Construction of a High-density Genetic Linkage Map and QTL Analysis of Resistance to Cucumber Mosaic Virus in *Luffa cylindrica* (L.) Roem. Based on Specific Length Amplified Fragment Sequencing

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Research article

Keywords: *Luffa cylindrica* L., Cucumber mosaic virus (CMV), high-density genetic map, SNPs, QTL analysis

Posted Date: October 10th, 2019

DOI: <https://doi.org/10.21203/rs.2.15852/v1>

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Abstract

Background: *Luffa cylindrica* L. is an economically important vegetable crop that is consumed globally. Cucumber mosaic virus (CMV) is an important virus affecting *Luffa* spp. No specific high-density maps have been constructed owing to a lack of efficient markers. Furthermore, no genes or quantitative trait loci (QTLs) are reportedly responsible for CMV resistance in *Luffa* spp. The development of next-generation sequencing has enabled discovery of single nucleotide polymorphisms and high-throughput genotyping of large populations.

Results: A total of 271.01 Mb pair-end reads were generated. The average sequencing depth was 86.19× in both maternal and parental lines, and 14.57× in each F₂ individual. When filtering low-depth specific locus amplified fragment (SLAF) tags, 100,077 high-quality SLAFs were detected, and 7,405 of them were polymorphic. Finally, 3,701 of the polymorphic markers were selected for genetic map construction, and 13 linkage groups were generated. The map spanned 1,518.56 cM with an average distance of 0.41 cM between adjacent markers. Our results also revealed that CMV resistance was regulated by QTLs. Based on the newly constructed high-density map, two loci located on chromosome 1 (100.072 ~ 100.457 cM) and 4 (42.475 ~ 44.398 cM) were identified to regulate CMV resistance in *L. cylindrica*. A gag-polypeptide of LTR copia-type retrotransposon was predicted as the candidate gene responsible for CMV resistance in *L. cylindrica*. **Conclusions:** A high-density linkage map of *L. cylindrica* was constructed using SLAF. QTL mapping based on CMV disease phenotypes of F₂ led to the identification of two QTL on chromosome 1 and 4, respectively. Kompetitive allele-specific PCR analysis of 60 F₂ individuals, which gave rise to F₂:3 individuals, was carried out. We found that the QTL on chromosome 1 was associated with CMV resistance. Mapping of CMV QTL combined with the transcriptomic sequence alignment identified a gag-polypeptide of LTR copia-type retrotransposon as the most likely causal gene.

Background

Luffa spp. (2n = 26), which belongs to the family Cucurbitaceae, is widely cultivated in tropical and subtropical regions, such as China, Malaysia, India, Korea, Thailand, Central America, and Africa [1, 2]. Among the nine *Luffa* species found worldwide, only *Luffa cylindrica* (L.) Roem. and *L. acutangula* (L.) Roxb. are domesticated [2, 3]. Furthermore, *L. cylindrica* has always been a preferred crop for cultivation because of its higher fruit set and bigger fruit size [4]. This species is grown principally for production of sponges, which can be used as bath sponges, or as an alternative biomaterial used for packaging, furniture, and plastic appliances, and also as an adsorbent of heavy metal contaminants from industrial waters, which makes it a cash crop [4–6]. The immature fruits are used as a vegetable and are rich in various nutrients [7]. Its medicinal value has also been reported [8, 9].

High-density genetic linkage maps play an important role in genome assembly, gene/ quantitative trait locus (QTL), fine mapping, and map-based gene cloning. One major requisite for high-density genetic map construction is the availability of a huge amount number of polymorphic markers. In *Luffa* spp., research concerning genetic linkage maps has only started recently, using sequence related amplified polymorphism (SRAP) markers. Cui et al. (2015) constructed an interspecific genetic linkage map using an F₂ population derived from a cross between *L. acutangula* (L.) Roxb. and *L. cylindrica* (L.) Roem [10]. Although this map was the first genetic linkage map of *Luffa* spp, 24 linkage groups more than 13 chromosomes were construct in Cui's study. Wu et al. (2016) constructed a more condensed interspecific genetic map, which consists of 177 EST-SSR markers distributed in 14 linkage groups (LG) with an average of 102.58 cM per LG [11]. Due to interspecific hybridization, there was a marked segregation distortion of molecular markers in this map. The markers were not well-distributed over the LGs. To our knowledge, based on *L. cylindrica* (L.) Roem genetic background, no higher-density genetic maps have been constructed with markers distributed evenly in the 13 chromosomes. The lack of abundant, stable, and polymorphism molecular markers, such as single nucleotide polymorphisms (SNPs), hampers the construction of high-density genetic maps.

With the advent of next-generation sequencing (NGS) technology, such as specific locus amplified fragment sequencing (SLAF-seq), it is now feasible to develop SNP markers on a large scale, which is useful for constructing a genetic map. With various advantages, such as its high-throughput, high accuracy, and low-cost, this technique has been employed for high-density genetic map construction in many species, such as common carp [12], sesame [13], soybean [14, 15], *Prunus mume* Sieb. et Zucc [16], cucumber [17, 18], wax gourd [19], and sweet osmanthus [20]. Moreover, SLAF-seq based genotyping does not depend on a reference genome sequence, which makes it particularly important for species without a sequenced genome, such as *Luffa* spp.

Cucumber mosaic virus (CMV) belongs to the Cucumovirus genus of the family Bromoviridae, which can be transmitted via >1200 plants and 60 aphid species [21]. Although several genotypes of *L. acutangula* have been found to be resistant to CMV [22], the genetic mechanism of their resistance is still unclear. In our study, SLAF-seq was employed to construct an SNP-based genetic map for *L. cylindrica* (L.), which spanned 1,518.56 cM, with an average of 0.41 cM among adjacent markers, and consisted 3,701 SNP markers distributed on 13 linkage groups. In addition, the QTLs associated with CMV resistance were mapped by using this genetic map, certified the usefulness of it. Furthermore, a gag-polypeptide of LTR copia-type retrotransposon as a candidate gene for such QTL was also predicted in this study. To our knowledge, this is the most high-density genetic map of *Luffa* spp. to date. The SNP markers and the genetic map reported in this study could prove to be valuable resources for future research. This is also the first report on detection of CMV resistance-associated QTLs and prediction of related candidate genes in *L. cylindrica* (L.).

Results

SLAF sequencing and genotyping

SLAF-seq is an efficient approach for large-scale genotyping, which is based on high-throughput sequencing technologies, and which could be applicable to various species and populations. DNA was isolated from parental genotypes 'P1–21' and 'P2–16', and 130 F₂ individuals generated by a cross between these genotypes, and used to create SLAF libraries. SLAF-seq yielded 271.01 M clean, trimmed 100 bp paired-end reads, among which the guanine-cytosine (GC) content was 39.58% and Q30 ratio (the ratio of reads with a quality score of >30) was 89.16%.

All SLAF pair-end reads were clustered based on sequence similarity, and sequences with over 90% identity were grouped as one SLAF locus. Table 1 shows an overview of the average degree of SLAFs and the SLAF sequencing depth in both parental lines and the F₂ population. SLAFs containing more than four tags and sequence depth less than 213 were filtered out as repetitive and low-depth SLAFs, respectively. Only the SLAFs with 2–4 tags were identified as polymorphic SLAFs. Finally, in this study, a total of 100,077 high-quality SLAFs were detected, among which 14,918 were polymorphic (14.91%) (Additional file 1). After filtering out markers lacking parental locus information among the polymorphic SLAFs, 11,788 markers were screened for genetic construction. Following the genotype encoding rule, the 11,788 SLAFs were classified into eight segregation patterns (ab × cd, ef × eg, hk × hk, lm × ll, nn × np, aa × bb, ab × cc, and cc × ab) (Fig. 1). Due to the homozygous genetic background of the two parental lines (P1–21 and P2–16), aa × bb segregation pattern was needed to follow.

There were 7,404 of 11,788 polymorphic SLAF markers followed the aa × bb segregation pattern, and were further selected to construct the linkage map (Fig. 1). While SLAFs markers with parental sequence depth of less than 10×, completeness less than 70%, and/or significant segregation distortion (p-value <0.001), were considered as low-quality and should be filtered. Then, 3,701 markers were finally selected for genetic map construction.

High-throughput linkage map construction and evaluation

After High Map linkage analysis (Liu et al., 2014), the 3,701 markers were mapped on 13 linkage groups (LGs) (Table 2, Fig. 2, Additional file 2). The average integrity of the mapped markers was 99.18% (Additional file 3). Detailed information of this genetic map is presented in Additional file 4 and Table 2.

The map consisted a total of 1,518.56 cM with an average distance of 0.41 cM among adjacent markers. The biggest and smallest LG was LG1 and LG10, which contained 380 and 98 markers, respectively. On average, there were 285 SLAF markers per LG. The length of individual LGs varies from 143.28 cM (LG 9) to 91.71 cM (LG 8), with the average intermarker distance ranging from 0.30 cM (LG 7) to 1.16 cM (LG 10). Furthermore, the maximum distance between markers among the 13 LGs ranged from 2.77 cM (LG 7) to 4.86 cM (LG 10) (Table 2).

Haplotype and heat maps were used to evaluate the quality of the newly constructed genetic map. The double crossovers in the mapping population showing in haplotype maps indicated the errors in genotyping. the relationship of recombination among markers on the same linkage group was reflected in the heat map, and can be used to find potential sorting errors of markers.

By using 3,701 SLAF markers, haplotype maps were generated for each of the F₂ individuals and the parental controls, which can intuitively display the recombination events within each individual as previously described [23] (Additional file 5). In this study, most of the recombination blocks were clearly defined and the percentage of double crossovers in all of the linkage groups was less than 0.18% (Table 3), which was lower than the describe of usually less than 3% [19]. Heat maps were also generated by using pair-wise recombination values for the 3,701 SLAF markers (Additional file 6). The SLAF markers in most LGs were well ordered.

QTL mapping and validation of SNP markers using KASP assay

The parental line P2–16 showed resistance to the CMV isolate, characterized by only subtle symptoms after CMV infection. Most P2–16 plants scored 1 or 3 and only one out of ten scored 0 (average 1.3). Most P1–21 plants scored 7 and only one got a score of 5 and other two plants got 9 (average 7.2). All F₁ plants scored 3 or 5 (average 4.4) (Fig. 3a). The disease rating of parental lines and their F₁ progeny suggested that the resistance to CMV in P2–16 might be incompletely dominant. In the segregating F₂ population, plants that were neither completely immune (disease score 0) nor severely infected (disease score 9) were found. The disease grades of F₂ plants were more or less equally distributed over 1, 5, and 7, whereas a slightly lower number of individuals scored 3 (Fig. 3b). If we consider plants with disease grade 1 as resistant, grades 3 and 5 as intermediately resistant, and grade 7 as susceptible, then among the 130 F₂ plants, 34 individuals were resistant to CMV, 61 were intermediately resistant, and 35 were susceptible ($\chi^2 = 0.507$ for 1R:2MR:1S). Therefore, we concluded that the resistance of genotype P2–16 to CMV cannot be explained by one single resistance gene. Sixty F₂ plants, including 30 resistant (29 plants scored as 1, and one as 3) and 30 susceptible plants (all scored 7), were chosen to produce F_{2:3} progenies. When we compared the disease index of F_{2:3} progenies generated from the resistant and susceptible F₂ groups, we found that they were extremely significant, which indicated that the disease index of F_{2:3} progenies corresponded to the disease score of their F₂ plants. The disease rating in the F_{2:3} populations from 60 F₂ single plants also showed a lower distribution on disease indices 33.4 ~ 55.6%, suggesting that the resistance to CMV in P2–16 was regulated by multiple quantitative genes (Fig. 3c).

Based on the high-density genetic map and phenotypic data of CMV resistance in the F₂ population, QTL mapping of CMV resistance was performed by Join Map 4.0 with the Kosambi mapping function. The threshold of significance ($P = 0.05$) for each marker after 1,000 permutations was set to 5.0 and based on logarithm of odds (LOD) 3.0 intervals, which identified two loci at 100.072 ~ 100.457 cM and 42.475 ~ 44.398 cM on LG 1 and LG 4, respectively (Fig. 4, Additional file 7). On LG 1, the genetic distance of markers in the flanking regions of CMV resistance loci included three markers at 100.072 cM and 27 markers at 100.457 cM. (Additional file 7). The estimated proportion of phenotypic variance explained by the locus at LG1 was 7.48–8.41%. On LG 4, only one marker was significantly linked to CMV resistance, explaining 9.75% of the phenotypic variance (Additional file 7).

To validate the QTL identified by SLAF-seq, a kompetitive allele-specific PCR (KASP) assay was used for genotyping. All the SLAF markers in the two loci intervals were considered to develop KASP marker. However, due to the lack of genomic database, not all the SLAF marker sequences were suitable to design KASP markers. Finally, only six significant markers on LG 1 for the KASP assay were identified successfully (Table 4 and 5, Fig 5). As to the loci on LG 4, sequences were unable to reveal KASP markers. The disease index from the F_{2:3} populations was used to represent the phenotypic values of individual F₂ plants in KASP analysis. All the KASP markers at 100.457 cM on LG 1 were significantly associated with CMV resistance in the 60 extremely resistant and susceptible F₂ plants with the phenotypic values from their pedigrees (Table 5).

Transcript sequencing and candidate gene prediction

To predict which genes were related to CMV resistance in *L. cylindrica*, a transcript sequencing database was built using the parental lines after CMV inoculation. The interval of marker sequences on LG1 (98.534 ~119.689 cM) and LG4 (42.475~54.66 cM) were chosen to conduct BLAST searches, including the markers most significantly linked to CMV resistance. The candidate genes were identified by alignment searches of mapped QTL marker sequences against the translated *L. cylindrica* reference database (Supplementary data sheet 4). By pfam, Swissprot, and nr annotation, zinc-binding in reverse transcriptase, retrovirus-related Pol polyprotein from transposon, probable disease resistance protein, serine hydrolase (FSH1), serine/arginine-rich splicing factor, and histidine kinase responded to the CMV inoculation in *L. cylindrica*. Retrovirus-related Pol polyprotein (gag-polypeptide of LTR copia-type; gag-pre-integrase domain) was most frequently identified in this study (Additional file 8).

Expression of the identified *L. cylindrica* defense gene annotation in the RNA-seq dataset was analyzed between treatments within each genotype and between genotypes within both treatment groups. The gene predicted by three markers (Marker 33125 on 98.534, Marker 18364 on 100.457 cM, and Marker 921 on 106.611cM) on LG1 was downregulated in CMV-inoculated plants in both susceptible and resistant parents, with no difference between genotypes during disease development. Marker 31030, marker 36013, and marker 42619 were downregulated in susceptible parent plants only at 2 days after CMV inoculation, when compared to the plants without inoculation; in resistant parental plants, marker 31030 and marker 36013 were downregulated comparing R0 with R6, while both were downregulated after inoculation, and they were observed to be downregulated when comparing R0 with R2, and upregulated when comparing R2 with R6. There was no significant difference between parental lines among both marker 31030 and marker 36013, while marker 42619 was upregulated. For marker 68384, there were no changes in expression in the susceptible plants, but a downregulation was observed in CMV-resistant plants. For marker 65023, no changes in expression were observed between treatments within each genotype or between genotypes within both treatment groups (Additional file 8). Due to lack of expression data, we did not analyze the other markers on LG1. Only markers on 100.457 cM were significant to CMV resistance in this interval. Although markers in interval 42.475~54.66 cM on LG4 were annotated to retrovirus-related Pol polyprotein, only marker 52122 was present on the QTL mapping interval. Due to inadequate expression data, the prediction on LG4 was not convincing.

Discussion

Saturated SNP-based high-density genetic linkage map in *Luffa*

Genetic linkage maps are useful tools for fine mapping and map-based cloning [17, 24, 25]. Two interspecific genetic maps have previously been constructed in *Luffa* [10,11]. Cui et al. (2015) reported the first sponge gourd genetic linkage map, which was constructed using SRAP markers [10]. Due to the low accuracy of SRAP markers, this map was not efficient to be used in the subsequent study of genes or QTL mapping. Whereas there are 13 pairs of chromosomes in *Luffa*, the genetic linkage map constructed by Cui et al. (2015) contained 24 small linkage groups, due to segregation distortion of molecular markers caused by the interspecific hybridization [10].

By using more accurate and stable extended sequence tag-simple sequence repeats (EST-SSR) markers through transcriptome sequencing, Wu et al. (2016) constructed a more comprehensive interspecific *Luffa* genetic linkage map with 177 EST-SSR markers distributed on 14 linkage groups [11]. Although the fact that an interspecific *Luffa* cross was used for this map ensures a wide genetic diversity, the distant relationship between the two parental lines and subsequent high level of segregation distortion caused an uneven marker distribution on the chromosome. Some linkage groups only contain a limited number of markers, hindering the utilization of this map in map-based cloning.

Some studies have indicated that genetic diversity within single *Luffa* species is narrow [26]. This situation would cause a low polymorphism rate for molecular markers, which then makes it difficult to construct a high-density genetic map in *Luffa cylindrica*. With the rapid development of sequencing technology, SLAF-seq is a good strategy for high-throughput SNP discovery and genotyping, especially for species for which there is no reference genome available, such as *Luffa* spp.

In the present study, we employed SLAF sequencing technology to obtain novel markers in *Luffa cylindrica* for linkage map construction and QTL mapping. In total, 271.01 Mb pair-end reads were generated, which corresponded to 100,077 high-quality SLAFs. Among them, 7,405 of SLAFs were polymorphic (7.4%). Eventually, 3,701 polymorphic markers were used to construct a genetic map, which contained 13 LGs. This map spanned 1,518.56 cM with an average distance of 0.41 cM between adjacent markers.

Cui et al. (2015) [10] and Wu et al. (2016) [11] used interspecific *Luffa* spp. crosses for their genetic maps, whereas we used an intraspecific *Luffa cylindrica* F₂ mapping population in our study, which avoided the marked segregation distortion of molecular markers in interspecific hybridization populations. Furthermore, we were able to select more polymorphic markers with high accuracy and stability, distributed uniformly over the whole genome. To our knowledge, this is the first high-density genetic linkage map in *Luffa cylindrica*. This could be a useful starting point for further genetic studies on this plant species.

QTL analysis of CMV resistance in *Luffa*

CMV causes serious diseases in a broad range of host plants globally. It is difficult to eradicate CMV by pesticides. Using CMV-resistant crop varieties is an economical and durable approach to defend against this virus. CMV is a very complex disease, symptoms of which can be affected by plant age, virus strain, environment, and inoculation methods, complicating the evaluation of promising genetic materials [27]. The study on CMV resistance in *Luffa* spp. is at its early stages. Rani et al. recently reported 12 genotypes of ridge gourd [*L. acutangula* (L.) Roxb], which were found to be resistant to CMV, being completely symptomless after inoculation [22]. To our knowledge, this is the only study on CMV resistance in *Luffa* spp.

The genetics of resistance to CMV are varied among species, comprising dominant, recessive, monogenic, and polygenic genes or QTLs. Monogenic dominant resistance genes have been identified, such as RCY in *Arabidopsis thaliana* C-24 [28], RT4-4 in common bean [29], and *Cmr 1* in pepper [30]. In *Arabidopsis*, two independent recessive genes (*cum1* and *cum2*) were also identified to be involved in translation and movement of the CMV virus [31,32]. CMV resistance in chili pepper (*Capsicum annuum* L.) was also found to be regulated by multiple genes and/or QTLs in pepper [33–35]. In potato, CMV resistance was reported to be regulated by one major QTL and several minor QTLs [36]. Among cucurbits, like cucumber, a study of the genetics of resistance to CMV in *Cucumis sativus* var. *hardwickii* revealed that it was regulated by a single recessive gene [37]. In melon, resistance to CMV in the Korean accession PI161375 (SC) was initially described as oligogenic, recessive [38], and quantitative [39], with a major QTL, named *cmv 1*, which was in fact a single gene responsible for resistance to some CMV strains, providing full resistance to strains P9 and P104.82. Another study [40] found that the resistance to CMV in melon SC was governed by one gene and at least two quantitative trait loci, the single gene, *cmvqw12.1*, co-located with *cmv 1* in linkage group (LG) XII, two QTLs in LGIII (*cmvqw3.1*) and LGX (*cmvqw10.1*) and showed interaction between *cmvqw12.1* and *cmvqw3.1*. Due to the lack of sufficient molecular markers and adapted genetic linkage map, no resistance gene or QTL has yet been mapped.

Presently, we employed SLAF-seq to construct the high-density genetic map in sponge gourd (Fig. 3). SLAF-seq is a low-cost, efficient high-throughput sequencing based technique, and does not depend on the availability of a reference genome sequence. SLAF-seq is a high-throughput technique that promoted the detection speed of QTLs associated with CMV resistance. *L. cylindrica* genotype P2-16 was found to exhibit partial CMV resistance. We were able to identify two QTLs underlying the resistance to CMV on linkage groups 1 and 4. These QTLs could explain 8.41% and 9.75% of the phenotypic variance respectively, with the LOD values of 3.56 and 3.28. The QTL on LG 1 was verified in subsequent $F_{2:3}$ lines using KASP markers developed based on the SLAF sequences.

The high-density *L. cylindrica* genetic map constructed in this study could serve as a valuable tool in many ways, such as gene mapping, marker-assisted breeding, map-based gene cloning, comparative mapping, and draft genome assembling. Furthermore, the resistance of CMV were mapped by using the high-genetic map constructed in this study, which certified the utility of it. Single type of segregated population and inefficiency of derived progeny may affect the detection of QTLs. Stable genetic populations, such as recombinant inbred lines (RILs), might be developed in the next step, which might lead to the detection of more QTLs.

Annotation of gene function of mapping intervals

Plants have evolved a series of mechanisms for defense against viral infections. Three main components were developed to confer resistance to CMV in plants, including restriction of virus installation in the host cells [41, 42], restriction of virus multiplication in the whole plant [43], and restriction of long-distance movement [44]. The most well-known examples are the studies that identified the eukaryotic translation initiation factors 4E(iso) (Eif(iso)4E) and 4E(elf4E) as the host factors required for potyvirus multiplication, which directly interacts with the viral factor of potyviral genome-linked protein (VPg) [45]. eIF4E and eIF(iso)4E are recessive genes conferring resistance to viruses through the viral-encoded protein VPg [46–50]. Resistance to RNA virus has been validated by silencing of the eif4E gene in tomato, melon, and cucumber [51–53]. QTLs, involved in the restriction of CMV in pepper, were also found to be associated with partial restriction of CMV long-distance movement [34]. A phloem protein in cucumber has also been reported to be involved in long-distance movement of CMV [54].

The plants attacked by the virus activate defenses at different levels. At first, they activate basal defenses involving pathogen-associated molecular patterns (PAMPs), which are recognized by plant transmembrane receptors [55]. In addition, some plants exhibit a second line of defense, known as effector-triggered immunity (ETI), to counter pathogens that have evaded the basal defenses [56]. In this step, genes associated with ETI, known as resistance (R) genes, such as nucleotide-binding site (NBS) and leucine-rich repeats (LRRs), are activated [55,56]. The RCY1 gene in *A. thaliana* [28,57], and RT4-4 in common bean [29], are NBS-LRR type genes against CMV. In addition to these R genes, RNA silencing plays a role in antiviral responses, including Dicer-like (DCL) proteins (DCL2/4) along with ARGONAUTE(AGO) protein AGO1/2 [58–61], RNA-dependent RNA polymerase 1 (RDR1/6) (59,62,63), and ENOR3[64], in *Arabidopsis thaliana*.

In this study, retrovirus-related Pol polyprotein (gag-polypeptide of LTR copia-type), which exhibits high similarity to the retrotransposon Tnt 1-94 of *Nicotiana tabacum*, was identified in the CMV resistance mapped location (Additional file 8). Retrotransposons are mobile genetic elements closely related to retroviruses [65]. Both types of elements share structural characteristics and are functionally related. The expression of the Tnt 1-94 element has been studied in detail and shown to be induced in association with plant defense mechanisms [66–68]. We speculated that the retrovirus-related Pol polyprotein plays an important function in *Luffa* spp. against CMV virus. Furthermore, a probable disease resistance protein At4g27220 in *A. thaliana* or At4g27190-like in *Cucumis melo*, serine hydrolase (FSH1), and serine/arginine-rich splicing factor, RSZ21, in *A. thaliana* were predicted very near to the mapped location. Conversely, the annotation of gene function of mapping intervals in this study also validated the effectiveness of the mapped QTLs.

Conclusion

In this study, we performed SLAF-seq on an F_2 population of *L. cylindrica*. A high-density genetic map was constructed, which spanned 1,518.56 cM with an average distance of 0.41 cM between adjacent markers. Two QTLs on LG 1 and 4 of CMV resistance were mapped based on this map, and QTL on LG 1 was verified by KASP markers. Based on the search of the sequences of the linked markers in an RNA-seq database, candidate genes were predicted to be likely a gag-polypeptide protein of LTR copia-type retrotransposon. Thus, we concluded that the QTLs on LG 1 and 4 most likely represent a retrovirus-related Pol polypeptide (gag-polypeptide of LTR copia-type) with resistance activity towards CMV in *Luffa* spp.

Methods

Plant material and genomic DNA extraction

Two advanced inbred lines of *L. cylindrica* (L.) Roem., 'P1–21' and 'P2–16', were selected as parental genotypes. 'P1–21' has palmate leaves and short stick-type immature fruit, and is susceptible to CMV. 'P2–16' has palmate deep cleft leaves and long stick-type immature fruit, and is resistant to CMV. To investigate the inheritance and to map the CMV resistance in *L. cylindrica*, a cross was made between 'P1–21' (female parent) and 'P2–16' (pollen donor) to create an F₁ population in 2014. The F₁ individuals were self-pollinated to generate an F₂ population in 2015. One hundred and thirty F₂ individuals were used as the mapping population. After inoculation with CMV and recording of the phenotype, F₂ individuals were self-pollinated to generate F_{2:3} lines in 2016. Only 60 F₂ individuals gave rise to F_{2:3} populations, as the other 70 F₂ individuals either died during the growing season or only had female or male flowers in one plant, which led to no self-progeny production.

Seedlings of the offspring and parents were planted in a greenhouse of the Vegetable Research Institute, Jiangsu Academy of Agricultural Sciences, Nanjing, China. Young healthy leaves from the two diploid parents as well as F₂ individuals were immediately frozen in liquid nitrogen and stored in a –80 °C freezer. Total genomic DNA was extracted from each leaf sample using the cetyltrimethylammonium ammonium bromide method [69]. DNA quality was evaluated by 1% agarose gel electrophoresis, and the concentration was estimated using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA).

CMV disease evaluation

The CMV isolate used in this study was CMV_{FNY} provided by Dr. Guo of the Institute of Vegetable crops, Jiangsu Academy of Agricultural Sciences. Response of F₂ population (130 individuals) to CMV inoculation was evaluated in a climate-controlled chamber in the lab in Nanjing, China in 2016. The F_{2:3} individuals (60 lines) were evaluated in 2017. The F_{2:3} families were arranged in a randomized complete block design with three replicates, and each replicate had ten plants. The parental lines and F1 plants were included in all experiments.

CMV was inoculated as previously described [37] with minor modifications. The CMV inoculum was isolated from infected leaves of *Nicotiana benthamiana* and 1 g of infected leaves was ground in 5 mL of 0.02 M phosphate buffer (pH 7.2). The sap was rub-inoculated using a pestle dusted with carborundum powder (600 mesh), at the 3–4 true leaf stage. After 5 min, the excess sap was washed off with distilled water. After inoculation, luffa seedlings were kept in a climate-controlled incubator maintained at 26 to 28 °C. Inoculated seedlings began to show symptoms after 1 week, and the disease grade of luffa plants was recorded at 3 weeks' post-inoculation.

Each plant was examined visually and assigned a disease grade based on the severity of mosaic and leaf distortion on a scale of 0 to 9 [70]. Disease grades were assigned as follows: 0 = no symptoms, 1 = a very mild mosaic and puckering on inoculated leaf, or few light and dark green areas associated with vines, 3 = mosaic, blistering, and puckering of leaves on the upper parts of the plant, 5 = strong mosaic and mild leaf distortion on the upper parts of the plant, 7 = severe leaf mosaic and distortion, mild stunting, 9 = severe stunting and leaf death (Fig. 6). Disease indices of a population were calculated from disease grades of individual plants in that population, according to the formula: disease indices (DI) = $\frac{\sum (\text{number of plants in the disease grade} \times \text{disease grade})}{9 \times \text{total number of plants}} \times 100$ [35].

SLAF library construction and high-throughput sequencing

The SLAF library construction and high-throughput sequencing were conducted as described by Sun et al. with few modifications [12]. The DNA samples of the 130 F₂ progenies and two parents were treated with two restriction enzymes, i.e. HaeIII and Hpy166II (NEB), to digest the genomic DNA. Subsequently, a single nucleotide (A) overhang was added to the digested fragments using Klenow Fragment (3' → 5' exo-) (NEB) and dATP at 37 °C. Then, duplex tag-labeled sequencing adapters (PAGE-purified, Life Technologies, USA) were ligated to the A-tailed fragments using T4 DNA ligase (NEB). Polymerase chain reaction (PCR) was performed using diluted restriction-ligation DNA samples, dNTP, Q5® High-Fidelity DNA polymerase, and PCR primers (Forward primer: 5'-AATGATACGGCGACCAACCGA-3', reverse primer: 5'-CAAGCAGAAGACGGCATACG-3') (PAGE-purified, Life Technologies). The PCR products were then purified and separated on a 2% agarose gel. Fragments with 300 to 500 base pairs (with indexes and adaptors) in size were excised and purified using a QIAquick gel extraction kit (Qiagen, Germany). Gel-purified products were diluted for sequencing. Then, pair-end sequencing (each end 125 bp) was performed on an Illumina HiSeq 2500 system (Illumina, Inc., USA) at the Biomarker Technologies Corporation in Beijing.

SLAF-seq data analysis and genotyping

SLAF marker identification and genotyping were performed as previously described [12]. Low-quality reads in each cycle (quality score < 30; a quality score of 30 represents a 0.1% chance of error) were discarded by real-time monitoring during sequencing. Then, all SLAF paired-end reads with clear index information were detected by BLAT (tileSize = 10 –stepSize = 5) [71]. The sequences with >90% similarity were grouped and identified as one SLAF locus.

In mapping populations of diploid species, such as *L. cylindrica*, one locus can contain at most four genotypes. Thus, the groups containing more than four tags were filtered out as repetitive SLAFs. Only the individuals that contained fewer than four tags, had an average sequence depth of 20-fold or more in the parents and 8-fold or more in the progenies, and exhibited >70% integrity in mapping population individuals were identified as high-quality SLAFs, and SLAFs containing 2 to 4 tags were considered as polymorphic markers. Polymorphic SLAF markers were classified into eight segregation patterns as follows: ab × cd, ef × eg, hk × hk, lm × ll, nn × np, aa × bb, ab × cc, and cc × ab. The alleles of each SLAF locus were defined according to the parental genotypes, and individuals were genotyped by sequence similarity to their parents [12]. Since the F₂ population was derived from two homozygous parents with a genotype of aa or bb, only the SLAF markers with segregation patterns of aa × bb was selected for subsequent map construction.

Linkage map construction

The modified LOD (MLOD) scores were calculated for the high-quality SLAFs from the F₂ population, which can be used for linkage clustering. Prior to ordering, markers with MLOD score <5 were filtered out. Map construction was achieved according to the method of High Map, as previously explained [72]. First, the markers with high qualities were grouped based on a pair-wise MLOD score for the recombination frequency. Second, those markers were ordered using Gibbs sampling, spatial sampling, and a simulated annealing algorithm. At the same time, the map distance was estimated. Third, the incorrect genotypes were recognized and eliminated through the k-nearest neighbor algorithm. Marker ordering and error correcting were carried out iteratively so that the markers could be ordered correctly. Several cycles later, accurate linkage maps were achieved. The map function of Kosambi was used in the process. At last, heat maps and haplotype maps were used to evaluate the map quality.

Mapping of CMV resistance genes and KASP assay

The phenotypic data on CMV resistance were collected as described above. Linkage analysis of the CMV resistance gene loci with SLAF markers was performed with the Kosambi mapping function of JoinMap 4.0 (Kyazma, NL).

SNP markers associated with CMV resistance identified by SLAF-seq were validated using a KASP assay. For each marker, two allele-specific primers (one for each SNP allele) and one common (reverse) primer were designed for each KASP assay using a tool provided by LGC Genomics (www.lgcgenomics.com) based on the SNP locus sequence. The KASP assays were designed by LGC Genomics and carried out according to the company's protocol (<http://lgcgenomics.com>).

RNA extraction, transcript sequencing, and candidate gene prediction

For RNA extractions, plants of parental lines were inoculated with CMV as described above. At 0, 2, and 6 days' post-inoculation, leaves of three biological replicates per genotype and treatment combination were sampled and stored at 80°C. Total RNA was isolated using the RNeasy Kit (Qiagen, Germany), and RNA samples were shipped on dry-ice to Biomix (Beijing) Biotech Co. Ltd.

Poly (A) mRNA was isolated using oligo-dT beads (Qiagen, Germany). The mRNA was broken into short fragments of approximately 300 nt. First-strand cDNA was synthesized using random hexamer-primed reverse transcription. Second-strand cDNA was generated using DNA polymerase I. The cDNA fragments were purified and washed for end repair and ligated to sequencing adapters. The cDNA fragments of suitable size were purified and enriched by PCR to obtain the final cDNA library. The cDNA library was sequenced using Illumina HiSeq equipment (Illumina, USA). Clean reads were selected after removing low-quality sequences, adapter sequences, and some ambiguous bases 'N'. The remaining high-quality reads were filtered for short reads below 25 bp, and repeat sequences were masked before assembling. The clean reads were *de novo* assembled using Trinity with default K-mers = 25 [73]. Contigs were obtained by joining the K-mers in an unambiguous path. Then, the reads were mapped back to contigs to construct unigenes with the paired-end information. Finally, the overlapping unigenes from the six libraries were assembled into a continuous sequence, and redundant sequences were removed to acquire non-redundant unigenes that were as long as possible.

The sequences of mapping markers on chromosomes 1 and 4 were aligned to the transcript sequencing database. Candidate gene prediction was performed using GO, Pfam, Swissprot, and nr annotation.

Abbreviations

CMV: Cucumber Mosaic Virus; DCL: Dicer-like; EST-SSR: Expressed sequence tag-Simple sequence repeats; ETI: Effector-triggered immunity; FSH1: serine hydrolase; KASP: Kompetitive allele-specific PCR; LG: Linkage group; LTR: Long terminal repeat; LRRs: Leucine-rich repeats; MLOD: Modified logarithm of odds; NBS: Nucleotide-binding site; NGS: Next-generation sequencing; PAMPS: Pathogen-associated molecular patterns; QTL: Quantitative trait locus; R gene: Resistance gene; Recombinant inbred lines: RILs; SLAF: Specific Length Amplified Fragment; SNP: Single nucleotide polymorphism; SRAP: Sequence related amplified polymorphism; VPg: potyviral genome-linked protein

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All the authors consent to publish.

Availability of data and materials

The datasets generated and analyzed during this study are available from the first and corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by the National Natural Science Foundation of China (No. 31501778).

Authors' contributions

Lou L designed, performed, analyses and drafted the experiments. With valuable suggestions by Su X as well as technical assistance by Liu Z and Liu X. All authors read and approved the manuscript.

Acknowledgements

We thank Dr. Jeroen A. Berg for valuable suggestions during the preparation of the manuscript. We also acknowledge the reviewers for their constructive comments to improve the manuscript. We would give thanks to Biomarker Technologies Co., Ltd., Beijing for technical support in bioinformatics. Also we would like to thank Biomix (Beijing) Biotech Co. Ltd for providing funding for the RNA-seq data.

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Tables

Table 1. Summary of SLAF markers.

Samples	SLAF Number	Total depth	Average depth
P2-16	88,606	5,153,236	58.16
P1-21	89,434	5,596,706	62.58
Offspring	68,636	842,043	12.27

Notes: Total Depth: Mapped Reads; Average depth: Number of reads per SLAF;

Offspring: Average value of all offspring for each parameter per sample.

Table 2. Basic information of sponge gourd genetic map.

Linkage	Marker number	Map	Marker interval (cM)	Max
		length (cM)		distance (cM)
LG1	380	124.00	0.33	4.01
LG2	348	118.78	0.34	4.40
LG3	252	114.95	0.46	3.03
LG4	269	119.84	0.45	3.31
LG5	329	102.80	0.31	4.01
LG6	275	119.91	0.44	4.64
LG7	379	111.50	0.30	2.77
LG8	191	91.71	0.48	3.78
LG9	315	143.28	0.45	4.09
LG10	98	113.91	1.16	4.86
LG11	322	126.80	0.39	3.27
LG12	271	97.70	0.36	2.93
LG13	272	133.40	0.49	4.40
Total	3,701	1518.56	0.41	4.86

Table 3. The double crossover and missing percent of all the linkage groups.

Linkage	Double crossover percent (%)	Missing percentage (%)
LG1	0.04	0.68
LG2	0.03	0.54
LG3	0.05	1.15
LG4	0.02	0.77
LG5	0.04	0.79
LG6	0.04	0.55
LG7	0.03	0.39
LG8	0.07	0.93
LG9	0.04	0.95
LG10	0.18	2.23
LG11	0.03	0.57
LG12	0.10	1.48
LG13	0.03	0.99

Table 4. The significant level of CMV resistance markers in the genetic linkage groups of the Luffa F_{2:3} populations.

Linkage Group	Genetic position (cM)	SNP markers	KASP markers	P value
1	100.457	Marker 7731	A009529	0.0030**
1	100.457	Marker 5105	A009530	0.0030**
1	100.457	Marker 9318	A009533	0.0045**
1	100.457	Marker 18971	A009534	0.0030**
1	100.457	Marker 58975	A009540	0.0030**
1	100.457	Marker 18364	A009544	0.0030**

** Significant at 0.01

Table 5. The sequences of KASP marker primers.

SNP marker	KASP marker	Primer_AlleleX	Primer_AlleleY	Primer_Common	AlleleX	AlleleY
Marker 7731	A009529	ATGAGTGAGGGAACAGAAAGATG	ATGAGTGAGGGAACAGAAAGATA	AGTCTTTACGGGTTAGCCTACAAAT	C	T
Marker 5105	A009530	GGGGAGTGCCTAAACCTCTT	GGGGAGTGCCTAAACCTCTA	CATCCGTAAGCTCACATTTCTCTG	A	T
Marker 9318	A009533	CAAGAAAAAACTCTCATTTACCATTGTC	CCAAGAAAAAACTCTCATTTACCATTGTT	CTGGAAGCACGAAAAGTTAGGGTTTTTTA	G	A
Marker 18971	A009534	GAGTCGTAAGTTGTATGGCGCTA	AGTCGTAAGTTGTATGGCGCTG	TCCAACATCTCTTTCTCAGAGACAATGTA	T	C
Marker 58975	A009540	CTGTAGATGACCCCTTTTGTAAATTGCG	CTGTAGATGACCCCTTTTGTAAATTGCA	AGTGTCCATCTTAAAITCTGGGCATCA	C	T
Marker 18364	A009544	AGCAAACTTTTTATGAAAATGGTTTATCTT	GCAAACTTTTTATGAAAATGGTTTATCTC	TCTCATACATTGAAAAGGACATGAAAAG	T	C

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

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