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Molecular Identification of Mildew Locus O (MLO1) Gene in Selected Watermelon (*Citrullus Lanatus* Thunb.) Cultivars

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ABSTRACT

The Mildew Resistance Locus "O" (MLO) is a gene family unique to plants. MLO1 exists in many plants but is also known as an important member of the MLO gene, which plays a negative regulatory role in plant disease resistance. This gene was first discovered in barley and the mutants of this gene produce broad-spectrum resistance to barley Powdery Mildew. No comprehensive study to identify (MLO1) homologs in adopted watermelon cultivars of Nigeria has been previously conducted. This study aimed at molecular identification of the MLO1 gene in selected Watermelon cultivars. MLO1 Gene was amplified to extract DNA of 10 selected watermelon varieties using PCR. The result of Multiple Sequence Analysis revealed 100% consistencies in the nucleotide consensus sequence of selected watermelon varieties concerning that of the MLO1 gene as reference sequence and SNP observed between two watermelon varieties (Pink Sweet and Heracles) at two different locations and studies on phylogeny revealed 100% relationship with the reference sequence of MLO1 gene. The findings of this research confirmed the presence of conserved MLO1 gene in 10 selected watermelon varieties.

Keywords: PCR =Polymerase chain reaction, MLO =Mildew Locus "O", SNPs = Single Nucleotide Polymorphisms, Powdery Mildew

INTRODUCTION

The Mildew Locus O (MLO) genes family encodes plant-specific proteins distributed among conserved clades and implicated in Powdery mildew resistance in monocots and dicots. Further analysis of the MLO genes and their deduced protein sequences revealed that many characteristics of the gene family, such as the presence of seven (7) transmembrane domains, the MLO functional domain, and particular amino acid positions, were present and well conserved (Pépin *et al.*, 2021). The Mildew Resistance Locus "O" (MLO) is a gene family unique to plants (Kim *et al*., 2002). It exists in many plants but is also known as an important member of the MLO gene, and plays a negative regulatory role in plant disease resistance, equivalent to the "disease susceptibility" gene in plants to a certain extent. This gene was first discovered in barley, and mutants of this gene produce broad-spectrum resistance to barley Powdery mildew (Schultheiss *et al*., 2002). Numerous studies have shown that the MLO gene has dual functions that are especially involved in plant disease resistance and plant leaf cell death (Piffanelli *et al*., 2002). The previously published studies believed that the MLO protein has seven Transmembrane domains (Kim *et al*., 2002). It has been found that the number of MLO protein Transmembrane domains varies in both lower and higher plants. In addition, MLO proteins with <5 transmembrane domains are usually found in lower plants, while MLO proteins with 6–8 transmembrane domains are found in higher plants (Zhang *et al*., 2018). Some studies have shown 12 to 19 members of the MLO gene family in Arabidopsis, grape, rice, peach, woodland strawberry, and tobacco (Appiano *et al*., 2015).

MLO genes were first described in grasses; some members of this family are also inferred to play a role in modulating host response to the pathogenic Powdery mildew fungus in dicots. For example, in Arabidopsis, the AtMLO2 gene shows significantly reduced susceptibility to *Golovinomyces orontii.* Two other closely related Arabidopsis genes, AtMLO6 and AtMLO12, are also mutated, along with AtMLO2, to achieve complete PM resistance (Consonni *et al*., 2006). Subsequently, the absence of MLO genes (SlMLO) was demonstrated to be the cause of Powdery mildew resistance in tomatoes (Catalano *et al*., 2008). Recently, (Pavan *et al*., 2011) demonstrated that pea Powdery mildew er1 resistance is associated with loss-of-function mutations at an MLO-homologous locus. A biologically active MLO may be a general requirement for Powdery mildew pathogenesis in both monocotyledonous and dicotyledonous plants. The interaction between MLO genes and Powdery mildew may have a special mechanism. Nevertheless, knowledge of the precise action mechanism of MLO proteins is limited. The function

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of MLO genes from plants and bacteria in the signal transduction process may be identified for determining the precise MLO mechanism. MLOs are calmodulin-binding proteins, which promote the susceptibility of barley to Powdery mildew (Kim *et al*., 2002b). Moreover, pharmacological studies suggest that the influx of Ca2+ ions is important for this MLO function (Kim *et al*., 2002a). Whereas MLO-mediated defense suppression might likely involve one or several small GTP-binding proteins of the ROP family (Schultheiss *et al*., 2002).

For many years extensive studies of MLO genes revealed that mutations of one or more specific MLO genes in *Vitis vinifera*, *Petunia hybrid*, *Pisum sativum*, *Capsicum annuum*, *and Triticum aestivum* were found to cause broad-spectrum resistance to PM (Pessina *et al*., 2016), because there is a possibility that Powdery mildew requires MLO proteins to invade host plant cells (Panstruga, 2005b). Therefore, deletion or mutation of the MLO gene can cause Powdery mildew Spores to not enter the plant cell wall (Panstruga, 2005b). Researchers have found that a recessive mutation in the MLO gene of barley shows resistance to a broad spectrum of powdery mildews, and the MLO mutant has been widely used as a resource for the breeding of wild barley in Europe (Lyngkjaer *et al*., 2000). The normal transcription and expression of the MLO protein are necessary for the successful invasion of powdery mildew (Zellerhoff *et al*., 2010). By inducing mutations, a change coding sequence of MLO can be changed to induce plant resistance to powdery mildew (Panstruga, 2005a).

This study aimed at the molecular identification of the Mildew Locus O (Mlo) gene in selected Watermelon (*Citrullus lanatus* Thunb) Cultivars with the view to amplify the identified MLO1 gene of the selected watermelon cultivars using polymerase chain reaction (PCR) and sequence the amplified MLO genes homologs and construct a phylogenetic tree for evolutionary relationship

MATERIALS AND METHODS

Plant Materials Sampling

A total of 10 watermelon varieties were selected for this study based on adoption by local farmers within Sokoto Nigeria. Information on names of variety, ecotype, and location was presented in Table 1.

Table 1: Watermelon varieties selected and location of the collection.

S/N	Genotypes	Place of Collection	Ecotype
1	Greey	IITA Kano Office	Improved
2	Sweet Sangaria	IITA Kano Office	Improved
3	Baraka	IITA Kano Office	Improved
4	Heracles	IITA Kano Office	Improved
5	Vinking F1	IITA Kano Office	Improved
6	Kayack	IITA Kano Office	Improved
7	Pink Sweet	IITA Kano Office	Improved
8	Sugar Dragon	IITA Kano Office	Improved
9	Dan Hassan	IITA Kano Office	Local
10	Dan Tsugu	IITA Kano Office	Local

Seed Germination for DNA Assays

Ten seeds of each variety were germinated in a sterilized petri -dish line with a paper towel in a laboratory conditions. Before germination, the laboratory bench was subjected to surface cleaning with 70% ethanol to avoid the risk of contamination. After germination, 10 days old seedlings were used for DNA isolation.

Genomic DNA Extraction

Fresh green leaves were collected, weighed (~100 mg), and immediately used for DNA isolation using CTAB mini-prep protocols. Leaf tissue was ground to fine powder after freezing with liquid nitrogen in a pre-chilled mortar. The finely powdered tissues were then transferred to a tube of Pre-warmed CTAB buffer (2.0% CTAB (w/v); 0.1 M Tris-Cl, pH 8; 0.02M of EDTA, pH 8; 1.4 M NaCl) and the mixture was incubated at 65°C for 20 min. The supernatant was collected after centrifugation, 500µl of Phenol/Chloroform (1:1) was added and centrifuged at 13,000rpm for 10 minutes under room temperature. The supernatant was transferred to a clean tube and 500ul of Chloroform was added, and centrifuged at 13000rpm for 10 minutes. The aqueous phase was collected, mixed with 500ul of isopropanol, and incubated for 1hr 30minutes at -20°C. Centrifugation was done to pellet down DNA. The pellet was washed with chilled 70% (v/v) ethanol two times, and air dried in laminar flow for 20 – 30 minutes then the DNA pellet was dissolved in nuclease-free water and incubated at -20°C for future use. The concentration and quality of purified DNA were checked in a Nanodrop spectrophotometer (Thermo Scientific, USA) employing 260/280 and 230/260 ratios and by 1% (w/v) agarose gel electrophoresis.

Sequence Retrieval
The amino acid sequences of Mildew-Locus-O (MLO1-15) genes in *Arabidopsis thaliana* were retrieved from the NCBI protein database and genomics database. The retrieved sequences were used as queries for BLASTP against the watermelon (*C. lanatus*) whole genome.

Primer Design
The retrieved nucleotide sequence of the identified MLO1 gene for powdery mildew resistance was used for primer design. Overlapping oligos of the genes were designed and thermodynamic properties of the oligos were checked using PRIMER 3 software for hairpin, primer dimer, optimum Tm values, and %GC content

Forward primer	**AACGACGCTGGAATTCACTC**
Reverse primer	**TGCACAAAACAGTAAGCAATGA**

Amplification of 750bp MLO1 Gene
The selected amplicon (750kb) of the identified MLO1 gene was amplified by PCR using gene-specific primers in a 50µl volume reaction using C1000 Touch Thermal Cycler (Bio-Rad, USA). Each 50 µl volume of reaction mixture contained 50 ng of genomic DNA as template, 5X Taq polymerase buffer, 25 mm $MgCl_2$, 0.2 mm dNTPs mix, 0.4 pm each of the forward and reverse primer, 1µ of Taq polymerase. The optimized condition is initial 5 minutes of incubation at 95°C for complete denaturation, followed by 34 cycles consisting of 95°C for 30 seconds, 54.4°C (vary with the primer pair) for 30 seconds, 72°C for 1 minute, and finally 72°C for 10 minutes. The PCR products were run on 1% agarose gel electrophoresis to check amplification using a constant voltage of 70V and 1X TAE (Tris Acetate EDTA) buffer system. The gel was stained with Florosafe (Axil Scientific Pte Ltd) and viewed under a UV trans-illuminator system.

MLO1 Sequencing
The PCR products were submitted for sequencing to Integrated DNA Technology Singapore.

Sequence Submission into NCBI and Cucurbits GenBank Database
The edited MLO1 gene sequence was submitted to NCBI and Cucurbits GenBank databases for accession number generation and archiving. Sequence accession numbers will be generated and sequences stored in NCBI GenBank databases.

Sequence Analysis and Phylogenetic Studies
The Molecular Evolutionary Genetic Analysis (MEGA) tool was used for multiple sequence Alignment and Multifurcating phylogenetic tree construction with about 99% bootstrapping value for the 10 consensus sequences along with the Reference sequence of the MLO1 gene.

RESULTS

Sequence Retrieval and Genomic DNA Extraction
Results on sequence retrieval show the identified MLO1 gene as Cla97C06G109800 in watermelon of 5,952 bp genomic sequence, with 1,552 bp coding sequence and 517 amino acid sequence. (Table 2)

Amplification of 750bp MLO1 Gene Fragment
MLO1 genes of 750bp were amplified in all the selected watermelon varieties and confirmed by gel runs with visible bands using 1kb DNA Ladder (Plate 2).

Sequence Analysis and Phylogenetic Studies
The multiple sequence alignment you performed showed high similarity across the selected watermelon varieties except that SNPs were observed at 2 different locations in 2 varieties (Plate 3). The phylogenetic tree results of selected watermelon showed a 100% relationship among the varieties across all clades (Figure 1).

Table 2: MLO1 gene family member Homology identified in *C. lanatus* whole genome.

S/N	Gene ID	Size (bp)		
		Gdna	CDS	Protein
1	Cla97C06G109800	5952	1552	517

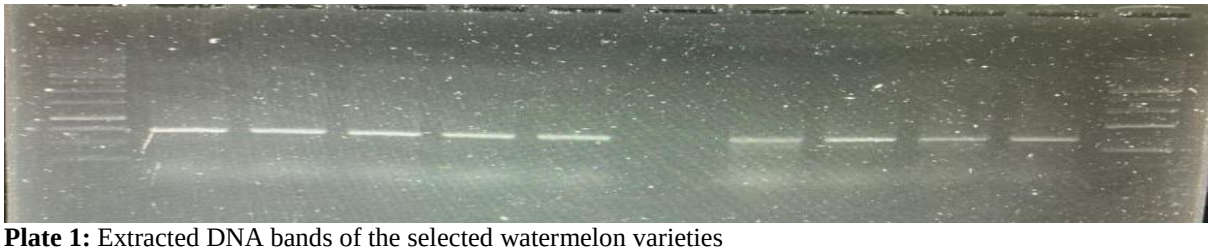
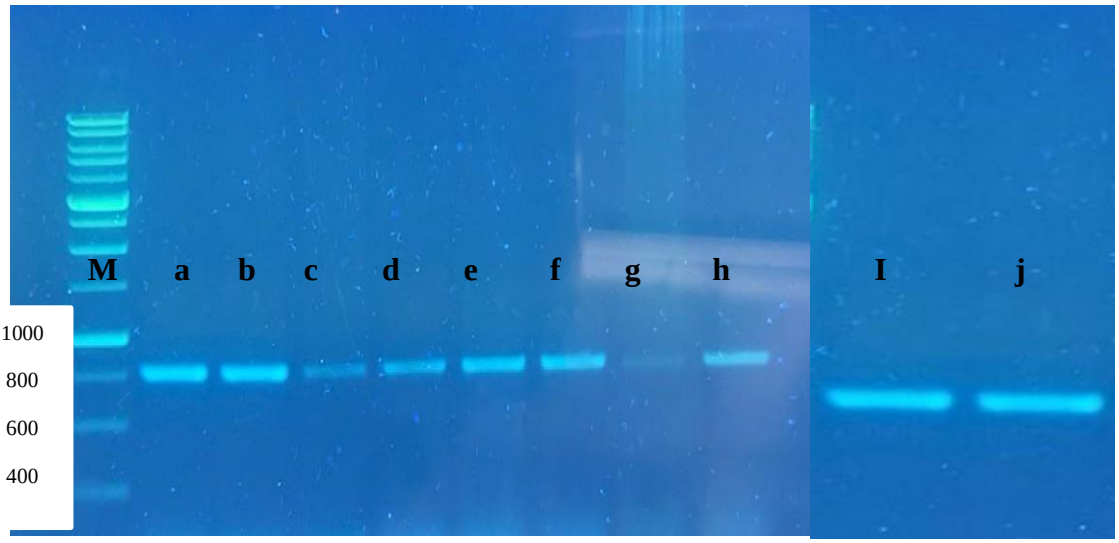


Plate 1: Extracted DNA bands of the selected watermelon varieties



Plates 2: MLO1 gene amplification in the selected 10 varieties using 1kb DNA ladder; Lane M = 1000bp; Lane a =Greey ; Lane b =Sweet Sangaria ; Lane c =Baraka ; Lane d =Heracles ; Lane e =Vinking F1 ; Lane f =Kayack ; Lane g =Pink Sweet ; Lane h=Sugar Dragon ; Lane I =Dan Hassan ; Lane j =Dan Tsugu



Figure 1: Figure 1: Multiple Sequence Alignments of MLO1genes obtained from selected 10 Watermelon varieties

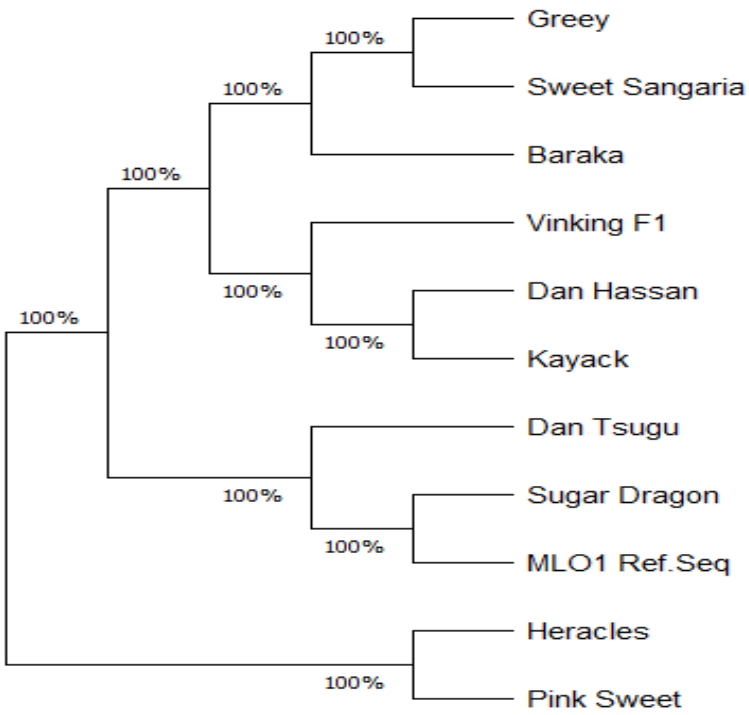


Figure 2: Phylogenetic tree showing the relationship among the Watermelon varieties

DISCUSSION

In recent years, many studies have shown that the MLO gene is significantly associated with PM resistance in plants, but the analysis of the MLO gene family in melon remains unclear (Zhang *et al*., 2023). But in this research MLO gene family member coded as MLO1 was identified in watermelon and this MLO protein resulted in having amino acid length (517) comparable to those of *Arabidopsis* at MLO homologs, ranging from 460 to 593 residues as reported by Devoto *et al*. (2003). This is consistent with previous genome-wide surveys in India that 1-14 MLO-like gene sequences in the genomes of *C. lanatus* in China (Paolo *et al*., 2015). Also, similar work was reported by (Zhang *et al*., 2023) that a total of 14 members of the MLO genes family were identified in melon

Multiple Sequence Analysis revealed 100% consistencies in the nucleotide consensus sequence of ten (10) selected watermelon varieties with that of the MLO1 gene as a reference sequence. In a similar work reported by (Zhang *et al*., 2023) the Multiple Sequence Analysis of four melons in Compared with the reference genome sequence in China, showed 98.4% homology. (Yong *et al*., 2012) reported Multiple Sequence Analysis results of twenty watermelon chromosomes corresponding to regions of about 99% homology with reference MLO gene reference. Phylogenetic analysis was performed showing 100% relationship among members across the all. Similar work reported by Paolo *et al*. (2015) that five watermelon proteins were clustered together in phylogenetic clade v.

CONCLUSIONS

The findings of this research confirmed the presence of conserved MLO 1 gene in the selected watermelon varieties and studies on phylogeny revealed a 100% relationship with the reference sequence, and SNPs were observed at two different locations along the consensus sequences of (Pink Sweet and Heracles occupied separate clade)

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