PLANT-BASED EXPRESSION OF RECOMBINANT ANTIMICROBIAL PEPTIDES

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Key Message

Using rational design and computational screening, antimicrobial peptides with physicochemical properties suitable for heterologous expression in plants were selected. Generated transgenic tobacco lines represent a platform for further evaluation of recombinant antimicrobial peptide accumulation and antimicrobial activity.

Introduction / Background

Climate change increasingly exposes plants to extreme environmental conditions such as drought and temperature fluctuations. These stresses weaken plant immunity and promote the rapid spread of bacterial and fungal pathogens, significantly increasing disease pressure in agricultural systems. At the same time, the growing demand for biologically active recombinant proteins requires scalable and cost-effective production platforms.

Antimicrobial peptides (AMPs) are small bioactive molecules involved in innate defense mechanisms across many organisms. Due to their cationic and amphipathic properties, AMPs interact with microbial membranes, disrupt cellular integrity, and inhibit pathogen growth.

Advances in plant genetic engineering enable the introduction of genes encoding AMPs into plant genomes, allowing plants to produce recombinant bioactive peptides directly in their tissues. This study investigates the use of transgenic plants as a platform for recombinant AMP production.

Materials and Method

Bioinformatic Modeling and Candidate Selection

Candidate AMPs were selected using the **Antimicrobial Peptide Database (APD)** and included representatives of bacterial, plant, and animal origin to ensure structural diversity. Physicochemical properties, including **GRAVY index and Boman index**, were calculated to predict membrane affinity (**Table 1**). Three-dimensional peptide structures were predicted using **AlphaFold** and visualized in **PyMOL**.

Vector Construction

For constitutive high-level expression, genes encoding selected AMPs were cloned into a binary plant expression vector under the control of the **Cassava Vein Mosaic Virus promoter (CsVMV)** and the **Cauliflower Mosaic Virus 35S terminator (CaMV 35S)**.

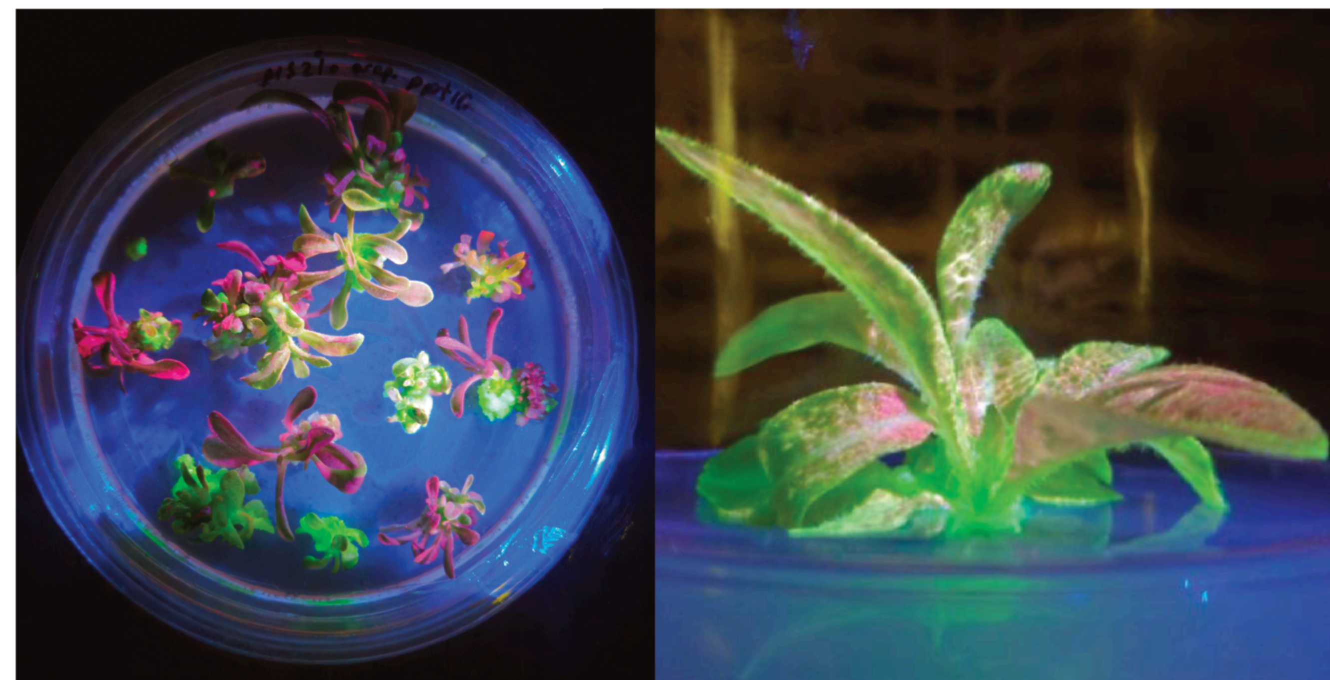

Fig. 2. Screening of transgenic *Nicotiana tabacum* plants grown on MS medium supplemented with phosphinothricin (PPT). ZsGreen fluorescence under 480 nm excitation allows rapid identification of transformed tissues.

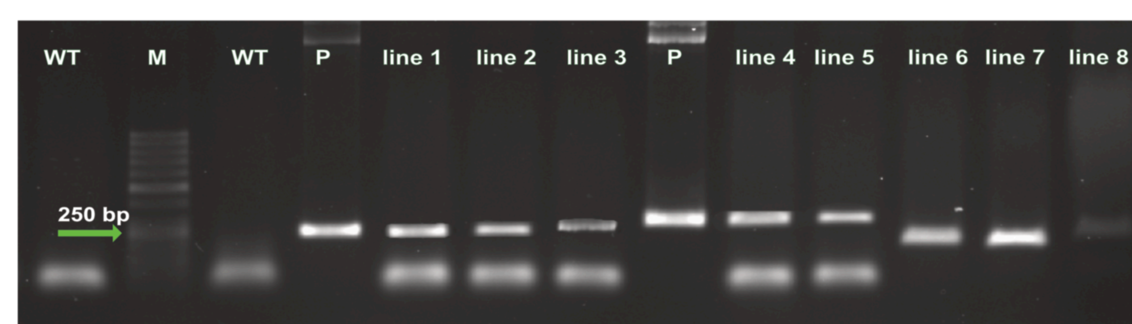

Fig.3. PCR verification of AMP transgene integration in transgenic *Nicotiana tabacum* plants. M – Thermo Scientific GeneRuler 50 bp DNA Ladder; WT – wild type control; P- positive control;lanes 1–X – independent transgenic lines.

Table 1. Panel of antimicrobial peptides (AMPs) used in this study and their predicted physicochemical properties. Amino acid substitutions in modified peptides are highlighted in green.

№	APD ID/name	Source	MW (Da)	Amino acid sequence	Charge	GRAVY	Boman Index (kcal/ mol)	APD hydrophobic ratio (%)	Form
1	AP00293	*Phyllomedusa bicolor*	3353.004	MAMWKDVLKKIGTVALHAGKAALGAVADTISQG	+2,25	0,47	-0,05	55	α-helix
2	AP00308	*Bufo bufo gargarizans*	2566.08	MTRSSRAGLQFPVGRVHRLLRK	+6,25	-0,52	3,08	36	α-helix
3	mod AP00293+ AP00308	modified	5769.87	MAMWKDVLKKIGTVALHAGKAALGAVADTISQGTRSSRAGLQFPVGRVHRLLRK	+8,5	0.037	1.26	46	α-helix
4	AP00479	*Phytolacca americana*	4066.706	MAGCIKNGGRCNASAGPPYCCSSYCFQIAGQSYGVCKNR	+4	-0,18	1,18	38	β-sheet
5	AP01330	*Vitis vinifera*	5486.332	MRTCESQSHRFKGTCVRQSNCAAVCQTEGFHGGNCRGFRRRCFCTKHC	+7,75	-0,74	3,17	35	α-helix + β-sheet
6	mod AP01330	modified	5483.416	MRTCESVSHRFKGTCVRQLNCAAVCQTEGFHGGNCRGFRRRCFCTKHC	+7,75	-0,48	2,8	40	α-helix + β-sheet
7	AP00206	*Bacillus subtilis*	3596.313	MWKSESLCTPGCVTGALQTCFLQTLTCNCKISK	+2	0,24	0,54	45	coil
8	mod AP00206	modified	3931.813	MWKRERLCTPGCVTGALQTCFLQTLTCNCKIRKK	+6	-0.20	1,7	44	α-helical fragment + coil
9	APs.SM1b	synthetic	6732.032	MKWKLFKKIGIGAVLKVLTTGLPALKAKARKEAEAAELAFQEARQREEAVRQLARQLKQA	+8	-0.34	1.69	48	α-helix
10	APs.SM2a	synthetic	7442.46	MSADGKVVIVIDGKMPVDTGAGGTGGGGGGKVGGTSESSAAIHATAKWSTAQLKKTLAE KAARERETAAAMAAAKAK	+4,25	-0,18	1,05	40	α-helix
11	APs.Ai	synthetic	6344.914	MKWKLFKKIGVVLAIVTLKKFGKAVKALAEKAQRQLEDAAKLVKAAIGGLRKKVVKAL	+13	0,25	0,49	55	α-helix

The expression cassette included ***zsgreen*** as a fluorescent reporter and the ***bar* selectable marker gene**, conferring resistance to **phosphinothricin (PPT) (Fig. 1).**

Plasmids were transferred in ***Escherichia coli*** using heat-shock method and subsequently introduced into ***Agrobacterium tumefaciens* strain GV3101** via electroporation.

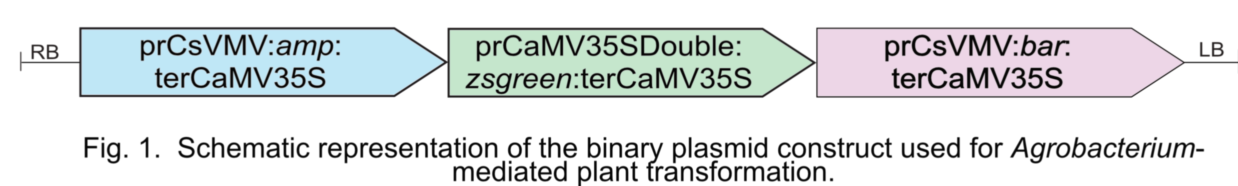

Fig. 1. Schematic representation of the binary plasmid construct used for *Agrobacterium*-mediated plant transformation.

Plant Transformation and Regeneration

Plant transformation was performed using ***Agrobacterium*-mediated transformation of *Nicotiana tabacum***. Leaf explants (1–2 cm²) from six-week-old in vitro plants were co-cultivated with *Agrobacterium* suspension (OD600 = 0.8) in **MS medium supplemented with 200mM acetosyringone**.

After co-cultivation, explants were transferred to **MS regeneration medium containing ceftriaxone to eliminate *Agrobacterium* and phosphinothricin for selection of transformed tissues**. Putative transgenic regenerants were rapidly identified using **ZsGreen fluorescence (480 nm excitation)**. Fluorescent shoots were transferred to rooting medium and subsequently acclimatized in greenhouse conditions (**Fig. 2**). The integration of AMP transgenes in regenerated plants was additionally verified using PCR analysis. Genomic DNA was isolated from putative transgenic *Nicotiana tabacum* plants.

PCR amplification was performed using a universal forward primer targeting the CsVMV promoter and gene-specific reverse primers for each AMP coding sequence. PCR products were analyzed by agarose gel electrophoresis (**Fig. 3**). Additionally, expression of the bar selectable marker protein was verified using phosphinothricin immunostrip assays, which detect the presence of the corresponding protein in plant extracts.

Results

To develop a plant-based platform for recombinant AMP production, we established a multi-stage biotechnological pipeline integrating computational peptide analysis with stable genetic transformation.

Comparative analysis of peptide properties revealed variation in **charge, hydrophobicity, and protein-binding potential** among the analyzed peptides (**Table 1**). Based on these characteristics, peptides with the most favorable predicted antimicrobial properties were selected for further experimental validation.

Predicted peptide structures are shown in **Fig. 4.1–4.9**, where the cartoon model is combined with a semi-transparent surface representation. Hydrophobic residues (Leu, Ile, Val, Phe, Met) are colored **yellow**, positively charged residues (Lys, Arg) **blue**, negatively charged residues (Asp, Glu) **red**, and remaining residues **white**.

To optimize antimicrobial potential, several rational design strategies were applied:

- **Charge enhancement:** In **mod AP00206**, the net charge increased from **+2 to +6**, strengthening electrostatic interactions with bacterial membranes.
- **Hydrophobicity tuning:** In **mod AP01330**, substitutions **Q7V and S17L** increased hydrophobicity while maintaining strong cationic character.
- **Hybrid design:** The peptide **AP00293+AP00308** achieved a near-neutral **GRAVY index (+0.037)**, supporting membrane interaction while reducing potential phytotoxicity.
- **Synthetic peptides:** Three fully synthetic peptides (named **APs.SM1b, APs.SM2a, APs.Ai**) with high cationic charge (**+4.25 to +13**) were included to broaden structural diversity and antimicrobial potential.

Binary expression constructs containing selected AMP genes were successfully generated and used for *Agrobacterium*-mediated transformation of ***Nicotiana tabacum* plants**. For each vector, **multiple independent stable transgenic lines** were obtained, and regenerants were identified based on **ZsGreen fluorescence** (**Fig. 1, bottom left**). Transformation was confirmed using four independent approaches: fluorescence screening, herbicide resistance, PCR detection, and BAR protein immunostrip assays.

These transgenic plant lines provide the basis for further evaluation of peptide accumulation and antimicrobial activity determination.

Discussion / Interpretation

Plant-based systems represent promising platforms for the production of recombinant bioactive proteins and peptides due to their scalability, biosafety advantages, and relatively low production costs. Antimicrobial peptides are particularly attractive candidates for molecular farming because of their small size, biological activity, and potential use in pharmaceutical and agricultural applications.

In this study, we generated a set of binary vectors carrying genes encoding antimicrobial peptides and successfully obtained stable transgenic Nicotiana tabacum lines. The inclusion of the fluorescent reporter ZsGreen enabled rapid visual identification of transformants and may facilitate transformation workflows in plant systems with lower transformation efficiency.

Importantly, the regenerated transgenic plants developed normally and did not exhibit visible phytotoxic effects associated with AMP expression. This observation suggests that the selected peptides are compatible with plant expression systems and can be further evaluated as recombinant bioactive molecules.

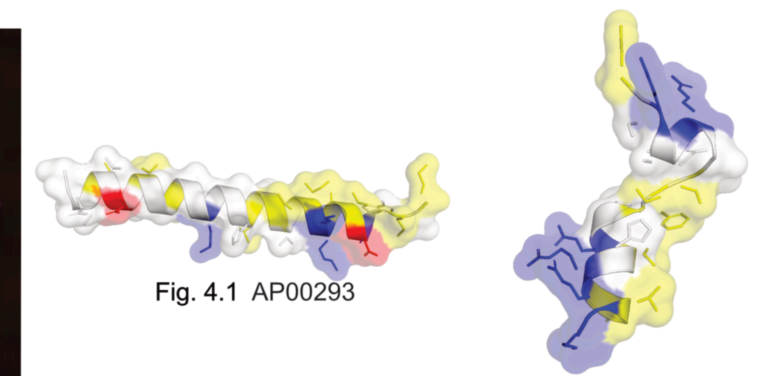

Fig. 4.1 AP00293

Fig. 4.2 AP00308

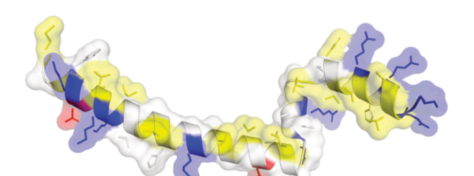

Fig. 4.3 mod AP00293+ AP00308

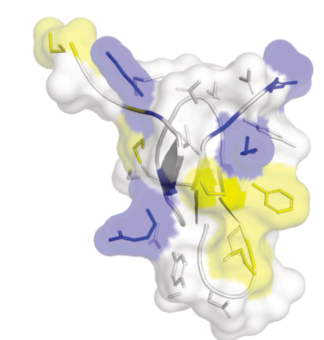

Fig. 4.4 AP00479

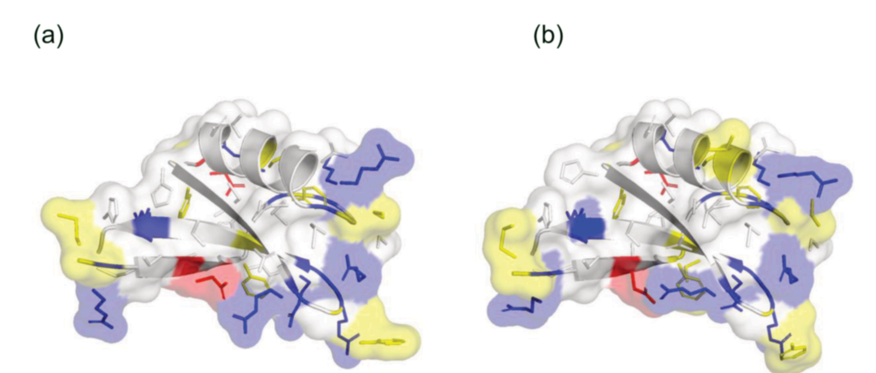

Fig. 4.5 AP01330 and modAP01330

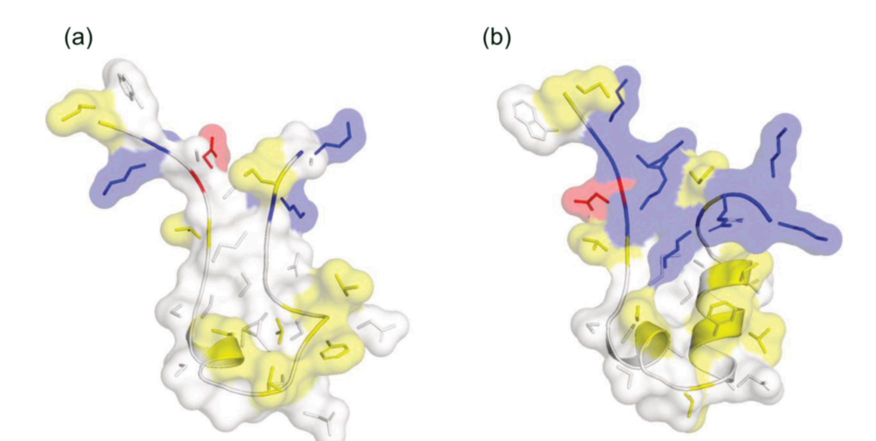

Fig. 4.6 AP00206 (a) and modAP00206 (b)

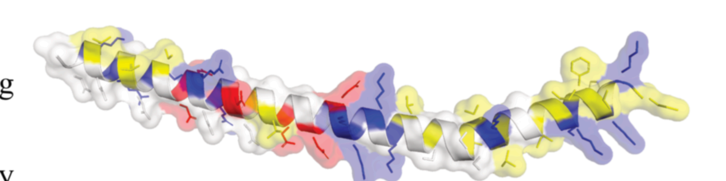

Fig. 4.7 APs.SM1b

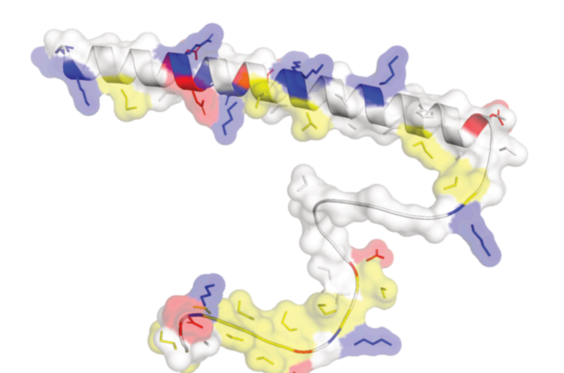

Fig. 4.8 APs.SM2a

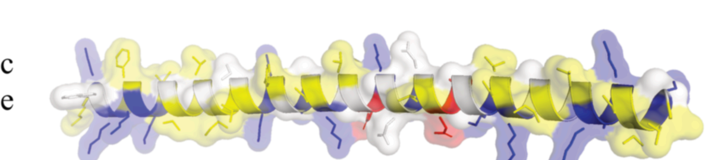

Fig. 4.9 APs.Ai