MALAYSIAN JOURNAL OF BIOCHEMISTRY & MOLECULAR BIOLOGY

The Official Publication of The Malaysian Society For Biochemistry & Molecular Biology (MSBMB)
http://mjbmb.org

IN SILICO CHARACTERIZATION OF DEFENSIN IN *Musa acuminata* DH PAHANG (MaDef) PROVIDES INSIGHT INTO POTENTIAL DEFENCE-RELATED PROTEIN

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History
Received: 6th May 2023
Accepted: 24th September 2023

Keywords:

Defensins; Wild banana; Cis-acting; Bioinformatics; Biotic stress

Abstract

Plant defensins are expressed in response to phytopathogens and various defence-related signalling molecules. They possess diverse biological properties such as antifungal, antibacterial and proteinase inhibitory, as well as playing roles in plant growth and development. Multiple defensin copies are identified in numerous plant species, such as *Arabidopsis*, *Brassica oleracea*, *Zea mays* and *Medicago truncatula*. To our knowledge, the multigene family of defensin has never been reported in bananas. In addition, specific banana defensin genes involved in the defence and stress responsiveness are yet to be identified. Thus, this study predicts specific copies of *Musa acuminata* DH Pahang (wild banana) defensins that are potentially involved with defence and biotic stress response using *in silico* analysis. A total of 6 defensin copies from *Musa acuminata* DH Pahang (wild banana) (MaDef) were identified and categorised under the Knottin_1 clan (CL0054). All of them except Ma07_t03680.1 carry conserved sequences of the gamma-thionin domain (PF00304). A total of 8 cysteines forming 4 disulfide bridges are found across all six MaDef peptide sequences. Using phylogenetic analysis, wild banana defensins are categorised under three clades with the predicted molecular weight of 8 to 9 kDa. Gene ontology (GO) revealed that all MaDefs except for Ma07_t03680.1 are involved in defence response. Furthermore, analysis of the promoter regions through PlantCARE shows Ma04_t36140.1 is associated with defence and stress responsiveness. Overall, this study contributes to a deeper understanding of defensins characteristics and functional predictions, which are critical for future crop advances against biotic challenges, notably in bananas.

INTRODUCTION

Banana (*Musa spp.*), including plantains, is one of the major staple food crops farmed in about 140 countries in the subtropics and tropics, with annual production of around 148 million tonnes, feeding approximately 500 million people [1]. Numerous factors, including biotic and abiotic stressors, decreased soil fertility, limited genetic variety in germplasm, and insufficient access to clean planting material among smallholder farmers, have a significant impact on banana production. The environmental elements, both biotic and abiotic, force the plants to develop survival strategies in order to withstand the stressors [2]. In response to these occurrences, a broad array of pathogenesis-related (PR) defence proteins, such as plant defensins [3] are expressed. Defensins are a broad family of cationic host defence peptides (HDP) that can be found in both plants and animals [4] [5] [6]. Due to their similarities in size (5 kDa, 45 to 54 amino acids) and cysteine content (usually 4, 6 or 8 cysteine residues), these proteins were formerly referred to as gamma-thionins [7]. Through detailed structural analysis, it was found that gamma-thionin proteins have structural characteristics common to animal defensins, including the gamma-core motif (GXCX3-9C) and other conserved locations of the sequence. However, there are significant sequence variations in plant defensins despite their structural resemblances and conserved cysteine residues [3] [8] [9] [10]. Several structure-activity studies point to the gamma-core motif as the primary site of antifungal and antibacterial activity in plant defensins [11] [12] [13] [14] [15]. The gamma-core motif peptides derived from defensins RsAFP-2 (*Rhapanus sativus*), MtDef4 and MtDef5 (*Medicago truncatula*), MsDef1 (*Medicago sativa*), So-D2 (*Spinacia oleracea*), Vu-Def (*Vigna unguiculata)*, BcDef (*Brugmansia x candida)*, PvD1 (*Phaseolus vulgaris)* and the tomato defensin, SolyC07g007760 exhibited antifungal and antibacterial properties at micromolar concentrations [11] [15] [16] [17] [18] [19] [20] [21]. In another study, MsDef1 and MtDef4 from *Medicago spp*., possessing a highly conserved gamma-core motif with different net positive charges, can inhibit the fungal growth via different modes of action. MsDef1 is classified as morphogenic antifungal plant defensin, which inhibits the fungal growth accompanied by increasing hyphal branching, unlike the non-morphogenic antifungal MtDef4. When the MsDef1's gamma-core motif was switched out for that of MtDef4, it almost had the same potency as MtDef4. This alteration also caused it to lose its method of antifungal action and fail to trigger fungal hyphae hyperbranching [11]. Sagaram et al. [11] further added that the gamma-core motif of MtDef4 alone managed to restrict the fungal growth, unlike the MsDef1's suggesting that the net positive charge and hydrophobicity contribute towards the functional significance of the motif and possibly defensins' mode of actions.

Most characterised plant defensins exhibit constitutive expression patterns that are induced in response to pathogen harm, destruction, and some abiotic stressors [22]. They are essential for the safety of germinating seeds and developing seedlings, and have been found in leaves, tubers, flowers, pods, and seeds [23]. Plant defensins are not only found in the parenchyma cells, but also in the xylem, stomata, and stomata cells [16] [24] [25]. As these tissues are thought to be the initial point of interaction with any possible pathogen, their presence is coherent with a protective role for these peptides. These localisations give rise to the hypothesis that these proteins play an important part in the suppression of entry points that could be exploited by potential adversaries [26]. Defensins exist as multigene families in plants. A total of 317 genes that encode defensin and defensin-like peptides were identified in the *Arabidopsis* genome [27] [28]. The genomes of *Medicago truncatula* and *Vitis vinifera* both contain 778 potential sequences [29] and 79 genes [30], respectively. Although plant defensin gene sequences have been identified and analysed in different plant species, information regarding the whole defensin gene family in wild banana and the specific defensin genes associated with defence and stress responsiveness have not been reported yet.

Therefore, the goals of this study are to catalogue all of the potential defensin genes in *Musa acuminata* DH Pahang (wild banana) and predict their roles using *in silico* methods such as gene ontology and cis-acting regulatory elements (CAREs) analyses with a specific focus on the defence-related proteins. The findings of this study lay the groundwork for further functional analysis to particularly address the biotic challenges faced by bananas.

MATERIALS AND METHODS

Identification of Defensin Family in *Musa acuminata* DH Pahang (Wild Banana) (MaDef)

Based on a literature search, a number of plant defensins with proven antimicrobial properties are associated with the gamma-thionin domain (PF00304) under the Knottin_1 clan (CL0054) when subjected to the Pfam databases (Supplementary Table 1). Thus, putative defensin proteins in *Musa acuminata* DH Pahang (wild banana) (MaDef) were also retrieved based on the known gamma-thionin (PF00304) in Knottin_1 clan (CL0054) of Pfam databases (http://pfam-legacy.xfam.org/). Firstly, the protein sequence under the accession number M0SU99_MUSAM (M0SU99) was retrieved from the UniProt database (https://www.uniprot.org/) and used as the query sequence in a BLASTp search against the *Musa acuminata* DH Pahang database (version 2) in Banana Genome Hub (BGH) (https://banana-genome-hub.southgreen.fr/). All the hit sequences were screened based on their E-value, the conserved domain and the clan that they belong to. All putative defensin sequences with significant blast hit (< 0.05) and associated with the Knottin_1 clan (CL0054) (with or without gamma-thionin domain (PF00304)) were short-

listed and subjected to domain verification using ScanProSite (https://prosite.expasy.org/scanprosite/). Once the domain was verified, the putative MaDef sequences were subjected to further *in silico* analysis.

Multiple Sequence Alignment and Phylogenetic Analysis of MaDefs

All MaDef amino acid sequences were aligned using ClustalW (https://www.ebi.ac.uk/Tools/msa/clustalo/), followed by the construction of a neighbour-joining tree with 1000 replication bootstraps using MEGAX 11. Partial deletion gaps and the Jones, Taylor, and Thornton (JTT) model were employed during the run. The phylogeny analysis also included defensin sequences from other plant species including *Capsicum annum* (CaDef2, J1-1), *Cicer arietinum* (Cadef1), *Medicago truncatula* (MtDef2), *Pentadiplandra brazzeana* (Brazzein), *Clitoria ternatea* (CtAMP), *Dahlia merckii* (DmAMP1), *Brassica oleracea* (BoPCP, PCP-A1), *Vigna unguiculata* (Cpthio2), *Vigna radiate* (VrD1), *Zea mays* (ZmESR6, ZmES2, ZmES1, ZmDef, $_{\gamma}$-Z2), *Nicotiana alata* (NaD1), *Arabidopsis thaliana* (LCR72), *Picea abies* (SPI1B), *Picea glauca* (PgD1), *Beta vulgaris* (AX1, AX2), *Sorghum bicolor* (SLα2), Triticum turgidum ($_{\gamma}$-puro1), *Spinacia oleracea* (SoD2) and *Vicia faba* (fabatin1, fabatin2) [18].

Analysis of Physicochemical Properties and Subcellular Localisation of MaDef Protein Sequences

All MaDef protein sequences were examined for their molecular weights and theoretical isoelectric point (pI) using ExPASy (https://web.expasy.org/compute_pi/). In addition, SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/index.php) was used to predict the presence of signal peptides. In order to characterise MaDefs physicochemical characteristics, estimation of the percentage of positive residues, negative residues, polar and hydrophobic residues, aliphatic index, and value of the grand average of hydropathicity (GRAVY) were performed using ExPASy-ProtParam tool (https://web.expasy.org/protparam/). Then, the bioinformatics tool, Cell-Ploc 2.0, was used to predict the subcellular localisation of MaDefs. The retrieved MaDefs protein sequences were inserted in the query section of the Plant-mPloc 2.0 website (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi) in FASTA format and were submitted.

Analysis of MaDef Protein Sequence Features

The percentage of protein sequence similarity of all MaDefs was determined through Sequence Identity and Similarity, SIAS (http://imed.med.ucm.es/Tools/sias.html) and the ungapped motifs in MaDefs were analysed using Multiple Em for Motif Elicitation (MEME) Suite 5.1.1 (http://meme-suite.org/) with the setting of 'Zero or One Occurrence per Sequence' and was set for more than ten sequences.

Analysis of Gene Ontology (GO) of MaDefs

The predicted biological function and cellular components of MaDefs were analysed using PANNZER2 (http://ekhidna2.biocenter.helsinki.fi/sanspanz/).

Analysis of the cis-acting Regulatory Elements (CAREs) of MaDefs

The promoter regions of identified *MaDef* genes (approximately 1.5 kbp upstream to the translation start site) were retrieved from BGH. The sequences were then subjected to the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to classify and further analyse the CAREs according to their respective functions.

RESULTS AND DISCUSSION

Sequence Retrieval and Identification of Defensin Family in *Musa acuminata* DH Pahang (Wild Banana) (MaDef)

A total of eighteen defensin candidates were obtained through BLASTp search of the protein sequence under the M0SU99_MUSAM accession number against the *Musa acuminata* DH Pahang (wild banana) v2.0 database (Supplementary File 2). After eliminating the hit sequences with an e-value higher than 0.05 and do not belong to the Knottin_1 clan (CL0054), a total of 6 defensin genes were identified in wild banana (*MaDef*s) (Table 1). All of them are classified under the gamma-thionin domain (PF00304) except for Ma07_t03680.1. Previous studies have shown that MsDef1 (*Medicago sativa*), MtDef4 (*Medicago truncatula*) [31], NaD1 (*Nicotiana alata*) [32] [33], Vv-AMP1 (*Vitis vinifera*) [34] and recombinant AhPDF1.1b (*Arabidopsis halleri*) [35] are some of the plant defensins that had been reported to have antifungal activity against *Fusarium oxysporum* which are associated with the gamma-thionin (PF00304) protein family under the Knottin_1 clan (CL0054). Antimicrobial defensins such as Pth-sf1 (*Solanum tuberosum*) [36], Cp-thionin II (*Vigna unguiculata*) [37] and fabatin (*Vicia faba*) [38] were also classified under the gamma-thionin domain (PF00304). Also known as the Scorpion toxin-like knottin superfamily, a variety of different toxin families that all have the same knottin structure belong to this clan. These families are derived from plants, arthropods, and scorpions. Knottins are tiny proteins that are distinguished by a cystine-knot. They make up a large group of structurally similar peptides with numerous biological properties, such as inhibitory, antimicrobial, and toxic properties. ScanProSite was utilised to further verify the domain and conserved region of these sequences where all of the five MaDefs' protein sequences

are associated with gamma-thionin family signature at the amino acid residue positions of 31 to 54 with the confidence level of 0, which strongly indicates that a portion of the wild banana defensins' protein sequences belong to the gamma-thionin family signature. According to Bruix, M. et al. [7], these proteins were initially termed gamma-thionins because their size (5 kDa, 45 to 54 amino acids) and the cysteine content (usually 4, 6, or 8 cysteine residues) which were discovered to be similar to thionins. Despite being classified under the Knottin_1 clan (CL0054), Ma07_t03680.1 produced a non-significant hit when screened for the gamma-thionin domain using Pfam and ScanProsite databases. Nevertheless, the Ma07_t03680.1 sequence was included in the subsequent analysis to aid in homology comparison with the other putative MaDefs.

Table 1. Description of wild banana defensins associated with the Knottin_1 clan (CL0054)

Accession number	Organism Hit	Function	Sequence Identities (%)	E-value
Ma07_t03680.1	DH-Pahang	Conserved hypothetical protein	30	1.00E-04
Ma02_t21840.1	DH-Pahang	Knot1 domain-containing protein	30	0.036
Ma08_t13660.1	DH-Pahang	Knot1 domain-containing protein	30	1.00E-06
Ma04_t36140.1	DH-Pahang	Knot1 domain-containing protein	36	6.00E-06
Ma11_t12930.1	DH-Pahang	Defensin J1-2	31	1.00E-06
Ma06_t12420.1	DH-Pahang	Defensin-like protein 2	37	0.006

Multiple Sequence Alignments Analysis of MaDefs

By analysing the plant defensin sequences from different plant species, Lay and Anderson [4] discovered that the plant defensin family has relatively little sequence conservation except for eight cysteine, two glycine, and one glutamic acid residues. According to Broekeart et al. [39], the eight cysteines, two glycines at positions 13 and 34, an aromatic residue at position 11, and a glutamic acid at position 29 are the only residues that are conserved throughout all sequences (numbering relative to Rs-AFP1 of *Raphanus sativus*). There are other common residues that are involved in the folding of plant defensins [40], which include a glutamate (at position 27), an aromatic residue (at position 10), and two glycine residues (at positions 12 and 32 (numbering related to the plant defensin NaD1 of *Nicotiana alata*) [41].

Multiple sequence alignment analysis revealed that all putative MaDefs possess eight conserved cysteine residues, which resulted in the formation of 4 disulfide bond linkages between cysteine (Figure 1). In plant defensins, the disulfide bonds are arranged as follows: Cys1-Cys8, Cys2-Cys5, Cys3-Cys6, and Cys4-Cys7 [4]. The presence of four disulfide bridges contributes to the pseudo-cyclic orientation of defensins that connects the N- and C-terminal, giving them a highly stable conformation under adverse chemical and temperature circumstances. The peptides with four disulfide bridges are referred to as 8C-plants, and they resemble other plant defensins such as NaD1 (defensin from *Nicotiana alata*), VrD1 (defensin from *Vigna radiate*), AlfAFP (antifungal protein from alfalfa), Ms-Def1 (defensin from *Medicago sativa*), ω-hordothionin (barley), Psd1 (defensin from *Pisum sativum*) and Rs-AFPs (antifungal proteins from *Raphanus sativus*). In addition, the presence of disulfide bonds stabilise the defensin structure, allowing it to function properly biologically [42] [43].

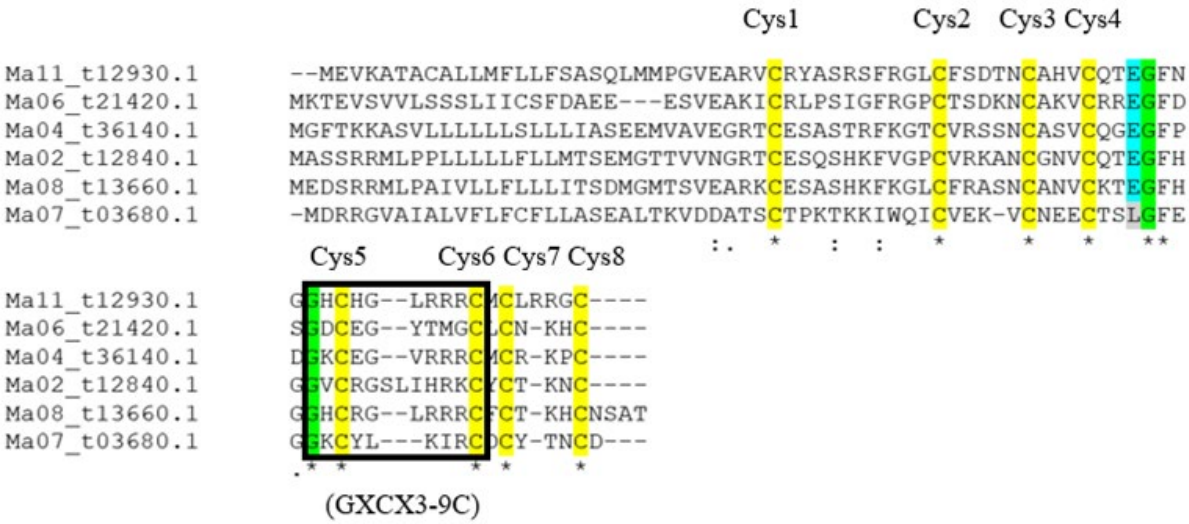

Figure 1. Multiple sequence alignment of MaDef peptide sequences from *Musa acuminata* DH Pahang (wild banana). The yellow highlight represents the conserved cysteine residues. Green, turquoise and grey highlights indicate the presence of glycine, glutamic acid and leucine residues, respectively. The black box represents the gamma-core motif in MaDefs.

Similar to the defensins from other plant species [21], [44] and [45], glycine residues in wild banana defensins are conserved in the gamma-core motif. Glutamic acid residues (E) are conserved in all MaDefs at position 57 (E_{57}) (turquoise) (relative to Ma02_t21840.1, Ma08_t13660.1 and Ma04_t36140.1) except for Ma07_t03680.1 in which leucine (L) (grey) was encoded instead. This phenomenon occurred probably due to point mutations within the Ma07_t03680.1 genomic sequence. According to Terras et al. [46], the primary structures of Rs-AFP1 and Rs-AFP2 only differ by two amino acids. Both Glu_5 (glutamic acid) and Asn_{27} (asparagine) from Rs-AFP1 have been replaced by glutamine (Gln) and arginine (Arg) residues, respectively, in Rs-AFP2. As a result, Rs-AFP2 loses one negative charge and gains one positive charge as a result of these natural substitutions. Compared to its native isoform, Rs-AFP1, Rs-AFP2 has enhanced cationicity, which is associated with increased antifungal activity in the presence of cations. However, there is no evidence that clarifies the effect of E57L (relative to Ma02_t21840.1, Ma08_t13660.1 and Ma04_t36140.1) substitutions on the biological role of plant defensin.

Carvalho and Gomez [26] discovered that the amino acid sequences in the primary structures of plant defensins from different plant species vary significantly. Although primary structure variability can be observed among these characterised plant defensins, their tertiary structure is highly conserved. Given that the primary structure dictates the tertiary structure, these variations in primary structure reflect small spatial variations of the three-dimensional structure, primarily in the size of the loops that provide overall structural diversity and contribute to the broad range of biological activities described for plant defensins [26].

In addition, the gamma-core motif can be observed as well in the sequence alignment (Figure 1). The consensus amino acid sequence of GXC3-9C, which is present in practically all types of cysteine-stabilised AMPs from prokaryotes and eukaryotes, makes up the gamma-core motif. It has been reported that the $_{\gamma}$-core motif is critical for antibacterial action in disulfide-stabilised peptides [47].

Phylogenetic Analysis of MaDefs

In this study, the neighbour-joining approach was used to deduce the evolutionary history of defensins in wild bananas and other plants. The evolutionary history of the taxa analysed was represented by the bootstrap consensus tree generated from 1000 replicates. The evolutionary distances were computed using the JTT matrix-based method and are in the units of the number of amino acid substitutions per site.

The original classification of defensins into functional groups given by Broekaert et al. [39] was based on the defensin's ability or inability to inhibit fungal growth, as well as the influence it had on fungal morphology during growth inhibition. The classification was done based on 14 different defensin sequences isolated from *Brassicaceae* (Rs-AFP1 and Rs-AFP2 from radish seeds), *Saxifragaceae* (Hs-AFPI from *Heuchera sanguinea* seeds), *Asteraceae* (Dm-AMPl from dahlia seeds), the *Fabaceae* (Ct-AMPl from *Clitoria tevnatea* seeds), *Hippocastan-aceae* (Ah-AMP1 from horse chestnut), *Poaceae* (including $_{\gamma}$l-P, $_{\gamma}$l-H, and Siα2) from wheat, barley, and sorghum seeds), PPT (*Petunia inflata*), FST (*Nicotiana alata*), pSAS10 (*Vigna unguiculata*), pI230 (*Pisum sativum*) and p322 (*Solanum tuberosum*), four of which differed by only seven amino acids [46] [48]. Since

the classification was derived solely based on the antifungal properties, the classification failed to account for the numerous additional functions carried out by defensins. In a more comprehensive evolutionary study of 139 plant defensins, the proteins were classified into 18 distinct groups [42]. Given that new defensins are continually being discovered and the proposed grouping may change in the future, this study revealed that defensins with comparable activity frequently cluster together [49]. Therefore, a maximum of two defensin sequences from each of the 18 distinct groups comprising different vascular plant species, which include both angiosperms and gymnosperms were selected for the evolutionary analysis with wild banana defensins to give more insight into their potential biological activities as proteins that share similar function tend to evolve together.

In this study, wild banana defensins (MaDef) can be classified into three major clades, although there are no specific functions cater to these clades (Figure 2). Ma02_t12840.1, Ma08_t13660.1 and Ma04_t36140.1 were categorised under Clade I together with other plant defensins such as from *Arabidopsis thaliana, Capsicum annum, Cicer arietinum, Medicago truncatula, Petandiplandra brazzeana, Clitoria ternatea, Dahlia merckii, Brassica oleracea, Petunia hybrid, Nicotiana alata, Picea abies and Picea glauca*. In particular, Ma02_t12840.1 and Ma08_t13660.1 share the closest relationship as they were both descended from a common ancestor, forming a one-to-one relationship. In addition, all MaDefs under Clade I share many-to-many relationships with the defensins of *Picea abies* (SPI1B), *Picea glauca* (PgD1) and *Arabidopsis thaliana* (LCR72). According to Weerden and Anderson [42], the antifungal activity of PgD1 from *Picea glauca* had been proven through in vitro assay, while LCR72 from *Arabidopsis thaliana* is yet to be characterised. Since structurally similar proteins tend to carry similar functions, it is also possible that Ma02_t12840.1, Ma08_t13660.1 and Ma04_t36140.1 may possess antifungal properties which can be verified through functional analysis.

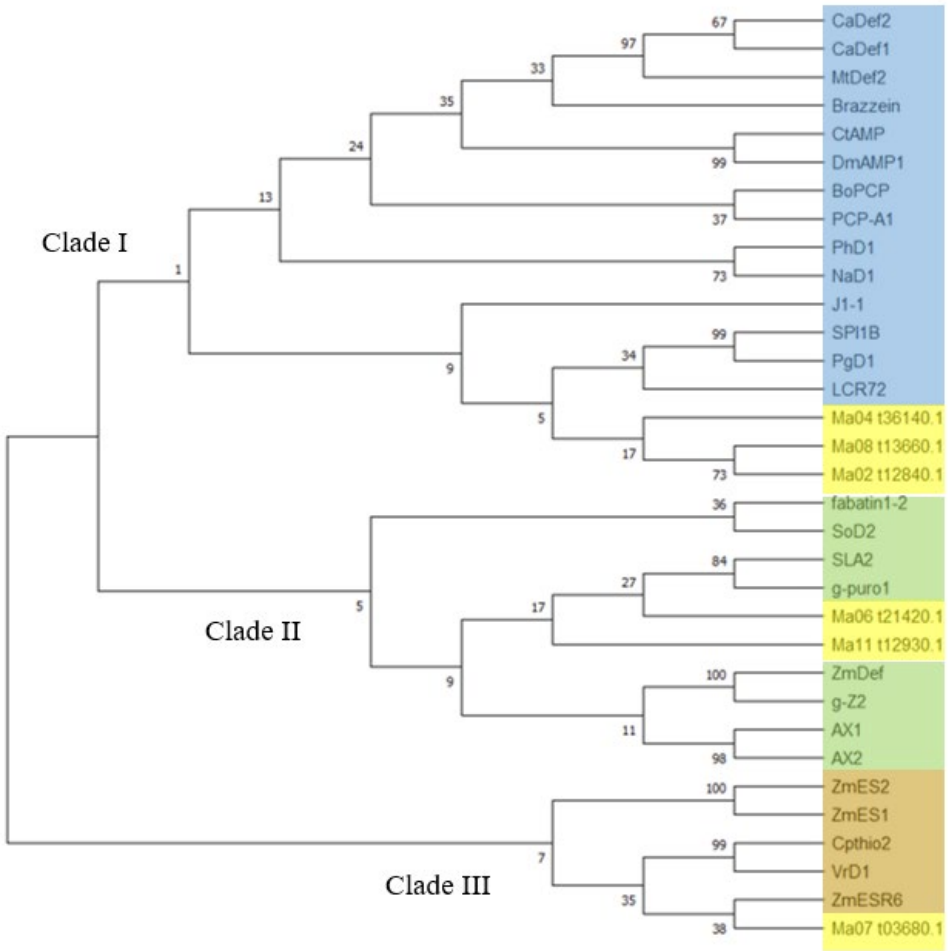

Figure 2. Phylogenetic tree construction where evolutionary analyses between defensins in wild banana and other plant defensins were conducted in MEGA 11, and the evolutionary history was inferred using the neighbour-joining method with bootstrap consensus tree inferred from 1000 replicates computed using JTT matrix-based method.

Ma11_t12930.1 and Ma06_t21420.1, which are classified under Clade II, share the same ancestral lineage with defensins from *Sorghum bicolour* (Slα1) and *Triticum turgidum* ($_{\gamma}$-puro1). It has been reported that these defensins are involved in protein synthesis inhibitors, α-amylase inhibitors, and sodium channel blockers [42], which might be the potential molecular function for Ma11_t12930.1 and Ma06_t21420.1.

Ma07_t03680.1 is the only wild banana defensin categorised under Clade III, where it shares the last common ancestor with the corn's ZmESR6. Weerden and Anderson [42] discovered that *Zea may's* ZMESR6 is active against both fungus and bacteria and is expressed with a C-terminal pro-peptide (CTPP). In developing maise kernels, this protein is expressed in the area around the embryo and builds up in the *placentochalaza* cells. In clade III, similar ancestral lineage can also be seen between Ma07_t03680.1 with ZmES1 and ZmES2 peptides. These peptides were discovered in the female gametophyte of *Zea mays* where it has been reported by Amien et al. [50] that interaction between these peptides with potassium channel causes the pollen tubes to burst.

Each member of the defensin family that was divided into distinct clusters has a specific function in plants, despite the fact that some of their functions and mechanisms are still unknown. As plants engage their defence mechanisms to survive, the genes can be biotically or abiotically induced, and they are differently expressed and triggered in accordance with the necessary circumstances [51]. This shows that the roles of defensins extend beyond defence and are crucial components of other cellular processes. In this study, only representative genes from each defensin group proposed by Weerden and Anderson [42] were included. To infer a more accurate orthologous link and elucidate the potential biological functions of MaDefs, the phylogenetic analysis should include all defensin members of the plant species analysed.

Analysis of Physicochemical Properties and Subcellular Localisation of MaDefs

The molecular weight, theoretical isoelectric point and signal peptides of MaDefs are summarised in Table 2. Overall, the predicted molecular weight of MaDefs ranges from 8 to 9 kilo Dalton (kDa), where Ma08_t13660.1 has the highest predicted molecular weight. In addition, SignalP-5.0 analysis indicated the presence of signal peptides in all six wild banana defensins, implying their extracellular localisation. Defensins are assisted by secretory signal peptides to locate in the extracellular space where they exhibit their biological action [52].

Table 2. Predicted molecular weight, theoretical isoelectric point and the presence of signal peptide of MaDefs

Number	MaDef	Molecular weight	Theoretical isoelectric points	Signal peptide (Sec/SPI)	Predicted locations (subcellular localisation)
1.	Ma07_t03680.1	8410.87	6.15	Yes	Nucleus
2.	Ma02_t21840.1	8679.28	9.27	Yes	Vacuole
3.	Ma08_t13660.1	9041.63	9.27	Yes	Vacuole
4.	Ma04_t36140.1	8320.86	8.92	Yes	Nucleus & Vacuole
5.	Ma11_t12930.1	8468.02	9.06	Yes	Vacuole
6.	Ma06_t12420.1	8046.23	5.26	Yes	Vacuole

According to Vriens et al. [44], plant defensins are classified into two classes based on the pre-protein structure. Class I defensin precursors include a signal peptide and a mature defensin domain, which is directed toward the secretory route from the endoplasmic reticulum. The first line of protection against encroaching plant pathogens in the extracellular space is provided by class I defensins. However, class I defensins lack C-terminal pro-protein (CTPP) compared to class II defensins which are derived from progenitors that are directed to the vacuole where the CTPP is removed. Class I defensins are only found in seeds, whereas class II plant defensins are discovered to be abundantly expressed in both reproductive and vegetative parts of the plant [53]. Plants expressing class II defensins without CTPP experienced development retardation, demonstrating the phytotoxicity of these defensin peptides [54, 55].

As the functions of proteins are closely determined by their locations in the cell, proteins must be transported to the correct locations to perform the roles assigned to them [56] [57]. While it is possible to gather this knowledge through biochemical assays, it is laborious and costly to individually determine the subcellular localisation of uncharacterised proteins. In a study conducted by Kong et al. [58], four *in silico* programmes, including Predotar [59], TargetP [60], Plant-mPLoc [61], and WoLF PSORT [62], were used to

predict the subcellular localisation of monovalent cation-proton antiporter superfamily in maize (ZmCPA) proteins. The prediction findings were more precise using Plant-mPLoc compared to other programmes as only 1/3 of the cation-proton antiporter proteins where their subcellular localisations were correctly predicted. Transient expression assays were carried out to further validate their subcellular localisations, and the results obtained were consistent with the *in silico* predictions. Therefore, Plant-mPloc was utilised to predict the subcellular localisations of MaDefs.

Plant AMPs are subcellularly localised to various areas of the cell based on their signal peptides and potential activities. It has been reported that plant defensins are localised either in extracellular areas [63] [64] or in the vacuole [53]. Analysis using Plant-mPloc 2.0 [65] predicted that all six MaDefs are localised at specific locations. Based on the Plant-mPloc computation result, only Ma04_t36140.1 was predicted to localise at two different locations: the nucleus and vacuole, while Ma07_t03680.1 was predicted to localise at the nucleus. Other MaDefs are potentially localised at the vacuole (Table 2). It has been proposed that defensin plays a role in peptide signalling to the vacuole, where floral defensins from *Nicotiana alata*, NaD1 was immunolocalised [53]. Plant defensins' putative vacuolar localisation does not rule out a function in defence mechanisms. Vacuolar defensins, like chitinases, can only be released when pathogens damage plant cells [66], concentrating them at the site of cell damage and delaying the development of plant defensin-resistance in the pathogen as a result of ongoing exposure in the intercellular space. Furthermore, plant defensin intracellular localisation may be related to (extra) *in vivo* activities of defensins not associated with plant defence.

The estimated values of the Grand Average of Hydrophaticity (GRAVY) of wild banana defensins (MaDefs) range from -0.109 to 0.158 (Table 3). Only Ma08_t13660.1 and Ma06_t21420.1 showed negative values. Negative values imply the hydrophilic nature of the proteins, which predicts a better interaction with water. Understanding the hydrophobicity nature of proteins gives insight into their protein folding and stability, which is essential for predicting the biomolecular interaction and possibly the proteins' functions [67]. Sagaram et al. [11] showed that the gamma-core motifs of MsDef1 and MtDef4 have a net positive charge and a hydrophobic phenylalanine (Phe) residue that potentially contributed to the antifungal activity of both proteins. Substitution of a hydrophobic phenylalanine residue at position 3 and cationic Arginine (R) at position 4 of GMA4-L (RGFRRR), respectively, in MsDef1, reduce the antifungal activity of the peptide as exemplified by two different variants, GMA4-L1 (RGARRR) and GMA4-L2 (RGFARR) [11].

Table 3. Percentage of positive, negative, polar and hydrophobic residues and value of Grand Average of Hydrophaticity (GRAVY) of MaDefs

	Residue charges (%)					
MaDef	**Positive residues**	**Negative residues**	**Polar residues**	**Hydrophobic residues**	**Aliphatic index**	**Grand average of Hydrophaticity**
Ma07_t03680.1	13.30	13.40	36.10	42.50	83.20	0.141
Ma02_t12840.1	12.70	3.80	35.50	45.60	73.92	0.019
Ma08_t13660.1	16.10	8.70	33.30	43.20	66.30	-0.105
Ma04_t36140.1	15.60	9.10	35.1	46.80	79.74	0.121
Ma11_t12930.1	13.10	5.20	30.10	48.70	65.53	0.158
Ma06_t21420.1	12.20	14.90	42.10	39.30	65.81	-0.109

Analysis of MaDef Protein Sequence and Motifs

SIAS analysis demonstrated the percentage of sequence similarity between two sequences. It is widely assumed that two proteins that share high sequence similarity may also have similar structures as well as functions [68]. This information, aided by other analyses such as phylogenetics tree, can help to infer the ancestral relationship between two proteins. Ma02_t12840.1 and Ma08_t13660.1 share the highest protein sequence identity and similarity (Supplementary File 3), which are 62.02 % and 70.88 %, respectively, which implies their homologous relationship. This was also reflected by their one-to-one relationship demonstrated in the phylogenetic tree analysis. The lowest percentage of identity and similarity belong to Ma07_t03680.1 and Ma06_t21420.1, which are 21.62 % and 31.08%, respectively. Phylogenetic analysis also suggests the low degree of similarity between Ma07_t03680.1 and Ma06_t21420.1 as these two proteins are classified under distinct clades. When the proportion of sequence identity is smaller than 30%, and little is known about the specific relationship between the two measures of similarity, this difference is statistically less trustworthy. Thus, the homologous relationship between the two sequences cannot be confirmed [68]. Nevertheless, additional analysis such as structural comparison can be performed to verify their ancestral relationship.

Using MEME 5.1.1, a total of ten conserved motifs were identified in MaDefs (Figure 3). It can be observed that the orange box (**LLLFLLLI**) and turquoise box (**GLRRRCYCTKH**) motifs are present across all MaDefs. Further exploration through InterProScan discovered that the orange motifs are potentially associated with signal peptides, while the turquoise motifs correspond to the gamma-thionin family. This motif was revealed in host defence peptides from phylogenetically distant organisms, implying that it plays a long-standing and fundamental role in effector molecules mediating host-pathogen interactions [69]. This motif may comprise the whole peptide or a portion of the protein [47]. Sathoff et al. [17] demonstrated that peptides chemically synthesised with the defensins' gamma-core motif might imitate portions of the biological activities of the full-length defensin. Muñoz et al. [70] demonstrated that the synthetic peptides generated from the gamma-core motif of MtDef4 and MsDef1 (from *Medicago truncatula* and *Medicago sativa*) have unique antifungal activities that set them apart from the parental defensin. Apart from these motifs, the red box motif (**SDMGMTAVEARTCESASHKFKGPCVRDSNCANV CQTEGFH**), which is potentially associated with the Knot1 domain is present across all MaDefs except for Ma07_t03680.1. The "knottin" fold is a stable cysteine-rich scaffold in which one disulfide bridge crosses the macrocycle created by two other disulfide bridges and the connecting backbone segments. Plant lectins and antimicrobial peptides, plant proteinase and amylase inhibitors, plant gamma-thionins, and arthropod defensins are all examples of members of this group [23].

In addition, Ma02_t12840.1 and Ma08_t13660.1 share the most similar motif patterns since both share the highest percentage of identity and sequence similarity along with the recent common ancestor. Furthermore, Ma07_t03680.1 and Ma06_t21420.1 share the most distinct motif patterns since they both share the lowest percentage of identity and sequence identity.

Figure 3. Block diagrams representation of different conserved regions in MaDefs using MEME Suite 5.1.1. where different colours represent different motifs.

Gene Ontology of MaDefs

A total of four biological processes and two molecular functions of MaDefs were predicted using PANNZER2 (Table 4). However, Ma07_t03680.1 were excluded as the annotation came out as an uncharacterised protein. Based on Table 4, all MaDefs are involved in the defence response. In addition, Ma02_t12840.1, Ma08_t13660.1, Ma04_t36140.1 and Ma11_t12930.1 were characterised with the killing of cells of another organism as well as response to fungus. Defensins from *Nicotiana alata* (NaD1), *Pachyrrhizus erosus* (SPE10), *Petunia hybrida* (PhD1), *Pisum sativum* (Psd1), *Raphanus sativum* (Rs-AFP1) and *Saccharum officinarum* (Sd5) are among the peptides with antifungal action whose structures have been established [42] [53] [71] [72] [73] [74] [75]. Three approaches or models—the "Carpet model", the "Barrel-stave model", and the "Toroidal pore model"—are utilised to describe the mechanism of antimicrobial peptides. The "Carpet model" peptides are electrostatically bound to negatively charged phospholipid head groups at various places across the membrane's surface. As they accumulate on the membrane surface, tension forms between the two leaflets of the bilayer. When this tension rises above a certain concentration, the bilayer is disrupted

in a detergent-like way, which ultimately causes the membrane to disintegrate or rupture [76] [77]. As for the "Barrel-stave model", the hydrophobic portion of the peptide aligns with the hydrophobic lipid acyl chains of the core of the bilayer, while the hydrophilic portion forms the lining of the interior region of the pore; this topology can be compared to a barrel with helical peptides as staves. In the "Toroidal-pore model," as opposed to the "Barrel-stave" model, antimicrobial peptide helices insert themselves perpendicularly into the membrane to relieve the curvature strain caused by peptide binding, causing the monolayers to continuously bend to ensure the water core is lined by both the inserted peptides and the lipid head groups [78] [79]. In addition to these models, another defensin mechanism has been described. Defensin interacts with specific phospholipids and promotes aggregation rather than directly generating pores. This allows tiny chemicals, ions, and peptides to permeate cells and produce reactive oxygen species, which finally limit microbial development [80] [81] [82] [83]. Plant defensins, such as Rs-AFP2, Hs-AFP1 (AFP from *Heuchera sanguine*), and Dm-AMP1 (AMP from *Dahlia merckii*), also may bind to specific binding sites termed receptors of the microbial membranes, resulting in the ion leakage and inflow, outflow of the positive ions like Ca^{2+} and K^{+} [84] [85] [86] [87].

In comparison with other MaDefs, only Ma04_t36140.1 was uniquely characterised with cadmium ion homeostasis. For the molecular function prediction, only Ma08_t13660.1 and Ma04_t36140.1 were characterised by protein and cadmium ion binding. Plants undertake a number of strategies to minimise cadmium toxicity in response to cadmium accumulation stress, including vacuolar separation, cytoplasmic chelation, and cell-wall detoxification. Cell wall adsorption, which stops cadmium from entering the cell, cytoplasmic chelation mediated by defensin and metallothionein, vacuolar compartmentation, and cytosolic cadmium efflux to the apoplast, as well as activating the signalling pathway for reactive oxygen species to reduce oxidative stress, are all components of the detoxification mechanism for cadmium in plant cells [88].

Table 4. The predicted biological and molecular functions of MaDefs through gene ontology (GO) analysis performed using PANNZER2

GO accession	Ontology	Function/component	MaDefs
GO:0006952	Biological process	Defence response	Ma02_t12840.1 Ma08_t13660.1 Ma04_t36140.1 Ma11_t12930.1 Ma06_t21420.1
GO:0031640	Biological process	Killing of cells of another organism	Ma02_t12840.1 Ma08_t13660.1 Ma04_t36140.1 Ma11_t12930.1
GO:0031640	Biological process	Response to fungus	Ma02_t12840.1 Ma08_t13660.1 Ma04_t36140.1 Ma11_t12930.1
GO:0055073	Biological process	Cadmium ion homeostasis	Ma04_t36140.1
GO:0005515	Molecular function	Protein binding	Ma08_t13660.1
GO:0046870 GO:0005515	Molecular function	Cadmium ion binding Protein binding	Ma04_t36140.1

Rice defensin-like proteins, CAL1 and CAL2, have been shown to interact with cytoplasmic cadmium to form complexes and excrete them to the xylem sap via long-distance transport, thereby regulating cadmium accumulation in the shoot [89] [90]. Cadmium chelation was also elucidated by Luo et al. [91] through *Arabidopsis* defensin, AtPDF2.5, which is found in the cell wall of xylem vascular bundles, causes cytoplasmic cadmium regulation and excretes the AtPDF2.5-Cd complex to the apoplast. In *Arabidopsis*, cadmium tolerance and accumulation were reduced when AtPDF2.5 function was lost [92]. Plant defensin-like protein BnPDFL, which is only present in samples that have been exposed to cadmium, was discovered through xylem sap proteomic investigations and may assist rapeseed (*Brassica napus*) in becoming more tolerant to cadmium [93]. Since the mechanism of MaDefs' molecular functions is still unknown, further exploration needs to be conducted to elucidate wild banana defensins' mechanisms.

Analysis of cis-acting Regulatory Elements (CAREs) of *MaDef*s

The promoter regions of target genes consist of small regulatory motifs known as cis-acting regulatory elements (CAREs), which are typically non-coding regions of DNA. CAREs are crucial regulatory components that regulate the

transcription of genes under different stress reactions or during plant development by interacting with the transcription factors or other regulatory molecules [94]. Through the PlantCARE database, cis-acting regulatory elements (CAREs) of all six *MaDef*s were analysed. A total of 36 types of CAREs with 15 distinct functions were discovered across all of the six wild banana defensins promoter regions (Figure 4). Light-responsive elements are the most prominent CARE elements found in *MaDef*s with Box 4 motif (ATTATT) present in Ma07_t03680.1, Ma02_t12840.1, Ma04_t36140.1 and Ma06_t21420.1. Research made by Ahmed et al. (2021) [95] in the banana RNA interference (RNAi) pathway gene families, namely Dicer-like (DCL), Agronaute (AGO) and RNA-dependent RNA polymerase (RDR) also discovered the Box 4 motif along with other important light-responsive motifs that include ACE, CAG motif, chs CMA1a, chs CMA2a, Gap box, GATA motif, G-box, G box1, GT1 motif, MRE, Sp1, TCCC motif, and TCT motif, all of which have CAREs shared by RNAi genes in banana. Furthermore, hormonal cis-acting regulatory elements such as auxin, methyl jasmonate (MEJA), abscisic acid (ABA) gibberellin and salicylic acid (SA) were also identified, which suggest their significant biological roles in the plant growth and development. Only Ma04_t36140.1 was predicted to have defence and stress-related CAREs which is TC-rich repeats motif (GTTTTCTTAC). Ahmed et al. (2021) [95] verifies the existence of TC-rich repeats motifs in bananas, making it a potential site for manipulation for the development of tolerant banana against diseases.

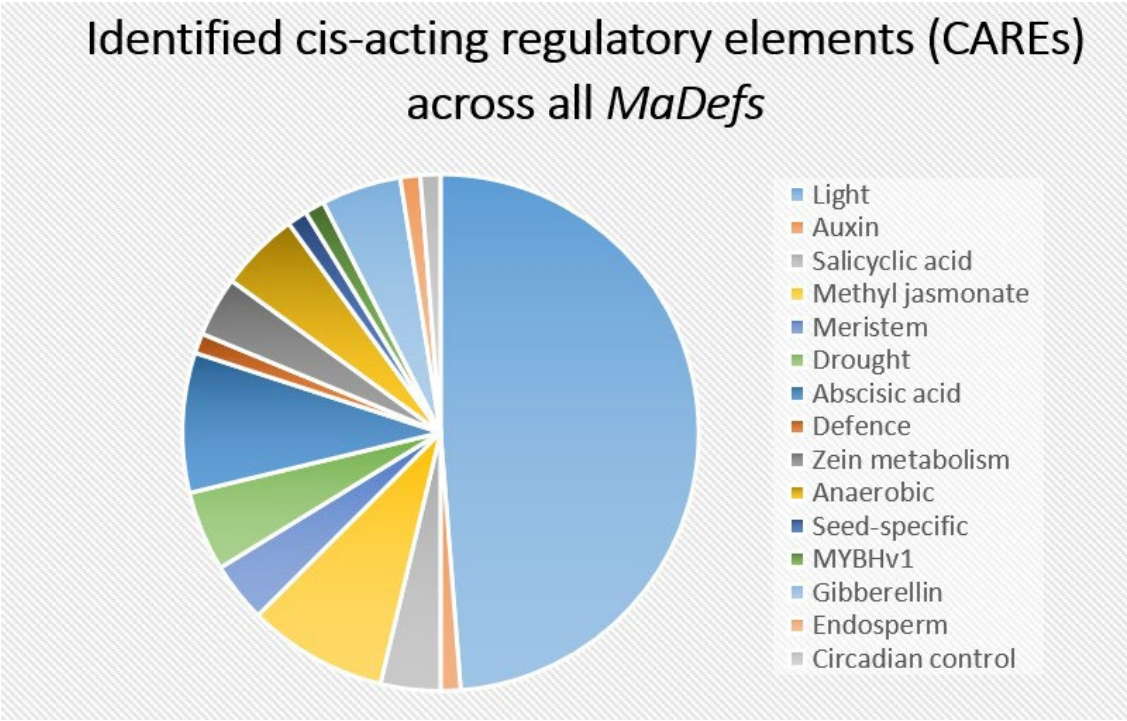

Figure 4. A pie chart of CAREs function distribution in all six wild banana defensins (*MaDef*s) analysed using PlantCARE database

CONCLUSION

In conclusion, a total of 6 defensin copies (MaDefs) categorised under the Knottin_1 clan (CL0054) from *Musa acuminata* DH Pahang (wild banana) were identified. Domain search and gene ontology (GO) analysis revealed that all MaDefs except for Ma07_t03680.1 are associated with gamma-domains (PF00304) and potentially involved in the defence response, respectively. Of these, Ma04_t36140.1 stands out as a potential defence-related defensin as the analysis on promoter regions through PlantCARE shows that Ma04_t36140.1 is associated with defence and stress responsiveness (TC rich repeats). Further analysis such as differential expression profile against phytopathogen and antimicrobial assays must be conducted to prove this prediction. Moving forward, defensins with anti-microbial potential can be integrated into plant genomes for improved tolerance against diseases. Overall, the *in silico* analysis of defensin genes sheds light on their potential activities and provides foundational knowledge for future functional research, all of which can aid in enhancing banana plants' response to a wide range of biological stresses.

ACKNOWLEDGEMENTS

The authors thank Ms. Afiqah Insyirah bt Lutfi (Plant Biotechnology Research Laboratory, Universiti Putra Malaysia) for her assistance in the sequence retrieval and software tutorials.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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