RT-PCR Analysis and Stress Response Capacity of Transgenic *gsh*I-Poplar Clones (*Populus* × *canescens*) in Response to Paraquat Exposure

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Stress response capacity (Fv/Fm at 690 nm and F690/F735 at F_{max}) of untransformed hybrid poplar, *Populus* × *canescens* (*P. tremula* × *P. alba*), and two transgenic lines overexpressing γ-ECS (γ-glutamylcysteine synthetase) either in the cytosol (cyt-ECS) or in the chloroplast (chl-ECS) was studied in response to the herbicide paraquat (4.0×10^{-9} to 4.0×10^{-6} M) for 21 days. Significant differences at sublethal (4.0×10^{-7} M) and bleaching (4.0×10^{-6} M) concentrations of paraquat were observed with about a two-fold and eight-fold decrease in the photosynthetic activity (Fv/Fm at 690 nm and F690/F735 at F_{max}), respectively. None of the *gsh*I transgenic lines (cyt-ECS, chl-ECS) with elevated GSH content exhibited significant tolerance to paraquat.

Semiquantitative RT-PCR of the cyt-ECS clone was used for gene expression analysis of the nuclear encoded *rbcS* gene and the stress responsive *gst* gene. Expression of the constitutively expressed *26SrRNA* ribosomal gene was probed as a control for all RT-PCR reactions. The relative intensities of gene expressions normalized to the level of *26SrRNA* intensity showed a 50% decrease in the nuclear encoded *rbcS* expression and a 120% increase in the stress responsive *gst* gene expression of the paraquat treated (4.0×10^{-7} M) samples of the transgenic poplar line (cyt-ECS).

Key words: cyt-ECS (*ggs*11), chl-ECS (*lgl*6), Paraquat Stress, *Populus* × *canescens*

Introduction

Hybrid poplar (*Populus* × *canescens* = *P. tremula* × *P. alba*) (2n = 4x = 38) has significant phytoremediation capacity which may be increased by cell manipulation and genetic transformation. Recently *P.* × *canescens* was transformed to overexpress the bacterial (*Escherichia coli*) gene *gsh*I encoding γ-glutamylcysteine synthetase (γ-ECS, EC 3.2.3.3). γ-ECS is the rate-limiting regulatory enzyme in the biosynthesis of the ubiquitous tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) thiol compound GSH (glutathione) (Arisi *et al.,* 1997; Noctor *et al.,* 1998; Schröder *et al.,* 2001). GSH plays a central role in the plant detoxification processes supplying the initial molecule for sulphur-rich peptides of metallothioneins (MTs), metal transporter proteins (MTPs) and phytochelatins (PCs). In the present study stress response capacity of the wild-type hybrid poplar *P.* × *canescens* and two transgenic lines overexpressing γ-ECS in the cytosol (cyt-ECS) and in the chloroplasts (chl-ECS) was studied in response to paraquat (4.0×10^{-9} to 4.0×10^{-6} M) *in vitro*.

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 *ggs*11 (also designated as cyt-ECS; Arisi *et al.,* 1997) and *lgl*6 (also designated as *Lggs*6 and chl-ECS; Noctor *et al.,* 1998) were micropropagated *in vitro* (Kiss *et al.*, 2001; Koprivova *et al.,* 2002).

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Paraquat treatment

Leaf discs (8 mm) were cut and placed onto the surface of aseptic tissue culture medium WPM (Lloyd and McCown, 1980) with low sucrose content (1%) and supplemented with a concentration series of paraquat (4.0×10^{-9} to 4.0×10^{-6} M). The discs were incubated for 21 d in a 16 h/8 h light/dark (40 μE m^2 s^{-1}) photoperiod.

Fluorometry

Photosynthetic activity was determined using a laser-induced (635 nm), two-wavelength fluorometer (CFM-636973) detecting chlorophyll fluorescence at 690 nm and 735 nm (Barócsi *et al.,* 2000). Chlorophyll fluorescence ratios of Fv/Fm [(Fm–Fo)/Fm] at 690 nm and F690/F735 at F_{max} were calculated according to Lichtenthaler and Rinderle (1988). Ten leaf discs were measured in each treatment.

RT-PCR analysis

Total RNA was extracted from 0.1 g leaf disc tissues using TRI-reagent (Sigma-T9424) following the manufacturer's protocol. The quality and quantity of extracted DNA samples were measured by a NanoDrop ND-1000 UV-Vis spectrophotometer which enabled highly accurate analyses of extremely small samples (2 μl DNA) with remarkable reproducibility (NanoDrop Technologies, Montchanin, DE, USA – BioScience, Budapest, Hungary). First strand cDNA was synthesized on the mRNA templates by RT (reverse transcriptase) using an oligo$(dT)_{18-23}$ primer following the protocol (K1622) of Fermentas-Biocenter (Szeged, Hungary). For gene expression analysis, cDNA (2.5 μl) samples were probed by gene specific primers (Heinze, 1998). A degenerative primer pair for *gst* (GST, glutathione-*S*-transferase) was designed using the *'Primer3'* computer program and blasting the *gst27* (*Zea mays*) gene on sequences of *P. tremula* × *tremuloides* (Populus Database, http://poppel.fysbot.umu.se): 5′-gca caa gaa aga gcc (a/g)tt cc-3′ and 3′-agc tcc cag ttc agc ttt ga-5′ (Fig. 3).

For the expression analysis of *rbcS* gene (RuBPCase SSU: the SSU, **s**mall **su**b**u**nit, of RuBPCase, ribulose-1,5-bisphosphate carboxylase) the primer pair used was: 5′-agc ttg taa gag atg gct tcc tc-3′ and 3′-cca cat agt cca gta gcg tcc at-5′; and for the *26SrRNA* gene expression analysis the primer pair used was*:* 5′-ttc cat ggt tcg atc ctt cc-3′ and 3′-gca ggg cga tcg tgt ttt tc-3′ according to Gray-Mitsumune *et al*. (2004).

The level of gene expressions was quantified under a densitometer (ChemiImager™ 4400, Alpha Innotech Inc., San Leandro, CA, USA) with a computer program (Phoretix 1D Advanced, Nonlinear Dynamics, Ltd., Newcastle upon Tyne, UK) directly analyzed on the agarose (1.2%) gels of RT-PCR runs.

Results and Discussion

Phytotoxicity

The herbicide paraquat (methyl viologen; 1,1′-dimethyl-4,4′-bipyridinium dichloride) primarily affects the electron transport chains located in the chloroplasts and mitochondria as electron acceptors (Böger, 1993; Will *et al.,* 2001). In our study a reduced sucrose content (1%) was used in the leaf disc culture media to stimulate the activity of the photosynthetic electron transport chain (PETC) which switches off at higher sucrose contents (2 to

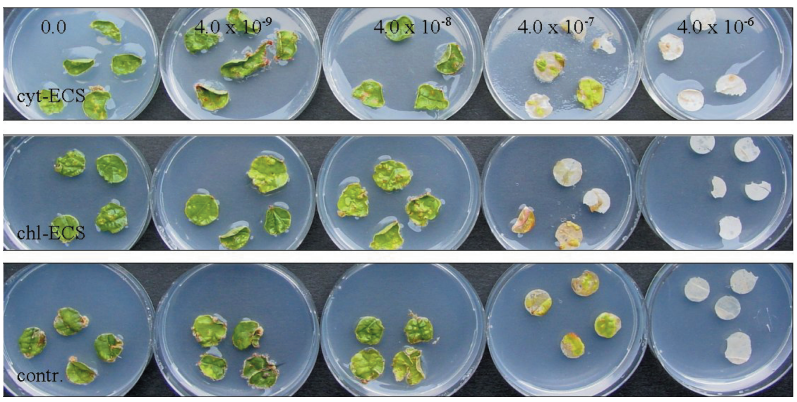

Fig. 1. The effect of paraquat (4.0×10^{-9} to 4.0×10^{-6} M) on leaf discs of hybrid poplar clones (*P.* × *canescens*) incubated on low sucrose (1%) WPM media. cyt-ECS (*ggs*11), transgenic clone overexpressing γ-ECS in the cytosol; chl-ECS (*lgl*6), transgenic clone overexpressing γ-ECS in the chloroplasts; contr., untransformed wild-type clone.

3%) (Lehoczki *et al.,* 1992). A concentration of 4.0×10^{-6} M paraquat led to bleaching of leaf discs, but with some growth activity retained in all the poplar clones. A concentration of 4.0×10^{-7} M paraquat caused chloroplast sublethality with a mixture of bleached and green spots on the leaf discs. No toxic effects were observed at lower paraquat concentrations in either of the clones (Fig. 1).

Photosynthetic activity

The phytotoxic effect of paraquat was determined by measuring chlorophyll fluorescence of leaf discs and calculating the ratio of P690/P735 at F_{max} (Lichtenthaler and Rinderle, 1988; Barócsi *et al.,* 2000). The results showed no significant differences at concentrations of 4.0×10^{-9} to 4.0×10^{-8} M compared to untreated samples (Fig. 2). Significant differences at sublethal (4.0×10^{-7} M) and bleaching (4.0×10^{-6} M) concentrations of paraquat were observed with about a two-fold and eight-fold (bleaching) decrease in the photosynthetic activity (Fv/Fm at 690 nm and F690/F735 at F_{max}), respectively (Fig. 2). Surprisingly, none of the *gsh*I transgenic lines (cyt-ECS, chl-ECS) with elevated GSH content exhibited significant tolerance to paraquat in our *in vitro* experiments under aseptic conditions similar to a results of an *ex vitro* paraquat exposure (Will *et al.*, 2001).

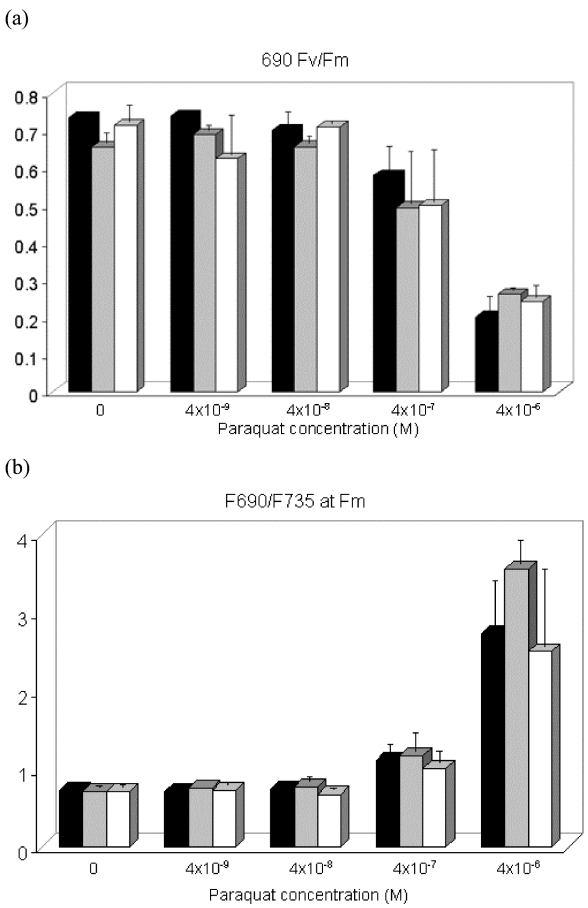

Fig. 2. The effect of paraquat (4.0×10^{-9} to 4.0×10^{-6} M) on the photosynthetic activity [Fv/Fm = (Fm –Fo)/Fm] at 690 nm (a) and chlorophyll fluorescence ratio F690/F735 at F_{max} (b) of leaf discs from hybrid poplar clones (*P.* × *canescens*). cyt-ECS (*ggs*11) (■), transgenic clone overexpressing γ-ECS in the cytosol; chl-ECS (*lgl*6) (▒), transgenic clone overexpressing γ-ECS in the chloroplasts; control (□), untransformed wild-type clone. Error bars show ± SD (means of 10 leaf discs).

RT-PCR analysis

In general, gene expression is controlled by regulatory processes of transcription (at a transcriptional level), pre-mRNA processing and mRNA turnover (at a post-transcriptional level), and translation (at translational and post-translational levels). The combined effect of these regulatory processes accounts for gene expression. Because of post-transcriptional events affecting mRNA stability and translation, expression levels of genes do not directly correlate with steady-state levels of mRNAs (Gygi *et al.*, 1999). Post-transcriptional gene silencing (PTGS), which operates at the level of sequence-specific RNA degradation, acts against transgenes (and also against endogenous genes and viruses) (Depicker and Van Montagu, 1997). An association between PTGS and methylation of the coding region in plants was also indicated by Jones *et al.* (1999). The instability of gene expression is also associates with transgene incorporation, as the results of multicopy integration of transgene at the same locus (Assaad *et al.*, 1993; Atkinson *et al.*, 1998), the position effects due to random integration, 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), and the environmental conditions (Meyer and Heidman, 1994). All these factors can generate different expression patterns of the gene from an overexpression to the total gene silencing (Koprek *et al.,* 2001).

In our study, gene expression analysis was carried out with RT-PCR using cDNA samples from

leaf discs of cyt-ECS (*ggs*11) clone after paraquat treatment at a sublethal concentration of 4.0×10^{-7} M compared to untreated samples (Fig. 3).

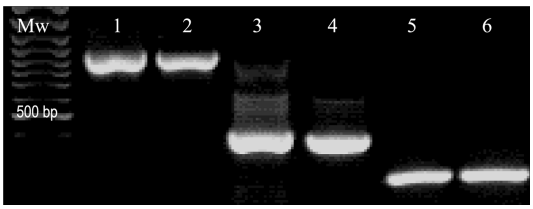

Fig. 3. RT-PCR analysis of gene expression of the constitutively expressed gene *26SrRNA* (1, 2), the nuclear encoded *rbcS* (3, 4), and the stress responsive *gst* (5, 6) in the cDNAs of the paraquat-treated (4.0×10^{-7} M) (2, 4, and 6) and untreated (1, 3, and 5) samples of transgenic poplar *P.* × *canescens*, cyt-ECS (*ggs*11).

The constitutionally expressed ribosomal gene *26SrRNA* was used as control for measuring the standard transcriptional activity (Gray-Mitsumune *et al.,* 2004) which obviously showed equal gene expression in the untreated and treated samples (Fig. 3). The expression of the nuclear encoded *rbcS* gene, as a marker for chloroplasts vitality (Gray-Mitsumune *et al*., 2004), showed slightly less activity in the paraquat-treated samples (Fig. 3). The stress responsive expression of *gst* in the paraquat-treated (4.0×10^{-7} M) samples showed slightly higher activity compared to untreated samples (Fig. 3).

As the differences of gene expressions between the paraquat-treated (4.0×10^{-7} M) and untreated samples showed slight differences visually observed on agarose gels (Fig. 3), a computer densitometry was applied for calculation (Table I). The relative intensities of gene expressions were normalized to the level of constitutively expressed *26SrRNA* intensity. The results showed a 50% decrease in the nuclear encoded *rbcS* expression, and a 120% increase in the stress responsive *gst* expression of the paraquat-treated (4.0×10^{-7} M) samples (cyt-ECS) (Table I). The 50% decrease of *rbcS* gene expression confirms the results obtained in the decreased photosynthetic activity (Fig. 2). The increase of *gst* gene activity can indicate an increased phytoextraction capacity of the *gsh*I transgenic poplar (cyt-ECS) as observed in an *in vitro* Zn-stress by Gyulai *et al.* (2005).

Table I. The relative gene expressions of *26SrRNA*, *rbcS* and *gst* in the untreated (0.0 M) and paraquat-treated (4.0×10^{-7} M) samples of transgenic *gsh*I-poplar clone (*P.* × *canescens*, cyt-ECS) analyzed by a computer densitometer.

Paraquat	Rel. gene expression		
	26SrRNA	*rbcS*	*gst*
0.0 M	1.0	1.0	1.0
4×10^{-7} M	1.0	0.5	1.2

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