AFLP Analysis and Improved Phytoextraction Capacity of Transgenic *gsh*I-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 (*ggs*11 and *lgl*6) were studied as *in vitro* leaf disc cultures. Presence of the *gsh*I-transgene in the transformed clones was detected in PCR reactions using *gsh*I-specific primers. Clone stability was determined by fAFLP (*f*luorescent *a*mplified DNA *f*ragment *l*ength *p*olymorphism) analysis. In total, 682 AFLP fragments were identified generated by twelve selective primer pairs after *Eco*RI–*Mse*I digestion. Four fragments generated by *Eco*AGT–*Mse*CCC 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 $ZnSO_4$ (10^{-1} to 10^{-5} M) incubated for 21 days on aseptic tissue culture media WPM containing 1 μM Cu. Zn^{2+} caused phytotoxicity only at high concentrations (10^{-1} to 10^{-2} M). The transgenic poplar cyt-ECS (*ggs*11) 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 *gsh*I-transgenic poplars may be suitable for phytoremediation of soils contaminated with zinc and copper.

Key words: Phytoextraction, cyt-ECS (*ggs*11), chl-ECS (*lgl*6)

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. termula* × *P. alba*) and two transgenic lines overexpressing γ-ECS either in the cytosol (line *ggs*11 of Arisi *et al.,* 1997) or in the chloroplasts (line *lgl*6 of Noctor *et al.,* 1998) following a 21 day exposure to $ZnSO_4$ (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 *gsh*I-transgene was detected using *gsh*I-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-

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ing the *gsh*I (*Escherichia coli*) gene product of glutathione (GSH) in the cytosol: line *ggs*11 (Arisi *et al.,* 1997) and in the chloroplasts: line *lgl*6, also designated as *Lggs*6 (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/8 h light/dark (1000 lx) photoperiod. Auxiliary shoots developed were dissected and transferred onto hormone-free WPM (Lloyd and McCown, 1980) media and incubated for additional 28 d for rooting according to Kiss *et al.* (2001). 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 (cethyltrimethylammonium 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 fAFLP 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 $MgCl_2$), dNTPs (200 μM each), 20 pmol of each 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 ChemilImager 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 fAFLP (*f*luorescent *a*mplified DNA *f*ragment *l*ength *p*olymorphism) analysis following the method of Vos *et al.* (1995) with modifications (Cresswell *et al.,* 2001; Skøt *et al.,* 2002). For digestion-ligation reactions, pairs of *Eco*RI–*Mse*I restriction endonucleases (REases) were used (Table I). The sequences of the preselective primers were: *Eco*A: gac tgc gta cca attc-a, and *Mse*C: gat gag tcc tga 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, -ccg, -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 *Eco*RI and frequent cutter *Mse*I), adaptors, sequence data of non-selective primer pair and the twelve active selective primer pairs (a) to (l) applied in fAFLP analysis.

	*Eco*RI	*Mse*I
Restriction sites		
	5′-NNNgaattcNNN-3′ 3′-NNNcttaagNNN-5′	5′-NNNttaaNNN-3′ 3′-NNNaattNNN-5′
Adaptor sequences		
	5′-ctcgtagactgcgtacc catctgacgcatggttaa-5′	5′-gacgatgagtcctgag tactcaggactcat-5′
Non-selective primers		
	*Eco*A: 5′-gac tgc gta cca attc-a-3′	*Mse*C: 5′-gat gag tcc tga gtaa-c-3′
Selective primers		
(a)	5′-gac tgc gta cca attc-aat-3′	
(b)	5′-gac tgc gta cca attc-acc-3′	5′-gat gag tcc tga gtaa-cac-3′
(c)	5′-gac tgc gta cca attc-agt-3′	
(d)		5′-gat gag tcc tga gtaa-caa-3′
(e)		5′-gat gag tcc tga gtaa-cag-3′
(f)		5′-gat gag tcc tga gtaa-cat-3′
(g)	5′-gac tgc gta cca attc-agt-3′	5′-gat gag tcc tga gtaa-ccc-3′
(h)		5′-gat gag tcc tga gtaa-cct-3′
(i)		5′-gat gag tcc tga gtaa-cga-3′
(j)		5′-gat gag tcc tga gtaa-cgc-3′
(k)		5′-gat gag tcc tga gtaa-cta-3′
(l)		5′-gat gag tcc tga gtaa-ctc-3′

Transgene detection

The *gsh*I-transgene (*E. coli*, NCBI No. X03954) in the transformed poplar clones was amplified by the *gsh*I-specific primer 5′-atcccggacgtatcacagg-3′ (position bp 341–359 in *gsh*I) and its reverse 3′-gatgcaccaaacagataagg-5′ (position bp 939–920 in *gsh*I) 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 $ZnSO_4$ (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 $CuSO_4 \cdot 7H_2O$ (M_r 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 *gsh*I-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-L-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 experimnts, the *gsh*I-transgene was found to be stable in all the tested poplar clones (*ggs*11 and *lgl*6) 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 (Smulders *et al.,* 2002). The most effective primer pair was found to be the *Eco*AGT–*Mse*CAT resulting in 35 AFLP fragments (Table II). In a comparative preliminary study with wheat (*Triticum aestivum* L.) the *Eco*AGT–*Mse*CAC produced the most ALF fragments (47).

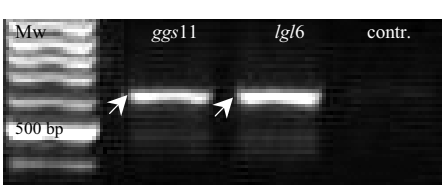

Figure 1. PCR detection of a part (561 bp) of the *gsh*I-transgene (*E.* coli. NCBI No. X03954) in the transformed *gsh*I-poplar (*Populus* × *canescens*) clones *ggs*11 (cyt-ECS), *lgl*6 (chl-ECS) and the non-transformed (contr.) clones. Primer pair was: 5′-atc ccg gac gta tca cag g-3′ (position bp 341–359 in *gsh*I) and 3′-gat gca cca aac aga taa gg-5′ (position bp 939–920 in *gsh*I). Arrows indicate the transgene.

Table II. Total numbers of the fAFLP fragments (rel. intensity over 100 units, at the range of 150–600 bp) of the *gsh*I-transgenic poplar (*P.* × *canescens)* clones of *ggs*11 (cyt-ECS) and *lgl*6 (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-CAA (d), -*Mse*-CAG (e), -*Mse*-CAT (f), -*Mse*-CCC (g), -*Mse*-CCT (h), -*Mse*-CGA (i), -*Mse*-CGC (j), -*Mse*-CTA (k) and -*Mse*-CTC (l).

Clones	No. of fAFLP fragments/ selective primer pairs (a to l)												
	a	b	c	d	e	f	g	h	i	j	k	l	total
*ggs*11	25	6	17	30	25	35	16	14	11	9	17	21	226
*lgl*61	25	6	17	30	25	35	19	14	11	9	17	21	229
contr.	25	6	17	30	25	35	17	14	11	9	17	21	227

These results appear to reflect some relationship between AFLP fragment number and genome size. The genome size of wheat (*Triticum aestivum*) is very large (2n = 6x = 42; 16×10^9 bp, which is equal to 16.58 pg as calculated by the equation 965 Mbp = 1 pg DNA) compared with the extremely small genome size of poplar (2n = 4x = 38; 5.5×10^8 bp; 2C = 1.1 pg) (Cervera *et al.,* 2001; Taylor, 2002).

Four AFLP fragments were polymorphic of the total 682 fragments (99.4% genetic similarity) which appeared in the untransformed clone and in the *gsh*I-transgenic *lgl*6 clone generated by *Eco*AGT–*Mse*CCC (Table II, Fig. 2). Despite trees with high bud mutation rate (Rohde *et al.,* 2002) such as apple, this result indicates an unexpectedly low level of bud mutation in *P.* × *canescens* cut clones thus providing genetically uniform plant material for phytoextraction purposes.

Phytoremediation in vitro

Field trials of transgenic plants are under strict regulation, hence an *in vitro* analysis for phytoextraction 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

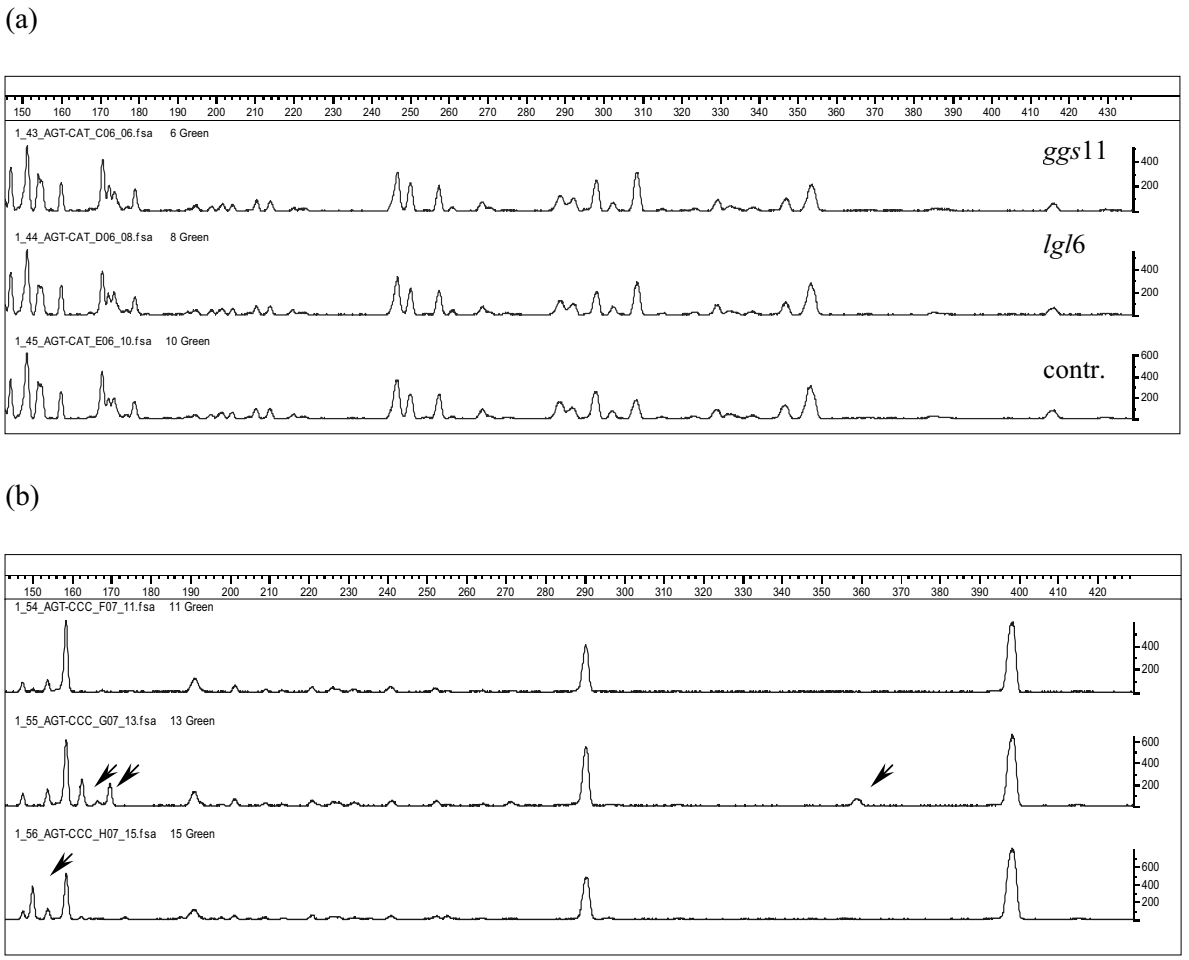

Fig. 2. Samples of fAFLP analysis with monomorphic (*Eco*AGT–*Mse*CAT) (a) and polymorphic (*Eco*AGT–*Mse*CCC) (b) fragment patterns (150 to 430 bp) of transgenic *gsh*I-poplar (*Populus* × *canescens*) clones of *ggs11* (cyt-ECS) and *lgl6* (chl-ECS), and the non-transformed (contr) clone (rel. intensity 200 to 400–600). Arrows indicate polymorphic fragments.

increased linearly with the exogenously applied concentrations of $ZnSO_4$ in all clones. None of the *gsh*I-transgenic clones (*ggs*11 and *lgl*6) showed elevated Zn uptake capacity.

An unexpected Zn-stimulated Cu uptake was observed in the transgenic cyt-ECS (*ggs*11) clone with a peak at 10^{-2} M $ZnSO_4$ concentration (331.6%). An enhanced Cd uptake in the *ggs*11 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 hyperaccumulating species. These species include *Thlaspi caerulescens*, as a known Ni and Zn hyperaccumulator, and *Brassica juncea* as a known Pb accumulator (Gleba *et al.*, 1999).

Reactive metal ions such as Cu^{2+} and Zn^{2+} can interfere with sulfhydryl (SH) groups of proteins, therefore should be inactivated in the cell through sequestration by cysteine-rich PCs, MTs and sulfide. The cysteinyl sulphur in the peptides and proteins function as ligands for the metal ion as supported by SO_4^{2-} of the zinc form ($ZnSO_4$) 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, *ggs*11 (cyt-ECS) and *lgl*6 (chl-ECS), after 21 d of exposure to concentration series of $ZnSO_4$ (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.

	Zn		Cu	
	[μg/g DM]	(%)	[μg/g DM]	(%)
contr.				
10^{-1}	53,643.37	100.0	32.47	100.0
10^{-2}	26,822.07	49.9	30.59	94.2
10^{-3}	8,434.80	15.7	13.96	43.0
10^{-4}	907.48	1.7	10.95	33.7
10^{-5}	171.97	0.3	9.31	28.7
*ggs*11				
10^{-1}	51,729.04	96.4	96.32	296.6
10^{-2}	32,124.33	59.9	107.76	331.9
10^{-3}	5,561.96	10.4	32.78	100.9
10^{-4}	1,213.45	2.1	21.16	59.7
10^{-5}	258.73	0.5	16.51	50.8
*lgl*6				
10^{-1}	50,751.02	94.9	37.17	114.5
10^{-2}	25,973.51	48.4	39.87	122.7
10^{-3}	8,601.29	16.0	31.77	97.8
10^{-4}	1,013.98	1.8	24.91	76.7
10^{-5}	218.90	0.4	15.56	47.9

ron and Harrison, 1998). Zn is considered to be an activator of PCs *in vitro* and *in vivo* (Grill *et al.,* 1988; Maitani *et al.,* 1996). The capacity to synthesize PCs is supposed to be present in all higher plants. Their synthesis from GSH and different forms of GSHs such as homo-glutathione, hydroxymethyl-glutathione or γ-glutamylcysteine is catalyzed by a constitutive transpeptidase enzyme, the phytochelatin synthase (PCS). PCS requires a post-translational activation by heavy metals, in particular Cd, Ag, Pb, Cu, Hg, Zn, Sn, Au, and As, both *in vivo* and *in vitro* (Schat *et al.,* 2002; Grill *et al.,* 1988; Maitani *et al.,* 1996; Chen *et al.,* 1997). Nevertheless, a PC-independent sequestration mechanism was also reported in plants (Schat *et al.,* 2002).

Our results confirm that under *in vitro* conditions the $ZnSO_4$ stimulated accumulation of Cu in the transgenic *ggs*11 (cyt-ECS) clone is improved by the *gsh*I-transgene which thus provides potential to develop transgenic phytoextractor poplar for *ex vitro* purposes.

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