Research article

Expression of *Pinellia ternata* leaf agglutinin under rolC promoter confers resistance against a phytophagous sap sucking aphid, *Myzus persicae*

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ABSTRACT

Background: Piercing/sucking insect pests in the order Hemiptera causes substantial crop losses by removing photoassimilates and transmitting viruses to their host plants. Cloning and heterologous expression of plant-derived insect resistance genes is a promising approach to control aphids and other sap-sucking insect pests. While expression from the constitutive 35S promoter provides broad protection, the phloem-specific rolC promoter provides better defense against sap sucking insects. The selection of plant-derived insect resistance genes for expression in crop species will minimize bio-safety concerns.

Results: Pinellia ternata leaf agglutinin gene (*pta*), encodes an insecticidal lectin, was isolated and cloned under the 35S and rolC promoters in the pGA482 plant transformation vector for *Agrobacterium*-mediated tobacco transformation. Integration and expression of the transgene was validated by Southern blotting and qRT-PCR, respectively. Insect bioassays data of transgenic tobacco plants showed that expression of *pta* under rolC promoter caused 100% aphid mortality and reduced aphid fecundity up to 70% in transgenic tobacco line LRP-9. These results highlight the better effectivity of *pta* under rolC promoter to control phloem feeders, aphids.

Conclusions: These findings suggested the potential of PTA against aphids and other sap sucking insect pests. Evaluation of gene in tobacco under two different promoters; 35S constitutive promoter and rolC phloem-specific promoter could be successfully use for other crop plants particularly in cotton. Development of transgenic cotton plants using plant-derived insecticidal, PTA, would be key step towards commercialization of environmentally safe insect-resistant crops.

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1. Introduction

Agricultural productivity meets the demand of a growing world population and influences the economy of all countries [1]. Annually, more than 10,000 species of insect pests cause 18–20% yield loss worldwide [2]. Aphids are the largest group of Hemiptera, having about 5000 species. It is the most economically significant and geographically distributed group of sap-sucking insect pests in the world [3]. The green peach aphid (*Myzus persicae*) is a diverse, common species that is responsible for worldwide losses of numerous crop species, including cotton, maize, soybean, potato, pea, and wheat [4,5]. Plant damage is

directly related to aphid density and the duration of its infestation [6]. Aphids drain the phloem sap that results the depletion of nutrients and sap in phloem tissues. Additionally, *M. persicae* causes indirect damage in plants by transmission of viral diseases [7]. It is a vector for more than 100 plant viruses [8], including luteovirus that cause 45% insect-born viral diseases in agricultural crops [9]. Aphid infestation releases honeydew on the plant surface that allows the growth of sooty molds that inhibit photosynthesis.

Plants counteract insect pests through their direct and indirect defenses. Effective mechanisms of plant defense include toxic secondary metabolites, amino acid deaminases, protein inhibitors, proteases, oxidases, and lectins [10]. Lectins are carbohydrate-binding proteins that interact reversibly with the saccharide residues of glycolipids, glycosides, and glycoprotein [11,12]. Plant lectins are divided into 12 groups based on their binding affinity including chitin-binding lectin,

type II ribosome-inactivating lectin, jacalin lectin, and mannose-binding lectin [13,14]. Lectins are resistant to proteolytic activity of the insect mid gut and bind to the glycosyl motifs of the insect epithelium lining or peritrophic membrane (PM) [15]. Lectin binding changes the pH of hemolymph, disturbs osmoregulation, and retards the nutrient assimilation in insects. Some lectins have an ability to cross into the hemolymph and hence can easily be deposited in ovarioles. These changes reduce growth, development, and fecundity, and even can lead to the mortality of insect pests [16].

Mannose-binding lectin *Pinellia ternata* leaf agglutinin (PTA) has been reported for its insecticidal activity [17,18]. PTA has been reported in Chinese medicinal plant, *P. ternata* that belongs to Araceae family [19]. Complete cds sequence of *pta* gene encodes homodimer protein of 29.4 kDa molecular weight [20]. The N-terminus of protein consists of a transmembrane region and signal peptide region for protein localization [21]. PTA has been introduced in several crop plants, including tobacco, potato, and wheat to target insect pests [18]. In-planta and artificial diet bioassays revealed the efficiency of PTA against aphids [22]. Artificial diet with 0.04–0.1% (w/v) PTA caused significant aphid mortality [13,23]. The novelty of *pta* gene could be governed by the choice of appropriate promoter. In the present study, the expression of *pta* gene under 35S and rolC promoter significantly reduced aphid survival and fecundity. Expression of *pta* under rolC promoter could benefit plants to directly target aphids and other phloem feeders with less metabolic payoff and pleotropic effects. Furthermore, the plant origin of *pta* makes it a promising approach for the development of commercial crops that are both insect-resistant and environmentally friendly.

2. Materials and methods

2.1. Plant material

Pinellia ternata tubers were obtained from Li Weishan Hu Hang, Luoyang, Henan, China and were grown in peat mix at 25–27°C and 54–67% relative humidity. Tubers grown into mature plants after 15 to 21 d.

Seeds of tobacco plants were sterilized with mixture of 20% chlorox plus and 0.02% Tween 20. After that, they were rinsed with distilled water and dried. Tobacco seeds were grown on Murashige and Skoog (MS) medium [24] under aseptic conditions at 27–28°C and 54–67% relative humidity. Young leaves of mature tobacco were used in *Agrobacterium*-mediated tobacco transformation.

2.2. In-silico analysis

The cis-regulatory elements within the 2X35S constitutive promoter and rolC phloem-specific promoter were identified and analyzed using PlantCARE and PLACE database. Signal peptide and subcellular localization of PTA protein was predicted by TargetP and SignalP server. Hypothetical weight and isoelectric point (pI) of protein was estimated using in-silico analysis. Protein domain was predicted by online PROSITE scan tool while protein structure was predicted by I-TASSER. Protein model having C-score -2.99, estimated TM-score 0.38 ± 0.13 and estimated RMSD 12.0 ± 4.4 Å was selected. Protein model predicted by I-TASSER was submitted to ConSurf server for prediction of evolutionary conserved residues. Phylogenetic analysis of PTA was conducted by MEGA 6.0 (Maximum Likelihood) while Motif prediction in all the orthologs of PTA was done using Motif finder.

2.3. Isolation and amplification of full-length pta gene

Total RNA was isolated from the leaves of *P. ternata* using Plant RNA Purification Reagent (Cat No.12322-012). The quality of the RNA was examined on a 1% agarose gel. Total RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized from 1 μg of total RNA using a H-Minus First Strand cDNA Synthesis Kit (Cat No. K1632, Thermo Fisher)

according to the manufacturer's protocol. RT-PCR was conducted to amplify 810 bp full-length *pta* gene (GenBank AY191305.1) using gene-specific primer pair, PTA-F3 and PTA-R3 (Table 1). A PCR reaction mixture of 50 μL was prepared using 1 μL of each primer (10 μM), 5 μL of *pfu* buffer, 4 μL of MgSO₄, 1 μL of dNTPs (2 mM), 0.5 μL of *pfu* DNA polymerase, 5 μL of cDNA (1 μg) and water to make up the final volume. RT-PCR was carried out using a thermocycler at 94°C for 5 min followed by 39 cycles of 94°C for 1 min, 63°C for 1 min, 72°C for 1 min and final extension of 72°C for 5 min. PCR product was eluted using a Gel Extraction Kit (Cat No. K2100-12, Invitrogen) that was further cloned in pTZ57R/T (Cat No. K1213, Thermo Scientific) for sequential cloning of *pta* in plant transformation binary vector pGA482.

2.4. Construct development in plant transformation vector

Full-length amplified *pta* gene having 3'dA overhangs was cloned in pTZ57R/T. Restriction analysis was carried out to detect the orientation and confirmation of *pta* in resulting clone pTZ-pta. Resulting clone was further confirmed by Sanger sequencing. Full-length *pta* was restricted from pTZ-PTA using *Hin*dIII and *Sma*I for its cloning in pJIT163 (Biovector Science Lab, Inc.) having 2X35S promoter to develop gene cassette (35S-PTA-CaMV). The resulting clone, pJIT163-PTA, was confirmed through restriction analysis. The gene cassette was carried out from pJIT163-PTA through restriction using *Kpn*I and *Eco*RV and cloned in *Kpn*I and *Hpa*I digested plant transformation binary vector, pGA482 [25]. The resulting plasmid, pGA482-35S-PTA, was confirmed through restriction analysis.

Sequential cloning of *pta* gene was performed to develop gene cassette rolC-PTA-CaMV. Full-length *pta* gene was restricted from pTZ-PTA using *Hin*dIII and *Sam*I for its cloning in pJIT163 having the rolC promoter. The resulting plasmid pJIT163-rolC-PTA was confirmed through restriction analysis. To develop gene cassette in pGA482, gene-terminator fragment *pta*-CaMV was restricted from pJIT163-rolC-PTA using *Xba*I and *Eco*RV and cloned in pGA482 under rolC promoter. The resulting plasmid was named as pGA482-rolC-PTA and confirmed through restriction analysis.

2.5. Agrobacterium-mediated tobacco transformation

Cultures of *Agrobacterium tumefaciens* LBA4404 were engineered, harboring gene construct pGA482-35S-PTA and pGA482-rolC-PTA, respectively. Young leaf discs of *Nicotiana tabacum* (cv. *Samsun*) [26] were co-cultivated with these cultures to develop transgenic tobacco plants having 35S-PTA and rolC-PTA, respectively. Co-cultivated discs were placed over solidified MS medium with 30 g/L sucrose and incubated in the dark at 26°C for 2 d. After this incubation period, leaf discs were gradually shifted on selection media, elongation media and rooting media after every 2–3 weeks. Selection media (4.3 g/L MS, 30 g/L sucrose, 0.5 mg/mL N⁶-benzyladine, 1 mg/mL α-naphthalene acetic acid, 0.6 g/L agar, 200 mg/mL kanamycin and 250 mg/mL timentin) allowed discs to turned into callus that were further differentiated into adventitious buds. These buds were shifted to the bud elongation agar medium (4.3 g/L MS, 30 g/L sucrose, 0.5 mg/mL N⁶-benzyladine, 200 mg/mL kanamycin, 250 mg/mL timentin) at 26°C and 16 h photoperiod.

Table 1
Primer sequences for amplification of *pta* gene, promoter-gene fragment and qRT-PCR.

Prime name	Primer sequence 5′-3′
35S-F1	CTGACGTAAGGGATGACGCAC
rolC-F2	ATCGATGCGAAAGCGGCATTGGCAAA
PTA-F3	AAGCTT ATGGCCTCCAAGCTCCTCCTCT
PTA-R3	GAATTCTTAATTCACCTTCTCCGT
qPTA-F5	CTACCTACTGTCCGGCGAAA
qPTA-R5	CTTGCAGTCTCGTCCTTTGTT
18S-F6	GTGGTGCATGGCCGTTCTTA
18S-R6	AACTTCCGCGGCCTAAAAGG

Shoots were generated that were shifted to $1/2\times$ MS agar rooting medium (2.15 g/L MS, 30 g/L sucrose, 200 mg/mL kanamycin, 250 mg/mL timentin). All putative transgenic plants were screened by PCR at the rooting stage. Transgenic plants were shifted to soil pots, kept in growth chamber at 25°C with 16:8 h light:dark photoperiod, and finally shifted to a greenhouse to collect T_1 seeds.

2.6. Molecular analysis of transgenic tobacco plants

DNA was isolated from putative T_0 tobacco plants expressing *pta* under the 35S and rolC promoters using cetyl trimethylammonium bromide (CTAB) method [27]. Specific primers were used to screen putative transgenic plants (Table 1). Promoter-gene fragment (35S-PTA and rolC-PTA) was confirmed using the construct-specific primer pair (35S-F1, PTA-R3 and rolC-F2, PTA-R3) in transgenic tobacco plants expressing *pta* under the 35S and rolC promoters, respectively. The gene-specific primer pair, PTA-F3 and PTA-R3, was used to confirm the presence of a full-length *pta* gene in transgenic tobacco plants. PCR was conducted using reaction mixture containing, 25 μL of Dream Taq Green PCR Master Mix (2X) (Cat No. K1081, Thermo Scientific), 1 μL of each primer pair (10 μM) and 100 ng of genomic DNA. The PCR product was checked on 1% agarose gel to screen transgenic tobacco plants containing *pta* cassettes.

2.7. Southern analysis

Southern blot analysis was performed to detect integration and copy number of *pta* in transgenic tobacco lines. DNA (100 μg) was isolated from the leaves of tobacco lines expressing *pta* under the 35S and rolC promoters. Genomic DNA from all samples was restricted using *Eco*RI. The restricted samples were fractioned by electrophoresis at 80 V and transferred to a positively charged nylon membrane. The nylon membrane was crosslinked with UV linker (0.240 J/cm^2) (Stratalinker) and hybridized with 810 bp long digoxigenin-labeled *pta* probe. The PCR-based probe was prepared using the gene-specific primer pair PTA-F3, PTA-R3. The nylon membrane was washed using Dig Wash and Blocking Buffer Set (Cat No.11585762001 Roche, Basel, Switzerland), signals were developed using color substrate NBT/BCIP.

2.8. qRT-PCR for pta gene expression

qRT-PCR of T_0 transgenic tobacco lines was conducted to evaluate the expression of the *pta* gene. RNA was isolated from the 3–4 leaf stage (45 d old) transgenic and non-transgenic plants using SV Total RNA Isolation System (Cat No. Z3101, Promega, Madison, WI, USA). Primers were designed using Primer3 program [28]. The qRT-PCR reaction mixture of 10 μL contained 2 μL of cDNA (200 ng), 10 μM of each primer (qPTA-F5 and qPTA-R5), Power SYBR Green PCR Master Mix (Thermo Scientific), and deionized water to make up the final volume. Each transgenic line with its three technical replicates using gene-specific primers and18S rRNA as internal control, was run in Quantstudio 6 Real-time PCR system (Thermo Fisher Scientific) using the following conditions; 95°C for 3 min, 40 cycles at 95°C for 30 s, 56°C for 30 s 72°C for 50 s. Gene expression of each transgenic tobacco line was calculated with ΔΔCt method.
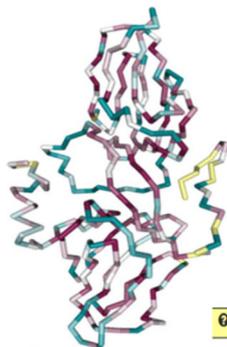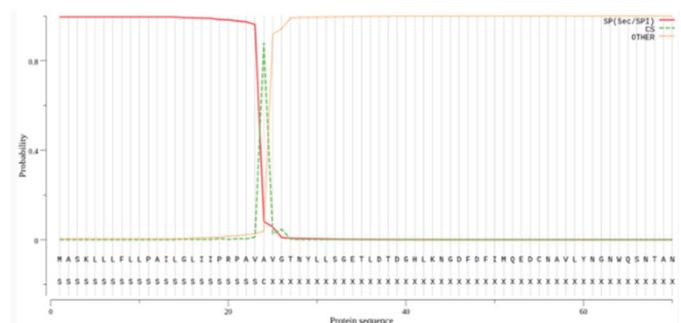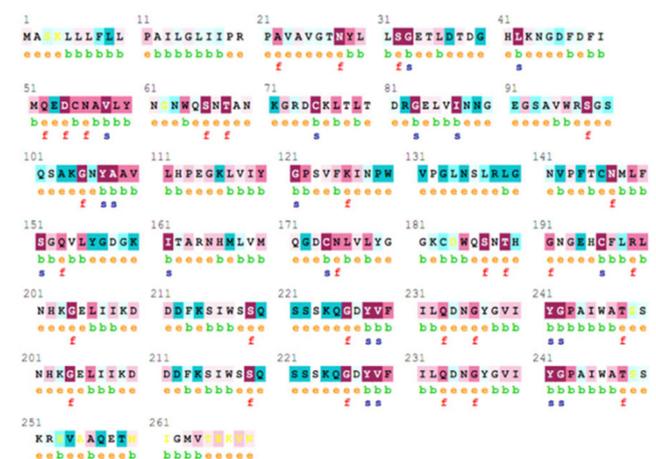
Fig. 1. In silico analysis of PTA. (A) Translated sequence of PTA having 269 amino acid residues, yellow color presents region of 24 residue long signal peptide, (B) cleavage site of signal peptide between 24 and 25 amino acid residue, (C) two bulb type lectin domains of PTA predicted through PROSITE tool, (D) ConSurf analysis for PTA predicted three dimensional structure of PTA protein having variable and conserved residues, (E) the amino-acid sequence of PTA is colored by their conservation grades using the color coding bar, e: Exposed residue, b: Buried residue, f: Predicted functional residue, s: Predicted structural residue, X: Insufficient data.

2.9. Aphid bioassay

A culture of a tobacco-adapted *M. persicae* strain [29,30] was established on tobacco plants at $25 \pm 1°C$, 70–80% relative humidity and 16 h light:8 h dark cycle. Aphid bioassays were performed on two biological replicates of each T_0 transgenic tobacco line versus non-transgenic tobacco lines. Detached leaves of three tobacco lines, LSP-2, LSP-3, and LSP-6, expressing *pta* under the 35S promoter and four tobacco lines LRP-8, LRP-9, LRP-14 and LRP-15 expressing *pta* under the rolC promoter were placed on petri plates containing 1% agar. Five 1st instar nymphs, which turned into adult aphids, were released on each leaf of transgenic and non-transgenic plants. Mortality and fecundity of adult aphids were observed after every 24 h for 13 d.

2.10. Statistical analysis

Significant difference in relative gene expression was determined between transgenic versus non-transgenic tobacco plants by Mann–Whitney *U*-tests using SPSS23x86 software. Dunnett's test was used to determine the significance of aphid mortality and fecundity on transgenic and control tobacco plants. Correlation of *pta* gene expression and aphid growth was performed using Pearson correlations in Microsoft Excel.

3. Results

3.1. Identification of cis-elements of 35S and rolC promoter

PlantCARE [31] and PLACE [32] databases identified different types of cis-elements in DNA sequence of 35S and rolC promoters that include phytohormone-regulating and stress-responsive elements. Dehydration-responsive elements DRE, E-box, MYC, CBF, ACGT sequence found as abundant stress-responsive elements in DNA sequence of 35S promoter. Phytohormone-responsive elements and factors (OS1, POLLEN1, PYRIMIDINEBOX, CACT) that are involved in the expression of genes in different tissues and organs are found in 35S promoter. rolC promoter contain phytohormone-responsive elements include abscisic acid-responsive elements (ABRE, ABRE3a, ABRE4), gibberellin-responsive elements (p-box), and salicylic acid recognition element (W-box). Major stress-responsive elements that were found in promoter sequence of rolC included anaerobic-responsive element (ARE), light-responsive elements (AT1, Box4, G-box, I-box, SORLIP, GA motif, GT1 motif and MRE), cold-responsive elements (LTRE) and drought-responsive element (DRE). Among these stress-responsive elements, light-responsive elements were found abundant in rolC promoter. In-silico analysis indicated presence of two G-box and SORLIP (sequences over represented in light-induced promoters) sequences (GCCAC) in rolC promoter. Member of transcription regulating superfamily were also found in both 35S and rolC promoter sequences. They include RAVI, WRKY, MYB and MYB like sequences.

3.2. In-silico analysis of PTA

Pinellia ternata agglutinin (PTA) was successfully isolated from *P. ternata* and sequentially cloned in pGA482. Sanger sequencing showed 99% identity of cloned *pta* with PTA (accession # AY191305.1) while amino acid sequence retains 98% similarity with AAP20876.1. Full-length *pta* gene comprises of 810 bp encodes agglutinated protein of 269 amino acids. Hypothetically, molecular weight of agglutinated protein PTA was estimated 29,396.48 Da having 6.58 isoelectric point (pI) [33]. Analysis of PTA using TargetP [34] suggested secretary nature of PTA protein. SignalP 5.0 [35] estimated 24 residues long signal peptide in PTA having cleavage site between residue numbers 24 and 25 (AVA-VG) with reliability class 1. Motif search and ScanProsite predicted two bulb lectin domains in PTA. First domain spans over 27–132 residues having di-sulphide bond between 55 and

75 cysteine residues. Other domain comprises of 146–253 residues and di-sulphide bond exists between 174 and 196 cysteine residues. ConSurf server [36] predicted three-dimensional structure of PTA that consists of several functionally and structurally, variable and conserved residues (Fig. 1A, Fig. 1B, Fig. 1C, Fig. 1D, Fig. 1E). Phylogenetic analysis revealed the homology of PTA with different members of Araceae family. Members of this family belong to mannose-binding lectin having bulb lectin domains (Fig. 2A, Fig. 2B).

3.3. Construct development in pGA482 under 35S and rolC promoters

Gene cassettes 35S-PTA-CaMV and rolC-PTA-CaMV were successfully developed in pGA482. Restriction of gene construct pGA482-35S-PTA using *Hind*III produced two fragments (1.5 kb and 13 kb), *Eco*RI produced three fragments (4 kb, 10 kb, and 27 bp), whereas *Sma*I also produced three fragments of 766 bp, 6.39 kb and 7.7 kb (Fig. 3A, Fig. 3B). Restriction analysis using *Hind*III, *Cla*I, *Xba*I and *Kpn*I confirmed the successful engineering of gene construct pGA482-rolC-PTA. *Hind*III have two restriction sites in pGA482-rolC-PTA and produced two fragments; 857 bp and 14 kb. Single digestion using *Cla*I linearized the pGA482-rolC-PTA while double digestion with *Cla*I and *Xba*I produced two fragments; one of 1.5 and other fragment of 13 kb. *Kpn*I restricted two fragments; gene cassette (rolC-PTA-CaMV) of 2.3 kb and vector backbone of 12.6 kb (Fig. 3C, Fig. 3D).

3.4. Screening and molecular analysis of transgenic tobacco plants

Transgenic tobacco plants were screened successfully using gene-specific and construct-specific primer pairs (Table 1). Gene-specific primer PTA-F3, PTA-R3 amplified full-length *pta* gene of 810 bp in four tobacco lines (LSP-2, LSP-3, LSP-6 and LSP-8; Fig. 4A) harboring 35S::PTA, while construct-specific primer pair 35S-F1 and PTA-R3 amplified 35S-PTA promoter-gene fragment of 911 bp in these transgenic tobacco lines (Fig. 4A). Full-length *pta* gene was amplified in eight tobacco lines (LRP-1, LRP-5, LRP-7, LRP-8, LRP-9, LRP-13, LRP-14 and LRP-15; Fig. 4B) transformed with rolC::PTA using gene-specific primer PTA-F3, PTA-R3, while construct-specific primer pair rolC-F2, PTA-R3 amplified rolC-PTA promoter-gene fragment of 1.6 kb in these tobacco lines (Fig. 4B).

Results of southern blot analysis of T_0 transgenic tobacco lines LSP-2, LSP-3, LRP-8, LRP-9 and LRP-15 confirmed the integration of *pta* gene and its copy number. Tobacco lines LSP-2, LRP-8, LRP-9 showed single insertion of transgene *pta* while tobacco lines LSP-3 and LRP-15 showed two insertion of transgene. The number of bands in Southern blot determined the copy number of transgene in transgenic tobacco plants (Fig. 5).

3.5. Gene expression analysis

qRT-PCR showed significant expression of *pta* in different transgenic tobacco lines (Fig. 6A, Fig. 6B). Higher relative gene expression was calculated in transgenic tobacco line LSP-3 that has four-fold higher gene expression than LSP-2. Transgenic tobacco line LSP-2 has eight-fold higher gene expression than LSP-6 (Fig. 6A). Relative gene expression of tobacco line LRP-9 showed 1.6, 1.5 and 2.3-fold higher gene expression than LRP-14 and LRP-15, and LRP-8 (Fig. 6B).

3.6. Aphid bioassays

Transgenic tobacco plants caused significant mortality of aphids and reduced their fecundity than non-transgenic tobacco plants. Transgenic tobacco LSP-2, LSP-3 and LSP-4 caused 60%, 80% and 20% aphid mortality over the period of 13 d (Fig. 7A). During this period, aphids produced less number of nymphs on transgenic tobacco than non-transgenic tobacco. Data analysis of aphid fecundity showed
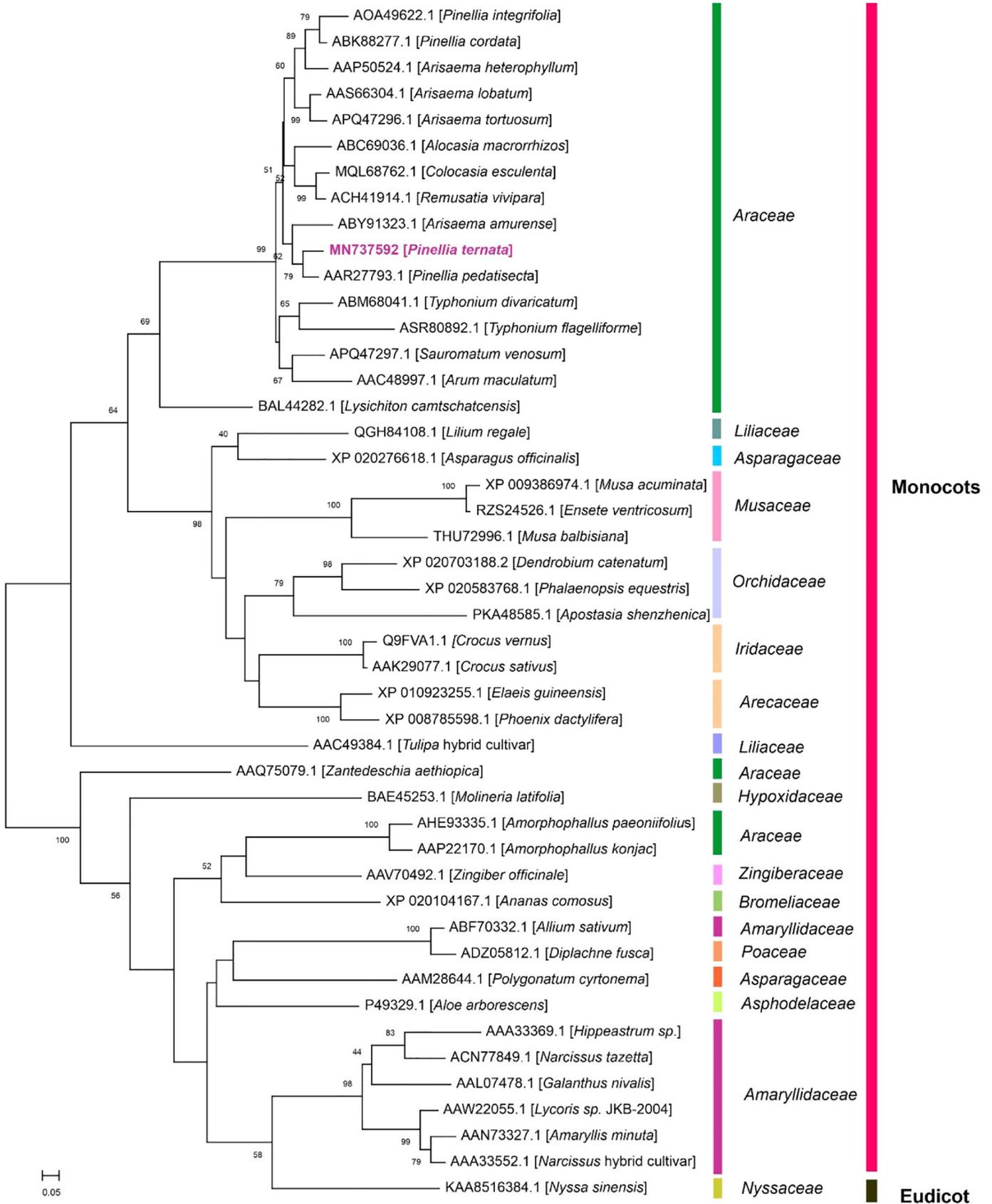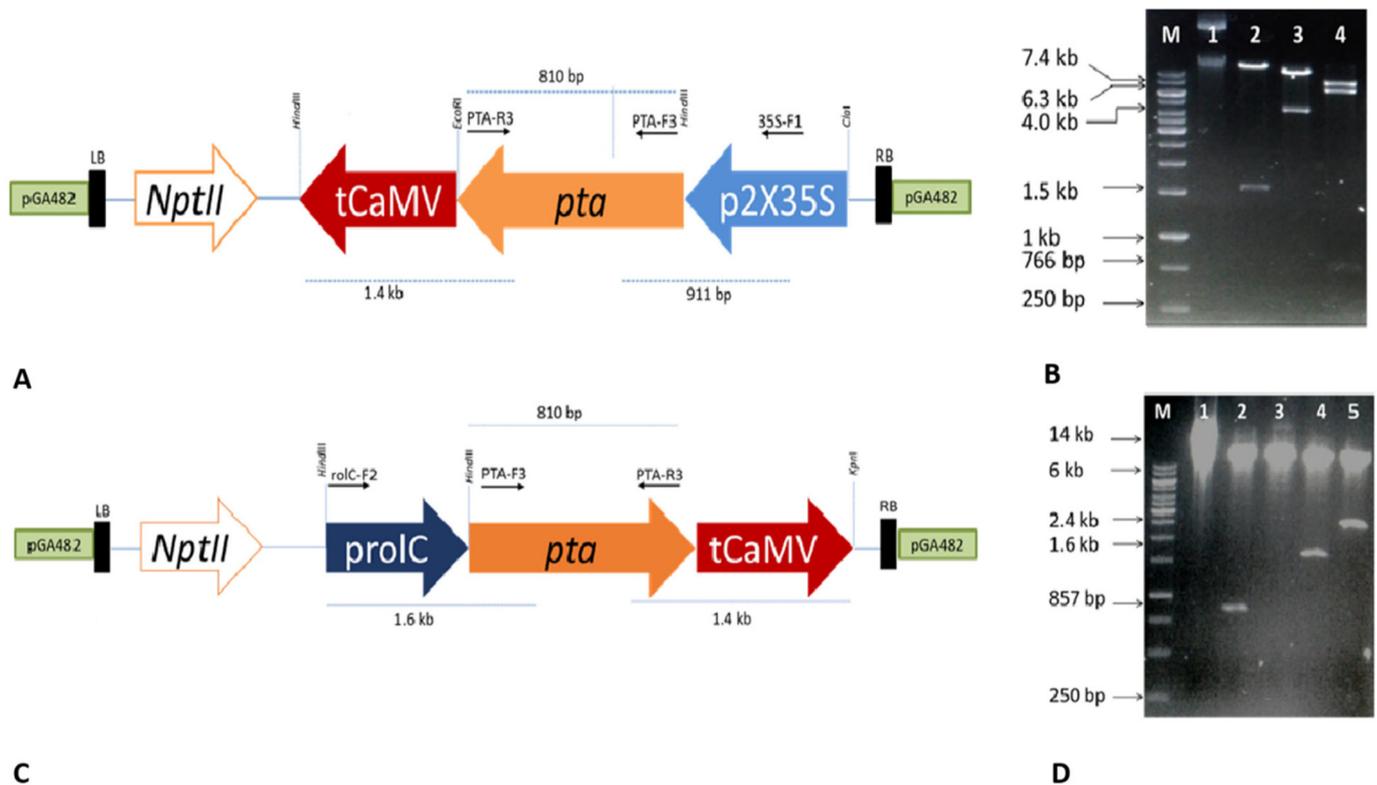
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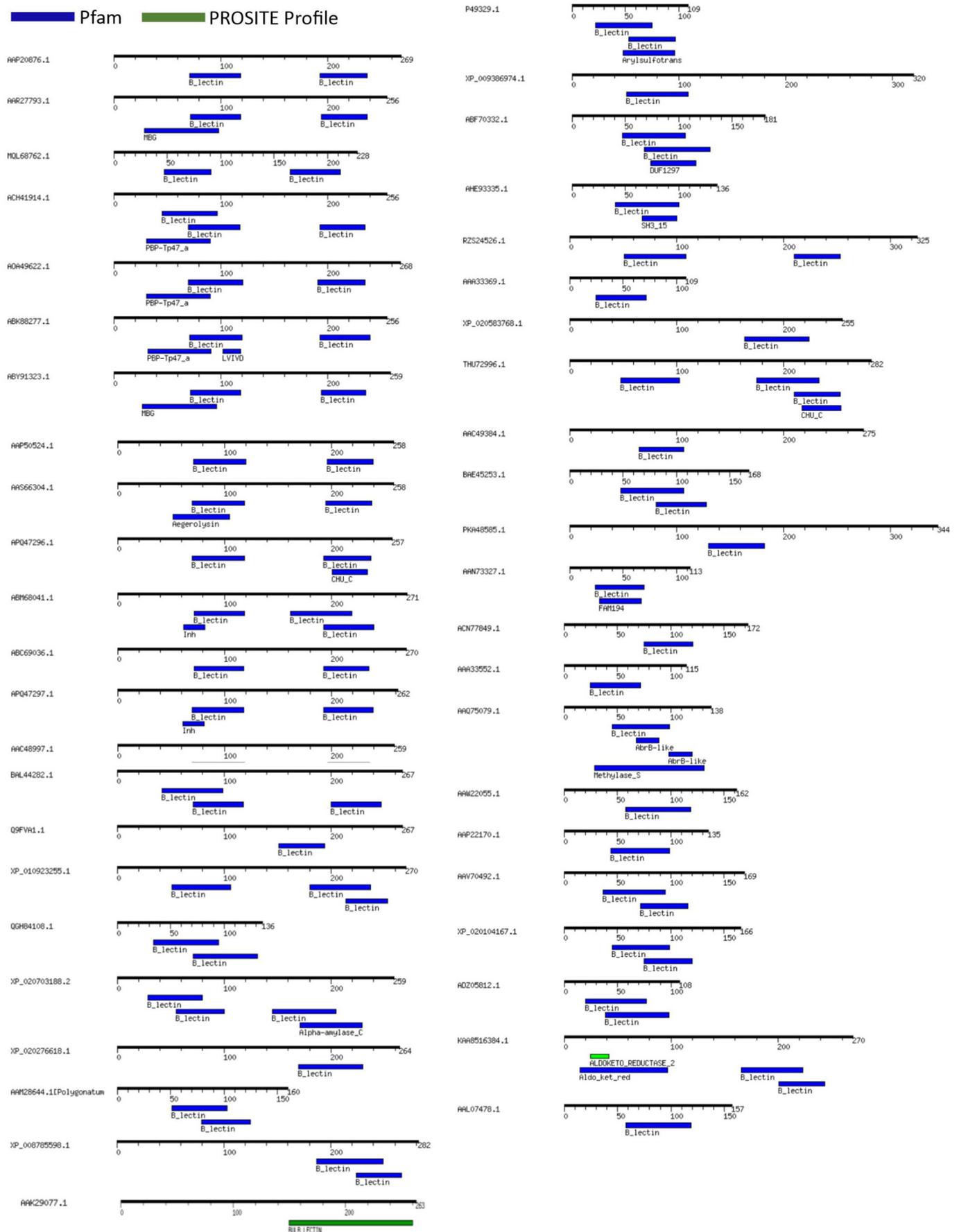
Fig. 2. Phylogenetic analysis and motif prediction of PTA protein. (A) Phylogenetic analysis of PTA with its homologs from different species. (B) Motif prediction in all the homologs of PTA by Motif finder.

Fig. 3. Confirmation of engineered plasmids pGA482-35S-PTA and pGA482-rolC-PTA through restriction analysis. (A) Vector map of pGA482-35S-PTA. (B) Restriction analysis of pGA482-35S-PTA plasmid. M: 1 kb DNA ladder. 1: Uncut plasmid. 2: Restriction using *Hind*III. 3: Restriction using *Eco*RI. 4: Restriction using *Sma*I. (C) Construct map of pGA482-rolC-PTA. (D) Restriction analysis of pGA482-rolC-PTA plasmid. 1: Uncut plasmid. 2: Restriction using *Hind*III. 3: Restriction using *Cla*I. 4: Restriction using *Cla*I and *Xba*I. 5: Restriction using *Kpn*I.

up to 42% less production of nymphs on transgenic tobacco than non-transgenic tobacco (Fig. 7B).

Data of aphid mortality and fecundity on transgenic tobacco expressing rolC::PTA showed that tobacco lines LRP-8, LRP-14 and LRP-15 showed up to 70% aphid mortality whereas 100% aphid mortality was recorded in tobacco line LRP-9 (Fig. 7C). Tobacco line LRP-9 also showed maximum efficiency to control aphid population and reduced 70% aphid fecundity than control tobacco. Other tobacco lines; LRP-8, LRP-14 and LRP-15 reduced aphid fecundity up to 41% than non-transgenic tobacco (Fig. 7D).

The *pta* transgene expression level, from both the 35S and the rolC promoters, was positively correlated with aphid mortality (Fig. 8A) and
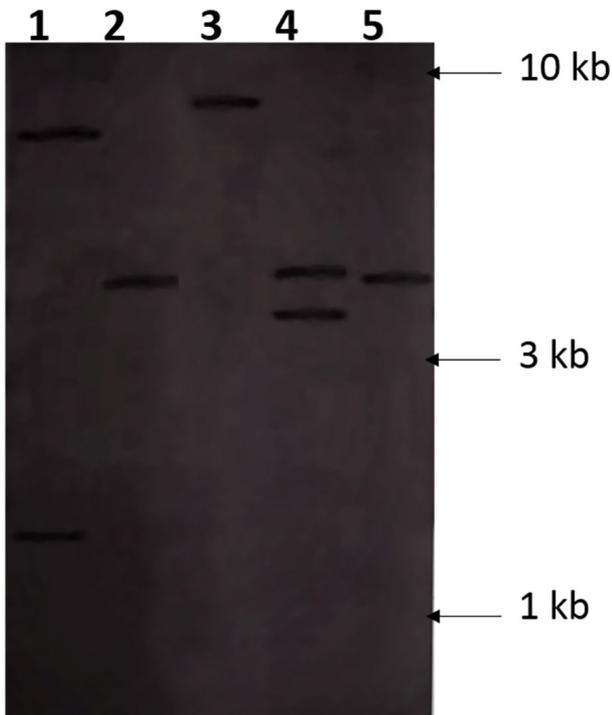
a negatively correlated the cumulative number of nymphs (Fig. 8B). However, due to the smaller sample size, these effects were not significant in the case of the 35S promoter (P > 0.05).

4. Discussion

Aphid infestation damages the productivity of crop plants worldwide. Development of transgenic plants is promising approach to reduce aphid infestations [37]. Plant-derived genes such as lectins can be used to develop insect resistant crop varieties [38]. PTA is reported as a novel plant derived insecticidal lectin to develop insect resistant plants [39]. In the present study, expression of *pta* under 35S constitutive promoter
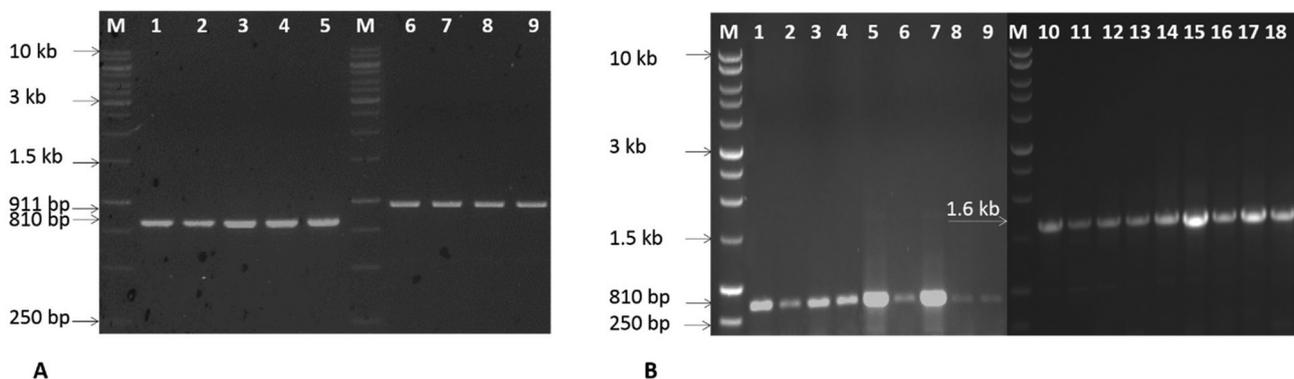
Fig. 4. PCR-based detection of transgenic tobacco plants expressing *pta*. (A) Amplification of full length *pta* gene (product 810 bp) and promoter-gene (35S-PTA) fragment (product 911 bp). M: 1 kb DNA ladder, Lane# 1–4: Transgenic tobacco lines LSP-2, LSP-3, LSP-6, LSP-8, Lane# 5: Positive control (plasmid), Lane# 6–9: Transgenic tobacco lines LSP-2, LSP-3, LSP-6, LSP-8. (B) Amplification of *pta* gene (product 810) and promoter-gene (rolC-PTA) fragment (product 1.6 kb). M: 1 kb DNA ladder, Lane# 1–8: Transgenic tobacco lines LRP-1, LRP-5, LRP-7, LRP-8, LRP-9, LRP-13, LRP-14, LRP-15, Lane# 9: Positive control (plasmid), Lane# 10–17: Transgenic tobacco lines LRP-1, LRP-5, LRP-7, LRP-8, LRP-9, LRP-13, LRP-14, LRP-15, Lane# 18: Positive control (plasmid).
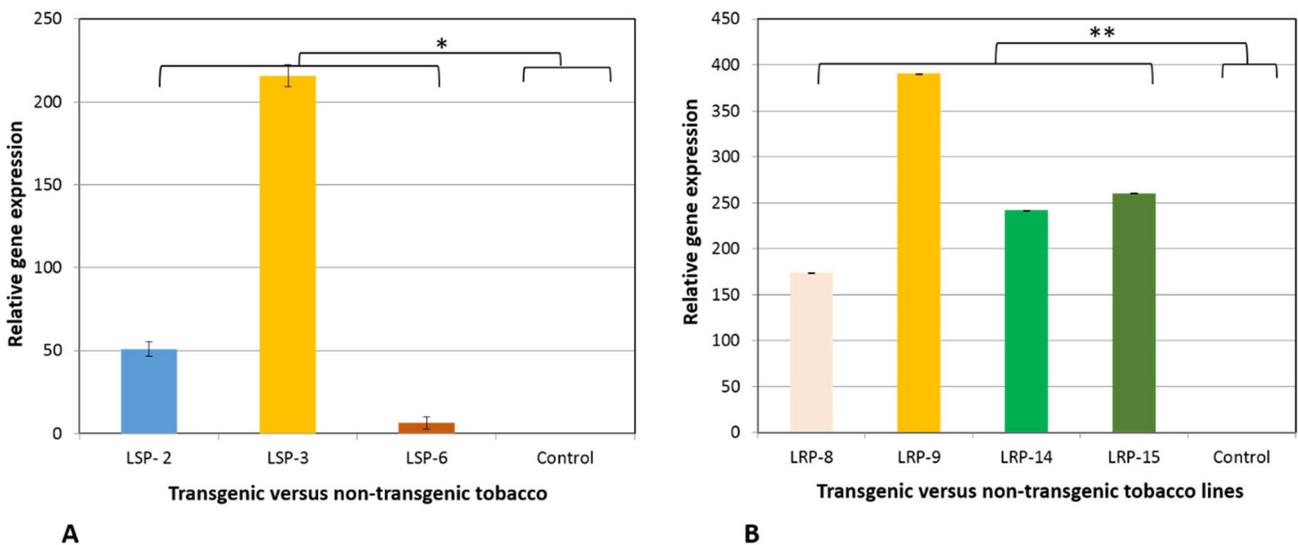
Fig. 5. Southern blot analysis to confirm transgene integration in genomic DNA of tobacco plants restricted with *EcoRI*. Lane# 1–2: Restricted DNA of transgenic tobacco lines LSP-3 and LSP-2 expressing *pta* under the 35S promoter. Lane# 3–5: Restricted DNA of transgenic tobacco lines LRP-9, LRP-15, LRP-8 expressing *pta* under the rolC promoter.

ABRE elements are found in rolC promoter that are reported as abscisic acid-responsive element. Abscisic acid-responsive element generates response to various environmental stresses including drought, cold and salinity. During this response, plant closes their stomatal opening and ABA induces gene expression [40]. DRE (dehydration-responsive element) and LTRs (light responsive element) with a core sequence of GCCGAC is found in rolC promoter. These are cold-responsive elements and are reported in upstream region of cold stress-induced genes [41]. In-silico analysis of rolC promoter indicated several light-responsive cis-elements (Box4, G-box, AT1, GT1, GA motif and MRE) in promoter sequence than 35S promoter. These light-responsive elements have been reported for light regulation during photosynthesis that synthesize more sap that is inducing factor for rolC promoter. Beside these light-responsive elements, GATA-box, I-box (GATAAG) and SORLIP (sequences over represented in light-induced promoters) are also found in rolC promoter. These elements are known to induce gene expression in light response. Transcription regulating factors RAVI, MYB and WRKY are found in both 35S and rolC promoter. MYB is a superfamily of transcription-regulating factor [42]. Myb sequences TAACTG/AACG are found in rolC promoter AACG sequence of *myb* element that has reported identical to MRE sequence, flanking at 5′ end of *myb* gene of *Hordeum vulgare*. W-box interacts with WRKY, superfamily of transcription factor that is induced by abiotic stress like cold, drought, salinity and biotic stress-like wounding [43].

Southern blot analysis determined the integration of transgene in tobacco plants. Single and double bands were visualized that indicated the copy number of transgene in host tobacco plants. Copy number of T-DNA in transgenic plants determines the effectiveness and zygosity of transgenic event [44]. The stability and level of transgene expression is influenced by its site of integration and copy number [45,46,47]. Multicopy integration of transgene could induce higher expression than single copy. In present study, it was observed that transgenic tobacco LSP-3 has two copies of integrated transgene that are consistent with its higher gene expression, higher aphid mortality, and lower aphid fecundity. However, double copy integration of transgene could also develop steric hindrance that reduces the effectivity of transgene. Results of southern analysis and qRT-PCR showed that transgenic tobacco LRP-9 had higher gene expression while having single copy of transgene. On the other hand, LRP-15 with two copies of integrated transgene had lower relative gene expression than LRP-9.

Several studies have supported the effectivity of *pta* in transgenic plants against insect pests and pathogens [48]. Transgene resistance

and rolC phloem-specific promoter give insight to target insect pests at broad spectrum and phloem-specific level. 35S promoter sequence include elements that are responsible for constitutive expression of transgene under 35S. These elements are absent in rolC promoter. CACT, tetra nucleotide is a cis element for mesophyll-specific gene expression. OSE1 is responsible for gene expression in infected cells of root nodules, Pollen1, induces gene expression in pollen cells and Pyrimidine-box causes gene expression in aleurone layers. rolC has been reported to drive gene expression in phloem tissue where phloem sap activates the expression of rolC promoter. Computational analysis of DNA sequence of rolC promoter revealed the presence of different cis-acting elements.

A

B

Fig. 6. Real time quantitative RT-PCR of *pta* expression under the 35S and rolC promoter in transgenic tobacco lines. (A) LSP-2, LSP-3, LSP-6: Transgenic tobacco lines expressing *pta* under the 35S promoter. Control: Non-transgenic tobacco lines. *$P < 0.05$, Mann–Whitney U-test; mean $\pm$ SE of N = 3. (B) LRP-8, LRP-9, LRP-14, LRP-15: Transgenic tobacco lines expressing *pta* under rolC promoter. Control: Non-transgenic tobacco line. **$P < 0.01$, Mann–Whitney U-test; mean $\pm$ SE of N = 3.
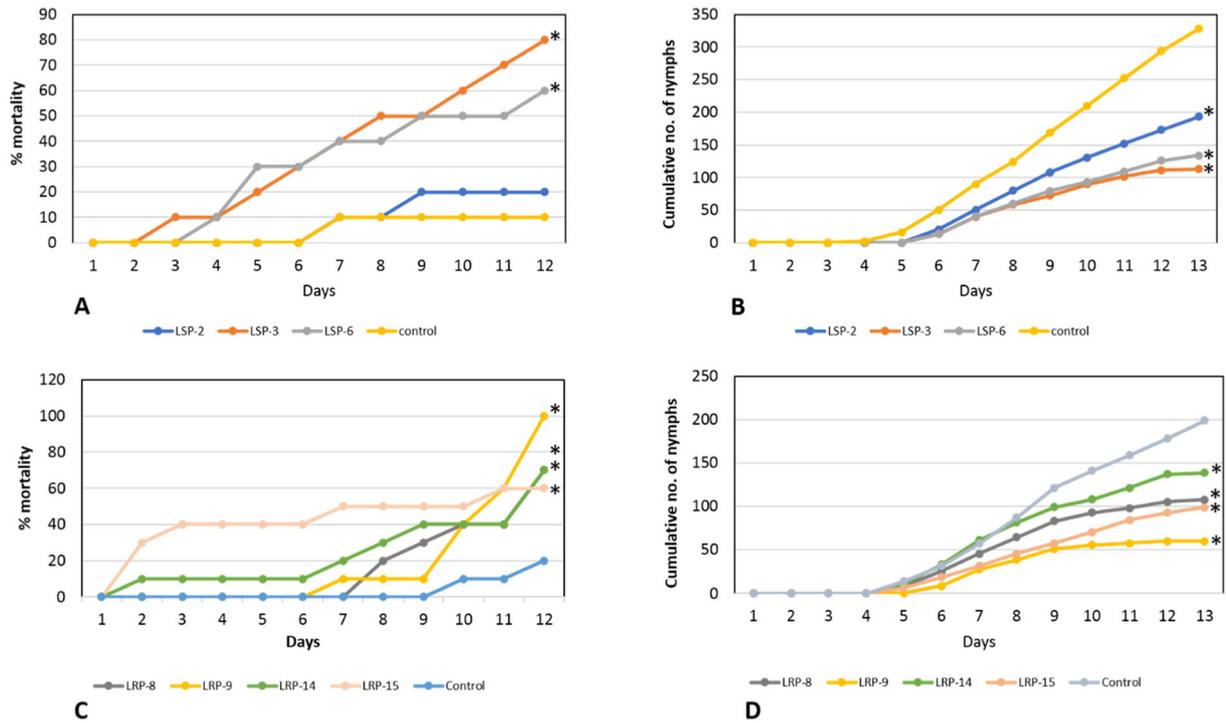
Fig. 7. Aphid bioassay of transgenic tobacco lines expressing *pta* gene under the 35S and rolC promoter. (A) % mortality of adult aphids on transgenic tobacco lines (LSP-2, LSP-3, LSP-6) versus non-transgenic tobacco during the period of 13 d, (B) Cumulative number of nymphs on transgenic tobacco lines versus non-transgenic tobacco, (C) % mortality of adult aphids on transgenic tobacco lines (LRP-8, LRP-9, LRP-14, LRP-15) versus non-transgenic tobacco during the period of 13 d, (D) Cumulative number of nymphs on transgenic tobacco lines versus control tobacco. *P < 0.05, Dunnett's relative to un-treated tobacco plants; mean ± SE of N = 10.

corresponds to its expression levels. Our findings suggested that LSP-3 and LRP-9 showed maximum resistance against aphid that is related to highest expression level of *pta* in these lines. These finding are consistent with similar studies that revealed aphidicidal effect of *pta* under constitutive promoter [48]. In present study, aphidicidal effect of *pta* was evaluated under phloem-specific rolC promoter and determined that transgenic tobacco expressing *pta* under rolC promoter caused up to 100% aphid mortality and reduced aphid fecundity by up to 70%.

In particular, cotton (*Gossypium hirsutum*) would be a good target for transformation with *pta*. Cotton contributes 46% to Pakistan's exports and has an impact on 35% of employment opportunities [49].

Sap sucking insect pests pose a serious threat to cotton and are responsible for 845 kg/ha annual yield losses [50]. Development of transgenic cotton plants using plant-derived insecticidal genes like *pta* would be key step towards commercialization of environmentally safe insect-resistant crops.

5. Conclusions

The present study scrutinized the insecticidal and expression profile of *Pinellia ternata* leaf agglutinin (PTA) under two different 35S constitutive and rolC phloem-specific promoters against aphids (sap-sucking insect-pest) in model plant *Nicotiana tabacum*. It is the first
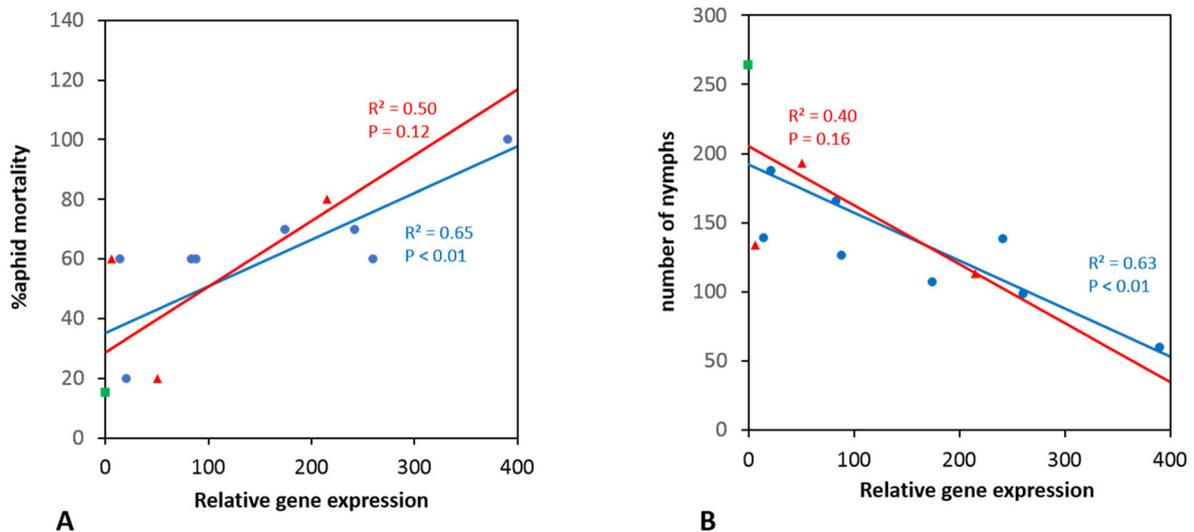
Fig. 8. Correlation of *pta* gene expression and aphid growth. (A) Aphid mortality is positively correlated with *pta* expression level. (B) Aphid progeny production is negatively correlated with *pta* expression level. Average of N = 2, N = 4 for the control samples. Red triangles = 35S promoter, blue circles = rolC promoter, green squares = non-transgenic control. P values are based on Pearson correlations. Lectins are resistant to insect gut proteolysis and can form complexes with gut proteins (likely glycosylated proteins) with high affinity.

report in which expression and effectivity of PTA has been determined under phloem-specific rolC promoter. Our findings suggested the potential of mannose binding PTA against aphids. Increase in insecticidal gene expression under rolC promoter and resulted mortality of target insect pest suggested the potential of the gene as well as selection of promoter. Therefore, it is concluded that PTA could be further used for the management of sucking insect pests.

Conflict of interest

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Supplementary material

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