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Full Length Article

Transcriptome and miRNA Profiling of a Hydroxyproline-Tolerant Peanut Mutant with Higher Grain Size and Oil Contents

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Abstract

Peanut is an important oilseed crop. In previous studies, a hydroxyproline (HYP)-tolerant peanut mutant, with increased oil contents and 100-grain weight, was introduced. In this study, transcriptome analysis and miRNA profile sequencing of seeds from the HYP-tolerant mutant and the original parent (Huayu 20, the control) were conducted to elucidate the molecular basis for higher grain size and oil contents. Major transcription factors linked to seed development and/or oil biosynthesis (including AP2/EREBP, WRKY, bZIP, DOF, B3 domain, MADS-box, bHLH, and MYB) were differentially expressed between the HYP-tolerant mutant and its parent. Moreover, differentially expressed genes related to seed development or oil biosynthesis like *IKU2*, oleosins, *LTPs*, *SSPs*, *ACCases*, *ACP*, and *BCCPs* were also identified. The miRNA profiling identified 116 differentially expressed miRNAs; and functional analysis emphasized that their target genes i.e., *SPL*, *KCS*, *PLC*, *B3* domain transcription factors played an important role during seed development. Findings of this study are highly helpful for further research on peanut seed development and oil biosynthesis. Moreover, this study also provided a potentially usable genomic resource material which can be used for breeding high yielding peanut varieties with more oil contents. © 2019 Friends Science Publishers

Keywords: Differentially expressed genes; Hydroxyproline tolerance; miRNA; Mutation breeding; Seed development

Introduction

Peanut (*Arachis hypogaea* L.) is an important oilseed crop in tropical and subtropical regions which is grown in more than 100 countries in the world (FAO, 2014). Seeds of peanut contain oil (40–56%), protein (20–30%), carbohydrate (10–20%), and several nutritional components such as vitamin E, calcium, magnesium, and potassium (Dean *et al*., 2009). About 50% of peanut grown in China are used to extract oil and therefore oil contents is an important quality trait targeted by breeders (Yu, 2008). Thus, a peanut germplasm with high oil contents and yield will raise the value of a peanut variety (Chen *et al*., 2014). However, peanut germplasm resources with high oil content are currently inadequate, thus impeding progress in cross breeding (Yu, 2008).

In a previous study *in vitro* mutagenesis was conducted on peanut embryonic leaflets from mature seeds using pingyangmycin as the mutagen and hydroxyproline (HYP) as the screening agent. Peroxidase (POD) and superoxide dismutase (SOD) activities in eight offspring from 11 regenerated plants were substantially increased relative to those in the mutagenised Huayu 20 parent after drought treatment of seedlings (Sui *et al*., 2015). The pod weights for some M_3-generation individuals were increased, and oil content was significantly higher in 19 M_3 individuals relative to that in the parent (Sui *et al*., 2015). One derivative had an oil content (60.5%) that was substantially higher than that the parent (50%). This high oil contents character was stable in the subsequent self-pollinated progenies (Sui *et al*., 2015).

However, little is known about the molecular mechanisms of this HYP-tolerant peanut mutant. High-throughput RNA and miRNA sequencing are effective technologies for the identification of differentially expressed genes (DEGs), miRNAs and their target genes (Karlova *et al*., 2013; Tombuloglu *et al*., 2015). These powerful technologies make it possible to study non-model plants such as peanut (Karlova *et al*., 2013; Tombuloglu *et al*., 2015).

In this study, the transcriptome and miRNA profiles of seeds from the HYP-tolerant mutant were compared with those from Huayu 20. Dynamic trends in transcriptome and

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microRNA (miRNA) profiles were recorded during seed development. Specific transcripts, miRNAs, and their target genes were identified and analyzed, and the molecular mechanism of the HYP-tolerant peanut mutant was explored.

Materials and Methods

Materials Treatment

Seeds of the HYP-tolerant peanut mutant characterized by high oil contents and high 100-grain weight (denoted by H) and the parent Huayu 20 as control (denoted by C) were grown in the field. The experimental field was ridged with 85 cm between adjacent ridges. Each ridge was planted with one row, with one seed per hole and 18 cm between adjacent holes. At 35, 55 75 days post planting and after the gynophores had penetrated into the soil, pods containing young seeds with similar size were sampled. The H samples were labeled as H-35, H-55, and H-75, and the C samples were labeled as C-35, C-55, and C-75 on the basis of sampling time. Three biological replications were conducted for every treatment.

Library Construction for Transcriptome Sequencing

The seeds of 35, 55 and 75 days samples from the HYP-tolerant mutant and the control were prepared after peanut gynophores penetrated the soil, then RNA was isolated from the samples using Trizol reagent and RNA sequencing was conducted by Novogene Bioinformatics Technology Co., Ltd. (Beijing).

Identification of differentially Expressed Genes (DEGs)

Within-genotype and between-genotype expression levels were compared. D series data sets were obtained by comparison between the H and C genotypes, and were denoted as D-35, D-55, and D-75, respectively. The dynamic discrepancies of DEGs in H, C, and D data sets were analyzed in accordance with standards mentioned below: the adjusted p value <0.05 and $|\log_2$ (fold change) $| \geqq 1$. Statistically significant pathways (FDR ≤ 0.05) were enriched with KEGG. Functional gene annotation was from the reference genome annotation information published by PeanutBase (https://peanutbase.org/).

Library Preparation for miRNA Sequencing and Identification of Conserved and Novel miRNAs

Sequencing libraries were generated, and the small RNA tags were mapped to a reference sequence using Bowtie (Langmead *et al*., 2009). Mapped small RNA tags were used to screen known miRNAs. The miREvo (Wen *et al*., 2012) and miRDeep2 (Friedlander *et al*., 2011) programs were used to predict novel miRNAs. The miRNA target gene was predicted by ps Robot_tar for plants (Wu *et al*., 2012). The adjusted p-value of 0.05 was used as the default threshold for detection of significantly differential expression.

Quantitative RT-PCR (qRT-PCR)

A real-time PCR detection system with the SYBR® Premix Ex Taq™ was used for qRT–PCR. Gene expression levels of the seeds (H and C) samplecd at 35, 55 and 75 days were analyzed. The relative expression level of each gene was calculated relative to the internal peanut *AhActin* reference gene using the $2^{-\triangle\triangle Ct}$ method. All reactions for each gene were conducted in 20 μL volumes with three replicates. The thermal cycling parameters were 95°C for 30 s, followed by 40 cycles at 95°C for 10 s and 50°C–56°C for 25 s. The primers used in the current experiment are listed in Table 1.

Results

Transcriptome Library Sequencing, Clustering Analysis of Samples, and Identification of DEGs

The total reads in the C genotype ranged from 40,914,282 to 52,140,220, and the total reads in the H genotype ranged from 41,573,154 to 50,804,756. Approximately 89.1–91.1% of the clean reads from each sample were mapped to the reference genome published by PeanutBase.

To obtain an overall view of the gene expression profiles, the 18 samples from the H and C genotypes were analyzed by cluster analysis and TreeView. Samples from the same time point gave very similar gene expression patterns (Fig. 1).

In current studies, after multidimensional comparisons, the DEGs were identified in accordance with the following standard: adjusted p-value < 0.05 and |log2 (fold change) $| \geqq 1$. The dynamic trends of DEGs in H, C, and D data sets were explored. Significant changes in gene expression profiling were screened. The highest number of DEGs was found between samples from 35 days to 75 days, whereas the lowest number of DEGs was found between samples from 55 days to 75 days in both H and C genotypes (Fig. 2). Totals of 353 DEGs in the C genotype and 647 DEGs in the H genotype were in common at the three aforementioned time points (Fig. 3a, b). A peak appeared at 75 days for all upregulated and downregulated DEGs in the D series data sets (Figs. 2, 3c). D-55 and D-75 had the least and most exclusive DEGs, respectively. In addition, 237 DEGs appeared in their neighboring data sets (Fig. 3c).

Annotation and KEGG Enrichment Analyses of DEGs Based on the Comparison of H vs. C genotypes (D Series Data Sets)

Functional annotation of DEGs was mainly referenced

Table 1: Primers for real-time PCR

miRNA or gene	Forward	Reverse
ahy-MIR3513	5' GAGTTTGTCATCTGAACTTC3'	5' AAACCAACAACACATGTAAC3'
novel_97	5' AACGCTTCATGCGACTGATG3'	5' AGAGTTCCCCCAAACACTTC3'
novel_67	5' AACGACCAGAACTTTCAGCT3'	5' GTTCCCCCAAACACTTCATA3'
novel_55	5' TTGATTGGTGGTTGTTTATG3'	5' TATTTGATTGCTGGTTGTTC3'
novel_216	5' TTCTTGGTTGAACCGATTCG3'	5' TCGGACCGGTCAGTCGAATC3'
novel_230	5' GATCTCTGAAATTATATTCG3'	5' AATAGAGACTAACCTGATTA3'
novel_194	5' AGGGAGAAGAAACGATGGAG3'	5' TAACTAGGCAAATGGAGTGC3'
novel_127	5' CCGTGCTATTATTCTTGCCG3'	5' AGCCCTATTTCCAGTTTGGT3'
novel_132	5' TAATTGAGTCCTTACACCAA3'	5' GGTCCTGCACTAATTTTTTT3'
107467011	5' TGATGATGAGCAGAAGCAAC3'	5' CAACATAAGGTAAGCCAGGG3'
107494415	5' AGCCTCCTCACTTCTCCTTC3'	5' ATGTCATTTGGTTTCCTTGC3'
107635517	5' GACATCAATTATATACGCCC3'	5' GTAGACACCCAAATACCCTC3'
107635569	5' CGAGCATGGAGAAGAAGAAG3'	5' TATGAGAGGGTGCCAGAGAG3'
107635517	5' TGGGATGTGGTCAGCAGACA3'	5' GTGGTTTCCTCGGATGGTTC3'
107467011	5' TGATGATGAGCAGAAGCAAC3'	5' CAACATAAGGTAAGCCAGGG3'
107612315	5' TAAGGGGAAAATGAAGAACG3'	5' CCAAGCGAGGAAGAAGAGAG3'
107610612	5' CTTTCTTGGTGAGGCTTTAC3'	5' CAATTCCGAGCTTCTTTATG3'
107464645	5' CATTTCTCACTGTGGTGGAC3'	5' TTTGTAGGTTGCAGGATTTC3'
Actin	5' GTGGCCGTACAACTGGTATCGT3'	5' ATGGATGGCTGGAAGAGAACT3'

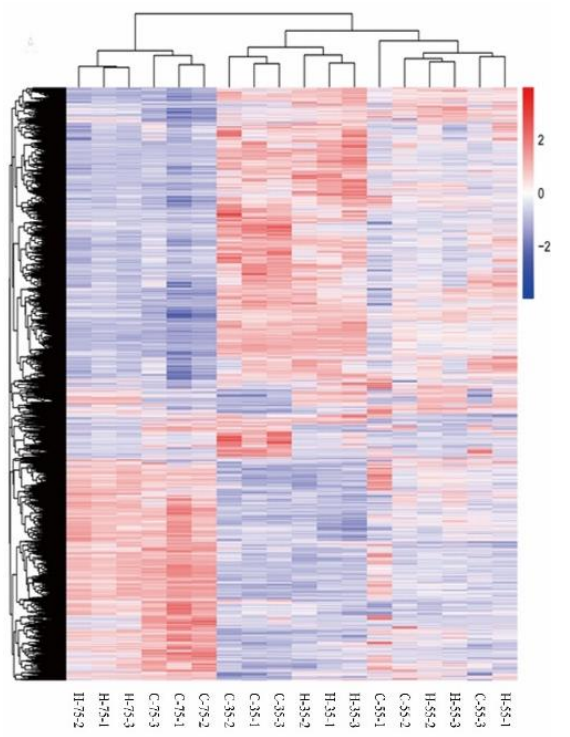

Fig. 1: Hierarchical cluster analysis of changes in gene expression during seed development. H denotes the hydroxyproline-tolerant mutant, and C denotes Huayu 20 (control); the first letter plus indicates the H or C sample, the first two numbers represent the time point after peanut gynophores penetrated the soil; and the last number represents the sample replicate number

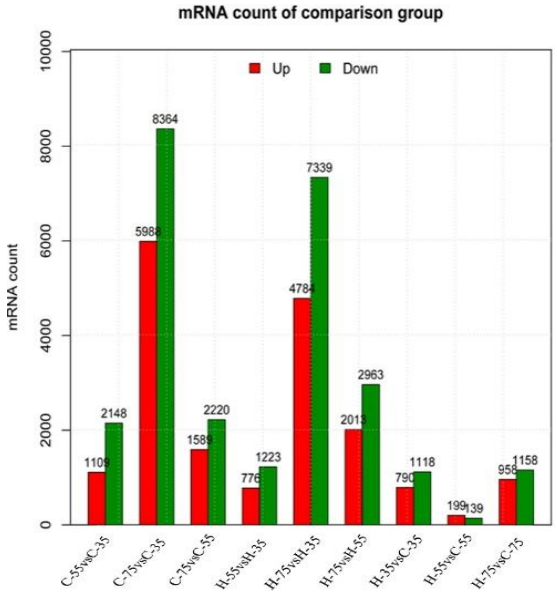

Fig. 2: Changes in DEGs during seed development in the hydroxyproline-tolerant mutant (H), control (C), and D (between H and C) series data sets. The Y axis represents the number of DEGs. Red indicates upregulation, and green indicates downregulation

by KEGG enrichment analyses. For D series data sets (H vs. C at the paired time points) at 35 days, the KEGG pathways "Glycerophospholipid metabolism" and "Ether lipid metabolism" were significantly enriched. Nine DEGs were assigned with KEGG pathways, including "Ether lipid metabolism," "Fatty acid biosynthesis, elongation and metabolism," and "Linoleic acid metabolism." At 75 days, the KEGG pathways — namely, "Fatty acid biosynthesis and metabolism," and "Carbon metabolism" — were enriched. A total of 12 DEGs were assigned KEGG pathways, including "Ether lipid metabolism," "Fatty acid biosynthesis, elongation, degradation and metabolism," and "Linoleic acid metabolism" (Fig. 4).

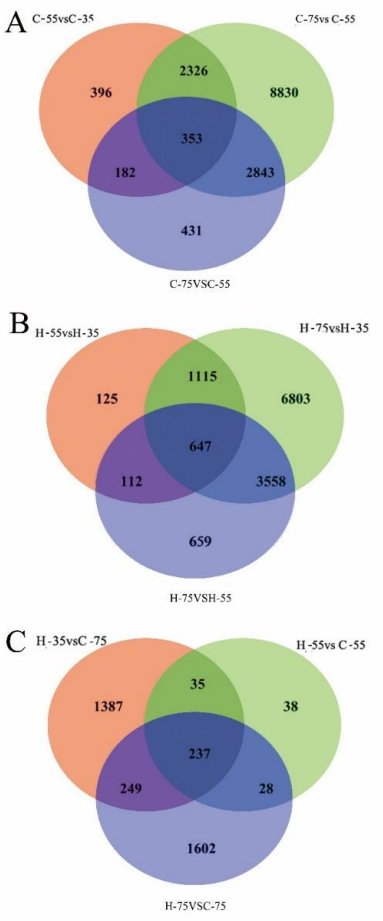

Fig. 3: Comparison between the numbers of DEGs found in C (A), H (B), and D (C) series data sets. The Venn diagram depicts the number of statistically significant DEGs. The number of DEGs exclusively expressed in each sample is indicated in each circle. The numbers of DEGs with common expression changes between two or three treatments are shown in the overlapping regions

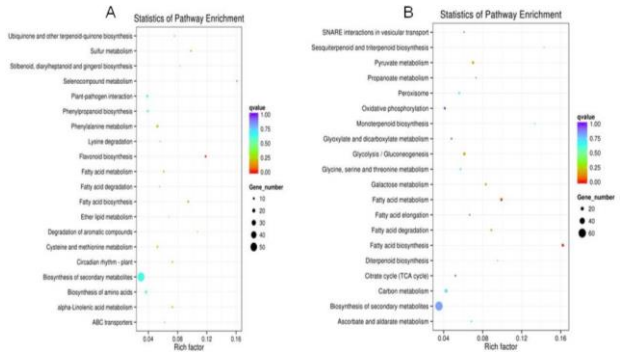

Fig. 4: KEGG pathway enrichment analyses of DEGs in D-35 (A) and D-75 (B) series data sets (H relative to C at 35 and 75 days)

In this study, several IFs (transcription factors) were differentially expressed in the D-35 data set, including: 2 *basic leucine zipper* (*bZIP*) IFs, 2 *MYC* IFs, 5 *MYB* IFs, 4 *basic helix–loop–helix* (*bHLH*) IFs, 5 *WRKY* IFs, 1 *B3 domain* TF, 10 *APETALA2* (*AP2*)/ *ethylene- responsive element-binding protein* (*EREBP*) TFs, and 3 *Dof* IFs. Several genes related to seed maturation were also differentially expressed. These included those that encoded late embryogenesis abundant proteins (LEAs), basic 7S globulin, lipid transfer proteins, receptor-like protein kinase HAIKU2 (IKU2), and several genes related to oil biosynthesis, including genes encoding three 3-ketoacyl-CoA synthases (KCS), a biotin carboxyl-carrier protein of acetyl-CoA carboxylase (BCCP), a stearoyl-[acyl-carrier-protein] 9-desaturase, a delta(8)-fatty-acid desaturase, and a linoleate 13S-lipoxygenase (Table S1).

In the D-75 data set, several IFs were also differently expressed, including 2 *bZIP* IFs, 2 *MADS-box* IFs, 9 *WRKY* IFs, 7 *B3 domain* TFs, 11 *AP2/EREBP* IFs, and 2 *NAC* IFs. The following were also found to be differentially expressed: 2 *LEA* genes; 3 basic 7S globulins; 1 legumin type B; 1 *LTP* gene. Some genes related to oil synthesis were also differentially expressed, including 4 oleosin genes, 2 acetyl-CoA carboxylase (ACCase), 4 acyl carrier proteins (ACP), 2 BCCPs, 3 KCSs, 1 peroxisomal fatty acid beta-oxidation multifunctional protein MFP2, 1 acetyl-coenzyme A carboxylase carboxyl transferase alpha subunit,1 3-oxoacyl-[acyl-carrier-protein] synthases, 1 long-chain acyl-CoA synthetase, and 2 fatty acid desaturase genes (Table S1).

Analysis of miRNA differences between the H and C Genotypes

To analyze differential expression of miRNAs between the H and C genotypes during seed development, seeds of two genotypes at the three different time points were used for miRNA library construction. Twenty miRNA precursors generated 28 known mature miRNAs, and 116 potential miRNA precursors with sizes ranging from 49 nt to 297 nt were predicted to form 110 mature miRNAs.

To associate related miRNAs with seed development we examined the differences in expression. There were 51, 86, and 42 differentially expressed miRNAs between the H and C genotypes at 35, 55, and 75 days post soil penetration by gynophores (Figs. 5a–c). Eleven differentially expressed miRNAs in the H and C genotypes were common to all three time points (Fig. 5d).

Prediction and Analysis of differentially Expressed miRNAs and their differentially Expressed Target Genes between the H and C genotypes

MiRNA-targeted genes were predicted as a means of exploring the functions of the miRNAs. Several miRNAs generally regulated a few target genes. For example, miR181 and ahy-miR156b-5p potentially targeted 4 genes. Most miRNAs targeted a single DEG (Table S2).

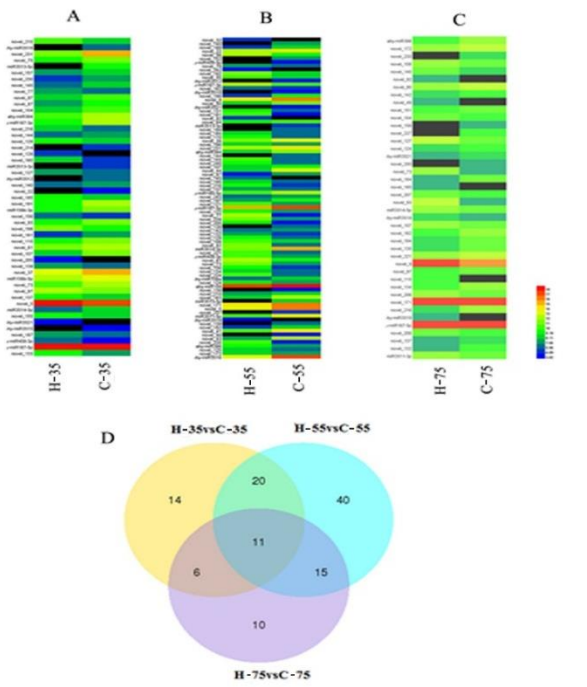

Fig. 5: Differentially expressed miRNA profiles during seed development and comparison of the number of miRNAs in the C, H, and D series data sets. Fold changes of miRNAs at 35 d (A); 55 d (B); and 75 d (C). Red indicates highly expressed miRNA, and blue indicates poorly expressed miRNA. D: Venn diagram depicting the numbers of statistically significant miRNAs. The overlapping regions exhibits the numbers of miRNAs with common expression changes between treatments

Moreover, some target genes of novel miRNAs participated in seed development and/or oil synthesis. Novel_37 targeted a gene encoding a non-specific phospholipase and a *KCS* gene; novel_67 targeted a gene encoding E3 ubiquitin-protein ligase; and novel_129 targets a *B3* domain IF. Three novel miRNAs (novel_92, novel_93, and novel_129) and one known miRNA (ahy-miR156b-5p) targeted genes involved in seed development; miR156 targeted a gene encoding SQUAMOSA promoter binding protein-like (SPL), previously demonstrated to modulate fruit ripening in tomato (Manning *et al*., 2006). An *SPL* gene as a target of miR156 (107468861) was downregulated in our data (Fig. 6). Several miRNA-targeted genes were involved in biotic and abiotic stress response and other processes. Some targeted genes were predicted to be uncharacterized or hypothetical proteins (Fig. 6).

Experimental Verification of DEGs and miRNAs

Nine miRNAs and 9 mRNAs were randomly selected for qRT-PCR to verify the reliability of differentially expressed DEGs and mRNAs. Except for some differences in fold change, sequencing data and qRT-PCR data showed similar gene expression trends for most miRNAs and mRNAs (Fig. 7).

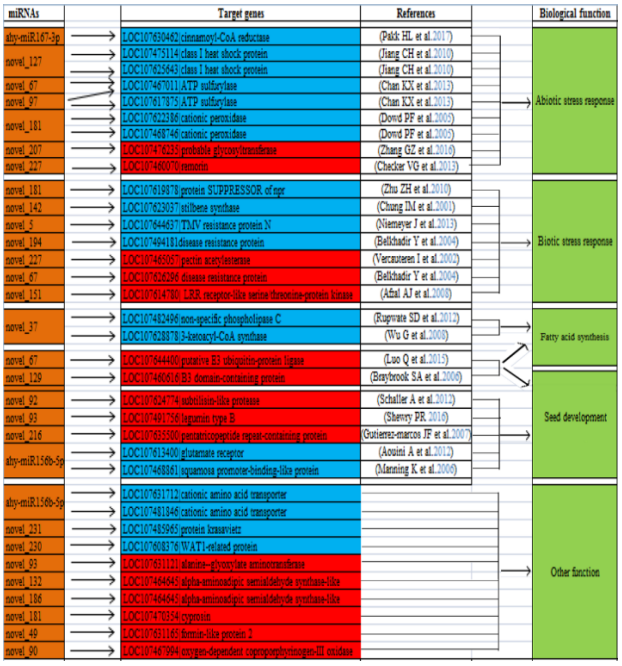

Fig. 6: Hypothetical model of the miRNA-mediated regulatory network during peanut seed development. Blue boxes, downregulated DEGs; red boxes, upregulated DEGs

Discussion

In the present study, 13 and 22 IFs (including the *B3 domain*, *bZIP*, and *MADS-box* IFs) were differentially expressed in the D-35 and D-75 data sets, respectively. Notably, one *FUS3* homologue (107494712) and 2 *ABI5* homologues (107469845 and 107623913) were differentially expressed between the H and C genotypes.

Several *DOF* IFs may play key roles in seed development and oil synthesis. The *DOF4* and *DOF11* IFs from soybean transferred into *Arabidopsis* caused increases in oil content and 1000-grain weight. Expression of these genes increased the activity of acetyl-coA carboxylase and long-chain acetyl-coA synthase, and the expression of genes encoding seed storage proteins (SSPs) were downregulated, thereby changing the direction of fatty acid synthesis (Wang *et al*., 2007). In the current work, 3 *DOF* IFs (107631382, 107468063 and 107631382) were differently expressed in the D-35 data set.

Some *MYB* and *WRKY* TFs were also reported to be related to seed development. *MYB56*, an R2R3 *MYB* TF, regulates seed development through maternal effects and influences the size and shape of seeds in *Arabidopsis*. Loss-of-function mutation of *MYB56* led to smaller seeds than those of the wild type (Zhang *et al*., 2013). *TRANSPARENT TESTA GLABRA 2* (*TTG2*), a type of *WRKY* IF, regulates cell elongation in *Arabidopsis*. In the *ttg2* mutant, the original elongation ability of bead cells was reduced, the space of seed cavity development was limited, and the

endosperm and embryo could not grow normally, resulting in a reduction in seed size (Garcia *et al*., 2005). Transcriptome analysis showed that 5 *MYB* TFs and 14 *WRKY* TFs were differentially expressed during seed development between the H and C genotypes (D data sets).

During seed maturation, several seed proteins such as LEAs, oleosins, LTPs, and SSPs are also massively accumulated, and some of them are regulated by aforementioned IFs. In *Arabidopsis*, *LEC1*, *LEC2*, and *ABI3* regulate the expression of oleosin genes (Crowe *et al*., 2000; Mendoza *et al*., 2005; Mu *et al*., 2008); *ABI3* and *LEC2* can also regulate expression of *SSP* genes (Stone *et al*., 2001; To *et al*., 2006); and *ABI5* can regulate the expression of *LEA* genes (Carles *et al*., 2002). Our results indicated that a few IFs mentioned above might interact with the LEAs, oleosins, LTPs, and SSPs to regulate seed development and/or oil biosynthesis in peanut.

The miRNA sequencing was conducted to elucidate the role of miRNAs during seed development in peanut, and 116 miRNAs differentially expressed between the H and C genotypes were identified. The functions of the differentially expressed target genes mainly involved in seed development, lipid synthesis, and biotic and abiotic stresses. For example, three novel miRNAs (novel_37, novel_67, and novel_129) were found to target genes that could be involved in seed development and/or oil biosynthesis. Novel_37 targeted a gene encoding non-specific phospholipase C (PLC, 107482496) and a *KCS* gene (107628878). PLC hydrolyzes phosphatidylinositol 4, 5-biphosphate to produce diacyglycerol and trisphosphate inositol (Rupwate and Rajasekharan, 2012). A very long-chain fatty acid (VLCFA) is widely present in some oilseed plants (Harwood, 1998) and KCS is the rate-limiting enzyme to catalyze VLCFA. The *FAE1* gene from the *KCS* family plays an important role in the formation of erucic acid in *Brassica napus* (Wu *et al*., 2008). Novel_67 targets a gene (107644400) encoding E3 ubiquitin-protein ligase, which is important for plant growth and development. AtPUB43 and AtPUB44 have roles in seed germination and early development. Ubiquitin ligase CrPUBs from *Chlamydomonas* involved in lipid metabolism, and silencing of the *CrPUB5* or *CrPUB14* genes resulted in significantly decreased lipid content (Luo *et al*., 2015). Novel_129 targets a gene (107460616) encoding a B3 domain IF, which might be involved in seed development and oil biosynthesis as previously mentioned. Our results showed that these miRNAs might affect seed development and oil biosynthesis by regulating their target genes in peanut.

Conclusion

RNA sequencing identified candidate TFs (*AP2*/*EREBP*, *WRKY*, *bZIP*, *DOF*, *B3 domain*, *MADS-box*, *bHLH*, *MYB*, and others) and genes (*IKU2*, oleosins, *LTPs*, *SSPs*, *ACCases*, *ACP*, and *BCCPs*) differentially expressed between the HYP-tolerant mutant and its control. The miRNA analysis indicated that several miRNAs and their target genes might regulate seed development and/or oil biosynthesis. The information obtained from this study could help to clarify the molecular mechanism of high oil content and high yield in this mutant and provide an important genetic resource for peanut breeding.

Acknowledgments

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