Genome-wide identification of *mlo* genes in the cultivated peanut (*Arachis hypogaea* L.)

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Abstract Powdery mildew disease caused by *Oidium arachidis* poses a threat to peanut production in Africa. Loss of function mutants of specific *Mlo* (Mildew Locus O) genes have provided broad-spectrum and durable resistance against pathogen in many crop species. Since there is huge potential to utilize susceptibility gene-mediated resistance in crop improvement, genome-wide mining of susceptibility genes is required for further researches. However, the susceptibility genes have not been characterized in peanut genome. In this research study, the genome of

the cultivated peanut was used as reference to identify the *AhMlo* loci. Our results revealed that 25 *AhMlo* loci were identified and distributed on the chromosomes of the cultivated peanut. Eleven *AhMlo* loci were located on the A-genome while the remaining 14 on the B-genome. Variable number of inserted intron sequences (4–14) and transmembrane helix (4–8) were observed in the coding sequence of the *AhMlo* loci. Furthermore, phylogenetic analysis of the *AhMlo* loci along with homologs from other species has clustered the *AhMlo* loci into six clades. Three *AhMlo* loci were clustered in the clade V known to regroup the powdery susceptibility loci in dicots. Additionally, four core promoters were predicted on the promoter region of the specific *AhMlo* along with *cis*-regulatory elements related to PM susceptibility. These results provided strong evidence of the identification and distribution of the *Mlo* loci in the cultivated peanut genome and the identified specific *AhMlo* loci can be used for loss of susceptibility study.

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Introduction

The resource of disease resistance genes (R) is essential prerequisite for development of resistant varieties

in plant. Much efforts have been made to exploit, clone, and transfer R genes in conventional resistant breeding programs. R genes encode proteins that recognize pathogen effectors to generate effector-triggered immunity system. The R-gene mediated disease resistance incorporated into elite cultivars provides an effective and eco-friendly approach for sustainable agriculture. Although R genes play a key role in effectively combating a diversity of pathogens and pests, they tend to have a narrow spectrum of pathogen recognition due to monoculture systems in modern agriculture (Langner et al. 2018). In addition, breeding of resistant varieties via introgression of R genes is time consuming, especially when R genes are linked to deleterious genes, requiring more generations to break the linkage drag (Berg et al. 2015; Yin and Qiu 2019).

Complementary to R genes, the exploitation of susceptibility genes (S) is an alternative source for disease resistance. S genes confer proteins that facilitate pathogen growth and suppress immune responses, or they can act as negative regulators of immunity (Pavan et al. 2010; Langner et al. 2018). Loss of function mutations in an S gene leads to durable, broad-spectrum, recessively inherited resistance. Pathogenesis is terminated by impaired S gene that fails to produce substances for pathogen growth. Identification of S genes and induction of mutants in S genes is becoming popular strategy for resistant breeding (Kusch and Panstruga 2017; Borrelli et al. 2018).

Powdery mildew (PM) is a widespread plant disease caused by obligate biotrophic fungi. As an airborne disease, it spreads easily and quickly, threatening crop plants and causing significant reduction in crop yield (Acevedo-Garcia et al. 2014; Murube et al. 2017). *Mildew locus O* (*Mlo*) genes are functionally negative regulated vesicle-associated actin-dependent defense pathways at the site of PM penetration (Pessina et al. 2016). *Mlo* genes encode proteins with seven transmembrane domains and a calmodulin-binding site and are conserved in monocots and dicots (Appiano et al. 2015) and have been phylogenetically divided into seven clades (Loieno et al. 2015). Monocot *Mlo* proteins in the clade IV were associated with PM susceptibility while dicot proteins sorted in the clade V (Polanco et al. 2018).

Powdery mildew disease caused by *Oidium arachidis* (Chorin) in peanut leads to a yield loss between

13 and 50% in Africa (Middleton et al. 1994). The sub-Saharan area is the largest peanut production region in Africa, including Nigeria producing 30% of total production in Africa, followed by Senegal at 8% and Ghana at 5% (OECD/FAO 2016). To reduce the loss of yield, fungicide application is the strategy to control powdery mildew disease. However, it could result in a negative impact on the environment and human health as well as increasing farming costs. The rapid evolution of fungal strains that can develop resistance to these fungicides is another worrisome aspect. Enhancing host resistance is the best sustainable approach to control the PM disease and beneficial to farmers and the entire peanut industry in Africa. The information on the resource of resistance to PM disease is limited in peanut. Therefore, the aim of this study was to localize all *Mlo* genes in the peanut genome and understand their gene structures and to identify specific *Mlo* genes that confer powdery mildew susceptibility. Identification of these specific *Mlo* susceptible genes would facilitate induction of mutagenesis for the loss of function through biotechnology, such as genome editing, which would prevent pathogen infection.

Materials and methods

Isolation and validation of the *mlo* genes in the cultivated peanut

Mlo DNA sequences were downloaded from the peanut genome assembly v1 for *Arachis hypogaea* var Tifrunner (http://www.peanutbase.org/gbrowse_peanut1.0). All sequences were used as query for amino acid sequence search using BLASTx tool from the NCBI database (<http://www.ncbi.nlm.nih.gov>). Prediction of TM helices of the putative AhMlo proteins was performed using TMHMM server 2.0 (<http://cbs.dtu.dk/services/TMHMM>).

Phylogenetic and gene structure analysis

Peanut *Mlo* protein sequences were aligned with the protein sequences of *Mlo* homologs from several legume species along with those from Arabidopsis and barley using the ClustalW method in MEGA7 (Kumar et al. 2016). The aligned sequences were used to generate a phylogenetic tree using neighbor-joining

method with 1000 times bootstrap replicates. All DNA sequences were also used for gene finding using soybean as genome-specific parameters in the software FGENESH-M (<http://linux1.softberry.com/berry.phtml?topic=fgenes%26group=programs%26subgroup=gfind>). Gene structure was visualized using Gene Structure Display Server GSDS2.0 (<http://gsds.cbi.pku.edu.cn>) (Hu et al. 2015).

Characterization of Mlo proteins

All exons identified from *AhMlo* DNA sequences belonged to the Clade V were subjected protein–protein BLAST (blastp) against NCBI protein datasets and those exons were aligned with their corresponding Mlo-like protein sequences to predict their locations in the protein structure. The upstream sequences (3 kb) of *AhMlo* in the clade V were predicted for core promoters from the link of BDGP (http://www.fruitfly.org/seq_tools/promoter.html) and identified for cis-acting regulatory elements (CRE) from New PLACE (Higo et al. 1999, <https://www.dna.affrc.go.jp/PLACE/?action=newplace>).

Results

Identification and distribution of *AhMlo* loci in peanut

To identify *Mlo* loci in the cultivated peanut, Mlo DNA sequences were searched from the reference genome of *A. hypogaea*. There were 107 homologous sequences related to Mlo. Among these sequences, some were at the same location but with different length of sequences, while some were same sequences but at different locations. After removed redundant sequences, a total of 34 *Mlo* locus sequences were used for analysis and 25 sequences were identified significantly homologous to Mlo-like protein by BLASTx searching against NCBI protein datasets. The predicted *AhMlo* loci were distributed on 14 chromosomes across the cultivated peanut genome (Supplemental file S1) and numbered 1–25 based on their chromosomal location (Fig. 1). These 25 *AhMlo* loci were hit to 7 Mlo-like proteins, like as *AhMlo*2, 7, 9, 18, 20, 23, 24 ascribed to Mlo1-like protein,

*AhMlo*4, 6, 12, 14 and 17 to Mlo4, *AhMlo*10, 16, and 21 to Mlo6, *AhMlo*3, 8, 13 and 19 to Mlo9, *AhMlo*1 and 25 to Mlo10, *AhMlo*11 and 22 to Mlo11, and *AhMlo*5 and 15 to Mlo13 (Table 1). Five *AhMlo* loci were located on the chromosome 8, three loci on chromosomes 14 and 17, two loci on chromosomes 4, 18 and 19, only one loci in each of the remaining chromosomes, and no Mlo loci were observed on chromosomes 6, 7, 9, 10, 12, and 16. Among the predicted loci, *AhMlo*7 and 8 clustered on chromosome 8 were separated by 30 kb without significant sequence similarity between two loci. Similarly, *AhMlo*18 and 19 clustered on chromosome 17 did not show sequence similarity although the distance between them was less than 60 kb. This indicated these clustered loci are not derived from tandem duplication events. Considering the A and B genomes, 11 *AhMlo* loci were identified from the A genome (chromosomes 1–10) while 14 loci belong to the B genome (chromosomes 11–20).

Cluster analysis of 25 *AhMlo* loci sequences resulted in two groups, in which corresponding MLO-like proteins 4 and 13 were presented only in one group and proteins 6, 10 and 11 formed in another group, while proteins 1 and 9 were included in both groups (Fig. 2). The TM helices detected using TMHMM server 2.0 showed that the number of TM domains varied from 5 in *AhMlo*9 to 8 in *AhMlo*1 and 25. Most Mlo-like proteins (17) had seven number of characteristic TM domains with three extracellular loops and three intracellular loops. There were four pairs of *AhMlo* locus that each pair corresponded to the same Mlo-like protein and the same number of TM domains was located in the corresponding pair of chromosomes of genome A and B. For instance, *AhMlo*3-*AhMlo*13 were located on chromosomes A3 and B3; *AhMlo*5-*AhMlo*15 on chromosomes A4 and B4; *AhMlo*6-*AhMlo*17 on chromosomes A5 and B5; *AhMlo*11-*AhMlo*22 on chromosomes A8 and B8, respectively, indicating they are homologous loci. Although *AhMlo*9 and *AhMlo*24 corresponded to the same protein as Mlo1, they possessed different number of TM domains and were located on chromosomes A8 and B9, suggesting they might be paralogous loci.

Fig. 1 Distribution of *AhMlo* loci across chromosomes in the cultivated peanut

Table 1 List of *AhMlo* loci and their corresponding Mlo-like proteins

Locus name	Chrom number	Gene ID	Locus location	Genbank accession	MLO-like protein	aa length	TM domains
AhMLO1	1	Arahy.FX71XI	12410103..12416163	XP_025696118.1	MLO10	569	8
AhMLO2	2	Arahy.T5DRVA	1468999..1475389	XP_025677222.1	MLO1	506	7
AhMLO3	3	Arahy.UZNM2C	22360366..22368270	XP_025687728.1	MLO9	594	7
AhMLO4	4	Arahy.GMF3XI	2909687..2913417	XP_025693651.1	MLO4	563	6
AhMLO5	4	Arahy.KKSD5Z	7498383..7502436	XP_025694173.1	MLO13	504	6
AhMLO6	5	Arahy.RCEZ9L	4675629..4679541	XP_025651700.1	MLO4	507	7
AhMLO7	8	Arahy.G3GLYO	4085358..4089080	XP_025612444.1	MLO1	558	7
AhMLO8	8	Arahy.ZD1HZA	4119346..4122883	XP_029144615.1	MLO9	503	7
AhMLO9	8	Arahy.PYV8LV	5088761..5094750	XP_025612522.1	MLO1	478	5
AhMLO10	8	Arahy.A9XXHW	28446004..28449295	XP_025616322.1	MLO6	529	7
AhMLO11	8	Arahy.W5M46W	39363386..39370571	XP_025614897.1	MLO11	559	7
AhMLO12	11	Arahy.3S7K56	16730385..16732166	XP_025693651.1	MLO4	563	6
AhMLO13	13	Arahy.HC6JW6	23672818..23680315	XP_025687728.1	MLO9	594	7
AhMLO14	14	Arahy.SY37LP	4223670..4227629	XP_025693651.1	MLO4	563	6
AhMLO15	14	Arahy.GX1WML	8971014..8974939	XP_025694173.1	MLO13	504	6
AhMLO16	14	Arahy.I7518D	57039852..57041928	XP_025647050.1	MLO6	573	7
AhMLO17	15	Arahy.8BXV5P	4673673..4677585	XP_025651700.1	MLO4	507	7
AhMLO18	17	Arahy.QBG8E9	52885233..52891148	XP_025612521.1	MLO1	515	7
AhMLO19	17	Arahy.ZZ7YX9	102932871..102936505	XP_029150517.1	MLO9	505	7
AhMLO20	17	Arahy.XPJ3VE	102993694..102998593	XP_025612444.1	MLO1	561	7
AhMLO21	18	Arahy.XB535B	4194515..4199944	XP_025616322.1	MLO6	529	7
AhMLO22	18	Arahy.XKDE21	24261540..24268641	XP_025614897.1	MLO11	559	7
AhMLO23	19	Arahy.TJ3DXD	10782040..10786812	XP_025678066.1	MLO1	501	7
AhMLO24	19	Arahy.73DSWE	10808014..10814928	XP_025677222.1	MLO1	507	7
AhMLO25	20	Arahy.ZL8L46	569206...574905	XP_025696118.1	MLO10	569	8

Gene structure and phylogenetic analysis

All *AhMlo* locus sequences were subjected for gene finding using FGENESH-M and GSDS tools. Gene structure analysis showed the structure differences

among *AhMlo* loci as depicted in Fig. 3. The number of exons varied from 4 (*AhMlo17*) to 14 (*AhMlo7*), while the majority (60%) of *AhMlo* loci harbored 9–13 exons. All *AhMlo* loci carried introns with a range of 4

Fig. 2 Groups of *AhMlo* loci with TM domains

Fig. 3 Structural representation of the *AhMlo* gene family in the cultivated peanut

(*AhMlo17*) to 14 (*AhMlo24*), and 25% of *AhMlo* loci retained 13 introns.

In order to predict specific *AhMlo* loci responsible for PM susceptibility, we performed a phylogenetic analysis using peanut Mlo-like protein sequences along with Mlo homologs of 6 legume species

(Supplemental file S2), including *Medicago truncatula* (Deshmukh et al. 2016), soybean (Shen et al. 2012; Deshmukh et al. 2014), Pigeonpea, chickpea, common bean, mungbean (Rispaal and Rubiales 2016), as well as Arabidopsis (Chen et al. 2006) and barley (Kusch et al. 2016). The phylogenetic tree based on the

multiple sequence alignment of Mlo proteins showed the presence of seven clades, in which seven peanut Mlo-like protein sequences were grouped in clade I and II, two in clade IV and VI, three in clade V, and non in clade VII (Fig. 4). As expected, most Mlo protein sequences from other species responsible for PM susceptibility were clustered in clade V, including *AtMlo2*, 6, 12, *MtMlo1*, *GmMlo6*, 12, *CaMlo2*, 6, *CcMlo1*, 3, *VrMlo1*, 2, 5, and *PvMlo6*, 12.

Characterization of peanut MLO proteins related to PM susceptibility

Three *AhMlo* loci (*AhMlo10*, 16, 21) in the clade V were homologous to Mlo-like protein 6 in peanut, where *AhMlo16* on chromosome B4 was homologous to XP_025647050 while *AhMlo10* and 21 on chromosomes A8 and B8 to XP_025616322. The protein sequences of XP_025647050 and XP_025616322 shared only 52.2% of identity, suggesting that they are different isoforms for

Mlo6 protein. In order to ascertain the important regions responsible for susceptibility in the Mlo protein, the exons of each locus were aligned with the Mlo6-like protein sequence to determine their locations on the structure of Mlo6 protein. In this study, *AhMlo16* contained 5 exons and four of them displayed significant similarity with Mlo6, while the fifth exon did not show homolog with any protein sequences in the NCBI protein datasets, indicating this locus may be truncated and be not used in the consequent analysis. Notably, 5 out of 10 exons in *AhMlo10* and 5 from 7 exons in *AhMlo21* were exactly same and identical to the protein sequences at different regions on the Mlo6 (XP_025616322). These five exons were located on extracellular loop 1, and intracellular loop 1 and 2. Other 5 exons from *AhMlo10* was pinned on intracellular loop 3, and calmodulin binding region, while the remaining 2 exons in *AhMlo21* located on intracellular loop 2 (Fig. 5).

Loss of susceptibility can be accomplished by disrupting the coding region of susceptibility gene,

Fig. 4 Phylogenetic tree analysis of the *AhMlo* along with MLO homologs from other crop species

Fig. 5 Schematic locations of exons identified from *AhMlo 10* and *21* loci in the Mlo6 protein sequence

also can be achieved through unique motif and regulatory element that mediate gene expression responsible for the susceptibility. To identify regulatory elements in the promoter region, 3,000 base sequence upstream of each locus were downloaded as putative promoter region. Five core promoters were predicted in *AhMlo10* and four in *AhMlo21*. The third and fourth core promoters in *AhMlo21* was identical with the second and third core promoters in *AhMlo10* with single SNP (A/T) and (T/G) variation, respectively. Identification of *cis*-acting regulatory elements (CREs) showed 101 CREs in *AhMlo10* and 110 CREs in *AhMlo21* (Table S1). Comparison of CREs between two loci suggested that the most abundant CREs were CAATBOX1 (ID:S000028), CACTFTPPCA1 (ID:S000449), and DFCOREZM (ID:S000265) in both putative promoter regions, which was consistent with the observation from other plant species (Andolfo et al. 2019). These CREs were recorded 39 and 56, 21 and 33, 21 and 30 times out of total of CREs in *AhMlo10* and *21*, respectively. In addition, we observed TC-box and Thymine rich motifs located at distal promoter of both loci (over 1.5 kb upstream of the transcriptional start site), which were presumed for PM susceptibility in different plant species (Shen et al. 2012; Andolfo et al. 2019).

Discussion

AhMlo loci distribution in the cultivated peanut

Taking the advantage of reference genome sequences of the cultivated peanut, we downloaded DNA sequences of *Mlo* loci and searched for their corresponding Mlo-like proteins by BLAST. Twenty-five *AhMlo* loci were identified and distributed in 14 of the

20 *A. hypogaea* chromosomes. The previous studies have reported that the number of Mlo homologs was varied from 13 to 20 in different legume species exception of 39 in soybean (Deshmukh et al. 2014, 2016; Rispail and Rubiales 2016) demonstrated that 14 potential peanut Mlo sequences were retrieved from the reference sequences of two wild species, *A. duranensis* and *A. ipaensis*, by BLAST using the *M. truncatula* Mlo CDS and protein sequences. The cultivated peanut reference genome sequences were not available until in 2019, the different number of Mlo sequences from the cultivated and wild species may indicate that gene content in the cultivated peanut genome has been expanded after tetraploidization. Zhuang et al. (2019) also reported that the B subgenome in the cultivated peanut showed 12.46% expansion in gene content compared with B genome in *A. ipaensis*.

Identification and characterization of specific mlo for PM susceptibility

The specific *Mlo* loci related to PM susceptibility function as a negative regulator of hypersensitive response upon PM infection in plant. Loss of function mutations in specific *Mlo* genes confer a broad-spectrum and durable disease resistance in various crops. Thus, genome-wide identification of susceptibility genes is a prerequisite for loss of function studies. In this study, two *AhMlo* loci were clustered with Mlo gene members from other plant species responsible for the susceptibility in the clade V. These two *AhMlo* loci were located on chromosome A8 and B8, respectively, as being homologous to the same Mlo-like protein 6, indicating they are homeologous loci. By aligning the exons identified from two *AhMlo* homeologous loci with Mlo6 protein, five exons were

same in both loci and pinpointed in extracellular loop 1, intracellular loop 1 and 2. They might be the intracellular domains responsible for the PM susceptibility. However, *AhMlo21* may have gone through an intragenic recombination event after the tetraploidization resulting in two additional exons in the intron region and the missing of five exons as identified in *AhMlo10*. Such recombination can result in deletion, duplication, inversion and fusion leading to a loss of function of the gene. A further function study by induction of mutations in *AhMlo21* will validate whether *AhMlo21* is a pseudogene. Nevertheless, each of these exons can be a target for disruption of these *AhMlo* loci to provide the basis for functional studies in peanut. Reinstadler et al. (2010) pointed out that the second and third cytoplasmic loops of Mlo protein are critical for the susceptibility-conferring activity of the Mlo protein in barley. The calmodulin binding region was identified as an important Ca^{2+} signal response motif that mediates defense reactions against powdery mildew (Kim et al. 2002). All these domains can be useful in the loss of susceptibility for the resistant breeding.

Many research studies have focused on the development of resistant lines for resistance to PM disease by targeting the Mlo homologs in the clade IV and V for monocots and dicots respectively. However, loss of function of all homologs in clade IV and V did not result in resistance to the PM disease. For instance, three tomato SIMLO were identified in clade V, but only one MLO (SIMLO1) line was resistant to PM disease by the loss of function (Andolfo et al. 2019; Zheng et al. 2016). Furthermore, four grapevine MLO (*VvMLO6*, 7, 11 and 13) were identified in clade V, however, silencing *VvMLO7* in combination with 6 and 11 reduced PM severity exception of *VvMLO13* (Pessina et al. 2016). Two *AhMlo* were grouped in clade V in this study; therefore, these two *AhMlo* loci can be targeted for the loss of function study. By analyzing the level of expression of these *AhMlo* can determine which *AhMlo* is playing an important function in PM susceptibility upon pathogen infection.

Even though the molecular mechanisms modulating the expression of *MLO* susceptibility genes are not well understood, by using “In Silico” study, Andolfo et al. (2019) have shown the conservation of two motifs among putative promoter regions of the MLO homologs of clade V related to PM susceptibility (Andolfo et al. 2019). In this study, in addition to the

coding regions, defense responsive cis-regulatory elements, TC-box and Thymine rich, were identified in the promoter region of the two *AhMlo*. These defense related CREs can be alternative targets for manipulation of gene expression. CRISPR/Cas9 approaches can be used to induce mutations in the coding and promoter regions to interfere with the compatibility between the host and the pathogens and provide durable disease resistance (Zaidi et al. 2018). For instance, disruption of promoter and coding regions of SWEET genes that encode sugar transporters using TALENs and CRISPR/Cas9 system has provided enhanced resistance to rice bacterial blight (Blanvillain-Baufume et al. 2017). Mutations induced in *Mlo* gene in tomato and *enhanced disease resistance 1 (EDR1)* in wheat, another PM-susceptibility gene, using CRISPR/Cas9 resulted in significant reduction of PM (Nekrasov et al. 2017; Zhang et al. 2017). The findings in this study provide a useful information on *Mlo* genes in peanut and suggest potential utilization of susceptibility genes for resistant breeding in peanut.

Conclusions

Susceptibility genes are favorite for pathogen growth by providing substances to pathogen or acting as negative regulators of immunity. Disruption of susceptibility genes can improve plant capability of resistance to pathogens as demonstrated in many plant species using specific *Mlo* genes. Therefore, susceptibility gene-mediated resistance is supplemental benefits in plant resistance breeding. The information on *Mlo* genes in peanut, especially specific *Mlo* genes for susceptibility of powdery mildew, is limited. In this study, 25 *AhMlo* loci were identified, two of which were putative specific genes for the susceptibility that can be used for loss of susceptibility study and resistance breeding in peanut.

Author contributions GH conceptualized the idea for the manuscript. ST, SH, and WX supported in bioinformatics. PB, XC, and FL analyzed the data. GH and ST wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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