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FUNCTIONAL PREDICTION OF PATHOGENESIS-RELATED 10 IN *Musa acuminata* DH PAHANG (MaPR-10) FOR TARGETED BANANA ADAPTATION AGAINST STRESSES

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Abstract

Pathogenesis-related 10 (PR-10) is a defense-related protein that provides adaptive responses to various biotic and abiotic stimuli. It was characterized by diverse roles due to its multigene property. Hence, the specific biological roles of PR-10 are still inconclusive. To date, there is no reported *in-silico* characterization of PR-10 in wild bananas yet. Thus, this study predicts the valuable *Musa acuminata* DH Pahang (wild banana) MaPR-10 copies which might be useful for the targeted functions. A total of ten putative MaPR-10 members were discovered which can be clustered into three major subgroups (intracellular PR-10 (IPR), S-norcoclaurine synthase (NCS) and major latex protein (MLP) through phylogenetic analysis. MEME suite analysis displayed distinguished motif arrangement among all three subgroups. Gene ontology (GO) revealed that all MaPR-10s exhibit similar functions such as plant defence, phosphoprotein phosphatase activity and activation of ABA signaling pathway except for Ma09_p15840.1 (an IPR) and Ma04_p33910.1 (NCS) which displayed distinct roles both in biological and molecular functional prediction. Furthermore, analysis of the promoter regions using PLACE presented a diverse regulation of *MaPR-10*s upon various biotic and abiotic stimuli. Altogether, this study contributes to a better perspective of MaPR-10s features and their functional predictions, which will be essential for future crop improvements against biotic and abiotic stresses, particularly in bananas.

INTRODUCTION

The threatening environmental factors including biotic and abiotic stimuli make plants strategize in perceiving the stresses and survival. The strategies include the generation of the functional traits to improve the plants' fitness [1] and the induction of several protective molecules which act as a part of the plant's defence mechanisms [2]. Pathogenesis-related (PR) protein is one of the defence proteins that is commonly induced upon both abiotic and biotic stresses. PR proteins consist of 17 groups (PR-1 until PR-17) and are widely recognized as the innate immune system in plants [3]. Each PR groups exhibit different enzymatic and antimicrobial activities upon induction [2].

Despite being initially discovered in pea [4] and parsley [5], pathogenesis-related 10 (PR-10) did not attract much attention until the birch allergen (*Betula verrucosa*) was cloned and showed high sequence similarity with the protein [6]. There was also a report of structural similarity between birch allergen and START domain that led to a classification

of both domains. Until then, the proteins were categorized to adopt similar helix-grip folds and have the same ligand-binding features under one big family [7]. Though it was an imperfect classification, the relationship between birch allergen and the START domain unfolded and recently led to a better grouping of domains. Under Bet V 1 clan (CL209) in Pfam database, PR-10 belongs to Bet v 1 family (PF00407) which is a type of major pollen allergen group.

PR-10 is a multigene family protein. They possess diverse roles and are responsive to plant stresses such as by possessing antibacterial [8], antiviral [9] and antifungal [10] activity. These proteins are also known as ribonuclease-like proteins due to their ability to degrade RNA [11,9,12] by recruiting helper proteins to bind and target RNA specifically in both pathogen and host [13]. However, there is no specific function that can be assigned to this group yet. Since PR-10 has various copy numbers in plants, it is difficult to determine their functions individually. Therefore, it would be a great benefit to elucidate the features and functions of individual PR-10 copies first through *in silico* analysis and provide a ground basis for further functional studies especially in major crops such as bananas.

Comprehensive knowledge of the functions of banana proteins is crucial to enhancing the adaptability of banana plants to various stresses. Hence, this research is conducted to identify all the putative PR-10 members in *Musa acuminata* DH Pahang (wild banana) as well as their predicted functions through *in silico* studies such as gene ontology and cis-acting regulatory elements (CAREs) analysis. The knowledge obtained from this study serves as an important foundation for future functional analysis to tackle various biotic and abiotic problems associated with bananas.

MATERIALS AND METHODS

Identification of Putative Pathogenesis-related 10 (*PR-10*) Gene Family in Wild Banana

Putative PR-10 proteins in *Musa acuminata* DH Pahang (wild banana) were first retrieved based on the known PR-10 domain, Bet V 1 using Pfam database under Bet_v_1 family (PF00407) in Bet_V_1 clan (CL0209). All retrieved protein sequences were then used as query sequences in the blastp program against the *Musa acuminata* DH Pahang genome database in Banana Genome Hub (BGH) (https://banana-genome-hub.southgreen.fr/) to verify the identity of all PR-10 family members. The sequences obtained from BGH (designated as MaPR-10) were then screened for Bet V 1 domain in the Interpro server (https://www.ebi.ac.uk/interpro/) as the domain is associated with PR-10. All putative MaPR-10 sequences were then compiled and subjected to subsequent *in silico* analysis.

Multiple Sequence Alignments and Phylogenetic Analysis of MaPR-10s

All putative MaPR-10 protein sequences were aligned using the Muscle algorithm in MEGA-X. A neighbor-joining tree with 1000 replication bootstraps was generated using MEGA-X. Jones, Taylor and Thornton (JTT) model and partial deletion gaps were applied during the run. PR-10 sequences from *Oryza sativa*, *Hordeurm vulgare*, *Lilium longiflorum*, *Zea mays*, *Elaeis guineensis*, *Gossypium hirsutum*, *Arabidopsis thaliana*, *Coptis japonica* and *Thalictrum flavum* were also included to represent different PR-10 clades while cytokinin-specific binding protein (CSBP) from *Vigna radiata* serves as the outgroup.

Analysis of Physicochemical Properties of MaPR-10 Protein Sequences

All putative MaPR-10 sequences were analyzed for their predicted molecular weight and theoretical isoelectric point (pI) using ExPASy (https://web.expasy.org/compute_pi/). The presence of signal peptide was predicted using SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/index.php).

The percentage of positive residues, negative residues, polar and hydrophobic residues, aliphatic index, and value of grand average of hydropathicity (GRAVY), were estimated using ExPASy-ProtParam tool (https://web.expasy.org/protparam/) to characterize MaPR-10 physicochemical characteristics.

Analysis of MaPR-10 Protein Sequence Features

The percentage of protein sequence similarity was determined using Sequence Identity and Similarity, SIAS (http://imed.med.ucm.es/Tools/sias.html) and the ungapped motifs in MaPR-10 were analyzed 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 10 sequences.

Analysis of Gene Ontology (GO)

The predicted biological function and cellular components of MaPR-10s were analyzed using PANNZER2 (http://ekhidna2.biocenter.helsinki.fi/sanspanz/).

Retrieval of Promoter Region Sequence and Cis-acting Regulatory Elements (CARE) Analysis

The promoter regions of *MaPR-10* genes (approximately 1.5 kbp upstream to the translation start site) were retrieved from BGH. The CARE elements in the promoter region of *MaPR-10*s were analyzed using PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace).

RESULTS AND DISCUSSION

Identification of *MaPR-10* Gene Family in *Musa acuminata* DH Pahang (Wild Banana)

Direct retrieval using Bet_v_1 family (PF00407) in Bet_V_1 clan (CL0209) from Pfam database yielded eight putative MaPR-10 members, namely M0SCU1, M0U0W1, M0SSJ6, M0SDG0, M0UCN1, M0U1E0, M0SCU0, and M0SCU2. The protein sequences from these accession numbers were used to do blastp search against the *Musa acuminata* DH Pahang database in BGH and also yielded eight MaPR-10 candidates. Further Bet V 1 domain screening in Interpro discovered two additional Ma-PR10 members. Taken together, a total of 10 putative *MaPR-10* members were identified in *M. acuminata* DH Pahang (wild banana) and their information such as annotated names, chromosome number, strand, CDS and protein sequence length were summarized in Table 1.

Since *PR-10* is a multigene family, the multiple copies of *MaPR-10* found in wild banana are in accordance with other studies. For instance, a total of 21 *PR-10* members have been reported in *Fragaria ananassa* [14], 17 in *Vitis vinifera* [15] and nine in *Lilium regale* Wilson [16]. PR-10s can be expressed either constitutively or by the biotic or abiotic induction. Since plants have multiple *PR-10* copies, each of them may specifically be expressed in different stress infection phases and organs [14].

Table 1. Description and characteristic of MaPR-10 sequences retrieved from Banana Genome Hub (BGH)

Num	Accession Numbers	Annotated Names	Chromosome Num	CDS Length (bp)	Protein Length (aa)
1	Ma08_p34150.1	Pathogenesis-related protein 1-like	8	483	160
2	Ma01_p19550.1	Pathogenesis-related protein 1-like	1	483	160
3	Ma08_p34160.1	Phytohormone-binding protein-like	8	564	187
4	Ma03_p08150.1	Pathogenesis-related protein 1-like	3	483	160
5	Ma03_p08160.1	Pathogenesis-related protein 1-like	3	483	160
6	Ma09_p15840.1	Pathogenesis-related protein 1-like	9	483	160
7	Ma03_p08140.1	Pathogenesis-related protein 1-like	3	483	160
8	Ma03_p10130.1	MLP-like protein 423	3	453	150
9	Ma04_p33910.1	S-norcoclaurine synthase-like	4	621	206
10	Ma09_p17720.1	MLP-like protein 423	9	426	141

Multiple Sequence Alignment Analysis

When plants are under pathogen attack, they will utilize a sequence-specific RNA binding protein to directly regulate the innate immunity of plants [17]. Glycine-rich (GXGGXGXXK) and KAXEXYL motifs are among the signature features of PR-10s (Figure 1) which are believed to act as the binding site for RNA although the specificity of the binding target is still unknown [2]. Based on multiple sequence alignment analysis, MaPR-10s have three different patterns of glycine-rich motifs which are GXGGXG (Ma04_p33910.1 and Ma03_p08160.1), GXGXXG (Ma03_p08150.1, Ma03_p08140.1, Ma09_p15840.1, Ma01_p19550.1, Ma08_p34150.1, and Ma08_p34160.1) and GXGXXXG (Ma03_p10130.1 and Ma09_p17720.1). These various and less conserved patterns are often correlated with the low activity of RNA degradation compared to the glycine-rich motif that has RNase activity, GXGGXGGG [18].

Glycine-rich motif is also often referred to as P-loop, a phosphate-binding loop that is commonly found in the nucleotide-binding protein of mostly adenine and guanine [19]. However, in PR-10, the so-called P-loop is conformationally different from the phosphate-binding P-loop [20]. But in some PR-10s, the ribonuclease activity of that P-loop was also predicted to be associated with the presence of binding sites for adenine and guanine [21]. In SPE-16, the P-loop motif of PR-10 was proven to be involved in the ribonuclease activity as the deletion of certain regions proved to lower the activity of RNase [22].

The position of conserved amino acids, E96, E148 and Y150 (as positioned in Bet v 1) were also found in four of the intracellular (IPR) MaPR-10s. The regions were located at the position of E97, E149 and Y151 of Ma03_p08160.1, Ma03_p08150.1, Ma03_p08140.1 and Ma09_p15840.1 (Figure 1) suggesting potential ribonuclease activity [21].

In *M. acuminata* DH Pahang, seven out of ten MaPR-10s have the KAXEXYL motif (Figure 1). The motif is only

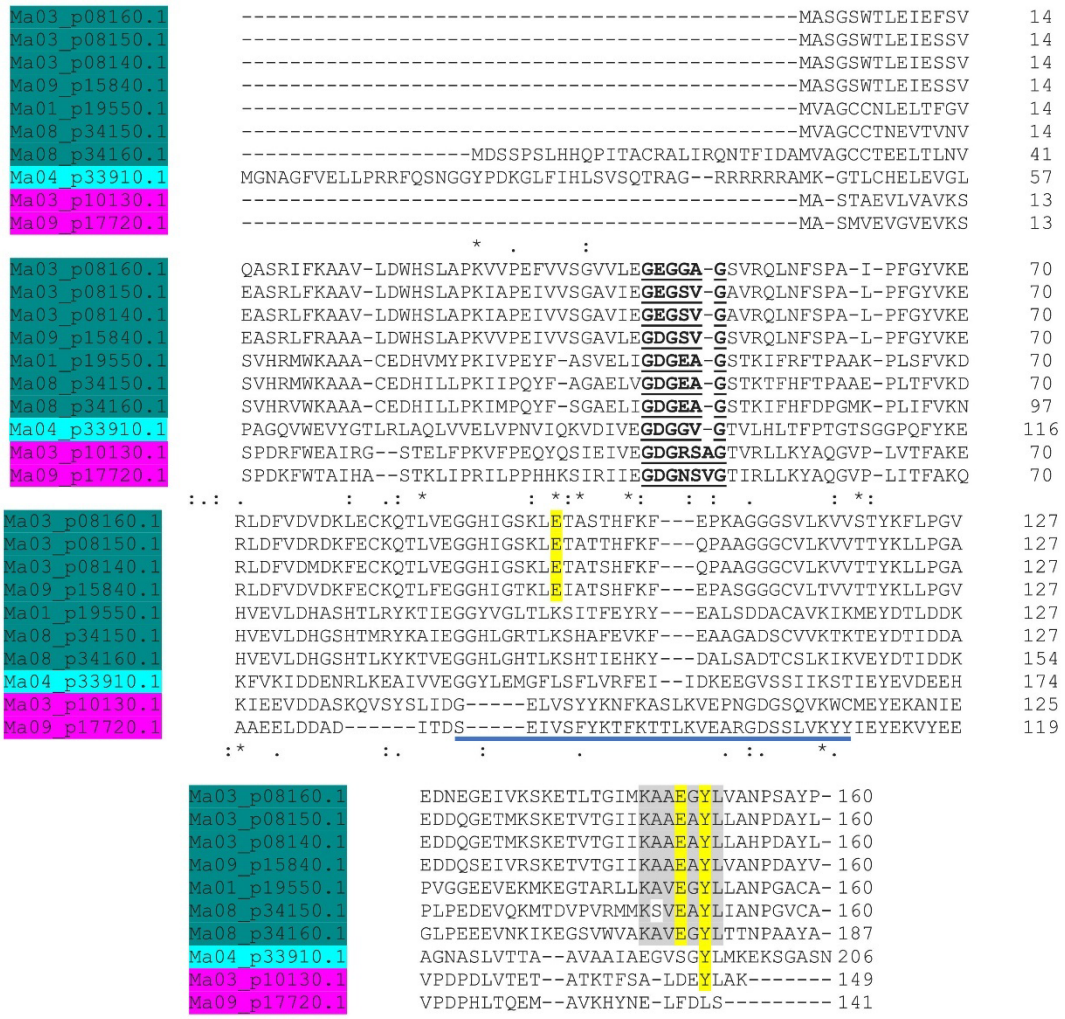

Figure 1. Multiple sequence alignments of all PR-10s in *Musa acuminata* DH Pahang (wild banana) (MaPR10s). The glycine rich (P-loop) motif (bolded and underlined), KAXEXEYL motifs (gray highlight) and Bet V 1 domain (blue line) are the signature of PR-10 proteins. E97, E149, and Y151 (yellow highlight) are the predicted ribonuclease catalytic site. The IPR, NCS, and MLP subgroup's accessions are highlighted in teal, turquoise and pink respectively.

found in the IPR subgroup of wild banana MaPR-10. Interestingly, despite having the KAXEXYL motif only, CsPR10 identified in *Crocus sativus* still displayed the RNase function [23] which suggests that the absence of glycine-rich motif does not affect RNase function. The role of PR-10 in RNase activity upon biotic and abiotic stress is described in detail by Sinha, Verma and Rastogi [24].

Phylogenetic Analysis

In wild bananas, MaPR-10s were divided into three major clades (Figure 2). The majority of the proteins belong to clade I. Clade I is an intracellular/classic PR10 (IPR) cluster that consists of two different subgroups which can be classified as monocot type 1 and monocot type 2 [6]. Ma04_p33910.1 is the only MaPR-10 member that is categorized under Clade II and belongs to the (S)-norcoclaurine synthase (NCS) family. According to [25], the NCS family is one of the functional PR-10 members which possesses enzymatic activity other than ribonuclease, phenolic oxidative coupling protein (Hyp-1), papain inhibitory activity as well as hydroxy nitrilelyase [26]. NCS is needed in catalyzing the first step of benzylisoquinoline alkaloids biosynthesis [27].

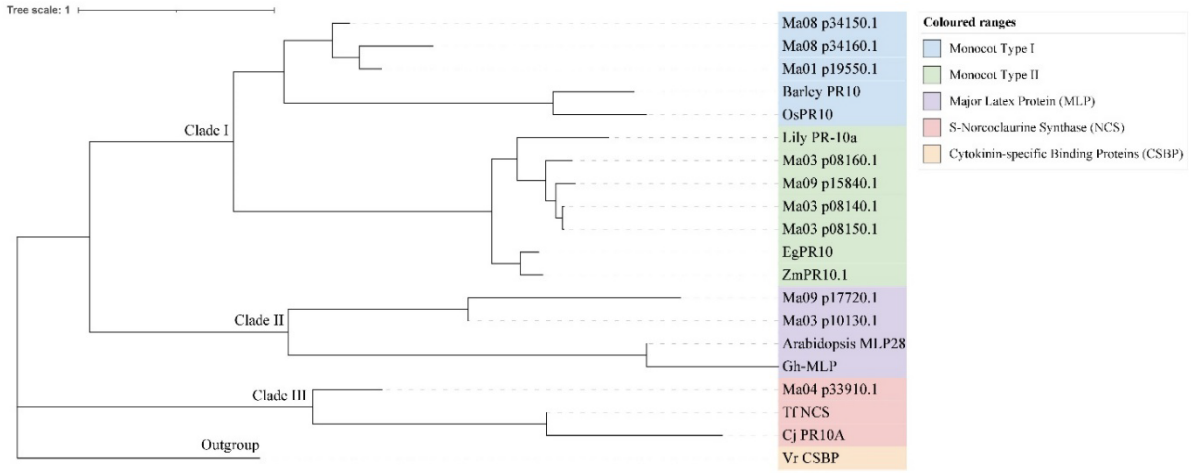

Figure 2. Three clades of MaPR-10s observed in phylogenetic tree analysis according to Radauer et al. (2008). The tree was generated using neighbor-joining tree with bootstrap 1000 replicates and Jones, Taylor and Thornton (JTT) model with Gamma (G) distribution were applied during the run using MEGA-X. Vr CSBP served as an outgroup in the tree. The tree was visualized and edited in Interactive Tree of Life (iTOL). Accession number: OsPR10: D38170 (*Oryza sativa*), Barley PR10: Q84QC7 (*Hordeurm vulgare*), Lily PR-10a: AAD17335.1 (*Lilium longiflorum*), ZmPR10.1: ADA68331.1 (*Zea mays*), EgPR10: AEB96227.1 (*Elaeis guineensis*), Gh-MLP: ABA01325.1 (*Gossypium hirsutum*), Arabidopsis MLP28:AT1G70830.1 (*Arabidopsis thaliana*), Cj PR10A: BAF45338.2 (*Coptis japonica*), Tf NCS: BAF45338.2 (*Thalictrum flavum*), Vr CSBP: BAA74451.1 (*Vigna radiata*).

Two MaPR-10 members (Ma03_p10130.1 and Ma09_p17720.1) are classified under Clade III which are the major latex protein-like (MLP-like). MLP-like is the second largest subgroup in plant PR-10s and may possess roles in responses towards stresses as well as defence [6]. In apple (*Malus domestica* Borkh.), MLP-like members showed responses towards biotic stress [28]. However, a further study proved that one of the MLP-like members in the 'Golden Delicious' apple variety, *MdMLP423* negatively regulates the defence response upon biotic stress by inhibiting the expression of transcriptional factors and genes of several pathogenesis-related members [29]. While in cotton, *Gh-MLP* more possibly responds to salt stress rather than the fungal infection [30]. The possibility of *Gh-MLP* being involved in biotic stress was not entirely rejected as it could play a minor role in the resistance or infection with other types of pathogens.

Additionally, in field pumpkin, MLP-like proteins were mainly expressed in roots and critical in translocating hydrophobic pollutants to the aerial parts of plants [31]. The translocation process serves as one of the steps involved in the bioaccumulation uptake in crops. On the other hand, MLP-like gene in *Arabidopsis thalian*a, *MLP43,* is responsive towards drought stress and the mediation of the abscisic acid (ABA) signaling pathway by acting as a positive regulator [32]. Yet to date, there is no reported function of MLP-like proteins in bananas. In this study, both MLP-like proteins observed in the *MaPR-10* gene family possess Bet v 1 domain which could possibly be linked to the pathogenic resistance response and also might involve stimuli responses. Further, functional studies are required to prove this functional speculation.

All MaPR-10 members that were grouped into different clusters have their own roles in plants. Although some of their roles and mechanisms are still unclear, evidence proved their responses to various stimuli when plants are under stress. It can be biotically or abiotically induced as plants activate their defence mechanism to continue surviving. They are also induced and differentially expressed depending on the warranted situations. Other than that, MaPR-10s are also involved in other biological functions in maintaining plant health and well-being. This suggests that PR-10's function is not only limited to defence, but they also play important roles in other biological mechanisms.

Physicochemical Properties of PR-10 Protein Sequences in Wild Banana

Overall, MaPR-10s encoded 141 – 206 amino acid (aa) residues and have predicted molecular weight ranging from 15.9 – 22.4 kilo Dalton (kDa) (Supplementary file 1). IPR subgroup encodes 151-162 amino acids (aa) with a predicted molecular weight of 15 – 18 kDa. (S)-norcoclaurine synthase (NCS) subgroup generally has a longer open reading frame (633 – 696 bp) and a higher molecular weight of 26 kDa [33]. Six out of seven IPR MaPR-10 share the same length of amino acids which are 160, except for Ma08_p34160.1 which has 187 aa. In wild banana, MLP-described proteins, Ma03_p10130.1 and Ma09_p17720.1 have the shortest amino acid sequence among all MaPR10s with a predicted

molecular weight of 15.9 and 16.7 kDa, respectively. Ma04_p33910.1 which encoded a NCS-described protein showed the longest residues (Table 1) and the highest molecular weight (Supplementary file 1) compared to other MaPR-10 subgroups which is in accordance with Agarwal and Agarwal [33]. Analysis using SignalP-5.0 predicted the absence of signal peptides in MaPR10s, suggesting their intracellular localization (Supplementary file 2).

The estimated values for the Grand Average of Hydropathicity (GRAVY) of MaPR10s range from -0.338 to 0.111 (Supplementary file 3). All GRAVY values showed negative values except for Ma03_p08160.1, Ma09_p15840.1, and Ma03_p08140.1. Negative values imply the hydrophilic nature of the proteins which predicts a better interaction with water.

Analysis of PR-10 Protein Sequence and Motifs

Ma03_p08140.1 and Ma03_p08150.1 share the highest protein sequence identity and similarity (Supplementary file 4) which are 98.12 % and 98.75 %, respectively. The lowest percentage identity belongs to Ma04_p33910.1 and Ma08_p34160.1 which is 17.64 %. According to the phylogenetic analysis, Ma04_p33910.1 and Ma08_p34160.1 belong to different subgroups thus possibly explaining the low percentage of identity they shared with each other.

Using MEME 5.1.1, a total of ten conserved motifs were identified in MaPR-10s (Figure 3). Remarkably, the motifs organization demonstrated inter- and intra-subgroups variation. The least-containing motif was from other than the

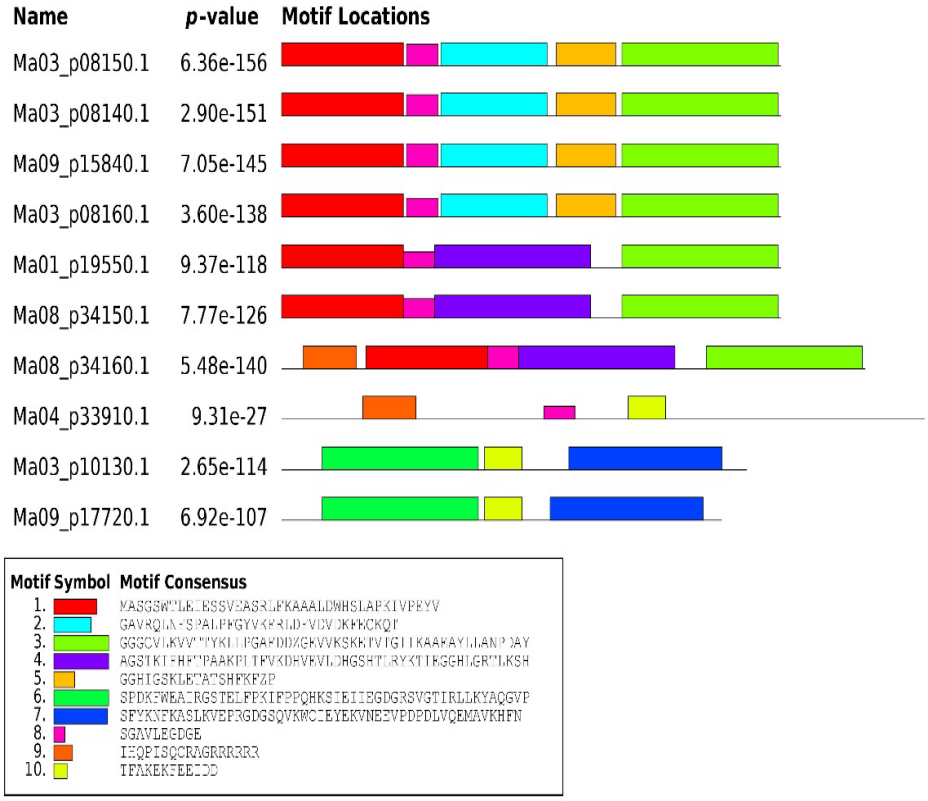

Figure 3. Block diagrams representation of different conserved region in MaPR-10s using MEME Suite 5.1.1. Different colours represent different motifs.

IPR subgroup which harbored three different motifs. The low number of motifs might indicate that the protein sequences will become less conserved during the evolution [34].

Gene ontology (GO)

A total of nine biological processes and six molecular functions of MaPR-10 members were predicted using PANNZER2 (Table 2). However, two MaPR-10s from MLP subgroups were excluded as the annotation came out as uncharacterized proteins. Based on Table 2, all MaPR-10s are involved in the ABA signaling pathway, phosphatase inhibitor binding and also in defence response whereas most of the IPR PR-10s respond to the biotic stimulus. Ma09_p15840.1 was uniquely characterized compared to other MaPR-10s. For the molecular function prediction, Ma09_p15840.1 was characterized for both RNA binding and ribonuclease activity. Even though the specific binding affinity of these sites remains unknown, this is consistent

with the PR-10's general function as a ribonuclease with the presence of highly conserved regions which are KAXEXYL domain and P-loop motif (GxG) [17]. Therefore, it can be suggested that Ma09_p15840.1 does possibly possess ribonuclease activity which can be further proven using functional analysis such as RNA degradation assay.

Table 2. The predicted biological and molecular functions through gene ontology (GO) analysis performed using PANNZER2

GO accession	Ontology	Function/Component	MaPR-10s
GO:0009738	Biological process	Activation of ABA signaling pathway	All MaPR-10s*
GO:0032515	Biological process	Phosphoprotein phosphatase negative regulation activity	
GO:0006952	Biological process	Defence response	
GO:0009607	Biological process	Biotic stimulus response	Ma08_p34150.1 Ma03_p08150.1 Ma09_p15840.1 Ma03_p08140.1
GO:0009646	Biological process	Absence of light response	Ma09_p15840.1
GO:0009751	Biological process	Salicylic acid response	
GO:0009735	Biological process	Cytokinin response	
GO:0009739	Biological process	Gibberellin response	
GO:0080163	Biological process	Phosphatase activity for serine/threonine	Ma04_p33910.1
GO:0010427	Molecular function	ABA binding protein	All MaPR-10s*
GO:0004864	Molecular function	Phosphatase inhibitor binding	All MaPR-10s*
GO:0038023	Molecular function	Signaling receptor activity	All MaPR-10s*
GO:0004540	Molecular function	Ribonuclease activity	Ma09_p15840.1
GO:0003723	Molecular function	RNA binding	Ma09_p15840.1
GO:0050474	Molecular function	(S)- norcoclaurine synthase activity	Ma04_p33910.1

*Except 2 MLPs

Cis-acting Regulatory Elements (CAREs)

A total of 46 different function types of cis-acting regulatory elements (CAREs) were identified in *MaPR-10*s promoter regions through PLACE (Figure 4). These CAREs might explain the interaction between *MaPR-10*s with the transcription factors and elucidate its regulation model upon induction by various situations [33]. The highest four *MaPR-10*s expression regulators identified are associated with light (35 elements), followed by seed-related (27 elements), ABA-related (16 elements) and water-related (16 elements) (Figure 5).

In *MaPR-10* promoters, there are several light-responsive elements (LREs) that singly belong to a certain gene. In Arabidopsis, a single LRE indicated that the gene might respond to a specific light wavelength compared to the combined LREs which respond towards a broader light wavelength [35].

The second highest CAREs in *MaPR-10*s, which is seed-related were mostly associated with seed storage protein (Supplementary file 5). Those seed storage elements are also known to be abundant in other plant species [36] and are favorable for transgene expression due to their stability which yields a successful recombinant protein [37].

The third-highest number of *MaPR-10*s regulatory elements are involved in the hormonal function which is an ABA-related function. ABA-related CAREs was found abundantly compared to other phytohormone elements such as salicylic acid, auxin and ethylene. Banana which is categorized as climacteric fruit, can ripe after the harvesting process with the help of ethylene [38]. However, the ethylene regulatory elements in bananas were found to be limited compared to other certain regulatory phytohormones. On the contrary, the abscisic acid regulatory elements were found abundantly in *MaPR-10*s. Despite being ethylene-dependent, it showed that ABA also helped in the ripening of bananas by increasing the ethylene sensitivity during the progress of the ripening process [39].

Besides, the activity of the *Bet v 1a* promoter was also positively regulated by abscisic acid in transgenic tobacco [40]. Abscisic acid regulatory elements are important hormonal regulatory elements probably in *Ma-PR10*s as well as they are involved in various signaling pathways and functions in plants [41].

Lastly, water-related CAREs such as dehydration responsive elements (DREs) were also prevalent in the promoter regions of *MaPR-10*s. Notably, drought is one of the threats that could heighten the food security concern. In the tropic region

with high temperatures, the combination of drought and heat stresses can worsen the banana condition as the water content will be reduced greatly from the leaves [42]. As a defence mechanism, plants will close their stomata to prevent water loss. However, closing the stomata will lead to reduced crop yield as carbon assimilation will also be reduced [43].

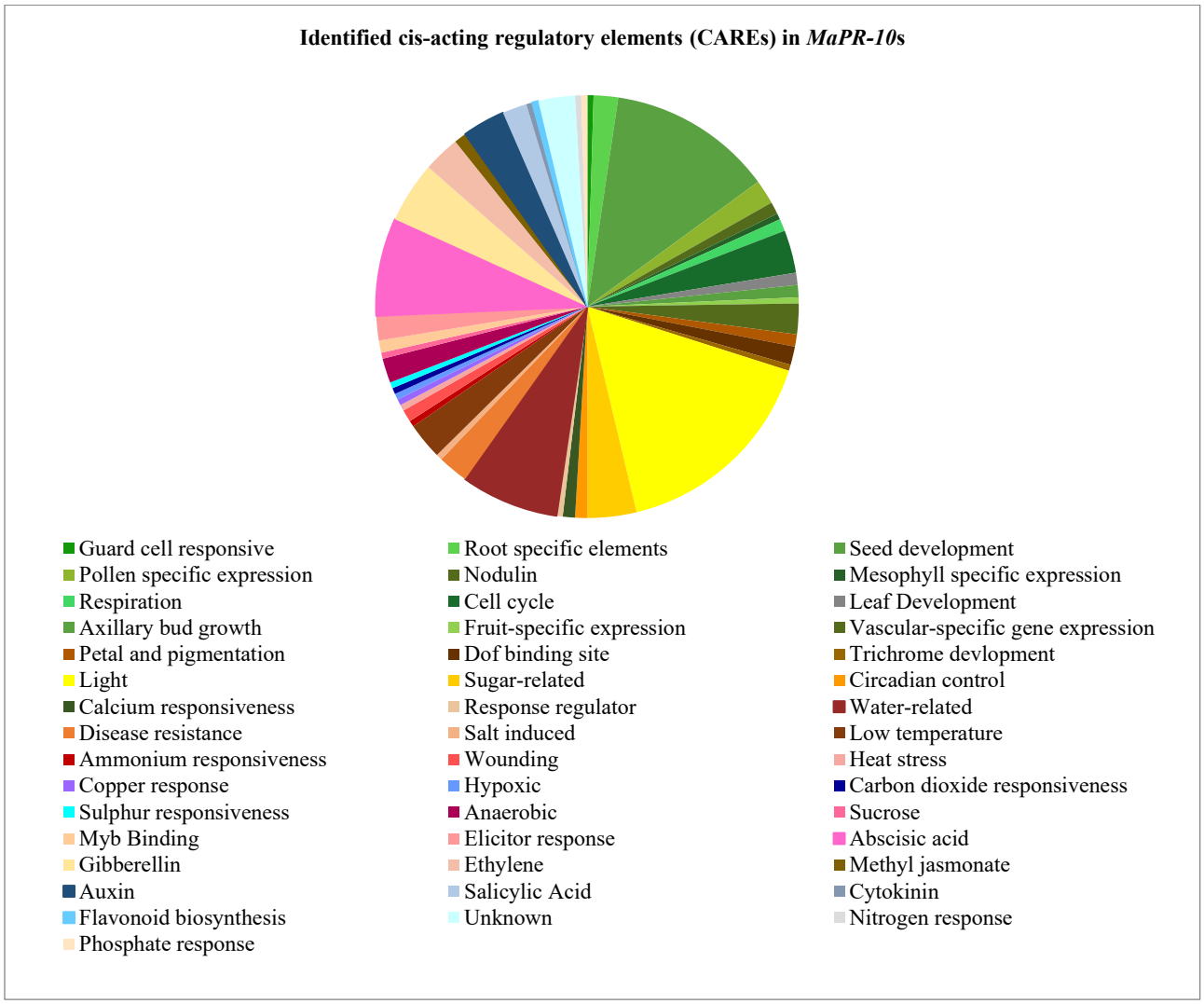

Figure 4. A pie chart of CAREs function distribution in all *MaPR-10*s analyzed using PLACE.

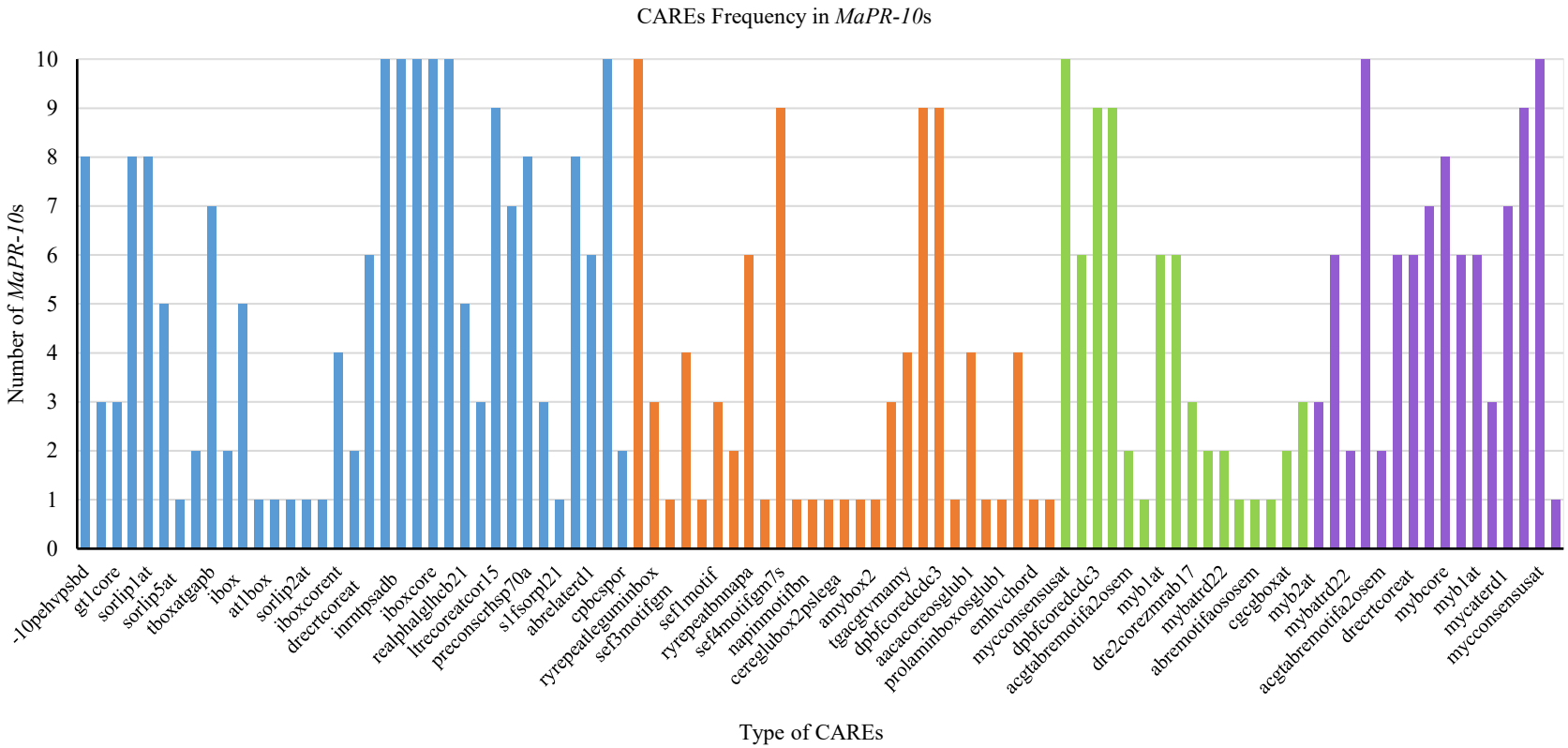

Figure 5. The frequency of the mostly identified CAREs in 1.5 kbp upstream to the translation start sites of *MaPR-10*s which comprise from light-, seed-, ABA- and water-related processes. The CAREs were identified through PLACE database and plotted in the number of *MaPR-10*s (y-axis) against type of CAREs (x-axis).

CONCLUSION

In conclusion, a total of ten MaPR-10s were identified in *M. acuminata* DH Pahang (wild banana) with small protein sizes and no signal peptide present. Most of these MaPR-10s are hydrophilic in nature, and several have the potential for antimicrobial activity. One of MaPR-10 family members, Ma09_p15840.1, was expected to function in the plant defence response by degrading the RNA pathogen as its mode of action. On the other hand, several MaPR-10s also were predicted to respond to biotic stimuli based on the reported features such as the presence of three signature amino acids and glycine rich motif (P-loop).

Overall, the *in silico* analysis of MaPR-10s provides insight regarding their putative functions and serves as fundamental knowledge for future functional studies which can help to improve the adaptation of banana plants against various biological and non-biological factors.

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CONFLICT OF INTEREST

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

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