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MaPep1 and MbPep1, as plant elicitor peptides in bananas, confer resistance to banana blood disease caused by *Ralstonia syzygii* subsp. *celebesensis*

Praphat Kawicha[1#], *Ladawan Rattanapolsan*[2#], *Ratri Boonruangrod*[3], *Yube Yamaguchi*[4], *Kusavadee Sangdee*[5], *Aphidech Sangdee*[2,6*], *Thanwanit Thanyasiriwat*[1*]

[1]*Plant Genome and Disease Research Unit, Department of Agriculture and Resources, Faculty of Natural Resources and Agro-Industry, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, Sakon Nakhon, Thailand*

[2]*Department of Biology, Faculty of Science, Mahasarakham University, Maha Sarakham, Thailand*

[3]*Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom, Thailand*

[4]*Graduate School of Agriculture, Department of Agricultural Biology, School of Agriculture, Osaka Metropolitan University, Sakai, Japan*

[5]*Preclinical Group, Faculty of Medicine, Mahasarakham University, Maha Sarakham, Thailand*

[6]*Microbiology and Applied Microbiology Research Unit, Faculty of Science, Mahasarakham University, Maha Sarakham, Thailand*

[#]*Authors contributed equally*

[*]*Corresponding authors: aphidech.s@msu.ac.th, thanwanit.t@ku.th*

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Abstract: Peptides play regulatory roles in various plant development and defence processes. They function as molecular messengers that detect threats and trigger defence responses. This study aimed to identify the genes encoding endogenous plant elicitor peptide precursors (*PROPEPs*) in bananas and their role in inducing resistance to *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*). Two precursor genes, *MaPROPEP1* and *MbPROPEP1*, were discovered and predicted to encode the precursor proteins of elicitor peptides, namely, MaPep1 and MbPep1. Both elicitor peptides contained 23 amino acids of the active elicitor peptide, which activated innate immune responses in banana resistance to *Rsc*. The disease assessment was conducted by inoculating banana plants with *Rsc* isolate MY4101 using the root-stabbing method. The results demonstrated that MaPep1 and MbPep1 pretreatment enhanced resistance to banana blood disease, as indicated by reduced disease severity and the absence of wilting for 7 days after infection. The expression of the *MaPROPEP1*, *MbPROPEP1*, *MaLOX7*, and *Pr-10* genes was evaluated using qPCR and found to be upregulated by MaPep1 and MbPep1 injection followed by *Rsc* infection in aboveground banana tissues within 7 days. These findings prove that MaPep1 and MbPep1 are members of the Pep family and exhibit conserved functions across various plant species. This approach may be used to develop strategies for enhancing disease resistance in banana cultivation.

Keywords: immunity; PROPEP genes; pathogenesis-related genes; plant immunity

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Peptides play crucial roles in regulating a wide range of processes related to both plant development and defence (Matsubayashi & Sakagami 2006). In defence mechanisms, peptides function as molecular messengers during plant interactions with other organisms, signalling plants about potential threats and triggering defence responses (Huffaker et al. 2011a). Defence responses are triggered by molecules from invading organisms and endogenous host-derived elicitor molecules released upon injury or infection, which are perceived as danger or alarm signals (Yamaguchi & Huffaker 2011). The defence mechanism occurs when plant cells recognise infectious microbe-associated molecular pattern (MAMP) molecules through pattern recognition receptors (PRRs). This recognition triggers a signalling cascade that produces molecules such as Ca^{2+}, reactive oxygen species (ROS), and mitogen-activated protein kinases (MAPKs). These molecules continue to induce a response against the pathogen, resulting in the production of proteins and secondary metabolites related to disease resistance, such as PROPEPs, jasmonic acid, salicylic acid, and ethylene (Yamaguchi & Huffaker 2011; Klauser 2014; Gowthami 2018).

Endogenous plant elicitor peptides (Peps) are short signalling peptides, 23–36 amino acids in length, contributing to broad-spectrum defences against biotic and abiotic stresses. They are post-translationally cleaved from precursor proteins called PROPEPs (Lee et al. 2018; Zelman & Berkowitz 2023) and often contain a glycine-enriched motif: (S/G)(S)Gxx(G/P)xx(N) (Tavormina et al. 2015). Originally identified in *Arabidopsis thaliana*, Peps share sequence similarity across plant species and activate defence responses by binding to Leu-rich repeat receptors (PEPRs), leading to the induction of defence genes and signalling molecules such as jasmonate and hydrogen peroxide (Huffaker et al. 2006; Yamaguchi et al. 2006; Huffaker & Ryan 2007). However, the role of plant elicitor molecules in banana immunity remains largely unexplored. These molecules have been shown to enhance plant defence against various pathogens in other plant species (Eder & Cosio 1994; Hahn 1996; Nürnberger 1999; Thakur & Sohal 2013; Badosa et al. 2022), but their potential for improving disease resistance in bananas requires further investigation.

Recently, researchers have been looking for ways to manage plant diseases that are safe for the environment and human health (Zelman & Berkowitz 2023). One promising approach is to study elicitor molecules. Previous reports have shown that bananas and plantains accounted for 155 mil. t of fruit production globally in 2018, are among the three most valuable fruit crops (FAO 2020). Still, their production faces significant challenges due to banana bacterial wilt disease, particularly banana blood disease (BBD), caused by *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*). This disease results in thick, reddish oozes in the vascular system of banana plants, causing severe damage to critical parts such as the pseudostem, branches, and fruit bunches (Badrun et al. 2017). BBD affects all banana varieties grown in affected areas, posing substantial threats to food security and the livelihoods of smallholder farmers who rely heavily on banana cultivation. Current methods for managing this disease mainly involve labour-intensive phytosanitary practices, and despite the proven effectiveness of disease-resistant varieties, their widespread adoption remains a challenge (Tripathi et al. 2022).

The external application of Peps has enhanced disease resistance in different plant species, suggesting that the Pep family is likely conserved in higher plants. Treatment of *Arabidopsis* plants with *At*Pep1 conferred resistance to *Pythium irregulare* and inhibited the growth of the bacterial leaf pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Yamaguchi et al. 2010). Huffaker et al. (2011a) showed that injecting ZmPep1 protein into maize leaves stimulated immunity, resulting in resistance against southern leaf blight and anthracnose stalk rot caused by *Cochliobolis heterostrophus* and *Colletotrichum graminicola*, respectively. Among the rice Peps, OsPep3 has been demonstrated to have diverse defensive effects on brown planthoppers (*Nilaparvata lugens*), fungi (*Magnaporthe oryzae*), and bacterial pathogens (*Xanthomonas oryzae* pv. *oryzae*) (Shen et al. 2022).

This study identified the precursor genes *MaPROPEP1* and *MbPROPEP1*, which encode the peptides MaPep1 and MbPep1 in bananas and activate pathogen defence mechanisms. Upon recognition by pattern recognition receptors (PRRs), MaPep1 and MbPep1 may trigger a signalling cascade involving secondary messengers such as calcium ions, reactive oxygen species (ROS), and phytohormones like jasmonic acid (JA) and salicylic acid (SA). This cascade ultimately activates defence-related gene expression, enhancing resistance against pathogens. These genes have been

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identified in both susceptible and resistant banana cultivars, 'Hin' and 'Khai Kasetsart 2'. Pretreatment of banana pseudostems with MaPep1 and MbPep1 before infection enhances resistance to *Rsc*. Additionally, MaPep1 and MbPep1 induce the expression of genes encoding jasmonate biosynthetic enzyme (*MaLOX7*), pathogenesis-related protein (*Pr-10*), and the peptide precursors *MaPROPEP1* and *MbPROPEP1*. This study highlights the significance of Pep signalling in plants for defending against bacterial pathogen infections, suggesting that MaPep1 and MbPep1 are promising crop immune boosters in agriculture against pathogen attacks. The potential future applications of MaPep1 and MbPep1 in banana disease management may involve foliar application, recombinant peptide production through genetic engineering, or peptide-based treatments.

MATERIAL AND METHODS

Plant materials and conditions

Two banana cultivars, 'Hin' and 'Khai Kasetsart 2', were used in this study. Both cultivars are economically important crops in Thailand. The 'Hin' banana (ABB genome), a widely cultivated local variety in southern Thailand, has been officially recognised as a Geographical Indication (GI) plant and is used for fresh consumption and processing. In contrast, 'Khai Kasetsart 2' (AA genome) is a gamma-irradiated mutant cultivar valued for its sweet taste and fragrance, making it a popular table banana exported to China, Hong Kong, and Vietnam. Their resistance to *Rsc* has been previously evaluated. 'Hin' banana plants exhibit susceptibility to *Rsc*, whereas Khai Kasetsart 2 plants exhibit resistance (Nitayaros et al. 2023; Kawicha et al. 2024). The susceptible 'Hin' banana variety was chosen to evaluate the efficacy of elicitor peptides in inducing immunity against *Rsc* infection. Both banana cultivars were micropropagated and subcultured in Murashige and Skoog (MS) media (Murashige & Skoog 1962) supplemented with 5 mg/L 6-benzylaminopurine (BAP) to generate multiple shoots for 4 months. The shoots were transferred to MS media to induce root formation for 1 month. The plantlets were transferred to seedling trays containing peat moss and acclimatised for one month in an evaporative greenhouse maintained at 30 °C and 60% humidity. The acclimatised plants were transferred to 3 × 6-inch plastic pots containing a double sterilised soil mixture (topsoil and rice husk charcoal in a 2 : 1 ratio) and kept in a greenhouse until 1 month before the next experiment started. Watering was applied daily with 50 mL of tap water per plant, and NPK 15-15-15 fertiliser was applied every 10 days.

Searching for genes controlling endogenous plant elicitor peptide precursor (*PROPEP*) in banana. The mature elicitor peptide (EgPep) sequence, IRTRRSRRPSRPPPSEGRGGQIN, from oil palm (*Elaeis guineensis*) (kindly provided by Associate Professor Dr. Alisa Huffaker) was used as a query to predict the banana peptide sequence. The prediction was conducted using a BLASTp search against *Musa* (taxid: 4640) within the National Center for Biotechnology Information (NCBI) database. The nucleotide sequence of a hit accession was then searched against *Musa* within NCBI using tBLASTn. The derived nucleotide sequence was predicted for the banana peptide using a BLASTn search in the NCBI and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) databases. The nucleotide and amino acid sequences of the open reading frame (ORF) of the predicted *PROPEP* gene were analysed. The bioactive peptide (Pep) region was analysed by alignment to known *PROPEP* sequences from Arabidopsis, maize, and oil palm.

Identification of the *MaPROPEP1* and *MbPROPEP1* genes in *Rsc*-susceptible and *Rsc*-resistant banana cultivars 'Hin' (ABB) and 'Khai Kasetsart 2' (AA). Young leaves of 'Hin' and 'Khai Kasetsart 2' bananas were harvested from three individual plants, specifically the third leaf from the basal portion of each stem. The collected leaf samples were cut into small pieces and homogenised. A 100 mg subsample of the composite leaf material was used for genomic DNA extraction following the CTAB method with modifications described by Porebski et al. (1997). The quality and quantity of the DNA were determined using a NanoDrop^{TM} Lite spectrophotometer (Thermo Fisher Scientific, USA) and agarose gel electrophoresis. The DNA samples were stored at –20 °C until use.

The target genes *MaPROPEP1* and *MbPROPEP1* were amplified via PCR with gene-specific primers (Ma-F2: 5'-AAAGGAAGAAGATAAGGGTTTTCA-3', Ma-R2: 5'-CAACTCACAGACAAATAGGCACA-3', Mb-F2: 5'-GGAAGAAGATAAGGGTTTGCATC-3', and Mb-R2: 5'-CAACTCACAGACAAATAGGGACA-3'). Each 20 µL PCR mixture contained 10 ng of DNA tem-

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plate, 1 × PCR buffer (Vivantis, Malaysia), 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.2 µM each primer, and 1 unit of *Taq* DNA polymerase (Invitrogen). The amplification protocol was carried out in a thermal cycler (PCRmax Alpha Cyler, Great Britain) with the following steps: *(i)* predenaturation at 96 °C for 5 min, *(ii)* amplification for 30 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s, and *(iii)* a final extension at 72 °C for 5 min. The PCR products were evaluated by agarose gel electrophoresis and visualised using ViSafe Green Gel Stain (Vivantis, Malaysia).

The PCR products were purified using a BioFact Gel & PCR Purification System (BIOFACT, South Korea) following the manufacturer's instructions. Standard nucleotide sequencing was conducted by Macrogen (Macrogen, Inc., South Korea) in both forward and reverse directions, employing the same primer pairs mentioned earlier. The resulting sequences were subjected to base quality evaluation, and both DNA strands were assembled using GAP4. Sequence alignment among *MaPROPEP1* and *MbPROPEP1*, as predicted from the databases and through gene amplification, was performed using CLUSTALW (www.ebi.ac.uk). The structures of the *MaPROPEP1* and *MbPROPEP1* genes in the *Rsc*-susceptible and *Rsc*-resistant banana cultivars 'Hin' and 'Khai Kasetsart 2', respectively, were then assessed.

Endogenous plant elicitor peptide synthesis. The 23-amino acid peptides corresponding to the predicted MaPep1 (VETKAARRPRRPPPSEGRGGQIN) and MbPep1 (FETKAARRPRRPPPSEGRGGQIN) were synthesised using the PepPower™ peptide synthesis technology platform provided by GenScript (GenScript USA, Inc.).

Pathogen inoculum preparation and inoculation. The methods for preparing the *Rsc* isolate MY4101 inoculum and conducting the inoculation were described by Nitayaros et al. (2023). The colonies were cultured in casamino acid-peptone-glucose (CPG) media (Kelman 1954) at 28 °C for 72 h. The inoculum was prepared by harvesting bacterial cells from the culture and suspending them in sterile distilled water. The concentration of the inoculum was adjusted to 10^8 CFU/mL.

Inoculation was carried out using the root-stabbing method after the peptides were injected into the pseudostem of the 'Hin' bananas. Before inoculation, the banana roots were wounded by stabbing with an 18 mm wide blade through the soil surface at a 5 cm depth and 2 cm distance from both sides of the stem. 10 mm of the *Rsc* inoculum at 10^8 CFU/mL concentration was inoculated into 1-month-old 'Hin' bananas. After inoculation, disease symptoms and disease severity score (DSS) were monitored and recorded. The DSS was assessed using a five-point severity scale ranging from 0–5, as Nitayaros et al. (2023) described. The severity scales included the following categories: (0) symptomless, (1) wilted leaves, (2) initial yellowing, (3) 2 to 3 chlorotic leaves, (4) 4 or more chlorotic leaves, and (5) plant death.

Evaluating the effectiveness of elicitor peptides in eliciting immunity against *Rsc* infections. The initial investigation to determine the optimal concentration of elicitor peptides for inducing resistance in 'Hin' bananas against *Rsc* infections involved concentrations of 0.5 and 0.75 mg/mL. The 0.50 mg/mL elicitors concentration was ultimately chosen for further experiments. One-month-old 'Hin' bananas were injected with MaPep1 and MbPep1 at a concentration of 0.5 mg/mL into the pseudostem at the axil of the first leaf. The injected plants were left for 18 h before further assessment. Sterile distilled water served as a control (ctrl 1). To assess the impact of MaPep1 and MbPep1 on enhancing resistance to pathogen infection, *Rsc* inoculation was conducted after peptide treatment using the method described earlier. Ten millilitres of 10^8 CFU/mL *Rsc* inoculum was applied to the wounded plants. Sterile distilled water was used as a control (ctrl 2). Disease symptoms and severity scores were monitored at 1, 3, 5, 7, 14, and 21 days postinoculation.

To confirm the sequence and function of active elicitor peptides in eliciting immunity, a crucial alteration was made to the 17th amino acid glycine (G), which was substituted with alanine (A). The mutated peptides MaPep1-G/A (VETKAARRPRRPPPSE**A**^{17}RGGQIN) and MbPep1-G/A (FETKAARRPRRPPPSE**A**^{17}RGGQIN) at a concentration of 0.5 mg/mL were injected into the stems of one-month-old 'Hin' bananas. Subsequently, the plants were inoculated with 10^8 CFU/mL *Rsc* inoculum using the same method. Disease symptoms and severity scores were assessed at 1, 7, 14, and 21 days postinoculation.

Quantification of gene expression induced by peptides in response to *Rsc* inoculation. The susceptible banana cultivar 'Hin' was subjected to MaPep1, MbPep1, or water injections followed by *Rsc* inoculation. The pseudostem samples,

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which were collected 3 cm above ground, were collected at 1, 3, 5, and 7 days postinoculation. Three plant samples per treatment were processed for total RNA extraction using the RNeasy Kit (QIAGEN, Germany). The extracted RNA was quantified using a spectrophotometer (NanoDrop™ Lite, ThermoFisher Scientific) and assessed for integrity by agarose gel electrophoresis, followed by cDNA synthesis using the SensiFAST™ cDNA Synthesis Kit (Bioline, Great Britain).

The expression levels of the *MaPROPEP1*, *MbPROPEP1*, *Pr-10* (pathogenesis-related protein), and *MaLOX7* (linoleate 13S-lipoxygenase 2-1 involved in the jasmonic acid pathway) genes were assessed via qPCR, with ribosomal protein S2 (RPS2) serving as an internal control. The sequences of primers used for gene expression analysis are provided in Table 1. Standard curves and threshold values (Cq) for all genes were generated using twofold serially diluted cDNA, and primer efficiency was calculated using the following equation: Efficiency (%) = $[10^{(-1/\text{slope})} - 1] \times 100$. Real-time PCR was performed with a PCR mixture containing 1 µL of diluted cDNA, 1X SensiFAST SYBR No-Rox Mix (Bioline, Great Britain), and 0.4 µM each of the forward and reverse primers. PCR was conducted in triplicate for each cDNA sample along with the no template control**.** All reactions were processed via PCRmax ECO 48 REAL TIME PCR PLATE (PCRmax) and run via PCRmax Eco 48 real-time PCR (PCRmax). The 2-step PCR cycling conditions consisted of an initial enzyme activation step at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 62 °C for 30 s. Dissociation curve analysis was performed after amplification to assess primer specificity. Gene expression was determined by calculating the difference (ΔCq = Cq target gene – Cq RPS2) between the Cq values of the target genes and the housekeeping gene at each time point. The relative gene expression levels were quantified using the $2^{-\Delta Cq}$ method, and fold changes relative to water-injected plants were calculated using the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen 2001). Two biological replicates were used for gene expression analysis. All collected data were analysed using analysis of variance (ANOVA) in Statistix (version 8.0), and means were compared using Tukey's mean comparison test at $\alpha = 0.05$.

RESULTS

Banana *MaPROPEP1* and *MbPROPEP1* peptide precursor genes are orthologous to the *PROPEP* gene in oil palm. Using the mature elicitor peptide sequence IRTRRSRRPSRPPPSEGRGGQIN from oil palm (*Elaeis guineensis*) as a query to predict the banana peptide sequence, the sequence of the hypothetical protein C4D60_Mb07t06480 (*Musa balbisiana*; THU59861.1) was matched with 94.12% sequence identity. This sequence was named *MbPROPEP1*. The nucleotide sequence of THU59861.1 was subsequently searched to query the National Center for Biotechnology Information and Phytozome Musa nucleotide sequence. The sequences of the identified lncRNAs (XR_001979034, *M. acuminata* subsp. *malaccensis* and GSMUA_Achr7G20910_001)

Table 1. Gene-specific sequences of primers used for quantifying the expression of elicitor peptide-inducible genes in response to *Ralstonia syzygii* subsp. *celebesensis* infection

Gene	Primer name	Primer sequence (5'-3')	Reference
MaPROPEP1	Ma-F Ma-R	GACAGGATCCTTTGGCTTTG GTGGGGTCCATGTCATTAGC	In this study
MbPROPEP1	Mb-F Mb-R	CAAAGGATCTCCTGCGACA GTGGGGTCCATGTCATTAGC	In this study
MaLOX7	Ma03_g11520-F Ma03_g11520-R	CTGATCACCGAGGAACTTATC GTGTCTTCCAGCTCTCTAATC	Tripathi et al. (2019)
Pathogen related protein (*Pr-10*)	Pr10-F Pr10-R	CTCCGAGAAGCAGTACTACGA GATGGCCGTGGACGAA	Munusamy et al. (2019)
Ribosomal protein S2 (*RPS2*)	RSP2-F RSP2-R	TAGGGATTCCGACGATTTGTTT TAGCGTCATCATTGGCTGGGA	Munusamy et al. (2019)

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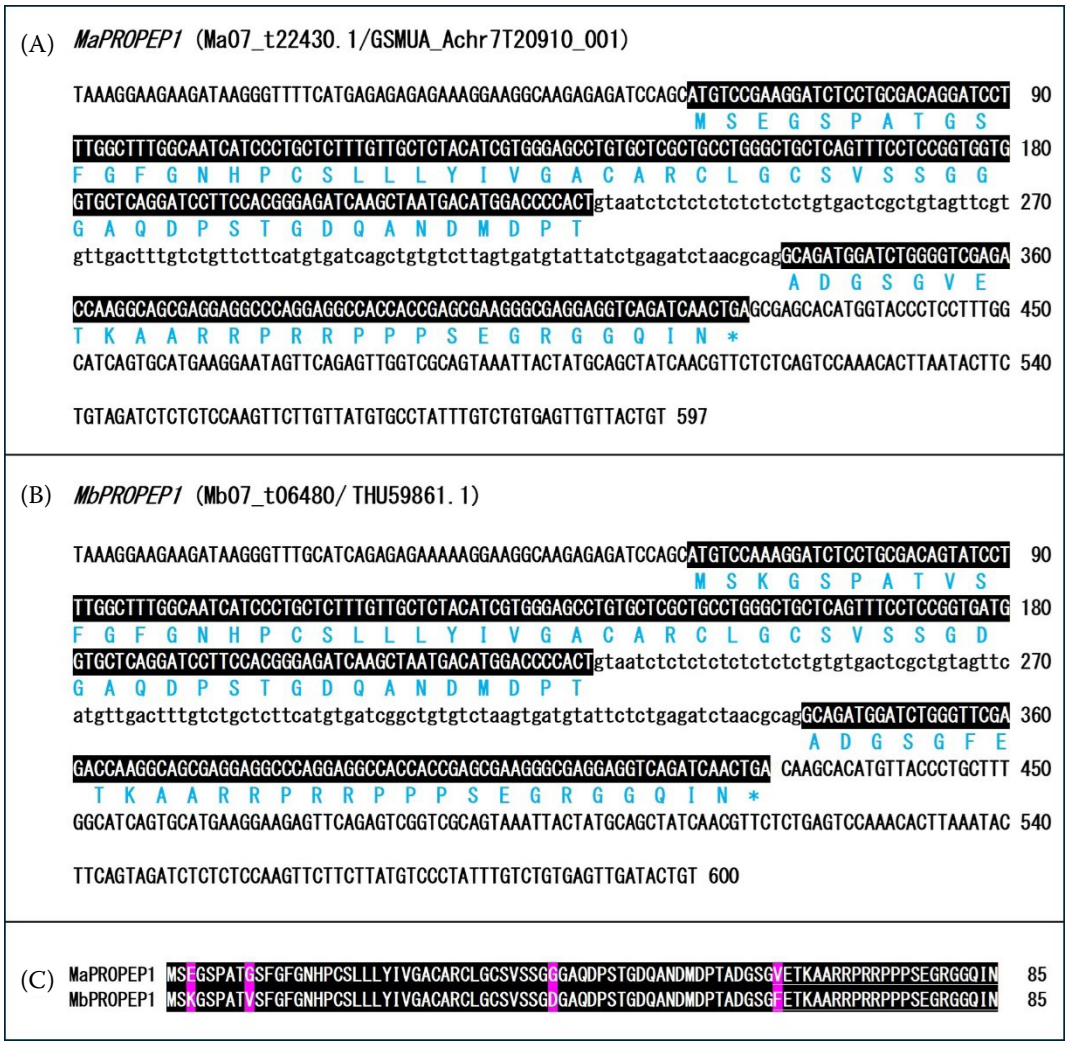

Figure 1. Nucleotide sequences and translated amino acids of elicitor peptides encoded by the genes
(A) *MaPROPEP1;* (B) *MbPROPEP1* genes and comparison of the translated amino acid sequences of *MaPROPEP1* and *MbPROPEP1* (C); amino acids highlighted in pink showed dissimilarity

had a sequence identity of 85.88%. This sequence was designated *MaPROPEP1*. These two genes are located on chromosome 7 of the banana genome.

The nucleotide sequences of *MaPROPEP1* and *MbPROPEP1* were 597 and 600 bases long, respectively, comprising a 5' untranslated region (5'UTR), 2 exons, an intron, and a 3' untranslated region (3'UTR). The transcript length was 258 bases, with 98.4% sequence identity. The translated *MaPROPEP1* and *MbPROPEP1* in exons 1 and 2 encoded 85 amino acids (Figures 1A and 1B). Amino acid sequence alignment revealed differences in the levels of 6 amino acids, namely, glutamic acid (E), lysine (K), glycine (G), valine (V), aspartic acid (D), and phenylalanine (F), between *MaPROPEP1* and *MbPROPEP1* (Figure 1C).

The bioactive elicitor peptide region at the C-terminus was identified by comparison with known amino acid sequences of *Arabidopsis*, maize, and oil palm. The results suggested that 23 amino acids, namely, VETKAARRPRRPPPSEGRGGQIN (MaPep1) and FETKAARRPRRPPPSEGRGGQIN (MbPep1), were most similar to the sequence of oil palm (EgPep). Several features of MaPep1 and MbPep1 were observed to be similar in *At*Peps and ZmPeps, although *At*Pep, ZmPep, and banana peptides were dissimilar. The features included many charged amino acids, such as glutamic acid (E), lysine (K), glycine (G), valine (V), aspartic acid (D), phenylalanine (F), proline-rich (P), and glycine (G) at the 17th amino acid and asparagine (N) or histidine (H) at the last amino acid (Figure 2, Table 2).

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Figure 2. The 23 conserved amino acid residues of the bioactive elicitor peptide at the C-terminus were compared across banana, *Arabidopsis thaliana*, maize, and oil palm

The common features included highly conserved charged amino acids, R, K, E, and D (red); proline-rich (P) (blue); glycine (G) at the 17th amino acid (green); and the last amino acid, N or H (orange)

The stop codon found in the *MbPROPEP1* gene could influence the functionality of the elicitor peptide in susceptible 'Hin' banana. The *MaPROPEP1* and *MbPROPEP1* genes were identified in *Rsc*-susceptible and *Rsc*-resistant banana cultivars 'Hin' (ABB) and 'Khai Kasetsart 2' (AA). The nucleotide sequences of *MaPROPEP1* were 530 bp and 511 bp long in 'Hin' and 'Khai Kasetsart 2', respectively, and were transcribed to 258 bp in length in both cultivars. Translation yielded 85 amino acids, with the active elicitor peptide identified as VETKAARRPRRPPPSEGRGGQIN at the C-terminus, showing similarity to sequences obtained from databases (Figures 3A and 3B). In addition, the *MbPROPEP1* gene was identified, measuring 562 bp and 573 bp in length in 'Hin' and 'Khai Kasetsart 2', respectively. Interestingly, a nonsense mutation at the 52nd base resulting in a stop codon (TGA) was found in the 258 bp transcript sequence of the 'Hin' banana but not in the 'Khai Kasetsart 2' (TGC), potentially affecting peptide translation and functionality in eliciting immunity to *Rsc* infection. The 85 translated amino acids, including the 23 amino acids FETKAARRPRRPPPSEGRGGQIN at the C-terminus, were identified as active elicitor peptides (Figures 4A and 4B). Notably, the first amino acid at the N-terminus of the active elicitor peptides differed between 'Hin' and 'Khai Kasetsart 2' compared to peptides obtained from databases. However, a conserved region was observed between the two cultivars.

Table 2. Comparison of bioactive elicitor peptides at the C-terminus in banana, *Arabidopsis thaliana*, maize, and oil palm

Elicitor peptide	Plant species	Amino acid sequence
MaPROPEP1	*Musa acuminata*	VETKAARRPRRPPPSEGRGGQIN
MbPROPEP1	*Musa balbisiana*	FETKAARRPRRPPPSEGRGGQIN
EgPROPEP	*Elaeis guineensis*	IRTRRSRRPSRPPPSEGRGGQIN
AtPROPEP1	*Arabidopsis thaliana*	ATKVKAKQRGKEKVSSGRPGQHN
AtPROPEP2		DNKAKSKKRDKEKPSSGRPGQTN
AtPROPEP3		EIKARGKNKTKPTPSSGKGGKHN
AtPROPEP4		GLPGKKNVLKKSRESSGKPGGTN
AtPROPEP5		SLNVMRKGIRKQPVSSGKRGGVN
AtPROPEP6		ITAVLRRRPRPPPYSSGRPGQNN
AtPROPEP7		VSGNVAARKGKQQTSSGKGGGTN
AtPROPEP8		GVIVKSKKAARELPSSGKPGRRN
ZmPROPEP1	*Zea mays*	VRRRPTTPGRPREGSGGNGGNHH
ZmPROPEP2		RRPRPRPPDHAREGSGGNGGVHH
ZmPROPEP3		TRTPPWPPCPPEEGSGGNGGSHN
ZmPROPEP4		LMRGPAPPGHPAEGAGGRGGSIH
ZmPROPEP5		RARGPTPPGLPAEGSGGNGGTKH

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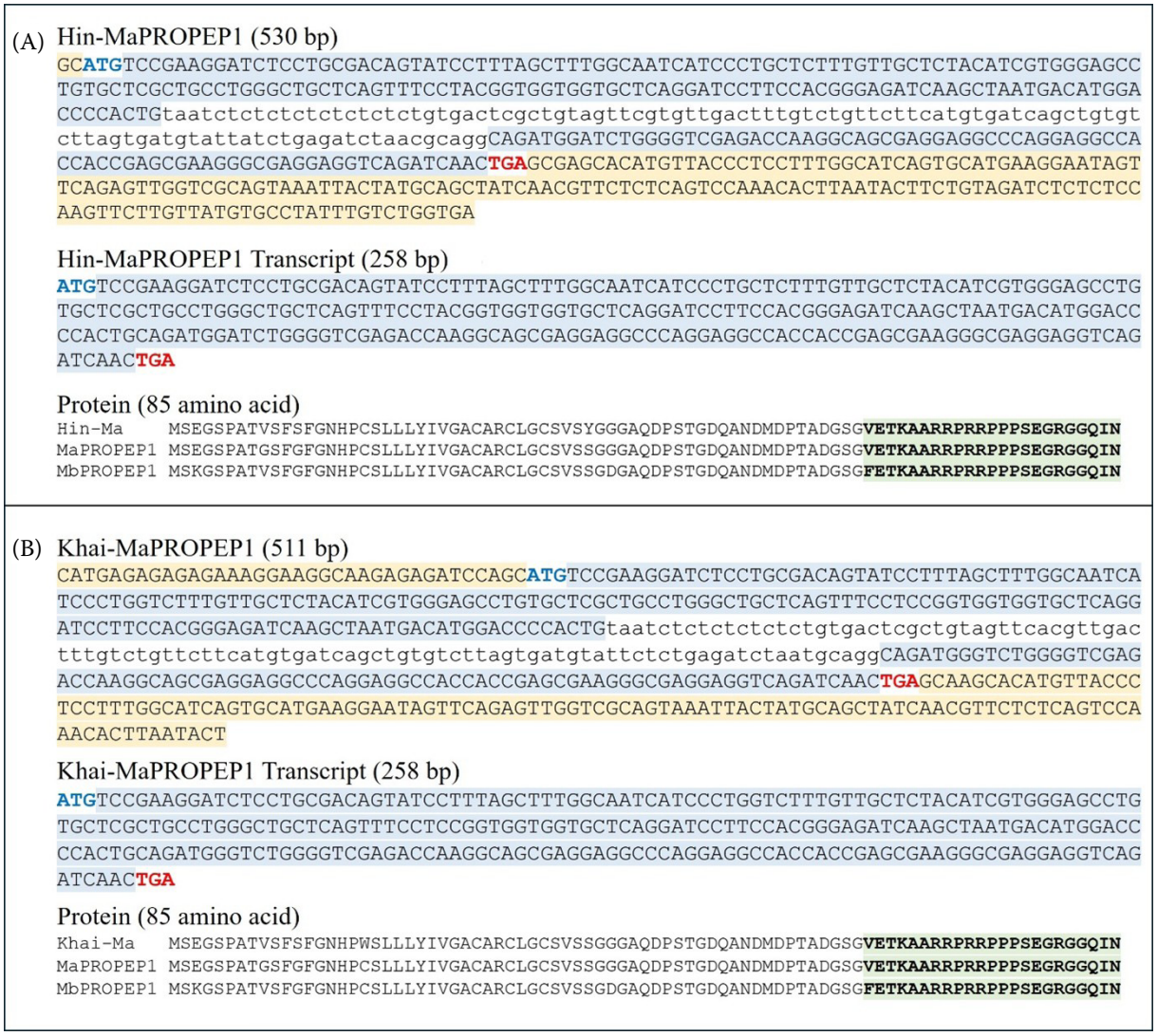

Figure 3. Nucleotide and amino acid sequences of *MaPROPEP1*

Identified from Hin (A) and Khai Kasetsart 2 (B) bananas. The nucleotides highlighted in blue represent exons, and those highlighted in yellow represent the 3'-UTR and 5'-UTR; the 23 amino acids are highlighted in green

The endogenous plant elicitor peptides MaPep1 and MbPep1 can immunise 'Hin' banana plants against *R. syzygii* subsp. *celebesensis* infection. The efficacy of the elicitor peptides MaPep1 and MbPep1 in inducing immunity against *Rsc* infection was evaluated by injecting these peptides into the pseudostem of 'Hin' bananas for 18 hours, followed by infection with water or *Rsc*. Disease symptoms and severity were monitored 1, 3, 5, 7, 14, and 21 days post-infection. The results revealed that 'Hin' bananas treated with both peptides (MaPep1 + *Rsc* and MbPep1 + *Rsc*) remained symptomless for the 7-day monitoring period, in contrast to the control (water + *Rsc*), which exhibited wilted leaves. The disease symptoms continued to progress until the plants died 21 days after *Rsc* infection in the control treatment, while the peptide-pretreated bananas showed leaf wilting and chlorosis (Figure 5A). The disease severity score (DSS) for the peptide-treated 'Hin' bananas ranged from 0 to 3.2, whereas the control had a DSS of 0.0–4.9 from days 1–21 after *Rsc* inoculation (Figure 5B).

Glycine is an important amino acid residue responsible for the biological functions of MaPep1 and MbPep1. The important functionality of the amino acid residue in the mature peptides was determined by substituting the alanine (A) residue with the glycine (G) residue at the 17th position of MaPep1 (VETKAARRPRRPPPSE**A**^{17}RGGQIN)

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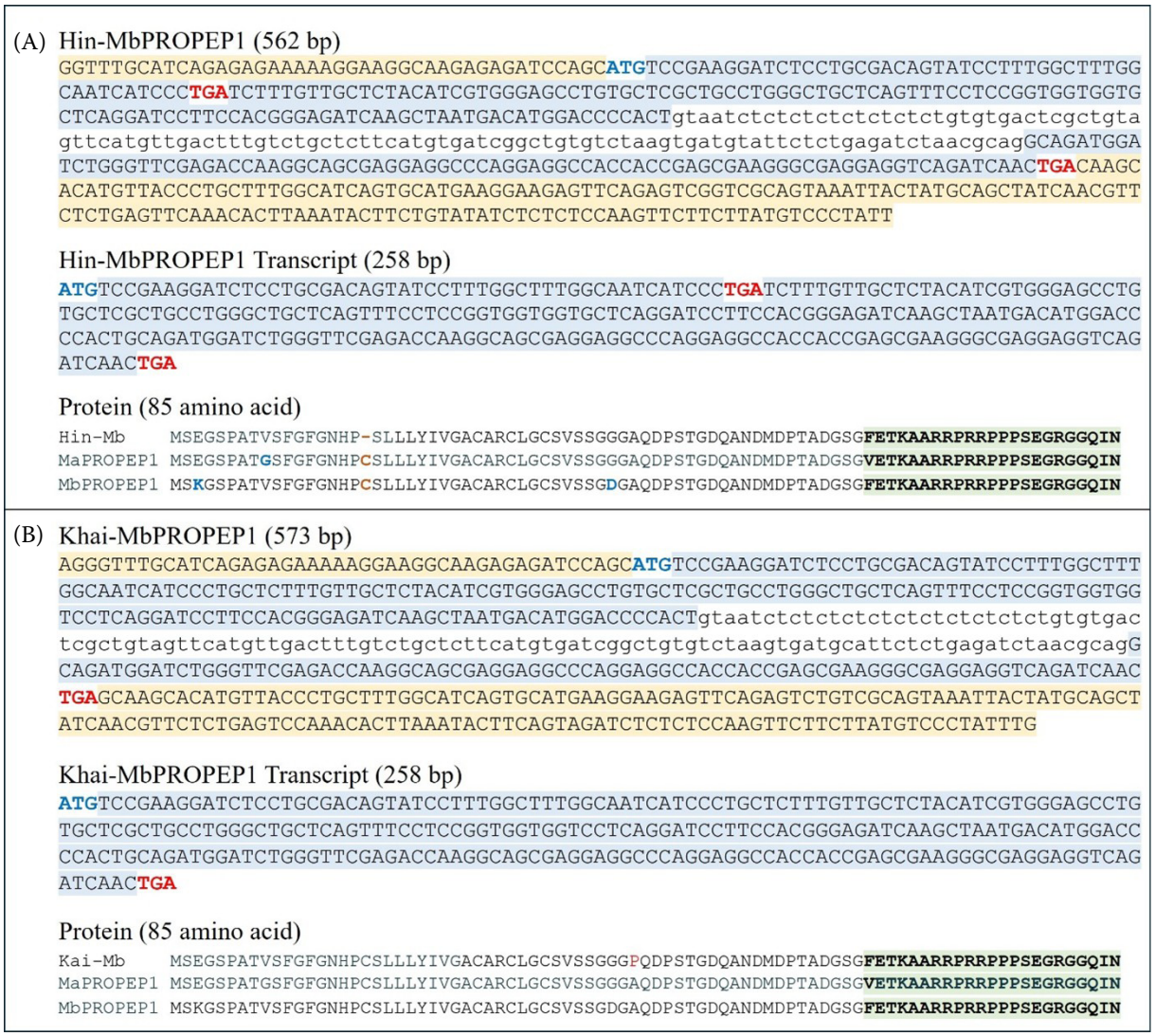

Figure 4. Nucleotide and amino acid sequences of *MbPROPEP1*

Identified from Hin (A) and Khai Kasetsart 2 (B) bananas; the nucleotides highlighted in blue represent exons, and those highlighted in yellow represent the 3'-UTR and 5'-UTR; the 23 amino acids are highlighted in green

and MbPep1 (FETKAARRPRRPPPSE**A**^{17}RGGQIN). The mutant peptides were injected into the pseudostem of 'Hin' bananas and infected with either water or *Rsc* inoculum. Disease symptoms and severity scores were determined after treatment for 1, 7, 14, or 21 days. The results indicated that the mutant peptides could not induce immunity against *Rsc* inoculation at 14–21 days compared to the water-treated 'Hin' bananas. The disease symptoms and severity scores across all treatments indicated normal plant growth on day 1 (DSS = 0), followed by symptom development between days 7 and 21, including wilted, chlorotic leaves and plant death. The DSS ranged from 0.4 ± 0.5 to 4.4 ± 0.5 in plants treated with mutant MaPep1 and from 0.8 ± 0.4 to 4.4 ± 0.9 in those treated with mutant MbPep1, compared to water-treated bananas, which had a DSS range of 0.8 ± 0.4 to 4.6 ± 0.5. No significant differences in DSS or symptoms were observed among treatments at any time (Figure 6).

The elicitor peptides MaPep1 and MbPep1 induced the expression of the precursor genes *MaPROPEP1* and *MbPROPEP1* and the jasmonic acid (JA) and pathogenesis-related protein 10 (*Pr-10*) genes in response to *R. syzygii* subsp. *celebesensis* infection. To assess the expression of the *MaPROPEP1*, *MbPROPEP1*, *MaLOX7*, and pathogenesis-related protein 10 (*Pr-10*) genes involved in response to *Rsc* pathogen infection in the susceptible banana cultivar, 'Hin', the peptides MaPep1

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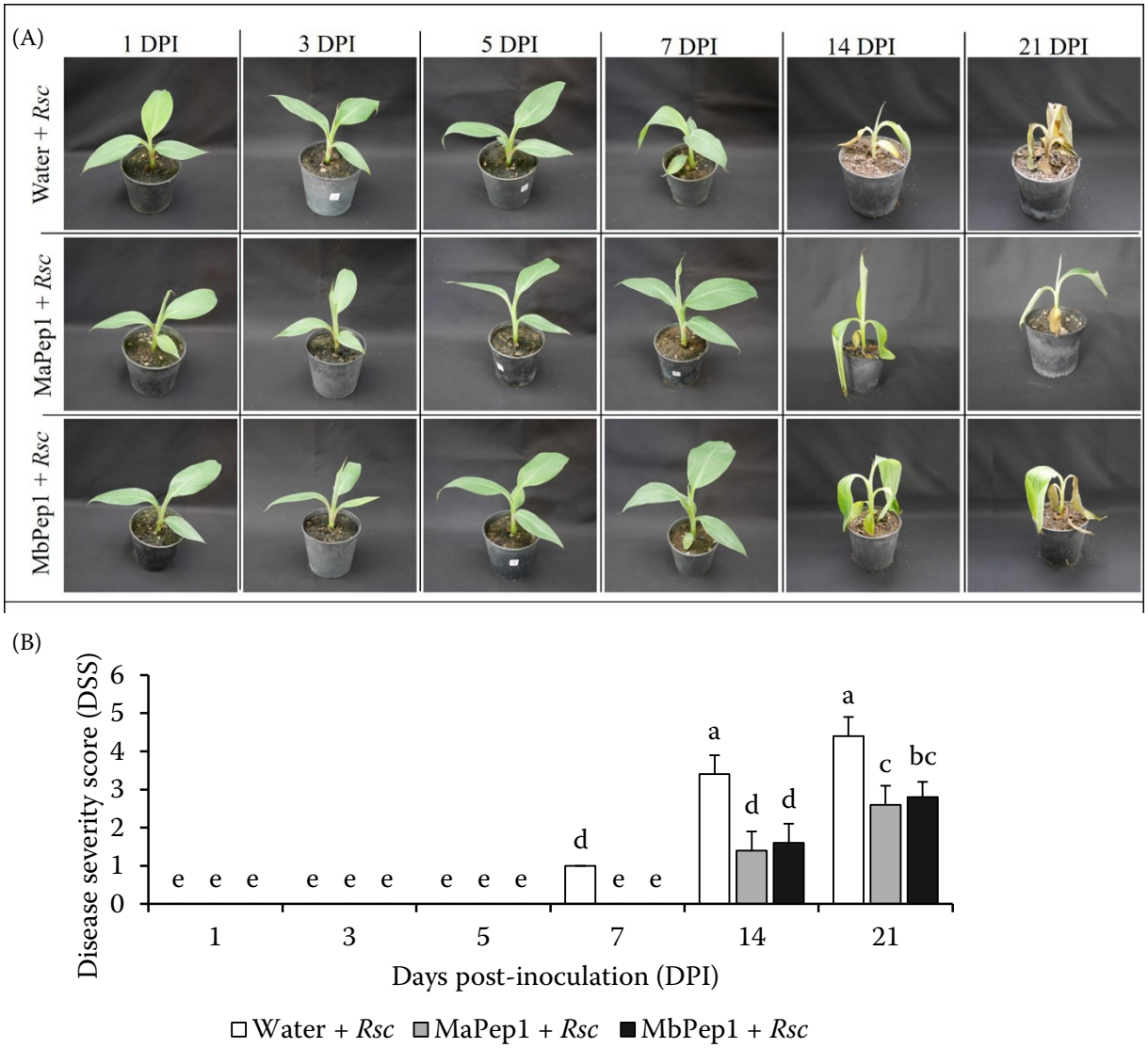

Figure 5. The response of 'Hin' bananas pretreated with peptides to infection with *Ralstonia syzygii* subsp. *celebesensis* for 21 days post-infection

The figure presents disease symptoms (A) and disease severity scores (B) for both peptide-treated and control bananas at 1, 3, 5, 7, 14, and 21 days post-infection

and MbPep1, or water was injected into the pseudostems, followed by *Rsc* inoculation. Aboveground pseudostem samples were collected at 1, 3, 5, and 7 days postinoculation. The results revealed that the expression of four genes was induced in peptide-pretreated 'Hin' bananas. Compared with those in water-treated 'Hin' bananas, *MaPROPEP1* transcript levels increased 1.47 ± 0.32-fold in MaPep1-treated stems (MaPep1+*Rsc*) on day 1 after inoculation. The expression levels decreased on days 3–5 and increased again on day 7. Compared with those in the water-treated plants, the number of *MaPROPEP1* transcripts in the plants treated with MbPep1 (MbPep1+*Rsc*) increased 1.36 ± 0.10-fold after 5 days of inoculation but gradually decreased on day 7. Although fluctuations in *MaPROPEP1* expression were observed in response to both peptides, statistical analysis indicated these differences were insignificant (α = 0.05) (Figure 7A).

The expression of *MbPROPEP1* transcripts tended to increase at 7 days after *Rsc* inoculation, showing a 1.59 ± 0.48-fold increase in MaPep1-pretreated stems (MaPep1 + *Rsc*) compared to the control (water + *Rsc*). Similarly, MbPep1 (MbPep1 + *Rsc*) induced the expression of the *MbPROPEP1* gene by gradually increasing the number of tran-

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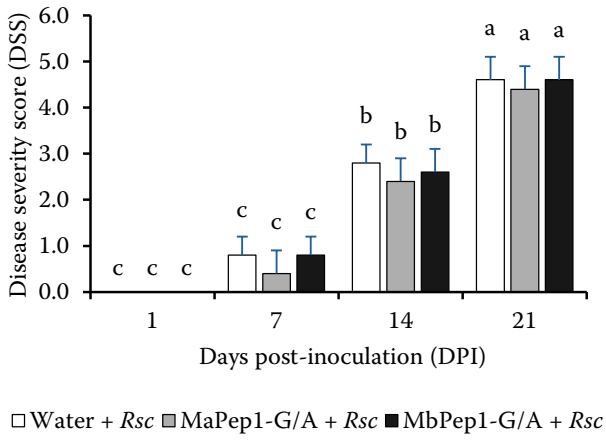

Figure 6. The disease severity scores in 'Hin' bananas pretreated with either water or mutant peptides in response to *Ralstonia syzygii* subsp. *celebesensis* inoculation at 1, 7, 14, and 21 days post-infection

scripts to 1.84 ± 0.16-fold in the treated samples at days 5–7 after *Rsc* infection. The expression pattern of the *MbPROPEP1* gene was progressive and induced by both peptides in response to pathogen infection for 7 days. However, although *MaPep1* and *MbPep1* induced *MbPROPEP1* expression, the increase was not significantly different from the control treatment, and both peptide treatments resulted in statistically similar responses. (Figure 7B).

Similarly to those in the MbPep1-treated plants, the *MaLOX7* transcript level in the MaPep1-treated stems decreased by 20.96 ± 4.77-fold at 7 days after *Rsc* inoculation, which was a 10.28 ± 3.24-fold increase compared to that in the control plants (Figure 7C). Similarly, the transcription of *Pr-10* was induced by MaPep1, MbPep1, and water injection at the early stage of treatment. At 5 days after

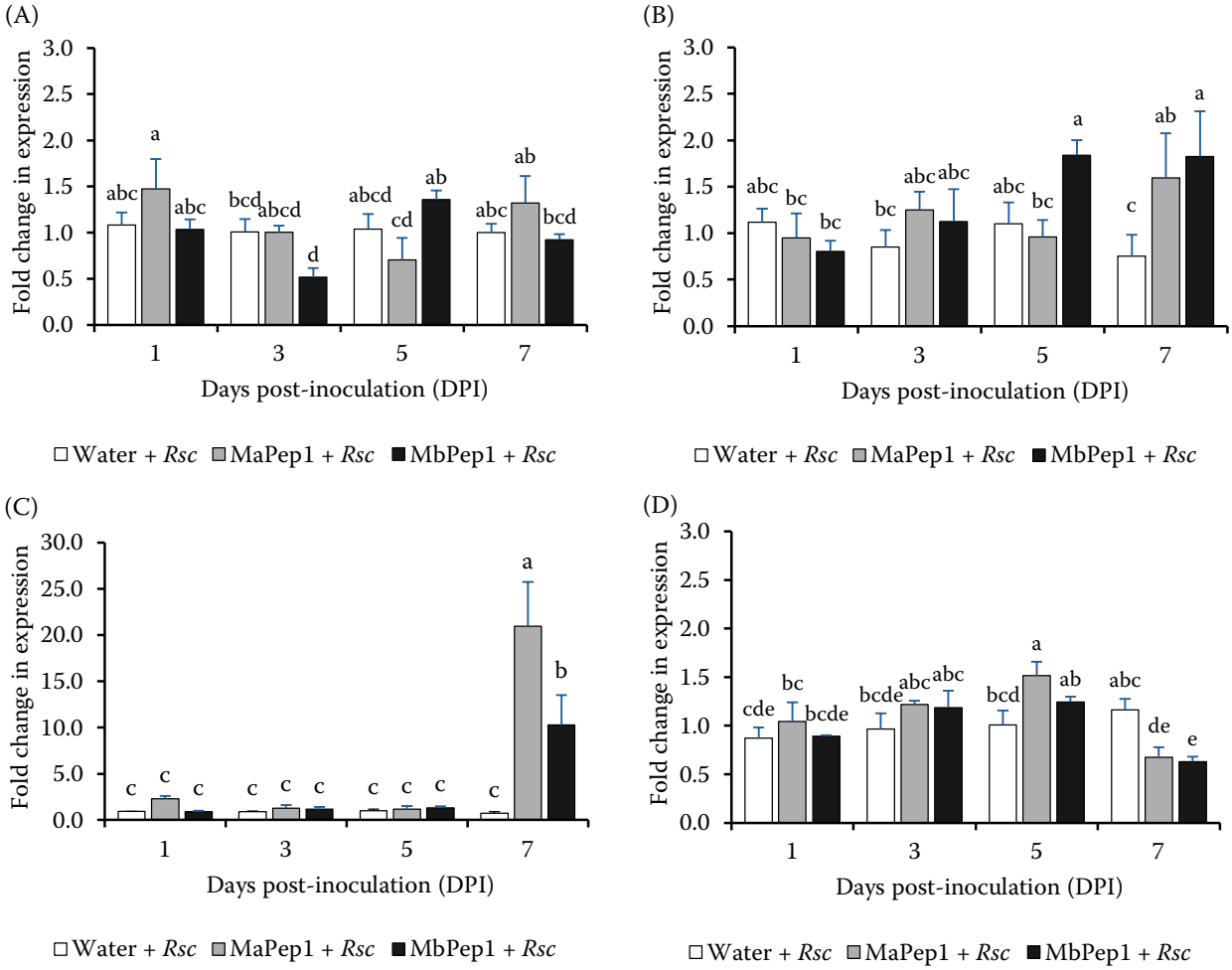

Figure 7. Expression of the *MaPROPEP1* (A), *MbPROPEP1* (B), *MaLOX7* (C), and *Pr-10* (D) genes induced by the peptides MaPep1, MbPep1, or water following *Ralstonia syzygii* subsp. *celebesensis* (*Rsc*) inoculation at 1, 3, 5, and 7 days post-infection

The data are presented as the means ± SDs (n = 3); the letters above the bars indicate significant differences at α = 0.05 according to the Tukey's test

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Rsc inoculation, the *Pr-10* transcript levels were 1.52 ± 0.14- and 1.24 ± 0.05-fold greater in the MaPep1- and MbPep1-pretreated stems than in the water-injected controls. However, the level of *Pr-10* transcripts in MaPep1- and MbPep1-pretreated stems gradually decreased at 7 days after *Rsc* infection compared to controls (Figure 7D).

DISCUSSION

Elicitors are extensively utilised to activate plant defenses, inducing responses that enhance resistance against diseases (Eder & Cosio 1994; Hahn 1996; Nürnberger 1999; Thakur & Sohal 2013; Badosa et al. 2022). Previous research has identified endogenous plant elicitor peptides (Peps) in various plant species, demonstrating their ability to activate the immune system and confer broad-spectrum defences against pests such as insects and disease pathogens (Lee et al. 2018). Peptides derived from precursor genes are plant elicitor peptide precursors (*PROPEPs*). Bioactive Peps are typically located on the C-terminal region and consist of 23–36 amino acids, with glycine being a major component [characterised by a glycine-rich motif: (S/G)(S)Gxx(G/P)xx(N)] (Tavormina et al. 2015). Similarly, this study identified the peptide precursor genes *MaPROPEP1* and *MbPROPEP1* in bananas, resulting in the bioactive Peps MaPep1 and MbPep1, respectively. These peptides exhibited a 23-amino acid bioactive region, specifically, VETKAARRPRRPPPSEGRGGQIN and FETKAARRPRRPPPSEGRGGQIN. Comparative analysis revealed a 73.9% similarity between the amino acid sequences of these banana Peps and those of oil palm. The conserved glycine (Gly-17) residue was found to be similar to those of *At*Peps (Huffaker et al. 2006) and ZmPeps (Huffaker et al. 2011a), which were identified in *Arabidopsis thaliana* and maize, respectively, and is known to be essential for plant immunity. The genes responsible for Pep synthesis belong to the gene family. For instance, *Arabidopsis thaliana* has 8 genes (*AtPROPEP1* to *AtPROPEP8*), while soybean possesses 6 genes, each located on different chromosomes, including *GmPROPEP1* (chromosome 10), *GmPROPEP2* (chromosome 20), *GmPROPEP3*, *GmPROPEP4* and *GmPROPEP5* (chromosome 13), and *GmPROPEP6* (chromosome 4) (Lee et al. 2018). Similarly, maize has 5 genes, and rice has 3 genes associated with this family (Huffaker et al. 2011a; Huffaker et al. 2013). In Bananas, two genes, *MaPROPEP1* and *MbPROPEP1*, are located on chromosome 7 of the *Musa acuminata* and *M. balbisiana* genomes.

Identification of the peptide precursor gene in *Rsc*-susceptible and resistant bananas was conducted. The nucleotide sequences were identical to those in the database. Interestingly, a stop codon was discovered in the *MbPROPEP1* gene, suggesting potential implications for the functionality of the elicitor peptide in susceptible 'Hin' bananas. This stop codon halts the translation process required to produce the mature peptide. In contrast, evidence from the *ZmPROPEP1* precursor gene, identified in the maize varieties Golden Queen and B73, revealed alternatively spliced introns in the sequences, suggesting the regulation of peptide processing with proteolytic activity to release the active peptide from the precursor (Huffaker et al. 2011a).

Previous studies have reported that *At*Pep1 homologues contain proline, glycine, and serine residues, with a conserved glycine at residue 17 in the C-terminal region, suggesting its significance for receptor recognition (Huffaker et al. 2006). Pearce et al. (2008) demonstrated the relationship between the structure and alkalinisation activity of *At*Pep1 and its receptor *At*PEPR1. The sequential removal of N-terminal amino acids led to a decrease in alkalinisation activity until 15 amino acids were removed, indicating the importance of the C-terminal end of *At*Pep1 for its activity. Additionally, the substitution of glycine with alanine at position 17 resulted in a more than 4 000-fold decrease in half-maximal activity compared to that of normal *At*Pep1. These findings support our observations, where the substitution of glycine with alanine at position 17 in banana Peps increased susceptibility to *Rsc* infection after treatment with mutated peptides, highlighting the importance of this residue for the bioactivity of MaPep1 and MbPep1. Recently, Cui et al. (2024) proposed that the N-terminal amino acids of *At*Pep1$^{(1–23)}$ are dispensable for primary root growth and ROS bursts, while the conserved C-terminal aspartic acid plays a significant role in its functionality. Removing 8 or 12 N-terminal amino acids inhibited primary root growth and induced a ROS burst, showing activity comparable to *At*PEP1$^{(1–23)}$. Truncated *At*Pep1$^{(13–23)}$ has the potential to induce plant resistance to pathogens without adversely affecting their growth and development. There-

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fore, *At*PEP1$^{(13–23)}$ shows promise for development as a potentially applicable biopesticide.

The observed increased resistance to *Rsc* after pretreatment with the MaPep1 and MbPep1 peptides was notable. The 'Hin' bananas resisted the *Rsc* pathogen, displaying no wilt symptoms (disease severity score = 0) even 7 days after infection. The prolonged resistance of 'Hin' bananas after MaPep1 and MbPep1 pretreatment is likely due to immune priming, which enhances defence signalling, activates resistance genes, and strengthens cell walls, delaying *Rsc* colonisation and preventing wilt symptoms. This finding aligns with the report of Yamaguchi et al. (2010), where the application of *At*Peps peptide to *Arabidopsis* leaves reduced the spread of *Pseudomonas syringae* pv. *tomato* DC3000 bacteria. Huffaker et al. (2006) also reported that overexpression of the *At*Pep1 peptide conferred resistance to the soil fungus *Pythium irregulare*. Moreover, several previous studies have established the critical role of elicitor peptides in activating innate immune responses and priming plants for defence against various pathogens (Zipfel & Felix 2005; Jones & Dangl 2006; Asai et al. 2008). For instance, Asai et al. (2008) demonstrated that MAPK signalling pathways and reactive oxygen species (ROS) production are known to be triggered upon elicitor peptide recognition, leading to the activation of defence-related genes and the production of antimicrobial compounds. Applying the ZmPep3 protein to maize resulted in resistance to *Spodoptera exigua* by producing secondary metabolites and enzymes that defend against insect bites (Huffaker et al. 2013). Injecting the ZmPep1 peptide into maize leaves stimulated immunity, resulting in resistance to southern leaf blight and anthracnose stalk rot caused by *Cochliobolus heterostrophus* and *Colletotrichum graminicola*, respectively (Huffaker et al. 2011a). Furthermore, Ruiz et al. (2018) sprayed peptides from the *Prunus* group, including PpPep1, PdPep1, and PpPep2, onto GF-677 peach tree leaf surfaces before inoculation with *Xanthomonas arboricola* pv. *pruni* (*Xap*) for varying durations (3 h, 24 h, and 48 h). This treatment prevented necrosis of peach leaf tissues compared to the control group after a 3-week inoculation period.

In this study, gene expression analyses supported the role of MaPep1 and MbPep1 peptides in modulating defence responses in banana plants. The results showed the upregulation of the *MaPROPEP1*, *MbPROPEP1*, *MaLOX7*, and *Pr-10* genes after peptide treatment and subsequent pathogen challenge. This suggests that Peps play a role in enhancing plant resistance by activating immune pathways. For instance, in *Arabidopsis*, MAMPs trigger immune signalling via LRR receptors (e.g., FLS2, EFR), activating MAPK cascades, calcium influx, and ROS production. This induces *PROPEPs*, generating *At*Peps that bind to PEPR1, amplifying defence responses through a feedback loop that further enhances jasmonic acid (JA) and salicylic acid (SA) signalling. JA plays a crucial role in activating transcription factors that regulate the expression of defence-related genes, including pathogenesis-related (*PR*) genes such as *PDF1.2* and *PR-1*, which encode antimicrobial proteins that strengthen immunity against pathogens (Ryan et al. 2007). This indicates banana plants' coordinated and specific defence response against bacterial pathogens (De Smet et al. 2009; Huffaker et al. 2013). In this study, the fluctuations in *MaPROPEP1* expression were observed in response to both peptides, but the differences were not statistically significant. This suggests a dynamic, time-dependent immune response, where elicitor peptides trigger early defence signalling, followed by transient downregulation and a secondary activation phase, reflecting the complexity of immune regulation. According to Li et al. (2020), signal molecules, including hormones, proteins, and peptides, transmit signals with high specificity, robustness, and durability, albeit at the expense of speed. These characteristics are essential for inducing and sustaining the second phase of distal immunity, during which plants synthesise large quantities of pathogen-inhibitory molecules to strengthen defence mechanisms. The conserved function of MaPep1 and MbPep1 across different plant species underscores their evolutionary significance in plant immunity (Bartels et al. 2013; Klauser et al. 2015). Similar elicitor peptides have been identified in various plant systems, indicating a common strategy plants employ to recognise and respond to pathogen attacks. This evolutionary conservation suggests that the mechanisms triggered by MaPep1 and MbPep1 are fundamental and have been finely tuned during plant evolution. Bartels et al. (2013) demonstrated that similar elicitor peptides activate pattern-triggered immune responses across diverse plant species, indicating a common defence strategy. Furthermore, Klauser

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et al. (2015) highlighted the role of the Arabidopsis Pep-PEPR system in mediating plant defence against herbivory, emphasising the versatility of elicitor peptides in combating various stresses.

The *MaLOX7* gene encodes a lipoxygenase enzyme involved in the biosynthesis of jasmonic acid, a key signalling molecule in plant defence against pathogens. Its transcript level gradually increased in MaPep1- and MbPep1-treated stems 7 days after *Rsc* inoculation. This parallels the action of ZmPep1, which activates both jasmonic acid biosynthetic genes and their accumulation (Huffaker et al. 2011a). The induction of *Pr-10* transcript accumulation was greater in the MaPep1- and MbPep1-pretreated stems at 5 days after *Rsc* inoculation, indicating the activation of systemic acquired resistance (SAR) pathways in response to MaPep1 and MbPep1 treatments. Huffaker et al. (2011b) reported that elicitor peptides induce the expression of genes encoding phytoalexins, which are important antimicrobial compounds in plant defence. The magnitude of gene expression changes may vary based on the pathogen type and elicitor peptide used. For example, *Pseudomonas syringae* induced a 3.2-fold increase in *AtPROPEP2* gene expression in *Arabidopsis*, while *P. infectans* led to a 64.4-fold increase in *AtPROPEP3* gene expression compared to the control (Huffaker et al. 2006). Additionally, genes encoding pathogenesis-related proteins (PR4, PR5-TLP2, and PR5-TLP3) showed heightened responses to *Xap* when exposed to the peptides PpPep1, PdPep1, and PpPep2 for 24 hours (Ruiz et al. 2018).

CONCLUSION

Based on these experimental results, it can be concluded that the identified endogenous plant elicitor peptide genes (*PROPEPs*), encoding MaPep1 and MbPep1, effectively enhance banana resistance to *Rsc*, delaying disease symptoms for up to 14 days and inducing the expression of the precursor genes *MaPROPEP1, MbPROPEP1*, *Pr-10*, and *MaLOX7*. The discovery of Peps in bananas represents a significant advancement in understanding plant defence mechanisms and offers promising agricultural applications for sustainable crop protection against wilt disease. Peptide-based biocontrol is the most probable translational approach; however, the high cost of peptide synthesis remains a major challenge for large-scale field applications. To address this, microbial-based peptide production and plant-integrated expression offer the most feasible long-term solutions, ensuring cost-effective and sustainable disease control. Future research should focus on elucidating the molecular mechanisms of MaPep1 and MbPep1, including receptor interactions, downstream signalling pathways, and their role in long-term immune priming. To enhance feasibility, cost-effective production strategies such as microbial-based peptide expression, plant-integrated expression, and synthetic peptide mimics should be explored. Large-scale field trials and integration into integrated pest management (IPM) strategies will be crucial for establishing peptide-based biocontrol as a scalable and sustainable approach for banana disease management.

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