

## Detection of Mulatexin in Local Mulberry Plants (*Morus* spp.) from Bogor, West Java, Indonesia

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### ABSTRACT

The *mlx56* gene family is found exclusively in the mulberry (*Morus* spp.) plant, and its role is to defend against insect herbivores, yet it had never been discovered from Indonesia, which has been cultivating mulberry silkworm's (*Bombyx mori*) feed in order to produce silks. This research focused on studying the expression of *mlx56* gene family in local mulberry plants from Bogor, West Java, using different plant organs which were leaf, stem, and root. Each sample was collected from four species of mulberry; *Morus alba* var. *kanva-2*, *M. alba* var. *multicaulis*, *M. bombycis* var. *lembang*, and *M. cathayana*. Genomic RNA samples were collected and synthesized into cDNA, amplification was done using eight primers of the *mlx56* gene family and the products were sequenced. Sequences were then analyzed by BLAST. Results showed diverse *mlx56* gene family expression among species of mulberry and plant organs. The *mlx56-2*, *mlx56-4*, and *mlx56-7*, were mostly expressed in all species and organs. The gene *mlx56-3*, which was not discovered in the previous study, was expressed in *M. cathayana* leaf and root also in the roots of other species. It was identified as chitinase-like protein LA along with *mlx56-5*, while other genes were identified as *mlx56* based on BLAST.

### 1. Introduction

Insect herbivores are biotic threats to plant which cause plant disease and crop loss. Application of insecticide is commonly used to contain these threats due to its low price and effectiveness. However, most insecticide contains numerous toxic chemicals that harm the targeted pest and the surrounding environment. Many insecticides effectively eradicate insect herbivores, but its continuous application in the long term may harm other living organisms. The plant itself is affected by the accumulation of toxic chemicals, and the remaining residues could lead to food security issues (Aktar *et al.* 2009). The insecticide directly impacts humans, resulting in serious health implications to the high-risk group; formulators, sprayers, production workers, and farm workers. These workers are at risk as they are mostly exposed to hazardous materials of insecticide. Studies had shown low-dose chemicals used in insecticide might have the activity to mimic or antagonize the natural hormone, resulted in metabolic abnormalities and

cancer (Hurley *et al.* 1998; Brouwer *et al.* 1999). The chemical residues contaminate soil, air, water environment and eventually harm non-target living organisms such as fish, birds, mammals, and microorganisms (Kannan *et al.* 1992; Kannan *et al.* 1995). Long-term application of insecticide drawn resistance, as the targeted pest develops several mechanisms to avoid toxicity. Most insect herbivore has a detoxification enzyme, which binds and metabolize different substrates as a protection mechanism against toxic compounds. A previous study showed that the resistance property was inheritable as the survivor and offspring remained tolerant of insecticide (Li *et al.* 2000). However, the detoxification enzyme expression was known to be repressed by the exposure of the plant to chemicals (Yu and Abo-Elghar 2000). Indication of plant resistance to insect herbivores using its natural compounds.

A recent study showed that mulberry plants could produce a toxic compound that harms some insect herbivores, except silkworm (*Bombyx mori*). This compound is found in mulberry plant latex, known as plant anti-herbivores protection (Konno *et al.*

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2006). The latex of mulberry contains an alkaloid, almost half of its total amount, which causes toxicity to insects. Published research discovered that alkaloid was not the only compound in latex that created toxicity but also found a protein that served as a plant defense system and the source of toxicity. This protein is named mulatexin and thus coded by the gene *mlx56*. This protein had been isolated and characterized from mulberry plant latex, and the result showed significant growth-inhibitory activity against *Mamestra brassicae* and *Samia ricini*, and was harmless to silkworm (Wasano *et al.* 2009). This indicated silkworm had developed its enzymatic adaptation to survive from its diet and avoid competitors (Hirayama *et al.* 2007). A different study discovered seven *mlx56* genes family in mulberry plants (Han *et al.* 2015). These genes shared similar characteristics as plant protection properties by genetic modification. Therefore, further research and studies on this matter are necessary.

Indonesia had been producing silks from silkworms for industrial needs to serve local demand and export purposes (Atmosoedarjo *et al.* 2000). Silk production involved the cultivation of local mulberry as the main diet of the silkworm, yet research upon plant protection genes to develop local mulberry had not been done. Therefore, this research focused on studying the expression *mlx56* gene family in local mulberry plants from Bogor, West Java, using different species and organs of the plant.

## 2. Materials and Methods

### 2.1. Plant Materials

Four species of local mulberry plants from Bogor, West Java; *Morus alba* var. *kanva-2*, *M. alba* var. *multicaulis*, *M. bombycis* var. *lembang*, and *M.*

*cathayana* were used as samples. These plants, taken from Sukamantri, University Farm, Bogor Agricultural University, were cultivated in the greenhouse, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia.

### 2.2. RNA Isolation

RNA isolation materials include leaves, stems, and roots samples from each mulberry species. Stages of genomic RNA isolation were carried out based on GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific Inc., MA, USA) protocol.

### 2.3. First-strand cDNA Synthesis

The cDNA synthesis step was conducted according to RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., MA, USA) protocol. Briefly, cDNA was synthesized using oligo dT as a primer. Reaction mixtures were incubated for 60 minutes at 42°C. After that, the reaction was terminated by heating at 70°C for 5 minutes. The cDNA was stored at -20°C before it is used.

### 2.4. Amplification with RT-PCR

Eight *mlx56* gene family primers (IDT; Singapore) used for amplification were as described in Table 1, *mlx56-0* primer was used to detect the original MLX56 protein in mulberry plants. GAPDH primers were used as the kit control, while 18S and *rbcl* primers were used as plant control. The reaction was conducted in 20 µl reaction mixture, which consisted of 1 µl diluted template cDNA, 1 µl of each forward and reverse primers, 9.5 µl of nuclease-free water, and 12.5 µl of KAPA2G Fast ReadyMix. The PCR consisted of 35 cycles of predenaturation at 94°C for 4 minute, denaturation at 94°C for 30 seconds,

Table 1. Forward and reverse primers of *mlx56* gene family (Han *et al.* 2015)

Gene	Primer forward	Primer reverse
<i>mlx56-0</i>	ATGAAGTTKAKAACTCTTTAATMR	CGAGCACCTTCYGAGAAATRCGTTT
<i>mlx56-1</i>	GGAGCTTGCTGCTTTCTTTGCTCAA	ATACCTTCATAGTGTAATACGGC
<i>mlx56-2</i>	CCCGATCACAGGAAGGTGTGGAAGA	TTGTCATTGCCATTGGTGGTCTCAT
<i>mlx56-3</i>	GGAGCTTGCTGCTTTCTTTGCTCAA	ATAGTACCCATTACTAGTAGTAATA
<i>mlx56-4</i>	CCTAGAATGACCGATGTGGAAGAGC	ATATCCCCATGCATATGGATCTTGT
<i>mlx56-5</i>	ACGGCAACCTGTGTTGTAGCCATTG	ATTAGAGCGAAAGGAGAGAATTCC
<i>mlx56-6</i>	CCCGCTCCACCAAGTCCACCTCCA	ATATCACCAGTAGTACCAAAATGAG
<i>mlx56-7</i>	TGTTGGAGTTCACCAAGCCACCTC	ATTAGAGCAGAAAGGAGAGATTCCC

annealing (18S and *rbcL* 51°C; GAPDH 58°C; *mlx56-0* 44°C; *mlx56-1* 53°C; *mlx56-2* 54°C; *mlx56-3* 44°C; *mlx56-4* 52°C; *mlx56-5* 52°C; *mlx56-6* 49°C; *mlx56-7* 55°C) for 30 seconds, extension at 72°C for 2 minutes and post-extension at 72°C for 7 minutes.

## 2.5. Visualization of PCR Product

The PCR products were then visualized by 2% agarose gel electrophoresis in 1x TAE solution at 80 Volts for 90 minutes. About 4 µl of samples (3 µl of sample mixed with 1 µl loading dye) and 3 µl of DNA ladder loaded into the gel. cDNA fragments measured by 1 kb DNA ladder GeneRuler (Thermo Fisher Scientific Inc., MA, USA). The agarose gel was then stained by ethidium bromide and visualized with UV light.

## 2.6. Sequencing of PCR Products

PCR products were sequenced using the service provided by PT Genetika Science, Indonesia. PCR products and primers sent to First Base, Malaysia, to be sequenced both by forward and reverse primers. The sequence results were managed using the SeqTrace application (Stucky 2012) and saved in the form of FASTA. The nucleotides were later analyzed by the Basic Local Alignment Search Tool (BLAST).

## 3. Results

The result of cDNA synthesis was confirmed in PCR reaction with 18S and *rbcL* primers at the annealing temperature 51°C, the visualization using gel electrophoresis as seen in Figure 1 and 2. This step was conducted to confirm synthesized cDNA had these housekeeping genes as plant samples.

The expression of *mlx56* gene family in different species and organs of mulberry plants as presented in Figure 3. Primers were determined to amplify the partial gene of each *mlx56* gene family, except *mlx56-0*, which was coded to isolate complete nucleotides of original MLX56 protein mRNA.

Isolated *mlx56* gene families from leaf samples were sent for sequencing. The sequences were then analyzed using BLASTN, and the results are as shown in Table 2.

## 4. Discussion

cDNA from each mulberry plant sample was confirmed using 18S and *rbcL* primers. 18S primer was applied to determine the presence of 18S ribosomal RNA (rRNA) sequence in plant samples, and this rRNA is the basic component of all eukaryotic cells

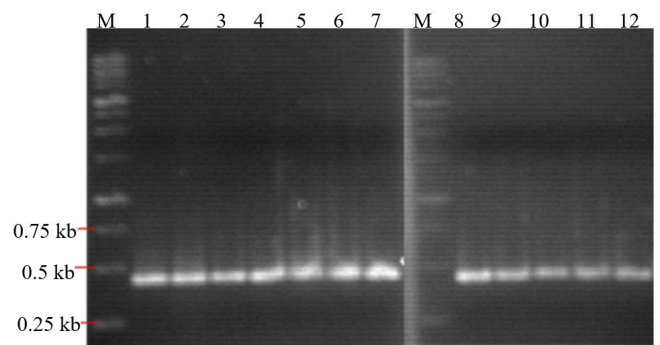


Figure 1. Confirmation of cDNA as plant samples using 18S primers. Lane M; 1 kb DNA ladder GeneRuler. Lane 1-12; cDNA from leaf, stem, and root of local mulberry plants from Bogor, West Java

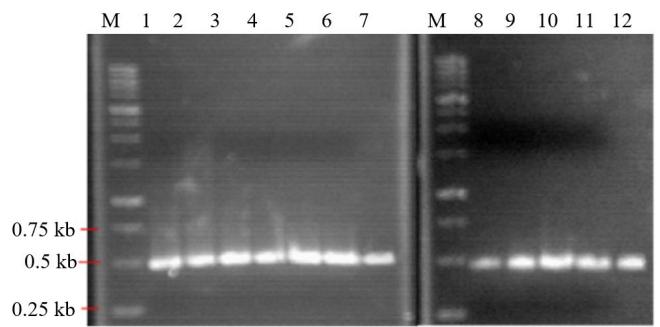


Figure 2. Confirmation of cDNA as plant samples using *rbcL* primers. Lane M; 1 kb DNA ladder GeneRuler. Lane 1-12; cDNA from leaf, stem, and root of local mulberry plants from Bogor, West Java

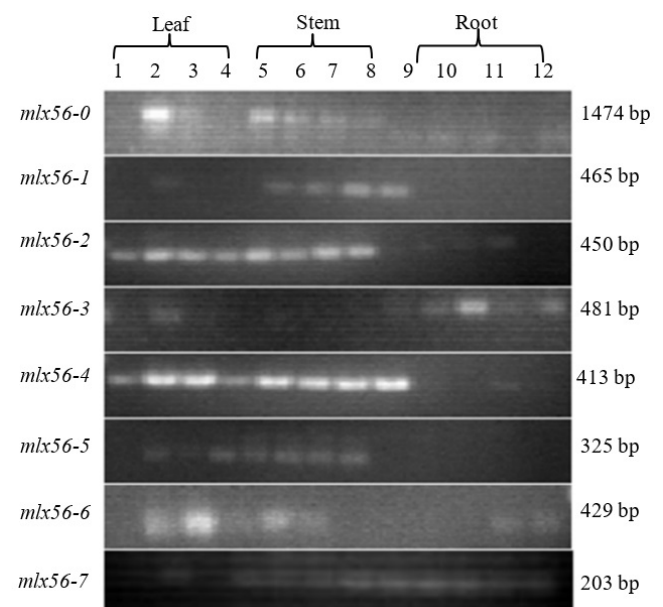


Figure 3. Reverse transcription PCR (RT-PCR) of *mlx56* gene family in Indonesian local mulberry plants. Lane 1, 5, and 9 were isolated from *Morus alba* var. *multicaulis*; lane 2, 6, and 10 from *M. cathayana*; lane 3, 7, and 11 from *M. bombycis* var. *lembang*; lane 4, 8, and 12 from *M. alba* var. *kanva-2*

Table 2. BLASTn result of isolated *mlx56* gene family

Gene	Sequence hit	Maximum score and total score	E Value	Identity (%)
<i>mlx56-0</i>	<i>Morus alba</i> var. <i>alba latex</i> protein mRNA complete cds (EF535852.2)	1969	0	97
<i>mlx56-1</i>	<i>Morus alba</i> var. <i>multicaulis</i> MLX56 mRNA, complete cds (JX432966.1)	756	0	99
<i>mlx56-2</i>	<i>Morus alba</i> var. <i>multicaulis</i> MLX56 mRNA, complete cds (JX432966.1)	778	0	100
<i>mlx56-3</i>	<i>Morus alba</i> mRNA for chitinase-like protein LA-ab-like protein, partial cds (AB639038.1)	819	0	99
<i>mlx56-4</i>	<i>Morus alba</i> var. <i>multicaulis</i> MLX56 mRNA, complete cds (JX432966.1)	737	0	100
<i>mlx56-5</i>	<i>Morus alba</i> mRNA for chitinase like protein LA-b, partial cds (AB6390337.1)	619	1e-173	99
<i>mlx56-6</i>	<i>Morus alba</i> var. <i>alba latex</i> protein MLX56-6 mRNA, complete cds (KJ496132.1)	713	0	100
<i>mlx56-7</i>	<i>Morus alba</i> var. <i>alba latex</i> protein MLX56-7 mRNA, complete cds (KJ496133.1)	375	2e-100	100

and thus should be existed in plant genomes (Wang *et al.* 2014). Meanwhile, the *rbcl* primer was used to determine the presence of genes coding ribulose-1.5-biphosphate carboxylase (RuBisCo) enzyme, which is discovered in the chloroplast DNA (Feller *et al.* 2008). Both genes were shown to be expressed in all mulberry plant samples, the size of amplified genes about 500 bp for 18S rRNA and *rbcl* (Figure 1 and 2). This result was according to the literature that stated the size of 18S rRNA gene is mostly 500 bp, while *rbcl* gene size varies from 1-599 bp (Smit *et al.* 1999; CBOL Plant Working Group 2009). The visualization of mulberry plants cDNA using 18S and *rbcl* primers showed similar intensity, thus suggesting the same quality of each cDNA sample.

Primers used to obtain *mlx56* gene family proven to amplify genes from each sample. As shown in Figure 3, there were different expressions of each gene family among organs of the mulberry plant. Most gene families were discovered in every mulberry species (*Morus alba* var. *kanva-2*, *M. alba* var. *multicaulis*, *M. bombycis* var. *lembang*, and *M. cathayana*), yet not every gene was expressed in every organ of the same plant. Gene family *mlx56-2* and *mlx56-4* were discovered in all species, yet both genes are only expressed in the leaves and stems of the mulberry plant. A similar condition was observed in *mlx56-1* and *mlx56-5*, which showed gene expression in the leaf and stem of some species, but not in their roots. Gene family *mlx56-7*, in contrast, existed in every species of mulberry and most organs of the plant. Unfortunately, in the leaf of *M. bombycis* var.

*lembang*, the intensity of *mlx56-7* gene expression was different. Gene family *mlx56-6* and *mlx56-3* were discovered in roots and expressed in some leaf and stem samples. This result was in accordance with a previous study that showed diverse expression of these genes in the mulberry plant, although some genes were not much discovered. The *mlx56-1* gene was discovered in only a few varieties of *M. alba* in the previous study, mostly found in the stem of local mulberry plants. The *mlx56-3* gene had been found in the leaf and the root of *M. cathayana* and the root of other species. The *mlx56-3* gene was not discovered in mulberry materials used in the previous study (Han *et al.* 2015). However, this study showed the presence of the gene in local mulberry from Bogor, West Java.

M35 and MLL1 genes were found and expressed in mulberry species from West Java. MLL1 gene might be expressed in the young leaves organ of *Morus cathayana*, *M. bombycis* var. *lembang*, and *M. alba* var. *kanva-2*. However, MLL1 might not be expressed in young leaves of *M. alba* var. *multicaulis*, and in the stems and roots organ of all mulberry plant species. Meanwhile, M35 gene might not be expressed on the young leaves, stems, and roots organ in all mulberry plant species (Wulandari *et al.* 2018). Some studies have found that lectin (MLL1) content of plants utilized as antibacterial, insecticide, anti herbivory, and antifungal to prevent pest attacks (Peumans and Damme 1995). Diverse expression of this gene family among plant organs resulted from specific environmental stress levels of each plant organ.



Biotic and abiotic conditions could result in distinct respond from each organ, causing the specific organ to develop its gene regulation based on its need. The gene family expression may also vary when different types of pathogens and/or herbivores exist (Carolino *et al.* 2003; Campos *et al.* 2007).

Primers from isolated MLX56 protein mRNA from *M. alba* (GenBank: EF535852.2) were also used in this study to observe the original protein presence in each mulberry plant sample. The gene was coded as *mlx56-0* in accordance with the other *mlx56* gene family. Results showed diverse MLX56 protein mRNA expression in each sample as seen in Figure 3. The result is due to different structures of the MLX56 protein in species and organs of mulberry plant through evolution (Han *et al.* 2015). As a chitinase-like protein, gene duplication and mutation event through evolution may cause lack of its chitinase activity, thus further studies is necessary to determine whether each gene family of *mlx56* still has the defense property or has developed other adaptive function independently (Bussink *et al.* 2007; Kesari *et al.* 2015).

Isolated *mlx56* gene family from leaf samples were sent for sequencing to represent other specific genes found in stem and root. The nucleotides were identified using BLASTn, and sequence hits were shown in Table 2. The isolated MLX56 protein mRNA showed similarity with its actual source, yet the highest sequence hit obtained was with Latex Abundant (LA) protein which might be caused by nonspecific primer as seen in Table 1 for *mlx56-0*. Gene family *mlx56-1*, *mlx56-2*, and *mlx56-4* were identified as the original MLX56 mRNA, suggesting that these genes had originated from the MLX56 mRNA itself and might be parts of this gene. Gene family *mlx56-6* and *mlx56-7* were identified as their complete nucleotides isolated before, while *mlx56-3* and *mlx56-5* had LA protein mRNA as their identity.

LA protein was discovered as another abundant protein present in the latex of the mulberry plant. A published study found four types of LA protein: LA-a, LA-b, LA-ab-like, and LA-c protein. These proteins had similarities in structure and function with MLX56, especially LA-a known to be identical to MLX56 with only one residue difference. These proteins also had similar chitin-binding activity to defend against insect herbivores. The mechanism itself, however not well understood. The LA-ab-like protein shown to be the identity of *mlx56-3* poses similarities with

both LA-a and LA-b protein structure, yet the specific function is rarely studied. Meanwhile, *mlx56-5* had the identity of LA-b protein, which was expressed more in unligified parts of the mulberry plant with two chitin-binding domains, along with LA-a protein.

In contrast, the LA-c protein was shown to be more abundant in lignified organs with antifungal activity. These diverse accumulations of LA proteins among organs probably happened due to laticifers' adaptation based on the enemies. Unligified organs, for example, leaves, were mainly invaded by Lepidoptera as their main diet. Therefore the laticifers in unligified organs required more chitin-binding protein to defend against insect herbivores. Lignified organs such as trunks are not a target for herbivores yet widely infected by fungi, which required antifungal activity such as in LA-c protein (Kitajima *et al.* 2010; Kitajima *et al.* 2012). These suggested that laticifer cells adaptation could also result in diverse expression of *mlx56* gene family respectively, and the LA protein gene might also be part of *mlx56* gene family.

In conclusion, The *mlx56* gene family had different expressions among organs of local mulberry plants from Bogor, West Java. Most mulberry plants expressed all *mlx56* gene families, but not every organ of the same plant expressed the genes. It was also in accordance with the diverse expression of the original MLX56 protein mRNA in samples used. The *mlx56-3* gene, which was not discovered in the previous study, was found in the leaf and root of *Morus cathayana* and root samples of other mulberry plants and was identified as LA-ab-like protein mRNA.

Further study of the gene function, regulation, and character would be needed to provide more understanding about *mlx56* gene family, and thus research of *mlx56* gene family expression in treated mulberry plants would develop the future use of these genes.

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