

The involvement of Chloride Intracellular Channel expression and binding interaction to Emamectin benzoate in *Plutella xylostella*.

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Abstract. *Plutella xylostella* collected from Tsaotun, Nantaou (TT) strains has resistance to emamectin benzoate for more than 400-fold. Results from previous studies, the target sites GABA receptors and glutamate receptors, were not detected any mutation. So that the next generation sequencing (NGS) was carried out and showed that the expression of Chloride Intracellular Channel (CLIC) in emamectin benzoate selected Tsaotun (TT_{sel}) strain was much higher than that of emamectin benzoate relax Tsaotun (TT_{rx}) strain. In this study, the quantitation-comparative cycle threshold ($\Delta\Delta C_T$) was analyzed. The 4th larvae of 45th generation of TT_{sel} and TT_{rx} using a qPCR machine were used as samples. The results showed that an increase in the expression of the CLIC gene in TT_{sel} strain was detected in comparison with TT_{rx} strain. The expression of the CLIC genes in female larvae is higher than that of male larvae, especially the CLIC X1 gene. It may be the cause of lepidopteran resistance to emamectin benzoate. The phylogenetic tree analysis showed that CLIC X1 and CLIC X2 genes are also preserved in some lepidopterans. There are indications the presence of nuclear localization signals (NLS) conserved only in CLIC X1 which is important in the resistance to emamectin benzoate.

1 Introduction

Chloride Intracellular Channel (CLIC) protein is one of the newer classes of putative intracellular ion channels. Although it is one class of putative intracellular channels, CLIC has different properties and compartments. Such differences include the presence of a single transmittance domain when conditions dissolve, and dimorphic when forming members [1,2]. The previous study has found the tissue of *D. melanogaster* CLICs distribute on the hemocytes and retina and have a function to the protective role during apoptosis and response to oxidative stress. The disruption of the CLICs effectively decreased its sensitivity to alcohol. On other invertebrates, the CLICs of *Caenorhabditis elegans* have response to heat stress. Under the heat shock, the CLICs translocate to the nucleus from the cytoplasm in the body wall muscle cells and intestinal cell. [3–5]. The CLIC1 which is integrated into the membrane can be influenced by the N-domain located in amino acids between 1-90bp. Supported by a helical structure that forms a putative transmission active around the redox active site [6]. The CLIC functions as a sensor during oxidative stress received in vertebrates. In addition, CLIC also acts as an effector in cells that experience oxidative stress. CLIC in vertebrates to respond to oxidative stress undergoes movement of soluble cytosol into a channel in the plasma member. Chanel in the plasma member provides conductivity to chloride to maintain cell balance. Cell excitability is caused by anionic flow which is produced excessively by active NADPH oxidase. Later, the anionic compound if not released out of the cell will cause superoxide formation supported by the enzyme [7]. Another study from Al Khamici et al. in 2015 concluded that CLIC also has enzymatic activities similar to

glutaredoxin-like as monothiol when dissolved. CLIC also has an additional role, CLIC proteins also have a role in cellular processes of detoxification and oxidoreduction [8].

Emamectin benzoate is an insecticide that is effective in lepidopterans and has a broad spectrum. This insecticide is considered effective for lepidopterans because it shows moderate resistance [9]. Emamectin benzoate can affect the chromatin condensation process in the nucleus and cell apoptosis. In addition, emamectin benzoate can accumulate Reactive Oxygen Species (ROS) and abnormal activities of the antioxidant enzymes that increased oxidative stress and mediate damage to Vitamin E. DNA in the insect cells [10,11]. Emamectin benzoate is one of the new antibiotic pesticides. Emamectin benzoate is an insecticide that works by entering into the leaf tissue of plants through translaminar transfer. This insecticide is an avermectin family with a modification to epi-methyl amino (-NHCH₃) into the hydroxyl (-OH) group at the 4th position in the disaccharide position and produced as benzoate salt. Emamectin benzoate is a mixture of two homologous compounds, consisting of 90% avermectin B1a (MAB1a) benzoate and <10% avermectin B1b (MAB1b) benzoate. The two avermectin have a structure of 4"-deox-4"-(epi-methyl amino) with a difference in MAB1b additional methylene units in the side chain on C-25. Emamectin benzoate (MK244) has better thermal stability because it has more water solubility and a broader spectrum of activity than avermectin. Currently emamectin benzoate is developed as a broader spectrum insecticide that is newer for vegetables and has a very low application level [12–14].

The *Plutella xylostella* collected from Tsaotun, Nantaou field has developed 414.51-fold resistance to emamectin benzoate in comparison with *P. xylostella* lab-susceptible population [15,16]. The results from previous studies, the target sites on emamectin benzoate, GABA receptor and glutamate receptor, do not mutate. So that the next generation sequencing (NGS) test was performed which showed that the 598-bp cDNA gene (CL450.Contig3_All) on the emamectin selected Tsaotun (TT_{sel}) strain was much higher (The log₂-fold change is about 11.9) than the emamectin benzoate relax Tsaotun (TT_{Rx}) strain. The CL450.Contig3_All gene is very homologous (97.4%) with C-terminal nucleotide residue 347 from the Chloride Intracellular Channel (CLIC) in *P. xylostella*. The results from BLAST on GenBank indicate that the CLIC has 2 variants with an accession number. XM_011552148.1 for variant X1 and XM_011552149.1 for variant X2 [17]. CLIC proteins come in two different forms, dissolved globular forms and integral membrane proteins. Based on the CLIC structure of *D. melanogaster*, *P. xylostella* CLICs (GenBank accession no. XP_011550450.1 and XP_011550451.1) do not include all 2 transmembrane terminal structures suspected of N- including helices 1 and β -sheet 1. Protein. the structure has 9 helices-a and 4-sheets containing concentrated C-terminal and N-terminal domains consisting of 260 amino acids [18]. Based on the results of cloning on cDNA that has been carried out by Chang 2018 [17], *P. xylostella* CLIC consists of two variants (variants X1 and X2) in TT_{sel} and TT_{Rx} strains. The two variants of CLIC was predicted two isomer of amino acid sequences. CLIC isomer X1 and CLIC isomer X2 has also been predicted to be preserved in TT_{sel} and TT_{Rx} strains. In an attempt to elucidate the role of CLIC involved in the molecular mechanism of Emamectin benzoate resistance in *P. xylostella*, the full-length cDNA of CLIC from *P. xylostella* was cloned. In addition, the molecular characteristics of CLIC variant X1 and X2 was analyzed, and its expression profiles in different sex were also examined by qPCR.

2 Material and Methods

2.1 Insect

The Emelectin benzoate-selection (TT_{Sel}) and relax (TT_{Rx}) strain of Diamondback moth (*P. xylostella*) were originally collected from Tsautsun, Caotun, Nantou, and were rearing in the laboratory with (TT_{Sel}) or without (TT_{Rx}) Emelectin benzoate-selection over 45 generations in the Laboratory of Insecticide Toxicology, Department of Entomology, National Chung Hsing University [15]. The sample was tested using Emelectin benzoate with a resistant ratio of TT_{Sel} and TT_{Rx} were 951 and 26.74-fold, compared with susceptible strain.

2.2 Molecular characteristics of CLIC variant X1 and X2

Sequence of PxCLIC obtained from the NGS data of TT_{Sel} and TTR_x was blasted using Basic Local Alignment Search Tool (BLAST) and the incomplete complementary DNA (cDNA) sequences of PxCLIC transcript variant X1 (XM_011552148) and variant X2 (XM_011552149) were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/>). By 5'- and 3'-race, the full length cDNAs of variant X1, (947 bp) and X2 (884 bp) including 5'- and 3'-untranslated regions, were obtained, and the translated regions encode a 281 amino acids of X1 and a 260 amino acids of X2 [17]. Based on these sequences, phylogenetic analyses, and homology modeling of *P. xylostella* were performed.

The *P. xylostella* CLIC sequence data were analyzed using Bioedit sequence alignment editor software and sequences analyzed, determined using AutoAssamblar (Perkin-Elmer Co.). The similarity / similarity with the closest reference channel on the DNA of Bank Data can be known by doing Blast analysis. Multiple Alignment of sequences was carried out with the Clustal W program (ver. 1.6). To construct phylogenetic trees, a neighbor-joining algorithm method is used [19]

The CLIC isoform X1 and CLIC isoform X2 protein were characterized with ExPASy Proteomic tools (<http://www.expasy.ch/tools/>). Homology-based structural modelling was performed by Swiss-Model (Arnold et al., 2006). The display of three-dimensional structure with PyMol (<https://pymol.org/2/>).

2.3 Molecular docking analysis

To find active sites, docking pockets, and comparing CLIC isoform X1 with Emelectin benzoate. Molecular docking was done using AutoDock Vina and its associated tools. The structure of protein PDB was constructed by eliminating water molecules and heteroatoms while optimizing the interaction inserted polar hydrogen. The three-dimensional grid of size 66 × 56 × 54 Å is used with 3D coordinates (4.402, -8.060, and 8.874) to define the region of interactions. The final prepared models and ligands were docked with AutoDock vina (<http://bioinfo3d.cs.tau.ac.il/PatchDock/patchdock.html>). The binding affinity and RMSD values were also calculated. A binding affinity of -7 kcal/mol was set as the threshold for screening the best-docked models. Models with binding affinity <-7 kcal/mol and 0.00 RMSD value were selected for further detailed study. The selected docked models were visualized in the discovery studio.

The Emelectin benzoate pesticides was built using the Gaussview5.0 program. Consequently, the electrostatic potential (ESP) charges of each ligand were computed by HF/6-31G(d) level of theory using Guassian09 program [20]

2.4 RNA extraction and cDNA synthesis

The total RNA from 5 fourth-instar larvae was extracted using TRIzol Reagent Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. The integrity of the total RNA was examined on 1% agarose gels, and the concentration was determined with NanoDrop 2000 (Thermo Scientific, Wilmington, USA). The cDNA for PCR was synthesized from 1 mg total RNA using Prime Script RT Reagent Kit with gDNA eraser (TaKaRa Biotechnology, Dalian, China) following the manufacturer's instructions.

2.5 Quantification of *P. xylostella* CLIC by RT-qPCR

The relative expression of *P. xylostella* CLIC in TT_{Sel} and TT_{Rx} populations were compared with that of the susceptible strain population by qRT-PCR using a Platinum® SYBR® Green qPCR SuperMix-UDG Kit (Invitrogen) following the manufacturer's instructions. A total of 10 third-instar larvae were randomly sampled for the total RNA isolation and cDNA synthesis. The total RNA of the above cold stored samples was isolated, and the cDNA was synthesized as described in Section 2.4. Then the qPCR was performed on an ABI 7500 Real Time PCR System (Applied Biosystems, Foster, CA). The cyclic thermal procedure contained an initial denaturation step at 95°C for 10 s, following 40 cycles at 95°C for 15 s and 72°C for 45 s, then a dissociation step was performed, the specificity of the PCR products was assessed by melting curve analysis for all samples. Primers used for RT-qPCR were listed in Figure 1, and ribosomal protein S13 was used as an internal control according to Li et al. (2017)[21]. Three biological replicates were set up for all treatments.

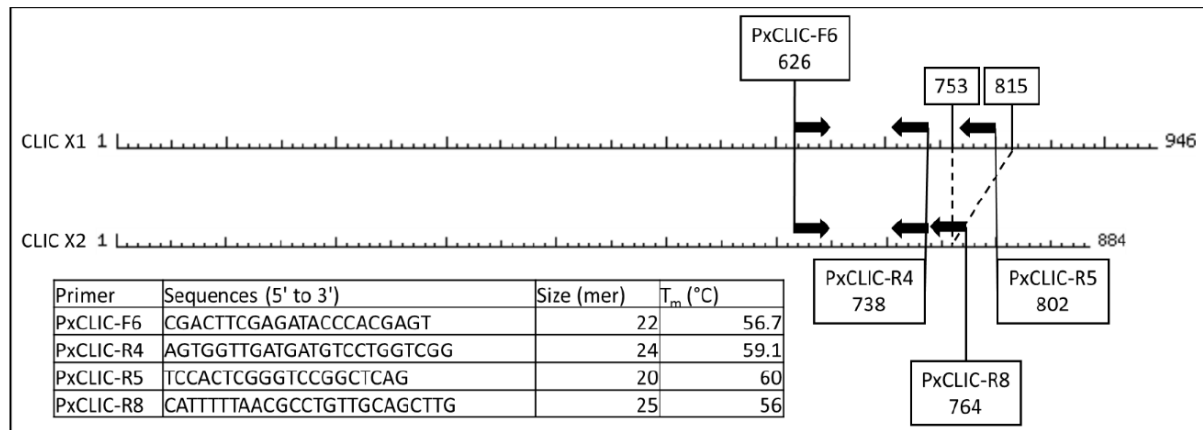


Figure 1. Primer design for gene expression analysis

3 Result

3.1 Phylogenetic analysis

Figure 2 show that CLICs isomer X1 and X2 are not only preserved in *P. xylostella* larvae. Another moths also express the two isomer of CLIC, like *P. polytes* (PpCLICX1 and PpCLICX2), *P. rapae* (PrCLICX1 and PrCLICX2), *V. tameamea* (VtCLICX1 and VtCLICX2), *A. transitella* (AtCLICX1 and AtCLICX2), *B. anynana* (BaCLICX1 and BaCLICX2), *G. mellonella* (GmCLICX1 and GmCLICX2), *H. armigera* (HaCLICX1 and HaCLICX2), *P. xuthus* (PaxCLICX1 and PaxCLICX2), and *H. kahanamoa* (HkCLICX1 and HkCLICX2). Which has the closest reality to the CLIC gene in *P. xylostella* is the CLIC gene in *D. melanogaster*. But not found 2 different isomers as shown by the CLIC moth gene in CLIC *D. melanogaster*.

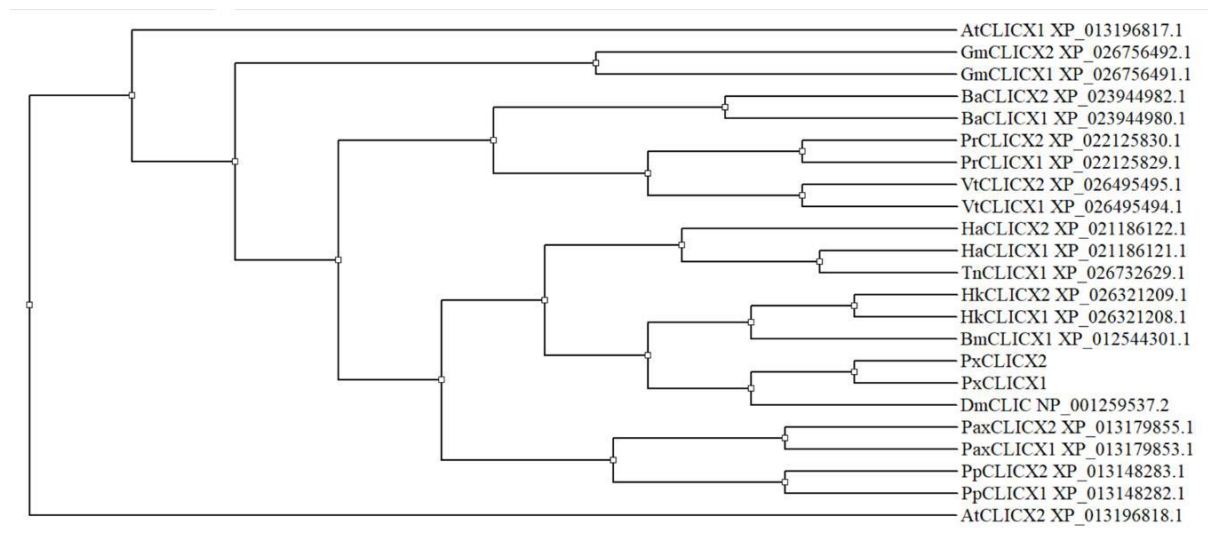


Figure 2. Phylogenetic tree of CLICs isoform X1 and CLICs isoform X2. *Papilio polytes* (Pp), *Pieris rapae* (Pr), *Vanessa tameamea* (Vt), *Amyelois transitella* (At), *Bicyclus anynana* (Ba), *Galleria mellonella* (Gm), *Helicoverpa armigera* (Ha), *Papilio xuthus* (Pax), *Hypomocoma kahamanoa* (Ha), *Drosophila melanogaster* (Dm), and *Plutella xylostella* (Px).

3.2 Primary structure comparison

The amino acid sequence of *P. xylostella* CLICs isoform X1 and isoform X2 was compared with CLICs from *D. melanogaster* using the Bioedit sequence alignment editor software (Figure 3). N-terminal glutaredoxin-like domain (residues 25-43) and a C-terminal GST like α -helical domain (residues 171-221) also consists among all the variant of *P. xylostella* CLIC. The amino acid sequence was carried out by BLAST analysis to find any relationships among other organisms. Two-cysteine thioredoxin/ glutaredoxin-like motif (C-L-F-C) was shown on the 25 until 28 bp and 168 until 171 bp. The CRAC (Cholesterol Recognition/interaction Amino acid Consensus) motif was shown on 29 until 33 bp (G-R-R-K-G) and 119 until 125 bp (L-I-E-N-L-Y-S-K). The 14-3-3-binding sites motif was shown on 97 until 104 bp (R-H-I-M-K-S-V-P). The two-cysteine thioredoxin/ glutaredoxin-like, CRAC and 14-3-3-binding sites motif was conserved on the both isoform of CLIC. The nuclear localization signal (NLS) is highly conserved only in CLIC isoform X1 (244 bp until 258 bp) with K-R-V-D-T-S-N-S-A-L-K-K-R.

3.3 3D structure of *P. xylostella* CLIC

The overall sequence alignment indicated that the *P. xylostella* CLICs isoform X1 and X2 were highly conserved on *D. melanogaster* CLICs (PDB template no 2yv7) with a high degree of identities (88.03 %). The three-dimensional structure of *P. xylostella* CLICs isomer X1 and X2 obtained using the homology-based modeling with *D. melanogaster* CLICs (PDB code: 2yv7) as the template. As shown in Fig. 6 C, *P. xylostella* CLIC consist of 9 helical domain and 4 β -sheet domains. The helix and β -sheet domain are signified by blue and yellow, respectively. Unlike the structures of the *D. melanogaster* CLICs and *P. xylostella* CLICs isoform X2, *P. xylostella* CLICs isoform X1 has some residue from 151 bp to 153 bp contain Serine, Alanine, and Leucine residues in the C terminal (Figure 3).

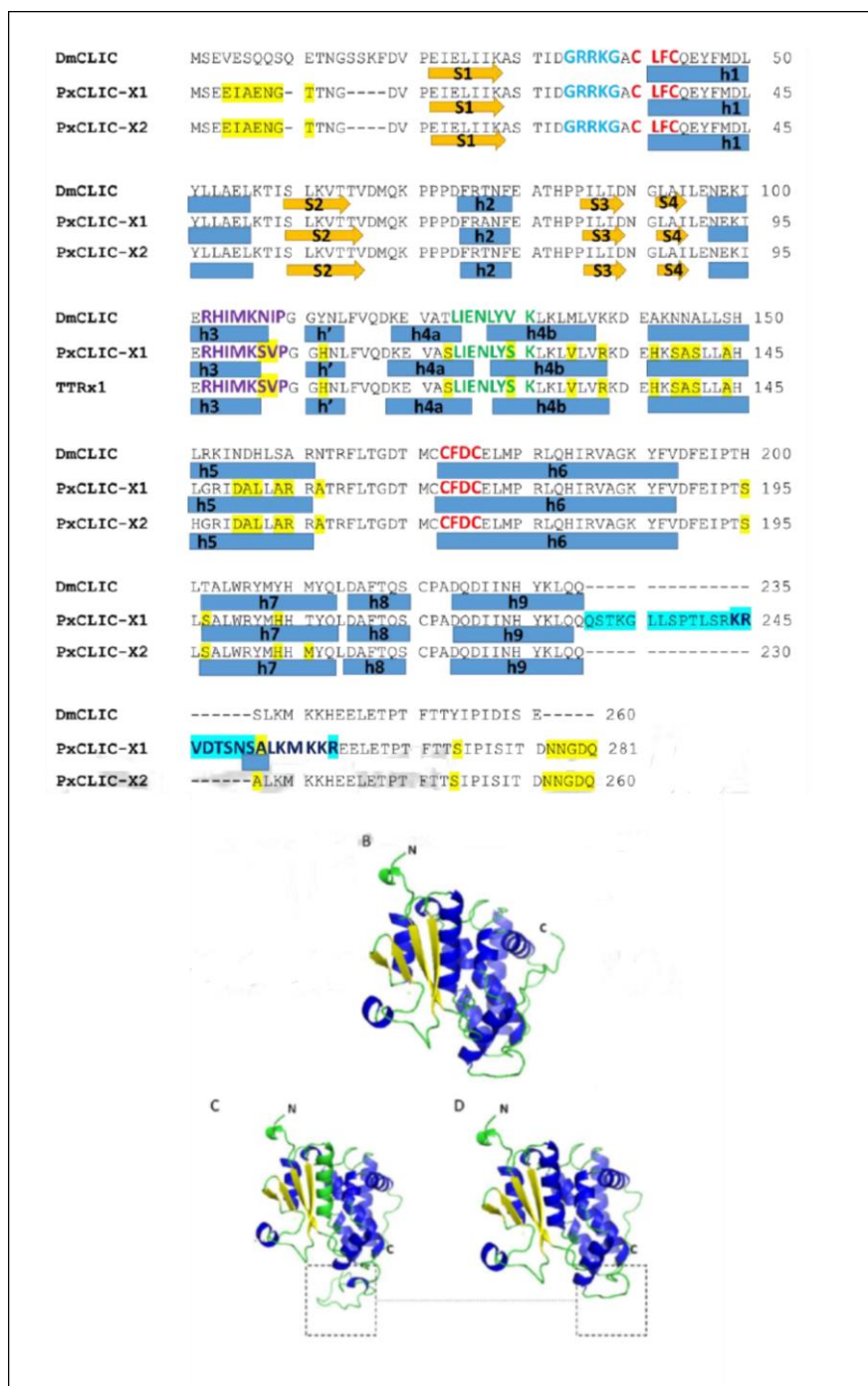


Figure 3. Homology modeling result of CLIC contain domain 4 β-sheet (s) and 10 helix (h) (A) Amino acid alignment with *D. melanogaster* CLICs (B) 3D structure of *D. melanogaster* CLICs (C) 3D structure of *P. xylostella* CLICs isomer X1 (D) 3D structure of *P. xylostella* CLICs exc- 4 isomer X2.

3.4 Docked emamectin benzoate binding to the *P. xylostella* CLIC X1

In this work, emamectin benzoate pesticides were docked into the substrate-binding site of the CLIC X1 and CLIC X2 using 100 docking runs. Based on these interactions, the docked conformers of CLIC are NLS motif in the form of arginine and valine amino acid. the NLS motive formed in the

intracellular is K-R-X(10-12)-K-(K/R)-(K/R). The NLS motif is always conserved at terminal C. With its location located on the C terminus, NLS forms a domain with an N terminus which serves to determine the subcellular location of CLIC. It can be noticed from Figure 4 that all emamectin benzoate have interaction energy of 100.83 kcal/mol, gave the good interaction energies with the CLIC X1.

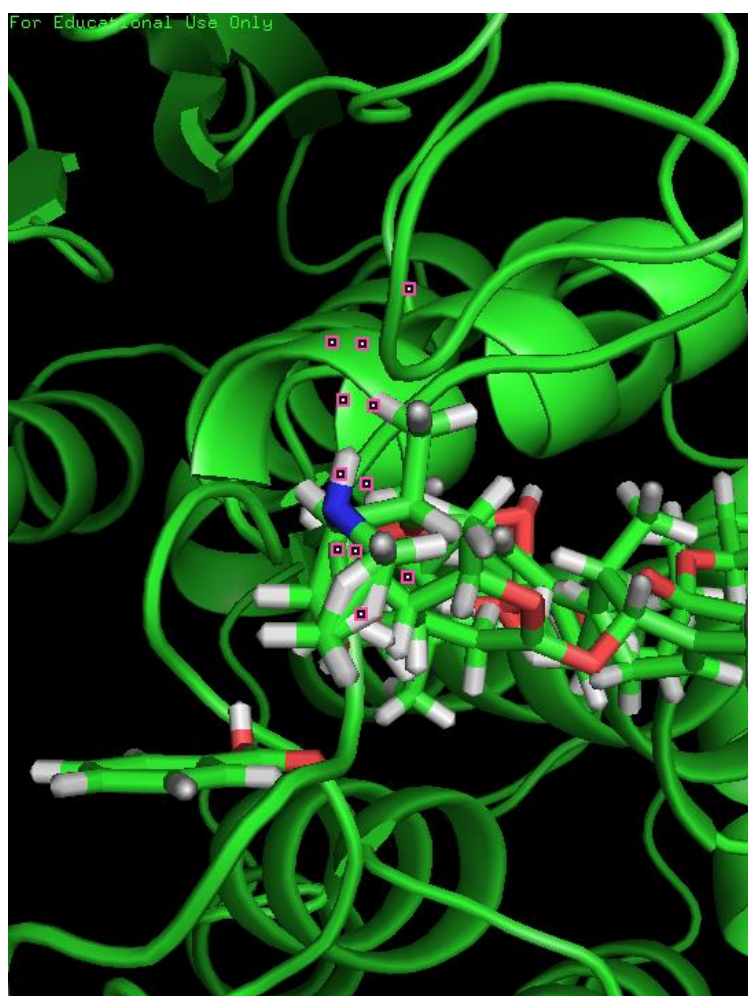


Figure 4. Docked emamectin benzoate binding to the *P. xylostella* CLIC X1

3.5 Expression profiles of CLIC from TT_{Sel} and TTR_x strain of *P. xylostella*

The quantitation-comparative C_T ($\Delta\Delta C_T$) results showed the increased expression of the CLIC gene from TT_{Sel} in comparison with that of TT_{Rx} (Figure 5). The Relative Quantitation (RQ) for CLIC X1 and X2 genes in TT_{Sel} was 25.95-fold higher than that of TT_{Rx} with a standard deviation of 12.2. The CLIC X1 gene showed an increase in expression in the TT_{Sel} sample is 13.36-fold higher than that of TT_{Rx} with a standard deviation is 8.5. The gene with the lowest increase in expression was CLIC X2, which showed an RQ of 5.37 more in TT_{Sel} higher than that of TT_{Rx}. The ANOVA statistic test showed the sample have a different with a P-value score of 0.003 between the three samples. However, after post-ANOVA tests using LSD, the increase in the expression of CLIC X1 has no significant difference with the increase in CLIC X2 in TT_{Sel} samples with TT_{Rx} as sample references.

In contrast to the expression of the CLIC X1 and X2 genes, there was a marked difference in the increase in expression compared to the CLIC X1 and CLIC X2.

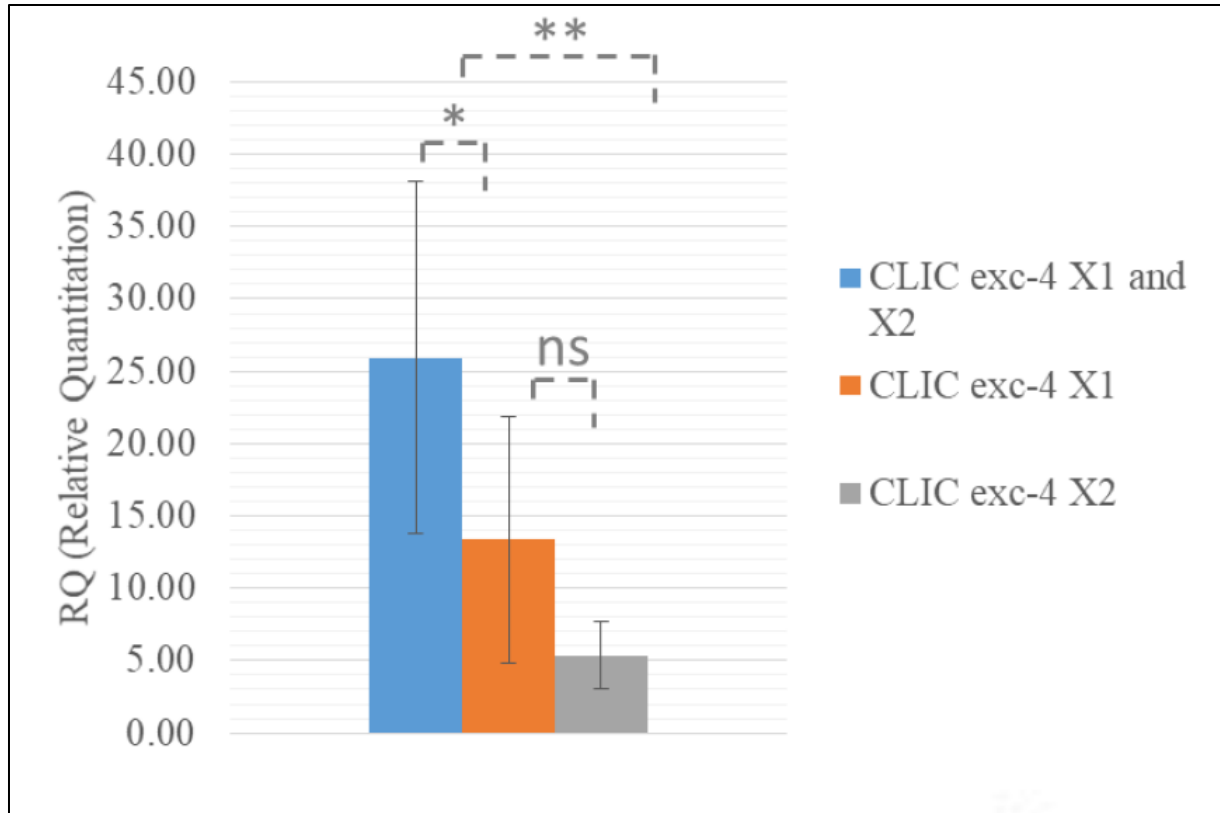


Figure 5. Increasing of CLIC X1, CLIC X2 and CLIC X1 and X2 genes (RQ) from TT_{Sel} for target sample, TT_{Rx} for control sample and RPS 13 for control gene. MLSD difference between means and significance of pairwise comparisons from the increasing of CLIC X1, CLIC X2 also CLIC X1 and X2 genes (RQ) replication of TT_{Rx} and TT_{Sel} strain. Differences larger than 8.80 are significant at the $\alpha = 0.05$ level and are indicated with *, differences larger than 13.06 are significant at the $\alpha = 0.01$ level and are indicated with **.

After sex separation between male and female larvae on *P. xylostela*, Figure 6 showed a marked difference in increased expression of the CLIC gene X1 and X2 (significant at the $\alpha = 0.01$) with RQ being 15.35-fold change in males and 36.56-fold changes in females. This was followed by an increase in the expression of the CLIC X1 gene which had differences between male and female larvae (significant at the $\alpha = 0.01$). Increased expression of CLIC X1 in females is 20.03-fold change and in males is 6.69-fold change. In contrast to the increased expression of the CLIC X2 gene, there is no significant difference between males and females. Increased gene expression based on TT_{Sel} as a sample target, and TT_{Rx} as a sample reference.

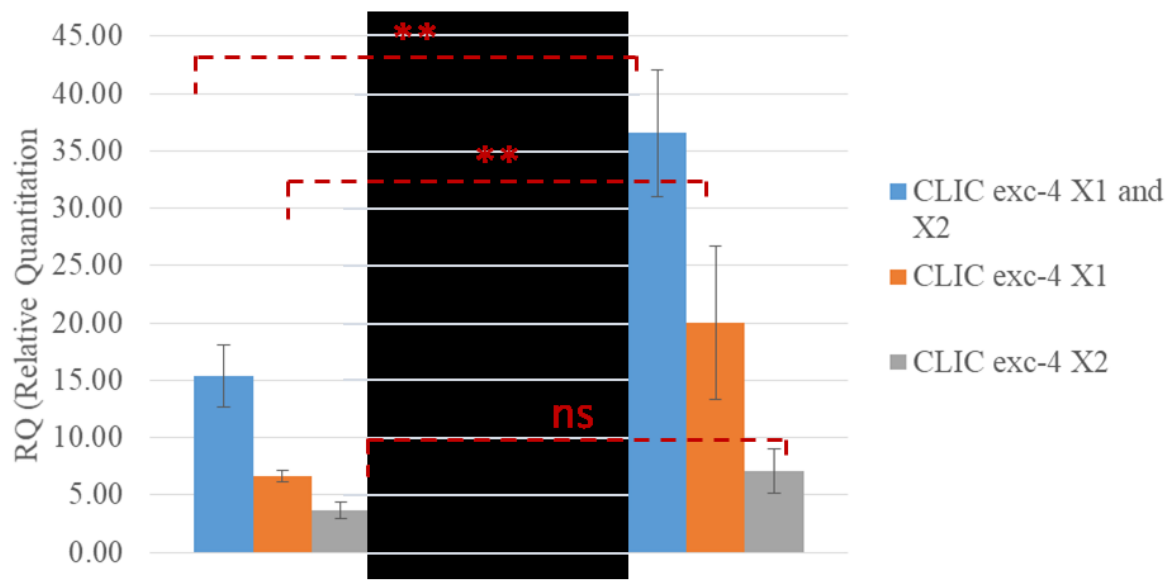


Figure 6. Increasing of CLIC X1, CLIC X2 and CLIC X1 and X2 genes (RQ) in the different sex from TT_{Sel} for target sample, TT_{Rx} for control sample and RPS 13 for control genes. MLSD difference between means and significance of pairwise comparisons from the increasing of CLIC X1, CLIC X2 also CLIC X1 and X2 genes (RQ) replication of TT_{Rx} and TT_{Sel} strain. Differences larger than 5.55 are significant at the $\alpha = 0.05$ level and are indicated with *, differences larger than 8.33 are significant at the $\alpha = 0.01$ level and are indicated with **.

4 Discussion

Results of quantitation-comparative C_T , $\Delta\Delta C_T$, showed an increase in the CLIC gene between *P. xylostella* in the TT_{Sel} strain in comparison with TT_{Rx} strain detected by SYBR green reagent with 40 times the PCR cycle. The highest increase occurred in the expression of CLIC X1 and X2 genes, then followed by CLIC X1 and CLIC X2. The highest increase occurred in CLIC X1 and X2 by 25.95 change fold because the primers used could detect both variants of the CLIC exc-4 gene. These results support the evidence of previous research using NGS which has an increased CLIC exc-4 gene in TT_{Sel} compared to TT_{Rx} strains. Although the results from the NGS data show an increase in CLIC gene expression by log2-fold change is about 11.9. Several factors affect this difference in expression. One of them is that the generation used in the sample is at a far different generation and has different resistance to emamectin benzoate.

In previous research [17], NGS was carried out when TT strain was in the 13th generation. TT_{Sel} strains in that generation had a resistance to emamectin benzoate of 1569.23 change fold. Whereas the TT_{Rx} has a resistance of 18.75 change fold. This thesis uses the same strain but in the 45th generation. TT_{Sel} strains in this generation have a resistance to emamectin benzoate of 951 change fold. Whereas the TT_{Rx} has a resistance of 26.74 change fold. After sex separation between male and female larvae on *P. xylostella*, the results showed that the expression of the CLIC X1 and X2 genes and then the CLIC X1 in female TT_{Sel} were greater than those of males, with TT_{Rx} as a control species. In contrast to increased expression in the CLIC X2 gene in females, which tends to have no difference with increased expression in males. This shows that CLIC X1 is a gene that is more sensitive to emamectin benzoate resistance in *P. xylostella*.

Based on the phylogenetic tree shows that the two isomers of the CLIC, CLIC X1 and CLIC X2 proteins are also founded in several other lepidopterans. Perhaps excessive expression of the CLIC gene is one of the mechanisms of lepidopteran defense in exposure to emamectin benzoate.

According to Gong et al. (2013) stated that emamectin benzoate is an insecticide antibiotic that has a specific spectral board in lepidopteran[14]. Also supported from research Luan et al. (2017) which states that emammectin benzoate can affect the chromatin condensation process in the nucleus and apoptotic cells [10]. While one of the functions of CLIC in insects is to the protective role during apoptosis [22]. The *P. xylostella* CLIC X1 and CLIC X2 proteins are also highly homologous (88.03%) to a number of *D. melanogaster* CLICs. So that the alignment of the amino acid sequences between CLIC isoform X1 and CLIC isoform X2 in *P. xylostella* with CLIC in *D. melanogaster*. This aims to determine the nature or character of the CLIC gene in *D. melanogaster* which also appears in the CLIC exc-4 in *P. xylostella*. There are several proteins in CLIC *D. melanogaster* which is also shown in CLC exc-4 in *P. xylostella*, including active redox cysteine, the CRAC, and 14-3-3 phosphoprotein binding protein.

The active redox cysteine residues presence oxidation process from a soluble form of CLIC (monomer) to become dimer. Where the CLIC experienced a transition by being non-covalent dimeric when forming ion channels. Both cysteine residues have a hydrophobic surface. Because of its highly hydrophobic nature, it helps to make the dimer interface turn CLIC into a dimer. In dimer conditions, it survives, thus creating chloride ion channels in artificial double layers and vesicles[23]. To facilitate CLIC dimers interact with plasma members, CRAC is also preserved. CRAC has a function as a CLIC domain in plasma members. Because plasma members are mostly covered with cholesterol, CRAC functions as a cholesterol binder[24]. 14-3-3s are also expressed in CLIC. It was reported that these proteins can influence the response of growth factors and stimuli to cells. So that 14-3-3s affect the behavior of cells which are structural components of the metabolic process [25].

One of the proteins not found in CLIC in *D. melanogaster* but shown in CLIC in *P. xylostella* is NLS. In the CLIC4 which has the function of actively participating and regulating the translocation of these proteins. Protein translocation occurs because it is caused by received signal stress. With its location located on the C terminus, NLS forms a domain with an N terminus which serves to determine the subcellular location of CLIC [26].

Three-dimensional structure homology is an analysis of early-stage protein structure as a predictor of bioinformatics for analyzing protein function. This study models *P. xylostella* CLIC X1 and CLIC X2 based on the crystal structure of CLIC *D. melanogaster*. In this modeling it has been found that the CLIC X1 and CLIC X2 protein contain all important structural elements in the form 10 α -helices and four β strands. The α -helix consists of h1, h2, h3, h4a, h4b, h5, h6, h7, h8, and h9. β strands consist of s1, s2, s3 and s4. In a soluble condition, similar to vertebrate CLIC, in invertebrates also have putative transmembrane domain sites and divalent metal binding that is between the α -helix- 1 and β -strand- s2 [3]. The main character that is the differentiator in *P. xylostella* CLIC X1 then CLIC on *D. melanogaster* is the helical domain consist of 3 residues in the C terminal. A polar and two hydrophobic, Serine- Aline- Leucine amino acids form the new helical domain.

5 Conclusion

There was an increase in the expression of the CLIC gene in *P. xylostella* which underwent emamectin benzoate selection compared to emamectin benzoate relax. Increased expression of CLIC X1 and X2 genes in females is more comparable to males, especially expression of the CLIC X1 gene. The expression of CLIC X1 and CLIC X2 genes preserved in some lepidopterans may be a

cause of lepidopteran resistance to emamectin benzoate. The NLS is conserved only in CLIC exc-4 isoform X1, which may be important on the resistance to emamectin benzoate. The suggestions that need to do more in-depth research on influences the NLS on the CLIC exc-4 mechanism resistance to Emamectin benzoate.

6 Reference

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