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ORIGINAL ARTICLE

Genetic transformation of common beans (*Phaseolus vulgaris* L.) through *Agrobacterium tumefaciens* carrying Cry1Ab gene

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Abstract

Background Seed beetles are one of the most important causes of yield loss in bean production. It is essential to develop resistant varieties in the fight against these pests. Agrobacterium-based gene transformation is the most widely used breeding method worldwide to develop insect-resistant varieties.

Methods and results Embryonic axes and plumule explants were obtained from Agrobacterium tumefciens treated mature zygotic embryos of low and high raw protein-based common bean cultivars Akman 98 and Karacaşehir 90. Agrobacterium tumefaciens contained a synthetic *Bacillus thuringiensis* insecticidal crystal protein gene (Bt Cry1Ab) controlled by the 35S promoter and NOS terminator sequences. The transformation event was genotype and explant dependent. The plumule explants could not withstand kanamycin-based selection pressure and died. It was possible to get two transgenic plants using embryonic axis explants of low protein cultivar Akman 98. These results were validated using GUS analysis, PCR, RT-PCR, bioassay analysis, and ELISA test from the samples taken from T_0 and T_1 generations. Bioassay tests showed that these plants were protected from the damage of legume seed insects (*Bruchus* spp.).

Conclusions The results are very encouraging and may help in producing better transgenic common bean germplasm leading to safe agriculture and reducing environmental pollutions.

Keywords Insect resistance · *Bacillus thuringiensis* · Beans · Legume seeds · Bruchus sp

Introduction

Common bean (*Phaseolus vulgaris* L.) is important edible legume energy and protein source, consumed both fresh and dry as food and feed [1]. It is well adapted to various climates and is cultivated on 33 million hectares of land with 29 million tons of production world over [2]. It is cultivated in all areas of Turkey and is tolerant to drought and heat [3]. Many insects are a major constraint to common bean production in Turkey. They attack plants at all growth and developmental stages with considerable yield losses [4]. The most destructive insect among them is the common bean seed beetle [*Acanthoscelides obtectus* Say., (Coleoptera: Chrysomelidae: Bruchinae)] [5]. These lethal and destructive beetles, continue to reproduce and multiply throughout the year in seeds under storage the world over [6]. These end up in high losses and reduce the quantity and quality of common bean seeds. This compels farmers to sell their produce early at low prices [6, personal observations 2021].

The studies have shown that breeding common bean for seed insect resistance through conventional breeding approaches is unlikely to provide feasible solutions to breed for insect resistance in the near future [7]. *A. obtectus* create cavities in common bean seeds; during storage by about more than 35–100% and are a great concern during postharvest conditions in terms of quality, yield, and seed germination [6, personal observations 2021].

These insects could be controlled by various cultural treatments like late sowing, storing clean seeds in sacks, using clean depots for storage, deep burying of crop residues, burning the plant residues left in the field after harvest, and using various chemical pesticides [8]. However, these practices increase environmental pollution and may end up

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in various degrees of risks to food security and human health [9].

There are several reports on common bean shoot regeneration by Martins and Sondahl [10] from shoot apex, by Mohamed et al. [11] and, Faranklin et al. [12] from cotyledon nodes with axillary buds, by Mariotti et al. [13] from cotyledon nodes without using axillary buds, by Delgado-Sánchez et al. [14] from embryonic axes, by Zambre et al. [15] from embryonic calluses and by Espinosa-Huerta et al. [16] from hypocotyl explants. However, no protocol among these is repeatable. No study has confirmed the transformation of viable plants during *Agrobacterium tumefaciens* mediated transformations.

Collado et al. [17] has reported transformation of *P. vulgaris* cv. CIAP7247F *using* primary green nodular calli using *A. tumefaciens* using bar gene for selection. However, the procedure is very complex and there is a need to simplify it. It is well established that callus explants could be multicellular in their origin, leading to development of shoots that are chimeric in origin and may restrict the selection of transgenic shoots [18].

Transforming common bean with cry genes is an active issue to control insects belonging to the order Coleoptera, Diptera, and Lepidoptera, [19] and presumed to play an important role in insect management in common bean and other legume crops in the future. Fearing et al. [20] reported that toxins from bt are relatively host-specific, that have physiological specific gut conditions and the receptor sites are highly sensitive to these toxins [21]. Many previous studies show that the Cry1Ab [19, 22] gene can be used in insect resistance with a high success percentage.

Therefore, this study was designed to develop a reproducible genetic transformation protocol against these insects using the Cry1Ab gene in two low and high protein common bean cultivars with high stability. It was presumed that this will help in safe agriculture without the use of undesired inputs like chemical pesticides and accumulation of their residues during storage reducing the environmental pollution.

Materials and methods

Plant materials and seed surface sterilization of the two cultivars

The seeds of common bean cv. Akman 98 and cv. Karacaşehir 90 were used in this study. Cv. Akman 98 has 23–26% protein (low protein cultivar) with a hard testa. Whereas, the seeds of cv. Karacaşehir 90 have a protein percentage of 28–30% (high protein cultivar) and soft testa [23]. These were obtained from the Transitional Zone Agricultural Research Institute, Eskişehir, Turkey.

The seeds of both cultivars were surface sterilized with 10 and 20% commercial bleach (Ace-Turkey, containing 5% NaOCl) at room temperature and after giving 4 °C treatment for 12 h or using 50% hydrogen peroxide (H_2O_2). After sterilization, the seeds were 3 × 5 min rinsed with tissue culture grade bidistilled sterilized water.

A magnetic stirrer was not used during sterilization. However, the sterilized beakers containing the seeds of each cultivar were shaken manually.

Preparation of explant

The embryos were collected from the seeds under aseptic conditions by gently removing the testa and holding seeds with forceps to open them with scalpel blades. These were pretreated for 4 on a 10 mg l^{-1} 6-benzylaminopurine (BAP) (These were separately modified in a separate experiment containing 5, 10, and 15 mg l^{-1} 6-benzylaminopurine (BAP) for 4 and 8 days- these results are not reported in this experiment). It was followed by excising the embryonic axis and plumule explants from the embryos.

Inoculation and transformation

The *A. tumefaciens* strain was multiplied using Nutrient Broth (NB) medium containing selective antibiotics (spectinomycin and rifampicin at 50 mg l^{-1} each) by inoculating a single colony of *A. tumefaciens* on Liquid NB medium (D (+)-glucose, 1 g l^{-1}; peptone, 15 g l^{-1}; sodium chloride, 6 g l^{-1} yeast extract, 3 g l^{-1}). The liquid NB medium was prepared by dissolving 25 g of broth in 1 l of tissue culture grade water before autoclaving.

Both embryonic axis and plumule were treated (for 30 min) with *A. tumefaciens* strain LBA4404 harboring recombinant binary vector pRGG bar that contained the herbicide (bar) and insecticide tolerance gene pRD400/35S/NOS-Cry1Ab harboring codon-optimized for insect resistance along with *uida* (GUS) coding β-glucuronidase under the control of 35S promoter by dipping them in a bacterial suspension—OD = 1.2. They were co-cultured for 24 h on agar solidified MS medium containing 30 g l^{-1} sucrose. Thereafter, these were transferred to the selection and regeneration medium containing, 0.50 mg l^{-1} 6-benzylaminopurine (BAP), 500 mg l^{-1} biostatic wide spectrum antibiotic Augmentin [a combination of amoxicillin and clavulanate potassium (a beta-lactamase inhibitör)] and 50 mg l^{-1} kanamycin monosulphate.

Subsequently, the 3.5–4 cm long regenerated putative transgenic shoots were excised and rooted on MS medium supplemented with 2.0 mg l^{-1} IBA [24], 50 mg l^{-1} kanamycin monosulfat, and 500 mg l^{-1} Augmentin.

Culture conditions

All cultures were incubated in a growth chamber at 24 ± 2 °C, using 42 µMol m^{-2} s^{-1} during transformation regeneration and rooting.

Histochemical GUS analysis

Putative transgenic plants were tested for *Gus* (*uidA*) analysis. The fresh leaves of putative transgenic plants (taken randomly) of T_0 generation were subjected to a histochemical GUS assay based on methods described by [25]. The leaf samples of putatively transformed shoots were obtained from newly developed leaves under greenhouse conditions and incubated at 38 °C for 24 h in 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.1% Triton X-100 and 1 mM 5-Bromo-4-chloro- 3-indolyl glucuronide (X-GLUC). The putatively transformed tissues were detected by continuous soaking in 96% ethanol for 3 days (36 h) to break up chlorophyll completely for easy detection of GUS activity in the tissues. The presence of GUS activity was indicated by blue staining of the leaf tissues.

DNA ısolation and PCR analysis

Putative transgenic gus positive plants were further screened using PCR analysis with Cry1Ab sequence-specific primers. Non transformed (control) and putative transgenic plants (confirmed by PCR and RT-PCR assay) were tested for the efficiency of transgenic plants' resistance against insect pests.

Genomic DNA was isolated using the CTAB method [26]. The isolated DNA was used for PCR analyses of the putative transgenic plants and their subsequent progenies.

PCR was performed in a total reaction mixture volume of 25 µL containing 1 × reaction buffer, 3 µl of DNA template, 25 mM (3 µl) MgCl2, 10 mM (2 µl) of each of the dNTPs, 2 µl of each primer, and 0.2 µl of Taq DNA polymerase, 2.5 µl 10X PCR tampon and 10.3 µl dH2O. Reactions are run at Biometra T-personal thermal cycler instrument. The PCR conditions were: initial denaturation at 95 °C for 10 min, denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and initial extension at 72 °C for 2 min, followed by 2 to 39 cycles, extension at 72 °C for 10 min and 4 °C pauses. Amplified DNA fragments were electrophoresed on 1.0% agarose gel at 70 Volt 1 h and visualized by 5 µl ethidium bromide staining under UV light.

RT-PCR expression analysis of Cry1Ab gene

Furthermore, the putative transgenic plants were analyzed by RT-PCR assay to confirm the presence of the introduced gene (Cry1Ab/Cry1AC). Genomic DNA was isolated from fresh common bean leaves using the CTAB method [27].

PCR was run using gene-specific primers for the presence of the Cry1Ab and bar gene to amplify internal fragments of 750 bp using forward 5′-TGG ATT GCA CGC AGG TTC TC-3′ and reverse 5′-CAA GAA GGC GAT AGA AGG CG-3′ as primers. DNA extracted from untransformed plants was used as the negative control and that of plasmid pRGG as the positive control.

Before using DNA samples, they were diluted 1/5, 1/10, 1/50, 1/100 and a standard curve was drawn to compare with positive samples. The Log Amplification curve was formed on the samples after obtaining the standard curve. The melting curve peaks were obtained after drawing a standard curve and log amplification according to the results. Analysis was carried out by the SYBR Green I assay format. The PCR conditions were annealing temperature at 60 °C for 6 s and extension temperature at 72 °C for 20 s. After obtaining the standard curve, the log amplification curve of the samples was extracted and the melting peaks were obtained according to the results obtained from these two curves.

ELISA based protein expression analysis

This qualitative ELISA kit was used to detect the presence of Bt-Cry1Ab protein expressed in transgenic plants. The test does not distinguish between Bt-Cry1Ab and Bt-Cry1Ac proteins. This assay is suitable for testing both seeds and leaves. The Bt Cry1Ab/1Ac ELISA is an immunoassay for the qualitative screening detection of Bt Cry1Ab protein residues. The Bacillus thuringiensis (Bt) Cry1Ab protein is an insecticidal crystalline protein expressed by the Cry1Ab/1Ac gene in genetically modified plants as described by the manufacturer (https://orders.agdia.com/bt-cry1ab-1ac-elisa-kit-psp-06200). The samples were considered positive when the absorbance value was above the absorbance value of the negative control reagent.

Insect bioassays

The insect bioassays were conducted following [28] using transgenic and nontransgenic seeds of the cultivars placed in separate glass Petri dishes under ambient conditions of temperature and moisture in the laboratory together with five males and females. These were collected from Prof. Dr. Mevlüt Emekci Department of Plant Protection, Ankara University, and were maintained in a mass-bred population maintained in the laboratory. 24–36 h old individuals

were put on separate transgenic and nontransgenic seeds maintained in separate Petri dishes for 10 days. After the stipulated time the females laid eggs. The experiments were performed at ambient conditions of temperature (25 ± 1 ℃ and relative humidity 70 ± 2%).

General culture conditions

The pH of all culture treatments was adjusted to 5.6–5.8 using 0.1 N KOH or 0.1 N HCl before autoclaving under the pressure of 105 kPa for 20 min at 121 °C. The sterilization and all tissue culture procedures were carried out under aseptic conditions. The glass Petri dishes were sterilized for 2 h in a drying oven at 160 °C.

Statistical analysis

A total number of 100 seeds divided equally into 5 replications were used in the experiment for each cultivar in a Completely Randomized Block Design. The frequency (%) of shoot regeneration, the mean number of shoots per explant, shoot length, and frequency of rooting were recorded and analyzed using univariate analysis of variance in a two factorial experimental randomized block design. SPSS 17.00 for Windows Computer software was used for statistical analysis. The post hoc tests were performed using the Duncans Multiple Range Test. Data given in percentages were subjected to arcsine transformation [29] before statistical analysis.

Results

Seed surface sterilization of the two cultivars

Surface sterilization of the seeds of both cultivars was highly genotype-dependent. It was possible to sterilize the seeds hard testa cv. "Akman 98" with 20% commercial bleach at room temperature (24 °C); whereas, the seeds soft testa cv. "Karacaşehir 90" did not allow the use of commercial bleach for surface sterilization. It damaged the seed testa, cotyledons, and embryos when using 10 and 20% NaOCl containing bleach. The seeds split during surface sterilization. The 10% solution worked partially well when the seeds were cold treated overnight at 4 °C. The percentage of damage to seeds was not completely reduced. The damage percentage reduced to about 65–67%. Therefore, these seeds were sterilized with 50% hydrogen peroxide (H_2O_2) without using a magnetic stirrer for 5 min. Treatment with H_2O_2 did not split the seeds during sterilization.

Inoculation and transformation

The plumule explants failed to induce shoot regeneration. The embryonic axis explants regenerated shoots. They did not show hyperhydricity or any kind of necrosis on the developing shoots or during the transfer of the shoots from the regeneration + selection medium. The results showed 40.00% and 66.66% shoot induction with 1.13 and 1.40 shoots per explant cv. Akman-98 and cv. Karacaşehir-90 in the same order. All of these were rooted and acclimatized for taking samples for GUS analysis. Only one plant resistant to kanamycin monosulphate of cv. Karacaşehir-90 has rooted. It was not confirmed transgenic through PCR. This plant was acclimatized in the greenhouse to external conditions for setting seeds. The recovery of transgenic plants in the presence of kanamycin has been greatly facilitated by the *npt II* gene. During genetic transformation, antibiotic kanamycin acts by binding to ribosomes by inhibiting protein synthesis due to the presence of the *nptII* gene.

Two regenerated shoots (plants) of cv. Akman-98 rooted; these two plants were confirmed transgenic through PCR. These were successfully acclimatized to the external conditions to set seeds.

GUS expression and PCR analysis

The common beans are important economic vegetable crops for Turkey and their genetic modification has significant importance. It is well known that the integration of GUS gene is either expressed stably or transiently in transformed plants [36, 37]. It is of interest to check their tissue-specific expression or activity.

The samples were taken from the two plants in cv. Akman-98 in T_0 were confirmed putative transgenic through GUS positive expression (Fig. 1). A total number of 17 seeds were produced from the T_0 generation. These were germinated in pots to produce T_1 generation. Histochemical analysis of the leaf samples obtained from newly developed putative transgenic plants of T_1 generation also proved GUS positive. Single plant obtained from cv. Karacaşehir-90 was not confirmed gus positive in T_0 or T_1.

Fig. 1 Leaf sample stained blue as a result of GUS test in gene transfer with GV2260:: p35GUSINT bacterial line in cv. Akman 98

Positive gus expression in Akman-98 and PCR results in T_0 and T_1 generations showed stable transformation. The GUS gene expression for stable and transient expression in the common bean is very important.

GUS gene expression was detected both in T_0 and T_1 plants that peaked in (1–5 days old) young leaves and declined in the old leaves. Strong GUS activity was observed in young leaves, young floral tissues, like mature anthers, stamens sepals, petals, leaves, and pollen grains of the seedlings of T_0 plants.

PCR analysis

The results were further confirmed and demonstrated by transgene integration into genomic DNA of putative transgenic Plants in T_0 and T_1 putative transformed common bean plants were observed.

Column 2 (K) Non-transformed plants did not show any hybridization signals as expected. Columns 1–6 show negative bean plants. Positive results were obtained in a total of two transformant plants (Columns 7 and 8) (Fig. 2.).

RT-PCR test

The data representing the exact quantification and the results obtained with the programs used are given schematically in Fig. 3. The RT PCR results also confirmed transgene integration into genomic DNA of putative transgenic Plants in T_0 and T_1 common bean plants.

It was determined that the putative transgenic plants obtained from the "plumule" explants treated with strains belonging to the LBA4404 line containing the *nptII* and *Cry 1Ab* genes of *A. tumefaciens* were RT-PCR positive.

ELISA based protein expression analysis

Quantitative evaluation of Cry1Ab delta endotoxins in T_0 and T_1 transformed common bean seeds of Akman 98 developed was observed using DAS-ELISA with their extracts. The mean quantity of Cry1Ab endotoxin among two T_0 transgenic plant seeds was 35 ng mg^{-1} TSP and 25–35 ng mg^{-1} TSP in T_0 plants showed an inconsistent level of toxin at seed setting (data not shown).

Only two transgenic plants obtained from a single cultivar in the current study showed that transformation was a difficult event in common bean. The current results further suggest that ELISA for Bt-Cry toxin in T_0 population results were better in T_0 compared to T_1 and reflected the level of insect resistance.

Insect bioassays

The bioassays were carried out on common bean seeds. The leaves of the Karacaşehir 90 were affected by oviposition (Fig. 4a). No oviposition was noted on the leaves of Akman 98. The transformed beans also did not show damage on the leaves (Fig. 4b). The transgenic seeds of the cultivar also did not show wounding in the feeding assay of transgenic cv. Akman-98. The results indicated that the Cry1Ab in transgenic common bean offered a protection shield against insects. The larval mortality on the T_0 leaves was very clear on the transgenic seed samples. A larval mortality rate of 89.48% was noted after a period of 24 h of bioassay. This mortality increased to 97.89% on seeds of transgenic plants after 36 h and 100% in 48 h. The mortality of larvae fed on transgenic leaves in T_1 was between 67.23 and 90.16% after 60 h and 100% after 84 h of feeding the seeds. Mortality was noted on larvae fed on the seeds of non-transgenic common bean plant leaves of cv. Karacaşehir-90 (Fig. 4).

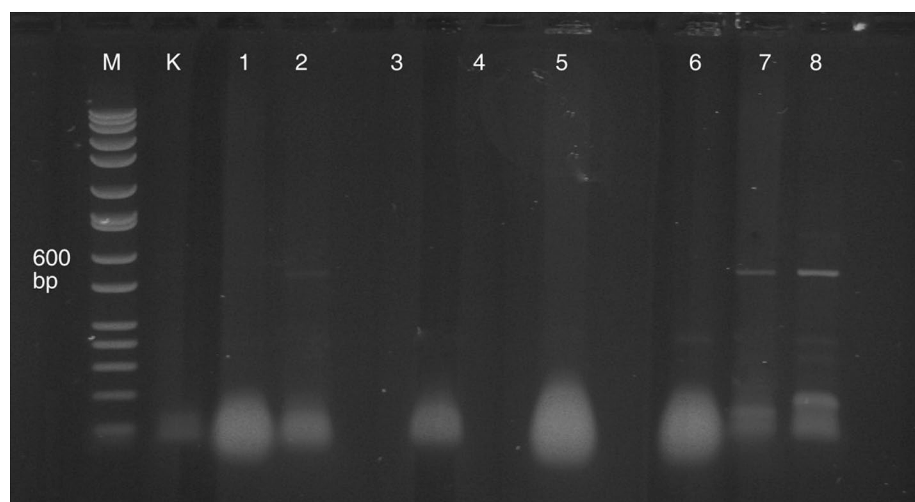

Fig. 2 Confirmation of the presence of *npt-II* gene by PCR in cv. Akman-98 grown in the greenhouse; (M) marker, (K) Cv. Akman–98 negative control, 1–6 Columns cry1Ab gene negative plants, 7–8. Columns transgenic bean carrying the Cry1Ab gene

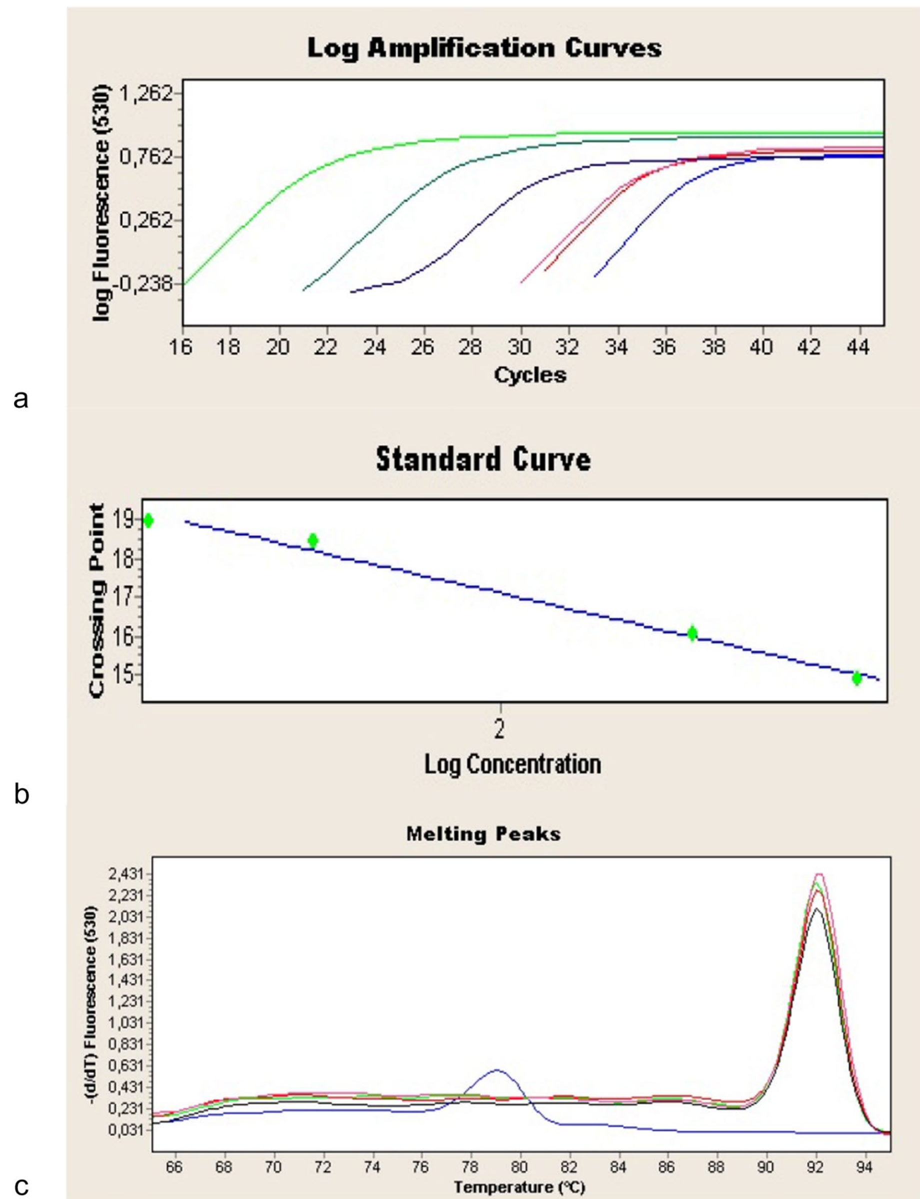

Fig. 3 Determination of nptII—Cry1Ab PCR product in cv. Akman-98 common bean by real-time PCR analysis. **a** Amplification curve using known amounts of pRD400. **b** Standard curve for data presented in **a**. **c** Real-time PCR melting curves showing overlapping peaks for known amounts of pRD400 and Cry1Ab-transformed plants

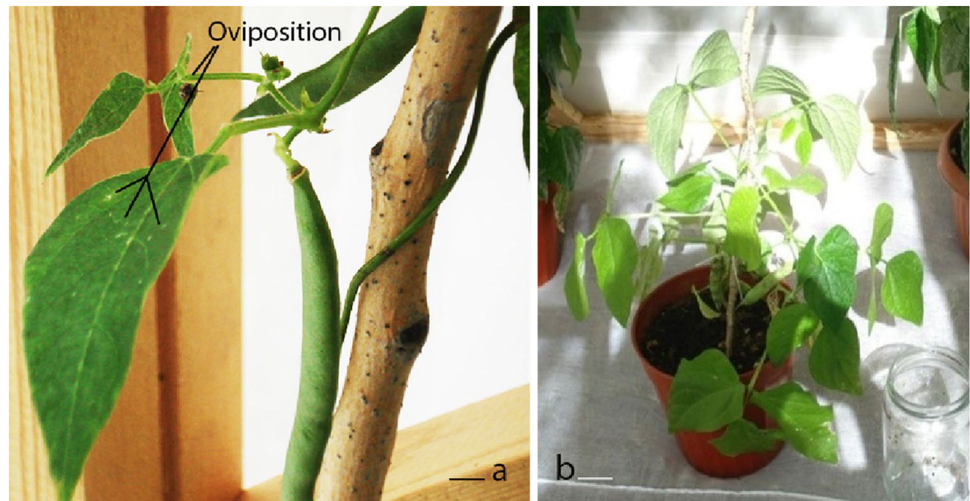

Fig. 4 Transformation of common bean. **a** Non transformed plant of Karacaşehir 90 with oviposition of insect **b** transformed plant of Akman 98 with mortality noted on larvae fed on the seeds of transgenic common bean plant Akman 98. Bar of **a** = 1 cm, **b** = 3.1 cm

Discussion

The results indicated that the seeds of low protein cv. Akman 98 were less sensitive towards the concentration and type of disinfectants compared to the seed coats of high protein cv. Karacaşehir 90. Findings from several studies have exhibited that NaOCl treatments influence the breaking of seed dormancy, germination, and viability of seeds of several plant species like Lens culinaris [30], *Amaranthus powellii* S. Wats [31], *Aconitum heterophyllum* Wall. [32]. The results are also in agreement with Khan and Zia [33], who reported that sodium hypochlorite reduced the effects of salinity under lower temperatures emphasizing that the activity of sodium hypochlorite was progressively lowered with a decrease in temperature. Therefore, care should be made in selection of the disinfectant. The disinfectant must be seed coat friendly to have optimum results.

The *Escherichia coli* based gus or uidA gene that encodes b-glucuronidase (GUS) [25] is a popularly used reporter gene in the genetic transformation of an innumerable number of plant species [38, 39]. It is well known that the integration of GUS reporter gene into the genome of transformed plants induces blue color in plants and facilitates in the analysis of gene expression without the need of expensive equipment and tedious chemical procedures. This gene enables easy quantified spectrophotometric analysis and is preferred in gene expression analysis. GUS gene expression in plant tissues is extremely useful to monitor the results immediately after genetic transformation the results of the study support induction of strong GUS activity on young leaves, and tissues in T_0 and T_1 plants. The results are in agreement with [40], while no Gus activity was noted in T_1 immature floral tissues of T_1 generation [41]. The results confirmed that the Gus expression in T_0 and T_1 plants were not transient.

Although the results showed a very low rate of genetic transformation, the results confirmed selection of transgenic shoots on kanamycin. That was reconfirmed through PCR and RT PCR from the plants setting seeds. Actually, transformation ends up catalyzing the phosphorylation of kanamycin as soon as it enters into plant cells by shutting down the protein synthesis machinery in plants. This enables transgenic plants to grow in the presence of kanamycin [34]. Inbuilt level of resistance in different plants species and varieties could vary and affect this reaction during transformation efficiency resulting in escapes during genetic transformation [35].

The results are in partial agreement with observations for differential expression of Bt-endotoxins in T_0 plants [42]. This variation in T_0 transgenic plants could be attributed to the position effect of gene integration ending up with physiological variations in the gene functions and foreign protein expression in the common bean plant tissues [42]. The efficiency level of this *Agrobacterium*-strain seems limited and restricted due to several limitations including the complex nature of the common bean genome [43, 44]. Expressing a higher Cry toxin of 1.0% of TSP in transgenic tomato, maize tobacco, cotton, and rice [45] was not noted for common bean. This suggests that transforming events in recalcitrant common bean is highly tedious like other grain legumes [46].

The results of bioassays in bean seeds showed that the leaves of the Karacaşehir 90 were more sensitive and were affected by oviposition on leaves of Akman 98 and any damage and offered protection by the Cry1Ab in transgenic common bean. The results are in agreement with previous studies, where the 2nd instar larvae showed mortality in feeding bioassays of other crop plants [47, 48].

Conclusion

This article discusses the developmental stages of Cry1Ab transgenic common bean lines and stable gene integration of Cry1Ab gene in common bean. This shows that the success of regeneration and gene transfer in the bean plants mainly depends on the explants, genotypes, and protein percentage along with other cofactors. The results of this study will influence future transgenic research on common beans and will help in improving and developing future common bean breeding technologies.

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Author contributions All authors contributed to the study conception and design. KMK and CYC conceived and supervised the research. SSY conducted the experiments. KMK, CYC and SSY analyzed the data. KMK and SSY wrote the manuscript. All authors reviewed and approved the final manuscript.

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Declarations

Conflict of interest Authors declare that they have no confict of interests.

Informed consent All authors agree for publication.

Research involved in human or animal rights No experiment was conducted on animals in this study.

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