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The developmental dynamics of the sweet sorghum root transcriptome elucidate the differentiation of apoplastic barriers

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ABSTRACT

Apoplastic barriers in the endodermis, such as Casparian strips and suberin lamellae, control the passage of water and minerals into the stele. Apoplastic barriers are thus thought to contribute to salt exclusion in salt-excluding plants such as sweet sorghum (*Sorghum bicolor*). However, little is known about the genes involved in the development of the apoplastic barrier. Here, we identified candidate genes involved in Casparian strip and suberin lamella development in the roots of a sweet sorghum line (M-81E). Three distinct developmental regions (no differentiation, developing, and mature) were identified based on Casparian strip and suberin lamella staining in root cross sections. Sequencing of RNA extracted from these distinct sections identified key genes participating in the differentiation of the apoplastic barrier. The different sections were structurally distinct, presumably due to differences in gene expression. Genes controlling the phenylpropanoid pathway, fatty acid elongation, and fatty acid ω-hydroxylation appeared to be directly responsible for the formation of the apoplastic barrier. Our dataset elucidates the molecular processes underpinning apoplastic barrier development and provides a basis for future research on molecular mechanisms of apoplastic barrier formation and salt exclusion.

Abbreviations: SHR, SHORTROOT; MYB, MYB DOMAIN PROTEIN; CIFs, Casparian strip integrity factors; CASP, Casparian strip domain proteins; PER, peroxidase; ESB1, ENHANCED SUBERIN1; CS, Casparian strip; RPKM, reads per kilobase per million reads; DEGs, differentially expressed genes; FDR, false discovery rate; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; RNA-seq, RNA sequencing; PAL, phenylalanine ammonia-lyase; CYP, cytochrome P450 monooxygenases; 4CL, 4-coumarate-CoA ligase; AAE5, ACYL-ACTIVATING ENZYME5; CCR, cinnamoyl CoA reductase; TKPR, TETRAKETIDE ALPHA-PYRONE REDUCTASE1; CAD, cinnamyl alcohol dehydrogenase; HST, shikimate *O*-hydroxycinnamoyltransferase; PMAT2, PHENOLIC GLUCOSIDE MALONYLTRANSFERASE2; CCOAOMT, caffeoyl-CoA *O*-methyltransferase; KCS, β-ketoacyl-CoA synthase; CUT1, CUTICULAR PROTEIN1; DET2, 5-alpha-reductase; TAX, 3′-*N*-debenzoyl-2′-deoxytaxol *N*-benzoyltransferase; CER1, ECERIFERUM1; FAR, fatty acyl reductase; AF-CoA, alcohol-forming fatty acyl-CoA reductase; ABCG, ATP-binding cassette, subfamily G; ERF, ethylene-responsive transcription factor; HSF, heat stress transcription factor; NTF, NUCLEAR TRANSCRIPTION FACTOR Y SUBUNIT B-5; GPAT, glycerol 3-phosphate acyltransferase.

Introduction

Sweet sorghum [*Sorghum bicolor* (L.) Moench], a variant of sorghum, has a rapid growth rate, high sugar content, and produces high amounts of biomass even when grown under environmental stresses such as salt and flooding.[1] Sweet sorghum is also considered an economically important crop because it can be used to produce biofuels such as ethanol,[2] in addition to being useful as feed for livestock.[1] The yields of most crops are severely reduced in saline environments; however, sweet sorghum can grow well even on barren saline soil, making this land suitable for the production of fuel and feed.[3]

Plants rely on transpirational pull to absorb water and mineral ions from the soil,[4] a process that is affected by various anatomical structures, growth conditions, and the age of the roots themselves.[5]

Root absorption relies on three major pathways: the symplastic pathway (via membrane-bound transporters and plasmodesmata), the apoplastic pathway (extracellular transportation), and the coupled transcellular pathway.[6] Casparian strips and suberin lamellae, the apoplastic barriers in the endodermal layer,[7,8] can effectively block water and inorganic salt transport through both the apoplastic and coupled transcellular pathways.

Biotic[9] and abiotic stresses[10,11] can enhance the biosynthesis of suberin, which is composed of both polyphenolic and polyaliphatic domains.[12] The ability of suberin to block water and solute transportation is particularly dependent on the polyaliphatic domain, which is mainly comprised of oxygenated fatty acids and their derivatives.[13,14] By contrast, the Casparian strip is mainly composed of lignin,[8] a hydrophobic compound biosynthesized

from the polymerization of syringyl, guaiacyl, and *p*-hydroxyphenol via the phenylpropanoid pathway.[15]

Several molecules involved in the formation of Casparian strip and suberin lamella in *Arabidopsis thaliana*.[16–18] For example, the transcription factor SHORTROOT (SHR) promotes Casparian strip formation by activating *MYB DOMAIN PROTEIN36* (*MYB36*) and *SCARECROW* expression. Two small peptides known as Casparian strip integrity factors (CIFs) participate in Casparian strip biosynthesis in Arabidopsis,[17] while MYB36 regulates the expression of genes encoding the Casparian strip membrane domain protein 1 (CASP1), PEROXIDASE64 (PER64), and ENHANCED SUBERIN1 (ESB1) and thereby regulates lignin polymerization.[19,20] Another MYB, MYB41, regulates the biosynthesis and transport of suberin lamella.[21]

Expression profiling has been widely used to identify candidate genes involved in important biological processes.[3,22–24] A recent study of the root transcriptome of sweet sorghum identified many genes putatively associated with apoplastic barrier development;[22] however, the developmental pattern of these barriers has not been fully elucidated. Moreover, all of the previously reported genes in sweet sorghum are annotated members of known gene families[22,25] and unknown functional genes may be important drivers of apoplastic barrier development.

To elucidate the genetic regulation of apoplastic barrier development, the exact region of the root in which this process takes place must be identified. In this study, we dyed root sections of the sweet sorghum inbred line M-81E to determine the positions at which Casparian strips and suberin lamellae formed. Three distinct developmental regions were observed and separately enriched in transcriptome analyzes to identify candidate genes involved in the differentiation of the apoplast barrier in sweet sorghum. These results may provide another perspective for further study on molecular mechanism of apoplastic barrier developemnt.

Materials and methods

Plant materials and growth conditions

The sweet sorghum inbred line M-81E was used in this study. The seeds were sterilized in 70% (v/v) ethanol for 1 min and 3% (v/v) NaClO for 10 min, after which they were washed with deionized water and germinated in the dark on filter paper in a Petri dish. After 2 days, seedlings with a 3-cm radicle and a 2-cm shoot were transplanted into a hydroponic culture using Hoagland solution, which was replaced each day. All seedlings were cultured at 28 ± 3°C/23 ± 3°C (day/night) at a light intensity of 600 μmol m^{-2} s^{-1} (15-h photoperiod) and 70% relative humidity.

Casparian strip and suberin lamella observations in the root

To identify regions of Casparian strip and suberin lamella development in the seminal root, freehand cross sections of the seminal root were made at 10-mm intervals along the root tip. After identifying the region (from 10 to 50 mm) of Casparian strip and suberin lamella development, sections

were cut every 5 mm for a more precise determination of the developmental positions.

The sections were cleared with lactic acid saturated with chloral hydrate for 2 h,[26] after which the sections were stained with 0.1% (w/v) berberine hemisulfate for 1 h and then with 0.5% (w/v) aniline blue for another hour.[27] The stained sections were observed with a fluorescence microscope (ECLIPSE 80i; Nikon, Japan) under an excitation wavelength of 450 to 490 nm to view the Casparian strip.

To observe the suberin lamella, the sections were stained with 0.01% (w/v) Fluorol Yellow 088 for 1 h[28] and then examined using a fluorescence microscope under ultraviolet (UV, C-SHG1; Nikon, Japan) excitation (330–380 nm). All images were directly recorded with a CCD camera (Nikon).

Casparian strip and suberin lamella development was analyzed using the Loess module in SPSS 22.0 (IBM, USA). Based on the different developmental stages, the number of epidermal cells, cortical cell layers, endodermal cells, and protoxylem and metaxylem layers was quantified, and the distribution of double protoxylem was measured.

RNA extraction, illumina library construction, and sequencing

Root sections displaying the three developmental stages of the Casparian strip and suberin lamella were separately enriched for RNA sequencing (RNA-seq). Each sample contains thirty roots in the corresponding stage and prepare them reference by Yang et al.[22] Total RNA was extracted using a Total Plant RNA Extraction Kit (Karroten, Beijing, China), and contaminating genomic DNA was removed in a digestion with DNaseI. Magnetic Oligo dT beads were used to collect the mRNA. A NEB fragmentation buffer was included in the thermomixer to cut the mRNA into short fragments at the right temperature. The broken mRNA was used as a template to generate the first-strand cDNA. The double-stranded cDNA was then synthesized by adding buffer, dNTPs and DNA polymerase I and RNase H, and the double-stranded cDNA was purified by AMPure XP beads. After the purified double-stranded cDNA was end-repaired, the A-tail was added and the sequencing linker was ligated. Fragments of 250–300 bp in length were amplified by PCR and used to build the cDNA library. The library quality was examined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), after which it was sequenced on an Illumina HiSeq 4000 (San Diego, CA, USA) at 1GENE. biotech.co.ltd (Hangzhou, China).

Read mapping and data quality assessment

The raw data in FASTQ format were filtered to remove reads containing adaptors, and low-quality reads with 30% or more of the bases having a quality value of ≤ 20 and reads with more than 5% unknown nucleotides were excluded. The clean reads were aligned to the reference data (ftp://ftp.ensemblgenomes. org/pub/plants/release-43/fasta/sorghum_bicolor/)[29,30] using SOAP2 (soap2.21)[31] and TopHat2 (tophat-2.0.11. Linux_x86_64).[32,33] The sequencing quality was assessed and the random read distribution and gene coverage were evaluated. Gene expression levels were calculated as reads per kilobase per million mapped reads (RPKM).[34]

Differentially expressed genes and functional annotation

The assembled sequences were annotated in Swiss-Prot.[35] Differentially expressed genes (DEGs) were identified using the edgeR function in Bioconductor (http://bioconductor. org), in which the count of the sequencing reads has a negative binomial distribution for each gene, providing a theoretical distribution basis for hypothesis testing.[36] The DEGs were identified according to the following criteria: | log2Ratio| ≥ 1 and false discovery rate ≤ 0.05. The Gene Ontology (GO) functional enrichment was performed using Bioconductor to determine the biological function of the DEGs.[37] A Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was also performed on the DEGs to identify any metabolic pathways or signal transduction pathways enriched in the DEGs.[38]

Quantitative real-time PCR analysis

Twelve DEGs possible involved in the formation of the apoplastic barrier were selected for quantitative real-time PCR analysis to verify the RNA-seq results. Primers were designed using Beacon Designer (version 7.0) (Table S1). The cDNA was generated from 1 μg total RNA using the cDNA Synthesis Kit (Vazyme, Nanjing, China). The housekeeping sorghum gene *β-ACTIN* (GenBank ID: X79378) was used as an internal standard. Real-time PCR was conducted with SYBR Premix Ex Taq (Takara, Beijing, China) using a LightCycler 96 system (Roche), as described by Sui et al.[3] The gene expression level was calculated using the $2^{-\triangle\triangle Ct}$ method.[39]

Accession numbers

The RNA-seq data generated in this study were deposited in the National Center for Biotechnology Information (NCBI) database under the accession numbers SRR8997148 (Stage I), SRR8997149 (Stage II), and SRR8997150 (Stage III).

Results

Localization of the Casparian strip and suberin lamella formation

We observed Casparian strip and suberin lamella development in root cross sections stained with berberine hemisulfate-aniline blue and Fluorol Yellow 088, respectively (Figure 1). The location of Casparian strip formation relative to the apex was determined based on the number of radial cell walls between two adjacent stained endodermis cells. Suberin lamella differentiation was characterized based on the number of stained endodermal cells. Based on these observations, the sweet sorghum seminal root apoplast barriers were divided into three developmental stages: Stage I (0–10 mm from the root apex), without any Casparian strip or suberin lamella; Stage II (20–25 mm from the root apex), with a developing Casparian strip and suberin lamella; and Stage III (35–45 mm from the root apex), with a developed Casparian strip and suberin lamella.

To reduce the overlapping expression patterns of genes involved in each stage of apoplastic barrier formation, a certain segment between the different stages was discarded (21–24 mm from the root apex for Stage II and 26–34 mm from the root apex for Stage III). To further elucidate apoplastic barrier development in the different root sections, we characterized the morphological structures of the seminal root sections at 5-mm intervals from the root apex. No apoplastic barriers were observed in Stage I (Figure 2). The Casparian strip and suberin were first detected in the root at a distance of 20 mm from the apex and were then found to intensively develop at 20 to 30 mm from the apex, particularly in Stage II. The endodermis and Casparian strip had completed their development by the time they were 35 mm from the root apex. The development of the morphological structures of the seminal root sections was further examined by tallying the number of protoxylem layers, metaxylem layers, epidermal cells, cortical cells, and endodermal cells present. There was significant change in the numbers of these morphological features as the distance from the root apex increased (Table S2). The root epidermis was composed of dense cells, with approximately 105 cells comprising the root circumference. Five to six cortical cell layers encircled about 31 endodermal cells (Fig. S1).

Analysis of DEGs associated with the developmental stages of Casparian strips and suberin lamellae

We divided the sweet sorghum roots into the three developmental sections based on the distances from the root tip, as described above. The number and quality of clean reads from each of the three root sections are presented in Table S3. In total, 24,344 annotated genes were expressed in Stage I, 24,501 in Stage II, and 24,423 in Stage III (Figure 3). A total of 23,246 genes were common among the three sections, while 414 genes were uniquely expressed in Stage I, 418 were unique to Stage II, and 408 were unique to Stage III (Figure 3; Fig. S2). These unique genes may determine the specific differentiation of each section of the root.

DEGs participating in lignin biosynthesis

Lignin biosynthesis and the deposition of monolignols are essential steps of Casparian strip formation.[8] Lignin biosynthesis involves the formation of precursors, monolignol biosynthesis and deposition. Sixty-one genes known to be involved in the phenylpropanoid pathway were differentially expressed between the three root sections (Figures 4 and 5; Tables S4 and S5), including 32 genes that were highly expressed in Stage I, 18 in Stage II, and 11 in Stage III.

During precursor biosynthesis, the lignin precursors syringyl, guaiacyl, and *p*-hydroxyphenol are fed into the phenylpropanoid biosynthesis pathway. Twenty-four of the genes encoding enzymes in this pathway were differentially expressed between the three root sections. Two genes (SORBI_3006G148800 and SORBI_3006G148900) encoding phenylalanine ammonia-lyases (PALs), which promote the conversion of phenylalanine into cinnamic acid and tyrosine into *p*-cinnamic acid, were highly expressed in the three root sections (Figure 4a); SORBI_3006G148800 was more highly expressed in Stage II, and SORBI_3006G148900 expression gradually increased throughout the three developmental stages but remained lower than the expression level of SORBI_3006G148800. This is the first step of Casparian strip precursor biosynthesis.
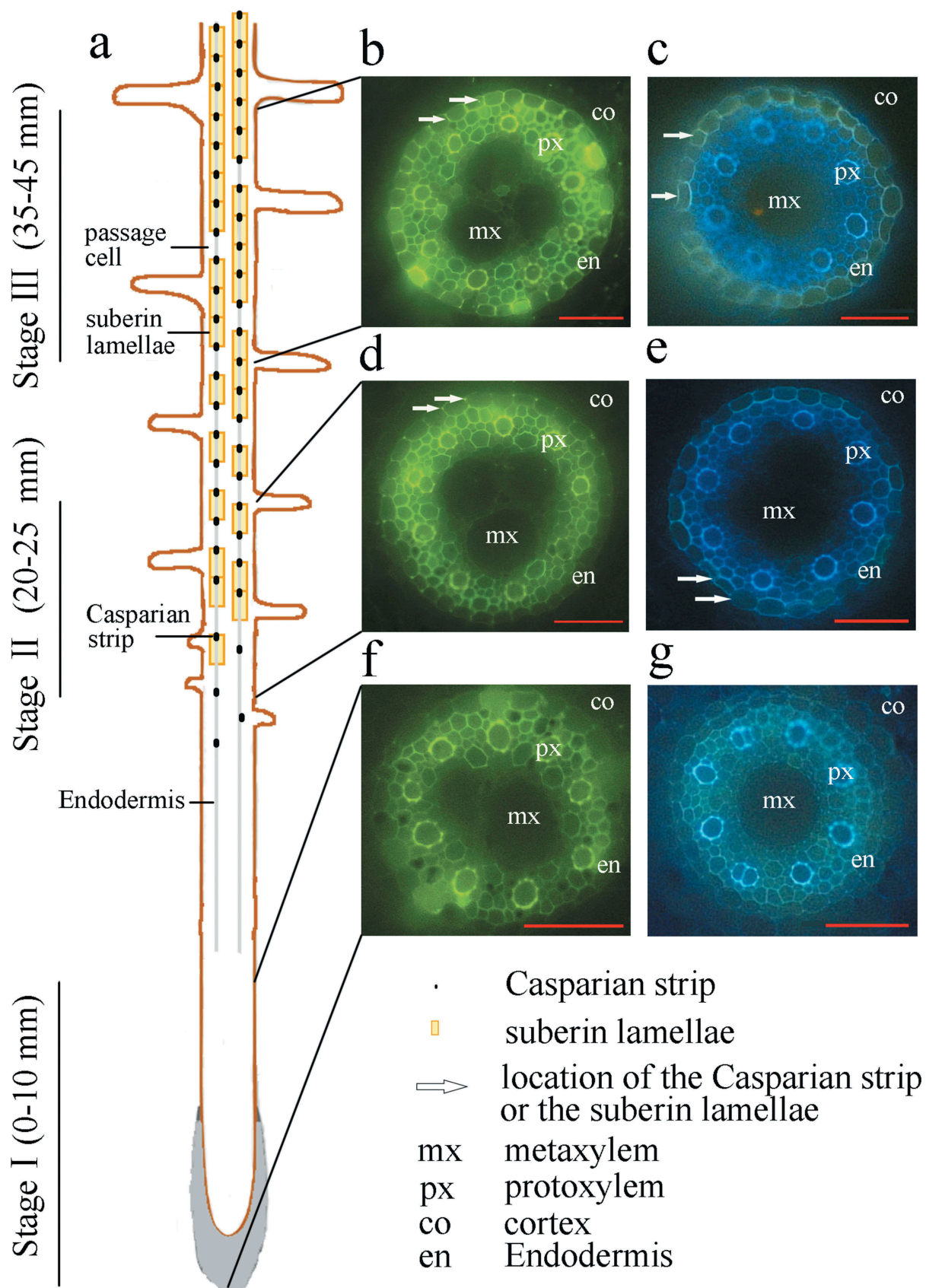
Figure 1. Localization and histochemical staining of the differentiating endodermis and developing Casparian strips and suberin lamellae of sweet sorghum seminal roots. Freehand cross sections were made using 10-day-old seminal roots grown in nutrient solution. (a) Schematic view of endodermal differentiation. Stage I (0–10 mm from the root tip), without any Casparian strips or suberin lamellae; Stage II (20–25 mm from the root tip), with developing Casparian strips and suberin lamellae; Stage III (35–45 mm from the root tip), with developed Casparian strips and suberin lamellae. (b, d, f) Root cross sections stained with berberine aniline blue to show the Casparian bands, viewed under blue light (450 nm). Arrows indicate Casparian strips. (c, e, g) Root cross sections were stained with FY088 to show the suberin lamellae, viewed under UV illumination (365 nm). Arrows indicate suberin lamellae. mx, metaxylem; px, protoxylem; co, cortex; en, endodermis. Bar = 50 μm.
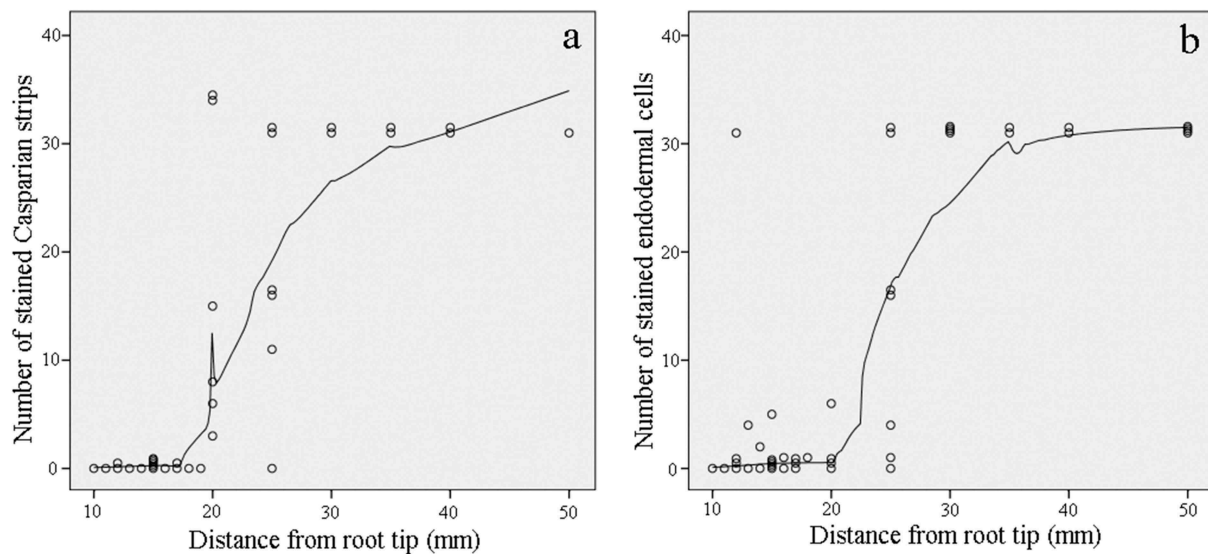
Figure 2. Number of Casparian strips and stained endodermal cells in cross sections along the sweet sorghum seminal root. (a) Number of stained Casparian strips between adjacent endodermis cells. The cells were stained using berberine hemisulfate-aniline blue dye. (b) Number of stained endodermal cells. The cells were stained using Fluorol Yellow 088. Each point represents the corresponding number at this location.
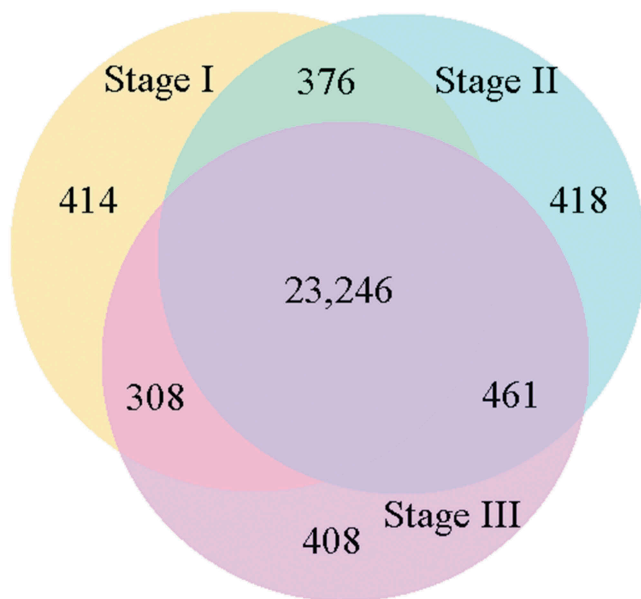

Figure 3. Venn diagram showing the number of shared and unique genes expressed in the three developmental sections of the root (expression threshold, RPKM > 1.0) .

The SORBI_3003G337400 gene encoding CYTOCHROME P450 MONOOXYGENASE 73A1 (CYP73A1), which promotes the conversion of cinnamic acid to *p*-coumaric acid, had decreased expression levels in the three root sections as development progressed (Figure 4b). The 4-coumarate:CoA ligase (4CL) enzymes catalyze the production of coenzyme A esters (Figure 4c). Three genes encoding 4CLs and related enzymes (SORBI_3001G500800, SORBI_3004G272700, and SORBI_3006G265000) showed differential expression levels in the development of the lignin pathway. The expression levels of SORBI_3001G500800 and SORBI_3004G272700, encoding 4CL1 and 4CL2, respectively, increased as development progressed through the three stages; however, the expression of SORBI_3006G265000, which encodes

ACYL-ACTIVATING ENZYME5 (AAE5), decreased as root development progressed and had its lowest expression level in Stage II. CINNAMOYL CoA REDUCTASE1 (CCR1) participates in the reduction of three cinnamate CoA esters to produce cinnamaldehyde and is the rate-limiting enzyme for lignin biosynthesis (Figure 4d). Four enzymes participate in the reduction reaction; SORBI_3002G250000 and SORBI_3003G116800 showed increasing levels of expression as root development progressed, SORBI_3004G272700 was most highly expressed in Stage I, and SORBI_3001G037800, encoding TETRAKETIDE ALPHA-PYRONE REDUCTASE1 (TKPR1), was most highly expressed in Stage II. Cinnamyl alcohol dehydrogenases family members (CADs) catalyze the biosynthesis of alcohol in lignin biosynthesis (Figure 4e). SORBI_3007G076000 showed decreased expression in Stage II, while SORBI_3006G014700 expression increased as root development progressed.

The peroxidases catalyze the last step of the formation of the lignin monomers (Figures 4f and 5). After lignin precursor biosynthesis, 37 peroxidase genes in the lignin biosynthesis pathway were found to be differentially expressed between the three root developmental stages outlined above. Eleven such genes were upregulated in Stage II, which suggests that they may participate in the formation of the apoplastic barrier. Seven genes encoding enzymes catalyzing two reactions that convert *p*-coumaroyl-CoA to caffeoyl-CoA, were differentially expressed (Figure 4g). Four genes encoding shikimate *O*-hydroxy-cinnamoyl transferase (HST) were differentially expressed in the three root systems, three of which were most highly expressed in Stage II. SORBI_3004G272700, encoding an acetyltransferase, was increasingly upregulated as the root developed. The expression of SORBI_3007G059400, encoding anthocyanin 5-aromatic acyltransferase, was slightly elevated in Stage II. The expression of SORBI_3002G041900, which encodes PHENOLIC GLUCOSIDE MALONYLTRANSFERASE2 (PMAT2), was similarly higher in Stages II and III than in Stage I. SORBI_3001G254800 encodes CYP78A1 and showed a decreasing expression trend across the
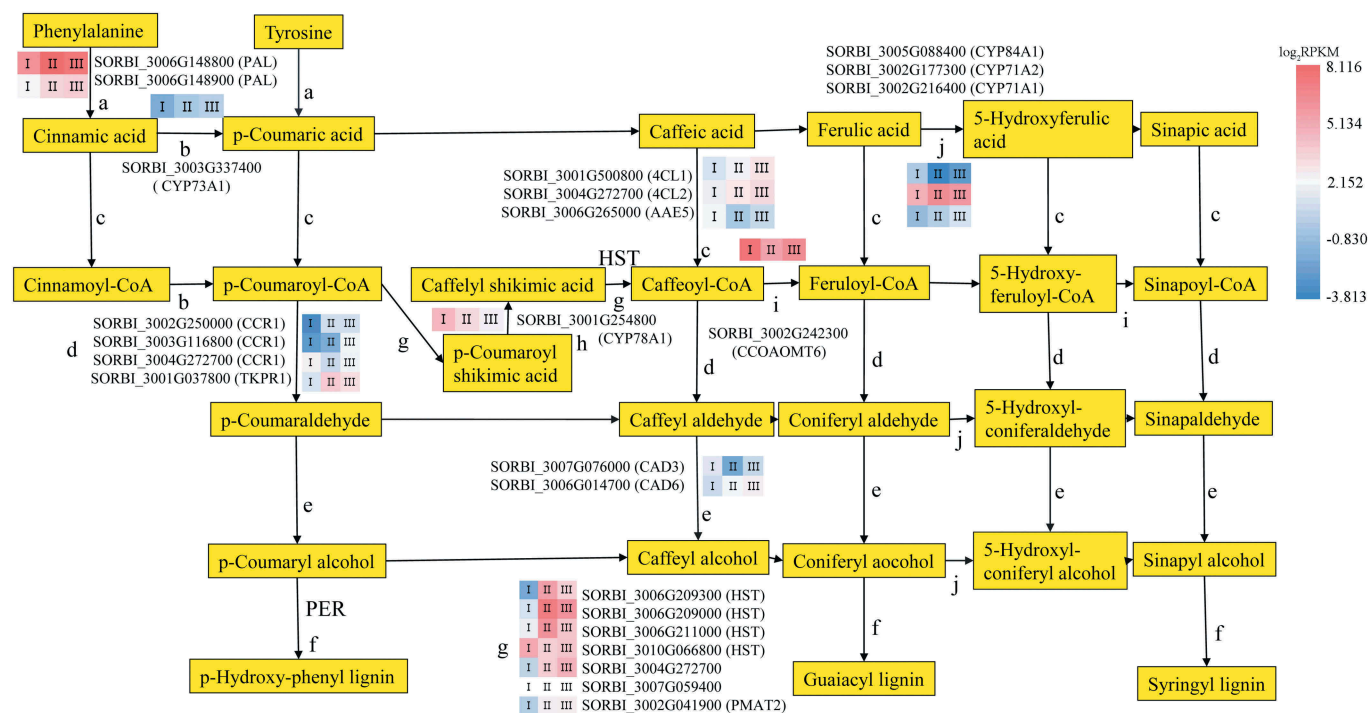
Figure 4. The DEGs mapped to the phenylpropanoid pathway (except peroxidase), with the expression levels in the three developmental sections marked as I, II, and III. The gene SORBI_3004G272700 is annotated as an uncharacterized acetyltransferase. The gene SORBI_3007G059400 is annotated as anthocyanin 5-aromatic acyltransferase. The encoded enzymes of the other genes are presented next to their accession numbers.
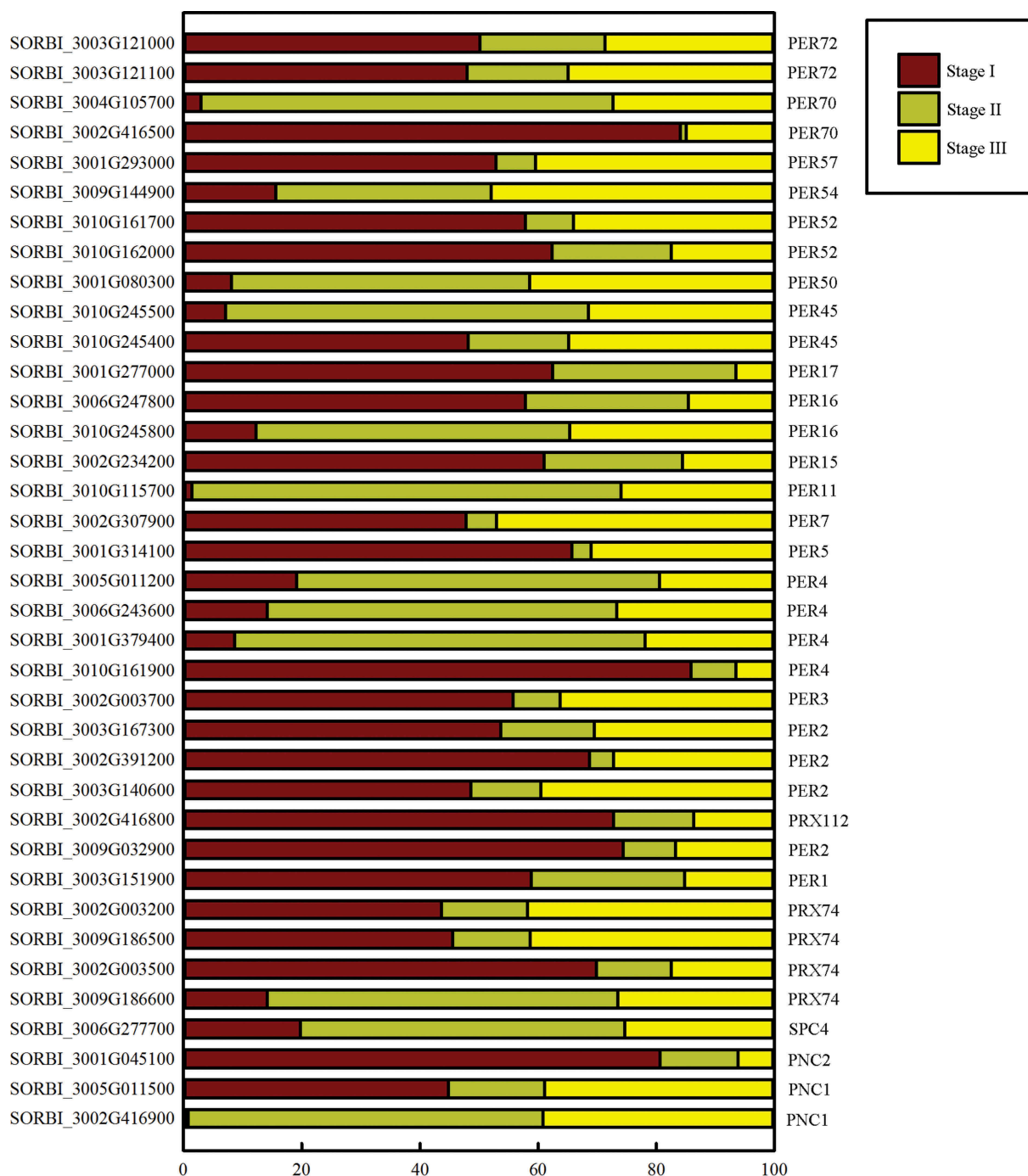
three Stages (Figure 4h), while SORBI_3002G242300, encoding caffeoyl-CoA *O*-methyltransferase (CCOAOMT), was most highly expressed in Stage I (Figure 4i). The above three enzymatic reactions (Figure 4g–i) promote the production of G-type and S-type lignin. These reactions depend on the CYP family of genes (Figure 4j), which promote the formation of S-type lignin. SORBI_3002G177300 (CYP71A2) was most highly expressed in Stage II, while SORBI_3005G088400 (CYP84A1) shows the contrasting trend. SORBI_3002G216400 (CYP71A1) was increasingly upregulated throughout root development.

DEGs involved in fatty acid elongation and hydroxylation

Fatty acid elongation and hydroxylation are key steps in suberin lamellae biosynthesis.[40,41] KCS is the rate-limiting enzyme in the fatty acid elongation pathway.[42] Nine DEGs were found to be involved in this process in sorghum (Figure 6; Table S6); the expression levels of six of these genes were elevated in Stage II. SORBI_3005G168700 expression peaked in Stage II, while SORBI_3002G179700 showed the lowest expression level at this stage. SORBI_3009G241000 expression increased as root development progressed. CUTICULAR PROTEIN1 (CUT1; also known as KCS6) was most highly expressed in Stage II. Another DEG in the fatty elongation pathway encodes a steroid 5-alpha-reductase (DET2), which functions downstream of the KCS enzyme. This gene was most highly expressed in Stage I, declined in Stage II, and finally increased again in Stage III.

The ω-hydroxylation of fatty acids is an enzymatic step in cuticular suberin and wax biosynthesis. Eleven genes in the

cytochrome family are differentially expressed between the different stages of root development (Figure 7; Table S7), five of which were most highly expressed in Stage II. SORBI_3001G213300 (CYP86B1) expression also peaked in Stage II. The expression levels of SORBI_3007G015200 and SORBI_3007G015100 declined in Stage II and then increased in Stage III, while SORBI_3001G137900 and SORBI_3010G197700 expression levels declined throughout root development. By contrast, SORBI_3003G040300 and SORBI_3001G510400 expression increased as root development progressed. Hydroxycinnamoyl-CoA omega-hydroxypalmitic acid O-hydroxycinnamoyltransferase (HHT) catalyzes the conversion of feruloyl-CoA to 16-feruloyloxypalmitic acid in the cutin, suberin, and wax biosynthesis pathway. Seven DEGs (SORBI_3003G408900, SORBI_3003G408900, SORBI_3003G408900, SORBI_3006G 211000, SORBI_3010G179900, SORBI_3008G068300, and SORBI_3007G059400) also participate in this enzymatic reaction. Six of these genes (excluding SORBI_3010G179900) were more highly expressed in Stage II, with SORBI_3003 G408900 showing the highest level of expression. SORBI_3010 G179900 (3′-*N*-debenzoyl-2′-deoxytaxol *N*-benzoyltransferase [TAX10]) was more highly expressed as root development progressed. SORBI_3004G342000 encodes ECERIFERUM1 (CER1), which can catalyze the conversion of a long-chain aldehyde to a long-chain alkane, and was most highly expressed in Stage II, while SORBI_3008G076200, also annotated as CER1, had its lowest level of expression in Stage II. This suggests that these two CER1 genes play opposing roles in

Figure 5. The distribution of the total expression of each gene in the peroxidase portion of the phenylpropanoid pathway. The gene expression level in each section is presented as a proportion of the total RPKM of the three sections.

hydroxylation. Three genes (SORBI_3005G063400, SORBI_3005G063300, and SORBI_3002G134100) were found to encode fatty acyl-CoA reductase (FAR), and all showed an increased level of expression in Stage II, which may be related to the biosynthesis of long-chain alcohols during suberin biosynthesis. The expression of the gene encoding alcohol-forming fatty acyl-CoA reductase (AF-CoA; SORBI_3007G169900) increased as root development progressed.

DEGs involved in the transport of lignin and fatty acid derivatives during the formation of Casparian strips and suberin lamellae

Members of the ATP-binding cassette subfamily G (ABCG) family are thought to mediate the transport of lignin and monolignol fatty acids outside cells to form the Casparian strips and suberin lamellae.[43] Five members in this family were
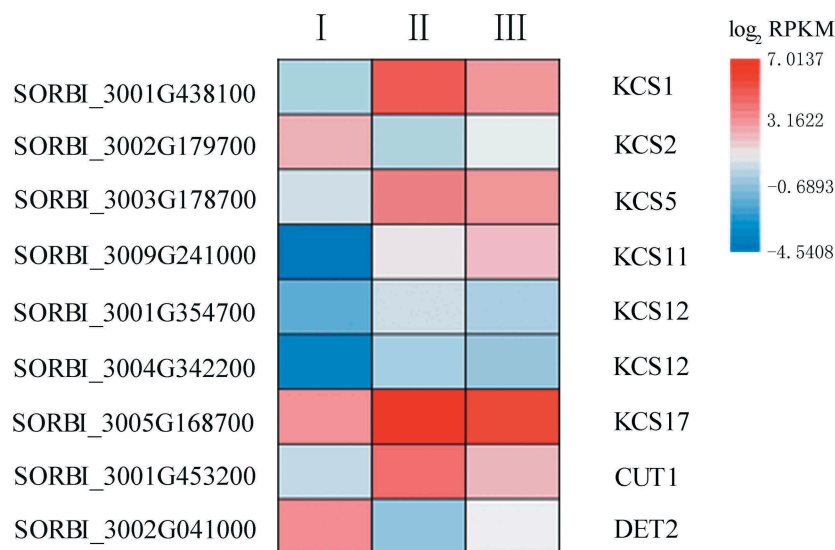
Figure 6. The expression of nine genes involved in fatty acid elongation and possible suberin development. The heatmap shows the $\log_2$ RPKM values of the expression levels.

differentially expressed throughout root development, with most of them being more highly expressed in Stage II. Two genes encoding ABCG16 showed higher expression than the other genes at each of the three stages. SORBI_3009G022000 showed equally high expression at each of the three stages, while SORBI_3001G413700 and SORBI_3003G348100 were most highly expressed in Stage II (Table S8).

Candidate transcription factors involved in the formation of Casparian strips and suberin lamellae

Forty DEGs encoding transcription factors were upregulated, while 28 DEGs were downregulated (Figure 8 and Table S9), including 12 WRKYs, 12 MYBs, 10 bHLHs, 12 NACs, 5 AP2s, 6 ethylene-responsive transcription factors (ERFs), 6 TFs, 3 heat stress transcription factors (HSFs), 1 GATA, and 1 nuclear transcription factor Y subunit B-5 (NTF). Previous studies have shown that MYBs and NACs are involved in the biosynthesis of the apoplastic barrier;[44] however, our data indicated that WRKYs may also participate in the formation of the apoplastic barrier, something that has not been reported previously. Two ERFs were found to have an increased level of expression in Stage II, while four genes encoding other transcription factors (SORBI_3008G172200, SORBI_3003G305000, SORBI_30 10G224200, and SORBI_3003G034300) showed increased levels of expression in Stages II and III. Three HSFs (SORBI_3001G496200, SORBI_3010G170600, and SORBI_ 3002G224200) and one NTF (SORBI_3003G417800) also showed increased expression levels in Stage II.

Other possible DEGs related to Casparian strip and suberin lamella development

The deposition of monolignols is a key step after the lignin monomers are transported out of the cells, with the CASPs known to be involved in the deposition of lignin on the cell wall.[45] Four of the DEGs we identified were annotated as CASP-

like proteins. SORBI_3010G214800 and SORBI_3009G088900 were most highly expressed in Stage II, with the latter having a higher expression level than the former. SORBI_3002G113100 and SORBI_3003G162100 expression levels increased during root development (Table S10).

Seven DEGs were annotated as encoding laccases, suggesting that they may contribute to lignin biosynthesis[46,47] (Table S11). *Laccase-4* and *Laccase-25* expression was lowest in Stage II, while *Laccase-14, Laccase-8*, and *Laccase-3* were most highly expressed in Stage II and may participate in the formation of the apoplastic barrier.

Suberin biosynthesis requires the formation and deposition of fatty acid precursors. We identified seven DEGs related to the fatty acid biosynthesis pathway (Table S12), more of which were upregulated than downregulated during root development. No obvious trends were detected in the genes responsible for the fatty acid metabolic pathways (Table S13). The gene encoding GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE5 (GPAT5) was also found to be differentially expressed throughout root development (Table S14). Three genes (SORBI_ 3004G010300, SORBI_3009G162000, and SORBI_3003G 360700) were highly expressed in Stage II, while two genes (SORBI_3001G099300 and SORBI_3001G250200) were highly expressed in Stage III.

qRT-PCR validation of RNA-seq

Based on the transcriptome results and the identified DEGs, 12 genes were randomly selected for qRT-PCR analysis to validate the RNA-seq data. The correlation between the RNA-seq and qRT-PCR data was high (R^2 = 0.82, Figure 9; Table S15), indicating that the transcriptomic data were valid.

Discussion

The current study is the first to identify the specific developmental sections of the sorghum root in which Casparian strips
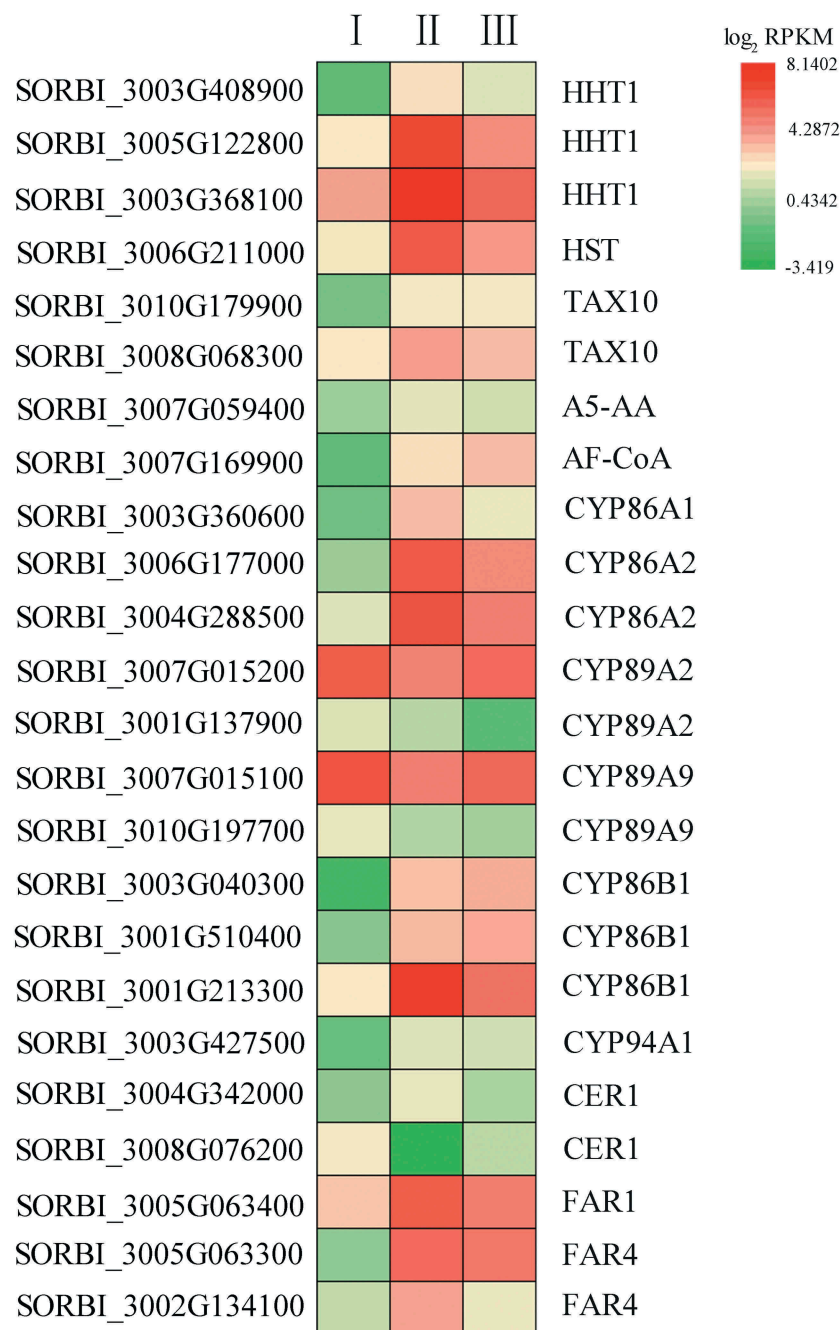
Figure 7. The expression of genes participating in fatty acid ω-hydroxylation and possible suberin development. The heatmap shows the log$_2$ RPKM values of the expression levels.

and suberin lamellae are formed. Using histochemical staining and morphological analyzes, we distinguished three distinct developmental sections, enabling the identification and analysis of putative candidate genes involved in Casparian strip and suberin lamella formation in the endodermis.

Cross sections taken at set distances from the apex of the sorghum seminal root showed no significant differences in morphological structures along the length of the root, which is consistent with the observation that water and inorganic salts are absorbed by the root tip.[7] Studies in rice and olive (*Olea europaea*)[11,48] and several research models[6,7,49] suggest that Casparian strips form before suberin lamellae. Barley (*Hordeum vulgare*) roots were previously divided into several

sections for the study of suberin lamellae;[5] however, in our study, both structures were found to form simultaneously. This rapid formation of both Casparian strips and the suberin lamellae may explain the salt tolerance of sweet sorghum.

We performed an RNA-seq analysis of the three distinct sections of the sorghum seminal root to identify putative candidate genes involved in Casparian strip and suberin lamella development. The formation of the Casparian strip is dependent on the polymerization of lignin,[8] which is produced via the phenylpropanoid pathway. CCR and 4CL are the key enzymes in this pathway[15] and were highly expressed in Stages II and III of the sorghum roots. These enzymes perform the initial reaction of lignin precursor formation. Members of the HST family,
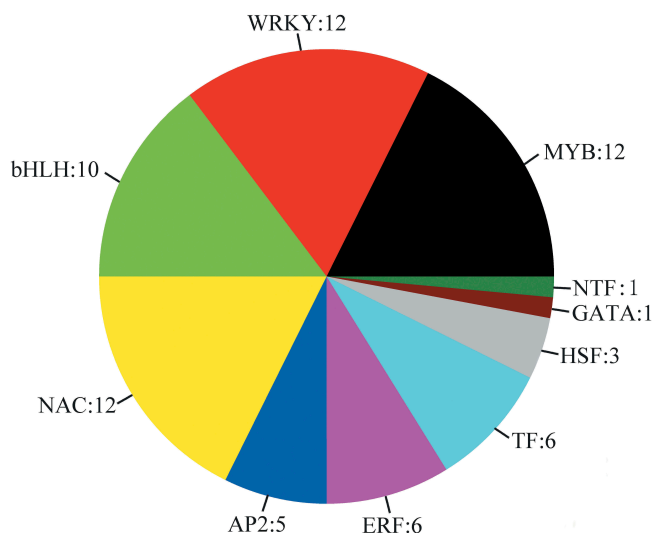
Figure 8. Differential expression of all transcription factors in the alignment of three developmental stages. The number of differentially expressed genes is specified.

SORBI_3002G242300 (CCOAOMT) and SORBI_3001G254800 (CYP78A1), then further promoted the biosynthesis of the subsequent products based on the lignin precursor. PER64,[45] PER02/25/71,[50] PER37,[51] and PER72[52] participate in Casparian strip biosynthesis in Arabidopsis. Here, we discovered many additional peroxidase-encoding genes that were also differentially expressed throughout root development.

The most important reactions in suberin lamella biosynthesis are the elongation and hydroxylation of fatty acids.[40,41] Many members of the KCS family are involved in these processes.[53–55] Here, *KCS2* was found to be highly expressed during Stage I of sorghum root development, consistent with the previous finding that the deletion of *KCS2* affects suberin lamella composition in Arabidopsis.[53] Recent studies have shown that the hydroxylation of the fatty acid ω sites is mainly catalyzed by the CYP86 and CYP94 families,[56–59] many of which were also found to be highly expressed in

Stage II. These results indicate that the high expression levels of the above genes may contribute to suberin lamella formation in Stage II. Moreover, *FAR* was most highly expressed during Stage II and was also previously shown to be involved in suberin lamella formation.[60]

Five members of the ABCG family were found to be highly expressed in Stage II of sorghum root development. These genes may function in the transport of lignin and fatty acids out of the cells during Casparian strip and suberin lamella formation.[61–63]

Although a series of genes were mapped to the pathways involved in Casparian strip and suberin lamella formation, most of the identified DEGs were annotated as functioning downstream of these pathways. Some of the upstream signaling genes, such as SHR,[17] were not identified as DEGs, possibly due to differences between monocotyledons and dicotyledons. Nevertheless, we identified other candidate genes participating in Casparian strip and suberin lamella development, including transcription factors and those encoding laccases. The 28 DEGs encoding transcription factors that were expressed during Stage I may be involved in initiating the development of the apoplastic barriers, while the 28 that were highly expressed in Stage II may regulate the developmental process.[20,44,64–67] The high expression levels of the NAC-type transcription factors in both Stages II and III likely participate in the regulation of the MYB transcription factors in the lignin biosynthesis pathway.[67]

CASPs play a critical role in the polymerization of the Casparian strip.[45,68] We found that most genes encoding CASPs were highly expressed in Stage II, which is consistent with the observation that a CASP-like protein functions in Casparian strip polymerization.[47] The laccases were also previously suggested to participate in Casparian strip biosynthesis.[47,69] In Arabidopsis, *gpat5* mutants had 50% less suberin lamellae than the wild type.[70] Here, we found that these genes are highly expressed in Stages II and III, during which Casparian strips form.

Among them, 16 genes (HSTs [SORBI_3006G209300, SORBI_3006G209000, and SORBI_3006G211000], CYP71A2,
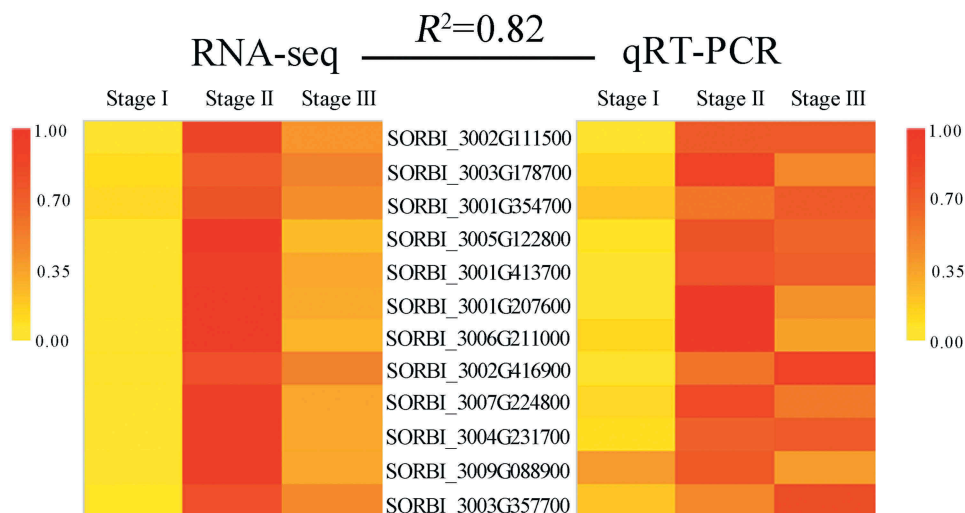

Figure 9. Validation of the RNA-seq results using qRT-PCR to analyze the expression of 12 representative candidate genes. R^2 represents the correlation between the RNA-seq (RPKM) and qRT-PCR data.

PER11, PER16, KCS17, HHT1 [SORBI_3005G122800 and SORBI_3003G368100], CYP86B1, MYB39 [SORBI_3007 G224800 and SORBI_3004G231700], WRKY70, ABCG16 [SORBI_3001G413700], CASP-like protein [SORBI_3009G 088900], and Laccase-3) are highly expressed in Stage II. KCS17 is a homolog of KCS9.[71] CYP86B1 can reduce the levels of 16:0, 18:0, and 18:1 ω-hydroxy acids and α,ω-DCAs in the root suberin.[58] AtMYB36 is a homolog of AtMYB39.[72] Phylogenetic analysis revealed that ABCG16 is closely related to ABCG2/6/20,

which have been reported to function in suberin transport.[63,73] The CASP-like proteins, laccases, and PERs also participate in the biosynthesis and transport of lignin.[47] The physical locations of the candidate genes on the chromosome has been shown in Figure 10.

Conclusion

The current research established a method for identifying the key genes involved in apoplastic barrier formation based on
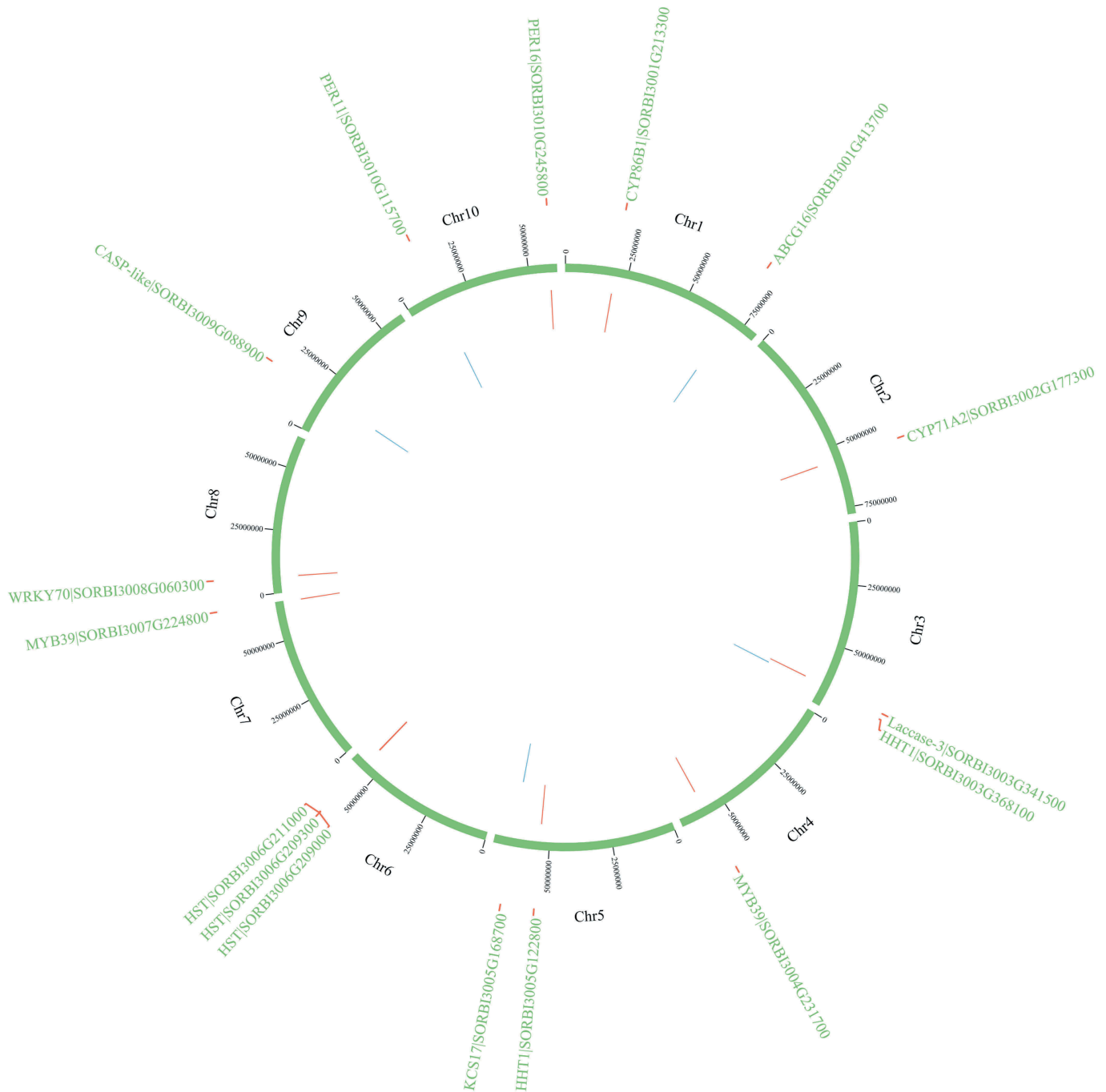

Figure 10. The physical locations of candidate genes involved on chromosomes based on BLAST results against the whole-genome sequencing dataset. Red color indicates the genes on the sense sequence and green color indicates the genes on the antisense strand.
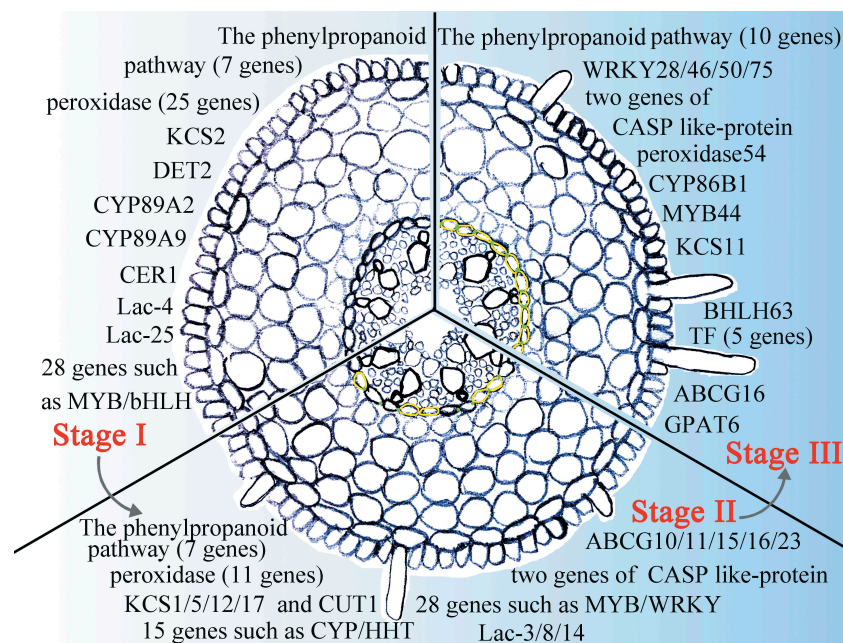
Figure 11. A model of sweet sorghum root Casparian strip and suberin lamella development. The presented genes display high levels of expression in Casparian strip- and suberin lamellae-related pathways in Stage I to Stage III of the root. Green between the endodermal cells indicates that the Casparian strip is stained in this section. The yellow endodermal cells indicate that the suberin lamellae of the cells in this section are stained.

different developmental stages of seminal root and the distance from the root apex. In total, 196 genes involved in Casparian strips and suberin lamellae development were identified in the sweet sorghum root (Figure 11). Different genes are expressed in various parts of the roots. Strikingly, 190 unknown genes were highly expressed in the three stages and likely function in apoplastic barrier formation in sweet sorghum (Table S16). Transformation systems[22] and CRISPR-Cas9[74] could be used to verify the functions of these candidate genes in apoplastic barrier formation.

Author Contributions

B.W. designed research. X.W. performed and wrote this manuscript. Z.Y., G.H., X.Z. and S.Y. participated in the writing and modification of this manuscript. F.Y. and B.W. revised the paper. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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