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Design, construction and cloning of pCAMBIA-MiAMP1 vector for enhancing disease resistance in plants using *Agrobacterium*-mediated transformation

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ABSTRACT
Objective: To design, construct and clone a pCAMBIA-MiAMP1 vector that can be used to enhance disease resistance in plants through *Agrobacterium*-mediated transformation.
Methodology and results: The cDNA sequence encoding MiAMP1 antimicrobial peptide as a defense resistance gene, which had been cloned into the cloning site of pGEM-T T-vector, was obtained from Australia. The pGEM-T-MiAMP1 was transformed into *E.coli* and the identification of transformed clones among antibiotic resistant bacteria carried out by *lacZ* expression using blue/white screening system. The presence of MiAMP1 gene was confirmed using PCR analysis with primers M13, restriction enzymes *Nco*I and *Bam*HI and DNA sequencing. Based on the sequence, the orientation and restriction map of target gene in plasmid was defined. After designing the specific PCR primers flanking the coding region of the MiAMP1 gene and amplifying the complete cDNA with additional restriction sites, either PCR product, or the binary vector pCAMBIA1305.1 were digested with restriction enzymes *Nco*I and *Bgl*II. Subsequently, the insert and the vector were ligated with T4 DNA ligase to produce binary vector pCAMBIA-MiAMP1 for genetic transformation of plants. This construction contains the full coding region of the MiAMP1 antimicrobial peptide and is flanked at its 5' end by the strong constitutive promoter of CaMV35S and at its 3' end by the polyadenylation sequence of NOS polyA. The designed expression vector also contains other elements that are useful for plant transformation such as a kanamycin resistance gene (*npt* II) and a hygromycin resistance gene (*hpt* II) that can be used as markers for selecting transformed plants, while the GUS*Plus*™ reporter gene contains the intron. The presence of MiAMP1 gene in the pCAMBIA-MiAMP1 construction was confirmed among antibiotic resistant colonies by PCR method, restriction enzymes analysis and DNA sequencing. The designed construction was transferred into *Agrobacterium tumefaciens* strain AGL0 by tri-parental mating method with the helper plasmid pRK2013.
Conclusion and application of findings: pCAMBIA-MiAMP1 construction can be used to genetically transform plants to enhance resistance to fungi e.g. *Leptosphaeria maculans* and *Sclerotinia sclerotiorum.* Currently, it is being used in *Agrobacterium-mediated* transformation of canola and sunflower.

Key words: Antimicrobial peptide, *Macadamia integrifolia,* MiAMP1 gene, binary vector, transformation.

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INTRODUCTION

Plant antimicrobial peptides (AMPs) are a large group of low molecular weight and cysteine rich short proteins commonly found in the seeds of many plant species, or are produced in the plants after infection. These peptides usually have broad antimicrobial activity against fungi, bacteria and viruses, and thus form part of the pathogen-defense mechanism in plants. Chemical control of plant diseases has been the method of choice until recently when the functions of AMPs against pathogenic infections were proven, with indications that they can be promising candidates for use in transgenic plants (Terras *et al*., 1994; Broekaert *et al*., 1997; Kazan *et al*., 1999). A number of studies have shown that expression of these defense related proteins enables plants to resist pathogen attacks (Kazan *et al*., 2002; Ponti *et al*., 2003; Yeaman & Yount, 2003; Asiegbu *et al*., 2003; Theis & Stahl, 2004; Bardan *et al*., 2004; Sanjeewani *et al*., 2005; Brogden, 2005; Castro & Fontes, 2005; Gloria *et al*., 2006; Wei *et al*., 2006; Ekramoddoullah *et al*., 2006; Jawahar *et al*., 2008).

Antifungal proteins with direct antimicrobial activity on pathogens are produced in the plants after infection. Expression of an antifungal gene encoding chitinase effectively enhanced the tolerance of transgenic canola to *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* (Grison *et al*., 1996) and *Rhizoctonia solani* (Broglie *et al*., 1991). However, large-scale exposure of transgenic plants expressing defensive proteins to pathogenic fungi may render these proteins less effective. Therefore, novel forms of resistance should be continuously identified for potential deployment in transgenic plants. Once new proteins with antimicrobial properties are identified, genes encoding such proteins can be cloned and expressed in transgenic plants (Kazan *et al*., 1999).

To identify new antifungal peptides that are effective against fungal pathogens of plants, Marcus *et al*. (1997) screened the crude extracts of approximately 250 accessions of Australian native plants, which represent relatively unexplored source of antimicrobial proteins. The survey resulted in identification, purification and cloning of a novel plant antimicrobial protein that was named MiAMP1 (*Macadamia integrifolia* antimicrobial peptide No. 1). The protein was isolated from the kernel of *Macadamia integrifolia* nut and had broad-range antimicrobial activity *in vitro*. MiAMP1 has a molecular mass of 8.1 kDa, is highly basic and its amino acid sequence contains a 26-amino acid signal peptide and a 76-amino acid mature peptide with six disulphide-linked cysteine residues (McManus *et al*., 1999). The structure of MiAMP1 adopts a Greek key β-barrel that is unique amongst plant antimicrobial proteins.

The use of antimicrobial peptides as a novel source of resistance in transgenic plants represents a potentially promising strategy for the management of plant diseases that are difficult to combat otherwise. However, for successful application of antimicrobial genes in engineering of disease resistant transgenic plants, AMPs should be evaluated for certain characteristics. Most importantly, the peptide should be toxic to the pathogen(s) of interest without having any toxicity to plant and mammalian cells. Correct processing and expression of the peptide at high levels in plants are also important for *in planta* antimicrobial activity. MiAMP1 meets these criteria (Kazan *et al*., 1999).

A study on the antimicrobial activity of MiAMP1 demonstrated that it inhibited several major taxonomic groups of microbes *in vitro*, including many plant pathogens. Purified MiAMP1 was active *in vitro* against *S. sclerotiorum* and *L. maculans* when fungal spores or hyphal fragments were incubated in microtitre plates in the presence of varying concentrations of MiAMP1 peptide. In these experiments, MiAMP1 had no apparent effect on the viability of plant and animal cell lines, and thus it could be used in transgenic plants as a novel resistance gene against *L. maculans* and *S. sclerotiorum* (Marcus *et al*., 1997; Kazan *et al*., 1999). Lack of toxicity towards plant and animal cells is particularly important if a protein is to be considered a suitable candidate for the control of pathogens by expression in transgenic food crops and for the design of new fungicides.

MiAMP1 exhibits no sequence homology to known proteins and therefore it represents a new

structural class of plant defense proteins. Despite this lack of sequence homology, MiAMP1 has some general features in common with other antimicrobial proteins. For example, its highly basic nature suggests a mode of action that involves contact with a negatively charged surface such as the plasma membrane (McManus *et al*., 1999).

The antimicrobial peptide MiAMP1 and the yeast killer toxin peptide WmKT from *Williopsis mrakii* are structural homologues. Comparative studies of yeast mutants were performed to test their sensitivity to these two antimicrobial peptides. No differences in susceptibility to MiAMP1 were detected between wild-type and several WmKT-resistant mutant yeast strains. A yeast mutant MT1 that was resistant to MiAMP1 but unaffected in its susceptibility to plant defensins and hydrogen peroxide, also did not show enhanced tolerance towards WmKT. It is therefore probable that the Greek key beta-barrel structure shared by MiAMP1 and WmKT provides a robust structural framework ensuring stability for the two proteins but that the specific action of the peptides depends on other motifs (Stephens *et al*., 2005).

Potentially exciting applications of MiAMP1 are associated with its use as a template for the design of new fungicides and the engineering of disease resistance in plants through expression of transgenes. Therefore, to express MiAMP1 peptides in transgenic plants, a binary vector pCAMBIA-MiAMP1 carrying MiAMP1 cDNA (including the 26 amino acid signal peptide) under the control of CaMV 35S promoter was constructed, to transform plants using an *Agrobacterium tumefaciens* mediated transformation technique.

MATERIALS AND METHODS

Bacterial strains and plasmids: *E.coli* strain DH10B was obtained from ABRC (USA). Cloning vector pGEM-T [Promega] containing target gene MiAMP1 (GenBank Accession # Y10903) was kindly provided from The CRC for Tropical Plant Protection, The University of Queensland, Australia. Binary vector pCAMBIA1305.1 was procured from CAMBIA vectors, Canberra, Australia. *A. tumefaciens* strain AGL0 containing plasmid pT1 Bo542 was used for transformation [Lazo *et al*., 1991], including helper plasmid pRK2013.

Enzymes and primers: All molecular biology enzymes and kits, antibiotics and PCR reactions were procured from Sigma Aldrich Chemicals, Fermentas, Sileks, DiaM, Syntol, SibEnzyme, Gosti Genetika and Helicon Pvt. Ltd. The experiments used primer M13 (5'-gtaaaacgacggccagt-3'), and designed specific primers Mia1 (5'- agatctgccatggcttccaccaagtt-gttc-3'), Mia2 (5'- gcagatctacgcattggatgaagatactcttc-3'), PCAM1 (5'-cttcgcaagacccttcctctat-3'), PCAM2, VirB1 (5'-ggctacatcgaagatcgta-tgaatg-3') and VirB2 (5'-gactatagcgatggttacgatgttgac-3').

Culture media: The *E.coli* strain was grown at 37°C and *Agrobacterium* at 28°C in liquid LB (Luria-Bertani) [5g/l Bacto-yeast extract, 10g/l Bacto-tryptone, 5g/l NaCl] and YEB (yeast extract) [5g/l Bacto-yeast extract, 5g/l peptone, 2 mM $MgSO_4.7H_2O$, 15g/l agar (Bacto-agar "DIFCO", USA)] media. pH was adjust to 7.0 before use.

Vector construction: The cDNA sequence encoding antimicrobial peptide MiAMP1 had been subcloned between restriction sites *Sac*II and *Spe*I of vector pGEM-T according to the standard T/A-cloning protocol. Starting from this construction, the MiAMP1 orientation in the plasmid and its restriction sites were first defined. Then subcloned vector pGEM-T-MiAMP1 was engineered by PCR mutation using the pair of primers Mia1 and Mia2, which efficiently amplified the MiAMP1 gene. The released target gene was used for designing and construction of expressin vector pCAMBIA-MiAMP1. The vector pCAMBIA1305.1 and the amplified gene were digested with restriction enzymes *Nco*I and *Bgl*II and further the digested DNAs were purified with GlassMilk gel extraction kit (Sileks Co.). The insert and the vector were ligated with T4 DNA ligase and the ligation was performed at 22°C overnight as per the standard protocol. Ligation was confirmed by PCR using the vector specific primers PCAM1 and PCAM2, and also by DNA sequencing.

Transformation: The constructed vector pCAMBIA-MiAMP1 was transformed into *E.coli* maintenance host DH10B by calcium chloride precipitation heat shock method. The next day, five random colonies were picked among antibiotic resistant colonies, and the DNA plasmid isolated. The presence of MiAMP1 gene in the pCAMBIA-MiAMP1 construction was confirmed by PCR with the specific primers Mia1, Mia2, PCAM1 and PCAM2, restriction enzymes *Spe*I and *Bam*HI, *Bst*EII and *Bam*HI, *Nco*I and *Bgl*II and also DNA sequencing. The transfer of pCAMBIA-MiAMP1 construction with the helper plasmid pRK2013 into *Agrobacterium* strain AGL0 was carried out by tri-

parental mating method. The transformed cells cultured on YEB agar medium at 28°C. The presence of *vir*-genes in transformed strain of *Agrobacterium* was defined using PCR-analysis of DNA with specific primers VirB1 and VirB2.

RESULTS AND DISCUSSION

The cDNA sequence encoding antimicrobial peptide MiAMP1 had been subcloned between restriction sites *Sac*II and *Spe*I of cloning vector pGEM-T. The pGEM-T-MiAMP1 was transformed into *E.coli* strain DH10B and the identification of desired clones among countless transformed and ampicillin resistant bacteria carried out by *lacZ* expression for β-galactosidase using blue/white screening system. White colonies on LB medium containing 50mg/l ampicillin supplemented with x-gal and IPTG reagents indicated bacteria transformed with pGEM-T-MiAMP1. Therefore, the presence of MiAMP1 gene amongst white colonies was checked and confirmed using PCR analysis with primers M13 (fig. 1a), restriction enzemes *Nco*I and *Bam*HI (fig. 1b) and DNA sequencing. Based on the sequence, MiAMP1 orientation in plasmid and its restriction map was defined (fig. 2).

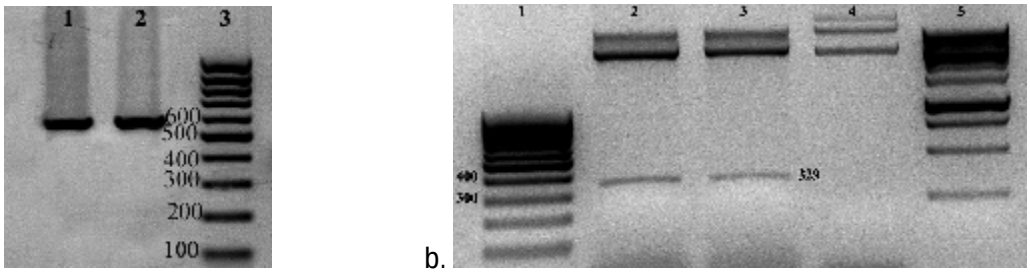


Figure 1: Confirmation of MiAMP1 gene in the pGEM-T-MiAMP1 using: a) PCR with primers M13. Lanes 1, 2: amplified colonies of pGEM-T-MiAMP1; lane 3: 100 bp ladder size marker; and b) digestion with restriction enzymes *Nco*I and *Bam*HI. Lane 1: 100 bp ladder size marker; lanes 2, 3: digested DNA colonies of pGEM-T-MiAMP1; lane 4: non-digested colony of pGEM-T-MiAMP1; lane 5: 1 kb ladder size marker.

For genetic transformation of plants, MiAMP1 gene was subcloned between restriction sites *Nco*I and *Bgl*II of binary vector pCAMBIA1305.1. For this purpose the following scheme was used: after defining the orientation of MiAMP1 gene in the vector pGEM-T and determination of its restriction map, specific primers Mia1 and Mia2 were designed, complementary with the gene MiAMP1 and also for creation of restriction sites *Nco*I and *Bgl*II at the beginning and the end of gene MiAMP1 (fig. 4), complementary with restriction sites of the binary vector pCAMBIA1305.1. Primer design is the single largest variable in PCR applications and the single most important factor in determining the success or failure of PCR reactions. To digest effectively, certain restriction enzymes require a greater number of bases surrounding the restriction site than are included in the design of the primers. To ensure efficient cleavage when using the enzymes, additional bases were added to the 5' ends of Mia1 and Mia2 primers. PCR conditions were then optimized for designed primers and the inclusion of gene MiAMP1 in the vector pGEM-T was defined using PCR analysis. The reaction produced a single major fragment of 325 bp (fig. 3).

For aligning the codon arrangement of DNA sequences of MiAMP1 gene with the amino acid frameworks, two nucleotides (GT) were added to the end of target gene before restriction site *Bgl*II. Also for expression of GUSPlus reporter gene together with the gene MiAMP, the stop-codon "tag" was removed from the end of MiAMP1 (fig. 4).

Figure 2: The restriction map of MiAMP1 gene in the vector pGEM-T for definition of gene orientation in plasmid and analysis of restriction sites at the beginning and the end of the target gene.

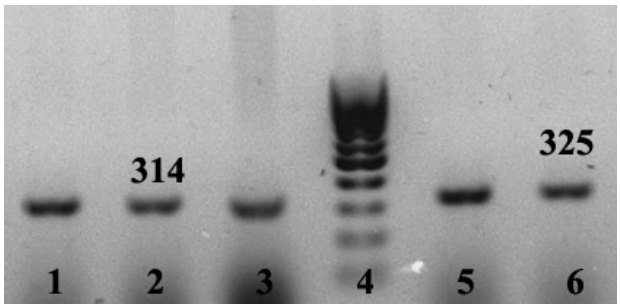


Figure 3: PCR product of pGEM-T-MiAMP1 construction with the specific primers Mia1 and Mia2; lanes 5, 6: purified and non-restricted DNA of amplified segment; lane 4: 100 bp ladder size marker; lanes 1-3: purified and restricted DNA of amplified segment with the endonucleases *Nco*I and *Bgl*II.

5'- AGA TCT GCC ATG GCT TCC ACC AAG TTG TTC TTC TCT GTC ATT ACT GTG ATG ATG CTC ATA GCA ATG GCA AGT GAG ATG GTG AAT GGG AGT GCA TTT ACA GTA TGG AGT GGT CCA GGT TGT AAC AAC CGT GCT GAG CGA TAT AGC AAG TGT GGA TGC TCA GCT ATA CAT CAG AAG GGA GGC TAT GAC TTC AGC TAC ACT GGA CAA ACT GCT GCT CTC TAC AAC CAG GCT GGA TGC AGT GGT GTT GCA CAC ACC AGG TTT GGG TCC AGT GCC AGG GCA TGC AAC CCT TTT GGT TGG AAG AGT ATC TTC ATC CAA TGC GTA GAT CTG C -3'

Figure 4: Nucleotide sequence of PCR product of pGEM-T-MiAMP1 using specific primers Mia1 and Mia2 (*Bgl*II - *Nco*I - MiAMP1 gene - *Bgl*II).

After digesting the amplified and purified fragments of MiAMP1 (325 bp) and binary vector pCAMBIA1305.1 (11846 bp) with the endonucleases *Nco*I and *Bgl*II, and extraction and purification from 1.5% agarose/ethidium bromide gel, they were ligated using T4 DNA ligase to construct the binary vector pCAMBIA-MiAMP1 (12153 bp) (fig. 5). The designed vector contains the coding region of gene MiAMP1

between CAMV35S promoter and terminator of cauliflower mosaic virus, providing constitutive expression of the MiAMP1 gene in plants, as well as other regulator sequences and elements of the binary vector pCAMBIA1305.1. The constructed vector was transformed into *E.coli* strain DH10B for cloning.

The presence of MiAMP1 gene in the vector pCAMBIA-MiAMP1 in kanamycin resistant colonies was confirmed by restriction analysis method, using restriction enzymes *Bst*EII and *Bam*HI (fig. 6a), *Spe*I and *Bam*HI (fig. 6b), and PCR analysis with the specific primers Mia1 and Mia2 (fig. 7).

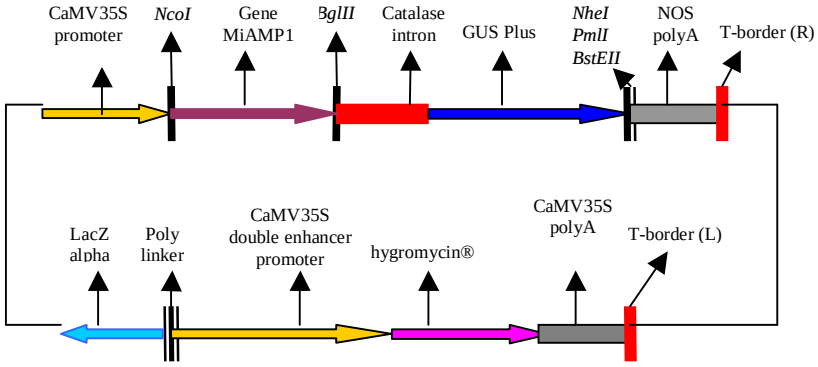


Figure 5: Diagram of the designed expression vector pCAMBIA- MiAMP1 (12153 bp), bearing *Macadamia integrifolia* antimicrobial peptide gene that is applicable in genetic transformation of plants.

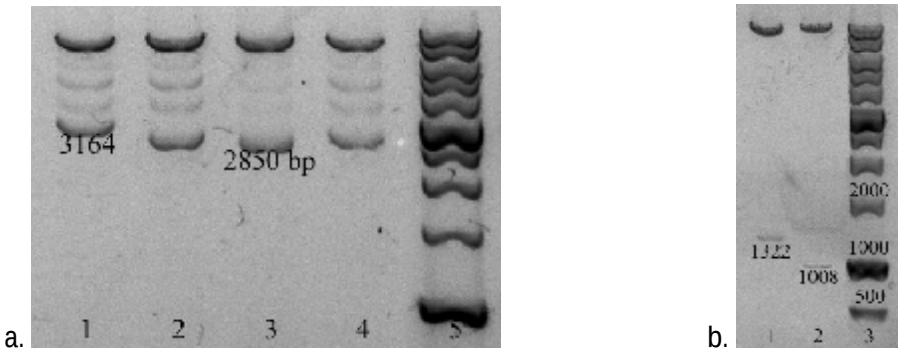


Figure 6: Confirmation of the presence of MiAMP1 gene in the designed vector pCAMBIA-MiAMP1 using: a) restriction enzymes *Bst*EII and *Bam*HI; lane 1: restricted DNA of pCAMBIA-MiAMP1; lanes 2-4: restricted DNA of pCAMBIA1305.1; lane 5: 1 kb ladder size marker; and b) restriction enzymes *Spe*I and *Bam*HI; lane 1: restricted DNA of pCAMBIA-MiAMP1; lane 2: restricted DNA of pCAMBIA1305.1; lane 3: 1 kb ladder size marker.

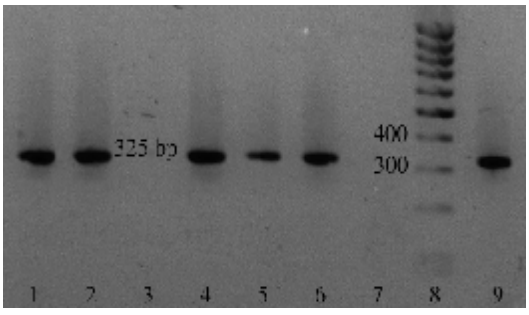
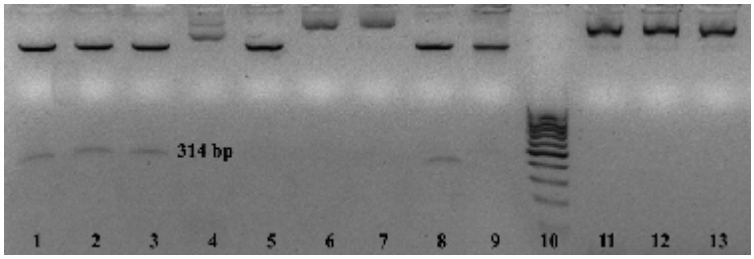


Figure 7: Amplification of the MiAMP1 gene in the designed vector pCAMBIA-MiAMP1 using specific primers Mia1 and Mia2; a 15 µl sample of PCR reaction was analyzed on a 1.5% agarose/ethidium bromide gel. Lanes 1-6: amplified colonies of pCAMBIA-MiAMP1. The MiAMP1 gene is in lanes 1, 2, 4, 5 and 6; lane 7: negative control (pCAMBIA1305.1); lane 8: 100bp ladder size marker; lane 9: positive control (amplified colony of pGEM-T-MiAMP1).

Based on results, a binary vector containing MiAMP1 gene was designed and constructed that is suitable for genetic transformation of plants. One of the clones was sequenced to confirm that the correct sequence was amplified and cloned. The designed vector pCAMBIA-MiAMP1 was transformed into *A. tumefaciens* strain AGL0, containing pBR322ori transfer system by tri-parental mating method with the helper plasmid pRK2013. After DNA extraction of selected plasmids among resistant agrobacterial colonies to rifampicin and kanamycin, containing vector pCAMBIA-MiAMP1, the presence of MiAMP1 gene was checked in the construction by restriction enzyme analysis (fig. 8).

a. 

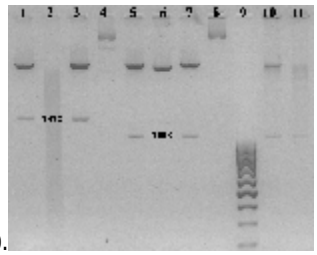
b. 

Figure 8: Selection of rifampicin and kanamycin resistant *Agrobacterium tumefasciens* colonies, containing vector pCAMBIA-MiAMP1 and determination of MiAMP1 gene in the vector using: a) restriction enzymes *Nco*I and *Bgl*II; lanes 1-9: digested plasmid DNAs of *Agrobacterium* colonies, transformed with pCAMBIA-MiAMP1. The 324 bp specific insert was revealed in the lanes 1-3 and 8; lane 10: 100 bp ladder size marker; lanes 11-13: plasmid DNAs of non-digested colonies; and b) restriction enzymes *Spe*I and *Bam*HI; lanes 1-7: digested plasmid DNAs of *Agrobacterium* colonies, transformed with pCAMBIA-MiAMP1. The gene MiAMP1 is in lanes 1 and 3; lane 8: plasmid DNA of non-digested colony of pCAMBIA-MiAMP1; lane 9: 100 bp ladder size marker; lanes 10, 11: digested DNA of vector pCAMBIA1305.1.

After confirmation of MiAMP1 gene in the *Agrobacterium* strain AGL0, specific primers PCAM1, complementary with CaMV 35S promoter, and PCAM2, complementary with a region of catalase intron site, were used for direct orientation of MiAMP1 in the binary vector pCAMBIA1305.1 which was used for DNA sequencing (fig. 9).

The MiAMP1 amplified fragment was sequenced. Nucleotide analysis of sequenced fragment showed that it fully coincided with the cDNA sequence encoding MiAMP1 antimicrobial peptide and had no mutation or deletion. Also for confirmation the presence of *vir*-genes in transformed *Agrobacterium* strain AGL0 specific primers VirB1 and VirB2 were designed, complementary with the *vir*-regions of *Agrobacterium* (fig. 10).

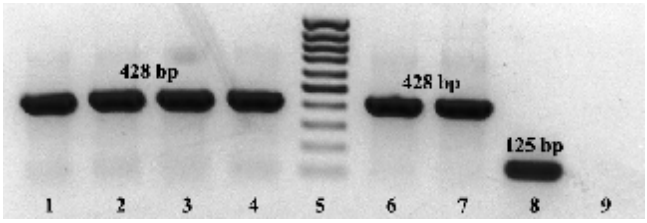


Figure 9: Agarose gel electrophoresis of MiAMP1 fragment in the *Agrobacterium* strain AGL0, containing the pCAMBIA-MiAMP1 construction using PCR analysis with specific primers PCAM1 and PCAM2; lanes 1-4: *Agrobacterium* strain AGL0, containing the pCAMBIA-MiAMP1; lane 5: 100 bp ladder size marker; lanes 6, 7: positive control (*E.coli* strain DH10B, containing the pCAMBIA-MiAMP1); lane 8: negative control (vector pCAMBIA1305.1); lane 9: negative control (a control that replaces the DNA template with PCR-grade distilled H_2O but still includes the primers).

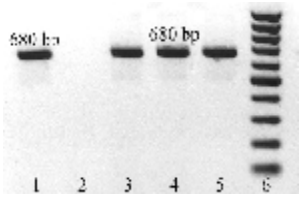


Figure 10: Confirmation of *vir*-genes in the *Agrobacterium* strain AGL0, containing vector pCAMBIA-MiAMP1 using PCR analysis with the specific primers VirB1 and VirB2; lane 1: *Agrobacterium* strain AGL0; lane 2: negative control (*E.coli* strain DH10B, containing pCAMBIA-MiAMP1); lanes 3, 4: *Agrobacterium* strain AGL0, containing pCAMBIA-MiAMP1; lane 5: positive control (DNA of VIR$^+$); lane 6: 100 bp ladder size marker.

Based on the received cDNA of antimicrobial peptide MiAMP1, the binary vector pCAMBIA-MiAMP1 was designed and constructed, carrying the complete coding region of MiAMP1 gene including the 26 amino acid signal peptide under the control of the strong constitutive CaMV35S promoter of cauliflower mosaic virus at its 5' end and the polyadenylation sequence of NOS polyA at its 3' end. The vector also contains other elements that are useful for plant transformation such as kanamycin resistance gene (*npt* II) as a marker for selection in bacteria, a hygromycin resistance gene (*hpt* II)) as a marker for selection in plants and also the reporter gene GUS*Plus*™ which contains the intron. The presence of MiAMP1 gene in pCAMBIA-MiAMP1 construction was confirmed among antibiotic resistant colonies by PCR, restriction enzyme analysis and DNA sequencing. The designed vector construct was successfully transferred into the *A. tumefaciens* strain AGL0 by tri-parental mating method with the helper plasmid pRK2013.

pCAMBIA-MiAMP1 is a new construction that can be used for genetic transformation of plants to enhance resistance to fungal pathogens, especially to *L. maculans* and *S. sclerotiorum*.. Currently, it is being utilized to carry out *Agrobacterium-mediated* transformation of canola and sunflower.

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