Progress in Biological Sciences
Vol. 2, No. 1, 50-58, Winter/Spring 2012

Glyphosate Tolerance in Transgenic Canola by a Modified Glyphosate Oxidoreductase (*gox*) Gene

Faranak Hadi[1,2], Amir Mousavi[*], Ali Hatef Salmanian[*], Kambiz Akbari Noghabi, Khosro Khajeh[3]

[1]*Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.*
[2]*Department of Biology, Faculty of Science, Lorestan University, Lorestan, Iran*
[3]*Department of Biochemistry, Tarbiat Modares University*

Received: 9 February 2012; Revised: 1 March 2012; Accepted: 10 March 2012

ABSTRACT

The engineering of transgenic canola (*Brassica napus* L.) to make tolerance to the broad-spectrum herbicide, glyphosate**,** is one of the most effective approaches for weed management. Glyphosate inhibits the enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) enzyme which functions in the shikimate pathway and has a key role in biosynthesis of aromatic amino acids required for survival of the plant. Induction of glyphosate tolerance in transgenic canola via introducing mutated *epsps* to the plant genome has been previously reported. By this strategy, enzyme's affinity for glyphosate is reduced. Applying glyphosate degrading enzyme of bacterial origin such as glyphosate oxidoreductase (GOX) in combination with a glyphosate-tolerant *epsps* is the ultimate approach to provide commercial rates of glyphosate tolerance. In this project, a synthetic geneencoding GOX enzyme with plant codon preferences was designed. The structure of the synthetic construct and its mRNA were analyzed by bioinformatic tools. This synthetic gene was subcloned and transformed into canola plant via *Agrobacterium* mediated transformation in order to investigate the potential roles in increasing glyphosate tolerance. The presence, copy numbers and expression of the transgene were confirmed by PCR, Southern blotting and RT-PCR analyses, respectively. The bioassay with glyphosate challenging showed that the transgenic plant tolerated glyphosate at a concentration of 1.5 mM whereas the non-transformed canola was unable to survive in the presence 0.5 mM glyphosate.

Keywords: Canola; Gene optimization; Glyphosate oxidoreductase; Glyphosate tolerance; Synthetic gene.

Introduction

For cultivation and production of canola as a slowly growing plant, weeds are considered as serious restriction factors (Kishore *et al.,* 1992; Kuiper *et al.,* 2000). Therefore, application of a broad-spectrum, non-selective herbicide, such as glyphosate (GP; N-phosphonomethyl glycine), that blocks plant growth by inhibiting EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), is one of the most effective approaches for weed elimination. This enzyme functions in the shikimate pathway and has a key role in biosynthesis of aromatic amino acids (Barry *et al.,* 1992; Padgette *et al.,* 1991). In spite of application of this herbicide for over three decades, the evolution of GP resistant (GR) weeds populations in non-GR crop has been very limited (Duke and Powles, 2009). Since this herbicide also affects rapeseeds performance, the engineering of GP-tolerant crops would result to higher yield and enhanced quality of the extracted oils.

*Corresponding authors: m-amir@nigeb.ac.ir; salman@nigeb.ac.ir
Tel.: +98 21- 44580365; fax: +98 21- 44580395

Furthermore, almost 90% of all transgenic crops grown worldwide has the GR trait and the adoption ofthese crops is increasing at a steady pace (Duke and Powles, 2008) lead to decrease of the cost brought following the expiration of the patent on the molecule itself in 2000 (Pollegioni *et al.,* 2011). Currently GR crops include soybean, maize, canola, cotton, sugar beet and alfalfa (James, 2011). Up to present time, 29 countries have approved cultivation of genetically modified crops and thirty more states have approved import of their products for food and feed applications. The strategy has been already common in existing commercial GP tolerance crop is the expression of mutated form of *epsps* or wild type *epsps (Cp4 epsps*) that is not inhibited by GP. One of the disadvantages of this strategy is that GP may remain and accumulate in plant tissues and may decrease crop yield by interfering with reproductive organs development. Hence, the removal of herbicidal residue may lead to more robust tolerance and allow spraying during period of reproductive development (Pline *et al.,* 2002; Pollegioni *et al.,* 2011). The glyphosate oxidoreductase (*gox*) gene has been isolated from *Ochrobactrum antrophi* strain LBAA; It encodes an enzyme which can degrade GP converting it to glyoxlate and aminomethylphosphonic acid (AMPA). This gene has been used to transform canola and maize in combination with *Cp4 epsps* to enhance GP tolerance in commercial events (Barry and Kishore, 1995), but the GOX enzyme has not been expressed alone in plant and the resistance to GP made by this enzyme was not evaluated. In this study, we optimized the codon of *gox* gene for optimal expression in engineered canola plant. Furthermore, in order to improve the efficiency of the transcription and translation steps, appropriate regulatory elements were added to *gox* sequence (Gustafsson *et al.,* 2004; Lim *et al.,* 2004; Mechold *et al.,* 2005). This gene was subsequently used to transform canola in order to establish the effect of the optimized synthetic *gox* (*synth-gox*) by itself on GP tolerance in transgenic lines.

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. Restriction enzymes and other DNA-modifying enzymes were obtained from Roche Biochemical and Fermentas (Germany). GP was supplied from Sigma (Germany).

Bacterial strains, plasmids and plant materials

E. coli DH5α and *Agrobacterium tumefaciens* LBA4404 bacteria were used for cloning and plant transformation experiments, respectively. pUC57 was used for routine cloning and sequencing and plant binary expression plasmid pBI121 (Clontech) was used for plant transformation procedure. *B. napus* L. cultivar R-line Hyola 308 seeds were obtained from the Seed and Plant Improvement Research Institute of Iran and used for genetic transformation.

In silico analysis of *synth-gox* gene

The sequence of the *gox* gene (accession number, GU214711.1) with the size of 1296 bp was subjected to codon gene optimization using Upgene (http://www. vectorcore. pitt. edu/upgene/upgene. html) and DNA20 (http://www. dnatwopointo. com/ commerce/misc/opt. jsp) softwares. The messenger RNA secondary structure of the synthetic gene was analyzed using the Mfold program (http://www. bio info. rpi.

edu/applications/mfold). The optimized *gox* gene was synthesized by Shine Gene Molecular Biotech, Inc. (Shanghai, China) and the final synthetic sequence (*synth-gox*) was submitted to the Genbank (accession number, HQ110097.1).

Plant transformation and selection

The *Synth-gox* gene was recovered as *Bam*HI/*Sac*I fragment from pUC57 and subcloned into the same sites of pBI121. By this strategy the *synth-gox* gene becomes placed under the control of cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (nos) terminator. This plasmid (pBI-synth-gox) contains the *nptII* gene encoding neomycin phosphotransferase II, which allows the selection of plant cell resistant to kanamycin. The authentic recombinant plasmids were selected by PCR and restriction enzymes analyses. The pBI-synth-gox was introduced into *A. tumefaciens* LBA4404 cells by the freeze-thaw transformation procedure (Sambrook *et al.,* 2001). Recombinant bacteria were confirmed by PCR analysis and were used for plants transformation. Seeds of canola were surface sterilized with 20% V/V commercial sodium hypochlorite and 0.1% Tween 20 as a surfactant for 5 min followed by five washes with sterile distilled water and germinated in solidified MS medium (Murashige and Skoog, 1962) containing 3% sucrose, pH 5.8. Plant transformation was carried out as described by Kahrizi *et al.* (2007) with some minor modifications. Cotyledonary explants were excised from 4-days old seedlings and used for transformation. *Agrobacterium* harboring pBI-synth-gox was grown overnight to an OD_{600} of 0.8 in liquid LB medium containing 100 mg/l rifampicin and 50 mg/l kanamycin. The culture was centrifuged and the pellet was suspended in MS medium (pH 5.2) with 0.05 mM acetosyringone as a *vir* gene inducer. Excised cotyledons were immersed in recombinant *A. tumefaciens* for 10 min, dried on filter paper and transferred to solidified (0.80% agar) MS medium (Moravec *et al.,* 2007) containing 4 mg/l 6-benzylaminopurine and 3% sucrose. After co-cultivation for 2 days at 25 °C in darkness, the infected cotyledonarys were transferred to regeneration medium containing MS supplemented with 200 mg/l cefotaxime and 20 mg/l kanamycin. The selection was continued for 2 weeks at 22 °C under a 16/8 h (light/dark) photoperiod. After regeneration, the kanamycin-resistant shoots were selected and transferred to root induction media (MS salt plus 15 g/l sucrose, 0.2 mg/l IBA and 200 mg/l cefotaxime). The rooted explants were transferred to soil and grown under greenhouse conditions (16/8 h light/dark at 20 °C) (Kahrizi *et al.,* 2007).

PCR and Southern blot analysis

Plant genomic DNA was extracted from non-transgenic and transgenic canola tissues using the cetyl-trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). The concentration of genomic DNA was measured at 260 nm in a UV spectrophotometer. The presence of the desired gene in transgenic plants was confirmed by PCR analysis using the *synth-gox* gene specific primers 5′-GGATCCACC ACCATGTCG-3′and 5′-AGCTCTCAGGA GGCAGGAC-3′resulting in amplification of a 1314 bp fragment. The amplification program consisted of initial denaturing at 94 °C for 5 min, followed by 35 cycles including 94 °C for 1 min, annealing at 59 °C for 1 min and extension at 72 °C for 1 min and a final extension step of 72 °C for 10 min. The PCR products were subjected to electrophoresis on a 1% agarose gel.

For Southern blot analysis, 40 µg of the genomic DNA was digested with *Bam*HI restriction enzyme, separated on a 1% agarose gel and then transferred onto

Hybond-N membrane (Roche, Germany) following standard procedures (Sambrook *et al.,* 2001). A DIG-labeled probe (1314 bp) was prepared by PCR DIG Labeling Mix (Roche, Germany) using *synth-gox* gene as template and specific *synth-gox* primers (above). Hybridization and immunological detection were performed with the DIG DNA labeling and detection kit (Roche, Germany).

RNA extraction and transcript analysis

Total RNA was extracted from leaves of transformed plants using Plant Total RNA Kit (Roche, Germany) and its quality and quantity were analyzed by agarose gel electrophoresis and spectrophotometer, respectively. Two microgram of isolated total RNA per sample was used to synthesize the first-strand cDNA using MMLV Reverse Transcriptase (Promega, USA) with oligo-dT primer. The cDNA was used as a template for RT-PCR to determine the presence of *synth-gox* mRNA in the transformed plants according to the instructions of manufacturer (Roche, Germany). Amplified tubulin-mRNA was used as an internal control in RT-PCR analysis.

Bioassay for GP resistance

Transformed lines were grown under controlled greenhouse conditions until the seed formation stage. The seeds were tested for GP resistance. The resistant T1 seedlings were examined on the MS media in the presence of two concentrations of GP (0.1 and 0.2 mM).

For *in vivo* assay, transgenic plantlets were propagated clonally using auxiliary buds replication. In this procedure, 12 independent transgenic canola lines were used. Seedlings from each line were used for evaluation of GP resistance with at least three independent replications. These plantlets were transferred to soil under greenhouse conditions and tested for GP resistance by spraying with four doses (0.5, 1.0, 1.5 mM) of Roundup (active ingredient, isopropylamine salt of GP, 41%). Non-transformed plants were used as negative control. The plants were evaluated for resistance to glyphosate one week after the second spraying.

Results

In-silico analysis of the wild type and optimized synthetic *gox* gene

The sequence of *gox* gene was designed according to the codon bias of canola (*Brasica napus* L.). To optimize the gene sequence, negatively *cis* acting motifs, the undesired splice sites, polyadenylation signal, instability elements and repeated sequences were avoided (Table 1). The Kozak sequence (Kozak, 1989) was added before the start codon in order to increase high and accurate expression of mRNA in a eukaryotic host. Furthermore, the suitable restriction enzyme sites (*Bam*HI and *Sac*I) were introduced at the 5′and 3′ends of the sequence, respectively for cloning purpose. Comparison between wild type and the modified sequence of gene demonstrated that the overall GC content was decreased from 56.46 to 53.64 which is expected to increase the overall stability of mRNA from the synthetic gene. Moreover, the optimized gene did not include rarely used plant codons as assessed by the codon adaptation index (CAI) developed by Sharp and Li (1987). This index is a measure of the synonymous codon usage bias for a DNA or RNA sequence and quantifies codon usage similarities. The value was 0.76 for optimized sequence compared to the wild type gene being 0.70 (Graf *et al.,* 2004).

Prediction of mRNA Secondary structure

A genetic algorithm-based RNA secondary structure prediction was combined with comparative sequence analysis to determine the potential folding of the synthetic gene. The minimum free energy for secondary structures formed by mRNA molecules was also predicted as -431.83 kcal. Among all twenty predicted structures, one structure of the beginning of mRNA is shown in Figure1. The data showed that the mRNA was stable enough for efficient translation in the new host (Zuker, 2003).

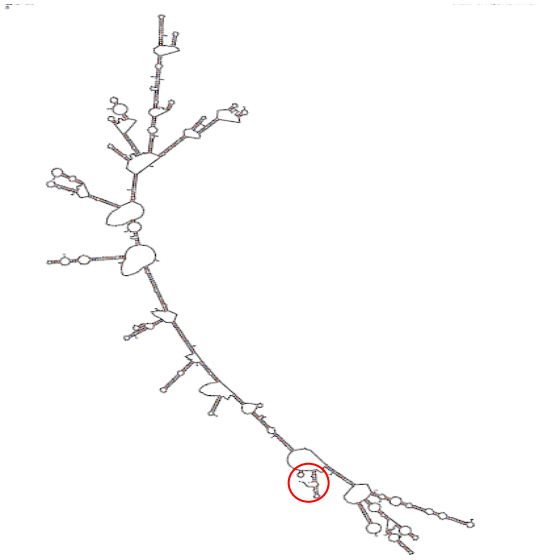

Figure 1. mRNA secondary structure prediction of codon optimized *synth-gox* gene. The initial region of the mRNA sequence is shown by a circle which is accessible for translation machinery.

Integration of the transgene in plant genome

Genomic DNA of putative transgenic and non-transgenic (control) plants were analyzed by PCR using transgene-specific primers to detect integration of the *synth-gox* gene. No amplification was observed in the control plants (Fig. 2).

Expression analysis of *synth-gox* gene in transgenic plant

Leaves of putative transgenic plants lines were characterized by RT-PCR using *synth-gox* gene-specific primers as demonstrated in Figure 3A. Analysis showed *synth-gox*-specific mRNAs of the expected size of approximately 1300 bp. Standard primers for amplification of tubulin house keeping gene were used as a control for mRNA purity. Due to the presence of a 200 bp within the intron in tubulin sequence, the expected size for amplified gene and cDNA products of tubulin are different (Fig. 3B).

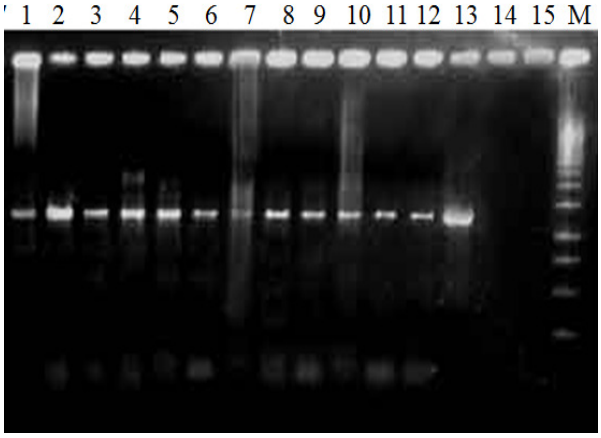

Figure 2. PCR amplification of the *synth-gox* gene in transgenic plants with the specific primers. Lanes. 1-12, transformed plant lines; 13, positive control (pBI-synth-gox); 14, non-transformed plant (Negative control); 15, ddH_2O and M, 1 kb Ladder (Fermentas). Expected amplicon was 1314 bp.

Southern blot analysis of transformed plant

Twelve transgenic lines and one non-transgenic line were analyzed by Southern blotting. Most of the transgenic plants were shown to carry one copy of transgene and only a few carried two or three copies (Fig. 4). *Bam*HI digested DNA of plants was hybridized with a 1314 bp long probe consisting of *synth-gox* gene sequences. As there is a *Bam*HI site in the recombinant T-DNA construct, the number of hybridization bands gives an indication of the number of integration copies.

Table 1. Comparison between restriction enzymes and *cis*-acting elements of optimized and original *gox* sequence.

	Optimized	Original
Splice site:		
GGATCC (*Bam*HI)	1	1
GAGCTC (*Sac*I)	1	2
Repeat Sequences:		
Max Direct Repeat:	Size, 10; Distance, 255: Frequency, 2	Size, 10; Distance, 886; Frequency, 2
Max Inverted Repeat	None	Size, 10; Tm, 43. 8; Start Positions, 1034, 1210

Bioassay with GP challenging

Treating plants with GP showed that there were significant differences between transgenic lines and non-transgenic control plants. At dose of 0.1 mM herbicide, both transformed and non-transformed plantlets survived but the latter plantlets were bleached after 21 days in 0.2 mM GP compared to transformed plant (Fig. 5).

Herbicide resistance under greenhouse conditions was also assayed too. Rooted plantlets propagated from selected transgenic canola lines were transferred to soil in the greenhouse and tested for GP resistance by spraying with Roundup at concentrations of 0.5, 1 and 1.5 mM. One week after the first application, no damage appeared in any transgenic plants containing *synth-gox*. After spraying a second dose with 0.5 and 1 mM GP, all *synth-gox*-transgenic plants survived and produced new leaves. The 1.5 mM concentration of GP caused bleaching of transgenic leaves. Non-transformed control plants were very sensitive and died seven days after the first spraying with even 0.5 mM GP (Fig. 6).

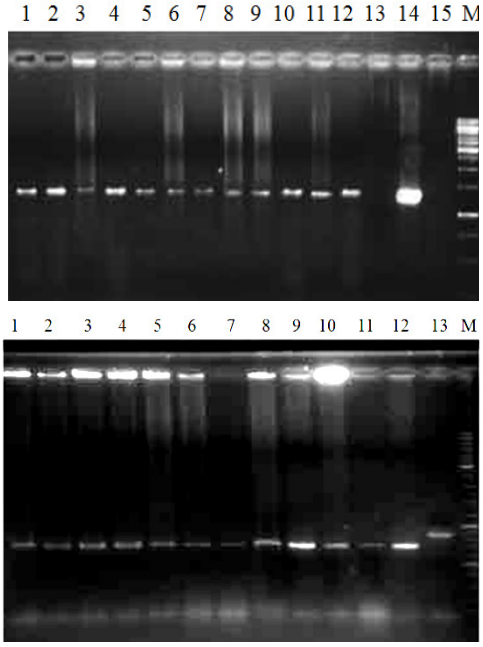

Figure 3. mRNA analysis of *synth-gox* transformed canola plant. A. RT-PCR analysis of *synth-gox* gene expression in transgenic plants. Lanes 1–12, putative transformed plants; 13, non transgenic plant as negative control, 14, pB1-synth-gox as a positive control, 15, ddH_2O and M, 1 kb Ladder (Fermentas); Expected amplicon was 1314 bp. B. Amplified tubulin transcripts. Lanes 1–12, transformed plant; 13, PCR from genomic DNA; M, 100 bp Mix ladder (Fermentas).

Figure 4. Southern blots of genomic DNA from 12 transgenic canola plants generated with the pBI-synth-gox plasmid. NT, non transgenic plant as a negative control; C+, pBI-synth-gox as a positive control; 1-12 transgenic plants; M, 100 bp Mix ladder (Fermentas)

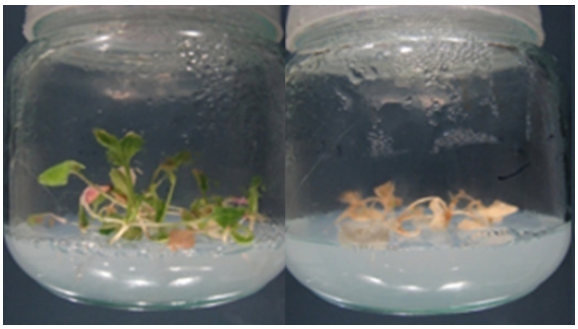

Figure 5. *In vitro* comparison of GP tolerance of transgenic plants (a) and untransformed control plants (b) at concentration of 0.2 mM GP after 21 days of application.

Discussion

Glyphosate is the most widely used herbicide in the world with minimal human and environmental toxicity (Dill, 2005). The herbicide inhibits EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) in shikimate pathway leading to the biosynthesis of aromatic amino acids and is required for survival of the plant (Padgette *et al.,* 1996; Dill, 2005). Transgenes have been used to achieve a commercial level of tolerance and allowing application of the herbicide to the crop (Tan *et al.,* 2006).

The most successful strategies used in existing commercial GP-tolerant crops is application of a native (*Agrobacterium* sp. CP4) or a mutated form of EPSPS that is not inhibited by GP and detoxification of the GP molecule (Pollegioni *et al.,* 2011). However, in previous study*, epsps* transgene obtained through site-directed mutagenesis with double mutations (Gly96Ala and $Ala^{183}Thr$) and was also used by stable insertion in a canola plant to achieve tolerance to GP (Kahrizi *et al.,* 2007). Most plants have little or no endogenous ability to detoxify GP (Dill, 2005). The *gox* gene isolated from *Ochrobactrum anthropi* LBAA (Barry and Kishore, 1995) was employed to detoxify GP in plants. As a result, GOX metabolized GP to glyoxylate and AMPA and reduces herbicide injury to the plant because of decreasing the amount of GP to inhibit target EPSPS enzyme (Dill, 2005; Padgette *et al.,* 1996). However, this was done by simultaneous use of the *CP4 epsps*gene. The study presented here is the first report in which resistance to glyphosate was evaluated after the GOX enzyme was expressed alone in the plant. In order to increase the expression of the bacterial gene in the plant host, we optimized *gox* gene sequences based on the canola plant codon preferences and eliminated the rare codons. Furthermore, adding Kozak sequence and decrease of negatively *ci*s element, (e. g. direct and invert repeat sequences), led to higher expression of *gox* mRNA in host plant. The *gox* gene became under the control of constitutive CaMV 35S promoter in the binary vector pBI121 to ensure high levels of gene expression in all tissues. Transformation was mediated by *Agrobacterium* and the cut surfaces of cotyledoneary as an ideal target for *Agrobacterium*-mediated transformation was performed (Kahrizi *et al.,* 2007).

PCR and Southern blot analysis of transgenic plants showed that the transformation of canola with *synth-gox* is stable. The transformation event had no negative effect on flowering and seed stage in T0 plants. Further confirmation of stability and heritability of the transgene was provided by analysis of T1 segregating plants by GP assay. The *synth-gox* transgenic plants indicated some levels of GP resistance, but not equal to commercial Roundup ready cultivars. We plan to apply this gene together with double mutant *epsps* gene which has been previously proved to be efficient (Kahrizi *et al.,* 2007) with the objective of obtaining yet higher levels of glyphosate resistance in our local cultivars.

Figure 6. Comparison of the GP tolerance of an untransformed plant (a), with transgenic lines containing *synth-gox* (b). Roundup was applied at the concentrations of 0.5, 1 and 1.5 mM (from left to right, respectively) twice after a one-week interval. Pictures were taken two weeks after the last application.

Acknowledgments

This project was funded by the National Institute of Genetic Engineering and Biotechnology (NIGEB), project no. 407-M.

References

1. Barry GF, Kishore G, Padgette S, Taylor S, Kolacz K, Weldon M, Re DB, Eichholtz DE, Fincher K, Halla L (1992) Inhibitors of amino acid biosynthesis: strategies for imparting glyphosate tolerance to crop plants In Biosynthesis and Molecular Regulation of Amino Acids in Plants Edited by Singh BK, Flores HE and Shannon JC pp, 139-145 American Society of Plant Physiologists, Rockville, MD.
2. Barry GF, Kishore GM (1995) Glyphosate tolerance crop plants US Patent 5463175.
3. CERA (2010) GM crop database Available: http://www. cera-gmc. org/?action=gm_crop_ database 2011.
4. Dill GM (2005) Glyphosate-resistant crops: history, status and future Pest Manag Sci 61, 219-224.
5. Duke SO, Powles SB (2008) Glyphosate: a once-in-a-century herbicide Pest Manag Sci 64, 319-325.
6. Duke SO, Powles SB (2009) Glyphosate-Resistant Crops and Weeds: Now and in the Future AgBioForum 12, 346-357.
7. Graf M, Deml L, Wagner R (2004) Codon-optimized genes that enable increased heterologous expression in mammalian cells and elicit efficient immune responses in mice after vaccination of naked DNA Methods Mol Med 94, 197-210.
8. Gustafsson C, Govindarajan S, Minshull J (2004). Codon bias and heterologous protein expression. Trends Biotechnol. 22, 346-353.
9. James C (2011) Global status of commercialized biotech/GM crops: 2010

ISAAA Brief 42, ISAAA, Ithica, New York.

10. Kahrizi D, Salmanian AH, Afshari A, Moieni A, Mousavi A (2007) Simultaneous substitution of Gly96 to Ala and Ala183 to Thr in 5-enolpyruvylshikimate-3-phosphate synthase gene of *E. coli* (k12) and transformation of rapeseed (*Brassica napus* L.) in order to make tolerance to glyphosate. Plant Cell Rep. 26, 95-104.
11. Kishore GM, Padgette SR, Fraley RT (1992) History of herbicide tolerant crops, methods of development and current state of the art-emphasis on Glyphosate tolerance. Weed Technol. 6, 626-634.
12. Kozak M (1989) The scanning model for translation: an update. J Cell Biol. 108: 229-241.
13. Kuiper HA, Kleter GA, Noordam MY (2000) Risks of the release of transgenic herbicide-resistant plants with respect to humans, animals, and the environment. Crop Prot. 19, 773-778.
14. Lim LH, Li HY, Cheong N, Lee BW, Chua KY (2004) High-level expression of a codon optimized recombinant dust mite allergen, Blo t 5, in Chinese hamster ovary cells. Biochem Biophys Res Commun. 316, 991-996.
15. Mechold U, Gilbert C, Ogryzko V (2005) Codon optimization of the BirA enzyme gene leads to higher expression and an improved efficiency of biotinylation of target proteins in mammalian cells. J Biotechnol. 116, 245-249.
16. Moravec T, Schmidt MA, Herman EM, Woodford Thomas T (2007) Production of *Escherichia coli* heat labile toxin (LT) B subunit in soybean seed and analysis of its immunogenicity as an oral vaccine. Vaccine. 25, 1647-1657.
17. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plantarum. 15, 473-479.
18. Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant. DNA Nucleic Acids Res. 8, 4321-4325.
19. Padgette SR, Re DB, Gasser CS, Eichholtz DA, Frazier RB, Hironaka CM, Levine EB, Shah DM, Fraley RT, Kishore GM (1991) Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. J Biol Chem. 266, 22364-22369.
20. Padgette SR, Taylor NB, Nida DL, Bailey MR, MacDonald J, Holden LR, Fuchs RL (1996) The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybeans. J Nutr. 126, 702-716.
21. Pline WA, Wilcut JW, Duke SO, Edmisten KL, Wells R (2002) Tolerance and accumulation of shikimic acid in response to glyphosate applications in glyphosate-resistant and nonglyphosate-resistant cotton (*Gossypium hirsutum* L.). J Agric Food Chem. 50, 506-512.
22. Pollegioni L, Schonbrunn E, Siehl D (2011). Molecular basis of glyphosate resistance-different approaches through protein engineering. Febs J. 278, 2753-2766.
23. Sambrook J, Maccallum P, Russell D (2001) Molecular Cloning: A Laboratory Manual. 3rd. Cold Spring Harbor Press, NY., 2344 p. ISBN 0-87969-577-3.
24. Sharp PM, Li WH (1987) The codon Adaptation Index--a measure of directional synonymous codon usage bias, and its potential applications. Nucleic Acids Res. 15, 1281-1295.
25. Tan S, Evans R, Singh B (2006) Herbicidal inhibitors of amino acid biosynthesis and herbicide-tolerant crops. Amino Acids. 30, 195-204.
26. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406-3415.