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2. **Title: Transgenic Tobacco Expressing Synthetic *EPSPS-CP4* Gene for Tolerance against Glyphosate Herbicides**
3. **Running Title**: Characterization of Synthetic Herbicide Tolerant Gene in Tobacco
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2. **Novelty statement**

Glyphosate herbicides are very effective weed killer chemicals, but unfortunately these cannot discriminate between plants and weeds. Hence, the ultimate solution is to develop resistance against glyphosate herbicides. We modify the nucleotide sequence of glyphosate tolerant bacterial gene using bioinformatics tools and synthetic gene was got synthetized commercially. The gene sequence was submitted to NCBI and accession number was allotted. Cloning was done in Gateway compatible fashion and genetic transformation in tobacco leaves was achieved by *Agrobacterium* mediated system. Molecular analysis and plant bioassay showed that synthetic gene is successfully introduced in tobacco and is fully functional. The synthetic gene is now ready for development of glyphosate tolerant character in economically important crops especially cotton.

**Abstract**

Glyphosate is an active ingredient of non-selective herbicides which cannot discriminate between main crops and weeds; hence it is necessary to develop tolerance in plants against glyphosate herbicides. Bacterial *EPSPS* is a well-known gene and its synthetic versions have been introduced successfully in many crops for tolerance against glyphosate. The goal of the current study was to modify bacterial *EPSPS* gene, characterization of synthetic gene in modal plant and verify the effectiveness of transgenics against glyphosate applications. Gateway cloning technology was adopted for cloning and development of expression cassette, final vector was introduced into sterile tobacco leaves using *Agrobacterium* mediated genetic transformation protocols. Seven putative transgenic shoots were recovered on selective media while stable transformation was achieved in two plants as verified by PCR and southern blotting. Transcriptional and translational studies of synthetic gene were carried-out using RT-PCR and protein specific strips respectively. Chemical resistance plant bioassay was conducted by spray the transgenic plants using 1.0% glyphosate and 0.1% of kanamycin separately. The bioassay results showed that *EPSPS* gene remained active at sufficient level to survive after two weeks of spray. These molecular analysis of synthetic gene showed that the current modified version of *EPSPS-CP4* gene is functionally equivalent to other genes being used for glyphosate tolerance in plants. Hence, it is recommended that this gene would be used in commercial cultivars against glyphosate herbicides and for effective weed control.

**Key words** Weed control; Glyphosate herbicides; Synthetic EPSPS; Tobacco; *Agrobacterium tumefaciens*; Transgenics

**Introduction**

Weeds are the major competitors of field grown crops and severely affecting the yield annually. They can compete for water, light and other nutrients required for proper growth and development of plants. A variety of management strategies and conventional approaches are being used to control the weeds worldwide. Furthermore, spray of different types of chemicals/herbicides is an effective, efficient and inexpensive weed controlling strategy, but at the same time it could create possible threats for human health and environment. Two types of herbicides selective and non-selective currently used to control the unwanted plants i.e. weeds. Selective herbicides could differentiate between the weeds and crops as they recognize the types of weeds i.e. narrow or broad leaves, while non-selective herbicides cannot differentiate the crops and weeds as they kill the entire flora and are very much effective for weed control. The main drawback in the use of herbicides is the lack of tolerance to herbicide by some major crops like wheat, rice, maize etc. (Cerdeira and Duke 2006). Therefore, it is necessary to develop a plant type which can tolerate the damaging effects of chemical herbicides. Selectivity of crops to a specific herbicide can be conferred only by the modification of genetic makeup and by the introduction of resistant genes. In this scenario, recombinant DNA technology has offered new prospects for engineering the valuable traits in crop plants. This technology facilitates by the introducing the foreign genes from any source into plants to alter the metabolic pathways and to generate the new products which includes also herbicide tolerant plants (Duke 2011). Glyphosate is a well-recognized and powerful broad spectrum herbicide which inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involve in shikimate metabolic pathway that connects the breakdown of carbohydrates to biosynthesis of an aromatic compounds (Herrmann 1995). The capability of plant genetic engineering to develop glyphosate tolerant crops was a scientific revolution that facilitated modernize weed controlling (Dill *et al*. 2008). For last two decades, glyphosate tolerant crops are the most favorable option to control weeds for farming community worldwide. The glyphosate tolerant crops made weed controlling easy, effective and cheap according to the demands of growers (Frisvold and Reeves 2010; Bonny 2011). A variety of herbicide tolerant genes like *bar, epsps, pat, bxn* etc. from various sources have been successfully introduced into targeted plants to develop broad spectrum resistance mechanisms (Ye *et al*. 2001; Zhou *et al*. 2006; Kahrizi *et al*. 2007). Roundup Ready resistant crops normally have a transgene isolated from *Agrobacterium* strain CP4 encoding a glyphosate-tolerant enzyme i.e. EPSPS. Transgenic plants with *EPSPS-CP4* gene, showed tolerance to glyphosate herbicides and enabling more effective weed control (Funke *et al*. 2006). The present research work describes the codon optimization of *EPSPS-CP4* according to cotton preferred codons, cloning of synthetic gene and integration into model plant for characterization and expression studies before genetic transformation into cotton.

**Materials and Methods**

**In-silico analysis and synthesis of glyphosate tolerant gene**

The nucleotide sequence of glyphosate tolerant *EPSPS*-*CP4* gene (1368bp) was retrieved from NCBI and subjected to codon optimization with the help of Geneius online software (<http://www.geneius.de/GENEius>). The secondary structures in mRNA were analyzed using the CLC Main Workbench 5.0.1. The Optimized *EPSPS*-*CP4* gene sequence was synthesized from GeneLink Company USA. The final synthetic gene sequence was submitted to the GenBank and awarded the accession number i.e. KP212901.

**Construction of *Agrobacterium* compatible plant transformation vector**

The synthetic *EPSPS*-*CP4* gene was ligated in a gateway system compatible binary vector pK2GW7,0 (Karimi *et al*. 2002). The ligation mixture then transformed in *E. coli* competent cells and allowed to grow on solid LB plates having spectinomycin @ 75µg/ml at 37oC for overnight. The appeared colonies again grown in LB liquid and plasmid DNA was extracted using GeneJET Plasmid isolation kit (Thermo Scientific, Lithuania). The resultant recombinant DNA was confirmed with a series of restriction enzyme.

**Preparation of plant material for genetic transformation**

Seeds of *Nicotiana tabacum* were disinfected using 70% ethanol for 3min and 10% Domestose (w/v) (containing 3-5.7% active hypochlorite) for 2 minutes. Seeds were carefully washed using germ-free water and dried under Laminar Flow Cabinet (ESCO, Australia). About 100 seeds were placed to grow on seed germinating medium (Murashige and Skoog 1962) and incubated at 25±2oC.

***Agrobacterium* inoculation and *in-vitro* selection of transformed tissues**

Binary vector pK2GW7,0 containing synthetic *EPSPS*-*CP4* gene was introduced into*Agrobacterium* strain LBA4404 using ECM-2001 Electroporation System (BTX Harvard Apparatus, USA) and confirmed with different molecular biology techniques. *Agrobacterium* containing synthetic *EPSPS*-*CP4* gene was grown on LB media supplemented with spectinomycin @ 75µg/ml at 28oC for 48 hours at 180rpm in shaking incubator (Taitec, Japan) and cells were permitted to multiply up to cell density (OD) of 1.0 at A600. Four weeks old *in-vitro* grown healthy tobacco leaves were sliced into small fragments of 5x5 cm and exposed to co-cultivation medium supplemented with 4.2g/L of MS salts, 30g/L sucrose, 1.0mg/L BAP, 0.1mg/L NAA and 2.7g/L phytagel. Leaves were incubated in dark for 48 hours at 28oC, later leaf pieces were thoroughly rinsed with 250mg/L of cefotaxime at least three times and sterile water. Leaf discs were transferred on regeneration media plates having 4.2g/L of MS salts, 30g/L sucrose, 1.0mg/L BAP, 0.1mg/L NAA and 2.7g/L phytagel, kanamycin 50mg/mL and cefotaxime 250mg/L for differentiation and selection. The plates were incubated at 25±2oC with 16/8 hours light/dark period in incubation room. Cells were started to proliferate and develop shoots after about 3 weeks of incubation. 3-4cm lengthy shoots were shifted in glass jars on MS media with 50mg/L of kanamycin to maintain the selection pressure and for root development.

**Genomic analysis of putative *EPSPS*-*CP4* transgenic tobacco clones**

Total cellular DNA from untransformed and antibiotic resistant putative transgenic tobacco plants was extracted using CTAB method with modifications (Nazir and Khan 2013) and quantified using Nanodrop Spectrophotometer (NanoDrop Technologies, USA). The isolated purified DNA (30ng) was used as template in PCR using Master Cycler Gradient (Eppendorf, Germany). The PCR programme was comprised of 35 cycles of 94oC for 30 seconds, 56oC for 45 seconds and 72oC for 1.0 minute. The resultant PCR product was separated using 1.0% agarose gel and visualized using UV equipped gel documentation system (GDS) (Photonyx Ultra, UK). *Pst*I digested 10.0µg of total cellular DNA was separated on 0.8% agarose gel and shifted onto nylon neutral transfer membrane (AppliChem, Germany) with the help of iBlot gel transfer system (Invitrogen, USA). The DNA was fixed on membrane with UV Cross Linker (CL-1000M, USA). An *EPSPS*-*CP4* gene specific probe was used in hybridization and detection was carried out with Biotin Chromogenic Detection Kit (Thermo Scientific, Lithuania) as described by Nazir and Khan (2012).

**Transcriptome analysis using RT-PCR**

Total RNA was extracted from PCR positive transgenic and wild type tobacco leaves with the help of GeneJET Plant RNA Purification kit (Thermo Scientific, Lithuania). Isolated RNA was treated with DNase-I enzyme for the removal of genomic DNA contamination and quantified. 1.0µg of purified RNA was used to generate cDNA with RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Lithuania). 2.0µl of cDNA was used in reverse transcriptase PCR to amplify the synthetic gene in 50µl of PCR reaction mixture. The PCR profile was used as denaturation for 30 seconds at 94oC, annealing at 56oC for 45 seconds and extension at 72oC for 1.0 minute for 35 cycles. The amplified product was resolved on 1.5% agarose gel and image was taken using GDS.

**Immunostrip strip test and chemical resistance bioassay**

Presence of EPSPS-CP4 transprotein in synthetic EPSPS-CP4 transgenic tobacco leaves were identified by using protein specific strips (Artron Laboratories Inc. Canada). Reaction result was noted as positive (+) and negative (-) on the base of test line looked on the strips. Around 20mg of leaf sample was ground in protein extraction buffer and test was performed as manufacturer’s instructions. Plant bioassay was performed by using kanamycin solution @ 0.1% (Phytotechnology, USA) was prepared in distilled water and swabbed on tobacco leaves. The reaction of plants was recorded after about two weeks of application. Moreover, commercial herbicide Roundup Ready having glyphosate as an active ingredient (Monsanto USA) 1.0 % (v/v) was sprayed on PCR positive potted plants maintained under controlled condition in incubation room. The effects of herbicide on plant’s vegetative growth were observed visually after two weeks of spray.

**Results**

**Plant transformation vector having synthetic *EPSPS*-*CP4* gene**

An *Agrobacterium* compatible gateway plant transformation vector was developed by introducing the synthetic gene in between the border sequences of plasmid pK2GW7,0. The plasmid containing the ccdB gene cassette was replaced with the synthetic gene with the help of BP clonase enzyme (Fig. 1). The genetic information in-between the left and right border was comprised on *npt*II selection marker gene which has its own regulatory elements. The second gene cassette was of ccdB which was replaced with 1368bp fragment of synthetic *EPSPS*-*CP4* gene. The synthetic gene is now regulated by CaMV35S promoter and T35S terminator. The total size of final transformation vector was 10851bp. The cloning of synthetic gene in final plant transformation vector was verified with the help of different restriction enzymes.

**Development and screening of transgenic tobacco plants**

The selection media was changed after every two weeks to maintain the selection pressure. After about 3-4 weeks the transformed cells started to proliferate while remaining cells bleached due to the selection pressure of antibiotic present in media. About seven plants were recovered on antibiotic containing selection medium. This was the first round of screening and regeneration of putative transgenic cells. The small green leaves from putative transgenic shoots were further screened on antibiotic supplemented regeneration medium for next round of selection and regeneration. Healthy shoots from second round of selection were shifted in glass jars for roots and shoots development. Transgenic tobacco plants were gradually exposed to natural environment for adaptation and seed formation in pots (Fig. 2).

**Molecular examination of putative transgenic clones**

Drug resistant putative transgenic tobacco clones were investigated at molecular level by isolating the total genomic DNA from the leaves of seven antibiotic resistant in-vitro grown plants. Isolated DNA was exposed to Polymerase Chain Reaction (PCR) using synthetic *EPSPS*-*CP4* gene specific primers. Amplification of 636bp product confirm the existence of transgene in tobacco plants as there was not any amplification of such fragment in WT tobacco plant’s DNA (Fig. 3A). From seven drug resistant clones, only two plants (Tr4 & Tr6) were found positive in PCR based verification of transgene integration in tobacco plants. In gel image DNA from wild type tobacco plant (un-transformed lane WT) was used as plant -ve control, while plasmid DNA was used as +ve control (lane +ve) in PCR. Synthetic gene positive plants were further verified using selection marker gene specific primers in PCR and successfully amplify 150bp fragment from both transgenic tobacco clones (Fig. 3B). These results confirmed that the transgenic cassette comprising on synthetic *EPSPS*-*CP4* and *npt*II selection marker gene have been successfully transfomred in two tobacco plants.

PCR positive tobacco plants were further analyzed at molecular level using southern hybridization technique. *Pst*I restricted cellular DNA from both transformed and untransformed plants was shifted to nylon membrane and hybridized with *EPSPS*-*CP4* gene-specific probe. Hybridization procedure was verified by using plasmid DNA as positive control. A PCR amplified 636bp fragment specific for synthetic gene was used as probe. Upon digestion of cellular DNA with *Pst*I enzyme, transgenic plants were expected to yield 1680bp fragment carrying synthetic gene, while no such fragment was expected in wild type tobacco plant’s DNA. Fig. 4 presenting the successful hybridization of *EPSPS*-*CP4* gene specific probe with its target sequence on the membrane, while no such signals were observed from non-transformed tobacco. These results also indicated the transgenic cassette has been successfully integrated in tobacco plants and both plants have only one copy of transgenic cassette.

Leaves from PCR positive transgenic tobacco plants were characterized by RT-PCR with gene specific primers. Amplification of 450bp fragment specific for synthetic *EPSPS*-*CP4* gene from cDNA of both transgenic tobacco plants sanctioned the transcription of transgene in tobacco plants while no such type of amplification was observed from non-transformed samples as shown in Fig. 5. These results also verifying that the regulatory elements i.e. promoters and terminators are working very successfully for the expression of transgene in the tobacco plants.

The existence of EPSPS protein was verified with specific strips in both transgenic and wild tobacco plants. The upper line indicating that the whole experimental procedure and strips are satisfactory, while lower line is protein specific (Fig. 6). These results showing that the synthetic gene is not only expressing itself at RNA level but also translated into protein as very strong signals were observed in PCR positive plants.

**Chemical Bioassay**

Bioassay is also very useful and powerful technology for the verification and expression analysis of transgene(s) in transgenic plants. Freshly prepared 0.1% kanamycin solution was spread on the upper side of selected leaves of transgenic and wild type tobacco. After about two week of application, it was noticed that the leaves of non-transformed tobacco turning to yellowish while no such indications were recorded on *EPSPS*-*CP4* transgenic tobacco leaves especially for Tr6. These results verifying that the gene used as selection marker for kanamycin i.e. *npt*II also expressing itself in transgenic tobacco plants but the response was different in both transgenics. PCR positive along with untransformed control potted plants were sprayed using 1.0% (v/v) Roundup Ready herbicide (40.6% w/w glyphosate salt as active ingredient). Normally 0.2% glyphosate is enough to destroy most of the weeds under open field conditions (Imran *et al.* 2017; Cakmak *et al*. 2009). It was observed that non-transformed wild-type control plants displayed sever chlorosis and subsequently leads to stunning and witling after one weeks of spray (Fig. 7). A variable response to glyphosate was also recorded between both transgenic tobacco plants after two weeks of treatment. These results also indicating that in addition to marker gene, the synthetic glyphosate tolerant gene also producing tolerant protein against glyphosate herbicide in tobacco leaves and ultimately tolerate the harsh effects of roundup ready spray.

**Discussion**

Genetically engineered crops tolerant for Roundup Ready herbicide have been extensively grown for over three decades. Roundup Ready crop varieties contains *EPSPS* gene isolated from soil living microbe i.e. *Agrobacterium* strain CP4. This gene encodes an enzyme (5-enolpyruvylshikimate-3-phosphate synthase) from shikimate pathway, catalyzes reversible reaction of phosphoenolpyruvate (PEP) and 5-hydroxyl of shikimate-3-phosphate (S3P) to procedure EPSP and phosphate (Padgette *et al*. [1995](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6267263/#CR34)). This enzyme is responsible for tolerance to the most effective group of herbicides i.e. glyphosate. All plant produced endogenously EPSPS enzyme, but its conformation is fairly dissimilar from EPSPS-CP4 product hence, this conformation reduces its attraction for glyphosate and helps the genetically engineered plants to persist (Dill *et al*. 2008). To maintain the adequate level of this enzyme in plants following glyphosate herbicide application, EPSPS-CP4 protein must express consistently and sufficiently. The high expression of transgene can be credited to the modifications in GC contents, codon usage bias and secondary structures (Liu *et al*. 2015). In eukaryotic system, *Agrobacterium* *EPSPS-CP4* gene can expressed itself thousand times more than other systems and such codon modified versions of genes have previously paved the way for crop enhancement in most of the nations around the globe. The transgenic cultivars developed for resistance to glyphosate herbicides are limited, which presenting a huge space is available for spreading this technology to different crops specifically for under-developed countries (Imran *et al*. 2017). Keeping in view the above scenario of herbicide tolerant technology and the importance of *EPSPS* gene, it was decided to modify the *Agrobacterium EPSPS* gene responsible for tolerance to Roundup Ready glyphosate herbicide for high expression in plants. For heterologous expression of bacterial genes in plants, modifications of codon usage is obligatory and is basic step towards transgenic development. First the nucleotide sequence of the bacterial *EPSPS-CP4* genes was optimized using codon usage table and rare codons were escaped at the expense of GC contents and codon adaptation index. During optimization and modification in codon sequence, removal of polyadenylation signals and potential RNA processing sequences is also a key requirement. To increase the stability and accumulation of trans-protein in receptor cells, codon optimization and accurate localization of signals sequence in gene are very much important step (Imran *et al*. 2017). The *EPSPS-CP4* gene sequence was optimized according to cotton codon table with the aim to introduce the herbicide tolerant trait in cotton crop. Optimized gene sequence was got synthesized commercially in gateway compatible fashion. The gateway cloning is very easy, time saving and very accurate system for cloning of transgenes and development of final plant transformation vector. For characterization and expression analysis of synthetic gene, it was decided to introduce this vector into model plant i.e. tobacco. Young aseptic tobacco leaves were used to transform the synthetic *EPSPS* gene construct using *Agrobacterium* mediated transformation technology. Genetic transformation using *Agrobacterium* is preferred method for transgene integration, because it is simple, precise and offers low insertions of transgene with higher expression (Rao *et al*. 2009). Integration of transgene was initially confirmed at molecular level with the help of PCR method and found that only two plants were positive for transgene integration out of seven drug resistant survived plants on selective media. The revived plantlets on screening media supposed to have the transgenes and known as putative transgenics. Since there are three opportunities that the developed plantlets may be (a) true transgenics (b) escapees (c) mutants (Nazir *et al*. 2019). Normally PCR is the very basic molecular technique which is very simple and fast method for the identification and screening of transgenic plants. Southern blot analysis also confirmed the successful transformation and showed that only one copy of transgene is integrated in both transgenic plants (Wang *et al*. 2014). Transgenic events with single copy insertion are usually preferred in the development of genetically modified plants. Southern is more reliable, precise and accurate system as compared to PCR for the verification of transgene at DNA level but it requires more time and expertise as compared to PCR (Nazir *et al*. 2019; Nazir and Khan 2012). Transcriptional analysis of synthetic *EPSPS*-*CP4* gene was verified in RT-PCR using mRNA as template and found that the gene is successfully transcribing itself in both plants. Qualitative ELISA i.e. strip test also successfully verifying that the synthetic gene is translating itself into protein as no signals were detected in control plant. Herbicide and kanamycin bioassay was performed on potted plants for the investigation of tolerance level of transgenic tobacco. Tolerance of transgenic tobacco to glyphosate herbicide was verified by spraying roundup ready @ 1.0 % (v/v). A similar dose of glyphosate herbicide was also used for the screening of transgenic tobacco and rice (Imran *et al*. 2017; Chhapekar *et al*. 2015). It was observed that transgenic tobacco plants showed variable response to Roundup Ready. Leaf necrosis and chlorosis was detected following the spray which was recovered in transgenic plants after some days. This variable response of tobacco plants might be due to different expression level of synthetic *EPSPS-CP4* gene in both transgenics. Variable and lower expression of *EPSPS* gene with chlorotic symptoms was also reported in other crops like soybean, maize, potato, tomato etc. (Dun *et al*. 2014). Moreover, bleaching of chlorophyll was also observed in non-transformed tobacco leaves by the application of 0.1% solution of kanamycin, while no such signs were detected on transgenic tobacco leaves. This confirmed that the marker gene is also expressing itself and producing the targeted protein resistance for antibiotic. Screening of plants using kanamycin is very easy, cheap and reliable plant bioassay for the screening of transgenic plants on the basis of selection marker gene i.e. *npt*II. These results are agreed with previous conclusions of Freitas-Astua *et al*. (2003) for the screening transgenic tobacco using kanamycin solution. These molecular characterization studies of transgenic tobacco plants indicating that the synthetic gene cassette for herbicide tolerance has been successfully integrated in tobacco. All regulatory elements i.e. promoters and terminators are working efficiently for the expression of synthetic *EPSPS-CP4* and *npt*II genes and translating into specific proteins as verified by strip test and plant bioassays. Hence, it is recommended that synthetic gene cassette would be used to develop glyphosate herbicide tolerance character in other commercial cultivars.

**Conclusion**

Nucleotide sequence of bacterial *EPSPS-CP4* gene was optimized according to cotton preferred codons and cloned in gateway compatible plant expression vector. For characterization and expression analysis, synthetic gene was introduced in tobacco leaves using *Agrobacterium* mediated genetic transformation protocol and initially screened using PCR and Southern blotting. The expression analysis of transgene was carried out using RT-PCR and strip test. Chemical plant bioassay showed that gene is functionally remained active and equivalent to already transformed glyphosate tolerant genes in various corps. Hence, it was concluded that this synthetic version of *EPSPS-CP4* gene would be used in commercial cultivars of cotton to develop tolerance against broad spectrum glyphosate herbicides.

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**Author contributions**

SN and IU conceived and designed research. MZI directed the project. SN, IU, and SJ conducted research work. SN, IU and SR analyzed data. SN wrote the manuscript. All authors read and participated in manuscript.

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**Fig.** **1** Physical map of *Agrobacterium* compatible plant expression vector having synthetic GT (Glyphosate Tolerant) *EPSPS-CP4* gene



**Fig. 2** *In****-****vitro* screening of *Agrobacterium* inoculated tobacco leaves on selective media. **a** Selection and regeneration on kanamycin @ 50mg/L. **b** Drug resistant shoots shifted in jars for roots and shoots formation. **c** Acclimatization of putative transgenic tobacco plants in pots



**Fig. 3** Molecular **c**onfirmation of transgenic cassette integration using PCR methodology. **a** PCR amplification using *EPSPS-CP4* gene specific primers: Lane M1Kb DNA ladder, Lane +ve having plasmid as control, Lane WT Non-transformed tobacco DNA, and Lanes TR1-TR7 having DNA from drug resistant tobacco plants. **b** PCR amplification using *npt*II marker gene specific primers: Lane M100bp DNA ladder, Lane +ve having plasmid as control, Lane WT non-transformed tobacco DNA, Lanes TR4 and TR6 having DNA from drug resistant tobacco plants



**Fig. 4** Southern-blot hybridization analysis to determine integration of transformed cassette into tobacco plants. **a** The 636bp fragment specific to synthetic GT gene was used as probe for hybridization. **b** Restricted genomic DNA with *Pst*I enzyme. **c** Hybridization results by using GT gene as probe; WT, non-transformed tobacco plant DNA, +ve having plasmid as control and Tr4, Tr6 are transformed tobacco plant’s DNA



**Fig. 5** RT-PCR analysis of transgenic tobacco plants. **a** Isolation of total RNA from transformed and wild type tobacco**. b** RT-PCR results using *EPSPS-CP4* gene specific primers using cDNA as template. Lane M100bp DNA ladder, Lane WT having cDNA from non-transformed tobacco and Lanes TR4 and TR6 having cDNA from transgenic tobacco plants



**Fig. 6** Strip test for the analysis of EPSPS protein. WT: non-transformed tobacco plants, TR4 and TR6 of EPSPS-CP4 transgenic tobacco plants



**Fig. 7** Chemical bioassay for the verification of tolerance in transgenic plants against glyphosate herbicide and kanamycin. Response of plants was checked after one week of interval. WT: non-transformed tobacco plant, Tr4 and Tr6 are EPSPS-CP4 transgenic tobacco plants