

DNA Profiling of Transgenes in Genetically Modified Plants

Bittsánszky A^{1,2}, Gabor G^{1*}, Gullner G and Komíves T

¹Plant Protection Institute, CAR, Hungarian Academy of Sciences, Hungary

²Inst Genetics and Biotechnology, Szent István University, Hungary

*Corresponding author: Gyulai Gabor, Inst Genetics and Biotechnology, Szent István University, 2103 Gödöllő, Páter K. u. 1. Godollo, Hungary, Tel: +36 28 522-069; E-mail: gyulai.gabor@mkk.szie.hu

Received date: April 20, 2016; Accepted date: May 25, 2016; Published date: June 02, 2016

Copyright: © 2016 Bittsánszky A, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Abstract

In silico sequence diversities of four orthologous plant gsh1 genes and their amino acid translates of GSH1 proteins (Glutathione Synthase) were compared to the non-orthologous prokaryotic gshI/GSHI gene/protein of *E. coli* (NCBI # X03954). Primer pair was designed and transgene detection was carried out in two types of gshI-transgenic poplar clones (*Populus x canescens*) of ggs11 (cyt-ECS) and lgl6 (chl-ECS). Usefulness of genetic modification technologies (GMO) is indicated.

Keywords: Biotechnology; DNA Profiling

Introduction

Transgenes represent genetic markers artificially introduced in laboratory motivated to improve crops. Detection of marker in the Genetically Modified Organism (GMO), and its vegetative or sexual progenies; and monitoring it in test and cultivated populations as well as in exposed non-target cross-pollinated populations is of fundamental and practical importance. The genetically modified state of an organism, i.e. the presence of the transgene, is verified essentially by DNA profiling. Selecting the DNA sequence for DNA profile is straightforward because a known sequence is introduced. Introduction of genes, self or foreign, into plants had prerequisites. The ability to select and identify desired genotypes in cells, tissues or intact plants laid the fundamentals for application of genetic transformation of plants and animals by tools of biotechnology.

The Biological Research Centre (Szeged, Hungary) can be considered as the Genius Loci [1] of the current plant biotechnology since methodologies of plant cell line selections for chloroplast mutants [2,3], cell fusion [4], genetic transformation [5,6] bacterial nitrogen fixation [7] and artificial chromosomes [8] were either fundamentally developed or highly improved there.

The first stable higher plant mutant, the antibiotic (i.e. streptomycin, SR) resistant (i.e. mutant) tobacco (SR1) was selected [2] *in vitro*, followed by the selection [9] and identification of SR1A15 [10] the first double mutant of higher plants, the albino (chloroplast) tobacco [9,11].

Later, as the early forms of gene transfer, protoplast cell fusion plants (i.e. cybrids) were developed in several laboratories [12-16].

Alternatives to the conventional haploid genome transfer (i.e. pollination), the technologies of single and pyramided gene transfer resulting in stable transgenic crops (i.e. GM - genetically modified, or GMO - genetically modified organism), were developed in four labs at the same time in 1983: GM *Nicotiana plumbaginifolia* (resistant to the antibiotic kanamycin) [17], other tobacco lines resistant to kanamycin and methotrexate (a drug used to treat cancer and rheumatoid

arthritis) [18], GM petunia resistant to kanamycin [19] and GM sunflower transformed by phaseolin gene isolated from bean [20].

The first field trial of GM cotton was carried out in 1990, followed by the first FDA-approved (Unites States Food and Drug Administration) transgenic food of Flavr-Savr tomato in 1994 [21]. A series of further GM crops were released in 1995, such as the canola oil seed rape (*Brassica napus*) with modified oil composition (*Calgene*), Bt (*Bacillus thuringiensis*) corn (*Ciba-Geigy*) resistant to the herbicide bromoxynil (*Calgene*), Bt cotton (*Monsanto*), GM soybeans resistant to herbicide glyphosate (*Monsanto*); virus-resistant squash (*Asgrow*), and a delayed ripening tomatoes (DNAP, *Zeneca/Peto* and *Monsanto*) [22,23]. Later, a series of woody plants were also bred by genetic transformation [24-29].

Here we present a case study of barcoding (i.e. detecting and monitoring GM plants) the CaMV-35S-gshI poplar (*Populus x canescens*) with techniques useful for both developing GM plants and for anti-GM purposes.

Materials and Methods

DNA extraction

Total DNA samples of 0.1 g leaf tissue in each case were extracted in CTAB, cethyltrimethylammonium bromide, buffer followed by RNase-A (from bovine pancreas, Sigma, R-4875, treatment) for 30 min at 37°C. DNA samples of ten individuals of each line were pooled in one bulk and subjected to PCR analysis.

Multiple sequence alignments for primer design

Nucleotide sequences of genes gsh1 were downloaded from the National Center for Biotechnology Information (NCBI) databases [30]. Multiple sequence alignments were applied *in silico* with the software programs BioEdit Sequence Alignment Editor (North Carolina State University, USA) [31], Multalin [32], Clustal W [33], FastPCR [34] and computer program MEGA4 [35].

Barcoding of the transgene

The *gshI*-transgene (*E. coli*, NCBI #X03954) in the transformed poplar clones was amplified by the *gshI* specific primer 5'-atccggacgtatcacagg- (position bp. 341-359 in *gshI*) and its reverse 3'-gatgcaccaaacagataagg-5' (position bp 939-920 in *gshI*) according to Koprivova et al. [36,37].

Hot Start PCR

Was combined with Touchdown PCR by using AmpliTaq Gold™ Polymerase. Reactions were carried out in a total volume of 10 µl, and 25 µl (transgene detection) respectively, containing 50 ng of genomic DNA. For transgene analysis 1xPCR buffer (2.5 mM MgCl₂), dNTPs (200 µM each), 20 pmol of each primer and 0.5 U of Taq polymerase were used.

Touchdown PCR

Was performed by decreasing the annealing temperature from 66 °C to 56 °C by 0.7 °C /30s increments per cycle in each of the initial 12 cycles (PE 9700, Applied Biosystems), followed by a 'touchdown' annealing temperature for the remaining 25 cycles at 56 °C for 30s with a final cycle of 60 °C for 45 min or 72 °C for 10 min (transgene detection) and hold at 4 °C. A minimum of three independent DNA preparations of each sample were used. Amplifications were assayed by agarose (1.8%, SeaKem LE, FMC) gel electrophoresis (Owl system), stained with ethidium bromide (0.5 ng/µl) after running at 80V in 1 X TAE buffer [37]. Each successful reaction with scorable bands was repeated at least twice. Transilluminated gels were analyzed by the ChemilImager v 5.5 computer program (Alpha Innotech). A negative control which contained all the necessary PCR components except template DNA was included in the PCR runs.

Results and Discussion

DNA profiling of CaMV-35S-*gshI* transgene in GM poplar (*P. x canescens*).

The phytoextraction and remediative capacity of poplars was improved significantly by genetic transformation of *Populus x canescens* (*P. tremula* x *P. alba*) to overexpress the bacterial gene coding for γ-glutamylcysteine synthetase (γ-ECS, EC 3.2.3.3), which is the rate-limiting regulatory enzyme in the biosynthesis of the ubiquitous tripeptide thiol compound glutathione (GSH, γ-L-glutamyl-L-cysteinyl-glycine) [24,25]. Here we show how *gshI* transgene is detected by using *gshI*-specific PCR primers [36,37]. The sequence differences between the eukaryotic plant *gshI* gene and the prokaryotic *gshI* transgene of *E. coli* (Figure 1) made it feasible to design transgene specific PCR primers.

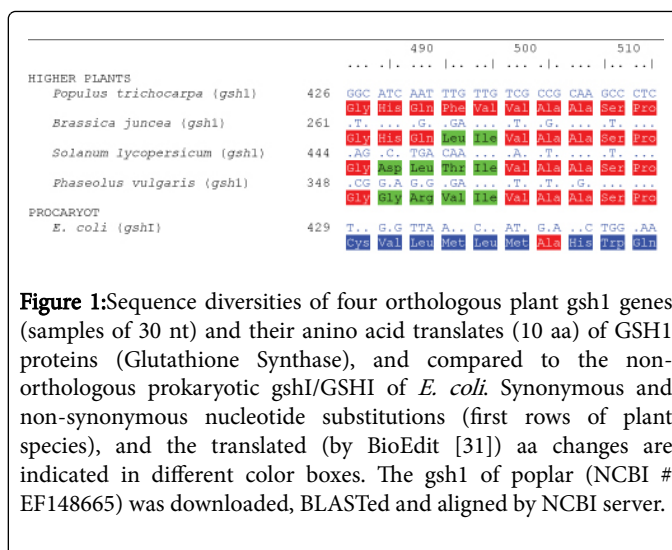


Figure 1: Sequence diversities of four orthologous plant *gshI* genes (samples of 30 nt) and their amino acid translates (10 aa) of GSH1 proteins (Glutathione Synthase), and compared to the non-orthologous prokaryotic *gshI*/GSHI of *E. coli*. Synonymous and non-synonymous nucleotide substitutions (first rows of plant species), and the translated (by BioEdit [31]) aa changes are indicated in different color boxes. The *gshI* of poplar (NCBI # EF148665) was downloaded, BLASTed and aligned by NCBI server.

Double strand breaks (DSBs) of DNA as the initial events of recombination occur not only in the meiotic but also in the somatic cells [38], which can cause transgene (Figure 2) elimination. In our study, the *gshI* transgene was found to be stably incorporated [24, 25] in the tested poplar lines (ggs11 and lgl6), and no transgene elimination or segregation was detected, which could occur during several cycles of micropropagation *in vitro* [39, 40] (Figure 3).

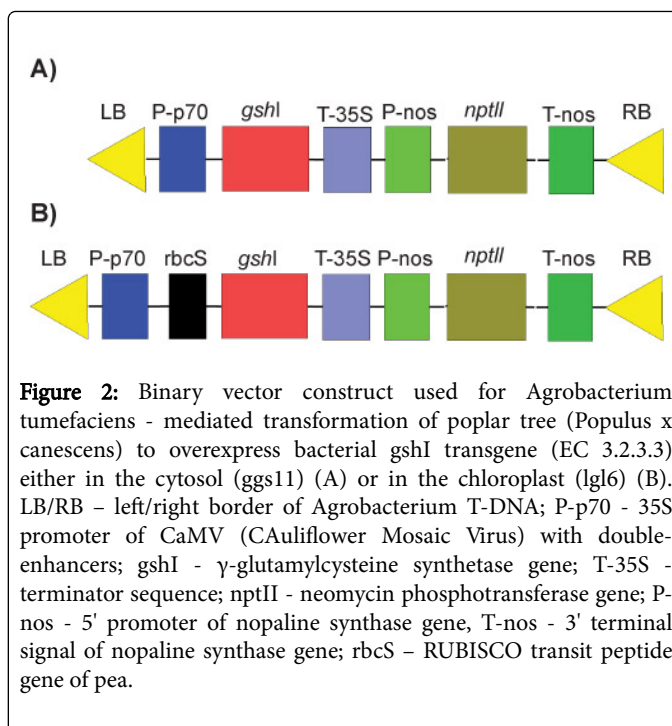


Figure 2: Binary vector construct used for *Agrobacterium tumefaciens* - mediated transformation of poplar tree (*Populus x canescens*) to overexpress bacterial *gshI* transgene (EC 3.2.3.3) either in the cytosol (ggs11) (A) or in the chloroplast (lgl6) (B). LB/RB – left/right border of *Agrobacterium T-DNA*; P-p70 - 35S promoter of CaMV (Cauliflower Mosaic Virus) with double-enhancers; *gshI* - γ-glutamylcysteine synthetase gene; T-35S - terminator sequence; *nptII* - neomycin phosphotransferase gene; P-nos - 5' promoter of nopaline synthase gene, T-nos - 3' terminal signal of nopaline synthase gene; *rbcS* - RUBISCO transit peptide gene of *pea*.

The RT-qPCR analysis confirmed that the transgene was not lost by revealing the high expression levels of the transgene CaMV-35S-*gshI* in poplar exposed to herbicides (Figure 3).

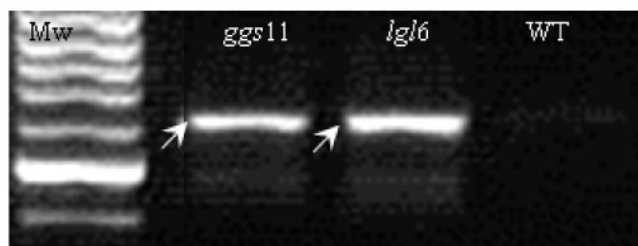


Figure 3: PCR detection of a partial sequence (598 bp indicated by arrows) of the *gshI*-transgene cloned from *E. coli* (NCBI # X03954) and transformed to 35S-*gshI*-poplar (*Populus x canescens*) clones *ggs11* (cyt-ECS) and *lgl6* (chl-ECS), and compared to the non-transformed (wild type, WT) clones (0.8% agarose gel). Primer pair was as 5'-atc ccg gac gta tca cag g-3' (position bp 341-359 nt of *gshI*) and 3'-gat gca cca aac aga taa gg-5' (position bp 939-920 nt of *gshI*). MW - Molecular weight markers are indicated.

Conclusions

By means of DNA profiling the transgenes, either coding or reporter genes, can be detected in the genetically transformed GMO plants for both GMO and anti-GMO purposes. The sequence differences between the foreign gene and the resident genes make it feasible to design GMO-specific barcodes. We should also emphasize that as opposed to more involved southern blotting and mapping of transgenes DNA barcoding is simple, cost effective and possibly accessible to the public and organizations through specialized commercial labs.

References

- Smil V, Loci G (2001) Nature. 409: 21-21.
- Maliga P, Sz Breznovits A, Márton L (1973) Streptomycin-resistant plants from callus culture of haploid tobacco. Nat New Biol 244: 29-30.
- Garab GL, Horváth G, Faludi-Dániel A (1974) Resolution of the fluorescence bands in greening chloroplasts of maize. Biochem Biophys Res Commun 56: 1004-1009.
- Dudits D, Rasko I, Hadlaczký G, Lima-de-Faria A (1976) Fusion of human cells with carrot protoplasts induced by polyethylene glycol. Hereditas 82: 121-3.
- Márton L, Wullems GJ, Molendijk L, Schilperoort RA (1979) In vitro transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*. Nature 277: 129-131.
- Koncz C, Kreuzaler F, Kalman Z, Schell J (1984) A simple method to transfer, integrate and study expression of foreign genes, such as chicken ovalbumin and alpha-actin in plant tumors. EMBO J 3: 1029-1037.
- Kiss GB, Dobo K, Dusha I, Breznovits A, Orosz L, et al. (1980) Isolation and characterization of an R-prime plasmid from *Rhizobium meliloti*. J Bacteriol 141: 121-128.
- Hadlaczký G (2001) Satellite DNA-based artificial chromosomes for use in gene therapy. Curr Opin Mol Ther 3: 125-132.
- Maliga P, Breznovits A Sz, Marton L Joo F (1975) Non-Mendelian streptomycin-resistant tobacco mutant with altered chloroplasts and mitochondria. Nature 255: 401-402.
- Svab Z, Maliga P (1986) *Nicotiana tabacum* mutants with chloroplast encoded streptomycin resistance and pigment deficiency. Theor Appl Genet 72: 637-643.
- Pay A, Smith MA (1988) A rapid method for purification of organelles for DNA isolation: self-generated percoll gradients. Plant Cell Rep 7: 96-99.
- Kao KN, Michayluk MR (1974) A method for high-frequency intergeneric fusion of plant protoplasts. Planta 115: 355-367.
- Melchers G, Labib G (1974) Somatic hybridisation of plants by fusion of protoplasts. MGG Mol Gen Genet 135: 277-294.
- Power JB, Frearson EM, Hayward C, George D, Evans PK, et al. (1976) Somatic hybridisation of *Petunia hybrida* and *P. parodii*. Nature 263: 500-502.
- Dudits D, Hadlaczký G, Lévi E, Fejér O, Haydu Z, et al. (1977) Somatic hybridisation of *Daucus carota* and *D. capillifolius* by protoplast fusion. Theor Appl Genet 51: 127-132.
- Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci U. S. A. 82: 6960-6964.
- Bevan MW, Flavell RB, Chilton MD (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304: 184-187.
- Herrera-Estrella L, Depicker A, Van Montagu M, Schell J (1983) Expression of chimaeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature 303: 209-213.
- Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, et al. (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci U. S. A. 80: 4803-4807.
- Murai N, Kemp JD, Sutton DW, Murray MG, Slightom JL, et al. (1983) Phaseolin gene from bean is expressed after transfer to sunflower via tumor-inducing plasmid vectors. Science 222: 476-482.
- Bruening G, Lyons JM (2000) The case of the FLAVR SAVR tomato. Calif Agric 54: 6-7.
- Conner AJ, Glare TR, Nap JP (2003) The release of genetically modified crops into the environment Part II Overview of ecological risk assessment. Plant J 33: 19-46.
- Noctor G, Arisi ACM, Jouanin L, Valadier MH, Roux Y, et al. (1997) Light-dependent modulation of foliar glutathione synthesis and associated amino acid metabolism in poplar overexpressing gamma-glutamylcysteine synthetase. Planta 202: 357-369.
- Arisi ACM, Noctor G, Foyer CH, Jouanin L (1997) Modification of thiol contents in poplars (*Populus tremula* x *P. alba*) overexpressing enzymes involved in glutathione synthesis. Planta 203: 362-372.
- Noctor G, Arisi ACM, Jouanin L, Foyer CH (1998) Manipulation of glutathione and amino acid biosynthesis in the chloroplast. Plant Physiol 118: 471-482.
- Bittsánszky A, Kómvés T, Gullner G, Gyulai G, Kiss J, et al. (2005) Ability of transgenic poplars with elevated glutathione content to tolerate Zinc (2+) stress. Environ Int 31: 251-254.
- Bittsánszky, Gyulai G, Malone RP, Bock I, Murenets LY, et al. (2015) Barcoding of transgenes in GM plants. Plant DNA Barcoding Phylogenetics, Lambert Academic Publ Germany 2015: 269-278.
- Gyulai G, Bittsánszky A, Gullner G, Heltai G, Pilinszky K, et al. (2012) Gene Reactivation Induced by DNA Demethylation in Wild Type and 35S-*gshI*-*rbcS* Transgenic Poplars (*Populus x canescens*). J Chem Sci Technol 1: 9-13.
- Gyulai G, Bittsánszky A, Szabó Z, Waters L, Gullner G, et al. (2014) Phytoextraction Potential of Wild Type and 35S-GSHI Transgenic Poplar Trees (*Populus x Canescens*) for Environmental Pollutants Herbicide Paraquat, Salt Sodium, Zinc Sulfate and Nitric Oxide In Vitro. Int J Phytoremediation 16: 379-396.
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95-98.
- Combet C, Blanchet C, Geourjon C, Deléage G (2000) NPS@: Network Protein Sequence Analysis. Trends Biochem Sci 25: 147-150.
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through

-
- sequence weighting position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-80.
34. Kalendar R, Lee D, Schulman AH (2009) FastPCR software for PCR primer and probe design and repeat search. *Genomes and Genomics* 3: 1-14.
35. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24: 1596-1599.
36. Koprivova A, Kopriva S, Jager D, Will B, Jouanin L, et al. (2002) Evaluation of transgenic poplars over-expressing enzymes of glutathione synthesis for phytoremediation of cadmium. *Plant Biol* 4: 664-670.
37. Gyulai G, Humphreys M, Bittsánszky A, Skot K, Kiss J, et al. (2005) AFLP analysis and improved phytoextraction capacity of transgenic gshI-poplar clones (*Populus x canescens* L.) for copper in vitro. *Z Naturforsch C* 60: 300-306.
38. Puchta H (1999) Double-strand break-induced recombination between ectopic homologous sequences in somatic plant cells. *Genetics* 152: 1173-1181.
39. Bittsánszky, Gyulai G, Humphreys M, Gullner G, Csintalan Z, et al. (2006) RT-PCR analysis and stress response capacity of transgenic gshI-poplar clones (*Populus x canescens*) in response to paraquat exposure. *Z Naturforsch C* 61: 699-703.
40. Gyulai G, Tóth Z, Bittsánszky A, Szabó Z, Gullner G, et al. (2008) Gene up-regulation by DNA demethylation in 35S-gshI-transgenic poplars (*Populus x canescens*). *Genet. Modif. Plants New Res Trends Nova Science Publisher Inc* 8: 1-22.