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DNA Profiling of Transgenes in Genetically Modified Plants

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Abstract

In silico sequence diversities of four orthologous plant gsh1 genes and their anino 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 37C. 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'-atcccggacgtatcacagg- (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 GoldTM 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 gsh1 gene and the prokaryotic gshI transgene of *E. coli* (Figure 1) made it feasible to design transgene specific PCR primers.

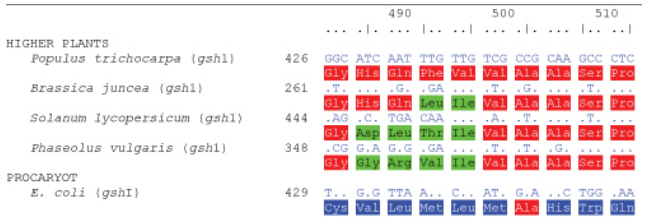

Figure 1: Sequence diversities of four orthologous plant gsh1 genes (samples of 30 nt) and their anino acid translates (10 aa) of GSH1 proteins (Glutathione Synthase), and compared to the non-orthologous prokaryotic gshI/GSHI of *E. coli*. Synonymous and non-synonymous nucleotide substitutions (first rows of plant species), and the translated (by BioEdit [31]) aa changes are indicated in different color boxes. The gsh1 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).

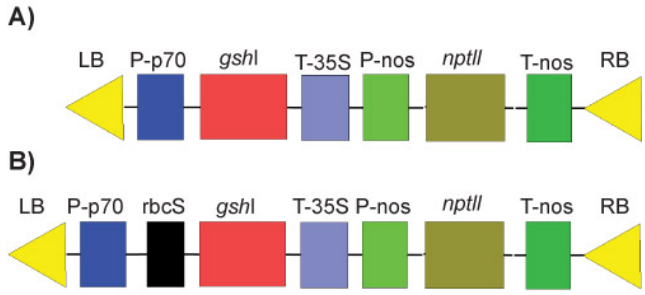

Figure 2: Binary vector construct used for Agrobacterium tumefaciens - mediated transformation of poplar tree (Populus x canescens) to overexpress bacterial gshI transgene (EC 3.2.3.3) either in the cytosol (ggs11) (A) or in the chloroplast (lgl6) (B). LB/RB – left/right border of Agrobacterium T-DNA; 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).

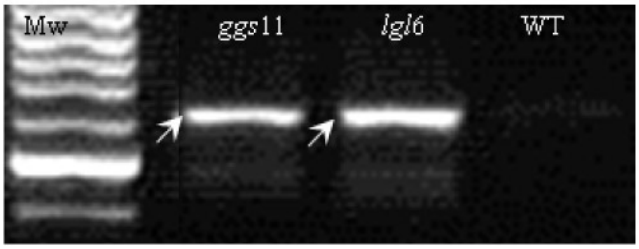

Figure 3: PCR detection of a partial sequence (598 bp indicated by arrows) of the gshI-transgene cloned from *E. coli* (NCBI # X03954) and transformed to 35S-gshI-poplar (Populus x canescens) clones ggs11 (cyt-ECS) and lgl6 (chl-ECS), and compared to the non-transformed (wild type, WT) clones (0.8% agarose 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 markes 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.

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