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PHYSIOLOGY AND BIOCHEMISTRY

Characterization and expression of an nsLTPs-like antimicrobial protein gene from motherwort (*Leonurus japonicus*)

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Abstract In screening for potent antimicrobial proteins (AMPs) from plant seeds, we had purified a heat-stable AMP, LJAMP2, from the seeds of a medicine herb, motherwort (*Leonurus japonicus* Houtt). In an in vitro assay, the protein can inhibit the growth of both fungi and bacteria. Then a cDNA encoding LJAMP2 was cloned by the rapid amplification of cDNA ends based on the N-terminal amino acid sequence determined. The deduced amino acid sequences of this cDNA show similarity to plant non-specific lipid transfer proteins. Northern blotting assay revealed that this nsLTP-like gene, designated *LJAMP2*, was expressed in seeds. Overexpression of *LJAMP2* in tobacco enhanced resistance to the fungal pathogen *Alternaria alternata* and the bacterial pathogen *Ralstonia solanacearum*, significantly, while no visible alteration in plant growth and development. Our data confirm the antifungal and antibacterial function of *LJAMP2* from motherwort seeds and suggest the potential of *LJAMP2* in improving disease resistance in plants.

Keywords Expression · Disease resistance · Antimicrobial protein · Phylopenetic analysis · Transgenic tobacco

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Introduction

Phytopathogens cause enormous losses in cultivated and stored crops worldwide and thereby threaten human sustenance (Oard and Enright 2006; Osusky et al. 2000). Since cultural practices, agrochemicals, and conventional breeding for disease resistance have not been sufficient, or permanently successful in keeping phytopathogens under control, alternative strategies for sustainable agriculture have attracted great attention during recent years (Langen et al. 2006). Plant genetic engineering in combination with antimicrobial proteins (AMPs) is regarded as an effective means (Gao et al. 2006; Oard and Enright 2006).

An increasing amount of defense-related genes have been characterized in some plant–pathogen systems (Lee et al. 2002). Proteins encoded by these genes are usually pathogenesis-related proteins or low-molecular-weight AMPs that may play an important role in the growth of pathogens in infected hosts. In screening of AMPs from plants, we have discovered an AMP, termed LJAMP2, in motherwort seeds (Yang et al. 2006). The partial N-terminal sequence determined has shown that the sequence revealed similarity with known plant non-specific lipid transfer protein (nsLTPs) sequences in data bank.

Plant nsLTPs, reported in various organs and tissues in many mono- and dicotyledonous species, form a multigene family of basic protein with 91–95 amino acid residues in their primary structure and a approximately 9 kDa molecular mass (Blein et al. 2002; Jung et al. 2005). They share several structural features, e.g. eight strictly conserved cysteine residues forming four disulfide bridges, which are responsible for the nsLTPs' compact folding (Blein et al. 2002). NsLTPs, as a member of plant AMPs, hold an internal hydrophobic cavity, which serves as the binding site for lipid-like molecule in structure (Carvalho and

Gomes 2007; Lin et al. 2005; Samuel et al. 2002). The structural, biochemical, and physiological data have confirmed that nsLTPs can bind not only lipids but also sterol molecules (Wang et al. 2007). Furthermore, these nsLTP/sterol complexes may interact with receptors at plant plasma membranes to trigger plant defense responses (Blein et al. 2002; Carvalho and Gomes 2007; Cheng et al. 2004). Evidence is accumulating that nsLTPs or nsLTP-like proteins display roles in plant defense against viral, bacterial, and fungal pathogens (Gomes et al. 2003; Maldonado et al. 2002; Molina and Garcia-Olmedo 1997; Park et al. 2002; Terras et al. 1995). However, it was also found that not all nsLTPs possess antimicrobial properties and accordingly defensive roles (Cammue et al. 1995). No antimicrobial activity was detected for Ta-nsLTP from *Triticum aestivum* seeds in the test at high concentration (200 μg/ml) (Cammue et al. 1995).

In previous work, we had purified a heat-stable AMP, termed LJAMP2, from seeds of motherwort (*Leonurus japonicus* Houtt), a medicine herb. In vitro experiments showed that this protein possesses inhibition activity on the growth of both fungi and bacteria (Yang et al. 2006). To further investigate the function of LJAMP2, in this paper, we cloned a cDNA encoding *LJAMP2* from motherwort, and validated its antimicrobial roles by challenging the transgenic tobacco with a fungal pathogen, *Alternaria alternata* and a bacterial pathogen, *Ralstonia solanacearum*, respectively.

Materials and methods

Plants and microbes

Motherwort (*L. japonicus* Houtt) seeds were obtained from a local medicine herb market, and plants were kept under natural condition. Tobacco plants (*Nicotiana tabacum* var. Xanthi) and its transgenic lines were grown in a greenhouse at 26–28°C under natural and additional artificial light (14/10 h photoperiod at 150 μmol m^{-2} s^{-1}). *A. alternata* and *R. solanacearum* were prepared as previously described (Peng et al. 2004).

Isolation of LJAMP2 cDNA and Northern blotting

Based on the N-terminal amino acid sequence of mature LJAMP2 (Yang et al. 2006), a degenerate primer, 5′-GCA ATA GGT TG(T/C) AA(T/C) AC(A/G/C/T) GT(A/G/C/T) GC-3′, was designed to amplify the cDNA using the rapid amplification of cDNA ends (3′-RACE) method with a 3′ full RACE kit (Takara Bio., Japan). Amplified DNA was purified and ligated into pGEM-T (Promega, WI, USA) plasmid for sequencing. To amplify the 5′-flanking sequence of the gene, a Y-shaped adaptor-dependent extension (YADE) method (Xiao et al. 2002) was employed. According to the nucleotide sequence of the 3′ RACE products, two nested primers (primer 1: 5′-GTAA GGAATGCTGACACCAC-3′ and primer 2: 5′-AGTACG ACTTGGCAAGCGTT-3′) were designed for the amplification of 5′ part sequence of the gene. The YADE product was purified and cloned into pGEM-T (Promega, WI, USA) for sequencing. The products of YADE and the 3′ RACE sequence were overlapped with SeqMan program of DNAStar (DNASTAR, Madison, WI, USA), and the contig sequence was further used to perform similarity search with BLASTX program to determine the putative initiator ATG. The coding region of *LJAMP2* gene was further amplified from the genomic DNA using the primers of 5′-TCCAATGGCTGCCTTGATCA-3′ and 5′-TCAGCCGGA GTACGACTTGGCAA-3′. The protein sequence of LJAMP2 was deduced using the Editseq program of DNASTAR software (DNASTAR, Madison, WI, USA) and it was aligned with 9 nsLTPs sequences from other plant species (http://www.ncbi.nlm.nih.gov/blast/) using ClustalW (v. 1.83) program provided online by DDBJ (http://clustalw.ddbj.nig.ac.jp) and GENEDOC computer programs. Analysis of posttranslational modifications was done using the tools and software packages in ExPASy provided online by the Swiss Institute of Bioinformatics (http://ca.expasy.org/) (Blom et al. 2004). The three-dimensional (3D) structure of LJAMP2 was simulated using the software package SWISS-MODEL provided online by SIB (Schwede et al. 2003).

Total RNAs were isolated from various motherwort tissues and tobacco leaves using a guanidinium thiocyanate method (Logemann et al. 1987). mRNA expression in motherwort tissues and tobacco were assessed by hybridizing a multiple tissue Northern blotting (containing 2 μg of polyadenylated RNA per lane) with a ^{32}P-labeled *LJAMP2* cDNA probe (Sambrook and Russell 2001).

Vector construction and plant transformation

The cDNA fragment encoding *LJAMP2* was cloned downstream of cauliflower mosaic virus (CaMV) 35S promoter. The *nos* transcription terminator was placed downstream. Then the expression cassette with the CaMV 35S promoter-*LJAMP2*-nos terminator and CaMV 35S promoter-*gus-nos* terminator and *nos* promoter-*npt* II-*nos* terminator were cloned in the binary vector pBIN19 (Frisch et al. 1995). The construct was delivered to *Agrobacterium tumefaciens* LBA4404 by freeze-thawing method (Hoekema et al. 1983) and the resulting *Agrobacterium* strain was used for transformation of tobacco leaf-disk by co-cultivation (Li et al.

1992). The transgenic plants were regenerated under kanamycin selection (100 mg/l, Sigma). GUS histochemical analysis was preformed as previously described (Jefferson et al. 1987)

Analyses on transgenic tobacco

For proteins analysis, after incubation at 26°C in a rotary shaker (170 rpm) for 3 days, the germinating tobacco seeds were grown in sterile soil in multi-well plastic containers that were kept in a growth chamber at 80% relative humidity, 25–28°C, under a 14/10 h photoperiod at 150 μmol m^{-2} s^{-1}. Fresh leaf tissues (10 g) of tobacco were homogenized with 10 ml of 20 mM sodium phosphate buffer (pH 6.2), containing 50 mM KCl, 5 mM EDTA, 1 mM aprotinin and 20 mM thiourea. To identify the transgene products, the samples were analyzed by SDS-PADE, and HPLC on a Source 5RPC column (4.6 × 150 mm, 15 μm, Amersham Biosciences, Sweden) equilibrated with 0.05% TFA using ÄKTA Explorer 10S (Amersham Biosciences, Sweden) with 0.05% TFA over 5 min, and a linear gradient of 0–45% acetonitrile in 0.05% TFA over 35 min at a flow rate of 1.0 ml/min.

To test the resistance of transgenic tobacco to infection by *A. alternata*, the bioassay was performed. *A. alternata* strain was grown on potato dextrose agar (PDA) at room temperature for 2–3 weeks. Hyphae, fragmented and combined with spores were harvested in sterile tap water with 0.05% (v/v) Tween 80. Phytopathogenic fungus inoculation of tobacco was carried out by placing 20 μl of the aqueous on the leaves of 4-week-old plants. The inoculated plants were placed in a growth chamber at 90% relative humidity and 28°C for 3 days. Then the inoculated plants were further incubated at 80% relative humidity and 28°C for 7 days. The development of symptoms was monitored and a five-class disease-severity-scale (DS) was evaluated by the previously described method (Yang et al. 2007). Disease resistance was expressed using a disease-index (DI). The DI was calculated using the following formula: DI (%) = ($\Sigma i \times j/4 \times n$) × 100, where i is the DS-class, j is the number of disease leaves in each class, and n is the total number of leaves. The experiments were repeated three times, and each replicate contained 18 plants.

To determine whether the overexpression of *LJAMP2* in tobacco can provide resistance to the bacterial pathogen, we infected the tobacco plants with a *R. solanacearum* isolate, which causes bacterial wilt disease on tobacco. The bacterium was grown for 48 h at 30°C on Luria–Bertani medium. The bacterial suspension for inoculation was prepared by washing the medium surface with sterile tap water, and the bacterial population was adjusted to 1×10^7 bacteria/ml. Tobacco plants (4 weeks old) were inoculated by dipping the roots into the bacterial suspensions. The plants were kept in a growth chamber at 95% relative humidity and 28°C for 2 days and then at 80% relative humidity and 28°C for 12 days. The number of wilting leaves was recorded for each plant daily, and a five-class DS was calculated by the previously described method (Cary et al. 2000). Each treatment was replicated three times, and each replicate contained 20 plants.

All data were analyzed by t test at $P \leq 0.05$ using the software of Origin v6 (OriginLab Co. MA, USA).

Results

Molecular cloning and analysis of the gene encoding LJAMP2

Based on the N-terminal amino acid sequence of LJAMP2 purified from motherwort seeds (Yang et al. 2006), a

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1    GACCTCCATGCTCACAAAACACCACACTCACAACAACTAAGAACCACTTTCATTTCCTAC
61   ACATAAATCAAAATTATCTTTCATCCATCCAATGGCTGCCTTGATCAAGTTGATGTGCAC
                              M  A  A  L  I  K  L  M  C  T

121  AATGCTGATCGTGGCGGCGGTGGTTGCTCCGCTGGCTGAGGCGGCGATAGGGTGCAACAC
41     M  L  I  V  A  A  V  V  A  P  L  A  E  A  A  I  G  C  N  T

181  GGTGGCTTCCAAGATGGCCCCATGTCTACCGTACGTCACCGGAAAAGGGCCGCTCGGCGG
61     V  A  S  K  M  A  P  C  L  P  Y  V  T  G  K  G  P  L  G  G

241  GTGCTGCGGTGGCGTAAAGGGTCTCATCGACGCCGCACGGACCACGCCGGATAGGCAGGC
81     C  C  G  G  V  K  G  L  I  D  A  A  R  T  T  P  D  R  Q  A

301  GGTTTGCAACTGCCTGAAAACGCTTGCCAAGTCGTACTCCGGCATCAACCTCGGCAACGC
101    V  C  N  C  L  K  T  L  A  K  S  Y  S  G  I  N  L  G  N  A

361  CGCCGGACTCCCCGGCAAATGTGGTGTCAGCATTCCTTACCAGATCAGCCCTAATACTGA
121    A  G  L  P  G  K  C  G  V  S  I  P  Y  Q  I  S  P  N  T  D

421  TTGCTCAAAGGTGCACTGAGCTAAAGTTCGATGGGAAGAGGAACTTCCTACAAGGCTACA
141    C  S  K  V  H  *

481  ATGATAATTAATTTACTTTCAGTAGTACTATGAAATAAAAGGAGGCCTTTTAAGACCTTT
541  TGAGTATCTTTCCAATGCTTTCTTTTGCAATGGATGTAGCAATATTTGCATTATCTTCAA
601  TGAAAGCGACGTTCGTCTCCAAAAAAAAAAAAAAA
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Fig. 1 Nucleotide sequence and deduced amino acid sequence of *LJAMP2* cDNA clone. The sequence corresponding to the determined N-terminal amino acid sequence of mature LJAMP2 is *italic*. The deduced signal peptide sequence is *bold*. The initiation codon (ATG) is *bold underlined*, and the poly (A) tail is *underlined*

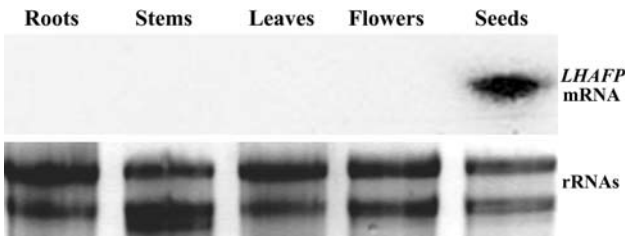

Fig. 2 RNA gel-blot analysis of the *LJAMP2* transcripts in different tissues of motherwort. Total RNAs were extracted from leaves, stems, flowers, roots of 5-week-old plants, and from the seeds at the 30th day after anthesis. RNAs electrophoresed in a 1% agarose gel containing 6% formaldehyde were hybridized with a ^{32}P-labeled *LJAMP2* cDNA probe. Hybridizing signals were visualized by exposing the membrane to X-ray film (*top*). The gel was stained with ethidium bromide to detect rRNAs as control (*bottom*)

degenerate primer corresponding to the sequence of AI-GCNTVA was designed for amplification of the 3′ cDNA. The nucleotide sequence of the fragment was determined and the N-terminal of the deduced amino acid sequence matched perfectly with the N-terminal amino acid sequence of LJAMP2 previously determined. To extend the 5′ part of the cDNA, two new primers were designed, and the YADE method (Xiao et al. 2002) was employed. The full-length cDNA of *LJAMP2* was constructed from the nucleotide sequences of the 5′ and 3′ parts. The gene contains a 345-bp open reading frame coding for 115 amino acids and a 3′ untranslated region of 181-bp up to the poly (A) tail (Fig. 1). The C-terminal portion of the deduced protein from the 25th to the 85th residue was identical to the amino acid sequence determined for the LJAMP2 protein. The first 24 residues had characteristic features of a signal peptide. The mature form of LJAMP2 is composed of 91 residues. According to the cDNA sequence, a genome DNA fragment was generated by PCR. By Comparing the PCR product sequence with the cDNA sequence, we found that the gene encoding LJAMP2, named *LJAMP2*, is an intronless gene. To determine *LJAMP2* transcript accumulations in various organs of motherwort, Northern blotting analysis was performed by using the cDNA probe and total RNA isolated from roots, stems, leaves, flowers and seeds. The hybridization signal was strong in seeds, but was not detected in roots, stems, leaves and flowers (Fig. 2), implying that *LJAMP2* is a seed-specific expression gene in motherwort.

The amino acid sequence of deduced mature LJAMP2 shows similarity to the family of plant nsLTPs-like proteins by comparing with the previously sequenced proteins from other plant species in the BLAST search (http://www.ncbi.nlm.nih.gov/BLAST), sharing 76% identity to a LTP from *Avicennia marina*, 70% identity to the LTP from *Sesamum indicum* (sesame), 67% to *Salvia miltiorrhiza*, 63% to *N. tabacum* (common tobacco) and *Oryza sativa*, and 61% to *Zea mays*, respectively. Thirty-three amino acids conserved residues, including eight cysteine residues, are present in all regions of the proteins (Fig. 3). Analyses of their evolutionary relationship indicated that the LJAMP2 from *L. japonicus* (Labiatae) has a close evolutionary relationship to nsLTPs from *A. marina* (Verbenaceae) and *S. indicum* (Pedaliaceae).

Analyses of posttranslational modifications using software programs in ExPASy provided online by (SIB)

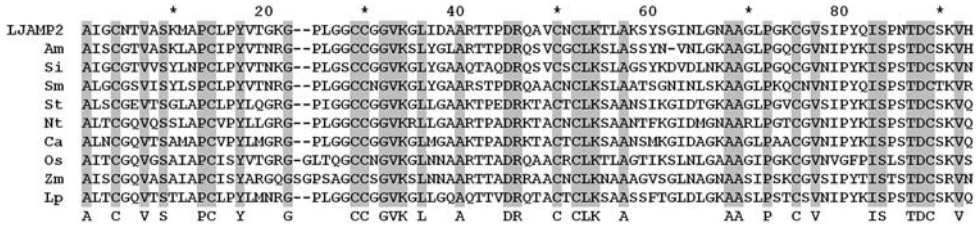

Fig. 3 Multiple sequence alignment of the deduced amino acid sequence of LJAMP2 with other nsLTPs from plants, available in the data banks. Protein sequences were aligned with the ClustalW algorithm. Gene bank numbers corresponding to these sequences are as follows: *Am A. marina* (AAK01293), *Ca C. annuum* (AAX20049), *Lp Lycopersicon pennellii* (AAB07487), *Nt N. tabacum* (AAM74206), *Os O. sativa* (EAY79716), *Si S. indicum* (ABQ53933) *Sm S. miltiorrhiza* (ABP01768), *Zm Z. mays* (A31779). The last line is the conserved amino acids residues

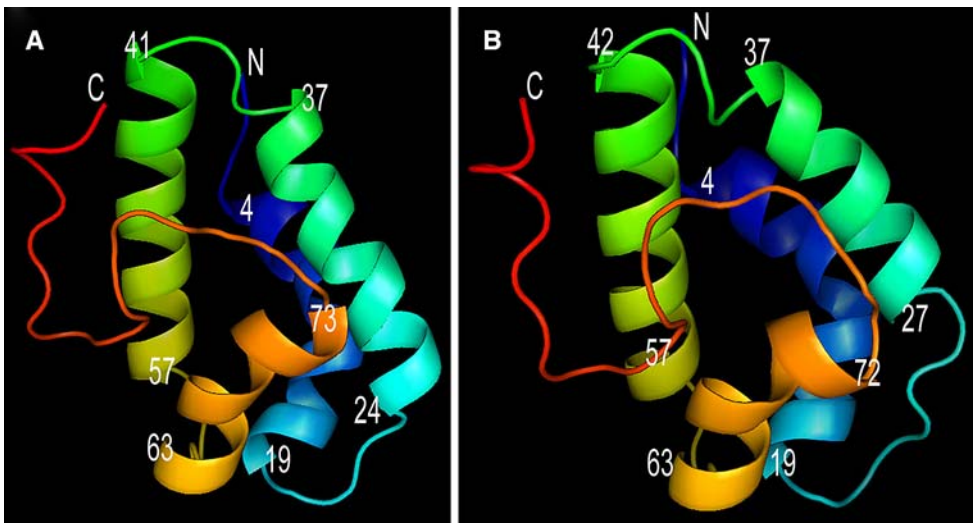

Fig. 4 The three-dimensional structure of LJAMP2 simulated using SWISS-MODEL software package. The structure was drawn with PYMOL software package (http://www.pymol.org/). **a**, LJAMP2; **b**, NLTP1 from tobacco (accession number: Q42952). *C* C-terminal; *N* N-terminal. *Numbers* indicate the sequence number of amino acid residues

indicated that five potential phosphorylation sites lie in LJAMP2 at positions Y17, T19, 40, 41, and S57 of the mature protein. Using the software package SWISS-MODEL provided online by SIB (Schwede et al. 2003), the 3D structure of LJAMP2 (Fig. 4a) was simulated. The 3D model suggests a global fold similar to the structure of NLTP1 (Fig. 4b) from tobacco (accession number: Q42952) (Da Silva et al. 2005), including an internal cavity and four large helixes involving residues Cys4-Thr19 (H1), Leu24 to Ala37 (H2), Thr41 to Ser57 (H3), and Leu63 to Cys73 (H4). The four helixes were stabilized by four disulfide bridges between the eight conserved cysteine residues (residue 4 with 50, 14 with 27, 28 with 73, 48 with 87, respectively).

Overexpression of *LJAMP2* in tobacco

To express *LJAMP2* in tobacco, a transgene expression plasmid was constructed for constitutive expression (Frisch et al. 1995), in which the LJAMP2 coding region was driven by a CaMV 35S promoter. The *npt* II and *gus* were used as the selectable marker gene and the reporter gene, respectively (Fig. 5a). This plasmid was introduced into tobacco via an *Agrobacterium*-mediated transformation method. As a control (Ct), tobacco plants were transformed with the empty vector. Kanamycin-resistant calli were regenerated and the T0 plants were screened by GUS histochemical analysis (Jefferson et al. 1987). The GUS-positive and kanamycin-resistant plantlets were grown in a greenhouse. Transgenic plants were self-pollinated to produce the second generation (T2).

We randomly selected GUS-expressing plantlets for subsequet molecular analyses. Hybridization with an *LJAMP2* cDNA probe indicated that the level of *LJAMP2* mRNA varied in the independent transgenic plants (Fig. 5b). A strong hybridization signal was seen in the transgenic line L5, a moderate signal in line L4, and weak signals in the lines L2 and L17. To detect the translation levels of *LJAMP2* in transgenic tobacco plants, the protein extracts from the leaves of all transgenic lines were subjected to HPLC, and the profiles were compared with that of the purified LJAMP2. The LJAMP2-specific peak, which displayed inhibition activity against *R. solanacearum* (data not shown), was detected in the lines L5 and L4, while this peak was not found in the line L2 (data not shown), L17 and Ct (Fig. 5c).

Disease resistance of transgenic tobacco

To assess the disease resistance of the transgenic tobacco, transgenic lines (T2), L2, L4, L5, L17, Ct (transformed with the empty vector), and wild-type tobacco (Wt) were challenged by the fungal pathogen, *A. alternata*. The resistance levels were evaluated by DI shown in Fig. 6a. Transgenic

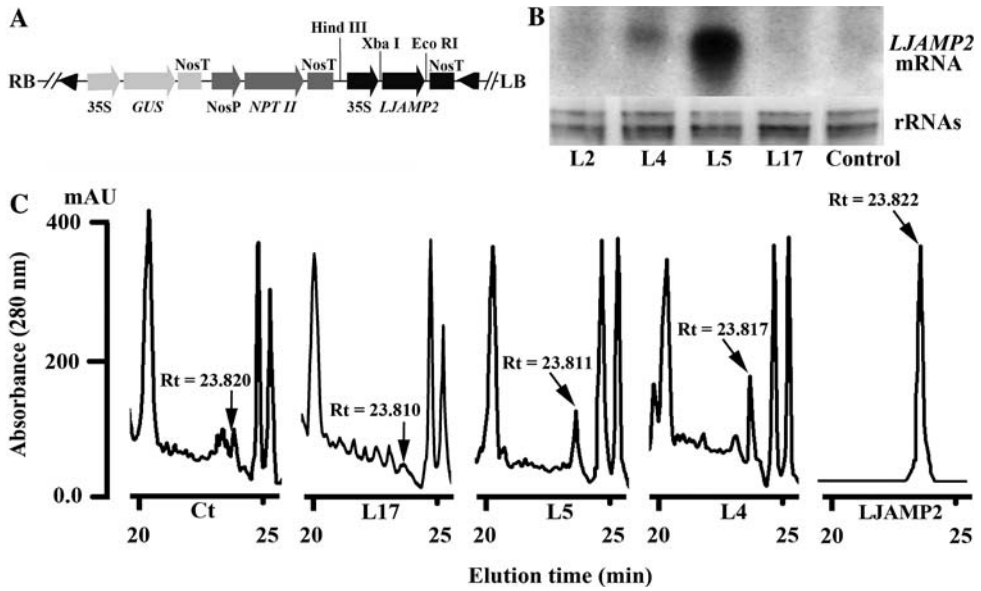

Fig. 5 The expression analysis of *LJAMP2* in transgenic tobacco seedlings. **a** Construct of plant expression vector pBIN-*LJAMP2*. *RB* right border, *NosP* nos promoter, *NPT II* neomycin phosphotransferase II, *NosT* nos terminator, *35S* cauliflower mosaic virus 35S promoter, *GUS* β-glucuronidase, *LB* left border. **b** RNA gel blot analysis of *LJAMP2* transcripts in four independent tobacco T1 transgenic lines and the control. Total RNA was prepared from leaves of transgenic plants. RNA samples were separated by denaturing formaldehyde-agarose gel electrophoresis, blotted, and hybridized with a ^{32}P-labeled *LJAMP2* cDNA probe. Hybridizing signals were visualized by exposing the membrane to X-ray film (*top*). The gel was stained with ethidium bromide to detect rRNAs as control (*bottom*). **c** HPLC profiles of proteins from transgenic lines control (*Ct*), L17, L5, L4, and the purified LJAMP2. The samples were analyzed by HPLC on a Source 5RPC column (4.6 × 150 mm, 15 μm) using ÄKTA Explorer 10S system. *Arrows* indicate the position of detected LJAMP2 peak. *Rt* retention time (min)

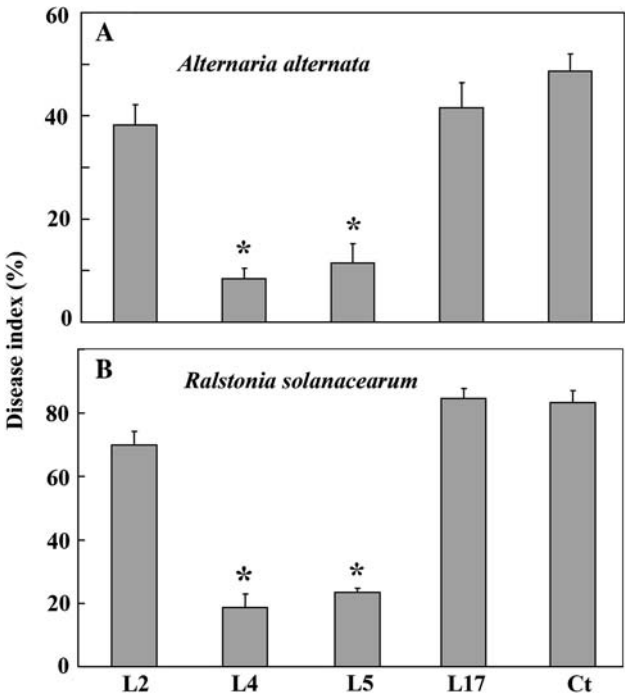

Fig. 6 Resistance of transgenic T2 tobacco plants inoculated with the fungal pathogen *A. alternata* and the bacterial pathogen *R. solanacearum*. **a** Fungal infection assays of tobacco plants holding *LJAMP2*. Mean values of disease index (%) estimated from three independent infection assays (18 plants per line) are shown. **b** Bacterial infection assays of tobacco transformed with *LJAMP2*. Mean values of disease index (%) estimated from three independent infection assays (20 plants per line) are shown. *Asterisks* indicate that the DI in transgenic lines are significantly different ($P \leq 0.05$ by t test) compared with that in the control. *Error bars* indicate standard deviation

lines L4 and L5 displayed higher disease resistance than L2, L17, and Ct, and the difference was statistically significant. The L2, L17, and Ct lines demonstrated increased disease resistance in comparison with that of Wt, but no significant difference among them was observed. When challenged, the tobacco plants with the bacterial pathogen, *R. solanacearum*, the results were very similar to that observed in that of *A. alternata* (Fig. 6b). The lines L4 and L5 showed significant enhanced resistance to *R. solanacearum*, while the resistance of lines L2 and L17 exhibited similarity with that of line Ct, which showed no significant difference in comparison with Wt. Furthermore, no visible phenotype alteration was observed in all transgenic tobacco (Fig. 7), suggesting that over-expression *of LJAMP2* might not bring about morphological changes in plants.

Discussion

In the previous work on screening for potent AMPs from plant seeds, we have isolated a novel heat-stable nsLTP-like AMP, LJAMP2, from motherwort (Yang et al. 2006). In the present study, cloning and characterization of this protein (LJAMP2) have further confirmed that it belongs to a member of plant nsLTP-like proteins. The deduced amino acid sequence of *LJAMP2* possesses a number of characteristics common to all other plant nsLTPs, such as low molecular mass, basic p*I* value, eight cysteine residues at the conserved positions and an N-terminal signal peptide. Analysis of the deduced amino acid sequence of *LJAMP2* indicates that the alanine residue at position 24, where the A-X(E)-A motif is located, is most likely cleaved to become a mature protein, which corresponds to the determined N-terminal sequence of LJAMP2.

It has been reported that the expression of nsLTP genes in various plants was different and most of these genes were temporally and spatially controlled (Gausing 1994; Molina et al. 1993). Expression of the LJAMP2 gene also appears to be organ-specific. *LJAMP2* gene transcripts are detected in seeds but are undetected in roots, stems, leaves and flowers of motherwort, indicating that the LJAMP2 protein may function mainly in the resistance of seeds against pathogens.

NsLTP's role in defense is mainly based on the antimicrobial activities that they exert in vitro and in planta (Jung et al. 2005; Jung et al. 2003; Molina and Garcia-Olmedo 1997). For instance, the overexpression of the barley LTP2 protein under the control of a constitutive promoter in tobacco and *Arabidopsis* enhanced resistance to *Pseudomonas syringae* pv. *tabaci* and *P. syringae* pv. *tomato*, respectively (Molina and Garcia-Olmedo 1997). Furthermore, expression of nsLTP-like AMP, *Ace*-AMP1, from onion in scented geranium shows transgenic plants have increased resistance to both fungal and bacterial pathogens (Bi et al. 1999; Patkar and Chattoo 2006). Considerable evidence suggests that the nsLTPs have a defensive role in the resistance of plants. The results reported in the previous work demonstrate in vitro, a potent inhibition of fungal and bacterial growth exerted by LJAMP2 (Yang et al. 2006).

To confirm the antimicrobial function of *LJAMP2* in planta, we let the gene overexpression in transgenic tobacco. Bioassays for fungal and bacterial pathogens indicated that transgenic tobacco plants had increased resistance to *A. alternata* and *R. solanacearum* (Figs. 6, 7). This evidence strongly supports the role of LJAMP2 in plant defense. *LJAMP2* could be a good candidate for transgenic overexpression in plants. One interesting finding is that the level of *LJAMP2* mRNA was inconsistent with the level of LJAMP2 protein found in the lines L4 and L5. This discrepancy may be explained as a result of transgenic suppression, which often was observed in plants, when the insertion of a transgene resulted in suppression (Cao et al. 1998; Stam et al. 1997). Here, we speculate that in L5 line, suppression takes place at the level of translation by an unknown mechanism. In addition, clearly, transgenic tobacco lines L4 and L5 differed in their levels of *LJAMP2* mRNA transcription and LJAMP2 protein expression, but

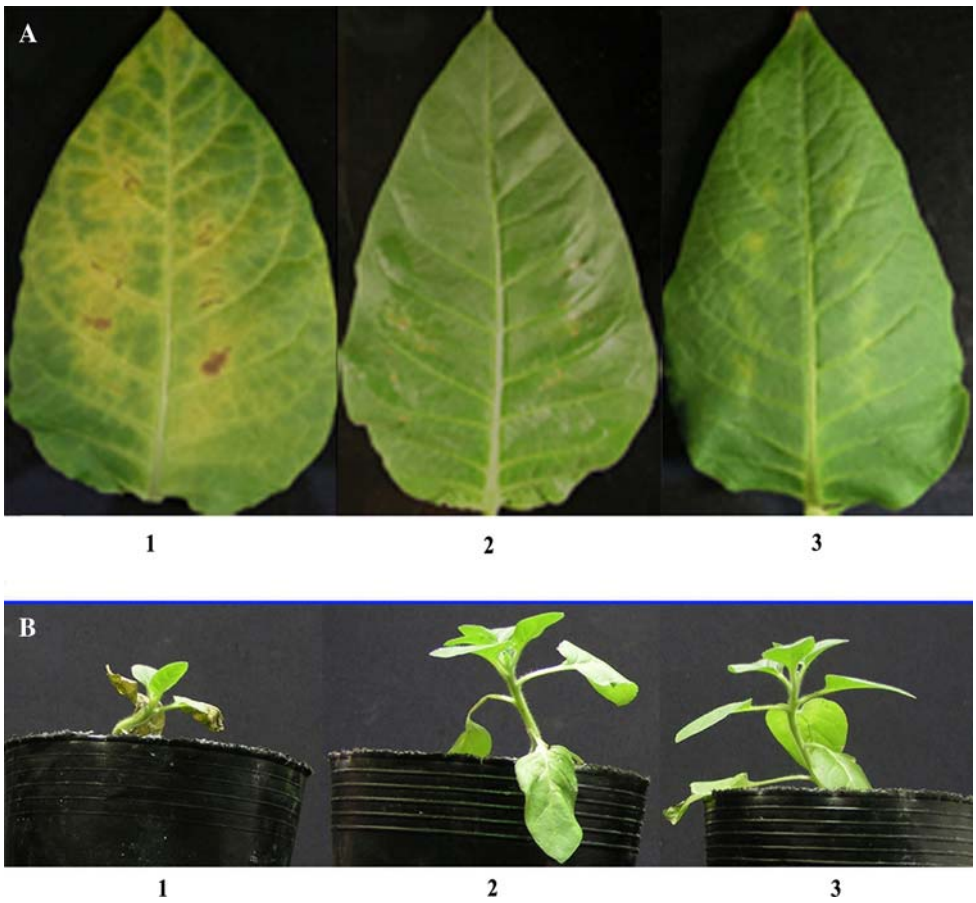

Fig. 7 Phenotype of transgenic and control tobacco plants infected with *A. alternata* and with *P. solanacearum*, respectively. **a** Tobacco leaves infected with *A. alternata* were photographed at 10 days after inoculation. **b** Tobacco plants were infected with *P. solanacearum* by dipping roots and photographed at 12 days after infected. L4 line was used as the representative plants to show the resistance against *P. solanacearum*. 1, Wild-type tobacco infected with *P. solanacearum*; 2, transgenic line L4, infected with *P. solanacearum*; 3, wild-type tobacco (uninfected)

the disease resistance was no significant difference. Instability and limitation of the transgenic protein may account for this phenomenon.

In conclusion, LJAMP2 cloned from motherwort exerted significant homology to nsLTPs-like protein from plants. The *LJAMP2* transgenic tobacco exhibited resistance to the bacterial pathogen, *R. solanacearum*, as well as the hyphomycete fungus, *A. alternata*. Furthermore, no visible changes were found in transgenic tobacco, implying that the over-expression of *LJAMP2* does not alter the plant growth and development. These results suggest that the role of LJAMP2 in motherwort seeds is against phytopathogens and *LJAMP2* has potential application in plant protection by gene engineering.

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