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Genome-wide association study of physical and microstructure-related traits in peanut shell

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Research Article

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Abstract

Peanut shell plays key roles in protecting the seed from diseases and pest infestation but also in the processing of peanut and is an important byproduct of peanut production. Most studies on peanut shell have focused on the utilization of its chemical applications, but the genetic basis of shell-related traits is largely unknown. A panel of 320 peanut (*Arachis hypogaea*) accessions including var. *hypogaea*, var. *vulgaris*, var. *fastigiata* and var. *hirsuta* was used to study the genetic basis of two physical and five microstructure-related traits in peanut shell. Significant phenotypic differences were revealed among the accessions of var. *hypogaea*, var. *hirsuta*, var. *vulgaris* and var. *fastigiata* for mechanical strength, thickness, three sclerenchymatous layer projections and main cell shape of the sclerenchymatous layer. We identified 10 significant single nucleotide polymorphisms (SNPs) through genome-wide association study ($P < 5.0 \times 10^{-6}$) combining the shell-related traits and high-quality SNPs. In total, 192 genes were located in physical proximity to the significantly associated SNPs, and 11 candidate genes were predicted related to their potential contribution to the development and structure of the peanut shell. All SNPs were detected on the B genome demonstrating the biased contribution of the B genome for the phenotypical make-up of peanut. Exploring the newly identified candidate genes will provide insight into the molecular pathways that regulate peanut shell-related traits and provide valuable information for molecular marker-assisted breeding of an improved peanut shell.

Introduction

Peanut (*Arachis hypogaea*) has high nutritional value and is an important oil and economic crop. China has a large peanut planting area and is one of the world's major peanut producing countries (Liang *et al.*, 2020). Peanut shell is an abundant agricultural byproduct in peanut production, accounting for about 30% of the total pod weight (Perea-Moreno *et al.*, 2018), and has important roles in the defence against diseases and insect pests during underground growth and later storage (Wee *et al.*, 2007). Peanut shell is rich in biopolymers such as cellulose, lignin and protein, as well as minerals and bioactive substances (Rico *et al.*, 2018; Adhikari *et al.*, 2019). Until now, studies on peanut shells have focused mainly on industrial applications, but studies on the genetic basis of shell-related traits are rare.

Plant organ growth and development involves a series of complex biological processes. Coordination of cell proliferation, differentiation and expansion is essential for the establishment of plant organs. The pattern of these biological processes has an important influence on the morphological characteristics of the mature organs (Harashima and Schnittger, 2010; Bundy *et al.*, 2012). Although environmental factors affect organ development, genetic control plays a key role in limiting the characteristics of mature organs (Hepworth and Lenhard, 2014). Various metabolic pathways involving genes that are regulated by transcription factors are necessary for cell growth and organ formation (Ma *et al.*, 2017). Cell walls are central in limiting plant cell growth, and enzymes involved in cell wall synthesis, such as cellulose synthase, play important roles in cell wall formation and remodelling (Arioli *et al.*, 1998; Wang *et al.*, 2018). Plant growth and development also are regulated by phytohormones (Pacifici *et al.*, 2015).

Mechanical or supporting tissue, which plays a major role in supporting and protecting plants, can be divided into the living collenchyma and the sclerenchyma, which can be found in the endocarp of mature peanut pods (Bercu *et al.*, 2011; Patil *et al.*, 2012). Sclerenchyma has a uniformly thickened secondary cell wall that originates from primary tissue, and the lignified secondary walls provide mechanical strength (Esau, 1960; Vallet *et al.*, 1996). The secondary cell wall is located between the plasma membrane and primary cell wall, and its principal components are cellulose, hemicellulose and lignin (Zhong and Ye, 2009). Cellulose and hemicellulose cross-link to form the main network in secondary cell

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walls, and lignin is impregnated to enhance mechanical strength and provide hydrophobicity (Reiter, 2002). Cellulose, an important structural component in both primary and secondary cell walls, is synthesized by the polymerization of β-1,4-glucan catalysed by a series of enzymes. Cellulose is the most widely distributed and abundant polysaccharide in nature, and has a very high application value as an important renewable energy material (Liu *et al*., 2012). Lignin is a complex phenolic polymer with abundant distribution in plant secondary cell walls. Lignin is assembled mainly by the polymerization of alcohol monomers, including p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, that are synthesized in the cytosol by the phenylpropanoid pathway; however, the process by which alcohol monomers are transported from the cytosol to the cell wall remains unknown (Alejandro *et al*., 2012; Yi Chou *et al*., 2018). Previous studies have confirmed that lignin biosynthesis changes when plants are resistant to biotic or abiotic stresses. A significant increase in lignin accumulation was observed in cultured *Cupressus lusitanica* cells on the fifth day of fungal and mechanical stresses (Alwis *et al*., 2014). In maize, analysis of the drought tolerance of different inbred lines showed that leaf lignin content could be used as an evaluation index of drought tolerance (Hu *et al*., 2009). Cellulose and lignin biosynthesis require high synergism among different parts of the cells and various enzymes. Gene expression regulates lignin and cellulose synthesis in sclerenchyma and thus affects the physicochemical properties of plant organs (Wu *et al*., 2017). Lignin was shown to have a significant effect on the mechanical strength of inflorescence stems in herbaceous peony (Zhao *et al*., 2012).

The peanut shell is part of the pod and is an effective protection barrier of the seed. Shell traits can influence the genetic expression of seed characteristics, such as seed size and duration of filling period (de Godoy and Norden, 1981). Peanut shell has developed sclerenchymatous supporting tissue that forms the skeleton structure of the shell. One ring of a sclerenchymatous layer with vertical Y-shaped projections is present in cross-sections of peanut shells, and vascular bundles are usually found in the grooves formed by the distal bifurcation of the Y-shaped projections. The Y-shaped projections are also important for the formation of the reticulate exterior surface of peanut shell (Halliburton *et al*., 1975). Mechanical strength and thickness are important physical characters of peanut shell that are closely related to the handling and processing of peanut, and to resistance against diseases and pests. A positive correlation has been found between peanut shell thickness and pod size (Patil, 1972; de Godoy and Norden, 1981). Many studies have focused on the regulation mechanisms of agronomic traits such as yield and seed quality of peanut, but there is still a lack of information about the regulation of the physical and microstructural characteristics of peanut shell at the molecular level.

The rapid development of next-generation sequencing technologies has promoted the application of genome-wide association studies (GWAS) to map a large number of phenotypic traits across all species including various crop plants. In rice, an association panel with 423 accessions was used in a GWAS of peduncle vascular bundle-related traits and six candidate genes, including one cloned gene (*NAL1*), were identified (Zhai *et al*., 2018). The genetic basis of 14 agronomic traits in rice was also uncovered by GWAS, and some of the identified peak signals were close to previously identified genes (Huang *et al*., 2010). In maize, the genetic architecture of husk-related traits and resistance to head smut have been preliminarily dissected by GWAS (Wang *et al*., 2012; Cui *et al*., 2016). In spring wheat, marker-trait associations significantly associated with the plant growth stage and yield components were identified by GWAS (Turuspekov *et al*., 2017). Also, the genetic architecture of agronomic traits and disease resistances has been dissected in peanut by GWAS. A panel with 192 peanut accessions was used to determine the genetic basis of seven peanut yield-related traits by GWAS, and a gene (*arahy.RI9HIF*) influencing yield was identified. This is supported by a homolog of *arahy.RI9HIF* identified in rice, which encodes a protein that is involved in rice yield (Wang *et al*., 2019). In another study, genetic factors for 11 agronomic traits of peanut have been dissected by GWAS and candidate genes were predicted (Zhang *et al*., 2017). Quantitative trait loci (QTLs) and candidate genes associated with leaf spot resistance in peanut have been identified by GWAS analysis (Zhang *et al*., 2020a).

In the present study, a panel of 320 peanut accessions with extensive genetic diversity was used in the GWAS analysis of two physical (mechanical strength and thickness) and five microstructural characteristics of peanut shell (Y-DBN, Y-PNBN, NYPN, SLCS and VBN), whereas Y-DBN is the number of Y-shaped distal bifurcations, Y-PNBN is the number of Y-shaped proximal ends with no bifurcations, NYPN is the number of non-Y-shaped projections, SLCS is the main cell shape of the sclerenchymatous layer excluding projections, and VBN is the number of vascular bundles. The panel was selected from more than 2000 worldwide germplasm resources stored in the Peanut Research Department of the Henan Academy of Crops Molecular Breeding (Zhengzhou, China). We used tunable genotyping-by-sequencing and selected from primarily 37,128 single nucleotide polymorphisms (SNPs) 10,004 high-quality SNPs (Zheng *et al*., 2018) with minor allele frequency (MAF) of $\geqslant$0.05. Here, we report the association mapping results related to the peanut shell traits and discuss the relevance of the resulting candidate genes.

Materials and methods

Plant material

A panel of 320 peanut accessions was used in this study, including 100 landraces and 133 breeding lines from China, and 87 accessions from the US mini-core collection (Belamkar *et al*., 2011). The panel comprised four botanical varieties, including 150 var. *hypogaea*, 104 var. *vulgaris*, 32 var. *fastigiata*, 20 var. *hirsuta* and 14 irregular type accessions (Supplementary Table S1). Detailed information about the 320 peanut accessions is provided in a previous study (Zheng *et al*., 2018).

Phenotyping

All the accessions were planted at two different locations (Shangqiu and Zhengzhou) in Henan Province, China in May 2018. Each accession was sown with 20 seeds in a 3 m-long and 0.4 m-wide single row plot with two replications using a randomized block design. The fields were managed according to local practices.

Observation and statistical analysis of the microstructure-related traits were conducted only in Shangqiu. Pods with consistent size were selected from each accession about 30 days after flowering. The part of the pod posterior chamber with the largest diameter was preserved in 70% formalin-acetic-alcohol fixative solution. After staining with Safranine O/Fast Green, the microstructure-related traits of peanut shell were captured using a Leika DM2500 microscope. Because the Y-shaped projections of the sclerenchymatous layer are

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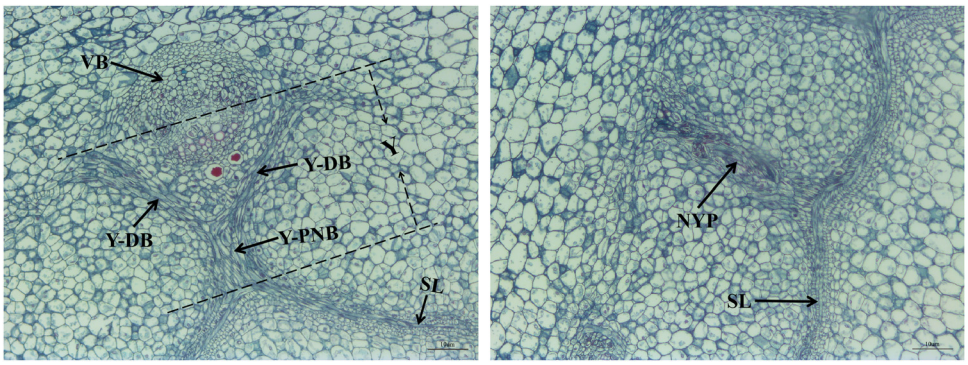

Fig. 1. Cross-section of representative peanut shell showing shell microstructure-related traits. Y, Y-shaped projection; Y-DB, Y-shaped distal bifurcation; Y-PNB, Y-shaped proximal end with no bifurcation; VB, vascular bundle; NYP, non-Y-shaped projection; SL, sclerenchymatous layer.

sometimes not completely realized and the function of the distal and proximal ends of the Y-shaped projections in formatting the reticulate exterior surface of peanut shell may follow different purposes, we evaluated the distal and proximal ends separately; the microstructure-related traits included a number of Y-shaped distal bifurcations (Y-DBN), number of Y-shaped proximal ends with no bifurcations (Y-PNBN), number of non-Y-shaped projections (NYPN), main cell shape of the sclerenchymatous layer (SLCS) excluding projections (not elongated (indicated by 1), half elongated (indicated by 1.5) or elongated (indicated by 2)) and the number of vascular bundles (VBN) (Fig. 1).

Ten mature and dried pods of similar size were collected from each accession for each replication in the two environments to measure the two physical characteristics of the peanut shell. Mechanical strength was measured by mechanical compression of the pod posterior chamber with an EZ-test texture analyser, Shimadzu (Tokyo, Japan). The loading speed of the mobile probe was set at 1 mm/s, and the compression distance was set at 2–4 mm according to the pod size and plumpness. The maximum force during this process was recorded and used for subsequent analysis (Ionescu *et al.*, 2016). Shell thickness was measured of the pod posterior chamber using a digital caliper (Terma CDA100). The average value of the traits of each accession was used for the GWAS analysis. The phenotypic data were analysed using GraphPad Prism 7 and SPSS Statistics 25 (Lazareno, 1994; Field, 2013).

Genotyping

Detailed information about DNA extraction, library preparation and resequencing are described in a previous study (Zheng *et al.*, 2018). Because the genome of the tetraploid cultivated peanut has not been published at that time, the genomes of two diploid peanut progenitors, *A. duranensis* and *A. ipaensis* (Aradu_v1.0.fa and Araip_v1.0.fa), were artificially combined to form a reference genome for sequence alignments with the GSNAP aligner. We used tunable genotyping-by-sequencing (Ott *et al.*, 2017) and identified 37,128 SNPs of which 10,004 high-quality SNPs with missing rate ⩽30% and MAF of ⩾0.05 were selected for GWAS.

Genome-wide association analysis and identification of candidate genes

GWAS was performed on all seven trait values of the 320 peanut accessions using the mixed linear model K + Q approach in TASSEL 5.2.13 (Bradbury *et al.*, 2007). In the K + Q approach, the genetic marker-based kinship matrix (K) is combined with population structure (Q) to avoid false-positive associations. In the present study, the Bonferroni test criterion with a significance level of 0.05 was used as the significance threshold, and a *P* value of 5.0×10^{-6} was obtained by calculating 0.05/n ($n = 10{,}004$). Manhattan and Q-Q plots were drawn using the R package (Turner, 2018). We used the 400 kb genomic regions on each side of SNPs that were significantly associated with the peanut shell traits as the candidate genome regions. All significant SNPs were located on the B genome, and candidate genes were identified by mapping the selected genomic regions to the genome sequence of the diploid progenitor peanut, *Arachis ipaensis* (https://peanutbase.org/data/public/Arachis_ipaensis/).

Results

Phenotypic variation and correlation

Extensive variation for all traits was observed among the 320 peanut accessions (Supplementary Table S1 and Table S2). The mechanical strength and thickness in the two environments showed continuous variation, but only the mechanical strength in Zhengzhou and thickness in both environments showed normal distribution. Mechanical strength in Shangqiu, the sclerenchymatous layer projection-related traits (Y-DBN, Y-PNBN, NYPN), and VBN were skewed to the right (Fig. 2). The SLCS of most of the accessions were not elongated (Supplementary Fig. S1(a)). Phenotype pairwise correlations between the traits revealed large differences. The mechanical strength and thickness were positively correlated in Shangqiu and Zhengzhou ($r = 0.64$ and 0.62). Y-DBN and Y-PNBN also were positively correlated ($r = 0.64$). The other traits showed weak or no correlation (Supplementary Fig. S2).

The association panel comprised four botanical varieties (var. *hypogaea*, var. *hirsuta*, var. *vulgaris* and var. *fastigiata*) and some irregular type accessions (Zheng *et al.*, 2018). To investigate the effect of population structure on the seven shell-related traits, phenotypic variation of the four botanical varieties was analysed. Significant differences in mechanical strength in Shangqiu were observed among the four varieties, and var. *vulgaris* had higher mean values than the other three varieties. However, no significant difference in mechanical strength in Zhengzhou was observed among the four varieties. Var. *fastigiata* showed significantly higher mean values for thickness than the other three

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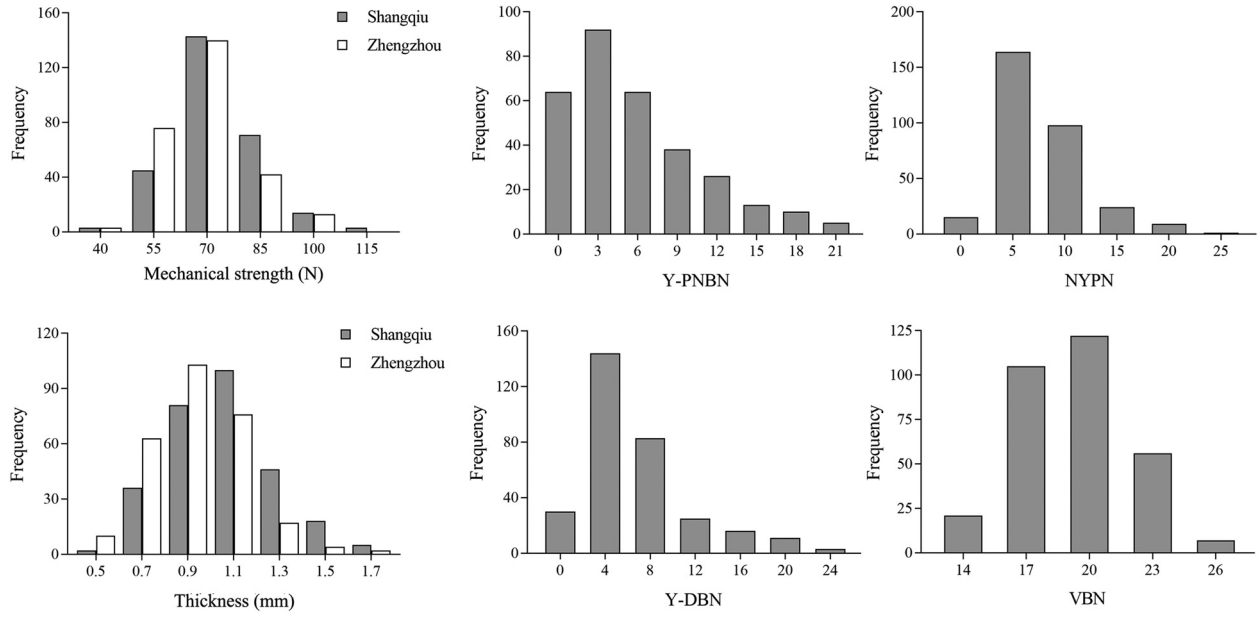

Fig. 2. Frequency distribution of peanut shell-related traits. Y-PNBN, number of Y-shaped proximal ends with no bifurcations; NYPN, number of non-Y-shaped projections; Y-DBN, number of Y-shaped distal bifurcations; VBN, number of vascular bundles. Shangqiu and Zhengzhou are the two trial locations.

varieties in both environments. The three sclerenchymatous layer projection-related traits (Y-DBN, Y-PNBN, NYPN) also showed highly significant differences among the four varieties (Supplementary Fig. S3). Y-DBN and NYPN showed significant higher values in var. *fastigiata*, followed by var. *vulgaris*, var. *hypogaea* and var. *hirsuta* with the lowest values. Similarly, Y-PNBN showed significant higher values in var. *fastigiata* than the other three varieties. The SLCS were significantly stronger elongated in the var. *fastigiata* and var. *vulgaris* than var. *hypogaea* (Supplementary Fig. S1(b)–(e)). No significant differences were observed for VBN among the four botanical varieties (Fig. 3).

Genome-wide association study and SNP detection

A total of 10 significant SNPs were detected, of which one was associated with mechanical strength in Zhengzhou, one with Y-DBN, seven with NYPN and one with SLCS. No SNP significantly associated with thickness, Y-PNBN or VBN was observed. All 10 SNPs were located on the *A. ipaensis* B genome (Fig. 4; Supplementary Fig. S4). The SNP associated with mechanical strength in Zhengzhou was located on B08 and explained 12.5% of the phenotypic variation. The SNP for Y-DBN was located on B07 and explained 11.6% of the phenotypic variation. Among the seven SNPs detected for NYPN, six were located on B09 and explained 8.6–15.8% of the phenotypic variation, and one was located on B02 and explained 10.1% of the phenotypic variation. The one SNP for SLCS was detected on B08 and explained 14.2% of the phenotypic variation (Table 1).

Candidate gene analysis

We defined a 400 kb genomic region of the *A. ipaensis* genome on each side of the significant SNPs and identified a total of 192 annotated genes; six were associated with mechanical strength, 14 with Y-DBN, 116 with NYPN and 56 were associated with SLCS (Supplementary Table S3). We further investigated the annotations to identify candidate genes with known functional relation to the underlying trait of the association and close proximity to the peak SNP.

For mechanical strength at Zhengzhou, the peak SNP B08-37288956 was about 124 kb away from *Araip.PJ60P*, which encodes a subtilisin-like serine protease 2. Subtilisin-like serine proteases were found to be present in components of the vascular bundle by *in situ* hybridization (Golldack *et al.*, 2010). For Y-DBN, the peak SNP B07-65047551 was located 345 kb away from *Araip.FG7C9*, which encodes an amino acid permease that is involved in the xylogenesis in poplar (Couturier *et al.*, 2010), and 183 kb away from *Araip.G84LS*, which encodes a sugar transporter that plays an important role in plant defence (Yamada *et al.*, 2016). For NYPN, 18 genes including *Araip.F83CP* which encodes a pyruvate kinase family protein were identified in the candidate region centred on the peak SNP B09-105682199. Pyruvate kinases play key roles in the glycolytic pathway and in fatty acid biosynthesis (Ambasht and Kayastha, 2002). *Araip.J3D1X*, which was located 137 kb away from SNP B09-72638166, encodes 6-phosphofructo-2-kinase/fructose-2, which is related to sucrose synthesis in leaves and to glycolysis (Furumoto *et al.*, 2001). A total of 62 candidate genes were identified in the candidate region centred on peak SNP B02-4772223 for NYPN. Among them, *Araip.VH6HT*, which was located about 55 kb away from SNP B02-4772223, encodes an MYB transcription factor. The MYB transcription factor family is one of the largest transcription factor families in plants and is closely related to lignin biosynthesis in secondary walls (Zhao *et al.*, 2019). Another gene, *Araip.HWR4Z*, which was located 120 kb away from SNP B02-4772223, encodes a bHLH13-like transcription factor. The bHLH transcription factors play regulatory roles in plant development and regulate genes involved in cell wall modification and lignin synthesis (Yan *et al.*, 2013). *Araip.Z6UY4*, which also was located in the candidate region centred on SNP B02-4772223, encodes a laccase that is involved in lignin biosynthesis (Lichao *et al.*, 2020). For SLCS, *Araip.3H9YN*, which encodes brassinosteroid signalling positive regulator (BZR1) family protein, was

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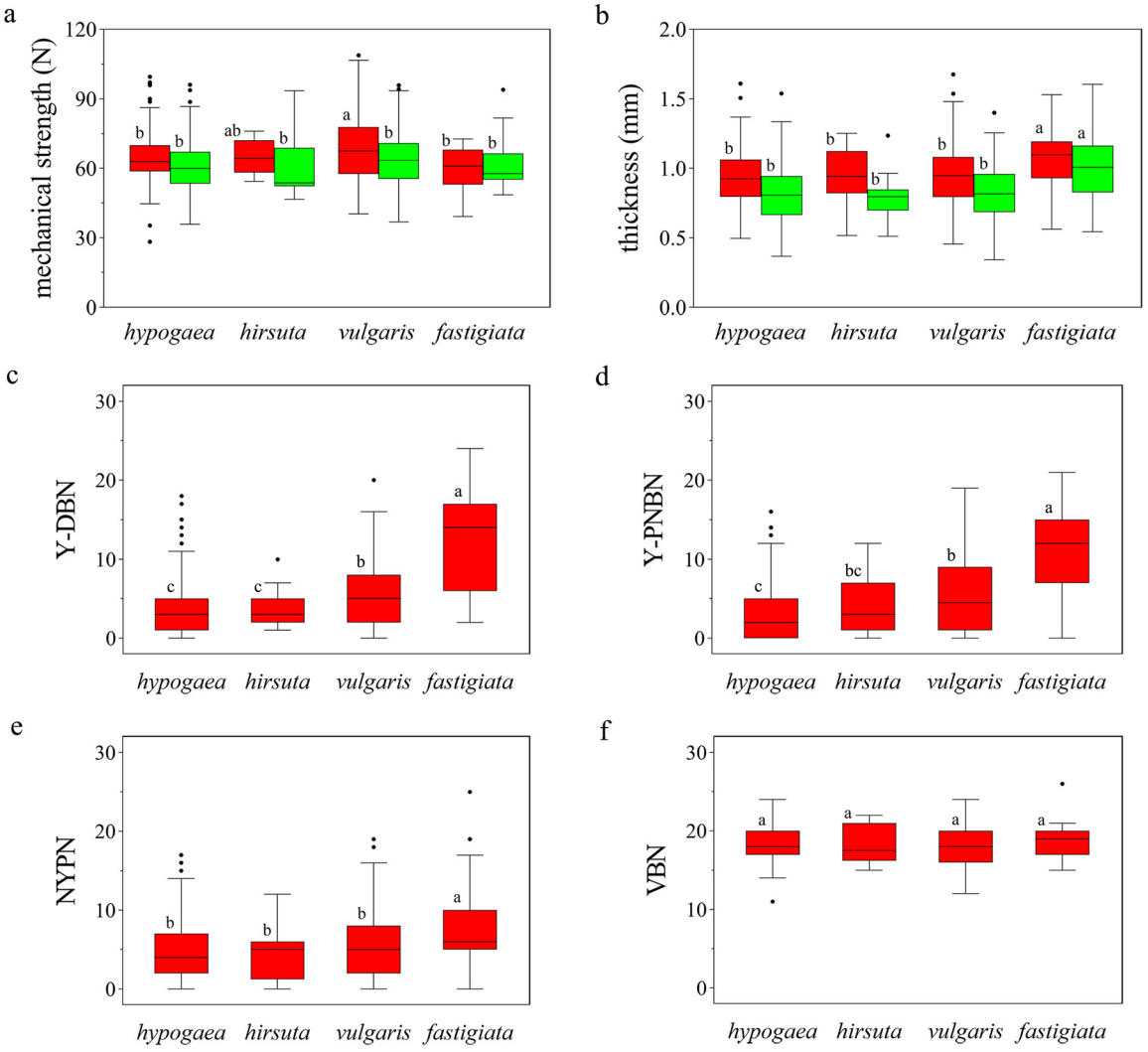

Fig. 3. Boxplot of shell-related traits in four botanical varieties of peanut. Analysis of variance (ANOVA) was applied to examine the differences of traits among the botanical varieties. Different letters (a, b and c) indicate significant difference at $P \leqslant 0.05$ (Tukey-HSD). Red and green colours indicate Shangqiu and Zhengzhou, respectively. a: mechanical strength; b: thickness; c: Y-DBN, number of Y-shaped distal bifurcations; d: Y-PNBN, number of Y-shaped proximal ends with no bifurcations; e: NYPN, number of non-Y-shaped projections; f: VBN, number of vascular bundles.

located 137 kb away from the peak SNP B08-125626766. Brassinosteroid signalling plays a key role in plant growth, and brassinosteroid is related to the formation of cell walls in plants (Sun *et al.*, 2015). Another gene, *Araip.67S5I*, which was located 337 kb away from SNP B08-125626766, encodes a cytochrome P450 superfamily protein. The cytochrome P450 superfamily is the largest enzymatic protein family in plants, and its members are involved in many complex metabolic pathways and regulate various important cellular processes that affect plant growth and development (Jun *et al.*, 2015). *Araip.C9SN6*, which was located 394 kb away from SNP B08-125626766, encodes translation elongation factor EF1B, which was reported to be involved in cell wall biosynthesis in Arabidopsis (Hossain *et al.*, 2012) (Table 2).

Discussion

Peanut shell is an important part of the pod that develops from the ovary wall and can be divided into exocarp, endocarp and mesocarp. The characteristics of peanut shells are important in handling, processing, resistance to pests and diseases, but also affect some seed traits (de Godoy and Norden, 1981). Despite the versatile importance of the peanut shell, most studies on peanut shell have focused on industrial utilization to reduce the waste of resources and prevent environmental pollution (Sareena *et al.*, 2012; Raj *et al.*, 2019). The genetic basis and molecular pathways underlying the characteristics of shell morphology and cellular structure are still largely unknown. In the present study, a diverse panel comprising four botanical varieties and some irregular type accessions was used to explore the natural variation of two physical and five microstructural characteristics of peanut shell. Significantly associated SNP loci were detected by GWAS analysis and candidate genes with functional relation to the peanut shell proposed.

Phenotypic correlation and the effect of population structure on phenotypes

The seven shell traits showed wide variation among the accessions in the association panel. Among them, mechanical strength and thickness were highly positively correlated, which

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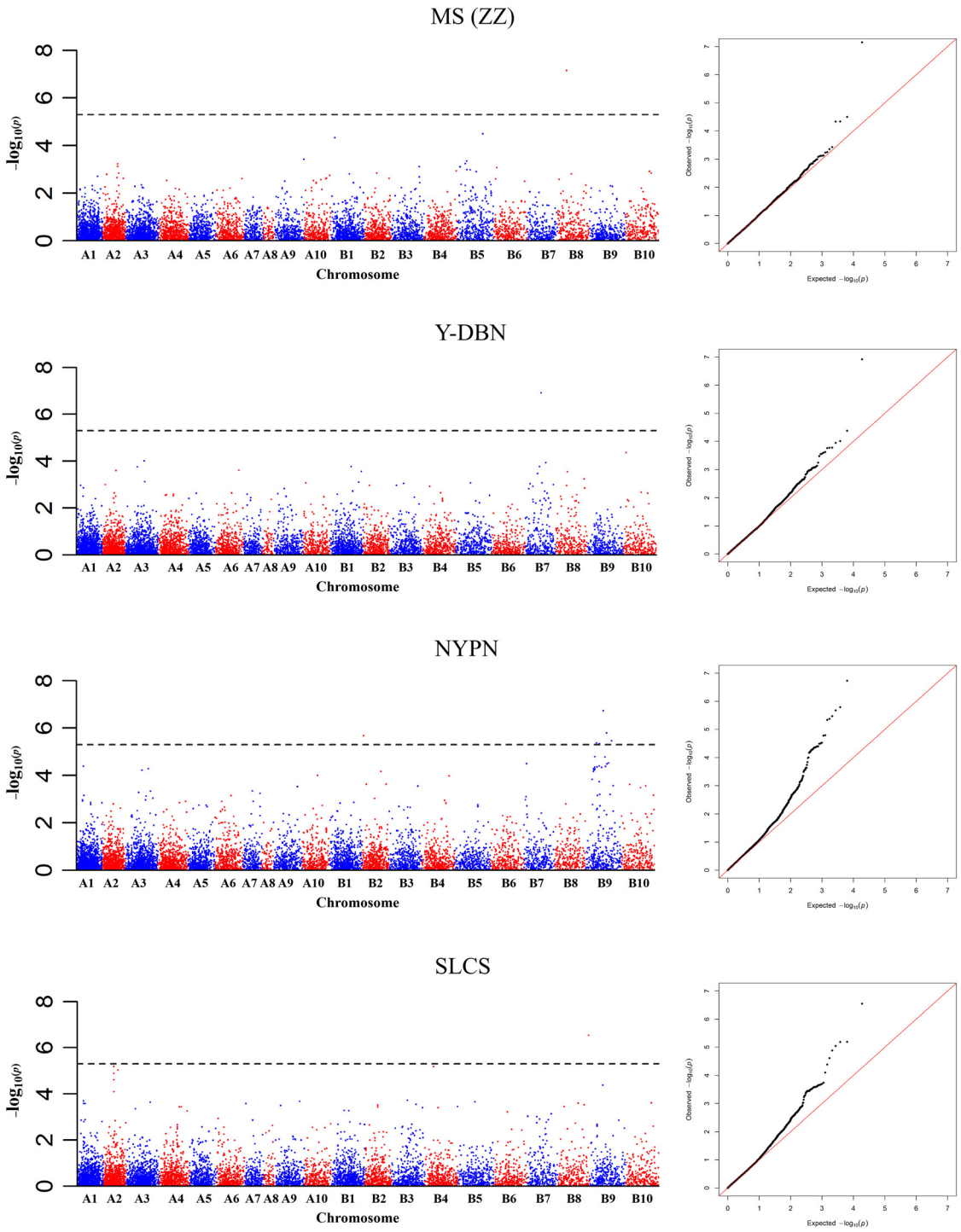

Fig. 4. Manhattan and Q-Q plots of peanut shell-related traits. Mechanical strength at Zhengzhou (MS (ZZ)), number of Y-shaped distal bifurcations (Y-DBN), number of non-Y-shaped projections (NYPN) and main cell shape of sclerenchymatous layer (SLCS). The dotted black line indicates the genome-wide significance threshold: −log10 (*P* value) = 5.3.

indicates that the shell thickness had a significant influence on the shell's load capacity. Y-DBN and Y-PNBN were also highly, positively correlated because they are both parts of the Y-shaped projections, but sometimes they do not occur together. Some vascular bundles were located in the open groove formed by the Y-shaped distal bifurcation (Supplementary Fig. S5). The Y-shaped projections are extensions of the sclerenchymatous layer and are related to the reticulate exterior surface of the peanut shell (Halliburton *et al.*, 1975). Vascular bundles also may be crucial in formatting the reticular surface. In this study, mechanical strength showed a weak correlation with the microstructure-related traits, which may be due to the complexity of mechanical strength, which may also be related to other more factors such as pod plumpness and shape. Further studies are needed for a better understanding of the mechanical strength of the peanut shell.

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Table 1. SNPs significantly associated with four traits identified by GWAS

Trait[a]	Environment	Chr	SNP location (bp)	Allele[b]	*P*	R^2 (%)[c]
MS	ZZ	ARAIP.B08	B08_37288956	A/G	7.07×10^{-8}	12.5
Y-DBN	SQ	ARAIP.B07	B07_65047551	C/A	1.21×10^{-7}	11.6
NYPN	SQ	ARAIP.B02	B02_4772223	C/T	2.10×10^{-6}	10.1
NYPN	SQ	ARAIP.B09	B09_44845009	C/T	4.21×10^{-6}	8.6
NYPN	SQ	ARAIP.B09	B09_56872263	G/A	4.58×10^{-6}	9.5
NYPN	SQ	ARAIP.B09	B09_72638166	G/A	1.87×10^{-7}	13.1
NYPN	SQ	ARAIP.B09	B09_83285193	G/A	1.74×10^{-9}	15.8
NYPN	SQ	ARAIP.B09	B09_86521541	C/A	1.62×10^{-6}	9.2
NYPN	SQ	ARAIP.B09	B09_105682199	T/A	3.37×10^{-6}	10.4
SLCS	SQ	ARAIP.B08	B08_125626766	C/T	2.85×10^{-7}	14.2

[a]MS, mechanical strength; Y-DBN, number of Y-shaped distal bifurcation; NYPN, number of non-Y-shaped projection; SLCS, main cell shape of sclerenchymatous layer; ZZ, Zhengzhou; SQ, Shangqiu.
[b]Major/minor allele.
[c]Phenotypic variance explained.

Table 2. SNPs and candidate genes significantly associated with peanut shell-related traits

Trait[a]	SNP location (bp)	Candidate genes	Distance to SNP (kb)	Functional annotation
MS	B08-37288956	Araip.PJ60P	124 (37,163,904–37,164,629)	Subtilisin-like serine protease 2
Y-DBN	B07-65047551	Araip.G84LS	183 (65,230,513–65,231,938)	Sugar transporter 1
		Araip.FG7C9	345 (64,700,309–64,702,511)	Amino acid permease
NYPN	B09-105682199	Araip.F83CP	0 (105,679,985–105,685,678)	Pyruvate kinase family protein
	B09-72638166	Araip.J3D1X	137 (72,498,597–72,500,898)	6-phosphofructo-2-kinase/fructose-2
	B02-4772223	Araip.VH6HT	55 (4,826,767–4,828,000)	myb transcription factor
		Araip.HWR4Z	120 (4,649,893–4,651,764)	Transcription factor bHLH13-like
		Araip.Z6UY4	146 (4,786,806–4,789,819)	Laccase 6
SLCS	B08-125626766	Araip.3H9YN	137 (125,763,619–125,764,263)	Brassinosteroid signalling positive regulator (BZR1) family protein
		Araip.67S5I	337 (125,245,790–125,249,298)	Cytochrome P450 superfamily protein
		Araip.C9SN6	394 (126,020,683–126,023,752)	Translation elongation factor EF1B

[a]MS, mechanical strength; Y-DBN, number of Y-shaped distal bifurcation; NYPN, number of non-Y-shaped projection; SLCS, main cell shape of sclerenchymatous layer.

The peanut shell thickness is a complex trait and may be interacting with other characters. Peanut shell thickness is positively correlated with pod size, while pod size is negatively correlated with shelling percentage (Patil, 1972; de Godoy and Norden, 1981). Therefore, only limited success can be achieved in selecting large pods with higher shelling percentage. At present, there are only a few studies on the correlation between shell thickness and shelling resistance in peanut, but it has been reported in other nuts. Peanuts with large kernels have an undesirable shelling percentage due to thicker shells (Patil, 1972). The rupture force increased linearly by increasing shell thickness in hazelnut and walnut (Koyuncu *et al.*, 2004; Kacal and Koyuncu, 2017). However, the breakage resistance was not significantly affected by shell thickness in cocoa pod (Maduako and Faborode, 1994).

Cultivated peanuts can be divided into two subspecies and six botanical varieties (Krapovickas and Gregor, 1994). The association panel used in the present study can be divided into four botanical varieties and the diversity analysis indicated that the population structure of the panel affected the shell-related traits. Mechanical strength of accessions planted in Shangqiu, thickness in the two environments, three sclerenchymatous layer projection-related traits (Y-DBN, Y-PNBN, NYPN) and SLCS showed significant differences among the four botanical varieties (var. *hypogaea*, var. *hirsuta*, var. *vulgaris* and var. *fastigiata*). Accessions of the var. *fastigiata* significantly deviated for thickness at both trial locations, Y-DBN, Y-PNBN, NYPN and SLCS from the other varieties. The var. *fastigiata* is a botanical type in subsp. *fastigiata*, which has pods that usually contain three to four seeds; the seeds are sweeter than those of other types, and var. *fastigiata* has higher quality and better resistance to leaf spot than other botanical varieties (Subrahmanyam *et al.*, 1989; Mannivannan *et al.*, 2007). Additionally to these favourable agronomic traits, the observed

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structural differences may pioneer breeding using var. *fastigiata* accessions as a plant genetic resource for the improvement of shell characteristics of the cultivated peanut.

Significant SNPs and candidate genes for peanut shell-related traits

Plant organ formation involves many biological processes, including cell proliferation, differentiation and expansion, and a diverse range of genes associated with regulatory processes or pathways (Harashima and Schnittger, 2010; Ma *et al*., 2017; Peng *et al*., 2017). GWAS analysis can help to reveal the genetic basis of complex trait variations and has been widely used in genetic studies of crop traits. In the present study, 10 significant SNPs were detected along with candidate genes for four of the traits (mechanical strength, Y-DBN, NYPN and SLCS). All 10 significant SNPs were only detected on the B genome, which may be caused by the differences between the A and B genomes. Cultivated peanut is an allotetraploid (AABB; $2n = 4x = 40$), which was probably derived from a single recent hybridization of two diploid progenitors (Bertioli *et al*., 2016). Molecular evidence indicates that *Arachis duranensis* and *Arachis ipaensis* are the two most likely progenitors that donated the A and B subgenomes, respectively (Moretzsohn *et al*., 2013). The cultivated peanut subgenomes have evolved asymmetrically, with the B subgenome resembling the ancestral state and the A subgenome undergoing more gene disruption, loss, conversion, and transposable element proliferation, and lacking genome-wide expression dominance (Chen *et al*., 2019). The cultivated peanut B subgenome has more genes and general expression dominance (Zhuang *et al*., 2019). In the present study, the significant SNPs detected need to be further verified and developed into DNA markers for breeders to use. The significant SNPs detected in this study will provide valuable information for molecular breeding of improved peanut shell.

In the candidate region centred on peak SNP B08-37288956, which was significantly associated with *mechanical strength*, six candidate genes were identified, including *Araip.PJ60P*, which encodes subtilisin-like serine protease 2. Subtilisin-like serine proteases are an enormous family of enzymes that play many roles in plant development and signalling (Othman and Nuraziyan, 2010). In Arabidopsis, a subtilisin-like serine protease was co-expressed with a pectin methylesterase that modifies pectin, which is the main component of the cell wall (Fabien *et al*., 2014). Another subtilisin-like serine protease that was located in the apoplast of *Arabidopsis thaliana* root, including xylem vessel, was suggested to be related to the stress response (Kuroha *et al*., 2010).

Araip.FG7C9, which encodes an amino acid permease, was found in the genomic region around peak SNP B07-65047551 that was significantly related to *Y-DBN*. As the main transport form of nitrogen, amino acids play fundamental and key roles in plant growth and development, and amino acid permeases are involved in the transport of amino acids. In poplar, an amino acid permease was reported to play an important role in supplying the proline required to synthesize cell wall proteins related to the formation of xylem (Couturier *et al*., 2010; Santiago and Tegeder, 2016).

Several candidate genes were in close association with the trait *NYPN*. *Araip.J3D1X* encodes 6-phosphofructo-2-kinase/Fructose-2, which is related to sucrose synthesis in leaves and to glycolysis. Sucrose is converted to UDP-glucose by sucrose synthase, and UDP-glucose is a substrate for cellulose synthesis (Furumoto *et al*., 2001; Fujii *et al*., 2010). *Araip.VH6HT*, which encodes an MYB transcription factor, was located about 55 kb from peak SNP B02-4772223. The MYB transcription factor family has a large number of members and is involved in the biosynthesis of secondary cell wall lignin in many plants. In *A. thaliana*, MYB transcription factors related to lignin biosynthesis have been reported, including MYB58 and MYB63 (Zhou *et al*., 2009), and MYB46 and MYB83 (McCarthy *et al*., 2009). In addition, ZmMYB31 and ZmMYB42 in maize (Fornalé *et al*., 2009), the R2R3-MYB transcription factors PtrMYB152 (Li *et al*., 2014), PtoMYB216 (Tian *et al*., 2013) and LTF1 (Gui *et al*., 2019) in poplar, and CmMYB19 in chrysanthemum (Wang *et al*., 2017) have been shown to be involved in lignin biosynthesis. *Araip.HWR4Z*, which encodes a bHLH13-like transcription factor, also was located in the genomic region centred on peak SNP B02-4772223. The bHLH transcription factors play vital roles in many biological processes, including plant growth, stress response and hormone regulation. In maize, bHLH transcription factors were found to be associated with the formation of secondary cell walls based on a QTL study (Courtial *et al*., 2013). In addition, bHLH transcription factors have been reported to regulate lignin biosynthesis (Yan *et al*., 2013). Another gene, *Araip.Z6UY4*, which is in the candidate region centred on peak SNP B02-4772223, encodes a laccase. Laccases are copper-containing oxidases that act on a wide range of phenolic compounds and have long been considered to be closely connected with lignin formation and lignification. An analysis of the role of laccases in lignin formation in *A. thaliana* showed that not all laccases have the same functions (O'Malley *et al*., 2010). Furthermore, several genes encoding receptor-like kinases were annotated in the genomic region around peak SNP B02-4772223. Receptor-like kinases are involved in maintaining the integrity of cell walls, which is necessary for plant growth and resistance to stress (Timo and Thorsten, 2014). MIK2/LRR-KISS is a leucine-rich repeat receptor kinase that belongs to the large receptor-like kinase superfamily and, in Arabidopsis, MIK2/LRR-KISS was reported to be an important regulator of the response to cellulose biosynthesis inhibition (Van der Does *et al*., 2017). Secondary cell wall thickening was inhibited when *AtVRLK1*, which encodes an Arabidopsis receptor-like kinase, was upregulated (Huang *et al*., 2018).

Araip.3H9YN encodes a brassinosteroid signalling positive regulator (BZR1) family protein and was located 137 kb away from the peak SNP B08-125626766 that was significantly related to *SLCS*. Brassinosteroid is an important steroid hormone that is essential for plant growth, and brassinosteroid signalling has been reported to be involved in the deposition of cellulose in the secondary cell wall of cotton fibres (Sun *et al*., 2015). A transcriptome analysis showed that brassinosteroid regulated cell wall-related genes and contributed to the rapid growth of bamboo shoots (Zhang *et al*., 2020b). Another gene, *Araip.67S5I*, which was located 337 kb away from SNP B08-125626766, encodes a cytochrome P450 superfamily protein whose members play complex and important roles in plant secondary metabolism by regulating the synthesis of secondary metabolites as signals of plant growth and development or by protecting plants against various stresses (Jun *et al*., 2015).

From a total of 192 candidate genes in the genomic intervals centred around the 10 observed significant SNP loci, 11 genes could be referenced to the observed traits. All these genes are located on the B genome demonstrating the biased contribution of the B genome for the phenotypical make-up of peanut. Further studies will be needed to reveal their function and contribution to the general microstructure and resilience of the peanut shell. This study provides phenotypic and genomic key elements

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and opens new directions to study the molecular mechanism and regulatory network of the peanut shell.

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