



# Transgenic Silkworms Overexpressing Relish and Expressing Drosomycin Confer Enhanced Immunity to Multiple Pathogens

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## Abstract

The sericulture industry faces substantial economic losses due to severe pathogenic infections caused by fungi, viruses, and bacteria. The development of transgenic silkworms against specific pathogens has been shown to enhance disease resistance against a particular infection. A single gene or its products that can confer protection against multiple pathogens is required. In an attempt to develop silkworms with enhanced immunity against multiple pathogens, we generated transgenic silkworm lines with an overexpressed NF- $\kappa$ B transcription factor, *Relish 1*, under two different promoters. Separately, a potent anti-fungal gene, *Drosomycin*, was also expressed in transgenic silkworms. Both *Relish 1* and *Drosomycin* transgenic silkworms had single copy genomic integration, and their mRNA expression levels were highly increased after infection with silkworm pathogens. The overexpression of the Relish 1 in transgenic silkworms resulted in the upregulation of several defense-related genes, *Cecropin B*, *Attacin*, and *Lebocin*, and showed enhanced resistance to *Nosema bombycis* (microsporidian fungus), Nucleopolyhedrovirus (*BmNPV*), and bacteria. The *Drosomycin* expressing transgenic silkworms showed elevated resistance to *N. bombycis* and bacteria. These findings demonstrate the role of Relish 1 in long-lasting protection against multiple pathogens in silkworms. Further, the successful introduction of a foreign gene, *Drosomycin*, also led to improved disease resistance in silkworms.

**Keywords** Silkworm · Pathogens · Relish · Overexpression · Disease resistance · Transgenic

## Introduction

Sericulture is an agro-based industry practiced in China, India, Brazil, and other developing countries. The majority of commercial silk produced in the world comes from the silkworm, *Bombyx mori*. It and other silkworms are susceptible to various infectious diseases caused by pathogens. The major pathogens infecting silkworms are microsporidia, including *Nosema bombycis*, nucleopolyhedrovirus (NPV), densovirus (DNV), infectious flacherie virus (IFV),

cytoplasmic polyhedrovirus (CPV), bacteria, and fungi, causing substantial crop loss in the sericulture industry [1, 2]. Therefore, the use of silkworm breeds resistant to diseases is a prerequisite for effective disease prevention and increased productivity. Conventional breeding strategies are labor-intensive, time-consuming and imprecise, whereas, through transgenesis, desired traits can be introduced into an organism with higher accuracy.

The use of transgenic technology to enhance disease resistance in silkworms is a promising strategy [3–5]. The development of individual transgenic silkworm lines for every pathogen to be controlled is challenging and not practical in sericulture. Hence, the need for a single gene or its products that can be used to develop transgenic silkworms conferring resistance to multiple pathogens is desirable. Several transgenic silkworms conferring protection against a particular pathogen such as *BmNPV*, *BmCPV*, *N. bombycis*, and bacteria by overexpressing endogenous/exogenous or by targeting pathogen-specific genes have been reported [6–13]. The Sprouty (Spry) protein

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is reported to regulate the activation of ERK and inhibit replications of different classes of silkworm viruses, NPV, CPV, and BDNV [14, 15]. To our knowledge, there are no reports on transgenic silkworms expressing a single gene to enhance immunity against multiple categories of pathogens (Fungi, viruses, and bacteria). Here, we show that overexpression of a single gene, *Relish 1*, can confer enhanced protection against multiple pathogens of silkworms. Analogously, we intended to look into whether a foreign gene and its promoter borrowed from *Drosophila melanogaster* would be functional in silkworms. Thus, transgenic silkworm expressing *Drosomycin*, a potent anti-fungal gene, was successfully developed and functionally tested.

Relish is a transcription factor that is an important part of the IMD (immunodeficient) signaling pathway. It is a key component in the induction of the humoral immune response in *Drosophila* and other insects, inducing anti-bacterial, anti-fungal, and anti-plasmodium factors [16–18]. The antimicrobial peptides (AMPs) under the regulation of Relish include Cecropin, Diptericin, Attacin, Defensin, and Metchnikowin [19]. *Relish 1* and *Relish 2* genes have been identified and characterized in insects, and *Relish 1* is found to be more active than *Relish 2* in silkworm, *B. mori* [20, 21]. Relish 1 consists of Rel homology domain (RHD), nuclear localization sequence (NLS), acidic and hydrophobic amino acid (AHAA)-rich region, ankyrin repeats, and death domain [20]. Relish 1 is present as an inactive form in the cytoplasm. It becomes active by endoproteolytic cleavage of ankyrin repeats and the death domain. In addition, Relish 1 lacking the ankyrin repeats (ANK) at the C-terminal region has been shown to strongly activate the promoters of antimicrobial genes in insect mbn-2 cells [20]. Hence, we chose *Relish 1* as a candidate gene to test whether transgenic silkworm overexpressing the same can enhance immunity against different pathogens. The *Drosomycin* was isolated from bacteria-infected flies [22], effective against fungal infection but inactive against bacteria. Its expression is regulated by the Toll pathway in the fat body of *D. melanogaster* [23]. It is a 44-residue peptide composed of one  $\alpha$ -helix and a twisted three-stranded  $\beta$ -sheet [24].

We have selected a productive and commercial breed of the silkworm, *B. mori*, CSR2, which is used in the sericulture industry in India, but susceptible to diseases. We generated transgenic silkworm lines expressing *Relish 1* or *Drosomycin* under the control of constitutive (*CMV*) and inducible (*Cecropin A* or *Drosomycin*) promoters. Next, we compared their expression levels and resistance against protozoan/fungus (microsporidian, *N. bombycis*), virus (*BmNPV*), and gram-negative and positive bacteria, the major pathogens infecting silkworms.

## Materials and Methods

### Silkworm

The silkworm *B. mori* breed, CSR2, was reared on fresh mulberry leaves at  $25 \pm 1$  °C with  $75 \pm 5\%$  relative humidity. Rearing and grainage activities were carried out as per standard protocols. All transgenic procedures and maintaining of transgenic silkworms were followed as per the Institute Biosafety Committee procedures (Approval No. CSB/SBRL/IBSC/2017-18-Project Code AIT 3540). Silkworm pathogens used in the study were as reported by us earlier [1].

### Vector Constructions

The *piggyBac* (PB) transposon vector (System Biosciences, USA) consists of *CMV* promoter for transgene expression and *green fluorescent protein* (*GFP*) reporter gene under the *elongation factor 1 $\alpha$*  (*EF1 $\alpha$* ) promoter, was used for making transgenes. The active form of *Relish 1* lacking ankyrin repeats and death domain was used separately under *CMV* and *Cecropin A* promoters. An 804 bp DNA fragment of *Cecropin A* promoter (Accession No. D84395.1, from nt 3 to 806) was amplified from genomic DNA using primer 1F & 1R with an additional NF $\kappa$ B site incorporated in the forward primer (Table 1). An 1881 bp *Relish 1* gene (Accession No. AB298441.1, from nt 324 to 2205) was amplified from fat body cDNA of *B. mori* larvae using primers, 2aF/2bF & 2R. For *Drosomycin* promoter, a 1168 bp DNA fragment, 5' upstream regulatory region of *Drosomycin* gene was amplified from genomic DNA of *D. melanogaster* using the primers 3F & 3R. A 213 bp *Drosomycin* gene (Accession No. NM\_079177.4 from nt 1 to 213) was amplified from fat body cDNA of *D. melanogaster* using primers, 4aF & 4aR or 4bF & 4bR. The *GFP* and SV40 poly A signal was individually amplified from PB plasmid employing primers 5F & 5R and 6F & 6R. The PCR amplification was set with an initial denaturation of 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 68 °C for 30 s and 72 °C for 1 min/kb using Platinum *Taq* DNA polymerase (Thermo Fisher Scientific, USA). For SV40 amplification, the annealing temperature was 52 °C. The amplified *Cecropin A* or *Drosomycin* promoter, *Relish 1* or *Drosomycin*, SV40 poly A signal and *GFP* after restriction digestions were ligated in-frame to PB plasmid separately to generate the vector constructs: PBCMV-*Relish1*-SV40, PBCecropinA-*Relish1*-SV40, PBCecropinA-*GFP*-SV40, PBCMV-*Drosomycin*-SV40, PBDrosomycin-*Drosomycin*-SV40 and PBDrosomycin-*GFP*-SV40], transformed in *E. coli* (*DH5 $\alpha$* ) and plasmids

**Table 1** List of primers used in the study. NFκB site is underlined in 1F

Primer	Sequence (5' to 3')	Product size (bp)
1F	CACTACGTAGGGATCTCCCGAACTCATACAAATGATTTGTTCCGGGC	833
1R	GCACGACGTCTTTACCGAAATTCTATAGTGCCGGG	
2aF	CCGCGCTAGCATGTCTACAAGTCCAGTGATCAGGAT	1902
2bF	GCACGACGTCATGTCTACAAGTCCAGTGATCAGGAT	
2R	ATTTAAATTTACAAATTCCTGTAGTCCAAGAGGTGG	
3F	CACTACGTAGTAGCTATTAAGGCGCAGCATGTAG	1187
3R	GCACGACGTCATTGGAAAAGTTCTCACGGAGCTTG	
4aF	CCGCGCTAGCATGATGCAGATCAAGTACTTGTTCCG	233
4aR	GCGCTTCGAATTAGCATCCTTCGCACCAGCACTTCAG	
4bF	GCACGACGTCATGATGCAGATCAAGTACTTGTTCCG	231
4bR	ATTTAAATTTAGCATCCTTCGCACCAGCACTTCAG	
5F	GCACGACGTCATGGAGAGCGACGAGAGCG	765
5R	GATTGTCGACTTAGCGAGATCCGGTGGAG	
6F	GGATTTAAATAACTAACTTGTTTATTGCAGCTTATAATGGTTACAA	155
6R	GCGCCTAGGCAGACATGATAAGATACATTGATGAGTTTGGACAA	
7F	TAGACAATGGCTCAAAGAGAAAC	1935
7R	GCGGAGCCAGTACACGACATCACT	
8F	TCTGCAACTGAATTCGACGG	178
8R	TGCAGAGTCGGTTTGCATC	
9F	TCGACGATGTGAAGCTCGAT	143
9R	CCTTGTTGTTACGGTGGCTCT	
10F	ATCCTTCGTCTTCGCTCTGG	161
10R	TGGCTTTAGCTGAACCGAGG	
11F	GTTGTTGTTGTTGTGCGCGT	153
11R	GCGCTGAGTACGTTCTTGTC	
12F	CTCTTCGCTGTCCTGATGCT	182
12R	CATCCTTCGCACCAGCACTT	
13F	CATCCGCGTCCCTGTTGCTAA	170
13R	ACCAATGAAGTCTGAGGACAC	
14F	CCTAAATGCACAGCGACGGA	~4 to 11 kb
14R	GACCAGATGAGCTTCTGATAG	
15F	TGATTATCTTTAACGTACGTCAC	
15R	ACTAGTATTATGCCAGTACATG	

were purified. The plasmids were sequenced to confirm their integrity and correctness of the frame. Finally, six transgenic PB vectors were constructed and designated as PBCMVPRelish1, PBCecAPRelish1, PBCecAPGFP, PBCMVPDr, PBDrsPDrs, and PBDrsPGFP. The list of primers and their product sizes which include the primer sequences are given in Table 1. Restriction enzymes (New England BioLabs, USA) and other reagents were purchased from Thermo Fisher Scientific, USA.

### Electroporation and Microinjection

Female moths were allowed to lay eggs post 4 h of copulation. Eggs were disinfected with 2% formalin for 5 min and then washed with water. To prevent the embryo from entering into a diapause state, eggs were treated with

HCl (specific gravity of 1.110) at 25 °C for 90 min and washed under running water and air-dried [25]. Transgenic silkworms were essentially generated using previously described methods [9, 26]. Eggs were collected in a 0.4 cm chilled electroporation cuvette containing 10:1 of vector and helper vector (System Biosciences, USA) in deionized water. Electroporation was performed using Gene Pulser (BioRad, USA) at constant resistance of 200 Ω, the capacitance of 25 μF giving single pulse at 1700 V. For microinjection, eggs were arranged on a glass slide and microinjected with vector and helper (5:1) in injection buffer (0.5 mM Na<sub>3</sub>PO<sub>4</sub> pH 7 and 5 mM KCl) using a microinjector (FemtoJet, Eppendorf, Germany). Electroporated/microinjected eggs were incubated at 25 °C and 75% relative humidity to allow hatching.

## Screening and Selection of Transgenic Silkworm

The hatched  $G_0$  larvae were reared on fresh mulberry leaves under standard conditions, and each surviving moth was mated with a parental CSR2 moth to generate  $G_1$ . Transgenic eggs, larvae, and pupae were screened for GFP at 480 nm under a fluorescent microscope (Euromex, Holland). The positive broods were inbred or backcrossed in subsequent generations. Apart from fluorescent screening, at each generation, the presence of *GFP*, *Relish 1*, and *Drosomycin* transgenes was confirmed by PCR using gene/vector-specific primers 5F & 5R, 7F & 7R, and 4aF & 4aR or 4bF & 4bR, respectively, using genomic DNA of moths.

## Quantitative Real-Time PCR Analyses

Total RNA was extracted from the fat body of 5th instar (day 1) infected and non-infected larvae using RNeasy Kit (Qiagen, Germany). Contaminating DNA was removed using RNase-free DNase I (Thermo Fisher Scientific, USA). First-strand cDNA was synthesized from 2  $\mu$ g of RNA using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, USA) employing oligo(dT)18 primers. Resultant cDNA was used as a template for qPCR. The qPCR analysis was carried out on an ABI StepOne Plus real-time PCR (Applied Biosystems, USA) with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. All the reactions were carried out using three biological replicates. Expression of the *Relish 1*, *Lebocin 4* (Accession No. AB003036.1), *Cecropin B* (Accession No. D11113) and *Attacin* (Accession No. NM\_001043541.1) was quantified using primers 8F & 8R, 9F & 9R, 10F & 10R and 11F & 11R, respectively (Table 1). *Drosomycin* was quantified using primers 12F & 12R. Relative quantification using a modified comparative Ct method [27] was employed for gene expression analysis. The relative expression levels of respective genes were calculated by normalization to GAPDH (Accession: NM\_001043921.1) using primers 13F & 13R.

## Inverse PCR

Genomic DNA was extracted from transgenic silkworms and digested with *KpnI* and *NdeI*, and self-ligated. The products were then used as templates for inverse PCR with primers specific for the transposon, pBacL (14F & 14R) and pBacR (15F & 15R) as reported [7, 28]. The PCR products were cloned into the pJET vector (Thermo Fisher Scientific, USA) or directly sequenced, then analyzed using the silkworm genome database, silkDB (<https://silkdb.bioinfotoolkits.net/>).

## Mortality Analyses

*B. mori* Nucleopolyhedrovirus (*BmNPV*, 3000 polyhedral/larva), *N. bombycis* (3000 spores/larva) or bacteria—*Escherichia coli* (JM 109) or *Staphylococcus aureus* (ATCC 25923) ( $10^4$  CFU/ml/larva) were injected into the hemocoel of transgenic and control larvae (5th instar, day 1) at  $G_7$ . Infected and non-infected non-transgenic larvae were used as controls. Surviving larvae were collected at 0, 6 and 12 h or 0, 4, and 8 h for gene expression analyses. The mortality rate was calculated for each group till moth emergence. The experiment was carried out in three groups with 100 larvae in each group. Microscopic examination was performed in the moth stage to determine the presence of injected pathogens. Comparative expression levels of transgenes were obtained by quantitative real-time PCR as above.

## Economical Characters

The economical characters of control and transgenic silkworms were recorded in  $G_7$ . Pupation rate was determined from the percentage of healthy pupae to the numbers of larvae at 5th instar, day 1, from 5 random batches. Fecundity was obtained by counting the number of eggs laid by 15 moths, and the hatching percentage was calculated by considering the number of larvae hatched from the total number of eggs laid. Cocoon and shell weight of 15 randomly selected cocoons were obtained.

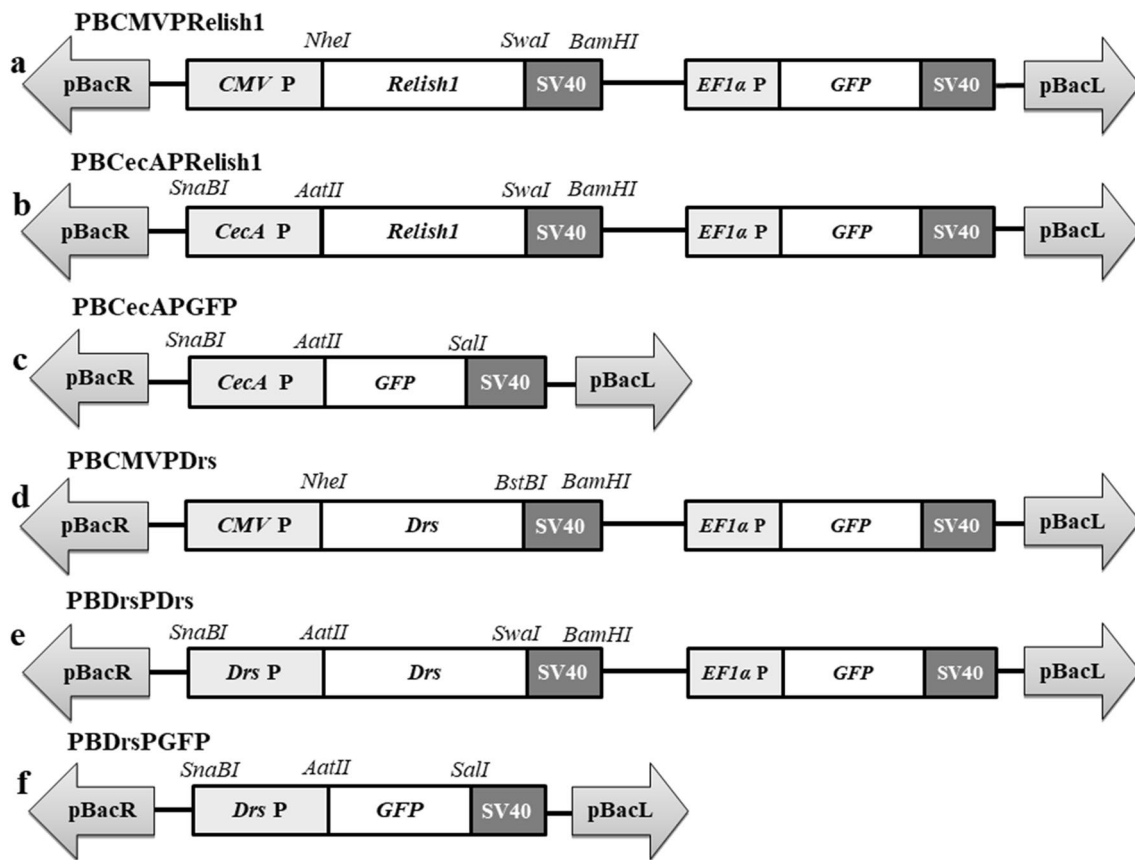
## Statistical Analyses

All statistical values are represented as the mean  $\pm$  SE and analyzed by Student's *t*-test at  $*p < 0.05$  and  $**p < 0.01$  significance levels. The experiments were performed in triplicates unless otherwise stated.

## Results

### Generation and Screening of Transgenic Silkworms Overexpressing *Relish 1* and Expressing *Drosomycin*

The transgenic vectors, PBCMVP*Relish1*, PBCecAP*Relish1*, PBCecAPGFP, PBCMVP*Drs*, PBD*DrsPDrs*, and PBD*DrsPGFP*, were generated for overexpression/expression of the *Relish 1* (active form) or *Drosomycin* under the control of constitutive and inducible promoters (Fig. 1). *GFP* under *EF1 $\alpha$*  promoter was used as a reporter to screen transgenic silkworms (Fig. 1). Each vector was mixed with transposase encoding a helper vector and was introduced into silkworm embryos of CSR2 breed by electroporation or microinjection. The  $G_0$  moth (s) was crossed with wild moth (s) to produce heterozygous  $G_1$  broods. The success



**Fig. 1** Generation of transgenic lines of *B. mori*. Schematic representation of overexpression/expression constructs in *piggyBac* (PB). **a** PBCMVPRelish1: *Relish1* without C-terminal domain under the control of *CMV* (Cytomegalovirus) promoter; **b** PBCecAPRelish1: *Relish1* without C-terminal domain under the control of *Cecropin A* promoter; **c** PBCecAPGFP: *GFP* under *Cecropin A* promoter; **d** PBCMVPDrs and **e** PBDrsPDrs: *Drosomycin* under the control

of *CMV* and *Drosomycin* promoters; **f** PBDrsPGFP: *GFP* under *Drosomycin* promoter; SV40: polyadenylation signal; pBacR and pBacL: right and left terminal inverted repeats of *piggyBac* vector; *CecA P*: *Cecropin A* promoter; *Drs P*: *Drosomycin* promoter; *Drs*: *Drosomycin*; *Ef1α P*: *Elongation factor-1alpha* promoter. Restriction enzyme sites are indicated

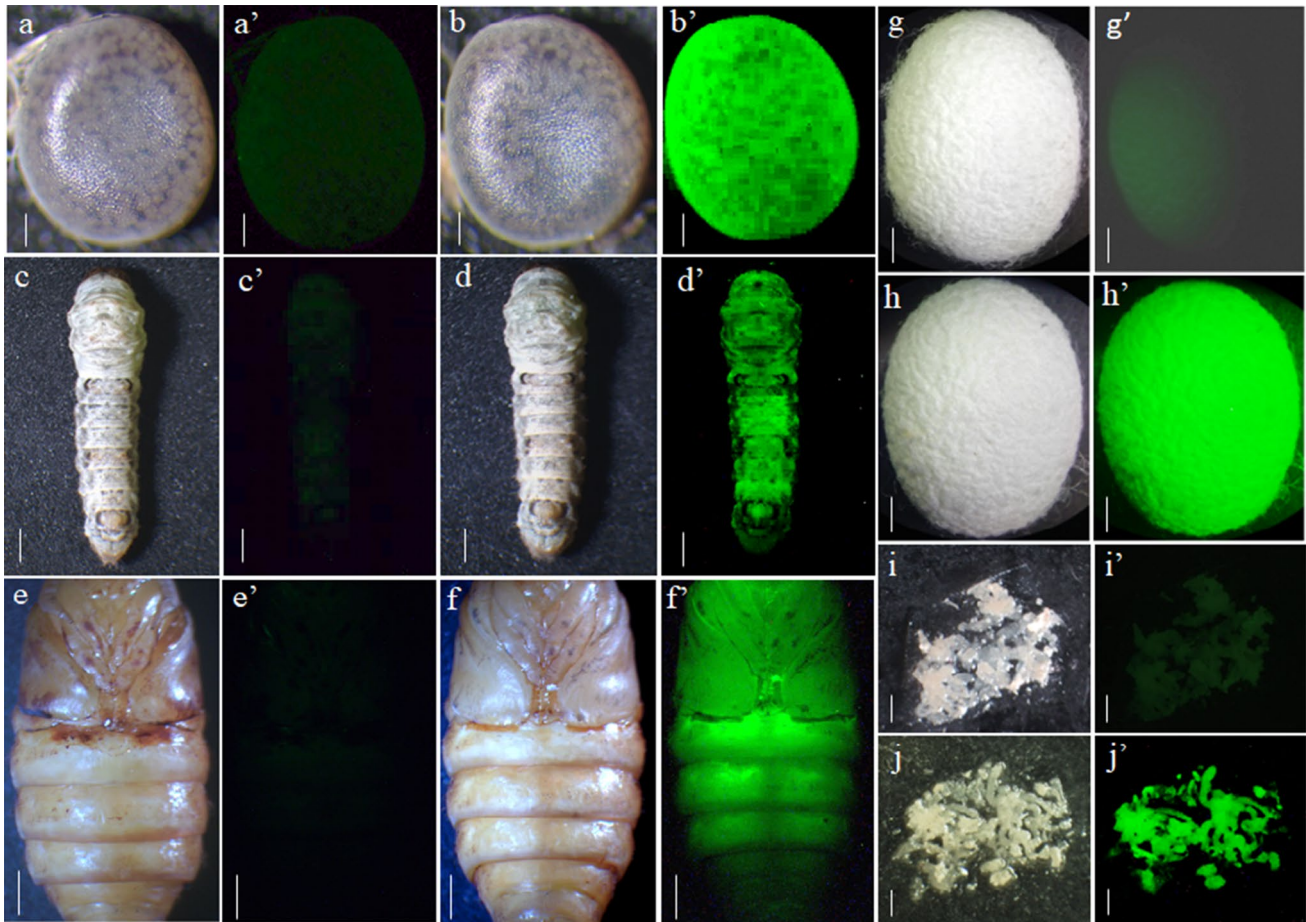
percentage was determined from the number of  $G_1$  broods having GFP-positive larvae to the number of  $G_0$  moths backcrossed. A difference in efficiency was observed in the transformation between the two gene delivery methods. In  $G_1$  broods, 16.6% and 9.5% of silkworms were found to be expressing GFP by electroporation and microinjection methods, respectively (Table 2). As shown in Fig. 2, GFP expression was observed in eggs (b'), 3rd instar larva (d'), pupae (f'), and cocoon (h') of transgenic silkworms compared with that of controls (a', c', e', and g'). The GFP expression in the fat body of CecAGFP transgenic larvae confirms that *Cecropin A* promoter (including an extra NFκB binding site) activated *GFP* transcription in the fat body (Fig. 2j'). The vector construction also included a GFP under the control of the *Drosomycin* promoter (Fig. 1f), which showed the same results. The  $G_1$  transgenic silkworms were inbred or backcrossed for generations to obtain stable transgenic lines. The transgenic lines showing good fitness, economical characteristics and

**Table 2** Comparative efficiency of exogenous DNA delivery into silkworm preblastoderm eggs using microinjection and electroporation

Treatment	No. of eggs injected / treated	No. of larva hatched	No. of moth ( $G_0$ )	No. of GFP-positive broods ( $G_1$ )	% of GFP-positive broods ( $G_1$ )
Microinjection	500	150	42	4	9.5
Electroporation	500	10	6	1	16.6

lifespan equivalent to controls were selected from each generation and continued breeding for further generations.

Apart from screening GFP expression microscopically, PCR amplification was carried out by *GFP*-specific primers using genomic DNA as templates. As shown in Fig. 3a–d, amplification of *GFP* resulted in a single band of 765 bp



**Fig. 2** GFP expression at different developmental stages of transgenic silkworms. Representative photographs were taken under white (a–j) or GFP (480 nm) (a'–j') in  $G_4$ . GFP fluorescence was observed in egg (b'), 3rd instar larval (d'), pupal (f') stages, cocoon (h') and larval fat body (j'). No GFP fluorescence was observed in egg (a'),

3rd instar larval (c'), pupal (e') stages, cocoon (g') and larval fat body (i'). Control silkworms: (a, a'), (c, c'), (e, e'), (g, g'), and (i, i'); Transgenic silkworms: (b, b'), (d, d'), (f, f'), (h, h'), and (j, j'). Scale bars—a, a', b, b': 0.2 mm; c, c', d, d': 2 mm; e, e', f, f': 3 mm; g, g', h, h': 5 mm; i, i', j, j': 4 mm

from CMVPrelish1, CecAPRelish1, CecAPGFP, and DrsPDrs transgenic silkworms, respectively. Further, to identify the *Relish 1* transgene, genomic DNA extracted from CMVPrelish1 and CecAPRelish1 and control silkworms were subjected to PCR using a combination of gene and vector-specific primers which gave a discrete band of 1935 bp only in the former two (Fig. 3e, f). Likewise, a single band of 233 or 231 bp was observed when genomic DNA from CMVPDrs and DrsPDrs were subjected to PCR using *Drosomycin* primers (Fig. 3g, h). All the PCR products were sequenced to confirm their authenticity (Data not shown).

### Analyses of Insertion Sites of the Transgenes

Inverse PCR was performed to detect and confirm the insertion sites of transgenes in silkworm lines. A single band was observed in PCR amplifications employing pBacL and pBacR primer pairs, indicating that transgenic insertion

occurred as a single copy (Supplementary Fig. S1). Sequencing analysis showed that all the transgenic insertions were in intergenic regions of the silkworm genome. CMVPrelish1 was inserted on chromosome 14, and the nearest left and right genes identified were *BGIBMGA009544* and *BGIBMGA009543*, which were 45.85 and 1.14 kb away from the insertion sites, respectively. The insertion site of CecAPRelish1 was found on chromosome 18, while the nearest genes on the left and right ends were *BGIBMGA008250* and *BGIBMGA008264*, which were 2.8 and 4.5 kb away from the insertion site, respectively. The CecAPGFP insertion was observed on chromosome 11, and the nearest genes identified were *BGIBMGA012123* and *BGIBMGA012124*, on the left and right sides that were 45.43 and 80.04 kb away from the insertion site, respectively. The CMVPDrs was inserted on chromosome 21, while the nearest gene on the left was *BGIBMGA012446*, and the gene on the right was *BGIBMGA012445* which were 45 and 37 kb away from the

insertion site, respectively. The insertion of DrsPDRs was found on chromosome 10, and the nearest genes to the left and right were *BGIBMGA006565* and *BGIBMGA013103*, which were 1.2 and 8.5 kb away from the insertion site, respectively. The insertion of DrsPGFP occurred on chromosome 3, while the nearest genes were *BGIBMGA007425* and *BGIBMGA007424*, which were 8.2 and 3.4 kb away from the insertion site on the left and right side, respectively (Fig. 3i).

### Transgenes Show Increased Expression and Enhanced AMP Production

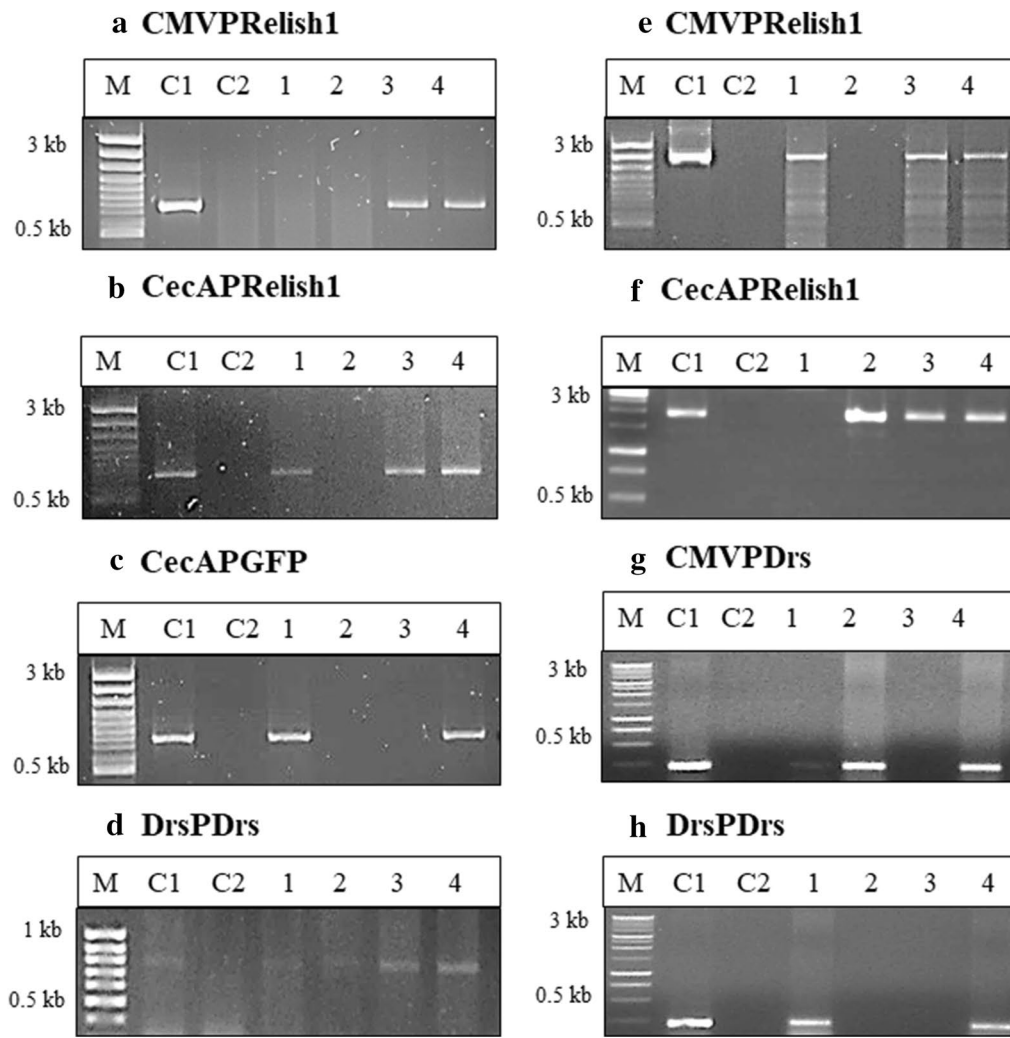
The expression level of *Relish 1* transcripts in 5th instar larvae was determined post-infection (p.i.) with *N. bombycis*, *BmNPV*, and bacteria (*E. coli* and *S. aureus*). Without the immune challenge, the expression levels of *Relish 1* transcripts were low in the larval fat body at 0 h of infection (Fig. 4). However, *Relish 1* mRNA was significantly increased in transgenic lines ( $p < 0.01$ ) compared to controls in response to *N. bombycis*, *BmNPV*, and bacterial challenges (Fig. 4a–d). In response to *N. bombycis* infection, elevated levels of *Relish 1* transcripts were observed at 6 h followed by a slight decline at 12 h p.i. (Fig. 4a). Further its expression was elevated by 5.28, 8.78, and 7.75-fold at 6 h p.i. and declined by 3.90, 6.20, and 6.00-fold at 12 h p.i. in control, CMVPrelish1, and CecAPRelish1, respectively (Fig. 4a). Interestingly, the level of expression of *Relish 1* mRNA was better manifested ( $p < 0.05$ ) in *CMV* promoter than that of *CecA* promoter at 6 h p.i. However, no significant level of its expression was found between *CMV* and *CecA* promoters at 12 h p.i. (Fig. 4a). Similar expression patterns were observed in the case of *BmNPV* infection (Fig. 4b). The transcript levels were elevated by 5.13, 9.28, and 8.50-fold at 6 h p.i. and declined by 3.76, 7.03, and 6.82-fold at 12 h p.i. in control, CMVPrelish1, and CecAPRelish1, respectively (Fig. 4b). Notably, the transcript levels in CMVPrelish1 and CecAPRelish1 transgenic silkworms were higher against *E. coli* than *S. aureus* 4 h and declined 8 h p.i. when compared to control silkworms (Fig. 4c, d). The transcripts of *Relish 1* were elevated by 42.56, 84.51, and 80.32-fold at 4 h p.i. and then declined to 33.36, 65.97, and 63.24-fold at 8 h p.i. in control, CMVPrelish1 and CecAPRelish1, respectively (Fig. 4c). The same in response to *S. aureus* was 9.76, 15.38, and 14.1-fold at 4 h p.i. and 5.98, 10.24, and 8.97-fold at 8 h p.i. (Fig. 4d). No significant difference in expression was observed between *CMV* and *CecA*Relish1 groups. Further, to confirm whether the overexpression of *Relish 1* activated the IMD pathway, the expression levels of AMP genes were quantified post 4 h of *E. coli* challenge. As shown in Fig. 4e and f, the transcript levels of AMPs-*Cecropin B*, *Attacin*, and *Lebocin 4* were significantly elevated in transgenic silkworms compared to

controls. Expression of these AMPs manifested better in CMVPrelish1 than in CecAPRelish1.

*Drosomycin* was found to be expressed in transgenic silkworms. The abundance of *Drosomycin* transcript was determined in response to *N. bombycis* and bacterial (*E. coli* and *S. aureus*) infections but not to *BmNPV* in CMVPrelish1 and DrsPDRs transgenic lines (Fig. 4g–j). Differing from *Relish 1*, *Drosomycin* under its promoter significantly ( $p < 0.01$ ) exhibited a higher expression level than under the *CMV* promoter. No significant difference was observed in the expression of *Drosomycin* between CMVPrelish1 and DrsPDRs at 4 or 6 h p.i. The expression levels of *Drosomycin* were not significantly altered against *BmNPV* infection (Fig. 4h). Transcript levels were significantly ( $p < 0.01$ ) elevated from 6 to 12 h in response to *N. bombycis* (Fig. 4g) and from 4 to 8 h in response to bacterial infection in DrsPDRs (Fig. 4i, j). Expression level of *Drosomycin* was elevated from 1.59 at 6 h p.i. to 6.36-fold at 12 h p.i. in DrsPDRs against *N. bombycis* (Fig. 4g). It was increased from 1.36-fold at 4 h p.i. to 4.82-fold at 8 h p.i. in response to *E. coli* (Fig. 4i) and from 2.06 at 4 h p.i. to 8.36-fold at 8 h p.i. in response to *S. aureus* (Fig. 4j). However, no significant difference in hike of expression was observed from 6 to 12 h in response to *N. bombycis* and from 4 to 8 h in response to bacterial infections in CMVPrelish1. Remarkably, the expression of *Drosomycin* was found to be more in DrsPDRs at 8 h against *S. aureus* infection (Fig. 4j). Together, these results indicated that the overexpression and expression of *Relish 1* and *Drosomycin*, respectively, were successful and led to an increased immune response in transgenic silkworms.

### Transgenic Silkworms Show Increased Resistance Against Pathogens and Thus Survivability

To determine the anti-pathogenic capacity of the transgenic silkworm, the mortality rate of transgenic and non-transgenic larvae was analyzed against *N. bombycis*, *BmNPV*, and bacterial infections. The fungal, viral or bacterial susceptibility of transgenic CMVPrelish1 and CecAPRelish1 silkworms were compared with non-transgenic silkworms, which showed higher mortality. The percentage of mortality of CMVPrelish1, CecAPRelish1 transgenic, and control silkworms was 42, 45, and 98 against *N. bombycis* (Fig. 5a), 51, 53, and 96 against *BmNPV* (Fig. 5b), 40, 43, and 80 against *E. coli* (Fig. 5c), and 46, 43, and 85 against *S. aureus* (Fig. 5d), respectively. Further, CMVPrelish1 showed a higher survival rate than CecAPRelish1 and was consistent with the high transcript levels observed. The *Drosomycin* was introduced into silkworms successfully and transgenic silkworms were challenged with *N. bombycis*, *BmNPV*, and bacteria. The transgenic lines CMVPrelish1 and DrsPDRs showed a mortality rate of 51 and 45% against *N. bombycis* compared to 97% in controls (Fig. 5e), whereas in *BmNPV*



- i** **CMVPrelish1 Chromosome 14**  
CGGGA~~ACTGTTATTATTAA~~-piggyBac-~~TTAATGATATCTTTTTTTT~~
- CecAPRelish1 Chromosome 18**  
AATTGATAATAAATGTTAA-piggyBac-~~TTAATTATTAAGTTATTGA~~
- CecAPGFP Chromosome 11**  
TATTGTTGATTTTTTTAA-piggyBac-~~TTAAGGGATTGATGACCT~~
- CMVPDrs Chromosome 21**  
TTAGTAGAAATAATATTAA-piggyBac-~~TTAAACGAGGGGGAGTTTG~~
- DrsPDrs Chromosome 10**  
AACGTTGATTTGTTTTTAA-piggyBac-~~TTAAACAGGTCAATGATCC~~
- DrsPGFP Chromosome 3**  
AAACATCCATGTGTATTAA-piggyBac-~~TTAACTGACATGGGCTCTT~~



**Fig. 3** Screening of transgene by PCR amplification using gDNA in G<sub>2</sub>. **a–d** *GFP* detection showing a band of 765 bp in CMVPrelish1, CecAPRelish1, CecAPGFP, and DrsPDrs, respectively; **e** and **f** A band of 1935 bp representing *Relish1* in CMVPrelish1 and CecAPRelish1, respectively; **g** and **h** *Drosomycin* detection yielded a band at 233 or 231 bp in CMVPDrs and DrsPDrs, respectively. Transgenic PB plasmids were used as positive control (C1) and gDNA from control moths (C2) was used as negative control. 1–4: Each lane shows the presence or absence of the respective transgene PCR product from random selection of moths; **i** Inverse PCR analysis of genomic insertion sites of CMVPrelish1, CecAPRelish1, CecAPGFP, CMVPDrs, DrsPDrs, and DrsPGFP

infection, mortality rates were high in transgenic lines (82 and 80%) similar to control (83%) (Fig. 5f). Mortality rates were 56, 49, and 80% against *E. coli* (Fig. 5g), and 50, 39, and 85% against *S. aureus* (Fig. 5h) for transgenic lines and controls, respectively. These results indicate significant protection of transgenic silkworms expressing *Drosomycin* against *N. bombycis* and bacterial infections.

### Commercial Traits are not Affected in Transgenic Silkworms

To evaluate whether the overexpression/expression of *Relish 1* or *Drosomycin* affected the economical characteristics of transgenic lines, we analyzed the shell rate, fecundity, pupation rate, and hatching in G<sub>7</sub>. The results are presented in Table 3. The transgenic silkworms were found weak in the initial few generations compared to control silkworms. After recurrent selective breeding, most economic traits were almost at par with control silkworms at G<sub>7</sub>. The pupation rate, fecundity, and hatching were slightly higher in control silkworms than that of transgenic ones. Our results show that the transgenic silkworms have commercial traits almost similar to that of non-transgenic lines.

### Discussion

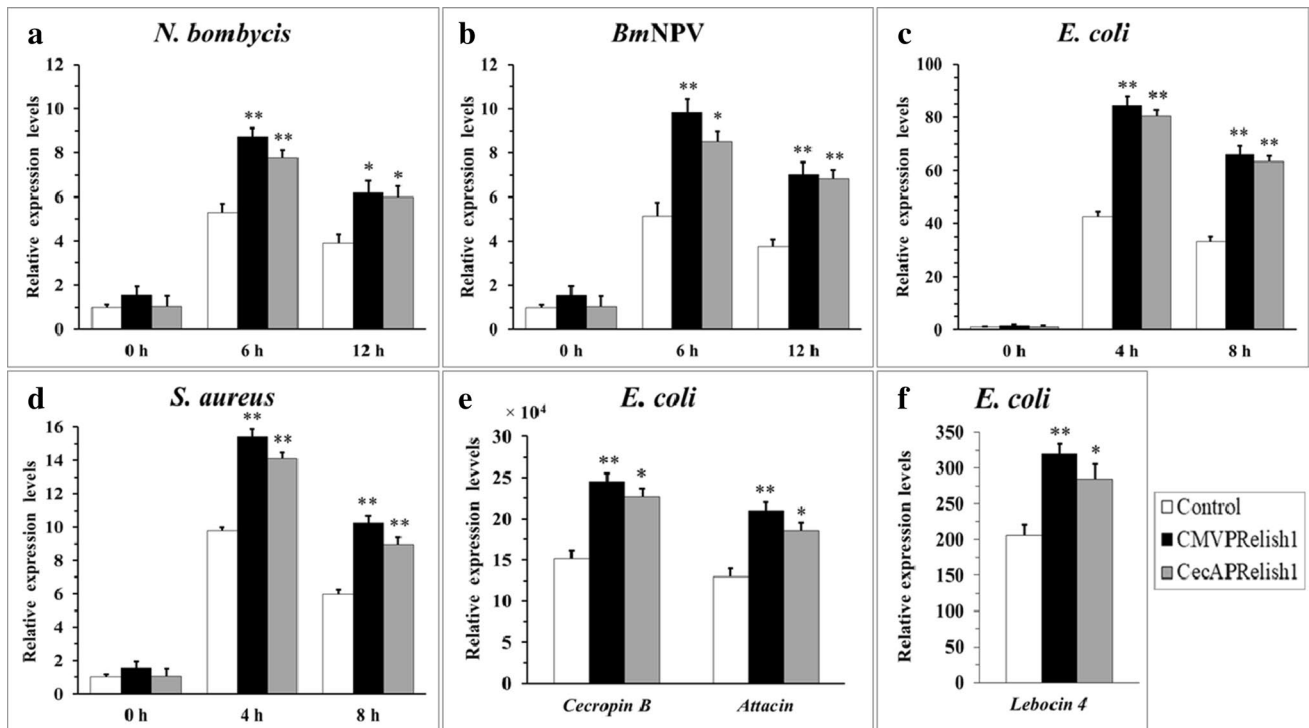
The silkworm, CSR2 breed is a bivoltine hybrid that is commercially used for silk production in India but is highly susceptible to infections. We used transgenic technology to confer enhanced immunity to the silkworm by overexpressing its *Relish 1* gene. We designed transgenic silkworms with the *BmRelish 1* gene lacking the C-terminal domain [20], an active form of Relish 1, under a constitutive and an inducible promoter, *CMV* and *Cecropin A*, respectively. Our results demonstrate that both promoters can drive the overexpression of *Relish 1* and the reporter gene, *GFP*. In the transgene delivery methods, the percentages of GFP-positive G<sub>1</sub> broods produced from electroporation and microinjection are 16.6 and 9.5, respectively. The efficiency of introducing a transgene into silkworm eggs was voltage-dependent and showed a high difference between polyvoltine and bivoltine

silkworms. Further, the acid treatment of the electroporated 54A embryos may be responsible for the low efficiency [29]. In the present study, we obtained an efficiency of 16.6% at 1700 V for the bivoltine CSR2 breed.

The overexpression of the active form of *Relish 1* in transgenic silkworms enhanced their anti-pathogenic activity to major pathogens of silkworms, microsporidian (*N. bombycis*), *BmNPV*, and gram-negative and positive bacteria. High levels of *Relish 1* mRNA are expressed in transgenic silkworms compared to the controls in response to infections. Injections of pathogens to silkworms result in quick immune responses as evident by the *Relish 1* expression at the early stages of infection. Our results are consistent with the expression patterns of *Relish 1* and other immune-related genes in response to infections with bacteria, *BmNPV*, and *N. bombycis* in silkworms [20, 30–32]. The *Relish 1* was able to induce the downstream expression of AMP genes, *Cecropin B*, *Attacin*, and *Lebocin 4* at higher levels than in control silkworms. Interestingly, both *BmNPV* infection and cGAMP stimulation resulted in the activation of the IMD pathway and further, *BmSTING* promoted the processing of Relish to activate an antiviral immune response in silkworms [32]. The *Relish* is also reported to be expressed in response to *N. bombycis* infection in silkworms [33]. Further, *BmSTING* regulates the infection of *N. bombycis* in an LC3 (microtubule-associated protein) -dependent manner in the early stage of infection and then activates Relish as a protective response to survive *N. bombycis* infection [34].

Mainly Toll pathway is activated by gram-positive bacteria, and the IMD pathway responds to gram-negative bacterial infection. The *Relish 1* expression in silkworm is upregulated stronger against *E. coli* than *S. aureus* [20]. The transgenic mosquitoes overexpressing IMD-Rel2 (a homologue of *Drosophila* Relish) show greater resistance to protozoan *Plasmodium* and gram-negative and positive bacteria [18, 35]. Further, they showed Rel2 overexpression resulted in transcriptional activation of Defensins, Cecropins, and other AMPs. Hence, most of the AMP genes can be regulated by either pathway [36, 37]. In *Tenebrio molitor*, knock-down of IMD transcript significantly reduced host resistance to *E. coli* and fungus *Candida albicans* [38]. Our results show that the overexpression of Relish 1 is effective against major pathogens of the silkworm, a protozoan/fungus (*N. bombycis*), a virus (*BmNPV*), and both gram-negative and positive bacteria showing its potential in combating multiple pathogens. Though both promoters effectively drive the expression of *Relish 1*, the *CMV* promoter performed comparatively better than *Cecropin A* promoter. The reasons for this difference require further study.

The effect of transgenic modification of Relish 1 on silkworm immunity is evaluated by a survival test. The overexpression of Relish1 in transgenic lines results in reducing the mortality rate significantly in response to infections by major



**Fig. 4** Quantitative expression analyses of *Relish 1*, AMPs and *Drosomycin* in transgenic silkworms. **a–d** expression levels of *Relish 1* against *N. bombycis*, *BmNPV*, *E. coli* and *S. aureus*, respectively. \* $p < 0.05$  and \*\* $p < 0.01$  with respect to control. **e–f** expression levels of *Cecropin B*, *Attacin*

and *Lebocin* against *E. coli*. \* $p < 0.05$  and \*\* $p < 0.01$  with respect to control. **g–j** expression levels of *Drosomycin* against *N. bombycis*, *BmNPV*, *E. coli*, and *S. aureus*, respectively. \* $p < 0.05$  and \*\* $p < 0.01$  with respect to CMVPrs. All data belong to G<sub>7</sub>

pathogens. On average, we observed the resistance of 56.5, and 48% against *N. bombycis* and *BmNPV*, respectively, and 58.5 and 52.5% to gram-negative and positive bacteria, respectively, in transgenic silkworms. Previous reports have shown enhanced resistance due to overexpression/expression of endogenous/exogenous genes and anti-pathogen genes in transgenic silkworms. Resistance to *BmNPV* is achieved by overexpression of *Bmlipase* and expression of exogenous *hycu-ep32* genes [7, 8], and knocking down of *BmNPV* genes using transgenic RNAi [6, 39]. In addition, RNAi-based downregulation of a nuclear receptor, *BmNHR96*, which promotes *BmNPV* entry into the silkworm cells, effectively enhanced survivability of silkworm against *BmNPV* infection [9]. The overexpression of the *BmPGRP-S2* gene resulted in the activation of *IMD*, *Relish*, and AMP genes *Attacin 2*, *Gloverin 2*, and *Moricin*, and has shown resistance against *BmCPV* (cytoplasmic polyhedrosis virus) [10]. Knockdown of *BmCPV* and *BmDENV* (Densovirus) genes enhanced the antiviral capacity of transgenic silkworms [40, 41]. Further, *BmSpry* is involved in resistance against three silkworm viruses [14, 15]. Congruent with these findings, in our study, overexpression of *Relish 1* resulted in increased resistance of transgenic silkworms to multiple pathogens. To our knowledge, this is the first study demonstrating

overexpression of a single gene can confer greater resistance to multiple pathogens of different categories in silkworms. The active *Relish 1* led to the upregulation of effector AMPs that protect against invading pathogens, and it may also be due to the production of other factors that are controlled by *Relish 1*. Further, our study shows it is a good strategy to improve disease resistance by developing transgenic silkworms overexpressing a transcription factor.

*Drosomycin* is a key antimicrobial peptide involved in Toll signaling and innate immune responses primarily against fungal infections in *Drosophila* [42]. Recombinant *Drosomycin* is shown to have an inhibitory effect on several fungi, yeast, and a protozoan parasite, *Plasmodium berghei* [43]. Microsporidia, including *N. bombycis*, which causes the destructive “pebrine” disease in silkworms, were earlier classified under protozoa but now reported to be closely related to fungi [44]. The successful expression of *Drosomycin* from *D. melanogaster* in transgenic silkworm effectively reduced the *N. bombycis* and bacterial infections. The level of resistance of immune-enhanced transgenic silkworm lines, CMVPrs, and DrsPrs to *N. bombycis* infection is 49 and 55%, respectively. The expression of *Drosomycin* in transgenic silkworms may also help to combat other fungi, such as *Beauveria bassiana* which causes

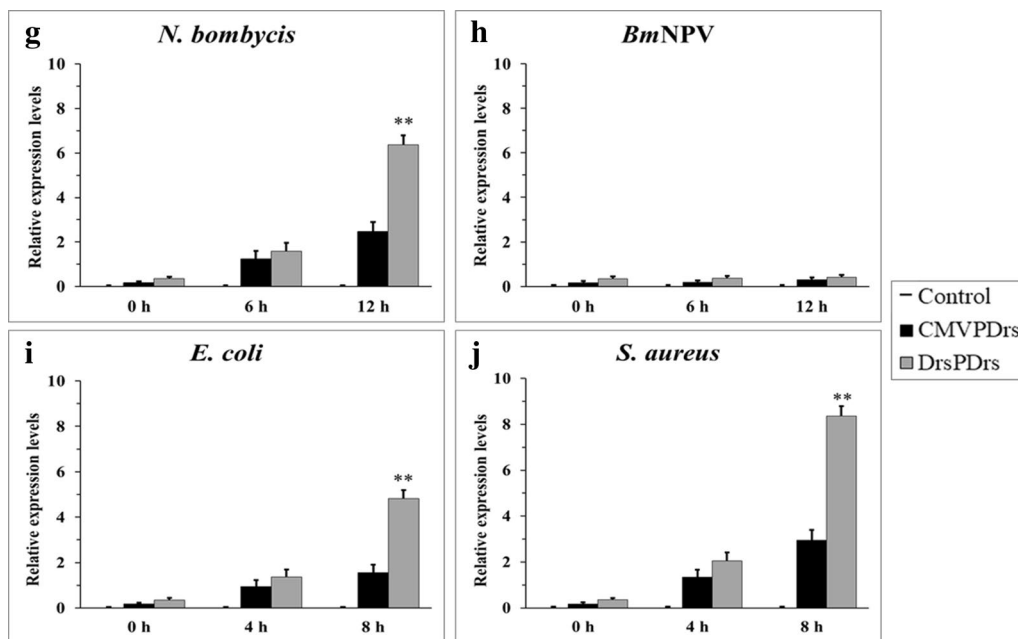
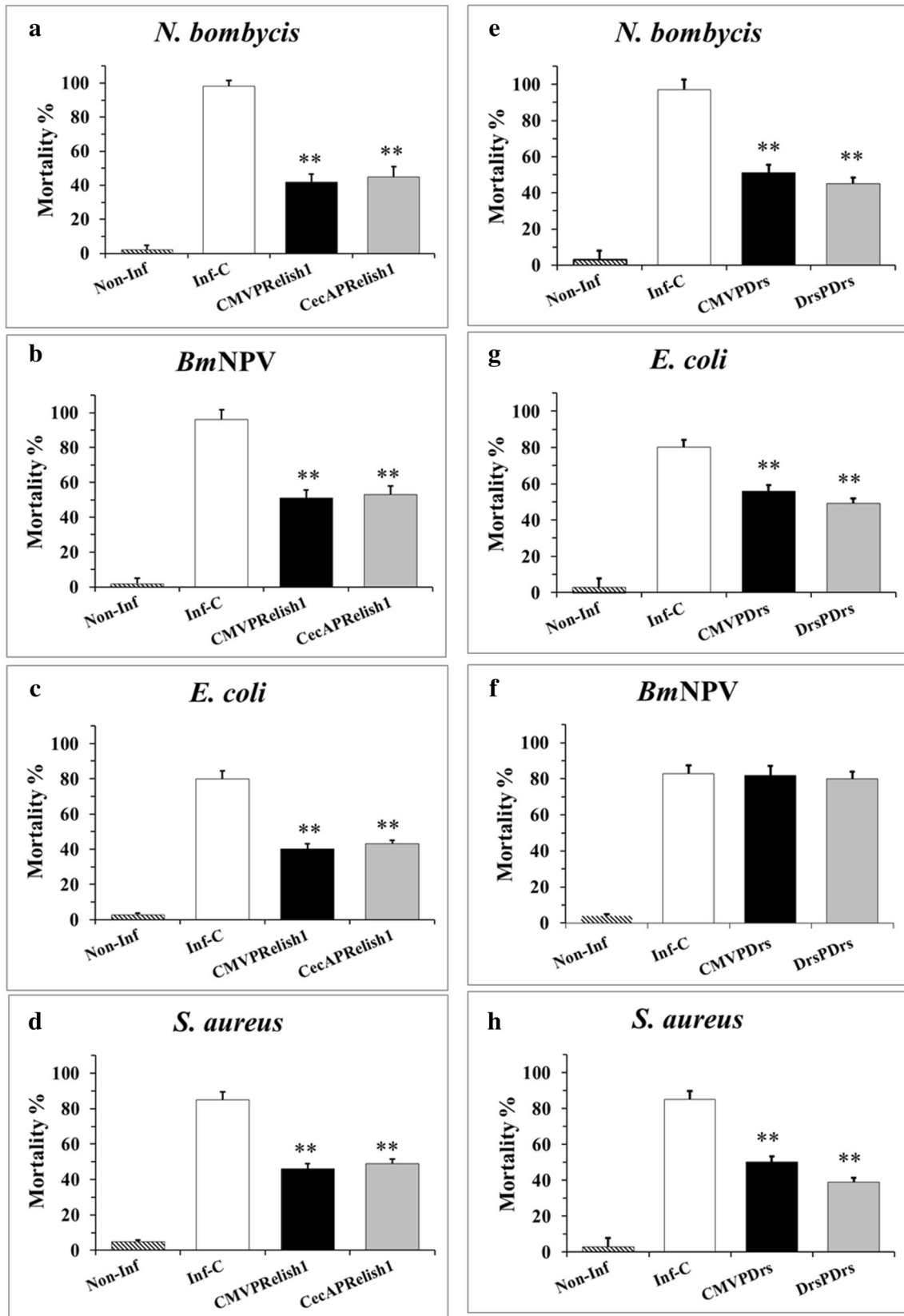


Fig. 4 (continued)

“muscardine” disease in silkworms. Drosomycin has been highly expressed in response to *B. bassiana* infection in *D. melanogaster* [45]. It may also help in combatting *Aspergillus spp.*, which causes aspergillosis in young silkworm larvae. Enhanced resistance to *A. fumigatus* was observed in transgenic *D. melanogaster* expressing Drosomycin peptide constitutively [46]. The transcript level of *Drosomycin* was higher in response to *S. aureus*, and hence it could be speculated that the transgenic silkworms may survive against flacherie disease caused by gram-positive bacteria. Toll pathway activation in response to gram-positive bacteria has been shown to control the expression of the *Drosomycin* gene [23, 47]. The AMPs including Gloverins, Lebocins, and Moricins are strongly upregulated against *N. bombycis* infection in silkworms and followed the Toll pathway [48]. However, Drosomycin is not found to be effective against *BmNPV* infection in silkworms. It has been shown

that unlike in fungal and bacterial infections, the expression of a single AMP provides no resistance to viral infections in *Drosophila* [49]. Our results demonstrate the enhancement of the silkworm innate immune system through the successful introduction of a foreign recombinant gene driven by its promoter.

The commercial traits such as cocoon and shell weight, silk yield, fecundity, and hatching in transgenic CSR2 lines were at par with control CSR2, after continuous selection and breeding. This process is continuing in our laboratory further to enhance the level of resistance among transgenic silkworms. Cross-breeding of transgenic lines will also help improve resistance. Overall, we have developed transgenic silkworms with enhanced immunity to major pathogens. Our findings also provide a strategy for enhancing disease resistance against multiple pathogens by the overexpression of a single gene.



**Fig. 5** Mortality analyses of transgenic silkworms. Non-Inf and Inf-C: control without and with infection, respectively. **a–d** Transgenic Relish 1 and **e–h** Drosomycin silkworms. Transgenic silkworm lines

show reduced mortality upon infection with various pathogens as indicated ( $G_7$ ). \*\* $p < 0.01$  with respect to infected control

**Table 3** Representative data for the economical parameters in transgenic and control silkworms

CSR2-G <sub>7</sub>	Control	Transgenic
Pupation rate (%)	90 ± 0.35	89 ± 0.25
Cocoon weight (g)	1.67 ± 0.15	1.64 ± 1.12
Shell weight (g)	0.29 ± 0.82	0.27 ± 0.75
Fecundity (No.)	480 ± 1.15	461 ± 0.41
Hatching (%)	95 ± 0.71	92 ± 0.10

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s12033-021-00438-0>.

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**Author Contributions** RSY performed experiments, data organization and analysis, and writing-first draft. DST, CM, and GR assisted in experiments, data organization and analysis, and writing-review. VK analyzed the data and review. RKM performed funding acquisition, resources and coordination. UN guided in experiments, supervision, writing-review and editing. RG did conceptualization, funding acquisition, guidance in experiments, supervision, and writing-review and editing.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

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