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Genome-Wide Association Study of Leaf Chlorophyll Content Using High-Density SNP Array in Peanuts (*Arachis hypogaea* L.)

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Abstract: The content of chlorophyll, a fundamental component required for photosynthesis in plants, has been widely studied across crop species. In this study, we aimed to evaluate the genetic diversity of 453 peanut accessions. We evaluated the evolutionary relationships using a genome-wide association study (GWAS) of leaf color data based on chlorophyll content analysis using the Axiom_Arachis array containing 58K single-nucleotide polymorphisms (SNPs). We identified seven SNPs as being significantly associated with leaf chlorophyll content on the chromosomes Aradu.A02, Aradu.A08, Araip.B02, Araip.B05, Araip.B06, and Araip.B08 in a GAPIT analysis. The SNP AX-176820297 on Araip.B05 was significantly linked with leaf chlorophyll content across the seasons. The Arahy.SDG4EV gene was detected to be in linkage disequilibrium (LD) with the significant SNPs, and its expression was significantly correlated with leaf chlorophyll content. The results of the current study provide useful and fundamental information with which to assess genetic variations in chlorophyll content and can be utilized for further genetic and genomic studies and breeding programs in peanuts.

Keywords: peanut; chlorophyll content; genetic diversity; population structure; genome-wide association study; linkage disequilibrium

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1. Introduction

The peanut (*Arachis hypogaea* L.) is a globally important oil seed and cash crop [1] and has been cultivated in more than 100 countries [2], including China, India, Nigeria, and the USA, with a global total production of 53,638,932 metric tons in 2019 (http://www.fao.org) (accessed on 21 December 2021). Peanut seeds contain high-quality oil, proteins, vitamins, and minerals and are used for oil extraction or in confectionary [3].

Chlorophyll is the fundamental material of photosynthesis in plant species. As a high level of photosynthetic efficiency is important for breeding [4], chlorophyll content has been widely studied in many crop species [5]. The chlorophyll content in leaves is an important index of photosynthesis, nutrient status, and response to diverse abiotic stresses, including drought and salinity [6], and is positively related to crop yield [7]; thus, a high chlorophyll content provides a material basis indicating improvements in the leaf photosynthetic rate [8]. It has been reported that the chlorophyll content in leaves is closely related to the photosynthetic capacity in many crops, including wheat [9], and abiotic

stresses could reduce the chlorophyll content and photosynthetic capacity [10,11]. As leaf photosynthesis is related to the chlorophyll content per leaf area, the SPAD chlorophyll meter has been widely used as a tool to screen genotypic variation in photosynthetic capacity [12].

Measurements of the chlorophyll content in peanut plants have commonly been used in studies of plant responses to abiotic stresses such as drought tolerance [13]. Previous studies have shown that there are a number of genetic variations in the chlorophyll content in legume species. A genome-wide association study (GWAS) using 332 diverse soybean genotypes identified the genomic regions responsible for diverse chlorophyll traits including those for chlorophyll a, chlorophyll b, total chlorophyll content, and chlorophyll a/b ratio and provided fundamental information about genetic diversity and breeding materials for soybean breeding programs [14]. Through measurements of the chlorophyll content in leaves from the seedling to the blooming stage, a total of 20 QTLs were mapped [15]. In addition, eight SNPs linked with a total of 168 soybean genotypes were reported that were used to detect SNPs associated with chlorophyll content and chlorophyll fluorescence using a SPAD meter [16].

Until now, few genetic studies on the chlorophyll characteristics of peanut plants have been reported. Chlorophyll content measurements in the peanut have been used to study the plant responses to abiotic stresses, such as drought tolerance. The International Crop Research Institute for the Semiarid Tropics (ICRISAT) assessed 269 wild *Arachis* species and identified some specimens with high SPAD chlorophyll meter readings [17]. It has been reported that genotypes with a high chlorophyll content could be used in further breeding programs specifically targeted to drought regions [18]. Additionally, studies on gene-encoding chlorophyll biosynthesis showed that AHGLK1, a transcription factor, upregulates the expression of AHPORA, encoding chlorophyll biosynthesis during post-drought recovery. This suggests that AHGLK1 may be involved in the recovery of peanut plants after drought stress and is a transcription factor that has a positive effect on growth during drought recovery by stimulating chlorophyll biosynthesis and photosynthesis [19].

Germplasms with high genetic diversity are fundamental resources for genetic and genomic research and breeding programs [20]. It has been reported that cultivated peanuts have a narrow genetic diversity, and most of the genetic resources in peanut germplasm have not been fully accessed and utilized in peanut breeding programs. Recently, effective methods for the evaluation and introduction of genetic diversity in germplasm resources have been applied in divers, and phenotypic information from the collection could thus be used as a starting point for the efficient use of the entire germplasm collection [21,22]. Establishment of a core or micro-core germplasm in a diverse crop species aims to facilitate e crop species. A core collection is a subset of the total germplasm resources in which most of the available genetic diversity in a species is preserved [23,24], and the genetic, genomic the efficient use of genetic resources and to identify germplasms with desirable characteristics.

Peanut core collections were developed by the US germplasm resource database [25]. The peanut mini-core collection was established by using the stratification strategy of the peanut germplasm resource center of the United States [26] to promote and improve the utilization of peanut germplasm resources in peanut breeding projects. Information about the core collections can be found on the Germplasm Resources Information Network (GRIN) (https://www.ars-grin.gov) (21 November 2021). Most of the mini-core collection comprises unrelated individuals that could be effectively used as starting materials for peanut association studies.

Cultivated peanuts are allotetraploid (2n = 4x = 40, AABB), derived from a relatively recent hybridization of *A. duranensis* (2n = 2x = 20, AA) and *A. ipaensis* (2n = 2x = 20, BB) [27–30]. Peanut subgenomes are very closely related [31,32], with an estimated repetition rate of 64% [1]. As a result of polyploidization, the genetic diversity of cultivated peanuts is extremely low [33], which makes the assembly of peanut genome sequences particularly difficult [1,30,34]. The genome sequences of diploid ancestors (*A. duranensis* and *A. ipaensis*) were reported in 2016 [30], and the cultivated peanut genome was then

sequenced in 2019 [35]. The genome sequences of cultivated peanut plants have provided new insights for biology, evolution, and genetic/genome studies on cultivated peanuts [36].

High-density SNP arrays have been used for high-resolution genetic mapping of target traits, genome selection, and genome-wide association studies (GWAS) [37–40]. In the case of peanuts, it was necessary to develop SNP chips with high-throughput genotyping because of the large genome size and low genetic diversity [38]. A total of 58,233 unique SNPs were selected to construct the Axiom_Arachis array [41]; then, the Axiom Arachis2 array [42] with 48K SNPs [42], including 1674 haplotype-based SNPs, was developed for *Arachis hypogaea*. The use of high-density SNP arrays can accelerate the progress of genetic/genomic studies and breeding programs in peanuts.

A genome-wide association study (GWAS) is an observational study of the genome-wide genetic variation in different individuals that investigates whether any variation is related to the target trait [40]. Currently, most GWASs have been performed using SNP arrays with a wide range of allele frequencies. A total of 158 peanut accessions that do not flower in the main stem or that flower in both the branches and the main stem were examined for 11 agronomic traits using a GWAS employing the specific-locus amplified fragment sequencing (SLAF-seq) method. Fifty-one association SNP peaks with 1429 candidate genes were found for marker-assisted selection (MAS) that could be used in future breeding programs [43]. To identify genomic regions that may be involved in seven yield-related traits, 195 peanut accessions were collected from 20 provinces in China for GWAS analysis using the genotyping-by-sequencing method with 13,435 SNPs. The GWAS analysis identified 93 SNPs that were associated with four yield-related traits [44]. Furthermore, the seed composition traits of 120 peanut mini-core collection accessions from the US peanut core collection with 13,382 single-nucleotide polymorphisms (SNPs) were investigated in a two-year genome-wide association study, where a total of 178 quantitative trait loci (QTLs) associated with seed composition traits were identified [45].

This study aimed to (1) evaluate the population structure and genetic diversity of 453 peanut germplasms using the Axiom_Arachis array with 58K SNPs, (2) conduct a GWAS of the chlorophyll content in the germplasms, and (3) identify candidate genes associated with leaf chlorophyll content. This study would provide useful molecular information for the improvement of peanut varieties with high photosynthetic efficiency.

2. Materials and Methods

2.1. Plant Materials

A total of 453 peanut germplasms were used in the current study (Supplementary Table S1). Among the germplasms, 353 peanut accessions that were widely distributed in Asia, Africa, North America (NA), South America (SA), Europe (EU), and the Australian continent (AU) were obtained from the core collections of the US Department of Agriculture (USDA) (Washington, District of Columbia, USA). In addition, 100 peanut accessions including cultivars, landraces, and breeding lines from the Korean National Agrobiodiversity Center, Rural Development Administration (RDA) Genebank Information Center (Wanju-gun, South Korea) were tested in this study. The peanut accessions were planted in the experimental field of Pusan National University, Miryang, South Korea, on 8 May 2019 (2019 season) and 28 April 2020 (2020 season). Each accession was planted in a two-row plot of 1 m length with 0.2 m row spacing. Each plot was spaced 0.4 m apart in the planting route with 0.9 m inter-row spacing to decrease cross-contamination. The seeds were planted at a density of two seeds/hole at a depth of 2 cm [46]. Conventional tillage, herbicide, insecticide, and fungicide practices were applied during both seasons.

2.2. DNA Extraction and Genotyping

A total of 453 peanut genomic DNA samples were extracted for each accession using the cetyltrimethylammonium bromide (CTAB) protocol. The quality and quantity of the extracted DNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and 1% agarose gel electrophoresis. The

high-density SNP array Axiom_Arachis (Thermo Fisher Scientific, Waltham, MA, USA) with 58K SNPs was used to obtain the genotyping data [37].

2.3. Measurement of Chlorophyll

The leaf chlorophyll content was measured on the third leaf from the top of the peanut plant using a SPAD-502Plus chlorophyll meter (Konica Minolta, Inc. Kanagawa, Japan) [47,48] at the early maturing stage on a clear day between 9:00 and 12:00 (Supplementary Table S2). The measurements were performed on three plants of each accession, and the average of the three values was calculated as the chlorophyll content of each accession. A one-way analysis of variance (ANOVA) was used to test the phenotypic variance among years.

2.4. Population Structure Analysis

Based on the SNPs derived from the high-density SNP array, the population structure was analyzed using ADMIXTURE software. Ten potential numbers of populations (K) were tested using a cross-validation procedure, and the lowest cross-validation error was chosen as the best K value (http://dalexander.github.io/admixture/admixture-manual.pdf) (21 November 2021) [49].

2.5. Genome-Wide Association Study (GWAS)

The GAPIT package of R software was used to conduct the GWAS, and the enriched compressed mixed linear model (ECMLM) was selected to analyze the association between the SNPs and the data for the phenotype of interest [50]. For single-locus analysis, the ECMLM involves algorithms to define the kinship between groups and increases statistical power; it calculates the kinship using several different algorithms and then chooses the best combination between the kinship algorithms and the grouping algorithms [51].

The suggested *p*-value threshold of 4.85×10^{-5} based on the Bonferroni correction [(1/20,623 (total SNPs)] [52] was used in the GWAS. The significant SNPs were also found using FDR-unadjusted *p*-value < 0.0001 if there were no significant SNPs found using the suggested *p*-value threshold. The genotype–phenotype association of candidate SNPs was evaluated by OriginPro 2018 C (9.5) software (Version 9.5, OriginLab Corporation, Northampton, MA, USA) (https://www.originlab.com/) using a total of 392 peanut accessions (21 November 2021).

2.6. Linkage Disequilibrium (LD) Analysis and Candidate Gene Identification

We performed linkage disequilibrium analysis for all possible SNP pairs with a minor allele frequency (MAF) greater than 0.01 and viewed the LD block with HaploView 4.2, which uses a permutation test to determine the *p*-values for each pairwise correlation. The measure for LD is r^2, the square of the correlation coefficient between two indicator variables [53].

Candidate genes within a 200 kb region upstream or downstream of the peak SNPs according to the previous linkage disequilibrium (LD) decay results were selected using the PeanutBase website tool (https://www.peanutbase.org) (21 November 2021) [37].

2.7. RNA Extraction, cDNA Synthesis, and Expression Analysis

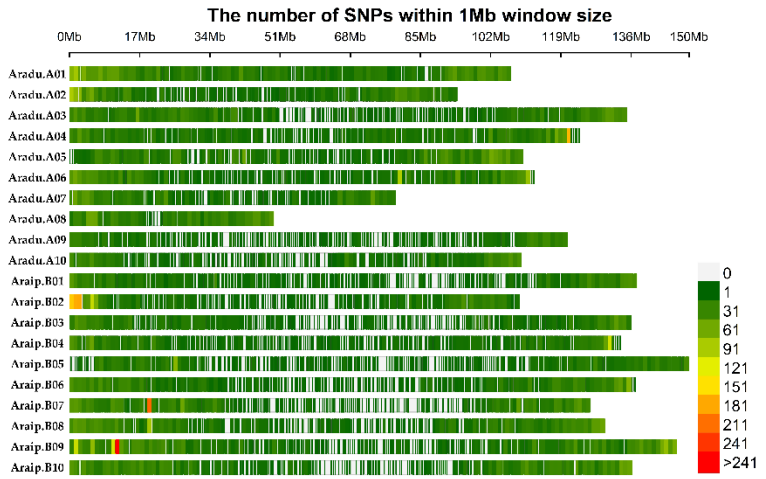
Gene expression was assessed in GWP174 (H), with high leaf-chlorophyll content, and GWP 411 (L), presenting low leaf-chlorophyll content. These two accessions were selected based on the phenotypic data collected over two years. At 20 and 60 days after planting, a young leaf—specifically the second leaf on the third branch of the main stem from each individual accession—was collected to extract RNA. Total RNA was extracted from fresh peanut leaves using a QIAGEN RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) and treated with QIAGEN RNase-Free DNase (QIAGEN, Hilden, Germany) to remove genomic DNA. The primer pairs for target candidate genes were designed using the NCBI Primer BLAST design tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) (21 November 2021). The SuperScript™ III First-

Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) was used to synthesize complementary DNA (cDNA), and PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania) with the Applied Biosystems QuantStudio 1 Real-Time PCR System (Applied Biosystems, Singapore) was used for qRT-PCR analysis; biological reactions were performed in triplicate. Transcript levels of genes were normalized to actin [54] (forward primer: 5′-TACCAGATGGACAGGTTATCACAAT -3′; reverse primer: 5′-TGGAACCACCACTCAAGACAAT -3′) for peanuts. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression, and actin expression was used as the baseline control for normalization. Amplifications were carried out in 20 µL reaction solutions containing 10 µL PowerUp SYBR Green Master Mix, 1 µL cDNA, 10 pmol/µL of each specific primer, and 7 µL of water. PCR conditions were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 1 min. A melting curve analysis was performed for each pair of primers at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression [55], and actin expression was used as the baseline control for normalization. The *t*-test in the Statistical Package for the Social Sciences (SPSS) 15.0 was used to compare the expressions of the target genes between H (GWP174) and L (GWP 411).

3. Results

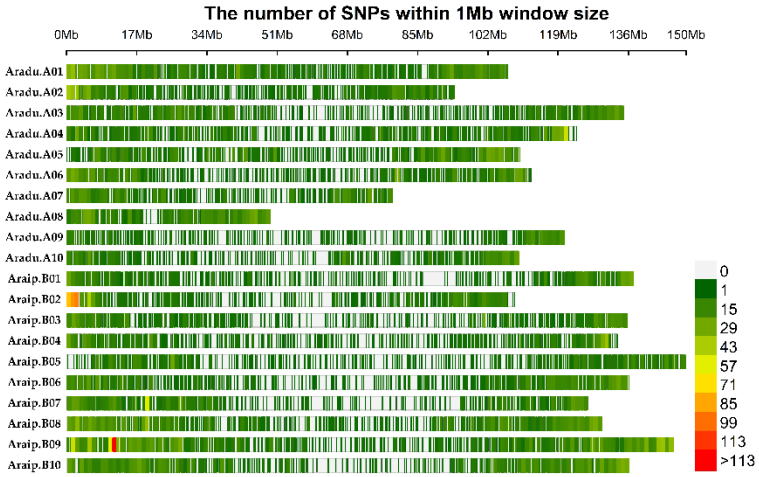
3.1. SNP Genotyping

Out of the 58K SNPs, a total of 47,837 SNPs presented polymorphisms (Supplementary Table S3 and Figure 1a). After eliminating SNPs with high levels of missing data (>20%), heterozygosity (>20%), or low minor allele frequency (MAF) (<0.01), a total of 20,623 SNPs were selected for future association analysis [37]. Of the 20,623 SNPs, 9370 and 11,253 SNPs were derived from subgenomes A and B, respectively. The majority of the SNPs were evenly distributed across the chromosomes; however, there were some relatively large gaps between SNPs on the chromosomes Aradu.A03, Aradu.A09, Aradu.A10, Araip.B01, Araip.B02, Araip.B03, Araip.B05, Araip.B06, Araip.B07, and Araip.B10 (Figure 1b). The overall SNP density across the chromosomes was 8.68 SNPs/Mb, with Aradu.A09 (5.26 SNPs/Mb) and Aradu.A08 (14.49 SNPs/Mb) showing the lowest and highest densities, respectively (Supplementary Table S4).



(**a**)

Figure 1. *Cont.*



(**b**)

Figure 1. Single nucleotide polymorphisms (SNP) distribution in the 20 chromosomes of the cultivated peanut. The horizontal axis shows the chromosome length (Mb); the shades of red represent SNP density. The vertical axis shows the 20 chromosomes. (**a**) Polymorphic SNPs (except for scaffold markers); (**b**) Polymorphic SNPs (except for scaffold markers) after filtering by GAPIT coding.

3.2. Phenotypic Data Analysis

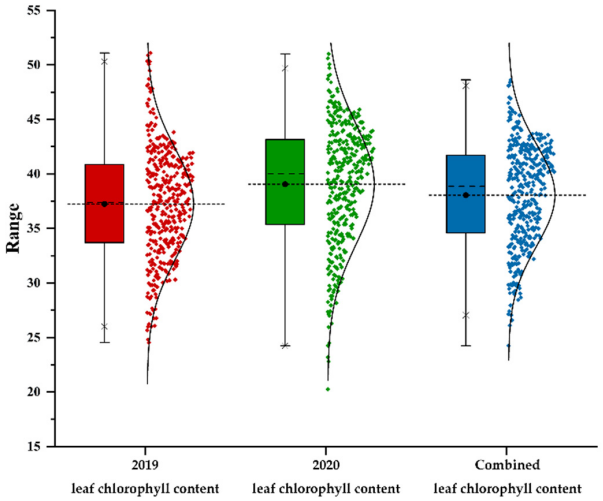
The minimum and maximum values of leaf chlorophyll content in the 2019 season were 2453 and 5110, respectively, and those in the 2020 season were 2025 and 5100, respectively. The minimum and maximum values for the combined leaf chlorophyll content across both seasons were 2425 and 4863, respectively (Figure 2a). The normal distribution test showed that the scatter points of the quantile–quantile (QQ plot) graph (Figure 2b) were clustered around the fixed line; therefore, we assumed that the data were normally distributed ($p = 0.05$). The one-way ANOVA test showed highly significant differences (p-value of 6.36×10^{-6}) between the 2019, 2020, and the combined phenotypic data, representing that environmental effects may exist.

3.3. Genetic Structure

The population structure of the peanut accessions was investigated using ADMIXTURE 3.1 software. Out of the 10 potential numbers (K: 1–10) of populations tested, K = 9 was the most sensible according to the cross-validation error, as it had the lowest value (Supplementary Figure S1a). The results for genetic structure show that the accessions from South Korea are genetically different from those found in peanuts from South America, where the cultivated peanut originates, although there are some genome crossover phenomena among the peanut accessions of different origins (Supplementary Figure S1b).

3.4. Genome-Wide Association Study

The data from the 20,623 filtered polymorphic SNPs and the leaf chlorophyll content were analyzed in a GWAS using GAPIT Version 3. Across the seasons, five SNPs showing significant associations (p-value threshold of 4.85×10^{-5}) with chlorophyll content were identified on four chromosomes (Aradu.A02, Aradu.A08, Araip.B02, and Araip.B05) (Table 1). The observed and expected distributions for each SNP in the QQ plot showed that the population structure and kinship relationship had good correspondence in the GWAS (Figure 3a). In the 2019 season, three significant SNPs, namely AX-176822908, AX-176820297, and AX-147230060, were identified. Meanwhile, three significant SNPs, namely AX-176823290, AX-176820297, and AX-147212224, were detected across the combined season. The SNP AX-176820297 on the chromosome Araip.B05 was detected in the 2019 season and the combined season.



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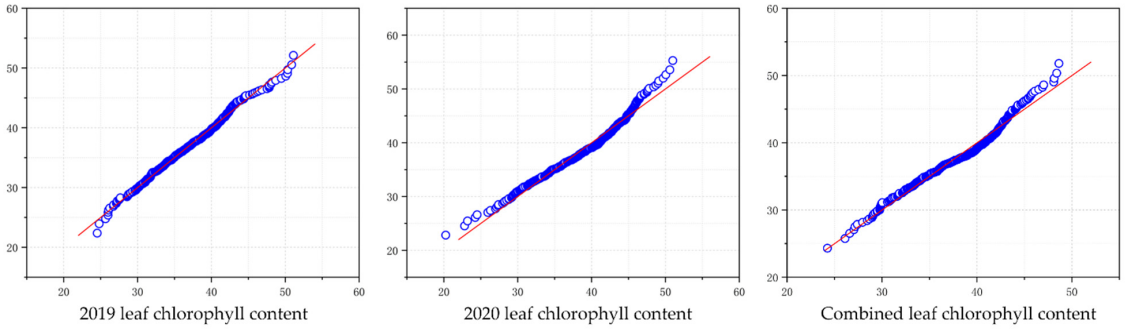


(**b**)

Figure 2. (**a**) Peanut leaf chlorophyll content histogram using the normal distribution test. The minimum and maximum values of leaf chlorophyll content in the 2019 season were 2453 and 5110, respectively, and those in the 2020 season were 2025 and 5100, respectively. The minimum and maximum values for the combined leaf chlorophyll content across both seasons were 2425 and 4863, respectively; (**b**) The normal distribution test showed that the scatter points of the quantile–quantile (QQ plot) graph were clustered around the fixed line for the 2019, 2020, and the combined leaf chlorophyll content (p = 0.05).

Table 1. Significant markers associated with the leaf chlorophyll content of peanuts identified using GAPIT analysis.

Group	SNP	Chromosome	Position (bp)	p-Value (p) [1]
2019	AX-176822908	Araip.B05	114145435	2.12×10^{-5}
	AX-176820297	Araip.B05	118278277	2.78×10^{-5}
	AX-147230060	Aradu.A08	15123765	3.65×10^{-5}
2020	AX-177644092	Araip.B08	80072452	6.49×10^{-5}
	AX-176794744	Araip.B06	103047540	6.54×10^{-5}
Combined	AX-176823290	Araip.B02	45735578	1.47×10^{-5}
	AX-176820297	Araip.B05	118278277	2.26×10^{-5}
	AX-147212224	Aradu.A02	533285	2.52×10^{-5}

[1] The significant SNPs were found in the 2019 data and the combined data for 2019 and 2020, with the p value threshold of 4.85×10^{-5} (suggestive threshold). The significant SNPs were found in the 2020 data with p value thresholds of FDR-unadjusted p-value < 0.0001.

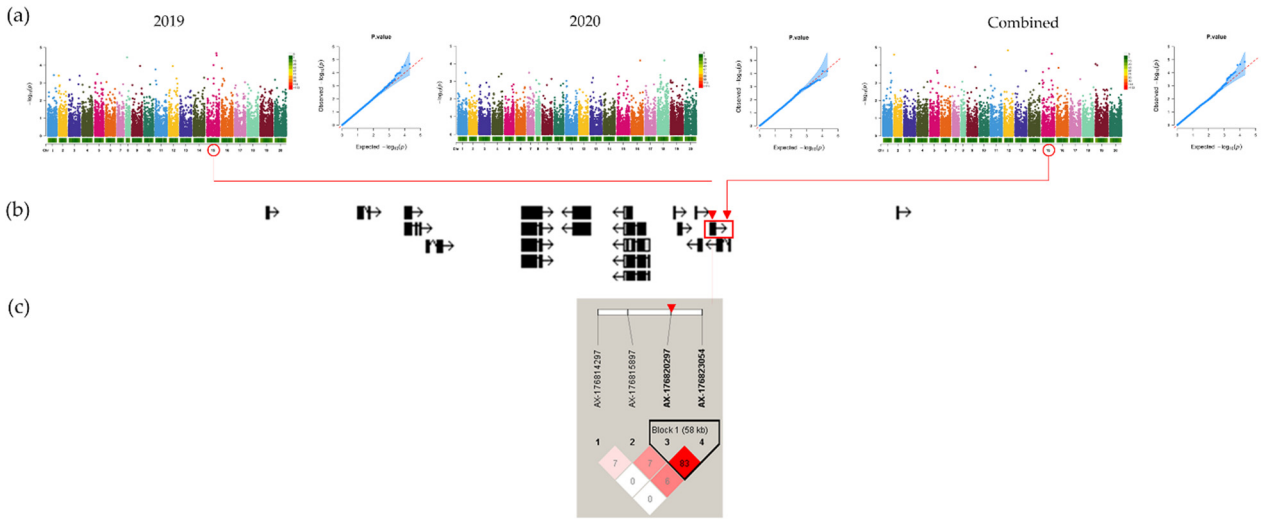
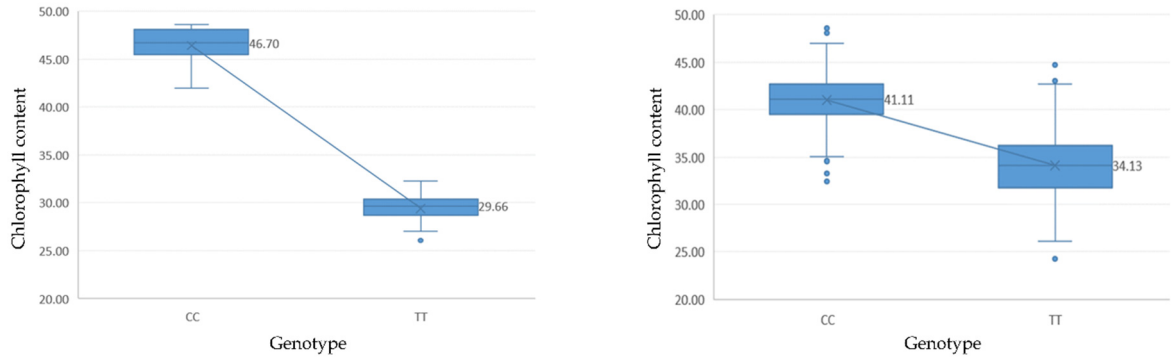


Figure 3. (**a**) A Manhattan plot of a genome-wide association analysis using GAPIT and a Q-Q (quantile–quantile) plot; (**b**) Genes located at the association regions based on the *Arachis hypogaea* Tifrunner 1.0 reference genome; the Arahy.SDG4EV gene is marked in a red square pattern showing that it was found in both the 2019 and the combined season GWAS results; (**c**) A linkage disequilibrium (LD) plot generated using HaploView software showing r^2 values for selected AX-176820297 SNP on chromosome Araip.B05 within diamonds. Significant combined *p* values are highlighted in red.

To evaluate the genotype–phenotype association of the SNP AX-176820297, we selected the 10 peanut accessions with the highest chlorophyll contents and the 10 with the lowest chlorophyll contents based on the chlorophyll phenotype data across the seasons. The results indicated that the accessions with the highest chlorophyll contents showed a CC genotype, whereas the accessions with the lowest chlorophyll contents had a TT genotype, presenting a statistically significant difference with a *p*-value of 5.07×10^{-17} (Figure 4a and Supplementary Table S5). Then, a total of 392 peanut accessions were used to determine the correlation between genotypes (CC or TT) and phenotypes, which was found to be statistically significant ($p < 0.001$) (Figure 4b and Supplementary Table S6).



(**a**) (**b**)

Figure 4. (**a**) The genotype–phenotype association of SNP AX-176820297 showing 10 peanut accessions with the highest chlorophyll content and 10 with the lowest chlorophyll content; (**b**) The genotype–phenotype association of SNP AX-176820297 with 392 peanut accessions. The average value of each set of data is marked in patterns.

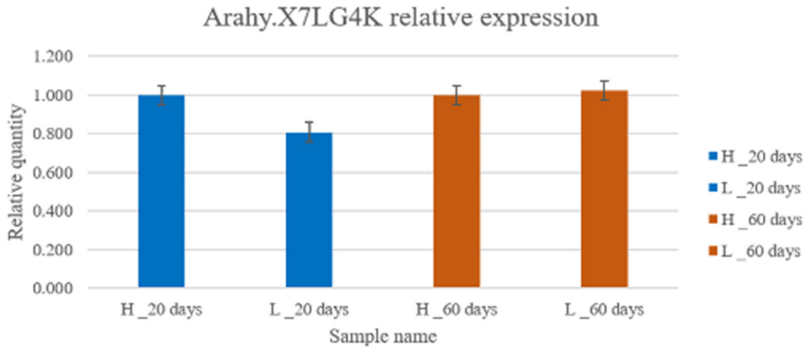
3.5. LD and Candidate Genes Analysis

The pattern of LD across the genome presented a number of haplotype blocks containing SNPs that can be used to determine the range of the candidate gene. The genomic locations harboring significant SNPs as indicated by the GWAS were investigated to identify putative candidate genes based on the peanut reference genome (*A. hypogaea* Tifrunner 1.0). Strong and extensive pairwise LDs were observed among highly significant SNPs around AX-176820297 on the chromosome Araip.B05, in which r^2 varied from 0 to 0.838 (Figure 3c and Supplementary Table S7).

Seventy-six annotated genes at the associated regions flanked by five SNPs were identified within the estimated ±200 kb window based on the reference genome (Figure 3b and Supplementary Table S8).

3.6. Gene Expression Analysis by qRT-PCR

After a series of comparisons of candidate genes with each gene annotation, referring to the published orthologous genes in the *Arabidopsis* genome, we selected two candidate genes, Arahy.X7LG4K and Arahy.SDG4EV, potentially associated with leaf chlorophyll content from 76 candidate genes (Supplementary Table S8) within a 200 kb region upstream or downstream of the peak SNPs. No statistical difference was detected in the expression levels of Arahy.X7LG4K between H (GWP174) and L (GWP 411) (Figure 5a). However, the expression level of Arahy.SDG4EV was statistically significant ($p < 0.05$) at two different time points (20 and 60 days) (Figure 5b). H (GWP174) presented a more than three-fold lower expression than L (GWP 411) (Supplementary Table S9).



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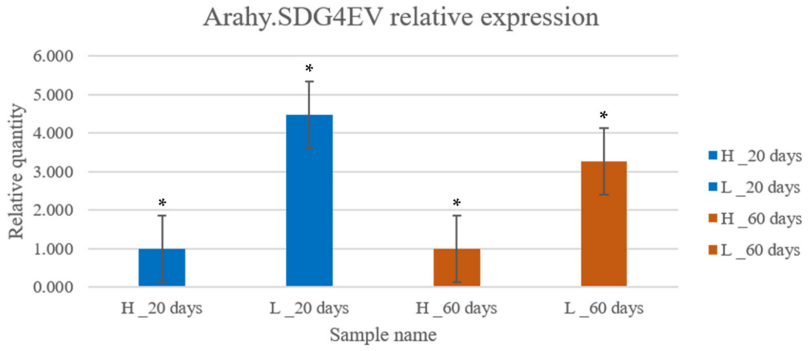

(**b**)

Figure 5. Gene Expression Analysis by qRT-PCR. Peanut accession H (GWP174) exhibiting high leaf-chlorophyll content and peanut accession L (GWP 411) showing low leaf-chlorophyll content at two different time points, 20 and 60 days. (**a**) Gene relative expression levels of Arahy.X7LG4K in H (GWP174) and L (GWP 411); (**b**) Gene relative expression levels of Arahy.SDG4EV in H (GWP174) and L (GWP 411). * Represents a significant difference in the two expression levels ($p < 0.05$).

4. Discussion

Genotypic data from the Axiom_Arachis array chip with 58K SNPs have played a pivotal role in understanding the evolutionary history and domestication of peanuts [56]. Applications of the array chip have also demonstrated that it is a powerful and reliable tool for the selection of peanut germplasm backgrounds [57]. Our efforts in finding genes associated with chlorophyll content were greatly assisted by the availability of the high-density SNP array chip and the publicly available peanut reference genome. A total of 20,623 SNPs derived from the 58K SNP array were used to perform the GWAS in the current research. The genome size of the cultivated peanut is estimated to be approximately 2.54 Gb [58], and the estimated LD decay distance of the 453 peanut accessions in our study was approximately 150–200 kb according to the applied options. Therefore, when calculated arithmetically, approximately 12,700–17,000 markers would be required for a GWAS analysis in peanuts, which is lower than the number of markers used in our GWAS analysis. Although it might not be perfect, there would likely be no major problem with performing a GWAS analysis with the number of SNPs used in the current study. In addition to high-density SNP markers, the use of two-year field phenotype data could also have played an important role in identifying candidate genes of interest and evaluating the effects of the candidate genes on the investigated phenotypes. False positives that are caused by population structure and family relatedness are a major issue in GWAS analysis, thus MLM-based methods have been developed to control false positives [59,60]. For the same reason, the ECMLM was used to identify the association between SNPs and phenotype data of interest in this study.

Of the 453 peanut germplasms used in the current study, the accessions from South Korea were genetically very different from those obtained from South America, where cultivated peanuts originated. The Korean accessions were also genetically independent from a large number of peanut accessions from the core collection. However, the Korean peanut accessions presented great genetic similarity with the exception of only two peanut accessions, which means that the Korean peanut accessions have very narrow genetic diversity. Therefore, to expand on genetic diversity, it would be necessary to introduce useful germplasms and traits from the core collection to develop new peanut cultivars in Korea.

Several studies have been performed to find genomic regions associated with chlorophyll content under different environmental conditions. For example, GWASs of PSII chlorophyll fluorescence in 283 rice accessions were conducted in multiple environments. One QTL on chromosome 9 was identified for photosynthetic parameter analysis, where energy absorption performance indicator (PIabs) phenotypes showed unique associations in the CH environment, and additional QTLs were identified in the ER environment [61]. In another study, QTL mapping was performed using 120 recombinant inbred lines (RILs) from two Chinese wheat cultivars (Longjian 19 × Q9086) in Lanzhou, China, from 2012 to 2014 under both drought stress and well-watered conditions. A total of 22 additive QTLs (A-QTLs) and 25 pairs of epistatic QTLs (AA-QTLs) were detected at different growth stages, and QTL expressions for the greenness of the flag leaf (a surrogate measure of leaf chlorophyll content) were assessed. Overall, 34 of 50 A-QTLs were identified at only one specific stage, but the remaining 16 loci were detectable at other stages [62]. Furthermore, several studies have shown that the traits that determine chlorophyll content are regulated by environmental factors such as the planting season or year, the cultivation site, and environmental stress in legume plants. Wang et al. [63] reported a total of 16 QTL hotspots for traits determining chlorophyll content and found that leaf chlorophyll content traits could be largely affected by environmental factors. Additionally, evaluations of 135 pea accessions, tested at multiple locations and in multiple years, yielded results revealing that the detection of significant SNPs/QTLs varied according to the environment [64]. The chlorophyll content in the peanut plants from the two seasons tested in the current study were somewhat different, and different significant SNPs were identified between the years despite the location being the same. Two significant SNPs, namely AX-177644092

and AX-176794744, were identified using an FDR-unadjusted p-value of 0.0001 [65] in the 2020 season, whereas we did not find any significant SNPs using the Bonferroni-corrected p-value threshold in the 2020 season (Table 1). Additionally, we identified the SNP AX-176820297 with a *p*-value of 0.0009 in 2020, which was already significant in 2019 and in the combination of seasons. As plants adjust their own physiological characteristics to adapt to different environments, climate and soil conditions may play an important role in regulating chlorophyll traits on a large scale [66]. It has been reported that chlorophyll synthesis requires many elements in the soil, such as nitrogen and phosphorus [67]. In addition, the synthesis of chlorophyll could also be affected by abnormal temperature, since a series of enzymatic reactions is involved in this process [68]. Therefore, extremely high or low temperatures can inhibit the enzyme reactions. Nevertheless, we found common SNPs between the 2019 and the combined seasons and also detected an SNP (AX-176820297) that was highly significant for each season alone and when combined.

Chlorophyll is a type of photosynthetic pigment, and it is an essential element in plant photosynthesis that converts solar energy into chemical energy, which results in carbohydrate production [69]. As its level is positively associated with photosynthetic capacity, an increase in chlorophyll content in crop species might be an effective way to elevate grain yield and biomass production [70]. Among the two genes, only Arahy.SDG4EV showed a significant difference in the gene expression analysis, revealing a negative correlation with chlorophyll content. In a previous study in *Arabidopsis thaliana*, the PHYTOCHROME-INTERACTING FACTOR 1 (PIF1) gene, which is likely associated with the Arahy.SDG4EV gene, has been shown to directly or indirectly negatively regulate the expression of a series of genes involved in controlling the chlorophyll biosynthetic pathway [71]. Moreover, the PIF1 gene interacts with the photoactivated conformation of phytochromes A and B to control the rate of chlorophyll biosynthesis and prevent photooxidative damage caused by excess free protochlorophyll exposed to light [72].

The measurements of the relative chlorophyll content were consistent with the observed gene expression level, and the gene of interest exhibited the same negative regulatory function as the known PIF gene; therefore, we can infer that the Arahy.SDG4EV gene may play a negative role in peanut chlorophyll biosynthesis. Furthermore, the Arahy.SDG4EV gene was located at 128,144–128,146 kb on the peanut chromosome Arahy.15, and the candidate SNP AX-176820297 was located at 128,030 kb on the same chromosome. The distance between the Arahy.SDG4EV gene and the candidate SNP is very close, indicating that they may be closely linked or that the SNP is within this gene. The SNP genotypes of the peanut accessions with the highest and lowest chlorophyll contents showed that the SNP AX-176820297 was significantly related to leaf chlorophyll content and could be used as a selection marker for chlorophyll content in peanuts. We are currently conducting ongoing studies to clone the candidate gene. Transformation of the gene from H to L, as well as genome editing of the gene in L according to the SNP information, might be needed to investigate the functions of the candidate gene and the interaction of the gene with other chromosome regions.

5. Conclusions

Since the peanut is one of the most important commercial crops, improving the quality and yield potential of the peanut plant is an important challenge and goal in breeding programs. Our study demonstrated the feasibility of GWAS analysis using the core germplasm and a high-density array chip to study leaf chlorophyll content in the peanut (Supplementary Figure S2). Five SNP markers that are significantly correlated with chlorophyll content in the peanut were identified, establishing a foundation for further research. The Arahy.SDG4EV gene is a promising candidate gene involved in chlorophyll biosynthesis and the photosynthesis processes. The results of the current study provide valuable and fundamental information on MAS for the development of cultivars with a high chlorophyll content as well as for the functional study and cloning of the candidate gene in the peanut.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12010152/s1; Figure S1a: Cross-validation error with K value; Figure S1b: ADMIXTURE subpopulations structure analysis of the genotype data from 453 peanuts; Figure S2: A study design flow chart; Table S1: Information on the 453 peanut accessions; Table S2: The leaf chlorophyll content of the 453 peanut accessions; Table S3: A high-density SNP array using Axiom_Arachis with 58 K SNPs; Table S4: Information on the peanut genome from the 58K SNP array chip; Table S5: The evaluation of the genotype frequencies of SNP AX-176820297 with 20 peanut accessions; Table S6: The evaluation of the genotype frequencies of SNP AX-176820297 with 392 peanut accessions; Table S7: Pairwise LD measurements on chromosome Araip.B05; Table S8: A list of genes in the significant region, with annotations, identified by the *Arachis hypogaea* Tifrunner 1.0 reference; Table S9: The expression level and correlation of the Arahy. SDG4EV and Arahy.X7LG4K genes.

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