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Substitution of Ala183Thr in *aro*A Product of *E. coli* (k12) and Transformation of Rapeseed (*Brassica napus*) with Altered Gene Confers Tolerance to Roundup

Danial Kahrizi[1,2*] • **Ali Hatef Salmanian**[3]

[1] Department of Agronomy and Plant Breeding, Faculty of Agriculture, P.Code 6715685438, Razi University, Kermanshah, Iran
[2] Biotechnology for Drought Resistance Dept., P.Code 6715685438, Razi University Kermanshah, Iran
[3] National Institute for Genetic Engineering and Biotechnology, P.O. Box 14155-6343, Tehran, Iran

Corresponding author: * **dkahrizi@yahoo.com**

ABSTRACT

Roundup is a non-selective broad-spectrum herbicide that inhibits the *aro*A gene product or 5-enolpyruvylshikimste 3-phosphate synthase (EPSPS), a key enzyme in the aromatic amino acid biosynthesis in microorganisms and plants. The manipulation of the bacterial *aro*A gene in order to reduce its affinity to Roundup and its transformation into plants is one of the most effective methods for production of Roundup-tolerant plants. In this research, we studied alanine183 of *E. coli* (k12) EPSPS enzyme. This amino acid is an important residue for EPSPS-Roundup binding. We used site-directed mutagenesis (SDM) to induce a point mutation in *E. coli aro*A gene in order to convert alanine183 to threonine (Ala183Thr). The manipulated *aro*A gene was cloned in pUC18 as a universal cloning vector and pBI121 as a plant expression vector. Molecular analysis and sequencing showed that the manipulated gene has been correctly changed and cloned in the correct orientation in both plasmids. Recombinant pBI121 containing an altered *aro* A gene was transferred to rapeseed (*B. napus*) via *Agrobacterium tumefaciens*-mediated transformation. Roundup tolerance was assayed in putative transgenic plants. Statistical analysis of Roundup challenging data showed that there is a significant difference between transgenic and control plants. The survival frequencies of transgenic plants in 1, 2.5, 5, 7.5 and 10 mM Roundup were 29, 22, 14, 5, and 0%, respectively whereas the non-transformed control plants were unable to survive even 1 mM Roundup. The presence and copy numbers of the construct in transgenic plants were confirmed by PCR and southern blot analysis, respectively.

Keywords: gene transformation, manipulated *aro*A, Roundup resistance, SDM

INTRODUCTION

Rapeseed or canola (*Brassica napus* L.) 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 decrease the effect of weeds on rapeseed cultivation, the use of the non-selective, broad-spectrum and post-emergence herbicide Roundup® or glyphosate [N (phosphonomethyl) Gly] is the most popular and suitable way. So production of Roundup-tolerant rapeseed is very important (Holt *et al.* 1993; Kuiper *et al.* 2000).

The primary mode of action *in planta* for Roundup is competitive inhibition of *aro* A gene product or EPSPS (enzyme 5-enolpyruvylshikimste 3-phosphate synthase) (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-phosphate (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 (Boocock *et al.* 1983; Sost *et al.* 1990; Haslam 1993; Franz *et al.* 1997; Gruys *et al.* 1999; Kahrizi *et al*. 2004; Kahrizi 2005).

Inhibition of EPSPS by Roundup appears competitive with respect to PEP. Roundup 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). The shikimate pathway is unique in bacteria, some fungi and plants, and leads to the biosynthesis of aromatic amino acids and other aromatic compounds. Sometimes it has been found spontaneous mutations in *aro*A gene in bacteria that induced Roundup-tolerant trait (Schulz *et al*. 1984). With inspired to this, the manipulation of the bacterial *aro*A gene in order to reduce its affinity to Roundup and transformation of this altered gene to plants is one of the most effective methods for production of Roundup-tolerant plants (Wang *et al.* 2003; Kahrizi *et al.* 2006). Roundup resistance was first reported in transgenic tobacco expressing the Pro-101 to Ser substitution mutant of *Salmonella typhimurium aro*A (Comai *et al.* 1985; Stalker *et al.* 1985). Anther report of a mutation in the *aro*A of *E. coli* conferring Roundup resistance in transgenic petunia involved the Gly96 to Ala substitution (Kishore *et al.* 1986; Padgette *et al.* 1991).

Site-directed mutagenesis (SDM) in coding sequence of enzymes is a powerful technique for investigation active structure-function relationships (Wagner and Benkovic 1990) and has also been used to verify the roles of active site residues (Padgette *et al*. 1991). Furthermore, SDM promises to provide valuable information about the distribution of fitness effects of mutants (Taylor *et al*. 1996). Procedures for site-specific mutagenesis have become increasingly important for the analysis of gene function, and many approaches are now available for changing primary DNA se-

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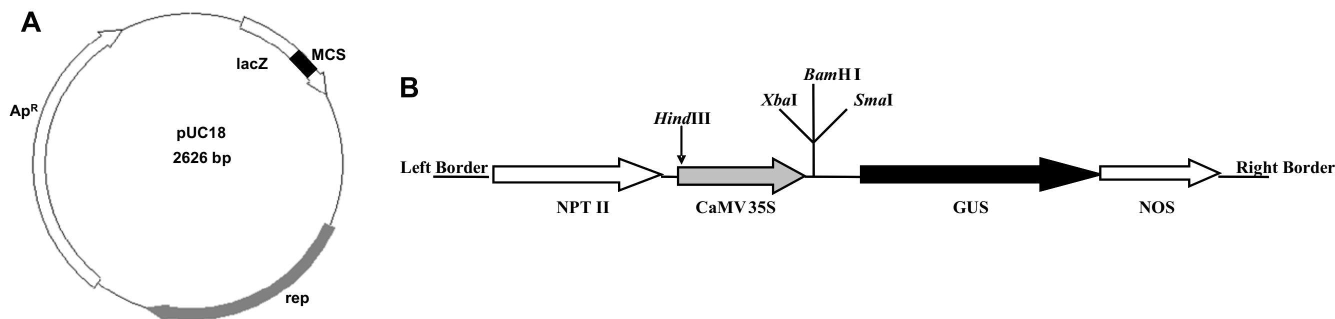

Fig. 1 Schematic representation of pUC18 plasmid (A), as a cloning vector and T-DNA region of pBI121 expression vector (B).

quences (Kunkel 1985). In the present study described the use of *in vitro* site-directed mutagenesis in *aro*A gene from *E. coli* (k12) in order to investigate the effect of amino acid alteration on enzyme affinity to Roundup. The amino acid changes were alanine to threonine at 183 residue to investigate the effect of amino acid alteration on enzyme affinity to Roundup in transgenic rapeseed.

MATERIALS 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 obtained from Roche Biochemical and MBI, Fermentas. Blunt ends PCR cloning kit was from Roche Biochemical. Roundup was supplied from Razor® Pro Herbicide 4467.

Bacterial strains, plasmids and plant materials

Escherichia coli DH5α (used for cloning and mutation experiments) and *Agrobacterium tumefaciens* LBA4404 (used for genetic transformation of plants) were cultivated in LB medium at 37 and 28°C, respectively. Plasmids pUC18 (MBI, Fermentas, **Fig. 1A**) and pBI121 (Novagen, **Fig. 1B**) 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 material. This universal cultivar that is an open pollination (OP) genotype prepared from Oilseed Research Department, Seed and Plant Improvement Institute (SPII), Karaj, Iran.

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 (Ebersberg, Germany).

*aro*A gene amplification and cloning

Isolation of genomic DNA from *E. coli* (K12) was carried out according to Sambrook *et al.* 2001. *aro*A gene was isolated using specific primers P1 forward 5′-CGGGATCC ATGGAATCCCTGA CGTTACAA-3′ and P2 reverse 5′-GCGGATCCTCAGGC TGCC TGGCTAATC-3′ with a *Bam*HI site at the 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 into pUC18 plasmid and sequenced in both directions with standard primers by dideoxy chain termination method.

Site-directed mutagenesis

To induce mutation (Ala188Thr), the SDM technique as described previously (Kahrizi *et al*. 2007) was used. For this purpose the following oligonucleotide pairs were used: P3 (forwards): 5′-GTTAT TGACTCGGCCTCTTACCCCGGAAGAT-3′ and P4 (reverse) 5′-ATCTTCCGGGGTAAGCGCAGTCATTAAC-3′, where underlined bases indicate the replaced nucleotides. For amplification of intermediate fragments, two separate PCR reactions were performed in 50 µl total volume containing 30-50 ng of sequenced *aro*A gene as template DNA, for fragment A (500 bp), P1 and P4 primers and for fragment B (800 bp), P2 and P3 primers 3 mM of Mg^{2+}, 200 µM of each dNTPs and 1.25 units of *pfu* DNA polymerase (MBI, Fermentas) (**Fig. 2**).

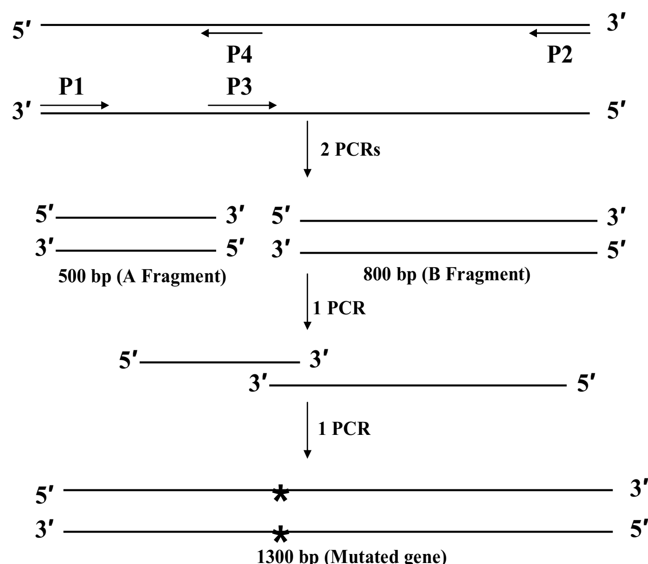

Fig. 2 Schematic representation of PCR-based SDM in order to introduce the mutation Ala183Thr into the *aro*A gene from *E. coli.*

Fragments amplified by internal primers were purified by agarose gel electrophoresis and using a DNA purification kit (Roche Diagnostic). Each pair of purified fragments was attached to each other in order to assemble the mutated gene. The attachment was carried out by the PCR based primer extension.

Cloning and sequencing of the mutated gene

Mutated genes were cloned into the *Bam*HI site of the vector pUC18. The recombinant plasmids were analyzed by agarose gel electrophoresis to see the displacement of heavier plasmids that contain the insert and further confirmed by restriction enzyme digestion and PCR. The plasmids with the desired inserts were sequenced by dideoxy chain termination methods (Sanger *et al.* 1977) and M13 standard primers. The mutated *aro*A gene sequence were compared with other sequence stored in gene bank (accession No. X00557) and aligned by using the Blast (www. ncbi.nlm.nih.gov) to confirm the fidelity of mutation as well as the absence of any undesired mutation.

The pBI121 plant binary vector was digested with *Xba*I and *Sac*I. Then the mutated genes were successfully cloned into this vector. The correct orientation for cloning into the expression vector (minus *gus*) was enzymatically tested. If there is the start codon near promoter and the stop codon near terminator gene, it will call

that the gene orientation is correct. This vector was transferred to *A. tumefaciens* LBA4404 in a standard method (Sambrook and Russel 2001).

Plant transformation and selection procedures

Seeds of *B. napus* 'PF-7045-91' were surface sterilized according to Moloney *et al*. (1989). Single colonies of *A. tumefaciens* harboring the modified binary vector were grown overnight at 28°C in LB medium supplemented with 50 mgl^{-1} kanamycin.

Plant transformation was carried out as described by Moloney *et al*. (1989). After root formation on selection medium, the plantlets were transferred to potting mix supplemented with liquid fertilizer. The plants were grown in a misting chamber (80% relative humidity) for 2-3 weeks at 25°C, with a 16-h photoperiod and light intensity of 60-80 $\mu Em^{-2}s^{-1}$. After 3 weeks, plants were transferred to the greenhouse and allowed to flower and set seed.

Bioassay with Roundup challenging under greenhouse conditions

Putative transgenic plantlets were clonally propagated (via auxillary bud culture) for Roundup treatment. In this experiment we used 10 independent transgenic rapeseed lines. 18 clones from each line were used for six Roundup doses in three replications. Then we used 180 transgenic plantlets for the Roundup challenging experiment (i.e. three clones form each line per dose). Then the statistic population was 10 lines × 3 clones × 6 doses = 180 samples. These plantlets were transferred to soil under greenhouse conditions and tested for Roundup tolerance by spraying them with the 5 doses (0, 1, 2.5, 5, 7.5 and 10 mM) of herbicide Roundup (active ingredient isopropylamine salt of Glyphosate, 41%). Spraying was done on leaves and applied herbicide in same volumes for each leaf area unit until witting the all area leaves of treated plants. Non-transformed plants were used as control. One week after the first application, the second round of Roundup spraying was carried out on surviving plants. For statistical analysis, the Student's *t*-test ($p < 0.01$) was used for comparison of Roundup resistance in the control and transgenic plants.

PCR screening of transformants

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 the desired gene into the plant genome was confirmed by PCR for CaMV 35S promoter/*aro*A and *aro*A /Nos terminator regions. Two pairs of specific primers were designed, first forward from the CaMV 35S region (35SF: 5′-GGCGAACA GTTCATACAGAGTCT-3′) and reverse from a mutated *aro*A region (ER: 5′-TCGCGTTGCGGCGTTACCGAGGA-3′) that amplify an 800 bp fragment; second forward from a transferred *aro*A region (P3: see above) and a reverse primer from the nopaline synthase (Nos) terminator region (NR: 5′-CGCGCGATAATTTAT CCTAGT-3′) that amplifies a 1030 bp fragment. Each of the above PCR products was digested with *Bam*HI for further confirmation. Digestion of the first PCR product produced 300 and 500 bp fragments and the second PCR product produced 1030 and 200 bp fragments.

Southern blot analysis

For Southern blot analysis, total genomic DNA was isolated from leaves of T_0 Roundup 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 transferred to nylon membranes. Prehybridization and hybridization were performed using a standard method (Sambrook and Russell 2001). A partial fragment (800 bp in size) was obtained from PCR amplification of the *aro*A gene using primers from the CaMV 35S promoter and the *aro*A gene and subjected to DIG DNA labeling (Roche Applied Science Gmbh, Germany) and used as a probe in hybridization experiments. It is worthy of mention that we analysed six independent transgenic lines in three separate southern blot experiments (in each experiment we tested only two transgenic lines and a control plant) but the results of one experiment have been shown.

RT-PCR analysis

To confirm the transcription of the mutated *aro*A gene, RT-PCR was performed according to the manufacturer's instructions (MBI Fermentas). Total RNA was extracted from leaves of Roundup resistant and control rapeseed plants using an RNA isolation kit (MBI, Fermentas). First strand cDNA was generated using the *E. coli aro*A-specific primer (5′-GCGGATCCTCAGGCTGCCTGGC TAATC-3′). PCR amplification of the 1300 bp fragment of the above gene was achieved by using the first strand synthesis as template with primers 5′-CGGGATCCATGGAATCCCTGACGTT ACAA-3′ and 5′-GCGGATCCTCAGGCTGCCTGGCTAATC-3′ under the following conditions: 30 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min.

RESULTS

The *aro*A gene of *E. coli* was amplified using specific primers P1 and P2. The 1300 bp fragment was subjected to restriction enzyme analysis using *Bgl*II, *Hinc*II and *Taq*I (data not shown). The gene was cloned and verified by sequencing. SDM was carried out using designed primers (P3 and P4). Two intermediate fragments were obtained, fragment A (500 bp) with P1 and P4 primers, and fragment B (800 bp) with P2 and P3 primers (**Figs. 2A, 2B**, **3**). The intermediate fragments (A+B) were attached to each other to ensure that the *aro*A gene carries a single mutation (Ala183Thr) by two-step PCR and primer extension method (**Figs. 2C, 3**). The mutated genes were cloned into the *Bam*HI site of the pUC18 plasmid. The positive clones were confirmed by PCR, restriction enzyme (*Bam*HI) analysis and sequencing (**Fig. 4**). The correct orientation for cloning into the expression vector was enzymatically tested. The sequencing data, including sequence with other sequence (accession No. X00557) in gene bank (www.ncbi.nlm.nih.gov)

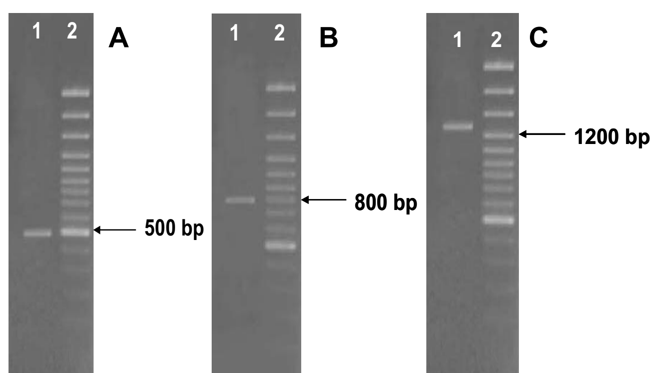

Fig. 3 Amplification of intermediate fragments in PCR-based SDM (Lanes 1 in all panels). Panels **A**, **B** and **C** show fragments that span 500 bp (A fragment), 800 bp (B fragment) and 1300 bp (C fragment), respectively. A MW marker (100 bp ladder) is shown in lane 2 of all panels.

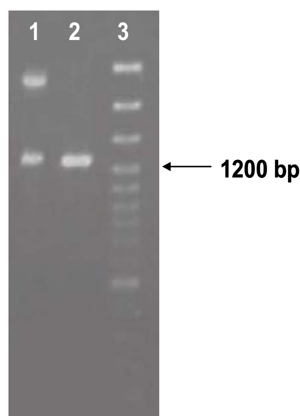

Fig. 4 Analysis of the pUC18 derivative carrying the mutated *aro*A gene. The PCR-amplified insert of the plasmid is shown in lane 2 and the *Bam*HI restricted plasmid DNA is shown in lane 1. A MW marker (100 bp ladder) is shown in lane 3.

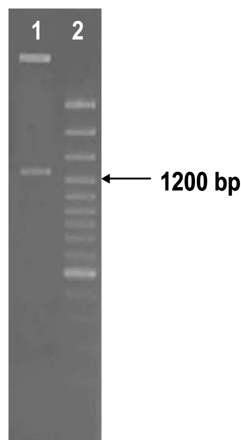

Fig. 5 Confirmation of cloning of 1300 bp gene in pBI121 (minus GUS). Analysis of cloned gene by restriction enzyme (*Bam*HI) digestion. The 1300 bp and heavier fragments are desired gene and pBI121 (minus Gus) plasmid respectively (Lane 1). A MW marker (100 bp ladder) is shown in lane 2.

verified the insertion of the desired mutations into the *E. coli aro*A gene.

The mutated genes were recovered and successfully cloned into the binary vector pBI121 (**Fig. 5**). *Agrobacterium*-mediated transfer was used to transform cotyledonary petioles of *B. napus.* Multiple shoots developed within 2-3 weeks from single explants. in regeneration medium (described by Moloney *et al*. 1989). Regeneration frequency was approximately 74% for explant growth in the absence of kanamycin. Regeneration was not observed for the non-transformed control plants, but the transgenic plants displayed a regeneration frequency of 28% in medium containing 25 mgL^{-1} kanamycin. The regenerated shoots were transferred to shoot elongation and root induction media (described by Moloney *et al*. 1989). After acclimatization of rooted plantlets to *in vivo* conditions, they were allowed to flower and set seed. The 50 independent transgenic plants were regenerated and established but used only 10 of them for Roundup challenging.

Selection and assay for Roundup tolerance

Statistical analysis of the data obtained by treating plants with Roundup showed that there were significant differences ($p < 0.01$) between putative transgenic lines and non-transgenic control plants. A number of kanamycin-resistant plants survived 2-times spraying of Roundup. Non-transformed control plants were very sensitive and died 5 days after the first spraying with even 1 mM Roundup (**Fig. 6**).

The survival frequencies of transgenic plants in 0, 1, 2.5, 5, 7.5 and 10 mM Roundup were 100, 29, 22, 14, 5, and 0%, respectively.

Stable integration of the transgene

Genomic DNA of putative transgenic and non-transgenic (control) plants were analysed by PCR for the presence of CaMV 35S promoter/*aro*A and *aro*A/Nos terminator regions, using two pairs of specific primers (35SF/ER and P3/NR, respectively). PCR amplification with primer combinations 35SF/ER and P3/NR yielded fragments of 800 and 1030 bp, respectively using DNA of transgenic plants as template. However, no amplification was observed in the control plants, with the above primers (**Fig. 7**).

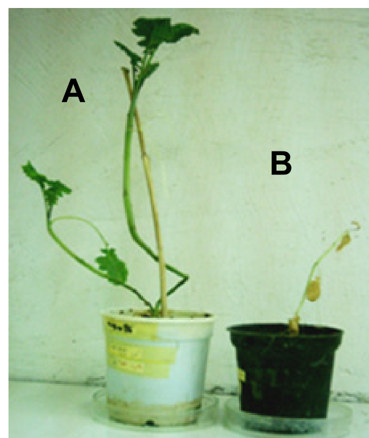

Fig. 6 Comparison of Roundup tolerance of transgenic plants (A) and untransformed control plants (B) at concentration of 7.5 mM Roundup after 15 days of application.

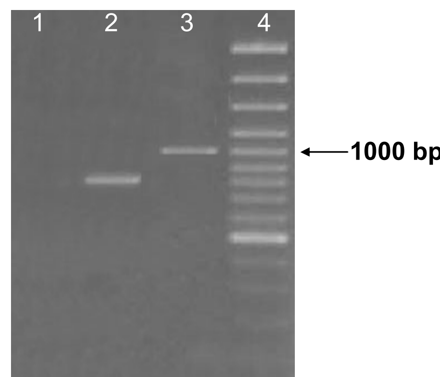

Fig. 7 PCR analysis of putative transformants. PCR amplification using non-transgenic plant (negative control) as template DNA with 35SF/ER primers (lane 1); PCR amplification using transgenic plant as template DNA with 35SF/ER primers (lane 2); PCR amplification using transgenic plant as template DNA with P3/NR primers (lane 3) and a MW marker (100 bp ladder) (lane 4).

The above two PCR products from putative transgenic plants were further analysed by restriction enzyme digestion. *Bam*HI digestion of the 800 and 1030 bp fragments resulted in the formation of 500, 300 bp and 800, 230 bp fragments, respectively.

Southern blot analysis of transformants

Six transgenic lines and one non-transgenic line were analysed by southern blotting in three experiments. We have shown results of only one experiment with two transgenic lines and one control plant, as results of the three experiments were the same in copy number insertion and Roundup tolerance. All transgenic lines carried a single copy of the mutated bacterial *aro*A (**Fig. 8**). *Hind*III digested DNA of plants was hybridized with an 800 bp-long probe that consisted of the CaMV35S promoter and *aro*A gene sequences. As there is a *Hind*III site in the recombinant T-DNA construct, the number of hybridization bands indicated the number of integration copies. The results of southern blotting analysis showed that the transformed plants had a single gene insertion.

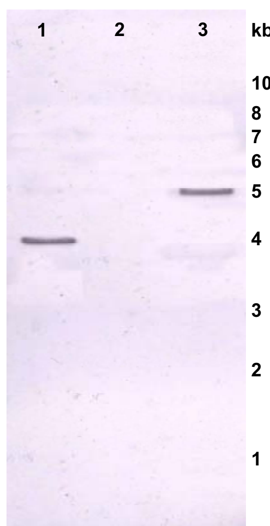

Fig. 8 Southern blot analysis of *Hind*III- digested DNA isolated from T_0 Round up-tolerant plants. Lane 2 is untransformed control plant and other lanes are transformed plants. Lanes 1 and 3: lines 58-1 and 58-2, respectively.

DISCUSSION

The last 50 years have witnessed a major shift with in medicine and agriculture towards almost total dependence on toxic chemicals designed to control unwanted organisms (Malik *et al.* 1989). Roundup 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 Roundup (Malik *et al.* 1989; Baerson *et al.* 2002).

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

Roundup-tolerant *Salmonella typhimurium* strain has been reported wherein the tolerance to Roundup 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 (Comai 1983; Stalker 1985; Padgette *et al*. 1991).

Among conserved amino acids of this region, alanine at residue 183 is an importation residue for EPSPS-Roundup interaction. Therefore substitution for this amino acid can decrease the affinity of Roundup for the enzyme. Based on this knowledge on the interaction between Roundup and its target enzyme, many scientists have tried to make plants resistant to Roundup (Eichholtz*et al*. 2001).

There are several distinct strategies for engineering herbicide resistance. The most efficient method uses gene(s) encoding naturally or artificially mutated protein. The logic behind this approach is to find a modified target protein that acts as a substitute for the native protein and is resistant to inhibition by the herbicide (Stalker 1985).

Eichholtz and coworkers (2001) described the modified gene encoding Roundup-tolerant EPSPS. By using M13 mutagenesis they introduced mutations (Gly96Ala and Ala183Thr) into a 660 bp fragment and replaced the wild type segment with it. These changes increased tolerance to Roundup and also exhibited lower Km values for phosphoenolpyruvate than other variant EPSPS enzymes.

In comparison to previous reports, SDM is simpler, more reliable and reproducible. In this technique, two fragments are joined together by overlap extension to produce mutated genes (Wagner and Benkovic 1990).

Studies have also shown that it is possible to reduce the affinity of the enzyme to Roundup by introducing changes in other amino acids e.g. Lys22 and Lys340 (Huynh *et al.* 1987) And Cys 408 (Padgette *et al*. 1988; Baerson 2002).

Identification of the active sites of EPSPS has been largely studied. Modification in Lys22 and Lys340 results in inactivation of the enzyme (Padegette 1988). Thus, due to the importance of these residues in enzyme activity, it is impossible to change them.

The cut surfaces of cotyledonary petioles were the target cells for these experiments. This 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 transformation efficiency in this study was about 28% (28 independent transformed plants that express the transgene from 100 explants. 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. Transformation efficiency in current research (28%) is higher than Radke *et al*. (1992) and Jun *et al*. (1995) that resulted 9 and 4%, respectively. However, in comparison with Moloney *et al.* (1989), who obtained 55%, our value was lower.

The survival frequencies of transgenic plants in 1, 2.5, 5, 7.5 and 10 mM Roundup were 29, 22, 14, 5, and 0%, respectively. The previous report that used simultaneously point mutation (Gly96 to Ala and Ala183 to Thr), this frequencies were 95, 93, 72, 64, and 51%, respectively for above concentrations (Kahrizi *et al*. 2007). These results showed that above double mutation is more efficient for roundup resistance than single. Furthermore simultaneously point mutation (Gly96 to Ala and Ala183 to Thr) has not any negative effects on EPSPS structure and function.

We examined the expression of a mutant *E. coli aro*A 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 the desired gene into the rapeseed genome. Southern blot analysis of six transgenic lines provided additional evidence for T-DNA integration (have been shown results of only two transgenic lines and one control plant) (**Fig. 8**). Furthermore, it was shown that the tested lines carried a single copy of the mutated bacterial *aro*A gene. It is desirable to have single gene insertion in plants as multiple copies of T-DNA influence the expression of the introduced gene (Stam *et al.* 1997). Moreover, multiple copies of the transferred gene are often associated with a reduction of transgene activity, affected by gene position or suppressed gene expression (Bhalla and Smith 1998). Our finding (i.e. the single copy insertion into the plant genome) is in agreement with the results of Bhalla and Smith 1998 that they used LBA444 *Agrobacterium* strain else in *Brassica olercea* var. *botrytis*, but is in disagreement with those of Moloney *et al.* (1989) who used EHA101 *Agrobacterium* strain, reported multiple copy insertion into the rapeseed cv. 'Westar' genome.

CONCLUSIONS

Site-directed mutagenesis (SDM) in coding sequence of enzymes is a powerful technique for investigation active structure-function relationships. The substitution of Ala183Thr in *aro*A product of *E. coli* (k12) and transformation of rapeseed (*Brassica napus*) with altered gene is effective for tolerance of plant to Roundup. Based on previous results the simultaneous substitution of Gly96Ala and Ala183Thr is more effective for roundup tolerance. All transgenic lines carried a single copy of transgene. The position effects of transgenes are active and suitable since transgenic plants express them.

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