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ORIGINAL ARTICLE

Efficacy of arginine kinase as a promising RNAi target in *Aphis gossypii* genome as revealed through aphid bioassay on field-grown transgenic cotton plants

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Abstract

The cotton aphid *Aphis gossypii* is a major agricultural pest of cotton that causes substantial damage to the crop not only by sucking sap but also through virus transmission. Globally adopted traditional and contemporary approaches to control aphid infestation have certain limitations and are hazardous to human health. RNA interference (RNAi) technology has unfolded its potential as an effective crop protection strategy against various pests. In this study, we adopted plant-mediated RNAi strategy to enhance aphid resistance in cotton by targeting arginine kinase (AK), which is a crucial enzyme responsible for energy homeostasis in insects. We selected a 312bp dsRNA fragment containing eight siRNAs and showing optimum GC content, Hb index, and stable secondary structure based on computational prediction studies. The binary construct expressing dsRNA was used to transform local cotton variety MNH886 and four transgenic lines were obtained in the T_1 generation. Out of the four T_1 transgenic cotton lines, dsA-7 exhibited the highest aphid mortality (73.3%), whereas, dsA-1, dsA-3 and dsA-6 revealed 60%, 61%, and 66.6% aphid mortality, respectively, in comparison to 13.3% mortality in the mock control cotton line. Moreover, significant knockdown in mRNA expression of AK was observed in aphids fed dsA-7 which was 79%. In comparison, 54%, 47%, and 45% downregulation was recorded in aphids which fed on dsA-6, dsA-3, and dsA-1 transgenic cotton lines, respectively. These results revealed that plant-mediated downregulation of aphid RNA induced significant RNA interference in *A. gossypii* which resulted in considerable aphid mortality and led to plant protection against aphids.

Keywords: arginine kinase, cotton aphid, RNAi target, transgenic cotton

Introduction

Sucking pests seriously threaten cotton crops and are one of the main causes of up to 37% decline in cotton yield (Ramalho *et al.* 2012). *Aphis gossypii* (Glover) (Hemiptera: Aphididae), commonly known as cotton aphid, is an important sap-sucking pest (Blackman and Eastop 2000; Ahmad *et al.* 2016; Qamar *et al.* 2019). *Aphis gossypii* infestation and its feeding on cotton crops can significantly damage cotton plants by

causing wilted and crinkled leaves (Eldesouky 2019). Hence, delayed plant growth, foliar alterations, less fruit setting, reduced fruit retention and less cotton lint weight has been observed (Raboudi *et al.* 2002). Furthermore, a sugar-rich substance (honeydew) secreted by cotton aphids facilitates the growth of black sooty mold on the leaves which eventually hinders photosynthesis and reduces plant growth (Godfrey *et al.* 2000).

Various cultural, biological and chemical approaches are being adopted across the globe to control and prevent insect pest infestation in cotton but the limitations of all mentioned approaches have highlighted the need of the development of an alternate effective and eco-friendly method to suppress aphid infestation in cotton crops (Mamta and Rajam 2017). Eukaryotic organisms have a special potential to modify the expression of a particular gene at a post-transcriptional level through RNAi technology (Taning *et al.* 2020). This interference in gene expression is mediated by double-stranded RNA, consequently developing resistance against pathogens through defense mechanisms (Christiaens *et al.* 2020). RNAi technology has been extensively used in plants to down-regulate the expression of endogenous and exogenous genes in order to study the function of a particular gene to enhance resistance (Mezzetti *et al.* 2020). Another interesting feature of RNAi is the mobility of siRNAs. siRNAs can travel in plants from the site of production to another site through the plant's vascular system. Studies using grafted plant systems have revealed that siRNAs produced in any plant part can move into the grafted segments to promote growth and development (Limera *et al.* 2017; Westwood and Kim 2017; De Francesco *et al.* 2020). Because of the high sequence specificity of RNA interference technology, RNAi-based GM plants are considered safe for the environment and non-targeted species as no protein is formed and released during the process (Casacuberta *et al.* 2015; Tan *et al.* 2016). This sequence-specific action of RNAi makes this approach more efficient and effective than other conventional agrochemical approaches to control insect pests and other pathogens (Gong *et al.* 2014; Taning *et al.* 2020; Jekayinoluwa *et al.* 2021).

In invertebrates, arginine kinase (AK) is a widely distributed enzyme that belongs to the category of phosphagen kinase (Uda *et al.* 2006). Arginine kinase is the main enzyme responsible for energy homeostasis in insects. Various enzymatic assays and RNA interference (RNAi) studies have described the fundamental role of AK in energy metabolism (Tanaka *et al.* 2007; Wu *et al.* 2007; Werr *et al.* 2009; Zhang *et al.* 2022). Its involvement in physiological responses and short-term memory has also been demonstrated (Chen *et al.* 2015; Bozzato *et al.* 2020). Being crucial for energy, homeostasis for insects, crustaceans, and some unicellular organisms, AK serves as a potential target candidate for RNAi to develop and enhance resistance in plants against insects. Studies have confirmed that in certain insects, growth and development can be impaired significantly by downregulating the AK gene (Kola *et al.* 2015; Shaheen *et al.* 2015; Andrade and Hunter 2017; Ma *et al.* 2022; Zhang *et al.* 2022). Various studies have been conducted to develop resistance against *Phyllotreta striolata* (F.), *Helicoverpa armigera*, *Diaphorina citri* Kuwayama, *Nylanderia fulva* (Mayr), *Plutella xylostella* (L.), and *Tuta absoluta* in different plants by targeting AK *through* RNAi technology (Zhao *et al.* 2008; Qi *et al.* 2015; Andrade and Hunter 2017; Bento *et al.* 2020; Fu *et al.* 2020; Meng *et al.* 2020) but so far, no study has been done on *A. gossypii*.

The current study was designed to develop independent transgenic cotton lines containing double stranded (ds)RNA fragments targeting AK of cotton aphid, *A. gossypii*. As *A. gossypii* cause serious damage to cotton crops leading to significant yield loss in Pakistan, we hypothesized that silencing of the crucial energy homeostatic enzyme gene AK in cotton aphid through dsRNA fragments would enhance the resistance ability of transformed cotton lines against *A. gossypii* to control its infestation.

Materials and Methods

Target gene selection and PCR amplification from the aphid genome

The arginine kinase (AK) gene fragment was amplified from a local *A. gossypii* isolate, through PCR with the help of gene specific primers 5′-CATTGAGCGGATTGGAAGGC-3′ and 5′-TCTTCGTTGCACCAAACCAA-3′. Total RNA was isolated from the aphid, of which 1 µg was used for cDNA synthesis that was used further as the template for reverse transcription PCR. Amplification was obtained using 1X PCR buffer, 1 mM dNTPs, 100 µM of both forward and reverse primers, 1 µl cDNA and 2 U of Thermo Prime Taq DNA polymerase (ThermoScientific). The cycling profile was comprised of 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 60°C, and extension for 45 s at 72°C, followed by a final extension for 10 min at 72°C. The amplified products were resolved in a 1% agarose gel stained with ethidium bromide at a final concentration of 0.5 $\mu g \cdot ml^{-1}$ and visualized under a UV transilluminator.

In-silico target prediction study

Percentage of GC content

GC content (%) was calculated for each siRNA present in the dsAK fragment using an oligonucleotide properties calculator (http://biotools.nubic.northwestern.edu/OligoCalc.html). The GC content of siRNA is an important parameter that represents the functionality of siRNA. The preferable range for siRNA %GC content is between 31.6% and 57.9% as the %GC content is negatively correlated to RNAi efficiency.

Minimum Free Energy of Hybridization

The designed siRNAs and target mRNA sequences were put into RNA hybrid software for estimation of minimum free energy of hybridization (MFE: kcal/mol). Minimum free energy is one of the criteria to rank the pre-designed siRNAs. The silencing efficiency of siRNA is estimated by its minimum free energy which can be calculated by analyzing the thermodynamics of siRNA-mRNA interaction.

mRNA Secondary Structure Prediction

The secondary structure of AK mRNA was built using the RNAfold tool http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi (accessible from ViennaRNA services) which is employed to predict the stable secondary structure of RNA by means of the dynamic programming algorithm initially presented by Zuker & Stiegler (1981). In addition, it also gives equilibrium base-pairing likelihoods calculated from John McCaskill's partition function algorithm.

Hydrogen bonding (Hb) index

For the predicted secondary structures, the Hb-index was calculated for individually predicted structures at the local regions of the structure. Calculations were based on the method defined by Luo and Chang (2004). Hb-index = Σ (P_H x N_{HB}) (P_H represents the probability of a nucleotide in mRNA forming a duplex with other nucleotides in the same mRNA, and N_{HB} is the number of H- bonds formed by that nucleotide, i.e., three for G and C, and two for A or and U). The Hb index gives a measure of the overall probability of nucleotides in the mRNA region, targeted by siRNA, interacting with the other regions of mRNA. The siRNA interaction with mRNA can be influenced by the local structure of mRNA, making it important to consider this parameter. Low Hb-index values indicate greater accessibility of the RISC-siRNA complex to the single-stranded nucleotides located in the target region of mRNA.

Construct design

Nucleotide sequence of *A.* gossypii AK (accession # GU937512.1) was retrieved from NCBI database and double-stranded RNA (dsRNA) was designed by using RNAi designer tool (https://rnaidesigner.thermofisher.com/rnaiexpress/design.do). A ~312bp mRNA region with eight potential siRNAs (revealed through in-silico studies) was selected as (ds)RNA target fragment. The selected region was subjected to a BLAST search to reveal any potential off-targets. The selected dsRNA target fragment was inserted in sense and antisense orientation, with a 1349bp CHS chalcone synthase gene as an intron in between them. This intron would give stability to the dsRNA in the transgenic plant. Hence, the double stranded arginine kinase (dsAK) hairpin fragment comprised of a 1975bp fragment [*XhoI*-312bp dsAK sense orientation-1349bp-CHS- 312bp dsAK antisense orientation-*XhoI*] (Fig 1). It was chemically synthesized from BioBasic®, Canada as a pUC57 clone. The commercially synthesized ~1975 bp dsAK fragment was ligated into the pCAMBIA1300 vector in the T-DNA region between *XhoI* sites by replacing the hygromycin gene. The dsAK fragment was under the eukaryotic expression control of the CaMV35S promoter and cloning was confirmed through restriction digestion with *XhoI* enzyme and the developed binary construct was named as pCAM-dsAK (Fig. 1).

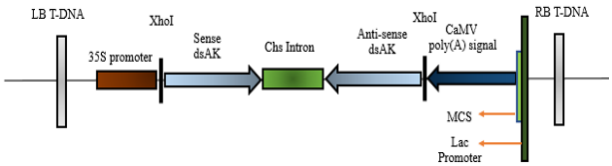


Fig. 1. A schematic diagram of pCAM-dsAK gene hairpin construct. The dsAK construct comprised of 312bp dsAK in sense, antisense orientation separated by CHS intronic loop while driven by CaMV35S promoter for constitutive expression in cotton

Cotton transformation

For transformation, the dsAK construct was mobilized initially into *Agrobacterium tumefaciens* strain GV3103, subsequently, the recombinant *Agrobacterium* was used to transform cotton. Cotton seeds (variety MNH886) were de-linted with sulphuric acid (100 ml/1 kg seeds), sterilized with 1% SDS and 0.1% $HgCl_2$ and soaked with distilled water and incubated in a sterilized flask wrapped with brown paper to facilitate germination. Two days post incubation, embryos were isolated and an incision was made in the shoot tips of the isolated embryos with the help of a sterilized surgical blade. The recombinant *Agrobacterium* cells harboring pCAM-dsAK construct were inoculated in 10 ml YEP media and grown for 48 h

at 28°C. The injured embryos were co-cultivated with an equal volume of MS broth and agrobacterium cell suspension harboring pCAM-dsAK construct for 2 hours on a gyratory shaker at 80 rpm. Later, the embryos were removed and shifted to MS media plates and co-cultivation was continued for 3 subsequent days. The plates were incubated in a growth room at 25°C ± 2°C with 16 / 8 light : dark cycle. After 72 h, the plantlets were shifted from Petri plates to a test tube containing MS media supplemented with cefotaxime (100 μg · ml $^{-1}$). The plantlets were subcultured into fresh MS media every 2 weeks. To acclimatize the plantlets, they were transferred to a pot containing sterilized soil: sand: silt (1 : 1 : 1) after 8–12 weeks. Post acclimatization, the putative transgenic cotton plants were transferred to the field in containment. The plants were then subjected to molecular analysis. As the mock control, cotton plants transformed with pCAMBIA1300 empty vector were generated simultaneously.

Confirmation of transgene integration and mRNA expression

The putative transgenic cotton plants with dsAK construct were analyzed for transgene presence by using different molecular techniques. Young leaves from transformed cotton plants were used for the isolation of genomic DNA according to the protocol described by Dellaporta *et al.* (1983) with few modifications. All regenerated cotton plants obtained from transformation were evaluated through PCR with primers; forward 5′-GCCACTTTTAAGCTCACCCA-3′ and reverse 5′-ACATAAACTCCAAGGCAAGTGT-3′ that amplify the 803bp region from the construct. PCR was performed using 1X PCR buffer, 1 mM dNTPs, 100 μM of both forward and reverse primers, 250ng genomic DNA and 2U of Thermo Prime Taq DNA polymerase (ThermoScientific®). The cycling profile was comprised of 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 60°C, and extension for 45 s at 72°C followed by final extension for 10 min at 72°C. The amplified products were resolved in a 1% agarose gel stained with ethidium bromide at a final concentration of 0.5 μg · ml^{-1} and visualized under a UV transilluminator.

The PCR positive T_0 generation transgenic cotton plants were grown to full maturity and seeds were collected and advanced to obtain T_1 generation cotton plants. The cotton seeds obtained from positive T_0 progenitor plants were grown in a tunnel under a contained environment as a transgenic line and were screened again for the presence of transgene (dsAK) as all the plants derived from a single T_0 progenitor would not be positive for a transgene due to segregation. Molecular analysis of T_1 cotton plants was done for the confirmation of successful gene transfer. The screening of all T_1 cotton plants was performed through PCR assay, employing the primers and the temperature profile as mentioned earlier.

Relative mRNA expression of the dsAK fragment in T_1 transgenic cotton lines was performed in the StepOne Realtime PCR system (BioRad). Total RNA was extracted by employing PureLink RNA Mini Kit (Ambion) with minor modifications. cDNA was synthesized from 1 μg RNA by using RevertAid First Strand cDNA Synthesis Kit (ThermoScientifc). Primers were designed from the sense fragment of the dsAK fragment. The mixture contained SYBR Green/fluorescein qPCR Master Mix 2X (Thermo Scientific), cDNA template, 1 μM primers 5′-TCGTTGCACCAAACCAAGAG-3′ and 5′-TGTTCAACAGCAACTCACCG-3′ and nuclease-free water in a total volume of 20 μl. The qRT-PCR cycling profile was initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 60°C, and extension for 45 s at 72°C. For each transgenic cotton line, three independent transgenic plants were taken as biological replicates. Beta actin was used as an internal control and the relative gene expression was calculated through the Livak method, termed the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Results were statistically analyzed by applying One-way ANOVA and post-multiple comparison test in GraphPad Prism software.

To reveal the genetic integration of dsAK fragment in transgenic cotton lines at T_1, Southern blotting hybridization assay was performed (Southern 1975). The gel-purified dsAK fragments of 1975 bp, obtained as digested products from pUC vectors, were used as a template to make biotin labeled probe, and 20 μg of denatured genomic DNAs from dsAK transgenic cotton lines were allowed to hybridize with biotin-labeled probes at 48°C. The probe-target hybrids were detected with alkaline phosphatase-conjugated antibodies in a chromogenic reaction. The pCAM-dsAK binary construct DNA was used as the positive control in this assay.

Plant-mediated Insect Bioassay

A total of 25 age-synchronized nymphs of *A. gossypii* per one biological replicate for each transgenic cotton line was used. A capturing device locally manufactured by our group was used to clip the nymphs to the dorsal side of fully expanded cotton leaves. The aphids were allowed to feed for 5 days. The mortality percentage of aphids was recorded for three biological replicates per transgenic cotton line. Cotton plants transformed with

empty pCAMBIA1300 vector were used as controls in all the experiments to rule out the effect of any other proteins of binary vector on insect mortality. The mortality was monitored every 24 hours and recorded for 5 days.

Quantification of mRNA expression of AK gene in *Aphis gossypii individuals* which fed on transgenic cotton lines

For quantification of mRNA expression of AK genes in *A. gossypii* which fed on transgenic cotton lines (T_1) after plant-mediated insect bioassay, live *A. gossypii* nymphs were collected post-bioassay and proceeded for RNA extraction. The downregulation in mRNA expression of AK genes in feeding aphids was revealed using cDNA as a template. Real-time RT-qPCR was performed in the StepOne Realtime PCR system (Applied Biosystems). For each insect sample, three biological replicates were used. For normalization, GAPDH was used as an internal control.

Statistical analysis

The data was analyzed statistically using One-Way ANOVA and post-Dunnett's test performed in Graph Padprism v.7.0. Prior to conducting a Dunnett test, essential assumptions were evaluated, i.e., the normality of data distribution was assessed using the Shapiro-Wilk test. The equality of variances across groups was examined through Bartlett's test. Unequal variances may necessitate adjustments like Welch's ANOVA or data transformations. Adherence to these assumptions was crucial for the validity of the Dunnett test, a method used to compare multiple treatment groups to a control group within the framework of analysis of variance. Consideration of alternative tests, i.e., the Kruskal-Wallis test is warranted if assumptions were substantially violated.

Results

In-silico target prediction studies

The dsAK fragment contains eight potential siRNAs as revealed in in-silico studies. The in-silico predicted GC content of each siRNA was calculated by using the OligoCalculator. The percentage of GC content of a particular siRNA is an important parameter to predict its efficacy. All of the siRNAs had a % GC content within the recommended range (31.6% to 57.9%), with the lowest being 36% and the highest being 52% (Fig. 2A, C). The exact position of the siRNA that would act on the target gene is depicted in Figure 2B. The minimum free energy of hybridization (kcal · mol $^{-1}$) for each designed siRNA was computed by using the Mfold program, and represented in Figure 2A, D. MFE of hybridization (kcal · mol $^{-1}$) provides an estimate of gene silencing efficiency based on the thermodynamics of siRNA-mRNA interactions. siRNA used in this study had perfect complementarity with mRNA, in the seed region. The Hb index value inversely correlates with the specificity of the siRNA molecules to interact with the targeted mRNA region. HB- index of all siRNAs have been shown in Figure 2E. The secondary structure of the mRNA of AK was computed by using the Mfold program and is shown in Figure 2F, while the targets of siRNAs in the mRNA of AK secondary structure are represented in Figure 2G.

Generation of transgenic cotton lines

The cotton plants which transformed initially (56 out of 500) and survived post acclimatization were subsequently shifted to the tunnel. A schematic diagram of cotton transformation events is shown in Figure 3C. The putative transgenic cotton plants were analyzed for the presence of the transgene through gene-specific amplification. Out of 56 acclimatized cotton plants obtained by transformation with pCAM-dsAK, only four plants named dsA-1, dsA-3, dsA-6 and, dsA-7 were found positive as shown in Figure 3B. Hence, the transformation efficiency was recorded as 0.92% (Fig. 3E). The PCR positive plants for each of the constructs were grown to maturity and seeds were harvested for T_1 plants in the following season. The cotton plants obtained from seeds of a single positive plant were referred to as a line. The cotton plants in T_1 were screened for transgene insertion and found that the number of transgene positive plants in each of the transgenic cotton lines (dsA-1, dsA-3, dsA-6 and dsA-7) was variable when analyzed through PCR. No segregation data was recorded. Moreover, not all progeny plants were positive for transgene insertion in each of the four transgenic cotton lines. We selected three plants from each of the transgenic cotton lines for insect feeding assay and subsequent knockdown studies. This selection was based on plant architecture and overall plant health and morphology.

It was observed that all three selected plants from four dsAK transgenic cotton lines (dsA-1, dsA-3, dsA-6 and dsA-7) showed chromogenic signals when hybridized with DIG-labeled dsAK probe (Fig. 3F).

The T_1 transgenic cotton lines containing dsAK transgene were evaluated for relative mRNA expression through quantitative real time PCR (RT-qPCR). The Livak $2^{-\Delta\Delta Ct}$ method was used for the relative analysis of gene expression. The relative analysis of gene expression in dsAK transgenic cotton lines revealed that dsA-7 exhibited the highest transcript level with an

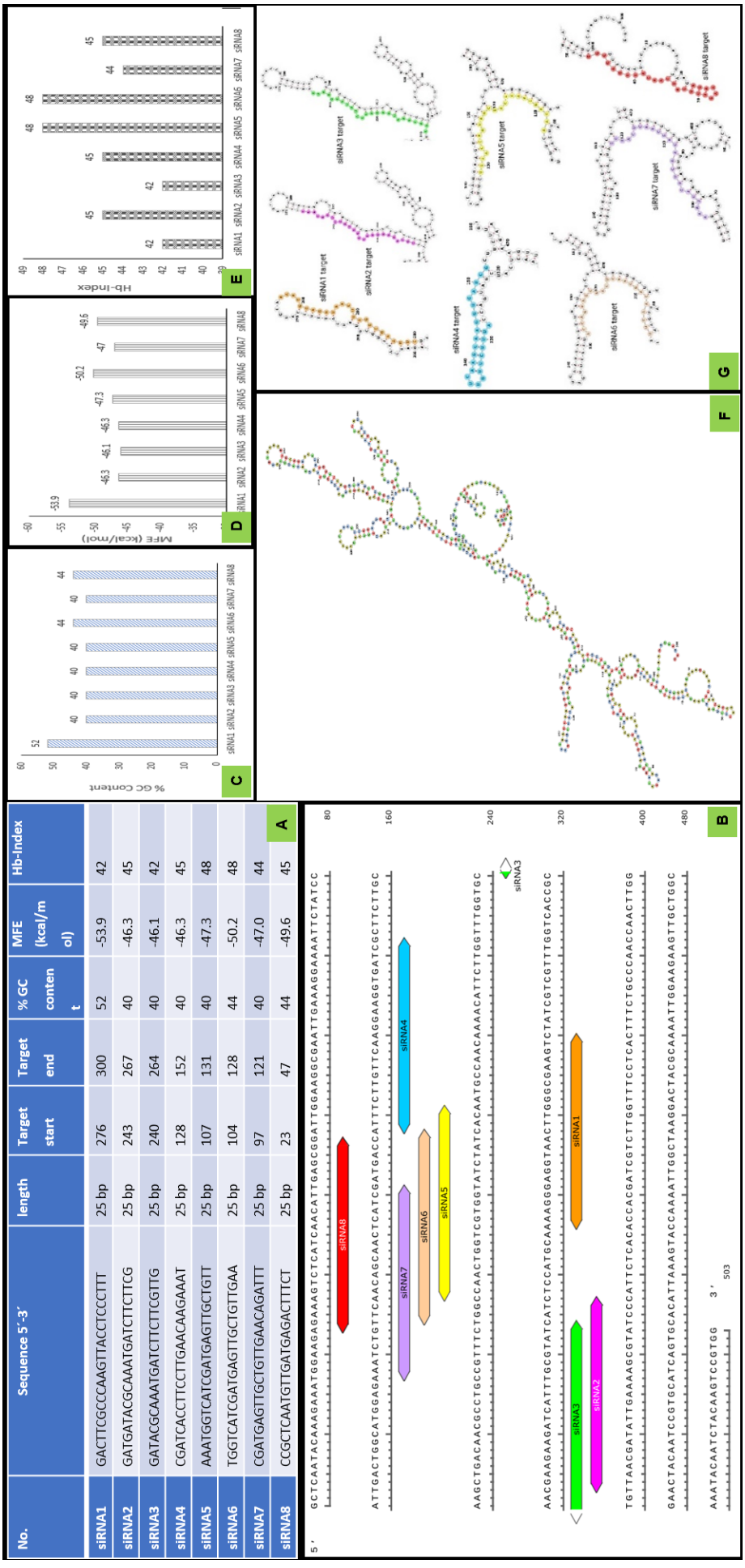
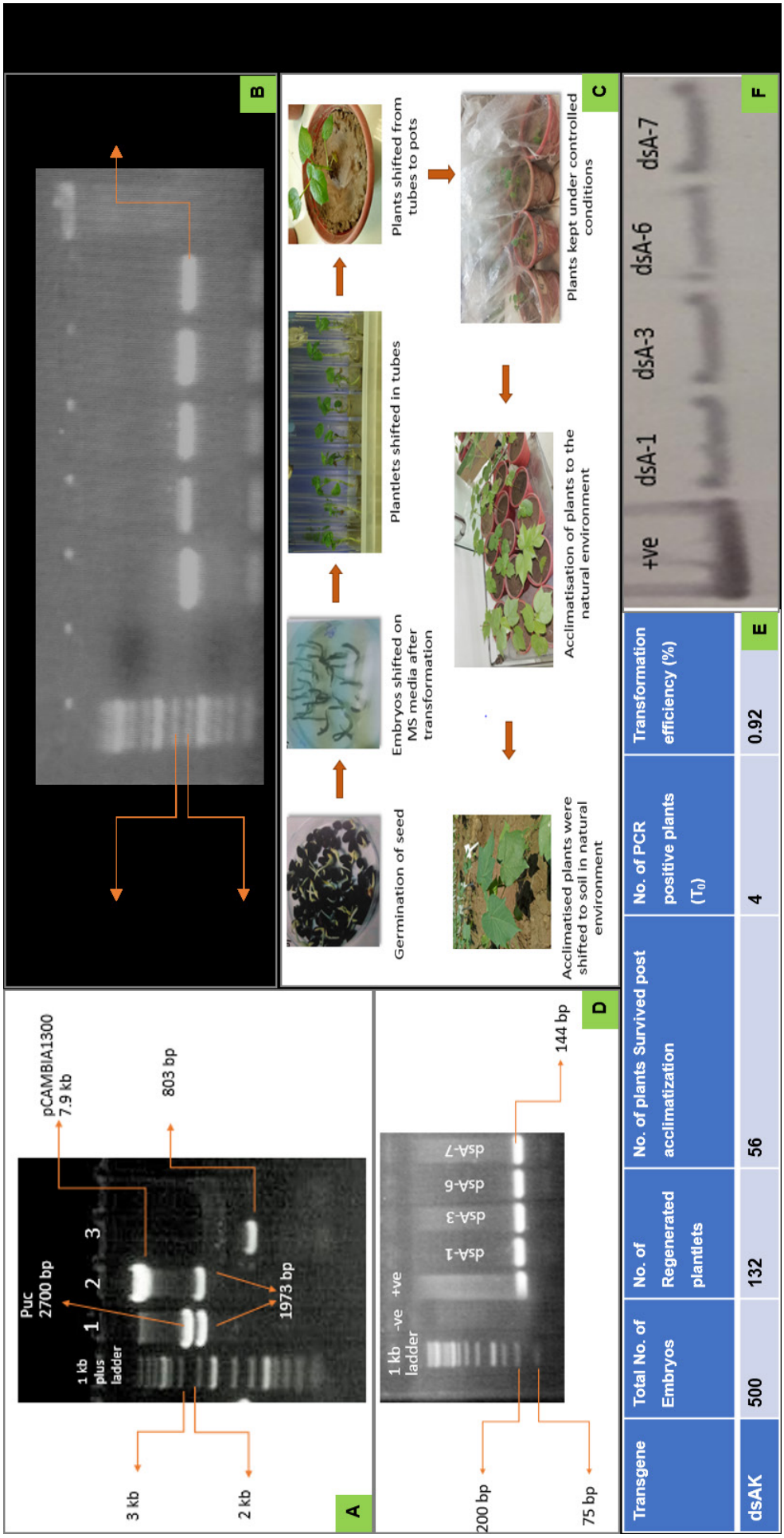


Fig. 2. A – graphical represenatation of properties of siRNAs present in dsAK fragment in tabular form; B – position of siRNA in AK mRNA; C – GC content of siRNAs; D – minimun free energy of hyberidization if siRNAs; E – HB index of siRNAs present in dsAK fragment; F – secondary structure of *Arginine Kinase* predicted by RNAFold. The free energy of the thermodynamic ensemble is –136.07 kcal · mol^{-1}; G – mRNA local secondary structures with target mRNA sequence for each designed siRNA of AK (highlighted)



Transgene	Total No. of Embryos	No. of Regenerated plantlets	No. of plants Survived post acclimatization	No. of PCR positive plants (T_0)	Transformation efficiency (%)
dsAK	500	132	56	4	0.92

E

Fig. 3. Generation of dsAK transgenic cotton lines. A – cloning of dsAK fragment in pCAMBIA1300 and its confirmation. Lane 1: dsAK fragment digested frompUC57 vector, fragment of ~1973bp represent dsAK fragment while ~2.7kb fragment represent pUC57 vcetor, lane 2: dsAK fragment cloned in pCAMBIA1300 vector, ~1973bp fragment represent dsAK while ~7.9kb fragment shows pCAMBIA1300 vector, lane 3: confirmation of dsAK presence in pCAM-dsAK construct through amplification of ~803bp specific amplicon. B – PCR confirmation of dsAK transgene (~803bp) in recombinant *Agrobacterium* cells. +ve: positive sample amplified from plasmid DNA (pCAM-dsAK construct), -ve: negative control; C – a schematic representation of transformation events of cotton; D – PCR confirmation of dsAK transgene in transformed cotton plants. +ve: positive sample amplified from plasmid DNA pCAM-dsAK. -ve: negative control; E – transformation efficiency; F – **s**outhern blot analysis of four dsAK modified transgenic cotton lines in T_1 generation to reveal the genetic integration of the transgene in cotton genome. Ant-DIG dsAK probe was used in hybridization with genomic DNA of subjected cotton plants. The positive sample depicted hybridization of dsAK transgene obtained through restriction digestion of pCAM-dsAK construct

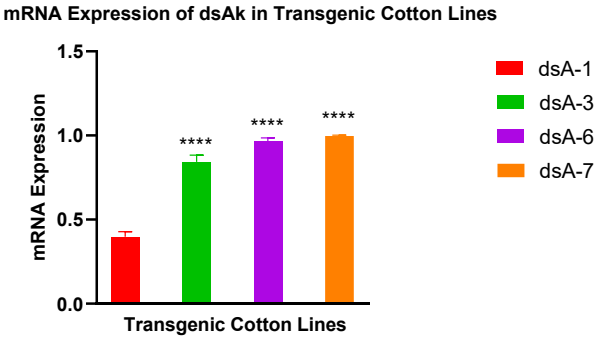


Fig. 4. Relative mRNA expression of dsAK transgene in dsAK modified T_1 transgenic cotton lines. The values were normalized with beta actin while Livak method was used to reveal relative mRNA expression among transgenic cotton lines

expression value of one while the lowest transcript level was revealed in dsA-1. Similarly, the mRNA expression of dsAK in transgenic cotton lines; dsA-3 and dsA-6 was 96% and 84% when compared with dsA-7 transgenic cotton line (Fig. 4).

Aphid feeding bioassay and expression levels of arginine kinase gene in aphid population post bioassay

The four T_1 transgenic cotton lines (dsA-1, dsA-3, dsA-6, and dsA-7) exhibited transgene positivity in more than three plants, but we selected only three individual plants for the in-planta aphid-feeding assay (Fig. 5A, B, C). The in-planta bioassay showed that transgenic line dsA-7 had the highest aphid mortality of 73.3% whereas dsAK modified transgenic cotton line, dsA-1, dsA-3 and dsA-6 revealed 60%, 61%, and 66.6% aphid mortality, respectively, while only 13.3% mortality of aphids was observed in the mock control cotton plants 5 days post feeding (Fig. 5D). The knockdown in mRNA expression of the targeted gene, AK, was measured in aphids which fed on dsAK modified T_1 transgenic cotton plants during in-planta bioassay. For

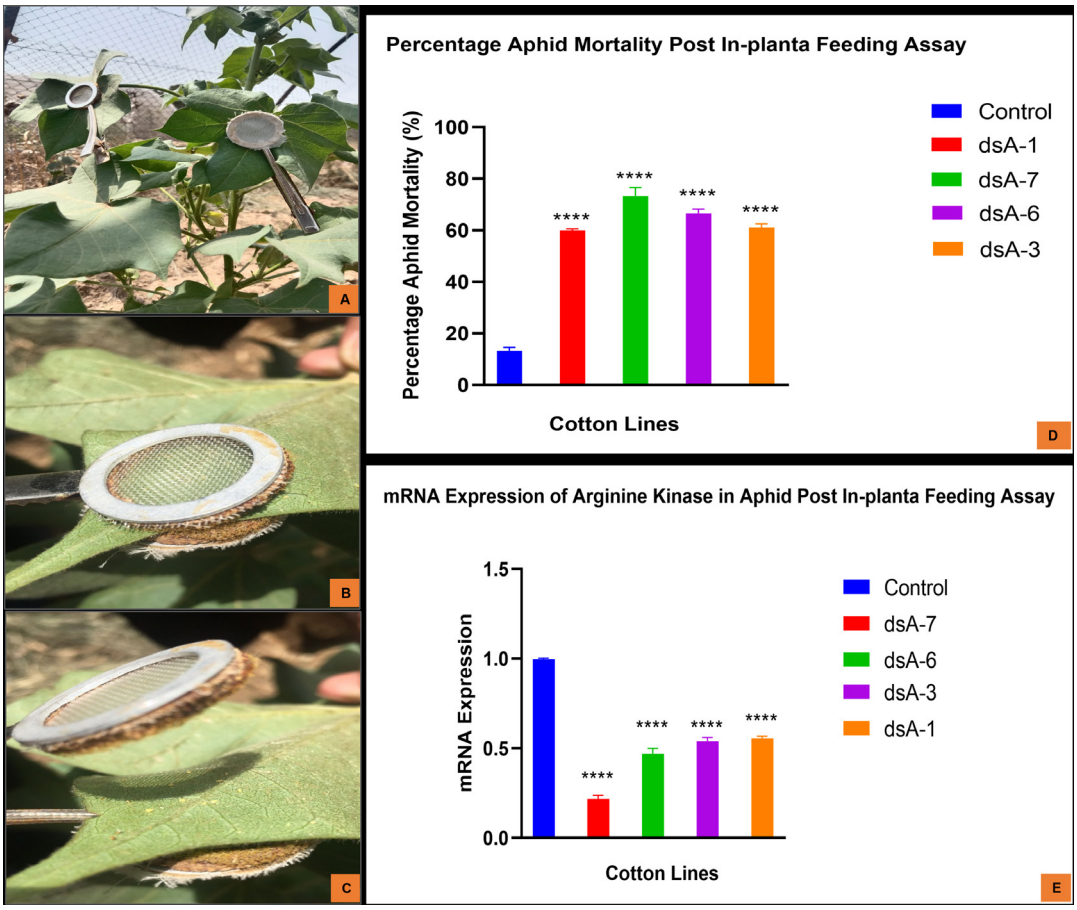


Fig. 5. Plant-mediated aphid bioassay. A–C – aphid bioassay setup. A local aphid capturing device was used to let aphids fed on dsAK T_1 transgenic cotton plants; D – graphical representation of percentage *Aphis gossypii* mortality in transgenic cotton lines modified with dsAK. Microsoft Excel and GraphPad Prism Software were used to apply. One-way ANOVA and post-Dunnet's test was applied ($p > 0.05$; $n = 3$); E – graphical representation of relative mRNA expression of *AK* gene in aphids fed on dsAK modified transgenic cotton lines. Three biological replicates were used for each transgenic line. Error bars show the standard deviation among three biological replicates

this, the live aphids post in-planta bioassay were used for RNA isolation. It was found that the mRNA expression of the AK gene was significantly downregulated in all aphid groups which fed on transgenic cotton lines expressing dsAK in comparison to the control. The maximum knockdown was 79% which was observed in aphids fed transgenic cotton line, dsA-7, while the mRNA expression of AK in aphids fed cotton plant modified with empty pCAMBIA1300 vector was taken as the mock control. However, the AK gene expression was downregulated to variable extents in aphids which fed on all other transgenic lines such as 54, 47 and 45% downregulation in mRNA expression of the AK gene in aphids which fed on dsA-6, dsA-3 and dsA-1 transgenic cotton lines, respectively (Fig. 5E). While the mRNA expression of AK in aphids fed the mock control cotton line was kept as the control that exhibited maximum mRNA expression while all others were compared to this (Fig. 5E).

Discussion

Significant scientific work has been done over the last two decades to manage pests by producing transgenic pest-resistant plants (Tian *et al.* 2015; Fatima *et al.* 2019). It is necessary to develop effective and environmentally acceptable methods to avert large economic losses and insecticidal resistance by insects. *A. gossypii* cause serious damage to cotton crops leading to significant yield loss not only in Pakistan but also in other cotton growing regions of the world. RNAi has the potential to be an effective and dependable approach to developing bioinsecticides. The best RNAi target genes are those that encode proteins with critical biological activities since they can induce mortality/lethality, obviously impaired growth and development, and phenotypic abnormalities (Shaheen *et al.* 2015). AK is the main energy enzyme responsible for energy homeostasis in insects. The initial evidence that AK may be successfully targeted by dsRNA was used to suppress psyllids and sharpshooters in citrus and grapevine trees (Zhang *et al.* 2022).

In this study using RNAi, dsRNA of the 312bp region from accession number GU937512.1 was selected and designed with sense and antisense of the selected dsRNA containing intron of 1349 bp of chalcone synthase gene were commercially synthesized. The dsRNA named dsAK was ligated in plant expression vector pCAMBIA 1300 under the CaMV35S promoter and poly A terminator. An agrobacterium-mediated transformation approach was used to transform the cotton variety MNH886 with dsAK construct and further Southern blot analysis confirmed the successful integration of dsAK in the genome of transgenic cotton lines.

The design of dsRNA is crucial in obtaining significant mortality of insects. Different parameters are to be considered before proceeding with the trial. The high GC content has the tendency to fold which will reduce the target accessibility (Chan *et al.* 2009). Secondary siRNA structure formation may hinder RISC-mediated target cleavage. As a result, predicting prospective secondary structures and determining the free energy of matching folding is critical. Lower free energy depicts that the selected dsRNA is more potent to the mRNA sequence than high free energy dsRNA molecules (Chowdhury *et al.* 2021). For all these purposes RNAi designer of thermos fisher was used to select the most potent dsRNA against the mRNA of arginine kinase.

The aphid nymphs exposed to transgenic cotton lines expressing dsRNA exhibited significant mortality as revealed during in-planta insect bioassays. We reported that the transgenic cotton line dsA-7 harboring transgene dsAK showed the highest aphid mortality of 73% with $p < 0.01$ in comparison to the mock control cotton plants. Similarly, Bento *et al.* (2019) observed significant decreases in transcript accumulation and an increase in larval mortality when AK dsRNA was fed to *Tuta absoluta* (Meyrick) larvae. Our results also differ from some of the studies such as Fu *et al.* (2020) who reported a 25% mortality rate in *Plutella xylostella* exposed to transgenic *Arabidopsis thaliana* expressing AK dsRNA.

In comparison to the mock control, mRNA expression of the AK gene from live aphids after the in-planta bioassay was carried out and it was observed that the AK gene was downregulated in all aphid groups fed transgenic cotton lines expressing dsAK. In aphids fed a transgenic cotton line, dsA-7, the greatest knockdown was 79%. Here we have documented significant downregulation of the AK gene of the *A. gossypii* fed respective transgenic cotton lines post in-planta bioassay which co-related to the mortality of aphids. According to Andrade and Hunter (2017), ingestion of double-stranded AK by psyllids reduced the mRNA level by 70% and the insect mortality rate by 53% which was significantly high. Murtaza *et al.* (2022) demonstrated that aphid mortality and inhibition of MIF1 gene expression do not correlate, which may be related to the insect's effective uptake of dsRNA.

This study proves that using AK dsRNA has the potential to be the target of choice for bioinsecticide-based RNAi approach.

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