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Functional Characterization of a Novel Synthetic Herbicide Resistance Gene in a Model Plant

(Pencirian Fungsian Gen Rintangan Herbisid Sintetik Novel dalam Model Tumbuhan)

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ABSTRACT

As crop losses can occur owing to the abrupt growth of uncontrollable weeds in the field, research is currently being conducted in Pakistan to eradicate herbs. To carry out our proposed research domain, we have designed a novel synthetically modified EPSPS gene that has a potent role in herbicide-resistance development. The novel codon-optimized synthesized mEPSPS sequence was inserted into the pXCSG-mYFP plant expression vector. Results of colony PCR (1400 bp) confirmed the integration of genes into bacteria. For functional validation of pXCSG-mYFP-EPSPS, transient expression in tobacco (*Nicotiana benthamiana*) in comparison with PBSF-16 was done. Benth infiltration results showed that transient expression was successfully confirmed through ELISA and western blot analysis via anti-YFP antibody in tobacco leaves. For further validation of transient expression, the stable transformation results of the pXCSG-mYFP-EPSPS vector showed that successful transformation was done via two days of co-cultivation followed by selection and regeneration of transformed tobacco plants. The regenerated tobacco plants were then confirmed through gene-specific-based PCR. After PCR-based confirmation, western blot further validates the 26 kDa anti-YFP antibodies expression in transformed tobacco plants. Another important finding of the study was the bar and PAT gene real-time expression elucidating that the bar gene was 4.9-fold more expressive under the *35S* promoter than the PAT gene under the *nos* promoter.

Keywords: EPSPS gene; functional characterization; molecular analysis; tobacco; transformation

ABSTRAK

Memandangkan kepentingan penyelidikan semasa di Pakistan adalah untuk menghapuskan herba kerana kehilangan hasil boleh berlaku disebabkan oleh pertumbuhan mendadak rumpai yang tidak terkawal dalam amalan lapangan. Untuk melaksanakan domain penyelidikan kami yang dicadangkan, kami telah mereka bentuk gen EPSPS yang diubah suai secara sintetik novel yang mempunyai peranan yang kuat dalam pembangunan tahan herbisid. Urutan mEPSPS tersintesis yang dioptimumkan kodon novel telah dimasukkan ke dalam vektor ekspresi tumbuhan pXCSG-mYFP. Keputusan koloni PCR (1400 bp) mengesahkan integrasi gen ke dalam bakteria. Untuk pengesahan fungsi pXCSG-mYFP-EPSPS ekspresi sementara dalam tembakau (*Nicotiana benthamiana*) berbanding dengan PBSF-16 telah dilakukan. Keputusan penyusupan Benth mendedahkan bahawa ekspresi sementara telah berjaya dilakukan melalui pengesahan ELISA dan analisis pemblotan western melalui antibodi anti-YFP dalam daun tembakau. Untuk pengesahan lanjut bagi ekspresi sementara, keputusan transformasi stabil bagi vektor pXCSG-mYFP-EPSPS menunjukkan bahawa transformasi yang berjaya dilakukan melalui penanaman bersama selama dua hari diikuti dengan pemilihan dan penjanaan semula tumbuhan tembakau yang telah diubah. Tumbuhan tembakau yang dijana semula kemudiannya disahkan melalui PCR berasaskan gen khusus. Selepas pengesahan berasaskan PCR, pemblotan western mengesahkan lagi ekspresi antibodi anti-YFP 26 kDa dalam loji tembakau yang diubah. Satu lagi penemuan penting dalam kajian ini ialah ekspresi masa nyata gen bar dan PAT yang menjelaskan bahawa gen bar adalah 4.9 kali ganda lebih ekspresif di bawah promoter *35S* berbanding gen PAT di bawah promoter *nos*.

Kata kunci: Analisis molekul; EPSPS; pencirian fungsi; tembakau; transformasi

Introduction

An induced inherent ability of some plant species to persist and reproduce after getting a lethal dose of herbicide is known as herbicide resistance (Wilson & Orloff 2008). Whereas, herbicide tolerance is related to the plant's inherent to cope with the herbicide and survive at a standard use rate (Vargas & Wright 2005). There are two mechanisms in herbicide resistance: Target site-based and non-target site-based. In the first type of resistance, the site of action is altered so that the modified enzyme can no longer bind with that site as the herbicide affinity is reduced (Owen, Goggin & Powles 2012). While in target site-based resistance, herbicides inhibit photosynthetic electron transfer at photosystem II, acetyl CoA carboxylase, acetoacetate synthase, and tubulin polymerization (Délye, Jasieniuk & Le Corre 2013). 5-enol pyruvate shikimate-3-phosphate (EPSP) synthase enzyme that is inhibited by glyphosate (Steinrucken & Amrhein 1980) is the sixth enzyme of shikimic-acid pathway and is essential for the biosynthesis of aromatic amino acids including tyrosine, tryptophan and phenylalanine as well as for production of ubiquinone, vitamin K, tetrahydrofolate, secondary aromatic metabolite including phytoalexins, auxins, lignin and anthocyanins (Kishore & Shah 1988). Phosphoenolpyruvate (PEP) and shikimate-3-phosphate undergo conversion through EPSP synthase enzyme to produce EPSP, and inorganic phosphate and amino acid pools reduce as the shikimic acid increases.

The increase in shikimic acid is also associated with dropping carbon fixation intermediates and reducing photosynthesis (Zobiole et al. 2010). Thus, inhibition of EPSP synthase resulted in plant death due to the shortage of all three aromatic amino acids. EPSPS inhibition results in bleaching, chlorosis, and stunted growth of plants. These symptoms are mainly concentrated in metabolically active sink tissues such as immature leaves, shot tips, buds, and roots. The yellowing from exposure to glyphosate starts with the older leaves and moves toward the younger leaves. The Agrobacterium sp. strain CP4, isolated from a waste-fed column at a glyphosate production facility, yielded a glyphosate-resistant, kinetically efficient EPSP synthase suitable to produce transgenic, glyphosate-tolerant crops. 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase enzyme that catalyzes the second last step of the shikimate pathway to produce aromatic amino acids is inhibited by glyphosate, which is a broad-spectrum herbicide and an active roundup-ready ingredient. Thus, the integration of foreign genes might become more acceptable in the genome of chickpeas by codon optimization of the CP4 EPSPS gene. The construction of synthetic gene circuits and even whole bacterial genome synthesis have become possible due to recent developments in the synthesis of an artificial gene (Hughes & Ellington 2017). Modulating already existing biological functions and the rise of novel cellular behaviors are possible outcomes when synthetic genes are introduced into any living system. Thus, *de novo* gene synthesis can help us eliminate the aforementioned abiotic constraint by non-target site-based resistance.

The agrobacterium infiltration is the preferred approach for transient gene transformation compared to biolistic bombardment and protoplast transformation (Zheng et al. 2021). Before transforming into the desired crop, the stable gene expression must be checked in a model plant. To date, Tobacco is the best choice for Agrobacterium-mediated genetic transformation. This old approach does not require expensive reagents or complicated protocols. The advantages of using this plant for genetic transformation are as follows: easy regeneration capability (Tyurin et al. 2020), short acclimatization time (Deb & Imchen 2010), high transporting survival percentage (100%), its ability to keep a hemizygous state with T-DNA cassette transfer. Considering the class II EPSP synthases result in herbicide resistance, we designed our study to synthetically modify the mutant of the CP4 EPSPS gene for the plant genome in general and specifically for chickpeas. Moreover, to clone the synthesized gene into a plant expression vector to evaluate the transient and stable gene expression in a model plant.

Materials and Methods

Codon optimization and secondary structure analysis

The CP4-EPSPS gene was retrieved using the codon usage table of *Cicer arietinum* L. via the software Optimizer. The restriction sites within the sequence were removed via online CLC Sequence viewer software. The signal peptide sequence insertion was made at 5'end for expression in the chloroplast organelle of cell. Sequence secondary structures were analyzed via CLC Main Workbench 8.0 software and obtained a suitable ΔG value. Finally, restriction sites were inserted at the 5' and 3' ends of the resultant sequence according to the multiple cloning sites of plant expression vector.

VECTOR CONSTRUCTION

The synthesized sequence was inserted into pXCSG-mYFP plant expression vector following gateway cloning (Invitrogen). The expression construct p35S-EPSPS-mYFP was developed by the gateway cloning approach (*Invitrogen*). *attB* sites were ligated with the herbicide resistance gene; the EPSPS gene was amplified with EPSPS primers containing *attB* sites at 5'-end. Bp reaction was performed to achieve the recombination of *aatB* sites attached with the EPSPS fragment and *attP* sites of pDONR™ (*Invitrogen*), using the manufacturer's protocol. Resultant positive entry clones were selected on zeocin plates and miniprep. The plant expression clone having EPSPS gene was formed by LR recombination reaction initiated between *attL* sites of entry clone and *attR* sites of binary destination vector pXCSG-mYFP (Feys et al. 2005), driven by CaMV *35S* promoter and with *phosphinothricin acetyltransferase* (PAT) as plant selectable marker under *nos* promoter. Positive clones were selected on ampicillin plate, followed by miniprep and PCR confirmation. The p35S-EPSPS-mYFP expression plasmid was transformed into *Agrobacterium tumefaciens* GV3101 (PMP90RK).

Vector function validation through transient method using tobacco as a model plant

The vector pBSF-16 was already used in tobacco and chickpeas for genetic transformation. The binary vector pXCSG-mYFP-EPSPS was compared with already reported vector pBSF-16 for the vector functional behavior via reporter gene expression. The vector pBSF-16 was used as a control in this experiment. The confirmed GV3101 colony of pXCSG-mYFP-EPSPS and pBSF-16 was cultured separately. The vector pXCSG-mYFP-EPSPS was evaluated for protein expression in model tobacco plant.

Nicotiana benthamiana infiltration for transient gene expression

A transient gene expression is a valuable approach for studying the functions of gene products. Therefore, to validate the synthetically designed vector (pXCSG-mYFP-EPSPS), the Benth infiltration method was used to evaluate transient gene expression. Before starting the transient gene transformation experiment, the 3-4 weeks-old tobacco plants (*Nicotiana benthamiana*) were watered. GV3101 culture was grown (Negative control; GV3101 cells with helper vector: PMP90RK) and other agro cells (pXCSG-mYFP-EPSPS plasmid, harboring EPSPS gene with antibiotic ampicillin) in 10 mL LB media for 2-3 days in 50 mL falcon tube. The culture was transferred to a 15 mL round-bottomed polycarbonate tube and centrifuged (5,000 rpm for ten min). 70 mL of infiltration buffer (LB) was prepared by adding 70 µL of 100 mM acetosyringone to the LB medium giving a final concentration of 100 uM. Each agro pellet was gently re-suspended in 5 mL of infiltration buffer (LB). It is recommended to re-suspend the pellet in 1 mL, add 4 mL (LB) and mix it. Transferred cells back to larger tubes. The tubes were shaken at 28 °C for 2-4 hours or until OD600 =.3. Gently infiltrated the *Nicotiana benthamiana* leaves (3 to 4 weeks old tobacco plants) using a 1 mL syringe in three replications per sample. The plants were allowed to grow for 2-3 days before dissecting leaf discs from each infected area. The leaf discs were weighed and ground in an Eppendorf tube with 10X the disc weight of ELISA protein extraction buffer (PBST). A Sandwich ELISA kit was used to measure Roundup Ready CP4 ELISA protein (Agdia RR) of genetically modified tobacco samples by following the kit protocol. A Western Blot analysis with (an anti-YFP antibody) was carried out to check the expression of protein from the infiltrated Tobacco plant.

Western Blot protocol for tobacco leaves

Sufficient Tris buffer Saline (TBS; 80 mL Tris B, 560 mL H2O, and 160 mL ethanol) was prepared to fill the transfer tank, equilibrate the gel and membrane, and wet the filter paper. The assembled cassette with membrane and the cassette holder was shifted into the transfer tank. The samples were loaded and run at 100 V for 45 min. The membrane was washed with the Towbin buffer (TTB) 4 times for 5 min each with agitation to remove the transfer buffer. The non-fat milk was poured into the tray with a membrane and was kept on shaking overnight. TTB was used to wash the membrane three to five times. Primary antibodies (mYFP) were added to the tray having a membrane and kept on shaking for 2 h. The membrane has been washed the membrane with TPB (10% tween, TBS) four to five times. The secondary antibody HRS (Horse Radish Peroxidase) was added to the washed membrane and kept in a shaker for 1 hour. The membrane was then washed with TTB and analyzed under chemiluminescence.

In vitro genetic transformation in Nicotiana benthamiana for stable gene expression

The stable gene transformation was also validated in the tobacco plant. In stable gene transformation, the

plasmid DNA is successfully integrated into the cellular genome and passed on to future cell generations. Explant (leaf segments) of Tobacco was transformed with the *Agrobacterium tumefaciens* GV3101 strain containing the pXCSG-mYFP-EPSPS plasmid and pBSF-16 plasmid and kept in the dark on co-cultivation media for two days. The co-cultivated explants were transferred to the first cycle of regeneration and selection medium (5 mg/L PPT) for two weeks and shoots developed slowly from the explants. Therefore, after two cycles (4 weeks) on regeneration and selection medium (2.5 mg/L PPT + meropenem to inhibit agrobacterium growth), the explants with regenerated shoots in Petri plates were transferred to the rooting medium. The regenerated plantlets were evaluated for transgene presence or absence via gene-specific PCR and reporter gene expression by western blotting to check whether the stable integration of genetic transformation is possible using pXCSG-mYFP-EPSPS.

Real Time gene expression of bar and PAT gene

The transgenic shoots (pXCG-mYFP-EPSPS) and (pBSF-16) were subjected to RNA extraction using the GeneJET Plant RNA Purification Mini kit (K0801). The extracted RNA was quantified and subjected to cDNA synthesis by RevertAid First Strand cDNA Synthesis Kit (K1621). The cDNA was further evaluated for the bar, and PAT gene relative fold expression to that of internal control 18S ribosomal RNA using SYBR™ Green PCR Master Mix (4309155).

Results

CODON OPTIMIZATION OF CP4 EPSPS SEQUENCE ACCORDING TO PLANT GENOME

The final optimized sequence was subject to the following protein translation using BLAST A. The final optimized DNA sequence has bankit NCBI submission ID: 2499043. The energy levels (Delta-G) were calculated for both the original DNA sequence and optimized sequence when converted to mRNA using CLC Main Workbench 8.0 software. The value of delta-G obtained for original and optimized sequences was found to be -627 and -490 cal/mol, whereas the value required must range between -200 to -500 cal/mol.

VECTOR CONSTRUCTION AND CLONING

The final outsized sequence was then synthesized synthetically from BGI. The resultant linear gene cassette after gateway cloning is shown in Figure 1. Figure 2 shows the positive clone of pXCSG-mYFP via PCR against mEPSPS gene-specific primers.

PCR analysis of cloned vector pXCSG-mYFP with synthetically designed EPSPS gene

The single colony was sorted and subjected to colony PCR analysis. Meanwhile, those colonies were cultured in a separate place to keep a backup. Among the six colonies, four were positive, and two were negative. Glycerol stock of positive colonies cultures was prepared and kept at -80 °C till further use. The positive clone's PCR gel picture was confirmed by Gel Documentation System (Syngene) (Figure 2).

FIGURE 1. Linear gene cassette LB= Left Border, RB= Right Border, PAT = Phosphinothricin acetyltransferase selectable marker gene, mYFP= modified Yellow Fluorescent protein, EPSPS = herbicide resistance gene

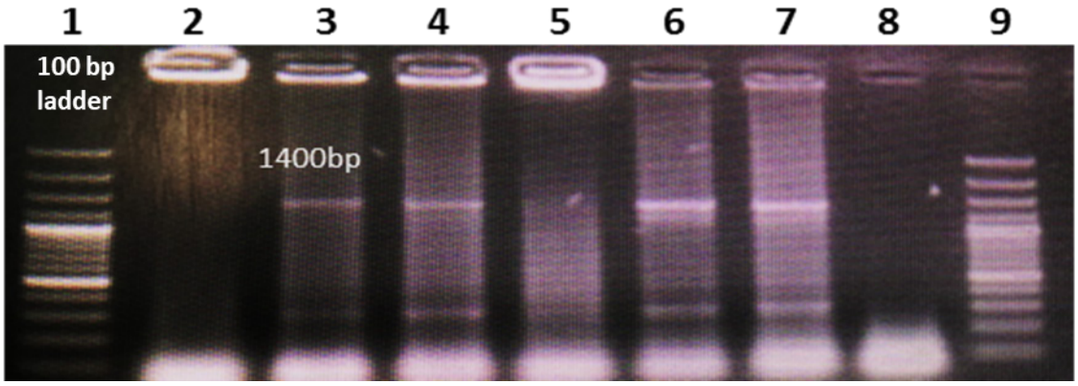

FIGURE 2. Confirmation of positive clones 1400 bp = Lane 3 and 4, Negative Colony = Lane 2 and 5, Negative Control = Lane 8, Positive Control = Lane 6 and 7, 100bp Ladder= Lane 1 and 9. Functional

Characterization of EPSPS gene in Tobacco

Following are the results discussed for transient and stable gene integration of transgene into tobacco plants.

Nicotiana benthamiana infiltration for transient gene expression assay

The *Nicotiana benthamiana* infiltration was performed to check and verify whether the pXCSG-mYFP-EPSPS vector expresses the integrated gene cassette into desired protein Figure 3. Initially, the ELISA O.D. for the seven samples were determined to depict that sample 1 and 6 showed negative results. However, the other samples showed intermediate results (Table 1). To validate these ELISA results further for protein expression more specific technique, western blot, confirms the protein expression. The western blot analysis was evaluated against reporter marker mYFP protein with (PMP90RK) vectors as a negative control. The pXCSG-mYFP-EPSPS shows a positive 26 kDa protein fragment size (Figure 3) when infiltrated plant parts were subjected to western blot analysis, while (PMP90RK) does not have any antigen antibodies interaction. It confirms that the vector pXCSG-mYFP-EPSPS expresses the protein and is suitable for further transformation.

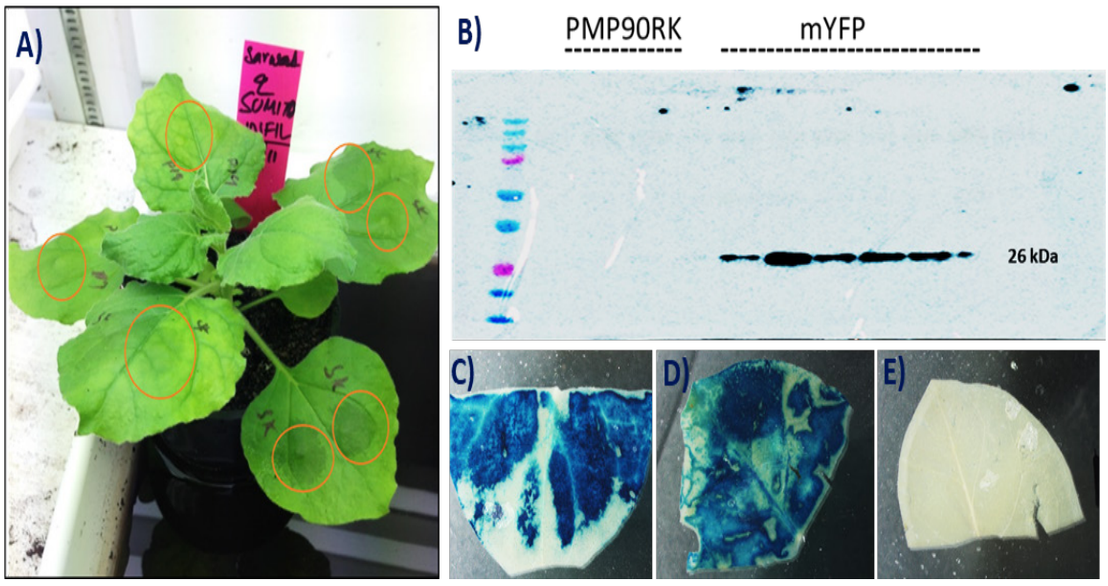

FIGURE 3. (A) Infiltrated Tobacco Plant (B) Western blot using anti-YFP antibodies (pXCSG-mYFP-EPSPS), M= Marker, PMP90RK = Negative control, mYEP= Positive samples, (C and D) GUS histochemical assay of infiltrated tobacco plants with PBSF-16, (E) Negative Control plant without infection (stained with X-Gluc)

TABLE 1. ELISA for roundup ready in tobacco plant infiltrated with pXCSG-mYFP-EPSPS

Sr. No	Samples	O. D
1	-ve Control	0.056
2	1	0.086
3	2	0.132
4	3	0.122
5	4	0.131
6	5	0.191
7	6	0.076
8	7	0.144

In vitro transformation in *Nicotiana benthamiana* for stable gene expression assay

Two different constructs: pXCSG-mYFP-EPSPS (PAT selectable marker gene) and PBSF16 (*bar* selectable marker gene), were used for stable transformation and functional characterization of reporter as well as plant selectable marker genes in tobacco plant (W38) in the presence of 5 mg/L PPT along with control 1, i.e., explant without selection and control 2, i.e., explant with selection Figure 4. Explants regenerated shoots on control 1 with regeneration and selection media in the absence of PPT. Explants that were not transformed with the plasmid died on control 2 in the presence of 5 mg/L PPT in the regeneration and selection media. Tobacco's explants (leaf segments) were transformed with the *Agrobacterium tumefaciens* GV3101 strain harboring the vector PBSF16 and kept in the dark on co-cultivation media for two days. The explants were then transferred to MS regeneration and selection media (2.5 mg/L PPT) for two weeks. Shoots are regenerated from leaf explants of tobacco (Figure 4).

Tobacco explants (leaf segments) of tobacco were transformed with the *Agrobacterium tumefaciens* GV3101 strain containing the pXCSG-mYFP plasmid and kept in dark on co-cultivation media for two days. When transferred to the first cycle of regeneration and selection medium (5 mg/L PPT) for two weeks, shoots developed slowly from the explants Figure 4. Therefore, after two cycles (4 weeks) on regeneration and selection medium (5 mg/L PPT), the explants were transferred to the regeneration medium without PPT selection Figure 4. This transfer was done so that the small shoots that developed from the explants might grow to test whether they were transformed with the EPSPS gene. Growth might have been slowed on PPT because the pat gene was not as highly expressed under the control of the *nos* promoter as the bar gene is under the control of the CaMV *35S* promoter in pBSF16. The EPSPS gene specific PCR analysis of developed shoots resulted in positive PCR results, as depicted in Figure 5.

Moreover, the positive western blot against mYFP protein (26 kDa) was observed in regenerated shoots after infection with the GV3101 strain harboring the pXCSG-EPSPS-mYFP (Figure 6). It confirms that the gene cassette the pXCSG-EPSPS- mYFP has been integrated into model plant and is suitable for further transformation into the desired crop. Simultaneously the +ve control tobacco plants infected with GV3101 harboring PBSF-16 having GUS reporter gene were run as positive control (Figure 6).

Comparison of real time expression of PAT of pXCSG-mYFP-EPSPS vector and BAR gene pBSF16 vector

It was observed from the *in vitro* results that the *pat* gene was not as highly expressed under the control of the *nos* promoter as the *bar* gene is under the control of the CaMV *35S* promoter in pBSF16. The real-time expression of the pat and bar gene was carried out to validate this speculation. The results showed 4.9-fold high expression of bar gene (pBSF16) when compared to the pat gene (pXCSG-mYFP-EPSPS of putative transgenic tobacco lines (Figure 7).

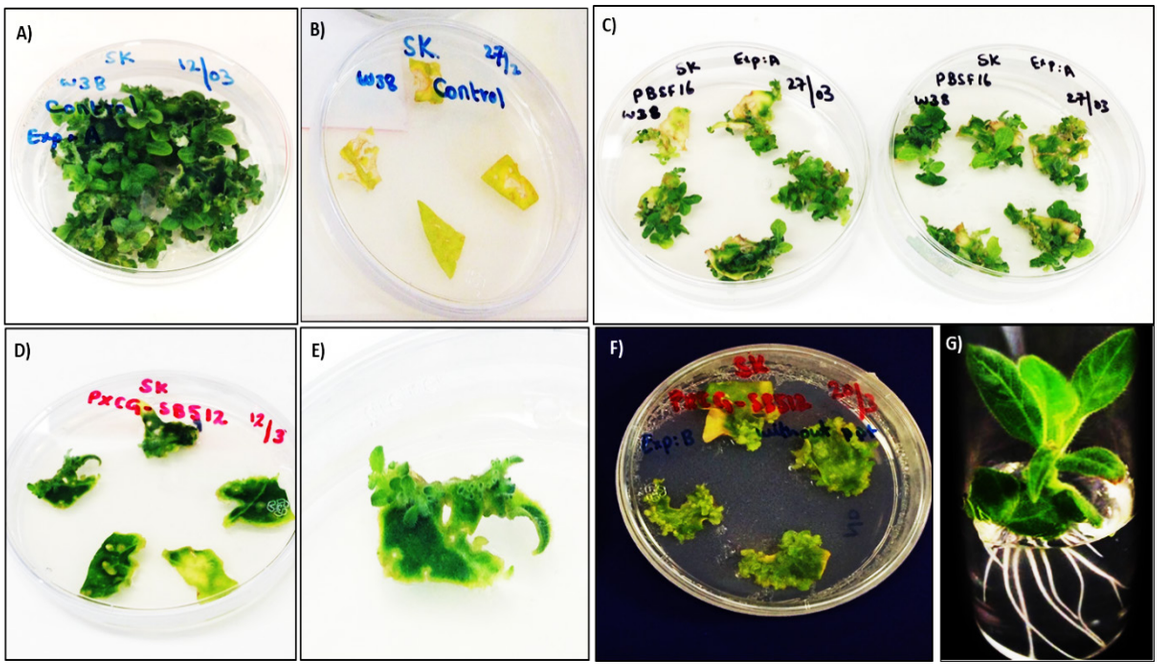

FIGURE 4. Control=1 Explants without selection; Control=2, Explants with selection; Development of transformed shoots (pBSF16); Development of transformed shoots (pXCSG-mYFP-EPSPS); Tobacco leaf explants transformed with pXCSG-mYFP without selection on regeneration media; Transformed shoots with roots

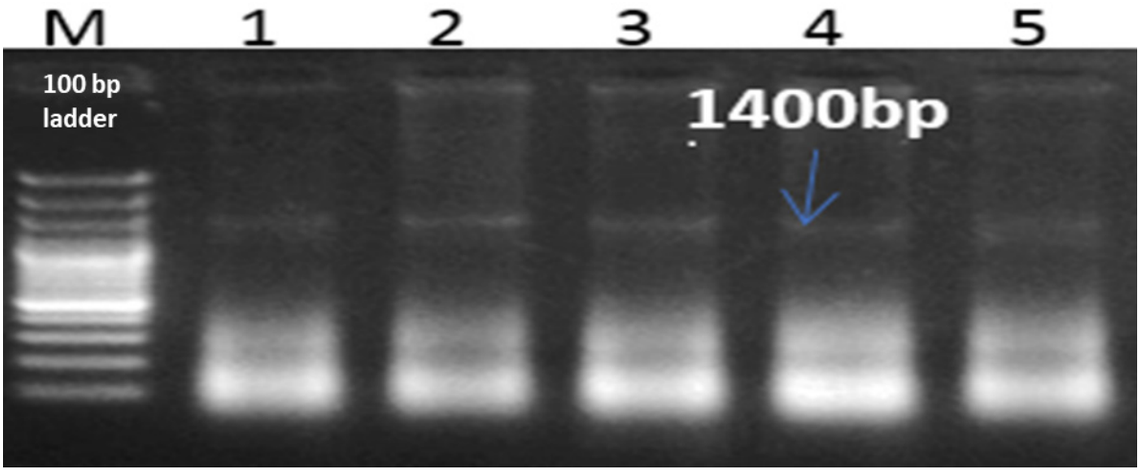

FIGURE 5. PCR analysis of putative transformed tobacco shoots M= 100bp Ladder, 1, 2, 3, 4, 5 = Transgenic shoots

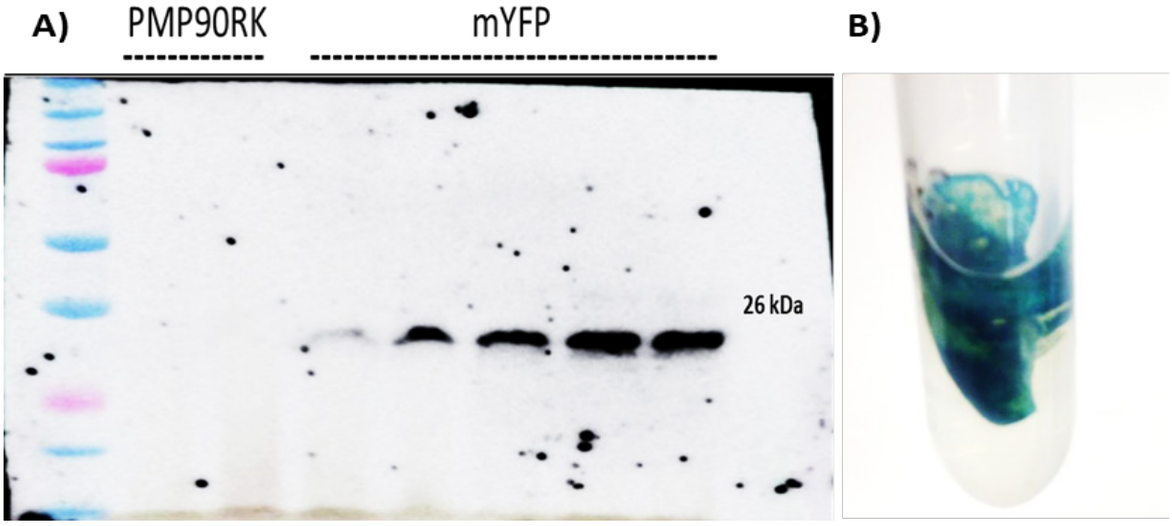

FIGURE 6. Western blot using anti-YFP antibodies (pXCSG-mYFP-EPSPS), M= Marker, PMP90RK = Negative control, mYFP= Positive samples

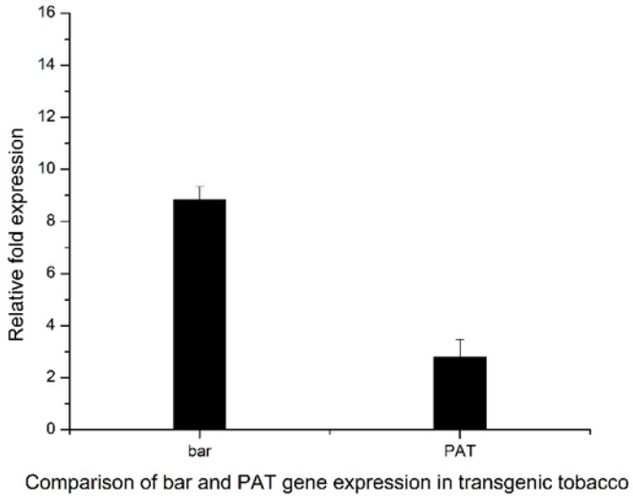

FIGURE 7. Real time expression of PAT of pXCSG-mYFP-EPSPS vector and BAR gene pBSF16 vector under two different promoters

DISCUSSION

One of the productive products of biotechnology, i.e., herbicide tolerance, has been initiated with a potential progressive role in the farming community (Webster & Sosnoskie 2010). Many scientific efforts have been reported to develop herbicide tolerance through, i.e., target gene overexpression, gene expression responsible for herbicide-detoxifying enzymes and changes in the target enzyme that may become nonfunctional in various crops (Fartyal et al. 2018). Tremendous success has

been achieved due to insensitive CP4-EPSPS because of their optimum catalytic activity and minute affinity for glyphosate (Quinn 1990). Codon optimization is a process used to improve gene expression and increase the translational efficiency of the EPSPS gene of the CP4 strain by accommodating the codon bias of the host organism. The current study is based on innovative findings for utilizing optimized codons of bacterial CP4-EPSPS. The synthetic codon optimization of bacterial origin CP4-EPSPS gene was performed with respect to the *Cicer arietinum* codon usage bias table. CP4 EPSPS gene was taken, and later on, the codon was optimized according to the chickpea codon usage table. Like our findings, Chou and Moyle (2014) also described the potential use of codon optimization of foreign gene sequences to improve transgene expression in other plants. Alteration in the target sequences of a desired transgene is crucial to imitate native coding sequences of the host plant used to elude prompting interaction of the host plant against foreign gene sequences. They also reported that codon usage modification increases mRNA translation. Recent schematic rules prevent known RNA destabilizing motifs and polyadenylation signals. Our results showed ΔG were calculated through WORKBENCH 8.0 software for successfully conversing optimized EPSPS gene into mRNA. In a similar pattern, Welch et al. (2009) also designed synthetic genes having high GC contents through redundant genetic codon usage to elevate the efficiency of protein synthesis and remove high AT sequence that harms mRNA stability.

Among other gene cloning methods, cloning of the sticky end is an effective and most frequently used method (Conze et al. 2009). To produce sticky ends in the cloning experiment, insert DNA and vector separately cut with the same restriction enzyme. Likewise, another method of DNA cloning, i.e., blunt-end, can generate the implanting of blunt-ended DNA or 5'-end-phosphorylated PCR product in linearized and blunt-ended vectors (Conze et al. 2009). The major drawbacks of blunt-end cloning are non-specific ligation and self-ligation in the vector. These factors make blunt-end cloning unsuitable for cloning and mutant libraries construction (Horvath et al. 2013). Through the TA cloning method, Taq DNA polymerase is required for non-template-dependent terminal transferase activity that can help to add a single deoxyadenosine (A) to the 3′ ends of PCR products. The resultant PCR product could be directly cloned into a linearized T-vector having single base 3'-T overhangs on each end (Osman et al. 2019). However, the limitation of TA cloning is the non-directional overhanging of T and A bases that can be flanked on each site of linearized vector. Invitrogen company has introduced an advanced method of cloning, i.e., Gateway® Technology, that can be used as a universal cloning strategy for site-specific recombination properties of bacteriophage lambda (Landy 1989). The unique properties of this technology can allow foreign gene transfer into various cloning vectors deprived of restriction enzymes and ligase uses (Reece & Walhout 2018). Gateway cloning has maintained the orientation and open reading frame of DNA insert due to their unique property of site-specific recombination (Pouyet et al. 2017). The required DNA insert enters the Gateway cloning system, so further, it can be shifted to other Gateway vectors, which offer cloning and sub-cloning of DNA inserts, functional characterization, and protein expression patterns. In this study, the pXCSG-mYFP-EPSPS vector was also constructed using the gateway cloning strategy, and PBSF-16 vector was acquired from CSIRO, Australia (Chitty, Allen & Larkin 2006). At first, the constructed vector pXCSG-mYFP-EPSPS was verified to determine whether it expressed the desired gene product. Similar vectors have already been used for cloning (Greenfield 2021). The vector validation was done by transformation experiments in a tobacco model plant with small genome size, and transformation can easily be achieved and validated.

The transgene's expression in the cell depends on the transgene construct design and transformation method used. The integration of transgenes into plant cells can be carried out either stably or transiently. As an expression of the transgene is dependent on construct design, it was a prerequisite to validating the newly designed vector pXCSG-mYFP-EPSPS. A transient transformation expressed the transgene transiently, and the transgene is not integrated into the plant genome. In the transiently transformed plant, the copy numbers of the transgene inserted remain as they are not replicated. These transgenes are expressed for a limited time, and the genes are lost after several days through cell division. The transient gene expression of pXCSG-mYFP-EPSPS was evaluated. *Nicotiana benth* infiltration showed positive western blotting results against mYFP antibodies, depicting that the vector is expressing the target region between the left and right border. A similar finding has been observed by Norkunas et al. (2018); they have reported different strategies to assess and optimize the increase in plant-made protein capacities in Nicotiana benthamiana using agro-infiltration.

In stable transformation, the process usually begins with the integration of transgenes into the nucleus of plant cells. Stable transformation is achieved when some transgenes integrate successfully into the cell genome. These transgenes then become a part of the genome and are replicated together, enabling the next generation to inherit and express the transgene. Likewise, for the stable integration of two vector systems were used pXCSG-mYFP-EPSPS and pBSF16 contained PAT and bar genes, respectively. The purpose of two different constructs was to evaluate the PAT and bar gene expression under two different promoters. Since the bar- and the pat gene products show an approximately 85% similarity in their amino acid sequence (Wohlleben et al. 1988). However, the bar gene was under *35S* promoter, whereas the pat gene was under *nos* promoter. The tobacco plants infected with pXCSG-mYFP-EPSPS showed slow growth on the first selection round (5 mg/L PPT); thus, the regenerated shoots were transferred to regeneration media without selection. The regenerated tobacco shoots gave a positive response to the EPSPS gene-specific PCR. It verifies and validates the vector pXCSG-mYFP-EPSPS working. However, we speculate that the difference in gene expression under two different promoters resulted in a low selectable marker gene expression level. pBSF16 express plant selectable marker gene under *35S* promoter was considerably high compared to the pXCSG-mYFP-EPSPS selectable marker gene under *nos* promoter. Wohlleben et al. (1988) findings were similar to our study as they reported that the plants containing the CaMV *35S* promoter had an average of 110-fold higher levels of NPTII enzyme activity than those containing the nopaline synthase promoter.

Leguminous crops are weak weed competitors because of their slow growth and modest stature. Our recently developed synthetically modified herbicide-tolerant vector can transform legume crops, primarily grown in underdeveloped nations, including chickpeas, lentils, and peanuts. However, overexpression in the target crop could be the limit as this is affected by many factors, including, i.e., host genome integration, gene position in the host genome, type of promoter used in gene cassette, and certain environmental conditions.

Conclusion

Our results demonstrate the efficient performance of the newly synthesized vector. The gene of interest has dual utility of being exploited for the genetic improvement of plant but also as an effective selectable marker selection for the recovery of transgenic plants. From this study, we have concluded the functional characterization of synthetically modified gene (codon optimized EPSPS gene for chickpea) in tobacco through molecular confirmation of transient gene expression. New innovative technology products are much capable enough to fulfill the food requirements of increasing world population are required to boost up crop production. Moreover, leguminous crops are poor competitor of weed during growth period because of slow growth and short stature. Our newly synthetically modified herbicide tolerance vector can produce legume crops such as chickpea, lentils, and groundnut which are mainly cultivated in developing country.

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