Transcriptome Analysis of a New Peanut Seed Coat Mutant for the Physiological Regulatory Mechanism Involved in Seed Coat Cracking and Pigmentation

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Seed-coat cracking and undesirable color of seed coat highly affects external appearance and commercial value of peanuts (Arachis hypogaea L.). With an objective to find genetic solution to the above problems, a peanut mutant with cracking and brown colored seed coat (testa) was identified from an EMS treated mutant population and designated as “peanut seed coat crack and brown color mutant line (pscb).” The seed coat weight of the mutant was almost twice of the wild type, and the germination time was significantly shorter than wild type. Further, the mutant had lower level of lignin, anthocyanin, proanthocyanidin content, and highly increased level of melanin content as compared to wild type. Using RNA-Seq, we examined the seed coat transcriptome in three stages of seed development in the wild type and the pscb mutant. The RNA-Seq analysis revealed presence of highly differentially expressed phenylpropanoid and flavonoid pathway genes in all the three seed development stages, especially at 40 days after flowering (DAF40). Also, the expression of polyphenol oxidases and peroxidase were found to be activated significantly especially in the late seed developmental stage. The genome-wide comparative study of the expression profiles revealed 62 differentially expressed genes common across all the three stages. By analyzing the expression patterns and the sequences of the common differentially expressed genes of the three stages, three candidate genes namely c36498_g1 (CCoAOMT1), c40902_g2 (kinesin), and c33560_g1 (MYB3) were identified responsible for seed-coat cracking and brown color phenotype. Therefore, this study not only provided candidate genes but also provided greater insights and molecular genetic control of peanut seed-coat cracking and color variation. The information generated in this study will facilitate further identification of causal gene and diagnostic markers for breeding improved peanut varieties with smooth and desirable seed coat color.

Keywords: peanut (Arachis hypogaea), seed-coat cracking, pigmentation, RNA-seq, flavonoid pathway
INTRODUCTION

A typical peanut (Arachis hypogaea L.) seed has three parts i.e., seedcoat (also known as testa), embryo and endosperm. Seedcoat is the outer protective layer of seed and one of its major roles is to provide protection to embryo and endosperm from external factors such as infection of insects, bacteria, fungi and virus, mechanical injuries, and even desiccation of the seed. In legumes including peanut, the seed coat, and endosperm develop first, followed by the embryo (Weber et al., 2005). Rapid cotyledon growth sometimes may not adequately match the expansion of the seed coat leading to formation of cracks in seed coat (Agarwal and Menon, 1974). In other words, the seed-coat cracking (SC) results from the separation of epidermal (palisade cells) and hypodermal tissues leading to exposure of the underlying parenchyma tissues (Wolf and Baker, 1972). The most adverse effect of SC is that seeds become more vulnerable to storage problems and field microorganisms, leading to seed rotting or pre- and post-emergence damping under high humid conditions. Although the reason for the physical separation of palisade and hypodermal cells is not well-known, genetic and environmental factors have been implicated for SC in other crops such as soybean (Stewart and Wentz, 1930; Woodworth and Williams, 1938; Liu, 1949; Schlub and Schmithenner, 1978; Duke et al., 1983, 1986) and watermelon (Hafez et al., 1981). The seed coat cracks in soybean were linked to physiological and ultimate structure of the cell wall (Kour et al., 2014).

Lignin is a complex and heterogeneous polymer that constitutes one of the major components of the secondary wall of xylem cells and fibers (Mellerowicz et al., 2001). Lignification confers not only the mechanical support and optimizes transport of water and solutes along vascular system but also protects against pathogens (Boerjan et al., 2003). Lignin is the second most abundant biological product in nature, and is formed by oxidative polymerization of three main constituents, namely monolignols p-coumaryl, coniferyl, and sinapyl alcohols through the phenylpropanoid pathway. Once incorporated in the lignin polymer, these precursors are known as p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) subunits, respectively (Anterola et al., October 2016 | Volume 7 | Article 1491). The same study also indicated that the genetic loci for both structural and regulatory genes were scattered across the Arabidopsis genome and were identified largely on the basis of mutations that abolish or reduce pigmentation in the seed coat. The major functional and regulatory genes in flavonoid metabolism include PAL, C4H, 4CL, CHS(TT4), CHI(TT5), F3H(TT6), F3’H(TT7), DFR(TT3), ANS(LODX(TT18), LAR/LCR, BAN(ANR), TT12, TT19(GST), TT10, FLS, and AHA10 etc. (Chapple et al., 1994; Winkel-Shirley, 2001; Abrahams et al., 2003). Regulatory proteins controlling flavonoid biosynthesis were also characterized e.g., MYB-bHLH-WDR (MBW) complex was found to be involved in biosynthesis of PAs and anthocyanins (Baudry et al., 2006; Lepiniec et al., 2006) and the R2R3-MYBs PRODUCTION OF FLAVONOL GLYCOSIDE (PFG1/MYB12, PFG2/MYB11, and PFG3/MYB111) positively regulated flavonol biosynthesis in root and the aerial parts (Dubos et al., 2010; Stracke et al., 2010a,b), whereas single repeat small MYBs CAPRICE (CPC) or MYBL2 was found to be involved in negatively regulating anthocyanin synthesis (Dubos et al., 2008; Zhu et al., 2009).

In peanut, previous studies mainly focused on identifying the antioxidant of seed coat and the extraction pigments (Wang et al., 2007; Ballard et al., 2009; Zhang et al., 2013; de Camargo et al., 2014; Ma et al., 2014). These studies showed that peanut seed coat with different colors were composed of different pigment composition. However, none of the above mentioned studies provided any information on peanut seed-coat cracking and pigmentation mechanism. In this study, we first identified a spontaneous seed coat-cracking and seed color mutant from Zhonghua16, designated as “pscb,” and then employed RNA-seq approach to develop better understanding of the mechanism of seed coat-cracking and brown color development in seed coat of peanut.

MATERIALS AND METHODS

Plant Materials and RNA Isolation
The seed coat crack and brown testa mutant pscb was isolated from an ethyl methanesulfonate (EMS)-mutated population originated from an improved peanut cultivar, Zhonghua 16, with high yield and high oil content. All plants were planted in the experimental farm at the Oil Crops Research Institute (OCRI) in Wuhan.
The wild type (WT) and pscb mutant (M6 generation) were planted in the same field (Wuhan, China). Seed coat samples were taken at 20, 40, and 60 days after flowering (DAF) from 10 different plants in 2014. Twelve representative seeds were sampled from each seedling at each developmental stage of both the wild type and the mutant. Three biological replicates were designed. The tests separated from the sample seeds was sliced. The sliced WT and the pscb mutant testa samples were then frozen rapidly in liquid nitrogen and kept at −80°C, and were later used for extracting the RNA using the Tiangen RNA extraction kit (category number DP432).

Seed Water Uptake and Germination Assays
Seeds used for permeability study were harvested in 2014. For the WT and pscb materials, 30 seeds were tested with three replicates. The seeds were weighed, immersed in tap water for each specific time, removed from the water, blotted with cellulose tissue, weighed again, and kept again into the water. Seeds were weighed at the intervals of 30-min and 60-min during the first 8 h; at 60-min intervals during the last 6 h; and a final measurement at 24 h. The rate of water uptake was calculated by expressing it as weight increase (g) per gram seed weight (initial).

For the seed germination test, seeds were incubated in petri dishes (9 cm diameter) over two layers of medium-speed qualitative filter papers. A total of 20 seeds were placed in each petri dish and added 12 ml of sterile water. Complete experiment was performed in three replications. The seeds were incubated in a 25°C incubator with darkness. Germination was determined based on the radicle breaking through the seed coat. The germination percentage was calculated and recorded at different time points.

Tissue Preparation and Light Microscopy Observations
Peanut seeds were harvested at 20, 30, 40, 50, and 60 DAF and immediately were fixed for 24 h at 4°C in a fixation solution containing 5% acetic acid, 5% formaldehyde, and 50% ethanol. Following fixation, seeds were dehydrated at 60 min intervals through a 20% step-graded series of ethanol-water mixtures, and ended at 100% ethanol. Then, the seeds were processed at 60 min intervals through a 30% step-graded series of ethanol-TBA (tert-butyl alcohol) mixtures, and ended at 100% TBA. Seeds were subsequently infiltrated over a 24 h period with saturated paraffin-TBA mixtures, and then embedded for 48 h period in paraffin. Blocks were completely polymerized at 4°C. Semi-thin (5–8 μm thick) sections were cut with a microtome blade KD-P (Zhejiang Jinhua Kedi Instrumental Equipment Co., LTD, China) and viewed under a stereo microscope (SZX12, Olympus, Japan). Sections were stained with TBO and observed with a Nikon ECLIPSE TI-SR microscope (Nikon Instruments, Japan).

Quantification of Lignin, Anthocyanin, Proanthocyanidin, and Phyto melanin Content
The lignin content was analyzed following Kirk and Obst (1988) and Hoebler et al. (1989), and the extraction of anthocyanins was performed as per Pang et al. (2009). For PA analysis, 0.5–0.75 g of ground samples were extracted using extraction solution containing 5 mL of 70% acetone/0.5% acetic acid. The samples were vortexed and then sonicated at room temperature for 1 h. Following centrifugation at 2500 g for 10 min, the residues were re-extracted twice following the same above mentioned procedure. The pooled supernatants were then extracted three times using chloroform, once with hexane. The supernatants (containing soluble PAs) and residues (containing insoluble PAs) from each sample were freeze dried separately and were then suspended in extraction solution. Total soluble PA content was determined using Spectrophotometer after reaction with DMACA reagent (0.2% [w/v] DMACA in methanol-3 N HCl) at 640 nm, with (+)-catechin as standard.

For quantification of insoluble PAs, 2 mL of butanol-HCl (95:5, v/v) was added to the dried residues and the mixtures were sonicated at room temperature for 1 h, followed by centrifugation at 2500 g for 10 min. The absorption of the supernatants was measured at 550 nm. The samples were then boiled for 1 h and cooled to room temperature, and were measured again. The values were recorded by subtracting the first value from the second. Absorbance values were converted into PA equivalents using a standard curve generated with procyanidin B1 (Indofine). The extraction and characterization of phytomelanins of mature seeds were described in detail by Park et al. (2007). The phytomelanin pigments were extracted from 1 g seeds in 5 mL of 0.5 M NaOH for 1 h. The extracts were purified, and were diluted 20 times with 0.5 M NaOH, and the dilutions were subjected to absorbance measurement at 820 nm using an Ultrospec EPOCH (BioTek, China).

RNA-Seq, Data Processing, and Gene Annotation
According to the mutant seed coat color and crack phenotype, the WT and pscb mutant seed coat were harvested at DAF20, DAF40, and DAF60 followed by RNA sequencing using Illumina HiSeqTM2500 platform at the Novogene company (Beijing) in 2014. Briefly, 3 μg of the total RNA of each sample was used to enrich the mRNA and to construct cDNA libraries. High quality reads (clean reads) were obtained by removing low-quality reads with ambiguous nucleotides, and cutting the adaptor sequences. Transcripts were assembled using Trinity (Grabherr et al., 2011) while gene expression levels were calculated using RPKM (reads per kb per million reads) method of RSEM (Li and Dewey, 2011). Gene function was annotated based on multiple databases namely Nr (NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam (Protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Swiss-Prot (A manually annotated and reviewed protein sequence database), KO (KEGG Ortholog database), and GO (Gene Ontology). The GO enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution (Young et al., 2010), which can adjust for gene length bias in DEGs. KOBAS (Mao et al., 2005) was used to perform KEGG pathway enrichment for the differential expression genes. Picard–tools (v1.41) and samtools
(v0.1.18) were used to sort, remove duplicated reads and merge the bam alignment results of each sample.

qRT-PCR Analysis

The reverse transcriptions were performed using an Invitrogen SuperScript Reagent Kit. The primer was designed using the Oligo6 software. For RT-PCR, the SYBR® Premix ExTaq™ (TAKARA) was used on a Bio-Rad IQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). Gene expression was analyzed for samples at 20, 40, and 60 DAF of WT and mutant. All reactions for each gene were performed in triplicate. The relative expression level of each gene among samples was calculated using the $2^{-\Delta \Delta C_t}$ method with normalization to the internal reference actin gene. The parameters of thermal cycle were 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 50–56°C for 25 s at a volume of 20 µl.

RESULTS

Phenotypic Variation between Seed Coat of Wild and Mutant Genotypes

A peanut mutant with cracked and brown color seed coat named ps cb was identified from a mutant population treated with 1.0% EMS in the background of an elite peanut cultivar Zhonghua16 (WT). In M3 generation, the ratio of ps cb mutant and the normal plants was 1:3 (69 ps cb and 206 normal). Although there was no difference in the seed coat of WT and the ps cb mutant at the early stage, however, few tiny brown points appeared in the seed coat of the ps cb mutant at the stage of DAF40 when the seed coat of WT turned pink gradually. Interestingly, the seed coat of ps cb turned totally brown while the WT were still in pink at DAF60. It was observed that the seed coat developed cracks when plants reached their physiological maturity and seeds were fully developed. At stage DAF60, seed coat cracks became more evident and wide (Figure 1). It is important to mention that cracks only appeared in the outer layer of the seed coat and not in the inner integument (Figure 1). The seed coat cracks in mutant appeared in all the growing conditions (3 years in Wuhan and 3 years in Zhanjiang) in varied intensities i.e., from a minute or invisible crack to several wide cracks. We observed that the mature seed coat of ps cb mutant was much thicker than the WT. In addition, the seed coat of ps cb mutant had 190% higher fresh weight and 150% higher dry weight than that of WT.

The seed coat (testa) of higher plants protects the embryo against adverse environmental conditions including germination control through dormancy imposition and by limiting the detrimental activity of physical and biological agents during seed storage. We subsequently speculated that there may be significant differences between WT and ps cb mutant in seed water uptake and germination. To test this hypothesis, the water uptake and germination experiments were carried out in the WT and the ps cb mutant. After keeping seeds immersed in water for 0.5 h, the ps cb mutant absorbed 11.19% of the seed dry weight water while it was only 7.87% in wild type. The water absorption in ps cb mutant was recorded 14.75, 17.69, 21.16, 24.37, 27.75, 38.94, 42.32, 44.06, 54.12, 54.12, and 54.12% at time interval of 1, 1.5, 2, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 18.5, 19.5, and 20.5 h, respectively. While at each paired time points, the water absorbed by the wild type was 1.58–6.43% lower than that in ps cb mutant (Figure 2A). After absorption of enough water, the seeds of ps cb mutant and wild type were transferred to a 25°C incubator. The germination rate of wild type was 29.40% as compared to only 15.4% observed in ps cb mutant, the differences enlarged till 24 h and 36 h and then narrowed at 48 h. Till 60 h, all the seeds germinated (Figure 2B) and only the length of bacon differed between wild type and ps cb mutant (Figure 2C). In other words, the results showed that the ps cb mutant had faster water uptake efficiency and delayed germination compared to WT.

Estimation of Lignin, Anthocyanin, and Proanthocyanidin Contents

It is well-known fact that the seed crack is related to physiological and ultimate structure of the cell wall. Since the lignin in the early stages of peanut seed coat is extremely low in quantity to detect, we analyzed the lignin content of the mature seed coat of the ps cb mutant and the WT. The results showed that the lignin content in ps cb mutant was 40.2% less as compared to WT (from 19.38 to 7.79 mg/g FW; Figure 3A). The ps cb mutant showed brown seed coat color from DAF40, indicating change in the seed coat pigments. Direct measurement of anthocyanins and PAs content confirmed significant difference between ps cb mutant and WT seed coat extracts (Figures 3B,D). The anthocyanins content was obviously lower i.e., 9.9 µg/g FW in ps cb mutant as compared to WT i.e., 231 µg/g FW (Figure 3B). To quantify PAs accumulation, we extracted soluble and insoluble (non-extractable) PAs separately. Soluble PAs content in WT was 0.415 µg/g FW whereas the level of soluble PAs in ps cb mutant was almost undetectable i.e., mere 0.0076 µg/g FW (Figure 3C). Measurement of insoluble PAs based on butanol-HCl hydrolysis showed that ps cb mutant had less non-extractable PAs (Figure 3D).

To determine the cellular distribution of polyphenol compounds in developing seeds of ps cb mutant and WT seed coat, a histochemical analysis was performed in the seed coat harvested at different developmental stages. TBO
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FIGURE 2 | Water uptake efficiency and germination in pscb mutant and WT. (A) Water uptake efficiency of pscb mutant and WT. (B) Germination time of pscb mutant and WT. (C) The radicals elongated faster in WT when compared with pscb mutant.

(toluidine blue O) staining of transverse sections of developing seeds revealed that at early seed development stages (DAF20 and DAF30), staining was similar in pscb mutant and WT (Figures 3e-1, e-2, f-1, f-2). At 40DAF, WT stained more intensely than the pscb mutant in the outer layer of the testa, indicating that more polymeric phenolic compounds had accumulated in WT (Figures 3e-3, f-3). The distribution of polymeric phenolic compounds was significantly different at the two late seed development stages (Figures 3e-4, e-5, f-4, f-5).

Seed Coat Transcriptome Differences between pscb Mutant and WT during Seed Coat Development

In order to understand the mechanism of seed coat development, we selected RNA samples of seed coat from three seed developmental stages i.e., DAF20, DAF40, and DAF60 showing different seed coat phenotype to perform the transcriptome analysis. The sequence data was deposited in the BioProject database of National Center for Biotechnology Information under the accession number PRJNA324725. One of the primary goals of transcriptome sequencing was to compare the gene expression levels in pscb mutant and WT. In this study, we used a stringent value of FDR ≤0.001 and fold change ≥2 as the threshold to judge the significant differences in the gene expression. A total of 5726 genes were found differently expressed between pscb mutant and WT (Table S1). Of these, 255 genes expressed at DAF20, 5443 genes at DAF40, and 341 genes at DAF60 (Figure 4). At DAF20, the number of up-regulated genes was more than the down-regulated genes (165:90). At DAF40, there was not much difference in the number of up-regulated and the down-regulated genes (2544:2899). At DAF60, the number of up-regulated genes was half of the number of the down-regulated genes (110:231). It was important to note that the number of DEGs increased significantly at the stage of DAF40, which was the vigorous growth stage during seed development (Figure 4).

To gain insights into the functional categories that were altered between pscb and WT (Zhonghua16), GO categories were assigned to the DEGs. Further, GO enrichment analysis of the DEGs in different developmental stages between the pscb mutant and WT (Zhonghua16) was performed for different developmental stages. Interestingly, no GO terms were enriched at DAF20, however, several significantly enriched terms in the biological process, molecular function, and cellular component categories were identified at DAF40 and DAF60 (Table 1). ADP binding (GO: 0043531), structural constituent of cell wall (GO: 0005199), plant-type cell wall organization or biogenesis (GO: 0071669), plant-type cell wall organization (GO: 0009664), and external encapsulating structure organization (GO: 0045229) were dominant terms at DAF60 in comparisons to DAF40. At DAF40, seed coat gets brown phenotype in
the \textit{pscb} mutant, indicating the difference between cell wall organizations might play an important role in the phenotype differentiation. At the DAF60, the main GO terms were related to fatty acid synthase and oxidoreductase activity (Table 1), including 3-oxoacyl-[acyl-carrier-protein] synthase activity (GO: 0004315), fatty acid synthase activity (GO: 0004312), fatty acid synthase complex (GO: 0005835), transferase activity, transferring acyl groups other than amino-acyl groups (GO: 0016747) etc. KEGG pathway analysis assigned the differential genes to 37, 287, and 46 metabolic pathways in three different developmental stages of \textit{pscb} and Zhonghua 16. The complete list of metabolic pathways is provided in Table S2. Table 2 lists the metabolic/biological pathways in common of \textit{pscb} compared with WT. Notably, among the 16 common KEGGs, 6 were involved in Tyrosine, Tryptophan, and Phenylalanine metabolism (Table 2).

**Verification of Differentially Expressed Genes during Seed Coat Development in \textit{pscb} Mutant and WT**

Transcriptional regulation revealed by RNA-seq data was confirmed in a biologically independent experiment using the quantitative reverse transcription PCR. A total of 17 genes related to cell wall organization were selected to design gene-specific primers (Table S3) for real time PCR analysis (Figure 5). A linear regression analysis showed an overall correlation coefficient of $R = 0.622$, which indicated a good correlation between transcript abundance assayed by real-time PCR and the transcription profile revealed by RNA-seq data (Figure 5B).
### TABLE 1 | Functional categorization of genes with significant transcriptional differences between pscb mutant and WT.

| GO accession       | Description                                             | Term type* | Corrected p-value | DEG item |
|-------------------|---------------------------------------------------------|------------|-------------------|----------|
| DAF40             |                                                        |            |                   |          |
| GO:0043531        | ADP binding                                             | MF         | 8.55E-08          | 100      |
| GO:0005199        | Structural constituent of cell wall                     | MF         | 0.00025097        | 15       |
| GO:0071669        | Plant-type cell wall organization or biogenesis         | BP         | 0.0061632         | 18       |
| GO:0096684        | Plant-type cell wall organization                       | BP         | 0.013663          | 17       |
| GO:0045229        | External encapsulating structure organization           | BP         | 0.031101          | 30       |
| GO:0071555        | Cell wall organization                                  | BP         | 0.041254          | 28       |
| GO:0080312        | External encapsulating structure                        | CC         | 0.041254          | 49       |
|                  |                                                        |            |                   |          |
| DAF60             |                                                        |            |                   |          |
| GO:0004315        | 3-oxoacyl-[acyl-carrier-protein] synthase activity      | MF         | 0.0066424         | 5        |
| GO:0004312        | Fatty acid synthase activity                           | MF         | 0.0066424         | 5        |
| GO:0016747        | Transferase activity, transferring acyl groups other than amino-acyl groups | MF         | 0.012673          | 12       |
| GO:0016706        | Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, 2-oxoglutarate as one donor, and incorporation of one atom each of oxygen into both donors | MF         | 0.012673          | 8        |
| GO:0016491        | Oxidoreductase activity                                | MF         | 0.012673          | 40       |
| GO:0016705        | Oxidoreductase activity, acting on paired donors, with incorporation, or reduction of molecular oxygen | MF         | 0.016231          | 15       |
| GO:0043115        | Precorrin-2 dehydrogenase activity                     | MF         | 0.016392          | 5        |
| GO:0003824        | Catalytic activity                                     | MF         | 0.020356          | 127      |
| GO:0008171        | O-methyltransferase activity                           | MF         | 0.020356          | 6        |
| GO:0016628        | Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor | MF         | 0.020356          | 6        |
| GO:0006633        | Fatty acid biosynthetic process                        | BP         | 0.012673          | 10       |
| GO:0072330        | Monocarboxylic acid biosynthetic process               | BP         | 0.016392          | 10       |
| GO:0019354        | Siroheme biosynthetic process                          | BP         | 0.016392          | 5        |
| GO:0046156        | Siroheme metabolic process                             | BP         | 0.016392          | 5        |
| GO:0008610        | Lipid biosynthetic process                             | BP         | 0.017694          | 19       |
| GO:0006631        | Fatty acid metabolic process                           | BP         | 0.020356          | 10       |
| GO:0044710        | Single-organism metabolic process                      | BP         | 0.020356          | 73       |
| GO:0055114        | Oxidation-reduction process                            | BP         | 0.028844          | 38       |
| GO:0006629        | Lipid metabolic process                                | BP         | 0.030943          | 26       |
| GO:0005835        | Fatty acid synthase complex                            | CC         | 0.0066424         | 5        |

The genes were categorized based on GO. "MF," molecular function; "BP," biological process; "CC," cellular component.

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The **pscb** Mutant Seed Coat Accumulates Phytomelanin Through Higher Level of Polyphenol Oxidases and Peroxidase Expression

Various methods were used to solubilize and characterize the molecule(s) imparting the seed coat color that contribute to the brown pigmentation in **pscb** mutant seed. Compounds comprised of anthocyanins and proanthocyanidin were eliminated as candidates since their contents in **pscb** mutant were much lower than WT. Both bleach and peroxide were capable of removing the brown testa color of the **pscb** mutant seed coat. The intransigent nature of the dark compound, particularly its stability under acid hydrolysis and its susceptibility to the two treatments mentioned above, were reported as hallmarks of melanin, a class of chemically resistant phenolic polymers.

WT and **pscb** seed coat, when hydrolyzed with NaOH, produced little or no precipitate when the hydrolysates were subsequently acidified to pH 2. Contrasted with hydrolysates, both the **pscb** and WT seed coat produced abundant precipitates upon acidification. Furthermore, the precipitates could be resolubilized in NaOH or in dimethyl sulfoxide (DMSO), consistent with the hypothesis that the black pigment was melanin in nature, means both the WT and **pscb** seed coat had melanin constituents. The melanin content in **pscb** was 64.3 mg/g FW as compared to WT i.e., 27.6 mg/g FW (Figure 6A). Among the DEGs of three different stages of **pscb** and WT, there were nine polyphenol oxidases (PPOD) (Figure 6B, Table S4) and 24 peroxidases (POD) (Figure 6C, Figure S2, Table S4) obviously increased during the late developmental stages especially at DAF60 when compared with the WT. Most of the PPOD and POD showed different expression patterns among the developing process between **pscb** mutant and WT. In the **pscb** mutant, these genes had higher expression level during the seed coat development, while these genes either declined or remained stable in WT.
TABLE 2 | KEGG pathways in common of pscb mutant compared with WT at DAF20, DAF40, and DAF60.

| KEGG pathway                        | Gene number |
|-------------------------------------|-------------|
|                                     | DAF20 | DAF40 | DAF60 |
| Fatty acid elongation                | 1     | 6     | 7     |
| Isoquinoline alkaloid biosynthesis   | 1     | 2     | 1     |
| Tropane, piperidine and pyidine      | 1     | 2     | 1     |
| Stilbenoid, dihydroxybenzene and     | 1     | 5     | 1     |
| flavonoid biosynthesis               |        |       |       |
| Tyrosine metabolism                 | 1     | 8     | 1     |
| Beta-Alanine metabolism              | 1     | 10    | 1     |
| Cysteine metabolism                 | 1     | 10    | 1     |
| Tryptophan metabolism                | 1     | 11    | 1     |
| Flavonoid biosynthesis               | 3     | 11    | 9     |
| Amino sugar and nucleotide sugar     | 3     | 13    | 1     |
| metabolism                          |        |       |       |
| Phenylalanine metabolism            | 2     | 20    | 6     |
| Pyrimidine metabolism               | 1     | 31    | 1     |
| Phenylpropanoid biosynthesis        | 2     | 33    | 6     |
| Oxidative phosphorylation           | 1     | 38    | 1     |
| Protein processing in endoplasmic   | 1     | 50    | 2     |
| reticulum                           |        |       |       |
| Plant hormone signal transduction   | 1     | 56    | 7     |

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| Amino sugar and nucleotide sugar     | 3     | 13    | 1     |
| metabolism                          |        |       |       |
| Phenylalanine metabolism            | 2     | 20    | 6     |
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| Phenylpropanoid biosynthesis        | 2     | 33    | 6     |
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| reticulum                           |        |       |       |
| Plant hormone signal transduction   | 1     | 56    | 7     |

Table 2 KEGG pathways in common of pscb mutant compared with WT at DAF20, DAF40, and DAF60. Bold means important KEGGs related to the phenotype.

Transcriptional Regulation of ABA and Ethylene Signal Transduction Related Genes during Peanut Seed Coat Development

The KEGG analysis of the RNA-seq data indicated significant change in the expression of ABA and ethylene signal transduction related genes in peanut seed coat, especially the genes of ABA signal transduction pathways. Previous study showed that ABA and ethylene in the maturation process play important roles, and between them there was a very close interaction. To better understand the transcriptional regulation of ABA and ethylene-related genes in peanut seed coat development, genes related to ABA and ethylene signal transduction were analyzed in the three different developing stages of WT and pscb mutant seed coat. At the early developing stage (DAF20), there was no DEG related to ABA and ethylene signal transduction. However, at DAF40, there were 13 DEGs in the ABA signal transduction between the pscb mutant and WT, including seven PYR/PYL family absicic acid receptors (down-regulated), five PP2C (three down-regulated and two up-regulated), and two SnRK2 proteins (one up-regulated and one down-regulated) (Table S5), showing that the ABA signal transduction was weak in the mutant. The analysis of ethylene signal transduction related genes revealed a differential regulation between pscb mutant and wild type. The overall expression patterns of the genes in ethylene signal transduction were almost the same which were increased or kept invariant in WT and down-regulated in pscb mutant (Table S5). Such as ethylene-responsive transcription factor 1 (c31399_g2), the expression level stabilized around 2 (2.68, 1.96, 2.51) FPRK in WT of three stages, while in pscb mutant, declined from 1.32 to 0.12 FPRK.

Candidate Genes Related to Peanut Crack and Brown Seed Coat in pscb

In order to identify candidate genes controlling seed-coat cracking and seed color, we analyzed common DEGs between WT and pscb mutant from the three different developmental stages. The above analysis resulted in identification of 62 unigenes in WT and pscb mutant (Figure S1, Table S6). Among the common DEGs, we found three putative candidate genes (c36498_g1:CCoAOMT1, c40902_g2:kinesin, and c33560_g1:MYB3), which were significantly down-regulated in the pscb (Table S6). In the seed coat of wild type, the FPRK value of c36498_g1 gene decreased from 10.96 (DAF20) to 3.38 (DAF40) and then to 8.59. In case of the pscb mutant, the FPRK value of c36498_g1 gene declined from 0.58 (DAF20) to 0.24 (DAF40) and then to 0.03 (DAF60). The expression of c40902_g2 gene in WT went up gradually from 5.31 (DAF20) to 7.29 (DAF40) and then 18.04 (DAF60). In contrast, the expression level of c40902_g2 in pscb mutant went down from 0.38 (DAF20) to 0.34 (DAF40) and then to 0.17 (DAF60). Very few reads of c33560_g1 gene were detected in pscb mutant, the FPRK were 0.08 (DAF20), 0.07 (DAF40), and 0 (DAF60), while in case of wild type, the FPRK value changed from 5.00 (DAF20) to 2.67 (DAF40) and then 2.77 (DAF60). C36498_g1 was a caffeoyl-CoA O-methyltransferase which was reported in Phenylalanine metabolism. The CCoAOMT1 gene from maize, medicago, jute, poplar, Zinnia elegans, Arabidopsis, and loblolly pine were involved in lignin biosynthesis and cell wall organization (Ye et al., 1994, 2001; Ye and Varner, 1995; Goujon et al., 2003; Zhou et al., 2010; Wagner et al., 2011; Li et al., 2013; Zhang et al., 2014). C40902_g2 encode a kinesin-4-like protein, and kinesin protein were reported functioned in cell wall organization (Zhong et al., 2002; Zhang et al., 2010; Fujikura et al., 2014; Kong et al., 2015). c33560_g1 was an R2R3-Myb transcription factor encoding gene. Previous studies showed R2R3-Myb factor worked combined with other transcription factors together to regulate flavonoids and phenylalanine metabolism further regulate the proanthocyanidin biosynthesis (Baudry et al., 2006; Quattrocchio et al., 2006). These genes that might lead to the brown seed color and crack seed coat phenotype need to be confirmed in further functional genomics studies.

DISCUSSION

The conventional understanding of the role of the seed coat is that it provides a protective layer for the developing zygote. It also acts as channel for transmitting environmental cues to the interior of the seed which helps seed to adjust its metabolism in response to changes in its external environment (Radchuk and Borisjuk, 2014). In peanut, flavonoid, and phenylpropanoid biosynthesis pathways were reported to be related with aflatoxin resistance (Garcia et al., 2013; Wang et al., 2016). Therefore, the research on seed coat cracking and pigmentation/color will not only help in understanding and improving the peanut seed quality, it will also help in understanding the genetic control for few seed borne diseases such as aflatoxin contamination. In the present study, RNA-seq was used to investigate the differences...
FIGURE 5 | qRT-PCR validation of differential expression. (A) Transcript levels of 17 genes, which were involved in plant cell wall organization (B) Comparison between the gene expression ratios obtained from RNA-seq data and qRT-PCR. The RNA-seq log2 value of the expression ratio (y-axis) has been plotted against the developmental stages (x-axis).
FIGURE 6 | The pscb mutant testa accumulates phytomelanin through higher level of peroxidase and polyphenol oxidase expression. (A) Phytomelanin contents in pscb mutant and WT matured seed coat. The phytomelanin content was expressed as the mg g⁻¹ fresh weight of seed coat. (B) Heatmaps represent the expression level of nine peroxidases in pscb mutant and WT seed coat of DAF20, DAF40, and DAF60. (C) Heatmaps represent the expression level of 24 polyphenol oxidase in pscb mutant and WT seed coat of DAF20, DAF40, and DAF60. The gene expression was scaled using Z-score of FPKM in the heatmap.

in the transcriptome between the three different stages of pscb mutant and its WT. Thousands of genes that were differently regulated during the three stages of seed coat development were identified by transcriptomic profiling.

Seed Coat Pigmentation was Redirected in pscb Mutant between Anthocyanin, Proanthocyanidin, Melanin, and Lignin

Flavonoids are the secondary metabolites that accumulate in plants and promote seed and pollen dispersal by contributing to color formation in fruits and flowers (Winkel-Shirley, 2001). Previously, researchers showed that epicatechin derivatives (Marinova et al., 2007; Zhao and Dixon, 2009) and a PA monomer (Holton and Cornish, 1995; Grotewold, 2006) are important flavonols in the synthesis of the seed coat of Arabidopsis and Medicago. Originally when we first observed the pscb mutant, we thought the pigmentation must be markedly enhanced when compared with WT for the sight of deeper color. However, compounds comprised of anthocyanins and lignins were eliminated as candidates, in contrast the production of anthocyanins and proanthocyanidins was reduced in the pscb mutant seed coats. We noticed that both bleach and peroxide were capable of removing the dark testa color of the pscb mutant seed and the dark compound was stable under acid hydrolysis, these features were reported as hallmarks of melanin (Fogarty and Tobin, 1996; Sava et al., 2001), a class of chemically resistant phenolic polymers. Melanin, was reported as black pigments, especially in the seed coat of composite, morning glory and many oilseed Brassica species (Park et al., 2007; Park and Hoshino, 2012; Yu, 2013). Surprisingly, the melanin content in the pscb mutant was more than twice as compared to WT. The seed coat crack and brown color might be contributing for the inhibition of flavonoid and lignin metabolism pathway, and the accumulation of the upper component of the aromatic amino acid converted to be melanin and compensating for the disadvantages of the lower anthocyanin and proanthocyanidin content.

Potential Mechanisms Underlying Seed Coat Color and Crack in Peanut

Flavonoids are plant secondary metabolites derived from the phenylpropanoid pathway. The flavonoid pathway in Arabidopsis has been characterized mainly using the mutants. Twenty-three genes have already been identified at the molecular level corresponding to several enzymes (CHS, CHI, F3H, F3'H, DFR, LDOX, FLS, ANR, LACCASE), transports (TT12, TT19, AHA10), and regulatory factors (TT1, TT2, TT8, TT16, TTG1, TTG2, PAP1, GL3, ANL2, FUSCA3, KAN4) (Baxter et al., 2005; Li et al., 2010). The transcriptome data generated in this study showed that the F3H, F3’H, DFR, and ANR were suppressed in the stage of DAF40. These results suggested that DAF 40 was the key growth stage for the anthocyanins and proanthocyanidins accumulation. Further, F3H, F3’H, DFR, and ANR are the key
genes underlying differences in seed coat pigmentation in pscb mutant and WT.

Melanin production resulting in black pigmentation proceeds by one of two pathways in plants. The first leads to compounds of the allomelanin variety deviating in the shikimate acid pathway at β-coumarate before the flavonoids (Goodwin and Mercer, 1983). The second produces eumelanin from Tyr via oxidation of DL-dioxy-Phe and is divorced from the shikimate pathway altogether. Polymerization of melanic compounds produces peroxide (Blois, 1978) and trapped free radicals that should lead to an up-regulation of PRX activity and an EPR signal, respectively. The transcriptome analysis revealed that many structural genes which are involved in the phenylalanine metabolism were found down-regulated in the pscb mutant (Figure 7) in addition to decrease of the lignin, anthocyanin, and proanthocyanidin content. In contrast, there were also nine polyphenol oxidases and 24 peroxidases obviously increased at the late developmental stages especially at DAF60 when compared with the WT.

It has already been reported that the down regulation of C3H, 4CL1, F5H, or COMT enzymes in A. thaliana affects the final lignin composition (Chapple et al., 1994; Lee et al., 1997; Meyer et al., 1998; Franke et al., 2002; Goujon et al., 2003). The repression of this set of genes in transgenic plant leads to a 70% reduction in the total lignin content and resulted in severe phenotypic effects. We recently described that the lignin content in the pscb mutant decreased by 60%, this strong reduction in lignin content can be related with the down regulation of genes involved in lignin synthesis pathway such as CAD, F5H, or COMT. The above results indicate that the synthesis mechanism of lignin in peanut is similar to other plants.

Previous study hold the idea that lignin is related to PA due to common steps in the phenylpropanoid pathway. The low lignin is found strongly associated with the unpigmented seed coat trait as lignin is usually accumulated within the cell wall whereas PA is usually accumulated in endoplasmic reticulum vesicles or in the plant vacuole. Lignin variability may influence seed coat pigment extractability, owing to the position of the highly lignified palisade cells adjacent to the inner integument in the seed coat, where pigment is initially deposited (Marles and Gruber, 2004). Here, our result showed that the anthocyanins, proanthocyanidins, and the lignin content were reduced while the melanin content was enhanced, indicating new regulation mechanism may exist in peanut seed coat.

Regulation Mechanisms Underlying the Biosynthesis of Seed Coat Pigment in Peanut

Plant hormone and transcription factors play an important role in plant seed development. Through the analysis of the

![Phenylalanine metabolism was down-regulated in pscb mutant seed coat](image)

**FIGURE 7 | Phenylalanine metabolism was down-regulated in pscb mutant seed coat.** PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeic acid O-methyltransferase; COAOCT, caffeoyl-CoA O-methyltransferase; F5H, ferulate-5-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3′H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; UGTs, UDP sugar glycosyltransferases; DFR, dihydroflavonol reductase.
In this study, one R2R3-MYB transcription factor (c33560_g1), the homolog in apple, was suggested to play a role in fruit anthocyanin biosynthesis (Vimolmangkang et al., 2013). Through RNA-seq data, we found that hormone-related genes in the pscb mutant seed coat during seed development changed greatly when compared with WT especially the ABA and ethylene signal transduction pathway. Plant hormone ABA has been suggested to play a role in fruit anthocyanin biosynthesis (Shen et al., 2014; Li et al., 2015). Our transcriptome data showed that all the seven ABA receptor PYR/PYL genes, which presented in the KEGG “plant hormone signal transduction,” were obviously down regulated in the pscb mutant indicating the ABA signal transduction might be strongly suppressed in the mutant leading to the lower anthocyanin and proanthocyanidin content. Ethylene is required for the onset of accumulation of anthocyanins (Chervin et al., 2004). In this study, one ETR1, three EBF1, two EIN3, and two ERF1 involved in ethylene signal transduction were down regulated at DAF40 in pscb mutant. Further, the three EBF1 and two ERF1 were also down-regulated in the stage of DAF60, which strongly demonstrate that the ethylene signaling was suppressed in the mutant. MYB transcription factors have been well-reported in plant development in different species (Appelhagen et al., 2011; Liu et al., 2014; An et al., 2015; Cavallini et al., 2015; Yoshida et al., 2015). Through analysis of the common DEGs of the three different stages, we identified a R2R3-MYB transcription factor (c33560_g1), with extremely low expression in the stages of DAF20 and DAF40 and none expression in the late stage of DAF60 in pscb mutant. By blasting the protein sequence of c33560_g1 in The Arabidopsis Information Resource (TAIR), AT1G22640 (MYB3) showed the highest similarity, while AT1G22640 was reported as an MYB-type transcription factor (MYB3) that represses phenylpropanoid biosynthesis gene expression (Rowan et al., 2009). The MdMYB3, the homolog in apple, was similarly identified as regulator of anthocyanin biosynthesis and flower development (Vimolmangkang et al., 2013), indicating the potential function of c33560_g1 in peanut seed coat pigmentation.

In the present study, the ABA pathway, ethylene pathway, and the R2R3-MYB transcription factor (c33560_g1) were all different between pscb mutant and WT and were selected as important candidate for the format of the cracking and brown seed coat phenotype. We hypothesize that the R2R3-MYB transcription factor (c33560_g1), ABA and ethylene signaling pathways interact cooperatively to suppress the anthocyanin, proanthocyanidin, and lignin synthesis related pathways’ genes to influence the anthocyanin, proanthocyanidin and lignin level. Simultaneously, the enhanced expression of POD and PPOD encoding genes further regulate seed coat pigmentation (Figure 8). The above does not prove the interaction relationship of the three factors responsible for seed-coat cracking and brown seed color trait of pscb mutant and requires further detailed study.

AUTHOR CONTRIBUTIONS
Conceived and designed the experiments: LW, YL, LY, HJ, BSL. Performed the experiments: LW, YW, BL. Analyzed the data: LW, MP. Contributed reagents/materials/analysis tools: GW, XD, YL, HJ. Wrote the paper: LW, MP, RV, BSL. All authors have read and approved the manuscript.

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SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01491

Figure S1 | Venn diagram showing the number of genes with increased and decreased expression of three different development stages.

Figure S2 | qPCR verification of the POD and PPOD change in pscb mutant compared with WT.

Table S1 | DEGs between pscb and WT at DAF20, DAF40, and DAF60.

Table S2 | KEGG pathway of the DEGs between pscb mutant and WT.

Table S3 | Gene-specific primers for real time PCR analysis.

Table S4 | Expression and annotation the differential expressed POD and PPOD genes.

Table S5 | Differentially expressed genes involved in ABA and ethylene signal transduction between pscb mutant and WT.

Table S6 | Common DEGs between WT and pscb mutant of the three different developmental stages.
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