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ORIGINAL ARTICLE

Novel-iridoviral kinase induces mortality and reduces performance of green peach aphids (*Myzus persicae*) in transgenic *Arabidopsis* plants

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Abstract

Insect pests are a serious threat to agricultural productivity. Genetically-modified crops offer a promising alternative, and *Bacillus thuringiensis* endotoxin genes have played a major role in this respect. However, to overcome insect tolerance issues and to broaden the target range, it is critical to identify alternative insecticidal toxins working through novel mechanisms. In our previous study, a kinase from *Chilo* iridescent virus (CIV) that has insecticidal activity was identified and designated as Iridovirus Serine/Threonine Kinase (ISTK). A 35 kDa truncated form of ISTK, designated iridoptin, was obtained during expression and purification of ISTK in the yeast system. This yeast-expressed CIV toxin induced 50% mortality in cotton aphids and 100% mortality in green peach aphids (GPA). In this study, codon optimized *ISTK* gene and *iridoptin* fragment (designated *oISTK* and *oIRI,* respectively) were designed by altering the codon usage features that are seldom present in plant exons. Codon-optimized gene(s) cloned into plant expression vectors were used to stably transform *Arabidopsis* plants. PCR analysis of genomic DNA of transformed plants confirmed the presence of the DNA insert (*oISTK*/*oIRI*) in selected transgenic lines. Further screening was performed by selecting the PCR positive lines, which showed expression of respective toxins at the polypeptide level, using Western blot analyses. Codon-optimized gene constructs resulted in significant improvement in levels of expression of ISTK and iridoptin polypeptide and confirmed its stability in planta. The stable lines expressing either of the two toxin forms induced moderate to very high mortality in GPAs and significantly affected GPA development and fecundity.

Keywords Transgenic insect-resistant plants · Green peach aphids · *Chilo* iridescent virus · Serine/threonine kinase · Plant-incorporated protectants

Introduction

Insect pests are a key factor in reduction of agricultural productivity. The economic impact of insect pests exceeds $200 billion globally (Pimental 2009) and is $33 billion for the U.S. (USBC 2001). Extensive use of chemical insecticides dominates the current insect control strategies. Genetically modified (GM) crops offer a promising alternative toward reducing the insecticide load in agricultural systems. Insect-resistant transgenic crops commercialized thus far almost always have utilized genes derived from the bacterium *Bacillus thuringiensis (Bt)*.

Aphids (Order: Hemiptera) are sap-sucking insects and a global crop pest. They cause serious economic damage via direct feeding and via transmission of viral diseases, resulting in crop loss estimated to be hundreds of millions of dollars (Head 1992; Yu et al. 2014). There are over 250

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species of aphids that are considered serious pests for agriculture (Blackman and Eastop 2000) and green peach aphids (GPAs; *Myzus persicae*) are key pests of many agriculturally important crops within this order. They are highly capable of transmitting more than 100 plant viruses, resulting in significant yield reductions (Blackman and Eastop 2000). Apart from the pest status, the rapidly developing chemical insecticide resistance has become a major problem in their control (Silva et al. 2012; Bass et al. 2014). *Bt* δ-endotoxins exhibit only low to moderate effects on aphid mortality and development (Faria et al. 2007; Lawo et al. 2009; Porcar et al. 2009; Li et al. 2011; Chougule et al. 2013). Transgenic approaches using lectin, proteinase inhibitor, plant derived R genes and RNAi control has been explored for aphid control with negligible to minimal success (Yu et al. 2014). However, so far, no transgenic approaches for host-plant resistance to aphids have been commercialized. Commercial breeding efforts have been hindered due to the limited availability of germplasm for plant resistance to aphids (Dogimont et al. 2010; Bhatia et al. 2012). Thus, there is a need for identification of new polypeptide toxins working through novel mechanisms for potential use in development of transgenic plants that are resistant to aphids.

Viruses of invertebrates are a potential source of insecticidal genes in the development of pest-resistant transgenic plants (Liu et al. 2006). *Chilo* iridescent virus or invertebrate iridescent virus 6 (CIV or IIV6) is a type species for genus *Iridovirus* and family *Iridoviridae*. CIV has been shown to induce mortality and deformity in cotton boll weevil (McLaughlin et al. 1972; Bilimoria 2001), significant reduction in cotton aphid (Bilimoria 2001) and citrus aphid (Hunter et al. 2001) populations, sub-lethal infections in scarab beetles (Jenkins et al. 2011) and covert infections in mosquito species (Marina et al. 2003). CIV virion protein extract (CVPE) induces apoptosis in spruce budworm and boll weevil cell lines which is dependent on JNK signaling pathway and apical caspase of the host cells (Bilimoria et al. 2001; Paul et al. 2007; Chitnis et al. 2008).

In our previous study, we showed that a full length iridovirus serine/threonine (s/t) kinase (ISTK) identified from CIV (ORF389L) and/or its truncated form (iridoptin) expressed in yeast induced apoptosis and/or host-protein shut off in insect cell lines (Chitnis et al. 2011). Iridoptin retains motifs for s/t kinase and an ATP-binding site, and Chitnis et al. (2011) demonstrated that kinase activity of this toxin is absolutely necessary for insecticidal activity, and that it presumably works through novel mechanism (Chitnis, Paul and Bilimoria, manuscript in preparation). Yeast-expressed and purified iridoptin induced mortality in green peach aphids (Becker, Ganapathy, and Bilimoria, unpublished) and cotton aphids (Parajulee and Bilimoria, unpublished).

These studies suggest an insecticidal role of ISTK and iridoptin, and the goal of this research was to assess the potential of transgenic plants expressing ISTK/iridoptin in aphid control. Expression of foreign genes in plants may be compromised due to mRNA instability and poor translational efficiency (Doran 2006; Jackson et al. 2014). This was overcome in the most successful *Bt* plants by codon optimization (Perlak et al. 1991). Many studies have indicated the necessity of redesigning genes of prokaryotic or non-plant eukaryotic origin for use in plant systems for proper expression (Perlak et al. 1990; Perlak et al. 1991; Sutton et al. 1992; Liu et al. 2003; Liénard et al. 2007; Wang et al. 2008; Wu et al. 2008; Webster et al. 2009; Sanahuja et al. 2011; Wu et al. 2011).

We hypothesized that viral ISTK/iridoptin nucleotide sequence requires codon optimization for robust expression in plants and ectopic expression of the optimized *ISTK* gene or iridoptin sub-fragment in plants will yield active and stable polypeptide in planta and this polypeptide will likely have aphicidal activity.

In this article, we present the development of transgenic *Arabidopsis* plants constitutively expressing the insecticidal iridoviral kinase, ISTK or their sub-fragment (iridoptin), and evaluate the aphicidal potential of these plants expressing the toxin by quantifying their effects on aphid growth and developmental duration, fecundity, and mortality. To the best of our knowledge, this is the first report of utilizing a novel- iridovirus-encoding kinase towards development of aphid-resistant transgenic plants.

Materials and methods

Codon-optimization and synthesis of the full-length *ISTK* gene sequence (1233 bp)

The native *ISTK* gene was optimized according to the plant codon usage (http://www.kazusa.or.jp/codon; *Arabidopsis thaliana* and *Nicotianna benthamiana*). The *ISTK* sequence was also modified to eliminate certain factors considered to be responsible for low mRNA stability and foreign-protein expression, and were proportionately replaced by their synonymous codons in the plant system. Splice sites were predicted using softberry FSPLICE (Find Splice Site in Genomic DNA) software accessible through http://www.softberry.com at 90 percent threshold and modified using the degenerate codons. Apart from the factors listed in Supplementary Table 1, localized AT rich regions in the viral gene sequences resembling plant introns were modified, consecutive strings of A + T or G + C nucleotides of 5 or more were avoided, G + C content in codon position 3 were preferred, pyrimidines (C, T) were used more frequently than purines (G, A) to mimic dicots. The final in-silico modified *ISTK*

sequence *(oISTK)* was artificially synthesized by Geneart (Life Technologies), Germany.

Construction of expression vectors

The *ISTK* gene (native and optimized; ~ 1.2 kb) and *iridoptin* sub-fragment (native and optimized; ~ 0.9 kb) were each cloned into plant binary vector constructed with a dual 35S enhancer promoter sequence extracted from the PRG229 vector generously donated by Dr. Roberto Gaxiola (Arizona State University) and introduced into the pBI121 vector backbone in place of the CaMV 35S promoter (in-house constructed vector designated pD35S). Apart from the constitutive promoter for the gene of interest (GOI), the vector also harbored 2X/3X flag tag at the 3′end of the GOI, and a *Npt* II (Neomycin phosphotransferase II*;* kanamycin resistance gene) selection marker. Cloned vectors were used to transform bacterial strain *DH5α* through chemical transformation. Cloning and gene sequences were confirmed by PCR, DNA sequencing, and restriction analysis. The confirmed vector constructs were used to transform *Agrobacterium tumefaciens* strain GV3101 by freeze/thaw shock treatment (Weigel and Glazebrook 2006).

Aphid rearing

GPAs were provided by Dr. Jyoti Shah (University of North Texas, Denton, TX). A GPA colony was then reared on an equal mixture of 2–3-week-old radish and mustard seedlings. Growth chamber conditions were maintained at 22 ± 2 °C with 14:10 h light:dark photoperiod and 50–60% relative humidity.

Plant transformation

Agrobacterium GV3101 clones containing the following plant binary vector constructs were used in the stable transformation of *Arabidopsis thaliana* (ecotype Columbia-0 or Col) plants: pD35S-*GUS*, pD35S-*oIRI*, and pD35S-*oISTK* using the modified floral dip method (Clough and Bent 1998). Mature T_1 seeds were harvested from the transformed plants. To select for putative stable and independent transformants (T_1 lines), the sterilized *Arabidopsis* seeds were plated on *Arabidopsis* seed germination media [1/2 Murashige Skoog (MS), 1% (w/v) Sucrose, 0.05% (w/v) MES, 0.8% (w/v) Agar, pH 5.8] containing 50 µg/mL kanamycin. To break dormancy, the sterilized seeds were kept at 4° C for 3 days before moving to room temperature (RT) under constant lighting. Selected transgenic T_1 lines were then moved to pots.

Analysis of transgenic plants by Polymerase Chain Reaction (PCR)

Genomic DNA was isolated from rosette leaves of 3–4-week-old putative transgenic lines (T_1) and PCR was carried out using GoTaq Green Master Mix (Promega). To ensure proper insertion of *oISTK* gene, forward primer was designed from the promoter region (5′ CTC GAG TGG CCA CCA TGG G 3′) and the reverse primer (5′ AAAA GAGCTCTCACTT GTC AT 3′) was gene sequence specific. For *oIRI* gene fragment, promoter-specific forward primer (5′ CTC GAG TGG CCA CCA TGG G 3′) and gene sequence-specific reverse primer (5′ AAAA GAGCTC TCACTT ATC GTC GTC 3′) were used. PCR conditions were set at 94 °C: 3 min; [94 °C: 45 s, 59.5 °C: 60 s, 72 °C: 90 s] 35 cycles; 72 °C: 5 min. Cloned pD35S-*oISTK* vector was used as a positive control for PCR amplification. Genomic DNA extracted from transgenic lines containing the *GUS* transgene was used as negative control. After PCR, samples were run on a 0.8% agarose gel, with 0.5 µg/mL ethidium bromide, for visualization of bands.

Screening T_1 lines using Western blot analysis

Arabidopsis rosette leaves (~ 10 mg; fresh weight) were grounded with 50 µL of protein extraction buffer [10 mM EDTA (pH 8.0), 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium lauryl sarcosine, 40 mM sodium phosphate buffer, pH 7.0, 10 mM β-mercaptoethanol, 1 µg/mL leupeptin, 1 µg/mL pepstatin A, and 1 mM phenylmethylsulfonyl fluoride], and incubated on ice for 45 min to extract total proteins (TP). After incubation, samples were centrifuged at 14,000 g for 20 min at 4 °C to collect supernatant. Protein concentration was determined using the Bradford assay (Bradford 1976). TP were separated and resolved in a 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After staining the blotted membrane with ponceau stain, it was blocked with blocking solution [5% (w/v) milk in Tris buffered saline- Tween 20 (TBS-T; 100 mM Tris–Cl, 154 mM NaCl; pH 7.5 with 0.1% Tween-20)] for 1 h (RT). Blocking was followed by primary antibody probing (Anti-flag antibody; Rockland, Cat # 600-401-383; 1:1000, with 2% milk-TBS-T) at 4 °C overnight and horse-radish peroxidase conjugated anti-rabbit secondary antibody (Novex, Cat # A16104; 1:5000; with 2% milk-TBS-T). Chemiluminescent substrate was used for detection.

Aphid bioassay

Several independent, transgenic T_1 lines, expressing ISTK/iridoptin at the protein level, were challenged with GPAs and investigated for their effects on survival, development, and reproductive rate of aphid populations via excised-leaf

bioassays. Fully expanded, young leaves were detached from the selected 5 to 6-week-old transgenic lines and placed in tightly sealed sterile petri dishes (Fisher Scientific, Cat # 09-720-503) with moist adsorbant pads at the bottom. Each leaf was inoculated with five newly born GPA nymphs (< 12 h old). Five replicate plates were used for each transgenic line resulting in 25 aphid nymphs per treatment. Every 3 days, old leaves were replenished with new leaves from respective transgenic lines. Mortality and reproductive rate (number of new nymphs produced by the initial inoculum after developing into adults) were recorded every 2–3 days. The insect mortality rate was used to measure the level of insect resistance of each independent transgenic line. Because normal aphid development takes about 6–7 days for a new born aphid to become an adult, the first day of reproductive phase of these initial cohorts was used as a cut-off point to assess nymphal development to adulthood [between 7 and 9 days after treatment (DAT)]. The percentage developmental success was calculated based on the number of initial aphid inoculum in the cohort that developed into adulthood at the cut-off DAT. Transgenic lines transformed with the *GUS* construct were used as negative controls. Bioassays were conducted in a growth chamber with conditions as detailed in aphid rearing. Aphid mortalities were analyzed as percentages in each replicate, and fecundity (parthenogenetic reproduction) was measured as the mean of the total number of newborn nymphs produced in each replicate. After counting, the neonate nymphs were removed from the experimental dish to avoid the crowding effect. The entire experiment was conducted in three sequential runs, but each run had a GUS negative control and the transgenic line with the highest mortality (positive control; oIRI line 23) as control treatments to ensure consistency across the runs. The sequential experiments were necessitated by the experimental logistics and the availability of the resources. The period during which the experiment was carried out was kept narrow and chamber conditions were kept identical.

Statistical analysis

Aphid mortality and fecundity data were all analyzed using a one-way analysis of variance, with transgenic line as a source of variation (SAS Institute, 2010). Treatment means were separated by Tukey–Kramer HSD test at the $\alpha = 0.05$ level. Data for three GUS control runs, three oIRI 23 runs and two oISTK 11 runs were pooled to verify the consistency of the experiment. Upon the verification that the three sequential runs were consistent, all runs were pooled for the final analysis (at 8 or 9 DAT).

Results

Native *ISTK/iridoptin* sequence analysis and optimization to better suit expression in plants

A wide range of factors that influence foreign gene expression in plants were considered in the in-silico modification of the native *ISTK* gene (1233 bp), resulting in a single-optimized gene sequence that could reach a detectable and improved expression level in plants. *Iridoptin* sequence is a sub-fragment of *ISTK* gene comprising of 1–909 bp. Nucleotide sequence analysis of the native *ISTK* gene revealed significant differences in codon usage between *ISTK* and plant genes, and presence of 23 potential mRNA degradation signals (ATTTA, ATTAA), five splice acceptor sites (AG) and five splice donor sites (GT) with reference to the plant system. The nucleotide sequence of the native *ISTK* gene was optimized by replacing the less preferred codon triplets with the respective highly preferred degenerate codons without altering the amino acid sequence. After codon optimization, the nucleotide sequence was again scanned and altered by modifying potential mRNA degradation signals, polyadenylation signals (AATAAA, AATAAT, AATTAA, and AACCAA), RNA polymerase II termination (polyA) signals (CAN[7–9]AGTNNA), splice acceptor sites (AG), splice donor sites (GT) and sites for common restriction enzymes that include *Bam* HI, *Sac* I, *Kpn* I, and *Not* I (Supplementary Table 1). Multiple negative cis-acting sites were successfully modified and eliminated wherever possible as mentioned in Supplementary Table 1. The sequence was simultaneously modified by increasing the frequency of codons with a C or G, in the third position, from ~ 16% to 58%. GC content was improved from ~ 26% in the native *ISTK* sequence to ~ 40% in the optimized sequence to prolong mRNA half-life. The *optimized ISTK (oISTK)* sequence contains 276 (out of 411) silent codon changes when compared to the respective native viral sequence. A value of 100 is set for a codon with the highest codon frequency and it was compared with less preferred codon triplets that are very likely to reduce the efficiency of expression using relative frequencies. About 60% of the total codons (~ 246 out of 411) of the *oISTK* gene showed a relative codon frequency between 91 and 100%. The codon adaptation index (or CAI) value describes how well the codons of this gene match with the codon usage of the host on a scale of 1. The *oISTK* sequence resulted in CAI of 0.86. Finally, after complete multi-parametric gene optimization, the native gene was 75% identical to the optimized sequence (Fig. 1a). In the optimization process, extreme care was taken to keep the protein sequence exactly the same while modifying only the nucleotides (Fig. 1b).

Construction of plant expression cassettes and creation of transforming *Agrobacteria*

A total of five plant expression vectors were developed, harboring the 1.2 kb native/optimized *ISTK*, 0.9 kb native/optimized *iridoptin* and native *GUS* nucleotide sequences in the backbone of pD35S plant-binary vector: 1. pD35S—*GUS*, 2. pD35S—*IRI*, 3. pD35S—o*IRI*, 4. pD35S—*ISTK*, 5. pD35S—o*ISTK* (Fig. 1c). The gene fragments were present under the control of the dual 35S promoter at the 5′ end and a 2X/3X/no flag tag at the 3′ end of the gene of interest. All constructs except the GUS construct contained a flag tag. The presence, size and orientation of respective gene inserts (GOI) in transformed *DH5α* cells were confirmed by PCR with gene-specific primers and primers for promoter and terminator sequence that flank the GOI; and also by restriction digestion analysis. *Agrobacterium tumefaciens* GV3101 clones transformed with these different vectors had the right size of inserts as confirmed by PCR. Sequencing of these PCR products was done to confirm orientation and the absence of any mutation in the gene inserts (data not shown).

Creation of T_1 generation transgenic *Arabidopsis* lines

A. tumefaciens GV3101 strains carrying the recombinant plant binary vectors with dual 35S enhancer promoter (pD35S-*oIRI*/ pD35s-*oISTK*/pD35S-*GUS*) were used to transform Arabidopsis plants (ecotype *Columbia-0*). In this study, lines transformed with GV3101 containing the *GUS* gene construct served as a negative control. Thirty to forty independent first generation (T_1) lines were identified by selection on kanamycin-containing media for each transformation.

Confirmation of integration of transfer DNA (T-DNA) into the *Arabidopsis* genome by PCR

PCR was carried out to demonstrate the integration of the right-sized gene insert into the plant genome in T_1 transgenic lines. PCR with genomic DNA, isolated from independent transgenic T_1 lines containing *oISTK*, using gene-specific/flanking region primers, yielded a band at the expected ~1200 bp region, and that of *oIRI* in ~900 bp region, when run on a 0.8% agarose gel (Fig. 2a, b). Cloned pD35S-*oISTK* vector was used as template for positive control and showed a ~ 1200 bp band. However, *GUS* negative control showed no band as expected (Fig. 2a).

Translation of *ISTK* and *iridoptin* in stable *Arabidopsis* transgenic lines

To facilitate the detection of transgene expression, a flag epitope tag was added to the 3′ end of *ISTK* and *iridoptin*. Optimization of the *ISTK/iridoptin* for expression in plant system resulted in detectable and significantly pronounced protein accumulation as compared to the corresponding native gene (which was undetectable) when transiently expressed in tobacco (Supplementary Fig. S1). This is the first evidence of detectable accumulation of the viral insecticidal polypeptides, ISTK and iridoptin, in the plant system.

Western blot analyses were carried out to demonstrate the expression and accumulation of ISTK and iridoptin at the polypeptide level in the transgenic T_1 *Arabidopsis* lines. Total proteins extracted from the 20–30 PCR-positive lines of each of the *oISTK* and *oIRI* transformants were tested for the presence of respective polypeptide accumulation. ISTK and iridoptin polypeptides were accumulated at the predicted molecular weight region (ISTK: 50 kDa; iridoptin: 37 kDa region) (Fig. 3a, b). Flag tags (2X/3X) add approximately 2–3 kDa to the actual polypeptide size. GUS lines served as a negative control and detected no band as expected (Fig. 3a, b). Immuno-precipitated ISTK and iridoptin from the transgenic plant protein extracts were identified using MALDI-TOF analysis (Supplementary Figs. S2 and S3). Hence, this serves as a confirmation of ectopic expression of ISTK and iridoptin in the plant cells after extensive optimization of the gene sequence to suit expression in plant system. The Western blot analysis also helped in the identification of ISTK/iridoptin expressing T_1 lines to carry out further bioassays.

Based on more Western blot analyses (data not shown) of samples from leaves of different lines, the transgenic T_1 lines expressing iridoptin used in the bioassays were placed in one of the three following categories with respect to iridoptin detection levels in total proteins: (1) oIRI lines 6, 8 and 13 were included in the "high expresser" group in which the iridoptin polypeptide levels were detectable when 50 µg total proteins (TP) were loaded in SDS-PAGE followed by Western blot analysis; (2) the "average expresser" category include lines that had detectable levels of iridoptin when 130 µg of TP were loaded in the gel (Lines 14, 20 and 27) and not with 50 µg of TP; 3) Lines that had detectable levels of iridoptin when more than 130 µg TP were loaded were categorized in "low expresser" group (Lines 5, 16, 23, 26, 28, 30) (Table 1).

Among the several ISTK-expressing T_1 lines, oISTK 31 was classified as high expresser and the remaining lines (except oISTK 35—data not available) were considered average/low expressers. Nevertheless, reliable information was not available to distinguish ISTK-expressing lines between low versus average expresser as in the case of oIRI lines.

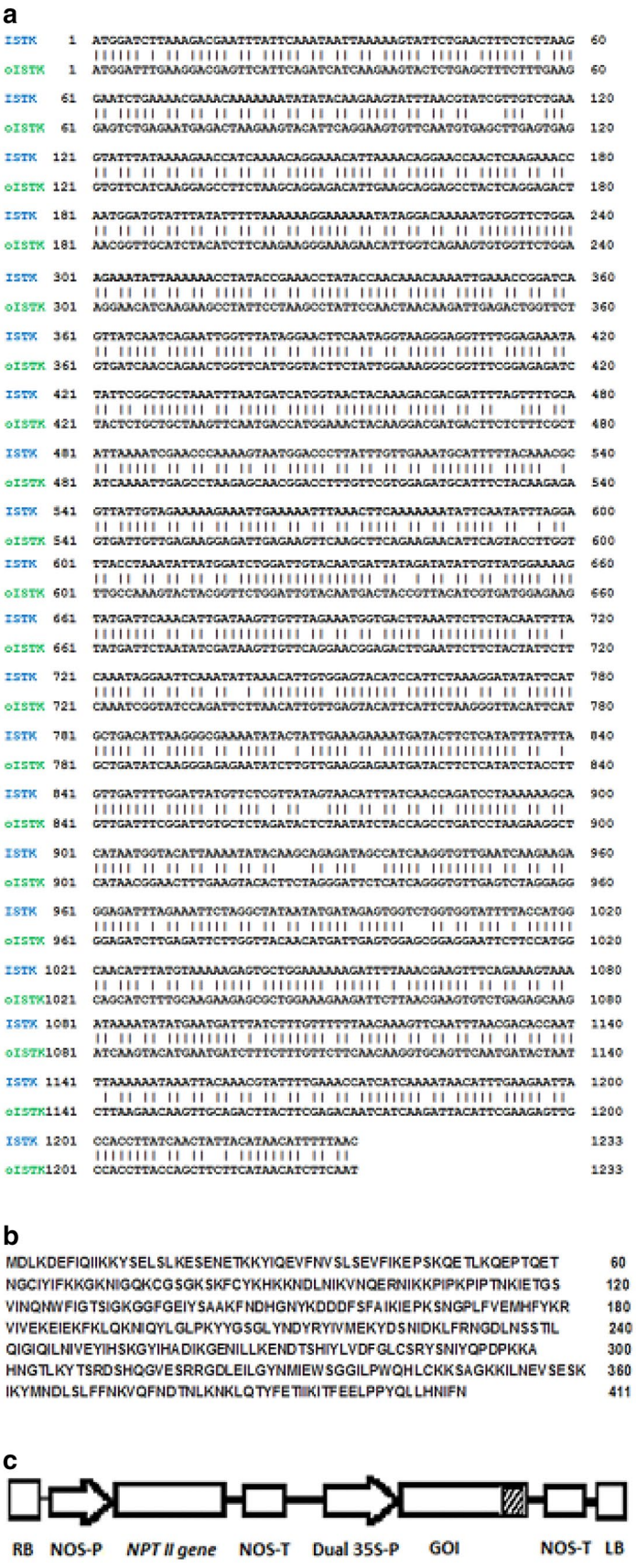

Fig. 1 DNA sequences of native and optimized *ISTK/iridoptin*, amino acid sequence of ISTK/iridoptin, and the recombinant expression vector construct containing *ISTK/iridoptin*. **a** Alignment of the full-length native (*ISTK/iridoptin*) and the plant codon-optimized *ISTK* (*oISTK/oIRI*) showing positions of nucleotide replacement (*ISTK:* 1–1233 bp; *iridoptin:* 1–909 bp). **b** Amino acid sequence of ISTK (1–411 aminoacid; iridoptin: 1–303 aminoacid) (Chitnis et al. 2011). **c** Schematic diagram of the transformation vector, pD35S containing the gene of interest (GOI). The GOI, such as *oIRI, oISTK,* or *GUS*, is under the control of the dual 35S promoter (Dual 35S-P) with 2X/3X flag tag ( ) or without flag tag at the 3′end of the gene insert. The *GUS* gene construct lacks the flag tag at the 3′ end of the gene. RB and LB, right and left borders of the T-DNA region. NOS-P and NOS-T, NOS promoter and terminator sequence. *NPT II* (kanamycin resistance) gene is the selective marker

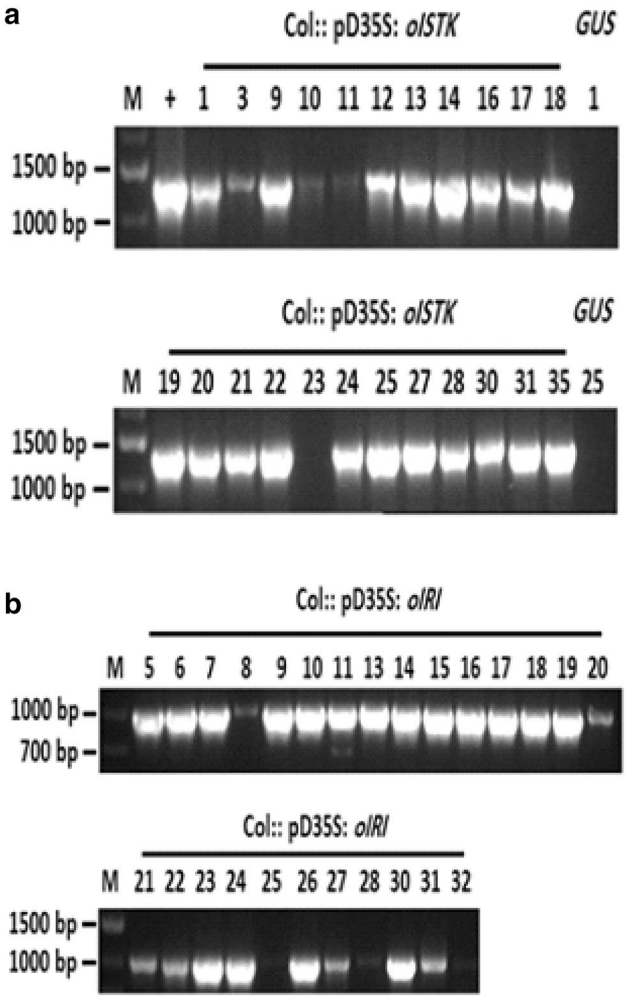

Fig. 2 PCR analysis of putative transgenic T_1 *Arabidopsis* lines transformed with the *oISTK* or *oIRI* construct. **a** Analysis of *oISTK* transgenic plants. Lanes: M, DNA Marker; +, PCR with the recombinant vector pD35-*oISTK* as template; GUS 1 and GUS 25, PCR with transgenic Arabidopsis lines (T_1) transformed with the pD35S-*GUS* vector; Col:: pD35S: *oISTK* (1–35), PCR with several independent putative transgenic *Arabidopsis* lines transformed with the pD35S-*oISTK* binary vector. **b** Analysis of *oIRI* transgenic plants. Col:: pD35S: *oIRI* (5–32), PCR with several independent putative transgenic *Arabidopsis* lines transformed with the pD35S-*oIRI* binary vector

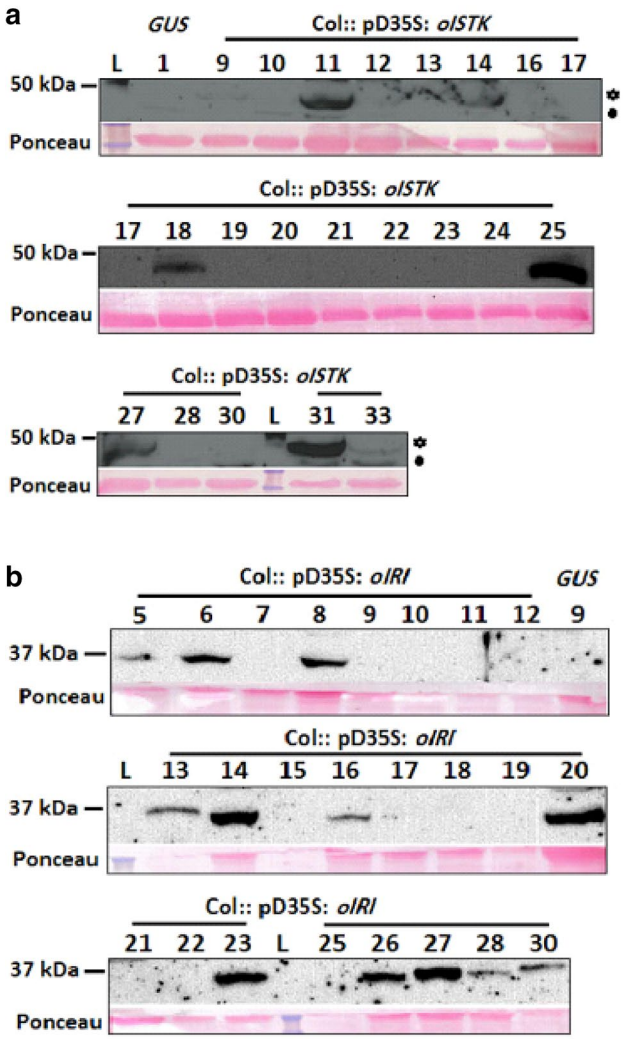

Fig. 3 Western blot analyses of T_1 transgenic *Arabidopsis* (*oISTK* or *oIRI*) lines using anti-flag antibody. **a** Analysis of *oISTK* transgenic plants. L, protein molecular weight markers; GUS, total protein extracts from transgenic line containing the *GUS* gene construct; Col:: pD35S: *oISTK* (1–33), total protein extracts from different T_1 transgenic lines containing *oISTK* gene construct. Star (✿), ISTK band detected at 50 kDa region. Ponceau, blot stained with Ponceau stain shows unequal loading in these lanes. Dot (●), non-specific band detected in all samples including GUS controls. **b** Analysis of *oIRI* transgenic plants. Col:: pD35S: *oIRI* (5–30), total protein extracts from different T_1 transgenic lines containing *oIRI* gene construct

Aphid resistance in iridoptin-expressing transgenic T_1 lines

Five similar-aged leaves from each of the different transgenic lines, together with *GUS*-containing lines (control) were challenged with less than 15 h old-GPA nymphs to investigate the effect of plant-expressed iridoptin on aphid survival, development, and fecundity/reproductive potential.

In the first run, several T_1 iridoptin-expressing lines (oIRI 20, oIRI 23, oIRI 26, oIRI 13) were examined. Aphid survival and fecundity were investigated over a period of 21 days after treatment (DAT). Line oIRI 23 showed significant reduction in the inoculated aphid population with an average of 92% mortality in less than 2 DAT and showed 100% mortality on 4 DAT. The oIRI 20 line showed over 65% mortality on 2 DAT and 96% mortality by 9 DAT, a period during which introduced aphids could have developed

Table 1 Summary of expression pattern and aphicidal activities of different T_1—oIRI/oISTK lines

Based on expression level	Col:: pD35S: *oIRI* (T_1) lines
High expressers	6(L), 8 (L), 13 (L)
Average expressers	14 (N), 20 (H), 27 (L)
Low expressers	5 (ND), 16 (ND), 23 (H), 26 (H), 28 (ND), 30 (ND)
Based on expression level	**Col:: pD35S: *oISTK* (T_1) lines**
High expressers	31 (L)
Rest (average and low expressers)	11 (H), 18 (H), 35 (H), 27 (N), 25 (L)

Labelled as *H* high insecticidal activity, *L* low insecticidal activity, *N* no insecticidal activity, *ND* not determined

into adults and undergone active reproduction. Lines oIRI 26 and 13 steadily reduced the aphid survival, with 30–50% mortality on 9 DAT (data not shown). Mortality rates were compared at 9, 13 and 21 DAT among the iridoptin-expressing lines evaluated (Fig. 4a). A significant increase in GPA mortality was observed on iridoptin-expressing leaves from lines 20, 23, and 26 compared with that in GUS control, for all 3 days analyzed. GUS control showed 0% mortality until 13 DAT and an average of 28% mortality by the end of the assay period which could be partly due to aging of the adult aphids. Data collection in line oIRI 13 was discontinued after 13 DAT due to lack of availability of leaf material to test. At the end of the assay period (21 DAT), remaining oIRI lines (20, 23 and 26) showed 100% mortality of inoculated aphids compared to only 28% mortality in GUS control. While being considered as a high expresser, oIRI 13 appeared to not perform as well as the low and average expressers (oIRI 20, 23 and 26) at 13 DAT. A direct correlation between the expression levels of iridoptin and the aphicidal activity was not observed.

Normally, a GPA takes about 6–7 days for a neonate aphid to become an adult. Once it becomes an adult, it starts parthenogenetic reproduction. Hence, 7–9 DAT (beginning of the aphid reproductive period) was used as a cut-off point to assess the nymphal development to adulthood. Because aphids were introduced into the treatment very early in their life cycle, effects of iridoptin on aphid's growth and development were also observed. At 9 DAT, there was a notable delay in the development of surviving, iridoptin-fed aphids, whereas 100% GUS control-fed aphids developed to adulthood (Fig. 4b).

Total fecundity of 86.6 ± 9.35 nymphs per replicate was observed in GUS control at 21 DAT. However, the fecundity was significantly reduced in all oIRI lines evaluated in

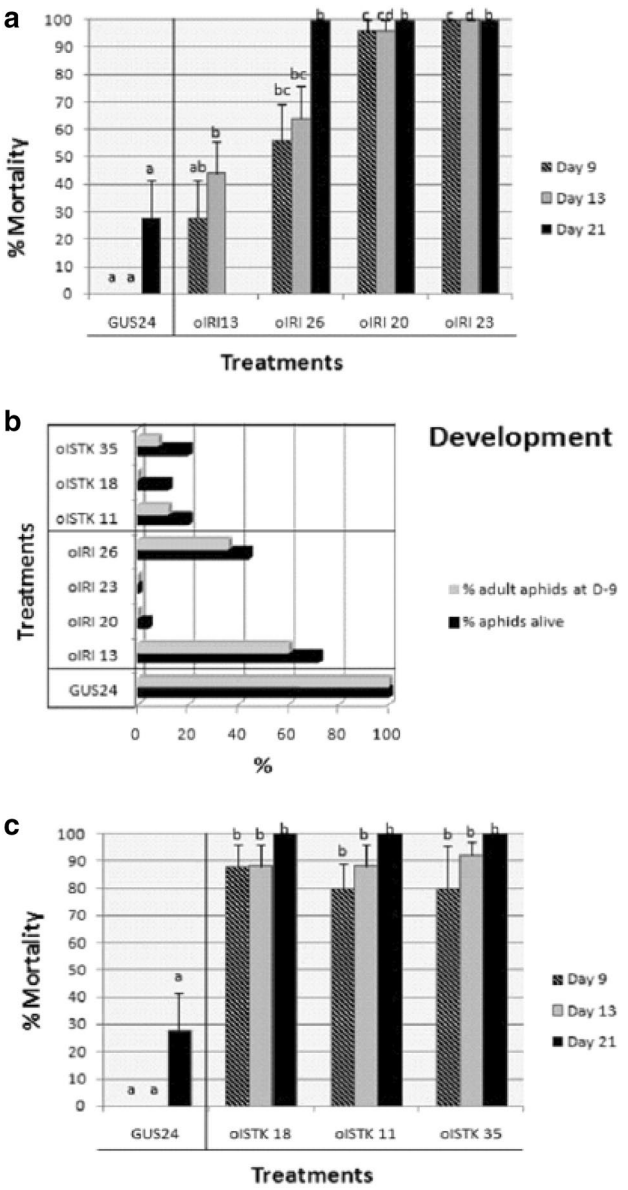

Fig. 4 Analyses of transgenic plants for their aphicidal activities. **a** and **c** Mean percentage mortality of green peach aphids in excised-leaf bioassay on iridoptin/ISTK-expressing transgenic T_1 lines at 9, 13 and 21 DAT. Note: no data available for oIRI 13 line at 21 DAT. Different letters represent significant difference between treatments in each day ($p < 0.05$); Points and bars represent means ± SE. **b** Comparison chart that summarizes the pattern of developmental delay that was observed in the growth of aphids, from nymphal to adult stage, that fed on different *iridoptin/ISTK*-expressing leaves, when compared to GUS control. Red, GUS control; Grey, oIRI lines; Blue, oISTK lines

this run (oIRI 20: 0.4 ± 0.4; oIRI 23: 0; oIRI 26: 12.6 ± 5.04 nymphs per replicate). The low/negligible fecundity on these iridoptin-fed aphids can be attributed to both increased mortality and developmental inhibition of mother aphids. Among the three oIRI lines, oIRI 26 resulted in reproduction rate of 0.51 ± 0.11 nymphs per aphid per day compared to

no reproduction on oIRI 20 and oIRI 23; the GUS control resulted in 2.26 ± 0.54 nymphs per aphid per day.

Because oIRI 23 line showed high mortality against GPA, it was then considered as a positive control to identify more iridoptin-expressing lines with aphicidal activity. In the second run of the experiment, evaluated at 8 DAT, an average expresser oIRI 14 showed no mortality (Supplementary Fig. S4a) or reproductive defects (data not shown) on GPA and was similar to the GUS control.

In the third run, line oIRI 27, another average expresser, exhibited moderate effects on aphid mortality (32 ± 10.19%, 8 DAT) compared to 0% mortality in GUS negative control and 100% mortality in the oIRI 23 line (positive control) (Supplementary Fig. S4b). However, significant difference in fecundity at 8 DAT between GUS and oIRI 27 was observed (GUS: 42 ± 5.26; oIRI 27: 7.6 ± 2.1 nymphs per replicate). A simultaneous analysis of development of aphids fed on oIRI 27 leaves showed a substantial delay in the development of aphids to adult stage. At 7 DAT, when 100% of aphids feeding on GUS control leaves developed into adults, < 50% of the surviving aphids had developed to adult stage in oIRI 27 leaves (Supplementary Fig. S4c); not all surviving aphids developed to adulthood even at 9 DAT (Supplementary Fig. S4d). Interestingly, high expressers such as oIRI 6 and 8 had no significant mortality or fecundity effects against GPA, further confirming a lack of direct correlation to the expression levels of iridoptin in the plants (data not shown).

The transgenic line oIRI 23 appeared to consistently perform superior across all lines evaluated and showed 100% mortality in less than 4 DAT in all three consecutive trials conducted within a period of 30 days. These lines also showed 100% mortality when GPA adults were directly exposed to the leaves in an excised-leaf bioassay (data not shown).

This study clearly demonstrated the inhibitory effect of iridoptin-expressing leaves on GPA survival, development, and fecundity. However, a direct relationship between expression levels and the inhibitory effects was not observed, for reasons that are currently not well understood (Table 1).

Aphid resistance in ISTK-expressing transgenic T_1 lines

Excised leaf bioassays with *oISTK*-expressing lines were conducted simultaneously with *iridoptin*-expressing lines as described above. *Experiment 1*: leaves from three oISTK lines (11, 18 and 35) showed significant reduction in the initial inoculum population when compared to the GUS control. At 9 DAT, the aphid mortality had increased to as high as 80–90% in these treatments, with 0% mortality in the GUS control (Fig. 4c). At 21 DAT, all oISTK treatments showed 100% mortality compared to 28% mortality in the GUS control (Fig. 4c). This demonstrates the complete inhibition of aphid survival in these *oISTK*-expressing leaves. Aphid fecundity was also significantly affected by these oISTK lines. Reproduction was noted at 9 DAT and steadily increased to a total of 86.6 ± 9.3 nymphs per replicate in GUS control at 21 DAT, whereas ISTK treatments resulted in a total of 4.8 ± 3.24 (oISTK 11), 0.8 ± 0.58 (oISTK 18) and 1.4 ± 0.97 (oISTK 35) nymphs per replicate. These data clearly demonstrated a significant inhibition of population growth when exposed to ISTK-expressing leaves. The reduction in fecundity could be attributed to the mortality effects on initial inoculum and slower development of surviving initial inoculum to the adult stage when fed on *ISTK*-expressing leaves (Fig. 4b).

Experiment 2 In the presence of a positive control (oIRI 23 line) and a negative control (GUS 9), additional oISTK lines were examined for aphicidal effects. The transgenic line oISTK 27 had no significant effect on GPA survival and was similar to the GUS control (Supplementary Fig. S4a). *Experiment 3:* oISTK lines 25 and 31 showed minimal/no mortality (Supplementary Fig. S4b) but significant decrease in fecundity of adult aphids (data not shown). There was a corresponding delay in the development of initial inoculum to the adult stage, when exposed to these lines (Supplementary Figs.S4c and S4d). The reproductive potential of aphids was 2.81, 0.32, and 1.37 nymphs/aphid/day for GUS, oISTK 31, and oISTK 25, respectively.

As observed in *iridoptin*-expressing T_1 lines, negative effects on aphid survival, development and fecundity were also observed when fed on *ISTK*-expressing leaves. However, they were not directly proportional to the expression levels of the respective toxins in plants (Table 1).

Comparison of effects of ISTK and iridoptin-expressing leaves on aphid survival and fecundity at 8 or 9 DAT

Three different effects on GPAs were observed when exposed to these different iridoptin/ISTK expressing transgenic lines. Lines oIRI 20, 23, 26 and oISTK 11, 18, 35 significantly increased mortalities and decreased fecundity of GPAs when compared to the GUS control (Fig. 5a, b). This could lead to a significant reduction in the population growth. Lines oIRI 13, oIRI 27, and oISTK 31 had no significant effects on aphid survival (Fig. 5a) but showed significant reduction in fecundity (Fig. 5b). However, lines oIRI 14, oISTK 25, and oISTK 27 had similar effects as GUS control on both aphid survival and fecundity (Fig. 5a, b).

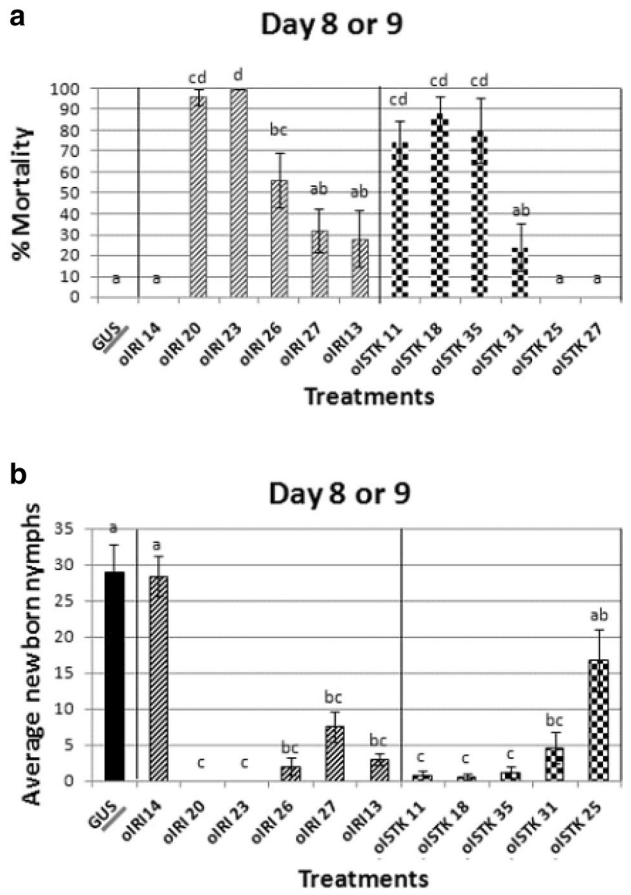

Fig. 5 Comparison chart combining multiple runs evaluating the effects of different iridoptin/ISTK-expressing T_1 lines on mortality (**a**) and fecundity (**b**) per replicate. GUS line was used as the negative control on 8 or 9 DAT. Data of the line oIRI23 resulted from the three independent runs and the data of oISTK 11 were from two independent runs. Data for rest of the lines were from a single run of the experiment

Discussion

Development of transgenic plants expressing foreign insecticidal genes would act as a valuable tool for generating plants toxic to the insect pests. Expression of the native, un-optimized *iridoptin* gene fragment in stable transgenic *Arabidopsis* lines showed expression at the RNA level, but not at the protein level, suggesting poor or no translation of this foreign mRNA in the plant system. A gene with the sequence adapted for an insect-virus may not have the appropriate coding sequence for efficient expression in plants. Efforts were taken to optimize *ISTK* and *iridoptin* DNA sequences to better suit expression in plants. Various factors were considered simultaneously during the gene optimization process and modified to achieve the detectable expression. However, it cannot be assured that the algorithm followed, in this gene optimization process, has achieved an optimal combination of sequence variables to result in the best possible-expression level.

These optimized constructs under the control of dual 35S promoter were introduced into *Arabidopsis* via the *Agrobacterium*-mediated floral dip method. Putative T_1 transgenic lines were obtained by screening the T_1 seeds on kanamycin selection media. PCR analyses were carried out to confirm the presence of the right sized gene inserts. Further screening of these PCR positive lines was carried out using Western blot analysis and transgenic lines (T_1) expressing the expected 50 kDa ISTK/37 kDa iridoptin at the polypeptide level were identified. Both optimized constructs (*oISTK/oIRI*) showed detectable levels of accumulation of respective polypeptides suggesting that the codon optimization of these genes were successful. Out of a large population of stable, transgenic T_1 plants screened for the expression of ISTK/iridoptin, ~45% of plants showed expression at the polypeptide level. The expression levels varied from barely detectable to strong bands in the expected molecular weight region using anti-flag antibody. These T_1 transgenic plants were comparable to wild-type morphology and growth, when grown on potted soil in growth chambers. Two different strong constitutive promoters, i.e., CaMV 35S and dual 35S promoters, were used to test expression of optimized *ISTK* and *iridoptin* constructs. However, there was no significant difference in the protein expression levels among them (data not shown).

The purpose of this study was to determine the possible utility of this novel, iridovirus-insecticidal kinase in insect control using transgenic technology. Here, as a first step, it has been demonstrated that it is possible to ectopically produce CIV's ISTK and a truncated form, iridoptin, in plants. Excised-leaf bioassays using T_1 transgenic plants expressing ISTK/iridoptin were used to determine the plant expressed toxin's effect on survival and reproductive potential of green peach aphids. Several transgenic T_1 lines expressing ISTK/iridoptin were shown to induce high mortality (60–100%) in as little as 4–8 days following exposure, accompanied by reduced fecundity of the surviving aphids. Developmental inhibition of surviving aphids was also observed in many of those lines. Increased pre-reproductive time clearly indicated retardation of aphid development. However, irrespective of the toxin expression levels, several lines showed very low or no inhibitory effects on GPAs. Lack of correlation between expression levels and respective insecticidal activities complicated comparisons between the different transgenic lines. However, the optimization strategy used in this study appears to have resulted in desirable levels of expression in stable transgenic *Arabidopsis* plants to show significant aphid mortality and suppression. In-planta expression of ISTK/iridoptin providing strong inhibitory effects on the survival and fecundity of GPAs was established in stable transgenic *Arabidopsis* plants. Based on these observations, it is likely not required to obtain higher accumulation of

these toxins in plants. An accurate estimation of the concentration range required for an adequate control of GPAs in these transgenic plants is warranted.

Bioassay experiments showed that surviving aphids that fed on the treatment leaves (expressing ISTK/iridoptin) frequently avoided feeding and were found to roam around in the petri plates. This was not observed in the GUS-expressing control leaves. More detailed studies would help to elucidate the mode of action of ISTK and iridoptin at the organismal level and the apparent avoidance behavior. The most studied *Bt* (Cry) toxins are shown to result in feeding inhibition due to paralysis of insect gut and mouth parts (Aronson et al. 1986; Deist et al. 2014). A few other observations were made during the bioassays: Some aphids continuously feeding on ISTK/iridoptin-expressing leaves had extremely small body sizes which ultimately succumbed to death compared to the normal body size in GUS control-feeding aphids. Also, some deformed and paralyzed aphids were found in the treatments. The encounters of such aphids were not significantly high but were only observed in aphids feeding on ISTK/iridoptin-expressing leaves.

The frequency at which the aphid-resistant lines were recovered was low (~ 3 out of 20–23 transgenic plants (T_1) screened, i.e., 13–15%). The analysis performed in this study did not show a direct correlation between the expression levels of the toxin and the insecticidal activity. In fact, some of the high expression lines showed low or no inhibitory effects on aphid survival and growth. Previous studies in our laboratory suggested that kinase activity of iridoptin is absolutely necessary for its insecticidal effects (Chitnis et al. 2011). The effects of plant-expressed ISTK and iridoptin on aphids, or any target-insect, might be dependent on the availability of these active forms of the toxins in transgenic plants, rather than dependence on just the levels of accumulation. Dose-independent effects of toxins in feeding-insect antibiosis have been reported with *Bt*-toxin expressing canola plants against a lepidopteran pest (Stewart et al. 1996). Another study over-expressing *Mi-1.2* gene in tomato conferred developmentally regulated resistance in potato aphids. Here, irrespective of the use of constitutive promoter, aphid resistance was developmentally regulated with resistance observed in the mature, flowering stage plants but lacked resistance in the seedling stage. Constitutive expression showed 3–eightfold increase in *Mi-1.2* mRNA accumulation levels when compared to that of native promoter of the gene. However, there was no significant difference in aphid numbers between these two types of plants (with native promoter versus constitutive expression) at the flowering stage (Goggin et al. 2004). A study on Bollgard cotton showed no correlation between bollworm survival and Cry1A(c) expression levels in different plant parts (Gore et al. 2001). Inconsistencies have been observed in the expression patterns of reporter genes under the control of constitutive CaMV35S-like promoters in different cell types (Yang and Christou 1990). Plethora of information is available on such dose-independent effects of insecticidal toxins against insect pests in transgenic plants (Chen et al. 2005; Jiang et al. 2006; Sadeghi et al. 2007).

Numerous approaches have been undertaken by researchers to improve different plant traits using plant transformation with foreign genes. However, the biggest concern is the unsolicited effects of integration of foreign gene in the host plant genome and subsequent expression effects (Tagashira et al. 2005; Li et al. 2006). In this study, observations showed reproductive defects such as lack of seeds in some aphid-resistant T_1 lines and expression silencing in the second generation plants of few aphid-resistant lines (data not shown). More careful and detailed studies on the ability of inheritance of stable expression and insecticidal activity of ISTK and iridoptin in these transgenic plants are warranted. Use of phloem-specific promoters or wound-inducible promoters, or a chimeric version with both, might be a more optimal and useful approach as this study aims at sap-sucking pests like aphids and hence would avoid any unwanted effects of constitutive, over-expression in plants (Smigocki et al. 1993; Duan et al. 1996; Rao et al. 1998; Godard et al. 2007; Sadeghi et al. 2007; Tiwari et al. 2011; Dutt et al. 2012; Will and Vicinskas 2013; Chandrasekhar et al. 2014). This approach would also reduce the metabolic cost of over-expression of foreign genes. Hence, further evaluation of ISTK/iridoptin with these promoters is warranted to expand the knowledge base on *ISTK, iridoptin*, and similar toxin genes for insect control.

This is the first report of utilizing a novel-iridoviral kinase towards development of aphid-resistant transgenic plants. The current study has generated proof-of-concept data that will facilitate the use of the optimized *ISTK/iridoptin* to successfully generate stable transgenic lines of agronomic importance. The analysis of exploiting the applicability of this viral kinase in aphid suppression in plants, although preliminary, will create a new niche for identification and exploration of similar toxins, as aphid control agents are of potential integrated pest management interest. This could be a torch bearer for rapid progress in utilization and screening of similar viral insecticidal genes, as PIPs, that can be isolated from the reservoir of invertebrate iridoviruses. Such insect-iridoviruses appear to be an untapped resource for identification of more potential insecticidal toxins for an extremely wide range of insect pests (Williams et al. 2005; Ince et al. 2018). Hence, this study would serve as a pioneer in the use of CIV for an effective and sustainable insect pest control in agriculture.

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Author contributions SG, SLB and HZ conceptualized the study and planned the transgenic plant related work. SG and MNP planned and designed the insect and bio-assay studies. SG, HZ, MNP and SLB secured the funding for this project. SG performed the experiments. SG, MSF, MNP and HZ interpreted the results and prepared the manuscript.

Compliance with ethical standards

Conflict of interest The authors of this manuscript declare no conflicts of interest. The funding sponsors had no role in the design and performance of the experiments, or in the decision to publish the results.

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