High-Density Genetic Linkage Map Construction Using Whole-Genome Resequencing for Mapping Qtls of Resistance to Aspergillus Flavus Infection in Peanut

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Abstract

Cultivated peanut (*Arachis hypogaea* L.) is rich in edible oil and protein, which is widely planted around the world as an oil and cash crop. However, aflatoxin contamination seriously affects the quality safety of peanut, hindering the development of peanut industry and threatening consumers’ health. Breeding peanut varieties with resistance to *Aspergillus flavus* infection is important for control the aflatoxin contamination, and understanding of the genetic basis of resistance is vital to its genetic enhancement. In this study, we report the QTL mapping of resistance to *A. flavus* infection of a well-known resistant variety J11. A recombination inbred line (RIL) population was constructed by crossing a susceptible variety Zhonghua 16 and J11. Through whole-genome resequencing, a genetic linkage map was constructed with 2,802 recombination bins and an average inter-bin distance of 0.58 cM. Combined with phenotypic data of infection index in four consecutive years, six novel resistant QTLs were identified and they explained 5.03-10.87% phenotypic variances. The favorable alleles of five QTLs were from J11 while that of one QTL were from Zhonghua 16. The pyramiding of these favorable alleles significantly improved the resistance to *A. flavus* infection. These results could contribute greatly to understanding of genetic basis of *A. flavus* resistance and could be meaningful in further resistance improvement in peanut.

Key Message

A high-density genetic map was constructed and novel QTLs of resistance to *A. flavus* infection were identified in a RIL population of peanut across four environments.

Introduction

The cultivated peanut, also known as groundnut, is one of the most important oil crops and widely cultivated in most tropical and sub-tropical regions. China, India, USA and Nigeria account for more than 90% in cultivated area of this crop. In China, it is grown on 4.50 million ha with a total production of 17.52 million tons (FAOSTAT 2019).

Aflatoxin contamination is a huge threat to peanut industry. It is caused by *A. flavus* which also infects other important crops such as corn, rice, and wheat (Mahato et al. 2019). The contaminated agricultural products would endanger the health of human beings and animals (Desjardins 2003; Kew 2013). Breeding of varieties with resistance to *A. flavus* infection has been one of the main objectives in the peanut breeding programs, and was recognized as the most cost-effective measure to solve the problem. However, only a few germplasms were found to possess the resistance, and the genetic basis of their resistance remain unclear. Breeding of novel varieties faces tough challenge in peanut. Thus, understanding the genetic pattern of resistance to *A. flavus* infection is vital to the enhancement of the resistance in peanut.
The Indian commercial peanut variety J11 is a well-known resistant germplasm to *A. flavus* infection, while the underlying genetic basis of its resistance remain largely unclear. It was firstly reported by Mehan et al. (1980) and was confirmed in different environments by Mehan et al. (1981) and Nigam et al. (2009). J11 was also reported to be resistant to *A. parasiticus* infection (Kisyombe et al. 1985). The infection resistance of J11 was reported to be related to drought stress and pod maturity (Mehan et al. 1986) as well as seed coat integrity (Asis et al. 2005). Nayak et al. (2017) deployed RNA-seq approach to understand the host-pathogen interaction and they found 4,445 differentially expressed genes (DEGs) at four critical stages after inoculation in J11 and JL 24 (a susceptible genotype). Zhao et al. (2019) conducted both transcriptomic and proteomic analysis to reveal changes that occurred during the infection of J11 by *A. flavus*, and 663 DEGs and 314 proteins were identified. However, the key genetic loci or genes responsible for the infection resistance of J11 were still unclear.

In recent years, QTL mapping was successfully used to identify QTLs with resistance to *A. flavus* infection from two newly identified resistant germplasm named ICG12625 (Yu et al. 2019) and Xinhuixiaoli (Khan et al. 2020). ICG12625 is a landrace from Ecuador, and Yu et al. (2019) constructed a recombination inbred line (RIL) population from the cross between ICG12625 and a susceptible variety Zhonghua 10. A genetic map with 1,219 SSR loci were constructed and two QTLs were identified on chromosomes A03 and A10 with 7.96% and 12.16% phenotypic variation explained (PVE), respectively. On the other hand, Xinhuixiaoli is a Chinese landrace with resistance to *A. flavus* infection and it was crossed with a susceptible variety Yueyou 92 to generated a RIL population (Khan et al. 2020). A SNP-based genetic map was constructed by specific length-amplified fragment sequencing (SLAF-seq), and a major QTL on A03 with 18.11% PVE and a minor QTL with 4.40% PVE on B04 were identified.

In order to reveal the underlying genetic basis of the durable resistance to *A. flavus* infection of the well-known variety J11 at QTL level, a susceptible variety Zhonhua 16 was crossed with J11 to develop a RIL population, upon which a high-quality genetic map was constructed by whole-genome resequencing and QTLs associated with resistance to *A. flavus* infection were identified in four environments in the present study.

**Materials And Methods**

**Plant materials**

Zhonghua 16, a variety susceptible to *A. flavus* infection was used as the female parent and crossed with the resistant variety J11 to develop a mapping population containing 200 recombinant inbred lines (RILs) using single seed decent (SSD) method. Four generations from F$_7$ to F$_{10}$ of the RIL population were used for phenotypic identification during 2017-2020 year in Wuhan, China. These trials were designated as WH2017, WH2018, WH2019 and WH2020 respectively. Field trials adopted a randomized complete block design with three replications. Each plot contained 10-12 plants in one row, with 10 cm between plants and 30 cm between rows. Field management followed standard agricultural practices.
Trait measurement and data analysis

The resistance of peanut seeds to *A. flavus* infection was identified according to the previous method with a slight modification (Yu et al. 2019). The resistance level to *A. flavus* infection of each seed was divided into four different grades: (1) grade 0: seed surface without spore coverage; (2) grade 1: seed surface has less than 1/4 spore coverage; (3) grade 2: seed surface covered 1/4-1/2 spore; (4) grade 3: seed surface exceeds 1/2 spore coverage. The percent seed infection index (PSII) was calculated according to the formula:

$$ PSII = \frac{n1+n2+2n3}{N} \times 100\% , $$

where \( n1, n2, n3 \) represent the number of seeds of grade 1, grade 2, grade 3, respectively, and \( N \) represent the total number. The SPSS 25.0 software was used for the statistical analysis of PSII. The broad sense heritability \( (H^2) \) for PSII was calculated using the equation

$$ H^2 = \frac{\sigma^2_e}{\sigma^2_e + \sigma^2_{xe}/n + \sigma^2_m} . $$

\( \sigma^2_e \) is genetic variance component, \( \sigma^2_{xe} \) is genotype-environment interaction variance component, \( \sigma^2_m \) is the residual (error) variance component, \( n \) and \( r \) were defined as the number of environments and replications.

Library construction and sequencing

Genomic DNA was isolated from young leaf tissues using a modified CTAB method. Paired-end libraries were constructed by a whole-genome resequencing strategy and the libraries were sequenced using Illumina HiSeq 2500 platform (Illumina, Inc; San Diego, CA, USA). High-quality reads were aligned to the tetraploid cultivated peanut reference genome version 1 (www.peanutbase.org) and the HaplotypeCaller module of GATK software was used to detect SNPs and InDels.

Linkage map construction

Raw SNPs and InDels were filtered with the following criteria: (a) minor allele frequency (MAF) \( \geq 0.2 \); (b) relative heterozygosity rate \( \leq 0.2 \) (calculated according to Wu et al. 2016); (c) the proportion of missing genotypes \( \leq 0.5 \); (d) polymorphism exists between parents. Then, the high-quality SNPs and InDels were used to construct a high-quality bin map using the MPR package (Xie et al. 2010). Linkage groups were named according to the location of each SNPs/InDels on the reference genome.

QTL analysis

QTL mapping of each phenotypic data was performed using the default setting of the BIP (QTL mapping in bi-parental populations) approach in IciMapping 4.2 software (Meng et al. 2015). The scanning step was set as 1 cM, and LOD threshold was set as 2.5 to detect additive QTLs. QTLs were named with initial letter “q” followed by the trait name “PSII” and linkage group. An English lowercase letter was added if
two or more QTLs were identified in the same linkage group. For instance, two QTLs for PSII detected on LG A08, and then they were named as \textit{qPSIIA08.a} and \textit{qPSIIA08.b}. If multiple QTLs overlapped on the same linkage group, they are considered to be a consistent QTL across environments and designated with the same name.

**Results**

**Phenotypic variation of resistance to \textit{Aspergillus flavus} infection**

The PSII of the RIL population were identified with seeds harvested from four consecutive years (2017-2020). PSII of the female parent Zhonghua 16 were significantly higher than the male parent J11. The PSII of Zhonghua 16 ranged from 87.04 to 93.00%, whereas J11 ranged from 51.04 to 55.97% in the four environments. PSII varied among RILs from 46.92 to 98.25%, 40.74 to 100%, 26.98 to 100%, 24.73 to 100% in the four environments (Table 1). Continuous distributions with transgressive segregation were observed, which indicated that both parents contain resistant genes against \textit{A. flavus} infection (Fig. 1). The results of ANOVA for PSII showed significant difference among genotypes, environments, and genotypes×environments interactions at $P<0.001$ (Table 2). The broad-sense heritability of PSII was estimated to be 0.76, indicating PSII was mainly controlled by genetic factors.

**Sequencing and construction of genetic map**

About 5 billion reads was generated from the 200 RILs and their parents. The female parent Zhonghua 16 was sequenced at 8.95× coverage and the male parent J11 at 8.35× coverage, while the RIL population individuals were sequenced at ~2.96× coverage approximately (Table S1). On average, 84.22% reads were uniquely aligned to the reference genome (Table S2). A total of 233,365 SNPs/InDels were used to construct a high-density genetic map. These markers were divided into 2,802 recombination bins, which generated the final genetic map covering 1573.85 cM with an average inter-bin interval of 0.58 cM (Table 3; Fig. 2). There were 1,257 bins for A sub-genome with a map length of 760.69 cM, and 1,545 bins for B sub-genome with a map length of 813.16 cM. The length of LGs varied from 57.28 cM (A06) to 96.64 cM (A09) and the number of bins in LGs ranged from 102 (A06) to 210 (B05).

**Evaluation of the genetic map**

To evaluate the quality of the high-density genetic map, the sources of bins in each RIL were analyzed and the results showed that the bins from each parent form continues fragments as expected (Fig. S1). The collinearity analysis, which compared the genetic position of all bins to their physical position on the reference genome, indicated that high collinearity between LGs and corresponding chromosomes (Fig. S2).
Detection of additive QTLs for resistance to *Aspergillus flavus* infection

Genome-wide QTL analysis was performed using the high-density genetic map and the phenotypic data of PSII from the 200 RILs in four consecutive years. A total of six additive QTLs were identified with 5.03-10.87% PVE (Table 4; Fig. 3). Their LOD value ranged from 2.63 to 5.97. Two QTLs were detected on B03, and the other four QTLs were on A05, A08, B01 and B10 respectively. QTL *qPSIIA08* was consistently detected in four years showing 6.91-10.58% PVE. QTL *qPSIIB03.a* and QTL *qPSIIB03.b* were repeatedly detected in two years with 9.16-9.23% PVE and 5.03-5.75% PVE respectively. The major QTL *qPSIIA08* was only detected in one year with 10.87% PVE. In addition, the minor QTLs *qPSIIA05* and *qPSIIB01* were only detected in one year.

Pyramiding of resistant alleles

The positive additive effects of *qPSIIA05*, *qPSIIA08*, *qPSIIB01*, *qPSIIB03.a* and *qPSIIB03.b* indicated that the alleles from J11 were resistant to infection. On the contrary, the negative additive effect of *qPSIIB10* showed that the alleles from Zhonghua 16 were responsible for improving the resistance to infection. The recombination of *qPSIIA05*, *qPSIIA08*, *qPSIIB01*, *qPSIIB03.a*, *qPSIIB03.b* and *qPSIIB10* were screened in the RIL population. As shown in Table 5 and Table S3, the genotypes of these QTLs derived from Zhonghua 16 and J11 were designated as “A” and “B”, respectively. RILs with genotype BBBBBA possessed all resistant alleles of the six QTLs from both parents, while those with genotype AAAAAAB possessed all susceptible alleles of the six QTLs. RILs with genotype BBBBBB and AAAAAA possessed resistant alleles from J11 and Zhonghua 16, respectively. Notably, RILs with genotype BBBBBB showed significantly lower PSII than RILs with other genotypes (BBBBBB, AAAAAA and AAAAAAB) in 2017-2020 year, indicating that the pyramiding of resistant alleles could enhance the resistance.

Discussion

With the change of peanut harvest methods and the aggravation of global warming, the risk of peanut contaminated by aflatoxin is also increasing. The most valid measure to control this disease is cultivating varieties with resistance to *A. flavus* attacks. However, breeding of novel resistant varieties is still a challenge in peanut and high-yield resistant varieties were still lack in peanut production, owing to the fact that genetic mechanism of resistance to *A. flavus* is still unclear. In the present study, the infection resistance of the RIL population derived from internationally recognized peanut variety J11 with resistance to *A. flavus* was analyzed to explore the quantitative genetic loci of resistance.

High-quality genetic map is the fundamental basis of QTL mapping of agronomic traits. Varshney et al. (2009) constructed the first map of the cultivated peanut, but this map only has 135 SSR markers. More genetic maps have been constructed with the development of abundant SSR markers. Recently, Luo et al. (2018) constructed three genetic maps involving 743, 830 and 817 SSR markers, respectively. But the
number of SSR markers is much lower than that of SNP markers in genome. Relying on reduced-representation genome sequencing technologies, SNP markers were unearthed for the construction of genetic map in peanut. For example, Hu et al. (2018) built a genetic map containing 2,334 SNP/SSR markers and Liu et al. (2020) built a genetic map comprising 2,595 SNPs. Recently, two reports used whole genome resequencing technology to develop genetic maps. The first one contained 2,156 recombination loci derived from 8,869 SNPs (Agarwal et al. 2018), and the second one involved 3,634 recombination bins (Liu et al. 2020). It is obvious that using the whole genome resequencing technology can obtain abundant SNP markers and denser maps than other methods. In the present study, we resequenced 200 RILs and both parents upon which 233,365 SNPs/InDels were identified and formed 2,802 recombination bins on the genetic map. Compared with the previous maps, this map was high-density and would provide convenience for the mapping of resistance to *A. flavus* infection and other traits.

Forty years ago, peanut variety J11 was discovered to be resistant to *A. flavus* infection. Subsequently, its stable infection resistance was proved in multiple researches. However, this precious material has not been fully utilized, for the resistant loci or genes of J11 were not found. In the present study, the QTL mapping of its resistance was firstly studied using a RIL population. Six additive QTLs for resistance to *A. flavus* infection were identified on chromosomes A05, A08, B01, B03 and B10. These QTLs were novel because the previously reported QTLs by Yu et al. (2019) and Khan et al. (2020) were on A03, A10 and B04. Notably, the favorable alleles of five of the six novel QTLs were from J11, but these QTLs were not stable across environments. Three of them were only identified in one environment and the other two QTLs were identified in two of four environments, indicating that the resistance to *A. flavus* infection of J11 might be controlled by multiple QTLs and was significantly interacted with environment. This phenomenon was consistent with the previous observation of Mehan et al. (1987). Surprisingly, the favorable alleles of the stable QTL *qPSIIB10* was from the susceptible parent Zhonghua 16, indicating that attentions should also be paid to susceptible germplasm since they might possess valuable loci for resistance to *A. flavus* infection. Another important finding is that the RILs pyramided favorable alleles of the six additive QTLs significantly improved their resistance levels, which was consistent with the existing of transgressive segregation. Therefore, both major QTLs and minor QTLs should be concerned in the genetic enhancement of resistance to *A. flavus* infection in peanut breeding.

**Conclusions**

The present study constructed a high-density genetic map using whole genome resequencing technology and six important infection-resistant QTLs were identified in four environments lay a foundation for further research on fine mapping and breeding application in peanut.

**Declarations**

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Conflict of Interest
The authors declare that there is no conflict of interest.

Author contributions
YJ, HL, YL, BL and HJ conceived, designed and supervised the experiments. YL, BL, and HJ developed the RIL population. YJ, HL, BY, YD, HZ, SX, XR, JG, LH, XZ, YC, WC and NL conducted field trials and phenotyping. YJ, BY, YD, HZ, SX, JG and WC performed DNA extraction and genotyping. YJ, HL, XZ, BL, and HJ performed the data analysis and interpreted the results. YJ, HL prepared the first draft and YJ, HL, YL, BL and HJ contributed to the final editing of manuscript. All authors read and approved the final manuscript.

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Tables

**Table 1** Phenotypic variations in parents and RILs for PSII

| Env    | Parents | RILs     | CV   |
|--------|---------|----------|------|
|        | Zhonghua 16 | J11 | Range | Mean ± SD |
| WH2017 | 87.04±9.44 | 55.97±8.57* | 46.92-98.25 | 80.58±10.57 | 0.13 |
| WH2018 | 91.15±6.26 | 53.83±6.68** | 40.74-100.00 | 83.74±11.20 | 0.13 |
| WH2019 | 93.00±7.06 | 51.04±6.70** | 26.98-100.00 | 83.35±11.55 | 0.14 |
| WH2020 | 91.73±5.80 | 51.65±6.76** | 24.73-100.00 | 67.87±16.18 | 0.24 |

PSII, percent seed infection index; Env, environment; SD, standard deviation; CV, coefficient of variation; *Difference is significant at p<0.05 level between parents; **Difference is significant at p<0.01 level between parents

**Table 2** Analysis of variance for PSII in the RIL population across four environments

| Source                 | df | Mean square | F value | P value |
|------------------------|----|-------------|---------|---------|
| Genotypes              | 199| 0.08        | 8.71    | < 0.001 |
| Environments           | 3  | 16.55       | 1776.57 | < 0.001 |
| Genotypes×Environments | 551| 0.02        | 2.37    | < 0.001 |
| Error                  | 1291| 0.01        |         |         |

PSII, percent seed infection index
### Table 3 Basic information of the high-density genetic linkage map

| Chr | Length(cM) | No. markers | No. bins | Marker interval(cM) | Bin interval(cM) | Max interval(cM) |
|-----|-------------|-------------|----------|---------------------|------------------|------------------|
| A01 | 83.85       | 3,420       | 119      | 0.025               | 0.71             | 4.19             |
| A02 | 61.50       | 13,895      | 124      | 0.004               | 0.50             | 3.99             |
| A03 | 72.62       | 3,185       | 110      | 0.023               | 0.67             | 4.49             |
| A04 | 86.66       | 2,042       | 120      | 0.042               | 0.73             | 12.34            |
| A05 | 70.59       | 3,169       | 116      | 0.022               | 0.61             | 2.15             |
| A06 | 57.28       | 2,409       | 102      | 0.024               | 0.57             | 1.87             |
| A07 | 68.56       | 5,634       | 122      | 0.012               | 0.57             | 3.30             |
| A08 | 93.76       | 5,298       | 181      | 0.018               | 0.52             | 2.63             |
| A09 | 96.64       | 6,017       | 147      | 0.016               | 0.66             | 2.76             |
| A10 | 69.23       | 15,859      | 116      | 0.004               | 0.60             | 4.80             |
| B01 | 88.76       | 22,268      | 164      | 0.004               | 0.55             | 2.43             |
| B02 | 59.01       | 14,568      | 110      | 0.004               | 0.54             | 3.88             |
| B03 | 89.19       | 5,201       | 167      | 0.017               | 0.54             | 2.62             |
| B04 | 93.57       | 3,352       | 116      | 0.028               | 0.81             | 9.10             |
| B05 | 93.00       | 23,930      | 210      | 0.004               | 0.45             | 2.43             |
| B06 | 59.62       | 21,306      | 143      | 0.003               | 0.42             | 2.43             |
| B07 | 79.98       | 20,055      | 164      | 0.004               | 0.49             | 3.30             |
| B08 | 80.99       | 17,578      | 148      | 0.005               | 0.55             | 3.00             |
| B09 | 95.53       | 24,980      | 174      | 0.004               | 0.55             | 5.74             |
| B10 | 73.51       | 19,199      | 149      | 0.004               | 0.50             | 2.91             |
| Whole | 1573.85   | 233,365     | 2,802    | 0.013               | 0.58             | 4.02             |

### Table 4 Additive QTLs for resistance to *Aspergillus flavus* infection across four environments
| QTL     | LG | Env  | Cl (cM)       | Marker interval       | LOD  | PVE(%) | Add   |
|---------|----|------|---------------|-----------------------|------|--------|-------|
| qPSIIA05| A05| WH2017| 56.5-57.5     | c05b092-c05b093      | 3.17 | 5.50   | 2.41  |
| qPSIIA08| A08| WH2017| 53.5-54.5     | c08b121-c08b122      | 5.97 | 10.87  | 3.39  |
| qPSIIB01| B01| WH2020| 43.5-44.5     | c11b078-c11b079      | 2.63 | 6.16   | 3.74  |
| qPSIIB03.a| B03| WH2017| 52.5-53.5     | c13b091-c13b092      | 5.16 | 9.16   | 3.07  |
|          |    | WH2020| 52.5-53.5     | c13b091-c13b092      | 3.91 | 9.23   | 4.58  |
| qPSIIB03.b| B03| WH2018| 36.5-37.5     | c13b049-c13b050      | 2.74 | 5.03   | 2.68  |
|          |    | WH2019| 36.5-37.5     | c13b049-c13b050      | 2.75 | 5.75   | 2.87  |
| qPSIIB10 | B10| WH2017| 32.5-33.5     | c20b057-c20b058      | 5.39 | 10.10  | -3.23 |
|          |    | WH2018| 32.5-33.5     | c20b057-c20b058      | 5.28 | 9.94   | -3.79 |
|          |    | WH2019| 32.5-33.5     | c20b057-c20b058      | 4.92 | 10.58  | -3.91 |
|          |    | WH2020| 32.5-33.5     | c20b057-c20b058      | 2.84 | 6.91   | -3.99 |

LG, Linkage group; Cl, Confidence interval of QTLs; PVE, Phenotypic variance explained; Add, Additive effect

**Table 5** Phenotypic effect of the pyramiding of six QTLs

| Genotype | No. lines | WH2017 (%) | WH2018 (%) | WH2019 (%) | WH2020 (%) |
|----------|-----------|------------|------------|------------|------------|
| BBBBBA   | 5         | 55.53±6.17<sup>a</sup> | 64.40±7.85<sup>a</sup> | 63.91±7.57<sup>a</sup> | 42.61±11.27<sup>a</sup> |
| BBBBBB   | 4         | 81.67±9.66<sup>b</sup> | 89.02±5.50<sup>b</sup> | 88.52±4.09<sup>b</sup> | 67.34±7.98<sup>b</sup> |
| AAAAAA   | 5         | 90.38±4.53<sup>b</sup> | 91.23±8.89<sup>b</sup> | 94.23±5.78<sup>b</sup> | 84.98±10.37<sup>b</sup> |
| AAAAAAB  | 10        | 87.37±6.63<sup>b</sup> | 93.27±5.37<sup>b</sup> | 93.58±5.27<sup>b</sup> | 75.63±6.84<sup>b</sup> |

“A” genotype of QTLs from Zhonghua 16, “B” genotype of QTLs from J11; The letter a, b after the phenotypic value are statistically different at p<0.05 based on ANOVA and Games-Howell multiple-comparison

**Figures**
Figure 1

Distribution of phenotype from 2017 to 2020
Figure 2

Distributions of bins on the high-density genetic linkage map
Figure 3

Distributions of QTLs for PSII. PSII, percent seed infection index

Supplementary Files

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