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RESEARCH ARTICLE

# *In silico* characterization and identification of putative defence-related non-specific lipid transfer proteins (nsLTPs) in *Musa acuminata* DH Pahang

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**Abstract**

Non-specific lipid transfer proteins (nsLTPs) are small proteins belonging to the pathogenesis-related (PR) 14 family, with eight conserved cysteines forming four disulfide bridges. These proteins play diverse roles in plants, including pathogen defence, and have been introduced into plant genomes to enhance resistance against various biotic stressors. Banana (Musa spp.), a key food crop of economic importance, continuously faces natural and biological threats. However, no studies have yet explored nsLTP gene family in banana. Moreover, despite persistent biotic challenges, particularly fungal diseases, potential defence-related nsLTP candidates in banana remain unidentified. This study employs bioinformatics to identify potential nsLTP candidates involved in the defence mechanisms of *Musa acuminata* DH Pahang. A total of 35 nsLTPs were identified in *M. acuminata* DH Pahang (MansLTPs) through domain searches for PF00234 or PF14368 domains. Based on Edstam's classification, MansLTPs are grouped into five main categories: Types 1, 2, C, D, and G. Gene ontology analyses predicted several biological processes associated with nsLTPs, including systemic acquired resistance. Ma11_t18240.1, Ma07_t23570.1, Ma01_t10510.1, Ma04_t17220.1 and Ma04_t17200.1 were identified as potential antifungal candidates due to their orthologous relationship and secondary structural similarities with known antifungal nsLTPs from other plants. In addition, another candidate, Ma10_t06730.1, was identified based on the analysis of the cis-acting regulatory elements for the presence of TC-rich elements. Molecular docking further supported these findings by revealing strong ligand–protein affinities with LPC-16 across all MansLTPs. Key conserved residues (Tyr77, Leu32, Leu49, Ile64, Ile67, Ile79) were identified as central to ligand stabilization, highlighting their potential roles in defence-related lipid binding. Further functional analyses are recommended to confirm the roles of these proteins in the defence mechanisms of banana plants. Gaining insights into the gene structures, evolutionary history, and *in vitro* antifungal properties of these proteins will significantly contribute to future crop improvement strategies.

**Keywords**: antifungal protein; banana; bioinformatics; biotic stress, molecular docking

## INTRODUCTION

Plants have developed effective mechanisms to protect themselves from pathogenic attacks, relying on a group of proteins known as pathogenesis-related (PR) proteins that play a crucial role in host defence responses. These proteins are induced under biotic and abiotic stress conditions and contribute to the plant's immune system. PR proteins can be classified into 17 subclasses (PR-1 to PR-17) with an additional two putative PR families (Joshi et al., 2021). Among these, the PR-14 group is linked to non-specific lipid transfer proteins (nsLTPs) and are regulated across different developmental stages, organs, and environmental stress conditions (Missaoui et al., 2022). They are called "non-specific" because they can transport a variety of lipid components, including fatty acyl-CoA, cutin monomers, phospholipids, and gangliosides (Sterk et al., 1991; Stillwell, 2016). nsLTPs are small proteins, ranging from 6 kDa to 10

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kDa, yet they are abundant in both monocotyledons and dicotyledons within the plant kingdom. These proteins are basic in nature and have an internal hydrophobic cavity that binds and transports lipid molecules between membranes (Liu et al., 2015; Hanada, 2018). A key characteristic of nsLTPs is the presence of eight conserved cysteine residues linked by four disulfide bonds (Liu et al., 2015). Furthermore, most plant nsLTPs contain N-terminal signal peptides, indicating their localization in the apoplast (Carvalho & Gomes, 2007; Salminen et al., 2016).

Beyond lipid translocation, nsLTPs play diverse roles in plants, including regulation of seed germination, stress resistance, signal transduction, and membrane stabilization (Liu et al., 2015). nsLTPs belong to a multigene family, which means multiple copies of nsLTPs can be found within a single species. For example, 83 nsLTP genes have been identified in *Solanum tuberosum* L. (Li et al., 2019), 64 in *Arachis duranensis* (Song et al., 2020), and 89 in *Brassica oleracea* var. capitata (Ji et al., 2018). Due to the multigene nature of nsLTPs, specific copies may carry out unique functions in plant systems (Lutfi et al., 2022). Notably, several plant nsLTPs have demonstrated antifungal properties (Bogdanov et al., 2016; Cai et al., 2016; Gizatullina et al., 2013; Schmitt, 2018; Zhu et al., 2012).

Banana is an economically important fruit crop that contributes significantly to the global agricultural trade market. In 2024, the worldwide export quantity of bananas was approximately 19.7 million tonnes, with Latin America and the Caribbean remaining the top exporters (Food and Agriculture Organization of the United Nations, 2025). Despite their high commercial and economic value, bananas are vulnerable to serious biological threats, particularly fungal diseases such as Fusarium wilt and black Sigatoka caused by *Fusarium oxysporum* f. sp. *cubense* (Foc) (Ploetz, 2015) and *Pseudocercospora fijiensis* (Yonow et al., 2019), respectively. Various management strategies, including fungicide application, crop rotation, and quarantine measures, have been implemented to combat these diseases; however, none have offered sustainable long-term solutions (Blomme et al., 2024; Ploetz, 2015). Developing resistant banana cultivars is considered a more sustainable approach, but this effort is hindered by the narrow genetic base of cultivated banana varieties and the polyploid nature of many cultivars, which complicates conventional breeding efforts (D'Hont et al., 2012).

To date, specific defence-related nsLTPs have not been identified in banana. However, transcriptomic analysis revealed that one *nsLTP* gene in Cavendish banana was differentially regulated in response to Foc Race 1 (R1) and Tropical Race 4 (TR4) (Li et al., 2013). Additionally, proteomic analysis of *Fusarium proliferatum*-infected banana fruit showed significant downregulation of two *nsLTP* genes (Ma04_p30830.1 and Ma10_p18990.1) (Xie et al., 2022). Given the multigene nature and functional diversity of nsLTPs, it is plausible that specific nsLTPs play a role in the plant's defence mechanisms. Therefore, a systematic *in silico* approach can be utilized to identify defence-related nsLTP candidates in a cost-effective and efficient manner. In this study, all nsLTP members in *Musa acuminata* DH Pahang will be identified, followed by the prediction of potential antifungal nsLTP candidates based on evolutionary relationships with known antifungal nsLTPs and secondary structural analyses. Specific banana nsLTPs associated with the plant's defence mechanism will also be identified through gene ontology prediction and analysis of the cis-acting regulatory elements in their promoter region. Molecular docking will also be performed to support the involvement of the potential MansTLPs in banana's defence mechanism. Information on the specific functions of nsLTPs is essential for designing strategies to enhance plant tolerance against various biotic stresses and for general crop improvement.

## MATERIALS AND METHODS

### *Sequence retrieval of non-specific lipid transfer protein from Musa acuminata DH Pahang (MansLTP)*

PF00234 and PF14368 were identified from literature review as Pfam accession numbers for non-specific lipid transfer protein (nsLTP) in plants (Bogdanov et al., 2016; Schmitt et al., 2018). These Pfam accessions were used to retrieve *Musa acuminata* DH Pahang (wild banana) non-specific lipid transfer protein sequences (MansLTPs) from the Pfam database (http://pfam.xfam.org/) (currently hosted by Interpo). The retrieved sequences were then used as queries in the BLAST search against *Musa acuminata* DH Pahang (version 2) database in Banana Genome Hub (https://banana-genome-hub.southgreen.fr/) (Droc et al., 2013). All the protein sequences from the BLAST results were compiled and redundant sequences were removed. Sequences that do not belong to Pfam accession PF00234 or PF14368 were also excluded.

### *Analysis of MansLTP sequences*

MansLTP sequences were checked for the presence of signal peptides using SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) (Almagro Armenteros et al., 2019) and manually scanned for the eight-

cysteine motif (8CM). The sequences that did not fulfil the requirements were omitted. Theoretical molecular weight and isoelectric point of MansLTP protein sequences were calculated using Compute pI/Mw (Gasteiger et al., 2005). Cell-Ploc 2.0 was used to predict the subcellular localization of MansLTPs. The MansLTPs protein sequences were uploaded in FASTA format to the query section of the Plant-mPloc 2.0 website (Chou & Shen, 2010) and submitted for analysis.

### *Evolutionary relationship of MansLTP*

Multiple sequences alignments were performed using MUSCLE alignment and a phylogenetic tree was constructed based on WAG model with Gamma Distributed with Invariant Sites (G+I). The tree was constructed using Maximum-likelihood method with 1000 bootstrap value in MEGA-X software (Kumar et al., 2018). nsLTPs from *Oryza sativa* and *Hordeum vulgare* were included as representatives for each nsLTP group based on the Edstem classification (Zhang et al., 2019) (Supplementary Table 1).

### *Identification of potential defence-related nsLTPs from M. acuminata DH Pahang*

*Functional annotation of MansLTP*

Functional annotation of MansLTPs was predicted using PANNZER2 (Protein ANNotation with Z-score) (http://ekhidna2.biocenter.helsinki.fi/sanspanz/). The output from PANNZER2 included sequence identifiers, theoretical functional descriptions, and gene ontology predictions for biological processes, molecular functions, and cellular components. All predictions were accompanied by color-coded probabilities, ranging from green for high-confidence predictions to red for low confidence (Törönen & Holm, 2022). The cut-off positive predictive value (PPV) used in this analysis was 0.7 as it indicates high confidence predictions (Törönen et al., 2018).

*Structure-based alignment and orthologous relationship analysis with proven antifungal nsLTPs from other plant species*

PROMALS3D (http://prodata.swmed.edu/promals3d/promals3d.php) was used to align MansLTPs together with proven antifungal nsLTPs retrieved from the literature (Pei, Kim & Grishin, 2008). Additionally, a phylogenetic tree was built with MEGA-X to have a clearer picture of the evolutionary relationship between MansLTP and proven antifungal nsLTP from other plant species. The list of proven antifungal nsLTPs from other plant species used is provided in Supplementary Table 2. To build an unbiased phylogenetic tree that illustrates the orthologous relationship between MansLTP (PF00234 domain) and proven antifungal nsLTP, nsLTP from other plant orders and their family members were retrieved too (Supplementary Table 3). nsLTPs from different plant orders were identified from published articles, Phytozome (https://phytozome-next.jgi.doe.gov/) (Goodstein et al., 2012), UniProt (https://www.uniprot.org/) (The UniProt Consortium, 2021), and Pfam database (http://pfam.xfam.org/) (Mistry et al., 2021). Family members of the nsLTPs were retrieved using Phytozome, and BlastP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Plants with proven antifungal nsLTPs were prioritised in selecting the representative from each order. All the sequences were screened for PF00234 or PF14368 accession, signal peptide and eight-cysteine motif (8CM). The phylogenetic tree was based on Jones-Taylor-Thornton (JTT) model with Gamma Distributed with Invariant Sites (G+I) with maximum likelihood method, at 1000 bootstraps test.

*Cis-acting regulatory elements (CARE) analysis of MansLTPs*

Cis-acting regulatory elements (CARE) analysis was conducted on the MansLTP sequences under PF00234 domain. The promoter region of 1.5 kbp upstream of the translation start site was retrieved from the Banana Genome Hub database. The sequences were analyzed using PlantCARE database (Rombouts, 1999). The output was compiled in Microsoft® Excel and analyzed.

*Molecular docking analysis of MansLTPs*

Molecular docking was performed to evaluate the interaction between MansLTPs and selected defence-related lipid ligands. Protein structures were generated using AlphaFold 3 (Abramson et al., 2024). A total of six ligands were retrieved from PubChem (Kim et al., 2025): 1-Palmitoyl-sn-glycero-3-phosphocoline (LPC-16) (CID:460602), linoleic acid (CID:5280450), palmitic acid (CID:985), myristic acid (CID:11005), oleic acid (CID: 445639) and 12-oxo-phytodienoic acid (OPDA) (CID:5280411). Docking was performed using AutoDock Vina version 1.5.7 (The Scripps Research Institute, La Jolla, California, USA) (Trott & Olson, 2010) to predict the optimal binding mode of the MansLTPs. The prepared protein file was saved in PDBQT (Protein Data Bank, Partial Charge, and Atom Type) format. AutoGrid was used to generate the grid map, with a grid spacing of 0.375 Å to facilitate ligand binding. The grid box was adjusted to specific dimensions (x = 40, y = 40, z = 40; centre x = 2.95, centre y = -4.795, centre z = 5.8) to position ligands at the conserved residues of MansLTPs. This site includes key residues (Tyr77/Phe80, Leu8, Leu32, Ile/Val64, Ile67 and

Ile79) which are critical for ligand stabilization of MansLTPs. Docking was conducted with an exhaustiveness of 8, and the best substrate binding pose was selected based on the lowest binding energy and optimal pose orientation. Docking results were visualized using the PyMOL Molecular Graphics System version 3.0.4 (Schrödinger, 2015) and LigPlot+ software version 2.3.1 (EMBL-EBI, Cambridge, UK) (Laskowski & Swindells, 2011) to identify the interactions involved.

## RESULTS AND DISCUSSION

### *Identification and in silico characterization of nsLTPs from M. acuminata DH Pahang (MansLTPs)*

Based on Pfam database, *M. acuminata* DH Pahang (wild banana) has 5 sequences under PF00234 and 25 sequences under PF14368. Blast result against *Musa acuminata* DH Pahang (version 2) in Banana Genome Hub resulted in 124 non-redundant sequences. Among the 124 protein sequences, 60 sequences do not belong to PF00234 nor PF14368. A total of 29 sequences did not possess either signal peptide or conserved eight cysteine motifs which are the basic characteristics of nsLTP. In conclusion, 35 protein sequences were identified as *Musa acuminata* DH Pahang nsLTPs (MansLTPs) (Table 1). Out of the 35 sequences, 9 sequences belong to PF00234, and 26 sequences belong to PF14368.

MansLTPs with PF00234 domain have protein length from 112 amino acids to 119 amino acids and isoelectric point ranging from 8.84 to 9.91. Their molecular weight falls between 11520.50 Da and 12157.19 Da. Contrastingly, MansLTPs with PF14368 domain has more variation. MansLTPs with PF14368 domain spans from 91 amino acids up to 199 amino acids. Similarly, their molecular weight ranges from 9440.21 Da to 20899.16 Da. Interestingly, PF14368 isoelectric points ranges from 4.08 to 9.98 as several copies possess acidic isoelectric point (Table 1). Although generally nsLTP are considered as small, basic proteins, it is common to find acidic nsLTP in plants too (Li et al. 2019; Schmitt et al., 2018). Basic amphipathic peptide is common among antimicrobial peptides thus the basic properties of nsLTP are thought to correlate with its antimicrobial activity. Basic amphipathic molecules bind to the negatively charged molecule in the microbial membrane, destroying the membrane structure leading to loss of function in the pathogen (Sun et al., 2008; Juhász et al., 2018). All MansLTPs were predicted to possess signal peptide, suggesting that these proteins are destined for secretion (Owji et al., 2018).

### *Subcellular localization of MansLTPs*

The functions and interactions of molecules within the cell are significantly influenced by their subcellular localization. For proteins to become fully functional, they must be correctly targeted to specific subcellular compartments. This targeting is largely directed by the N-terminal signal peptide, which plays a crucial role in determining the subcellular localization of nsLTPs (Missaoui et al., 2022). According to Table 1, a total of 13 MansLTPs were predicted to be localized at the cell wall, whilst 19 nsLTPs were possibly localized at the cell membrane. Three MansLTP, namely Ma06_t08070.1, Ma08_t09250.1, and Ma04_t30830.1, might be localized either at the cell membrane or the cell wall. The findings highlight the dynamic nature of nsLTP localization.

In this study, all MansLTPs under the PF00234 domain and four under PF14368 domain were potentially localized in the cell wall. The localization of nsLTP in the cell wall may suggest its role in combating pathogen attacks and adapting to stress. Salcedo et al. (2007) indicated that nsLTP is prominently expressed in the peripheral cell layers encasing aerial organs, which comprise the cell wall and cuticle of epidermal tissue. This further substantiates the idea about the role of nsLTP as a plant defence mechanism against external microbial invasion and the deposition of extracellular cutin. The defensive mechanism of nsLTP involves preserving the adhesion integrity between the cuticle and the underlying cell wall (Gao et al., 2022).

As expected, most of the MansLTPs were predicted to be localized in the cell membrane. The localization of MansLTP in the cell membrane supports the main role of this protein as they participate in lipid transport, which is critical for maintaining membrane integrity (Deeken et al., 2016, Missaoui et al., 2022). According to a study conducted by Edqvist et al. (2018), majority of the nsLTP localization occurred external to the plasma membrane. Several investigations indicated that nsLTP, localized in both the cell wall and cell membrane, mediates connections between both structures (Edstam et al., 2011). The lipid transport process, the stabilisation of membranes, and the defence against pathogens are all dependent on this interaction (Missaoui et al., 2022).

**Table 1.** Characteristics of non-specific lipid transfer proteins in *Musa acuminata* DH Pahang (MansLTPs)

| PFAM | Gene Accession | Length (Amino acid) | Molecular Weight (Da) | Isoelectric Point (pI) | Subcellular localization |
|---|---|---|---|---|---|
| PF00234 | Ma11_t18240.1 | 117 | 11719.62 | 8.84 | Cell wall |
| | Ma07_t23570.1 | 114 | 11520.50 | 9.30 | Cell wall |
| | Ma01_t10510.1 | 117 | 11758.80 | 9.32 | Cell wall |
| | Ma04_t17220.1 | 116 | 11581.42 | 9.18 | Cell wall |
| | Ma04_t17200.1 | 117 | 11502.54 | 9.44 | Cell wall |
| | Ma04_t17190.1 | 119 | 12157.19 | 9.16 | Cell wall |
| | Ma04_t17240.1 | 119 | 12157.14 | 9.03 | Cell wall |
| | Ma10_t06730.1 | 112 | 11874.33 | 9.24 | Cell wall |
| | Ma04_t34910.1 | 116 | 12122.20 | 9.91 | Cell wall |
| PF14368 | Ma01_t00360.1 | 121 | 12905.12 | 8.42 | Cell wall |
| | Ma02_t21140.1 | 124 | 13473.90 | 7.60 | Cell wall |
| | Ma02_t21850.1 | 117 | 12081.09 | 6.53 | Cell wall |
| | Ma02_t21910.1 | 115 | 11712.48 | 5.68 | Cell wall |
| | Ma06_t22400.1 | 109 | 11559.52 | 8.88 | Cell membrane |
| | Ma10_t25670.1 | 165 | 16680.37 | 8.60 | Cell membrane |
| | Ma07_t12130.1 | 196 | 20899.16 | 9.20 | Cell membrane |
| | Ma02_t19200.1 | 166 | 16503.73 | 5.05 | Cell membrane |
| | Ma10_t02380.1 | 118 | 12318.73 | 9.01 | Cell membrane |
| | Ma10_t22460.1 | 118 | 12095.28 | 8.82 | Cell membrane |
| | Ma10_t17100.1 | 97 | 10049.97 | 6.04 | Cell membrane |
| | Ma07_t24450.1 | 145 | 15332.58 | 7.47 | Cell membrane |
| | Ma03_t07430.1 | 139 | 15022.51 | 6.06 | Cell membrane |
| | Ma06_t08190.1 | 104 | 10692.75 | 8.69 | Cell membrane |
| | Ma02_t19230.1 | 179 | 17707.39 | 8.95 | Cell membrane |
| | Ma04_t07630.1 | 170 | 16578.81 | 4.78 | Cell membrane |
| | Ma06_t08070.1 | 109 | 11631.35 | 9.86 | Cell membrane/ Cell wall |
| | Ma05_t02850.1 | 199 | 20221.77 | 8.48 | Cell membrane |
| | Ma09_t21930.1 | 91 | 9513.32 | 9.00 | Cell membrane |
| | Ma08_t09250.1 | 129 | 13940.69 | 9.98 | Cell membrane/ Cell wall |
| | Ma02_t08320.1 | 91 | 9678.43 | 9.30 | Cell membrane |
| | Ma09_t18270.1 | 91 | 9594.42 | 9.08 | Cell membrane |
| | Ma02_t16400.1 | 92 | 9688.46 | 8.64 | Cell membrane |
| | Ma10_t26820.1 | 173 | 18581.81 | 6.27 | Cell membrane |
| | Ma07_t10090.1 | 142 | 15248.49 | 4.08 | Cell membrane |
| | Ma04_t30830.1 | 91 | 9440.21 | 9.11 | Cell membrane/ Cell wall |

***Classification of MansLTP***

Based on Edstam classification, there are five major groups (Type 1, 2 C, D and G) and five minor groups (Type E, F, H, J and K) to classify nsTLPs. nsLTPs from both monocotyledons and dicotyledons can be grouped into the five major types (Type 1, 2 C, D and G). In addition, nsLTPs from dicotyledons can also be classified into Type E (Edstam et al., 2011). According to Zhang et al. (2019), *Oryza sativa* and *Hordeum vulgare* nsLTPs were classified into five types: Type 1, Type 2, Type C, Type D and Type G. This further confirms that monocotyledons nsLTP are classified into only the five major groups. Since banana plant is a monocotyledon, representatives of each class of *Oryza sativa* and *Hordeum vulgare* nsLTPs were included in the clustering analysis of MansLTPs. A total of 13 MansLTPs copies belong to Type 1 which becomes the main clade of MansLTPs classification followed by Type G (7 copies) (Figure 1). Coincidently, all 9 MansLTP with PF00234 domain are grouped under Type 1 nsLTP.

Even by looking at the spacing pattern of 8-cystein motifs (Table 2) of Type 2 and D MansLTPs, they only differ by one or two amino acid residues between the cysteine molecules. The 8 cysteine residues are arranged in the sequence C-Xn-C-Xn-CC-Xn-CXC-Xn-C-Xn-C and is conserved across all LTPs (Zaidi et al., 2020). The 4 disulphide bonds are responsible in folding the protein into compact and functional alpha helixes (H1, H2, H3 and H4)

connected by short loops (L1, L2 and L3) (Liu et al., 2010). Ma08_t09250 and Ma06_t08070 did not group into any existing major groups so there is a possibility that they either belong to the minor groups or independently form a new group.

### *Identification of potential defence-related MansLTP*

*Functional annotation of MansLTPs*

Based on PANNZER2 analysis (Table 3), majority of the MansLTPs are predicted to be involved in lipid transport and lipid binding. In addition, three unique MansLTP copies (Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1) are associated with several biological processes that include seed trichome differentiation, unidimensional cell growth cell morphogenesis involved in differentiation, cutin-based cuticle development, plant-type secondary cell wall biogenesis, symbiotic interaction, macromolecule biosynthetic process and response to abscisic acid. Various studies have demonstrated the roles of LTPs in plant physiology and development. The accumulation of LTP-GFP fusion protein in the plasma membrane/cell wall of epidermal cells of transgenic Arabidopsis overexpressing maize *LTP* suggested the potential role of LTP in cuticle biosynthesis as those are the sites for cuticle component biogenesis and translocation (Qiao et al., 2020). Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1 are also predicted to have one molecular function which is transcription factor binding. In addition, Ma05_t02850.1 is the only copy that is predicted to be involved in proteolysis and possess peptidase activity. With regards to the defence roles and response to biotic stress environments, only Ma06_t22400.1, Ma10_t02380.1, Ma10_t22460.1, Ma10_t17100.1, and Ma06_t08190.1 are associated with the systemic acquired resistance in wild banana. nsLTPs exhibit direct antimicrobial activity against a wide range of pathogens, including fungi, which may be attributed to their fundamental ability to bind and transport lipids (McLaughlin & Tumer, 2025).

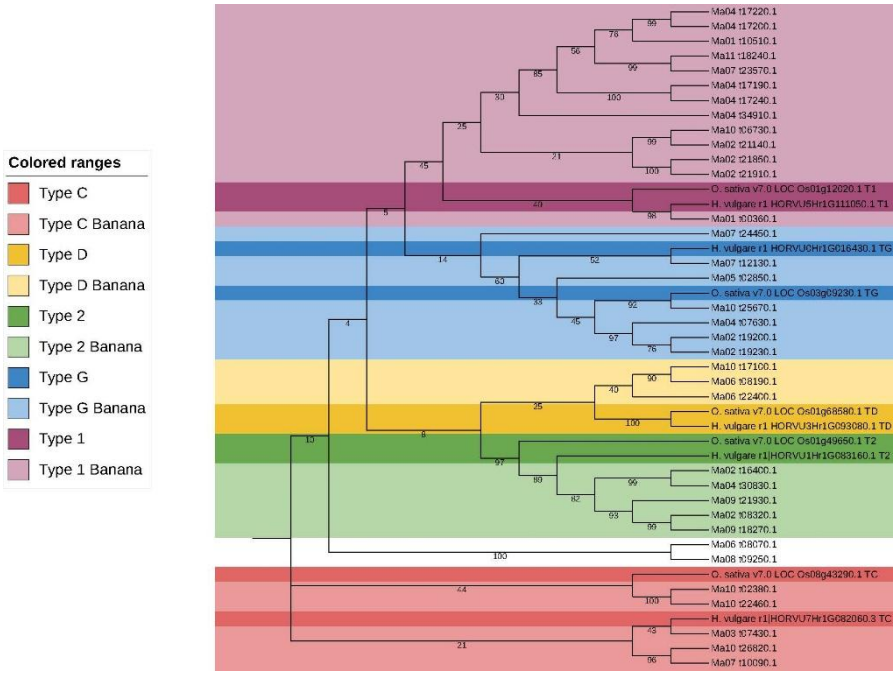



**Figure 1.**1000 bootstrap test phylogenetic tree of *Musa acuminata* DH Pahang nsLTPs (MansLTPs), *Oryza sativa* nsLTPs (OsnsLTPs) and *Hordeum vulgare* nsLTPs (HvnsLTPs). Sequences aligned using MUSCLE and built using Maximum-likelihood method based on WAG model with Gamma Distributed with Invariant Sites (G+I). Different clades of nsLTPs are represented by different colours.

**Table 2.** Spacing pattern of eight cysteine motifs (8CM) in different types of nsLTPs identified in *Musa acuminata* DH Pahang

| Type | Spacing Pattern | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Type 1 | C | $X_{9}$ | C | $X_{12\text{-}15}$ | CC | $X_{19}$ | C-1-C | $X_{21\text{-}23}$ | C | $X_{13,14}$ | C |
| Type 2 | C | $X_{7}$ | C | $X_{13}$ | CC | $X_{8}$ | C-1-C | $X_{21,23}$ | C | $X_{6}$ | C |
| Type C | C | $X_{6,14}$ | C | $X_{13,14}$ | CC | $X_{11,12}$ | C-1-C | $X_{24,25}$ | C | $X_{8\text{-}10}$ | C |
| Type D | C | $X_{9}$ | C | $X_{15,16}$ | CC | $X_{9}$ | C-1-C | $X_{24}$ | C | $X_{7,8}$ | C |
| Type G | C | $X_{9,10}$ | C | $X_{14,16,17}$ | CC | $X_{12,13}$ | C-1-C | $X_{24,25,26,28,29}$ | C | $X_{8,9}$ | C |

"X" represents any amino acid, and the number following "X" represents the number of amino acid residues. The spacing patterns are manually derived.

**Table 3.** Gene ontology analysis of *Musa acuminata* DH-Pahang non-specific lipid transfer protein (MansLTP) through PANZERR 2 database

| GO Accession | Ontology | Function/Component | MaLTPs |
|---|---|---|---|
| GO:0006869 | Biological Process | lipid transport | All MaLTPs except Ma06_t22400.1, Ma10_t02380.1, Ma10_t17100.1, Ma10_t26820.1, Ma07_t10090.1, Ma03_t07430.1, Ma06_t08190.1, Ma06_t08070.1, Ma08_t09250.1 and Ma02_t19200.1 |
| GO:0090376 | Biological Process | seed trichome differentiation | Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1 |
| GO:0009826 | Biological Process | unidimensional cell growth | Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1 |
| GO:0000904 | Biological Process | cell morphogenesis involved in differentiation | Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1 |
| GO:0160062 | Biological Process | cutin-based cuticle development | Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1 |
| GO:0009834 | Biological Process | plant-type secondary cell wall biogenesis | Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1 |
| GO:0009737 | Biological Process | response to abscisic acid | Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1 |
| GO:0045165 | Biological Process | cell fate commitment | Ma11_t18240.1 |
| GO:0044403 | Biological Process | biological process involved in symbiotic interaction | Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1 |
| GO:0009059 | Biological Process | macromolecule biosynthetic process | Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1 |
| GO:0009627 | Biological Process | systemic acquired resistance | Ma06_t22400.1, Ma10_t02380.1, Ma10_t22460.1, Ma10_t17100.1, and Ma06_t08190.1 |
| GO:0006508 | Biological Process | proteolysis | Ma05_t02850.1 |
| GO:0008289 | Molecular Function | lipid binding | All MaLTPs except Ma06_t22400.1, Ma10_t02380.1, Ma10_t22460.1, Ma10_t17100.1, Ma02_t16400.1, Ma10_t26820.1, Ma03_t07430.1, Ma08_t09250.1, Ma06_t08190.1, Ma07_t10090.1, Ma02_t19200.1, Ma02_t19230.1 and Ma02_t08320.1 |
| GO:0008134 | Molecular Function | transcription factor binding | Ma11_t18240.1, Ma04_t17190.1, and Ma04_t17240.1, |
| GO:0005504 | Molecular Function | fatty acid binding | Ma06_t08190.1, Ma06_t22400.1, Ma10_t02380.1, Ma10_t22460.1, and Ma10_t17100.1 |
| GO:0008233 | Molecular Function | peptidase activity | Ma05_t02850.1 |

The mechanisms underlying their antimicrobial action are diverse. Madni et al. (2019) proposed that nsLTPs can remove lipid components from microbial cell membranes, leading to membrane porosity. Supporting this, Chen et al. (2024) reported that StLTPs from potato bind to lipids in the plasma membrane of *P. infestans* hyphal cells, increasing membrane permeability and limiting pathogen spread. In addition to pore formation and membrane permeabilization, other proposed mechanisms include ion leakage, inhibition of pathogen enzymes, and disruption of key metabolic pathways (Melnikova et al., 2022). Notably, the antimicrobial activity of nsLTPs is generally directed towards pathogens rather than host tissues, suggesting that these proteins may specifically recognize and target lipid molecules unique to pathogen membranes (McLaughlin & Tumer, 2025).

Finkina et al. (2016) proposed that nsLTP were secreted into the apoplast and then binds with lipid molecules from plants or invading microorganism. nsLTP then interact with receptor in plant such as serine/threonine protein kinases that contain an extracellular leucine-rich repeat (LRR) domain, besides a transmembrane region and a cytoplasmic protein kinase (PK). This could trigger a series of signal transduction and leads to acquired systemic resistance (SAR) in plants (Amador et al., 2021). This may also suggest that Ma06_t22400.1, Ma10_t02380.1, Ma10_t22460.1, Ma10_t17100.1, and Ma06_t08190.1 are involved in a broader aspect of plant immunity, rather than directly disrupting the cell membrane.

*Orthologous relationship analysis between MansLTPs and proven antifungal nsLTPs*

Orthologous relationship with proven antifungal nsLTP from other plant species was analyzed to infer which *Musa acuminata* DH Pahang nsLTPs potentially have antifungal properties because orthologs descend from the same ancestral sequence and separate at speciation event, but they retain the same gene function (Moreira, 2014). Therefore, theoretically, those MansLTPs that form orthologous relationship with any proven antifungal nsLTP can be taken into consideration as antifungal protein too. Proven antifungal nsLTP from monocots all possessed PF00234 domain as none bearing PF14368 domain were discovered in the literature. Therefore, only nsLTPs with PF00234 domain were used to build the phylogenetic tree. The phylogenetic tree was built using maximum likelihood method, Jones-Taylor-Thornton (JTT) model with Gamma Distributed with Invariant Sites (G+I) at 1000 bootstraps test. Outgroup, fern, basal angiosperm, gymnosperm, monocots, and eudicots were all included in building the tree to have a better insight into the evolutionary relationship. The whole gene family of the selected plant species was also included to avoid bias. Analysis of the phylogenetic tree demonstrated that Ma11_t18240.1, Ma07_t23570.1, Ma01_t10510.1, Ma04_t17220.1 and Ma04_t17200.1 formed an orthologous relationship with antifungal *Triticum aestivum* nsLTP (Figure 2), thus can be identified as potential antifungal MansLTP candidates. Even though bootstrap values of 54 is not significant, however, this is the highest bootstrap value after optimization of the tree (Supplementary Table 4). Optimization of tree was done by trying out different combination of plants species when building the tree especially those with proven antifungal nsLTP. Different types of alignment, MUSCLE alignment and ClustalW alignment was also performed to optimize the final tree.

*Structural alignment analysis of MansLTPs and proven antifungal nsLTPs*

Further analysis using PROMALS3D was conducted to support the results obtained in Figure 2. PROMALS3D was chosen because it includes additional secondary structure prediction in the alignment which increases the fidelity of clustering antifungal proteins together since protein structure and function are closely related. Based on PROMAL3D output, three MansLTP (Ma01_10510, Ma04_t17220, Ma04_17200) was found to be clustered together with the proven antifungal nsLTPs from *Oryza sativa*, *Triticum aestivum* and *Horgeum vulgare*. What is more interesting is that they only clustered with proven antifungal nsLTPs from monocot. None of MansLTP was found to group with any proven antifungal nsLTPs from PF14368 or dicotyledon plants (Figure 3A). Further analysis on the structural alignments of Ma01_10510, Ma04_t17220, Ma04_17200 predicted only alpha-helices structures, which is in accordance with other reports of nsLTPs (Figure 3B). All plant nsLTPs, particularly of type 1, share a conserved fold consisting of four α-helices forming a compact domain, connected by short loops and followed by an extended C-terminal region (Madni et al., 2019; Megeressa et al., 2020; Missaoui et al., 2022). This structure is stabilized by eight conserved cysteine residues forming four disulfide bonds, which contribute to the overall compactness and confer high thermal stability as well as resistance to simulated gastroduodenal proteolysis (Abdullah et al., 2016). The full secondary structure alignments of MansTLPs with the proven antifungal LTPs from selected plant species were provided as Supplementary Table 5.

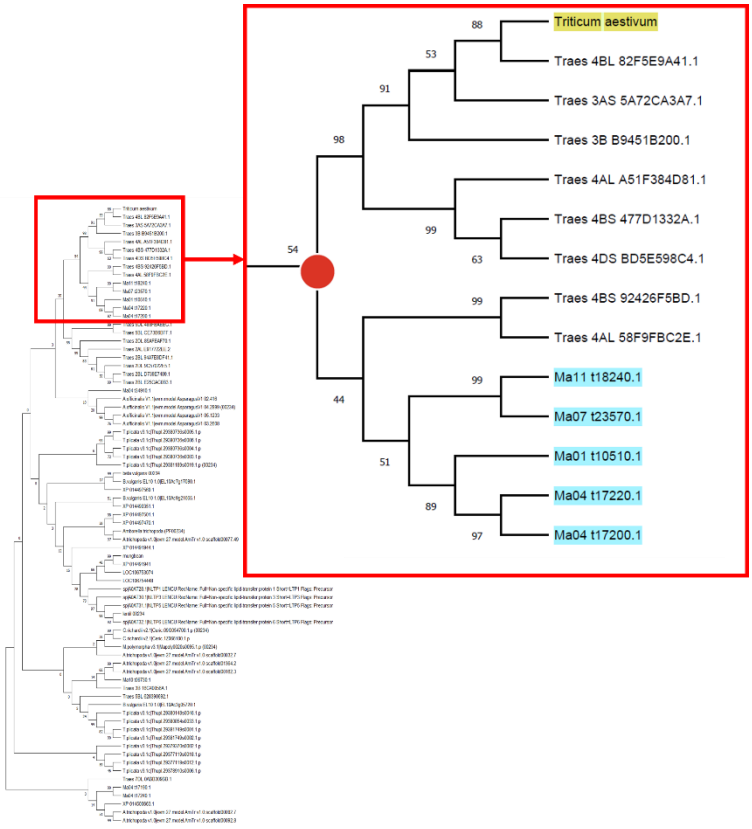



**Figure 2.** Fragment of phylogenetic tree showing orthologous relationship between MansLTP and antifungal nsLTP from *Triticum aestivum*. Antifungal *Triticum aestivum* nsLTP (TansLTP) is highlighted in yellow while MansLTPs forming many-to-many relationship with TansLTP are highlighted in blue. Red dot indicates the node that builds orthologous relationship between MansLTPs and antifungal nsLTP from *Triticum aestivum*. The full phylogenetics tree is provided as Supplementary Table 4.


**Figure 3.** PROMALS3D analysis of MansLTPs and proven antifungal nsLTPs from *Brassicca rapa, Triticum aestivum, Pisum sativum, Oryza sativa, Hordeum vulgare* and *Helianthus annus.* A) PROMALS3D outcome of Ma01_10510, Ma04_t17220 and Ma04_17200 with proven antifungal nsLTP from *Oryza sativa*, *Triticum aestivum* and *Horgeum vulgare* (shown in red box). Magenta names indicate representative sequences. Black-named sequences below a magenta-named one are members of the same sequence group (or cluster) as that representative. (B) Full secondary structure alignment of potential anti-fungal MansLTPs (Ma01_10510, Ma04_t17220 and Ma04_17200) clustered with the proven antifungal proteins from monocots (*Triticum aestivum, Oryza sativa,* and *Hordeum vulgare)*. Red fonts indicate predicted alpha-helices structure. The full PROMALS3D results are provided as Supplementary Table 5.

*Analysis of the cis-acting regulating element in the promoter region of MansLTP genes from PF00234 domain*

The cis-acting elements that are located upstream of the transcriptional start site are primarily responsible for controlling the regulation of gene expression at the promoter level (Nayak et al., 2025). By using PlantCARE database, a total of 15 cis-acting regulatory elements and 36 motifs were identified in the promoter regions of *MansLTP* genes under PF00234 domain (Supplementary Table 6). Based on Figure 4, the highest number of cis-acting regulatory elements identified in *MansLTP*s (PF00234 domain) are related to light responsiveness. There are 20 motifs for light responsiveness present across 9 *MansLTPs* such as G-box, TCT-motif, GATA-motif and AE-box motif (Supplementary Table 6). GATA-binding proteins have been demonstrated to play a role in the regulation of genes involved in light-dependent activities. One such GATA-binding protein, GATA2, has been linked to the control of genes required for the formation of photoreceptors, the plant structures that detect light (Luo et al., 2010). Hormonal cis acting elements were also identified across multiple *MansLTPs*. Methyl jasmonate (MeJA)-responsive and gibberellin acid-responsive elements are the most abundant plant hormone-related motifs that are present in almost all *MansLTP* (PF00234 domain). The MeJA-responsive elements are represented by two motifs which are CGTCA-motif and TGACG-motif. Simultaneously, gibberellin acid-responsive elements are represented by GARE-motif and TATC-box. Many elements of plant development, including in the stem growth and flowering, are thought to be influenced by gibberellins (GAs) (Shan et al., 2007). The TATC-box has also been demonstrated to regulate genes involved in the response to abiotic stress, such as drought and high salinity (Lv et al., 2017). Abscisic acid-cis acting regulatory element, ABRE, is found in all *MansLTP* in the PF00234 domain. The ABRE motif has a role in the regulation of genes involved in seed dormancy, germination, and senescence, as well as other plant responses to stress. Abiotic stress response-related cis acting elements such as drought and low-temperature responsiveness are represented by MBS and LTR motif, respectively.

With regards to biotic response, *Ma10_t06730.1* is the only Musa *nsLTP* that was predicted to possess the defence and stress cis-acting regulatory element, represented by TC-rich repeats motif. The TC rich repeats motif is based on ATTCTCTAAC nucleotide sequences located at around 500 bp of the promoter region, upstream from the coding sequence region of the gene. TC-rich repeats may be involved in the immune system of some organisms, according to some studies. It has been discovered, for instance, that plant genes involved in defence against pathogens often include TC-Rich repeats in their promoters (Agarwal et al., 2013). In response to pathogen infection, the repetitions also function as cis-acting regulatory elements that regulate the expression of defence-related genes. Similar motif was also identified in the promoter region of *LTP7* from *Gossypium hirsutum* L. (Masood et al., 2019). Further functional analysis such as using antimicrobial assay can be performed to verify the role of *Ma10_t06730.1* in the plants' defence system.

*Molecular docking analysis of MansLTPs*

Based on the orthologous relationship and secondary structural similarities with known antifungal nsLTPs from other plants, as well as the CARE analysis, a total of six potential antifungal MansLTPs (Ma11_t18240.1, Ma07_t23570.1, Ma01_t10510.1, Ma04_t17220.1, Ma04_t17200.1 and Ma10_t06730.1) were identified. Docking analysis of six different ligands across these six potential antifungal MansLTPs were performed to further evaluate the potential involvement of these proteins in the bananas' defence mechanism. The result demonstrated distinct patterns of ligand selectivity, reflecting differences in cavity geometry and residue composition among the MansLTPs (Table 4). LPC-16 (1-palmitoyl-sn-glycero-3-phosphocholine) consistently exhibited the strongest binding energies across all MansLTPs possibly due to its structural advantage which are the long hydrophobic palmitoyl chain that fits deeply into the hydrophobic cavity while the phosphocholine headgroup forms stabilizing polar and electrostatic contacts at the cavity entrance (Goufo & Cortez, 2020; Lim et al., 2017). This dual-interaction mode explains why LPC binds more strongly than all the other ligands tested. This behaviour aligns with LPC's established biological functions in which phosphatidylcholine-derived lipids participate in membrane restructuring, ROS-mediated defence signalling and the activation of antifungal responses during pathogen invasion (David et al., 2021; Hidalgo et al., 2021). Linoleic acid (C18:2) showed the next highest affinity particularly for Ma11_t18240.1, Ma07_t23570.1, Ma01_t10510.1 and Ma04_t17220.1, due to its two cis double bonds that introduce conformational flexibility and facilitate enhanced fitting into diverse protein cavities (McClelland et al., 2016). Functionally, linoleic acid is a key player to plant immunity as a precursor of jasmonic acid that triggers antifungal defences, oxidative bursts and defence protein expression (Zhu et al., 2021). Palmitic acid (C16:0) is bound strongly to Ma07_t23570.1, Ma01_t10510.1, Ma04_t17220.1 and Ma04_t17200.1, despite its lack of unsaturation. However, it interacted less effectively with

Ma11_t18240.1 due to the absence of interaction with the essential Tyr77 gate residue. It also interacted poorly with Ma10_t06730.1. The weak performance in Ma10_t06730.1 reflects its altered gate residue (Phe instead of Tyr) and shifted polar anchors, which reduce stabilization of simple saturated fatty acids. Myristic acid (C14:0) displayed moderate to high affinity for Ma11_t18240.1, Ma07_t23570.1, Ma01_t10510.1, Ma04_t17220.1 and Ma04_t17200.1, consistent with the ability of medium-chain fatty acids to fit efficiently within the MansLTPs cavity. However, its binding decreased sharply in Ma10_t06730.1, again highlighting the restrictive nature of this protein's pocket. Palmitic and myristic acids relied primarily on strong hydrophobic contacts due to their saturated aliphatic chains and carboxyl-group polar interactions where both fatty acids are documented for their anti-pathogenic activity through membrane disruption and synergistic enhancement of defence enzymes (Guimarães & Venâncio, 2022; Zhu et al., 2021). Oleic acid (C18:1), despite its biological importance as a signalling fatty acid regulating disease resistance, achieved strong binding only for Ma11_t18240.1 and Ma07_t23570.1 due to limited flexibility from its single unsaturation (Goufo & Cortez, 2020). OPDA (12-Oxo-phytodienoic acid), a key oxylipin intermediate in jasmonate-related immunity, binds strongly only to Ma11_t18240.1 and shows low binding energy towards the other MansLTPs (Mano et al., 2019). The binding energy patterns correlate not only the physicochemical compatibility of each ligand with the MansLTPs cavities but also align closely with their established biochemical roles in antifungal activity and plant defence signalling pathways which highlight the functional relevance of these lipid–protein interactions in plant innate immunity.

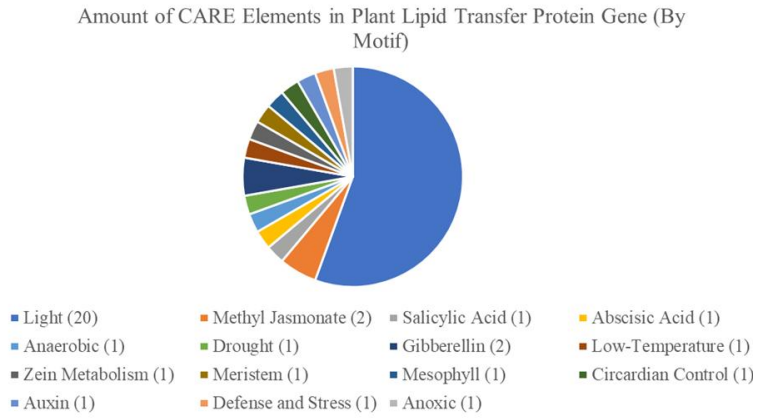



**Figure 4.** Cis-acting regulatory elements in *MansLTP* genes from PF00234 domain by motif

**Table 4.** Binding energies (kcal/mol) of six ligands docked to Ma11_t18240.1, Ma07_t23570.1, Ma01_t10510.1, Ma04_t17220.1, Ma04_t17200.1 and Ma10_t06730.1

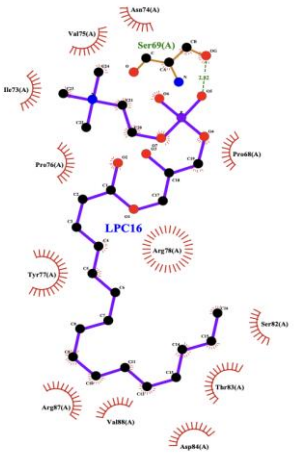
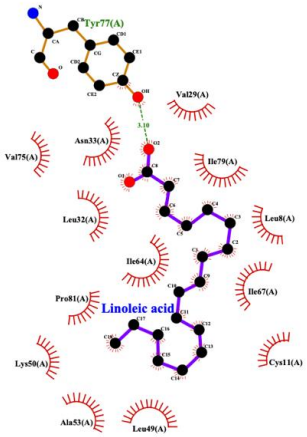
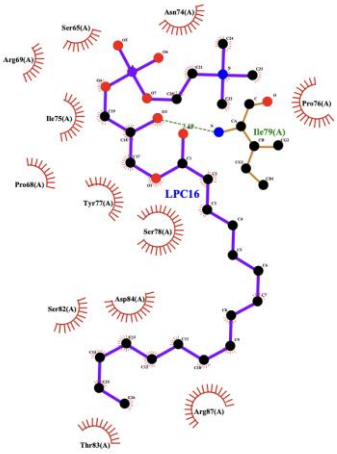
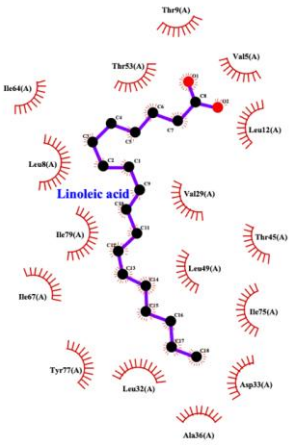
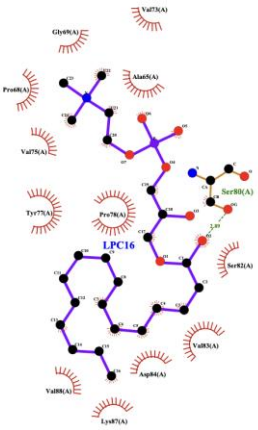
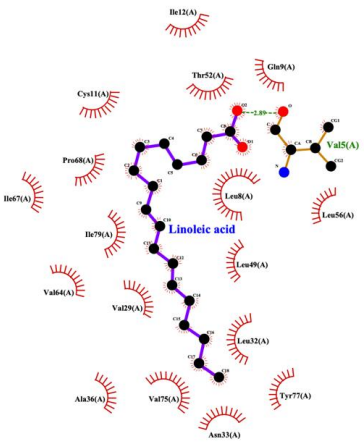
| Ligand | Binding Energy (kcal/mol) | | | | | |
|---|---|---|---|---|---|---|
| | **Ma11_t18240.1** | **Ma07_t23570.1** | **Ma01_t10510.1** | **Ma04_t17220.1** | **Ma04_t17200.1** | **Ma10_t06730.1** |
| LPC-16 | -9.3 | -7.4 | -8.1 | -6.8 | -8.1 | -7.5 |
| Linoleic acid | -6.9 | -7.0 | -7.5 | -7.7 | -4.4 | -4.0 |
| Palmitic acid | -6.2 | -6.7 | -7.2 | -7.0 | -7.1 | -3.4 |
| Myristic acid | -5.9 | -6.3 | -7.2 | -6.7 | -6.7 | -3.6 |
| Oleic acid | -6.9 | -7.0 | -3.4 | -3.5 | -3.5 | -3.9 |
| OPDA | -6.9 | -4.2 | -4.6 | -4.0 | -3.8 | -4.6 |

Analysis of the binding interactions among the MansLTPs with LPC-16 and linoleic acid reveals several residues that consistently contribute to ligand stabilization and affinity (Table 5). For LPC-16, interacting residues are reported for all MansLTPs but in linoleic acid only Ma11_t18240.1, Ma07_t23570.1, Ma01_t10510.1 and Ma04_t17220.1 are shown because the other two MansLTPs exhibited very low binding affinities. Tyr77 emerges as a central residue in MansLTPs because tyrosine residues are often noted for their involvement in the binding site of nsLTPs. According to Jain and Salunke (2017), Tyr79 in rice nsLTP is critical for maintaining the lipid binding environment by reorienting to facilitate effective lipid binding. Therefore, Tyr77 in this MansLTPs may perform a similar role in stabilizing the ligand within the hydrophobic cavity. The aromatic side chain of tyrosine favors π–π stacking and hydrophobic contacts with unsaturated fatty acids such as oleic and linoleic acids (Carrillo-Tripp et al., 2023; Jain & Salunke, 2017). Tyr77 is present in all the five MansLTPs even when tested with different ligands (Supplementary Table 7). Surrounding Tyr77 is a cluster of hydrophobic residues across all the ligands tested which are Leu8, Leu32, Leu 49, Ile/Val64, Ile67 and Ile79 that likely form a hydrophobic pocket contributing significantly to the interactions within the MansLTPs and enhancing its affinity for hydrophobic ligands such as fatty acids and phospholipids (Fadaka et al., 2019; Nazeer et al., 2019). The involvement of leucine, isoleucine and valine in ligand stabilization is well supported across nsLTPs. In Ajwain (*Trachyspermum ammi*) seeds, the presence of Val7, Leu11, Leu12, Ala55, Ala69, Leu70, Leu78, and Ile82 increases binding affinity and helps form optimal configurations for stable interactions (Nazeer et al., 2019). Structural and molecular dynamic studies further demonstrate that the geometry of nsLTP binding pockets accommodate leucine, isoleucine and valine effectively, reinforcing their roles in ligand-binding mechanism (Bansal et al., 2019). Their strong conservation across nsLTPs reflects evolutionary pressure to maintain efficient hydrophobic interactions (Nazeer et al., 2019). A study by Wang et al. (2012) emphasizes the functional importance of these residues where mutating a single residue of Leu8, Phe36 or Val49 to alanine will disrupt cavity integrity, illustrating their essential contribution to ligand specificity and structural stability (Wang et al., 2012). Although the pocket is predominantly hydrophobic, Asn/Asp33 may serve critical functions as polar residues that can partake in hydrogen bonding and electrostatic interactions with various ligands. The ability of these residues to form ionic interactions with the negatively charged groups of lipid molecules facilitates a stable binding conformation which is essential for the transport activity of the MansLTPs. For example, the binding of charged and polar lipids is significantly influenced by the orientation and properties of Asn and Asp residues located in the binding cavities of nsLTPs (Nazeer et al., 2019; Scheurer & Schülke, 2018). Research has shown that aspartate participates actively in forming crucial interactions with potential ligands, enhancing the specificity and efficiency of lipid transfer (Amador et al., 2021). The presence of Asp in specific motifs often associated with the involvement in ATP binding (Pavlopoulou et al., 2019). In contrast, asparagine while similar, is often more geared towards maintaining structural stabilization rather than direct involvement in binding events because its side chain can participate in maintaining the conformational dynamics of the nsLTPs (Dubiela et al., 2019). While not every residue is universally present across all MansLTPs, the recurrence of residues such as Tyr77, Leu8, Leu32, Leu49, Ile/Val64, Ile67, Ile79 and Asn/Asp 33 across the MansLTPs sequences indicates that these residues form a coordinated network of aromatic gating, hydrophobic packing and selective polar anchoring that defines ligand affinity, orientation and specificity in MansLTPs.

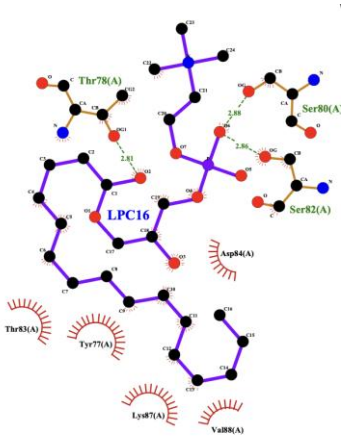
## CONCLUSION

In conclusion, a total of 35 nsLTP sequences, in which 9 sequences belong to PF00234, and 26 sequences belong to PF14368 were identified in *Musa acuminata* DH Pahang (wild banana) (MansLTPs). MansLTPs can be classified into 5 clades, namely Type 1, 2, C, D and G. All MansLTPs possess internal hydrophobic spaces and have 8 conserved cysteine molecules linked by four disulphide bridges. As expected, most of the MansLTPs are predicted to be involved in lipid transport and lipid binding. Several MansLTPs potentially playing role in the defence response of banana were identified, namely Ma10_t06730.1, Ma06_t22400.1, Ma10_t02380.1, Ma10_t22460.1, Ma10_t17100.1, Ma06_t08190.1, Ma11_t18240.1, Ma07_t23570.1, Ma01_t10510.1, Ma04_t17220.1 and Ma04_t17200.1 based on the orthologous relationship with the proven antifungal nsTLPs from other species, functional annotation, analysis of the cis-acting regulatory elements and molecular docking analysis.

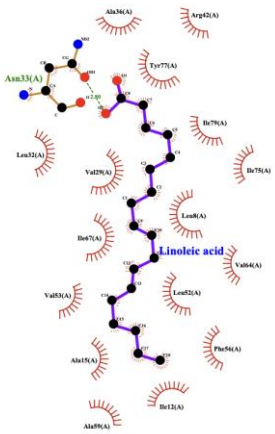
**Table 5.** Molecular interaction profile of 1-Palmitoyl-sn-glycero-3-phosphocholine (LPC-16) and Linoleic Acid with Ma11_t18240.1, Ma07_t23570.1, Ma01_t10510.1, Ma04_t17220.1, Ma04_t17200.1 and Ma10_t06730.1

| Accession Number | Interacting Residue | |
|---|---|---|
| | 1-Palmitoyl-sn-glycero-3-phosphocholine (LPC-16) | Linoleic Acid |
| Ma11_t18240.1 |  P1-LPC16 Complex |  P1-Linoleic Complex |
| Ma07_t23570.1 |  P2-LPC16 Complex |  P2-Linoleic Complex |
| Ma01_t10510.1 |  P3-LPC16 Complex |  P3-Linoleic Complex |

Ma04_t17220.1

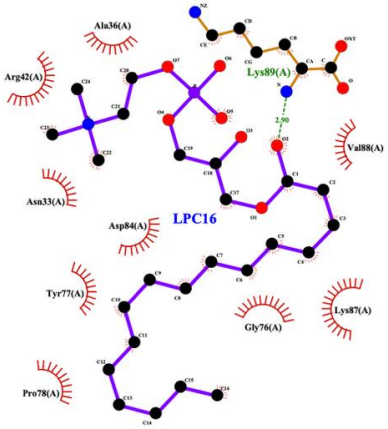



P4-LPC16 Complex



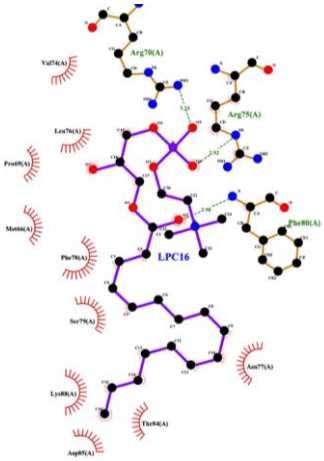

P4-Linoleic Complex

Ma04_t17200.1




P5-LPC16 Complex

Ma10_t06730.1




P6-LPC16 Complex

*The interacting residues between Ma04_t17200.1 and Ma10_t06730.1 with Linoleic Acid are not shown due to low binding energy.

Further analysis such as differential expression profile against phytopathogen and antifungal assay must be conducted to prove this prediction. Overall, the *in silico* analysis of MansLTPs offers insights into their potential functions and lays a foundation for future functional studies, ultimately contributing to improved banana plant responses against various biological stresses.

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## CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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