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ORIGINAL PAPER

Transgenic potato lines expressing hairpin RNAi construct of molting-associated *EcR* gene exhibit enhanced resistance against Colorado potato beetle (*Leptinotarsa decemlineata, Say)*

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Abstract Most of the commercialized insect resistant transgenic crops express *cry* gene(s) isolated from *Bacillus thuringiensis;* however, intensive cultivation of *Bt* crops over almost two decades has been questioned regarding its sustainability and durability in pest management. The present study focused on silencing of highly specific molting-associated Ecdysone receptor (*EcR*) gene of Colorado potato beetle (CPB) using RNA interference (RNAi) approach. The partial cDNA of *EcR* gene of CPB was amplified using specific primers in sense and anti-sense orientations, and cloned in pRNAi-GG vector flanked by an intronic sequence (*pdk*). Leaf and internodal explants of Agria and Lady Olympia potato cultivars were infected with Agrobacterium strain LBA4404 harboring constructs under the control of *CaMV 35S* promoter. Standard molecular analysis of primary transformants showed proper integration of T-DNA in plant genome. The transgenic plants of both cultivars were evaluated for their efficacy against first, second and third instar CPB larvae. The leaf biotoxicity assays revealed 15–80% of CPB mortality. A significantly lower fold-change (0.87–4.14×) in larval weight was observed in insects fed on transgenic plants compared to the ones fed on control plants (1.87–6.53×). Furthermore, CPB larvae fed on transgenic plants exhibited reduced *EcR* transcripts, indicating the functionality of dsRNA *EcR* in silencing EcR gene expression. This study is an excellent example of the integration of an alternative, effective and reliable method to cope with potato insect pests that incur significant losses to potato production in the world.

Keywords Ecdysone receptor · Transgenic technology · Insect resistance · Molting

Introduction

Extensive usage of insecticides in pest management contaminates the environment and costs billions of dollars. Due to co-evolution, insect pests have developed resistance against many commercial insecticides. As of 2018, more than 550 arthropod species have developed resistance to at least one type of insecticides (Gould et al. 2018). As an alternative to chemical insecticides, transgenic crops have been integrated to the pest management strategies. Most of the insect resistant transgenic crops express insecticidal

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gene(s) from the bacteria *Bacillus thuriengenesis* (*Bt*) under the control of Cauliflower mosaic virus 35S promoter that induce expression of genes at high levels in all types of tissues at different growth stages (Carrière et al. 2016). However, sustainability of *Bt* crops has been questioned due to the increase in pests' resistance against these transgenic crops (Gassmann et al. 2011; Zhang et al. 2011; Tabashnik et al. 2013; van den Berg et al. 2013; Tabashnik and Carrière 2017). The cases of pest resistance to *Bt* crops increased more than five folds in 12 years (Tabashnik and Carrière 2017). Therefore, integration of novel alternative biotechnological tools is required in crop pest management.

Recently, RNA interference (RNAi) emerged as an eco-friendly, efficient and reliable tactic for the control of insects in economic crops (Mamta and Rajam 2017). This is a gene silencing strategy that uses double stranded RNA (dsRNA) to hinder the normal gene function directly against a specific gene sequence or promoter region of messenger RNA (mRNA) (Mansoor et al. 2006). Hence, the transcript of target insect gene is silenced when dsRNA is ingested by the insects through RNAi pathway, which in turn may lead to insect growth or developmental defects, or morbidity, or mortality (Zhang et al. 2017). The recent studies of plant-mediated RNAi by Baum et al. (2007), Mao et al. (2011), Zhu et al. (2012), Yao et al. (2013) and Mao and Zeng (2014) in different crops have paved the pathways to use this technology against notorious insect pests of crops. RNAi-based silencing of vital insect pest genes could be advantageous in decreasing dependence on chemical insecticides as well as in fighting pest resistance development against chemical insecticides and *Bt* crops.

Potato (*Solanum tuberosum* L.) is a significant crop globally among other food crops regarding production and ranks 4th in position after wheat, rice and maize (FAOSTAT 2017). It is one of the most promising crop plants to overcome the challenges of hunger and poverty around the globe due to its high yield potential (Bagri et al. 2018). Moreover, it is very nutritious since tubers are enriched with proteins, carbohydrates, minerals (K, Mg, Mn, Cu, Fe, P) and vitamins (C, B3, B1, K, B6, folate, pantothenic acid) (Çalışkan et al. 2010). Various biotic and abiotic stresses limit productivity and growth of potato. Estimated quantitative losses of potato due to insect pests are 34% (Oerke 2006). Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, is economically the most important potato pest in many parts of the world, including Asia, Europe and North-America. Co-evolution with Solanaceous crops, which produce diverse secondary metabolites, has enhanced the detoxification mechanism of CPB to survive a variety of complex natural and synthetic chemicals. Therefore, there is an urgent need of exploring new alternative methods to control the CPB infestation in the fields (Zhu et al. 2011).

Many breeding attempts have been made to develop insect resistant potato lines (Flanders et al. 1992). Due to narrow genetic base of potato, conventional breeding strategies are usually inefficient (Douches et al. 1996). Biotechnology has assisted the classical breeding by providing alternatives for improvement of potato in area of insect resistance. Insect pest resistance, herbicide resistance, and biofortification of crop plants are the common applications of genetic engineering (Bakhsh et al. 2015).

Growth stages of insect larvae are marked by series of molts, essential for hardening and expansion of cuticle as the larva grow. These molts are initiated by the surges of steroid hormone ecdysone that is converted to an active form of 20-hydroxyecdysone (Sehnal 1989). The interaction of 20-hydroxyecdysone with its receptor initiates various physiological and developmental phenomena in insects including molting and metamorphosis (Schwenke et al. 2016). Ecdysone receptor gene (*EcR*) is one of the members of nuclear receptor (NR) superfamily and is ligand-inducible nuclear transcription factor (Hopkins 2009). Ecdysone signaling synchronizes the entire network of germline stem cell development in the ovary of *Drosophila melanogaster* in pre-adult stages (Belles and Piulachs 2015). Therefore, ecdysteroids are critically significant for the growth, development, reproduction, and regeneration and molting of crustaceans (LeBlanc 2007). As the ecdysone factor 20E and its nuclear receptor EcR-USP are insect-associated, both are absolute requirements for the insect development.

Therefore, we hypothesized that the transgenes which express dsRNA *EcR* in transgenic plants can be a promising and efficient way of bringing improvement in terms of pest resistance in potato against CPB. The insects feeding on two transgenic potato lines (Agria and Lady Olympia) pressing *EcR* dsRNA showed up to 80% of mortality, and could not

complete their life cycles since dsRNA reduced *EcR* transcript accumulation via RNAi pathway in CPB feeding on transgenic potato lines. The findings of this study show that RNAi-based silencing of a vital insect gene could be used to control the potato pest CPB as an alternative tactic.

Materials and methods

Development of RNAi constructs (pRNAi-CPB and pRNAi-GFP)

The construct development was carried out using Golden Gate protocol as described by Yan et al. (2012). For this purpose, the whole bodies of third instar larvae of Colorado potato beetle (CPB) were subjected to total RNA isolation using Omega E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek Georgia). Following proper quantification by nano-spectrophotometer (Shimadzu), the first strand cDNA was synthesized from total RNA according to the manufacturer's instructions using Thermoscientific RevertAid First Strand cDNA Synthesis Kit (Cat. No. K1622). cDNA was used as template to amplify 445 bp internal fragment of EcR-B1 gene (Accession No. AB211192) using specific primers containing overhangs of BsaI sites for cloning into pRNAi-GG vector. PCR was performed in the reaction volume of 20 µL containing 20 ng of cDNA template, 50 pM of forward and reverse primers, 100 µM of dNTPs, 1× PCR Buffer (1.5 mM of MgCl2, 50 mM of KCl and 10 mM of Tris–HCl) and 1 unit of Pfu Polymerase (Thermo Scientific™).

The PCR conditions were set as follows: 4 min at 94 °C, 15 s at 94 °C, 15 s at 60 °C and 30 s at 72 °C for 34 cycles, followed by final extension at 72 °C for 7 min. Likewise, a 390 bp fragment of *Green Fluorescent Protein* (*GFP*) gene was also amplified with specific primers containing overhangs of BsaI sites to be used as negative control construct in further experiments. Table S1 shows the list of primers, their annealing temperatures and product sizes used in the study.

The Golden Gate reaction for making ihpRNA constructs was set up by combining 50 ng of purified PCR product, 200 ng of pRNAi-GG vector, 5 units of BsaI enzyme and 10 units of T4 DNA ligase in a total volume of 10 µl in 1× ligation buffer in a tube. Then, the restriction-ligation was incubated at 37 °C for 2 h, followed by incubation for 5 min at 50 °C (final digestion) and then 5 min at 80 °C (heat inactivation). Next, 5 µl of the mixture was transformed into *E. coli* DH5α competent cells and plated on Luria–Bertani (LB) medium containing 25 mg/L of kanamycin and 5 mg/L of chloramphenicol to select the recombinants. The pRNAi-CPB and pRNAi-GFP constructs were maintained in *E.coli* DH5α and DB3.1, and they were further electroporated to Agrobacterium strain LBA4404 by using Gene Pulser Xcell™ Electroporation Systems (Cat. No. 1652660). Clones were confirmed using standard molecular analyses, including colony PCR, restriction enzyme digestion and sequencing. All DNA manipulations were performed according to the standard protocols (Sambrook et al. 2001).

Plant material and genetic transformation

One processing (Lady Olympia) and one ware (Agria) commercial varieties of potato growing zone in Turkey were selected for the studies as both cultivars have good agronomic characteristics and yield potential in the area but are susceptible to insect pest damage. First, the shoot cultures of both cultivars were established from tuber sprouts and propagated in vitro using single node stem explants on basal Murashige and Skoog (MS) medium (Murashige and Skoog 1962). The cultures were incubated in growth chamber at 25 °C and 60% humidity under 16/8 h of light/dark photoperiod.

The overall transformation procedure was followed as described by Beaujean et al. (1998) with modifications. Briefly, leaf discs and internodes (4–6 mm) were excised from the propagated plants in vitro and inoculated with the suspension (O.D 0.6) of Agrobacterium strains containing targeted constructs (pRNAi-CPB and pRNAi-GFP) for 30 min with mid-shaking in LB liquid medium without antibiotics, followed by further incubation in co-cultivation medium (MSO supplemented with 50 mM acetosyringone) for 3 days. Following co-cultivation, the explants were washed with broad spectrum antibiotic (Sulcid), dried and cultured on regeneration selection media (MS salts supplemented with 2 mg/L of BAP, 0.2 mg/L of NAA, 1 mg/L of kinetin, 2 mg/L of trans-zeatin and 100 mg/L of kanamycin). The data for callus induction and number of shoots per explant was recorded.

With well-developed calli, explants were transferred to shoot induction medium (MS salts supplemented with 1 mg/L of BAP, 0.1 mg/L of GA_3, and 100 mg/L of kanamycin) for sub-culturing. The regenerating shoots with a length of 1–2 cm were excised and transferred to bigger magenta boxes for rooting. Furthermore, putative transgenic plantlets with well-developed shoots and roots were shifted to pots filled with a mixture of perlite and peat moss (1:3) for acclimatization, earlier in growth chamber and later in green house. In order to determine the transformation efficiency, all plantlets were used in PCR-based screening of constructs integration into plant genome.

PCR-based confirmation of primary transformants

The putative transgenic plants developed from Agria and Lady Olympia was subjected to molecular analyses to confirm transgene integration, expression and efficacy. DNA extractions were carried out using Plant Genomic DNA Extraction Kit (Thermo Scientific, Cat. No. K0792) according to the manufacturer's instructions. PCR analysis of transgenic plants was conducted with different primers to confirm the presence of *EcR* gene fragment in sense (primers of P21–P24) and antisense orientations (primers of P22–P25). Integration of *EcR* and *GFP* genes were further determined by gene specific primers and plant selectable marker gene (*nptII*) in the host genomes. Agrobacterium contamination was determined by PCR using *ChvA* gene specific primers in primary transformants. List of primers are given in Table S1.

Southern-blot analyses of primary transformants

Southern-blot was carried out according to modified method of Southern (1975) to confirm transgene integration in plant genome. Approximately 10 µg of genomic DNA from transgenic plants were digested with HindIII and BamH1 to release the *EcR* gene fragment. The digested DNA samples were resolved in 1% agarose gel electrophoresis using 1× TBE buffer at 20 V for 10 h, and transferred to Hybond-N membrane (Amersham). Membrane was placed in hybridization tube along with pre-hybridization solution and was treated for an hour at 65 °C. PCR amplified *EcR* fragment was used as probe, and it was labeled by Biotin DecaLabel™ DNA Labeling Kit (Fermentas, Cat. No. K0652) following instructions provided in the manual. Then, hybridization with the probe was done for 18 h at 65 °C temperature. The detection procedure was followed according to the manual instructions of Biotin Chromogenic Detection Kit (Fermentas, Cat. No. K0662)

EcR gene expression analyses of primary T_0 transformants

Among positive primary transformants, 10 randomly selected transgenic plants were subjected to quantitative real time PCR (qRT-PCR) analysis to analyze the *EcR* transcript levels. Total RNA was extracted from the leaves of transgenic plants using RiboZol™ RNA Extraction Reagent (Amresco). Following quantification (by spectrophotometric measurement) and quality confirmation (by agarose gel electrophoresis) of total RNA samples, 1 µg of total RNA was used to synthesize 1st strand cDNA according to the instructions of cDNA synthesis kit (Fermentas, Cat. No. K1622). qRT-PCR reaction mixture included SYBR green master mix (2×), 1 µM of each of forward and reverse primers, RNase-free sterile distilled water and 1:10 diluted cDNA as template. The reaction mixture was incubated at 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s, 55 °C for 15 seconds, 72 °C for 20 s by using Rotor-Gene Q. After the amplification is completed the melting curve analysis was performed by incubation at 99 °C to 70 °C with a transition rate of 1.0 °C/min. For normalization, *ELONGATION FACTOR 1-α* (*EF1α*) was used as reference housekeeping gene for normalization (Nicot et al. 2005). The Ct values of samples in target gene expression analysis were determined by Rotor-Gene Q Software. According to the qRT-PCR results, the standard deviations of Ct values of the samples were calculated by using Microsoft Excel program and the expression levels of the genes were determined according to the $2^{-\Delta\Delta Ct^*}$ proportional calculation method (Livak and Schmittgen 2001). The fold changes in *EcR* transcript levels are given relative to the control plants.

Insect rearing

Leaf bio-toxicity assays and EcR transcript analysis in CPB

Leaf feeding bioassays were used to evaluate the efficacy of *EcR* dsRNA against CPB. The fresh leaves

from primary T_0 transformants were taken in petri plates containing moist filter paper; then first, second and third instar larvae of CPB were allowed to feed on transgenic plant leaves along with the controls in three biological replications. For each feeding assay, 10 larvae were used. After 3 days of feeding, leaf and insect pictures were taken by camera and mortality rates of CPB larvae were recorded. The larvae released on the plants were weighed before and after the experiments and fold change in larval weight was recorded according to Swift (1997).

Following 72 h of leaf feeding bioassays, the total RNA was extracted from the 1st, 2nd and 3rd instar CPB whole larval bodies using RiboZol^{TM} RNA Extraction Reagent (Amresco) following instruction in manual. qRT-PCR was performed to analyze *EcR* transcript levels as describe earlier. *Ribosomal 18S* gene of CPB was used as reference housekeeping gene for normalization (Yang et al. 2015). The fold changes in *EcR* transcript levels in insects feeding on transgenic plants were given relative to the ones fed on control plants.

Statistical analyses

All statistical analyses were performed with Statistix 8.1 software (Analytical Software, Tallahassee, Florida, USA). Significance of variance was determined after the one-way ANOVA ($p < 0.05$) followed by Tukey's multiple comparison test (n = 3) and is presented in all graphs as mean ± SE.

Results

Generation of potato transformants expressing ihpRNA constructs

Following protocol of Yan et al. (2012), RNAi constructs were developed, named as pRNAi-CPB and pRNAi-GFP and were transformed to potato using Agrobacterium mediated transformation (Fig S1–S2). In each potato variety, a total of 1500, 1000 and 1000 explants were transformed with pRNAi-CPB, pRNAi-GFP and pRNAi-GG (mock) constructs, respectively. The optimized regeneration selection medium exhibited good results, though the response among cultivars varied (Table S2). Approximately 55 to 75% of calli induction and 1.95 to 3.77 average shoots per explants were recorded. In general, the calli induction rate was slightly higher in Lady Olympia than Agria. No visible problems were encountered regarding rooting of regenerated transgenic shoots. A total of 210 PCR positive transformants derived from the cultivars were first acclimatized in pots in a controlled growth chamber for 2 weeks and then transferred to greenhouse. T_0 transgenic plants looked similar in morphology and physiology to the control plants, indicating normal plant development and growth (Fig S2). Based on the results of total PCR positive plants, overall transformation efficiency was calculated to be 2.8% with respect to total inoculated explants though the efficiency varied (Table S3). The transformation efficiency was slightly higher in Lady Olympia than Agria, suggesting that the transformation efficiency, callus induction and transformed plant generation is dependent on potato cultivars.

In order to select for the positive primary T_0 transformant potato plants, genomic DNA was extracted from their leaves. PCR analysis of putative transgenic plants was conducted with different primers to confirm the presence of *EcR* gene in sense orientation (with P21–P24 primers) and antisense orientation (with P22–P25 primers) (Fig S2). P21–24 and P22–25 showed required bands in sense and antisense orientations, respectively, while amplification with gene (*EcR* and *GFP*) specific primers further confirmed the results. Only the plants positive for both orientations along with the marker genes were selected and subjected to further assays. Agrobacterium ability depends on the activation of *vir* genes along with *ChvA* genes to infect the host plant. ChvA act as a promotor for the attachment of host cell to bacteria (Douglas et al. 1985). Findings of PCR should show amplicon of about 890 bp using *ChvA* gene-specific primers in case of Agrobacterium contamination in primary transformants (Nain et al.2005). Therefore, the putative transgenic plants were also analyzed by PCR using specific primers for *ChvA* gene to determine any possible Agrobacterium contaminations. The plants that showed positive amplification with *ChvA* gene were discarded and not considered for further studies. The positive plants were selected and grown in greenhouse.

Potato transformants expressing dsRNA *EcR*

The PCR positive plants were selected in random to determine the integration and copy number of dsRNA

EcR transgene in the genome of T_0 transformant potato plants (Fig. 1a). Southern-blot integration analysis was performed using PCR amplified *EcR* fragment as a probe. All PCR positive plants also showed signal on membrane indicating integration of introduced cassette in host genomes. All tested selected plants showed one copy of the construct. Quantitative real time PCR (qRT-PCR) was used to analyze the transcript levels of *EcR* in primary T_0 transformant leaves. Results showed higher *EcR* transcript levels in primary transformants as compared to the control plants (Fig. 1b). In Lady Olympia transformants, 2–34 folds of higher expression were recorded whereas 5–23 folds of higher expression were recorded in case of Agria transformants. Interestingly, Lady Olympia showed both slightly higher transformation efficiency and *EcR* expression than did Agria, suggesting a positive effect of transformation efficiency on the expression of gene of interest. Overall, four T_0 potato transformants efficiently expressing dsRNA *EcR* with single copy insertions were selected in both Lady Olympia and Agria background.

Potato transformants expressing dsRNA *EcR* were more tolerant to CPB

In order to determine the efficient usage of *EcR* dsRNA expression in potato against CPB, CPB larvae at three different developmental stages were fed to T_0 transgenic plant leaves along with the controls in petri dishes for up to 72 h. According to the observations, there was a significant difference regarding the percent mortality among different transgenic plants expressing dsRNA (Table 1, Fig. 2a). After 24 h of feeding, a significantly higher mortality percentage (20%) of 1st instar larvae was recorded when fed on Ag1, Lo2, Lo4 lines. On the other hand, a significantly lower mortality was observed in insects feeding on Ag2, Ag3, Ag4, Lo1 and Lo3, ranging between 5 and 15%. After 48 h of feeding, a higher mortality rate (25–30%) was observed in insects feeding on Ag1, Lo2, Lo3 and Lo4, whereas a lower mortality was observed in rest of the observed lines ranging between 15 and 20%. With the passage of time (after 72 h), higher mortality (70–80%) of first instar larvae were recorded feeding on Lo2 and Lo4 while a lower mortality (20%) of larvae was observed feeding on Ag2 and Ag3 plant leaves.

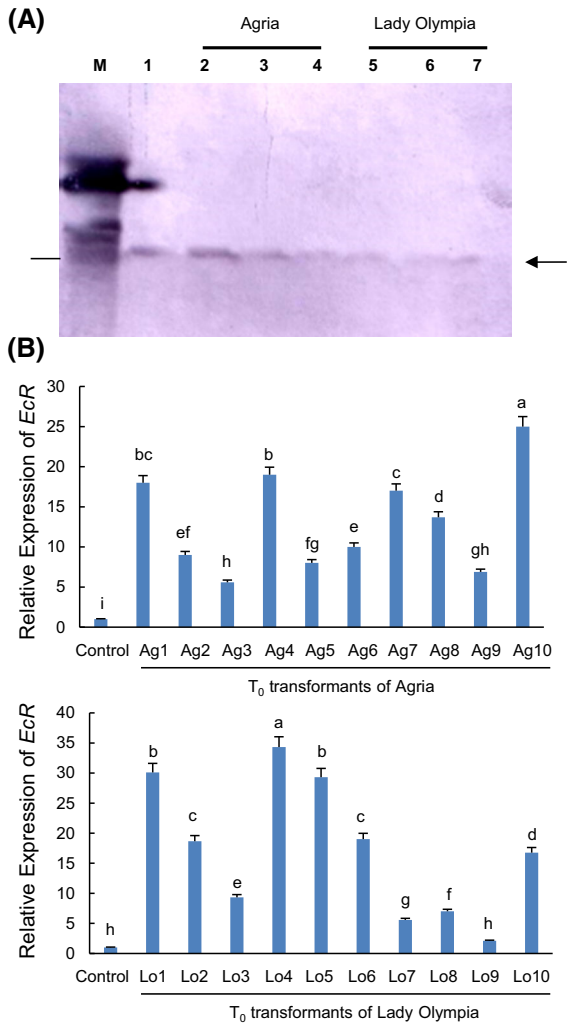


Fig. 1 The copy number and mRNA expression for *EcR* detected in primary transformants **a** Southern-blot analysis of PCR positive plants with EcR specific probe. M: Lambda HindIII marker (Thermo Scientific), lane 1: positive control (plasmid 35SpRNAi-CPB), lanes 2–7: putative T0 transgenic plants of Lady Olympia and Agria. **b** qRT-PCR analysis of *EcR* transcript levels in T0 transgenics compared to control plants. Control: pRNAi-GFP expressing Agria or Lady Olympia. Significant differences ($p < 0.05$) are indicated with different letters detected by one-way ANOVA analysis after Tukey's multiple comparison test (n = 3)

In case of second instar larval analysis, no mortality was recorded after 24 h; however, a significant mortality (20%) of larvae was noticed feeding on Ag1, Lo1 and Lo4 plant leaves after 48 h of incubation. A significantly lower mortality (10–15%) of larvae was observed that were feed on rest of the transgenic plant leaves (Ag3, Ag4, Lo2 and Lo3)

Table 1 Mortality percentage (%) of CPB larvae feeding on T_0 transgenic plants at different developmental stages

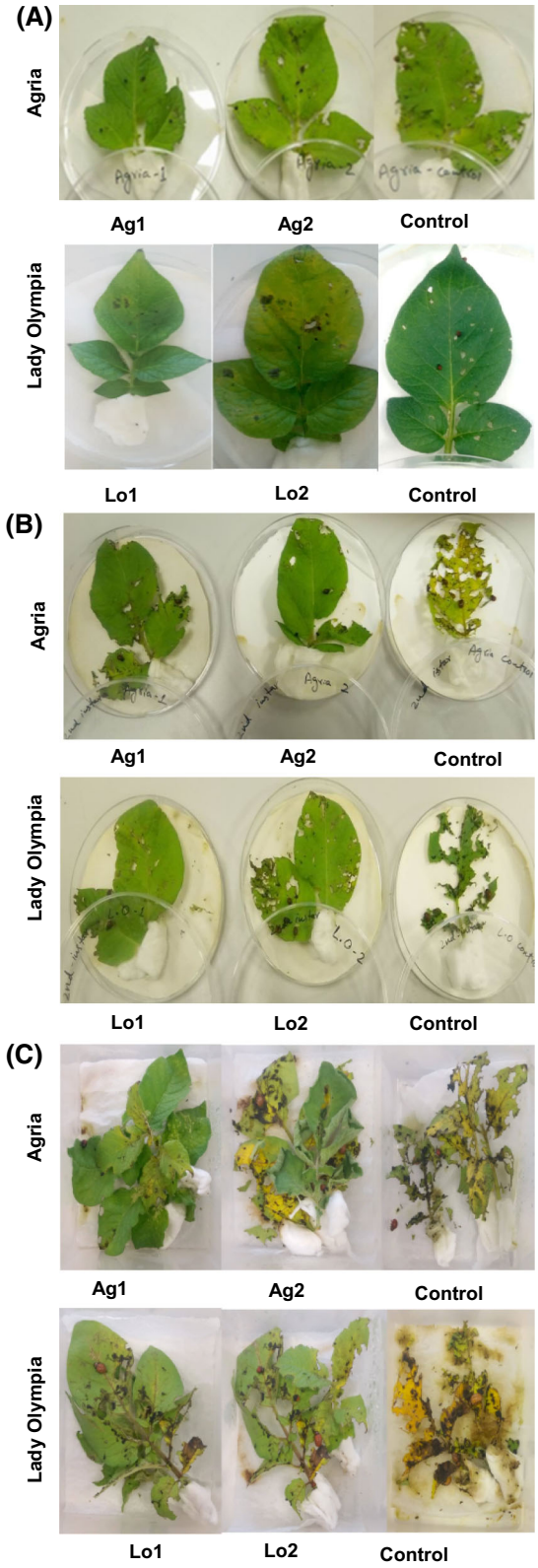
Feeding duration (h)	1st Instar			2nd Instar			3rd Instar		
	24	48	72	24	48	72	24	48	72
Varieties									
Agria									
Ag1	20.0a	30.0a	40.0b	0.0a	20.0 a	30.0a	0.0a	15.0ab	40.0b
Ag2	10.0ab	20.0ab	20.0 cd	0.0a	0.0b	20.0ab	0.0a	0.0b	30.0b
Ag3	5.0ab	15.0 ab	20.0 cd	0.0a	15.0 ab	15.0 ab	0.0a	15.0ab	25.0bc
Ag4	10.0ab	20.0ab	30.0bc	0.0a	15.0 ab	25.0ab	0.0a	10.0ab	40.0b
Lady Olympia									
Lo1	10.0ab	20.0ab	40.0b	0.0a	20.0a	30.0a	0.0a	20.0a	60.0a
Lo2	20.0a	30.0a	80.0a	0.0a	15.0ab	25.0ab	0.0a	10.0ab	40.0ab
Lo3	15.0ab	25.0a	45.0 bc	0.0a	10.0ab	20.0ab	0.0a	15.0ab	30.0b
Lo4	20.0a	30.0a	70.0a	0.0a	20.0a	40.0a	0.0a	10.0ab	50.0ab
Control	0.0b	0.0b	0.0d	0.0a	0.0b	0.0b	0.0a	0.0b	0.0c

Numbers with same letters within column are not significantly different from each other according to LSD test at 5% level of significance

while no mortality was observed of insects feeding on Ag2 and control plants. Similarly, increased mortality rates (30–40%) of larvae were recorded after 72 h of continues feeding on Ag1, Lo1 and Lo4 plant leaves whereas lower rates of 15–25% were observed in insects feed on other primary transformants with no mortality in control plants (Table 1, Fig. 2b).

Third instar CPB larval analysis showed no mortality after 24 h; however 20% of mortality of larvae feeding on Lo2 was observed after 48 h of feeding while larvae feeding on other transformants showed non-significant mortality (10%). After 72 h of feeding, the mortality rate was recorded as 35–60% in larvae feeding on Ag1, Ag4, Lo1, Lo2, and Lo4 plant leaves. However, mortality of 25–30% of larvae was recorded feeding on Ag2, Ag3 and Lo3 (Table 1, Fig. 2c). Taken together, the leaf bioassays suggested that the mortality rate of CPB was significantly enhanced when fed on transgenic potato plants expressing dsRNA *EcR*, and this increase is more pronounced in younger insects fed for a longer period of time, suggesting the accumulatory effects of dsRNA *EcR* with the time in developmentally-vulnerable pest insects.

The CPB larvae feeding on potato gained weight. Therefore, the CPB larvae were weighed after leaf bioassays to determine the inhibitory effects of dsRNA *EcR* in insect feeding on primary T_0 transformant leaves. There was a significant difference in fold increase of weight in different larval instars when fed on transgenic plants compared to the control plants (Fig. 3). In first instar, a significant increase of up to 6.53-folds in weight was observed in insects feeding on Agria control plants while 6.45-folds of increase was observed in insects fed on Lady Oylmpia control plants (Fig. 3a). On the other hand, the ones feeding on Ag4 and Lo4 lines showed 1.83-folds and 2.44-folds of higher body weight, respectively. Similarly, 2nd instar larvae feeding on Ag1 and Lo1 transgenic plants showed only 1.35- to 2.65-folds of increase in weight whereas insects feeding on control plants gained 3.03 to 7.94 times more weight (Fig. 3b). Likewise, 3rd instar larvae feeding on Ag4 and Lo4 transgenic plants exhibited reduced fold weight of 0.87-fold and 1.13-folds, respectively, as compared to the ones fed on controls (Fig. 3c). These results suggest that the insects feeding on control plants gained more weight while the ones fed on transgenic plants expressing dsRNA *EcR* gained less weight. Taken together, results of leaf feeding bioassays indicated that potato transformants expressing dsRNA *EcR* were more resistant to CPB and the level of resistance is related directly to the expression level of dsRNA *EcR* in plant leaves.



◀**Fig. 2** Representative images of leaf biotoxicity assay of CPB larvae feeding on T0 transformants of Lady Olympia and Agria after 72 h of feeding. a First instar CPB larvae. **b** Second instar CPB larvae. **c** Third instar CPB larvae

CPB fed on primary transformant potato leaves showed morbidity due to silencing of *EcR*

It was believed that molting defects in CPB will be resulted from the suppression of its *EcR* mRNA level after feeding on leaves of the transgenic potato plants expressing dsRNA *EcR*. To counter-check this hypothesis, *EcR* transcript levels in CPB larvae was determined by qRT-PCR after feeding on primary T_0 transformant leaves (Fig. 4). The results showed a higher suppression of *EcR* transcript in the first instar larvae fed on transgenic plants compared to the controls (Fig. 4a). Similarly, transcript levels of *EcR* in the second instar larvae feeding on transgenic leaves also decreased significantly (Fig. 4b). This decrease was much higher especially in the insects feeding on Ag1, Ag4, Lo1 and Lo4 lines. In the third instar, there was less change in overall transcript levels after 3 days of feeding on transgenic plants. Relatively lower expression (0.28) was observed in Ag4, while significantly higher expression (0.88) was recorded in Lo3 (Fig. 4c). These results suggest that CPB fed on primary transformant potato leaves expressing the *EcR* dsRNA showed suppression of *EcR* transcript. The *EcR* suppression was dependent on potato genotype and was more significant in younger insects feeding on transgenic plants.

In order to determine the effectiveness of the silencing of *EcR* in CPB pests, the insects were photographed after 72 h of leaf feeding on primary T_0 transformants (Fig. 5). As expected, silencing of *EcR* transcript in insects due to the feeding on transgenic plants expressing *EcR* dsRNA significantly altered the size and morphology of the insects, especially in earlier developmental stages. In general, insects fed on transgenic plants expressing dsRNA *EcR* in higher levels (Lo2) were smaller than the ones fed on control plants or transgenic plants expressing dsRNA *EcR* in lower levels (Lo1). Overall, these results indicate that the higher expression of dsRNA resulted in less weight gain due to retarded insect growth.

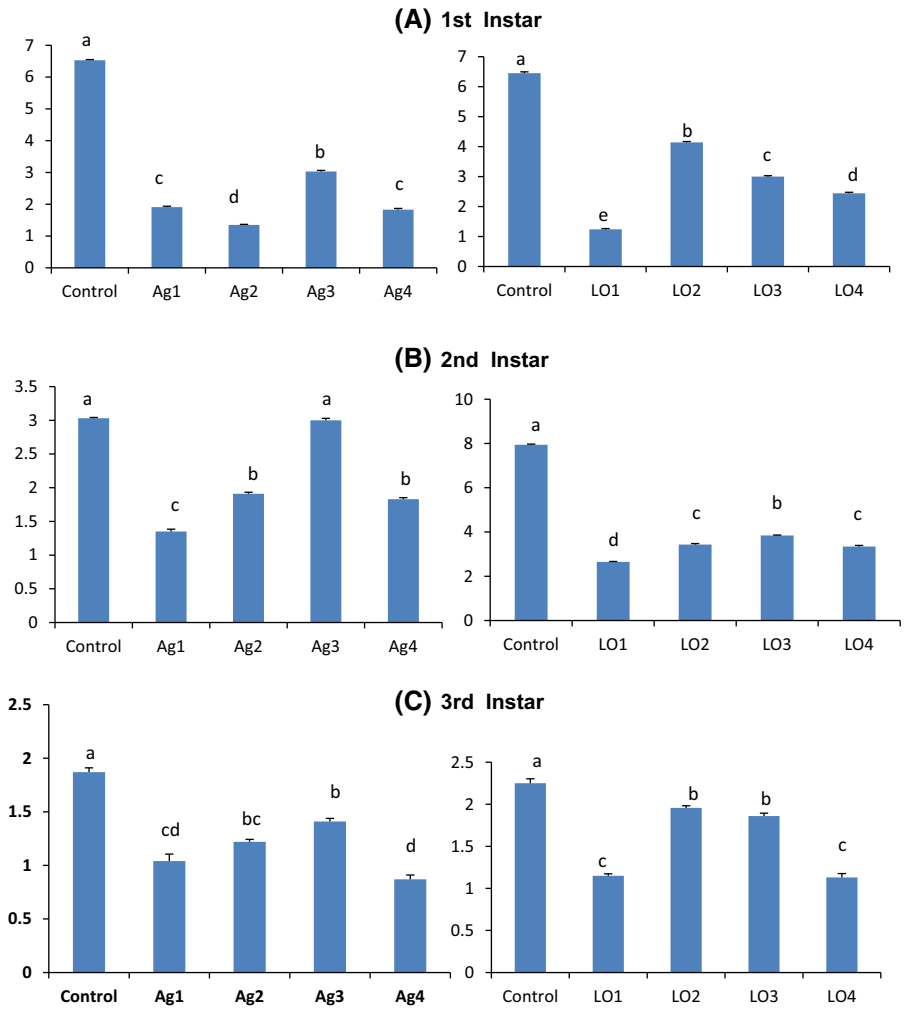


Fig. 3 Fold change larval weight analysis of Ist instar (**a**), 2nd instar (**b**) and 3rd instar (**c**) CPB larvae fed on primary transformants of Lady olympia and Agria along with control. Significant differences ($p < 0.05$) are indicated with different letters detected by one-way ANOVA analysis after Tukey's multiple comparison test (n = 3)

Discussions

RNA interference is a sequence specific silencing mechanism that is catalyzed by the introduction of transgenes in the form of inverted repeats to result in dsRNA transcripts (Lindbo and Dougherty 2005). Transgenic plants expressing non-endogenous dsRNA can be created via *Agrobacterium tumefaciens* mediated transformation, which either can make a single sequence consisting of a long hairpin (Guo and Lee 2007; Mamta et al. 2015), or two complementary sequences in the cytoplasm (Kumar et al. 2012). The present study exhibited the functionality of ecdysone receptor (*EcR*) dsRNA in transgenic potato plants to encode resistance against CPB, resulting in a promising approach for efficient pest management. Using RNAi technique, transgenic potato lines expressing *EcR* dsRNA were generated. This *EcR* dsRNA lowered the transcript levels of target *EcR* gene when digested by the insect pests. Plant-mediated RNAi technology successfully triggered silencing of the targeted gene in the insects, and hence caused significant mortality of CPB.

For RNAi, proper selection of target gene is challenging in order to obtain encouraging results (Yu et al. 2014). As no EcR homologues have been reported in genomes of higher organisms (Nakagawa and Henrich 2009; Zhu et al. 2012), it can be assumed

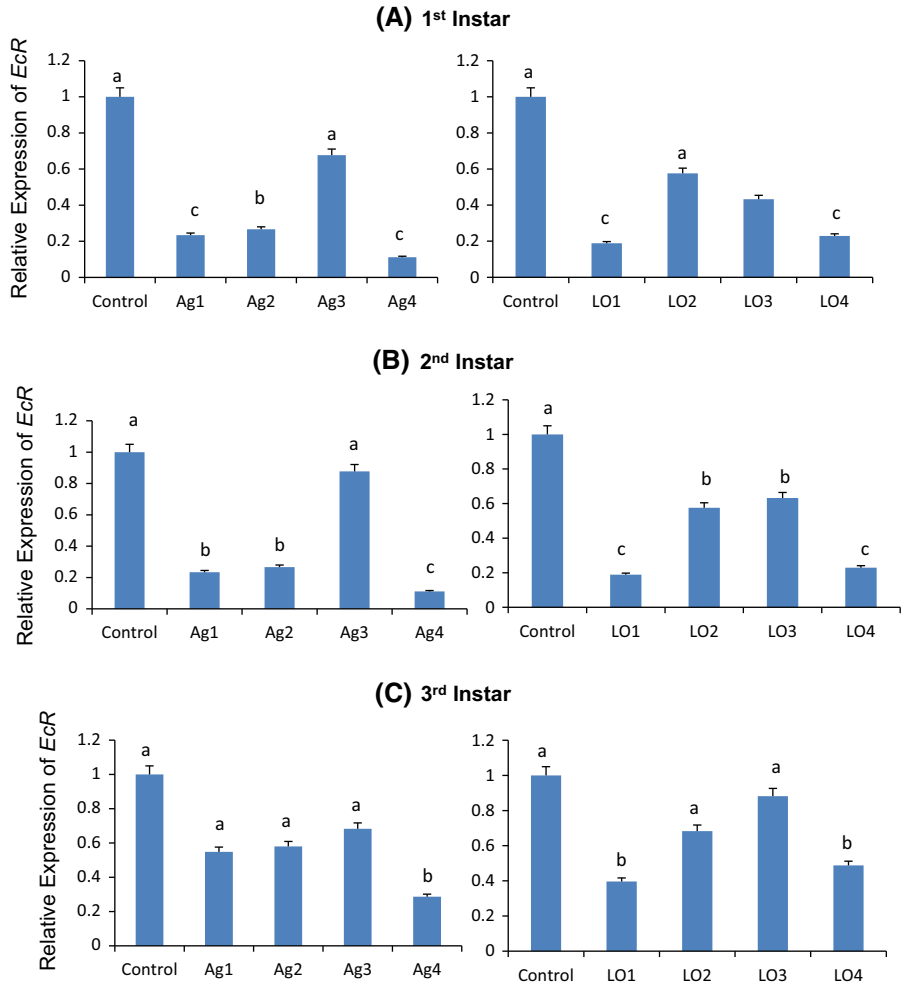


Fig. 4 qRT-PCR analysis of *EcR* transcript levels in CPB larvae after 72 h of feeding on T0 transgenic plant leaves. **a** Transcripts levels of *EcR* in 1st instar larvae fed on primary transformants of Agria and Lady Olympia, **b** Transcripts levels of *EcR* in 2nd instar larvae fed on primary transformants of Agria and Lady Olympia, **c** Transcripts levels of *EcR* in 3rd instar larvae. Control: pRNAi-GFP expressing Agria or Lady Olympia. Significant differences ($p < 0.05$) are indicated with different letters detected by one-way ANOVA analysis after Tukey's multiple comparison test (n = 3)

that designing hairpin dsRNA for targeting of insect associated gene EcR can be a better strategy for CPB control in potato.

Genetic engineering of potato has been achieved by *Agrobacterium*-mediated transformation (Van Eck, 2018). In present study, Agrobacterium-mediated transformation of potato was performed as described by Beaujean et al. (1998) with some modifications adopted in our laboratory. The researchers have reported different transformation efficiencies in potato using various explants (Beaujean et al. 1998; Soto et al. 2007; Veale et al. 2012; Hameed et al. 2017). Overall transformation efficiency in our experiments was calculated as 2.8%. According to some previous reports there are different ways to calculate genetic transformation efficiency of different plants (Wang et al. 2011; Bakhsh et al. 2012). We calculated it by dividing the number of PCR positive plants to the total number of inoculated plants used in the study. The various factors, such as the type of vector and Agrobacterium strain, explant type, varietal genetic background and gelling agent, can affect the efficiency of Agrobacterium mediated transformation in crops (Frary and Earle 1996; Rao et al. 2009; Bakhsh et al. 2014, 2018). A total of 210 PCR positive plants

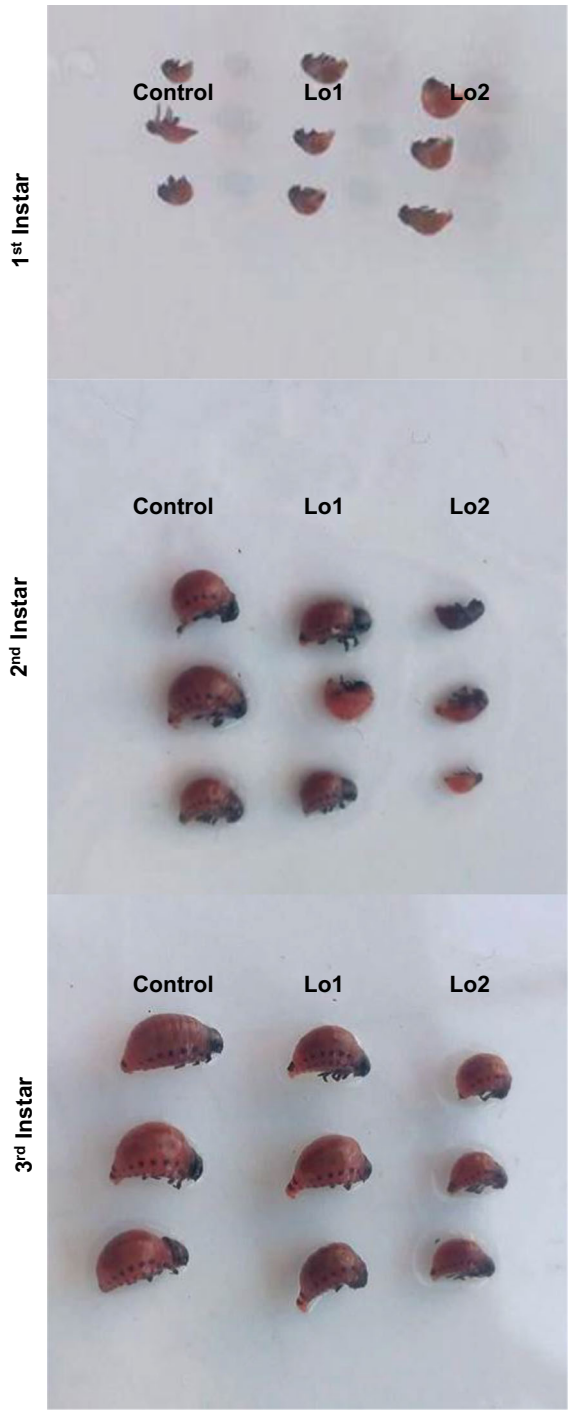


Fig. 5 Representative CPB larvae after 72 h of feeding on Lady Olympia T0 transgenic plant leaves

belonging to different constructs were transferred to the greenhouse and confirmed by PCR assays.

Southern-blot analysis of PCR positive plants revealed the integration of transgene in plant genome. A detection of 445 bp fragment on the membrane confirmed the integration of *EcR* gene in transgenic plants. The presence of expected hybridization signal in transformed plants showed that EcR gene was successfully integrated in the potato genome (Zha et al. 2011). qRT-PCR analysis was performed to analyze the expression levels of CPB *EcR* gene in transgenic plants. PCR positive plants showed a remarkably high expression of *EcR* transcripts in transgenic plants as compared to control plants. Expression level of gene was different in various transgenic plants (Fig. 1) though the expression varied among plants. Our results are in agreement with previous studies of Li et al. (2017) who reported the expression level of any genes can vary in different transgenic lines.

Once transgenic plants were confirmed for transgene integration and expression, leaf biotoxicity assays were performed by allowing feeding of 1st, 2nd and 3rd instar CPB larvae on these transgenic plants. A variation in mortality of CPB was recorded with different instars (Table 1). Mortality of CPB larvae established the functionality of dsRNA *EcR* in CPB although the efficiency of dsRNA remained variable among different instars and different transgenic plants. Earlier reports are also in agreement with our results. Zhu et al. (2012) reported mortality of *Helicoverpa armigera* up to 40% when transgenic tobacco lines expressing ds*EcR* were fed to the pest. RNAi-expressing transgenic plants gave satisfactory protection against western corn rootworm and American bollworm in the initial experiments conducted by Baum et al. (2007) and Mao et al. (2007), respectively. Following these initial studies, various achievements have been made by different researchers to target various insect-pests such as *Myzus persicae* (Mao and Zeng 2014), *N. lugens* (Yu et al. 2014), *S. avenae* (Xu et al. 2014), *H. armigera* (Liu et al. 2015) and aphids (Pitino et al. 2011; Coleman et al. 2015, 2016) by using RNAi-based silencing of target genes.

Following 72 h of feeding, overall trend showed more mortality rates of 1st and 2nd instar larvae compared to 3rd instar larvae (Table 1, Fig. 2). The higher mortality in 1st and 2nd instars larvae can be attributed to the lower metabolism and higher susceptibility of earlier instars (Ferro and Lyon 1991). However the decreased mortality rates of 3rd instar

CPB larvae can possibly be due to the difference in bioactivity of digestive proteinase during different growth stages of the CPB. Our findings are comparable to the results of Michaud et al. (1995) who found that earlier instars were more prone to rice cysteine proteinase inhibitor oryzacystatin I as compared to the 3rd and 4th larval instars. A unique digestive proteolytic system is found in growing stages of CPB which became more active during greater instars.

The incubated CPB larvae on transgenic plants were analyzed for the weight gain before and after the experiment. There was a significantly higher fold-increase in larval weight ranging between 1.87 and 6.53 times in insects fed on control plants. On the other hand, weight gain ranged between 0.87 and 4.14 times in insects fed on transgenic plants expressing dsRNA *EcR*. These results are comparable to the findings of Zhu et al. (2011), Xiong et al. (2013) and Jin et al. (2015). Higher expression of dsRNA resulted in more weight loss due to retarded growth owing to the fact that *EcR* gene is crucial for insect growth and development (Zhu et al. 2011, 2012).

The transcript levels of *EcR* genes were analyzed in CPB larvae fed on transgenic plants as well as control plants to evaluate the effect of ds*EcR* in efficient silencing of the target gene. qRT-PCR results revealed significant reduction of *EcR* transcripts in larvae after 72 h of feeding on transgenic plant leaves (Fig. 4). Our results established that the expression of dsRNA in transgenic plants was directly proportional to the percent mortality of insects (Upadhyay et al. 2011; Wuriyanghan et al. 2011; Zha et al. 2011). These findings are in accordance with the previous studies (Zha et al. 2011; Xiong et al. 2013; Xu et al. 2014). Higher expression also affected the weight gain in different larval instars of CPB (Mao et al. 2011). These results are in agreement with the previous findings of Jin et al. (2015), Mao et al. (2011) and Asokan et al. (2014), where reduction in larval weight was reported when insect pests were fed to transgenic plants expressing dsRNA of insect target gene.

The present study was focused on the suppressed transcripts level of highly expressive *Ecdysone receptor* (*EcR*) gene of Colorado potato beetle (CPB) (*Leptinotarsa decemlineata, Say)* using plant-mediated RNAi approach. The leaf bioassays of transgenic plants performed with different instars of CPB exhibited 20–80% of mortality of the pest insects. Our results show that CPB larvae feeding on transgenic potato plants expressing *EcR* dsRNA showed halted metamorphosis, lower body weight, and larvae were not able to shift to their next instar. These results are very encouraging in order to control notorious potato pest by an alternative, effective and reliable method since enhanced CPB resistance has been reported against almost every group of insecticides in the last decade worldwide.

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Author contributions AB designed the study and secured funding for the project. TH constructed recombinant vectors, optimized transformation protocol for potato cultivars, conducted leaf bioassays. AB, EA and TH interpreted the results, and prepared the manuscript. AB and MEÇ supervised overall activities of the work.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

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