Abnormal swelling of the peritrophic membrane in Eri silkworm gut caused by MLX56 family defense proteins with chitin-binding and extensin domains

*Graphical Abstract

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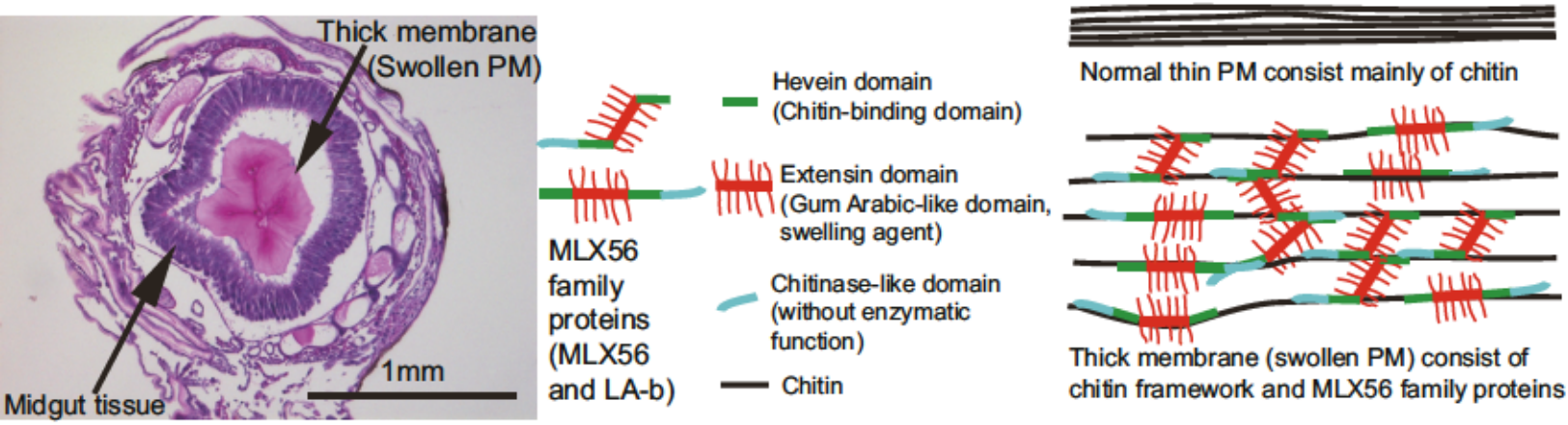
Kotaro Konno*, Sachiko Shimura, Chihiro Ueno, Toru Arakawa, and Masatoshi Nakamura

MLX56 family anti-insect chitin-binding defense proteins, MLX56 and LA-b, from mulberry latex, exert unique defensive activities by binding to gut peritrophic membrane with hevein domains and swelling it into abnormally thick gel-like membrane.



Highlights

MLX56 family proteins in mulberry latex, exert unique anti-insect defense activities.

Gut peritrophic membrane (PM) of Eri silkworms fed MLX56 and LA-b swells abnormally.

A hard gel-like swollen PM made of chitin, MLX56, and LA-b inhibits insect growth.

Chimera lectin MLX56 binds to PM by hevein domain and swells PM by extensin domain.

Abnormal swelling of the peritrophic membrane in Eri silkworm gut caused by MLX56 family defense proteins with chitin-binding and extensin domains

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Abstract

MLX56 family defense proteins, MLX56 and its close homolog LA-b, are chitin-binding defense proteins found in mulberry latex that show strong growth-inhibitions against caterpillars when fed at concentrations as low as 0.01%. MLX56 family proteins contain a unique structure with an extensin domain surrounded by two hevein-like chitin-binding domains, but their defensive modes of action remain unclear. Here, we analyzed the effects of MLX56 family proteins on the peritrophic membrane (PM), a thin and soft membrane consisting of chitin that lines the midgut lumen of insects. We observed an abnormally thick (>1/5 the diameter of midgut) hard gel-like membrane consisted of chitin and MLX56 family proteins, MLX56 and LA-b, in the midgut of the Eri silkworms, *Samia ricini*, fed a diet containing MLX56 family proteins, MLX56 and LA-b. When polyoxin AL, a chitin-synthesis-inhibitor, was added to the diet containing MLX56 family proteins, the toxicity of MLX56 family proteins disappeared and PM became thinner and fragmented. These results suggest that MLX56 family proteins, through their chitin-binding domains, bind to the chitin framework of PM, then through their extensin-domain (gum arabic-like structure), which functions as swelling agent, expands PM into an abnormally thick membrane that inhibits the growth of insects. This study shows that MLX56 family proteins are plant defense lectins with a totally unique mode of action, and reveals the functions of extensin domains and arabinogalactan proteins as swelling (gel-forming) agents of plants.

Key words: *Morus alba*, Moraceae, MLX56 family proteins, arabinogalactan protein, chitin-binding lectin, extensin domain, plant latex defense protein, peritrophic membrane, plant-insect interaction, swelling (gel-forming) agent

1. Introduction

Plant latex plays important defensive roles against herbivorous insects, and contains various defense chemicals and defense proteins (Dussourd and Eisner, 1987; Farrell et al., 1991; Konno et al., 2004; Agrawal and Konno, 2009; Konno, 2011). Mulberry trees (*Morus spp.*, Moraceae), which are known as host plants of the silkworm *Bombyx mori*, exude latex to defend themselves against non-specialist herbivores (Konno et al., 2006).

We previously detected, purified and characterized two types of defense substances in mulberry latex; the first type consisted of several sugar-mimic alkaloids such as DNJ (1-deoxynojirimycin) and D-AB1 (1,4-dideoxy-1,4-imino-D-arabinitol) (Konno et al., 2006), which function as inhibitors of sugar-metabolizing enzymes such as sucrase and trehalase and are therefore toxic to generalist insects (Hirayama et al., 2007), and the second type consisted of MLX56, a defense protein (lectin) with unique characteristics. MLX56 is a 56 kDa glycosylated protein composed of 394 amino acids (Wasano et al., 2009). MLX56 is highly toxic to generalist insects, and shows a strong growth-inhibitory activity against lepidopteran larvae such as those of the Eri silkmoth (*Samia ricini*, Saturniidae) or cabbage armyworm, (*Mamestra brassicae*, Noctuidae), even when added to the artificial diet at very low concentrations of 0.01-0.03% (Wasano et al., 2009). This is much stronger than the growth inhibition achieved by protease inhibitors or other well-studied plant defense proteins, which must be added to the artificial diet at concentrations of 1-2% to reduce the growth of caterpillars (Bell et al., 2001). Sequence analyses of MLX56 have shown that it has a unique structure: the N-terminal region consists of an extensin domain surrounded by two hevein-like chitin-binding domains, and the C-terminal region consists of a chitinase-like domain

which has no or weak activity presumably because of a mutation in the catalytic domain (Wasano et al., 2009). As expected due to its hevein-like chitin-binding activity, MLX56 shows strong binding activity to chitin. MLX56 is highly resistant to digestion by proteases and digestive juices of insects (Wasano et al., 2009), which is commonly seen in many plant defense proteins that act inside insect midgut (Chen et al., 2007).

Soon after we purified and characterized MLX56 (Wasano et al., 2009), another group of scientists, who had been analyzing mulberry latex proteins, identified and characterized two very closely related latex proteins, LA-a (estimated size, 50 kDa) and LA-b (estimated size, 46 kDa), both of which are very abundant (equally abundant) major proteins in petiole latex of mulberry, and have strong lethal effects on the larvae of the fruit fly, *Drosophila melanogaster* (Kitajima et al., 2010, 2012). The sequence data showed that LA-a is identical to MLX56 with 394 amino acids, while LA-b (389 amino acids) is a very close homolog of LA-a (MLX-56) that shows 95% sequence identity with LA-a (MLX56) (Kitajima et al., 2012). The sequence data further showed that LA-b share a unique structure with MLX56: the N-terminal region consists of an extensin domain surrounded by two hevein-like chitin-binding domains, and the C-terminal region consists of a chitinase-like domain which a mutation in the catalytic domain (Kitajima et al., 2012; Wasano et al., 2009). The above similarities between MLX56 and LA-b suggested that these proteins can be treated as a family (Kitajima et al., 2012).

In spite of the strong defense activity of MLX56 family proteins, MLX-56 and LA-b, and the available information on their molecular structures, the modes of their defensive functions of MLX56 family proteins remain unclear. It would be reasonable to suppose that the chitinase-like domain in the C-terminal region of MLX56 family proteins

functions like chitinase and damages insect structures made of chitin, including the peritrophic membrane and cuticle. However, this explanation seems unlikely, since MLX56 family proteins have very weak or no chitinase activity, presumably because of the mutations in amino acids observed in their catalytic sites, and this is particularly true under the alkaline conditions in the caterpillar midgut (Wasano et al., 2009; Kitajima et al., 2010, 2012). Another possible explanation is that the chitin-binding domains (hevein domains) in the N-terminal region bind to the peritrophic membrane (PM), a thin membrane composed of chitin and proteins in the midgut lumen of insects (Wang and Granados, 2001), and cause some abnormality in the PM. This seems possible, since MLX56 family proteins are resistant to digestion by protease and retain chitin-binding activity under the alkaline conditions in the caterpillar midgut (Wasano et al., 2009). However, even if the binding of MLX56 family proteins to PM takes place, it is still difficult to explain the mode of the toxic activity of MLX56 family proteins. Further, it is necessary to explain the meaning of the unique structure of the N-terminal region of MLX56 family proteins, i.e., the unique sandwich-like composition of domains, namely the "hevein domain (chitin-binding domain) – extensin domain – hevein domain" structure (Wasano et al., 2009; Kitajima et al., 2012). What is the meaning of this structure, and why does it include an extensin domain? Extensin domains are composed of repeats of (Ser-Pro-Pro-Pro-Pro) sequences that are highly glycosylated by arabinose oligomers in proline residues (Stafstrom and Staehelin, 1986; Shpak et al., 2001). Extensin domains are frequently found in proteins in the plant cell wall (Estévez et al., 2006; Lamport et al., 2011), and are known to be involved in root hair formation (Baumberger et al., 2001), in the pollen tube growth and guidance (Nguema-Ona et al., 2012; Pereira et al., 2015; Mizukami et al., 2016) and to be included in plant exudates

(Goodrum et al., 2000; Dror et al., 2006). In acacia, highly arabinosilated glycoproteins that are composed mostly of extensin domains together with structurally closely related arabinogalactan and other arabinogalactan proteins comprise the major components of gum arabic, a well-known exudate that is discharged from damaged points of plants (Goodrum et al., 2000; Dror et al., 2006). Despite their unique but uniform structures of extensin domains, the common physiological or physical functions of the extensin domains in above proteins, which are diverse in biological functions, have not been explained.

Therefore, the purpose of the present study was to elucidate the mode of the defensive function of MLX56 family proteins against insects, and to clarify the role of each domain in this defensive activity, by observing and biochemically analyzing the PM of caterpillars fed an MLX56 family protein-containing diet. We succeeded in revealing the unique effect of MLX56 family proteins on the PM that is responsible for the defensive activity of MLX56 family proteins (i.e., the thickening of the PM), and in clarifying the important role played by the extensin domains as swelling agents.

2. Results

2.1. Abnormally thick membrane in the midgut lumen of Eri silkworm larvae fed an MLX56 family protein-containing diet

Neonate larvae of the Eri silkworm that were fed an artificial diet containing

MLX56 family proteins, MLX56 and LA-b, at a concentration of 10% latex equivalent (or 0.02%) for 48 h showed a significant growth inhibition compared to the larvae that were fed the control diet (L4M) (Table 1).

In order to examine the effects of MLX56 family proteins on the PM, neonate larvae of the Eri silkworm (*Samia ricini*, Saturniidae) were fed an artificial diet containing MLX56 family proteins (MLX56 and LA-b) at a concentration of 20% latex equivalent (or 0.04%) for 24 h, then the larvae were fixed with paraformaldehyde, cut into sections, and were PAS-stained. Then, the sections were observed using an optical microscope, and were compared with the samples prepared from larvae that were fed a diet that did not contain MLX56 family proteins. A normal thin PM was observed throughout the midgut lumen of the Eri silkworm larvae fed a control diet (Fig. 1 a-e). In contrast, an abnormal thick membrane was observed in the midgut lumen of the Eri silkworm fed an artificial diet that contained a 20%-latex-equivalent concentration (0.04%) of MLX56 family proteins (Fig. 1f-j). The longitudinal vertical section showed that the membrane was particularly thick in the posterior part of the midgut (Fig. 1j), and transverse sections showed that the thickness of the thick membrane reached 1/5 the diameter of the midgut lumen (Fig. 1h and i) or even more (Supplementary Fig. S1a and b). The thick membrane could be recovered from the 3rd instar larvae of the Eri silkworm fed the MLX56 family protein-containing diet (0.04% or 20% latex equivalent for 24 h) by dissection (Fig. 2). While the PM collected from Eri silkworm fed the control diet was thin, soft, and wavering in the buffer (Fig. 2a and b), the membrane collected from the MLX56-fed larvae was thick, hard, gel-like, and not wavering, and retained its tubular shape even when moved in the buffer (Fig. 2c and d). The images of thick membranes of MLX56 protein-fed larvae in Fig. 2f-j may appear somewhat fragmented and

discontinuous with a lot of cracks, but it is likely to have resulted from paraformaldehyde fixation processes, because both the thick membranes fixed with Carnoy's solution (Supplementary Fig. S1a and b) and the thick membranes collected by dissection had continuous membrane-like structures.

2.2 Association of PM thickened by MLX56 family proteins with defensive function of these proteins

In order to examine the mode of the growth-inhibitory effect of MLX56 family proteins against caterpillars, and in particular, whether the thickened PM in the midgut lumen of the caterpillars fed MLX56 family proteins is involved in the strong toxicity of MLX56 family proteins, experiments were performed by feeding caterpillars MLX56 family proteins, MLX56 and LA-b together with polyoxin AL, an inhibitor of chitin synthesis. Polyoxin AL is a mixture of polyoxins, with polyoxin B being the major component (Arakawa et al., 2008). Polyoxins and the structurally related nikkomycins are potent inhibitors of chitin synthesis, because they function as analogs of UDP-*N*-acetylglucosamine (UDP-GlcAc), a precursor of chitin synthesis by chitin synthase (Cohen, 1987). Polyoxins and nikkomycins are strongly toxic (lethal) to insects because they inhibit the synthesis of chitin in cuticles during the molting stages (Cohen, 1987; Arakawa et al., 2008). However, during the intermolting stages they are not toxic, and reduce the growth of insects only slightly (Arakawa et al., 2008). Nevertheless, the insects fed these UDP-GlcNAc analogs during the intermolting stages showed interesting symptoms such as total or partial loss and disorder of the PM (Cohen, 1987; Arakawa et al., 2010). Therefore, we performed bioassays using polyoxin

AL to assess the involvement of the PM in the toxicity of MLX56 family proteins (Table 1). The neonate Eri silkworm larvae (1.4 mg) which were fed a diet containing polyoxin AL (0.1%) for two days grew well and the larval mass (9.07 ± 2.70 mg) was not significantly different from that of the larvae fed the control L4M diet (10.96 ± 1.19 mg), while the larvae that were fed a diet containing 10% latex equivalent or 0.02% of MLX56 family proteins showed significant growth reduction (6.92 ± 0.51 mg) (Table 1). Surprisingly, the toxicity of MLX56 family proteins disappeared when the larvae were fed a diet containing both polyoxin AL and MLX56 family proteins, and the larval mass of the larvae (9.22 ± 1.29 mg) was not significantly different from that of the larvae fed a control diet, but was significantly larger than that of the larvae fed an MLX56 family protein-containing diet (6.92 ± 0.51 mg) (Table 1). This result is compatible with the idea that MLX56 family proteins exert growth-inhibitory activity by modifying the PM into a structure having growth-inhibitory activity, and with the idea that a normal PM made of chitin is necessary for MLX56 family proteins to exert growth-inhibitory activity. In agreement with these ideas, the microscopic image of the midgut section of the Eri silkworm fed polyoxin AL and MLX56 family proteins (Fig. 1p-t) showed a membrane-like structure that was thicker than the PM of larvae fed a control diet (Fig. 1a-e) or a diet containing polyoxin AL alone (Fig. 1k-o), but that was much thinner (Fig. 1p-t) than the thickened PM of the larvae fed a diet containing MLX56 family proteins alone (Fig. 1f-j). These results indicated that the thickened membrane in MLX56 family protein-fed larvae was related to the strong growth inhibitory effects of MLX56 family proteins. In order to examine whether the digestion and absorption of proteins are involved in defensive activities, we fed a cellulose-based artificial diet consisting of glucose alone and no protein/amino acid-sources to

Eri-silkworms for one day in the presence or absence of MLX56 family proteins, and found that MLX56 family proteins still exhibited a strong growth-inhibitory activity when added to a diet without protein (Supplementary Table 1). This result shows that some process other than protein digestion, might be disrupted by the thick membrane.

2.3. Biochemical analyses of the thick membrane of MLX56 family protein-fed larvae

In order to examine whether the thick membrane observed in the gut lumen of the MLX56 family protein-fed Eri silkworms was a structure related to the PM or a structure with no relationship to the PM, the biochemical components of the thick membrane were analyzed. When the thick membrane collected from the 3rd instar larvae fed an artificial diet containing MLX56 family proteins, MLX56 and LA-b, at a concentration of 20% latex equivalent (or 0.04%) for 24 h was boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer, the thick, hard (Fig. 2c and d), gel-like membrane become a thin soft wavering membrane (Fig. 2e and f) that was similar to the ordinary PM (Fig. 2a and b). Proteins extracted from the thick membrane after boiling in the SDS-PAGE buffer contained a large amount of pure MLX56 family proteins, MLX56 (56 kDa) and LA-b (50 kDa) (Fig. 3, lane 6), although MLX56 family proteins were minor components in the MLX56 family protein-containing artificial diet that was fed to the larvae (Fig. 3, lane 4). No bands corresponding to MLX56 family proteins were detected either for the PM of the larvae fed the control L4M diet (Fig. 3, lane 5) or for the control L4M diet itself (Fig. 3, lane 3). This result indicated that MLX56 family proteins, MLX56 and LA-b, are the major proteinaceous components of the thick membrane, and that MLX56 family proteins

were specifically concentrated in the thick membrane. It is estimated from quantitative data that approximately 17 % of the MLX56 family proteins fed to the Eri silkworm existed in the thick membrane, whereas MLX56 family proteins consisted approximately 5 % of the dry mass of the thick membrane. On the other hand, the thin membrane that remained after the boiling of the thick membrane in SDS-PAGE buffer was digested and dissolved when the thin membrane was incubated with chitinase solution (from *Trichoderma viride*, 0.1% solution) and finally disappeared within 2-20 h (Fig. 2g-k), which indicated that the framework of the thick membrane consisted of chitin. The above results are shown in Fig. 2 and Fig. 3. Taken together, they indicate that the thick membrane is a structure composed of a large amount of MLX56 family proteins bound to a framework made of chitin, and suggest that MLX56 family proteins modify and convert the framework of the PM into a thick membrane.

3. Discussion

3.1. MLX56 family proteins are plant defense proteins with a unique mode of action

The present study shows a unique and novel mode of action of MLX56 family defense proteins, i.e., the induction of abnormal thickening or swelling of the PM. Several studies have suggested that the PM is a potential target of plant defense proteins (Wang and Granados, 2001), and that proteins which interact with chitin, a major component of PM, function as plant defense proteins against herbivorous insects. Among those are chitinase (Kramer and Muthukrishnan, 1997), chitin-binding lectins

such as WGA (Hopkins and Harper, 2001), and chitin-binding proteases such as Mir1 (Pechan et al., 2002). In insects which were fed chitinase or Mir1, the PM was damaged and contained many small holes (Pechan et al., 2002), and therefore these proteins were suggested to exert defensive activities primarily by damaging the PM. The effect of MLX56 family proteins on the PM, which is the formation of an abnormally thickened (or swollen) continuous membrane that occupies a considerable part of the midgut lumen (Fig. 1f-j, Supplementary Fig. S1a,b), is strikingly different from the effects of the above chitin-interacting proteins that weaken, thin, fragment, and/or make innumerable holes (Kramer and Muthukrishnan, 1997;Hopkins and Harper, 2001; Wang and Granados, 2001; Pechan et al., 2002). Our experiments using polyoxin AL (Fig. 2p-t, Table 1) indicated that the thick membrane functions as a defense and inhibits the growth of insects. One possible mechanism by which the thickened membrane inhibits the growth is that the swollen membrane functions as an obstacle to digestive processes in the midgut, such as movement and absorption of nutrients from the gut lumen to midgut cells and secretion of digestive enzymes from midgut cells to the midgut lumen. We fed a cellulose-based artificial diet consisting of glucose alone and no protein/amino acid-sources to Eri-silkworms for one day in the presence or absence of MLX56 family proteins, and found that MLX56 family proteins still exhibited a strong growth-inhibitory activity when added to a diet without protein (Supplementary Table 1). This result shows that some process other than protein digestion is inhibited by the thick membrane. One candidate process for such inhibition is water absorption. Another possible mechanism is that the thick membrane, by physically occupying a large portion (space) of the gut lumen (Supplementary Fig. S1b), simply decreases the available space for food material and thereby decreases the uptake of food material. These points

should be clarified in the future.

3.2. Why do MLX56 family proteins thicken the PM?: The primary function of the extensin domain as a swelling agent and/or gel-forming agent and implicated function of arabinogalactan proteins

The above results lead to several important questions. Why do MLX56 family proteins thicken the PM? What are the functions of the domains in MLX56 family proteins, and the extensin domain in particular? It is quite noteworthy that both MLX56 family proteins and gum arabic share swelling and gel-forming characteristics. When the gum arabic-containing exudates discharged from the damaged points of Acacia plants dry out and then absorb water from dew or rain, they swell into a unique ball-shaped hard-gel of up to several centimeters' diameter. If there is excess water, the gum arabic gel finally dissolves, but when the gum arabic molecules are artificially crosslinked and polymerized, the gum arabic polymer forms an insoluble superabsorbent hydrogel that can absorb an extremely large amount of water and can swell to 500 hundred times the original volume of the dry polymer (Favaro et al., 2007). Since gum arabic is made up of proteins having extensin domains in which arabinose oligomers are attached to a $(Ser\text{-}Pro\text{-}Pro\text{-}Pro\text{-}Pro)_n$ backbone, together with arabinogalactans, which also have a structure with arabinose oligomers attached to a galactose oligomer backbone (Goodrum et al., 2000; Dror et al., 2006), it seems reasonable to expect that the extensin domain of MLX56 family proteins confers the gel-forming / swelling ability to these molecules.

Therefore, the likely scenario by which MLX56 family proteins thicken the PM is as

follows (Fig. 4). First, MLX56 family proteins bind to a chitin-based framework by their chitin-binding (hevein) domains. Second, the local concentration of the extensin domains (of MLX56 family proteins) become high as in gum arabic. Under the moist conditions in the midgut lumen, the extensin domains swell and form a thick gel-like structure (i.e., the swollen membrane observed in the midgut lumen of the insects which were fed MLX56 family proteins, MLX56 and LA-b). Unlike the gel consisting of gum arabic, which dissolves in water, the thick gel (swollen membrane) formed by MLX56 family proteins bound to the chitin framework will never dissolve in water, because the extensin domains of MLX56 family proteins that form the gel are firmly bound to the chitin framework, and the chitin framework itself is insoluble to water. This insoluble thick membrane may have similarity with the superabsorbent hydrogel composed of crosslinked gum arabic polymer (Favaro et al., 2007). Our observation that the thick membrane collected from the MLX56 family protein-fed caterpillars by dissection became thin again when boiled in SDS-PAGE buffer (Fig. 2c-f) on detachment of MLX56 family proteins, MLX56 and LA-b, from the membrane under this treatment (Fig. 3, lane 6) supports the idea that proteins with an extensin domain may function as swelling / gel-forming agents.

The MLX56 family protein-preparation that were fed to the Eri silkworm in the bioassays and biochemical analyses in the present study contained MLX56 and LA-b as major components in comparable concentrations with some other contaminant proteins, and was not pure (Fig. 3, lane 2). Further, the MLX56-family protein-containing artificial diet contained a lot of proteins derived from soybean together, and MLX56 and LA-b were not even major components in this diet (Fig. 3, lane 4). However, the proteins detached from the thick membrane was pure MLX56 family proteins mixture

free of other contaminant proteins, and MLX56 and LA-b existed in comparable concentration again (Fig. 3, labe 6). These facts, together with the facts that MLX56 and LA-b share a very high sequence identity (> 95 %), and that the both proteins shares the unique sandwich-like composition of domains, namely the "hevein domain (chitin-binding domain) – extensin domain – hevein domain" structure (Wasano et al., 2009; Kitajima et al., 2012), suggest that both MLX56 and LA-b contributes to thickening of the PM and growth inhibitory effects against the Eri silkworms by the same mode of action. However, the observed PM-thickening effects and growth inhibitory effects of MLX56 family protein-preparation are supposed to be the means of the effects of two MLX56 family proteins, MLX56 and LA-b, and there remains the possibility that the effects of MLX56 and LA-b might be slightly different. This possibility should be clarified in the future.

Moreover, this defensive activity involving thickening and swelling of the chitin-based structure may not be limited to MLX56 family proteins. Since Solanaceae lectins, including tomato lectin and potato lectin, share with MLX56 family proteins the sandwich-like "hevein domain-extensin domain-hevein domain" structure (Peumans et al., 2003; Van Damme et al., 2004), it is possible that Solanaceae lectin may have a defensive activity similar to that of MLX56 family proteins. This possibility should be examined in the future.

It is also expected that other proteins with an extensin domain function as swelling / gel-forming agents, and the resulting formed gels would be insoluble to water if the proteins had bound to insoluble supports such as the chitin-membrane and cellulose membrane or if the proteins had become crosslinked with each other. Extensin proteins in the cell wall of plants may function as swelling agents that swell the cellulose-based

thin backbone of the cell wall, rendering the wall sufficiently bulky and strong to support the forces prevailing under wet conditions. Extensin proteins and structurally related arabinogalactan proteins (AGP), both of which are highly arabinosylated, have been suggested to play important roles in the formation of root hairs and in the pollen tube growth and guidance, but the detailed modes of the actions of these remains mysteries (Baumberger et al., 2001; Nguema-Ona et al., 2012; Pereira et al., 2015; Mizukami et al., 2016). However, some of these functions can be reasonably understood if the gel formation takes place in the root hair and pollen tubes; the formed gel would exert inner and/or outer force or pressure that would control the extending pattern and direction change of root hair and pollen tube (Baumberger et al., 2001; Nguema-Ona et al., 2012; Pereira et al., 2015; Mizukami et al., 2016). The functions of extensin proteins and arabinogalactan proteins as swelling agents or gel-forming agents of plants should be examined in detail in future studies.

3.3. Implications for the modes of the biological actions of lectins and chimera lectins

Although the defensive functions of plant lectins against insect herbivores have long been studied, and the binding of lectins to PM and the disorder of microvilli in midut cells have been reported, the detailed molecular modes of these actions remain mostly unexplained (Chrispeels and Raikhel, 1991; Vandenborre et al., 2011; Li-Byarlay et al., 2016; Lagarda-Diaz et al., 2017). The present study about MLX56 family proteins clearly shows the detailed molecular mode of action of lectin as a defense protein. Our

results showed that MLX56 family proteins are not simple lectins with chitin-binding moieties, but lectins with an additional functional moiety, an extensin domain, with swelling activity, and MLX56 family proteins function as swelling agents when they bind to chitinous structures. Although the mere binding of a simple lectin with a chitin-binding domain to the chitinous structures may only have minimal effects, the lectin activities (chitin-binding activities) of MLX56 family proteins are crucial for their physical and biological activities (PM swelling and growth inhibition against insects); the lectin activities of MLX56 family proteons (hevein domains) localize and anchor the functional moieties of the defense proteins (extensin domains) at the site of the target molecules (PM). Lectins with additional functional moieties, known as chimera lectins, are also found in other cases. For example, Mir1, a chimeric protein with a hevein domain and a cysteine protease domain, inhibits the growth of caterpillars at very low concentrations and disrupts the PM (Pechan et al., 2002), which suggests that the protease moiety localized on the PM anchored by the chitin-binding activity of the lectin moiety digests the PM proteins which are necessary to maintain the structure and function of the PM. Therefore, at least in some lectins, the carbohydrate-binding moieties of lectins localize and anchor the functional moieties at the positions (carbohydrate-containing structures) optimal for their function, and this idea may be important to understand the biological functions of some lectins, including the defensive functions of plant lectins against herbivores.

4. Conclusions

We showed that MLX56 family proteins, MLX56 and LA-b, which are defense

proteins in mulberry latex against herbivorous insects, have a unique mode of defensive action; the induction of swelling of the peritrophic membrane (PM). We found that MLX56 family proteins, when fed to insect caterpillars, modify the peritrophic membrane (PM), a thin membrane consisting of chitin and protein in the insect midgut lumen, into an abnormally thick gel-like membrane consisting of MLX56 family proteins and chitin, which leads to inhibition of caterpillar growth. Our results suggest that MLX56 family proteins, with their chitin-binding domains, bind to the chitin framework of the PM, then use their extensin domains (a gum arabic-like structure) as swelling agents to expand the PM into an abnormally thick membrane. This study shows a novel mode of action (PM-thickening) of plant defense proteins (plant lectins), and discovered the function of extensin domains and arabinogalactan proteins as swelling (gel-forming) agents of plants.

5. Experimental

5.1. MLX56 family protein preparation from mulberry latex

Mulberry latex was collected from a cultivar Shin-Ichinose that belongs to the species *Morus alba* (Moraceae). Latex was collected by cutting the petioles of young leaves, then catching the exuded latex in ice-cold screw tubes (15 ml); the tubes were then kept at -20 °C. For preparation of the MLX56 family protein solution used in the experiments and bioassays, 4.5 ml of latex was mixed with 4.5 ml of 100% ethanol. Then the mixture was centrifuged (2,000 g, 25 °C, 15 min) and the pellet was collected by discarding the supernatant. The pellet was washed by adding 10 ml of 70% ethanol

with mixing, and after centrifugation (2,000 g, 25 °C, 15 min), the supernatant was discarded and the pellet was collected. The washing process was repeated twice. In this way, low molecular-mass defense chemicals such as sugar-mimic alkaloids (Konno et al., 2006) were removed. Then the pellet was lyophilized, weighed, and dissolved in a 4.5 ml (original latex volume) of PBS. This solution contained MLX56 (56 kDa) and its close homolog LA-b (50 kDa) as its major components in similar concentration to each other, although it contained some other proteins as minor components (cf. Fig. 3, lane 2). The defense activities (growth-inhibitory activities) of MLX56 family proteins were not lost by this preparation procedure. This MLX56 family protein preparation (MLX56 family protein solution), which had the same volume as the original latex, contained approximately 0.2% (wt/wt) of active MLX56 family proteins, and was used in all the experiments and bioassays in this study.

5.2. Insects used in experiments

A line of the Eri silkmoth, *Samia ricini* (Saturniidae), that is maintained at our institute as an experimental insect line was used for the bioassays and analyses to verify the effects of MLX56 family proteins on growth and on the structure and composition of the PM. Eri silkmoth larvae have been successfully used in bioassays to detect novel plant defense factors and mechanisms, such as cysteine proteases in papaya latex (Konno et al., 2004), sugar-mimic alkaloids (Konno et al., 2006) and novel chitin-binding defense protein MLX56 (Wasano et al., 2009) in mulberry latex, defense protein (lectin) BPLP in the phloem exudate of cucurbit plants (Ota et al., 2013), and the synergistic defense mechanism between raphides and protease in kiwifruits (Konno et

al., 2014). The neonate larvae or the third instar larvae that were reared on an L4M artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) used for polyphagous insects based on soybean powder were used in the experiments and bioassays. The L4M artificial diet, which was purchased as a dry powder, was mixed with a 2.5 times its mass of distilled water and then steamed for 30 min. Then, after cooling and solidification, the wet L4M diet was used for rearing the Eri silkworms and for the bioassays and experiments involving them. Tungjitwitayakul and Tatun (2017) reported that a small amount (4%) of mulberry leaf powder contained in L4M diet has moderate growth retarding effect on the Eri silkworm which is presumably caused by sugar-mimic alkaloids derived from mulberry leaf powder. In regard to MLX56 family proteins, however, no effects, neither thickening of the PM nor elution of MLX56 family proteins from the PM, were observed in Eri silkworm larvae that were fed the control L4M diet (cf. Fig. 1a-e; Fig. 2a, b; Fig. 3, lane 5), which means that the effects of MLX56 family proteins derived from the L4M diet can be neglected.

5.3. Bioassay of the growth-inhibitory effect of MLX56 family proteins and the rescue effect of polyoxin AL

Three hundred mg of one of the following L4M diets was fed to five neonate Eri-silkmoth larvae at 25 °C: (1) a control L4M diet; (2) an L4M diet containing 0.02% (or 10% latex-equivalent) of MLX56 family proteins (i.e. an L4M diet containing 10% of MLX56 family protein preparation); (3) an L4M diet containing 0.1% of polyoxin AL; (4) an L4M diet containing both 0.02% of MLX56 and 0.1% of polyoxin AL. Forty-eight hours later the masses of larvae were measured individually. The bioassays

were performed in duplicate (total 10 larvae for each treatment). Polyoxin AL (water soluble powder), a mixture of polyoxins, potent inhibitors of chitin synthesis, with polyoxin B being the major component (Arakawa et al., 2008), was purchased from Kaken Pharmaceutical (Tokyo, Japan).

5.4. Histochemical observation of the midgut and peritrophic membrane

Neonate Eri silkmoth larvae were fed one of the following diets for 24 h at 25 °C: (1) a wet L4M diet (100 mg); (2) a wet L4M diet (100 mg) containing 0.02% (or 10% latex-equivalent) of MLX56.; (3) a wet L4M diet (100 mg) containing 0.03% polyoxin AL; (4) a wet L4M diet (100 mg) containing both 0.02% (or 10% latex-equivalent) of MLX56 and 0.03% polyoxin AL. The larvae were fixed as follows. Fixing solution (4% paraformaldehyde) was injected into the bodies of larvae (hemolymph) using a syringe. Then the heads and tails were cut off from the larvae, and the body parts including the midguts were soaked and kept in fixing solution (4% paraformaldehyde) overnight. Then, the fixed samples were dehydrated and n-butanol-substituted by soaking the body samples sequentially in phosphate-buffered saline (PBS), for 5 min, 3 times; 1-Butanol (BtOH):Ethanol (EtOH):Distilled water (DW)=10:40:50, 30 min; BtOH:EtOH:DW=20:50:30, 30 min; BtOH:EtOH:DW=35:50:15, 30 min; BtOH:EtOH:DW=55:40:5, 30 min; BtOH:EtOH=75:25, 30 min; BtOH 100%, 30 min, 3 times, at room temperature. Paraffin-embedding of the samples was performed by keeping the samples sequentially in BtOH:paraffin=1:1 for 1 h, 3 times; 100% paraffin, 1 hour; 100% paraffin, overnight, at 63 °C; followed by cooling and molding. Samples embedded in paraffin were then sliced into cross and longitudinal sections of 5 μm

thickness, placed on glass slides, kept at 42 °C for a while to allow the slices to extend on the glass slide, and then kept under 4 °C until staining. The periodic acid-Schiff (PAS) staining of the samples on the glass slides was performed by treating the samples as follows: 100% Lemosol (Wako Pure Chemical Industries, Japan), 1 min, twice; Lemosol:xylene=1:1, 1 min; 100% EtOH, 1 min, 4 times; ion-exchanged water 5 min; 1% periodic acid, 5 min; washing with DW, 3 times; Schiff reagent, 15 min; washing with DW, 5 min; 95% EtOH, 1 min; 100% EtOH, 1 min; 100% EtOH, 5 min, twice; EtOH:Lemosol=1:1, 15 min; 100 Lemosol, 15 min, 3 times. After the inclusion, the PAS-stained samples were observed using an optical microscope. The method used to take the images presented in Supplementary Fig. S1 was identical to that used for the images in Fig. 1 except that Carnoy's solution (ethanol:chloroform:acetate = 6:3:1) was used for fixation as follows. After the head and tail parts were trimmed from the paraformaldehyde-injected larvae, the remaining body parts were soaked in Carnoy's solution for 10 min and then twice for 1 h. Then, the body parts were washed twice in 100% EtOH for 1 h at 25 °C. After washing, the samples were soaked in 100% EtOH at 4 °C overnight. The samples were then dehydrated and paraffin-embedded using EtOH:Lemosol=1:1, 15 min, 25°C; EtOH:Lemosol=1:3 15 min, 25 °C; 100% Lemosol, 15 min, 4 times, 25 °C; xylene:paraffin=1:1, 60 min, 63 °C; 100% paraffin, 60 min, 2 times, 63 °C; followed by cooling and molding.

5.5. Biochemical analyses of the peritrophic membrane

Newly molted third instar larvae of the Eri silkmoth were fed either an L4M diet (control diet) or an L4M diet containing 0.04% MLX (20% latex-equivalent of MLX56)

(MLX56-containing diet), for 24 h at 25 °C. The larvae were dissected in PBS using scissors, and the tubular peritrophic membranes (PMs) inside the midgut lumen were collected using forceps. The collected PMs were washed twice in PBS to eliminate all gut contents, including materials from artificial diets and the digestive fluid, until the PMs became colorless. Approximately 6.2 mg wet mass (0.2 mg dry mass) of the PM was collected from three larvae for each diet. After taking photos of the collected PMs floating in PBS using an optical microscope, the PMs were dried (lyophilized). The dried PMs (0.2 mg for each diet) were then boiled in 50 µl of SDS-PAGE buffer (containing 0.5M Tris-HCl pH 6.8, 5 µl; SDS, 0.5 mg; glycerol 10 µl; bromophenol blue (BPB) 5 µg; dithiothreitol (DTT), 0.39 mg) in a microtube (1.5 ml) for 5 min at 96 °C. After cooling on ice, the SDS-PAGE buffer (containing proteins extracted from the PM) was collected using a micropipette, and kept at -20 °C for SDS-PAGE analysis. The boiled PMs from the larvae fed an MLX56-containing diet, which were very thin and soft after boiling but were thick and hard before boiling, were collected and boiled with the same SDS-PAGE buffer again. Then the PMs were collected, washed twice with 1 ml of PBS, and suspended in PBS in a petri dish. After taking photos using an optical microscope, the boiled PMs were suspended in PBS containing 0.1% chitinase from *Tricoderma viride* (C-8241, SIGMA; 1,300 units / g solid) at 25 °C, and observed using an optical microscope in order to examine whether the thin membrane was digested and whether the thin membrane consisted of chitin. SDS-PAGE of proteins was performed using a 15% gel (ATTO Corp., Tokyo, Japan). After electrophoresis, the gels were stained with Bio-Safe™ Coomassie brilliant blue G-250 stain (Bio-Rad Laboratories Inc., Hercules, CA). Precision Plus Protein™ Standards (Bio-Rad Laboratories Inc.) were used as standards.

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Appendix A. Supplementary data

Supplemantary Fig. S1 Additional images of the extremely swollen peritrophic membrane (PM) observed in the midgut lumen of the Eri silkworm fed an MLX56 family protein-containing diet.

Supplemantary Table 1 The growth-inhibitory effect of MLX56 family proteins added to a non-protein diet.

Figure captions

Fig. 1. Abnormal thickening of the peritrophic membrane (PM) observed in the midgut lumen of Eri-silkworms fed MLX56 family protein-containing diets and the effects of polyoxin AL, an inhibitor of chitin synthesis. Microscopic images of PAS-stained paraffin sections of 1st instar larvae of the Eri-silkworm that were fed either a control diet (a-e), a diet containing MLX56 family proteins (20% latex equivalent or 0.04%) (f-j), a diet containing polyoxin AL (0.03%) (k-o), or a diet containing both MLX56 family proteins and polyoxin AL (p-t) are shown. The images (a-d, f-i, k-n, p-s) show cross sections and the images (e, j, o, t) show longitudinal sections. The vertical lines in longitudinal images indicate the approximate positions where cross sections were made. Arrows indicate the PM or related membrane-like structures observed in the midgut lumen. mc, midgut cells; cu, cuticle. The bars indicate 0.5 mm. Abnormally thick membrane-like structures were observed in the midgut lumen of larvae fed the MLX56 family protein-containing diet instead of the thin PM observed in the midgut

lumen of the larvae fed the control diet. When polyoxin AL was fed to the larvae together with MLX56 family proteins, the membrane in the midgut lumen became thinner, cracked, and discontinuous.

Fig. 2. Characterization of the thick membrane collected from the midgut lumen of Eri-silkworms fed an MLX56 family protein-containing diet. The ordinary PM collected from third instar larvae of the Eri-silkworm fed the control diet was a very thin membrane that fluttered in water when observed under an optical microscope (a, b). In contrast, the membrane collected from the midgut lumen of 3rd instar larvae fed the MLX56 family protein-containing diet (20% latex equivalent or 0.04%) had thick, hard, and rigid-gel like features (c, d). After boiling in SDS-PAGE buffer, the thick membrane turned into a very thin and soft membrane (e, f). This thin membrane was digested and disappeared when incubated with chitinase (from *Trichoderma viride*, 0.1%) for 2-20 h, indicating that the framework of the thick membrane consisted of chitin. The bars indicate 2 mm.

Fig. 3. SDS-PAGE analysis of the protein composing the thick membrane. The following samples were applied to SDS-PAGE. Lane 1: Molecular mass standard; Lane 2: MLX56 family proteins (MLX56 protein-preparation which was added to the diet, and which contains MLX56 and LA-a as major components) (1 μg or 0.5 μl latex equivalent); Lane 3: Control L4M artificial diet (soybean-based) without MLX56 family proteins) (40 μg as dry matter); Lane 4: MLX56 family protein-containing diet

(L4M diet + MLX56 family proteins) (40 μg dry matter diet containing 56 ng MLX56 or 28 nl latex equivalent MLX56 family proteins); Lane 5: Proteins extracted from the normal PM (40 μg as dry matter) of the third instar larvae fed a control L4M diet without MLX56 family proteins by boiling in the SDS-PAGE buffer; Lane 6: Proteins extracted from the thick membrane (40 μg dry matter of membrane) in the midgut of the third instar larvae fed the MLX56 family protein-containing diet. Although MLX56 family proteins, MLX56 and LA-b, were minor components in the MLX56 family protein-containing diet (lane 4), MLX56 family proteins, MLX56 and LA-b, were by far the most abundant proteins in the thick membrane, and MLX56 proteins, MLX56 and LA-b, were extracted from the thick membrane in a pure form (lane 6). This result and the result in Fig. 2 indicate that the thick membrane is composed of a large amount of MLX56 family proteins, MLX56 and LA-b, bound to the framework made of chitin.

Fig. 4 A proposed model for the swelling effect of MLX56 family proteins and the function of the extensin domain. The structure of MLX56 family proteins includes an extensin domain sandwiched between two hevein (chitin-binding) domains. The extensin domain is a major component of gum arabic. Gum arabic is known to have a unique character that dry form of it swells greatly and form a steady gel after absorbing as it absorbs a large amount of water until it finally dissolves in water when it gets excess water. The extensin domain in MLX56 family proteins may well have similar swelling or gel-forming property in the presence of moisture. MLX56 family proteins also have hevein domains which bind to chitin. When a large amount of MLX56 family protein molecules bind to an insoluble chitin framework, which alone is a thin membrane, the local concentration of extensin domains will be sufficiently high to form

a steady gel, but the formed gel will be insoluble to water because the MLX56 family protein molecules are bound to an insoluble chitin framework. It is likely that the thick membrane (thickened PM) is formed by MLX56 family proteins in this way. Further, it was suggested that the primary role of extensin domains in various extensin domain-containing proteins is to function as swelling or gel-forming agents.

Table 1. The growth-inhibitory effect of MLX56 family proteins on the Eri silkworms and the rescue effect of polyoxin AL

	Larval mass (mg)
Control diet (L4M diet)	10.96 ± 1.19^{a}
+MLX56 family proteins (0.02% or 10% latex equivalent)	6.92 ± 0.51^{b}
+Polyoxin AL (0.1%)	9.07 ± 2.70^{a}
+MLX56 family proteins (0.02% or 10% latex equivalent)+Polyoxin AL (0.1%)	9.22 ± 1.29^{a}

Neonate Eri-silkmoth larvae (1.5 mg) were fed the above diets for 2 days (48 h), and the larval mass (average ± SD, $n = 10$) was measured. The values of larval mass not followed by the same letters were significantly different ($P < 0.05$; Tukey's test for multiple comparisons).

Figure 1

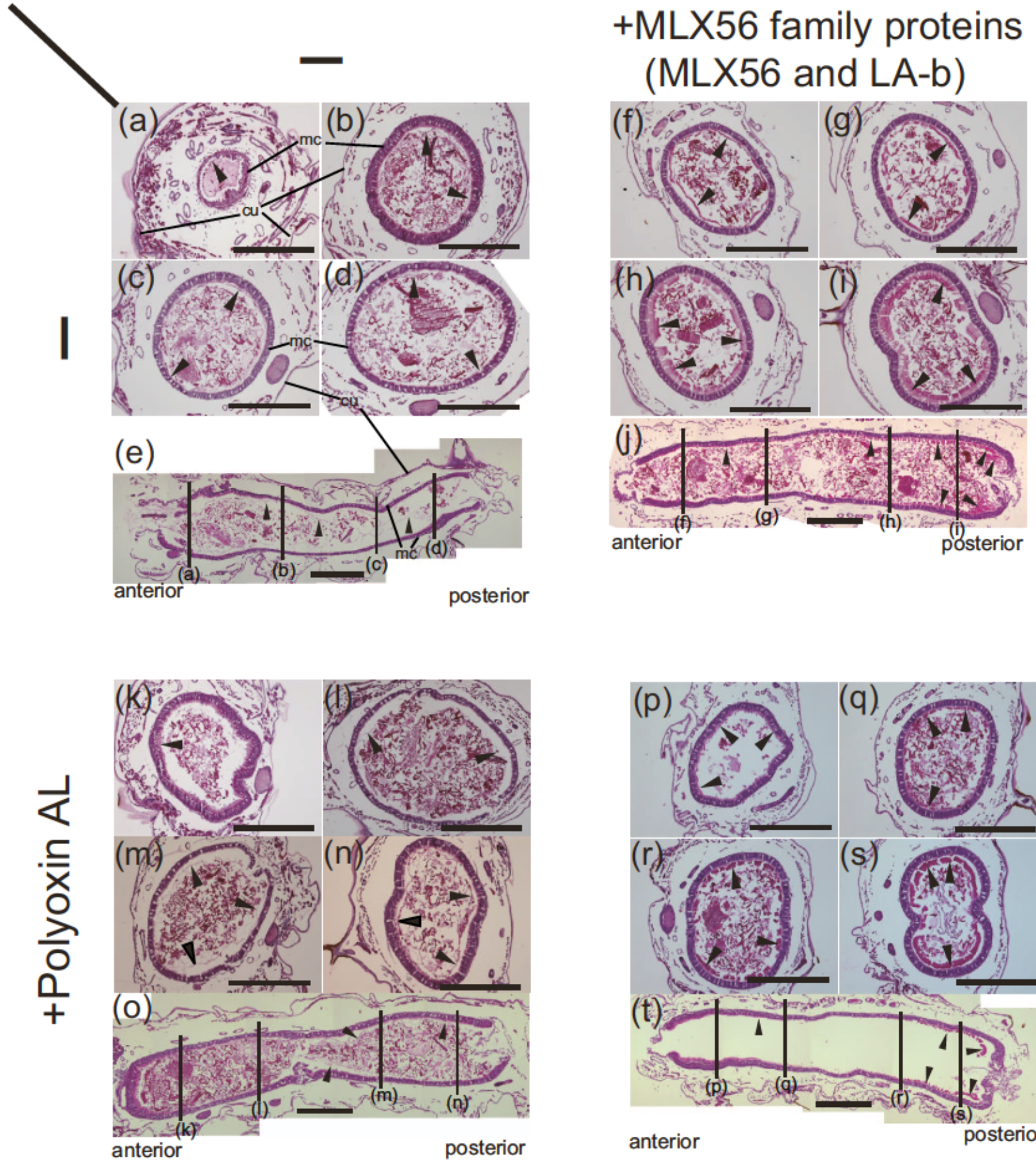

+MLX56 family proteins
(MLX56 and LA-b)
+Polyoxin AL
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)
(n)
(o)
(p)
(q)
(r)
(s)
(t)
mc
cu
anterior
posterior

Figure 2

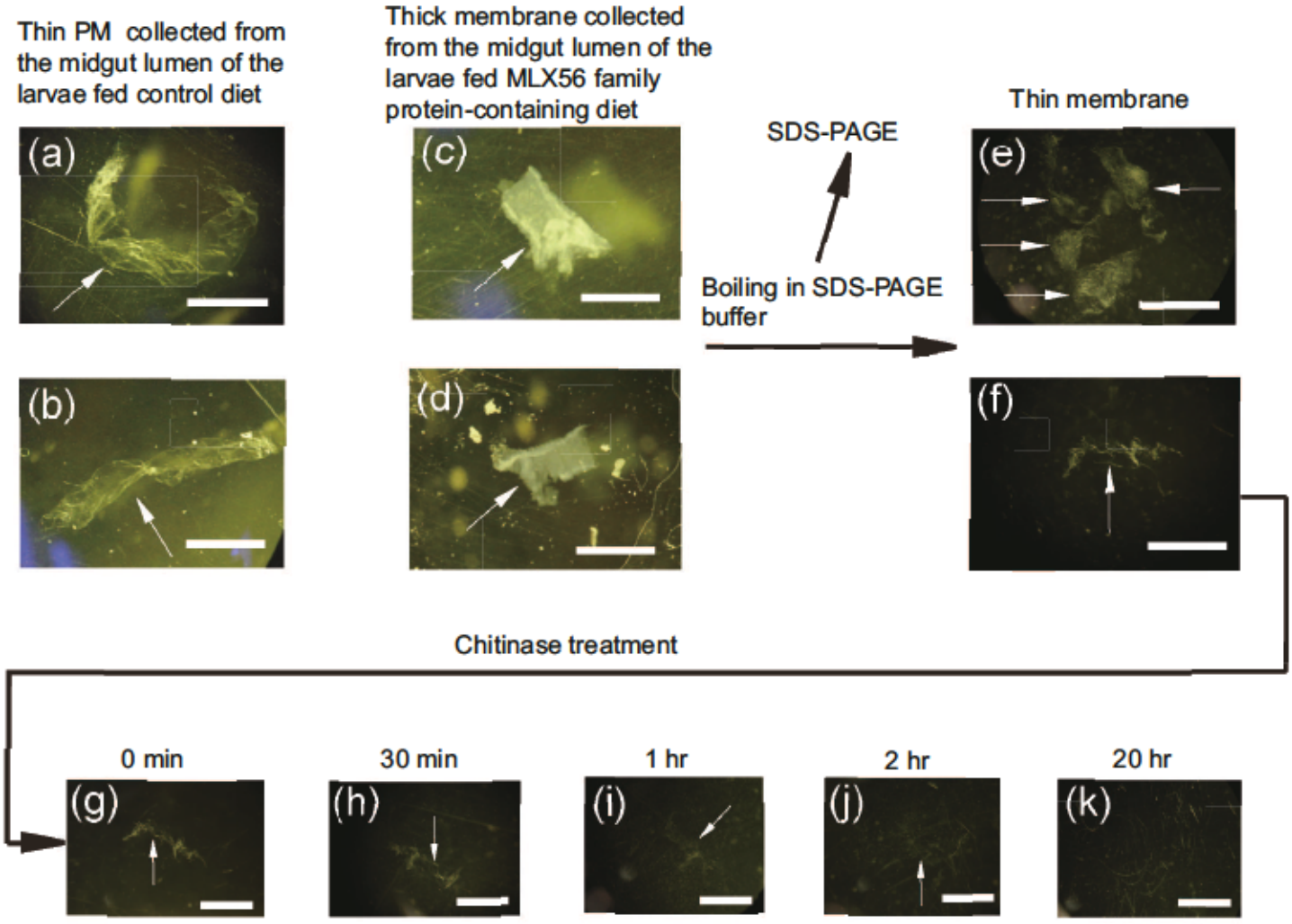

Thin PM collected from the midgut lumen of the larvae fed control diet
Thick membrane collected from the midgut lumen of the larvae fed MLX56 family protein-containing diet
SDS-PAGE
Thin membrane
(a)
(c)
(e)
Boiling in SDS-PAGE buffer
(b)
(d)
(f)
Chitinase treatment
0 min
30 min
1 hr
2 hr
20 hr
(g)
(h)
(i)
(j)
(k)

Figure 3

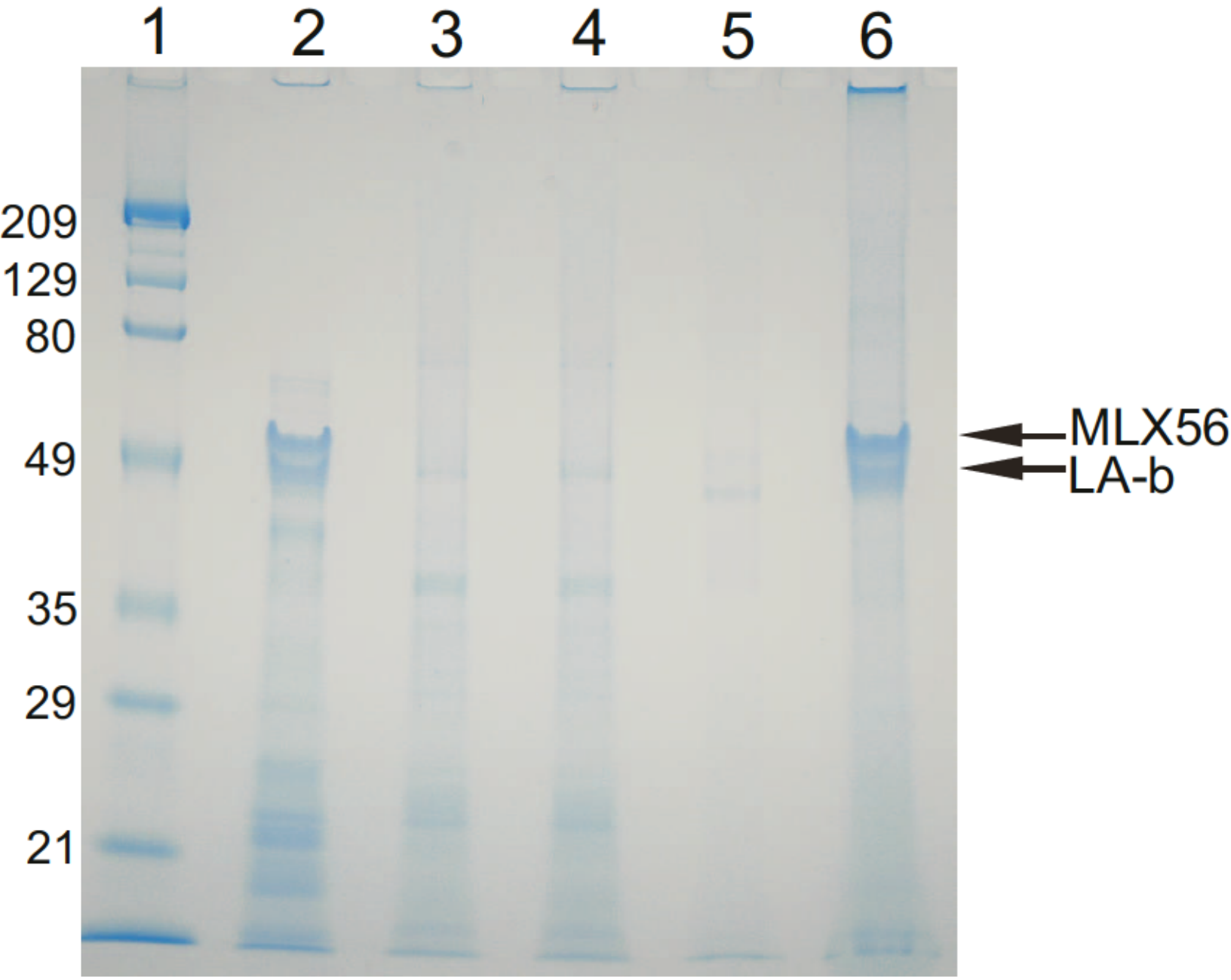
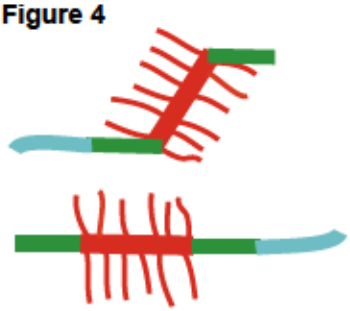


Figure 4



MLX56 family proteins (MLX56 and LA-b)

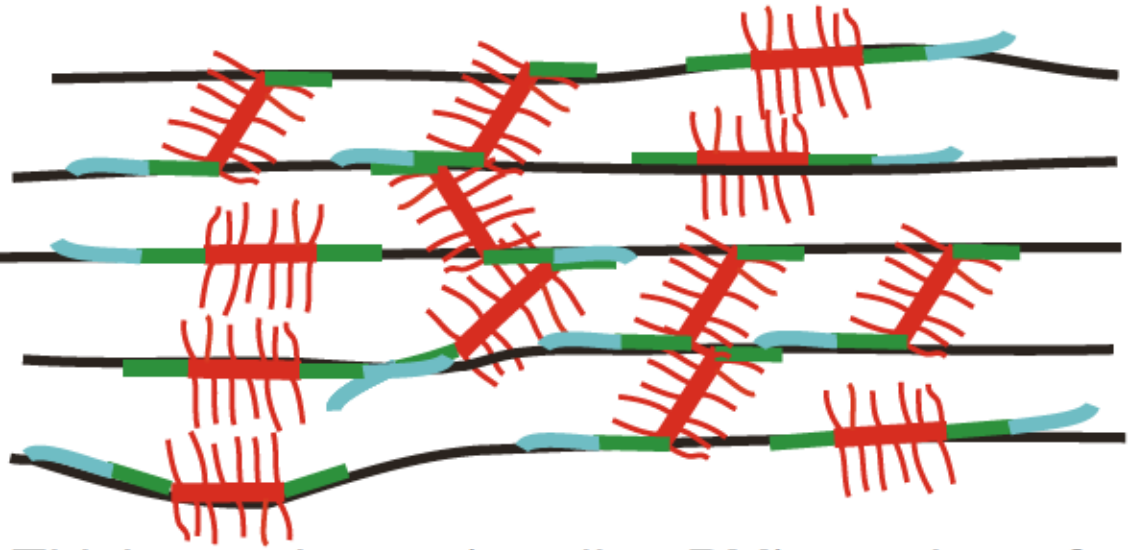
Hevein domain (Chitin-binding domain)

Extensin domain (Gum Arabic-like domain, swelling agent)

Chitinase-like domain (without enzymatic function)

Chitin

Normal thin PM consists mainly of chitin



Thick membrane (swollen PM) consists of chitin framework and MLX56 family proteins bound to it (Extensin domain or Gum Arabic-like structure swells the membrane in wet conditions while chitin framework makes the swollen structure insoluble to water)

Supplementary Information

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