AFLP Analysis and Improved Phytoextraction Capacity of Transgenic gshl-Poplar Clones (Populus × canescens L.) for Copper in vitro

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Clone stability and in vitro phytoextraction capacity of vegetative clones of P. × canescens (2n = 4x = 38) including two transgenic clones (ggs11 and lgl6) were studied as in vitro leaf disc cultures. Presence of the gshl-transgene in the transformed clones was detected in PCR reactions using gshl-specific primers. Clone stability was determined by fAFLP (fluorescent amplified DNA fragment length polymorphism) analysis. In total, 682 AFLP fragments were identified generated by twelve selective primer pairs after EcoRI–MseI digestion. Four fragments generated by EcoAGT–MseCCC were different (99.4% genetic similarity) which proves an unexpectedly low bud mutation frequency in P. × canescens. For the study of phytoextraction capacity leaf discs (8 mm) were exposed to a concentration series of ZnSO4 (10−1 to 10−5 M) incubated for 21 days on aseptic tissue culture media WPM containing 1 µM Cu. Zn2+ caused phytotoxicity only at high concentrations (10−1 to 10−2 M). The transgenic poplar cyt-ECS (ggs11) clone, as stimulated by the presence of Zn, showed elevated heavy metal (Cu) uptake as compared to the non-transformed clone. These results suggest that gshl-transgenic poplars may be suitable for phytoremediation of soils contaminated with zinc and copper.

Key words: Phytoextraction, cyt-ECS (ggs11), chl-ECS (lgl6)

Introduction

Poplars (Populus ssp.) are known to take up and detoxify pollutants from the soil, such as atrazine and chloroacetanilide herbicides, organic pollutants such as trinitrotoluene and trichloroethylene, as well as heavy metals such as mercury and selenium (Gullner et al., 2001). This remediative capacity of poplars may be significantly increased by cell and genetic manipulations. P. × canescens was transformed recently to overexpress the bacterial gene encoding 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) which plays a central role in plant detoxification processes (Kömives and Gullner, 2000; Schröder et al., 2001). In the present study phytoextraction activity was investigated in the wild-type poplar hybrid P. × canescens (P. tremula × P. alba) and two transgenic lines overexpressing γ-ECS either in the cytosol (line ggs11 of Arisi et al., 1997) or in the chloroplasts (line lgl6 of Noctor et al., 1998) following a 21 day exposure to ZnSO4 (10−1 to 10−5 M) in vitro. Prior to the experiments genetic stability of the poplar cut clones was determined by AFLP analysis. The presence of the gshl-transgene was detected using gshl-specific primers.

Materials and Methods

Plant material

Clones of the untransformed INRA 717–1-B4 hybrid poplar P. × canescens (P. tremula × P. alba) and two genetically transformed lines overexpress-
ing the gshI (Escherichia coli) gene product of glutathione (GSH) in the cytosol: line ggs11 (Arisi et al., 1997) and in the chloroplasts: line lgs6, also designated as Lggs6 (Noctor et al., 1998) were micropropagated and maintained in aseptic shoot culture in vitro (Kiss et al., 2001; Koprivova et al., 2002).

Shoot culture

Poplar clones were micropropagated by nodal segments using a two step protocol. First, shoot segments (0.5 cm) were laid onto WPM media (Lloyd and McCown, 1980) supplemented with benzyl adenine (BA, 0.5 mg/l) and naphthalene acetic acid (NAA, 0.2 mg/l) followed by incubation for 28 d in a 16 h/h light/dark (1000 lx) photoperiod. Auxiliary shoots developed were dissected and transferred onto hormone-free WPM media and incubated for additional 28 d for rooting according to Kiss (Lloyd and McCown, 1980) media and incubated for 2002). Leaves of rooted shoots were used for leaf disc cultures according to Gyulai et al. (1995).

DNA extraction

Total DNA samples of 0.1 g leaf tissue in each case were extracted in CTAB (cethyltrimethyl-ammonium bromide) buffer (Murray and Thompson, 1980; Doyle and Doyle, 1990) followed by RNase-A (from bovine pancreas) treatment (Sigma, R-4875), for 30 min at 37 °C. DNA samples of ten individual of each line were pooled in one bulk according to Michelmore et al. (1991). The undiluted genomic DNA samples were subjected to PCR and tAFLP analysis.

PCR

Hot Start PCR (Erlich et al., 1991) was combined with Touchdown PCR (Don et al., 1991) using AmpliTaq Gold™ Polymerase. The reactions were carried out in a total volume of 10 µl (AFLP) and 25 µl (transgene detection), respectively, containing genomic DNA of 50 ng. For transgene analysis 1 × PCR buffer (2.5 mM MgCl2), dNTPs (200 µM each), 20 pmol each of primer and 0.5 U of Taq polymerase was used (Heinze, 1998). Touchdown PCR was performed by decreasing the annealing temperature from 66 °C to 56 °C by 0.7 °C/30 s increments per cycle with 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 30 s with a final cycle of 60 °C for 45 min (AFLP) or 72 °C for 10 min (transgene detection) and a hold at 4 °C. A minimum of three independent DNA preparations of each sample was used. Amplifications were assayed prior to AFLP analysis by agarose (1.8 %, SeaKem LE, FMC) gel electrophoresis (Owl system), stained with ethidium bromide (0.5 ng/µl) after running at 80 V in 1 × TAE buffer. Each successful reaction with scorable bands was repeated at least twice. Transilluminated gels were analyzed by the ChemiImager v 5.5 computer program (Alpha Innotech Corporation – Bio-Science Kft, Budapest, Hungary). A negative control which contained all the necessary PCR components except template DNA was included in the PCR runs.

AFLP analysis

Bulked DNA samples of ten individual shoots of each line (wild-type, cyt-ECS and chl-ECS) were pooled (5.5 µl) and subjected to tAFLP (fluorescent amplified DNA fragment length polymorphism) analysis following the method of Vos et al. (1995) with modifications (Cresswell et al., 2001; Skot et al., 2002). For digestion-ligation reactions, pairs of EcoRI–MseI restriction endonucleases (REases) were used (Table I). The sequences of the preselective primers were: EcoA: gag tgc gta cca attc-a, and MseC: gat gac tgc gtaa-c. For selective amplification 24 primer combinations were used with JOE (green) fluorescent labeled *Eco*-primers. In primer combinations 1 to 12 the primer Mse-CAC was combined with labeled primers of *Eco* -aaa, -aac, -aag, -aat, -aca, -acc, -agg, -act, -aga, -agc, -agg, -agt. In primer combinations 13 to 24 the labeled primer *Eco*-AGT was combined with primers of Mse -caa, -cag, -cat, -cca, -ccc, -cgc, -cct, -cga, -cgc, -cgg, -cgt, -cta. All oligonucleotides were supplied by Sigma Genosys and enzymes by Roche Diagnostics. Digestion of DNA, adapter ligation, non-selective and selective amplifications are described in detail by Cresswell et al. (2001) and the sequences of adapters and primers used are listed (Table I). PCR-amplified AFLP fragments were subsequently denaturated at 98 °C for 5 min, and kept at 60 °C for 30 min to allow DNA heteroduplex formation, or directly forwarded to an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using a G5 filter set in two repetitions, and analyzed by ABI PRISM Genotyper 3.7 NT software.
Table I. Restriction sites of the enzymes (rare cutter EcoRI and frequent cutter MseI), adaptors, sequence data of non-selective primer pair and the twelve active selective primer pairs (a) to (l) applied in fAFLP analysis.

<table>
<thead>
<tr>
<th>EcoRI restriction sites</th>
<th>MseI restriction sites</th>
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<tbody>
<tr>
<td>5′-NNNgaaattcNNN-3′</td>
<td>5′-NNNtttaaNNN-3′</td>
</tr>
<tr>
<td>3′-NNNcctttgaNNN-5′</td>
<td>3′-NNNaattNNN-5′</td>
</tr>
</tbody>
</table>

Adaptor sequences

5′-ctcgtagactgcgtacc
   catctgacgcatggttaa-5′

3′-nttaag-NNNcttaag-NNN-5′
   gacgatgagtcctgag

Non-selective primers

EcoA: 5′-gactgctgaacaa-3′
MseC: 5′-gatggttccgtaa-3′

Selective primers

(a) 5′-gactgctgaacaa-3′
(b) 5′-gactgctgaacaa-3′
(c) 5′-gactgctgaacaa-3′
(d) 5′-gactgctgaacaa-3′
(e) 5′-gactgctgaacaa-3′
(f) 5′-gactgctgaacaa-3′
(g) 5′-gactgctgaacaa-3′
(h) 5′-gactgctgaacaa-3′
(i) 5′-gactgctgaacaa-3′
(j) 5′-gactgctgaacaa-3′
(k) 5′-gactgctgaacaa-3′
(l) 5′-gactgctgaacaa-3′

Transgene detection

The gshI-transgene (E. coli, NCBI No. X03954) in the transformed poplar clones was amplified by the gshI-specific primer 5′-atcccggacgtatcacagg-3′ (position bp 341–359 in gshI) and its reverse 3′-gatgcaccaaacagataagg-5′ (position bp 939–920 in gshI) according to Koprivova et al. (2002). Amplification reactions were run at a volume of 25 µl containing 50 ng DNA by a PE-9700 thermocycler.

Phytoextraction

Leaves were taken from the aseptic shoot cultures and discs (8 mm) were cut and placed onto the surface of tissue culture media WPM (Lloyd and McCown, 1980) with supplementation of a concentration series of ZnSO₄ (10⁻¹ to 10⁻⁵ M) followed by incubation for 21 d according to Gyulai et al. (1995). Eight leaf discs per Petri dish (10 cm) were applied at each concentration in three repetitions. Zn-free WPM basal medium contained 63.6 µg (1.0 µM) Cu applied as CuSO₄ · 7H₂O (Mᵣ 287.4)

ICP analysis

After 21 d exposure of discs heavy metal contents (Zn and Cu) mean values of three independent measurements were determined by inductively coupled plasma emission spectrometry (Zarcinas et al., 1987).

Statistics

At least three independent parallel experiments were carried out in each case. Differences between mean values were evaluated by Student’s t-test at P = 0.05.

Results and Discussion

The in situ application of transgenic plants for phytoremediation purposes needs an in vitro vegetative multiplication period with the possibility of transgene elimination as a result of bud mutation or gene segregation. Transgenic poplar clones used in the present study were micropropagated in aseptic in vitro shoot culture by nodal segments for several years (Arisi et al., 1997; Noctor et al.,
1998) which prompted a study of the genetic stability.

The gshI-poplar (P. × canescens) was developed to overexpress the bacterial gene encoding γ-glutamylcysteine synthetase (γ-ECS, EC 3.2.3.3) which is the rate-limiting regulatory enzyme in the biosynthesis of GSH (γ-glutamyl-l-cysteinyl-glycine) (Arisi et al., 1997; Noctor et al., 1998). The increased production of GSH contributes to the antioxidative protection of plant cells against oxidative stress (Böger and Wakabayashi, 1995) caused by various environmental pollutants such as heavy metals, herbicides, fungicides (Kömives and Gullner, 2000; Koprivova et al., 2002).

gshI -transgene stability

Double strand breaks (DSBs) of DNA as the initial events of recombination occur not only in the meiotic cells but also in the somatic cells (Puchta, 1999; Frewen et al., 2000; Rohde et al., 2002) which can cause a transgene distortion. In our experiments, the gshI-transgene was found to be stable in all the tested poplar clones (ggs11 and lgl6) indicating no transgene elimination or segregation (Fig. 1).

AFLP analysis

Cut clone stability was analyzed by fAFLP. Twelve of the 24 selective primer combinations applied were effective in producing sharp and reproducible AFLP patterns (Fig. 2). A total of 682 common AFLP fragments were detected (Table II). The average number of ALF fragments per selective primer pair was 56.6 which falls in the same range as was observed in an analysis of black poplar (P. nigra) clones with a total of 104 AFLP fragments generated by two primer pairs (Smul-

Table II. Total numbers of the fAFLP fragments (rel. intensity over 100 units, at the range of 150–600 bp) of the gshI-transgenic poplar (P. × canescens) clones of ggs11 (cyt-ECS) and lgl6 (chl-ECS) compared to the non-transformed (contr.) clone. The selective AFLP primer combinations (see Table I) were: Mse-CAC combined with -Eco-AAT (a), -Eco-ACC (b), and -Eco-AGT (c); and Eco-AGT combined with -Mse-CA (d), -Mse-CAG (e), -Mse-CAT (f), -Mse-CCC (g), -Mse-CCT (h), -Mse-CGA (i), -Mse-CCG (j), -Mse-CTA (k) and -Mse-CTC (l).

<table>
<thead>
<tr>
<th>Clones</th>
<th>No. of fAFLP fragments/ selective primer pairs (a to l)</th>
</tr>
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<tbody>
<tr>
<td>ggs11</td>
<td>a b c d e f g h i j k l total</td>
</tr>
<tr>
<td></td>
<td>25 6 17 30 25 35 16 14 11 9 17 21 226</td>
</tr>
<tr>
<td>lgl6</td>
<td>25 6 17 30 25 35 19 14 11 9 17 21 229</td>
</tr>
<tr>
<td>contr.</td>
<td>25 6 17 30 25 35 17 14 11 9 17 21 227</td>
</tr>
</tbody>
</table>

Table II: Total numbers of the fAFLP fragments (rel. intensity over 100 units, at the range of 150–600 bp) of the gshI-transgenic poplar (P. × canescens) clones of ggs11 (cyt-ECS) and lgl6 (chl-ECS) compared to the non-transformed (contr.) clone. The selective AFLP primer combinations (see Table I) were: Mse-CAC combined with -Eco-AAT (a), -Eco-ACC (b), and -Eco-AGT (c); and Eco-AGT combined with -Mse-CA (d), -Mse-CAG (e), -Mse-CAT (f), -Mse-CCC (g), -Mse-CCT (h), -Mse-CGA (i), -Mse-CCG (j), -Mse-CTA (k) and -Mse-CTC (l).

Phytoremediation in vitro

Field trials of transgenic plants are under strict regulation, hence an in vitro analysis for phytoremediation capacity as done in the present study is essential prior to any field test. The heavy metal contents of leaf discs analyzed in the poplar clones following Zn stress (10^{-1} to 10^{-5} m) showed a complex pattern (Table III). The Zn uptake

Figure 1. PCR detection of a part (561 bp) of the gshI-transgene (E. coli. NCBI No. X03954) in the transformed gshI-poplar (Populus × canescens) clones ggs11 (cyt-ECS), lgl6 (chl-ECS) and the non-transformed (contr.) clones. Primer pair was: 5′-ata cgc gta tca cag g-3′ (position bp 341–359 in gshI) and 3′-gat gca cca aac aga taa gg-5′ (position bp 939–920 in gshI). Arrows indicate the transgene.
increased linearly with the exogenously applied concentrations of ZnSO₄ in all clones. None of the gshI-transgenic clones (ggs11 and lgl6) showed elevated Zn uptake capacity.

An unexpected Zn-stimulated Cu uptake was observed in the transgenic cyt-ECS (ggs11) clone with a peak at 10⁻² M ZnSO₄ concentration (331.6%). An enhanced Cd uptake in the ggs11 poplar clone was also found in ex vitro experiments (Koprivova et al., 2002). With regard to metallothioneins (MTs), metal transporter proteins (MTPs), phytochelatins (PCs) and the precursor of sulphur-rich peptides like glutathione (GSH), no genes conferring metal resistance have been identified in any of the naturally occurring hyper-accumulating species. These species include Thlaspi caerulescens, as a known Ni and Zn hyper-accumulator, and Brassica juncea as a known Pb accumulator (Gleba et al., 1999).

Reactive metal ions such as Cu²⁺ and Zn²⁺ can interfere with sulphhydryl (SH) groups of proteins, therefore should be inactivated in the cell through sequestration by cysteine-rich PCs, MTs and sulfo-de. The cysteiny1 sulphur in the peptides and proteins function as ligands for the metal ion as supported by SO₄²⁻ of the zinc form (ZnSO₄) applied in the present study. Ultimately, the metals are bound in polynuclear metal thiolate clusters (Dame-
Table III. The mean values (n = 3) of heavy metal (Zn and Cu) contents (µg/g dry matter, DM) in aseptic leaf discs of untransformed poplar clones P. × canescens (contr.) and two transgenic lines, ggs11 (cyt-ECS) and lgl6 (chl-ECS), after 21 d of exposure to concentration series of ZnSO₄ (10⁻¹ to 10⁻⁵ M) using in vitro leaf disc cultures on Zn-free WPM basal medium containing 63.6 µg/g (1.0 µM) Cu.

<table>
<thead>
<tr>
<th></th>
<th>Zn µg/g DM</th>
<th>(%)</th>
<th>Cu µg/g DM</th>
<th>(%)</th>
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<tr>
<td>contr.</td>
<td>53,643.37</td>
<td>100.0</td>
<td>32.47</td>
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<td>107.76</td>
<td>331.9</td>
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<td>10.4</td>
<td>32.78</td>
<td>100.9</td>
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<td>10⁻⁴</td>
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<td>21.16</td>
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<tr>
<td>ggs11</td>
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<td>114.5</td>
</tr>
<tr>
<td>10⁻¹</td>
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<td>94.9</td>
<td>37.17</td>
<td>114.5</td>
</tr>
<tr>
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<td>16.0</td>
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<td>10⁻⁵</td>
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<td>0.4</td>
<td>15.56</td>
<td>47.9</td>
</tr>
</tbody>
</table>

Ours results confirm that under in vitro conditions the ZnSO₄ stimulated accumulation of Cu in the transgenic ggs11 (cyt-ECS) clone is improved by the gsh1-transgene which thus provides potential to develop transgenic phytoextractor poplar for ex vitro purposes.

Acknowledgements

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