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ORIGINAL ARTICLE

High-density SNP map facilitates fine mapping of QTLs and candidate genes discovery for *Aspergillus flavus* resistance in peanut (*Arachis hypogaea*)

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Abstract

Key message **Two novel resistant QTLs mapped and candidate genes identified for *Aspergillus flavus* resistance in cultivated peanut using SLAF-seq.**

Abstract Aflatoxin contamination in peanuts caused by *Aspergillus flavus* is a serious food safety issue for human health around the world. Host plant resistance to fungal infection and reduction in aflatoxin are crucial for mitigating this problem. Identification of the resistance-linked markers can be used in marker-assisted breeding for varietal development. Here we report construction of two high-density genetic linkage maps with 1975 SNP loci and 5022 SNP loci, respectively. Two consistent quantitative trait loci (QTL) were identified as *qRAF-3-1* and *qRAF-14-1*, which located on chromosomes A03 and B04, respectively. QTL *qRAF-3-1* was mapped within 1.67 cM and had more than 19% phenotypic variance explained (PVE), while *qRAF-14-1* was located within 1.34 cM with 5.15% PVE. While comparing with the reference genome, the mapped QTLs, *qRAF-3-1* and *qRAF-14-1*, were located within a physical distance of 1.44 Megabase pair (Mbp) and 2.22 Mbp, harboring 67 and 137 genes, respectively. Among the identified candidate genes, six genes with the same function were found within both QTLs regions. In addition, putative disease resistance RPP13-like protein 1 (RPP13), lipoxygenase (Lox), WRKY transcription factor (WRKY) and cytochrome P450 71B34 genes were also identified. Using microarray analysis, genes responded to *A. flavus* infection included coding for RPP13, pentatricopeptide repeat-containing-like protein, and Lox which may be possible candidate genes for resistance to *A. flavus*. The QTLs and candidate genes will further facilitate marker development and validation of genes for deployment in the molecular breeding programs against *A. flavus* in peanuts.

Introduction

Peanut (*Arachis hypogaea* L.) is an annual allotetraploid ($2n = 4x = 40$) legume, probably originated through hybridization of *A. duranensis* (A-genome) and *A. ipaensis* (B-genome) followed by polyploidization (Bertioli et al. 2016). Peanuts are a good source of balanced diet for humans, providing both edible oil and proteins in significant amount. The hulls are used as feed for animals. As a

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legume, peanuts play a vital role in the soil improvement through nitrogen fixation (Varshney et al. 2017). However, the production and quality of peanuts is greatly threatened by aflatoxin contamination, which is carcinogenic produced by *Aspergillus flavus* (*A. flavus*) and *Aspergillus parasiticus* (*A. parasiticus*). Humans as well as animals face adverse health complications due to the consumption of aflatoxin-contaminated peanuts, including liver damage leading to liver cancer, retarded growth, immune suppression and speed up the progression of acquired immune deficiency syndrome (AIDS), where aflatoxin exposure may add to high viral loads, badly effecting the normal liver functioning in HIV-positive people and thus promoting disease progression. Aflatoxin B_1 could cause acute aflatoxicosis and hepatitis B and C chronic infections (Gong et al. 2004; Jolly et al. 2011; Wild and Montesano 2009). Aflatoxin contamination reduces economic value of the crop in the international trade market (Fountain et al. 2014; Pandey et al. 2019; Shephard 2008). Currently, over 5 billion people around the globe are at serious risk of chronic exposure (> 1000 ppb) to aflatoxin through consumption of the contaminated foods (Shephard 2003; Williams et al. 2004; Strosnider et al. 2006).

It has been elucidated that the aflatoxin production is linked not merely to the infection rate of *A. flavus* but to a combination of changes in environmental conditions, including drought dress, especially when *A. flavus* infection occurs during the pod maturation stage (Davidson et al. 1983; Priyadarshini and Tulpule 1978), and also the interaction with other soil-borne pathogens. Peanuts are contaminated with aflatoxin during seed development (at pre-harvest stage) in the field, harvesting process, post-harvest operations, storage and transportation (Upadhyaya et al. 2002; Waliyar et al. 2015). A combined approach of host resistance followed by pre- and post-harvest management practices is required (Pandey et al. 2019). The seed coats and outer shells play vital roles in protecting the seeds from mechanical damage, pest infestation and surviving harsh weather conditions (Souza and Marcos-Filho 2001). To develop peanut cultivars resistant to *A. flavus* infection with reduced aflatoxin contamination, there is a possible defense mechanism at three stages: against fungal invasion in the pericarp, aflatoxin production in the seed coat and against aflatoxin production in cotyledons (Mixon 1986). Using cultivars resistant to kernel infection by *A. flavus* is one of the promising ways to reduce aflatoxin contamination. Unfortunately, there are no so-called resistant cultivars released to public use yet.

Various efforts have been made to phenotype peanut germplasm, and only a few lines have been reported to show moderate-to-high resistance against *A. flavus* infection (Pandey et al. 2019; Zhuang et al. 2007). To know the resistance mechanism, several studies in the area of physiology, transcriptomic and proteomics provided an effective explanation of the resistance (Korani et al. 2018; Fountain et al. 2016, 2018; Nayak et al. 2017; Wang et al. 2016; Pandey et al. 2019). Using a two-dimensional difference gel electrophoresis proteomic approach, several proteins corresponding to aflatoxin production were identified in peanut using toxigenic strains of *A. flavus* (Wang et al. 2012a, b). A proteomic approach was applied to gain insights into the regulatory mechanism of pathogen involved during oxidative stress, similar to drought stress in peanut (Fountain et al. 2018). This knowledge has empowered researchers and breeders to develop aflatoxin contamination-free transgenic maize (Thakare et al. 2017) and peanut (Sharma et al. 2018) via host-induced gene silencing. However, there are only two reports that have identified quantitative trait loci (QTLs) for *A. flavus* infection in peanut. The first study of QTL mapping for *A. flavus* infection reported six QTLs on chromosomes LG1, LG2, LG3, LG4, LG15 and LG18 using genetic linkage maps based on simple sequence repeat (SSR) markers (Liang et al. 2009). Furthermore, a recent study has reported QTLs for aflatoxin AFB_1, AFB_2 and PSII in peanuts using SSR linkage map. Two QTLs on A03 and A10 chromosomes for PSII, seven QTLs for AFB_1 including two major QTLs on chromosomes A05 and B06 and seven QTLs for AFB_2 on chromosomes A07, B05, B06 and B07, respectively, were identified (Yu et al. 2019). As these studies used low-density SSR genetic maps, it has not been possible to fine map-resistant QTLs for candidate gene discovery and thus hinder identification of genotypes with reliable resistance.

Genomics-assisted breeding (GAB), specially marker-assisted selection (MAS) and marker-assisted backcrossing (MABC), can drastically reduce the time for developing new varieties; however, the linked markers for target traits are needed for deploying GAB (Pandey et al. 2016; Varshney et al. 2018). In this context, mapping of QTLs for *A. flavus* resistance and identification of putative candidate-resistant genes is critical to deploy GAB for developing superior varieties for aflatoxin resistance. In past, several genetic maps have been developed, marker density has been very poor. For instance, the first SSR-based genetic linkage map for cultivated peanut had 135 mapped SSR loci (Varshney et al. 2009). Subsequently, comparatively dense genetic maps with 1114 mapped SSR loci (Shirasawa et al. 2012) and 1219 SSR loci (Huang et al. 2016) were developed. Low polymorphism has been the main reason for the lack of high-density genetic maps and high-resolution trait mapping in cultivated peanut (Bertioli et al. 2016; Pandey et al. 2012; Samoluk et al. 2015; Varshney et al. 2013).

The single nucleotide polymorphism (SNP) markers are preferred molecular markers for several genetic studies due to its wide distribution throughout the genome and occurrence in higher numbers compared to SSR markers (Liao and Lee 2010). Moreover, the current progress of next-generation sequencing (NGS) technologies, such as specific length-amplified fragment sequencing (SLAF-seq), has

significantly facilitated the development of a large number of molecular markers and QTL mapping in cultivated peanut. Various studies have been carried out using bi-parental populations to map QTLs for different traits of interest in peanuts (Leal-Bertioli et al. 2016; Luo et al. 2018; Pandey et al. 2017; Varshney et al. 2014; Zhou et al. 2016). In the current study, we sequenced a large RIL population and an HDGM based on SNP markers was constructed using SLAF-seq approach. In parallel, many resistant lines were identified with promising phenotypic resistance to *A. flavus* infection. Finally, two resistant QTLs and candidate genes of *A. flavus* resistance were identified that can be deployed in future peanut breeding applications.

Materials and methods

Plant materials

In this study, the resistance level of two parents, Xinhuixiaoli (XHXL) and Yueyou 92 (YY92), and the RIL populations were examined under laboratory conditions through artificial inoculation using a high toxicogenic strain of *A. flavus* AF2. XHXL has red color of testa, small kernel size, usually contains two to three kernels per pod with 42.40 grams of 100 kernels weight, while YY92 with pink color of testa, comparatively bigger in its kernel size, usually contains one to two kernels per pod with 100 kernels weight of 67.68 grams. The two parents were highly contrasted in resistance to *A. flavus;* XHXL showed high resistance level, while YY92 showed high susceptibility. RIL mapping population named *R* population at F_{10} generation consisted of 314 RILs, was used in this study, developed through single seed descent (SSD) method by crossing parents, XHXL and YY92. The male parent XHXL is highly resistant to *A. flavus* infection, a landrace belonging to *A. h. ssp. fastigiata var. fastigiata*, collected at Guangdong Province of China. It was identified by our group showing stable resistance to *A. flavus* both at the pre-harvest stage in the fields and post-harvest inoculation for many years. It is registered in the Chinese Crop Germplasm Resources Information System (http://www.cgris.net/) under accession number Zh.h0341. The female parent YY92, *A. h. ssp. fastigiata var. vulgaris*, highly susceptible to *A. flavus* infection is a Chinese cultivar developed by Guangdong Academy of Agricultural Sciences, China (Fig. 1). The RILs were grown in the research fields of Fujian Agriculture and Forestry University at Pingtan (located between 25° 15′–25° 45′ north latitude and 119° 32′–120° 10′ east longitude) and at Yangzhong (located at 26.71° north latitude, 119.37° east longitude), Fujian, China, using a randomized complete block design (RCBD) with three replications. The two parents were planted as a control and standard agricultural practices applied during the entire plant growth stages.

Fig. 1 Pods and kernels phenotypes of XHXL and YY92. Where **a–c** shows the pods and kernels of XHXL, the resistant parent with the red color of testa, small kernel size, usually contains two to three kernels per pod and 100 kernels weight of 42.40 grams, while **d–f** represents YY92, susceptible parent with pink color of testa, comparatively bigger kernel size, usually contains one to two kernels per pod with 100 kernels weight of 67.68 grams, g and I is the comparison of XHXL and YY92 length and width wise, respectively (color figure online)

Assessment of phenotypic variation among RILs to *A. flavus* infection

From each line and the parents, 15 healthy kernels without any apparent damage were selected and blanched. Then the selected kernels were immersed well in 75% ethanol for 1 min and surface sterilized with 0.1% $HgCl_2$ for 5 min followed by five washes with distilled water. The first 4 washes lasted 5 min each, while the 5th wash lasted for 10 min. The kernels were then transferred to 9-cm-diameter disposable sterilized petri plates. The entire RIL population along with the parents were phenotyped under laboratory conditions via inoculation. Potato dextrose agar (PDA) medium (Potato 300 g/l, glucose 20 g/l, agar 16 g/l) was used to culture a highly toxic *A. flavus* strain AF2 for about one week to obtain the desired concentration of spores for inoculation. Then 50 ml sterile water with 0.01% Tween 20 was added to the obtained spores, which were grown in 100-ml conical flasks and the solution was mixed thoroughly with a glass rod until the spores were evenly distributed. The average number of conidia per milliliter (ml) was approximately 1.9×10^6 (Li et al. 2006). To each petri plate, 500 microliters (µl) *A. flavus* spore solution was added and vigorously shaken to ensure all kernels surface contact with the spores. All 15 kernels were arranged separately on each plate, and the plates were then incubated at 28 °C for 14 days. For all the RILs and parents, the analysis was performed in three replicates. After 14 days, resistance and susceptibility levels were scored according to the previously described method (Mehan et al. 1981) with some modifications. Resistance was divided into six different levels: (1) level 0: peanut kernels with no apparent spores of *A. flavus*, (2) level 1: > 0–10% of the kernel area covered with *A. flavus* spores, (3) level 2: > 10–20% of the kernel area covered with *A. flavus* spores, (4) level 3: > 20–50% of the kernel area covered with *A. flavus* spores, (5) level 4: > 50–80% of the kernel area covered with *A. flavus* spores and (6) level 5: > 80–100% of the kernel covered with *A. flavus* spores. The infection index was calculated according to the formula:

$$\text{Infection index} = (10\%\, n1 + 20\%n2 + 50\%n3 + 80\%n4 + 100\%n5) \times 100/N.$$

where $n1$, $n2$, $n3$, $n4$, and $n5$ are the number of kernels corresponding to levels 1, 2, 3, 4 and 5, respectively. Based on the kernel resistance characterization, the resistant grades were classified into six levels as follows: highly resistant (HR): no kernel showed apparent infection; resistant (R): > 0–10% of kernels were infected with spores; moderately resistant (MR): > 10–25% of kernels were infected with spores; moderately susceptible (MS): > 25–50% of kernels were infected with spores; susceptible (S): > 50–75% of kernels were infected with spores; and highly susceptible (HS): > 75% of kernels were infected with spores (Mehan et al. 1981; Zhuang et al. 2007).

Statistical analysis

Statistical analysis of the data was carried out using Microsoft Excel 2016, IBM SPSS statistics 22 (IBM SPSS 2013), while for figures drawing Sigma plot 14 was used. The broad sense heritability (H^2) was calculated according to the following formula:

$$H^2 = \sigma_g^2 / \left(\sigma_g^2 + \sigma_{ge}^2 / n + \sigma_e^2 / rn \right)$$

where σ_g^2 is genetic variance, σ_{ge}^2 is the interaction variance between genotype and environment, σ_e^2 is the residual (error) variance, r is the number of replications in each environment and n is the number of environments.

Genomic DNA extraction

Fresh leaves from RILs and their parents were collected, frozen in liquid nitrogen and stored at − 80 °C refrigerator. Genomic DNA was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) method of Murray and Thompson with minor modifications (Murray and Thompson 1980). Leaf tissue was ground in liquid nitrogen, CTAB extraction solution was added, and samples were incubated in a 65 °C water bath for 25 min. Then, the samples were centrifuged at 12,000 rpm for 10 min, 1 ml of the supernatant was transferred to another centrifuge tube and the residual sample was discarded. The same volume of chloroform–isoamyl alcohol (24:1) was added, shaken and centrifuged for 10 min at 12,000 rpm. The supernatant was transferred to a new centrifuge tube. The last two steps were repeated to ensure the complete removal of polysaccharides. Then, 1/3 volume of 10% lithium chloride (LiCl) solution was added, gently inverted to properly mix it and incubated at − 80 °C refrigerator for 2 to 3 h. After incubation, the samples were centrifuged, the resulting supernatant was discarded, and the pellet was washed with 75% ethanol three times via centrifugation at 12,000 rpm for 5 min each. After drying, 50 µl of sterilized water and RNase A were added and incubated at 37 °C for approximately 1 h. The DNA quality and quantity were determined by 1% agarose gel electrophoresis compared with standard DNA ladder and NanoDrop spectrophotometer 2000 (Thermo Fisher Scientific Inc., USA).

Library construction and high-throughput sequencing

For genotyping RILs and both parents, the SLAF-seq approach was used. SLAF library was constructed using genomic DNA extracted from stable F_{10} RILs population and both parents. Moderate dense genetic linkage map (MDGM) and HDGM were constructed from genomic DNA of the same 314 F_{10} RILs in two efforts by SLAF-seq according to the previously described method of Sun et al. (2013). To maintain the sequence depth uniformity in different fragments, a tight length of 314–414 bp was selected. Then a trial PCR amplification was performed to check the reduced representation library (RRL) (Hyten et al. 2010) features in targeted fragment length size (314–414 bp). Following digestion, adenine and duplex tag-labeled sequencing adapters were added to the selected DNA fragments. The extracted genomic DNA of all samples was treated with HaeIII (NEB, Ipswich, MA, USA), T4 DNA ligase (NEB), ATP (NEB) and HindIII adapter at 37 °C. The genomic DNA extracted from RILs and their parents were incubated at 37 °C with MseI (New England Biolabs, NEB), T4 DNA ligase (NEB), ATP (NEB) and MseI adapter. The restriction-ligation reaction solutions were diluted and mixed with dNTPs, Taq DNA polymerase (NEB) and MseI primer containing barcode 1 for PCR reactions. Then restriction-ligation reactions were heat-inactivated at 65 °C, followed by restriction via additional AluI restriction enzyme at 37 °C. The PCR reaction was carried out using diluted restriction ligation samples, dNTPs, Taq DNA polymerase (NEB) and MseI primer-containing barcode1. Then the PCR amplicons were purified using E.Z.N.A.® Cycle Pure Kit (Omega) and pooled. The pooled sample was incubated again at 37 °C with MseI, T4 DNA ligase, ATP and Solexa adapter. The samples were then purified using a Quick Spin column (Qiagen) and run on a 2% agarose gel. The targeted fragments were isolated via Gel Extraction Kit (Qiagen) and subjected to PCR amplification using Phusion Master Mix (NEB) and Solexa Amplification primer mix to add barcode 2. The Phusion PCR was carried out in the same way listed in the Illumina sample preparation guide. Then the samples were gel purified, excising the targeted fragment size, diluted for sequencing. Pair-end sequencing was carried out for the selected SLAFs markers using Illumina HiSeq 2500 high-throughput sequencing platform (Illumina, Inc; San Diego, CA, USA) following the manufacturers' recommendations. Real-time monitoring was implemented for each cycle during sequencing and the ratio of raw high-quality reads having quality scores more than Q30, the quality score of 30 specifies a 0.1% chance of procurement an error, and thus 99.9% confidence level, and the guanine-cytosine (GC) contents were calculated for quality control. All sequences clustered together were defined as a SLAF locus. Both parents were sequenced separately at a high sequencing depth of approximately 107.84×, while the RILs were sequenced at 13.52×. The pre-design experiment was changed in the case of the amplification of non-specific bands. The SLAF library was then constructed according to the evaluated pre-design scheme (Sun et al. 2013) (Fig. S1).

Grouping and genotyping of the sequenced data

Previously described methods and software (Sun et al. 2013) were used for genotyping the current RIL population and their parents. All SLAF paired-end reads with clear index information were clustered based on sequence similarity, detected via one-to-one alignment by BLAT (Kent 2002). The reads with more than 90% sequence similarity were grouped into one SLAF locus, while the identical reads were merged (Sun et al. 2013). In each SLAF locus, alleles were defined using minor allele frequency (MAF) evaluation, i.e., SNPs with MAF of > 0.05 were targeted. The sequence error rate was assessed using the rice data as a control for preventing false-positive results. Normally the true genotypes must have higher MAF values than those containing sequence errors. After MAF evaluation, the SLAFs with sequence errors were corrected compared to the most similar genotype and the data efficiency was improved. In the mapping population, one locus can contain mostly 4 genotypes, so the groups with above 4 SLAF tags were considered as repetitive SLAFs and filtered out from the final SLAFs. Only SLAFs groups having suitable depth with less than 4 tags were considered as high-quality SLAFs. The obtained SLAF were filtered with the following criteria: (a) SLAF with less than 10× parental sequence depth, (b) SLAF with a sufficient degree below 30%, (c) SLAF with serious distorted segregation (p value < 0.05), and (d) SLAF that were heterozygous in the both parents. It was further clarified that only the SLAF with less than four alleles were considered polymorphic and used as potential markers for genetic map construction. The Bayesian approach was applied for genotypic scoring, and those markers that had less than 30% data were further filtered out before genetic map construction, as described previously (Zhang et al. 2015). After these quality filtrations, the obtained polymorphic SLAF markers were sorted into eight segregation patterns: $ab \times cd$, $ef \times eg$, $hk \times hk$, $lm \times ll$, $nn \times np$, $aa \times bb$, $ab \times cc$, and $cc \times ab$. Since our mapping population was derived from two homozygous parents with aa and bb genotypes, only the $aa \times bb$ segregation patterns of SLAF markers were selected for current genetic maps construction (Fig. S2).

Genetic linkage maps construction

The obtained SLAF markers were distributed throughout the genome. Based on their locations, they were assigned to

20 LGs. Keeping in mind the risk that NGS may have some genotypic deletions or errors that will cause a reduction in the quality of the genetic map, the high map strategy was used to correct these errors (Liu et al. 2014). Join Map 4.0 software was used to construct both MDGM and HDGM for the 314 RIL F_{10} population and both parents (Van Ooijen 2006). After genotyping of the sequenced data, high-quality SLAFs and SNP markers were allocated to 20 LGs based on their locations on the respective chromosomes. All LGs were further processed, first using the primary markers to locate all the LGs according to their location on chromosomes following the relationship between the ordered markers. Then the genotyping errors or deletions were corrected via the SMOOTH algorithm, the minimum spanning treemap was used to order the maps, and then the SMOOTH algorithm was used to correct all the newly ordered genotypes. After repeating these corrections for four or more cycles, 20 high-quality maps were obtained. Using the Kosambi mapping function the map distances were calculated (Kosambi 2016). During both linkage maps construction, highly distorted and unlinked markers were neglected. For the adjacent two markers, the value of MLOD was calculated according to the methods of Vision et al. (2000), and then by Marker HighMap, the markers with MLOD values less than 3 were filtered. This filtration was carried out through all the 20 LGs, and for each pair of adjacent markers, the genetic distances were calculated (Liu et al. 2014). For constructing both MDGM and HDGM, the same procedures were followed but with difference in sequencing depths (Fig. S3).

QTL analysis

For mapping resistance QTLs against *A. flavus*, we performed QTL mapping using the phenotyping data generated for two years. For the first time, MDGM consisted of 1975 SNP markers and mean phenotypic data of 208 F_{10} RILs were used for mapping. For closely locating the QTLs to identify putative candidate-resistant genes, the phenotypic data of 209 F_{10} RILs and HDGM consisted of 5022 SNP markers were used in second time mapping. An interval mapping model with a LOD score of 2.5 for potential QTLs was used for current QTL detection. Two resistant QTLs were mapped repeatedly located on the same respective chromosomes, A03 and B04. For mapping, these resistance QTLs against *A. flavus*, R/QTL software and the method of internal mapping were used. According to the Bayes Interval Method (Sen and Churchill 2001), QTLs at 95% confidence intervals were calculated using the same R/QTL software. The total LOD score, the additive effect and the proportion of total phenotypic variation explained (PVE) by both detected QTLs were obtained by fitting a multiple linear regression model that included both detected QTLs for the studied trait.

Candidate genes discovery and evaluation by microarray

Candidate genomic regions for the two QTLs were located by BLASTN using the flanking marker sequences of the mapped QTLs in the HDGM with 5022 SNP loci. Gene loci located within two regions were searched and submitted to the Nr dataset by BLASTX, Gene Ontology (GO) and UniProt annotations. Bioinformatics analysis was performed to find the possible association of candidate genes with resistance to *A. flavus*.

Peanut cultivar, Minhua 6, which is a widely planted cultivar in Southern China, showing resistance to *A. flavus* (Zhuang et al. 2007), was grown in pots under the normal conditions with 20–30 °C. At 40 days after pegging, the plants were treated with drought, drought plus inoculation with *A. flavus* (drought is a necessary factor for *A. flavus* infection), and normal watering as a control. The drought stress was given by stopping water supply, the normal watering treatment was performed with daily water supply, and the *A. flavus* treatment was carried out by inoculating the drought-stressed plants. Inoculation was done by spreading *A. flavus* spores above the soil surface. Pericarps samples were collected from all the above treatments at 3*d*, 6*d*, and 12*d* after inoculation for RNA extraction. Total RNA was extracted using the CTAB-LiCl method of Chen et al. (2016), double strands were synthesized and purified for chip hybridization. In silico, analysis was performed for gene expression within the QTL regions. The slide containing unigene probes of 60 base oligonucleotides with a density of 12 × 135 K probes was used for analysis. The expression data of genes were generated using the Robust Multichip Average (RMA) algorithm (Irizarry et al. 2003). For all experiments, two replicates were performed.

Results

Phenotypic variation among RILs against *A. flavus* resistance

Significant differences of *A. flavus* infection between XHXL and YY92 were observed across three environments. XHXL revealed consistently high resistance to *A. flavus* infection showing lower infection rate while YY92 showed high susceptibility and infection rate (Table 1; Table S1; Fig. 2). The broad range of phenotypic variations was observed among the RILs against *A. flavus* infection. The highest infection index distribution was found within the range of 10–20%, showing the potential-resistant RILs against *A. flavus* infection (Table 1; Table S1; Figs. 2, 3). One-way analysis of variance (ANOVA) revealed significant difference in infection level of *A. flavus* among RILs in 2016 spring [ANOVA

Table 1 Phenotypic variations in parents and RILs for *A. flavus* infection

Trait	Seasons	XHXL		YY92		Range	RILs		*P*-value	H^2
		Mean ± SD	CV	Mean ± SD	CV		Mean ± SD	CV		
A. flavus infection	2016S	20.00 ± 0.00	0.00	70.00 ± 5.65	0.08	1.33–89.33	27.43 ± 4.51	0.21	< 0.001	0.89
	2017S	16.85 ± 4.60	0.27	80.89 ± 12.32	0.15	0.44–87.00	28.04 ± 12.82	0.53	< 0.001	
	2017A	5.14 ± 4.88	0.95	83.83 ± 10.25	0.12					

S spring, *A* autumn, *SD* standard deviation, *CV* coefficient of variation, H^2 broad-sense heritability

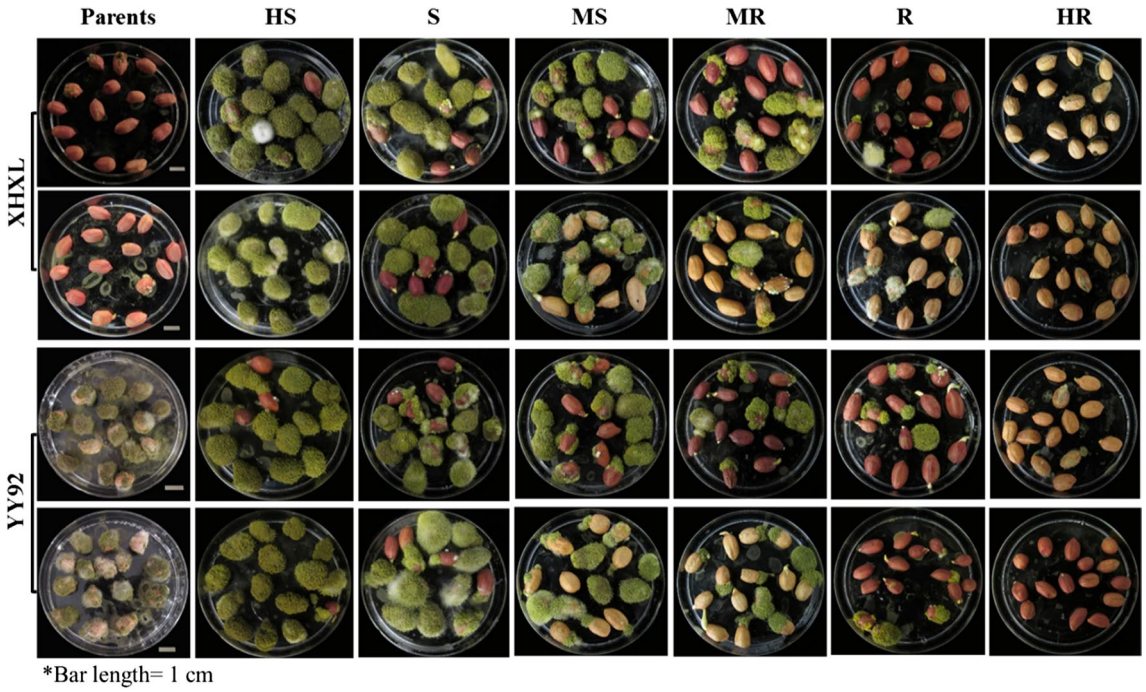

Fig. 2 Phenotypic discrepancies between parents and among RILs. From right in the first column (parents), the first two petri plates are resistant XHXL, while the last two represent the susceptible parent YY92. The second column represents highly susceptible (HS), followed by susceptible (*S*), moderate susceptible (MS), moderate resistant (MR), resistant (*R*) and highly resistant (HR) RILs

$F_{(207,416)} = 23.987$] ($p < 0.001$) and 2017 spring [ANOVA $F_{(208,418)} = 4.174$] ($p < 0.001$). Wide segregation and continuous distribution in the RIL populations against *A. flavus* infection was detected, predicting the presence of resistance alleles in two parents. A significant correlation ($r = 0.465$**) was found among the matched phenotypic data between 2016 and 2017. The broad-sense heritability for the studied trait was estimated to be relatively high (89%) indicating more control of genetic factors and fewer environmental effects, credited to the control environmental conditions during both years (Table 1).

SLAF sequencing, SNP identification and construction of genetic maps

For MDGM construction in 2016, a total of 1398 million reads (M reads) of raw data was generated within the range of 314–414 bp with Q30 values of 86.64% and 43.62% GC content, after DNA SLAF-seq of 314 F_{10} RILs and parents. There were about 14 M reads from the female parent with GC contents of 43.73% and Q30 of 85.14%, while the contribution from the male parent was 17 M reads, consisted of 43.09% GC contents and 88.03% Q30 value. The average numbers of reads from RILs were 1366 M reads with 43.62% GC contents and 86.64% Q30 (Table 2a). As the RIL population was developed through continuous self-pollination of the *F*1 cross of two homozygous parents with aa and bb genotypes, only aa and bb genotype were selected for genetic linkage map construction from the eight segregation patterns, *ab* × *cd*, *ef* × *eg*, *hk* × *hk*, *lm* × *ll*, *nm* × *np*, *aa* × *bb*, *ab* × *cc*, and *cc* × *ab*. Initially a total of 371,638 SLAF markers with segregation pattern of *aa* × *bb* were found, in which the contribution of offspring was 90,437 markers, while male and female contributed 141,869 and

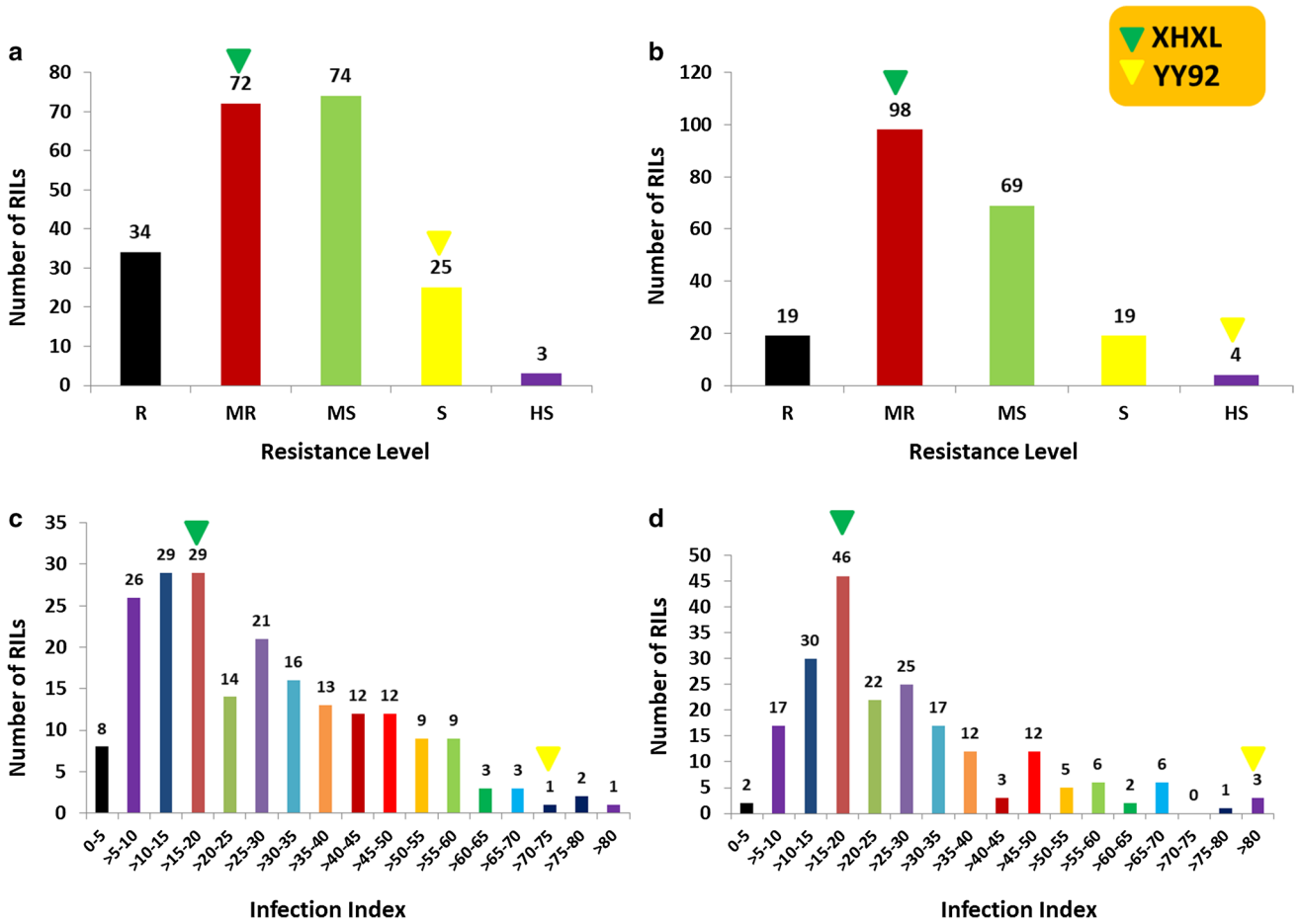

Fig. 3 Infection Index of RILs and parents against *A. flavus*. Where **a**, **b** represent the number distribution of RILs including parents in various resistance and susceptibility levels, while **c**, **d** show the distribution of infection index among different levels evaluated in 2016 and 2017, respectively

Table 2 Summary of SLAF-seq data of parents and RILs compared with control

	Sample ID	BMK ID	Total reads	GC %	Q30%
A	XH	P	17,191,760	43.09	88.03
	YY92	M	14,459,351	43.73	85.14
	Offspring	Offspring	1,366,647,609	43.62	86.64
B	XH	P	32,561,321	42.94	84.90
	YY92	M	35,173,168	43.01	84.26
	Offspring	Offspring	1,366,647,609	43.62	86.64

A = data used in MDGM, while B = data used in HDGM construction

134,626 SLAF markers, respectively. In total, 4561 markers fall into the *aa* × *bb* segregation pattern. Finally, the SLAF with low quality, a parental sequence depth of less than 10×, completeness of less than 70%, and significant segregation distortion ($p < 0.001$) was discarded. After these quality-based filtrations, the remaining 1975 SNP markers were used to construct the MDGM.

For HDGM construction in 2017, a total of 1434 M reads of raw data were generated initially within the range of 314–414 bp with Q30 values of 86.64% after DNA SLAF-seq of parents with more sequencing depth. There were 33 M reads from the female parent with GC contents of 42.94% and Q30 of 84.90%, while the contribution from the male parent was 35 M reads, consisting of 43.01% GC contents and 84.26% Q30 value. The same sequenced data of 1366 M read from RILs in MDGM were used (Table 2b). The number of SLAF markers in male and female parents were 655,327 and 656,743 with an average sequencing depth of 107.84×. The SLAFs number in the RIL population was 329,812, with an average sequencing depth of 8.03-fold. The total number of high-quality SLAFs found was 790,026, of which 56,011 (7.09%) were polymorphic. Out of 56,011 polymorphic SLAFs, 33,003 were sorted into eight segregation patterns as above, and 19,064 markers were found with *aa* × *bb* segregation pattern, which were 56.76% of the total (Fig. 4). After

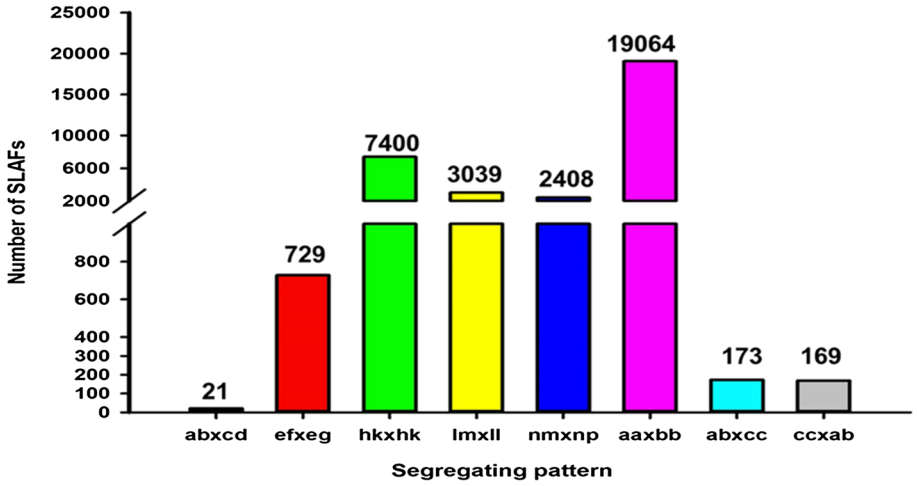

Fig. 4 SLAF marker distribution among different segregation patterns. The one with $aa \times bb$ type of SNPs which were 57.76% of the eight types of SNPs were used in linkage map construction

filtering non-qualified markers, the remaining 5022 SNP markers were used to construct the current HDGM.

Basic features of the constructed MDGM and HDGM

The MDGM consisted of 1975 SNP markers derived from a total of 214,002 SLAF tags and 4561 polymorphic markers. The 1975 SNP markers distributed throughout the genome covering all 20 LGs (Table S2a, Fig. S4). The total genetic map distance covered by these markers was 979.91 cM, with an average distance of 0.59 cM between adjacent markers. The largest among all 20 LGs was B05, on which 307 marker loci were mapped covering a total distance of 92.28 cM, with 0.30 cM as an average adjacent marker distance. LG A06 was the smallest among all LGs, with a 0.57 cM length and an average adjacent marker distance of 0.14 cM containing 4 marker loci. Throughout the map, the largest gap of 19.32 cM was found on LG B03 (Table 3).

To construct HDGM, a total of 790,026 SLAF tags were initially obtained, of which 56,011 were polymorphic distributed throughout the genome covering all 20 LGs (Table S2b, c; Fig. 5a, Fig. S5). After some quality filtration,

Table 3 Basic characteristics of 20 LGs in both genetic maps

LGs	SNP No.		Total distance (cM)		Avg. distance (cM)		Largest gap (cM)	
	MDGM	HDGM	MDGM	HDGM	MDGM	HDGM	MDGM	HDGM
A01	22	82	15.29	90.64	0.70	1.11	7.96	6.73
A02	72	177	25.45	100.38	0.35	0.57	3.6	15.07
A03	93	215	28.1	130.41	0.30	0.61	3.46	9.77
A04	50	193	44.13	95.67	0.88	0.50	6.11	4.35
A05	34	121	64.43	99.11	1.90	0.82	15.37	9.13
A06	4	20	0.57	50.52	0.14	2.53	0.19	10.06
A07	49	145	34.78	108.48	0.71	0.75	5.95	3.35
A08	34	61	20.43	52.93	0.60	0.87	2.58	3.66
A09	52	110	39.84	47.79	0.77	0.43	6.41	5.85
A10	107	226	95.14	96.02	0.89	0.42	15.32	3.24
B01	64	194	28.07	73.96	0.44	0.38	2.51	4.62
B02	102	419	52.52	137.55	0.51	0.33	3.7	3.4
B03	106	228	82.05	162.81	0.77	0.71	19.32	13.28
B04	244	464	126.77	246.18	0.52	0.53	3.58	6.61
B05	307	574	92.28	150.52	0.30	0.26	3.67	2.56
B06	19	45	13.32	24.1	0.70	0.54	4.63	4.21
B07	275	540	64.69	136.14	0.24	0.25	2.16	3.6
B08	26	503	7.19	103.27	0.28	0.21	2.72	13.22
B09	302	592	139.95	265.03	0.46	0.45	4	4.34
B10	13	113	4.91	59.74	0.38	0.53	4.91	4.62
Total	1975	5022	979.91	2231.25	0.59	0.64	19.32	15.07

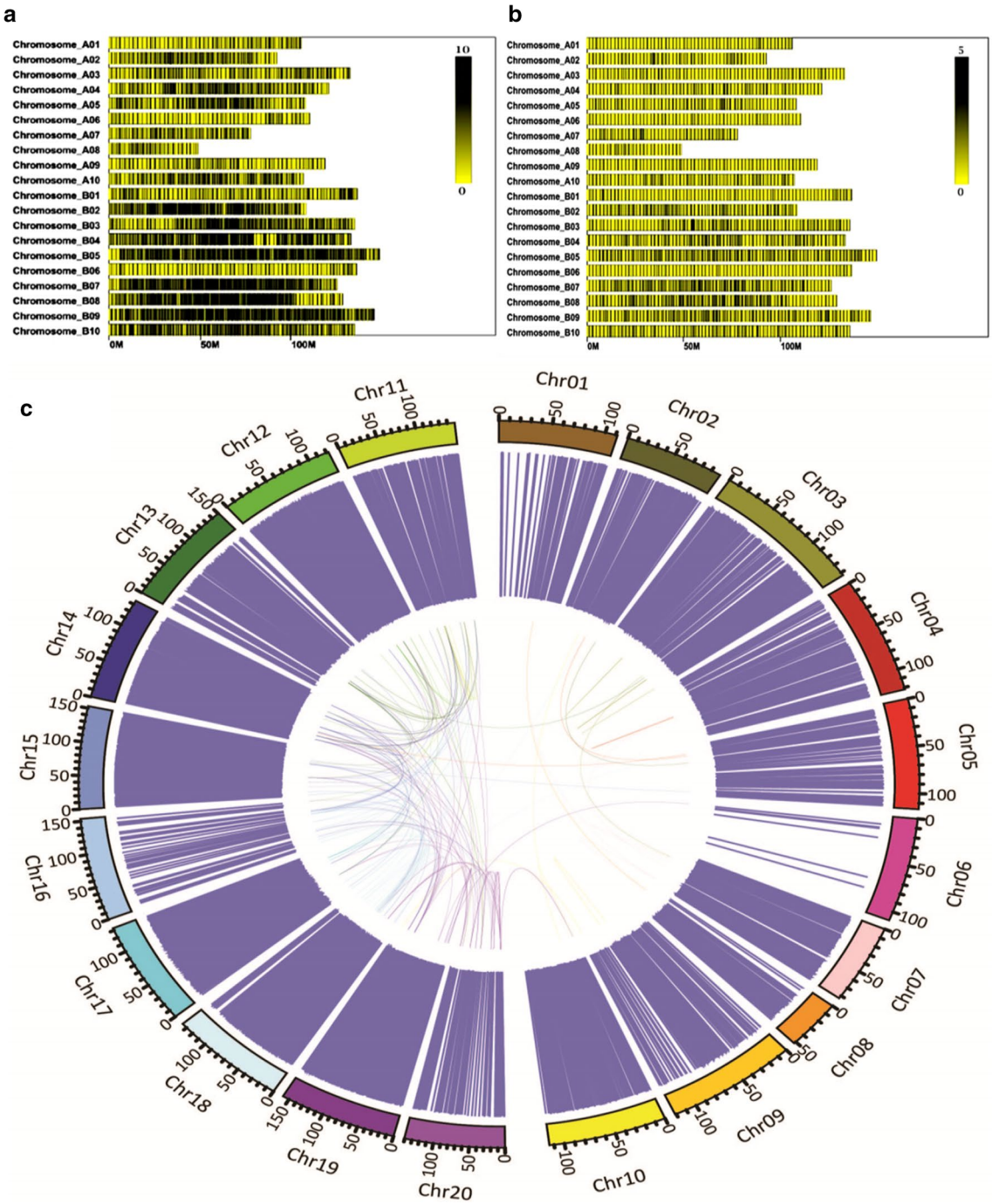

Fig. 5 SLAFs and SNPs distribution among the 20 LGs. Where **a**, **b** show SLAFs and SNPs distributions throughout the genome, respectively. Each yellow band represents a chromosome. The more the number of SLAFs and SNPs, the darker the color is. The **c** represents circos plot showing the distribution of SNPs. Different colored blocks represent the different LGs. The outer circle indicates chromosomes length and the inner circle shows physical positions of SNP makers. Fifty-eight out of 5022 SNP markers contain two or more repeats, which are linked with lines between and most of the repeat SNP markers located within the same subgenome (color figure online)

the final linkage map was consisted of 5022 SNP marker loci distributed throughout the genome covering all 20 LGs (Fig. 5b). The total genetic map distance covered by these marker loci was 2231.25 cM taking 2.3082 Gb (92.08%) of the total length of reference chromosomes (Zhuang et al. 2019; Table S2 d; Fig. 5c) and with an average distance of 0.64 cM between adjacent marker loci. By BLASTing, the reference genome using 5022 markers sequences, only 58 SNP markers contained 2–8 exact identical repeats with a total of 163 repeats, most of which located at the same

subgenome (Fig. 5c). The largest among all 20 LGs was B09, to which 592 marker loci were mapped covering a total distance of 265.03 cM with 0.45 cM an average adjacent marker distance. LG A06 was the smallest among all, with a length of 50.52 cM and an average adjacent marker distance of 2.53 cM containing 20 SNP marker loci. Throughout the map, the largest gap of 15.07 cM was found on LG A02 (Table 3).

QTL analysis of *A. flavus* resistance

For the first time, QTL mapping using the MDGM, mean phenotypic data in 2016 generated on 208 F_{10} RILs and genotypic data based on F_{10} population were used for QTL discovery. QTL analysis resulted in the identification of two QTLs, *qRAF-3-1* and *qRAF-14-1*, with PVE of 17.7 and 3.64% and LOD values of 9.66 and 2.41, respectively (Table 4a, Fig. S6a). QTL *qRAF-3-1* was located on LG A03 between Marker-7617 and Marker-7609 with 20.960–21.343 cM intervals, while QTL *qRAF-14-1* was located on LG B04 between Marker-1842 and Marker-1857 with an interval of 1.390–4.873 cM. The additive effect of *qRAF-3-1* was positive with 17.17% of PVE, while the additive effect of *qRAF-14-1* was negative with 3.64% contribution in PVE.

For confirmation and closely locating the identified QTLs, the HDGM of 5022 SNP markers obtained from genotypic data of 314 F_{10} RILs and phenotypic data in 2017 based on the 209 F_{10} RILs population were utilized. QTL analysis resulted in the confirmation of these two QTLs, *qRAF-3-1* and *qRAF-14-1*, with PVE of 19.04% and 5.15% on their respective chromosomes, A03 and B04. The LOD value of *qRAF-3-1* was 10.54 while 2.85 for *qRAF-14-1* with clear LOD peaks on respective locations (Table 4b, Fig. 6, S6b). QTL *qRAF-3-1* was located on LG A03 between Marker-8555604 at 111.9 cM, and Marker-8633509 at 113.6 cM. The second QTL on LG14, *qRAF-14-1*, was located between Marker-4230399 at 29.006 cM and Marker-4413338 at 34.460 cM. Due to the high PVE and LOD value, *qRAF-3-1* was regarded as a major effect QTL.

Putative candidate genes associated with *A. flavus* resistance

QTL *qRAF-3-1* located on LG A03 was flanked by marker-8555604 and marker-8633509, covering a 1.44 Mbp region. A total of 67 genes were found in this region coding for different proteins, including WRKY, ABC and TGA4 transcription factors, CDPK-related and shikimate kinase 1% 2C chloroplastic (CDPK), putative disease resistance RPP13-like protein 1 (RPP13), cytochrome P450 71B34, stress enhanced protein (SEP), E3 ubiquitin-protein ligase RKP (RKP), pectin lyase-like superfamily protein (PLLS), pentatricopeptide repeat-containing-like protein (PPR), tetratricopeptide repeat (TPR)-containing-like protein (TPR), heavy metal transport/detoxification superfamily

Table 4 Locations of currently mapped QTLs

QTL	LG	Position (cM)	Marker interval	LOD	Additive effect	PVE (%)
A						
qRAF-3-1	A03	20.960–21.343	Marker7617–Marker7609	9.66	7.52	17.17
qRAF-14-1	B04	1.390–4.873	Marker1842–Marker1857	2.41	−3.46	3.64
B						
qRAF-3-1	A03	111.902–113.575	Marker8555604–Marker8633509	10.54	7.94	19.04
qRAF-14-1	B04	29.006–34.460	Marker 4230399–Marker4413338	2.85	−4.13	5.15

A = QTLs mapped using MDGM, B = QTLs mapped using HDGM

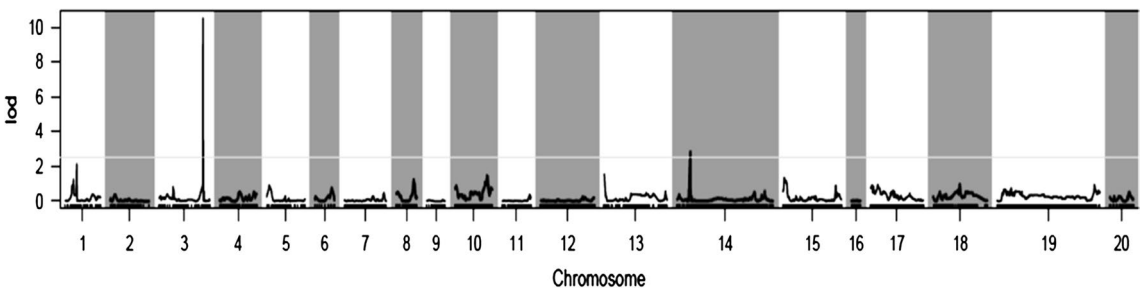

Fig. 6 LOD peaks of identified QTLs on respective chromosomes. The two QTLs located on Chromosome A03 and B04. Another QTL found on chromosome A01 but was below the desired LOD value

protein (HMT/DP), cupredoxin superfamily protein (CDSP), and lipase/lipoxygenase (Table S3b), etc. QTL *qRAF-14-1* located on LG B04 was flanked by marker-4230399 and marker-4413338, encompassing a physical region of 2.22 Mbp. In this region, a total of 137 genes were found. Interestingly, several genes of the same function, such as RPP13, RKP, PLLS, PPR, TPR and HMT/DP were found in the genomic regions of both QTLs, indicating that they are somewhat related. Other genes also found coding for proteins with functional similarities, like calcium-dependent protein, some transcription factors such as Zinc fingers and oxidases/peroxidases, etc. Besides, several important resistance-related genes like LRR receptor-like kinase protein, serine/threonine receptor kinases, LRR proteins GDSL esterase/lipase and copper transport protein family (CTPF) were also found in the QTLs harbored genomic regions (Table S3c).

Candidate genes response to *A. flavus* challenge

Resistance to *A. flavus* infection and aflatoxin production is a quantitative trait. To further characterize the regions, we determine the expression level of the putative candidate genes while the plants were challenged by drought with or without *A. flavus* infection along with control. Most of the identified candidate genes responded to *A. flavus* infection in the form of different expression levels compared with control. Among the putative candidate genes found in the region covered by the major QTL, *qRAF-3-1*, those coding for WRKY transcription factor (WRKY), CDSP, cytochrome P450 71B34, flavonoid 3′-monooxygenase (FM) and acyl-coenzyme A oxidase 2% 2C peroxisomal (AOP) were upregulated with 3.252-, 3.032-, 2.932-, 2.932- and 2.445-fold, respectively, when challenged by *A. flavus* under drought compared to those droughts only conditions. Under drought only condition, in the absence of *A. flavus*, genes coding for CDSP and protein NEN4 were found upregulated with 4.527- and 4.276-fold, respectively, compared with control (Table S3b).

Among the putative candidate genes found in the flanking region of QTL *qRAF-14-1*, the genes coding for CTPF, PPR, peroxidase 54, MATE efflux family protein LAL5 (MEFP) and S-adenosyl-L-methionine-dependent methyl transferases superfamily protein (SMTSP) were found upregulated with 6.062-, 5.756-, 4.747-, 4.598- and 3.290-fold, respectively, when challenged with *A. flavus* under drought stress compared with drought only. Substantial upregulation was observed in genes coding for PPR, peroxidase 54, MEFP, SAMDMTSP and RPP13 with 17.267-, 5.178-, 4.336-, 3.643- and 2.256-fold, respectively, under drought stress condition in the absence of *A. flavus* compared with control (Table S3c).

Discussion

Aflatoxin contamination of peanuts is a major food safety challenge, hazardous to both humans and livestock worldwide, affecting various biological pathways via interaction of epoxide with DNA and proteins. Aflatoxin exposure also negatively affects nutrition as well as the economy, social and political aspects particularly of poor people living in the developing countries. It is considered that aflatoxin causes aflatoxicosis and exists in two forms of acute intoxication, leading to liver damage and chronic subsymptomatic exposure (Krishnamachari et al. 1975). Currently, the global load of aflatoxin-driven hepatocellular carcinoma (HCC) is around 25%, most predominant in developing countries of the globe (Liu and Wu 2010; Valery et al. 2018). The best solution to cope with aflatoxin contamination is to equip the available elite cultivars with genetic resistance to aflatoxin. This study is among the pioneering work constructing an HDGM, mapping two resistant QTLs against *A. flavus* infection, and identifying candidate-resistant genes in cultivated peanuts. So far, only two studies available in cultivated peanut in this direction (Yu et al. 2019; Liang et al. 2009). The mapped QTLs and its harbored putative-resistant candidate genes in this study will provide a solid base for future genetic and genomic studies against *A. flavus* infection. Besides, the HDGM will provide great assistance mapping other important traits in peanuts.

HDGM via SLAF-seq for QTL mapping and breeding application

Genetic Linkage maps, especially HDGMs, are crucial tools for genomic and genetic studies, providing basic role in the genetic breeding of economically important crops. An HDGM provides a vital foundation for QTL mapping (Esteras et al. 2012; Petroli et al. 2012; Song et al. 2013), QTLs fine localization facilitates the genetic assistant selection and breeding for important traits in different crops. The scarcity of available molecular markers with low polymorphism hinders the construction of an HDGM in peanuts via conventional approaches. An alternative approach of genotyping by sequencing is a high throughput sequencing using SNP markers that unlock the doors for the efficient development of large numbers of markers in a very short time, generating enough polymorphic markers for an HDGM construction. The success in constructing HDGM in this study is mainly accredited to the mature pipeline of SLAF-seq technology, where comparatively large population size took it to the climax. Compared to other crops, the cultivated peanut is very young

from the perspective of genetic linkage map construction. The discovery of SSR markers in the twenty-first century made it possible to construct genetic linkage maps in the cultivated peanut (Cuc et al. 2008; Ferguson et al. 2004; He et al. 2003; Hopkins et al. 1999; Koilkonda et al. 2012; Luo et al. 2005; Proite et al. 2007; Zhao et al. 2012). Over 1000 polymorphic SSR markers have been established but its mapped number in populations of bi-parental crosses were limited to less than 200–400 marker loci, where the main hurdle was the narrow genetic base and large genome size of cultivated peanut (Hong et al. 2008; Qin et al. 2012; Varshney et al. 2009; Wang et al. 2012a). Efforts were made to combine genetic linkage maps of wild and cultivated peanut, a genetic linkage map containing 3693 loci was constructed (Shirasawa et al. 2013). Although the constructed genetic linkage map was comparatively denser than previous ones still this approach couldn't manage to fine map QTL of interest (Pandey et al. 2012, 2016; Varshney et al. 2013). In this respect, the development of SNP markers by SLAF-seq has offered great support for the construction of an HDGM and QTL/gene discovery. SLAF-seq is a new sequencing technology that has attained auspicious achievements in very limited time because of its highly automated nature based on high-throughput sequencing. This technology combines locus-specific amplification and high-throughput sequencing to effect de novo SNP discovery and large-scale genotyping. SLAF-seq is measured by sequencing the paired ends of the sequence-specific restriction fragment length, which ensures its better repeatability than its counterparts, such as restriction-site-associated DNA sequencing (RAD-seq) (Miller et al. 2007) and double digest RAD-seq (ddRAD-seq) (Peterson et al. 2012). SLAF-seq offers significant advantages, making it appropriate for an HDGM construction and QTL mapping in crops with complex genomes and low levels of polymorphism. Among the best features of SLAF-seq are high numbers of marker development with great accuracy and less sequencing depth compared to that of the past conventional sequencing techniques (Xie et al. 2010). SLAF-seq has been subjected to a sequence of critical trials to assure its high accuracy, efficiency and density. This approach has been effectively applied to an HDGM construction, QTL mapping and identification of candidate genes in several plants and animals species (Huang et al. 2013; Qi et al. 2014; Wei et al. 2014; Xu et al. 2015; Zhang et al. 2013, 2015). SNP markers are more useful among genetic markers due to their abundance and stability for the identification of genetic discrepancies in most genomes (Liu et al. 2012). Owing to the aptness of SLAF-seq using SNP markers to complex genomes, here we have sequenced a stable F_{10} RIL population, mapped QTLs and identified candidate genes in cultivated peanut with potential resistance to *A. flavus* infection.

The RIL population was derived from two highly contrasting parents, resistant XHXL and susceptible YY92 to *A. flavus* infection, which led to the construction of MDGM consisting of 1975 markers that were used to map the current two QTLs for the first time. Based on cautious scrutiny of the genomic GC content, repeat conditions and genome length of *A. duranensis and A. ipaensis*, HaeIII was selected for genomic DNA digestion. Then SLAFs within fragment size of 314–414 bp were selected in a pilot experiment for further paired-end sequencing. A pre-designed scheme and a pilot experiment were led to guarantee the density, uniformity and efficiency of the marker development. Then via deeper sequencing for the second time, an HDGM consisting of 5022 SNP markers was constructed. Using HDGM, the previously identified QTLs were confirmed and closely located. For HDGM construction, 19,064 SLAF markers were used, which were homozygous in both parents with more than 20-fold sequence depth and SLAF tag integrity over 70%. The constructed genetic map was consisted of 5022 SNP loci with a total map distance of 2231.25 cM and an average markers distance of 0.64 cM/loci covering all 20 LGs. BLASTing the reference genome with the 5022 markers sequences showed that they covered a physical length of 92.08% of total reference genome size and only 58 SNP markers had over two exact repeats in the genome, thereby could provide a complete QTL mapping. To our knowledge, the HDGM presented in this paper is the densest map to date for cultivated peanut following the map constructed by Agarwal et al. (2018) in cultivated peanut. The average distance attained in this map is the shortest yet reported, compared with previous similar studies conducted in cultivated peanut (Agarwal et al. 2018; Chen et al. 2017). Except LGs A01 and A06, in all other LGs, the inter-marker distances were less than 1 cM (Table 3), where larger gaps among markers affecting fine-mapping of QTL of interest. The shorter inter-marker distances are the indication of the density and quality of our constructed HDGM. Thereby, our results reveal that SLAF-seq is an efficient strategy for large-scale genotyping of plants with complex genome like cultivated peanut. Furthermore, SNP markers are sequence-tagged markers which are codominant in inheritance and are appropriate for comparative genomic studies (Luo et al. 2009), as well as association mapping (Chan et al. 2010; Cogan et al. 2006). The HDGM and SNP markers in this study provide mass markers and data for genes/QTLs fine-mapping, map-based gene cloning and molecular-breeding programs of cultivated peanut.

De novo QTLs and candidate-resistant genes for *A. flavus* resistance in peanut

The resistance to *A. flavus* infection is a complex trait influenced by numerous non-genetic factors including

water stress, temperature, plant physiologic status and density of microorganisms in the soil. The extensive phenotyping efforts of the large scale germplasm for multiple seasons acknowledged some lines with lowest *A. flavus* infection but still the problem is craving for stable solution. Host natural resistance is an indispensable trait for cost-effective and environment friendly approach to cope with the challenge of aflatoxin contamination. *A. flavus* is a facultative microbe that especially infects peanuts and corn, producing the most toxic B_1 and B_2 carcinogenic substances in both living and/or dead states. This greatly thwarts the solution of aflatoxin contamination (Pittet 1998; Reddy et al. 2010). The resistant phenotype to *A. flavus* demonstrates a quantitative nature, which should be controlled by several genes or loci with some effect. Through MDGM and HDGM with medium and high-density markers, we have mapped two novel QTLs, *qRAF-3-1* and *qRAF-14-1*, located on LGs A03 and B04, consistently. The PVE by these QTLs was 19.04% and 5.15%, respectively (Table 4). Comparing with the QTLs for *A. flavus* infection resistance identified in previous studies (Liang et al. 2009; Yu et al. 2019), the currently mapped QTLs are novel with significantly shorter QTL interval. QTL *qRAF-3-1* with positive additive effect was considered as major effect QTL, while *qRAF-14-1* was found with a negative additive effect for the trait of resistance to *A. flavus* infection. Showing opposite additive effects assumed that both mapped QTLs were not originated from the resistant parent (XHXL) and vice versa. Due to harbored high resistant genes, XHXL should be the donor of the main QTL, *qRAF-3-1*. Among the RILs, many lines displayed higher resistance than XHXL as well as higher susceptibility than YY92, demonstrating transgressive segregation in resistance (Fig. 2; Table S1). In the previous two studies on QTL mapping for resistance to *A. flavus* infection in cultivated peanut, one did not provide the specific position information based on data from a single environment (Liang et al. 2009), the other mapped the resistance on chromosome A03 and A10 of different genomic intervals compared to our study. We mapped the resistance on chromosome A03 at 123.80–125.24 bp, i.e., 1.44 Mbp, while in the previous study they located resistant QTL on chromosome A03 at 5.9–9.4 Mb (Yu et al. 2019). Furthermore, the genetic linkage maps used in both previous studies were lower dense maps of SSR markers, while our study was based on an HDGM constructed of SNP markers, which will provide vital assistance in future breeding programs. In the current study, 67 candidate-resistant genes (Table S3b) in the genomic region of major QTL *qRAF-3-1* were identified. These genes are putative candidate-resistant genes for *A. flavus* resistance. Till now, no other studies available with mapped QTLs in such a narrow region in cultivated peanut for *A. flavus* infection resistance.

Cloning QTLs affecting complex traits have remained the main challenge faced by plant breeders and molecular biologists. To tickle this issue via the classical approach of map-based cloning is very troublesome and time-consuming. Using NGS based on SNP markers and microarray analysis, we were able to identify candidate genes prevailing both mapped QTLs for the trait of *A. flavus* infection resistance in peanut. Mapping-resistant QTLs and identifying candidate genes of *A. flavus* infection resistance is the first step toward resistance enhancement. Referring to our sequenced genome for cultivated peanut (Zhuang et al. 2019), we have identified 67 and 137 candidate functional genes within the genomic regions of QTLs *qRAF-3-1* and *qRAF-14-1*, respectively. Interestingly, six genes among these candidates were located within both mapped QTLs. Including RPP13 reported to be involved in protection of plants against pathogens invasion via triggering a specific defense system (Bittner-Eddy et al. 2000), RKP, that mediates E2-dependent, 'Lys-48'- and/or 'Lys-63'-linked polyubiquitination of substrates and may play a role in diverse biological processes (Smith et al. 2013), PLLS, which are a group of enzymes contributing in numerous biological processes, including degradation of pectin (Cao 2012), PPR, required for the intergenic processing between chloroplast rsp7 and ndhB transcripts (Hashimoto et al. 2003), TPR, which can be observed in almost every kingdom of life, involved in regulating diverse biological processes including organelle targeting and biomineralization (Zeytuni and Zarivach 2012) and another important gene coding for HMT/DP were found in the genomic region of both mapped QTLs. The presence of genes related to plant development and stresses under both QTLs is an indication of their team work in response to *A. flavus* infection. Moreover, several other important candidate genes were identified related to *A. flavus* resistance within the genomic regions of mapped QTLs. Including lipoxygenase (Lox) identified in *qRAF-3-1* had been reported to catalyze the oxidation of polyunsaturated fatty acids, creating fatty-acid hydroperoxides (oxylipins), signal molecules regulating normal *A. flavus* growth and aflatoxin production (Gao et al. 2009; Scarpari et al. 2014). Many maize Lox genes have been identified in previous studies (Ogunola et al. 2017), and strong Lox expression activity was associated with higher resistance to *A. flavus* infection (Song et al. 2016). The transcription factor TGA4, reported playing a key role in the induction of systemic acquired resistance (SAR) and providing basal resistance (Kesarwani et al. 2007) was also found in the genomic regions of currently mapped QTLs. Other genes found coding for cytochrome P450 71B34, CDPK-related kinase 5, and WRKY known for the response to biotic and abiotic stresses (Jun et al. 2015; Xiao et al. 2017)

and disease resistance (Bakshi and Oelmüller 2014), were also identified in the genomic region covered by the major QTL *qRAF-3-1*. In the genomic region of QTL *qRAF-14-1*, several LRR receptor-like protein kinases genes were identified, involved in the interaction with a wide array of various processes including disease resistance and different stress responses (Goring and Walker 2004; Dufayard et al. 2017). Some peroxidase responding to various oxidative stresses and pathogen invasion (Birben et al. 2012) were also found linked with the resistance to *A. flavus* infection in peanut (Liang et al. 2009).

Resistant QTL to *A. flavus* infection is quite large and hosts tens and hundreds of candidate genes, thus making it challenging to incorporate such a large region into susceptible cultivars. An alternative is to check the expression of those genes upon *A. flavus* infection and pinpoint the highly responsive ones for later on breeding application. In the current study by using microarray analysis to validate the candidate genes, we found RPP13 showed significant response to *A. flavus* invasion in both QTLs, downregulated in *qRAF-3-1* while upregulated in *qRAF-14-1*, respectively (Fig. 7a, b, Table S3). PPR displayed a significant downregulation under drought plus *A. flavus* stress compared with the drought alone, where upregulation of 17-fold was noticed compared with control. Significant upregulation was noticed in genes coding for the WRKY, cytochrome P450 71B34 and CTPF in response to the *A. flavus* infection under drought stress compared with drought only condition (Fig. 7). Several resistance-related genes also responded to *A. flavus* and drought stress found either down- or upregulated as compared with the control condition (Fig. 7a, b, Table S3). Owing to the previously described important functions of genes found in the genomic regions of currently mapped QTLs and their significant responses to *A. flavus* challenge in current study, Genes coding for *Lox*, *RPP13*, and *PPR* may be the key candidates to deploy for further investigation. These candidate genes may be responsible for *A. flavus* resistance and phenotypic variation. Taken together, this was the first report for candidate genes discovery toward resistance enhancement to *A. flavus* infection in peanuts.

Conclusion

This study deployed SLAF-seq technology to construct an HDGM for the cultivated peanut which is the cost-effective sequencing strategy for large-scale genotyping in crops with complex genomes like cultivated peanut. Two QTLs

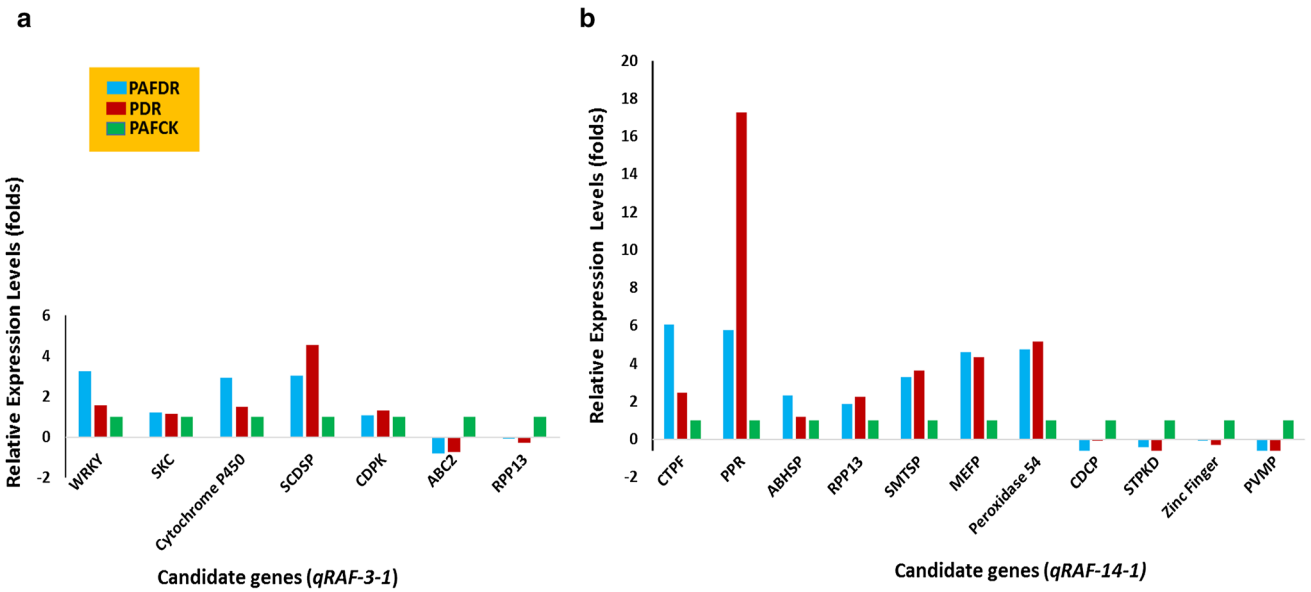

Fig. 7 In silico analysis of genes expression in genomic regions of the two QTLs under stresses of *A. flavus* + drought (PAFDR, blue), drought (PDR, red) and normal watering condition as a control (PAFCK, green), where **a** represents candidate genes expression found in the genomic region of QTL *qRAF-3-1* including WRKY (Probable WRKY transcription factor 51), SKC (Shikimate kinase 1% 2C chloroplastic), Cytochrome P450 (Cytochrome P450 71B34), SCDSP (cupredoxin superfamily protein), CDPK (CDPK-related kinase 5), ABC2 (ABC2 homologue13) and RPP13 (putative disease resistance RPP13-like protein 1), while **b** shows the candidate genes expression found in the genomic region of QTL *qRAF-14-1*, including CTPF (copper transport protein family), PPR (pentatricopeptide repeat-containing-like protein), ABHSP (alpha/beta-hydrolase super family protein), RPP13, SMTSP (S-adenosyle-L-methionine-dependent methyl transferase superfamily protein), MEFP (MATE efflux family protein LAL5), peroxidase 54, CDCP (cell division control protein 48 homologue C), STPKD (serine/threonine protein kinase D6PKL2), zinc finger (Zinc finger (CH4-type RING finger family protein) and PVMP (putative vesicle-associated membrane protein 726) (color figure online)

(*qRAF-3-1* and *qRAF-14-1*) against *A. flavus* infection were identified, confirmed and fine mapped to linkage groups A03 and B04 by comprehensive genetic analysis through phenotyping and genotyping. Of these QTLs, *qRAF-3-1* alone contributed more than 19% of the total PVE, potential genomic region to be used in molecular breeding using linked markers. The QTL region harbored important genes coding for different kinases, transcription factors and disease resistance. The identified resistant QTLs/genomic regions against *A. flavus* infection will facilitate further research on resistance gene cloning and aflatoxin-resistant cultivar development in cultivated peanut.

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Author contribution statement SAK and CH performed the research, generated data and wrote the manuscript. YC helped in experiment and resistance identification in 2016. ZC, DY and TC constructed the RIL mapping population used in the current research. NA and GM participated resistance evaluation and field experiment. GB and RKV contributed in data analysis and writing the manuscript. ZW designed the research project, supervised the experiments and modified the manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all contributing authors of the manuscript, the corresponding author states that there is no conflict of competing interest.

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