Overexpression of the mulberry latex gene MaMLX-Q1 enhances defense against *Plutella xylostella* in *Arabidopsis thaliana*

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Abstract: Purified mulberry latex chitinase (MLX) has a role in defense against some lepidopteran insects. In this study, a full length chitinase gene, *MaMLX-Q1,* of 1405 bp with a 1140 bp open reading frame, was obtained from mulberry leaves by the degenerate primers and rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) procedure. The gene encoded a mature protein with the predicted molecular mass of 39.38 kDa and an isoelectric point (pI) of 6.43; it contained two chitin-binding domains and a hydrolase family 19 chitinase domain. Sequence alignment and phylogenetic analysis grouped it in the class I chitinase protein group. Heterogeneous expression of this MaMLX-Q1 in *Arabidopsis* showed non-visible alterations in growth phenotype, except for the higher transcriptional expression of *MaMLX-Q1* when compared to that of wild-type *Arabidopsis*. This ectopic *MaMLX-Q1* exhibited toxicity to the growth and development of *Plutella xylostella* larvae, causing significantly lower weight gain and higher mortality. These results indicate an application of *MaMLX-Q1* as an insecticide for plant protection.

Key words: chitinase; *Plutella xylostella*; transgenic *Arabidopsis thaliana*; latex, mulberry

INTRODUCTION

Latex laticifers are widely found in angiosperms (more than 20000 species from some 40 families, e.g. the *Asteraceae* family, the poppy family, greater *Celandine* and rubber trees) [1-3], and act in the plant defense system against microbes or herbivores [4,5]. Many toxic compounds incorporated in latices are well characterized, such as the neurotransmitter dopamine in the Persian poppy (*Papaver bracteatum*), the narcotic alkaloid morphine in the opium poppy (*Papaver somniferum*), cysteine protease in the latex of wild fig (*Ficus virgata latex*) and papaya (*Carica papaya*) [6]. Such compounds are often 50-1000 times more concentrated in latex than in leaf tissues [4]. There is increasing research into the biological functions of latex.

Mulberry (*Morus spp.,* Moraceae) is a deciduous tree that mainly grows in China and other Asian countries. Its leaves have been used for rearing the silkworm *Bombyx mori,* an economically very important insect, for thousands of years. Mulberry plants are regularly pruned to encourage leaf growth in industry. They usually ooze a milky latex after injury. This latex is supposed to exert rapid wound closure, thereby preventing infection by pathogens [7]. It contains high concentrations of alkaloidal sugar-mimic glycosidase inhibitors, such as 1,4-dideoxy-1,4-imino-d-arabinitol (D-AB1), 1-deoxynojirimycin (DNJ) [8-10], and insect-toxic high-molecular-weight factors like chitinase-like protein [9,11]. Chitinases (latex abundant proteins, LA) purified from mulberry displayed insecticidal activities on the larvae of D*rosophila melanogaster,* and a 56-kDa defense protein in mulberry latex (chitinase MLX56) retarded the growth of Eri silkworm, *Samia cynthia ricini* (*Saturniidae*) and the cabbage armyworm, *Mamestra brassicae* (*Noctuidae*) [11].

Since laticifers are not present in well-studied model plants such as *Arabidopsis*, rice, wheat and tobacco, it is of interest to explore the potential effects of heterogeneous expression of latex proteins *in vivo*. Here, we isolated a chitinase gene from the mulberry variety Qiangsang 1 (Q1) using PCR technology, and analyzed its toxicity to *Plutella xylostella* after ectopic expression in *Arabidopsis thaliana*. Based on the growth reduction of *P. xylostella* larvae fed on these transgenic *Arabidopsis*, we conclude that MaMLX-Q1 protein is a potential biochemical insecticide that could be used for crop protection.

MATERIALS AND METHODS

Plant materials

A wild type of Col-0 *Arabidopsis thaliana* was used in the present study. Col-0 seeds were dipped in 70% (v/v) ethanol for 1 min, followed by 10% (v/v) sodium hypochlorite for 5 min, and rinsed five times in sterile distilled water. The seeds were culture-grown on 1/2 Murashige-Skoog (MS) medium (Hopebio, China) under a 14/10 (light/dark) photoperiod at 22°C. The humidity of the climate chambers was maintained at 65%. After 2 weeks, the plantlets were transplanted into pots with common garden soil nutrition for further growth. The mulberry variety Qiangsang 1 (Q1) was planted according to a standard cultivation protocol in the permanent nursery of the Zhejiang Academy of Agricultural Science. Latex exuded from cut petioles was collected and kept at -20°C.

Insects

Plutella xylostella larvae were kindly provided by the Institute of Plant Protection and Microbiology of the Zhejiang Academy of Agricultural Science. Larvae were maintained in a laboratory growth cabinet at 25±1°C and 70% room humidity, with a light, dark photoperiod of 16 and 8 h, respectively. Experimental larvae were cultivated on a 10-cm plate with a water-dipped cotton ball alongside.

RNA extraction

Total RNA was isolated from the mulberry leaves using the TransZol RNA extraction kit (TransGen Biotech, China) according to the manufacturer's instructions. RNA concentration and purity were verified by UV absorption spectra and electrophoresis on agarose gels. Then the total RNA was diluted to 0.5 μg/μL with RNase free water and stored at -80°C for further use.

Rapid amplification of *MaMLX-Q1* cDNA of 3' and 5' ends

After DNase I treatment, the above RNA was reverse-transcribed using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China) at 42°C for 60 min. The degenerate primers MLX-P1 (5′ AGYAYCYWTARWTRSTGYG-GCA 3′, Y=T+C, W=A+T, R=A+G, S=G+C) and MLX-P2 (5' TTATCYCCAKRGCTCACWCCCA 3′, K=T+G) were designed according to the conserved nucleotide sequences of the reported mulberry chitinase-like latex protein in the NCBI database (http://www.ncbi.nlm.nih.gov/). A partial cDNA fragment of MLX-Q1 gene was amplified with the cDNA sample and degenerate primers as follows: 94°C for 2 min, 30 cycles at 94°C for 30 s, 55°C for 30 s and then 72°C for 1.5 min. Amplified products were separated by gel electrophoresis on 1.2% agarose gels at 100 V for approximately 1 h using a 1× TAE buffer (40 mM Tris acetate and 2 mM EDTA in water). After electrophoresis, the PCR products were retrieved by a Gel Extraction Kit (TransGen Biotech, China). Then the amplified PCR products were ligated into the pEASY-T vector (TransGen Biotech, China) and subsequently sequenced by Shanghai Biosune Biotech Co. Ltd. (Shanghai, China). The specific gene primers of MLX-P3 (5′TTGTCATTTCCATTGGTGGTCTC3′, for 5′RACE) and MLX-P4 (5′TTTAGGACCGCC-GTATGGTTTTG3′, for 3′RACE) were synthesized and used in 5′- and 3′RACE, respectively, using the SMARTer® RACE cDNA Amplification Kit according to manufacturer's instructions (Clontech, USA). Positive recombinants were sequenced.

Analysis of nucleotide and deduced amino acid sequences of *MaMLX-Q1*

The obtained intermediate fragments, 3′ and 5′ end sequences of *MaMLX-Q1* were assembled by the online program CAP3 (http://pbil.univ-lyon1.fr/cap3.php). The similarity of the obtained sequence was blasted with the BLAST program (http://www.ncbi.nlm.nih.gov/). The nucleotide sequence was translated into amino acid with the EditSeq software of the DNAStar software package 5.0. Molecular weight and isoelectric point were acquired with Isoelectric (http://isoelectric.ovh.org/). The signal peptide was analyzed by the online SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Conserved domains were searched by the NCBI Conserved Domain Search program (http://www.ncbi.nlm.nih.gov/Structure/cd-d/wrpsb.cgi).

The secondary structure of the MaMLX-Q1 protein was analyzed with an online program (https://www.predictprotein.org/), and the transmembrane

structure domain was analyzed with the online TM-PRED program (http://web.expasy.org/protscale/). Homology of the amino acid sequence was aligned using the DNAMAN software package 5.2.2.

Construction of the recombinant *Agrobacterium tumefaciens* and transformed *Arabidopsis*

A pair of primers (MLX-P5: 5′GG**CTCGAG**AT-GAAGTTTAGAACTCTTTTAATC3′, sense with an *XhoI* site; MLX-P6: 5′GA**GGATCC**TTACATTC-GAGCAACTTCCGAG3′, antisense with a *BamH I* site) was designed to amplify the full length CDS of MaMLX-Q1. PCR products were purified and digested with *XhoI* and *BamHI*, and ligated into the same sites of pFGC5941 under the control of the cauliflower mosaic virus (CaMV) 35S promoter. After verification by sequencing, the recombinant plasmid pFGC-MaMLX-Q1 was transformed into the *Agrobacterium tumefaciens* strain *GV3101* and used for plant transformation. The genetic transformation procedure was based on the *Agrobacterium*-mediated floral-dip method reported everywhere [12]

Screening the positive transformed *Arabidopsis*

Seeds from the transformed *Arabidopsis* were germinated in 1/2 Murashige and Skoog (1/2 MS) agar plates supplemented with 10 μg/mL Basta. The seeds were stratified at 4°C for 2 days and transferred to a growth chamber with 14 h of light and 10 h of darkness at 22°C. Plants that grew well in agar plates containing Basta were transferred to gardening soil (Hawita Ltr, Germany). Transformed *Arabidopsis* were then characterized by PCR to demonstrate the positive insertion of MaMLX-Q1 using the specific primer pair of MLX-P5/MLX-P6.

qRT-PCR analysis of MaMLX-Q1 in transformed *Arabidopsis* lines.

Quantitative real-time PCR (qRT-PCR) assays were performed to analyze the expression level of MaMLX-Q1 mRNA in positive transformed lines of *Arabidopsis*. RNA was extracted and reversed with TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China). The specific primers MLX-P7 (5′GCTTGCTGCTTTCTTTGCTCA3′), MLX-P3

for MaMLX-Q1 qRT-PCR and F-Actin (5′CCCTGC-TATGTATGTGGCTAT3′), R-Actin (5′GCTGTG-GTGGTGAAAGAGTAA3′) for actin (AT2G37620) qRT-PCR were designed to amplify 116 bp and 225 bp cDNA. The amplification of the above two fragments was performed using a StepOnePlus PCR instrument (ABI, USA) with TransStart Top Green qPCR SuperMix (TransGen Biotech, China). The amplifications were conducted by the following procedures: 95°C for 1 min for predenaturation, then 45 cycles at 95°C for 10 s, 56°C for 10 s and 72°C for 45 s. Three replicate biological experiments were conducted. Relative expression levels of *MaMLX-Q1* mRNA were calculated according to the $2^{-\Delta\Delta CT}$ method [13].

Bioassays of ectopic MLX-Q1 against *Plutella xylostella*

To confirm the toxicity to insects of the ectopically expressed MLX-Q1 protein, bioassays were performed using the 3rd instar larvae of *Plutella xylostella*. Detached mature leaves from transgenic and wild-type *Arabidopsis* were prepared and fed to larvae for seven days. Two groups were named: Con (control group) and MaMLX-Q1 (treated group), respectively. Each group contained 36 larvae (12 larvae×3 replicates) at 25°C. Following oral administration, both weight and mortality of the tested larvae were monitored daily.

Statistical analysis

Data were reported as the means±SD. Statistical differences were analyzed with Turkey's multiple comparison tests after one-way ANOVA.

RESULTS

Analysis of the structure and function of *MaMLX-Q1*

A 1405 bp-full-length MaMLX-Q1 cDNA was obtained from mulberry leaf that had a open reading frame (ORF) 1140 bp in length encoding 379 amino acids, and flanked by a 38-bp 5′-untranslated region (UTR) and 227-bp 3′UTR including a poly (A) sequence (GenBank accession: KU757309) (Fig. 1). The deduced amino acids of MaMLX-Q1 had 7.92% of
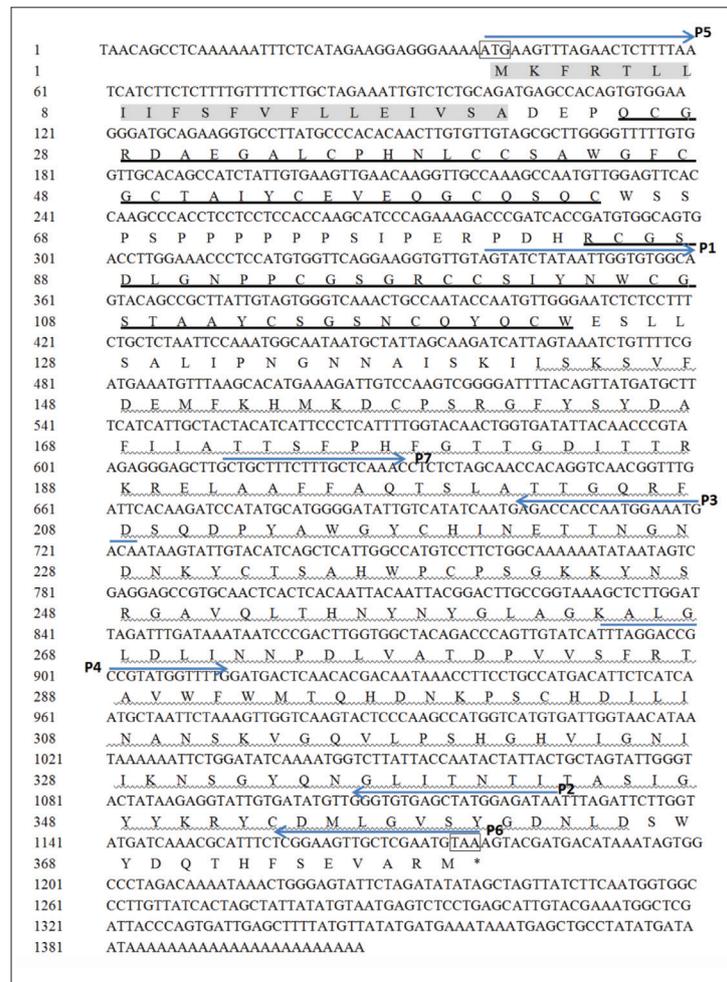
Fig.1. The full-length cDNA nucleotide and deduced amino acid sequence of MaMLX-Q1 are shown above (GenBank accession: KU757309). Initiation codons and stop codons are shown in frames, primers are shown with arrow marks, signal peptides are shadowed. Conserved chitin-binding domains (CBD) and hydrolase family 19 chitinase domain of the MaMLX-Q1 are underlined with black segments and grey squiggly lines, respectively.

helix, 4.22% of strand and 87.86% of loop (https://www.predictprotein.org/). MaMLX-Q1 had a signal peptide of 21 amino acids in length, as reported by the SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) program and deduced as a secretory protein by the TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) program. The mature protein of MaMLX-Q1 contained 358 amino acids with a predicted molecular weight of 39.38 kDa and a calculated isoelectric point of 6.44. It had two typical conserved chitin-binding domains (CBDs) (25-64, 84-123), as well as one glycoside hydrolase family 19 chitinase domain (142-365) (Fig. 1), which is highly consistent with reported findings for other chitinases [11]. Align-

ment analysis showed that MaMLX-Q1 was member of the class I chitinase protein group, and it had more than 89.9% identity (Fig. 2) with latex chitinase proteins from mulberry [9,11].

The identification of recombinant and transformed *Arabidopsis*

The *MaMLX-Q1* gene was placed downstream of the CaMV 35S promoter region in PGFC-5941 vectors and produced pFGC-MaMLX-Q1. Twenty-three independent transgenic lines were generated by agrobacterium-mediated transformation in *Arabidopsis*. In the preliminary screen, six of these lines were identified with
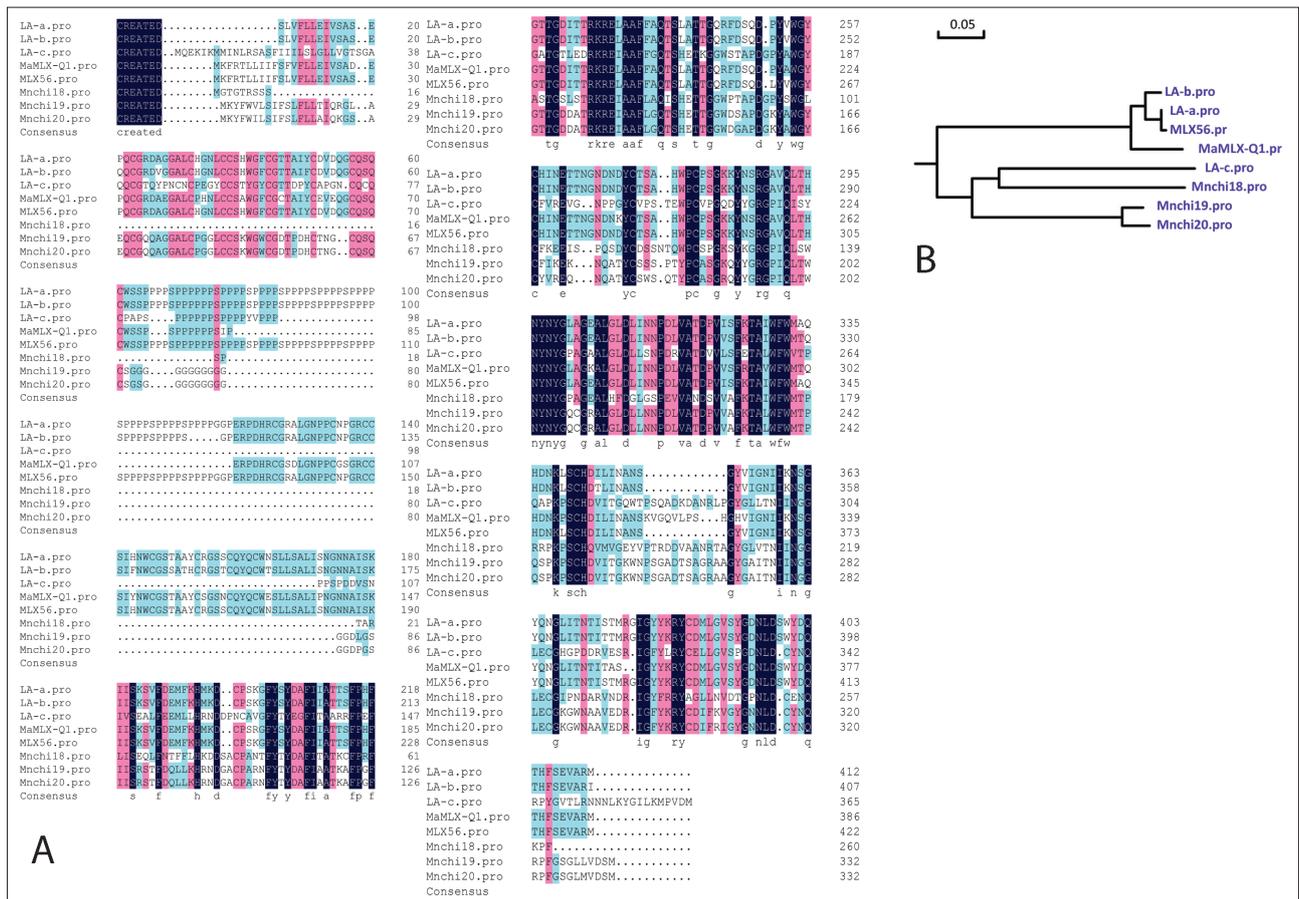
Fig.2. Comparison of the genes encoding mulberry chitinases. **A** – alignment of deduced amino acids sequence of MaMLX-Q1 with other relevant sequences. Identical amino acid residues among the nine sequences are denoted by black boxes, and partially identical residues are denoted by red and blue boxes. **B** – molecular phylogenetic analysis of class I chitinases isolated from mulberry tree. These chitinases included Mnchi18~Mnchi20 [14], MLX56 [11], La-A/La-B [9], and La-C [15].
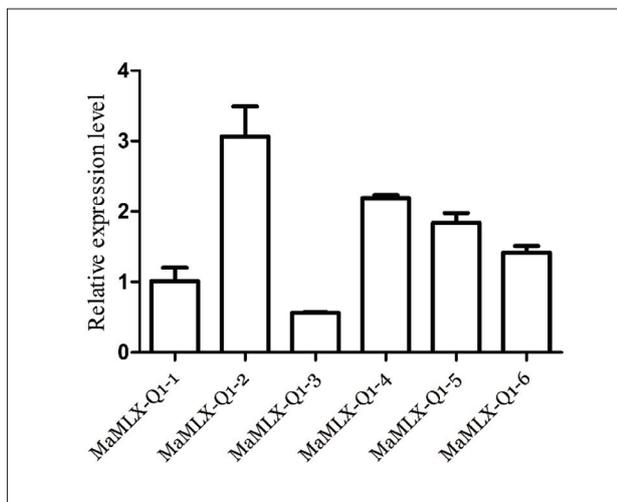
Fig.3. Relative transcriptional expression level of the *MaMLX-Q1* in six different transgenic *Arabidopsis* lines. *Arabidopsis* MaMLX-Q1-5# was used for bioassay assessment on larvae of *Plutella xylostella*.

significant expression level of *MaMLX-Q1* by quantitative PCR. The growth of these transgenic plants showed no observed difference to that of the wild type (data not shown). The transgenic line MaMLX-Q1-5#, with highest transcriptional expression level of *MaMLX-Q1*, was used in bioassay analysis (Fig. 3).

Plutella xylostella growth affected by ectopic MaMLX-Q1 in transgenic *Arabidopsis*

When fed with transgenic *Arabidopsis* leaves from MaMLX-Q1-5# for one week, the *P. xylostella* larvae appeared listless and their growth was significantly reduced. Compared to the control group, larvae exhibited lower weight and higher mortality after ingestion of transgenic *Arabidopsis* leaves containing the ectopic MaMLX-Q1 protein (Fig. 4). In contrast to the 66.46%
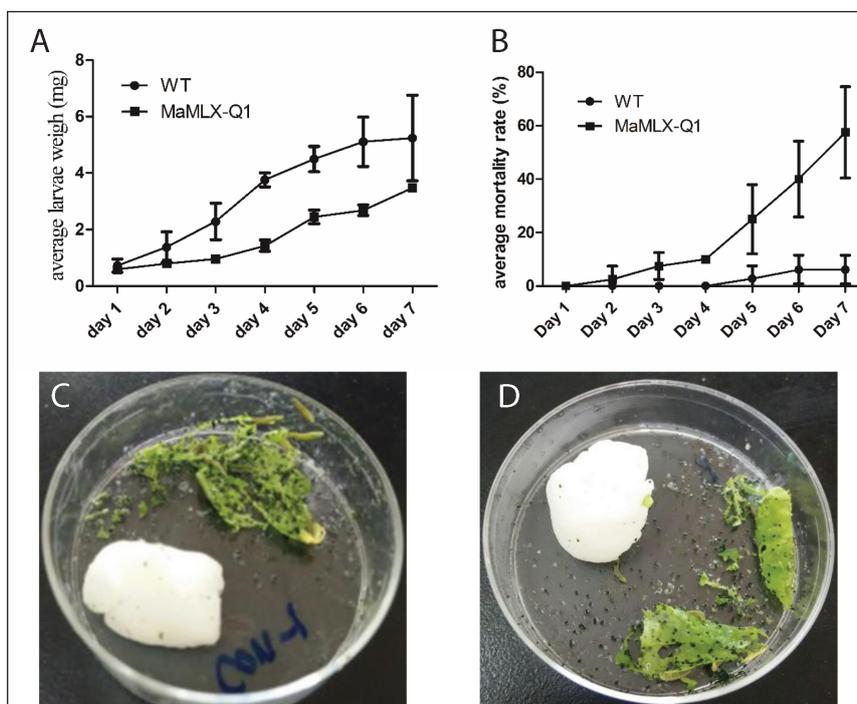
Fig.4. Toxicity tests of ectopic MaMLX-Q1 protein in *Arabidopsis* leaves against larvae of *Plutella xylostella*. Fresh leaves collected from wild-type and transgenic lines (MaMLX-Q1) were randomly divided into three portions to perform the bioassay, respectively. **A** – average weights (mg) of the surviving larvae fed wild-type and transgenic *Arabidopsis* leaves. **B** – average mortality rate of *P. xylostella* larvae fed on wild-type and transgenic *Arabidopsis* leaves. **C** – *P. xylostella* larvae fed on wild-type *Arabidopsis* leaves for 3 days. **D** – *P. xylostella* larvae fed on MaMLX-Q1 transgenic *Arabidopsis* leaves for 3 days. Bioassays were performed in triplicate, with 12 larvae used in each replicate. Error bars indicate ±SD. Scale bar = 10mm.

pupation rate in the control larvae group, there was only a 20.50% pupation rate in the MaMLX-Q1 group, indicating a huge reduction (69.15%) in the pupation rate after ingestion of MaMLX-Q1 leaves. This result strongly suggested that ectopic MaMLX-Q1 was responsible for the toxicity of transgenic *Arabidopsis* to *P. xylostella*.

DISCUSSION

In the present study, a chitinase gene *MaMLX-Q1* was cloned from the mulberry variety Qiangsang 1 that was catalogued to family 19 and class I category [14]. This MaMLX-Q1 shared a higher match (89.6%~96.6%) with chitinase proteins from mulberry latex [9,11,14], and a relatively lower match (44.7%~51.6%) with LA-c (latex abundant protein c) [15] and MnChi18~20 (*Morus nigra L.* chitinases, MnChi) [14] (Fig. 2). Compared to the reported

nucleotide sequence of *MLX56*, *MaMLX-Q1* was found with a 60 bp deletion near its N-terminal (Fig. 2), which indicated that chitinases exhibit diversity within the same clade. Bioassay results indicated that MaMLX-Q1 exerted significant toxicity to *P. xylostella* (Fig. 4). Both the growth rate and pupation rate were greatly reduced after larvae were fed with transformed *MaMLX-Q1* transgenic leaves. Our results were similar to previous reports indicating that purified mulberry latex chitinase protein had toxicity to cabbage armyworm (*Mamestra brassicae*) [11], Eri silkworm (*Samia cynthia ricini*) [11] and *Drosophila melanogaster* [9], but not to the mulberry specialist silkworm (*Bombyx mori*) [11]. LA proteins were supposed to exert insecticidal activity by cooperation with chitinase and some unidentified biochemical activity [9], while MLX56 was highly resistant to the activities of the digestive juices of lepidopteran larvae and of proteases such as chymotrypsin and trypsin after 24-h treatments[11]. As regards MaMLX-Q1,

the conserved CBDs for chitin recognition or binding were supposed to play a role in destroying the insect's cuticle and peritrophic membrane in the midgut lumen. However, details of the mechanism of insect toxicity caused by MaMLX-Q1 is still unknown at this stage. Due to its long history of sericulture, mulberry-silkworm has become one of the oldest and best recognized plant-insect interactions. Mulberry latex and its defensive ingredients play crucial roles, which show no harm to the growth of silkworm but insect toxicity to generalist lepidopteron caterpillars not specialized in mulberry trees [1,7,11]. Some attempts were made to address the molecular bases of the mulberry-silkworm interactions. Unlike some specialists that developed their abilities to avoid consuming latex and to block the flow of latex by cutting veins or using resins when fed on latex-producing plants [16-18], the silkworm is thought to have evolved some sophisticated physiological adaptive mechanism [7]. One possible explanation could be the sugar-mimic alkaloid-insensitive β-fructofuranosidase-type sucrose in *B. mori*. This digestive enzyme was different to the α-glucopyranoside-type sucrase adopted by most insects as their digestive enzyme in the midgut [7,19]. In addition, mulberry micro RNAs (miRNAs) found in the tissues of silkworm were also proposed as evidence of plant-herbivore interactions at a molecular level [20]. However, the overall interaction mechanism between mulberry and silkworm is unclear and still under investigation.

In summary, a chitinase-like gene from the mulberry variety Qiangsang 1 was identified and categorized as class I in the chitinase family 19, and exhibited significant toxicity to the growth and development of *P. xylostella*. This study suggests that mulberry latex MaMLX-Q1 might be a promising insecticide for plant defense in the future.

Authors' contribution: Yan Liu, Zhiqiang Lv, and Dongfeng Ji designed the experiments. Yan Liu carried out the experiments, analyzed the data and wrote the manuscript. Jine Chen and Yan Zhu performed the bioassay of MaMLX-Q1 on *P. xylostella,* and Jia wei, Tianbao Lin took part in screening the transformed *Arabidopsis*.

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