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Identification of Hotspot Regions for Candidate Genes Associated with Peanut (*Arachis hypogaea* L.) Pod and Seed Size on Chromosome A05

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Abstract: The size of peanut pods and seeds, which directly affects yield and quality, also has significant implications for mechanized production and market efficiency. Identifying relevant loci and mining candidate genes is crucial for cultivating high-yield peanut varieties. In this study, we employed advanced generation recombinant inbred lines developed by crossbreeding Huayu 44 and DF12 as the experimental material. Quantitative trait locus (QTL) mapping for traits related to pod and seed size was conducted across six environments. A total of 44 QTLs were detected, distributed on chromosomes A02, A05, B04, B08, and B10. An enrichment region for multiple QTLs was also identified on chromosome A05 (19.28~52.32 cm). In this region, 10 KASP markers were developed, narrowing the enrichment area to two candidate gene hotspot regions of 600.9 kb and 721.2 kb. By combining gene prediction and functional annotation within the intervals, 10 candidate genes, including those encoding cytochrome P450 protein, polyamine synthase, mannose-1-phosphate guanylyltransferase, pentatricopeptide repeat protein, and E2F transcription factor, were identified as regulators of pod and seed size. This study provides technical support for the genetic improvement and key gene identification of peanut pod and seed size.

Keywords: candidate genes; QTL mapping; whole-genome re-sequencing

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1. Introduction

Peanut (*Arachis hypogaea* L.) is a globally significant oilseed and economic crop. Owing to its rich content of protein, fat, and other nutrients, it is widely used in food and oil production, making it an important source of plant-based protein for human consumption. Peanuts are primarily cultivated in the vast region between 40° S latitude and 40° N latitude, with major producing countries including China, India, Nigeria, the United States, and Indonesia. As of 2021, the global peanut cultivation area was 32.72 million hectares, with a total production of 53.93 million tons [1].

Cultivated peanuts (2n = 4x = 40) originated in South America. They evolved gradually through distant hybridization between two wild diploid species, *A. duranensis* (AA genome) and *A. ipaensis* (BB genome), and spontaneous chromosome doubling [2–6]. The seeds of the wild diploid species are very small, weighing only 0.1 to 0.3 g [7]. During the early domestication of peanuts, the size of the pods and seeds directly influenced yield and harvesting efficiency. People tend to select individuals with larger seeds and pods, increasing the size of the seeds and pods in cultivated peanuts. Larger pods not only provide more space and nutrients for the seeds, but also enhance their adaptability

and resistance to adverse conditions. Therefore, they are considered advantageous for increasing yield [8]. In modern agriculture, larger pods facilitate mechanized harvesting, thereby improving efficiency. Larger seeds typically accumulate more nutrients, and during seed maturation, the accumulation of proteins and fats within the seeds increases. The pod-filling process is considered complete when the seeds accumulate sufficient nutrients and reach their maximum size [9]. Additionally, when the size of the pods and seeds reaches an appropriate ratio, shelling becomes easier and the shelling percentage is higher. Therefore, effectively regulating this trait can not only enhance the yield and quality of peanuts but also optimize processing and utilization, expanding the application range of peanut products; this maximizes the value of peanuts as an economically significant crop.

Peanut pod and seed sizes are complex quantitative traits regulated by multiple genes and influenced by genetic factors and environmental conditions [10]. Marker-assisted selection (MAS) technology, an important tool for quantitative trait locus (QTL) mapping, has played a crucial role in the screening and detection of QTLs related to peanut pod and seed size. Hopkins et al. (1999) discovered many CT and GT repeat sequences in the cultivated peanut genome. Owing to their high polymorphism, simple sequence repeats (SSR) are particularly well suited for developing molecular markers [11]. Subsequently, Varshney et al. integrated 135 SSR loci to establish the first relatively comprehensive SSR-based molecular linkage map of peanuts [12], significantly advancing the research progress in QTL mapping. With extensive development and application of many SSR markers, researchers have successfully identified numerous QTLs associated with pod and seed size in peanuts [13–18]. However, SSR markers are limited by factors such as low polymorphism and marker density, resulting in a lack of precision and efficiency in the localization of candidate genes.

With the rapid advancement of high-throughput sequencing technology, the genome sequences of the tetraploid peanut cultivars A. hypogaea 'Shitouqi' [19], A. hypogaea 'Fuhuasheng' [20], and A. hypogaea 'Tifrinner' [21] have been published to ensure the accuracy of genetic map construction and candidate gene identification. Applying whole-genome re-sequencing and reduced representation sequencing technologies (e.g., RAD-seq and SLAF-seq) has significantly promoted the development of single nucleotide polymorphism (SNP) markers in the peanut genome. High-density SNP markers provide a more refined genomic map and allow for the accurate identification and localization of numerous genomic regions related to pod and seed size [9,22–25].

Recent studies have identified several candidate genes associated with peanut pod and seed size. Yang et al. finely mapped the major quantitative trait locus qAHPS07 related to pod size to a 36.46 kb interval on chromosome A07. They identified *AhRUVBL2* as the most likely candidate gene within this interval [26]. Zhao et al. located the major QTL controlling pod size within an approximately 1.17 Mb interval on chromosome 07 and fine-mapped it to a 20.4 kb interval using $F_{6:7}$ and $F_{6:8}$ populations, which contained only one predicted nonsynonymous gene mutation [27].

However, previous QTL mapping studies on traits related to peanut pod and seed size primarily relied on SNP chips and reduced representation sequencing technologies. In contrast, the present study employed whole-genome re-sequencing technology, a more precise and comprehensive approach, which resulted in a genetic map that is more detailed and accurate. This high-resolution genetic map not only aids in the precise localization of key QTL, but also provides a deeper understanding of the underlying genetic mechanisms. The candidate genes linked to peanut pod and seed size remain incompletely discovered, and existing research offers only a restricted comprehension of the genetic foundation of these characteristics. Although several QTL mapping investigations have been carried out, only a limited number of candidate genes have been identified due to the use of somewhat restricted techniques. The complexity of these qualities has posed a challenge in fully elucidating the genetic mechanisms that underlie them. The objective of this work is to conduct high-resolution mapping of quantitative trait loci (QTLs) associated with pod and seed size. The goal is to find more potential genes and enhance the knowledge of the genetic control mechanisms that govern these characteristics.

This study used a high-generation recombinant inbred line (RIL) population derived from a cross between Huayu 44 and DF12. A high-density genetic map was constructed based on whole-genome re-sequencing, and QTL analysis was conducted for hundred pod weight (HPW), hundred seed weight (HSW), pod length (PL), pod width (PW), seed length (SL), and seed width (SW) across six environments. A total of 44 QTLs related to pod and seed size were detected. Notably, a QTL enrichment region was identified at the distal end of chromosome A05. This enrichment region was narrowed down by developing molecular markers for two candidate gene hotspot regions of 600.9 kb and 721.2 kb. Ten candidate genes that potentially regulate pod and seed size were identified from these regions. The experimental results provide a theoretical basis for the genetic improvement of peanut pod and seed size traits through MAS and identifying genes associated with these traits.

2. Materials and Methods

2.1. Plant Materials and Development of RIL Population

By selecting Huayu 44 (a large-seed variety) as the female parent and DF12 (a small-seed variety) as the male parent, a high-generation RIL population comprising 807 lines was constructed using the Single-Seed descent method. Huayu 44 was created by the Shandong Academy of Agricultural Sciences using interspecific hybridization of the farmed species Arachis hypogaea with the biologically incompatible wild species Arachis glabrata Benth. The pods of this cultivar are somewhat huge and it is classified as the ordinary type. DF12 was created by the Industrial Crops Research Institute of the Henan Academy of Agricultural Sciences through a cross between Kaifeng Xuan 01-6 and Baisha 1016. It is an ordinary small-podded kind (Figure 1). The 807 lines and their parents were planted in six different environments. Those grown at the Fen-Yang (FY) experimental station (37°42′ N, 111°79′ E) in 2021, 2022, and 2023 are referred to as 21FY, 22FY, and 23FY, respectively. Those used for the experiment conducted at the Le-Dong (LD) experimental station (18°43′ N, 109°00′ E) in 2021 and 2023 are referred to as 21LD and 23LD, respectively. Experiments were also conducted at the Nan-Bin (NB) experimental station (18°38′ N, 109°21′ E) in 2021, and those grown there are referred to as 21NB (Table S1). Experimental planting in each environment followed a replicate randomized block design, with each row containing 15–20 plants spaced 15 cm apart within rows and 25 cm between rows. Standard agricultural practices were followed for field management during planting.

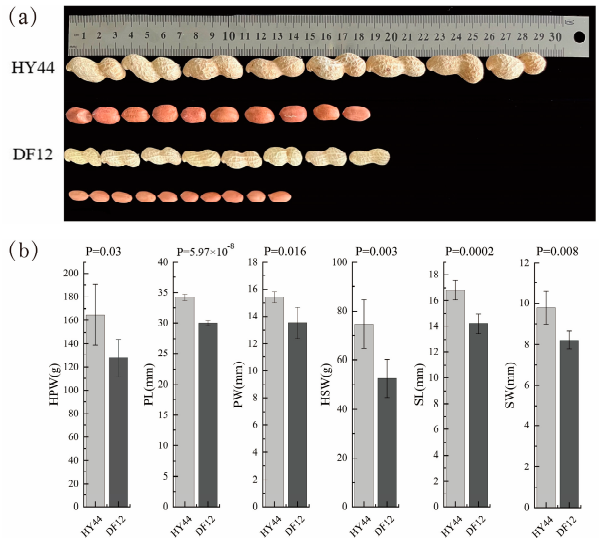

Figure 1. Phenotypic characterization of seeds from the female parent Huayu 44 and the male parent DF12: (**a**) Pod and seed morphologies of the two parents. (**b**) Comparisons of hundred pod weight (HPW), pod length (PL), pod width (PW), hundred seed weight (HSW), seed length (SL), and seed width (SW) between Huayu 44 and DF12. Data are shown as mean ± standard error. *p* values were generated using Student's *t*-test.

2.2. Trait Measurements and Data Analysis

After physiological maturity, RIL populations and their parents were harvested individually in rows. After drying in the sun, ten full pods and 20 intact seeds were selected from each family. The traits of the parents and families across the six environments were measured using a Wanshen SC-G Automatic Seed Analysis Instrument (Model: SC-G). The equipment was manufactured by Hangzhou Wanshen Testing Technology Co., Ltd., located in Hangzhou, China.

Based on image recognition principles, the six traits measured were hundred pod weight, pod length, pod width, hundred seed weight, seed length, and seed width. Basic statistical analyses of both the pod and seed traits of the parents and the RIL population, including mean values, standard deviations, and coefficients of variation, were performed using SPSS 20.0. Additionally, correlation coefficients between the six traits were calculated. Normal distribution graphs were plotted using Origin 7.0.

2.3. QTL Analysis

We used the high-density genetic linkage map previously constructed in our laboratory [28]. Combining the phenotypic data of six traits related to pod and seed size from 200 re-sequenced peanuts of the RIL population and the two parents across six planting environments, QTL identification was performed using the composite interval mapping (CIM) model in the R/qtl package [29], with 1000 permutation tests. A log of the odds (LODs) threshold of 3.0 at the 95% significance level was used to declare the presence of a QTL. Positive and negative additive effects indicated favorable alleles in Huayu 44 and DF12, respectively. QTLs with a variance contribution rate of $\geq$10% were considered major QTLs, while others were considered minor QTLs [18]. A QTL detected in two or more environments was considered stable. QTL naming followed previous conventions, starting with the letter "q" followed by the trait name and chromosome number [30]. If multiple QTLs were detected for the same trait and chromosome, they were distinguished by numbers, such as qHPWA05.1 and qHPWA05.2 for two QTLs related to HPW on chromosome A05.

2.4. Further QTL Mapping Based on KASP Genotyping

Twelve KASP markers were developed based on the identified QTL enrichment regions (Table S2), covering the main QTL enrichment area and its flanking regions. For KASP genotyping, 607 families from the RIL population were selected after excluding 200 re-sequenced families. Ten of the twelve KASP molecular markers were successfully genotyped (Figure S1). The genotyping results and corresponding phenotypic data were used to construct a local genetic linkage map using the MAP module in IciMapping v4.2. QTL mapping was performed using the BIP module with the parameters set to step (cm) = 0.1000.

2.5. Prediction of Candidate Genes in Hotspot Regions

Narrowed intervals on chromosome A05 were defined as hotspot regions for candidate genes regulating peanut pod and seed size. Genes within these hotspot regions were predicted based on the "Tifrunner.gnm2.J5K5" genome sequence. The Candidate genes were annotated using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Non-Redundant Protein (NR) databases. The potential functions of these genes were categorized and analyzed to identify potential candidate genes.

3. Results

3.1. Agronomic Performance of the Parents and RIL Individuals

Using Huayu 44 and DF12 as parental lines, an RIL population consisting of 807 families was constructed. The female parent, Huayu 44, has larger pods and seeds, whereas the male parent, DF12, is a small-seeded variety (Figure 1a). Across the six environments, Huayu 44 showed higher values for HPW, PL, PW, HSW, SL, and SW than DF12 (Figure 1b). The six traits related to pod and seed size in the RIL population exceeded the mean values

of the two parents, indicating a wide range of variation and transgressive segregation (Table 1). The population exhibited a continuous distribution and rich genetic diversity. Most phenotypic data had absolute kurtosis and skewness of <1. Combined with the frequency distribution histograms (Figure 2), all traits displayed a normal distribution, characteristic of typical quantitative traits. Significant positive correlations were observed among most traits across the six environments (Table 2), particularly between HPW and HSW, which showed strong correlations in all six environments (0.36~0.95, $p < 0.001$). The highest correlation was 0.95 ($p < 0.001$) in the 23LD environment, with HSW and SW, PL and SL, and HPW and SW also highly correlated in this environment with correlation coefficients of 0.87, 0.82, and 0.79, respectively ($p < 0.001$).

Table 1. Phenotypic variations for six traits of the RIL population and their parents in six environments.

Trait	Env.	Parents		RIL Population					
		DF12	HY44	Max	Min	Mean ± SD	CV/%	Kurt	Skew
HPW (g)	20FY	132.64	158.65	356.15	77.80	140.62 ± 35.51	25.25	14.93	3.24
	21FY	162.97	192.86	203.23	65.50	149.30 ± 24.69	16.54	0.02	−0.36
	21LD	127.86	154.54	220.92	36.10	126.57 ± 26.24	20.73	1.48	−0.02
	21NB	112.37	140.68	180.24	62.71	123.27 ± 21.10	17.11	0.03	−0.12
	22FY	94.01	126.99	179.81	77.10	126.52 ± 19.51	15.43	0.33	0.06
	23LD	135.66	213.91	236.88	111.26	166.74 ± 23.77	14.25	0.02	0.32
PL (mm)	20FY	29.81	33.44	43.85	26.62	34.40 ± 3.08	8.96	−0.17	0.10
	21FY	31.22	34.65	39.40	27.06	33.44 ± 2.80	8.36	−0.73	−0.14
	21LD	29.85	34.78	41.99	15.70	32.15 ± 3.58	11.12	1.98	−0.63
	21NB	29.77	33.69	41.77	22.40	33.10 ± 3.46	10.44	−0.31	−0.18
	22FY	29.49	34.75	40.04	25.27	31.94 ± 2.64	8.26	−0.10	0.09
	23LD	29.78	33.87	37.72	25.49	32.25 ± 2.52	7.83	−0.29	−0.26
PW (mm)	20FY	12.48	14.85	19.04	11.70	14.91 ± 1.15	7.74	0.94	0.48
	21FY	15.98	16.29	18.07	12.25	14.98 ± 1.34	8.95	−0.81	0.21
	21LD	14.45	15.46	17.90	10.70	14.54 ± 1.26	8.69	0.06	0.23
	21NB	12.25	14.77	17.46	11.89	14.59 ± 1.07	7.31	0.23	0.42
	22FY	12.57	15.67	17.84	11.56	14.48 ± 1.23	8.49	−0.23	0.26
	23LD	13.37	15.43	17.24	11.88	14.59 ± 0.97	6.62	−0.10	0.19
HSW (g)	20FY	54.15	74.36	77.46	33.15	56.45 ± 8.58	15.20	−0.40	−0.05
	21FY	60.34	75.87	85.82	33.26	65.07 ± 8.69	13.36	0.22	−0.20
	21LD	48.12	85.85	92.30	32.30	61.50 ± 12.26	19.93	−0.39	0.04
	21NB	62.57	76.45	81.93	29.80	55.49 ± 9.43	17.00	0.02	−0.04
	22FY	33.19	44.69	66.09	10.29	46.48 ± 8.12	17.48	1.46	−0.38
	23LD	55.70	89.94	98.24	44.38	68.54 ± 10.00	14.60	−0.10	0.29
SL (mm)	20FY	14.92	16.24	18.55	10.89	15.27 ± 1.27	8.30	0.05	−0.15
	21FY	15.47	16.60	18.75	12.34	15.81 ± 1.21	7.65	−0.43	−0.17
	21LD	13.79	18.11	18.41	10.58	15.47 ± 1.46	9.44	−0.15	−0.30
	21NB	13.91	17.79	18.13	11.68	15.22 ± 1.30	8.53	−0.53	−0.23
	22FY	12.90	15.35	17.37	11.83	14.48 ± 1.14	7.85	−0.37	0.04
	23LD	14.52	16.79	18.01	12.85	15.50 ± 1.10	7.10	−0.47	−0.23
SW (mm)	20FY	8.43	10.01	9.75	6.32	8.62 ± 0.54	6.28	1.08	−0.56
	21FY	8.81	10.32	10.81	7.54	9.31 ± 0.51	5.44	0.49	0.04
	21LD	8.18	10.22	10.49	6.55	8.78 ± 0.70	7.99	0.14	−0.22
	21NB	8.12	9.35	10.92	6.76	8.73 ± 0.64	7.35	0.53	−0.17
	22FY	6.96	7.80	9.35	6.47	8.06 ± 0.55	6.83	0.05	−0.24
	23LD	8.77	11.04	11.10	7.87	9.54 ± 0.63	6.63	−0.30	0.18

The female parent is Huayu 44 and the male parent is DF12. Env. is the environment. The unit of measurement for HPW and HSW is grams. The unit of measurement for PL, PW, SL, and SW is centimeters. Max, maximum; Min, minimum; SD, standard deviation; cv is the coefficient of variation; Skew, skewness; Kurt, kurtosis.

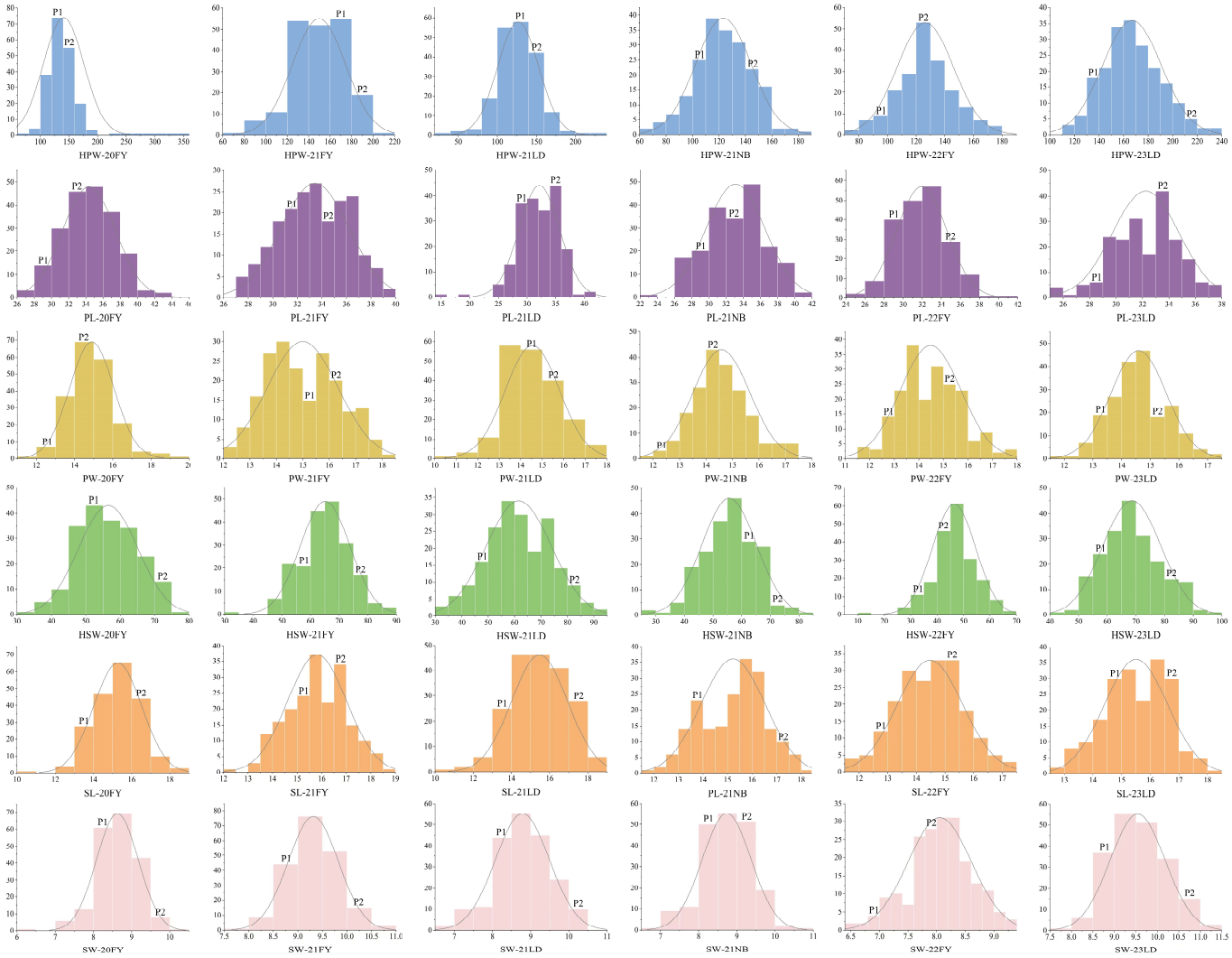

Figure 2. Phenotypic distributions of six traits related to pod and seed size in the RIL population under six environments. P1 represents the phenotypic distribution of DF12, and P2 represents the phenotypic distribution of Huayu 44.

Table 2. Correlation analysis of six traits in the RIL population under six environments.

Environment	Trait	HPW	PL	PW	HSW	SL	SW
20FY	HPW	1					
	PL	0.20 **	1				
	PW	0.37 ***	0.64 ***	1			
	HSW	0.36 ***	0.39 ***	0.44 ***	1		
	SL	0.28 ***	0.70 ***	0.52 ***	0.76 ***	1	
	SW	0.28 ***	0.045	0.20 **	0.83 ***	0.38 ***	1
21FY	HPW	1					
	PL	0.66 ***	1				
	PW	0.65 ***	0.65 ***	1			
	HSW	0.85 ***	0.62 ***	0.61 ***	1		
	SL	0.70 ***	0.86 ***	0.65 ***	0.77 ***	1	
	SW	0.68 ***	0.26 ***	0.44 ***	0.83 ***	0.41 ***	1

Table 2. *Cont.*

Environment	Trait	HPW	PL	PW	HSW	SL	SW
21LD	HPW	1					
	PL	0.71 ***	1				
	PW	0.68 ***	0.68 ***	1			
	HSW	0.83 ***	0.55 ***	0.57 ***	1		
	SL	0.69 ***	0.77 ***	0.59 ***	0.78 ***	1	
	SW	0.65 ***	0.19 **	0.42 ***	0.84 ***	0.42 ***	1
21NB	HPW	1					
	PL	0.58 ***	1				
	PW	0.64 ***	0.65 ***	1			
	HSW	0.82 ***	0.51 ***	0.57 ***	1		
	SL	0.44 ***	0.63 ***	0.44 ***	0.52 ***	1	
	SW	0.46 ***	0.07	0.27 ***	0.61 ***	0.25 ***	1
22FY	HPW	1					
	PL	0.65 ***	1				
	PW	0.62 ***	0.72 ***	1			
	HSW	0.90 ***	0.53 ***	0.48 ***	1		
	SL	0.74 ***	0.85 ***	0.64 ***	0.71 ***	1	
	SW	0.77 ***	0.23 ***	0.35 ***	0.81 ***	0.43 ***	1
23LD	HPW	1					
	PL	0.54 ***	1				
	PW	0.63 ***	0.69 ***	1			
	HSW	0.95 ***	0.44 ***	0.55 ***	1		
	SL	0.63 ***	0.82 ***	0.63 ***	0.61 ***	1	
	SW	0.79 ***	0.093	0.37 ***	0.87 ***	0.29 ***	1

The correlations were calculated with Pearson correlation coefficients, and the p values are indicated as follows: **, $p < 0.01$; ***, $p < 0.001$.

3.2. QTL Mapping for Traits Related to Pod and Seed Size

Our laboratory previously performed whole-genome re-sequencing on 200 of the RIL population and the two parents using the genome of the cultivated peanut variety Tifrunner.gnm2.J5K5 as a reference to construct a genetic linkage map. The total length of this linkage map was 1216.32 cm, comprising 20 linkage groups and 2494 bin markers, which included 447,528 SNPs and 220,443 indels. The average distance between adjacent markers was 0.49 cm. We conducted QTL mapping analysis for traits related to pod and seed sizes. A total of 44 QTLs associated with six traits were detected (Table 3, Figure 3) and distributed across chromosomes A02, A05, B04, B08, and B10. Among these, 40 were major QTLs and four were minor QTLs. The additive effects of these QTLs ranged from −5.94% to 12.8%, LOD values ranged from 4.17 to 22.72, and the phenotypic variance explained (PVE) ranged from 9.16% to 40.73%. These relatively high values reflect the strong stability and reliability of these QTLs. On chromosomes A05, B08, and B10, seven QTLs related to HPW were detected. Among them, qHPWA05.1, which was repeatedly detected in two environments (21FY and 21LD), showed high positive additive effects (12.8% and 11.77%) and explained 26.63% and 19.39% of the phenotypic variance, respectively. Therefore, qHPWA05.1, considered a major and stable QTL, is a key target region for breeding efforts to increase HPW. Eight QTLs related to PL were detected on chromosomes A02, A05, and B04, with all but qPLB04 showing positive additive effects. Among these, qPLA05.2 had the highest PVE for PL at 35.85%, making it the most significant contributor to PL variation. For PW, six QTLs were detected, all located on chromosome A05. qPWA05.2 exhibited the highest positive additive effect (1.37%), explaining 30.65% of the phenotypic variance, thus making the most significant contribution to PW. Eight QTLs related to HSW were detected on chromosomes A05 and B10, all showing positive additive effects (2.48–4.97%). qHSWA05.2 had the highest PVE at 32.20%, whereas qHSWB10.2 had an explained variance of 9.61%, classifying it as a minor QTL. qHSWA05.1, qHSWA05.3, qHSWA05.4, qHSWA05.5, qHSWB10.1, and qHSWB10.3 explained 14.08%, 17.75%, 21.66%,

16.68%, 12.82%, and 10.52% of the phenotypic variance, respectively, all being major QTLs. Six QTLs were detected for SL, located on chromosome A05 and showing positive additive effects. qSLA05.2 had the highest phenotypic variance (40.73%), the highest among all the QTLs for SL. qSLA05.1, qSLA05.3, qSLA05.4, qSLA05.5, and qSLA05.6 explained 22.08%, 37.86%, 25.39%, 24.31%, and 10.54% of the phenotypic variance, respectively. Nine QTLs related to seed width SW were detected on chromosomes A02, A05, B04, B08, and B10, with PVE ranging from 10.54% to 16.00%, all of which were major QTLs. Among these, qSWA05.1 had the highest contribution, explaining 16.00% of the phenotypic variance.

Table 3. QTL information for traits related to pod and seed size.

Trait	QTL	Chr.	Range (cm)	LOD	Env.	Additive	PVE%
HPW	qHPWA05.1	A05	29.91–49.52	13.45	21FY	12.80	26.63
	qHPWA05.2	A05	34.81–49.52	11.71	22FY	9.52	23.64
	qHPWA05.3	A05	29.91–49.42	9.36	21LD	11.77	19.39
	qHPWA05.4	A05	30.17–48.99	11.16	23LD	11.41	22.66
	qHPWA05.5	A05	35.34–48.72	5.95	21NB	7.68	12.81
	qHPWB08	B08	0–0.80	4.18	22FY	−5.94	9.18
	qHPWB10	B10	23.66–24.76	4.17	21FY	7.50	9.16
PL	qPLA02	A02	67.60–68.37	4.21	21NB	1.05	9.24
	qPLA05.1	A05	24.34–48.19	7.88	20FY	1.26	16.59
	qPLA05.2	A05	25.14–52.32	19.28	21FY	1.68	35.85
	qPLA05.3	A05	21.43–49.52	13.48	22FY	1.37	26.82
	qPLA05.4	A05	27.78–48.19	9.41	21LD	1.61	19.49
	qPLA05.5	A05	27.78–49.52	12.62	23LD	1.28	25.22
	qPLA05.6	A05	26.73–52.32	9.36	21NB	1.55	19.38
	qPLB04	B04	35.65–37.53	4.83	21FY	−0.91	10.52
PW	qPWA05.1	A05	34.02–48.19	7.88	20FY	0.47	16.60
	qPWA05.2	A05	29.91–49.52	15.89	21FY	1.37	30.65
	qPWA05.3	A05	30.96–49.52	13.26	22FY	0.63	26.31
	qPWA05.4	A05	25.14–52.32	15.51	21LD	0.70	30.04
	qPWA05.5	A05	35.87–49.52	10.91	23LD	0.46	22.22
	qPWA05.6	A05	34.02–52.32	10.26	21NB	0.49	21.04
HSW	qHSWA05.1	A05	34.81–46.33	6.59	20FY	3.29	14.08
	qHSWA05.2	A05	27.8–49.52	16.88	21FY	4.97	32.20
	qHSWA05.3	A05	35.34–47.39	8.49	22FY	3.44	17.75
	qHSWA05.4	A05	29.91–49.52	10.60	23LD	4.70	21.66
	qHSWA05.5	A05	34.02–49.52	7.92	21NB	3.90	16.68
	qHSWB10.1	B10	20.64–35.72	5.95	21FY	3.13	12.82
	qHSWB10.2	B10	23.12–26.91	4.39	22FY	2.48	9.61
	qHSWB10.3	B10	20.64–30.13	4.83	21NB	3.10	10.52
SL	qSLA05.1	A05	30.17–52.32	10.83	20FY	0.60	22.08
	qSLA05.2	A05	21.17–52.32	22.72	21FY	0.77	40.73
	qSLA05.3	A05	19.84–52.32	20.66	22FY	0.70	37.86
	qSLA05.4	A05	23.82–49.52	12.72	21LD	0.74	25.39
	qSLA05.5	A05	21.17–48.19	12.10	23LD	0.54	24.31
	qSLA05.6	A05	38.22–44.25	4.84	21NB	0.42	10.54
SW	qSWA02	A02	66.49–68.37	5.35	23LD	−0.21	11.60
	qSWA05.1	A05	30.70–46.59	7.57	21FY	0.20	16.00
	qSWA05.2	A05	36.92–43.73	5.18	22FY	0.18	11.25
	qSWA05.3	A05	37.18–48.99	5.88	21LD	0.25	12.67
	qSWA05.4	A05	38.75–45.55	5.90	23LD	0.23	12.69
	qSWB04.1	B04	47.79–51.78	4.73	22FY	0.18	10.31
	qSWB04.2	B04	32.43–51.25	5.40	21LD	0.24	11.70
	qSWB08	B08	0–1.07	4.92	22FY	−0.18	10.71
	qSWB10	B10	20.64–35.45	4.84	21FY	0.17	10.54

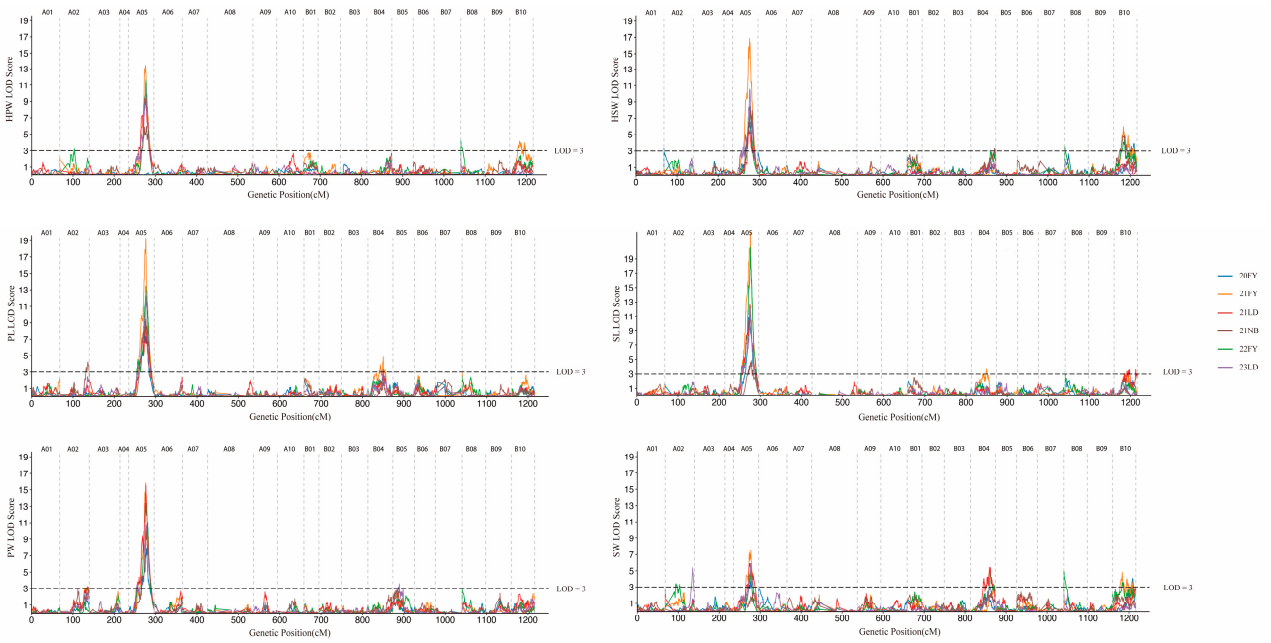

Figure 3. QTL mapping for traits related to pod and seed size.

3.3. Further Identification of QTL Enrichment Regions on Chromosome A05

The preliminary QTL mapping results indicate an enriched region of multiple QTLs on chromosome A05 (Figure 4). To further confirm and narrow down the QTL on chromosome A05 related to HPW, PL, PW, HSW, SL, and SW, we constructed a local genetic linkage map based on 10 successfully genotyped KASP molecular markers and further refined the QTL enrichment region on chromosome A05 (Figure 5). Six QTLs were located (Table 4), with additive effect values ranging from −9.89 to −0.20. The negative additive effect values indicated that the favorable alleles for these traits originate from the male parent DF12. High LOD values (7.08 to 20.26) indicated a strong association between the QTL and the target traits. At the same time, the PVE ranged from 10.75% to 27.92%, indicating significant contributions of these major QTLs to the target traits. qHPWA05.6 explained 16.69% of the phenotypic variance for HPW, qPLA05.7 explained 23.90% of the phenotypic variance for PL, qPWA05.7 explained 18.06% of the phenotypic variance for PW, qSLA05.7 explained 18.36% of the phenotypic variance for SL, and qSWA05.5 explained 10.75% of the phenotypic variance for SW. These QTLs were co-located between the markers Ah900023 and Ah900027. The co-located interval was named after the QTL with the highest contribution rate (qPLA05.7). Genes within this interval regulated multiple traits, including HPW, PL, PW, SL, and SW, indicating pleiotropic effects. qHSWA05.6, located between the markers Ah900027 and Ah900026, explained 27.92% of the phenotypic variance in HSW.

Table 4. Information on QTL related to pod and seed size after further narrowing of the enriched region.

Trait	Chromosome	Marker Interval	LOD	Add	PVE(%)
qHPWA05.6	A05	Ah900023–Ah900027	11.31	−9.89	16.69
qPLA05.7	A05	Ah900023–Ah900027	16.82	−1.32	23.90
qPWA05.7	A05	Ah900023–Ah900027	12.43	−0.56	18.06
qHSWA05.6	A05	Ah900027–Ah900026	20.26	−0.65	27.92
qSLA05.7	A05	Ah900023–Ah900027	12.15	−4.10	18.36
qSWA05.5	A05	Ah900023–Ah900027	7.08	−0.20	10.75

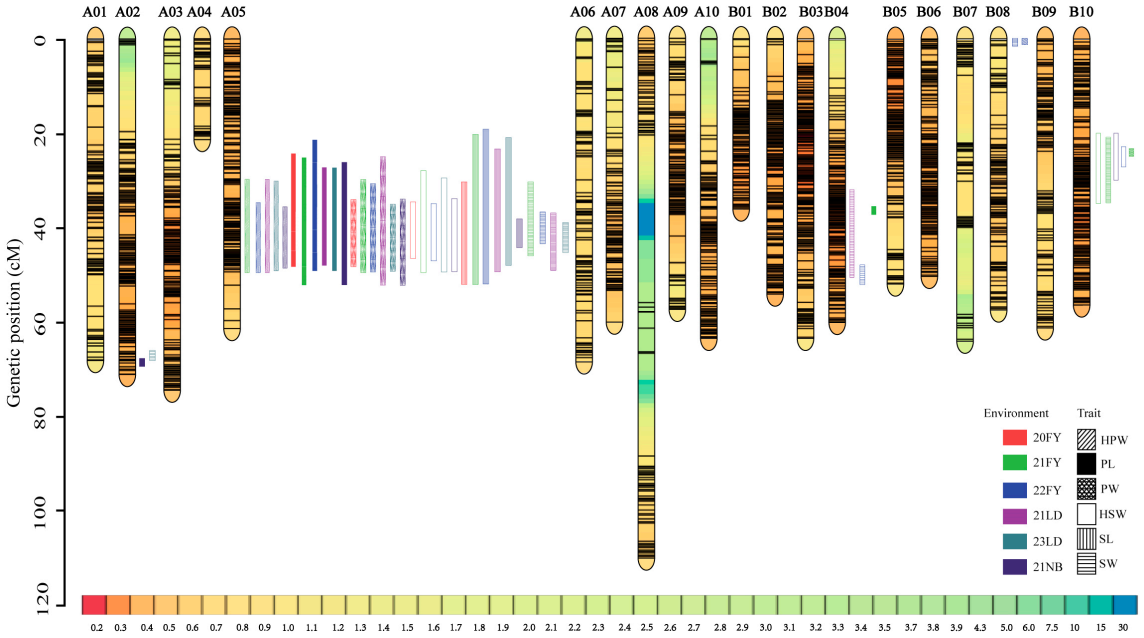

Figure 4. Distribution of QTLs related to pod and seed size on the genetic linkage map. The distributions of recombination hotspots and coldspots on the genetic map are shown by the color blocks along the horizontal axis. Recombination density increases with color; conversely, it decreases with color. Redder colors indicate higher recombination densities.

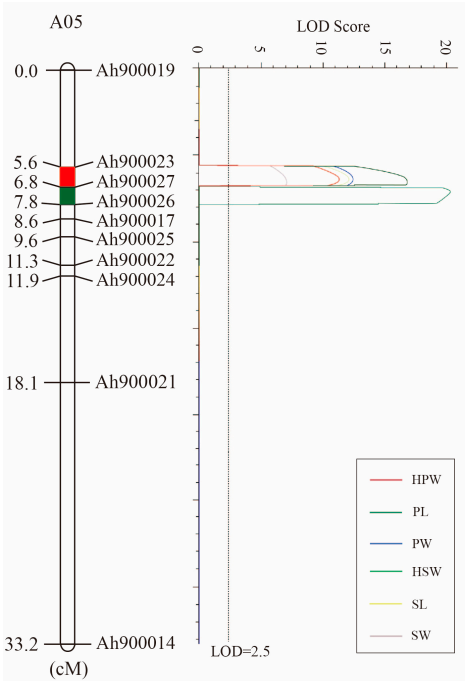

Figure 5. Further narrowing of the QTL related to pod and seed size in the enriched region. The red region represents qPLA05.7, and the green region represents qHSWA05.6.

3.4. Confirmation and Identification of Candidate Gene Hotspot Regions on Chromosome A05

Under the QTL enrichment region on chromosome A05, we developed ten KASP markers, refining the enrichment area to two QTL regions: qPLA05.7 (721.2 kb) and qHSWA05.6 (600.9 kb). These regions have been identified as hotspots for candidate genes associated with pod and seed size. We annotated 22 candidate genes within the qPLA05.7 region and 27 candidate genes within the qHSWA05.6 region using the GO, KEGG, and NR

databases (Table S3). Through GO annotation, 36 of the 49 candidate genes were assigned at least one GO term. The 36 genes were classified into three GO categories: "cellular component", "molecular function", and "biological process." The biological processes these genes are involved in include the methionine biosynthetic process (GO:0009086), threonine biosynthetic process (GO:0009088), isoleucine biosynthetic process (GO:0009097), embryo development ending in seed dormancy (GO:0009793), regulation of transcription, DNA-templated (GO:0006355), sugar-mediated signaling pathway (GO:0010182), protein ubiquitination (GO:0016567), ubiquitin-dependent protein catabolic process (GO:0042787), response to auxin (GO:0009733), response to jasmonic acid (GO:0009753), and polyamine biosynthetic process (GO:0006596). These biological processes involve amino acid synthesis, embryo development, transcription regulation, sugar signal transduction, protein degradation, and plant hormone response. KEGG analysis revealed that these genes encode proteins including chloroplast-like ferredoxin-thioredoxin reductase catalytic chain, 40S ribosomal protein S10, mannose-1-phosphate guanylyltransferase, E3 ubiquitin-protein ligase, cyclic nucleotide-gated ion channel, spermidine synthase, Cullin-1, E2F transcription factor, and lysine-specific histone demethylase. These genes are involved in protein synthesis, modification of cell signaling, and metabolic regulation, highlighting their potential roles in pod and seed development. Using the NR database, we identified the following homologies for the candidate genes: 18 candidate genes were homologous to *Glycine max*, nine to *Cicer arietinum*, eight to *Medicago truncatula*, and seven to *Phaseolus vulgaris*. Additionally, one gene was homologous to *Fragaria vesca* subsp. *vesca*, *Pisum sativum*, *Glycine soja*, and *Lotus japonicus*.

4. Discussion

The present study constructed a new RIL population through the cross between Huayu 44 and DF12. The extensive variation among offspring provided a rich diverse foundation for QTL mapping, facilitating the identification of different genes affecting these traits. Some offspring exhibit trait values beyond the extremes of their parents, which helps in identifying potentially favorable gene combinations and interactions. The six traits also exhibited a normal distribution across the six different environments, providing a solid population foundation for accurately mapping the QTL-controlling pod and seed size. Whole-genome re-sequencing technology covers the entire genome, avoiding gene loss or bias and ensuring the completeness and accuracy of the constructed map. We constructed a high-density genetic map based on whole-genome re-sequencing in the present study. We conducted QTL mapping to ensure the precision and accuracy of the identified QTL.

A total of 44 QTLs were detected across six environments, with 40 major QTLs and four minor QTLs with PVE values close to 10%, indicating a high contribution to the target traits. The high additive effects and PVE values of these QTLs are important for breeding practices because selecting these QTLs can substantially improve target traits. High LOD scores provided reliable statistical evidence for the significance of the detected QTL, laying the foundation for further functional validation and application. Previous studies have identified numerous QTLs related to pod and seed size on chromosome A05. We identified 32 additive QTLs with PVE values up to 40.73% on chromosome A05, all of which were major QTLs, emphasizing the central role of chromosome A05 in the genetic control of pod and seed size. These QTLs highly overlapped, with peaks at the same or adjacent positions, suggesting the presence of one or more gene clusters on chromosome A05 that collectively influence pod and seed sizes through complex genetic mechanisms. Several stable and consistent QTLs detected in the initial mapping had widespread effects on multiple traits of peanut pod and seed size. This phenomenon was also observed in the subsequent validation and fine mapping, indicating that the strong correlations between HPW, PL, PW, HSW, SL, and SW were the main reasons for this phenomenon.

By developing 10 KASP markers on chromosome A05 to cover the overlapping major QTL enrichment region and its flanking areas, we narrowed down the overlapping section to intervals of approximately 600.9 kb between markers Ah900026 and Ah900027

(106,625,271–107,226,203 bp) and approximately 721.2 kb between markers Ah900027 and Ah900023 (107,226,203–107,947,402 bp). Comparatively, these two narrowed intervals overlap with the QTL region of 101.70–111.64 Mb on chromosome A05 identified by Wang [31] and the QTL reported by Luo et al. [18,32]. Identifying similar or overlapping QTL regions in populations with different genetic backgrounds demonstrates that these QTLs generally influence pod and seed size traits rather than being specific to a particular parental genetic background. This further suggests that these regions may contain genes crucial for pod and seed size that are conserved across different parents; that is, the position and function of these genes did not change significantly among the parents. This consistency in gene position and function across different genetic backgrounds indicates that these genes and their effects have been preserved throughout evolution.

Numerous QTLs associated with peanut pod and seed size have been reported on chromosomes A02, A03, A05, A07, A09, A10, B02, B03, B06, B07, and B08 [14,16,22,33,34]. However, in our recent study, five new QTLs were detected on chromosome B10. These findings are significant because QTLs associated with pod and seed traits on chromosome B10 have rarely been reported. Therefore, chromosome B10 is crucial for studying peanuts' pod- and seed-related traits. This warrants further research with an increased density of molecular markers for precise localization.

Using GO, KEGG, and NR databases annotated 49 candidate genes within the identified hotspot regions. By comparing these genes with homologous genes in other species with related functions, we identified 10 candidate genes that may regulate pod and seed size. These genes encode cytochrome P450, spermidine synthase, mannose-1-phosphate guanylyltransferase, pentatricopeptide repeat proteins, and E2F transcription factors. The genes *Arahy.QW591P*, *Arahy.Q9F5SX*, *Arahy.Z6EAYS*, *Arahy.4A049R*, and *Arahy.TNFT39* encode proteins belonging to the cytochrome P450 (CYP450) family. As one of the largest plant protein families, CYP450 proteins have been found to play a significant role in seed development in various crops, such as wheat [35], tomato [36], and rapeseed [37]. Adamski et al. found that the CYP450 protein encoded by *KLUH* determines the growth potential of the seed coat by promoting cell proliferation in both the inner and outer integuments, thereby affecting final seed size [38]. Fang et al. discovered that EOD3 encodes a CYP450 protein in Arabidopsis, and its overexpression significantly increases seed size [39]. Ma et al. found that the activity of wheat *TaCYP78A3* is positively correlated with the final seed size, as it influences the number of cells in the seed coat. Silencing *TaCYP78A3* reduces seed coat cell numbers, resulting in an 11% decrease in seed size, whereas overexpression of *TaCYP78A3* in Arabidopsis induces more seed coat cells, increasing seed size by 11–48% [35]. Liu et al. utilized multi-omics analysis to identify a candidate gene encoding the CYP450 protein associated with increased peanut seed size. They hypothesized that the significant upregulation of CYP450 may contribute to larger seed size [40]. Based on existing research evidence, we hypothesize that the genes mentioned above encoding CYP450 proteins (*Arahy.QW591P*, *Arahy.Q9F5SX*, *Arahy.Z6EAYS*, *Arahy.4A049R*, and *Arahy.TNFT39*) may significantly influence seed size by regulating the number and size of cells within the seed coat and other related organs during seed development.

Arahy.DY91G5 encodes spermidine synthase (SPDS). Studies have shown that the *OsSPMS1* gene in rice plays a crucial role in converting spermidine (SPD) to spermine. Overexpression of *OsSPMS1* leads to the excessive consumption of SPD, thereby negatively regulating seed germination, grain size, and yield of rice [41]. Recent studies have also found that the overexpression of the TaSPDS-7D1 gene, which encodes SPDS in wheat, significantly increases the thousand-grain weight of wheat seeds. Therefore, the SPD expression level has a crucial effect on seed size [42].

Arahy.Q67Z2R encodes an E2F transcription factor that plays a vital role in cell-cycle regulation. Studies have shown that the activators E2FA and E2FB primarily regulate cell cycle progression. These factors also inhibit the premature expression of seed maturation-related genes and coordinate the orderly progression of cell proliferation and maturation

during seed development [43]. Therefore, E2F transcription factors are critical regulators of seed development.

Arahy.SEJH4Y encodes mannose-1-phosphate guanylyltransferase, which mediates N-glycosylation during cellulose biosynthesis. Cellulose is a crucial component of peanut seed coats and pods [44]. Thus, mannose-1-phosphate guanylyltransferase may influence the development of seed coats and pods by regulating cellulose synthesis and affecting peanut pod size.

Arahy.7TY6PE and *Arahy.X5R2L4* encode pentatricopeptide repeat (PPR) proteins, whose functional impairments can lead to abnormal phenotypes related to embryonic development, photosynthetic capacity, and seed pigment deposition [45]. In maize, *qKW9,* which encodes a PPR protein, regulates kernel size and weight by influencing photosynthesis and grain filling [46]. Recent studies have identified several PPR-encoding genes as candidates for regulating seed size in peanuts. Gangurde et al. identified several candidate genes related to peanut SW using linkage and association analyses, one of which was predicted to encode a PPR protein [44]. Using transcriptome analysis combined with previously reported QTL data, Li et al. predicted the PPR protein-encoding gene *EVM0025654* to be a candidate gene associated with peanut seed size variation [45]. Therefore, the PPR protein-encoding genes *rahy.7TY6PE* and *Arahy.X5R2L4* identified in this study may be potential candidate genes associated with peanut pod and seed size variations.

Notably, several key candidate genes identified in this study, including those encoding SPDS, mannose-1-phosphate guanylyltransferase, PPR proteins, and E2F transcription factors, were also mentioned by Gangurde et al. in their identification of candidate genes related to peanut pod and seed weight [44]. Although we have identified several candidate genes within the hotspot regions that may regulate peanut pod and seed size, there remains some uncertainty due to the lack of functional validation. Functional validation of these candidate genes is crucial in confirming their role in trait regulation. It is possible to further elucidate whether and how these genes influence pod and seed size through functional experiments such as gene knockout, overexpression, or gene editing.

5. Conclusions

This study constructed a high-density genetic map through whole-genome re-sequencing using a high-generation RIL population derived from a Huayu 44 and DF12 cross. QTL analysis was then employed to identify potential genomic regions associated with pod and seed size traits found on five chromosomes (A02, A05, B04, B08, and B10). In addition, a QTL-rich region was discovered on chromosome A05. To further narrow the interval, ten molecular markers were developed to cover the QTL-rich region and its flanking regions, reducing the interval lengths to 600.9 kb and 721.2 kb. These two QTL regions were identified as candidate gene hotspots regulating pod and seed size. Through prediction and annotation of candidate genes within these hotspot regions, 10 genes were proposed as candidate genes for regulating pod and seed size. These include those encoding CYP450 protein (*Arahy.QW591P*, *Arahy.Q9F5SX*, *Arahy.Z6EAYS*, *Arahy.4A049R*, and *Arahy.TNFT39*), polyamine synthase (*Arahy.DY91G5*), mannose-1-phosphate guanylyltransferase (*Arahy.SEJH4Y*), pentapeptide repeat proteins (*Arahy.7TY6PE* and *Arahy.X5R2L4*), and E2F transcription factor (*Arahy.Q67Z2R*). Identifying and characterizing these candidate genes will enhance our understanding of peanuts' molecular mechanisms underlying pod and seed size traits. Additionally, the molecular markers developed in this study can serve as potential selection markers, laying the foundation for marker-assisted breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14091634/s1, Table S1: annual average temperature and annual average precipitation in six environments; Table S2: The primers of 12 SNP-based KASP markers; Table S3: Annotation of candidate genes on spot region on chromosome A05; Figure S1: Genotyping profiles of the 12 developed KASP markers.

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Abbreviation

Abbreviation	Full Term
CIM	Composite Interval Mapping
KASP	Kompetitive Allele-Specific PCR
PVE	Phenotypic Variance Explained
QTL	Quantitative Trait Loci
RAD-seq	Restriction site-Associated DNA sequencing
RIL	Recombinant Inbred Line
SLAF-seq	Specific-Locus Amplified Fragment sequencing
SNP	Single Nucleotide Polymorphism

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