Regeneration of glyphosate-tolerant *Nicotiana tabacum* after plastid transformation with a mutated variant of bacterial *aro*A gene

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Abstract

Presence of antibiotic resistance markers has always been considered as one of the main safety concerns in transgenic plants and their derived products. Elimination of antibiotic selectable markers from transgenics is a major hurdle for finding efficient and safe candidates. Herbicide tolerance genes might be attractive alternatives. In this study, a variant form of the 5-enoylpyruvyl shikimate-3-phosphate synthase (EPSPS) gene that harbors glycine at position 96 to alanine and alanine 183 to threonine substitutions and confers higher resistance to the broad-spectrum herbicide, glyphosate, was substituted against the spectinomycin resistant gene as a sole selectable marker for plastid transformation of *Nicotiana tabacum*. Plastid transformation was carried out using the biolistic delivery procedure while delivery parameters such as rupture disk pressure, bombardment distance, etc had been optimized first. A previous study showed that the glyphosate herbicide imposes lethal effects on the structure and integrity of the plastid membrane, even at low concentrations. In order to overcome this problem, a modified procedure for selection of transplastomic cells was used. A long preculture incubation period followed by a gradual increased in glyphosate concentration led to sufficient expression of the transgene. Tolerant calli were thus regenerated through direct selection of transformed plastids in the presence of the glyphosate.

Keywords: Plastid transformation; *Nicotiana tabacum*; Glyphosate; 5-enoylpyruvyl shikimate-3-phosphate synthase

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INTRODUCTION

Genetic information of plants is distributed among three cellular compartments: the nucleus, the mitochondria and the plastid. Each of these compartments carries its own genome and consequently expresses heritable traits (Ruf *et al*., 2001; Siguira, 1992). Recently, plastids have become attractive targets for genetic engineering efforts. Transformation of the plastids' genome (plastome) has several advantages over nucleus transformation. Since plastids in most agronomically important plant species are inherited maternally, the introduction of foreign genes into the plastome prevents pollen mediated outcrossing (Bock *et al*., 2004). Additionally, polyploidy of the plastid genome leads to high level protein production when transformed with a transgene. Furthermore, possibility of polycistronic operon expression and the absence of epigenetic effects and gene silencing are other noticeable advantages of plastid transformation (Maliga *et al*., 2003; Sidorov *et al*., 1999). Svab and colleagues (1990) reported the first successful plastid transformation of higher plants in 1990 for *Nicotiana tabacum*, since then; numerous reports have successfully proved the feasibility of plastid transformation in *Arabidopsis thaliana* (Sikdar *et al*., 1998), potato (Sidorov *et al*., 1999), *Lesquerella fendleri*, a kind of oilseed Brassicacea (Skarjinskaia *et al*., 2003) and oilseed rape (Hou *et al*., 2004).

Since the beginnings of transgenic plant commercialization (1996 to 2007), herbicide tolerance has consistently been considered as the dominant trait. In

2007, a total of 114.3 million hectares was used for cultivation of biotechnological products; a portion of 63% or 72 million hectares was dedicated to herbicide-tolerant plants (International Service for the Acquisition of Agri-biotech Application, 2007). Among different herbicides, glyphosate (under the Roundup® trade name) is a broad-spectrum, safe and effective herbicide that blocks plant growth by inhibiting the production of aromatic amino acids, which leads to arrest of protein synthesis. Glyphosate inhibits 5-enoylpyruvyl shikimate-3-phosphate synthase (EPSP synthase) activity, which is a nuclear-encoded, plastid-localized enzyme in the shikimic acid pathway of plants and microorganisms (Eschenburg *et al*., 2002; Alibahai and Stallings 2001; Schonbrunn *et al*., 2001; Sost *et al*., 1990). Previously, a variant form of *Escherichia coli* K12 EPSP synthase gene with reduced affinity for glyphosate was made through simultaneous Gly96Ala and Ala183Thr substitutions (Haghani *et al*., 2008; Salmanian *et al*., 2006). The efficiency of this mutated gene for tolerance induction in model plant has also been reported (Kahrizi *et al*., 2007). The amino glycoside 3-adenylyltransferase *(aad*A) gene, which confers dual resistance to spectinomycin-streptomycin antibiotics, is still the most efficient and routinely used selectable marker for plastid transformation (Ye *et al*., 2001; Ye *et al*., 2003; Maliga, 2004). Not only the antibiotic resistant markers are not needed in the final transgenic products, but also their presence imposes some biosafety concerns for consumers. Therefore, elimination of selectable marker genes from transgenic plants is highly recommended. Different methods including marker excision (*Cre-lox* site-specific recombination system) and co-transformation followed by independent segregation of antibiotic and herbicide genes have been successfully used to produce marker free transgenic plants (Ye *et al*., 2003; Corneille *et al*., 2001; Hajdukiewicz *et al*., 2001; Zou, 2001).

This study has focused on the production of high-level glyphosate tolerant plants (*N. tabacum*) through biolistic transformation of plastids by introduction of a mutated herbicide-tolerant gene coding for EPSP synthase. It has previously been reported that glyphosate, represents a lethal selection that kills plastids even in the early stages of transformation therefore, attempts were unable to produce homoplasmic, glyphosate-tolerant plants upon direct selection on the herbicide(Ye *et al*., 2003). Hence, in this study, it was decided to modify the selection procedure within two manners: The selection procedure was started with sublethal doses of glyphosate and the incubation period was increased from 2 days to 1 week. As a result, glyphosate tolerant plantlets were regenerated without the use of antibiotic resistant selectable markers. For this purpose, different parameters of the chloroplast transformation procedure were optimized first by using the chloroplast transformation vector, pKCZ (Zou *et al*., 2003; Zou, 2001).

MATERIALS AND METHODS

Optimization of plastid transformation: In order to evaluate and optimize all the parameters of plastid transformation, *Nicotiana tabacum* cultivar Samsun was transformed with the intact pKCZ vector (a generous gift from Prof. H.U. Koop; Ludwig-Maximilians Universitat, Munchen) through biolistic bombardment of well-grown young leaves. The vector harbors the *aad*A gene, which confers simultaneous spectinomycin/streptomycin resistance. Two regions, INSL (Insertion Site left) and INSR (Insertion Site Right), are exactly copied from tobacco plastid genome therefore; the gene of interest that could be cloned between them will be integrated in plastid genome through homologous recombination (Fig. 1). Plastid transformation of tobacco was carried out using the particle bombardment device (PDS-1000/He, Bio-Rad Biolistic system, USA). The gold particle (0.6 µm) coating procedure was performed by using standard protocols provided by the Bio-Rad manual. Different parameters such as the rupture disk pressure (ranging from 900 to 1300 psi), distance of the target tissue from the stopping screen (3, 6 and 9 cm) and vacuum pressure (ranging from 20 to 25 inches Hg) were considered. The optimum condition involved the use of 0.6 µm gold particles, 900 Psi rupture disk, a vacuum pressure of 24 inches Hg and a 6 cm distance between the stopping screen and the target tissue. Selection was started two days after bombardment with segmented leaf pieces (0.5 × 0.5 cm) on RMOP medium (MS + 6-benzylaminopurine (BAP) 1 mg/l + 1-Naphthalene acetic acid (NAA) 0.1 mg/l) which contained spectinomycin. The antibiotic (spectinomycin) concentration was adjusted to 500 mg/l. In order to achieve homoplasmy, three rounds of selection and regeneration were performed. Each round involved approximately 2 months .During each round, the primary shoots of resistant transformants were dissected into small pieces (2.5 × 2.5 mm) and placed on new selective medium (RMOP plus spectinomycin). In

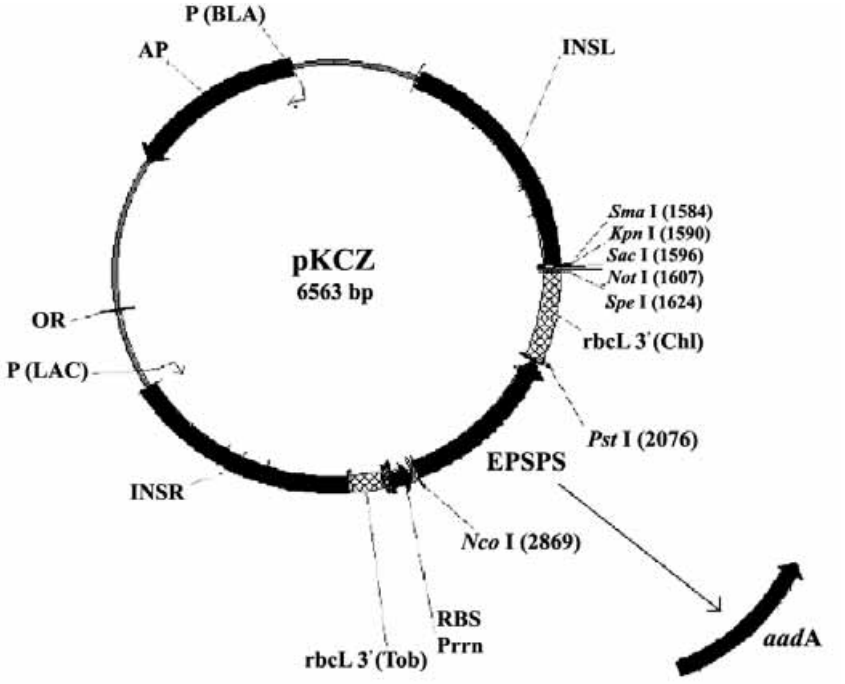

Figure 1. Substitution of the *aad*A gene with the mutated *epsp* synthase using the *Nco*I and *Pst*I digestion sites. *epsp* synthase integrates into the plastid genome through homologous recombination. INSL and INSR are identical to the flanking regions of the insertion site. For more details see materials and methods section.

order to eliminate spontaneous mutations which make the plastids ribosome resistant to spectinomycin, the regenerated plantlets were transferred onto RMOP medium containing streptomycin (500 mg/l).

PCR analysis of transplastomic plants: The presence of foreign gene in the plastome was confirmed with PCR amplification using specific primers. One of the primers was designed on the basis of *aad*A gene which introduced to chloroplast genome and had the following sequence; aadAR: 5′-CACAGTGATATTGATTTGCTGG-3′, while the other primer was located on the right flanking region of the insertion site in the plastid genome, INSRF: 5′-GTAGCTCAGAGGATTAGAGCAC-3′. The latter primer was designed due to the native sequence of plastid genome.

Construction of the plastid transformation vector: pKCZ had been previously constructed for plastid transformation of tobacco (Zou *et al*., 2003). This vector contains the *aad*A gene under the control of the rRNA operon promoter (Prrn) and the 3' UTR of the *Chlamydomonas rbcl* gene as a terminator (rbcl3'chl). The mutated Enolpyruvyl shikimate 3-phospho (EPSP) synthase (Salmanian *et al*., 2006) was isolated from pUC18-EPSP and substituted the *aad*A in pKCZ, using *Nco*I/*Pst*I restriction endonucleases (Fig. 1).

Plant material: Sterile tobacco plants were grown on MS medium in a phytochamber (Snijder Scientific, the Netherland) (16 h light/8 h dark/25ºC). For biolistic transformation, fully expanded leaves were harvested and placed overnight on RMOP medium with the upper side up, under the above conditions. Chloroplast transformation with pKCZ containing the gene coding for EPSP synthase was performed several times with parameters, which had been optimized previously.

Selection and regeneration of EPSP synthase transformants: For the selection procedure, two different strategies were applied. In the first, two days after bombardment, leaves were cut into approximately 0.5 × 0.5 cm^2 pieces and placed onto regeneration medium (RMOP) containing glyphosate as selectable marker. In this selection procedure, serial dilutions (0.1-1 mM) of glyphosate were prepared. After two weeks, non transformed (bleached) parts were removed and the remaining green explants transferred to fresh medium

with the same glyphosate concentration. The second procedure of selection was carried out on sub-lethal doses of glyphosate. In this procedure, one week after bombardment, explants were cut and transferred onto RMOP medium containing 5 µM glyphosate (one tenth of lethal dose). Glyphosate concentration was doubled every two weeks up to the lethal dose (50 µM). Each time the swelled leaf explants were partitioned into smaller pieces.

RESULTS

Optimization of plastid transformation: The precultured leaves were bombarded under optimized conditions with pKCZ. Two days later, selection of the segmented explants was started on RMOP supplemented with spectinomycin as selectable marker. After several rounds of selection, the first antibiotic-resistant explants were regenerated 12 weeks after bombardment (Fig. 2). The antibiotic-tolerant plantlets were able to generate green shoots and expanded roots (Fig. 3). The well-grown plants were transferred to pots under greenhouse conditions, to allow flowering and set seeds.

In order to distinguish between spontaneously mutated and transplastomic plants, regenerated plants were transferred to RMOP medium with streptomycin (500 mg/l). Three weeks later, the transformed plantlets were still green and no noticeable adverse effect was obvious, while the wild type control was bleached completely (Fig. 4).

PCR analysis of transplastomic plants: In order to prove the correct orientation of the *aad*A gene, a pair of primers was designed. Amplification of the desired fragment (approximately 1300 bp) was analyzed on 1% (w/v) agarose gel (Fig. 5 A-B).

Construction of the EPSP synthase-containing vector: Replacement of the *aad*A gene with mutated EPSP synthase was achieved and the desired construct was prepared using the same cassette: Prrn/*epsp* synthase/ rbcl3'chl. The presence of foreign genes in the vector was confirmed both with restriction enzyme analysis and PCR (Data not shown).

Selection of the transformed plastid with the altered EPSP synthase gene: To improve the efficiency of selection two different selection procedures were used. In the first strategy, where 0.1 to 1 mM of glyphosate was used, leaf explants were highly sensitive and bleached completely in less than 1 month and no callus formation was observed. In the second pattern, leaf explants were cut and placed on selection medium containing 5 µM of glyphosate, one week after bombardment. Herbicide concentration was doubled every two weeks. Primary glyphosate resistant calli were selected in the presence of 50 µM herbicide.

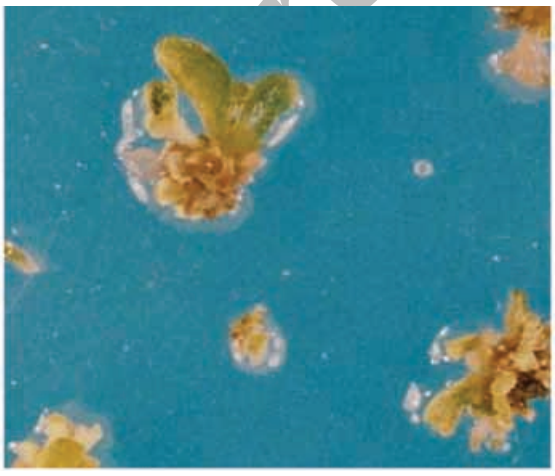

Figure 2. The first regenerated tobacco plant, 3 months after bombardment with pKCZ in RMOP medium containing spectinomycin as selectable marker. For more details, see the text.

Figure 3. The first antibiotic-resistant transplastomic plant in RMOP medium containing 500 mg/l of spectinomycin. Formation of shoots and roots is apparent.

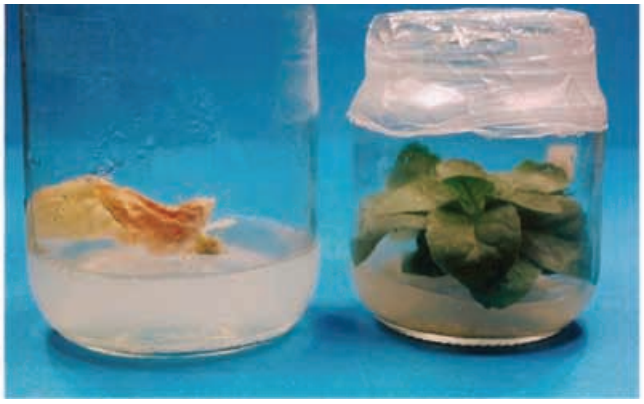

Figure 4. Simultaneous transfer of wild type (left) and transformed (right) plants into RMOP medium with streptomycin. The transformed plant stayed green and grew completely.

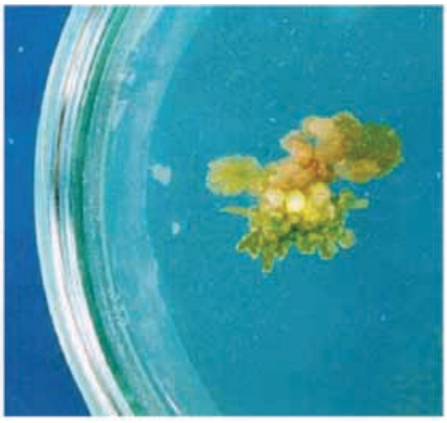

Figure 6. Callus induction and initial leaf formation in selective medium containing glyphosate (1 mM).

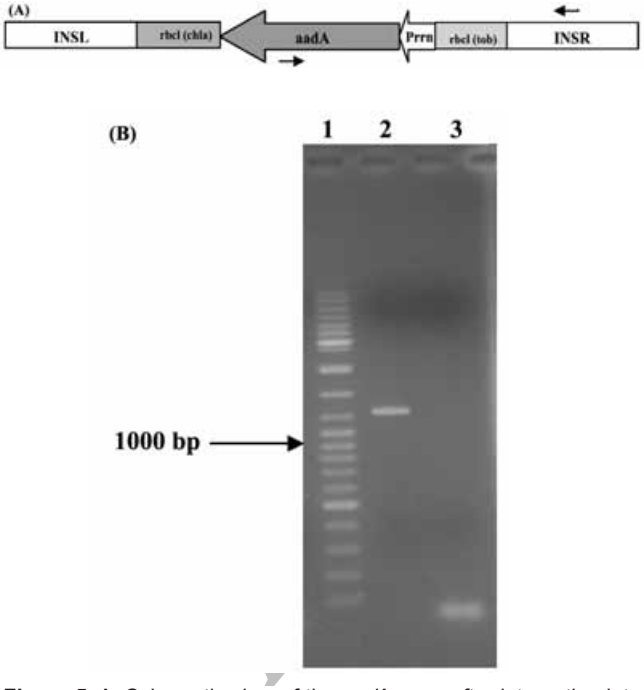

Figure 5. A: Schematic view of the *aad*A gene after integration into the plastid genome. Arrows show the location of forward and reverse primers. B: PCR amplification of a 1300bp fragment. Lane 1: 100bp molecular weight marker (Fermentas), 2: transgenic plant, 3: wild type plant.

These calli were also tolerant to 0.1 mM glyphosate, but regeneration did not occur completely. Small, green plantlets were unable to form roots (Fig. 6).

DISCUSSION

Glyphosate [N-(phosphonomethyl) glycine], the active ingredient of weed control agent, Round up® is known as one of the most successful commercial broad-spectrum herbicides. The EPSP synthase has been identified as a highly selected target for glyphosate. Efforts to achieve herbicide resistance in crop plants were started in the 1980s with isolation of EPSP synthase in various glyphosate-resistant organisms (Eschenburg *et al*., 2002). In previous research, in order to produce more stable and active variants of this enzyme, two amino acid substitutions (Gly96Ala and Ala183Thr) were introduced simultaneously into the EPSP synthase gene (Kahrizi *et al*., 2007).

Glyphosate resistant crops, which are generated through nuclear transgenic technology, are currently used commercially in several countries (ISAAA 2007). So far, it has been clearly shown that transplastomic technology has tremendous advantages over nuclear transformation including high-level foreign protein accumulation and reduction in the risk of foreign gene flow into environment. Hence, in this study, efforts were made to induce glyphosate tolerance in *N. tabacum* through plastid transformation with the new mutated gene.

Replacement of *aad*A with the EPSP synthase mutated gene changed the expression cassette of the pKCZ vector. Therefore, EPSP synthase was localized under the control of the chloroplast's rRNA operon promoter. This strong promoter causes high levels of transcript accumulation in plastids. The insertion site of the transgene is located between two tRNA genes (Asparagine and Argenine) in the inverted repeats of

the plastid genome. The flanking regions of this site are exactly copied in the chloroplast vector pKCZ, therefore; each plastome could be transformed twice through homologous recombination. High Copy numbers of plastid DNA in the homoplasmic transformed plants and double transformation of each genome with the altered EPSP synthase, could produce *N. tabacum* with high levels of resistance to glyphosate.

The *aad*A gene has been used as a main selectable and efficient marker in previous plastid transformation studies. The *neo* and *aph* genes, which confer resistance to kanamycin and betaine aldehyde dehydrogenase (BADH) (that induces resistance toward toxic compounds like betaine aldehyde (BA), have also been reported as successful selection markers for transplastomic plants. Since the antibiotic resistant genes are not desirable in the final products, different strategies have been applied to eliminate the necessity of using these kinds of selectable markers (Maliga 2004, 2003).

Herbicide tolerant markers are considered as highly attractive alternatives and are currently used in nuclear transformation, but transplastomic selection only in the presence of herbicides seems to be inefficient (Mannerlof *et al*., 1997; Zhou *et al*., 1995; Barry *et al*., 1992). The low success of herbicides like glyphosate and phosphinothricin in achieving plastid transformations is supposed to be the result of their lethal effects on plastids. It has been claimed that their remarkable lethality hinders the small fraction of transformed plastids to stay alive and divide efficiently. Therefore, plant cells will be killed at the early stages of transformation before the resistant gene has enough time for expression. Ye and co-workers (2003) have investigated the effects of herbicides on the ultrastructure of plastids using transmission electron microscopy (TEM). The results have shown that the ultrastructures of plastids become damaged in the presence of glyphosate. Plastids lose their reticulated network of thylakoids and the photosynthetic membranes become disintegrated.

In the present research, the aim was to create transplastomic plants possessing high-level glyphosate tolerance, without using antibiotic resistance genes as selectable markers. However, after several transformation experiments using pKCZ containing EPSP synthase under optimized conditions, it was not possible to regenerate glyphosate tolerant plants on selective medium containing serial dilutions of glyphosate (0.1 mM to 1mM). Due to these findings, another selection scheme was carried out with non-lethal concentrations of glyphosate. The incubation time before selection was extended up to two weeks in order to provide enough time for plastids to amplify sufficiently and express the resistance gene. Selection was then initiated with one tenth of the lethal dose (50 μM). Media were changed each two weeks with doubled concentrations of glyphosate. By using such a selection with gradual increase in glyphosate concentrations and prolonged incubation, before initiation of selection, small, green and dense calli were generated and leaf formation was observed. These plantlets showed resistance to 0.1 mM glyphosate. The preliminary results of this study have shown that by using this altered selection procedure, it is possible to produce tolerant calli with an herbicide resistant gene as the only selectable marker. It is believed that the lack of completely formed shoots or roots can be solved with appropriate tissue culture treatments.

Acknowledgments

We highly thank Professor Hans Ulrich Koop for preparation of the plastid transformation vector, pKCZ. This work was supported by a grant # 278 provided by the National Institute of Genetic Engineering and Biotechnology (NIGEB), Iran.

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