Gene Reactivation Induced by DNA Demethylation in Wild Type and 35S-*gsh*I-*rbc*S Transgenic Poplars (*Populus x canescens*)

Novel plant sources for phytoremediation

Gábor Gyulai[1], András Bittsánszky[2], Gábor Gullner[2], György Heltai[3], Katalin Pilinszky[2], Erik Molnár[3], Tamás Kömíves[2]

[1]St. István University, Institute of Genetics and Biotechnology, Gödöllő H-2103 Hungary

[2]Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest H-1525 Hungary

[3]St. István University, Department of Chemistry and Biochemistry, Gödöllő H-2103 Hungary

[1]gyulai.gabor@mkk.szie.hu; [2]bittsanszky.andras@agrar.mta.hu; [2]gullner.gabor@agrar.mta.hu; [3]heltai.gyorgy@mkk.szie.hu; [2]pilinszky.katalin@agrar.mta.hu; [3]molnar.erik@mkk.szie.hu; [2]komives.tamas@agrar.mta.hu

***Abstract*- For gene reactivation DNA demethylating agent DHAC (5,6-dihydro-5'-azacytidine hydrochloride) (10^{-4} M for 7 days) was applied in aseptic leaf cultures of wild type (WT) and 35S-gshI-rbcS GM (genetically modified) transgenic (TR) poplar (Populus x canescens) clone lgl6. Gene expression levels were determined by RT-qPCR (reverse transcriptase quantitative PCR) measuring the mRNA levels of the prokaryotic gshI-rbcS-mRNA (γ-glutamylcysteine synthetase) cloned from E. coli, and two endogenous poplar genes of gsh1-mRNA and gst-mRNA (glutathione S-transferase). 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. After DHAC treatment the expression levels of 35S-gshI-rbcS transgene showed a double (1.8 - times) increment. The endogenous poplar gene gsh1 increased by 19.7-fold in the WT, and by 8.7-fold in the TR clone. The endogenous gst gene showed a 4.9 - times (in WT) and a 2.9-times (in TR) increment. Sequence analysis of DNA methylating enzymes were analyzed in silico and significant distinction was found among the three main plant DNA methylases (METases) of METs (maintenance methyltranferase), CMTs (chromomethylases) and DRMs (de novo domains rearranged DNA methylases). The DHAC-treated WT poplars with increased gene expressions of gsh1 and gst might provide novel plant sources for application for detoxification and soil remediation concerning general public frightened by GM poplars.**

Keywords- Populus x canescens; DNA Demethylation; RT-qPCR

I. INTRODUCTION

Poplars (*Populus* ssp.) are capable of removing and degrading toxic substances from the polluted soils through phytoextraction and phytoremediation due to the extensive root system, high water uptake capacity, rapid growth and large biomass production [13, 17, 28, 29].

This phytoremediation capacity of *Populus x canescens* has been significantly increased by genetic transformation with the prokaryotic 35S-*gsh*I gene, which encodes for γ-glutamylcysteine synthetase (γ-ECS, EC 6.3.2.2) [4, 31, 33]. Gene *gsh*I was cloned from *E. coli* (NCBI X03954) [53]. The transgene construct included an additional targeting sequence (32 to 202 of 206 bp; NCBI M25614) of transit peptide (57 amino acids, *Pisum sativum*) gene of *rbc*S (RuBPCase SSU: *s*mall *sub*unit of RuBPCase, *r*ib*u*lose-1,5-*b*isphos*p*hate *c*arboxyl*ase*), which aimed to target the cytosolically synthesized RBCS-GSH complex into chloroplasts [31]. The transformed poplar clones showed higher contents of both *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 [35, 36].

Transgenic clone studied here (*TRlgl*6) has been maintained in aseptic shoot cultures for about a decade without 35S-*gsh*I-*rbc*S transgene elimination [7, 20, 21]. However, transgenes have been exposed to gene silencing processes either in the region of the constitutive *CaMV-35*S promoter, or in the coding regions [14, 18]. By the application of a *DNA*-demethylating agent in the study presented, this natural gene silencing process was reversed, and both genes and transgene were reactivated.

II. MATERIALS AND METHODS

A. Clones

The genetically transformed (INRA 717-1-B4) poplar line (*TR lgl*6) [4, 31, 33] overexpressing 35S-*gsh*I-*rbc*S (γ-glutamylcysteine synthetase (EC 6.3.2.2) cloned from *Escherichia coli* (1557 bp; NCBI X03954) coupled with transit peptide *rbc*S (from *Pisum sativum* NCBI M25614) [23] was used in the control of *WT* (*Populus x canescens* = *P. tremula* x *P. alba*; 2n = 4x = 38; 4.5 x 10^8 bp) [44, 47].

B. Shoot cultures

Nodal segments of poplar clones were micropropagated and maintained *in vitro* [19] (Fig.1).

Fig. 1 Clones of *Populus* x *canescens*, *WT* and *TR* (35S-*gsh*I-*rbc*S) in glass houses (*a*), propagated in aseptic shoot culture (*b*) and sources of explants (petiole, nodal segments, and leaf discs) prepared for the experiments (*c*)

C. RT-qPCR

Relative gene expression levels of 35S-*gsh*I-*rbc*S transgene (NCBI #X03954; *E. coli*) [23] and the endogenous poplar gene *gsh*1 (γ-glutamylcysteine synthetase; EC 6.3.2.2)

and *gst* (glutathione S-transferase*;* EC 2.5.1.18) 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). *First strand cDNA*s. Reverse transcription of first strand c*DNA* was synthesized on the *mRNA* templates by *RT* (reverse transcriptase of Moloney Murine Leukemia Virus: M-MuLV) with primer oligo$(dT)_{18}$ (0.5 µg) following the manufacturer's protocol (# K1622; Fermentas – Biocenter, Szeged, Hungary). First strand c*DNA*s (2.5 µl) were directly applied in *RT-qPCR* (25 µl) and probed by gene specific primers (400 nM). *Primer pairs* were as follows: for transgenic gene 35S-*gsh*I-*rbc*S: 5'-aggtcaggacatcgaactgg-3' and 5'-gatgcaccaaacagataagg-3' which the amplified a fragment of 273 nt of the incorporated transgene (from 667 nt to 939 nt). For endogenous *gsh*1: 5'-agttccgaggctgacatgat-3' and 5'-cagcacggttgttgtcagta-3'; for endogenous *gst*: 5'-gcacaagaaagagcc(a/g)ttcc-3' and 5'-agctcccagttcagctttga-3'; for control *α-tubulin* (poplar): 5'-taaccgccttgtttctcagg-3' and 5'-cctggggtatggaaccaagt-3'; and *actin* gene (poplar): 5'-aatggtaccggaatggtcaa-3' and 5'-cccaacatacgcatcctttt-3' were applied [9, 21]. The DyNAmo HS SybrGreenI *qPCR* kit (#F-410L, Finnzymes, Finland – Izinta, Hungary) was used for *qPCR* analyses. *qPCR* reactions were performed by Rotor Gene 6000 cycler (Corbett Research, Australia – Izinta, Hungary) in forty cycles (95 °C / 20 sec, 60 °C / 20 sec, 72 °C / 20 sec) prior to a hold at 95 °C for 10 min, and a final hold at 4 °C. *Data analysis*. For both calibration and quantification of reactions, ten-fold serial dilutions (1 x, 10^{-1} x, 10^{-2} x, 10^{-3} x) of *cDNA*s 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 [32]. *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 $Ct_{a\text{-}tubulin}$ [32]. *ΔΔCt values*: ΔΔCt values were determined as mean $Ct_{untreated}$ minus mean $Ct_{treated}$.

D. Lipoxygenase (LOX) Activity

Cell-free extracts of leaf tissues of *TR* and *WT* clones were prepared before and after the *DHAC* treatment and the enzymatic activities of LIPOXYGENASE (LOX; EC 1.13.11.12) were determined at pH range 5.0 – 9.5 according to [3, 8].

E. Multiple Sequence Alignments

Sequences were analyzed *in silico* by programs of BioEdit Sequence Alignment Editor (North Carolina State University, USA) [22], MULTALIN [12], CLUSTALW [45] and FastPCR [27]. For BLAST (*Basic Local Alignment Search Tool*) analysis the NCBI (*National Center for Biotechnology and Information*) server was used [1]. Molecular cladograms were edited by MEGA4 [42].

III. RESULTS AND DISCUSSION

A. DNA Methylation

DNA methylation is a natural enzymatic process of *TGS* (transcriptional gene silencing) catalyzed by *DNA* methyltransferase enzymes which results in the meiotically heritable methylation pattern ('*inprints*') [34]. *DNA* methylation is not universal, as in the insect fruit fly *Drosophyla* has not been detected [25].

In *Arabidopsis*, there are at least three classes of *DNA* methyltransferases (*MET*ases). These are *MET*s (maintenance DNA methyltranferase), *CMT*s (chromomethylase 3) and *DRM*s (*de novo* domains rearranged *DNA* methylases) [16].

The *MET*1 genes are similar in sequences, and homologues in functions to mammalian *Dnmt*1. The *CMT*3 is specific to the plant kingdom and contain a chromo domain [24].

*CMT*s transfer a methyl group (CH_3) mainly from S-adenosyl methionine (AdoMet-dependent methyltransferases) mainly to the position of cytosine-C_5 (EC 2.1.1.73), and also of cytosine-N_4 (E.C. 2.1.1.13) [11, 38] and adenine-N_6 by adenine *DNA* methyltransferases (E.C. 2.1.2.72). The first eukaryotic adenine *DNA* methyltransferase was isolated from plants (wheat) and was found mainly responsible for the methylation of mitochondrial *DNA* [15].

The *DRM* class of genes include *DRM*1 (624 amino acid - aa) and *DRM*2 (626 aa) (syn. *DNA-MET*ase) (both EC 2.1.1.37) and contain catalytic domains which shows sequence similarity to mammalian *de novo Dnmt*3 [10]. The conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine were discovered recently in mammalian DNA [43]. In *Arabidopsis*, the same enzyme (*DRM*2) can methylate both cytosine and adenine nucleotides [49].

A process of *RdDM* (siRNA and micro-RNA directed DNA methylation) also occur in eukaryotes which was also observed first in plants [52].

B. Triggering of DNA Methylation

Gene expression and *TGS* can be triggered *in vitro* by up/down regulation of *DNA* methylase genes (syn.: re/activation, hypo/hyper/de methylation) [18]. For induced gene up-regulation, *MT*ase-inhibitors such as the structurally modified cytosine analogues zebularine, 5-azacytidine (5-*azaC*), 5-aza-2'-deoxycytidine (5-*azadC*) and 5,6-dihydro-5'-azacytidine hydrochloride *DHAC* have been shown to be highly effective [11]. Alternatively, the drug 3-aminobenzamide has been used for gene down-regulation in a series of genes [55]. Gene reactivation through the application of thymidine analogues can also occur in demethylation-independent gene up-regulations [14].

The exogenously applied *MT*ase-inhibitors act via covalent complex formation [14, 54] when present either in the cytosol or when incorporated into *DNA* as *DNA* base analogue [6, 26].

The study presented here aimed to upregulate simultaneously both the prokaryotic gene 35S-*gsh*I-*rbc*S and the endogenous eukaryotic poplar gene *gsh*1 and *gst* in both clones *WT* and *TR* after *DHAC*-treatment (10^{-4} M, for 7 days). Both genes *gsh* and *gst* play central role in detoxifications [9].

Reverse transcription (*RT*) followed by *qPCR* analysis was found to be an exceptionally sensitive method for gene expression analyses compared to *RNA-DNA* hybridizations

(Northern blots) [2] in both cases of absolute and relative quantification [50]. In the study presented, relative quantification was used as it is more relevant than absolute quantification to compare expression levels of different treatments [32, 37, 46].

Gene expression of transgene 35S-*gsh*I-*rbc*S increased from a high relative expression level (13.5 relative units) to 23.7 with about a two-fold increment compared to the endogenous *gsh*1 which increased from a lower level (1.3 rel. unit) to 13.9 with an 8.7-fold increment after *DHAC*-treatment (Fig.2).

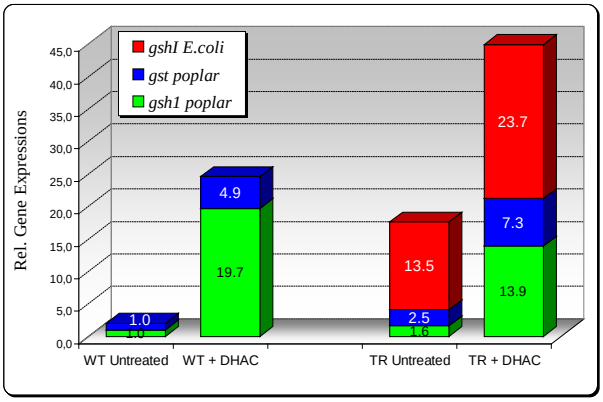

Fig. 2 Cumulative gene expression levels (*RT-qPCR*) of *gsh*1, *gst* and 35S-*gsh*I-*rbcl*S (cloned from *E. coli*) in the *WT* and transgenic (*TR*) poplars (*Populus x canescens*) exposed to *DHAC* (at 10^{-4} M, for 7 days)

Gene expression of the endogenous *gsh*1 of the *WT* clone showed also high responsiveness to *DHAC*-treatment with an extremely high expression (19.8 - fold). This result indicates a difference in *DNA* methylating capacity between transgenes and proper wild type genes as a type of cosuppression [5, 30]. The endogenous poplar *gst* gene also showed *DHAC*-inducibility with a 4.9 - times (*WT*) and a 2.9 - times increment (from 2.5 rel. unit to 7.3 in the *TR* clone). Increased levels of *gsh*1-*mRNA* (syn.: γ-ECS-*mRNA*) has also been reported in *Brassica juncea* [40], *Brassica napus* [41], *Arabidopsis thaliana* [23] exposed to cadmium stress. The moss *Physcomitrella patens* also showed a high level of γ-ECS overexpression (5.7 – 7.9 - fold increase) in response to 10 µM Cd^{2+} [39].

For functional analysis, enzyme activities of LIPOXYGENASE (LOX, EC 1.13.11.34), which catalyzes the conversion of arachidonic acid to 5-*HPETE* [48] were measured. The LOX activity was higher in the *DHAC*-treated *WT* clones than in the transgenic clone at all pH levels (Fig.3).

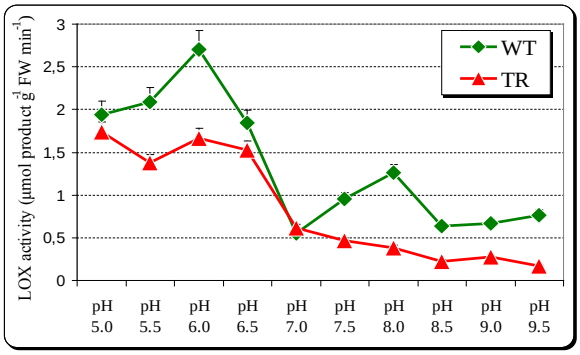

Fig. 3 LIPOXYGENASE (LOX) activity of the *DHAC* treated (at 10^{-4} M, for 7 days) *WT* (wild type) and *TR* (transgenic 35S-*gsh*I-*rbc*S) poplars (*Populus* x *canescens*) at the range of pH 5.0 - 9.5

The LOX activity curves with two pH optima between the ranges of pH 5.0 – 9.5 indicate that two LOX izozymes are present in poplar. The promoter region of *lox* gene was found to be downregulated by *DNA* methylation in human U937 and HL-60TB cells [48], however in our experiments LOX measured at pH 6 and pH 8 in *DHAC* (10^{-4} M and 10^{-5} M) treated poplar did not show concentration dependent increment in the activity (detailed elsewhere). Concentration independent morphogenetic effect of *DHAC* treated poplar leaf discs were also found in adventitious root development at all concentrations (10^{-8} M to 10^{-6} M) with the same rooting capacity [21].

C. Multiple Sequence Analyses

Protein sequence analyses were used to study *MET*ases identified in trees, which are still rarely available in gene banks. Trees (*Populus, Prunus, Malus Elaeis*), woody *Vitis* and herbaceous species were compared, and the results revealed distinctive groups between *MET*s (*DNA* methyltransferases) and *CMT*s (chromomethylases) (Fig.4), and compared to the third group of *MET*ases of mammalian *Dnmt*3 homologue *DRM*s (domains rearranged *DNA* methylases) (Fig.5). These results indicate an extreme molecular diversity of *DNA* methylases with indications for the possibility of site specific (single gene directed) *DNA* demethylation [51].

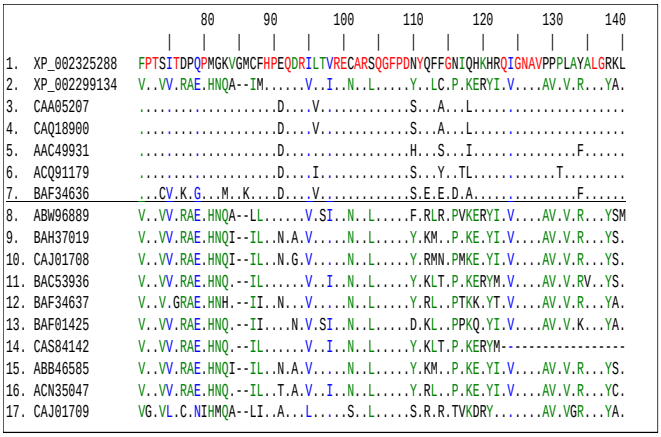

Fig. 4 Partial amino acid (70-140 aa) sequence alignments of *MET*s (*DNA*-methyltransferases, *1-7*) and *CMT*s (chromomethylases, *8-17*) blasted to XP_002325288 (*Populus trichocarpa*) from NCBI server [1], and edited by Multalin server [12]. Consensus aa (*dots*) are indicated. Accession numbers: **XP_002325288** (*MET*, *Populus trichocarpa*, 1549 aa). **XP_002299134** (*MET*, *Populus trichocarpa*, 973 aa). CAA05207 (*MET*, *Solanum lycopersicum*, 1559 aa). CAQ18900 (*MET*, *Nicotiana sylvestris*, 1558 aa). AAC49931 (*MET*, *Pisum sativum*, 1554 aa). ACQ91179 (*MET*, *Fragaria x ananassa*, 1557 aa). BAF34636 (*MET*1b, *Brassica rapa*, 1519 aa). // **ABW96889** (*CMT*, *Elaeis guineensis*, 925 aa). BAH37019 (*CMT*, OsMET2a, *Oryza sativa* Japonica Group, 907 aa). CAJ01708 (*CMT*1, *Hordeum vulgare*, 735 aa). BAC53936 (*CMT*, *Nicotiana tabacum*, 741 aa protein). BAF34637 (*CMT*, *Brassica rapa*, 805 aa). CAS84142 (*CMT*, *Nicotiana tomentosiformis*, 500 aa). ABB46585 (*MET*2a, *Oryza sativa* Japonica, 371 aa). ACN35047 (*Zea mays*, 329 aa). BAF01425 (*CMT*, *Arabidopsis thaliana*, 839 aa). CAJ01709 (*CMT*2, *Hordeum vulgare* subsp. *vulgare*, 187 aa)

IV. CONCLUSIONS

To conclude, since *DNA* methylation patterns are inherited ('epigenetic memory') [6], the *DHAC*-treated *WT* poplars regenerated from leaf discs and with increased gene expression levels of endogenous genes *gsh*1 and *gst* might provide novel plant sources for the application to air and soil detoxification and remediation concerning the general public frightened by genetically modified (*GM*) organisms.

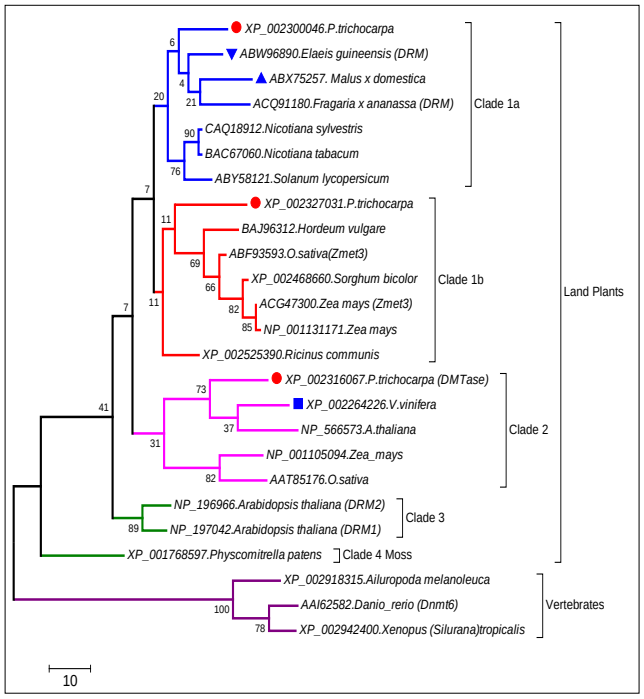

Fig. 5 Protein phylogram (Max. Parsimony by MEGA4) [42] of enzyme *MET*s (*DNA* - methyltransferases) (654 aa each) of 25 plant species. Trees (*Populus*, *Malus*, *Elaeis*) and woody *Vitis* signed with symbols, main clades, rel. genetic distance (*scale*, 10 aa substitutions per scale), branch information of boot strap values (x1000) and accession numbers are indicated

ACKNOWLEDGMENT

Poplar clones were kindly provided by H Rennenberg (Freiburg Univ, D) and CH Foyer (Newcastle upon Tyne, UK). The financial support of the Hungarian Scientific Research Fund (OTKA grant K77641, K72926 and PD75169) is gratefully acknowledgedof the.

REFERENCES

[1] S. F. Altschul, T. L. Madden, A. A. Schaffer, J. H. Zhang, Z. Zhang, W. Miller, D. J. Lipmand, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," *Nucleic Acids Research*, vol. 25, pp. 3389–3402, 1997.

[2] J. C. Alwine, D. J. Kemp, G. R. Stark, "Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes," *Proc Natl Acad Sci USA*, vol. 74, pp. 5350–5354, 1997.

[3] B. Axelrod, T. M. Cheesbrough, Laakso S, "LIPOXYGENASE from soybeans," *Methods in Enzymology* vol. 71, pp. 441–451, 1981.

[4] A.-C. M. Arisi, G. Noctor, C. F. Foyer, L. Jouanin, "Modification of thiol contents in poplars (*Populus tremula* x *P. alba*) overexpressing enzymes involved in glutathione synthesis," *Planta*, vol. 203, pp. 362–372, 1997.

[5] N. Baumberger, D. C. Baulcombe, "Arabidopsis ARGONAUTE1 is an RNA slicer that selectively recruit microRNAs and short interfering RNAs," *Proc Natl Acad Sci USA*, vol. 102, pp. 11928–11933, 2005.

[6] A. Bird, "DNA methylation patterns and epigenetic memory," *Genes and Development*, vol. 16, pp. 6–21, 2002.

[7] A. Bittsánszky, T. Kőmíves, G. Gullner, G. Gyulai, J. Kiss, L. Heszky, L. Radimszky, H. Rennenberg, "Ability of transgenic poplars with elevated glutathione content to tolerate Zinc (2+) stress," *Environment International*, vol. 31, pp. 251–254, 2005.

[8] A. Bittsánszky, G. Gyulai, G. Gullner, J. Kiss, Z. Szabó, Gy. Kátay, L. Heszky, T. Kőmíves, "In vitro breeding of grey poplar (*Populus* x *canescens*) for phytoremediation purposes,"*J Chemical Technol and Biotechnol*, vol. 84, pp. 890–894, 2009.

[9] A. Bittsánszky, G. Gullner, G. Gyulai, T. Kömíves, "A Case Study: Uptake and Accumulation of Persistent Organic Pollutants in *Cucurbitaceae* Species," in *Organic Xenobiotics and Plants: From Mode of Action to Ecophysiology*. P. Schröder, C. D. Collins Ed., Berlin: Springer-Verlag, pp. 77–85, 2011.

[10] X. Cao, S. E. Jacobsen, "Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes," *Proc Natl Acad Sci USA*, vol. 99, pp. 16491–16498, 2002.

[11] X. Cheng, R. J. Roberts, "AdoMet-dependent methylation, DNA methyltransferases and base flipping," *Nucleic Acid Research*, vol. 29, pp. 3784–3795, 2001.

[12] C. Combet, C. Blanchet, C. Geourjon, G. Deléage, "NPS@: Network Protein Sequence Analysis," *Trends in Biochemical Sci*, vol. 25, pp. 147–150, 2000.

[13] D. Di Baccio, R. Tognetti, L. Sebastiani, C. Vitagliano, "Responses of *Populus deltoides* x *Populus nigra* (Populus x euramericana) clone I-214 to high zinc concentrations," *New Phytologist*, vol. 159, pp. 443–452, 2003.

[14] J. Fan, E.-I. Kodama, Y. Koh, M. Nakao, M. Matsuoka, "Halogenated Thymidine Analogues Restore the Expression of Silenced Genes without Demethylation," *Cancer Research*, vol. 65, pp. 6927–6933, 2005.

[15] L. I. Fedoreyeva, B. F. Vanyushin, "N(6)-Adenine DNA-methyltransferase in wheat seedlings," *FEBS Letters*, vol. 514, pp. 305–308. 2002.

[16] E. J. Finnegan, K. A. Kovac, "Plant DNA methyltransferases," *Plant Molecular Biology*, vol. 43, pp. 189–201, 2000.

[17] C. H. Foyer, G. Noctor, "Redox homeostasis and antioxidant signalling: a metabolic interface between stress perception and physiological responses," *The Plant Cell*, vol. 17, pp. 1866–1875, 2005.

[18] J. Goffin, E. Eisenhauer, "DNA methyltransferase inhibitors—state of the art," *Annals of Oncology*, vol. 13, pp. 1699–1716. (2002)

[19] G. Gyulai, Z. Jekkel, E. Kiss, J. Kiss, L. Heszky, "A selective auxin and cytokinin bioassay based on root and shoot formation in vitro," *Journal of Plant Physiology*, vol. 145, pp. 379–382, 1995.

[20] G. Gyulai, M. Humphreys, A. Bittsánszky, K. Skøt, J. Kiss, L. Skøt, G. Gullner, S. Heywood, Z. Szabó, A. Lovatt, L. Radimszky, H. Roderick, M Abberton, H. Rennenberg, T. Kőmíves, L. Heszky, "AFLP analysis and improved phytoextraction capacity of transgenic *gsh*I-poplar clones (*Populus canescens* L.) in vitro," *Z Naturforschung*, vol. 60c, pp. 300–306, 2005.

[21] G. Gyulai, Z. Tóth, A. Bittsánszky, Z. Szabó, G. Gullner, J. Kiss, T. Kőmíves, L. Heszky, "Gene up-regulation by DNA demethylation in 35S-*gsh*I-transgenic poplars (*Populus* x *canescens*)," in *Genetically Modified Plants: New Research Trends*. T. Wolf, J. Koch, Eds., New York: Nova Science Publisher, pp. 173–191, 2008.

[22] T. A. Hall, "BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT," *Nuclec Acids Symposium Series*, vol. 41, 95–98, 1999.

[23] E. Harada, Y. Yamaguchi, N. Koizumi, S. Hiroshi, "Cadmium stress induces production of thiol compounds and transcripts for enzymes involved in sulfur assimilation pathways in *Arabidopsis*, " *J Plant Physiol*, vol. 159, pp. 445–448, 2002.

[24] S. Henikoff, L. Comai, "A DNA methyltransferase homolog with a chromodomain exists in multiple polymorphic forms in *Arabidopsis*," *Genetics*, vol. 149, pp. 307–318, 1998.

[25] H. Hirochika, H. Okamoto, T. Kakutani, "Silencing of retrotransposons in *Arabidopsis* and reactivation by the ddm1 mutation," *The Plant Cell*, vol. 12, pp. 357–368, 2000.

[26] J. P. Issa, H. Kantarjian, "Azacitidine," *Nature Reviews Drug Discovery*, May. Suppl. S6–7, 2005.

[27] R. Kalendar, D. Lee, A. H. Schulman, "FastPCR Software for PCR Primer and Probe Design and Repeat Search," *Genes, Genomes and Genomics*, vol. 3, pp. 1–14, 2009.

[28] S. J. Kays "Phytoremediation of indoor air – Current state of the art," in: *The Value Creation of Plants for Future Urban Agriculture*, KJ Kim Ed., Suwon, Korea: Nat. Inst. Hort. Herbal Science RDA, 2011, pp. 3-21.

[29] T. Kőmíves, G. Gullner, Z. Király, "Role of glutathione and glutathione-related enzymes in response of plants to environmental stress. Stress of Life," *From Molecules to Man*, vol. 851, pp. 251–258, 1998.

[30] A. R. Krol, L. A. Mur, M. Beld, J. N. Mol, A. R. Stuitje, "Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression," *The Plant Cell*, vol. 2, pp. 291–299, 1990.

[31] J. C. Leple, A. C. M. Brasileiro, B. F. Michel, F. Delmotte, L. Jouanin, "Transgenic poplars - Expression of chimeric genes using 4 different constructs," *Plant Cell Reports*, vol. 11, pp. 137–141, 1992.

[32] K. J. Livak, T. D. Schmittgen, "Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method," *Methods*, vol. 25, pp. 402–408, 2001.

[33] G. Noctor, A.-C. M. Arisi, L. Jouanin, K. J. Kunert, H. Rennenberg, C. H. Foyer, "Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants," *J Exp Botany*, vol. 49, pp. 623–647, 1998.

[34] Y.-D. Park, I. Papp, E. A. Moscone, V. A. Iglesias, H. Vaucheret, A. J. M. Matzke, M. A. Matzke, "Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity," *The Plant Journal*, vol. 9, pp. 183–194, 1996.

[35] A. D. Peuke, H. Rennenberg, "Phytoremediation with transgenic trees," *Z Naturforschung*, vol. 60c, pp. 199–207, 2005.

[36] A. D. Peuke, S. Kopriva, L. Stroukova, L. Ivanova, R. Baumbach, H. Tintemann, G.-J. Krauss, R. Wennrich, H. Rennenberg,"Metal accumulation in wildtype and transgenic *Poplars* overproducing glutathione on different contaminated field sites: results from a release experiment in Germany and Russia," *Environmental and Experimental Botany* (in press)

[37] M. W. Pfaffl, "A new mathematical model for relative quantification in real-time RT-PCR," *Nucleic Acids Research*, vol. 29, E45:1–6, 2001.

[38] J. Pósfai, A. S. Bhagwat, Gy. Pósfai, R. J. Roberts, "Predictive motifs derived from cytosine methyltransferases," *Nucleic Acids Research*, vol. 17, pp. 2421–2435, 1989.

[39] M. Rother, G.-J. Krauss, G. Grass, D. Wesenberg, "Sulphate assimilation under Cd^{2+} stress in *Physcomitrella patens* – combined transcript, enzyme and metabolite profiling," *Plant, Cell and Environment*, vol. 29, pp. 1801–1811, 2006.

[40] H. J. Schäfer, A. Haag-Kerwer, T. Rausch, "cDNA cloning and expression analysis of genes encoding *GSH* synthesis in roots of the heavy-metal accumulator *Brassica juncea* L. evidence for Cd-induction of a putative mitochondrial gammaglutamylcysteine synthetase isoform," *Plant Mol Biol*, vol. 37, pp. 87–97, 1998.

[41] X. Sun, X. M. Sun, Z. M. Yang, S. Q. Li, J. Wang, S. H. Wang, "Expression of *Brassica napus* L. γ-glutamylcysteine synthetase and low-and high-affinity sulfate transporters in response to excess cadmium," *J Integrative Plant Biol*, vol. 47, pp. 243–250, 2005.

[42] K. Tamura, J. Dudley, M. Nei, S. Kumar, "MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0," *Molecular Biology and Evolution*, vol. 24, pp. 1596–1599, 2007.

[43] M. Tahiliani, K. P. Koh, Y. Shen, W. A. Pastor, H. Bandukwala, Y. Brudno, S. Agarwal, L. M. Iyer, D. R. Liu, L. Aravind, A. Rao, "Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1," *Science*, vol. 324, pp. 930–935, 2009.

[44] G. Taylor, "*Populus*: Arabidopsis for forestry. Do we need a model tree?," *Annals of Botany*, vol. 90, pp. 681–689, 2002.

[45] J. D. Thompson, D. G. Higgins, T. J. Gibson, "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice," *Nucleic Acids Research*, vol. 22, pp. 4673–4680, 1994.

[46] A. Tichopad, M. Dilger, G. Schwarz, M. W. Pfaffl, "Standardized determination of real-time PCR efficiency from a single reaction set-up," *Nucleic Acids Research*, 31, $N^{0.}$ 20 e122, pp. 1–6, 2003.

[47] G. A. Tuskan et al, "The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray)," *Science*, vol. 313, pp. 1596–1604. (2006)

[48] J. Uhl, N. Klan, M. Rose, K. D. Entian, O. Werz, D. Steinhilber, "The 5-LIPOXYGENASE promoter is regulated by DNA methylation," *Journal of Biological Chemistry*, vol. 277, pp. 4374–4379, 2002.

[49] B. F. Vanyushin, "DNA methylation in plants," *Current Topics Microbiology and Immunology*, vol. 301, pp. 67–122, 2006.

[50] G. Veres, R. A. Gibbs, S. E. Scherer, C. T. Caskey, "The molecular basis of the sparse fur mouse mutation," *Science*, vol. 237, pp. 415–417, 1987.

[51] W.-S. Wang, Y.-J. Pan., X.-Q. Zhao, D. Dwivedi, L.-H. Zhu, J. Ali, B.-Y. Fu, Z.-K. Li, "Drought-induced site-specific DNA methylation and its association with drought tolerance in rice (*Oryza sativa* L.)," *Journal of Experimental Botany*, vol. 62, 1951–1960, 2011.

[52] M. Wassenegger, "RNA-directed DNA methylation. *Plant Molecular Biology*, vol. 43, pp. 203–220, 2000.

[53] K. Watanabe, Y. Yamano, K. Murata, A. Kimura, "The nucleotide sequence of the gene for gamma-glutamylcysteine synthetase of Escherichia coli," *Nucleic Acids Research*, vol. 14, pp. 4393–4400, 1986.

[54] A. P. Wolfe, M. A. Matzke, "Epigenetics: Regulation through repression," *Science*, vol. 286, pp. 481–486, 1999.

[55] G. Zardo, S. Marenzi, M. Perilli, P. Caiafa, "Inhibition of poly (ADP-ribosyl) ation introduces an anomalous methylation pattern in transfected foreign DNA," *The FASEB Journal*, vol. 13, pp. 1518–1522, 1999.