

Expression of a novel NaD1 recombinant antimicrobial peptide enhances antifungal and insecticidal activities

1. Signal peptide- RGS(HIS)6- Xa factor- NaD1- CBDa- CBDb- CBDc- CBDd
2. Signal peptide- RGS(HIS)6- Xa factor- NaD1- linker (EAAAK)2- CBDa- CBDb- CBDc- CBDd
3. Signal peptide- RGS(HIS)6- Xa factor- NaD1- linker (EAAAK)4- CBDa- CBDb- CBDc- CBDd
4. Signal peptide- RGS(HIS)6- Xa factor- NaD1- linker (EAAAK)6- CBDa- CBDb- CBDc- CBDd
5. Signal peptide- RGS(HIS)6- Xa factor- CBDa- CBDb- CBDc- CBDd - NaD1
6. Signal peptide- RGS(HIS)6- Xa factor- CBDa- CBDb- CBDc- CBDd - linker (EAAAK)2- NaD1
7. Signal peptide- RGS(HIS)6- Xa factor- CBDa- CBDb- CBDc- CBDd - linker (EAAAK)4- NaD1
8. Signal peptide- RGS(HIS)6- Xa factor- CBDa- CBDb- CBDc- CBDd - linker (EAAAK)6- NaD1

Figure S1. Different modes of recombinant protein to check the appropriate length of the linker for the recombinant protein

Modes 1-4 are structures with different lengths of the EAAAK linker sequence in the mode where chitin-binding domains are in the C-terminal of the NaD1 peptide, Mode 5-8 structures with different lengths of the EAAAK linker sequence in the mode where chitin-binding domains are at the N-terminal of the NaD1 peptide.

Proteins	Without Linker		Linker					
			2 Repeat		4 Repeat		6 Repeat	
	CBD-NaD1	NaD1-CBD	CBD-NaD1	NaD1-CBD	CBD-NaD1	NaD1-CBD	CBD-NaD1	NaD1-CBD
4AML	0.95	0.92	0.92	0.99	0.98	0.95	0.99	0.96
1MR4	0.98	0.99	0.99	0.98	0.98	0.99	0.99	0.98

Table S1. Determining the length of EAAAK linker based on *Modeller* software

Seq. ID.	Class	AMP Probability
NaD1-(CBD)4	AMP	1.000
(CBD) ₄ - NaD1	AMP	1.000
NaD1	AMP	0.978

Table S2. The result of investigating the antimicrobial properties of the NaD1-(CBD)4, NaD1 -(CBD)4 and NaD1 cassettes by the Support Vector Machine (SVM) classifier algorithm

Seq. ID.	Class	AMP Probability
NaD1-(CBD)4	AMP	0.7875
(CBD) ₄ - NaD1	AMP	0.799
NaD1	AMP	0.8935

Table S3. The result of investigating the antimicrobial property of NaD1-(CBD)4, NaD1 -(CBD)4 and NaD1 cassettes by Random Forest Classifier algorithm

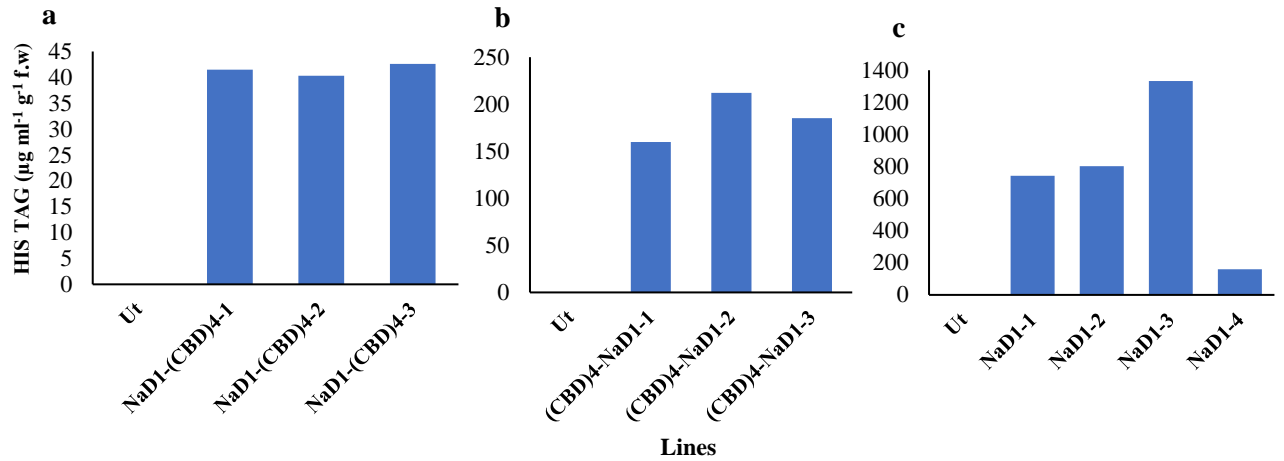
Seq. ID.	Class
NaD1-(CBD)4	AMP
(CBD) ₄ - NaD1	AMP
NaD1	AMP

Table S4. the result of investigating the antimicrobial property of NaD1-(CBD)4, NaD1 -(CBD)4 and NaD1 cassettes by the Artificial Neural Network (ANN) classifier algorithm

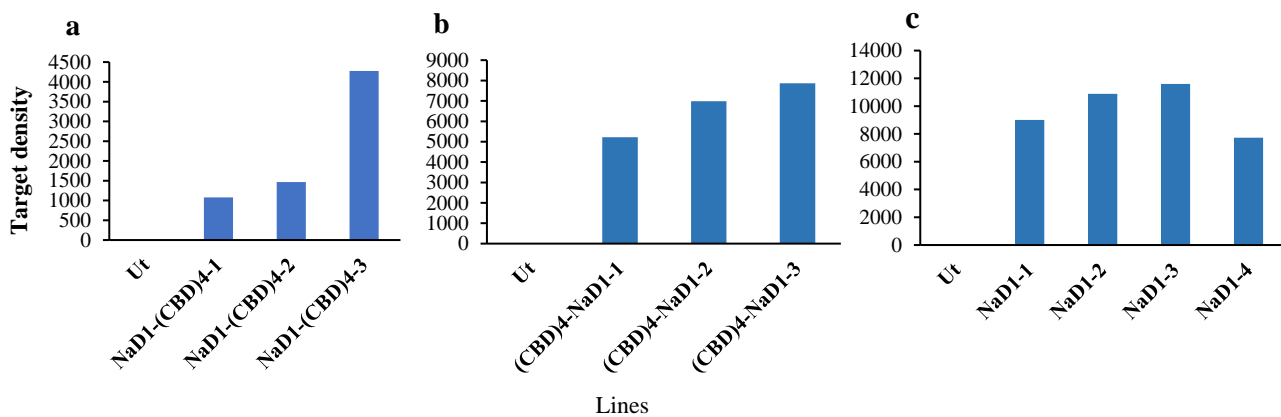
Seq. ID.	Class	AMP Probability
NaD1-(CBD)4	AMP	1.000
(CBD) ₄ - NaD1	AMP	1.000
NaD1	AMP	0.974

Table S5. The result of investigating the antimicrobial property of NaD1-(CBD)4, NaD1 -(CBD)4 and NaD1 cassettes by the Discriminant Analysis classifier algorithm

Step	Temperature (C°)	Time	Cycles
Initial denaturation	95	5 min	
Denaturation	95	45 Sec	35
Annealing	Table 1	30 Sec	
Extension	72	30 Sec	

Table S6. PCR, colony PCR and RT-PCR reaction program for all genes**Figure S2.** Production of the recombinant protein containing Histidine tag sequence, (His)₆ by ELISA test.

The recombinant peptides were identified using a His-Tag Protein ELISA kit (AKR-130 of Cell Biolabs, Inc.). Transgenic HRs (a) expressed (His)₆-NaD1-(CBD)₄; (b) expressed (His)₆- (CBD)₄- NaD1, (c) Transgenic HRs expressed (His)₆-NaD1 peptide. Ut: represents non-transgenic HRs.

**Figure S3.** Quantification of immunoblots using the image j 1.62 software.

The recombinant peptides were identified by utilizing the Anti-His monoclonal antibody sc-8036 (Santa Cruz Biotech). Transgenic HRs (a) expressed (His)₆-NaD1-(CBD)₄; (b) expressed (His)₆- (CBD)₄- NaD1, (c) Transgenic HRs expressed (His)₆-NaD1 peptide. Ut: represents non-transgenic HRs.



Figure S4. Assessment of bacterial contamination by checking *Vir D1* gene from *Agrobacterium rhizogenes*.

Samples 1 to 4 correspond to transgenic HRs with the *NaD1*-*(CBD)₄* cassette, samples 5 to 8 to those with the *(CBD)₄*-*NaD1* cassette, and samples 9 to 12 to those with the *NaD1* cassette. Sample 13 is the control HR, which lacks the gene fragment. The image also displays the negative control and the amplification band of *Vir D1* at 441 bp, serving as the positive control (*Agrobacterium rhizogenes* PCR). Ladder is 100 bp.

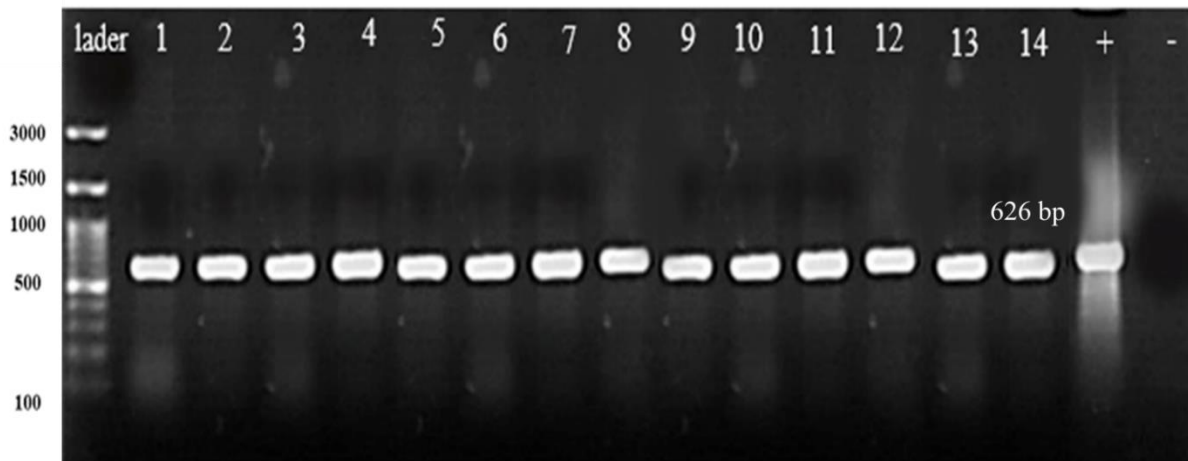


Figure S5. Amplifying of *rol C* gene to confirm the formation of HRs.

Amplifying a 626 bp PCR product of HRs with specific *rol C* gene primers on the 1% agarose gel. Numbers 1 to 5 belong to transgenic HRs containing the *NaD1*-*(CBD)₄* cassette, numbers 6 to 9 belong to transgenic HRs containing *NaD1*-*(CBD)₄* cassette, numbers 10 to 13 belong to transgenic HRs containing *NaD1* cassette, number 14 belong to the control HR (lacking the gene fragment). The end of the negative control image and the *rol C* gene bp fragment 626 amplification band are shown as positive control (*Agrobacterium rhizogenes* PCR). The ladder is 100 bp.

S.O.V	DF	Ms			
		2nd day	5th day	7th day	10th day
Protein extracts	3	485.9**	1620.8**	755.4**	1131.2**
Concentrations	2	817.3**	8.8 n.s	681.5**	470.1**
Protein extracts *Concentrations	6	6.3 n.s	27.3**	9.2 n.s	34.6**
Erorr	24	8.3	3.1	9.4	8.11
Total	35				
CV	9.61				

Table S7. Variance analysis of growth inhibition rate of mycelium (*Pyricularia oryzae*) growing on the PDA medium containing concentrations of 20, 50, and 100 µg/ ml NaD1-(CBD)₄, (CBD)₄-NaD1 and NaD1 and protein extract after 2, 5, 7, and 10 days of culture

. *Significant $\alpha=0.05$ ** Significant $\alpha=0.01$ n.s: Non- Significant

S.O.V	DF	Mean squares
Protein extracts	4	606.116**
Time	2	450.986**
Protein extracts* Time	8	79.129**
Erorr	30	0.068**
Total	44	
CV	3.23	

Table S8. Statistical analysis of antifungal activity of the HR protein extracts, by using the disc diffusion approach against *Pyricularia oryzae*.

*Significant $\alpha=0.05$ ** Significant $\alpha=0.01$ n.s: Non- Significant

S.O.V	DF	Mean squares
Protein treatment	4	7030**
Time	2	2802.222**
Protein treatment* Time	8	471.66667**
Error	30	66.66667
Corrected Total	44	
CV	11.62	

Table S9. Variance analysis of the survival percentage of third instar larvae of *Chilo suppressalis* feeding by the recombinant protein extracts after 72 hours.

*Significant $\alpha=0.05$ ** Significant $\alpha=0.01$ n.s: Non- Significant

S.O.V	Mean squares						
	DF	CAT	POD	APX	SOD	TRY	CTR
Protein treatment	4	0.000011**	112.2**	0.811**	0.000016**	47.88**	28.77**
Error	10	0.0000006	0.017	1.72	0.00000016	1.43	0.64
Total	14						
CV		17.73	14.39	18.6	8.30	12.19	9.88

Table S10 Variance analysis of the activities of CAT, POD, APX, SOD, TRY and CTR enzymes in the third instar larvae of *Chilo suppressalis* feeding by the recombinant protein extracts from tobacco HRs after 72 hours.

*Significant $\alpha=0.05$ ** Significant $\alpha=0.01$ n.s: Non- Significant