Substitution of Ala-183 to Thr in 5-enolpyruvylshikimste 3-phoshpate synthase of *E. coli* (k12) and Transformation of Rapeseed (*Brassica napus*) with altered gene to tolerance of plant to Glyphosate

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Abstract

Glyphosate is a non-selective broad-spectrum herbicide that inhibits 5-enolpyruvylshikimste 3-phoshpate synthase (EPSPS), a key enzyme in the aromatic amino acid biosynthesis in microorganisms and plants. The manipulation of bacterial EPSPS gene in order to reduce its affinity to glyphosate and transformation of it to plants is one of the most effective methods for production of glyphosate tolerant plants. In this research, we study on alanine183 of *E. coli* (k12) EPSPS enzyme. This amino acid is an important residue for EPSPS-Glyphosate configuration. We used site directed mutagenesis (SDM) method to inducing a point mutation in *E. coli* EPSPS gene to convert alanine183 to threonine (Ala183Thr). The manipulated EPSPS gene was cloned in pUC18 as a universal cloning vector and pBI121 as a plant expression vector. The molecular analysis and sequencing showed that the manipulated gene has been correctly changed and cloned in correct orientation in both plasmids. Recombinant pBI121 containing altered EPSPS gene was transferred to rapeseed (*B. napus*) via *Agrobacterium tumeifaciens* mediated transformation method. The transformed explants were screened in 25 mgL^{-1} kanamycin containing shoot induction medium. Glyphosate tolerance was assayed in putative transgenic plants. Statistical analysis of data showed that there is a significant different between transgenic and control plants. Presence and copy numbers of construct in transgenic plants were confirmed with PCR and southern blotting analysis respectively.

Key words: Rapeseed (*Brassica napus* L.), Glyphosate resistance, SDM, Manipulated EPSPS and Gene transformation

Introduction

Rapeseed is an important oil seed crop in the world (ANZFA, 1999). Effective weed control is a major problem in canola production. The presence of weeds in rapeseed plantation reduces crop yield quantity and quality (Kishore *et al*., 1992; Kuiper et al., 2000). In order to decreasing the effect of weed on rapeseed cultivation, the use of The non-selective, broad-spectrum and post-emergence herbicide glyphosate [N (phosphonomethyl) Gly] is the most popular and suitable way. So production of glyphosate tolerant rapeseed is very important (Kuiper *et al*., 2000; Holt *et al*., 1993).

The primary mode of action in planta for glyphosate which described is competitive inhibition of enzyme 5-enolpyruvylshikimste 3-phoshpate synthase (EPSPS; E.C.2.5.1.19), which catalyses the penultimate and main step of the prechorismate part of the plastid-localized shikimate pathway (Steinrucken and Amrhein, 1980). EPSPS converts shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to 5-enolpyruvylshikimste 3-phoshpate (EPSP) and inorganic phosphate. Of the several known PEP dependent enzymatic reactions, EPSPS is the only enzyme that interacts with PEP as an enzyme-substrate complex (E.S3P) and not as the free enzyme (Padgett et al., 1991). In plants, as much as 20% of all fixed carbon flows through the shikimate pathway leading to the formation of aromatic amino acids such as tyrosine, phenylalanine and tryptophan, as well as tetrahydrofolate, ubiquinon and vitamin K and E (Haslam, 1993; Franz et al., 1997; Sost et al., 1990; Gruys et al., 1999; Boockock et al., 1983).

Inhibition of EPSPS by glyphosate appears competitive with respect to PEP. Glyphosate forms a stable but non-covalent ternary complex with the enzyme and S3P (Ream *et al*., 1992; Marzabadi *et al*., 1996; McDowell *et al*., 1996). Shikimate pathway is unique in bacteria, some fungi and plants, and leads to the biosynthesis of aromatic amino acids and other aromatic compounds. The manipulation of bacterial EPSPS gene in order to reduce its affinity to glyphosate and transformation of this altered gene to plants is one of the most effective methods for production of glyphosate tolerant plants (Wang et al., 2003). Glyphosate resistance was first reported in transgenic tobacco expressing the Pro-101 to Ser substitution mutant of Salmonella typhimurium EPSPS (Comai *et al*., 1985; Stalker *et al*., 1985). Other report of a mutant EPSPS used to confer glyphosate resistance to transgenic petunia was Gly-96 to Ala substitution mutant of *Escherichia coli* enzyme (Kishore *et al*., 1986; Padgette *et al*., 1991). In the present study we substitued Ala-183 to Thr in *E. coli* (k12) EPSPS with SDM technique in order to investigate the effect of amino acid alteration on enzyme affinity to glyphosate in transgnic Rapeseed (*Brassica napus* L.).

Material and methods

Enzymes and chemicals

All chemicals, culture media, plant growth regulators and antibiotics were purchased from Merck (Germany) at the

highest purity available, unless stated otherwise. Reaction enzymes and other DNA-modifying enzymes were procured from Roche Biochemical and MBI, Fermentas. Blunt ends PCR cloning kit was from Roche Biochemical.

Bacterial strains, plasmids and plant materials

Escherchia coli DH5α (was used for cloning and mutation experiments) and *Agrobacterium tumefaciens* LBA4404 (was used for gene transformation to plants) that cultivated in LB medium at 37 °C and 28 °C respectively. Plasmids pUC18 (MBI, Fermentas) and pBI121 (Novagen) were used for routine cloning, sequencing and as a binary plant expression vector. *Brassica napus* L. cv. PF7045/91 was used as the experimental plant materials.

Recombinant DNA technologies

Unless stated otherwise, standard DNA technologies were used (Sambrook *et al*., 2001). Oligonucleotide synthesis and DNA sequencing reactions were carried out at MWG-Biotech (Ebensburg, Germany).

EPSPS gene amplification and cloning

Isolation of genomic DNA from *E. coli* (K12) was done according to Sambrook *et al*. 2001. EPSPS gene was isolated using specific primers P1 forward 5′-CGGGATCC ATGGAATCCCTGACGTT ACAA-3′ and P2 reverse 5′-GCGGATCCTCAGGC TGCCTGGCTAATC-3′ with *Bam*HI site at 5′ end of each primer (underlined). Restriction enzyme analysis was carried out using *Bgl*II, *Hinc*II and *Taq*I. The authentic PCR product was cloned in pUC18 plasmid and sequenced in both directions with standard primers by dideoxy chain termination method.

Site-directed mutagenesis

To induce mutation (Ala188Thr), the following oligonucleotide pairs were used: P3 (forwards): 5′ -GTTATTGACTCGGCCTCTTACCCCGGAAGAT-3′ and P4 (reverse) 5′-ATCTTCCGGGGTA AGCGCAGTCATTAAC-3′, where underline indicate the replaced nucleotides. For amplification of intermediate fragments, tow separate PCR reactions were performed in 50µl total volume containing 30-50ng of sequenced EPSPS gene as a template DNA, for fragment A (500bp), P1 and P4 primers and for fragment B (800bp), P2 and P3 primers were used, (see fig.1).

Site-directed mutagenesis was generated with performing PCR. Amplified fragments were purified by agarose gel electrophoresis and using DNA purification kit (Roche diagnostic). Each of the purified fragments was attached to make mutated gene. The attachment was done by primer extension based on PCR methods.

Cloning and sequencing the gene

Mutated genes were cloned in *Bam*HI site of the vector pUC18. The recombinant plasmids were analyzed by agarose gel electrophoresis to see the jumption of heavier plasmids that contain the insert and further confirmed by restriction enzyme digestion and PCR. The plasmids with desired inserts were sequenced by dideoxy chain termination methods and standard primers. Gene sequences were compared with other sequences stored in gene bank and aligned by using the Blast to confirm due mutations.

Mutated and cloned genes in pU18 with correct direction, and pBI121 plant binary vector were digested in *Xba*I and *SacI* sites. Then mutated genes were cloned in pBI121 (minus Gus) in above sites. This vector was transferred to *Agrobacterium tumefaciens* LBA4404 in a standard method (Sambrook and Russel).

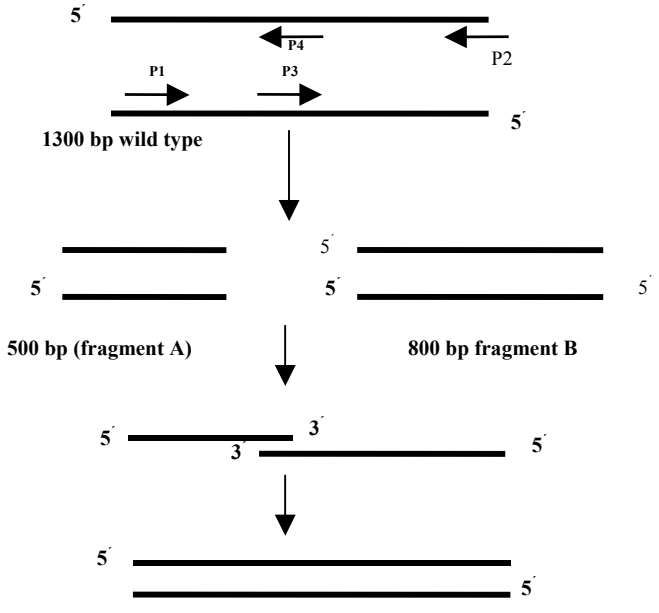

Figure 1. Schematic of site-directed mutagenesis method for inducing a point mutation in the 1300 bp fragment.

Plant tissue culture procedures

Seeds of *B. napus* cv. PF-7045-91 were surface sterilized in 1.5% sodium hypochlorite for 10 min. The seeds were washed in sterile distilled water 3 times and planted on germination medium (MS minimal organics medium with 3% sucrose, agar 0.8% and pH 5.8). Seeds were germinated at 25 ^{o}C in a 16 h light/8 h dark photoperiod. After 5 days the cotyledons were excised in such a way that they included 2mm petiole at the base. Care was taken to eliminate the apical meristem, which sometimes adheres to petioles. The excised cotyledons were placed on shoot induction medium (MS medium with 3% sucrose enriched with 20 μM benzyladenine, agar 0.8% and pH 5.8). The petioles were embedded in this solid medium. The explants were maintained on shoot induction medium under light and temperature conditions specified above for 2-3 weeks. During this time many shoots appeared on over half the explants. The shoots were counted and transferred to elongation (the same as previous medium minus the benzyladenine) and root induction (MS medium with 2% sucrose, 2 mg/l indole butric acid, pH 8) media. After root formation the plantlets were transferred to potting mix supplemented with liquid fertilizer. The plants were grown in a missing chamber (average relative humidity 80%) for 2-3 weeks at 25 ^{o}C, 16 h light/8 h dark photoperiod, and intensity 60-80 $\mu Em^{-2}s^{-1}$. Under these conditions plants established rapidly. After 3 weeks plants were to the greenhouses and allowed to flower and set seed.

Transformation procedure

Single colonies of *Agrobacterium tumefaciens* strain LBA4404 containing a modified binary plasmid pBI121 were grown overnight at 28 ^{o}C in LB medium supplemented with 50 mgl^{-1} kanamycin. This vector carries the mutated EPSPS gene under CaMV 35S promoter activity. The bacterial suspension was pelleted by centrifugation for 10 min at 2700 rpm then resuspended in MS medium containing 3% sucrose and pH 5.8. Individual excised cotyledons were taken from plates described above and the cut surface of their petioles was immersed into this bacterial suspension for a few seconds. They were immediately returned to same MS plates from which they had been taken. The cotyledons were co-cultivated with the *Agrobacterium* for 72 h.

After co-cultivation, the cotyledons were transferred to shoot induction medium plus 25 mg/l kanamycin and 200 mg/l cefotaxim. Again the petioles were carefully embedded in the agar. Another medium (elongation and root formation) are the same as previous that described plus above antibioses.

Bioassay with glyphosate challenging under greenhouse conditions

Rooted putative transgenic plantlets propagated from 50 selected transgenic rapeseed were transferred to soil in a greenhouse and tested for glyphosate tolerance by sparing them with Roundup at 5 doses of glyphosate (1, 2.5, 5, 7.5 and 10 mM). The control plants were non-transgenic plants. One week after the first application, the second glyphosate treatment was done on survived plants. For statistical analysis, t-test ($p < 0.01$) was used for comparison of the control and transgenic plants.

PCR and digestion analysis for integrated constructs

Genomic DNA was prepared from young leaves of green putative transgenic and control (non-transgenic) plants by the cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thampson, 1980). Integration of desired gene to plant genome was confirmed by PCR for CaMV 35S Promoter/EPSPS and EPSPS/Nos terminator regions. Two pair specific primers were designed, first forward from a CaMV 35S region (35SF: 5′-GGCGAACAGTTCATACAGAGTCT-3′) and reverse from a mutated EPSPS region (ER: 5′-TCGCGTTGCGGCGTTACCGAGGA-3′) that amplify an 800 bp fragment. Second forward from a transferred EPSPS region (P3: previous described) and reverse from a Nos terminator region (NR: 5′-CGCGCGATAATTTATCCTAGT-3′) that amplifies a 1030 bp fragment. Each of above PCR products was digested in *Bam*HI site for more confirmation. Digestion of 1st PCR product produces 300bp and 500bp fragments and 2nd PCR product produces 1030bp and 200bp fragments.

Southern blot analysis

For Southern blot analysis, total genomic DNA was isolated from leaves of T_0 glyphosate resistant and non-transformed control plants. Genomic DNA (15 μg) was digested with *Hind*III and separated on 0.7% (w/v) agarose gels, then transformed to nylon membranes. Prehybridization and hybridization performed using a standard method (Sambrook and Russell, 2001). A partial fragment from CaMV 35S and EPSPS that amplify an 800 bp fragment was subjected to DIG DNA labeling (Roche Applied Science Gmbh, Germany) and use as probe in hybridization experiments.

RT-PCR analysis

For confirming of mutated EPSPS gene transcription, RT-PCR was performed according to the manufacturer's instructions (MBI Fermentas). Total RNA was isolated from leaves of glyphosate resistant and control rapeseed plants using a RNA isolation kit and DNase treatment. First strand cDNA was generated using the bacterial EPSPS specific primer (5′-GCGGATCCTCAGGCTGCCTG GCTAATC-3′). PCR amplification of 1300 bp fragment of above gene was achieved using the first strand reaction with primers 5′-CGGGATCCATGGAATCCCTGACGTTACAA-3′ and 5′-GCGGATC CTCAGGCTGCCTGGCTAATC-3′ under the following conditions: 30 cycles of 94 ^{o}C for 1 min, 63 ^{o}C for 1 min and 72 ^{o}C for 1min, and a final extension at 72 ^{o}C for 10 min.

Results

Total genomic DNA was isolated from *E. coli* and EPSPS gene was cloned using specific primers P1 and P2. The 1300

bp fragment was subjected to restriction enzyme analysis using *Bgl* II, *Hinc* II and *Taq* I enzymes (data not shown). The gene was cloned and verified by sequencing. Site-directed mutagenesis was carried out using designed primers (P3 and P4). Tow intermediate fragments were obtained, fragment A (500 bp) with P1 and P4 primers, and fragment B (800 bp) with P2 and P3 primers (Fig 1, 2-a and 2-b). The intermediate fragments (A+B) were attached to each other to make EPSPS gene caring single mutation (Ala183Thr) by two-step PCR and primer extension method (Fig 1 and 2-c). The mutated gene was cloned in pUC18 plasmid in *Bam*HI site. The positive clones were confirmed by jumption, PCR and restriction enzymes (*Bam*HI) and further sequenced (Figure 3). Correct orientation for cloning in expression vector was enzymatically tested. By the results of sequencing and comparing with other sequences in the gene bank, it was verified that mutations were inserted.

For cloning in pBI121 plant binary vector, pUC18 (caring the mutated and cloned genes in correct orientation), and pBI121 vectors were digested in *Xba*I and *SacI* sites. This digestion causes elimination of Gus gene in pBI121. Then mutated genes (with 2 ends for *Xba*I and *SacI* sites) were cloned in pBI121 (minus Gus) in these sites (fig 4). The pBI121 vector that caring the mutated and cloned genes in correct orientation in front of CaMV 35S promoter, was mobilized from *Escherchia coli* DH5α to *Agrobacterium tumefaciens* LBA4404 (Hoekea *et al*., 1983) in freeze-thaw standard method (Höfgen and Willmitzer, 1988).

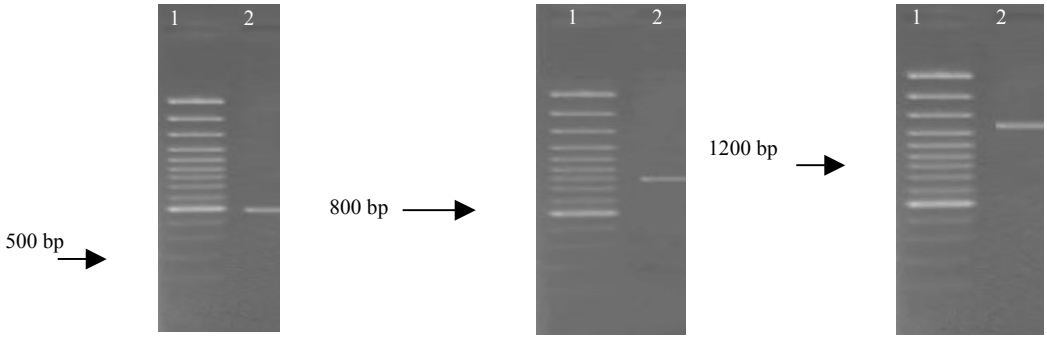

Figure 2. Amplification for 2 intermediate fragments and Construction of mutated EPSPS gene.

2-a: Amplification of 500 bp fragment.
2-b: Amplification of 800 bp fragment.
2-c: Combination and amplification of fragment 500 bp and 800 bp for Construction of mutated EPSPS gene (1300 bp fragment)

In all figures lane 1 is 100 bp ladder.

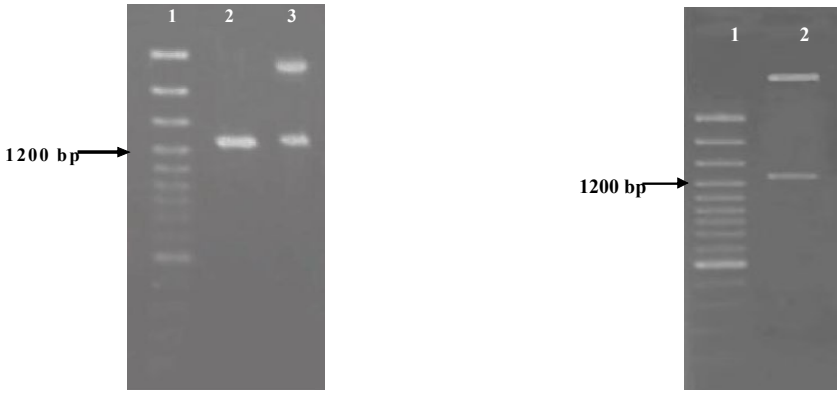

Figure 3. Confirmation of cloning by checking PCR and digestion.

Lane 1: 100 bp ladder.
Lane 2: Amplification of cloned EPSPS gene by specific primers.
Lane 3: Analysis of cloned gene by restriction enzyme (*Bam*HI) digestion.

Regeneration capacity of target tissue

The target cells for these experiments were those at the cut surfaces of cotyledonery petioles. In Figure 5 the development of shoots from these explants is shown. This shoot development is in 2-3 weeks. Regeneration frequency for this explants without selection are approximately 74%. On selection using 25 mgl^{-1} kanamycin, no green shoots obtained with explants, which derived from control (were not treated with *Agrobacterium*) plants. Transformation efficiency in treated petioles was about 28%. An important aspect of this procedure is that multiple shoot often from a single explant (Fig 5-a). The regenerated shoots were transferred to shoot elongation and root induction media. After acclimatization of rooted plantlets in *in vivo* conditions, they transferred, allowed to flower and set seed.

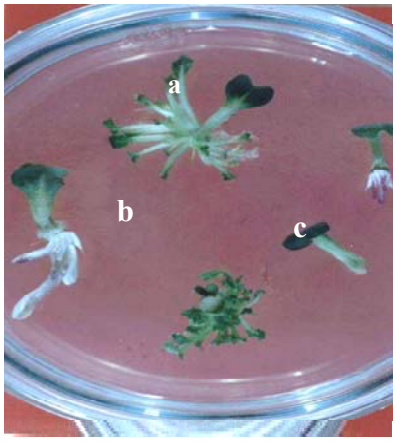

Figure 5. Development of shoots from explants.

a: Regeneration of multiple green shoot from a single explant.
b: Regeneration of a white shoot from a single explant.
c: Unregenerated explant.

Figure 6. Comparison of the glyphosate tolerance an untransformed plant (a), with transgenic plant containing mutated EPSPS gene (b).

Selection and assay for glyphosate tolerance

Data statistical analysis of treated plants with glyphosate showed that there were significant differences ($p < 0.01$) between putative transgenic plantlets and control (non-transgenic plants). About 51% of kanamycin-resistance plants survived in the presence of glyphosate up to 10 mM. Untransformed control plants were very sensitive to glyphosate and could not survive on medium containing 1 mM glyphosate and were completely killed 5 days after the first sparing (Fig 6).

The transgenic plants survived 95, 93, 72, 64, and 51% in 1, 2.5, 5, 7.5 and 10 mM respectively.

PCR and digestion analysis for integrated constructs

Prepared genomic DNA of putative transgenic and control (non-transgenic) plants was analyzed by PCR for CaMV 35S promoter/EPSPS and EPSPS/Nos terminator regions with two pair specific primers (35SF/ER and P3/NR respectively). In putative transgenic plants, 35SF/ER and P3/NR primers amplify an 800 bp and 1030 bp fragments respectively. In control plants no fragments amplify with above primers (Fig 7). Above two PCR products from putative transgenic plants were analyzed with *Bam*HI digestion. 800 bp fragment was divided two pieces (500 and 300 bp) and 1030 bp fragment was digested two pieces (800 and 230 bp) (fig 8).

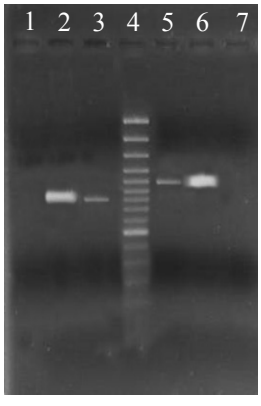

Figure 7. PCR analysis for integrated constructs.

Lane 1: PCR for non-transgenic plant with 35SF/ER primers (negative control). Lane 2: PCR product for recombinant pBI121 plasmid containing desirable gene with 35SF/ER primers (positive control). Lane 3: PCR product for transgenic plant with 35SF/ER primers. Lane 4: 100bp ladder. Lane 5: PCR product for transgenic plant with P3/NR primers. Lane 6: PCR product for recombinant pBI121 plasmid containing desirable gene with P3/NR primers (positive control). Lane 7: PCR for non-transgenic plant with P3/NR primers (negative control).

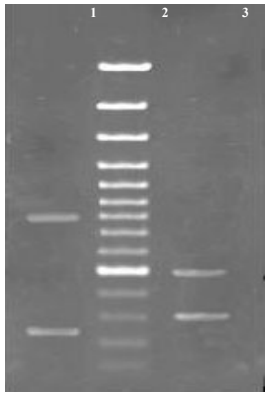

Figure 8: Digestion analysis (with *Bam*HI) for integrated constructs.

Lane 1: *Bam*HI Digestion of the 1030 bp fragment was digested two pieces (800 and 230 bp).
Lane 2: 100bp ladder.
Lane 3: *Bam*HI Digestion of the 800 bp fragment was divided two pieces (500 and 300 bp).

Southern blot analysis of transformants

The southern blot shown in fig 9 includes of the putative transgenic and control plants. This genomic blot of transformed and untransformed plants provides evidence of insertion of the T-DNA in each putative transformant. It also provides evidence of a range of copy number insertions into the genomes of these plants. Insertions appear to be between one to three copies. Separate digestions of putative transgenic and control genomic DNA were attempted using *Hind* III (data not shown), which cuts only once between the T-DNA borders of pBI121.

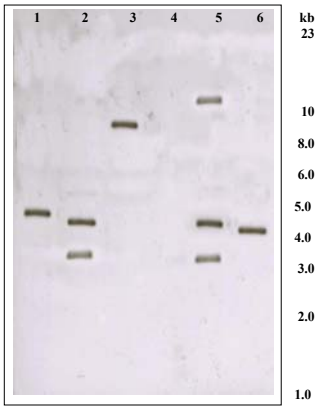

Fig 9

Figure 9: Southern blot analysis of *Hind* III- digested DNA isolated from T0 glyphosate-tolerant plants. Lane 4 is untransformed control plant and others lane is transformed plants.

Discussion

The last fifty years have witnessed a massive transition in medicine and agriculture toward almost total dependence on toxic chemicals designed to control unwanted organisms (Malik *et al*., 1989). In the farming, chemicals have special roles and among these chemicals, herbicides and specially glyphosate are very important. Glyphosate is a post-emergence, nonselective herbicide used in weed control programs around the world since its commercialization in 1974. Despite its widespread and long-term use, weeds have evolved limited resistance to glyphosate (Malik *et al*., 1989; Baerson et al., 2002).

The shikimate pathway, which occurs in plants and microorganisms, couples with the specificity of glyphosate as an inhibitor of EPSPS, and contributes in large part to glyphosate's lack of toxicity to animals (Baerson *et al*., 2002). For the first time, Padgette and his coworkers reported the isolation of an *E. coli* B variant, containing a highly glyphosate-tolerant EPSPS. The further analysis on glyphosate- tolerant EPSPS revealed that the altered affinity for glyphosate was the result of a single amino acid substitution of alanine for glycine at residue 96.

Glyphosate-tolerant *Salmonella typhimurium* strain has been reported wherein the tolerance to glyphosate results from a single amino acid substitution of serine for glycine at position 101(Stalker, 1985; Comai, 1985).

Alignment of the amino acid sequences of EPSPS from different prokaryotes and plants shows that these two amino acids are in a highly conserved region, but not located in the active site of the enzyme.

Among conserved amino acids of this region, alanine at residue 183 is a importation residue for EPSPS-glyphosate interaction. Therefore substitution for this amino acid can decrease the affinity of glyphosate for the enzyme. Due to the knowledge, which exists about the interaction between glyphosate and its target enzyme, many of scientists try to make resistant plants to glyphosate.

There are mainly several distinct strategies for engineering herbicide resistance. Using naturally or artificially mutated protein is the efficient method. The logic behind this approach is to find a modified target protein that substitute for the native protein and which is resistant to inhibition by the herbicide (Stalker, 1985). Here we used the SDM technique, which is related to this strategy.

Eichholtz and his coworkers (2001) have described the modified gene encoding glyphosate-tolerant 5-enolpyruvyl-3-phosphoshikimate synthase. By using M13 mutagenesis method they introduced mutations (Gly96Ala and Ala183Thr) in 660 bp fragment and replaced the wild type segment with it. Studies have shown that these changes increased tolerance to glyphosate herbicide and also exhibited lower Km values for phosphoenolpyruvate than other variant EPSPS enzymes.

In comparison to previous reports, the site-directed mutagenesis method is more simple, reliable and reproducible. In this technique, two fragments are joined together by overlap extension to produce mutated genes.

Studies have also shown that it is possible to reduce the affinity of enzyme for glyphosate by introducing changes in other amino acids (Baerson, 2002).

Identification of the active sites of EPSPS has been largely studied. The data indicate that modification in Lys22 and Lys340 results in inactivation of the enzyme (Padegette, 1988). Thus, in spite of the importance of these residues in enzyme

activity, it is impossible to change them. But in this research two residues in a conserved motif of EPSPS gene were chosen to change which although cause decrease in the affinity of glyphosate for enzyme, it doesn't make any changes in enzyme activity.

The cut surfaces of cotyledonery petioles were the target cells for these experiments. Result showed that this target is a vigorous source of new shoot material and shoot development is very rapid. The origin of these shoots has been shown by Sharma (1987) to be cells around the cut end of petioles. This cut surface is an ideal target for *Agrobacterium*-mediated transformation as the cells undergoing organogenesis are those most readily accessed by the Sharma (1987) that the explant must include most or all of the medium results.

The value of *Agrobacterium*-mediated plant transformation is measured by the number of independent transformed plants expressing the gene of interest per explant used. This can be a function of genotype of species to be transformed, the strain (virulence) of *Agrobacterium*, the selectable marker, regeneration capacity of the target cells and the accessibility of the bacterium to the regenerable cells. An additional less-frequently quantified variable is the amount of labour required to maintain cultures until transformed shoots are obtained.

We examined the expression of mutant *E. coli* EPSPS gene in transgenic rapeseed. Furthermore, we used the CaMV 35S promoter to ensure high expression levels in all tissues.

Genome analysis (PCR and digestion) confirmed integration of desired gene to rapeseed genome. Southern analysis showed that most of the transgenic plants carried one copy of the bacterial EPSPS gene and only a few carried two or three copies. This is in agreement with the results of Wang *et al.,* 2003. The level of gene expression appeared to be correlated to the number of integration (Wang *et al*., 2003). There was no apparent relationship between copy number and the level of the resistance to glyphosate. Similar multiple inserts with brassicaceae may be a feature intrinsic and it may be dependent on the selection schemes and the levels or type of antibiotics used (Moloney *et al*., 1989).

Acknowledgments

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