Allium Sativum Leaf Agglutinin (ASAL) Endowed Enhanced Resistance Against *Myzus Persicae* under Both Constitutive and Phloem Specific Promoters

Noroza Umer
National Institute for Biotechnology and Genetic Engineering
Rubab Zahra Naqvi
National Institute for Biotechnology and Genetic Engineering
Imran Rauf
National Institute for Biotechnology and Genetic Engineering
Naveed Anjum
National Institute for Biotechnology and Genetic Engineering
Hamid Anees Siddiqui
National Institute for Biotechnology and Genetic Engineering
Syed Najeebullah
National Institute for Biotechnology and Genetic Engineering
Shaheen Asad
National Institute for Biotechnology and Genetic Engineering
Muhammad Asif (asif.biosafety@gmail.com)
National Institute for Biotechnology and Genetic Engineering https://orcid.org/0000-0001-5209-2953

Research Article

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Abstract

Globally, aphid, *Myzus persicae* is an economically significant, polyphagous crop pest that feeds on more than 400 plant species and transmits more than 100 plant viruses. Aphid infestation is mostly managed by insecticides that cause heavy environmental contamination and insect resistance. Cloning of plant derived insecticidal genes to develop transgenic plants under suitable promoter is a promising technology. In the present study, ASAL (MN820725) was isolated from native garlic and cloned in plant transformation vector, pGA482 through *Agrobacterium* mediated tobacco transformation. PCR of genomic DNA of transgenic tobacco plants using gene specific primers confirmed the presence of *asal* gene of 546 bp. To detect the integration of gene Southern blot analysis was conducted that revealed stable integration of *asal* gene while, gene expression was analyzed through qRT-PCR that showed variable expression of *asal* gene in transgenic tobacco plants. Efficacy of *asal* gene was evaluated through aphid bioassay. Aphid bioassay revealed that transgenic tobacco lines LS-17, LS-20, LR-1, and LR-7 exhibited 100% aphid mortality and significantly reduced the aphid population. These findings suggested the potential of ASAL against aphids that can be further used against other notorious sap sucking pests.

Introduction

Insect pest control has become a challenge for sustainable agriculture. Physical and molecular defense of plants have evolved against biotic and abiotic stress. Meanwhile, insect pests have also adopted themselves against defense system of plants (Napoleao et al. 2018). Although morphological and chemical defense of plants alter the preference (host plant selection, feeding behavior) and performance (growth and development) of insect complex (Furstenberg-Hagg et al. 2013) but still they are responsible for 40-50% qualitative and quantitative losses in crop plants. Sucking insect pest, aphid is a key pest that penetrate its stylet like mouth part into phloem tissue by probing through epidermis and mesophyll cells to drain free amino acids and amides of plants (Salman et al. 2011; Kaloshian and Walling 2016; Xu et al. 2019). Aphids are responsible of about 13% agricultural losses, globally. They can transmit about 50% plant-viruses as vector and secret honeydew that allow the growth of sooty molds on plant surface. Aphid feeding damages the strength of plants and stunt their growth. Growth of sooty mold covers the plant surface that retards the process of photosynthesis and distorts the plants (Will et al. 2007). In addition of short life cycle, female adults are capable to produce nymphs sexually, asexually or both (Dedryver et al. 2013). Aphid infestation triggers phosphorylation, calcium flux, and production of reactive oxygen species (ROS) that fluctuate the production of phyto-hormone and interrupt the transcriptional regulation of plants (Liang et al. 2015; Shah et al. 2017).

Different control strategies based on physical barriers, pesticides, biotic agents and host-plant resistance have been used to combat sucking pests (Shukla et al. 2016). Transgenic technology is an economical and environment friendly approach to manipulate host plant resistance and incorporate insecticidal genes in plants (Arora and Sandhu 2017). *Allium sativum* leaf agglutinin (ASAL) is a mannose-binding lectin that has been reported in garlic (*Allium sativum*). It targets sucking insect pests and binds to their

glycosylated receptors including alkaline phosphatase (ALP), aminopeptidase-N (APN), cadherin-like proteins, polycalins, sucrase, symbionin, crosses the hemolymph and deposits in the ovarioles and/or in fat bodies. These processes disrupt the membrane integrity, inhibit feed digestion and retard nutrient absorption that ultimately lead towards insect mortality (Upadhyay and Singh 2012). It has been reported that deposition of ASAL in ovarioles of aphid retards circulation of nutrients that could reduce aphid's fecundity up to 74%. Aphid fecundity is also reduced when ASAL binds with NADH quinone oxidoreductase (NQO), a key component of electron transport chain, insect defense response and gametogenesis (Roy et al. 2014). In present study, ASAL has been introduced in tobacco plants to confer resistance against aphids and other sucking insect pests. ASAL was isolated from garlic leaves and cloned in plant transformation vector, pGA482 for *Agrobacterium* mediated tobacco transformation. Expression of ASAL was achieved under 2X35S constitutive promoter and rolC phloem specific promoter to specifically target phloem feeders, aphids. Molecular characterization and aphid bioassay of transgenic tobacco plants revealed insecticidal effectivity of ASAL. These findings could be useful to develop environment friendly sucking-pest resistant crop varieties.

Material And Methods

In-silico characterization of promoters and *asal* gene

The cis-regulatory elements within the 2X35S constitutive promoter and rolC phloem specific promoter were identified using PlantCARE software for comparative analysis (Lescot et al. 2002).

The nucleotide sequence of ASAL was translated through Swiss Bioinformatics Server (Artimo et al. 2012). Signal peptide and subcellular localization of protein was predicted by TargetP 1.1 Server (Emanuelsson et al. 2000). Molecular weight and isoelectric point of protein was computed using in-silico analysis (Gasteiger et al. 2005). Domain was predicted by online PROSITE scan tool (Sigrist et al. 2012). Protein structure was predicted by I-TASSER (Roy et al. 2010; Yang et al. 2015). Protein model having C-score =-2.99, Estimated TM-score 0.38±0.13 and Estimated RMSD = 12.0±4.4Å was selected. Protein Model predicted by I-TASSER was submitted to ConSurf server (Ashkenazy et al. 2010; Ashkenazy et al. 2016) for prediction of evolutionary conserved residues. Phylogenetic analysis of ASAL was conducted by MEGA 6.0 (Maximum Likelihood) while Motif prediction in all the orthologues of ASAL was identified by Motif finder.

Plant materials

Bulblets of *Allium sativum* (garlic) were obtained from Ayub Agricultural Research Institute (AARI), Faisalabad, Pakistan and grew them in peat moss under control conditions at 27-28 °C and 54-67% relative humidity. Seeds of *Nicotiana tabacum* were sterilized with mixture of 20% chlorox plus, 0.02% Tween 20, rinsed with distilled water and dried to sow on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) under aseptic conditions at 27-28 °C and 54-67% relative humidity.

Construct development (35S-ASAL-CaMV and rolC-ASAL-CaMV)

Total RNA was isolated from garlic using Plant RNA Purification Kit (Cat No.12322-012, Invitrogen) to synthesis cDNA using H-Minus First Strand cDNA Synthesis Kit (Cat No. K1632, Thermo Fisher) according to the manufacturer's protocol. Primers were designed in-silico using Primer3 program (Untergasser et al. 2012) to amplify full length *asal* gene from garlic. RT-PCR of 50 µl reaction mixture was conducted using proofreading DNA polymerase, *pfu* (2.5 U), and cDNA (1 µg) to amplify 546 bp full length *asal* gene using 10 µM of gene-specific primer pair, ASAL-F3 and ASAL-R3 (Table 1). Reaction mixture was run in thermal cycler under following conditions; initial denaturation 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. Amplified gene was eluted using a Gel Extraction Kit (Cat No. K2100-12, Invitrogen) and cloned in PCR blunt cloning vector (Cat No. K2700-20, Invitrogen). Resulting clone was confirmed through restriction analysis and Sanger sequencing.

Full length *asal* gene was restricted from PCR blunt vector using *Hin*dIII and *Sma*I and cloned in pJIT163 having 2X35S promoter and CaMV terminator. Gene cassette, 35S-ASAL-CaMV was restricted from resulting plasmid pIT163-ASAL using *Sac*I and *EcoR*V and cloned in plant transformation vector, pGA482. The resulting plasmid, pGA482-35S-ASAL was confirmed through restriction analysis.

2X35S promoter of pIJT163 was replaced with rolC promoter to clone full length *asal* gene under phloem specific rolC promoter. Engineered gene cassette (rolC-ASAL-CaMV) was cloned in pGA482. The resulting plasmid, pGA482-rolC-ASAL was confirmed through restriction analysis.

Agrobacterium mediated tobacco transformation

Plasmids pGA482-35S-ASAL and pGA482-rolC-ASAL were separately, electroporated in *Agrobacterium tumefaciens* strain, LBA4404 and their cultures were developed. For stable transformation, leaf discs of *Nicotiana tabacum* (cv. *Samsun*) (Amaya 1997) were co-cultivated with engineered cultures of *A. tumefaciens*. and kept over the regeneration media containing MS salt and sucrose to develop transgenic tobacco plants..

Molecular analysis of tobacco plants

All putative transgenic tobacco plants were screened using gene specific and construct specific primer pairs (Table 1). CTAB (cetyl trimethylammonium bromide) method (Doyle and Doyle 1987) was used to isolate genomic DNA from leaves of tobacco plants expressing ASAL under 35S and rolC promoters, respectively. PCR reaction mixture of 25 µl was prepared using 100 ng of DNA, 12.5 µl of Dream Taq Green PCR Master Mix (2X) (Cat No. K1081, Thermo Scientific), and 1 µl of each primer (10 µM). Gene specific primers were used to confirm the presences of *asal* gene while construct specific primers were

used to detect the promoter-gene fragment (35S-ASAL or rolC-ASAL) in transgenic tobacco plants. PCR reaction mixture was run in thermal cycler at 94°C for 5 min followed by 35 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. The PCR amplified products were checked on 1% agarose gel to screen transgenic tobacco plants.

Integration and copy number of transgene (*asaI*) was detected in T_0 tobacco lines through Southern blotting. For this genomic DNA was extracted from the transgenic tobacco expressing ASAL under 35S and rolC promoters. DNA (100 µg) samples were restricted using *Eco*RI at 37°C overnight and then subjected to the electrophoresis using 0.8% agarose gel at 25 volts overnight. Fractionated DNA fragments were transferred to the positively charged nylon membrane. Nylon membrane was crosslinked with UV linker (0.240 J/cm^2) (Strata linker) and hybridized with DIG (digoxigenin) labelled probe. Nylon membrane was washed using Dig Wash and Blocking Buffer Set (Cat No.11585762001 Roche) and signals were developed using color substrate NBT/BCIP.

qRT-PCR for gene expression analysis

qRT-PCR was conducted for relative gene expression analysis of *asaI* gene in T_0 transgenic tobacco lines. RNA was isolated from the 3-4 leaf stage (45 days old) transgenic and non-transgenic tobacco plants using SV Total RNA Isolation System (Cat No. Z3101, Promega). Primers were designed using Primer3 program. Concentration of primers were optimized, and qPCR reaction mixture was prepared containing, cDNA (200 ng), Power SYBR Green PCR Master Mix (Thermo Scientific), gene specific primers (qASAL-F5, qASAL-R5) and 18S rRNA as internal control (Table 1). Three replicates/sample were loaded on 96 well plate to run in Quantstudio 6 Real-time PCR system (Thermo Fisher Scientific) using following conditions; 95 ^oC for 3 min, 39 cycles at 95 ^oC for 30 sec, 56 ^oC for 30 sec and 72 ^oC for 50 sec. Relative gene expression of each transgenic tobacco line was measured with ΔΔCt method.

Aphid bioassay

A culture of a tobacco-adapted *M. persicae* strain (Ramsey et al. 2007; Ramsey and Jander 2008) leaves of tobacco lines, LS-15, LS-17, LS-18, LS-20, LS-21 and LS-25 expressing ASAL under 2X35S promoter and tobacco lines, LR-1, LR-3, LR-7, LR-10, and LR-12 expressing ASAL under the rolC promoter were placed on Petri plates containing 1% agar. Detached leaves of non-transgenic tobacco were set parallel to transgenic tobacco lines as control. Five adult aphids were released on each leaf of transgenic and non-transgenic plants. Mortality and fecundity of adult aphids were observed after every 24 hours for thirteen days.

Statistical analysis

Significant difference of relative gene expression was determined between transgenic and non-transgenic tobacco plants using ANOVA followed by LSD. Mann-Whitney *U*-tests was used to calculate significant difference of mortality and fecundity between aphid feeding on transgenic and non-transgenic tobacco.

Results

Cis-regulatory elements of promoters

PlantCARE software identified several motifs in 35S and rolC promoter sequence. Phytohormone responsive elements like auxin, methyl jasmonic acid and abscisic acid responsive elements were identified in both 35S and rolC promoter. Light responsive elements were frequently found dispersed throughout the rolC promoter. The predicted regulatory elements of both promoters have been illustrated in Table 2.

In-silico analysis of *asal* gene

Signal peptide of ASAL consist of 30 residues and belongs to the secretary protein with Reliability Class 2. The theoretical weight of ASAL was estimated at 19.25 kDa with an isoelectric point (pI) 9.05. PROSITE tool predicted bulb-type lectin domain in ASAL that spanned over 31-140 residues that is D-mannose specific domain and contains disulfide bond between 59 and 83 residues. ConSurf results estimated the evolutionary conservation of amino acid positions in ASAL protein molecule based on the phylogenetic relations between homologous sequences (Fig. 1A-B). The server results were predicted in the range of scale 1–9, where scale 1 is highly variable and scale 9 is highly conserved. Besides the conservation scale, the server also predicts whether the amino acid is functional or structural or exposed or buried (Fig. 1C). Phylogenetic analysis revealed the homology of ASAL protein among different species of Allium and other related families of plant species (Fig. 1D).

Construct development for plant transformation

Full length *asal* gene of 546 bp was successfully cloned in plant transformation vector pGA482 under 2X35 promter (Fig. 1E). Restriction analysis of resulting plasmid pGA482-35S-ASAL using *Hin*dIII produced two fragments (1.3 kb and 13.3 kb), *Eco*RI produced two fragments (3.8 kb and 10.8 kb), while *Sma*I produced three fragments of 766 bp, 6.3 kb, 7.4 kb (Fig. 1E).

Successful cloning of full length *asal* gene of 546 bp under rolC promoter in pGA482 confirmed in resulting plasmid pGA482-rolC-ASAL using *Hin*dIII that produced two fragments (857 bp, and 13.8 kb). Restriction with *Cla*I linearized the plasmid while *Cla*I and *Xba*I digestion produced two fragments of 1.33 kb, and 13.4 kb (Fig. 1F).

Molecular analysis of transgenic tobacco

Gene specific primer (ASAL-F3, ASAL-R3) amplified full-length *asal* gene of 546 bp and construct-specific primer pair (35S-F1, ASAL-R3) amplified promoter-gene fragment of 643 bp in transgenic tobacco lines LS-15, LS-17, LS-18, LS-20, LS-21, LS-25 expressing ASAL under 2X35S promoter. (Fig. 2A-B). PCR of transgenic tobacco lines expressing ASAL under rolC promoter using gene specific primers confirmed the presence of *asal* gene of 546 bp in tobacco lines LR-1, LR-3, LR-7, LR-10, LR-12 while, *asal* gene was not amplified in LR-8 and LR-9 (Fig. 3A). Promoter gene fragment (rolC-ASAL) of 1.3 kb was amplified in tobacco lines LR-1, LR-3, LR-7, LR-10, LR-12 using construct specific primer pair (Fig. 3B). Results of Southern blot analysis of T_0 transgenic tobacco lines LS-18, LS-21, LR-10, and LR-12 confirmed integration of transgene *asal* in host tobacco genome (Fig. 4).

Findings of qRT-PCR showed that each transgenic tobacco line had variable expression of ASAL under 35S promoter. The maximum expression was observed in transgenic tobacco line, LS-21 followed by LS-18, LS-15, LS-17, LS-25 and LS-20. Transgenic tobacco LS-21 has 1 fold, 1.2 folds, 4 folds, 6 folds and 16 folds higher gene expression than LS-18, LS-15, LS-17, LS-25 and LS-20 respectively (Fig. 5). Variable, relative gene expression was also observed in transgenic tobacco lines expressing ASAL under rolC promoter. Transgenic tobacco, LR-1 has 1.2 folds, 1.7 folds, 5.8 folds, 9.6 folds higher gene expression than LR-12, LR-10, LR-3 and LR-7, respectively (Fig. 6).

Aphid bioassays

Data of aphid bioassay revealed entomotoxic effect of ASAL expressed under 35S promoter or rolC promoter. Survival and fecundity of adult aphids reduced during feeding on transgenic tobacco. Tobacco lines expressing ASAL under 35S showed considerable level of resistance against aphids than non-transgenic tobacco (Fig. 7A-7C). Tobacco lines LS-17 and LS-20 showed 100% aphid mortality while 80-90% mortality was showed by LS-15, LS-18, LS-21 and LS-25 (Fig. 7D). Different transgenic lines showed lethal effects on aphids during different time interval. LS-18 showed aphid mortality after 48 hours while LS-21 showed aphid mortality after 72 hours. Expression of ASAL in transgenic tobacco under 35S promoter also reduced the population of aphids and retarded the aphid fecundity. Transgenic tobacco lines showed up to 63% reduction in aphid fecundity than non-transgenic tobacco plants (Fig. 7E).

Transgenic tobacco lines expressing ASAL under rolC promoter showed significant resistance against aphids than non-transgenic tobacco (Fig. 8A-C). Results of bioassay revealed that tobacco lines, LR-1 and LR-7 showed 100% aphid mortality while transgenic lines, LR-3, LR-10 and LR-12 showed 50-70% aphid mortality (Fig. 8D). Aphid's fecundity reduced up to 78% on transgenic tobacco expressing ASAL under rolC promoter (Fig. 8E).

Discussion

Aphids are deleterious crop pest that drain off phloem sap, transmit plant viruses and secret honeydew that allow the growth of molds (Eid et al. 2018). Expression of insecticidal genes in transgenic plants is an effective strategy to control sap sucking insect pests. Lectins have emerged as promising and eco-friendly insecticidal proteins for the development of insect resistance crop plants. Lectins can tolerate proteolytic effect of insect mid gut and confer entomotoxic effect against sap sucking insect pests, aphids (Caccia et al. 2012). In present study we have evaluated the efficacy of a mannose binding lectin, ASAL under 2X35S constitutive promoter and rolC phloem specific promoter.

Choice of promoter is an effective criterion to achieve higher expression levels of a gene in transgenic plants (Hu et al. 2003). In the present study, we used 2X35 and rolC promoter to drive the expression of *asal* gene in transgenic tobacco plants. In silico study detected different cis-acting elements in both promotes. Presence of core transcriptional, metabolism, light responsive, temperature and phytohormones related cis-elements in 2X35S promoter showed its constitutive nature as studied earlier (Kay et al. 1987; Lam 1994; Benfey et al. 1990). Several cis-acting elements detected in the promoters of 2X35 and rolC were common. However, light responsive elements like AT1-motif, Box 4, Box II, LAMP element, Pc-CMA2c, and Sp1 were abundant in rolc promoter. It is reported that phloem tissues require light energy for conductivity of sap (Epron et al. 2019), therefore frequent distribution of light responsive elements in rolC sequence might contribute towards the phloem specificity of this promoter. The presence of these motifs and a canonical 13bp element phloem specific signatures in rolC promoter is in agreement with another study where they characterized rolC promoter using motif-based approach combined by EMSA (Saha et al. 2007). Moreover, phloem specific motifs found here in rolC promoter are concurrent with the previous studies of tissue specific promoters characterization (Hehn and Rohde 1998; Srivastava et al. 2014).

Allium sativum leaf agglutinin (ASAL) is a mannose-binding lectin having potent insecticidal activity against sucking complex as well as bollworms (Ghosh et al. 2016). Previous studies have reported 50% hydrophobic nature of signal peptide of ASAL that allows its synthesis on endoplasmic reticulum and follows the secretory pathway (Damme et al. 1998). Our results of ConSurf model (Fig. 1A-D) prediction also indicated 50% part of signal peptide is exposed (hydrophilic) while 50% part is embedded (hydrophobic) while subcellular localization of ASAL through TargetP 1.1 indicated its secretory nature. In-silico analysis of *asal* gene predicted N-terminal signal peptide and B-type lectin domain. The degree to which an amino acid position is evolutionarily conserved is strongly dependent on its structural and functional importance. Thus, conservation analysis of positions among members from the same family often revealed the importance of each position for the protein's structure or function. Lectin engineering could be used to enhance their toxicity and spectra (Van Holle et al. 2017; Hu et al. 2015; Bendre et al. 2018). Phylogenetic analysis revealed that ASAL and its orthologue are from important spice, ornamental and medicinal plants. So, it is inferred that ASAL lectin might have evolutionary importance for plant’s own defense against insect-pests. Moreover, due to distribution of this ASAL and other lectin proteins in mostly edible and medicinally important crops may show its safe history. This indicates that ASAL and its homologs are used safely to develop transgenic plants. The phylogenetic analysis revealed that the translated *asal* is related to B-lectin family and present across different families in the plant kingdom and

may have a widespread defensive role against pest-complex. A bulb lectin super-family (Amaryllidaceae, Orchidaceae and Aliaceae) contains a ~115 residue long domain. Each bulb-type lectin domain consists of three sequential β-sheet subdomains (I, II, III) that are inter-related by pseudo three-fold symmetry.

Tobacco lines expressing 35S-ASAL and rolC-ASAL were developed through *Agrobacterium* mediated transformation. ASAL expression levels in transgenic tobacco plants were considerably high as indicated by aphid bioassays. Tobacco lines, expressing ASAL under 35S promoter and rolC promoter showed considerable aphidicidal effects. It is reported that mannose binding lectin, *Galanthus navlis* agglutinin (GNA) showed equivalent insecticidal effects against sap sucking pests under both constitutive and phloem specific promoter (Rao et al. 1998). Earlier findings suggested that ASAL under 35S constitutive promoter and rolC phloem specific promoter showed less or more equivalent rate of mortality and reduction in fecundity of sap sucking insect pests (Chakraborti et al. 2009). Our study also supported the similar results as we found that expression of ASAL in transgenic tobacco caused significant aphid mortality and reduced aphid fecundity during aphid bioassays. Maximum tobacco lines (LS-17, LS-20, LR-1and LR-7) showed up to 100% aphid mortality and reduced aphid fecundity up to 78%.

qRT-PCR is a sensitive and quick approach to detect the expression of transgene. Results of qRT-PCR on showed variable expression of ASAL among different tobacco lines. Such variations in gene expression level have already been reported due to accumulation of variable level of transcripts in plants. This difference in level of gene expression and accumulation of transcript may due to positional effect of integrated transgene. It may cause detrimental effect on transgene expression. In tobacco lines, LS-17, LS-20, LS-25, LR-3 and LR-7 inverse relationship was observed between expression and resistance against aphid. Recombination and DNA methylation of transgene could affect the colinear relationship between gene expression and its resistance (Naqvi et al. 2017).

Copy number of selected transgenic tobacco lines were detected using southern blot analysis. Hybridization bands of >3.5 kb was detected in transgenic tobacco lines, LS-15, LS-18 and LS-21, harboring ASAL under 35S promoter and transgenic lines, LR-10 and LR-12 harboring ASAL under rolC promoter that reveled the stable integration of *asal* gene in each tobacco line. Presence of single hybridization band represented integration of single copy of T-DNA into tobacco genome. Single copy transgene integration in T_0 generation caused segregation of transgene in T_1 generation according to Mendelian inheritance (3:1) (Travella et al. 2005). Transgenic plants having single copy of introducing gene could preferably selected in breeding programs to develop commercialized varieties of plants. While, integration of multiple copy of T-DNA on single or multiple locus of host genome could be associated with co-suppression, non-Mendelian inheritance and instability of the transgenes (Matzke and Matzke 1998).

In the present study, the expression of ASAL was achieved under 35S promoter and rolC promoter to evaluate the efficiency of both promoters. It was found that irrespective to the type of promoters the expression and efficiency of ASAL was equivalent effective against aphids. Previous studies reported that expression of transgene under constitutive promoter could be high and/or equivalent to phloem specific

promoter (Nakasu et al. 2014). It is also reported that mannose binding lectins expressing under both constitutive and phloem specific promoter showed equivalent insecticidal activity against sap sucking pests (Saha et al. 2007). Therefore, we conclude that ASAL expressed under both 2X35S and rolC promoters could be used for transformation of other crops. Furthermore, ASAL could be used in combination with other insecticidal genes for durable and sustainable insect pest control.

Conclusions

Evaluation of ASAL (MN820725) under 35S and rolC promoter, respectively revealed its entomotoxicity against aphid, *Myzus persicae*. Results of qRT-PCR verified significant expression of ASAL in transgenic tobacco lines. While, aphid bioassay reconnoitered that expression of ASAL in tobacco lines LS-17, LS-20, LR-1, LR-7 caused up to 100% aphid mortality and reduced their fecundity than control tobacco. These findings suggested the potential of ASAL that could be effectively used to engineer insect resistant crop varieties against various sucking insect pests.

Abbreviations

ASAL: *Allium sativum* leaf agglutinin, **CTAB**: Cetyl trimethylammonium bromide, **PCR**: Polymerase chain reaction, **qRT**: Quantitative reverse transcriptase, **kb**: kilo bites, **bp**: Base pair, **kDa**: kilo Dalton

Declarations

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Conflict of interest

Authors declare that they have no conflict of interest.

Availability of data and material

Not applicable

Code availability

Not applicable

Author contributions

MA conceived and supervised this study. NU, MA and RZN were involved in designing the constructs and vectors. NU performed all the cloning experiments, analyzed data, interpreted the results, and wrote first draft of this manuscript. NA and HAS performed *in silico* analysis and its interpretation. NU and IR conducted aphid bioassays and analyzed the results. NU and RZN analyzed and interpreted qRT-PCR data. MA keenly revised and shaped the final manuscript. SA and SN participated in tissue culture experiments and gave their suggestions and recommendations in this regard.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

All authors are agree to submit this publication

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Tables

Table 1

Primers used for cloning, PCR and qRT-PCR

Prime name	Primer sequence 5— 3
35S-F1	CTGACGTAAGGGATGACGCAC
rolC-F2	ATCGATGCGAAAGCGGCATTGGCAAA
ASAL-F3	AAGCTTATGGGTCCTACTACTTCATC
ASAL-R3	GAATTCTCAAGCAGCACCGGTGCCAA
qASAL-F5	CCAATCACTGGATGTAGAACA
qASAL-R5	CGTCCGTTAACATCGTAGA
18S-F6	GTGGTGCATGGCCGTTCTTA
18S-R6	AACTTCCGCGGCCTAAAAGG

Table 2

Cis-acting elements associated with 35S promoter and rolC promoter

Promoter	Motif name	Organism	Position	Sequence	Function
Cis-acting elements found common in both 35S and rolC promoter	ABRE	*Arabidopsis thaliana*	610	ACGTG	cis-acting element involved in the abscisic acid responsiveness
	CAAT box	*Nicotiana glutinosa*	60	CAAT	common cis-acting element in promoter and enhancer regions
	CGTCA motif	*Hordeum vulgare*	436	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness
	DRE box	*Arabidopsis thaliana*	98	GCCGAC	dehydration responsive element
	G box	*Zea mays*	610	CACGTC	cis-acting regulatory element involved in light responsiveness
	G box	*Zea mays*	271	CACGTC	cis-acting regulatory element involved in light responsiveness
	TATA box	*Arabidopsis thaliana*	700	TATATA	core promoter element around -30 of transcription start
	TGA box	*Brassica oleracea*	766	AACGAC	auxin-responsive element
	TGACG box	*Hordeum vulgare*	436	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness
Cis-acting elements found in 35S	AuxRR-bov	*Nicotiana tabacum*	222	GGTCCAT	cis-acting regulatory

promoter					element involved in auxin responsiveness
	LTR	*Hordeum vulgare*	72	CCGAAA	cis-acting element involved in low-temperature responsiveness
	LTR	*Hordeum vulgare*	411	CCGAAA	cis-acting element involved in low-temperature responsiveness
	O2 site	*Zea mays*	112	GATGA(C/T) (A/G)TG(A/G)	cis-acting regulatory element involved in zein metabolism regulation
	O2 site	*Zea mays*	451	GATGA(C/T) (A/G)TG(A/G)	cis-acting regulatory element involved in zein metabolism regulation
Cis-acting elements found in rolC promoter	A- box	*Petroselinum crispum*	147	CCGTCC	cis-acting regulatory element
	ARE	*Zea mays*	359	AAACCA	cis-acting regulatory element essential for the anaerobic induction
	AT1	*Solanum tuberosum*	457	AATTATTTTTTATT	part of a light responsive module
	Box 4	*Petroselinum crispum*	85	ATTAAT	part of a conserved DNA module involved in light responsiveness
	Box 4	*Petroselinum crispum*	702	ATTAAT	part of a conserved DNA module involved in light responsiveness

BoxII	*Solanum tuberosum*	223	TGGTAATAA	part of a light responsive element
LAMP element	*Pinacia oleracea*	204	CCTTATCCA	light responsive element
Pc-CMA2C	*Pisum sativum*	282	GCCCACGCA	light responsive element
Sp1	*Oryza sativa*	287	GGGCGG	light responsive element
TATC-box	*Oryza sativa*	387	TATCCCA	cis-acting element involved in gibberellin-responsiveness
13 bp element	*Nicotiana tabacum*	405	TTAAGAGACCCTA	Cis-acting element involved in phloem specificity

Figures

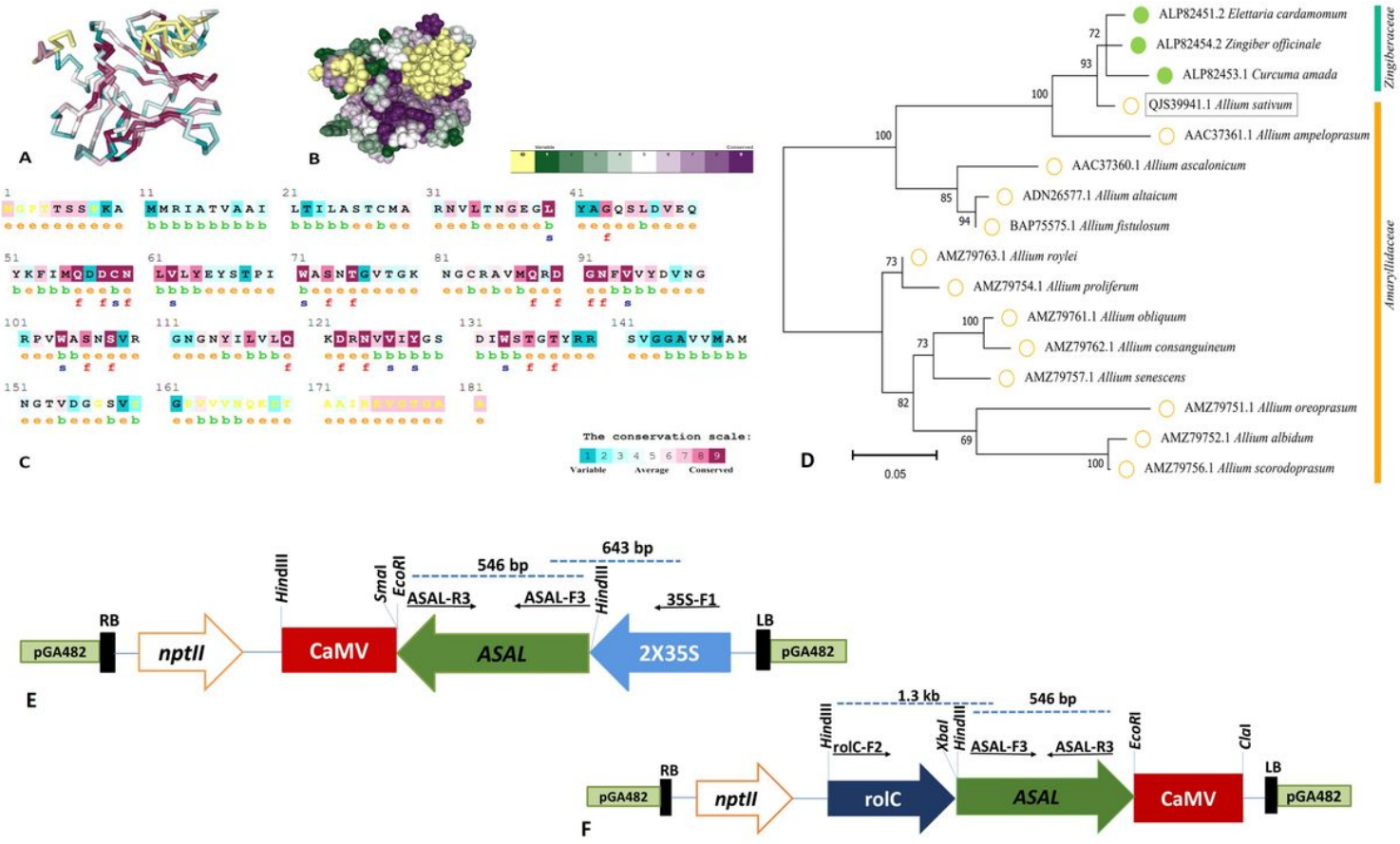

Figure 1

In-silico analysis and vector map of ASAL under 2X35S and rolC promoter in pGA482. (A) ConSurf analysis for the ASAL predicted backbone style, (B) space fill style, (C): The amino-acids are 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 (D) Phylogenetic analysis of ASAL protein and its orthologous from different species. (E)Vector map of plasmid pGA482-35S-ASAL to represent cloning of asal gene under 35S promoter in pGA482. (F) Vector map of plasmid pGA482-rolC-ASAL to represent cloning of asal gene under rolC promoter in pGA482.

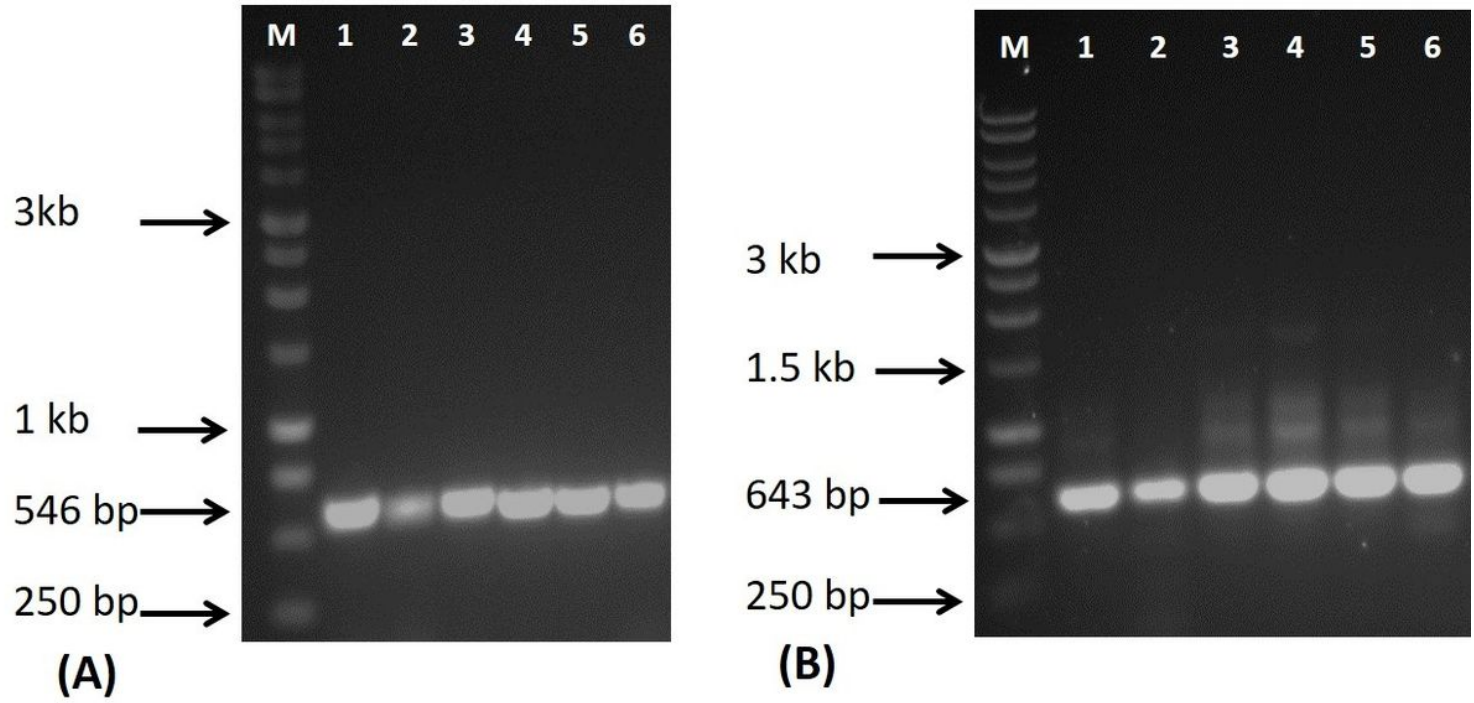

Figure 2

PCR based detection of transgenic tobacco plants having 35S-ASAL. (A) PCR amplification of full length asal, gene using ASAL-F3 and ASAL-R3 primer pair (product 546 bp). M: 1 kb DNA ladder. Lane# 1-6: Transgenic tobacco lines LS-15, LS-17, LS-18, LS-20, LS-21, LS-25. (B) PCR amplification of promoter-gene (35S-ASAL) fragment using primer pair 35S-F1 and ASAL-R3 (product 643 bp). M: 1 kb DNA ladder. Lane# 1-6: Transgenic tobacco lines, LS-15, LS-17, LS-18, LS-20, LS-21, LS-25.

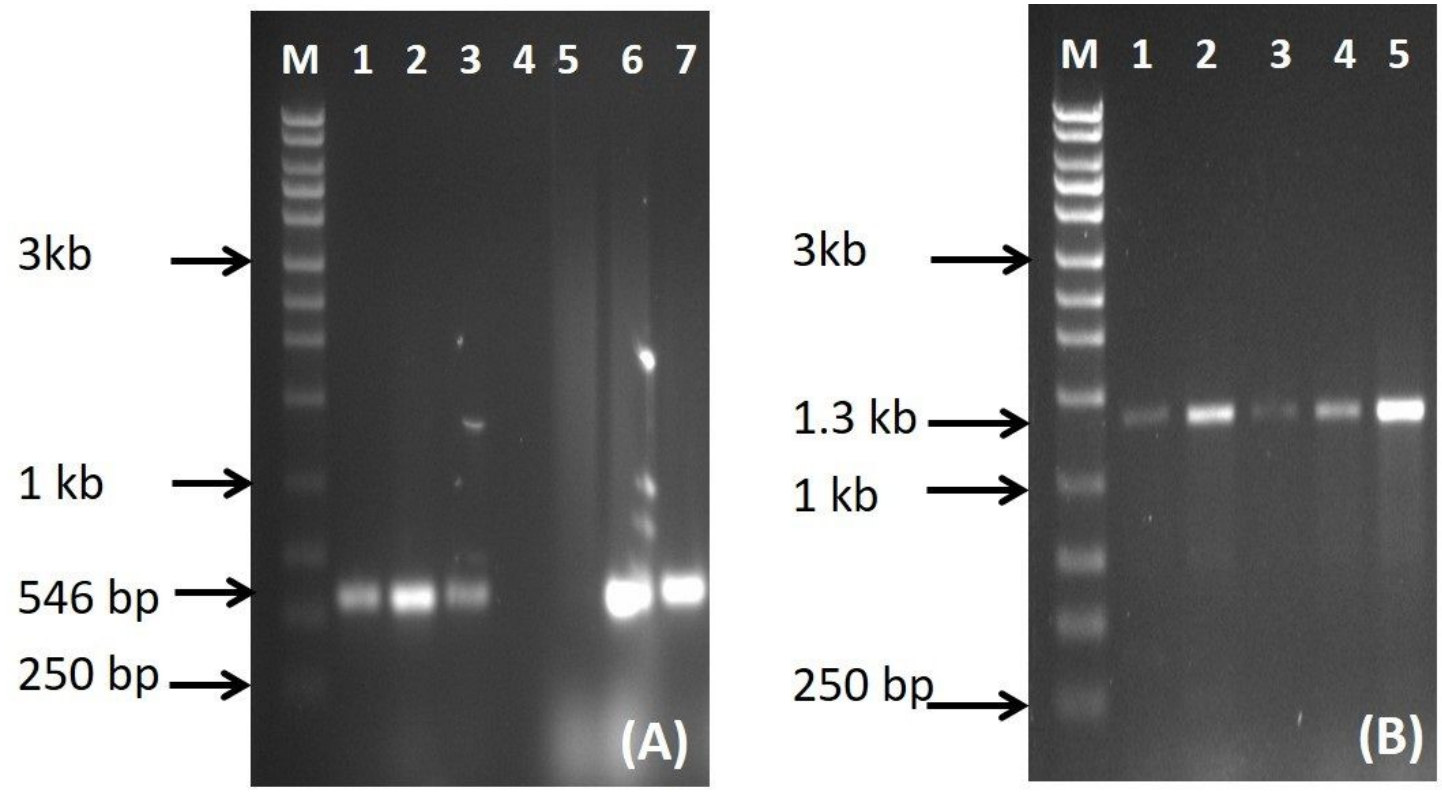

Figure 3

PCR based detection of transgenic tobacco plants having rolC-ASAL. (A) PCR amplification of asal, gene using ASAL-F3 and ASAL-R3 primer pair (product 546). M: 1 kb DNA ladder. Lane# 1-7: Putative transgenic tobacco lines LR-1, LR-3, LR-7, LR-8, LR-9, LR-10, LR-12. (B) PCR amplification of promoter-gene (rolC-ASAL) fragment using rolC-F2 and ASAL-R3 (product 1.3 kb). Lane# 1-5: Transgenic tobacco lines LR-1, LR-3, LR-7, LR-10, LR-12.

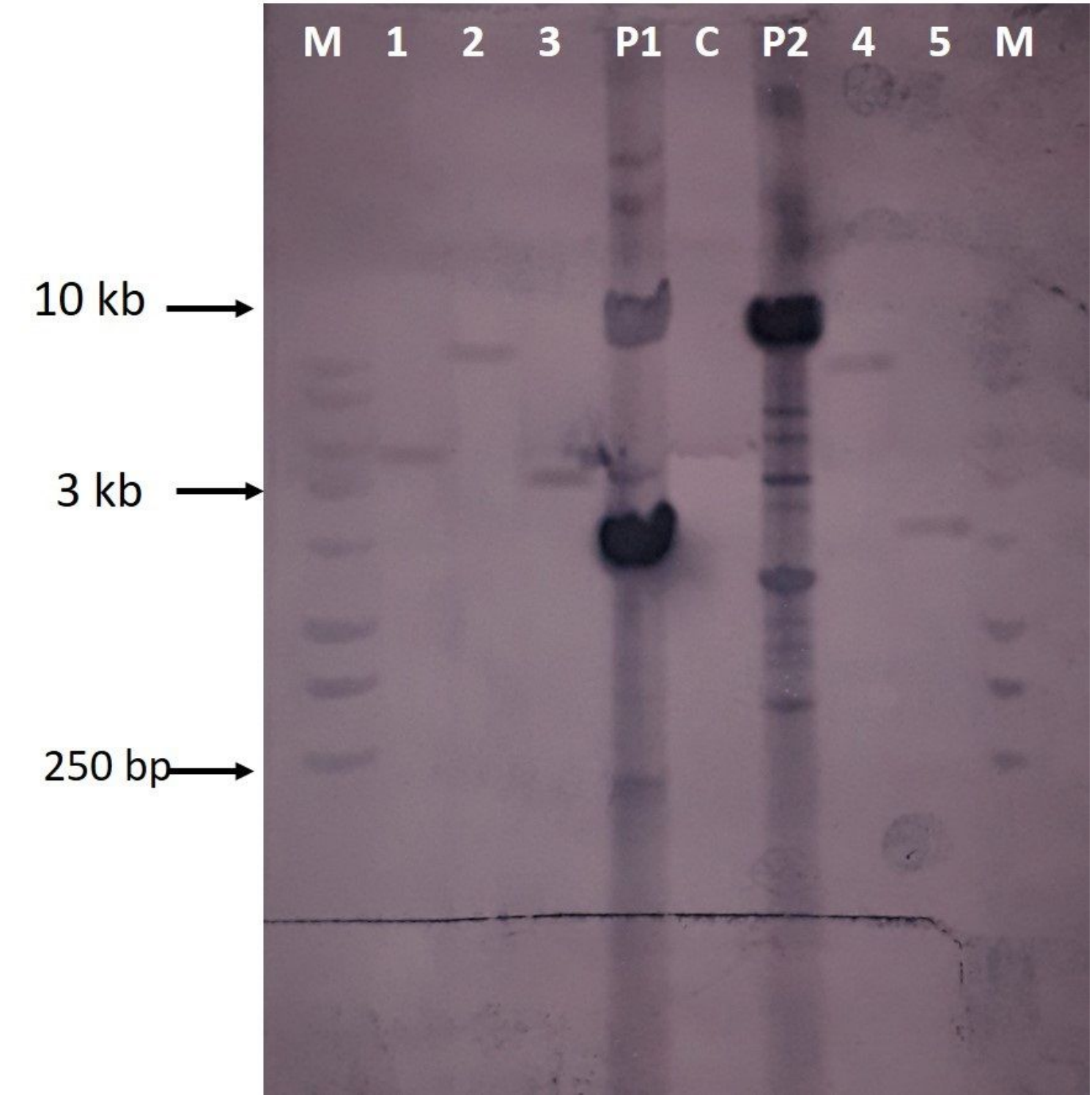

Figure 4

Southern blot analysis to confirm integration of transgene in genomic DNA of tobacco plants restricted with EcoRI. M: 1 kb DNA ladder. P1: Plasmid (pGA482-35S-ASAL). P2: Plasmid (pGA482-rolC-ASAL). C: Restricted genomic DNA of non-transgenic tobacco. Lane # 1-3: 35S-ASAL expressing tobacco lines, LS-15, LS-18, and LS-21. Lane # 4-5: rolC-ASAL expressing tobacco lines LR-10, and LR-12.

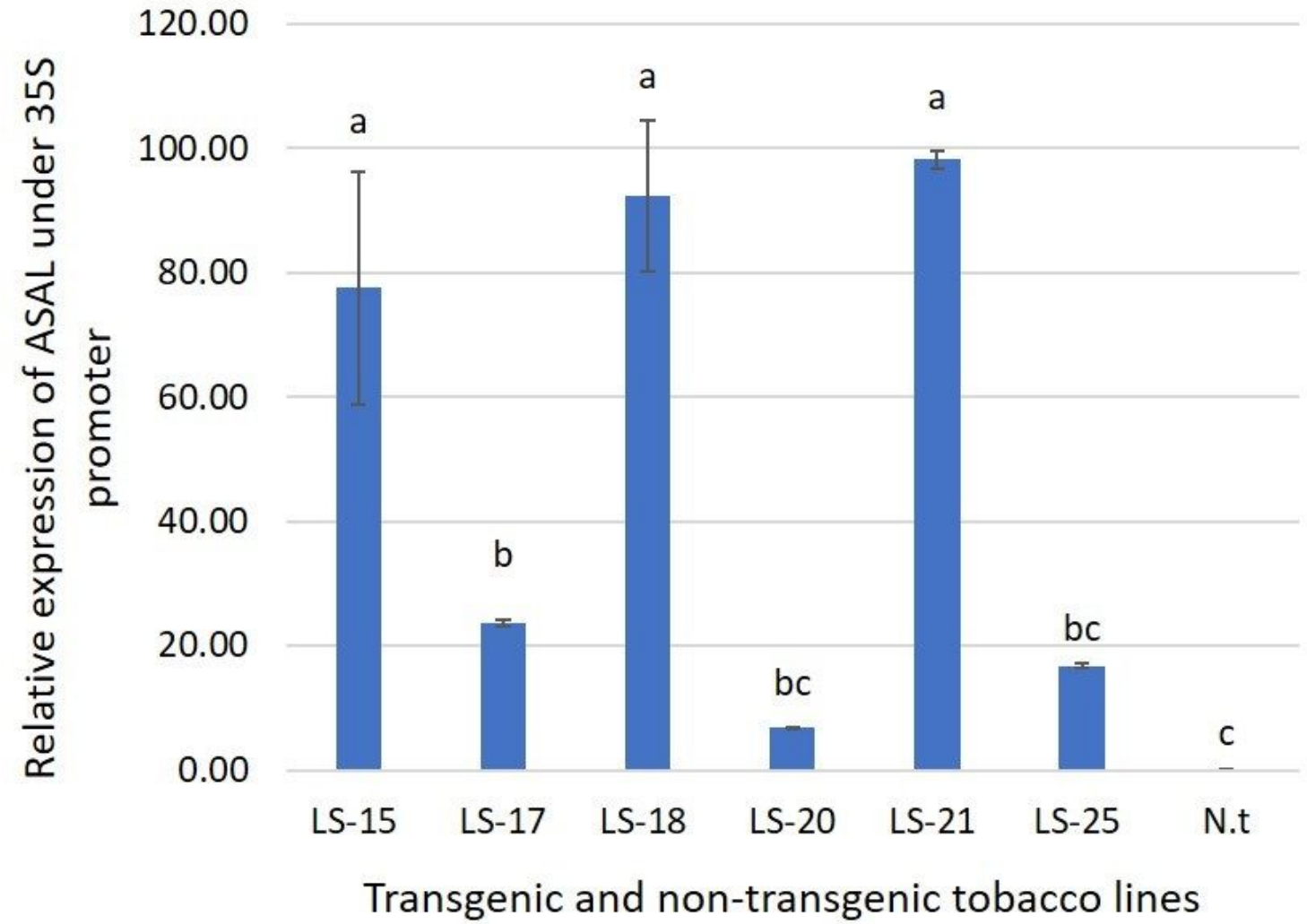

Figure 5

qRT-PCR for gene expression analysis of ASAL under 35S promoter in transgenic tobacco lines. LS-15, LS-17, LS-18, LS-20, LS-21 and LS-25: Transgenic tobacco lines expressing ASAL under 35S promoter. N.t: Non-transgenic tobacco line. Column height shows expression level of transcript, ASAL. Alphabets on error bars indicate significant differences of expression among transgenic and non-transgenic tobacco at $p<0.01$ using ANOVA followed by LSD ; Mean ± SE of N = 3.

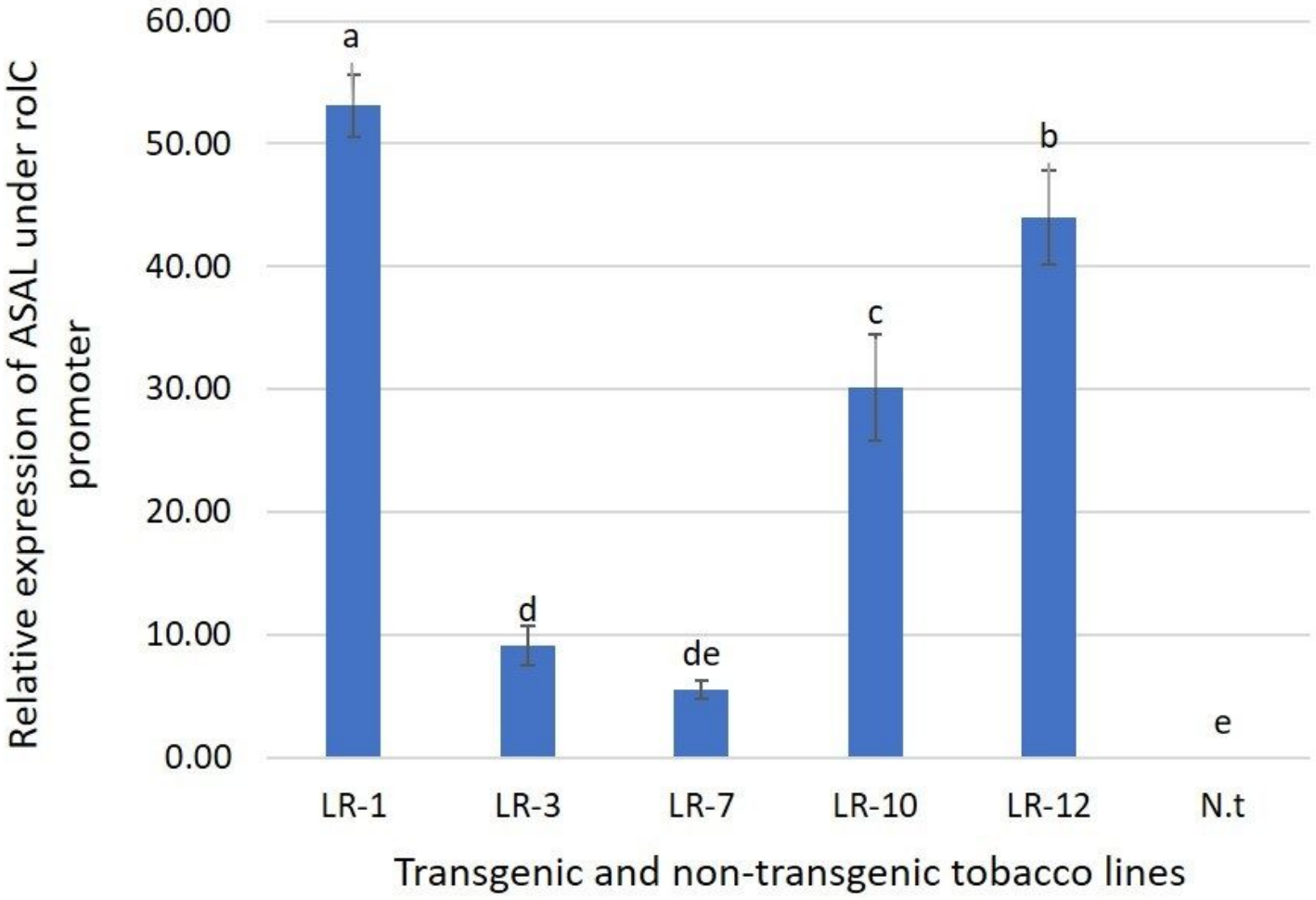

Figure 6

qRT-PCR for gene expression analysis of ASAL under rolC promoter in transgenic tobacco lines. LR-1, LR-3, LR-7, LR-10 and LR-12: Transgenic tobacco lines expressing ASAL under rolC promoter. N.t: Non-transgenic tobacco lines. Column height shows expression level of transcript, ASAL. Alphabets on error bars indicate significant differences of expression among transgenic and non-transgenic tobacco at $p<0.01$ using ANOVA followed by LSD ; Mean ± SE of N = 3.

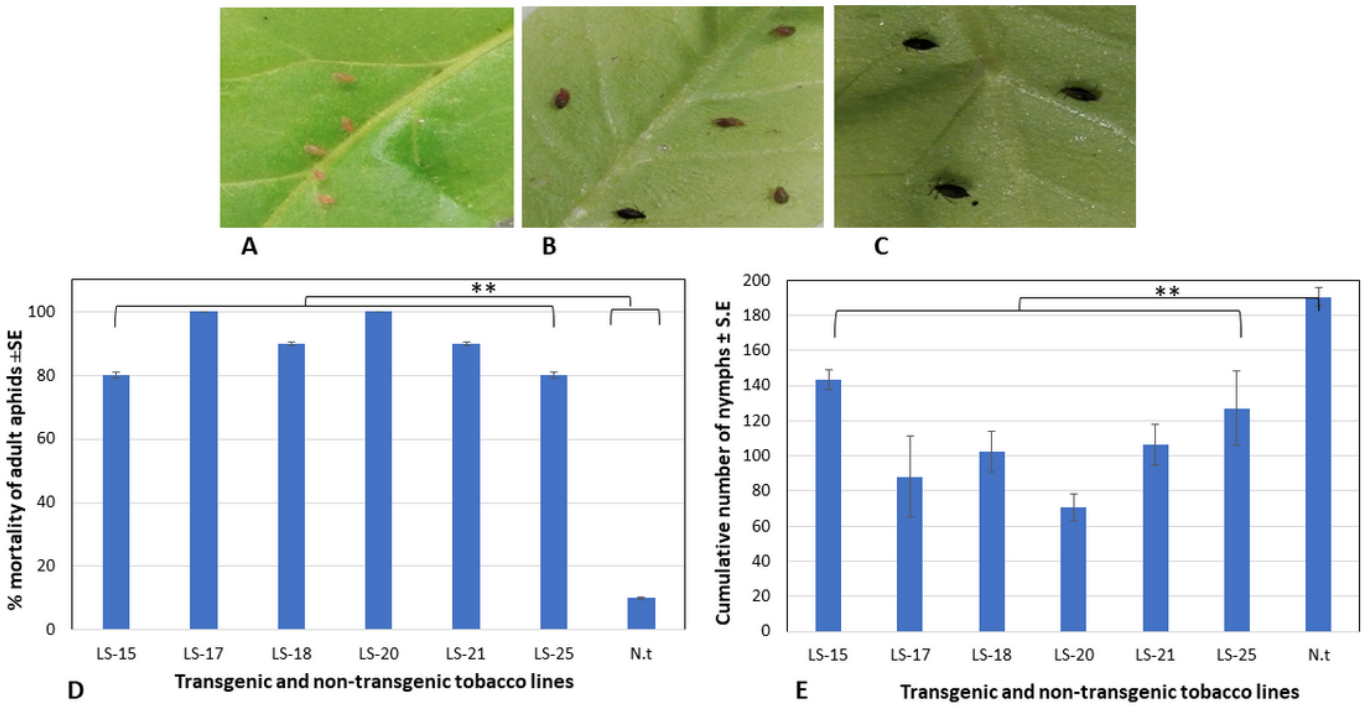

Figure 7

Aphid bioassay of transgenic tobacco lines expressing ASAL under 35S promoter. (A) Adult aphids feeding on non-transgenic plants showed no mortality. (B-C) Aphid mortality during feeding on transgenic detached leaves. (D) Percent mortality of adult aphids on tobacco lines (LS-15, LS-17, LS-18, LS-20, LS-21, and LS-25) versus control tobacco after every 24 hours for thirteen days. (E) Number of cumulative nymphs released by adult aphids from day 5-13 on transgenic tobacco lines (LS-15, LS-17, LS-18, LS-20, LS-21, and LS-25) versus control tobacco plants. Double steric represent the significant difference among 35S-ASAL tobacco lines and non-transgenic tobacco using Mann-Whitney U-test; Mean ± SE of N = 2, **P < 0.01.

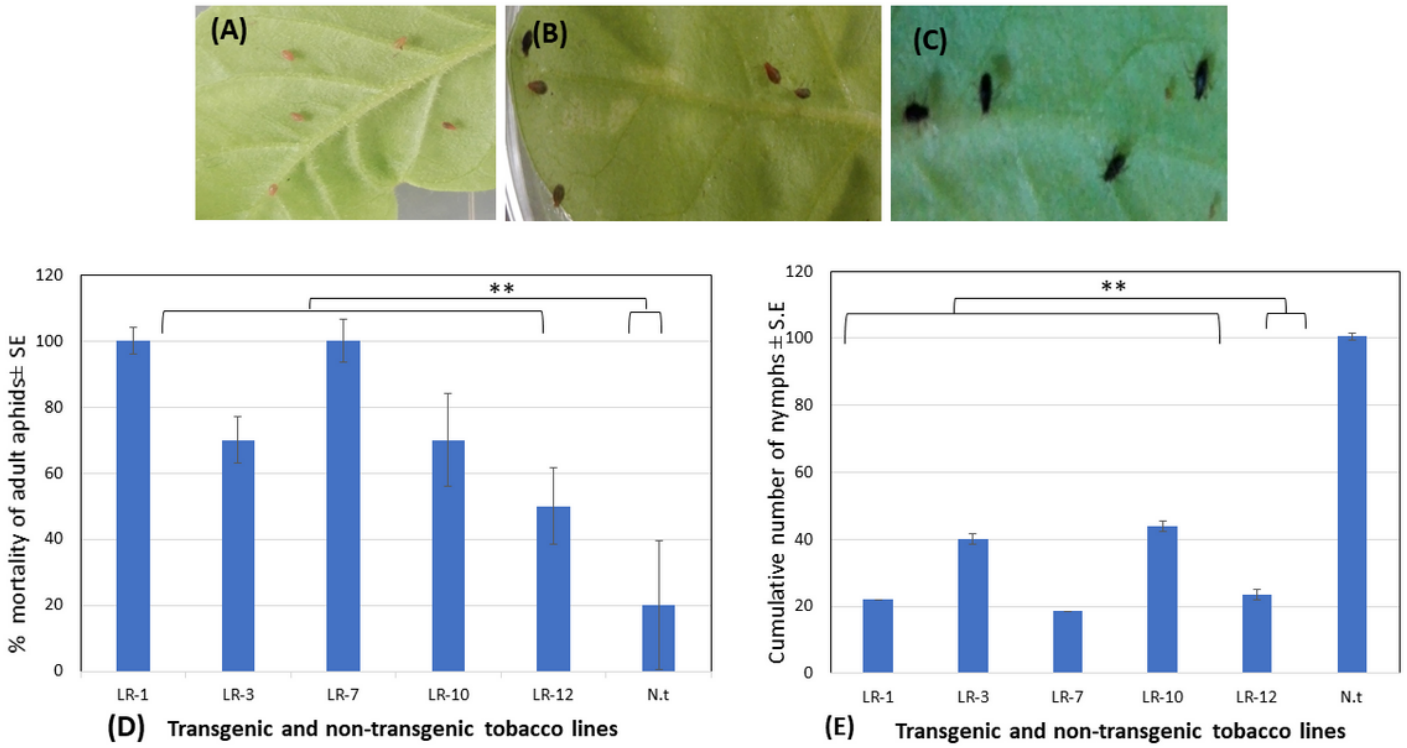

Figure 8

Aphid bioassay of transgenic tobacco lines expressing ASAL under rolC promoter. (A) Adult aphids feeding on non-transgenic plants showed no mortality. (B-C) Aphid mortality during feeding on transgenic detached leaves. (C) Percent mortality of ten adult aphids on each tobacco lines (LR-1, LR-3, LR-7, LR-10 and LR-12) versus control tobacco after every 24 hours for thirteen days. (D) Number of cumulative nymphs produced by adult aphids on transgenic tobacco lines (LR-1, LR-3, LR-7, LR-10 and LR-12) versus control tobacco plants from day 5-13 after 24 hours. Double steric show the significant difference among rolC-ASAL tobacco lines and non-transgenic tobacco using Mann-Whitney U-test; Mean ± SE of N = 2, **P < 0.01.