacking CSE show an altered morphology, confirming the crucial role of CSE in the formation of the G and S lignin monomers (Vanholme *et al.*, 2013).

CSE orthologs have been identified in several plants, including *Populus trichocarpa*, *Eucalyptus grandis*, *Oryza sativa*, and *Panicum virgatum* (Wang *et al.*, 2014). CSE could represent a new target for modulating the recalcitrance of lignocellulosic biomass to hydrolysis. Escamilla - Treviño *et al.* (2014) suggested that the classical two - step involvement of HCT in the shikimate shunt is unlikely to occur in switchgrass (*Panicum virgatum*) for the G and S monolignol biosynthesis, and that a more likely pathway will involve a switchgrass ortholog of CSE. In this pathway, CSE hydrolyzes caffeoyl shikimic acid to caffeic acid, which in turn can be converted to caffeoyl - CoA by 4 - coumaric acid:coenzyme A ligase (4CL). The conversion of caffeoyl shikimic acid to caffeoyl - CoA by HCT may be bypassed in this pathway. Recent research further identified CSE as a critical enzyme for normal lignification in Medicago truncatula, *Populus deltoids*, and *Panicum virgatum*. However, the model grass *Brachypodium distachyon* and corn (*Zea mays*) do not possess orthologs of the currently characterized *CSE* genes (Ha *et al.*, 2016). Future studies should determine whether the CSE - mediated pathways have distinct tissue and cell type - specific biological roles in plant lignification.

Enzymes associated with lignin biosynthesis are activated during early development in peach fruit. Transcriptome studies showed that expression levels of phenylalanine ammonia - lyase (PAL), 4 - coumarate:CoA ligase (C4H), caffeoyl - CoA O - methyltransferase (CCoAOMT), and POX (Peroxidases) were increased during the process of lignin accumulation in peach endocarps (Dardick *et al.*, 2014). In a previous study, we showed that the expression level of PAL was up - regulated in the peach endocarp during lignification (Hu *et al.*, 2011) and an earlier study demonstrated the key role of PAL in lignin biosynthesis (Schuster and Rjetey, 1995). In the present study, a *cse* gene was cloned from the peach endocarp and designated PpCSE. The expression levels of PpCSE at the mRNA and protein levels varied by tissue type and developmental stage and were closely associated with the degree of lignin accumulation in the peach endocarp, suggesting the central role of CSE in endocarp lignification in peach fruit. Our preliminary results demonstrate that CSE is a key enzyme involved in the lignification process in the peach endocarp and provide important clues to explore the monolignol biosynthesis pathway in peach.

Han *et al.* (2015) showed that pit - splitting in peach fruit was related to weakening of the endocarp. Peach cultivars that have rapid fruit growth before the endocarp has completely hardened are more likely to show pit - splitting. Tani *et al.* (2007) found that the expression of *SHATTERPROOF* (SHP) in a pit - splitting - resistant variety was lower during lignification. The factors contributing to abnormal endocarp development and pit - splitting are complex and include gene expression, planting methods, and climate. Future studies should aim to reveal the influences of specific genetic mechanisms and environmental factors on pit - splitting.

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