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Heterologous expression of maize-derived antimicrobial peptide ZmES4 in *Escherichia coli* for potential plant pathogen control

Abstract. ZmES4 is a plant-derived antimicrobial peptide (AMP) from maize that shows promise as a biocontrol agent against plant pathogenic organisms. In the context of growing challenges in sustainable agriculture, AMPs like ZmES4 represent innovative alternatives to chemical pesticides. This study focuses on the structural characterization and heterologous expression of the ZmES4 peptide in *Escherichia coli* (*E. coli*). The gene encoding ZmES4 was obtained from the maize female gametophyte (NCBI Reference Sequence: NM_001112150.3) and cloned into the pET24d(+) expression vector using NcoI and XhoI restriction sites. Transformation into *E. coli* BL21 (DE3) cells enabled recombinant expression upon induction with isopropyl β-D-1-thiogalactopyranoside (IPTG). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford assays confirmed the expression of ORF-ZmES4, with protein concentrations ranging from 14.647 to 63.606 mg/mL. The successful expression of ZmES4 in *E. coli* highlights its potential application as a recombinant AMP for future plant disease management strategies.

Keywords: Recombinant protein production · Defensin-like peptides · Biological control agent · Crop protection

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Introduction

The current challenges in agricultural cultivation have become increasingly complex due to the intensification of both biotic and abiotic stresses. Biotic stresses, such as pests and plant diseases, can significantly reduce crop yields, while abiotic stresses, including drought, excessive rainfall, and fluctuating humidity levels, also negatively impact plant growth and productivity. Climate change exacerbates these stressors, demanding the development of innovative and sustainable solutions to ensure food security (Pandey et al., 2021; Zhu et al., 2020). While the use of resistant crop varieties remains a key strategy in disease management, it is often limited by the emergence of new pathogen races and environmental instability.

An emerging and promising approach to complement existing disease management strategies is the use of antimicrobial peptides (AMPs) derived from plants. In addition to their natural role in plant immunity, certain AMPs like ZmES4 from maize have shown potential when heterologously expressed in microbial systems such as *E. coli* (Bej et al., 2021; Montesinos & Bardají, 2020), enabling their broader application in biocontrol strategies.

Plant-derived AMPs are small, positively charged molecules typically consisting of 20–60 amino acids with amphipathic structures and high cysteine content. These peptides play a crucial role in plant innate immunity by inhibiting the growth of phytopathogenic bacteria, fungi, and oomycetes. Their primary modes of action include disrupting microbial membrane integrity, binding to specific lipid components, or triggering oxidative stress in pathogens (Goyal & Mattoo, 2021). Due to their structural stability and specificity, AMPs have attracted attention as promising candidates for engineering disease-resistant crop varieties across different agricultural sectors, including cereals, vegetables, and industrial crops (Li et al., 2023).

Several AMP genes have been isolated and characterized in the past five years from various plant species such as *Oryza sativa* (rice), *Capsicum annuum* (chili pepper), *Medicago truncatula* (barrel medic), and *Solanum tuberosum* (potato). Each AMP exhibits antimicrobial activity against specific pathogens. For instance, OsDEF7 from rice effectively suppresses *Magnaporthe oryzae*, the causative agent of rice blast, while Snakin-1 from potato shows broad-spectrum activity against both bacterial (*Erwinia*) and fungal (*Fusarium*) pathogens (Singh et al., 2022; Jha et al., 2021). These peptides can be integrated into pest and disease management programs in staple crops, horticultural commodities like chili and tomato, as well as estate crops such as cocoa and oil palm, offering an eco-friendly alternative to chemical pesticides (Yuan et al., 2020; Santos et al., 2023). Ten plant-derived AMPs, their functions, and sources (2019–2024) are shown in Table 1.

The ZmES genes in maize encode peptides that exhibit significant structural homology to plant defensins, a class of small, cysteine-rich proteins known for their antimicrobial functions. Interestingly, these genes are specifically expressed in the cells of the female gametophyte, particularly in the synergid cells adjacent to the egg apparatus, suggesting a specialized role in reproductive processes such as pollen tube guidance or sperm cell release. The tissue-specific expression and structural characteristics of the ZmES peptides highlight their potential function in mediating intercellular communication during fertilization in maize (Cordts et al., 2001). *ZmES4* is one of the member families of these genes.

The potential of ZmES4 and other plant-derived AMPs lies in their integration into crop protection strategies via transgenic expression or bioformulation approaches. As climate change intensifies abiotic stresses such as drought, heat, and irregular rainfall, the vulnerability of crops to disease increases. While the development and deployment of disease-resistant elite varieties remain essential, these strategies alone are not sufficient. There is an urgent need for integrated disease management approaches that combine conventional methods with innovative tools, including the application of AMPs for enhancing plant immunity (Li et al., 2022; Natarajan et al., 2021). These peptides offer a sustainable and environmentally friendly alternative to synthetic pesticides and can play a crucial role in building future crop resilience.

Table 1. Plant-derived antimicrobial peptides (AMPs), their functions, and sources (2019–2024)

No	Gene/ Peptide Name	Plant Source	Target Pathogen(s)	Function/ Mechanism	Reference (Year)
1	OsDEF7	*Oryza sativa*	*Magnaporthe oryzae*	Antifungal; disrupts membrane integrity	Jha et al. (2021)
2	CaAMP1	*Capsicum annuum*	*Colletotrichum gloeosporioides*	Inhibits hyphal growth and spore germination	Li et al. (2023)
3	MtDef4	*Medicago truncatula*	*Fusarium oxysporum*	ROS induction; antifungal	Goyal & Mattoo (2021)
4	RsAFP2	*Raphanus sativus*	*Botrytis cinerea*	Targets fungal glucosylceramides	Yuan et al. (2020)
5	Snakin-1	*Solanum tuberosum*	*Erwinia carotovora*, *Fusarium* spp.	Antimicrobial spectrum broad; membrane disruption	Singh, et al. (2022)
6	NbAMP1	*Nicotiana benthamiana*	*Phytophthora infestans*	Induces systemic resistance	Wang et al. (2023)
7	TcDef	*Theobroma cacao*	*Moniliophthora perniciosa*	Inhibits germination and colonization	Santos et al. (2023)
8	AtPDF1.2	*Arabidopsis thaliana*	*Alternaria brassicicola*, *Botrytis cinerea*	JA/ET-pathway regulated peptide; antifungal	Chen et al. (2020)
9	PaAMP	*Persea americana*	*Pseudomonas syringae*, *Phytophthora cinnamomi*	Broad-spectrum antimicrobial	Morales et al. (2022)
10	EgAMP	*Elaeis guineensis*	*Ganoderma boninense*	Induces apoptosis-like cell death in pathogen	Rahman et al. (2023)

Materials and Methods

Open reading frame (ORF) of ZmES4 gene was extracted from NCBI Reference Sequence: NM_001112150.3 of Zea mays female gametophyte-specific protein ES4 precursor (LOC5426230). ORF fragment (276 base pairs) was inserted between NcoI and XhoI restriction enzyme into plasmid pET24d(+) as vector (Figure 1a and 1b). This plasmid pET-24d(+) was provided by Novagen Specialty Limited, (2024). E. coli BL21 (DE3) (Merck KGaA, Darmstadt, Germany) was used as the expression host. Bacterial strains were cultured in Luria-Bertani medium (supplemented with 50 µg/mL kanamycin when required).

Expression of ORF-*ZmES4* in *E. coli* BL21. Following the transformation of *E. coli* BL21 (DE3) competent cells with pET24-ORF-*ZmES4* plasmid by the standard CaCl2 protocol, expression of recombinant ORF-ZmES4 protein was induced by the addition of isopropyl-β-d-thio-galactoside (IPTG) to a final concentration of 1 mM at a bacterial concentration of OD600 = 0.6. Bacterial culture was incubated for 4 h at 37°C in the presence of IPTG on a rotary shaker incubator at 150 rpm. Subsequently, the optical density of bacterial suspensions was measured at 600 nm for each sample right before they were collected by centrifugation. After the washing steps, the pellets were saved at − 70°C for further analyses.

SDS-PAGE analyses. The bacterial pellet was resuspended in an appropriate volume of Laemmli buffer based on their measured OD600 at the time of their collection to normalize the amount of loaded sample (i.e. equalizing their OD measures for the same value) and boiled at 100°C for 10 min and analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To visualize the protein bands, the gel was stained with Coomassie brilliant blue. After image acquisition by a flatbed scanner (Scanjet™ 3800, HP), the yield of expressed recombinant protein was determined by image analysis. To obtain the protein expression yield, the area under the peaks was divided by the total area under the curve.

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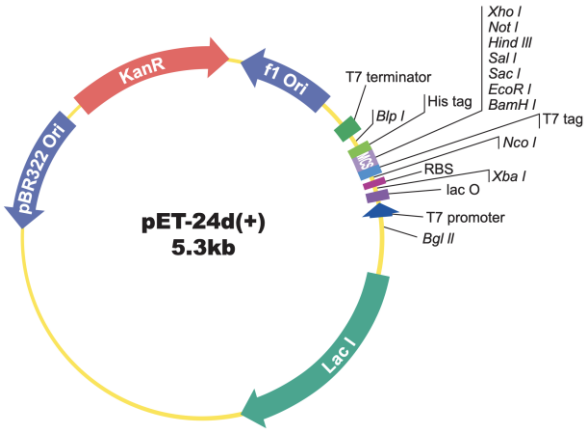

Figure 1a. Plasmid pET24d(+) for inserting ORF of *ZmES4*

Figure 1b. ORF of *ZmES4* inserted in Plasmid pET24d(+)

Results and Discussion

Plasmid Isolation and Quantification. Three mL of cultures were isolated by Tianprep Rapid Mini Plasmid Kit (alkaline lysis methods). It is shown in Table 2. that Colony 1 and Colony 4 are the best colonies and were sent for Single Pass DNA sequencing. Quantification of plasmid was done through Nanodrop-spectrophotometry methods, at A260 nm. Colony 1 and Colony 4 were determined as the best colonies based on the highest plasmid DNA concentration parameters (202.156 ng/µL and 204.555 ng/µL, respectively, as shown in Table 2). This parameter indicates successful transformation, making them the most suitable candidates for further analysis.

Assessment of ORF-ZmES4 expression by SDS-PAGE. To evaluate the ability of bacterial colonies to express the recombinant ORF-ZmES4 protein, several randomly selected kanamycin-resistant colonies of BL21 (after transformation by pET24-ORF-ZmES4 recombinant vector) were induced for protein expression by the addition of IPTG, and the expression level of these colonies was assessed by SDS-PAGE. The result of SDS-PAGE did not show a visible ZmES4 protein band, likely because the protein is a minor component, as shown in Figure 2. Therefore, purification using Ni-Sepharose was necessary to accumulate the band. Colonies 1 and 4 showed higher expression levels of the ORF-ZmES4 protein compared to the other colonies (Table 2).

Table 2. The concentration of the plasmid containing *ZmES4*

No.	Colony	Concentration (ng/µL)
1	pET24d(+)_ZmES4 Colony 1	202.156
2	pET24d(+)_ZmES4 Colony 2	166.306
3	pET24d(+)_ZmES4 Colony 3	161.749
4	pET24d(+)_ZmES4 Colony 4	204.555
5	pET24d(+)_ZmES4 Colony 5	189.393
6	Control	33.069

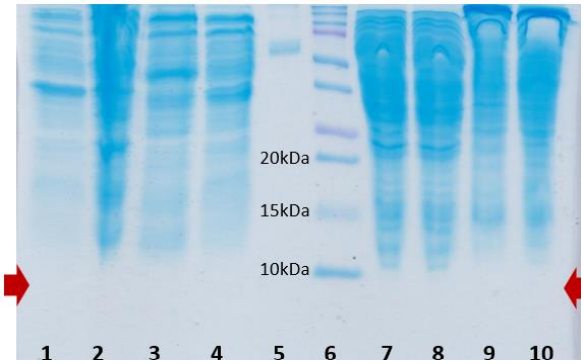

Figure 2. Test Expression Result of ORF-ZmES4 protein

The total protein of ORF-ZmES4 (mg/mL) by Bradford-spectrophotometry methods at A590 nm from ten samples (induced and uninduced) was shown in more detail in Table 3. The protein concentration in the induced sample was lower than that in the uninduced one, likely because the sample contained total protein, not only the target protein.

The maize-derived antimicrobial peptide ZmES4 has gained attention for its role in inhibiting the growth of various plant pathogens, particularly through its cysteine-rich structure that promotes membrane disruption in target microbes. Compared to AMPs from other plants, such as defensins from Arabidopsis thaliana or thionins from wheat, ZmES4 exhibits a unique expression profile in the female gametophyte and has shown effective antifungal activity when heterologously

expressed in microbial systems (Bej et al., 2021). While thionins and defensins tend to be broadly expressed across plant tissues and show general antimicrobial activity, ZmES4 appears to have a more targeted expression and may function in a specialized context, offering opportunities for tissue-specific disease management applications (Kim et al., 2022).

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Table 3. Total protein of the several samples

No	Sample	Total Protein Concentration (mg/mL)*
1	SF *E. coli* BL21(DE3) Induced	14.647
2	SF *E. coli* BL21(DE3) Uninduced	61.710
3	**SF *E. coli* BL21(DE3)-ZmES4 Induced**	**26.857**
4	SF *E. coli* BL21(DE3)-ZmES4 Uninduced	22.299
5	BSA	-
6	Protein Marker	-
7	IB *E. coli* BL21(DE3) Induced	72.808
8	IB *E. coli* BL21(DE3) Uninduced	27.116
9	**IB *E. coli* BL21(DE3)-ZmES4 Induced**	**63.606**
10	IB *E. coli* BL21(DE3)-ZmES4 Uninduced	70.788

Note: SF = Soluble Fraction, IB = Insoluble Fraction

By implementing the expression of antimicrobial peptides from plants, i.e., maize in *E. coli*, researchers can now exploit new tools for producing recombinant proteins for controlling crop diseases research, and applications. Targeted antimicrobial peptide delivery and viral vector modifications are other new areas of interest that can particularly benefit from this technology (Lesch et al., 2010).

The future potential of ZmES4 and other plant-derived AMPs lies in their integration into crop protection strategies via transgenic expression or bioformulation approaches. For example, recent advances in peptide engineering and delivery systems enable the use of synthetic AMPs with enhanced stability and efficacy under field conditions (Roy et al., 2023). Compared to traditional chemical pesticides, AMPs like ZmES4 are environmentally friendly, biodegradable, and pose a lower risk of resistance development in pathogens. As biotic stressors become increasingly complex due to climate change, AMPs offer a versatile and sustainable tool for the next generation of disease-resistant crops.

Conclusions

1. The ORF-ZmES4 protein was successfully produced using the pET24d(+)-ORF-ZmES4 plasmid construct. The total protein concentration ranged from 14.647 to 63.606 mg/mL after induction.
2. Implementing the expression of antimicrobial peptides from plants, i.e., maize in *E. coli*, is a new tool to produce recombinant proteins for future plant pathogen control.

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References

Bej A, Pradhan S, Nayak DK. 2021. Antimicrobial peptides in plant disease management: A review. Plant Gene, 27: 100302.

Chen X, Liu Y, Zhang S. 2020. Plant defensins and their roles in defense against pathogens. Frontiers in Plant Science, 11: 1025.

Cordts S, Bantin J, Wittich PE, Kranz R, Lörz H, Dresselhaus T. 2001. ZmES genes encode peptides with structural homology to defensins and are specifically expressed in the female gametophyte of maize. The Plant Journal, 25: 103–114.

Goyal RK, Mattoo AK. 2021. Multitasking antimicrobial peptides in plant defense. Plants, 10: 555.

Jha S, Roy S, Mandal S. 2021. Characterization of a rice defensin OsDEF7 for resistance against *Magnaporthe oryzae*. Journal of Plant Pathology, 103: 547–555.

Kim JH, Kim YJ, Lee HJ. 2022. Comparative analysis of antimicrobial peptides in crops: Structure, function, and application in agriculture. Frontiers in Agronomy, 4: 874321.

Lesch HP, Kaikkonen MU, Pikkarainen JT, Ylä-Herttuala S. 2010. Avidin-biotin technology in targeted therapy. Expert Opinion on Drug Delivery, 7: 551–564.

Li Y, Liu L, Zhang H. 2022. Plant antimicrobial peptides: Structures, functions, and applications in agriculture. Journal of Integrative Agriculture, 21: 715–728.

Li H, Niu J, Wang X, Niu M, Liao C. 2023. The contribution of antimicrobial peptides to immune cell function: a review of recent advances. Pharmaceutics, 15(9): 2278.

Montesinos E, Bardají E. 2020. Synthetic antimicrobial peptides as agricultural pesticides for plant-disease control. Chemical and Biological Technologies in Agriculture, 7: 1–11.

Morales L, Diaz ML, Gomez-Lopez D. 2022. Avocado-derived antimicrobial peptides against bacterial and oomycete pathogens. Phytopathology Research, 4: 17.

Natarajan B, Patel A, Verma S. 2021. Engineering crops for abiotic stress tolerance and disease resistance using antimicrobial peptides. Biotechnology Advances, 53: 10783.

Novagen Specialty Limited. 2024. Novagen: Renewable Energy MGA. [Internet]. [cited 2025 May 1]. Available from: https://novagenrenewables.com

Pandey P, Irulappan V, Bagavathiannan MV, Senthil-Kumar M. 2021. Impact of combined abiotic and biotic stresses on plant growth and avenues for crop improvement by exploiting physio-morphological traits. Frontiers in Plant Science, 11: 617954.

Rahman AM, Zainal Z, Rosli R. 2023. Functional analysis of Elaeis guineensis AMP gene against Ganoderma boninense. International Journal of Molecular Sciences, 24: 1289.

Roy A, Saha S, Ghosh A. 2023. Recent advances in plant-derived antimicrobial peptides and their potential use in sustainable crop protection. Journal of Agricultural Biotechnology and Sustainable Development, 15: 12–24.

Santos MA, Luz EDMN, Lima M. 2023. TcDef: A cocoa defensin with activity against Moniliophthora perniciosa. Brazilian Journal of Plant Physiology, 35: e2356.

Singh R, Mishra S, Verma P. 2022. Functional validation of Snakin-1 peptide in potato defense. Molecular Biotechnology, 64: 129–138.

Wang L, Xie T, Zhou Y. 2023. Induction of AMP NbAMP1 in tobacco confers late blight resistance. Plant Cell Reports, 42: 777–788.

Yuan S, Li M, Fang Y. 2020. RsAFP2 peptide inhibits Botrytis cinerea in radish. Journal of Agricultural and Food Chemistry, 68: 8223–8231.

Zhu J, Zhang J, Tang Q. 2020. Abiotic stress signaling and responses in plants. Cellular and Molecular Life Sciences, 77: 2931–2951.