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Chapter 8

GENE UP-REGULATION BY DNA DEMETHYLATION IN 35S-*GSHI* TRANSGENIC POPLARS (*POPULUS* X *CANESCENS*)

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ABSTRACT

Gene expression levels of transgene 35S-*gsh*I (γ-glutamylcysteine synthetase) cloned from *E. coli*, and the endogenous gene *gsh*1 of poplar (*Populus* x *canescens*) were up-regulated by the DNA demethylating agent DHAC (5,6-dihydro-5'-azacytidine hydrochloride) (10^{-4} M for 7 days) in aseptic leaf discs cultures. Two 35S-*gsh*I-transgenic (6*lgl* and 11*ggs*) and wild type (WT) poplar clones were used. The efficiency of gene upregulation was also analyzed under herbicide paraquat stress (4 x 10^{-7} M). Levels of *gsh*I-mRNA and *gsh*1-mRNA were determined by RT-qPCR (reverse transcriptase quantitative PCR) after cDNA synthesis. For internal control, the constitutively expressed housekeeping poplar genes *α-tubulin* and *actin* were used, and the $2^{-\Delta\Delta Ct}$ method was applied for data analysis. In long term DHAC treatment (21 days), a morphogenetic response of *de novo* root development was observed on leaf discs in a wide concentration range of DHAC (10^{-8} to 10^{-6} M). Adventitious shoots (11*ggs* clone) also emerged from leaf discs after a combined treatment with DHAC (10^{-4} M) and paraquat (10^{-7} M). Shoots were dissected, rooted and transplanted in glass houses for further analyses for phytoremediation capacity. Since DNA methylation patterns are inherited (*epigenetic memory*), these poplar plants with increased gene expression levels of both transgene 35S-*gsh*I and endogenous gene *gsh*1 provide novel plant sources for *in situ* application.

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ABBREVIATIONS:

5-mC: *5-*methyl*C*ytosine;
AdoMet: S-*Ad*en*o*syl methionine dependent *Met*hyltransferases;
C: *C*ytosine nucleotide of DNA;
BA: *B*enzyl *A*denine;
cDNA: *C*opia *DNA*;
DHAC: 5,6-*Dih*ydro-5'-*A*za*C*ytidine hydrochloride;
E. coli: bacterium *Escherichia coli*;
GMO: *G*enetically *M*odified *O*rganism;
γ-ECS: γ-*G*lutamyl*C*ysteine *S*ynthetase enzyme encoded by gene *gsh*;
GSH: glutathione tripeptide (γ-L-glutamyl-L-cysteinyl-glycine);
MTases: DNA *M*ethyl*T*ransfer*ases*;
NAA: *N*aphthalene *A*cetic *A*cid;
PCR: *P*olymerase *C*hain *R*eaction;
NTC: *N*on *T*emplate *C*ontrol;
PQ: *P*ara*Q*uat (*N,N'-Dimethyl-4,4'-bipyridinium dichloride*; *syn*.: methylviologen);
RT-qPCR: *R*everse *T*ranscription based *Q*uantitative PCR;
*rbc*S: gene for RBCS (*syn*.: RuBPCase SSU, *R*ib*u*lose-1,5-*B*isphos*p*hate *C*arboxylase *S*mall *Sub*unit);
TGS: *T*ranscriptional *G*ene *S*ilencing;
PTGS: *P*ost-*T*ranscriptional *G*ene *S*ilencing;
6*lgl* and 11*ggs*: *gsh*I-transformed poplar (*Populus* x *canescens*) clones;
WT: *W*ild *T*ype.

INTRODUCTION

Poplars (*Populus* ssp) are capable of removing and degrading toxic substances and heavy metals from polluted soils through phytoremediation due to the extensive root system, high water uptake capacity, rapid growth and large biomass production (Kőmíves *et al.* 1998; Burken and Schnoor 1998; Di Baccio *et al.* 2003; Gyulai *et al.* 2005). The oxidative stress tolerance capacity of *Populus* x *canescens* clones has been significantly increased recently by genetic transformation with the 35S-*gsh*I gene, which encodes for γ-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2) (Leple *et al.* 1992; Arisi *et al.* 1997; Noctor *et al.* 1998) cloned from *E. coli* (Watanabe *et al.* 1986; NCBI X03954). The transformed poplar clones (6*lgl* and 11*ggs*) showed higher levels of GSH and its precursor of γ-L-glutamyl-L-cysteine (γ-EC) than the WT, which led to an improved detoxification capacity against various environmental pollutants (Noctor *et al.* 1998; Peuke and Rennenberg 2005).

Transgenic clones (6*lgl* and 11*ggs*) studied have been maintained in aseptic shoot cultures for about a decade without 35S-*gsh*I transgene elimination (Gyulai *et al.* 2005; Bittsánszky *et al.* 2006). However, transgenes have been exposed to gene silencing processes either in the region of the constitutive CaMV-35S promoter, or in the coding region of the genes. The CaMV-35S promoter is considered to be a constitutive promoter; nevertheless its expression might be modulated by photoperiod, temperature, developmental stages and DNA methylation (Benfey and Chua 1990; Zardo *et al*. 1999; Schnurr and Guerra 2000; Obertello *et al.* 2005; Yang *et al.* 2005). By the application of a DNA-demethylation treatment, as in the study presented here, this natural gene silencing process can be reversed.

DNA Methylation:

DNA methylation of plant genes is a general endogenous process for gene silencing catalyzed by nuclear enzyme family MTases (DNMT - *DN*A *m*ethyl*t*ransferases in mammals; and DNMT-orthologue MET - *me*thyl*t*ransferases, CMT - *c*ytosine *m*ethyl*t*ransferases, and DRM in plants). These enzymes transfer a methyl group (CH_3) mainly from S-*ad*en*o*syl *met*hionine (AdoMet-dependent methyltransferases) either to the position of cytosine-C_5 (EC 2.1.1.73), cytosine-N_4 (E.C. 2.1.113) or adenine-N_6 of DNA (E.C. 2.1.2.72) (Pósfai *et al.* 1989; Cheng and Roberts 2001; Vaucheret 2006). The cytosine methylation to 5-methylcytosine (5mC) is the most frequent process resulting in down regulation of both genes and transgenes. DNA methylation is frequently directed by RdDM (*R*NA-*d*irected *D*NA *m*ethylation) (Linn *et al.* 1990; Kumpatla *et al.* 1997; Castilho *et al.* 1999; Mathieu and Bender 2004).

Genetically, DNA methylation patterns are associated with two types of gene silencing in plants, the *t*ranscriptional *g*ene *s*ilencing (TGS) caused by methylation in the promoters of genes, and the *p*ost-*t*ranscriptional *g*ene *s*ilencing (PTGS; called RNA interference in animals, RNAi) caused by methylation in the coding region of genes (Elmayan and Vaucheret 1996; Kooter *et al*. 1999). DNA methylation is not universal, as in the insect fruit fly *Drosophyla* where is does not occur (Hirochika *et al.* 2000). The meiotically heritable TGS (Park *et al.* 1996) results in methylation '*inprints*', whereas PTGS, which is not heritable, affects the stability of the RNA transcripts, at a translational level under the control of RNAi (Napoli *et al.* 1990; Krol *et al.* 1990; Fire *et al*. 1998; Agrawal *et al.* 2003). The PTGS (RNAi) system allows cells to control endogenous nucleic acids (e.g. transposons) and exogenous (e.g. virus, transgenes) invaders (Vaucheret 2006).

DNA methyltransferases can be blocked exogenously by MTase-inhibitors, which act via covalent complex formation (Wolfe and Matzke 1999; Fan *et al.* 2005) when present either in the cytosol or when incorporated into DNA (as base analogues) resulting in gene reactivation (Bird 2002; Issa and Kantarjian 2005). Gene reactivation through the application of thymidine analogues can also occur in demethylation-independent gene up-regulations (Fan *et al.* 2005).

The study presented here aims to achieve the simultaneous analysis of gene up-regulation of both the prokarytic gene 35S-*gsh*I and the endogenous eukaryotic poplar gene *gsh*1 of two 35S-*gsh*I-transformant (6*lgl* and 11*ggs*) poplar clones (Leple *et al.* 1992; Arisi *et al.* 1997; Noctor *et al.* 1998) and the WT, in response to DHAC-treatment (10^{-4} M) combined with paraquat (4×10^{-7} M) stress.

CONCLUSION

Sequence Analysis of *gsh* Genes:

Comparative analysis of sequence alignments (Figure 1), DNA nucleotide diversities (Figure 2), amino acid contents (Figure 3) and dendrogram analyses based on nucleotide and amino acid sequences of both *gsh*1 and *gsh*I genes of prokaryotic and eukaryotic plant species (Figure 4) reflects different rates of DNA nucleotide substitutions due to the consequences of evolution and adaptation (Baldwin 1896; Amirnovin 1997).

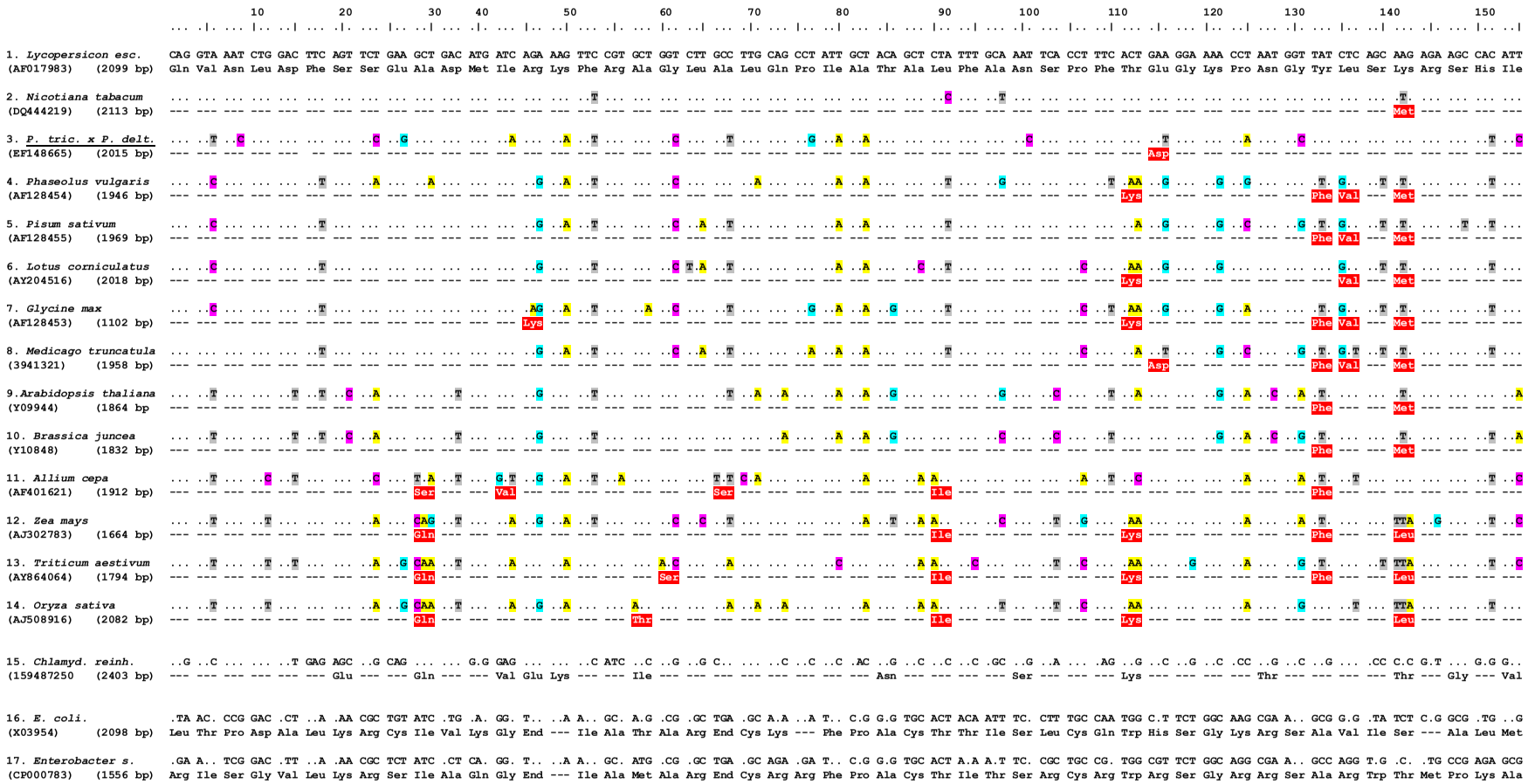

Figure 1. Multiple sequence alignments and box shade of parts (150 nt) of *gsh*1-cDNA triplets (top lines) of plants (1 – 14); alga *Chlamydomonas reinhardi* (15.); and the non-homologous *gsh*I-cDNAs of prokaryotes of *Escherichia coli* (16.) and *E. sakazaki* (17.) aligned with translated amino acid (50) sequences (below). Synonymous and non-synonymous (amino acid red box) nucleotide substitutions are indicated. Sequences of NCBI databases (Altschul *et al.* 1997) were analyzed by BioEdit program (Hall 1999).

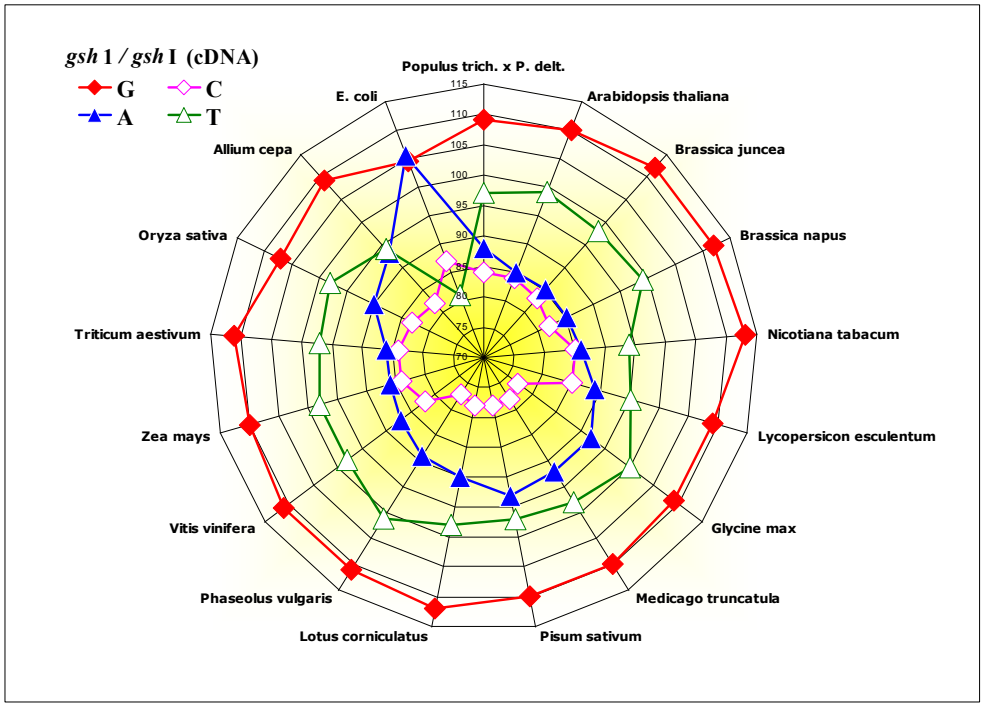

Figure 2. Nucleotide (G, C, A, T) compositions of *gsh*1-cDNAs of sixteen plant species and the non-homologous *gsh*I-cDNA of prokaryote *E. coli*. (accession # are indicated in Figure 1).

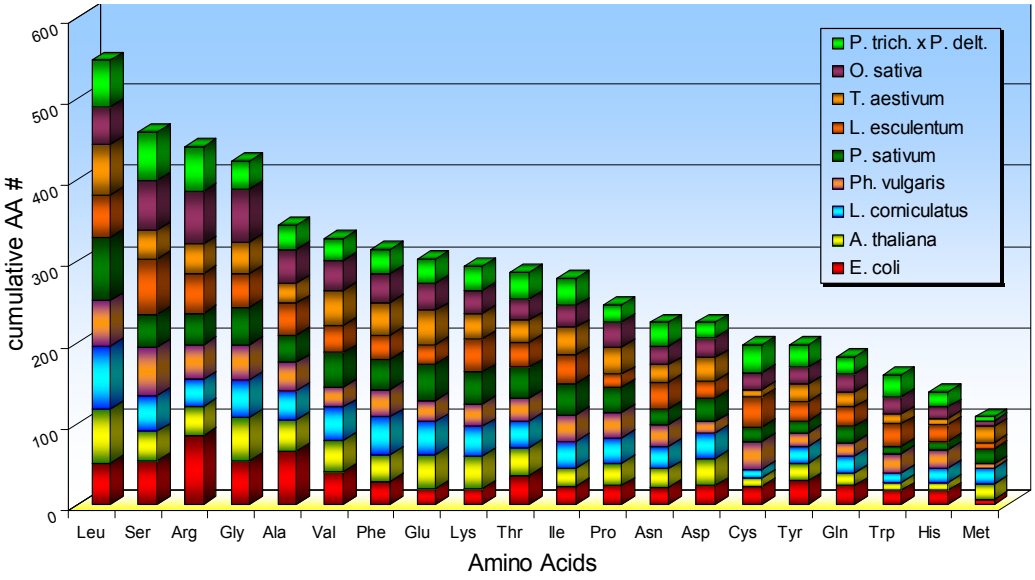

Figure 3. Comparative amino acid (AA) content of complete sequences of the leucine (Leu)-rich GHS1 enzymes of eight plant species compared to non-homologous GSHI of *E. coli* (accession # are indicated in Figure 1).

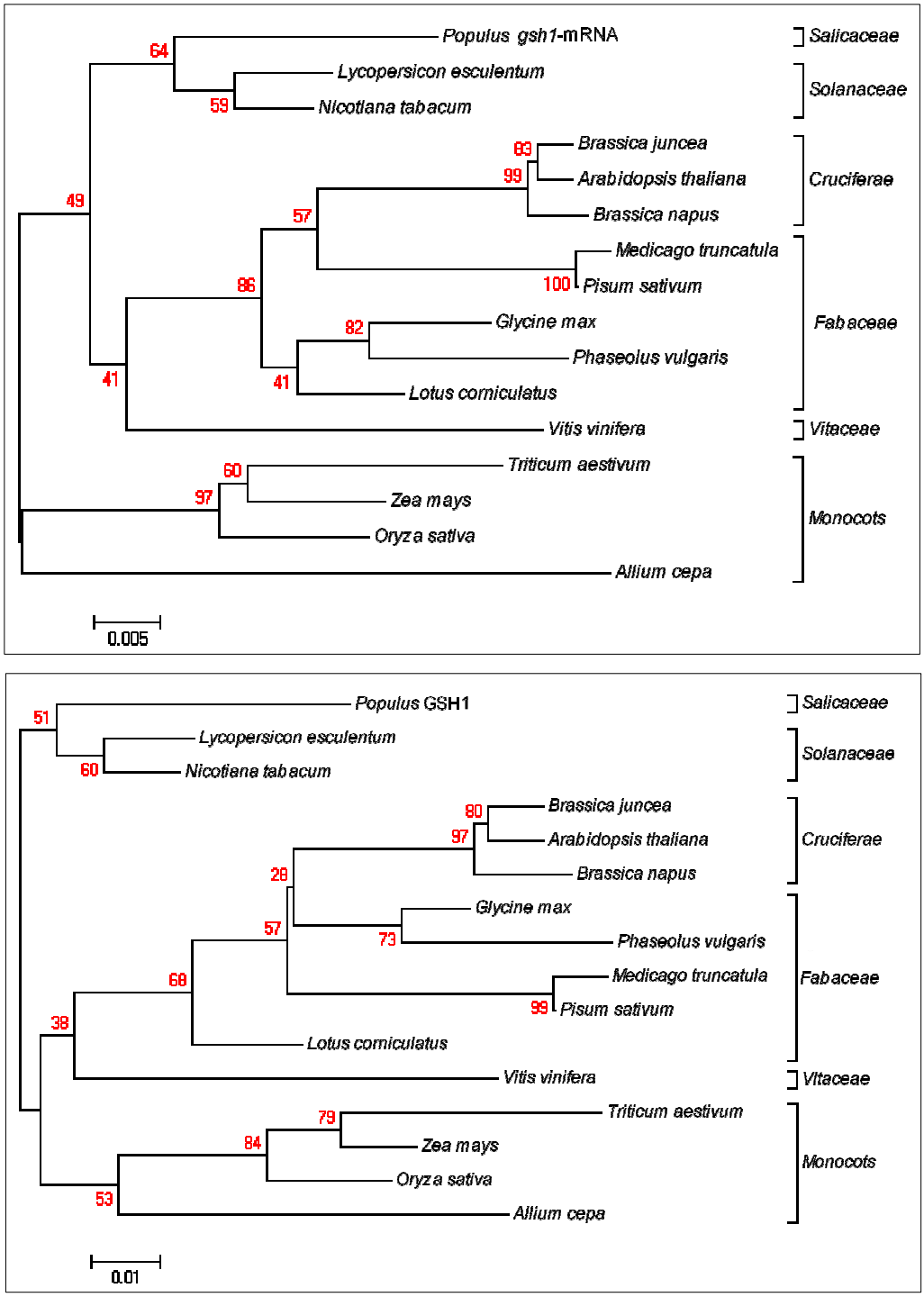

Figure 4. Distance trees (MEGA4; Tamura *et al.* 2007) of sixteen plant *gsh*1-cDNA (derived form *gsh*1-mRNAs) (left), and the translated GSH1 protein sequences (right) with boot strap analyses (1000). Size bars indicate numbers of substitutions per locus (accession numbers are indicated in Figure 1

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As a result of redundancy of genetic code, which provides protection against mutations, synonymous (*silent*) nucleotide substitutions (*point mutation*) in the protein coding triplets do not cause amino acid replacement in the protein sequences (Wong 1981 in 1988). Sample sections of the plant DNA and cDNA of *gsh*1 genes and the non-homologous prokaryotic *gsh*I gene of *E. coli* revealed several synonymous and non-synonymous nucleotide substitutions (Figure 1). In general, nucleotide substitution is a neutral processes as certain codons are translated more frequently (*codon use preferences*) which might be related to adaptation capacity (*fitness*) of the species (Palmer 1997); however, distance tree of *gsh*1 sequences of plant species showed correlation with taxonomical lineages, with dominance of *Brassicaceae* species (Figure 4). Both cDNA and protein sequences of poplar showed the closest linkage to *Solanaceae* species (tomato and tobacco), however nucleotide substitution scale (0.005) showed double level of that of amino acids (0.01) (Figure 4).

The Role of Reduced Glutathione (GSH) in Plant Cell:

Reduced glutathione (GSH), the major non-protein, tripeptide thiol compound (Meister and Anderson 1983), present in high concentrations (mM) of plant tissues, is regarded as one of the major determinants of cellular redox homeostasis (Rusznyák and Szent-Györgyi 1936) with a key role in the detoxification processes (Strohm *et al.* 1995; Foyer and Noctor 2005; Mullineaux and Rausch 2005; Wachter *et al.* 2005). Glutathione becomes reactive as the thiol group of cysteine residue releases electrons to unstable electron acceptor molecules, such as reactive oxygen species. The two reduced GSH molecules form glutathione disulfide (GSSG) which regenerates to GSH again by the catalysis of gluathione reductase. Cells under regular conditions ('healthy cells') have a GSH / GSSG ratio over about 90 %. Changes in the GSSG / GSH ratio indicates that plants are exposed to oxidative stress.

Up-Regulation of *gsh* Genes by DNA Demethylation:

Gene expression levels can be easily triggered *in vitro* by up-regulation (*syn*.: reactivation, hypomethylation and demethylation) or down-regulation (*syn*.: silencing, hypermethylation and suppression) of the gene of interest (Goffin and Eisenhauer 2002; Columbus 2006). For gene up-regulation, MTase-inhibitors such as the structurally modified cytosine analogues zebularine, 5-azacytidine (5-azaC), 5-aza-2'-deoxycytidine and DHAC have been shown to be highly effective (Chen and Pikaard 1997; Cao *et al.* 2000; Cheng and Roberts 2001). Alternatively, the drug 3-aminobenzamide has been used for gene down-regulation in a series of genes by DNA hypermethylation (Zardo *et al.* 1999). In the study presented here, DHAC was applied due to its high chemical stability in aqueous solutions of long term treatments (Goffin and Eisenhauer 2002).

RT-qPCR:

Reverse transcription (RT) followed by qPCR analysis has proven to be an exceptionally sensitive method compared to RNA-DNA hybridization (*Northern blot*) (Alwine *et al*. 1977)

for both absolute and relative quantification of gene expressions (Veres *et al*. 1987). In the study presented here, relative quantification was used as it is more relevant to compare expression levels of different treatments rather than absolute quantification (Pfaffl 2001; Livak and Schmittgen 2001; Tichopad *et al*. 2003).

The relative gene expression level of 35S-*gsh*I transgene in the 6*lgl* clone showed a 13.5-fold increase over the 11*ggs* clone (1.0) which was doubled (1.8-fold) in the DHAC-treated 6*lgl* samples (23.7) but not in the 11*ggs* clone (0.4-fold) (Figure 5). This expression pattern was contrary to the observation of relative copy numbers of the transgene 35S-*gsh*I as it was lower in the 6*lgl* clone (1.0) than in the 11*ggs* samples (1.69) (Table 1). These results might be due to the 35S-*gsh*I transgene construct of the 6*lgl* clone which included an additional targeting sequence (from 32nd nt to 202nd nt of the total 206 bp; NCBI M25614) of a transit peptide (TP) (57 amino acids) gene of *rbc*S (RuBPCase SSU: small subunit of RuBPCase, ribulose-1,5-bisphosphate carboxylase) (Leple *et al.* 1992; Arisi *et al*. 1997; Noctor *et al*. 1998; Bittsánszky *et al*. 2007). TPs as N-terminal extensions of transgene-products facilitate targeting and translocation of the cytosolically synthesized RBCS-GSH complex into plastids, which has also been found to be more effective in transgenic tobacco compared to regular transgene cassettes (Creissen *et al*. 1996; Bruce 2001; Wachter *et al*. 2005).

Gene expression levels of the endogenous poplar gene *gsh*1 also showed high responsiveness to DHAC-induced demethylation with an extremely high expression in the untransformed WT poplar clone (19.8-fold). A competition in the reactivation capacity between transgene 35S-*gsh*I and poplar *gsh*1 of 6*lgl* clone occurred as the gene expression of transgene 35S-*gsh*I increased from a high rel. expression level (13.5) up by about a two-fold (1.8 times) rate to 23.7 compared to the poplar *gsh*1 gene that increased by an 8.7-fold increment from a lower level (1.3) to 13.9 rel. unit (Figure 5). These results might indicate differences in DNA methylating capacity between transgenes and proper wild type genes as a type of cosuppression (Krol *et al*. 1990; Anand *et al*. 2003; Baumberger and Baulcombe 2005).

The phenomenon of co-suppression was originally discovered in plants (Napoli *et al*. 1990; Krol *et al*. 1990). Napoli *et al*. (1990) intended to up-regulate the activity of a gene for chalcone synthase (*chs*A), an enzyme involved in the production of anthocyanin pigments. Some of the transgenic petunia plants harboring the *chs*A coding region under the control of a CaMV-35S promoter lost both endogene and transgene chalcone synthase activity. As a result, many of the flowers were variegated in color or developed white sectors (Napoli *et al*. 1990). The molecular analysis discovered that transgene expression led to the formation of dsRNA, which, in turn, initiated PTGS (Metzlaff *et al*. 1997).

The interaction between transgenes and endogenous genes might also have resulted either from multicopy integration of transgene at the same locus (Assaad *et al*. 1993), the position effects due to random integration (Yang *et al*. 2005), the AT/CG composition of the transgene (Matzke and Matzke 1998), the presence of inverted repeats in the integration site (Stam *et al*. 1997), the overexpression of the transgene (Que *et al*. 1997), or the environmental conditions (Meyer and Heidman 1994).

Selective methylation capacity of transgene homologues has also been observed in supertransformants (a type of transgene pyramiding) where the resident transgene promoter *Ubi*1 from the first transformation remained unmethylated whereas the incoming 35S transgene promoter of the subsequent transformation was silenced in rice (Yang *et al*. 2005).

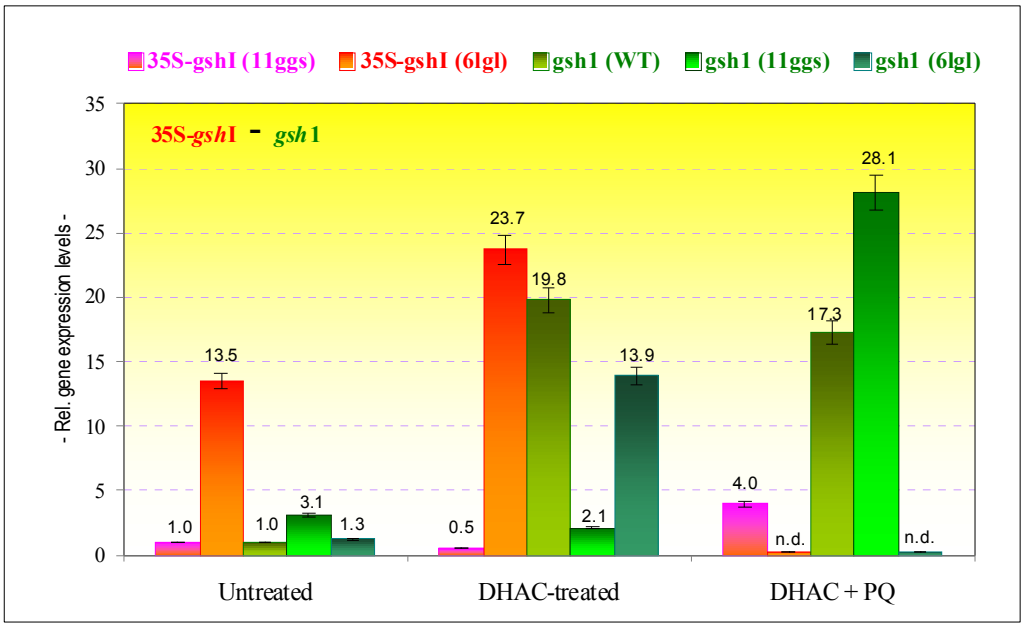

Figure 5. Relative gene expression levels (RT-qPCR) of transgene 35S-*gsh*I (*E. coli*) and endogenous poplar (*Populus x canescens*) gene *gsh*1 in the 35S-*gsh*I-transgenic clones of 11*ggs*, 6*lgl* and WT exposed to DHAC (10^{-4} M; 7 days) and DHAC combined with paraquat (PQ) (4 x 10^{-7} M; 7 days) (n = 6) (n.d. - not detected).

Table 1. Relative copy number of transgene 35S-*gsh*I (*E. coli*) in the transgenic poplar (*P.* x *canescens*) clones 6*lgl* and 11*ggs* determined by RT-qPCR analysis using the $2^{-\Delta\Delta Ct}$ method with the internal control of constitutively expressed poplar gene *actin* (three repetitions of two samples in each case, n = 6)

Poplar	35S-*gsh*I		*actin*		ΔCt		ΔΔCt		35S-*gsh*I copy # ($2^{-\Delta\Delta Ct}$)		
lines	Ct	SD	Ct	SD	Value	SD	Value	SD	-/+ range		Value
6*lgl*	23.75	0.14	23.41	0.24	0.34	0.21	0.0	0.21	0.86	1.16	1.00
11*ggs*	22.50	0.26	22.91	0.14	-0.41	0.13	-0.75	0.13	1.54	1.85	1.69

Paraquat Stress:

Paraquat (*N,N'-Dimethyl-4,4'-bipyridinium dichloride*; *syn.*: methylviologen) primarily acts as an electron acceptor in the electron transport chains located in chloroplasts (Gyulai 1984; Lehoczki *et al.* 1992; Szigeti *et al.* 2001). The paraquat, at bleaching concentrations totally impairs chloroplast function by reduction of the bleached tissues generating superoxide radicals that react with unsaturated lipids in membranes (Bittsánszky *et al.* 2005). This deleterious effect of paraquat was effectively eliminated by the DHAC treatment, as the endogenous poplar gene *gsh*1 of the 11*ggs* clone did not show DHAC responses but under paraquat stress it increased by 8-fold (from 3.1 to 28.1) along with co-expressing the

transformed 35S-*gsh*I gene with 4-fold increment (from 1.0 to 4.0) (Figure 5). In contrast, endogenous *gsh*1 of the WT clone showed extreme upregulation in response to DHAC-induced DNA-demethylation with 20-fold increase (from 1.0 to 19.8) but no further responses to paraquat stress (17.3) (Figure 5).

Increased *gsh*-activity with elevated levels of *gsh*-mRNA (syn.: γ-ECS-mRNA) has also been reported in *Brassica napus* (Sun *et al*. 2005), *Brassica juncea* (Schäfer *et al.* 1998), and *Arabidopsis thaliana* under different stress conditions (Xiang and Oliver 1998; Harada *et al.* 2002). The moss *Physcomitrella patens* also showed a high level of γ-ECS overexpression (5.7 – 7.9-fold increase) in response to heavy metal (10 μM Cd^{2+}, for 3 days) stress (Rother *et al.* 2006). These results indicate a wide stress-response capacity of *gsh*1 genes not only to herbicides but also to heavy metals.

DHAC-Induced Morphogenesis:

A *de novo* root development was observed on DHAC-treated poplar leaf discs incubated for long term (21 days) treatment, which indicates a multi-target action site of DHAC at especially the auxin-related root initiating genes (Figure 6).

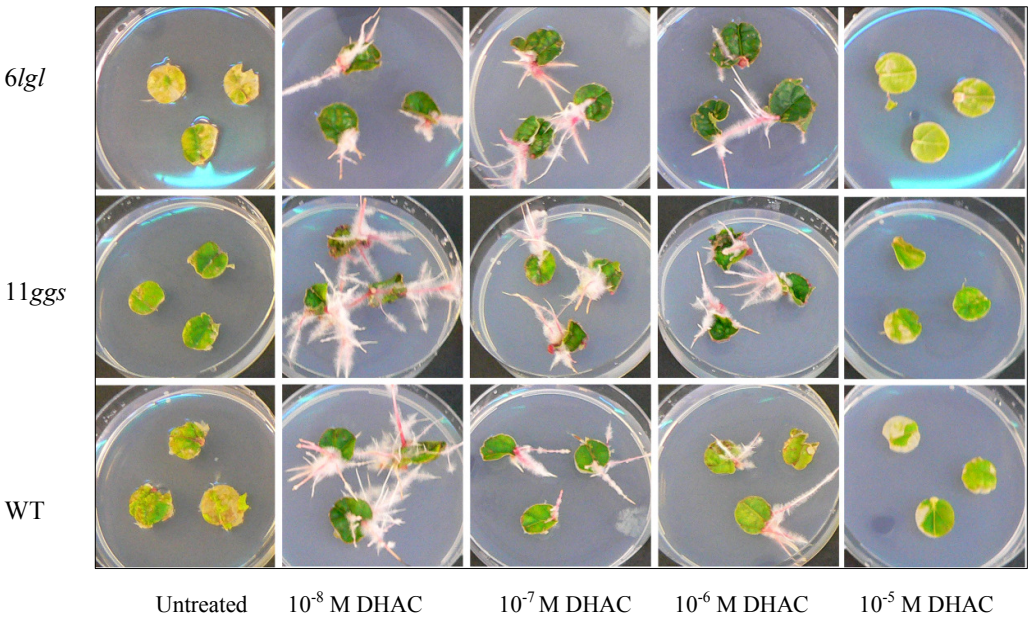

Figure 6. Root initiation capacity of the DNA demethylating agent DHAC (10^{-8} M to 10^{-6} M) on leaf discs of untransformed (WT) and 35S-*gsh*I-transformed poplar (*Populus* × *canescens*) clones (6*lgl* and 11*ggs*) incubated on long-term (21 days) aseptic agar media.

The morphogenetic capacity of DNA demethylating agents on plant development has been reported in different organs (Finnegan and Kovac 2000; Xiao *et al.* 2006). Flower induction of *Arabidopsis* (Finnegan *et al.* 1998) and shoot development of *Petunia* (Prakash and Kumar 1997; Prakash *et al.* 2003) were initiated by DNA-demethylating agents. Early flower bud

development (vernalization, *remembering winter*) in *Arabidopsis* was found to be coupled with low levels of DNA methylation (Finnegan *et al.* 1998, 2005; Henderson and Dean 2004).

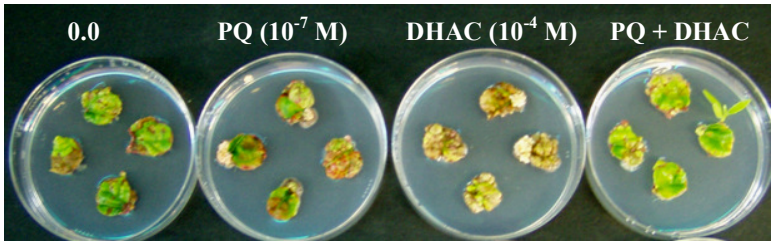

Figure 7. Samples of shoot regeneration from leaf discs of 35S-*gsh*I-transgenic poplar (*Populus* × *canescens*) clone (11*ggs*) treated with DNA demethylating agent DHAC (10^{-4} M) combined with paraquat (10^{-7} M) stress and incubated on aseptic agar media for 21 days.

Low levels of methyl-cytosine were also associated with organogenetic capability in sugarbeet (*Beta vulgaris altissima*) (Causevic *et al.* 2005). Contrary to morphogenesis, incremental DNA methylation levels occurred during bud dormancy (Horvath *et al.* 2003; Law and Suttle 2003). Methylation also plays a key role in the chromosome modelling as it turnes out that the Pc-G (*P*oly*c*omb *G*roup) protein complexes encoded by *pcg-g* genes (*p*oly*c*omb *g*roup *g*enes) control flowering in plants, which genes are similar to the PRC2 (Polycomb Repressive Complex 2) in animals, and functions as a histone methyltransferase (Chanvivattana *et al.* 2004).

Adventitious shoots were also developed from leaf discs of the 11*ggs* clone treated with DHAC (10^{-4} M) and paraquat (10^{-7} M) in long term cultur (Figure 7). Shoots (three in total of the four repetition experiments) were micropropagated and rooted *in vitro* according to Gyulai *et al*. (2005), and transplanted in glass houses (86 lines) for further analyses for phytoremediation capacity. As DNA methylation patterns are inherited (*epigenetic memory*), these poplar plants with increased gene expression levels of both transgene 35S-*gsh*I and endogenous gene *gsh*1 provide novel plant sources with elevated stress capacity.

MATERIALS AND METHODS

Plant material: Clones (INRA 717-1-B4) of the untransformed (WT) poplar (*Populus* x *canescens* = *P. tremula* x *P. alba*; 2n = 4x = 38; 4.5 to 5.5 x 10^8 bp) (Taylor 2002) and the genetically transformed lines overexpressing 35S-*gsh*I (γ-glutamylcysteine synthetase; EC 3.2.3.3; cloned from *Escherichia coli*; 1.557 bp) (Watanabe *et al*. 1986) (NCBI X03954) gene product, glutathione (GSH) either in the cytosol (line 11*ggs*) or in the chloroplasts (line 6*lgl*) were used. Gene constructs are driven by a copy of the CaMV-35S promoter (Leple *et al*. 1992; Arisi *et al*. 1997; Noctor *et al*. 1998).

Shoot culture in vitro: Nodal segments of poplar clones were micropropagated and maintained in aseptic shoot cultures *in vitro* (Gyulai *et al*. 1995; Kiss 1999; Gullner *et al*. 2005).

Detection of gene expression levels by RT-qPCR analysis, RNA isolation: Relative gene expression levels of 35S-*gsh*I-transgene (*E. coli*) and the endogenous poplar gene *gsh*1 were analyzed by RT-qPCR in the control of constitutively expressed housekeeping poplar gene *α*-

tubulin and *actin*. Total RNA was extracted from 0.05 g leaf disc tissues using the Absolutely RNA Miniprep Kit (# 400800, Stratagene, USA - Biomedica, Hungary) following the manufacture's protocol. Three individual leaf discs were analyzed in duplicate measurements (n = 6) in each case. The quality and quantity of extracted RNA samples (2 µl) were measured by NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA – BioScience, Budapest, Hungary).

cDNA synthesis: Reverse transcription of first strand cDNA was synthesized on the mRNA templates by RT (reverse transcriptase of Moloney Murine Leukemia Virus: M-MuLV) with oligo(dT)$_{18}$ (0.5 µg) as the primer following the manufacturer's protocol (# K1622; Fermentas – Biocenter, Szeged, Hungary).

Gene expression analysis: First strand cDNA samples (2.5 µl) were directly applied in RT-qPCR (25 µl) and probed by gene specific primers (400 nM). Primers were as follows: 35S-*gsh*I: 5'-aggtcaggacatcgaactgg-3' and 5'-gatgcaccaaacagataagg-3'; *gsh*1-poplar: 5'-agttccgaggctgacatgat-3' and 5'-cagcacggttgttgtcagta-3'; α-tubulin (poplar): 5'-taaccgccttgtttctcagg-3' and 5'-cctggggtatggaaccaagt-3'; and actin (poplar): 5'-aatggtaccggaatggtcaa-3' and 5'-cccaacatacgcatcctttt-3' according to Bittsánszky *et al*. (2007).

Kit of DyNAmo HS SybrGreenI qPCR kit (# F-410L, Finnzymes, Finland – Izinta, Hungary) was used. Reactions were performed in forty cycles (95 °C/20sec, 60 °C/20sec, 72 °C/20sec) prior to a hold at 95 °C for 10 min, and a final hold at 4°C. Reactions were run by Rotor Gene 6000 cycler (Corbett Research, Australia – Izinta, Hungary).

Detection of relative copy number of 35S-*gsh*I-transgene: Relative copy number of 35S-*gsh*I-transgene in clones of 11*ggs* and 6*lgl* were determined under the control of constitutively expressed housekeeping gene *actin* by using selective primer pairs used for RT-PCR. The levels of *gsh*I-mRNA were detected by the amplified fragment (273 nt) of the incorporated transgene (from 667 nt to 939 nt).

Data analysis of real-time RT-qPCR: For both calibration and quantification of reactions, ten-fold serial dilutions (1x, 10^{-1}x, 10^{-2}x, 10^{-3}x) of cDNAs were applied including controls of NTC (non DNA-template control) and ddH_2O. Data were analyzed by relative quantification of the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Ct values (threshold cycle): The threshold of fluorescence value (dR) of the amplified PCR products was determined manually above the background of fluorescence signals. Standard curve correlating Ct values to log amount of DNA were plotted at high R^2- ratio (0.976 to 0.987). ΔCt: ΔCt values were calculated as Ct_{gshI} minus $Ct_{a\text{-}tubulin}$ and Ct_{gsh1} minus $Ct_{a\text{-}tubulin}$ according to (Livak and Schmittgen 2001). ΔΔCt values: ΔΔCt values were determined as mean $Ct_{untreated}$ minus mean $Ct_{treated}$ (Livak and Schmittgen 2001).

Multiple sequence alignments were carried out by BioEdit Sequence Alignment Editor (NCSU, USA) (Hall 1999) and CLUSTALW EMBL-EBI (Thompson *et al*. 1994) software programs. BLAST (Basic Local Alignment Search Tool) analysis (Altschul *et al*. 1997) was carried out by a computer program of NCBI (National Center for Biotechnology Information). Distance trees based on *gsh*1 sequences were edited by either CLUSTALW EMBL-EBI (Thompson *et al*. 1994) or MEGA4 (Tamura *et al*. 2007). For MEGA4 the following steps were applied: Bootstrap Test of Phylogeny (1000); Neighbor-Joining; Gaps (Complete deletions); Substitution model (Nucleotide Maximum Composite Likelihood) according to Tamura *et al*. (2007). Diagrams were edited by Microsoft Office Excel program (9625 West 76th Street, Eden Prairie, MN 55344, USA).

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